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LABORATORY OF MOLECULAR ENTOMOLOGY

Functional analysis of insecticide resistance conferred by detoxification enzymes or target insensitivity mutations

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Acknowledgements

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Abstract

Agricultural pests consist a real threat because they attack crops and cause extensive damage. Crop protection is mainly based on insecticides; however, their extensive use has led to development of high levels of resistance. In the present study, resistance-associated genes are evaluated *in vivo*, on their ability to confer resistance, using *Drosophila melanogaster* as a model. The thesis consists of two parts:

The first part is referred to metabolic resistance, conferred by increased expression levels of P450s. More specifically, the study focuses on the role of two P450 enzymes, the CYP6BQ23 from the pollen beetle, *Meligethes aeneus* and the CYP6A51 from the Mediterranean fruit fly, *Ceratitis capitata*. These genes are overexpressed in resistant populations and have been associated with pyrethroid resistance. In order to validate the contribution of each gene in the resistant phenotype, we attempted to ectopically express both P450s via the GAL4/UAS system in the model organism *Drosophila melanogaster*. The integration of the UAS transgene was achieved through phiC31 site-specific integrase in the genome of attP background susceptible flies and overexpression was accomplished by crossing the UAS line with a relevant GAL4 driver line. The susceptibility of transgenic flies to pyrethroids was evaluated with contact bioassays. In the case of CYP6BQ23 enzyme, we succeed to generate UAS.CYP6BQ23 transgenic *Drosophila* lines and indicate that overexpression of CYP6BQ23 in HR-GAL4 x UAS.CYP6BQ23 flies confers significant levels (~ 7-fold) of pyrethroid resistance. Moreover, in the case of CYP6A51, we performed biochemical and functional analyses of bacterially expressed CYP6A51. We achieved isolation of functional CYP6A51 enzyme by heterologous expression in *E. coli* and showed that it is capable to metabolize lambda-cyhalothrin *in vitro*, in the presence of a NADPH regenerating system. Additionally, we generated UAS.CYP6A51 flies to be used in future experiments of ectopic expression of CYP6A51 in *Drosophila* and contact bioassays with lambda-cyhalothrin, in order to investigate its contribution to resistance.

The second part is focused on target-site resistance and attempts to study the role of a G4946E mutation in ryanodine receptor (RyR), which has been related to diamide resistance in the diamondback moth, *Plutella xylostella*. *D. melanogaster* was used as a model system in order to introduce the G4946E mutation in the ryanodine receptor, using the CRISPR/Cas9 technology, a recently developed tool for targeted genome editing. The evaluation of susceptibility to diamides and the possible effect of this point mutation to resistant phenotype is relied on feeding bioassays. However, although the introduction of the G4946E mutation in *D. melanogaster* was achieved, the generation of homozygous

flies, which was a prerequisite for conducting bioassays considering the recessive character and monogenic inheritance of the relevant mutation, was not feasible.

In conclusion, this work as part of a broader research framework, provides a better understanding of insecticide resistance mechanisms and can be used to inform appropriate strategies to manage resistance.

1. Introduction

Agricultural crops are essential for food production and play a critical role in many countries' economy. However, agricultural pests that attack crops, consist a real threat that endangers economies, food security and human health. Thus, crop protection is a critical practice to prevent or manage plant diseases, weeds and pests that cause severe damage (FAO, 2010). The protection of agricultural crops is mainly based on the application of chemical insecticides in order to prevent or minimize insect damage.

1.1 Insecticides

Insecticides act by disrupting vital biological processes of insects, though not all of them are efficient against all insects. The IRAC committee, which provides guidelines and strategies to proactively maintain insect susceptibility and delay the evolution of resistance, has classified insecticides into groups depending on their mode of action (www.irac-online.org/modes-of-action/).

In this study, we will deal with two insecticide classes, namely pyrethroids and diamides.

1.1.1 Pyrethroids

Pyrethroids are synthetic organic compounds which are commonly used as insecticides (Robert *et al.*, 2002) (Figure 1) and are classified by IRAC into category 3. They are similar to pyrethrins, which are natural compounds found in extracts made from chrysanthemum plants. Pyrethroid insecticides usually contain a mix of pyrethrins, pyrethroids and synergists that are combined in a single formula in order to increase their efficacy. A common synergist used with pyrethroids is the piperonyl butoxide, PBO, which inhibits cytochrome P450 enzymes from metabolizing pyrethroids, resulting in a more efficient insecticide.

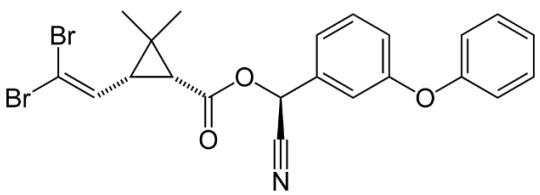


Figure 1. Deltamethrin, a common pyrethroid insecticide.

Pyrethroids affect the nervous system of insects by preventing the closure of the voltage-gated sodium channels in nerve cells. Pyrethroids keep the channels in their open state resulting in a repeated propagation of action potential, which finally leads the insect to paralysis and death (Soderlund *et al*., 2002).

1.1.2 Diamides

Diamides are a recently invented chemical class of insecticides with a distinct mode of action that are classified by IRAC into category 28. Chemically, diamides are either Phthalic, or Anthranilic organic compounds (Tohnishi et al., 2005; Lahm *et al*., 2007). They are available in commercial formulations and act effectively against a wide spectrum of pests and especially against Lepidoptera, with low impact on the environment (Hirooka *et al*., 2007; Lahm *et al.*, 2007). Currently, there are three different diamide compounds; Flubendiamide, which was discovered first, Chlorantraniliprole and its analogue Cyantraniliprole, as seen in Figure 2.

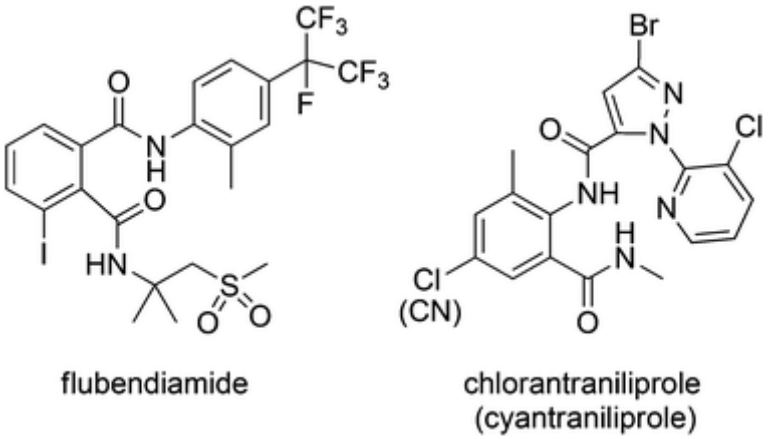


Figure 2. Chemical structures of Flubendiamide, Chlorantraniliprole and its analogue Cyantraniliprole, which are the three commercially available diamide compounds.

Diamides are considered ryanodine receptor modulators and their mode of action affects calcium homeostasis. They bind to the ryanodine receptors (see below) and cause the calcium channel to remain open, leading to an uncontrolled release of calcium stores (Ebbinghaus-Kintscher *et al.*, 2006). Since calcium is involved in a variety of important cell processes, this loss of ability to regulate calcium leads to lethargy, feeding cessation and eventually death (Cordova *et al.*, 2006; Jeanguenat *et al*., 2012).

The ryanodine receptor (RyR, Figure 3), the molecular target of diamides, is a calcium release channel which is located in the sarcoplasmic and endoplasmic reticulum membrane of excitable tissues, like muscle and nervous tissue (Otsu *et al*., 1990). The receptor was named after the plant alkaloid ryanodine, which has an extremely high affinity to RyR and was originally used as an insecticide. RyR modulates the release of calcium from intracellular stores, thereby mediating many cellular and

physiological activities such as neurotransmission and muscle contraction (Sattelle *et al.*, 2008). When ryanodine is present at nanomolar concentrations, it locks the ryanodine receptor in a half-open state, resulting in the release of calcium stores, followed by a massive muscle contraction, while at micromolar concentrations, ryanodine fully closes the receptor, leading to paralysis (Meissner *et al*., 1986; Lai *et al*., 1989; McGrew *et al*., 1989).

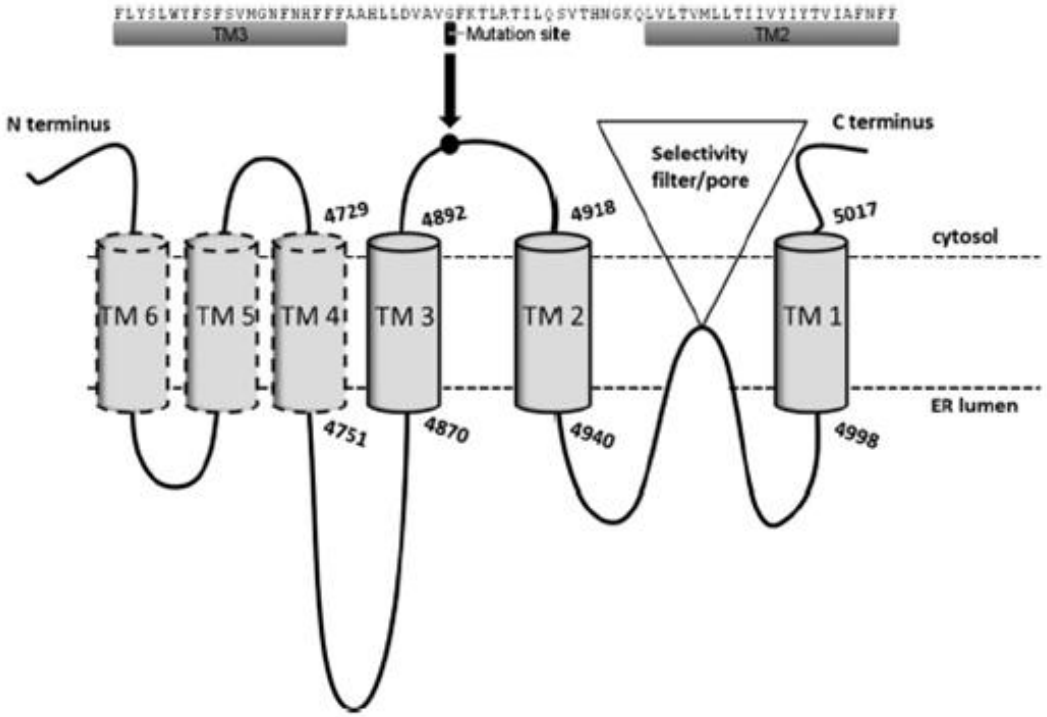


Figure 3. The insect ryanodine receptor (RyR). The sequence on top is derived from *Plutella xylostella* RyR; the arrow indicates the position of the target G4946E mutation (Troczka *et al*., 2012)

Insects contain a single RyR gene, while mammals have three different isoforms of RyR (Rossi and Sorrentino, 2002) with low amino acid identity to insect RyR (Takeshima *et al*., 1994; Vazquez-Martinez *et al*., 2003). The insect RyR is a large tetrameric channel found in sarcoplasmic and endoplasmic reticulum membrane in muscle and nervous tissue (Sattelle *et al*., 2008). It consists of a transmembrane domain at the C-terminal of the protein, where it contains the ion-conducting pore and a large N-terminal cytoplasmic domain (Xu *et al*., 2000; Lanner *et al*., 2010).

1.2 Insecticide resistance

The extensive use of insecticides facilitates the development of resistance, which is a major problem and makes insecticides ineffective. Insecticide resistance is "the natural ability of a population of an organism to survive exposure to an insecticide dose that would normally be lethal for an individual of that species" (Denholm *et al*., 2001).

The application of insecticides creates a selection pressure, which can artificially select for resistant pests. As shown in Figure 4, the first generation F_0 of pest population happens to have a number of insects with a heightened resistance to a given pesticide (insects in red colour). After pesticide application, sensitive pests (insects in blue) have been selectively killed. However, the resistant insects survive, multiply and spread. Thus, the descendants of the resistant pests now represent a larger proportion of the population. After repeated applications, the majority of the population consists of resistant pests.

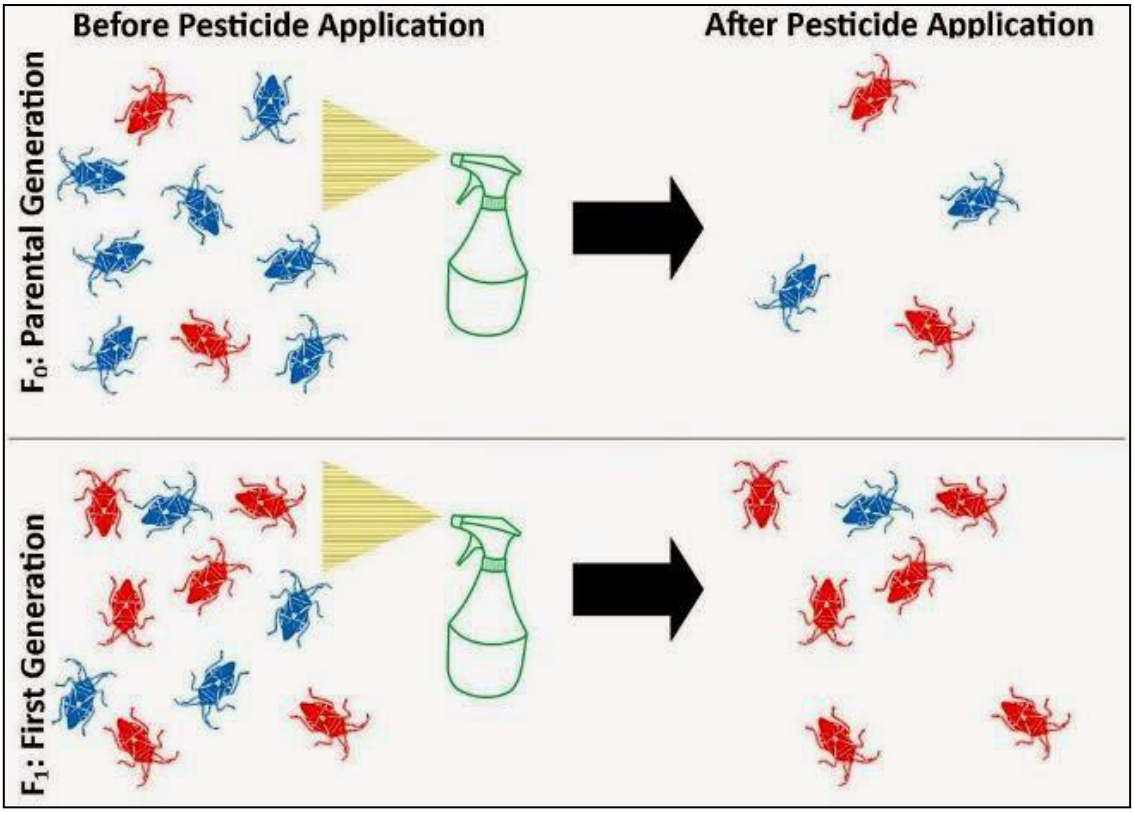


Figure 4. Evolution of insecticide resistance. A change in genetic characteristics of the insect population is inherited from one generation to the next, under insecticide selection pressure. Red: Resistant insects. Blue: Susceptible insects (Image from http://rsandss.blogspot.gr/2013/09/pesticide-resistance.html).

Consequently, when the frequency of resistant pests increases to a certain level in field populations, the control of population demands higher doses of the insecticide, which not only becomes economically unsustainable, but also endangers environment and human health.

In order to address the problem of insecticide resistance, it is essential to study the molecular mechanisms that cause the resistant phenotypes, as well as to elucidate the reasons that make an insecticide ineffective.

1.3 Mechanisms of insecticide resistance in insects

Resistance mechanisms can be divided into four categories: behavioral resistance, penetration resistance, metabolic resistance and target-site resistance.

- The behavioral resistance, refers to development of behaviors to sense and avoid exposure to toxic compounds.
- The penetration resistance, refers to structural changes in insect cuticle that slows the absorption of a chemical compound, thus, reducing the dose that reaches its target site.
- The metabolic resistance, refers to the ability of an insect to metabolize insecticides to less toxic compounds that eventually are excreted, thus, reducing the dose that reaches its target site.
- The target-site resistance, refers to structural changes in the target site of insecticides that lower the binding affinity of the insecticide and reducing it toxic action.

In this study, we deal with the latter two types of resistance, and more specifically with metabolic resistance to pyrethroids through overexpression of P450s, and target-site resistance to diamides.

1.3.1 Metabolic resistance to pyrethroids

Resistance to pyrethroids can be conferred by two main mechanisms, either by metabolic resistance, or by target-site resistance (Feyereisen, 1995; Li *et al*., 2007; Heckel, 2012). Target-site resistance is caused by mutations in the voltage-gated sodium channel, known as kdr (knock-down) mutations and is considered the common mechanism that confers pyrethroid resistance (Nauen *et al*., 2012). On the other hand, metabolic resistance is caused by overexpression of detoxification enzymes, such as cytochrome P450 monoxygenases.

Cytochrome P450s enzymes (CYPs) are heme-containing monooxygenases that catalyze metabolism of various endogenous and exogenous compounds. CYPs form an enzyme superfamily and are

characterized as hemoproteins, because of the heme group that exists in their molecule. CYPs are found in nearly all organisms from bacteria, to plants and humans (Werck-Reichhart and Feyereisen, 2000). They can metabolize a wide range of substances and especially in insects, P450s are known for their role in metabolism and detoxification of xenobiotic compounds (Feyereisen, 2012). P450 enzymes took their name due to their absorption peak of the cytochrome-carbon monoxide complex (Fell-CO) at 450 nm (Omura and Sato, 1964).

P450 monooxygenases catalyze many reactions, but the most common is the monooxygenase reaction, in which P450 enzymes, using electrons from NADPH, transfer an atom of molecular oxygen into a hydrophobic organic substrate, while the other oxygen atom is reduced to water:

$$\text{Substrate} + \text{NADPH} + H^+ + O_2 \rightarrow \text{Substrate (O)} + NADP^+ + H_2O$$

The required electrons are transferred from NADPH into the "P450-Substrate" complex, either via cytochrome P450 reductase (CPR), or via cytochrome b5 reductase (Berge *et al.,* 1998).

This monoxygenase activity of CYPs is related to insecticide metabolism in insects, resulting in their detoxification (Scott *et al*., 1999). In many cases, the detoxification of insecticides takes place to that extend that the insecticide is degraded and cannot find its molecular target, resulting in resistant insects (Berge *et al*.,1998). This type of insecticide resistance is mainly caused by changes in the expression levels of CYPs, and only rarely by mutations in CYPs amino acid sequence (Van Leeuwen *et al*., 2005, Scott *et al*., 1999).

Cytochromes P450 enzymes have been reported to confer resistance to many insecticides including pyrethroids (Sogorb and Vilanova, 2002; Feyereisen, 2012). The detection of high expression levels of several P450 genes in resistant strains, the *in vitro* enzymatic activity in pyrethroid metabolism and the ability of the synergist PBO to suppress resistant phenotype consist evidence for the key role of P450s in pyrethroid resistance (Scott and Georghiou, 1986; Hemingway *et al.,* 1993). The resistant phenotype can also result from the overexpression of more than one P450 genes at the same time (Liu *et al.*, 2011; Feyereisen, 2012). Moreover, ectopic expression of P450 genes in heterologous systems was enough to metabolize the pyrethroid and reduce its toxicity (Muller *et al*., 2008; Duangkaew *et al.*, 2011; Pavlidi *et al*., 2012). The high expression levels of P450 genes in resistant strains can be either constitutive or induced (Festucci-Buselli *et al.,* 2005; Zhu *et al*., 2008; Liu *et al.,* 2011).

1.3.2 Target-site resistance to diamides

Diamides are classified by IRAC into category 28 and are considered ryanodine receptor modulators. They act by binding to ryanodine receptor, keeping it in its open state and causing an extended and disorganized muscle contraction which leads to death (Ebbinghaus-Kintscher *et al.*, 2006). Diamides are mainly used against lepidopteran pests. However, their repeated and extensive use led to development of resistance for both diamides, as well as, cross-resistance between flubendiamide and chlorantraniliprole, for example in the diamondback moth, *Plutella xylostella* (Wang and Wu, 2012).

Although, the knowledge of diamide resistance mechanisms has not been yet fully elucidated, it is known that there detoxification enzymes that contribute to resistant phenotype through a metabolic detoxification, for example P450 enzymes (Liu *et al*., 2015), as well as target-site mutations on the RyR gene that are involved in resistance (Troczka *et al*., 2012, Guo *et al*. 2014a,b). A non-synonymous point mutation, resulting in a glycine to glutamic acid substitution (G4946E) in RyR (Figure X), that has been found in a resistant strain (Troczka *et al*., 2012), is a major candidate mutation whose contribution to the emergence of target-site resistance requires validation.

1.4 Agricultural Insect Pests and their management

1.4.1 The pollen beetle, *Meligethes aeneus*

The pollen beetle or *Meligethes aeneus Fab*. is a major pest of oilseed rape (*Brassica napus*) through Europe (Alford *et al*., 2003). Crops of oilseed rape and especially the winter oilseed rape crops are of great economic importance for many European countries, like France, Germany, Poland and the UK (Richardson, 2008). Pollen beetles migrate to oilseed rape crops between March and April. When the oilseed crops are in the flowering stage, the beetles migrate to the open flowers, feed on pollen and become pollinators. However, this flowering stage, when the buds have a green-yellow color, is a damage-susceptible stage. Pollen beetles migrate to crops and cause damage because they bite the buds to lay their eggs. The hatching larvae feed on the sexual parts of flowers, causing the flower to abort (Figure 5).

Figure 5. Left: Adult Pollen Beetle, *Meligethes aeneus.* (Source: www.davidkennardphotography.com), Right: Hatching larvae of pollen beetle, feeding on oilseed rape buds (Source: www.bayercropscience.co.uk).

Every year, the control of pollen beetles population is based on the use of chemical insecticides and mainly on pyrethroids, like deltamethrin, cypermethrin, lambda-cyhalothrin, alpha-cypermethrin, bifenthrin, tau-fluvalinate and zeta-cypermethrin (Heimbach *et al*., 2013). Pyrethroids are considered to be fast-acting insecticides due to their neurotoxic activity and thus effective even in low concentrations (Khambay and Jewess, 2005). However, the extensive use of insecticides against pollen beetle has led to development of resistance to pyrethroids (Hansen, 2003; Thieme *et al*., 2010; Slater et al., 2011). Pyrethroid resistance can be conferred by two main mechanisms, either by target site mutations in the voltage-gated sodium channel, known as kdr mutations, resulting in lower binding affinity of the insecticide, or by increased expression levels of detoxification enzymes resulting in metabolic resistance (Feyereisen, 1995; Li *et al*., 2007; Heckel, 2012).

However, resistant populations of pollen beetle from many European countries, except from Denmark and Sweden, lack kdr mutations. This suggests that the main mechanism is the enhanced detoxification of pyrethroids due to detoxification enzymes (Zimmer and Nauen, 2011; Nauen *et al*., 2012). A recent study showed that the effect of pyrethroid can be synergized by PBO, a P450 inhibitor, but not by DEF or DEM, an esterase and a glutathione inhibitor, respectively (Zimmer and Nauen, 2011; Philipou *et al*., 2011). In addition, *in vitro* assays with microsomal preparations from resistant populations showed a significant deltamethrin degradation in the presence of NADPH, which was inhibited by PBO, tebuconazole and 1-aminobenzotriazole, known inhibitors of P450s (Zimmer and Nauen, 2011). Furthermore, analysis with LC-MS/MS of 4-hydroxy deltamethrin formation in microsomes from resistant and susceptible populations was correlated with pyrethroid resistance (Zimmer and Nauen, 2011). Finally, a quantitative PCR approach was used in order to examine the expression levels of

P450 genes in different resistance pollen beetle populations and this study revealed a P450 gene, the CYP6BQ23, which is highly overexpressed (up to ~900-fold) in resistant pollen beetle adults and larvae (Zimmer *et al.*, 2014). This study was combined with expression of CYP6BQ23 together with CPR in Sf9 insect cell line which showed efficient metabolism of deltamethrin to 4-hydroxy deltamethrin (Zimmer *et al.*, 2014). These results suggest that the high expression levels of CYP6BQ23 are responsible for the resistant phenotype in pyrethroids in European pollen beetle populations.

1.4.2 The Mediterranean fruit fly, *Ceratitis capitata*

The Mediterranean fruit fly (medfly), *Ceratitis capitata*, is a destructive pest and causes extensive damage to fruit crops. *C. capitata* can rapidly colonize a wide range of fruit and vegetable crops (Liquido *et al*., 1991) and due to its wide distribution around the world, it can tolerate lower temperatures. Thus, due to crop damage and economical cost for its management, *C. capitata* is considered one of the most economically important pests in the world.

C. capitata originated in sub-Saharan Africa, but since then it has spread throughout Africa, the Mediterranean region, southern European countries, the Middle East, South and Central America and the Hawaiian Islands (Malacrida *et al*., 1992; Szyniszewska *et al*., 2014)

C. capitata is a major pest of citrus, but it can feed on most deciduous and tropical fruits and some vegetables (Figure 6). Adult medflies lay their eggs below the skin of the host fruit (Knapp, 1981). The larva develops inside the fruit by feeding on its pulp, until it reaches the next developmental stage of pupa, when it falls to the ground leaving the fruit mashed, decomposed by invasion of microorganisms and inedible.

The control of *C. capitata* is relied on treatments based on the use of insecticides. However, the European legislation recommends the sustainable use of insecticides in combination with other control methods, more environmentally acceptable. These methods include cultural practices, trapping methods, the sterile insect technique and chemosterilant. However, the monitoring and the identification of resistance status of the field populations is necessary for developing appropriate control strategies.

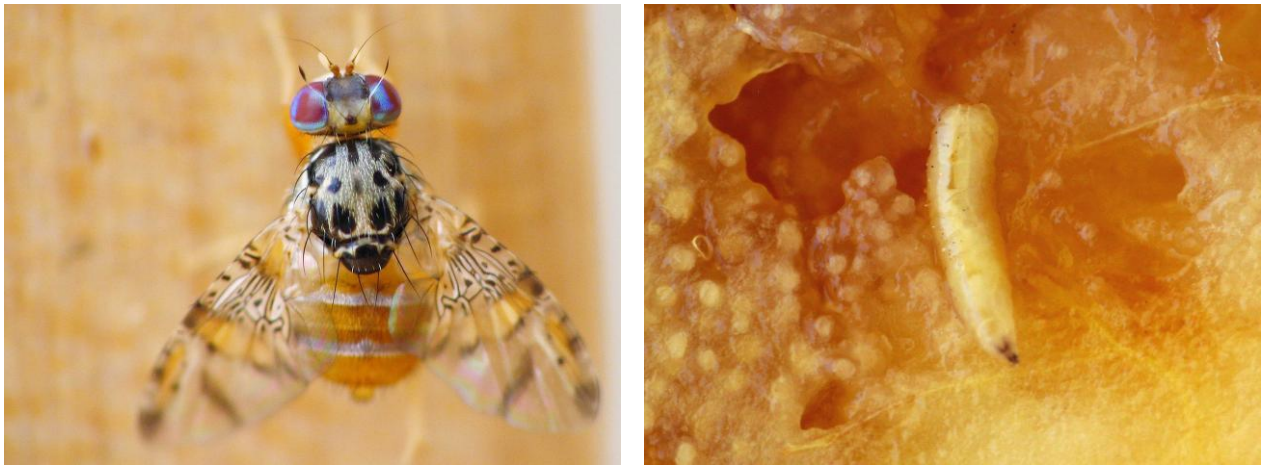


Figure 6. Left: Adult Mediterranean fruit fly, *C. capitata*. Right: Larvae of *C. capitata*, feeding on fruit. (Source: http://www.globalspecies.org/ntaxa/514529)

In the past decades, the main control method for *C. capitata* was the application of insecticides. The most common used insecticide was malathion aerial and ground treatments. However, malathion was withdrawn in the European Union in 2009, and the insecticides that are currently used, include spinosad, lambda-cyhalothrin, etofenprox, methyl-clorpyrifos and lufenuron. Among these insecticides, malathion and methyl-clorpyrifos are organophosphate compounds, lambda-cyhalothrin and etofenprox are pyrethroids and spinosad is a mixture of two natural compounds with insecticidal properties. Except for lufenuron, the other insecticides target the nervous system of the insect and eventually lead the insect to the death by paralysis.

