Innovative methods for insect control and insecticide resistance management

Doctoral Thesis by
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&
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ΙΝΣΤΙΤΟΥΤΟ ΜΟΡΙΑΚΗΣ ΒΙΟΛΟΓΙΑΣ ΚΑΙ ΒΙΟΤΕΧΝΟΛΟΓΙΑΣ

Διδακτορική Διατριβή

Ανάπτυξη καινοτόμων μεθόδων για την καταπολέμηση βλαβερών εντόμων και την αντιμετώπιση της ανθεκτικότητας τους σε εντομοκτόνα

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List of Abbreviations

βFTZ-F1	Fushi tarazu
ABC transporters	ATP-binding cassette transporters
Ace	acetylcholinesterase
BC domain	Biotin Carboxylase domain
BSA	Bovine Serum Albumin
BrC	Broad Complex
Cad	Caudal
CRISPR-Cas9	Clustered Regularly Interspaced Palindromic Repeats-CRISPR-associated Protein 9
dsRNA	double-stranded RNA
DAPI	4′,6-diamidino-2-phenylindole
DCJW	Decarbomethoxyllated JW062
DTT	dichlorodiphenyltrichloroethane
EcR	Ecdysone Receptor
Eip74/75	Ecdysone induced protein 74/75
ER	Endoplasmic reticulum
HR3	Hormone response gene 3
IRAC	Insecticide Resistance Action Committee
IRM	Insecticide Resistance Management
kdr	Knockdown resistance
Lab	Labial
MoA	Mode of Action
OATP74D	Organic Anion Transporting Polypeptide 74D
para	*paralytic* gene of *Drosophila melanogaster*
P450	P450 mono-oxygenase
PtxI	Pituitary homeobox I
qPCR	quantitative Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
RH	Relative Humidity
RNAi	RNA interference
RR	Resistance Ratio
SDS	Sodium dodecyl sulfate
SLC	Solute Carrier transporters
Tsp2A	Tetraspanin 2A
Usp	Ultraspiracle
VGSC	Voltage Gated Sodium Channel
WHO	World Health Organization

Table of Contents

Περίληψη

Ως έντομα εχθροί ορίζονται τα έντομα που προκαλούν σοβαρές επιπτώσεις στις αγροτικές καλλιέργειες αλλά και στη δημόσια υγεία. Ο έλεγχος αυτών πραγματοποιείται κατά κύριο λόγο με τη χρήση των εντομοκτόνων. Παρόλα αυτά η αλόγιστη χρήση στο πεδίο έχει οδηγήσει στη φυσική επιλογή πολλών διαφορετικών ανθεκτικών φαινοτύπων. Η λειτουργική ανάλυση των μοριακών μηχανισμών ανθεκτικότητας είναι απαραίτητη προϋπόθεση για τη διαχείριση και την αντιμετώπιση του φαινομένου, για παράδειγμα μέσω της ανάπτυξη μοριακών διαγνωστικών ή βελτιωμένων σκευασμάτων.

Στα πρώτα δύο κεφάλαια χρησιμοποιήσαμε τον οργανισμό μοντέλο *Drosophila melanogaster* για να μελετήσουμε συγκεκριμένους μηχανισμούς που έχουν συσχετιστεί με την ανθεκτικότητα σε δύο κλάσεις νευροτοξικών εντομοκτόνων, τα πυρεθροειδή καθώς και τα Sodium Channel Blocker Insecticides (SCBIs). Συγκεκριμένα στο πρώτο κεφάλαιο μελετήθηκαν δύο μεταλλαγές του τασεοευαίσθητου καναλιού νατρίου (F1845Y ή V1848I), οι οποίες έχουν συσχετιστεί με την ανθεκτικότητα ενάντια στα εντομοκτόνα indoxacarb και metaflumizone, που ανήκουν στην κατηγορία των SCBIs. Για το σκοπό αυτό, κατασκευάστηκαν αμιγή στελέχη μυγών που φέρουν σε ομόζυγη κατάσταση μία από τις δύο μεταλλαγές, χρησιμοποιώντας την τεχνική γονιδιωματικής τροποποίησης CRISPR/Cas9. Εν συνεχεία πραγματοποιήθηκαν βιοδοκιμές τοξικότητας με σκοπό την αξιολόγηση της συνεισφοράς της εκάστοτε μεταλλαγής στην ανθεκτικότητα ενάντια στα δύο εντομοκτόνα, που έδειξαν ότι και οι δύο μεταλλαγές συνεισφέρουν στην ανθεκτικότητα ενάντια στο indoxacarb και στο metaflumizone, με διαφορετική ωστόσο ένταση στο φαινότυπο. Στο επόμενο κεφάλαιο μελετήθηκε η πιθανή συνεργιστική αλληλεπίδραση δύο διαφορετικών μηχανισμών ανθεκτικότητας ενάντια στα πυρεθροειδή, μία κλάση εντομοκτόνων που χρησιμοποιείται ευρέως για την καταπολέμηση εντόμων φορέων ασθενειών όπως είναι τα κουνούπια *Aedes aegypti*. Ανάμεσα στην πληθώρα γενετικών αλληλομόρφων που έχουν ταυτοποιηθεί στο είδος *Aedes aegypti*, τα πιο συνήθη φαίνεται να είναι οι μεταλλαγές στο τασεοευαίσθητο κανάλι νατρίου (π.χ. η μεταλλαγή στο σημείο V1016G) καθώς και η υπερέκφραση ενζύμων αποτοξικοποίησης (π.χ. η μονοοξυγενάση του κυτοχρώματος P450 CYP9J28). Για το σκοπό αυτό κατασκευάστηκαν διαγονιδιακά στελέχη μυγών που φέρουν τη μεταλλαγή V1016G στο γονίδιο *para* (κανάλι νατρίου) σε συνδυασμό με την ιστοειδική υπερέκφραση του ενζύμου CYP9J28. Βιοδοκιμές τοξικότητας υπέδειξαν τη συνεργιστική αλληλεπίδραση μεταξύ αυτών των δύο μηχανισμών που οδηγεί σε μεγαλύτερο συντελεστή ανθεκτικότητας, σε σχέση με το γινόμενο των επιμέρους επιπέδων ανθεκτικότητας ($RR_{combination}$: 19.85 >> $RR_{CYP9J28}$:1.78 X RR_{V1016G}: 3.00). Τα αποτελέσματα των δύο πρώτων κεφαλαίων μας βοηθούν στην κατανόηση νέων μηχανισμών ανθεκτικότητας και στην αξιολόγηση της αξίας επιμέρους μοριακών διαγνωστικών που εφαρμόζονται για την παρακολούθηση του φαινομένου.

Δεδομένης της ανθεκτικότητας, κρίνεται απαραίτητο να αναπτυχθούν και εφαρμοστούν εναλλακτικές μέθοδοι (όπως βιοτεχνολογικές) διαχείρισης των εντόμων και στο πλαίσιο αυτό εντάσσονται τα επόμενα δύο κεφάλαια της διατριβής. Στο κεφάλαιο 3 ταυτοποιήθηκαν υποκινητές εξαρτώμενοι από τις RNA πολυμεράσες II και III του είδους *Helicoverpa armigera*, με σκοπό την βιοτεχνολογική εφαρμογή τους. Συγκεκριμένα ταυτοποιήθηκαν 4

υποκινητές που εμφανίζουν υψηλή ενεργότητα σε κυτταρική σειρά προερχόμενη από το μεσέντερο λεπιδοπτέρων (RP-HzGUT-AW1(MG)), καθώς και 4 υποκινητές που έχουν την ικανότητα να επάγουν την έκφραση μικρών RNAs (π.χ. shRNAs και sgRNAs), τα οποία αποτελούν βασικό συστατικό τεχνικών γενετικής τροποποίησης (RNAi και CRISPR). Στο κεφάλαιο 4 πραγματοποιήθηκε λειτουργική ανάλυση της πρωτεΐνης OATP74D ως πιθανού μεταφορέα εκδυσόνης καθώς και χαρακτηρισμός της ως πιθανού στόχου για εντομοκτόνα. Αρχικά με τη χρήση των πλασμιδίων CRISPR που κατασκευάστηκαν στο κεφάλαιο 3, κατέστη εφικτή η κατασκευή μεταλλαγμένης για το γονίδιο *HzOatp74D* μονοκλωνικής κυτταρικής σειράς RP-HzGUT-AW1(MG). Η απαλοιφή του γονιδίου οδήγησε σε αδυναμία ενεργοποίησης του μονοπατιού της εκδυσόνης, δεδομένου ότι δεν παρατηρήθηκε αλλαγή στα επίπεδα έκφρασης των γονιδίων στόχων παρουσία της ορμόνης, συγκριτικά με την κυτταρική σειρά αγρίου τύπου. Επιπλέον η υπερέκφραση των ορθόλογων γονιδίων του *Oatp74D* από τα λεπιδόπτερα *H. armigera* και *Spodoptera frugiperda*, υπέδειξε ότι είναι ικανά να επαναφέρουν την ευαισθησία των κυττάρων στην εκδυσόνη. Εν συνεχεία κατασκευάστηκαν κυτταρικές σειρές που υπερεκφράζουν μόνιμα τις πρωτεΐνες HaOATP74D και SfOATP74D με σκοπό τη χρήση τους ως πλατφόρμα για τον έλεγχο πιθανών αναστολέων, όπως η ριφαμπικίνη, που βρέθηκε να είναι ικανή να αναστείλει την είσοδο της εκδυσόνης ενδοκυτταρικά. Μερική απαλοιφή του γονιδίου *Oatp74D* στο λεπιδόπτερο *S. frugiperda* (*in vivo*), υπέδειξε την αναγκαιότητα του γονιδίου αυτού για την ανάπτυξη των εντόμων. Καταλήγοντας, είναι η πρώτη φορά που αποδεικνύεται ότι η μεταφορά εκδυσόνης πραγματοποιείται μέσω του μεταφορέα OATP74D στα λεπιδόπτερα. Επιπλέον αποδείχθηκε ότι η πρωτεΐνη αυτή έχει ουσιαστικό ρόλο στην ανάπτυξη των εντόμων *H. armigera* και *S. frugiperda*, και ότι η δράση της μπορεί να ανασταλεί από χημικούς αναστολείς, ευρήματα τα οποία αναδεικνύουν το ρόλο της μελλοντικά ως πιθανό στόχο εντομοκτόνων.

Abstract

Insect pests pose devastating consequences in both agriculture and public health. Most of the control strategies employed rely on the use of chemical insecticides. However, the heavy use of the same chemical compounds have led to the natural selection of insecticide resistance phenotypes in the field. Functional validation of insecticide resistance mechanisms is a necessary step to manage and cope with this phenomenon, through the development of diagnostic molecular tools and/or generation of efficient insecticidal compounds.

In the first two chapters we used *Drosophila melanagaster* as a tool to functionally validate mechanisms that have been associated with resistance against Sodium Channel Blocker Insecticides (SCBIs) and pyrethroids, two different classes of neurotoxic insecticides. In the first chapter we dealt with two certain point mutations in the voltage gated sodium channel that have been previously associated with resistance against SCBIs. To validate and measure the size of the resistance that each of them confers, we employed CRISPR/Cas9 technology to generate *Drosophila* strains harboring each of the two mutations (F1845Y or V1848I) in the voltage gated sodium channel (*para*) gene. Toxicity bioassays have revealed that both of them confer resistance against indoxacarb and metaflumizone, when compared to wild type control lines, but their effect size was not uniform. In the second chapter we sought to validate a putative synergistic interaction between metabolic and target site resistance mechanisms against pyrethroids, a class of insecticides that is frequently used for the control of mosquito-vectors such as *Aedes aegypti.* Several resistance loci found in this species have been associated and functionally correlated (individually) with pyrethroid resistance. However, the most prevalent are mutations in the *para* gene (e.g. V1016G) and increased pyrethroid detoxification mediated by overexpressed P450s (e.g. CYP9J28). Herein, we combined standard genetics and CRISPR/Cas9 system, to generate *Drosophila* strains that combine both of these resistance alleles in the same genetic background. Toxicity bioassays have revealed that there is a synergistic interaction between these pyrethroid resistance alleles, yielding resistance ratios greater than the product of the resistance ratios obtained for the individual mechanisms ($RR_{combination}$: 19.85 >> $RR_{CYP9J28}$:1.78 X RR_{V1016G}: 3.00). The first two chapters provided essential information to understand novel mechanisms of resistance to insecticides, as well as to evaluate and interpret the validity of the available molecular diagnostics that they are used for monitoring resistance in the field.

Considering the impacts of insecticide resistance in pest control management, it is necessary to develop alternative methods (such as biotechnology based) for effective and sustainable pest control, and the next two chapters are under this framework. In Chapter 3, we tried to develop a genetic toolkit for the non-model species *Helicoverpa armigera.* In particular we have identified and functionally characterized 4 strong RNA-Pol II promoters with strong activity in the midgut derived cell line RP-HzGUT-AW1(MG). Additionally, we characterized 4 functional U6 promoters (RNA-Pol III promoters), able to induce the expression of shRNAs and sgRNAs for RNAi and CRISPR respectively. In Chapter 4 we opted to functionally characterize the OATP74D of two lepidoptera pests, as a putative ecdysone transporter and insecticide target. First, we generated a mutant clone of the cell line RP-HzGUT-AW1(MG),

that harbors a deletion in the *Oatp74D*. Disruption of this gene lead to inability of the activation of the ecdysone pathway, as treatment of the mutant cells with ecdysone did not alter the expression of ecdysone responsive genes, in regard with the wild type cells. Moreover, we generated cell lines overexpressing the *Oatp74D* of *H. armigera* and *S. frugiperda*, which indicated that both of them are sufficient to induce ecdysone dependent gene transcription. Inhibition analysis also denoted that both of these proteins are sensitive to inhibitors of organic anion transporters. Moreover, CRISPR mediated partial disruption of the gene in *S. frugiperda* increased the lethality rates, compared to the negative control. Taken all together, we conclude that lepidoptera OATP74D is an essential protein, sensitive to inhibitors that could be used in the future as a potent insecticide target. In this context we have also generated a cell-based platform for screening candidate chemical compounds against these proteins - targets.

General Introduction

Insects as a threat to humans

Insects represent one of the largest taxa of the animal kingdom, including 1,013,825 species as described in the Catalogue of Life from Zhang (Stork, 2017). A large part of the scientific community focuses on insect pests which are species with adverse effects on cultivated plants causing significant problems on the economy. However, the definition includes also insect vectors of several human diseases which pose severe threats to public health.

Agricultural pests

Agricultural pests are insects that threaten cultivated species causing severe problems in crops but also in food security. A very thorough study of the global distribution of approximately 1,300 invasive insects has revealed that several countries of America and Asia contain numerous of those species imposing severe consequences in their economy (40$billion per year only in the United States) (Paini et al., 2016). There are multiple species threatening broad numbers of plant species, however, of interest are those belonging to the lepidoptera group, like *Plutella xylostella*, *Tuta absoluta*, *S. frugiperda* and *H. armigera*. *P. xylostella* is one of the most destructive pests threatening cruciferous plants worldwide. It is estimated that the annual economic loss is US$4-5 billions (Zalucki et al., 2012). According to CABI this species is distributed almost universally, wherever its cruciferous hosts develop. One of the most cosmopolitan pest species is *H. armigera* which attacks more than 200 plant species, including many of high economic importance like cotton, tomato crops, corn and tobacco. It is distributed in Africa, Asia, Australasia, South and central America and there is an increased risk assessment for extending its distribution to North America that would be followed by severe economic consequences (Kriticos et al., 2015). *S. frugiperda* is another polyphagous pest able to parasitize more than 60 plant species (e.g. maize, rice) and is present in Africa, Asia, Oceania and widely distributed in South America (Parra et al., 2021). Given the dynamic range of distributions which is highly dependent on the climate conditions, it is foreseen that their consequences will be exacerbated. Therefore, chemical control strategies need to be reconsidered and sustainable management plans need to be developed for more effective control.

Insect-Vectors of human diseases

Vector-borne diseases account for more than 17% of all infectious diseases, with more than three billion people at risk, bringing about 700,000 deaths per annum (WHO, 2014, Girard et al., 2020). Overall, nine different species have been officially recorded as vectors by the World Health Organization (WHO), and eight of them are insects (WHO, 2014). The most important and dangerous vectors are mosquitoes and especially species of the genera *Anopheles, Aedes* and *Culex*. *Aedes aegypti* and *Aedes albopictus* are two of the most consequential anthropophilic mosquitoes globally distributed and thriving in urban locations. They exhibit high vectorial capacity

for several human diseases including the yellow fever, Chikungunya, Dengue fever and the Zika virus, which all cause high disease burden in worldwide scale (Kraemer et al., 2015). Chikungunya and dengue fever are severe illnesses with high impact on public human health; more than 2.5 billion people are at risk upon infection with dengue virus being manifested in more than 100 countries globally including Europe and the Americas, while chikungunya virus seems to occur mainly in Africa and Asia (WHO, 2014). Yellow fever is another disease transmitted by *Aedes* species, being responsible for 29,000-60,000 deaths/ year until 2013 (WHO, 2014). Although there is no specific cure, vaccination along with vector control and the Eliminate Yellow fever Epidemics strategy have significantly reduced the spread of the disease. Zika virus has been associated with several consequences during pregnancy (microcephaly) but also with Guillain-Barre syndrome (WHO, 2014).

Malaria is the most significant vector borne disease caused by the parasite *Plasmodium falciparum*, which is transmitted by the female mosquitoes of the genus *Anopheles* (Cowman et al., 2017). Although several malaria incidents have been recorded in Asia and South America, sub-Saharan Africa has been always the continent with the highest number of cases and deaths annually, accounting for 95% of cases and 96% of deaths globally (WHO malaria report, 2020). From 2000 to 2015 malaria deaths have declined steadily by almost 37% mainly due to the use of insecticides (Bhatt et al., 2015). Insecticides treated nets (ITNs), inner residual spraying (IRS) and treatment of malaria with artemisinin are the most common practices for intervention in malaria cases.

Insecticides: An effective method for pest control

One of the most efficient weapons in the humans' arsenal for pest control is the use of insecticides. Today, there is a big list of several insecticides which are classified in five large classes based on the physiological functions affected and in 34 main groups based on their mode of action. The 34 main groups of insecticides are summarized in Figure 1. Further below we discuss about the most frequently used insecticides based on their targets, focusing more on the ones that are related with the Chapters of this thesis.

The most widely known neurotoxic insecticides are Dichlorodiphenyltrichloroethan (DDT) and pyrethroids. Both of them target the insects' sodium channel, an essential protein for the generation and propagation of action potentials in neurons and other excitable cells. Its role in regulating membrane excitability is profound and therefore it comprises a target of many different neurotoxins, including four main categories of insecticides; DDT (1,1,1-trichloro-2-2-di-(p-chlorophenyl)ethane), pyrethroids and pyrethrins and sodium channels blocker insecticides (Davies et al., 2007). DDT, pyrethrins and pyrethroids share similar mode of action. In particular they bind with high affinity at the open (activated) state of the sodium channel prohibiting the transition of the channel to the inactivation state, resulting in prolonged opening of the channel and persistent influx of sodium current, leading to repetitive firing and membrane depolarization which consequently brings about disruption of electrical signaling and nerve function (Bloomquist, 1996). On the contrary Sodium Channel Blocker Insecticides (SCBIs) bind preferentially to the slow inactivated state of the channel shifting the voltage dependence of slow inactivation to more negative potentials which obscures the transition to the activation state (block of inward sodium currents, for further details see Chapter 1).

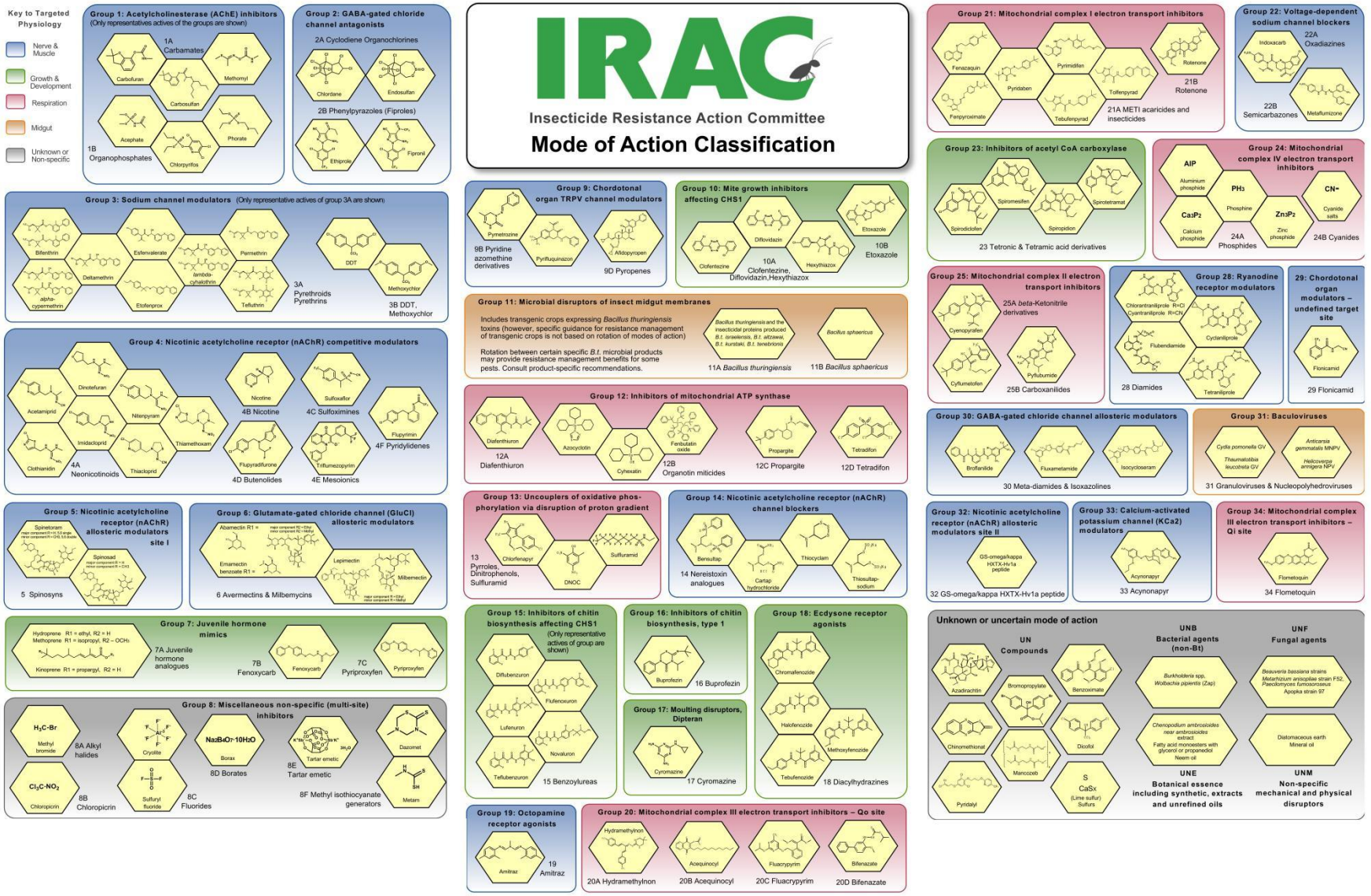


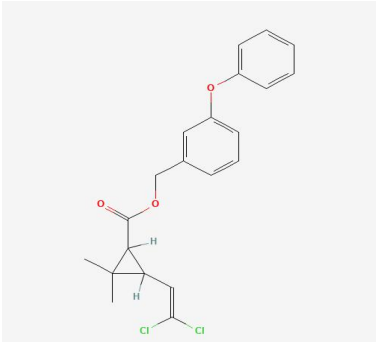
Figure 1. Illustration of the 34 groups of insecticides based on their modes of actions, adapted from IRAC MoA (https://irac-online.org).

DDT is one of the first synthetic insecticides developed in the 1940s for the control of vector borne disease outbreaks such as malaria and yellow fever (Davies et al., 2007). Although highly efficient in controlling such diseases (Roberts, 2001, Berg et al., 2017), DDT use was prohibited by the US Environmental Protection Agency (EPA) in 1972, due to the suspected impacts of this chemical on the environment but also on human health. However, it still remains an effective weapon in vector-borne diseases control arsenal in some regions in the world (Berg et al., 2017). Nevertheless, alternative solutions for pest control had to be found like switching to use of pyrethroids.

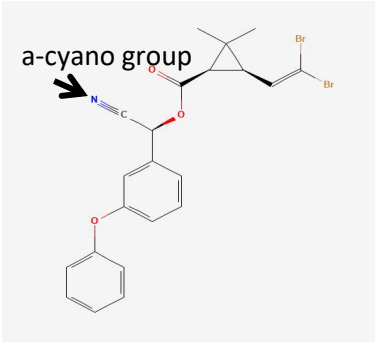
Pyrethroids are synthetic analogs of natural pyrethrins, generated as a need to increase their stability in the air and light. As previously mentioned, they target sodium channel and they are known for their fast-acting and high insecticidal activities, but also for their low mammalian toxicity (WHO, 2014). Therefore, they are systemically used in the cases of Insecticide Treated Nets (ITNs) and Long Lasting insecticides Treated Nets (LLINs) as a mean for the control of malaria, leishmaniasis, dengue fever and other vector borne diseases (WHO, 2014, WHO malaria report, 2021). There are two main types of pyrethroids based on the presence (type II pyrethroids) or absence (type I pyrethroids) of an a-cyano group at the 3-phenoxybenyl alcohol group (Figure 2) (Ensley, 2007).

Several studies have tried to delineate the possible binding sites of pyrethroids at the voltage gated sodium channel. O' Reilly had proposed a possible pyrethroid receptor site to the lipid exposed interface that is formed between the linker of the segments 4 and 5 of the domain II (IIL4,5), the IIS5 and the IIIS6 domains (O'Reilly et al., 2006). Based on this model and site-directed mutagenesis, specific residues of the IIIS6 domain have been correlated with lower binding affinity with pyrethroids such as permethrin. However, as Du mentioned in his report, point mutations correlated with resistance against pyrethroids (e.g. the mutation L1014F) are found in domains that are not included in the O'Reilly suggested pyrethroid site (Du et al., 2013). *In vitro* electrophysiology studies coupled with site directed mutagenesis and computational biology have indicated that there are two major pyrethroid receptor sites (PyR1 and PyR2) which are formed in between two homologous lipid exposed interfaces; the one formed among the linker of 4 and 5 helices of domain II, the IIS5, the IIS6 and IIIS6 and the second formed among the IL4,5 linker, the IS5, the IS6 and the IIS6 domains (Figure 2) (Du et al., 2016). Pyrethroids exhibit high selectivity against insects and low mammalian toxicity and this is attributed to the residue differences between the two proteins. Site directed mutagenesis has revealed that residues of the PyR2 receptor site are critical for the high selectivity of pyrethroids towards insects (Du et al., 2013).

A



Permethrin (PubChem ID: 40326)



Deltamethrin (PubChem ID: 40585)

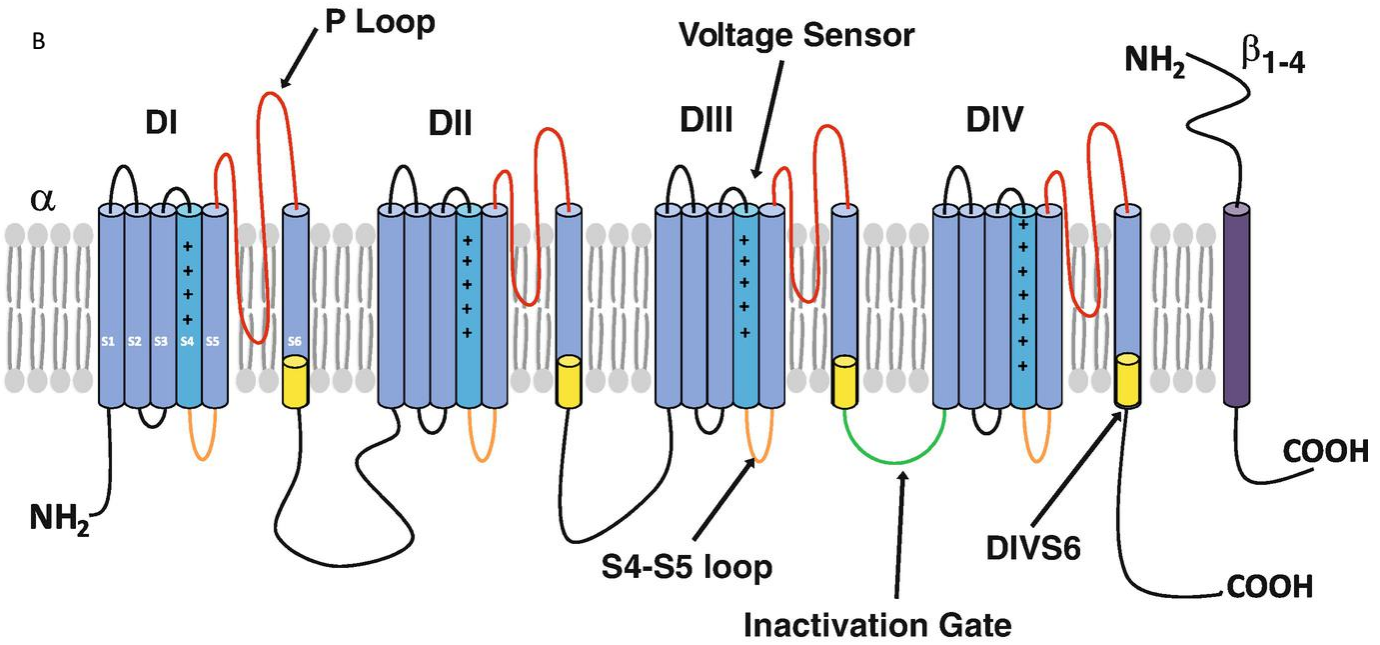


Figure 2. A. Illustration of the chemical structure of permethrin (type I pyrethroid) and deltamethrin (type II pyrethroid). B. Illustration of the four domains of alpha-subunit of Voltage gated sodium channel and the beta-subunit (O'Leary et al., 2017)

Another interesting chemical group of insecticides are those related with disruption of the endocrine system, such as the Ecdysone Receptor agonists. Ecdysone Receptor (EcR) is an insect specific nuclear receptor that interacts with another nuclear receptor, Ultraspiracle, to form a heterodimer. Upon binding with their ligand 20-hydroxyecdysone (20-HE), they act as transcription factor to regulate the expression of genes that are related with insect development (larval molting and metamorphosis). Certain insecticides (such as chromafenozide, halofenozide, methoxyfenozide and tebufenozide) have been developed to target EcR and act as agonists leading to molting associated endocrine disruption (precocious molting and incomplete ecdysis) which leads eventually to death (Song et al., 2017). The EcR agonists diacylhydrazines, such as the chromafenozide and tebufenozide, have been commercialized since 1990s and used for the control of several pests like lepidoptera (Wang et al., 2017). Ecdysone pathway is crucial for insect development and drugs targeting EcR exhibit high selectivity towards insects, therefore in terms of IRM and pest control developing novel drugs against components of this pathway could be a very good practice (for further information see Chapter 4).

Despite the different modes of action, pest control is still a major problem to cope with and the most important reason is the selection of resistance phenotypes due to the persistent use of the same pesticides in the field.

Insecticide Resistance

One of the most major problems in pest control strategies is the emergence of insecticide resistance, an evolutionary adaptive process varying in space and time regulated by several biological, genetic and environmental factors. Insecticide resistance could be defined as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected levels of control when used according to the

label recommendation for that pest species" (IRAC, 2021). The first case of resistance was documented almost 100 years ago, but a significant outbreak happened in the 1940s and afterwards, probably due to the intensive use of synthetic insecticides, like DDT, carbamates and organophosphates (Sparks et al., 2021). Until today, the systemic use of several of the aforementioned classes of insecticides for insect pests' control has led to the emergence of resistant populations reducing dramatically the efficiency of the current control strategies, with diverse impacts on both agriculture and public health. For example, malaria incidents were gradually reducing over the years since 2000. From 2015 to 2019 the number of cases and deaths were stable but between 2019 and 2020 the number of deaths increased from 534,000 to 602,000 (WHO malaria report, 2021). This increase could be probably associated with the outbreak of COVID-19 disease, which impacted dramatically the healthcare systems in 2020. Nevertheless, one of the most prominent and profound reasons for the reduced efficacy of malaria control strategies during the last seven years is the emergence of resistance against insecticides used in Insecticide Treated Nets (ITN) and Indoor Residual Spraying (IRS) practices.

Evolutionarily, it is believed that due to the standing genetic variation in natural insect populations, there are rare individuals carrying one or more resistance alleles in low frequency (polymorphisms) correlated with insecticide resistance (Hawkins et al., 2019). These kinds of polymorphisms could be the result of random mutagenesis and might have some other function prior to their role in resistance (ffrench-Constant, 2013). Introduction and application of insecticides against a field population of insects can lead to the demise of sensitive individuals but survival of the resistant ones. Thereafter the population is constituted by individuals carrying the resistance alleles which shall be maintained in heterozygous or homozygous state (depending on the intensity of insecticides use). The persistence of the selective pressure can lead to the permanent establishment (fixation) of the mutation associated with the resistance within the population.

Insecticide resistance mechanisms

Identification of the molecular mechanisms underlying insecticide resistance is essential for monitoring and prevention of this phenomenon. To date four different resistance mechanisms have been reported: a) Target site resistance, b) Metabolic resistance, c) Penetration resistance and d) Behavioral resistance.

I. Target site resistance

Target site resistance is one of the best known and reported mechanisms against several insecticides, especially against pyrethroids. This mechanism is related with specific non-synonymous mutations that lead to reduction of the affinity between the insecticide and its target. Target site resistance to organochlorines (DDT) and pyrethroids was firstly reported in *Musca domestica* in 1976 (Naqqash et al., 2016), which comprised the substitution L1014F in the S6 segment of the II domain of the voltage gated sodium channel, also known as kdr (knock-down resistance). Since then, many different species of agricultural pests and disease vectors have been found to carry this mutation alone or in combination with other alterations in the Voltage Gated Sodium Channel (VGSC) sequence. Apart from L1014F mutation, several mutations in other residue sites have been mapped and correlated with pyrethroid resistance. M918T is another mutation that although it did not have great effect in

resistance when found alone, its combination with L1014F causes the super-kdr phenotype by leading to greater reduction of sodium channel sensitivity against pyrethroids. To date several kdr mutations have been identified in the sequence of VGSC associated with pyrethroid and DTT resistance (Smith et al., 2016). However, there are species, like *Aedes aegypti* and *Aedes albopictus,* which although are highly resistant against pyrethroids, they were never found to harbor the L1014F mutation but instead they carried other mutations associated with the resistance phenotype (Scott, 2019). These mutations are in different domains of the protein, like the F1534C at the IIIS6 and the V1016G at the IIS5 (Scott, 2019).

The contribution of VGSC mutations to resistance against pyrethroids has been verified by heterologous expression of sodium channel in *Xenopus* oocytes and electrophysiology experiments (Dong et al., 2014, Du et al., 2016). Whether these mutations do only affect the binding affinity of the pyrethroids with the protein or not is still under investigation. There are *in vitro* studies indicating that mutations in the VGSC could alter the gating properties (e.g. shifting the voltage dependence of both activation and slow inactivation to more positive potentials) of the channel in a manner that could slow the rates of activation or accelerate the deactivation (Silver et al., 2014). Given that pyrethroids show a high preference for binding at the open/activated gate of the sodium channel, such changes could reduce the sensitivity to the insecticide.

II. Metabolic resistance

Another major mechanism of resistance against insecticides is the increased metabolic – detoxifying activity performed by specific enzymes. The most important enzyme families involved in detoxification are esterases, cytochrome P450-dependent monooxygenases and glutathione S-transferases (GSTs) (Casida, 2017) but in here we are going to focus on cytochrome P450s. The two main mechanisms by which those enzymes mediate resistance in the insects are: a) gene overexpression for their overproduction in order to sequester the toxic agents they are exposed to or by b) point mutations in the catalytic activity of the detoxifying enzyme which increases the affinity with its substrate (although the latter is less frequently addressed) (Nauen et al., 2022).

Cytochrome P450 monooxygenases are a superfamily of proteins found in all living organisms (Feyereisen, 2005). The role and the diversity of cytochrome P450s have been well described in several reviews (Feyereisen, 2005, Feyereisen, 2012). These enzymes are also known as heme thiolate proteins, since they comprise a characteristic cysteine axial ligand to the heme iron which is located toward to the C-terminus of the protein in a highly conserved region (Feyereisen, 2005). Several P450 genes have been identified to serve essential functions related to insect development (*Drosophila* CYP314A1 or else *shade* with an essential role in the hydroxylation of ecdysone to 20-hydroxyecdysone, Feyereisen, 2012), insect communication and desiccation resistance (mosquitoes and *Drosophila* CYP4G, Balabanidou et al., 2018, Kefi et al., 2019), host-plant adaptation and xenobiotic resistance (Feyereisen, 2012, Vontas et al., 2020). Given the diversity of P450s among insects, our knowledge of insect P450s function is very limited and therefore there is a lot of effort by the insect scientific community to shed light in their regulation, molecular physiology and their relatedness with insecticide resistance.

There are two key mechanisms leading to P450-linked insecticide resistance; increased expression of the P450 protein available to metabolize the insecticide and qualitative changes that increase the affinity of the enzyme with the substrate. The first mechanism can be manifested by two significant types of changes: a) constitutive changes leading to resistance exhibited from generation to generation (heritable change) or b) reversible changes displayed

temporarily (induction) increasing the tolerance of insects (Nauen et al., 2022). Changes in the promoter sequence of P450s linked with resistance to xenobiotics have been associated with the regulation of gene expression (*cis*-regulation) and they are describing well the case of constitutive heritable changes. A characteristic example is the *Drosophila Cyp6g1* which conferring resistance to DDT (Daborn et al., 2001) and was found that its up-regulation was caused by *cis*-acting elements inserted in the promoter sequence of the gene by the retrotransposon Accord (Chung et al., 2007). Another example is the regulation of the *Tetranychus urticae* P450 CYP392A16 which is associated with abamectin resistance in and its expression has been functionally correlated with *cis*-acting elements in the promoter of the gene (Papapostolou et al., 2022).

These enzymes are known for their wide substrate specificity and the numerous activities they are able to catalyze, with the most prevalent and related with insecticide resistance to be the one of monooxygenase (Feyereisen, 2005). The major metabolic pathway against pyrethroids is the 4'-hydroxylation, which is common among insect P450s. The hydroxylation is carried out with the transfer of one atom of oxygen to the substrate (insecticide) and the reduction of the second atom of oxygen to form one molecule of H_2O. NADPH is required as electron donor, which is mediated by the cytochrome P450 reductase (CPR) (Panini et al., 2016). This reaction renders the compound less toxic, more hydrophilic and more excretable (David et al., 2013, Nauen et al., 2022). The contribution of this protein family to insecticide resistance has been verified in many resistant insects and especially in mosquitoes of *Anopheles* and *Aedes* species (David et al., 2013, Liu, 2015, Vontas et al., 2020). Overall, it is crucial to functionally validate the roles of certain P450s that are associated with resistance to insecticides and there are several *in vivo* and/or *in vitro* methods developed nowadays (reviewed in Nauen et al., 2022, Vontas et al., 2020).

III. Penetration resistance

Insects make physical (tarsal) contact with an area treated with insecticides. Once the insect is exposed, the insecticide is believed to pass through the cuticle, in order to reach its target. However, there are many cases of insects such as *A. gambiae* (Balabanidou et al., 2016) which develop a physico-chemically altered structure of the cuticle, rendering the uptake of the insecticide more difficult. These mosquitoes have been found to express certain enzymes, like CYP4G16 and CYP4G17, which share similar function with the CYP4G1 of *Drosophila* in hydrocarbon biosynthesis (Kefi et al., 2019). Those hydrocarbons are believed to play a certain role in the thickening of the cuticle compared to the susceptible counterparts. This mechanism of resistance, also known as penetration resistance, protects the insects from a wide range of insecticides, specifically when it acts in synergism with other mechanisms such as metabolic resistance.

IV. Behavioral resistance

Insects exhibit the ability to escape from an area treated with insecticides, often without lethal consequence. This response can be further divided into a) "direct excitation" which involves the physical contact of the insect with the insecticide and b) "non-contact spatial repellency". In the last case no physical contact of the insect with the chemical is taking place. This ability is referred as behavioral resistance to insecticides (Chareonviriyaphap et al., 2013).

Means to overcome resistance & implement effective control strategies

Given the tremendous effects of the exponential phase of resistance cases reported by the years (Sparks et al., 2021) novel pest control strategies have to be generated. The repetitive failure for successful pest control during the 1960s and 1970s turned the scientific community and crop protection companies to propose strategies for effective insect (pests & vectors) control. The Insecticide Resistance Action Committee was founded in an attempt to circumvent the effects of insecticide resistance setting as major goal the development of Insecticide Resistance Management (IRM) strategies, in order to retain the efficacy of the already commercial compounds and support sustainable agriculture and improved public health. Three major practices employed for resistance management are rotations, mosaics and mixtures of insecticides with different mode of actions, so that to avoid the case of being targeted by the already existing resistance mechanisms (Dusfou et al., 2019). However prior to proceeding to integrated IRM strategies there are some criteria that need to be considered: a) quantification of resistance levels of resistance phenotype (by performing toxicity bioassays), b) understanding and functional validation of putative mechanisms underpinning resistance, c) understanding of the evolutionary outcome of these resistance mechanisms in terms of insects fitness, which is generally considered in the framework of rotation-based use of insecticides for IRM (given that resistance alleles manifesting fitness cost will decrease in frequency in the absence of selection), d) monitoring resistance in the field by using molecular markers of validated mechanisms as diagnostics. Regardless of IRM, another serious caveat for successful pest and vector control is the dearth of commercially available insecticides with different Modes of Action. To sidestep this drawback, the research community has started putting a lot of effort on the identification of novel insecticide targets for novel drugs (biopesticides, RNAi-plant mediated control etc). The functional validation of insecticide resistance mechanisms and the identification of novel drug targets are the major two topics of this thesis, therefore below we introduce each topic separately.

Functional validation of insecticide resistance mechanisms

To better understand the mechanisms underlying insecticide resistance, it is important to identify the genetic loci (including mutations or genes) that are related with the resistance phenotype. From classic genetic approaches to -omics, several alleles have been associated with resistance to certain insecticides. However, association of the phenotype with a possible genetic loci (highlighted by allele frequency in resistant populations or expression levels) is not enough for drawing inferences about their actual contribution, given two specific caveats; lacking of susceptible strains of the same genetic background for direct pairwise comparison and possible existence of confounding or synergistically acting mechanisms in the resistant populations. Therefore, functional validation of the contribution of a putative mechanism to resistance is necessary and is also the basis for the generation of molecular diagnostics which are usually used for monitoring resistance in the field (Van Leeuwen et al., 2020). Model species such as *Drosophila* along with several functional approaches and tools developed (e.g. RNAi-silencing, genetic knock-out and knock-in via CRISPR, *in vivo* overexpression) are widely used for validating and measuring the effect size of certain mechanisms of resistance (Douris et al., 2020, Nauen et al., 2022).

I. Drosophila: a tool to study insecticide biology

The fly *Drosophila melanogaster* has been widely used as a tool for investigating the genetic and molecular underpinnings of basic biological processes. Certain advantages like the short life cycle (approximately 10 days at optimal temperature conditions), the almost fully sequenced and annotated genome and transcriptome (spatiotemporally), the accessibility to large libraries with RNAi stocks, knock-out mutants, balancer and driver lines in combination with the UAS-ORFeome initiative (Bischof et al., 2013), make this model species almost inevitable in research (Scott and Buchon et al., 2019). In the context of insect toxicology, *Drosophila* provides all the necessary means to reveal the mechanisms underlying the penetration of the insecticide through the insect cuticle or gut, distribution of the compound into the insect body, detoxification/metabolism, insecticide-target interaction and finally excretion (Scott and Buchon, 2019). However, its contribution is also enormous in functional validation of putative mechanisms of resistance (Perry and Batterham, 2018). The established molecular genetic toolbox such as the bipartite GAL4-UAS system in *Drosophila*, has allowed the overexpression of certain enzymes for validating their *in vivo* metabolic activity, such as CYP9J28 (Pavlidi et al., 2012), CYP6A51 (Tsakireli et al., 2019) and CYP392A16 (Riga et al., 2020a) and several other P450s (reviewed in Nauen et al., 2022, Vontas et al., 2020). Furthermore, employing CRISPR in *Drosophila* has contributed to the functional validation of specific mutations with resistance to insecticides (see further below). The ability of generating several mutants with a well-defined genetic background is of the most critical reasons for using *Drosophila* as a tool in insecticide biology, since any putative resistant allele could be studied in a background free of any other resistance mechanisms.

II. Reverse genetics: model and non-model organisms

One of the most prevalent and widely used methods for genome modification is CRISPR/Cas9 (Clustered, regularly interspaced, short palindromic repeats). Cas9 is an endonuclease originally discovered in bacteria as a defense system, that generates Double Stranded Breaks (DSBs) in the genome, in a sequence specific manner mediated by a single-guide 20nt RNA sequence (sgRNA) (Wiedenheft et al., 2012, Jinek, et al., 2012). DSBs induced by the complex of Cas9-sgRNA lead to the recruitment of either of two mechanisms for DNA repair, the Non-Homologous End Joining pathway (NHEJ) and the Homologous-Directed Repair (HDR) (Basset et al., 2014). Single nucleotide modifications can be mediated by the employment of HDR, driving to substitution of a gene region, containing the modifications of interest. CRISPR/Cas9 has been widely used in insecticide biology mostly in the cases of analyzing the contribution of point mutations associated with resistance to insecticides (Douris et al., 2020, also see Chapters 1 and 2). Similarly, it has been also performed for unraveling the relevance of a whole P450 gene cluster with insecticide resistance (Wang et al., 2018).

Identification of novel drug targets

The reduction in the efficiency of pest/vector control is underlined by the emergence of insecticide resistance as previously described, however there are two other factors as the lack of novel insecticidal compounds and the dearth of diversity in the modes of action, that altogether highlight the necessity for the identification of novel compounds and insecticide targets with high selectivity. From 1950s there was a slow yet steady increase in the list of insecticide targets, reaching in total 22 known protein targets at 2018 (Swale, 2019). However, the

number is still limited in comparison with the known drug targets of human pharmaceuticals which count more than 700 (Swale et al., 2019). Therefore, the research community that focus on novel insecticide chemistries have to follow the example and protocols of human pharmaceuticals.

Identification of novel targets is a rather complicated and daunting task, given that there are several considerations that have to be taken into account prior to selecting a candidate target: a) essentiality of the protein, b) high selectivity towards insect species of interests: evolutionary conservation of the protein (in regard with humans and beneficial insects), c) accessibility of the protein to the putative drug (tissue localization, subcellular localization, membranous or intracellular), d) druggability of the protein (prediction of possible binding pockets, druggability scores, computational models with docking simulations) and finally e) assayability (assay development for screening different inhibitors/drugs). Further below we are discussing the methodologies that can be used for the identification of novel insecticide targets and we are referring to methods available for addressing their essentiality, conservation and assayability.

Methods employed for characterization of novel targets

I) Identification of the targets

One approach for identification of novel drug targets is the translation of human targets to insecticide targets (Swale, 2019). This approach transfers the existing knowledge for druggable human targets to insects, but it suffers from one major shortcoming, the low selectivity towards insects. Another approach for identification of novel insecticide targets is by inferring knowledge from omic technologies like comparative genomics, which allow the identification of small differences between species leading to the design of drugs targeting essential proteins with high species selectivity (targeting only the species of interest based on the target conservation) (Ngai et al., 2017). Genomics and transcriptomics have been successfully used to find novel targets, like the identification and functional characterization of G-protein coupled receptors (GPCRs) (Liu et al., 2021). A noteworthy example is the identification of the human ortholog Neuropeptide Y (NPY), a GPCR-like receptor, in the mosquito *Aedes aegypti* which controls host seeking and blood feeding and is modulated by certain small molecules agonists, which is suggestive of its use as a putative insecticide target (Duvall et al., 2019).

Another approach for identification of novel insecticide targets is the use of genome-wide RNAi screenings from model species like *Drosophila* and *Tribolium* (Liu et al., 2016, Ulrich et al., 2015). Such lists provide the basis for identifying essential genes in the context of novel drug target identification. Using comparative genomics and transcriptomic approaches we are able to identify whether the genes inferred from these lists, are expressed in the target organisms and finally proceed to phylogenetic analysis in order to check for their conservation. In Chapter 4 we have followed a similar approach. First, we identified that a *Drosophila* gene essential for larval development is implicated in the ecdysone pathway, which comprise already a target of insecticides. Subsequent phylogenetic analysis denoted the existence of this gene in our species of interest, while transcriptomic data indicated its expression at all larval stages, which finally let us proceed with further functional characterization.

II) Characterization of essentiality

The key point of targets is that the protein has to be essential for the insects' development and survival. Neural targets, such as the VGSC, are essential proteins for the insect survival leading to fast acting lethal phenotypes upon inhibition of their function. Provided this condition, industrial research has focused a lot on targets related with this tissue and other tissues which will yield similar effects like rapid immobility and cessation of feeding. However, there are also other essential tissues that could be taken account of, like the insects' midgut and salivary glands, with several essential proteins related with development and survivorship. To characterize the essentiality of particular proteins, several methodologies have been used, with the most prevalent to be RNAi and CRISPR. RNAi has been extensively used for screening several candidate genes as putative insecticide targets in several pests, such as *Tribolium castaneum* (Baum et al., 2007), in *Nezara viridula* (Riga et al., 2020b), in *Myzus perscicae* (Tzin et al., 2015), in *Bemisia tabaci* (Thakur et al., 2013) and *Locusta migratoria* (Liu et a., 2022). The CRISPR/Cas9 system is another versatile transgenesis technique that could be employed for analyzing the essentiality of genes in non-model species. Recently Zhu et al. (2020) have demonstrated its application for analyzing the phenotype of certain genes in a single generation. Similarly, CRISPR has been utilized for analysis of gene essentiality in several other insects (reviewed in Ngai et al., 2017).

III) Screening platforms

One major barrier in the novel drug target characterization is the identification of chemistries with selectivity against the protein of interest. Therefore, the development of *in vitro* platforms destined for high-throughput screening of several compounds is mandatory. *In vitro* screening methods are widely used in the target discovery by human pharmaceuticals (Swale, 2019). Cell cultures show significant merit for *in vitro* drug target characterization. Cell lines amenable to transfection, over-expression of proteins and high-throughput screening methodologies are of particular interest. A well-suited example is the use of the HEK-293 cell line for over-expression of the inward rectifier potassium channel Kir, which served as a platform for screening different chemical structures (Lewis et al., 2009). Similarly, Swevers et al. (2004) have generated a stable lepidoptera cell line expressing an ecdysone induced gene reporter, aiming to be used for screening different ecdysone analogs. Employing *in vitro* screening systems would rather facilitate narrowing down the lists of the selective candidate chemical leads for the protein-target of interest.

It is worth noting that *in vitro* assays display some limitations in regard with *in vivo* state, such as lack of information regarding the bioavailability, delivery and metabolism. Direct screening of a chemical lead *in vivo* sidesteps these barriers and provides various information about the molecular properties that are required for insecticide action (penetration through the cuticle, toxicity induced etc). However, it is not informative with respect to the specification regarding the target identity, which raises the need for further downstream analysis. Therefore, it seems that a combination of *in vitro* and *in vivo* screenings addresses a more integrative strategy for novel drug target identification.

Midgut: A primary target of novel insecticides

An explosion in research of RNAi mediated pest control has occurred over the past years in many different insect species (reviewed in Liu et al., 2020). The main idea is that control of pests is mediated through transgenic

plants expressing dsRNAs or toxins targeting essential genes expressed in the midgut of the insect (Liu et al., 2020). This technique has been already applied in Canada for the generation and cultivation of transgenic corn that express Bt toxins along with dsRNAs that target an essential gene of *Diabrotica rigifera* (Head et al., 2017). In that case the dsRNAs are delivered orally and the first tissue that will come in contact with the dsRNA is the midgut, an essential tissue for insect development, food digestion, nutrient absorption and immune responses (Denecke et al., 2018). Given the extent of the biological processes taking place, the midgut entails a large repertoire of essential genes and hence is of particular interest in the insecticide target discovery. The midgut is the target of microbial disruptors of the midgut membrane, like the Bt-toxins (Wu et al., 2008). Moreover, it is targeted by several protease Inhibitors (PIs) and lectins (proteins that bind to carbohydrates), which are both naturally occurring in plants from which insect pests are feeding. Both PIs and lectins have been studied a lot for their actions against the midgut of insects and have been associated with developmental arrest and growth impairment as well as with reduction of survival (reviewed in Napoleao et al., 2019). Several other proteins of the midgut have been identified to serve as putative drug targets: a) the proton pump V-ATPase (Liu et al., 2022), b) the Snf-7 (member of the ESCRT III complex, sorting the transmembrane proteins destined for lysosomal degradation) (Ramaseshadri et al., 2013), c) osmoregulatory genes like aquaporins and sugar transporters, mediating the regulation of osmotic pressure in midgut cells (Tzin et al., 2015), and d) genes encoding proteins like *mesh* and *ssk*, which are essential for the formation of the smooth septate junctions, one of the subtypes of septate junctions identified in *Drosophila*, crucial for the connections between epithelial cells lining the midgut and for controlling the paracellular pathway (in between the cells of the epithelium) (Hu et al., 2016).

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Aim of the thesis

This Phd thesis is divided in four chapters and aims to address two main topics. The first topic focuses on the functional analysis of certain resistance alleles found in insects of agricultural and medical importance. In the first chapter we sought to analyze the contribution of two point mutations of the voltage gated sodium channel to resistance against SCBIs. These mutations (F1845Y and V1848I) have been identified in resistant populations of two agricultural pests. However, the levels of resistance conferred by those two mutations against different SCBI active ingredients *in vivo* was not previously established. Using genome engineering in *Drosophila*, we endeavored to generate a causal relationship between the two point mutations with the resistance phenotype and showed that they confer different levels of resistance against different active ingredients of the same insecticide class.

Although several alleles have been functionally related with resistance as individual mechanisms, a possible interaction between different alleles has yet to be delineated. Individual mechanisms seem to contribute to resistance, which is far too less than the resistance ratios observed in the resistant field populations, hence there is a hypothesis of synergistic effects between different resistant mechanisms. Using *Drosophila melanogaster* and state-of-the-art technologies we analyzed the relationship of specific resistance loci (either individually or in combination) with resistance against pyrethroids as well as measured their possible fitness costs, in a background that is lacking any confounding genetic factor.

The next two chapters aimed at the development of tools for biotechnological applications against pests and for the characterization of a novel drug target in lepidoptera species, respectively. In Chapter 3 we opted to identify lepidopteran specific promoters of biotechnological interest, that could be used for reverse genetics methodologies such as CRISPR/Cas9. The final Chapter of this thesis focused on the identification of a putative insecticide target. A recent study in *Drosophila* has demonstrated that the insect steroid hormone ecdysone does not passively diffuse through the cellular membranes of the target cells, but rather is transported by a transmembrane protein, the OATP74D. *Drosophila Oatp74D* is essential for larval development and survival, which renders it a good candidate as a drug target. Based on these hypotheses we proceeded to the functional characterization of the *Oatp74D* orthologs of two lepidopteran pests, *H. armigera* and *S. frugiperda.* Using *in vivo* and *in vitro* approaches we analyzed the function of the protein as an ecdysone importer and its possible role as a novel insecticide target.

Chapter 1

"Functional validation of two novel target site resistance mutations against sodium-channel blocker insecticides (SCBIs) via genome engineering in *Drosophila*"

Samantsidis, G.-R., O'Reilly, A.O., Douris, V., and Vontas, J. (2019). Functional validation of target-site resistance mutations against sodium channel blocker insecticides (SCBIs) via molecular modeling and genome engineering in Drosophila. Insect Biochemistry and Molecular Biology *104*, 73–81.

Authors' contribution:

VD and J.V conceived the project and designed the experiments, G-R.S performed the CRISPR in *Drosophila*, generated fly lines and performed the toxicity bioassays, A.O'R. performed the molecular modelling. The manuscript was written and revised by all authors.

Abstract

Sodium Channel Blocker Insecticides (SCBIs), like indoxacarb and metaflumizone, offer an alternative strategy for Insecticide Resistance Management (IRM) against several insect pests which have developed resistance to other chemical compounds. However, resistance has already been reported against this class of insecticides in several pests like *Tuta absoluta* and *Plutella xylostella*. Two specific mutations (F1845Y and V1848I) in the IV S6 domain of Voltage Gated Sodium Channel (VGSC), the primary target of SCBIs, have been reported to be associated with resistance against both indoxacarb and metaflumizone. In this chapter we used the genome engineering technology CRISPR/Cas9 to generate *Drosophila* lines harboring homozygous F1845Y or V1848I mutations in the *para* (voltage-gated sodium channel) gene. Toxicity bioassays indicated that these mutations reduce sensitivity against both indoxacarb and metaflumizone. In particular, we observed that both mutations conferred moderate resistance to indoxacarb (RRs: 6-10.2) and V1848I to metaflumizone (RR: 8.4), with respect to the wild type control line of the same genetic background. Interestingly, the mutation F1845Y displayed a striking effect against metaflumizone (RR: >3400) compared to the background strain. Our study provides useful information about the actual contribution of either of these two mutations *in vivo*, in a genetic background devoid of any confounding resistance mechanisms.

1.1 Introduction

Voltage-gated sodium channel (VGSC) is an essential transmembrane protein in almost all excitable cells, responsible for the influx of sodium ions that lead to the generation and propagation of electrical signals (comprised of action potentials), which enables the communication among the nerve cells and overall the response to several environmental stimuli (Carnevale and Klein, 2017). VGSC α-subunits are comprised of four homologous domains (I-IV), each having six membrane spanning helical segments (S1-S6) (Catterall, 2017). Each channel encompasses two distinct modules, the voltage sensing module which is formed by the segments S1-S4 and the pore domain which is formed by the S5 and S6 segments of the four homologous domains. The S4 segment harbors positively charged amino acids, usually Lys and Arg, which act as voltage sensors and upon membrane depolarization these residues detect sodium ions and move outwards, towards the extracellular side of the membrane, changing the conformation of the protein leading to the open state of the gate (Carnvale and Klein, 2017, Dong et al., 2014).

Provided their necessary function for the generation of action potentials and cell-cell communication, VGSC are primary targets for many drugs, exemplified by local anesthetics (LAs). Those compounds act as blockers since they bind to the channel and block the influx of sodium, suppressing the high-frequency discharges in excitable cells (Gawali et al., 2015). The insect VGSC is also a target of several insecticide classes such as pyrethroids, DDT and sodium channel blocker insecticides (SCBIs). Pyrethroids and DDT bind at the open activated state of the channel leading to continuous influx of sodium ions (Dong et al., 2014). However local anesthetics and oxadiazines like SCBIs bind at the central cavity of the inner pore of the channel, during the slow inactivation state of the channel following prolonged depolarization, impeding recovery to the resting state, blocking its activation and terminating intracellular sodium influx by shifting the voltage dependence of slow inactivation state to more negative potentials (Dong et al., 2014, von Stein et al., 2013).

