Ταυτοποίηση και χαρακτηριμός γονιδίων που ενέχονται στην ανθεκτικότητα σε νεονικοτινοειδή εντομοκτόνα στη *Drosophila melanogaster*

Identification and characterization of genes involved in resistance to neonicotinoid insecticides in *Drosophila melanogaster*

M.Sc. Predrag Kalajdžić

Ph.D. Thesis

July, 2012

Faculty of Medicine

University of Crete, Greece

I hereby declare that this thesis has not been submitted, either in the same or a different form, to this or any other University for a degree.

Signature:

Περίληψη:

Η ανθεκτικότητα στα εντομοκτόνα αποτελεί ένα πολύ σημαντικό εμπόδιο για τον αποτελεσματικό έλεγχο εντόμων που είναι επιβλαβή στη γεωργία ή είναι φορείς ασθενειών. Ανθεκτικότητα σε όλες τις μεγάλες κατηγορίες εντομοκτόνων (οργανοφωσφορικά, καρβαμιδικά και πυρεθροειδή) έχει εμφανιστεί, σχετικά σύντομα μετά την εισαγωγή της χρήσης τους, σε πληθυσμούς πολλών ειδών εντόμων.

Τα νεονικοτινοειδή αποτελούν μία από τις πλέον υποσχόμενες κατηγορίες εντομοκτόνων, με την Ιμιδακλοπρίδη ως το πιο επιτυχημένο μέλος της ομάδος, με τις μεγαλύτερες πωλήσεις παγκοσμίως. Τα νεονικοτινοειδή δρουν ως αγωνιστές των μετασυναπτικών νικοτινικών υποδοχέων ακετυλοχολίνης στο κεντρικό νευρικό σύστημα του εντόμου. Παρά το ότι τα νεονικοτινοειδή παραμένουν εξαιρετικά αποτελεσματικά, μελέτες πεδίου δείχνουν σημαντική αύξηση εμφάνισης ανθεκτικότητας σε διάφορα είδη επιβλαβών εντόμων.

Η κατανόηση των μηχανισμών ανθεκτικότητας σε εντομοκτόνα είναι απαραίτητη για την περαιτέρω ανάπτυξη εργαλείων και παρεμβάσεων που μπορούν να βελτιώσουν τον έλεγχο των επιβλαβών εντόμων. Μέχρι τώρα έχουν χαρακτηριστεί δύο κύριοι μηχανισμοί οι οποίοι, μόνοι ή σε συνδυασμό, είναι υπεύθυνοι για την ανθεκτικότητα στα εντομοκτόνα: η ανθεκτικότητα στόχου και η μεταβολική ανθεκτικότητα. Η ανθεκτικότητα στόχου είναι αποτέλεσμα μεταλλαγών στο μόριο-στόχο του εντομοκτόνου. Μεταβολική ανθεκτικότητα προκαλείται συνήθως από την ενεργοποίηση ή την υπερέκφραση ενζύμων που εμπλέκονται στην αποτοξίνωση του εντομοκτόνου. Βιοχημικές μελέτες έχουν δείξει ότι η μεταβολική ανθεκτικότητα συνδέεται γενικά με τρεις οικογένειες ενζύμων: οξειδάσες μεικτής λειτουργίας (κυτοχρώματα P450) καρβοξυλ-εστεράσες και S-τρανσφεράσες γλουταθειόνης (GST). Η μοριακή βάση της ανθεκτικότητας στόχου είναι αρκετά καλά μελετημένη και κατανοητή, αντίθετα οι βασικοί μοριακοί μηχανισμοί της μεταβολικής ανθεκτικότητας παραμένουν σε μεγάλο βαθμό άγνωστοι.

Ένα σημαντικό μέρος της γνώσης μας σχετικά με τους μηχανισμούς ανθεκτικότητας προέρχεται από τη μελέτη φυσικών πληθυσμών που έχουν αναπτύξει ανθεκτικότητα. Η επαγωγή ανθεκτικότητας σε εργαστηριακούς πληθυσμούς μέσω μεταλλαξιγένεσης αποτελεί μια εναλλακτική πειραματική προσέγγιση, η οποία έχει δώσει πρόσφατα

σημαντικές πληροφορίες. Στην παρούσα μελέτη, χρησιμοποιήθηκε μεταλλαξιγένεση μέσω του μεταθετού στοιχείου *Minos* για τη δημιουργία ανθεκτικών στελεχών σε έναν εργαστηριακό πληθυσμό του εντόμου-μοντέλου *Drosophila melanogaster*.

Το μεταθετό στοιχείο (ΜΣ) *Minos*, μέλος της υπεροικογένειας Tc1/*mariner*, έχει χρησιμοποιηθεί για μεταλλαξιγένεση ή/και διαγένεση σε διάφορα είδη ευκαρυωτικών οργανισμών, και κυρίως στη Δροσόφιλα. Το γεγονός ότι τρανσποζόνια με βάση το *Minos,* μετά από κινητοποίηση μέσω της *Minos* τρανσποζάσης, παράγουν σταθερές ενθέσεις στα χρωματοσώματα με υψηλή απόδοση και με τελείως τυχαίο τρόπο έχει αναδείξει πρόσφατα την μεταλλαξιγένεση μέσω του στοιχείου αυτού ως ένα εξαιρετικά χρήσιμο εργαλείο γενετικής και γονιδιωματικής ανάλυσης.

Η Δροσόφιλα, αν και δεν είναι επιβλαβές είδος, έχει χρησιμοποιηθεί εκτενώς ως πειραματικό μοντέλο στην έρευνα για την κατανόηση των μηχανισμών ανθεκτικότητας σε εντομοκτόνα. Μελέτες σε φυσικούς και εργαστηριακούς πληθυσμούς της *D. melanogaster* έχουν καταδείξει ότι το είδος αυτό μπορεί να αναπτύξει ανθεκτικότητα σε ένα ευρύ φάσμα εντομοκτόνων. Κύριος στόχος της παρούσης μελέτης είναι η ταυτοποίηση γονιδίων της Δροσόφιλας που εμπλέκονται στην ανθεκτικότητα σε νεονικοτινοειδή εντομοκτόνα, με απώτερο στόχο την κατανόηση των σχετικών μοριακών μηχανισμών.

Η παρουσίαση των αποτελεσμάτων χωρίζεται σε δύο μέρη. Το πρώτο μέρος αφορά σειρά πειραμάτων τα οποία καταδεικνύουν την καταλληλότητα ενός αποδοτικού γενετικού συστήματος με βάση το ΜΣ για τη δημιουργία τυχαίων ενθέσεων οι οποίες ενεργοποιούν παρακείμενα γονίδια. Το σύστημα αυτό στηρίζεται στο τρανσποζόνιο *Minos* TREP, το οποίο περιέχει έναν χιμαιρικό υποκινητή που επάγεται *in trans* από τον μεταγραφικό ενεργοποιητή tTA. Στο δεύτερο μέρος παρουσιάζεται η τοξικολογική, βιοχημική, γονιδιωματική και γενετική ανάλυση ενός μεταλλαγμένου στελέχους ανθεκτικού στην Ιμιδακλοπρίδη και το DDT, το οποίο απομονώθηκε από μια σάρωση του γονιδιώματος της Δροσόφιλας με τη χρήση του TREP.

Για την ανάπτυξη συστήματος σάρωσης του γονιδιώματος χρησιμοποιήθηκε το διαγονιδιακό στέλεχος TREP 2.30, το οποίο περιέχει μια ένθεση του τρανσποζονίου TREP στο χρωματόσωμο 4. Η ένθεση αυτή σε ομόζυγη κατάσταση δεν έχει φαινότυπο, αλλά είναι θανατογόνος σε ετερόζυγη κατάσταση, σε συνδυασμό με ένα

διαγονιδιακό χρωματόσωμο που εκφράζει τον μεταγραφικό ενεργοποιητή tTA. Δεδομένου ότι το τρανσποζόνιο TREP είναι γενετικά σεσημασμένο με το γονίδιο-δείκτη *white,* είναι εύκολο να επιλεγούν έντομα στα οποία έχει συντελεστεί μετακίνηση του TREP από την αρχική του θέση στο γονιδίωμα σε άλλη θέση. Η συχνότητα της μετακίνησης του τρανσποζονίου TREP στους γαμέτες του στελέχους TREP 2.30, μετά από επαγωγή μέσω έκφρασης της *Minos* τρανσποζάσης, αποδείχτηκε εξαιρετικά υψηλή (92%) με τα 2/3 των νέων ενθέσεων να εντοπίζονται στα 3 μείζονα χρωματοσώματα (X, 2ο και 3ο). Για τον εντοπισμό γονιδίων που ενέχονται στην ανθεκτικότητα, διενεργήθηκε μεγάλης κλίμακας σάρωση του γονιδιώματος κατά την οποία παρήχθησαν περίπου 12900 νέες ενθέσεις TREP, που αντιστοιχούν σε στόχευση του 35% των γονιδίων της Δροσόφιλας. Τα αποτελέσματα αυτά καταδεικνύουν ότι το σύστημα TREP 2.30 είναι ένα χρήσιμο εργαλείο για μεγάλης κλίμακας γενετικές σαρώσεις του γονιδιώματος της Δροσόφιλας. Κατά τη διάρκεια της σάρωσης έγινε επιλογή ενός θηλυκού εντόμου με μεγάλη ανθεκτικότητα στην Ιμιδακλοπρίδη, από το οποίο μετά τις κατάλληλες διασταυρώσεις ιδρύθηκε ένα ανθεκτικό στέλεχος, το MIT[w-]3R2. Γενετική ανάλυση του χρωματοσώματος MIT[w-]3R2 έδειξε ότι ο χαρακτήρας της ανθεκτικότητας εντοπίζεται στο χρωματόσωμα 2, αλλά δεν έδειξε σύνδεση με το τρανσποζόνιο TREP, υποδηλώνοντας ότι η μεταλλαγή οφείλεται πιθανώς σε φαινόμενο "hit and run", δηλαδή ένθεση του τρανσποζονίου μετά την οποία έγινε εκτομή. Το στέλεχος MIT[w-]3R2 αναλύθηκε περαιτέρω με τη χρήση τοξικολογικών, βιοχημικών, γονιδιωματικών και γενετικών προσεγγίσεων.

Η διασταυρούμενη ανθεκτικότητα του στελέχους MIT[w-]3R2 στο DDT, που έχει διαφορετικό στόχο αλλά παρόμοιο μηχανισμό αποτοξίνωσης με την Ιμιδακλοπρίδη, αποτέλεσε σοβαρή ένδειξη ότι η ανθεκτικότητα στο στέλεχος MIT[w-]3R2 έχει μεταβολική βάση. Τοξικολογική και βιοχημική ανάλυση, με τη χρήση του αναστολέα του P450 PBO και τον προσδιορισμό της ενζυμικής δραστικότητας P450 αντίστοιχα, απέδειξαν ότι η ανθεκτικότητα οφείλεται, εν μέρει τουλάχιστον, σε αυξημένη δραστηριότητα του P450 στο στέλεχος MIT[w-]3R2 σε σύγκριση με το ισογονιδιακό στέλεχος iso31 (w^{1118}_{iso}; 2_{iso}; 3_{iso}) που χρησιμοποιήθηκε ως στέλεχος αναφοράς. Δεν εντοπίστηκε αυξημένη δραστηριότητα της GST και εστερασών στο ανθεκτικό στέλεχος. Μοριακή ανάλυση έδειξε ότι το στέλεχος MIT[w-]3R2 χαρακτηρίζεται από

υπερέκφραση των mRNA των τριών γονιδίων P450 (Cyp6g1, Cyp6a2 και Cyp12d1) που είναι ήδη γνωστό ότι ενέχονται σε ανθεκτικότητα σε νεονικοτινοειδή και το DDT.

Μεταγραφωματική ανάλυση του ανθεκτικού στελέχους και του ευαίσθητου στελέχους-μάρτυρα μέσω βαθειάς αλληλούχισης cDNA (cDNA deep sequencing) με την τεχνολογία Illumina διενεργήθηκε με στόχο την ποσοτικοποίηση των διαφορών στην έκφραση όλων των γονιδίων στα δύο στελέχη. Ταυτοποιήθηκαν 357 διαφορετικά γονίδια, από τα οποία 150 ήταν υπερ-εκφραζόμενα, και 207 υπο-εκφραζόμενα στο ανθεκτικό στέλεχος. Ταξινόμηση και ομαδοποίηση των διαφορικά εκφραζόμενων γονιδίων με βάση λειτουργικές ομοιότητες εντόπισαν τρεις λειτουργικές ομάδες υπερ-εκφραζομένων γονιδίων και δύο λειτουργικές ομάδες υπο-εκφραζομένων γονιδίων. Στην πρώτη κατηγορία υπερεκπροσωπούνται γονίδια που κωδικοποιούν οξειδάσες μεικτής λειτουργίας P450, πρωτεολυτικά ένζυμα και πρωτεΐνες με δραστικότητα πεπτιδάσης. Στην κατηγορία των υπο-εκφραζόμενων γονιδίων υπερεκπροσωπούνται γονίδια που κωδικοποιούν πρωτεΐνες του χορίου των ωοκυττάρων και μια ομάδα γονιδίων για πρωτεΐνες με δραστικότητα πεπτιδάσης.

Τα πλέον έντονα υπερ-εκφραζόμενα γονίδια P450 που εντοπίστηκαν με αυτήν την προσέγγιση και επιβεβαιωθηκαν με real time PCR, είναι τα Cyp4p2, Cyp6a2 και Cyp6g1. Η συμμετοχή των Cyp6a2 και Cyp6g1 στο μηχανισμό ανθεκτικότητας σε εντομοκτόνα στη Δροσόφιλα έχει ήδη τεκμηριωθεί και τα αποτελέσματά μας επικυρώνουν τη χρησιμότητα της μεταγραφωματικής προσέγγισης για την ανίχνευση γονιδίων που εμπλέκονται στην ανθεκτικότητα στα εντομοκτόνα. Αυξημένη έκφραση του γονιδίου Cyp4p2 σε έντομο ανθεκτικό στην Ιμιδακλοπρίδη και το DDT αναφέρεται για πρώτη φορά.

Ο ρόλος των πρωτεολυτικών ενζύμων και των ενζύμων με δραστικότητα πεπτιδάσης στην ανθεκτικότητα δεν είναι πλήρως κατανοητός. Οι πρωτεάσες πιθανώς να συμμετέχουν στην τροποποίηση της διαμόρφωσης ενζύμων και στη βιοσύνθεση πρωτεϊνών, προκειμένου να καλυφθούν οι ενεργειακές ανάγκες κατά τη διάρκεια του ξενοβιοτικού στρες. Η ελαττωμένη έκφραση ενός αριθμού γονιδίων που κωδικοποιούν δομικές πρωτεϊνες του χορίου θα μπορούσε να προκύψει ως

αποτέλεσμα της γενικής απάντησης στο στρες που προκαλείται από το ενεργοποιημένο σύστημα αποτοξίνωσης.

Λειτουργική ομαδοποίηση των διαφορικά εκφραζόμενων γονιδίων με βάση την πρόβλεψη της βιολογικής τους λειτουργίας εντόπισε 10 υπερ-εκπροσωπούμενες ομάδες υπερ-εκφραζόμενων γονιδίων και 13 υπερ-εκπροσωπούμενες ομάδες υπο-εκφραζόμενων γονιδίων στο ανθεκτικό στέλεχος. Ανάμεσα στα υπερ-εκφραζόμενα γονίδια ξεχώρισαν οι λειτουργικές ομάδες "*οξειδοαναγωγική δραστηριότητα*", "*μιτωτικός διαχωρισμός αδελφών χρωματίδων*", "*δραστηριότητα μεταφοράς ηλεκτρονίων*" και "*απάντηση σε βλάβες DNA*". Στα υπο-εκφραζόμενα γονίδια περιλαμβάνονταν οι ομάδες "*μεταβολισμός χιτίνης και αμινογλυκανών*", "*απάντηση σε μόλυνση με βακτήρια*" και "*δραστηριότητα ανοσολογικής απάντησης*". Η οξειδοαναγωγική δραστηριότητα παίζει σημαντικό ρόλο στην αποτοξίνωση, ενώ οι άλλες βιολογικές διεργασίες θα μπορούσε να είναι μια ένδειξη γενικής αντίδρασης στο στρες από το ανθεκτικό έντομο.

Η απουσία σύνδεσης του τρανσποζονίου TREP με τον γενετικό τόπο της ανθεκτικότητας υποδηλώνει ότι η μεταλλαγή πιθανώς οφείλεται σε φαινόμενο "hit and run", δηλαδή αρχική ένθεση του τρανσποζονίου το οποίο ακόλουθα αποκόπηκε, προκαλώντας μια τοπική χρωματοσωμική ανωμαλία (τοπικό έλλειμα, μικρή ένθεση, ή συνδυασμό τους). Ως εκ τούτου, η χαρτογράφηση του γενετικού τόπου της ανθεκτικότητας έγινε με κλασσική γενετική ανάλυση (ανάλυση ανασυνδυασμού με χαρτογραφημένες ενθέσεις του ΜΣ *P*) και με ανάλυση πολυμορφισμών μοναδικών νουκλεοτιδίων (SNPs) οι οποίες προέκυψαν από την αλληλούχιση του μεταγραφώματος. Ο γενετικός τόπος εντοπίζεται στο δεξιό σκέλος του δεύτερου χρωμοσώματος, σε μια περιοχή μικρότερη από 1Mb. Μέσα στην ίδια περιοχή βρίσκεται και το γονίδιο Cyp6g1, το οποίο είναι ένα από τα πλέον ισχυρά υπερ-εκφραζόμενα γονίδια στο στέλεχος MIT[w-]3R2 και έχει ήδη περιγραφεί ως βασικός παράγοντας ανθεκτικότητας στη Ιμιδακλοπρίδη.

Ανάλυση της αλληλουχίας του mRNA του Cyp6g1 δεν έδωσε ένδειξη για ύπαρξη μεταλλαγής στην κωδική περιοχή του γονιδίου στο ανθεκτικό στέλεχος. Είναι πιθανόν να έχει συντελεστεί μια μεμονωμένη μεταλλαγή σε *cis*-ρυθμιστικό στοιχείο του γονιδίου Cyp6g1, η οποία οδηγεί σε αύξηση της έκφρασης του γονιδίου, που με

τη σειρά της επηρεάζει την έκφραση άλλων γονιδίων που εμπλέκονται σε ανθεκτικότητα. Εναλλακτικά, η μεταλλαγή μπορεί να αφορά ένα γονίδιο που αντιστοιχεί σε έναν παράγοντα μεταγραφής ή ένα microRNA, που ρυθμίζει πολλαπλά γονίδια P450. Μέχρι στιγμής, δεν υπάρχουν στοιχεία που να υποστηρίζουν την τελευταία υπόθεση, δεδομένου ότι μια *in silico* αναζήτηση απέτυχε να εντοπίσει κοινά μοτίβα προσδεσης, είτε για κάποιον από τους γνωστούς παράγοντες μεταγραφής, είτε για κάποιο microRNA, στα γονίδια P450 τα οποία υπερ-εκφράζονται. Μελλοντική ανάλυση της αλληλουχίας γονιδιωματικού DNA στην περιοχή όπου εντοπίζεται ο γενετικός τόπος, πιθανώς να αποκαλύψει τη μοριακή βάση της ανθεκτικότητας στο στέλεχος MIT[w-]3R2.

Abstract:

Insecticide resistance is a serious, long term problem that impacts agricultural production and health of animals and humans. Resistance to all major insecticide classes, including neonicotinoids, arose in numerous and diverse insect field populations. Imidacloprid, the most prominent neonicotinoid, has been widely used during the last decade in controlling different insect pests. *Drosophila melanogaster*, although not a pest species, is a widely used model organism and a promising model system for insecticide resistant research. In our study we have analyzed a *Drosophila* laboratory mutant which is resistant to Imidacloprid and cross-resistant to DDT. The mutant has been retrieved in a genome-wide *Minos*-based insertional mutagenesis screen. The resistant line was characterized using genetic, toxicological, molecular and transriptomic analysis. Genetic analysis mapped resistance to the right arm of the second chromosome. Toxicologal analysis showed higher activity of P450 enzymes, while molecular analysis revealed higher expression of three unlinked P450 genes in the resistant line compared to the susceptible line. Deep sequencing transcriptomic analysis showed changes in several groups of genes involved in metabolic processes. Taken together, these results strongly suggest that the mutation results in upregulation of several unlinked genes involved in metabolic detoxification of the insecticides. The exact molecular mechanism remains to be elucidated.

Acknowledgments:

This work was carried out mainly at the Insect Molecular Biology Laboratory, Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology (IMBB–FoRTH), Heraklion, Crete. A small part of the project was done at the Institute of Cellular and Developmental Biology, Biomedical Science Research Center (BSRC) "Alexander Fleming", Athens. This research was supported by a scholarship of the Greek State Scholarship Foundation (IDRYMA KRATIKON YPOTROFION – IKY) (2006 – 2010), as well as a scholarship of the Biomedical Science Research Center "Alexander Fleming" (October 2010 – February 2011).

Firstly, I want to thank my supervisor Prof. Dr. Charalambos Savakis for giving me the opportunity to work with *Minos* genomic tools and his excellent guidance through all the stages of my PhD thesis. Furthermore, I would like to thank him for his moral support, immense optimism, helpfulness in all matters and the scientific and general life experience, which he generously shared with me.

I am grateful to all the members of the committee for reviewing my PhD thesis and providing very helpful comments: Prof. Dr. Σαββάκης Χαράλαμος (thesis supervisor), Prof. Dr. Καραγωγέως Δόμνα, Prof. Dr. Δελιδάκης Χρήστος, Prof. Dr. Μαυροθαλασσίτης Γεώργιος, Assistant Prof. Dr. Βόντας Γιάννης, Lecturer Dr. Ηλιόπουλος Γιάννης and Assistant Prof. Dr. Μαθιόπουλος Κώστας.

I thank our collaborators Dr. Martin Reczko and Prof. Dr. Artemis Hatzigeorgiou for their help with the bioinformatical analysis of the deep sequencing data.

I am very grateful to Prof. Dr. John Vontas, for providing access to his laboratory for toxicological and molecular analysis, as well as for his professional and personal support. I also thank all the people from Prof. Dr. Vontas laboratory, especially Dr. Evangelia Morou, for scientific assistance, personal help and warm friendship.

I thank all the people at the Institute of Molecular Biology and Biotechnology (IMBB-FoRTH), especially the members of Prof. Dr. Christos Delidakis΄ and Prof. Dr. Michalis Averof΄s labs, as well as from the members of Dr. Efthimios Skoulakis΄ lab (at the BSRC "Alexander Fleming") for scientific help, the always pleasant atmosphere, and their friendship.

My special thanks go to the members of Prof. Dr. Charalambos Savakis´ laboratory (Insect Molecular Biology Laboratory, IMBB–FoRTH) for their general help and the pleasant atmosphere, which I enjoyed throughout all the time.

I owe gratitude to Dr. Alexandros Kiupakis for theoretic and experimental guidance during the beginning of my thesis.

I want to thank Mary Adamaki, Secretary of the Medical School, University of Crete, for the always warm reception and immediate help with various administrative issues.

There are two people to whom I especially want to express my sincerest gratitude. Many thanks to Dr. Maria Markaki-Stefanou for generously sharing her immense scientific and experimental knowledge. I consider myself lucky that I had such a warm hearted person with a pure soul as a mentor in the laboratory, who was as well a friend. Also, many thanks for Dr. Stefan Oehler for teaching me in practice what science is all about, always unselfishly sharing with me his tremendous scientific and technical knowledge. It was my greatest pleasure to learn, discuss different aspects of science and life and to have continuous generous professional support from a person with such deep interest in science, and at the same time to have such a supportive and sincere friend.

Finally, I want to thank all my friends near and far who made my stay in Crete and in Greece unforgettable.

Ενα μεγάλο ευχαριστό για ολα...

1. INTRODUCTION

1.1. Insecticides and insecticide resistance

The increase in productivity of the agricultural industry in the last century can to a large extent be attributed to an increased use of synthetic chemical insecticides. As a consequence of the strong long-term selective pressure, insecticide-resistant field populations of many insect pest species appeared. During the second half of the twentieth century, the number of resistant species increased to more than 500 worldwide (Gut *et al*., 2007).

One of the definitions of resistance describes it as “the inherited ability of a strain of some organism to survive doses of a toxicant that would kill the majority of individuals in a normal population of the same species” (WHO, 1957). Insecticide resistance can be diagnosed “when there is a repeated failure of an insecticide to achieve the expected level of control of insects when used according to the product label recommendations and where problems of product storage, application and unusual climatic or environment conditions can be eliminated as causes of the failure” (IRAC, 2005). Insecticide resistance is an ever increasing problem compromising the reliable control of insect pests of medical, veterinary and agricultural impact. The effect of insecticide resistance includes drastic changes in agriculturally relevant insect communities and has even medical implications. Much research has been directed toward understanding the changes that allow global populations of insects to lose susceptibility to pesticides. Numerous studies have documented evolution of resistance in field populations as the result of selection of already existing mutations in the nature. In contrast to field pest populations, which often possess a highly heterogeneous genetic background, the possibility for the generation of single mutations in a known and characterized background would substantially facilitate the identification of resistance-associated changes. Understanding resistance in more detail will provide the necessary knowledge for rational approaches to combat the detrimental effects of insecticides and to increase their specificity and efficiency.

Insecticides can be classified in several ways, but the biologically most useful method of classification is by mode of action (MoA), in which insecticides are grouped based on their biological targets (IRAC, 2005). Using this grouping, there are around 29

different MoA by which insects attain resistance. The major insecticide biological targets groups, depicted in table 1, can be divided into:

- Neurotoxins
- Microbial or derived disruptors of insect midgut membranes
- Cuticle Synthesis, Moulting and Metamorphosis disruptors
- Disruptors of Various other Metabolic Processes
- Repellents, attractants and other modifiers of insect behaviour
- Non-specific, Unknown and Miscellaneous MoA

Table 1. Major insecticide classes target site groups (source IRAC international MoA working group 2010)

Mode of action (MoA)	Insecticide class
Neurotoxins	
Acetylcholinesterase (AChE) inhibitors	Carbamates, Organophosphates
GABA-gated chloride channel antagonists	Cyclodienes and other organo-chlorines (OCs), Phenylpyrazoles (Fiproles)
Sodium channel modulators	Pyrethrins, pyrethroids, DDT
Acetylcholine receptor (nAChR) agonists	Neonicotinoids, nicotine
nAChR agonists: Allosteric	Macrocyclic lactones (Spinosyns)
Chloride channel activators	Avermectins, Milbemycins
nAChR channel blockers	Nereistoxin analogues
Voltage dependent sodium channel blocker	Oxadiazine
Microbial or derived disruptors of insect midgut membranes	
Mode of action (MoA)	Insecticide class
Disruption of biological membranes	Toxins derived from bacterium *Bacillus thuringiensis* (*Bt*): Bt sprays and Cry proteins expressed in transgenic Bt crop varieties
Cuticle synthesis, moulting and metamorphosis disruptors	
Mode of action (MoA)	Insecticide class and insecticides
Juvenile hormone mimics and analogues	Methoprene, pyriproxyfen
Inhibitors of chitin biosynthesis (insect growth regulators (IGRs))	Novaluron, buprofezin, cyromazine
Ecdysone agonist/molting disruptors	Diacylhydrazines, Azadirachtin
Disruptors of Various other Metabolic Processes	
Mode of action (MoA)	Insecticide class and insecticides
Inhibitors of oxidative phosphorylation, disruptors of ATP formation (inhibitors of ATP synthase)	Diafenthiuron Organotin acaricides
Uncouplers of oxidative phosphorylation via disruption of proton gradient	Chlorfenapyr, DNOC
Octopaminergic (nervous system) agonist acaricide and insecticide (probably loss of feeding and adhesion)	Amitraz
Mitochondrial complex III electron transport inhibitors	Hydramethylnon, acequinocyl, fluacrypyrim
Mitochondrial complex I electron transport inhibitors	Rotenone, METI acaricides
Inhibitors of lipid synthesis	Tetronic acid derivatives
Mitochondrial complex IV electron transport inhibitors	Precursors of fumigant: phosphine (PH_3)
Ryanodine receptor modulators: sustained contraction of insect muscle	Diamides
Non-specific, Unknown and Miscellaneous MoA	
Mode of action (MoA)	Insecticide class and insecticides
Inorganic fumigants with non-specific MoA	Methyl bromide, chloropicrin, sulfuryl fluoride
Various compounds of non-specific mode of action (selective feeding blockers)	Cryolite, pymetrozine, flonicamid
acaricidal growth inhibitors	Clofentezine, hexythiazox, etoxazole
Synergists P450-dependent mono oxygenase inhibitors, Esterase inhibitors	Piperonyl butoxide, tribufos (DEF)
Unknown mode of action	Dicofol, pyridalyl
Repellents, attractants and other modifiers of insect behaviour	
Mode of action (MoA)	Insecticide class and insecticides
Insect repellents	DEET, citronella oil
Pheromones	Specific many
Baiting attractants	methyl eugenol

Neurotoxins are insecticides that act specifically on nerve and muscle targets, usually by interacting with ion channels or neurotransmitter receptors. The main insecticide classes from this group are carbamates, organophosphates, pyrethroids and neonicotinoids (table 1). “Microbial or derived disruptors of insect midgut membranes” are protein toxins that induce pore formation in the midgut membrane, resulting in ionic imbalance and septicemia (table 1). Protein toxins from this class are derivatives of a *Bacillus thuringiensis* toxin. Insecticides that interfere with growth and development are in the group of the “cuticle synthesis, moulting and metamorphosis disruptors”. They act by mimicking ecdysone or juvenile hormone, or by directly affecting cuticle formation/deposition or lipid biosynthesis (table 1). Several insecticides are known to interfere with mitochondrial respiration through inhibition of electron transport and/or oxidative phosphorylation. They have been assigned to the somewhat arbitrary “group of disruptors of various other metabolic processes”. The “non-specific, unknown and miscellaneous MoA” group collects insecticides that affect less well-described target-sites or functions, or act non-specifically on multiple targets (table 1).

1.1.1. Neonicotinoids

The neonicotinoids form one of the most promising insecticide classes. They were introduced in the early 1990s and became one of the most widely used classes of insecticides worldwide (Jeschke and Nauen, 2008). Both neonicotinoids and nicotine are neurotoxins and belong to group four of the IRAC insecticide MoA classification (IRAC, 2005). Nicotinic acetylcholine receptors (nAChRs) are a family of neurotransmitter-gated ion channels that play an important role in nerve signaling at the post-synaptic membrane of both vertebrates and invertebrates. Neonicotinoids act as agonists of the nicotinic acetylcholine receptor (nAChR), opening the channel and causing continuous depolarization and firing of postsynaptic neurons, resulting in paralysis and death (Bai *et al.*, 1991; Zhang *et al.*, 2000; Nauen *et al.*, 2001). They act selectively on insect nAChRs, while exhibiting only low binding affinity and activity on vertebrate nAChRs (Tomizawa *et al.*, 2000). As a result of their specific MoA,

there is no cross-resistance to the long-established conventional insecticide classes (Nauen and Denholm, 2005). Neonicotinoids currently in use as pesticides include Acetamiprid, Clothianidin, Dinotefuran, Imidacloprid, Nitenpyram, Thiacloprid and Thiamethoxam (figure 1). Based on the pharmacophore moiety, the seven commercialized neonicotinoids can be divided into open-chain compounds and neonicotinoids having ring systems such as five- and six-membered compound which differ in their molecular characteristics (figure 1).

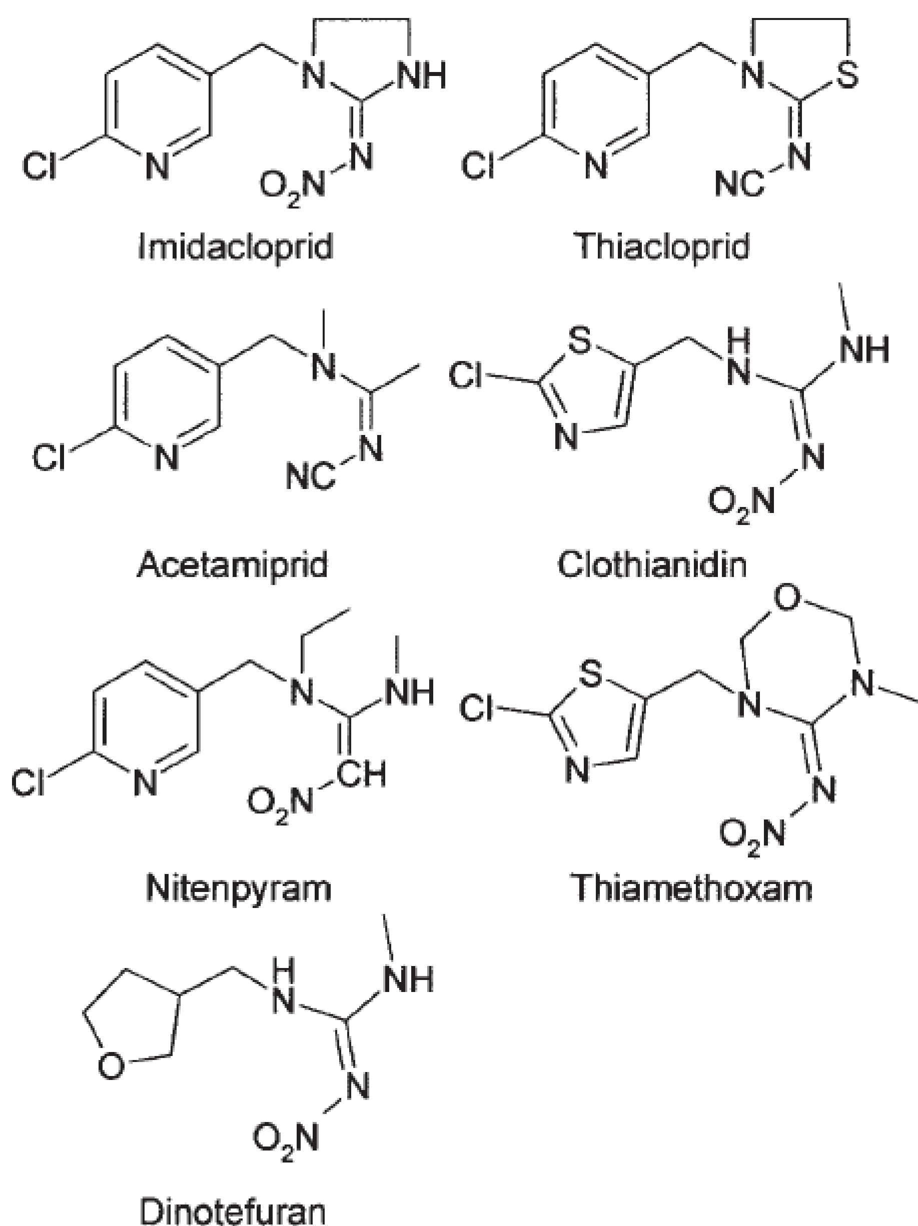


Figure 1. Neonicotinoids currently used as pesticides (figures adapted from Wikipedia)

Imidacloprid ($C_9H_{10}ClN_5O_2$; IUPAC Name: N-[1-[(6-chloropyridin-3-yl) methyl]-4,5-dihydroimidazol-2-yl] nitramide), the first commercially introduced neonicotinoid, became fast the most successful and best-selling insecticide worldwide (Mencke and Jeschke, 2002). Imidacloprid alone had an annual turnover on the insecticide market of over 1 $ billion in 2008 (2010 Bayer Annual Report, www.annualreport2010.Bayer.com). According to the pharmacophore classification they are N-nitroguanidines with a five-member ring system pharmacophore moiety (Jeschke *et al.*, 2001). The overall chemical structure of Imidacloprid consists of the bridging fragment [R1–R2] (figure 2a), CPM group (6-**C**hloro-**p**yridin-3-yl**m**ethyl) (figure 2a and 2b) and the functional group [=X–Y] (X-Y=N-NO_2) as part of the pharmacophore type [–N–C(E)=X–Y] (-N-C(NH)=N-NO_2) (figure 2a) (Jeschke, 2007; Nauen *et al.*, 2001). Comparative molecular field analysis (CoMFA) yielded a binding model for Imidacloprid in which a nitrogen of the CPM (6-chloro-pyridin-3-ylmethyl) (figure 2b) moiety interacts with a hydrogen donor of the *n*AChR. The nitrogen at position 1 of the imidazolidine ring is predicted to interact with the negatively charged basic residues (Okazawa *et al.*, 2000). Generally, the nitrogen-containing hetarylmethyl group as *N* substituent, such as CPM of Imidacloprid, has a strong influence on the insecticidal activity. Imidacloprid is effective on a wide range of targeted insects including sucking insects, beetles, lepidoptera, leafminers, some diptera, termites, locusts and fleas (Cloyd and Bethke, 2011). Some studies show that Imidacloprid is highly toxic for bees, and can harm them due to its presence in pollinated plants (Cresswell, 2011).

Figure 2a. Chemical structural segments of neonicotinoids (Imidacloprid).

R1–R2 bridging fragment; CPM - 6-**C**hloro-**p**yridin-3-yl**m**ethyl; –N–C(E)=X–Y stands for -N-C(NH)=N-NO_2 (modified after Jeschke and Nauen, 2008)

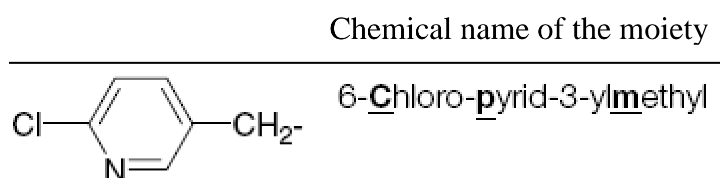


Figure 2b. Structural segment of Imidacloprid.

CPM - 6-**C**hloro-**p**yridin-3-yl**m**ethyl (modified after Jeschke and Nauen, 2008)

The extraordinary spectrum of target species, long-lasting effect, versatile uses and applications, and low cost of Imidacloprid have promoted its worldwide usage.

Although Imidacloprid is still an invaluable tool for managing some of the world's most destructive crop pests, sporadic cases of resistance to neonicotinoinds have been reported worldwide in the last 10 years (Jeschke and Nauen, 2008).

1.1.2. Mechanisms of insecticide resistance

During the last decades, extensive biochemical and molecular studies have been conducted to elucidate insecticide resistance mechanisms. Resistance against all insecticide groups is conferred by a limited number of mechanisms in all insects analyzed to date (IRAC). Often, resistant insects utilize more than one of these mechanisms at the same time.

There are four general insecticide resistance mechanisms in insects:

- Behavioural resistance
- Penetration resistance
- Metabolic resistance
- Altered target-site resistance

Metabolic and altered target-site resistance mechanisms are the major mechanisms of resistance in insects (Hemingway, 2000). The other two, behavioural and penetration resistance, appear to be an additional mechanisms.

Behavioural resistance

Behavioural resistance has been demonstrated in insects which modify their behaviour so that they avoid the insecticide treated areas. Although the evidence is controversial, some instances of specific avoidance behaviour in presence of insecticides have been documented for different insects (Rowland, 1991, Pluthero and Threlkeld, 1981, Sparks *et al.*, 1989). For example, certain behavioural characteristics that are different between resistant and susceptible *Anopheles gambiae* have been reported (Rowland, 1991). Also, avoidance of insecticide has been documented in field populations of *Drosophila*, but a strong correlation with resistance could not be established (Pluthero and Threlkeld, 1981). Behavioural resistance has been reported for several classes of insecticides, including organochlorines, organophosphates, carbamates and pyrethroids (IRAC).

Penetration resistance

Toxins can penetrate into insects through the cuticle, the respiratory system or the gut. Resistance to toxins, including insecticides, can occur when any of these entry routes is blocked. In general, penetration resistance develops when the insect outer cuticle slows down absorption of toxins. One of the classic examples is the *pen* gene in *Musca domestica,* which lowers the penetration rate of insecticides through the cuticle (Plapp and Hoyer, 1968). Reduced cuticular penetration is also documented for various insecticides in the tobacco budworm (*Heliothis virescens*) (Lanning *et al.*, 1996; Ottea *et al.*, 2000) the cotton bollworm (*Helicoverpa armigera*) (Ahmad *et al.*, 2006; Gunning *et al.*, 1994), and mosquitoes (*Aedes aegypti* and *Culex pipiens*) (Pan *et al.*, 2009; Matsumura and Brown, 1961; Shrivastava, *et al.*, 1970). High levels of resistance are, however, only seen in combination with another resistance mechanism (Sawicki, 1970).

Altered target-site resistance

Target site resistance is the second major mechanism of toxin resistance of insects. The reduction of toxicity of the chemical results from an alteration in the target molecule (binding site), making the insecticide less effective or even completely ineffective. The change of the target protein is caused by a mutation of the coding

gene, which lowers the protein-toxin binding affinity. Such target molecule modification has been identified as a main resistance mechanism in several cases, covering a wide range of species and types of chemicals (Mutero *et al.*, 1994; Vaughan *et al.*, 1997; Williamson *et al.*, 1996; Martin *et al.*, 2000; ffrench-Constant *et al.*, 2000). Resistance of this class has been found for nervous system targets (Oakeshott *et al.*, 2003), as well as for developmental targets (Ashok *et al.*, 1998; Wilson and Ashok, 1998). Nervous system targets of different insecticides include voltage-gated sodium channels, GABA receptors, acetylcholinesterase and nicotinic acetylcholine receptor (ffrench-Constant *et al.*, 2004). Developmental toxins against which resistance develops include juvenile hormone analogues (JHAs) such as methoprene (Met), which mimic endogenous hormones (Wilson and Ashok, 1998).

A well illustrated example of resistance caused by structural modifications of the insecticide binding site is a ‘‘knockdown’’ (*kdr*) resistance of the house fly to pyrethroids. These insecticides, like DDT, interact with sodium channel proteins, disrupting the gating kinetics of action potentials, resulting in rapid paralysis (termed ‘‘knockdown’’) and subsequent death (Soderlund and Knipple, 2003). Molecular analysis shows that different levels of resistance (*kdr*, *super-kdr*) occur due to different point mutations in the sodium channel gene (Williamson *et al.*, 1996; Soderlund and Knipple, 2003).

Acetylcholinesterase (AChE) is critical for hydrolysis of acetylcholine at cholinergic nerve synapses. Certain mutations in the AChE gene confer resistance to organophosphates and carbamates in many insects (Anthony *et al.*, 1995; Alout *et al.*, 2007).

Mutated GABA receptor (the inhibitory neurotransmission channel in insects) can become resistant to avermectins and cyclodiene (Bloomquist, 1994).

Neonicotinoid resistance is also due to target-site modification. In a laboratory-selected insect colony of *Nilaparvata lugens,* target-site modification (Y151S) of the two alpha subunits of the nicotinic acetlycholine receptor (nAChR) confers resistance (Liu *et al.*, 2005).

Juvenile hormone analogues (JHAs) can cause a hormonal imbalance, leading to insect death (Wilson *et al.*, 2006). A mutation in the Methoprene-tolerant (Met) bHLH-PAS gene in *Drosophila melanogaster* results in resistance to the toxic and morphogenetic effects of JHA and JHA-agonist insecticides, such as methoprene (Wilson *et al.*, 2006).

Target site resistance alone can lead to a high level of resistance in different laboratory and field populations (Oakeshott *et al.*, 2003).

Metabolic resistance

Metabolic resistance is based on detoxification of insecticides (or any other xenobiotics), which includes sequestration or active degradation of targeted molecules (Oakeshott *et al.*, 2003). Enhanced metabolism of the insecticide before it can affect its target is probably one of the most common types of resistance found in insects (Scott, 1991). Biochemical analysis has shown that three major gene families - esterases, glutathione-S-transferases and cytochrome P450 monooxygenases, alone or in combination, are involved in detoxification of insecticides (Hemingway, 2000). In most cases, enhanced transcription of coding genes leads to overexpression of these enzymes in resistant insects (Hemingway, 2000).

Xenobiotic metabolism is often divided into three phases: modification (phase I), conjugation (phase II) and excretion (phase III) (Xu *et al.*, 2005). Modification and conjugation involve metabolizing enzymes while phase III involves transporters, which are members of the ATP-binding transporter family (Xu *et al.*, 2005). Cytochrome P450 monooxygenases and esterases are phase I enzymes, while glutathione-S-transferases are phase II enzymes. Phase II enzymes often act in conjunction with phase I enzymes (Hemingway *et al.*, 1991). In phase I, P450s add a functional group (mostly a hydroxyl group) to the xenobiotic, and protein-protein interactions move the metabolite to the catalytic site of the transferase without releasing it from the protein complex. In phase II, the transferase catalyses the conjugation of a bulky substituent molecule, such as glutathione, to the functional group (Gibson and Skett, 2001). This cooperative metabolic detoxification system is more efficient than independent systems and therefore of great importance in insecticide resistance.

Esterases

The esterases are phase I enzymes that catalyse hydrolysis of a chemical bond. Many hydrolases are believed to use a two-step reaction mechanism based on a ‘‘catalytic triad”. An alcohol group of the substrate is released, forming a covalent linkage to the active site of the hydrolase. In the second step, cleavage of this linkage results in a hydrolysed compound (Ollis *et al.*, 1992; Oakeshott *et al.*, 1999). Based on substrate inhibitor specificity, esterases are classified into three groups: carboxylesterases (CE), arylesterases (ArE) and cholinesterase (ChE) (Yoo *et al.*, 1996).

The majority of resistance-conferring esterases are overexpressed through gene amplification (Devonshire and Field, 1991; Vaughan and Hemingway, 1995). Esterase gene amplification is well documented in resistant strains of the aphid *Myzus persicae*, the mosquitoes *Culex quinquefasciatus, C. pipiens, C. tarsalis* and *C. tritaeniorhynchus* and the brown planthopper (*Nilaparvata lugens*) (Karunaratne *et al.*, 1998; Mouches *et al.*, 1986; Field and Devonshire, 1998; Small and Hemingway, 2000). Single point mutations in structural genes can dramatically alter the substrate specificities of the enzyme. This is documented for the E3 malathion carboxylesterase from the sheep blow fly *Lucillia cuprina* (Campbell *et al.*, 1998) and the *Musca domestica* alpha E7 gene (Claudianos *et al.*, 1999). Resistance to malathion is caused by a single (Trp251-Leu) substitution in esterase E3, while a Gly139-Asp substitution in E3 confers broad spectrum cross-resistance to a range of organophosphates, excluding malathion, in the blow fly (Campbell *et al.*, 1998). In *M. domestica,* this Gly-Asp substitution is also found to cause resistance to organophosphates (Claudianos *et al.*, 1999). Although the main cause of esterase resistance is amplification of specific esterase genes, a few cases of esterase gene overexpression through a combination of gene upregulation and amplification have also been described (ffrench-Constant *et al.*, 2004; Paton *et al.*, 2000). In the peach-potato aphid *Myzus persicae*, gene amplification of esterase E4 is accompanied by DNA methylation, altering transcriptional gene regulation in the resistant line, such that the amount of protein relative to gene copy number is decreased (Field and Devonshire, 1998; Field *et al.*, 1999). *C. quinquefasciatus* mosquitoes show co-amplification of esterases α2 and β2 in a resistant strain, with α2 and β2 mRNA expression ratio of 10 : 1 respectfully. A protein level ratio for the α2 : β2 transcripts has been found to be 3

: 1. This indicates that the expression of these amplified genes in insecticide-resistant mosquitoes is regulated in both transcriptional and translational level (Paton *et al.*, 2000).

Glutathione-S-transferases (GST)

Transferases are a superfamily of detoxification enzymes, whose role is to conjugate glutathione, sulfuric acid, or glucuronic acid to exogenous hydrophilic substrates, facilitating their excretion (Gibson and Skett, 2001). Glutathione-S-transferases (GSTs) are the predominant large multifunctional group of the transferases superfamily, involved in the detoxification of a wide range of xenobiotics, including insecticides (Salinas and Wong, 1999). There are at least 25 groups of GST or GST-like proteins, with one major clade containing the currently recognized mammalian, arthropod, helminth, nematode and mollusc GST classes (Snyder and Maddison, 1997). Based on their gene structure and amino acid sequence, GSTs belong to two main groups, class I and class II. This classification does not extend to the substrate specificities, which are wide and varied. The class I insect GSTs are encoded by a multigene family in *Anopheles* mosquitoes, *D. melanogaster* and *Musca domestica* (Toung *et al.*,1993; Zhou and Syvanen, 1997; Ranson *et al* ., 2002). The class II insect GSTs, in contrast, is encoded by a single gene in all species studied to date (Beall *et al.*, 1992; Reiss and James, 1993; Snyder *et al.*, 1995).

