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[Λειτουργική ανάλυση της συνεισφοράς της ανθεκτικότητας δύο μεταλλαγών του τασεοευαίσθητου καναλιού νατρίου ενάντια στα εντομοκτόνα indoxacarb και metaflumizone με τη χρήση της τεχνικής CRISPR-Cas9 στη *Drosophila melanogaster*]

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THESIS

[Functional validation of the contribution of two Voltage Gated Sodium Channel (VGSC) target site mutations in Sodium Channel Blocker Insecticides resistance by CRISPR-Cas9 genome modification in *Drosophila melanogaster*]

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Abstract

Insecticide resistance emergence have rendered laborious the counteract of agricultural pests and vector borne diseases the last decades. *Plutella xylostella* and *Tuta absoluta* comprise two major agricultural pests having a tremendous potential to develop resistance against insecticides such as Sodium Channel Blocker Insecticides (SCBIs, Indoxacarb and Metaflumizone). Two amino acid alterations, F1845Y and V1848I (*P.xylostella* numbering), in the sixth segment of the forth domain of voltage gated sodium channel have been correlated with high levels of indoxacarb and metaflumizone resistance. Although electrophysiological studies have shown the correlation of those mutations with resistance against SCBIs, no *in vivo* experimental data have been recorded so far. In this study we have managed to introduce F1845Y and V1848I mutations in *Drosophila melanogaster* sodium channel gene (*para*), with the CRISPR-Cas9 genome editing technique, in order to investigate functionally the contribution to SCBIs resistance. F1845Y and V1848I mutations seem to confer higher resistance to metaflumizone (3460X and 9.3X respectively) than to indoxacarb (12.5X and 6.6X respectively). Moreover we had the question why F1845Y and V1848I mutations have never been found in the same allele in field populations of *P.xylostella* and *T.absoluta*. For this reason we tried to introduce both mutations in *cis*, in order to investigate whether the phenotype is lethal or not. These results provided useful information about the actual contribution of those mutations to indoxacarb and metaflumizone resistance and suggest that both indoxacarb and metaflumizone exhibit stronger binding affinity to the 1845Y than to 1848I.

Περίληψη

Η εμφάνιση του φαινομένου της ανθεκτικότητας σε εντομοκτόνα έχει καταστήσει προβληματική την αντιμετώπιση των πληθυσμών εντόμων που αποτελούν πλήγμα για την παγκόσμια αγροτική οικονομία καθώς και για τη δημόσια υγεία. Τα έντομα *Tuta absoluta* και *Plutella xylostela* αποτελούν είδη απειλή των αγροτικών παραγωγών και φαίνεται να αναπτύσσουν υψηλά επίπεδα ανθεκτικότητας σε εντομοκτόνα, όπως είναι τα indoxacarb και metaflumizone. Μελέτες σε ανθεκτικούς σε αυτά τα εντομοκτόνα πληθυσμούς υποδεικνύουν την ύπαρξη δύο μεταλλαγών, F1845Y και V1848I (αρίθμηση με βάση την αλληλουχία του γονιδίου *para* του είδους *P.xylostella*), οι οποίες εντοπίζονται στην έκτη διαμεμβρανική περιοχή της τέταρτης υπομονάδας του καναλιού νατρίου. Η συνεισφορά των μεταλλαγών αυτών στην ανθεκτικότητα έχει πιστοποιηθεί με προσεγγίσεις *in vitro*. Ωστόσο μέχρι και σήμερα δεν έχουν σημειωθεί *in vivo* πειραματικά δεδομένα που να αναδεικνύουν τη σημασία των μεταλλαγών αυτών στην ανθεκτικότητα. Σε αυτή τη μελέτη πραγματοποιήθηκε επιτυχής εισαγωγή των μεταλλαγών αυτών στο γονίδιο του τασεοευαίσθητου καναλιού νατρίου *para* στο δίπτερο *Drosophila melanogaster*, χρησιμοποιώντας την τεχνική CRISPR-Cas9, ώστε τελικά να πραγματοποιηθεί λειτουργική ανάλυση της συνεισφοράς της ανθεκτικότητας των μεταλλαγών F1845Y και V1848I στα εντομοκτόνα indoxacarb και metaflumizone. Τα αποτελέσματα των βιοδοκιμών στα ομόζυγα για τις μεταλλαγές ανθεκτικά στελέχη των διαγονιδιακών ατόμων *Drosophila* κατέδειξαν ότι και οι δύο μεταλλαγές σχετίζονται με υψηλότερα επίπεδα ανθεκτικότητας έναντι του εντομοκτόνου metaflumizone (F1845Y: 3460X και V1848I: 9.9) συγκριτικά με την ανθεκτικότητα στο εντομοκτόνο indoxacarb (F1845Y: 12.5X και V1848I: 6.6X). Επιπλέον τέθηκε το ερώτημα για το ποιος είναι ο λόγος που οι δύο μεταλλαγές δεν συνεντοπίζονται στο ίδιο αλληλόμορφο (*cis*) στους φυσικούς πληθυσμούς των ανθεκτικτικών εντόμων. Για το λόγο αυτό δημιουργήθηκαν διαγονιδιακά στελέχη εντόμων που έχουν τις δύο μεταλλαγές *in cis* ώστε να εξακριβωθεί εάν ο φαινότυπος είναι θνησιγόνος ή όχι. Τα αποτελέσματα της μελέτης αυτής παρέχουν χρήσιμες πληροφορίες σχετικά με την ακριβή ανθεκτικότητα που προσδίδουν οι μεταλλαγές αυτές έναντι των εντομοκτόνων indoxacarb και metaflumizone σε πληθυσμούς εντόμων και επιπλέον προτείνουν ότι τα εντομοκτόνα αυτά εμφανίζουν ισχυρότερη συγγένεια πρόσδεσης με τη μεταλλαγή F1845Y παρά με τη V1848I.

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Contents

1. General Introduction

1.1 Insecticides: From the past to date

Insect pests represent a serious threat for agricultural production and vector disease control, so the need of insecticides in order to counteract these two problems is pretty high. Most of the insecticides used in the past were based on botanical extracts such as *Dalmatian pyrethrum* flowers which contain up to 1.5% pyrethrin, a widely used insecticide to date (Davies et al., 2007). Another example is an aqueous tobacco extract which was used as insecticide for the control of aphid populations (Oberemok et al., 2015). Natural and synthetic compounds have been used as insecticides since many years ago since they have been found to be more effective than botanical extracts. The most well known synthetic compound was DDT (dichlorodiphenyltrichloroethan), synthesized by Othmar Tseidler, in 1874 (Davies et al., 2007). Although the toxicity effect against insects was high, it was found that DDT is also toxic against other organisms such as reptiles, mammals etc. In order to overcome the problem of toxicity effects several compounds have been synthesized, such as organophosphates, carbamates and so on. To date several different compounds have been synthesized for efficient control of insect populations that threaten public health and agriculture.

1.2 Need of insecticide use against vector-borne diseases

Public health has been threatened since many years ago by several diseases such as dengue fever, Leishmaniasis, Zika, malaria etc which are transmitted by insects, characterized as vectors. According to World Health Organization (WHO) vectors are living organisms that can transmit infectious diseases among humans or from animals to humans. These kinds of insects are bloodsucking and ingest disease-causing microorganisms during a blood meal from an infected host (animal or human), which they later inject it into a new host.

Malaria is an infectious hematologic disease, caused by *Plasmodium falciparum* parasite. It has been a worldwide dreadful disease for many years in the past and still causes more than 400,000 deaths annually especially in the countries of the third world, according to the WHO, February 2016. Transmission of this parasite to humans is accomplished by vectors of the genus *Anopheles*. Counteract of malaria disease has been based on two strategic tools developed: a) use of insecticide with nets impregnated with insecticides (Insecticide Treated Nets and Long Lasting Insecticide Treated Nets. for vector control and b) malaria diagnosis and development of effective medicines (Silva, 2014). Four main groups of neurotoxic insecticidal agents have been used for public health purposes, which are classified according to their chemical nature and mode of action: organochlorines, organophosphates,

carbamates and pyrethroids (Silva et al., 2014). However resistance[1] to those insecticides has been developed in many insects which renders the control of the populations difficult.

1.3 Insecticides: Classification and mode of action

As previously mentioned, several natural or synthetic compounds have been developed for the efficient control of insect pests. Development of resistance against most of these compounds is observed in the field populations, thus it is critical to elaborate the mode of action and target of each insecticide. Insecticide Resistance Action Committee (IRAC) has been founded in order to maintain the major problem of insecticide resistance spreading, aiming towards supporting sustainable agriculture and improved public health (Sparks et al., 2015). In table 1, we sum up the most important categories of insecticides according to their target and the mode of action.

Most of the insecticides developed target the nervous system of insects. A reason why nervous system is selected over other tissues could be probably the fact that it contains at least 11 different targets for neuroactive insecticides. Moreover these compounds exhibit primary target selectivity to insects rather than humans, rendering them safe for commercial use (Casida et al., 2013). Nerve cells in animals communicate with each other through chemical and electrical signals. These signals are transmitted via synapses which are formed between the post-synaptic and pre-synaptic nerve cells. The presynaptic cells are stimulated and release neurotansmitters, such as acetylcholine (ACh). This substance binds to nicotinic acetylcholine receptor sites which are located in the post-synaptic membrane and acts as an agonist which leads to conformational change of the receptor resulting to ion channel opening (Casida et al.,2013). This leads to Na^+ influx and K^+ efflux, resulting in membrane depolarization and electrical signal generation. Nicotine, derived from *Nicotiana tabacum*, is the oldest insecticide used and shares the same target site with ACh (Sparks et al., 2015). High concentration of nicotine can lead to overstimulation of the nerve cell or else termed as cholinergic overactivity (Marrs et al., 2013). which leads to insect death. This effect might be caused by allosteric modulators of nACh receptors, like spinosyns (Biondi et al., 2012). Another group of insecticides comprise the inhibitors of Acetylcholinesterase (AChe); the hydrolytic enzyme of acetylcholine regulates negatively the transmission of the electrical signal from the pro-synaptic to post-synaptic cell (Alout et al., 2012). Carbamates and organophosphates inhibit AChE, by forming a complex which leads to continuous activation of the post-synaptic cell (Casida et al.,2013). γ-Aminobutyric Acid gated (GABA) chloride channels are protein complexes consisting of chloride ion channels and the receptor of the ligand GABA. Once GABA binds to its receptor site, the channel is opening, leading to influx of Cl^-, which results to hyperpolarization of the nerve cell membrane, inhibiting the generation of an action potential.