However, the extensive use of insecticides led to the development of resistance to most commonly used compounds, resulting in a major problem of economic importance (Vontas *et al*., 2011). Resistance to insecticides was first reported in Spanish field populations of *C. capitata* after intensive use of malathion in citrus crops (Magana *et al*., 2007). After the withdrawal of malathion, the most used insecticides were lambda-cyhalothrin and spinosad. However, resistance to lambda-cyhalothrin has also been reported in Spanish field population of *C. capitata* (Arouri *et al*., 2014).

The resistance to lambda-cyhalothrin in Spanish populations appears to be mediated by P450 enzymes (Arouri *et al*., 2014). Bioassays with the P450 inhibitor PBO showed suppression of the resistant phenotype, indicating the key role of P450s. In addition, expression studies revealed a P450 gene, CYP6A51, which is overexpressed (13-18 fold) in the resistant strain (Arouri *et al*., 2014). Furthermore, cross-resistance to etofenprox and deltamethrin, also pyrethroids, was demonstrated in a laboratory selected resistant population (Arouri *et al*., 2014).

1.4.3 The diamondback moth, *Plutella xylostella*

The diamondback moth, *Plutella xylostella* is an important lepidopteran pest of cruciferous crops around the world and is considered one of the most difficult pests to control (Figure 7). *Plutella xylostella* attacks in cultivated and wild plants of the family Cruciferae. *P. xylostella* is a cosmopolitan species, which is found over North and South America, southern Africa, Europe, India, Southeast Asia, New Zealand, and in some regions in Australia.

Figure 7. Left: Adult *Plutella xylostella*. Right: Larvae of *Plutella xylostella*. (Source: http://www.ukmoths.org.uk/species/plutella-xylostella)

P. xylostella causes damage to plants due to larval feeding on leaves of cultivated cruciferous plants. The adult lays its eggs on the leaf, and although, larvae have a very small size, when they are numerous, they can completely consume all the plant's foliage, except the leaf veins. This damage disrupts the head formation in cabbage, broccoli, and cauliflower resulting in a severe injury.

The control of *P. xylostella* populations is based on the application of insecticides. The economic losses for its control globally is estimated to be more than a billion US Dollars (Talekar and Shelton, 1993). However, insecticide resistance has already been reported worldwide.

Diamides are a new chemical class of insecticides that is globally used for the control of *P. xylostella* due to its high efficacy. However, in 2011 the first diamides inefficacy was reported in China and further studies confirmed high levels of resistance in populations of *P. xylostella* (Wang and Wu, 2012). Generally, it is known that *P. xylostella* can rapidly develop resistance to insecticides that are intensively used for crop protection. (Talekar and Shelton, 1993; Zhao *et al*., 2006; Sparks *et al*., 2012). Different mechanisms that confer resistance to various insecticides including high expression levels of

detoxification enzymes, target-site mutations and reduced penetration have been reported for *P. xylostella* (Noppun *et al*., 1989; Baxter *et al*., 2010; Sonoda, 2010), but the possible mechanism that confers diamide resistance has not been yet known (Lai *et al*., 2011; Sial *et al*., 2011).

A more recent study revealed a point mutation in the proposed region for diamide binding site of the RyR gene in resistant population of *P. xylostella* (Troczka *et al*., 2012). These *P. xylostella* populations were collected from the Philippines and Thailand and the resistance level, to both chlorantraniliprole and flubendiamide, was over 200-fold, compared to susceptible strains,. A non-synonymus point mutation, resulting in a glycine to glutamic acid substitution (G4946E) in RyR, was found in the resistant strain (Troczka *et al*., 2012). There are facts that provide evidence for this assumption: the mutation is found in a critical region for diamide binding, this region is highly conserved among insects and the substitution of glycine with a glutamic acid, a large and negatively charged amino acid, is likely to cause critical structural changes in RyR of *P. xylostella*, resulting in a binding inefficacy of diamides (Troczka *et al*., 2012).

1.5 Methods to study insecticide resistance

The development of insecticide resistance and its management is usually a major problem with economic and environmental aspects that have to be considered. The knowledge of resistance mechanisms and the elucidation of insecticides mode of action are essentials for the design of management strategies to prevent the evolution of resistance in field populations and extend the useful life and efficacy of insecticides.

There are a lot of methods and tools that are used for the investigation of insecticide resistance mechanisms, including *in vitro* screening systems (for example, see Troczka *et al*., 2015), electrophysiology (Dong *et al*., 2015), direct ligand-insecticide either *in vivo* (Steinbach *et al*. 2015) or *in silico* (O'Reilly *et al*., 2016), functional expression of enzymes (Nauen et al., 2015; Riga et al., 2015) or genetic linkage analysis (Heckel, 2003; Van Leeuwen *et al*., 2012). However, there are inherent limitations in each method that call for innovative use of new technologies in order to elucidate contribution of specific genes and validate candidate mutations in an efficient manner.

In the present study, methods for the ectopic heterologous expression and targeted genome editing of resistance related genes, using *Drosophila melanogaster* as model organism are explored.

1.5.1 Drosophila melanogaster as a model organism

Drosophila melanogaster or the common fruit fly is a classical model for biological research studies. It is used because of its small size that makes it an inexpensive and easy to handle species. It has also a short lifecycle and its physiology and genetics is well studied through years. *Drosophila* can be used for the ectopic expression of resistance associated genes, in order to study the contribution of these genes in the phenotype of resistance.

1.5.2 The GAL4-UAS system in *Drosophila melanogaster*

The GAL4-UAS system is a well-established powerful tool to study gene expression in organisms like *Drosophila melanogaster*. This expression system combines two components; the GAL4 transcriptional activator and a transgene of interest, under the control of a UAS artificial promoter (Brand *et al*., 1993).

The *Gal4* gene encodes a yeast protein which acts as a regulator of genes induced by galactose. GAL4 directly binds to sites located upstream of these loci and are defined as Upstream Activating Sequences (UAS). The GAL4/UAS system for targeted gene expression in *Drosophila* was initially developed by Fischer et al., in 1988, who demonstrated, that GAL4 expression in *Drosophila* could stimulate the transcription of a reporter gene under the UAS control (Fischer *et al*., 1988).

In more detail, this technique consists of the responder and the driver that are maintained as separate lines:

- The responder line consists of flies that carry the gene of interest under the control of a UAS site, but the gene is not expressed.
- The driver line, consists of flies that express the GAL4 protein, in a specific tissue and there are many available drivers, which express the GAL4 in specific tissues (Brand *et al*., 1993).

After the development of the responder line, by injecting *Drosophila* embryos with a plasmid that carries the gene of interest, the flies are crossed with the driver line (Figure 8). In their offsprings, gal4 will be expressed, resulting in the expression of the gene of interest.

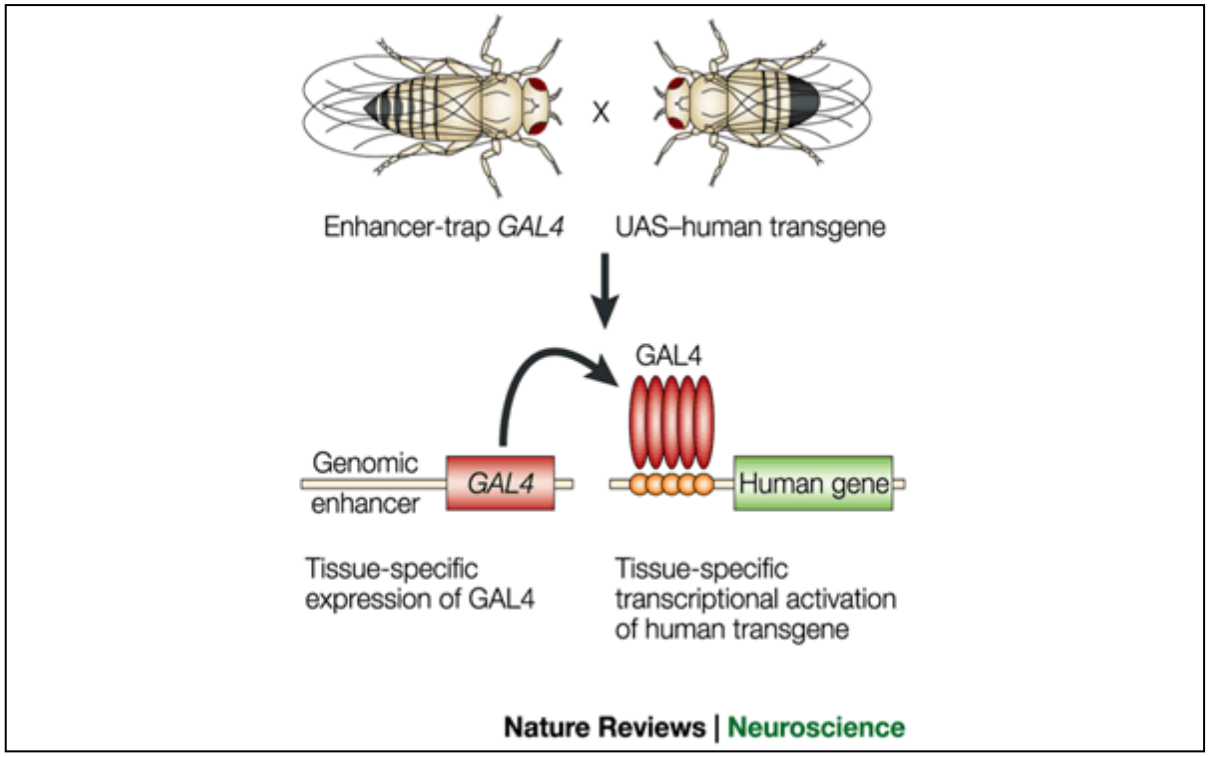


Figure 8. The GAL4/UAS system in *D. melanogaster*. This system allows the ectopic expression of a transgene in a specific tissue. The expression of the transgene will be activated in the progeny of driver and responder cross (Source: Nature Reviews Neuroscience 3, 237-243 (March 2002) doi:10.1038/nrn751).

1.5.3 Site-specific integration into *Drosophila*'s genome with phiC31 integrase

The phiC31 integrase is a site-specific recombinase which is used for the integration of the gene of interest into *Drosophila*'s genome (Thorpe *et al*., 1998).

The phiC31 integrase system is presented in Figure 9. The phiC31 integrase is a sequence-specific recombinase isolated from the bacteriophage phiC31 and normally mediates recombination between two sequences termed attachment sites (att). The one attachment site is located in phage's genome (attP), while the other in the bacterial host (attB). As seen in Figure 9, in the presence of phiC31 integrase, an attB-containing donor plasmid can be integrated into a target genome with attP sites (Thorpe *et al*., 2000). The phiC31 integrase can integrate a plasmid of any size, as a single copy, and requires no cofactors. The integrated transgenes are stably expressed and heritable (Groth *et al*., 2000; Thyagarajan *et al*., 2001; Groth *et al*., 2004).

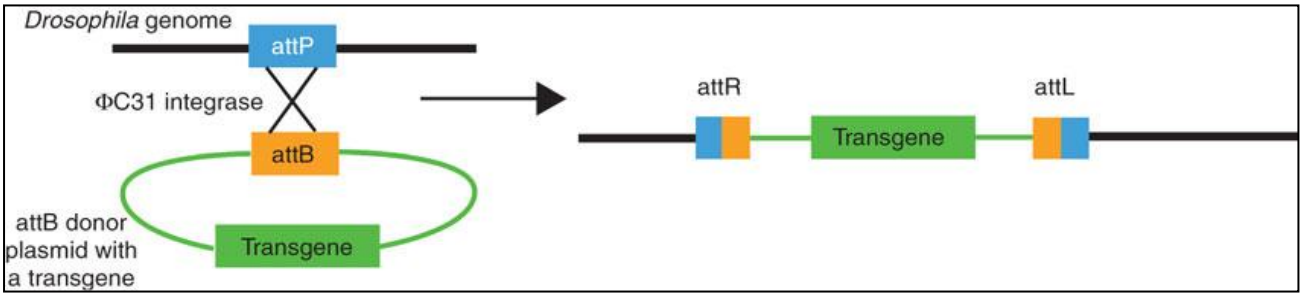


Figure 9. Site-specific integration mediated by phiC31 integrase into *Drosophila*'s genome. The phiC31 integrase can mediate the recombination between an attB-containing donor plasmid and an attP site in *Drosophila*'s genome, resulting in the integration of the transgene into the attP site. (Source: Nature Protocols 2, 2325 - 2331 (2007) doi:10.1038/nprot.2007.328)

The big advantage of this system is that the insertion of the gene of interest does not occur randomly, but in a specific site in the genome. With this approach the position effects are avoided and the resulting lines are comparable because the positioning of transgenes is controlled (Groth *et al.*, 2004; Bateman *et al.*, 2006)

1.5.4 The CRISPR-Cas9 technology

The CRISPR/Cas9 system is, currently, the most popular tool for targeted genome editing. The CRISPR system or Clustered Regularly Interspaced Short Palindromic Repeats is based on a prokaryotic immune system which provides an adaptive immunity against foreign genetic material (Horvath & Barrangou, 2010; Marraffini & Sontheimer, 2010). However, this system that has been modified and now is used as a tool for genome engineering, in order to effectively generate precise changes in the genome. The CRISPR/Cas9 system generates targeted double-stranded breaks (DSBs) in DNA, resulting in the activation of either non-homologous end joining or homology directed repair mechanism (Jinek *et al.*, 2013).

The system version triggering homology directed repair consists of three components, as seen in Figure 10:

- The "guide" RNA (gRNA), which is synthesized in order to contain a sequence, necessary for Cas9-binding and a spacer sequence which defines the genomic target to be modified (Jinek *et al.*, 2013). The DNA target has to be directly upstream of a Protospacer Adjacent Motif (PAM), which is usually an "NGG" tri-nucleotide.

- The CRISPR-associated endonuclease Cas9, which was firstly isolated from *Streptococcus pyogenes* (Jinek *et al*., 2012). Cas9 uses the gRNA in order to find and cut the DNA target. The Cas9 endonuclease is non-specific and its specificity is defined by the sequence of the gRNA.

- The donor DNA, which can be a single stranded oligo or a double stranded plasmid, which is a DNA template containing the desired change, between additional homologous sequences (homology arms).

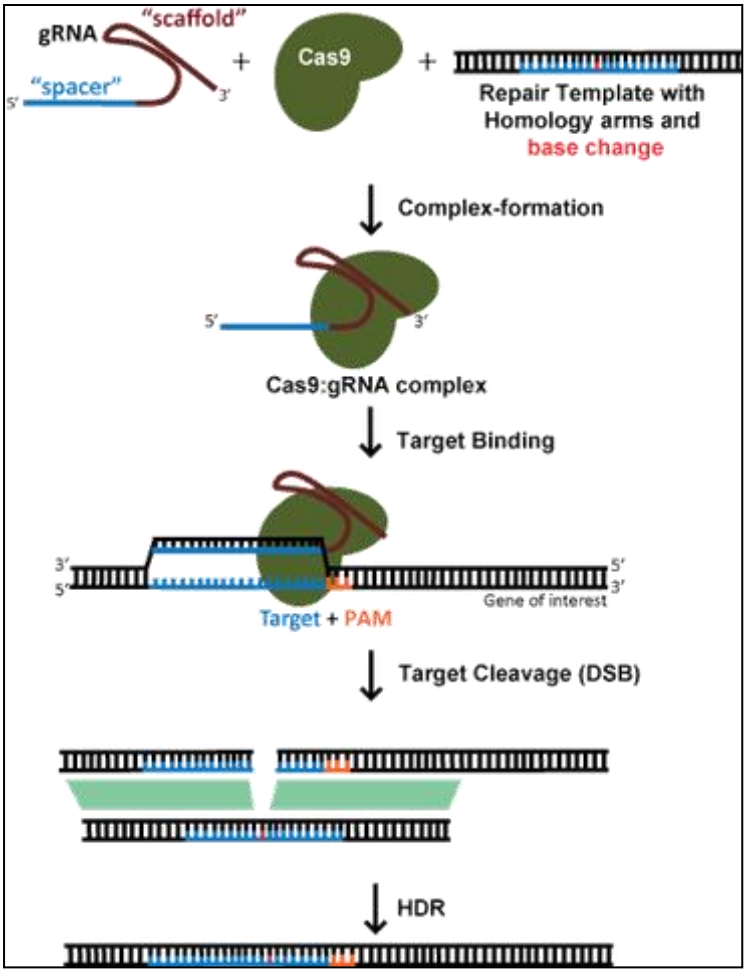


Figure 10. The CRISPR-Cas9 technology for genome editing. The Cas9 endonuclease binds to the gRNA and introduces a DSB on DNA. The target site recognition is based on gRNA sequence, found upstream a PAM sequence. The DSB can be repaired by HDR pathway, resulting in the integration of donor sequence in the genome. (Source: https://www.addgene.org/crispr/guide/)

As described in Figure 10, when the gRNA is expressed, it binds the Cas9 endonuclease resulting in a riboprotein complex. After the formation of this complex, the Cas9 molecule undergoes a conformational change that enables it to bind DNA. The Cas9-gRNA complex is able to bind the DNA sequence that is indicated by the spacer sequence of gRNA and the PAM sequence and to detect if there is sequence homology between the gRNA and the target sequence. Then, if the homology is sufficient, Cas9 cleaves the target DNA on the one strand. A subsequent conformational change makes Cas9 able to cleave the opposite strand of the target DNA, resulting in a double strand break.

The resulting double strand break can be repaired either by the Non-Homologous End Joining (NHEJ) pathway, which is efficient but error-prone, or by the Homology Directed Repair (HDR) pathway, which is less efficient but provides high-fidelity. Generally, the NHEJ repair pathway is activated first and rapidly repairs double strand breaks. However, this mechanism frequently results in small nucleotide insertions or deletions at the repairing site. On the other hand, the HDR pathway has a lower frequency and its efficiency is generally low. However, only the double strand breaks that are repaired through the Homology Directed Repair pathway, having the donor plasmid as a template, will carry the desired edited allele. Finally, it is important to have a diagnostic method in order to confirm the presence of the desired change.

The CRISPR/Cas9 system can be used to target the genome of living cells or living organisms. In order to be functional and effective, it requires all the above mentioned components. The gRNA that indicates the genome target is co-expressed with the Cas9 endonuclease and it has to be a unique sequence, in order to avoid any off-target effects.

1.6 Thesis objectives

Agricultural pests exhibit a high potential to develop resistance to most insecticides used for their control. The elucidation of mechanisms underlying resistance is powerful knowledge for designing the appropriate management strategies and avoiding the evolution of resistance in field populations.

The objective of the present thesis is the study of target-site resistance, derived from point mutations, as well as, metabolic resistance, mediated by increased insecticide detoxification. The work presented is divided in two parts:

1. Evaluation of P450s associated with metabolic resistance: Ectopic expression of a P450 enzyme, CYP6BQ23 of *Meligethes aeneus* in the model organism *Drosophila melanogaster* and evaluation of susceptibility of transgenic flies to pyrethroids. Another P450 enzyme, CYP6A51 of *Ceratitis capitata*, is studied, biochemical and functional characterization is performed, and transgenic lines for ectopic expression are generated.

2. Validation of a mutation potentially associated with target-site resistance: Targeted genome editing with CRISPR/Cas9 technology, in order to introduce the point mutation G4946E in the Ryanodine Receptor gene of *Drosophila melanogaster* and evaluation of susceptibility to diamides.

2. Materials and methods

Cyp6bq23 gene from *Meligethes aeneus* and *Cyp6a51* gene from *Ceratitis capitata* were cloned into a modified *Drosophila* cloning vector pH_Stinger.attB (Appendix, Figure 1) under a hsp70 basal promoter and the Upstream Activation Sequence, UAS (Bischof *et al*, 2006). Transgenic flies were generated using the *Drosophila* strain *yw;nos-int;attP40*, which expresses phiC31 integrase during oogenesis under control of *nanos* regulatory sequences and contains the attP40 landing site in the second chromosome. dPelican-attP vector contains the reporter *mini-white* gene for the selection of the transgenic lines and also the attachment site (attB). PhiC31 integrase catalyzes the recombination between the attP40 and attB site, mediating the integration of the gene of interest into *Drosophila* genome. The induction of the CYP6BQ23 was carried out by crossing this line with a constitutive HR-GAL4 driver line (Chung *et al*., 2007). Finally, the progeny that overexpress the above genes, was tested for deltamethrin susceptibility levels, with contact bioassays and the LD_{50} values were calculated. The *yw;nos-int;attP40* strain, as well as, the dPelican-attP vector, originally modified by Pawel Piwko (unpublished), were provided by the lab of Professor Christos Delidakis, IMBB/FORTH. The HR-GAL4 driver line was part of the host lab's stock collection (Pavlidi et al., 2012) and was originally developed by Chung *et al*., 2007. The embryo injections were performed by Mr. Ioannis Livadaras, IMBB/FORTH.

D. melanogaster stocks were maintained at 25 °C in 70% humidity, under a 12-hour light-dark cycle, into vials with standard cornmeal-agar-yeast medium. For this study, the following *Drosophila* lines were used:

- the *yw* strain (Vontas lab stock collection IMBB/FORTH)
- the *yw; nos-int; attP40* strain which contains an attP40 site on the second chromosome, marked with $yellow^+$ and the phiC31 integrase gene on X chromosome under the *nanos* promoter. This strain was kindly provided by Pawel Piwko, Delidakis Lab, IMBB/FORTH.
- the w^{1118} strain (Stock collection IMBB/FORTH)
- the HR-GAL4 strain (Chung *et al*., 2007) which expresses GAL4 in the midgut, the malpighian tubules and the fat body in adults and larvae (Vontas Lab stock collection IMBB/FORTH), and
- the *yw; CyO/ Sco*, which carries the Curly marker gene on the second balancer chromosome *CyO* and the scutoid marker. (Stock collection IMBB/FORTH).

2.1 Ectopic expression of *Meligethes aeneus Cyp6bq23* gene in *Drosophila melanogaster* and investigation of pyrethroid susceptibility in transgenic lines

2.1.1 Bioinformatics Analysis

The gene sequence of Cyp6bq23 from *Meligethes aeneus*, version KC840055.1, was acquired by gene database of NCBI. The protein sequence had many ambiguities that were evaluated. The final codons were estimated by taking into consideration the codon usage bias in *Drosophila melanogaster* and additional information on protein sequence polymophisms, provided by Ralf Nauen, Bayer CropScience AG, R&D, Germany (personal communication).

2.1.2 Construct design, codon optimization and gene synthesis for *Meligethes aeneus* P450 monooxygenase CYP6BQ23

The DNA coding sequence of the cytochrome P450 monooxygenase 6BQ23 from *Meligethes aeneus*, GenBank version KC840055.1, was synthesized by GenScript (USA) with the following sequential modifications: DNA gene sequence was optimized for the preferred codon usage in *Drosophila melanogaster*, a Kozac consensus sequence CACC was added right before the first ATG (starting methionine) codon, the cleavage site, 5'-ACGCGT-3', for the restriction endonuclease *MluI*, an hsp70 basal *Drosophila* promoter, the UAS sequence for Gal4 binding and cleavage sites for the restriction endonuclease *NotI*, flanking the ends of these sequences, as shown in Figure 11 (see also Figure 2, Appendix). The restriction site for *MluI* was added, so that the gene sequence could be exchanged easily with another gene sequence in future constructs.

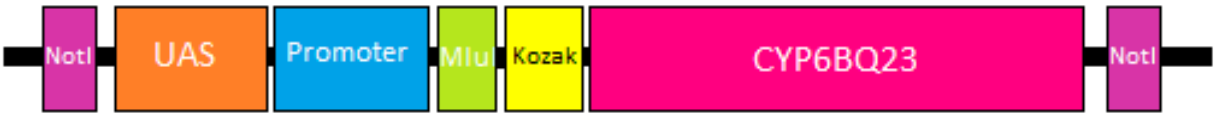


Figure 11. Schematic of the construct for CYP6BQ23 overexpression in *D. melanogaster*.

2.1.3 Restriction digest with *Not*I for the sub-cloning of UAS.CYP6BQ23 insert into the pH_Stinger.attB vector

The UAS.CYP6BQ23 gene was synthesized by GenScript (USA) into a pUC57 vector, flanked by *NotI* restriction sites. The plasmid vector was delivered lyophilized and diluted in 20μl dH_2O, according to the manufacturer's protocol.

The pUC57.UAS.CYP6BQ23 plasmid was digested with *NotI* restriction enzyme (MINOTECH Biotechnology) in order to acquire the UAS.CYP6BQ23 fragment, flanked with *NotI* restriction sites (insert). The digestion (Table 1) was mixed with loading buffer and electrophoresed in 1% w/v agarose gel in 0.5X TBE and purified from gel with NucleoSpin® Gel and PCR Clean-up (MACHEREY NAGEL), according to the manufacturer's protocol.

The pH_Stinger.attB vector was also digested with the same restriction enzyme, *Not*I, in order to remove a GFP expression cassette and acquire a vector consisting of pH_Stinger.attB with an SV40 terminator (poly-A signal), bearing compatible ends for the sub-cloning of the UAS.CYP6BQ23 insert (see also Appendix, Figure 1) The digest reaction was performed, as previously described. The digestion was electrophoresed in 1% w/v agarose gel in TBE and the linear vector was purified from gel with NucleoSpin® Gel and PCR Clean-up (MACHEREY NAGEL), according to the manufacturer's protocol.

Reaction Mix	Volume
10X *Not*I Buffer	2 μl
*Not*I Restriction enzyme (10Units/μl)	1 μl
pUC57.UAS.CYP6BQ23 plasmid vector (2000ng)	5.5 μl
dH_2O	11.5 μl
Total volume	**20 μl**

Table 1. Reaction mix components for *NotI* digest of pUC57.UAS.CYP6BQ23 plasmid vector. The sample was incubated at 37°C for 2 hours and the enzyme was deactivated at 65°C for 20 minutes

2.1.4 Dephosphorylation of linearized plasmid vector

The linearized pH_Stinger.attB.*Not*I.SV40 vector was dephosphorylated prior to ligation in order to prevent recircularization and self-ligation. The reaction was performed with Alkaline Phosphatase, Calf Intestinal (CIAP) (Promega, Part# 9PIM182), according to the manufacturer's protocol.

The reaction was performed as seen in Table 2.

Reaction Mix	Volume
linearized pH_Stinger.attB.*NotI*.SV40 vector	17 µl
10X CIAP Buffer	5 µl
CIAP (0,01 Units/µl per pmols of DNA ends)	1 µl
dH_2O	27µl
Total volume	**50 µl**

Table 2. Reaction mix components for dephosphorylation of the linearized pH_Stinger.attB. *NotI*.SV40 vector.

- Incubation at 37°C for 15 minutes
- Incubation at 56°C for 15 minutes
- Add another aliquot of 1µl CIAP (0,01 Units/µl)
- Incubation at 37°C for 15 minutes
- Incubation at 56°C for 15 minutes

2.1.5 Ligation reaction of pH_Stinger.attB.*Not*I.SV40 vector with the UAS.CYP6BQ23 insert

The ligation reaction was performed in order to covalently connect the insert DNA into the plasmid vector. The molar ratio of vector: insert DNA used in this reaction was 1:3 and was calculated with the following formula.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

The reaction was performed with T4 DNA ligase (Promega, Part# 9PIM180). Control reactions was also set up and run in parallel, as seen in Table 3. The reaction was incubated at 4°C, overnight.

