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Functional analysis and characterization of insecticide target and detoxification proteins in insect pests and disease vectors

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Abstract

Insect pests are major threats for public health and agriculture, as they transmit various diseases and attack agricultural crops. Their control has been largely relied on the use of chemical insecticides. However their intensive use has resulted in many problems including the development of resistance – mainly achieved by detoxification enzymes and target site resistance mutations – effects in human health and environmental impacts. Thus, understanding the mechanisms underlying insecticide resistance as well as developing novel ways for insect control, more efficient, selective and environmentally safe are a clear and urgent need. In this thesis, we used techniques of molecular biology, protein expression approaches and biochemical/functional techniques to a) investigate detoxification mechanisms of major mosquito vectors against insecticides and b) attempt to functionally express a pest chitin deacetylase to further evaluate it as a potential target for novel pesticides.

First, three mosquito UGTs, previously associated with insecticide resistance in two major vectors of human diseases, were cloned and expressed in insect cells using the baculovirus expression system and were tested for their ability to glycosylate known insecticide metabolites. AgUGT1 from *Anopheles gambiae*, the major malaria vector, associated with resistance to insecticides of the pyrethroid class, were functionally expressed and tested for their activity towards the pyrethroid metabolites 3-Phenoxybenzyl alcohol (PBAlc) and 3-Phenoxybenzoic acid (PBA). Aealbo_UGT1 and Aealbo_UGT2 from *Aedes albopictus*, the Asian Tiger mosquito, vector of arboviruses such as dengue, associated with resistance to the organophosphate temephos were expressed and, as well as AgUGT1, were active showing glucosyltransferase activity towards the common UGT substrate a-naphthol.

In the second chapter, we tried to express and characterize a possible chitin deacetylase from the cotton bollworm *Helicoverpa armigera* to further study it as a possible target for the design and development of novel pesticides.

Περίληψη

Τα επιβλαβή έντομα αποτελούν απειλή για τη δημόσια υγεία και την γεωργία καθώς μεταφέρουν εντομομεταδιδόμενες ασθένειες και καταστρέφουν τις καλλιέργειες. Η καταπολέμησή τους βασίζεται κυρίως στη χρήση χημικών εντομοκτόνων. Ωστόσο, η συχνή τους εφαρμογή έχει οδηγήσει σε πολλά προβλήματα, μεταξύ των οποίων η ανάπτυξη ανθεκτικότητας στα εντομοκτόνα – η οποία επιτυγχάνεται κυρίως μέσω της δράσης των ενζύμων αποτοξικοποίησης και μεταλλαγές στις πρωτεΐνες-στόχους των εντομοκτόνων – καθώς και επιδράσεις στην υγεία και το περιβάλλον. Έτσι, η κατανόηση των μηχανισμών που διέπουν την ανθεκτικότητα στα εντομοκτόνα καθώς και η ανάπτυξη νέων μεθόδων για την καταπολέμηση των εντόμων, πιο αποτελεσματικών, επιλεκτικών και φιλικών προς το περιβάλλον αποτελούν επείγουσα ανάγκη. Σε αυτήν την εργασία, χρησιμοποιήσαμε μοριακές τεχνικές, προσεγγίσεις για την έκφραση πρωτεϊνών καθώς και βιοχημικές/λειτουργικές μελέτες για α) τη διερεύνηση των μηχανισμών αποτοξικοποίησης εντομοκτόνων ουσιών σε κουνούπια-φορείς ανθρώπινων ασθενειών και β) την προσπάθεια λειτουργικής έκφρασης του ενζύμου απακετυλάση χιτίνης από παράσιτο της γεωργίας ώστε να μπορέσουμε να αξιολογήσουμε περαιτέρω το ρόλο του ενζύμου ως πιθανό στόχο για νέα παρασιτοκτόνα.

Αρχικά, τρεις UGTs από κουνούπια-φορείς ανθρώπινων ασθενειών, οι οποίες έχουν συσχετιστεί με την ανθεκτικότητα σε εντομοκτόνα, κλωνοποιήθηκαν και εκφράστηκαν ετερόλογα σε κύτταρα εντόμων με τη χρήση του συστήματος έκφρασης με βακουλοϊούς και ελέγχθηκαν για την ικανότητά τους να γλυκοζυλιώνουν γνωστούς μεταβολίτες εντομοκτόνων. Από το κουνούπι *Anopheles gambiae*, τον κύριο φορέα της ελονοσίας, η πρωτεΐνη AgUGT1 η οποία έχει συσχετιστεί με την ανθεκτικότητα σε πυρεθροειδή εντομοκτόνα εκφράστηκε επιτυχώς και ελέγχτηκε ως προς την δράση της έναντι στους μεταβολίτες πυρεθροειδών: 3-φαινοξυβένζυλ αλκοόλη (PBAlc) και 3-φαινοξυβενζοϊκό οξύ (PBA). Οι πρωτεΐνες Aealbo_UGT1 και Aealbo_UGT2 από το ασιατικό κουνούπι τίγρης *Aedes albopictus*, φορέα ιών όπως ο δάγκειος πυρετός, οι οποίες έχουν συσχετιστεί

με την ανθεκτικότητα στο οργανοφωσφορικό εντομοκτόνο τέμεφος, εκφράστηκαν επιτυχώς και όπως η AgUGT1, έδειξαν ενεργότητα γλυκοζυλτρανσφεράσης προς το ένα γενικό υπόστρωμα για τις UGTs, το a-naphthol.

Στο δεύτερο κεφάλαιο, προσπαθήσαμε να εκφράσουμε και να χαρακτηρίσουμε λειτουργικά μια πιθανή απακετυλάση χιτίνης από το σκουλήκι του βαμβακιού *Helicoverpa armigera* ώστε να την μελετήσουμε περαιτέρω ως πιθανό στόχο για τον σχεδιασμό και την ανάπτυξη νέων φυτοφαρμάκων.

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Abbreviations

ABC transporter	ATP-Binding Cassette transporter
Ache	Achetylcholinesterase
BLAST	Basic Local Alignment Search Tool
CCE	Carboxylesterase
CSI	Chitin Synthesis Inhibitors
CDA	Chitin Deacetylase
CYPs	Cytochromes P450
DTT	Dichlorodiphenyltrichloroethane
ER	Endoplasmic Reticulum
ESI	Electrospray Ionization
GFP	Green Fluorescent Protein
GlcUA	Glucuronic acid
Glc	Glucose
GSH	Glutathione
GST	Glutathione S-tranferase
HPLC	High Performance Liquid Chromatography
IGRs	Insect Growth Regulators
IRAC	Insecticide Resistance Action Committe
IRS	Indoor Residual Spraying
ITN	Insectiside Treated Nets
IVM	Integrated Vector Management
MS	Mass Spectrometry
NCBI	National Center for Biotechnology Information

NTC	No Template Control
ORF	Open Reading Frame
P450	Cytochrome p450 mono-oxygenase
PBA	3-Phenoxybenzoic acid
PBAlc	3-Phenoxybenzyl alcohol
PM	Peritrophic Matrix
P_{PH}	Polyhedrin Promoter
qPCR	quantitative Polymerase Chain Reaction
RIDL	Release of Insects carrying a Dominant Lethal
RNAi	RNA interferece
SIT	Sterile Insect Technique
UDP	Uridine diphosphate
UGT	UDP-glycosyltransferase
VGSC	Voltage-Gated Sodium Channel
WGS	Whole Genome Sequencing
WHO	World Health Organization

General Introduction

Insects are a class of invertebrate animals within the arthropod phylum. They are the most diverse group of animals on the planet, including more than a million described species and representing more than half of all known living organisms. Insects play significant roles in the ecology of the world and in the preservation of life in the planet due to their interaction with plant life, other organisms and the environment. Some of their beneficial roles are the pollination of plants, the recycling of nutrients by decaying organic matter acting as decomposers, the production of several good for the benefit of human such as silk and honey and of course they are important part of the food chain (https://www.britannica.com/animal/insect).

In spite of all their positive attributes, some insects can cause severe problems and are considered deadly enemies of humanity. Insect pests can transmit serious diseases and attack agricultural crops threatening public health, food security and global economy. For example mosquitoes are responsible for the transmission of infectious diseases such as malaria, filariasis, dengue, chikungunya and yellow fever causing severe mortality and morbidity annually (WHO, 2015). Insect pests are also responsible for huge crop losses worldwide damaging food production representing an enormous threat for humans. An example of agricultural pest is the cotton bollworm *Helicoverpa armigera* the larvae of which is feeding on many important cultivated crops such as cotton , tomato, chickpea, rice and many others (Tay et al., 2013).

The efficient control of insect pests is vital for the protection of public health, agriculture and economy. The primary means of their control is through the application of chemical insecticides. Chemical insecticides can either have neurotoxic effects targeting the central nervous system or act as growth regulators interfering with fundamental physiological pathways of insect pests (Sparks and Nauen, 2015). Even though such interventions are capable of rapidly killing a range of pests, and insecticides can be characterized as typically convenient, fast acting and inexpensive, their extended and frequent application has resulted in several problems, including

reduced efficacy (insecticide resistance), outbreaks of secondary pests and impacts on human health and environment.

The development of insect pest resistance to chemical insecticides is an evolutionary procedure driven by natural selection and it is considered as one of the most important limitations for an effective insect pest control in our days. It is most commonly evolved by the excessive and/or wrong usage of insecticides in the field and usually arises within ten years after the introduction of an insecticide in the market (Stenersen, 2004). Resistance mechanisms can decrease the amount of insecticide that finally reaches the target with the over-production of detoxification enzymes (metbolic resistance), and/or reduce the affinity of the target with the insecticide with mutations occuring in the insecticide targets (target-site resistance), resulting in resitant strains compromisisng the efficiency of the control programs (Nauen, 2007). Therefore, understanding molecular mechanisms in resistant populations can give valuable insight to be able to control the development and spread of resistant insects.

The development of insecticide resistance in insect pests is one major problem caused by the extended use of chemical insecticides but it is not the only one. Other problems resulted from their intense use is environmental pollution and impacts in human health. Regarding environmental impacts, pesticides contribute to air, water and soil pollution, they decrease the general biodiversity having effects in non-target species and have some direct harmful effects on plants. Their usage has also effects on animals (birds, aquatic life, amphibians) decreasing their population (Damalas and Eleftherohorinos 2011). As far as human health is concerned, exposure to insecticides can range from mild skin irritation to birth defects, tumors, genetic changes, blood and nerve disorders, several immune disorders, coma or death (Gilden et al., 2010). Taking into account the increasing insecticide resistance along with the growing concerns about health and environmental impacts of chemical control, there is a growing need for novel, effective, sustainable, environmental-friendly and safe methods for insect pest control.

The present study aims on the one hand to investigate the role of specific enzymes (UGTs), associated with insecticide resistance, in metabolic resistance and detoxification of insecticides in mosquitoes-major vectors of human diseases, and on the other hand the evaluation and validation of chitin deacetylases as potential targets for novel insecticides starting from the overexpression and characterization of a potential chitin deacetylase from the agricultural pest *Helicoverpa armigera.* The thesis is divided into two main parts:

I. The functional characterization of UDP-glucosyltransferases associated with insecticide resistance in mosquito vectors of human diseases. Baculovirus-mediated insect cell expression was used for the heterologous expression of mosquito UGTs from *Ae. albopictus* (up-regulated in a temephos resistant strain) and *An. gambiae* (up-regulated in pyrethroid resistant mosquitoes), in order to funtionally characterize them and test their ability in vitro to metabolize insecticide metabolites.

II. The overexpression of the potential chitin deacetylase HaCDA5a from the agricultural pest *Helicoverpa armigera* to further investigate it as potential target of novel insect growth regulators. Baculovirus-mediated insect cell expression was used for the heterologous expression of the potential chitin deacetylase HaCDA5a from *Helicoverpa armigera* in order to firstly, validate the chitin deacetylase activity and secondly, evaluate its importance in insect's biological cycle so as to develop and design new enzyme inhibitors as novel pesticides.

PART I

Functional characterization of UDP-glucosyltransferases associated with insecticide resistance in mosquito vectors of human diseases

1.1. Introduction

1.1.1. Mosquito—borne diseases

Mosquito-borne diseases represent an immense threat for public health and are among the leading causes of human deaths. The three most important genera of mosquitoes (Diptera: Culicidae) are *Anopheles*, which transmits malaria; *Culex*, which is responsible for the transmission of filariasis and several arboviruses, such as West Nile virus; and *Aedes*, which transmits dengue, Zika, chikungunya and yellow fever viruses. With millions of deaths annually caused by these diseases, where malaria alone is causing 400.000 deaths every year globally and with approximately 3 billion people in endemic areas around the world being at risk of potential infection, mosquitoes can be characterized as one of the deadliest animals in the world (data retrieved from WHO, 2015). Two major vector species for human diseases are the African malaria mosquito *Anopheles gambiae*, which is the primary and most efficient mosquito vector responsible for malaria and Asian Tiger mosquito *Aedes albopictus* (Figure 1.1.1.) transmitting pathogens responsible for viral diseases and currently the most invasive vector species worldwide (della Torre et al., 2005; Bonizzoni et al., 2013 respectivey). Besides premature mortality, the diseases spread by mosquitoes have also detrimental effects on fertility and population growth and represent a major burden in terms of economy and development worldwide. It has been estimated that every year, malaria causes a gross domestic product loss of 12 billion USD to Africa (WHO, 2010). Furthermore, the increasing globalization of trade and human movement along with environmental changes facilitates the introduction and establishment of invasive mosquito species outside their natural geographical sites (Medlock et al., 2012). To date, there are no approved vaccines neither for malaria nor dengue and specifically in the case of dengue there are no antiviral therapies as well (Simmons et al., 2012). In addition, even though there are drugs available for malaria, problems with drug resistant and affordability make them a non-universal solution (Price and Nosten, 2001). In the absence of vaccines or therapies available the control of the vectors, i.e. the mosquitoes, is the most effective and realistic option available to reduce disease transmission.

A 	**B** 

Figure 1.1.1. Two of the major mosquito vectors for human diseases. **A.** The Asian Tiger mosquito *Aedes albopictus* and **B**. The African malaria mosquito *Anopheles gambiae*

1.1.2. Mosquito Control Strategies

Mosquito control is achieved through a number of different approaches, which can be classified in 5 main categories: environmental methods, mechanical methods, biological methods, genetic methods and chemical methods (Baldacchino et al., 2015). Integrated Vector Management (IVM) is the combination of the different management approaches available, aiming to confer efficient control minimizing the damage to humans and the environment and the cost (WHO 2004). The different approaches for mosquito control are described briefly below:

Environmental methods: include methodologies that aim to reduce potential mosquitos' breeding sites. This strategy is based on removing temporary water containers and covering the permanent ones. Source reduction can affect the distribution of both native *Culex sp.* and invasive species such as *Ae. albopictus*, which breed in artificial containers (Baldacchino et al., 2015).

Mechanical methods: include the use of odour baits and traps targeting gravid or host-seeking females and have been suggested as a means to reduce adult populations of mosquitoes (Baldacchino et al., 2015).

