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DEPARTMENT OF BIOLOGY

DOCTORAL THESIS

Adaptation of arthropod pests in plant allelochemicals and pesticides, with emphasis on the role of detoxification

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ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ

ΤΜΗΜΑ ΒΙΟΛΟΓΙΑΣ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

Μελέτη προσαρμογής των αρθροπόδων στις τοξίνες των φυτών (plant allelochemicals) και τα εντομοκτόνα, με έμφαση στον ρόλο των μηχανισμών αποτοξικοποίησης

ΝΕΝΑ ΠΑΥΛΙΔΗ

ΕΠΙΒΛΕΠΩΝ ΚΑΘΗΓΗΤΗΣ: ΙΩΑΝΝΗΣ ΒΟΝΤΑΣ

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List of publications

1. Vontas J., Kioulos I., **Pavlidi N.**, Morou E., della Torre A., Ranson H. (2012) Insecticide resistance in the major dengue vectors *Aedes albopictus* and *Aedes aegypti,* Pesticide Biochemistry and Physiology, 104: 126-131

2. **Pavlidi N**, Monastirioti M., Daborn P., Van Leeuwen T., Ranson H.,Vontas J.(2012)Transgenic expression of the *Aedes aegypti* CYP9J28 confers pyrethroid resistance in *Drosophila melanogaster*, Pesticide Biochemistry and Physiology, 104: 132-135

3. Kalajdzic P., Oehler S., M.,**Pavlidi N.**, Vontas J., Hatzigeorgiou A.G., Savakis C. (2012)Use of Mutagenesis, Genetic Mapping and Next Generation Transcriptomics to Investigate Insecticide Resistance Mechanisms, PloS One 7

4. Morou E., Lirakis M., **Pavlidi N**., Zotti M., Nakagawa Y., Smagghe G., Vontas J., SweversL. (2012) A New Dibenzoyl hydrazine with Insecticidal Activity against Anopheles Mosquito Larvae, Pest Management Science, 69: 827-833

5. **Pavlidi N.**, Dermauw W, Rombauts S, Chrisargiris A, Van Leeuwen and John Vontas(2013) Analysis of the olive fruit fly *Bactrocera oleae* transcriptome and phylogenetic classification of the major detoxification gene families. PLoS One 8 (6): e66533. doi:10.1371/journal.pone.0066533

6. **Pavlidi N.**, Tseliou V., Riga M., Nauen R., Van Leeuwen T., Labrou N. and Vontas J. (2015) Functional characterization of glutathione S-transferases associated with insecticide resistance in *Tetranychus urticae*, Pesticide Biochemistry and Physiology, 120: 53-60

7. Halon E., Eakteimana G., Moshitzkya P., Elbaza M., Alon M., **Pavlidi N.,** Vontas J., and Morin S. (2015) Only a minority of broad-range detoxification genes respond to a variety of phytotoxins in generalist *Bemisia tabaci* species (under second revision in Scientific reports)

8. Walsh T., Joussen N., ... **Pavlidi N.***et al.* (2015) Global Patterns of a synthetic Pyrethroid Resistance Mechanism in *Helicoverpa armigera*: Using CYP337B3 as a Selected Gene to Infer Movement (in preparation)

9. **Pavlidi N**. *et al*. (2015), Molecular analysis of olive fruit fly– olive interactions, during larval development in olive flesh, with and without symbiotic bacteria (in preparation)

10. **(Pavlidi N.***, Dermauht P.* - equal first authors), Dermauw W., Labrou N., Vontas J., Van Leeuwen T., (2015) Characterization of GST- based cyflumetofen resistance in Tetranychus urticae (in preparation for Journal of Biological Chemistry)

11. **Pavlidi N.** *et al*. (2015) Molecular analysis of pyrethroid resistance in *Bactrocera oleae* (in preparation for Insect Biochem Mol Biol)

Ευχαριστίες

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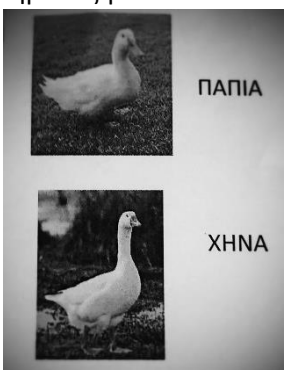

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Abstract

Arthropod pests seriously threaten food security and human health, as they attack agricultural crops and transmit various diseases. Their control has been largely based on chemical insecticides. However the intense use of insecticides has led in the development of resistance, mainly achieved by target site resistance mutations and detoxification, enzymes, such as the Cytochrome P450s, the Glutathione S-transferases GSTs and the Carboxylesterases (CCEs). The same detoxification enzymes also participate in the adaptation of arthropod pests to their hosts, as they also metabolize – inactivate phytotoxins. In this thesis, I used transgenic approaches, bioassays, transcriptomics and biochemical/ functional techniques to investigate detoxification mechanisms of major arthropod pests, such as the *Aedes aegypti*, the *Bactrocera oleae* and the *Tetranychus urticae*, against xenobiotics.

First, the *A. aegypti* cytochrome P450 *CYP9J28* was successfully expressed in *Drosophila melanogaster* and shown to confer significant levels of resistance *in vivo*, providing solid evidence for its role in pyrethroid resistance and showing that ectopic expression in *D. melanogaster* may be a robust approach for validation of candidate resistance genes.

Second, a large transcriptomic dataset of *B. oleae* was generated and more than 130 putative detoxification genes were identified and phylogenetically classified. The transcriptome was used for the construction of a microarray tool, which was used, in its pilot application, to study detoxification and adaptation mechanisms of the olive fly against insecticides and olive flesh/phytotoxins, respectively. The pyrethroid resistance study indicated the association of two cytochrome P450 genes with the phenotype. Several detoxification and digestive genes were found over-expressed upon development in olives (green versus black versus artificial diet), providing a useful starting point for further investigation.

In the last chapter, my study dealt and focused on *T. urticae* GSTs that had been associated with insecticide resistance by microarray studies. Four GSTs were functionally expressed and characterized. TuGSTd14 was found to interact with

abamectin, supporting earlier work that GSTs are may play a role in abamectin resistance. Strong evidence were provided that TuGSTd05 catalyzes the conjugation of glutathione (GSH) to cyflumetofen *in vitro* and the possible site of attack as well as key amino acids possibly implicated in the interaction were identified. This study represents the first functional convincing report for the implication of an acari GST in resistance.

Περίληψη

Τα αρθρόποδα παράσιτα αποτελούν απειλή για την ασφάλεια των τροφίμων και τη δημόσια υγεία καθώς καταστρέφουν τις καλλιέργειες και μεταφέρουν εντομομεταδιδόμενες ασθένειες. Ο έλεγχός τους βασίζεται κυρίως στη χρήση εντομοκτόνων. Ωστόσο, η συχνή τους εφαρμογή έχει οδηγήσει στην ανάπτυξη ανθεκτικότητας, η οποία επιτυγχάνεται κυρίως μέσω μεταλλαγών στις πρωτεΐνες-στόχους των εντομοκτόνων και μέσω της δράσης των ενζύμων αποτοξικοποίησης, όπως είναι οι κυτοχρωμικές P450 μονοοξυγενάσες (P450s), οι μεταφοράσες της γλουταθειώνης (GSTs) και οι καρβοξυλεστεράσες (CCEs). Τα ίδια ένζυμα αποτοξικοποίησης συμμετέχουν και στην προσαρμογή των αρθροπόδων στα φυτά-ξενιστές, καθώς έχουν την ικανότητα να μεταβολίζουν- απενεργοποιούν τις φυτοτοξίνες. Στην παρούσα διατριβή χρησιμοποιήθηκαν προσεγγίσεις με διαγονιδιακούς οργανισμούς, βιοδοκιμές, μεθόδοι ανάλυσης μεταγραφώματος καθώς και βιοχημικές/ λειτουργικές μελέτες για τη διερεύνηση των μηχανισμών αποτοξικοποίησης ξενοβιοτικών ουσιών στο κουνούπι *Aedes aegypti*, το δάκο της ελιάς *Bactrocera oleae* και το φυτοφάγο άκαρι *Tetranychus urticae*.

Αρχικά, το γονίδιο *CYP9J28* του *A. aegypti* που κωδικοποιεί για κυτοχρωμική οξειδάση P450 εκφράστηκε επιτυχώς στη *Drosophila melanogaster* και δείχθηκε ότι προσδίδει σημαντικά επίπεδα ανθεκτικότητας *in vivo*, παρέχοντας ισχυρές αποδείξεις για το ρόλο του στη ανθεκτικότητα στα πυρεθροειδή και δείχνοντας ότι η έκφραση γονιδίων στη *D. melanogaster* είναι μια ισχυρή προσέγγιση για την επιβεβαίωση του ρόλου υποψηφίων γονιδίων στην ανθεκτικότητα.

Στο δεύτερο κεφάλαιο της διατριβής, δημιουργήθηκε ένα μεγάλο σύνολο δεδομένων μεταγραφώματος για το *B. oleae* και ταυτοποιήθηκαν και κατηγοριοποιήθηκαν φυλογενετικά πάνω από 130 πιθανά γονίδια αποτοξικοποίησης. Τα δεδομένα του μεταγραφώματος χρησιμοποιήθηκαν για την κατασκευή μιας πλατφόρμας μικροσυστοιχιών, η οποία εν συνεχεία χρησιμοποιήθηκε πιλοτικά για την μελέτη των μηχανισμών αποτοξικοποίησης και προσαρμογής του δάκου έναντι των εντομοκτόνων και του ελαιοκάρπου/ φυτοτοξινών, αντίστοιχα. Η ανάλυση της ανθεκτικότητας σε πυρεθροειδή υπέδειξε

τη συσχέτιση δύο κυτοχρωμικών οξειδασών P450 με το φαινότυπο. Επίσης, μελετήθηκαν οι μοριακοί μηχανισμοί προσαρμογής στον ελαιόκαρπο και η αξιοποίηση του μεσοκαρπίου. Αρκετά γονίδια αποτοξικοποίησης και γονίδια της πέψης βρέθηκαν να υπερ-εκφράζονται κατά τη διάρκεια της ανάπτυξης στον ελαιόκαρπο, παρέχοντας ένα χρήσιμο σημείο εκκίνησης για περαιτέρω διερεύνηση.

Στο τελευταίο κεφάλαιο της διατριβής εκφράστηκαν λειτουργικά και χαρακτηρίστηκαν τέσσερις μεταφοράσες της γλουταθειώνης (GSTs) του *T. urticae*, οι οποίες είχαν προηγουμένως συσχετιστεί με την ανθεκτικότητα σε εντομοκτόνα από μελέτες μικροσυστοιχιών. Η TuGSTd14 βρέθηκε να αλληλεπιδρά με το abamectin, ενισχύοντας προηγούμενες μελέτες που υποστηρίζουν ότι οι GSTs ενδεχομένως εμπλέκονται στην ανθεκτικότητα στο abamectin. Ισχυρές αποδείξεις δόθηκαν για την *in vitro* κατάλυση της σύζευξης της γλουταθειώνης (GSH) στο cyflumetofen από την TuGSTd05 και αναγνωρίστηκε το πιθανό σημείο της προσβολής καθώς και σημαντικά αμινοξέα που πιθανόν να συμμετέχουν στην αλληλεπίδραση. Η συγκεκριμένη μελέτη αποτελεί την πρώτη πειστική αναφορά για τη συμμετοχή μιας GST από ακάρεα στην ανθεκτικότητα.

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Abbreviations

ABC transporter	ATP-Binding Cassette transporter
AChe	Acetylcholinesterase
BSA	Bulk Segregant Analysis
CDNB	1-chloro-2,4-dinitrochlorobenzene
CCE	Carboxylesterase
CRISPR	Clustered Regularly Interspaced
CuOOH	Cumene Hydroperoxide
DCNB	1,2-dichloro-4-nitrobenzene
DEF	S, S, S- tributyl phosphorotrithioate
DEM	Diethyl Maleate
DMC	Dosage Mortality Curve
EC	Enzyme Commission
ESI	Electrospray Ionization
FC	Fold Change
GluCl	Glutamine-gated Chlorine channels
GMQS	Global Model Quality Score
GSH	Glutathione
GSSG	Oxidized Glutathione Disulfide
GST	Glutathione S-transferase
HPLC	High Performance Liquid Chromatography
IC50	Inhibition Concentration 50
IPM	Integrated Pest Management
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRAC	Insecticide Resistance Action Committee
IRS	Insecticide Residual Spray
IVM	Integrated Vector Management
K_{cat}	Catalytic Constant
KEGG	Kyoto Encyclopedia of Gene and Genome
K_i	Inhibition Constant
K_m	Michaelis Constant
LC_{50}	Lethal Concentration 50
LD_{50}	Lethal Dose 50

MACE	Modified ACEtylcholinesterase
METI	Mitochondrial Electron Transport Inhibitor
MS	Mass Spectrometry
nAChR	Nicotinic Acetlycholinic Receptor
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBD	Nucleotide Binding Receptors
NCBI	National Center for Biotechnology Information
Ni-NTA	Nickel- Nitrilotriacetic acid
NTC	No Template Control
OP	Organophosphate
P450	Cytochrome P450 mono-oxygenase
PBO	Piperonyl Butoxide
PI	Protease Inhibitor
PTP	Picotiterplate
qPCR	quantitative Polymerase Chain Reaction
RLR	Recommended Label Rate
ROS	Reactive Oxygen Species
RR	Resistance Ratio
RyR	Ryanodine Receptors
S. D.	Standard Deviation
SNP	Single- nucleotide polymorphism
U	Enzyme Unit
UAS	Upstream Activation Sequence
UGT	UDP-glycosyltransferase
VGSC	Voltage Gate Sodium Channel

Chapter 1

General introduction

1.1. The threats posed by arthropod pests

Arthropods are invertebrate animals that represent the largest group of animals in the Earth, including more than 80% of all known species worldwide [1]. More than one million species have been described as yet and typical examples of arthropods are insects, which is the major category of this phylum (750,000 species have been described), mites (30,000 species have been described), spiders and crabs (http://www.britannica.com/animal/arthropod). Arthropods are of great importance for humans. The production of several goods such as silk and honey and the pollination of flowering plants[2] are only a few of the great benefits of arthropods for humans. However, numerous arthropod species are deadly enemies of humanity. They can transmit serious diseases and attack agricultural crops, therefore; they severely threaten economy, food security and global health.

A number of arthropod pests, such as mosquitoes, ticks and sundflies, is responsible for the transmission of infectious and deadly diseases. Vector-borne diseases cause more than one million deaths every year[3], making arthropod pest vectors the biggest killers for humanity. One of the worst examples, the mosquito *Anopheles gambiae* transmits the malaria parasite, which causes more than 600,000 deaths per year, in particular in children under 5 years old[3]. Aedes mosquitoes also represent a major threat, transmitting various diseases such as chikungunya, dengue and yellow fever, infecting 50-100 million people per year[4]. Moreover; arthropod pests are responsible for huge crop losses world-wide, the 20-50% of potential production is damaged annually due to arthropod pest infection. For example, the Tetranychidae family contains over 6,000 species of plant feeding spider mites that attack and destroy a wide variety of field and greenhouse crops[5-7] and the olive fruit fly, *Bactrocera oleae,* represents the major pest of olive orchards worldwide. It is estimated that by 2050, the human population will be increased by 34% and, in order for the food to be sufficient, the crop production needs to duplicate[8]. In this context, agricultural arthropod pests represent an enormous threat.

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1.2. Arthropod pest management

The efficient control of arthropod pests is vital for the protection of public health, agriculture and economy. Arthropod pest management is achieved through a number of different approaches, which can be classified in 5 main categories: 1. physical control, 2. cultural control, 3. pest-resistant plants, 4. biological control and 5. chemical control[2, 9]. Integrated Pest Management (IPM) or 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[10].

Briefly, the different approaches for arthropod pest control are described below:

Physical control: includes methodologies that physically block the access of arthropod pests to their hosts, including barriers that keep pests away from plants in the greenhouses as well as bed nets, extensively used in Africa for the prevention of mosquito biting[2].

Cultural control: includes several practices that aim to make the environment less preferable for arthropod pests via sanitation, crop rotation, trap cropping, use of pest-free seed etc[2, 9] .

Pest-resistant plants: includes the use of plant varieties showing increased resistance to arthropod pests and has been successfully used in order to control arthropod pests. A number of resistant varieties have been selected (e.g. by traditional breeding), such as rice, corn and apple varieties resistant to many pests[2, 9].

Biological control: is achieved by the usage of natural enemies of the pests. Natural enemies may be other arthropods or microbes and this control method is successfully used for the management of a number of insects and mites[2, 9].

Chemical control: is achieved by the use of natural or synthetic chemicals in order to either directly kill the pests or act via interfering with fundamental activities of the arthropod such as feeding and mating[2]. Chemical control is the predominant and most efficient approach recruited for the control of all arthropod pests.

1.2.1 Chemical control

Chemical control is further discussed below since it is in direct correlation with the objectives of this thesis.

There is no doubt that the chemical control and usage of insecticides contribute significantly in the arthropod pest control. For example, it is estimated that without the use of chemical insecticides the food supplies would fall to 30-40%[11]. Chemicals protect the availability of food as well as prevent the spread of pest-borne diseases thus, they play a decisive role in the quality of human life.

The vast majority of chemicals targets the central nervous system of the arthropod pest causing paralysis and finally death, while others act as growth regulators, energy metabolism disruptors (e.g. mitochondrial electron transport inhibitors, METIs) or act in the midgut membranes [12, 13] (**Figure 1.1 A**). Among the main categories of insecticides/ acaricides with neural/ muscle action are the organophosphates (OPs), pyrethroids, neonicotinoids, avermectins and diamides [13]. METIs represent the main category of energy metabolism disruptors currently used[13] (**Figure 1.1 B and C**).

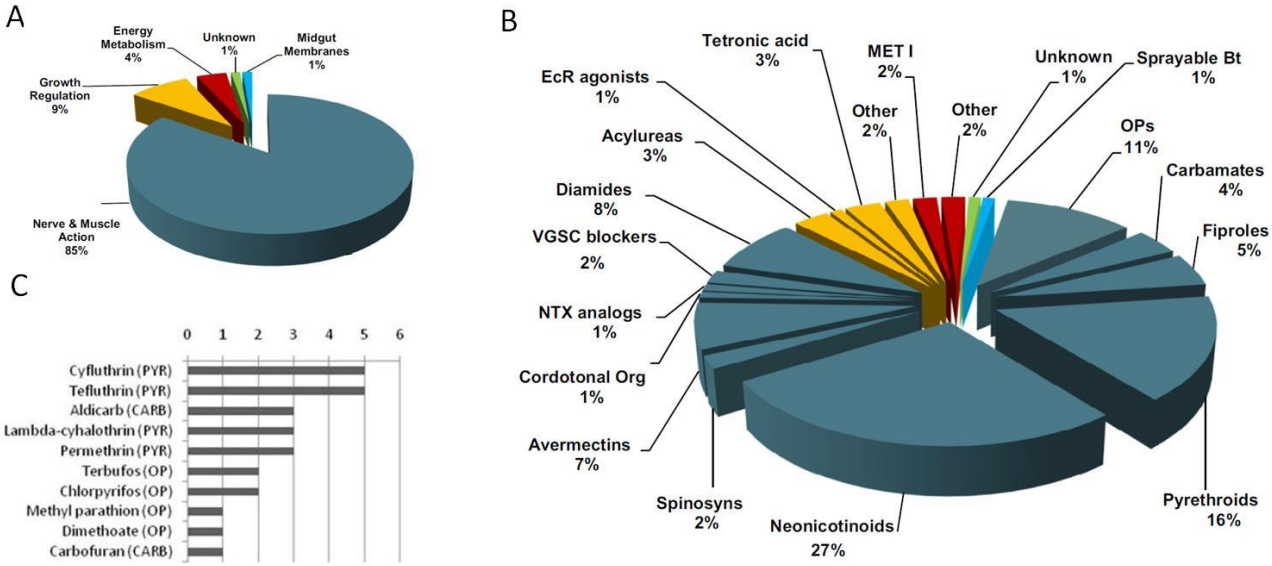


Figure 1.1. A. **Percentage distribution of the total insecticide sales by general mode of action for the year 2013** (figure from Ref. 13). **B**: **Percentage of distribution of total insecticide sales (percent of total value) by Insecticide Resistance Action Committee (IRAC) mode of action**

for the year 2013 (Figure from Ref. 13). **C**. **Distribution of active ingredient use in millions of acres in the USA, 2001.** PYR: pyrethroid, CARB: carbamate, OP: organophosphate (Figure from Ref.10).

Organophosphates (OPs): one of the oldest group of insecticides, were first used in early '40s and more than 100 different active ingredients have been used[14]. They target the central nervous system by binding to the enzyme acetylcholinesteraste (AChe), leading to the disruption of the hydrolysis of acetylcholine and the continuous nerve stimulation causing paralysis and eventually death[15]. OPs are used for the control of a large number of pests, such as species of Diptera (e. g mosquitoes, flies) Coleoptera, Lepidoptera, Hemiptera, nematodes and mites[16]. OPs is the most successful group of insecticides however; they are currently in a replacement procedure by other compounds as they display toxicity to vertebrates, including humans[17-19].

Pyrethroids: were first used in 1970s and represent an alternative of the OPs as they are effective without displaying toxicity to non-target species, such as humans[20]. They are synthetic derivatives of the naturally existing insecticidal molecules pyrethrins, derived from the extract of the flower *Chrysanthemum cinerarifolium*[21]. Pyrethroids bind to the voltage-gated sodium channel (VGSC) of the neuronal cells and affect ion flow[15, 22]. They are divided in 2 classes, type I pyrethroids, which cause hyperactivity resulting in lack of pest coordination and type II pyrethroids, which cause depolarization resulting in pest paralysis[23]. Pyrethroids are used against a wide variety of pest species, i.e. Lepidoptera, Coleoptera, aphids and mites as well as against several mosquito species[24].

Neonicotinoids: were started to be used in the beginning of 90s[25]. Nowadays (after 2008), they represent the most extensively used insecticides world-wide[13] (based on Agranova[26]).They also target the pest nervous system. In particular, they bind and block the nicotinic acetylcholine receptors (nAChRs) causing paralysis and death[27]. Neonicotinoids are systemic insecticides, distributed in plant tissues and they are used extensively for the control of piercing-sucking insect pests, such as aphids and whiteflies[28] as well as against several other pests.

Avermectins: were first used in 1980 and they are a group of eight compounds produced by the microorganism *Streptomyces avermitilis* during the fermentation process[29]. They activate the glutamate-gated chloride channels (GluCls). They bind to the GluCls causing a slow and irreversible open of the channel, which leads to hyper- or de- polarization eventually causing paralysis and death[30, 31]. Avermectins are used for the control of a wide range of pest species of Coleoptera, Homoptera, Diptera, Orthoptera, Isoptera, Hymenoptera, and Lepidoptera as well as mite species[32].

Diamides: is a relatively new class of insecticides, introduced in 2008, which is particularly active against lepidopteran species[33-35]. Diamides bind and activate the ryanodine receptors (RyR) affecting the calcium ions flow [36] and causing feeding cessation, paralysis and finally death[34].

Mitochondrial electron transport inhibitors (METIs): were first introduced in 90's and they act as inhibitors of the mitochondrial electron transport chain causing the rapid knockdown of the pest[37]. They are used for the control of mite species as they display high acaricidal activity[37]. Cyflumetofen is a novel METI acaricide that acts as inhibitor of complex II in the mitochondrial electron transport chain[38, 39].

1.3 Arthropod pests develop resistance to insecticides/ acaricides

The extended and frequent application of insecticides has led to the rapid development of insecticide resistance, which seriously threaten the sustainability of arthropod pest management efforts.

Insecticide resistance is defined by the Insecticide Resistance Action Committee [12] 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's recommendation for that pest species". Alternatively, resistance is "a heritable decrease in susceptibility of a population to a toxin caused by exposure of the population to the toxin" [40]. Resistance is most commonly evolved by the excessive and/or wrong usage of insecticides in the field and usually arises within 10 years after the introduction of an insecticide in the market[14].

The development of arthropod pest resistance to chemical insecticides is an evolutionary procedure driven by natural selection. Populations are polymorphic and genetic variation between individuals exists. Random mutations are caused which under normal conditions, are very rare as they have a fitness cost. Therefore, mutant individuals represent a small number in the population. However when a selection pressure is applied, as in the case of the insecticide application, a mutation may become advantageous for survival and reproduction. The insecticide kills off the susceptible genotypes while resistant mutants gain a selective advantage thus, after a few generations, resistant alleles spread into the population and the resistant phenotypes increase in number leading to the development of insecticide resistance.

The first case of resistance was reported in 1914, in the insect *Quadraspidiotus perniciosus* (Comstock) which evolved resistance to lime sulfur[41] and since 1950's more and more species started to become resistant and the number of reported cases as well as the number of insecticides in which resistance has evolved, dramatically increased[42-45]. To date, 11,403 cases of the resistance have been recorded in 586 arthropod pest species against 325 chemicals[13, 46] (**Figure 1.2**) and the numbers continue to rise.

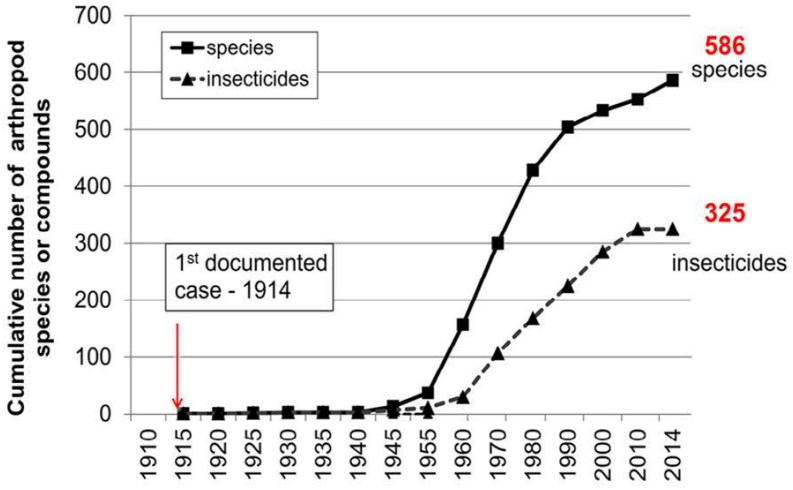


Figure 1.2 Cumulative number of insecticide resistant species and insecticides against of which resistance has arisen. The first resistance case was documented in 1914 and since then, 586 arthropod pest species have developed resistance in 325 insecticides. Figure from [13].

The development of insecticide/ acaricide resistance is considered as one of the most important limitations for an effective arthropod pest control in our days. Resistance mechanisms can decrease the amount of insecticide that finally reaches the target and/or reduce the affinity of the target with the insecticide. Based on IRAC [12] resistance is divided in 4 major types: 1. behavioral, 2. penetration, 3. target-site and 4. metabolic resistance (**Figure 1.3**).

Behavioral resistance: occurs when the arthropod pest recognizes and does not get in touch/ avoids the chemical compound (e.g. by moving into another area)[12]. This type of behavior is genetically controlled and has been involved in resistance in a number of insecticides such as organophosphates and pyrethroids[12].

Penetration resistance: occurs when upon contact with the insecticide, the penetration rate is reduced[12]. This type of resistance is conferred by changes in the arthropod cuticle and usually coexists and assists other types of resistance, in terms of providing more time to the other resistance mechanisms to act[12].

Target-site resistance: is caused by mutation that lead to the production of a target protein form with reduced affinity for the insecticide, thus preventing its binding[12]. This type has been found to confer resistance in several insecticides including pyrethroids and avermectins[12].

Metabolic resistance: In this type of resistance, detoxification enzymes like cytochrome P450 mono-oxygenases (P450s), carboxylesterases (CCEs) and glutathione S-transferases (GSTs) detoxify the insecticide into non-toxic compounds[12]. Metabolic resistance occurs due to over-production or more catalytically efficient forms of metabolic enzymes.

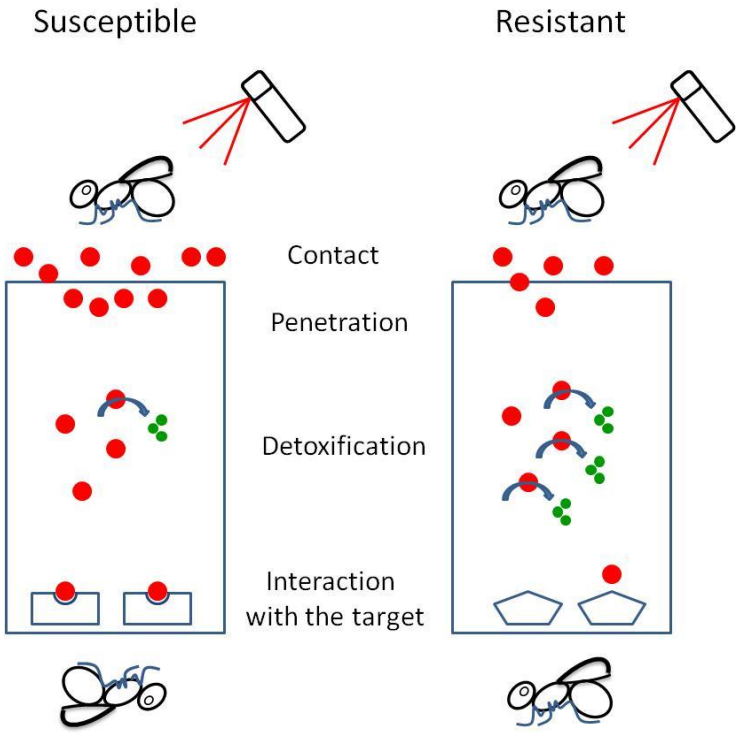


Figure 1.3 Major categories of insecticide resistance. Resistance may occur via behavioral adaptations by which the arthropod pest avoids the contact with the insecticide (red cycles), reduced penetration of the insecticide due to cuticle changes, enhanced metabolism of the insecticide to non-toxic compounds (green cycles) by detoxification enzymes (blue arrows) and finally, mutations in the target (blue rectangle/ polygon) which prevent the binding of the insecticide. (Figure inspired by [47]).

Apart from their involvement in resistance to insecticides/ acaricides, detoxification enzymes also participate in the adaptation of arthropod pests to their host, as they are capable of metabolizing phytotoxins.

1.4 Detoxification genes are also major weapons in the fight of arthropod pests against plants

In order to prevent herbivore attack, plants have evolved defense systems, which interfere with principle pest activities, such as feeding, oviposition and development[48-50]. These defenses often include chemical compounds, known as

phytotoxins or allelochemicals, which are secondary metabolites normally synthesized during development and are stored or induced during herbivore attack[50-52]. A large number of phytotoxins have been identified to date, such as alkaloids, glucosinolatea, phenolic compounds, terpenoids, protease inhibitors etc [50, 51].

On the other hand, herbivore pests have also evolved adaptation mechanisms to cope with plant phytotoxins and successfully feed upon their host. Adaptation mechanisms can be divided in five main categories: 1. behavioral adaptation, where the arthropod avoids the contact with the phytotoxin, 2. host manipulation, where enzymes in saliva neutralize the defense, 3. excretion and sequestration of the phytotoxin, 4. metabolic adaptation, where the phytotoxin in metabolized by detoxification enzymes and 5. target- site mutations which hinders the binding of the phytotoxin to its target[48, 49, 53, 54].

The metabolism or inactivation via binding by detoxification enzymes represents the major weapon by which arthropod pests fight against plants and cope with phytotoxins as well as is also among the reasons responsible for arthropod plasticity, which allowed the phylum Arthropoda to be the most successful animal group[33, 49, 55]. Arthropod pests metabolize phytotoxins by detoxification enzymes which mainly include members of the super-families of P450s, GSTs, CCEs, UDP-glucosyltransferases (UGTs) and ABC transporters[42, 53]. Metabolic resistance is conferred due to increased levels of detoxification enzymes, usually induced by the exposure to the phytotoxin, as well as from mutations in detoxification genes, resulting in enzyme forms catalytically more active towards phytotoxins[42, 53].

1.5 Detoxification genes, enzymes and mechanisms

In general, the detoxification of a xenobiotic (i.e. e. insecticide or plant toxin) is divided in three phases[56-58]. In phase I enzymes, i.e. P450s and CCEs, modify the xenobiotic (e.g. via hydroxylation) and the modified xenobiotic is recognized and conjugated to polar compounds by phase II enzymes [58, 59], which are transferases such as GSTs [60, 61] and UGTs [56, 62, 63]. 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[64, 65].

1.5.1 Cytochrome P450s monooxygenases (P450s)

Cytochrome P450 monooxygenases (P450s), encoded from CYP genes, are present in all living organisms and represent one of the largest super-families[66]. They are 45- 55 kDa heme-thiolate enzymes bound in the membranes and they are divided in 4 major clades, named CYP2, CYP3, CYP4 and mitochondrial. Although they can catalyze more than 60 reactions[66], P450s are best known for catalyzing the insertion of one atom of oxygen in an organic compound, while the other atom of oxygen is reduced to water[67]:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

In this reaction, the so called monooxygenase reaction, NADPH serves as the electron donor, which is transferred to the P450 by NADPH-cytochrome P450 reductase (CPR) and/ or cytochrome b5[68, 69, 70] (**Figure 1.4**).