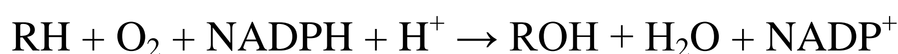
Elevated activity of one or more GST enzymes has been associated with resistance to all major insecticide classes (Prapanthadara *et al.*, 1993; Huang *et al.*, 1998; Vontas *et al.*, 2001). In resistant flies, increased GST levels are in most cases caused by an increased transcriptional rate rather than gene amplification or qualitative change of individual enzymes (Grant and Hammock, 1992; Ranson *et al.*, 2001).

Glutathione-S-transferases are responsible for many cases of organophosphate resistance in different insect species (Hayes and Wolf, 1988; Huang *et al.*, 1998; Wei *et al.*, 2001; Rodríguez *et al.*, 2010). Although there is no direct evidence of involvement of GSTs in the metabolism of pyrethroid insecticides, some reports suggest that GSTs may play an important role in resistance also to this insecticide class (Vontas *et al.*, 2001; Vontas *et al.*, 2002). On the other hand, several GSTs that are overexpressed in DDT-resistant strains of *Anopheles gambiae* were shown to be

able to metabolise DDT (Ranson *et al.*, 2001; Ortelli *et al.*, 2003). In many cases, overexpression of one or more GSTs in resistant lines appears to be controlled by a mutation in a trans-acting regulator (Grant and Hammock, 1992; Ranson *et al.*, 2000).

Cytochrome P450 monooxygenases

Cytochrome P450 monooxygenases are phase I metabolic enzymes and are important for the detoxification of a vast variety of xenobiotics, including insecticides (Scott and Kasai, 2004). These enzymes also play a crucial role in regulation of the levels of endogenous compounds such as hormones, pheromones, fatty acids and steroids (Scott, 1999). Cytochrome P450 monooxygenases have been found in virtually all aerobic organisms, including insects, plants, mammals, birds and bacteria (Stegeman and Livingstone, 1998). There are at least 70 families, with 127 subfamilies, of P450 monooxygenases genes in different organisms (Scott, 1999). Insect genomes alone contain from 46 to over 150 P450 genes, each encoding a different P450 enzyme (Feyereisen, 2006; Nelson, 2009). Due to the presence of numerous P450s in each species, as well as the broad substrate specificity, cytochrome P450 monooxygenases have an immense capacity for metabolizing different substrates (Scott and Wen, 2001). The most common reaction catalyzed by cytochromes P450 is a monooxygenase reaction. One atom of oxygen (O_2) is incorporated into an organic substrate (RH) while the other atom is reduced to water (Scott and Wen, 2001):



$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

Cytochrome P450 monooxygenases can be divided into four classes, depending on how electrons are delivered to the catalytic site from NADPH. Class I enzymes require both an FAD-containing reductase and an iron sulphur redoxin, while class II enzymes require only FAD/FMN-containing P450 reductases. Class III proteins are self-sufficient (require no electrons) and class IV proteins receive electrons directly from NADPH (Werck-Reichhart and Feyereisen, 2000).

The insect cytochrome P450 monooxygenases are multifunctional enzymes involved in growth, development, feeding, insecticide resistance and tolerance to plant toxins (Scott, 1999). Several authors suggest that resistance mediated by P450 monooxygenases could be the most frequent type of metabolism based insecticide

resistance (Scott, 1999). Upregulated transcription of one or more P450 genes appears to be the general molecular mechanism which increases levels of the enzymes in resistant individuals (Scott, 1999; Karunker *et al.*, 2008; Karunker *et al.*, 2009; Daborn *et al.*, 2002). An exception has recently been reported for a resistant *Myzus persicae* (Puinean *et al.*, 2010) strain. Here, amplification of a P450 monooxygenase gene causes overexpression of the enzyme.

The analysis of P450-dependent resistance is made complicated by the variable expression of individual P450s, as well as the wide range of tissues in which they are expressed (Chung *et al.*, 2009; Giraudo *et al.*, 2010). The highest monooxygenase activities are usually associated with the midgut, fat bodies and Malpighian tubules (Hodgson, 1983). Recently research in *Drosophila* focuses increasingly on P450 expression patterns in flies, whose resistance is mainly monooxygenase-dependent (Giraudo *et al.*, 2010; Chung *et al.*, 2009).

1.2. Genome-wide insertional mutagenesis

The completion of whole genome sequencing projects has provided the full complement of genes of many organisms. One of the main goals of modern genetics is to link the thousands of sequenced genes of model organisms to their function. The function and interactions of most of these genes in different biological phenomena, including insecticide resistance, however, remains largely unknown. In analyzed resistant *Drosophila* flies this is, in part, due to the fact that flies derived from field populations with preexisting genetic variations, which are not easy to characterize. The generation of the mutations in a defined genomic background in laboratory insect lines should simplify the characterization of insecticide resistance factors.

One of the most powerful techniques for genetic and functional genomic analysis is mutagenesis with mobile elements. This technique can achieve disruption, overexpression or mis-expression of single genes. One of the main advantages of insertional mutagenesis over the classical method of chemical mutagenesis is the ease which the targeted gene can be identified, since it carries the transposon as a tag.

Insertional mutagenesis using transposable elements has been an exceptionally efficient method to create mutants in various organisms (Ivics and Izsvák, 2010). Many transposons like the P-element, *mariner*, *hobo*, *piggyBac*, *Hermes* and *Minos* have been used successfully in insects for this purpose (Adams and Sekelsky, 2002; Pavlopoulos *et al.*, 2007).

1.3. Transposable elements

Transposable elements (TEs) are DNA sequences that have the capacity to change their genomic locations by excision and insertion into new loci. They are widely distributed in living organisms in both prokaryotes and eukaryotes (Ling and Cordaux, 2010). TEs are divided into two main classes, according to their structural organization and mechanism of transposition (Finnegan, 1989; Capy *et al.*, 1997). Class I elements encoding a reverse transcriptase (RT) and employ an RNA-mediated mode of transposition, using a copy and paste mechanism of transposition. Class II elements use a DNA-mediated cut and paste mode of transposition.

1.3.1. The transposable element *Minos*

The transposon *Minos* has been identified as a repetitive element in the genome of the fruit fly *Drosophila hydei* (Franz and Savakis, 1991). The element is approximately 1,8 Kb long with 254 base pair (bp) identical inverted terminal repeats flanking a single gene encoding a transposase (figure 3). The *Minos* transposase gene consists of two exons interrupted by a 60 bp long intron (Franz and Savakis, 1991). The *Minos* element (Class II) is a member of the Tc1/*mariner* superfamily of eukaryotic transposons. The insertion of *Minos*, like that of the other Tc1/*mariner* elements (Plasterk *et al.*, 1999), occurs into a TA dinucleotide. The *Minos* transposase catalyzes excision and re-integration of the element, which leaves 6 bp long footprints without excision of flanking DNA (Loukeris *et al.*, 1995a; Arca *et al.*, 1997).

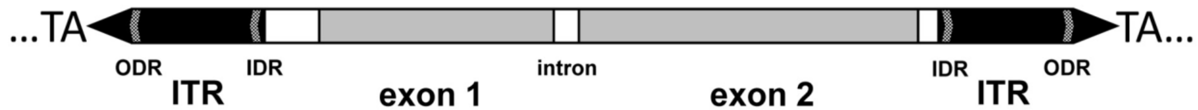


Figure 3. Structure of the *Minos* element isolated from *Drosophila hydei*.
The transposase gene is interrupted by a 60 base pair long intron. Not all features are drawn to scale. IDR: inner direct repeat, ODR: outer direct repeat, ITR: inverted terminal repeat: TA duplicated target dinucleotide (modified after Pavlopoulos *et al.*, 2007)

Minos has been shown to create stable insertions in germ line chromosomes of embryos of several insect species and ascidians (Loukeris *et al.*, 1995a; Loukeris *et al.*, 1995b; Catteruccia *et al.*, 2000a; Catteruccia *et al.*, 2000b; Shimizu *et al.*, 2000; Sasakura *et al.*, 2003; Pavlopoulos *et al.*, 2004). Also, it is active in cultured insect and mammalian cells, as well as in somatic and germ cells of mice (Pavlopoulos *et al.*, 2007; Klinakis *et al.*, 2000a; Klinakis *et al.*, 2000b Zagoraiou *et al.*, 2001; Drabek *et al.*, 2003).

The wide range of host organisms in which *Minos* is active makes it a versatile tool for screens of very different genetic model systems. The fact that transposition produces stable transformants with high efficiency (Kapetanaki *et al.*, 2002), allowing genome-wide mutagenesis in insects (Metaxakis *et al.*, 2005) and mammalian cells (Klinakis *et al.*, 2000a) makes *Minos* a versatile transgenesis tool (Bellen *et al.*, 2011).

1.4. *Drosophila* as a model organism

A model organism can be defined as “a species that is extensively studied to understand particular biological phenomena, with the expectation that discoveries made in this organism will also provide insight into the workings of other organisms” (Fields and Johnston, 2005). *Drosophila melanogaster* has been one of most commonly used model organisms in biology for the last 100 years (Morgan, 1915; Beckingham *et al.*, 2005.). It has some classical advantages like the small number of chromosomes, rapid life cycle and easy rearing and maintenance. Availability of a vast array of mutant stocks and genetic tools (Bloomington, 2010), highly detailed

cytological maps of polytene chromosomes (Pardue, 1986) and a large body of well described protocols for genetic and molecular analysis (Sullivan *et al.*, 2000) are additional, more recent advantages. The full genome sequence (Adams *et al.*, 2000; Tweedie *et al.*, 2009) and the availability of large numbers of cDNA clones for microarrays (White *et al.*, 1999) makes *Drosophila* an excellent model organism for genomic research.

Insecticides are primarily used to target pest species, but in many cases non-targeted field populations, like *Drosophila*, are affected too. *Drosophila melanogaster* has been proposed as a model organism for insecticide resistance research in the late 1980s (Wilson 1988). Utilization of comprehensive and refined methods for resistance mechanism analysis in *Drosophila* in most cases is not possible in other non-targeted insects (Wilson, 2001).

Although not a pest species, *Drosophila melanogaster* has been lately increasingly used as a model organism for toxicology and insecticide resistance studies (Giraudo *et al.*, 2010; Perry *et al.*, 2011), due to the many molecular and genomic tools available for this insect.

1.5. Insecticide resistance in *Drosophila*

Examinations of laboratory and field populations of *Drosophila* show that this species can develop resistance to a broad range of insecticides (Feyereisen, 1995; Wilson, 2001; Hemingway *et al.*, 2002). These insecticides belong to different target site classes, including neurotoxins and moulting and metamorphosis disruptors (Willoughby *et al.*, 2006).

Of the four major insecticide resistance mechanisms, metabolic and target-site resistance have been detected in various *Drosophila* populations (Wilson, 2005). Although behavioural and penetration resistance are suggested as additional resistance mechanisms (Wilson, 2001), there is no firm experimental evidence for these mechanisms in *Drosophila*.

Target site mechanisms have been described in different resistant *Drosophila* flies for insecticides acting on different targets including GABA receptor, chloride and sodium channels and acetylcholine receptor (Wilson, 2005).

Reports for *Drosophila* show a positive correlation between resistance to different insecticides and overexpression of one or more cytochrome P450, glutathione–S-transferase and esterase genes (Maitra *et al.*, 2000; Brandt *et al.*, 2002; Campbell *et al.*, 2003; Pedra *et al.*, 2004; Festucci-Buselli *et al.*, 2005; Le Goff *et al.*, 2006; Bhaskara *et al.*, 2006; Willoughby *et al.*, 2006). Biochemical and molecular analyses of DDT resistant *Drosophila* lines showed that at least 4 genes from the cytochrome P450 monooxygenases family are involved in resistance to DDT (Maitra *et al.*, 1996, Festucci-Buselli, *et al.*, 2005, Pedra *et al.*, 2004).

1.5.1. Cytochrome P450-mediated resistance in *Drosophila*

The correlation between overexpressed individual P450 genes and resistance to different insecticides has been analyzed (Le Goff *et al.*, 2003; Daborn *et al.*, 2007) with various transgenesis techniques (Venken and Bellen, 2005). Resistant DDT transgenic flies, over-expressing Cyp6g1, showed cross-resistance to three different neonicotinoids (Imidacloprid, Acetamiprid and Nitenpyram) (Le Goff *et al.*, 2003). Also, an increased survival rate on Nitenpyram and Diazinon was found for flies overexpressing Cyp6g2 (Daborn *et al.*, 2007). A low level of DDT resistance was detected in transformed *Drosophila* overexpressing Cyp6g1, as well as in flies overexpressing Cyp12d1 (Daborn *et al.*, 2007).

The midgut, Malphighian tubules and fat body are the major sites of cytochrome P450-mediated detoxification in insects (Hodgson, 1985; Scott and Lee, 1993). Resistant *Drosophila* carrying a fragment of the *Accord* transposable element located upstream of Cyp6g1 show tissue specific expression of this gene, localized in gastric cecum, midgut, Malpighian tubules, and fat body (Chung *et al.*, 2007). Moreover, it has been shown that expression of the Cyp6g1 in Malpighian tubules is critical for conferring DDT resistance in *Drosophila* (Yang *et al.*, 2007). Spatial expression

analysis of P450 genes shows that tissue specific expression is critical in determining the toxicodynamics of insecticides that are metabolized by P450 enzymes (Giraudo *et al.*, 2010).

In mammals, the regulation of cytochrome P450 genes involved in xenobiotic detoxification is very well understood (Xu *et al.*, 2005, Pavek and Dvorak, 2008). In insects on the other hand, while the cytochrome P450 monooxygenases enzyme family has been associated with insecticide resistance, the role of individual enzymes, as well as the regulation of their genes, is largely unknown (Giraudo *et al.*, 2010). Functional analysis of the cis-acting control elements of genes Cyp6g1 and Cyp6a8 indicates that transcriptional regulation of insect P450 genes is different from that of P450 genes in mammals (Morra *et al.*, 2010). Experiments on resistant insects suggest that mutations in cis and/or trans acting regulators activate detoxification mechanisms (Giraudo *et al.*, 2010).

1.6. Deep sequencing

Whole genome sequencing, combined with adequate annotation, will identify a nearly-complete set of genes of a species. It will not, however, provide on its own information about levels of expression for any gene. To acquire this information, genome-wide transcription profiling is a powerful approach, which can shed light on transcriptome variants and gene interaction networks. A fast developing and promising tool for the generation of genome-wide transcriptional profiles of individual strains is high-throughput deep sequencing (Lister *et al.*, 2009). This method is classified according to the genome annotation constraints in the family of "open" technologies (Green *et al.*, 2001). In contrast to "closed" technologies like microarrays, "open" technologies transcriptome analysis does not require biological or sequence information of the analyzed organism. This technology is very suitable for discovering new transcribed sequences, as well as sequences that are not well studied (Hanriot *et al.*, 2008). Also it gives information about new variations of the genes and confirmation of newly discovered genes. The method enables rapid parallel

sequencing of large cDNA libraries with several millions of tags. Genome-wide transcription profiling as a final result gives a complete genome transcriptome footprint of differently expressed sequences in the analyzed organism or tissue.

1.7. Aims of the project:

In this project, *Drosophila melanogaster*, one of the best characterized model organisms in biology, is used for the analysis of insecticide resistance. The work presented in this thesis is divided in two parts.

In the first part, a genome-wide mutagenesis screen of *Drosophila* with the *Minos* based TREP transposon was used as a proof of principle for the TREP promoter-delivery element, a promising new genomic tool.

The second part is the analysis of a resistant *Drosophila* mutant retrieved from this screen with genetic, biochemical and molecular tools, in order to further characterize the resistance mechanism. Transcriptomic footprint analysis revealed expression patterns and gene groups that could be involved in the mechanism of insecticide resistance.

2. MATERIALS AND METHODS

2.1. *Drosophila melanogaster* strains and lines

D. melanogaster stocks were maintained on standard cornmeal-agar-yeast medium at 24°C under a 12-hour light/12-hour dark cycle. The following *Drosophila* strains were used: TREP 2.30 (Kiupakis, Oehler and Savakis, manuscript in preparation), BOEtTA 6.24 (Koukidou *et al.*, 2006) and iso31 [SM6, MiT 2.4]/Sco (Metaxakis *et al.*, 2005), Bloomington deletion kit lines, as well as SM6, y,w; ci^{94}/ey^{D}, iso31, and strains 5906 and 5907. All strains, except strains SM6 and y,w; ci^{94}/ey^{D}, are isogenic for all chromosomes and were obtained from the Bloomington Drosophila Center, Ind, USA (Bloomington, 2010). Strain SM6 (stock collection IMBB-FoRTH) is a balancer strain carrying a Curly marker gene on the second chromosome. Line ci^{94}/ey^{D} (stock collection IMBB-FoRTH) carries an eyeless marker gene on the fourth chromosome. Line iso31 (Ryder *et al.*, 2004) is a standard strain isogenic for chromosomes X, 2 and 3 (w^{1118}). Strains 5907 and 5906 are balancer strains derived from iso31. Strain 5907 (Ryder *et al.*, 2004) is isogenic for chromosomes X and 3, with the Curly marker gene on the second balancer chromosome ($w^{1118}{}_{iso}$/Dp(1;Y)y^{+}; noc^{Sco}/SM6a). Strain 5906 (Ryder *et al.*, 2004) is isogenic for chromosome X and 2, with the Stubble marker gene on the third balancer chromosome (w^{1118}/Dp(1;Y)y^{+}; TM2/TM6C, Sb^{1}).

Line iso31 [SM6, MiT 2.4]/Sco carries the Curly marker and the *Minos* transposase gene on the second balancer chromosome. This way, *Minos* transposase gene located on the second chromosome can be easily traced by following the *Cy* phenotype.

TREP 2.30 is a homozygous line (TREP 2.30/TREP 2.30) carrying a *Minos* based TREP (**t**etracycline **r**egulatable **e**nhancer **p**romoter) transposon element inserted into the 4th chromosome (Kiupakis, personal communication). The TREP construct carries a minimal *hsp70* promoter under the control of *tetO* element (figure 4). In the presence of tTA transcriptional activator protein, the TREP transposon directs ectopic overexpression of the next gene downstream of the minimal *hsp70* promoter. As a transformation marker, this element carries a *mini-white* gene (*w^{+})*, which confers a red eyes phenotype in a *white* background (figure 4).

Line BOEtTA 6.24 carries the P-element based transposon BOEtTA located on the sex (X) chromosome. This construct together with the TREP transposon element forms a promoter delivery system. The TREP-BOEtTA promoter delivery system is depicted in figure 4. The BOEtTA transposon element carries a tTA gene (source of tTA-transcriptional activator). As a transformation marker, it has an *egfp* (enhanced green fluorescent protein) gene which confers green eyes to the flies under UV illumination. Also, it carries a *mini-white* marker gene (w^+), which is nonfunctional in this line. A specific feature of the TREP 2.30 line is lethality of the flies, in the presence of the BOEtTA 6.24 construct. Thus, in the presence of the BOEtTA construct, the only viable flies will be the ones with TREP excision events.

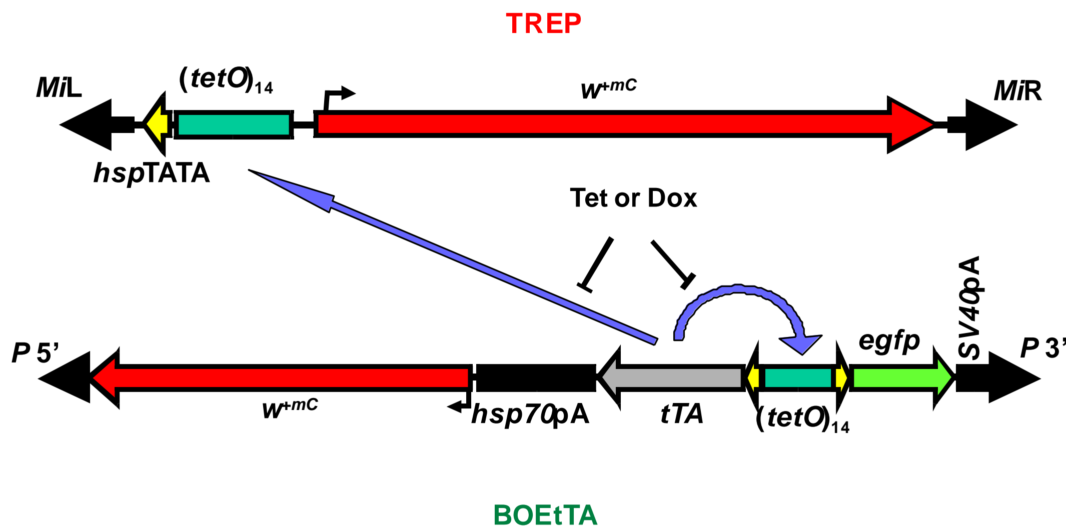


Figure 4. Schematic of the TREP and BOEtTA constructs and the activation of the TREP-borne minimal promoter.

2.2. 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 susceptible line iso31 were set up. Individual larvae produced in these crosses were microscopically analyzed for the presence of aberrations of all 5 polytene chromosomes (X, 2L, 2R, 3L and 3R).

2.3. Toxicological analysis

2.3.1. Lethal concentration (LC50) analysis

2.3.1.a. Determination of the lethal concentration 50 (LC50) for Imidacloprid and DDT

Resistance was measured by comparing LC50 values, which represent the lethal concentration of an insecticide that kills 50% of treated individuals. TREP 2.30 (line with the initial TREP insertion) and iso31 (used as a *D. melanogaster* insecticide-susceptible strain) flies, together with MIT[w^-]3R2 resistant flies were tested for Imidacloprid and DDT LC50's. The lethality of different concentrations of Imidacloprid was tested by analyzing egg to adult viability of the flies. Flies were massed-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. Egg to adult viability was analyzed by counting the number of the emerged flies for each concentration of Imidacloprid. For DDT analysis, 3 days post-eclosion males and females were used in a contact assay. DDT was coated to the inside of 35 ml glass vials 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 used, with 100 flies per concentration in total. For both Imidacloprid and DDT assays, the control mortality in the absence of insecticide was determined and corrected for.

2.3.1.b. Exposure to piperonyl butoxide (PBO)

A quantity of 2 µl of PBO (95%, SIGMA-ALDRICH) was added to 200 µl of acetone and immediately transferred to 35 ml glass vial. Each vial was rolled horizontally, until the acetone was evaporated. The controls were prepared the same way, omitting the PBO. Twenty flies (10 males and 10 females) were transferred to each vial and left for 3 hours prior to 48h Imidacloprid exposure.

2.3.1.c. LC50 calculation and construction of dose-response curves

For both insecticides (Imidacloprid and DDT), as well as the PBO assay, flies were tested on at least, 4 different concentrations plus control. The LC50 values were calculated with computer program SPSS 16.0 using the regression probit 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.

2.3.1.d. Insecticides and PBO

Bioassays were carried out with active ingredients diluted in acetone. Imidacloprid (98.7%) was kindly provided by Bayer CropScience GmbH- Germany, while DDT (4,4' – DDT PESTANAL®) and PBO (95%) were purchased from SIGMA-ALDRICH Laborchemikalien GmbH.

2.3.1.e. Paraquat assay

Two to three days old resistant mutant and iso31 flies were collected. Ten males and ten females from each line were placed into vials with different concentrations of paraquat (SIGMA-ALDRICH, PESTANAL®, analytical standard), including a control lacking paraquat. Two replicas for each concentration and control were set up. Paraquat was applied to a paper filter disc mixed with a 1% sucrose solution, and put in plastic vials. To each paper filter (1.5 cm diameter) disc, 1 ml of paraquat in 1% sucrose was applied. In the control, 1 ml of 1% sucrose without additive was applied. Three different concentrations of paraquat, 5%, 10% and 12.8% were used. The mortality was scored after 24 hours.

2.3.2. Biochemical assays

All protocols were used as previously described in Roditakis *et al.* (2009), except for P450 activity in live larvae. This was done according to the protocol described in Inceoglu *et al.* (2009). Activity of cytochrome P450 dependent monooxygenases was determined in adult microsomes and in live larvae. Heads of the 3-5 days old males and females were cut on ice, and abdomens (30 flies per sample) were used for microsome extraction. Third instar larvae were used for the P450 activity assay. For the esterase and glutathione S-transferase activity assays, 3-5 days old males and females were used. For all assays, activity was measured at 25˚C on microplate reader SpectraMax M2 with software SoftMax prov5 (Molecular Devices, Sunnyvale, CA).

2.4. Molecular analysis

2.4.1. Standard PCRs

2.4.1.a. Preparation of genomic DNA

Genomic DNA was extracted using a modified BDGP (http://www.fruitfly.org/about/methods/inverse.pcr.html) protocol (Bellen *et al.*, 2004). Adult flies (3-5 days old) were collected, pooled and transferred to 1.5 ml Eppendorf tubes (~15 flies per Eppendorf tube). To each tube, 400 µl of Buffer A (1 M Tris.HCl-pH7.5, 500 mM EDTA-pH8.0, 4 M NaCl, 10% SDS) were added and flies were homogenized on ice, using plastic grinders. Tubes with homogenized flies were incubated for 30 min at 65ºC. In the next step, 800 µl of LiCl/KAc solution was added, tubes were inverted several times to mix and incubated for 10 min on ice. After incubation, tubes were centrifuged at 14,000 rpm for 15 min at room temperature. 1 ml of supernatant was transferred to new 2 ml Eppendorf tubes (leaving floating solids behind) supplemented with 800 µl of isopropanol (MERCK), and tubes were inverted several times to mix. Tubes were then spun at 14,000 rpm for 10 min at room temperature. Following centrifugation, the supernatant was discarded.

The pellet was washed with 500 µl of ice-cold 70% ethanol (MERCK). Tubes were spun at 14,000 rpm for 5 min at room temperature, and the supernatant was discarded. The pellet was air dried for ~15 min and resuspended in 75 µl TE overnight at 4ºC. An aliquot of 1µl from each sample was analyzed on a 1% agarose-gel, as well as with a nanodrop analyzer. Samples were stored at -20 ºC.

2.4.1.b. PCR reactions for detection of mini white gene, Minos transposase gene and adjacent mini white and Minos transposase genes

All reactions were performed in 25µl volumes and repeated in order to confirm results. For each sample, 200 ng of template DNA was used. To a mixture of 5 µl of template DNA, 2.5 µl of 10 x PCR Buffer (Minotech) with 15mM of $MgCl_2$, 1 µl of PCR primers (25 pmole) and 2.5 µl of dNTP mix (2 mM) was added. Then, 1 unit of Taq Polymerase (Minotech) was added. Mixture was heated to 94ºC for 3 min. Thirty-five cycles of PCR amplification followed (denaturation for 30 sec at 94ºC, annealing for 30 sec at 58ºC and extension for 3 min at 72ºC). After the 35th cycle, the mixture was incubated for 5 min at 72ºC. For the *Minos* transposase gene, the following primers were used: forward 5'-CGATGGTTCGTGGTAAACCT-3' and reverse 5'-AACTCGTTTTGGCATTGAGC-3' with the expected 1037 bp product size. For the mini-*white* gene, forward 5'-ATGACCTTTCAAAACGTCTTTGC-3' and reverse 5'-AGCTTTTTGAGGGGGCAATA-3' primers with the expected 803 bp product size. For adjacent *Minos* transposase and mini-*white* genes forward 5'-ATGACCTTTCAAAACGTCTTTGC-3' and reverse 5'-GCTTAAGAGATAAGAAAAAAGTGACC-3' with the expected 1348 bp product size. The PCR amplification was done on a MJ Research PTC-200 machine, and PCR products were analyzed on 1 % agarose gels.

2.4.2. Semi-quantitative and quantitative real time PCRs for the gene analysis of relative mRNA expression in resistant and susceptible lines

2.4.2.a. RNA extraction

Total RNA from *Drosophila melanogaster* flies was extracted using a standard Trizol RNA isolation protocol (http://quantgen.med.yale.edu/). Adult flies (3 days old) were

anesthetized, transferred to 1.5 ml Eppendorf tubes (~40 flies per tube), flash frozen in liquid nitrogen and stored at -80 °C. In the next step, samples were thawed on ice and 500 µl of Trizol was added. Samples were carefully homogenized on ice for 30-60 sec, using plastic grinders. Depending of the amount of material, up to 500 µl of Trizol more was added. Eppendorf tubes with the homogenizate were centrifuged at 13,000 rpm for 10 min at 4ºC to pellet debris. After centrifugation, 200 µl of chloroform was added, tubes were shaken vigorously for 15 sec. and incubated at room temperature for 2-3 min. Tubes were then centrifuged at 13,000 rpm for 15 min at 4ºC. The upper phase (~0.6 ml) was carefully removed to a new RNAse-free tube. After that, 0.7 volumes of isopropanol (~0.5 ml) were added to each tube to precipitate RNA. Tubes were incubated for 1 hour at -20ºC and then centrifuged at 13,000 rpm for 15 min at 4ºC. The supernatant was discarded, and the RNA pellet was washed with 1 ml of 70% ethanol/DEPC-treated MilliQ water. Tubes were centrifuged at 13,000 rpm for 10 min at 4ºC. After a second centrifugation, the supernatant was removed, and the tubes were briefly centrifuged again. The last of the supernatant was removed carefully with a micropipette. The pellet was air dried for ~10 min. In the last step, the pellet was resuspended in an appropriate volume of DEPC MilliQ water (20 to 50 µl). An aliquot from each sample was analyzed on a 1% agarose-gel. DNAse I treatment was done following the protocol of the RNAqueous®-Micro instruction manual. To 10 µl of total RNA, 5 µl of 10 x DNase I Buffer, 1 µl of DNase I and 34 µl of RNAse free water were added and mixed gently. The DNase reaction was performed at 37ºC for 20 min. After incubation, DNase I was inactivated by adding 5.6 µl of resuspended DNase inactivation reagent. The reaction was stored for 2 min at room temperature, vortexing once during this interval to disperse the DNase Inactivation reagent. The reaction was centrifuged for 1.5 min at maximum speed, and the total RNA was transferred to a fresh RNAse free tube and stored at -20 ºC. The quality of the RNA samples was verified with standard quality control/assessment protocols. Synthesis of the First-Strand cDNA was done following the AccuScript® High Fidelity RT-PCR System protocol. For each sample, 1.5 µg of total RNA was used for synthesis of the first-strand cDNA. The cDNA reaction was set up by mixing 4.4 µl of RNAse free water, 1.0 µl of 10 x AccuScript RT Buffer, 0.9 µl of oligo(dT) primers, 1.0 µl of dNTP mix (10mM each dNTP) and 1.0 µl of total RNA preparation. The reaction was incubated at 65ºC for 5 min and cooled to

room temperature to allow primers to anneal to the RNA (approximately 5 min). After this step, 1.0 µl of 100mM DDT and 1.0 µl of AccuScript RT were added to the reaction. The total reaction volume was 10 µl. Tubes were placed in a temperature-controlled thermal block at 42ºC, and reactions were incubated for 30 min. The completed first-strand cDNA synthesis reactions were stored at -20ºC.

2.4.2.b. Semi-quantitative PCR

Relative mRNA expression of genes Cyp6g1, Cyp6a2, Cyp6a8 and Cyp12d1 was measured between resistant and susceptible lines reared on standard medium and medium with Imidacloprid. Relative expression of each Cyp gene was measured in reference to the housekeeping ribosomal protein gene Rp49. Flies from susceptible line were maintained on medium with 0.4 µg/ml of Imidacloprid, and resistant flies were maintained on 3 µg/ml of Imidacloprid. The PCR reactions were performed on a MJ Research PTC 200 Peltier Thermal Cycler machine. For this purpose, 5 sets of primers were designed. In order to obtain specific cDNA products, primers were designed to span exon-intron junctions. For Cyp6g1: forward 5'-ACCCTTATGCAGGAGATTG-3' and reverse 5'-TAGGCTGTTAGCACGAATG-3' primers, with an expected product size of 159 bp. Cyp6a2: forward 5'-GTTACTGCCTGTATGAGTTGG-3' primer and reverse primer 5'-TAGAGCCTCAGGGTTTCTG-3', with an expected product size of 160 bp. Cyp6a8: forward 5'-CCTTTGTGTTCTTCATTGCT-3' and reverse 5'-GTTTCATCTAAAACCTGATTGA-3' primers, with an expected product size of 196 bp. Cyp12d1: forward 5'-AAGGATTGGTGGCTTCAC-3' and reverse 5'-GTAAAATCTTCGGGGACTTC-3' primers, with an expected product size of 184 bp. Primers for the control housekeeping ribosomal protein gene Rp49: 5'-CGGTTACGGATCGAACAAGCG-3' and reverse 5'-TTGGCGCGCTCGACAATCT-3', with an expected product size of 174 bp. For each sample, two biological replicates were analyzed with two technical repetitions. Reaction mixtures for the Cyp genes were as follows. 1 µl of cDNA reaction, 5 µl of 10xPCR Minotech Buffer with 15 mM of $MgCl_2$ (for Cyp12d1, the $MgCl_2$ concentration was increased to 3 mM), 1 µl PCR primers (25 pmole) and 1 µl dNTP mix (10 mM), 2 units of Taq polymerase (Minotech) (0.5µl) and 41.5 µl of dd H_2O. The total volume of the reaction mixture was 50 µl. The reaction mixture for Rp49 was the same, except that 20 pmoles of

primers were used. Cycling conditions were: 95ºC for 5 min, then 40 cycles of 95ºC for 30 sec, 56ºC for 30 sec and 72ºC for 30 sec. PCR products were analyzed on 2 % agarose gels every 5th cycle, between the 20th and the 40th cycle.

2.4.2.c. Quantitative real time RT-PCR

All samples and primers used in the semi-quantitative PCR analysis were also used for quantitative real time RT-PCR analysis. Quantitative real time RT-PCRs were performed using the QIAGEN SYBR green kit on the DNA Engine Opticon TM MJ Research analyzer. Standard samples for each gene were made from RT-PCR products isolated from 2.5% agarose-gels. The efficiency of RT-PCR amplification for each gene-specific primer pair was analyzed with five serial dilutions in three technical replicates. Cycling conditions were: 94°C for 5 min, then 37 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec (plate reading at 78°C, 80°C and 82°C). Data were analyzed with the MJ Opticon Monitor 3.1 analysis software. Calculation was done with software REST-MCS (Pfaffl and Horgan, 2001). Additionally, relative expression of the Cyp4p2 in the resistant line was analyzed. Quantitative real time PCR for Cyp4p2 was performed using same protocols as for other Cyp genes analyzed. Flies maintained for more than 25 generations on standard medium after deep sequencing and Cyp6g1, Cyp6a2 and Cyp6a8 expression analysis, were used for Cyp4p2 expression analysis. The forward and reverse primer sequences were as follows: Cyp4p2 - 5' CTGAAAAGGCATCCTTACGC 3' and 5' TTGGGATCGATAACAGGCAG 3'. Quantitative real time PCR was performed on the Bio-Rad CFX analyzer with cycling conditions: 95°C for 2 min, then 35 cycles of 95°C for 15 sec, 55°C for 30 sec and 60°C for 30 sec (melt curve 60 to 95 C, increment 1.0 C).

2.4.2.d Quantitative real time RT-PCRs for the analysis of gene amplification in the resistant line

Genomic DNA from 3 days old *Drosophila melanogaster* flies was extracted using the DNAzol® Reagent protocol. Pooled DNA samples were extracted for the analysis of genes Cyp4p2, Cyp6g1 and Cyp6a2. Three biological samples (10 males and 10 females per sample) were prepared for each line. Amplification of all the genes was measured relative to the housekeeping ribosomal protein gene Rp49. For this purpose,

four sets of primers were designed. Rp49: forward primer 5'-CGGTTACGGATCGAACAAGCG-3' and reverse primer 5'-TTGGCGCGCTCGACAATCT-3' with an expected product size of 174 bp. Cyp4p2: forward primer 5'-GGCCATACTTGTGGTCATCC-3' and reverse primer 5'-TGATCATGGGCACTAAGCTG-3', with an expected product size of 125 bp. Cyp6g1: forward primer 5'-GCCTTCGAAGCCTCACTATG-3' and reverse primer 5'-TCTGCATCTCTGGATGCTTG-3', with an expected product size of 140 bp. Cyp6a2: forward primer 5'-AGCACCTGTTCAACCTGGAC-3', reverse primer 5'-GCCATCAGCTCCTTGATCTC-3', with an expected product size of 193 bp.

Each experiment was performed on three biological replicates, with three technical replicates each. Quantitative real time RT-PCRs were performed using the GoTaq® qPCR Master Mix kit (Promega) on a Bio-Rad CFX analyzer. For each sample, 200 ng of genomic DNA were used. Cycling conditions were: 94°C for 3 min, then 35 cycles of 94°C for 30 sec, 60°C for 20 sec and 72°C for 3 min (plate reading at 78°C, 80°C and 82°C). For each technical triplicate, the average and the standard deviation of the individual efficiencies were calculated. Technical triplicates with a ratio between average and the standard deviation higher than 0.03 were excluded from further data analysis. The mean RT-PCR efficiency per amplicon and the Cq value per sample were used to calculate a starting concentration per sample, expressed in arbitrary fluorescence units. Analysis of data was performed with the LinRegPCR quantitative SYBR Green qPCR software (Ruijter *et al.*, 2009).

2.5. Deep sequencing analysis

Total RNA was extracted as in the previous mRNA expression real time RT-PCR analysis experiments. Preparation of cDNA for sequencing was done according to the Illumina mRNA Seq V2 protocol (Illumina, Inc, 2010). Formation of single molecule arrays, cluster growth and sequencing was all done according to the standard protocols of Illumina, Inc. Sequencing was performed on a 2008 Illumina Genome Analyzer, version 2 (GA2).

This method involves several steps that are designed to convert total RNA into a library of template molecules suitable for high throughput DNA sequencing (Seq V2 protocol, Illumina, Inc., 2010). The first step involves purifying the poly-A containing mRNA molecules using poly-T oligo-attached magnetic beads. Following purification, the mRNA is fragmented into small pieces, using divalent cations at elevated temperature. Then, the cleaved RNA fragments are copied into first strand cDNA, using reverse transcriptase and a high concentration of random hexamer primers. This is followed by second strand cDNA synthesis using DNA Polymerase I and RNaseH. These cDNA fragments then go through an end-repair process, where the overhangs are converted into blunt ends using T4 DNA polymerase and Klenow DNA polymerase. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs, and the polymerase activity fills in the 5'-overhangs. After that, DNA fragments are prepared for ligation to the adapters by adding a single ‘A’ nucleotide to the 3'-end of the blunt phosphorylated DNA fragments, using the polymerase activity of the Klenow fragment (3' to 5' exo minus). The adapters, which have a single ‘T’ base overhang at their 3' end, will ligate to the ends of the DNA fragments, preparing them to be hybridized to a flow cell. Ligated products are then purified on a gel selecting a size range of templates for following PCR. Next step is PCR amplification of the cDNA in the cDNA library. The PCR is performed with two primers that anneal to the ends of the adapters. In the next step, quality control analysis on the sample library is performed to check the size, purity, and DNA concentration of the sample. Finally, the library is prepared for sequencing on the Illumina Genome Analyzer (Seq V2 protocol, Illumina, Inc., 2010).

Mapping of the 51 nt long sequencing reads of both lines, MiT[w^-]3R2 and iso31, to the reference genome (*Drosophila* release 5 sequence assembly Flybase) (Tweedie *et al.*, 2009) was performed with the RMAP (version 2.05) software (Smith *et al.*, 2008). Genes with 10 or less reads for one line and 50 reads or less for the other line were excluded from further analysis. The minimum difference threshold between lines was set to 2-fold. Analysis of upregulated and downregulated genes was performed with the DAVID 6.7 BETA release bioinformatics resources (Huang *et al.*, 2009a; Huang *et al.*, 2009b).

Also, a comparison of single nucleotide polymorphisms (SNP) of the deep sequence data between resistant MiT[w$^-$]3R2 and susceptible iso31 line for all chromosomes was obtained. Genomic SNP analysis of the pooled assembly of the resistant and the susceptible strains reads have been done with Gigabayes SNP discovery algorithm (improved PolyBayes algorithm version (Marth *et al.*, 1999)) and MOSAIC algorithm (Gonzalez *et al.*, 2011) using all Refseq mRNA transcripts of the dm3 assembly (Pruitt *et al.*, 2009) as a reference. A polymorphism probability threshold of 0.9 is used, with alleles requiring a minimal overall coverage of 10 and of 5 for the minor allele. A SNP density track with the number of SNPs in 1000nt (1Kb) tiling windows have been created. The SNP density was visualized with the UCSC Genome Browser on *D. melanogaster* release 5 (http://genome.ucsc.edu/) (Kent *et al.*, 2002) and presented for each chromosome (X, 2L, 2R, 3L, 3R and 4).

An *in silico* search of overrepresented transcription factor binding sites, using the JASPAR CORE Incesta database (Wasserman and Sandelin, 2004), was conducted. All upregulated and downregulated genes in the resistant line were analyzed for the presence of common transcription factor binding sites. The same was done for the subset of upregulated and downregulated Cyp genes separately, as well as all for Cyp genes irrespective of regulation. The sequences of all genes were retrieved from Flybase (*Drosophila* release 5 sequence assembly) (Tweedie *et al.*, 2009). For each gene, the upstream region of 3kb and the downstream region of 1kb, as well as the 3'UTR region sequences, were retrieved and analyzed.

A survey of predicted targets of microRNA in the 3'UTR of all upregulated and downregulated genes, as well as in the subsets of upregulated and downregulated CyP genes was performed with DIANA-microT (version 3.0) (Maragkakis *et al.*, 2009).

Also, we compared differently expressed Cyp gene sequences of resistant and susceptible line retrieved from deep sequencing analysis for nucleotide differences. Comparison of the DNA sequences and translation to amino acids was done with the software Ape (Davis, 2003).

3. RESULTS

3.1. *Minos*-based genome-wide insertional mutagenesis

3.1.1. The TREP element

The *Minos*-based TREP element (figure 4, Materials and methods) was used for genome-wide insertional mutagenesis of the *Drosophila melanogaster* genome. Estimation of new TREP insertions generated during the screen is based on the insertional efficiency and the percentage of local jumps of the TREP element. Insertion efficiency and percentage of local jumps of the TREP element in line TREP 2.30 were determined for this purpose.

3.1.1.a. The TREP element in TREP 2.30 line shows high integration efficiency

TREP line 2.30 carries a TREP element insertion on the 4th chromosome. The mobilization of the TREP construct and generation of flies with new insertions was performed with a standard “jumpstarter” system (Cooley *et al.*, 1988). In order to test the mobilization efficiency of the TREP construct, a procedure was adapted to utilize the specific features of the TREP 2.30 insertion in the presence of BOEtTA 6.24, hence two groups of crosses were set up. The first (Control group) and the second group (Jumpstarter group) of crosses are depicted in figures 5 and 6 respectively. In the Control group, crosses were set up to confirm lethality of the TREP 2.30 in the presence of BOEtTA, e.g. the TREP construct was not mobilized. In the Jumpstarter group, the TREP 2.30 construct located on the 4th chromosome (original insertion in the TREP 2.30 line) was mobilized. Transposition frequency was scored by counting the number of emerging flies with new jumps in the presence of BOEtTA. In the Control group, virgin TREP 2.30 female flies (red eyes phenotype) were mass-crossed with SM6 (standard balancer line without *Minos* transposase gene, carrying a *Cy* marker on the second balancer chromosome) males. Embryos were heat shocked every day on 37°C for an hour, until first pupae appeared. Red-eyed (original TREP 2.30 insertion), *Cy* virgin female progeny were selected and individually crossed with BOEtTA 6.24 male flies (carriers of the tTA source). All the female progeny from this cross carry the original TREP insertion in chromosome 4 and are not expected to develop into adults in the presence of BOEtTA element (figure 5). In the Jumpstarter

group, the crossing procedure was equivalent, except that the virgin TREP 2.30 female flies were mass-crossed with iso31 [SM6, MiT 2.4]/Sco (*Cy* marker on the second balancer chromosome with a heat shock-inducible *Minos* transposase source within the same inversion) males. In the Jumpstarter group, all the viable red eyes (TREP) females in the presence of the BOEtTA element are expected to be flies with new TREP insertions (figure 6).

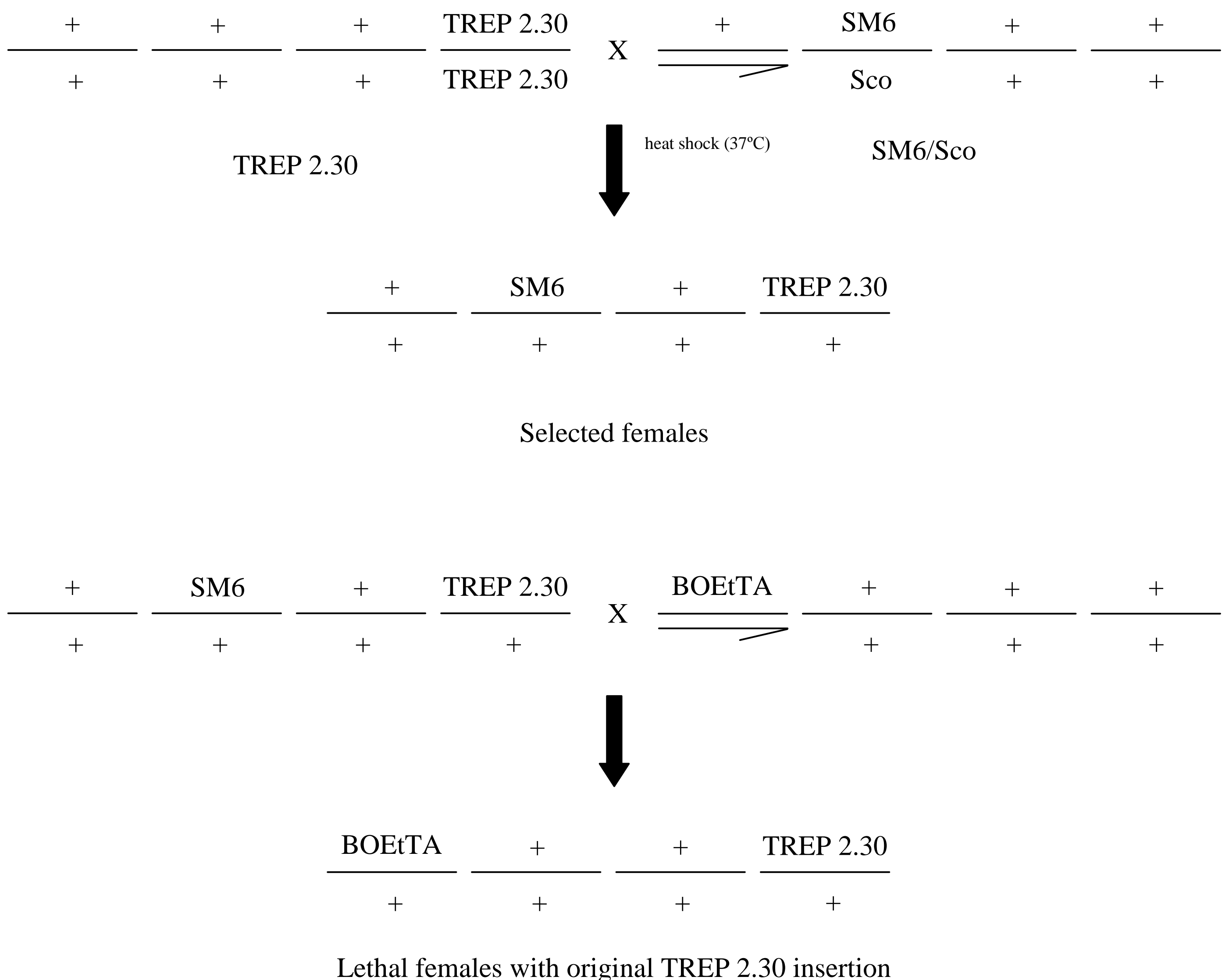


Figure 5. Crossing scheme of the Control group

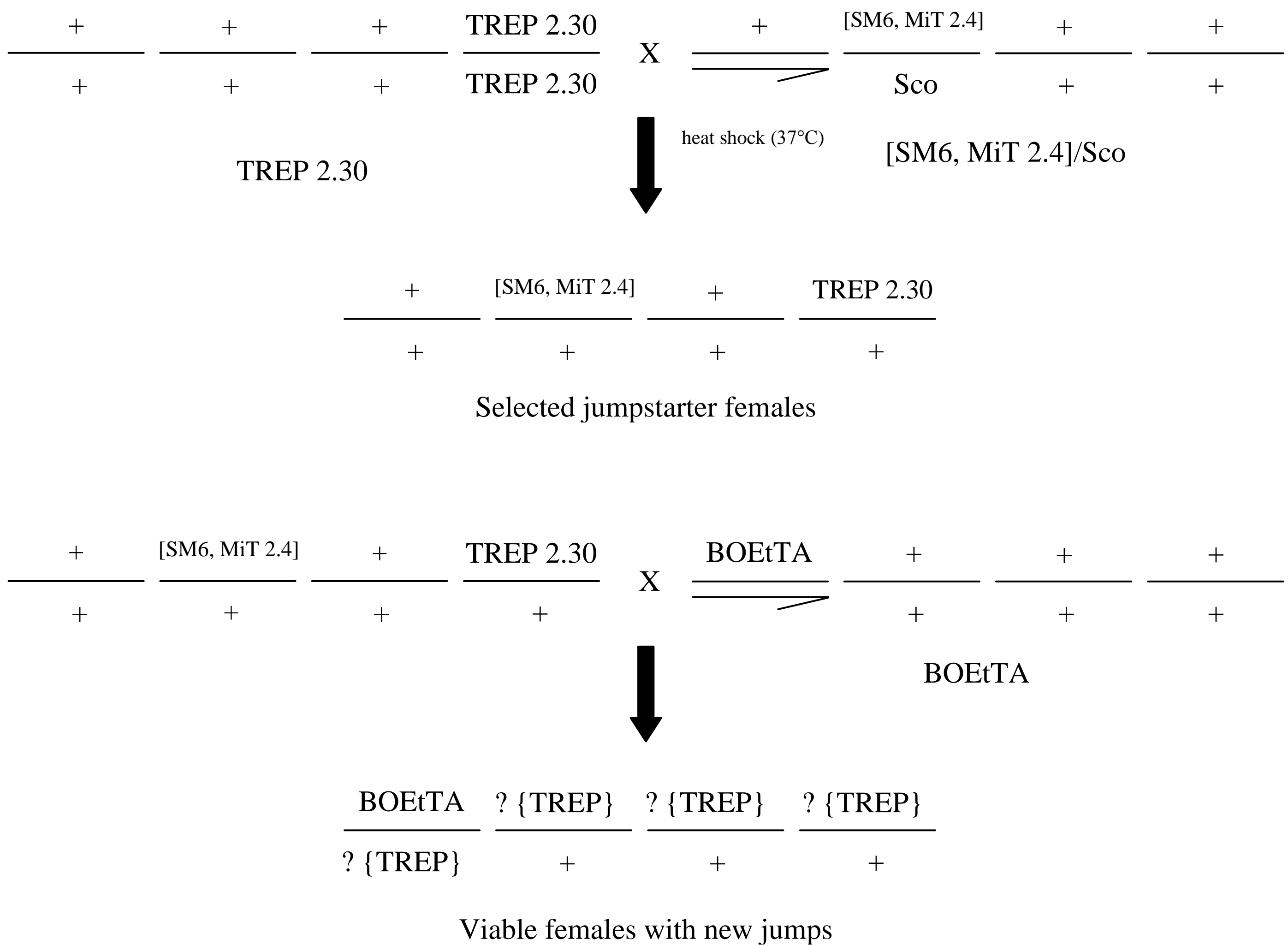


Figure 6. Crossing scheme of the Jumpstarter group

Legend for figures 5 and 6:
TREP 2.30 – minimal promoter, enhancer trap, w^+ marker
BOEtTA – tTA source, *egfp* marker
[SM6, MiT 2.4]/Sco - *Minos* transposase source, *Cy* marker
SM6/Sco – no transposase source, *Cy* marker

Progeny from two groups of crosses, a Jumpstarter and a Control group, was analyzed for the presence of new TREP insertions. The total number of flies analyzed for the presence of new insertions in the Control and the Jumpstarter group is given in table 2.