Biological methods: include the usage of natural enemies of the mosquitoes or natural products with larvicide activity. Natural enemies may be other invertebrate species such as the crustacean copepods which are used as predators of mosquito larvae or microbes such as the well-known bacterium *Bacillus thuringiensis*, entomopathogenic fungi (e.g. *Metarhizium anisopliae* and *Beauveria bassiana*) or

the endosymbiotic bacterium *Wolbachia pipientis* which is present in many mosquito species (Baldacchino et al., 2015). *Wolbachia* infects the gonads and can induce reproductive alterations leading to sterile progenies. Also it reduces the ability of certain pathogens to replicate in insects (Iturbe-Ormaetxe et al., 2011). Another method for mosquito biological control is the use of natural products such as essential oils or spinosad as larvicides (Dias and Morraes, 2014 and Hertlein et al., 2010 respectively).

Genetic methods: include the usage of genetic-based approaches, enabled by advances in mosquito genetics, aiming either to reduce the number of individual vector mosquitoes –population suppression– or to reduce the ability of individual mosquitoes within the population to transmit pathogens –population replacement (Alphey et al., 2013). In the first case, a familiar approach is SIT (Sterile Insect Technique) which relies on the release of sterile males, sterilized either by irradiation or RNAi, leading in the death of most of their offsprings. In the same category is RIDL technique (Release of Insects carrying a Dominant Lethal) which has been successfully tested in the field in the Cayman Islands (Harris et al., 2011). Genetic-based approaches are extremely species-specific due to the vertical transmission of heritable elements (Alphey et al., 2013). Research for developing novel strategies for pest management includes another very important genetic approach, which is the silencing of key genes, important for growth and development, using RNA interference as an alternative (Das et al., 2015; Mysore et al., 2013) or complementary method (Zhang et al., 2010) of chemical control.

Chemical methods: include the use of chemicals in order to either directly kill the mosquitoes or via interfering with some of their fundamental activities such as molting and metamorphosis (Sparks and Nauen, 2015; Baldacchino et al., 2015). Chemical control is the predominant and most efficient approach recruited for the control of mosquitoes.

All the above mentioned control methods are represented in **Fig. 1.1.2.** with Aedes mosquitoes as an indicated example.

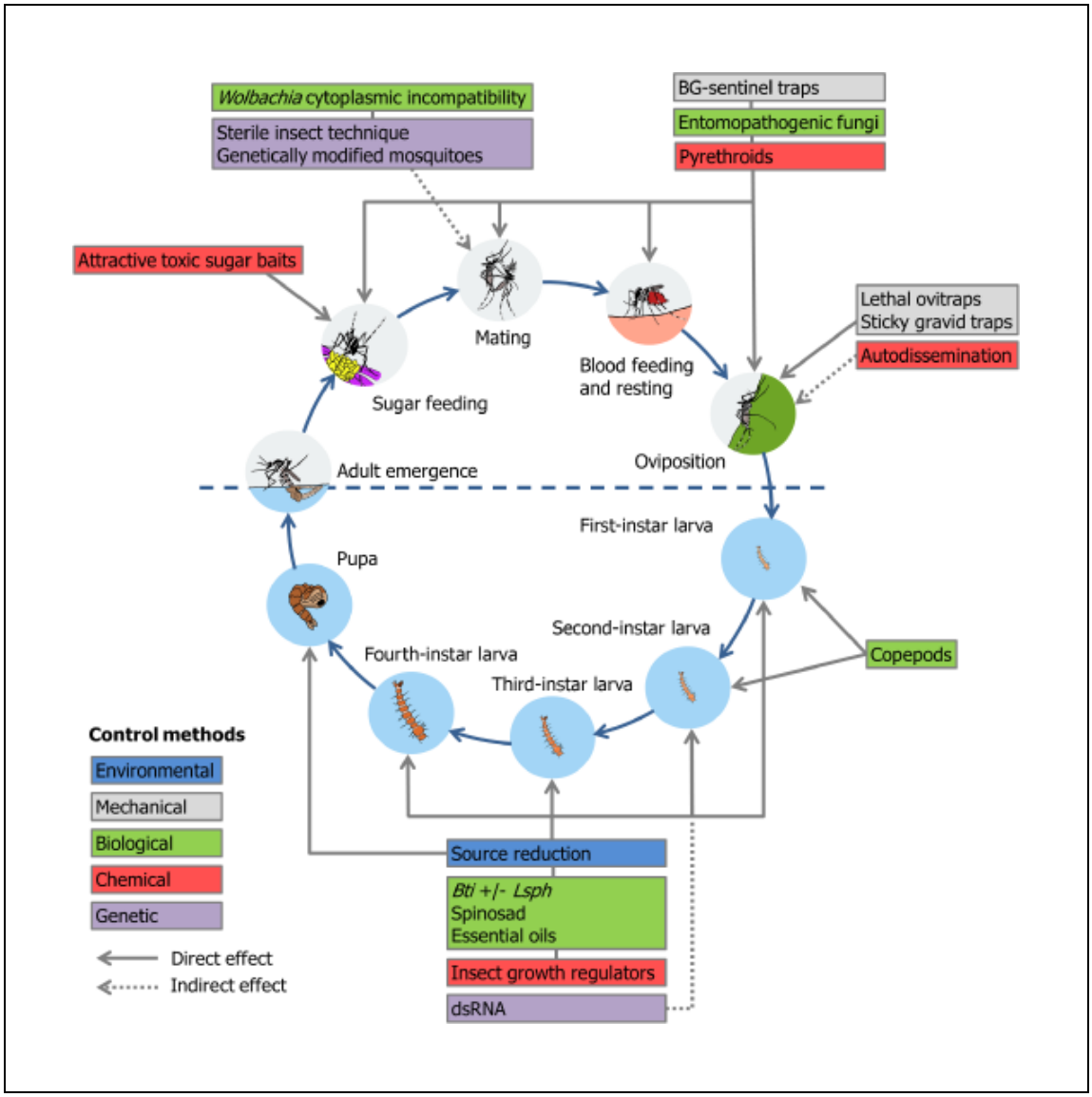


Figure 1.1.2. Control methods available against invasive Aedes mosquitoes. *Bti*: *Bacillus thuringiensis* var. *Israelensis*; *Lsph*: *Lysinibacillus sphaericus*; dsRNA: doublestranded RNA **.** Image adapted from Badacchino et al., 2015.

1.1.2.1. Chemical control

Chemical control, as mentioned above, includes the use of chemicals in order to either directly kill the mosquitoes or via interfering with some of their fundamental activities. The insect central nervous system is the main target of the vast majority of synthetic chemicals (insecticides) causing paralysis and finally death, while others act as growth regulators (e.g. juvenile hormone agonists, chitin synthases inhibitors) (Sparks and Nauen, 2015). Undoubtedly, a significant contribution in mosquito

control is achieved by the usage of insecticides. There are four chemical classes of synthetic insecticides with neural/muscle action used for mosquito control to date, pyrethroids, organophosphates, carbamates and organochlorines (which are now banned in most countries) and they exhibit two different modes of action. Synthetic pyrethroids and organochlorines such as DTT inhibit the voltage-gated sodium channels (VGSC), where organophosphates and carbamates inhibit acetylcholine esterase, Fig 1.1.3. (Nauen, 2007; David et al., 2013). Common interventions with insecticides include insecticide treated nets (ITNs) and indoor residual spraying (IRS) targeting indoor night biters such as *An. gambiae* and space spraying (fogging) or larviciding for outdoor day-time biters such as *Ae. albopictus* and *Ae. aegypti* (van den Berg et al., 2012). Synthetic pyrethroids are the only insecticide class recommended for insecticide treated nets (ITN) due to their low toxicity in mammals (Wollansky and Harrill, 2008) and together with organophosphates they play an important role in vector control.

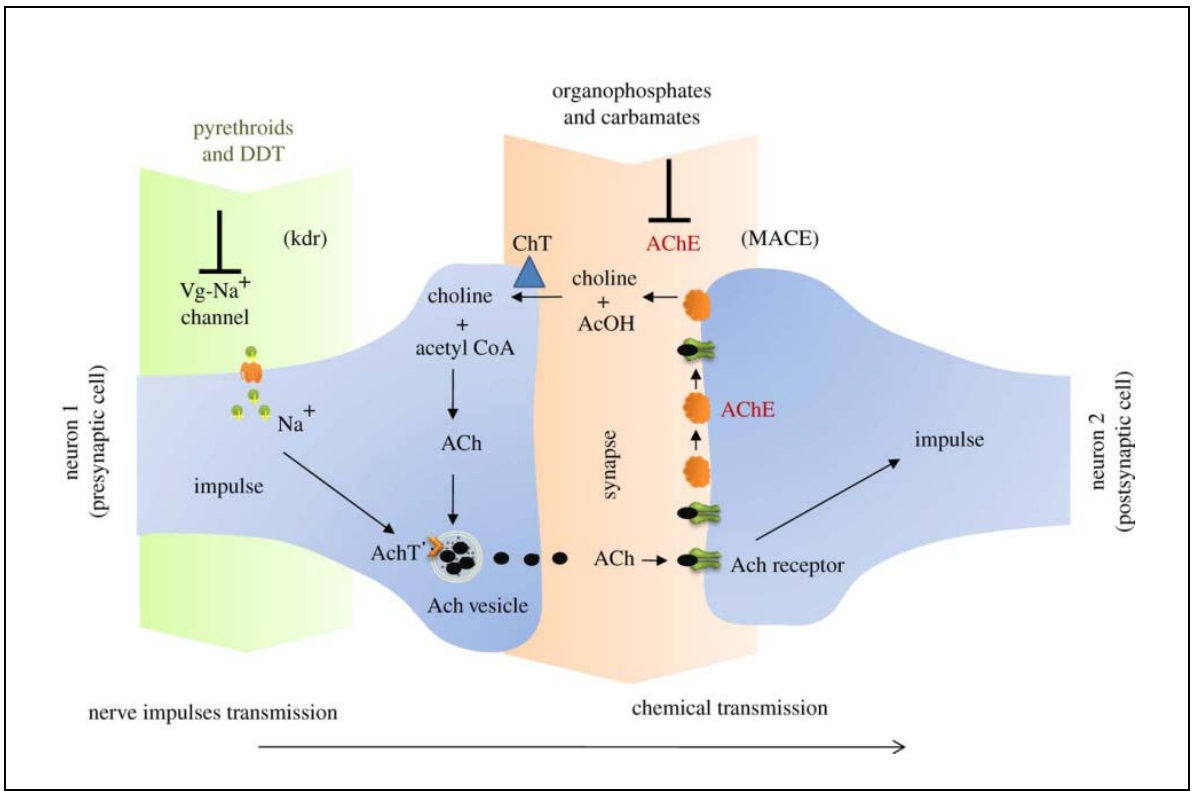


Figure 1.1.3. Biochemical target sites of synthetic insecticides. Pyrethroids and DDT exert their toxic effect by blocking the voltage-gated sodium channels, which generally produces fast knock-down properties (kdr). Organophosphate (OP) and carbamate insecticides inhibit acetylcholinesterase (AChE) which plays an important role in

terminating nerve impulses. Reduced sensitivity of AChE as a result of a gene mutation (MACE) causes resistance to OP and carbamate insecticides). ACh, acetylcholine; AchT, Ach transporter; AcOH, acetic acid; ChT, choline transporter; MACE, modified acetylcholinesterase; Vg-Na^+ channel, voltage-gated sodium channel; kdr, knock-down resistance. Acquired from David et al., 2013.

Insect growth regulators (IGR), on the other hand, are a group of chemicals that possess growth-retarding or growth-inhibiting properties and are highly effective against mosquito larvae, therefore considered promising vector control alternatives (Mulla 1995). They have been used as chemical larvicides but they also possess ovicidal properties, and can inhibit egg hatching, depending on their mode of action, the dose applied and the mosquito species (Suman et al., 2013). They are also considered more selective as well as environmentally safe comparing to common neurotoxic insecticides since they target specific physiological pathways of insect pests (Graf, 1993). Known IGRs are chitin synthesis inhibitors (CSI) which interfere with the insect molting process. They belong to the benzoylurea family and their mode of action was described recently (Douris et al., 2016). The fact that chitin is present in invertebrates and fungi and the important role that plays in structures such as cuticles and peritrophic membranes (PM), the enzymes that take part in its synthesis (chitin synthases) or degradation (chitinases) are well established targets for pest control (Sun et al., 2015; Naqpure et al., 2014). In the same context, chitin deacetylases (CDAs) are chitin modifying enzymes which convert chitin to chitosan which in contrast to chitin synthases and chitinases have not been examined as potential targets for novel insecticides (Zhao et al., 2010). The evaluation, validation and prioritization of CDAs as potential insecticide targets of novel IGRs is one of the goals of our lab in collaboration with Enzyme Biotechnology Group/IMBB.

1.1.3. Insecticide resistance in mosquito-vectors of human diseases

In agreement with the general global strategies designed to prevent vector-borne diseases where the control methods mainly target the vectors, mosquito control programs, mostly through the use of insecticides, have an important role for the management and prevention of the diseases they spread (WHO, 2006; Hemingway,

2003). The extensive application of insecticides, though, has contributed to the development of resistance compromising the efficiency of the control interventions against mosquitoes (Nauen, 2007; Ranson et al., 2011; Vontas et al., 2012; Yewhalaw et al. 2011).

According to IRAC, insecticide resistance can be described as 'a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species'. In the molecular level, there are two major mechanisms conferring resistance to insecticides, target-site resistance, through structural modifications and/or mutations that alter the targets of insecticides in the nervous system –namely, AChEs, sodium channels, and GABA receptors – thus decreasing the sensitivity of the target to the insecticide and metabolic resistance, referring to alterations in the levels or activities of detoxification enzymes (Hemingway et al., 2004) (**Fig. 1.1.4.**). Among the most common detoxification enzymes are members of the cytochrome P450 family, glutathione transferases (GSTs) carboxylesterases (CCEs) and UDP-glucosyltransferases, with members of the first three categories being well studied in mosquitoes (reviewed in Liu 2015). Resistance may also occur via physiological changes in mosquitoes' cuticle resulting in lower insecticide penetration (cuticular resitance) (Balabanidou et al., 2016).