SCBIs (indoxacarb and metaflumizone) represent a new class of neurotoxic insecticides developed in an effort to reduce the toxicity against other organisms (e.g. mammals) by increasing the primary target selectivity to insects. SCBIs seem to share similar or overlapping binding sites with local anesthetics at the mammalian sodium channels, as indicated by mutation analysis coupled with *in vitro* electrophysiology studies (Silver and Soderlund, 2007). Mutation analysis in the IVS6 segment of the rat sodium channel has demonstrated that the mutation F1579A (which corresponds to the position 1845 of *P. xylostella*, see further below) at the $Na_v1.4$ significantly reduces the binding affinity of the activated metabolite of indoxacarb, Decarbomethoxyllated JW062 (DCJW), to the sodium channel (Silver and Soderlund, 2007). However, the same mutation in the respective site of the cockroach sodium channel did not have any effect in the inhibition by indoxacarb or its activated metabolite (Silver et al., 2010). Of note, this mutation enhanced the inhibition by metaflumizone and also accelerated the recovery of the mutated sodium channels (Silver et al., 2010). Therefore, it was postulated that the binding site of SCBIs in the insect sodium channels consists of residues which are distinct from those in mammalian sodium channels (Silver et al., 2010).

Indoxacarb is an insecticidal oxadiazine characterized as a pro-insecticide since it has to be converted to the activated metabolite DCJW, a secondary product generated by the hydrolyzing activity of insect esterases or amidases, which underlies the action selectivity against insects (Zhang et al., 2016). Indoxacarb is used against moths, beetles, leafhoppers, weevils, flies and other pests mainly causing cessation of feeding, un-coordination and

paralysis (Silver et al., 2010). It has been shown that spraying treatment of *Drosophila melanogaster* with DCJW is effective and eventually causes mortality (Zhang et al., 2013). Metaflumizone belongs to the category of semicarbazones which are ring-opened dihydropyrazoles (von Stein et al., 2013). Metaflumizone exhibits low toxicity to mammals and high selectivity towards insects (Hempel et al., 2007).

Resistance against SCBIs has been recorded in many insect species, such as *Musca domestica* (Shono et al., 2004), *Plutella xylostella* (Khakame et al., 2013, Wang et al., 2016), *Spodoptera exigua* (Tian et al., 2014), *Helicoverpa armigera* (Bird et al., 2017), *Tuta absoluta* (Roditakis et al., 2017) and *Blatella germanica* (Liang et al., 2017). There is evidence for synergistic effects of metabolic inhibitors on SCBI toxicity, which implicates metabolic resistance mechanisms involving the activity of esterases and oxidases (Wang et al., 2016, Zhang et al., 2017). However, the use of synergists only partially reduced the levels of resistance against indoxacarb in *T. absoluta* (Roditakis et al., 2017) and metaflumizone in *S. exigua* (Su and Sun, 2014). High resistance against SCBIs is positively correlated with the increased frequencies of two point mutations in the IVS6 domain of the voltage-gated sodium channel gene, F1845Y (corresponding site to the mammalian F1579 position of $Na_v1.4$) and V1848I, found in two field resistant populations of *P. xylostella* (Figure 1.1) (Wang et al., 2016). Another agricultural pest resistant to SCBIs is *Tuta absoluta*, collected from tomato greenhouses from Italy and Greece, in which F1845Y and V1848I mutations were also identified (Roditakis et al., 2017). Both mutations were tested using electrophysiology studies of heterologous expressed *B. germanica* VGSCs in *Xenopus* oocytes, and it was found that F1845Y and V1848I (but not V1848A) reduced almost equally the inhibition of sodium current by indoxacarb, DCJW (the activated metabolite of indoxacarb) and metaflumizone. This indicates that these mutations might contribute to non-selective target-site resistance against both SCBIs, but whether they might contribute to resistance *in vivo* has yet to be determined.

In the final decade, CRISPR/Cas9 technology has been frequently used to address several biological questions, including the validation of insecticide resistance mechanisms (Douris et al., 2020). Employing this technique in model insect species like *Drosophila* or directly in the species of interest, has provided useful information about the association of specific mutations with resistance against several insecticide classes, like spinosyns that target nicotinic acetylcholine receptor (Somers et al., 2015; Zimmer et al., 2016), etoxazole and benzoylureas targeting chitin synthase (Douris et al., 2016; Grigoraki et al., 2017) and diamides targeting ryanodine receptor (Douris et al., 2017; Zuo et al., 2017). In here, we used the CRISPR/Cas9 technology in order to generate *Drosophila* lines that bear the mutations F1845Y or V1848I (*P. xylostella* numbering) at the gene *para* and performed toxicity bioassays to analyze their contribution to resistance against SCBIs, in a genetic background lacking any confounding resistance mechanisms.

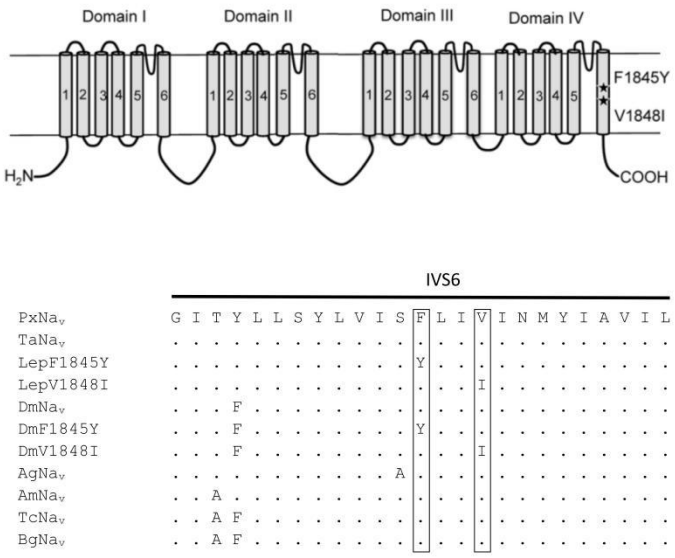

Figure 1.1 Positions of the sodium channel mutations (F1845Y and V1848I) in the voltage- gated sodium channel. Top Schematic presentation of the four homologous domains (I-IV) of the sodium channel each one consisting of six transmembrane segments (S1-S6). The mutations F1845Y and V1848I related to sodium channel blocker insecticides resistance are identified in the IVS6 segment of the sodium channel. The amino acid positions are numbered based on a *Plutella xylostella* sequence (GenBank accession no. KM027335). **Bottom** Sequence alignment of a part of the IVS6 domain of sodium channel of different species PxNav: *P. xylostella* (GenBank accession no. KM027335); TaNav: *Tuta absoluta* susceptible strain (Roditakis et al., 2017); LepF1845Y: Lepidopteran (*P. xylostella* and *T. absoluta*) sequence with mutation F1845Y); LepV1848I: Lepidopteran (*P. xylostella* and *T. absoluta* sequence with mutation V1848I); DmNav: *Drosophila melanogaster* (AAB59193.1); DmF1845Y: *D. melanogaster* sequence with mutation F1845Y; DmV1848I: *D. melanogaster* sequence with mutation V1848I. AgNav: *Anopheles gambiae* (CAM12801.1); AmNav: *Apis mellifera* (NP_001159377.1); TcNav: *Tribolium castaneum* (NP_001159380.1).BgNav: *Blattella germanica* (AAC47484.1). The mutations F1845Y and V1848I relative to *P. xylostella* numbering are shown in rectangles.

1.2 Materials and Methods

1.2.1 Chemicals

For contact toxicity bioassays we used the chemicals Indoxacarb (Sigma-Aldrich, PubChem CID: 107,720) and metaflumizone (Sigma-Aldrich, PubChem CID: 11614934). The formulations used for feeding bioassays were Steward 30 WG (DuPont) for indoxacarb, and Alverde 24 SC (BASF) for metaflumizone.

1.2.2 Fly strains

For CRISPR/Cas9 genome modification injections were performed in preblastoderm embryos of the lab strain y1M{nos-Cas9.P}ZH-2A w*, where Cas9 is expressed under the control of nanos promoter (Port et al., 2014) (herein referred as nos.Cas9, #54591 in the Bloomington Drosophila stock center). Strain w+oc/FM7yBHw (kindly provided by Professor Christos Delidakis, IMBB and University of Crete) which contains the X chromosome balancer FM7c was used for genetic crosses and for keeping heterozygous mutants. The flies were kept at 25 °C temperature, at 60–70% humidity and 12:12 h photoperiod on a typical fly diet.

1.2.3 Strategy for genome editing

To generate *Drosophila* strains bearing either one or both homozygous mutations in the gene *para* (equivalent to the F1845Y and V1848I found in *P. xylostella* and *T. absoluta*), we employed an *ad hoc* CRISPR/Cas9 strategy coupled with Homology Directed Repair (HDR).

1.2.3.1 Amplification and sequencing of the target region of para

Prior to designing CRISPR targets it was necessary to verify the sequence of *para* gene proximal to the region of interest in the nos.Cas9 strain. Genomic DNA from nos.Cas9 *Drosophila* adults was extracted with DNAzol (MRC) following the manufacturer instructions. Three sets of primers (Inv1F/R, Inv2F/R and Inv3F/R, Table 1.2) were designed based on the *para* gene sequence in order to amplify three overlapping fragments (Inv1-3) that add up to a 3134bp region encompassing genomic region X:16,466,144–16,463,017 of the *Drosophila* genome sequence (numbering according to BDGP6 genome assembly). The amplification reactions were performed using KapaTaq DNA Polymerase (Kapa Biosystems). The conditions were 95^{o}C for 2 min for initial denaturation followed by 30–35 cycles of denaturation at 95^{o}C for 30 s, annealing at 61^{o}C-66^{o}C for 15 s, extension at 72^{o}C for 45–90 s and a final extension step for 2 min. The PCR products were purified with a PCR clean-up kit (Macherey-Nagel) according to manufacturer's instructions and sequenced from both ends.

1.2.3.2 CRISPR strategy (target identification and construct generation)

To generate the lines F1845Y (further below referred as FY), V1848I (further below referred as VI) and F1845Y-V1848I (encompassing both mutations in the same allele, FYVI) we used the same CRISPR targets but with different donor plasmids for Homology Directed Repair. Based on the genomic sequence of *para* obtained for strain nos.Cas9, several CRISPR targets in the desired region were identified using the Optimal Target Finder online tool (Gratz et al., 2014, http://tools.flycrispr.molbio.wisc.edu/targetFinder). Two target sequences found upstream (Lpara) and downstream (Rpara) of the desired region in *para* gene were selected (Figure 1.2) with no predicted off-target effects. In order to generate sgRNAs targeting those sequences, two different RNA expressing plasmids were generated based on the vector pU6-BbsI chiRNA (Gratz et al., 2013) following digestion with BbsI and ligation of two double stranded oligos, (dsLpara and dsRpara), which were generated by annealing single stranded oligos RparaF/RparaR and LparaF/LparaR (Table 1.2) respectively. Following ligation and transformation, single colonies for each construct were picked and checked for the correct insert by performing colony PCR using T7 universal primer and the reverse primer for each dsDNA. The sequence of each sgRNA expressing plasmid was verified by sequencing (Macrogen, Amsterdam).

To introduce the mutations FY, VI and FYVI in the *Drosophila* genome we used three different donor plasmids (synthesized *de novo*, GenScript) in order to facilitate homologous directed repair. The newly synthesized sequences (Figure S1) were subcloned into the pUC57 vector in the EcoRV restriction site. The two sgRNA targets Lpara and Rpara (Table 1.2, Figure 1.2) spanned a region of 228bp (target region), flanked by symmetric homology arms of ~1000bp. The target region was designed to bear the mutations of interest, as well as of additional synonymous mutations serving as molecular markers to facilitate molecular screening but also to prevent unwanted Cas9 mediated digestion of the donor template.

1.2.4 Molecular Screening and genetic crosses

Preblastoderm embryos of nos.Cas9 strain were injected with mixes containing 100ng/µl donor plasmid and 75ng/µl of each of the sgRNA expression plasmid. Hatched first instar larvae were transferred into standard artificial fly food and incubated at 25ºC. G_0 adult survivors were collected and backcrossed with nos.Cas9 flies. CRISPR positive events were screened by PCR in the genomic DNA exctracted from pools of ~30 from the G_1 generation of each cross. Two different screening methodologies were employed: A) Allele Specific PCR; A specific primer pair (ParaSpecF/R, Table 1.2) was designed based on the synonymous mutations introduced in the two sgRNA target sequences in all donor templates, yielding a product of 250bp that is specific only to the genome modified alleles. B) PCR-RFLP; 2µg of genomic DNA was digested with restriction enzymes that digest only the wild type alleles but not the mutated (HindIII in the case of FY and FYVI crosses and BsrGI in the case of VI crosses). The generated digestion product was used as template (~30ng) for PCR amplification with a generic primer pair (ParaGenF/R, Table 1.2) and the respective

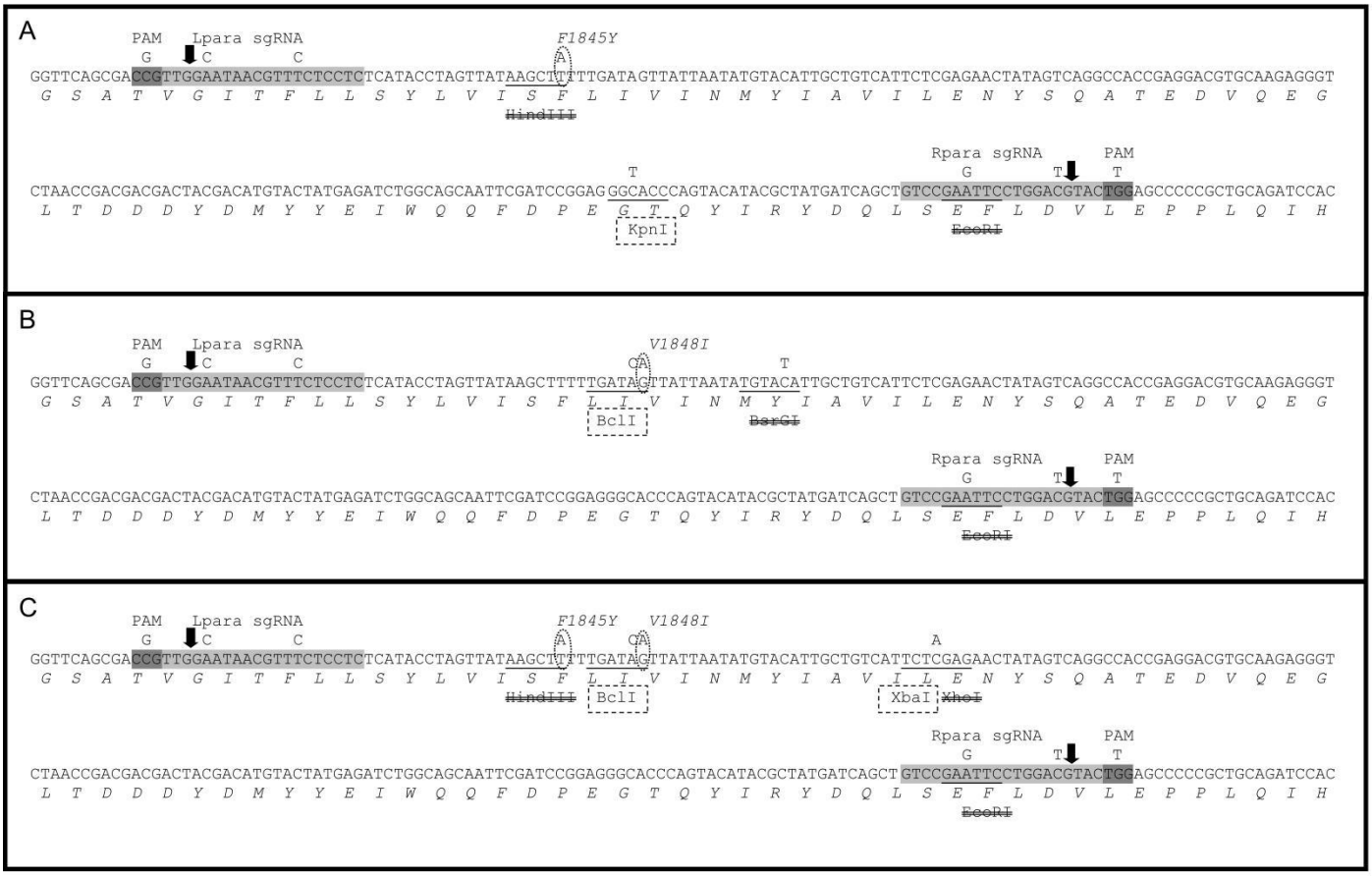

Figure 1.2 CRISPR/Cas9 strategies employed for the generation of genome modified flies bearing the mutations A. F1845Y, B. V1848I and C. FYVI (which corresponds to the double mutant harboring both F1845Y and V1848I at the same allele). Nucleotide and deduced amino acid sequence of a 258 bp fragment of para (corresponding to reverse complement of X: 16358465–16358722 at the BDGP6 genome assembly), flanking positions 1845 and 1848 (*P. xylostella* numbering) of the *Drosophila melanogaster* amino acid sequence. Light gray areas indicate the CRISPR/Cas9 targets selected (Lpara sgRNA, Rpara sgRNA), while dark gray areas indicate the corresponding PAM (-NGG) sequences. Vertical arrows indicate the CRISPR/Cas9-induced double stranded breaks. Ovals mark non-synonymous differences between target (wild-type) and donor (genome modified) sequences. Synonymous mutations incorporated for diagnostic purposes, as well as to avoid cleavage of the donor plasmid by the CRISPR/Cas9 machinery, are shown above the nucleotide sequence. Restriction sites abolished because of the genome modification are shown with double strikethrough letters (used for diagnostic purposes as described in detail in the 1.2.3.2 section) and the corresponding sequence is underlined. Restriction sites introduced because of the genome modification are shown in dashed boxes and the corresponding sequence is also underlined.

PCR product (752bp) that may be derived by either wild type (if still present, given the initial enzymatic cleavage of the template DNA mix) or genome modified alleles, was further digested with diagnostic restriction enzymes that recognize sites existing only in the mutant DNA. Specifically, we used the enzymes KpnI for F1845Y (producing two diagnostic fragments of 536 bp and 217 bp), BclI for V1848I (producing two diagnostic fragments of 405 bp and 347 bp) and XbaI for FYVI (producing two fragments of 437 bp and 315 bp).

Crosses that proved positive for mutant alleles were used and further explored to identify genome modified flies to establish homozygous lines bearing the mutations of interest (Figure 1.3). Specifically, G_1 flies from the positive G_0 crosses were backcrossed with nos.Cas9 flies and after generating G_2 progeny they were sacrificed for genotyping, as previously described. From the positive G_1 crosses, G_2 female individuals were selected and outcrossed with the males carrying a balancer X chromosome (Fm7c) which carries the phenotypic eye marker *bar*. Similarly, after generating the G_3 progeny, the G_2 female flies were collected and underwent molecular screening. Then from the positive crosses, G_3 females (potentially carrying the mutant allele opposite of the Fm7 balancer chromosome) were collected and crossed again with Fm7c males to produce G_4 progeny. After genotyping the G_3 parents, the positive crosses were chosen and G_4 heterozygous females were intercrossed with G_4 hemizygous males of the same line to generate the G_5 progeny from which the final homozygous lines were established for each mutation. Both males and females were collected from each positive line and used for DNA extraction, followed by PCR amplification (using the primer pair ParaGenF/R) and sequence verification (Macrogen, Amsterdam).

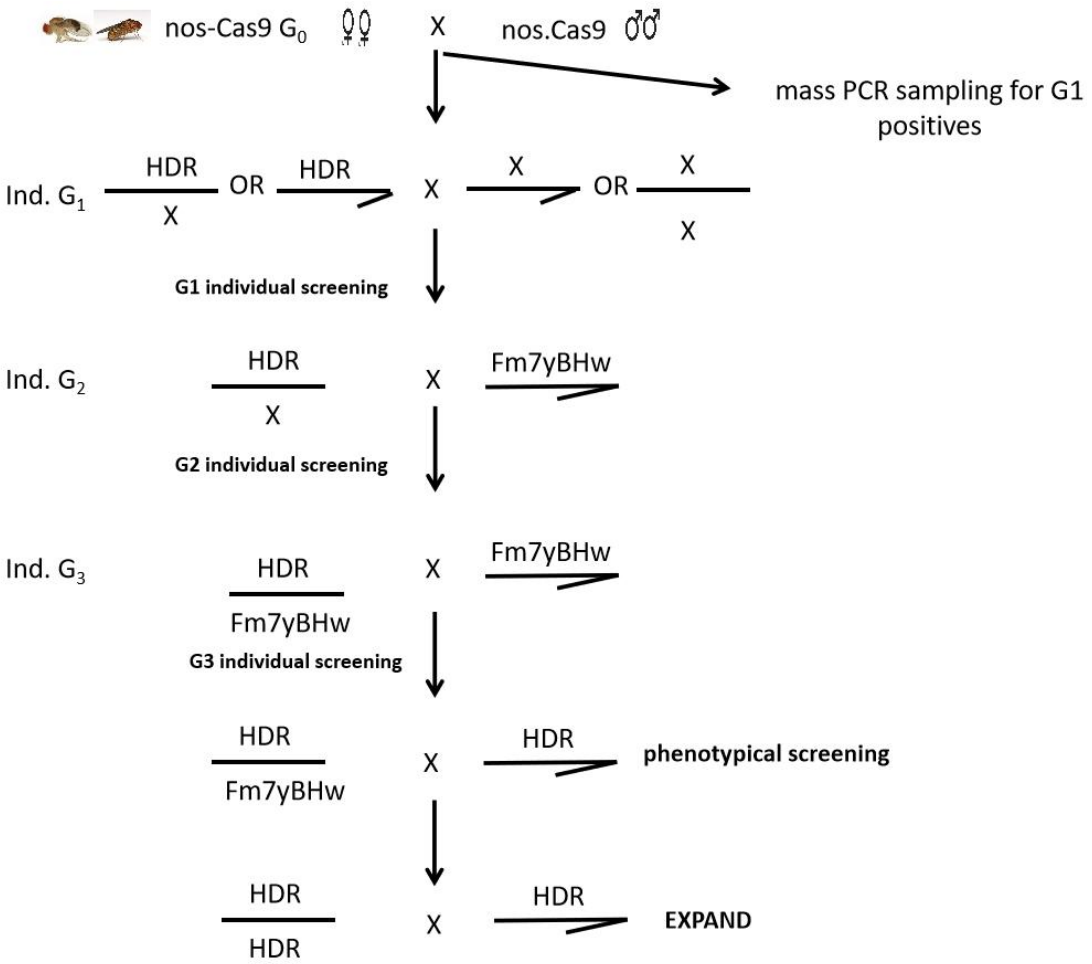

Figure 1.3 Scheme of the genetic crosses performed for the generation of *Drosophila* lines bearing homozygous mutations F1845 or V1848I or FYVI, as written in the section 1.2.4. Transgenic female flies will carry a genome modified allele marked as

HDR, Homology Directer Repair, according to which the donor plasmid encompassing the mutations of interest flanked by ~1kb Homology arms will substitute the target region through homologous recombination.

1.2.5 Toxicity bioassays

Contact toxicity bioassays were performed in order to evaluate the toxicity levels of both indoxacarb and metaflumizone in the strain nos.Cas9, following a similar procedure as previously described (Daborn et al., 2001). In brief, glass scintillation vials were filled with 0.5ml of acetone based solution containing several concentrations of each insecticide (technical grades) and let on a roller at room temperature, so that the insecticide to be evenly coated on the glass surface. Twenty female adult flies (1-3 days old) per technical replicate were transferred from plastic vials into the glass vials. Serial dilutions of 6-7 concentrations were used for dose response bioassays, while vials coated only with acetone served as control. The vials were plugged with cotton soaked with 5% sucrose solution. For each compound, each concentration was assayed in three replicates, while the bioassays were performed several times. The vials were maintained at room temperature and flies were exposed for 24-96hrs.
However, no lethality was observed at any of the two insecticides tested. For this purpose, feeding toxicity bioassays were performed using formulation insecticides. Adult flies were capped with cherry juice-agar plates supplemented with yeast, left to cross overnight and after plate replacement the flies were left to lay eggs for 5-6 hrs. Then the adults were removed and the plates were incubated for another 42hrs. Early second instar larvae were transferred in batches of 20 into freshly prepared standard artificial fly food supplemented with several concentrations of insecticide formulation solutions. Larval development, mortality, pupal eclosion, pupal size and adult survival were monitored and measured for 7-10 days. Each bioassay consisted of five to seven different concentrations and tested in triplicates. At each experiment, the control line nos.Cas9 was tested along with the genome modified flies (FY and VI), always including as a negative control fly food without insecticide.

1.2.6 Statistical analysis

Concentration-response data of each bioassay setup were collected and analyzed with ProBit analysis using PoloPlus (LeOra Software, Berkeley, California) in order to calculate Lethal Concentrations of the 50% of the population subjected to the experiment (LC50 values), 95% fiducial limits (FL), linearity of the dose-mortality response, construction of mortality curves and statistical significance of the results.

1.3 Results

1.3.1 Generation of homozygous mutated *Drosophila* lines.

The two mutations F1845Y and V1848I (according to *P. xylostella* numbering) associated with resistance against SCBIs, were introduced in the genome of *Drosophila melanogaster* via CRISPR/Cas9 technology coupled with Homology Directed Repair. Furthermore, we tried to generate flies bearing both mutations in the same allele (namely FYVI). Embryos of nos.Cas9 strain were injected with three different injection mixes each containing the two

sgRNA expression constructs targeting a 228bp region flanking the positions 1845 and 1848, along with one of the donor plasmids (FY, VI and FYVI).

For the F1845Y mutation, 55 adult G_0 founders were crossed individually with nos.Cas9 flies and only 46 were fertile. Molecular screening of the G_1 progeny (as described in the 1.2.4 section) showed that 6 out of the 46 crosses were positive for HDR and for the mutation FY (Figure 1.4B). Concerning the V1848I mutation 55 adult flies were crossed with nos.Cas9 flies and only 34 gave G_1 progeny, from which eight found to be positive for HDR. For the combination of the two mutations, 71 crosses were set, 56 were fertile and six were positive for HDR. Following the crossing strategy (Figure 1.3) by using the balancer line Fm7c, six homozygous fly lines were generated for the mutation FY and four for the mutation VI. All homozygous lines were verified for the mutations by sequencing. Although we were able to create homozygous lines bearing each of the mutations individually, no homozygous flies bearing both mutations in the same allele (FYVI) were ever generated. Therefore, the FYVI allele was kept as heterozygote over the Fm7 balancer chromosome.

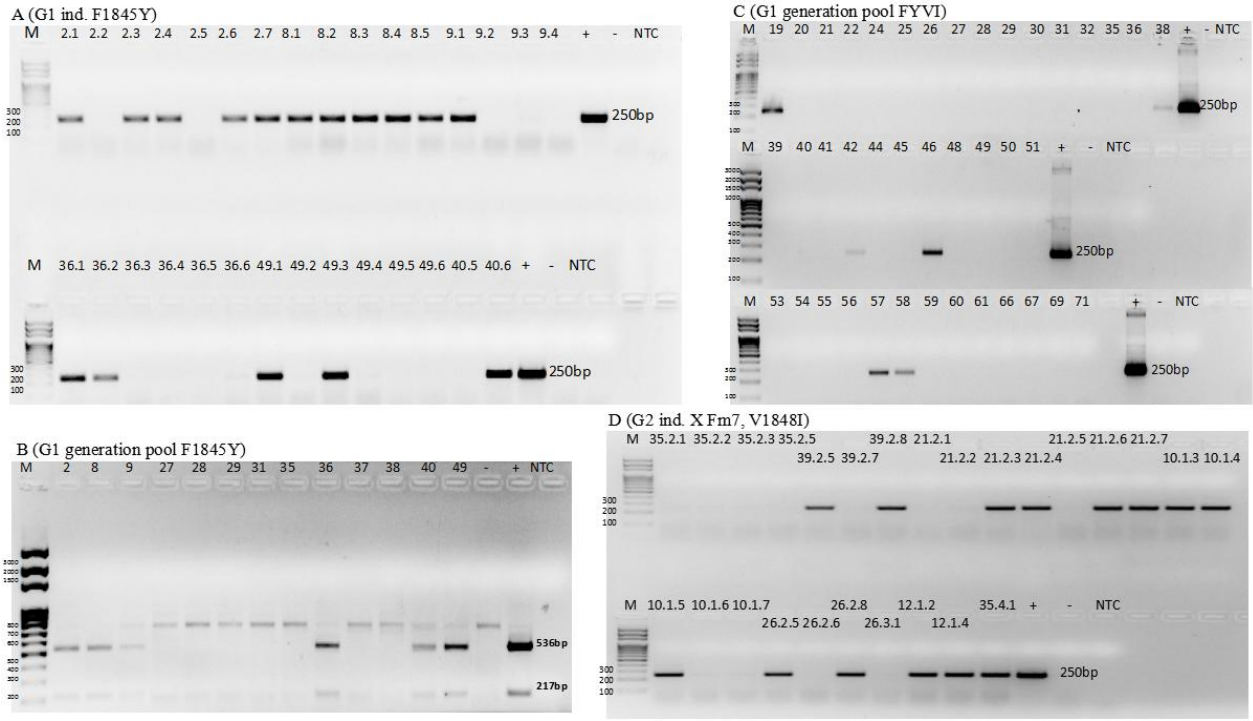

Fig. 1.4. Indicative diagnostic molecular screening with primers yielding diagnostic PCR products in 2% agarose gel electrophoresis. (A) PCR screening yielding a 250 bp product of G_1 individuals backcrossed with nos.Cas9 originating from each original line (G_0) for the F1845Y mutation. (B) Diagnostic KpnI digestion of PCR product (752 bp) amplified with generic primers for *en masse* screening of G_1 progeny samples of G_0 flies backcrossed with nos.Cas9, yielding two diagnostic fragments of 536 bp and 217 bp. (C) PCR screening with specific primers (250 bp product) in pools of G1 progeny of the original injected flies for the dual mutations FYVI. (D) PCR screening with specific primers (250 bp) of G_1 individuals for the mutation V1848I after cross with flies bearing balancer FM7c. M: molecular weight marker (100 bp ladder); +: positive control (PCR using as template the relevant donor plasmid for each mutation); -: negative control (PCR using as template DNA from non-injected nos.Cas9 flies; NTC: blank (no DNA template).

1.3.2 Contribution of each mutation to resistance against SCBIs

To evaluate the toxicity of SCBIs against *Drosophila*, contact toxicity bioassays were performed in 1-3 day old adult nos.Cas9 flies. Six different concentrations of indoxacarb and metaflumizone (maximum 1000μg/ml) were tested but no mortality was observed even after 96hrs of continuous exposure.

Feeding toxicity bioassays were performed as an alternative method for administration at early L2 instar larvae. Larvae were collected and transferred in batches of 20 in standard artificial fly food supplemented with several concentrations of either indoxacarb or metaflumizone. The larvae were incubated for 7-10 days and were continuously exposed to the food containing the insecticide. Toxicity effects such as cessation of feeding, larval paralysis, prolonged development and reduction of the size of pupae were observed. To evaluate and quantify the mortality rates, provided that dead larvae cannot be visible inside the fly food, molting to pupae was considered as a measurable proxy of survival (most pupae eclose normally 7–10 days after the bioassay is initiated). Survival data underwent probit analysis and the corresponding LC_{50} values and resistance ratios versus the control (nos.Cas9) flies, along with 95% fiducial limits and associated statistics are shown in Table 1.1.

Table 1.1 Feeding toxicity bioassay responses of the genome modified flies bearing the mutations F1845Y or V1848I and the respective control line (nos.Cas9) against the insecticides Indoxacarb and Metaflumizone.

Compound	*Drosophila* strain	Slope ±se	LC_{50} (95% CI) ug/ml	X^2 (df)	RR vs nos.Cas9
Indoxacarb	nos.Cas9	4.012 ±0.360	2.756 (2.416-3.133)	17.406 (14)	1
	F1845Y	3.901 ±0.370	28.202 (25.547-31.209)	14.782 (17)	**10.2**
	V1848I	4.270 ±0.352	16.658 (15.124-18.434)	14.555 (22)	**6**
Metaflumizone	nos.Cas9	4.983 ±0.598	0.525 (0.479-0.575)	9.375 (10)	1
	F1845Y	5.906 ±0.798	1816.675 (1627.624-2017.529)	8.748 (16)	**3441.2**
	V1848I	2.964 ±0.331	4.412 (3.763-5.131)	12.111 (13)	**8.4**

Based on these results, flies bearing the mutation V1848I in homozygous (females)/hemizygous (males) state exhibit moderate levels of resistance against both SCBIs; 6-fold resistance against indoxacarb and 8.4-fold resistance against metaflumizone, compared to the wild type control line (nos.Cas9). Of note, the mutation F1845Y seems to contribute rather differently to resistance against the two insecticides. While FY flies exhibited only 10.2-fold resistance against indoxacarb, they displayed much higher levels of resistance against metaflumizone (RR: 3441.2) compared to the nos.Cas9 control line. These results were confirmed in several experimental procedures using

different fly lines bearing the respective mutations, with limited LC_{50} variation among different experiments, within the fiducial limits shown in Table 1.1.

1.4 Discussion

Two novel mutations at the IVS6 domain of the voltage gated sodium channel (F1845Y and V1848I, *P. xylostella* numbering) have been identified in resistant populations of *P. xylostella* and *T. absoluta* (Wang et al., 2016, Roditakis et al., 2017). Both of them have been associated with resistance against sodium channel blocker insecticides (SCBIs) via *in vitro* electrophysiology studies, where each of these mutations were introduced in the *B. germanica vgsc* expressed in *Xenopus* oocytes (Jiang et al., 2015). In here we have engineered the *para* gene of *Drosophila* so that to bear either the F1845Y or V1848I mutations at the IVS6 domain in homozygous state, employing the CRISPR/Cas9 genome editing technology. Using the homozygous genome modified fly strains, we performed feeding toxicity bioassays against two commercial SCBIs, indoxacarb and metaflumizone.

Our results provide a functional *in vivo* validation of the actual contribution of both mutations to resistance against SCBIs, since they have been introduced in a genetic background lacking any confounding resistance mechanisms. Dose-response data of the bioassays against indoxacarb indicated that both F1845Y and V1848I confer low to moderate levels of resistance (RR_{FY}: 10.2 and RR_{VI}: 6) compared to the wild type control line (nos.Cas9). Although toxicity bioassays indicated that the effect of these mutations against indoxacarb was uniform, their response against metaflumizone was quite divergent. While fly strains bearing the mutation V1848I exhibit 8.4-fold resistance against metaflumizone, flies harboring the homozygous F1845Y mutation display a striking resistance phenotype increasing the ratios of resistance by 3441.2-fold compared to the control line.

The relative binding affinity of SCBIs with the position F1845 has been studied previously in the *B. germanica* sodium channel through *in vitro* electrophysiology studies in *Xenopus* oocytes, which showed that mutation F1845A does not have any effect against indoxacarb and metaflumizone (Silver et al., 2010). A similar approach was followed for studying the effects of the mutations F1845Y and V1848A/I against SCBIs (Jiang et al., 2015). Based on their results it was shown that both mutations reduce the inhibitory effect of both insecticides. In particular, while 1uM of DCJW inhibited the sodium currents by 77% when tested in oocytes expressing the WT sodium channel of *B. germanica*, a 39% of inhibition was observed in oocytes expressing the cockroach sodium channel bearing either the F1845Y or the V1848I mutation (Jiang et al., 2015). However, 10uM of DCJW reached a ~77% inhibition of sodium currents in oocytes expressing the mutated sodium channels, which was comparable with the inhibition levels observed by the 1uM of DCJW on the WT (Jiang et al., 2015). This indicated that mutants were almost 10-fold less sensitive to the activated metabolite of indoxacarb (Jiang et al., 2015). This is almost in line with what we observed in our study, where the F1845Y and V1848I exhibited 10.2 and 6-fold resistance respectively, when exposed to several concentrations of indoxacarb, in respect to the control line (Jiang et al., 2015).

It is noteworthy to mention that although the *in vitro* work showed that the levels of reduction in the sensitivity of the cockroach sodium channel was not substantially different between the combinations of mutations with metaflumizone (Jiang et al., 2015), the *in vivo Drosophila* bioassay results demonstrated a sharp difference in the response of each mutation against this insecticide (F1845Y flies were almost 400 times more resistant to

metaflumizone than the V1848I flies). Docking simulation studies of the *Drosophila* wild type and the mutated versions of voltage-gated sodium channels with metaflumizone, indicated that the resistance to this compound is possibly attributed to a steric clash (Samantsidis et al., 2019). The steric clash was found in both V1848I and F1845Y sodium channels. Moreover, in the F1845Y mutation the introduction of a hydrophilic hydroxyl group raises the possibility that hydrophobic repulsion may take place with the hydrophobic moiety of metaflumizone, impeding the ligand binding. In particular, the steric clash was noticed to take place between the tyrosine and the 4-cyanophenyl moiety of metaflumizone which does not exist in indoxacarb, and this possibly explains the difference in the response of the F1845Y flies against the two compounds (Samantsidis et al., 2019).

Our effort to generate a homozygous fly strain carrying both mutations at the same allele (in *cis*) was not successful; although such a "dual" allele has been generated by CRISPR/Cas9 coupled with homologous recombination, it was always found in heterozygotes and no homozygous flies bearing both mutations in *cis*. Interestingly, heterozygotes from resistant populations of *P. xylostella* have been found to always have the two mutations at different chromosomes (*in trans*) and never *in cis* ("dual" allele; Wang et al., 2016), which was also identified in resistant populations of *T. absoluta* (data not shown; samples from Roditakis et al., 2017). This is a strong indication that the two mutations are mutually exclusive, i.e. that the "dual" allele bearing both mutations is not viable, leading to a non-functional VGSC.

In here we have functionally validated the actual contribution of two point mutations to resistance against SCBIs, a class of insecticides used as an alternative method for pest control, given the large amount of pyrethroid resistance incidents. *Drosophila* provides a powerful toolkit which allows several questions to be addressed in a common genetic framework (Scott and Buchon 2019, Perry et al., 2018). The establishment of genome modification in insecticide resistance studies (Douris et al., 2020), in combination with standard genetic engineering may facilitate validation of synergistic interaction between target-site with metabolic resistance mechanisms against SCBIs, as soon as candidate genes are available for investigation. An example of such synergistic interaction between these two different mechanisms of resistance is described in the following Chapter.

Tables - Chapter 1

Table 1.2 List of primers used in this study.

Primer name	Primer sequence (5'->3')	Experimental use
Tuta_F	GTGCTGGACGGCATCATCAA	Amplification from *Tuta absoluta* DNA samples
Tuta_R	CTCGAGAATGACGGCGATGT	
LparaF	CTTCGAGGAGAAACGTTATTCCAA	Generation of sgRNA expression plasmids
LparaR	AAACTTGGAATAACGTTTCTCCTC	
RparaF	CTTCGTCCGAATTCCTGGACGTAC	

RparaR	AAACGTACGTCCAGGAATTCGGAC	
ParaSpecF	AATTGTGGTTCAGCGACGGTTGGC	Molecular screening for genome modified flies
ParaSpecR	GGGGCTCAAGTACATCCAGGAAC	
ParaGenF	TCGCACAACTGCCAATCCTA	
ParaGenR	CACCAATCTCACCCGTCTCC	
Ind1F	CCTCTGTCTATCTGTCTGCC	Sequencing of overlapping fragments of *para* genomic region
Ind1R	ATACGAGCGTGTTACCGATT	
Ind2F	GCCCACATACGAACACTCCG	
Ind2R	CGTATGTACTGGGTGCCCTC	
Ind3F	ATCCACCCGACAACGACAAA	
Ind3R	TACCGTCATTTGCTCGCCAT	

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Chapter 2

"Functional validation of synergism between metabolic and target site pyrethroid resistance mechanisms identified in *Aedes aegypti*"

Samantsidis, G.-R., Panteleri, R., Denecke, S., Kounadi, S., Christou, I., Nauen, R., Douris, V., and Vontas, J. (2020). 'What I cannot create, I do not understand': functionally validated synergism of metabolic and target site insecticide resistance. Proc. R. Soc. B. *287*, 20200838.

Authors' contributions:

V. D. and J.V. conceived the project and designed the experiments; G.-R.S., R.P, V.D., S.K. and I.C. generated fly lines; G.-R.S. and R.P. performed toxicity bioassays and qPCR; G.-R.S. performed fitness cost experiments; V.D., S.D., R.N. and J.V. analyzed the data, contributed to scientific discussion and wrote the manuscript, which has been revised and approved by all authors.

Abstract

In this chapter the scope of study was to functionally validate the interaction between target site and metabolic detoxification as two major pyrethroid resistance mechanisms. The putative synergistic interaction between target-site mutations and enhanced metabolic detoxification has been postulated as a major mechanism of pyrethroid resistance imposing severe consequences in vector-borne diseases incidences. Using both genetic transformation and CRISPR/Cas9 mediated genome modification, we generated *Drosophila* strains expressing the *Aedes aegypti CYP9J28* in a genetic background encompassing the mutation V1016G in the sequence of voltage-gated sodium channel (*para*), also known as *kdr* (knock-down resistance). Toxicity bioassays indicated that the resistance ratio mediated by the interaction of these two mechanisms was more than the multiplicative product of each individual mechanism ($RR_{combination}$:19.85 > $RR_{Cyp9J28}$:1.77 X RR_{V1016G}: 3). Furthermore, *Drosophila* strains combining the overexpression of the CYP9J28 while carrying the mutation V1016G, displayed a significant developmental delay as well as a reduction in total eggs laid, raising the point that although existence of both mechanisms in the same insects increase the resistance against pyrethroids, they also put their fitness at a disadvantage. These are two major findings that should be considered for Insecticide Resistance Management and vector control.

2.1 Introduction

Insecticides have been considered for many years one of the most effective means for the control of insects which are vectors of numerous human disease-causing parasites and viruses, such as malaria and yellow fever virus. One of the most prominent classes of insecticides used for the control of such pest species has been pyrethroids, a major class of neurotoxic insecticides, which has contributed to the reduction of malaria cases by >50% since 2000 (Vontas et al., 2020). *Aedes aegypti*, the principal vector of several arboviruses like dengue, Zika, chikungunya and yellow fever viruses, originated in Africa but has been expanded in a global scale the last three decades (Kraemer et al, 2015). Insecticide based control strategies (mainly based on the use of pyrethroids for the control of adult mosquitoes) along with the developing vaccination programs have reduced somehow the severity of the arboviruses transmitted by *Aedes aegypti* (WHO, 2011). However, some of these diseases like yellow fever still cause significant burden in Africa and South America (Kraemer et al., 2015), which is in part attributed in the ability of *Aedes aegypti* populations to adapt to fast environmental changes, like the pressure of insecticide use. Therefore, elucidating the mechanisms underpinning pyrethroid resistance and understanding the evolutionary outcome of the emergence of these mechanisms in the absence of the insecticide pressure is more than necessary for Insecticide resistance management (IRM) and for developing novel strategies for slowing down the development of resistance.

Overexpression of metabolic detoxification enzymes like the cytochrome P450s and alterations in the sequence of the insecticide target have been identified as major mechanisms in pyrethroid resistant *Aedes aegypti* populations (Vontas et al., 2020, Smith et al., 2016). Transcriptomic approaches have been used extensively for unraveling genes and detoxification pathways potentially conferring the resistance phenotype of several populations of *Aedes aegypti* (Bariami et al, 2012, Seixas et al., 2017). Although several CYPs have been found up-regulated in resistant populations of this species (Vontas et al., 2020), there are a few genes that have been detected more consistently, such as the *AaegCYP9J28* (Bariami et al, 2012, Seixas et al., 2017, Zheng et al., 2018), which is suggestive of its association with the resistance against pyrethroids like permethrin and deltamethrin. Both *in vitro* and *in vivo* studies have functionally validated its actual contribution to pyrethroid (deltamethrin) resistance (Pavlidi et al., 2012, Stevenson et al., 2011). Furthermore, certain alterations in the sequence of the voltage gated sodium channel (the orthologues to the *para* gene in *Drosophila*) have been associated with resistance against pyrethroids. Although the most consistent *voltage gated sodium channel* (*vgsc*) mutation associated with pyrethroid resistance is L1014F/H, it has never been detected in *Aedes* species, presumably due to codon bias (the 1014 codon is CTA, and any alteration does not lead to the generation of Phenylalanine) (Scott 2019). Nevertheless, other mutations like V1016G and F1534C have been identified in populations of *Aedes* mosquitoes and are associated with pyrethroid resistance (Smith et al., 2016). The V1016G and F1534C mutations have been found either individually or in combination with the S989P mutation in resistant *Aedes* mosquitoes (Smith et al., 2016). Their contribution to resistance against pyrethroids has been demonstrated via *in vitro* electrophysiology studies, indicating that both increase insensitivity levels against deltamethrin and permethrin (Du et al., 2013, Du et al, 2016).

Although there has been a big effort to analyze and functionally validate the contribution of individual mechanisms in insecticide resistance (Samantsidis et al., 2019, Douris et al., 2020), functional analysis of a possible interaction between different resistant loci has yet to be performed. A possible synergism between different alleles

could support the high levels of resistance obtained in field populations, considering the much lower contribution of individual mechanisms to pyrethroid resistance. Although a putative synergistic epistasis of the metabolic and target site resistance loci has been considered, it has only been tested by crossing lines with different resistance factors together in order to see their effect (Hardstone et al., 2009). Using nearly isogenic lines in *Aedes aegypti* by multiple backcrossing of resistant strains with a susceptible lab strain, a putative synergistic interaction manifested by *kdr* mutations and upregulated cytochrome P450s has been demonstrated, increasing by far the resistance levels against pyrethroids compared to the susceptible counterparts but also to the lines bearing each mechanism individually (Hardstone et al., 2009, Smith et al., 2019). However, this approach introduces a large amount of unrelated genetic variation, which complicates inferences drawn about the specific resistant loci being studied. Model organisms like *Drosophila melanogaster* provide the leverage of a controlled genetic background to study the contribution of individual or interacting mechanisms, which could utterly minimize any differences obtained by genetic variation.

Another consideration in IRM is the fitness cost related with insecticide resistance mechanisms, which emerge as high energetic costs or disadvantages that diminish the insects' fitness in the absence of insecticides (ffrench-Constant and Bass 2017). Genes with high costs tend to revert back to their susceptible form once the selective pressure of the insecticide application is removed, that is the frequency of resistant alleles is progressively reduced while the one of susceptible is increased upon cessation of pesticide application. Fitness cost studies associated with insecticide resistance has been performed in many athropod species, such as mosquitoes and mites (Smith et al., 2021, Bajda et al., 2018, Brito et al., 2013), highlighting that particular resistance alleles may be able to bring about severe consequences in terms of development and reproduction when they are found in certain genetic backgrounds. Given that epistatic effects of different resistance alleles may provide increased tolerance in the presence of insecticides determination of their possible evolutionary outcome is rather valuable in terms of Insecticide resistance management.

In this study we used transgenic flies already existing in our lab, bearing individual mechanisms identified in many studies of pyrethroid resistant populations of the species *Aedes aegypti*, in order to bring the two mechanisms in the same genetic background. In particular we generated transgenic *Drosophila* flies overexpressing the gene *Cyp9j28* in a genetic background where we have introduced by CRISPR/Cas9 the mutation V1016G in the sequence of *para*. This strategy enabled us to determine and measure the relative contribution of each mechanism individually and of their interaction to deltamethrin resistance, but also to address any possible fitness cost imposed, with limited confounding genetic effects.

2.2 Materials and Methods

2.2.1 Fly strains

Drosophila strains used in this study are shown in table 2.2. Strain yw nos int; attP40 (Markstein et al., 2008) was a gift by Pawel Piwko and Christos Delidakis (IMBB/FORTH). CRISPR/Cas9 genome modification was performed at strain y1M{nos-Cas9.P}ZH-2A w*, where Cas9 is expressed under the control of nanos promoter (Port et al., 2014) (herein referred as nos.Cas9, #54591 in the Bloomington Drosophila stock center); the homozygous strain for the

mutation V1016G in the *para* sequence was generated by Iason Christou, MSc. Background strain yellow white (*yw*) and several balancer lines (Table 2.2) are part of the IMBB/FORTH facility fly collection (kindly provided by Prof. Christos Delidakis, IMBB and University of Crete). The *yw*; attP40 strain was generated and kindly provided by Dr. Vassilis Douris (Samantsidis et al., 2020). The HR-GAL4 driver line is previously described (Chung et al., 2007), while the responder line UAS.AaegCYP9J28 (herein referred as UAS-CYP9J28) was generated also as described previously (Pavlidi et al, 2012). All flies were kept at a temperature of 25°C, humidity 60–70% and 12 : 12 h photoperiod on a standard fly diet.

2.2.2 Toxicity Bioassays

Contact toxicity bioassays were performed as previously described in the 1.2.1 section. Each deltamethrin concentration was assayed in three replicates, while the bioassays were performed several times. Knock-down effect was scored for 180min with 15-30min intervals and lethality was measured after 24hrs. Given the high variability in contact bioassay deltamethrin responses observed among different technical replicates, topical application was performed as an alternative methodology for insecticide administration to minimize variability. Topical application of deltamethrin was performed on 1–3 day old female flies. Deltamethrin was dissolved in acetone and serial dilutions were made to generate the appropriate concentrations. Each insecticide concentration (or acetone as negative control) was applied in a dose of 1 µl per fly using a 10 µl Hamilton syringe. Flies were immobilized by keeping them on an ice cold slide. For each concentration 40 flies were tested. Following insecticide application, the flies were transferred into glass scintillation vials covered with cotton moisturized with 5% sucrose solution. The vials were maintained in a 25°C incubator while mortality was scored after 24 h. For all bioassays performed nos.Cas9 and *yw*; attP40 strains were used as control lines and resistance ratio was calculated versus both lines.

The calculation of concentration-response data were collected and analyzed with ProBit analysis using the software PoloPlus (Leora Software, Berkeley, CA) in order to calculate lethal concentrations of the 50% of the population subjected to the experiment (LC_{50} values), 95% fiducial limits (FL), linearity of the dose-mortality response and statistical significance of the results. A χ^2-test was used to assess how well the individual LC_{50} values observed in the bioassays agree with the calculated linear regression lines. The LC_{50} values and resistance ratio (RR) are considered significant if the 95% FL did not include 1.

2.2.3 Generation of homozygous recombinant yw; HR-GAL4>UAS-CYP9J28 (2N) strain

It has been previously demonstrated that overexpression of CYP9J28 in Malpighian tubules, midgut and fat body of *Drosophila* using the driver HR-GAL4, increases resistance ratio by almost 7-fold when compared to the respective control line (Pavlidi et al., 2012). However, this phenotype was not observed when flies were subjected to topical application with deltamethrin. To this end, flies bearing two copies of both driver and responder transgenes were generated as shown in Figure 2.2A. Both transgenes are located in the second chromosome of *Drosophila*, therefore we opted to bring them in *cis* via genetic recombination. Specifically, we crossed the strains HR-GAL4 and UAS-CYP9J28 which will generate a heterozygous for both loci progeny (*yw*; HR-GAL4>UAS-CYP9J28). The emerging

female adults (in which chromosomal cross-over is feasible) were collected *en masse* and outcrossed with males of the *yw*;*CyO*/*Sco* balancer line. From the resulting progeny females or males with strong red eyes (indicative of the HR-GAL4 transgene) were collected and crossed individually again with the balancer for the second chromosome line *yw*; *CyO*/*Sco*. The females were collected and screened for the UAS-CYP9J28 transgene by PCR (primers used are summarized in the table 2.3). The positive for the transgene crosses were collected and intercrossed to generate and establish the homozygous for the two transgenes flies, *yw*; HR-GAL4>UAS-CYP9J28 (2N).

2.2.4 Generation of driver and responder lines in a V1016G genetic background

The voltage gated sodium channel (*para*) gene in *Drosophila* is located at the X chromosome, while the HR-GAL4 and UAS-CYP9J28 transgenes are located at the second chromosome. To generate flies that will bear two copies of the transgenes HR-GAL4 and UAS-CYP9J28 and at the same time to be homozygous for *para*^{V1016G} locus, we employed a simple strategy of genetic crosses as depicted in the Figure 2.1.

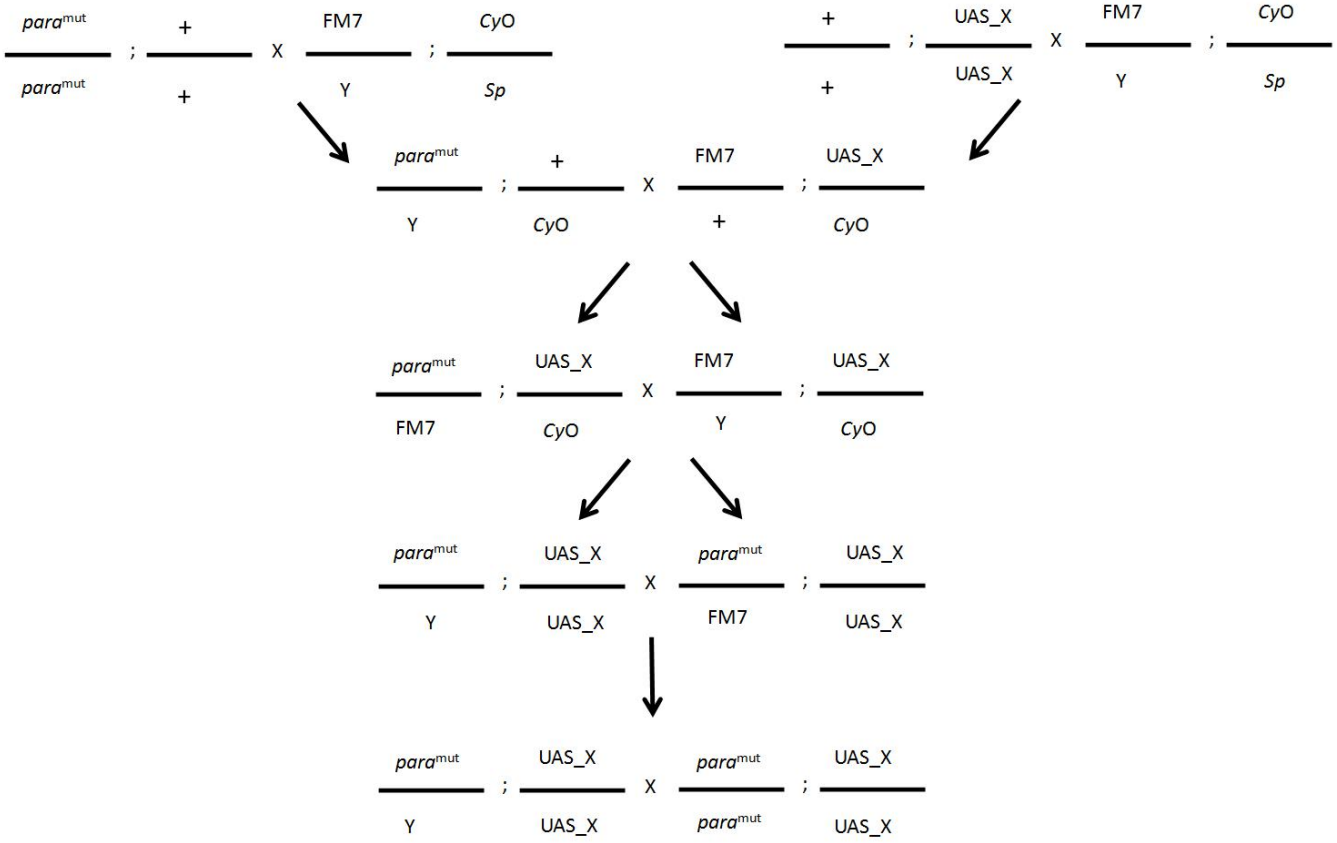

Figure 2.1 Genome engineered females bearing *para* mutations (*para*^{mut}) are crossed to FM7;CyO/Sp males in order to generate *para*^{mut}; CyO/+ males, while females from genetically transformed lines with a UAS insertion (UAS_X) are also crossed to FM7;CyO/Sp males in order to generate FM7/+;UAS_X/CyO females that are crossed to the *para*^{mut}; CyO/+ males. Following the series of crosses depicted, we can generate strains bearing any transgene insertion located in chromosome 2 together with any X-linked *para* mutation of interest, as for example the [HR-GAL4_UAS-CYP9J28] linked alleles in a *para*^{V1016G} genetic background.

2.2.5 Extraction of RNA, cDNA synthesis, reverse transcription PCR and quantitative RT-PCR

To verify that *Cyp9J28* transgene was expressed in the heterozygous flies bearing one and/or two copies of both HR-GAL4 and UAS-CYP9J28, reverse transcription PCR was performed. Total RNA was extracted from 20 female flies (1-3 days old) using the Trizol reagent (MRC). The extracted samples were treated with Turbo DNAse (Ambion) to remove the genomic DNA and 2μg of RNA was used as template for first strand synthesis using oligo-dT_{20} primers with the Supercript III reverse transcriptase kit (Invitrogen). The PCR reaction was performed using primers specific for each transgene and for the *rpl11* (ribosomal protein L11) as a reference gene (Table 2.3), while one microliter of cDNA samples was used as template. The conditions of the reactions were 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and final extension for 2 min.

To analyze the levels of expression of the UAS-CYP9J28 in strains bearing one copy of the transgene, two copies of the transgene in a wild-type for *para* background and two copies of the transgene in a V1016G background, a quantitative RT-PCR was performed. qPCR was conducted using the Kapa SYBR Fast qPCR Master Mix kit (Kapa Biosystems) and the reactions were carried out in the Bio-Rad CFX Connect using the following conditions: 95°C for 2 min, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. The efficiency of the qPCR reaction for each primer pair was assessed in 10-fold dilution series of pooled cDNA samples. The experiment was performed using three biological and two technical replicates. Relative expression was normalized to the reference genes rpl11 and rp49, while the analysis was conducted as previously described (Pfaffl 2001). All primer sequences are shown in the table 2.3. A two-tailed unpaired student's t-test (using GraphPad Prism) was carried out in order to compare relative expression in qPCR data.

2.2.6 Life table parameters

Determination of life table parameters was performed as previously described (Douris et al., 2016). To determine developmental time and sex ratio, cages with 50 virgins (1–3 day old) and 20 males were capped with cherry juice-agar plates supplemented with yeast, left to cross overnight and after plate replacement the flies were left to lay eggs for 4–5 h. Eggs were transferred into vials with standard fly food in batches of 50 eggs (10 replicates for each genotype). Pupation, adult emergence time and total number of males/females were scored daily from day 7. To monitor daily and total fecundity, fifteen females from each genotype were mated individually with three males, transferred in small cages capped with 35 mm yeast supplemented cherry agar plates, and the number of eggs laid by each female was counted daily for 25 days in total. Life table parameter data (electronic supplementary material Samantsidis et al., 2020) were analyzed for significant differences between strains with one-way ANOVA followed by post-Dunett multiple comparison test using the software GRAPHPAD PRISM 8.0.2.

2.3 Results

2.3.1 Generation of *Drosophila* lines over-expressing CYP9J28 in a *kdr* background

We used a previously generated genome engineered *Drosophila* strain bearing the mutation *para*^{V1016G} in a homozygous state as well as a transgenic line for the heterologous expression of *AaeCyp9j28* under the control of HR-GAL4 driver (Pavlidi et al., 2012). However, overexpression of this P450 showed a marginal effect when flies were subjected to topical application with deltamethrin. For this reason, we employed a standard genetic approach to generate a fly strain that will bear two copies of both driver and responder transgenes, via genetic recombination, (Figure 2.2A) provided that both transgenes are located in the second chromosome. Using strong red colour of the eyes as a phenotypic marker for HR-GAL4 transgene and a diagnostic PCR for UAS-CYP9J28 we generated three different lines bearing both in *cis* (Figure 2.2B).

As indicated by qRT-PCR the expression levels of *Cyp9j28* were increased by 3.1-folds in the *yw*; HR-GAL4>UAS-CYP9J28 (2N) strain compared to flies bearing one copy of each of the trasngenes (Figure 2.3A). To analyze the relative contribution of CYP9J28 to deltamethrin resistance when it is overexpressed in a *kdr* background, we employed a strategy based on standard genetic crosses so that to generate a strain that will continuously over-express the *Cyp9j28* while it will carry the mutation V1016G in the *para* gene (Figure 2.1). Resulted flies were successfully over-expressing CYP9J28 and were also homozygous for the mutation (Figure 2.3B and 2.3C). Furthermore, quantitative RT-PCR indicated that stains *yw*; HR-GAL4>UAS-CYP9J28 (2N) and *para*^{V1016G}; HR-GAL4>UAS-CYP9J28 (2N) did not display any significant differences regarding the expression levels of *Cyp9j28* (Figure 2.3A).