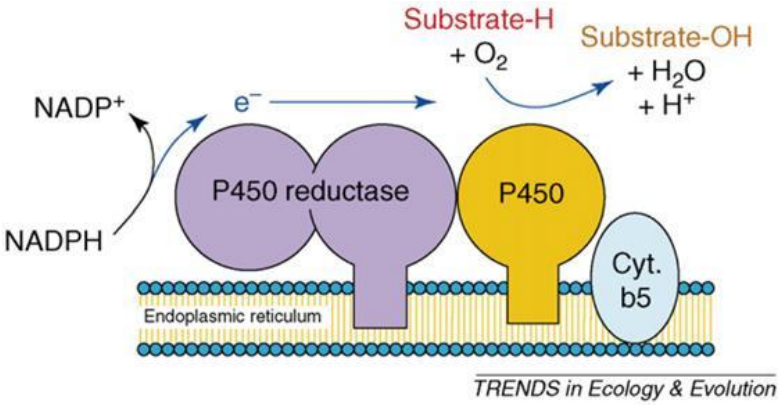


Figure 1.4 The cytochrome P450 system (Figure from Ref. 53).

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[66] and phytotoxins[71], thus playing a dominant role in

insecticide resistance and arthropod adaptation to plants. 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[66]. 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)[72, 73]. CYP6A2 from *Drosophila melanogaster* metabolizes DDT, aldrin (cyclodiene), dieldrin and DDT (organochlorides) as well as diazinon (organophosphate)[74, 75]. *T. urticae* CYP392E7 and CYP392E10 were found to metabolize spirodiclofen (ketoenol)[76], CYP392A16 was found to metabolize abamectin (avermectin)[77] and the recently characterized CYP392A11 which is capable of metabolizing cyenopyrafen (mitochondrial complex II electron transport inhibitor) and fenpyroximate (mitochondrial complex I electron transport inhibitor)[78].

Arthropod P450s have also been shown to metabolize several plant toxins such as furanocoumarins, terpenoids, glucosinolates and flavonoids[66, 71, 79]. An example is the involvement of the P450 CYP6B1 in the adaptation of Papilionidae Lepidoptera to furanocoumarin. *CYP6B1* gene is specifically induced by xanthotoxin (a liner furanocoumarin) encoding for an enzyme capable of efficiently metabolizing furanocoumarins [80-83].

1.5.2 Glutathione S- transferases (GSTs)

Glutathione S-transferases (GSTs) is a large super-family of enzymes present in all aerobic organisms[84]. They are divided in 2 main categories based on their sub-cellular localization, microsomal and cytosolic. Insect GSTs are divided in 7 classes, named delta (δ), epsilon (ε), omega (ο), sigma (σ), theta (θ), zeta (ζ) and microsomal GSTs. In the spider mite *T. urticae* also an additional class has been identified, named mu [85]. GSTs catalyze numerous reactions, such as glutathione (GSH) conjugation reaction, dehydrochlorination, and reduction of hydroperoxides and isomerization of unsaturated molecules[84]. Moreover GSTs may also display non-catalytic function via passive, sacrificial binding[84]. The major reaction catalyzed by the GSTs in the

conjugation of electrophilic compounds with the thiol group of reduced glutathione[86] (**Figure 1.5**). This conjugation increases the solubility of the substrate facilitating its subsequent excretion from the cell[87].

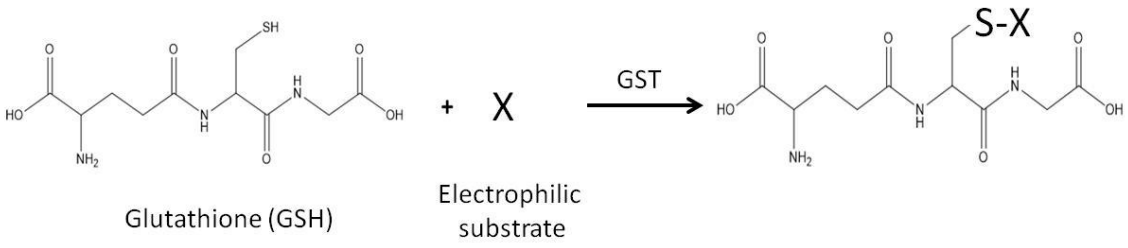


Figure 1.5. The conjugation of reduced GSH to electrophilic moieties, catalyzed by GSTs.

Cytosolic GSTs are 25 kDa proteins and act as homodimers or heterodimers. The 3D structure of each GST monomer consists of two domains which are linked by a short linker[88, 89] (**Figure 1.6**). An N-terminal domain (amino acids 1-80) consisting of both α- helices and β-sheets arranged in a βαβαββα secondary structure as well as a larger C-terminal domain consisting of a variable number of α-helices[88, 89]. The N-terminal domain forms the G-site, which is the binding site of glutathione (GSH) while the C-terminal domain mainly forms the H-site, which is the binding site of electrophilic substrates, such as insecticides and phytotoxins [88, 89]. The H-site is much more variable from the G-site giving in the different GSTs variable substrate specificities [88, 89]. Despite the fact that each monomer acts independently, the homo or hetero-dimerization is needed for activity [90].

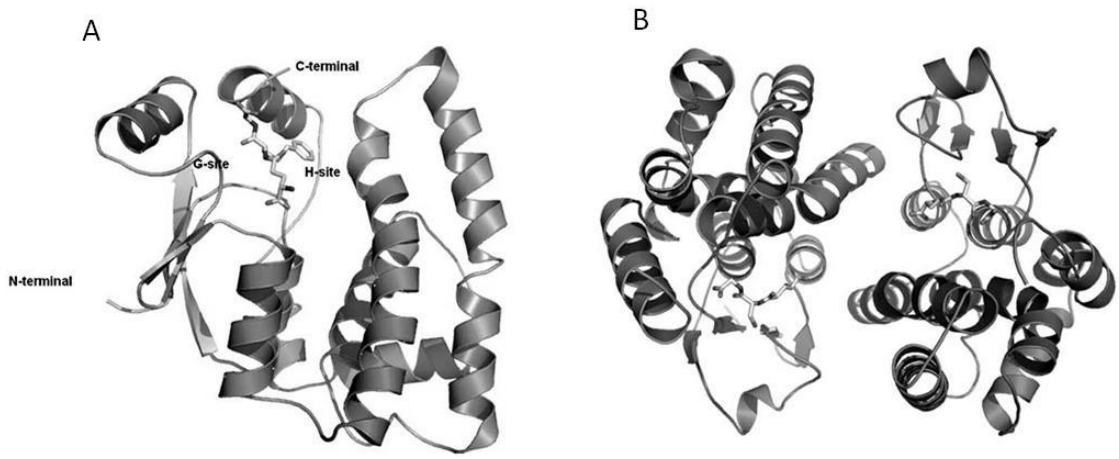


Figure 1.6 The 3D structure of a cytosolic GST (GmGSTU4-4). A. Ribbon representation of the monomer. The *S*-(p-nitrobenzyl)-glutathione, bound in the active site is shown as stick. The G-site and H-site are mentioned. B. Ribbon representation of the dimer. The *S*-(p-nitrobenzyl)-glutathione, bound in the active site is shown as stick. (Figure from Ref. 88).

GSTs are implicated in phase II metabolism of both endogenous compounds as well as xenobiotics. To date, elevated GST activity has been associated with resistance to all main categories of insecticides, such as pyrethroids, OPs, organochlorides and abamectin in many arthropod pests [91-94], however; the precise functional characterization at the protein level and the identification of specific GSTs capable to metabolize insecticides *in vitro* has not kept similar pace. The best known example of insecticide metabolism is the metabolism/ dehydrochlorination of DDT by GSTe2 from *Anopheles gambiae* [95, 96]. The *Manduca sexta* GST-6A was also found to conjugate GSH to the OPs methyl parathion and lindane[97], while GST3 and GST4 from *Plutella xylostella* metabolize parathion, methyl parathion and paraoxon (OPs)[42, 98, 99]. GST activity was found to participate in pyrethroid resistance via sequestration of pyrethroids [100] and detoxification of lipid peroxidation products [101] but not to direct detoxification of pyrethroids themselves. Finally, there is a strong association of elevated GST activity with abamectin resistance[94, 102], however the putative role of the GSTs in conferring resistance to abamectin has not been investigated as yet.

The involvement of arthropod GSTs in metabolism of plant toxins has also been investigated. Several GSTs have been found to detoxify coumarins and

furanocoumarins, glucosinolates, isothiocyanates, organothiocyanates and α, β-unsaturated carbonyls[103-107].

1.5.3 Carboxylesterases (CCEs)

Carboxylesterases (CCEs) is a ubiquitous and super-family. In the super-family belong non- catalytic proteins such as neurotactins, enzymes with a wide range of substrates and enzymes with very specified substrates such as acetylcholinesterase[108]. CCEs 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[108] (**Figure 1.7**).

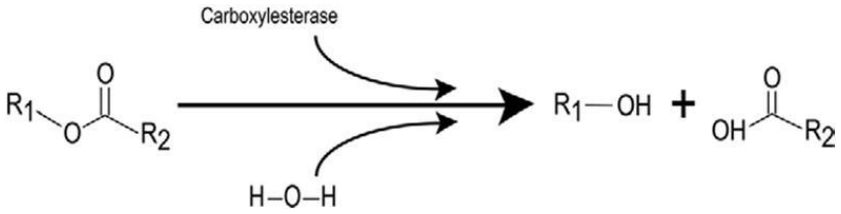


Figure 1.7. Hydrolysis reaction catalyzed by CCEs. (Figure from Ref. 108)

Arthropod CCEs are involved in the detoxification of xenobiotics and are considered as phase I enzymes. In insecticide resistance, are mainly implicated in resistance to OPs, carbamates and pyrethroids, where act either via hydrolysis or/ and sequestration [43, 109]. For example, esterases were shown to slowly hydrolyze the insecticide permethrin (pyrethroid) in *Nilaparvata lugens* [93]. The *Lucilia cuprina* E3 esterase was shown to confer resistance to malathion (OP)[110].

The role of CCEs in the adaptation of insects to plant toxins has not been extensively studied yet [53]. Their involvement in metabolism of phenolic glycosides[111, 112] as well as tannins[113] have been reported.

1.5.4. UDP-glucosyltransferases (UGTs)

UDP-glucosyltransferases are a ubiquitous super-family of enzymes that catalyze the conjugation of a hexose group (glucose, galactose, xylose, glucuronic acid) to their substrates [114], increasing their solubility and facilitating their excretion from the cell [115]. UGTs are implicated in the detoxification of endogenous and exogenous compounds, such as phytotoxins and insecticides[116-118]. For example, UGT activity is involved in resistance to organophosphates in tobacco budworms *Heliothis virescens*[117].

Glucosylation by UGTs has been associated with adaptation of insect pests to phenolic compounds. A phenol UGT from the silkworm *Bombyx mori*, BmUGT1 [119], was shown to catalyze the glucosidation of phenolics and phenolic-derived compounds [119]. Metabolism of plant compounds by UGTs has also been reported in *Manduca sexta*[116].

1.6 Techniques and approaches for investigating detoxification mechanisms in insects and mites

Metabolic resistance mainly occurs due to the overproduction of one or more detoxification enzymes and in some cases due to mutations that lead to the production of enzyme forms catalytically more efficient towards the insecticides[42].

A number of techniques have been used for the study of metabolic resistance:

Bioassays with synergists: they are employed for the first metabolic resistance investigation. Synergists, such as, the P450 inhibitor piperonyl butoxide (PBO), the GST inhibitor diethyl maleate (DEM) and the esterase inhibitor S, S, S-tributyl phosphorotrithioate (DEF) are used in bioassays with insecticides giving a first indication for the detoxification mechanisms involved[120]. For example synergism bioassays were employed to associate pyrethroid resistance with P450- mediated metabolism in *B. oleae*[121].

Biochemical studies: they are conducted in crude homogenates or pest extracts to investigate the relative role of specific metabolic systems in resistance. The

total activity of detoxification enzymes i.e. P450, GST, esterase activity, is measured in order to correlate elevated activity levels with resistance phenotype in many cases.

Gene expression studies: these are ranging from the examination of the expression of small number of candidate genes to high-throughput expression profiling analysis [122] and have been employed for the analysis of metabolic resistance at the gene transcript level. A large number of genes associated with insecticide resistance and/ or adaptation to host plants have been identified to date, in several arthropod species including flies, mosquitoes and mites[123-125]. Next generation sequencing technologies also allowed the investigation of both expression levels and the identification of sequence polymorphisms revealing in parallel quantitative and qualitative changes in resistant strains.

Genetic studies: genetic crosses between different phenotypes in conjunction with the genotyping of the segregated progenies are also employed for the investigation of metabolic resistance. For example, genetic crosses were employed to associate the involvement of two esterases gene amplification, *CCEae6a* and *CCEae3a*, with resistance to temephos (OP) in *Aedes albopictus* [126].

However association studies represent only a first, preliminary indication for the putative role of a candidate gene in resistance. *In vitro* and *in vivo* validation is required. *In vitro* metabolism studies have been employed and a large number of detoxification genes from numerous arthropod species have been functionally expressed in heterologous systems such as *Escherichia coli*, baculovirus and yeast and confirmed their ability to metabolize insecticides *in vitro* (examples are described in section 1.5).

The *in vivo* validation stage is much more challenging. RNAi and transgenic methodologies have been employed for the *in vivo* validation task. RNAi has been used to validate the role of candidate detoxification genes in insects in some cases. In particular, the knockdown of *CYP6BG1* via RNAi in *Plutella xylostella* confirmed its role to permethrin (pyrethroid) resistance[127] while the knockdown of the *Tribolium castaneum CYP6BQ9* confirmed its role in deltamethrin (pyrethroid) resistance[128]. RNAi was also applied in the mosquito *A. aegypti* and validated the role of *GSTe7* and *GSTe2* in resistance to deltamethrin (pyrethroid)[129]. However, with the exception of *T.*

castaneum, the lack of a systemic RNAi response, as the dsRNA does not effectively travel between cells[130], as well as a number of disadvantages with the RNAi injection procedure such as insect injury, differential gene silencing in alternative tissues and side effects may complicate the routinely use of the approach.

In vivo validation could also be achieved through transgenic over-expression or mis-expression methodologies. However transgenesis in arthropod pests is limited at the moment due to the lack of genomic information in a number of species, the lack of validated promoters to drive the transgene expression in species where the genome is sequenced and the lack of established methodologies to create transgenic lines consistently. Instead, the model organism *Drosophila melanogaster* could serve as an effective alternative for over- or mis-expression. Over-expression in *D. melanogaster* has been successfully used, as for example validated the role of *Cyp6g1* in resistance to several insecticides[131], and has been proposed as an alternative for functional validation of insecticide resistance genes [132].

1.7 Overall aim of the study

The aim of the study is the understanding of adaptation of arthropod pests to chemical insecticides and phytotoxins, with emphasis on the role of detoxification. A number of techniques, including classical bioassays, transcriptomics biochemical/ functional studies and transgenic techniques were used. Analysis of xenobiotic detoxification in three major arthropod pests, the mosquito *Aedes aegypti*, the major dengue and yellow fever vector, the olive fruit fly, *Bactrocera oleae,* the major pest of olive orchards worldwide and the two-spotted spider mite, *Tetranychus urticae,* an extremely polyphagous pest was conducted.

More specifically, the thesis is divided in 3 chapters:

1. *The transgenic expression of the A. aegypti cytochrome P450 CYP9J28 in Drosophila melanogaster*: The GAL4/UAS system was used for the transgenic over-expression of *A. aegypti CYP9J28* (a gene which is up-regulated in pyrethroid resistant mosquitoes and encodes an enzyme capable of metabolize pyrethroids *in vitro*[73]) in

order to investigate if it is capable to confer resistance *in vivo* as well as test the utility of the Drosophila model in evaluating the role of mosquito P450s in pyrethroid resistance.

2. *The understanding of the molecular base of insecticide resistance in B. oleae and its adaptation to olives, with emphasis on the role of detoxification:* A large transcriptomic dataset was generated in order to identify and phylogenetically classify the *B. oleae* detoxification gene repertoire as well as to construct a molecular tool (microarray chip). The microarray chip was subsequently used to analyze the gene expression of olive fruit flies resistant to the pyrethroid insecticide α-cypermethrin in order to understand the underlying mechanisms of pyrethroid resistance. Moreover the gene expression of larvae during their development in olives was analyzed, as an attempt to understand the mechanisms of adaptation to the olive fruit and identify genes potentially involved in the overcoming of plant defense and utilization of the olive flesh.

3. *The functional characterization of GSTs associated with insecticide/ acaricide resistance in T. urticae*: The *T. urticae* GSTs, TuGSTd10, TuGSTd14 and TuGSTm09, previously associated with resistance to abamectin were expressed in *E. coli* and their catalytic properties against model substrates, as well as their potential to interact with abamectin and other insecticides were examined. Also, TuGSTd05, previously associated with resistance to cyflumetofen, was functionally expressed and characterized and the interaction and capability of metabolizing cyflumetofen *in vitro* were studied.

1.8 References

1. http://www.britannica.com/animal/arthropod.
2. Mahr, D.L., Whitaker, P. & Ridgway, N.M. Biological Control of Insects and Mites: An Introduction to Beneficial Natural Enemies and Their Use in Pest Management (University of Wisconsin-Extension, 2008).
3. http://www.who.int.
4. World Health Organization, G.I.U.f.V.-B.D., Control. A 10-Year assessment (2000–2009), t.e., World Health & Organization.
5. Evans, G.O. Principles of acarology (CAB International, 1992).
6. Jeppson, L.R., Keifer, H.H. & Baker, E.W. Mites Injurious to Economic Plants (University of California Press, 1975).
7. Krantz, G.W. & Walter, D.E. A Manual of Acarology (Texas Tech University Press, 2009).
8. http://www.fao.org.
9. Assessment, U.S.C.O.o.T. Pest management strategies (Congress of the United States, Office of Technology Assessment, 1979).
10. http://www.epa.gov.
11. Saravi, S.S. & Shokrzadeh, M. Role of Pesticides in Human Life in the Modern Age: A Review in "Pesticides in the Modern World - Risks and Benefits" (2011).
12. http://www.irac-online.org/.
13. Sparks, T.C. & Nauen, R. IRAC: Mode of action classification and insecticide resistance management. *Pestic Biochem Physiol* **121**, 122-8 (2015).
14. Stenersen, J. Chemicals pesticides: mode of action and toxicology (CRC Press, 2004).
15. Kerkut, G.A. & Gilbert, L.I. Comprehensive Insect Physiology, Biochemistry, and Pharmacology: Nervous system: structure and motor function (Pergamon Press, 1985).
16. Siegfried, B.D. & Scharf, M.E. Biochemical Sites Important in Insecticide Action and Resistance (Springer-Verlag, 2001).
17. Minton, N.A. & Murray, V.S. A review of organophosphate poisoning. *Med Toxicol Adverse Drug Exp* **3**, 350-75 (1988).
18. Eddleston, M., Buckley, N.A., Eyer, P. & Dawson, A.H. Management of acute organophosphorus pesticide poisoning. *Lancet* **371**, 597-607 (2008).
19. Pandit, V. et al. A case of organophosphate poisoning presenting with seizure and unavailable history of parenteral suicide attempt. *J Emerg Trauma Shock* **4**, 132-4 (2011).
20. Wolansky, M.J. & Harrill, J.A. Neurobehavioral toxicology of pyrethroid insecticides in adult animals: a critical review. *Neurotoxicol Teratol* **30**, 55-78 (2008).
21. Casida, J.E. Pyrethrum flowers and pyrethroid insecticides. *Environ Health Perspect* **34**, 189-202 (1980).
22. Narahashi, T. Nerve membrane Na+ channels as targets of insecticides. *Trends Pharmacol Sci* **13**, 236-41 (1992).
23. Gammon, D.W., Brown, M.A. & Casida, J.E. Two classes of pyrethroid action in the cockroach. *Pestic Biochem Physiol* **15**, 181-191 (1981).
24. Khambay, B.P.S. & Jewess, P. Pyrethroids in Insect contro, Biological and synthetic agents (Academic Press, 2010).
25. Kollmeyer, W.D. et al. Discovery of the nitromethylene heterocycle insecticides. Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor (Springer-Verlag, 1999).
26. http://www.a2os.com/.
27. Goulson, D. An overview of the environmental risks posed by neonicotinoid insecticides. *J Appl Ecol* **50**, 977-987 (2013).
28. Tomizawa, M. & Casida, J.E. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annu Rev Entomol* **48**, 339-64 (2003).
29. Fisher, M.H. & H., M. Chemistry in ivermectin and abamectin (Springer, 1989).
30. Wolstenholme, A.J. & Rogers, A.T. Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. *Parasitol* **131** (2005).

31. Lynagh, T. & Lynch, J.W. Molecular mechanisms of Cys-loop ion channel receptor modulation by ivermectin. *Front Mol Neurosci* **5**, 60 (2012).
32. Putter, H. et al. Avermectins: novel insecticides, acaricides and nematicides from a soil microorganism. *Exp* **37**, 963-964 (1981).
33. Howe, G.A. & Jander, G. Plant immunity to insect herbivores. *Annu Rev Plant Biol* **59**, 41-66 (2008).
34. Tohnishi, M. et al. Flubendiamide, a novel insecticide highly active against lepidopterous insect pests. *J Pestic Sci* **30**, 354-360 (2005).
35. Lahm, G.P. et al. Rynaxypyr: a new insecticidal anthranilic diamide that acts as a potent and selective ryanodine receptor activator. *Bioorg Med Chem Lett* **17**, 6274-9 (2007).
36. Ebbinghaus-Kintscher, U. et al. Phthalic acid diamides activate ryanodine-sensitive Ca2+ release channels in insects. *Cell Calcium* **39**, 21-33 (2006).
37. Marcic, D. Acaricides in modern management of plant-feeding mites. *J Pestic Sci* (2012).
38. Takahashi, N., Nakagawa, H., Sasama, Y. & Ikemi, N. Development of a new acaricide, cyflumetofen. *J Pestic Sci* **37**, 263-264 (2012).
39. Hayashi, N., Sasama, Y., Takahashi, N. & Ikemi, N. Cyflumetofen, a novel acaricide - its mode of action and selectivity. *Pest Manag Sci* **69**, 1080-1084 (2013).
40. Tabashnik, B.E., Van Rensburg, J.B. & Carriere, Y. Field-evolved insect resistance to Bt crops: definition, theory, and data. *J Econ Entomol* **102**, 2011-25 (2009).
41. Melander, A.L. Can insects become resistant to sprays? *J Econ Entomol* **7**, 167-173 (1914).
42. Li, X., Schuler, M.A. & Berenbaum, M.R. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* **52**, 231-53 (2007).
43. Hemingway, J. & Ranson, H. Insecticide resistance in insect vectors of human disease. *Annu Rev Entomol* **45**, 371-91 (2000).
44. Van Leeuwen, T., Vontas, J., Tsagkarakou, A., Dermauw, W. & Tirry, L. Acaricide resistance mechanisms in the two-spotted spider mite Tetranychus urticae and other important Acari: a review. *Insect Biochem Mol Biol* **40**, 563-72 (2010).
45. Vontas, J., Hernandez-Crespo, P., Margaritopoulos, J.T., Ortego, F. & Feng, H.-T. Insecticide resistance in Tephritid flies. *Pestic Biochem Physiol* **100**, 199-205 (2011).
46. http://www.pesticideresistance.com.
47. Georghiou, G. Principles of insecticide resistance management. *Phytoprotection* (1994).
48. Walling, L.L. The Myriad Plant Responses to Herbivores. *J Plant Growth Regul* **19**, 195-216 (2000).
49. War, A.R. et al. Mechanisms of plant defense against insect herbivores. *Plant Signal Behav* **7**, 1306-20 (2012).
50. Furstenberg-Hagg, J., Zagrobelny, M. & Bak, S. Plant Defense against Insect Herbivores. *Int J Mol Sci* **14**, 10242-97 (2013).
51. Gatehouse, J.A. Plant resistance towards insect herbivores: a dynamic interaction. *N Phytol* **156**, 145-169 (2002).
52. Mazid, M., Khan, A. & Mohammad, F. Role of secondary metabolites in defense mechanisms of plants, Biology and Medicine. *Biol Med* **3**, 232-249 (2011).
53. Despres, L., David, J.P. & Gallet, C. The evolutionary ecology of insect resistance to plant chemicals. *Trends Ecol Evol* **22**, 298-307 (2007).
54. Sarmento, R.A., Sarmento, A. & Sarmento, A. A herbivore that manipulates plant defence. *Ecol Lett* (2001).
55. Agrawal, A. Current trends in the evolutionary ecology of plant defence. *Funct Ecol* **25**, 420-432 (2011).
56. Meyer, U.A. Overview of enzymes of drug metabolism. *J Pharmacokinet Biopharm* **24**, 449-59 (1996).
57. Rushmore, T.H. & Kong, A.N. Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes. *Curr Drug Metab* **3**, 481-90 (2002).
58. Xu, C., Li, C.Y. & Kong, A.N. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res* **28**, 249-68 (2005).
59. Jakoby, W.B. & Ziegler, D.M. The enzymes of detoxication. *J Biol Chem* **265**, 20715-8 (1990).
60. Moscow, J.A. & Dixon, K.H. Glutathione-related enzymes, glutathione and multidrug resistance. *Cytotechnology* **12**, 155-70 (1993).

61. Tew, K.D. & Ronai, Z. GST function in drug and stress response. *Drug Resist Updat* **2**, 143-147 (1999).
62. King, C.D., Rios, G.R., Green, M.D. & Tephly, T.R. UDP-glucuronosyltransferases. *Curr Drug Metab* **1**, 143-61 (2000).
63. Tukey, R.H. & Strassburg, C.P. Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annu Rev Pharmacol Toxicol* **40**, 581-616 (2000).
64. Glavinas, H., Krajcsi, P., Cserepes, J. & Sarkadi, B. The role of ABC transporters in drug resistance, metabolism and toxicity. *Curr Drug Deliv* **1**, 27-42 (2004).
65. Dermauw, W. & Van Leeuwen, T. The ABC gene family in arthropods: comparative genomics and role in insecticide transport and resistance. *Insect Biochem Mol Biol* **45**, 89-110 (2014).
66. Feyereisen, E. Insect cytochrome P450. In Comprehensive Molecular Insect Science (Elsevier, 2005).
67. Hamdane, D., Zhang, H. & Hollenberg, P. Oxygen activation by cytochrome P450 monooxygenase. *Photosynth Res* **98**, 657-66 (2008).
68. Guzov, V.M., Houston, H.L., Murataliev, M.B., Walker, F.A. & Feyereisen, R. Molecular cloning, overexpression in Escherichia coli, structural and functional characterization of house fly cytochrome b5. *J Biol Chem* **271**, 26637-45 (1996).
69. Zhang, M. & Scott, J.G. Purification and characterization of cytochrome b5 reductase from the house fly, Musca domestica. *Comp Biochem Physiol B Biochem Mol Biol* **113**, 175-83 (1996).
70. Megias, A., Saborido, A. & Muncio, A.M. NADH-cytochrome b5 reductase from the insect Ceratitis capitata. Enzyme properties and membrane binding capacity. *Comp Biochem Physiol B Biochem Mol Biol* **77**, 679-685 (1984).
71. Schuler, M.A. The role of cytochrome P450 monooxygenases in plant-insect interactions. *Plant Physiol* **112**, 1411-9 (1996).
72. Karunker, I. et al. Structural model and functional characterization of the Bemisia tabaci CYP6CM1vQ, a cytochrome P450 associated with high levels of imidacloprid resistance. *Insect Biochem Mol Biol* **39**, 697-706 (2009).
73. Stevenson, B.J., Pignatelli, P., Nikou, D. & Paine, M. Characterization of cytochrome P450s associated with pyrethroids resistance in Aedes aegypti. *PLoS Negl Trop Dis* **6** (2012).
74. Dunkov, B.C. et al. The Drosophila cytochrome P450 gene Cyp6a2: structure, localization, heterologous expression, and induction by phenobarbital. *DNA Cell Biol* **16**, 1345-56 (1997).
75. Amichot, M. et al. Point mutations associated with insecticide resistance in the Drosophila cytochrome P450 Cyp6a2 enable DDT metabolism. *Eur J Biochem* **271**, 1250-7 (2004).
76. Demaeght, P. et al. Molecular analysis of resistance to acaricidal spirocyclic tetronic acids in Tetranychus urticae: CYP392E10 metabolizes spirodiclofen, but not its corresponding enol. *Insect Biochem Mol Biol* **43**, 544-54 (2013).
77. Riga, M. et al. Abamectin is metabolized by CYP392A16, a cytochrome P450 associated with high levels of acaricide resistance in Tetranychus urticae. *Insect Biochem Mol Biol* **46**, 43-53 (2014).
78. Riga, M. et al. Functional characterization of the Tetranychus urticae CYP392A11, a cytochrome P450 that is up-regulated resistant mites, and is capable to hydroxylate the METI acaricides cyenopyrafen and fenpyroximate. *Insect Biochem Mol Biol (submitted)* (2015).
79. Fogleman, J.C. The molecular basis of adaptation in Drosophila: the role of cytochrome P450s. *Evol Biol* **30**, 15-77 (1998).
80. Harrison, T.L., Zangerl, A.R., Schuler, M.A. & Berenbaum, M.R. Developmental variation in cytochrome P450 expression in Papilio polyxenes in response to xanthotoxin, a hostplant allelochemical. *Arch Insect Biochem Physiol* **48**, 179-89 (2001).
81. Cohen, M.B., Schuler, M.A. & Berenbaum, M.R. A host-inducible cytochrome P-450 from a host-specific caterpillar: molecular cloning and evolution. *Proc Natl Acad Sci U S A* **89**, 10920-4 (1992).
82. Wen, Z., Pan, L., Berenbaum, M.R. & Schuler, M.A. Metabolism of linear and angular furanocoumarins by Papilio polyxenes CYP6B1 co-expressed with NADPH cytochrome P450 reductase. *Insect Biochem Mol Biol* **33**, 937-47 (2003).
83. Li, W., Schuler, M.A. & Berenbaum, M.R. Diversification of furanocoumarin-metabolizing cytochrome P450 monooxygenases in two papilionids: Specificity and substrate encounter rate. *Proc Natl Acad Sci U S A* **100 Suppl 2**, 14593-8 (2003).

84. Sherratt, P.I. & Hayes, J.D. GLutatione S-transferases in Enzyme Systems that Metabolize Drugs and Other Xenobiotics (John Wiley and Sons Ltd, 2001).
85. Grbic, M. et al. The genome of Tetranychus urticae reveals herbivorous pest adaptations. *Nature* **479**, 487-92 (2011).
86. Atkins, W.M., Wang, R.W., Bird, A.W., Newton, D.J. & Lu, A.Y. The catalytic mechanism of glutathione S-transferase (GST). Spectroscopic determination of the pKa of Tyr-9 in rat alpha 1-1 GST. *J Biol Chem* **268**, 19188-91 (1993).
87. Habig, W.H., Pabst, M.J. & Jakoby, W.B. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**, 7130-9 (1974).
88. Chronopoulou, E.G. & Labrou, N.E. Glutathione transferases: emerging multidisciplinary tools in red and green biotechnology. *Recent Pat Biotechnol* **3**, 211-23 (2009).
89. Sheehan, D., Meade, G., Foley, V.M. & Dowd, C.A. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem J* **360**, 1-16 (2001).
90. Danielson, U.H. & Mannervik, B. Kinetic independence of the subunits of cytosolic glutathione transferase from the rat. *Biochem J* **231**, 263-7 (1985).
91. Prapanthadara, L.A. & Ketterman, A.J. Qualitative and quantitative changes in glutathione S-transferases in the mosquito Anopheles gambiae confer DDT-resistance. *Biochem Soc Trans* **21 (Pt 3)**, 304S (1993).
92. Huang, H.S. et al. Molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the diamondback moth, Plutella xylostella. *Insect Biochem Mol Biol* **28**, 651-8 (1998).
93. Vontas, J.G., Small, G.J. & Hemingway, J. Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens. *Biochem J* **357**, 65-72 (2001).
94. Konanz, S. & Nauen, R. Purification and partial characterization of a glutathione S-transferase from the two-spotted spider mite, Tetranychus urticae. *Pestic Biochem Physiol* **79**, 49-57 (2004).
95. Ranson, H., Prapanthadara, L. & Hemingway, J. Cloning and characterization of two glutathione S-transferases from a DDT-resistant strain of Anopheles gambiae. *Biochem J* **324 (Pt 1)**, 97-102 (1997).
96. Ranson, H. et al. Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector Anopheles gambiae. *Biochem J* **359**, 295-304 (2001).
97. Wei, S.H., Clark, A.G. & Syvanen, M. Identification and cloning of a key insecticide-metabolizing glutathione S-transferase (MdGST-6A) from a hyper insecticide-resistant strain of the housefly Musca domestica. *Insect Biochem Mol Biol* **31**, 1145-53 (2001).
98. Chiang, F.M. & Sun, C.N. Glutathione transferase isozymes of diamondback moth larvae and their role in the degradation of some organophosphorus insecticides. *Pestic Biochem Physiol* **45**, 7-14 (1993).
99. Ku, C.C., Chiang, F.M., Hsin, C.Y., Yao, Y.E. & Sun, C.N. Glutathione transferase isozymes involved in insecticide resistance in diamondback moth larvae. *Pest Biochem Physiol* **50**, 191-197 (1994).
100. Kostaropoulos, I., Papadopoulos, A.I., Metaxakis, A., Boukouvala, E. & Papadopoulou-Mourkidou, E. Glutathione S-transferase in the defence against pyrethroids in insects. *Insect Biochem Mol Biol* **31**, 313-9 (2001).
101. Vontas, J.G., Small, G.J., Nikou, D.C., Ranson, H. & Hemingway, J. Purification, molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the rice brown planthopper, Nilaparvata lugens. *Biochem J* **362**, 329-37 (2002).
102. Stumpf, N. & Nauen, R. Biochemical markers linked to abamectin resistance in Tetranychus urticae (Acari: Tetranychidae). *Pest Biochem Physiol* **72**, 11-121 (2002).
103. Francis, F., Vanhaelen, N. & Haubruge, E. Glutathione S-transferases in the adaptation to plant secondary metabolites in the Myzus persicae aphid. *Arch Insect Biochem Physiol* **58**, 166-74 (2005).
104. Nitao, J.K. et al. Characterization of furanocoumarin metabolites in parsnip webworm, Depressaria pastinacella. *J Chem Ecol* **29**, 671-82 (2003).