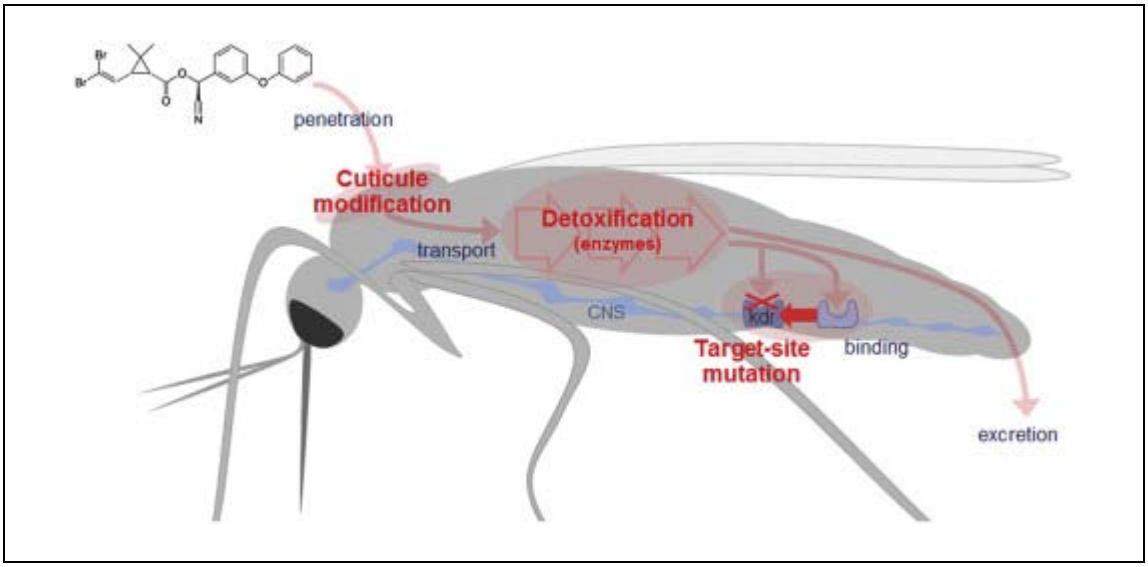


Figure 1.1.4. Resistance mechanisms in mosquitoes. CNS: Central Nervous System. The molecule represented is the synthetic pyrethroid deltamethrin. Acquired from Nkya et al., 2013**.**

1.1.3.1. Metabolic resistance in mosquitoes and detoxification enzymes

Metabolic detoxification of insecticides in mosquitoes is achieved through gene overexpression/amplification or structural mutations in detoxification enzymes that result in their decreased biodegradation. In general, the detoxification of a xenobiotic such as an insecticide is divided in three phases (Xu et al., 2005). In phase I enzymes, P450s and CCEs, modify the xenobiotic (e.g. via hydroxylation) and the modified xenobiotic is recognizes and conjugated to polar compounds by phase II enzymes, which are transferases such as GSTs and UGTs (Xu et al., 2005). The polar compounds increase xenobiotic solubility as well as act as a tag for phase III, where the xenobiotic is recognized from membrane transporters (e.g. ABC transporters) and excreted from the cell (Dermauw and Van Leeuwen, 2014). The major families of detoxification genes involved in mosquitoes' metabolic resistance are further described below:

Cytochrome P450s monooxygenases (P450s): They are phase I enzymes, present in all living organisms, encoded from CYP genes and they are best known for catalyzing the so-called monooxygenase reaction where one of the atoms from an oxygen molecule (O_2) is inserted in an organic compound, while the other is reduced to

water. P450s are involved in the metabolism of a wide range of endogenous and exogenous molecules. They have been extensively studied due to their ability to metabolize insecticides thus playing a dominant role in insecticide resistance. P450s represent the only detoxification super-family that can metabolize and confer high levels of resistance to all classes of insecticides, such as OPs, pyrethroids, organochlorides and carbamates (Feyereisen, 2005). Several P450s have been identified to date to metabolize insecticides, in particular enzymes belonging to the CYP6 and CYP9 clades. Selected examples are CYP9J24, CYP9J26, CYP9J28 and CYP9J32 from *A. aegypti* which metabolize deltamethrin and permethrin (pyrethroids) (Stevenson et al., 2012).

Carboxylesterases (CCEs): CCEs belong to an ubiquitous enzyme superfamily that hydrolyze ester bonds from substrates containing a carboxylic ester with the subsequent formation of an alcohol and a carboxylate product, in a two-step reaction, and are considered phase I enzymes (Montella et al., 2012). In insecticide resistance, are mainly implicated in resistance to OPs, pyrethroids and carbamates, where they act either via hydrolysis or/and sequestration (Hemingway and Ranson, 2000). The ability of esterases to metabolize pyrethroids it is well known in mammals (Godin et al., 2006; Nakamura et al., 2007; Takako et al., 2011). In mosquitoes, the capacity of *Ae. aegypti* CCEs to metabolize pyrethroids has been demonstrated in vitro (Somwang et al., 2011). Likewise, a member of the CCE family, previously ascociated with resistance to the organophosphate insecticide temephos in the major arbovirus vectors *Aedes aegypti* and *Aedes albopictus,* was found to metabolize temephos *in vitro* (Grigoraki et al., 2016). Up-regulation of carboxylesterases associated in temephos resistance is due to gene amplification (Grigoraki et al., 2017).

Glutathione S – transferases (GSTs): GSTs are enzymes that catalyze numerous reactions. Regarding metabolism, they are phase II enzymes that are known for the catalysis of glutathione (GSH) conjugation reaction. This conjugation increases the solubility of the substrate facilitating in subsequent excretion from the cell (Sherratt and Hayes, 2001). In mosquitoes, elevated levels of GSTs have been frequently

involved in insecticide resistance (David et al., 2005; Macrombe et al.,2012; Muller et al., 2007; Strode et al., 2008). Furthermore, a recent study showed that the partial RNA interference-mediated knock down of *Ae. aegypti GSTe7* and *GSTe2* genes led to an increased susceptibility to the pyrethroid deltamethrin (Lumjuan et al., 2011).

UDP-glucosyltransferases (UGTs): UGTs are phase II enzymes that play a very important role in detoxification and have been associated with adaptation of insect pests to phenolic compounds (Luque et al., 2002). Regarding insecticide biodegradation, in mammals it is known that UGTs participate in pyrethroid metabolism, since glucoside conjugates of their metabolites (i.e. pheboxybezoic acid) have been found in human biological samples (Takaku et al., 2011). Even though glucuronidation of xenobiotics from enzymes of the UDP-glucosyltransferase family is a major pathway for their biotransformation and excretion in most mammalian species (Ouzzine et al., 2003; Parkinson 2001), in mosquitoes their role in resistance is not fully understand. Due to the objectives of the study, UGTs are further discussed below.

1.1.4. UDP-glucosyltransferases (UGTs)

UDP-glucosyltrasferases are a superfamily of enzymes that catalyze the conjugation of a hexose group (glucose, galactose, xylose, glucuronic acid), donated from a nucleotide sugar, to a variety of small lipophilic molecules. This sugar conjugation increases the polarity and thus the solubility of the substrate facilitating its subsequent excretion from the cell (Bock 2003; Meech and Mackenzie 1997). The importance of these enzymes is reflected by the fact that UGT-orthologues are present in all living organisms (vertebrates, invertebrates, plants, bacteria) and some viruses (Bock 2016). In most organisms UGTs are implicated in the detoxification of noumerous endogenous and exogenous compounds and can be considered as “phase II” enzymes (Iyanagi 2007). Although considered to play an important role in all organisms, UGTs are best studied in mammals where an extra interest is showing due to their ability to metabolize drugs. Specifically in humans, cytochromes P450

(CYPs) together with UGTs are responsible for the detoxification of more than 90% of drugs that are dependent on hepatic clearance (Rowland et al., 2013; Miners et al., 2004).

1.1.4.1. Mammalian UGTs

Mammalian UGTs are divided in 4 families UGT1, UGT2, UGT3 and UGT8 (Meech et al. 2012). The UGT1 and UGT2 families are better studied and linked with detoxification of various endogenous and exogenous compounds. They mainly use glucuronic acid (GlcUA), as the UDP-sugar donor. Some substances found to be substrates for human UGTs are the endogenously produced bilirubin (during the metabolism of heme), thyroid hormones, bile acids and fatty acids (Radominska et al., 1993). Many drugs are also substrates of UGTs such as morphine, aldose reductase inhibitor (used for prevention of diabetic complications) and paracetamole (Toide K et al., 2004). Regarding the tissue localization of these enzymes, liver shows the highest and broadest level of UGT expression. This is in agreement with their role in metabolism and detoxification (reviewed in Mackenzie et al., 2010). In addition to mammalian UGTs, large UGT families have also been identified in insects (Ahn et al., 2012), non-insect arthropods such as the spider mite *Tetranychus urticae* (Ahn et al., 2014) and plants (Bowles et al., 2006). The reaction catalyzed by mammalian UGTs is shown in **Figure 1.1.5.** the underlying mechanism of which is a second order nucleophilic substitution (Radominska-Pandya et al., 2010).

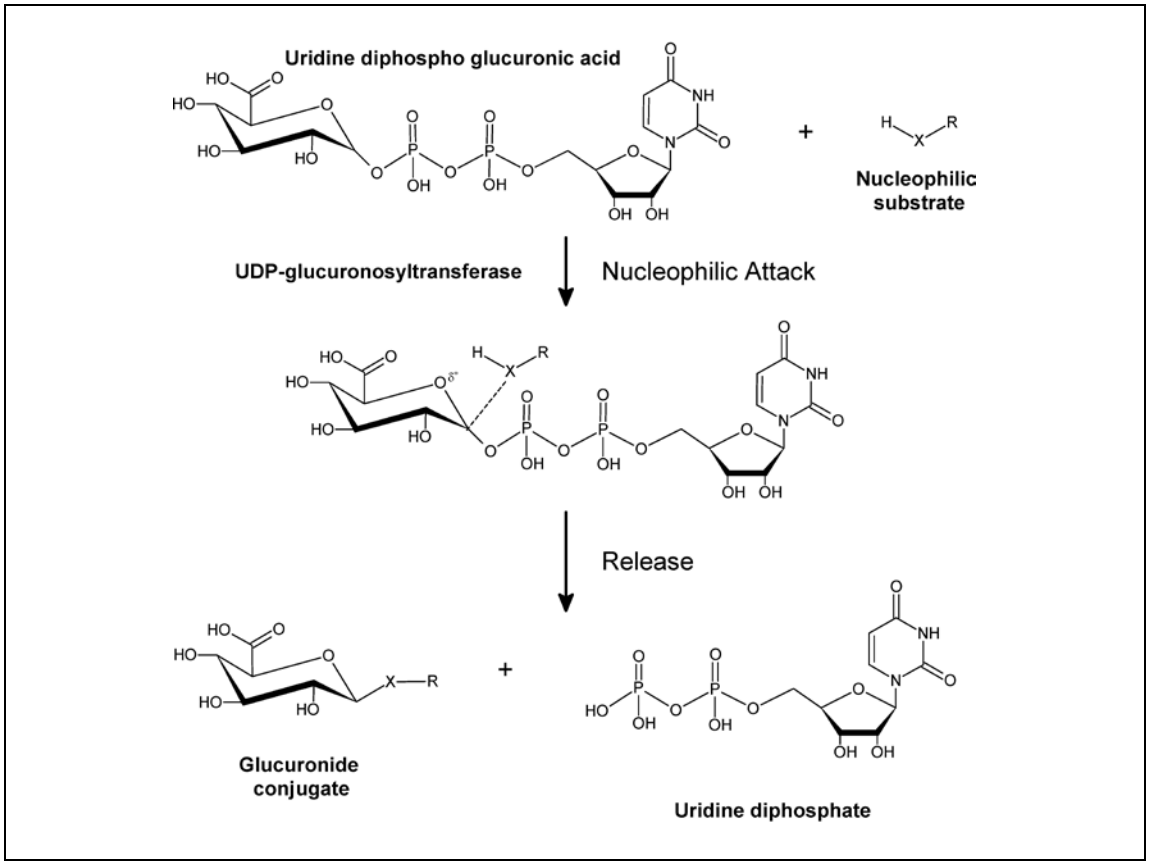


Figure 1.1.5. Reaction catalysed by mammalian UGTs. Image from Rowland et al., 2013.

Mammalian UGTs catalyze the transfer of glucuronic acid from UDP-glucuronic acid (UDPGA) to hydroxyl, carboxyl, or amine groups of hydrophobic compounds (Oda et al., 2015). Their topology in ER, together with the other important detoxification enzymes and their possible substrates are shown in **Figure 1.1.6.**

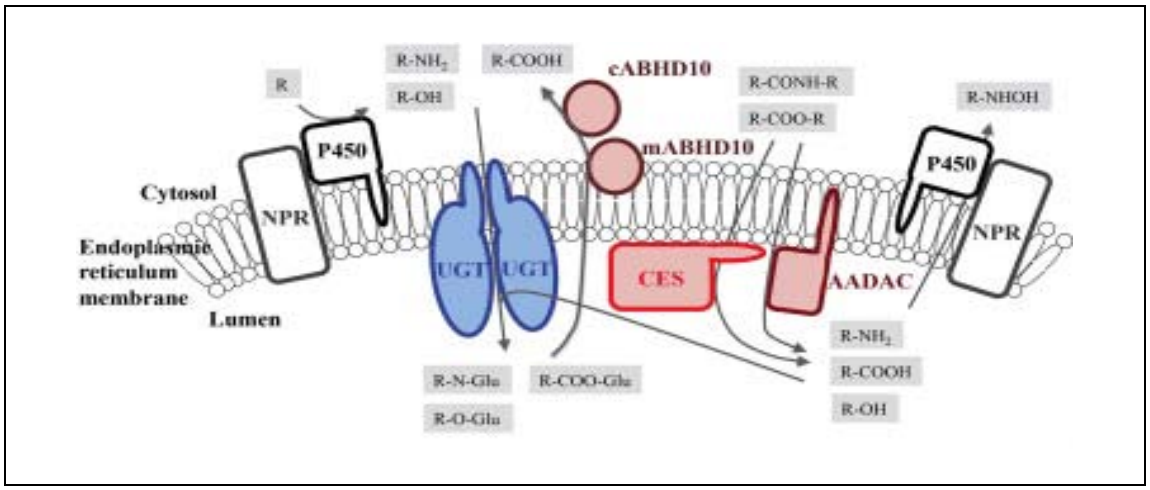


Figure 1.1.6. Topology of mammalian UGTs in the ER together with the other important detoxification enzymes and possible substrates. Image acquired from Oda et al., 2014 .

1.1.4.2. Insect UGTs

As far as insect UGTs are concerned, they mainly use glucose (Glc) as the UDP-sugar donor and their enzyme activities are detected in the fat body, midgut, and other tissues (Ahmad and Hopkins, 1993). They were found to possess multiple functions in different biological processes such as pigmentation (Xu et al., 2013), UV shielding (Daimon et al., 2010), endobiotic modulation (Svodoba and Weirich 1995), and the clearance of odorants and xenobiotics (Bozzolan et al., 2014). Specifically for xenobiotic compounds, several studies have shown the important role of UGTs in detoxification of plant toxins encoundered by insects in their diet. The first functional characterization of a insect UGT was with BmUGT1, a UGT of the silkworm *Bombyx mori*, an important species due to its use in silk production, where it was functionally characterized to metabolize several plant allelochemicals, specially phenolic compounds (Luque at al., 2002). Also UGTs from the cotton pest *Helicoverpa armigera* were fuctionally expressed and shown to metabolize gossypol, a toxic compound from cotton, via glycosylation (Krempl et al., 2016). Furthermore glycosylated gossypol was found in the feces of *Helicoverpa armigera* and *Heliothis virescens,* another important agricultural pest, showing that glycosylation might be an important defense mechanism of these herbivores pest species (Krempl et al., 2016). Regarding the role of UGTs in insecticide detoxification, a recent study showed that the over-expression of a UGT gene was involved in chlorantraniliprole resistance in *Plutella xylostella*. The toxicity to chloranthraniliprole, an insecticide of the ryanoid class, was dramatically increased after knocking-down of this gene by RNA interference (Li et al., 2016).

1.1.4.3. Cellular localization of animal UGTs

In respect to the cellular localization of animal UGTs, they are transmembrane proteins of the endoplasmic reticulum (ER) as well as inner and outer nuclear membrane. Their catalytic side faces the ER lumen and their UDP-sugar donors are thought to be transported into the ER lumen through specific transmembrane proteins (transporters). After the conjugation of the hexose group to the aglycone substrate the complex is excreted out of the ER, again through transmembrane proteins (Rowland et al., 2013) (**Fig. 1.1.7.**).