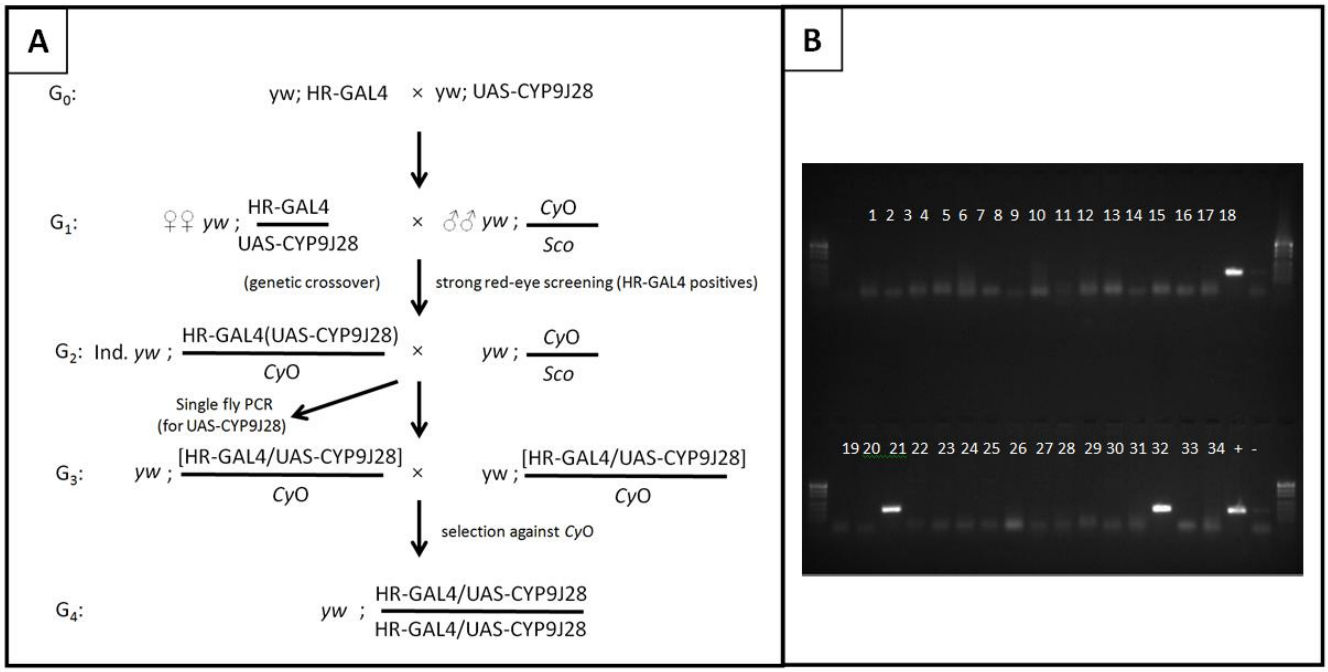

Figure 2.2: Generation of homozygous recombinant strain *yw;*HR-GAL4>UAS-CYP9J28(2N). **A**: Crossing scheme for the generation of a strain bearing both HR-GAL4 and UAS-CYP9J28 in the 2nd chromosome, following a cross between lines HR-GAL4 (Daborn et al., 2007) and UAS-CYP9J28 (Pavlidi et al. 2012) that produces a heterozygous genotype (y)w;HR-GAL4>UAS-CYP9J28. Since both strains were originally generated by P-element mediated transgenesis at random (unknown) positions at the 2nd chromosome, the frequency of genetic crossover is proportional to the distance between the relevant insertion positions. Virgin (y)w;HR-GAL4/UAS-CYP9J28 females were crossed with *yw*;*CyO*/*Sco* balancer males and the *Cy* progeny was monitored for the characteristic red-eye phenotype (derived from the w^+ marker expression marking the HR-GAL4 transgene) in contrast to orange-eye phenotype (derived from the *miniwhite* marker expression marking the UAS-CYP9J28 transgene). Selected individuals, expected to have the HR-GAL4 transgene opposite to a *Cy*O balancer, were crossed to *yw*;*CyO*/*Sco* balancer flies and after giving progeny they were individually screened for the presence of the UAS-CYP9J28 transgene by PCR amplification (B). Heterozygous yw; [HR-GAL4_UAS-CYP9J28] / CyO flies were then intercrossed to give the homozygous yw; HR-GAL4>UAS-CYP9J28(2N) strain following selection against *Cy* marker. **B**: Single fly PCR genotyping for the presence of the UAS-CYP9J28 transgene using primer pair Cyp9j28_RT_F/R. The presence of the diagnostic fragment indicates a genetic recombination event in three out of the 34 individuals examined.

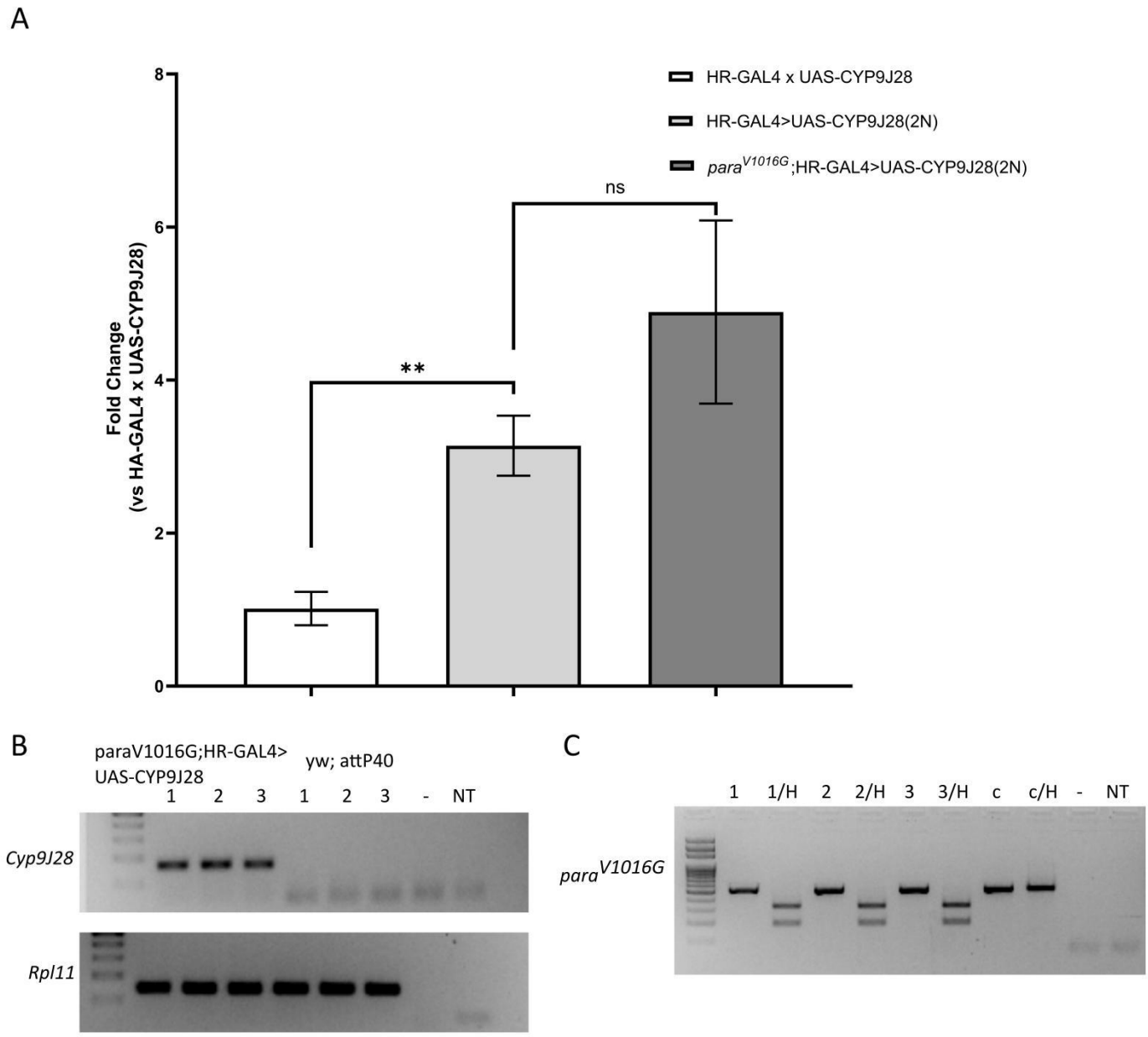

Figure 2.3 P450 overexpression in the flies bearing the *kdr* mutations. A. Using the progeny of HR-GAL4 x UAS-CYP9J28 (bearing one copy of driver and responder transgene) as basal expression, the Ct values of strains *yw*;HR-GAL4>UAS-CYP9J28(2N) and *para*^{V1016G}; HR-GAL4>UAS-CYP9J28(2N), each bearing two copies of driver and responder transgenes in the absence or presence of the V1016G mutation respectively, was calculated. No significant difference in expression between strains *yw*;HR-GAL4>UAS-CYP9J28(2N) and *para*^{V1016G}; HR-GAL4>UAS-CYP9J28(2N) was observed (p= 0.07), while a significant difference was observed between the *yw*;HR-GAL4>UAS-CYP9J28(2N) and HR-GAL4 x UAS-CYP9J28 (**p= 0.0053). B. (top) *Cyp9j28* expression is

confirmed by reverse transcription and PCR amplification of cDNAs. Lanes *para*^{V1016G}; HR-GAL4>UAS-CYP9J28(2N) (1–3) indicate three biological replicates of the flies tested for the overexpression of the transgene. Lanes yw; attP40 (1–3) indicate the three biological replicates of the control line. The same cDNAs were used to amplify the housekeeping gene rpl11 as a reference gene (bottom). -: no reverse transcription control (to monitor for genomic DNA contamination); NT: no template control. C. The presence of V1016G mutation in the same flies is tested by PCR of genomic DNA with generic primers (kdrF/kdrR, Table 2.3) yielding a fragment of 516bp and subsequent digestion of the product with HindIII (/H) which produces two bands of 324bp and 192bp. c: yw; attP40 negative control DNA.

2.3.2 Toxicity assays postulate a synergistic interaction between different resistant mechanisms

Slow uptake contact bioassay experiments (results are summarized in the Table 2.4) were performed for all strains used in this study (*yw*; attP40, nos.Cas9, *para*^{V1016G}, *yw*;HR-GAL4>UAS-CYP9J28(2N) and *para*^{V1016G}; HR-GAL4>UAS-CYP9J28(2N)) and showed that resistance levels were extremely high in flies combining both resistance alleles in the same genetic background (LC_{50} 2441.675 (1611.612-3816.105) µg/vial) compared to the control flies nos.Cas9 (LC_{50} 5.45 (2.4-8.57) µg/vial), or the flies bearing any of the two mechanisms individually (RR_{V1016G}: 54.23 and $RR_{Cyp9J28}$: 2.52 compared to relevant control lines nos.Cas9 and *yw*; attP40, respectively). Although these results could imply a possible synergistic interaction between these two mechanisms, the substantial difference between the LC_{50} values of the control lines nos.Cas9 and *yw*; attP40 (Table 2.4) was a significant caveat. However, this difference was significantly minimized when these strains were subjected to topical application with deltamethrin, as shown in Table 2.1 (nos.Cas9 LC_{50} 3.33 (1.3-5.1) ng/fly and *yw*; attP40 LC_{50} 3.1 (2.65-3.51) ng/fly). Therefore, topical application was performed in order to measure the resistance conferred by each mechanism individually as well as in combination. As shown in Table 1, the *para*^{V1016G} strain displayed modest levels of resistance against deltamethrin (RR_{V1016G}: 3.00), while the strain consistently over-expressing the *Cyp9J28* (two copies of the transgene) also exhibited moderate resistance ($RR_{Cyp9J28}$: 1.77) compared to both control lines. However, the strain *para*^{V1016G}; HR-GAL4>UAS-CYP9J28(2N) displayed a more striking resistance phenotype significantly greater than the product of individual RRs (RR_{combo}:19.85>> RR_{V1016G}: 3.00 X $RR_{Cyp9J28}$: 1.77).

Table 2.1 Topical application deltamethrin bioassay responses of transgenic flies expressing pyrethroid metabolizing CYP9J28 alone or along the engineered V1016G mutation in their voltage gated sodium channel (*para*).

Strain/Cross	LC_{50} (ng/fly)	(95% CI)	Slope (±SE)	RR
HR-GAL4 x *yw*; attP40	3.10	(2.65-3.65)	3.59(±0.47)	**1**
[1]HR-GAL4>UAS-CYP9J28 (x2)	5.49	(4.051 - 6.60)	4.4(±1.09)	**1,77**
para^{V1016G}	9.30	(4.98– 14.55)	1.696(±0.36)	**3,00**
[2]*para*^{V1016G} ; HR-GAL4>UAS-CYP9J28 (x2)	61.53	(47.48 –78.50)	4.851(±0.80)	**19,85**
nos.Cas9	3.33	(1.3-5.1)	2.259(±0.39)	**1.07**

[1]: homozygous recombinant HR-GAL4>UAS-CYPJ28 (two copies of driver and responder)
[2]: *para*^{V1016G} ; HR-GAL4>UAS-CYPJ28 (two copies of driver and responder)

2.3.3 The interaction of two different resistance alleles possible impose some fitness disadvantages

To analyze the evolutionary outcome of the epistatic interaction between metabolic and target site resistance mechanisms, we used the set of transgenic flies we generated and analyzed a certain array of fitness cost associated parameters (developmental time, sex ratio and total fecundity).

I. Developmental time

Development time was determined as pupation time and scored as number of pupae emerged at day 7 and day 8 post egg laying. While the lines bearing individual mechanisms of resistance (*para*^{V1016G} and *yw*; HR-GAL4>CYP9J28(2N)) did not exhibit any significant difference with respect to the control lines (nos.Cas9 and *yw*;attP40), the line overexpressing the CYP9J28 in the *para*^{V1016G} background showed a significant cost (Figure 2.4A). In particular, at days 7 and 8 only ~38% of the *para*^{V1016G}; HR-GAL4>UAS-CYP9J28(2N) flies reached pupation, in contrast with the control lines and the strains bearing individual mechanisms (average pupae >60% in respect with the initial number of eggs per vial at days 7 and 8, N=50). Statistical analysis by using one-way ANOVA indicated that this difference was significant (p-values of all comparisons made are summarized in Table 2.5), further implying that this was not attributed to any of the individual mechanisms but possibly due to their combination.

II. Sex ratio

Another parameter analyzed was the sex ratio, therefore we analyzed the number of males and females emerged post egg laying. However, no statistical significant difference was observed among the five different genotypes.

III. Total fecundity

To analyze if these mechanisms individually or in combination impose any significant effect in reproduction, we sought to analyze the number of eggs laid by females of each genotype. Therefore, we set almost fifteen individual crosses (one female crossed with three males of the same genotype) and measured the total number of eggs laid at a daily basis. A significant reduction in the total number of eggs was observed in the case of the combination of the two mechanisms compared to all control lines (one-way ANOVA followed by post Dunett multiple comparison test, $p_{comboVSV1016G}$ =0.0001, $p_{comboVScyp9j28}$ =0.0153, $p_{comboVSnos.Cas9}$<0.0001 and $p_{comboVSyw;attP40}$<0.0001). Overall, it was demonstrated that combination of overexpression of CYP9J28 in the *para*^{V1016G} background impose a significant developmental delay but also seems to reduce the reproduction rate of the flies in terms of the number of eggs laid.

2.4 Discussion

Insects-vectors of human diseases have the ability to develop resistance, an evolutionary adaptation process that poses a severe problem with dramatic consequences to public human health. The overuse of the same classes of insecticides can lead to multigenic resistance as a result of strong selection pressure, with each genetic locus to contribute via different mechanisms to the resistance phenotype. *Aedes aegypti* is one of the most important vectors transmitting several arboviruses displaying increased tolerance against several insecticides like pyrethroids, usually found to carry more than one putative resistance mechanisms (Aponte et al., 2013, Smith et al, 2016, Seixas et al, 2017, Bariami et al., 2012). Although several resistance factors that have been detected consistently in this

species have been functionally resolved individually, either *in vivo* or *in vitro* (Pavlidi et al., 2012, Du et al., 2013, Du et al., 2016), their combinatorial effect has yet to be resolved. Here we used *Drosophila melanogaster* in order to analyze and functionally validate the putative synergistic interaction between target site resistance mutations and enhanced detoxification due to upregulated P450s. Flies overexpressing CYP9J28 in addition with the target site mutation V1016G displayed a RR greater than the product of the RRs obtained from each mechanism alone. Our results are in line with previous studies reporting a synergistic interaction between different resistance loci, which found that the combination of different mechanisms result mostly to a multiplicative effect (Hardstone et al., 2010).

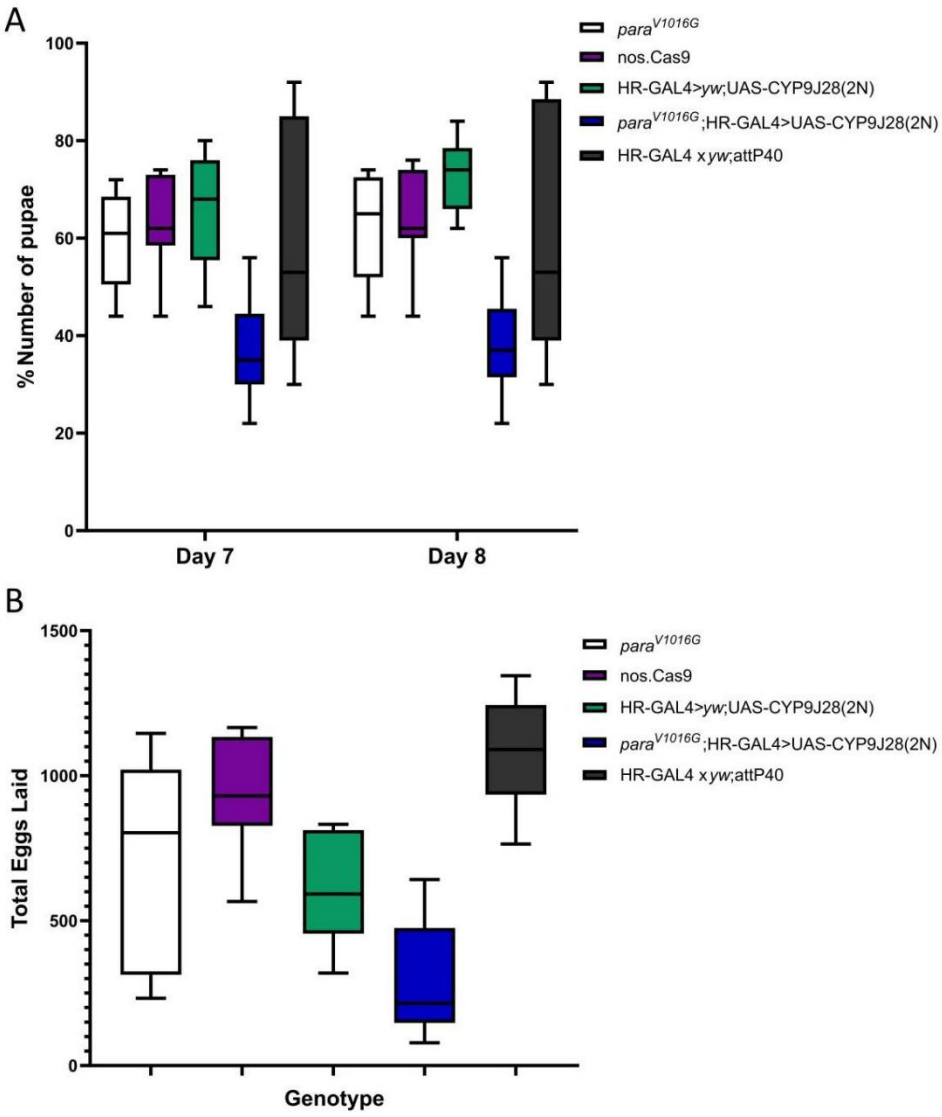

Figure 2.4 Fitness cost analysis in "super-resistant" fly lines. A. Determination of development time of strains *para*^{V1016G}, nos.Cas9, yw;HR-GAL4>UAS-CYP9J28(2N), *para*^{V1016G};HR-GAL4>UAS-CYP9J28(2N) and yw; attP40. For each strain fifty eggs were transferred in batches in standard fly food and let incubate. Pupation time was scored at days 7 and 8 post egg laying. One-way ANOVA test was used to determine statistically significant differences between the super-resistant flies (combining both mechanisms) and the rest of the lines (wild type or bearing individual mechanisms). The p-values for all comparisons performed are summarized in the Table 2.4. **B)** The total number of eggs laid by all strains was determined after measuring at a daily basis for 20 days. The strain combining both the expression of the mosquito P450 and the *kdr* mutation laid significantly less eggs than the rest of the genotypes, as indicated by one-way ANOVA analysis followed by post Dunnett multiple comparison test (pcomboVSV1016G =0.0001, pcomboVScyp9j28 =0.0153, pcomboVSnos.Cas9<0.0001 and pcomboVSyw;attP40<0.0001).

The synergistic interaction between metabolic detoxification and target site resistance mutations has been previously reported in mosquitoes (*Culex pipiens*), however, their strategy introduced a lot of genetic variation that limited any inferences that could be drawn (Hardstone et al., 2009). In this single study, the authors have demonstrated that a non-additive effect was exhibited when P450 upregulation was combined with a *kdr* mutation, regardless of the state of homozygosity. Moreover, they have noticed that the resistance phenotype of *kdr* genotype was altered between same genotypes generated by reciprocal crosses, which possibly indicates the different genetic backgrounds among the strains used in their study (Hardstone et al., 2009). The use of *Drosophila melanogaster* provides the advantage of limiting any confounding genetic factors arising from different genetic backgrounds, that could lead to wrong estimations. Moreover, genetic background is rather important in insect toxicology since it is known that it can alter and cause substantial variation in wild-type lines (Chandler et al., 2013). A similar attempt has been performed also for the mosquitoes *Aedes aegypti* by the same group, in which they have used congenic lines by multiple backcrossing of a resistant strain harboring known resistance mechanisms to a susceptible strain (Smith et al, 2019). This study showed that interaction between elevated expression of CYPs and target site resistance mutations (V1016G+S989P in the voltage gated sodium channel) exhibited a greater RR than the sum of the RRs obtained by individual mechanisms against five different pyrethroids (not deltamethrin), which further corroborates our findings. However, their study lags behind on the specification of the CYPs responsible for the insecticide metabolism, given that they have not yet determined the gene identity and even the number of CYP genes involved (Smith et al., 2019, Smith et al, 2021).

The exact mechanism of the synergistic epistasis between the metabolic and target site resistance remains elusive. There are two possible models that could describe the mechanistic background of synergism. One hypothesis is that the existence of the *kdr* mutation lowers the binding affinity of the parental compound with the target which gives to the enzyme additional time to perform detoxification and thus to avoid saturation (Samantsidis et al., 2020). This hypothesis is corroborated by comparing slow uptake contact bioassays to the fast uptake topical application (Tables 2.1 and 2.4) described in here but also in a similar array of mechanisms (mutation *kdr* L1014F and the over-expression of CYP6BQ23) (see publication Samantsidis et al., 2020). In the slow uptake bioassays, the insecticide is absorbed through the insect cuticle in a much slower rate, therefore the P450 enzyme alone is not saturated so fast metabolizing more insecticide compound and the presence of kdr mutation gave to P450 more time for further detoxification. Alternatively, the synergistic interaction between P450s and kdr mutations against pyrethroids, could be explained better by another model, which supports that the deltamethrin metabolites generated by the P450 activity, probably bind to the mutated voltage gated sodium channel disproportionately less effectively, preventing the actual parental compound to reach and bind the target. However, it is currently unknown if pyrethroid metabolites have any affinity with the target and this hypothesis needs further investigation.

Another aspect, that the insecticide resistance community should focus on, is the evolutionary outcome of insecticide resistance mechanisms in terms of insects' fitness. We analyzed if the combination of the two mechanisms impacts fitness by measuring three of the most commonly measured parameters, developmental time, sex ratio and fecundity (Freeman et al., 2021). Although none of the strains carrying individual resistance mechanisms displayed any significant difference in neither of these parameters compared to the control lines, overexpression of CYP9J28 in a *kdr* background seem to exert some effects. In particular, flies carrying both

resistance alleles displayed a significant developmental delay and reduction in total fecundity against multiple controls contributing to the genetic background (Figure 2.4). Whether the combination of two resistance mechanisms exert fitness costs or not has been also examined previously. Mosquitoes carrying two target site resistance mutations (V1016G+S989P) with elevated levels of a P450s did not show any significant difference in the life table parameters measured compared to the susceptible counterparts, except for the body size and the sex ratio (Smith et al., 2021). These data collectively suggest epistatic effects between different resistance mechanisms and highlight the need for fitness cost assessment to be done in multiple backgrounds. Further work will thus be needed to establish and characterize the evolutionary significance of these resistance alleles in the field.

Pyrethroid resistance mechanisms do not always induce fitness costs when certain life table parameters or life history traits are measured (Bajda et al., 2018, Freeman et al., 2021), however, in most cases reversion of resistance is taking place. This raises the question of whether fitness cost is sensitively and accurately measured by determination of a group of parameters, rather than by examination of traits depending on the type of resistance mechanism. Given that voltage gated sodium channel is essential for neuronal function (Perrier et al., 2021), it is hypothesized that any mutation could alter the protein's function and thus fitness analysis could be determined by analyzing traits like behavior, locomotion, mating success and under different environmental conditions (experiments in the field are preferred for costs to manifest) (Freeman, et al., 2021, ffrench-Constant 2017). Moreover, it is worth noting that in some cases costs induced by certain resistance mechanisms (like mutations in insecticide targets) are ameliorated by other mutations that act as fitness modifiers, therefore it is also very important to study fitness cost in certain backgrounds. This is rather difficult to perform by using congenic or nearly isogenic lines, as used in many different studies (reviewed in Freeman et al., 2021). To this end, using *Drosophila melanogaster* as a model species or genetic engineering via CRISPR/Cas9 directly into the pest species of interest (if feasible) seems rather a solution to this problem in order to assess the possible fitness costs of specific resistance mechanisms.

Understanding the contribution and the interaction between different mechanisms to insecticide resistance is rather important so that to unravel and understand the molecular and the evolutionary basis of this complex phenomenon, which overall may pave the way for designing integrated and targeted control strategies. Our results have direct application for IRM practices, since the "super-resistant" flies we generated could be immediately used as a platform for screening chemicals which are the most or least effective in insect-populations harboring these particular mechanisms of resistance.

Tables - Chapter 2

Table 2.2 *Drosophila* strains used in this study.

Strain	Description	Source
y^1 M{nos-Cas9.P}ZH-2A w* (referred as nos.Cas9)	Expresses Cas9 in germ line under *nanos* promoter	Bloomington Drosophila Stock Center #54591
yw; TM3 *Sb e*/TM6B *Tb Hu e*	Balancers for 3rd chromosome (TM3 with *Stubble* marker. TM6B with *Tubby* and *Humoral* markers)	IMBB flyroom stock
yw; *Cy*O/*Sco*	Balancer (CyO) for 2nd chromosome, marked with *Curly*.	IMBB flyroom stock
(FRT)w^+ / FM7c *Hw w B*	Balancer (FM7c) for X chromosome marked with *Bar*	IMBB flyroom stock
FM7 ; *Cy*O / *Sp*	Balancer (FM7c) for X chromosome marked with *Bar*; balancer for 2nd chromosome (CyO) marked with *Curly*, non-balancer 2nd chromosome marked with *Sternopleural*	IMBB flyroom stock
yw *nos*.int; attP40	Expresses Phic31 integrase under *nanos* promoter; contains attP40 landing site in 2nd chromosome, *yw* genetic background	Markstein et al., 2008
UAS-CYP9J28	Bears a *Cyp9j28* transgene under UAS at random P-element insertion point at 2nd chromosome, *yw* genetic background.	Pavlidi et al., 2012
HR-GAL4	Bears HR-GAL4 transgene (marked with miniwhite) at P-element insertion site in 2nd chromosome	Chung et al., 2017a
$para^{V1016G}$	Bears a V1016G mutation in *para;* X-chromosome derived from nos.Cas9, other chromosomes from *yw* background.	This study
HR-GAL4;*yw*> UAS-CYP9J28(2N)	Bears both HR-GAL4 and UAS-CYP9J28 at the 2nd chromosome, derived from genetic crossover, other chromosomes from *yw* background.	This study
*yw;*attP40	Contains empty attP40 landing site in 2nd chromosome, *yw* genetic background (same as UAS-CYP6BQ23 but without the transgene)	This study
$para^{V1016G}$;HR-GAL4> UAS-CYP9J28(2N)	X-chromosome derived from $para^{V1016G}$, 2nd chromosome from *yw*;HR-GAL4>UAS-CYP9J28(2N), other *yw*	This study

Table 2.3 Primers used in this study.

Primer Name	Sequence (5' – 3')	Use	Reference
kdrF	TCGCTCCAAATCCAACTGAT	Sequencing, allele screening	This study
kdrR	ACCGACTTTATGCACAGCTT		This study
Cyp9j28_RT_F	CTCCACGTTCATTCAGACGCT	qRT-PCR, Diagnostic reasons for genetic recombination	Pavlidi et al., 2012
Cyp9j28_RT_R	CTCGAGTTCCCAAATACCTGC		
RPL11_Dm_F	CGATCCCTCCATCGGTATCT		Daborn et al., 2012
RPL11_Dm_R	AACCACTTCATGGCATCCTC		
Rp49F	TACAGGCCCAAGATCGTGAA		Sadern et al., 2009
Rp49R	TCTCCTTGCGCTTCTTGGA		

Table 2.4: Contact bioassay deltamethrin responses of transgenic flies expressing pyrethroid metabolizing P450s alone or along engineered target site resistance mutations in their voltage gated sodium channel (*para*)

Strain/Cross	LC_{50} (µg/vial)	(95% FL)	Slope (±SE)	RR (vs HR-GAL x yw.attP40)	RR (vs nos. Cas9)
HR-GAL4 x *yw*; attP40	1.689	(0.65-2.61)	1.406(±0.270)	1	
[1]HR-GAL4> *yw*; UAS-CYP9J28(2N)	4.249	(2.83- 6.814)	2.9(±0.33)	2.52	
nos.Cas9	5.45	(2.4- 8.57)	2.748(±0.514)	3.23	1
$para^{V1016G}$	295.567	(222.252-440.5)	2.27(±0.42)	174.99	54.23
[2]$para^{V1016G}$; HR-GAL4>UAS-CYP9J28(2N)	2441.675	(1611.612-3816.105)	2.67(±0.44)	1445.63	448.13

[1]: homozygous recombinant yw; HR-GAL4>UAS-CYPJ28(2N) contains two copies of driver and responder

[2]: $para^{V1016G}$; HR-GAL4>UAS-CYPJ28(2N) contains two copies of driver and responder in $para^{V1016G}$ X-chromosome background

Table 2.5: P-values of One-Way ANOVA test (Figure 3) for pupation time (Day 7 above diagonal, Day 8 below diagonal) among different genotypes. Significant values are shown in bold.

Genotype	*para*^{V1016G}	nos.Cas9	HR-GAL4>*yw*;UAS-CYP9J28(2N)	*para*^{V1016G};HR-GAL4>UAS-CYP9J28(2N)	HR-GAL4>*yw*;attP40
para^{V1016G}		0.9802	0.8092	**0.0110**	>0.9999
nos.Cas9	0.9993		0.9896	**0.0039**	0.9869
HR-GAL4>*yw*;UAS-CYP9J28(2N)	0.4952	0.6988		**0.0004**	0.8399
para^{V1016G};HR-GAL4>UAS-CYP9J28(2N)	**0.0048**	**0.0045**	**<0.0001**		**0.0093**
HR-GAL4>*yw*;attP40	0.9995	0.9920	0.3678	**0.0089**	

2.5 References

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Chapter 3

"Identification of *Helicoverpa armigera* promoters for biotechnological applications"

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Authors contribution:

J.V and S.D conceived the project; G-R.S and S.D designed the experiments; G-R.S performed the experiments and analyzed the data; the original draft was written by G-R.S; S.D, J.V, G.S, L.S and S.G revised and edited the manuscript which has been approved by all authors.

Abstract

Helicoverpa armigera is a highly polyphagous agricultural pest worldwide and its control is largely based on the use of insecticides. However, new, effective, and environmentally friendly control tools are required to be developed and validated. In an effort to develop advanced biotechnological tools for this species, that will take advantage of new powerful molecular biology techniques like CRISPR/Cas9, we used available transcriptomic data and literature resources, in order to identify putative midgut-specific and U6 promoters. Following functional analysis in insect cell lines, four RNA polymerase II promoters from the genes *HaLabial*, *HaTsp-2A*, *HaPtx-I* and *HaCaudal* were found to exhibit high transcriptional activity in the midgut derived cell line RP-HzGUT-AW1(MG). The HaTsp-2A promoter did not exhibit any activity in the non-midgut derived cell lines Sf-9 and Hi-5, suggesting that it may function as a midgut specific promoter. Furthermore, considering the utility of RNA polymerase III U6 promoters in methodologies such as RNAi and CRISPR/Cas9, we identified and evaluated four different U6 promoters of *H. armigera*. *In vitro* experiments based on luciferase and GFP reporter assays indicate that these U6 promoters are functional and can be used to experimentally silence or knockout target genes through the expression of shRNAs and sgRNAs respectively. Taking our findings together, we provide a set of promoters useful for the genetic manipulation of *Helicoverpa* species*,* that can be used in various applications in the context of agricultural biotechnology.

3.1 Introduction

Throughout the last decades, significant progress has taken place in the field of insect genetic manipulation, beginning from the application of random integration techniques and continuing with precise genome editing methodologies (Fraser 2012, Gantz et al., 2020). Insect transgenics has been a valuable tool for both functional characterization of genes and the development of biotechnological methods to control the populations of harmful insects (Douris et al., 2020, Fraser 2012). Importantly, population control approaches based on genetically modified insects, like sterile insect technique (Lees et al., 2015) and gene drive (McFarlane et al., 2018) hold the promise of more effective and safe pest management.

Reverse genetic methodologies are largely based on the use of promoters. The RNA polymerase II promoters (RNA-pol II) are DNA sequences containing *cis*-acting elements, capable of driving gene expression in a ubiquitous or in a tissue specific manner. A large number of studies focuses on the identification and characterization of constitutive promoters of non-model insects (Lu et al., 1997, Tsubota et al., 2014, Chen et al,2020a, Chen et al,2020b, Bleckman et al., 2015, Miyata et al., 2019). Promoters of differing strengths are often useful for different applications, and the same promoter often works differently depending on the organism, tissue, and cell line under investigation. Apart from the RNA-Pol II, RNA-pol III promoters, which are known to drive the expression of small RNAs (e.g. small nuclear RNAs, tRNAs etc) are also very useful in the field of transgenesis. Specifically, U6 promoters, which comprise a subset of RNA-pol III promoters that regulate the expression of U6 small nuclear RNAs (Hernandez et al., 2007), are extensively used in techniques such as RNAi and CRISPR/Cas9, for the synthesis of short hairpin RNAs (shRNAs) and single-guide RNAs (sgRNAs) respectively (Huang et al., 2017, Port et al., 2014). Generally, insect U6 promoters exhibit high sequence divergence within and between species (Hernandez et al., 2007). However, there are two regulatory sequences that are crucial for the activity of U6 promoters and are highly conserved throughout Insecta: the Proximal Sequence Element A (PSEA) and the TATA box (Hernandez et al., 2007). Although there are some types of versatile U6 promoters, which can function across different insect species, high levels of expression are usually achieved only when endogenous U6 promoters are used (Huang et al., 2017, Mabashi-Asazuma et al., 2017). Nevertheless, in several important pest insect species, endogenous U6 promoters have not yet been characterized.

The cotton bollworm *Helicoverpa armigera* (Hübner) and the corn earworm *Helicoverpa zea* are allopatric species capable of interbreeding and they are considered as agricultural pests threatening several cultivated plants (Haile et al., 2021, Reay-Jones 2019). Their control is based on microbial or chemical insecticides that are usually delivered orally while the insects are at the larval stages of their life cycle (Wu et al., 2008, Haile et al., 2021). Most of the orally delivered insecticides either have to pass through or directly target the midgut epithelium, which highlights the importance of this tissue in pest control (Denecke et. al 2018; Heckel 2020, Syed et al., 2019). The lepidopteran midgut comprises one of the largest tissues of the lepidopteran body, with a predominant role in insects' physiology (nutrient digestion and absorption). It is an endodermally derived highly complex tissue, composed of a single cell epithelial layer where midgut cells are connected tightly with smooth septate junctions (sSJs), mediated by genes like *Tsp-2A* (Izumi et al., 2016, Izumi et al, 2021). Moreover, midgut compartmentalization in both larval and adult stages is another important characteristic that increases its complexity. Certain genes such as

the homeobox- containing genes *Labial*, *Caudal* and P*tx-l* have been correlated with differentiation, specification as well as regionalization of the gut during development in *Drosophila* (Buchon et al., 2013).

Given the difficulties in working with tissues like midgut *in vivo*, several research groups have developed and used lepidopteran cell lines that may retain *in vivo* properties, in order to study insect biology in a more tractable system (Swevers et al., 2021, Arunkarthick et al., 2017). Well-suited examples are the development of RP-HzGUT-AW1(MG) (Goodman et al., 2004) and its further characterization (Vorgia et al., 2021) as well as two recently developed midgut derived cell lines of *S. frugiperda* (Zhou et al., 2020). Genetic tools are thus needed to further study these cell lines across a range of organisms. For example, the identification and characterization of a range of RNA Pol II promoters in Sf9 cells and a midgut derived cell line from *Spodoptera frugiperda* has greatly expanded the range of possible projects undertaken on these cell lines (Chen et. al 2020; Chen et. al 2020b). Furthermore, the application of the CRISPR/Cas9 technology in lepidopteran cell lines like *Plutella xylostella* and *Bombyx mori*, has been substantially facilitated by the use of customized expression vectors carrying species specific U6 promoters (Huang et al., 2017, Mabashi-Asazuma et. al 2017). However, such transgenesis tools remain to be identified in other important non-model species like *H. armigera* or *H. zea*.

Here, by using available tissue specific transcriptomic datasets and literature resources, we identified RNApol II and RNApol III promoters and we have functionally characterized them in cell lines including a midgut derived cell line from *H. zea*. It is hoped that this work will provide an enhanced genetic toolkit for *Helicoverpa* cell lines and provide a basis for performing more advanced *Helicoverpa* transgenics *in vivo.*

3.2 Materials And Methods

3.2.1 Identification of U6 promoters and putative midgut-active promoters

To select putative midgut-active promoters we employed two complementary approaches. The first approach was “literature driven”: five genes which have been previously associated with midgut physiology were selected for analysis of their promoters. The second approach attempted to identify genes with expression specifically in the midgut as these would theoretically both be active in midgut derived cell lines but may also have future use in generating midgut specific expression in vivo. This second approach relied purely on expression data of *H. armigera* midgut of L2, L3 and L4 larval stages (Ioannidis et al., 2022) and led to the selection of eight genes from a subset of the 20 most abundant (high TPM, transcripts per million) and most midgut-specific (FC, fold change against carcass) genes, which were filtered after using a threshold with value 100 for both TPM and FC. For the 13 selected genes, an approximately 2,000bp region directly upstream of the translation initiation codon was considered as a putative promoter.

To identify the U6 snRNA gene(s) of *H. armigera*, we searched the genome (GCF_002156985.1) using BLASTn with the 102nt sequence of *Bombyx mori* U6 snRNA gene (Accession Number AY309084.1) as a query (Hernandez et al., 2007). Orthologous midgut promoters from other lepidopteran species were also identified using Blastn, selecting the most significant hits in terms of E-value. All multiple sequence alignments were performed using ClustalW implemented in Bioedit 7.2 with default parameters.

3.2.2 Plasmids for the analysis of the midgut-active promoters

A modified version of pBluescript SK(+) was initially engineered, in order to insert a PCR fragment of the multiple cloning site of the pSLfa1180fa vector (Horn et al., 2003) with SacI. This multiple cloning site contains the recognition sites of the 8-cutter restriction enzymes FseI and AscI, which can be used for cloning the promoter sequences of any gene of interest. This modified vector was named as pBSk^{+}FA. A 2.7Kb PCR fragment (amplified with Phusion High Fidelity Polymerase, NEB) containing a firefly luciferase-SV40 poly(A) cassette (derived from pGL2-Basic Vector, Promega), was cloned between the AscI and SpeI sites of the pBSk^{+}FA (primer sequences are presented in the Table 3.1) to create the plasmid pBSk^{+}FA[FFluc-SV40]. All the promoters and the respective truncated versions, were amplified with PCR (Phusion High Fidelity Polymerase, NEB) using primers bearing the restrictions sites FseI and AscI, they were cloned into the pGEM-T-easy Vector (Promega) and verified by sequencing (CeMia, Greece). Subsequently, each promoter was cloned into the vector pBSk^{+}FA[FFluc-SV40] directly upstream of the luciferase reporter gene (Figure S3). All of the plasmids were purified using the NucleoSpin Plasmid, Mini kit for plasmid DNA (Mancherey Nagel). The pBmAc3-Renilla luciferase construct was used as control for normalization of transfection efficiency in the luciferase assays and was generated as follows: the ORF of *Renilla* luciferase was cloned as PCR fragment (from the pSis-Check2 plasmid) between the BamHI and NotI sites of the pBmAc3 vector (a modified version of pEIA plasmid, lacking the hr3 enhancer and the IE1 cassette, kindly provided by Dr. Kostas Iatrou (NCSR "Demokritos") (Douris et al., 2006)).

3.2.2 Plasmids for assessing the ability of the U6 promoters to drive the expression of shRNAs

To assess the ability of the U6 promoters to drive the expression of shRNAs, a 63bp fragment coding for a shRNA that targets *Renilla* luciferase (Tanaka et al., 2008), which was generated by annealing the single-stranded oligos Rluc_shRNA_F and Rluc_shRNA_R (Table 3.1), was cloned between the NotI and XhoI sites of the pSLfa1180fa vector. Four out of the five putative U6 promoters were managed to be PCR amplified from *H. armigera* genomic DNA using forward and reverse primers that contain a BamHI and a NotI restriction site respectively (Table 3.1). The PCR fragments were cloned upstream of the fragment coding for the shRNA and the final plasmids were named pSL[HaU6:1-shRNA-Rluc], pSL[HaU6:2-shRNA-Rluc], pSL[HaU6:2a-shRNA-Rluc] and pSL[HaU6:3a-shRNA-Rluc]. The constructs were verified by sequencing at each step of the cloning and prior to transfection.

3.2.3 Plasmids for assessing the ability of the U6 promoters of to drive the expression of sgRNAs

To generate a Lepidoptera-specific CRISPR-vector, we initially modified the Cas9-T2A-mCherry cassette (Addgene #64324) by replacing the coding sequence of the T2A peptide with a fragment coding a GSG-P2A peptide, which has been previously shown to exhibit higher cleavage efficiency in lepidopteran cells (Wang et al., 2015). The fragment coding for the GSG-P2A peptide, which was generated by annealing the single-stranded oligos GSG-P2A-F and GSG-P2A-R, was ligated between the HindIII and NheI sites of the Cas9-T2A-mCherry vector. The Cas9-GSG-P2A-mCherry cassette was excised with the AgeI and EcoRI restriction enzymes and was cloned between the XmaI and EcoRI sites of the pSLfa1180fa plasmid (Figure 3.1). The expression of Cas9-GSG-P2A-mCherry cassette was regulated by a ubiquitous-acting promoter (either the BmNPV immediate early gene 1 (IE1) promoter (Huybrechts et al., 1992) or the promoter of the silkworm A3 cytoplasmic actin gene (BmAc3) (Johnson, et al., 1992)). Each of these two promoters was cloned as a PCR fragment, with BamHI and AgeI restriction sites (Figure 3.1). The SV40 poly(A) signal was ligated to the vector with EcoRI. The sgRNA scaffold was synthesized with PCR using Phusion Polymerase with no

template; one oligonucleotide carrying the NotI (bold) and BbsI (underlined) restrictions sites (gRNAscaffold_NotI_F: GTAC**GCGGCCGC** GAAGACCTGTTTTAGAGCTAGAAATAGCAAGTTAAAAtaaggctagtccgttatcaacttg) and another oligonucleotide carrying XhoI cut site (GTACCTCGAGCTCTGTACAAAAAAAGCACCGACTCGGTGCCACTTTTT caagttgataacggactagcctta), both sharing 24 complementary ends (lowercase letters), were used in 100ul PCR reactions and cloned between the NotI and XhoI sites of the plasmid. PCR reactions were performed as follows: 98 ^{o}C for 30 s, followed by 30 cycles of 98 ^{o}C for 10 s, 60 ^{o}C for 30 s and 72 ^{o}C for 15 s, followed by final extension at 72 ^{o}C for 2 min. Finally, each of the U6 promoters was cloned in the vector with AgeI and NotI as PCR fragments (Figure 3.1). Two BbsI restriction sites were introduced in between the end of the U6 promoters and the beginning of the sgRNA scaffold in 5'->3' direction (Figure 3.1), in order to facilitate the cloning of any CRISPR target of interest as dsDNA oligos (Figure 3.1).

3.2.3 Cell lines and Gene reporter assays

To functionally validate and quantify the activity of the 13 putative midgut-specific promoters we performed luciferase assays in cell lines. The cell lines used in this study were sub-cultured twice a week. The *H. zea* midgut cell line RP-HzGUT-AW1(MG), generously provided by Dr. Cynthia L. Goodman (Biological Control of Insects Research, U.S, Department of Agriculture, Agriculture Research Service), was routinely maintained as adherent culture in Excell 420 insect serum-free medium (Sigma Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO, Thermo Fisher Scientific), 100U/ml of penicillin and 0.1mg/ml streptomycin. The *Trichoplusia ni* embryo cell line HighFive(Granados et al., 1994), and the *Spodoptera frugiperda* ovarian cell line Sf-9 were maintained as suspension and adherent cultures respectively, in the insect serum free medium SF-900 II SFM (Thermo Fisher Scientific), while the medium of Sf-9 cells was additionally supplemented with 10% FBS. All of the aforementioned cell lines were kept in T-25 flasks in an incubator at 28^{o}C. The RP-HzGUT-AW1(MG), Hi-5 and Sf-9 cells were transfected using Escort IV Transfection Reagent (Sigma Aldrich), according to the manufacturer instructions. The cells were seeded in the appropriate density in 12-well or 24-well plates and transfected with 500ng and 250ng of DNA respectively.

The promoters were analyzed using the dual-luciferase reporter assay system (Promega), according to the manufacturer instructions. For the luciferase assays each cell line was co-transfected with the experimental reporter constructs and the control vector (pBmAc3-Renilla luciferase) at a ratio of 200:1. As a negative control we used an empty (promoterless) vector that contained the firefly luciferase gene. The Relative Luciferase Activity (Firefly Luciferase/ Renilla Luciferase, RLA) was further normalized to obtain a fold change with respect to the promoterless vector. Transfections and subsequent luciferase assays were performed in triplicates or quadruplicates and in at least three independent experiments.

To measure the ability of the *H. armigera* U6 promoters to drive the expression of the shRNA that targets Renilla luciferase, the RP-HzGUT-AW1(MG) and the Sf-9 cells were co-transfected with a plasmid carrying each U6 promoters driving shRNA expression, the pBSk^{+}FA[iE1_FFluc-SV40] or pBSk^{+}FA[HaLabial_FFluc-SV40] and pBmAc3-Renilla luciferase, at a ratio of 200:200:1. The pBSk^{+}FA[iE1:FFluc-SV40] plasmid was used as a normalizer to control

for transfection efficiency in RP-HzGUT-AW1(MG), while pBSk⁺FA[HaLabial_FFluc-SV40] was used to control for transfection efficiency in Sf-9 cells.

To analyze the competence of the U6 promoters for sgRNA expression, we used the RP-HzGUT-AW1(MG) cell line, which was co-transfected at a ratio 1:1 with the pEIA-GFP plasmid (Vorgia et al., 2021) and each of the U6-driven CRISPR vectors. The cells were observed under an inverted fluorescence microscope 72-hrs post transfection. The CRISPR efficiency was analyzed by calculating the percentage of cells emitting green fluorescence in at least 3 random fields and the pictures were analyzed using the software Image J (Rueden et. al 2017).

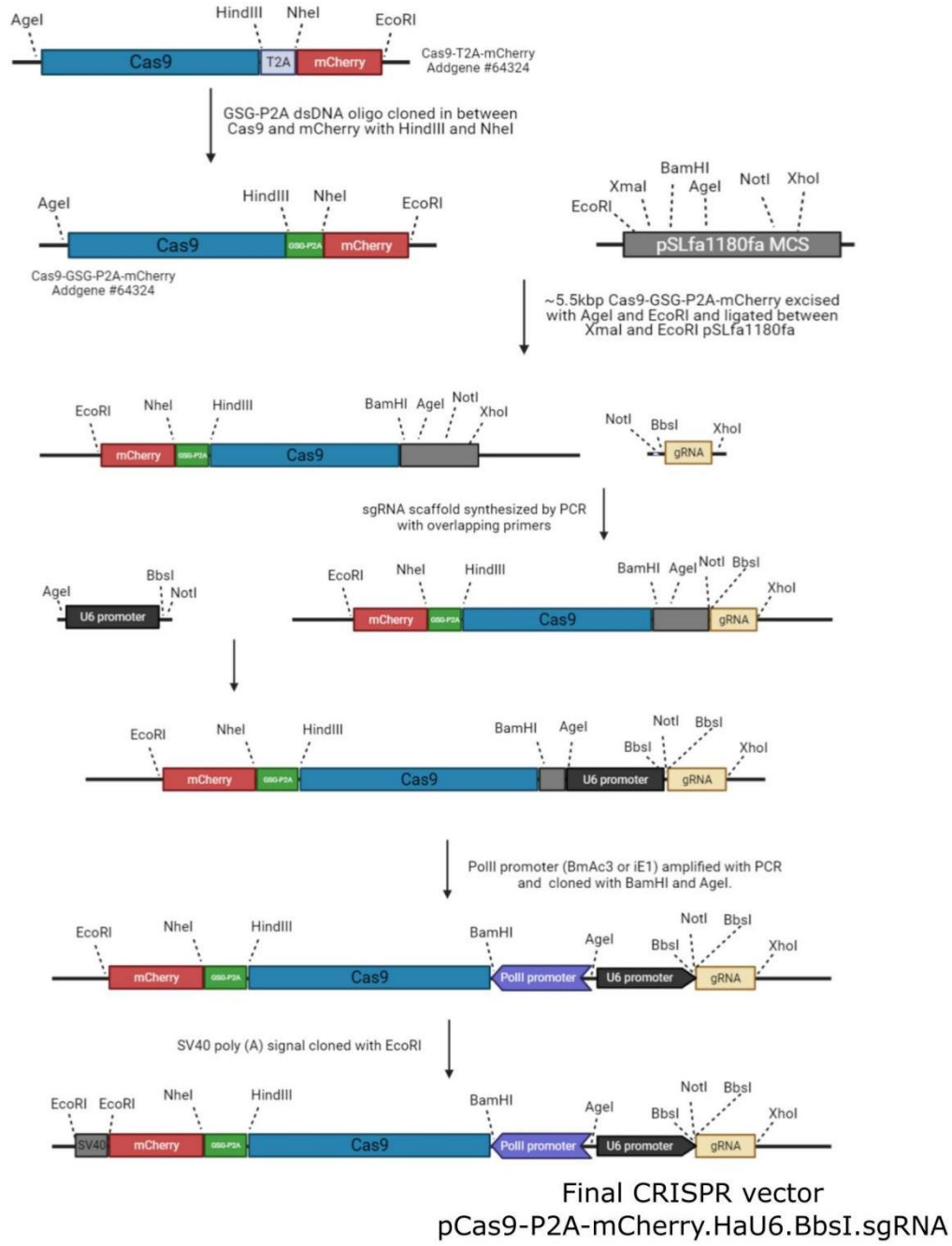

Figure 3.1. Cloning strategy for the preparation of CRISPR vectors. The initial template of pU6BbsI-Cas9-T2A-mCherry was modified so that to replace the T2A coding sequence with GSG-P2A. The whole cassette (Cas9-GSG-P2A-mCherry) was excised with AgeI and EcoRI and inserted between XmaI and EcoRI of pSLfa1180fa vector. The gRNA scaffold was synthesized with PCR by using overlapping long primers. Each of the HaU6 promoters were cloned with AgeI and NotI upstream of the gRNA scaffold, while two BbsI restriction sites were introduced in between. A Pol II promoter (either BmAc3 or IE1) was cloned upstream of Cas9-P2A-mCherry cassette with AgeI and BamHI. Finally, SV40 poly(A) signal was ligated downstream of the Cas9 cassette with EcoRI.

3.2.4 Statistical analysis

The statistical analysis for significance was calculated with One-Way ANOVA, non-parametric test using the software GraphPad Prism 3.0.1.

3.2.5 Western blot analysis

To further validate the knock-out of GFP in RP-HzGUT-AW1(MG), the cells co-transfected with each of U6-driven CRISPR vectors and pEIA-GFP were harvested 72-hrs post transfection and lysed with RIPA buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 0.5% Sodium-Deoxycholate, 0.1% SDS and 1% NP-40) supplemented with Protease Inhibitors (Sigma-Aldrich) and 1mM PMSF. Protein concentration was measured with Bradford Assay. Approximately 30μg of total protein/cell lysates were loaded onto a 10% SDS-PAGE and electrotransferred to nitrocellulose membrane for 1.5hrs at 350mA at 4^{o}C. The membrane was blocked with 5% milk (ROTH) in 1X TBST buffer for 1 hr and then incubated with 1:1000 dilution of mouse Anti-GFP antibody (Cell Signalling), 1:1000 dilution anti-beta-tubulin (Sigma-Aldrich) as a loading control and also with 1:2,500 dilution of anti-Flag (Sigma-Aldrich) for detection of Cas9 which is tagged with a 3xFLAG peptide at the N-terminus.

3.3 Results

3.3.1 Selection of putative Midgut active promoters for analysis

In order to identify promoters which would be i) active in midgut cell lines and ii) potentially used for future *in vivo H. armigera* midgut specific transgenics in *H. armigera*, we used as a starting material a set of 637 genes which are commonly over-expressed in the midgut of L2, L3 and L4 larvae with respect to the carcass tissues (Ioannidis et al., 2022). Based on the consensus view that very strong promoters are probably reflected by highly expressed genes, from the set of 637 genes we selected those with FC>100 for tissue-specificity and TPM>100 for absolute expression and finally obtained the 20 most highly up-regulated genes compared to the carcass tissues (Figure 1A). From the subset of the 20 most highly up-regulated genes (Figure 1A), the 8 displaying TPM and FC values above the median at all life stages were selected for functional analysis of their promoters (Table 1, NCBI GeneIDs: 110377047 (serine protease 3-like), 110384513 (brachyuran-like), 110379024 (trypsin, alkaline C-like), 110372923 (actin cytoskeleton regulatory complex PAN1-like), 110380915 (cilia- and flagella-associated protein 58-like), 110381636 (uncharacterized protein), 110381625 (uncharacterized protein) and 110370997 (uncharacterized protein)). However, we could not amplify with PCR the promoters of the genes with IDs: 110381636, 110381625 and 110370997 possibly due to sequence polymorphisms in our strain.

In parallel, we selected 5 additional genes from set of 637 which were also known from the literature to be expressed in the midgut of *Helicoverpa* or in closely related insect species. The genes *Labial*, *Tetraspanin-2A*, *Ptx-I* and *Caudal*, which are highly expressed in the midgut of *Drosophila* (Buchon et al., 2013), share only one ortholog with *Helicoverpa* (NCBI GeneIDs 110371850, 110378176, 110377322 respectively) and were found to be included in the dataset of 637 midgut upregulated genes. The promoter of *mucin-I*, which based on proteomic dataset drives the

expression of one of the most abundant proteins of the peritrophic matrix of *H. armigera* (Campbell et al., 2008), was also included in the analysis.

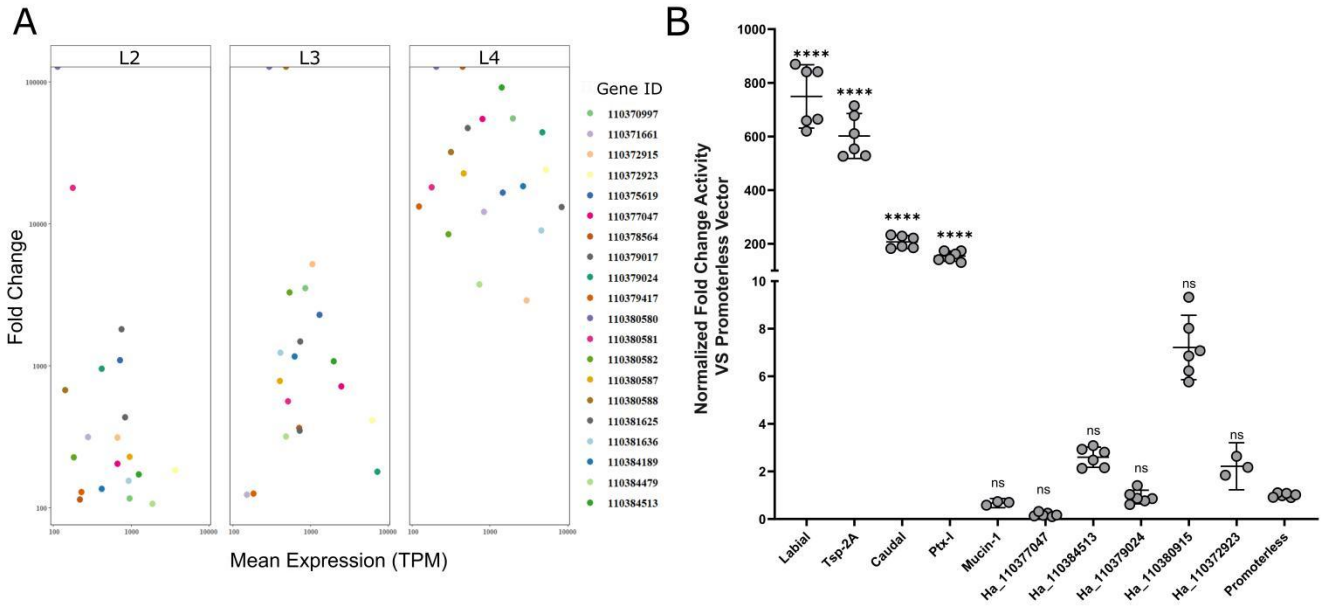

Figure 3.2. Identification and functional characterization of midgut strong promoters. A) Distribution of the 20 most highly upregulated genes, displaying the highest absolute expression (Transcript Per Million, TPM) and tissue specificity (Fold Change with respect to the carcass tissues) of *H. armigera* midgut from L2, L3 and L4 instar larvae, as defined by the transcriptomic dataset. B) Functional analysis of the transcriptional activity of transcriptomic (Ha_110377047, Ha110384513, Ha110379024, Ha110380915 and Ha110370923) and literature (HaLabial, HaTsp-2A, HaPtx-I,HaCaudal and HaMucin-1) driven promoters in RP-Hz-GUT-AW1 cells. Fold change luciferase activity is expressed as ratio of the Relative Luciferase Activity (RLA) of each promoter against the RLA of promoterless vector and values are expressed as means with 95% CIs. The fold change luciferase activity for each promoter was compared against the promoterless vector (negative control) and data were analyzed with one-way ANOVA followed by post-hoc Dunnett test. Different replicates are represented as individual dots. ****: p-value<0.001, ns: not significant.

