"Functional validation of target-site resistance mutations against Sodium Channel Blocker Insecticides (SCBIs) via genome engineering in *Drosophila*"

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Abstract

Voltage-gated sodium channels are the target of several insecticides including DTT, pyrethroids, and SCBIs like indoxacarb and metaflumizone. SCBIs are an alternative insecticide resistance management (IRM) strategy against several pests 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 bearing homozygous F1845Y or V1848I mutations in the *para* (voltage-gated sodium channel) gene of *Drosophila melanogaster.* 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), a finding that may be related to the specific binding of each insecticide to its target.

1. Introduction

Voltage-gated sodium channels (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, leading to the generation and propagation of electrical signals facilitating the response to several environmental stimuli (see review by Carnevale and Klein, 2017). The VGSC α-subunits are comprised by four homologous domains (I-IV), each having six membrane spanning helical segments (S1-S6); sodium ions are sensed by positively charged aminoacids of the S4 transmembrane segments (Duclohier, 2009). In response to membrane depolarization, the S4 segments move to the extracellular side of the cell membrane, triggering an allosteric alteration to the coupling between the sensor module and the gate that is contained within the assembly of the S5 and S6 transmembrane helices. This leads to pore opening, initiating the influx of sodium ions (Dong et al, 2014; Carnevale and Klein, 2017). Soon after, the sodium channel undergoes inactivation through two different modes, 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 α-subunit and containing a characteristic IFM amino acid motif; Goldin, 2003) and another mode of slow inactivation whose molecular basis is still not elaborated (Kass, 2004; Silva, 2014).

Since they are major players of cell excitability in the nervous system, VGSCs are the primary targets of many chemical substances such as local anesthetics (analgetics, antirrhythmic drugs) in vertebrates as well as chemical insecticides in insects, which are used in order to suppress the cells' excitability and their high frequency discharges (Gawali et al., 2015). These chemicals' efficiency lies on their affinity to the inactivated

state of the sodium channels. Many types of insecticides such as DDT and pyrethroids target the nervous system, by prolonging the channel's open conformation state, resulting to the increase of Na^+ influx and finally to cell hyperexcitability (Wakeling et al., 2012). Indoxacarb (a pyrazoline type insecticide) and metaflumizone belong to the family of Sodium Channel Blocker Insecticides (SCBIs; von Stein et al., 2013) that has a different mode of action (Group 22A in the IRAC classification system). Both target the sodium channel in the slow-inactivated state in a fashion similar to local anesthetics, by binding to the opened channel pore when the membrane is still depolarized and causing a shift in the voltage dependence of slow inactivation to more negative currents. Thus, VGSCs are stabilized in the inactivated state leading to hindrance of the intracellular sodium influx (Silver & Soderlund, 2007; Silver et al., 2010; 2017; Jiang et al., 2015, Zhang et al, 2016).

Indoxacarb is an insecticidal oxadiazine 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 formulations are used either by spraying or by digestion and the desirable effects (cessation of feeding, lack of coordination and paralysis) are observed from within few minutes to four hours. It is used against moths, beetles, leafhoppers, weevils, flies and other pests (Silver et al., 2010) and it has been shown that spraying treatment of *Drosophila* 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), and it is a different SCBI developed by BASF in an effort to overcome the side effects of dihydropyrazoles. Metaflumizone exhibits low toxicity to mammals and selectivity towards insects (Hempel et al., 2007).

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The use of SCBIs has been a significant alternative insecticide resistance management (IRM) strategy against several pests resistant to other compounds. However, cases of resistance against SCBIs have been reported in insects such as the housfly *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 limited Resistance Ratio (RR) increase for metaflumizone (Roditakis et al., 2017), while earlier studies of indoxacarb-resistant populations of *P. xylostella* indicate no cross-resistance to metaflumizone (Khakame et al., 2013). Conversely, a report of a population of *Spodoptera exigua* developing 942-fold resistance to metaflumizone, but only 16-fold resistance to indoxacarb (Su & 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).

In some cases there is evidence for synergistic effects of metabolic inhibitors on SCBI toxicity, implicating metabolic resistance mechanisms through 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), while their use suggested a limited role of detoxification in metaflumizone resistance in Spodoptera exigua (Su & Sun, 2014).