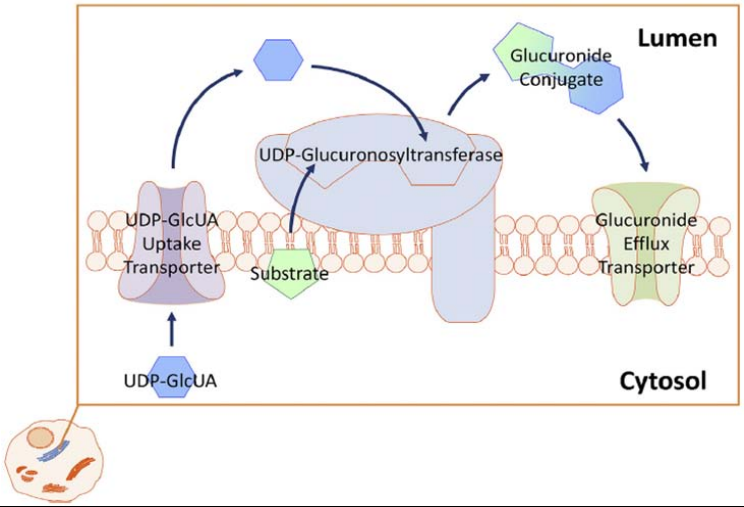


Figure 1.1.7. Animal UGTs cellular localization. UGTs are imported to ER facing the lumen. Their lipophilic substrates diffuse through ER membrane and after conjugation with sugars by UGTs they are exported through specific transporters. Adapted from Rowland et al., 2013.

1.1.4.4. Protein structure of animal UGTs

The protein structure of animal UGTs is divided into two main parts: the N-terminal aglycone substrate binding domain and the C-terminal UDP-glycoside binding domain, the latter is highly conserved and is defined by a signature sequence of 44 aminoacids responsible for binding the UDP-sugar. The N-terminal end of the animal UGTs has a signal peptide mediating the integration of the protein precursor into the ER compartment. The signal peptide is subsequently cleaved and then the protein is

N-glycosylated. The mature protein is retained in the ER membrane by its hydrophobic transmembrane domain at the C-terminal end, followed by a short cytoplasmic tail (Magdalou et al., 2010) (**Fig. 1.1.8.**) Comparison of protein primary sequences showed that insect UGTs share the same domains with mammalian, in contrast with the bacterial, plant and *T.urticae* UGTs that lack the signal peptide and are present in cytosol (Ahn et al., 2014).

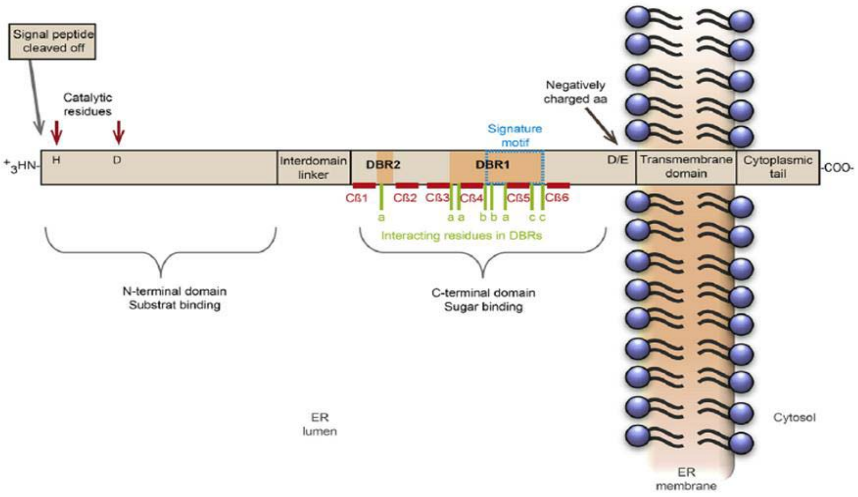


Figure 1.1.8. General structure of a membrane bound animal UGT. Image from Krempl et al. (2016)

1.1.4.5. Mosquito UGTs

In the genomes of the mosquitoes, Anopheles gambiae and Aedes albopictus, they have been identified 25 and 34 *ugt* genes respectively (**Figure 1.1.9.**). However the substrates for most of those remain unknown.

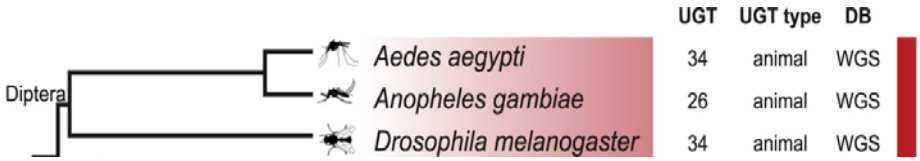


Figure 1.1.9. Numbers of UDP-glycosyltransferase genes in the genomes of *D. melanogaster* and the mosquitoes *An. gambiae* and *Ae. aegypti,* identified from genome-wide surveillance and manual annotations. Image from Ahn et al., 2012.

Transcriptomic data have associated UGT transcripts with insecticide resistance, although their functional characterization has not yet been reported. In particular, in a previous study from our lab an *Ae. albopictus* strain resistant to temephos was shown to overexpress three UGT transcripts compared to a susceptible strain (Grigoraki et al., 2015). In *An.gambiae* mosquito, the main malarian vector, UGTs have also been implicated in resistance where data from whole-transcriptome analysis from two studies have shown up-regulation of a specific UGT transcript (AGAP007990-RA) from two different pyrethroid resistant strains, one from Burkina Faso (Kwiatkowska et al., 2013) and the other from Western Kenya (Vontas et al., 2005). The abovementioned data are coming from whole body gene expression analysis of the resistant strains compared to the susceptible strains. Supporting the role in detoxification, the overexpression of the same *An. gambiae* UGT transcript (AGAP007990-RA) was observed in the malpigian tubules of both the resistant (Tiassalé) and susceptible (N'Gousso) strain tested compared to the whole organism about 8 times, in a study where transcriptome from dissected body parts was analyzed (Ingham et al., 2014). In this study we are referring to this UGT from *Anopheles gambiae* as Agam_UGT1. Agam_UGT1 has been designated UGT308G1 (Ingham et al., 2014) following the rules by the Nomenclature Committee for UGTs (Mackenzie at al., 1997).

1.1.5. Aim of the study

The **aim** of the study was to functionally express the candidate UGTs found to be up-regulated in resistant mosquitoes and detect their possible role in the resistant phenotype.

1.2. Materials and Methods

1.2.1. Mosquito strains

The mosquito strains that were used in this work are the following:

1. "Tem-GR" which is an *Ae. albopictus* strain resistant to temephos. This strain is originated from a parental population collected in Greece ("Par-GR") further selected in our lab (Grigoraki et al, 2015),

2. "Tiassalé" which is an *An. gambiae* strain. This is a multiresistant strain originated from Côte D'Ivoire showing high levels of resistance to pyrethroids permethrin and deltamethrin (Ingham et al., 2014).

3. "Par-Gr" and "Lab" are *Ae. albopictus* strains, susceptible to insecticides were used as references for qRT-PCR experiments.

1.2.2. Selection of candidate UGTs from microarray data and in silico sequence analysis

For *Ae. albopictus*, candidate UDP-glycosyltransferase (UGT) genes significantly up-regulated in "Tem-GR" larvae were selected from the microarray data. Contigs derived from the microarray data set were individually blasted against NCBI GenBank to identify putative *Aedes aegypti* orthologues. To design primers for the amplification and subsequent expression of candidate UGTs, the open reading frame of each UGT was predicted using the ORFfinder tool of NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). Signal peptides and cleavage sites were predicted using SignaIP4.1 (http://www.cbs.dtu.dk/services/SignalP/) and transmembrane domains with TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

For *Anopheles gambiae* UGT, complete coding sequence was derived from NCBI GenBank and Vector Base (**AGAP007990**) and primers were designed against both possible sequences. Similar in silico analysis was performed also for this sequence.

For protein structure prediction, a multiple alignment was performed using the ClustalIW multiple alignment function of the BioEdit software (**Figure 1.3.2.**)

1.2.3. Quantitative real time PCR

The levels of the 3 selected UGT transcripts identified from the microarray analysis were further assessed by quantitative PCR (qPCR) to validate their expression pattern. A fivefold dilution series of pooled cDNA was used to establish standard curves for each gene in order to assess qPCR efficiency and quantitative differences between samples. Amplification reactions of 25µl final volume were performed on a MiniOpticon Two-Color Real-Time PCR Detection System (BioRad) using 100ng of cDNA from each sample, 0.2µM primers and Kapa SYBR FAST qPCR Master Mix (Kapa-Biosystems) in a 3-step program involving a denaturation at 95 °C for 2 min followed by 35 cycles of 15 sec at 95 °C, 30 sec at 58 °C and 30sec at 60°C. A no-template control (NTC) was also included to detect possible contamination and a melting curve analysis was done in order to check the presence of a unique PCR product. Experiments were performed using four biological replicates and two technical replicates for each reaction. The relative expression and fold-change of each target gene in Tem-GR strain relative to Lab-GR was calculated according to Phaffl (Pfaffl, 2001) after normalization with the housekeeping gene histone 3 (H3).

1.2.4. Cloning of mosquito candidate UGTs

Full length coding sequences of *An.gambiae* and *Ae.albopictus* UGTs of interest were amplified by PCR from total larval cDNA from Tiassalé strain and Tem-GR respectively, using the Phusion Hot Start High-Fidelity DNA Polymerase (Thermo Scientific, USA) and gene-specific primers (**Table 1.2.1**). Forward primers include a 5' Kozak sequence (ACC), as in some cases this sequence acts as an enhancer, and reverse primers lack the stop codon for His-tag fusion expression. Melting temperatures were measured with Thermo's Multiple Primer Analyser software

following polymerase manufacturer's instructions. PCR products were separated via gel electrophoresis and excised from gel using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany). Purified Blunt-End PCR products were cloned into pFastBac/CT-TOPO vector (Invitrogen Life Technologies, USA), which contains (among others) a strong polyhedrin (P_H) promoter for high-level baculovirus-based protein expression in insect cells upstream the TOPO Cloning site, a C-terminal polyhistidine tag allowing the detection by western blot and a mini-Tn7 elements for site-specific transposition into the bacmid DNA (pFastBac/CT-TOPO vector map in **Figure 1.2.1. B**).

Table 1.2.1. Primers used for the cloning of mosquito UGTs

Gene	Primer	Sequence (5'-3')	Prod. size (bp)
AgUDP-GT7990	AgaUGT-VF	ACCATGGGTACGGCCACAGT	1614
	AgaUGT-R	CTCAACCTTCGATTTTGTGC	
Ae_alboUGT 84756seq1	ugt84756_1F	ACCATGAATTTCAAAGCTCTTGTCTC	1566
	ugt84756_2R	GCTTTGCTTCGCTTTTAC	
Ae_alboUGT 84756seq2	ugt84756_2F	ACCATGAATTTCAAATTATTTCTCTCA	1566
	ugt84756_2R	GCTTTGCTTCGCTTTTAC	

Ligation reactions were used to transform **One Shot Mach1-T1** competent cells and the resulting colonies that were selected on ampicillin plates were screened using cloning primers. Plasmids from positive clones were extracted using NucleoSpin Plasmid kit (Macherey-Nagel, Germany). To screen for insert directionality purified plasmids from 3 different positive clones were sent for sequencing using the Polyhedrin forward primer provided from the Bac-to-Bac kit which binds upstream the TOPO Cloning site (**Table 1.2.2.**).

Table 1.2.2. Primer pairs for the analysis of recombinant pFast Bac plasmids and bacmids.

application	Primer	Sequence
analyzing recombinant plasmids	Polyhedrin Forward primer	5’-AAATGATAACCATCTCGC-3’
	SV40 polyA Reverse primer	5’-GGTATGGCTGATTATGATC-3’
analyzing recombinant bacmids	pUC/M13 Forward primer	5’-CCCAGTCACGACGTTGTAAAACG-3
	pUC/M13 Reverse primer	5'AGCGGATAACAATTTCACACAGG-3’

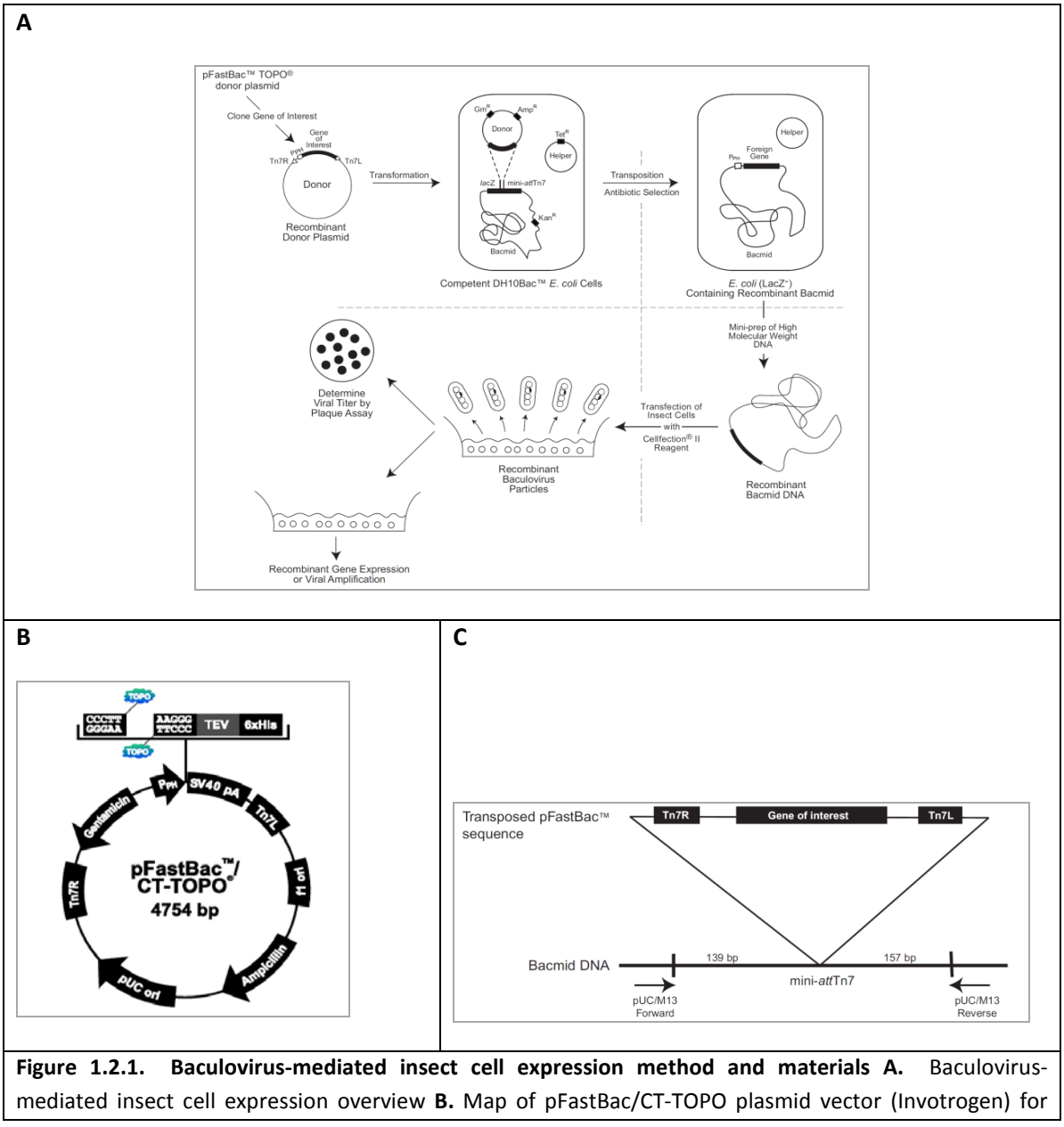


Figure 1.2.1. Baculovirus-mediated insect cell expression method and materials A. Baculovirus-mediated insect cell expression overview **B.** Map of pFastBac/CT-TOPO plasmid vector (Invotrogen) for

blunt end insertion and cloning. **C.** mini-Tn7 elements for site-specific transposition into the bacmid DNA.