[1] According to IRAC (Insecticide Resistance Action Committee) resistance to insecticides is 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 level of control when used according to the label recommendation for that pest species".

Insecticides acting as antagonists of these channels, lead to hyper-excitation of insects' nerve and muscle cells.

Table 1 Most important insecticides classified according to insect target. NR: non recorded (Sparks et al., 2014).

Target	Primary site of Action	Chemical subgroup	1st year
Nervous system			
1	Acetylcholinesterase (AChE) inhibitor	Carbamates Organophosphates	1950 1944
2	Voltage gated sodium channel (VGSC) modulators[2]	Pyrethroids, pyrethrins, DDT and analogs	1977 1944
3	Voltage gated sodium channel (VGSC) blocker	Oxadiazines Semicarbazones	1997 2007
4	Nicotinic acetylcholine receptor (nAChR) agonist[3]	Neocotinoids, nicotine, sulfoximines, burenolides	1990, 1763 2013 2014
5	GABA gated chloride channel (GGCC) antagonist	Cyclodienes, Fiproles	1950 1990
6	Nicotinic acetylcholine receptor allosteric[4]	Spinosyns	1997
Muscle system	Ryanodine Receptor	Diamides	2008
Insect Growth and development regulators			

[2] Antagonist is a substance that shares a similar structure with a ligand, which eventually binds to the receptor but blocks the biological response rather than provoking it as the ligand does.

[3] Agonist is a substance that causes the opposite biological response than an antagonist does.

[4] Allosteric is defined as the case that a substance acts at a different site of the receptor than the ligand does, and eventually that may lead either to the inhibition of the receptor or to its activation.

7	Juvenile Hormone Receptor	Juvenoids, Pyriproxifen	1973 1995
8	Ecdysone Receptor	Diacylhydrazines	1993
9	Mitochondrial Electron Transport	Phosphines, Cyanides, β-ketonitriles derivatives	NR, 1877 2007
10	Chitin Synthase inhibitor	Benzoylureas, Buprofezins	1975 1984
11	Oxidative Phosphorylation uncouplers	Chlorfenapyr	1892
12	Mite Growth Inhibitors (MGIs)	Oxazoles, Clofentezine	1998 1983
13	Midgut target	*Bacillus thuringiensis*	1970

VGSCs are important transmembrane proteins, in the cells of nervous system in animals, since they are responsible for the passage of sodium ions across the plasma membrane which leads to the generation and propagation of electrical signals facilitating the response to several environmental stimuli (Carnevale et al., 2017). Specifically sodium ions are sensed by positively charged aminoacids of the S4 transmembrane segment of the four homologous domains that comprise VGSCs (Fig. 1) (Duclohier 2009). In response to a membrane depolirization, the S4 segment moves to the extracellular side of the cell membrane (Dong et al, 2014). This triggers an allosteric change to the coupling between the sensor module and the gate that is contained within the assembly of the S5 and S6 transmembrane helices and leads to the pore opening initiating the influx of sodium ions (Carnevale et al., 2017). After milliseconds the sodium channel undergoes inactivation through two different models, the one of fast inactivation which is served by the inactivation particle occluding the cytoplasmic end of the pore (an intracellular loop linking domains III and IV of the a-subunit and containing the characteristic IFM amino acid motif) (Goldin 2003). and the other of slow inactivation whose molecular basis is still not elaborated (Silva 2014, Kass 2004). VGSCs are a well studied target of neuroactive insecticides. Pyrethrins, pyrethroids, DDT and other compounds target the sodium channel as modulators since they bind to the channel in specific state of activity, altering its gating properties and finally they keep it in open conformation for unusually long time (Liu, 2015).

VGSCs are a target for several compounds used as drugs, like local anesthetics (LAs), antiarrhythmics, analgesics, antepiliptics. 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). Oxadiazines and other insecticidal chemical substances have been found to act like LAs against sodium channels and they are proposed to exhibit high affinity binding to specific states during the firing of action potentials. Specifically they show to bind preferentially to the slow inactivated state of the channel, hindering the restoration of excitability which prolongs the inactivation (von Stein et al., 2013). Indoxacarb and metaflumizone, or else termed as Sodium Channel Blocker Insecticides (SCBIs) are the major compounds belonging to this group of insecticides and share the same mode of action. These two types of insecticides have been developed in an effort to reduce the toxicity against other organisms (e.g mammals) by increasing the primary target selectivity to insects. In this study we have worked with SCBIs in an effort to decipher resistance against them using the model organism of *Drosophila melanogaster*. The forms of insecticide resistance are discussed in detail in the following section.

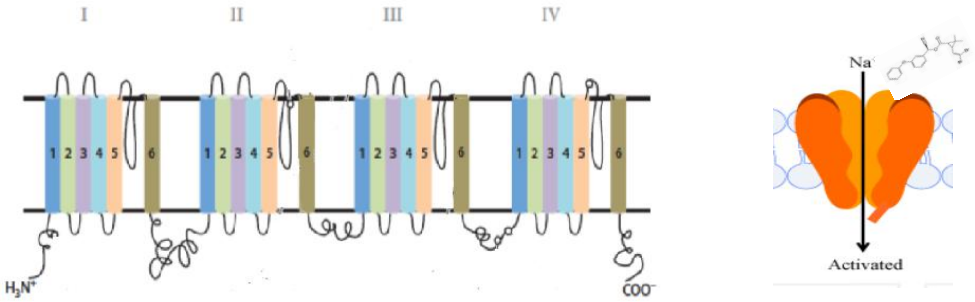

Fig. 1 Voltage gated sodium channel scheme representing the 4 homologous domains and each transmemembrane segment (A) Voltage gated sodium channel in the activated state in which deltamethrin (pyrethroid) is bound on the receptor site of the channel, resulting in continuous influx of sodium ions (Wakeling et al., 2012).

1.4 Insecticide Resistance and Mechanisms

The phenomenon of resistance against toxic agents is observed in most of the living organisms, from bacteria to humans. Resistance is the phenomenon in which a living organism has developed tolerance against a xenobiotic substance and this tolerance can be inherited from one generation to another. Insects belong to the organisms being able to develop resistance against toxic agents like insecticides. However, there is a query that many scientists tried to elucidate: Resistance pre-dates insecticide introduction in the field or not? According to the minireview of ffrench-Constant, 2007, single mutations conferring resistance to malathion (organophosphate) were found in a field population of blowflies of the genus *Lucilia*, which affect the sheep in Australia. However these mutations have been already present before the introduction of the insecticide (ffrench-Constant, 2007). Currently 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, prior to its exposure to a stressor (Liu, 2015). 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 (selective pressure) can lead to the demise of sensitive individuals but survival of the resistant ones (Fig. 2). After that the population is comprised of individuals carrying the resistance alleles either as heterozygous or homozygous. The existence of the selective pressure can lead to the permanent establishment (fixation) of the mutation in the population. In general, according to IRAC four different mechanisms of resistance against insecticides can be employed: a) metabolic resistance, b) behavioral resistance , c) penetration resistance and d) target site resistance.

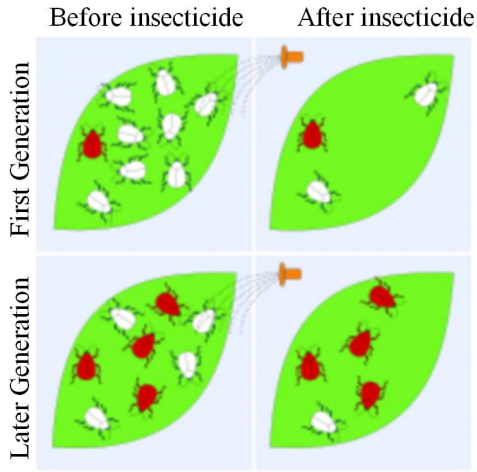

Fig. 2 Graphic representation of the selective pressure that insects undergo after exposure to insecticide. Before the treatment of a field population with insecticide, only individuals carrying the resistant alleles (red bugs) survive. In the later generations the frequency of those alleles will increase, establishing them permanently in the population.

1.4.1 Metabolic resistance

A 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). 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, b) single aminoacid alteration which increases the affinity of the detoxifying enzyme with its substrate.

Many insect species found to be resistant against carbamates and organophosphates, due to the high activity of esterases. Esterases catalyse the hydrolysis of ester insecticides into their corresponding acids and alcohol groups. Carboxylesterases seem to be the most important class of esterases that

mediate metabolic resistance. This is more clear in cases of resistant populations of *Cimex lectularius* in which high levels of esterases mRNA have been documented which was verified by either RNA sequencing or qRT-PCR (Dang et al., 2017). Moreover, there are cases in which typical esterases (carboxylesterases) can be converted into insecticide hydrolases by a single aminoacid alteration. For instance Organophosphorus resistant strains of sheep blowflies have been found to carry a single mutation (glycine to aspartic acid) in the residue site 137 of the carboxylesterase (*LcaE7*), converting the esterase to an organophosphate hydrolase (Claudianos et al., 1999).

Micorosomal Cytochrome P450 monooxygenases are a superfamily of proteins found in all living organisms. They 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, 1999). More than 1700 genes encoding P450s monooxygenases have been characterised from many insect species (Dang et al., 2017). The biochemical pathway of P450-mediated degradation of insecticides is not yet fully understood. 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 more hydrophilic and more excretable from the organism, thus less toxic (David et al., 2013). The contribution of this protein family to insecticide resistance has been verified in many resistant insects and especially in mosquitoes of *Anopheles* species (David et al., 2013, Liu, 2015).