In the Control group, the progeny derived from 23 TREP females was checked for the presence of new TREP insertions. In total, 5520 male and female progeny was

analyzed in this group. Progeny derived from 49 TREP/BOEtTA 6.24 females in the Jumpstarter group was also analyzed. In total, 13710 male and female progeny was checked for the presence of new TREP insertions in this group (table 2).

The presence of the BOEtTA element is necessary for the selection of new TREP insertions, hence only female progeny was analyzed. The results from the Control and Jumpstarter group analysis are given in table 3. In total, 1600 survived female progeny was analyzed for the presence of transpositions in the Control group. All of 1600 surviving female progeny analyzed was without TREP element (table 3). In the Control group, female progeny carrying both the original TREP insertion and the BOEtTA 6.24 construct were not viable. In the Jumpstarter group, 4193 female progeny, derived from 49 TREP/Transposase females, was analyzed for the presence of new insertions. At least 100 female progeny from each of the 49 individual TREP/Transposase females were analyzed for the presence of red eyes (e.g. presence of new TREP insertions). Detection of at least one red eyes female progeny indicates transposition of the TREP element in the germ line of analyzed TREP/Transposase female. Viable female progeny with new TREP insertions were detected in 45 out of 49 TREP/Transposase females analyzed (table 3). Transposition efficiency (TE) was calculated as the percentage of the TREP/Transposase females with new TREP insertions in the total number of TREP/Transposase females analyzed. Percent of the gametes with transposition event is calculated from the formula (B/(B+2A))*100, where A represents total number of female progeny without transposition while B is total number of female progeny with transposition.

Table 2. Number of analyzed flies in the Control and Jumpstarter groups

	Total analyzed	Total number of male and female progeny
Control group (TREP females)	23	5520
Jumpstarter group (TREP/Transposase females)	49	13710

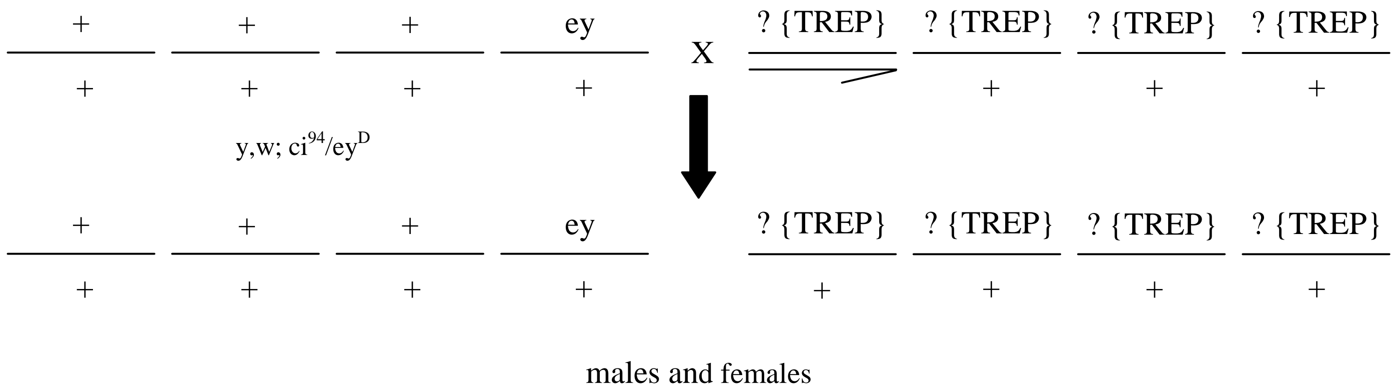
Table 3. Transposition efficiency (TE) of the TREP element of line TREP 2.30 in the Control and Jumpstarter groups

	New insertion in progeny	No new insertion in progeny	TE (%)	Female progeny without transposition	Female progeny with transposition	Total	Gametes with transposition (%)
Control group (TREP females)	0	23	0	1600	0	1600	0
Jumpstarter group (TREP/Transposase females)	45	4	~92	3962	231	4193	2.83

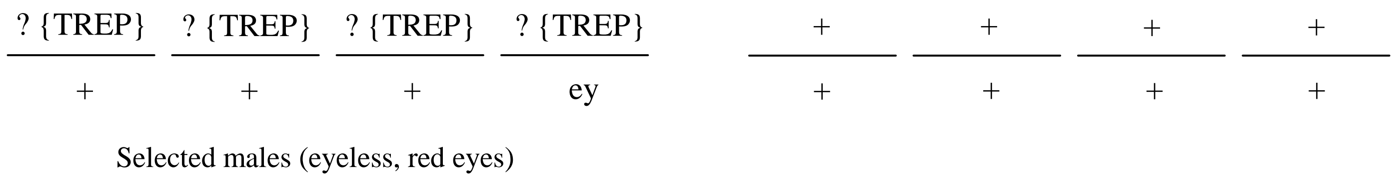
The summarized results of the analysis show that the TREP element of line TREP 2.30 has a transposition frequency of around 92 percent. These results also show that TREP/BOEtTA 6.24 females have on average one new transposition event in 2.8 percent of the gametes (table 3). In addition, lethality of the TREP 2.30 in the presence of BOEtTA is also confirmed.

3.1.1.b. One third of total jumps of the TREP 2.30 element are jumps on the 4th chromosome (local jumps)

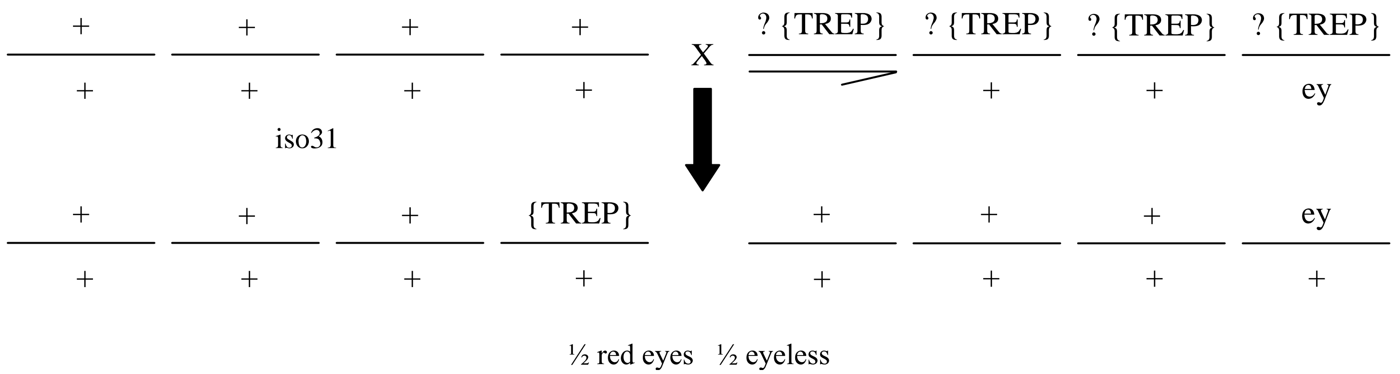
Following the transposition efficiency analysis, the frequency of local jumps of the TREP 2.30 element was analyzed. Males with new insertions, each selected from individual Jumpstarter group crosses, were individually crossed with virgin y,w; ci^{94}/ey^{D} females, carrying an eyeless marker on the 4th chromosome (stock collection IMBB-FoRTH). Eyeless, red eyed male progeny from this cross were selected and crossed with iso31 virgin female flies. New insertions of the TREP 2.30 element on the 4th chromosome were determined by analyzing the phenotype ratios of the progeny as presented in figures 7 and 8.



males and females



Selected males (eyeless, red eyes)



½ red eyes ½ eyeless

Figure 7. Jump on the 4th chromosome

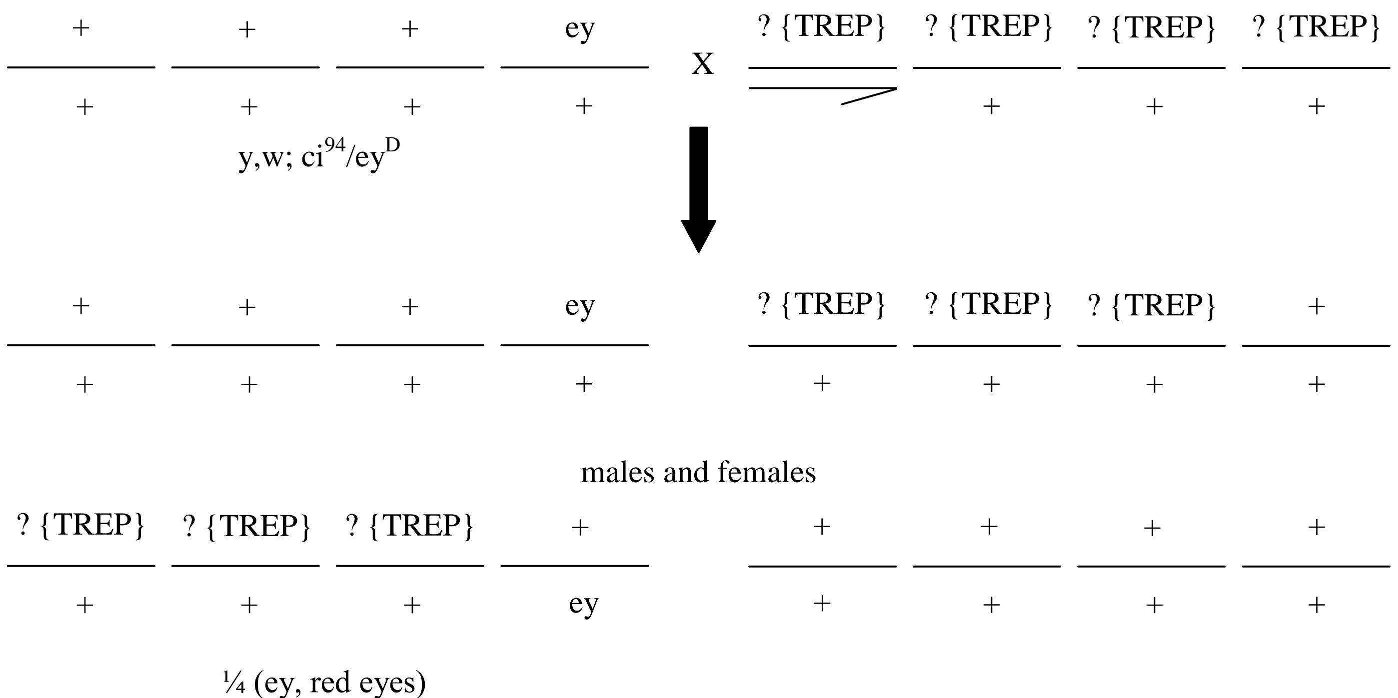


Figure 8. Jump on the sex, second or third chromosome

Male progeny from 34 TREP/Transposase females were used for determination of the frequency of local jumps (table 4). Insertion on the 4th chromosome was detected in eleven out of thirty-four male progeny. Thus, about one third of all insertions were insertions on the 4th chromosome (local jumps) (table 4).

Table 4. Distribution of jumps of the TREP 2.30 element on the 4th and the other three chromosomes of *D. melanogaster*

	Total	Local jumps	X, 2nd and 3rd chromosome	Local jump (%)	X, 2nd and 3rd chromosome (%)
TREP/Transposase females	34	11	23	32.35	67.65

Legend:

Total – total number of TREP/Transposase females of the Jumpstarter group
Local jumps – number of TREP/Transposase females with jumps of the TREP 2.30 element on the 4th chromosome (local jumps)
X, 2nd and 3rd chromosome – number of TREP/Transposase females with jumps of the TREP 2.30 element on the X, 2nd and 3rd chromosome
Local jumps (%) – percentage of local jumps of TREP/Transposase females
X, 2nd and 3rd chromosome (%) – percentage of jumps of the TREP 2.30 element on the X, 2nd and 3rd chromosome in TREP/Transposase females

Overall results show that about 32 percent of the jumps were local ones, with 2/3 of jumps on the other three chromosomes (X, 2nd and 3rd) (table 4).

3.1.2. *Minos*-based genome-wide insertional mutagenesis to identify genes involved in insecticide resistance

Transgenic *Drosophila* flies generated during the insertional mutagenesis will be selected on specific Imidacloprid concentration. The insecticide concentration used in the genome-wide screen has to be selected to be toxic enough to prevent a high number of escapers, but not so toxic as to prevent the survival of transgenics exhibiting resistance. Thus, Imidacloprid lethal concentration for the lines that would be used in this screen had to be determined.

3.1.2.a. Imidacloprid lethal concentration shows approximately same values for all Drosophila lines used in insertional mutagenesis

The iso31 (used as *D. melanogaster* insecticide-susceptible line) flies were tested for their susceptibility to Imidacloprid. The Imidacloprid lethality was tested by analyzing *Drosophila* egg to adult viability. The crossing scheme is given in figure 9. Flies were massed-crossed. They were placed into fly cages, allowing females to lay eggs on the cherry juice medium. Eggs were collected within 24 hours and placed into vials (50 eggs per vial) containing medium with different Imidacloprid concentrations. Flies were tested on two concentration ranges. The first range included concentrations of 5 µg/ml, 1 µg/ml, 0.2 µg/ml, 0.04 µg/ml, 0.008 µg/ml, 0.00016 µg/ml and 0.00032 µg/ml. The second range included concentrations of 1.5 µg/ml, 1 µg/ml, 0.5 µg/ml and 0.25 µg/ml of Imidacloprid. For each concentration, 8 replicas were set up, hence the total number of eggs was 400 per concentration. Egg to adult viability was analyzed by counting the number of emerged flies for each concentration of Imidacloprid. For this analysis, the control mortality in the absence of insecticide was determined and taken into account.

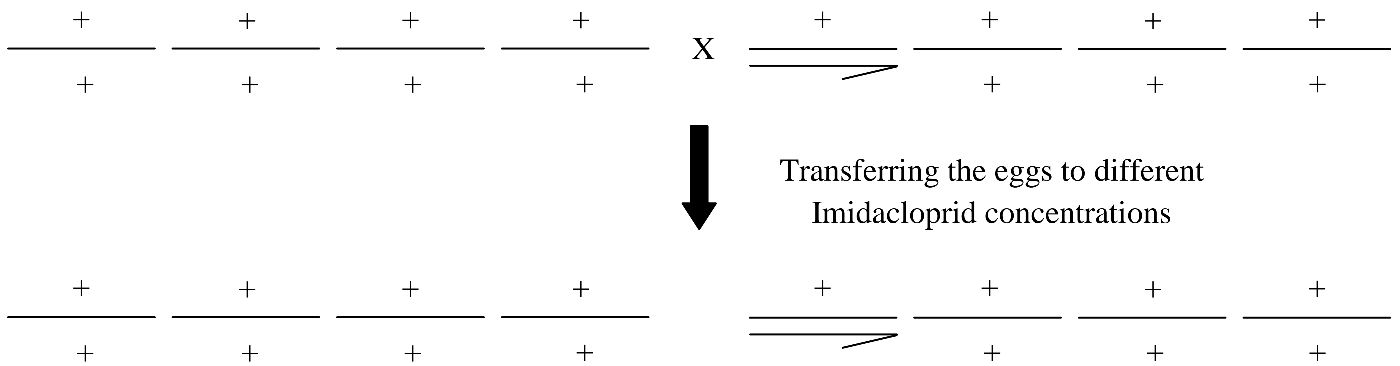


Figure 9. Crossing scheme for lethality testing of iso31 flies

It was determined that about 1 µg/ml is the threshold lethal Imidacloprid concentration for iso31 (figures 10 and 11). Three other lines (TREP 2.30, BOEtTA 6.24 and iso31[SM6,MiT2.4]/Sco), which were used for the insertional mutagenesis were also tested for lethality, by putting flies directly on medium with 1 µg/ml of Imidacloprid.

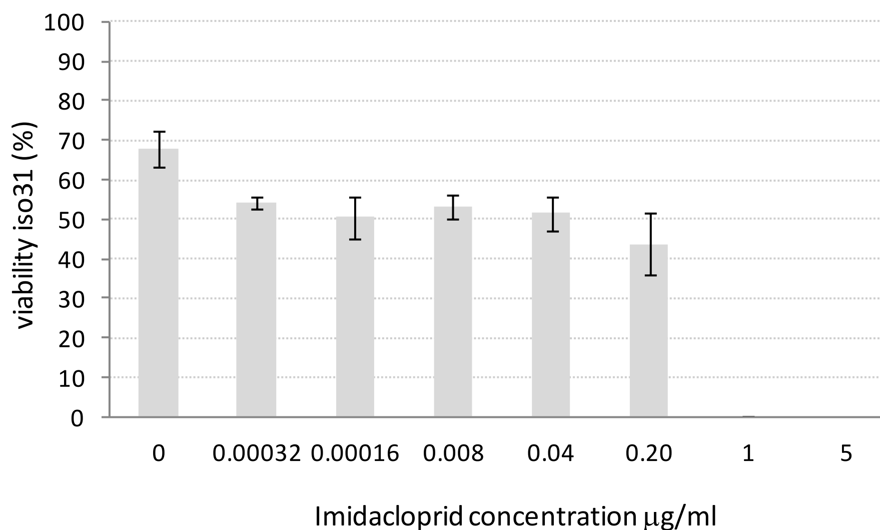


Figure 10. Survival of iso31 flies on food with the indicated Imidacloprid concentrations

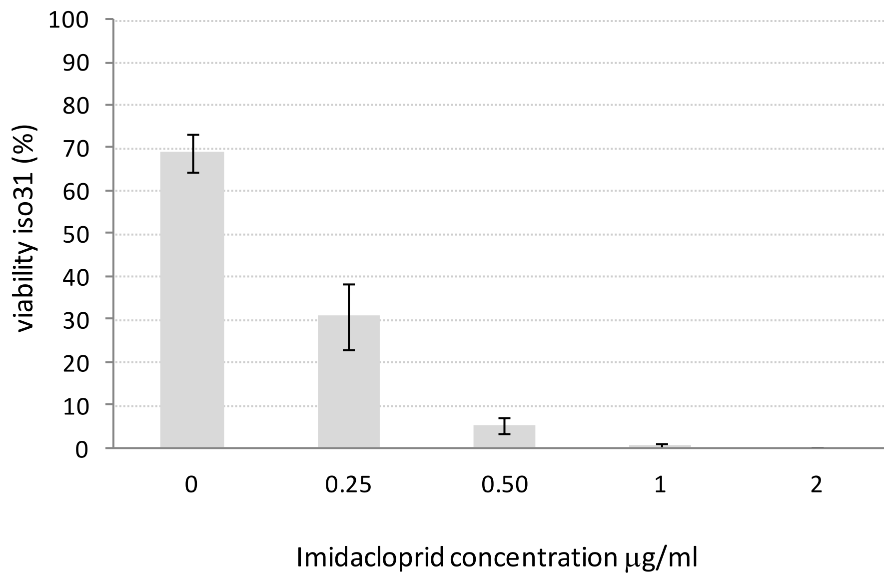


Figure 11. Survival of the iso31 flies on food with the indicated Imidacloprid concentrations.

Summarized results show that except from iso31, 1 μg/ml of Imidacloprid is also lethal for TREP 2.30, BOEtTA 6.24 and iso31[SM6,MiT2.4]/Sco lines.

3.1.2.b. Minos-based genome-wide insertional mutagenesis screen

Three lines (TREP 2.30, BOEtTA 6.24 and iso31[SM6,MiT2.4]/Sco) analyzed for the Imidacloprid LC50 were used for genome-wide insertional mutagenesis. The crossing scheme of the genome-wide insertional mutagenesis system is given in figure 12. "Jumpstarter" flies were generated by crossing twenty virgin TREP 2.30 females with ten iso31 [SM6, MiT 2.4]/Sco males (source of *Minos* transposase). Flies were placed into vials and females were left to lay eggs on standard *Drosophila* medium. Virgin jumpstarter females (red eyes, *Cy*) from this cross were selected, mass-crossed with BOEtTA 6.24 (source of tTA) males and left to lay eggs on cherry juice medium. To each cage, 100 virgin jumpstarter females were put together with 50 BOEtTA 6.24 males. Within 24 hours, eggs were collected and transferred (~300 eggs per vial) on medium with 3μl/ml of Imidacloprid. This concentration of Imidacloprid (3 times higher than the LC99 of the susceptible line) was carefully selected to be toxic enough to prevent high number of escapers, but not so toxic as to prevent survival of mutants

exhibiting higher resistance levels. Surviving TREP female offspring were tested for their level of resistance to Imidacloprid.

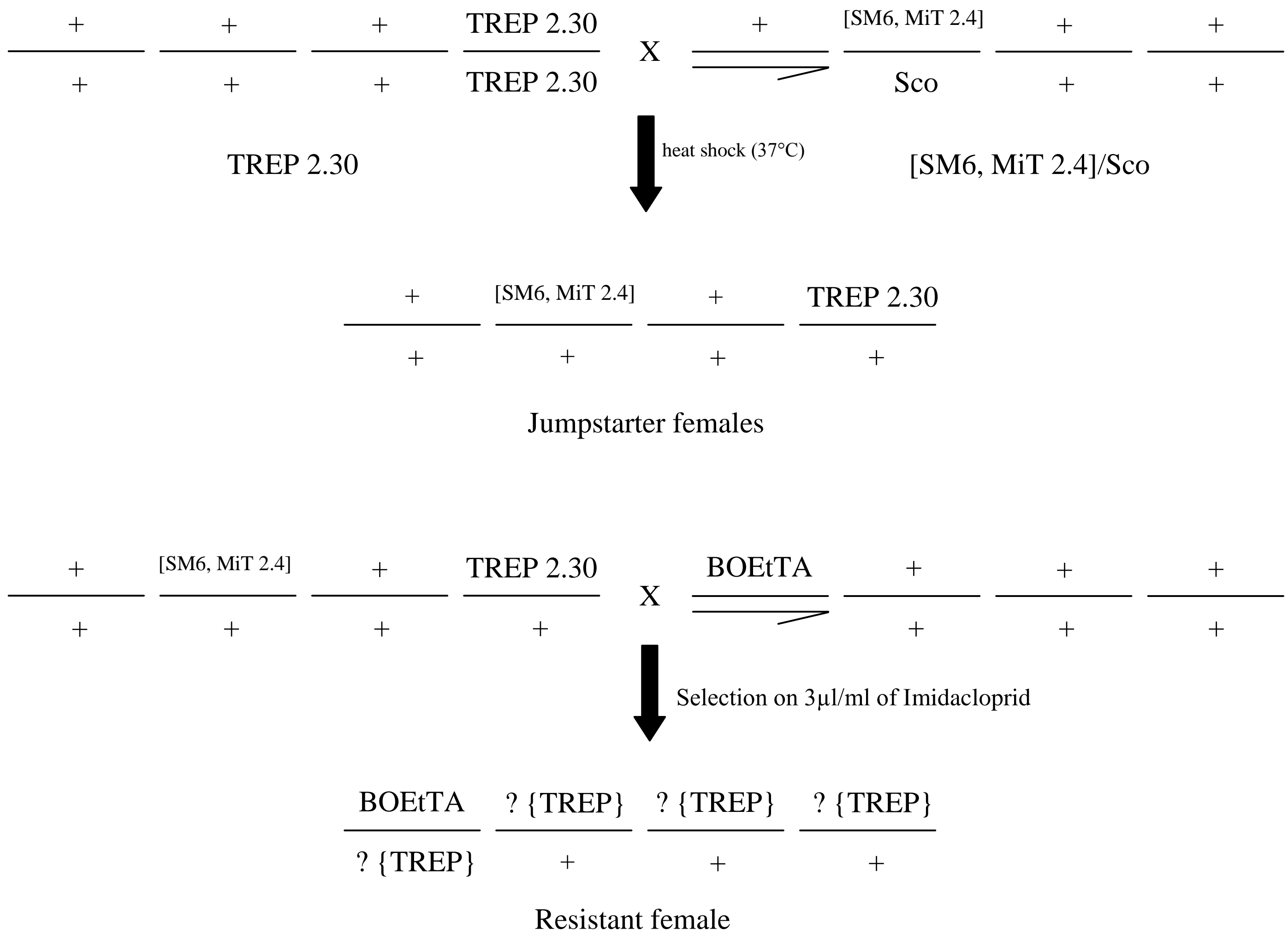


Figure 12. Crossing scheme of the genome-wide insertional mutagenesis system

During the genome-wide insertional mutagenesis, about 12900 new TREP insertions were generated (table 5). Insertional sites analysis has shown that 47% of total *Minos* insertions were found to be within or close to (2 kb upstream) known or predicted genes (Metaxakis *et al.*, 2005). Hence, during this screen 6063 insertions (47% of 12900 new TREP insertions) are expected to be within or close to (2 kb upstream) known or predicted genes including introns. Excluding introns, 3767 insertions (29.2% of 12900 new TREP insertions) are expected to be in known or predicted genes. Increased number of insertions in genome-wide insertional mutagenesis calls

for correction of estimation with respect to multiple insertions into the same genes (Pollock and Larkin, 2004). The analysis of transposition events has shown that *Minos* insertions into the *D. melanogaster* genome can be considered random (Metaxakis *et al.*, 2005). The Poisson distribution has been used for the multiple insertions into the same genes correction (Pollock and Larkin, 2004). It is expected, calculated with the Poisson distribution that 26% of 6063 insertions will hit the same gene two times or more, thus 4487 insertions (74% of the 6063 insertions) in this screen are expected to hit gene once including introns. The same calculation for the insertions excluding introns (74% of the 3767 insertions) results in 2788 insertions expected to hit gene once. The *D. melanogaster* genome is estimated to have approximately 13000 known or predicted genes (Adams *et al.*, 2000). Theoretically, during this screen it is expected that approximately 35% of known or predicted genes in *Drosophila melanogaster* genome are hit once including introns ((4487 hit genes /13000 known or predicted genes)*100) (table 5). Excluding introns, approximately 22% of the genes are expected to be hit once ((2788 hit genes /13000 known or predicted genes)*100) (table 5). Out of ~1400000 embryos transferred to medium containing 3 μg/ml of Imidacloprid, 708 survivors with different phenotypes emerged (Table 5). Thus, the lethality of the selected flies was 99.95 %.

Table 5. Genome-wide insertional mutagenesis results

Embryos transferred to Imidacloprid medium	Emerged survivors	New jumps (92%)	Known or predicted genes hit once in *D. melanogaster* genome (%)	Lethality of transgenic flies selected on Imidacloprid (%)
1400000	708	~12888	~35%	99.95

The different phenotypes of the 708 male and female survivors are listed in table 6. There are differences in the distribution of phenotypes of the survivors. A lower number of survivors is detected for male escapers with and without new TREP (w^+) insertion and carrying the *Minos* transposase chromosome (Cy). The same is true for female survivors without new TREP insertion, but carrying the *Minos* transposase chromosome (table 6). Surviving males carrying TREP and the *Minos* transposase chromosome were 3.4-fold less compared to males carrying just the TREP construct.

Males carrying only the *Minos* transposase chromosome are 2.2-fold less abundant compared to surviving males with neither a TREP insertion, nor the transposase chromosome (table 6). Female escapers carrying the *Minos* transposase construct but no TREP insertion were 2.5 fold fewer compared to females without both a TREP insertion and the *Minos* transposase chromosome. The number of female escapers without new TREP insertions was lower compared to the number of males without new TREP insertions (around 3.9 fold). The same is true for female survivors carrying the *Minos* transposase chromosome, compared to the male escapers with the same chromosome (about 4.5-fold difference). The same number of females carrying a TREP element with the *Minos* transposase versus without the *Minos* transposase chromosome was found (table 6).

The summarized differences in the distribution of phenotypes of the survivors mostly show a decrease in viability of the flies with the presence of specific construct or combination of the constructs. This difference can be detected within and between the sexes. On the other hand, specificity of the TREP-BOEtTA system, where TREP 2.30 is lethal in the presence of BOEtTA, can be clearly observed with lower survivor of females compared to males, since all females carry BOEtTA construct.

Table 6. All emerged flies with different phenotypes selected on medium with 3 µg/ml of Imidacloprid

	non-Cy, w^+	Cy, w^+	non-Cy, w	Cy, w	Total
Males	236	70	218	98	622
Females	**4**	**4**	56	22	86
Total	240	74	274	120	708

Cy-Curly wings (marker of the *Minos* transposase chromosome)
w^+- red eyes (marker of the TREP element)

Eight female TREP-carrying survivors were retrieved from the insertional mutagenesis. The EGFP marker could not be detected under UV-illumination in four out of the eight individuals. The progeny of all eight individuals was retested for resistance.

Crosses for the testing of the survivors are schematically presented in figure 13. Each female escaper was crossed with 3 iso31 susceptible males. Progeny was selected on

medium with 3µl/ml of Imidacloprid and scored for the combination of markers and the presence of a new TREP insertion with and without BOEtTA driver. If all emerged progeny carry a TREP insertion, the resistance is correlated with that insertion. If progeny both with and without a TREP element emerged, the resistance was not correlated with the insertion.

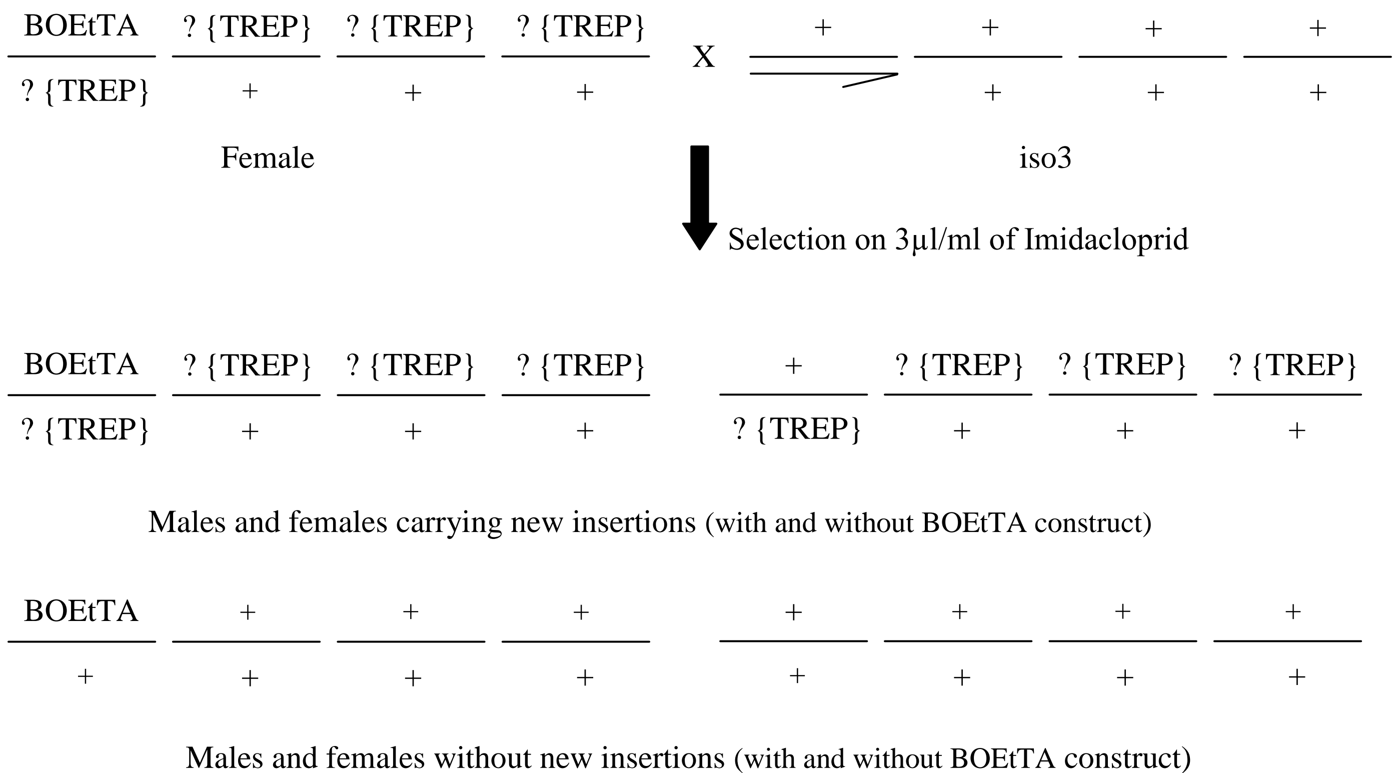


Figure 13. Crossing scheme for testing the female survivors

Three out of eight survivors had progeny that survived on 3 µg/ml of Imidacloprid, confirming inherited resistance to Imidacloprid. The progeny from these three females were used to establishing three isofemale lines carrying new TREP insertion. The LC50 of Imidacloprid was determined for these three lines, and the line with the highest resistance was selected for further analysis.

Table 7. LC50 values of 3 lines retrieved from *Minos*-based insertional mutagenesis, calculated with program SPSS 16

	LC50 (µg/ml) (95% confidence limits)	Slope ± S.E.	RR (resistance ratio)
Line 3 MiT[W-]3R2	2.6 (0.8-4.2)	-1.20±0.57	5.2
Line 2 MiT[W-]2R2	0.5 (0.1-1.0)	0.81±0.36	1.0
Line 1 MiT[W-]1R2	0.6 (0.1-1.1)	0.74±0.36	1.2

RR (resistance ratio) – LC50 value of the line with the highest resistance/LC50 value of the line with the lower resistance

Two of the three lines showed moderate resistance to Imidacloprid (LC50 = ~ 0.5 µg/ml) (table 7). The line (line 3) with the highest resistance to Imidacloprid (LC50 = 2.6 µg/ml with 95% confidence limits of 0.8 to 4.2 µg/ml) was selected for further analyses (table 7).

3.2. Genetic analysis of the resistant line

3.2.1. Obtaining and establishing the resistant line

The line with the highest resistance to Imidacloprid was further analyzed to narrow down the chromosomal location of the TREP insertion. For this purpose, individual crosses with lines (ITE stock collection) carrying standard balancer chromosomes were set up. The TREP element was mapped to the X chromosome, and the resistant line MiT[w^+]3RX was established. This resistant line was crossed with the susceptible line iso31, and progeny was tested for the linkage between the TREP element (with and without BOEtTA driver) and the Imidacloprid resistance. There was no correlation between the Imidacloprid resistance and the TREP element in the presence, or in the absence of the BOEtTA. In addition to the expected phenotype (red eyes), derived from TREP element, resistant flies with two different phenotypes

(orange eyes and white eyes) were detected. The orange eye (w^+ marker) of the first line was mapped to the second chromosome. Both derived lines were homozygous lethal for the second chromosome. Two resistant isofemale lines, both heterozygous for the second chromosome, one with orange eyes (MiT[orange]3R) and the other with white eyes (MiT[w^-]3R), were established.

Both lines (MiT[orange]3R and MiT[w^-]3R) were analyzed for the chromosomal location of the resistance.

3.2.2. Mapping the resistance to the second chromosome in line MiT[orange]3R

In order to map the resistance in the MiT[orange]3R line, the orange eyes phenotype was used as a marker. Crosses are schematically depicted in figures 14 and 15. MiT[orange]3R flies heterozygous for the second chromosome where crossed with flies carrying a second chromosome balancer. Five virgin females carrying a balancer chromosome and three males from the MiT[orange]3R line were set up. Equivalent crosses were performed with a balancer of the third chromosome. Female flies were left to lay eggs on cherry juice medium. Eggs were transferred to medium with 3µg/ml of Imidacloprid (50 eggs per vial) and progeny were selected during development (egg to adult). Emerged progeny was scored for the “resistance” and non “resistance” chromosome derived from line MiT[orange]3R. As control, progeny from the same crosses were maintained on standard medium during development. For each cross, five replicas were set up.

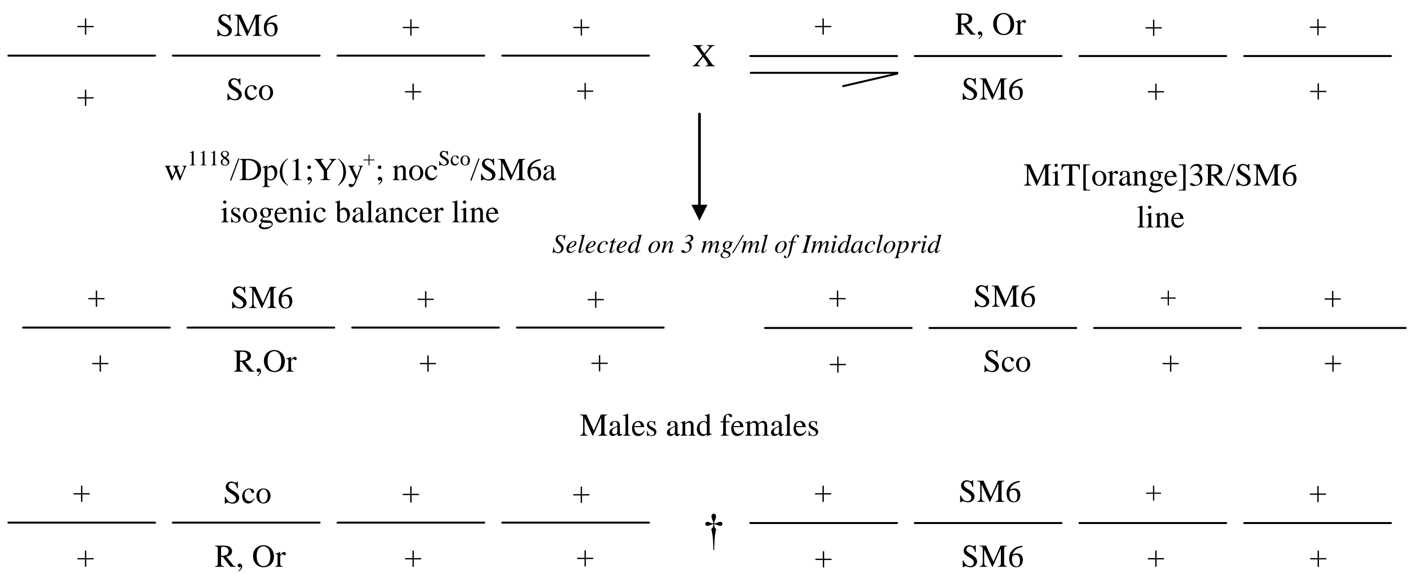


Figure 14. Crossing scheme for the second chromosome

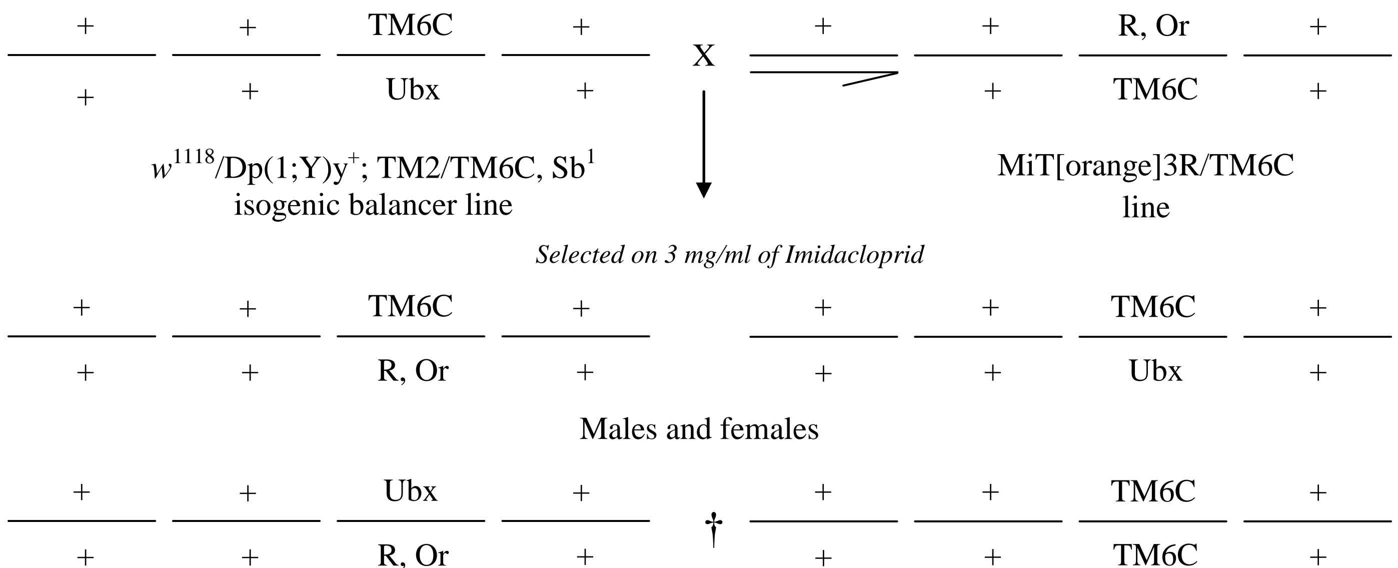


Figure 15. Crossing scheme for the third chromosome

Results of the crosses with the second and third balancer chromosome are presented in the table 8 and 9 respectively. Progeny carrying a second chromosome derived from MiT[orange]3R2 (orange eyes progeny) was detected, while progeny carrying both second balancer chromosomes (white eyes progeny) did not survive (table 8). Hence, the resistance locus resides on the second chromosome in line

MiT[orange]3R2. Also, this result was confirmed by the result of third chromosome analysis where progeny carrying all possible combinations of the third chromosome emerged (table 9). The orange eyes marker and the lethality mapped to the second chromosome.

Table 8. Viability of second chromosome combinations in progeny emerged on standard medium and medium with 3 µg/ml of Imidacloprid

	Medium with 3µg/ml of Imidacloprid	Standard medium
SM6/R,Or	viable	viable
SM6/Sco	lethal	viable
Sco/R,Or	viable	viable

SM6– Curly marker
Or – Orange eyes
Sco – Scutoid marker
R – Resistance chromosome

Table 9. Viability of third chromosome combinations in progeny emerged on standard medium and medium with 3 µg/ml of Imidacloprid

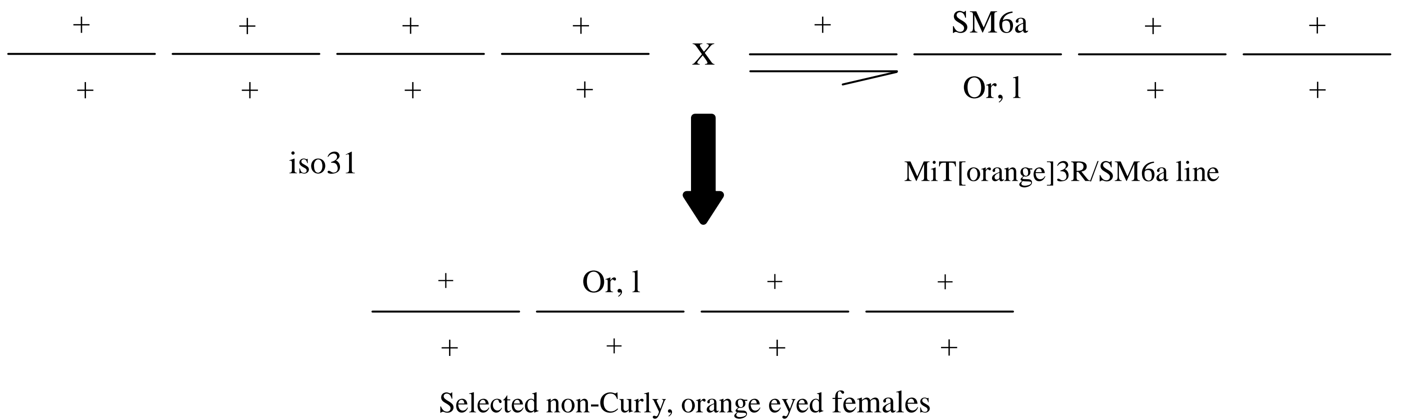
	Medium with 3µg/ml of Imidacloprid	Standard medium
TM6C /R,Or	viable	viable
TM6C /Ubx	viable	viable
Ubx/R,Or	viable	viable

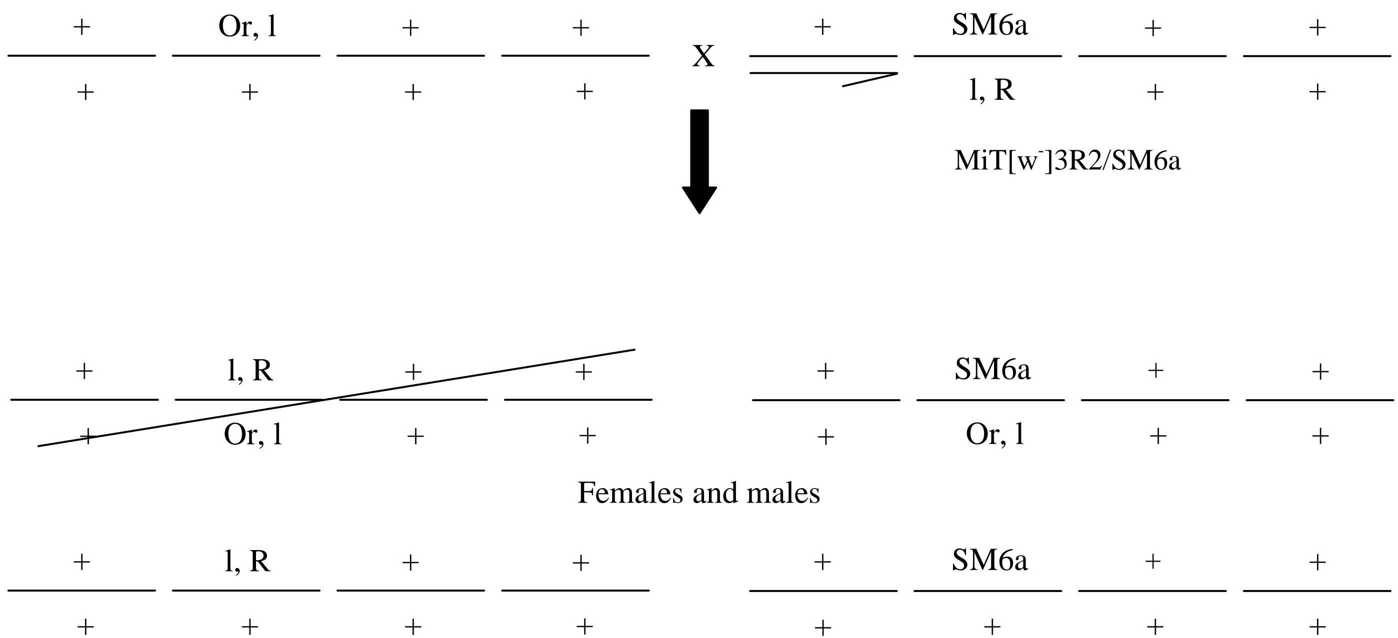
TM6C – Stubble marker
Or – Orange eyes
Ubx – Ultrabithorax marker
R – Resistance chromosome

3.2.2.1.a. Recombination test shows no correlation between lethality and orange marker eyes in resistant line MiT[orange]3R

Further experiments were performed to test a possible linkage between the lethality locus and the w^+ marker in resistant line MiT[orange]3R. The analysis is based on the determination of genetic distance by measuring the recombination rate between these genes. Crosses are schematically depicted in the figure 16. Resistant virgin female MiT[orange]3R flies were mass crossed with iso31 males (20 females with 10 males in 5 replicas). Only non-Curly, orange eyed virgin females were selected for the next cross. These females were mass-crossed with MiT[w^-]3R2/SM6a males and the progeny was scored for the different phenotypic classes. We assume that the lethality

in MiT[orange]3R and MiT[w^-]3R2/SM6a lines mapps to the same location since both lines derive from the same initial line. In each cage, 100 non-Curly, orange eyed virgin females were crossed with 50 MiT[w^-]3R2/SM6a males. Five replicas of this cross were made. Presence of offspring with non-Curley wings and orange eyes would show the linkage between lethality and orange eyes (w^+) marker.