Resistance levels to both SCBIs were significantly correlated to the frequencies of two sodium channel mutations, F1845Y and V1848I, identified in the domain IV S6 segment of the voltage-gated sodium channel, (Figure 1) in two field populations of *P. xylostella* exhibiting resistance to indoxacarb (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).

An *in vitro* approach for the investigation of F1845 and V1848 mutations (*P. xylostella* numbering) with SCBIs binding was performed through heterologous expression of modified german cockroach *B. germanica* voltage gated sodium channel in *Xenopus* oocytes for electrophysiological experiments where F1845Y and V1848I/A mutants were generated (Jiang et al., 2015). This *in vitro* assessment suggested that single amino acid mutations F1845Y and V1848I (but not V1848A) in the cockroach sodium channel reduced almost equally the inhibition of sodium current by indoxacarb, DCJW (an active metabolite of indoxacarb) and metaflumizone, indicating that both these specific 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 these strains in order to functionally validate resistance to SCBIs *in vivo*.

2. Materials and Methods

2.1 Chemicals

Chemical compounds used for contact bioassays were indoxacarb (Sigma-Aldrich, PubChem CID: 107720) 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.

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 25ºC temperature, at 60-70% humidity and 12:12 hour 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 1) 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 sec, annealing at 61°C-66°C for 15 sec, extension at 72°C for 45-90 sec 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 (relevant to F1845Y and V1848I found in *P. xylostella* and *T. absoluta*) in the *para* gene (voltage-gated sodium channel). 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 (Figure 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) 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 generations of strains F1845Y, V1848I and FYVI respectively (relevant insert sequences shown in Figure S1). Each plasmid contained two ~1000 bp homology arms flanking the 228 bp target region between the two sgRNA targets Lpara and Rpara. The target region was specifically designed (see Figure 2 for details) in order to contain the desired in each case combination of desired mutations along with certain synonymous mutations serving either as molecular markers in order 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 100ng/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, 2μg of gDNA was 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 Figure 4 for the whole crossing scheme). Individual G_1 flies from positive original G_0 crosses were back-crossed 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

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 hours.

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

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.

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 voltage-gated sodium channel of *Drosophila*, *paralytic* or *para* was aligned to the lepidopteran and other insect orthologs (Fig.1B) and the target region identified. A genome modification strategy was designed in order to introduce the mutations under study (Fig. 2) and carried out as described in Materials and Methods.

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. 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 Experimental Procedures. 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. 3) and then balanced in order to establish homozygous fly lines for each mutation (overall crossing scheme shown in Figure 4). 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 hours 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.

4. Discussion

Two mutations at the S6 segment of domain IV of voltage-gated sodium channel (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 to 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* (sodium channel) gene of *Drosophila melanogaster* whose IVS6 sequence is very similar to the sequence of the two lepidopteran pests (Figure 1B). 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 characterisation 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 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 (Jiang et al., 2015; Table 2), 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.

The specific conformational changes induced by each mutation (and thus the resulting resistance observed) may be very much dependent on the protein sequence context. Jiang et al. (2015) suggest that the SCBIs interact with both valine V1848 and phenylalanine F1845 (*P. xylostella* numbering), and propose a homology model based prediction for DCJW binding, according to which "the [V1848] sidechain faces the inner pore, whereas the [F1845] sidechain may move between the inner pore and the III/IV domain interface. […] SCBIs bind in the inner pore and may expand a hydrophobic moiety into the III/IV domain interface." (Jiang et al., 2015). In a follow-up study that includes docking simulations of metaflumizone (Zhang et al., 2016), the two positions are described as "SCBI sensing residues" and it is proposed that the SCBI receptor includes certain residues in IVS6 (F1845, V1848 and possibly Y1852), as well as at least one other residue in IIS6 that faces the pore, but does not contribute to LA binding, while it contributes to the metaflumizone receptor (Zhang et al., 2016; Silver et al., 2017). Other residues may also participate in binding SCBIs to VGSCs (Silver et al., 2017).

Our finding that F1845Y mutation in genome modified flies has a much greater impact on metaflumizone resistance compared either to indoxacarb or to the impact of V1848I against both commercial SCBIs, provided it mirrors the actual situation in lepidopteran pest, might be indicative of a much more critical role of residue 1845 for metaflumizone binding to the SCBI receptor. Indoxacarb and metaflumizone belong to the same IRAC class and have a common target, but they belong to different chemical classes and their structure is substantially different; thus, there may be differences in their specific binding sites and respective binding properties as already documented for at least one residue that affects channel sensitivity to metaflumizone, but not indoxacarb or DCJW (von Stein & Soderlund, 2012). Detailed *in silico* analysis and additional experiments are necessary in order to fully understand the specific interactions, but the outcome of this study may already have important implication in IRM with impact on crop production.