Glutathione - S transferases are enzymes playing critical role in many organisms, being involved in biosynthesis of hormones, protection against xenobiotics via degradation and protection against oxidative stress. The major pathway via which they contribute resistance against insecticides in insects is the conjugation of the sulphhydryl group of endogenous reduced glutathione (GSH) on electrophilic center of a range of xenobiotic compounds (Panini et al., 2016). This reaction increases the solubility of the insecticide which finally is excreted by the formation of mercapturic acid derivatives (Panini et al., 2016).

1.4.2 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). Behavioral resistance might involve some mechanisms (chemosensors). for the detection of the insecticides at very low concentrations. This was indicated when resistant insects could evade an area treated with low concentrations of insecticide, in contrast with sensitive insects which seemed to be unable to evade (Panini et al., 2016).

1.4.3 Penetration resistance

Insects make physical (tarsal) contact with an area treated with insecticides. Once the insect is exposed, the insecticide must get 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. 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.

1.4.4 Target-site resistance

Each insecticide has a specific target, that could be a specific amino-acid residue of the protein target. If this aminoacid is altered to another one, then potentially the binding affinity of the insecticide is reduced, thus leading to resistance. Target site alteration can provide high levels of resistance, but this tends to be specific for a particular chemical class of insecticides (Panini et al., 2016). However many cases of target site mutations have been documented which can lead to cross-resistance[5] emergence. Target site resistance to organochlorines (DDT) and pyrethroids was firstly reported in *Musca domestica* in 1976 (Naqqash et al., 2016). Dang and co-workers (2017) summarized four main types of target site insensitivity: a) kdr (knock-down resistance) which involves specific alterations in the sodium channel sequence conferring resistance to pyrethroids and DTT, b) altered AChEs, c) rdl-mutations which are correlated with alterations in the sequence of the GABA receptor and d) altered nAChRs conferring resistance to neonicotinoids (Dang et al., 2017). However, mutations associated with the Ryanodine receptor (muscle tissue) have been also reported to contribute to diamide resistance (Douris et al., 2017).

VGSC amino acid sequence has been found to comprise many different mutations have been documented, despite the high conservation it exhibits among species. Those mutations have been associated with pyrethroid and DTT resistance, thus they are characterised as kdr mutations (Dong et al., 2014). The most important kdr mutation is the convertion of leucine to phenylalanine in the residue site 1014 (using *M. domestica* numbering), in the S6 segment of the II domain of VGSC, which has been originally found in *M.domestica*. Since then, many different species of agricutltural pests and diseases vectors have been found to carry this mutation alone or in combination with other alterations in the VGSC sequence. Apart from L1014F mutations, several divergent mutations in other residue sites have been mapped and correlated with pyrethroid resistance. M198T mutation is a mutation that when it occurred alone, it did not have great effect in resistance, but when it is together with L1014F it causes the super-kdr phenotype by leading to greater reduction of sodium channel sensitivity against

[5] According to IRAC cross resistance occurs when a specific mechanism provides resistance to one insecticide but also confers resistance to another insecticide of different class, especially when the insect has never been exposed to the latter. Metabolic mechanisms of resistance are known to confer cross resistance in insects, but target site mutations rarely contribute to this phenomenon.

pyrethroids. The contribution of those mutations to resistance against pyrethroids have been verified by heterologous expression of sodium channel cDNA in *Xenopus* oocytes in combination with electrophysiological experiments (Dong et al., 2014). Dong and co-workers (2014) summarized all the kdr mutations found in most of the insect species in a review for sodium channels and pyrethroid resistance (Dong et al., 2014).

1.5 Sodium Channel Blocker Insecticides (SCBIs) and target site resistance

SCBIs (Indoxacarb and metaflumizone) had been introduced in order to overcome the resistance that many insects developed against pyrethroids. Indoxacarb (Fig. 4A) is an insecticidal oxadiazine and it was the first commercially registered SCBI from DuPont company. It was developed in their effort to limit the toxicological side effects, the photoinstability and the reduced persistence in soil that dihydropyrazoles exhibit (von Stein et al., 2013). Indoxacarb is characterized as a proinsecticide since it has to be converted to the active metabolite N-decarbomethoxyllated JW062 (DCJW), a secondary product generated by the hydrolysing activity of insect esterases or amidases, which underlies the action selectivity against insects (Zhang et al., 2016). According to the manufacturer's labeling, Steward (Oken et al., 1982) the commercial name of indoxacarb, is used either by spraying or by digestion and the desirable effects (cessation of feeding, un-coordination and paralysis) are observed within a few minutes to 4 hours. Although indoxacarb was proposed to be active and used against lepidopteran insects, it has been shown that spraying treatment of diptera (*Drosophila melanogaster*) with DCJW is effective and finally causes mortality (Zhang et al., 2013). Metaflumizone (Fig.4B) belongs to the category of semicarbazones and is another SCBI developed by BASF company, in the effort to overcome the side effects of dihydropyrazoles. Finally they have managed to obtain semicarbazones which are ring-opened dihydropyrazoles (von Stein et al., 2013). Metaflumizone exhibits low toxicity to mammals and selectivity to insects (Hempel et al., 2007).

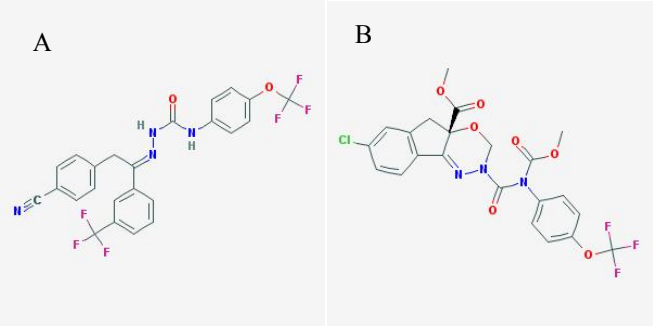

Fig. 4 Chemical structure of (A) metaflumizone and (B) indoxacarb. (Pubchem)

Indoxacarb and metaflumizone target the sodium channel in the slow-inactivated state as local anesthetics. Specifically, they bind to the opened pore of the channel when the membrane is still depolarised and they cause a shift in voltage dependence of slow inactivation to more negative currents. Thus VGSCs are stabilised in the inactivated state leading to hindrance of sodium influx intracellularly (Silver, 2007).

Although SCBIs had been introduced as more potent, effective and less toxic for the humans insecticides in order to deal with pyrethroid resistance emergence, the improvident and widespread use of these specific insecticides led to the emergence of resistance in many insect species, such as *Plutella xylostela* (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., 2016) and *Blatella germanica* (Liang et al., 2017). Target site resistance against these insecticides has been found specifically in the S6 segment of the IV domain of sodium channel in *Tuta absoluta* (Roditakis et al., 2016) and *Plutella xylostela* (Wang et al., 2016). Cross-resistance has been observed in many of the above mentioned pests which was result of either metabolic or-and target site resistance. In the case of *P. xylostela*, the diamondback moth, two different field strains exhibiting cross resistance to metaflumizone and indoxacarb have been isolated (Wang et al., 2016). Metabolic mechanisms of resistance have been implicated in resistance to SCBIs, which was assured after the treatment of the insects with insecticide combined with synergists for different types of hydrolysing enzymes (P450s, GSTs and esterases) (Wang et al., 2016, Zhang et al., 2017). Even though metabolic resistance is implicated in those resistant populations, two target site alterations in the S6 segment of the voltage-gated sodium channel gene (*para*), F1845Y and V1848I, are correlated with resistance as well (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., 2016). Although synergists reduced the LC_{50} values against indoxacarb, there is no strong experimental evidence for the contribution of those two mutations to SCBIs resistance, *in vivo*.

An *in vitro* approach for the investigation of F1845 and V1848 alterations with SCBIs binding was the heterologous expression of modified german cockroach voltage gated sodium channel in *Xenopus* oocytes for electrophysiological experiments (Jiang et al., 2015). Dong and coworkers generated the mutants F1845Y and V1848I/A in order to elucidate their hypothesis that Local Anesthetics share common receptor sites with SCBIs. Their work was based on the binding assays in transgenic rat sodium channels (F1764A and Y1771) expressed in *Xenopus oocytes* which revealed high affinity of SCBIs at LAs' receptor site in sodium channel (Silver et al., 2007). Patch-clamp recording in oocytes expressing transgenic (F1845Y and V1848I) VGSCs in presence and absence of SCBIs revealed that insecticide perfusion does not affect the flow of sodium current, as in the case of the wild type in absence of the insecticide (Jiang et al., 2015). However no *in vivo* experimental data have been documented in order to validate the significance and the actual contribution of these mutation in indoxacarb and metaflumizone resistance. In this case *Drosophila melanogaster* provide a well studied tool in order to investigate the correlation of the existence of the mutations F1845Y and V1848I with SCBIs resistance.

1.5 Why *Drosophila*?

Drosophila melanogaster, the common fruitfly has been employed as a model organism for genetic concepts and techniques for several years. *Drosophila* has been used as a tool for insecticide development since it can give information about the mode of action of chemical compounds, it can

determine the source and the extent of resistance and finally it can give information about developing novel targets of pesticides.

Forward genetics and reverse genetics in *Drosophila* can be used for unraveling the mode of action of any insecticide. Genetic screening can be used for finding recessive or dominant mutations conferring resistance to insecticides (Schneider et al., 2000). This way the mechanism of action of the insecticide can be unraveled. However in the era of genomics and genome editing in combination with *Drosophila'*s high genetic accessibility unraveling the mode of action of insecticides has been rendered easier. For example genetic screening for finding of recessive mutations, led to the understanding of the mode of action of methoprene (juveline hormone mimic) and unravelled the singificance of a juveline hormone-binding protein as a target of this insecticide (Schneider et al., 2000).