Reaction Mix	Ligation	Control 1	Control 2
pH_Stinger.attB.*NotI*.SV40 vector	3 µl	-	3 µl
UAS.CYP6BQ23 insert	1.5 µl	1.5 µl	-
10X Ligase Buffer	1 µl	1 µl	1 µl
T4 DNA ligase (1–3u/µl)	0.5 µl	0.5 µl	0.5 µl
dH_2O	4 µl	7 µl	5.5 µl
Total volume	**10 µl**	**10 µl**	**10 µl**

Table 3. Reaction mix components for ligation reaction of pH_Stinger.attB.*Not*I.SV40 vector with the UAS.CYP6BQ23 insert

2.1.6 Transformation of *E.coli* and selection of transformants

E.coli DH5alpha competent cells were transformed with the 10µl ligation reaction, as well as, with the control reactions, using the heat shock protocol. The transformant cells were plated on agar plates containing 100µg/ml of ampicillin. The plates were incubated at 37°C for 12-16 hours (overnight incubation). The following day, several colonies were selected for further investigation. The selected colonies were inoculated in LB medium with ampicillin and incubated at 37°C, overnight.

2.1.7 Alkaline lysis plasmid preparation

Plasmid DNA was isolated from the selected colonies using the alkaline lysis protocol. Firstly, bacteria were pelleted and resuspended in resuspension buffer (50 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0, 50 mM glucose). Then, bacteria were lysed with a buffer containing 0.2 M NaOH and 1 % SDS. Subsequently a neutralization buffer (3 M potassium acetate pH 4.8) was added which resulted in the precipitation of the genomic DNA, the SDS and the denatured proteins, while the soluble plasmid DNA remained in the supernatant. After centrifugation, the soluble plasmid DNA was purified from the solution by isopropanol precipitation.

2.1.8 Diagnostic digests of pH_Stinger.attB.UAS.CYP6BQ23 plasmid DNA with *NotI* and *Hind*III

Initially, plasmid DNA from different colonies was digested with *Not*I in order to examine whether the colonies had integrated the UAS.CYP6BQ23 insert in their DNA. The colonies that have incorporated the insert (positive colonies) should give a diagnostic DNA fragment at 1983 bp.

Then, plasmids from positive colonies, were further digested with *Hind*III, in order to verify the correct orientation of the UAS.CYP6BQ23 insert. The digest reactions were performed as seen in Table 4.

Reaction Mix	Volume
Plasmid DNA	2 μl
*Not*I or *Hind*III (10Units/μl)	0.5 μl
10X Buffer	2 μl
dH_2O	15.5 μl
Total volume	**20 μl**

Table 4. Reaction mix components for *Hind*III or *Not*I digest.

2.1.9 Injection mix preparation and embryo micro-injections

The plasmid mixture was micro-injected into the posterior end of *Drosophila yw;nos.int;attP40* embryos. The plasmid for injections was prepared using the Qiagen® Plasmid Purification Kit. The injection mix contained 400 ng/μl of vector. After recovering from injection, the hatched first instar larvae were placed into regular larval food.

The injection mix consists of plasmid DNA diluted in dH_2O in final concentration of 400ng/μl.

2.2 Functional study of *C. capitata* cytochrome CYP6A51 and ectopic expression of Cyp6a51 gene in *Drosophila melanogaster* for investigation of pyrethroid susceptibility in transgenic lines

2.2.1 Functional analysis of *C. capitata* cytochrome CYP6A51 using heterologous expression in *E.coli*

2.2.1.1 Cloning procedure of CYP6A51 for heterologous expression in *E.coli*

Total RNA was extracted from individual *Ceratitis capitata* adults (laboratory strains from Crete), using the RNeasy Mini kit (Qiagen). First strand cDNA was synthesized by using an oligodT_{20} primer and SuperScriptIII reverse transcription kit (Invitrogen). All procedures were performed by following manufacturers' instructions. The full length *Cc*CYP6A51 gene was amplified from the susceptible laboratory strain using the Kapa DNA polymerase (KapaBiosystems, UK) with the primers CYP6A51_exp_F, introducing a *Sac*I restriction site before the ATG codon and CYP6A51_exp_R, complementary to the 3 prime end of the CYP6A51 cDNA, introducing an *Xba*I restriction site (primer sequences listed in Appendix, Table 1). In order to express functional *Ceratitis capitata* CYP6A51 in *E. coli* cells, a pCWompA plasmid provided by Dr. Mark Paine (Liverpool School of Tropical Medicine, UK) was used (McLaughlin *et al*., 2008). The resulting fragments with *Sac*I and *Xba*I compatible ends, were ligated into pCW.ompA vector to create pCW-CYP6A51.

2.2.1.2 Preparation of *E.coli* membranes and functional expression of CYPA51

Competent *E. coli* BL21STAR cells were co-transformed with the pCW_ompA.CYP6A51 plasmid and the pACYC.AgCPR plasmid, which contains the P450 reductase from *Anopheles gambiae*. Transformed cells were grown in Terrific broth medium with ampicillin (50 μg/ml final concentration) and chloramphenicol (34 μg/ml final concentration) selection at 37 ^{o}C with shaking, until the optical density at 595 nm reached ~1 cm^{-1}, whereupon the heme precursor δ-aminolevulinic acid (ALA) was added to a final concentration of 1 mM. Induction was initiated with the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM The cultures after the addition of ALA and IPTG transferred at 25 ^{o}C for further incubation. After 24 hours, the cultures were harvested by centrifugation at 4000 rpm for 20 minutes at 4^{o}C.

Spheroplasts were prepared by adding TSE buffer (0.1 M Tris acetate pH 7.6, 0.5 M sucrose, 0.5 mM EDTA) containing 0.25 mg/ml lysozyme to the cell pellet and gently mixing for 60min at 4 ^{o}C. The solution was centrifuged at 2800 g for 25 min at 4 ^{o}C and the spheroplast pellet was resuspended in

spheroplast resuspension buffer (0.1 M potassium phosphate buffer, pH 7.6, 6 mM magnesium acetate, 20% glycerol) containing the protease inhibitors 0.1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mg/ml aprotinin and 1 mg/ml leupeptin. The suspension of spheroplasts was sonicated and the membrane fraction was pelleted by ultracentrifugation at 64.000 rpm for 60 min at 4^{o}C. Finally, the membrane preparations were diluted in TSE buffer and stored at -80^{o}C.

2.2.1.3 Carbon monoxide (CO) spectrum assay for P450 detection on membranes preparations

Cytochromes P450 are hemoproteins. The heme group that exists in their molecule contains a Fe^{3+}, which can be reduced to Fe^{2+}, in the presence of the reducing agent sodium hydrosulfate, which enables the cytochrome P450 to form a complex with CO. The resulting P450-CO complex gives a characteristic absorption spectrum at 450 nm, which can be measured (Omura and Sato, 1964). A peak at 450nm indicates that P450 molecules exists in the membrane preparation.

Firstly, a pinch of the reducing agent sodium Dithionite was mixed with 1 ml spectrum buffer (100 mM Tris-HCl pH 7.4, 20% glycerol, 1 mM EDTA) and 25 μl of membrane preparations sample into a plastic cuvette. The solution was saturated with CO gas, in order to start the P450-CO complexes formation. The absorption spectrum was measured at a spectrophotometer at 400 to 500 nm, at room temperature.

2.2.1.4 Cytochrome P450 reductase (CPR) activity assay

Cytochrome P450 Reductase (CPR) is a membrane protein that mediates the electron transfer from NADPH to cytochrome P450 in the endoplasmic reticulum and is required for the function of all P450 enzyme (Sevrioukova and Peterson, 1995). Therefore, the Cyp6a51 gene of *C. capitata* was co-expressed with CPR gene from *Anopheles gambiae*, so that the two proteins can work together. The CPR activity of the isolated membranes was estimated spectrophotometrically by measuring the oxidation of cytochrome *c* at 550 nm, at 25 ^{o}C (Strobel and Dignam, 1978).

Two buffers were prepared: buffer A, which contains 50 μM cytochrome c in 0.3M potassium phosphate pH 7.7 and buffer B, which contains 5 mM NADPH in ddH_2O. Firstly, 1 ml of buffer A was mixed with 10 μl of membrane preparations sample into a plastic cuvette. The ratio of oxidation was measured at 550 nm, for 1 minute and this measurement was considered as the control (blank).

Then, 10 μl of buffer B were added into the cuvette and after a quick stirring, the ratio of oxidation was measured at 550 nm, for 1 minute.

The catalytic activity of the enzyme was calculated with the following function, where the constant 21.4 is related with the diameter of the plastic cuvette:

$$\boldsymbol{catalytic\ activity} = \frac{\dfrac{\boldsymbol{Vmax\ (in\ mUnits/min)}}{\boldsymbol{21.4}}}{\boldsymbol{Protein\ concetration\ (in\ mgr/ml)}}$$

2.2.1.5 Glo activity assay with cytochrome CYP6A51 enzyme

In order to determine if CYP6A51 on membrane preparations was active, membrane preparations were tested with luciferin-based substrates (P450 Glo™ proluciferin substrates, Luciferin-H, Luciferin-ME, Luciferin-CEE, Luciferin-H EGE, Luciferin-PFBE, Luciferin-PPXE, Luciferin-ME EGE and Luciferin-IPA Promega). The reaction was performed by incubating the P450 sample with the luminogenic substrate. The P450 enzyme is considered to have catalytic activity when it can convert the substrates to luciferin, which in turn reacts with luciferase and produces light. The amount of light produced can be measured by a luminometer and it is directly proportional to P450 catalytic activity.

The activity measurements were performed in 50 mM potassium phosphate pH 7.4, 5 mM luminogenic substrate, 1 pmol of CYP6A51 (membranes preparation) and 0.1 mM NADPH. Luciferin reactions were incubated for 10 min at 37°C before adding NADPH. After addition of NADPH, samples were incubated for 30 min at 37°C. Finally, after adding the detection buffer, the signal was measured by a single tube luminometer.

2.2.1.6 Cytochrome P450 assay of lambda-cyhalothrin metabolism

CYP6A51 catalytic activity to metabolize pyrethroids was tested against lambda-cyhalothrin. The substrate lambda-cyhalothrin (20\μM) was incubated with Tris-HCl Buffer (0,2M, pH 7.4), 25pmoleCYP6A51 enzyme (25 pmole of membrane peparations) and 200pmole b5 either in the presence or in absence of NADPH generating system at 30 ºC, with 1250 rpm shaking for 0 hour and 2 hours The final concentration of acetonitrile in the reaction mixtures was 2%. The reactions were stopped at time points of 0 and 2 hours, upon addition of 100ul acetonitrile and incubated for further 30 min, in order to ensure that all amount of lambda-cyhalothrin was dissolved. The quenched reactions were centrifuged at 10.000 rpm for 10min and the supernatant of each reaction sample was transferred into HPLC vials. Lambda-cyhalothrin insecticide (100 ppm) was, then, added in each vial,

as an internal control for the elimination of injection error. 100ul of the supernatant were injected at a flow rate of 1.25 ml/min. The reactions were run with an isocratic program 80% Acetonitrile and 20% H_2O, for 20 min with a flow rate 1,25 ml/min with 250 mm 5um UniverSil HS C18 column. Various concentrations of lambda-cyhalothrin were also tested (10-60 μM), as well as various concentrations of CYP6A51 p450 enzyme preparation (5-40 pmole).

2.2.2 Ectopic expression of *C. capitata* cytochrome P450 CYP6A51 in *Drosophila melanogaster*

2.2.2.1 Construct design for *Ceratitis capitata* cytochrome P450 monooxygenase CYP6A51

The design of plasmid construct for the overexpression of *Ceratitis capitata* P450 monooxygenase CYP6A51 in *Drosophila melanogaster* was differentiated from the strategy used for CYP6BQ23. The DNA coding sequence of CYP6A51 from *Ceratitis capitata*, GenBank version KF305738.1, was already available into a pGEM plasmid vector and provided by Dimitra Tsakireli, Vontas Lab. The UAS sequence was also available into a pUAST vector and provided by Vassilis Douris, Vontas Lab. The general strategy to construct a pH_Stinger.attB vector containing the UAS.CYP6A51 sequence was to work in ‘‘functional blocks’’. The first step was to amplify the UAS sequence from pUAST vector with specific primers containing *Sac*II sites and insert this fragment into the pGEM.CYP6A51 vector, at a *Sac*II site right before the gene ORF. The pGEM vector contains cleavage sites for NotI flanking the UAS and CYP6A51 ORF region. Thus, the UAS.CYP6A51 fragment was excised through a restriction digest with *Not*I enzyme and inserted into the pH_Stinger.attB vector (see also Figure 3, Appendix).

2.2.2.2 Generation of UAS expression plasmid

The coding sequence of CYP6A51 from *Ceratitis capitata* was available into a pGEM plasmid vector, provided by Dimitra Tsakireli, Vontas Lab. The 5xUAS_basal promoter sequence was also available into a pUAST vector, provided by Vassilis Douris, Vontas Lab.

First, the 5xUAS_basal promoter region was amplified from the vector pUAST.Casper.A16 with the specific primers, UAS2F and UAS2R (Appendix, Table 1). Primers were designed so as to introduce a restriction site for *Sac*II, on both ends of the UAS amplicon.

The reaction was performed in 25μl volume and contained 25 ng of template DNA. The polymerase used was the KAPA Taq DNA Polymerase, KAPA BIOSYSTEMS. The reaction components and the

cycle conditions are seen in Table 5 and 6, respectively. The PCR amplification was done on a BioRad PTC-200 Thermocycler, and the PCR product was analyzed on 1 % w/v agarose gel.
The PCR product was purified with NucleoSpin® Gel and PCR Clean-up (MACHEREY NAGEL), according to the manufacturer's protocol.

Reaction components	25 µl reaction
DNA template (pUAST.Casper.A16)	4 µl
PCR Buffer 10x	5 µl
dNTPs (10 mM)	1 µl
Primer UAS2F (10 µM)	2 µl
Primer UAS2R (10 µM)	2 µl
KAPPA Taq polymerase (5 Units/ µl)	0.5 µl
dH2O	35.5 µl

Table 5. PCR reaction components for amplification of UAS sequence from pUAST.Casper.A16 vector

Step	Temperature (°C)	Duration (minutes)	Cycles
Initial denaturation	95	3	1
Denaturation	95	30	35
Annealing	62	30	
Extension	72	1	
Final extension	72	5	1
Hold	4	∞	1

Table 6. PCR cycle conditions for amplification of UAS sequence from pUAST.Casper.A16 vector

2.2.2.3 Sub-cloning procedure of UAS insert into pGEM.CYP6A51 vector

The UAS PCR fragment and the pGEM.CYP6A51 plasmid were digested with the isoschizomer of *Sac*II, the restriction enzyme *Sgr*BI (MINOTECH Biotechnology). The pGEM.CYP6A51 plasmid was also digested with *Sgr*BI , in order to linearize the plasmid at the unique restriction site right upstream of the gene ORF, in order to be used as cloning vector for subcloning of the 5xUAS_basal promoter amplified fragment. Subsequently, the two samples were electrophoresed in 1% agarose gel in TBE and purified from gel with NucleoSpin® Gel and PCR Clean-up (MACHEREY NAGEL), according to the manufacturer's protocol. The digest reactions were performed as seen in Table 7. The samples were incubated at 37°C for 2 hours and the enzymes were deactivated at 65°C for 20 minutes.

Reaction Mix	Volume
10X *Sgr*BI Buffer	2 µl
*Sgr*BI Restriction enzyme (10Units/µl)	1 µl
pGEM.CYP6A51 plasmid vector (2000ng) or UAS fragment	5.5 µl
dH2O	11.5 µl
Total volume	20 µl

Table 7. Reaction mix for *Sgr*BI digest of UAS insert and pGEM.CYP6A51 plasmid.

Subsequently, the linearized pGEM.CYP6A51 vector was dephosphorylated and ligation reaction was performed with the appropriate control reactions. Finally, *E.coli* DH5alpha competent cells were transformed with the ligation reaction.

2.2.2.4 Colony PCR for selection of positive transformants

A colony PCR reaction was set up in order to detect positive colonies that had incorporated the UAS insert into their genome. The standard primer T7 and UAS2R were used. The reaction was performed as seen in Table 8. The total reaction volume was 25µl. Individual colonies were picked using a sterile tip, dipping into the PCR mix. Subsequently, the tip was dipped into 3 ml LB medium with ampicillin and the cultures were incubated at 37 °C overnight. The cycle conditions are seen in Table 9. The PCR products were analyzed on 1 % w/v agarose gel.

Reaction components	25 µl reaction
DNA template	single colony
PCR Buffer 10x	5 µl
dNTPs (10 mM)	0.5 µl
Primer T7 (10 µM)	1 µl
Primer UAS2R (10 µM)	1 µl
KAPPA Taq polymerase (5 Units/ µl)	0.3 µl
dH2O	19.7 µl

Table 8. Colony PCR reaction components for detection of positive colonies

Step	Temperature (°C)	Duration (minutes)	Cycles
Initial denaturation	95	10	1
Denaturation	95	30	35
Annealing	52	30	
Extension	72	1	
Final extension	72	5	1
Hold	4	∞	1

Table 9. Cycling protocol for colony PCR reaction

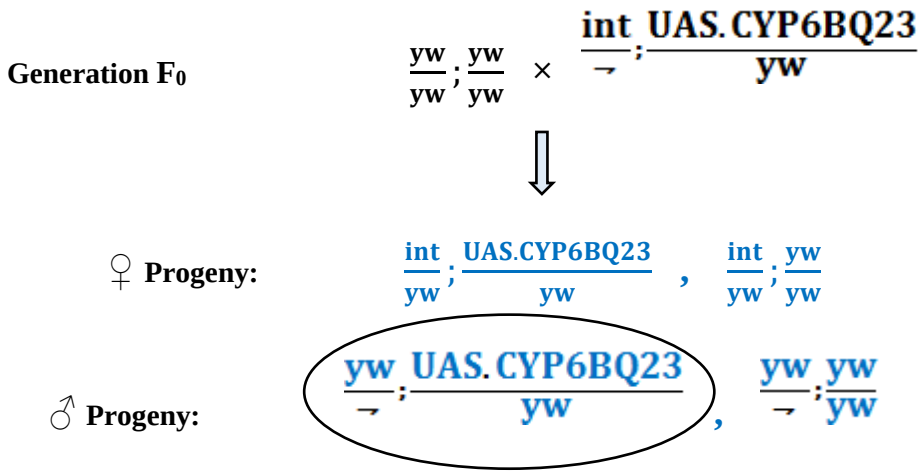
Plasmid DNA was isolated from the positive colonies using the alkaline lysis protocol. The plasmid DNA molecules were digested with *Sgr*BI and *Hind*III, in order to test whether the colonies had incorporated the UAS insert with the correct orientation, into their genome. The diagnostic digests were performed as described in section 2.1.9. Finally, the DNAs of the positive clones were sequenced (Macrogen, The Netherlands) in order to confirm the correct insertion of UAS fragment.

2.2.2.5 Sub-cloning procedure of UAS.CYP6A51 insert into pH_Stinger.attB.*Not*I.SV40 vector

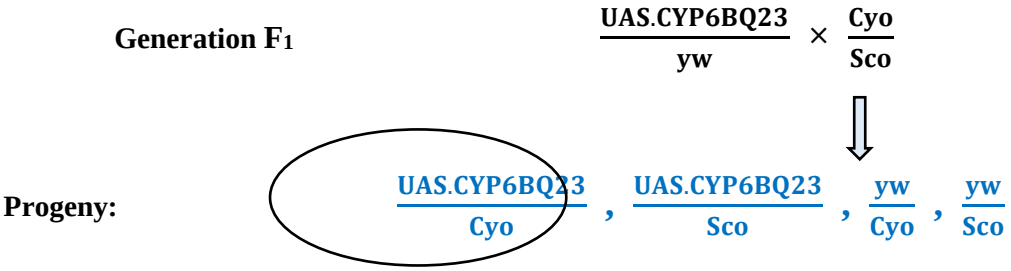
The sub-cloning procedure of UAS.CYP6A51 insert into pH_Stinger.attB vector was carried out as described. The pH_Stinger.attB.*Not*I.SV40 vector was generated after *Not*I digestion, as described. The pGEM.UAS.CYP6A51 vector was also digested with *Not*I, so that the fragment UAS.CYP6A51 would have compatible ends with the linearized pH_Stinger.attB.*Not*I.SV40 vector. The UAS.CYP6A51 insert was also electrophoresed in 1% w/v agarose gel in TBE and purified from gel. A ligation reaction was set up with a molar ratio 1:5 for vector and UAS.CYP6A51 insert respectively. The ligation mix was used for *E.coli* DH5alpha competent cells transformation. Plasmid DNA was isolated from selected colonies according to the alkaline lysis protocol and diagnostic digests with *Not*I and *Hind*III, were performed in order to check the UAS insertion. Finally, a positive clone was selected for plasmid midiprep, using the Qiagen® Plasmid Purification Kit. The injection mix was prepared with plasmid diluted in dH_2O in final concentration of 400ng/μl. The micro-injections were performed in *yw;nos.int;attP40 Drosophila* embryos as described previously.

2.3 Genetic crosses to get transgenic flies expressing CYP6BQ23 and CYP6A51.

Following the *Drosophila* embryo injections, first instar larvae were collected and transferred into regular larval food until they develop into adult flies. Initially, the injected flies (G_0) were outcrossed with *yw* flies. The injected flies carry the phiC31 integrase gene on the X chromosome, which is not necessary for the final transgenic flies. Thus, the male progeny are preferred for the crosses because by using a male positive G_0 fly, the desired progeny appear earlier. The crosses are depicted below. The progeny of each cross are seen in blue colour and the desired progeny are circle



The G_1 progeny with red eyes that have the gene insertion on attP40 site were selected and crossed with the balancer flies *yw; CyO/Sco*. The crosses with balancer line are necessary in order to prevent recombination and facilitate the genetic mating. The cross is seen below.



The desired flies from these crosses, which have red eyes and curly wings, were crossed again with the balancer flies *yw; CyO/Sco*. The resulting flies that have red eyes (w^+) and curly wings (*Cy*), were heterozygous (UAS_geneX marked with w^+ / *Cy*O) and were crossed with flies with the same genotype of the opposite sex in order to obtain homozygous UAS.CYP6BQ23 or UAS.CYP6A51 flies.

Generation F_3 $\quad \frac{\text{UAS.CYP6BQ23}}{\text{UAS.CYP6BQ23}} \times \frac{\text{UAS.CYP6BQ23}}{\text{UAS.CYP6BQ23}}$

⇩

Progeny: $\quad \frac{\text{UAS.CYP6BQ23}}{\text{UAS.CYP6BQ23}}$

These flies have the Cyp6bq23 (or Cyp6a51) transgene present in the 2nd chromosome, but they do not overexpress it. UAS.CYP6BQ23 homozygous flies were further crossed with the GAL4 driver, HR-GAL4. The resulting flies overexpress the CYP6BQ23 in the fat body, malpighian tubules and midgut and they were used for contact bioassays.

2.4 Insecticides

The insecticide used for this study was technical grade deltamethrin (≥98% pure).

2.5 Bioassays for determination of the lethal concentration 50 (LC50) for Deltamethrin

Resistance was measured by comparing LD_{50} values of control and mutant flies. LD_{50} represents the lethal dose of an insecticide that kills half population of treated individuals. The resistance levels of flies to deltamethrin were measured with contact bioassays.

The contact bioassay is conducted with adult flies. The transgenic UAS.CYP6BQ23 (or UAS.CYP6A51) flies were crossed with HR-GAL4 flies and 1-3 days-old adults were used for contact bioassay. Furthermore, UAS.CYP6BQ23 (or UAS.CYP6A51) flies were crossed with w^{1118} flies and 1-3 days-old adults that normally do not overexpress the P450, were used as a negative control for the bioassay. For contact bioassay, glass vials were coated with different concentrations of deltamethrin diluted in acetone. 500 µl of diluted deltamethrin was applied into each vial by rolling the vials on a roller machine, until the acetone was evaporated. The vials were plugged with cotton wool. 20 flies (both males and females) were flipped in each vial and remained there for 1.5 hours. Control vials were also set up and were treated only with acetone. Subsequently, the flies were flipped into other vials covered only with acetone. The bottom of each vial was covered with cotton soaked in 5% sucrose. Mortality was scored after 24 hours. The individuals were considered to be dead when no movement was observed. Three replicates were tested for each deltamethrin concentration.

2.5.1 Data analysis

LC_{50} value represents the estimated concentration which causes 50% mortality of the test population during the observation period of 24h. Bioassay derived data were used to estimate the LC_{50} values by probit analysis using the computer program PoloPlus, LeOra Software Company, which were corrected for the control mortality using the Abbott's formula (Abbott, 1987). Resistance ratios (RR) represent a ratio of the LC_{50} of mutant (resistant) strains with respect to the LC_{50} of control (susceptible) strains.

2.6 Targeted genome editing with CRISPR/Cas9 technology in *Drosophila* genome and evaluation of susceptibility to diamides

2.6.1 Drosophila melanogaster strains and lines

For this study, two *Drosophila* strains were used:

- the nanos-Cas9 (stock number 54591 in Bloomington Stock Center) strain, which expresses Cas9 endonuclease during oogenesis under control of *nanos* regulatory sequences (Port *et al*., 2014).
- the *yw; Cy*O/ *Sco* which carries the Curly marker gene on the second balancer chromosome and the scutoid marker. (Stock collection IMBB/FORTH).

2.6.2 Genome editing of ryanodine receptor gene in *Drosophila melanogaster*

A region spanning ~2.5 kb around the position of the desired point mutation was sequenced in order to ensure the matching between the published genome sequence and the genomic sequence of nanos-Cas9 flies.

The gRNA target sequence (Appendix, Figure 5) was designed using the CRISPR Optimal Target Finder program (Gratz *et al*., 2014) (http://tools.flycrispr.molbio.wisc.edu/targetFinder/), and it was selected because it has no off-targets and it is close to the site of the desired mutation. The gRNA target sequence was constructed as complementary phosphorylated oligonucleotides (Invitrogen, Thermo Fisher Scientific), flanked with *Bbs*I compatible sites for cloning into the appropriate *Bbs*I digested vector. The oligonucleotides were annealed and cloned into the pU6-BbsI-chiRNA plasmid (Addgene, #45946) (Gratz *et al*., 2014), under the U6 promoter which is recognized by RNA polymerase III.

The design of the donor plasmid (Figure 4, Appendix) was conducted by taking into consideration the homology of the region around the desired mutation so that it can serve as a template for homology directed repair, as well as the presence of molecular tools for mutant screening. The G4946E mutation was introduced by changing the GGC codon to GAG leading to an amino acid substitution from a Glycine to Glutamic acid. A silent mutation was also introduced in order to change the PAM sequence in the donor plasmid, so that it is not targeted by the CRISPR/Cas9 process. In addition, two restriction sites were engineered with silent mutations; a site for *Aat*II was introduced, in order to be present only in mutant alleles, while a site for *Pst*I was mutated, in order to be present only in wild type alleles. The donor plasmid was synthesized by GenScript (USA) into a pUC57 vector.

2.6.3 Sequencing of Ryanodine Receptor gene of *Drosophila melanogaster*

The last exon of RyR gene of *Drosophila melanogaster* nanos-cas9 #54591 strain was sequenced with three pairs of primers (RyR1F, RyR1R, RyR2F, RyR2R RyR3F, and RyR3R) that were designed in order to amplify a 2543 bp region. The primers are seen in Table 1 in Appendix. The PCR product was purified using the NucleoSpin® Gel and PCR Clean-up (MACHEREY NAGEL). The sequencing was done by Macrogen, the Netherlands.

2.6.4 Generation of gRNA containing plasmid

The two single-stranded, complementary oligonucleotides (Figure 5, Appendix) were firstly resuspended in sterile dH_2O. Subsequently, they were mixed in equal molar concentration (20 μM) together in 1x NEB 3.1 buffer (100mM NaCl, 50mM Tris-HCl, 10mM $MgCl_2$, 100μg/ml BSA, pH 7.9, at 25°C). The mixed oligos were heated to 95 °C for 5 minutes and let to gradually cool until reach the room temperature.