1.2.5. Cell culture and transfection

Spodoptera frugiperda Sf21 were grown in IPL-41 insect cell culture medium, supplemented with 10% fetal bovine serum and 50ug/ml gentamycin (Gentamycin Sulfate, BioChemica). *Sf*9 and *Trichoplisia ni* BTI-TN-5B1-4 HighFive cells (Hi5) were grown in ESF 921 insect cell culture medium (Expression Systems, USA) in the absence of serum with 50ug/ml gentamycin. Cell cultures were maintained at 27°C and subcultured weekly. Transfection was performed with Cellfectin (Invitrogen) or Escort IV (Sigma) reagents according to standard protocols.

1.2.6. Expression of recombinant UGTs in insect cells

Generation of recombinant baculoviruses was carried out using Bac-to-Bac Baculovirus expression system (Invitrogen Life Technologies, USA), according to manufacturer's instructions. pFastBac/CT-TOPO vectors harbouring the UGTs of interest were transformed into DH10Bac *E.coli* cells and colonies with recombinant bacmids were selected on kanamycin/tetracycline/gentamycin plates by blue-white selection. Recombinant bacmids were isolated from overnight cultures from white colonies. Bacterial cells were harvested by centrifuge (14,000xg, 1min) and the resulted cell pellets were resuspended in 300ul of Qiagen plasmid purification solution I (15mM Tris-HCl, pH 8.0, 10mM EDTA, 100µg/ml RNAase A). Samples were incubated for 5min at RT after the addition of 300ul of Qiagen plasmid purification solution II (0,2N NaOH, 1% SDS). Next, samples were incubated on ice for 10 min after the addition of 300ul of 3M potassium acetate pH 5.5 and centrifuged for 10 min at 14000xg. Supernatant was transferred to a clean tube containing 800ul isopropanol and incubated on ice for 10min. Samples were centrifuged for 15min at 14000xg and the cell pellet washed with 500ul of 70% ethanol. After a centrifugation for 5min at 14000xg pellet was air-dryed for 10 min and dissolved in 30ul TE buffer, pH 8.0. Recombinant bacmids were verified with PCR and sequencing using

pUC/M13 primer pairs suggested by the Bac-to-Bac expression system kit (**Table 1.2.2.**).

DNA from positive colonies (or recombinant bacmids) was used to transfect *Sf*21 insect cells using cellfectin reagent and cells were inspected under microscope for visual signs of infection. Recombinant baculovirus was collected 5-7 days after transfection. Recombinant baculovirus that expresses Green Fluorescent Protein (GFP) was used as a negative control in all expreriments. To check for ugt expression, sf21 cells were infected with P1 baculovirus stock at a multiplicity of infection dictated from manufacturer's manual. Cells were checked for visual signs of infection in a reverse phase microscope. Three days after infection, cells were collected by centrifugation (2000rpm, 5min) and cell pellets as well as supernatants were analyzed by Western blot. Cell pellets were resuspended in phosphate buffered saline (PBS), frozen at -70°C for 30 min and centrifuged (13.300rpm, 15min) to separate the soluble protein fraction from the insoluble fraction. Sample preparation for Western included the addition of cracking buffer (0.125M Tris pH 6.8, 5% β-mercaptoethanol, 2% SDS, 4M Urea; Ralle et al. 1991) and boiling at 100°C for 5-15 min. Proteins were separated via SDS polyacrylamide electrophoresis at 120V. Then, proteins were electro-blotted onto a nitrocellulose membrane, which was blocked with 5% milk (in PBS-T) for 1h. Detection of recombinant protein was done using an anti-His antibody (Qiagen) at a dilution of 1:2000 (5% milk in PBS-T). Antibody binding was detected with 1:10.000 rabbit anti-mouse IgG HRP-linked secondary antibody.

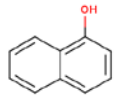
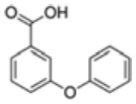
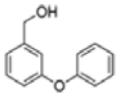
1.2.7. Crude microsomal extraction from infected insect cells expressing UGTs

Insect cells, both Sf9 and Hi5, were infected with the recombinant baculoviruses. After 72h cells were harvested and centrifuged at 2000rpm for 5min. The cell were washed twice with PBS (pH 7.4) and centrifuged at 2000rpm for 10 min. The cell pellet was resuspended in hypotonic buffer (20mM Tris, 5mM EDTA, 1mM DTT, and 20%($^{v}/_{v}$) glycerol at pH 7.5) and incubated for 20 min on ice. The cells were

homogenized with pestles and subsequently mixed with an equal volume of sucrose buffer (20mM Tris, 5mM EDTA, 1mM DTT, 500mM sucrose and 20%($^{v}/_{v}$) glycerol at pH 7.5). After centrifugation at 1,200 x g for 10min at 4°C, the supernatant was centrifuged at 100,000 x g for 1h at 4°C. The cell pellet containing microsomes, plasma membrane and cell organelles was resuspended in sodium phosphate buffer (0.1M; pH 6.4). Samples were aliquoted and snap frozen in liquid nitrogen and stored at -80°C. Successful expression of UGTs in ER membranes was validated by Western blot analysis using anti-His as primary antibody and anti-mouse IgG HRP-linked as secondary.

1.2.8. Enzyme assays with a general substrate and insecticide metabolites

The glycosylation activity of the heterologously expressed UGTs was tested with 1-naphthol (Sigma Aldrich) as general substrate. Twenty-five ug of total protein of the crude microsomal extract from each enzyme were used. For each of the UGT extracts a control consisting of a GFP-infected crude microsomal extract with the same total protein amount was prepared. UDP-glucose (1mM), 1-naphthol (5ppm in DMSO), MnCl2 (20mM) and sodium-phosphate buffer (0.1M; pH 6.4) were added to the proteins resulted in a total volume of 250ul and incubated in glass micro-inserts for 90min at 30°C. The reaction was stopped with 240ul of methanol followed by a centrifugation step at 3,000 x g for 3 min at room temperature. Supernatants of the samples from a-naphthol were analyzed via HPLC.

Table 1.2.3. Chemical compounds used in the study.		
Substrate name	**Structure**	**M.W.**
1-naphtol		144.173 g/mol
PBA (**3-Phenoxybenzoic acid)**		214.22 g/mol
PBAIc (**3-Phenoxybenzyl alcohol)**		200.23 g/mol

1.3. Results and Discussion

1.3.1. Validation of the microarray up-regulation of *Ae. albopictus* UGT genes with qPCR

Transcriptomic analysis between a temephos resistant *Ae. albopictus* strain and a reference susceptible strain showed the overexpression of 5 UGT trascripts, the three of which showed striking up-regulation. This three were selected to be further tested with qPCR analysis with cDNA synthesized from 3rd instar larval RNA. (**Figure 1.3.1.**). Out of the three ugt84756 was found to be approx. 5 folds overexpressed in the temephos resistant strain. With further sequence analysis, we found that there were two different transcripts of this UGT which will be further mentioned as Aealbo_UGT1 and Aealbo_UGT2.

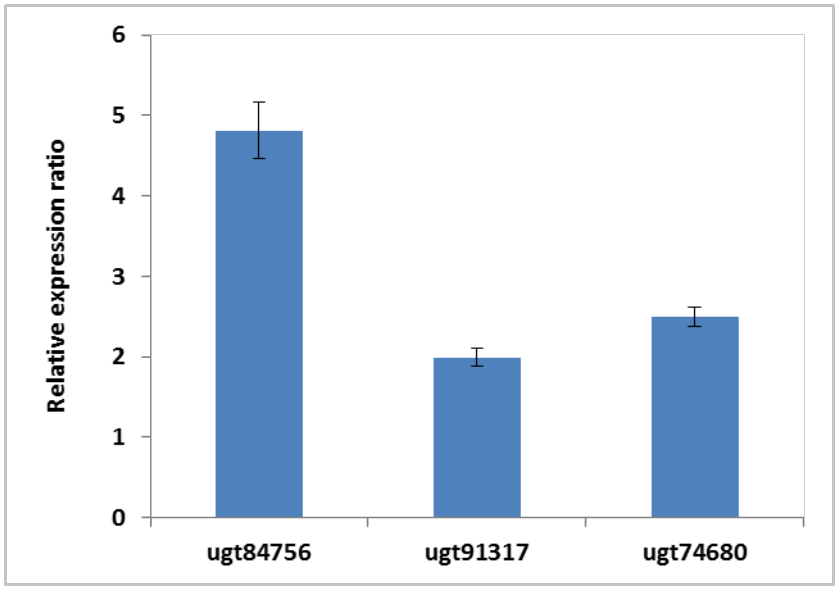


Figure 1.3.1. qRT-PCR expression profile of the three candidate genes in *Ae. albopictus* 3rd instar larvae of the resistant strain Tem-GR and the susceptible strain Lab.

1.3.2. In silico sequence analysis of candidate UGTs from *Aedes albopictus*

Extracted UGT sequences (contigs) from *Ae. albopictus* from microarry analysis (Grigoraki et al., 2015) were blasted and aligned to *Ae. aegypti* UGTs to detect putative orthologues. A multiple alignment of the UGT amino acid sequences and in

silico analyses provided further structure information (**Figure 1.3.2.**). All three ugts, two from *Ae. albopictus* and one from *An. gambiae* validated with qPCR were shown to have typical UGT structure. An aminoterminal signal peptide for the import of UGTs to endoplasmic reticulum, the UGT signature motif, responsible for the binding of UDP-sugar, a transmembrane domain and a small cytoplasmic tail.

Figure 1.3.2. In silico sequence analysis of candidate mosquito UGTs

1.3.3. Molecular cloning of mosquito UGTs, isolation of recombinant bacmids and production of recombinant baculoviruses

Three mosquito UGTs (**Table 1.3.1**), previously identified as up-regulated in insecticide resistant strains, two from *Aedes albopictus*, up-regulated in a temephos resistant strain and one from *Anopheles gambiae*, up-regulated in a pyrethroid resistant strain were pcr-cloned from cDNA from 3rd instar larvae and adult mosquitoes respectively (**Figure 1.3.3. A**). Isolated fragments were purified and subsequently cloned in a pFastBac/CT-TOPO vector, with a C-terminal polyhistidine tag. Recombinant plasmids were used to transform **One Shot Mach1-T1** competent cells which included a viral DNA (bacmid) and a helper plasmid to assist transposition.

Table 1.3.1. Mosquito UGTs used in this study and relative information.

Species	Draft Name	Unigene ID (Contig)	Transcript	Microarray FC	qRT-PCR FC	Source
Aedes albopictus	Aealbo_ UGT1	comp84756_c0 _seq1	AAEL003079	16.7	4.7	Grigoraki et al., 2015
	Aealbo_ UGT2	comp84756_c0 _seq2	AAEL003079	16.7	4.7	Grigoraki et al., 2015
Anopheles gambiae	AgUGT1	-	AGAP007990	7.7	7.7	Kwiatkowska et al.,

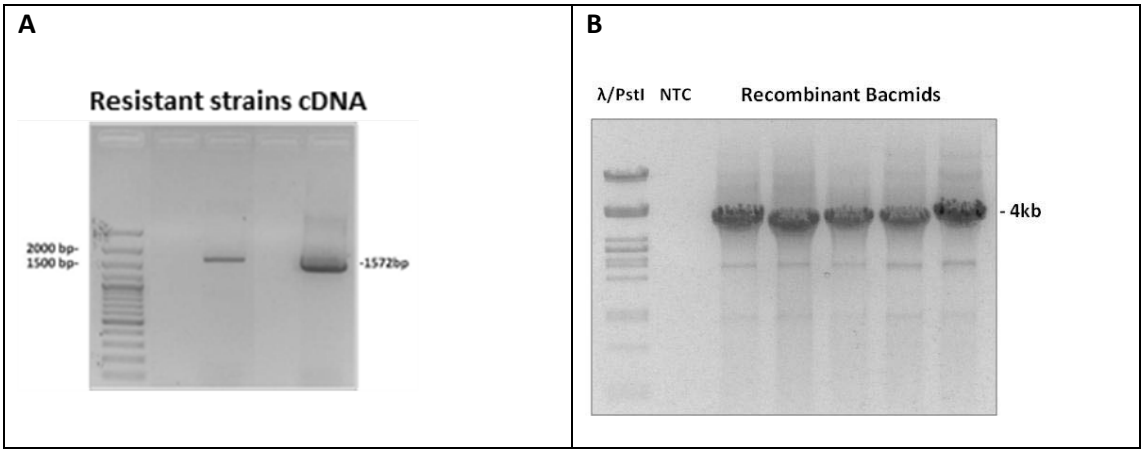


Figure 1.3.3. Molecular cloning of mosquito UGTs (A), and isolation of recombinant bacmids (B).

Recombinant bacmids (**Figure 1.3.3. B**) were used to transfect *Sf*21 insect cells to produce recombinant baculoviruses. Cells were inspected under microscope for visual signs of infection. Successful transfection was verified under reverse phase microscope where cells were showing signs of viral infection 4 days post transfection. Infected cells have increased cell diameter and they have granular appearance comparing to uninfected ones. Cell division seems to be arrested and cells are not dense in the monolayer comparing to the untreated control due to cell lysis (**Figure 1.3.4. A, B, C**). Successful transfection with GFP recombinant bacmids was verified using fluorescent microscopy (**Figure 1.3.4. D**).