3.3.2 Functional Analysis of the Midgut-Specific Promoters

A. Transcriptional strength

To characterize strong midgut promoters, we performed luciferase assays in the *H. zea* midgut cell line RP-HzGUT-AW1(MG) (Goodman et al., 2004). Although the 5 *Helicoverpa* midgut specific genes selected from the transcriptomic data had very high expression, in the luciferase assays the activity of their promoters was only between ~2 to ~7-fold higher than the promoterless vector (110384513, 110372923 and 110380915) or undetectable (110379024 and 110377047) (Figure 3.2B). On the contrary, with the exception of the *mucin-I*, the promoters of the other four genes selected from the literature (*HaLabial*, *HaTsp-2A*, *HaPtx-I and HaCaudal*), exhibited very high transcriptional activity ranging from ~150 to ~750-fold higher than the promoterless vector (Figure 1B).

B. Deletion analysis

To determine the minimum length necessary for high transcriptional activity of each strong promoter, we performed deletion analysis. Our results, presented in Figure 3.3, indicate that the 400-bp proximal region of the *HaTsp-2A* and

HaPtx-I promoters retained full activity. Full activity was also retained by the 1600bp proximal region of the *HaCaudal* promoter, while shorter fragments (800bp and 400bp) exhibited reduced activity (by approximately 33% and 45% respectively). In the case of the *HaLabial* promoter deletion analysis indicated that 1480bp fragment upstream of the start codon displayed almost similar activity with the 2000bp. Furthermore, deletion analysis suggested the presence of two positive regulatory elements within the sequence of *HaLabial* promoter: one is located within the 1480bp to 1170bp region, and another is located within the 597bp to 400bp region (numbers indicate distances upstream from the ATG initiation codon).

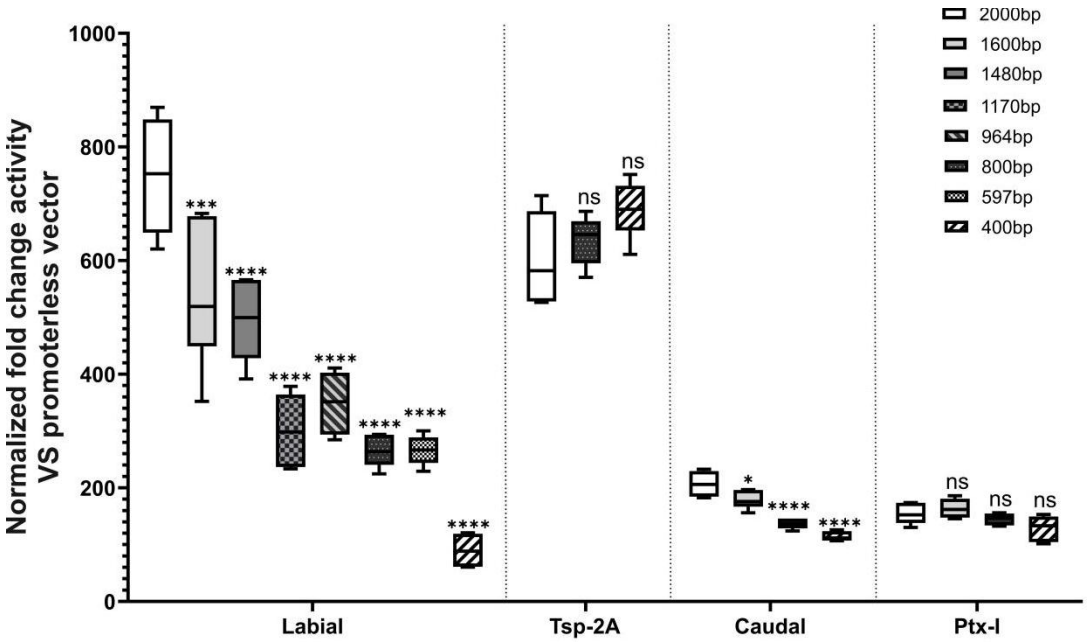

Figure 3.3. **Deletion analysis for the 4 highly active literature driven promoters of the genes *HaLabial*, *HaTsp-2A*, *HaCaudal* and *HaPtx-I* in RP-Hz-GUT-AW1 cells.** Fold change luciferase activity is expressed as ratio of the Relative Luciferase Activity (RLA) of each promoter against the RLA of promoterless vector. Deletions analysis data of each group (promoter) were analyzed by comparing the mutated promoters with the initial size, using one-way ANOVA followed by post-hoc Dunnett test. (Labial ***: p-value=0.0001, Caudal *: p-value=0.0124, ****: p-value<0.0001, ns: not significant).

C. Comparison of midgut promoters with ubiquitous Promoters

To evaluate the strength of the four midgut-specific promoters, we compared their activity with that of two versatile yet exogenous ubiquitous-acting promoters, the BmNPV IE1 and BmAc3 which are active in many lepidoptera cell lines (Tamura et al., 2000, Masumoto et al., 2017). Although the activity of the 4 midgut-specific promoters was >80% less compared with the BmAc3, they display significantly stronger activity than BmNPV IE1 (Figure 3.4A). Additionally, we checked the activity of the putative endogenous promoter of Ha*Ef1a*, which is highly expressed based on the transcriptomic data. Despite the high expression levels of this gene, its promoter seems to be less active with respect to the promoters of the genes *HaLabial*, *HaTsp-2A*, *HaCaudal* and *HaPtx-I*. Specifically the promoters of midgut enriched genes displayed approximately 4.7-23.1X greater activity than the 2000bp fragment upstream of the start codon of *HaEf1a* (Figure 3.4A).

D. Activity of HaLabial, HaPtx-I, HaCaudal and HaTsp-2A promoters in other insect cell lines

In order to analyze the evolutionary conservation of the four strong promoters, we functionally characterized them in the non-midgut lepidopteran cell lines Hi-5 and Sf-9. The luciferase assays showed that *HaLabial*, *HaPtx-I* and *HaCaudal* promoters exhibit low to moderate activity in these cell lines, ranging from 5-22 fold in Hi-5 and almost 25 fold in Sf-9 cells (Figure 3.4B). However, HaTsp-2A was not functional in either Sf-9 or in Hi-5 cells. To explain why some of these promoters are functional in other insect cells of different species and tissue origin, we compared their sequence with the putative promoters (~2kb upstream of start codon) of their orthologs from five insect species (*Trichoplusia ni*, *Spodoptera litura*, *Spodoptera frugiperda*, *Bombyx mori* and *Drosophila melanogaster*). Multiple sequence alignment of the *HaLabial* putative promoter with the respective putative promoters of *SlLabial*, *SfLabial* and *TnLabial* indicated an overall sequence identity of ~52% throughout the 2000bp sequence (Table 3.2), with the region -800bp to exhibit more than 70% homology (Samantsidis et al., 2022). Furthermore, the sequence alignments for the *HaPtx-I* and *HaCaudal* promoters indicated a sequence identity of approximately 35% and 45% respectively with the promoters of their orthologs in *S. litura*, *S. frugiperda* and *T. ni* (Table 3.2). The low activity in cell lines derived from different lepidoptera species conforms with the partial sequence conservation of the promoters and is probably explained by conserved regulatory elements found in the first 800bp of the promoters (Samantsidis et al., 2022). Sequence identity analysis for *Tsp-2A* putative promoters indicated very high conservation among the lepidopteran species *Helicoverpa*, *Trichoplusia* and *Spodoptera* (72% and 76% identity of *Helicoverpa* with *Trichoplusia* and *Spodoptera* respectively, Table 3.2). However, functional analysis of the *HaTsp-2A* promoter in Hi-5 and Sf-9 cells showed that promoter is not active, raising the possibility that this promoter is active only in midgut cells and thus acting as a midgut specific promoter (Figure 3.4B).

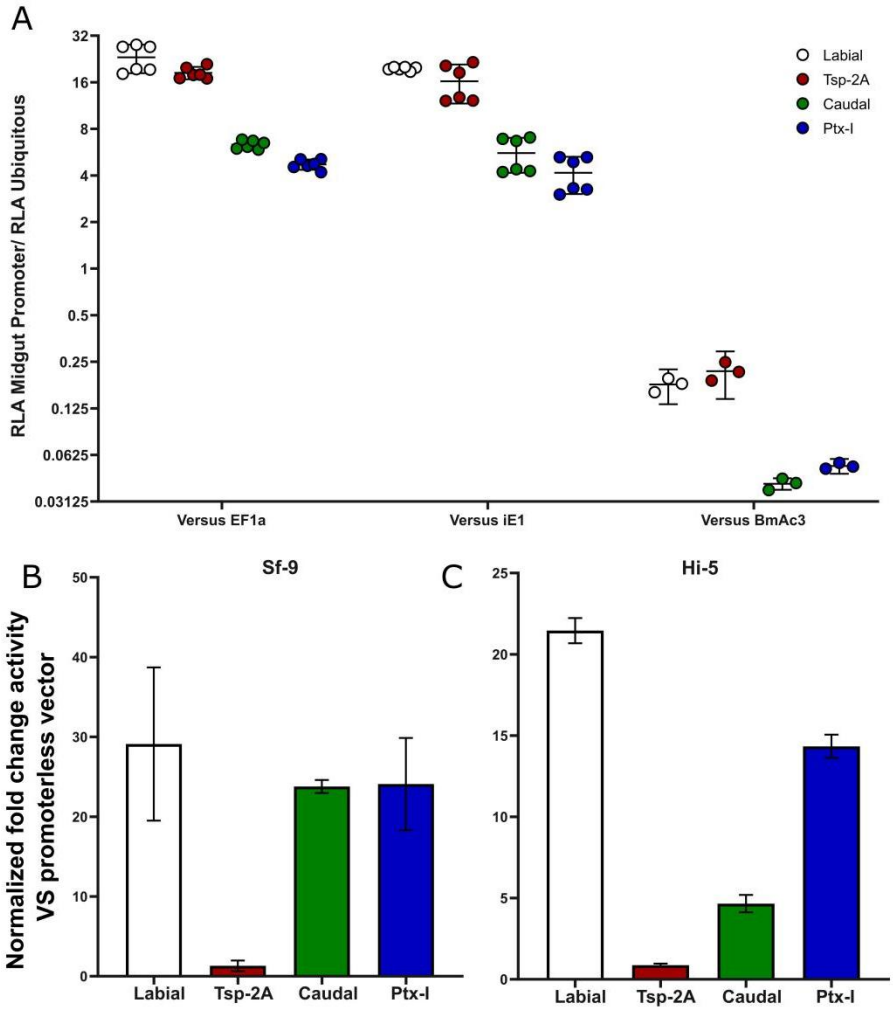

Figure 3.4. Characterization of the 4 highly active literature driven promoters. A) Comparative analysis of the activity of *HaLabial*, *HaTsp-2a*, *HaCaudal* and *HaPtx-I* promoters with the activity of constitutive promoters (HaEF1a, BmNPV IE1 and BmAc3) in RP-Hz-GUT-AW1 cells. The RLA of each Ha Midgut specific promoters was divided with the RLA of the ubiquitous-acting promoters. B,C) Conservation analysis of the 4 promoters. The promoters were analyzed for their activity in B) Sf-9 and C) Hi-5 cell lines. Normalized fold change activity is expressed as ratio of the Relative Luciferase Activity (RLA) of each promoter against the RLA of promoterless vector. Data were analyzed for significance with one-way ANOVA among groups followed by post-hoc Tukey test. The p-values per comparison are summarized in the Table 3.3.

3.3.3 Identification and functional analysis of *H. armigera* U6 promoters

Five different copies of the U6 snRNA gene were identified with 100% identity in the *H. armigera* genome, localized in three different scaffolds. The U6 promoters (~500bp upstream of U6 TSS) were named numerically arbitrarily as follows, U6:1 (scaffold 14:457024-457588), U6:2 (scaffold 263:211789-212407), U6:2a (scaffold 263:206977-207533), U6:3 (scaffold 62:261765-262314) and U6:3a (scaffold 62:314253-314733). Of these five promoters, four were successfully amplified with PCR. Alignment of the four sequenced *H. armigera* U6 promoters with four functional U6 promoters of other lepidoptera species suggested that the 5' half of the PSEA seemed to be conserved (Figure 3.5A). No distinct TATA box motifs were identified in the sequences of the *H. armigera* U6 promoters, whereas the sequences of *B. mori*, *S. frugiperda* and *P. xylostella* U6 promoters contain this motif ~13 bp downstream of the PSEA (Figure 3.5A). Despite the fact that the existence of TATA box motifs is a parameter for selecting functional U6 promoters (Hernandez et al., 2007), we proceeded to their characterization by testing their ability to drive shRNAs for RNAi and sgRNAs for CRISPR in the RP-Hz-GUT-AW1 cell line.

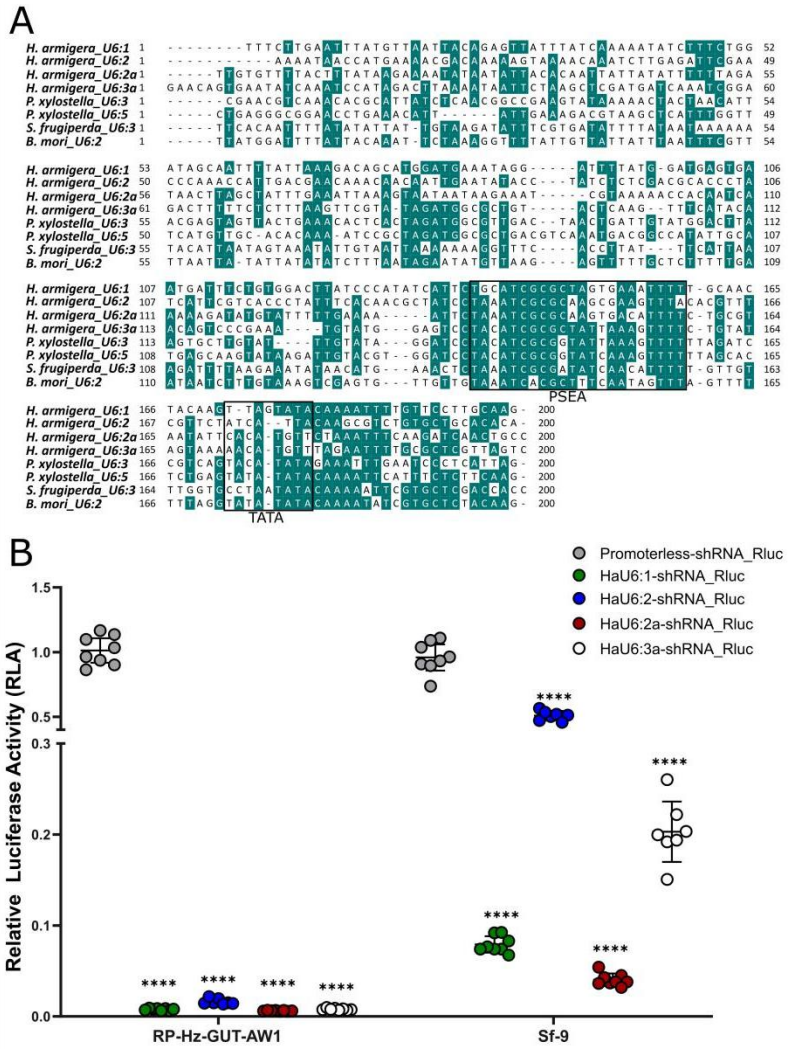

Figure 3.5. Identification of *H. armigera* U6 promoters and functional analysis of their silencing efficiency. A) Sequence alignment of 200bp upstream of the TSS of *H. armigera* U6 snRNA gene with the respective regions of *P. xylostella*, *S. frugiperda* and *B. mori* U6 promoters. Dark green highlights more than 60% sequence similarity. Sequences in frames highlight the regulatory sequence PSEA and TATA-box. B) Functional characterization of the silencing activity of the 4 *H. armigera* U6 promoters by analyzing the expression of shRNAs targeting *Renilla* luciferase in RP-Hz-GUT-AW1 and Sf-9 cells. Relative Luciferase Activity is calculated as ratio of the normalized RLUs of *Renilla* of the experimental condition against the normalized RLUs of *Renilla* obtained by the U6 promoterless expression vector (negative control). Multiple comparison among different groups was performed using one-way ANOVA with post-hoc Tukey test. The p-values are summarized in Table 3.3.

According to the luciferase assay, in which *Renilla* luciferase was targeted by shRNA produced by each of the four HaU6 promoters, all of the *Helicoverpa* U6 promoters were functional. More than 98% reduction of luminescence was observed for all of the four U6 promoters tested in RP-Hz-GUT-AW1, compared to the negative control which contains only the sequence for shRNA against *Renilla* (Figure 3.5B). Analysis of these promoters in Sf-9 cells has indicated a statistically significant reduction of *Renilla* luciferase expression mediated by HaU6:1 and HaU6:2a (92% and 96% of inhibition respectively). Additionally, we tested the ability of the U6 promoters for expression of sgRNAs by targeting GFP in a plasmid expressing a Cas9-GSG-P2A-mCherry cassette under either BmAc3 or iE1 promoter (Figure 3.1). Significant reduction of the GFP fluorescence intensity was observed for all of the *Helicoverpa* U6 promoters, when Cas9 was expressed under BmAc3 promoter. Specifically, sgRNA targeting the 5' region of GFP sequence exhibited almost complete loss of signal (Figure 3.6A). The efficiency of GFP knock-out was less in the case of HaU6:2a promoter, since a small amount of GFP protein could still be detected with specific antibody, whereas it was almost absent when HaU6:1 and HaU6:3a promoters were used (Figure 3.6B). Less efficient,

yet effective was Cas9 when expressed under IE1 regulatory elements leading to lower reduction of GFP compared to BmAc3, which is attributed to the lower expression of the endonuclease, as it is indicated by Western-blot analysis (Figure 3.6B).

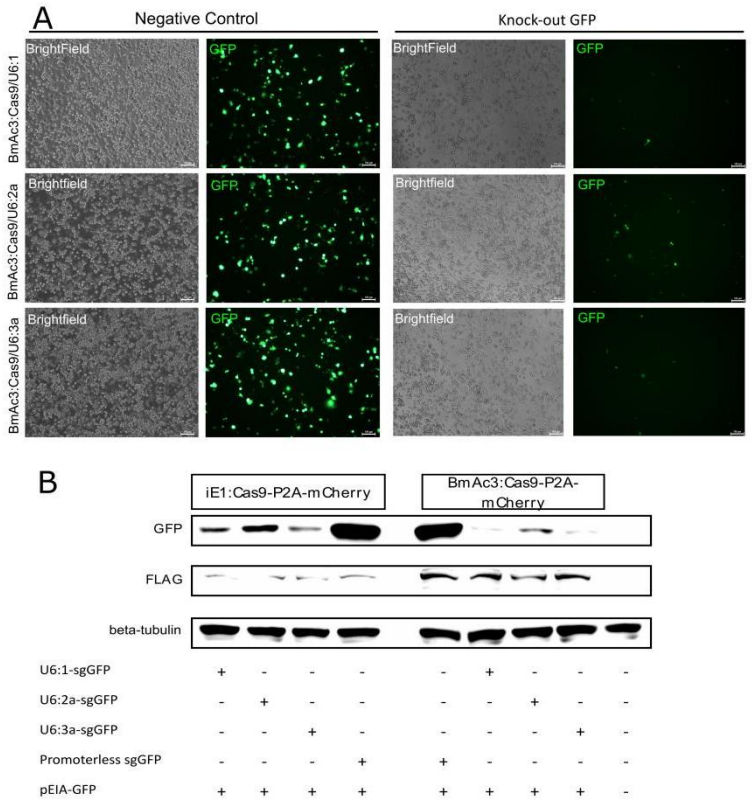

Figure 3.6. Functional analysis of *H. armigera* U6 promoters in a proof of principle CRISPR mediated knock-out . A) Knock-out of GFP via CRISPR/Cas9 in the RP-Hz-GUT-AW1 cells using *H. armigera* U6 promoters carrying CRISPR vectors. The RP-Hz-GUT-AW1 cells were transiently transfected with pEIA-GFP and a CRISPR vector (BmAc3:Cas9-mCherry). As negative control a CRISPR vector without sgRNAs was used. Scale bars, 100uM. B) Western-Blot analysis of CRISPR against GFP (~27kDa) in RP-Hz-GUT-AW1 cells analyzing the efficiency of the vectors at a protein level. Both IE1 and BmAc3 promoters were tested for their efficiency in driving the expression of Cas9-P2A-mCherry. Beta-tubulin (~55kDa) was used as a loading control. Cas9 (~160kDa) which is tagged in the N-terminus with a 3X FLAG tag was detected using anti-Flag. Non-treated cells were used as a second negative control.

3.4 Discussion

The midgut of pests like *H. armigera* and *H.* zea is the site of nutrient digestion and absorption, as well as other biological processes like xenobiotic metabolism and parasite interactions (Denecke et al., 2018, Caccia et al., 2019). The difficulty in studying many of these processes *in vivo* has led to significant effort towards generating midgut derived cell lines for exploration of midgut like properties *in vitro*. In particular, the further characterization of midgut derived cell lines such as RP-Hz-GUT-AW1 (Vorgia et. al 2021), has set the stage for its use in future studies, but a lack of genetic tools in this species precludes its widespread use. Here, we characterized *Helicoverpa* Pol II and Pol III promoters in the RP-Hz-GUT-AW1 cell line in order to provide systems for precisely regulating gene expression in this cell line.

RNA polymerase II promoters

Analysis of promoters derived from five of the most highly larval midgut up-regulated genes indicated these genes show low transcriptional activity in the midgut derived cell line of *H. zea* RP-Hz-GUT-AW1 (Figure 1B). This is in accordance with a recent work in which the putative promoter of *S. frugiperda acpp1*, along with other putative promoters of highly expressed genes of the midgut, have been found to be also non-functional in the midgut derived cell line Sf17 (Chen et al., 2020b). A reasonable explanation could be that the activity of these promoters is probably governed by tissue specific enhancers localized in genomic regions further than the ~2kb sequence upstream of the start codon. Another explanation could be that high levels of expression of those genes are caused by high mRNA stability, as has been reported previously in an analogous study (Bleckman et al., 2015).

Furthermore, it needs to be considered that, while the RP-HzGUT-AW1(MG) cell line was derived from midgut tissue (Goodman et al., 2004), considerable de-differentiation may have occurred during adaptation to *in vitro* culture, as is typically observed for insect cell cultures (Swevers et al., 2021). Genes that are associated with highly specialized functions such as secreted proteases, cytoskeleton elements that maintain the columnar epithelial structure, and microvilli are expected to become turned off when cells start dividing and become a continuously multiplying population. The low activity of such promoters is therefore not unexpected and may illustrate the relatively low differentiation status of the employed midgut-derived cell line. This is supported by the transcriptomic analysis of RP-HzGUT-AW1(MG) cells, which has revealed the expression of specific genes that are suggestive of an "arrested" differentiation status between Intestinal Stem cells (ISCs) and fully differentiated cells (Vorgia et al., 2021). Further research efforts are required to establish the mechanisms by which (at least partial) differentiation can be induced in insect midgut-derived cell lines (Swevers et al., 2021).

The putative promoters of four *Helicoverpa* genes, that were characterized as midgut enriched in other insect species, yielded very high levels of transcriptional activity in RP-HzGUT-AW1 cells. Among the four, the promoters of *Tsp-2A* and *Labial* genes exhibited the highest activity, but HaTsp-2A seems to be especially interesting, since it was not active in cell lines that are not related with midgut tissue origin (Sf-9 and Hi-5). Given the conservation of HaTsp-2A promoters among *Helicoverpa*, *Spodoptera* and *Trichoplusia* species (Table S2), a possible explanation for the low activity of HaTsp-2A could be the existence of tissue specific *cis*-acting elements in the promoter sequence, limiting its activity only in midgut derived cells. Further analysis of its activity either in a non-midgut derived cell line of *Helicoverpa* or through *in vivo* validation would be necessary to establish these promoters as truly midgut specific.

Several tissue specific promoters have been identified in the silkworm *B. mori*, such as the germ-line specific promoter nanos (Xu et al., 2019), the middle-silk gland Ser1 promoter (Liu et al., 2006, Ye et al., 2015), the midgut specific APN4 promoter (Jiang et al., 2015) and the fat body specific Bmlp3 promoter (Deng et al., 2013). The latter was analyzed with luciferase assay in two different cell lines and found to exhibit ~80 to ~140-fold stimulation of transcriptional activity, but also was tested *in vivo* and found to drive high levels of RFP expression (Deng et al., 2013). This would suggest that the four strong promoters identified in this study which displayed similar activity with Bmlp3, could also be used in the future for *in vivo* transgenics. To further validate the strength of these promoters, we analyzed them in comparison with already known constitutive promoters, like BmNPV-IE1 and BmAc3. Although BmNPV-IE1 has been used in many previous works as a strong ubiquitous-acting promoter (Fujita et al.,

2015,Masumoto et al., 2012), its activity seems to be statistically significantly less than *HaLabial*, *HaTsp-2A*, *HaPtx-I* and *HaCaudal* promoters in RP-Hz-GUT-AW1 cells. On the contrary, BmAc3 exhibited much higher activity. Even though very strong promoters are generally preferred in the field of transgenesis, increased levels of protein expression mediated by a very strong promoter could lead to cellular stress or even lethality. This has been reported *in vivo* by Okamoto and co-workers, by observing embryonic lethality when OATP74D protein was expressed ubiquitously in *Drosophila* using Act5C-gal4 and daughterless-gal4 driver lines, while less expression by the weaker driver line armadillo-gal4 was viable in all life stages (Okamoto et al., 2018). Similarly cellular stresses could also be observed in cell cultures (in either transient or stable transfections) by using very strong promoters that lead to high levels of protein expression affecting downstream experimental procedures. Using promoters of milder activity could circumvent this limitation. Hence, the weaker yet active promoters of this study might be an excellent tool for balanced expression and suitable for *in vitro* studies and presumably *in vivo* use.

RNA polymerase III promoters

The spliceosomal snRNA U6 promoters are exceptionally interesting, since they are required to drive the expression of small RNAs like sgRNAs and shRNAs. We showed that all of the U6 promoters analyzed are capable of mediating both RNAi (Figure 4B) and CRISPR-Cas9 (Figure 5) with high efficiency. The first report of insect U6 promoters used in CRISPR-Cas9 was in *Drosophila melanogaster* (Gratz et al., 2013). Since then, several U6 promoters of non-model insect species have been identified and used in the context of CRISPR such as mosquitoes (Anderson et al., 2020), *Plutella xylostella* (Huang et al., 2018), *Spodoptera frugipeda* (Mabashi-Asazuma et al., 2017) and *Drosophila suzukii* (Ahmed et al., 2019). Our analysis indicated that HaU6:1 and HaU6:3a were able to reduce GFP by more than 90%, while HaU6:2a promoter was slightly less efficient (Figure 5B). Differential cleavage efficiency mediated by the same sgRNAs expressed by different U6 promoters has been reported in *P. xylostella* and *D. melanogaster*, in which only PxU6:3 and DmU6:3 promoters respectively, were found to exhibit the highest activity (Huang et al., 2017, Port et al., 2014).

Similarly, high efficiency of HaU6 promoters (>98%) was displayed when shRNA targeting *Renilla* luciferase was tested in RP-Hz-GUT-AW1 (Figure 5C), which compares favorably to reports using *B. mori* and *P. xylostella* cell lines (Tanaka et al., 2009, Huang et al., 2017). Strikingly, luciferase assays indicated that HaU6:1 and HaU6:2a promoters displayed 92% and 95% reduction of *Renilla* luciferase expression in Sf-9 cells. This is probably suggestive that their function is not species specific, which is supported by a similar study in which *Spodoptera* U6 promoter was shown to be functional in Hi-5 and BmN cell lines (Mabashi-Asazuma et al., 2017). However, the SfU6 promoter was found to be the only functional promoter in Sf-9 cells while homologous promoters of *B. mori*, *T. ni* and *D. melanogaster* were not (Mabashi-Asazuma et al., 2017). This possibly broadens the application and utility of *Helicoverpa* U6 promoters in other cell lines. Moreover, generating the Cas9-P2A-mCherry (Figure S2) facilitates screening procedures, especially when it comes with cell lines which can be used in combination with Fluorescence-Activated Cell sorting (FACS) for establishing clonal knock-out cells (Fu et al., 2018). Further, *in vivo* analysis of HaU6 promoters indicated their functionality in *H. armigera in vivo* (Figure S5); to the authors' knowledge this is the first use of plasmid based CRISPR vectors in this species. Overall, based on our results we suggest that these constructs

carrying either HaU6:1 or HaU6:3a, in combination with BmAc3 promoter driving the expression of Cas9-P2A-mCherry, can be used for genetic manipulation *in vitro* and *in vivo*.

Tables - Chapter 3

Table 3.1 Primers used in this study.

Name	Sequence (5'-3')	Used for
FFluc_AscI_F	GTACGGCGCGCCATGGAAGACGCCAAAAACATAAAG	
FFluc_SpeI_R	GTACACTAGTATCCAGACATGATAAGATAC	
Ha_Tsp2A_FseI_F	GTACGGCCGGCCTCTTGGTTCGTGTATTAAGGTTGAC	
Ha_Tsp2A_AscI_R	GTACGCGGCCGCCGCACTTTAAAAACACTTTACAACAC	
Ha_Caudal_FseI_F	GTACGGCCGGCCGGTCCCTAAGTTCCTCTACTCATG	Literature Driven Promoters
Ha_Caudal_AscI_R	GTACGCGGCCGCATCACCGTCCGAGCATCG	
Ha_Labial_FseI_F	GTACGGCCGGCCTTGACACCATTGACTGTTTCGGA	
Ha_Labial_AscI_R	GTACGCGGCCGCGTTTGTCACGATCACCGTGTCTG	
Ha_PtxI_FseI_F	GTACGGCCGGCCATTGTTCTAAGCGTCCGGTGT	
Ha_PtxI_AscI_R	GTACGCGGCCGCACTTTGACAGTGGACATACTCACAG	
Ha_110384513_FseI_F	GTACGGCCGGCCTGAGAAAAACTTATTACGAGGGATACG	
Ha_110384513_AscI_R	GTACGCGGCCGCACTTAGCTCTACTTCAGGCAAAC	
Ha_110372915_FseI_F	GTACGGCCGGCCGTGTCCTCATGTCCTCAGACC	
Ha_110372915_AscI_R	GTACGCGGCCGCTTTGCTGTAACAAGAGAAAAATTATTGTTT	
Ha_110372923_FseI_F	GTACGGCCGGCCAACAAAAGCTGAAAGACAATGATGT	
Ha_110372923_AscI_F	GTACGCGGCCGCAGAGGCGATGACGAACAAGG	
Ha_110377047_FseI_F	GTACGGCCGGCCAACCCATGCTTCCGATTGC	Transcriptomic Derived Promoters of 5 most-highly up-regulated genes
Ha_110377047_AscI_R	GTACGCGGCCGCTGGTATTCCTACACTTTGTCAACTG	
Ha_110377024_FseI_F	GTACGGCCGGCCATGCGTGTGGGGTAAACAG	
Ha_110377024_AscI_F	GTACGCGGCCGCTAGGAAGCCATTGTAGTTCTGC	
Ha_110381636 _FseI_F	GTACGGCCGGCCCTCCTCTGAAGTCAGCTATTTGAC	
Ha_110381636 _AscI_R	GTACGCGGCCGCCTGGAAGACAAAATAGTGTACTGATGAG	
Ha_110381625 _FseI_F	GTACGGCCGGCCAAGCACCAACCGACAGAACA	
Ha_110381625 _AscI_R	GTACGCGGCCGCTAAGTCCCGTGGAGGTGTCC	
Ha_110370997 _FseI_F	GTACGGCCGGCCTACCTGGTTTAACAGAGAGTACCTC	
Ha_110370997 _AscI_R	GTACGGCGCGCCATGGCTCCCACACTCAAGACG	
Ha_Caudal_FseI_F_1900	GTACGGCCGGCCATGTCTTCTTCATGTCACCTTCAG	
Ha_Caudal_FseI_F_1500	GTACGGCCGGCCTAACGGAAGCTATTTCTAAGTGTTAAG	
Ha_Caudal_FseI_F_800	GTACGGCCGGCCACTAGGTACGATACCATAGACTGATAC	
Ha_Caudal_FseI_F_400	GTACGGCCGGCCAGACAGATTCATCATTTCGTTCATAAAC	
Ha_PtxI_FseI_F_1500	GTACGGCCGGCCCTAACTCCACCACTCTATACATCATC	
Ha_PtxI_FseI_F_1200	GTACGGCCGGCCACTTCTGCCCGTGAAGTGTG	
Ha_PtxI_FseI_F_800	GTACGGCCGGCCACAACGCCATAATTGCCTTCATT	Deletion Analysis
Ha_PtxI_FseI_F_400	GTACGGCCGGCCCGACGGATACATGAATGTTCTTTAGA	
Ha_Lab_FseI_F_1600	GTACGGCCGGCCAGACGATGTCAGTCACAG	
Ha_Lab_FseI_F_800	GTACGGCCGGCCAGTACAAAACCAGTAGGCGATAC	
Ha_Lab_FseI_F_400	GTACGGCCGGCCACGAAATCATCTACCGTGACTAC	
Ha_Tsp2A_FseI_F_800	GTACGGCCGGCCACCAGACGACTGTAGAATTTTCAC	
Ha_Tsp2A_FseI_F_400	GTACGGCCGGCCCTGAGATCGTTCTCTCGTATG	
Ha_EF1a_FseI_F	GTACGGCCGGCCTCTTTGTTCTCGGCAATTTTCTACAT	Amplification of Ef1a promoter of *H. armigera*
Ha_EF1a_AscI_R	GTACGGCGCGCCGCCTCGCCCTATCTTGACATTT	
Rluc_BamHI_F	GTACGGATCCATGGCTTCCAAGGTGTACGAC	Construction of *Renilla* luciferase Vector
Rluc_NotI_R	GTACGCGGCCGCCACACAAAAAACCAACACACAGATG	
shRNA_RlucF_NotI	GGCCGCCTACGAGCACCAAGACAAGAATGTGCTGTCCTCTTGTCTTGGTGC TCGTAGGTTTTT	Construction of shRNA targeting *Renilla* luciferase
shRNA_RlucR_XhoI	TCGAGAAAAACCTACGAGCACCAAGACAAGAGGACAGCACATTCTTGTCT TGGTGCTCGTAGGC	
gRNA_scaffold_F_NotI	GTACGCGGCCGCGAAGACCTGTTTTAGAGCTAGAAATAGCAAGTTAAAAT AAGGCTAGTCCGTTATCAACTTG	Generation of a BbsI-gRNA scaffold
gRNA_scaffold_R_XhoI	GTACCTCGAGCTCTGTACAAAAAAAGCACCGACTCGGTGCCACTTTTTCAA	

	GTTGATAACGGACTAGCCTTA	
P2A_Forward_HindIIIPhospo	AGCTTGGCAGCGGCGCTACTAACTTCAGCCTGCTGAAGCAGGCTGGAGACGTGGAGGAGAACCCCGGCCCTG	Generation of a GSG-P2A dsDNA
P2A_Reverse_NheIPhopho	CTAGCAGGGCCGGGGTTCTCCTCCACGTCTCCAGCCTGCTTCAGCAGGCTGAAGTTAGTAGCGCCGCTGCCA	
IE1_Promoter_AgeI_F	GTACACCGGTGATTTGCAGTTCGGGACATAAATG	Amplification of BmNPV-IE1 promoter
IE1_Promoter_BamHI_R	GTACGGATCCAGTCGTTTGGTTGTTCACGATC	
BmAc3_Promoter_AgeI_F	GTACACCGGTGGAGCTCTTACACCCATCTAC	Amplification of BmAc3 promoter
Bmac3_Promoter_BamHI_R	GTACGGATCCTCGATACCGTCTACTACCAAC	
Ha_U6:1_BamHI_F	GTACGGATCCTTCAAAACCTATCGCAAAACTTACAAT	Amplification of U6 promoters
Ha_U6:2_BamHI_F	GTACGGATCCATTACCATTCCTTTTGCAGTTCA	
Ha_U6:2a_BamHI_F	GTACGGATCCAGAAACAAGGCTGTGCCTACA	
Ha_U6:3_BamHI_F	GTACGGATCCTTACAAAGCGGGTCAGATATGC	
Ha_U6:3a_BamHI_F	GTACGGATCCATGCGGAGGTTTAACAGTTTCG	
Ha_U6_generic_R	CGATTTTGCGTGTCATCCTTG	Reverse generic primer for sequencing of the U6 promoters
Ha_U6:1_BbsI_NotI_R	GTACGCGGCCGCGAAGACCACTTGCAAGGAACAAAATTTTGTATAC	Amplification of U6 promoters with BbsI site for cloning sgRNAs in the final CRISPR vector
Ha_U6:2a_BbsI_NotI_R2	GTACGCGGCCGCGAAGACCCGGCAGTTGATCTTGAAATTTAGAACAT	
Ha_U6:2_BbsI_NotI_R2	GTACGCGGCCGCGAAGACCATGTGTGCAGCACAGACG	
Ha_U6:3a_BbsI_NotI_R2	GTACGCGGCCGCGAAGACCCGACTAACGAGCGCAAAATTCTAAACATG	
sgRNA_GFP_R	AAACTCAGCTCGATGCGGTTCACC	Primers for generation of dsDNAs for gRNA targeting GFP
sgRNA_GFP_F_U6:1	CAAGGGTGAACCGCATCGAGCTGA	
sgRNA_GFP_F_U6:2a	TGCCGGTGAACCGCATCGAGCTGA	
sgRNA_GFP_F_U6:3a	AGTCGGTGAACCGCATCGAGCTGA	
sgRNA_OATP74D_U62aF	TGCCGTGTCCCTACTGTATCATCAG	Primers for generation of dsDNAs for gRNA targeting *HaOatp74D*
sgRNA_OATP74D_U63aF	AGTCGTGTCCCTACTGTATCATCAG	
sgRNA_OATP74D__U61F	CAAGGTGTCCCTACTGTATCATCAG	
sgRNA_OATP74D_R	AAACCTGATGATACAGTAGGGACAC	

Table 3.2. Percent sequence identity of each promoter (Labial, Caudal, Ptx-I and Tsp-2A) among *H. armigera*, *T. ni*, *S. litura*, *S. frugiperda*, *B. mori and D. melanogaster* calculated using BioEdit Software.

		Caudal					
	Species	***H.armigera***	***T.ni***	***S.litura***	***S.frugiperda***	***B.mori***	***D.mel***
Labial	*H.armigera*		52.79	56.71	55.73	42.27	34.68
	T.ni	41.92		52.81	51.63	41.13	30.95
	S.litura	49.71	43.5		64.42	42.36	34.25
	S.frugiperda	50.75	43.4	67		40.98	34.43
	B.mori	40.09	35.53	39.94	38.7		31.39
	D.mel	31.22	27.03	31.26	31.93	30.86	

		Tsp-2a					
	Species	***H. armigera***	***T.ni***	***S.litura***	***S.frugiperda***	***B.mori***	***D.mel***
Ptx-I	*H.armigera*		35.86	35.31	35.29	35.97	30.24
	T.ni	72.5		74.52	74.18	61.31	36.46
	S.litura	76.07	69.73		91.33	62.35	35.73
	S.frugiperda	76.63	69.06	90.03		61.65	35.39
	B.mori	39.48	40.45	39.36	39.75		34.08
	D.mel	33.1	31.2	30.84	31.91	31.32	

Table 3.3. Adjusted p-values (One-way ANOVA post-hoc Tukey analysis) for: A) conservation analysis of the four promoters; below the diagonal indicate the comparisons for the figure 3B (Sf-9) while above the diagonals refer to the Figure 3C (Hi-5), and B) for functional analysis of silencing activity of *H. armigera* U6 promoters (Figure 4B).

A. Adjusted p-values from promoters conservation analysis				
Promoter	**HaLabial**	**HaTsp-2A**	**HaPtx-I**	**HaCaudal**
HaLabial		<0.0001	0.0001	<0.0001
HaTsp-2A	0.0332		<0.0001	0.0086
HaPtx-I	0.9195	0.0805		<0.0001
HaCaudal	0.9059	0.085	>0.9999	

B. Adjusted p-values from silencing activity of U6 promoters					
Promoter	**promoterless**	**HaU6:1**	**HaU6:2**	**HaU6:2a**	**HaU6:3a**
promoterless		<0.0001	0.0001	<0.0001	<0.0001
HaU6:1			0.9976	>0.9999	>0.9999
HaU6:2				0.9954	0.9978
HaU6:2a					>0.9999
HaU6:3a					

3.5 References

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Chapter 4

"Functional Characterization of two lepidoptera pests OATP74D as a putative ecdysone transporter and insecticide target"

Samantsidis, G.R., Fotiadou, M., Tzavellas, S., Giebel, S., Nauen, R., Swevers, L., Denecke, S. and Vontas, J. (2022) Functional characterization of two lepidoptera pests OATP74D as an ecdysone transporter and a putative insecticide target. Manuscript in preparation

Authors' Contributions

S.D and J.V conceived the project, S.D. and G-R.S designed the experiments, G-R.S performed the CRISPR in cell lines and generated stable cells, G-R.S and S.T. performed the luciferase assays in cell cultures, G-R.S performed the gene expression, cell death analysis and immunofluorescence in cell lines, S.D. and M.F. performed the phylogenetic analysis, M.F. performed the CRISPR in *S. frugiperda*, G-R.S, S.D and J.V analyzed the results. G-R.S, M.F, S.D and J.V. wrote the manuscript. The manuscript has been revised and accepted by all authors.

Abstract

In this chapter we opted to functionally characterize the role of the Organic anion transporting polypeptide 74D (Oatp74D), an essential for *Drosophila* development ecdysone transporter, in the two major lepidopteran pests *Helicoverpa armigera* and *Spodoptera frugiperda*. Using *in vivo* and *in vitro* approaches we surveyed the essentiality of the protein in animal development and survival but also we surveyed the actual role of HaOATP74D and SfOATP74D in the ecdysone pathway in order to address if these proteins function as ecdysone transporters. Using CRISPR/Cas9 technique to knock-out the *SfOatp74D*, we observed a significant decrease of egg hatching and larval survival with respect to the negative control. To further characterize the physiological role of the lepidoptera OATP74D, we knocked-out the *Oatp74D* gene in the RP-HzGUT-AW1(MG) cell line (hereafter referred as HzAW1). In particular, we have observed that *Oatp74D* of the HzAW1 is necessary for the transcriptional regulation of certain ecdysone responsive genes. Additionally, we observed that knock-out of the gene renders cells rather resistant to ecdysone since treatment with high concentrations of 20-hydroxyecdysone did not induce the upregulation of caspase-3 and hence apoptotic cell death. Furthermore, we proved that both HaOATP74D and SfOATP74D are sufficient for ecdysone dependent gene transcription and their activities are inhibited by Rifampicin, a well known organic anion transporter inhibitor. Taken all together, we have characterized the lepidopterab orthologs' of *DmOatp74D* function as ecdysone transporters and our data indicate that these proteins could be used as putative insecticide targets.

4.1 Introduction

Steroid hormones are molecules acting as chemical cues governing and coordinating several biological processes in animal physiology, metabolism and development. In hemi- and holometabolous insects the most critical and well studied steroid hormone is ecdysone, which acts as a critical regulator of development, controlling insects' molting, growth, metabolism and reproduction (Yamanaka et al., 2013). Ecdysone, as a typical steroid hormone, acts either by membrane receptors, which lead to the initiation and modulation of signaling transduction pathways, predominantly by their cognate nuclear receptors, which act as transcription factors to selectively regulate target gene expression (Sever and Glass 2013, Norman et al., 2004).

During insects' life cycle, precisely controlled and tightly regulated ecdysone pulses (of either low or high titers) occur in order to regulate transitions between developmental stages, beginning from the egg hatching stage until pupation (Lavrynenko et al., 2015, Ou et al., 2016). Ecdysone is produced in the insect prothoracic gland and released into the larval hemolymph (Yamanaka et al., 2013) to be activated by a monooxygenase into its activated form, the 20-Hydroxyecdysone (20-HE) (Feyereisen et al., 2012). The hormone is then incorporated into the target cells and initiates a complex signaling pathway, by binding to the nuclear receptors Ecdysone Receptor (EcR) and Ultraspiracle (USP) which form a heterodimeric transcription factor that initiates a gene expression cascade (Yamanaka et al., 2013, Zhao et al., 2020). Among a large number of tissue specific genes implicated in insect development and regulated by ecdysone, there is a core set of ubiquitously expressed transcription factors induced by the steroid hormone (Truman et al., 2019). These are the early response genes such as the *Eip74A* and *Eip75B*, which in turn lead to the activation of the early late response genes like *HR3*, the zinc finger protein *Broad*, the nuclear receptor *Ftz-F1* and the *E93* (Broadus et al., 1999, Ruaud 2010). This regulatory hierarchy of genes respond to 20-HE and function as molecular determinants of developmental timing and amplification of the hormone signal in order to ensure successful molting and metamorphosis by initiating different and tissue specific-dependent biological processes (King-Jones and Thummel 2005).

In holometabolous insects a high titer of ecdysone that is released at the final larval stage is necessary for the development of adult structures. While it can promote differentiation and pattern specification through cell cycle regulation in imaginal tissues (Ninov et al., 2009, Nijhout et al., 2015, Guo et al, 2016, Chen et al., 2017), it can also initiate programmed cell death in certain larval tissues that will not be required in the adult stage. Secretion of ecdysone commits larvae to pupariation and cessation of growth by orchestrating processes such as proliferation, differentiation and cell death to ensure the proper development of insects. With regard to cell death, ecdysone regulates the proper activation of programmed cell death (autophagy and apoptosis) in obsolete larval tissues like abdominal muscles, midgut and salivary glands in holometabolous insects like *Drosophila melanogaster* (Cakouros et al., 2004, Zirin et al., 2013), *Bombyx mori* and *Helicoverpa armigera* (Romanelli et al., 2016, Tetamanti et al., 2019). Furthermore, ecdysone induced programmed cell death seems to be necessary for other tissues that undergo remodeling during larval-to-pupal transition, like fat body and certain types of neurons (Xu et al., 2020). Activation of autophagy and apoptosis related genes expression is tightly controlled by ecdysone induced transcription factors like EcR, BR-C, βFtz-F1, E75A and E75B (Xu et al., 2020). For example, the expression of the *Drosophila* caspase *dronc* is directly regulated by EcR transcription factor which binds to the promoter region of the gene (Cakouros et al., 2004),

while *Broader complex* (*BR-C*) has been shown to regulate the expression of the death activators *reaper* and *head involution* (*hid*) (Jiang et l., 2000). Studies in lepidoptera also indicated that ecdysone is involved in the regulation of both autophagy and apoptosis genes and blockage of the process compromises insects development by causing a severe delay in metamorphosis and lethality (Li et al., 2018, Chen et al, 2017).

Although a large part of the regulatory network of ecdysone signaling has been resolved, there was until recently limited knowledge about ecdysone transport mechanisms. For many years, a general theory for simple passive diffusion of steroid hormones prevailed, but this started to be rejected when genetic screens in *Drosophila* identified the presence of transporters that mediate the transport of ecdysone. E23, a member of the ATP-Binding Cassette G (ABCG) protein subfamily, mediates the export of ecdysone in order to regulate the concentration of the hormone into the target cells after executing its function (Hock et al., 2000). *Atet*, which also belongs to the ABC protein family, was detected in the prothoracic gland of *Drosophila* and was shown to be involved in importing ecdysone into vesicles which are released by calcium stimulated exocytosis to reach hemolymph (Yamanaka et al., 2015). Furthermore, additional work indicated that target cells use an active transport mechanism for ecdysone uptake (Neuman and Bashirulah 2018, Okamoto et al., 2018). In particular, Organic anion transporting polypeptide 74d (OATP74D), which belongs to the protein superfamily of Solute Carrier (SLC) transporters, was found to be critical for larval development in *Drosophila*, suggested by the larval arrest observed at the L1 stage when the gene was eliminated, a phenotype that resembled EcR loss of function (Okamoto et al., 2018, Okamoto et al., 2020). Furthermore, OATP74D was found to regulate the ecdysone signaling pathway and to be necessary for ecdysone dependent gene expression in cultured cells (Okamoto et al., 2018). It is noteworthy to mention that although three additional organic anion transporters mediating ecdysone import have been identified in *Drosophila*, they were dispensable compared to OATP74D (Hun et al., 2021). Ecdysone was suggested as substrate by different organic anion transporters, and this has been proved also in mosquitoes which lack an OATP74D ortholog (Hun et al., 2021). Another study in *Tribolium castaneum* showed that knockdown of the closely related OATP4C1 transporter led to developmental arrest during metamorphosis, a similar phenotype observed by targeting the EcR (Rosner et al., 2021). Considering that human OATPs have been shown to use hormones and other amphipathic organic compounds as substrates (Hagenbuch et al., 2013, Stieger et al., 2014), it could be suggested that the mechanism of cellular uptake of hormones via OATPs is conserved between insects and mammals. However, this requires further functional proof given that a) the SLCO transporter family differs significantly among species (Schafer et al., 2021) and b) the case of hormonal transport in insects has been functionally validated in *Drosophila* and more recently in mosquitoes (Hun et al., 2021), yet limited information exists for other insect species.

Helicoverpa armigera and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) are two major agricultural pests damaging several economically important cultivated crops around the world (Sparks 1977, Haile et al., 2021). Most of the control strategies employed to date rely on the use of microbial or small molecule insecticides which are administered orally during the larval stages (Haile et al., 2021, Paredes-Sanchez et al., 2021). Among the several existing compounds, those targeting insect development (such as insect growth regulators, IGRs) are preferred considering that they are less harmful for humans and other non-target species. IGRs include the EcR agonists which target the nuclear receptor in order to activate ecdysone signaling precociously, leading to developmental defects and finally death (Paredes-Sanchez et al., 2021). Although there are several reports regarding the developmental

role of ecdysone pathway in lepidoptera pests (Zhao et al., 2020), the knowledge about transport and cellular uptake of the steroid hormone in these species is limited.

Here, we have tried to analyze the role of the lepidopteran OATP74D as a putative ecdysone importer. In particular, employing a series of *in silico*, *in vitro* and *in vivo* experimental approaches we show that the OATP74D orthologs of *H. armigera* and *S. frugiperda* are responsible for ecdysone uptake by target cells and are necessary for regulating ecdysone receptor mediated gene expression. Additionally, *Oatp74D* is essential for lepidopteran development and survival. Overall, this work sheds more light on ecdysone uptake mechanisms across insect species and broadens our knowledge in the physiological roles of Organic anion transporters in the transportation of endogenous substrates.

4.2 Materials and Methods

4.2.1 Insects and cell lines

A *Spodoptera frugiperda* population was obtained from Bayer CropScience, maintained in the lab as a quarantine pest for several generations. The insects were reared at 24±1°C with a 16:8 hour photoperiod on a standard artificial food (based on corn flour).

Two different cell lines were used in this study in order to analyze the role of lepidoptera OATP74D in 20-HE transport. The Sf-9 cell line was obtained from Sigma and maintained as adherent culture in the insect serum free SF900 II SFM (Thermo Fisher Scientific) medium supplemented with 10% heat inactivated fetal bovine serum (FBS, GIBCO, Thermo Fisher Scientific) and 100U/ml of penicillin and 0.1mg/ml streptomycin. The *Helicoverpa zea* midgut cell line RP-HzGUT-AW1(MG) (referred hereafter as HzAW1) was generously given by Dr. Cynthia L. Goodman (Biological Control of Insects Research, U.S, Department of Agriculture, Agriculture Research Service). The cell line was routinely maintained as adherent culture in Excell 420 insect serum-free medium (Sigma Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO, Thermo Fisher Scientific) and 100U/ml of penicillin and 0.1mg/ml streptomycin. Both cell lines were kept in a humidified incubator at 27°C.

4.2.2 Plasmid construction

Plasmids for transient OATP74D over-expression in insect cell lines

The open reading frames of *SfOatp74D* (Gene ID: 118271297, 2109bp) and *DmOatp74D* (Gene ID: 39954, 2460bp) were PCR amplified using Phusion polymerase (NEB) from cDNA templates of 3rd instar larvae of *S. frugiperda* and adults of *D. melanogaster* respectively. The primer pairs used for PCR amplification were Sf-OATP74D-XbaI-F/Sf-OATP74D-NotI-R and Dm-OATP74D-XbaI-F/Dm-OATP74D-NotI-R (Table 4.1), respectively. The PCR reactions for both genes were performed as follows: 98°C for 30sec initial denaturation, followed by 30cycles of 98°C for 10sec, 63°C for 30sec, 72°C for 1min10sec, followed by final extension at 72°C for 5min. Both PCR products were purified with a PCR clean-up kit (Macherey-Nagel) according to manufacturer's instructions. Both fragments were cloned into the shuttle vector pGEM-T easy (Promega) and verified by Sanger sequencing. The *HaOatp74D* (2136bp) ORF was

synthesized *de novo* (Genscript, Piscataway, NJ) based on the alignment of both NCBI reference sequence and the *de novo* transcriptome assembly of *H. armigera* (Ioannidis et al., 2022). The newly synthesized sequence was subcloned between the BamHI and NotI restriction sites of pFastBac1 vector. The *SfOatp74D* and *DmOatp74D* were finally cloned in between the XbaI and NotI sites of the lepidoptera specific expression vector pBmAc3 (Samantsidis et al., 2022) while *HaOatp74D* was cloned between BamHI and NotI sites.

Plasmids for stable cell line generation

The pEIA vector (Douris et al., 2006) was modified with the Gibson assembly methodology in order to replace the BmNPV-IE1 ORF with Puromycin N-acetyltrasferase (PAC). The primers used to amplify the pEIA plasmid were pEIA-Fgibson and pEIA-Rgibson and the PCR reaction was performed using Phusion polymerase (NEB). The ORF of puromycin resistance gene was amplified using Phusion polymerase and pEA-PAC as a template (kindly provided by Dr. Luc Swevers, NCSR "Demokritos") and the primer pair used for the PCR reaction were PAC-Fgibson-AscI/PAC-Rgibson-NcoI (Table 4.1). Both primers introduce the restriction sites of the unicutters AscI and NcoI to facilitate cloning of any other gene of interest downstream of the BmNPV-IE1 promoter. Both PCR products were used for constructing the final vector with Gibson assembly Master Mix (NEB), according to the instructions of the manufacturer. The final vector was verified by sequencing (Genwiz, Germany) and named as piE1:puro-BmAc3. To replace puromycin N-acetyltransferase with Zeocin resistance gene (*Sh ble*), the pPICZa vector was digested with NcoI and EcorV. The generated 439bp fragment was cloned into the vector piE1:puro-BmAc3 digested with AscI, followed by treatment with Klenow fragment (Minotech) and subsequent digestion with NcoI. The ORF of *SfOatp74D*, *DmOatp74D* and *HaOatp74D* were cloned into the final vector (piE1:Zeocin-BmAc3) using the same strategy as used in the case of pBmAC3 vector.

To tag both *SfOatp74D* and *HaOatp74D* with a V5 epitope (GKPIPNPLLGLDST) at the C-terminus of the protein, both ORFs were amplified with PCR using the primer pairs Sf-OATP74D-XbaI-F/Sf-OATP74D-BspEI-V5-NotI-R and Ha-OATP74D-BamHI-F/HaOATP74D-BspEI-R, respectively (Table 4.1). The SfOATP74D insert was cloned in between XbaI and NotI sites of piE1:Zeocin-BmAc3 vector, harboring a BspEI restriction site upstream of the V5 epitope sequence. The *HaOatp74D-V5* PCR fragment was cloned between the BamHI and BspEI of the pBmAc3-SfOATP74D-V5 vector. A linker sequence (Gly-Ser-Gly) was used to separate the C-terminus of each of the two proteins with the V5 epitope.

4.2.3 CRISPR mediated knock-out of HzOatp74D in HzAW1 cell line

A CRISPR-Cas9 strategy was employed to knock-out the *HzOatp74D* in the HzAW1 cell line. Several CRISPR targets were identified in the first exon of the gene based on the *de novo* transcriptome assembly of HzAW1 cell line (Vorgia et al., 2021), using the online version of the target finder chopchop (Labun et al., 2021). Two different target sequences were selected displaying the minimal predicted off-target effects and the highest predicted efficiency. Single guide RNA sequences were annealed as single stranded oligos (Table 4.1) and ligated into the CRISPR vector pBmAc3:Cac9-HaU6:1 (Samantsidis et al., 2022) following digestion with BbsI.

The HzAW1 cell line was co-transfected with the two sgRNA expressing vectors and the pEA-PAC plasmid at a molecular ratio of 10:10:1. Specifically, one million cells were seeded in 6-well plates and co-transfected with 1μg of total DNA using the ESCORT IV transfection reagent (Sigma) following the instructions of the manufacturer. To positively select the transfected and possibly mutant cells, selection with 25ug/ml of puromycin was carried out for 10 days. Genotyping of the two generated cell lines was performed with PCR using primers flanking the targeted region (HzOATP74D-F-5UTR and HzOATP74D-R-exon1, Table 4.1) yielding a fragment of 912bp corresponding to the wild type allele. PCR reactions were performed using Taq DNA polymerase (EnzyQuest, Greece) on genomic DNA extracted from both transfected cell lines with DNAzol reagent (Molecular Research Center); the conditions of the PCR were as follows: 95^{o}C for 3min initial denaturation, followed by 30cycles of 95^{o}C for 30sec, 50^{o}C for 30sec, 72^{o}C for 30sec, followed by final extension at 72^{o}C for 5min. The combination of sgRNAs yielded two distinct products corresponding to the wild type (912bp) and the mutated allele (~800bp) (Figure 4.1C). Each of the generated PCR fragments was purified and sequenced to validate the existence of mutated OATP74D isoforms.

Once the existence of mutated OATP74D alleles were verified by Sanger sequencing, single cell cloning was initiated to isolate a clonal line encompassing a unique isoform of mutated OATP74D gene. Limiting dilution method was employed in order to isolate clonal cell lines in a 96 well plate. From the 96 well plate, 16 wells were found to contain colonies of cells proliferating and only one of them were found to bear a single mutated OATP74D isoform. The monoclonal cell line was subsequently scaled up and used for downstream assays.

4.2.4 Analysis of 20-HE induced cell death in HzAW1 cell lines

In order to assess the role of OATP74D in ecdysone mediated cell death, 4x10^{5} cells of both HzAW1^{WT} and HzAW1$^{\Delta OATP74D}$ cells were seeded in 6 well plates and treated with 5μM of 20-HE for 48hrs. Each condition consisted of three biological replicates. The cells were harvested and 10^{5} cells from each replicate were used for Fluorescence Activated Cell Sorting following staining with Annexin-PI (BD Pharmigen). The rest of the cells were used for protein and RNA extraction. Caspase-3 activity was calculated using the Caspase-3 assay kit (BD biosciences) following the instructions of the manufacturer. Furthermore, the Ac-DEVD-CHO (BD Biosciences) was used as a potent Caspase-3 inhibitor to validate that fluorescence is mediated by caspase specifically and not by other serine proteases like cathepsins. Fluorescence was measured using the spectramax plate-reader with an excitation wavelegnth of 380nm and an emission wavelength range of 420-460nm (with 5nm increment). For each condition three biological and two technical replicates were used.

Extracted RNA from each sample was used for cDNA synthesis for qRT-PCR to analyze the expression of the genes *HzCaspase-3* and *HzCaspase-8* as previously described (NCBI Gene IDs: 110374006 (*HaCaspase-3*) and 110369675 (*HaCaspase-8*)). Primer sequences used for both genes are shown in the Table 4.1. Relative expression was normalized against *HzGadph* and *HzRps3a*.