The annealed oligos were mixed with pU6-BbsI-chiRNA vector in 1:3 molar ratio. The oligos have been designed so as to have *Bbs*I-compatible restriction ends. The pU6-BbsI-chiRNA vector was already digested with *Bbs*I , dephosphorylated and a ligation reaction was incubated at 4 °C, overnight. Subsequently, the ligation mix was transformed into *E.coli* DH5alpha competent cells, using the heat shock protocol and incubated at 37 °C overnight. Afterwards, 12 colonies were tested with colony PCR using the standard primer T7 and one of the oligonuneclotides at the complementary strand. Positive colonies were selected and the extracted DNA was sequenced (Macrogen, The Netherlands) and proved to have the correct insertion of the gRNA fragment.

2.6.5 Injection mix preparation and embryo micro-injections

Donor plasmid with gRNA expression plasmid mix was micro-injected into the posterior end of nanos-Cas9 (#54591) *Drosophila* embryos. The gRNA plasmid was prepared using the Qiagen® Plasmid Purification Kit. The injection mix consists of donor plasmid (100 ng/µl) and gRNA plasmid (75 ng/µl) diluted in injection buffer (2 mM Sodium phosphate pH 6.8-7.8, 100 mM KCl). After recovering from injection, the hatched first instar larvae were placed into regular larval food until they develop into adult flies.

2.6.6 Genetic Crosses

The adult injected flies (G_0) were crossed with nanos-Cas9 (#54591) flies and the G_1 progeny were screened. The screening process includes DNA extraction in pools of 30 larvae followed by PCR with primers specific for the desired mutations. The positive G_1 flies were subsequently crossed with the balancer *yw;CyO/Sco*. *Cy*O is a balancer for the second chromosome and is phenotypically seen by curly wings. The Sco marker, which is not on a balancer chromosome, phenotypically results in missing bristle sockets from scutellum and it is not used for the selection of progeny. This cross with balancer line is necessary in order to prevent recombination between the mutated allele and the opposite chromosome and facilitate the genetic mating. At this stage the molecular screening was done in individual G_1 parental flies and it shows that if a positive allele is found, then the relevant fly is heterozygous. The desired G_2 progeny from this cross have curly wings, which means that they carry the balancer chromosome. However, given that the parent is heterozygous, only half of the progeny with curly wings would carry the mutated allele. Hence, the G_2 progeny with curly wings were crossed again with the balancer *yw;Cy*O*/Sco*. The parental flies were screened and the positive flies are revealed. In this cross, half of the G_3 progeny with curly wings carry the mutated allele, while the other half carries the scutoid allele, which can easily be recognized. Eventually, the G_3 heterozygous flies with curly wings and wild type scutellum are sub-mated. The resulting G4 homozygous flies are easily recognized because their wings are normal, and are expected to carry the target site mutation G4946E in RyR gene, which has to be confirmed with sequencing. The genetic crosses are described in brief in Table 10.

	Cross	Description
F0	$\frac{M/\mathrm{wt}}{\mathrm{wt}} \times \frac{wt}{wt}$	This cross rescues the mutation and ensures the viability of the progeny
Progeny	$\frac{\mathrm{M/wt}}{\mathrm{wt}}$, $\frac{\mathrm{wt}}{\mathrm{wt}}$	Molecular screening confirms if the genotype is $\frac{\mathrm{M}}{\mathrm{wt}}$ or $\frac{\mathrm{wt}}{\mathrm{wt}}$
F1	$\frac{M/\mathrm{wt}}{\mathrm{wt}} \times \frac{\mathrm{Cyo}}{\mathrm{Sco}}$	This cross ensures that no recombination occurs between the mutated and the wild type allele
Progeny	$\frac{\mathrm{M/wt}}{\mathrm{Cyo}}$, $\frac{\mathrm{M/wt}}{\mathrm{Sco}}$, $\frac{\mathrm{Cyo}}{\mathrm{wt}}$, $\frac{\mathrm{Sco}}{\mathrm{wt}}$	Molecular screening confirms the genotype of parental fly. The desired balanced progeny have curly wings. It is impossible to discriminate between the M and wt
F2	$\frac{M/\mathrm{wt}}{\mathrm{Cyo}} \times \frac{\mathrm{Cyo}}{\mathrm{Sco}}$	This cross rules out the wild type allele
Progeny	$\frac{\mathrm{M}}{\mathrm{Cyo}}$, $\frac{\mathrm{M}}{\mathrm{Sco}}$, $\frac{\mathrm{Cyo}}{\mathrm{Cyo}}$, $\frac{\mathrm{Cyo}}{\mathrm{Sco}}$	Molecular screening confirms the genotype of parental fly. The desired balanced progeny have curly wings and they carry the mutated allele
F3	$\frac{M}{\mathrm{Cyo}} \times \frac{\mathrm{M}}{\mathrm{Cyo}}$	This cross results in homozygous flies
Progeny	$\frac{\mathrm{M}}{\mathrm{M}}$, $\frac{\mathrm{M}}{\mathrm{Sco}}$, $\frac{\mathrm{Cyo}}{\mathrm{Cyo}}$	No molecular screening is needed. Flies with curly wings and wild type scutellum are homozygous
F4	$\frac{M}{\mathrm{M}} \times \frac{\mathrm{M}}{\mathrm{M}}$	This cross ensures the establishment of the new homozygous population

Table 10. Crosses for rescue of G4946E mutation in RyR gene

2.6.7 Molecular screening: PCR and restriction digest based screening methods

In the above genetic mating scheme, there is no phenotypic maker linked with the desired mutated allele. Therefore, a screening method that can distinguish between the wild type and the mutated allele is necessary. The donor plasmid was designed in a way that the mutated flies would have specific traits like restriction sites that are not present in wild type flies, and these traits can be used for molecular screening. In this case, two screening methods were used for the detection of the mutated allele and they are presented below:

- **PCR based method:**

This method includes genomic DNA isolation from individual flies or from pools of 30 larvae. The DNA extraction is conducted by grinding and homogenizing flies in DNAzol®, Molecular Research Center. The resulting DNA is used for polymerase chain reaction with a primer set specific for the mutation. More specifically, the forward primer Specific Forward (Appendix, Table 1) is specific for the mutated alleles because it contains four nucleotides that are not present in the wild type allele; two nucleotides that create the position for *Aat*II digest, one nucleotide which changes the PAM sequence and one nucleotide of the desired point mutation, as seen in Figure 12. The reverse primer GENEREV (Appendix, Table 1) is not allele-specific. Finally, the PCR product is separated on an agarose gel. The desired amplicon is expected at 585 base pairs.

5′- ATCTGCTCGA**C**GT**C**GC**T**GTGG**A** - 3′

Figure 12. The specific primer. Green: nucleotides that create the restriction site for *Aat*II. Blue: nucleotide that changes the PAM sequence. Yellow: nucleotide that introduces the desired point mutation.

- **Restriction digest based method:**

This method also includes genomic DNA isolation from individual flies or from pools of 30 larvae. Firstly, DNA is digested with *Pst*I. The restriction site for *Pst*I exists only in wild type flies. Secondly, a PCR with a generic set of primers amplifies only the undigested molecules. The generic primer set GENEFOR and GENEREV (Appendix, Table 1) is common for both mutant and wild type allele and amplifies a region with all the inserted changes. However, due to wild type allele digest, mostly mutated alleles are amplified. The PCR product can be loaded on an agarose gel. The desired amplicon

is expected at 900 base pairs. Furthermore, a digest with *Aat*II, which digests only the mutated allele, can confirm the presence of the desired mutations. The *Aat*II digest results in 2 bands, the one at 328 base pairs and the other one at 572 base pairs.

2.6.8 Insecticides

The insecticides (formulations) used for this study were: Belt, which is a diamide based insecticide produced by Bayer, which contains Flubendiamide 24% w/w and Altacor, which is also a diamide based insecticide produced by DU PONT and it contains Chlorantraniliprole 35% w/w.

2.6.9 Feeding bioassay

Bioassays were used to measure the resistance levels of flies to diamides, by estimating and comparing LC_{50} values of wild type and mutant flies. The feeding bioassay was conducted with 2^{nd} instar *Drosophila* larvae. The nanos-Cas9 (#54591) strain was used as control. Regular size vials were prepared with larval food containing different concentrations of diluted insecticide. 20 larvae were transferred into the food and the vials were plugged with cotton wool. Mortality was scored after 10 days. Each insecticide concentration was tested on three replicates. Finally, the LC_{50} values were calculated with the computer program PoloPlus, LeOra Software Company.

3. Results

3.1 Generation of UAS.CYP6BQ23 expression construct

3.1.1 Restriction digest of pUC57.UAS.CYP6BQ23 and pH_Stinger.attB vectors with *Not*I

The UAS.CYP6BQ23 region was synthesized by GenScript (USA) into a pUC57 vector, flanked by *Not*I restriction sites. The plasmid was resuspended in 20μl dH_2O and its concentration was 492.7 ng/μl, as measured by NanoDrop™ spectrophotometer. The plasmid was used to transform *E. coli* DH5alpha cells. Single colonies were picked and cultured and the overnight bacterial culture was used for plasmid extraction with QIAprep Spin Miniprep Kit, Qiagen.

In order to subclone the UAS.CYP6BQ23 region and generate the expression vector, it is necessary for pH_Stinger.attB vector to be also digested with *Not*I, in order to remove the existing GFP expression cassette and replace it with the UAS.CYP6BQ23 sequence. The restriction digest was performed with plasmid DNA, incubated at 37°C for 2 hours and the enzyme was deactivated at 65°C for 20 minutes. The digest's products were electrophoresed in a 1% w/v agarose gel (Figure 13).

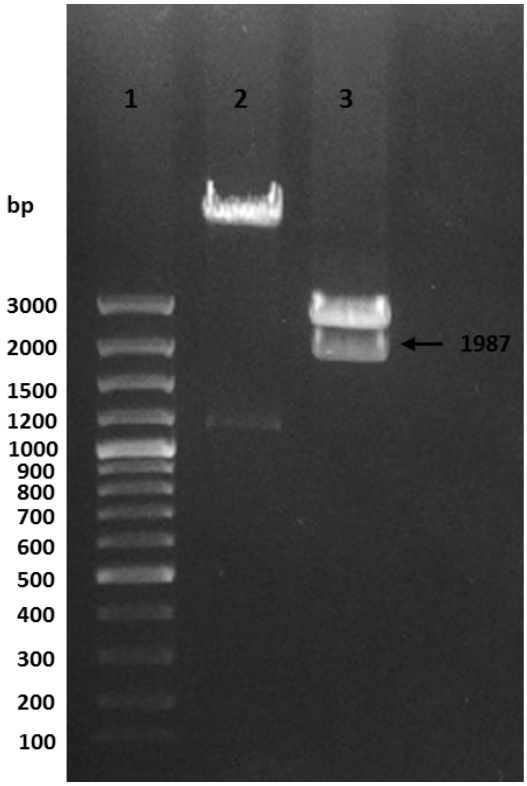


Figure 13. Electrophoresis of *Not*I digest products. Lane 1: DNA Ladder GeneRuler Plus (Fermentas), Lane 2: *Not*I digested pH_Stinger.attB, Lane 3: *Not*I digested pUC57.CYP6BQ23

As seen on the agarose gel in Figure 13, both the two plasmids, the pH_Stinger.attB and the pUC57.CYP6BQ23, were digested with *Not*I. In Lane 2, the upper zone is the linearized pH_Stinger.attB.*Not*I.SV40 plasmid (11475 bp), and the lower zone is the GFP-expressing region, which was already cloned into the vector. In Lane 3, the upper zone is the linearized pUC57 vector and the lower zone is the UAS.CYP6BQ23 fragment (1987 bp).

3.1.2 Dephosphorylation and gel extraction of linearized plasmid vector

The vector was digested with *Not*I and dephosphorylated in order to prevent recircularization and self-ligation. The preparative gel is seen in Figure 14.

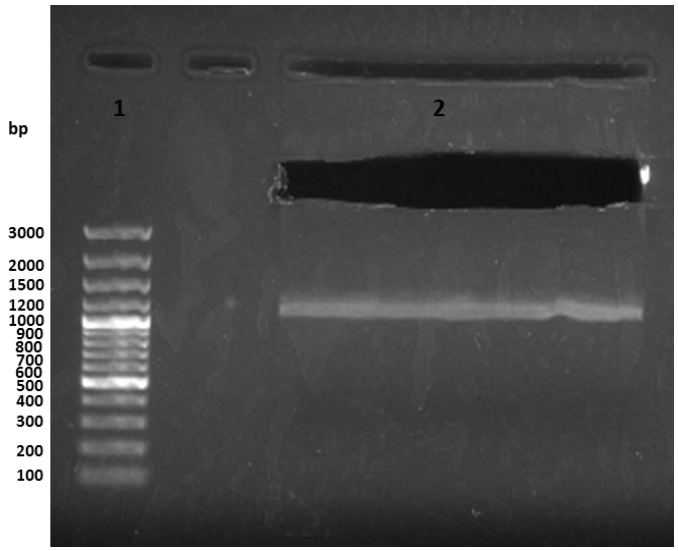


Figure 14. Gel extraction of pH_Stinger.attB vector. Lane 1: DNA Ladder GeneRuler Plus (Fermentas), Lane 2: The (missing) extracted band of vector and the GFP zone.

3.1.3 Ligation of pH_Stinger.attB.*Not*I.SV40 vector with the UAS.CYP6BQ23 insert, transformation in *E.coli* cells and detection of positive colonies

The pH_Stinger.attB.*Not*I.SV40 linearized vector and the UAS.CYP6BQ23 insert with *Not*I compatible ends were ligated. The ligation reaction was used to transform competent cells. 24 colonies were selected for alkaline lysis plasmid preparation and restriction digest screening. The plasmid DNA was digested with *Not*I and *Hind*III.

The *Not*I digest was performed in order to examine whether the colonies had integrated the UAS.CYP6BQ23 insert. Positive colonies are expected to have a DNA zone at 1987 bp. The digest products are seen in Figure 15.

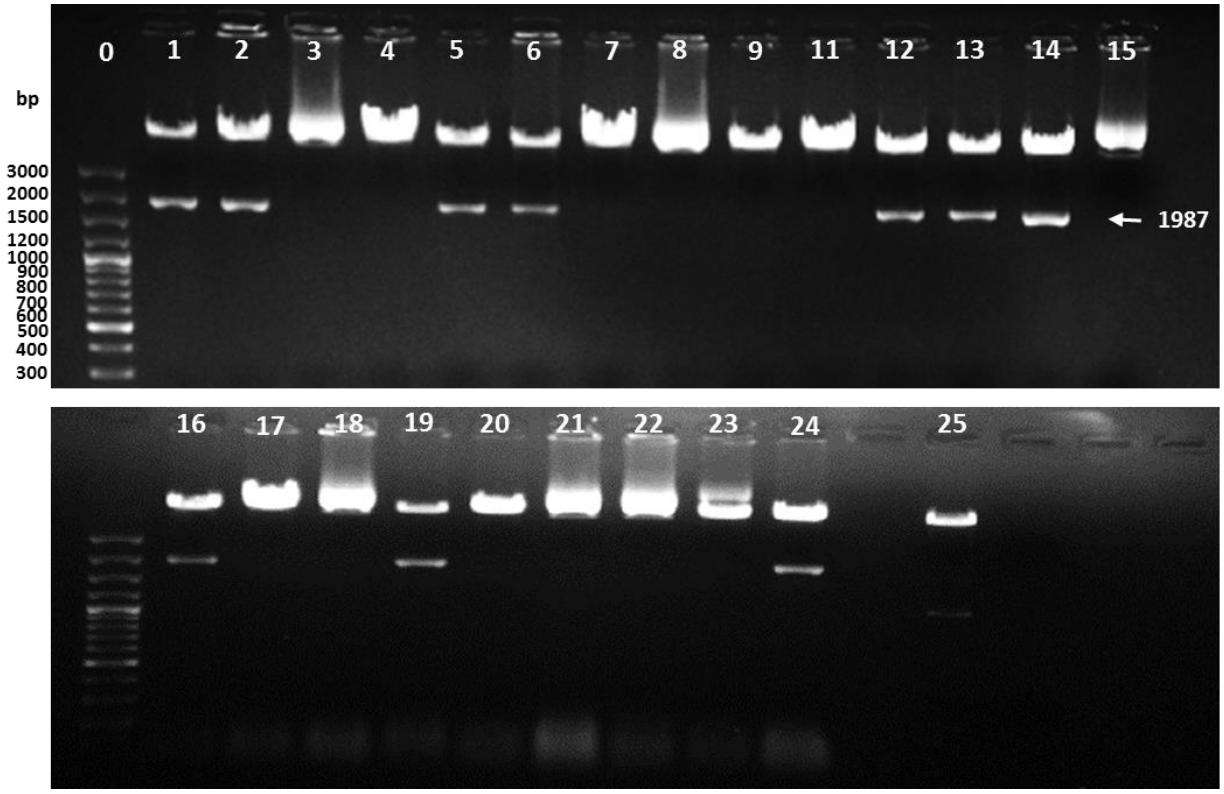


Figure 15. Diagnostic digests of pH_Stinger.attB.UAS.CYP6BQ23 plasmid with *Not*I. Lane 0: DNA Ladder GeneRuler Plus (Fermentas), Lanes 1-23: *Not*I digest products, Lane 24: pPelican.GFP digested with *Not*I.

The clones 1, 2, 5, 6, 12, 13, 14, 16, 19 and 24 were considered positive, due to the DNA band at 1987 bp, which indicates that they carry the UAS.CYP6BQ23 insert in their plasmid DNA. Furthermore, a *Hind*III digest was performed in order to verify whether the UAS.CYP6BQ23 insert has been inserted with the correct orientation.

The recombinant plasmid pH_Stinger.attB.UAS.CYP6BQ23 has 4 restriction sites for *Hind*III. If the UAS.CYP6BQ23 fragment has been inserted in one copy with the correct orientation, the *Hind*III digest must result in 4 DNA bands at 383, 483, 3318 and 7293 bp. On the contrary, if the UAS.CYP6BQ23 fragment has been inserted in the opposite orientation, the *Hind*III digest will also result in 4 DNA bands at 383, 2446, 3318 and 5328 bp. The digest results are seen in Figure 16.

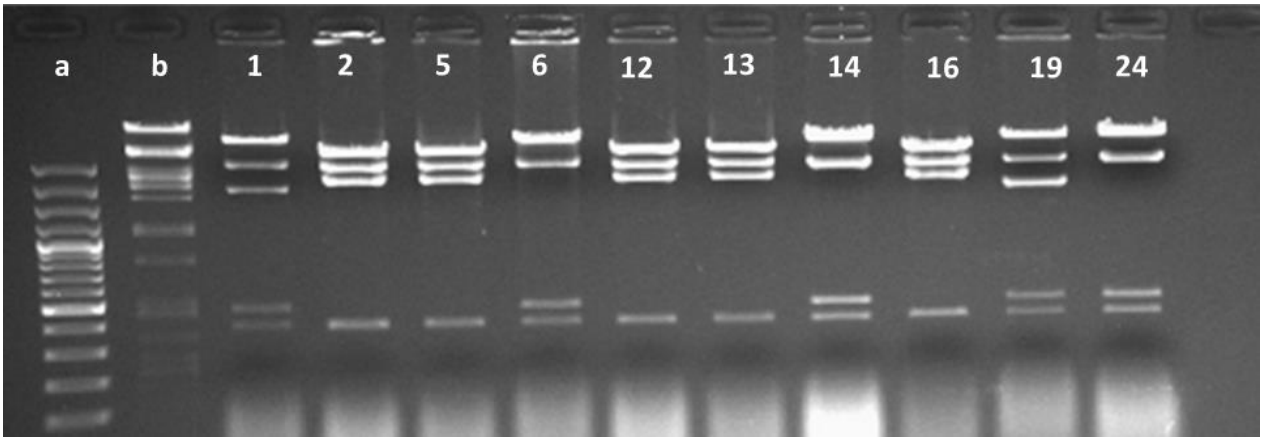


Figure 16. Diagnostic digests of pH_Stinger.attB.UAS.CYP6BQ23 candidate plasmid clones with *Hind*III. Lane a: DNA Ladder GeneRuler Plus (Fermentas), Lane b: Lambda DNA/ *Pst*I digested Ladder, Lanes 1-24: *Hind*III digested pH_Stinger.attB.UAS.CYP6BQ23.

The clones 6, 14 and 24 are positive due to the DNA bands at 483 and 7293 bp. These clones carry the UAS.CYP6BQ23 insert in the correct orientation, integrated in their plasmid DNA.

Subsequently, clone 24 was selected for plasmid midiprep extraction.

3.2 Functional analysis of *C. capitata* cytochrome CYP6A51 and generation of UAS.CYP6A51 construct

3.2.1 Functional analysis of C. capitata cytochrome CYP6A51

3.2.1.1 Functional expression of *C. capitata* CYP6A51 along with *Anopheles gambiae* Cytochrome P450 reductase (CPR) in *E. Coli*

The cytochrome P450 CYP6A51 with *Anopheles gambiae* CPR were functionally co- expressed in *E. coli* and the proteins were directed to the inner bacterial membrane using the signal sequence ompA. As seen in Figure 17, the characteristic peak at 450 nm indicated that CYP6A51 was expressed predominately in its P450 form, which is indicative of a good-quality, functional enzyme (Omura and Sato, 1964).

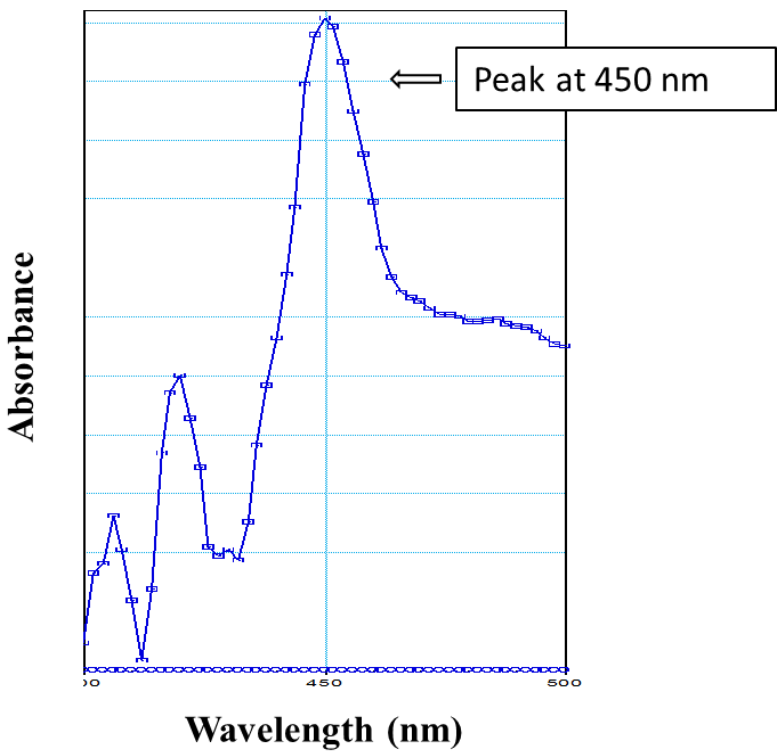


Figure 17. CO difference spectrum of bacterial membranes of *Ceratitis capitata* CYP6A51-AgCPR complex with 5.25 μM. P450 concentration (image courtesy of D. Tsakireli)

All the isolated bacterial membranes have the characteristic peak at 450nm, containing approximately 5,25μM P450 CYP6A51, whereas the cytochrome P450 reductase activity was in 205,4 μmole cytochrome c/min/g protein, as seen in Figure 18.

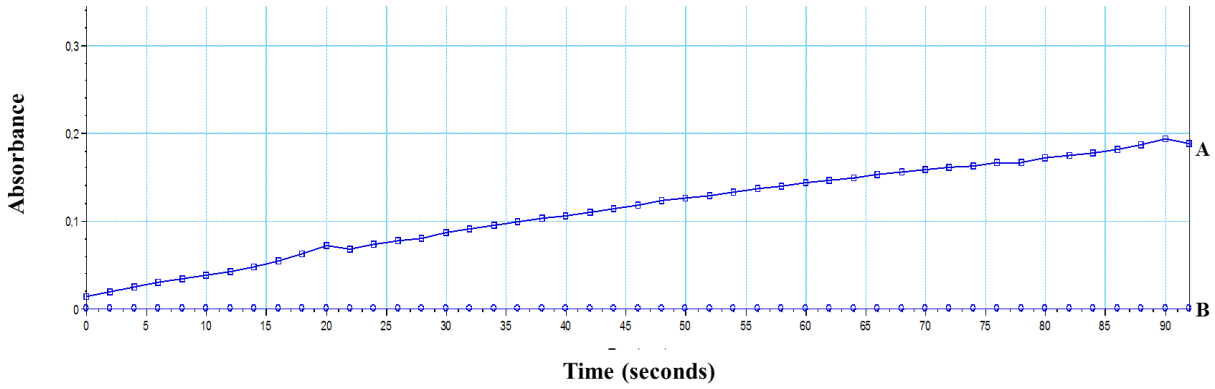


Figure 18. Kinetic diagram at 550nm of CYP6A51 bacterial membranes.
A: Sample with NADPH, B: Sample without NADPH.

3.2.1.2 Investigation of *C. capitata* CYP6A51 substrates using Glo assay

The metabolic activity of CYP6A51 was examined against model P450 fluorescent and chemi-luminescent substrates in order to verify the catalytic activity of recombinant enzyme and to identify

appropriate enzyme substrates. CYP36A51 showed a high rate of metabolism of the luciferin compound L-ME-EGE while the other substrates were metabolized at very lower rates, as seen in Figure 19.

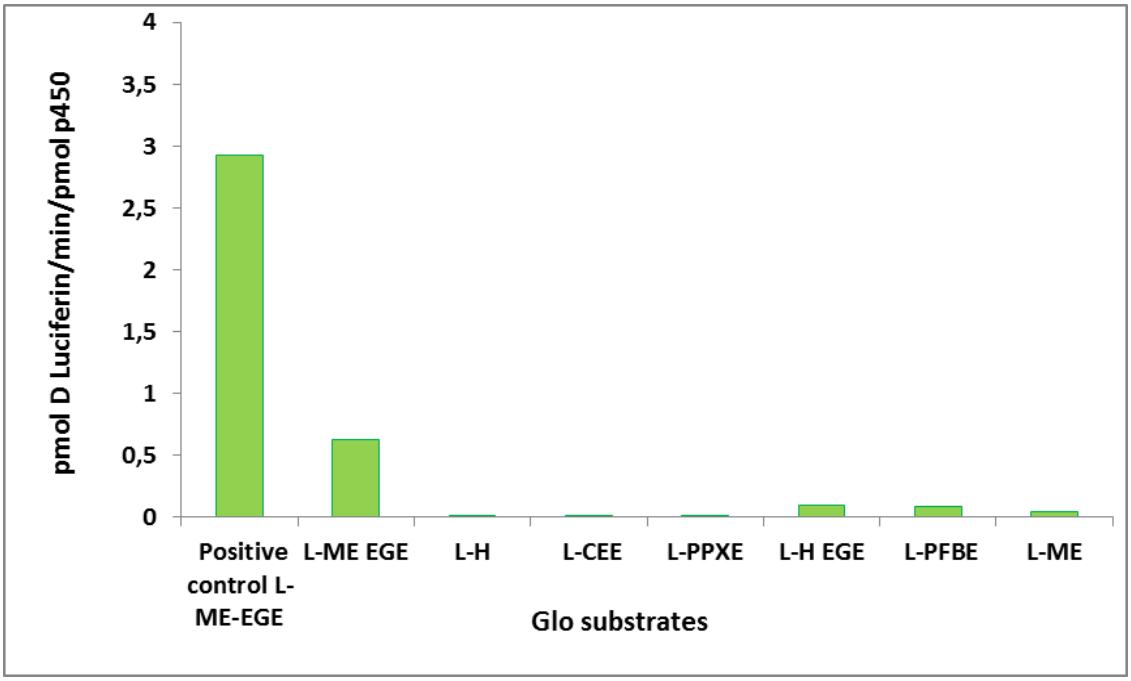


Figure 19. Glo assay of CYP6A51 membrane preparation (colony 1).The diagram indicates the metabolism rate of L-ME-EGE mediated by CYP392A16 used as a control and the metabolism rate of other Glo substrates mediated by CYP6A51.