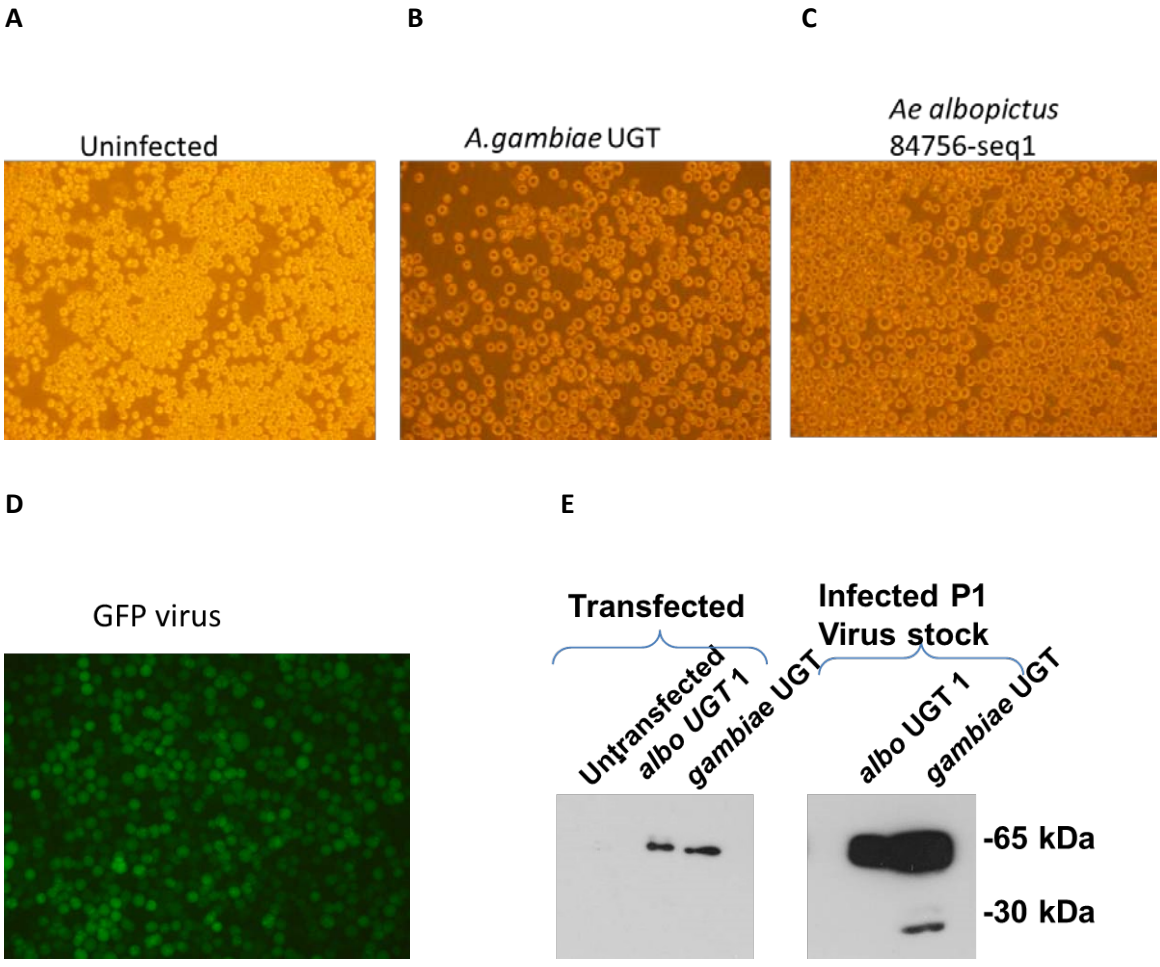


Figure 1.3.4. Insect cells transfected with recombinant bacmids expressing UGTs and GFP as control. A) Uninfected cells, **B)** Cells expressing *An. gambiae* UGT, **C)** Cells expressing *Ae. albopictus* UGT, **D)** Cells transfected with GFP recombinant bacmid and **E)** Western blot results after transfection with recombinant bacmids and infection with recombinant virus showing UGT expression.

Recombinant baculoviruses were collected 7 days post transfection and used to infect *Sf*21 insect cells to amplify the virus stock. Cell pellets post transfection and post infection were analyzed with western blot to verify the successful expression (**Figure 1.3.4. E**).

1.3.4. Heterologous expression of mosquito UGTs in insect cells using recombinant baculoviruses

The expression of all three UGTs were examined in Hi5 and *Sf*9 cells in three different time points, 24h, 48h and 72h post infection (**Figure 1.3.5.** *An. gambiae* UGT indicative results). Immunoblotting using an antibody against the His epitope revealed the presence of the UGT of interest at approximately 65 kDa. In silico analysis showed that UGTs have approximately 520 aminoacids and their predicted molecular weight was around 59-60kDa. The western blot results that showed a high intense signal at 65 kDa corresponds to the His-tagged versions of the proteins.

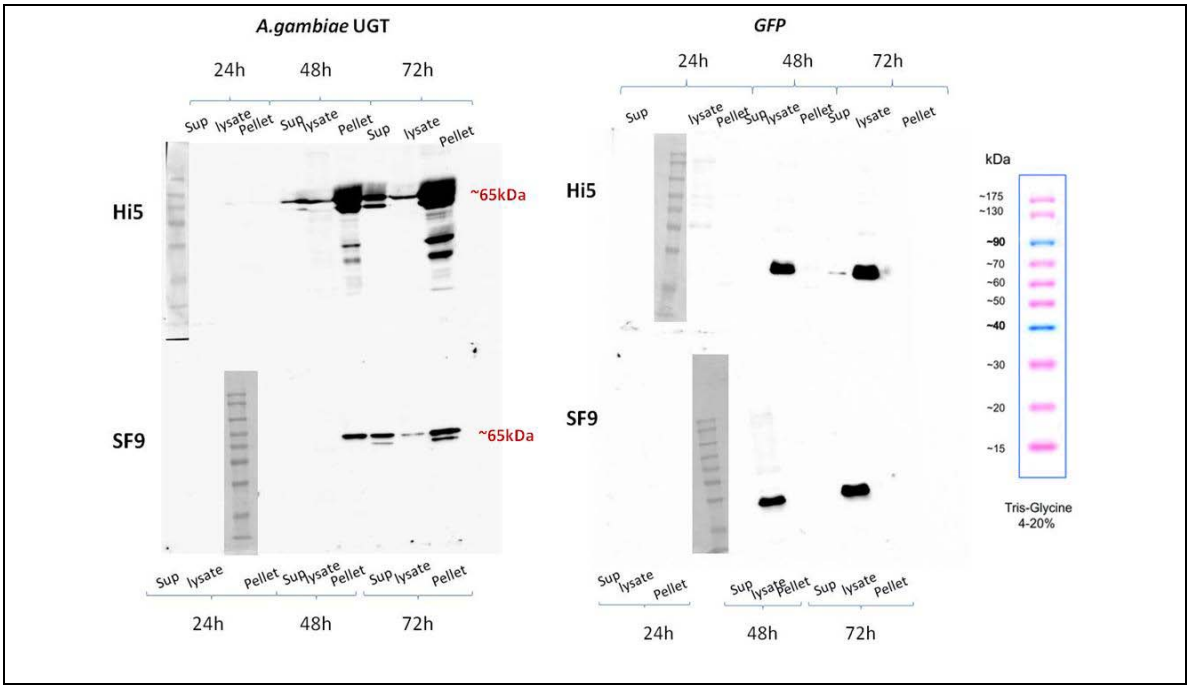


Figure 1.3.5. Infection with recombinant baculoviruses expressing *An. gambiae* UGT and GFP. Time-course experiment comparing two different insect cell types, Hi5 and *Sf*9, in three different time points, 24h, 48h and 72h post infection.

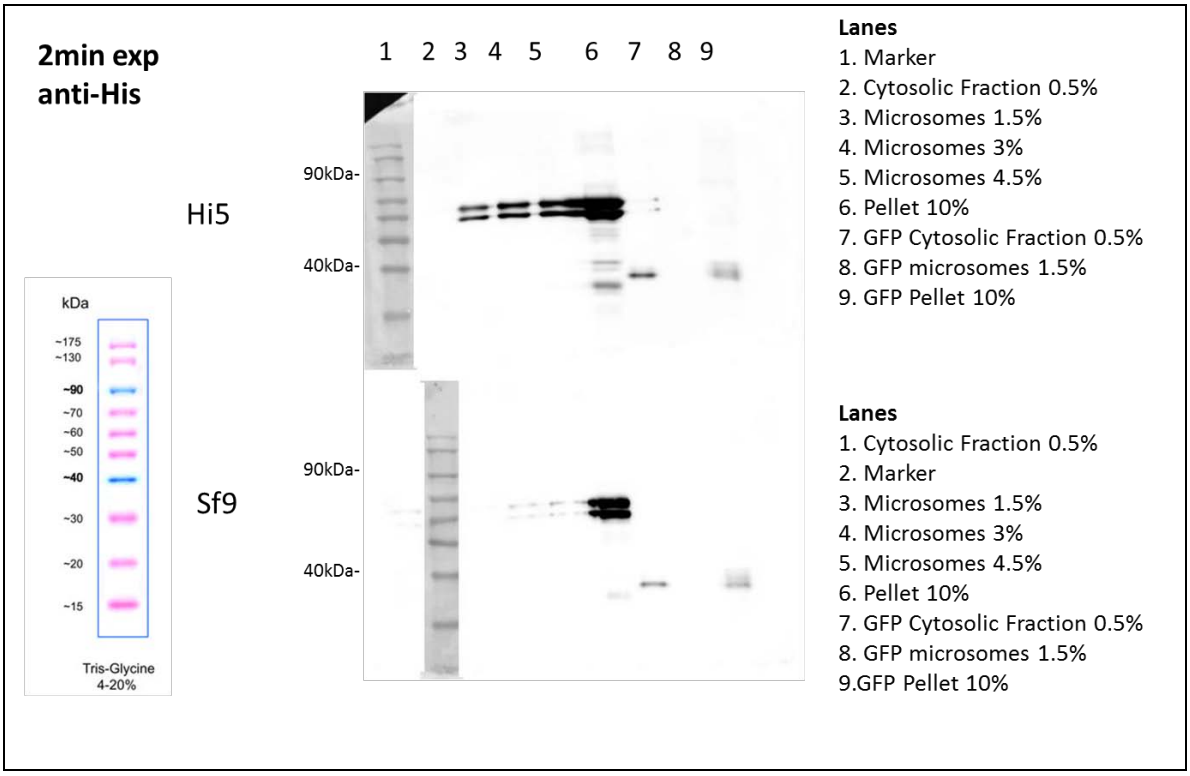


Figure 1.3.6. *A. gambiae* UGT microsomal extracts from two different types of insect cells, Hi5 and *Sf9* – 72hrs post infection analyzed via western blot.

After successfully expression of UGTs in cell lysate we continued with infections in both types of insect cells and isolation of crude microsomal extracts with ultracentrifuge and high sucrose concentration. UGTs are, as mentioned membrane-bound proteins anchored in ER membrane and after successful expression they were supposed to be present in microsomal extracts. After western blot analysis all three mosquito UGTs were successfully expressed in the ER membranes of insect cells. Hi5 cells showed higher protein yield and we continued the expression experiments with that type of insect cells (**Figure 1.3.6.**). As shown in **Figure 1.3.7.** where we compared the cytosolic fraction and the microsomal extracts of the UGTs of interest we detected expression only in the microsomes. The presence of two bands instead of one is most probably the N-glycosylated and non glycosylated forms of the proteins since UGTs are N-glycosylated after import to ER (Luque et al., 2002). Also GFP was detected in the cytosolic fraction as supposed. Subsequently, we proceeded with enzymatic assays and HPLC analysis to examine the glycosyltransferase activity of the mosquito UGTs.

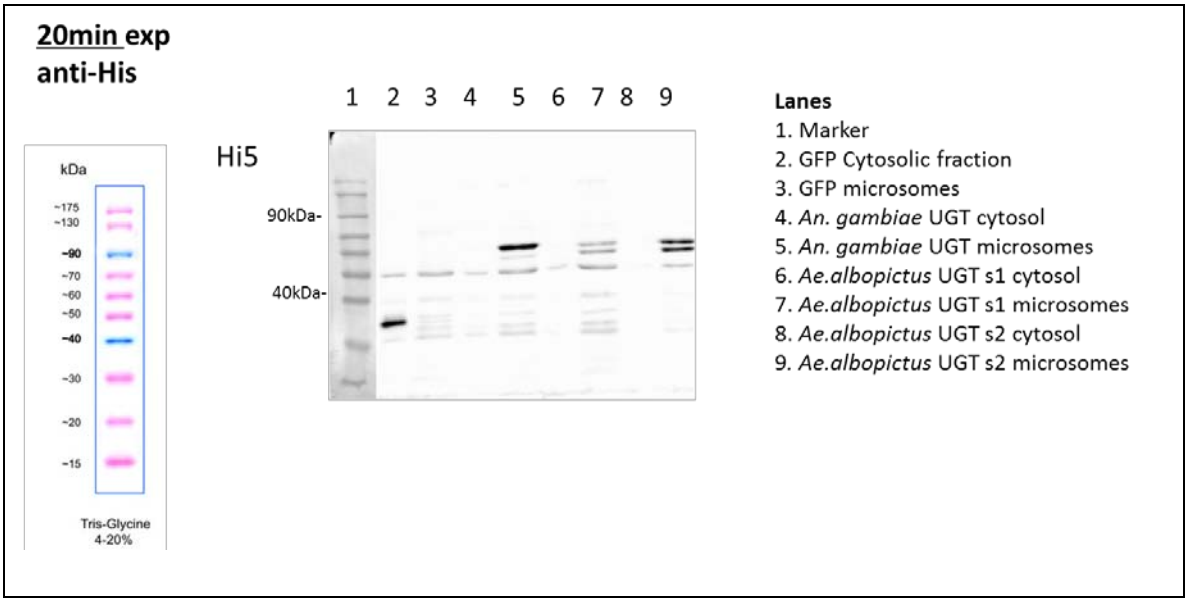
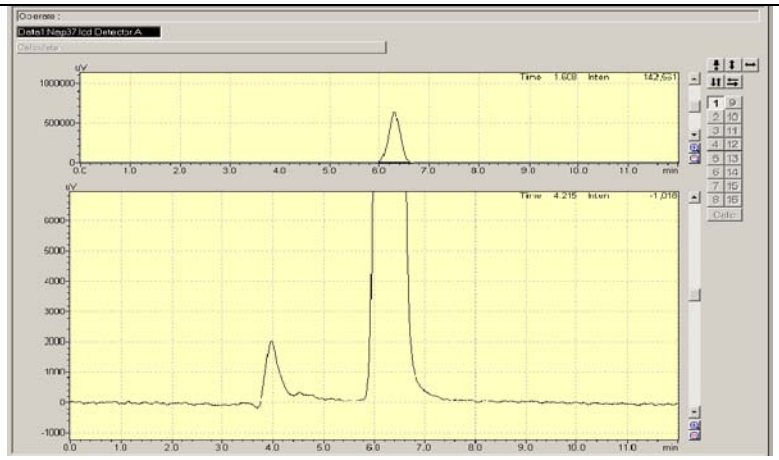


Figure 1.3.7. Microsomal extracts from insect cells infected with recombinant baculoviruses expressing mosquito UGTs and GFP.

1.3.5. Production of catalytically active UGTs

Enzymatic activity of all recombinant UGTs were tested using UDP-glucose as sugar donor and a-naphthol, a general UGT substrate, as an acceptor. Due to endogenous UGT production by the insect cells used, we also used microsomal extracts isolated from insect cells infected with GFP-expressing virus as internal control. The reaction products were monitored by HPLC as new peaks corresponding to the glycosylated products (**Figure 1.3.8.**). The retention time of a-napthol is between 6-7 min and the retention time of the glycosylated products is supposed to be between 4-4.5 min. Samples will be analyzed further with MS to identify the peaks that resulted after the enzymatic reactions.