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 sodium channel conformation.

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.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

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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)	6.45

Table S1: List of primers used in this study

No.	Primer name	Primer sequence (5'->3')	Experimental use
1	Tuta_F	GTGCTGGACGGCATCATCAA	Amplification from *Tuta absoluta* DNA samples
2	Tuta_R	CTCGAGAATGACGGCGATGT	
3	LparaF	CTTCGAGGAGAAACGTTATTCCAA	Generation of sgRNA expression plasmids
4	LparaR	AAACTTGGAATAACGTTTCTCCTC	
5	RparaF	CTTCGTCCGAATTCCTGGACGTAC	
6	RparaR	AAACGTACGTCCAGGAATTCGGAC	
7	ParaSpecF	AATTGTGGTTCAGCGACGGTTGGC	Molecular screening for genome modified flies
8	ParaSpecR	GGGGCTCAAGTACATCCAGGAAC	
9	ParaGenF	TCGCACAACTGCCAATCCTA	
10	ParaGenR	CACCAATCTCACCCGTCTCC	
11	Ind1F	CCTCTGTCTATCTGTCTGCC	Sequencing of overlapping fragments of *para* genomic region
12	Ind1R	ATACGAGCGTGTTACCGATT	
13	Ind2F	GCCCACATACGAACACTCCG	
14	Ind2R	CGTATGTACTGGGTGCCCTC	
15	Ind3F	ATCCACCCGACAACGACAAA	
16	Ind3R	TACCGTCATTTGCTCGCCAT	

Figure Legends

Figure 1: 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).

Figure 2: 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.

Figure 3: Indicative diagnostic screening with specific primers yielding a 250 bp PCR product 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 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 (250bp) of G_1 individuals for the mutation V1848I after cross with flies bearing balancer FM7.

Figure 4: Crossing scheme for *Drosophila*. Several nos.Cas9 G_0 embryos are injected and surviving adults back-crossed to nos.Cas9 The G_1 progeny is sampled (n≈30) and if positive, individual G_1 flies are crossed to nos.Cas9 and then screened with single fly PCR for homologous directed repair (HDR). Individual G_2 feamles are crossed to males of a strain bearing X chromosome balancer FM7c marked with *Bar* and then screened for HDR. Individual G_3 females with heterozygous *Bar* phenotype are crossed to the balancer strain males and then screened for HDR. G_4 females with *Bar* phenotype (bearing the desired mutation opposite to FM7c) are crossed with male siblings selected against *Bar* (i.e. hemizygous for the genome modified chromosome bearing the HDR-derived allele) and their progeny (G_5) is selected against *Bar* to generate homozygous lines bearing the desired mutation.

A

Domain I Domain II Domain III Domain IV

F1845Y
V1848I

H₂N— ... —COOH

B

IVS6
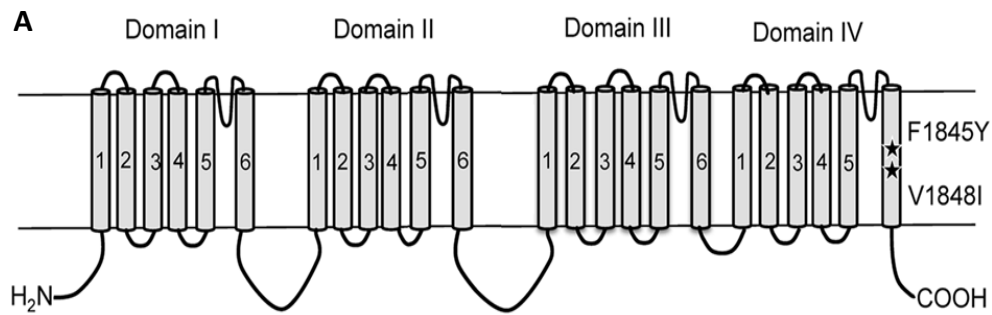
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
PxNa$_v$	G	I	T	Y	L	L	S	Y	L	V	I	S	F	L	I	V	I	N	M	Y	I	A	V	I	L
TaNa$_v$	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
LepF1845Y	.	.	.	.	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.
LepV1848I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
DmNa$_v$	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DmF1845Y	.	.	.	F	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.
DmV1848I	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
AgNa$_v$	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
AmNa$_v$	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TcNa$_v$	.	.	A	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BgNa$_v$	.	.	A	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