Only non Curly, white eyes – Lethality is linked with marker (orange eyes)

+	l, R	+	+		+	SM6a	+	+
+	Or, l	+	+		+	Or, l	+	+

Females and males

+	l, R	+	+		+	SM6a	+	+
+	+	+	+		+	+	+	+

Non-Curly, orange eyes – Lethality is not linked with marker (orange eyes)

Figure 16. Crosses for the recombination test for analysis of the correlation between the lethality locus and the orange eyes marker for resistant line MiT[orange]3R

In total, 14473 flies were analyzed for recombination events (table 10). Presence of all 4 classes of the phenotypes was detected. The results indicate that the w^+ marker is at least 4,5 cM away from the lethality locus (table 10).

Table 10. Recombinant and non recombinant progeny

Phenotype	Or, l/SM6a	SM6a /++	Or, l/R, l	R, l/++	Total number
Number of flies analyzed	4083	4282	2802	3306	14473

Or, l/SM6a – Curly wings, Orange eyes
SM6a/++ - Curly wings, White eyes
Or, l/R, l – Normal wings, Orange eyes
R, l/++ - Normal wings, White eyes

Recombination analysis did not show correlation between lethality and orange marker eyes. Further analysis were performed to test a possible linkage between the resistance locus and the w^+ marker in resistant line MiT[orange]3R.

3.2.2.1.b. Genetic analysis failed to link the resistance locus with the orange eyes marker in resistant line MiT[orange]3R

Non-Curly, orange eyed male progeny from recombination cross were analyzed for a correlation between the resistance locus and the orange eyes marker on the second chromosome. The crosses are depicted in figure 17. Ten Non-Curly, orange eyed male

progeny were crossed with five virgin iso31 females in ten replicas. Male progeny heterozygous for orange eyes were selected and crossed again with iso 31 females, using the same number of individuals and replicas as in the previous cross. Progeny from this cross was selected during development on 3µg/ml of Imidacloprid. After emerging, they were scored for the presence or absence of orange eyes.

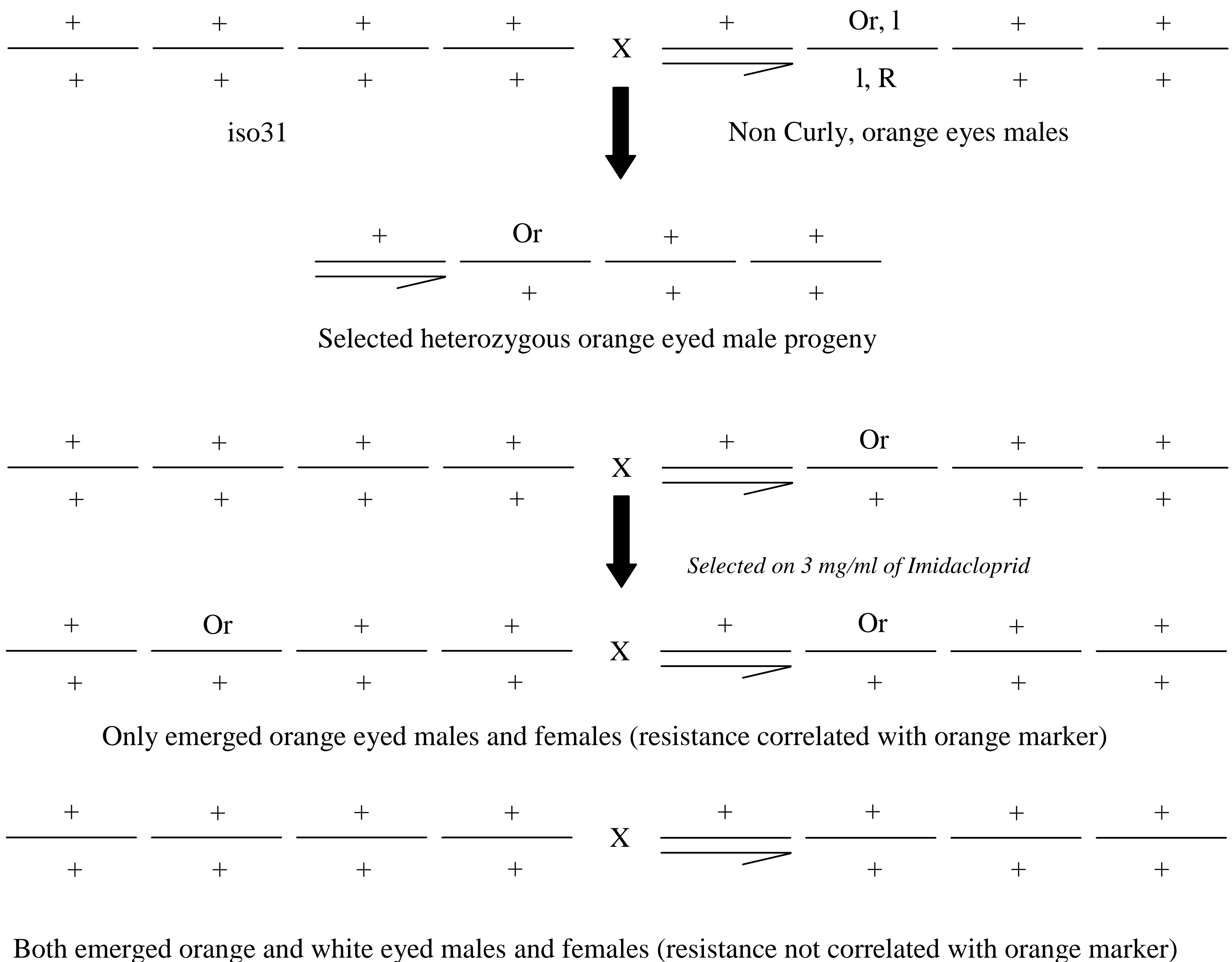


Figure 17. Crossing scheme for analysis of the correlation between the resistance locus and the orange eyes marker in resistant line MiT[orange]3R

Results show that all progeny reared on medium with Imidacloprid died, while flies reared on standard medium emerged normally. This demonstrated that the resistance was not linked with the w^+ marker gene in the resistant line MiT[orange]3R2.

3.2.3. Mapping the resistance to the second chromosome in line MiT[w⁻]3R

Chromosome mapping of the resistance in line MiT[w⁻]3 was done in parallel with analysis for the chromosomal location of the resistance in line MiT[orange]3R. The crosses depicted in figures 18 and 19 were set up in order to map the resistance to a chromosome in line MiT[w⁻]3R. Male MiT[w⁻]3R flies were crossed with virgin flies carrying a balancer chromosome (balancers for the second or for the third chromosome). Progeny heterozygous for the "resistance" chromosome from both crosses were selected on Imidacloprid during development. Male progeny heterozygous for the "resistance" chromosome were individually crossed (one male with 2 females) with virgin iso31 females. Progeny from this cross were again selected on medium with 3µg/ml Imidacloprid. Five replica of each cross were set up. Emerged male and female progeny was scored for the presence or absence of the balancer chromosome. Absence of a balancer chromosome in the emerged progeny maps the resistance to the respective balanced chromosome.

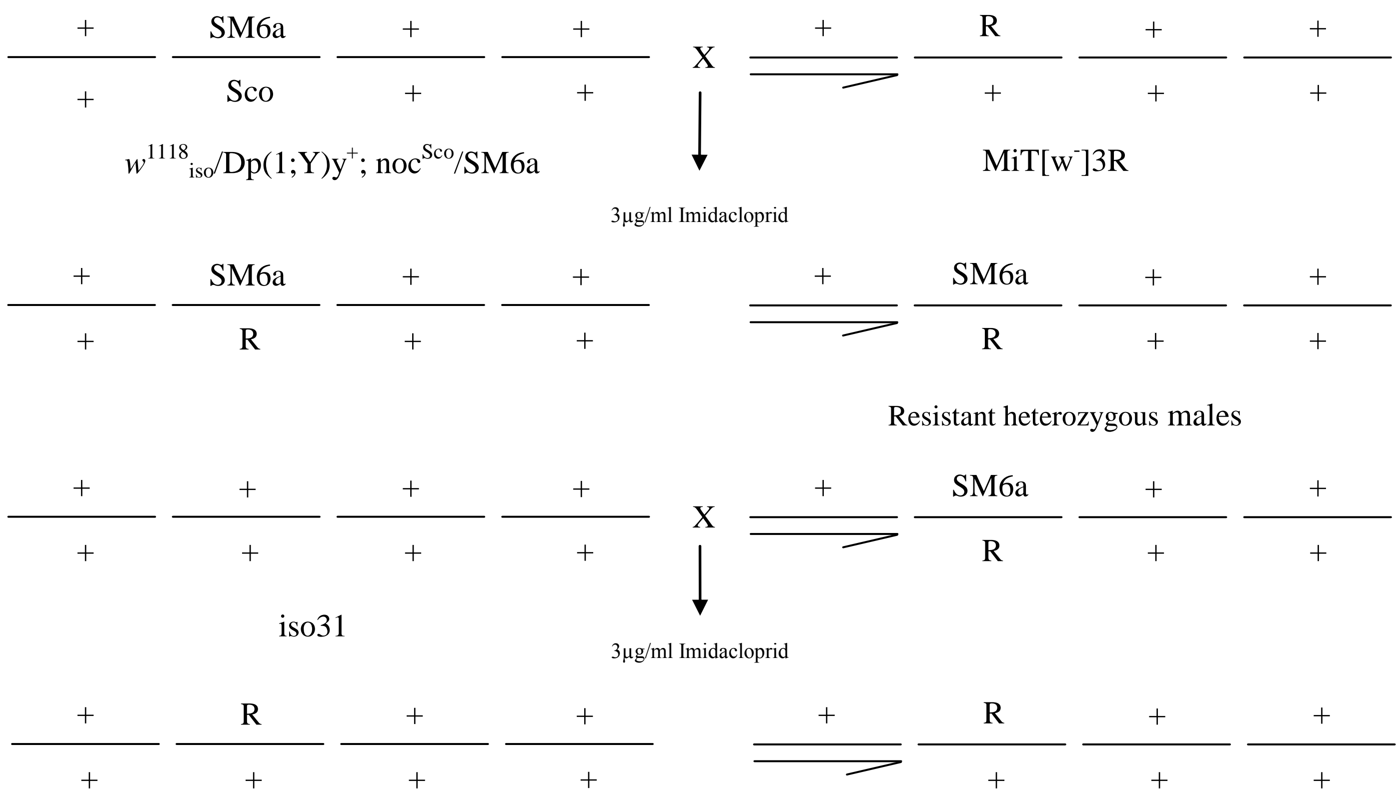


Figure 18. Second chromosome crossing scheme of the mapping of the resistance locus

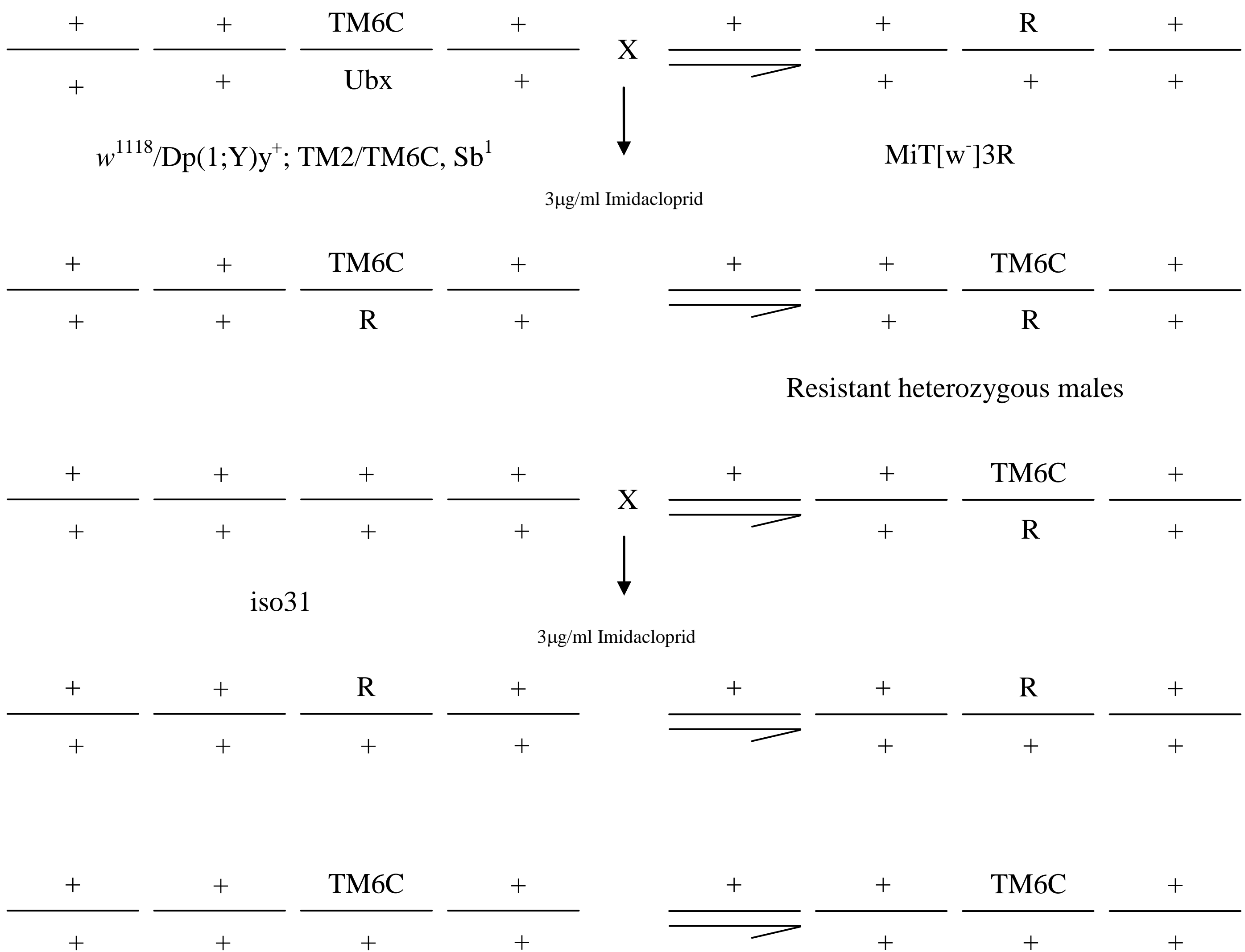


Figure 19. Third chromosome crossing scheme of the mapping of the resistance locus

There was no sex bias in the emerged flies, thus the resistance does not map to the sex chromosome. The second chromosome analysis yielded in all five replicas progeny carrying the second chromosome derived from line MiT[w$^-$]3R (table 11). Progeny carrying the balancer chromosome derived from the iso31 balancer line was not viable. This maps the resistance to the second chromosome of line MiT[w$^-$]3R. The third chromosome analysis confirmed that resistance maps to the second chromosome (table 12). In all five replicas of this experiment, progeny with third chromosomes derived from the resistant line, as well as from the iso31 balancer line were detected (table 12). The resistant line MiT[w$^-$]3R2/SM6 was established.

Table 11. Number of adult progeny with and without the second chromosome from MiT[w$^-$]3R (non-Cy) after selection on medium with 3 µg/ml of Imidacloprid

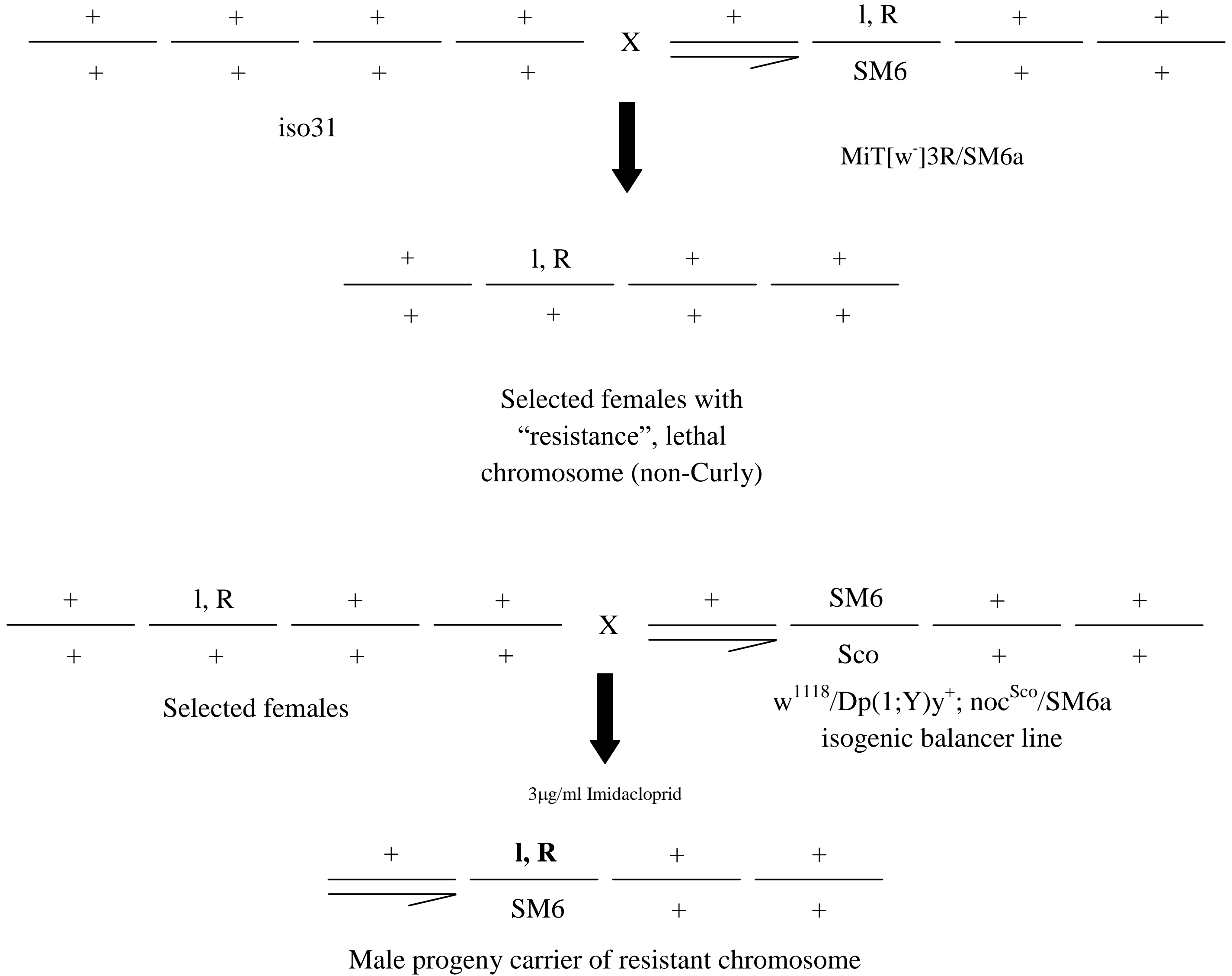
Replica	Non Cy ♀ 2. chromosome	Non Cy ♂ 2. chromosome	Cy ♀ balancer (Curly) chromosome	Cy ♂ balancer (Curly) chromosome
I	11	14	0	0
II	15	17	0	0
III	12	9	0	0
IV	13	11	0	0
V	10	12	0	0

Table 12. Number of adult progeny with and without the third chromosome from MiT[w$^-$]3R (non Sb) after selection on medium with 3 µg/ml Imidacloprid

Replica	Non Sb ♀ 3. chromosome	Non Sb ♂ 3. chromosome	Sb ♀ balancer (Stubble) chromosome	Sb ♂ balancer (Stubble) chromosome
I	13	14	12	12
II	12	11	14	15
III	12	13	13	11
IV	13	14	10	12
V	14	16	16	15

As for MiT[orange]3R2, correlation between resistance and lethality was analyzed in line MiT[w$^-$]3R2/SM6a by determining recombination frequencies (figure 20). Virgin female iso31 flies were mass-crossed with males from the resistant line (20 females with 10 males in 5 replicas). Female progeny with the "resistance" chromosome (non-Curly phenotype) were selected and mass-crossed with males carrying the Curly balancer chromosome (100 females with 50 males per cage, 5 cages in total). From each cage, 4000 eggs were transferred to medium with Imidacloprid. From this cross, 400 resistant Cy male progeny were selected and mass crossed with virgin MiT[w$^-$]3R/SM6a females. Progeny from this cross were scored for the different phenotype

combinations. Correlation between resistance and lethality was observed by analyzing progeny of recombinant females carrying a heterozygous resistance chromosome.



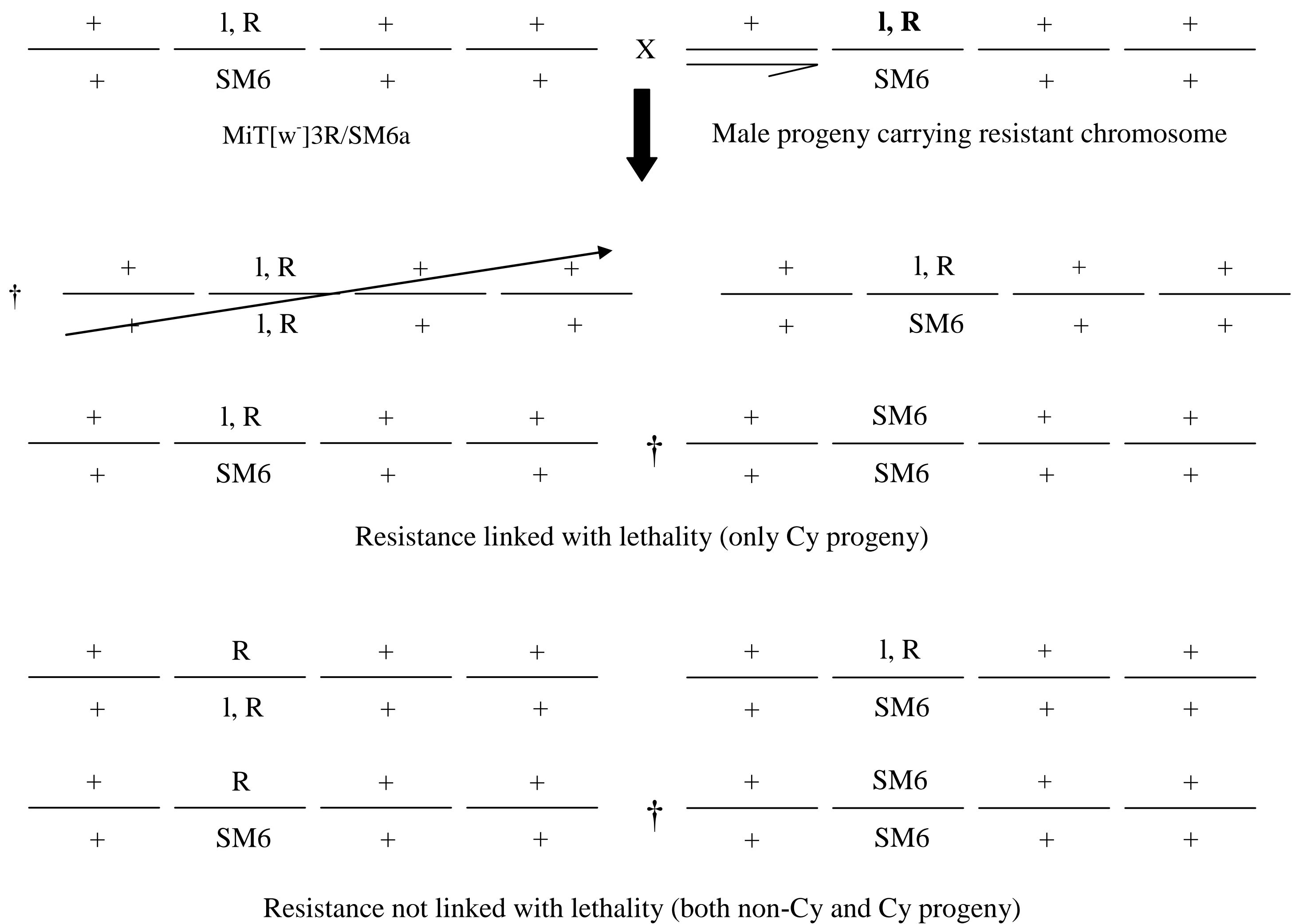


Figure 20. Crossing scheme for recombination analysis of correlation between resistance and lethality in line MiT[w$^-$]3R2

The presence or absence of non-Curly progeny from the second cross will indicate if there is linkage between lethality and resistance. If the lethality were linked to the resistance locus, progeny homozygous for the second "resistance" chromosome would not be viable (non-Curly phenotype would not be detected). The presence of the non-Curly phenotype shows that there is no close linkage between resistance and lethality. Resistant flies homozygous for the second chromosome (non-Curly) were used to establish the homozygous resistant line MiT[w$^-$]3R2.

Data from this experiment were used for an approximate determination of the genetic distance between the resistance and lethality loci. In total, 600 flies were analyzed for

recombinants. Distance was calculated by dividing the total number of recombinant flies by the total number of flies and expressed in centimorgans (cM) (Sturtevant, 1913). This calculation does not give a precise distance, because the non-viable flies can not be counted. The distance between resistance and lethality was estimated to be around 15.8 cM (table 13).

Table 13. Approximate number of recombinants emerged on medium with Imidacloprid

MiT[w-]3R2/ SM6a recombinant x MiT[w-]3R2/ SM6a original	total number of flies analyzed	number of recombinant flies	percentage of recombination (cM)
I	100	18	0.18 (18)
II	100	17	0.17 (17)
III	100	12	0.12 (12)
IV	100	14	0.14 (14)
V	100	18	0.18 (18)
VI	100	16	0.16 (16)
average	600	15.83	0.1583 (15.83)

Line MiT[w^-]3R2 carries a lethal mutation, which was mapped to the same chromosome as the "resistance" locus. In order to narrow down the chromosomal position of the resistance locus, lethality was used as a marker. For this purpose, 111 fly lines covering whole second chromosome with lethal deletions were used (Bloomington Stock Fly center deficiency kit for the second chromosome; Data stored electronically on CD - Fly stock 1 deletion kit file). Mass crosses between resistant flies and flies from second chromosome deficiency kit were performed (figure 21). A combination of the resistance chromosome carrying the lethality locus and a chromosome from the kit with deletion covering the same locus will cause lethality. Thus, if all viable progeny carry the Cy marker, lethality is mapped to the interval of the second chromosome spanned by the deletion.

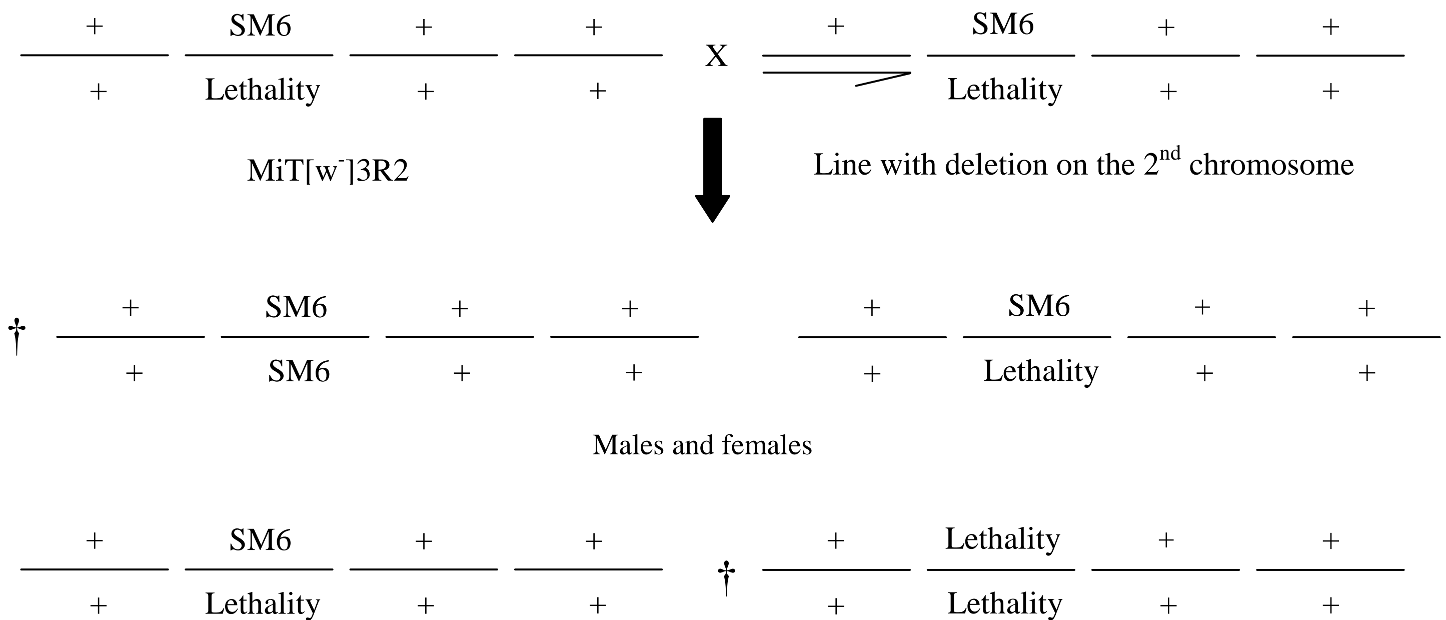


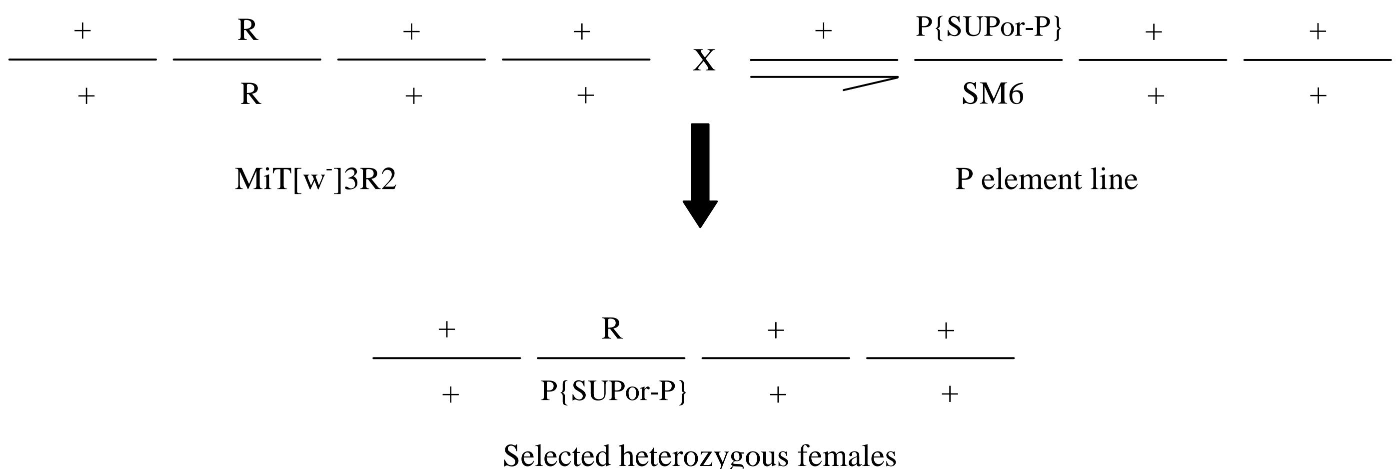
Figure 21. Crossing scheme of MiT[w$^-$]3R2 and deletion kit flies

The right arm of the second chromosome has a length of ~21.1 Mb, while the left arm is ~23.0 Mb long (Tweedie *et al.*, 2009). Lethality locus is mapped to the right arm of the second chromosome (2R), to the region between 49C1 (8.5 Mb) -50D2 (9.9 Mb). An imprecise genetic mapping placed resistance up to 15.8cM from the lethality locus, suggesting that the resistance is located on the same chromosome arm (2R) as lethality.

3.2.3.a. Genetic mapping relative to P element insertions narrows down the resistance locus

To narrow down the resistance locus on the right arm of the second chromosome, four lines with inserted P elements were employed (Bloomington Stock Fly center; Data stored electronically on CD - Fly stock 2 P element kit file). The specific crosses are schematically depicted in figure 22. The resistant MiT[w$^-$]3R2 flies do not carry any visible marker gene, while all flies carrying P element insertions have w$^+$ as phenotypic marker. Resistant flies were mass crossed with flies carrying the P element insertion. Virgin female progeny with red eyes (one chromosome deriving

from the resistant line and the other from the P element line) were collected and crossed with iso31 males. For each experiment, 50 female flies, heterozygous for the resistance chromosome were crossed with 25 iso31 males, per replica. Each experiment had eight replicas with a total number of 250 females crossed for each P element line. Crossed flies were kept on standard medium for 2-3 days. After that period, all flies were transferred to medium with 3 µg/ml of Imidacloprid. Progeny was scored for recombination events, e.g. presence of the P element marker gene w^+. At least 1000 emerged flies with different phenotypes were analyzed per replica. Recombination rates were calculated as the ratios of the total number of recombinant flies over the total number of emerged flies. The distance between the P-element insertions and resistance was calculated in centimorgans (cM), from which the physical distance was calculated using estimates of the local recombination rates at the sites of the P-element insertions after Fiston-Lavier *et al* (2010) and Singh *et al* (2005). This estimate was not possible for one of the P-elements, which is too close (about 0.5 Mb) to the centromere. Here, the recombination rate for the interval between the P element and the average position of the resistance locus, as determined relative to the other three P-elements, was calculated.



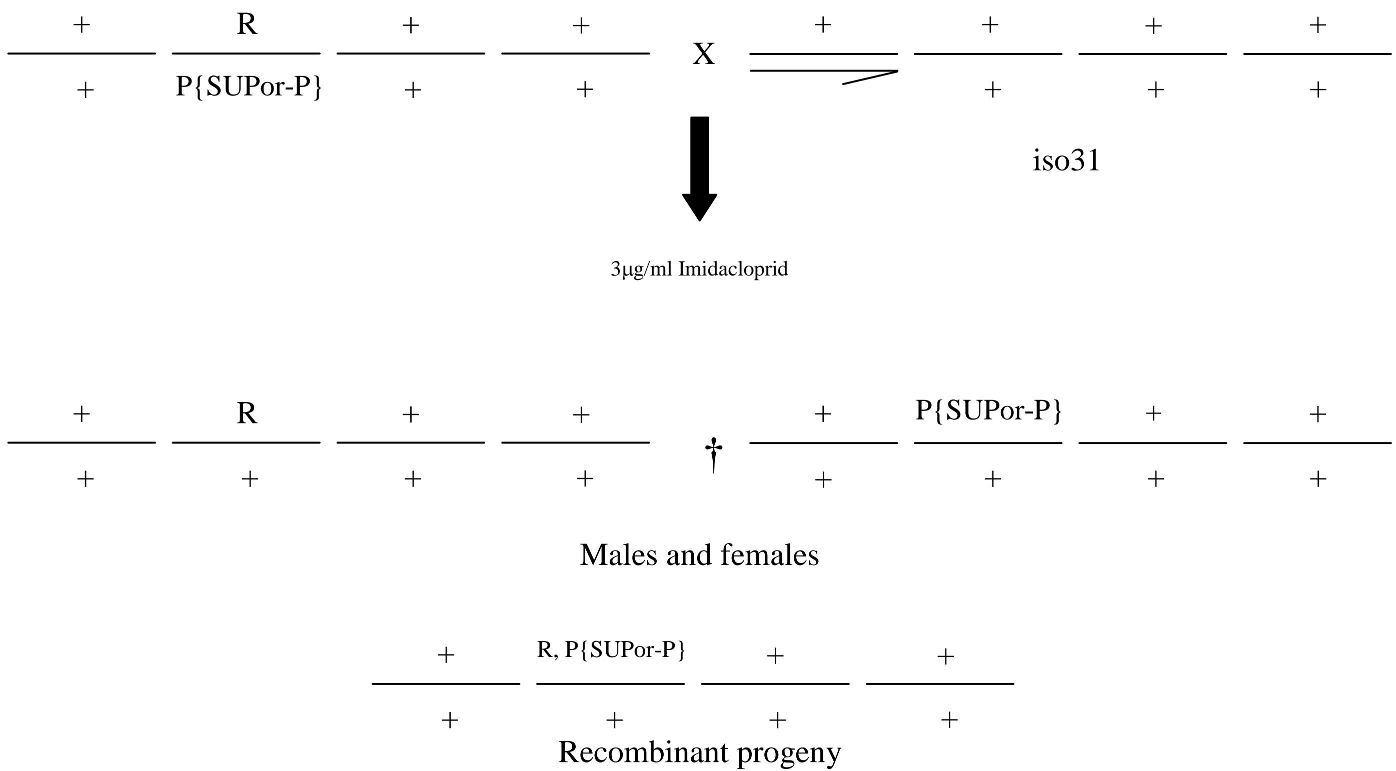


Figure 22. Crossing scheme of P element resistance mapping

In order to confirm the resistance mapping using lethality as a marker, a more precise P element mapping of the resistance on the 2R chromosome was performed (figure 23, table 14). The distance between a P element located at ~ 0.5 Mb (P element line 12973 with insertion location 504496) and the resistance locus is 8.2 cM (table 14, figure 23). The distance between a P element located at ~ 6.1 Mb (P element line 14341with insertion location 6189895) and the resistance locus is 3.5 cM. The distance between a P element located at ~ 6.5 Mb (P element line 13840 with insertion location 6560770) and the resistance locus is 2.8 cM. The distance between P element located at ~ 11.2 Mb (P element line 13763 with insertion location 11210503) and the resistance locus is 3.0 cM (table 14, figure 23).

Table 14. P element insertion coordinates and distance between insertion and resistance region on the right arm of the second chromosome

P element line	Insertion location (coordinates depicted in base pairs)	Distance between P element and resistance locus calculated from recombination rate (depicted in centimorgans (cM))	Calculated corrections of the local recombination rate for each P-element insertions on the 2R chromosome (Fiston-Lavier *et al.*, 2010 and Singh *et al.*, 2005) (depicted in centimorgans (cM))	Distance between P element and resistance locus corrected with estimated corrections rates of the local recombination rate (depicted in centimorgans (cM))
12973	504496	9.3	1.13	8.2
14341	6189895	6.9	1.99	3.5
13840	6560770	5.9	2.14	2.8
13763	11210503	10.5	3.46	3.0

Schematic representation of the right arm of the second chromosome, size in megabase (Mb) from 0-20 Mb

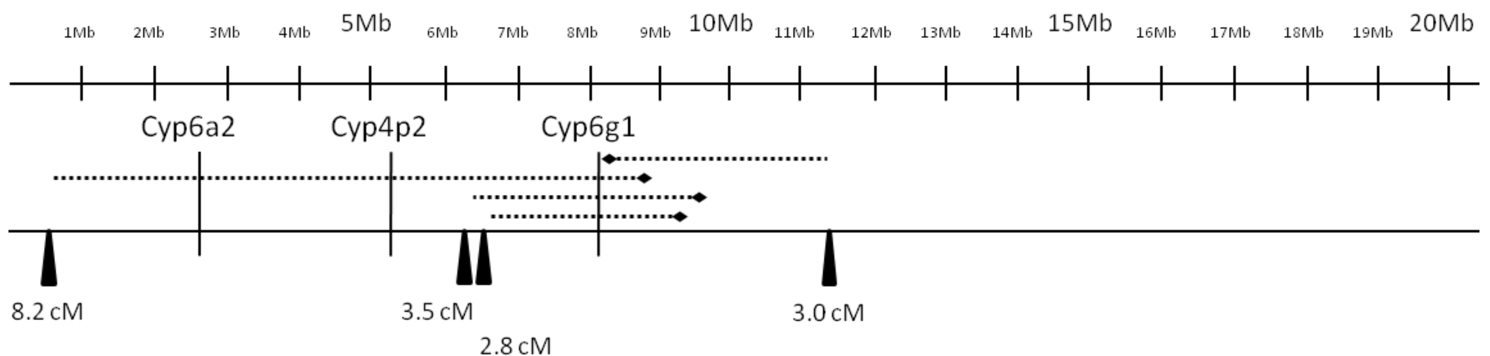


Figure 23. Location of the P element insertions (black filled triangles) and distance between insertion and resistance region (interrupted lines) on the right arm of the second chromosome

The genetic mapping relative to the P-element insertions places the 8Mb and 9.7 Mb on the right arm of the second chromosome (table 14, figure 23). The three most highly overexpressed p450 genes (Cyp4p2, Cyp6a2 and Cyp6g1) are also located on the same chromosome arm (figure 48). Interestingly, the upregulated p450 gene Cyp6g1 is located within the region where resistance is mapped (figure 48).

3.2.4. Karyotype analysis shows no structural changes of the polytene chromosomes in resistant line

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). Therefore, the resistant *Drosophila* line was also analyzed for the presence of inversions. The karyotype of the salivary glands of larvae from a cross between resistant MiT[w^-]3R and susceptible iso31 line was microscopically analysed for the presence of inversions on all five polytene chromosome (figure 24). All five polytene chromosomes (X, 2L, 2R, 3L, 3R) show the standard banding patterns, with no obvious rearrangements (figure 24).

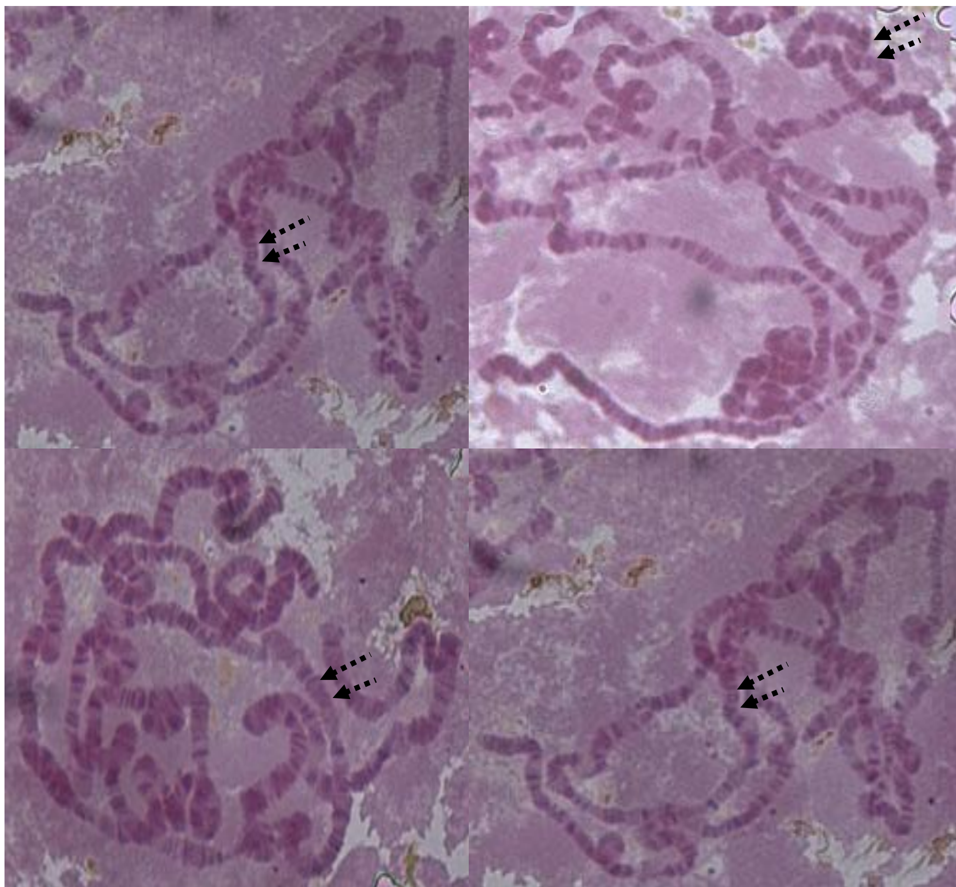


Figure 24. Salivary gland polytene chromosomes of larvae progeny from the cross between resistant and susceptible line, prepared with a squash technique (dashed arrows surround the region where the resistance locus is mapped)

3.3. Toxicological analysis

3.3.1. Resistance to Imidacloprid

In order to get more accurate data about the degree of resistance, the lethal concentration 50 (LC50) for Imidacloprid was determined for line MiT[w$^-$]3R2 by analyzing egg to adult viability. In total, 400 eggs per concentration were transferred to medium with different concentrations of Imidacloprid. Calculations were done with PROBIT statistics, using the software SPSS 16. Dose response curves were constructed from at least six concentrations.

Dose-response curves and LC50 values for TREP 2.30, iso31 (e.g. susceptible lines) and the resistant lines are shown in figures 25 and 26, and table 15. Susceptible lines have a significantly lower LC50, ranging from 0.15 to 0.18 µg/ml (with 95% confidence limits of 0.06µg/ml – 0.26µg/ml and 0.15µg/ml to 0.21 µg/ml, respectively). Lines (MiT[W$^-$]3R2/SM6 and MiT[orange]3R2/SM6), derived from the original resistant line, heterozygous for the second chromosome carrying the resistance locus, have an LC50 of 2.15 and 2.03 µg/ml (with 95% confidence limits of 1.59µg/ml – 2.61µg/ml and 1.63µg/ml to 2.91 µg/ml), respectively. Further analyses were only performed with resistant line MiT[W$^-$]3R2. A much increased LC50 was found for flies homozygous for the "resistance" second chromosome. The LC50 was ~18-fold higher than that of the wild-type line iso31 (the LC50 for MiT[w$^-$]3R2 was 3.32µg/ml, with 95% confidence limits of 1.91µg/ml and 4.12µg/ml; and iso31 was 0.18 µg/ml (0.15µg/ml and 0.21 µg/ml) (table 15).