Calculation of the proportion of apoptotic cells after treatment with 20-hydroxyecdysone with Annexin-PI staining was conducted using FlowJo V10 software (BD, Lifesciences). All the results for fluorescence-based estimation of caspase-3 activity and the fold change relative expression of *HzCaspase-3* and *HzCaspase-8* were

graphed and analyzed by unpaired t-test for individual comparisons between the treated and untreated cells, using the software GraphPad Prism 8.0.

4.2.5 Gene reporter assays

Luciferase assay in insect cell lines

To verify that OATP74D of *H. zea* acts as an ecdysone importer, an *in vitro* approach based on luciferase was employed. Specifically, the HzAW1^{WT} and HzAW1$^{\Delta Oatp74D}$ cell lines were transfected with 1ug of the plasmid ERE-b.act.luc (Soin et al., 2008) in 6-well plates using ESCORT IV transfection reagent, following the instructions of the manufacturer. Both cell lines were incubated for 72 hrs after transfection, after which 100µl of the transfected cells were seeded into 48-well plates and incubated for 2-3 hrs, followed by treatment with 0.1uM and 1uM of 20-hydroxyecdysone (TCI chemicals, #1480). Twenty-four hours post treatment the cells were lysed and analyzed for luminescence using the Luciferase Assay system (Promega; Cat #E1500). Normalization among different technical replicates and conditions was carried out by normalizing the Relative luminescence units (RLUs) against total protein content (calculated with the Bradford protein assay, BioRad). Each condition was measured in quadruplicates and each experiment was performed at least twice.

Luciferase assay in HzAW1^{WT} and Sf-9 cells

For the OATP74D overexpression experiments 800ng of piE1:zeocinBmAc3-empty or piE1:zeocinBmAc3-*HaOatp74D* or piE1:zeocinBmAc3-*SfOatp74D* or piE1:zeocinBmAc3-*DmOatp74D* were co-transfected along with 200ng of ERE-b.act.luc plasmid in HzAW1^{WT} and Sf-9 cells, using the ESCORT IV transfection reagent in 6-well plates. Luciferase expression was analyzed following exactly the same procedure as described in the previous section.

Luciferase assay in HzAW1$^{\Delta Oatp74D}$ stably overexpressing HaOATP74D and SfOATP74D

For the OATP74D overexpression experiments, piE1:zeocinBmAc3-empty, piE1:zeocinBmAc3-*HaOatp74D,* piE1:zeocinBmAc3-*SfOatp74D,* or piE1:zeocinBmAc3-*DmOatp74D* were transfected along with ERE-b.act.luc plasmid in the HzAW1$^{\Delta Oatp74D}$ cell line. The *DmOatp74D* and empty vector were used as positive and negative control respectively. Three days later 200 µl of the transfected cells were seeded into new 6-well plates treated with 0.01% poly-L-Lysine (Sigma), followed by selection with 1mg/ml of Zeocin (Invitrogen). The medium was refreshed every 4 days while selective concentration was reduced to 500ug/ml after 4 weeks of selection. Furthermore, a similar procedure was followed for the piE1:zeocinBmAc3-*HaOatp74D-V5* or piE1:zeocinBmAc3-*SfOatp74D-V5*, which both bear a V5 epitope tag at the C-terminus of the protein, in order to validate the expression of OATP74D in HzAW1$^{\Delta Oatp74D}$ cell line. Validation of *HaOatp74D* and *SfOatp74D* was performed with Western blot and immunofluorescence, as described below.

After propagating the cell lines of each genotype, the cells were tested for responsiveness to 20-HE with luciferase assay, following the same procedure as previously described. 10^5 cells were seeded in 48-well plates coated with 0.01% poly-L-lysine (Sigma), followed by treatment with 20-HE overnight and were then tested for luciferase expression. Each condition was measured in eight independent technical replicates and each experiment was performed at least twice.

Inhibition assay

To analyze the potency of broad-spectrum inhibitors of organic anion transporters to inhibit the function of lepidopteran OATP74D, HzAW1$^{\Delta OATP74D}$ cells stably overexpressing the lepidoptera OATP74D were pre-treated with several concentrations of telmisartan (Sigma Aldrich) or rifampicin (Sigma Aldrich) in the presence of 0.1uM 20-HE. Both inhibitors were tested at concentrations that do not impact cellular viability using the luciferase assay described above. Results were analyzed using the one-way Anova statistical test with Dunnet's multiple comparison test.

4.2.6 Western Blot and Immunofluorescence

For western blots, cell lines stably over-expressing *HaOatp74D-V5* and *Sf-Oatp74D-V5* were harvested and lysed with RIPA lysis buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 0.5% Sodium-Deoxycolate, 0.1% SDS and 1% NP-40) supplemented with 1X cocktail Protease Inhibitors (Sigma-Aldrich) and 1mM PMSF, followed by centrifugation for 10min at 4^{o}C at 6,000g. Protein concentration was measured with Bradford assay (BioRad). Approximately 30ug of total protein was loaded onto 10% SDS-PAGE and subsequently transferred to a nitrocellulose membrane. A mouse anti-V5 antibody (Cell signaling) was used at a dilution of 1:2500 in 1% milk dissolved in 1X TBST buffer for detection of either HaOATP74D-V5 or SfOATP74D-V5 proteins. Anti-beta tubulin (Santa-Cruz) was also used at a 1:1000 dilution as loading control.

Cells over-expressing the epitope tagged lepidopteran OATP74D were used for immunostaining. Specifically, cells were incubated on round shaped coverslips in 24-well plates. The cells were washed with 1X PBS and blocked for 1hr at room temperature with PBT solution, containing 2% BSA and 0.1% Triton-X100 in 1X PBS. The cells were incubated with 1:250 of primary antibody (mouse anti-V5) diluted in the blocking solution for overnight at 4^{o}C. The cells were incubated with 1:1000 of anti-mouse secondary antibody conjugated with Alexa Fluor 555 for 1hr at room temperature. Nuclei were counterstained with DAPI and mounted with Vectashield Antifade mounting medium. Samples were observed using a Leica SP8 Inverted confocal microscope.

4.2.7 CRISPR mediated Knock-out of Oatp74D in *S. frugiperda*

In order to somatically disrupt the *Oatp74D* gene *in vivo*, CRISPR-Cas9 was performed by injecting *S. frugiperda* eggs according to a previously established protocol (Zhu et. al 2020). Briefly, egg batches were (Hun et al., 2021) collected shortly after the onset of the scotoperiod and transferred to double sided tape using a whetted paintbrush. Eggs were then injected under air-dry conditions with a solution containing 300ng/µl of recombinant Cas9 Nuclease (NEB) and 100ng/ul each of four sgRNAs targeting the first exon of the *Oatp74D* gene (Table 4.1). Two days post-injection, wheat powder was sprinkled on top of the tape, which prevented the larvae from sticking once emerged. Survivorship and the number of days until pupation were measured across the lifespan of the emerging larvae. DNA samples obtained from healthy and weak larvae were sent for amplicon sequencing (GeneWiz) using primers flanking the four sgRNA cut sites (Table 4.1). As a control for normalizing lethality due to technical handling during microinjections, *S. frugiperda* eggs were injected with sgRNAs targeting the *Scarlet* gene, which does not impact insect development and survival (Khan et al., 2017).

4.3 Results

4.3.1 *S. frugiperda* OATP74D is essential for larval development

This specific part of the *in vivo* characterization of *SfOatp74D*, was performed by Melina Fotiadou as a part of her Master thesis under my supervision, and the following results were kindly provided for this PhD thesis chapter. It was speculated that the lepidopteran OATP74D homologs are necessary for ecdysone uptake, regulating transcription of ecdysone responsive genes as well as processes like apoptotic cell death. Therefore, it was hypothesized that the lepidopteran OATP74D would be essential for survival at early developmental stages. To address this question, a CRISPR/Cas9 strategy was employed in order to knock-out the *Oatp74D* of *S. frugiperda*. Four different sgRNAs were used in order to ensure high mutation frequency displayed at G_0 mosaic insects (Figure 1A). As shown in figure 2B, targeting *SfOatp74D* lead to a significant decrease in hatching rate (10%) compared to eggs injected targeting *SfScarlet* (38%). Furthermore, considering only hatched eggs, a lower proportion of *Oatp74D* injected larvae survived during larval development (25%) compared to the control larvae (86%) The overall survival was calculated and it appeared that a considerably lower percentage of *Oatp74D* injected eggs (25%) completed larval transitions compared to S*carlet* injected eggs (85%) (Figure 4.1). Moreover, *Oatp74D* target region was sequenced in a number of injected larvae that turned to adults and were found to be wild type. Taken all together, although G_0 animals were mosaic for the deletion of *Oatp74D,* the mutation frequency was probably enough to affect survival highlighting the essentiality of the gene even from the egg stages.

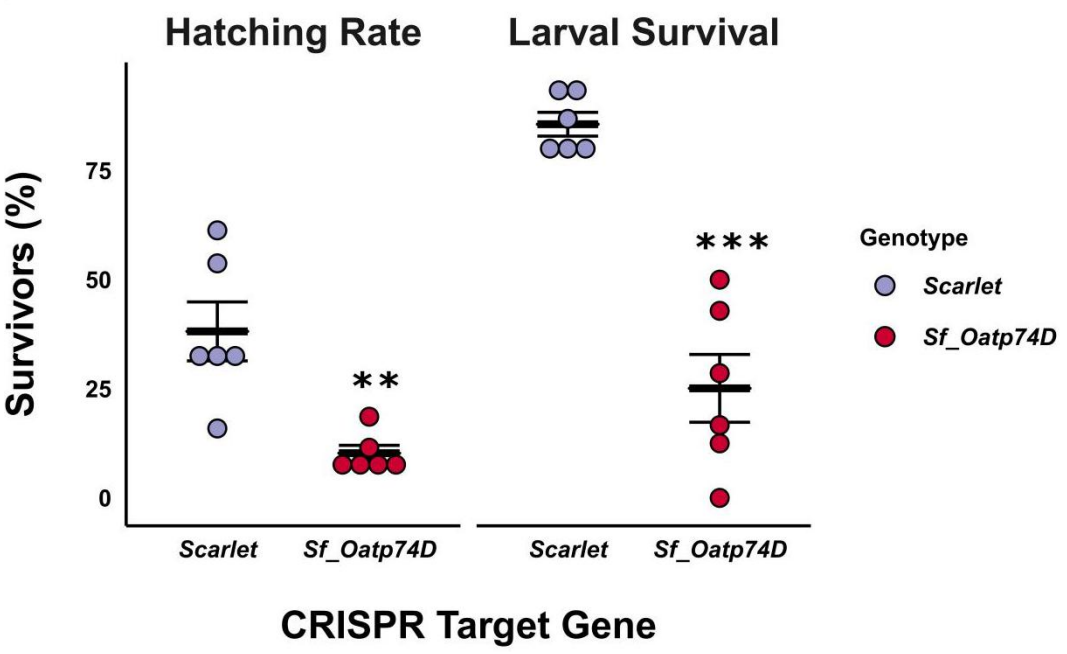

Figure 4.1 Analysis of *SfOatp74D* essentiality in *Spodoptera frugiperda*. Left: Hatching rate percentiles of hatched G_0 mosaic eggs after injection with Cas9-sgRNAs complexes were calculated by the number of first instar larvae emerged against the total number of eggs per replicate (petri dish). Right: Larval survival rates post injection with Cas9-sgRNAs complexes, calculated as the number of larvae survived until the fifth instar. As a negative control for animal survival, *S. frugiperda* eggs were injected with Cas9-sgRNAs targeting the gene *Scarlet*. Each dot represents a separate technical replicate (petri dish) consisting of 80-100 injected eggs in total. (Experiment and figure was performed by MSc Melina Fotiadou in the context of her Master thesis)

4.3.2 *Helicoverpa zea* OATP74D is necessary for the genomic function of ecdysone pathway

The high mortality rates at the embryonic stages of *S. frugiperda* could not let any further characterization of the role of SfOATP74D *in vivo*. Therefore, we opted to analyze the role of the lepidopteran OATP74D in the ecdysone pathway in cell cultures. The HzAW1 and Sf-9 cells were used and analyzed for the expression of the endogenous *Oatp74D*. Both cell lines seem to express the *Oatp74D* (Figure 4.2A). Given that *Helicoverpa* specific U6 promoters have been identified and customized in lepidoptera specific CRISPR vectors (see Chapter 3), the HzAW1 cell line was used and genome modified via CRISPR/Cas9, targeting the first exon of *HzOatp74D.* Transfection of the HzAW1 cells with two sgRNA expression vectors and subsequent selection with puromycin, lead to the generation of a 100bp deletion indicative of the distance between the two target regions (Figure 4.2B, 4.2C). Serial dilution method was further employed in order to generate a clonal cell line that will harbor one mutant allele of the *Oatp74D*. A clonal cell line harboring a 4-bp deletion at the first exon of the gene was isolated and verified by PCR and sequencing (Figure 4.2D). Elimination of these 4bp shortly after the start of translation are predicted to lead to a truncated protein.

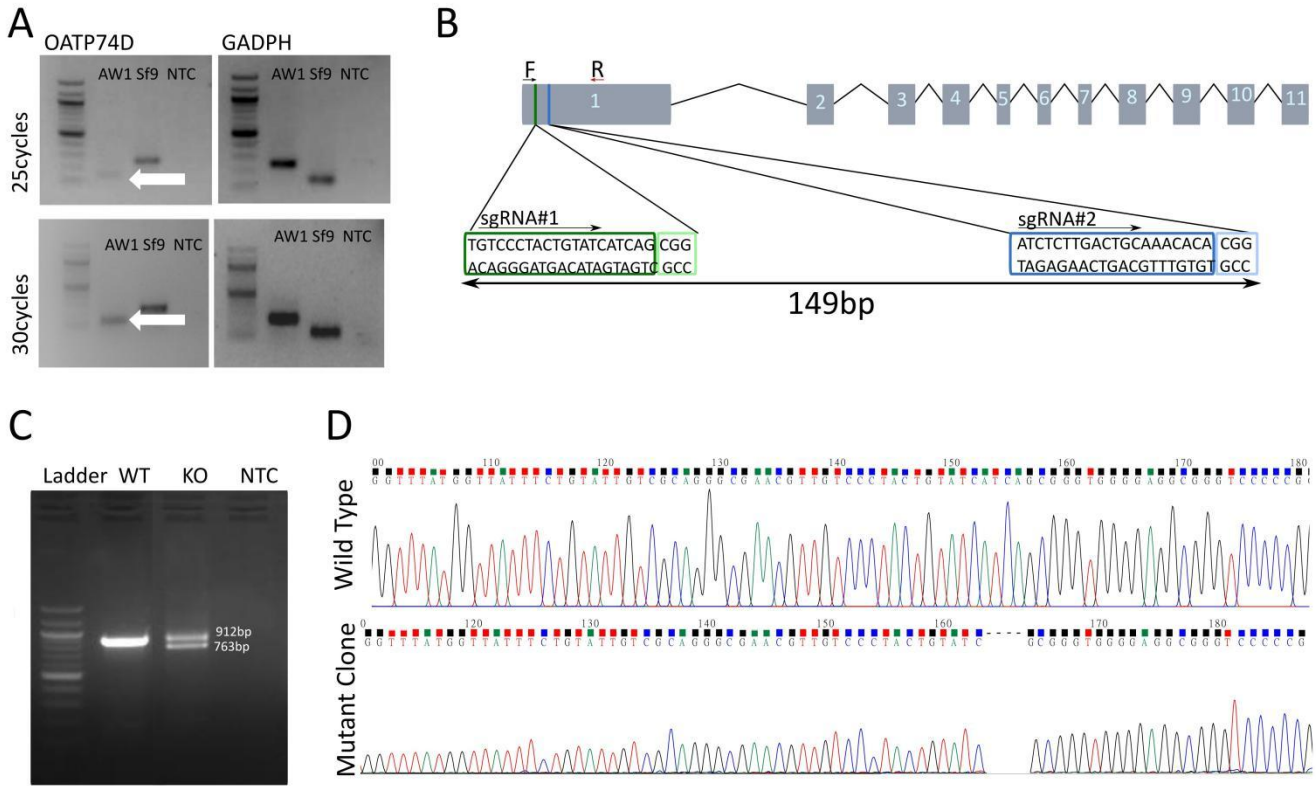

Figure 4.2 Characterization of *Oatp74D* in HzAW1 cell line. A) *Oatp74D* is expressed in HzAW1 and Sf-9 lepidoptera cell lines as indicated by RT-PCR. Same cDNA samples were used to amplify the housekeeping gene *gadph* as a reference, for 25 and 30 cycles (top and bottom panels respectively). B) Schematic representation of the *HzOatp74D* gene consisting of 11 exons.Two sgRNAs (#1 and #2) designed to target the first exon of the gene spanning a region of 149bp. F and R indicate the forward and reverse primers respectively used in PCR for diagnostic reasons. C) Diagnostic PCR indicating the expected deletion of 149 bp after transfection of HzAW1 cells; WT and KO indicate the wild type and knock-out cells, NTC: non-template control. D) Sequencing chromatogram of the target region close to the region of sgRNA#1 in wild type and mutant clone, indicating the deletion of the 4 bp (5'-ATCA-3').

Knock-out of *HzOatp74D* inhibits differential expression of ecdysone responsive genes

To address if the *HzOatp74D* is implicated in the ecdysone pathway for the regulation of gene expression cascades, four different ecdysone responsive genes were analyzed for their expression following treatment of both wild type and genome modified HzAW1 cell lines with 1μM of 20-HE for four different time points. As depicted in Figure 4.3A *EcR* was down-regulated at 9hrs and 12hrs of treatment in the wild type cells. However, the expression levels of this gene remained at the same levels of expression in the *HzOatp74D* knocked-out cells at both of these time points compared to the untreated cells. Furthermore, *Eip74A* and *Eip75B,* which both belong to the group of early response genes of the ecdysone pathway, were upregulated by 1.5X - 2X with respect to the negative control, at 9 and 12hrs of treatment (Figure 4.3A). Moreover, *Eip74A* was upregulated by 3-folds at 24hrs of treatment compared to the untreated wild type cells. On the contrary, the HzAW1$^{\Delta Oatp74D}$ cells did not display any significant difference at the expression levels of both of these genes upon treatment with 20-HE with respect to the untreated knocked-out cells (Figure 4.3A). Finally, expression analysis of the *Hr3* (*Hormone response 3*) revealed that treatment of wild type cells with 1uM of 20-HE induced the expression of the gene almost by ~9-folds at 6hrs of treatment with respect to untreated cells. Longer exposure of the cells with the steroid hormone increased the expression of the gene up to 17-folds compared to the negative control. On the contrary *Hr3* expression increased only by ~2-folds in HzAW1$^{\Delta Oatp74D}$ cells, while its expression was uniform among all time points tested. Overall, these results indicated that *HzOatp74D* deletion obstructed the activation of the canonical pathway of 20-HE in the context of regulation of gene transcription.

***HzOatp74D* is essential for regulating 20-HE mediated cell death via Caspase-3 activation**

One of the physiological functions of the ecdysone pathway at the onset of metamorposis is the induction of apoptotic cell death (Cakouros et al., 2003, Yamanaka et al., 2013). To this end both wild type and knock-out cell lines were treated with 5uM of 20-HE for 48hrs. Cells were analyzed using flow cytometry followed by staining with Annexin-PI. Treatment of wild type cells with 20-HE for 48hrs increased the percentage of early apoptotic cells (+ Annexin, - PI) by 2.9-folds compared to the untreated wild type cells (Figure 4.3b). In contrast, the percentage of early apoptotic HzAW1$^{\Delta Oatp74D}$ cells was at same levels with the respective negative control (untreated cells harboring the 4-bp deletion at *Oatp74D*).

Apoptotic cell death was further validated in the wild type cells via relative quantification of the active caspases using a fluorometric approach. As Figure 4.3C indicates HzAW1^{WT} exhibited a 5-fold increase of activated caspases upon treatment with 5uM of 20-HE for 48hrs, compared to the untreated wild type cells. However, the HzAW1$^{\Delta Oatp74D}$ did not display any significant difference when treated with the steroid hormone. Given that ecdysone pathway regulates the expression of caspases in *Drosophila* directly through EcR (Cakouros et al., 2004), two different caspases were analyzed for their expression in the treated and untreated wild type and knocked-out cells. Relative expression analysis of the two caspases (*caspase-3* and *caspase-8*) of *Helicoverpa* indicated that 20-HE induced the expression of caspase-3 in the wild type cells by almost 3.7-folds with respect to the untreated cells (Figure 4.3D). On the other hand, no difference was observed when HzAW1$^{\Delta Oatp74D}$ cells are exposed to the steroid hormone, which further support Annexin-PI staining and fluorometric analysis results.

Taken together these results demonstrate that HzOATP74D is necessary for 20-hydroxyecdysone to induce differential expression of ecdysone target genes and apoptotic cell death in the midgut derived cell line HzAW1.

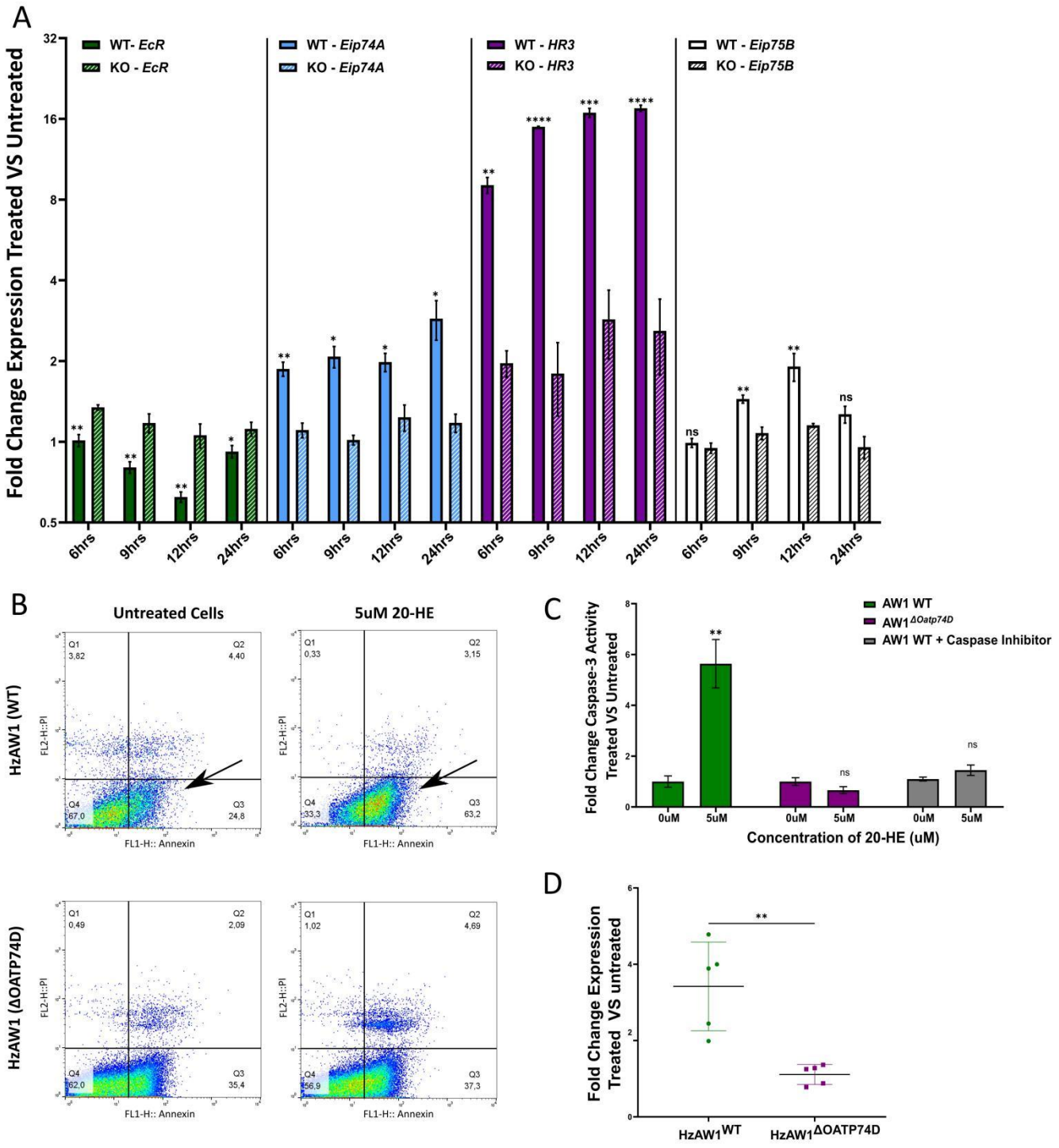

Figure 4.3 *H. zea Oatp74D* regulates the ecdysone induced signaling pathway. A) Gene expression analysis of ecdysone responsive genes in HzAW1^{WT} and HzAW1$^{\Delta Oatp74D}$ cells followed by treatment with 1uM of 20-HE for 6, 9, 12 and 24hrs. Bars represent the mean ± SE fold change gene expression of the treated versus untreated cells. B) Flow cytometry analysis of FITC-Annexin V and propidium iodide (PI) stained cells after treatment of both Wild type and monoclonal knock-out cell lines with 5uM of 20-HE for 48hrs; Q1: Annexin$^-$/PI$^-$ (live cells), Q2: Annexin$^+$/PI$^-$ (early apoptotic cells), Q3: Annexin$^+$/PI$^+$ (late apoptotic cells), Q4: Annexin$^-$/PI$^+$ (dead cells). Flow cytometry plots represents one of the three biological replicates. C) Fold Change Caspase-3 activity in HzAW1^{WT} and HzAW1$^{\Delta Oatp74D}$ cells at the same conditions as in B. AW1^{WT} cells were also incubated in the presence of Caspase-3 inhibitor (Ac-DEVD-CHO) during the assay in order to exclude the non-specific cleavage of the synthetic tetrapeptide DEVD. D) *HzCaspase-3* expression analysis in both wild type and knock-out cell lines post treatment with 5uM of 20-HE for 48 hrs. Bars represent the mean ± SE fold change gene expression of the treated versus untreated cells. Asterisks

indicate statistically significant differences between the wild type and knock-out cell lines by un-paired t-tests (For A:*p<0.0321, **p<0.0046 and ***p<0.0002, for C: **p=0.009 and for D: **p=0.0025, summarized in table 4.2).

4.3.3 *H. armigera* and *S. frugiperda* OATP74D are sufficient for ecdysone dependent gene transcription in HzAW1$^{\Delta Oatp74D}$

To analyze if other lepidoptera orthologs of Ecdysone Importer are implicated in 20-hydroxyecdysone uptake, a luciferase assay system was implemented similar to that reported previously for the *Drosophila Oatp74D* (Okamoto et. al 2018). Prior to overexpression of *Oatp74D* orthologs in cell lines, the assay was first performed in the wild type and knocked-out cells. The luciferase assay indicated that HzAW1^{WT} exhibited a significant proportionally increased luciferase activity upon treatment with 0.1uM and 1uM of 20-HE, almost by 3.6-folds and 6.6-folds respectively, compared to the untreated cells (Figure 4.4). On the contrary treatment of the HzAW1$^{\Delta Oatp74D}$ with the same concentrations of 20-HE did not induce any increase, keeping the levels of luciferase activity at baseline (Figure 4.4).

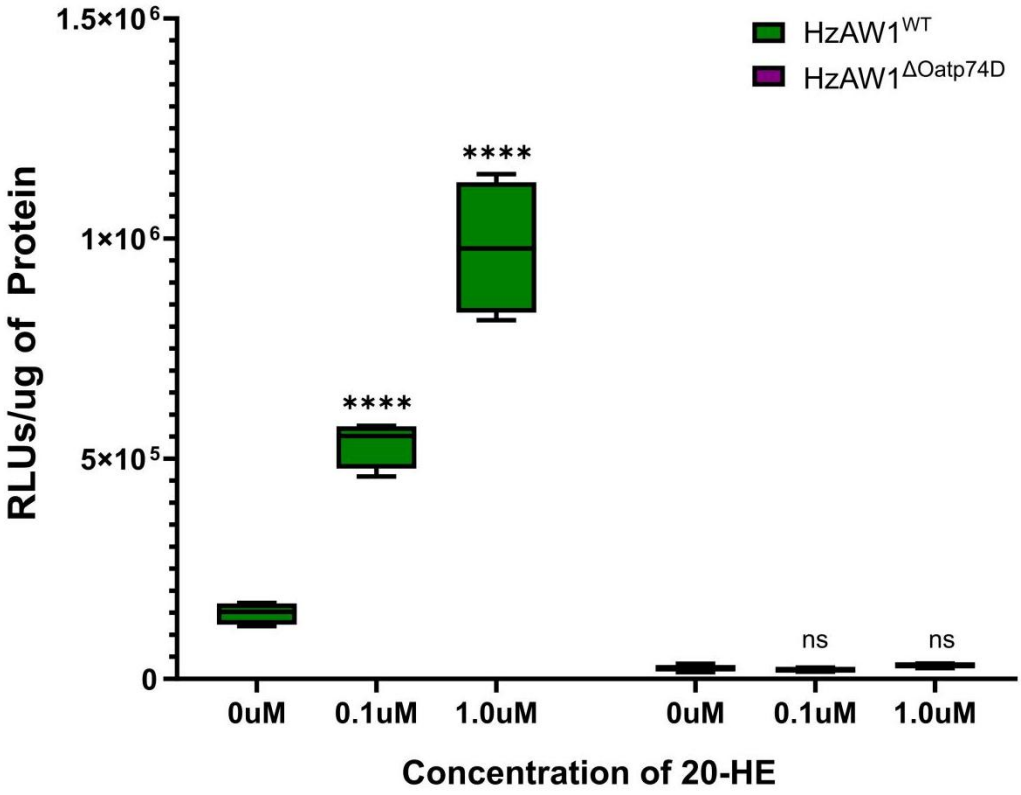

Figure 4.4 Analysis of ecdysone induced luciferase expression upon treatment with 20-hydroxyecdysone in HzAW1^{WT} and HzAW1$^{\Delta Oatp74D}$ cells post transfection with 1ug ERE-b.act.luc construct. The relative luminescence units were calculated against the total protein content per reaction. Each box represent the values obtained from 4 different replicates. ****p<0.0001 with one-way ANOVA followed by post-hoc Dunnett test., ns: statistically non-significant.

Therefore, HzAW1$^{\Delta Oatp74D}$ cell line was preferred to be used for stable expression of other *Oatp74D* orthologs along with the ecdysone responsive firefly luciferase construct, in order to analyze their potency for responsiveness to the steroid hormone measuring luciferase activity. To verify if *HaOatp74D* and *SfOatP74D* are actually expressed in the cells, both of them were tagged with a V5 epitope and checked for their expression at a protein level via western blot. As indicated in Figure 4.5b, lepidopteran OATP74D were expressed and identified at the predicted molecular weight, around ~75KDa. Furthermore, immunostaining of HzAW1$^{\Delta Oatp74D}$ and Sf9 cells expressing *HaOatp74D-V5* and

SfOat74D-V5 indicated that both proteins are localized at the cellular membrane of the cells as delimited in the brightfield (Figure 4.5A, Figure 4.5C).

The DmOATP74D was over-expressed and used as a positive control to test if luciferase activity would be induced upon treatment with different concentrations of 20-HE in a similar fashion as depicted in the schematic representation (Figure 4.5D). An increase of luciferase activity by 2.24- and 3.9-folds was observed when cells were treated with 0.1uM and 1uM of the steroid hormone respectively, compared to the untreated cells. Stable cells expressing HaOATP74D displayed significant increase of luciferase activity by 1.7-folds and 2.54-folds, upon treatment with 0.1uM and 1uM of 20-HE respectively, with respect to the untreated (Figure 4.5E). Finally, treatment of stably expressing SfOATP74D cells with the same concentrations of 20-HE induced the expression of luciferase by 3.43 and 5.36-fold. Cells transfected with an empty vector instead did not display any difference upon treatment with 0.1uM and 1uM of 20-HE compared to the untreated cells.

Lepidoptera OATP74D are inhibited by known Organic Anion Transporters' Inhibitors

Rifampicin and telmisartan, two well known inhibitors of Organic anion transporters, were used in order to test whether OATP74D could be pharmacologically inhibited. Although telmisartan did not impact the function of any of the OATP74Ds (Figure S4C), rifampicin inhibited the ecdysone induced luciferase activity when tested in stable cells treated with 0.1uM of 20-hydroxyecdysone (Figure 5d). In particular 10μM of rifampicin inhibited SfOATP74D by 30%, but did not affect the activity of *Drosophila* and *Helicoverpa* proteins. Conversely 50μM and 100μM of rifampicin lead to significant reductions of luciferase activity by >50% and >90% respectively, when tested against each of the OATP74D proteins (Figure 3d).

4.4 Discussion

Ecdysone uptake mechanism by the target cells has been vague until three recent reports rebutted the consensus of the simple passive diffusion of the steroid hormone across the lipid bilayer of the cellular membrane (Neuman et al., 2018, Okamoto et al., 2021, Hun et al., 2021). The organic anion transporter Oatp74D was identified as the major transporter implicated in the transport of 20-HE and found to be essential for larval development in *Drosophila* (Okamoto et al., 2018). Although a clear ortholog of *Drosophila* Oatp74D was identified in some arthropod species (Okamoto et al., 2018), the theory for transporter mediated ecdysone uptake cannot be generalized to all insects without concrete proof. Therefore, functional characterization of Oatp74D orthologs is mandatory in order for this mechanism to be established in other insect species.

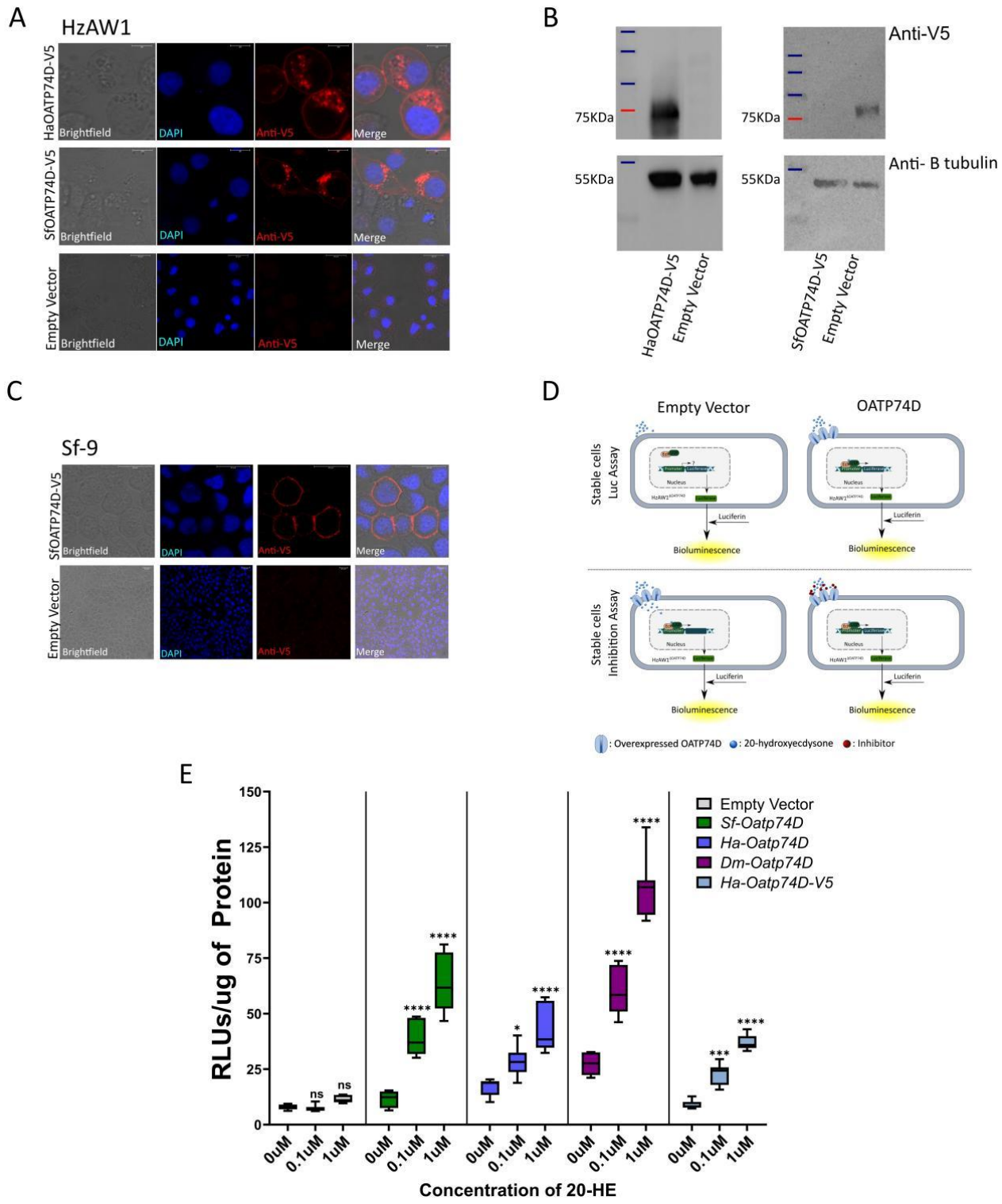

Figure 4.5 *SfOatp74D* and *HaOatp74D* are sufficient to regulate ecdysone induced gene expression in cell cultures. A) Subcellular localization of both SfOATP74D and HaOATP74D tagged with V5 epitope in transiently transfected HzAW1 cells. Blue indicates DAPI which counterstains nuclei, while Red indicates anti-V5, scale bar 20µM. B) Western blot analysis of SfOATP74D-V5 and HaOATP74D-V5 in HzAW1 stable cell lines. Empty vector stable cells were used as negative control. Top panels represent blots HaOATP74D-V5 (left) and SfOATP74D-V5 (right) along with empty vector stably transfected cells using anti-V5. Bottom panel represent beta-tubulin used as loading control (~55kDa). C) Subcellular localization of Sf-OATP74D-V5 in transiently transfected Sf-9 cells. Blue indicates DAPI which counterstains nuclei, while Red indicates anti-V5, scale bar 20µM. D) Schematic representation of luciferase assay in stable cell lines expressing OATP74D orthologs, in absence or presence of inhibitors. E) Analysis of ecdysone induced luciferase expression in stable cell lines over-expressing *SfOatp74D*, *HaOatp74D*, *DmOatp74D* and *HaOatp74D-V5*, upon treatment with 0.1 and 1uM of 20-HE for 24hrs. Values are calculated as ratio of Relative luminescence units (RLUs) against the total protein content. Asterisks indicate statistically significant differences between treated and untreated cells, *p=0.0149, ***p=0.0004, ****p<0.0001, calculated with one-way ANOVA followed by post-hoc Dunnett test.

Lepidoptera OATP74D are essential for insect development and survival

Partial disruption of *Oatp74D* in mosaic *S. frugiperda* animals (Figure 4.1) had a severe impact on insect development in respect to the egg hatching rate. This was not unexpected, since the ecdysone pathway is essential during embryogenesis, as indicated in *Drosophila melanogaster* embryos which seem to express the major biosynthetic enzymes of ecdysone and require EcR-USP nuclear receptors for normal development and survival (Chavez et al., 2000, Kozlova and Thummer 2003). Heterologous expression of a dominant negative allele of EcR in a heterozygous mutant background for the endogenous *EcR* increased the lethality as well as the penetrance of germ band retraction defects, indicating the necessity of the pathway overall in the development and morphogenesis of embryos (Kozlova and Thummer 2003). Moreover, null mutant flies of other components of the ecdysone pathway like the β*FTZ-F1* and *DHR3* failed to hatch since they exhibited severe defects in Ventral Nerve Cord condensation and also an inability to fill their tracheal system with air (Ruaud et al., 2010). Additional studies in lepidoptera species, like *Manduca sexta* and *Bombyx mori*, have documented the expression of ecdysteroidogenic enzymes during embryogenesis (Sonobe et al., 2004, Yamanaka 2021). Therefore, the reduced egg hatching rate caused by *SfOatp74D* disruption (Figure 4.1) would be explained if the protein is implicated in ecdysone transport. Similar results were observed when disruption of the organic anion transporter EcI-2 of *Aedes aegypti* reduced significantly egg survival (Hun et al., 2021). Interestingly, *DmOatp74D* null mutants did not exhibit any significant embryonic lethality, which contradicts with the increased embryonic lethality induced by knocking out *SfOatp74D* (Figure 4.1) and EcI-2 of *Aedes aegypti* (Okamoto et al., 2018, Hun et al., 2021). This raises the question then if there is a different mechanism for ecdysone uptake by the target cells of *Drosophila* embryo.

High mortality observed in *SfOatP74D* injected individuals at larval stages (Figure 2B) further suggests an essential role for this gene in development. This is in line with *DmOatp74D*, which seems to be essential for the development of larval stages since homozygous mutant flies arrested at L1 stage, failing to molt to the second larval stage (Okamoto et al., 2018). Our results are also consistent with a previous study in *Tribolium castaneum*, in which decreased larval survival and failure of pupation was observed upon silencing of *TcOATP4-C1*, a putative ortholog of the *Drosophila Oatp74D* (Rosner et al., 2021). Increased larval mortality was also observed in *Aedes aegypti* upon silencing of EcI-2 which induced 70-80% lethality (Hun et al., 2021). It is noteworthy to mention that knock-out of other organic anion transporters of *Drosophila* which were shown to mediate ecdysone uptake *in vitro*, did not impact animal development and survival, indicating the predominant role of *DmOatp74D* (Hun et al., 2021). Taken together *SfOatp74D* is essential for embryo hatching, larval molting and overall survival, although the existence of other lepidoptera organic anion transporters functioning as ecdysone transporters cannot be ruled out.

HzOatp74D is essential for the regulation of canonical ecdysone pathway and activation of programmed cell death

In parallel to *in vivo* work, an *in vitro* approach was taken by isolating a mutant clone of *Oatp74D*. Expression analysis suggested that *OatP74D* is necessary for the transcriptional regulation of four different ecdysone responsive genes, *HzEcR*, *HzEip74A*, *HzEip75B* and *HzHR3* (Figure 4.3A). Differential expression analysis of these genes between the wild type and knock-out cell lines upon treatment with the hormone (Figure 4.3A) highlighted the role of the *HzOatp74D* gene in the activation of the ecdysone pathway and are in agreement with other studies in which knock-out of *Oatp74D* affected the expression of ecdysone responsive genes (Okamoto et al., 2018, Hun et al., 2021).

It is well established that the molting hormone ecdysone is implicated in apoptotic cell death of larval tissues like midgut and salivary glands during the larval to pupal transition (Kang et al., 2019, Zirin et al., 2013). Previous studies have also indicated the role of certain G-protein coupled receoptors (GPCRs) in the regulation of caspases expression as a response to 20-HE inducing apoptotic cell death (non genomic function of ecdysone) (Zhao 2020, Li et al., 2017, Kang et al., 2019, Chen et al., 2017). Certain caspases, like the *Drosophila dronc*, *reaper* and *hid* are upregulated at the onset of metamorphosis in tissues like the salivary glands and midgut as a response to the ecdysone pathway through immediate binding of EcR/USP transcriptional complex on the promoter region of these genes (Cakouros et al., 2004, Jiang et al., 2000). However, the role of OATP74D remained unknown in the induction of apoptosis by the steroid hormone. Given the lethal phenotype of mutant for the OATP74D *S. frugiperda*, even from the egg stages, we decided to analyze the involvement of the OATP74D in cell death by using the cell line HzAW1, provided that the knock-out of the gene did not affect significantly the viability of the cells. Treatment of the knock-out and wild type cells with ecdysone indicated a clear difference in the number of early apoptotic cells as well as in the expression levels and activity of *caspase-3* (Figure 4.3B-D), indicating the necessity of the transporter in 20-HE induced apoptosis. Several studies have documented that the interplay between the genomic and the non-genomic pathway in *H. armigera* epidermal cell line (HaEpi cells) is mediated by G-protein coupled receptors, which modulate gene transcription via regulating EcR and USP phosphorylation (Chen et al., 2017, Wang et al., 2015, Kang et al., 2019). Based on these results, it is proved that OATP74D function is necessary for the activation of the ecdysone pathway and the downstream physiological effects (e.g triggering of apoptosis), despite the possible activation of the non-genomic pathway by 20-HE in the lepidoptera cells.

It has to be considered that although HzAW1 cell line is derived from the midgut tissue of *Helicoverpa zea* (Goodman et al., 2004), a substantial de-differentiation may have occured (Swevers et al., 2021) rendering difficult to transfer the knowledge directly from *in vitro* to *in vivo*. Nevertheless, the role of the OATP74D in the ecdysone pathway could still be resolved, given that genes of the pathway are expressed in these cells under physiological conditions (Vorgia et al., 2021). A possible explanation to this could be that ecdysone or other ecdysteroids with similar structure is one of the signaling molecules that in very low doses promotes proliferation and growth in insect cultured cells, thus most of the insect cell lines maintain high levels of expression of the ecdysone related genes (Fallon et al., 2010, Nijhout et al., 2015). Moreover, this is also a possible explanation to why *HzOatp74D* is expressed in HzAW1 cells.

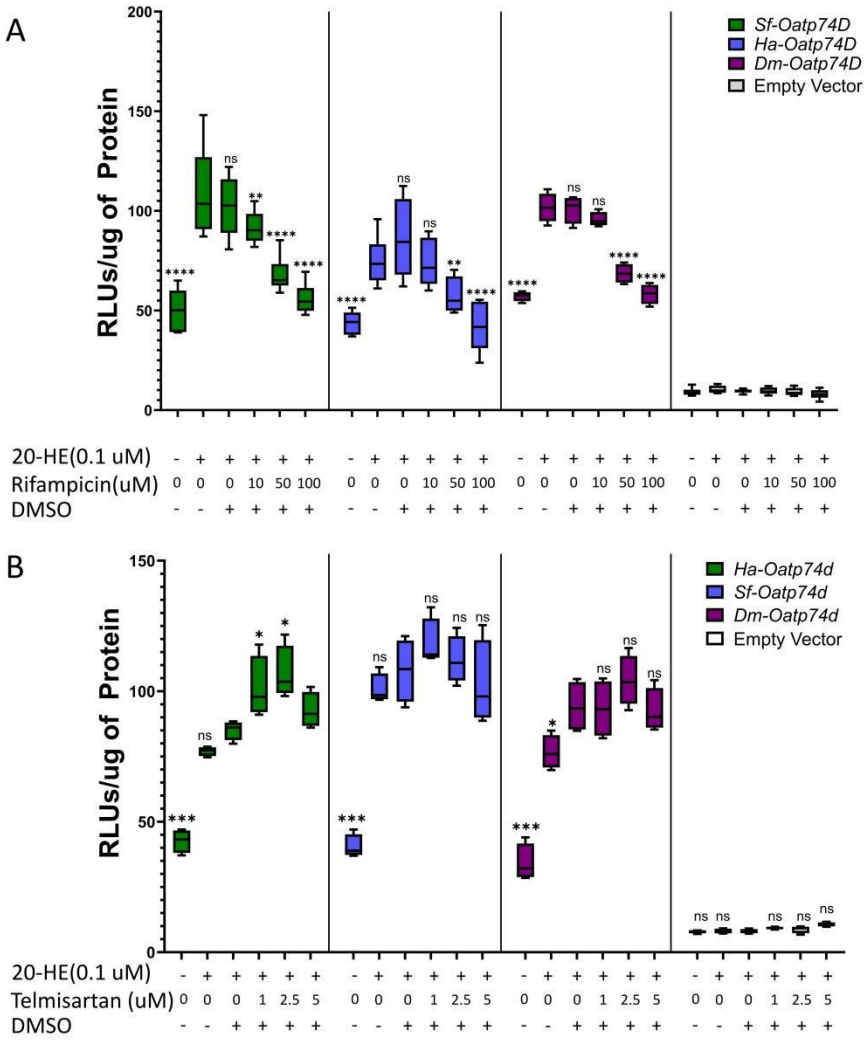

Figure 4.6 Testing Inhibitory activity of two organic anion transporter inhibitors, rifampicin and telmisartan. Stable cells were incubated in medium supplemented with 0.1uM of 20-HE and different increasing concentrations of A) rifampicin and B) telmisartan. As negative control cells were incubated with 0.1µM of 20-HE and 0.1% DMSO (solvent of the inhibitors). Moreover, cells were incubated with 0.1uM of 20-HE but without DMSO to test its impact in the luciferase assay. Asterisks indicate statistical significance between the different conditions and treatment with 20-HE+ 0.1% DMSO, A: **p<0.0035, ****p<0.0001, B: *p=0.02, **p=0.0095, ***p=0.0008, ****p<0.0001, with one-way ANOVA followed by post-hoc Dunnett test.

SfOATP74D and HaOATP74D import ecdysone to regulate gene expression

Indirect measurement of ecdysone importation was also accomplished using an ecdysone sensitive luciferase reporter assay with a panel of *OatP74D* manipulated cell lines. Removal of endogenous *OatP74D* decreased ecdysone response (Figure 4.4) while re-expression of any ortholog rescued sensitivity and was sensitive to the inhibitor rifampicin (Figure 4.5E). To further characterize these transporters, rifampicin and telmisartan were tested for their efficiency to inhibit the function the lepidopteran OATP74D. Both of them have previously been characterized to act as inhibitors of mammalian organic anion transporters (Patik et al., 2015, Dolberg et al., 2018, Li et al., 2015). Although telmisartan had no impact on stable cells expressing OATP74D, rifampicin was shown to inhibit successfully the ecdysone induced luciferase expression when tested in cells overexpressing SfOATP74D and HaOATP74D, which is indicative that both function as typical organic anion transporters mediating cellular uptake of

20-HE. Of note, to the authors' knowledge this is the first time that an ecdysone transporter is proved to be inhibited by certain chemical compound, which is also suggestive of their druggability.

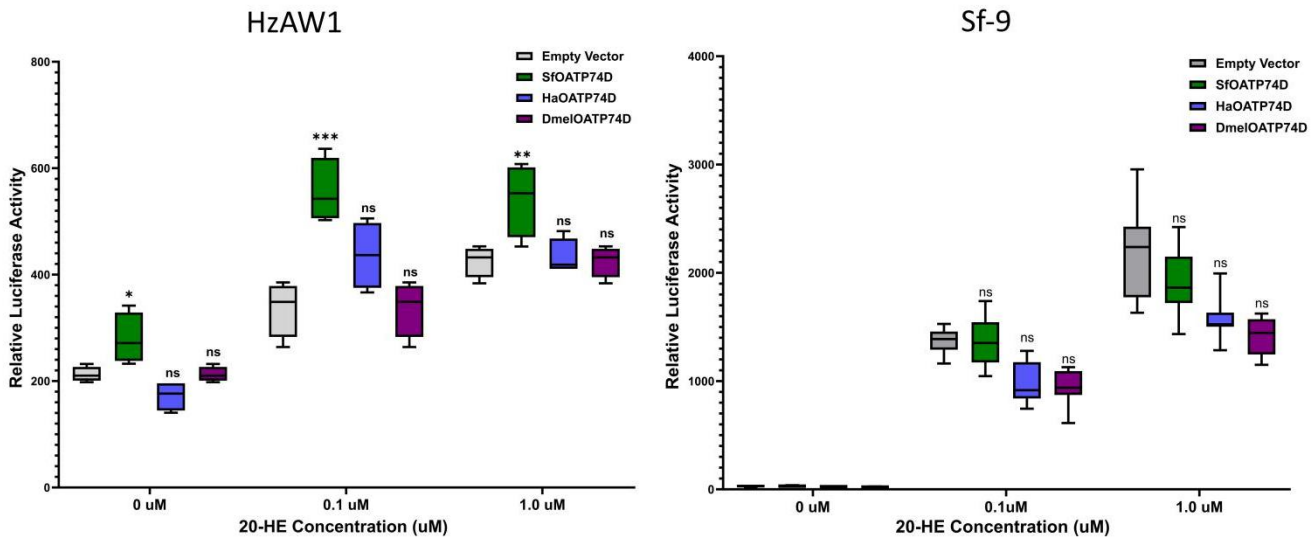

Figure 4.7. A) Functional characterization of lepidopteran OATP74D transiently transfected in HzAW1WT (left) and Sf-9 cells (right). Cells were treated with several concentrations of 20-HE 72hrs post transfection and tested for luciferase expression 24hrs post treatment. Each group consists of four technical replicates and statistical significance was calculated by one-way ANOVA with post-Dunett's test comparing cells overexpressing OATP74D with cells transfected with empty vector, *p<0.033, **p<0.0012, ***p<0.0008.

Ecdysone induced luciferase assays have been used extensively for the characterization of the DmOATP74D as well as of other organic anion transporters of both *Drosophila* and *Aedes* in the S2 and the mammalian HEK293 cell lines (Okamoto et al., 2018, Hun et al., 2021). In the case of S2 cells, overexpression of *DmOatp74D* did not exhibit large differences compared to the empty vector transfected cells in the luciferase assay (Okamoto et al., 2018). Similarly, we found only minor differences when wild type HzAW1 cells were transfected with exogenous *OatP74D* (Figure S4) suggesting that endogenous *OatP74D* was masking observable measurements (Fu et. al 2018; Shu et. al 2017). Previous studies performing the assay in mammalian HEK293 cells indicated a clear difference with the negative control given that mammals are unlikely to express any ecdysone importer (Okamoto et al., 2018). Nevertheless, using HEK293 cells for this assay is more laborious since it requires the co-transfection of two major components, a modified version of EcR and the RXR (Okamoto et al., 2018). Thus, HzAW1$^{\Delta OATP74D}$ cells have considerable advantages for the characterization of other OATP74D orthologues given that they lack endogenous *OatP74D* while possessing the nuclear receptors necessary for ecdysone response.

In line with the essentiality of ecdysone transporters and their "susceptibility" to certain inhibitors, it could be hypothesized that the lepidopteran OATP74D could be used as putative pesticide targets. Molting associated endocrine disruption is already used for pest control, as in the case of Ecdysone Receptor agonists which lead to precocious activation of the ecdysone pathway (Song et al., 2017). However, targeting OATP74D for blocking ecdysone signaling would be more promising, as membrane proteins are more accessible to extracellular compounds, compared to cytosolic ones. Insecticide resistance cases in the field along with the reduction of the efficiency of pest control have increased the need for developing alternative and more integrated strategies. For this purpose,

identification of novel drug targets in conjunction with strategies for pest control, like plant mediated RNAi, is rather promising (Poreddy et al., 2017). To this end, targeting essential proteins in the midgut, which is the first epithelial tissue encountered by oral insecticides (Denecke et al., 2018) would be rather effective. Analysis of the structure of the OATP74D protein would be rather helpful to facilitate predictions of drug-protein interaction as well as designing of drugs based on *in vitro* data.

Conclusion

A very old enigma in steroid hormone uptake mechanism has been recently resolved in three case studies in *Drosophila*. Even though OATP74D of most insect species seem to follow the same evolutionary direction, the existence of other ecdysone transporters cannot be ruled out, as already shown in *Drosophila* (Hun et al., 2021). Therefore, unraveling the role of OATPs in other insects' physiology will further enable understanding of the ecdysone uptake mechanisms. Our study provides useful information about the function of *H. armigera* and *S. frugiperda* OATP74D in the incorporation of 20-HE hormone and proves that both regulate the initiation of the canonical ecdysone pathway. Moreover, it is proved that *SfOatp74D* is an essential protein for animal survival even from the embryonic stages. This is the first time reported that ecdysone transporters are able to be inhibited by a classic inhibitor of Organic anion transporters which overall points that both could be used in the future as prominent insecticide targets. Furthermore, the HzAW1$^{\Delta Oatp74D}$ cell line developed in this study can be utilized as a platform for heterologous expression of other ecdysone transporters and subsequent screening of chemical compounds.

Tables - Chapter 4

Table 4.1 Primers used in this study.

Prmer Name	Sequence	Experimental Use
Sf_OatP74D_CDS_F	ATGGATAGACGGCCAATAAAA	seq of CRISPR target
Sf_OatP74D_CR_R	CCATGTAAAGTGGTGACTGCC	
Sf_oatp74D_amplicon.seq.F	CAGGTTTGTAAATACCTAGTG	amplicon seq of CR. target
Sf_oatp74D_amplicon.seq.R	GACCACACCCACCGCCAGCAC	
CRISPR universal	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	sgRNA synthesis
Sf_OatP74D_long_1	GAAATTAATACGACTCACTATAGGTCGCTACAGTATCATCAGCGTTTTAGAGCTAGAAATAGC	
Sf_OatP74D_long_2	GAAATTAATACGACTCACTATAGGAGCAGGAACGGCTAGCAACGTTTTAGAGCTAGAAATAGC	
Sf_OatP74D_long_3	GAAATTAATACGACTCACTATAGGTCGTTACACTGACGAAGTGTTTTAGAGCTAGAAATAGC	
Sf_OatP74D_long_4	GAAATTAATACGACTCACTATAGGCGCGGGCACCGGCCGCGAGTTTTAGAGCTAGAAATAGC	
Sf-OATP74D-NotI-F	GAATTGGGAATTCGTTAACAGATCTGCGCGGCCGCATGACGGCGAACGTTGTC	Cloning of Sf_oatp74D in pUAST-attB
Sf-OATP74D-XbaI-R	ATCCTCTAGAGGTACCCTCGAGCCGCTCTAGATCAGAGTTGTGTATCGGATGGGTTTG	

Sf-OATP74D-XbaI-F	GTACtctagaATGACGGCGAACGTTGTC	Cloning *SfOatp74D* in pBmAc3
Sf-OATP74D-NotI-R	GTACgcggccgcTCAGAGTTGTGTATCGGATGGGTTTG	
Sf-OATP74D-BspEI-**V5**-NotI-R	GTACgcggccgcTCA**GGTAGAGTCCAGACCCAGCAGAGGGTTAGGGATAGGCTTACC**TCCGGAACCGAGTTGTGTATCGGATGGGTTTG	Cloning of tagged with V5 epitope *SfOatp74D* in pBmAc3
Dm-OATP74D-XbaI-F	GTACtctagaATGACGAAGAGCAATGGCGATG	Cloning *DmOatp74D* in pBmAc3
Dm-OATP74D-NotI-R	GTACgcggccgcCTAGACCGTCGTGTCCGGC	
Ha-OATP74D-BamHI-F	GTACggatccATGACGGCGAACGTTGTC	Cloning of tagged with V5 epitope *HaOatp74D* in pBmAc3
HaOATP74D-BspEI-R	GTACtccggaACC GAGCTGAGTGTCTGGACGAG	
pEIA-Fgibson	AGTCGTTTGGTTGTTCACG	Primers used for the synthesis of the novel plasmid piE1:puro-BmAc3 (small letters indicate complementary sequences with the pEIA vector)
pEIA-Rgibson	TTATACATATCTTTTGAATTTAATTAATTATACATATATTTTATATTATTTTTG	
PAC-Fgibson-AscI	*taaattcaaaagatatgtataa*ggcgcgcc*TCAGGCACCGGGCTTGCG*	
PAC-Rgibson-NcoI	*gtgaacaaccaaacgact*ccatgg*ATGACCGAGTACAAGCCCACG*	
Hz_OATP74D_F_sgRNA#1	**CAAG**GCTATTCCTGACGTACCTGG	Phosphorylated primers used for generating dsDNA for cloning into BbsI digested pBmAc3:Cac9-HaU6:1 vector
Hz_OATP74D_R_sgRNA#1	**AAAC**CCAGGTACGTCAGGAATAGC	
Hz_OATP74D_F_sgRNA#2	**CAAG**GTGTCCCTACTGTATCATCAG	
Hz_OATP74D_R_sgRNA#2	**AAAC**CTGATGATACAGTAGGGACAC	
Hz_OATP74D_F_sgRNA#3	**CAAG**GATCTCTTGACTGCAAACACA	
Hz_OATP74D_R_sgRNA#3	**AAAC**TGTGTTTGCAGTCAAGAGATC	
HzOATP74D-F-5UTR	GGTCACATAGACTTGATAGCATAG	Genotyping of CRISPR mediated deletion yielding a PCR fragment equal to 912bp
HzOATP74D-R-exon1	GTTGTCCCTACTGTATCATCAGC	
Hz_HR3_F	CTCTTGAAATCTGGCTCGTTCG	Primers used for gene expression analysis of ecdysone responsive genes
Hz_HR3_R	CACACATTCTCTGATGGACAGCAC	
Hz_E74A_F	GTGGAGTCGTCTTCATCAGG	
Hz_E74A_R	CTGGTGGTGCTGGTAGAAG	
Hz_EcR_F	CAACAACCAGGCGTACACTC	
Hz_EcR_R	CAGCGTGTTCAGGTAATATCTCTGGAT	
Hz_Eip75B_F	CCTCAACGGCGTGGTGAAA	
Hz_Eip75B_R	GAGTGGGTTGCGAGTAGGTG	
caspase-3 qPCR_F	ATGTGTGTCACTATCCTAAGCCAC	
caspase-3 qPCR_R	AGCATCCATACTAGCACCTCTG	
caspase-6 qPCR_F	GCTGTGATCAGTGCTACGGAT	
HzAW1_GADPH_F	GAACATCATTCCCGCCTCCA	
HzAW1_GADPH_R	TCGGATGACACAACCTGCTC	
HzAW1_RPS3A_F	GCTCATCCCCGACTCCATTG	
HzAW1_RPS3A_R	CTTGCCACCACCACCTTCTC	
caspase-6 qPCR_R	CCGAATCAGCTGCATACATT	
Sf9_EcI_q-RT-PCR_F	ACTGACAGACAAGACAAAGCGATG	RT-PCR validation of *Oatp74D* expression in Sf-9 and HzAW1 cells
Sf9_EcI_q-RT-PCR_R	CCTTGCTCCACACCAAAATGTC	
HzAW1_EcI_q-RT-PCR_F	ACTGATAAACAAGACAAAGCGATGG	
HzAW1_EcI_q-RT-PCR_R	AGGAACATTAGGGTTGCTGATAG	

Table 4.2: P-values of Student's t-test for un-paired comparisons in gene expression analysis between the HzAW1^{WT} and HzAW1$^{\Delta Oatp74D}$ cells.