3.2.1.3 Lambda-cyhalothrin metabolism assay

CYP6A51 was tested on its ability to metabolize lambda-cyhalothrin, due to its strong association with pyrethroid resistance.

The first experiment examined the ability of CYP6A51 to metabolize lambda-cyhalothrin and showed strong metabolism. Catalytic activity was assessed by measuring substrate depletion and formation of metabolites. NADPH-dependent depletion of lambda-cyhalothrin, eluted at 15.7 min and its metabolite, eluted at 7.1 min was observed after incubating the compound with the CYP6A51. Lambda-cyhalothrin elution was monitored by absorption at 230 nm and quantified by peak integration (Chromeleon, Dionex). The depletion of lambda-cyhalothrin was time-dependent, as seen in Table 13. The reactions were also carried out in the absence of a NADPH regenerating system and did not show any change compared to the control chromatogram.

Time	Substrate depletion %
0 hour	6,8 %
2 hour	93 %

Table 13. Depletion of lambda-cyhalothrin through incubation time mediated by CYP6A51.

The second experiment examined the ability of CYP6A51 to metabolize different concentrations of lambda-cyhalothrin. CYP6A51 (25 pmole of spheroplasts preparation) showed the higher catalytic activity at 10 μM of lambda-cyhalothrin in 2 hours incubation time, as seen on Table 14.

Lambda-cyhalothrin concentration (μM)	0 hours incubation Depletion %	2 hours incubation Depletion %
10	0	99.65
20	4	80.65
40	4	52
60	8	57

Table 14. Metabolism of Different concentrations of lambda-cyhalothrin mediated by CYP6A51.

The third experiment examined different concentrations of CYP6A51 on their ability to metabolize lambda-cyhalothrin. CYP6A51 showed catalytic activity against lambda-cyhalothrin (60 μM)**,** which was directly proportional to its concentration, as seen on Table 15.

CYP6A51 concentration (pmole)	0 hours incubation Depletion %	2 hours incubation Depletion %
5	0	13.3
10	1.6	21.4
25	9.6	26.4
40	8.5	40.5

Table 15. Metabolism of lambda-cyhalothrin mediated by different concentrations of CYP6A51.

The HPLC chromatogram indicate that in the presence of a NADPH regenerating system, metabolites of lambda-cyhalothrin are 10 times higher, than in the absence of NADPH, as seen in Figure 20.

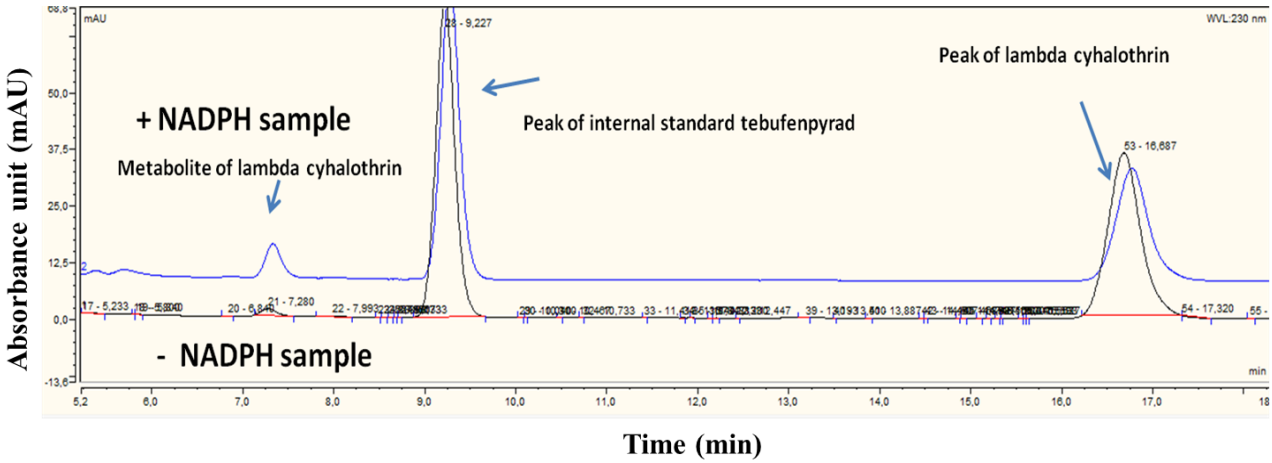


Figure 20. HPLC-UV chromatogram of the lambda cyhalothrin analysis with CYP6A51 p450. (Image courtesy of D. Tsakireli)

3.2.2 Generation of UAS.CYP6A51 expression construct

3.2.2.1 PCR amplification of UAS region from pUAST.Casper.A16

The UAS region was amplified from the vector pUAST.Casper.A16 with the primers UAS2F and UAS2R. As seen in Figure 21, the UAS amplicon has the expected size of 400 base pairs.

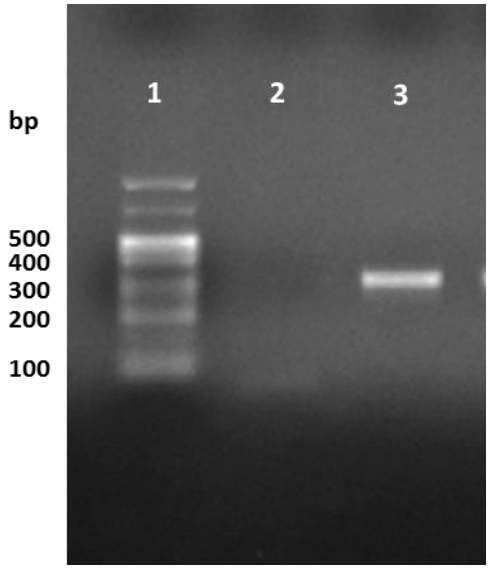


Figure 21. PCR for amplification of UAS region from pUAST.Casper.A16. Lane 1: DNA Ladder GeneRuler Plus (Fermentas), Lane 2: Negative control without DNA, Lane 3: UAS amplicon.

3.2.2.2 Restriction digest of UAS amplicon and pGEM.CYP6A51 vector with *Sgr*BI

The UAS amplicon and the pGEM.CYP6A51 plasmid were digested with *Sgr*BI. A relevant gel is seen in Figure 22.

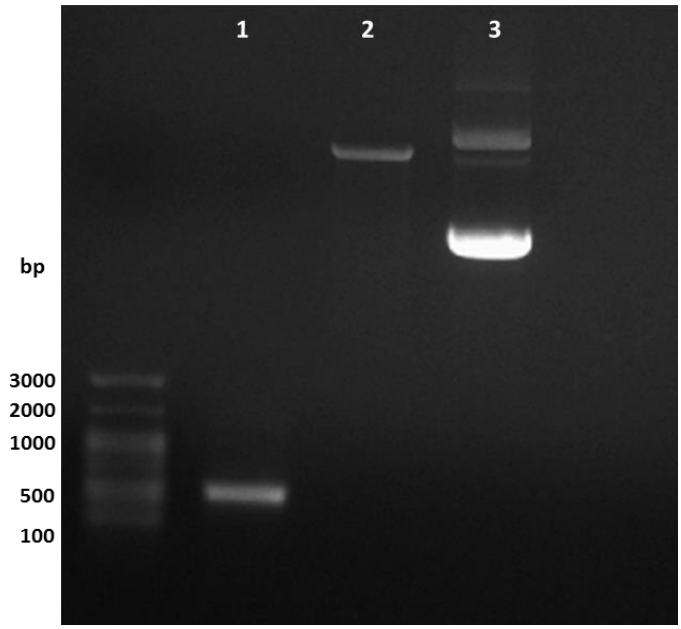


Figure 22. Restriction digest of UAS amplicon and pGEM.CYP6A51 vector with *Sgr*BI. Lane 1: UAS amplicon. Lane 2: undigested pGEM.CYP6A51 plasmid, Lane 3: pGEM.CYP6A51 plasmid *Sgr*BI digested.

Then, the linearized pGEM.CYP6A51 vector was dephosphorylated and in parallel with the UAS insert they were purified from gel as previously described.

3.2.2.3 Ligation of pGEM.CYP6A51 vector with the UAS insert, transformation in *E.coli* cells and detection of positive colonies

The pGEM.CYP6A51 vector and the UAS insert with *Sgr*BI compatible ends were ligated. The ligation reaction was transformed in bacteria. For the detection of colonies that had integrated the insert, 26 colonies were selected for colony PCR screening. The primers used in colony PCR were the T7 and the UAS2R. The PCR products were electrophoresed in 1% w/v agarose gel and the results are seen in Figure 23.

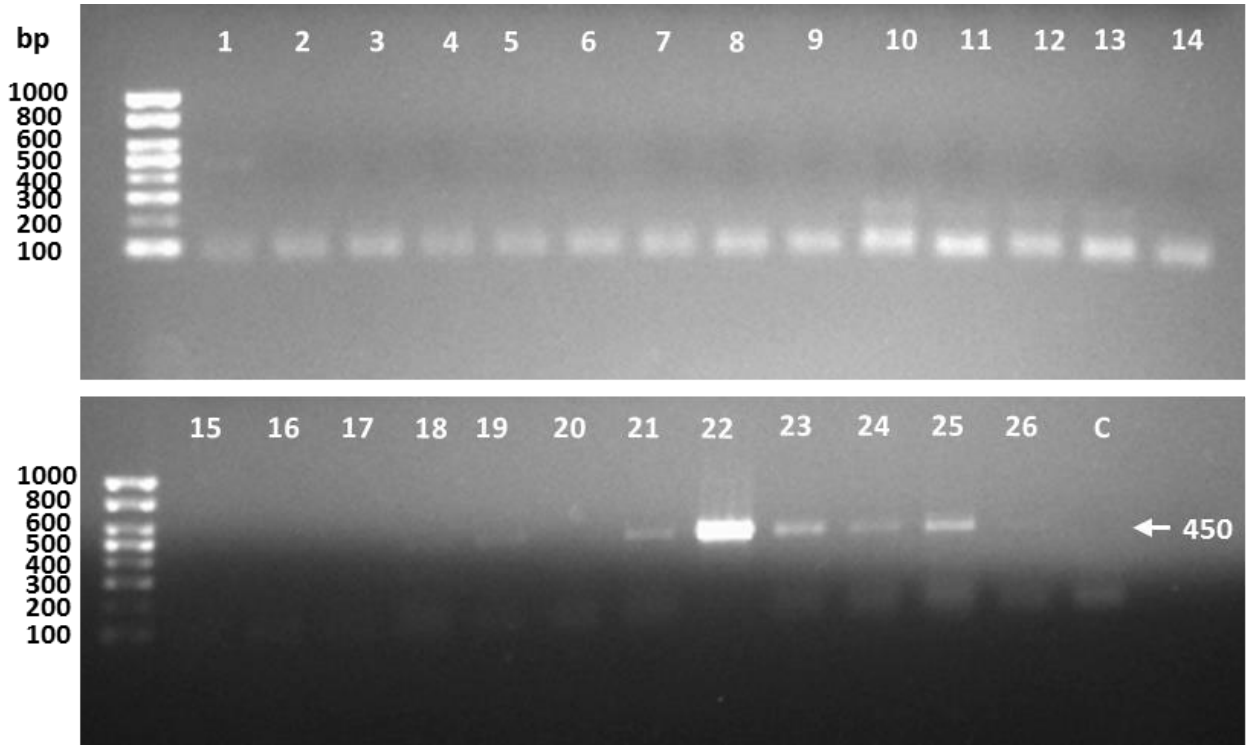


Figure 23. Colony PCR with the primers T7 and UAS2R for the detection of positive colonies.

As seen in Figure 23, the colonies 1, 19, 21, 22, 23, 24, 25 and 26 were positive, because they had a DNA band at 450 base pairs. The colony 22 was selected for plasmid preparation and sequencing with T7 primer.

The sequencing verified that the recombinant plasmid contained the expected 5xUAS_basal promoter sequence at the correct orientation upstream of the CYP6A51 ORF, thus we have generated plasmid pGEM.UAS.CYP6A51.

3.2.2.4 Restriction digest of pGEM.UAS.CYP6A51 vector with *Not*I

The pGEM.UAS.CYP6A51 plasmid was digested with *Not*I, resulting in a UAS.CYP6A51 fragment flanked with *Not*I restriction ends. The digest's product was electrophoresed in 1% w/v agarose gel and the corresponding band was excised and purified as described previously. The preparative gel is seen in Figure 24.

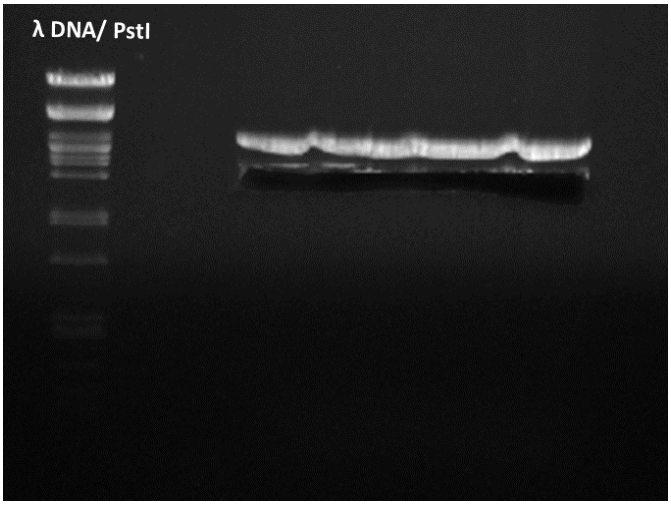


Figure 24. Gel extraction of linearized UAS.CYP6A51 fragment.

3.2.2.5 Ligation of pH_Stinger.attB.*Not*I.SV40 vector with the UAS.CYP6A51 insert, transformation in *E. coli* cells and detection of positive colonies

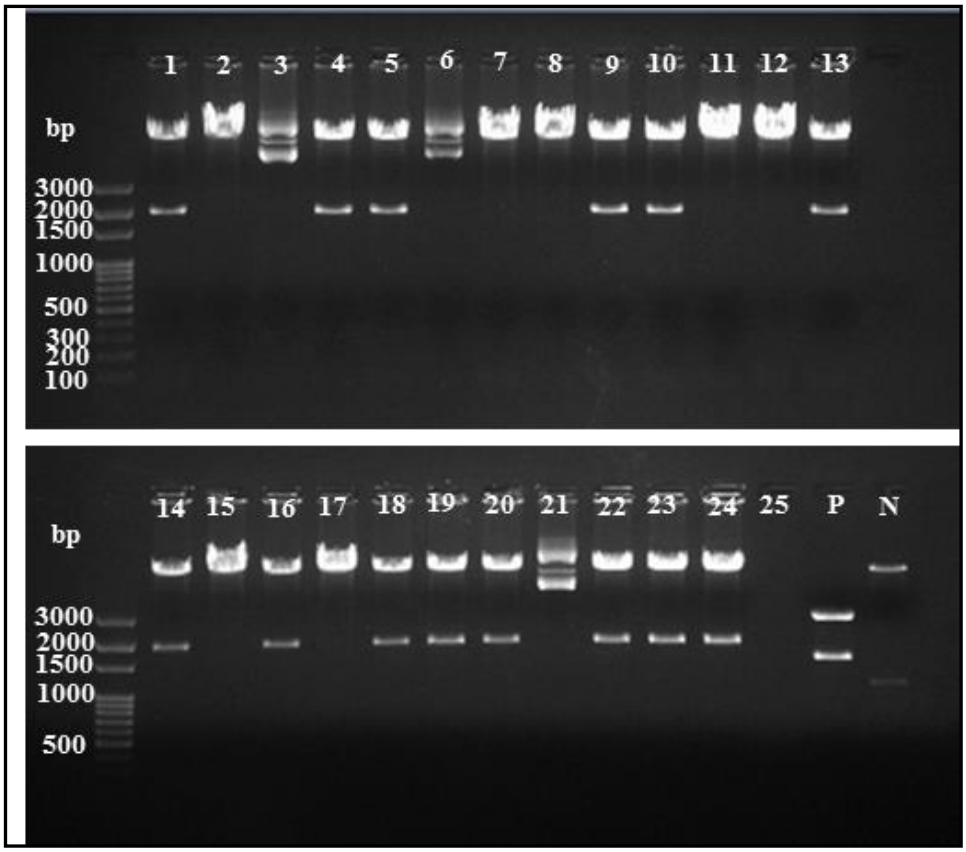
The linearized with *Not*I, dephosphorylated vector pPelican.attB.NotI.SV40 and the UAS.CYP6A51 insert with *Not*I compatible ends were ligated and the ligation reaction was transformed in *E. coli.*

24 colonies were selected for alkaline lysis plasmid preparation and restriction digest screening. The plasmid DNA was digested with *Not*I and *Hind*III.

The *Not*I digest was performed in order to examine whether the colonies had integrated the UAS.CYP6A51 insert. Positive colonies are expected to have a DNA zone at 1931 bp.

On the other hand, the subsequent *Hind*III digest in positive clones was performed in order to verify whether the UAS.CYP6A51 insert has been inserted with the correct orientation. Colonies with the correct orientation of the insert are expected to have 4 DNA zones at 383, 483, 3318 and 5957 bp. The digest products are seen in Figure 25.

a



b

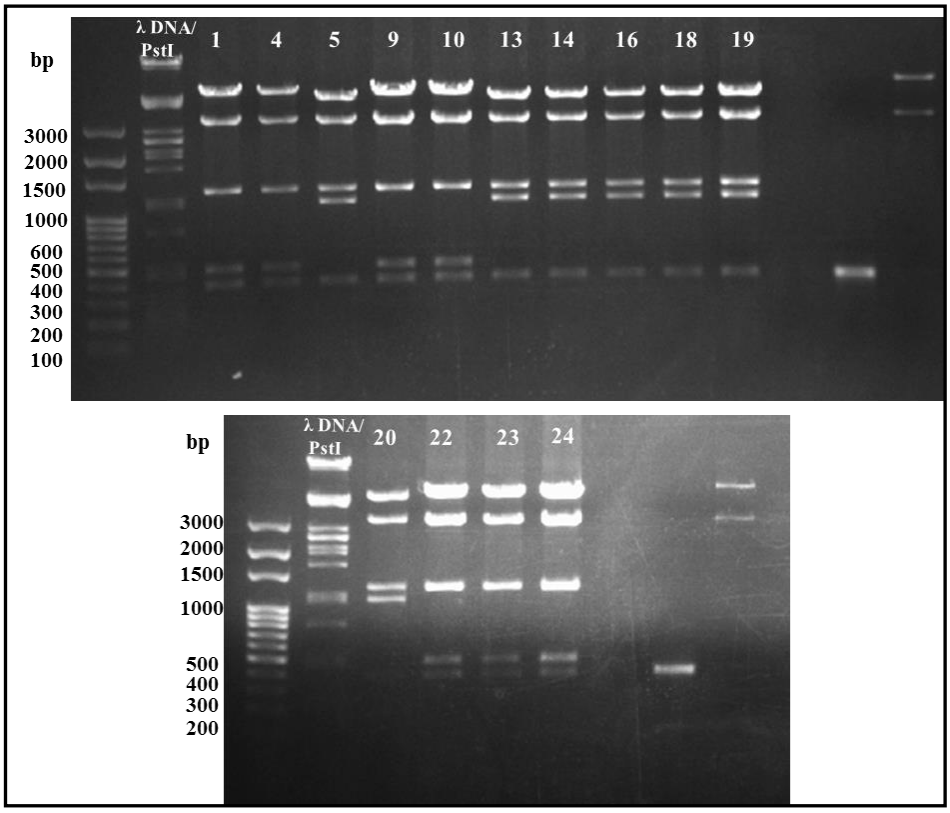


Figure 25. Restriction digest for the detection of positive colonies. a: *Not*I digest. b: *Hind*III digest.

As seen in Figure 25, only the plasmids that confirmed to be positive after *Not*I digest (colonies 1, 4, 5, 9, 10 and 13) were further digested with *Hind*III. The plasmid DNA from colony 1 was selected for plasmid midiprep. Finally, the purified plasmid was used for injection mixture preparation.

3.3 Ectopic expression of CYP6BQ23 and CYP6A51 in *Drosophila melanogaster*

Starting from male injected flies, four transgenic UAS.CYP6BQ23 and one UAS.CYP6A51 lines were generated. The resulting populations have the transgene present in the 2nd chromosome, but they do not overexpress it. The UAS.CYP6BQ23 lines were further crossed with the HR-GAL4 driver, in order to overexpress the transgene in the fat body, malpighian tubule and midgut of the progeny, to be used for contact bioassays.

3.4 Contact bioassays with Deltamethrin

The bioassay was conducted with the progeny of the cross of homozygous flies UAS.CYP6BQ23 Line 33 (generated from male injected fly) with HR-GAL4 driver. In addition, the progeny of the cross of w^{1118}, the genetic background strain of HR-GAL4 driver, with homozygous UAS.CYP6BQ23 flies were used as a control.

The resistance levels of mutant flies to deltamethrin were measured with contact bioassays. Each Deltamethrin dose was tested on triplicates. Resistance was measured by comparing LC_{50} values of control and mutant flies. LC_{50} represents the lethal concentration of an insecticide that kills half the population of treated individuals.

Strain	LC_{50} (mg/L)	95% CI	Slope ± SE	χ^2 (DF)	RR
Control	109.128	73.293 – 214.052	1.886 ± 0.207	56.820 (16)	1
Mutant UAS.CYP6BQ23	715.791	267.142 – 4750.611	1.012 ± 0.182	71.382 (22)	6.559

Table 16. Bioassay with Deltamethrin. LC_{50}: Lethal dose 50. CI: Confidence interval. DF: Degrees of freedom. RR: Resistance ratio.

As seen in Table 16, the LC_{50} for deltamethrin of control flies (progeny of UAS.CYP6BQ23 x w^{1118}) was 109.128 while the LC_{50} of mutant flies (progeny of UAS.CYP6BQ23 x HR-Gal4) was 715.791.

Additionally, the flies that overexpress the CYP6BQ23 (expression in the fat body, malpighian tubules and midgut) have a resistance ratio 6.559 higher compared to control flies.

In Figure 25, a diagram which shows the response of control and mutant flies in deltamethrin is presented. Control flies survived the dose of 200 mg/L, while mutant flies survived even the dose of 1000 mg/L.

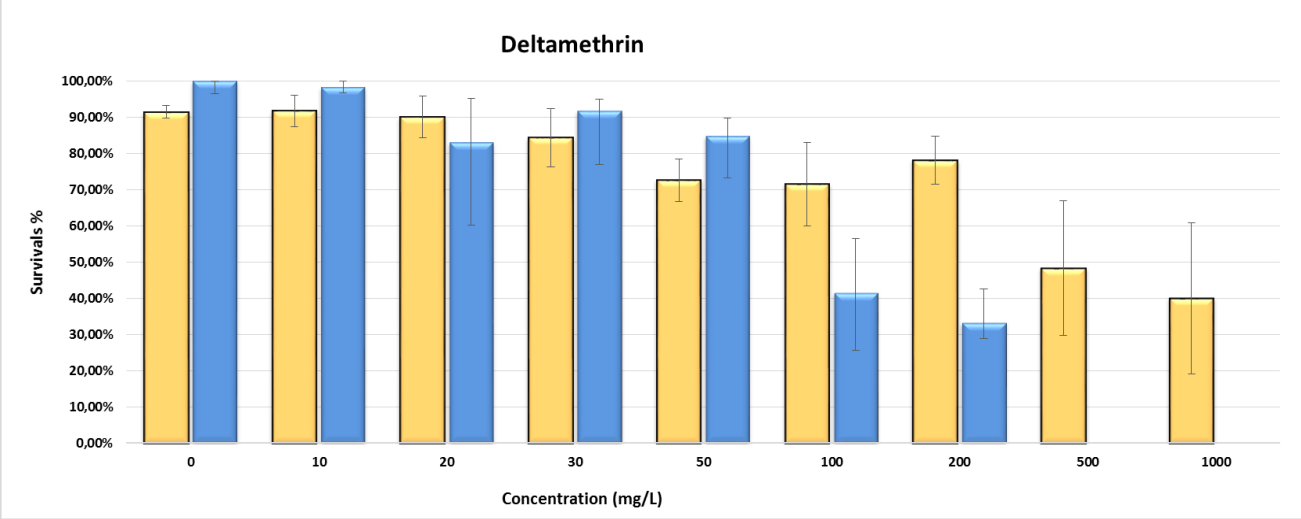


Figure 25. Bioassay with Deltamethrin. Yellow: Progeny of UAS.CYP6BQ23 x HR-Gal4 cross. Blue: Progeny of 0.

3.5 Targeted genome editing using CRISPR/Cas9 system for introduction of *Plutella xylostella* G4946E mutation in *Drosophila melanogaster*

3.5.1 Construction of gRNA containing plasmid

The last exon of RyR gene of *Drosophila melanogaster* was sequenced in order to ensure the matching between the published genome sequence and the genomic sequence of strain used. The donor plasmid which contains the RyR region with the desired changes, was synthesized by GenScript (USA) into a pUC57 vector.

The gRNA was generated as described in Materials & Methods, subcloned into a pU6-BbsI-chiRNA vector and transformed into *E.coli.* Afterwards, 12 colonies were tested with colony PCR using the standard primer T7 and the oligo 4946R. The PCR products were electrophoresed in a 1% w/v agarose gel and are seen in Figure 26.

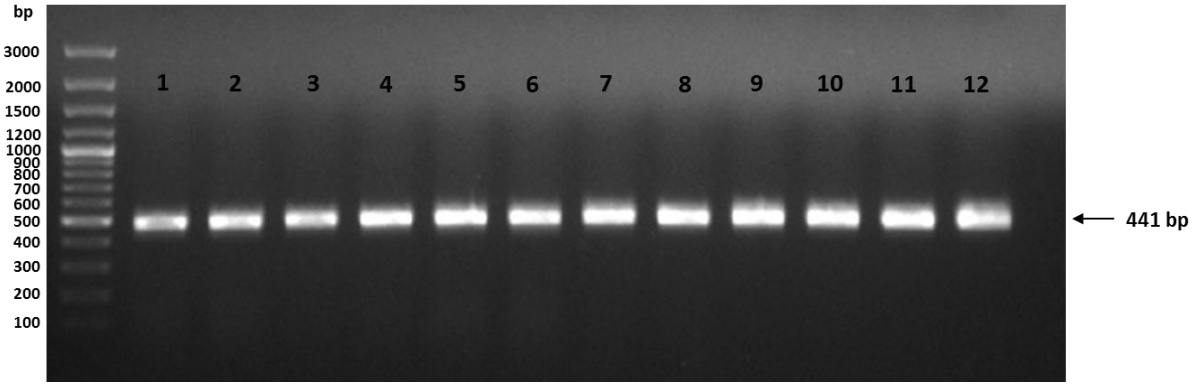


Figure 26. Colony PCR for gRNA-containing plasmid

As seen on Figure 26, all clones were positive, because they all had the diagnostic DNA band at 441 bp meaning that they have integrated the gRNA target sequence in their plasmid DNA. The first four clones were selected and the extracted DNA was sequenced.

The sequencing confirmed the correct insertion of the gRNA sequence fragment for all clones. The plasmid DNA of clone number 1 was used for the preparation of the injection mix.

3.5.2 Genetic crosses to generate flies with the point mutation G4946E in RyR

After injections, first instar larvae were picked and placed into regular larval food and 19 of them recovered the injections and became adults. The adult injected flies (G_0) were crossed with nanos-Cas9 (#54591) flies and the G_1 progeny were screened. The screening process includes DNA

extraction in pools of 30 larvae followed by PCR with primers specific for the desired mutations. The cross was performed as follows:

$$\frac{\mathbf{M/wt}}{\mathbf{wt}} \times \frac{\mathbf{wt}}{\mathbf{wt}}$$

The letter "M" indicates the mutation, and the "wt" is an abbreviation for wild type, referring to the non-mutated allele. The gel in Figure 27, shows the molecular screening applying the two methods.