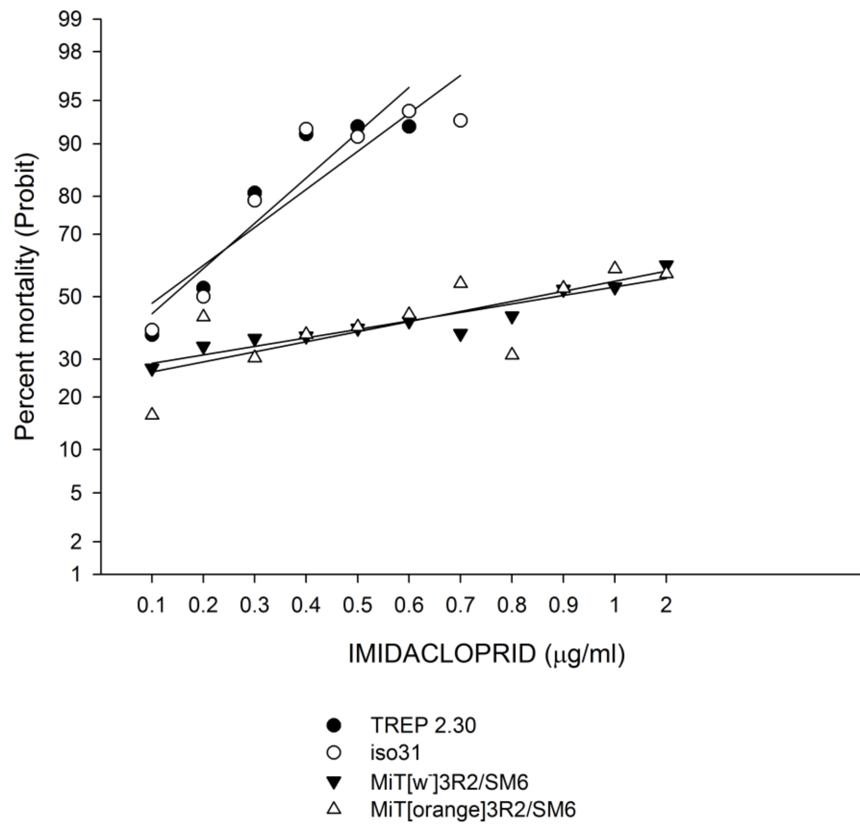


Figure 25. Dose-response curves to Imidacloprid of two susceptible and resistant lines (heterozygous for the second chromosome)

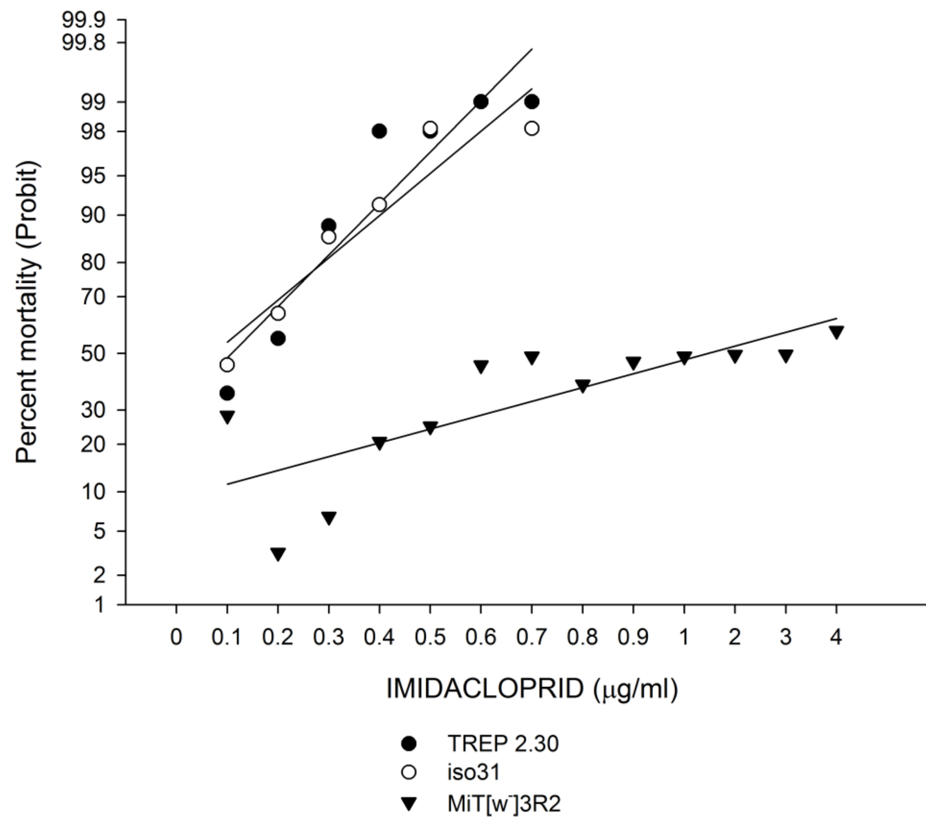


Figure 26. Dose-response curves to Imidacloprid of two susceptible lines and one resistant line (homozygous for the second chromosome).

Table 15. LC50 values for Imidacloprid of susceptible and resistant lines.

lines	LC50 (µg/ml) (95% confidence limits)	RR (resistance ratio)
TREP	0.16 (0.06 – 0.26)	1.00
iso31	0.15 (0.06 – 0.26)	1.00
MiT[W⁻]3R2/SM6 (heterozygous)	2.15 (1.59 -2.61)	14.33
MiT[orange]3R2/SM6 (heterozygous)	2.03 (1.63 – 2.91)	13.53
TREP	0.18 (0.15 – 0.22)	1.00
iso31	0.18 (0.15 – 0.21)	1.00
MiT[W⁻]3R2 (homozygous)	3.32 (1.91 – 4.12)	18.44

RR (resistance ratio) – LC50 value of the resistant line /LC50 value of the susceptible line

3.3.2. Cross-resistance to DDT

Toxicological studies show that DDT resistant field and laboratory *Drosophila* lines also confer resistance to different neonicotinoids, including Imidacloprid (Daborn *et al.*, 2001; Le Goff *et al.*, 2003; Daborn *et al.*, 2007). Thus, the MiT[W^-]3R2 line resistant to Imidacloprid was 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. 100 adults per concentration were analyzed on different DDT concentrations. Calculations were performed with PROBIT statistics using the SPSS 16 software. Dose response curves were constructed from at least four DTT concentrations (plus control).

Dose-response curves and LC50 values for the susceptible lines and resistant line MiT[W^-]3R2 are given in figures 27 and 28 and table 16, respectively. Both susceptible lines have significantly lower LC50 values than the resistant one. Line TREP 2.30 has a higher LC50 value (1.16 µg/ml with 95% confidence limits of 0.22 µg/ml – 2.65 µg/ml) than iso31 (0.35 µg/ml with 95% confidence limits of 0.07 µg/ml – 0.77 µg/ml). As in the case of Imidacloprid, there is a positive correlation between the number of second "resistance" chromosomes and the LC50 in MiT[W^-]3R2. 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 and 0.65 µg/ml; and for MiT[w^-]3R2 37.50 µg/ml (32.20 µg/ml and 41.90 µg/ml) (table 16).

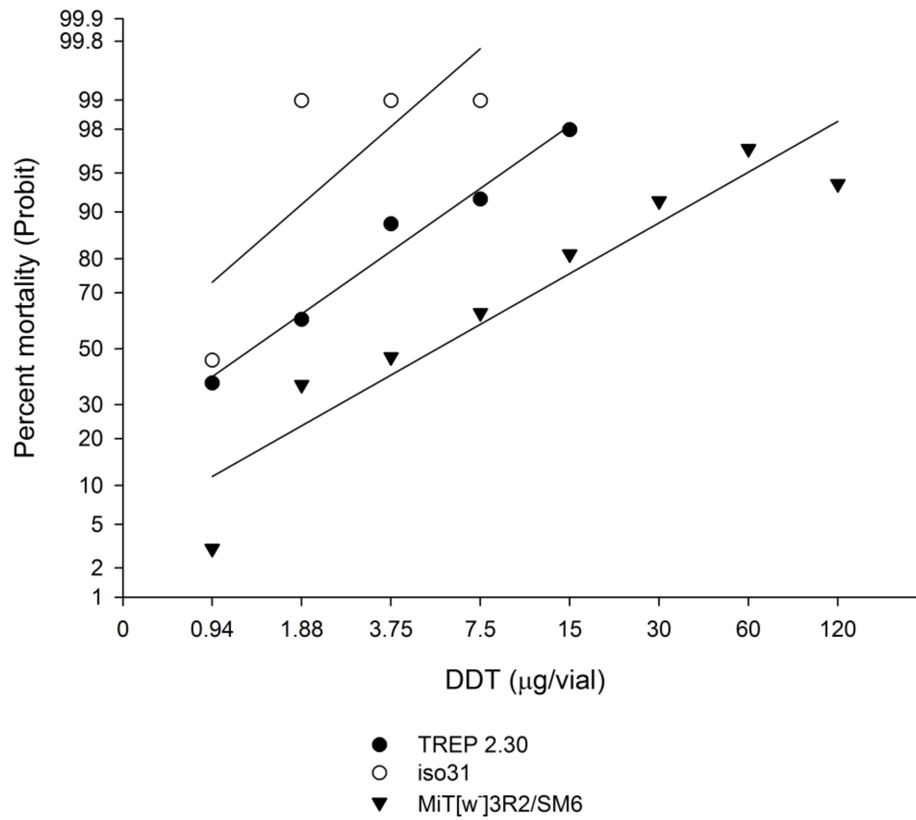


Figure 27. DDT dose-response curves of two susceptible lines and a resistant line (heterozygous for the second chromosome)

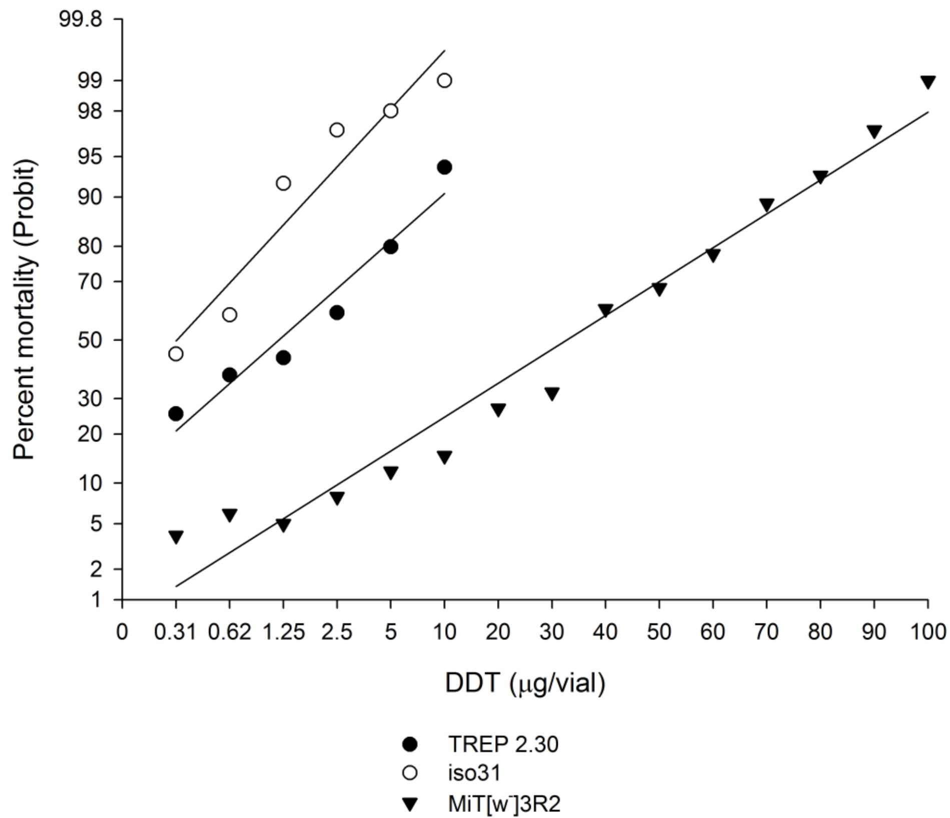


Figure 28. DDT dose-response curves of two susceptible lines and a resistant line (homozygous for the second chromosome)

Table 16. LC50s for DDT of susceptible and resistant lines (homozygous and heterozygous, respectively, for the second chromosome)

lines	LC50 (µg/vial) (95% confidence limits)	RR (resistance ratio)
TREP 2.30	1.16 (0.22 – 2.65)	
iso31	0.35 (0.07 – 0.77)	1.0
MiT[W-]3R2/SM6 (heterozygous)	5.5 (0.1 – 18.2)	15.7
TREP 2.30	1.63 (0.59 – 3.02)	
iso31	0.37 (0.15 – 0.65)	1.0
MiT[W-]3R2 (homozygous)	37.5 (32.2 – 41.9)	101.4

RR (resistance ratio) – LC50 value of the resistant line /LC50 value of the susceptible line

Dose-response curves show that the resistance to both Imidacloprid and DDT is positively correlated with the number of the resistant second chromosomes (figures 25, 26, 27 and 28).

3.3.3. Piperonyl butoxide (PBO) analysis suggests involvement of cytochrome P450 genes in resistance mechanism

Resistance to two insecticides with different mode of action suggested involvement of metabolic resistance mechanisms as potential mechanism of resistance in line MiT[W^{-}]3R2. One of the major gene families involved in metabolic insecticide resistance is the group of cytochrome P450 genes. Piperonyl butoxide (PBO) is a potent cytochrome P450 inhibitor and pesticide synergist (Hodgson and Levi, 1998). In order to test for the involvement of cytochrome P450 genes in the resistance of line MiT[W^{-}]3R2, a PBO assay was performed. Flies were tested for susceptibility to Imidacloprid in 48 hours contact assays. All data were analyzed as for the DDT contact assay. For each concentration, 50 individuals were analyzed. Dose response curves were constructed from at least four concentrations (plus control).

Treatment of the resistant mutant with PBO reduced its resistance to Imidacloprid from ~2.2-fold to ~1.4-fold, compared to the susceptible line (figure 29, table 17). The LC50 of the non-treated mutant is 9.4 µg/vial, with a 95% confidence interval of 6.4 to 12.8 µg/vial, while for the PBO treated mutant, the LC50 is 5.7 µg/vial, with a confidence interval of 3.4 to 7.2 µg/vial (table 17).

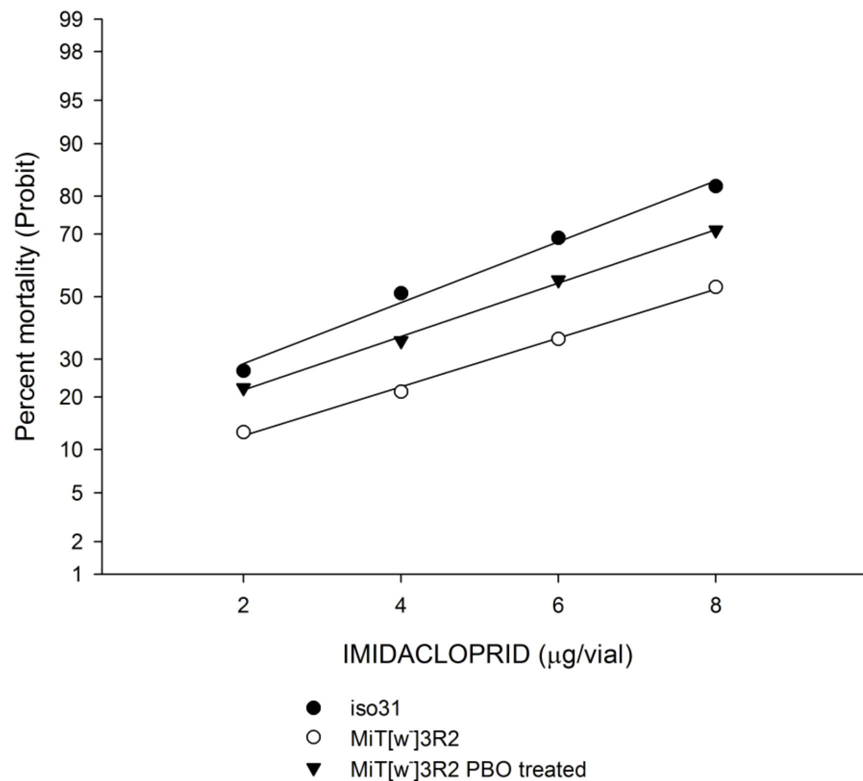


Figure 29. Imidacloprid dose-response curves of the susceptible and resistant lines treated with PBO and of the non-treated resistant line

Table 17. Imidacloprid LC50s of a susceptible and a resistant line treated with PBO and of the untreated resistant line

Line	LC50 (μg/vial) (95% confidence limits)	RR (resistance ratio)
iso31	4.2 (2.3- 5.3)	1.0
MiT[w$^-$]3R2 – PBO	5.7 (3.4 – 7.2)	1.4
MiT[w$^-$]3R2	9.4 (6.4 – 12.8)	2.2

RR (resistance ratio) – LC50 value of the resistant line /LC50 value of the susceptible line

PBO has no effect on the slope of the dose-response curve of the treated line compared to non treated resistant flies (figure 29). Results of the PBO analysis established the involvement of P450s in the resistance mechanism of the MiT[w$^-$]3R2 line.

3.3.4. Biochemical assays show increased activity of the P450 in the resistant line compared to susceptible line

Further analysis of the activity of cytochrome P450 monooxygenases, as well as analysis of esterases and glutathione S-transferases (two more enzymes involved in metabolic mechanism) was performed in resistant and susceptible lines (Table 18). Esterase activity was measured using *α* and *β naphthol,* while glutathione S-transferases activity was measured using *1-chloro-2,4-dinitrobenzene*. For both enzyme activities, no significant difference was detected in the resistant line compared to line iso31 (table 18). Cytochrome P450-dependent monooxygenase activity was determined by *O-deethylation* of *7-ethoxycoumarin* in adult microsomes and living larvae. The activity of cytochrome P450 was higher in the resistant line MiT[W$^-$]3R2, for both adults and larvae, compared to the susceptible line (table 18). Activity of P450s in MiT[W$^-$]3R2 flies was ~3-fold higher in live larvae (2.1 (± 0.1) pg/min/larvae) compared to susceptible third instar larvae (0.72 (± 0.05) pg/min/larvae) (table 18).

Table 18. Activities of detoxification enzymes of resistant and susceptible lines

	Cytochrome P450 monooxygenase (adults,microsomes) (pg/min/mg protein) ±(SD)	Cytochrome P450 monooxygenase (alive larvae) (pg/min/larvae) ±(SD)	Esterase (nmol a-naphthol produced/min/mg) ±(SD)	Esterase (nmol b naphthol produced/min/mg) ±(SD)	GST (μmole/min/mg) ±(SD)
MiT[W$^-$]3R2	1400 ±201	2.10±0.10	73±1.60	27±2	0.13±0.04
iso31	800 ±60	0.72±0.05	53±4	32±4	0.12±0.04
Fold difference MiT[W$^-$]3R2/ iso31	1.75	2.92	1.36	0.84	1.02

Summarized results of this analysis suggested that the resistance mechanism in line MiT[W$^-$]3R2 is mainly P450-based.

3.3.5. Paraquat assay fails to detect oxidative stress in line MiT[w$^-$]3R2

Unusual behavior, which manifested itself in upright wing posture and seizure-like episodes was observed in resistant adults. An oxidative stress-mediated toxicity could

cause such behaviour. Flies were analyzed for their resistance to paraquat in order to test if there is a decrease in antioxidant defense.

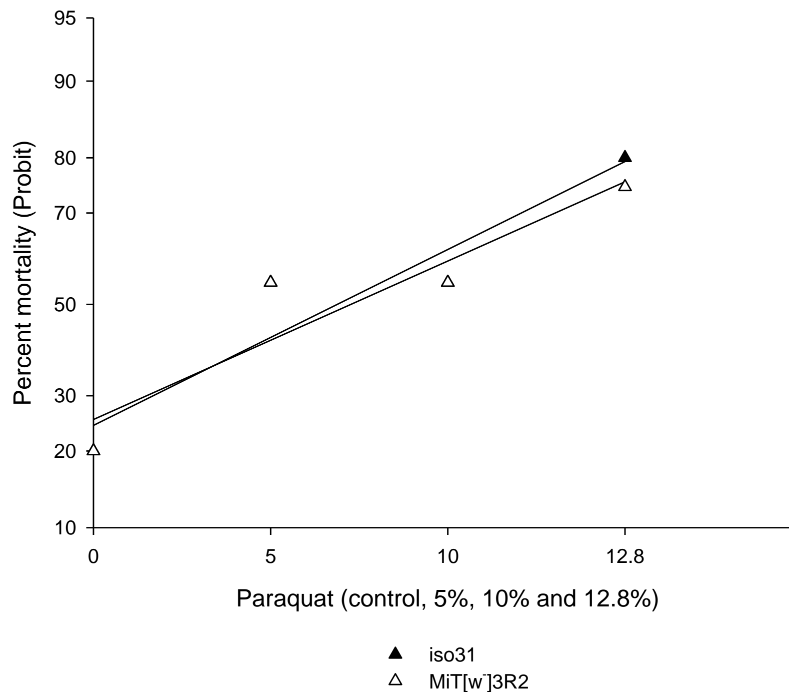


Figure 30. Dose-response curves of the susceptible and resistant lines on 5%, 10% and 12.8% concentrations of paraquat

Table 19. 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

The analysis did not yield any significant difference in survival between the resistant and the susceptible line (figure 30, table 19), thus there is no indication of a decrease in antioxidant defense in the resistant line MiT[W-]3R2.

3.4. Molecular analysis

3.4.1. Standard PCR analysis

3.4.1.a. Nature of orange eyes phenotype in MIT[orange]3R2 resistant line remains unclear

The line MIT[orange]3R2 and line MIT[w^-]3R2 were derived from the original resistant line identified in a insertional mutagenesis screen. During the generation of the resistant flies, three constructs (TREP, BOEtTA and MiT 2.4), carrying a *Mini white* gene were used, which can, depending on the chromosomal location, cause an orange eyes phenotype in a *white* background. It is unlikely that the orange marker derives from the BOEtTA 6.24 insertion, since this is located on the X chromosome and, as a P-element based construct, was not mobilized during the screen. Although the original TREP2.30 insertion was located on the 4th chromosome, this element was mobilized and thus could be a possible source of the orange phenotype gene. However, in the original resistant line the TREP insertion was genetically mapped to the X chromosome, while the orange marker was mapped to the second chromosome. Flies from the iso31[SM6, MiT2.4]/Sco line carry the P-element-based MiT2.4 construct (*Minos* transposase source) on a second chromosome balancer. In order to obtain more information about the nature of the orange marker gene, standard PCR analysis was initially used. A *Mini white* gene, which produces in this case an orange eyes phenotype, could derive either from the TREP construct, or from the MiT 2.4 insertion that carries a *Minos* transposase gene in the line iso31[SM6, MiT 2.4]/Sco. Iso31[SM6, MiT 2.4]/Sco, MiT[w^+]3Rx (TREP) and plasmid MiT 2.4 samples were used as positive controls. Line MiT[w^-]3R2/SM6 (without marker gene) and line iso31 were used as negative controls. PCR products were analyzed on 1% agarose gels, as depicted in figure 32. The expected PCR product was detected in all three positive controls. In the negative control (iso31 line), no PCR product was generated. However, also for both lines (MiT[w^-]3R2/SM6 and MIT[orange]3R2/SM6) derived from the original resistant line, no PCR product was detected (figure 31).

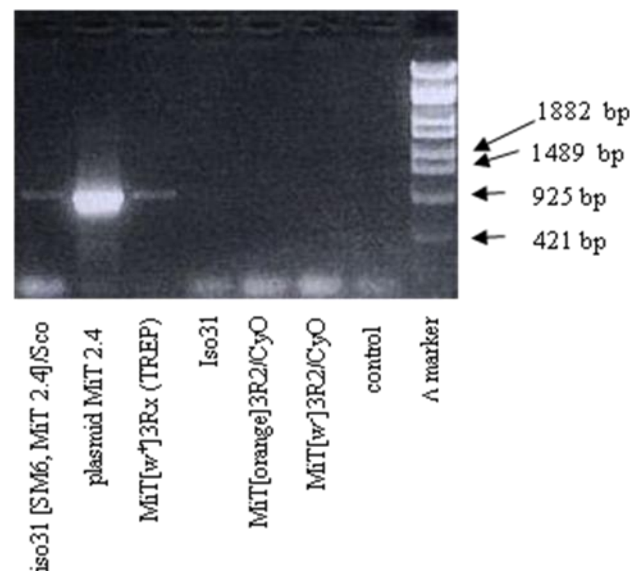


Figure 31. Agarose gel (1%) for PCR detection of a *Mini white* gene fragment in lines iso31[SM6, MiT 2.4]/Sco, MiT[w^+]3Rx (TREP), iso31, MIT[orange]3R2/SM6 (MIT[orange]3R2/CyO), MiT[w^-]3R2/SM6 (MiT[w^-]3R2/CyO) and in plasmid MiT 2.4

The *Mini white* gene is joined to a *Minos* end in the TREP construct (figure 4). PCR primers were designed to yield a product containing part of a *Minos* end and part of the *Mini white* gene. Line MiT[w^+]3Rx (TREP) was used as a positive control, while lines iso31 and MiT[w^-]3R2/SM6 served as negative controls. PCR products were analyzed on 1% agarose gel as depicted in figure 32. The expected PCR product was detected in line MiT[w^+]3Rx (TREP) (positive control). In the negative controls, as well as in the resistant line MiT[orange]3R2/SM6, however, no products were detected (figure 32).

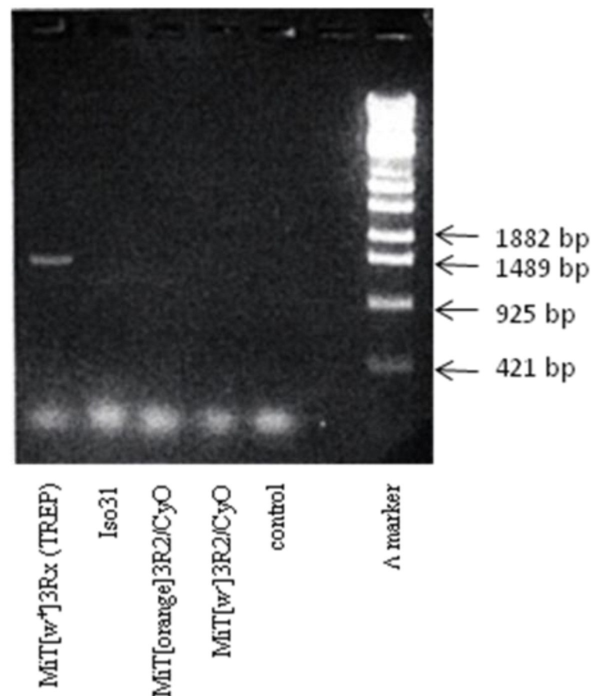


Figure 32. Agarose gel (1%) for PCR product detection of joined *Minos* end and *Mini white* gene sequence product in lines MiT[w$^+$]3Rx (TREP), iso31, MIT[orange]3R2/SM6 (MIT[orange]3R2/CyO) and MiT[w$^-$]3R2/SM6 (MiT[w$^-$]3R2/CyO)

The *Mini white* gene could also derive from construct MiT 2.4 of line iso31[SM6, MiT 2.4]/Sco, which carries a *Minos* transposase gene. A standard PCR analysis for the detection of *Minos* transposase gene was performed. DNA samples from line iso31[SM6, MiT 2.4]/Sco and plasmid MiT 2.4 were used as positive controls. Lines MiT[w$^+$]3Rx (TREP), iso31 and MiT[w$^-$]3R2/SM6 were used as negative controls. PCR products were analyzed on 1% agarose gels, as presented in figure 33. In all positive controls, the expected PCR products were detected, while in the negative controls, as well as in line MiT[orange]3R2/SM6 PCR, no PCR product was formed (figure 33).

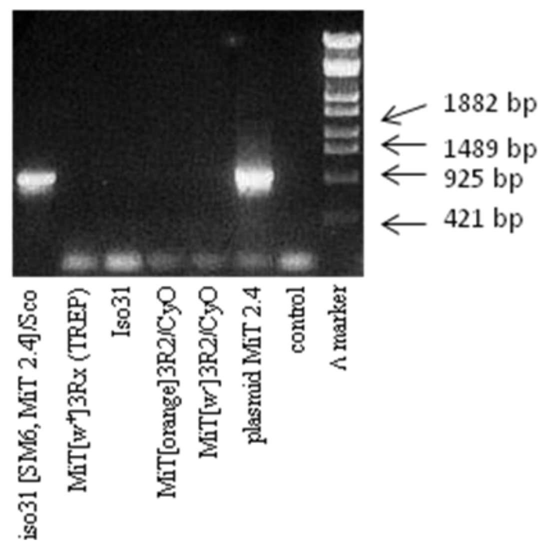


Figure 33. Agarose gel (1%) of PCR reactions for detection of the *Minos* transposase gene in lines iso31 [SM6, MiT 2.4]/Sco, MiT[w$^+$]3Rx (TREP), iso31, MIT[orange]3R2/SM6 (MIT[orange]3R2/CyO), MiT[w$^-$]3R2/SM6 (MiT[w$^-$]3R2/CyO) and in plasmid MiT 2.4

A *Mini white* gene, which in this case produces an orange eyes phenotype in the MIT[orange]3R2/SM6 line, could not be detected with used primers and obtained PCR technique. This result however does not reject the hypothesis that the orange marker derives either from the TREP construct or the MiT 2.4 insertion that carries a *Minos* transposase gene. Additional analysis is required to obtain more information about the nature of orange marker.

3.4.1.b. Overexpression of individual P450 genes is observed in Imidacloprid resistant line

Results of the P450 activity assays show increased activity of these enzymes in the resistant line compared to susceptible line. Recent studies of neonicotinoid resistance show that overexpression of one or more P450s appears to be additional or even primary resistance mechanism to neonicotinoid in different insect species (Puinean *et al.*, 2010; Karunker *et al.*, 2009). Thus, overexpression of individual representative P450 genes in the resistant line was analyzed. For this purpose genes already known to be overexpressed in resistant *Drosophila* lines (Daborn *et al.*, 2001; Le Goff *et al.*, 2003; Daborn *et al.*, 2007) have been chosen for this analysis. Semi-quantitative PCR of reverse transcribed total RNA was performed to analyze the relative expression of

the representative cytochrome P450 genes Cyp6g1, Cyp6a2, Cyp6a8 and Cyp12d1 in the resistant and susceptible lines. Flies used for semi-quantitative PCR were reared on standard medium and medium with Imidacloprid. Induction of different P450 genes (including Cyp6g1, Cyp6a2, Cyp6a8 and Cyp12d1) by several compounds including insecticides was reported in different *Drosophila* lines (Morra *et al.*, 2010; Giraudo *et al.*, 2010). Flies selected on Imidacloprid were used to analyze possible Imidacloprid-mediated induction of the representative P450 genes. Samples of the PCR reactions were analyzed on 2% agarose gels, starting from the 20th until the 40th cycle. Products were detected for all genes (including control gene Rp49) in both lines, except for gene Cyp12d1, which was not detected in the susceptible line (table 20).

Table 20. PCR fragment production of genes Cyp6g1, Cyp6a2, Cyp6a8, Cyp12d1 and Rp49 from a resistant and a susceptible line, analyzed on 2% agarose gels at the 25th cycle

	Cyp6g1	Cyp6a2	Cyp6a8	Cyp12d1	Rp49
MiT[W^{-}]3R2	+	+	+	+	+
iso31	+	+	+	-	+

The difference between product amounts between the two lines was analyzed at the 25th cycle on 2% agarose gels (figures 34, 35, 36 and 37). Housekeeping gene (quantitative control samples) PCR products were detected at high and equal amounts for both lines, reared on standard and Imidacloprid medium (figure 34).

RP49 housekeeping

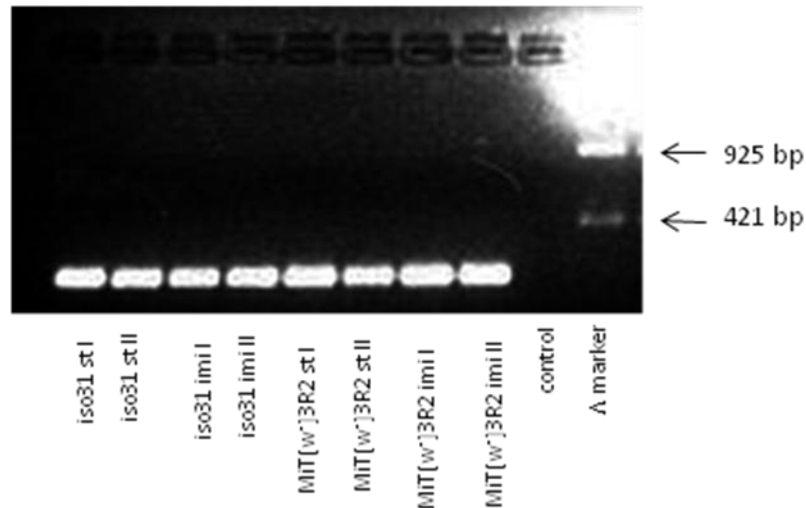


Figure 34. Semi-quantitative RT-PCR (on a 2% agarose gel) detection of control gene RP49 mRNA in susceptible iso31 and resistant MiT[w^{-}]3R2 flies raised on standard medium (st) and medium with Imidacloprid (imi)

In resistant flies, maintained on both media, higher amounts of RT-PCR products of genes Cyp6g1 and Cyp6a2 were detected in resistant versus susceptible flies (figure 35).

The Cyp12d1 RT-PCR product was detected only in the resistant line MiT[w^{-}]3R2 and only at a low quantity, under both rearing conditions (figure 36).

The amount of Cyp6a8 PCR product was about the same in both lines and independent of rearing conditions (figure 37).

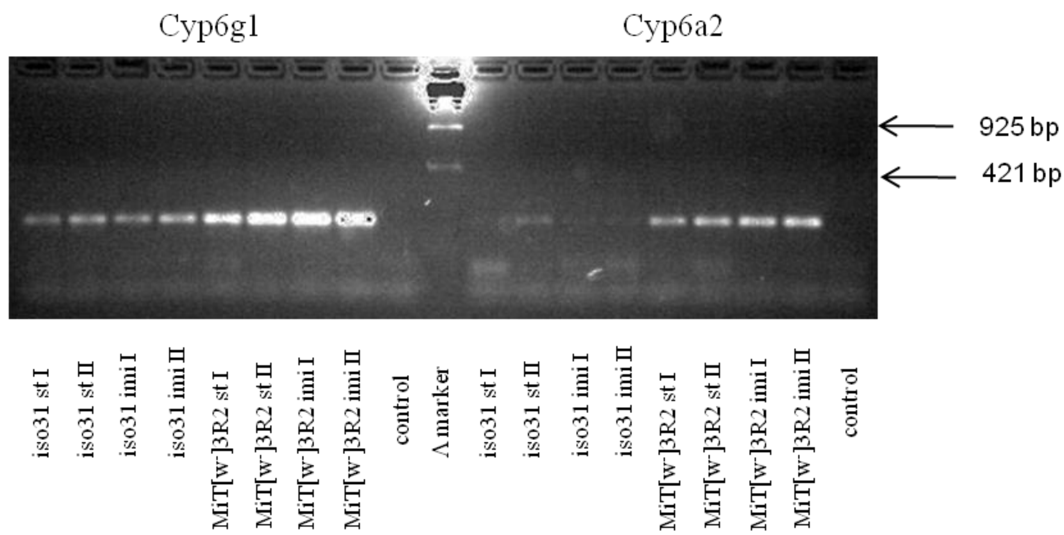


Figure 35. Semi-quantitative RT-PCR (on a 2% agarose gel) detection of Cyp6g1 and Cyp6a2 mRNAs in susceptible iso31 and resistant MiT[w$^-$]3R2 flies raised on standard medium (st) and medium with Imidacloprid (imi)

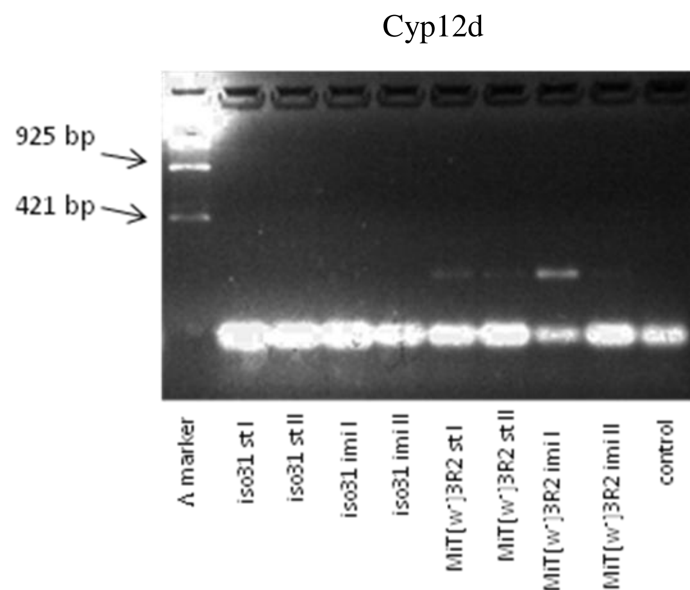


Figure 36. Semi-quantitative RT-PCR (on 2% agarose gel) detection of Cyp12d1 mRNA in susceptible iso31 and resistant MiT[w$^-$]3R2 flies raised on standard medium (st) and medium with Imidacloprid (imi)

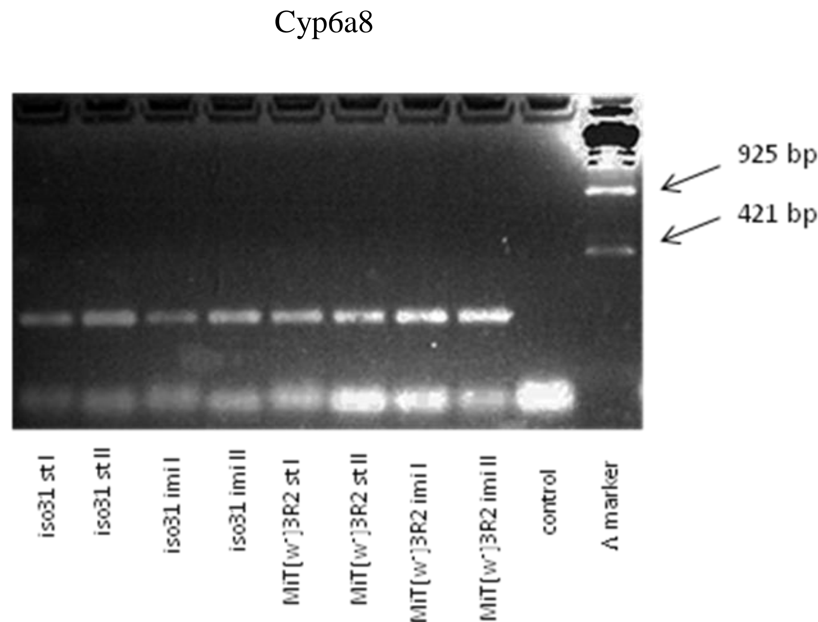


Figure 37. Semi-quantitative RT-PCR (on 2% agarose gel) detection of Cyp6a8 mRNA in susceptible iso31 and resistant MiT[w$^-$]3R2 flies raised on standard medium (st) and medium with Imidacloprid (imi)

The summarized results of the analysis show higher expression of the Cyp6g1, Cyp6a2, and Cyp12d1 in the resistant line compared to susceptible line. Following semi-quantitative RT-PCR analysis, in order to more accurately quantify the relative expression of the representative cytochrome P450 genes in the two lines, quantitative real time RT-PCR was used.

3.4.2. Real time RT-PCR shows increased levels of expression of some representative cytochrome P450 genes in the resistant line

The relative expression of the cytochrome P450 genes was measured between the resistant line MiT[w$^-$]3R2 and the susceptible line iso31, maintained both on standard medium and medium with Imidacloprid. The real time RT-PCR analysis, like in the previous semi-quantitative PCR analysis, did not detect Cyp12d1 expression in iso31.

It was therefore not possible to analyze the relative expression of this gene between line MiT[W⁻]3R2 and the susceptible line.

Elevated expression of Cyp6g1 was detected in the resistant line compared to the susceptible line (figure 38, Appendix - table 1). The MiT[W⁻]3R2 resistant flies, reared on both media, had ~ 8-fold higher expression of Cyp6g1 compared to susceptible flies reared on standard medium. Resistant flies maintained on standard medium and medium with Imidacloprid had a 7-fold higher expression of Cyp6g1 compared to susceptible flies reared on medium with Imidacloprid (figure 38, Appendix - table 1). There were no significant changes of Cyp6g1 expression between Imidacloprid-reared and standard medium-reared resistant flies. The same is true for the susceptible iso31 flies reared on standard medium and medium with Imidacloprid (figure 38, Appendix - table 1).

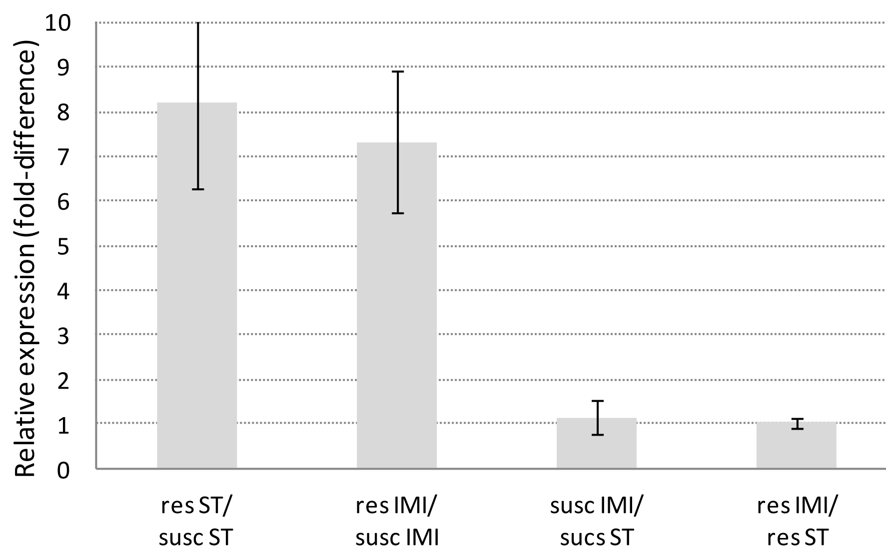


Figure 38. Expression difference of gene Cyp6g1 between two lines maintained on standard medium and medium with Imidacloprid (res – resistant line; susc – susceptible line; ST – standard medium; IMI – medium with Imidacloprid)

As for Cyp6g1, an elevated expression of Cyp6a2 was detected in the resistant line MiT[w⁻]3R2 compared to the susceptible line iso31 (figure 39, Appendix - table 2). Resistant flies maintained on standard medium had 10-fold higher Cyp6a2 expression compared to susceptible flies maintained on the same medium. The same lines, maintained on Imidacloprid medium, show an 8-fold higher Cyp6a2 expression in the resistant line (figure 39, Appendix - table 2). Resistant flies maintained on standard

medium did not have a significantly different Cyp6a2 expression compared with resistant flies maintained on medium with Imidacloprid. The same is true for flies from the susceptible line (figure 39, Appendix - table 2) reared on the two different media.

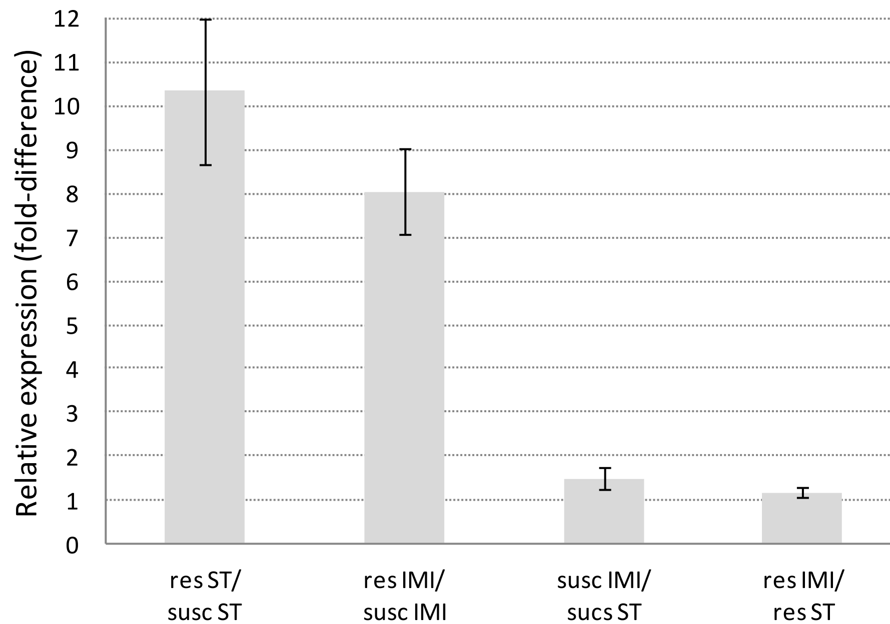


Figure 39. Expression difference of gene Cyp6a2 between two lines maintained on standard medium and medium with Imidacloprid (res – resistant line; susc – susceptible line; ST – standard medium; IMI – medium with Imidacloprid)

There was no significant difference of Cyp6a8 expression between the resistant and susceptible lines, maintained on different media (table 21).

Table 21. Expression difference of gene Cyp6a8 between two lines maintained on standard medium and medium with Imidacloprid (res – resistant line; susc – susceptible line; ST – standard medium; IMI – medium with Imidacloprid)

Cyp6a8	res ST/ susc ST	res IMI/ susc IMI	res IMI/ susc ST	res ST/ susc IMI	susc IMI/ susc ST	res IMI/ res ST
Fold difference	1.13 ± (0.25)	1.43 ± (0.27)	1.44 ± (0.21)	1.12 ± (0.28)	1.01 ± (0.21)	1.28 ± (0.25)

In general, quantitative real time RT-PCR analysis detected significant overexpression (more than 5-fold) of the Cyp6g1 and Cyp6a2 genes in the resistant line compared to susceptible line. Imidacloprid had no significant effect on inducibility of Cyp6g1 and Cyp6a2.

3.4.3. Quantitative PCR analysis shows no amplification of the Cyp4p2, Cyp6g1 and Cyp6a2 genes in the resistant lines

It appears that the overexpression of P450 genes in a different resistant insect species is exclusively attributed to one molecular mechanism - increased transcription (Scott, 1999). A recent report of P450 gene amplification associated with neonicotinoid resistance in the aphid *Myzus persicae* shows the existence of another molecular mechanism apart from increased transcription, that causes elevated P450 levels (Puinean *et al.*, 2010). Deep sequencing analysis detected 3 highly overexpressed genes (Cyp4p2, Cyp6g1 and Cyp6a2) in the MiT[w$^-$]3R2 resistant line compared to susceptible line iso31. In order to test if there is relative amplification of the three Cyp genes between resistant line MiT[w$^-$]3R2 and susceptible line iso31, quantitative PCR analysis was performed on genomic DNA. No relative amplification of these genes in resistant line MiT[w$^-$]3R2 compared to susceptible line iso31 was detected (table 22).

Table 22. Quantitative real time PCR analysis results for amplification of Cyp4p2, Cyp6g1 and Cyp6a2 genes in resistant line compared to susceptible line.

	Gene amplification: fold difference ±(SD) (resistant/susceptible)
Cyp4p2	1.39 ± (0.23)
Cyp6g1	1.25 ± (0.15)
Cyp6a2	1.27 ± (0.13)

In conclusion, results show that amplification is not mechanism that brings to increased expression of analyzed P450 genes in the resistant MiT[w$^-$]3R2 line.