105. Yu, S.J. Biochemical characteristics of microsomal and cytosolic glutathione S-transferases in larvae of the fall armyworm, Spodoptera frugiperda. *Pestic Biochem Physiol* **72**, 100-110 (2002).
106. Wadleigh, R.W. & Yu, S.J. Glutathione transferase activity of fall armyworm larvae toward alpha-, beta-unsaturated carbonyl allelochemicals and its induction by allelochemicals. *Insect Biochem* **17**, 759-764 (1987).
107. Yu, S.J. & Abo-Elgar, O.E. Allelochemicals as inhibitors of glutathione S-transferases in the fall armyworm. *Pestic Biochem Physiol* **68**, 173-183 (2000).
108. Montella, I.R., Schama, R. & Valle, D. The classification of esterases: an important gene family involved in insecticide resistance--a review. *Mem Inst Oswaldo Cruz* **107**, 437-49 (2012).
109. Kadous, A.A., Ghiasuddin, S.M., Matsumura, F., Scott, J.G. & tanaka, K. Difference in the picrotoxinin receptor between the cyclodiene-resistant and susceptible strains of the German cockroach. *Pest Biochem Physiol* **19**, 157-166 (1983).
110. Campbell, P.M., Newcomb, R.D., Russell, R.J. & Oakeshott, J. Two different amino acid substitutions in the aliesterase, E3, confer alternative types of organophosphorus insecticide resistance in the sheep blowfly, Lucilia cuprina. *Insect Biochem Mol Biol* **28**, 139-150 (1998).
111. Lindroth, R.L. Biochemical detoxication: mechanism of differential tiger swallowtail tolerance to phenolic glycosides. *Oecologia* **81**, 219-224 (1989).
112. Lindroth, R.L. & Weidbrod, A.V. Genetic variation in ,response of the gypsy moth to aspen phenolic glycosides. *Biochem System Ecol* **19**, 97-103 (1991).
113. Rey, D., Cuany, A., Pautou, M.-P. & Meyran, J.-C. Differential Sensitivity of Mosquito Taxa to Vegetable Tannins. *J Chem Ecol* **25**, 537-548 (1999).
114. Mackenzie, P.I. et al. The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* **7**, 255-69 (1997).
115. Miners, J.O. & Mackenzie, P.I. Drug glucuronidation in humans. *Pharmacol Ther* **51**, 347-69 (1991).
116. Ahmad, S.A. & Hopkins, T.L. β-glycosylation of plant phenolics by phenol β- glucosyltransferase in larval tissues of the tobacco hornworm, Manduca sexta (L.). *Insect Biochem Mol Biol* 581-589 (1993).
117. Bull, D.L. & Whitten, C.J. Factors influencing organophosphorus insecticide resistance in tobacco budworms. *J Agric Food Chem* **20**, 561-4 (1972).
118. Ahmad, S. & Forgash, A.J. Nonoxidative enzymes in the metabolism of insecticides. *Ann Clin Biochem* **13**, 141-64 (1976).
119. Luque, T., Okano, K. & O'Reilly, D.R. Characterization of a novel silkworm (Bombyx mori) phenol UDP-glucosyltransferase. *Eur J Biochem* **269**, 819-25 (2002).
120. in Encyclopedia of Entomology (ed. Capinera, J.L.) (Springer Nethrlands, 2008).
121. Margaritopoulos, J.T. et al. Efficacy of the pyrethroid alpha-cypermethrin against Bactrocera oleae populations from Greece, and improved diagnostic for an iAChE mutation. *Pest Manag Sci* **64**, 900-8 (2008).
122. Vontas, J., Ranson, H. & Alphey, L. Transcriptomics and disease vector control. *BMC Biol* **8**, 52 (2010).
123. Hsu, J.C. et al. Discovery of genes related to insecticide resistance in Bactrocera dorsalis by functional genomic analysis of a de novo assembled transcriptome. *PLoS One* **7**, e40950 (2012).
124. Dermauw, W. et al. A link between host plant adaptation and pesticide resistance in the polyphagous spider mite Tetranychus urticae. *Proc Natl Acad Sci U S A* **110**, E113-22 (2013).
125. Vontas, J. et al. Insecticide resistance in the major dengue vectos Aedes albopictus and Aedes aegypti *Pest Biochem Physiol* **104**, 126-131 (2012).
126. Grigoraki, L. et al. Transcriptome Profiling and Genetic Study Reveal Amplified Carboxylesterase Genes Implicated in Temephos Resistance, in the Asian Tiger Mosquito Aedes albopictus. *PLoS Negl Trop Dis* **9**, e0003771 (2015).
127. Bautista, M.A., Miyata, T., Miura, K. & Tanaka, T. RNA interference-mediated knockdown of a cytochrome P450, CYP6BG1, from the diamondback moth, Plutella xylostella, reduces larval resistance to permethrin. *Insect Biochem Mol Biol* **39**, 38-46 (2009).
128. Zhu, F. et al. 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**, 8557-62 (2010).

129. Lumjuan, N. et al. The role of the Aedes aegypti Epsilon glutathione transferases in conferring resistance to DDT and pyrethroid insecticides. *Insect Biochem Mol Biol* **41**, 203-9 (2011).
130. Price, D.R. & Gatehouse, J.A. RNAi-mediated crop protection against insects. *Trends Biotechnol* **26**, 393-400 (2008).
131. Chung, H. et al. Cis-regulatory elements in the Accord retrotransposon result in tissue-specific expression of the Drosophila melanogaster insecticide resistance gene Cyp6g1. *Genetics* **175**, 1071-7 (2007).
132. Perry, T., Batterham, P. & Daborn, P.J. The biology of insecticidal activity and resistance. *Insect Biochem Mol Biol* **41**, 411-22 (2011).

Chapter 2

Transgenic expression of the *Aedes aegypti* cytochrome P450 CYP9J28 in *Drosophila melanogaster*

This chapter was redrafted from Pavlidi N., Monastirioti M., Daborn P., Van Leeuwen T., Ranson H., Vontas J. (2012)Transgenic expression of the *Aedes aegypti* CYP9J28 confers pyrethroid resistance in *Drosophila melanogaster*, Pesticide Biochemistry and Physiology, 104: 132-135.

2.1 Introduction

2.1.1 Insecticide resistance in the mosquito *Aedes aegypti*

The mosquito *A. aegypti* (**Figure 2.1**) represents a severe threat for global health as it is the major vector of dengue and yellow fever throughout tropics and subtropics worldwide[1]. It is estimated that 390 million dengue fever infections[2] and 200,000 infections of yellow fever occur every year causing thousands of deaths, particularly in Africa[1]. Prevention of mosquito-borne diseases depends largely on insecticide based vector control interventions. A range of applications, including larvicides, insecticide residual sprays (IRS), space sprayings and treated/impregnated materials have been employed[1]. However, the emergence and spread of insecticide resistance poses a serious threat to the sustainability of control efforts. Several insecticide classes such as pyrethroids show reduced efficacy in controlling *A. aegypti*[3, 4]. Identification of the factors responsible for insecticide resistance is an important prerequisite for the subsequent development of resistance management tactics.

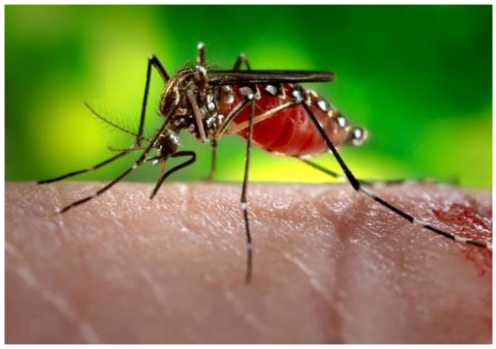


Figure 2.1. The mosquito *A. aegypti* (Photo by James Cathany[5]).

Resistance to pyrethroids, the most common class of insecticides used against mosquitoes, in *A. aegypti* has been attributed to mutations in the sodium channel gene, the target site of these insecticides and to increased rates of insecticide detoxification[4]. Pyrethroid resistance has been attributed to a number of mutations in the voltage gated sodium channel, such as the amino acid substitutions I1011M/V[6,

[7], G923V[6], L982W[6], V1016G/I[6, 7], D1794Y[8] and F1534C[9]. Also, many genes belonging to P450 superfamily have been found to be overexpressed in *A. aegypti* pyrethroid resistance strains. The majority are members of the CYP9 family, such as *CYP9J9* and *CYP9J10*, *CYP9J26*, *CYP9J27* and *CYP9J28*. Members of CY6Z subfamily, such as *CYP6Z6*, *CYP6Z8* and *CYP6Z9* have also been found overexpressed in resistant mosquitoes[4].

2.1.2 *Drosophila melanogaster* as a model organism for insecticide resistance studies

Drosophila is widely used in research due to the many advantages it has such as low dietary and requirements, allows easy observation and manipulation at most developmental stages, produces a large number of progeny, has as small life cycle and contains a small number (4) of chromosomes as well as a large number of genetic tools have been developed for this organism[10]. *Drosophila* has also been proposed as a model organism for studying insecticide resistance[11]. Although insecticides are designed to target pest species, in many cases they can also affect non-target species such as *Drosophila*. Even though *Drosophila* is not a pest the availability of genetic, genomic and molecular tools facilitate insecticide resistance studies.

The GAL4/UAS system (**Figure 2.2**) is a robust technique for gene expression in *Drosophila* and it is based in the yeast GAL4 transcriptional regulator (activator) which binds in the upstream activation sequence (UAS) of target genes causing the initiation of transcription[12]. The two components, the GAL4 and UAS, are carried in separate Drosophila lines. The GAL4 lines, also known as "enhancer-trap" lines contain the GAL4 gene in random positions in the genome under the control of genomic enhancers or promoters which may drive the expression ubiquitously or in a tissue-specific manner[12]. There are plenty of GAL4 lines available, which allow the expression of GAL4 in tissue-specific even cell-type specific pattern[12]. The second fly line, the "responder" line carries the gene of interest under the control of UAS[12]. The 2 components come together with a single fly cross and the progeny express the transgene only in tissues that GAL4 is expressed. In other words, the expression of the

gene of interest can be driven in any of the tissue/cell-type pattern by crossing the appropriate GAL4 line with flies carrying the UAS-transgene[13].

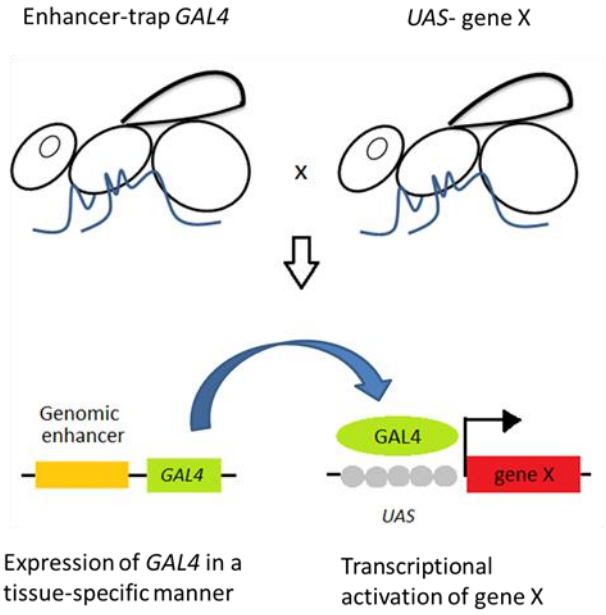


Figure 2.2. The GAL4/UAS system. The enhancer trap GAL4 line contains the GAL4 under a tissue-specific promoter/enhancer and the UAS-gene X (gene of interest) line contains the gene of interest under UAS site. In their progeny the GAL4 activates the transcription of gene X in a tissue-specific pattern (Figure adapted from Ref. 14).

Strong evidence for genes being involved in resistance can be obtained by transgenic approaches in *D. melanogaster* utilizing the GAL4/UAS system, which provides temporal and spatial control of gene expression[15]. Over-expression of the *D. melanogaster Cyp6g1* in the larval midgut, malpighian tubules and fat body has been used to validate the role of this gene in insecticide resistance[16]. In a similar way, resistance gene candidates from other species have been transgenically expressed in *D. melanogaster* and their insecticide resistance capacity has been tested. For example, over-expression of *CYP6BQ9* from *Tribolium castaneum* specifically in the *D. melanogaster* brain confers deltamethrin resistance[17]. However, the approach has not been tested for validating candidate resistance genes in mosquitoes.

2.1.3 Aim of the study

Here, we report the use of *D. melanogaster* and the GAL4/UAS system as a model for establishing the role of the *A. aegypti CYP9J28* P450, a gene which is upregulated in pyrethroid resistant mosquitoes and encodes an enzyme capable of metabolize pyrethroids *in vitro*[18, 19]. The Aaeg*CYP9J28* was selected among four P450s implicated in *A. aegypti* pyrethroid resistance in a recent study[19], as one example, in order to test the utility of the drosophila model in evaluating the role of mosquito P450s in pyrethroid resistance.

2. 2 Materials and Methods

2.2.1 Construction of transgenic *Drosophila* strains over-expressing Aaeg*CYP9J28*

A Kapa Taq DNA polymerase (Kapa Biosystems) was used to PCR amplify DNA corresponding to *CYP9J28* gene from *A. aegypti* (Isla Mujeres strain, Gene bank accession number of the cDNA clone JF924910, [13]) using the primers 5'-GGAATTCATGGAGGTTAATCTGTTCTATTTCG-3' and 5'-AAGGAAAAAAGCGGCCGCCT ACTTCTTAGGTCTAGG- 3'. PCR conditions were 95^{o}C for 2 min, followed by 30 cycles of 95^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 1 min and 40 sec. PCR product was purified using NucleoSpin Extract II kit (MACHEREY-NAGEL) and digested with EcoRI and NotI digestive enzymes. The digested PCR product was inserted into the PUAST vector[20] (**Figure 2.3**), which is a p-element transformation vector, pre-digested with the same restriction enzymes and transformed into DH5a competent cells. Plasmid DNA was prepared from the resulting clones using NucleoSpin Plasmid (MACHEREY-NAGEL) and 3 different clones were sequenced. A clone of the correct DNA sequence was transformed into the germ-line of *D. melanogaster* yellow-white strain using standard

techniques[15]. *D. melanogaster* transformation was conducted by I. Livadaras, IMBB, FORTH.

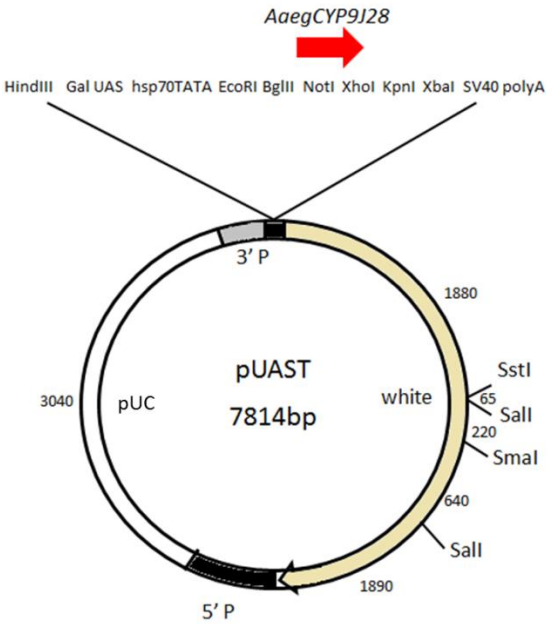


Figure 2.3. The P-elment-based transformation vector pUAST[20]. The vector contains 5 arrayed GAL4 upstream activation sequences (UAS) for GAL4 binding, followed the hsp70 TATA box and transcription start, a polylinker consisting of resitriction sites for EcoRI, BglII, NotI, XhoI, KpnI and XbaI, an SV40 small T intron and a polyadenylation site. These features are between the P element ends (3'P, 5'P). The vector also contains the ampicillin resistance genes as well as the white gene which serves as a phenotypical marker for monitoring the incorporation of the gene of interest into the *Drosophila* genome.

Two independent transformed lines (one homozygous and one stable heterozygous) where crossed with balancers of the 2nd chromosome (Cyo/Gla; yw) and the 3rd chromosome (Db/DgL3; yw) and the insertion of the construct was mapped in the 2nd chromosome for both of them. The HR-GAL4[16] was used to drive the expression of Aaeg*CYP9J28* in the midgut, malpighian tubules and fat body. The driver is constructed in the w^{1118} strain and it is homozygous for the 6g1HR-GAL4 construct inserted on the 3rd chromosome, which contains the regulatory region of Dmel*Cyp6g1* that leads the production of GAL4 in the midgut, malpighian tubules and fat body. Transgenic UAS-Aaeg*CYP9J28* virgin females were crossed to HR-GAL4 males

and the progeny assayed for deltamethrin survival. As a control, UAS-Aaeg*CYP9J28* virgin females were crossed to w^{1118} males and the progeny, which does not over-express the P450 transgene, was used as control in the toxicity bioassays (**Figure 2.4**).

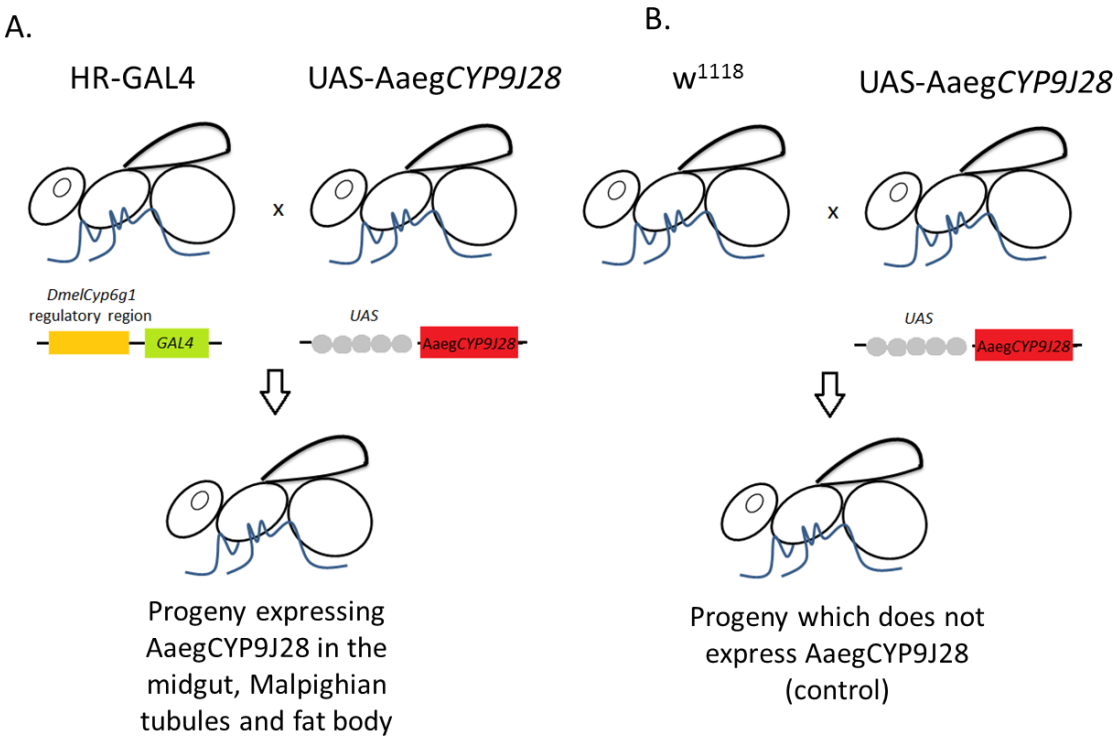


Figure 2.4. Crossing scheme for the creation of AeaegCYP9J28-expressing and control progenies. A. UAS-Aaeg*CYP9J28* x HR-GAL4 progeny express the GAL4 transcription factor under the regulatory elements of Dmel*Cyp6g1* which enables the expression in midgut, Malpighian tubules and fat body. The GAL4 binds to the UAS leading to the expression of AaegCYP9J28 transgene only in these tissues. **B.** UAS-Aaeg*CYP9J28* x w^{1118} share the same genetic background with UAS-Aaeg*CYP9J28* x HR-GAL4, however not expressing the Aaeg*CYP9J28*.

2.2.2 Confirmation of expression by RT-PCR

RT-PCR was used to confirm the successful expression of the Aaeg*CYP9J28* gene in transgenic *Drosophila*. For each cross, total RNA was extracted from 20 adults (ten males and ten females) using RNeasy mini kit (QIAGEN) and three biological

replicates were performed for each experiment. Each RNA sample was treated with Turbo DNA-free (Ambion). Reverse transcription was performed on 1 µg of RNA for each sample, in 20 µL reaction using Superscript III Reverse Transcriptase (Invitrogen Life technologies) and oligo $(-dT)_{17}$ primer. One microliter of each cDNA was used in RT-PCR, using specific primers for Aaeg*CYP9J28* gene (5'-CTCCACGTTCATTCAGACGCT-3' and 5'-CTCGAGTTCCCAAATACCTGC-3') and the reference housekeeping gene RpL11 (5'-CGATCCCTCCATCGGTATCT-3'[21] and 5'-AACCACTTCATGGCATCCTC-3'[21]. The reaction conditions were 95°C for 2 min, followed by 25 cycles of 95°C for 15 sec, 58°C for 30 sec and 72°C for 40 sec and PCR products were run on a 1% agarose gel.

2.2.3 Insecticide bioassays

A contact adult bioassay with both males and females 1–3 days post-eclosion was conducted as previously described[22]. Briefly, deltamethrin was coated on the inside of glass vials by applying 500 µl of acetone containing varying concentrations (0-2048 ppm) of deltamethrin (CHEM SERVICE) and rolling the vials until the acetone had evaporated. Vials were plugged with cotton wool soaked in 5% sucrose, and 20 flies (10 males and 10 females) were placed in each vial. After 90 min exposure, flies were transferred to acetone only treated vials and mortality was scored after 24 h. Each dose was assayed in 3 replicates and control mortality in the absence of deltamethrin was taken into account. *A. aegypti* CYP9J28 was considered to have insecticide resistance potential when the over-expression through crosses with HR-GAL4 caused increased survival compared to the control cross, for both independent transformed lines. Dosage-mortality curves (DMCs) were generated with PoloPlus Version 2.0., using a single transgene. At least 5 concentrations that caused mortality in the range of 5–95% were used.

2.3 Results and Discussion

2.3.1 Transgenic expression of the *Aedes aegypti CYP9J8* in Drosophila

Approximately 350 embryos were injected with the Aaeg*CYP9J28* in pUAST construct. Out of them, 212 larvae survived and finally seven adults were successfully transformed in the gametic line. Two transformed adults were back-crossed with w^{1118} flies and the progeny with red eyes (carrying the transgene) where then crossed with balancers of the 2nd chromosome (CyO/Gla;yw) and the 3rd chromosome (Db/Dgl3) in order to create homozygous stable lines as well as to map the insertion of the construct. One stable homozygous and one stable heterozygous lines were created and the insertion was mapped in the 2nd chromosome for both. Thus, *Drosophila* transgenic strains carrying a UAS-AaegCYP9J28 transgene were successfully generated. The UAS-AaegCYP9J28 flies were subsequently crossed to the HR-GAL4 line[16], which is known to drive the expression of the Aaeg*CYP9J28* in the midgut, Malpighian tubules and fat body of the progeny, based on previous studies[6], tissues that it is believed that detoxification takes place. The mRNA levels of Aaeg*CYP9J28* were determined in 3 replicates of 1–3 days old adults progeny by RT-PCR, confirming the expression of the transgene in the UAS-Aaeg*CYP9J28* x HR-GAL4 flies (progeny expressing the Aaeg*CYP9J28*) (**Figure 2.5**).

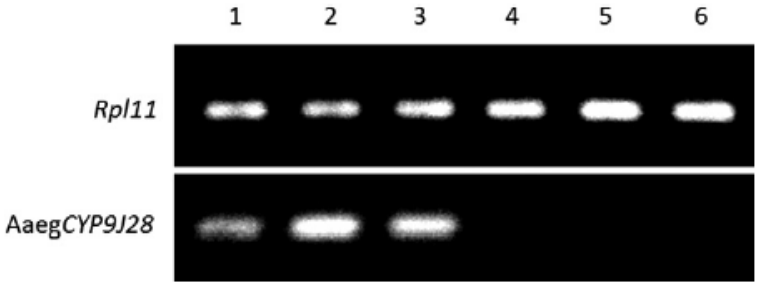


Figure 2.5. Confirmation of the expression of Aaeg*CYP9J28* in *D. melanogaster* adult's body using the GAL4/UAS system by RT-PCR. Lanes 1, 2 and 3 represent three biological replicates of UAS-Aaeg*CYP9J28* x HR-GAL4, transgenic flies expressing the Aaeg*CYP9J28*. Lanes 4,5 and 6 represent three biological replicates of UAS-Aaeg*CYP9J28* x w1118, flies with similar genetic background not expressing the AaegC*YP9J28*. The Aaeg*CYP9J28* was successfully expressed in

UAS-Aaeg*CYP9J28* x HR-GAL4 progeny but not in control flies (UAS-Aaeg*CYP9J28* x w^{1118}). Primers for the housekeeping gene *Rpl11* were also used as control.

2.3.2 Bioassay data

In order to investigate if the expression of Aaeg*CYP9J28* confers resistance to the pyrethroid insecticide deltamethrin, a contact bioassay in the presence of varying concentrations of deltamethrin was conducted. For the bioassay, adult progeny (1–3 days old) of two crosses, UAS-Aaeg*CYP9J28* x HR-GAL4 (progeny expressing the AaegCYP9J28) and UAS-Aaeg*CYP9J28* x w^{1118} (fly progeny with similar genetic background not expressing the Aaeg*CYP9J28*) were assayed. The comparison of survival at different concentrations of deltamethrin between the two types of progeny showed a significant resistant phenotype in the Aaeg*CYP9J28* overexpressing line (**Figure 2.6**), and provided strong in vivo evidence for the role of *A. aegypti CYP9J28* in conferring deltamethrin resistance. Dosage-mortality analyses determined the UAS-Aaeg*CYP9J28* x HR-GAL4 LD50 at 260 mg/L of deltamethrin (95% confidence limits: 150–531 mg/ L), while the LD50 of the UAS-Aaeg*CYP9J28* x w^{1118} was determined at 38 mg/L (95% confidence limits: 30–49 mg/L). The resistance ratio (RR) of Aaeg*CYP9J28* overexpressing progeny compared to the control line was approximately 7-fold.

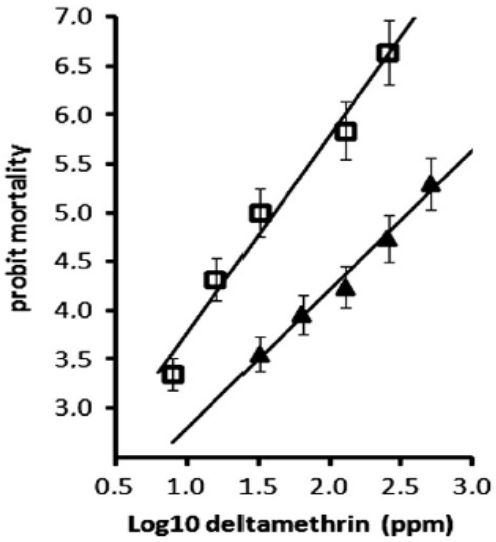


Figure 2.6. Dosage – mortality responses of transgenic Drosophila melanogaster adults expressing the Aedes aegypti CYP9J28 using the GAL4/UAS system. Black triangle: UAS-Aaeg*CYP9J28* x HR-GAL4, transgenic flies expressing the Aaeg*CYP9J28*; Empty square: UAS-Aaeg*CYP9J28* x w^{1118}, flies with similar genetic background not expressing the Aaeg*CYP9J28*. Values are means of three replicates ± S.D. A significant resistant phenotype in the Aaeg*CYP9J28* overexpressing line was observed.

The RR is higher than that previously reported for transgenic lines over-expressing *D. melanogaster* cytochrome P450s, *Cyp6g1, Cyp6g2* and *Cyp12d1* which confer resistance to DDT, nitenpyram, dicyclanil and diazinon[21]. The expression of *Cyp6g1* resulted in a RR of 4.06 folds for DDT, 1.96 folds for nitenpyram and 2.23 folds for dicyclanil. The expression of *Cyp6g2* resulted in a RR of 4.23 folds for nitenpyrad and 1.68 for diazinon while in the case of *Cyp12d1* a RR of 5.96 folds for DDT and 1.48 for dicyclanil were observed[21]. However, the RR of Aaeg*CYP9J28* overexpressing flies was still generally lower than that observed in *A. aegypti* mosquitoes which range from 30 to >1000 folds[4]. Reconstituted enzyme complex/system that may not be optimum, might explain this discrepancy.

2.4. Conclusions

We successfully expressed the *A. aegypti* cytochrome P450 *CYP9J28*, a gene which is upregulated in resistant adults of this major dengue vector and encodes an enzyme capable of metabolizing pyrethroids[18, 19] in *D. melanogaster* and showed that it confers significant levels of pyrethroid resistance. The Aaeg*CYP9J28* was selected among four P450s implicated in *A. aegypti* pyrethroid resistance in a recent study[19], as one example, in order to test the utility of the *Drosophila* model in evaluating the role of mosquito P450s in resistance. Our study provides functional *in vivo* evidence for the role of *CYP9J28* in deltamethrin resistance in *A. aegypti*. It confirms that ectopic expression in *D. melanogaster* can be a robust approach for validating candidate resistance genes[15] and extends its application to mosquito species for the first time.

2.5 References

1. http://www.who.int/en/.
2. Bhatt, S. et al. The global distribution and burden of dengue. *Nature* **496**, 504-7 (2013).
3. Ranson, H., Buhrani, J., Lumjuan, N. & Black, W.C. Insecticide resistance in dengue vectors. *TropIKA.net J.* **1** (2010).
4. Vontas, J. et al. Insecticide resistance in the major dengue vector Aedes albopictus and Aedes aegypti. *Pestic. Biochem. Physiol.* (2012).
5. https://www.vectorbase.org/imagegallery/Aedes%20Species/A.%20aegypti.
6. Brengues, C. et al. Pyrethroid and DDT cross-resistance in Aedes aegypti is correlated with novel mutations in the voltage-gated sodium channel gene. *Med Vet Entomol* **17**, 87-94 (2003).
7. Saavedra-Rodriguez, K. et al. A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American Aedes aegypti. *Insect Mol Biol* **16**, 785-98 (2007).
8. Chang, C. et al. A novel amino acid substitution in a voltage-gated sodium channel is associated with knockdown resistance to permethrin in Aedes aegypti. *Insect Biochem Mol Biol* **39**, 272-8 (2009).
9. Yanola, J. et al. High-throughput assays for detection of the F1534C mutation in the voltage-gated sodium channel gene in permethrin-resistant Aedes aegypti and the distribution of this mutation throughout Thailand. *Trop Med Int Health* **16**, 501-509 (2011).
10. Sullivan, W., XXXXXXXX, x. & XXXXXXXX, x. Drosophila protocols (NY, Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press, 2000).
11. Wilson, T.G. Drosophila melanogaster (Diptera: Drosophilidae): a model insect for insecticide resistance studies. *J Econ Entomol* **81**, 22-7 (1988).
12. Duffy, J.B. GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *Genesis* **34**, 1-15 (2002).
13. Johnston, D. The art and design of genetic screens: Drosophila melanogaster. *Nat Rev Genet* **3**, 176-88 (2002).

14. Karunker, I. et al. Structural model and functional characterization of the Bemisia tabaci CYP6CM1vQ, a cytochrome P450 associated with high levels of imidacloprid resistance. *Insect Biochem Mol Biol* **39**, 697-706 (2009).
15. Perry, T., Batterham, P. & Daborn, P.J. The biology of insecticidal activity and resistance. *Insect Biochem Mol Biol* **41**, 411-22 (2011).
16. Chung, H. et al. Cis-regulatory elements in the Accord retrotransposon result in tissue-specific expression of the Drosophila melanogaster insecticide resistance gene Cyp6g1. *Genetics* **175**, 1071-7 (2007).
17. Zhu, F. et al. 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 (2010).
18. Bariami, V., Jones, C.M., Poupardin, R., Vontas, J. & Ranson, H. Gene amplification, ABC transporters and cytochrome P450s: unraveling the molecular basis of pyrethroid resistance in the dengue vector, Aedes aegypti. *PLoS Negl Trop Dis* **6**, e1692 (2012).
19. Stevenson, B.J., Pignatelli, P., Nikou, D. & Paine, M.J. Pinpointing P450s associated with pyrethroid metabolism in the dengue vector, Aedes aegypti: developing new tools to combat insecticide resistance. *PLoS Negl Trop Dis* **6**, e1595 (2012).
20. Brand, A.H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-15 (1993).
21. Daborn, P.J. et al. Evaluating the insecticide resistance potential of eight Drosophila melanogaster cytochrome P450 genes by transgenic over-expression. *Insect Biochem Mol Biol* **37**, 512-9 (2007).
22. Daborn, P., Boundy, S., Yen, J., Pittendrigh, B. & ffrench-Constant, R. DDT resistance in Drosophila correlates with Cyp6g1 over-expression and confers cross-resistance to the neonicotinoid imidacloprid. *Mol Genet Genomics* **266**, 556-63 (2001).