Insecticide resistance can be studied either *in vitro* or *in vivo*. *In vitro* experiments for the study of resistance involve the heterologous expression of a gene of interest that might be implicated in resistance to a specific insecticide in systems like *Escherichia.coli* or *Saccharomyces.cerevisiae. E.coli* has been initially used as an heterologous system for the expression of a GST from *M.domestica* in order to facilitate the isolation of the protein and study the resistance it can confer with biochemical approaches (Wang et al., 1991). On the other hand, *in vivo* functional validation of an insecticide resistance mechanism is necessary. However, sensitive and resistant strains of insects compared in fitness studies may not share a common genetic background since backcrossing is time consuming (ffrench-Constant et al., 2007). Moreover, knock-down and knock-out techniques are not always applicable in non-model organisms. *Drosophila melanogaster* has been used for several years as a tool for genetical studies, since it can be easily reared in order to produce large numbers of progeny in a short time period. Moreover its whole genome has been sequenced since 2000 and is easily accessible through genome databases, e.g. ensembl and Flybase, which is making genome editing techniques design easier (Adams et al., 2000). Furthermore *Drosophila* eggs are easily manipulated for procedures like injection in order to accomplish the insertion of exogenous DNA. Thus, *Drosophila* comprises the best tool so far, for the *in vivo* study of insecticide resistance. Metabolic resistance mechanisms have been studied thoroughly in *Drosophila* via heterologous overexpression. For example, *GSTE2* gene was managed to be expressed of *A.gambiae* in *Drosophila*, validating the resistance that conferred to DTT, after the performance of toxicity bioassays (Liu et al., 2015). Target site resistance can be also validated *in vivo* with *Drosophila*. However this demands targeted mutagenesis techniques that have been quite expensive and time consuming. TALENs and ZFNs have been developed for targeted mutagenesis, but those two techniques have been considered as too expensive and with low efficiency. On the other hand, CRISPR-Cas9 technique is characterised as less expensive and with high efficiency. This technology has been widely used for studying insecticide resistance in *Drosophila* (ffrench-Constant et., 2007, Douris et al., 2016, 2017, Zimmer et al., 2016).

1.6 CRISPR-Cas9 technique for target site mutagenesis

In the era of CRISPR (Clustered, regularly interspaced, short palindromic repeats)-Cas9 technology, genome modification has been rendered low cost and more effective. Cas-9 is an endonuclease originally discovered in bacteria as a defense system and employed in order to generate 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, in the haploid germ line cells of *Drosophila*. Invoking of HDR mechanism requires the consideration of an homologous template, which can be managed with the use of a donor plasmid containing two homology arms (each ~1Kb) flanking the region of interest (Beumer et al., 2013, Beumer et al.,2014). After the generation of transgenic homozygous flies, toxicity bioassays can be performed for the evaluation of the insecticide resistance with the insecticides of interest (Fig.3).

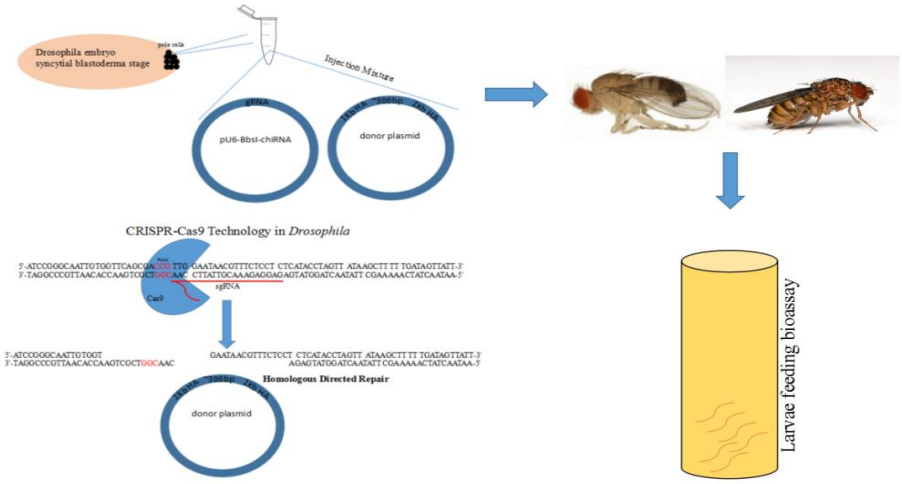

Fig. 3 Workflow of CRISPR-Cas9 technology for the generation of transgenic lines, targeting the gene *para*.

2. Scope of this study

In this study we managed to validate functionally the contribution of F1845Y and V1848I alterations in resistance against indoxacarb and metaflumizone by performing genome engineering using *Drosophila melanogaster* as a model organism. Specifically we have designed a CRISPR strategy targeting the gene of voltage-gated sodium channel for generating DSBs in order to achieve substitution of the region that contain the amino-acids F1845 and V1848 in susceptible population of *Drosophila melanogaster*. Through this technology we have managed to elucidate the molecular basis of target site resistance against SCBIs in *para* and assay modified fly lines to reveal the relative contribution of each single mutation (F1845Y and V1848I) to resistance phenotype. Moreover we tried to elucidate the reason why both mutations have never been found *in cis* in the field populations of *P.xylostella* and *T.absoluta* thus we tried to generated modified fly lines bearing the mutations in the same allele (FYVI). It is shown that F1845Y exhibit different binding affinity with indoxacarb and metaflumizone if compared with V1848I mutation. We found also that both mutations in homozygous transgenic flies confer high levels of resistance against indoxacarb and metaflumizone and which seems to be in accordance with the resistance phenotypes characterised in lepidopteran species.

3. Materials and Methods

3.1 Chemicals

Chemical compounds used for the bioassays are indoxacarb (Sigma Aldrich CAS Number 144171-61-9), metaflumizone (Sigma aldrich CAS Number 139968-49-3), Alverde 24 SC (BASF) which is a formulated version of metaflumizone (24% active ingredient) and Steward (DuPont) which is a formulated version of indoxacarb (30% active ingredient).

3.2 Lab strains and DNA extraction

The injections for genome modification of *Drosophila* were performed in eggs of the lab strain y1 M{nos-Cas9.P}ZH-2A w*, in which the endonuclease Cas9 is expressed under the control of the promoting element nanos (further below referred as nanos-Cas9; #54591, Bloomington *Drosophila* stock center, Port et al., 2014). Moreover this strain was used for outcrossing individually the G_0 adults generated from the injection. Strain w+oc/Fm7yBHw (balancer stock was kindly provided by professor Christos Delidakis, IMBB and University of Crete) which contains an X chromosome balancer, was used for genetical crosses and for keeping the mutations at heterozygous state. The flies were cultured at 25^{o}C temperature, at 60-70% humidity and 12:12 hour photoperiod on a standard fly diet.

DNA from *Drosophila* was extracted with DNAzol (MRC) following the instructions of the manufacturer.

3.3 Sequencing of *para* gene in the *Drosophila* genome

The whole sequence of *para* gene was obtained from Ensembl Metazoa (link) and three sets of primers (Table 2) were designed based on that. The sets of the primers and nanos-Cas9 DNA template were used for the amplification of a 3134bp region by performing three separate PCR reactions. The PCR reaction was performed according to the standard protocol of KapaTaq DNA Polymerase (Kapa Biosystems). The conditions of the reaction were 95^{o}C for 2 min for the initial denaturation followed by 25-35 cycles of denaturation at 95^{o}C for 30sec, annealing at 61^{o}C-66^{o}C, extension at 72^{o}C for 45sec-1m30sec and a final extension step for 2min. The products of PCR were purified by using columns for PCR clean-up (PCR clean-up gel-extraction kit, Mancherey Nagel) according to the manufacturer's instructions.

3.4 Cloning and sequencing of *Tuta absoluta* region

Two different samples of *Tuta absoluta*, collected from Italy (Roditakis et al., 2017) were found to be heterozygous for the S6 sodium channel mutations. A region of 168bp from two different samples (T9 and T13) of genomic DNA of *Tuta absoluta* was amplified with Go-Taq DNA polymerase (Promega Corporation), by performing a PCR reaction at a final volume of 50ul (primers used for the PCR are listed in Table 2). The conditions for the PCR were 95^{o}C for 5min for an initial denaturation followed by 40cycles of denaturation at 95^{o}C for 30sec, annealing at 60^{o}C for 30sec, extension at 72^{o}C for 30sec and a final step of extension at 72^{o}C for 1min. Both PCR products were purified by using the PCR clean-up gel extraction kit (Macherey Nagel) and then the products were cloned into pGEM-T easy vector according to the manufacturer's protocol (Promega Corporations). The ligation reaction was transformed into DH5a competent cells which then were cultured at 37^{o}C overnight on Luria Bertani agar plates (in presence of 100ug/ml Ampicillin, 0.5mM IPTG and 50mg/ml Xgal). Blue colonies were considered as negative concerning the cloning. Single non-blue colonies were picked in order to create liquid cultures from which plasmid DNA was isolated by using alkaline lysis protocol. pGEM-T easy vector contains a poly-linker site in which EcoRI cuts twice in between the insert. After the screening of several preps with EcoRI (Minotech), two positive clones from each sample were sent for sequencing (StarSeq) and the sequencing was performed with T7 promoter universal primer.

3.5 Strategy of genome editing

The CRISPR-Cas9 strategy was designed in order to generate two single mutations (relevant to the ones found in *P.xylostella* and *T.absoluta,* F1845Y, V1848I) in the *para* gene of nanos-Cas9. Those two mutations were never found in *cis* conformation in the samples of *P.xylostella* (Wang et al., 2016). So we wondered what would be the phenotype concerning the resistance, if both mutations would be found in the same allele. For this reason we designed a construct containing both F1845Y and V1848I

(further below referred as FYVI). The constructs we have used for CRISPR mediated substitutions in *para* gene are depicted in figure S1.