3.5. Transcriptomic profiling identified a high number of differently expressed genes between resistant and susceptible line

In order to get more information on transcriptome variation and gene interaction networks in the resistant line, deep sequencing transcriptomic analysis was performed. Whole genome transcriptional profile analyses of the of resistant line MiT[w$^-$]3R2 and the susceptible line iso31 was performed with the Illumina deep sequencing technique (Illumina Inc., 2010). Deep sequencing yielded in total 16344712 high quality reads for line MiT[w$^-$]3R2 and 16859384 high quality reads for line iso31 (Data uploaded to GEO site; Appendix – table 3 and table 4). All 51 nt long reads from both, resistant and susceptible lines, were mapped to the *Drosophila* reference genome (*Drosophila* release 5 sequence assembly Flybase). The alignment of the reads to the *Drosophila* reference genome identified 18963 distinct transcripts for the susceptible line and 18967 distinct transcripts for the resistant line (Data uploaded to GEO site; Appendix - table 5). Using a minimum difference threshold of 2-fold, a total of 357 transcripts were found to be differently expressed between lines MiT[*w*$^-$]3R2 and iso31 (Data uploaded to GEO site; Appendix - table 5). 150 genes were upregulated and 207 genes were downregulated in the resistant line compared to the susceptible line (figure 40, Data stored electronically on CD – table 6 and table 7 excel files). The highest upregulated gene (251 fold) encodes Chorion protein 38, and the highest downregulated gene (140 fold) encodes lectin-37Da (figure 40, Data stored electronically on CD – table 6 and table 7 excel files).

Figure 40. Volcano plot of up- and down-regulated genes in the resistant line (as compared to the susceptible line)

X axis - log2 (number of reads in resistant line) / (number of reads in susceptible line)
Y axis - log2 (number of reads in resistant line) + (number of reads in susceptible line)

Of the 150 upregulated genes, eight are members of the P450 gene family (figure 40, Data stored electronically on CD – table 6 excel file). The three highest upregulated genes were Cyp4p2 (100-fold), Cyp6a2 (19.85-fold) and Cyp6g1 (16.31-fold) (figure 41, table 23). The other five are Cyp6w1 with 5.97-fold, Cyp4e3 with 5.21-fold, Cyp309a2 with 4.38-fold, Cyp6g2 with 2.85-fold and Cyp4d14 with 2.35-fold upregulation in the resistant line. Three of the 207 downregulated genes are also from the P450 family - Cyp9b1 with 9.33-fold, Cyp4d21 and Cyp4p1 both with 4.77-fold lower expression in the resistant line (figure 41, table 23). Among the 357 differentially expressed genes, neither glutathione-S-transferase nor esterase genes were detected (figure 40, Data stored electronically on CD – table 6 and table 7 excel files).

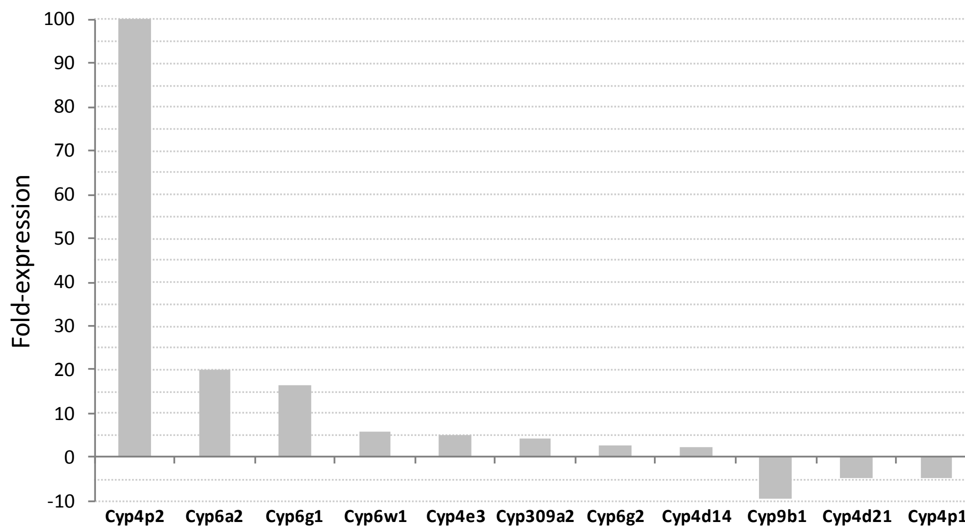


Figure 41. Up- and downregulated Cyp genes in the resistant line (with respect to the susceptible line)

Table 23. Up- and downregulated Cyp genes in the resistant line (with respect to the susceptible line)

Upregulated		
Symbol	Location (arm)	Fold-change
Cyp4p2	2R	100.00
Cyp6a2	2R	19.85
Cyp6g1	2R	16.31
Cyp6w1	2R	5.97
Cyp4e3	2L	5.21
Cyp309a2	2L	4.38
Cyp6g2	2R	2.85
Cyp4d14	X	2.35
Downregulated		
Symbol	Location (arm)	Fold-change
Cyp9b1	2R	9.33
Cyp4d21	2L	4.77
Cyp4p1	2R	4.77

Deep sequencing of cDNA of the resistant line shows high expression levels of major chorion genes Cp38 (251-fold), Cp36 (89-fold), Cp7Fc (150-fold) and Cp7Fb (57-

fold). Also, yellow-g2 and yellow-g are expressed 37 and 8 times higher in the resistant MiT[w$^-$]3R2 line compared to susceptible iso31 line (figure 40, Data stored electronically on CD – table 6 excel file).

An odorant binding protein (Obp19c) is found among the first 20 highly expressed genes, with approximately 15-fold higher expression in line MiT[w$^-$]3R2 (figure 40, Data stored electronically on CD – table 6 excel file).

Gene functional classification analysis by grouping genes based on functional similarities identified three functional groups in the upregulated genes (table 24) and two functional groups in the downregulated genes (table 25). Cyp P450 genes, proteolytic genes and genes showing peptidase activity were overrepresented in the upregulated genes. The enrichment score (the geometric mean (in log scale) of the members' p-values in a corresponding annotation cluster, used to rank their biological significance statistically measured by Fisher Exact test; Huang *et al.*, 2009a) of the gene functional group showing proteolytic activity is 5.10, while the enrichment score for the Cyp P450 gene group is 3.73. The gene group that includes metallocarboxypeptidase activity, biopolymer catabolic process and macromolecule catabolic process has an enrichment score of 2.32 (table 24). Cuticular protein genes and genes showing peptidase activity were overrepresented in the downregulated genes. The enrichment score of structural constituents of chitin-based cuticle group of genes is 2.52, and the enrichment score of genes showing peptidase activity and proteolysis is 1.05 (table 25).

Table 24. Gene functional groups in the up-regulated genes (analyzed with the DAVID 6.7 BETA bioinformatics resource)

Peptidase activity, proteolysis		Cytochrome P450 (Cyp) genes		Metallocarboxypeptidase activity, biopolymer catabolic process, macromolecule catabolic process	
Enrichment Score: 5.10		Enrichment Score: 3.73		Enrichment Score: 2.32	
Gene name	Kappa*	Gene name	Kappa*	Gene name	Kappa*
CG31219	1.00	Cyp309a2	0.99	CG8539	0.89
Jonah 65Ai	1.00	Cyp6w1	0.99	CG8560	0.84
CG10469	1.00	Cyp4p2	0.96	CG15254	0.65
CG9676	1.00	Cyp6g2	0.96	CG2493	0.62
CG7829	0.97	Cyp4d14	0.94	CG31918	0.59
Jonah 25Biii	0.97	Cyp4e3	0.93		
CG32277	0.97	Cyp6g1	0.87		
CG4259	0.97	Cyp6a2	0.84		
Jonah 74E	0.94				
CG10477	0.94				
Jonah 25Bii	0.91				
CG11911	0.91				
CG4812	0.91				
Jonah 25Bi	0.84				
CG31918	0.59				
CG2493	0.56				

Table 25. Gene functional groups in the down-regulated genes (analyzed with DAVID 6.7 BETA bioinformatics resource)

Structural constituent of chitin-based cuticle		Peptidase activity, proteolysis	
Enrichment Score: 2.52		Enrichment Score: 1.05	
Gene name	Kappa*	Gene name	Kappa*
CG1252	1.00	CG17234	1.00
CG2360	1.00	CG18180	0.97
CG2341	1.00	CG18179	0.97
Cuticular protein 56F	0.91	CG11037	0.94
Cuticular protein 47Ef	0.83	Jonah 66Ci	0.94
		Serine protease 12	0.88
		CG34043	0.80

*Kappa score – The Kappa value quantitatively measures the degree to which genes share similar annotation terms (the higher the Kappa, the stronger the functional similarity)

Functional annotation clustering identified 10 groups with similar predicted biological functions in the upregulated genes and 13 groups in the downregulated genes (Data stored electronically on CD – table 8 and table 9 excel files). Among the functional groups in the upregulated genes, four clusters are connected to peptidase activity and three functional clusters are connected to P450 gene family activity. There were also overexpressed genes significantly overrepresented in other functional groups like oxidoreductase activity, mitotic sister chromatid segregation, electron carrier activity and response to DNA damage (Data stored electronically on CD – table 8 excel file). In the downregulated genes, groups like nutrient reservoir activity, chitin and aminoglycan metabolic processes, response to bacteria and immune response activity were identified (Data stored electronically on CD – table 9 excel file).

Deep sequencing transcription profiling detected significant number of differently expressed genes between resistant and susceptible lines, suggesting a complex insecticide resistance mechanism. Gene ontology analysis identified several overrepresented functional gene groups that are differentially expressed in the resistant *Drosophila* line. Eight cytochrome P450 were significantly overrepresented in the upregulated genes suggesting their potential role in the resistance mechanism, as well as confirming P450-based resistance mechanism of the MiT[w^-]3R2 line. Additional bioinformatics analysis of the deep sequencing data was further performed for more information about the nature of the mutation that causes resistance.

3.5.1. *In silico* analysis of deep sequencing data of the resistant and susceptible lines

It has been suggested that mutations of *trans*-regulating factor/s, or of cis-acting elements of some of the Cyp genes are responsible for insecticide resistance in *Drosophila* (Maitra *et al.*, 2000; Morra *et al.*, 2010; Giraudo *et al.*, 2010). The deep sequencing information was further explored using bioinformatics analysis tool for identification of a resistance mutation or putative regulating factor in the MiT[w^-]3R2 line.

3.5.1.a. Deep sequencing data bioinformatics analysis failed to detect common regulatory factor linked with the resistance in line MiT[w$^-$]3R2

Comparison of the sequences of the Cyp genes differently expressed in the resistant versus the susceptible line showed no sequence changes of the P450 proteins. The flanking sequences of the differentially expressed genes were also analyzed for possible common transcription factor binding sites using the JASPAR database (Wasserman and Sandelin, 2004). The sequence of all genes was retrieved from Flybase (*Drosophila* release 5 sequence assembly). For each gene, the upstream 3kb, downstream 1kb and 3'UTR sequence were retrieved and analyzed. *In silico* analysis did not detect common transcription factor binding sites either just for the Cyp genes or for all overexpressed genes. A survey of predicted targets of microRNAs in the 3'UTRs sequences of all upregulated was performed with program DIANA-microT (version 3.0) (Maragkakis *et al.*, 2009). The analysis did not identify any common target site neither for all genes, nor for all Cyps.

Summarized results show that the bioinformatics sequence analysis of the significantly up- or downregulated genes did not detect putative mutation that could be linked to the resistance mechanism.

3.5.1.b. Single nucleotide polymorphism analysis of differently expressed genes sequences mapped the resistance locus within ~1Mb region in line MiT[w$^-$]3R2

Analysis of genetic variation on nucleotide level between MiT[*w*$^-$]3R2 and iso31 lines was performed with the sequences obtained from deep sequencing expression profiling, for more accurate mapping of the resistance mutation.

The sequences from the deep-sequencing analysis, as well as the Cyp gene sequences were compared between lines MiT[w$^-$]3R2 and iso31 for single nucleotide polymorphisms (SNP). MiT[*w*$^-$]3R2 was derived from the original resistant line (MiT[*w*$^-$]3X) using *Drosophila* lines with different genetic backgrounds (TREP 2.30 and BOEtTA have a yw background, while [SM6a, MiT 2.4]/Sco] is an iso31 derivative). In order to replace the genetic background of the resistant mutant with that of the susceptible control line, MiT[*w*$^-$]3R2 was back-crossed with iso31 for 6

generations under selection with 3 µg/ml of imidacloprid. SNP analysis was carried out with resistant MiT[*w*$^-$]3R2 line homogenized for iso31 background.

There were no significant differences in SNP of Cyp genes analyzed between resistant and susceptible line. A total of 12718 SNP are detected in the pooled assembly of the resistant and the susceptible strain reads. On the X chromosome 944 SNPs, on the 2L chromosome 4293, on the 2R chromosome 1309, on the 3L chromosome 4833, on the 3R chromosome 1311, and on the 4th chromosome 28 SNPs were detected. A SNP density track with the number of different SNPs per 1000nt (1Kb) between the resistant and the susceptible line is presented for each chromosome (X, 2L, 2R, 3L, 3R and 4) on the UCSC Genome Browser (Kent *et al.*, 2002) (figure 42, 43, 44, 45, 46 and 47). A list of all SNPs showing differences between the resistant and the susceptible line is stored electronically on CD – table 10 txt file.

In general, on all chromosomes single nucleotide polymorphism can be detected, with different number of polymorphic nucleotides per 1Kb (figure 42, 43, 44, 45, 46 and 47).

The line MiT[*w*$^-$]3R2, homozygous for the resistance chromosome, derives from the mutant line MiT[*w*$^-$]3R2/SM6 heterozygous for the second chromosome carrying both resistance and lethality. Genetic analysis placed resistance to the right arm of the second chromosome together with the lethality, thus the focus of the SNP analysis was 2R chromosome arm. On the right arm of the second chromosome two regions with different SNP densities can be detected which indicates a recombination event (figure 44; Data is stored electronically on CD – table 10 txt file). Resistant line was back-crossed with line iso31 and selected on Imidacloprid for 6 generation in order to homogenize genetic background. SNP analysis suggests a hybrid origin of the 2R chromosome where the right half probably comes from iso31 background, while the left half comes from a different line, most likely yw. Moreover, the position of the lethality (between 8.5Mb – 9.9Mb), resistance and recombination break point show that the recombination event occurred between the resistance and lethality loci (figure 48). The SNP analysis and P-element recombination mapping (which mapped resistance locus between 8Mb and 9.7Mb) data suggest that the resistance is located to the left of the recombination break on the 2R chromosome (figure 48). The three most

highly overexpressed P450 genes (Cyp4p2, Cyp6a2 and Cyp6g1) are also located left of the recombination brake. Combined mapping results of the SNP analysis and P-element recombination data overlap in the region of around 1 Mb between 8Mb and 8.7Mb, placing resistance locus within this region (figure 48). Interestingly, the highly over-expressed Cyp6g1 gene is located within the mapped resistance region.

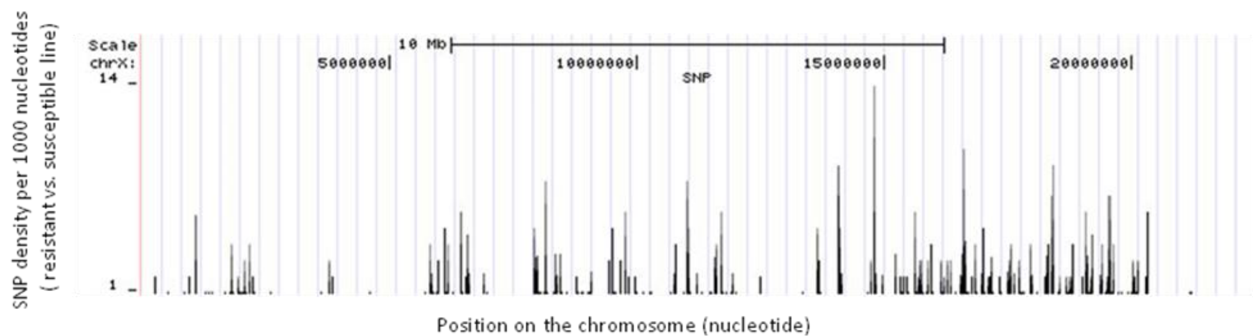


Figure 42. Single nuclear polymorphisms (SNP) density (per 1Kb) on the X chromosome between resistant line MiT[w$^-$]3R2 and susceptible line iso31

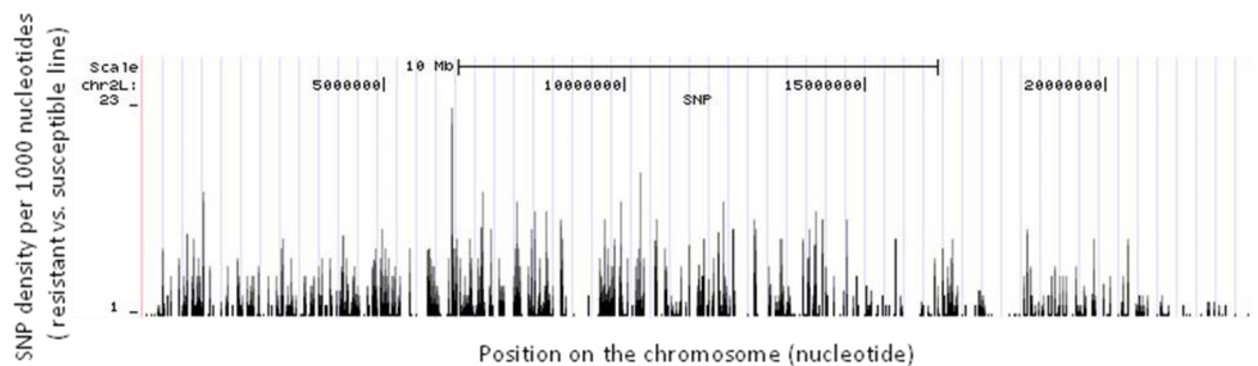


Figure 43. Single nuclear polymorphisms (SNP) density (per 1Kb) on the 2L chromosome between resistant line MiT[w$^-$]3R2 and susceptible line iso31

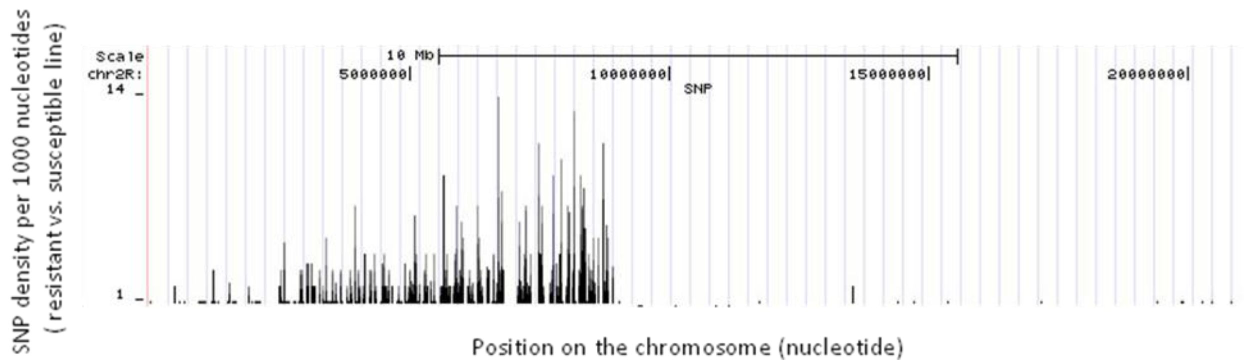


Figure 44. Single nuclear polymorphisms (SNP) density (per 1Kb) on the 2R chromosome between resistant line MiT[w-]3R2 and susceptible line iso31

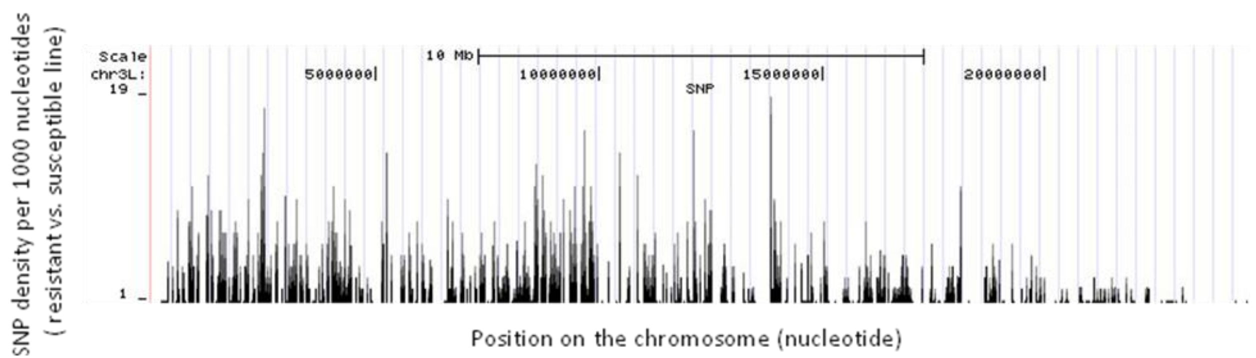


Figure 45. Single nuclear polymorphisms (SNP) density (per 1Kb) on the 3L chromosome between resistant line MiT[w-]3R2 and susceptible line iso31

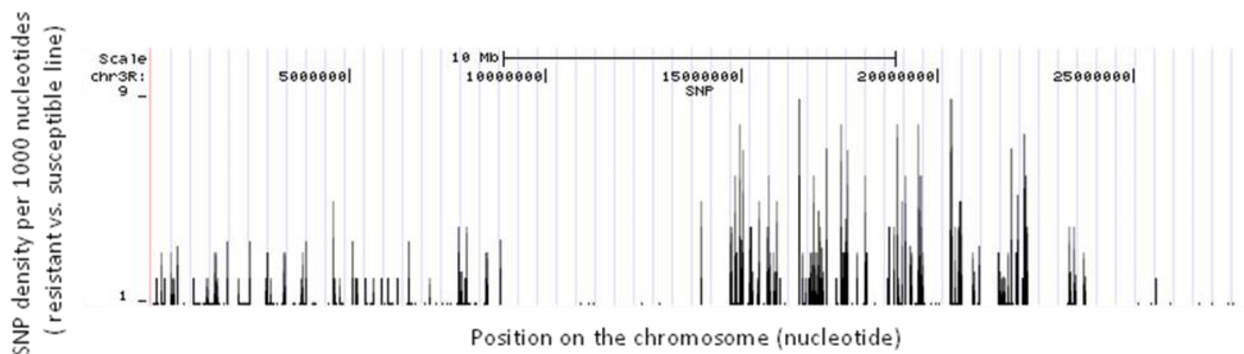


Figure 46. Single nuclear polymorphisms (SNP) density (per 1Kb) on the 3R chromosome between resistant line MiT[w-]3R2 and susceptible line iso31

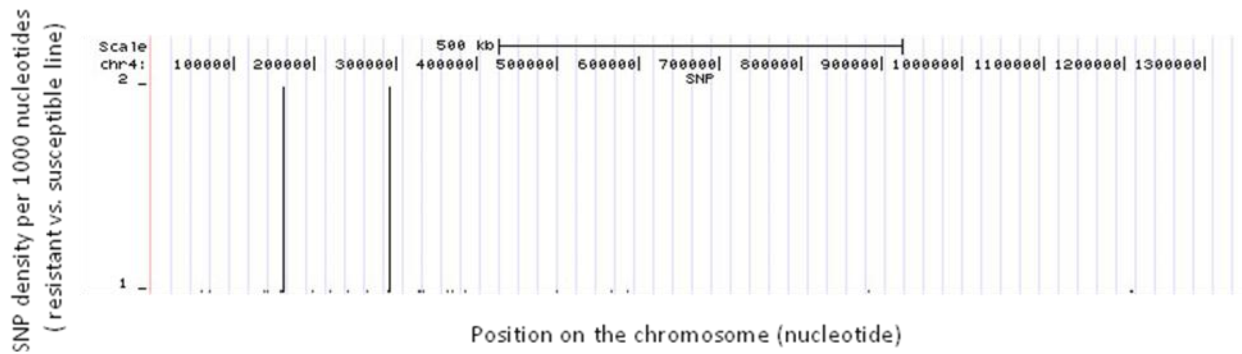


Figure 47. Single nuclear polymorphisms (SNP) density (per 1Kb) on the 4th chromosome between resistant line MiT[w-]3R2 and susceptible line iso31

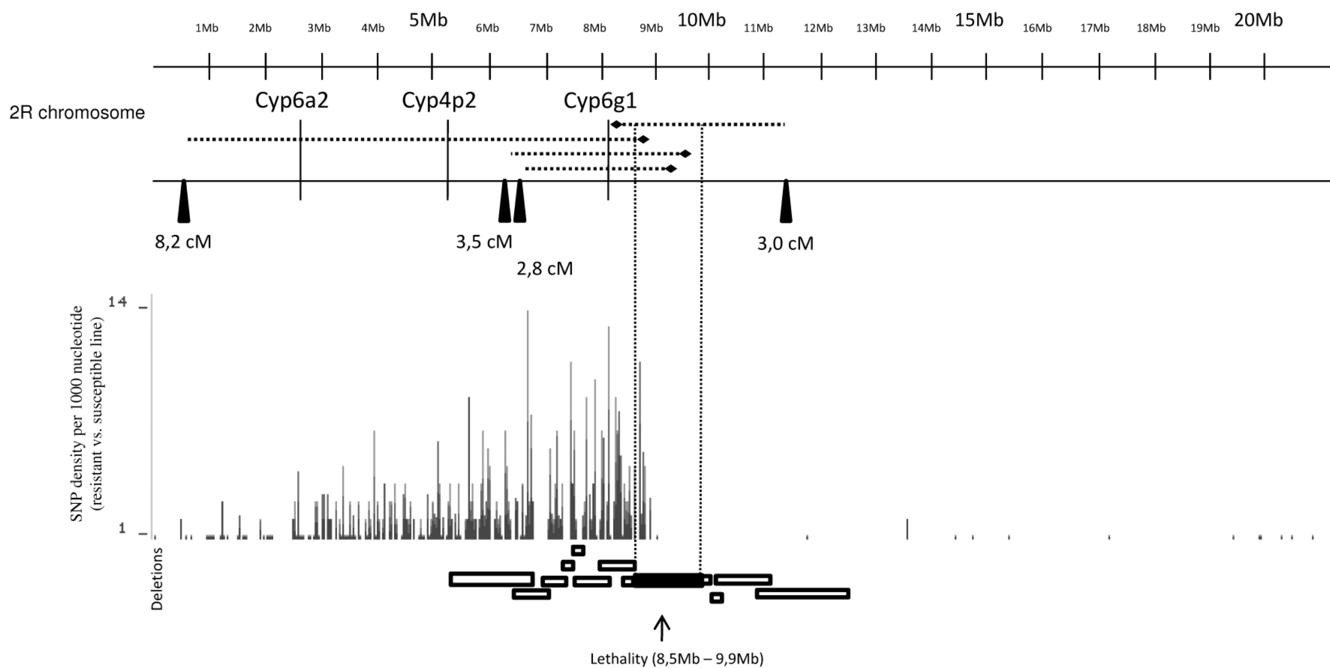


Figure 48. The resistance locus was mapped relative to P element insertions to a region between 8Mb and 8.7Mb (black arrows on the second scale, distance between insertion and resistance region is indicated with dotted horizontal lines). The location of the three highly expressed P450 genes (Cyp6a2, Cyp6g1 and Cyp4p2) in the resistant MiT[*w*-]3R2 line is indicated. Below is a comparison of single nucleotide polymorphism (SNP) density (per 1 Kb) between resistant line MiT[*w*-]3R2 and susceptible line iso31. At the bottom, Bloomington deletions overlapping lethality locus (filled box) and flanking the lethality locus (open boxes) (lethality maps to the region between 8.5 Mb and 9.9 Mb, close to the place of recombination).

The summarized results of the single nucleotide polymorphism analysis confirmed that the resistance locus is located on the right arm of the second chromosome. Moreover, genetic and SNP analysis narrowed the position of the resistance locus, close to the recombination brake point, within a ~1Mb region (8Mb and 8.7Mb). The highly over-expressed Cyp6g1 gene, already known to be involved in Imidacloprid resistance, is located within the mapped resistance region.

4. DISCUSSION

The main goal of this project was the identification of genes involved in Imidacloprid resistance, using *Drosophila melanogaster* as the model organism. Resistant mutants were to be generated by genome-wide insertional mutagenesis, using the *Minos* based transposon element TREP (**t**etracycline **r**egulatable **e**nhancer **p**romoter) as a mutagenesis vector (figure 4). Individuals with novel TREP insertions and carrying the *Minos* transposase expressing insertion BOEtTA 6.24 were selected on medium with 3 μg/ml of Imidacloprid. One *Drosophila* mutant resistant to Imidacloprid was retrieved in this screen and subjected to further analysis. The mutant was characterized using genetic, toxicological, molecular and transcriptomic approaches.

4.1. Minos-based genome-wide insertional mutagenesis

4.1.1. The TREP-BOEtTA system and conditions for screening for Imidacloprid resistance

A combination of features of the TREP and BOEtTA constructs give the TREP-BOEtTA system unique advantages for genome-wide mutagenesis. The TREP element is a transposon *Minos*-based construct for promoter-delivery in *Drosophila melanogaster*. BOEtTA is a P-element based construct which produces tetracycline trans-activator (tTA), which activates the minimal promoter on the TREP construct. In the transgenic *Drosophila* line TREP 2.30, a TREP insertion is located on the 4th chromosome. The fourth chromosome of *D. melanogaster* is the shortest one of this species (Bridges, 1935) with negligible recombination due to, most probably, its mainly heterochromatic nature (Arguello *et al.*, 2010). The insertion in line TREP 2.30 is phenotypically easily detectable, due to the presence of a *Mini white* marker gene. An important feature of this insertion is the lethality of the TREP 2.30 chromosome in the presence of a chromosome carrying the BOEtTA trans-activator expressing construct. This property permits the selection of flies that have lost the TREP 2.30 insertion as a result of transposase-induced excision of the TREP element. This allows detection of progeny of new insertions of TREP, since all viable progeny of jumpstarter flies (TREP 2.30 - transposase double heterozygotes) carrying the

BOEtTA activator and expressing the *white* marker will carry a new insertion. In order to test the transposition efficiency, as well as to confirm the lethality of TREP 2.30 in the presence of BOEtTA, two groups of crosses (Control and Jumpstarter group) were set up. In the Control group, derived from 23 TREP 2.30 females, no progeny with loss of TREP insertion was detected (table 3). This result confirms the lethality of the TREP2.30 insertion in the presence of BOEtTA. The re-integration efficiency of the TREP element was analyzed in the offspring of 49 jumpstarter females. Insertional efficiency was calculated as the percentage of TREP/Transposase females with progeny carrying new TREP insertions (at least one out of 100 progeny *per* female) of the total number of jumpstarter females analyzed. The re-integration efficiency of the TREP construct was found to be high: 92% of jumpstarter females gave progeny with new insertions on all four chromosomes in *Drosophila melanogaster* genome (table 3). This is in agreement with a previous report of the transposition efficiency of *Minos*-based constructs (Metaxakis *et al.*, 2005). Jumpstarter females were also analyzed for the average percentage of gametes with a new insertion. The analyzed females exhibit high mobilization of the TREP vector in germ cells, having on average 2.83 % of gametes with new insertions (table 3). This result demonstrates that TREP 2.30 can be used efficiently for large scale, genome wide insertional mutagenesis screens.

Next, the TREP transposon was tested for the frequency of local jumps. The term "local jumps" refers to transposition events where the element re-inserts into the same chromosome from which it is excised (in line TREP 2.30, the element is located on the 4th chromosome). In total, 34 males carrying new insertions were analyzed for the chromosomal location of the TREP vector. Insertion on the 4th chromosome (local insertion) was found in about 1/3 of the analyzed flies (table 4).

The minimal Imidacloprid concentration that induces 100 % lethality in iso31 flies was determined to be 1 µg/ml. In order to prevent high number of escapers but still allow the survival of mutants exhibiting high levels of resistance, 3 times the minimal lethal concentration (3 µg/ml of Imidacloprid) was chosen for the selection of resistant individuals with novel TREP insertions.

4.1.2. Genome-wide insertional mutagenesis

Approximately 1400000 embryos (deriving from approximately 14000 jumpstarter females) were selected on medium with 3 μg/ml Imidacloprid during the genome-wide mutagenesis screen (table 5). Since 92% of jumpstarter females produce offspring with transposed TREP elements, it is estimated that about 12900 new insertions were generated during the screen. Insertional sites analysis has shown that 47% of total *Minos* insertions were found to be within or close to (2 kb upstream) known or predicted genes (Metaxakis *et al.*, 2005). Hence, during this screen 6063 insertions (47% of 12900 new TREP insertions) are expected to be within or close to (2 kb upstream) known or predicted genes including introns. Although the analysis of transposition events has shown that *Minos* insertions into the *D. melanogaster* genome can be considered random (Metaxakis *et al.*, 2005), the Poisson distribution has been used for the multiple insertions into the same genes correction (Pollock and Larkin, 2004). According to the Poisson distribution it is calculated that 26% of 6063 insertions will hit the same gene two times or more, thus 4487 insertions (74% of the 6063 insertions) in this screen are expected to hit gene once including introns. With estimated 13000 known or predicted genes in *Drosophila* (Adams *et al.*, 2000), in this screen 35% ((4487 hit genes /13000 known or predicted genes)*100) of genes are expected to be targeted once by a TREP element insertion (table 5). Insertional sites analysis shown that 29.2% of total *Minos* insertions were found to be within or close to (2 kb upstream) known or predicted genes, excluding introns (Metaxakis *et al.*, 2005). Thus, approximately 22% of known or predicted genes of *Drosophila* genome were hit at least once, directly or within 2 Kb upstream and downstream, excluding introns (Metaxakis *et al.*, 2005). Calculations were performed similarly, as for the *Minos* insertions including introns (29.2% of 12900 new hits is 3767 insertions with the Poisson distribution showing that 2788 insertions are expected to hit gene once (74% of the 3767 insertions results) which makes in total 22% ((2788 hit genes /13000 known or predicted genes)*100))). Although there is no apparent preference for insertions into genes, there is certain preference of *Minos* transposon for insertion into introns vs. exons (Metaxakis *et al.*, 2005).

Only TREP element insertions where the outwards-pointing promoter lies in the same direction as the promoter of the targeted gene will be able to overexpress the gene or gene fragment downstream of insertion (in presence of the BOEtTA construct). Assuming that 50% of the insertions will be in the correct orientation, in 17.5% of the known or predicted genes one TREP insertion of the correct orientation is expected. It is expected that approximately 22% of known or predicted genes, excluding introns, were hit at least once. Taking into account presented calculations we can estimate that up to 11% of the genes in *Drosophila* genome were "functionally" targeted (one TREP insertion of the correct orientation in known or predicted genes excluding introns is expected).

Surviving *Drosophila* individuals exhibiting high resistance to Imidacloprid correlated with a TREP element insertion are expected to overexpress genes (or trunctated genes) involved in resistance to neonicotinoids. Three major gene families - esterases, glutathione S-transferases and cytochrome P450 monooxygenases - are involved in detoxification of insecticides (Hemingway, 2000). A target-site modification (replacement Y151S) in the two alpha subunits of the nicotinic acetylcholine receptor (nAChR) confers resistance to neonicotinoids (Liu *et al.*, 2005). The most commonly found family of genes involved in metabolic mechanisms of insecticide resistance is the cytochrome P450 gene superfamily (Scott and Kasai, 2004). It has been estimated that the cytochrome P450 gene superfamily is represented by 89 genes in the *Drosophila melanogaster* genome (Tijet *et al.*, 2001). Not all of these genes are involved in xenobiotic metabolism (including insecticides). Scott (2008) estimated that the fraction of P450s involved in xenobiotic metabolism processes of insects is 30 percent. For *Drosophila melanogaster,* this would make approximately 27 cytochrome P450 genes that are involved in detoxification. The TREP element coverage is estimated to be 35%, hence at least nine P450 genes are expected to be targeted by one TREP insertion, half of which have the TREP-delivered promoter in transcription direction. Excluding introns, where the TREP element coverage is estimated to be approximately 22%, six P450 genes are expected to be targeted by one TREP insertion, half of which have the TREP-delivered promoter in transcription direction. In *Drosophila melanogaster*, 39 glutathione S-transferases, 35 carboxylesterases, as well as 14 genes that code for different nicotinic

acetylcholine receptors (nAChR) have been identified (Low *et al.*, 2007; Ranson *et al.*, 2002; Tweedie *et al.*, 2009). If all genes expected to be involved in detoxification response are included, one can expect that around 40 detoxification response genes are targeted by one TREP element insertion. Half of the targeted detoxification response genes will have new TREP insertion in the correct orientation. Excluding introns, approximately 25 detoxification response genes are targeted by one TREP element insertion, in which half of them will have new TREP insertion in the correct orientation.

During the screen, a total of 708 surviving individuals emerged, out of 1400000 embryos transferred to medium with Imidacloprid (table 5). Thus, the overall lethality of the transgenic flies selected on 3 μg/ml of Imidacloprid was 99.95%.

During the genome-wide mutagenesis screen, a total of eight Imidacloprid resistant females with new TREP element insertions were detected. The number of female survivors with a TREP element is much lower than the number of male escapers carrying a TREP element (see above).

In four of the eight female survivors, the GFP marker (marker of the BOEtTA transposon construct on the X chromosome) could not be detected. This could have several reasons, like suppression of GFP gene expression on the BOEtTA construct, or lack of the BOEtTA X chromosome. A contamination of the TREP 2.30, iso31 [SM6, MiT 2.4]/Sco and/or BOEtTA lines that were used for the generation of the mutants could be the cause of this phenomenon. On the other hand, a thorough examination of the used stocks did not reveal any such contamination. During the genome-wide screen, around 14000 virgin TREP females were crossed with BOEtTA males. It cannot be excluded that accidentally a few already mated TREP 2.30 females were crossed with male carriers of BOEtTA 6.24.

The primary aim was to generate *Drosophila* mutants highly resistant to Imidacloprid. That is why all eight female survivors, regardless of their genotype, were retested on high concentration of Imidacloprid (3μg/ml). Three out of eight survivors produced progeny resistant to 3μg/ml of Imidacloprid carrying TREP insertion and BOEtTA driver. This progeny was used to establish three isofemale lines. All three lines were analyzed for their LC50, and the line with the highest resistance was selected for

further analysis. Progeny from two isofemale lines showed mild resistance to Imidacloprid (LC50 = ~ 0.50 µg/ml) (table 7). The third line with the highest resistance to Imidacloprid (line MiT[w^+]3RX, LC50 = 2.60 µg/ml) was chosen for further analysis (table 7).

4.2 Mechanism of resistance in the MiT[w^-]3R2 mutant line

The TREP element was mapped to the X chromosome in the resistant MiT[w^+]3RX mutant. Further analysis of this line show no correlation between the Imidacloprid resistance and the TREP element in the presence or absence of the BOEtTA driver. The isofemale line (MiT[w^-]3R/SM6) without TREP insertion derived from analyzed Imidacloprid-resistant mutant (MiT[w^+]3RX) was established. While the resistance and the lethality loci both map to the second chromosome in line MiT[w^-]3R/SM6, there is no genetic linkage between the two loci, as determined by recombinational mapping (table 13). Resistant individuals homozygous for the second chromosome were retrieved during analysis, and the homozygous line MiT[w^-]3R2 was established.

Line MiT[w^-]3R2 was analyzed for the insecticide resistance mechanism using genetic, toxicology, biochemical and molecular methods, as well as transcriptomics.

The level of resistance to Imidacloprid of the MiT[w^-]3R resistant individuals, homozygous and heterozygous for the second chromosome, was analyzed. There was a high level of resistance to Imidacloprid of the homozygous, as well as the heterozygous individuals. The lethal concentration (LC50) of flies heterozygous, for the “resistance” chromosome was 2.1 µg/ml, with 95% confidence limits of 1.6 – 2.6 µg/ml. Individuals homozygous for the “resistance” second chromosome show an increased resistance with a LC50 of 3.3 µg/ml with 95% confidence limits of 1.9 – 4.1 µg/ml. Resistant heterozygous flies had about ~14 fold higher LC50 compared to iso31 and TREP 2.30 flies, both susceptible lines. Flies homozygous for the resistance locus increased their resistance to ~18 fold compared to the susceptible lines.

Drosophila melanogaster has been used in studies of chemical mutagenesis and selection for resistance to different insecticides (Kikkawa, 1964; Wilson and Fabian, 1986; Adcock *et al.*, 1993; Daborn *et al.*, 2001). The present study is the first on *Drosophila* resistant flies from an insertional mutagenesis screen using a transposon element and with selection on Imidacloprid. 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 flies heterozygous for the second resistant chromosome show thus a more than 3-fold higher resistance compared to these EMS mutants. The resistance increases in individuals homozygous for the second "resistance" chromosome to about 5-fold higher compared to the EMS mutants.

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). MiT[w^-]3R2 flies were thus also checked for cross-resistance to DDT. As for Imidacloprid, MiT[w^-]3R2 flies, both homozygous and heterozygous for the second "resistance" chromosome, show higher resistance to DDT compared to susceptible lines. Flies heterozygous for the MiT[w^-]3R2 chromosome were ~16-fold more resistant compared to iso31 flies. This factor increases to ~100 fold in flies homozygous for the resistance locus. MiT[w^-]3R2 flies also show higher resistance to DDT than the EMS mutants (Daborn *et al.*, 2001).

Resistance to two insecticides (Imidacloprid and DDT) with different modes of action (MoA) suggests metabolic detoxification as the major resistance mechanism in resistant line MiT[w^-]3R2. Additionally, insects exhibiting very high level of resistance usually have target site resistance as a major resistance mechanism. Lower level of resistance in MiT[w^-]3R2 mutant also indicates metabolic rather than target site resistance.

Piperonyl butoxide (PBO) is an insecticide synergist known to inhibit the activity of cytochrome P450 enzymes (Hodgson and Levi, 1998). It was used to test flies for the involvement of cytochrome P450 family genes in the resistance of line MiT[w^-]3R2.

Resistant flies treated with PBO had a lower LC50 for Imidacloprid (5.7 µg/vial), which was closer to the LC50 of susceptible *Drosophila* (4.2 µg/vial), compared to untreated resistant flies (LC50 = 9.4 µg/vial) (figure 29, table 17). This is further evidence for the involvement of the cytochrome P450 enzyme family in the resistance mechanism of line MiT[w^-]3R2, although involvement of other mechanisms cannot be ruled out.

In order to confirm an involvement of P450s on the biochemical level, adult flies and larvae of line MiT[w^-]3R2 were analyzed for cytochrome P450 enzymatic activity. The resistant line was also analyzed for the activity of glutathione-S-transferases and esterase enzymes. This analysis confirmed a higher activity of cytochrome P450 enzymes in resistant adults and larvae compared to the susceptible line. There was no increased glutathione-S-transferases enzyme activity or activity of α and β esterases in the resistant line.

The presence of increased amounts of P450 enzymes in resistant insects is in most cases correlated with increased expression of P450 genes (Scott, 1999; Karunker *et al.*, 2008; Karunker *et al.*, 2009). The relative expression of the representative cytochrome P450 genes Cyp6g1, Cyp6a2, Cyp6a8 and Cyp12d1 was measured for resistant MiT[w^-]3R2 and susceptible iso31 flies, both maintained on standard medium, as well as on medium with Imidacloprid. In semi-quantitative PCR analysis, products for all genes were detected in both lines, except for Cyp12d1. Cyp12d1 expression was only detected in the resistant line MiT[w^-]3R2, but not in iso31, in samples maintained on both media. Brandt and colleagues (2002) failed to detect Cyp12d1 mRNA in Northern blot analysis, due to the low expression in the insecticide susceptible Canton-S line. A comprehensive microarray-based atlas of adult gene expression in multiple *Drosophila* tissues shows a generally low expression of the Cyp12d1-d gene in Canton-S flies (Chintapalli *et al.*, 2007; http://flyatlas.org). Interestingly, higher expression of this gene was detected in tissues involved in detoxification, like midgut, Malpighian tubules and fat body (Chintapalli *et al.*, 2007; http://flyatlas.org). Additional analyses would be required for a more detailed investigation of the correlation between tissue specific expression of Cyp12d1 and Imidacloprid resistance in line MiT[w^-]3R2.

A higher quantity of mRNA of two genes (Cyp6g1 and Cyp6a2) was detected in semi-quantitative RT-PCR in resistant flies compared to susceptible flies, again independent of the presence of Imidacloprid (figure 35).

In order to quantify expression differences of the representative cytochrome P450 genes between resistant and susceptible lines, quantitative real time PCR was employed. Real time PCR analysis confirmed the absence of detectable Cyp12d1 expression in the line iso31. It was therefore not possible to analyze the relative expression of this gene between line MiT[w^-]3R2 and the susceptible line.

Real time RT-PCR analysis confirmed overexpression of genes Cyp6g1 and Cyp6a2 in resistant MiT[w^-]3R2 flies. An elevated expression of genes Cyp6g1 and Cyp6a2 in different DDT resistant *Drosophila* lines has been documented (Pedra *et al.*, 2004).

Resistant MiT[w^-]3R2 flies maintained on standard medium and medium with Imidacloprid had an about 8-fold higher expression of Cyp6g1 compared to susceptible flies maintained on standard medium (figure 38, Appendix - table 1).

Increased expression of Cyp6a2 was found for resistant MiT[w^-]3R2 flies compared to susceptible iso31 individuals (figure 39, Appendix - table 2). Resistant flies reared on standard medium have a 10-fold elevated expression of Cyp6a2 compared to the iso31 line reared on the same medium. When reared on Imidacloprid, resistant flies have an about 8-fold higher Cyp6a2 expression compared to susceptible flies maintained with Imidacloprid. Comparison of Cyp6a2 expression between resistant lines reared on different media did not yield any variation of relative expression. It is known that the Cyp6a2 gene is inducible by at least five compounds (phenobarbital, pentobarbital, organochlorines (DDT and aldrin), trans-stilbene oxide and limonene), and constitutive overexpression has been causally linked to resistance (Giraudo *et al.*, 2010). It has been shown that Imidacloprid induces mixed-function oxidases (MFO) in the liver of rats (Pauluhn, 1988). To date, Imidacloprid induction of cytochrome P450 genes in insects, including *Drosophila*, has not yet been documented. Real time RT-PCR analysis of Cyp6a2 expression in the susceptible line iso31, as well as in the resistant line MiT[W^-]3R2 are in concordance with data regarding Imidacloprid as an inducer.

Real time RT-PCR analysis did not indicate significant differences in expression of Cyp6a8 between resistant and susceptible lines, neither in the presence nor the absence of Imidacloprid (table 21).

Due to their mode of action as agonists of postsynaptic nicotinic acetylcholine receptors (nAChRs), the main resistance mechanism to neonicotinoids is target site resistance (Nauen *et al.*, 2001). Recent studies of neonicotinoid resistance in different insect species suggest that overexpression of one or more P450s is an auxiliary or even the primary resistance mechanism to neonicotinoids (Karunker *et al.*, 2009; Puinean *et al.*, 2010). Genetic and toxicology analyses suggest metabolic resistance as the main or at least a major mechanism of resistance to Imidacloprid (neonicotinoid) in mutant line MiT[w$^-$]3R2.

These results, combined with the observation that the Imidacloprid resistant line exhibits cross-resistance to DDT, lead to the conclusion that insecticide resistance in line MiT[W$^-$]3R2 is based on metabolic detoxification, rather than on target site resistance.

4.3 Transcriptomic analysis of line MiT[W$^-$]3R2

A genomic approach was used in order to quantify differences in expression of all genes between resistant line MiT[w$^-$]3R2 and susceptible line iso31. Out of 357 genes which were differentially expressed, 150 were upregulated and 207 were downregulated in the resistant line with respect to the susceptible line (figure 40, Data stored electronically on CD – table 6 and table 7 excel files). Transcriptional profiling of the resistant and susceptible lines revealed interesting differences.

Gene ontology classification yielded three significantly overrepresented upregulated and two significantly overrepresented downregulated functional groups of genes in the resistant line (table 24 and 25). Upregulated were genes of the P450 family and two groups of genes coding for peptidase activity. Downregulated were cuticular protein genes and another group of peptidase genes.

The cytochrome P450 gene family plays an important role in insecticide resistance because of their variety and the broad substrate specificity of several P450 genes (Scott and Kasai, 2004). The *D. melanogaster* genome contains around 89 putative P450 genes (Tijet *et al.*, 2001), of which only a restricted subset is likely to be involved in xenobiotic metabolism (Scott, 2008; Chung *et al.*, 2009). The involvement of P450s in Imidacloprid resistance in the described mutant was established by toxicological analysis using the P450 inhibitor PBO (figure 29, table 17). Pre-treatment with PBO reduced the resistance of line MiT[w^{-}]3R2 to Imidacloprid. Biochemical analysis of adults and third instar larvae showed increased P450s activity in the resistant line compared to the susceptible line. Assays of glutathione-S-transferases and esterase activities, however, did not show significant differences between the resistant and susceptible lines (table 18).