Chapter 3

Understanding the molecular basis of insecticide resistance in *Bactrocera oleae* and its adaptation to olives, with emphasis on the role of detoxification

A part of this chapter was redrafted from: Pavlidi N., Dermauw W, Rombauts S, Chrisargiris A, Van Leeuwen and John Vontas (2013) Analysis of the olive fruit fly *Bactrocera oleae* transcripto me and phylogenetic classification of the major detoxification gene families. PLoS One 8(6): e66533. doi:10.1371/journal.pone.0066533.

The qPCR analysis of contig00436 was performed by Vasileios Tseliou (M. Sc. thesis).

3.1. Introduction

3.1.1. The olive fruit fly, *Bactrocera oleae*

The olive fruit fly, *B. oleae* (Diptera: Tephritidae) (**Figure 3.1**), is the most destructive pest of olive orchards worldwide. At the larval stage, *B. oleae* is strictly monophagous on olives of the genus *Olea*, such as *Olea europaea* *Olea verrucossa* and *Olea chrysophylla*[1]. The olive fruit fly infestation has been recorded in regions around the Mediterranean basin from ancient times[2, 3]. In our days, infestation has also been recorded in Central and South Africa, Near and Middle East, Central America and California[4]. The female fly lays its eggs in ripening olive fruits. Each female can lay around 250 eggs and usually do not deposit more than one egg per drupe with the exception of cases of overpopulation[5]. The female punctures the fruit with the ovipositor and deposits the egg inside the olive mesocarp and the growing larva feeds upon mesocarp until pupation[5].

Olive fruit fly infestation significantly reduces olive production and therefore, causes extensive economical loses. During feeding, the larva forms tunnels inside the drupe destroying the pulp and this activity causes the rejection of entire harvests of table olives which become unsuitable for consumption as well as significantly reduces the overall oil production[4]. Moreover, larval feeding enables the secondary infestation of the fruit by bacteria and fungi, such as bacteria of the genus Xanthomonas, Candida yeasts, Fusarium molds etc resulting in inceased oil acidity, severely affecting the quality and value of the olive oil[4]. It has been estimated that *B. oleae* infestation results in the destruction of the 5% of the total olive production and 800 million dollars per year get lost[4]. In Greece, 30–35% economic losses due to *B. oleae* have been recorded, and the annual cost for its control exceeds 2 million euros.

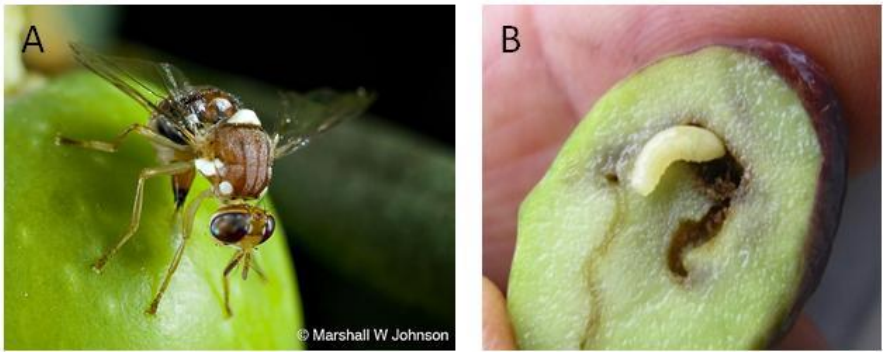


Figure 3.1 The olive fruit fly, Bactrocera oleae. A. A female olive fruit fly laying its egg in the olive fruit. (Image by **Marshall W. Johnson[6]). B.** An olive fruit fly larva feeding upon mesocarp forming tunnels (Image by Ref. 7).

3.1.2 Control of *B. oleae*

Chemical insecticides represent the major control method. For more than 40 years the control of *B. oleae* has been relied in the use of organophosphate insecticides (OPs) and particularly dimethoate and fenthion, but the intense use of these insecticides, as baits or cover sprays, has resulted in the selection of resistance which negatively impacts on the ability to control *B. oleae*[8]. Modified acetylcholinesterase (MACE) resistance mechanism has been studied extensively[8], and specific mutations in the *B. oleae Ache* gene (the target of OPs) which reduce sensitivity to organophosphate insecticides have been identified and characterized[9, 10].

As an alternative chemical control tool, pyrethroid insecticides (such as α-cypermethrin, deltamethrin, cyfluthrin) are also used in many countries[11, 12]. However resistance to pyrethroids has already been reported[13].

In 2008, the resistance levels of 20 *B. oleae* populations from different regions in Greece to the pyrethroid insecticide α-cypermethrin were monitored, showing that moderate resistance had already been selected in some regions[13]. The resistance mechanisms were studied and no target-site mutations were identified but the phenotype was associated with elevated P450 activity[13]. Recently, a new method for the estimation of insecticide effectiveness was developed and many field populations

collected from Crete (Greece), a region with extended cultivation of olives, were tested for resistance to α-cypermethrin[14]. Both the lethal concentration needed to kill off half of the sample (LC50) and the percentage mortality in the recommended label rate (RLR), were calculated and was revealed that the field *B. oleae* populations from Crete have evolved resistance to α-cypermethrin ranging from 66- 530- folds compared to the susceptible strain[14]. The understanding of molecular mechanisms by which *B. oleae* develops resistance to α-cypermethrin is crucial for the sustainability of efficient control. However the analysis of molecular mechanisms and the identification of specific P450 (and/or other) genes implicated in *B. oleae* pyrethroid resistance has been hampered by the lack of genomic information and molecular tool for this insect species.

3.1.3 Adaptation of *B. oleae* to olives

The olive tree, *Olea europaea*, is one of the most important trees cultivated in the Mediterranean basin[15]. In our days, it is extensively cultivated in all over the world, with a harvested area of more than 10 million hectares and a more than 20 million tons of olive production per year (based on FAOSTAT[16]). *B. oleae* (at the larval stage) is strictly monophagous and represents the only insect that feeds upon olives. In contrast to other Tephritidae species it feeds on solid flesh instead of consuming hydrolyzed compound of decaying substrates, meaning that it has the unique ability to hydrolyze olive proteins and utilize the nutrients of the olive flesh as well as to overcome the presence of very high levels of phenolic compounds in the olive fruits, particularly the immature - green olives. Phenolic compounds are produced and accumulated during development[17] and are present in high amounts, reaching up to 14% of the dry fruit weight[17-19]. The phenolic composition is complex and varies depending on the olive variety, season, geographical region, cultivation practices and the olive maturation stage[20]. During maturation, the content in phenolic compounds is reduced and as olive maturation reaches the black stage the content gets down to

half[21]. Oleuropein is the most abundant phenolic compound in the olive fruit, representing the major constituent of unripe- green olives[22, 23].

The molecular mechanism by which *B. oleae* overcomes these high levels of phenolic compounds and utilizes the olive flesh has not been fully elucidated as yet. It has been associated with symbiotic bacteria which mediate the development in green olives but are not essential for the development in black olives[24-26]. The precise role of symbiosis has not been elucidated as yet but it has been proposed that symbionts may be involved in the enzymatic hydrolysis of dietary proteins, the degradation of defense molecules, overcome the negative effects of oleuropein and the synthesis of amino acids[24, 27-29].

The molecular interactions between *B. oleae* and olives have been recently studied from the plant perspective[30]. Transcriptomic and proteomic approaches were used for the analysis of the molecular response of olive fruits attacked by *B. oleae*. It was shown the upregulation of genes involved in the production of Reactive Oxygen Species (ROS), activation of stress response pathways as well as the production of compounds implicated in direct defense. Among the latest, trypsin inhibitors (Trypsin protease inhibitor II, Trypsin/Chymotrypsin inhibitor) were identified. In general, plants have evolved protease inhibitors (PIs) against insect proteases as a defense mechanism. To overcome the effects of inhibitors, insects may use of PI-insensitive proteases and gene expression of PI-insensitive proteases seem to be upregulated[31].

3.1.4 Aim of the study

The aim of this study was to 1. to generate transcriptomic recourses and molecular tools for *B. oleae*, a species which lacked behind remarkably in regard to the availability of genomic resources for molecular research and 2. to use these resources and tools to analyze pyrethroid resistance and gene expression levels of olive fly larvae upon development in olive flesh.

3.2. Analysis of the olive fruit fly *Bactrocera oleae* transcriptome

Until study was conducted (April 2013), only 802 *B. oleae* nucleotide and 876 protein sequences had been deposited in the NCBI database[32], with less than 10 detoxification gene homologues in total; a data set clearly not sufficient for the investigation of metabolism-based insecticide resistance mechanisms, or the study of molecular interactions between the olive and the fruit fly *B. oleae*. Therefore, we used the 454-pyrosequencing technology to characterize the *B. oleae* (pooled stages) transcriptome and to identify and phylogenetically classify a large number of genes potentially encoding detoxification enzymes. Based on the obtained sequences we designed and constructed a microarray chip.

3.2.1 Materials and methods

3.2.1.1 Insects

In order to obtain a large and broad transcriptome data set, RNA was extracted from a pool of different life stages of *B. oleae*, including mixed lab strains[9] and field caught insects (collected from Herakleion, Crete in 2011–2012) fed on artificial diet and olives (equal representation in each stage), with a proportion: 10 eggs; 4 instar larva, 3 pupae; 4 adults (two males and two females, 4 and 20 day old). This pooled sample was snap frozen in liquid nitrogen until sent to sequencing service (LGC genomics, Berlin, Germany).

3.2.1.2 mRNA isolation, cDNA library preparation, sequencing and assembly

The mRNA isolation, cDNA library preparation, sequencing and assembly were performed by LCG Genomics, Berlin (Germany).

"Total mRNA was isolated using an mRNA-Only Eukaryotic mRNA isolation kit (Epicente, USA). cDNA synthesis and amplification was performed using Mint-Universal cDNA Synthesis kit (Evrogen, Russia) and 1 mg of *B. oleae* mRNA. About 800 ng of amplified cDNA were used as starting material in the normalization reaction using the Trimmer kit (Evrogen, Russia). Normalized material was re-amplified for 18 cycles and subsequently digested with 10 Units SfiI for 2 hours at 48^{o}C. Fragments larger than 800 bp were isolated from a Low Melting Point agarose gel and purified using the MinElute Gel Extraction kit (Qiagen, Germany). Purified cDNA fragments (200 ng) were ligated into 100 ng of a dephoshorylated pDNR-lib Vector, pre-digested with SfiI (Clontech, USA) using the Fast Ligation kit (New England Biolabs, USA). Ligations were desalted by ethanol precipitation and re-dissolved in 10 µl water. Threefold desalted ligation was used to transform NEB10b competent cells (New England Biolabs, USA).

Roughly a million clones were plated on LB-Cm plates, scrapped off the plates and stored as glycerol stocks at -70^{o}C. One half of the cells were used to inoculate a 300 ml Terrific Broth/Cm culture, which was grown for 5 hours at 30^{o}C. Plasmid DNA was prepared using standard methods (Qiagen, Germany). Purified plasmid DNA (200 µg) was digested with 100 Units SfiI for 2 hours at 48^{o}C. cDNA inserts were gel-purified (LMPAgarose/ MinElute Extraction kit) and ligated to high-molecular weight DNA using a proprietary SfiI-linker.

Library generation for the 454 FLX sequencing was carried out according to the manufacturer's standard protocols (Roche/ 454 life sciences, USA). In short, the concatenated inserts were sheared randomly by nebulization to fragments ranging in size from 400 to 900 bp. These fragments were end polished and the 454 A and B adaptors that are required for the emulsion PCR and sequencing were added to the ends of the fragments by ligation. The resulting fragment library was sequenced on a

picotiterplate (PTP) on the GS FLX using the Roche/454 Titanium chemistry" (materials and methods provided by LGC genomics, Germany).

3.2.1.3 Degenerate PCR

A degenerate PCR strategy for insect P450s [31] had been initiated prior to the transcriptomic study, to amplify orthologous regions from *B. oleae*. cDNA synthesis was carried out using the SuperScript III protocol and the PCR products were isolated, sub-cloned into pGEMT-easy vector (Promega) and sequenced in both directions. Obtained *B. oleae* P450 partial sequences were deposited in the NCBI-database (GenBank accession numbers: KC917331, KC917335, KC917340, KC917344, KC917345).

3.2.1.4 Blast homology searches and sequence annotation

For blast homology searches and sequence annotation Blast2GO software v.2.6.0 [32] was used, as previously described for the analysis of *Manduca sexta* and *Trialeurodes vaporariorum* transcriptomes[33, 34]. For homology searches, all *B. oleae* contigs larger than 200 bp were searched using blastx against the NCBI non-redundant (nr) protein database, using an e-value cut-off of $1E^{23}$. The sequences that did not give any blastx hit were subsequently searched using blastn against the NCBI nr nucleotide database using an e-value cut-off of $1E^{-10}$.

For gene ontology (GO) mapping, Blast2GO recovers all the GO terms associated to the hits obtained by the blast search. After the mapping step, results were subjected to GO annotation, a process of selecting GO terms from the GO pool and assigning them to the query sequences. The sequences were further annotated using InterPro. The functionality of InterPro annotation in Blast2GO allows retrieving domain/motif information in a sequence-wide manner. GO terms corresponding to these Interpro domains, were then transferred to the sequences and merged with already existent GO terms. GO terms were modulated using the annotation

augmentation tool ANNEX[35], followed by GOSlim. As described in the Blast2GO tutorial ''the ANNEX approach uses uni-vocal relationships between GO terms from different GO categories to add implicit annotation and GOSlim is a reduced version of the GO that contains a selected number of relevant nodes''. In this study, the generic GOSlim mapping term was used. Finally, Enzyme classification (EC) codes and the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway annotations were obtained through the direct mapping of GO terms to the corresponding enzyme codes.

3.2.1.5 Analysis of genes related to xenobiotic detoxification

B. oleae contigs (>200 bp) encoding P450s, GSTs, CCEs and ABC transporters were identified using tblastn (E-value cutoff $<1E^{-3}$) and *D. melanogaster* and *Bactrocera dorsalis* protein sequences of P450S, GSTs, CCEs and ABCs as query (*D. melanogaster* sequences were downloaded from GenBank and *B. dorsalis* sequences were taken from Ref. 36. Each contig encoding a detoxification gene was manually curated for frame shifts and sequencing errors. Contigs were translated and trimmed using BioEdit version 7.1.9 and searched by blastx (E-value, $1E^{23}$) against all the assembled contigs. Results with more than 99% similarity were considered as allelic variants. Muscle 3.8.31[37] was used to perform multiple sequence alignment of *B. oleae* P450, GST, CCE or ABC protein sequences with a representative dataset of their counterparts in other species[36, 38, 39].

For each detoxification family, only protein sequences showing no misalignment were used in the final alignment for phylogenetic analysis. Since N- and C-termini of CCEs are variable, all CCE protein sequences were trimmed as previously described in Ref. 37. Only the nucleotide binding domains (NBDs) of the ABC transporters were used for phylogenetic analysis. NBDs were extracted using the ScanProsite facility[40] in combination with the PROSITE profile PS5089. When an ABC protein sequence contained 2 NBDs, the N- terminus NBD was selected for further analysis.

Model selection was performed with ProtTest 2.4[41] and the optimum model for phylogenetic analysis was selected according to Akaike information criterion (P450s: LG+G+F, GSTs: LG+I+G, CCEs: LG+I+G, ABC B: LG+I+G, ABC D: LG+G, ABC EF: LG+I+G). A maximum likelihood analysis was performed using Treefinder (version of March 2011[42]) and a bootstrap analysis with 1000 pseudoreplicates (LR-ELW) was performed to evaluate the branch strength of each tree. The resulting tree was midpoint rooted and edited with MEGA 5.0 software[43].

3.2.1.6 Microarray design

A custom Superprint G3 genome-wide G3 Gene Expression 8 x 60L microarray ws designed using Agilent eArray online platform (Agilent Technologies[44]) based on the *B. oleae* sequences obtained by 454 pyrosequencing of the transcriptome. Three alternate probes of 60 nucleotides per gene were designed with a Tm of 81^{o}C and parameters set to “best probe design” and “no 3’ bias”. Thirty one *B. oleae* sequences (annotated as actins, ribosomal proteins etc.) were used as internal controls as well as standard Agilent features such as spike-ins were added. The slide layout contains eight arrays allowing the accomplishment of two comparisons in four replicates each. The array design ID is 045129.

Microarray chip was designed in collaboration with Laboratory of Agrozoology, Department of Crop protection, Ghent University, Belgium (Dr. Wannes Dermauw and Prof. Thomas Van Leeuwen).

3.2.2 Results and discussion

3.2.2.1 454 FLX titanium sequencing and assembly

A library of pooled life stages of *B. oleae* were sequenced, using 454 pyrosequencing, in a single run on a picotiterplate (PTP). This resulted in 482,790

aligned reads with an average read length of 421 nucleotides and a total of 147,882,767 bases (**Table 3.1**). These reads were assembled into 14,204 contigs. More than 60% of the contigs (8,630 contigs) were larger than 500 base pairs (bp), with a total of 8,675,718 bases. The average contig size was 1,005 bp and largest contig size was 6,318 bp. The remaining contigs (5,574, 39.25%) ranged between 100–500 bp with a total of 10,240,327 bases. 126,383 reads could not be assembled and were classified as singletons while 363,905 and 11,980 reads were categorized as repeats and outliers, respectively. Compared to the previously reported *B. oleae* transcriptome dataset, consisting of 195 ESTs only and derived by single pass sequencing of a *B. oleae* adult cDNA library[45], our 454 pyrosequencing represents a substantial expansion to the genomic resources available for this species.

Table 3.1. Summary of run statistics and assembly.

Run statistics	
Total number of reads	1,012,155
Number of aligned reads	482,790
Average aligned read length	421 nucleotides
Total number of aligned bases	147,882,767
454-Newbler Assembly	
Number of full assembled reads	414,125
Number of partial assembled reads	68,529
Number of Singletons	126,383
Number of Repeats	363,905
Number of Outliers	11,980
Number of Too short reads	27,233
Large contigs (>500bp)	
Number of contigs	8,630
Number of bases	8,675,718
Average contig size	1,005
N50* Contig Size	1,087
Largest contig size	6,318
All contigs (>100bp)	
Number of contigs	14,204
Number of bases	10,240,327

*size above which 50% of the assembled sequences can be found.

3.2.2.2 Homology searches

A total of 8,129 sequences of all contigs (65.36%) from the *B. oleae* transcriptome returned an above cut-off blast hit to the NCBI non-redundant protein database. When an e-value cutoff of $1E^{-3}$ was used for blastx, 7,368 (59.24%) blast results were obtained, while when the e-value cut-off $1E^{-10}$ was used for blastn, 760 (6.12%) additional blast results were obtained. Blast statistics are presented in **Figure 3.3**. 83.88% (6,819 sequences) of the top blast hits correspond to Diptera, 5.75% (468 sequences) to other Arthropoda (except Diptera), 7.56% (615 sequences) to Fungi, 0.42% (34 sequences) to Bacteria and 2.37% (193 sequences) to other organisms (**Figure 3.3 A**). From the *B. oleae* contigs having their best blast hit with Fungi, 317 encoded for ribosomal RNA, 296 for hypothetical proteins, one for a transposase (contig07917) and one for a mitochondrial protein (contig03600). These 615 ''fungi'' contigs were considered as contamination and excluded from further analysis.

Analyzing the blast statistics more into detail revealed that the majority of *B. oleae* sequences returned its best blast hit for *Drosophila* species (5,299 sequences, 65.18%). Out of these species, *Drosophila virilis* returned the majority of blast hits (751 sequences, 9.23%) followed by *Drosophila willistoni* (696 sequences, 8.56%) and *Drosophila mojavensis* (672 sequences, 8.26%) (**Figure 3.3.B**). Among other Diptera, 695 (8.54%) sequences had their best hit for *Glossina morsitans*, 94 (1.15%) for *Aedes aegypti* and 55 (0.67%) for *Culex quinquefasciatus*. The distribution is in accordance with that obtained by transcriptome analysis of the closely related Tephritidae species, *B. dorsalis*[36], where approximately 80% of the genes were most closely related to Drosophila homologues. Notably, only 65 (0.79%) sequences had their best match with *B. oleae* sequences subjected to NCBI, reflecting the lack of genetic information for this insect species.

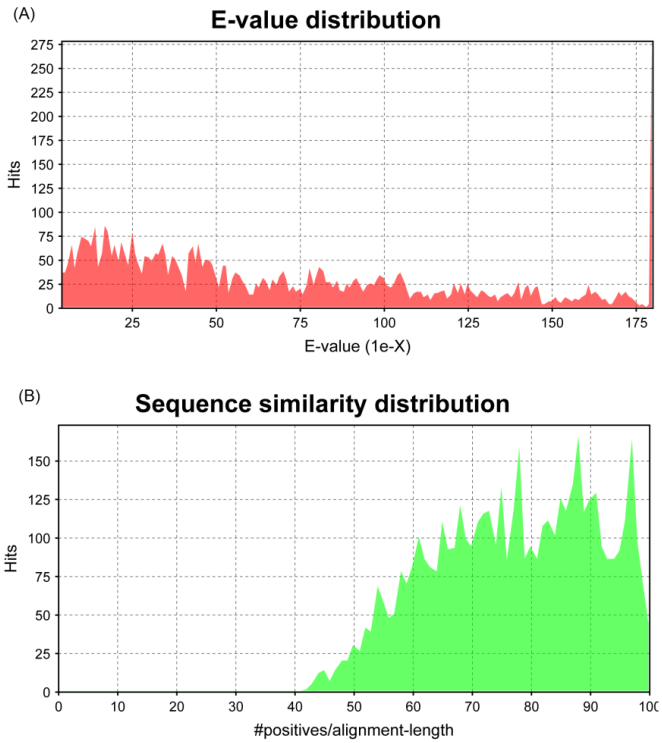
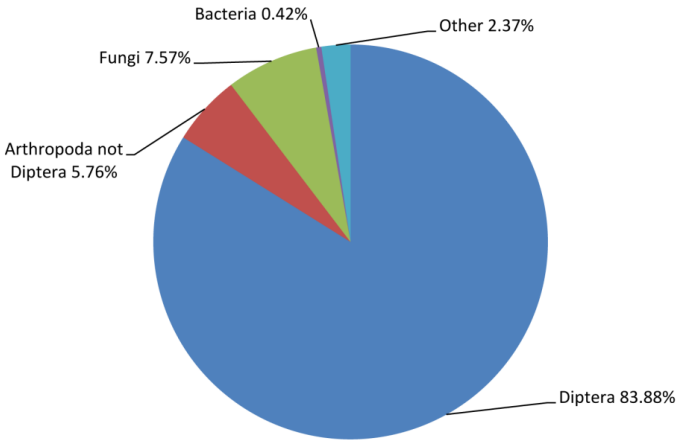


Figure 3.2. Blast statistics. A: E-value and **B:** percentage similarity distribution.

(A)



(B)

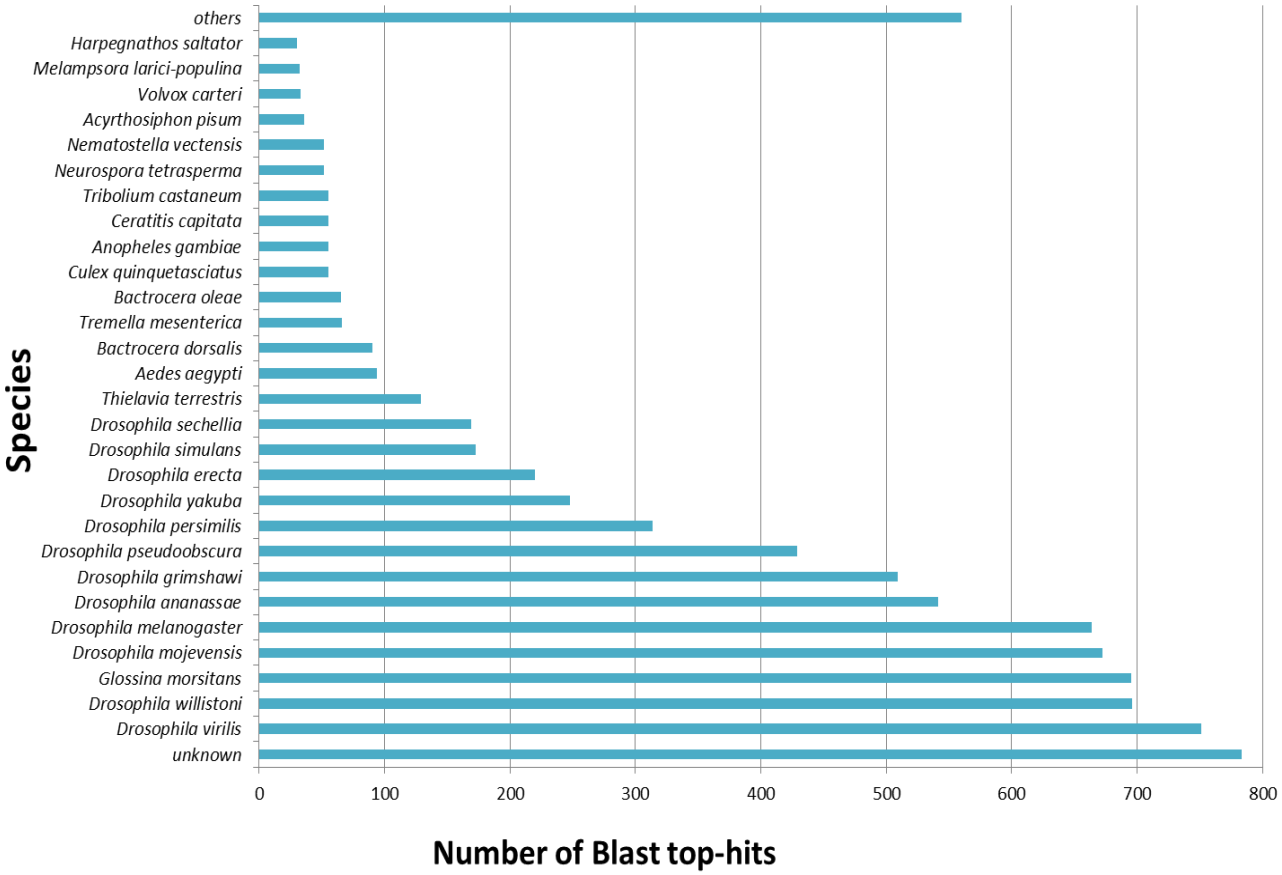


Figure 3.3. Analysis of the distribution of the 8,129 top blast hits obtained by blast against the nr database (NCBI). A. Percentage distribution within different taxonomic groups. **B.** Top hit species distribution.

3.2.2.3 Gene ontology (GO) analysis

Gene Ontology (GO) terms were used for the functional categorization of the 5,426 predicted *B. oleae* proteins (38.2% of the total number of contigs). In most cases more than one term was mapped to the same predicted protein. 10,098 terms for biological process categories, 3,662 for molecular function categories and 4,234 for cellular component categories were emerged. The sequences were categorized to 12 molecular function, 15 biological process and 7 cellular component categories in GO level 2 (general function categories) (**Figure 3.4**).

The majority of the molecular function GO terms were involved in binding (3,133 sequences, 44%), followed by catalytic activity (2,592 sequences, 36.76%), transporter activity (357 sequences, 5.06%), structural molecule activity (357 sequences, 4.96%), enzyme regulator activity (194 sequences, 2.75%), receptor activity (111 sequences, 1.57%), electron carrier activity (105 sequences, 1.49%), nucleic acid binding transcription factor activity (102 sequences, 1.45%), molecular transducer activity (67 sequences, 0.95%), antioxidant activity (23 sequences, 0.33%), translation regulation (14 sequences, 0.20%) and protein tag (1 sequence, 0.01%) (**Figure 3.4 A**).

Most of the biological process GO terms were involved in metabolic process (3,064 sequences, 18.84%), followed by cellular process (2,917 sequences, 17.94%), developmental process (1,626 sequences, 10.00%), biological regulation (1,601 sequences, 9.85%), multicellular process (1,482 sequences, 9.11%), cellular component organization or biogenesis (1,319 sequences, 8.11%), response to stimulus (1,221 sequences, 7.51%), localization (938 sequences, 5.77%), signaling (909 sequences, 5.59%), reproduction (551 sequences, 3.39%), death (230 sequences, 1.41%), growth (199 sequences, 1.22%), cell proliferation (172 sequences, 1.06%), viral reproduction (21 sequences, 0.13%) and multi-organism process (11 sequences, 0.07%) (**Figure 3.4 B**).

Cellular component GO terms are distributed in cell (3,333 sequences, 40.09%), organelle (2,254 sequences, 27.11%), macromolecular complex (1,502 sequences, 18.07%), membrane-enclosed lumen (491 sequences, 5.91%), membrane (384 sequences, 4.62%), extracellular region (317 sequences, 3.81%) and extracellular matrix (33 sequences, 0.40%) (**Figure 3.4 C**).

The GO classification results are in line with the recently sequenced transcriptomes of *B. dorsalis*[36, 38, 39], *T. vaporariorum*[33] and *Musca domestica*[46] where binding, cell and metabolic processes were the three largest groups, suggesting that 454 pyrosequencing technology provided a comprehensive representation of the *B. oleae* transcriptome. Although another Tephritid species, the Mediterranean fruit fly *Ceratitis capitata*, may also be related to *B. oleae*, a comparison with that species was not possible at the time, as the only published genomic dataset for *C. capitata* contains

a small number of ESTs derived from adult head and embryos[47], which is of limited use for annotation studies, such as the one conducted here.

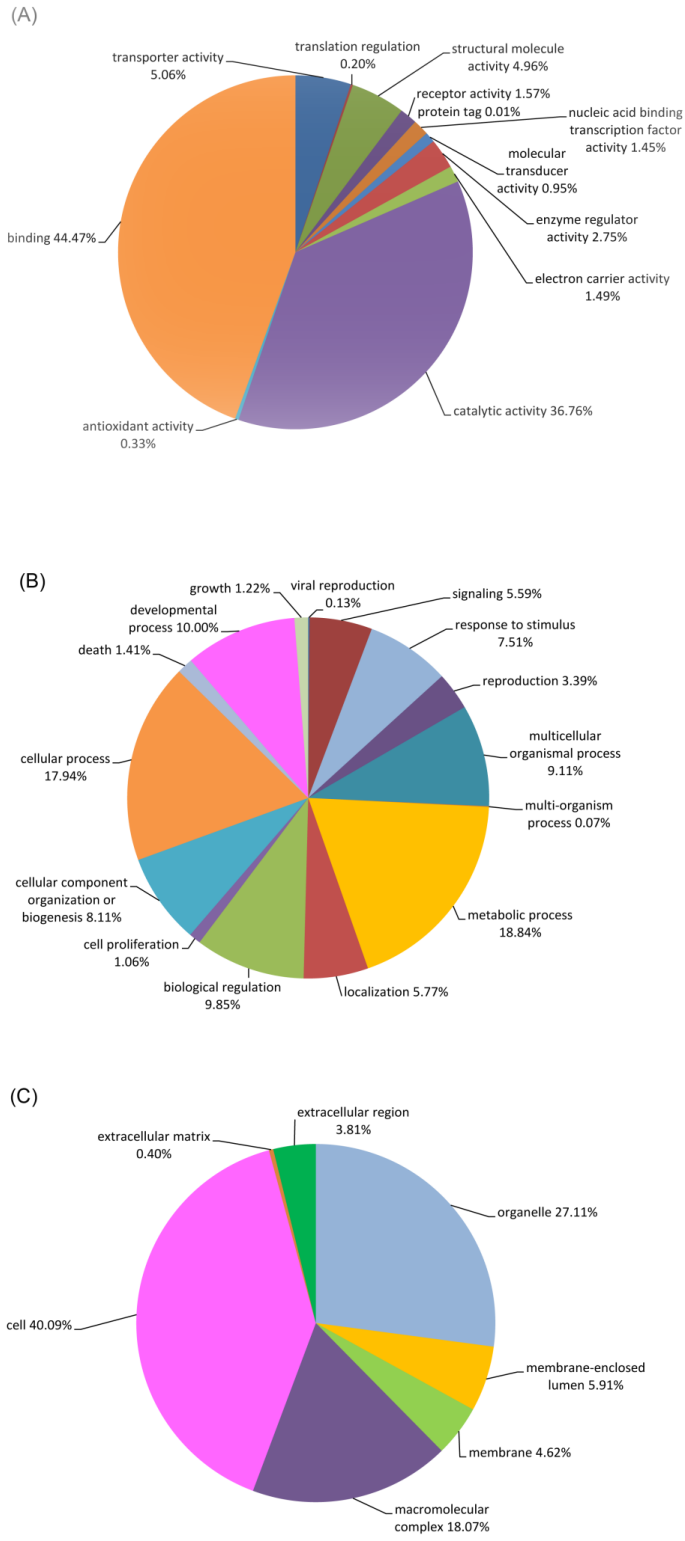


Figure 3.4. GO terms (level 2) distribution of *B. oleae* transcriptome. A. Molecular function, **B.** biological process, **C.** cellular component.

3.2.2.4 Enzyme classification and KEGG pathway analysis

After initial bioinformatic analysis of gene functions the potential enzymes were further characterized based on the chemical reaction they catalyze. This was achieved using the predictions of Enzyme Commission (EC) numbers for each sequence. Enzyme classification revealed that hydrolases are the largest group of *B. oleae* enzymes (38%, 644 enzymes), followed by transferases (30%, 519 enzymes), oxidoreductases (17%, 286 enzymes), ligases (7%, 129 enzymes), isomerases (4%, 64 enzymes) and lyases (4%, 61 enzymes). (**Figure 3.5 A**). The distribution/ relative proportion of each enzyme category is similar to that determined in the white fly *T. vaporariorum*[33] and *B. dorsalis*[39] transciptomes, with the possible exception of transferases and hydrolases, which were slightly over- and under- represented respectively (**Figure 3.5 B**).