To begin with, we had to obtain the sequence of *para* gene of nanos-Cas9 strain in order to design the desired mutations. Based on the sequence we obtained, several CRISPR targets in the desired region were obtained by using the online tool Optimal Target Finder (Gratz et al., 2014) (http://tools.flycrispr.molbio.wisc.edu/targetFinder). Two targets found upstream (Lpara) and downstream (Rpara) of the desired region in *para* gene were selected with no predicted off-target effects. According to those two targets, two different RNA expressing plasmids were constructed. Specifically, we have designed single stranded oligos (Table 2) according to the target region and ordered from invitrogen. Those ssDNA oligos have been used in order to generate double stranded DNA oligos (dsDNA oligos) by annealing, in order to obtain the ds DNA oligos, Lpara and Rpara (Fig.S2). Both of the dsDNA oligos have 5' and 3' single stranded overhangs based on the sequence of the sticky ends that digestion with BbsI enzyme generates. These overhangs have been generated in order to facilitate ligation into dephosphorylated gRNA vector pU6-BbsI chiRNA (Gratz et al., 2013), after digestion with BbsI. After the ligation, the constructs were transformed into DH5a competent cells following the standard protocol of transformation. After overnight culturing, 5 single colonies from each construct were picked and they were checked for the insert by performing colony PCR using T7 universal primer and the reverse oligo for each dsDNA. The sequence of each construct was checked by sequencing (Macrogen sequencing facility, Amsterdam). For the generation of single amino acid mutations our strategy was based on the Homologous Directed Repair. To facilitate HDR we had to construct three *de novo* (Genscript) donor plasmids which contained two ~1000bp homology arms flanking the region of the target that corresponds to 228 bp (region in between the two gRNAs) (Fig.S1). The target region was designed to contain several synonymous mutations (except for the main desired mutations) which served two purposes: a) generate molecular markers in order to facilitate the screening of the CRISPR events and b) mutations in the gRNA and in the PAM sequence in order to prevent CRISPR induced DSB in the donor plasmid and/or HDR-modified flies.

3.6 Screening and genetic crosses for the generation of modified flies

Injection of approximately 500 *Drosophila* eggs (nanos-Cas9 strain) was performed by Yannis Livadaras (IMBB/FORTH) with an injection mix containing at final concentration 75ng/ul of Lpara gRNA expressing vector, 75ng/ul of Rpara gRNA expressing vector and 75 or 100ng/ul of donor template[6]. First instar larvae were collected 24 hours after injection. The larvae were transferred into standard fly artificial diet. After 9-13 days, fly adults were collected and they were backcrossed individually with nanos-Cas9 strain (each individual cross was taken as a different line) (Fig. 5). Cas-9 is expressed under the control of promoting elements of nanos, which is an embryonic marker expressed in the posterior pole of the egg during oogenesis and specifically in the pole cells (Dahanukar et al., 1996).. Thus, HDR is taken place only in the pole cells of the egg during the early

[6] For the mutations F1845Y and V1848I we used donor template at a final concentration of 75ng/ul, while in the case of FYVI we used 100ng/ul of donor template.

development of the egg. Since G0 flies are not modified, they had to be backcrossed with nanos-cas9 strain in order to obtain their progeny which they carry the mutations. Pupae of G_1 generation of each line were pooled into batches of 30 and genomic DNA (gDNA) extraction was performed in order them to be screened with two different methods. Initially, 2ug of gDNA was digested with HindIII (Minotech) (F1845Y and FYVI) or BsrGI (New England Biolabs) (V1848I) (that cuts only the wild type[7] which reduces the amplification of the wild type target region).

A)PCR amplification with specific primers.

PCR was performed with 0.2μM of specific forward and reverse primers(Table 2), 0.2mM dNTPs, 1U of Kapa Taq polymerase (Kapa Biosystems) in presence of 1X assay buffer and 60ng of template DNA for 25 cycles. Specific primers were designed according to the mutations that we introduced in the two gRNA sequences in the donor templates, in order to generate a diagnostic fragment of 250bp. This set of primers was used in all of the CRISPRs we performed for the generation of the indoxacarb resistance related mutations.

B)PCR amplification with generic primers

PCR was performed with 0.2μM of generic forward and reverse primers and same as in the specific PCR conditions. Generic primers (ParaGenF and ParaGenR, Table 2) were designed in order to amplify a region 752 bp which can amplify both the wild type and the mutant flies' gDNA. After 35 cycles of PCR, the product was checked in an agarose gel and then it was digested with KpnI (Minotech) (F1845Y; yielding two diagnostic fragments of 536bp and 217bp), BclI (Minotech) (V1848I; yielding two restriction fragments of 405bp and 347bp) and XbaI (Minotech) (FYVI; yielding two bands of 437bp and 315bp). Those restriction enzymes can cut only the product amplified from the mutant gDNA and not from the wild type. The digestion generated a diagnostic cleavage pattern for the presence of mutated alleles.

As it is depicted in figure 5 G_1 flies from the same original cross positive for the mutations were backcrossed individually with nanos-Cas9, in order to generate the G_2 generation. Each G_1 fly was screened individually and crosses positive for the modification were established. The 50% of the G_2 generation is expected to be positive for the mutations. Individual G_2 flies were crossed with flies carrying one balancer chromosome for the X chromosome (w+oc/ FM7yBHw). G_2 flies crossed over w+oc/Fm7yBHw were individually screened for establishing the positive G_3 progeny lines against the balancer chromosome. Afterwards single flies derived by the G_3 progeny (50% of the progeny are modified) were back-crossed with the same balancer fly stock. Final molecular screening was carried

[7] The target region in the donor templates were designed in order to carry synonymous mutations for a) altering one restriction site of a specific "single-cutter" restriction enzyme and b) for generating one new restriction site that does not cut anywhere else in this region. So in the first case an enzyme cannot cut the target region in the mutated flies, while it can digest only the wild type. In the second case, another enzyme can digest the target region of the mutated flies but cannot cut the wild types' region.

out and from the positive lines adults were collected and pooled in order to take several homozygous flies for the establishment of a population. DNA was extracted from homozygous male and female adults and amplified by using primers yielding a fragment of ~700bp, which was then purified and sent for sequencing (Macrogen Sequencing Facility, Amsterdam).

Each experiment concerning the molecular screening of the modified flies was performed along with positive control (the donor plasmids used for the injections), negative control (the gDNA of nanos-Cas9) and blank (no template).

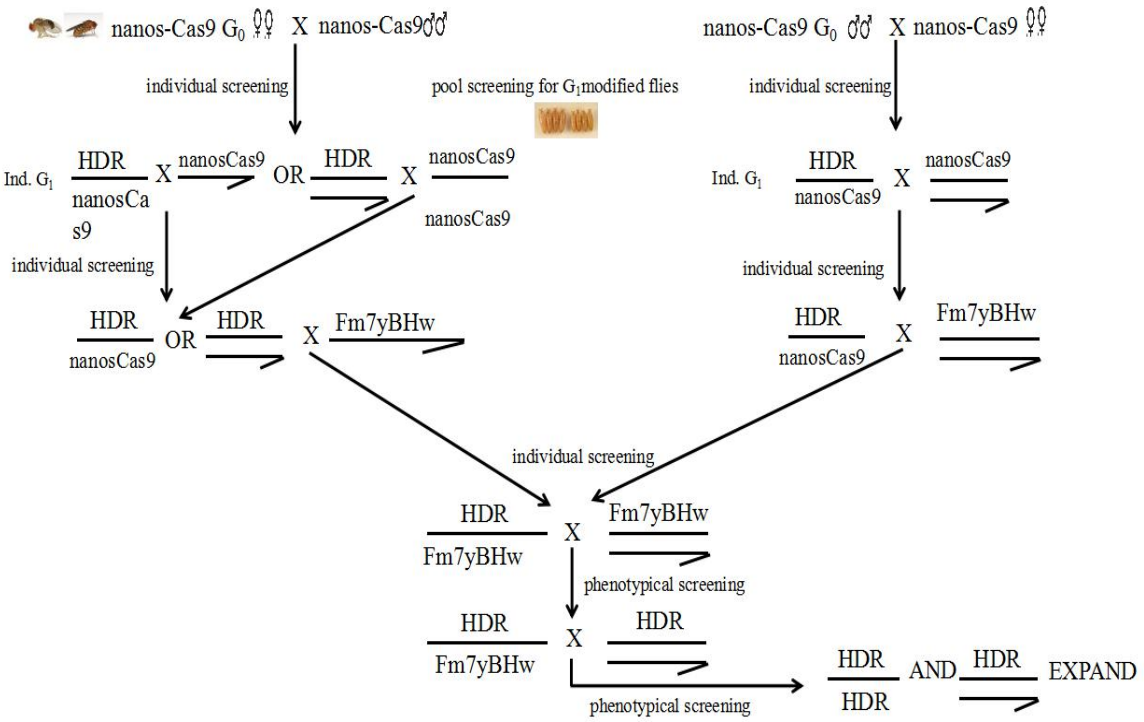

Fig.5 Scheme of the workflow of the genetical crosses of G_0 CRISPR events in order to generate the homozygous modified flies.

Table 2 List of the primers used in this study.

No.	Primer name	Primer sequence (5'->3')	Experimental use
1	Tuta_F	GTGCTGGACGGCATCATCAA	Cloning into pGEM
2	Tuta_R	CTCGAGAATGACGGCGATGT	
3	LparaF	CTTCGAGGAGAAACGTTATTCCAA	dsDNA synthesis-gRNA construction
4	LparaR	AAACTTGGAATAACGTTTCTCCTC	
5	RparaF	CTTCGTCCGAATTCCTGGACGTAC	
6	RparaR	AAACGTACGTCCAGGAATTCGGAC	
7	ParaSpecF	AATTGTGGTTCAGCGACGGTTGGC	Screening of modified flies
8	ParaSpecR	GGGGCTCAAGTACATCCAGGAAC	
9	ParaGenF	TCGCACAACTGCCAATCCTA	
10	ParaGenR	CACCAATCTCACCCGTCTCC	
11	Ind1F	CCTCTGTCTATCTGTCTGCC	Sequencing of overlapping fragments of *para* gene
12	Ind1R	ATACGAGCGTGTTACCGATT	
13	Ind2F	GCCCACATACGAACACTCCG	
14	Ind2R	CGTATGTACTGGGTGCCCTC	
15	Ind3F	ATCCACCCGACAACGACAAA	
16	Ind3R	TACCGTCATTTGCTCGCCAT	

3.7 Bioassays and Statistical Analyses

Contact Bioassays

In order to test if both insecticides are active against adult flies of nanos-Cas9 strain we performed a bioassay by residual contact application (Hardstone et al., 2006). Test insecticides were dissolved in acetone and after the preparation of the desired concentrations with several dilutions, 500ul of each one were applied into glass scintillation vials. For each concentration there were 3 technical replicates. The vials were put on a roller for overlaying all of their surface, for 30-40min, under a fume hood. After evaporation of the acetone, flies were transferred into each vial in a batch of 20 flies (10 males and 10 females). Each vial was covered with a piece of cotton soaked into a solution of 5% sucrose. Vials were left lying on the bench at room temperature and flies were exposed from 24 hours up to 96 hours.