Deep sequencing analysis detected eight members of the P450 family, Cyp4p2, Cyp6a2, Cyp6g1, Cyp6w1, Cyp4e3, Cyp309a2, Cyp6g2 and Cyp4d14 with elevated expression in the resistant line. Genes encoding glutathione-S-transferases, as well as esterases, did not show elevated expression in the resistant line (figure 40, Data stored electronically on CD – table 6 excel file). Small quantity of Cyp12d1 mRNA was detected in both lines, but there was no expression difference (with a threshold of 2-fold) between resistant and susceptible line (Data stored electronically on CD – table 6 excel file).

The Cyp4p2 gene was 100-fold overexpressed in MiT[w^{-}]3R2 compared to iso31. Over-expression of this gene was confirmed with quantitative real time PCR showing 4.9 (± 0.3) fold higher expression in the resistant line. This discrepancy (100-fold according to deep sequencing and 4.9 –fold according to real time PCR) can be attributed to the different techniques and different biological samples that were analyzed. Namely, Cyp4p2 expression was analyzed in resistant flies maintained for more than 25 generations on standard medium, after deep sequencing analysis. Reduction of the Cyp4p2 expression fold could be a consequence of the fitness cost imposed by long term maintenance of resistant line. An interesting feature of this gene is its elevated level of expression exclusively in the fat body in the standard isogenic y; cn bw sp *D. melanogaster* strain (Chung *et al.*, 2009). Fat body together with Malpighian tubules and midgut are tissues in which metabolism of xenobiotics is most

likely to take place in insects (Dow and Davies, 2006; Hoshizaki, 2005, Chahine and O`Donnell, 2011). Although there is no experimental evidence of the involvement of this gene in insecticide resistance, sequence similarity and P450 expression pattern analysis predict involvement of Cyp4p2 gene in breakdown of different xenobiotics, including insecticides (Chung *et al.*, 2009). This is the first report of the overexpression of the Cyp4p2 gene in a *Drosophila* line resistant to Imidacloprid and DDT. The correlation between overexpression of the Cyp4p2 gene and resistance to Imidacloprid (neonicotinoids) and DDT suggests an involvement of this gene in the resistance mechanism.

Two other P450 genes, Cyp6a2 and Cyp6g1, have also an elevated expression in the resistant line, of about 20- and 16-fold, respectively. Overexpression of both genes was confirmed with quantitative real time RT-PCR, showing a 10-fold higher level for Cyp6a2 and an 8- fold higher level for Cyp6g1. There is a difference in overexpression of the two genes, as determined by deep sequencing analysis and quantitative real time RT-PCR, while the relative expression between the two genes remains the same. This 2-fold discrepancy can be attributed to the different techniques used for expression analysis. The detoxification function of the CYP6A2 and CYP6G1 encoded proteins in *Drosophila* is well established. The Cyp6a2 gene is highly expressed in different insecticide resistant *Drosophila* strains (Waters *et al.*, 1992; Maitra *et al.*, 1996; Dombrowski *et al.*, 1998; Pedra *et al.*, 2004). Moreover, the CYP6A2 protein can metabolize various different insecticides, which include organochlorine, organophosphorus, dimethylbenzanthracene and aflatoxin B1 (Dunkov *et al.*, 1997; Saner *et al.*, 1996). A mutant form of this P450 gene has been reported to metabolize DDT as well (Amichot *et al.*, 2004). Homology modeling suggests that different CYP6A2 structural protein variants can metabolize different substrates (Jones *et al.*, 2010). The results presented here support the involvement of Cyp6a2 in DDT resistance and also suggest an involvement of this gene in neonicotinoid resistance in *Drosophila melanogaster*. Overexpression of Cyp6g1 in *Drosophila* confers resistance to DDT and neonicotinoids (Daborn *et al.*, 2002; Daborn *et al.*, 2007; Chung *et al.*, 2007). Also, it has been shown by heterologous expression in cell suspension cultures of *Nicotiana tabacum* L., that the CYP6G1

encoded enzyme is capable of metabolizing DDT and Imidacloprid (Joussen *et al.*, 2008). Our results support this function of the Cyp6g1 gene.

Five other P450 genes (Cyp6w1, Cyp4e3, Cyp309a2, Cyp6g2 and Cyp4d14), are also overexpressed in the resistant line. Genes Cyp6w1 and Cyp6g2 have been experimentally linked to insecticide resistance. Microarray analysis has shown that expression of Cyp6w1 is elevated in a DDT resistant *Drosophila* strain (Pedra *et al.*, 2004). Overexpression of Cyp6g2 confers resistance to diazonin and nitenpyram in transgenic *Drosophila* (Daborn *et al.*, 2007). To date, no experimental evidence of implication in insecticide resistance are available for Cyp4e3, Cyp309a2 and Cyp4d14.

Analysis of the deep sequencing results detected significantly overrepresented up- or downregulated genes belonging to different functional groups (Data stored electronically on CD – table 8 and table 9 excel files). Significantly overrepresented upregulated genes are associated with oxidoreductase activity process, establishment of chromosome and organelle localization and cellular response to DNA damage stimulus. Significantly overrepresented downregulated genes associated with nutrient reservoir activity, response to bacteria, biotic stimulus and immune response biological processes were also detected. Downregulation of genes involved in immune response has not previously been seen in another DDT resistant *Drosophila* line (Pedra *et al.*, 2004). Oxidoreductase activity is a part of detoxification process activity, while other biological processes could be indication of general stress response caused by the upregulation of detoxification enzymes.

The identification of a group of 21 upregulated genes involved in peptidase activity is consistent with microarray analysis of DDT-resistant *Drosophila*, where genes coding for peptidase activity are also significantly overexpressed (Pedra *et al.*, 2004). The role of proteolytic genes and genes showing peptidase activity in insecticide resistance is still poorly understood and under investigation (Silva *et al.*, 2010a; Silva *et al.*, 2010b; Kaiser-Alexnat, 2009; Yang *et al.*, 2010). There is increasing evidence of involvement of protein metabolism in insecticide resistance in different insect species (Ahmed *et al.*, 1998; Mushtaq *et al.*, 2003; Araujo *et al.*, 2008; Lopes *et al.*, 2010). It has been suggested that proteases are part of the detoxification response mitigating

fitness costs in insecticide resistant lines (Araujo *et al.*, 2008). Ahmed and colleagues (1998) hypothesized that, in order to cover energy requirements during xenobiotic stress, proteases may be involved in modification of the conformation of enzymes and altered protein biosynthesis. Future investigation of proteases in insect resistant lines should elucidate their possible specific role in resistance mechanisms.

Genes encoding cuticular proteins were significantly overrepresented among the downregulated genes of resistant line MiT[w^{-}]3R2. This could occur as a result of the general stress response induced by the upregulated detoxification system. Reduced cuticular penetration of insecticide, although it does not appear to be an important resistance mechanism, has been reported as an additional feature that can contribute to resistance in some insect species (Scott and Georghiou, 1986a; Scott and Georghiou, 1986b; Apperson and Georgiou, 1975). Hence, it is not likely that the down-regulation of cuticular protein genes plays a role in the insecticide resistance mechanism of laboratory MiT[w^{-}]3R2 line.

The second significantly overrepresented down-regulated group of genes in line MiT[w^{-}]3R2 encodes enzymes involved in peptidase activity. Downregulation of seven genes showing peptidase activity could be a consequence of the general stress response induced by the upregulated detoxification system.

Also, an odorant binding protein (Obp19c) was found to be upregulated about 15-fold in MiT[w^{-}]3R2. The role of odorant binding proteins and the molecular mechanisms of their control are poorly understood. Studies in mosquitoes show that Obps are primarily involved in odour binding and transport (Andronopoulou *et al.*, 2006). It has been suggested that Obps play a role in the controlled inactivation of odourants and contribute to the desensitization and/or protection of olfactory neurons from toxic chemicals (Andronopoulou *et al.*, 2006). High concentrations of insecticide molecules, in this case Imidacloprid, could cause excitation of specific olfactory neurons in treated *Drosophila* individuals. Upregulation of Obp19c in the resistant mutant could play a role in mitigation of the toxic effect of Imidacloprid. Further analysis of the possible correlation between the odorant binding protein and high concentration of Imidacloprid in MiT[w^{-}]3R2 is needed.

Deep sequencing analysis shows high expression levels of major chorion genes Cp38, Cp36, Cp7Fc and Cp7Fb, as well as yellow-g and yellow-g2 in the resistant line (figure 40). Major chorion genes Cp38, Cp36, Cp7Fc and Cp7Fb form one cluster located on the X chromosome (Parks *et al.*, 1986). Genes yellow-g and yellow-g2 are located next to each other on the left arm of the third chromosome of *Drosophila* genome (Claycomb *et al.*, 2004). The major chorion proteins are classified as developmentally early, middle, and late, according to the choriogenic stages at which they are synthesized (Cavaliere *et al.*, 2008). Both, the cluster of the major chorion genes and genes yellow-g and yellow-g2 are expressed during early stages of chorion formation (Parks *et al.*, 1986; Parks and Spradling, 1987; Claycomb *et al.*, 2004). High quantities of specific structural proteins are required in a limited period of time for normal development of the eggshell (Cavaliere *et al.*, 2008). Gene products of Cp38, Cp36, Cp7Fc and Cp7Fb are required for normal eggshell assembly, while the products of genes yellow-g and yellow-g2 are essential for rigid eggshells (Cavaliere *et al.*, 2008; Claycomb *et al.*, 2004). High production of the major chorion gene cluster and yellow-g genes suggests the production of thicker eggshells. Thicker eggshell formation can decrease penetration of insecticide during exposure of the eggs to high concentration of Imidacloprid. To date there is no evidence of penetration resistance mechanisms in insects resistant to Imidacloprid. It is conceivable, however, that overexpression of chorion protein genes (Cp38, Cp36, Cp7Fc and Cp7Fb) and genes yellow-g and yellow-g2 leads to production of a more impenetrable eggshell, thus protecting the embryo from Imidacloprid. The *Minos* insertion in the resistant line occured into the X chromosome. Although not likely, “hit and run” effect of the *Minos* transposon could be a cause of increased expression of the chorion genes. During the mutagenesis individuals were selected on medium with Imidacloprid throughout the whole development (egg to adult). Individuals with thicker eggshell will have higher chance of survival due to a decreased Imidacloprid penetration. It is conceivable that a preexisting mutation occurred in the pool of selected embryos and was favored on high Imidacloprid concentration. Regardless of the nature of the event, higher expression of the chorion genes should result in a thicker eggshell. One can hypothesize that the thicker eggshell in embryos could be an additional mechanism of resistance to Imidacloprid. Additional investigations of the resistant line are needed to obtain more insight into this matter.

Detoxification in general can be divided into three phases: modification (phase I), conjugation (phase II) and excretion (phase III) (Xu *et al.*, 2005). Cytochrome P450 monooxygenases are phase I metabolic enzymes that generally exert modification by incorporating one atom of oxygen (O_2) into an organic substrate (RH) (Scott and Wen, 2001). These enzymes are involved in the activation and detoxification of a vast variety of xenobiotics, including insecticides (Scott and Kasai, 2004). In most cases, increased quantities of these enzymes, due to over-transcription of their genes, can be detected in resistant insects compared to susceptible ones (Hemingway, 2000). A recent report of P450 gene amplification associated with neonicotinoid resistance in the aphid *Myzus persicae* shows the existence of another molecular mechanism apart from increased transcription, that causes elevated P450 levels (Puinean *et al.*, 2010). Genomic DNA dosage differences of genes Cyp4p2, Cyp6g1 and Cyp6a2 between MiT[w$^-$]3R2 resistant line and susceptible line iso31 were analyzed. No amplification of these genes was found, in agreement with the assumption that increased quantities of P450 enzymes are in most cases due to increased transcription.

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 discernible chromosomal aberrations of any of the five polytene chromosome arms (X, 2L, 2R, 3L, 3R) (figure 24).

It has been suggested that oxidoreductase enzymes, including the P450 cytochromes, could all 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 check 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 an existing oxidative stress in the

analyzed individuals, treatment with paraquat should increase their lethality compared to a control. The analysis show that there is no significant increase of lethality in the treated resistant flies compared to treated iso31 susceptible flies (figure 30, table 19). Thus, no decrease in antioxidant defense of the resistant line was substantiated.

Genetic analysis of line MiT[w$^-$]3R2 placed the lethality to the right arm of the second chromosome, between position 49C1-4; 50C23-D2 (8.5Mb – 9.9Mb) (figure 48). A comparison of single nucleotide polymorphisms (SNP) of the deep sequencing data between the resistant line MiT[*w*$^-$]3R2 and the susceptible line iso31 was done for chromosome 2R (figure 44). Resistant line MiT[*w*$^-$]3R2 had been back-crossed with line iso31 under selection with 3 µg/ml of Imidacloprid in order to homogenize the genetic background. The SNP comparison indicates a hybrid origin of the 2R chromosome, where the right half comes from iso31, while the left half comes from a different line, most likely yw (figure 44, figure 48). This result indicates a recombination event on 2R, close to the region between 8.5 Mb and 9.9 Mb, to which the lethality was mapped (figure 48). The resistance locus was genetically mapped relative to P-element insertions to the 2R chromosome, as well between 8Mb and 9.7 Mb. Moreover, the SNP analysis, lethality mapping and P-element recombination mapping data taken together suggest that the recombination event occurred between resistance and lethality on the 2R chromosome (figure 48). P element analysis narrows down the resistance roughly to a range of approximately 1 Mb, between 8Mb and 8,7Mb on the 2R chromosome (figure 48). While this is an interesting coincidence, one can only speculate about a connection between this recombination event and the insecticide resistance, until the exact nature of the locus that confers resistance is known. Flies carrying homozygous or heterozygous combinations of the second “resistance” chromosome both show resistance to Imidacloprid as well as DDT. This is not the first DDT resistant *Drosophila* line in which resistance maps to the second chromosome. Genetic analysis mapped resistance to the second chromosome in two mutant *Drosophila* lines, generated with chemical mutagenesis, both resistant to Imidacloprid and cross-resistant to DDT (Daborn *et al.*, 2001). In the same study the resistance of field derived DDT-resistant *Drosophila* strains was mapped close to a cluster of overexpressed Cyp genes on the 2R chromosome, suggesting that the Cyp6g1 gene may be responsible for resistance. Chung and

colleagues (2007) showed that a truncated *Accord* element insertion is the resistance-associated mutation which leads to increased expression of Cyp6g1 gene in DDT resistant flies. Although a high correlation of Cyp6g1 gene expression and resistance to DDT has been showed in some *Drosophila* strains derived from field populations, there is no direct evidence that single mutation events at this locus are responsible for resistance (Kuruganti *et al.*, 2007). Interestingly, seven out of eight upregulated cytochrome P450 genes are located on the second chromosome in MiT[w$^-$]3R2. Five of them are located on the right arm of the second chromosome and three out of these five are overexpressed more than 15-fold (Cyp4p2 – 100-fold, Cyp6a2 – 19.85-fold and Cyp6g1 – 16.31-fold) in the resistant line. Although involvement of the cytochrome P450 monooxygenases genes in insecticide resistance is well documented, molecular studies of their regulation did not reveal general mechanisms of cytochrome P450 gene regulation in insects (Giraudo *et al.*, 2010). On the other hand, regulation of cytochrome P450 genes involved in xenobiotic detoxification in mammals is very well understood (Xu *et al.*, 2005, Pavek and Dvorak, 2008). P450 induction with phenobarbital (PH) identified constitutive androstane receptor (CAR) and pregnane X receptor (PXR) as key transcription factors in mammals (Sueyoshi & Negishi, 2001; Timsit & Negishi, 2007). The ortholog of these receptors in *Drosophila* is the xenobiotic receptor 96 (DHR96). King-Jones and colleagues (2006) analyzed a *Drosophila* DHR96 null mutant and suggested that the DHR96 receptor could play a role in detoxification in insects. A recent analysis of the promoter region shows that DHR96 plays a role in Cyp6d1 induction by phenobarbital in *Drosophila* S2 cells (Lin *et al.*, 2011). The xenobiotic receptor 96 (DHR96) maps to the 3R chromosome in *D. melanogaster* genome outside of the region where resistance is mapped in our mutant. There is no evidence in support of DHR96 involvement in the resistance mechanism of MiT[w$^-$]3R2 mutant.

Bhaskara and colleagues (2008) analyzed caffeine induction of Cyp6a2 and Cyp6a8 in transgenic *Drosophila melanogaster* flies. The caffeine induction of these two Cyp6 genes is modulated by cAMP and D-JUN protein levels. The same is true for caffeine induction of same genes in transfected SL-2 cells (Bhaskara *et al.*, 2008). It has been suggested that mutations of *trans*-regulating factors or of *cis*-acting elements of some of the Cyp genes are responsible for P450 dependent resistance (Maitra *et al.*, 2000;

Morra *et al.*, 2010; Giraudo *et al.*, 2010). So far, all the evidence from these studies is, however, inconclusive.

Combination of genetic and SNP analysis maps resistance locus to the 2R chromosome where three highest upregulated P450 genes (Cyp4p2, Cyp6a2 and Cyp6g1) are located. Moreover, our results suggest that the resistance locus lies within a 1 Mb interval (between 8Mb and 8.7Mb) where upregulated gene Cyp6g1 is located (figure 48). This line was retrieved during a *Minos*-based insertional mutagenesis, but is not associated with a *Minos* insertion. Although the mutation which causes the resistance remains to be identified, it is conceivable that a “hit and run” *Minos* insertion might be responsible for the mutation, where the transposon integrated and re-excised. In *Drosophila*, *Minos* often leaves upon excision either a characteristic six bp “footprint” or a deletion around the site of insertion behind (Arca *et al.*, 1997), both of which can be mutagenic in genes and regulatory sequences. A recent report suggests that a single mutation event in a specific enhancer can modulate Cyp6g1 tissue-specific induction in *Drosophila* flies (Chung *et al.*, 2011). One might thus speculate that a single mutation event occurred in a *cis*-acting element of the Cyp6g1 gene, increasing the expression of this gene. This in turn could activate other Cyp genes involved in resistance. An alternative possibility which we cannot exclude is that the mutation affects a *trans*-regulating factor within the mapped region (8Mb-8.7Mb). If a common regulatory factor is controlling induction of the multiple members of P450 family, a mutation in this factor could account for resistance respond with the elevated activity of a number of different P450 genes. There is no evidence in support of this hypothesis, since an *in silico* search failed to identify common transcription factor motifs regulating the overexpressed P450 genes. The same is true for common predicted microRNA targets in the 3’UTRs. This does not rule out the possibility, however, that these genes are regulated by an as yet unidentified common transcription factor or microRNA(s). The exact location of the mutation would be needed in order to obtain more information about the event and its consequences. Further study should involve sequencing of the suggested resistant region and identifying the exact location of the mutation.

5. Conclusions

- ✓ A genome-wide insertional mutagenesis of the *Drosophila* genome with the *Minos*-based TREP element showed high mobilization efficiency, providing a proof of principle for this and similar constructs as promising tools for insertional mutagenesis.

- ✓ A novel *Drosophila melanogaster* mutant (MiT[w$^-$]3R2) resistant to Imidacloprid and DDT was retrieved during the screen. The mutation was not associated with a *Minos* insertion, possibly being the result of a hit-and-run (insertion/excision) event.

- ✓ Toxicological, genetic and molecular analyses of line MiT[w$^-$]3R2 suggests that metabolic detoxification is the major resistance mechanisms in this line.

- ✓ The resistance locus maps to the right arm of the second chromosome, in the vicinity of the Cyp6g1 gene.

- ✓ Transcriptomic analysis identified a high number of differently expressed genes in the resistant line compared to a susceptible line, suggesting a complex insecticide resistance mechanism.

- ✓ Transcriptomic analysis of the resistant line revealed the upregulation of eight cytochrome P450 genes (Cyp4p2, Cyp6a2, Cyp6g1, Cyp6w1, Cyp4e3, Cyp309a2, Cyp6g2 and Cyp4d14) that should to be further analyzed regarding their individual roles in the mechanism of resistance.

- ✓ Significantly overrepresented upregulated peptidase genes, as well as significantly overrepresented downregulated cuticular protein and peptidase genes also need to be further analyzed for their possible role in the resistance mechanism.

Table of Contents

Περίληψη……………………………………………………..………..…….…II

Abstract…………………………………………………………..…...............…VIII

Acknowledgments…………………………………………………..…....…IX

1. Introduction……………………………………………….……..…….…2

1.1. Insecticides and insecticide resistance……………………….…...….....3

1.1.1. Neonicotinoids…………..……………….…………………...…….…......6

1.1.2. Mechanisms of insecticide resistance……………………..…....…..…….9

1.2. Genome-wide insertional mutagenesis………………………….……..…...16

1.3. Transposable elements………………………………………..…………..17

1.3.1. The transposable element *Minos*…………………….…………………..….17

1.4. *Drosophila* as a model organism……………………………………..……..18

1.5. Insecticide resistance in *Drosophila*…………………………………….…......19

1.5.1. Cytochrome P450-mediated resistance in *Drosophila*………………………20

1.6. Deep sequencing…………………………………………………………….…….21

1.7. Aims of the project…………………………………..………………...…23

2. Materials and methods……………………………………………….....25

2.1. *Drosophila melanogaster* strains and lines……………………………………27

2.2. Karyotype analysis of polytene chromosomes……………………………...28

2.3. Toxicology analysis………………………………………………..…...…29

2.3.1. Lethal concentration (LC50) analysis………………………………….….29

2.3.1.a. Determination of the lethal concentration 50 (LC50) for Imidacloprid and DDT……………………………………………………………………...……….29

2.3.1.b. Exposure to piperonyl butoxide (PBO)………………………………….....30

2.3.1.c. LC50 calculation and construction of dose-response curves……………....30

2.3.1.d. Insecticides and PBO……………………………………...……….....30

2.3.1.e. Paraquat assay…………………………………………….……….......30

2.3.2. Biochemical assays……………………………………………………...…31

2.4. Molecular analysis……………………………………………………………..…31

2.4.1. Standard PCRs………………………………………………...………......31

2.4.1.a. Preparation of genomic DNA…………………………………………..…31

2.4.1.b. PCR reactions for detection of *mini white* gene, *Minos* transposase gene and adjacent *mini white* and *Minos* transposase genes………………………………….32

2.4.2. Semi-quantitative and quantitative real time PCRs for the gene analysis of relative mRNA expression in resistant and susceptible lines……………………........32

2.4.2.a. RNA extraction…………………………………………….……….....32

2.4.2.b. Semi-quantitative PCR………………………………………….........34

2.4.2.c. Quantitative real time RT-PCR……………………………….…........35

2.4.2.d. Quantitative real time RT-PCRs for the analysis of gene amplification in the resistant line ………………………………………………….…………..…...35

2.5. Deep sequencing analysis…………………………………………….......36

3. Results…………………………………………………..…...……......41

3.1. *Minos*-based genome-wide insertional mutagenesis……………………........43

3.1.1. The TREP element……………………………………….…………......43

3.1.1.a. The TREP element in TREP 2.30 line shows high integration efficiency ……………………………………………………………………….....…...43

3.1.1.b. One third of total jumps of the TREP 2.30 element are jumps on the 4th chromosome (local jumps) ………………………………………………………47

3.1.2. *Minos*-based genome-wide insertional mutagenesis to identify genes involved in insecticide resistance…………………………………………………….....50

3.1.2.a. Imidacloprid lethal concentration shows approximately same values for all *Drosophila* lines used in insertional mutagenesis……………………………..…...50

3.1.2.b. *Minos*-based genome-wide insertional mutagenesis screen…………..…...52

3.2. Genetic analysis of the resistant line…………………………………..…....57

3.2.1. Obtaining and establishing the resistant line……………………...……....57

3.2.2. Mapping the resistance to the second chromosome in line MiT[orange]3R...58

3.2.2.1.a. Recombination test shows no correlation between lethality and orange marker eyes in resistant line MiT[orange]3R…………………………………....60

3.2.2.1.b. Genetic analysis failed to link the resistance locus with the orange eyes marker in resistant line MiT[orange]3R…………………………………………....62

3.2.3. Mapping the resistance to the second chromosome in line MiT[w-]3R……..64

3.2.3.a. Genetic mapping relative to P element insertions narrows down the resistance locus…………………………………………………………………………....70

3.2.4. Karyotype analysis shows no structural changes of the polytene chromosomes in resistant line...74

3.3. Toxicology analysis……………………………………………………..…...75

3.3.1. Resistance to Imidacloprid………………………………………….......75

3.3.2. Cross-resistance to DDT…………………………………………………...78

3.3.3. Piperonyl butoxide (PBO) analysis suggests involvement of cytochrome P450 genes in resistance mechanism……………………………………………….…..…81

3.3.4. Biochemical assays show increased activity of the P450 in the resistant line compared to susceptible line…………………………………………………......83

3.3.5. Paraquat assay fails to detect oxidative stress in line MiT[w-]3R2.................83

3.4. Molecular analysis………………………...……………………………....85

3.4.1. Standard PCR analysis…………………………………...………….…..85

3.4.1.a. Nature of orange eyes phenotype in MIT[orange]3R2 resistant line remains unclear…………………………………………………………………………....85

3.4.1.b. Overexpression of individual P450 genes is observed in Imidacloprid resistant line…………………………………………………………………………......88

3.4.2. Real time RT-PCR shows increased levels of expression of some representative cytochrome P450 genes in the resistant line……………………………………..92

3.4.3. Quantitative PCR analysis shows no amplification of the Cyp4p2, Cyp6g1 and Cyp6a2 genes in the resistant lines…………………………………………....95

3.5. Transcriptomic profiling identified a high number of differently expressed genes between resistant and susceptible line…………………………………….….96

3.5.1. *In silico* analysis of deep sequencing data of the resistant and susceptible lines……………………………………………………………………...…101

3.5.1.a. Deep sequencing data bioinformatics analysis failed to detect common regulatory factor linked with the resistance in line MiT[w-]3R2………..…..…102

3.5.1.b. Single nucleotide polymorphism analysis of differently expressed genes sequences mapped the resistance locus within ~1Mb region in line MiT[w-]3R2……………………………………………………………………….102

4. Discussion…………………………………………………...……......109

4.1. *Minos*-based genome-wide insertional mutagenesis…………………….....111

4.1.1. The TREP-BOEtTA system and conditions for screening for Imidacloprid resistance……………………………………………………………………………...111

4.1.2. Genome-wide insertional mutagenesis………………………………..…....113

4.2. Mechanism of resistance in the MiT[w-]3R2 mutant line…………………………...116

4.3 Transcriptomic analysis of line MiT[W-]3R2……………………………………..120

5. Conclusions…………………………...………………...……………...….131

Table of contest……………………………………...……………………….....135

List of Tables

Table 1. Major insecticide classes target site groups (source IRAC international MoA working group 2010)

Table 2. Number of analyzed flies in the Control and Jumpstarter groups

Table 3. Transposition efficiency (TE) of the TREP element of line TREP 2.30 in the Control and Jumpstarter groups

Table 4. Distribution of jumps of the TREP 2.30 element on the 4th and the other three chromosomes of *D. melanogaster*

Table 5. Genome-wide insertional mutagenesis results

Table 6. All emerged flies with different phenotypes selected on medium with 3 μg/ml of Imidacloprid

Table 7. LC50 values of 3 lines retrieved from *Minos*-based insertional mutagenesis, calculated with program SPSS 16

Table 8. Viability of second chromosome combinations in progeny emerged on standard medium and medium with 3 μg/ml of Imidacloprid

Table 9. Viability of third chromosome combinations in progeny emerged on standard medium and medium with 3 μg/ml of Imidacloprid

Table 10. Recombinant and non recombinant progeny

Table 11. Number of adult progeny with and without the second chromosome from MiT[w^-]3R2 (non-Cy) after selection on medium with 3 μg/ml of Imidacloprid

Table 12. Number of adult progeny with and without the third chromosome from MiT[w^-]3R2 (non Sb) after selection on medium with 3 μg/ml of Imidacloprid

Table 13. Approximate number of recombinants emerged on medium with Imidacloprid

Table 14. P element insertion coordinates and distance between insertion and resistance region on the right arm of the second chromosome

Table 15. LC50 values for Imidacloprid of susceptible and resistant lines

Table 16. LC50s for DDT of susceptible and resistant lines (homozygous and heterozygous, respectively, for the second chromosome)

Table 17. Imidacloprid LC50s of a susceptible and a resistant line treated with PBO and of the untreated resistant line

Table 18. Activities of detoxification enzymes of resistant and susceptible lines

Table 19. Mortality (%) of the susceptible and resistant lines treated with different concentrations of paraquat

Table 20. PCR fragment production of genes Cyp6g1, Cyp6a2, Cyp6a8, Cyp12d1 and Rp49 from a resistant and a susceptible line, analyzed on 2% agarose gels at the 25th cycle

Table 21. Expression difference of gene Cyp6a8 between two lines maintained on standard medium and medium with Imidacloprid (res – resistant line; susc – susceptible line; ST – standard medium; IMI – medium with Imidacloprid)

Table 22. Quantitative real time PCR analysis results for amplification of Cyp4p2, Cyp6g1 and Cyp6a2 genes in resistant line compared to susceptible line.

Table 23. Up- and downregulated Cyp genes in the resistant line (with respect to the susceptible line)

Table 24. Gene functional groups in the up-regulated genes (analyzed with the DAVID 6.7 BETA bioinformatics resource)

Table 25. Gene functional groups in the down-regulated genes (analyzed with DAVID 6.7 BETA bioinformatics resource)

List of Illustrations

Figure 1. Neonicotinoids currently used as pesticides (figures adapted from Wikipedia)

Figure 2a. Chemical structural segments of neonicotinoids (Imidacloprid). R1–R2 bridging fragment; CPM - 6-**C**hloro-**p**yridin-3-yl**m**ethyl; –N–C(E)=X–Y stands for -N-C(NH)=N-NO_2 (modified after Jeschke and Nauen, 2008)

Figure 2b. Structural segment of Imidacloprid. CPM - 6-**C**hloro-**p**yridin-3-yl**m**ethyl (modified after Jeschke and Nauen, 2008)

Figure 3. Structure of the *Minos* element isolated from *Drosophila hydei*. The transposase gene is interrupted by a 60 base pair long intron. Not all features are drawn to scale. IDR: inner direct repeat, ODR: outer direct repeat, ITR: inverted terminal repeat: TA duplicated target dinucleotide (modified after Pavlopoulos *et al.*, 2007)

Figure 4. Schematic of the TREP and BOEtTA constructs and the activation of the TREP-borne minimal promoter

Figure 5. Crossing scheme of the Control group

Figure 6. Crossing scheme of the Jumpstarter group

Figure 7. Jump on the 4th chromosome

Figure 8. Jump on the sex, second or third chromosome

Figure 9. Crossing scheme for lethality testing of iso31 flies

Figure 10. Survival of iso31 flies on food with the indicated Imidacloprid concentrations

Figure 11. Survival of the iso31 flies on food with the indicated Imidacloprid concentrations

Figure 12. Crossing scheme of the genome-wide insertional mutagenesis system

Figure 13. Crossing scheme for testing the female survivors

Figure 14. Crossing scheme for the second chromosome

Figure 15. Crossing scheme for the third chromosome

Figure 16. Crosses for the recombination test for analysis of the correlation between the lethality locus and the orange eyes marker for resistant line MiT[orange]3R

Figure 17. Crossing scheme for analysis of the correlation between the resistance locus and the orange eyes marker in resistant line MiT[orange]3R

Figure 18. Second chromosome crossing scheme of the mapping of the resistance locus

Figure 19. Third chromosome crossing scheme of the mapping of the resistance locus

Figure 20. Crossing scheme for recombination analysis of correlation between resistance and lethality in line MiT[w^-]3R2

Figure 21. Crossing scheme of MiT[w^-]3R2 and deletion kit flies

Figure 22. Crossing scheme of P element resistance mapping

Figure 23. Location of the P element insertions (black filled triangles) and distance between insertion and resistance region (interrupted lines) on the right arm of the second chromosome

Figure 24. Salivary gland polytene chromosomes of larvae progeny from the cross between resistant and susceptible line, prepared with a squash technique (dashed arrows surround the region where the resistance locus is mapped)

Figure 25. Dose-response curves to Imidacloprid of two susceptible and resistant lines (heterozygous for the second chromosome)

Figure 26. Dose-response curves to Imidacloprid of two susceptible lines and one resistant line (homozygous for the second chromosome)

Figure 27. DDT dose-response curves of two susceptible lines and a resistant line (heterozygous for the second chromosome)

Figure 28. DDT dose-response curves of two susceptible lines and a resistant line (homozygous for the second chromosome)

Figure 29. Imidacloprid dose-response curves of the susceptible and resistant lines treated with PBO and of the non-treated resistant line

Figure 30. Dose-response curves of the susceptible and resistant lines on 5%, 10% and 12.8% concentrations of paraquat

Figure 31. Agarose gel (1%) for PCR detection of a *Mini white* gene fragment in lines iso31 [SM6, MiT 2.4]/Sco, MiT[w^+]3Rx (TREP), iso31, MIT[orange]3R2/SM6 (MIT[orange]3R2/CyO), MiT[w^-]3R2/SM6 (MiT[w^-]3R2/CyO) and in plasmid MiT 2.4

Figure 32. Agarose gel (1%) for PCR product detection of joined *Minos* end and *Mini white* gene sequence product in lines MiT[w^+]3Rx (TREP), iso31, MIT[orange]3R2/SM6 (MIT[orange]3R2/CyO) and MiT[w^-]3R2/SM6 (MiT[w^-]3R2/CyO)

Figure 33. Agarose gel (1%) of PCR reactions for detection of the *Minos* transposase gene in lines iso31 [SM6, MiT 2.4]/Sco, MiT[w^+]3Rx (TREP), iso31, MIT[orange]3R2/SM6 (MIT[orange]3R2/CyO), MiT[w^-]3R2/SM6 (MiT[w^-]3R2/CyO) and in plasmid MiT 2.4

Figure 34. Semi-quantitative RT-PCR (on a 2% agarose gel) detection of control gene RP49 mRNA in susceptible iso31 and resistant MiT[w^-]3R2 flies raised on standard medium (st) and medium with Imidacloprid (imi)

Figure 35. Semi-quantitative RT-PCR (on a 2% agarose gel) detection of Cyp6g1 and Cyp6a2 mRNAs in susceptible iso31 and resistant MiT[w^-]3R2 flies raised on standard medium (st) and medium with Imidacloprid (imi)

Figure 36. Semi-quantitative RT-PCR (on 2% agarose gel) detection of Cyp12d1 mRNA in susceptible iso31 and resistant MiT[w^-]3R2 flies raised on standard medium (st) and medium with Imidacloprid (imi)

Figure 37. Semi-quantitative RT-PCR (on 2% agarose gel) detection of Cyp6a8 mRNA in susceptible iso31 and resistant MiT[w^-]3R2 flies raised on standard medium (st) and medium with Imidacloprid (imi)

Figure 38. Expression difference of gene Cyp6g1 between two lines maintained on standard medium and medium with Imidacloprid (res – resistant line; susc – susceptible line; ST – standard medium; IMI – medium with Imidacloprid)

Figure 39. Expression difference of gene Cyp6a2 between two lines maintained on standard medium and medium with Imidacloprid (res – resistant line; susc – susceptible line; ST – standard medium; IMI – medium with Imidacloprid)

Figure 40. Volcano plot of up- and down-regulated genes in the resistant line (as compared to the susceptible line) X axis - log2 (number of reads in resistant line) / (number of reads in susceptible line) Y axis - log2 (number of reads in resistant line) + (number of reads in susceptible line)

Figure 41. Up- and downregulated Cyp genes in the resistant line (with respect to the susceptible line)

Figure 42. Single nuclear polymorphisms (SNP) density (per 1Kb) on the X chromosome between resistant line MiT[w^-]3R2 and susceptible line iso31

Figure 43. Single nuclear polymorphisms (SNP) density (per 1Kb) on the 2L chromosome between resistant line MiT[w^-]3R2 and susceptible line iso31

Figure 44. Single nuclear polymorphisms (SNP) density (per 1Kb) on the 2R chromosome between resistant line MiT[w^-]3R2 and susceptible line iso31

Figure 45. Single nuclear polymorphisms (SNP) density (per 1Kb) on the 3L chromosome between resistant line MiT[w^-]3R2 and susceptible line iso31

Figure 46. Single nuclear polymorphisms (SNP) density (per 1Kb) on the 3R chromosome between resistant line MiT[w^-]3R2 and susceptible line iso31

Figure 47. Single nuclear polymorphisms (SNP) density (per 1Kb) on the 4th chromosome between resistant line MiT[w^-]3R2 and susceptible line iso31

Figure 48. The resistance locus was mapped relative to P element insertions to a region between 8Mb and 8.7Mb (black arrows on the second scale, distance between insertion and resistance region is indicated with dotted horizontal lines). The location of the three highly expressed P450 genes (Cyp6a2, Cyp6g1 and Cyp4p2) in the resistant MiT[w^-]3R2 line is indicated. Below is a comparison of single nucleotide polymorphism (SNP) density (per 1 Kb) between resistant line MiT[w^-]3R2 and susceptible line iso31. At the bottom, Bloomington deletions overlapping lethality locus (filled box) and flanking the lethality locus (open boxes) (lethality maps to the region between 8.5 Mb and 9.9 Mb, close to the place of recombination

References

Adams, M. D., S. E. Celniker, R. A. Holt, C. A. Evans, J. D. Gocayne, P. G. Amanatides, S. E. Scherer, P. W. Li, R. A. Hoskins, R. F. Galle, R. A. George, S. E. Lewis, S. Richards, M. Ashburner, S. N. Henderson, G. G. Sutton, J. R. Wortman, M. D. Yandell, Q. Zhang, L. X. Chen, R. C. Brandon, Y. H. Rogers, R. G. Blazej, M. Champe, B. D. Pfeiffer, K. H. Wan, C. Doyle, E. G. Baxter, G. Helt, C. R. Nelson, G. L. Gabor, J. F. Abril, A. Agbayani, H. J. An, C. Andrews-Pfannkoch, D. Baldwin, R. M. Ballew, A. Basu, J. Baxendale, L. Bayraktaroglu, E. M. Beasley, K. Y. Beeson, P. V. Benos, B. P. Berman, D. Bhandari, S. Bolshakov, D. Borkova, M. R. Botchan, J. Bouck, P. Brokstein, P. Brottier, K. C. Burtis, D. A. Busam, H. Butler, E. Cadieu, A. Center, I. Chandra, J. M. Cherry, S. Cawley, C. Dahlke, L. B. Davenport, P. Davies, B. de Pablos, A. Delcher, Z. Deng, A. D. Mays, I. Dew, S. M. Dietz, K. Dodson, L. E. Doup, M. Downes, S. Dugan-Rocha, B. C. Dunkov, P. Dunn, K. J. Durbin, C. C. Evangelista, C. Ferraz, S. Ferriera, W. Fleischmann, C. Fosler, A. E. Gabrielian, N. S. Garg, W. M. Gelbart, K. Glasser, A. Glodek, F. Gong, J. H. Gorrell, Z. Gu, P. Guan, M. Harris, N. L. Harris, D. Harvey, T. J. Heiman, J. R. Hernandez, J. Houck, D. Hostin, K. A. Houston, T. J. Howland, M. H. Wei, C. Ibegwam, M. Jalali, F. Kalush, G. H. Karpen, Z. Ke, J. A. Kennison, K. A. Ketchum, B. E. Kimmel, C. D. Kodira, C. Kraft, S. Kravitz, D. Kulp, Z. Lai, P. Lasko, Y. Lei, A. A. Levitsky, J. Li, Z. Li, Y. Liang, X. Lin, X. Liu, B. Mattei, T. C. McIntosh, M. P. McLeod, D. McPherson, G. Merkulov, N. V. Milshina, C. Mobarry, J. Morris, A. Moshrefi, S. M. Mount, M. Moy, B. Murphy, L. Murphy, D. M. Muzny, D. L. Nelson, D. R. Nelson, K. A. Nelson, K. Nixon, D. R. Nusskern, J. M. Pacleb, M. Palazzolo, G. S. Pittman, S. Pan, J. Pollard, V. Puri, M. G. Reese, K. Reinert, K. Remington, R. D. Saunders, F. Scheeler, H. Shen, B. C. Shue, I. Siden-Kiamos, M. Simpson, M. P. Skupski, T. Smith, E. Spier, A. C. Spradling, M. Stapleton, R. Strong, E. Sun, R. Svirskas, C. Tector, R. Turner, E. Venter, A. H. Wang, X. Wang, Z. Y. Wang, D. A. Wassarman, G. M. Weinstock, J. Weissenbach, S. M. Williams, WoodageT, K. C. Worley, D. Wu, S. Yang, Q. A. Yao, J. Ye, R. F. Yeh, J. S. Zaveri, M. Zhan, G. Zhang, Q. Zhao, L. Zheng, X. H. Zheng, F. N. Zhong, W. Zhong, X. Zhou, S. Zhu, X. Zhu, H. O. Smith, R. A. Gibbs, E. W. Myers, G. M. Rubin and J. C. Venter (2000). "The genome sequence of Drosophila melanogaster." Science **287**(5461): 2185-2195.

Adams, M. D. and J. J. Sekelsky (2002). "From sequence to phenotype: reverse genetics in Drosophila melanogaster." Nat Rev Genet **3**(3): 189-198.

Adcock, G. J., P. Batterham, L. E. Kelly and J. A. McKenzie (1993). "Cyromazine resistance in Drosophila melanogaster (Diptera: Drosophilidae) generated by ethyl methanesulfonate mutagenesis." J Econ Entomol **86**(4): 1001-1008.

Ahmad, M., I. Denholm and R. H. Bromilow (2006). "Delayed cuticular penetration and enhanced metabolism of deltamethrin in pyrethroid-resistant strains of Helicoverpa armigera from China and Pakistan." Pest Manag Sci **62**(9): 805-810.

Ahmed, S., R. M. Wilkins and D. Mantle (1998). "Comparison of proteolytic enzyme activities in adults of insecticide resistant and susceptible strains of the housefly M. domestica L." Insect Biochem Mol Biol **28**(9): 629-639.

Alout, H., A. Berthomieu, A. Hadjivassilis and M. Weill (2007). "A new amino-acid substitution in acetylcholinesterase 1 confers insecticide resistance to Culex pipiens mosquitoes from Cyprus." Insect Biochem Mol Biol **37**(1): 41-47.

Amichot, M., S. Tares, A. Brun-Barale, L. Arthaud, J. M. Bride and J. B. Berge (2004). "Point mutations associated with insecticide resistance in the Drosophila cytochrome P450 Cyp6a2 enable DDT metabolism." Eur J Biochem **271**(7): 1250-1257.

Andersen, J. K. (2004). "Oxidative stress in neurodegeneration: cause or consequence?" Nat Med **10 Suppl**: S18-25.

Andronopoulou, E., V. Labropoulou, V. Douris, D. F. Woods, H. Biessmann and K. Iatrou (2006). "Specific interactions among odorant-binding proteins of the African malaria vector Anopheles gambiae." Insect Mol Biol **15**(6): 797-811.

Anthony, N., T. Rocheleau, G. Mocelin, H. J. Lee and R. ffrench-Constant (1995). "Cloning, sequencing and functional expression of an acetylcholinesterase gene from the yellow fever mosquito Aedes aegypti." FEBS Lett **368**(3): 461-465.

Apperson, C. S. and G. P. Georghiou (1975). "Mechanisms of resistance to organophosphorus insecticides in Culex tarsalis." J Econ Entomol **68**(2): 153-157.

Araujo, R. A., R. N. Guedes, M. G. Oliveira and G. H. Ferreira (2008). "Enhanced activity of carbohydrate- and lipid-metabolizing enzymes in insecticide-resistant populations of the maize weevil, Sitophilus zeamais." Bull Entomol Res **98**(4): 417-424.

Arca, B., S. Zabalou, T. G. Loukeris and C. Savakis (1997). "Mobilization of a Minos transposon in Drosophila melanogaster chromosomes and chromatid repair by heteroduplex formation." Genetics **145**(2): 267-279.

Arguello, J. R., Y. Zhang, T. Kado, C. Fan, R. Zhao, H. Innan, W. Wang and M. Long (2010). "Recombination yet inefficient selection along the Drosophila melanogaster subgroup's fourth chromosome." Mol Biol Evol **27**(4): 848-861.

Ashburner, M. (1989). Drosophila: laboratory manual, Cold Spring Harbor Laboratory.

Ashok, M., C. Turner and T. G. Wilson (1998). "Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators." Proc Natl Acad Sci U S A **95**(6): 2761-2766.

Bai, D., Lummis, S. C. R., Leicht, W., Breer, H. and Sattelle, D. B. (1991). "Actions of imidacloprid and a related nitromethylene on cholinergic receptors of an identified insect motor neurone." Pesticide Science **33**(2): 197-204.

Beall, C., C. Fyrberg, S. Song and E. Fyrberg (1992). "Isolation of a Drosophila gene encoding glutathione S-transferase." Biochem Genet **30**(9-10): 515-527.

Beckingham, K. M., J. D. Armstrong, M. J. Texada, R. Munjaal and D. A. Baker (2005). "Drosophila melanogaster--the model organism of choice for the complex biology of multi-cellular organisms." Gravit Space Biol Bull **18**(2): 17-29.

Bellen, H. J., R. W. Levis, G. Liao, Y. He, J. W. Carlson, G. Tsang, M. Evans-Holm, P. R. Hiesinger, K. L. Schulze, G. M. Rubin, R. A. Hoskins and A. C. Spradling (2004). "The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes." Genetics **167**(2): 761-781.

Bhaskara, S., M. B. Chandrasekharan and R. Ganguly (2008). "Caffeine induction of Cyp6a2 and Cyp6a8 genes of Drosophila melanogaster is modulated by cAMP and D-JUN protein levels." Gene **415**(1-2): 49-59.

Bhaskara, S., E. D. Dean, V. Lam and R. Ganguly (2006). "Induction of two cytochrome P450 genes, Cyp6a2 and Cyp6a8, of Drosophila melanogaster by caffeine in adult flies and in cell culture." Gene **377**: 56-64.

Bloomington. (2010). from http://flystocks.bio.indiana.edu/Inst/history.htm.

Bloomquist, J. R. (1994). "Cyclodiene resistance at the insect GABA receptor/chloride channel complex confers broad cross resistance to convulsants and experimental phenylpyrazole insecticides." Arch Insect Biochem Physiol **26**(1): 69-79.

Botella, J. A., F. Bayersdorfer, F. Gmeiner and S. Schneuwly (2009). "Modelling Parkinson's disease in Drosophila." Neuromolecular Med **11**(4): 268-280.

Brandt, A., M. Scharf, J. H. Pedra, G. Holmes, A. Dean, M. Kreitman and B. R. Pittendrigh (2002). "Differential expression and induction of two Drosophila cytochrome P450 genes near the Rst(2)DDT locus." Insect Mol Biol **11**(4): 337-341.

Bridges, C. B. (1935). "The mutants and linkage data of chromosome four of Drosophila melanogaster." Journal Biology (Moscow) **4**: 401--420.

Brooke, B. D., R. H. Hunt, F. Chandre, P. Carnevale and M. Coetzee (2002). "Stable chromosomal inversion polymorphisms and insecticide resistance in the malaria vector mosquito Anopheles gambiae (Diptera: Culicidae)." J Med Entomol **39**(4): 568-573.

Bus, J. S. and J. E. Gibson (1984). "Paraquat: model for oxidant-initiated toxicity." Environ Health Perspect **55**: 37-46.