A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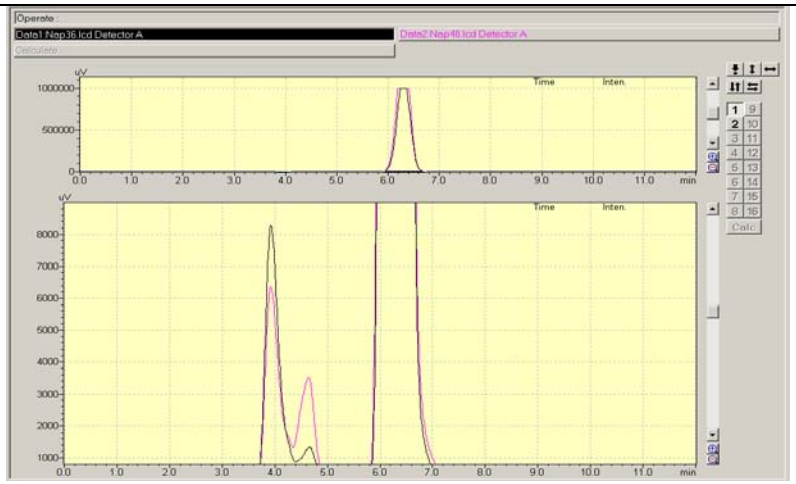
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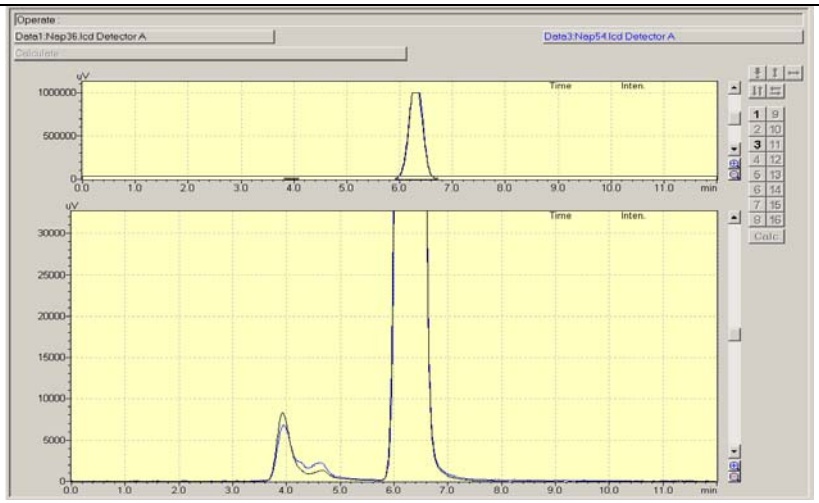
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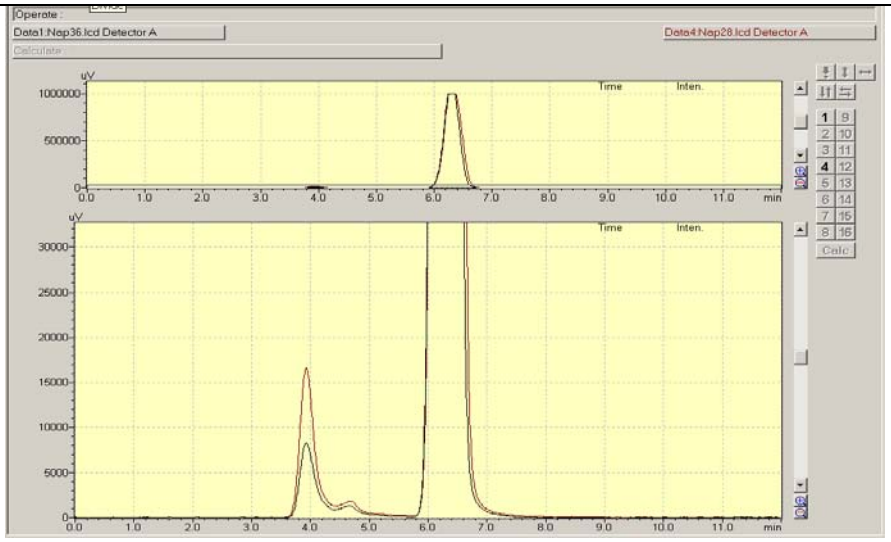

Figure 1.3.8. HPLC analysis of enzymatic reactions with recombinant proteins A, Enzymatic reaction with heat-inactivated protein, **B,C,D** Enzymatic reactions with crude microsomal extracts from insect cells expressing UGTs versus enzymatic reaction with crude microsomal extract from insect cells expressing GFP. **B.** *An. gambiae* UGT (pink) versus GFP (black), **C.** *Ae. albopictus* UGT1 (blue) versus GFP (black),**D.** *Ae. albopictus* UGT2 (orange) versus GFP (black).

1.4. Conclusions and Future Plans

In this study we describe the identification, production and attempt for functional characterization of three mosquito UGTs, two from *Ae. albopictus* and one from *An. gambiae* previously associated with insecticide resistance to the organophosphate temephos and to pyrethroids respectively. The hypothesis that the identified genes encode UGTs was prompted by their homology with known insect UGTs and the presence of the UGT signature motif and the domains that indicate proper UGT structure. The open reading frames (ORFs) of the three mosquito UGTs were identified with bioinformatic tools and were pcr-amplified from mosquito cDNA and cloned to expression vectors for heterologous expression in the eykaryotic system of insect cells. The baculovirus-mediated insect cell expression system was used because of the post-translational modifications of the proteins (signal peptide cleavage, N-glycosylation) and the fact that the proteins of interest are normally produced in the ER membranes of insect cells. After the successful production of all three recombinant baculoviruses expressing UGTs and a control recombinant baculovirus expressing GFP, all three mosquito UGTs were successfully produced and detected in the ER membranes after isolation and analysis of insect cells microsomal extracts, with Hi5 insect cells producing higher protein yield than *Sf*9. Glycosyltransferase activity of the heterologously produced proteins were examined after enzymatic assays using isolated crude microsomal extracts, UDP-glucose as sugar donor and a-naphthol, a general UGT substrate as sugar acceptor. The reactions' products were analyzed with HPLC and potential glycosylated products were identified as new peaks. Further analysis of the reaction products with MS will give the conclusive answer for the identity of the new peaks. Subsequently, catalytically active UGTs will be examined for their potential to glycosylate insecticide metabolites. UGTs originated from *Ae. albopictus* will be tested against temephos metabolite resulting after temephos-oxon incubation with estarases since this metabolite can be theoretically glycosylated. In the same context, the UGT originated from *An. gambiae* will be examined for its potential to glycosylate the known pyrethroid metabolites PBA (3-Phenoxybenzoic acid) and PBAlc (3-Phenoxybenzoic acid) since these metabolites have side groups that can be

theoretically glycosylated, in contrast with the synthetic pyrethroids. To further investigate the role of the mosquito UGTs used in this study to the detoxification of insecticides, genes will be silenced via RNA interference and we will test in vivo the effect of the gene knock-downs to the mosquito susceptibilities towards insecticides. Last but not least, specific antibodies for each UGT will be produced for tissue localization studies.

PART II

Overexpression of the potential chitin deacetylase HaCDA5a from the agricultural pest *Helicoverpa armigera* to further investigate it as potential target of novel insect growth regulators

2.1. Introduction

2.1.1. Chitin as target for pesticides

Chitin is an abundantly produced extracellular biopolymer deposited as orderly oriented microfibrils notably in exoskeletons of arthropods and in cell walls of most fungi species. It is synthesized from units of N-acetyl-D-glucosamine linked with β-1,4 glucosidic bonds. Its main function is to contribute to the strength and rigidity of structural elements of the organism. Being part of structures such as cuticles, peritrophic membranes (PM) or cell walls, any interference with chitin deposition or its untimely degradation is detrimental to the organisms involved. Enzymes involved in chitin synthesis and degradation (chitin synthases, chitinases) are well established targets for pest control. Chitosan on the contrary is a linear polysaccharide composed of D-glucosamine, it is the deacetylated form of chitin and it is soluble in water.

2.1.2. Development of novel control agents

The number of selective insect pests targets is limited and their exploration is expensive and time consuming, thus the development of novel chemical active ingredients is limited. Although biological control approaches have been proposed as substitutes for chemicals their use is limited since most microbes show a narrow spectrum of activity that enables them to kill only certain insect species. Moreover they have low environmental persistence and they require precise application practices, since many of these pathogens are specific to young insect larval stages or are sensitive to irradiation. The development of biotechnology and informatics, however, provides numerous modern tools for the employment of new approaches for designing and developing novel means of insect control. These advances have been achieved by combining new knowledge derived from basic molecular biology and comparative genomic studies with technical developments that enable comprehensive screens of potential insecticide targets.

2.1.3. Chitin deacetylases

Chitin deacetylases (CDAs) are specific chitin modifying enzymes that convert chitin to chitosan (**Figure 2.1.1.**) and they have been detected in several insects, including *Anopheles gambiae*, *Apis mellifera*, *Bombyx mori, Drosophila melanogaster*, ***Helicoverpa armigera***, *Mamestra configurata*, *Tribolium castaneum* & *Trichoplusia ni* (Zhao et al., 2010).

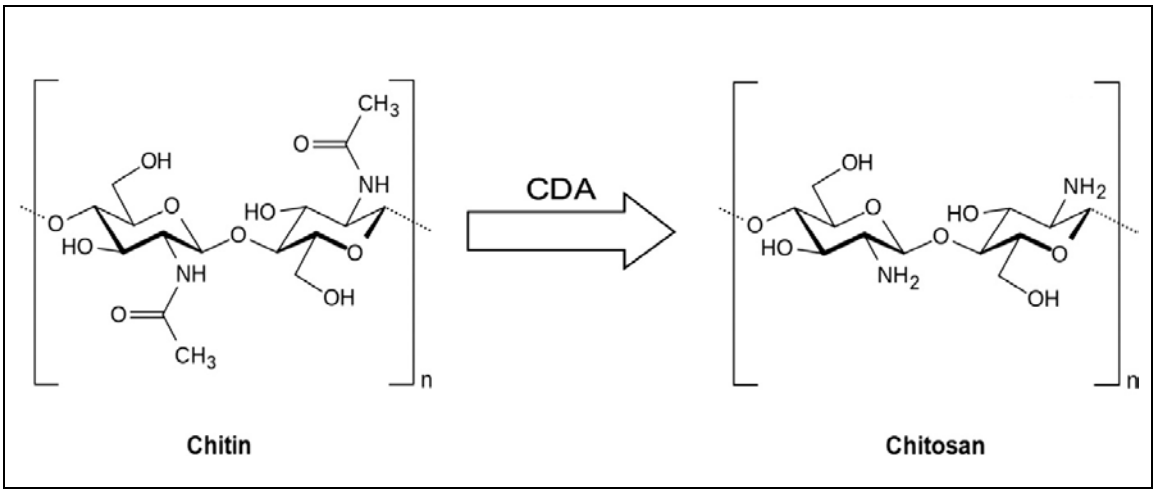

Figure 2.1.1. Reaction catalyzed by chitin deacetylases.

Insect CDAs have been classified into 5 discrete classes (I-V) (**Figure 2.1.2**) based on phylogenetic relation and structural domain organization. Expression of type V CDAs has been reported in the gut of several lepidopterans, including *H. armigera* (Jakubowska et al., 2010; Campbell et al. 2008), *Mamestra configurata* (Toprak et al. 2008) and *Bombyx mori* (Zhong et al., 2014), and is likely associated with the peritrophic matrix. It is implicated to PM stability and permeability to potential pathogens like baculovirus (Jakubowska et al., 2010). HaCDA5a is a CDA like protein identified from transcriptome analysis in *Helicoverpa armigera*. It has been selected to be heterologously expressed and functionally characterized due to the vital role that seems to play in PM.

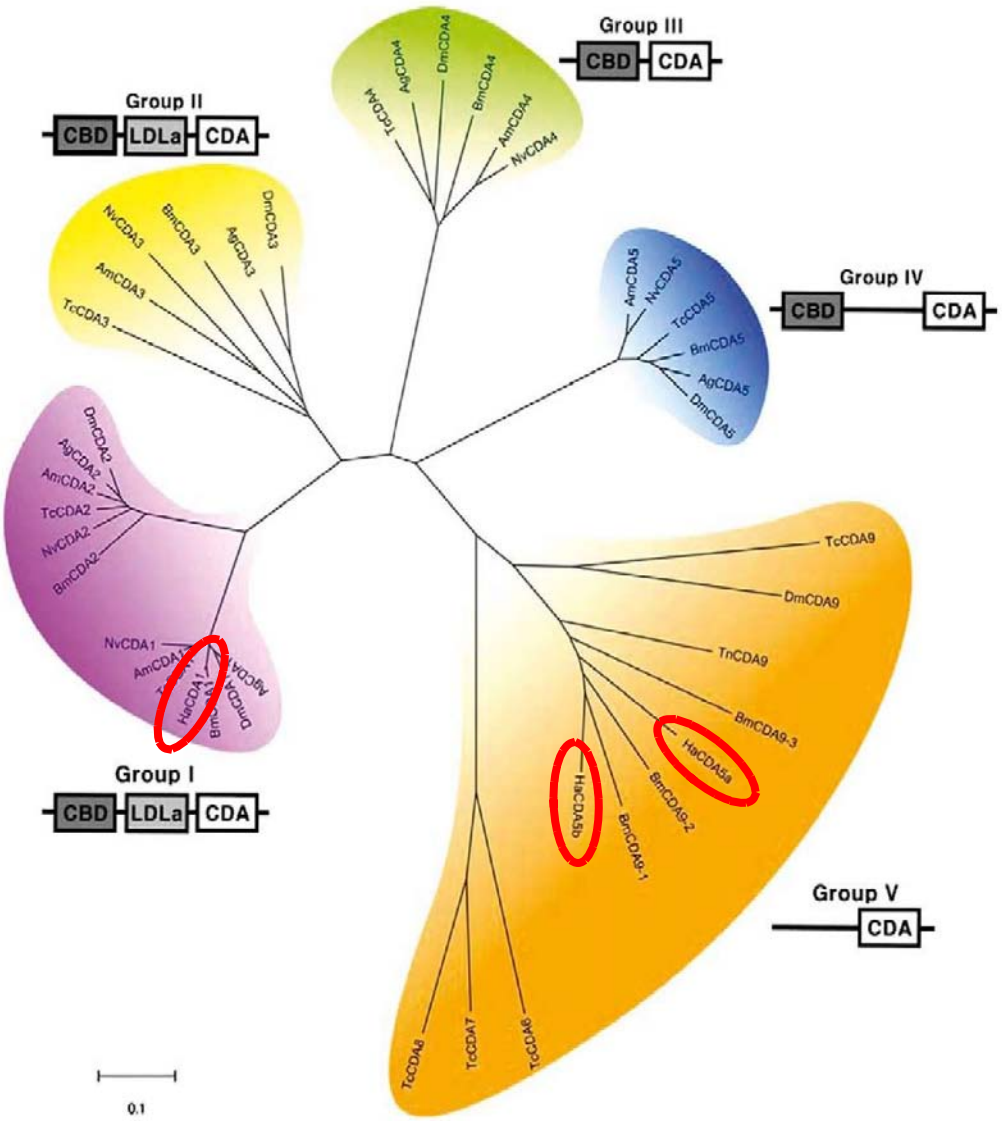

Figure 2.1.2. Insect CDAs are classified into 5 discrete classes (I-V) based on phylogenetic relation and structural domain organization.

2.1.4. Aim of the study

Our **aim** was the overexpression and functional characterization of a potential chitin deacetylase from the insect pest *Helicoverpa armigera,* which was shown to play a vital role in insect's peritrophic matrix to further investigate it as potential target for the development of new pesticides.