Feeding Toxicity Bioassays

For the feeding toxicity bioassays, 2nd instar larvae were transferred in batches of 20 into fresh standard fly artificial food, supplemented with several concentrations of insecticide solutions (Steward and Alverde). Larval development, mortality, pupal eclosion, pupal size and adult survival were monitored and measured after 7-10 days. Each experimental procedure comprised five to seven different concentrations, tested in triplicates. Sensitive population (nanos-Cas9) was tested every time along with the modified populations (F1845Y, V1848I and FYVI) and for each insecticide negative controls (distilled water instead of insecticide) were included in the experiments.

Statistical analyses

Concentration-response data of each experimental procedure were collected and analysed with the program PoloPlus (LeOra Software, Berkeley, California) 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 were calculated with this program.

4. Results

4.1 Sequencing of ~168bp of *T.absoluta* gDNA

In order to validate if the mutations can be found in *cis* or not in heterozygous mutants, DNA from an individual of *T.absoluta* (T9) resistant to indoxacarb. This strain, which was found previously to be heterozygous for both of the mutations with PCR-RFLP, was used for cloning. Five clones were derived and sequenced in order to identify if the mutations are in *cis* or in *trans* and the results reveal that they were not found in the same allele (Fig. 6).

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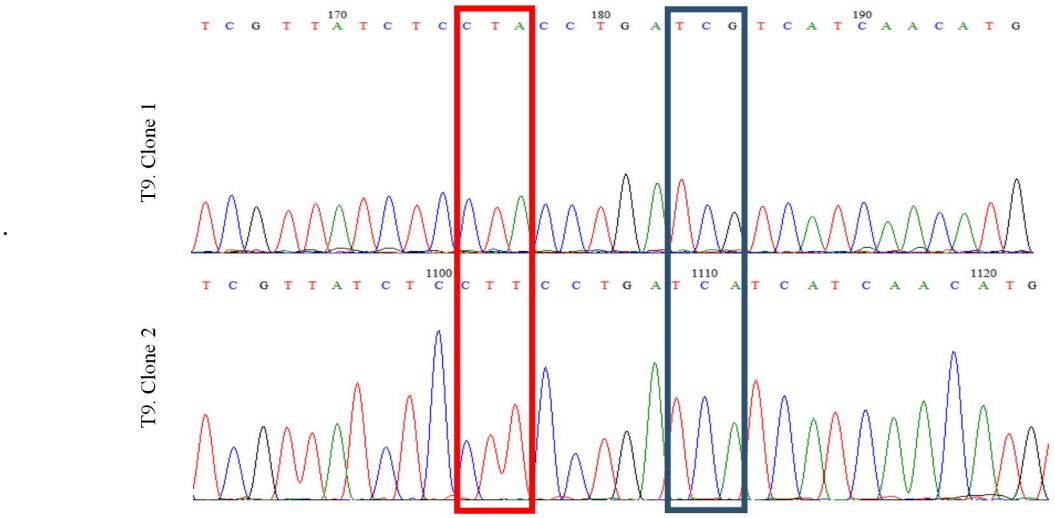

Tuta_9_Wild ATCTCCTTCCTGATCGTCATCAACATGTACATCGCCGTCATTCTCGAG--

Tuta_9_T9.14 ATCTCCTACCTGATCGTCATCAACATGTACATCGCCGTCATTCTCGAGAA

Tuta_9_T9.12 ATCTCCTTCCTGATCATCATCAACATGTACATCGCCGTCATTCTCGAGAA

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Fig. 6 A. Chromatograms pinpointing the mutations F1845Y (red frame) and V1848I (blue frame) in an heterozygote individual of *T.absoluta*. B. Alignment among the wild type sequence of *T.absoluta* (Roditakis et al. year) and the two clones of T9 individual pinpointing the single mutations at F1845Y (T->A) and V1848I (G->A)

4.2 CRISPR mediated substitution of F1845Y, V1848I and FYVI in *Drosophila*

Our strategy was to create modified flies that carry mutations F1845Y and V1848I in the *Drosophila para* gene and finally to validate and elucidate their actual contribution to the resistance to SCBIs. We obtained the whole amino acid sequence of *para* gene from four different insect species by using NCBI site (*P. xylostella* AJR27944.1, *Apis mellifera* NP_001159377, *Musca domestica* NP_001273814.1, *D. melanogaster* ADV37728.1) and we aligned them with Clustal Omega(1.2.4)

EMBL-EBI (Fig. 7). We found the orthologue *para* gene in *Drosophila* and based on the alignment we identified the target region in order to design the mutations under study.

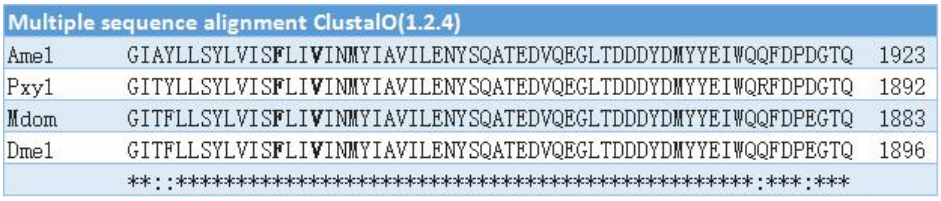

Fig. 7 Sequence alignment of the para amino-acid sequence among four different insects (*Apis mellifera*, *Plutella xylostella*, *Musca domestica* and *Drosophila melanogaster*) emphasizing F1845 and V1848 (in bold).

For the mutation F1845Y, 55 G_0 adult flies were backcrossed with nanos cas9 strain and 9 out of them were sterile. The G_1 progeny of the remaining 46 were screened with either "specific[8]" primers or digestion after amplification with generic primers. Six out of the 46 crosses were found to give progeny positive for the HDR, while two of them probably underwent NHEJ since they had a deletion of ~250bp. The results regarding the rest of the mutations are summarized in table 3.

Table 3. Results of the genetical crosses performed for homozygous lines generation of each mutation.

	F1845Y	V1848I	FYVI
Adults G_0	55	55	71
Sterile	9	21	15
Positive (HDR)	6	8	6
Deletion (NHEJ)	2	3	2

G_1 individuals originating from the original positive lines (G_0) were crossed with nanos-Cas9 adults and they were then screened in order to identify positive heterozygotes. The screening in individual adults is performed with PCR using "specific" primers (Fig.5), which yields a fragment of 250bp in the three cases of mutations, as it is depicted in figure 8. G_2 progeny from the crosses containing a G_1 positive parent were crossed with balancer flies for the X chromosome and molecular screening was carried out in order to distinguish the crosses that came from positive G_2 flies. After the final crosses in order to obtain homozygous modified flies, several lines for each mutation were established. Specifically, we generated 6 lines for the mutation F1845Y, 4 lines for the mutation V1848I and 5 lines for the dual mutations FYVI. All of the lines were checked by sequencing in order to verify that HDR mechanism worked successfully which is depicted in figure 9.

[8] Term specific refers to the primers that comprises of the specific mutations we potentially generate in *Drosophila.*

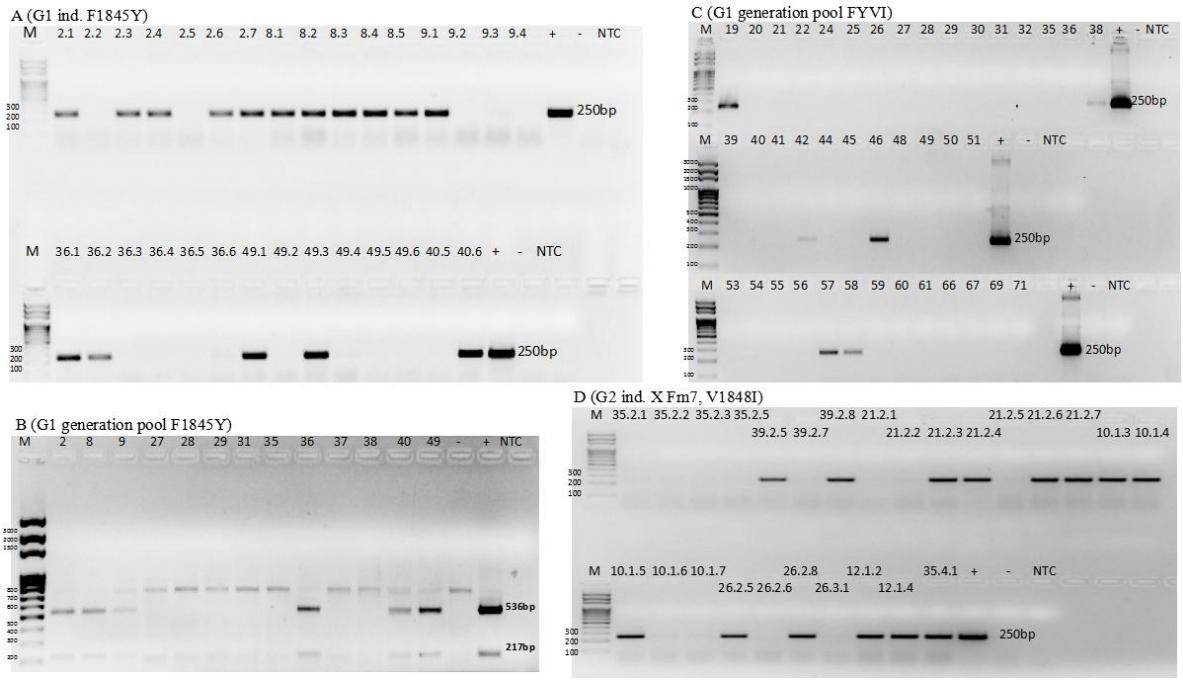

Fig. 8 Diagnostic screening with specific primers yielding 250bp product running in 2% agarose gel. M indicates the ladder while + indicates the positive control (donor plasmid used for each mutation) used for the experiments , - indicates the negative control of the experiment which was the non-injected nanos-Cas9 gDNA and NTC is the blank in which distilled water replaces the DNA template (A) PCR screening with specific primers (250bp) of G_1 individuals backcrossed with nanos-Cas9 originating from each original line (G_0) for the mutation F1845Y (B) Diagnostic digestion of PCR product (752bp) with generic primers with restriction enzyme KpnI for massively screening the G_1 generations of G_0 lines yielding two diagnostic fragments of 536bp and 217bp (C) PCR screening with specific primers (250bp) in pupae pools of the original lines for the dual mutations FYVI (D) PCR screening with specific primers (250bp) of G_1 individuals originating from each original line (G_0) for the mutation V1848I crossed with balancer Fm7yBHw.