	HzEcR	*HzHr3*	*HzEip74A*	*HzEip75B*
6hrs	0.0053	0.0032	0.0046	0.4597
9hrs	0.0048	<0.0001	0.0321	0.0011
12hrs	0.0031	0.0002	0.0451	0.003
24hrs	0.0379	<0.0001	0.026	0.0744

4.5 References

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General discussion

Functional validation of Insecticide resistance mechanisms using *Drosophila*

In the first chapter of this thesis, we clarified the contribution of two target site resistance mutations against Sodium channel blocker insecticides (SCBIs), a class of neurotoxic compounds frequently used for the control of agricultural pest species (Silver et al., 2014). Two mutations in IVS6 domain of the sodium channel, F1845Y and V1848I (*P. xylostella* numbering), have been identified in resistant populations of two lepidopteran pests *T. absoluta* and *P. xylostella* and associated with resistance against SCBIs based on the high frequency rates they were identified in the field (Wang et al., 2016, Roditakis et al., 2017). Toxicity bioassays in genome modified flies revealed that both of these mutations confer low-to-moderate resistance against indoxacarb. In the case of metaflumizone, the V1848I mutation seemed to impose a low effect, in sharp contrast with the F1845Y mutation which had a much stronger impact by several orders of magnitude. Our results do not just point out the correlation of the mutations with the phenotype but they actually reveal their effect size against each chemical compound, which is extremely important for resistance management. Herein we have proved that the F1845Y mutation has a greater effect to metaflumizone compared to indoxacarb. This is suggestive of the possible failure of metaflumizone to achieve the expected levels of control in field populations exhibiting high frequencies of this allele, while indoxacarb might be more effective. This knowledge facilitates monitoring the genetic diversity of an insect population (e.g. through the use of diagnostics) prior to the application of an insecticide to make the right decisions regarding what compound should be used.

Our results concerning the relative resistance of the mutations against indoxacarb were corroborated by an *in vitro* study performed in *Xenopus oocytes* (Jiang et al., 2015); however, they exhibited a different trend concerning metaflumizone resistance. It is worth to mention that the *in vitro* study was based on electrophysiology experiments performed in *Xenopus* oocytes overexpressing wild type and mutated sodium channel of cockroach (Jiang et al., 2015). Although *Drosophila para* shares almost ~78% identity with the VGSC of the cockroach, the different effects posed by the mutant cockroach and *Drosophila* sodium channels against metaflumizone could be attributed to different channel-specific residues that potentially alter the gating and kinetic properties in a manner that affects the accessibility of metaflumizone. This has been proposed for the L1014F (*kdr*) mutation as an alternative explanation of the lower accessibility of pyrethroids to the sodium channel (Silver et al., 2014). Therefore *in vitro* electrophysiology studies need to be performed to delineate whether there are inherent differences in the pharmacology of cockroach versus *Drosophila* sodium channels towards SCBIs.

Functional validation of the *in vivo* contribution of these mutations to resistance against indoxacarb and metaflumizone is the basis for developing molecular diagnostics that can be used for monitoring the resistance profile in the field (Van Leeuwen et al., 2020). This task is a very important issue for making the right decision in the use of insecticides to finally manage effective pest control (Van Leeuwen et al., 2020). Both F1845Y and V1848I mutations have been found in high frequencies in resistant populations of *T. absoluta* and *P. xylostella* (Wang et al., 2016, Roditakis et al., 2017), and their contribution to SCBIs resistance has been validated *in vitro* and *in vivo*

(Samantsidis et al, 2019, Jiang et al., 2015). Therefore, both mutations could be utilized for the generation of DNA-based molecular diagnostics with high predictive value of SCBI resistance. An example is the development of PCR-RFLP based diagnostic for identification of either F1845Y or V1848I mutations in *T. absoluta* (Roditakis et al., 2017). Using such molecular diagnostics could readily predict the abundance of these mutations and in advance resistance levels in the field, indicating whether SCBIs could be used or should be replaced by other chemical class.

It needs to be considered, though that such molecular diagnostics have to be carefully designed given that insecticide resistance is a complex evolutionary phenomenon and usually is manifested as polygenic, which means that more than one genetic factors can be correlated with the final resistance phenotype (ffrench-Constant, 2013). Two SCBIs resistant *P. xylostella* populations were tested for metabolic resistance mechanisms by using metabolic synergists, demonstrating the possible interplay of P450s and esterases for resistance against indoxacarb (Wang et al., 2016). This was also observed in the indoxacarb resistant populations of *T. absoluta* (Roditakis et al., 2017). Hence, there might be additional mechanisms that could confer resistance against these insecticides and potentially interact with these target site mutations augmenting resistance ratios to levels similar to what is observed in the field. This kind of putative synergistic epistasis between different resistance mechanisms has been reviewed for several insects, like *Musca domestica* and *Culex quinquefasciatus* (Hardstone et al., 2010), however a functional validation has yet to be performed.

In the next chapter we sought to functionally validate the combined effect of different mechanisms of pyrethroid resistance and more specifically between target-site resistance mutations and overexpressed cytochrome dependent P450 monooxygenases, derived from the major anthropophilic mosquito *Aedes aegypti*. A previous study in mosquitoes has indicated the multiplicative interaction between target site and metabolic resistance mechanisms, simply by crossing different strains bearing individual mechanisms (Hardstone et al., 2009). However, this approach could not eliminate the possible existence of confounding genetic factors that could arise from the different genetic backgrounds. In an attempt to circumvent the limitations that different genetic backgrounds may impose, we used *Drosophila melanogaster* in order to investigate the effect of the interaction between the sodium channel mutation V1016G and the P450 CYP9J28, two of the most consistently detected resistance mechanisms in this species (Vontas et al., 2020, Smith et al., 2016). Toxicity bioassays revealed a synergistic interaction between these two mechanisms given that the final resistance ratio was many times greater than the resistance ratio of each mechanism alone. A synergistic epistasis was also observed in another combination of resistance mechanisms, the *kdr* mutation L1014F along with the overexpression of CYP6BQ23 (Samantsidis et al., 2020). Furthermore, our results are corroborated by a very recent report, in which L1014F mutation was combined with the over-expression of a Glutathione S transferase (*Gste2*), increasing the levels of tolerance to permethrin compared to mosquitoes carrying each mechanism alone (Grigoraki et al., 2021). Therefore, it seems highly possible that striking resistant phenotypes observed in the field are potentially attributed to the interaction of these two mechanisms when found in the same genetic background. This has significant ramifications for the molecular diagnostics already used in the field. Molecular diagnostics are typically used for monitoring resistance in the field as an alternative method to diagnostic bioassays, as long as the gene markers used for diagnosis are functionally related with the resistance levels (Van Leeuwen et al., 2020). Most of the diagnostic platforms are based on single gene-markers ignoring other resistance loci. Our results here showed that single resistance alleles display minor effects, while the existence of two loci

increased dramatically the phenotype. Therefore, the available diagnostics could infer wrong estimates about the real resistance status in insect populations. This raises the need for establishment of multiplex platforms that use more than one gene-marker, which first have to be validated for their contribution to resistance, as we have performed herein.

Moreover, the interaction of these different alleles imposed significant disadvantages in the insects' fitness, most importantly it affected the developmental time (pupation) but also the total fecundity. Similarly, the combination of two *kdr* mutations with up-regulated P450s has been proved to exert significant fitness costs in *Aedes aegypti*, affecting the development, fecundity and adult longevity (summarized in Smith et al., 2016). Fitness cost analysis is not a trivial task to address, because there are many different traits that a certain mechanism could affect. For example, *kdr* mutations in *Aedes aegypti* have not been found to affect any specific measurable trait (Brito et al., 2013); however, population cage analysis indicated a significant reversion to the susceptible form after several generations without pyrethroid selection (Smith et al., 2021). Therefore, several experiments need to be performed to identify the existence of fitness costs posed by a certain mechanism, for which it is important to work in a well defined genetic background. *Drosophila* is a well suited tool reducing confounding genetic factors arising from different genetic backgrounds that would compromise any inferences drawn.

Our work is of major significance since we can now understand why highly resistant populations in the field entail mechanisms that individually confer low to moderate levels of resistance. This has been observed for example in *Tetranychus urticae*, where using marker assisted inbreeding the P450 CYP392A16 was introgressed in a susceptible background and finally proved to confer low resistance ratios against abamectin, in sharp contrast with the high resistance levels of the initial resistant strain which also entail other mechanisms of resistance (Papapostolou et al., 2022). The striking resistance phenotypes along with the possible fitness costs induced by the interaction of different resistance alleles should really be considered by Insecticide Resistance Management strategies. Resistance alleles that are associated with fitness costs tend to revert back to their susceptible form after the cessation of the selective pressure (Freeman et al., 2021). Thus, this should be taken into account for considering practices such as rotation of different modes of action in order to achieve effective control of a population. Furthermore, the synergistic epistasis between different resistance alleles may also reduce the efficacy and the predictive value of the available molecular diagnostic tools, that have been designed for monitoring resistance levels in the field. Therefore, it is necessary these tools to be carefully interpreted and calibrated, in a manner that they will be able to identify more than one resistance mechanisms and to explain the largest part or the complete phenotype. Our work provides a proof of principle for the functional validation of the synergistic interactions between different resistance mechanisms and enables to gain insight on their specific role to the resistance phenotype.

Functional characterization of novel insecticide targets in non-model lepidoptera species

Pest control is getting more and more ineffective over the years; insecticide resistance cases are exponentially increasing while the portfolio of available eco-friendly and harmless to human insecticides is gradually diminishing (Sparks et al., 2021). Therefore, there is an increasing need for identifying novel targets for insecticides

aiming to more integrated and sustainable control strategies with high selectivity. Herein we functionally characterized the role of the *Oatp74D* ortholog of two lepidopteran pests, *H. armigera* and *S. frugiperda*, with the prospect to use this protein as a putative insecticide target, provided that it functions as an ecdysone importer and is actually essential for the development of these species. Targeting developmental stages of such species could counteract the consequences they impose on the plants they infest.

We have proved that HaOATP74D and SfOATP74D are sufficient for uptake of ecdysone into the target cells. This observation highlights that the lepidopteran OATP74Ds function as primary regulators in the canonical ecdysone pathway. Inhibition of the ecdysone pathway (e.g. inhibition of ecdysone biosynthesis) leads generally to developmental defects, changes in the final body size and even lethality (Kannangara et al., 2021). Under this hypothesis we wondered if the OATP74D would be actually essential for the development of lepidoptera species. CRISPR mediated disruption of the gene in the G_0 generation of *S. frugiperda* indicated that the gene is essential for embryo hatching as well as for the developmental transitions of larvae. This underscores that inhibition of the protein *in vivo* would lead to lethality even from the early developmental stages, which is corroborated by a similar approach performed in the mosquito species *Aedes aegypti* (Hun et al., 2021). Therefore, any inhibitor targeting specifically this protein could lead to early developmental arrest. Moreover, heterologous expression of the lepidoptera OATP74D in HzAW1 cells and subsequent immunostaining indicated that both proteins are localized at the membrane of the cells. This underscores their higher accessibility to inhibitors compared to an intracellular protein like EcR, that is any compound could be designed to target specifically the extracellular domains without being necessary to pass through the lipid bilayer of the cellular membranes.

Another requirement that a putative drug target should meet is the sensitivity to inhibition of their function by certain chemical compounds. To this end we generated stable cell lines that over-express either of the two lepidopteran OATP74D and we tested two inhibitors of mammalian Organic anion transporters, rifampicin and telmisartan (Dolberg et al., 2018, Li et al., 2014). Rifampicin seemed to be promising given that it leads to reduction of the ecdysone mediated luciferase expression, indicating lower uptake of the hormone. However, the mechanism of inhibition mediated by this drug remains elusive. The present results indicate that these proteins are susceptible to inhibition to certain chemical classes (only to rifampicin and not to telmisartan). Although rifampicin is active against human OATPs, synthetic chemistry (such as produgs) may increase the selectivity towards to the species of interest and avoid non target species. For instance, indoxacarb is a synthetic pro-insecticide (prodrug) which requires biotransformation mediated by insect specific enzymes to yield the more potent VGSC inhibitor DCJW062, while in humans the prodrug is immediately metabolized producing less toxic products (Silver et al., 2014). The cell based platform we generated has a biotechnological aspect with applicability for the synthesis and screening of such novel compounds.

In the context of drug target characterization it is important to underline the biological pathways affected when the function of the protein of interest is blocked or prevented. An adverse outcome pathway (AOP) has been reported for Ecdysone Receptor agonists, in order to describe the key events that may occur between the block of the target and the final phenotype induced (Song et al., 2017). Establishment of adverse outcome pathway is necessary for making a causal relationship between the disruption of the function of a protein (biological target) to the adverse outcomes imposed, and may be used along with *in silico* approaches (conservation of the target) to

predict the environmental hazards and risks of chemicals (Song et al., 2017, Bal-Price et al., 2017). In here we have demonstrated that block of this protein inhibits ecdysone uptake and disrupts the transcriptional regulation of early ecdysone responsive genes, as well as of genes implicated in biological processes related with cell death. Our study along with studies in *Drosophila*, *Tribolium* and mosquitoes (Okamoto et al., 2018, Rosner et al., 2021, Hun et al., 2021) provide the basis for initiating the construction of an AOP. However, further experiments *in vivo*, including pharmacological evidence, are required to generate an integrated AOP.

Another important criterion for an insecticide target is to share low conservation with non-target species, such as beneficial insects and humans. The insect ecdysone pathway and *Oatp74D* have no orthologs in humans, indicative of its selectivity towards arthropods. Considering though other ecdysozoan species, phylogenetic analysis in SLCO transporters has indicated that several arthropods like *Apis mellifera* and *Daphnia magna* share one-to-one ortholog with *Drosophila* and lepidoptera species (Samantsidis et al., in preparation). Nevertheless, *A. mellifera* and *D. magna* OATP74D share less than 40% aminoacid identity with the *H. armigera* and *S. frugiperda*, which indicates a low probability for cross-reaction effects by any inhibitor. In the era of next generation pesticides, selectivity and target specificity can be increased, especially by the use of plant mediated RNAi (Birgul Iyison et al., 2021). However, it is not known yet if lepidoptera species are susceptible to plant-mediated RNAi, given their intrinsic property to degrade dsRNAs (Liu et al.,2020, Terrenius et al., 2011). Until then, carefully designed species-specific chemical insecticides (and even pro-insecticides) could be developed against this protein. Even more, oral delivery methods along with application on the plant-hosts of these pests may also be considered to increase safety for avoiding off-target effects. Of note, *Oatp74D* is expressed in lepidopteran midgut tissues (Ioannidis et al., 2022) which is the first tissue encountered by orally delivered xenobiotics (Denecke et al., 2018). This shall reduce the time of action of the oral insecticides and the risk of detoxification mechanisms to act against the compound but also may decrease the necessity for high bioavailability.

Our study provided also fundamental knowledge for the ecdysone signaling pathway. It is hypothesized that ecdysone signaling is activated by both the non-genomic and the genomic pathways (Zhao et al., 2020). The non-genomic pathway entails several ecdysone responsive GPCRs that regulate post-translational modifications of EcR and USP (Chen et al., 2017, Kang et al., 2017), while the genomic pathway involves the incorporation of ecdysone into the target cells to trigger target gene expression through the EcR-USP transcription factor. Ecdysone has been proved to be taken up by the target the cells through the OATP74D in *Drosophila*, but this mechanism has not been generalized to other insect species. Phylogenetic analysis has demonstrated that *Drosophila* shares one-to-one ortholog with many insect species, including *S. frugiperda* and *H. amrigera* (Okamoto et al., 2018). Herein we have functionally proved that both lepidopteran OATP74D mediate the incorporation of ecdysone into the target cells, and apart from that we have demonstrated that elimination of OATP74D completely inhibits the ecdysone pathway, regardless of the GPCRs function.

Overall, this study provided useful information about the role of OATP74D in ecdysone pathway in the context of both fundamental and applied biological research. Most importantly i) we have found that OATP74D is an essential protein for two major lepidopteran pests ii) we have demonstrated that lepidopteran OATP74D regulates the ecdysone signaling pathway and iii) we have generated a cell based platform for screening novel insecticidal compounds, to conclude that this protein may serve as a novel and promising insecticide target.

Conclusions

Taken all together, we believe that with this thesis we provided useful information in the research community regarding insecticide resistance mechanisms and pest management. We showed how different mutations may confer resistance against different compounds of the same class and measured their effects, highlighting their significance for monitoring resistance in the field. We provided concrete proof that addresses an old enigma in insect toxicology, which is significant in the context of both basic and applied biology. In terms of basic biological research we showed that epistatic effects between resistance mechanisms may manifest in the field as an evolutionary response to the use of pesticides. We proved that interaction between different alleles not only increased the tolerance against the pressure posed by the stressor but may impose significant changes in the fitness of the insects, affecting developmental time as well as reproduction success. The main fundamental output of this research was underlined by the mechanistic model we proposed which underlines a possible antagonistic effect between the metabolic derivatives of the parent compound mediated by the activity of the cytochrome P450 and the actual parental compound to bind on the modified target. If this hypothesis is validated in the future, then new gates in insecticide biology may open. Nevertheless, significance is also underscored in the context of application in the field. As we mentioned, the synergistic interaction between target site and metabolic resistance comes to explain the huge resistance displayed by field populations to the insecticides when these carry alleles with minor effects.

We generated a promoter-based genetic toolkit which can be employed in the future for transgenic lepidoptera species. Most importantly we constructed ready to use plasmids for performing CRISPR and RNAi in lepidoptera cell lines but also *in vivo* (only CRISPR). Finally, we endeavored to characterize a putative drug target. We highlighted the significance of the OATP74D in the development of the lepidoptera species *S. frugiperda*, and we provided evidence supporting that this protein is responsible for the uptake of the steroid hormone ecdysone by the target cells. This work strengthened the hypothesis that steroid hormones like ecdysone require a transporter for uptake, a knowledge that cannot be generalized solely based on studies on *Drosophila* and mosquitoes. In addition we provided a biotech-tool for use in the future, that is the knock-out cell line which lacks the ecdysone transporter and may be employed for several assays in both applied and basic research. This integrated study might be the beginning for a new and promising target for insecticides.

Future directions

In the first chapter of the PhD, we observed different responses against indoxacarb and metaflumizone, yet the underlying mechanism still remains elusive. It would be rather interesting to validate the contribution of these mutations directly in the species of interest. A proof-of-concept for CRISPR in *P. xylostella* has been already performed (Huang et al., 2017), therefore it would be rather interesting to study the contribution of these mutations to resistance against SCBIs directly in this species, eliminating confounding genetic and evolutionary factors distinct between *Drosophila* and Lepidoptera that would complicate inferences drawn. Furthermore, given that resistant field population might entail both target site and metabolic resistance mechanisms against SCBIs, it would be rather interesting to validate such an interplay between the mutations F1845Y or V1848I and any putative metabolic resistance gene that would come up from future studies.

The second chapter provided a proof-of-principle for the functional validation of the synergistic epistasis between metabolic and target site resistance mechanisms. However further analysis in the future should be performed in order to shed light on the mechanistic background of synergism theory. Heterologous expression of both wild type and mutated sodium channels in *Xenopus* oocytes would help to support our theory about the binding affinity of 4-hydroxy-deltamethrin with the target and whether it antagonizes the binding of the parental compound or not. Furthermore, it would be interesting to combine these resistance alleles with other mechanisms, such as penetration resistance, as soon as candidate genes for this mechanism become available for investigation.

In Chapter 3 we tried to identify Pol II promoters with midgut activity. It would be interesting to analyze *in vivo* the activity of the Pol II promoters we identified, however lack of techniques for germ-line transformation does not allow such an analysis. In the future we would like to establish a germ line transformation system in *H. armigera*, like the Piggy-bac transposon-based which has been described in *P. xylostella* (Martins et al., 2012), *Bombyx mori* (Tamura et al., 2000), *Spodoptera frugiperda* (Chen et al., 2021). Validating the activity of these promoters also *in vivo* and identifying a midgut specific promoter, would be very valuable for performing several techniques like tissue specific exogenous expression or RNAi (using systems like the binary GAL4-UAS system). RNAi is a valuable technique in drug target identification; however, RNAi mediated by injections using dsRNAs is highly labile in lepidoptera species (Terrenius et al., 2011, Liu et al., 2020). To this end the use of the GAL4-UAS system would be a perfect alternative augmenting robustness and reproducibility of experimental procedures.

Furthermore, given that both HaOATP74D and SfOATP74D seemed to be sensitive to a certain compound, we would like to screen more chemical compounds and proceed with *in vivo* tests of these compounds. Moreover, we would like to use the cell based platform and generate stable cells expressing an array of OATP74D orthologs of beneficial insects, e.g. *Apis mellifera*, so that to identify chemical compounds with high selectivity towards the species of interest. Finally given that there are several different analogs of ecdysone, like makisterone, ponasterone-A, which share similar structure with ecdysone, we would like to test whether these are substrates of OATP74D or not.

The increased awareness regarding the safety of the chemical insecticide-based pest control (concerning the safety for both environment and humans) has turned the scientific community to the development of alternative and sustainable (biotechnology based) methodologies, such as gene drive. Gene drives are systems that enable the inheritance of a certain allele at super-Mendelian frequency, that is more than 50% of the time. These systems have been proposed to be employed either for the replacement of an insect population (e.g. spreading alleles for eliminating resistance, or expressing anti-parasitic factors) or for suppressing the population (Price et al., 2020). Several systems have been described for insect population manipulation, such as transposable elements, Medea-like systems, genetic incompatibility systems, homing endonucleases and others (Price et al., 2020). Homing endonucleases are characterized as low-throughput drive systems, that is only a small number of individuals is required so that to take over in a population, and CRISPR/Cas belongs to this category (Bier, 2022). The main idea relies on the use of a cassette, expressing Cas9 under the regulation of a specific promotes along with sgRNAs targeting the region of interest. Additionally, this cassette may also include other possible genes e.g. genes expressing proteins that target a parasite. Such a technology would have great application for the elimination of resistance in the field or for the extinction of human disease vectors, such as the mosquitoes of the genus *Anopheles*. A proof of principle gene drive was successfully developed in *Anopheles stephensi*, where the authors described the introduction of an antimalarial effector gene into a wild type population under laboratory conditions (Gantz et al., 2015). Similarly, this technology was also applied in *Anopheles gambiae*, where the drive was employed to induce recessive mutations at genes responsible for the fertility of the insects, thus enabling the transmission of the drive in the field that will finally lead the wild type population in collapse (Hammond et al., 2016). Although promising, the application of this system still has some risks. Specifically, there is always a risk for target site resistance against the driver, which entail mutations in the target region which do not allow the hybridization of the gRNA. To this end careful design, identification of highly conserved genes and several field simulating experiments have to be performed in order to employ this method for pest/vector control.

Another mean for pest control with less risk for posing environmental impacts compared to the gene drive systems is the use of microbial biopesticides, like *Bacillus thuringiensis*. This species has been successfully used for control of several pests due to their ability to produce toxins (e.g. Cry toxins), which target the midgut of insects. However, there are several other bacterial species that have been found to produce toxins with a broad spectrum of insecticidal activity, such as *Yersinia entomophaga* and *Pseudomonas entomophila*, which produce toxins or/and chitinases (all commercialized bacteria species are reviewed in Ruiu, 2018). Another type of microbial biopesticides are also viruses like the baculoviruses, yet their high cost for production and the low stability in the environment limit their use in pest management. Fungi and nematodes have also been used for pest control. Most of these biopesticides exhibit a narrow range of target species compared to the chemical insecticides, for example specific Cry toxins target only a limited number of lepidopteran species. Therefore, they provide a more eco-friendly and generally safe means for effective pest control and certainly further research should focus on this field in order to increase their stability and efficacy.

References: General discussion & Future directions

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Curriculum Vitae

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From: 28/05/2016 To: 25/07/2016

Masters Rotation II
PI: Christos Tsatsanis, Laboratory of Clinical Chemistry, School of Medicine, UoC
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- Papapostolou, K.M., Riga, M., **Samantsidis, G.R.**, Skoufa, E., Balabanidou, V., VanLeeuwen, T., Vontas, J. **(2022)** Overexpression *in cis* of the midgut P450 CYP392A16 contributes to abamectin resistance in *Tetranychus urticae.* Insect Biochemistry and Molecular Biology. 142. doi.org/10.1016/j.ibmb.2021.103709
- **Samantsidis, G.R.**, Denecke, S., Skavdis, G., Swevers,L., Geibel, S., Vontas, J. (**2022**) Identification of *Helicoverpa armigera* promoters for biotechnological applications. Insect Biochemistry and Molecular Biology 142, 103725
- **Samantsidis G-R**, Panteleri R, Denecke S, Kounadi S, Christou I, Nauen R, Douris V, Vontas J. **(2020)** 'What I cannot create, I do not understand': functionally validated synergism of metabolic and target site insecticide resistance. Proc. R. Soc. B 287: 20200838. 10.1098/rspb.2020.0838
- **Samantsidis, G.R.**, O'Reilly, A., Douris, V., Vontas, J.**(2019)** Functional validation of target site resistance mutations against Sodium Channel Blocker Insecticides (SCBIs) via molecular modeling and genome modification in Drosophila*.* Insect Biochemistry and Molecular Biology, (104), 73-81. doi: 10.1016/j.ibmb.2018.12.008.

Conferences

- **George-Rafael Samantsidis**, Andrias O. O'Reilly, Vassilis Douris, John Vontas. (2019) *Functional validation of the contribution of sodium channel mutations F1845Y and V1848I against Sodium Channel Blocker Insecticides (SCBIs) via CRISPR-Cas9 genome engineering in Drosophila melanogaster.* Eighth International Symposium on Molecular Insect Science, 7-10 July, Sitges, Barcelona, Spain.
- Douris V, Papapostolou KM, **Samantsidis GR**, Panteleri R, Christou IK, Riga M, Nauen R, Van Leeuwen T, Vontas J. (2018) *Dissecting insecticide resistance via genetic manipulation and genome modification in Drosophila.* XI European Congress of Entomology (ECE2018), 2-6 July, Naples, Italy.
- V. Douris, M. Riga, A. Ilias, R. Panteleri, I.K. Christou, S. Kounadi, K.M. Papapostolou, **G.R. Samantsidis**, M. Kefi, T. Van Leeuwen and I. Vontas. (2017) *Investigation of the contribution of different molecular mechanisms of insecticide resistance through heterologous expression of genes and targeted genome editing in Drosophila.* 17th Hellenic Conference of Entomology 19-22 September at Agricultural University of Athens, Greece.
- Saishyam, N., Gustafsson, C., **Samantsidis G.R.**,Cohn, M. (2014) *In vitro assessment of the protective role of Cdc13 against degradation of telomeric single-stranded 3' overhang,* Proceeding in the international conference on **"Telomeres, Telomerase and disease"** 30 April - 4 May at Husa President Park Hotel, Brussels, Belgium.
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- Immunofluorescence and Cryotome using
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Publications

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Functional validation of target-site resistance mutations against sodium channel blocker insecticides (SCBIs) via molecular modeling and genome engineering in *Drosophila*

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ABSTRACT

Sodium channel blocker insecticides (SCBIs) like indoxacarb and metaflumizone offer an alternative insecticide resistance management (IRM) strategy against several pests that are resistant to other compounds. However, resistance to SCBIs has been reported in several pests, in most cases implicating metabolic resistance mechanisms, although in certain indoxacarb resistant populations of *Plutella xylostella* and *Tuta absoluta,* two mutations in the domain IV S6 segment of the voltage-gated sodium channel, F1845Y and V1848I have been identified, and have been postulated through *in vitro* electrophysiological studies to contribute to target-site resistance.

In order to functionally validate *in vivo* each mutation in the absence of confounding resistance mechanisms, we have employed a CRISPR/Cas9 strategy to generate strains of *Drosophila melanogaster* bearing homozygous F1845Y or V1848I mutations in the *para* (voltage-gated sodium channel) gene. We performed toxicity bioassays of these strains compared to wild-type controls of the same genetic background. Our results indicate both mutations confer moderate resistance to indoxacarb (RR: 6–10.2), and V1848I to metaflumizone (RR: 8.4). However, F1845Y confers very strong resistance to metaflumizone (RR: > 3400). Our molecular modeling studies suggest a steric hindrance mechanism may account for the resistance of both V1848I and F1845Y mutations, whereby introducing larger side chains may inhibit metaflumizone binding.

1. Introduction

Voltage-gated sodium channels (VGSCs) conduct sodium ions across the plasma membrane of neurons to generate and propagate electrical signals, which facilitate an animal's movement and response to various environmental stimuli (see review by Carnevale and Klein, 2017). VGSC α-subunits are comprised of four homologous domains (I-IV), each having six membrane spanning helical segments (S1−S6) (Catterall, 2017). Recent VGSC structures determined by cryo-electron microscopy reveal how pore gating is coupled with movement of the voltage sensors that detect transmembrane potential changes (Shen et al., 2017, 2018; Yan et al., 2017). A continuous ion-conducting pathway through the pore is found in the open-state electric eel VGSC structure (Yan et al., 2017) as the pore-lining S6 helices are dilated at the cytoplasmic entrance.

VGSCs are the primary targets of many inhibitory chemicals such as local anesthetics (analgesics, antirrhythmic drugs) in vertebrates as well as chemical insecticides in insects that suppress neurons' excitability and their high-frequency discharges (Gawali et al., 2015). Indoxacarb (a pyrazoline type insecticide) and metaflumizone (Fig. 1) belong to the family of Sodium Channel Blocker Insecticides (SCBIs; von Stein et al., 2013) that bind to the open channel pore when the membrane is still depolarized and cause a shift in the voltage dependence of slow inactivation to more negative potentials. Thus, VGSCs are stabilized in the inactivated state leading to termination of the intracellular sodium influx (Silver and Soderlund, 2007; Silver et al., 2010, 2017; Jiang et al.,

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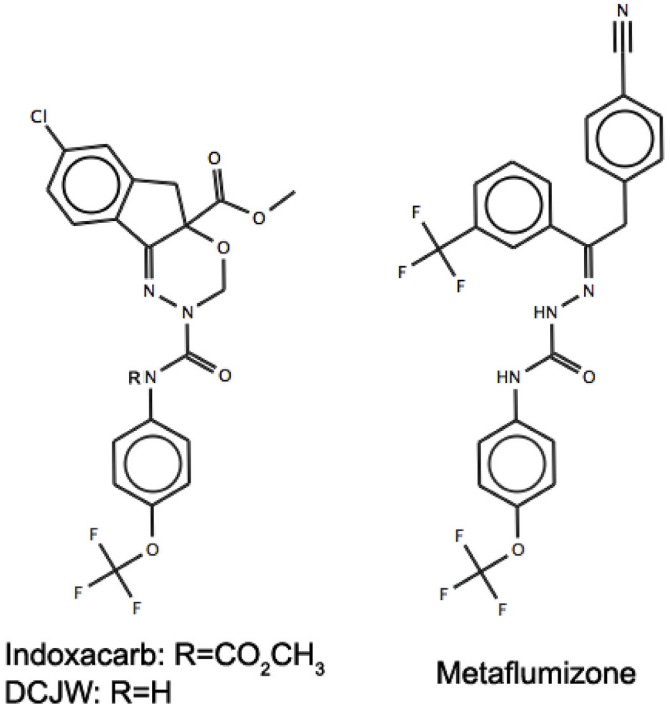


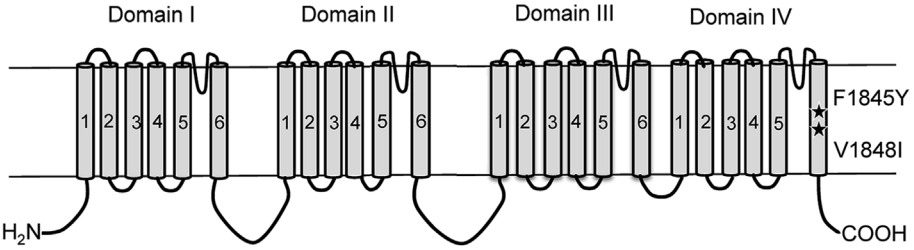
Fig. 1. Chemical structures of sodium channel blocker insecticides.

2015; Zhang et al., 2016).

Indoxacarb is an insecticidal oxadiazine (Fig. 1) characterized as a pro-insecticide since it has to be converted to the active metabolite N-decarbomethoxylated JW062 (DCJW), a secondary product generated by the hydrolyzing activity of insect esterases or amidases, which underlies its action selectivity against insects (Zhang et al., 2016). Indoxacarb is used against moths, beetles, leafhoppers, weevils, flies and other pests (Silver et al., 2010). Spraying treatment of *Drosophila* with DCJW is also effective and eventually causes mortality (Zhang et al., 2013). Metaflumizone belongs to the category of semicarbazones, which are ring-opened dihydropyrazoles (von Stein et al., 2013) (Fig. 1). Metaflumizone exhibits low toxicity to mammals and selectivity towards insects (Hempel et al., 2007) and, unlike indoxacarb, it does not require metabolism to produce the active compound.

Resistance against SCBIs have been reported in insects such as the housefly *Musca domestica* (Shono et al., 2004), the lepidopteran pests *Choristoneura rosaceana* (Ahmad et al., 2002), *Plutella xylostella* (Khakame et al., 2013; Wang et al., 2016; Zhang et al., 2017), *Spodoptera exigua* (Tian et al., 2014), *Helicoverpa armigera* (Bird et al., 2017) and *Tuta absoluta* (Roditakis et al., 2017) and the cockroach *Blatella germanica* (Liang et al., 2017). The cross-resistance spectrum between indoxacarb and metaflumizone is not clear: indoxacarb-selected *T. absoluta* strains exhibit only a limited Resistance Ratio (RR) increase for metaflumizone (Roditakis et al., 2017) whereas earlier studies of indoxacarb-resistant populations of *P. xylostella* indicate no cross-resistance to metaflumizone (Khakame et al., 2013). Conversely, a population of *Spodoptera exigua* with 942-fold resistance to metaflumizone exhibits only 16-fold resistance to indoxacarb (Su and Sun, 2014). On the other hand, selection of indoxacarb in the field confers cross-resistance to metaflumizone in at least one population of *P. xylostella* (Wang et al., 2016). There is evidence for synergistic effects of metabolic inhibitors on SCBI toxicity, implicating metabolic resistance mechanisms involving esterases or oxidases (Wang et al., 2016; Liang et al., 2017). However, synergists only partially reduced resistance against indoxacarb in *T. absoluta* (Roditakis et al., 2017) and their use suggested a limited role of detoxification in metaflumizone resistance in *Spodoptera exigua* (Su and Sun, 2014).

Resistance levels to both indoxacarb and metaflumizone are significantly correlated to the frequencies of two VGSC mutations, F1845Y and V1848I, identified in the domain IV S6 segment (Fig. 2), in two field populations of *P. xylostella* (Wang et al., 2016). The same mutations were identified in SCBI-resistant populations of *T. absoluta*, collected from tomato greenhouses from Italy and Greece (Roditakis et al., 2017). When F1845 and V1848 (*P. xylostella* numbering) mutations were tested using electrophysiology studies of heterologous expressed *B. germanica* VGSCs in *Xenopus* oocytes, it was found that F1845Y and



IVS6

PxNa$_v$	G	I	T	Y	L	L	S	Y	L	V	I	S	**F**	L	I	**V**	I	N	M	Y	I	A	V	I	L
TaNa$_v$	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
LepF1845Y	.	.	.	.	.	.	.	.	.	.	.	.	**Y**	.	.	.	.	.	.	.	.	.	.	.	.
LepV1848I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**I**	.	.	.	.	.	.	.	.	.
DmNa$_v$	.	.	.	**F**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DmF1845Y	.	.	.	**F**	.	.	.	.	.	.	.	.	**Y**	.	.	.	.	.	.	.	.	.	.	.	.
DmV1848I	.	.	.	**F**	.	.	.	.	.	.	.	.	.	.	.	**I**	.	.	.	.	.	.	.	.	.
AgNa$_v$	.	.	.	.	.	.	.	.	.	.	.	**A**	.	.	.	.	.	.	.	.	.	.	.	.	.
AmNa$_v$	.	.	**A**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TcNa$_v$	.	.	**A**	**F**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BgNa$_v$	.	.	**A**	**F**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Fig. 2. Positions of sodium channel mutations in the voltage-gated sodium channel (modified from Wang et al., 2016) and sequence alignment of the IVS6 segment. **A**: The sodium channel consists of four main domains (I–IV) and six transmembrane segments (S1−S6) within each domain. The two mutations in IVS6 related to sodium channel blocker insecticide resistance are shown. The amino acid positions are numbered based on a *Plutella xylostella* sequence (GenBank accession no. KM027335). **B**: Sequence alignment of the IVS6 segment of sodium channels from different insects. The mutation sites (F1845Y and V1848I) are shown in boxes. PxNa$_v$: *P. xylostella* (GenBank accession no. KM027335); TaNav: *Tuta absoluta* susceptible strain (Roditakis et al., 2017); LepF1845Y: Lepidopteran (*P. xylostella* and *T. absoluta*) sequence with mutation F1845Y); LepV1848I: Lepidopteran (*P. xylostella* and *T. absoluta* sequence with mutation V1848I); DmNa$_v$: *Drosophila melanogaster* (AAB59193.1); DmF1845Y: *D. melanogaster* sequence with mutation F1845Y; DmV1848I: *D. melanogaster* sequence with mutation V1848I. AgNa$_v$: *Anopheles gambiae* (CAM12801.1); AmNav: *Apis mellifera* (NP_001159377.1); TcNav: *Tribolium castaneum* (NP_001159380.1).BgNav: *Blattella germanica* (AAC47484.1).

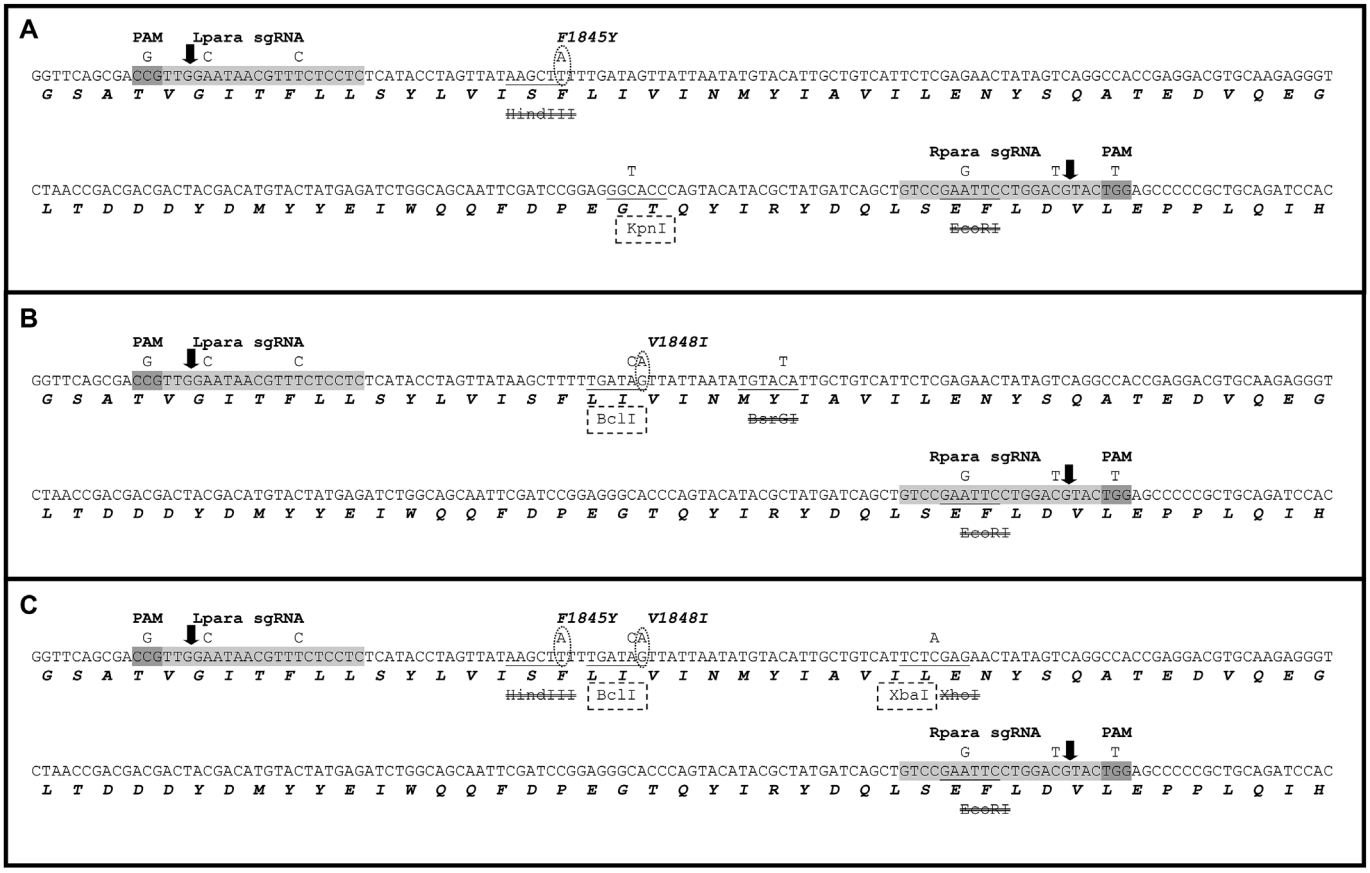


Fig. 3. CRISPR/Cas9 strategies for generation of genome modified flies bearing mutations F1845Y (A), V1848I (B), or both (C). Nucleotide and deduced amino acid sequence of a 258 bp fragment of *para* (corresponding to reverse complement of X: 16358465–16358722 at the BDGP6 genome assembly), flanking positions 1845 and 1848 (*P. xylostella* numbering) of the *Drosophila melanogaster* amino acid sequence. Light gray areas indicate the CRISPR/Cas9 targets selected (LPara sgRNA, Rpara sgRNA), while dark gray areas indicate the corresponding PAM (-*NGG*) triplets. Vertical arrows denote break points for CRISPR/Cas9-induced double stranded breaks. Ovals mark non-synonymous differences between target (wild-type) and donor (genome modified) sequences. Synonymous mutations incorporated for diagnostic purposes, as well as to avoid cleavage of the donor plasmid by the CRISPR/Cas9 machinery, are shown above the nucleotide sequence. Restriction sites abolished because of the genome modification are shown with double strikethrough letters and the corresponding sequence is underlined. Restriction sites introduced because of the genome modification are shown in dashed boxes and the corresponding sequence is also underlined.

V1848I (but not V1848A) reduced almost equally the inhibition of sodium current by indoxacarb, DCJW (an active metabolite of indoxacarb) and metaflumizone. This indicates that both these mutations might contribute to non-selective target-site resistance against both SCBIs. However, *in vivo* genetic functional validation of these mutations has not been documented so far.

In recent years, genome engineering through CRISPR/Cas9 technology has been employed in several insecticide resistance studies in model systems like *Drosophila* or in pest species where the technology has been established (reviewed in Perry and Batterham, 2018; Homem and Davies, 2018), providing useful information about the association of specific mutations with resistance against several insecticide classes, like spinosyns that target nicotinic acetylcholine receptor (Somers et al., 2015; Zimmer et al., 2016), etoxazole and benzoylureas targeting chitin synthase (Douris et al., 2016; Grigoraki et al., 2017) and diamides targeting ryanodine receptor (Douris et al., 2017; Zuo et al., 2017). In this study we have employed a CRISPR/Cas9 strategy in order to generate *Drosophila* strains bearing homozygous F1845Y or V1848I mutations in the *para* (voltage-gated sodium channel) gene, and performed toxicity bioassays on these strains in order to functionally validate resistance to SCBIs *in vivo*. We have also used molecular modeling studies to investigate SCBI interactions in the channel pore.

2. Materials and Methods

2.1. Chemicals

Chemical compounds used for contact bioassays were indoxacarb (Sigma-Aldrich, PubChem CID: 107,720) and metaflumizone (Sigma-Aldrich, PubChem CID: 11614934) (Fig. 1). The formulations used for feeding bioassays were Steward 30 WG (DuPont) for indoxacarb, and Alverde 24 SC (BASF) for metaflumizone.

2.2. Fly strains

The injections for genome modification of *Drosophila* were performed in preblastoderm embryos of the lab strain y1 M{nos-Cas9.P} ZH-2A w*, in which Cas9 is expressed under the control of *nanos* promoter (Port et al., 2014; further below referred as nos.Cas9, #54591 in Bloomington *Drosophila* stock center). Strain w + oc/FM7yBHw (kindly provided by Professor Christos Delidakis, IMBB and University of Crete) which contains the X chromosome balancer FM7c was used for genetic crosses and for keeping heterozygous mutants. The flies were kept at 25 °C temperature, at 60–70% humidity and 12:12 h photoperiod on a typical fly diet.

2.3. Amplification and sequencing of para target region

DNA from nos.Cas9 *Drosophila* adults was extracted with DNAzol (MRC) following the manufacturer instructions. Three sets of primers (Inv1F/R, Inv2F/R and Inv3F/R, Table S1) were designed based on the *para* gene sequence in order to amplify three overlapping fragments (Inv1-3) that add up to a 3134bp region encompassing genomic region X:16,466,144–16,463,017 of the *Drosophila* genome sequence (numbering according to BDGP6 genome assembly). The amplification reactions were performed using KapaTaq DNA Polymerase (Kapa Biosystems). The conditions were 95 °C for 2 min for initial denaturation followed by 30–35 cycles of denaturation at 95 °C for 30 s, annealing at 61°C-66 °C for 15 s, extension at 72 °C for 45–90 s and a final extension step for 2 min. The PCR products were purified with a PCR clean-up kit (Macherey-Nagel) according to manufacturer's instructions. Sequencing of the products was performed from both ends at StarSeq (Maintz, Germany).

2.4. Strategy for genome editing

An *ad hoc* CRISPR-Cas9 strategy was implemented in order to generate *Drosophila* strains bearing either one or both mutations (equivalent to F1845Y and V1848I found in *P. xylostella* and *T. absoluta*) in the *para* gene. We used the same CRISPR targets but different donor constructs for homologous-directed repair for the generation of each strain, containing either F1845Y or V1848I (or both, further below referred as FYVI). Based on the genomic sequence of *para* obtained for strain nos.Cas9, several CRISPR targets in the desired region were identified using the Optimal Target Finder online tool (Gratz et al., 2014, http://tools.flycrispr.molbio.wisc.edu/targetFinder). Two target sequences found upstream (Lpara) and downstream (Rpara) of the desired region in *para* gene were selected (Fig. 3) with no predicted off-target effects. In order to generate sgRNAs targeting those sequences, two different RNA expressing plasmids were generated based on the vector pU6-BbsI chiRNA (Gratz et al., 2013) following digestion with BbsI and ligation of two double stranded oligos, (dsLpara and dsRpara), which were generated by annealing single stranded oligos RparaF/RparaR and LparaF/LparaR (Table S1) respectively. Following ligation and transformation, single colonies for each construct were picked and checked for the correct insert by performing colony PCR using T7 universal primer and the reverse primer for each dsDNA. The sequence of each sgRNA expressing plasmid was verified by sequencing (Macrogen, Amsterdam).

Three different donor plasmids, paraF1845Y, paraV1848I and paraFYVI were synthesized *de novo* (Genscript) to facilitate Homologous Directed Repair for generation of strains F1845Y, V1848I and FYVI respectively (newly synthesized sequences were subcloned in pUC57 vector EcoRV site; relevant insert sequences for each donor plasmid are shown in Fig. S1). Each plasmid contained two ~1000 bp homology arms flanking the 228 bp target region between the two sgRNA targets Lpara and Rpara (Fig. 3). The target region was specifically designed in order to contain the desired mutations (F1845Y, V1848I or both in donor plasmids paraF1845Y, paraV1848I and paraFYVI respectively) along with certain additional synonymous mutations (see Fig. 3 for details) serving either as molecular markers (to facilitate molecular screening of CRISPR events), or to prevent unwanted CRISPR digestion of the donor itself.

2.5. Molecular screening and genetic crosses

Injection of nos.Cas9 pre-blastoderm embryos was performed at the IMBB/FORTH facility with injection mixes containing 75 ng/μl of each sgRNA expressing vector and 100 ng/μl of donor template. Hatched larvae were transferred into standard fly artificial diet and after 9–13 days G_0 surviving adults were collected and individually backcrossed with nos.Cas9 flies. In order to screen for CRISPR events, G_1 generation progeny from each cross were pooled into batches of ~30 and genomic DNA extraction was performed *en masse* in order to be screened with two different ways. Initially, 2 μg of genomic DNA were digested with HindIII (for F1845Y and FYVI crosses) or BsrGI (for V1848I); these enzymes cut only the wild type alleles but not potential mutant alleles in each DNA pool. Then, one strategy for screening consists of amplification with specific primers ParaSpecF/R (Table S1) that were designed taking into account the synonymous mutations introduced in the two sgRNA target sequences in all donor templates, in order to generate a diagnostic fragment of 250bp that is specific to genome modified alleles, but not wild-type ones. PCR was performed with Kapa Taq polymerase as previously described using ~60 ng of digested template DNA mix. An alternative strategy consists of PCR amplification with the "generic" primer pair ParaGenF/R (Table S1) which were designed in order to amplify a fragment of 752 bp that may be derived by either wild type (if still present, given the initial enzymatic cleavage of the template DNA mix) or genome modified alleles. Following PCR amplification, the product was digested with diagnostic enzymes introduced in the HDR donor sequence, namely KpnI for F1845Y (producing two diagnostic fragments of 536 bp and 217 bp), BclI for V1848I (producing two diagnostic fragments of 405 bp and 347 bp) and XbaI for FYVI (producing two fragments of 437 bp and 315 bp).

Crosses that proved positive for genome modified alleles were further explored in order to identify individual flies bearing mutant alleles and establish homozygous lines (see Fig. S2 for the whole crossing scheme). Individual G_1 flies from positive original G_0 crosses were backcrossed with nos.Cas9 and after generating G_2 progeny, they underwent molecular screening as previously described. Positive crosses now contain the mutant allele in 50% of the G_2 progeny. Individual female G_2 flies were then crossed with male flies carrying a balancer X chromosome (FM7c) with a characteristic phenotypic marker (*Bar*). After producing G_3 progeny, the female G_2 flies were again individually screened to identify positive crosses, and female G_3 flies potentially carrying the mutant allele opposite of an FM7c balancer were again back-crossed with male flies carrying FM7c balancer to produce G_4 progeny. One final round of molecular screening was performed to identify balanced lines containing the genome modified allele against FM7c, and G_5 adults were collected following phenotypic selection against the *Bar* marker and pooled in order to establish homozygous strains. DNA was extracted from several homozygous female and hemizygous male adults, amplified by using primers ParaGenF/R and the relevant amplification fragment was sequence verified (Macrogen, Amsterdam).

2.6. Toxicity bioassays

2.6.1. Contact bioassays

Insecticidal activity against adult flies was tested by residual contact application on nos.Cas9 flies. Test insecticides were dissolved in acetone and serial dilutions were prepared to make desired concentrations. A volume of 500 μl of each one was applied into glass scintillation vials. For each concentration there were 3 technical replicates. The vials were put on a roller for overlaying their entire surface for 30–40 min under a fume hood. Following the evaporation of acetone, 20 flies (10 males and 10 females, 1–3 day adults) were transferred into each vial. Individual vials were covered with a piece of cotton soaked into a solution of 5% sucrose. Vials were maintained at room temperature and flies were exposed for 24–96 h.

2.6.2. Feeding toxicity bioassays

For feeding bioassays, 2nd instar larvae were transferred in batches of 20 into fresh standard fly artificial food, supplemented with several concentrations of insecticide formulation solutions. Larval development, mortality, pupal eclosion, pupal size and adult survival were monitored and measured for 7–10 days. Each bioassay consisted of five to seven different concentrations, tested in triplicates. The control

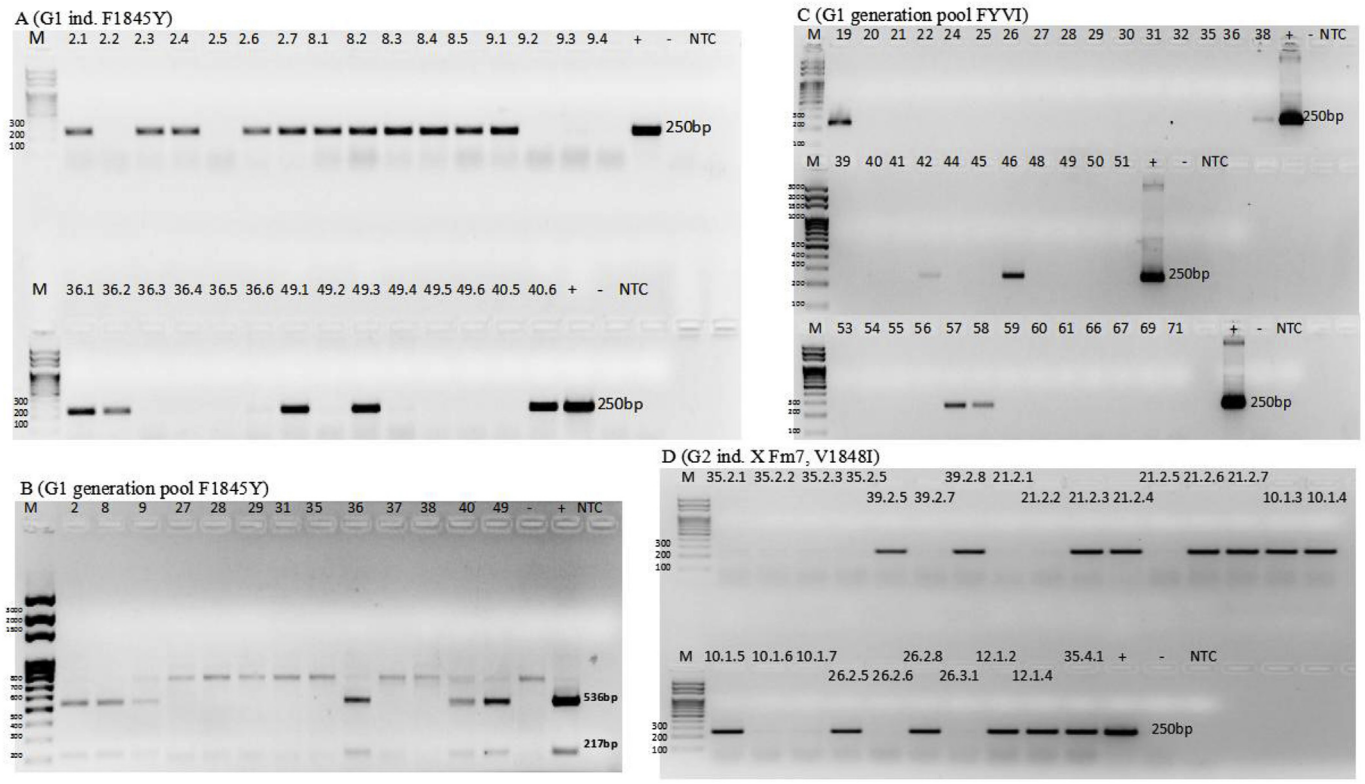


Fig. 4. Indicative diagnostic screening with specific primers yielding diagnostic PCR products in 2% agarose gel electrophoresis. M: molecular weight marker (100 bp ladder); +: positive control (PCR using as template the relevant donor plasmid for each mutation); -: negative control (PCR using as template DNA from non-injected nos.Cas9 flies; NTC: blank (no DNA template). (A) PCR screening yielding a 250 bp product of G_1 individuals backcrossed with nos.Cas9 originating from each original line (G_0) for the F1845Y mutation. (B) Diagnostic KpnI digestion of PCR product (752 bp) amplified with generic primers for massively screening G_1 progeny samples of injected G_0 flies yielding two diagnostic fragments of 536 bp and 217 bp. (C) PCR screening with specific primers (250 bp product) in pools of G_1 progeny of the original injected flies for the dual mutations FYVI. (D) PCR screening with specific primers (250 bp) of G_1 individuals for the mutation V1848I after cross with flies bearing balancer FM7c.

population (nos.Cas9) was tested along with the genome modified populations (F1845Y and V1848I); for each insecticide negative controls (no insecticide) were also included.

2.6.3. Statistical analyses

Concentration-response data of each bioassay setup were collected and analyzed with ProBit analysis using PoloPlus (LeOra Software, Berkeley, California) in order to calculate Lethal Concentrations of the 50% of the population subjected to the experiment (LC_{50} values), 95% fiducial limits (FL), linearity of the dose-mortality response, construction of mortality curves and statistical significance of the results.

2.7. Homology modeling and automated ligand docking

A homology model of the *Drosophila* para VGSC (UniProtKB accession P35500) was generated using the electric eel VGSC structure (PDB code 5XSY) as template. Sequences were aligned using ClustalW (Thompson et al., 1994) and are shown in Fig. S3. MODELLER (Eswar et al., 2007) was used to produce 50 initial homology models. The internal scoring function of MODELLER was used to select 10 models, which were visually inspected and submitted to the VADAR webserver (Willard et al., 2003) for assessment of stereochemical soundness in order to select the best model.

The 3-dimensional structure of metaflumizone was generated *ab initio* using MarvinSketch (version 5.9.1) of the ChemAxon suite (http://www.chemaxon.com). AutoDockTools (version 1.5.4) (Molecular Graphics Laboratory, Scripps Research Institute, La Jolla, CA, USA) was used to define rotatable bonds and merge non-polar hydrogens for metaflumizone. Automated ligand docking studies of metaflumizone with the *Drosophila para* model were performed using Auto-Dock Vina (version 1.1.2) (Trott and Olson, 2010) with a grid of 30 × 30 × 30 points (1 Å spacing) centered on the channel pore. F1845 and V1848 (*Plutella xylostella* numbering) side chains were allowed to flex during the docking run and subsequent docking predictions for metaflumizone were screened by selecting poses where the ligand was < 4.5 Å distance from these residues. Mutant channels with either the F1845Y or V1848I substitution were generated using Swiss-PdbViewer (Guex et al., 1999). Figures were produced using PyMOL (DeLano Scientific, San Carlos, CA, USA).