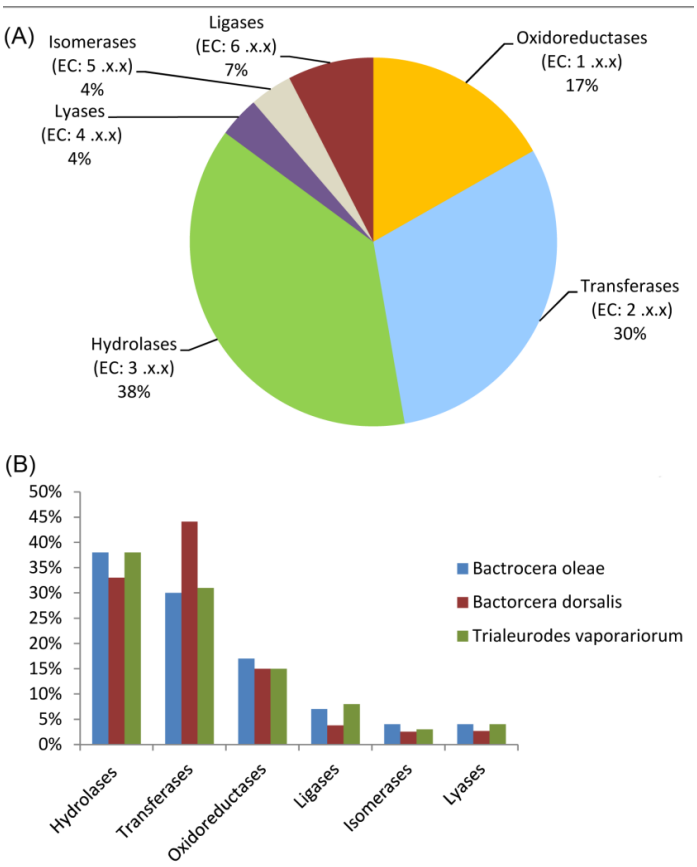


Figure 3.5 Enzyme Classification (EC) analysis of the transcriptome of *B. oleae*. A. Distribution of EC number in general EC terms, **B.** percentage of EC number distribution of *B. oleae*

compared to that from transcriptome sequencing of *B. dorsalis*[39], *M. domestica*[46] and *T. vaporariorum*[33].

The 1,700 sequences having EC numbers were further characterized by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The predicted enzymes are distributed in 122 pathways. The best matches of KEGG mapping are presented in Table 3.2. Interestingly, a large number of contigs were found to be associated with drug metabolism (58 with the drug metabolism-cytochrome P450 pathway; 52 with the drug metabolism-other enzymes pathway), possibly indicating the evolution of a multi-gene system in *B. oleae*, which might have a role in xenobiotic/phytotoxin detoxification.

Table 3.2 Summary of KEGG pathway mapping of *B. oleae* contigs.

Pathway ID	Pathway	Number of contigs in Pathway
230	Purine metabolism	127
190	Oxidative phosphorylation	101
910	Nitrogen metabolism	91
240	Pyrimidine metabolism	79
480	Glutathione metabolism	61
982	Drug metabolism - cytochrome P450	58
980	Metabolism of xenobiotics by cytochrome P450	58
520	Amino sugar and nucleotide sugar metabolism	56
983	Drug metabolism - other enzymes	52
10	Glycolysis / Gluconeogenesis	47
500	Starch and sucrose metabolism	47
627	Aminobenzoate degradation	44
330	Arginine and proline metabolism	43
260	Glycine, serine and threonine metabolism	42
620	Pyruvate metabolism	40
970	Aminoacyl-tRNA biosynthesis	40
561	Glycerolipid metabolism	39
71	Fatty acid metabolism	35

3.2.2.5 Transcripts encoding putative P450s

P450s are one of the largest super-families, playing a dominant role in plant-insect interactions and insecticide/xenobiotic metabolism. They are divided in 4 major clades, named CYP2, CYP3, CYP4 and mitochondrial. Eighty-eight P450s have been identified in the genome of *D. melanogaster*[48, 49], while 90 P450s were found in the transcriptome of the closely related *B. dorsalis*[36, 39]. A total of 55 contigs were identified as P450 encoding genes in the *B. oleae* transcriptome, and no allelic variants were found among P450 protein sequences encoded. Five additional P450 sequences were obtained by a degenerate PCR study.

The total number of P450s identified in the *B. oleae* transcriptome is similar to those found in the genomes of *D. melanogaster* and *B. dorsalis* but much lower than those of other dipteran species like e.g. *A. aegypti* and *C. quinquefasciatu*s having 160 and 170 P450 genes respectively[36, 39, 49-51] (**Table 3.3**). However, it is possible that additional P450 genes may await discovery in *B. oleae* genome. Notably, only one P450 sequence of *B. oleae* was available in the NCBI database prior to the present study.

Based on phylogenetic analysis with other known insect P450s or the identification of closest blastp hits in the NCBI nr database in the case of misaligning protein sequences, the B. oleae P450s were assigned to appropriate P450 clades and families. Representatives of all 4 major insect P450 clades were found in this dataset. The majority of *B. oleae* P450s (28 out of 55) identified in this study belong to the CYP3 clade, 2 to the CYP2, 17 to the CYP4 and 13 to the mitochondrial clade (**Figure 3.5**).

Members of the CYP3 and CYP4 clades in other insect species are most commonly involved in environmental response/detoxifying functions against xeniobiotics and phytotoxins in other insects[49, 52], and the over-representation of members of those clades possibly indicates an enhanced defense mechanism present in *B. oleae* against such compounds. Orthologues of major insecticide detoxification genes in other species, such as the *Bemisia tabaci CYP6CM1*, the first P450 from an agricultural pest that was demonstrated that is capable to detoxify neonicotinoid insecticides[53], and the *D. melanogaster CYP4G1*, an insect- specific P450 oxidative

decarbonylase for cuticular hydrocarbon biosynthesis[54] were among the list of genes that were identified in this study (**Figure 3.6**).

Table 3.3. Comparison of P450s in different insect species*.

Cytochrome P450 clan	*Bactrocera oleae*	*Bactrocera dorsalis*	*Drosophila melanogaster*	*Anopheles gambiae*	*Aedes aegypti*	*Apis mellifera*	*Tribolium castaneum*	*Myzus persicae*	*Trialeurodes vaporariorum*
CYP2	2	6	7	10	12	8	8	3	3
CYP3	28	50	36	40	82	28	72	63	34
CYP4	17	30	32	46	57	4	45	48	13
mitochondrial	13	4	11	9	9	6	9	1	7
Total P450s	**60**	**90**	**88**	**105**	**160**	**46**	**134**	**115**	**57**

*Numbers were derived from Refs 33, 39, 49 and this study.

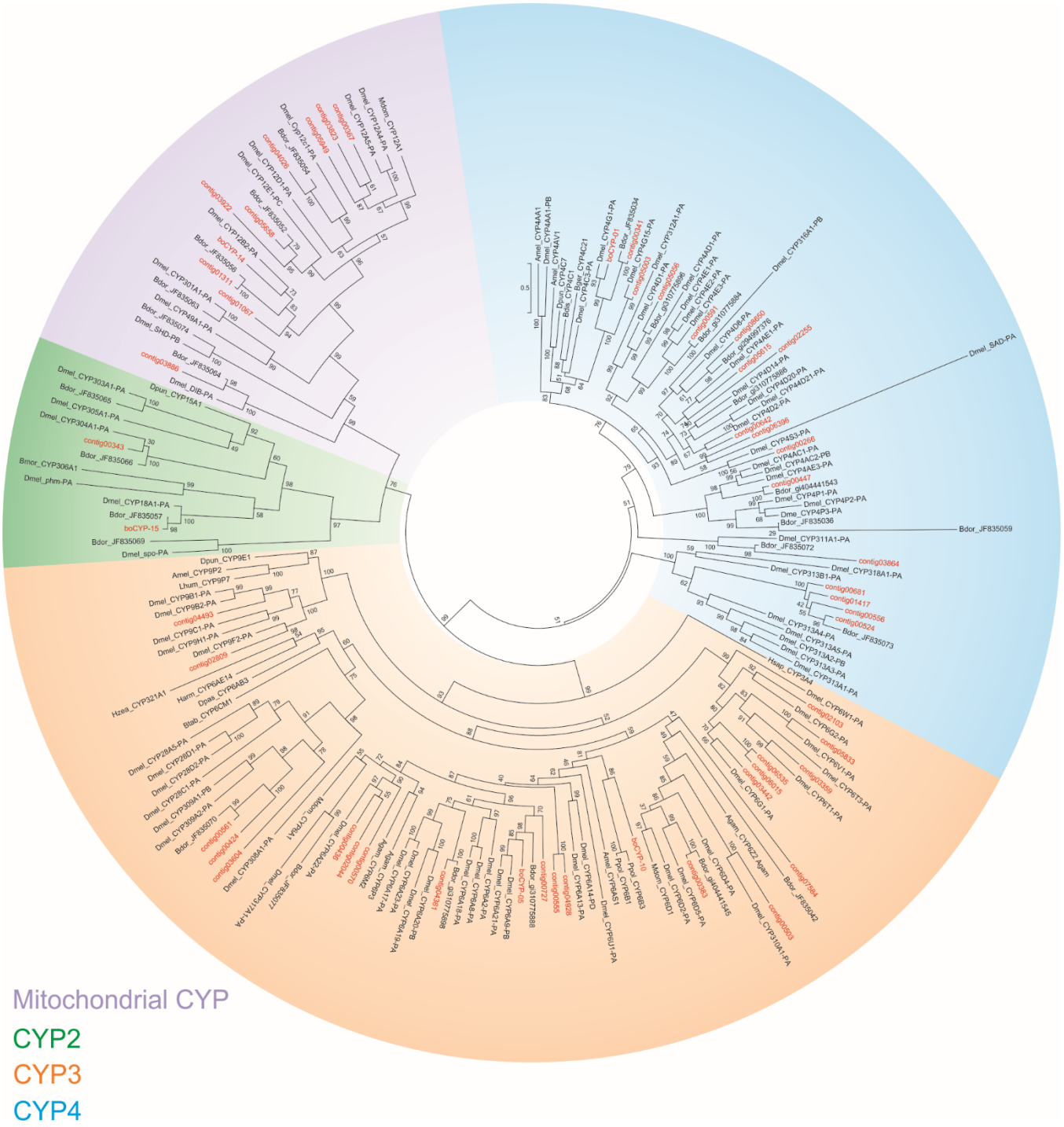


Figure 3.6. Phylogenetic analysis of *B. oleae* putative P450s. *B. oleae* P450s clustered within the 4 major insect CYP clades. Aech: *Acromyrmex echinatior,* Agam: *Anopheles gambiae*, Amel: *Apis mellifera,* Bdor: *Bactrocera dorsalis* ("JF" sequences were obtained from Shen *et al*., 2011), Bger: *Blatella germanica,* Bmor: *Bombyx mor*i, Btab: *Bemisia tabaci*, Dmel: *Drosophila melanogaster,* Dpas: *Depressaria pastinacella*, Dpun*: Diploptera punctata*, Harm:

Helicoverpa armigera, Hsap*: Homo sapiens*, Hzea: *Helicoverpa zea*, Mdom: *Musca domestica*, Ppol: *Papilio polyxenes*.

3.2.2.6 Transcripts encoding putative GSTs

The GST super-family has also been involved in the resistance to phytotoxins and insecticides[55]. Insect GSTs belong to seven classes, named delta (δ), epsilon (ε), omega (ο), sigma (σ), theta (θ), zeta (ζ) and microsomal GSTs. Thirty seven GSTs have been identified in *D. melanogaster*[56] while 42 putative GSTs have been identified in the transcriptome of *B. dorsalis*[39]. A total of 43 contigs encoding GSTs were identified in the *B. oleae* transcriptome. Three genes with amino-acid homology 99% at the protein level and two additional ones with identical sequences at the amino-acid level but several silent SNPs, which might represent putative allelic variants were found among these sequences: contig07254, contig08723, contig09900 are allelic variants of contig07812, contig08608 and contig09901, respectively, and contigs 09162 and 09177 are identical.

Based on phylogenetic analysis with other known insect GSTs or the identification of closest blastp hits in the NCBI nr protein database in the case of misaligning protein sequences, *B. oleae* GSTs were assigned to the delta, epsilon, omega, sigma, theta zeta and microsomal classes: out of 39 unique GSTs, 8 belong to the delta class, 12 to the epsilon class, 3 to omega, 1 to sigma, 4 to theta, 3 to zeta and 6 to microsomal class (**Figure 3.7**). The remaining 2 GSTs are described as delta/epsilon since they could not be assigned particularly to delta or epsilon GST class. A comparative summary of the cytosolic GSTs identified in *B. oleae* transcriptome versus those identified in other insect species is presented in **Table 3.4**. The number and distribution of cytosolic GSTs within classes in *B. oleae* is similar to that of other Diptera, such as *D. melanogaster*[56] and *A. gambiae*[57], with the possible exception of epsilon GSTs which are overrepresented in *B. oleae* (**Table 3.4**). The delta and epsilon GST classes are unique in insect species and seem to be implicated in xenobiotic detoxification[50]. For example, GSTE2 of *A. gambiae* (Agam_gi12007373 in **Figure 3.7**),

a glutathione transferase with DDTase activity, is responsible for conferring DDT resistance in *A. gambiae*[58]. More than half (22 out of 43) of GSTs identified in the transcriptome of *B. oleae* belong to delta and epsilon classes, which might indicate an enhanced potential for xenobiotic metabolism.

xenobiotic metabolism.

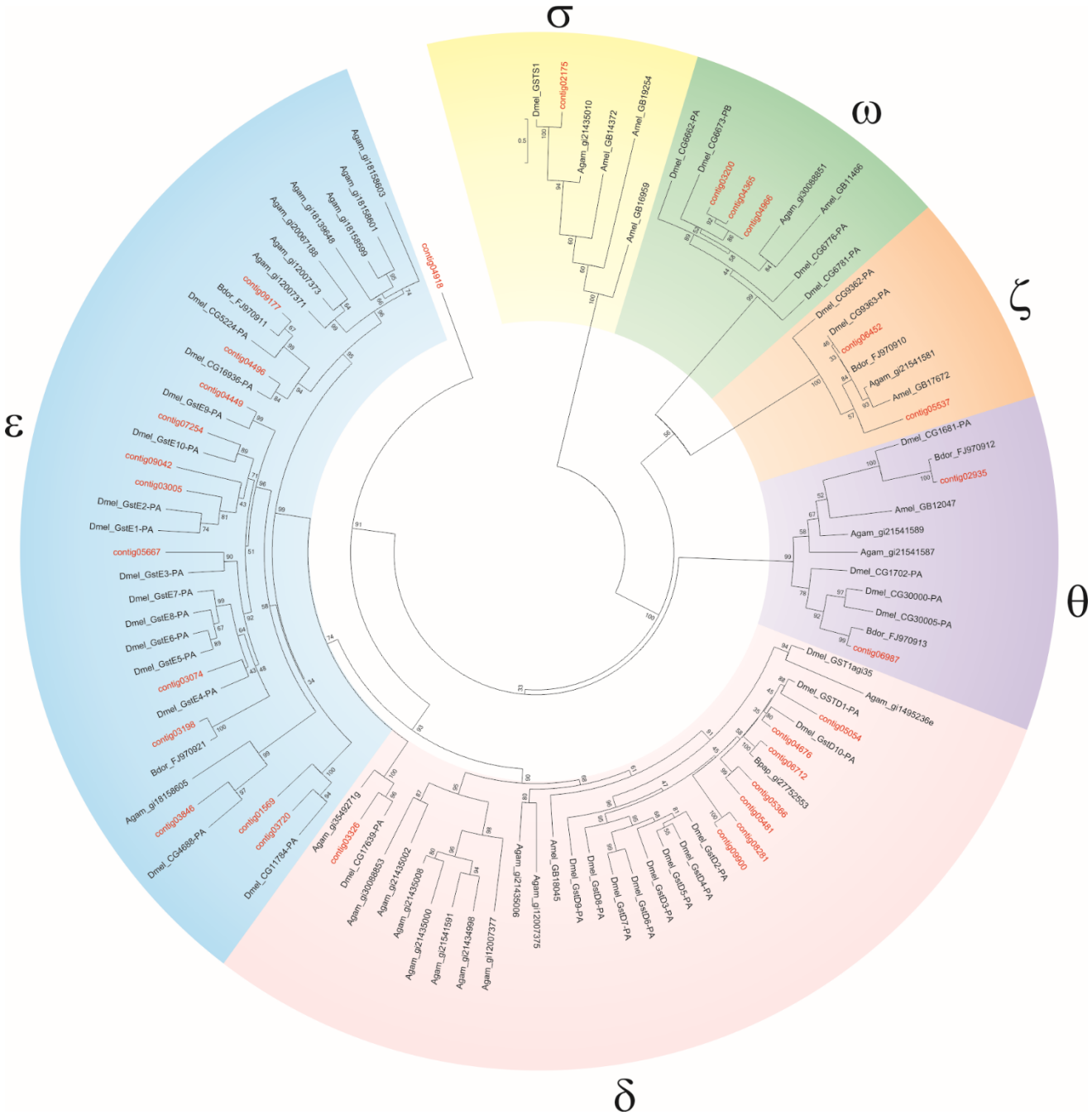


Figure 3.7. Phylogenetic analysis of *B. oleae* putative GSTs. *B. oleae* sequences, corresponding to cytosolic GSTs, clustered within classes. δ: delta class, ε: epsilon class, ζ: zeta

class, θ: theta class, σ: sigma class, ω: omega class. Agam: *Anopheles gambiae*, Amel: *Apis mellifera*, Bdor: *Bactrocera dorsalis*[36], Bpap: *Bactrocera papaya*.

Table 3.4: Comparison of cytosolic GSTs in different insect species*.

Cytosolic GST class	*Bactrocera oleae*	*Bactrocera dorsalis*	*Drosophila melanogaster*	*Anopheles gambiae*	*Aedes aegypti*	*Apis mellifera*	*Tribolium castaneum*	*Myzus persicae*	*Trialeurodes vaporariorum*
Delta	8	14	11	12	8	1	3	8	9
Epsilon	12	7	14	8	8	-	19	-	1
Omega	3	6	5	1	1	1	4	-	-
Sigma	1	1	1	1	1	4	7	8	5
Theta	4	3	4	2	4	1	1	2	-
Zeta	3	-	2	1	1	1	1	-	1
Other	-	1	-	3	3	-	-	-	
Delta/Epsilon	2	6	-	-	-	-	-	-	-
Total cytosolic GSTs	**33**	**39**	**37**	**28**	**27**	**8**	**35**	**18**	**16**

*Numbers were derived from Refs. 33, 39, 59, 60 and this study.

3.2.2.7 Transcripts encoding putative carboxylesterases (CCEs)

CCEs have been shown to be involved in the detoxification of insecticides as well as the metabolism of plant derived allelochemicals[55]. The CCEs can be divided into 13 clades[50, 61], including acetylcholinesterases (AChE). These clades can in turn be organized into 3 classes, i.e. the dietary detoxification enzymes (clades A–C), the generally secreted enzymes (clades D– G) and the neurodevelopmental CCEs (clades I–M). Thirty-five CCEs have been identified in the genome of *D. melanogaster*[62] while recently, 38 putative CCEs have been identified in the transcriptome of *B. dorsalis*[39]. A total of 15 contigs putatively encoding CCEs were identified in *B. oleae* transcriptome and no allelic variants were found among these sequences.

Based on phylogenetic analysis with other known insect CCEs or the identification of closest blastp hits in the NCBI nr database in the case of misaligning

or short CCE protein sequences, CCEs were assigned to respective clades and classes. Representatives of dipteran microsomal a-esterases (C clade), integument esterases (D clade), b-esterases and pheromone esterases (E clade) and glutactins and glutactin-like enzymes (H clade) were found in this dataset. Out of the 15 identified CCEs, 7 belong to the C clade, 2 to D clade, 1 to E clade and 2 to H clade (**Figure 3.8**). The remaining 3 CCEs could not be assigned to any particular CCE clade. Comparative analysis (**Table 3.5**) with CCEs from other known insect species shows that the number of identified CCEs is considerably less than those from other insects. However, the majority of them (7 out of 15) are assigned to the dietary class, which might indicate a possible association of this gene superfamily with the ability of olive fly to cope with substances present in the olive flesh.

Table 3.5. Comparison of CCEs in different insect species*.

Carboxyl/cholinesterases**	***Bactrocera oleae***	***Bactrocera dorsalis***	***Drosophila melanogaster***	***Anopheles gambiae***	***Aedes aegypti***	***Apis mellifera***	***Tribolium castaneum***	***Myzus persicae***	***Trialeurodes vaporariorum***
Dietary class		19							
A clade	-		-	-	-	5	-	5	11
B clade	-		13	16	22	3	14	-	-
C clade	7		-	-	-	-	12	-	1
Hormone/semiochemical processing		1							
D clade	2		3	-	-	1	2	-	-
E clade	1		2	4	2	2	7	12	6
F clade	-		3	6	6	2	2	-	-
G clade	-		0	4	6	-	-	-	-
Neurodevelopmental		4							
H clade	2		5	10	7	1	1	-	1
I clade	-		1	1	1	1	1	1	1
J clade	-		1	2	2	2	2	3	2
K clade	-		1	1	1	1	1	1	1
L clade	-		4	5	5	5	5	-	3
M clade	-		2	2	2	1	2	-	1
Unclassified	3	14	-	-	-	-	-	-	-
Total CCEs	**15**	**38**	**35**	**51**	**54**	**24**	**49**	**21**	**27**

* Numbers were derived from[33, 39, 59, 60] and this study

** For full CCE clade names, see legend of **Figure 3.8.**

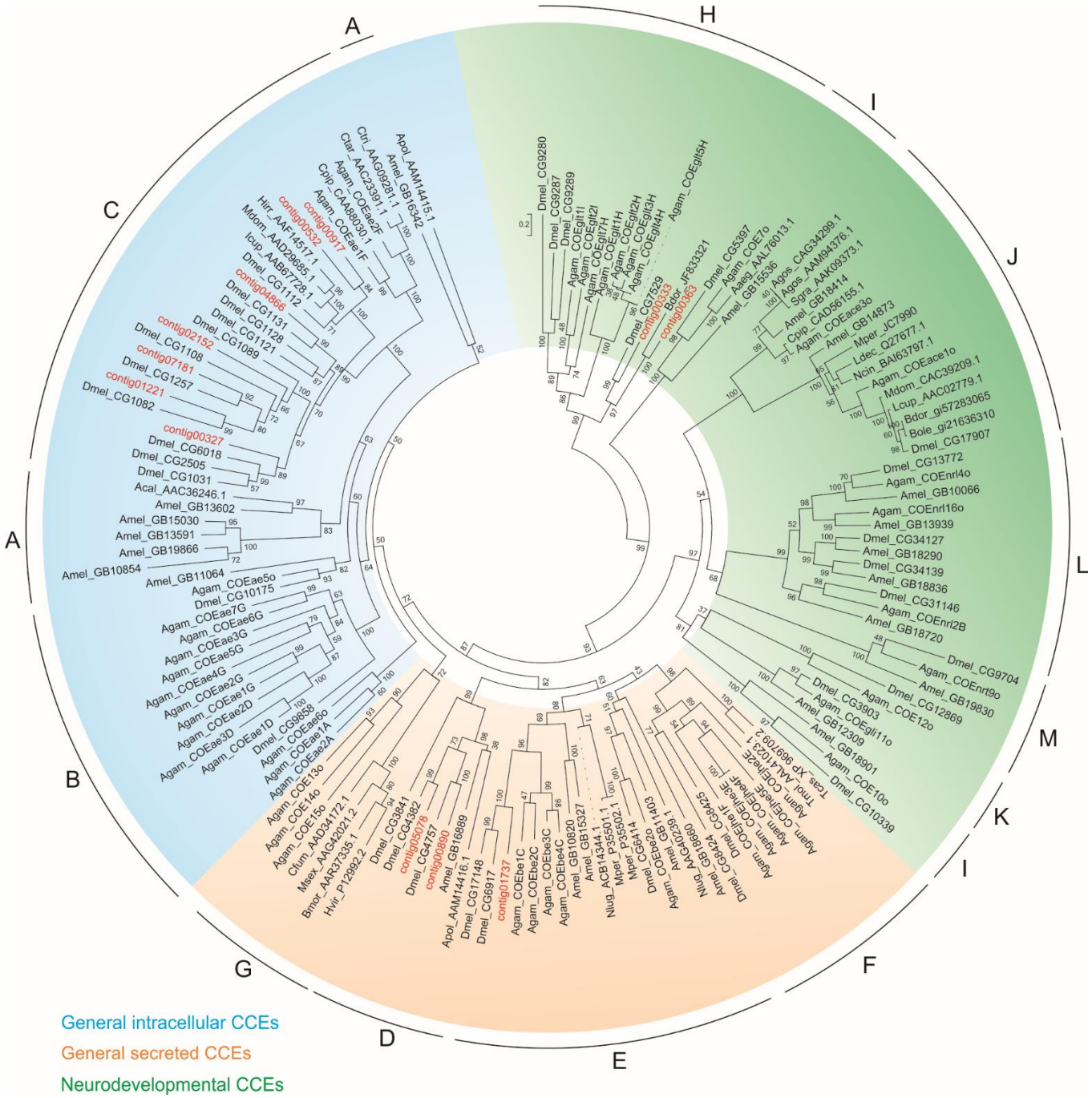
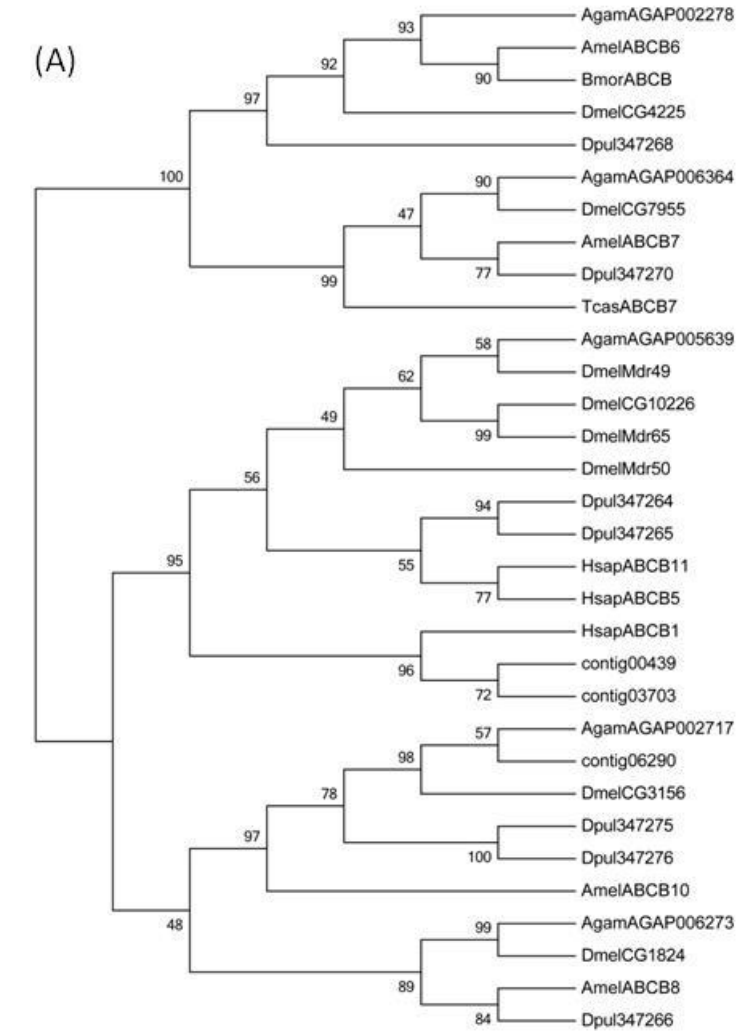


Figure 3.8. Phylogenetic analysis of *B. oleae* putative CCEs. *B. oleae* CCEs clustered within clades. A: hymenopteran radiation with related group containing odorant degrading esterases, B: dipteran mitochondrial, cytosolic and secreted esterases, C: dipteran microsomal, α-esterases, D: integument esterases, E: β-esterases and pheromone esterases, F: dipteran juvenile hormone esterases, G: lepidopteran juvenile hormone esterases, H: glutactin and glutactin-like enzymes, I: uncharacterized group, J: acetylcholinesterases, K: gliotactins, L: neuroligins, M: neurotactins. Aaeg: *Aedes aegypti*, Acal: *Anisopteromalus*

calandrae, Agam: *Anopheles gambiae*, Agos: *Aphis gossypii*, Amel: *Apis mellifera*, Apol: *Antheraea polyphemus*, Bdor: *Bactrocera dorsalis*, Bmor: *Bombyx mori*, Bole: *Bactrocera oleae*, Cfum: *Choristoneura fumiferana*, Cpip: *Culex pipiens*, Ctar: *Culex tarsalis*, Ctri: *Culex tritaeniorhynchus*, Dmel: *Drosophila melanogaster*, Hirr: *Haematobia irritans*, Hvir: *Heliothis virescens*, Lcup: *Lucilia cuprina*, Ldec: *Leptinotarsa decemlineata*, Mdom: *Musca domestica*, Mper: *Myzus persicae,* Nlug: *Nilaparvata lugens*, Sgra: *Schizaphis graminum*, Tcas: *Tribolium castaneum,* Tmol: *Tenebrio molitor*.

3.2.2.8 Transcripts encoding putative ABC transporters

The ATP-binding cassete (ABC) transporter superfamily is considered to play a major role in the ability of insects to cope with xenobiotics[63]. Fifty six ABC transporters have been identified in *D. melanogaster*[64]. A total of 18 contigs encoding ABCs were identified in the *B. oleae* transcriptome. No allelic variants were found among those sequences. Based on phylogenetic analysis or, in the case where the nucleotide binding domain (NBD) was missing, based on the closest blastp hits in the NCBI nr database, ABC transporters were assigned to different subfamilies. The number of identified ABC transporters is less than those from other insects[63]. Out of 18 *B. oleae* ABCs, four belong to the B subfamily of which only two are full transporters (those clustering with hsABCB1) (**Figure 3.9 A**), two to the C subfamily and four to the G subfamily. Interestingly, these families are believed to be the most relevant to xenobiotic detoxification[63] and are about half of the ABC transporters identified in this study. Furthermore, three ABC transporters belong to the A family, one to the D subfamily, one to the E subfamily and three to the F subfamibly (**Figure 3.9 B and C**). A comparative summary of the ABCs identified in *B. oleae* transcriptome versus those identified in other insect species is presented in **Table 3.6**.


(A)
AgamAGAP002278
AmelABCB6
BmorABCB
DmelCG4225
Dpul347268
AgamAGAP006364
DmelCG7955
AmelABCB7
Dpul347270
TcasABCB7
AgamAGAP005639
DmelMdr49
DmelCG10226
DmelMdr65
DmelMdr50
Dpul347264
Dpul347265
HsapABCB11
HsapABCB5
HsapABCB1
contig00439
contig03703
AgamAGAP002717
contig06290
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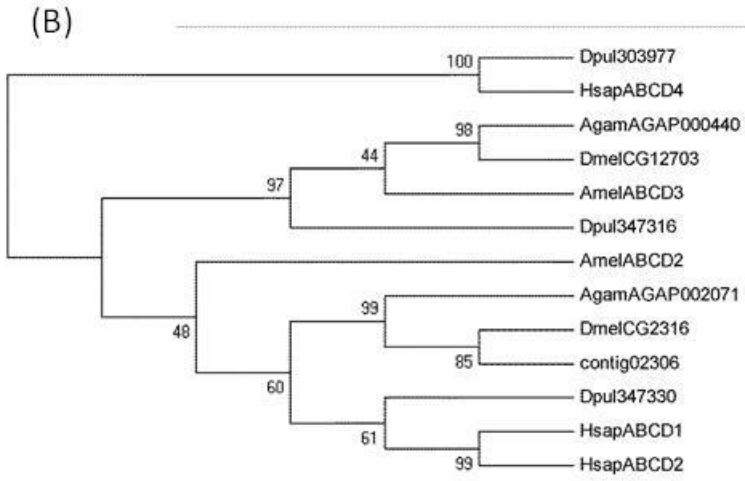
DmelCG1824
AmelABCB8
Dpul347266


(B)
Dpul303977
HsapABCD4
AgamAGAP000440
DmelCG12703
AmelABCD3
Dpul347316
AmelABCD2
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DmelCG2316
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Dpul347330
HsapABCD1
HsapABCD2

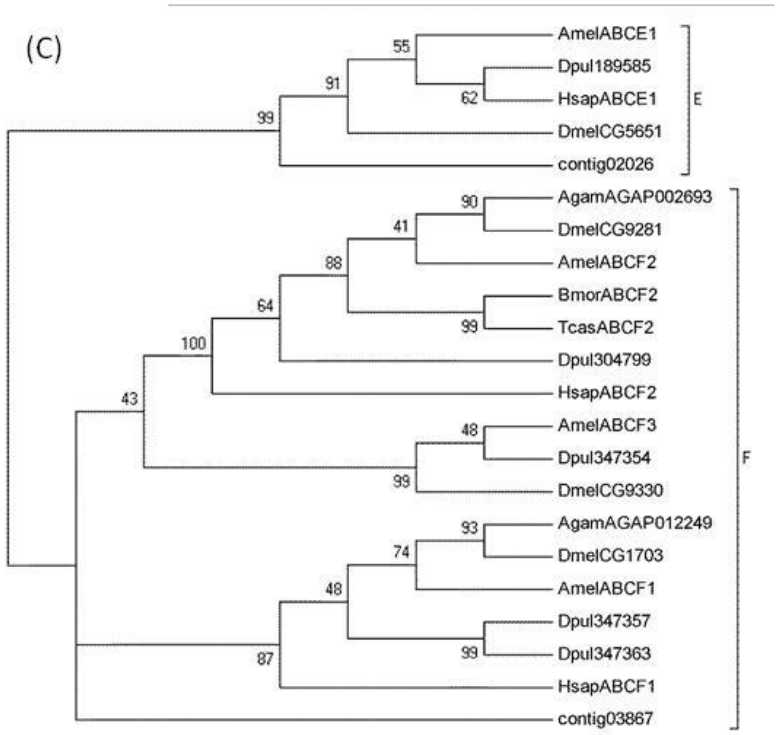

Figure 3.9. Phylogenetic analysis of *B. oleae* putative ABC transporters. (A) sub-family B. 4 *B. oleae* ABC sequences are clustered in B subfamily. **(B) sub-family D.** One *B. oleae* ABC sequence is clustered in D subfamily. **(C) sub-families E and F**. One *B. oleae* ABC sequence is clustered in E and one in F subfamily. Agam: *Anopheles gambiae*, Amel: *Apis mellifera,* Bmor: *Bombyx mori*[65]*,* Dpul*: Daphnia pulex*[66], Dmel: *Drosophila melanogaster*, Dpul: *Daphnia pulex*, Hsap: *Homo sapiens*, Tcas: *Tribolium castaneum*.