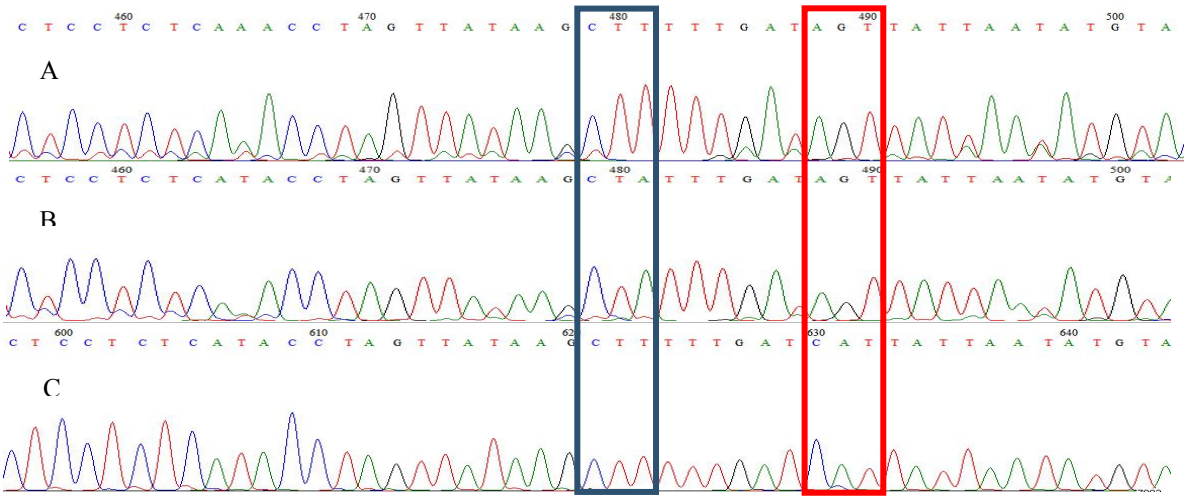

Fig. 9 Chromatograms of the sequencing results of homozygous lines established for each mutation. F1845 and V1848 amino acids are indicated with blue and red frames respectively. The mutation F1845Y is depicted in

chromatogram B, while mutation V1848I is depicted in chromatogram C. Chromatogram A shows the relevant sequencing of the wild type population of nanos-Cas9.

In order to study the contribution of each mutation to resistance to the SCBIs and perform toxicity bioassays, we selected one of the several modified homozygous lines based on the sequencing results.

4.3 Toxicity contact bioassay in *Drosophila*

According to manufacturer (DuPont) Indoxacarb (technical) is known to be absorbed by the cuticle. However, we performed contact bioassays in the 2-3 days old adult nanos-Cas9, reaching a concentration of 1000ppm, but no mortality was observed even after 96 hours of continuous exposure. Same procedure was followed for metaflumizone as well, but no mortality was observed. For this reason feeding toxicity bioassays were performed using the formulation compounds Steward and Alverde.

4.4 F1845Y and V1848I mutations confer high resistance to SCBIs in *Drosophila*

Drosophila larvae were continuously in contact with the food supplemented with the insecticides and toxicity effects such as cessation of feeding, larval paralysis, delay of development and reduction of the size of puppae were observed. Seven days after the performance of the bioassay, in the case of the lowest concentrations of the insecticide most of the pupae had eclosed. But this was not observed in the case of higher concentrations (>20ppm in the case of F1845Y and >10ppm in the case of V1848I). Probably in the lowest concentrations of insecticides the larvae seem to be fed normally without any developmental problems. In the highest concentrations, resistant larvae manage to survive but the developmental problems they exhibit (delay in development and small size) are probably caused by difficulty in feeding because of the effect from the insecticide.

Since dead larvae cannot be readily visible inside the fly food, larvae molting to pupae was considered a measurable proxy of eventual survival. Data have been analysed with polo plus software and the LC_{50} values for the calculation of the resistance ratios versus the control (nanos-Cas9), along with fiducial limits 95%, chisquare values and the slope of the curve are shown in Table 4 for every strain against the two insecticides.

According to the results, a single mutation from phenylananine to tyrosine in the aminoacid 1845 of the sodium channel renders *Drosophila* larvae more resistant to SCBIs, than the wild type strain. Specifically, the strain F1845Y exhibits 12.5 fold higher resistance when it is compared with nanos-Cas9 wild type strain. On the other hand it seems that metaflumizone is less effective against *Drosophila* larvae bearing the mutation F1845Y (RR: ~3460x with respect to sensitive nanos-Cas9 larvae). *T.absoluta* resistant strains, after selection with indoxacarb, showed extremely high resistance ratio against indoxacarb but very low resistance ratio agaisnt metaflumizone (Roditakis et al., 2016). However, the same mutations generated in *Drosophila* caused the reverse results; larvae bearing the mutation V1848I exhibited much less resistance to SCBIs, comparing the RRs of this mutation with the

RRs of F1845Y. Specifically V1848I strain seemed to be 6.6x resistant to indoxacarb and 9.9x to metaflumizone with respect to nanos-Cas9 strain. This difference in the contribution of resistance between the two strains potentially indicates lower affinity of 1845Y with the SCBIs, than the one exhibited by the 1848I mutation. Interestingly, these mutations seem to exhibit different levels of resistance against SBCIs among the field populations of *T.absoluta* and *P.xylostella* and the laboratory strain *D.melanogaster.* The reason why there are differences in the contribution of these two mutations in resistance to SCBIs among *D.melanogaster*, *T.absoluta* and *P.xylostella* are discussed in chapter 5.

Both mutations have never been found to be in *cis* in the field populations of lepidopteran insect pests. We wondered then what the phenotype would be if they were in the same chromosome. For this reason we attempted to generate *Drosophila* mutants bearing F1845Y/V1848I mutations and we obtained one homozygous line which is in progress for validating the success of HDR with sequencing.

Table 4 Log-dose probit-mortality data for indoxacarb and metaflumizone against larvae of *Drosophila* genome modified strains F1845Y, V1848I and FYVI versus control (nanos-Cas9).

Compound	Drosophila strain	Slope±se	LC50(95%CI) ug/ml	X^2(df)	RR (95%CI) vs nanos-Cas9
Indoxacarb	nanos-Cas9	5.084±0.638	2.041(1.548-2.554)	26.900	
	F1845Y	3.9±0.352	25.881(21.661-30.583)	26.394	12.5X
	V1848I	3.698±0.390	13.608(11.909-15.499)	17.739	6.6X
	FYVI		Still in progress	Still in progress	Still in progress
Metaflumizone	nanos-Cas9	4.98± 0.598	0.525(0.416-0.505)	9.375	
	F1845Y	5.906±0.798	1816.75(1627.624-2017.529)	8.748	3460X
	V1848I	2.496±0.241	4.882(3.899-5.903)	27.239	9.3X
	FYVI		Still in progress	Still in progress	Still in progress

5. Discussion

Metabolic resistance against SCBIs has been studied and identified in many insect pests, such as *Choristoneura rosaceana* (Ahmad et al., 2002), *Musca domestica* (Shono et al., 2014), *P.xylostella* (Zhang et al., 2017, Wang et al., 2016) and *Tuta absoluta* (Roditakis et al., 2016). Significant levels of resistance to SCBIs in lepidopteran insect pests have been correlated with two target site mutations in the S6 helix segment of domain IV in the voltage gated sodium channel, notably F1845Y and V1848I (Wang et al., 2016, Roditakis et al., 2017). One population of *P.xylostella* collected from a district of China, exhibited high levels of resistance to indoxacarb (750X) but lower levels against metaflumizone (70X), which was in accordance with the extent of the use of the respective insecticides in this area (Wang et al., 2016). Synergist effects lower the LC_{50} values of two *Plutella* populations, with the higher pooled mutation frequency, against indoxacarb and metaflumizone, which indicates the contribution of metabolic resistance, pinpointing the contribution of esterase (Wang et al., 2016). The mutation frequency of this population for V1848I was higher (42.5%) than the frequency of F1845Y (10%). Target site mutations and especially V1848I seemed to contribute to high levels of resistance against indoxacarb since the mutation frequency for this mutation in the highly resistant population was enriched comparing to the mutation F1845Y. After Pearson correlation analysis it has been suggested that there is significant association between the two target site alterations and the resistance ratio values against metaflumizone and indoxacarb but they were never validated *in vivo* (Wang et al., 2015).

Furthermore, correlation of target site mutations in sodium channel with resistance to SCBIs has been documented in two populations of the tomato invasive pest *T.absoluta* collected from Greece and Italy (Roditakis et al., 2017) Resistance of these populations to SCBIs was the synergistic effect of metabolic mechanisms of resistance (indicated by the treatment with synergists) with target site resistance. Target site resistance was proved with genotyping of those two populations, which revealed the existence of the mutations F1845Y and V1848I. Moreover enrichment of F1845Y mutation (21.1%) versus the V1848I (2.3%) was shown. Specifically after selection of the population collected from Greece with indoxacarb, the mutation frequency for F1845Y mutation increased to 66.7%, while V1848I was not present at all. Feeding bioassay of the selected population with indoxacarb showed high levels of resistance against indoxacarb (1794X versus the susceptible lab strain) and lower levels against metaflumizone (40X versus the susceptible lab strain) (Roditakis et al., 2016). So it seems that F1845Y mutation renders *T.absoluta* population quite resistant to indoxacarb and less resistant to metaflumizone. Those results pinpoint the differential contribution of those two mutations to indoxacarb resistance if compared to *P.xylostella* bioassay data.