3. Results

3.1. Generation of Drosophila strains bearing mutations F1845Y and/or V1848I at the para gene

The mutations F1845Y and V1848I (*P. xylostella* numbering) in segment S6 of domain IV were introduced in *Drosophila* via a CRISPR/Cas9 coupled with Homologous Directed Repair (HDR) genome modification strategy. The *Drosophila para* VGSC sequence was aligned to the lepidopteran and other insect orthologs (Fig. 2B) and the target region identified. A genome modification strategy was designed in order to introduce the mutations under study (Fig. 3) and carried out as described in Materials and Methods (see 2.4 above).

Embryos of nos.Cas9 flies (expressing Cas9 under *nanos* promoter) were injected with three different plasmid mix combinations, each containing two sgRNA target plasmids (Lpara, Rpara) and one of the donor plasmids paraF1845Y, paraV1848I or paraFYVI (Fig. S1). For the F1845Y mutation, 55 adult flies derived from injected embryos (G_0)

were crossed with nos.Cas9 flies. Nine crosses were sterile, while the progeny of the remaining 46 (G_1) were screened with two different molecular screening approaches as described in 2.5. Six out of the 46 crosses were found to be positive for HDR. Regarding the V1848I mutation, 55 G_0 flies were crossed to nos.Cas9 and 21 of them were sterile. The remaining 34 crosses that provided G_1 progeny were screened and eight were positive for HDR. Finally, for FYVI (bearing both mutations), 71 crosses were set, 56 gave G_1 progeny and were screened, and six were found to be positive for HDR.

G_1 individuals originating from the original positive lines (G_0) were crossed, screened (Fig. 4) and then balanced in order to establish homozygous fly lines for each mutation (overall crossing scheme shown in Fig. S2). Following the final crosses in order to obtain homozygous modified flies, six lines homozygous for the F1845Y mutation and four lines homozygous for the V1848I mutation were established and sequence verified. However, for all five FYVI lines that were eventually generated bearing both mutations in the same allele, no homozygous females or hemizygous positive males were ever generated, and the FYVI allele had to be kept as heterozygote over balancer chromosome.

3.2. Validation of ability of F1845Y and V1848I mutations to confer resistance to SCBIs in Drosophila

In order to validate toxicity of SCBIs in *Drosophila*, contact bioassays were performed in 2–3 day old adult nos.Cas9 flies. No mortality was observed even after 96 h of continuous exposure to a concentration of 1000 μg/ml of either indoxacarb or metaflumizone.

Then, feeding toxicity bioassays were performed with 2nd instar larvae that were collected and transferred into fresh food containing several concentrations of each insecticide. *Drosophila* larvae were continuously in contact with the food supplemented with the insecticides. Toxicity effects such as cessation of feeding, larval paralysis, prolonged development and reduction of the size of pupae were observed. Since dead larvae cannot be readily visible inside the fly food, molting to pupae was considered a measurable proxy of eventual survival (most pupae eclose normally 7–10 days after the bioassay is initiated). Survival data underwent probit analysis and the corresponding LC_{50} values and resistance ratios versus the control (nos.Cas9) flies, along with 95% fiducial limits and associated statistics are shown in Table 1.

According to these findings, flies bearing the F1845Y mutation in homozygous (female)/hemizygous (male) state, exhibit 10.2-fold resistance to indoxacarb compared to nos.Cas9 wild type controls. On the other hand, the same flies exhibit much higher resistance to metaflumizone (RR: > 3400 with respect to nos.Cas9). Flies bearing the mutation V1848I, show similar moderate levels of resistance both to indoxacarb (RR: 6) and to metaflumizone (RR: 8.4) compared to wild-type (nos.Cas9) controls. These results were confirmed in several experiments using different fly lines bearing the mutations, with limited LC_{50} variation among different experiments, within the fiducial limits shown in Table 1.

3.3. Interactions of metaflumizone with wildtype and mutant para homology models

A homology model of the wild type *Drosophila* para VGSC was generated using the open-state electric eel VGSC structure (Yan et al., 2017). These channels share 57% sequence identity, as shown in the sequence alignment (Fig. S3) used for model generation. Docking predictions for metaflumizone in the open pore were generated and screened for interactions with the F1845 and V1848 residues. The top docking pose in terms of estimated binding energy (−10.6 kcal/mol estimated free energy of binding (ΔG_b)) is shown in Fig. 5.

Metaflumizone makes the majority of its binding contacts with residues on the DIV S6 helix, which include F1845, V1848, I1849 and Y1852 (*P. xylostella* numbering). These binding contacts are predominantly hydrophobic in nature, such as the interaction with the I1849 side chain that is orientated towards the DIII-DIV interface (Fig. 5C). One potential polar interaction is between the metaflumizone carbonyl group positioned near the side chain hydroxyl group of Y1852 (Fig. 5E), which raises the possibility of a hydrogen bond. The F1845 side chain and metaflumizone 4-cyanophenyl group are positioned to form an edge-to-face aromatic-aromatic interaction (Fig. 5C). Introducing the F1845Y substitution adds a hydroxyl group that extends the length of this side chain and consequently a steric clash was encountered with the docked ligand (Fig. 5D). The V1848 side chain interacts with the aromatic ring on the other end of metaflumizone molecule – the (trifluoromethoxy)phenyl group – and a steric clash was also found when V1848 was substituted with the larger V1848I side chain (Fig. 5E–F).

4. Discussion

Two mutations at the S6 segment of domain IV of VGSC (F1845Y and V1848I, *P. xylostella* numbering) have been reported in resistant populations of two pest species, *Plutella xylostella* (Wang et al., 2016) and *Tuta absoluta* (Roditakis et al., 2017) and have been implicated in SCBI resistance through *in vitro* studies where the relevant mutations are introduced in cockroach sodium channels expressed in *Xenopus* oocytes (Jiang et al., 2015). In the present study, we employed a reverse genetics approach to induce these mutations through CRISPR/Cas9 genome modification at the *para* gene of *Drosophila melanogaster* whose IVS6 sequence is very similar to the sequence of the two lepidopteran pests (Fig. 2B). We generated genome modified fly strains bearing each mutation and performed toxicity bioassays against two commercial SCBIs, indoxacarb and metaflumizone.

Our results (Table 1) provide direct *in vivo* confirmation that both F1845Y and V1848I have an effect on resistance against both commercial SCBIs. However, in contrast to previous *in vitro* characterization studies (Jiang et al., 2015), this effect is not uniform for each mutation/insecticide combination. Toxicity bioassays against different concentrations of indoxacarb indicate that both F1845Y and V1848I confer comparable, low to moderate ratios of resistance compared to wild-type controls (RR: 10.2 and 6 respectively). On the contrary, toxicity bioassays against metaflumizone indicate that although V1848I also confers resistance of similar scale (RR: 8.4), the F1845Y mutation has a much stronger impact by several orders of magnitude (RR: 3441.2), a result obtained in several independent experiments.

Although available *in vitro* evidence suggests that both mutations reduce the sensitivity of the cockroach channel to both insecticides (Jiang et al., 2015), the level of reduction is not substantially different

Table 1
Log-dose probit-mortality data for indoxacarb and metaflumizone against larvae of *Drosophila* genome modified strains F1845Y and V1848I versus nos.Cas9 control.

Compound	*Drosophila* strain	Slope ± SE	LC_{50} (95% CI) ug/ml	X^2 (df)	RR vs nos.Cas9
Indoxacarb	nos.Cas9	4.012 ± 0.360	2.756 (2.416–3.133)	17.406 (14)	1
	F1845Y	3.901 ± 0.370	28.202 (25.547–31.209)	14.782 (17)	10.2
	V1848I	4.270 ± 0.352	16.658 (15.124–18.434)	14.555 (22)	6
Metaflumizone	nos.Cas9	4.983 ± 0.598	0.525 (0.479–0.575)	9.375 (10)	1
	F1845Y	5.906 ± 0.798	1816.675 (1627.624–2017.529)	8.748 (16)	3441.2
	V1848I	2.964 ± 0.331	4.412 (3.763–5.131)	12.111 (13)	8.4

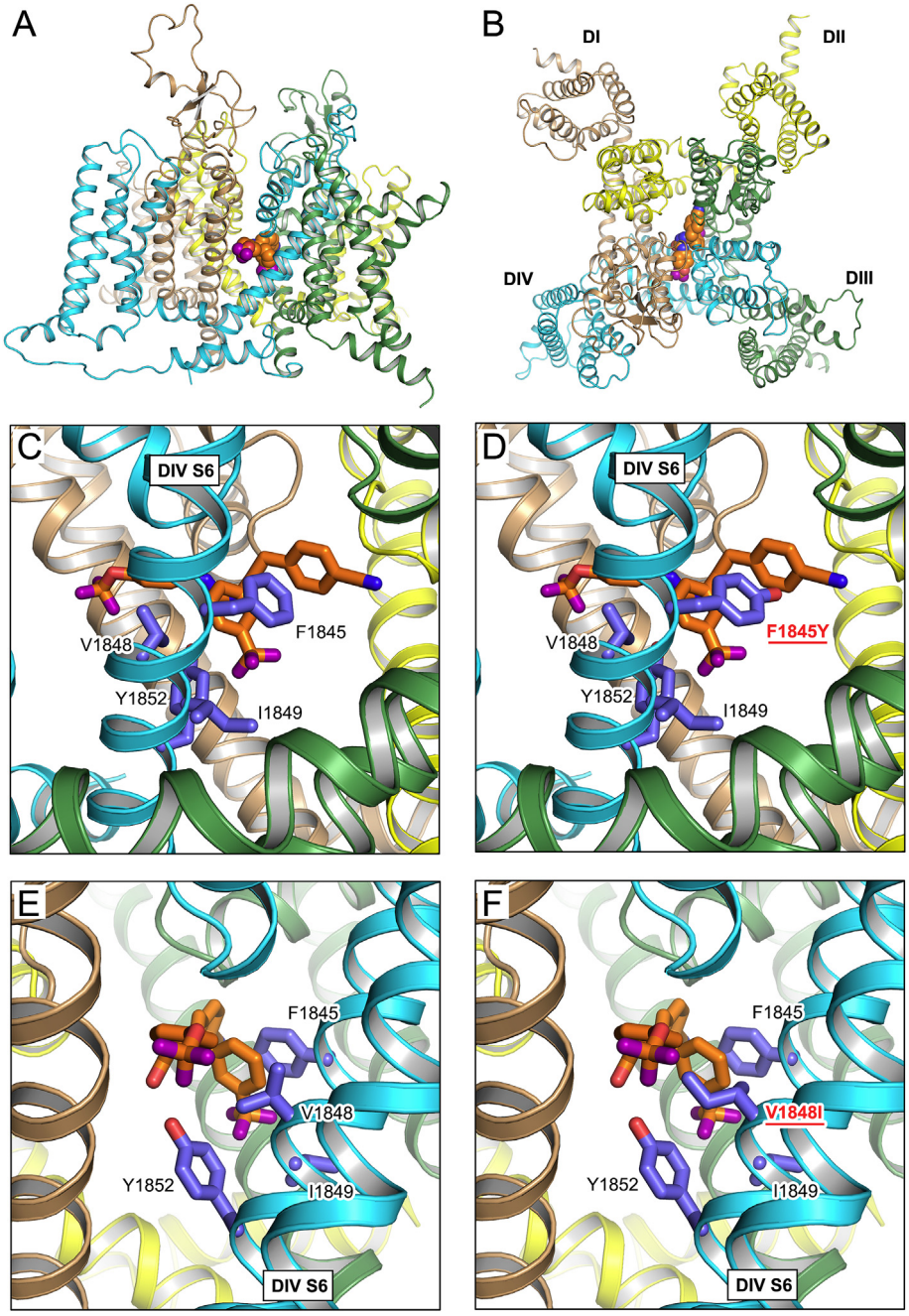


Fig. 5. Docking prediction of metaflumizone with *Drosophila para* VGSC homology models. (A–B) Transmembrane (A) and extracellular (B) view of the channel with each domain as differently colored ribbon and metaflumizone as orange spacefill. (C–D) Interaction of F1845 (C) and the substitution F1845Y (D) with metaflumizone. (E–F) Interaction of V1848 (E) and the substitution V1848I (F) with metaflumizone. Residues are numbered according to the *Plutella xylostella* channel. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

among different mutation/insecticide combinations. Although the two approaches are not readily comparable, it is noteworthy that *in vitro* the percentage of inhibition by metaflumizone in F1845Y and V1848I mutant cockroach channels is virtually the same (Table 2 in Jiang et al., 2015), i.e. both mutations induce approximately the same reduction of sensitivity, in sharp contrast with the *in vivo Drosophila* bioassay results where F1845Y flies are > 400 times more resistant to metaflumizone compared to V1848I ones.

Given that the largest RR (> 3400) was found with metaflumizone and the F1845Y mutant, we employed molecular modeling studies to explore binding interactions of this ligand. Our docking pose for metaflumizone in the channel pore differs from that reported by Zhang et al. (2016), which used a homotetrameric bacterial VGSC structure as template for homology modeling, which was then opened *in silico* so that the pore adopted a conformation resembling that of the activated-state Kv1.2 channel. Our model is based on the open-state eukaryotic eel VGSC structure (Yan et al., 2017) that, in comparison with the Zhang model, has a narrower cytoplasmic entrance to the pore that metaflumizone is unlikely to fit through. Metaflumizone may still be able to access the pore through the large fenestration at the domain III-IV interface in our model, which was proposed by Zhang et al. (2016) as a possible ingress route given that metaflumizone can bind to resting sodium channels (von Stein et al., 2013). When docked in our model, metaflumizone did not make contact with a number of residues ($W^{1p52}A$, $V^{2i18}K$, $F^{3p44}A$, $T^{3i18}A$, $L^{4i8}A$; numbered according to Zhang et al. (2016)) that decrease the inhibition effect of metaflumizone when

mutated (von Stein and Soderlund, 2012; Zhang et al., 2016). This identifies a potential limitation of our model, which is that binding contacts present in the more widely-opened pore described by Zhang et al. (2016) may not be positioned to interact with metaflumizone in our model. Conversely, in our different conformation of the open pore, we identified potential new interactions between DIV S6 residues and metaflumizone, including a possible hydrogen bond with Y1852 and also an interaction with I1849, which was previously identified as a possible determinant of SCBI species-selectivity as it is not conserved as isoleucine in mammals (Zhang et al., 2016). As previously suggested by Zhang et al. (2016), the resistance associated with the V1848I mutation may be due to a steric hindrance mechanism that inhibits metaflumizone binding; a steric clash with the ligand was found when this mutation was introduced into our model. A steric clash was also found with the F1845Y mutation and the introduction of the hydrophilic hydroxyl group on this side chain raises the possibility that hydrophobic repulsion with metaflumizone may also impede ligand binding. The F1845Y clash is with the 4-cyanophenyl moiety of metaflumizone that is absent from indoxacarb/DCJW (Fig. 1), which may explain the difference between the RR > 3400 of metaflumizone versus RR 10.6 for indoxacarb in flies with F1845Y. As mentioned, a major difference between the inhibitory effect of SCBIs on the equivalent F1845Y mutant of the cockroach VGSC was not found by Jiang et al. (2015). Future electrophysiology studies may determine if there is some inherent difference in the pharmacology of BgNav1-1 vs *para* VGSCs towards SCBIs, despite the sequences of their DIV S6 segments being essentially identical.

Our effort to generate a homozygous fly strain carrying both mutations at the same allele (in *cis*) was not successful; although such a "dual" allele has been generated by CRISPR/Cas9 coupled with homologous recombination, it was always found in heterozygotes and no homozygous flies bearing both mutations in *cis* could be generated. Interestingly, heterozygotes from resistant populations of *P. xylostella* have also been found to always have the two mutations in *trans* (single) and never in *cis* ("dual" allele; Wang et al., 2016), and similarly in resistant *T. absoluta* (data not shown; samples from Roditakis et al., 2017) This is a strong indication that the two mutations are mutually exclusive, i.e. that the "dual" allele bearing both mutations is not viable, leading to a non-functional VGSC.

Drosophila is versatile system that enables multiple questions to be addressed in a common genetic framework, providing the sophisticated toolkit required for such an operation. The establishment of genome modification technology in insecticide resistance studies in combination with standard genetic engineering may facilitate validation of target-site resistance to SCBIs (as in this study) as well as co-existing synergistic mechanisms of metabolic resistance as soon as candidate genes for these become available for investigation.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2018.12.008.

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'What I cannot create, I do not understand': functionally validated synergism of metabolic and target site insecticide resistance

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The putative synergistic action of target-site mutations and enhanced detoxification in pyrethroid resistance in insects has been hypothesized as a major evolutionary mechanism responsible for dramatic consequences in malaria incidence and crop production. Combining genetic transformation and CRISPR/Cas9 genome modification, we generated transgenic *Drosophila* lines expressing pyrethroid metabolizing P450 enzymes in a genetic background along with engineered mutations in the voltage-gated sodium channel (*para*) known to confer target-site resistance. Genotypes expressing the yellow fever mosquito *Aedes aegypti Cyp9J28* while also bearing the *para*^{V1016G} mutation displayed substantially greater resistance ratio (RR) against deltamethrin than the product of each individual mechanism ($RR_{combined}$: 19.85 > $RR_{Cyp9J28}$: 1.77 × RR_{V1016G}: 3.00). Genotypes expressing *Brassicogethes aeneus* pollen beetle *Cyp6BQ23* and also bearing the *para*^{L1014F} (*kdr*) mutation, displayed an almost multiplicative RR ($RR_{combined}$: 75.19 ≥ $RR_{Cyp6BQ23}$: 5.74 × RR_{L1014F}: 12.74). Reduced pyrethroid affinity at the target site, delaying saturation while simultaneously extending the duration of P450-driven detoxification, is proposed as a possible underlying mechanism. Combinations of target site and P450 resistance loci might be unfavourable in field populations in the absence of insecticide selection, as they exert some fitness disadvantage in development time and fecundity. These are major considerations from the insecticide resistance management viewpoint in both public health and agriculture.

1. Introduction

The prevention of vector-borne diseases and the protection of agricultural production largely relies on the control of pest insects through the use of insecticides. However, insects display a striking ability to develop resistance, an intriguing evolutionary adaptation to a very fast environmental change, with dramatic consequences. For example, the number of malaria cases increased in 2018 after many years of decline, indicative of a failure of pyrethroid based intervention strategies [1].

To mitigate against the failure of insecticide-based control tools, the mechanisms by which insects have evolved resistance must be elucidated and understood. Mutations at the insecticide target site which reduce insecticide binding affinity, and metabolic detoxification which inactivates and sequesters insecticidal active ingredients, are the most common mechanisms of insecticide

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resistance [2]. However, it has been widely hypothesized that it is only the synergism of different mechanisms in the same insect population that causes a real operational control failure in many cases [3–6]. This has important ramifications on insecticide resistance management (IRM) strategies. For example, the synergist piperonyl butoxide when incorporated into bednets seems to restore their efficacy even in areas with fixed target site resistance alleles [7], while the value of molecular diagnostics for IRM might be different depending on the presence or absence of additional mechanisms in the same mosquito population [8,9]. Although a putative synergistic epistasis of the metabolic and target site resistance loci has been considered [6], it has only been tested by crossing lines with different resistance factors together in order to see their effect. However, this process introduces a large amount of unrelated genetic variation, which complicates inferences drawn about the specific loci being studied. While there have been several efforts to isolate the contribution of resistance alleles by introducing them into model organisms like *Drosophila*, many of these studies have only managed to recapitulate a fraction of the total resistance levels observed in the field [10,11].

An additional factor in IRM strategies are the evolutionary fitness costs imposed by resistance alleles. Alleles that pose high costs will tend to revert back to their susceptible form once the selective pressure (pesticide) is removed. Variants that do not pose such a cost can persist indefinitely. Fitness costs related to drug resistance have long been the subject of investigation in clinical settings with resistant bacteria and cancer lines [12,13], and have also been subjected to investigation in insects [14]. From these studies it has become clear that the severity, and indeed presence, of a fitness cost brought about by a given resistance allele depends on the particular allele and the genetic background in which it is observed. However, the understanding of the costs of each variant and their epistatic effects are poorly understood.

Of particular interest to the insecticide community are the mechanisms underpinning pyrethroid resistance and their resulting evolutionary implications. This large class of structurally related insecticides targets the voltage-gated sodium channel (the orthologues to the *para* gene in *Drosophila*), and representative pyrethroids such as deltamethrin have been widely used in both agricultural and public health related pest control since the 1970s. Widespread use was followed by the appearance of several independent resistance mechanisms. Among several examples, the P450 *Cyp6BQ23* was found to be overexpressed in the pollen beetle *Brassicogethes aeneus* [15], while other resistant strains carried the *kdr* (L1014F) substitution in *para* [16]. In the mosquito *Aedes aegypti*, the main vector of yellow fever worldwide, a similar array of mechanisms have been identified including the overexpression of *Cyp9J28;* [17] and another mutation in *para* (V1016G). However, the interaction of these alleles *in vivo* has not, to our knowledge, been studied, neither in terms of contribution to pyrethroid resistance nor any resulting fitness cost.

Here, we report the generation of transgenic *Drosophila melanogaster* lines expressing pyrethroid metabolizing cytochrome P450 enzymes from major mosquito vectors (*A. aegypti*) and agricultural pests (*B. aeneus*) in a genetic background where we have engineered by CRISPR/Cas9 specific homozygous target-site resistance mutations in the voltage-gated sodium channel (*para*), also found in these insects. This strategy enabled us to directly measure the resulting resistance phenotypes, encountering either the contribution of both mechanisms or each one separately, with very limited confounding genetic effects.

2. Material and methods

(a) *Drosophila* strains

Drosophila strains used in this study are shown in the electronic supplementary material, table S1. Strain *yw nos int;* attP40 [18] was a gift by Pawel Piwko and Christos Delidakis (IMBB/FORTH). CRISPR/Cas9 genome modification was performed at strain y1 M{nos-Cas9.P}ZH-2A w*, where Cas9 is expressed under the control of *nanos* promoter [19] (herein referred as nos.Cas9, #54591 in the Bloomington *Drosophila* stock centre). Background strain *yellow white* (*yw*) and several balancer lines (see the electronic supplementary material, table S1) are part of the IMBB/FORTH facility fly collection (kindly provided by Prof. Christos Delidakis, IMBB and University of Crete). The HR-GAL4 driver line is previously described [20], while the responder line UAS.AaegCYP9J28 (herein referred as UAS-CYP9J28) was generated also as described previously [17]. All flies were kept at a temperature of 25°C, humidity 60–70% and 12 : 12 h photoperiod on a standard fly diet.

(b) Amplification and sequencing of *para* target regions

DNA from nos.Cas9 *Drosophila* adults was extracted with DNAzol (MRC, Cincinnati, OH) following the manufacturer's instructions. Several primers (paraInF, paraInR, kdrF, kdrR, exoF, exoR, electronic supplementary material, table S2) were designed based on the *para* gene sequence in order to amplify and sequence overlapping fragments that correspond to a 2585 bp sequenced genomic region of strain nos.Cas9 (X: 16485234:16487819, numbering according to BDGP6.22 genome assembly) which contains the exons that harbour positions L1014 and V1016, respectively. The amplification reactions were performed using KapaTaq DNA polymerase (Kapa Biosystems, Wilmington, MA). The conditions were 95°C for 2 min for initial denaturation followed by 30–35 cycles of denaturation at 95°C for 30 s, annealing at 54–60°C for 15 s, extension at 72°C for 90 s and a final extension step for 2 min. The polymerase chain reaction (PCR) products were purified with a PCR clean-up kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Sequencing of the products was performed from both ends at Macrogen (Amsterdam, The Netherlands).

(c) Strategy for genome editing

An ad hoc CRISPR–Cas9 strategy was implemented in order to generate *Drosophila* strains bearing either mutation (equivalent to L1014F and V1016G according to housefly *Musca domestica* numbering) in the *para* gene. Based on the genomic sequence of *para* obtained for strain nos.Cas9 several CRISPR targets in the desired region were identified using the Optimal Target Finder online tool [21] (http://targetfinder.flycrispr.neuro.brown.edu/). We selected three CRISPR targets in total, which had minimal predicted off-target effects. Targets para935 and para406 were used to obtain L1014F, while targets para406 and para205 were used for V1016G (figure 1). In order to generate single guide RNAs (sgRNAs) targeting those sequences, three different RNA-expressing plasmids were generated (sgRNA935, sgRNA406 and sgRNA205, respectively) based on the vector pU6-BbsI chiRNA [22] following digestion with BbsI and ligation of three relevant double-stranded oligonucleotides, which were generated by annealing single stranded oligonucleotides 935F/935R, 406F/406R and 205F/205R, respectively (electronic supplementary material, table S2). Following ligation and transformation, single colonies for each construct were picked and checked for the correct insert by performing colony PCR using T7 universal primer and the reverse

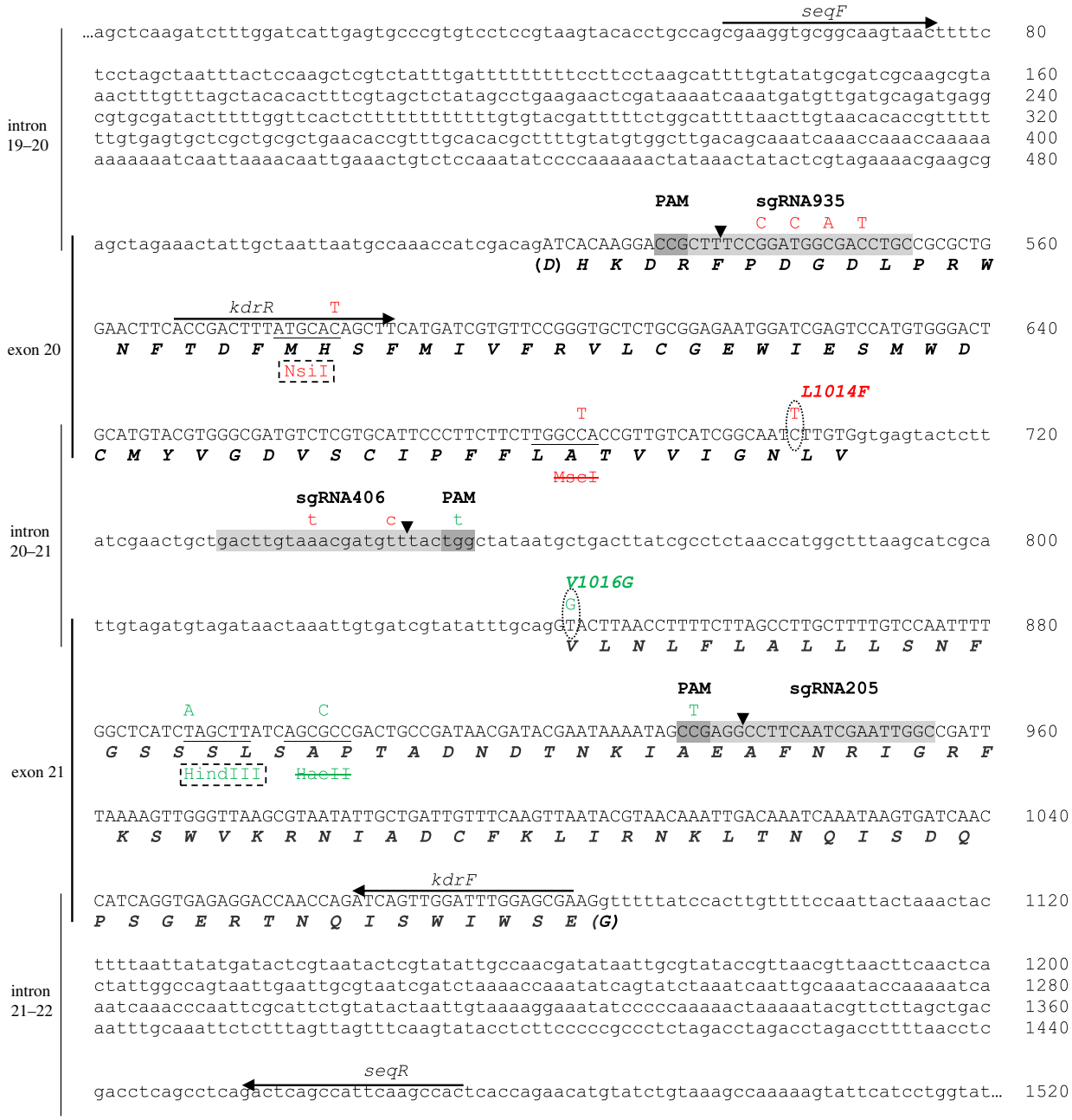

Figure 1. CRISPR/Cas9 strategies for the generation of genome modified flies bearing mutations L1014F and V1016G. Nucleotide and deduced amino acid sequence of a 1520 bp fragment of *para*, encompassing exons 20 and 21 that contain positions 1014 and 1016, respectively (*M. domestica* numbering) of the *Drosophila melanogaster* amino acid sequence. Light grey areas indicate the CRISPR/Cas9 targets selected (sgRNA935, sgRNA406 and sgRNA2015), while dark grey areas indicate the corresponding PAM (*-NGG*) triplets. Vertical arrows denote break points for CRISPR/Cas9-induced double-stranded breaks. Red lettering indicates the differences introduced in exon 20 for the generation of L1014F, while green lettering indicates the differences introduced in exon 21 for the generation of V1016G. Ovals mark non-synonymous differences between the target (wild-type) and donor (genome modified) sequences. Synonymous mutations incorporated for diagnostic purposes, as well as to avoid cleavage of the donor plasmid by the CRISPR/Cas9 machinery, are shown above the nucleotide sequence. Restriction sites abolished because of the genome modification are shown with strikethrough letters and the corresponding sequence is underlined. Restriction sites introduced because of the genome modification are shown in dashed boxes and the corresponding sequence is also underlined. Horizontal arrows indicate the positions of primer pairs kdrF/kdrR and seqF/seqR (electronic supplementary material, table S2) used for sequencing of the genome modified alleles. (Online version in colour.)

primer for each double stranded DNA. The sequence of each sgRNA expressing plasmid was verified by sequencing (Macrogen).

Two different donor plasmids, vgscL1014F and vgscV1016G were synthesized de novo (Genscript, Piscataway, NJ) to facilitate homologous directed repair for the generation of strains L1014F and V1016G, respectively (newly synthesized sequences were subcloned in the pUC57 vector EcoRV site; relevant insert sequences for each donor plasmid are shown in the electronic supplementary material, figure S1). Each plasmid contained two approximately 900 bp homology arms flanking the target region between sgRNA targets para935 and para406 (for L1014F) or para406 and para205 (for V1016G) (figure 1). The target regions were specifically designed in order to contain the desired mutations along with certain additional synonymous mutations (figure 1) serving either as molecular markers (to facilitate molecular screening of CRISPR events), or to prevent unwanted CRISPR digestion of the donor itself.

(d) Molecular screening and establishment of genome modified lines

Injection of nos.Cas9 pre-blastoderm embryos was performed at the IMBB/FORTH facility with injection mixes containing

75 ng µl^{-1} of each sgRNA plasmid vector and 100 ng µl^{-1} of donor template as previously described [23]. Hatched larvae were transferred into standard fly artificial diet and after 9–13 days G_0 (generation zero) surviving adults were collected and individually backcrossed with nos.Cas9 flies. In order to screen for CRISPR events, G_1 progeny from each cross were pooled into batches of approximately 30 and genomic DNA extraction was performed en masse in order to be screened with two different strategies. Initially, 2 µg of pooled genomic DNA (gDNA) were digested with MscI (for L1014F crosses) or HaeII (for V1016G crosses); these enzymes cut only the wild-type alleles but not potential mutant alleles in each DNA pool. Then, the strategy for screening for L1014F mutants consists of amplification with specific primers 1014UP/1014DOWN (electronic supplementary material, table S2) that were designed taking into account the synonymous mutations introduced in the relevant target sequences in donor template vgscL1014F, in order to generate a 234 bp diagnostic fragment that is specific to genome modified alleles, but not wild-type ones (electronic supplementary material, figure S2A). PCR was performed with Kapa Taq polymerase as previously described using approximately 60 ng of digested template DNA mix. For screening of V1016G mutants, an alternative strategy was used, which consists of PCR amplification with the 'generic' primer pair kdrF/kdrR (electronic supplementary material, table S1) which was designed in order to amplify a 516 bp fragment that may be derived by either wild-type (if still present, given the initial enzymatic cleavage of the template DNA mix) or genome modified alleles. Following PCR amplification, the product was digested with the diagnostic enzyme HindIII introduced in the vgscV1016G donor plasmid sequence, producing two diagnostic fragments of 324 and 192 bp (electronic supplementary material, figure S2B). Crosses that proved positive for genome modified alleles were further explored in order to identify individual flies bearing mutant alleles and establish homozygous lines. DNA was extracted from several homozygous female and hemizygous male adults, amplified by using primers kdrF/kdrR or seqF/seqR (electronic supplementary material, table S2) and the relevant amplification fragments were sequence verified (Macrogen) for the presence of the desired mutations (electronic supplementary material, figure S2C).

(e) Generation of transgenic *Drosophila* expressing *Cyp6BQ23*

In order to generate a transgenic *D. melanogaster* strain conditionally expressing *Cyp6BQ23*, a GAL4/UAS strategy was employed. The responder strain, UAS-CYP6BQ23, was generated by PhiC31 integrase mediated attB insertion at an attP40 landing site [24]. An ad hoc integration vector, dPelican-attB-UAS_CYP6BQ23 was generated by replacing the insert of plasmid dPelican-attB-UAS_CYP6A51 we had previously generated [25]. We performed de novo synthesis (Genescript) of the CYP6BQ23 coding sequence (GenBank acc. no. KC840055.1) with some modifications in order to optimize for expression in *Drosophila*, i.e. introducing a CACC Kozac-consensus sequence just upstream of the initiation codon and taking into account codon usage optimal for *Drosophila* (full construct sequence shown in the electronic supplementary material, figure S1). An MluI/XhoI fragment encompassing the CYP6BQ23 coding sequence was subcloned into dPelican-attB-UAS_CYP6A51 [25] plasmid backbone that had been digested with MluI and XhoI so that the existing CYP6A51 expression cassette was removed and replaced by the CYP6BQ23 fragment downstream of 5xUAS and just upstream of an SV40 polyadenylation sequence, to produce the final recombinant plasmid dPelican.attB.UAS_CYP6BQ23. This plasmid also contains a mini-*white* marker gene for *Drosophila*. The sequence was verified using sequencing primers pPel_uas F and pPel_sv40 R [26] and the recombinant plasmid was used to inject pre-blastoderm embryos of the *D. melanogaster* strain *yw nos int;* attP40. Injected G_0 flies were outcrossed with *yw* background flies and G_1 progeny was screened for w^+ phenotypes (red eyes) indicating integration of the recombinant plasmid. Independent transformed lines were crossed with a strain bearing a balancer for the second chromosome (*yw; CyO/Sco*), and G_2 flies with red eyes and *Cy* phenotype were selected and crossed among themselves to generate homozygous UAS-CYP6BQ23 flies used to establish the transgenic responder line population.

(f) Generation of null background strain *yw*;attP40

In order to generate a *Drosophila* line that is fully equivalent to the UAS-CYP6BQ23 strain in terms of genetic background and can be used as null control in downstream experiments, male non-injected flies of the *D. melanogaster* strain *yw nos int;* attP40 were outcrossed with female *yw* background flies, and male G_1 progeny (not carrying the *yw nos int* chromosome) was crossed with females bearing a balancer for the second chromosome (*yw; CyO/Sco*). G_2 flies with *Cy* phenotype were selected and crossed among themselves to generate homozygous *yw; attP40* flies that have essentially the same genetic background with the transgenic responder line population, apart from the UAS-CYP6BQ23 expression cassette.

(g) Generation of homozygous recombinant *yw*; HR-GAL4>UAS-CYP9J28(2N) strain

We generated a strain bearing both HR-GAL4 and UAS-CYP9J28 in the second chromosome by genetic recombination, as shown in the electronic supplementary material, figure S3. This was performed via a cross between lines HR-GAL4 [20] and UAS-CYP9J28 [17] that produces a heterozygous genotype (*y*)*w*;HR-GAL4>UAS-CYP9J28. However, while this genotype produces a detectable resistant phenotype in contact bioassays [17], preliminary topical application bioassays indicated that only marginal (i.e. not always significant) changes in resistance were detected (data not shown). Thus, heterozygous (*y*)*w*;HR-GAL4>UAS-CYP9J28 females (where chromosomal crossover is feasible) were crossed to *yw*; *CyO/Sco* balancer flies and the progeny screened for genetic recombination events as shown in the electronic supplementary material, figure S3. We identified heterozygous *yw*; [HR-GAL4_UAS-CYP9J28]/CyO recombinant flies bearing both HR-GAL4 and UAS-CYP9J28 transgenes in the second chromosome and these were intercrossed to generate the homozygous *yw*; HR-GAL4>UAS-CYP9J28(2N) strain, which contains two copies of both driver and responder transgenes.

(h) Generation of driver and responder lines in genome modified (mutant) background

Taking into account that the voltage-gated sodium channel (*para*) gene in *Drosophila* is located at the X chromosome, while the HR-GAL4 driver strain used in our laboratory, the *Cyp9J28* insertion site, as well as the attp40 insertion site bearing the *Cyp6BQ23* transgene, are all located in the second chromosome, we devised a simple genetic crosses strategy (electronic supplementary material, figure S4) to: (i) introduce both the HR-GAL4 driver and the UAS-CYP6BQ23 responder transgene in a $para^{L1014F}$ genetic background in order to generate strain $para^{L1014F}$; HR-GAL4>UAS-CYP6BQ23 that represents the 'beetle' allele combination, and (ii) generate a strain bearing the linked [HR-GAL4_UAS-CYP9J28] chromosome 2 (see (g) above) in a $para^{V1016G}$ genetic background (strain $para^{V1016G}$;HR-GAL4>UAS-CYP9J28(2N)) that represents the 'mosquito' allelic combination. As shown in the electronic supplementary material, figure S4, the crossing scheme results in lines where the X chromosome is derived from nos.Cas9 strain, the second chromosome from the

respective transgenic line (HR-GAL4, *yw nos int;* attP40 or UAS-CYP9J28) and the other chromosomes from *yw* (note that all balancer lines as well as nos.Cas9, *yw nos int;* attP40 and UAS-CYP9J28 had been originally generated in *yw* or extensively outcrossed to it in the IMBB fly facility).

(i) GAL4/UAS-expression in *Drosophila melanogaster*

In order to drive conditional expression of *Cyp9J28* or *Cyp6BQ23* in wild-type or mutant genetic backgrounds we used the HR-GAL4 driver [20] which drives expression in specific tissues related to detoxification (malpighian tubules, midgut and fat body). Transgenic responder virgin females were crossed with HR-GAL4 males and the progeny was used in toxicity bioassays with deltamethrin in order to validate the potential of each line to confer insecticide resistance. Crosses of *yw;* attP40 or nos.Cas9 virgin females with HR-GAL4 males were used as negative controls.

(j) Extraction of RNA, complementary DNA synthesis, reverse transcription polymerase chain reaction and quantitative polymerase chain reaction

Reverse transcription PCR was performed in order to confirm expression of *Cyp6bq23* or *Cyp9j28* in the progeny. Total RNA was extracted from pools of 20 adult *Drosophila* flies (1–3 day old) using Trizol reagent (MRC), according to the manufacturer's instructions. Extracted RNA samples were treated with Turbo DNase (Ambion, Foster City, CA) to remove genomic DNA and 2 µg of treated RNA was used to generate first strand complementary DNA (cDNA) using oligo-dT_{20} primers with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). One microliter of cDNA was used in the PCR reaction using specific primers for each transgene and for *rpl11* (ribosomal protein L11) which served as a reference gene (electronic supplementary material, table S1). The conditions of the reactions were 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and final extension for 2 min.

A two-step quantitative-reverse transcriptase PCR was performed in order to analyse the expression levels of *Cyp6BQ23* and *Cyp9j28* between genotypes bearing the relevant expression trangenes in either wild-type or genome modified *para* background (figure 2*c*,*d*). Quantitative PCR (qPCR) was conducted using the Kapa SYBR Fast qPCR Master Mix kit (Kapa Biosystems) and the reactions were carried out in the Bio-Rad CFX Connect using the following conditions: 95°C for 2 min, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. The efficiency of the qPCR reaction for each primer pair was assessed in 10-fold dilution series of pooled cDNA samples. The experiment was performed using three biological and two technical replicates. Relative expression was normalized to the reference genes *rpl11* and *rpl32*, while the analysis was conducted as previously described [27]. All primer sequences are shown in the electronic supplementary material, table S2.

(k) Toxicity bioassays

Deltamethrin (99.6%) of technical grade was purchased from Sigma Aldrich (Darmstadt, Germany) and used in contact assays and topical applications. Contact assays were performed as described previously [28]. More specifically, 20 adult female flies (1–3 days old, as per [20,28]) were used for each toxicity assay. Flies were collected in plastic vials and transferred in scintillation vials coated with insecticide. Serial dilutions of 6–7 concentrations of technical grade deltamethrin in acetone were used for dose response bioassays, while vials coated only with acetone served as control. The vials were plugged with cotton that was kept moist with 5% sucrose solution. Each deltamethrin concentration was assayed in three replicates. Knockdown was scored for 180 min with 15–30 min intervals and mortality was scored after 24 h. Topical application of deltamethrin was performed on 1–3 day old female flies. Deltamethrin was dissolved in acetone and serial dilutions were made to generate the appropriate concentrations. Each insecticide concentration (or acetone as negative control) was applied in a dose of 1 µl per fly using a 10 µl Hamilton syringe. Flies were immobilized by keeping them on an ice cold slide. For each concentration 40 flies were tested. Following insecticide application the flies were transferred into glass scintillation vials covered with cotton moisturized with 5% sucrose solution. The vials were maintained in a 25°C incubator while mortality was scored after 24 h.

(l) Life table parameters

Determination of life table parameters was performed as previously described [23]. To determine developmental time and sex ratio, cages with 50 virgins (1–3 day old) and 20 males were capped with cherry juice-agar plates supplemented with yeast, left to cross overnight and after plate replacement the flies were left to lay eggs for 4–5 h. Eggs were transferred into vials with standard fly food in batches of *ca* 50 (10 replicates for each genotype). Pupation, adult emergence time and total number of males/females were scored daily from day 8. To monitor daily and total fecundity, c. 15 females from each genotype were mated, transferred in small cages capped with 35 mm yeast-supplemented cherry agar plates, and the number of eggs laid by each female was counted daily.

(m) Statistical analyses

Concentration-response data of each bioassay setup were collected and analysed with ProBit analysis using PoloPlus (LeOra Software, Berkeley, CA) in order to calculate lethal concentrations of the 50% of the population subjected to the experiment (LC_{50} values), 95% fiducial limits (FL), linearity of the dose-mortality response and statistical significance of the results. A χ^2-test was used to assess how well the individual LC_{50} values observed in the bioassays agree with the calculated linear regression lines. The LC_{50} values and resistance ratio (RR) are considered significant if the 95% FL did not include 1 [29]. Life table parameter data (electronic supplementary material, dataset 1) were analysed for significant differences between strains with one-way ANOVA using the software GraphPad Prism 8.0.2. A two-tailed unpaired student's *t*-test (also using GraphPad Prism) was carried out in order to compare relative expression in qPCR data.

3. Results

(a) Generation of *Drosophila* lines expressing detoxification enzymes in a genetic background bearing target-site resistance mutations in *para*

We used CRISPR/Cas9 genome engineering (figure 1) to generate strains bearing homozygous target-site resistance mutations in the voltage-gated sodium channel (*para*) of *D. melanogaster* (strain $para^{L1014F}$ bearing mutation L1014F (*kdr*) and strain $para^{V1016G}$ bearing mutation V1016G). Additionally, we employed GAL4/UAS for the expression of known detoxification enzyme CYP6BQ23 from the pollen beetle *B. aeneus*. We also used a previously generated strain for expression of CYP9J28 from the mosquito *A. aegypti* under the control of HR-GAL4 driver. We used genetic recombination to bring HR-GAL4 and UAS-CYP9J28 in the same chromosome, resulting in a strain bearing two copies of each transgene (*yw*; HR-GAL4>UAS-CYP9J28(2N)) in

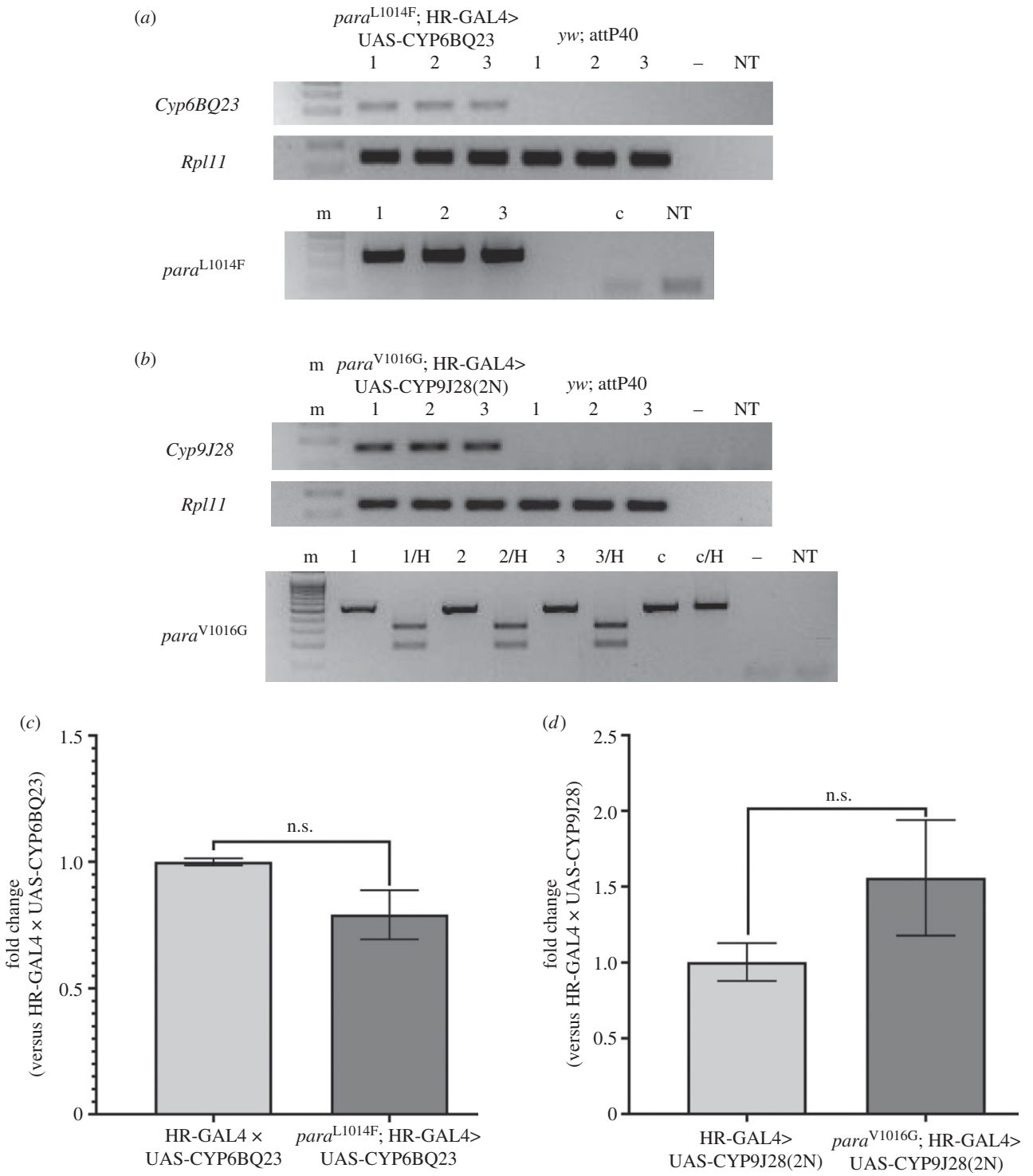

Figure 2. P450 overexpression in the flies bearing the *kdr* mutations. (*a*) (top) *Cyp6bg23* expression is confirmed by reverse transcription and PCR amplification of cDNAs. Lanes *para*^{L1014F};HR-GAL4>UAS-CYP6BQ23 (1–3) indicate three biological replicates of the flies tested for the overexpression of the transgene. Lanes *yw*; attP40 (1–3) indicate the three biological replicates of the control line. The same cDNAs were used to amplify the housekeeping gene *rpl11* as a reference gene. —: no reverse transcription control (to monitor for genomic DNA contamination); NT: no template control. (bottom): The presence of L1014F mutation in the same flies is tested by PCR of genomic DNA with allele-specific primers. c: *yw*; attP40 negative control DNA. (*b*) (top) *Cyp9j28* expression is similarly confirmed. Lanes *para*^{V1016G};HR-GAL4>UAS-CYP9J28(2N) (1–3) indicate three biological replicates of the flies tested for the overexpression of the transgene, while lanes *yw*; attP40 (1–3) indicate the three biological replicates of the control line. The same cDNAs were used to amplify the housekeeping gene *rpl11* as a reference gene. —: no reverse transcription; NT: no template. (bottom): The presence of V1016G mutation in the same flies is tested by PCR of genomic DNA with generic primers and subsequent digestion of the product with HindIII (/H). c: *yw*; attP40 negative control DNA. (*c*,*d*) qRT-PCR for evaluation of P450 expression levels in different strains. The Ct values of strains expressing CYP6BQ23 (*c*) and CYP9J28 (*d*) were calculated in the absence or presence of the relevant *para* mutations. No significant difference in expression was observed ($p = 0.0618$ for CYP6BQ23, $p = 0.1161$ for CYP9J28).

order to obtain higher expression levels providing a readily detectable effect in topical application assays (see below). Standard genetic crosses enabled *Cyp6BQ23* transgenic expression in *para*^{L1014F} genetic background and *Cyp9J28* transgenic expression in *para*^{V1016G} background so that both mechanisms were combined (figure 2).

Table 1. Topical application deltamethrin bioassay responses of transgenic flies expressing pyrethroid metabolizing P450s alone or along engineered target-site resistance mutations in their voltage-gated sodium channel (*para*).

strain/cross	LD_{50} (ng fly^{-1})	(95% FL)	slope (±s.e.)	RR
HR-GAL4 × *yw*; attP40	3.10	(2.65–3.65)	3.59 (±0.47)	1
HR-GAL4 × UAS-CYP6BQ23	17.8	(12.50–21.65)	4.27 (±0.87)	5.74
yw;HR-GAL4>UAS-CYP9J28(2N)[a]	5.49	(4.051–6.60)	4.4 (±1.09)	1.77
nos.Cas9	3.33	(1.3–5.1)	2.259 (±0.39)	1.07
$para^{L1014F}$	39.49	(23.1–53.95)	2.949 (±0.39)	12.74
$para^{V1016G}$	9.30	(4.98–14.55)	1.696 (±0.36)	3.00
$para^{L1014F}$; HR-GAL4>UAS-CYP6BQ23[b]	233.08	(161.70–333.85)	1.508 (±0.21)	75.19
$para^{V1016G}$; HR-GAL4>UAS-CYP9J28(2N)[c]	61.53	(47.48–78.50)	4.851 (±0.80)	19.85

[a]homozygous recombinant *yw*; HR-GAL4>UAS-CYPJ28(2N) contains two copies of driver and responder.
[b]$para^{L1014F}$; HR-GAL4 × $para^{L1014F}$; UAS-CYP6BQ23.
[c]$para^{V1016G}$; HR-GAL4>UAS-CYPJ28(2N) contains two copies of driver and responder in $para^{V1016G}$ X-chromosome background.

(b) Toxicity bioassays in *Drosophila* indicate synergistic action of different resistance mechanisms

Slow uptake contact bioassay experiments (all results shown in the electronic supplementary material, table S3) showed that resistance levels were extremely high in $para^{L1014F}$;HR-GAL4>UAS-CYP6BQ23 flies (totally insensitive at deltamethrin doses exceeding 5000 µg $vial^{-1}$), compared to the control flies nos.Cas9 (LC_{50} 5.45 (2.40–8.57) µg $vial^{-1}$) or the flies bearing any of the resistant mechanisms alone (RR_{L1014F}: 158.9; $RR_{Cyp6BQ23}$: 9.68 compared to relevant control flies nos.Cas9 and HR-GAL4 × *yw*; attP40, respectively).

Thus, in order to more precisely quantify the intensity of resistance phenotype, topical application bioassays were used. The results are shown in table 1. $para^{L1014F}$ flies exhibit 12.74-fold resistance to deltamethrin, while flies expressing *Cyp6BQ23* in wild-type *para* background exhibit 5.74-fold resistance compared to controls (nos.Cas9 and HR-GAL4 × *yw*; attP40), which had an absolutely similar response to deltamethrin toxicity.

Flies expressing *Cyp6BQ23* in $para^{L1014F}$ background displayed an almost multiplicative RR compared to the control ($RR_{combined}$: $75.19 \geq RR_{Cyp6BQ23}$: $5.74 \times RR_{L1014F}$: 12.74). In the case of the resistance alleles known from *A. aegypti* an even more striking effect was found: $para^{V1016G}$ flies show modest levels of resistance (RR: 3.00), while flies stably expressing *Cyp9J28* following genetic recombination between the UAS-CYP9J28 responder with the HR-GAL4 driver (bearing two copies of each) also exhibit modest resistance (RR: 1.77) compared to controls. However, the $para^{V1016G}$;HR-GAL4>UAS-CYP9J28(2N) flies displayed a significantly greater RR than the product of the individual RRs ($RR_{combined}$: $19.85 \gg RR_{Cyp9J28}$: $1.77 \times RR_{V1016G}$: 3.00).

(c) The presence of multiple resistance alleles may be associated with some fitness disadvantage

By contrast to the lines bearing only one resistant mechanism, which (with a possible exception on $para^{L1014F}$ fecundity) exhibited no statistically significant difference compared to the control lines (electronic supplementary material, dataset 1), both the 'super-resistant' lines, $para^{L1014F}$;HR-GAL4>UAS-CYP6BQ23 and $para^{V1016G}$;HR-GAL4>UAS-CYP9J28(2N) showed a significant cost in development time as indicated by one-way ANOVA of pupation time (figure 3 and electronic supplementary material, table S4, pupation after 7–8 days). For the 'beetle' allelic combination, $para^{L1014F}$;HR-GAL4>UAS-CYP6BQ23 flies exhibit some developmental delay compared to nos.Cas9 controls ($p_{d7} = 0.0249$), as well as to $para^{L1014F}$ ($p_{d7} = 0.0044$ and $p_{d8} = 0.0274$). Regarding the 'mosquito' combination, this is also evident in comparisons of $para^{V1016G}$;HR-GAL4>UAS-CYP9J28(2N) flies against nos.Cas9 ($p_{d7} = 0.0039$, $p_{d8} = 0.0045$), $para^{V1016G}$($p_{d7} = 0.0110$, $p_{d8} = 0.0048$), *yw*;HR-GAL4>UAS-CYP9J28 ($p_{d7} = 0.0004$, $p_{d8} < 0.0001$) and HR-GAL4>*yw*;attP40 ($p_{d7} = 0.0093$, $p_{d8} = 0.0089$). Furthermore, $para^{V1016G}$;HR-GAL4>UAS-CYP9J28(2N) flies also exhibit a significant cost in total fecundity after 20 days, compared to all controls tested (figure 3 and electronic supplementary material, table S5).

4. Discussion

An old enigma in insect toxicology, the putative synergistic action of target site resistance mutations and upregulated cytochrome P450s in pyrethroid resistance of major disease vectors and agricultural pests, has been functionally resolved. Specifically, field evolved P450s conferring pyrethroid resistance and target site resistance alleles were individually introduced in a single *in vivo* system. Genotypes overexpressing P450s in addition to the target-site resistance displayed a multiplicative RR equal or greater than the product of the RRs obtained for the individual resistance mechanisms. This is in line with previous studies focusing on synergism associated with insecticide resistance alleles (reviewed in [6]), which found that the combination of resistance alleles was mostly multiplicative.

While the work presented here then largely agrees with prior estimations of P450 target site synergism, the use of *D. melanogaster* in this study provides several advantages. Most critically *Drosophila* reduces confounding genetic factors arising from different backgrounds which are known to cause substantial variation in 'wild-type' lines [30]. While the flies used in this study were not completely isogenic, the backgrounds were much more similar compared to previous studies considering synergism in model organisms [6] and

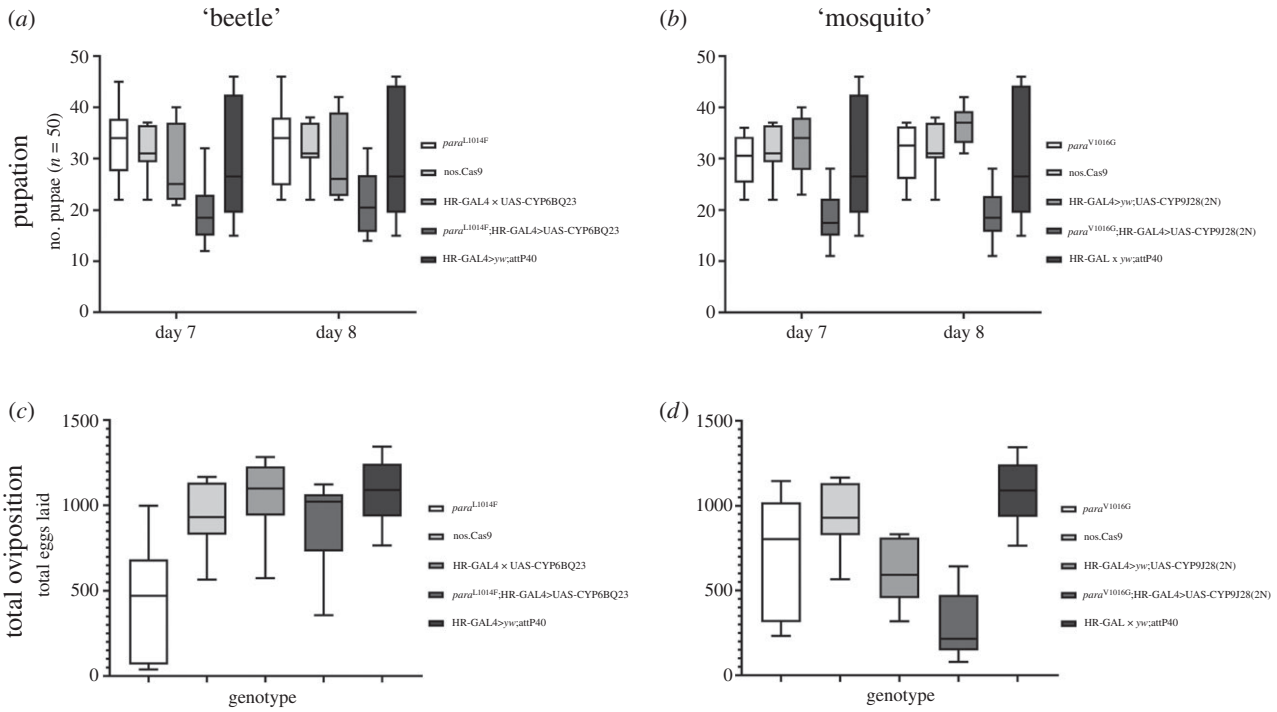

Figure 3. Evaluation of 'super-resistant' fly lines fitness. (*a*,*b*): one-way ANOVA of pupation after 7–8 days in strains bearing both resistance alleles for the 'beetle' (*a*) and 'mosquito' (*b*) allele combinations compared to controls, indicating an extended developmental time when both alleles are present. All *p*-values are shown in the electronic supplementary material, table S4. (*c*,*d*): one-way ANOVA of total oviposition in strains bearing both resistance alleles for the 'beetle' (*c*) and 'mosquito' (*d*) allele combinations. Oviposition is significantly reduced in *para*^{V1016G}; HR-GAL4>UAS-CYP9J28(2N) flies against all controls, indicating reduced overall fecundity. All *p*-values are shown in the electronic supplementary material, table S5. For the full life table parameters dataset, see the electronic supplementary material, dataset 1.

provide similar levels of resistance in topical application bioassays (table 1). When genetic background was controlled for in pest species, it has only been done by backcrossing individual variants such as the 1016 mutation in *A. aegypti* [31]. *Drosophila* provides an easier alternative, but caution must be taken between interpreting findings across species, taking into account not only the powers but also the limitations of this system (for a detailed relevant discussion see [32,33]).