Table 3.6: Comparison of ABC genes in different insect species*

ABC subfamily	*Bactrocera oleae*	*Drosophila melanogaster*	*Anopheles gambiae*	*Apis mellifera*	*Tribolium castaneum*
A	3	10	9	3	9
B	4	8	5	7	6
C	2	14	13	9	31
D	1	2	2	2	2
E	1	1	1	1	1
F	3	3	3	3	3
G	4	15	16	15	13
H	-	3	3	3	3
Total ABC transporters	**18**	**56**	**52**	**43**	**68**

*Numbers were derived from[65, 66] and this study

3.2.2.9 Construction of Agilent microarray chip for *B. oleae*

The Agilent eArray platform (Agilent Technologies) was used for the design of a microarray chip for *B. oleae* based on the obtained transcriptome dataset. 13,164 sequences of the transcriptome were used and 235 NCBI sequences were added to this dataset. Thus 13,398 sequences were submitted to eArray for creating 60-mer probes, using best probe distribution, no 3 bias, sense probes settings and aiming at 5 probes per sequence. In total 61,358 probes were created with an average Tm of 81 (± 3.5) ^{o}C. 154 probes were created for 31 control genes and 15 replicates of this control-probe group were selected and additional probes for the default set of Agilent controls were created. 88% of the sequences had at least 3 probes, which were randomly distributed on the 8 x 60K array.

3.2.3 Conclusions

A *B. oleae* transcriptome dataset containing 14,204 contigs was generated. This dataset represents a very significant expansion of the number of cDNA sequences currently available for *B. oleae*. Although the function of the majority of the assembled sequences is unknown, it is likely that new ongoing projects will facilitate their future annotation and our efforts to understand their role in the physiology and fundamental biology of the olive fruit fly. At least 132 putative major detoxification genes (60 P450s, 39 GSTs, 15 CCEs, 18 ABC transporters) involved in the metabolism of xenobiotics, such as plant phytotoxins and insecticides, was identified and phylogenetically classified in this study. Based on the obtained sequences an Agilent microarray chip was designed and constructed to be used for the analysis of pyrethroid resistance and adaptation to olives. The chip could also serve as a useful molecular tool for the scientific community working on molecular/ biological aspects of the olive fruit fly.

3.3 Transcriptomic analysis of pyrethroid resistance

In this study, I investigated the α-cypermethrin resistance mechanisms of *B. oleae* populations from Crete, at the molecular level. Target-site resistance was investigated and the gene expression of two α-cypermethrin resistant populations from Crete was analyzed, compares to susceptible insects, using the Agilent microarray slide that was developed in 3.2.

3.3.1 Materials and methods

3.3.1.1. Insects

Five populations of *B. oleae* were used for the purposes of this study. Populations AN12 and AN14 were collected from Agios Nikolaos (Crete, Greece) in 2012 and 2014 respectively, from olive orchards regularly treated with pyrethroid insecticides for the past decades. Fokida population was collected from Fokida region (Central Greece) in 2014 from olive orchards which do not undergo insecticidal treatment and was used as the field-derived susceptible reference strain. Two laboratory strains were also used as susceptible reference strains: LAB (Demokritos laboratory strain) which had not been exposed to insecticides for over 35 years as well as HYB, which is also not exposed to insecticides for several years. Laboratory strains were maintained under common rearing conditions[67] at 25^{o}C and 16:8 h light:dark photoperiod. For field populations, olives infested by *B. oleae* were collected from each sampling region, transferred to the laboratory and maintained in petri dishes containing a layer of sawdust (pupation substrate) at 25^{o}C and 16:8 h light: dark photoperiod. Upon pupation, pupae were transferred in plastic cages and the emerging adults were kept under common rearing conditions.

3.3.1.2 Bioassays with α-cypermethrin

One to three days old adults were subjected to bioassays with the pyrethroid insecticide α-cypermethrin (Sigma-Aldrich), using the topical application method. Insecticide was applied in 1μl of acetone on the dorsal thorax, using a 10 μl Hamilton syringe. Insects were anaesthetized by short exposure to carbon dioxide prior insecticide application. Different concentrations of α-cypermethrin (Sigma-Aldrich) were used (range 0.1-10 ppm) and 15-20 individuals (with approximately equal representation of males and females) were tested in each concentration. The treated adults were kept in plastic cages at 25^{o}C and 16:8 h light:dark photoperiod under standard rearing conditions and mortality was scored 48 h post-treatment. Control mortality in the absence of α-cypermethrin (acetone only) was taken into account. Bioassay data were analyzed using PoloPC (LeOra Software, Berkeley, CA). Individuals that survived bioassays were collected separately and stored at -80^{o}C for detection of target site mutations (see section 3.3.1.3).

3.3.1.3 Investigation of target-site resistance

To investigate the presence or not of the amino acid mutations M918T, L925I, T929I, L932F, I1011M, L1014F, V1016G that have been previously associated with kdr resistant phenotype, sequencing of para sodium channel (domain IIS4-IIS6) gene region was performed. Individual adults from AN12 and AN14 populations, which survived in high doses of α- cypermethrin (3-10 ppm) were tested. Genomic DNA (gDNA) was extracted from thirty two individuals using DNeasy Blood and Tissue kit (Qiagen). Specific primers (**Table 3.7**) were used to amplify a ~ 1300 bp sequence of IIS4-IIS6 domain of para sodium channel (EU253453.1[32]). PCR amplifications from 50 ng gDNA were performed using KAPAtaq polymerase (KAPAbiosystems, USA) and 10 μM of each primer. PCR conditions were 94^{o}C for 5min, followed by 29 cycles of: 95^{o}C for 30 sec, 52^{o}C for 2 min, 72^{o}C for 1.5 min and finally 72^{o}C for 2 min. The PCR products were identified in 1% agarose gel, purified using QiaQuick columns (Qiagen) and

directly sequenced (Starseq, Germany) using the appropriate primers (**Table 3.7**). Sequence data were analyzed using BioEdit software.

3.3.1.4 RNA preparation for microarray experiments

For each one of the 5 populations (2 resistant and 3 susceptible) total RNA was extracted from 4 biological replicates of 10, one to three days old, females using the RNeasy mini kit (Qiagen). RNAs were treated with Turbo DNA-free (Ambion) to remove any contaminating DNA. RNA quantity was measured using Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies) and RNA quality was judged in a 1.5% agarose gel.

One hundred nanograms of each RNA were used to generate Cy3- and Cy5-labeled cRNA using Agilent Low-input Quick Labeling kit (Agilent Thechnologies) and RNA spike-in controls (Agilent Technologies) were added to RNA samples before cRNA synthesis. The labeled cRNA was purified with RNeasy mini kit (Qiagen) and its concentration, as well as the concentration of dye, were measured using Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies).

3.3.1.5 Microarray hybridization and analysis

Cy3- and Cy5- labeled cRNAs were pooled and hybridized for 17 hours in a rotating (20 rpm) hybridization oven at 65^{o}C, using the Gene Expression Hybridization kit (Agilent Life Technologies). In total, six experimental groups (with four biological replicates each) were analyzed and hybdridized in three comparisons: (1) Cy5-labeled AN12 cRNA,Cy3- labeled HYB cRNA, (2) Cy5- labeled AN14 cRNA and (3) Cy3-labeled LAB cRNA, Cy5-labeled AN14 cRNA and Cy3-labeled FOKIDA cRNA. After hybridization, slides were washed using the Gene Expression Wash Buffer kit (Agilent Life Technologies). Subsequently, all slides were scanned by an Agilent Microarray High-Resolution Scanner (default settings for 8 x 60K GE microarrays). Data were extracted

(Agilent Feature Extraction software v 10.5) and transferred to the Bioconductor framework for final statistical analysis. Background correction and within- and between-array normalization was performed on the grouped data. Final data quality was assessed using arrayQualityMetrics[68]. Differentially expressed genes were identified using cut-offs of FDR-corrected p-values and log2FC at 0.05 and 1, respectively[69]. The microarray hybridization and analysis were performed in collaboration with Agrozoology laboratory, Ghent University, Belgium (Dr. Nicky Wybouw and Prof. Thomas Van Leeuwen).

3.3.1.5 Microarray validation by RT-PCR

Selected microarray data were validated using quantitative reverse transcription PCR (RT-qPCR). Primers were designed using Primer – BLAST online analysis software[70], based on cDNA sequences retrieved from pyrosequencing and are listed in **Table 3.7**. Reverse transcription was performed on 2 µg of pooled RNAs (used in microarray) for each condition, using Superscript III (Invitrogen Life Technologies) following the manufacturer's instructions. PCR reactions of 25 µl performed on a MiniOpticon two-color Real-Time PCR detection system (BioRad), using a 1 to 15 cDNA dilutions, 0.20 µM primers and KapaSYBR FAST qPCR master mix (Kapa Biosystems). A 5-fold dilution series of pooled cDNA was used assess the efficiency of the qPCR reaction for each gene-specific primer pair. A no template control (NTC) was also included to detect possible contamination. Melting curve analysis was performed to test the specificity of amplicons. Experiments were performed in 4 biological replicates for each gene. The fold-change of each target gene was normalized to the 40S ribosomal protein (GAKB01005984.1) and beta-Actin (GAKB01001968.1) and relative expression levels was calculated[71].

Table 3.7 Primers used in this study.

Sequence Name	GenBank ID	Primer (am, s, q, c)[a]	Sequence (5'-3')	Tm (°C)
Para sodium channel	EU253453.1	F (am)	TGGCTAAGTCATGGCCTACA	52
		R (s)	ATCAAGAAAGGCTTATCAACG	52
		F (s)	ATGGTGATTTGCCTCGCTGG	52
		R (am)	ACCTGTTTTCGTTTCGATTC	52
Contig00436	GAKB01000438.1	F (q)	GAAAGCGAATACCGAACGGC	60
		R (q)	CCATCCTTTCCGTCCTTGGT	60
Contig02103	GAKB01002105.1	F (q)	CTGCCAAAGCGTCTTTCTCC	60
		R (q)	TTGCGCGAAACAATCAAGCA	60
Bo-CYP06	KC917336.1	F (q)	ATCTAACGCGCTTAGCCGTCG	60
		R (q)	GGTGCAAAGCGTTCAGGGTCG	60
Contig09051	GAKB01009022.1	F (q)	TGGTTTTCGCCAGATTCTCCA	60
		R (q)	GAATTGCAGACCTCCATTGGC	60
Contig00503	GAKB01000505.1	F (q)	TAAGGGCTTCCCAACCGAAC	60
		R (q)	GGTATCTCGAGGACAGTGCG	60
Contig06987	GAKB01006983.1	F (q)	TCCACTCGGCGATTTTTGGA	60
		R (q)	ACCGAAACCTAGCGCTGAAA	60
Contig00917	GAKB01000919.1	F (q)	CCCACTTTGGTGGTGATCCA	60
		R (q)	TAAAACCGGCTAGCTTGGCA	60
Contig03442	GAKB01003443.1	F (q)	ATTGCCACCATCGCATTTGG	60
		R (q)	AGCTGAACTCCGGCGTAAAA	60
Contig01417	GAKB01001419.1	F (q)	TACAGTTGCGTGGACCCTTC	60
		R (q)	GACACACACGAACTTTGCCC	60
Contig01966 (beta-Actin)	GAKB01001968.1	F (q)	CGGTATCCACGAAACCACAT	60
		R (q)	ATTGTTGATGGAGCCAAAGC	60
Contig05987 (40S Ribosomal Protein S7)	GAKB01005984.1	F (q)	TTCGGTAGCAAGAAGGCTGT	60
		R(q)	GGTAGGTTTGGGCAGGATTT	60

[a] am: primers used for amplifying SII-S4 domain of para sodium channel gene; s: Primers used for sequencing; q: primers used for real time qPCR.

3.3.2 Results and discussion

3.3.2.1. Response of *B. oleae* populations to α-cypermethrin

The resistance levels of 3 field-collected populations (AN12, AN14, Fokida) and 2 laboratory strains (HYB, LAB) were determined and the lethal dose that kill half individuals (LD50) as well as the percentage mortalities in the diagnostic dose of 3 ppm

are presented in **Table 3.8**. For AN14, due to limitation to the biological material, only the percentage mortality in the diagnostic dose was calculated. The laboratory strains displayed the highest susceptibility to α-cypermethrin. Among the field-collected populations, Fokida displayed moderated susceptibility levels while AN12 and AN14 displayed significant resistance to α-cypermethrin. AN14 showed increased resistance compared to AN12 (22% mortality in 3 ppm of α-cypermethrin compared to 40%, respectively). The two populations were collected in two years' time difference from the same field region (Agios Nikolaos, Greece), which is routinely sprayed with pyrethroids (2-3 sprays per year). The variation in resistance levels points out a potential tendency for increment of resistance during time, due to the continuous and extensive application of α-cypermethrin in the field.

Table 3.8 Response of *B. oleae* populations from the field (AN12, AN14, Fokida, Episkopi) and laboratory strains (HYB, LAB) to α-cypermethrin by topical application bioassays.

Population	Collection date	N[a]	n[b]	Diagnostic dose (% mortality)	LD50 (ppm) (95% CI)	Slope	χ^2	RR[c]
AN12	2012	111	5	40	3.284 (2.611-3.880)	6.650	6.942	21.748
AN14	2014	-	-	22	-	-	-	-
Fokida	2014	127	5	65.5	1.322 (0.808-2.276)	1.168	5.062	8.754
HYB	2012	38	5	100	0.449 (0.305-0.672)	2.666	6.220	2.973
LAB	2014	118	5	100	0.151 (0.054-0.660)	1.522	27.739	1

[a] N: number of individual tested

[b] n: number of doses used in each bioassay (including control)

[c] RR: Resistance ratio, ED_{50} of population tested divided by LD_{50} of LAB (the most susceptible one)

3.3.2.2 Screening for resistance mutations within the domain II (IIS4-IIS6) of para-sodium channel

Thirty two individuals (both females and males) from AN12 and AN14 populations survived in high doses of α-cypermethrin (3-10 ppm) were genotyped to investigate the possible existence of resistance-associated mutations within the domain II (IIS4-IIS6) of the para- sodium channel gene. Sequence analysis showed that none of the individuals tested possessed any of the point mutations previously associated with target-site resistance[72] indicating no link between resistance of *B. oleae* to α-cypermethin and mutations in para sodium channel gene domain II. However, the existence of mutations in other regions of para sodium channel gene cannot not be excluded.

3.3.2.3 Detoxification genes differentially transcribed between olive fruit flies resistant and susceptible to α-cypermethrin

Given that target-site based resistance mechanisms were not detected in AN12 and AN14, we focused on the investigation of genome-wide gene expression differences between the resisatnt and susceptible populations. Microarray gene expression studies were performed to investigate detoxification (and/or other) genes associated with olive fruit fly resistance to α-cypermethrin. We analyzed the gene expression of the 2 resistant (AN12, AN14) and the 3 susceptible (HYB, LAB, Fokida) populations. In total 3 hybridization experiments were conducted: 1. AN12 compared to HYB, 2. AN14 compared to LAB and 3. AN14 compared to Fokida. The first hybridization experiment was conducted with a 2-year difference with the second and third.

We identified 1,084 genes differentially expressed between AN12 and the laboratory susceptible strain HYB. Five hundred ninety six are related to genes with known function, while the rest remain of unknown function. Four hundred eighteen

genes were over-transcribed, of which 205 had a predicted function, while 666 were under-transcribed, 391 of which had a blast description. Interestingly, among the 205 genes with a blast description several detoxification genes were included. Thirteen detoxification genes were identified, i.e. 10 genes encoding for P450s, 2 for GSTs and 1 for a CCE (**Table 3.9**). The contigs 00436 and 02103, which encode for putative CYP6 P450s were the strongest hits. Contig00436 is a putative homologue of *Drosophila ananassae Cyp6a23* and contig02103 is a putative homologue of *D. ananassae Cyp6g2*Using real-time quantitative PCR (qPCR), we validated the expression levels for selected detoxification genes (**Figure 3.7**). The levels of differential expression derived from the microarray analysis was in accordance with the levels obtained by qPCR and 7 out of 9 genes tested were found up-regulated indicating that the microarray experiment is reliable.

The up-regulated P450 encoding genes represent the approximately 5% of the total number of up-regulated genes with a predicted function indicating that P450-based metabolism may be a major mechanism engaged for resistance to α-cypermethrin.

Table 3.9 Over-expressed detoxification genes in AN12 resistant population compared to the susceptible HYB. The sequence name, the GenBank ID, the top blast hit description in the nr database, the hit accesion number (AAC), the folds of up-regulation (in log_2) and the p-value are indicated. Gene expression values are the average of four biological replicate hybridizations.

Sequence Name	GenBank ID	Top BLAST hit description (NCBI nr database)	Hit AAC/ Species	Gene family	Log_2 (FC)	P-value
contig00436	GAKB01000438.1	c6a23|drome ame: full=probable cytochrome p450 6a23 ame: full=cypvia23	XP_001958951 (*D. ananassae*)	P450	3.51	2.81E-06
contig02103	GAKB01002105.1	cp6g2|drome ame: full=probable cytochrome p450 6g2 ame: full=cypvig2	XP_001959362 *(D. ananassae)*	P450	2.84	1.96E-05
BoCYP-06	KC917336.1	-	-	P450	2.32	5,58E-07
contig00343	GAKB01000345.1	cytochrome p450	ADD19880 (*G. morsitans morsitans*)	P450	2.12	0.000408

contig09051	GAKB01009022.1	cytochrome p450 monooxygenase	AAC13308 (*C. capitata*)	P450	2.07	2.54E-06
contig00503	GAKB01000505.1	cytochrome p450	XP_001961628 (*D. ananassae*)	P450	1.49	6.29E-07
contig06987	GAKB01006983.1	glutathione s-transferase	XP_002017725 (*D. persimilis*)	GST	1.39	2.66E-05
contig05949	GAKB01005946.1	cytochrome p450 cyp12a3	XP_002072231 (*D. willistoni*)	P450	1.38	0.00244
BoCYP-13	KC917243	-	-	P450	1.35	0.000909
contig09718	GAKB01009654.1	glutathione-s-transferase gst	XP_001958610 *(D. ananassae)*	GST	1.30	1.08E-05
contig00917	GAKB01000919.1	alpha esterase partial	ACR56068 (*Cochliomyia hominivorax*)	CCE	1.25	0.000628
contig03442	GAKB01003443.1	cyp6g1	XP_001995137 (*D. grimshawi*)	P450	1.20	0.000127
contig01417	GAKB01001419.1	cp134|drome ame: full=probable cytochrome p450 313a4 ame: full=cypcccxiiia4	XP_001993731 (*D. grimshawi*)	P450	1.00	0.000628

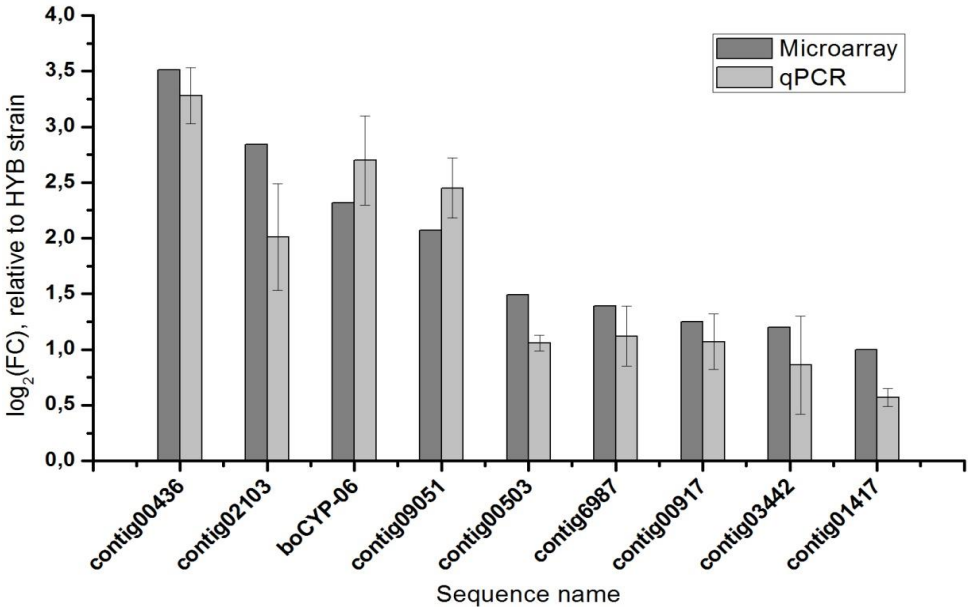

Figure 3.10. Microarray validation by real-time qPCR of selected putative detoxification genes over-transcribed in AN12 compared to HYB. Error bars represent the standard deviation of four biological replicates.

The expression levels of AN14 were also compared to LAB as well as Fokida. The AN14 samples were collected 2 years after AN12 samples from the same region,

which continued to be sprayed by pyrethroids 2 to 3 times per year. Bioassays in the diagnostic dose indicated that AN14 displays increased resistance compared to AN12. The gene expression of AN14 was compared to a second laboratory strain (LAB) but also to a field-collected susceptible population (Fokida), aiming to identify genes specific for the α-cypermethrin reistance excluding, as much as possible, and the differences in gene expression due to genetic variation between wild and laboratory-reared flies.

We identified 902 genes differentially expressed between AN14 and the laboratory susceptible strain LAB. Four hundred eighty nine are related to genes with known function, while remaining 413 are of unknown function. Five hundred forty nine genes were over-transcribed, of which 332 had a predicted function, while 350 were under-transcribed, 157 of which had a blast description. Fifteen putative detoxification genes were found over-transcribed (**Table 3.10**). The majority of the genes (14) encode for P450s, indicating also P450-based metabolism as the possible mechanism of resistance, while 1 gene encodes for a GST. The contigs 00436 and 02103 represent the most striking hits.

Table 3.10 Over-expressed detoxification genes in AN14 resistant population compared to the susceptible LAB. The sequence name, the GenBank ID, the top blast hit description in the nr database, the hit accession number (AAC), the folds of up-regulation (in log_2) and the p-value are indicated. Gene expression values are the average of four biological replicate hybridizations.

Sequence Name	GenBank ID	Top BLAST hit description (NCBI nr database)	Hit AAC/ Species	Gene family	Log_2 (FC)	P-value
contig00436	GAKB01000438.1	c6a23|drome ame: full=probable cytochrome p450 6a23 ame: full=cypvia23	XP_001958951 (*D. ananassae*)	P450	4.02	7.66E-03
contig02103	GAKB01002105.1	cp6g2|drome ame: full=probable cytochrome p450 6g2 ame: full=cypvig2	XP_001959362 *(D. ananassae)*	P450	3.66	2.04E-01
contig04493	GAKB01004493.1	cytochrome p450-9b2	XP_001986708 (*D. grimshawi*)	P450	2.32	1.88E-07
contig10502	GAKB01010316.1	cytochrome p450-9b2	XP_002059979 (*D. virilis*)	P450	1.82	1.60E-07
contig01067	GAKB01001069.1	cytochrome p450	XP_001987732 (*D. grimshawi*)	P450	1.67	1.01E-06

contig05056	GAKB01005054.1	cytochrome p450	ADP22308 (*B. dorsalis*)	P450	1.50	2.35E-06
contig00341	GAKB01000343.1	cytochrome P450	XP_001982391 (*D. erecta*)	P450	1.44	3.12E-07
BoCYP-01	KC917331	-	-	P450	1.33	6.41E-04
contig05003	GAKB01005001.1	cytochrome p450	NP_727531 (*D. melanogaster*)	P450	1.27	1.58E-05
contig05658	GAKB01005655.1	c12e1_drome ame: full=probable cytochrome p450 mitochondrial ame: full=cypxiie1 flags: precursor	AAY55552 (*D. melanogaster*)	P450	1.21	3.47E-06
BoCYP-02	KC917332	-	-	P450	1.17	5.52E-08
BoCYP-06	KC917336	-	-	P450	1.13	1.72E-02
contig01417	GAKB01001419.1	cp134_drome ame: full=probable cytochrome p450 313a4 ame: full=cypcccxiiia4	XP_001993731 (*D. grimshawi*)	P450	1.11	2.70E-02
contig08281	GAKB01008274.1	glutathione s-transferase d1	XP_001358020 (*D. pseudoobscura pseudoobscura*)	GST	1.09	9.94E-06
contig00503	GAKB01000505.1	cytochrome p450	XP_001961628 (*D. ananassae*)	P450	1.01	1.80E-04

When the gene expression of AN14 was compared to Fokida 809 genes were found differentially transcribed, of which 478 has a predicted function. Four hundred sixty seven genes were over-transcribed, 294 of which had a predicted function, while 342 were under-transcribed, 185 of which had a blast description. Ten detoxification genes were found among the up-regulated genes with a blast description (**Table 3.11**). Nine P450s and 1 GST were identified, pointing out P450-mediated detoxification as the possible primary mechanisms engaged for resistance to α-cypermethrin. The contigs 02103 and 05003 (encoding for P450s) were the most striking hits.

Table 3.11 Over-expressed detoxification genes in AN14 resistant population compared to the field Fokida. The sequence name, the GenBank ID, the top blast hit description in the nr

database, the hit accesion number (AAC), the folds of up-regulation (in log_2) and the p-value are indicated. Gene expression values are the average of four biological replicate hybridizations.

Sequence Name	GenBank ID	Top BLAST hit description (NCBI nr database)	Hit AAC/ Species	Gene family	Log_2(FC)	P-value
contig02103	GAKB01002105.1	cp6g2|drome ame: full=probable cytochrome p450 6g2 ame: full=cypvig2	XP_001959362 *(D. ananassae)*	P450	2.96	8,26E-02
contig05003	GAKB01005001.1	cytochrome p450	NP_727531 (*D. melanogaster*)	P450	1.64	3,75E-03
contig00341	GAKB01000343.1	cytochrome P450	XP_001982391 (*D. erecta*)	P450	1.54	1,24E-07
contig06396	GAKB01006393.1	c4d14_drome ame: full=probable cytochrome p450 4d14 ame: full=cypivd14	XP_001984690 (*D. grimshawi*)	P450	1.37	8,28E-07
contig01067	GAKB01001069.1	cytochrome p450	XP_001987732 (*D. grimshawi*)	P450	1.23	9,25E-07
BoCYP-01	KC917331	-	-	P450	1.22	2,57E-05
contig00436	GAKB01000438.1	c6a23|drome ame: full=probable cytochrome p450 6a23 ame: full=cypvia23	XP_001958951 (*D. ananassae*)	P450	1.21	0.0008
contig08281	GAKB01008274.1	glutathione s-transferase d1	XP_001358020 (*D. pseudoobscura pseudoobscura*)	GST	1.20	2,61E-06
contig04928	GAKB01004926.1	cytochrome p450	XP_002002750 (*D. mojavensis*)	P450	1.19	3,75E-07
contig03359	GAKB01003360.1	cyp6g1	XP_002002534 (*D. mojavensis*)	P450	1.07	7,38E-08

All three microarray hybridization experiments indicate that resistance to α-cypermethrin is conferred by P450s. The over-expression of a set of *CYP* genes, in particular members of the CYP6 sub-family, is in line with several other studies in different insects which associate the over- expression of *CYP* genes and P450- based detotification with resistance to pyrethroids. For instance, several members of CYP6 and CYP9 sub-families in the mosquitoes *A. aegypti* and *A. gambiae* and several P450 genes in *M. domestica* and *C. quinquefasciatus* have been associated with pyrethroid resistance[73-75].

3.3.2.4 Detailed expression analysis of the P450 encoding genes contig00436 and contig02103

Although population-specific mechanisms may also contribute to resistance, we consider that the overlap in *CYP* gene expression between the 3 comparisons is a robust approach to identify specific P450 encoding gene candidates correlated to resistance. **Figure 3.11** summarizes the number of *CYP* genes that were up-regulated in all 3 comparisons. Two P450s, contig00436 and contig02103, were commonly up-regulated in the three independent pairwise comparisons, making them top candidates and suggesting their possible involvement in the α-cypermethrin resistance.

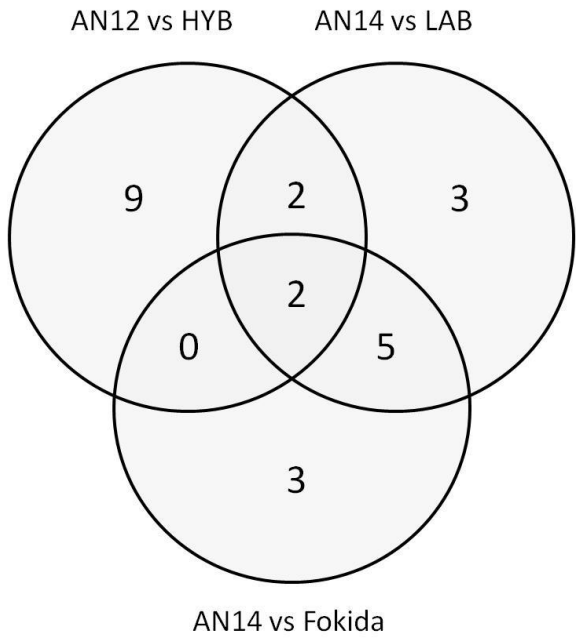

Figure 3.11. Venn diagram summarizing the number of CYP genes over-transcribed in all microarray comparisons, AN12 compared to HYB, AN14 compared to LAB, and AN14 compared to Fokida. Two CYP genes were commonly up-regulated in the 3 comparisons.

To further validate the over-transcription of contigs 00436 and 02103 in the resistant populations, detailed qPCR analysis was performed and the expression levels of each one of the resistant populations (AN12, AN14) were compared to the

expression levels of each one of the three susceptible populations (HYB LAB, Fokida) (**Figure 3.12**) (qPCR analysis of contig00436 was performed by V. Tseliou, M.Sc. thesis). The over-expression of contig 00436 and 02103 was confirmed in all possible resistant versus susceptible comparisons, showing an association of those genes with α-cypermethrin resistance phenotype in AN12 and AN14 populations.