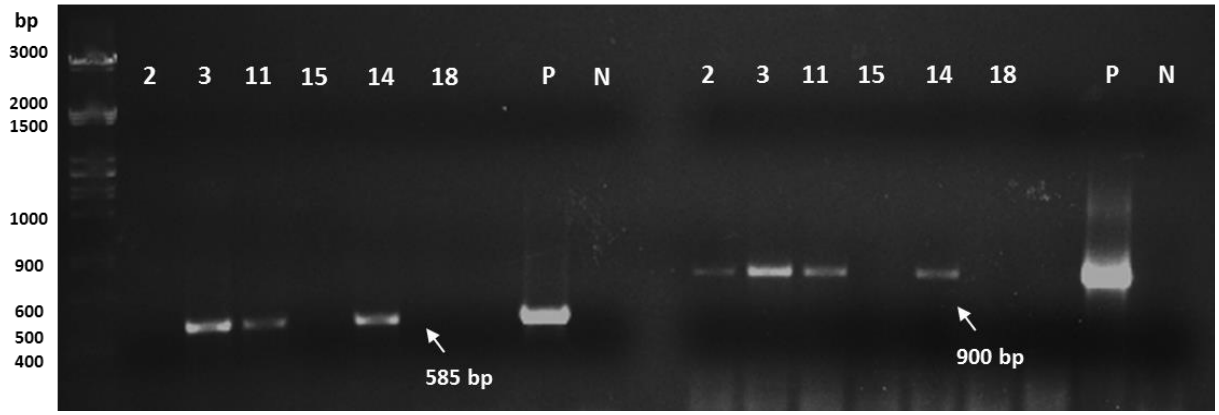


Figure 27. Molecular screening. Left: PCR with the specific primer GENEFOR, which detects the point mutation G4946E and GENEREV. Right: PCR with the generic set of primers, GENEFOR and the primer GENEREV, after the restriction digest of the template DNA with *Pst*I, which cleaves only the wild type allele. Lines 2, 3, 11 and 14 are positive for the mutation. P stands for positive control (donor plasmid) and N stands for negative control (nanos-Cas 9 genomic DNA).

As seen in Figure 26, the lines 3, 11 and 14 are considered positive, either by the PCR method with specific primers, or by the method based on *Aat*II Restriction digest. The lines 2, 15 and 18 are negative, as confirmed by both methods. Line 2, is positive with the *Aat*II digest method, but negative with the PCR method. However, line 2 was considered as positive and used for further crosses.

The genotype of injected flies can be either $\frac{\mathbf{M}}{\mathbf{wt}}$ (mutated), if a CRISPR/HDR event took place, or $\frac{\mathbf{wt}}{\mathbf{wt}}$ (wild type), if the CRISPR event did not take place. It is important to mention that the injected flies, even if they are CRISPR-modified, they do not actually carry the desired mutation in their somatic cells, but only in some cells of their germ line, where the Cas9 is expressed. However, these flies can produce progeny heterozygous for the mutation.

The potentially positive G_1 flies, from lines 2, 3, 11 and 14, were subsequently crossed with the balancer *yw;CyO/Sco*, as follows.

$$\frac{M}{wt} \times \frac{CyO}{Sco}$$

At this stage the molecular screening was done in individual parental flies and it indicates that if a positive allele is found then the parental fly was heterozygous for the desired mutation. The gel of a PCR-based screening is seen in Figure 28.

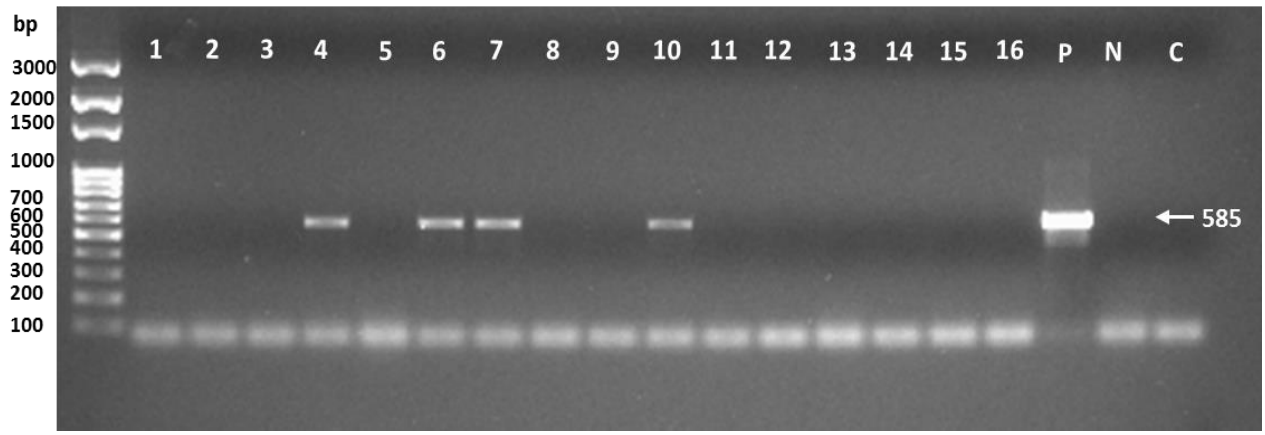


Figure 28. Molecular screening of Line 2. PCR approach with the specific primer GENEFOR and the primer GENEREV. Individuals 4, 6, 7 and 10 are positive for the mutation. P stands for positive control (donor plasmid) and N stands for negative control (nanos-Cas 9 genomic DNA).

In Figure 28, G_1 parental flies were screened with the PCR method. These results refer to line 2, but the picture is indicative for the other lines. In line 2, four G_1 flies were found to be positive for the desired mutation. Lines 3, 11 and 14 also had positive G_1 flies.

After this point, the selection of progeny was based on phenotypic traits. The desired G_2 progeny from this cross have curly wings, which means that they carry the balancer chromosome. However, given that the parent is heterozygous, only half of the progeny with curly wings would carry the mutated allele.

The F2 progeny with curly wings were crossed again with the balancer *yw;CyO/Sco*, as follows:

$$\frac{M}{wt} \times \frac{CyO}{Sco}$$

The parental flies were screened for the mutation and the results are shown in Figure 29.

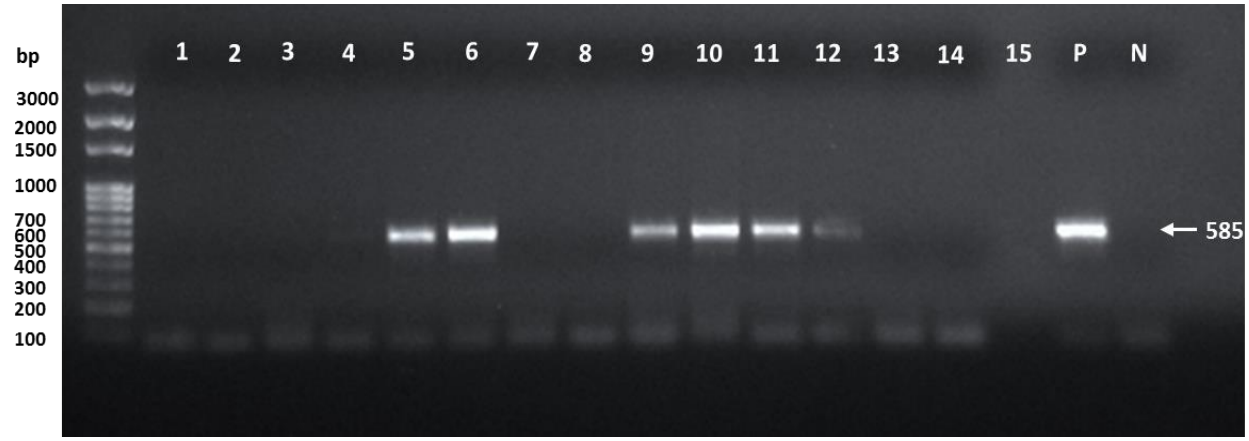


Figure 29. Molecular screening of Line 2. PCR approach with the specific primer GENEFOR and the primer GENEREV. Individuals 5, 6, 9, 10, 11 and 12 are positive for the mutation. P stands for positive control (donor plasmid) and N stands for negative control (nanos-Cas 9 genomic DNA).

In Figure 29, G_2 parental flies were screened with the PCR method. The results refer to line 2 and reveal flies positive for the mutation. The other three lines were tested accordingly.

In this cross, the selection of progeny is also based on phenotype. Half of the G_3 progeny with curly wings carry the mutated allele, while the other half carries the scutoid allele, which can easily be recognized.

The G_3 heterozygous flies with curly wings and wild type scutellum are selected and sub-mated, as follows.

$$\frac{M}{CyO} \times \frac{M}{CyO}$$

All the above crosses are briefly depicted in Figure 30.

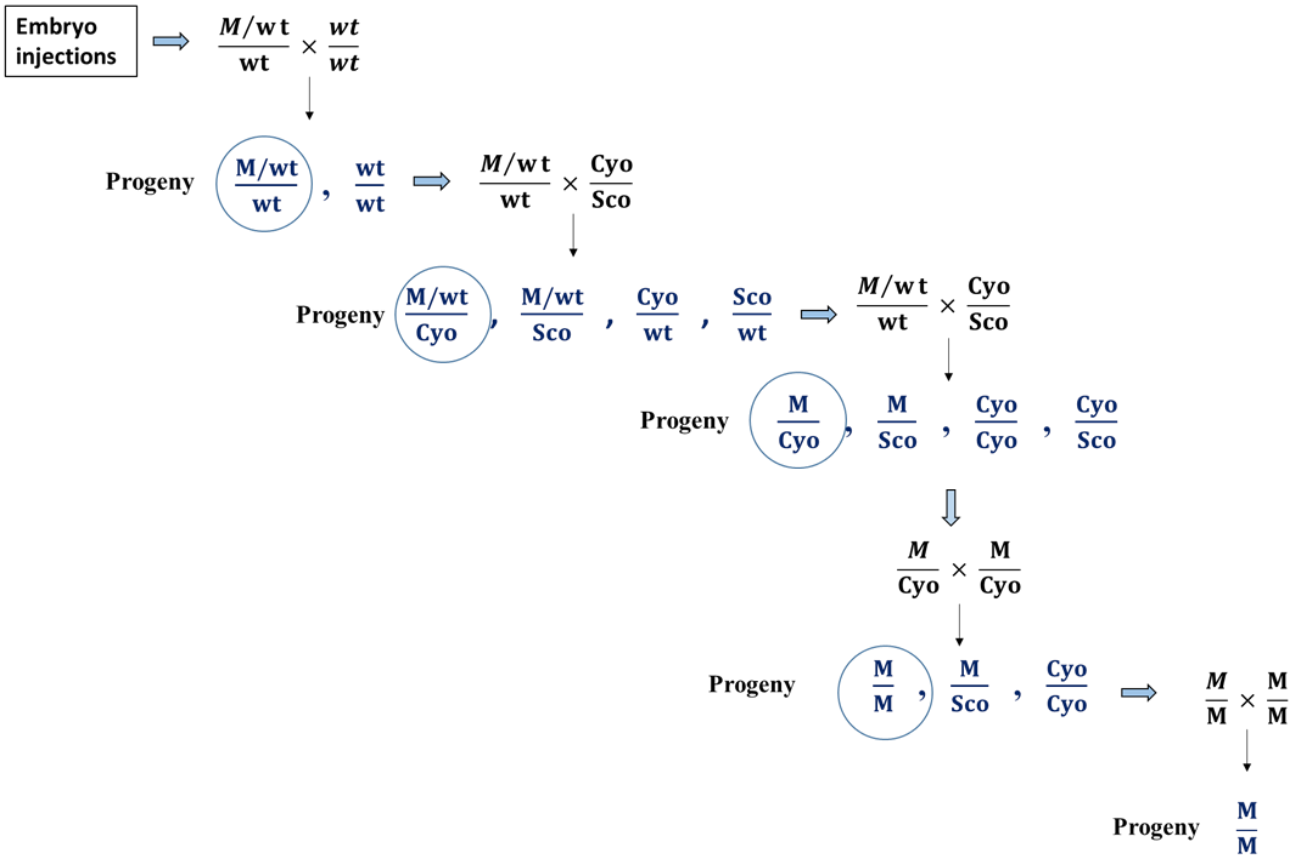


Figure 30. The crossing scheme for rescue of G4946E mutation in RyR gene. The progeny of each cross are depicted blue. The desired progeny are circled.

However, this cross did not result in homozygous flies and all the progeny were heterozygous. Individual progeny were sequenced and the heterozygous status was confirmed, as seen in Figure 31.

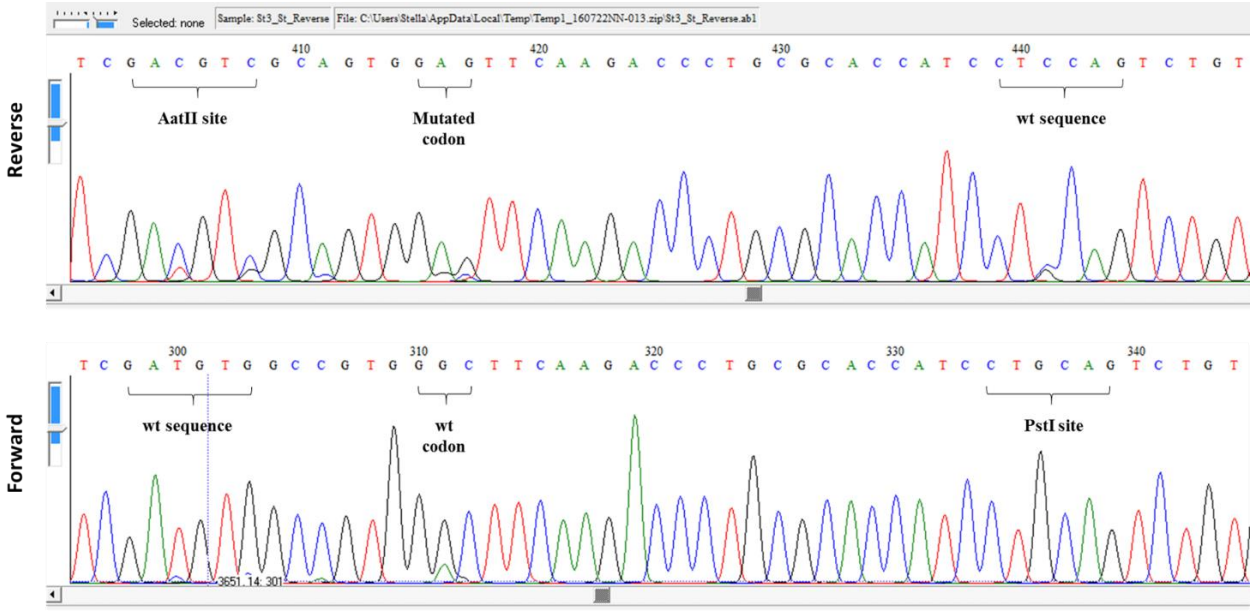


Figure 31. Sequencing of RyR of heterozygous flies. The sequencing confirmed the heterozygosity.

3.5.3 Feeding bioassays with Diamides

The resistance levels of control nanos-Cas9 (#54591) flies to diamides were measured with feeding bioassays. Each insecticide dose was tested on triplicates. LC_{50} represents the lethal concentration of an insecticide that kills half population of treated individuals. The LC_{50} values were calculated with the program PoloPlus, LeOra Software Company.

On Table 17, LC_{50} values of control nanos-Cas9 (#54591) flies to both diamides are described.

Insecticide	LC_{50} (ppm)	95% CI	Slope ± SE	χ^2 (DF)	RR
Flubendiamide	7.118	6.514 – 7.643	10.578 ± 1.188	44.982 (22)	1
Chlorantraniliprole	0.468	0.409 – 0.533	0.353 ± 1.047	20.314 (22)	1

Table 17. Bioassay with Diamides. LD_{50}: Lethal dose 50. CI: Confidence interval. DF: Degrees of freedom. RR: Resistance ratio.

As shown in Table 16, the LC_{50} for flubendiamide of control nanos-Cas9 (#54591) flies was 7.118 while for chlorantraniliprole was 0.468.

In Figures 32 and 33, the diagrams show the response of control nanos-Cas9 (#54591) flies in flubendiamide and chlorantraniliprole, rspectively. The nanos-Cas9 (#54591) flies survived exposure in flubendiamide until the dose of 14.7 mg/L, while for chlorantraniliprole, they survived the dose of 0.4 mg/L.

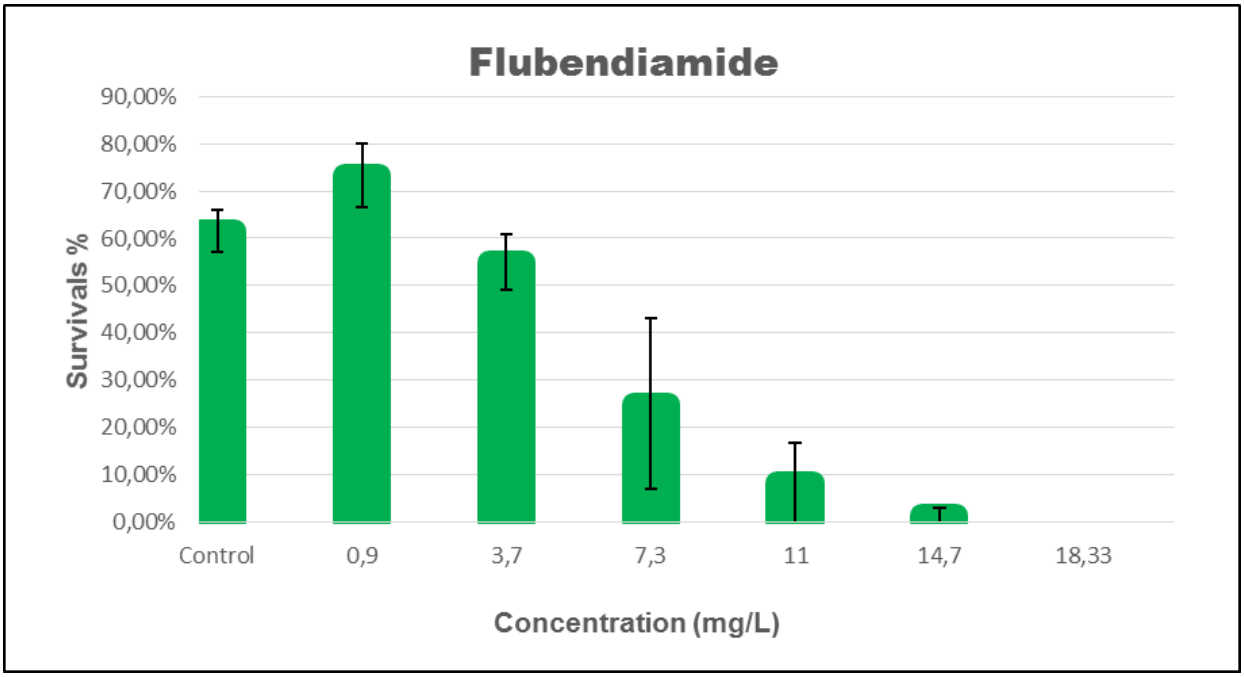


Figure 32. Bioassay with Flubendiamide of control nanos-Cas9 (#54591) flies.

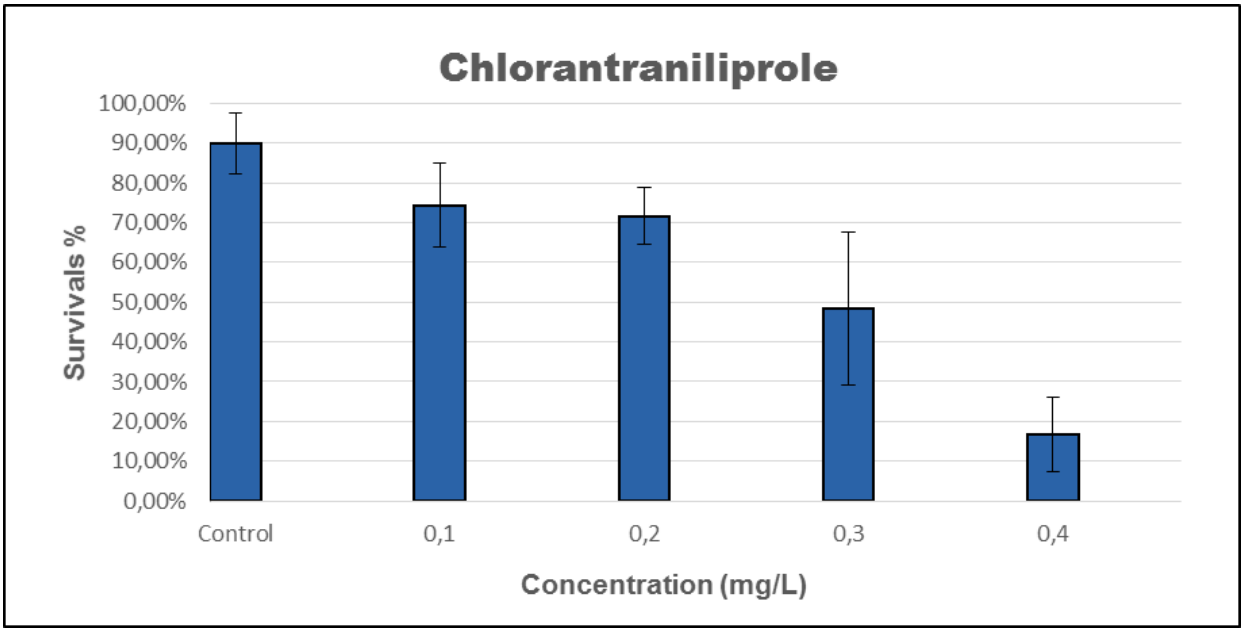


Figure 33. Bioassay with Chlorantraniliprole of control nanos-Cas9 (#54591) flies.

4. Discussion

4.1 Ectopic expression of P450s in *Drosophila melanogaster*

The objective of this part was the ectopic expression of two P450 genes, Cyp6bq23 of pollen beetle (*Meligethes aeneus*) and Cyp6a51 of the Mediterranean fly (*Ceratitis capitata),* in the model organism *Drosophila melanogaster* and the evaluation of susceptibility of transgenic flies to pyrethroids. Both *Meligethes aeneus* and *Ceratitis capitata* are destructive agricultural pests and they have long developed pyrethroid resistance (Arouri *et al*., 2014; Hansen, 2003; Slater et al., 2011; Thieme *et al*., 2010).

The Cyp6bq23 gene of *Meligethes aeneus* was selected for ectopic expression in *Drosophila*, because in a recent study, it was revealed that Cyp6bq23 was highly overexpressed in resistant populations of pollen beetle in Europe, implying that CYP6BQ23 is associated with the resistance phenotype (Zimmer *et al*., 2014). In the same study, microsomal preparations of pollen beetle resistant strains showed that CYP6BQ23 was able to metabolize deltamethrin and when it was functionally expressed in Sf9 insect cells together with the CPR, it was also able to metabolize deltamethrin and tau-fluvalinate. In addition, docking simulations of the predicted CYP6BQ23-deltamethrin binding showed that deltamethrin docks close to heme center of CYP6BQ23 active site, implying a possible mechanism of deltamethrin metabolism (Zimmer *et al*., 2014).

Furthermore, a protein sequence alignment showed that CYP6BQ23 of pollen beetle is more similar to cytochromes P450 of the red flour beetle, *Tribolium castaneum* (Zimmer *et al*., 2014). However, in Coleoptera, until now, only a single P450 gene, the CYP6BQ9 of *T. castaneum*, has been identified to confer pyrethroid resistance (Zhu *et al*., 2010). CYP6BQ9 is a brain-specific P450 and was found to be over 200-fold overexpressed in deltamethrin-resistant *T. castaneum* populations and was also shown to metabolize deltamethrin *in vitro* (Stevenson *et al*., 2011, Stevenson *et al*., 2012).

In the present study, we successfully expressed the *M. aeneus* cytochrome P450 CYP6BQ23, in *Drosophila melanogaster*. Homozygous *Drosophila* lines with Cyp6bq23 gene integrated into the second chromosome were obtained, using the site-specific phiC31 integrase technology (Bischof *et al*., 2007; Groth *et al*., 2004). In order to ectopically express CYP6BQ23, we used the GAL4/UAS system. One line was selected and crossed to HR-GAL4 driver (Chung *et al*., 2007), which drives expression in the midgut, malpighian tubules and fat body of the progeny. These tissues were selected

for overexpression because they are considered as the primary detoxification organs, where most insect P450s are expressed (Feyereisen, 2005).

A contact bioassay with deltamethrin was conducted, using a) 1–5 days old adult progeny of UAS.CYP6BQ23 x HR-GAL4 cross that overexpress CYP6BQ23 and b) 1–5 days adult old progeny of UAS.CYP6BQ23 x w^{1118} cross that have a similar genetic background and do not overexpress CYP6BQ23. The progeny that express CYP6BQ23 showed a significant resistant phenotype compared with the progeny of the control cross (Table 5). This finding suggests strong evidence for the role of *M. aeneus* CYP6BQ23 in conferring deltamethrin resistance. Analysis of doses mortality showed that UAS.CYP6BQ23 x HR-GAL4 progeny have a deltamethrin LD_{50} at 716 mg/L (95% confidence limits: 461-1011 mg/L), while UAS.CYP6BQ23 x w^{1118} progeny have a LD_{50} at 109 mg/L (95% confidence limits: 73-214 mg/L). The resistance ratio of flies overexpressing CYP6BQ23 was 6.5 times higher compared to control. This resistance ratio is consistent with that, previously reported for transgenic *Drosophila* lines over-expressing the cytochrome P450 CYP9J28 of *Aedes aegypti* (Pavlidi et al., 2012) and also higher than that of transgenic lines over-expressing *D. melanogaster* cytochrome P450s, responsible for resistance in DDT, nitenpyram, dicyclanil and diazinon (Daborn *et al*., 2007)

In order to further decipher the role of CYP6BQ23, a contact bioassay with tau-flluvalinate needs to be conducted in order to *in vivo* evaluate the susceptibility of transgenic flies, since it has been reported that tau-flluvalinate is metabolized when incubated with microsomes from Sf9 insect cell line that express recombinant CYP6BQ23 (Zimmer *et al*., 2014). Furthermore, mRNA levels of *M. aeneus* CYP6BQ23 need to be determined in progeny of UAS.CYP6BQ23 x HR-GAL4 cross by RT-PCR, in order to confirm the expression of the transgene. Subsequently, taking into consideration that CYP6BQ23 shows high amino acid identity to *T. castaneum* CYP6BQ9, which is a brain-specific cytochrome P450, it would be critical to establish a transgenic *Drosophila* line that would overexpress CYP6BQ23 in the nervous system. This could be achieved by crossing the UAS.CYP6BQ23 flies with nervous system specific drivers, like the neuronal driver ELAV. The progeny of this cross will be tested in contact bioassays regarding its susceptibility to deltamethrin or tau-fluvalinate. Moreover, the establishment of a transgenic line that would ectopically express the CYP6BQ23 in isogenic flies with kdr mutations, which are also linked to pyrethroid resistance in pollen beetle (Rinkevich *et al*., 2013; Nauen *et al*., 2012), would help in the investigation of multifactorial resistance and would facilitate the elucidation of the contribution of each mechanism in the resistant phenotype.

Finally, the ectopic expression *M. aeneus* CYP6BQ23 in *Drosophila melanogaster* demonstrates a successful example of its utility as a model in order to evaluate the role of resistance associated genes. This study provides functional *in vivo* evidence for the role of CYP6BQ23 in deltamethrin resistance

of pollen beetle and can be used as a robust approach in similar studies, along with classical approaches for studying insecticide resistance. In addition, the establishment of transgenic *Drosophila* lines that overexpress resistance genes, may also contribute to test the efficacy of novel compounds, prior to their application in field. These approaches, along with resistance monitoring can inform appropriate management strategies, such as alternation of insecticides with different mode of action, and could help to avoid positive selection of resistant phenotypes in field and prolong the insecticides useful life.