A

PAM   Lpara sgRNA   *F1845Y*

G   C   C   A

GGTTCAGCGACCGTTGGAATAACGTTTCTCCTCTCATACCTAGTTATAAGCTTTTTGATAGTTATTAATATGTACATTGCTGTCATTCTCGAGAACTATAGTCAGGCCACCGAGGACGTGCAAGAGGGT

G S A T V G I T F L L S Y L V I S F L I V I N M Y I A V I L E N Y S Q A T E D V Q E G

HindIII

Rpara sgRNA   PAM

T   G   T   T

CTAACCGACGACGACTACGACATGTACTATGAGATCTGGCAGCAATTCGATCCGGAGGGCACCCAGTACATACGCTATGATCAGCTGTCCGAATTCCTGGACGTACTGGAGCCCCCGCTGCAGATCCAC

L T D D D Y D M Y Y E I W Q Q F D P E G T Q Y I R Y D Q L S E F L D V L E P P L Q I H

KpnI   EcoRI

B

PAM   Lpara sgRNA   *V1848I*

G   C   C   CA   T

GGTTCAGCGACCGTTGGAATAACGTTTCTCCTCTCATACCTAGTTATAAGCTTTTTGATAGTTATTAATATGTACATTGCTGTCATTCTCGAGAACTATAGTCAGGCCACCGAGGACGTGCAAGAGGGT

G S A T V G I T F L L S Y L V I S F L I V I N M Y I A V I L E N Y S Q A T E D V Q E G

BclI   BsrGI

Rpara sgRNA   PAM

G   T   T

CTAACCGACGACGACTACGACATGTACTATGAGATCTGGCAGCAATTCGATCCGGAGGGCACCCAGTACATACGCTATGATCAGCTGTCCGAATTCCTGGACGTACTGGAGCCCCCGCTGCAGATCCAC

L T D D D Y D M Y Y E I W Q Q F D P E G T Q Y I R Y D Q L S E F L D V L E P P L Q I H

EcoRI

C

PAM   Lpara sgRNA   *F1845Y*   *V1848I*

G   C   C   A   CA   A

GGTTCAGCGACCGTTGGAATAACGTTTCTCCTCTCATACCTAGTTATAAGCTTTTTGATAGTTATTAATATGTACATTGCTGTCATTCTCGAGAACTATAGTCAGGCCACCGAGGACGTGCAAGAGGGT

G S A T V G I T F L L S Y L V I S F L I V I N M Y I A V I L E N Y S Q A T E D V Q E G

HindIII BclI   XbaI XhoI

Rpara sgRNA   PAM

G   T   T

CTAACCGACGACGACTACGACATGTACTATGAGATCTGGCAGCAATTCGATCCGGAGGGCACCCAGTACATACGCTATGATCAGCTGTCCGAATTCCTGGACGTACTGGAGCCCCCGCTGCAGATCCAC

L T D D D Y D M Y Y E I W Q Q F D P E G T Q Y I R Y D Q L S E F L D V L E P P L Q I H

EcoRI

A (G1 ind. F1845Y)

C (G1 generation pool FYVI)

B (G1 generation pool F1845Y)

D (G2 ind. X Fm7, V1848I)

G_0: inj. ♀♀♂♂ nos.Cas9 × nos.Cas9

mass PCR sampling for G_1 positives

ind. G_1: $\dfrac{X\_HDR}{X}$ or $\dfrac{X\_HDR}{Y}$ × $\dfrac{X}{X}$ or $\dfrac{X}{Y}$

single fly PCR

ind. G_2: $\dfrac{X\_HDR}{X}$ × $\dfrac{FM7c}{Y}$

single fly PCR

ind. G_3: $\dfrac{X\_HDR}{FM7c}$ × $\dfrac{FM7c}{Y}$

single fly PCR   selection for non-*Bar* males

G_4: $\dfrac{X\_HDR}{FM7c}$ × $\dfrac{X\_HDR}{Y}$

selection against *Bar*

G_5: $\dfrac{X\_HDR}{X\_HDR}$ or $\dfrac{X\_HDR}{Y}$