The mechanism for the apparent synergism between P450s and target site mutations is still not fully understood. It may involve only the parent compound, i.e. reduced binding affinity for the target site could simply give P450s additional time to perform insecticide metabolism and avoid saturation. This is thought to be the case with P-glycoprotein CYP3A4 synergism in humans, whereby the former increases the time a compound spends in the intestine and thereby increases its chance of being metabolized by the latter [34]. The higher $RR_{combined}$ in the slow uptake compared to the fast-uptake topical application seems to corroborate this hypothesis (electronic supplementary material, table S3 and table 1, respectively). Alternatively, the less toxic P450-generated metabolites might bind the mutant target receptors disproportionately less effectively, thus manifesting in a synergistic phenotype. It is currently unknown what, if any, affinity the P450 derived metabolites of deltamethrin would have for *para*, but it has been suggested that their accumulation is likely to be detrimental in mosquitoes and that their metabolism by certain P450s, such as the *Anopheles gambiae* CYPZ8 and CYP6Z2, is a secondary but important mechanism of insecticide resistance [35]. In addition, examples from other compounds may provide some guidance. Imidacloprid is 'detoxified' by P450s into a variety of metabolites which still show levels of toxicity that would probably be relevant *in vivo* [36,37]. Nevertheless, there is currently no data regarding the binding of various pyrethroid metabolites for different alleles of *para*, so such a hypothesis awaits functional validation.

In addition to differences in resistance level, fitness costs were also observed for the various genotypes used in this study. While genotypes carrying a single resistance allele behaved similarly to the control lines (with a possible exception regarding *para*^{L1014F} fecundity), the combination of these alleles significantly increased the developmental time both in the 'beetle' and 'mosquito' genotype combinations and significantly reduced fecundity in the 'mosquito' combination against multiple controls contributing to the genetic background (figure 3). It thus seems possible that these alleles, may exert a fitness cost only in certain backgrounds, an implication which has far reaching ramifications for IRM. This hypothesis is also supported by studies done directly on these pest species. The combination of high level *Cyp6BQ23* expression and *kdr* mutations is extremely rare in pollen beetle populations, supporting the fitness cost theory [16], but the mechanism underpinning this phenomenon is not known. By contrast, the results presented here on the V1016G mutation contradict those obtained by backcrossing the mutation into a susceptible *A. aegypti* background, although the precise mutation in that study was different [31]. These data collectively suggest epistatic effects between different resistance mechanisms and highlight the need for fitness cost assessment to be done in multiple backgrounds. Further work will thus be needed to establish and characterize the evolutionary significance of these resistance alleles in the field.

Several groups are currently developing and applying DNA-based technologies for insecticide resistance monitoring [8,9]. Our study shows that these molecular diagnostics need

careful calibration, integration and interpretation, as an apparent epistasis (i.e. different phenotype depending on the genetic background) is present, and thus they may or may not diagnose the importance of resistance in the field. The reconstruction of complex resistance phenotypes by reverse-genetics based simultaneous introduction of individual mechanisms in a susceptible genetic background enhances our ability to elucidate the contribution of each individual molecular mechanism in the resistance phenotype; a concept that is perhaps best represented by the famous quote found at the blackboard of R. P. Feynman (1918–1988) at the time of his death ('*What I cannot create, I do not understand*'). Although significant research effort remains to be done, the present study provides a 'proof of principle' of the applicability of such a reconstructed resistance 'network of interactions' within a model *Drosophila* 'test tube' that enables the validation of hypotheses regarding the molecular mechanisms contributing to insecticide resistance phenotypes in field populations; in other words, to (re)create the interactions among different mechanisms, so that we can gain insight on their specific role.

Data accessibility. All data generated or analysed during this study are available in this manuscript and its electronic supplementary material.

Authors' contributions. V.D. and J.V. conceived the project and designed the experiments; G.-R.S., R.P, V.D., S.K. and I.C. generated fly lines; G.-R.S. and R.P. performed toxicity bioassays and qPCR; G.-R.S. performed fitness cost experiments; V.D., S.D., R.N. and J.V. analysed the data, contributed to scientific discussion and wrote the manuscript, which has been revised and approved by all authors.

Competing interests. The authors declare no competing interests.

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Identification of *Helicoverpa armigera* promoters for biotechnological applications

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ABSTRACT

Helicoverpa armigera and *Helicoverpa zea* are highly polyphagous major agricultural pests with a global distribution. Their control is based on insecticides, however, new, effective, and environmentally friendly control tools are required to be developed and validated. In an effort to facilitate the development of advanced biotechnological tools in these species that will take advantage of new powerful molecular biology techniques like CRISPR/Cas9, we used available transcriptomic data and literature resources, in order to identify RNA polymerase II and III promoters active in RP-HzGUT-AW1(MG), a midgut derived cell line from *Helicoverpa zea*. Following functional analysis in insect cell lines, four RNA polymerase II promoters from the genes *HaLabial*, *HaTsp-2A*, *HaPtx-1* and *HaCaudal* were found to exhibit high transcriptional activity *in vitro*. The HaTsp-2A promoter did not exhibit any activity in the non-midgut derived cell lines Sf-9 and Hi-5 despite high sequence conservation among Lepidoptera, suggesting that it may function in a gut specific manner. Furthermore, considering the utility of RNA polymerase III U6 promoters in methodologies such as RNAi and CRISPR/Cas9, we identified and evaluated four different U6 promoters of *H. armigera*. *In vitro* experiments based on luciferase and GFP reporter assays, as well as *in vivo* experiments targeting an essential gene of *Helicoverpa*, indicate that these U6 promoters are functional and can be used to experimentally silence or knockout target genes through the expression of shRNAs and sgRNAs respectively. Taking our findings together, we provide a set of promoters useful for the genetic manipulation of *Helicoverpa* species, that can be used in various applications in the context of agricultural biotechnology.

1. Introduction

Throughout the last decades, significant progress has taken place in the field of insect genetic manipulation, beginning from the application of random integration techniques and continuing with precise genome editing methodologies (Fraser, 2012; Gantz and Akbari, 2018). Insect transgenics has been a valuable tool for both functional characterization of genes and the development of biotechnological methods to control the populations of harmful insects (Douris et al., 2020; Fraser 2012). Importantly, population control approaches based on genetically modified insects, like sterile insect technique (Lees et al., 2015) and gene drive (McFarlane et al., 2018) hold the promise of more effective and safe pest management.

Reverse genetic methodologies are largely based on the use of promoters. The RNA polymerase II promoters (RNA-polII) are DNA sequences containing *cis*-acting elements, capable of driving gene expression in a ubiquitous or in a tissue specific manner. A large number of studies focuses on the identification and characterization of constitutive promoters of non-model insects (Lu et al., 1997; Tsubota et al., 2014; Chen et al.,2020a, 2020b; Bleckmann et al., 2015; Miyata et al.,

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2019). Promoters of differing strengths are often useful for different applications, and the same promoter often works differently depending on the organism, tissue, and cell line under investigation. Apart from the RNA-PolII, RNA-polIII promoters, which are known to drive the expression of small RNAs (e.g. small nuclear RNAs, tRNAs etc) are also very useful in the field of transgenesis. Specifically, U6 promoters, which comprise a subset of RNA-polIII promoters that regulate the expression of U6 small nuclear RNAs (Hernandez et al., 2007), are extensively used in techniques such as RNAi and CRISPR/Cas9, for the synthesis of short hairpin RNAs (shRNAs) and single-guide RNAs (sgRNAs) respectively (Huang et al., 2017; Port et al., 2014). Generally, insect U6 promoters exhibit high sequence divergence within and between species (Hernandez et al., 2007). However, there are two regulatory sequences that are crucial for the activity of U6 promoters and are highly conserved throughout Insecta: the Proximal Sequence Element A (PSEA) and the TATA box (Hernandez et al., 2007). Although there are some types of versatile U6 promoters, which can function across different insect species, high levels of expression are usually achieved only when endogenous U6 promoters are used (Huang et al., 2017, Mabashi-Asazuma and Jarvis, 2017). Nevertheless, in several important pest insect species, endogenous U6 promoters have not yet been characterized.

The cotton bollworm *Helicoverpa armigera* (Hübner) and the corn earworm *Helicoverpa zea* are allopatric species capable of interbreeding and they are considered as agricultural pests threatening several cultivated plants (Haile et al., 2021; Reay-Jones 2019). Their control is based on microbial or chemical insecticides that are usually delivered orally while the insects are at the larval stages of their life cycle (Haile et al., 2021; Wu et al., 2008). Most of the orally delivered insecticides either must pass through or directly target the midgut epithelium, which highlights the importance of this tissue in pest control (Denecke et al., 2018; Heckel 2020, Syed et al., 2020). The lepidopteran midgut comprises one of the largest tissues of the lepidopteran body, with a predominant role in insects' physiology. It is an endodermally derived highly complex tissue, composed of a single cell epithelial layer where midgut cells are connected tightly with smooth septate junctions (SSJs), mediated by genes like *Tsp-2A* (Izumi et al., 2016, 2021). Moreover, midgut compartmentalization in both larval and adult stages is another important characteristic that increases its complexity. Certain genes such as the homeobox-containing genes *Labial*, *Caudal* and P*tx-I* have been correlated with differentiation and regionalization of the gut in *Drosophila* (Buchon et al., 2013).

Given the difficulties in working with tissues like midgut *in vivo*, several research groups have developed and used lepidopteran cell lines that may retain *in vivo* properties, in order to study insect biology in a more tractable system (Swevers et al., 2021; Arunkarthick et al., 2017). Well-suited examples are the development of RP-HzGUT-AW1(MG) (Goodman et al., 2004) and its further characterization (Vorgia et al., 2021) as well as two recently developed midgut derived cell lines of *S. frugiperda* (Zhou et al., 2020). Genetic tools are thus needed to further study these cell lines across a range of organisms. For example, the identification and characterization of a range of RNA PolII promoters in Sf9 cells and a midgut derived cell line from *Spodoptera frugiperda* has greatly expanded the range of possible projects undertaken on these cell lines (Chen et al., 2020a; Chen et al., 2020b). Furthermore, the application of the CRISPR/Cas9 technology in lepidopteran cell lines like *Plutella xylostella* and *Bombyx mori*, has been substantially facilitated by the use of customized expression vectors carrying species specific U6 promoters (Huang et al., 2017, Mabashi-Asazuma and Jarvis, 2017). However, such transgenesis tools remain to be identified in other important non-model species like *H. armigera* or *H. zea*.

Here, by using available tissue specific transcriptomic datasets and literature resources, we identified RNApolII and RNApolIII promoters and we have functionally characterized them in cell lines including a midgut derived cell line from *H. zea*. It is hoped that this work will provide an enhanced genetic toolkit for *Helicoverpa* cell lines and provide a basis for performing more advanced *Helicoverpa* transgenics *in vivo*.

2. Materials and methods

2.1. Identification of U6 promoters and putative midgut-specific promoters

To select putative midgut-active promoters we employed two complementary approaches. The first approach was "literature driven": five genes which have been previously associated with midgut physiology were selected for analysis of their promoters. The second approach attempted to identify genes with expression specifically in the midgut as these would theoretically both be active in midgut derived cell lines but may also have future use in generating midgut specific expression *in vivo*. This second approach relied purely on expression data of *H. armigera* midgut of L2, L3 and L4 larval stages (Ioannidis et al., 2022) and led to the selection of eight genes from a subset of the 20 most abundant (high TPM, transcripts per million) and most midgut-specific (FC, fold change against carcass) genes, which were filtered after using a threshold with value 100 for both TPM and FC. For the 13 selected genes, an approximately 2,000bp region directly upstream of the translation initiation codon was considered as a putative promoter.

To identify the U6 snRNA gene(s) of *H. armigera*, we searched the genome (GCF_002156985.1) using BLASTn with the 107 nt sequence of *Bombyx mori* U6 snRNA gene (NCBI GeneID: 119628772) as a query (Hernandez et al., 2007). Orthologous midgut promoters from other lepidopteran species were also identified using Blastn, selecting the most significant hits in terms of E-value. All multiple sequence alignments were performed using ClustalW implemented in Bioedit 7.2 with default parameters.

2.2. Construction of plasmids

2.2.1. Plasmids for the analysis of the midgut-active promoters

A modified version of pBluescript SK(+) was initially engineered, in order to insert a PCR fragment of the multiple cloning site of the pSLfa1180fa vector (Horn et al., 2003) with SacI. This multiple cloning site contains the recognition sites of the 8-cutter restriction enzymes FseI and AscI, which can be used for cloning the promoter sequences of any gene of interest. This modified vector was named as pBSk^{+}FA. A 2.7 Kb PCR fragment (amplified with Phusion High Fidelity Polymerase, NEB) containing a firefly luciferase-SV40 poly(A) cassette (derived from pGL2-Basic Vector, Promega), was cloned between the AscI and SpeI sites of the pBSk^{+}FA (primer sequences are presented in the Supplementary Table S1) to create the plasmid pBSk^{+}FA[FFluc-SV40](Fig. S3). All the promoters and the respective truncated versions, were amplified with PCR (Phusion High Fidelity Polymerase, NEB) using primers bearing the restrictions sites FseI and AscI. They were cloned into the pGEM-T-easy Vector (Promega) and verified by sequencing (CeMia, Greece). Subsequently, each promoter was cloned into the vector pBSk^{+}FA[FFluc-SV40] directly upstream of the luciferase reporter gene (Fig. S3). All of the plasmids were purified using the NucleoSpin Plasmid, Mini kit for plasmid DNA (Mancherey Nagel). The pBmAc3-Renilla luciferase construct was used as a control for normalization of transfection efficiency in the luciferase assays and was generated as follows: the ORF of Renilla luciferase was cloned as a PCR fragment (from the pSis-Check2 plasmid) between the BamHI and NotI sites of the pBmAc3 vector (a modified version of pEIA plasmid, lacking the hr3 enhancer and the IE1 cassette, kindly provided by Dr. Kostas Iatrou (NCSR "Demokritos") (Douris et al., 2006)).

2.2.2. Plasmids for assessing the ability of the U6 promoters of to drive the expression of shRNAs

To assess the ability of the U6 promoters to drive the expression of shRNAs, a 63bp fragment coding for a shRNA that targets Renilla luciferase (Tanaka et al., 2009), which was generated by annealing the

single-stranded oligos Rluc_shRNA_F and Rluc_shRNA_R (Supplementary Table S1), was cloned between the NotI and XhoI sites of the pSLfa1180fa vector. Four out of the five putative U6 promoters were able to be PCR amplified from *H. armigera* genomic DNA using forward and reverse primers that contain a BamHI and a NotI restriction site respectively (Supplementary Table S1). The PCR fragments were cloned upstream of the fragment coding for the shRNA and the final plasmids were named pSL[HaU6:1-shRNA-Rluc], pSL[HaU6:2-shRNA-Rluc], pSL[HaU6:2a-shRNA-Rluc] and pSL[HaU6:3a-shRNA-Rluc]. The constructs were verified by sequencing at each step of the cloning and prior to transfection.

2.2.3. Plasmids for assessing the ability of the U6 promoters of to drive the expression of sgRNAs

To generate a Lepidoptera-specific CRISPR-vector, we initially modified the Cas9-T2A-mCherry cassette (Addgene #64324) by replacing the coding sequence of the T2A peptide with a fragment coding a GSG-P2A peptide, which has been previously shown to exhibit higher cleavage efficiency in lepidopteran cells (Wang et al., 2015). The fragment coding for the GSG-P2A peptide, which was generated by annealing the single-stranded oligos GSG-P2A-F and GSG-P2A-R, was ligated between the HindIII and NheI sites of the Cas9-T2A-mCherry vector. The Cas9-GSG-P2A-mCherry cassette was excised with the AgeI and EcoRI restriction enzymes and was cloned between the XmaI and EcoRI sites of the pSLfa1180fa plasmid (Fig. S4). The expression of Cas9-GSG-P2A-mCherry cassette was regulated by a ubiquitous-acting promoter (either the BmNPV immediate early gene 1 (IE1) promoter (Huybrechts et al., 1992) or the promoter of the silkworm A3 cytoplasmic actin gene (BmAc3) (Johnson et al., 1992)). Each of these two promoters was cloned as a PCR fragment, with BamHI and AgeI restriction sites (Fig. S4). The SV40 poly(A) signal was ligated to the vector with EcoRI. The sgRNA scaffold was synthesized with PCR using Phusion Polymerase with no template; one oligonucleotide carrying the NotI (bold) and BbsI (underlined) restrictions sites (gRNAscaffold_NotI_F: GTAC**GCGGCCGC** GAAGACCTGTTTTAGAGCTAGAAATAGCAAGTTA AAAtaaggctagtccgttatcaacttg) and another oligonucleotide carrying XhoI cut site (GTACCTCGAGCTCTGTACAAAAAAAGCACCGACTCG GTGCCACTTTTT caagttgataacggactagcctta), both sharing 24 complementary ends (lowercase letters), were used in 100ul PCR reactions and cloned between the NotI and XhoI sites of the plasmid. PCR reactions were performed as follows: 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 15 s, followed by final extension at 72 °C for 2 min. Finally, each of the U6 promoters was cloned in the vector with AgeI and NotI as PCR fragments (Fig. S4). Two BbsI restriction sites were introduced in between the end of the U6 promoters and the beginning of the sgRNA scaffold in 5'->3′ direction (Fig. S4), in order to facilitate the cloning of any CRISPR target of interest as dsDNA oligos (Fig. S4).

2.3. Cell lines and gene reporter assays

To functionally validate and quantify the activity of the 13 putative midgut-specific promoters we performed luciferase assays in cell lines. The cell lines used in this study were sub-cultured twice a week. The *H. zea* midgut cell line RP-HzGUT-AW1(MG), generously provided by Dr. Cynthia L. Goodman (Biological Control of Insects Research, U.S, Department of Agriculture, Agriculture Research Service), was routinely maintained as adherent culture in Excell 420 insect serum-free medium (Sigma Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO, Thermo Fisher Scientific), 100U/ml of penicillin and 0.1 mg/ml streptomycin. The *Trichoplusia ni* embryo cell line Hi-5 (Granados et al., 1994), and the *Spodoptera frugiperda* ovarian cell line Sf-9 were maintained as suspension and adherent cultures respectively, in the insect serum free medium SF-900 II SFM (Thermo Fisher Scientific), while the medium of Sf-9 cells was additionally supplemented with 10% FBS. All of the aforementioned cell lines were kept in T-25 flasks in an incubator at 28 °C. The RP-HzGUT-AW1(MG), Hi-5 and Sf-9 cells were transfected using Escort IV Transfection Reagent (Sigma Aldrich), according to the manufacturer instructions. The cells were seeded in the appropriate density in 12-well or 24-well plates and transfected with 500 ng and 250 ng of DNA respectively.

The promoters were analyzed using the dual-luciferase reporter assay system (Promega), according to the manufacturer instructions. For the luciferase assays each cell line was co-transfected with the experimental reporter constructs and the control vector (pBmAc3-Renilla luciferase) at a ratio of 200:1. As a negative control we used an empty (promoterless) vector that contained the firefly luciferase gene. The Relative Luciferase Activity (Firefly Luciferase/Renilla Luciferase, RLA) was further normalized to obtain a fold change with respect to the promoterless vector. Transfections and subsequent luciferase assays were performed in triplicates or quadruplicates and in at least three independent experiments.

To measure the ability of the *H. armigera* U6 promoters to drive the expression of the shRNA that targets Renilla luciferase, the RP-HzGUT-AW1(MG) and the Sf-9 cells were co-transfected with a plasmid carrying each U6 promoters driving shRNA expression, the pBSk^{+}FA[iE1_FFluc-SV40] or pBSk^{+}FA[HaLabial_FFluc-SV40] and pBmAc3-Renilla luciferase, at a ratio of 200:200:1. The pBSk^{+}FA[iE1:FFluc-SV40] plasmid was used as a normalizer to control for transfection efficiency in RP-HzGUT-AW1(MG), while pBSk^{+}FA[HaLabial_FFluc-SV40] was used to control for transfection efficiency in Sf-9 cells.

To analyze the competence of the U6 promoters for sgRNA expression, we used the RP-HzGUT-AW1(MG) cell line, which was co-transfected at a ratio 1:1 with the pEIA-GFP plasmid (Vorgia et al., 2021) and each of the U6-driven CRISPR vectors. The cells were observed under an inverted fluorescence microscope 72-hrs post transfection. The CRISPR efficiency was analyzed by calculating the percentage of cells emitting green fluorescence in at least 3 random fields and the pictures were analyzed using the software Image J (Rueden et al., 2017).

2.4. Statistical analysis

The statistical analysis for significance for all comparisons was calculated with One-Way ANOVA, non-parametric test using the software GraphPad Prism 3.0.1. For comparisons of multiple treatments against a common control (e.g. truncation assays on promoter fragments), a Dunnett post-hoc test was used. In cases where all treatments were compared against each other, Tukey's post-hoc corrections were made.

2.5. Western blot analysis

To further validate the knock-out of GFP in RP-HzGUT-AW1(MG), the cells co-transfected with each of U6-driven CRISPR vectors and pEIA-GFP were harvested 72-hrs post transfection and lysed with RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% Sodium-Deoxycolate, 0.1% SDS and 1% NP-40) supplemented with Protease Inhibitors (Sigma-Aldrich) and 1 mM PMSF. Protein concentration was measured with Bradford Assay. Approximately 30 μg of total protein/cell lysates were loaded onto a 10% SDS-PAGE and electrotransferred to nitrocellulose membrane for 1.5hrs at 350 mA at 4 °C. The membrane was blocked with 5% milk (ROTH) in 1X TBST buffer for 1 h and then incubated with 1:1000 dilution of mouse Anti-GFP antibody (Cell Signalling), 1:1000 dilution anti-beta-tubulin (Sigma-Aldrich) as a loading control and also with 1:2500 dilution of anti-Flag (Sigma-Aldrich) for detection of Cas9 which is tagged with a 3xFLAG peptide at the N-terminus.

2.6. In vivo functional validation of H. armigera U6 promoters in G0 animals

In order to verify the activity of the U6 promoters *in vivo*, we designed one sgRNA targeting the *HaOatp74D* gene and confirmed for mutations by amplicon sequencing. Double-stranded DNA oligos (Table S1) were cloned in the vectors as previously described (section 2.2.3, Fig. S4). *H. armigera* egg batches were collected shortly after the onset of the scotoperiod, transferred to double sided tape. The eggs were then inected with 400 ng/μl of each U6 promoter vector. The eggs were visually inspected for mCherry expression under a fluorescent microscope, three days post injection. Genomic DNA was extracted from eggs injected for each construct, using DNAzol and used as template for amplification of the region of interest using Phusion polymerase. The primers used were HaOATP74DF/HaOATP74DR in each of the three sdifferent conditions (A. Eggs injected with U6:1-vector, B.Eggs injected with U6:2a-vector and C. Eggs injected with U6:3a-vector). The products were purified and sent for amplicon sequencing (GeneWiz).

3. Results

3.1. Selection of putative midgut active promoters for analysis

In order to identify promoters which would be i) active in midgut cell lines and ii) potentially used for future *in vivo H. armigera* midgut specific transgenics in the *H. armigera*, we used as a starting material a set of 637 genes which are commonly over-expressed in the midgut of L2, L3 and L4 larvae with respect to the carcass tissues (Ioannidis et al., 2022). Based on the consensus view that very strong promoters are probably reflected by highly expressed genes, from the set of 637 genes we selected those with FC > 100 for tissue-specificity and TPM>100 for absolute expression and finally obtained the 20 most highly up-regulated genes compared to the carcass tissues (Fig. 1A). From the subset of the 20 most highly up-regulated genes (Fig. 1A), the 8 displaying TPM and FC values above the median at all life stages were selected for functional analysis of their promoters (Table 1, NCBI GeneIDs: 110377047 (serine protease 3-like), 110384513 (brachyuran-like), 110379024 (trypsin, alakaline C-like), 110372923 (actin cytoskeleton regulatory complex PAN1-like), 110380915 (cilia- and flagella-associated protein 58-like), 110381636 (uncharacterized protein), 110381625 (uncharacterized protein) and 110370997 (uncharacterized protein)). However, we could not amplify with PCR the promoters of the genes with IDs: 110381636, 110381625 and 110370997 possibly due to sequence differences in our strain.

Table 1
Selected genes for functional analysis of RNA-PolII promoters.

	NCBI GeneIDs	Gene Annotation
Highly up-regulated midgut genes	110384513	brachyurin-like
	110379024	trypsin, alkaline C-like
	110377047	serine protease 3-like isoform X1
	110372923	actin cytoskeleton-regulatory complex protein PAN1-like isoform X1
	110972915	cilia- and flagella- associated protein 58-like
	110381636	uncharacterized protein
	110381625	uncharacterized protein
	110370997	uncharacterized protein
Literature derived genes	110370350	mucin-1
	110371850	tetraspanin-2a
	110375077	labial
	110377322	caudal
	110378176	ptx-1

In parallel, we selected 5 additional genes from set of 637 which were also known from the literature to be expressed in the midgut of *Helicoverpa* or in closely related insect species. The genes *Labial*, *Tetraspanin-2A*, *Ptx-I* and *Caudal*, which are highly expressed in the midgut of *Drosophila* (Buchon et al., 2013), share only one ortholog with *Helicoverpa* (NCBI GeneIDs: 110371850, 110378176, 110377322 respectively) and were found to be included in the dataset of 637 midgut upregulated genes. The promoter of *mucin-I*, which based on proteomic dataset drives the expression of one of the most abundant proteins of the peritrophic matrix of *H. armigera* (Campbell et al., 2008), was also included in the analysis.

3.2. Functional analysis of the midgut promoters

A. Transcriptional strength

To characterize midgut promoters, we performed luciferase assays in the *H. zea* midgut cell line RP-HzGUT-AW1(MG) (Goodman et al., 2004). Although the 5 *Helicoverpa* midgut specific genes selected from the transcriptomic data had very high expression, in the luciferase assays the activity of their promoters was only between ~2 and ~7-fold higher than the promoterless vector (110384513, 110372923 and 110380915) or undetectable (110379024 and 110377047) (Fig. 1B). On the contrary, with the exception of the *mucin-I*, the promoters of the other four genes selected from the literature (*HaLabial*, *HaTsp-2A*, *HaPtx-I and HaCaudal*), exhibited very high transcriptional activity ranging from

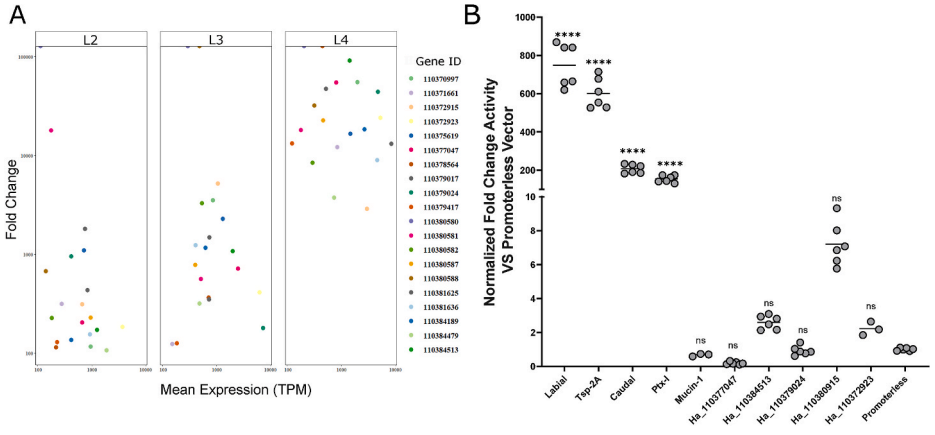

Fig. 1. Identification and functional characterization of midgut strong promoters. A) Distribution of the 20 most highly upregulated genes, displaying the highest absolute expression (Transcript Per Million, TPM) and tissue specificity (Fold Change with respect to the carcass tissues) of *H. armigera* midgut from L2, L3 and L4 instar larvae, as defined by the transcriptomic dataset. B) Functional analysis of the transcriptional activity of transcriptomic (Ha_110377047, Ha110384513, Ha110379024, Ha110380915 and Ha110370923) and literature (HaLabial, HaTsp-2A, HaPtx-I, HaCaudal and HaMucin-1; see section 3.1) driven promoters in RP-Hz-GUT-AW1 cells. Fold change luciferase activity is expressed as ratio of the Relative Luciferase Activity (RLA) of each promoter against the RLA of promoterless vector and values are expressed as means with 95% CIs. The fold change luciferase activity for each promoter was compared against the promoterless vector (negative control) and data were analyzed with one-way ANOVA followed by post-hoc Dunnett test. Different replicates are represented as individual dots. ****: p-value<0.001, ns: not significant.

~150 to ~750-fold higher than the promoterless vector (Fig. 1B).

B. Deletion analysis

To determine the minimum length necessary for high transcriptional activity of each strong promoter, we performed deletion analysis. Our results, presented in Fig. 2, indicate that the 400-bp proximal region of the *HaTsp-2A* and *HaPtx-I* promoters retained full activity. Full activity was also retained by the 1600bp proximal region of the *HaCaudal* promoter, while shorter fragments (800bp and 400bp) exhibited reduced activity (by approximately 33% and 45% respectively). The *HaLabial* promoter deletion analysis indicated that the 1480bp fragment upstream of the start codon displayed almost similar activity with the 2000bp fragment. Furthermore, deletion analysis suggested the presence of two positive regulatory elements within the sequence of *HaLabial* promoter: one is located within the 1480bp to 1170bp region, and another is located within the 597bp to 400bp region (numbers indicate distances upstream from the ATG initiation codon).

C. Comparison of midgut promoters with ubiquitous promoters

To evaluate the strength of the four active promoters, we compared their activity with that of two versatile yet exogenous ubiquitous-acting promoters, the BmNPV iE1 and BmAc3 which are active in many lepidoptera cell lines (Tamura et al., 2000, Masumoto et al., 2012). Although the activity of the 4 midgut-derived promoters was >80% less compared with the BmAc3, they display statistically significantly stronger activity than BmNPV iE1 (Fig. 3A). Additionally, we checked the activity of the putative endogenous promoter of Ha*Ef1a*, which is highly expressed based on the transcriptomic data. Despite the high expression levels of this gene, its promoter seems to be less active with respect to the promoters of the genes *HaLabial*, *HaTsp-2A*, *HaCaudal* and *HaPtx-I*. Specifically the promoters of midgut enriched genes displayed approximately 4.7–23.1X greater activity than the 2000bp fragment upstream of the start codon of *HaEf1a* (Fig. 3A).

D. The activity of HaLabial, HaPtx-I, HaCaudal and HaTsp-2A promoters in other insect cell lines

In order to analyze the evolutionary conservation of the four strong promoters, we functionally characterized them in the non-midgut lepidopteran cell lines Hi-5 and Sf-9**.** The luciferase assays showed that *HaLabial*, *HaPtx-I* and *HaCaudal* promoters exhibit low to moderate activity in these cell lines, ranging from 5 to 22 fold in Hi-5 and almost 25 fold in Sf-9 cells (Fig. 3B). However, HaTsp-2A was not functional in either Sf-9 or in Hi-5 cells. To explain why some of these promoters are functional in other insect cells of different species and tissue origin, we compared their sequence with the putative promoters (~2 kb upstream of start codon) of their orthologs from five insect species (*Trichoplusia ni*, *Spodoptera litura*, *Spodoptera frugiperda*, *Bombyx mori* and *Drosophila melanogaster*). Multiple sequence alignment of the *HaLabial* putative promoter with the respective putative promoters of *SlLabial*, *SfLabial* and *TnLabial* indicated an overall sequence identity of ~52% throughout the 2000bp sequence (Table S2), with the region −800bp to exhibit more than 70% homology (Additional Files). Furthermore, the sequence alignments for the *HaPtx-I* and *HaCaudal* promoters indicated a sequence identity of approximately 35% and 45% respectively with the promoters of their orthologs in *S. litura*, *S. frugiperda* and *T. ni* (Table S2). The low activity in cell lines derived from different lepidoptera species conforms with the partial sequence conservation of the promoters and is probably explained by conserved regulatory elements found in the first 800bp of the promoters (Additional Files). Sequence identity analysis for *Tsp-2A* putative promoters indicated very high conservation among the lepidopteran species *Helicoverpa*, *Trichoplusia* and *Spodoptera* (72% and 76% identity of *Helicoverpa* with *Trichoplusia* and *Spodoptera* respectively, Table S2). However, functional analysis of the *HaTsp-2A* promoter in Hi-5 and Sf-9 cells showed that this promoter is not active, raising the possibility that this promoter is active only in midgut cells and thus acting as a midgut specific promoter (Fig. 3B).

3.3. Identification and functional analysis of H. armigera U6 promoters

Five different copies of the U6 snRNA gene were identified with 100% identity in the *H. armigera* genome, localized in three different scaffolds. The U6 promoters (~500bp upstream of U6 TSS) were named numerically arbitrarily as follows, U6:1 (scaffold 14:457024-457588), U6:2 (scaffold 263:211789-212407), U6:2a (scaffold 263:206977-207533), U6:3 (scaffold 62:261765-262314) and U6:3a (scaffold 62:314253-314733). Of these five promoters, four were successfully amplified with PCR. Alignment of the four sequenced *H. armigera* U6 promoters with four functional U6 promoters of other lepidoptera species suggested that the 5' half of the PSEA seemed to be conserved (Fig. 4A). No distinct TATA box motifs were identified in the sequences of the *H.armigera* U6 promoters, whereas the sequences of *B.mori*, *S. frugiperda* and *P.xylostella* U6 promoters contain this motif ~13 bp downstream of the PSEA (Fig. 4A). Despite the fact that the existence of TATA box motifs is a parameter for selecting functional U6 promoters (Hernandez et al., 2007), we proceeded to their characterization by

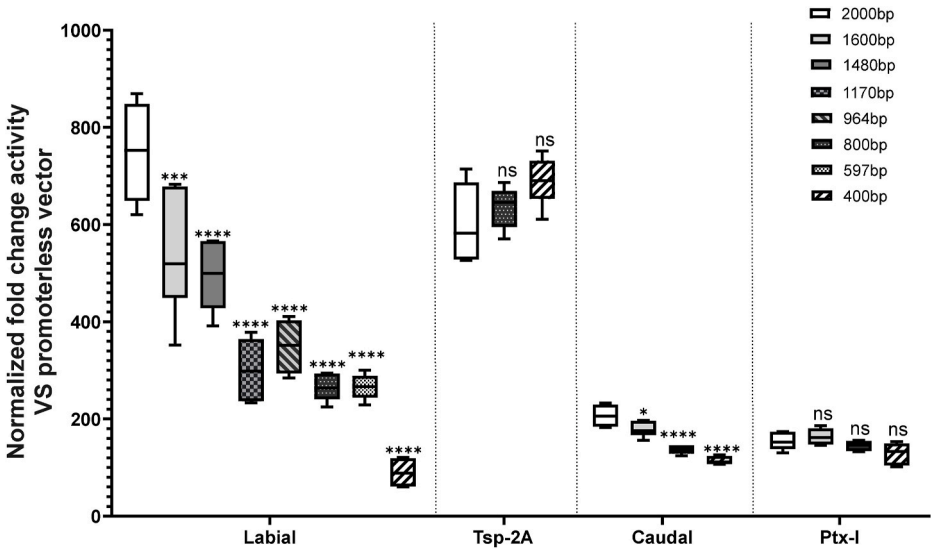

Fig. 2. Deletion analysis for the 4 highly active literature driven promoters of the genes *HaLabial*, *HaTsp-2A*, *HaCaudal* and *HaPtx-I* in RP-Hz-GUT-AW1 cells. Fold change luciferase activity is expressed as ratio of the Relative Luciferase Activity (RLA) of each promoter against the RLA of promoterless vector. Deletions analysis data of each group (promoter) were analyzed by comparing the mutated promoters with the initial size, using one-way ANOVA followed by post-hoc Dunnett test. (Labial ***: p-value = 0.0001, Caudal *: p-value = 0.0124, ****: p-value<0.0001, ns: not significant).

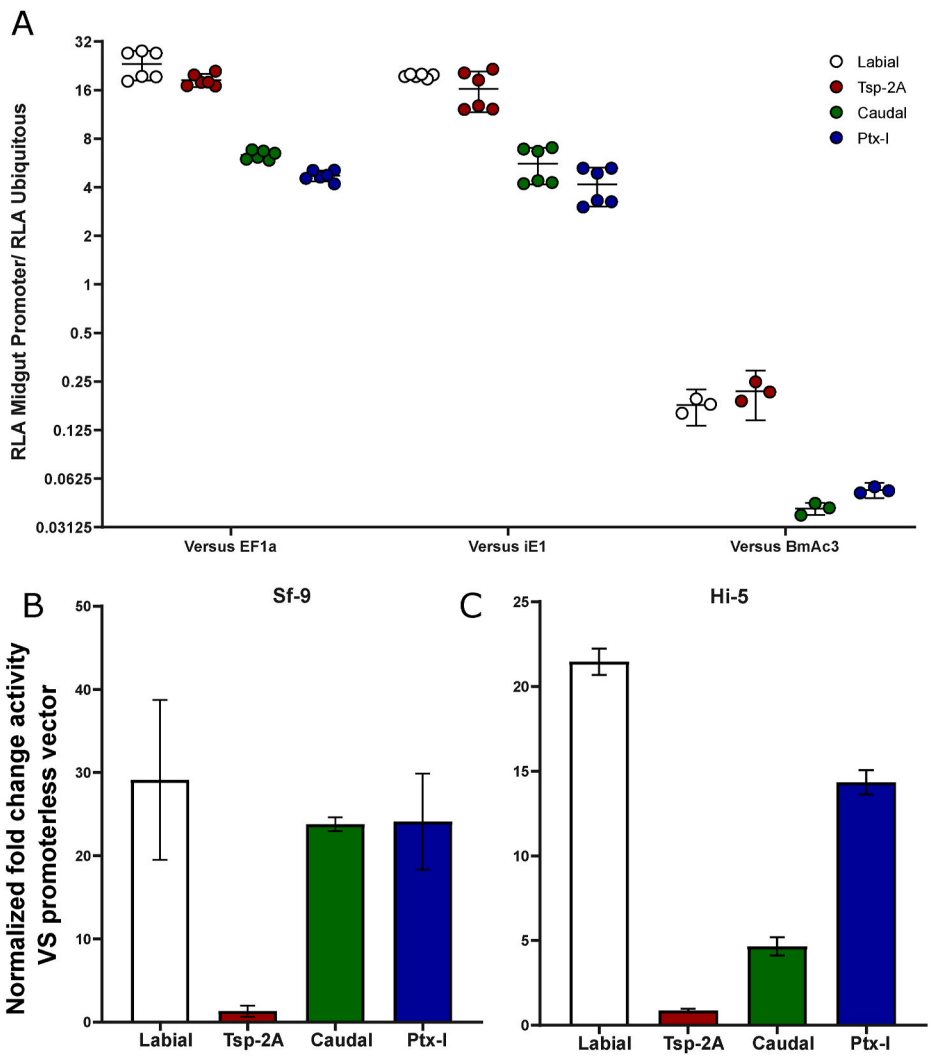

Fig. 3. Characterization of the 4 highly active literature driven promoters. A) Comparative analysis of the activity of *HaLabial*, *HaTsp-2a*, *HaCaudal* and *HaPtx-I* promoters with the activity of constitutive promoters (HaEF1a, BmNPV iE1 and BmAc3) in RP-Hz-GUT-AW1 cells. The RLA of each Ha Midgut specific promoters was divided with the RLA of the ubiquitous-acting promoters. B,C) Conservation analysis of the 4 promoters. The promoters were analyzed for their activity in B) Sf-9 and C) Hi-5 cell lines. Normalized fold change activity is expressed as ratio of the Relative Luciferase Activity (RLA) of each promoter against the RLA of promoterless vector. For B and C: data were analyzed for significance with one-way ANOVA among groups followed by post-hoc Tukey test. The p-values per comparison are summarized in the Table S3.

testing their ability to drive shRNAs for RNAi and sgRNAs for CRISPR in the RP-Hz-GUT-AW1 cell line.

According to the luciferase assay, in which Renilla luciferase was targeted by shRNA produced by each of the four HaU6 promoters, all of the *Helicoverpa* U6 promoters were functional. More than 98% reduction of luminescence was observed for all of the four U6 promoters tested in RP-Hz-GUT-AW1, compared to the negative control which contains only the sequence for shRNA against Renilla (Fig. 4B). Analysis of these promoters in Sf-9 cells has indicated a statistically significant reduction of Renilla luciferase expression mediated by HaU6:1 and HaU6:2a (92% and 96% of inhibition respectively). Additionally, we tested the ability of the U6 promoters for expression of sgRNAs by targeting GFP in a plasmid expressing a Cas9-GSG-P2A-mCherry cassette under either BmAc3 or iE1 promoter (Fig. S4). Significant reduction of the GFP fluorescence intensity was observed for all of the *Helicoverpa* U6 promoters, when Cas9 was expressed under BmAc3 promoter. Specifically, the sgRNA targeting the 5' region of GFP sequence exhibited almost complete loss of signal (Fig. 5A). The efficiency of GFP knock-out was less in the case of HaU6:2a promoter, since a small amount of GFP protein could still be detected with specific antibody, whereas it was almost absent when HaU6:1 and HaU6:3a promoters were used (Fig. 5B). Less efficient, yet effective was Cas9 when expressed under iE1 regulatory elements leading to lower reduction of GFP compared to BmAc3, which is attributed to the lower expression of the endonuclease, as it is indicated by Western-blot analysis (Fig. 5B). To further explore the efficiency and the applicability of these vectors *in vivo*, we performed microinjections of each of these constructs in *H. armigera* eggs, targeting the gene *Oatp74D* (Gene ID: 11377536). The region of interest was amplified and analyzed with amplicon sequencing to search for mutations proximal to the Cas9 target. All the CRISPR vectors were functional considering the different mutated alleles identified in each amplicon (Fig. S5).

4. Discussion

The midgut of pests like *H. armigera* and *H.* zea is the site of nutrient digestion and absorption, as well as other biological processes like xenobiotic metabolism and parasite interactions (Denecke et al., 2018; Caccia et al., 2019). The difficulty in studying many of these processes *in vivo* has led to significant effort towards generating midgut derived cell lines for exploration of midgut like properties *in vitro*. In particular, the further characterization of midgut derived cell lines such as RP-Hz-GUT-AW1 (Vorgia et al., 2021), has set the stage for its use in future studies, but a lack of genetic tools in this species precludes its widespread use. Here, we characterized *Helicoverpa* PolII and PolIII promoters in the RP-Hz-GUT-AW1 cell line in order to provide systems for precisely regulating gene expression in this cell line.

4.1. RNA polymerase II promoters

Analysis of promoters derived from five of the most highly larval midgut up-regulated genes indicated these genes show low

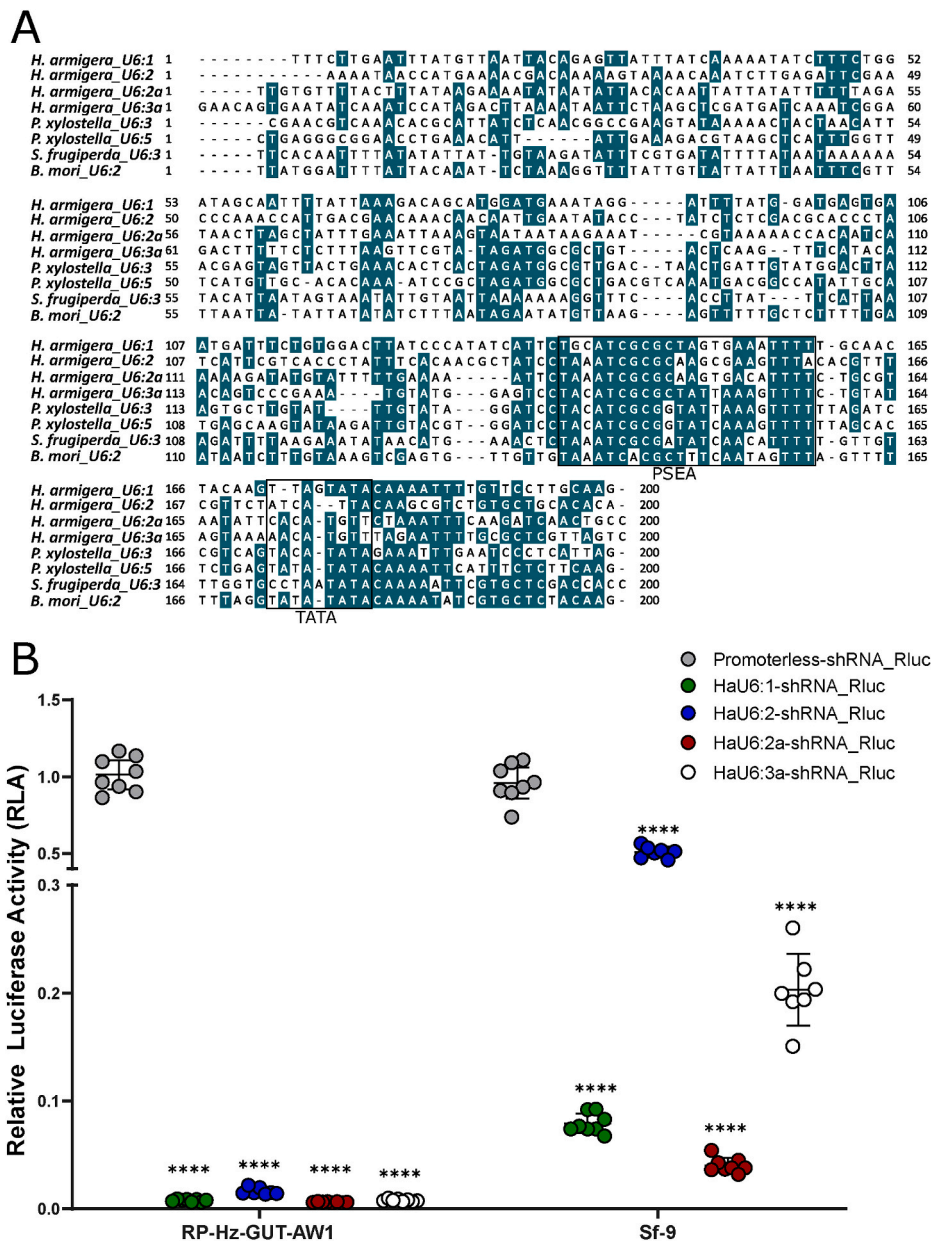

Fig. 4. Identification of *H. armigera* U6 promoters and functional analysis of their silencing efficiency. A) Sequence alignment of 200bp upstream of the TSS of *H.armigera* U6 snRNA gene with the respective regions of *P.xylostella*, *S.frugiperda* and *B.mori* U6 promoters. Dark green highlights more than 60% sequence similarity. Sequences in frames highlight the regulatory sequence PSEA and TATA-box. B) Functional characterization of the silencing activity of the 4 *H. armigera* U6 promoters by analyzing the expression of shRNAs targeting Renilla luciferase in RP-Hz-GUT-AW1 and Sf-9 cells. Relative Luciferase Activity is calculated as ratio of the normalized RLUs of Renilla of the experimental condition against the normalized RLUs of Renilla obtained by the U6 promoterless expression vector (negative control). Multiple comparison among different groups was performed using one-way ANOVA with post-hoc Tukey test. The p-values are summarized in the Table S3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

transcriptionally activity in the midgut derived cell line of *H. zea* RP-Hz-GUT-AW1 (Fig. 1B). This is in accordance with a recent work in which the putative promoter of *S. frugiperda acpp1*, along with other putative promoters of highly expressed genes of the midgut, have been found to be also non-functional in the midgut derived cell line Sf17 (Chen et al., 2020b). A reasonable explanation could be that the activity of these promoters is probably governed by tissue specific enhancers localized in genomic regions further than the ~2 kb sequence upstream of the start codon. Another explanation could be that high levels of expression of those genes are caused by high mRNA stability, as has been reported previously in an analogous study (Bleckmann et al., 2015).

Furthermore, it needs to be considered that, while the RP-HzGUT-AW1(MG) cell line was derived from midgut tissue (Goodman et al., 2004), considerable de-differentiation may have occurred during adaptation to *in vitro* culture, as is typically observed for insect cell cultures (Swevers et al., 2021). Genes that are associated with highly specialized functions such as secreted proteases, cytoskeleton elements that maintain the columnar epithelial structure, and microvilli are expected to become turned off when cells start dividing and become a continuously multiplying population. The low activity of such promoters is therefore not unexpected and may illustrate the relatively low differentiation status of the employed midgut-derived cell line. This is supported by the transcriptomic analysis of RP-HzGUT-AW1(MG) cells, which has revealed the expression of specific genes that are suggestive of an “arrested” differentiation status between Intestinal Stem cells (ISCs) and fully differentiated cells (Vorgia et al., 2021). Further research efforts are required to establish the mechanisms by which (at least partial) differentiation can be induced in insect midgut-derived cell lines (Swevers et al., 2021).

The putative promoters of four *Helicoverpa* genes, that were characterized as midgut enriched in other insect species, yielded very high levels of transcriptional activity in RP-HzGUT-AW1 cells. Among the four, the promoters of *Tsp-2A* and *Labial* genes exhibited the highest activity, but HaTsp-2A seems to be especially interesting, since it was not active in cell lines that are not related with midgut tissue origin (Sf-9 and Hi-5). Given the conservation of HaTsp-2A promoters among *Helicoverpa*, *Spodoptera* and *Trichoplusia* species (Table S2), a possible explanation for the low activity of HaTsp-2A could be the existence of

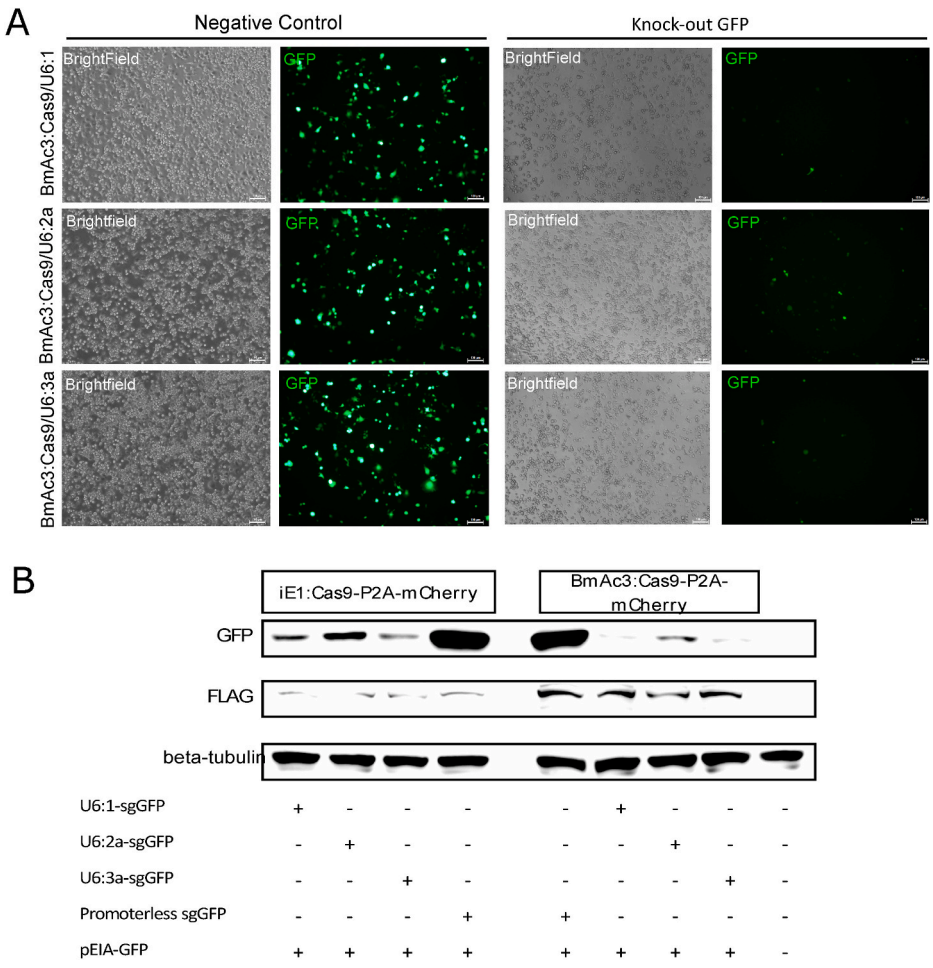

Fig. 5. Functional analysis of *H. armigera* U6 promoters in a proof of principle CRISPR mediated knock-out . A) Knock-out of GFP via CRISPR/Cas9 in the RP-Hz-GUT-AW1 cells using *H.armigera* U6 promoters carrying CRISPR vectors. The RP-Hz-GUT-AW1 cells were transiently transfected with pEIA-GFP and a CRISPR vector (BmAc3:Cas9-mCherry). As negative control a CRISPR vector without sgRNAs was used. Scale bars, 100uM. B) Western-Blot analysis of CRISPR against GFP (~27 kDa) in RP-Hz-GUT-AW1 cells analyzing the efficiency of the vectors at a protein level. Both iE1 and BmAc3 promoters were tested for their efficiency in driving the expression of Cas9-P2A-mCherry. Beta-tubulin (~55 kDa) was used as a loading control. Cas9 (~160 kDa) which is tagged in the N-terminus with a 3X FLAG tag was detected using anti-Flag. Non-treated cells was used as a second negative control.

tissue specific *cis*-acting elements in the promoter sequence, limiting its activity only in midgut derived cells. Further analysis of its activity either in a non-midgut derived cell line of *Helicoverpa* or through *in vivo* validation would be necessary to establish these promoters as truly midgut specific.

Several tissue specific promoters have been identified in the silkworm *B. mori*, such as the germ-line specific promoter nanos (Xu et al., 2019), the middle-silk gland Ser1 promoter (Liu et al., 2006; Ye et al., 2015), the midgut specific APN4 promoter (Jiang et al., 2015) and the fat body specific Bmlp3 promoter (Deng et al., 2013). The latter was analyzed with luciferase assay in two different cell lines and found to exhibit ~80–~140-fold stimulation of transcriptional activity, but also was tested *in vivo* and found to drive high levels of RFP expression (Deng et al., 2013). This would suggest that the four strong promoters identified in this study which displayed similar activity with Bmlp3, could also be used in the future for *in vivo* transgenics. To further validate the strength of these promoters, we analyzed them in comparison with already known constitutive promoters, like BmNPV-iE1 and BmAc3. Although BmNPV-iE1 has been used in many previous works as a strong ubiquitous-acting promoter (Fujita et al., 2015; Masumoto et al., 2012), its activity seems to be statistically significantly less than *HaLabial*, *HaTsp-2A*, *HaPtx-I* and *HaCaudal* promoters in RP-Hz-GUT-AW1 cells. On the contrary, BmAc3 exhibited much higher activity. Even though very strong promoters are generally preferred in the field of transgenesis, increased levels of protein expression mediated by a very strong promoter could lead to cellular stress or even lethality. This has been reported *in vivo* by Okamoto and co-workers, by observing embryonic lethality when OATP74D protein was expressed ubiquitously in *Drosophila* using Act5C-gal4 and daughterless-gal4 driver lines, while less expression by the weaker driver line armadillo-gal4 was viable in all life stages (Okamoto et al., 2018). Similarly cellular stresses could also be observed in cell cultures (in either transient or stable transfections) by using very strong promoters that lead to high levels of protein expression affecting downstream experimental procedures. Using promoters of milder activity could circumvent this limitation. Hence, the weaker yet active promoters of this study might be an excellent tool for balanced expression and suitable for *in vitro* studies and hypothetically *in vivo* use.

4.2. RNA polymerase III promoters

The spliceosomal snRNA U6 promoters are exceptionally interesting, since they are required to drive the expression of small RNAs like sgRNAs and shRNAs. We showed that all of the U6 promoters analyzed are capable of mediating both RNAi (Fig. 4B) and CRISPR cas9 (Fig. 5) with high efficiency. The first report of insect U6 promoters used in CRISPR-Cas9 was in *Drosophila melanogaster* (Gratz et al., 2013). Since then, several U6 promoters of non-model insect species have been identified and used in the context of CRISPR such as mosquitoes (Anderson et al., 2020), *Plutella xylostella* (Huang et al., 2017), *Spodoptera frugipeda* (Mabashi-Asazuma and Jarvis, 2017) and *Drosophila suzukii* (Ahmed et al., 2019). Our analysis indicated that HaU6:1 and HaU6:3a were able to reduce GFP by more than 90%, while HaU6:2a promoter was slightly less efficient (Fig. 5B). Differential cleavage efficiency mediated by the same sgRNAs expressed by different U6 promoters has been reported in *P. xylostella* and *D. melanogaster*, in which only PxU6:3 and DmU6:3 promoters respectively, were found to exhibit the highest activity (Huang et al., 2017; Port et al., 2014).

Similarly, high efficiency of HaU6 promoters (>98%) was displayed when shRNA targeting Renilla luciferase was tested in RP-Hz-GUT-AW1 (Fig. 5C), which compares favorably to reports using *B. mori* and *P. xylostella* cell lines (Tanaka et al., 2009; Huang et al., 2017). Strikingly, luciferase assays indicated that HaU6:1 and HaU6:2a promoters displayed 92% and 95% reduction of Renilla luciferase expression in Sf-9 cells. This is probably suggestive that their function is not species specific, which is supported by a similar study in which *Spodoptera* U6 promoter was shown to be functional in Hi-5 and BmN cell lines (Mabashi-Asazuma and Jarvis, 2017). However, the SfU6 promoter was found to be the only functional promoter in Sf-9 cells while homologous promoters of *B. mori*, *T. ni* and *D. melanogaster* were not (Mabashi-Asazuma and Jarvis, 2017). This possibly broadens the application and utility of *Helicoverpa* U6 promoters in other cell lines. Moreover, generating the Cas9-P2A-mCherry (Fig. S2) facilitates screening procedures, especially when it comes with cell lines which can be used in combination with Fluorescence-Activated Cell sorting (FACS) for establishing clonal knock-out cells (Fu et al., 2018). Further, *in vivo* analysis of HaU6 promoters indicated their functionality in *H. armigera in vivo* (Fig. S5); to the authors' knowledge this is the first use of plasmid based CRISPR vectors in this species. Overall, based on our results we suggest that these constructs carrying either HaU6:1 or HaU6:3a, in combination with BmAc3 promoter driving the expression of Cas9-P2A-mCherry, can be used for genetic manipulation *in vitro* and *in vivo*.

Authors contribution

J.V and S.D conceived the project; G-R.S and S.D designed the experiments; G-R.S performed the experiments and analyzed the data; the original draft was written by G-R.S; S.D, J.V, G.S, L.S and S.G revised and edited the manuscript which has been approved by all authors.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2022.103725.

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“It is not easy to convey, unless one has experienced it, the dramatic feeling of sudden enlightenment that floods the mind when the right idea finally clicks into place. One immediately sees how many previously puzzling facts are neatly explained by the new hypothesis. One could kick oneself for not having the idea earlier, it now seems so obvious. Yet before, everything was in a fog.”

— Francis Crick, What Mad Pursuit