4.2 Heterologous expression of *C. capitata* CYP6A51 in *E.coli* and ectopic expression of cytochrome P450 CYP6BQ23 in *Drosophila melanogaster*

Field populations of *C. capitata* have been reported to be resistant to malathion and other organophosphate insecticides (Couso-Ferrer *et al*., 2011; Magana *et al*., 2007). However, after the withdrawal of malathion use in Europe in 2009, *C. capitata* has also been reported to be resistant to lambda-cyhalothrin in Spanish population of citrus crops, exhibiting an LD50 value higher than the recommended dose of lambda-cyhalothrin for field applications (Arouri *et al*., 2014). In general, resistance to pyrethroids has also been reported in field populations for many other species of the Tephritidae family (Vontas *et al*., 2011).

Pyrethroid resistance is known to be developed by target-site mutations, increased metabolic detoxification or due to thicker cuticles that block insecticide penetration (Hemingway *et al*., 2004; Li *at al*., 2007). In the case of *C. capitata*, the resistance to lambda-cyhalothrin is associated with P450 metabolic detoxification, based on data from bioassays with the P450 inhibitor, PBO that showed a complete suppression of resistant phenotype (Arouri *et al*., 2014). In the same study, an analysis by qPCR, of fifty three P450 genes of *C. capitata*, revealed a single gene, the Cyp6a51 (GenBank accession number XM_004534804) that is overexpressed in a laboratory-selected resistant strain, compared to susceptible. The expression of CYP6A51 was higher in resistant strain compared to susceptible (Arouri *et al*., 2014) and was also induced in adults, after treatment with lambda-cyhalothrin, as it happens with other P450 genes linked with resistance (Festucci-Buselli et al., 2005; Zhu et al., 2008; Liu et al., 2011).

In the present study, we tried to elucidate the role of CYP6A51 in pyrethroid resistance. For this reason, we performed metabolic assays with recombinant CYP6A51 enzyme. We also attempted to evaluate *in vivo* the role of CYP6A51 in resistance, by generating transgenic *Drosophila* lines that over express the CYP6A51, based on GAL4/UAS system.

We succeed isolation of CYP6A51 enzyme in high yields, by heterologous expression in *E. coli*, which was able to form a functional complex with the CPR enzyme of *Anopheles gambiae* (AgCPR), also co-expressed in *E.coli*. The recombinant CYP6A51 was screened for activity against a number of luminescent substrates, and showed remarkable metabolism of Luciferin-ME EGE, confirming its catalytic activity. In addition, the recombinant CYP6A51 was also tested on its ability to metabolize lambda-cyhalothrin. Our results showed significant lambda-cyhalothrin metabolism in a time-dependent manner, in the presence of a NADPH regenerating system. More specifically, our experiments showed a strong interaction between the cytochrome CYP6A51 of *C.capitata* and the substrate lambda-cyhalothrin and also an important depletion of the initial amount of lambda-cyhalothrin, after 2 hours incubation. These findings indicate a strong association of CYP6A51 to lambda-cyhalothrin metabolism.

In addition, our future experiments include the ectopic expression of CYP6A51 in *Drosophila melanogaster*, as described above for CYP6BQ23 of pollen beetle. The transgenic flies that will overexpress the CYP6A51 will be tested on lambda-cyhalothrin contact assay, in order to investigate the susceptibility levels to lambda-cyhalothrin or other pyrethroids. Eventually, we believe that this approach will add to our knowledge about CYP6A51 contribution to lambda-cyhalothrin resistance. This knowledge could be useful for the design of new resistance management strategies in field populations of *C. capitata*.

4.3 Targeted gene editing using CRISPR/Cas9 system for introduction of *Plutella xylostella* G4946E mutation in *Drosophila melanogaster*

The aim of this part was the evaluation of susceptibility to diamides in *Drosophila melanogaster* carrying the *P. xylostella* G4946E point mutation in Ryanodine Receptor, introduced using the CRISPR/Cas9 genome editing technology.

Diamide insecticides consist a recently developed class of insecticides that are used for the control of a broad range of agricultural pests, and particularly Lepidoptera (Nauen, 2006). The diamides that are now available include the anthranilic compounds chlorantraniliprole and cyantraniliprole and the phthalic compound flubendiamide, as well as, new anthranilic diamides, cyclaniliprole and tetraniliprole that are currently under development (Sparks and Nauen, 2015). However, the over reliance and extensive use of diamides, particularly against Lepidoptera pests, has led to development of resistance, extremely diminishing their efficacy (Teixeira and Andaloro, 2013).

The diamondback moth *Plutella xylostella*, a major agricultural pest of cruciferous vegetables has exhibited diamide resistance, especially in Asia populations (Teixeira and Andaloro, 2013). Populations of *P. xylostella* larvae collected in Philippines and Thailand, were also found to exhibit over 200-fold resistance to all diamides, when compared to susceptible strains (Troczka et al., 2012). In both cases of resistance, a non-synonymous glycine to glutamic acid substitution (G4946E), resulting from a single point mutation (GGG to GAG) was identified in ryanodine receptor (Troczka et al., 2012). In the same study, it is remarkable that another non-synonymous mutation found in a diamide resistant population from Thailand, which has two nucleotide changes in the same codon (GGG to GAA), also results in exactly the same amino acid substitution. The difference in codon usage for glutamic acid substitution in position 4946 in the two resistant strains, found in distant countries, suggests that the mutations have been independently evolved and do not result from migration of *P. xylostella* resistant populations (Troczka et al., 2012).

Results from genetic crosses between susceptible and homozygous for G4946E mutation *P. xylostella* adults showed that diamide resistance is inherited as a recessive autosomal trait (Troczka et al., 2012). The same study supports the monogenic control of diamide resistance, providing strong evidence for a causal association of G4946E mutation with the resistant phenotype (Troczka et al., 2012 and 2015).

Furthermore, the G494E mutation is found in a critical region of the RyR channel, the C-terminal membrane-spanning domain, which is highly conserved among insects and is supposed to harbor the diamides binding site (Kato et al., 2009; Troczka et al., 2012). More specifically, G4946E mutation is considered to be located in a linker domain, between two transmembrane regions (Figure 3, Introduction) (Krogh et al., 2001; Yan et al., 2015; Zalk et al., 2015). Moreover, another study revealed the importance of this region for diamide binding, using *Drosophila melanogaster* as a model, where a 46-amino acid region in the *Drosophila* RyR C-terminal domain was replaced by nematode's corresponding region, resulting in insensitivity to diamides (Tao et al., 2013).

In addition, the substitution of glycine to glutamic acid causes significant structural consequences for the *P. xylostella* ryanodine receptor, because a small uncharged residue is replace by a large one, negatively charged, influencing the efficacy of diamide binding. Besides, a recent study revealed that chlorantraniliprole and flubendiamide bind at different but allosteric sites (Isaacs et al., 2012). It has been also shown that radioligand binding assays with microsomal preparations from thorax of resistant *P. xylostella* strain indicated specific binding of RyR to diamide analogs, compared to susceptible strain (Steinbach et al., 2015).

In the present study, we tried to generate an allele carrying the G4946E mutation in *Drosophila melanogaster* RyR, using the CRISPR-Cas9 technology, in order to evaluate the susceptibility levels

of mutants to diamides with bioassays. This system is a reliable and effective tool for genome editing in *Drosophila* (Bassett and Liu, 2014; Gratz et al., 2013; Harrison et al., 2014; Kondo, 2014). In our approach, we used a transgenic *Drosophila* line that expresses the Cas9 endonuclease in the germline under the control of the nanos promoter, in order to achieve efficient inheritance of modified allele in the progeny. Initially, we sequenced the target-site of *Drosophila* RyR in order to confirm the gRNA sequence. The donor plasmid contained along with G4946E mutation, further synonymous modifications to facilitate the molecular confirmation of genome engineered flies. The Cas9-expressing transgenic flies were co-injected with plasmid to express gRNA and the donor plasmid. In each genetic cross, we used a molecular confirmation to identify engineered flies.

However, the generation of homozygous lines for the G4946E mutation was not feasible. The heterozygosity was confirmed with sequencing, which also showed that apart from the changes that we had introduced, the examined region did not carry any other modification of mutation that could be responsible for not achieving homozygosity. Besides, the design of gRNA was done with the program Optimal Target Finder, in order to exhibit maximum sequence stringency for avoiding any potential off-target effects. Thus, due to recessive mode of inheritance of G4946E mutation in RyR, bioassays with heterozygous flies could not be performed, as they have the susceptible phenotype and no result could be obtained.

However, considering the reasons for which homozygosity was not achieved, one must consider the following:

- The G4946E mutation is a substitution of a glycine to glutamic acid, which is a bigger, polar amino acid compared to glycine. Considering that *Drosophila* has only one gene of RyR in its genome, a mutation in this part of the protein maybe is not tolerable. A similar strategy was followed in the laboratory in order to introduce a G4946V in the same transgenic *Drosophila* line and eventually achieved homozygous flies with the modified allele. This mutation is found in the exact same position, 4946 in RyR, but it is a substitution of a glycine to valine, which is a slightly bigger, non-polar amino acid compared to glycine. This fact could be an indication of a lethal effect of G to E substitution, compared to G to V, and since *Drosophila* and all insects have only one copy of ryanodine receptor gene, a destructive mutation like this could not be tolerable.
- Moreover, a possible reason is an unpredicted off-target effect, which happened in *Drosophila* genome that we cannot detect. This change maybe results in the disruption of the function of an important gene resulting in lethality. However, the fact that we could not get homozygotes from four different lines derived from different injection events, does not corroborate this hypothesis,

given that off-targets are expected to happen in a stochastic manner and much lower frequency than normal CRISPR targeting.

- Another possible reason is the presence of a lethal allele floating in the original nanos.Cas9 population, which might be linked to the chromosome of the desired mutation. Thus, in case one or more lethal alleles exist in the second chromosome, where the G4946E mutation is found, and is tolerable in heterozygous state but not in homozygous state, this could account to some extend for our inability to acquire a homozygous line.

5. References

- Abbott W. S. 1987. A method of computing the effectiveness of an insecticide. Journal of the American Mosquito Control Association. 3:302-303.
- Alford, D. V., Nilsson, C., Ulber B. 2003. Insect Pests of Oilseed Rape Crops. In Alford, D. V. (ed.): Biocontrol of Oilseed Rape Pests. Blackwell Science. Oxford, UK, 355 pp.
- Arouri R, Le Goff G, Hemden H, Navarro-Llopis V, M'saad M, Castañera P, Feyereisen R, Hernández-Crespo P, Ortego F. 2014. Resistance to lambda-cyhalothrin in Spanish field populations of *Ceratitis capitata* and metabolic resistance mediated by P450 in a resistant strain. Pest Manag Sci. 71(9):1281-91.
- Bassett AR, Liu JL. 2014. CRISPR/Cas9 and genome editing in *Drosophila*. Journal of genetics and genomics. 41:7–19.
- Bateman J. R., A. M. Lee, C. Wu. 2006. Site-specific transformation of *Drosophila* via ϕc31 integrase-mediated cassette exchange. Genetics 173 (2); 769-777.
- Baxter, S.W., Chen, M., Dawson, A., Zhao, J.Z., Vogel, H., Shelton, A.M., Heckel, D.G., Jiggins, C.D., 2010. Mis-spliced transcripts of nicotinic acetylcholine receptor alpha6 are associated with field evolved spinosad resistance in *Plutella xylostella* (L.). PloS Genet.; 6(1): e1000802.
- Berge J.B, Feyereisen R., Amichot M., 1998. Cytochrome P450 monooxygenases and insecticide resistance in insects. Phil.Trans.R.Soc.Lond. B, 353, 1701-1705.
- Bischof J, Maeda RK, Hediger M, Karch F, Basler K. 2007. An optimized transgenesis system for *Drosophila* using germ-line-specific phiC31 integrases. Proc Natl Acad Sci USA.27;104(9):33127.
- Brand, A. H. & Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118; 401–415.
- Brand, A.H. & Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118; 401–415.
- Chung H., Bogwitz M.R., McCart C., Andrianopoulos A., Ffrench-Constant R.H., Batterham P., Daborn P.J., 2007. Cis-regulatory elements in the Accord retrotransposon result in tissue-specific expression of the *Drosophila melanogaster* insecticide resistance gene Cyp6g1. Genetics 175; 1071–1077.
- Cordova D, Benner E.A., Sacher M.D., Rauh J.J., Sopa J.S., Lahm G.P., Selby T.P., Stevenson T.M., Flexner L., Gutteridge S., Rhoades D.F., Wu L., Smith R.M., Tao Y.. 2006. Anthranilic

diamides: A new class of insecticides with a novel mode of action, ryanodine receptor activation, Pesticide Biochemistry and Physiology: 196–214.

- Couso-Ferrer, F., Arouri, R., Beroiz, B., Perera, N., Cervera, A., Navarro-Llopis, V., Castaniera, P., Hernandez-Crespo, P., and Ortego., F., 2011. Cross-resistance to insecticides in a malathion-resistant strain of *Ceratitis capitata* (Diptera: Tephritidae). J. Econ. Entomol. 104: 1349-1356.
- Daborn P.J., Lumb C., Boey A., Wong W., Ffrench-Constant R.H., Batterham P., 2007. Evaluating the insecticide resistance potential of eight *Drosophila melanogaster* cytochrome P450 genes by transgenic over-expression, Insect Biochem. Mol. Biol. 37; 512–519.
- Denholm, I and Devin, G. 2001. Insecticide resistance. In: Levin SA [ed.], Encyclopedia of Biodiversity. Academic Press, San Diego, pp. 465-477.
- Dong K, Du Y, Rinkevich F, Nomura Y, Xu P, Wang L, Silver K, Zhorov BS. 2014. Molecular biology of insect sodium channels and pyrethroid resistance. Insect Biochem Mol Biol.; 50:1-17.
- Duangkaew, P., Kampa, D., and Rongnoparut, P. 2011. Protective efficacy of *Anopheles minimus* CYP6P7 and CYP6AA3 against cytotoxicity of pyrethroid insecticides in *Spodoptera frugiperda* (Sf9) insect cells. Trop Biomed 28: 293-301.
- Ebbinghaus-Kintscher, U., Luemmen, P., Lobitz, N., Schulte, T., Funke, C., Fischer, R., Masaki, T., Yasokawa, N., Tohnishi, M., 2006. Phthalic acid diamides activate ryanodine-sensitive Ca^{2+} release channels in insects. Cell Calcium. 39, 21-33.
- Eto, Morifusa 1974. Organophosphorus pesticides; organic and biological chemistry, CRC Press, Cleveland, Ohio.
- FAO (Food and Agriculture Organization of the United Nations). In: How to feed the world in 2050.2010.http://www.fao.org/fileadmin/templates/wsfs/docs/expertpaper/How_to_Feed_the_World_in_2050.pdf
- Festucci-Buselli, R. A., Carvalho-Dias, A. S., de Oliveira-Andrade, M., Caixeta-Nunes, C., Li, H. XL, Stuart, J. J., Muir, W., Scharf, M. E., and Pittendrigh, B. R. 2005. Expression of Cyp6g1 and Cyp12d1 in DDT resistant and susceptible strains of *Drosophila melanogaster*. Insect Mol. Biol. 14: 69-77.
- Feyereisen R., 2005. Insect cytochrome P450. Comprehensive Molecular Insect Science, eds Gilbert LI, Iatrou K, Gill SS (Elsevier, Oxford), Vol 4, pp 1–77
- Feyereisen, R., 1995. Molecular biology of insecticide resistance. Toxicol. Lett. 82, 89-90.
- Feyereisen, R., 2012. Insect CYP genes and P450 enzymes. In: Gilbert, L.L [Eds], Insect Molecular Biology and Biochemistry. Elsevier, Oxford, pp. 236-316.
- Fischer, J. A., Giniger, E., Maniatis, T. & Ptashne, M. 1988. GAL4 activates transcription in *Drosophila*. Nature 332; 853–856.

- Gratz SJ, Ukken FP, Rubinstein CD, Thiede G, Donohue LK, Cummings AM, O'Connor-Giles KM. 2014. Highly Specific and Efficient CRISPR/Cas9-Catalyzed Homology-Directed Repair in *Drosophila.* Genetics. 196:961–971.
- Groth A. C., Fish M., Nusse R. and M. P. Calos, 2004. Construction of transgenic *Drosophila* by using the site-specific integrase from phage φC31. Genetics 166: 1775–1782.
- Groth, A. C., E. C. Olivares, B. Thyagarajan and M. P. Calos, 2000. A phage integrase directs efficient site-specific integration in human cells. Proc. Natl. Acad. Sci. USA 97: 5995–6000.
- Guo, L., Liang, P., Zhou, X., Gao, X., 2014b. Novel mutations and mutation combinations of ryanodine receptor in a chlorantraniliprole resistant population of Plutella xylostella (L.). Scientific Reports 4, 6924.
- Guo, L., Wang, Y., Zhou, X., Li, Z., Liu, S., Pei, L., Gao, X., 2014a. Functional analysis of a point mutation in the ryanodine receptor of Plutella xylostella (L.) associated with resistance to chlorantraniliprole. Pest. Manag. Sci. 70, 1083−1089.
- Hansen, L.M., 2003. Insecticide-resistant pollen beetles (Meligethes aeneus F) found in Danish oilseed rape (Brassica napus L) fields. Pest Manage. Sci. 59, 1057-1059.
- Hanukoglu, Israel. 1996. Electron Transfer Proteins of Cytochrome P450 Systems. Advances in Molecular and Cell Biology. Advances in Molecular and Cell Biology. 14: 29–56.
- Harrison MM, Jenkins BV, O'Connor-Giles KM, Wildonger J. 2014. A CRISPR view of development. Genes & development. 28:1859–1872.
- Heckel DG. 2003. Genomics in pure and applied entomology. Annu Rev Entomol.; 48:235-60.
- Heckel, D.G., 2012. Insecticide resistance after silent spring. Science 337, 1612-1614.
- Heimbach U, Müller A. 2013. Incidence of pyrethroid-resistant oilseed rape pests in Germany. Pest Manag Sci.; 69(2):209-16.
- Hemingway, J., Dunbar, Si., Monro, A.G., and Small, G.J. 1993. Pyrethroid resistance in German cockroaches (Dictyoptera Blattelidae): resistance levels and underlying mechanisms. J. Erna Entomol. 86: 1631-1638.
- Hemingway, J., Hawkes, N. J., McCarroll, L., and Ranson, H., 2004. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem. Molec. Biol. 34: 653-665.
- Hirooka, T., Nishimatsu, T., Kodama, H., Reckmann, U., Nauen, R., 2007. The biological profile of flubendiamide, a new benzenedicarboxamide insecticide. Pflanzensch. Nachr. Bayer 60, 183-202.
- Horvath, P., & Barrangou, R. 2010. CRISPR/Cas, the immune system of bacteria and archaea. Science, 327(5962), 167-170.

- Isaacs, A.K., Qi, S., Sarpong, R., Casida, J.E., 2012. Insect ryanodine receptor: distinct but coupled insecticide binding sites for [N-C3H3]chlorantraniliprole, flubendiamide, and [3H]ryanodine. Chem. Res. Toxicol. 25, 1571-1573.
- Jeanguenat Andre. 2012. The story of a new insecticidal chemistry class: the diamides, Pest Manage. Sci. 69: 7–14
- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096):816–821.
- Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J. 2013. RNA-programmed genome editing in human cells. Elife 2:10.7554/eLife.00471.
- Kato, K., Kiyonaka, S., Sawaguchi, Y., Tohnishi, M., Masaki, T., Yasokawa, N., Mizuno, Y., Mori, E., Inoue, K., Hamachi, I., Takeshima, H., Mori, Y., 2009. Molecular characterization of flubendiamide sensitivity in the lepidopterous ryanodine receptor Ca^{2+}. Release Channel.Biochemistry 48, 10342−10352.
- Khambay, B.P.S., Jewess, P.J., 2005. Pyrethroids. In: . In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), Comprehensive Molecular Insect Science, first ed., vol. 6. Elsevier Ltd, pp. 1-29.
- Knapp, J. L. 1981. The Mediterranean fruit fly. Ent. 54. Coop. Ext. Serv., Inst. Food Agric. Sci., Univ. Florida. 3 p., 5 fig.
- Kondo S. 2014. New horizons in genome engineering of *Drosophila melanogaster*. Genes & genetic systems. 89:3–8.
- Krogh, A., Larsson, B., von Heijne, G., Sonnhammer, E.L.L., 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305, 567-580.
- Lahm, G.P., Stevenson, T.M., Selby, T.P., Freudenberger, J.H., Cordova, D., Flexner, L., Bellin, C.A., Dubas, C.M., Smith, B.K., Hughes, K.A., Hollinghaus, J.G., Clark, C.E., Benner, E.A., 2007. Rynaxypyr: a new insecticidal anthranilic diamide that acts as a potent and selective ryanodine receptor activator. Bioorg. Med. Chem. Lett. 17, 6274-6279.
- Lai FA, Misra M, Xu L, Smith HA, Meissner G. 1989. The ryanodine receptor $Ca2^{+}$ -release channel complex of skeletal muscle sarcoplasmic reticulum. Evidence for a cooperatively coupled, negatively charged homotetramer. J Biol Chem 264: 16776–16785.
- Lai, T., Li, J., Su, J., 2011. Monitoring of beet armyworm *Spodoptera exigua* (Lepidoptera: Noctuidae) resistance to chlorantraniliprole in China. Pestic. Biochem. Physiol. 101, 198-205.

- Lanner, J.T., Georgiou, D.K., Joshi, A.D., Hamilton, S.L., 2010. Ryanodine receptors: structure, expression, molecular details, and function in calcium release. Cold Spring Harb Pespect Biol. 2(11), a003996.
- Liquido NJ, Shinoda LA, Cunningham RT. Host plants of the Mediterranean fruit fly (Diptera, Tephritidae). An annotated world list. Ann Entomol Soc Am. 1991;77:1–57.
- Liu X., Wang H.Y., Ning Y.B., Qiao K., Wang K.Y. 2015. Resistance selection and characterization of chlorantraniliprole resistance in *Plutella xylostella* (Lepidoptera: Plutellidae). J. Econ. Entomol.,; 108: 1978–1985.
- Liu, N., Li, T., Reid, W. R., Yang, T., and Zhang, L., 2011. Multiple cytochrome P450 genes: their constitutive overexpression and permethrin induction in insecticide resistant mosquitoes, *Culex quinquefasciatus*. PlosOne 6: e23403.
- Magana, C., Hernandez-Crespo, P., Ortego, F., and Castanera, P. 2007. Resistance to malathion in field populations of *Ceratitis capitata*. J. Econ. Entomol. 100: 1836-1843.
- Malacrida AR, Guglielmino CR, Gasperi G, Baruffi L, Milani R. 1992. Spatial and temporal differentiation in colonizing populations of Ceratitis capitata. Heredity. 69:101–111.
- Marraffini LA, Sontheimer EJ. 2010. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. Nature reviews Genetics. 11(3):181-190.
- McGrew SG, Wolleben C, Siegl P, Inui M, Fleischer S. 1989. Positive cooperativity of ryanodine binding to the calcium release channel of sarcoplasmic reticulum from heart and skeletal muscle. Biochemistry 28: 1686–1691.
- McLaughlin LA, Niazi U, Bibby J, David JP, Vontas J, Hemingway J, Ranson H, Sutcliffe MJ, Paine MJ. 2008. Characterization of inhibitors and substrates of *Anopheles gambiae* CYP6Z2. Insect Mol Biol.,17(2):125-35.
- Meissner G, Darling E, Eveleth J. (1986). Kinetics of rapid calcium release by sarcoplasmic reticulum. Effects of calcium, magnesium, and adenine nucleotides. Biochemistry 25: 236–244.
- Muller, P., Warr, E., Stevenson, B. J., Pignatelli, P. M., Morgan, J. C., Steven, A., Dawson, A. E., Mitchell, S. N., Ranson, H., Hemingway, J., Paine, M. J. I., and Donnelly, K J. 2008. Field caught permethrin-resistant *Anopheles gambiae* overexpress CYP6P3, a P450 that metabolises pyrethroids. PLoS Genet. 4: e1000286.
- Nauen R., Wölfel K., Lueke B., Myridakis A.,Tsakireli D., Roditakis E., Tsagarakou A., Stephanou E., Vontas J. 2015. Development of a lateral flow test to detect metabolic resistance in Bemisia tabaci mediated by CYP6CM1, a cytochrome P450 with broad spectrum catalytic efficiency. Pestic. Biochem. Physiol.; 121:3-11.

- Nauen, R., 2006. Insecticide mode of action: return of the ryanodine receptor. Pest. Manag. Sci. 62, 690−692.
- Nauen, R., Vontas, J., Kaussmann, M., Wölfel, K., 2013. Pymetrozine is hydroxylated by CYP6CM1, a cytochrome P450 conferring neonicotinoid resistance in *Bemisia tabaci*. Pest Manage. Sci. 69, 457-461.
- Nauen, R., Zimmer, C.T., Andrews, M., Slater, R., Bass, C., Ekbom, B., Gustafsson, G., Hansen, L.M., Kristensen, M., Zebitz, C.P.W., Williamson, M.S., 2012. Target-site resistance to pyrethroids in European populations of pollen beetle, Meligethes aeneus F. Pest. Biochem. Physiol. 103, 173-180.
- Noppun, V., Saito, T., Miyata, T., 1989. Cuticular penetration of S fenvalerate in fenvalerate-resistant and susceptible strains of the diamondback moth *Plutella xylostella* L. Pestic. Biochem. Physiol. 33, 83-87.
- Omura, T., Sato, R., 1964. Carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239; 2370–2378.
- O'Reilly AO, Khambay BP, Williamson MS, Field LM, Wallace BA, Davies TG. 2006. Modelling insecticide-binding sites in the voltage-gated sodium channel. Biochem J.; 396:255-63.
- Otsu K, Willard HF, Khanna VK, Zorzato F, Green NM, MacLennan DH. 1990. Molecular cloning of cDNA encoding the Ca2þ release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. J Biol Chem 265: 13472–13483.
- Pavlidi N., Monastirioti M., Daborn P., Livadaras I., Van Leeuwen T., Vontas J. 2012. Transgenic expression of the *Aedes aegypti* CYP9J28 confers pyrethroid resistance in *Drosophila melanogaster*. Pesticide Biochemistry and Physiology 104 (2012) 132–135.
- Philipou, D., Field, L.M., Wegorek, P., Zamojska, J., Andrews, M., Slater, R., Moores, G.D., 2011. Characterising metabolic resistance in pyrethroid insensitive pollen beetle (Meligethes aeneus F.) from Poland and Switzerland. Pest Manage. Sci. 67, 239-243.
- Port, F., Chen, H.-M., Lee, T., & Bullock, S. L. 2014. Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in *Drosophila*. Proceedings of the National Academy of Sciences of the United States of America, 111(29), E2967–E2976.
- Richardson, D.M., 2008. Summary of findings from a participant country pollen beetle questionnaire. OEPP/EPPO Bulletin 38, 68-72.
- Riga M., Myridakis A., Tsakireli D., Morou E., Stephanou E.G., Nauen R., Van Leeuwen T., Douris V., Vontas J. 2015. Functional characterization of the Tetranychus urticae CYP392A11, a cytochrome P450 that hydroxylates the METI acaricides cyenopyrafen and fenpyroximate. Insect Biochem Mol Biol.; 65:91-99.