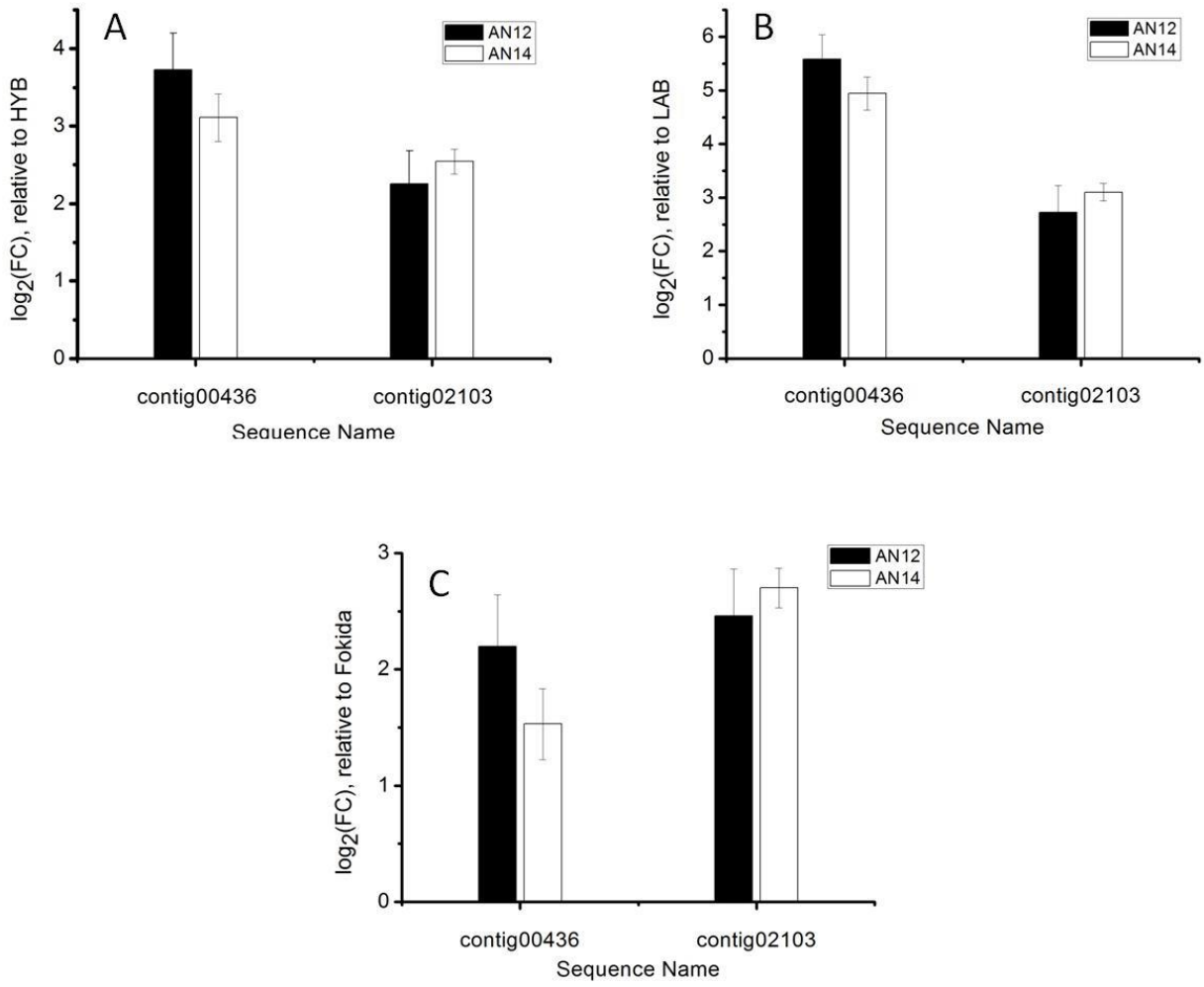

Figure 3.12. Real-time qPCR expression analysis of contig00436 and contig02101 in the α-cypermethrin resistant populations AN12 and AN14. Expression levels (in log_2(FC)) relative to A. HYB strain, B. LAB strain, C. Fokida population. Data are means of four biological replicates ± S.D. Analysis of contig00436 was performed by V. Tseliou (M.Sc thesis).

These two genes encode for putative P450 of CYP6 sub-family. Members of CYP6 sub-family have been previously strongly associated with resistance in several insects. For example, *CYP6G1* confers resistance in *D. melanogaster, CYP6P3* was found up-regulated in pyrethroid resistant populations of *A. gambiae* and encodes for an enzyme capable of metabolizing α-cyano and non α-cyano pyrethroids and *CYP6BG1* confers pyrethroid resistance in *Plutella xyllostella*[76, 77].

The over-expression of contigs 00436 and 02103 in AN12 and AN14 resistant populations is only a first indication for their involvement in resistance. Moreover, more gene expression analysis experiments including also additional α-cypermethrin resistant populations are needed. Since when other populations with higher levels of resistance were tested, the expression levels of contig00436 and contig02103 were not correlated with resistance levels (A. Kabouraki 2015, unpublished data). Further functional investigation is required to determine the possible role in resistance, i.e. *in vitro* metabolism studies in order to assess their capacity to metabolize α-cypermethrin *in vitro* as well as *in vivo* approaches, such as RNAi or over-expression in *Drosophila*, in order to verify their role in resistance.

3.3.3 Conclusions

I investigated the molecular mechanisms that confer resistance to the pyrethroid insecticide α-cypermethrin in the olive fruit fly, the most significant pest of olive orchards. No target-site resistant associated mutations were identified in the resistant populations, AN12 and AN14. I subsequently analyzed the differential gene expression among resistant and susceptible *B. oleae* populations and several over-transcribed detoxification genes were identified. Among them, two CYP genes, contig00436 (a putative homologue of *D. ananassae Cyp6a23*) and contig02103 (a putative homologue of *D. ananassae* Cyp6g2), were shown to be over-expressed in all resistant versus susceptible comparisons. This study provided further evidence and supported earlier work[13] that P450s are likely to play a role in α-cypermethrin resistance. However, further functional work, and possibly more association RT-PCR

studies are required to establish the link between these genes and pyrethroid resistance. The elucidation of underlying pyrethroid resistance mechanisms is crucial for the sustainability of efficient control for this important pest, as pyrethroids are among few registered insecticides for use against *B. oleae*.

3.4 Understanding the adaptation of olive fruit fly to olives

Gene expression of wild larvae during their development in green and black olives and artificial diet was analyzed. This study is the first to apply transcriptomic techniques in *B. oleae* for the identification of molecular mechanisms possibly implicated in the overcoming of olive defense and utilization of olive flesh, especially in the more toxic green ones. Genes that were associated with the development of larvae in green versus black olives and versus artificial diet were identified and they are discussed.

3.4.1 Materials and methods

3.4.1.1 Sample collections

For the purposes of this study the gene expression of wild 2nd instar larvae fed upon green olives, black olives and artificial diet was analyzed. Immature (green) olives (Kalamon variety) infested by *B. oleae* were collected from an olive orchard in Heraklion (Greece) in September 2012. Infested olives were transferred in the laboratory and 2nd instar larvae were picked up from the mesocarp. Specimens were preserved in RNAlater (Sigma-Aldrich) and stored at -80°C until used. Second instar larvae from mature (black) olives were collected in January 2013 from the same olive orchard following the same procedure as described for green olives. To obtain sample from artificial diet, infested olives were collected from the olive orchard, transferred to the laboratory and maintained in petri dishes containing a layer of sawdust (substrate for pupation) at 25°C and 16:8 h light: dark photoperiod. Upon pupation, pupae were transferred in plastic cages (8 x 8 x 8 cm) and the emerging females were allowed to lay eggs in wax substrate. The newly hatched larvae were transferred in artificial diet containing 550 ml distilled water, 20 ml extra virgin olive oil, 7.5 m; Tween-80, 0.5 gr potassium sorbate, 2 gr Nipagin, 20 gr sugar, 75 gr Brewer's yeast, 30 gr soy hydrolysate, 30 ml hydrochloric acid 2N and 75 gr cellulose powder. When attained 2nd instar, larvae were collected and stored at -80°C upon treatment with RNAlater (Sigma-Aldrich).

3.4.1.2 RNA preparation for microarray experiments

For each one of the 3 conditions (2nd instar larvae fed upon green, black olives and artificial diet) total RNA was extracted from 4 biological replicates of 20 individuals. RNA preparation was performed as described in section 3.3.1.4.

3.4.1.3. Microarray Hybridization and data analysis

Microarray hybridization and data analysis were conducted as described in section 3.3.1.5., in collaboration with Agrozoology laboratory, Ghent University, Belgium (Dr. Nicky Wybouw and Prof. Thomas Van Leeuwen). In total, two hybridization experiments, in four replicates each, were performed: Cy5-labeled cRNA derived from larvae fed upon green olives/Cy3- labeled cRNA derived from larvae fed upon black olives and Cy5- labeled cRNA derived from larvae fed upon green olives/Cy3-labeled cRNA derived from larvae fed upon artificial diet.

Using limma[78], a non-connected design was incorporated in the linear modeling of the data, enabling all against all comparisons.

3.4.1.4 Microarray validation by RT-PCR

Selected microarray data were validated using quantitative reverse transcription PCR (RT-qPCR). Primers were designed using Primer – BLAST online analysis software[70] and are listed in **Table 3.12**. Reverse transcription, real-time PCR and data analysis were conducted as described in section 3.3.1.5. Experiments were performed in 4 biological replicates for each gene.

Table 3.12 qPCR primers used in this study.

Sequence Name	GenBank ID	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (°C)	Product size (bp)
contig03604	GAKB01003604.1	GCTTTCTGCGGCACATTGAA	AATGCGTGACAGTGCTCAGA	60	156
contig04090	GAKB01004090.1	CGTTCCCCTGTCACCATCTC	ACCACCAGAGTCACCATTGC	60	192
contig04528	GAKB01004528.1	GTCGTGTCGTTGTTATGGCG	TGTTGGAGCTGTTCGGTCAA	60	226
contig02711	GAKB01002713.1	TCACGGCACTGTTCTTCTGG	GTTGATGTGAGTCCCGCTGT	60	161
contig03020	GAKB01003021.1	CAAATAGCGCACGGAACCAG	GGATGAACCAAAACGAGCGG	60	153
contig01887	GAKB01001889.1	AGGGAACGGCTACATTGCTC	TACTCAAGCCAACCGCACAT	60	166
contig06851	GAKB01006847.1	TGGCTCGTTGGGCTTGTATT	TGGGTTCGATGTTGTCGGTT	60	201
contig04367	GAKB01004367.1	TAAGCCCAGGACACCAAACC	ACCGAAGACGGACAGAAGTG	60	240
contig07649	GAKB01007645.1	ACATACTGCCGAACTGCCC	TCGGACGCAATTCAAAGCAG	60	240
contig04596	GAKB01004596.1	CGGCTCTGAAATCAGGGATA	TTCATCGGCAACATCACTGT	60	175
contig03768	GAKB01003768.1	ATTACCAACGGCAACAAAGC	GTGCTGCCCAGATAGACGAT	60	176
contig04001	GAKB01004001.1	ACAAACGCTTCGCCATTTAC	TAGCTGCATTTTGCTGATGG	60	207
contig04849	GAKB01004847.1	ATCCTTACCAATGGCAGCAG	CGGTTGGGGTACACTCTCAT	60	160
contig00850	GAKB01000852.1	ATGCAAGGCTGTTCAGAGGT	AGCGTCTATGTCGGGTGAGT	60	162
contig01966 (beta-actin)	GAKB01001968.1	CGGTATCCACGAAACCACAT	ATTGTTGATGGAGCCAAAGC	60	159
contig05987 (40S Ribosomal Protein S7)	GAKB01005984.1	TTCGGTAGCAAGAAGGCTGT	GGTAGGTTTGGGCAGGATTT	60	156

3.4.2 Results and discussion

3.4.2.1 Genes differentially transcribed between olive fly larvae developed in immature green olives (high phenol & non hydrolysed proteins) and mature black olives

In order to identify genes that are associated with the ability of *B. oleae* to develop in the green olive, the differential gene expression of larvae fed upon green olives compared to larvae fen upon black olives was studied. A total of 485 transcripts were differentially regulated. Out of them, 345 genes were related to a known

function. Three hundred and fifty one (279 of which with a blast description) were up-regulated while 103 (62 of which with a blast description) were down-regulated.

A large set of genes encoding for putative enzymes involved in digestion were up-regulated (**Table 3.13**) and the over-expression of selected genes was further confirmed by qPCR (**Figure 3.13**). Twenty four digestive-related genes were found, including genes potentially encoding for lysozymes (contig06851, contig08507), many proteases such as serine proteases (e.g. contig04367, contig04849, contig06115, contig04313, contig01887), metalloproteases (e.g. contig04956, contig00850), a phospholipase (contig04753), nucleases (e.g. 00776, contig01101, contig01241) and the digestion facilitator peritrophin (contig06046). The up-regulated genes related to digestion represent the 8.6% of the up-regulated genes with a predicted function, suggesting a possible machinery engaged by *B. oleae* larvae for the digestion of dietary proteins and development in green olives. Except from protein digestion, the induction of peptidase activity may be due to the increased needs for protein degradation in order to encounter energy requirements during stress response[79] or protein biosynthesis during induction processes[80] during *B. oleae* response to plant defenses. Moreover, the elevated peptidase activity may be an adaptive mechanism to overcome the negative effects of protease inhibitors produced by olives upon larval attack[30].

Six genes encoding for putative detoxification enzymes were also up-regulated, including a putative ABC transporter (cotig03867), an alpha-esterase (contig02152), 3 UGTs (contig04001, contig05006, and contig06187) and a beta-galactosyltransferase (a type of glucosyltrasferase). The over-expression of selected genes was further confirmed by qPCR (**Figure 3.13)**. These genes may play a role in the detoxification of phenolic compounds mainly present in the green olives. The over-expression of a set of glycosyltransferases is in line with other studies which associate glycosylation with detoxification of plant phenols and adaptation to host. For example, the BmUGT1 has been associated with elimination of effects of ingested plant phenols in *Bombyx mori*[81] while beta-glycosylation by beta-glycosyltransferases in *M. sexta* larvae has been correlated with detoxification of toxic phenols present in the larval diet[82].

Table 3.13 Over-expressed digestive and detoxification genes in larvae developed in green olives compared to larvae developed in black olives. The sequence name, the GenBank ID, the top blast hit description in the nr database, the hit accession number (AAC), the folds of up-regulation (in log_2) and the p-value are indicated. Gene expression values are the average of four biological replicate hybridizations.

Sequence Name	GenBank ID	Top BLAST hit description (NCBI nr database)	Hit AAC/ Species	Log_2(FC)	P-value
Digestion					
contig06851	GAKB01006847.1	lysozyme precursor	XP_002046431 (*D. virilis*)	2.15	0.000876571
contig04956	GAKB01004954.1	sperm-leucylaminopeptidase 1	XP_001971629 (*D. erecta*)	1.89	0.006708674
contig04367	GAKB01004367.1	midgut-specific serine protease 2	AAC39131 (*Stomoxys calcitrans*)	1.74	0.000104349
contig07649	GAKB01007645.1	cbpa1_drome ame: full=zinc carboxypeptidase a 1 flags: precursor	XP_002003983 (*D. mojavensis*)	1.61	0.001499507
contig04849	GAKB01004847.1	trydg_drome ame: full=trypsin delta gamma flags: precursor	XP_002138762 (*D.pseudoobscura pseudoobscura*)	1.58	0.000459965
contig06115	GAKB01006112.1	chymotrypsin-like serine protease precursor	AAF91345 (*G. morsitans morsitans*)	1.54	0.006603217
contig01887	GAKB01001889.1	low quality protein: tripeptidyl-peptidase 2-like	XP_002008256 (*D. mojavensis*)	1.54	0.003466457
contig00776	GAKB01000778.1	GK10387 [Drosophila willistoni]	XP_002068164 (*D. willistoni*)	1.49	0.004301274
contig00850	GAKB01000852.1	sperm-leucylaminopeptidase 3	XP_001972281 (*D. erecta*)	1.41	0.011112137
contig04603	GAKB01004603.1	GJ23580 [Drosophila virilis]	XP_002052990 (*D. virilis*)	1.32	0.000680422
contig04313	GAKB01004313.1	chymotrypsin-like serine protease precursor	AAF91345 (*G. morsitans morsitans*)	1.30	0.000494491
contig01101	GAKB01001103.1	GH19648 [Drosophila grimshawi]	XP_001997399 (*D.grimshawi*)	1.25	0.004294265
contig08507	GAKB01008495.1	lysozyme p	XP_002046428 (*D.virilis*)	1.24	0.003827647
contig01809	GAKB01001811.1	deoxyribonuclease i	XP_315045 (*A. gambiae*)	1.24	0.001396779
contig07006	GAKB01007002.1	jonah 65aiv	XP_002026421 (*D.persimilis*)	1.23	0.000103858
contig05361	GAKB01005358.1	GI22367 [Drosophila mojavensis]	XP_002000721 (*D. mojavensis*)	1.19	0.000319494
contig01241	GAKB01001243.1	deoxyribonuclease i	XP_001991936 (*D. grimshawi*)	1.17	0.004482746
contig05196	GAKB01005193.1	lethal 34fc	ADD19550 (*G. morsitans morsitans*)	1.16	0.004246222

contig06773	GAKB01006770.1	jonah 65aiv	XP_001957954 (*D.ananassae*)	1.13	0.000139611
contig02463	GAKB01002465.1	serine protease	XP_001978038 (*D. erecta*)	1.02	0.000763823
contig04632	GAKB01004632.1	serine protease 6	XP_002025085 (*D. persimilis*)	1.02	0.00211044
contig04753	GAKB01004753.1	phospholipase a1 member a	XP_002014726 (*D. persimilis*)	1.00	0.004980305
contig06046	GAKB01006043.1	type c peritrophin cb42	ADP89906 (*Chrysomya bezziana*)	1.00	0.005499954
Detoxification					
contig04001	GAKB01004001.1	n-acetyllactosaminide beta--n-acetylglucosaminyltransferase-like	XP_002063489 (*D. willistoni*)	2.45	0.000103858
contig03867	GAKB01003867.1	atp-dependent transporter	XP_002047542 (*D. virilis*)	1.20	0.000104349
contig01274	GAKB01001276.1	glucosyl glucuronosyl transferases	ADD20616 (*G. morsitans morsitans*)	1.08	0.003496896
contig05006	GAKB01005004.1	isoform a	XP_002037740 (*Drosophila sechellia*)	1.04	0.000459965
contig02152	GAKB01002154.1	alpha-esterase- isoform b	XP_002019532 (*D. persimilis*)	1.02	0.002997295
contig06187	GAKB01006184.1	beta- -galactosyltransferase brn	XP_001964323 (*D. ananassae*)	1.01	0.000401926
contig04142	GAKB01004142.1	GF16731 [Drosophila ananassae]	XP_001954440 (*D. ananassae*)	1.00	0.019480065

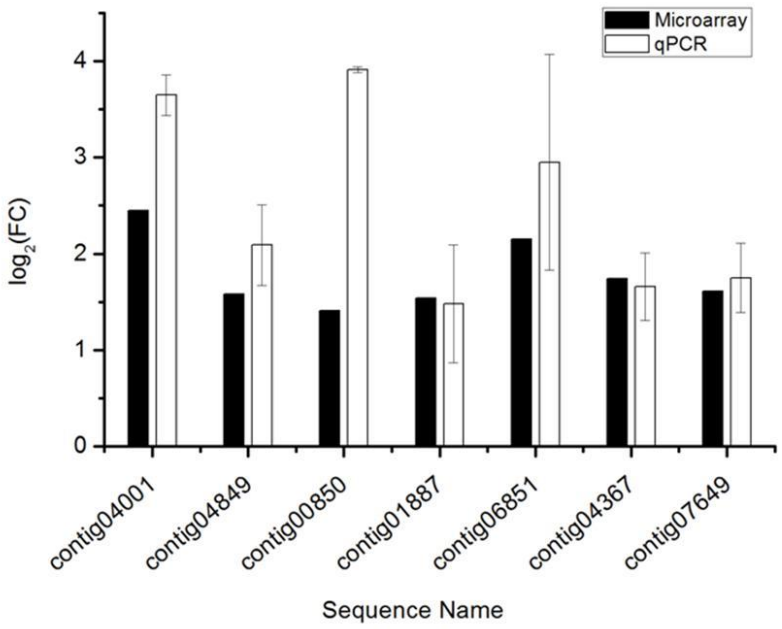

Figure 3.13 Microarray validation of selected putative detoxification and digestive genes up-regulated in larvae fed upon green olives compared to larvae fed upon black olives. Error bars represent the standard deviation of four biological replicates.

3.4.2.2 Genes differentially transcribed between olive fly larvae developed in olives and artificial diet

To investigate genes associated with adaptation of *B. oleae* larvae to olive fruits, we analyzed the gene expression of wild 2nd instar larvae during their development in green and black olives and artificial diet. Larvae fed upon green olives were compared to artificial diet directly by pairwise microarrays, while meta- analysis the comparison of expression levels of larvae developed in black olives to those developed in artificial diet.

Two hundred sixteen genes that were differentially transcribed between larvae fed upon green olives and artificial diet were identified. One hundred and one are related to genes with known function, while the rest are of unknown function. One hundred and fifty eight genes, 65 of which had a predicted function, were over-transcribed and 58, 36 of which had a predicted function, were under-transcribed. Among the 65 over-transcribed genes with a blast description, several genes encoding for enzymes related to food digestion and detoxification of xenobiotics were identified (**Table 3.14**). Nine putative serine-type proteases, such as midgut trypsins (e.g. contig04090, contig05378) as well as other proteases and a maltase (contig02414) were over-expressed in larvae developed in green olives. The over-production of digestive enzymes could represent the mechanism of digestion of proteins in the olives flesh but also may serve as a mechanism for overcoming the negative effects of trypsin inhibitors induced by the olive fruit upon *B. oleae* attack[30]. The contigs 04849 and 01887 (serine proteases) were also found up-regulated in the comparison of gene expression of larvae fed upon green olives versus black olives, enhancing their possible role in the adaptation to green olives. Four putative detoxification genes encoding for a UDP-glucosyltransferase (contig4001, which was also found in the comparison of

larvae fed on green olives versus black olive), a cytochrome P450 (contig03604) a N-acetyltransferase (contig03020) and a glycine methyltransferase (contig03118) were up-regulated upon development in green olives compared to artificial diet and the role of these detoxification genes could be in the de-activation of phenolic compounds present in the olive flesh. The up-regulation of several of the above genes was also confirmed by real-time qPCR (**Figure 3.14**).

Table 3.14 Over-expressed digestive and detoxification genes in larvae developed in green olives compared to larvae developed in artificial diet. The sequence name, the GenBank ID, the top blast hit description in the nr database, the hit accession number (AAC), the folds of up-regulation (in log_2) and the p-value are indicated. Gene expression values are the average of four biological replicate hybridizations.

Sequence Name	GenBank ID	Top BLAST hit description (NCBI nr database)	Hit AAC/ Species	Log_2(FC)	P-value
Digestion					
contig04596	GAKB01004596.1	trypsin-like serine protease precursor	P51588 (*Neobellieria bullata*)	2.63	1.73159E-06
contig03768	GAKB01003768.1	jonah 65aiii	XP_002093869 (*D. yakuba*)	1.74	0.002777149
contig04849	GAKB01004847.1	trydg_drome ame: full=trypsin delta gamma flags: precursor	XP_002138762 (*D. pseudoobscura pseudoobscura*)	1.53	0.000788508
contig04090	GAKB01004090.1	midgut trypsin	XP_002010361 (*D. mojavensis*)	1.41	0.001100079
contig04528	GAKB01004528.1	GG17383 [Drosophila erecta]	XP_001980845 (*D. erecta*)	1.39	0.000605486
contig02711	GAKB01002713.1	midgut-specific serine protease 1	NP_787956 (*D. melanogaster*)	1.39	0.000731639
contig04099	GAKB01004099.1	serine protease 6	XP_001355599 (*D. pseudoobscura pseudoobscura*)	1.26	0.001159644
contig02414	GAKB01002416.1	maltase a2	XP_002063228 (*D. willistoni*)	1.15	0.00397041
contig05378	GAKB01005375.1	midgut trypsin	XP_001958748 (*D. ananassae*)	1.02	0.005662067
contig01887	GAKB01001889.1	low quality protein: tripeptidyl-peptidase 2-like	XP_002008256 (*D. mojavensis*)	1.02	0.033848938
Detoxification					
contig04001	GAKB01004001.1	n-acetyllactosaminide beta--n-acetylglucosaminyltransferase-like	XP_002063489 (*D. willistoni*)	1.85	0.000196768
contig03604	GAKB01003604.1	cytochrome p450-28a1	XP_002089240 (*D. yakuba*)	1.54	0.01647456

contig03020	GAKB01003021.1	hypothetical conserved protein	XP_002056917 (*D. virilis*)	1.37	0.004155874
contig03118	GAKB01003119.1	glycine n-methyltransferase	XP_002000915 (*D. mojavensis*)	1.00	0.00108932

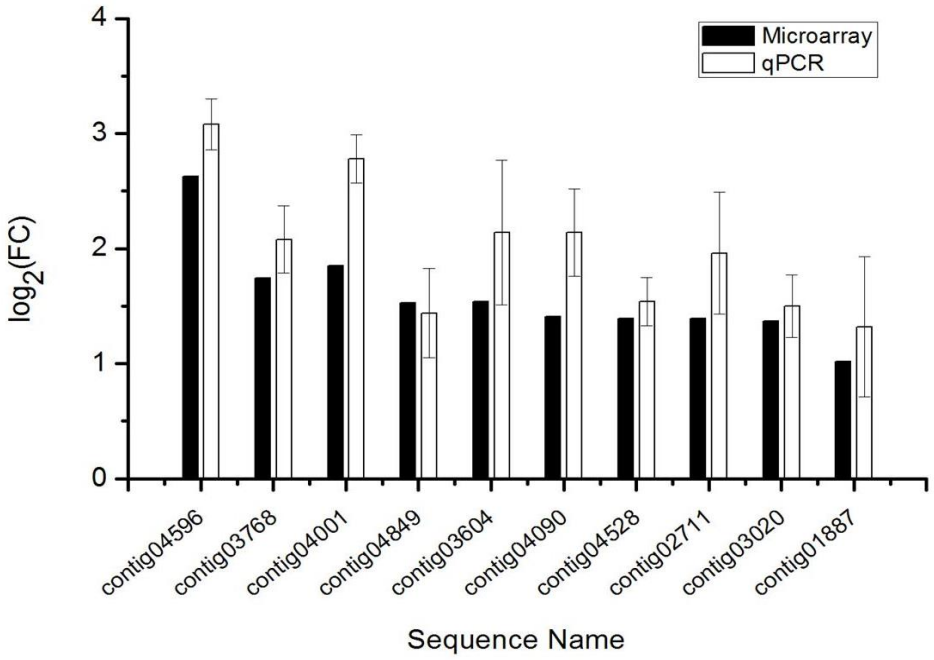

Figure 3.14 Microarray validation of selected putative detoxification and digestive genes up-regulated in larvae fed upon green olives compared to larvae fed upon artificial diet. Error bars represent the standard deviation of four biological replicates.

Meta- analysis of microarray results also allowed the comparison of expression levels of larvae developed in black olives to those developed in artificial diet. When the gene expression of larvae developed in black olives was compared with that of larvae developed in artificial diet, a total of 185 genes were differentially regulated, 86 of which had a predicted function. Sixty three genes (22 of them with a blast description) were up-regulated, while 122 (64 of them with a blast description) were down-regulated. Among the up-regulated genes with a predicted function, 2 serine proteases were identified (contig04596, contig02711) and 5 detoxification genes, including 2 genes encoding for a P450 (contig03604, contig10157), 2 encoding for a

GST (contig09042, contig07812) and an N-acetyltransferase (contig03020) (**Table 3.15**). The up-regulation of some of these genes was also confirmed by qPCR (**Figure 3.15**). The contigs 04596 an 02711 (encoding for serine proteases), 03604 (encoding for a P450) and 03020 (encoding for N-acetyltransferase) were also found up-regulated in the microarray comparison of larvae fed upon green olives versus artificial diet, potentially showing their association with adaptation of olive fruit fly to olives. The number of differentially transcribed genes upon development of larvae in black olives was relatively lower than that of larvae developed in green olives. This may reflect the composition of the artificial diet which is much closer to the ripe olives, as it contains high levels of hydrolyzed proteins (soy and yeast hydrolysates are used as amino acid sources), but not phenolic compounds.

Table 3.15 Over-expressed digestive and detoxification genes in larvae developed in green olives compared to larvae developed in artificial diet. The sequence name, the GenBank ID, the top blast hit description in the nr database, the hit accession number (AAC), the folds of up-regulation (in log_2) and the p-value are indicated. Gene expression values are the average of four biological replicate hybridizations.

Sequence Name	GenBank ID	Top BLAST hit description (NCBI nr database)	Hit AAC/ Species	Log_2(FC)	P-value
Digestion					
contig04596	GAKB01004596.1	trypsin-like serine protease precursor	P51588 (*Neobellieria bullata*)	2.07	0,000238595
contig02711	GAKB01002713.1	midgut-specific serine protease 1	NP_787956 (*D. melanogaster*)	1.14	0,015452759
Detoxification					
contig03604	GAKB01003604.1	cytochrome p450-28a1	XP_002089240 (*D. yakuba*)	2.19	0,028111227
contig10157	GAKB01010049.1	cytochrome p450	XP_001869955 (*C. quinquefasciatus*)	1.17	0,005104516
contig09042	GAKB01009014.1	glutathione s-transferase	AAD54938 (*M. domestica*)	1.16	0,017031325
contig07812	GAKB01007808.1	glutathione s transferase e10	XP_002074759 (*D. willistoni*)	1.11	0,027005205
contig03020	GAKB01003021.1	hypothetical conserved protein	XP_002056917 (*D. virilis*)	1.10	0,046910468

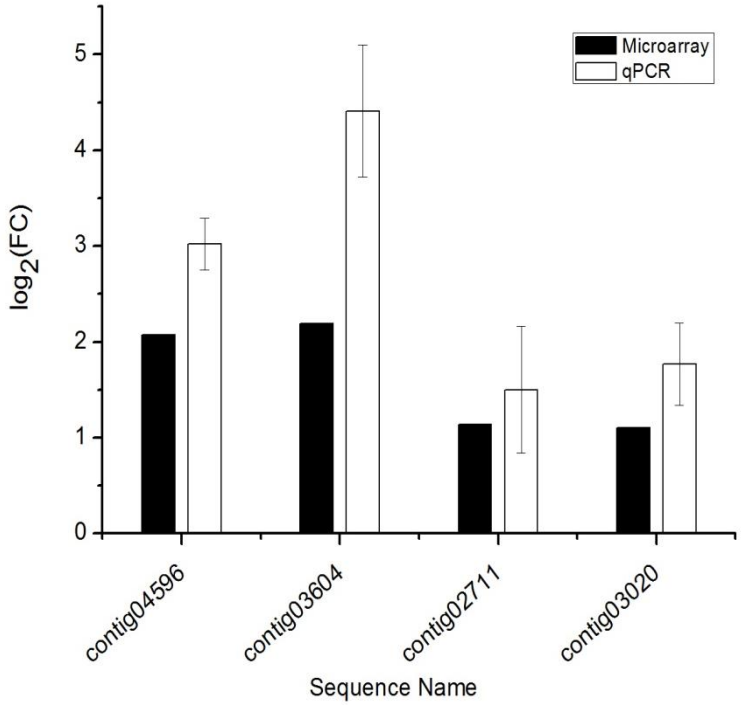

Figure 3.15 Microarray validation of selected putative detoxification and digestive genes up-regulated in larvae fed upon black olives compared to larvae fed upon artificial diet. Error bars represent the standard deviation of four biological replicates.

3.4.3 Conclusions

In this study genome-wide gene expression analysis in olive fruit fly larvae upon their development in green and black olives and artificial diet was conducted, for the first time. Genes possibly associated with the unique ability of *B. oleae* to develop in the olive fruits, especially in green immature olives, such as digestive genes as well as detoxification genes, were identified to be differentially regulated. In the comparison of larvae fed upon green olives versus larvae fed upon black olives, 24 digestive-related genes were found and 6 genes encoding for putative detoxification enzymes were also up-regulated. When the expression of larvae developed in green olives was compared with larvae developed in artificial diet, 9 putative serine-type proteases and 4 putative detoxification genes were up-regulated. Meta- analysis of microarray results also allowed the comparison of expression levels of larvae

developed in black olives to those developed in artificial diet. Among the up-regulated genes 2 serine proteases were identified as well as 5 detoxification genes.

The over-transcription of digestive enzymes could represent the mechanism of digestion of proteins in the olives flesh but also may be correlated to the adaptation to olives via protein degradation in order for larvae to cover energy and induction requirements[79, 80]. Increased peptidase activity may also serve as a mechanism for overcoming the negative effects of trypsin inhibitors induced by the olive fruit upon *B. oleae* attack[30]. The over-transcription of detoxification genes, in particular UGTs which were up-regulated in green olives, may be involved in the deactivation of phenolic compounds, mainly present in green olives. The over-expression of UGTs provides further indications and supports earlier studies that UGTs are implicated in the detoxification of plant phenols[81]. This analysis is a useful starting point for understanding the molecular mechanisms of *B. oleae* larvae adaptation to olives.

3.4.4 References

1. Zohary, D. The wild genetic resources of the cultivated olive. *Acta Hortic* **356** (1994).
2. Tzanakakis, M.E. Seasonal development and dormancy of insects and mites feeding on olive: a review. *Neth J Zool* **52** (2003).
3. Tzanakakis, M.E. Insects and Mites Feeding on Olive: Distribution, Importance, Habits, Seasonal Development and Dormancy (Brill Academic Publications, 2006).
4. Daane, K.M. & Johnson, M.W. Olive fruit fly: managing an ancient pest in modern times. *Annu Rev Entomol* **55**, 151-69 (2010).
5. Fletcher, B.S. The biology of dacine fruit flies. *Annu Rev Entomol* **32** (1987).
6. http://cisr.ucr.edu.
7. www.actaplantarum.org.
8. Vontas, J., Hernandez-Crespo, P., Margaritopoulos, J.T., Ortego, F. & Feng, H.-T. Insecticide resistance in Tephritid flies. *Pestic Biochem Physiol* **100**, 199-205 (2011).
9. Vontas, J.G. et al. Resistance-associated point mutations of organophosphate insensitive acetylcholinesterase, in the olive fruit fly Bactrocera oleae. *Insect Mol Biol* **11**, 329-36 (2002).
10. Kakani, E.G. et al. A small deletion in the olive fly acetylcholinesterase gene associated with high levels of organophosphate resistance. *Insect Biochem Mol Biol* **38**, 781-7 (2008).
11. Collier, T.R. & Van Steenwyk, R. Olive fruit fly in California: prospects for integrated control. *Calif Agric* **57**, 28-32 (2003).
12. Haniotakis, G.E. in WPRS Conference for Integrated Protection of Olive Crops (Chania, Crete, Greece, 2003).
13. Margaritopoulos, J.T. et al. Efficacy of the pyrethroid alpha-cypermethrin against Bactrocera oleae populations from Greece, and improved diagnostic for an iAChE mutation. *Pest Manag Sci* **64**, 900-8 (2008).
14. Roditakis, E. et al. in 7th meeting of the IOBC/wprs WG "Integrated Protection of Olive Crops" (Kalamata, Greece, 2015).