Campbell, P. M., Q. R. G. C. de, L. N. Court, S. J. Dorrian, R. J. Russell and J. G. Oakeshott (2003). "Developmental expression and gene/enzyme identifications in the alpha

esterase gene cluster of Drosophila melanogaster." Insect Mol Biol **12**(5): 459-471.
Campbell, P. M., R. D. Newcomb, R. J. Russell and J. G. Oakeshott (1998). "Two different amino acid substitutions in the ali-esterase, E3, confer alternative types of organophosphorus insecticide resistance in the sheep blowfly, Lucilia cuprina." Insect Biochemistry and Molecular Biology **28**: 139–150.
Capy, P., C. Bazin, D. Higuet and T. Langin (1997). Dynamics and evolution of transposable elements. Austin, TX, Landes Bioscience.
Catania, F., M. O. Kauer, P. J. Daborn, J. L. Yen, R. H. Ffrench-Constant and C. Schlotterer (2004). "World-wide survey of an Accord insertion and its association with DDT resistance in Drosophila melanogaster." Mol Ecol **13**(8): 2491-2504.
Catteruccia, F., T. Nolan, C. Blass, H. M. Muller, A. Crisanti, F. C. Kafatos and T. G. Loukeris (2000). "Toward Anopheles transformation: Minos element activity in anopheline cells and embryos." Proc Natl Acad Sci U S A **97**(5): 2157-2162.
Catteruccia, F., T. Nolan, T. G. Loukeris, C. Blass, C. Savakis, F. C. Kafatos and A. Crisanti (2000). "Stable germline transformation of the malaria mosquito Anopheles stephensi." Nature **405**(6789): 959-962.
Cavaliere, V., F. Bernardi, P. Romani, S. Duchi and G. Gargiulo (2008). "Building up the Drosophila eggshell: first of all the eggshell genes must be transcribed." Dev Dyn **237**(8): 2061-2072.
Chahine, S. and M. J. O'Donnell (2011). "Interactions between detoxification mechanisms and excretion in Malpighian tubules of Drosophila melanogaster." J Exp Biol **214**(Pt 3): 462-468.
Chintapalli, V. R., J. Wang and J. A. Dow (2007). "Using FlyAtlas to identify better Drosophila melanogaster models of human disease." Nat Genet **39**(6): 715-720.
Chung, H., M. R. Bogwitz, C. McCart, A. Andrianopoulos, R. H. Ffrench-Constant, P. Batterham and P. J. Daborn (2007). "Cis-regulatory elements in the Accord retrotransposon result in tissue-specific expression of the Drosophila melanogaster insecticide resistance gene Cyp6g1." Genetics **175**(3): 1071-1077.
Chung, H., T. Sztal, S. Pasricha, M. Sridhar, P. Batterham and P. J. Daborn (2009). "Characterization of Drosophila melanogaster cytochrome P450 genes." Proc Natl Acad Sci U S A **106**(14): 5731-5736.
Claudianos, C., R. J. Russell and J. G. Oakeshott (1999). "The same amino acid substitution in orthologous esterases confers organophosphate resistance on the house fly and a blowfly." Insect Biochem Mol Biol **29**(8): 675-686.
Claycomb, J. M., M. Benasutti, G. Bosco, D. D. Fenger and T. L. Orr-Weaver (2004). "Gene amplification as a developmental strategy: isolation of two developmental amplicons in Drosophila." Dev Cell **6**(1): 145-155.
Cloyd, R. A. and J. A. Bethke (2011). "Impact of neonicotinoid insecticides on natural enemies in greenhouse and interiorscape environments." Pest Manag Sci **67**(1): 3-9.
Cooley, L., R. Kelley and A. Spradling (1988). "Insertional mutagenesis of the Drosophila genome with single P elements." Science **239**(4844): 1121-1128.
Cresswell, J. E. (2011). "A meta-analysis of experiments testing the effects of a neonicotinoid insecticide (imidacloprid) on honey bees." Ecotoxicology **20**(1): 149-157.
Daborn, P., S. Boundy, J. Yen, B. Pittendrigh and R. ffrench-Constant (2001). "DDT resistance in Drosophila correlates with Cyp6g1 over-expression and confers cross-resistance to the neonicotinoid imidacloprid." Mol Genet Genomics **266**(4): 556-563.
Daborn, P. J., C. Lumb, A. Boey, W. Wong, R. H. Ffrench-Constant and P. Batterham (2007). "Evaluating the insecticide resistance potential of eight Drosophila melanogaster cytochrome P450 genes by transgenic over-expression." Insect Biochem Mol Biol **37**(5): 512-519.
Daborn, P. J., J. L. Yen, M. R. Bogwitz, G. Le Goff, E. Feil, S. Jeffers, N. Tijet, T. Perry, D. Heckel, P. Batterham, R. Feyereisen, T. G. Wilson and R. H. ffrench-Constant (2002). "A single p450 allele associated with insecticide resistance in Drosophila." Science **297**(5590): 2253-2256.
Davis, W. M. (2003). A plasmid Editor v1.17.

Devices, M. (2009). SoftMax software. SoftMax prov5 software. Sunnyvale, CA, Molecular Devices
Devonshire, A. L. and L. M. Field (1991). "Gene amplification and insecticide resistance." Annu Rev Entomol **36**: 1-23.
Dombrowski, S. M., R. Krishnan, M. Witte, S. Maitra, C. Diesing, L. C. Waters and R. Ganguly (1998). "Constitutive and barbital-induced expression of the Cyp6a2 allele of a high producer strain of CYP6A2 in the genetic background of a low producer strain." Gene **221**(1): 69-77.
Dow, J. A. and S. A. Davies (2006). "The Malpighian tubule: rapid insights from post-genomic biology." J Insect Physiol **52**(4): 365-378.
Drabek, D., L. Zagoraiou, T. deWit, A. Langeveld, C. Roumpaki, C. Mamalaki, C. Savakis and F. Grosveld (2003). "Transposition of the Drosophila hydei Minos transposon in the mouse germ line." Genomics **81**(2): 108-111.
Dunkov, B. C., V. M. Guzov, G. Mocelin, F. Shotkoski, A. Brun, M. Amichot, R. H. Ffrench-Constant and R. Feyereisen (1997). "The Drosophila cytochrome P450 gene Cyp6a2: structure, localization, heterologous expression, and induction by phenobarbital." DNA Cell Biol **16**(11): 1345-1356.
Festucci-Buselli, R. A., A. S. Carvalho-Dias, M. de Oliveira-Andrade, C. Caixeta-Nunes, H. M. Li, J. J. Stuart, W. Muir, M. E. Scharf and B. R. Pittendrigh (2005). "Expression of Cyp6g1 and Cyp12d1 in DDT resistant and susceptible strains of Drosophila melanogaster." Insect Mol Biol **14**(1): 69-77.
Feyereisen, R. (1995). "Molecular biology of insecticide resistance." Toxicol Lett **82-83**: 83-90.
Feyereisen, R. (2006). "Evolution of insect P450." Biochem Soc Trans **34**(Pt 6): 1252-1255.
Ffrench-Constant, R. H., N. Anthony, K. Aronstein, T. Rocheleau and G. Stilwell (2000). "Cyclodiene insecticide resistance: from molecular to population genetics." Annu Rev Entomol **45**: 449-466.
Ffrench-Constant, R. H., P. J. Daborn and G. Le Goff (2004). "The genetics and genomics of insecticide resistance." Trends Genet **20**(3): 163-170.
Field, L. M., R. L. Blackman, C. Tyler-Smith and A. L. Devonshire (1999). "Relationship between amount of esterase and gene copy number in insecticide-resistant Myzus persicae (Sulzer)." Biochem J **339 (Pt 3)**: 737-742.
Field, L. M. and A. L. Devonshire (1998). "Evidence that the E4 and FE4 esterase genes responsible for insecticide resistance in the aphid Myzus persicae (Sulzer) are part of a gene family." Biochem J **330 (Pt 1)**: 169-173.
Field, L. M., A. L. Devonshire and B. G. Forde (1988). "Molecular evidence that insecticide resistance in peach-potato aphids (Myzus persicae Sulz.) results from amplification of an esterase gene." Biochem J **251**(1): 309-312.
Fields, S. and M. Johnston (2005). "Cell biology. Whither model organism research?" Science **307**(5717): 1885-1886.
Finnegan, D. J. (1989). "Eukaryotic transposable elements and genome evolution." Trends Genet **5**(4): 103-107.
Finney, D. J. (1971). Probit analysis. London/ New York, Cambridge Univ. Press.
Franz, G. and C. Savakis (1991). "Minos, a new transposable element from Drosophila hydei, is a member of the Tc1-like family of transposons." Nucleic Acids Res **19**(23): 6646.
Gibson, G. G. and P. Skett (2001). Introduction to Drug Metabolism, United Kingdom: Nelson Thornes.
Girardot, F., V. Monnier and H. Tricoire (2004). "Genome wide analysis of common and specific stress responses in adult drosophila melanogaster." BMC Genomics **5**: 74.
Giraudo, M., G. C. Unnithan, G. Le Goff and R. Feyereisen (2010). "Regulation of cytochrome P450 expression in Drosophila: Genomic insights." Pestic Biochem Physiol **97**(2): 115-122.
Gonzalez, J. R., B. Rodriguez-Santiago, A. Caceres, R. Pique-Regi, N. Rothman, S. J. Chanock, L. Armengol and L. A. Perez-Jurado (2011). "A fast and accurate method to detect allelic genomic imbalances underlying mosaic rearrangements using SNP array

data." BMC Bioinformatics **12**(1): 166.

Grant, D. F. and B. D. Hammock (1992). "Genetic and molecular evidence for a trans-acting regulatory locus controlling glutathione S-transferase-2 expression in Aedes aegypti." Mol Gen Genet **234**(2): 169-176.

Green, C. D., J. F. Simons, B. E. Taillon and D. A. Lewin (2001). "Open systems: panoramic views of gene expression." J Immunol Methods **250**(1-2): 67-79.

Griffin-Shea, R., G. Thireos and F. C. Kafatos (1982). "Organization of a cluster of four chorion genes in Drosophila and its relationship to developmental expression and amplification." Dev Biol **91**(2): 325-336.

Gunning, R. V., I. G. Ferris and C. S. Easton (1994). "Toxicity, penetration, tissue distribution, and metabolism of methyl parathion in Helicoverpa armigera and H. punctigera (Lepidoptera: Noctuidae)." J Econ Entomol **87**(5): 1180-1184.

Gut, L., A. Schilder, R. Isaacs and P. McManus (2007). Chapter 2: Managing the Community of Pests. Fruit Crop Ecology and Management. Michigan Michigan State University Extension.

Hanriot, L., C. Keime, N. Gay, C. Faure, C. Dossat, P. Wincker, C. Scote-Blachon, C. Peyron and O. Gandrillon (2008). "A combination of LongSAGE with Solexa sequencing is well suited to explore the depth and the complexity of transcriptome." BMC Genomics **9**: 418.

Hayes, J. D. and C. R. Wolf (1988). Role of glutathione transferase in drug resistance. Glutathione Conjugation: Mechanisms and Biological Significance H. Sies and B. Ketterer. London, Academic Press Ltd**:** 315–355.

Hemingway, J. (2000). "The molecular basis of two contrasting metabolic mechanisms of insecticide resistance." Insect Biochem Mol Biol **30**(11): 1009-1015.

Hemingway, J., L. Field and J. Vontas (2002). "An overview of insecticide resistance." Science **298**(5591): 96-97.

Hemingway, J. and S. H. Karunaratne (1998). "Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism." Med Vet Entomol **12**(1): 1-12.

Hemingway, J., J. Miyamoto and P. R. J. Herath (1991). "A possible novel link between organophosphorus and DDT insecticide resistance genes in Anopheles: supporting evidence from fenitrothion metabolism studies." Pesticide Biochemistry and Physiology **39**(1): 49-56.

Hemingway, J. and H. Ranson (2000). "Insecticide resistance in insect vectors of human disease." Annu Rev Entomol **45**: 371-391.

Hodgson, E. (1983). "The significance of cytochrome P-450 in insects." Insect Biochemistry **13**: 237–246.

Hodgson, E. (1985). Microsomal monooxygenases. Comprehensive Insect Physiology, Biochemistry and Pharmacology. G. A. Kerkut and L. I. Gilbert. Oxford, Pergamon**:** 225–331.

Hodgson, E. and P. E. Levi (1998). Interactions of Piperonyl Butoxide with Cytochrome P450. Piperonyl Butoxide: The Insecticide Synergist. D. G. Jones. San Diego, CA, Academic: San Diego, CA**:** 41-53.

Hoffmann, M. P. and A. C. Frodsham (1993). Natural Enemies of Vegetable Insect Pests. Ithaca, NY, Cooperative Extension
Cornell University.

Hoshizaki, D. K. (2005). Fat-cell development. Comprehensive Molecular Insect Science. L. I. Gilbert, K. Iatrou and S. S. Gill. Amsterdam, Elsevier Amsterdam. **2:** 315–345.

Huang da, W., B. T. Sherman and R. A. Lempicki (2009b). "Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists." Nucleic Acids Res **37**(1): 1-13.

Huang, D. W., B. T. Sherman and R. A. Lempicki (2009a). "Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources." Nature Protocol **4**(1): 44-57.

Huang, H. S., N. T. Hu, Y. E. Yao, C. Y. Wu, S. W. Chiang and C. N. Sun (1998).

"Molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the diamondback moth, Plutella xylostella." Insect Biochem Mol Biol **28**(9): 651-658.

Illumina, I. (2010) "Deep-sequencing protocol."

Inceoglu, A. B., T. D. Waite, J. A. Christiansen, R. D. McAbee, S. G. Kamita, B. D. Hammock and A. J. Cornel (2009). "A rapid luminescent assay for measuring cytochrome P450 activity in individual larval Culex pipiens complex mosquitoes (Diptera: Culicidae)." J Med Entomol **46**(1): 83-92.

IRAC. (2005). "insecticide resistance action committee Mode of action classification v5.1." from www.irac-online.org.

Ivics, Z. and Z. Izsvak (2010). "The expanding universe of transposon technologies for gene and cell engineering." Mob DNA **1**(1): 25.

Jeschke, P. (2007). Chemical structural features of commercial neonicotinoids. Modern Crop Protection Compounds

W. Krämer and U. Schirmer. Weinheim, Germany, Wiley VCH**:** 958–961.

Jeschke, P., K. Moriya, R. Lantzsch, H. Seifert, W. Lindner, K. Jelich, A. Gohrt, M. Beck and W. Etzel (2001). "Thiacloprid (Bay YRC2894) – a new member of the chloronicotinyl insecticide (CNI) family." Pflanzenschutz-Nachrichten Bayer **54**(2): 147–160.

Jeschke, P. and R. Nauen (2008). "Neonicotinoids-from zero to hero in insecticide chemistry." Pest Manag Sci **64**(11): 1084-1098.

Jones, R. T., S. E. Bakker, D. Stone, S. N. Shuttleworth, S. Boundy, C. McCart, P. J. Daborn, R. H. ffrench-Constant and J. M. van den Elsen (2010). "Homology modelling of Drosophila cytochrome P450 enzymes associated with insecticide resistance." Pest Manag Sci **66**(10): 1106-1115.

Joussen, N., D. G. Heckel, M. Haas, I. Schuphan and B. Schmidt (2008). "Metabolism of imidacloprid and DDT by P450 CYP6G1 expressed in cell cultures of Nicotiana tabacum suggests detoxification of these insecticides in Cyp6g1-overexpressing strains of Drosophila melanogaster, leading to resistance." Pest Manag Sci **64**(1): 65-73.

Kaiser-Alexnat, R. (2009). "Protease activities in the midgut of Western corn rootworm (Diabrotica virgifera virgifera LeConte)." J Invertebr Pathol **100**(3): 169-174.

Kapetanaki, M. G., T. G. Loukeris, I. Livadaras and C. Savakis (2002). "High frequencies of Minos transposon mobilization are obtained in insects by using in vitro synthesized mRNA as a source of transposase." Nucleic Acids Res **30**(15): 3333-3340.

Karunaratne, S. H., J. Hemingway, K. G. Jayawardena, V. Dassanayaka and A. Vaughan (1995). "Kinetic and molecular differences in the amplified and non-amplified esterases from insecticide-resistant and susceptible Culex quinquefasciatus mosquitoes." J Biol Chem **270**(52): 31124-31128.

Karunaratne, S. H., A. Vaughan, M. G. Paton and J. Hemingway (1998). "Amplification of a serine esterase gene is involved in insecticide resistance in Sri Lankan Culex tritaeniorhynchus." Insect Mol Biol **7**(4): 307-315.

Karunker, I., J. Benting, B. Lueke, T. Ponge, R. Nauen, E. Roditakis, J. Vontas, K. Gorman, I. Denholm and S. Morin (2008). "Over-expression of cytochrome P450 CYP6CM1 is associated with high resistance to imidacloprid in the B and Q biotypes of Bemisia tabaci (Hemiptera: Aleyrodidae)." Insect Biochem Mol Biol **38**(6): 634-644.

Karunker, I., E. Morou, D. Nikou, R. Nauen, R. Sertchook, B. J. Stevenson, M. J. Paine, S. Morin and J. Vontas (2009). "Structural model and functional characterization of the Bemisia tabaci CYP6CM1vQ, a cytochrome P450 associated with high levels of imidacloprid resistance." Insect Biochem Mol Biol **39**(10): 697-706.

Kent, W. J., C. W. Sugnet, T. S. Furey, K. M. Roskin, T. H. Pringle, A. M. Zahler and D. Haussler (2002). "The human genome browser at UCSC." Genome Res **12**(6): 996-1006.

Kikkawa, H. (1964). "Genetical studies on the resistance to parathion in Drosophila melanogaster. II. Induction of a resistance gene from its susceptible allele.

." Botyu-Kagaku **2**: 37-42.
King-Jones, K., M. A. Horner, G. Lam and C. S. Thummel (2006). "The DHR96 nuclear receptor regulates xenobiotic responses in Drosophila." Cell Metab **4**(1): 37-48.
Klinakis, A. G., T. G. Loukeris, A. Pavlopoulos and C. Savakis (2000a). "Mobility assays confirm the broad host-range activity of the Minos transposable element and validate new transformation tools." Insect Mol Biol **9**(3): 269-275.
Klinakis, A. G., L. Zagoraiou, D. K. Vassilatis and C. Savakis (2000b). "Genome-wide insertional mutagenesis in human cells by the Drosophila mobile element Minos." EMBO Rep **1**(5): 416-421.
Koukidou, M., A. Klinakis, C. Reboulakis, L. Zagoraiou, N. Tavernarakis, I. Livadaras, A. Economopoulos and C. Savakis (2006). "Germ line transformation of the olive fly Bactrocera oleae using a versatile transgenesis marker." Insect Mol Biol **15**(1): 95-103.
Lanning, C. L., R. L. Fine, J. J. Corcoran, H. M. Ayad, R. L. Rose and M. B. Abou-Donia (1996). "Tobacco budworm P-glycoprotein: biochemical characterization and its involvement in pesticide resistance." Biochim Biophys Acta **1291**(2): 155-162.
Le Goff, G., S. Boundy, P. J. Daborn, J. L. Yen, L. Sofer, R. Lind, C. Sabourault, L. Madi-Ravazzi and R. H. ffrench-Constant (2003). "Microarray analysis of cytochrome P450 mediated insecticide resistance in Drosophila." Insect Biochem Mol Biol **33**(7): 701-708.
Le Goff, G., F. Hilliou, B. D. Siegfried, S. Boundy, E. Wajnberg, L. Sofer, P. Audant, R. H. ffrench-Constant and R. Feyereisen (2006). "Xenobiotic response in Drosophila melanogaster: sex dependence of P450 and GST gene induction." Insect Biochem Mol Biol **36**(8): 674-682.
Lin, G. G., T. Kozaki and J. G. Scott (2011). "Hormone receptor-like in 96 and Broad-Complex modulate phenobarbital induced transcription of cytochrome P450 CYP6D1 in Drosophila S2 cells." Insect Mol Biol **20**(1): 87-95.
Ling, A. and R. Cordaux (2010). "Insertion sequence inversions mediated by ectopic recombination between terminal inverted repeats." PLoS One **5**(12): e15654.
Lister, R., B. D. Gregory and J. R. Ecker (2009). "Next is now: new technologies for sequencing of genomes, transcriptomes, and beyond." Curr Opin Plant Biol **12**(2): 107-118.
Liu, N. and J. W. Pridgeon (2002). "Metabolic detoxification and the kdr mutation in pyrethroid resistant house flies, Musca domestica (L.)." Pesticide Biochemistry and Physiology **73**: 157-163.
Liu, Z., M. S. Williamson, S. J. Lansdell, I. Denholm, Z. Han and N. S. Millar (2005). "A nicotinic acetylcholine receptor mutation conferring target-site resistance to imidacloprid in Nilaparvata lugens (brown planthopper)." Proc Natl Acad Sci U S A **102**(24): 8420-8425.
Lopes, K. V., L. B. Silva, A. P. Reis, M. G. Oliveira and R. N. Guedes (2010). "Modified alpha-amylase activity among insecticide-resistant and -susceptible strains of the maize weevil, Sitophilus zeamais." J Insect Physiol **56**(9): 1050-1057.
Loukeris, T. G., B. Arca, I. Livadaras, G. Dialektaki and C. Savakis (1995a). "Introduction of the transposable element Minos into the germ line of Drosophila melanogaster." Proc Natl Acad Sci U S A **92**(21): 9485-9489.
Loukeris, T. G., I. Livadaras, B. Arca, S. Zabalou and C. Savakis (1995b). "Gene transfer into the medfly, Ceratitis capitata, with a Drosophila hydei transposable element." Science **270**(5244): 2002-2005.
Low, W. Y., H. L. Ng, C. J. Morton, M. W. Parker, P. Batterham and C. Robin (2007). "Molecular evolution of glutathione S-transferases in the genus Drosophila." Genetics **177**(3): 1363-1375.
Maitra, S., S. M. Dombrowski, M. Basu, O. Raustol, L. C. Waters and R. Ganguly (2000). "Factors on the third chromosome affect the level of cyp6a2 and cyp6a8 expression in Drosophila melanogaster." Gene **248**(1-2): 147-156.
Maitra, S., S. M. Dombrowski, L. C. Waters and R. Ganguly (1996). "Three second

chromosome-linked clustered Cyp6 genes show differential constitutive and barbital-induced expression in DDT-resistant and susceptible strains of Drosophila melanogaster." Gene **180**(1-2): 165-171.
Maragkakis, M., P. Alexiou, G. L. Papadopoulos, M. Reczko, T. Dalamagas, G. Giannopoulos, G. Goumas, E. Koukis, K. Kourtis, V. A. Simossis, P. Sethupathy, T. Vergoulis, N. Koziris, T. Sellis, P. Tsanakas and A. G. Hatzigeorgiou (2009). "Accurate microRNA target prediction correlates with protein repression levels." BMC Bioinformatics **10**: 295.
Marth, G. T., I. Korf, M. D. Yandell, R. T. Yeh, Z. Gu, H. Zakeri, N. O. Stitziel, L. Hillier, P. Y. Kwok and W. R. Gish (1999). "A general approach to single-nucleotide polymorphism discovery." Nat Genet **23**(4): 452-456.
Martin, R. L., B. Pittendrigh, J. Liu, R. Reenan, R. ffrench-Constant and D. A. Hanck (2000). "Point mutations in domain III of a Drosophila neuronal Na channel confer resistance to allethrin." Insect Biochem Mol Biol **30**(11): 1051-1059.
Matsuda, K., S. D. Buckingham, D. Kleier, J. J. Rauh, M. Grauso and D. B. Sattelle (2001). "Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors." Trends Pharmacol Sci **22**(11): 573-580.
Matsumura, F. and A. W. A. Brown (1961). "Biochemistry of malathion resistance in Culex tarsalis." Journal of Economic Entomology **54**: 1176-1185.
Mencke, N. and P. Jeschke (2002). "Therapy and prevention of parasitic insects in veterinary medicine using imidacloprid." Curr Top Med Chem **2**(7): 701-715.
Metaxakis, A., S. Oehler, A. Klinakis and C. Savakis (2005). "Minos as a genetic and genomic tool in Drosophila melanogaster." Genetics **171**(2): 571-581.
Morgan, T. H. (1915). The Mechanism of Mendelian heredity, H. Holt and company, Harvard University.
Morra, R., S. Kuruganti, V. Lam, J. C. Lucchesi and R. Ganguly (2010). "Functional analysis of the cis-acting elements responsible for the induction of the Cyp6a8 and Cyp6g1 genes of Drosophila melanogaster by DDT, phenobarbital and caffeine." Insect Mol Biol **19**(1): 121-130.
Mouches, C., N. Pasteur, J. B. Berge, O. Hyrien, M. Raymond, B. R. de Saint Vincent, M. de Silvestri and G. P. Georghiou (1986). "Amplification of an esterase gene is responsible for insecticide resistance in a California Culex mosquito." Science **233**(4765): 778-780.
Mushtaq, A. S., W. M. Richard, M. David and A. R. Shakoori (2003). "Effect of starvation on proteases in insecticide-resistant and susceptible strains of Tribolium castaneum." Pakistan journal of zoology **35**(3): 197-204.
Mutero, A., M. Pralavorio, J. M. Bride and D. Fournier (1994). "Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase." Proc Natl Acad Sci U S A **91**(13): 5922-5926.
Nauen, R. and I. Denholm (2005). "Resistance of insect pests to neonicotinoid insecticides: current status and future prospects." Arch Insect Biochem Physiol **58**(4): 200-215.
Nauen, R., U. Ebbinghaus-Kintscher, A. Elbert, P. Jeschke and K. Tietjen (2001). Acetylcholine receptors as sites for developing neonicotinoid insecticides. Biochemical sites important in insecticide action and resistance. . I. Ishaaya. New York, New York : Springer Verlag. **:** 77-105.
Nelson, D. R. (2009). "The cytochrome p450 homepage." Hum Genomics **4**(1): 59-65.
Nigatu, W., C. F. Curtis and M. Lulu (1995). "Test for association of DDT resistance with inversion polymorphism in Anopheles arabiensis from Ethiopia." J Am Mosq Control Assoc **11**(2 Pt 1): 238-240.
Oakeshott, J. G., C. Claudianos, R. J. Russell and G. C. Robin (1999). "Carboxyl/cholinesterases: a case study of the evolution of a successful multigene family." Bioessays **21**(12): 1031-1042.
Oakeshott, J. G., I. Home, T. D. Sutherland and R. J. Russell (2003). "The genomics of insecticide resistance." Genome Biol **4**(1): 202.
Okazawa, A., M. Akamatsu, H. Nishiwaki, Y. Nakagawa, H. Miyagawa, K. Nishimura and T.

Ueno (2000). "Three-dimensional quantitative structure activity relationship analysis of acyclic and cyclic chloronicotinyl insecticides." Pest Management Science **56**(6): 509-515.

Ollis, D. L., E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S. M. Franken, M. Harel, S. J. Remington, I. Silman, J. Schrag and et al. (1992). "The alpha/beta hydrolase fold." Protein Eng **5**(3): 197-211.

Ortelli, F., L. C. Rossiter, J. Vontas, H. Ranson and J. Hemingway (2003). "Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide-resistance locus from the malaria vector Anopheles gambiae." Biochem J **373**(Pt 3): 957-963.

Ottea, J. A., S. A. Ibrahm, A. M. Younis and R. J. Young (2000). "Mechanisms of pyrethroid resistance in larvae and adults from a cypermethrin-selected strain of Heliothis virescens (F.). ." Pesticide Biochemistry and Physiology **66**: 20-32.

Pan, C., Y. Zhou and J. Mo (2009). "The clone of laccase gene and its potential function in cuticular penetration resistance of Culex pipiens pallens to fenvalerate." Pesticide Biochemistry and Physiology **93**: 105-111.

Pardue, M. L. (1986). In situ hybridisation to DNA of chromosomes and nuclei. Drosophila: A practical approach. D. B. Roberts, Oxford: IRL press.

Parks, S. and A. Spradling (1987). "Spatially regulated expression of chorion genes during Drosophila oogenesis." Genes Dev **1**: 497-509.

Parks, S., B. Wakimoto and A. Spradling (1986). "Replication and expression of an X-linked cluster of Drosophila chorion genes." Dev Biol **117**(1): 294-305.

Paton, M. G., S. H. Karunaratne, E. Giakoumaki, N. Roberts and J. Hemingway (2000). "Quantitative analysis of gene amplification in insecticide-resistant Culex mosquitoes." Biochem J **346 Pt 1**: 17-24.

Pauluhn, J. (1988). "Technical Grade Imidacloprid (NTN 33893) study for Acute Inhalation Toxicity in the Rat." Bayer AG. Fachbereich Toxikologie Wuppertal, Germany. **DPR Vol. 51950-0002**(Study No. 99806.): 119449.

Pavek, P. and Z. Dvorak (2008). "Xenobiotic-induced transcriptional regulation of xenobiotic metabolizing enzymes of the cytochrome P450 superfamily in human extrahepatic tissues." Curr Drug Metab **9**(2): 129-143.

Pavlopoulos, A., A. J. Berghammer, M. Averof and M. Klingler (2004). "Efficient transformation of the beetle Tribolium castaneum using the Minos transposable element: quantitative and qualitative analysis of genomic integration events." Genetics **167**(2): 737-746.

Pavlopoulos, A., S. Oehler, M. G. Kapetanaki and C. Savakis (2007). "The DNA transposon Minos as a tool for transgenesis and functional genomic analysis in vertebrates and invertebrates." Genome Biol **8 Suppl 1**: S2.

Pedra, J. H., L. M. McIntyre, M. E. Scharf and B. R. Pittendrigh (2004). "Genome-wide transcription profile of field- and laboratory-selected dichlorodiphenyltrichloroethane (DDT)-resistant Drosophila." Proc Natl Acad Sci U S A **101**(18): 7034-7039.

Perry, T., P. Batterham and P. J. Daborn (2011). "The biology of insecticidal activity and resistance." Insect Biochem Mol Biol.

Pfaffl, M. W. and G. W. Horgan (2001). "Calculation Software for the Relative Expression in real time PCR using Pair Wise Fixed Reallocation Randomisation Test." Nucleic Acids Research **29**(9): 45.

Plapp, F. W., Jr. and R. F. Hoyer (1968). "Insecticide resistance in the house fly: decreased rate of absorption as the mechanism of action of a gene that acts as an intensifier of resistance." J Econ Entomol **61**(5): 1298-1303.

Plasterk, R. H., Z. Izsvak and Z. Ivics (1999). "Resident aliens: the Tc1/mariner superfamily of transposable elements." Trends Genet **15**(8): 326-332.

Pluthero, F. G. and S. F. Threlkeld (1981). "Genetic differences in malathion avoidance and resistance in Drosophila melanogaster." J Econ Entomol **74**(6): 736-740.

Pollock, D. D. and J. C. Larkin (2004). "Estimating the degree of saturation in mutant screens." Genetics **168**(1): 489-502.

Prapanthadara, L., J. Hemingway and A. J. Ketterman (1993). "Partial purification and characterization of glutathione S-transferase involved in DDT resistance from the mosquito Anopheles gambiae." Pesticide Biochemistry and Physiology **47**: 119–133.

Pruitt, K. D., T. Tatusova, W. Klimke and D. R. Maglott (2009). "NCBI Reference Sequences: current status, policy and new initiatives." Nucleic Acids Res **37**(Database issue): D32-36.

Puinean, A. M., S. P. Foster, L. Oliphant, I. Denholm, L. M. Field, N. S. Millar, M. S. Williamson and C. Bass (2010). "Amplification of a cytochrome P450 gene is associated with resistance to neonicotinoid insecticides in the aphid Myzus persicae." PLoS Genet **6**(6): e1000999.

Ranson, H., C. Claudianos, F. Ortelli, C. Abgrall, J. Hemingway, M. V. Sharakhova, M. F. Unger, F. H. Collins and R. Feyereisen (2002). "Evolution of supergene families associated with insecticide resistance." Science **298**(5591): 179-181.

Ranson, H., B. Jensen, X. Wang, L. Prapanthadara, J. Hemingway and F. H. Collins (2000). "Genetic mapping of two loci affecting DDT resistance in the malaria vector Anopheles gambiae." Insect Mol Biol **9**(5): 499-507.

Ranson, H., L. Rossiter, F. Ortelli, B. Jensen, X. Wang, C. W. Roth, F. H. Collins and J. Hemingway (2001). "Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector Anopheles gambiae." Biochem J **359**(Pt 2): 295-304.

Reiss, R. A. and A. A. James (1993). "A glutathione S-transferase gene of the vector mosquito, Anopheles gambiae." Insect Mol Biol **2**(1): 25-32.

Report, B. A. (2011). 2010 Bayer Annual Report. C. G. C. O. Jörg Schäfer. 51368 Leverkusen, Germany, Bayer AG**:** 273.

Roditakis, E., M. Grispou, E. Morou, J. B. Kristoffersen, N. Roditakis, R. Nauen, J. Vontas and A. Tsagkarakou (2009). "Current status of insecticide resistance in Q biotype Bemisia tabaci populations from Crete." Pest Manag Sci **65**(3): 313-322.

Rodriguez, M. A., D. Bosch, B. Sauphanor and J. Avilla (2010). "Susceptibility to organophosphate insecticides and activity of detoxifying enzymes in Spanish populations of Cydia pomonella (Lepidoptera: Tortricidae)." J Econ Entomol **103**(2): 482-491.

Rowland, M. (1991). "Activity and mating competitiveness of gamma HCH/dieldrin resistant and susceptible male and virgin female Anopheles gambiae and An.stephensi mosquitoes, with assessment of an insecticide-rotation strategy." Med Vet Entomol **5**(2): 207-222.

Ruijter, J. M., C. Ramakers, W. M. Hoogaars, Y. Karlen, O. Bakker, M. J. van den Hoff and A. F. Moorman (2009). "Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data." Nucleic Acids Res **37**(6): e45.

Ryder, E., F. Blows, M. Ashburner, R. Bautista-Llacer, D. Coulson, J. Drummond, J. Webster, D. Gubb, N. Gunton, G. Johnson, C. J. O'Kane, D. Huen, P. Sharma, Z. Asztalos, H. Baisch, J. Schulze, M. Kube, K. Kittlaus, G. Reuter, P. Maroy, J. Szidonya, A. Rasmuson-Lestander, K. Ekstrom, B. Dickson, C. Hugentobler, H. Stocker, E. Hafen, J. A. Lepesant, G. Pflugfelder, M. Heisenberg, B. Mechler, F. Serras, M. Corominas, S. Schneuwly, T. Preat, J. Roote and S. Russell (2004). "The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in Drosophila melanogaster." Genetics **167**(2): 797-813.

Salinas, A. E. and M. G. Wong (1999). "Glutathione S-transferases--a review." Curr Med Chem **6**(4): 279-309.

Saner, C., B. Weibel, F. E. Wurgler and C. Sengstag (1996). "Metabolism of promutagens catalyzed by Drosophila melanogaster CYP6A2 enzyme in Saccharomyces cerevisiae." Environ Mol Mutagen **27**(1): 46-58.

Sasakura, Y., S. Awazu, S. Chiba, S. Kano and N. Satoh (2003). "Application of Minos, one of the Tc1/mariner superfamily transposable elements, to ascidian embryos as a tool for insertional mutagenesis." Gene **308**: 11-20.

Sawicki, R. M. and K. A. Lord (1970). "Some properties of a mechanism delaying penetration

of insecticides into houseflies." Pest Management Science **1**(5): 213-217.
Schmidt, J. M., R. T. Good, B. Appleton, J. Sherrard, G. C. Raymant, M. R. Bogwitz, J. Martin, P. J. Daborn, M. E. Goddard, P. Batterham and C. Robin (2010). "Copy number variation and transposable elements feature in recent, ongoing adaptation at the Cyp6g1 locus." PLoS Genet **6**(6): e1000998.
Scott, J. G. (1991). Insecticide resistance in insects. Handbook of Pest Management in Agriculture. D. Pimentel. Boca Raton, CRC Press. **2:** 663.
Scott, J. G. (1999). "Cytochromes P450 and insecticide resistance." Insect Biochem Mol Biol **29**(9): 757-777.
Scott, J. G. (2008). Insect cytochrome P450s: Thinking beyond detoxification., Research Signpost
Scott, J. G. and G. P. Georghiou (1986a). "The biochemical genetics of permethrin resistance in the Learn-PyR strain of house fly." Biochem Genet **24**(1-2): 25-37.
Scott, J. G. and G. P. Georghiou (1986b). "Mechanisms responsible for high levels of permethrin resistance in the house fly." Pestic. Sci. **17**: 195-206.
Scott, J. G. and S. Kasai (2004). "Evolutionary plasticity of monooxygenase-mediated resistance." Pesticide Biochemistry and Physiology **78**: 171-178.
Scott, J. G. and S. S. Lee (1993). "Tissue distribution of microsomal cytochrome P-450 monooxygenases and their inducibility by phenobarbital in the insecticide resistant LPR strain of house fly, Musca domestica L." Insect Biochem Mol Biol **23**(6): 729-738.
Scott, J. G. and Z. Wen (2001). "Cytochromes P450 of insects: the tip of the iceberg." Pest Manag Sci **57**(10): 958-967.
Shimizu, K., M. Kamba, H. Sonobe, T. Kanda, A. G. Klinakis, C. Savakis and T. Tamura (2000). "Extrachromosomal transposition of the transposable element Minos occurs in embryos of the silkworm Bombyx mori." Insect Mol Biol **9**(3): 277-281.
Shrivastava, S. P., G. P. Georghiou, R. L. Metcalf and T. R. Fukuto (1970). "Carbamate resistance in mosquitos. The metabolism of propoxur by susceptible and resistant larvae of Culex pipiens fatigans." Bull World Health Organ **42**(6): 931-942.
Sigma Plot 10.0 software (2007). Systat Software Inc., San Johe, CA
Silva, L. B., A. P. Reis, E. J. Pereira, M. G. Oliveira and R. N. Guedes (2010a). "Partial purification and characterization of trypsin-like proteinases from insecticide-resistant and -susceptible strains of the maize weevil, Sitophilus zeamais." Comp Biochem Physiol B Biochem Mol Biol **155**(1): 12-19.
Silva, L. B., A. P. Reis, E. J. Pereira, M. G. Oliveira and R. N. Guedes (2010b). "Altered cysteine proteinase activity in insecticide-resistant strains of the maize weevil: purification and characterization." Comp Biochem Physiol B Biochem Mol Biol **157**(1): 80-87.
Small, G. J. and J. Hemingway (2000). "Molecular characterization of the amplified carboxylesterase gene associated with organophosphorus insecticide resistance in the brown planthopper, Nilaparvata lugens." Insect Mol Biol **9**(6): 647-653.
Smith, A. D., Z. Xuan and M. Q. Zhang (2008). "Using quality scores and longer reads improves accuracy of Solexa read mapping." BMC Bioinformatics **9**: 128.
Snyder, M. J. and D. R. Maddison (1997). "Molecular phylogeny of glutathione-S-transferases." DNA Cell Biol **16**(11): 1373-1384.
Snyder, M. J., J. K. Walding and R. Feyereisen (1995). "Glutathione S-transferases from larval Manduca sexta midgut: sequence of two cDNAs and enzyme induction." Insect Biochem Mol Biol **25**(4): 455-465.
Soderlund, D. M. and D. C. Knipple (2003). "The molecular biology of knockdown resistance to pyrethroid insecticides." Insect Biochem Mol Biol **33**(6): 563-577.
Sparks, T. C., J. A. Lockwood, R. L. Byford, J. B. Graves and B. R. Leonard (1989). "The role of behavior in insecticide resistance." Pest Management Science **26**(4): 383-399.
Spradling, A. C. (1981). "The organization and amplification of two chromosomal domains containing Drosophila chorion genes." Cell **27**(1 Pt 2): 193-201.
SPSS (1999). SPSS Base 10.0 for Windows SPSS Inc., Chicago IL.

Stegeman, J. J. and D. R. Livingstone (1998). "Forms and functions of cytochrome P450." Comp Biochem Physiol C Pharmacol Toxicol Endocrinol **121**(1-3): 1-3.

Sturtevant, A. H. (1913). "The linear arrangement of six sex-linked factors in Drosophila, as shown by their mode of association." Journal of Experimental Zoology **14**: 43-59.

Sueyoshi, T. and M. Negishi (2001). "Phenobarbital response elements of cytochrome P450 genes and nuclear receptors." Annu Rev Pharmacol Toxicol **41**: 123-143.

Sullivan, W., M. Ashburner and R. S. Hawley (2000). Drosophila protocols. NY, Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press.

Sykiotis, G. P. and D. Bohmann (2010). "Stress-activated cap'n'collar transcription factors in aging and human disease." Sci Signal **3**(112): re3.

T.G., W. (2005). "Drosophila: Sentinels of Environmental Toxicants." Integrative and Comparative Biology **45**: 127–136.

Tijet, N., C. Helvig and R. Feyereisen (2001). "The cytochrome P450 gene superfamily in Drosophila melanogaster: annotation, intron-exon organization and phylogeny." Gene **262**(1-2): 189-198.

Timsit, Y. E. and M. Negishi (2007). "CAR and PXR: the xenobiotic-sensing receptors." Steroids **72**(3): 231-246.

Tomizawa, M. and J. E. Casida (2005). "Neonicotinoid insecticide toxicology: mechanisms of selective action." Annu Rev Pharmacol Toxicol **45**: 247-268.

Tomizawa, M., D. L. Lee and J. E. Casida (2000). "Neonicotinoid insecticides: molecular features conferring selectivity for insect versus mammalian nicotinic receptors." J Agric Food Chem **48**(12): 6016-6024.

Toung, Y. P., T. S. Hsieh and C. P. Tu (1993). "The glutathione S-transferase D genes. A divergently organized, intronless gene family in Drosophila melanogaster." J Biol Chem **268**(13): 9737-9746.

Tweedie, S., M. Ashburner, K. Falls, P. Leyland, P. McQuilton, S. Marygold, G. Millburn, D. Osumi-Sutherland, A. Schroeder, R. Seal and H. Zhang (2009). "FlyBase: enhancing Drosophila Gene Ontology annotations." Nucleic Acids Res **37**(Database issue): D555-559.

Vaughan, A. and J. Hemingway (1995). "Mosquito carboxylesterase Est alpha 2(1) (A2). Cloning and sequence of the full-length cDNA for a major insecticide resistance gene worldwide in the mosquito Culex quinquefasciatus." J Biol Chem **270**(28): 17044-17049.

Vaughan, A., T. Rocheleau and R. ffrench-Constant (1997). "Site-directed mutagenesis of an acetylcholinesterase gene from the yellow fever mosquito Aedes aegypti confers insecticide insensitivity." Exp Parasitol **87**(3): 237-244.

Venken, K. J. and H. J. Bellen (2005). "Emerging technologies for gene manipulation in Drosophila melanogaster." Nat Rev Genet **6**(3): 167-178.

Vontas, J. G., G. J. Small and J. Hemingway (2001). "Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens." Biochem J **357**(Pt 1): 65-72.

Vontas, J. G., G. J. Small, D. C. Nikou, H. Ranson and J. Hemingway (2002). "Purification, molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the rice brown planthopper, Nilaparvata lugens." Biochem J **362**(Pt 2): 329-337.

Wasserman, W. W. and A. Sandelin (2004). "Applied bioinformatics for the identification of regulatory elements." Nat Rev Genet **5**(4): 276-287.

Waters, L. C., A. C. Zelhof, B. J. Shaw and L. Y. Ch'ang (1992). "Possible involvement of the long terminal repeat of transposable element 17.6 in regulating expression of an insecticide resistance-associated P450 gene in Drosophila." Proc Natl Acad Sci U S A **89**(11): 4855-4859.

Wei, S. H., A. G. Clark and M. Syvanen (2001). "Identification and cloning of a key insecticide-metabolizing glutathione S-transferase (MdGST-6A) from a hyper insecticide-resistant strain of the housefly Musca domestica." Insect Biochem Mol Biol **31**(12): 1145-1153.

Werck-Reichhart, D. and R. Feyereisen (2000). "Cytochromes P450: a success story." Genome Biol **1**(6): REVIEWS3003.

White, K. P., S. A. Rifkin, P. Hurban and D. S. Hogness (1999). "Microarray analysis of Drosophila development during metamorphosis." Science **286**(5447): 2179-2184.

(WHO), W. H. O. (1957). Expert committee on insecticides.

Williamson, M. S., D. Martinez-Torres, C. A. Hick and A. L. Devonshire (1996). "Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides." Mol Gen Genet **252**(1-2): 51-60.

Willoughby, L., H. Chung, C. Lumb, C. Robin, P. Batterham and P. J. Daborn (2006). "A comparison of Drosophila melanogaster detoxification gene induction responses for six insecticides, caffeine and phenobarbital." Insect Biochem Mol Biol **36**(12): 934-942.

Wilson, T. (1988). "Drosophila melanogaster (Diptera: Drosophilidae): a model insect for insecticide resistance studies." Journal of Economic Entomology **81**(1): 22-27.

Wilson, T. G. (2001). "Resistance of Drosophila to toxins." Annu Rev Entomol **46**: 545-571.

Wilson, T. G. and M. Ashok (1998). "Insecticide resistance resulting from an absence of target-site gene product." Proc Natl Acad Sci U S A **95**(24): 14040-14044.

Wilson, T. G. and J. Fabian (1986). "A Drosophila melanogaster mutant resistant to a chemical analog of juvenile hormone." Dev Biol **118**(1): 190-201.

Wilson, T. G., S. Wang, M. Beno and R. Farkas (2006). "Wide mutational spectrum of a gene involved in hormone action and insecticide resistance in Drosophila melanogaster." Mol Genet Genomics **276**(3): 294-303.

Xu, C., C. Y. Li and A. N. Kong (2005). "Induction of phase I, II and III drug metabolism/transport by xenobiotics." Arch Pharm Res **28**(3): 249-268.

Yan, S., F. Cui and C. Qiao (2009). "Structure, function and applications of carboxylesterases from insects for insecticide resistance." Protein Pept Lett **16**(10): 1181-1188.

Yang, J., C. McCart, D. J. Woods, S. Terhzaz, K. G. Greenwood, R. H. ffrench-Constant and J. A. Dow (2007). "A Drosophila systems approach to xenobiotic metabolism." Physiol Genomics **30**(3): 223-231.

Yang, Y., Y. C. Zhu, J. Ottea, C. Husseneder, B. R. Leonard, C. Abel and F. Huang (2010). "Molecular characterization and RNA interference of three midgut aminopeptidase N isozymes from Bacillus thuringiensis-susceptible and -resistant strains of sugarcane borer, Diatraea saccharalis." Insect Biochem Mol Biol **40**(8): 592-603.

Yoo, C. M., C. B. Bak and H. C. Lee (1996). "Substrate and inhibitor specificities of esterase in Lucilia illustris." Korean J Zool **39**: 190-197.

Zagoraiou, L., D. Drabek, S. Alexaki, J. A. Guy, A. G. Klinakis, A. Langeveld, G. Skavdis, C. Mamalaki, F. Grosveld and C. Savakis (2001). "In vivo transposition of Minos, a Drosophila mobile element, in mammalian tissues." Proc Natl Acad Sci U S A **98**(20): 11474-11478.

Zhang, A., H. Kayser, P. Maienfisch and J. E. Casida (2000). "Insect nicotinic acetylcholine receptor: conserved neonicotinoid specificity of [(3)H]imidacloprid binding site." J Neurochem **75**(3): 1294-1303.

Zhou, Z. H. and M. Syvanen (1997). "A complex glutathione transferase gene family in the housefly Musca domestica." Mol Gen Genet **256**(2): 187-194.

Appendix

Table 1. Overexpression fold difference of Cyp6g1 gene between two lines maintained on standard medium and medium with Imidacloprid (res – resistant line; susc – susceptible line; ST – standard medium; IMI – medium with Imidacloprid)

Cyp6g1	res ST/ susc ST	res IMI/ susc IMI	res IMI/ susc ST	res ST/ susc IMI	susc IMI/ susc ST	res IMI/ res ST
Fold difference	8.20 ± (1.94)	7.32 ± (1.58)	8.42 ± (0.68)	7.13 ± (1.21)	1.15 ± (0.40)	1.03 ± (0.12)

Table 2. Expression differences of Cyp6a2 gene between two lines maintained on standard medium and medium with Imidacloprid (res – resistant line; susc – susceptible line; ST – standard medium; IMI – medium with Imidacloprid)

Cyp6a2	res ST/ susc ST	res IMI/ susc IMI	res IMI/ susc ST	res ST/ susc IMI	susc IMI/ susc ST	res IMI/ res ST
Fold difference	10.34 ± (1.65)	8.04 ± (0.99)	12.00 ± (2.07)	6.92 ± (0.72)	1.49 ± (0.27)	1.16 ± (0.11)

Table 3. Overall number of 51nt reads sequences in the MiT[w^-]3R2 resistant line

Data uploaded on the Gene Expression Omnibus (GEO) site http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM707197

Supplementary file: GSM707197_Resistant_s_1_READS.txt.gz

Table 4. Overall number of 51nt reads sequences in the iso31 susceptible line

Data uploaded on the Gene Expression Omnibus (GEO) site http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM707198

Supplementary file: GSM707198_Susceptible_s_2_READS.txt.gz

Table 5. Total number of transcripts

Data uploaded on the Gene Expression Omnibus (GEO) site http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28560

Supplementary file: GSE28560_total_number_of_transcripts.txt.gz