- Rinkevich FD, Du Y, Dong K. 2013. Diversity and Convergence of Sodium Channel Mutations Involved in Resistance to Pyrethroids. Pestic Biochem Physiol.; 106(3):93-100.
- Robert L. Metcalf "Insect Control" in Ullmann's Encyclopedia of Industrial Chemistry" Wiley-VCH, Weinheim, 2002.
- Rossi D, Sorrentino V. 2002. Molecular genetics of ryanodine receptors Ca2+-release channels. Cell Calcium.;32(5-6):307-19.
- S.J. Gratz, A.M. Cummings, J.N. Nguyen, D.C. Hamm, L.K. Donohue, M.M. Harrison, J. Wildonger, K.M. O'Connor-Giles. 2013. Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. Genetics, 194; 1029–1035
- S.J. Gratz, F.P. Ukken, C.D. Rubinstein, G. Thiede, L.K. Donohue, A.M. Cummings, K.M. O'Connor-Giles. 2014. Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in *Drosophila*. Genetics, 196; 961–971.
- Sattelle DB, Cordova D, Cheek TR. 2008. Insect ryanodine receptors: molecular targets for novel pest control chemicals. Invert Neurosci. 8(3):107-19.
- Scott J.G. 1999. Cytochromes P450 and insecticide resistance, Insect.Bioch.Mol.Biol.29:757-777
- Scott, J. G. and Georghiou, G. P. 1986. Mechanisms responsible for high levels of permethrin resistance in the house fly. Pestic. Sci 17: 195-206.
- Sevrioukova, I. F. and Peterson, J. A. 1995. NADPH-P-450 reductase: structural and functional comparisons of the eukaryotic and prokaryotic isoforms. Biochimie 77, 562-572.
- Sial, A.A., Brunner, J.F., Garczynski, S.F., 2011. Biochemical characterization of chlorantraniliprole and spinetoram resistance in laboratory-selected obliquebanded leafroller, Choristoneura rosaceana (Harris) (Lepidoptera: Tortricidae). Pestic. Biochem. Physiol. 99,274-279.
- Slater, R., Ellis, S., Genay, J.P., Heimbach, U., Huart, G., Sarazin, M., Longhurst, C., Müller, A., Nauen, R., Rison, J.L., Robin, F., 2011. Pyrethroid resistance monitoring in European populations of pollen beetle (*Meligethes* spp.): a coordinated approach through the Insecticide Resistance Action Committee (IRAC). Pest Manage. Sci. 67, 633-638.
- Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, Stevens JT, Weiner ML. 2002. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment, Toxicology: 171, 3-59.
- Sogorb, MA., and Vilanova, E. 2002. Enzymes involved in the detoxification of organophosphorous, carbamate and pyrethroid insecticides through hydrolysis Toxicol. Lett. 128: 215-228.

- Sonoda, S., 2010. Molecular analysis of pyrethroid resistance conferred by target insensitivity and increased metabolic detoxification in *Plutella xylostella*. Pest Manag. Sci. 66, 572-575.
- Sparks, T. C., Nauen, R., 2015. IRAC: Mode of action classification and insecticide resistance management. Pestic Biochem Physiol. 2015 Jun;121:122-8.
- Sparks, T.C., Dripps, J.E., Watson, G.B., Paroonagian, D., 2012. Resistance and crossresistance to spinosyns e a reviewand analysis. Pestic. Biochem. Physiol.102,1-10.
- Steinbach D., Gutbrod O, Lümmen P, Matthiesen S, Schorn C, Nauen R. 2015. Geographic spread, genetics and functional characteristics of ryanodine receptor based target-site resistance to diamide insecticides in diamondback moth, *Plutella xylostella*. Insect Biochemistry and Molecular Biology 63, 14–22 .
- Stevenson, B.J., Bibby, J., Pignatelli, P., Muangnoicharoen, S., O'Neill, P.M., Lian, L.-Y., Müller, P., Nikou, D., Steven, A., Hemingway, J., Sutcliffe, M.J., Paine, M.J.I., 2011. Cytochrome P450 6M2 from the malaria vector *Anopheles gambiae* metabolizes pyrethroids: sequential metabolism of deltamethrin revealed. Insect Biochem. Mol. Biol. 41, 492-502.
- Stevenson, B.J., Pignatelli, P., Nikou, D., Paine, M.J.I., 2012. Pinpointing P450s associated with pyrethroid metabolism in the dengue vector, *Aedes aegypti*: developing new tools to combat insecticide resistance. PLOS Neglected Trop. Dis. 6, e1595.
- Strobel, H.W., Dignam, J.D., 1978. Purification and properties of NADPH cytochrome P-450 reductase. Meth. Enzymol. 52, 89-96.
- Szyniszewska AM, Tatem AJ. 2014. Global assessment of seasonal potential distribution of Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). PLoS One. 9(11):e111582.
- Takeshima, H., Nishi, M., Iwabe, N., Miyata, T., Hosoya, T., Masai, I., Hotta, Y., 1994. Isolation and characterization of a gene for a ryanodine receptor/calcium release channel in *Drosophila melanogaster*. FEBS Lett. 337, 81-87.
- Talekar, N.S., Shelton, A.M., 1993. Biology, ecology and management of the diamondback moth. Annu. Rev. Entomol. 38, 275-301.
- Tao, Y., Gutteridge, S., Benner, E.A., Wu, L., Rhoades, D.F., Sacher, M.D., Rivera, M.A., Desaeger, J., Cordova, D., 2013. Identification of a critical region in the *Drosophila* ryanodine receptor that confers sensitivity to diamide insecticides. Insect Biochem. Mol. Biol. 43, 820−828.
- Teixeira, L. A. & Andaloro, J. T. 2013. Diamide insecticides: Global efforts to address insect resistance stewardship challenges. Pesticide Biochemistry and Physiology 106, 76–78.
- Thieme, T., Heimbach, U., Müller, A., 2010. Chemical control of insect pests and insecticide resistance in oilseed rape. In: Williams, I.H. (Ed.), Biocontrol-based Integrated Management of Oilseed Rape Pests. Springer, London, UK, pp. 313-335.

- Thorpe HM, Smith MC. 1998. In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. Proc Natl Acad Sci USA 95:5505–5510.
- Thorpe HM, Wilson SE, Smith MC. 2000. Control of directionality in the site-specific recombination system of the *Streptomyces* phage phiC31. Mol Microbiol 38:232–241.
- Thyagarajan, B., E. C. Olivares, R. P. Hollis, D. S. Ginsburg and M. P. Calos. 2001. Site-specific genomic integration in mammalian cells mediated by phage phiC31 integrase. Mol. Cell. Biol. 21: 3926–3934.
- Tohnishi, M., Nakao, H., Furuya, T., Seo, A., Kodama, H., Tsubata, K., Fujioka, S., Kodama, H., Hirooka, T., Nishimatsu, T., 2005. Flubendiamide, a novel insecticide highly active against lepidopterous insect pests. J. Pestic. Sci. 30, 354-360.
- Troczka B., Williams A. J., Williamson M. S., Field L. M., Lüemmen P & Davies T.G. Emyr. 2015. Stable expression and functional characterisation of the diamondback moth ryanodine receptor G4946E variant conferring resistance to diamide insecticides. Scientific Reports 5:14680.
- Troczka B., Zimmer CT., Elias J, Schorn , Bass C., Davies TG, Field LM, Williamson MS, Slater R, Nauen R. 2012. Resistance to diamide insecticides in diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae) is associated with a mutation in the membrane-spanning domain of the ryanodine receptor. Insect Biochemistry and Molecular Biology 42, 873–880.
- Van Leeuwen T., Demaeghta P., Osborne E.J., Dermauw W., Gohlkec S., Nauen R., Grbić M., Tirry L., Merzendorfer H., Clark R.M. 2012. Population bulk segregant mapping uncovers resistance mutations and the mode of action of a chitin synthesis inhibitor in arthropods. Proc. Natl. Acad. Sci. USA.; 109: 4407-4412.
- Van Leeuwen T.,Van Pottelberge S.,Tirry L. 2005. Comparative acaricide susceptibility and detoxifying enzyme activities in field-collected resistant ans susceptible strains of *Tetranychus urticae*. Pest Manage. Sci. 61: 499-507.
- Vazquez-Martinez, O., Canedo-Merino, R., Diaz-Munoz, M., Riesgo-Escovar, J.R., 2003. Biochemical characterization, distribution and phylogenetic analysis of *Drosophila melanogaster* ryanodine and IP3 receptors, and thapsigargin sensitive Ca^{2+} ATPase. J. Cell. Sci. 116, 2483-2494.
- Vontas J., Hernandez-Crespo, P., Margaritopoulos, J. T, Ortego, F., Fang, H. T, Mathiopoulos, K. D., and Hsu, J. C. 2011. Insecticide resistance in Tephritid flies. Pestic. Biochem. Physiol. 100: 199-205.
- Wang X, Wu Y. 2012. High levels of resistance to chlorantraniliprole evolved in field populations of *Plutella xylostella*. J Econ Entomol; 105:1019–1023.

- Wang, X., Wu, Y., 2012. High levels of resistance to chlorantraniliprole evolved in field populations of *Plutella xylostella*. J. Econ. Entomol. 105, 1019-1023.
- Werck-Reichhart, D., and Feyereisen, R., 2000. Cytochromes P450: A success story. Genome Biology 1: 1-3003.9
- Xu, X., Bhat, M.B., Nishi, M., Takeshima, H., Ma, J., 2000. Molecular cloning of cDNA encoding a *Drosophila* ryanodine receptor and functional studies of the carboxyl-terminal calcium release channel. Biophys. J. 78, 1270-1281.
- Yan Z, Bai XC, Yan C, Wu J, Li Z, Xie T, Peng W, Yin CC, Li X, Scheres SH, Shi Y, Yan N. 2015. Structure of the rabbit ryanodine receptor RyR1 at near-atomic resolution. Nature; 517: 50-55.
- Zalk R, Clarke OB, des Georges A, Grassucci RA, Reiken S, Mancia F, Hendrickson WA, Frank J, Marks AR.. 2015. Structure of a mammalian ryanodine receptor. Nature 517, 44–49.
- Zhao, J.Z., Collins, H.L., Li, Y.X., Mau, R.F.L., Thompson, G.D., Hertlein, M., Andaloro, J.T., Boykin, R., Shelton, A.M., 2006. Monitoring of diamondback moth (Lepidoptera: Plutellidae) resistance to spinosad, Indoxacarb, and emamectin benzoate. J. Econ. Entomol. 99, 176-181.
- Zhu, F., Li, T., Zhang, L., and Liu, N., 2008. Co-up-regulation of three P450 genes in response to permethrin exposure in permethrin resistant house flies, *Musca domestica*. BMC Physiol. 25; 8-18.
- Zhu, F., Parthasarathy, R., Bai, H., Woithe, K., Kaussmann, M., Nauen, R., Harrison, D.A., Palli, S.R., 2010. A brain-specific cytochrome P450 responsible for the majority of deltamethrin resistance in the QTC279 strain of *Tribolium castaneum*. Proc. Natl. Acad. Sci. U S A 107, 8557-8562.
- Zimmer C.T., Bass C., Williamson M.S., Kaussmann M., Wölfel K., Gutbrod O., Nauen R. 2014. Molecular and functional characterization of CYP6BQ23, a cytochrome P450 conferring resistance to pyrethroids in European populations of pollen beetle, Meligethes aeneus. Insect Biochemistry and Molecular Biology 45: 18-29.
- Zimmer, C.T., Nauen, R., 2011. Cytochrome P450 mediated pyrethroid resistance in European populations of *Meligethes aeneus* (Coleoptera: Nitidulidae). Pest. Biochem. Physiol. 100, 264-265.
- Zorzato F, Fujii J, Otsu K, Phillips M, Green NM, Lai FA, Meissner G, MacLennan DH. 1990. Molecular cloning of cDNA encoding human and rabbit forms of the Ca2þ release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. J Biol Chem 265: 2244–2256.

6. Appendix

Primer Name	Sequence (5'-3')
UAS2F	TTCCGCGGAAGCTTGCATGCCTGCAGGT
UAS2R	TTCCGCGGCCCAATTCCCTATTCAGAGT
RyR1F	ATCACCGCCGTCAAAGAAGG
RyR1R	TTACCAGCTTGTCCCAGTGC
RyR2F	ACATGGAACACGTTCTCCGC
RyR2R	AGTTGTGCTCCTTCTGGACG
RyR3F	GGAGGTGGACAAAAAGTGCC
RyR3R	AACCAGCCTTGATATTTGAGATGAC
GENEFOR	GGGACAAGCTGGTAATTTCGGC
GENEREV	GGAGTATACCGGCCAGGAGA
Specific Forward	ATCTGCTCGACGTCGCTGTGGA
CYP6A51_exp_F	GAGCTCATGAGCGTGTTTCTGGCT
CYP6A51_exp_R	TCTAGACTATACACGCTCCACACGTAG

Table 1. Primers used in the present study.

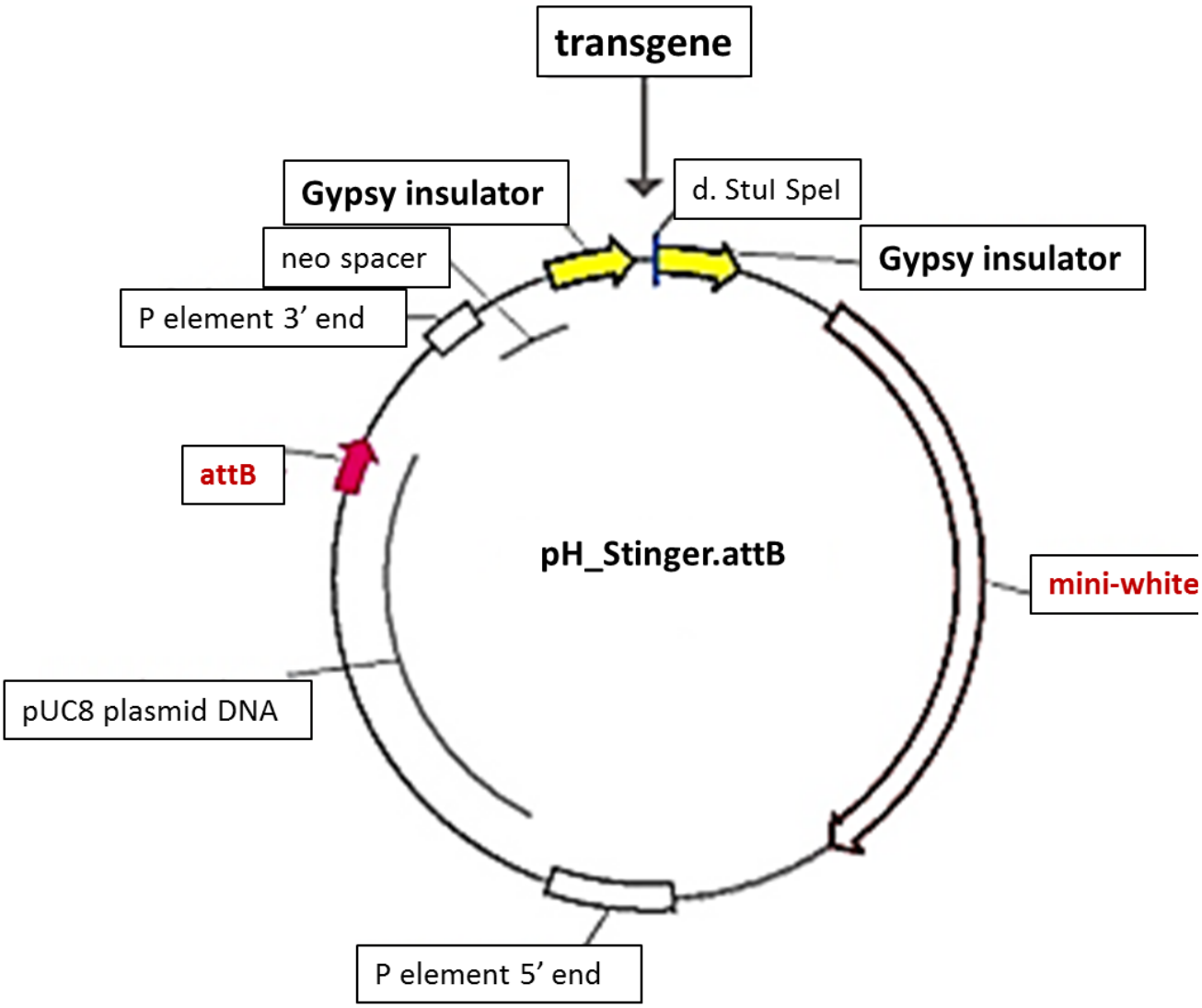


Figure 1. The plasmid vector pH_Stinger.attB, which consists a modification of pPelican (Barolo *et al*., 2000)

GCGGCCGCAAGCTTGCATGCCTGCAGGTCGGAGTACTGTCCTCCGAGCGGAGT
ACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCG
GAGTACTGTCCTCCGAGCGGAGACTCTAGCGAGCGCCGGAGTATAAATAGAGG
CGCTTCGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCG
CTAAGCGAAAGCTAAGCAAATAAACAAGCGCAGCTGAACAAGCTAAACAATCT
GCAGTAAAGTGCAAGTTAAAGTGAATCAATTAAAAGTAACCAGCAACCAAGTA
AATCAACTGCAACTACTGAAATCTGCCAAGAAGTAATTATTGAATACAAGAAG
AGAACTCTGAATAGGGAATTGGGACGCGTCACCATGGTGCTGATCACCAACAC
CCTGACCTACGACCTGGGCATCTTCCTGGCCACCCTGATCACCGCCTTCGTGA
TCTACATCAAGTGGGTGTACACCTACTGGCAGAAGAAGGGCCTGGCCACCGAG
CCCACCGTGATCCCCTTCGGCAACGGCGCCAAGGTGGCCACCTGCCAGCAGAA
CATCGGCGAGCTGTTCAGCGACATGTACTTCAAGTTCAAGAGCAAGGGCCTGA
AGCACGGCGGCGCCTACTTCCTGATGAAGCCCTTCTACGTGCCCGTGGACCTG
GAGATCGTGAAGGGCATCATGCAGAACGACTTCGGCAACTTCGTGAACAGGGG
CATGTACGTGAACGAGAAGGCCGACACCATGAGCACCAACCTGTTCAACCTGG
AGGGCGGCAAGTGGAAGAGCTTGAGGGCCAAGCTGAGCCCCACCTTCACCAGC
GGCAAGCTGAAGATGATGTTCAACACCCTGAGCGAGTGCGGCCTGGGCCTGAA
CGACATGATCGAGGAGTACGCCAGGACCGGCGAGGAGCTGAACATCAAGGACA
TCCTGCAGAGGTTCAGCATCGACATCATCGGCAGCGTGAGCTTCGGCATCGAG
TGCAACAGCATCAAGAACCCCGAGAGCGAGTTCTTCCTGAACGGCAAGAGGCT
GTTCAGCCTGGAGTTCACCAACATCCTGAGGTTCCTGAGCCCCATCGCCCTGC
CCCACAGCGTGCTGAAGTTCTTCAACTTCAACAGCTTCAACGTGAGCGTGACC
AACTTCTTCGACAACATCATCAAGCAGAACGTGGAGTACAGGGAGAAGAACAA
CATCACCAGGCCCGACTTCTTCCACCTGCTGCTGCAGCTGAAGAACAGGGGCA
AGGTGTGCGACGACGACAAGCTGCAGGACACCAACAAGAGCACCAAGGAGAGC
GCCCTGACCCTGGACGAGCTGACCGCCCAGAGCTTCGTGTTCTTCATCGCCGG
CTACGAGACCACCAGCACCACCATGACCTTCGCCCTGAGCGAGCTGGCCCTGA
ACCAGGAGATCCAGGAGAAGGCCAGGAAGGAGATCGAGATGGTGGCCGCCAAG
CACAACGGCGAGCTGAGCTACGAGGCCATCAAGGACATGACCTACATGGAGCA
GATCATCAACGAGACCCTGAGGAAGTACCCCCCCGTGCCCATCCTGCTGAGGA
AGTGCAACAAGACCTACCCCGTGCCCGGCACCGACGTGATCATCGAGAAGGAC
GACATGGTGGCCATCAGCTGCCTGGCCATCCAGAACGACCCCGAGATCTACGA
GAACCCCGAGAAGTTCGACCCCGACAGGTTCAGCGCCAAGAACACCGCCAGCA
GGCACCAGTTCGCCCACATCCCCTTCGGCGAGGGCCCCAGGATCTGCATCGGC
ATGAGGTTCGCCCTGATGGAGGCCAAGGTGGGCATGGCCGCCATCCTGAAGAA
CTACAACATCACCCTGAGCAAGAGGACCAAGGTGCCCATCCAGCTGGACCCCA
AGAGCTTCATCAGCGCCCCCAAGGACGGCATCTGGATCCACGCCAAGAGGATC
AGCACCGACTAACTCGAGGCGGCCGC

Figure 2. DNA sequence of CYP6BQ23 expression construct. Pink: *Not*I restriction sites. Yellow: Linker DNA. Green: Upstream activation sequence. Blue: hsp70 basal promoter for *Drosophila melanogaster*. Grey: kozak sequence. White: *M. aeneus* Cyp6bq23 gene sequence.

GCGGCCGCGGAAGCTTGCATGCCTGCAGGTCGGAGTACTGTCCTCCGAGCGGA
GTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAG
CGGAGTACTGTCCTCCGAGCGGAGACTCTAGCGAGCGCCGGAGTATAAATAGA
GGCGCTTCGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGT
CGCTAAGCGAAAGCTAAGCAAATAAACAAGCGCAGCTGAACAAGCTAAACAAT
CTGCAGTAAAGTGCAAGTTAAAGTGAATCAATTAAAAGTAACCAGCAACCAAG
TAAATCAACTGCAACTACTGAAATCTGCCAAGAAGTAATTATTGAATACAAGA
AGAGAACTCTGAATAGGGAATTGGGCCGCGGGAATTCGATTGAGCTCATGAGC
GTGTTTCTGGCTTTGCTCGTGCTCAGTGTTACGATCTTTGGGTTATTCCTCAA
GTACCGTCATGGTTTTTGGCAACGACGCGGCATACCACATGAAGTCCCCAGCT
TTCCCATGGGCGATTTTAAGGAATGGCGCACTACAAAGGGGTTCTTTGAGATA
ATCGGGCCTATATATAAGAAATACAAGGGCACAGCCCCATTTGCCGGCATGTT
TCTAGTCTTCAGACCCGTGGCACTTATACTCGATATGGACTTAGTGAAAAATA
TACTCATTAAAGACTTCAATAATTTTCGTGACCGTGGCGTATTCAGCAATCAA
CGAGACGATCCGCTAACGGGGCATCTCTTCGCTTTGGATGGTACTAAATGGCG
TGATTTGCGTCAAAAACTCACATCGGTCTTCAGTTCAGGCAAAATGAAGTACA
TGTACCCGACCGTCATTAAAGTGGCTGAAGATTTTAGAAAAGTTTTGGAGGAA
AAGCAGGCAACAGCCGTCGGTGGTGTTATTGAGATGAGCGAACTGCTCTCCCG
TTTCACTGCCGATGTCATTGGCGTATGCGCTTTCGGCATCGATTGCAATTGTC
TACATGACCCGCAAGCAGAGTTCGTATCAATGTGCAAGCGCGCAGTTATCGAG
CGTAGACATAACAAATTCATCGATTCCTTAATGGAGGGGGCCCCGAAGTTGGC
GCGTACGCTGCGCATGCGTCAACTGCCGCAAGAGGTGCACGACTTCTACATGG
GCATTGTGCATAAAACTATTGAGTATAGAGAGCAGAATAATGTGAAGCGCAAT
GATCTAATGGATTTGCTCTTAGAATTGAAAAACAAAGGCGACAAAAACTTCGC
CTTAACGGTAAATGATATAGCCGCCCAAGCTTTCGTCTTTCTCATAGCCGGTT
TGGAGACCTCCTCAACAACAATGGGCTTCGCTCTCTATGAGTTGGCTAAAAAC
CAGGCTATACAGGACAAATTGCGTGGGGAGATCAGTGATGTGCTCGAGCGACA
TAATAACGAGTACACCTACGAAGCCATGCAGAATATGCGTTATTTAGATCAAG
TGTTTTCAGAAACTCTACGCAAATATCCAATAGTGCCACATTTGGTACGCGAA
GCGCAAGCTGACTATAAGACCAAAGATCCAAAGTTTATAATCGAGAAAGACAT
GATGATCATCGTACCCGTGTATCAAATCCAACATGATCCGGAATACTTCCCTG
ATCCAGAAAAGTTTGATCCGGAACGCTTCACCGAAGCAGAGATCCATAAACGT
CCCGCTTGTACTTGGCTTCCCTTTGGCGATGGTCCACGCAATTGTATCGGTAT
GCGTTTCGGCAGGATGCAGTCGTTGGTCGGTCTCACATATTTACTGAGAAATT
TCAAATTTTTTCCTTCTGCCGAAACCGATGAGCAACTATCCTATGACGTAGAA
AAAGTTCTACTGCAATCGGCGAATGGCATTAATCTACGTGTGGAGCGTGTATA
GTCTAGAAATCACTAGTGAATTCGCGGCCGCC

Figure 3. DNA sequence of CYP6A51 expression construct. Pink: *Not*I restriction sites. Yellow: Linker DNA. Green: Upstream activation sequence. Blue: hsp70 basal promoter for *Drosophila melanogaster*. White: *C. capitata* Cyp6a51 gene sequence.

GGAGGATCGGGCGATGGCGAAATGGAGGACGAGATACCGGAACTTGTGCACGTGGACGAAGACTTCTTCTACATGGAAC
ACGTTCTCCGCATTGCGGCATGTCTGCACTCACTTGTCTCCCTGGCCATGTTGATTGCCTACTACCACCTCAAGGTTCC
ATTGGCCATCTTCAAGCGGGAAAAAGAGATTGCCCGCCGGCTGGAGTTCGAGGGATTGTTCATTGCAGAGCAGCCGGAG
GATGACGACTTCAAGTCGCACTGGGACAAGCTGGTAATTTCGGCGAAAAGTTTCCCGGTGAACTACTGGGACAAGTTCG
TGAAGAAGAAGGTGCGCCAAAAGTACAGCGAGACCTACGACTTTGATTCGATCTCTAATCTGCTGGGCATGGAGAAGAG
CACGTTCGCGGCTCAGGAGAGCGAGGAAACGGGCATCTTCAAGTACATCATGAACATCGACTGGCGCTATCAGGTGTGG
AAGGCTGGCGTCACCTTCACGGACAACGCCTTCCTCTACTCGCTGTGGTACTTCAGCTTCTCGGTGATGGGTAACTTCA
ACAACTTCTTCTTCGCCGCCCATCTGCTCGACGTCGCTGTGGAGTTCAAGACCCTGCGCACCATCCTCCAGTCTGTGAC
CCACAACGGCAAGCAACTGGTGCTCACCGTGATGCTGCTTACCATCATAGTGTACATCTACACTGTGATCGCGTTCAAC
TTCTTCAGGAAGTTCTACATCCAGGAGGAGGACGAGGAGGTGGACAAAAAGTGCCACGACATGTTGACCTGCTTCGTGT
TCCATCTGTACAAGGGTGTGAGAGCGGGCGGTGGAATAGGCGACGAGATCGGGGATCCAGATGGAGACGACTACGAGGT
CTACCGCATCATCTTCGATATCACGTTCTTCTTCTTCGTTATTATTATCCTGCTGGCCATTATCCAGGGTCTGATCATC
GACGCCTTCGGCGAGCTGCGTGACCAACTGGAGTCGGTGAAGGACAACATGGAGTCCAACTGCTTCATCTGCGGGATGG
GCAAGGACTTCTTCGACATAGTACCGCACGGCTTCGACACGCACGTCCAGAAGGAGCACAACTTAGCCAACTACATGTT
CTTCCTGATGCATTTGATTAACAAGCCGGACACGGAGTATACCGGCCAGGAGACGTACGTGTGGAACATGTACCAGCAG
CGCAGCTGGGACTTCTTCCCAGTGGGAGACTGCTTCCGCAAGCAATACGAGGATGAGCTTTCCGGCGGAGGCGGCGGCG
GCTAAATGACTGCGGATTCGCCGCGTCTGATTTCTGGGCACAATCACACTGGCACTGGACGGTCCAAGGACTCTCGGGA
TCCAATTTTGTATCGGTAGTTTGACAACATCTGGAATTTTTACCTAGTCAACTTTAGCAATGTATTTTAAGCA

Figure 4. Donor plasmid for genome editing in RyR gene in *Drosophila*. Pink: Generic primers. Green: *Aat*II restriction site. Blue: Mutated PAM sequence. Yellow: The point mutation G4946E; Glycine (GGC) to Glutamic acid (GAG) substitution. Grey: *Pst*I restriction site.

Sense oligo: CTTCGCGCAGGGTCTTGAAGCCCA
Antisense oligo: AAACTGGGCTTCAAGACCCTGCGC

Figure 5. The gRNA target sequence flanked with *Bbs*I sites