In our study we emphasize to the contribution of each one mutation to SCBIs resistance in *Drosophila*. Since the contact toxicity bioassays for SCBIs seemed to be not effective for *Drosophila* adults, we assumed that either *Drosophila* flies cannot absorb indoxacarb through their cuticle or they absorb the insecticide but they cannot metabolize the initial compound to the drastic secondary metabolite (Decarbomethoxyllated JW062) in order to act against sodium channels of the neurons.

Thus we performed an alternative toxicity bioassay by providing the insecticide to *Drosophila* by feeding 2nd instar larvae. Each mutation confers different levels of resistance against indoxacarb and metaflumizone. Specifically we show that F1845Y mutation confers higher levels of resistance against metaflumizone (>500X) than to indoxacarb (~12.5X). This is concordant with the resistance phenotype of *T.absoluta*, although in this case F1845Y mutation was shown to confer higher resistance to indoxacarb (Roditakis et al., 2016). The same pattern of resistance against the two SCBIs was observed in the case of V1848I. We assume that this probably has to do with the affinity of each compound with 1845Y (metaflumizone has lower affinity with tyrosin than indoxacarb). This result is confirmed by the electrophysiological studies performed in transgenic oocytes carrying the mutations F1845Y and V1848I (Jiang et al., 2015). In presence of metaflumizone the sodium current flowing inside the transgenic oocytes (F1845Y and V1848I) is higher than the current's infux into oocytes perfused with the same concentration of indoxacarb. Thus metaflumizone in both cases of the mutations probably has lower affinity with 1845Y and 1848I than indoxacarb. The resistance that both mutations conferred against SCBIs probably lies in the fact that hydrophobic moieties of SCBIs cannot actually interact with the hydrophilic hydroxyl of tyrosine which leads to the repulsion of the insecticide from the inner pore while in the case of V1848I, isoleucine's larger size might probably lead to the repulsion of the insecticide (Jiang et al., 2015).

Our results show that F1845Y mutation might confer higher resistance against SCBIs than V1848I. This conclusion is not in concordance with the bioassay data of indoxacarb resistant population of *P.xylostella*, which showed that V1848I mutation is more important for the resistance phenotype. Moreover, the wild type *Drosophila* larvae exhibited opposite sensitivity to each insecticide if LC_{50} values (Table 2) are compared with those of *T.absoluta* and *P.xylostella*. Therefore metaflumizone has been found to be more active against *Drosophila* larvae (Table 2) than indoxacarb. The above data could lie in some specific differences located in sodium channel amino acid sequences among those three insects, despite the high conservation that voltage gated sodium channel gene exhibits.

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7. Supplementary

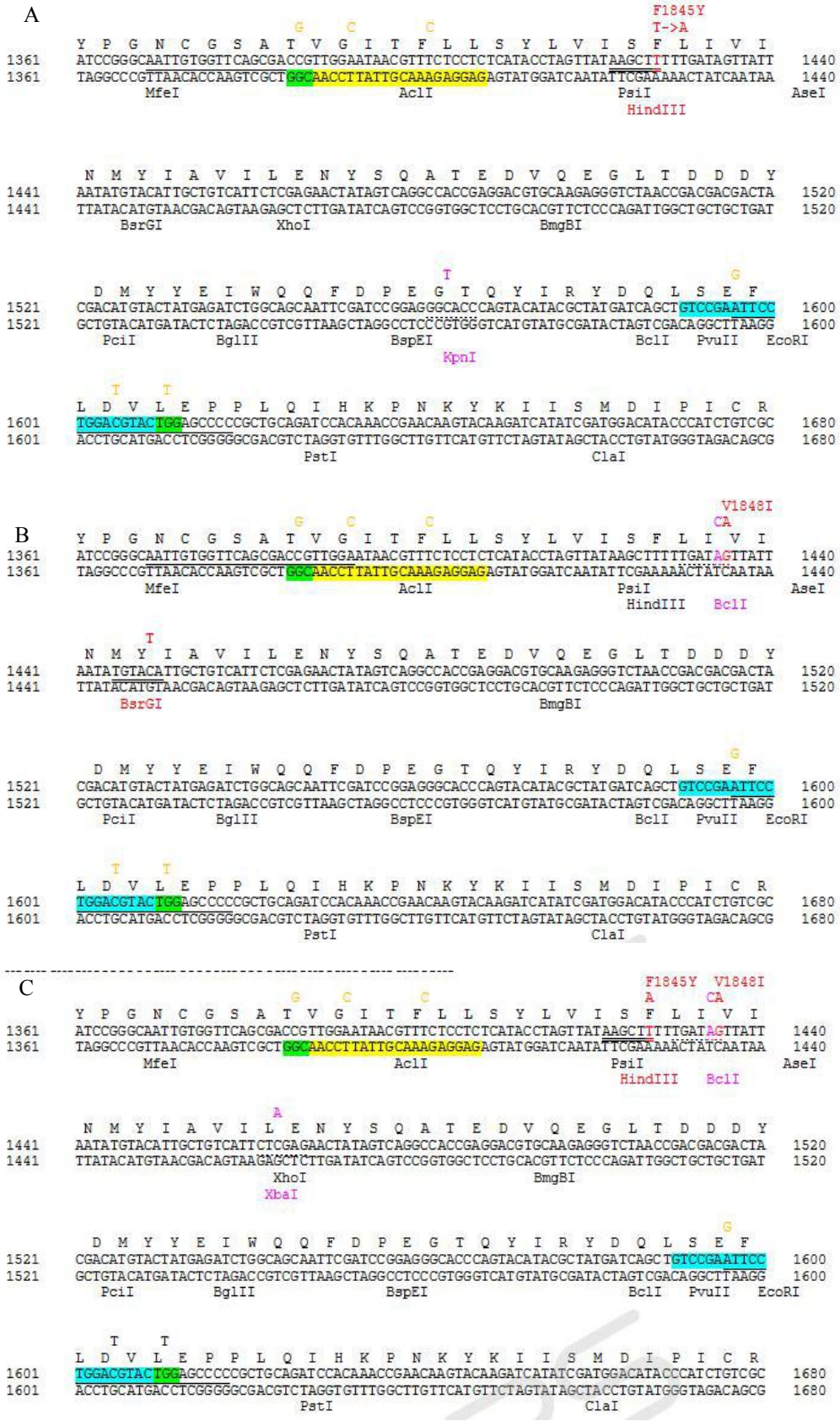

Fig. S1 Nucleotide sequence of the donor templates used in order to generate CRISPR mediated mutant flies bearing A.F1845Y, B.V1848I and C.FYVI. Highlighting with yellow indicates the gRNA's Lpara target sequence, while the blue one indicates the gRNA's Rpara target sequence. The green highlighting indicates the PAM sequence in 5'->3' direction. Sequences underlined with simple line refers to the specific primers used for the PCR reactions as diagnostics. The sequences underlined with dot-lines match the new restriction enzyme cutting motifs generated with the introduction of a single nucleotide synonymous mutation, which was used in the diagnostic strategy that was followed. The sequences underlined with double lines refer to the restriction enzyme cutting motif that was disrupted with the alteration of a single synonymous mutation.

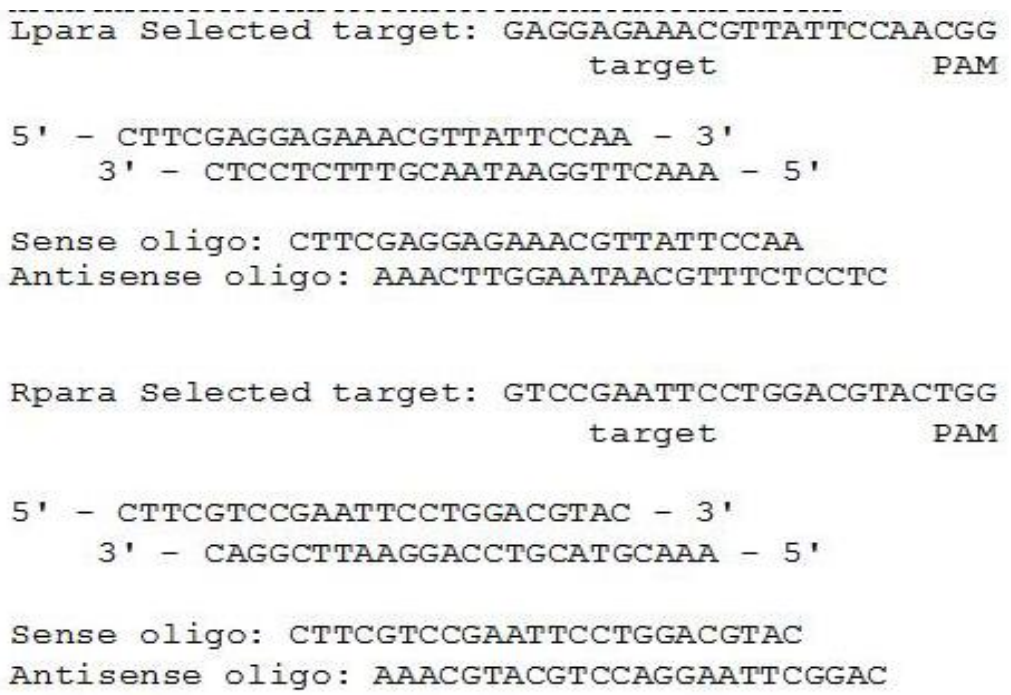

Fig. S2 Nucleotide sequences of the gRNA target sequences Lpara and Rpara with the 5' ssDNA overhangs. For the generation of the dsDNA two ssDNA oligos have been used, sense and antisense oligos.