2.2. Materials and Methods

2.2.1. Expression of recombinant HaCDA5a in insect cells

The recombinant baculovirus expressing HaDCA5a was kindly provided by Prof. Bouriotis/Enzyme Biotechnology Group, UoC. Recombinant baculovirus that expresses Green Fluorescent Protein (GFP) was used as a negative control in all expreriments. To check for HaCDA5a expression, sf21 cells were infected with P1 baculovirus stock at a multiplicity of infection dictated from manufacturer's manual. Cells were checked for visual signs of infection in a reverse phase microscope. Three days after infection, cells were collected by centrifugation (2000rpm, 5min) and cell pellets as well as supernatants were analyzed by Western blot. Cell pellets were resuspended in phosphate buffered saline (PBS), frozen at -70°C for 30 min and centrifuged (13.300rpm, 15min) to separate the soluble protein fraction from the insoluble fraction. Sample preparation for Western included the addition of cracking buffer (0.125M Tris pH 6.8, 5% β-mercaptoethanol, 2% SDS, 4M Urea; Ralle et al. 1991) and boiling at 100°C for 5-15 min. Proteins were separated via SDS polyacrylamide electrophoresis at 120V. Then, proteins were electro-blotted onto a nitrocellulose membrane, which was blocked with 5% milk (in PBS-T) for 1h. Detection of recombinant protein was done using an anti-His antibody (Qiagen) at a dilution of 1:2000 (5% milk in PBS-T). Antibody binding was detected with 1:10.000 rabbit anti-mouse IgG HRP-linked secondary antibody. The P2 baculovirus stock that was collected was used to infect insect cells, both Sf9 and Hi5 and the HaCDA5a expression was tested in three different time points 24h, 48h and 72h post infection. After that baculovirus stock was amplified, after infection of sf21 cells insect cells. P3 baculovirus stock produced after amplification was used to infect Hi5 insect cells. Cells were analyzed as mentioned above.

2.3. Results and Discussion

2.3.1. Transfection with recombinant bacmid containing HaCDA5a and production of recombinant baculovirus

The recombinant bacmid containing the gene coding for HaCDA5a, a chitin deacetylase from the agricultural pest *H. armigera*, along with an N-terminal His-tag was used to transect *Sf*21 insect cells to produce recombinant baculoviruses. The predicted molecular weight of HaCDA5a was approximately 44kDa. The expression of HaCDA5a tagged N-terminally with a His epitope has an extra molecular weight of 3-4 kDa and the protein migrates in an SDS-PAGE gel at approximately 48kDa (**Figure 2.3.1.**). P1 baculovirus stock was collected for insect cell infection.

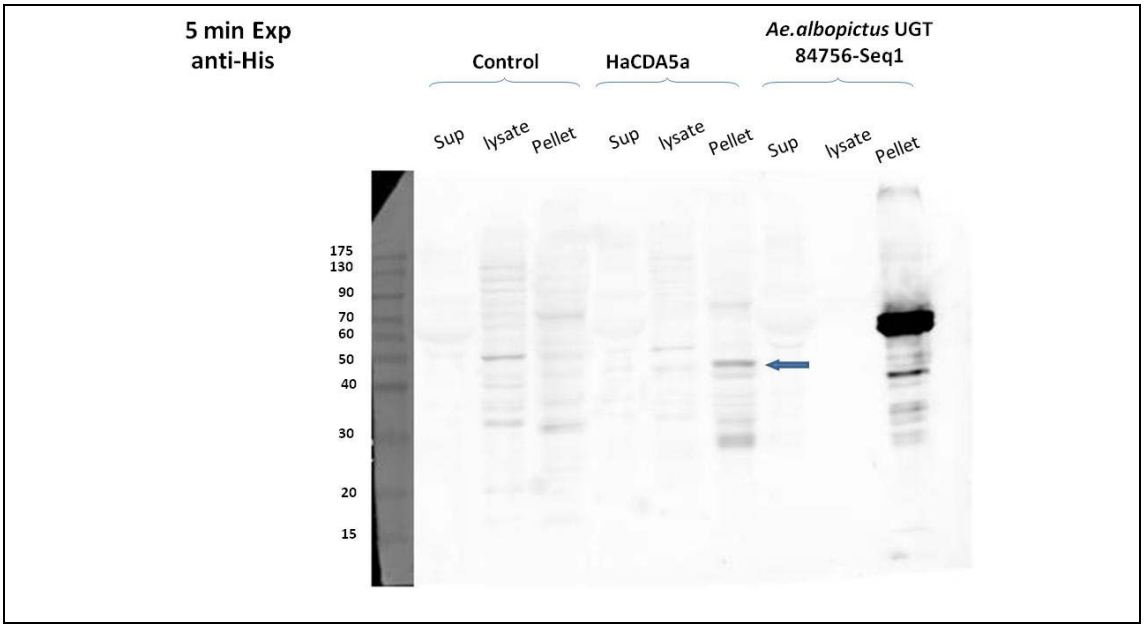

Figure 2.3.1. Western blot analysis of insect cells transfected with recombinant baculoviruses expressing HaCDA5a and Aealbo_UGT1**.**

2.3.2. Infection of insect cells with recombinant baculovirus expressing HaCDA5a and virus amplification

The expression of HaCDA5a was initially examined in Hi5 and *Sf*9 cells infected with the recombinant baculovirus (P1) in three different time points, 24h, 48 and 72h post infection. As shown in **Figure 2.3.2.**, immunoblotting of whole cell extracts (lysate) using an antibody against the His epitope, revealed the presence of the specific product at approximately 48kDa and the presence of several non specific products in Hi5 cells. The signal *Sf*9 cells were not so strong. GFP recombinant baculovirus was used as control which gave strong, intact signal in the cell extract sample (lysate) both in Hi5 and *Sf*9 cells insect cells.

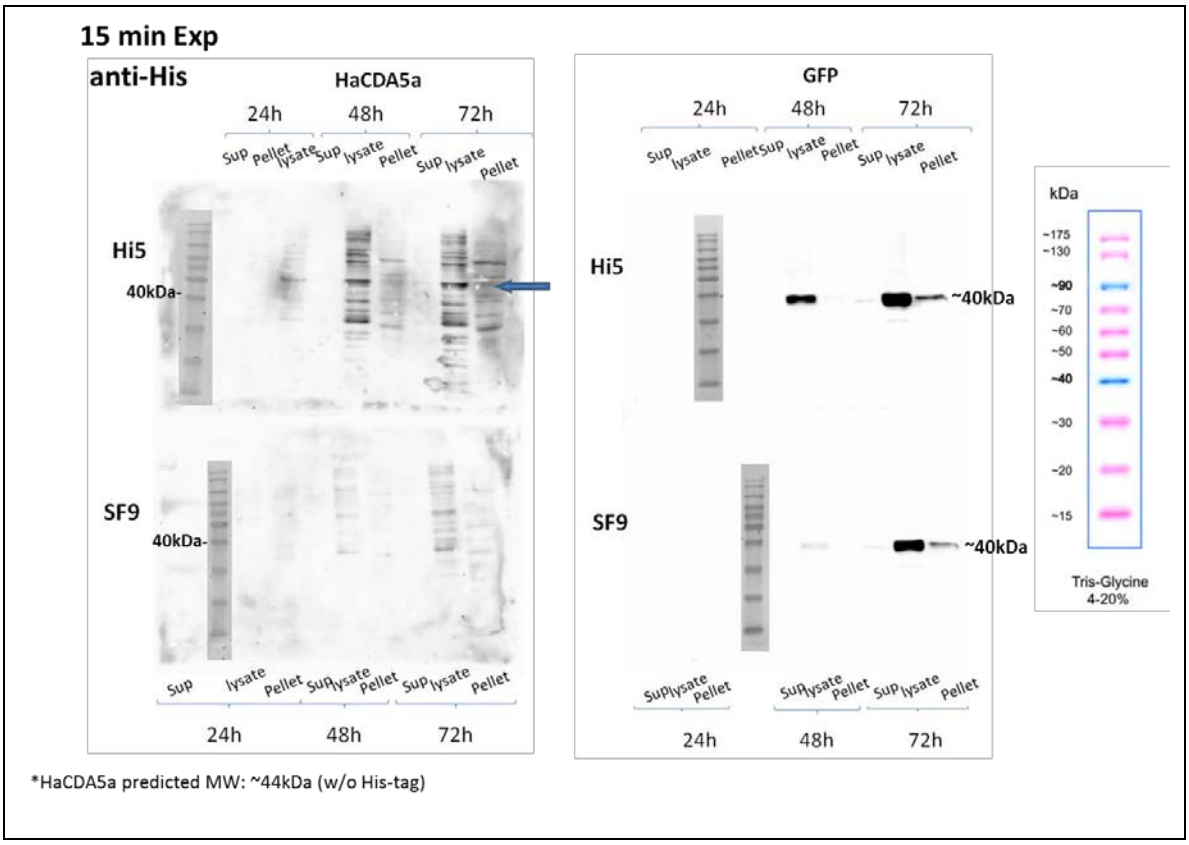

Figure 2.3.2. Infection with recombinant baculoviruses expressing HaCDA5a and GFP. Time-course experiment comparing two different insect cell types, Hi5 and Sf9, in three different time points, 24h, 48h and 72h post infection.

After infection with P1 baculovirus stock we used the recombinant virus to infect *Sf*21 cells so as to amplify the virus stock. As shown in **Figure 2.3.3** cells showed clear

signs of viral infection after observation in reverse phase microscope. Infected cells have increased cell diameter and they have granular appearance comparing to uninfected ones. Cell division seems to be arrested and cells are not dense in the monolayer comparing to the untreated control due to cell lysis. Successful infection with GFP recombinant baculovirus was verified using fluorescent microscopy. After isolation of the amplified virus it was used to infect Hi5 insect cells but no signal was detected as opposed to the amplified signal in GFP production in the control resulting from the virus amplification (data not showed). This result led us to the conclusion that probably HaCDA5a was not the only gene that has been transposed during transposition and bacmid production therefore we were not able to detect a clear enhanced signal after virus amplification and further optimization of the expression vector is most probably needed

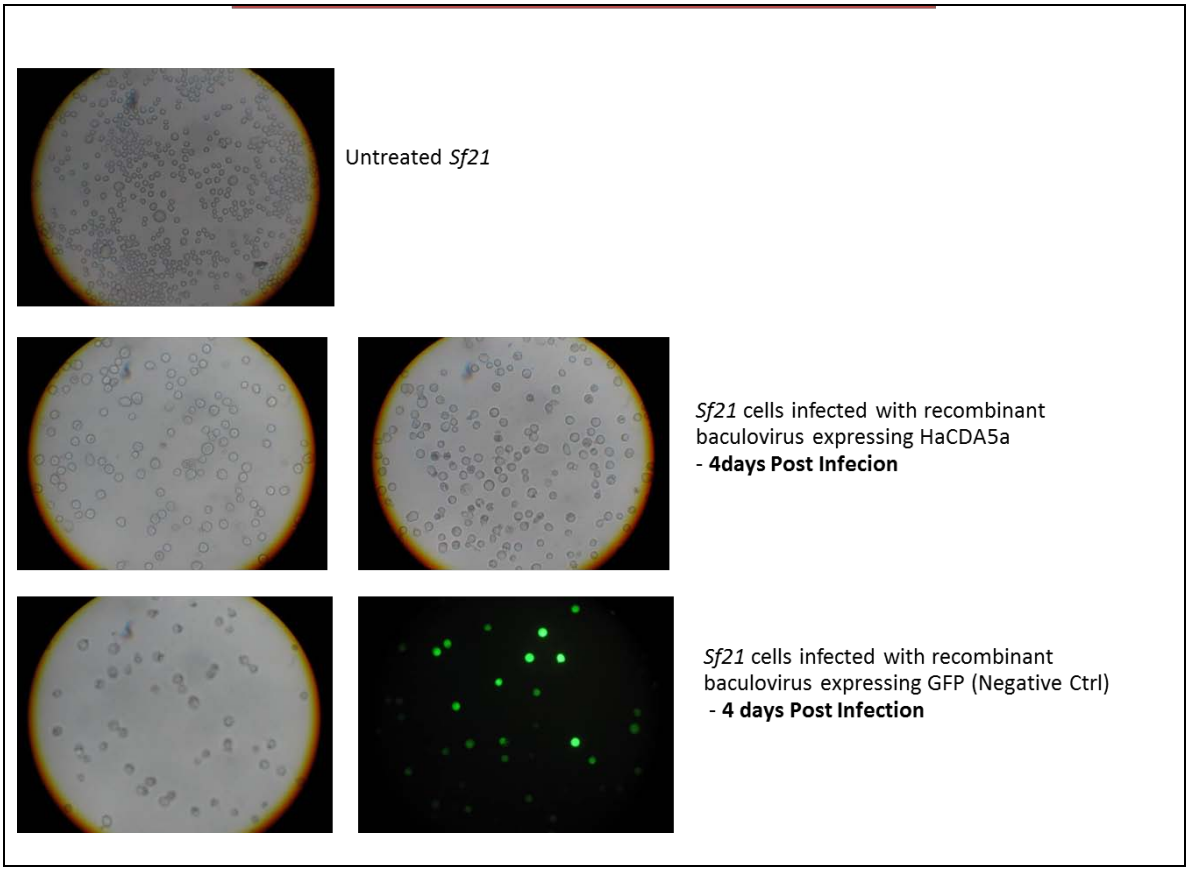

Figure 2.3.3. Virus amplification. Insect cells inspected under reverse phase microscope for visual signs of infection. Infected cells have increased cell diameter and they have granular appearance comparing to uninfected ones. Cell division seems to be arrested and cells are not dense in the monolayer comparing to the untreated control due to cell lysis.

2.4. Conclusions and Future Directions

Chitin deacetylases are chitin modifying enzymes that convert chitin to chitosan and they have been detected in many insects. This conversion influences the mechanical and permeability properties of structures such as the cuticle and peritrophic matrices. These enzymes have not been previously examined as targets of novel pesticides, comparing with the other enzymes involved in chitin synthesis and/or degradation (chitin synthases, chitinases) which are well established targets for pest control. In this study wee attempted to overexpress a chitin deacetylase from the cotton bollworm *H. armigera*, HaCDA5a, that has been shown to play a vital role in peritrophic matrix in order to functionally characterize it and evaluate its biological role in insect's life cycle. As a system for its heterologous expression we used baculovirus mediated insect cell expression, since previous attempts in *E.coli* and *P.pastoris* were unsuccessful. Also the insect cells used are lepidopteran cells, cells that are normally infected by baculoviruses and we hypothesized that this system will provide the best conditions for the production and proper folding of the protein. However, we were not able to produce high amounts of the protein to homogeneity. After western blot analysis, HaCDA5a was correctly detected in cytosolic fragment but the protein yield was low and a great non specific signal was also detected. These results along with the results after the observation of the cells during infection under the microscope, which were shown clear signs of infection led us to the conclusion that probably HaCDA5a was not the only gene that has been transposed during transposition and bacmid production therefore we were not able to detect only the HaCDA5a specific band in western blots. Further optimization of the expression vector is needed to be able to produce the protein of interest in high yield and homogeneity in insect cells so as to functionally express and test it for its deacetylase activity. After functional expression, crystallization and structural determination of the target enzyme, and the co-crystallization of the enzyme-possible inhibitors complexes will enhance the ability of understanding the interactions between the inhibitor and the protein target (HaCDA5a) leading to optimized inhibitors.

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