15. Loumou, A. & Giourga, C. Olive groves: ``The life and identity of the Mediterranean''. *Agric Hum val* **20** (2003).
16. www.faostat3.fao.org.
17. Amiot, M.J., Fleuriet, A. & Macheix, J.J. Accumulation of oleuropein derivatives during olive maturation. *Phytochemistry* **28** (1989).
18. Lauzon, C.R. Symbiotic relationships of tehritids, In Insect symbiosis (CRC Press, 2003).
19. Alagna, F. et al. Olive phenolic compounds: metabolic and transcriptional profiling during fruit development. *BMC Plant Biol* **12**, 162 (2012).
20. Ghanbari, R., Anwar, F., Alkharfy, K.M., Gilani, A.H. & Saari, N. Valuable nutrients and functional bioactives in different parts of olive (Olea europaea L.)-a review. *Int J Mol Sci* **13**, 3291-340 (2012).
21. Conde, C., Delrot, S. & Geros, H. Physiological, biochemical and molecular changes occurring during olive development and ripening. *J Plant Physiol* **165**, 1545-62 (2008).
22. Soler-Rivas, C., Epspin, J.C. & Wichers, H.J. Oleuropein and related compounds. *J Sci Food Agric* **80**, 1013-1023 (2000).
23. Andrews, M.-J., Busch, J., Joode, T.D., Groenewegen, A. & Alexandre, H. Sensory properties of virgin olive oil polyphenols: identification of deacetoxy-ligstroside agglycon as a key contributor topungency. *J Agric Food Chem* **51**, 1415-1420 (2003).
24. Hagen, K.S. Dependence of the olive fruit fly Dacus oleae larvae on symbiosis with Pseudomonas savastanoi for the utilization of olive. *Nat* **209** (1966).
25. Savva- Dimopoulou, C. & Fytizas, E. Accumulation des anides libres dans le mesocarpe de l olive apres conservation a basse temperature. *Canadian J Biochem* **45** (1967).
26. Fytizas, E. & Tzanakakis, M.E. Some effects of streptomycin, when added to the adult food, on theadultsof Dacus oleae (Diptera: Tephritidae) and their progeny. *Ann Entomol Soc Am* **59**, 269-273 (1966).
27. Manousis, T. & Ellar, D.J. Dacus oleae microbial symbionts. *Microbiol Sci* **5**, 149-52 (1988).
28. Tzanakakis, M.E. & Stavrinides, A.S. Inhibition of development of larvae of the olive fruit fly Dacus oleae (Diptera, Tephritidae) in olives treated with streptomycin. *Entomol Experim Applic* **16**, 39-47 (1973).
29. Ben-Yosef, M., Pasternak, Z., Jurkevitch, E. & Yuval, B. Symbiotic bacteria enable olive fly larvae to overcome host defenses. *R Soc open cci* (2015).
30. Corrado, G. et al. Molecular interactions between the olive and the fruit fly Bactrocera oleae. *BMC Plant Biol* **12**, 86 (2012).
31. Bolter, C. & Jongsma, M.A. The adaptation of insects to plant protease inhibitors. *J Insect Physiol* **43**, 885-895 (1997).
32. http://www.ncbi.nlm.nih.gov.
33. Karatolos, N. et al. Pyrosequencing the transcriptome of the greenhouse whitefly, Trialeurodes vaporariorum reveals multiple transcripts encoding insecticide targets and detoxifying enzymes. *BMC Genomics* **12**, 56 (2011).
34. Pauchet, Y. et al. Pyrosequencing the Manduca sexta larval midgut transcriptome: messages for digestion, detoxification and defence. *Insect Mol Biol* **19**, 61-75 (2010).
35. Myhre, S., Tveit, H., Mollestad, T. & Laegreid, A. Additional gene ontology structure for improved biological reasoning. *Bioinformatics* **22**, 2020-7 (2006).
36. Shen, G.M. et al. Transcriptome analysis of the oriental fruit fly (Bactrocera dorsalis). *PLoS One* **6**, e29127 (2011).
37. Edgar, R.C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**, 1792-7 (2004).
38. Zheng, W., Peng, T., He, W. & Zhang, H. High-throughput sequencing to reveal genes involved in reproduction and development in Bactrocera dorsalis (Diptera: Tephritidae). *PLoS One* **7**, e36463 (2012).
39. Hsu, J.C. et al. Discovery of genes related to insecticide resistance in Bactrocera dorsalis by functional genomic analysis of a de novo assembled transcriptome. *PLoS One* **7**, e40950 (2012).
40. http://expasy.org/tools/scanprosite.
41. Abascal, F., Zardoya, R. & Posada, D. ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* **21**, 2104-5 (2005).

42. Jobb, G., von Haeseler, A. & Strimmer, K. TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. *BMC Evol Biol* **4**, 18 (2004).
43. Tamura, K. et al. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731-9 (2011).
44. https://earray.chem.agilent.com/earry.
45. Tsoumani, K.T. et al. Isolation, annotation and applications of expressed sequence tags from the olive fly, Bactrocera oleae. *Mol Genet Genomics* **285**, 33-45 (2011).
46. Liu, F., Tang, T., Sun, L. & Jose Priya, T.A. Transcriptomic analysis of the housefly (Musca domestica) larva using parallel pyrosequencing. *Mol Biol Rep* **39** (2012).
47. Gomulski, L.M. et al. Gene discovery in an invasive tephritid model pest species, the Mediterranean fruit fly, Ceratitis capitata. *BMC Genomics* **9**, 243 (2008).
48. Tijet, N., Helvig, C. & Feyereisen, R. The cytochrome P450 gene superfamily in Drosophila melanogaster: annotation, intron-exon organization and phylogeny. *Gene* **262**, 189-98 (2001).
49. Feyereisen, E. Insect cytochrome P450. In Comprehensive Molecular Insect Science (Elsevier, 2005).
50. Ranson, H. et al. Evolution of supergene families associated with insecticide resistance. *Science* **298**, 179-81 (2002).
51. Strode, C. et al. Genomic analysis of detoxification genes in the mosquito Aedes aegypti. *Insect Biochem Mol Biol* **38**, 113-23 (2008).
52. Berenbaum, M.R. Postgenomic chemical ecology: from genetic code to ecological interactions. *J Chem Ecol* **28**, 873-96 (2002).
53. Karunker, I. et al. Over-expression of cytochrome P450 CYP6CM1 is associated with high resistance to imidacloprid in the B and Q biotypes of Bemisia tabaci (Hemiptera: Aleyrodidae). *Insect Biochem Mol Biol* **38**, 634-44 (2008).
54. Qiu, Y. et al. An insect-specific P450 oxidative decarbonylase for cuticular hydrocarbon biosynthesis. *Proc Natl Acad Sci U S A* **109**, 14858-63 (2012).
55. Li, X., Schuler, M.A. & Berenbaum, M.R. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* **52**, 231-53 (2007).
56. Tu, C.P. & Akgul, B. Drosophila glutathione S-transferases. *Methods Enzymol* **401**, 204-26 (2005).
57. Ding, Y., Ortelli, F., Rossiter, L.C., Hemingway, J. & Ranson, H. The Anopheles gambiae glutathione transferase supergene family: annotation, phylogeny and expression profiles. *BMC Genomics* **4**, 35 (2003).
58. Ranson, H. et al. Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector Anopheles gambiae. *Biochem J* **359**, 295-304 (2001).
59. Hayes, J.D., Flanagan, J.U. & Jowsey, I.R. Glutathione transferases. *Annu Rev Pharmacol Toxicol* **45**, 51-88 (2005).
60. Oakeshott, J.G. et al. Metabolic enzymes associated with xenobiotic and chemosensory responses in Nasonia vitripennis. *Insect Mol Biol* **19 Suppl 1**, 147-63 (2010).
61. Claudianos, C. et al. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. *Insect Mol Biol* **15**, 615-36 (2006).
62. Oakeshott, J., Claudianos, C., Campbell, P.M., Newcomb, R.D. & Russel, R.J. Biochemical genetics and genomics of insect esterases (Elsevier Oxford, 2005).
63. Labbe, R., Caveney, S. & Donly, C. Genetic analysis of the xenobiotic resistance-associated ABC gene subfamilies of the Lepidoptera. *Insect Mol Biol* **20**, 243-56 (2011).
64. Dassa, E. & Bouige, P. The ABC of ABCS: a phylogenetic and functional classification of ABC systems in living organisms. *Res Microbiol* **152**, 211-29 (2001).
65. Sturm, A., Cunningham, P. & Dean, M. The ABC transporter gene family of Daphnia pulex. *BMC Genomics* **10**, 170 (2009).
66. Liu, S. et al. Genome-wide identification and characterization of ATP-binding cassette transporters in the silkworm, Bombyx mori. *BMC Genomics* **12**, 491 (2011).
67. Tsitsipis, J.A. & Kontos, A. Improved solid adult diet for the olive fruit fly Dacus oleae. *Entomol Hellenica* **1** (1983).
68. Kauffmann, A., Gentleman, R. & Huber, W. arrayQualityMetrics--a bioconductor package for quality assessment of microarray data. *Bioinformatics* **25**, 415-6 (2009).

69. Benjamini, Y. & Hockber, Y. Multiple hypotheses testing with weights. *Scand J Stat*, 407-418 (1997).
70. http://www.ncbi.nlm.nih.gov/tools/primer-blast/).
71. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45 (2001).
72. Davies, T.G., Field, L.M., Usherwood, P.N. & Williamson, M.S. DDT, pyrethrins, pyrethroids and insect sodium channels. *IUBMB Life* **59**, 151-62 (2007).
73. Vontas, J. et al. Insecticide resistance in the major dengue vector Aedes albopictus and Aedes aegypti. *Pestic. Biochem. Physiol.* (2012).
74. Zhu, F. & Liu, N. Differential expression of CYP6A5 and CYP6A5v2 in pyrethroid-resistant house flies, Musca domestica. *Arch Insect Biochem Physiol* **67**, 107-19 (2008).
75. Zhu, F., Li, T., Zhang, L. & Liu, N. Co-up-regulation of three P450 genes in response to permethrin exposure in permethrin resistant house flies, Musca domestica. *BMC Physiol* **8**, 18 (2008).
76. Vontas, J., Ranson, H. & Williamson, M.S. A1 Addendum: Pyrethroid Insecticides and Resistance Mechanisms in Insect control, Biological and synthetic agents (Academic Press, 2010).
77. Bautista, M.A., Miyata, T., Miura, K. & Tanaka, T. RNA interference-mediated knockdown of a cytochrome P450, CYP6BG1, from the diamondback moth, Plutella xylostella, reduces larval resistance to permethrin. *Insect Biochem Mol Biol* **39**, 38-46 (2009).
78. Smyth, G.K. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**, Article3 (2004).
79. Pedra, J.H., McIntyre, L.M., Scharf, M.E. & Pittendrigh, B.R. Genome-wide transcription profile of field- and laboratory-selected dichlorodiphenyltrichloroethane (DDT)-resistant Drosophila. *Proc Natl Acad Sci U S A* **101**, 7034-9 (2004).
80. Ahmed, S., Wilkins, R.M. & Mantle, D. Comparison of proteolytic enzyme activities in adults of insecticide resistant and susceptible strains of the housefly Musca domestica L. *Insect Biochem Mol Biol* **28**, 629-639 (1998).
81. Luque, T., Okano, K. & O'Reilly, D.R. Characterization of a novel silkworm (Bombyx mori) phenol UDP-glucosyltransferase. *Eur J Biochem* **269**, 819-25 (2002).
82. Ahmad, S.A. & Hopkins, T.L. β-glycosylation of plant phenolics by phenol β- glucosyltransferase in larval tissues of the tobacco hornworm, Manduca sexta (L.). *Insect Biochem Mol Biol* 581-589 (1993).

Chapter 4

Functional characterization of GSTs associated with insecticide/acaricide resistance in *Tetranychus urticae*

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The functional expression and characterization as well as the interaction studies with acari/insecticides of TuGSTm09 were performed by Vasileios Tseliou (M.Sc. thesis).

Tetranychus urticae (Koch), the two-spotted spider mite (**Figure 4.1**), is one of the most damaging pests worldwide. It is an extremely polyphagous herbivore species, with more than 1,000 plant hosts including a number of economically important crops such as tomatoes, cotton, maize, sweet pepper, fruits and a wide range of ornamentals[1]. *T. urticae* damages its host plants during feeding, where uses a specialized stylet to penetrate into parenchyma cells removing chlorophyll and other cell ingredients[2, 3]. The removal of chlorophyll leads in the discoloration of leaf tissue (**Figure 4.1**) and the reduction of photosynthetic capacity and production of nutrients[3].

Figure 4.1. The two-Spotted Spider Mite (*T. urticae*) and the damage caused. Left: A female feeding on a bean leaf, SEM (Image by Wannes Dermauw and Stephane Rombauts[4]). Right: Leaf discoloration, after removal of chlorophyll, caused by *T. urticae* feeding (Image by Corey Eilhardt[5])

The use of chemical insecticides and acaricides is the major method for the control of *T. urticae* in the field. A wide variety of active ingredients of different chemical structures and mode of actions have been applied for its control. Among them, neurotoxic insecticides, such as pyrethroids, carbamates and organophosphates, specific acaricides, such as Mitochondrial Electron Transport Inhibitors (METIs), as well as novel compounds such as ketoenols[6-8]. However *T. urticae* develops resistance to pesticides very quickly. The frequent application of chemicals together with the small life cycle, the high fecundity, the inbreeding, the

arhenotokous reproduction and the high number of eggs per female individual resulted to the development of resistance to most pesticide groups[9]. The problem of insecticide resistance in *T. urticae* is enormous, as the species is the "most resistant species in terms of the total number of pesticides to which populations have become resistant"[10] (**Table 4.1[10]**).

Table 4.1. Top ten resistant arthropods (Table from Ref. 10).

Species	Taxonomy	Pest type	No. active ingredients	Cases
Tetranychus urticae	Acari: Tetranychidae	Crop	92	367
Plutella xylostella	Lepidoptera: Plutellidae	Crop	81	437
Myzus persicae	Homoptera: Aphididae	Crop	73	320
Leptinotarsa decemlineata	Coleoptera: Chrysomelidae	Crop	51	188
Musca domestica	Diptera: Muscidae	Urban	47	195
Blattella germanica	Blattodea: Blattellidae	Urban	43	213
Boophilus microplus	Acari: Ixodidae	Lifestock	43	151
Helicoverpa armigera	Lepidoptera: Noctuidae	Crop	42	608
Bemisia tabaci	Homoptera: Aleyrodidae	Crop	42	281
Panonychus ulmi	Acari: Tetranychidae	Crop	42	181

Glutathione S- transferases (GSTs) are a major family of detoxification enzymes mainly involved in phase II metabolism. They catalyze the conjugation of glutathione (GSH) to the electrophilic center of xenobiotics thus increasing their water solubility and aiding excretion from the cell[11]. The GSTs have been involved in insecticide detoxification by mediating the O-dealkylation or O-dearylation of organophosphorus insecticides[11, 12], catalyzing the dehydrochlorination of organochlorines[13], as well as primary insecticide metabolism or lipid peroxidation byproducts[14]. GSTs may also contribute to insecticide resistance by binding insecticide molecules (such as pyrethroids) via a sequestration mechanism[15]. Thirty one GSTs have been identified in the *T. urticae* genome, belonging to the classes delta (16 GSTs), mu (12 GSTs), omega (2 GSTs) and theta (1 GST)[16]. The role of TuGSTs as well as its potential implication in insecticide/acaricide resistance have not been studied yet.

4.1 Functional characterization of GSTs associated with resistance to abamectin

4.1.1. Introduction

4.1.1.1 Study of abamectin resistance in *T. urticae*

Abamectin (molecular formula: $C_{48}H_{72}O_{14}$, **Figure 4.2**) belongs to the family of avermectins and it is a natural fermentation product of the soil bacterium *Streptomyces avermitilis*[17] and it has been successfully used for the control of insects and mites[18], including *T. urticae*. It has neurotoxic activity by activating the invertebrate glutamate-gated chloride channels (GluCls)[19, 20] causing paralysis and eventually, death. It shows low toxicity to non-target beneficial arthropods, therefore it is suitable for Integrated Pest Management (IPM)[21]. Abamectin is applied in a large number of crops world-wide, such as ornamental plants, citrus, cotton and vegetable crops[21] representing one of the most important pesticides worldwide.

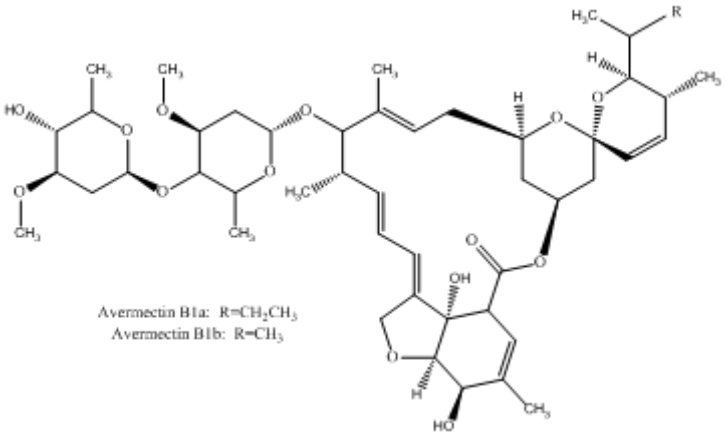


Figure 4.2. The chemical structure of abamectin.

Abamectin resistance in *T. urticae* is caused both from target site mutations and increased detoxification. Kwon et al[22] showed that a G323D point mutation in the glutamate-gated chloride channel is associated with moderate resistance levels (17-

fold). Phylogenetic analysis of the cys-loop ligand gated chloride channel family revealed that *T. urtica*e exhibits 6 orthologous GluCL genes, which may all be targets of abamectin and two point mutations, G314D in GluCL1 and G326E in GluCL3, were strongly associated with extremely high levels of abamectin resistance in Greek resistant strain (Mar-ab)[23].

Genome-wide gene expression analysis in Mar-ab strain, which was isolated from a rose greenhouse near Athens and exhibited >1600-fold resistance to abamectin, as well as high levels of resistance to other pesticides, identified a set of genes that are associated with the phenotype. This included several detoxification genes, such as several members of the cytochrome P450 mono-oxygenases (P450s) and the GST gene families[24]. Some of these genes were also up-regulated in another multi-resistant strain isolated from Belgium[24]. The functional role of some P450s present in the consensus has been already elucidated[25]. It has been shown that CYP392A16, which was highly over-expressed in Mar-ab strain compared to the susceptible strain, is capable of metabolizing abamectin *in vitro* resulting in a less toxic compound as confirmed by bioassay data[25]. However, the putative role of the GSTs, in conferring resistance against abamectin has not been investigated, although a strong association of elevated GST activity with abamectin resistance has been demonstrated in several occasions in *T. urticae*[26, 27]. For example, synergism data with the GST inhibitor diethyl maleate (DEM) have confirmed the implication of GSTs in abamectin resistance in mite strains from the Netherlands, Brazil and Colombia[27].

Two TuGSTs of the delta class, the TuGSTd10 and the TuGSTd14, and one of the mu class, the TuGSTm09, have been strongly associated with the abamectin/ multiple resistance phenotype[24]. Delta class GSTs have been involved in insecticide detoxification in insects[28], while mu class GSTs have been involved in detoxification of reactive oxygen species in mammals[29, 30]. The role of GSTs in conferring resistance against abamectin, has not been investigated, although a strong association of elevated GST activity with abamectin resistance has been demonstrated in several occasions in this pest[26].

4.1.1.2 Aim of the study

In this study, TuGSTd10, TuGSTd14 and TuGSTm09 were functionally expressed in *Escherichia coli* and their catalytic properties against model substrates, as well as their potential to interact with abamectin and other insecticides were examined.

4.1.2 Materials and Methods

4.1.2.1 Cloning, functional expression and purification of recombinant GSTs

The cDNA sequences encoding for TuGSTd10 (TeturID: tetur26g02802), TuGSTd14 (TeturID: tetur29g00220) and TuGSTm09 (TeturID: tetur05g05260) were amplified from Mar-ab cDNA[25]. For cDNA preparation, total RNA of adult spider mites was extracted using RNeasy mini kit (Qiagen, USA), treated with Turbo DNA-free (Ambion Life Technologies, USA) and reverse transcribed using Superscript III reverse transcriptase (Invitrogen Life Technologies, USA) and oligo(-dT)17 primer. For PCR amplification of TuGSTs, Pfu DNA polymerase (Thermo Scientific, USA) and specific primers (listed in **Table 4.2**) were used. PCR conditions were 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 sec, 61 °C for 30 sec and 60 °C for 30 sec. PCR products were cloned into pET100/D-TOPO vector (Invitrogen Life Technologies, USA, **Figure 4.3**), which allow expression of recombinant protein with an N-terminal 6x His tag, following manufacturer's instructions. Ligation reaction was used to transform DH5a competent cells and the resulting colonies were screened using cloning primers. Plasmid was extracted using NucleoSpin Plasmid (Macherey- Nagel, Germany) and 3 different clones were sent for sequencing. A clone of the correct DNA sequence was selected for downstream experiments.

Table 4.2. Primers used for the cloning of TuGSTs.

Gene	TeturID	Primer	Sequence (5'-3')	Prod.Size (bp)
Tu*GSTd10*	tetur26g02802	Tu*GSTd10*F	CACCATGTCAGTCCAATTATATCACCATAATC	660
		Tu*GSTd10*R	TTTCTC TTATGAATTTTTAGAAGCCATTGATTTGAGT AAATCTG	
Tu*GSTd14*	tetur29g00220	Tu*GSTd14*F	CACCATGGTGATTGAACTGTATCAAGTTCCC	642
		Tu*GSTd14*R	A TTAAAGTTTACTTTGAAGAAAATCTCGAAAT TCC	
Tu*GSTm09*	tetur05g05260	Tu*GSTm09*F	CACCATGGCACCAGTTATCGGTTATTGG	684
		Tu*GSTm09*R	TCAATATGGCTTTTGAATTGTGTCATTTCC	

The values presented in table are means of three independent experiments ± S.D. [a]Refers to the amount of product produced per minute per mg of the total enzyme at 25^{o}C. [b]One unit (U) is the amount of enzyme that catalyzes the reaction of 1 μmol of substrate per minute at 25^{o}C.

For heterologous expression of TuGSTd10 and TuGSTd14, *Escherichia coli* BL21(DE3) competent cells, harboring corresponding plasmids were grown at 37 °C in 2 lt LB containing 100 μg/ml ampicillin. The synthesis of GSTs was induced by the addition of 1 mM isopropyl β-D-1-thiogalactoside (IPTG) when the absorbance at 590 nm was at 0.7– 1. Four hours after induction, cells were harvested by centrifugation at 5,000 g for 20 min, re-suspended in sodium phosphate buffer (20 mM sodium phosphate buffer, 40 mM imidazole, 500 mM NaCl, pH 7.4), sonicated and centrifuged in 10,000 g for 30 min at 4 °C. The supernatant was collected and the GST was purified via Ni-NTA chromatography (Qiagen, USA) following manufacturer's instructions. For TuGSTm09, BL21(DE3) competent cells harboring corresponding plasmid were grown at 37 °C and when absorbance at 590 nm was at 0.7–1 the induction was performed with 0,5 mM IPTG for 4 hours at 28 °C. Protein concentration was determined by Bradford assay[31] and purity was judged by SDS-PAGE gel. In order to verify that recombinant GSTs are active, GST activity against 1-chloro-2, 4 dinitrobenzene (CDNB) was measured according to the method described in Ref. 32.

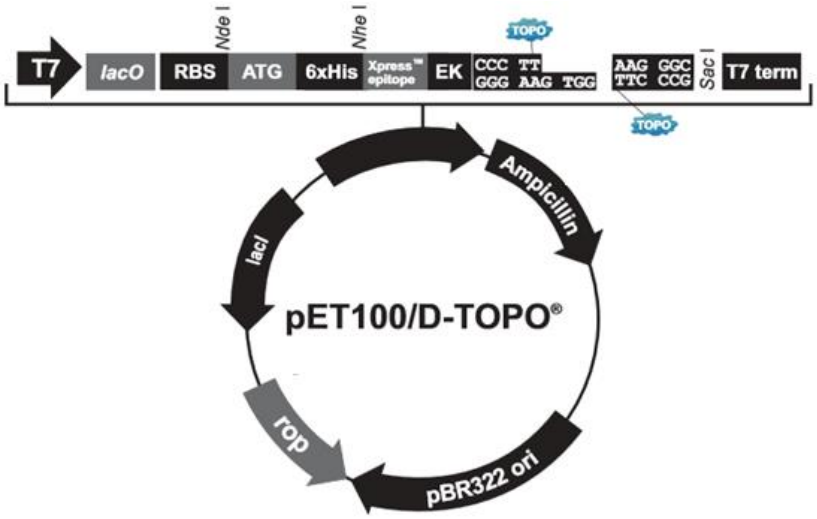


Figure 4.3. The pET100/D-TOPO vector (Invitrogen Life Technologies). The vector contains a T7 promoter (T7), followed by a lac operator (*lac*O), a ribosome binding site (RBS), an initiation ATG and a polyhistidine region (6xHis) which enable the production of recombinant protein with an N- terminal His tag. Vector enables inducible expression upon addition of IPTG.

4.1.2.2 Determination of substrate specificities for model substrates and kinetic studies

GST activity toward 1-chloro-2, 4 dinitrobenzene (CDNB, Sigma- Aldrich, UK) and 1, 2-dichloro-4-nitrobenzene (DCNB, Sigma- Aldrich, UK) was measured at 25 °C according to the method described in Ref. 32. Glutathione peroxidase activity was determined at 25 °C by coupling the reduction of Cumene hydroperoxide (CuOOH, Sigma Aldrich, UK) by GSH to the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) by oxidized glutathione disulfide (GSSG) with glutathione reductase according to the method described in Ref. 16. Kinetic measurements were performed at 25 °C in 0.1 M potassium phosphate buffer, pH 6.5. Initial velocities were determined in the presence of 2.475 mM GSH, and CDNB was used in the concentration range of 0.03–3 mM. Alternatively, CDNB was used at a fixed concentration (0.99 mM) and GSH was used in the concentration range of 0.075–15 mM. All the measurements were carried out in 96-well plates (NuncMaxiSorp, Thermo

Scientific, USA) using a SpectraMaxM2e multimode microplate reader (Molecular Devices, Berkshire, UK). The kinetic parameters k_{cat} and K_m were determined by fitting the steady-state data to the Michaelis–Menten equation using GraFit3 software (Ericathus Software Ltd., Version 3.06).

4.1.2.3 Enzyme–acaricides/insecticides interaction studies

The potential interaction of TuGSTs with abamectin (98.7% purity, 80% avermectin B1a/ 20% avermectin B1b, Sigma-Aldrich, UK), hexythiazox (99.9% purity, Sigma-Aldrich, UK), clofentezine (99.9% purity, Sigma-Aldrich, UK), bifenthrin (98.6% purity, Sigma-Aldrich, UK) and pyridaben (99.7% purity, Sigma-Aldrich, UK), active ingredients which showed reduced toxicity against the multi-resistant Mar-ab *T. urticae* strain[25], was determined by analyzing the inhibition of activity toward CDNB, in the presence of 0.05 mM of each acaricide/insecticide (in 5–10% final concentration of methanol or acetone). In the case of TuGSTd14 and its interaction with abamectin, for IC50 calculation, percentage inhibition of TuGSTd14 activity was determined at different concentrations of abamectin (in a range of 12.5–200 μM) in the presence of 10% methanol and 0.99 mM CDNB (concentration below K_m). IC50 was determined using Grafit3 software (Ericathus Software Ltd., Version 3.06). Dixon plot analysis was performed at 3 different concentrations of CDNB (0.1, 1 and 2 mM), using 4 different concentrations of abamectin (0, 25, 50, 100 μM) in the presence of 10% methanol in order to determine the type of inhibition. All the measurements were carried out in 96-well plates (NuncMaxiSorp, Thermo Scientific, UK) using a SpectraMaxM2e multimode microplate reader (Molecular Devices, Berkshire, UK) at 25 °C based on the method described in Ref. 32.

4.1.3 Results and Discussion

4.1.3.1 Cloning, heterologous expression and purification of TuGSTs

The sequences encoding for TuGSTd10, TuGSTd14 and TuGSTm09 were amplified from cDNA template prepared from RNA isolated from adults of Mar-ab strain. Coding sequences were successfully cloned into pET100/D-TOPO vector (Invitrogen Life Technologies, USA, **Figure 4.2**). The corresponding constructs were sequenced and it was ensured that no errors had been introduced during PCR amplification. Induction of the constructs with 1 mM IPTG for 4 hours at 37 °C in *E. coli* expression cells resulted in good levels of protein production. The majority of recombinant TuGSTd10 and TuGSTd14 were found to be in the soluble fraction; however, TuGSTm09 was primarily expressed at the insoluble fraction (inclusion bodies). By reducing the concentration of IPTG to 0.5 mM and the induction temperature to 25 °C we managed to obtain sufficient protein production sequestered in the soluble fraction. All three TuGSTs were purified successfully using metal chelate affinity chromatography (see indicative SDS-PAGE gel, **Figure 4.4**) and the recombinant proteins were found to be catalytically active.

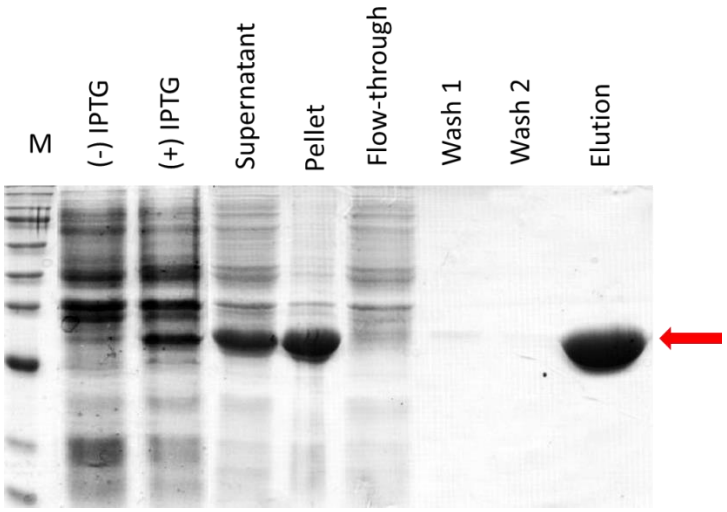


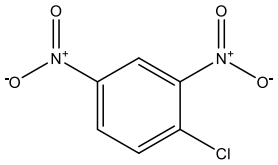
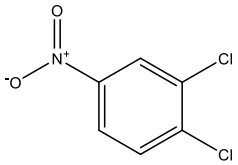
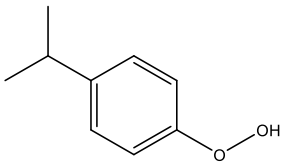
Figure 4.4. SDS-PAGE analysis of the GST expression and purification procedure. The expression and purification of TuGSTmd10 (red arrow) is shown. The visualization of protein bands occurred using Coomasie Brilliant Blue R-250. M: protein marker (BlueStar Prestained Protein Marker, GmbH), (-) IPTG: bacterial lysate before the addition of IPTG, (+) IPTG: bacterial lysate after 4 hours induction with IPTG, supernatant: soluble fraction after cell lysis

by sonication, pellet: insoluble fraction after cell sonication, Washes 1, 2: washes of Ni-NTA column with binding buffer, Elution: fraction containing the purified TuGST.

4.1.3.2 Substrate specificities and kinetic properties of recombinant TuGSTd10, TuGSTd14 and TuGSTm09

Recombinant TuGSTs were assayed toward selected model substrates to investigate if they exhibit glutathione transferase activity (toward CDNB and DCNB) and glutathione peroxidase activity (toward CuOOH). The results are presented in **Table 4.3**.

Table 4.3. Substrate specificities of TuGSTd10, TuGSTd14 and TuGSTm07.

		Specific activity[a] (Unit[b]/mg)		
Substrate	**Structure**	**TuGSTd10**	**TuGSTd14**	**TuGSTm09**
1-Chloro-2,4dinitrobenzene (CDNB)		1.10 ± 0.25	0.69 ± 0.05	15.94 ± 0.70
1,2-Dichloro-4-nitrobenzene (DCNB)		0.01 ± 0.00	0.08 ± 0.01	0.09 ± 0.00
Cumene hydroperoxide (CuOOH)		0.34 ± 0.16	0.10 ± 0.01	3.34 ± 0.39

The values presented in table are means of three independent experiments ± S.D. [a]Refers to the amount of product produced per minute per mg of the total enzyme at 25°C. [b]One unit (U) is the amount of enzyme that catalyzes the reaction of 1 µmol of substrate per minute at 25°C.

All three recombinant TuGSTs were active as they were capable of conjugating both CDNB and DCNB substrates to GSH. TuGSTd10 and TuGSTd14 displayed low specific activities for CDNB, compared to other delta GSTs derived from insects, such as, the AgGSTd1-5 (56.44 ± 8.7 µmol/min/mg) and the AgGSTd1-6 (195 ± 11.9 µmol/ min/mg) from *Anopheles gambiae* and the AdGSTd1 (174 ± 4.86 µmol/ min/mg) and the AdGSTd2 (39.9–43.3 µmol/min/mg) from *Anopheles dirus*[33]. The specific activity of the TuGSTd10 and TuGSTd14 for DCNB was also lower compared to *A. gambiae* GSTd1-5 (0.33 ± 0.03 µmol/min/mg), GSTd1-6 (0.64 ± 0.03 µmol/ min/mg) and *A. dirus* GSTd1 (0.28 ± 0.01 µmol/min/mg) and GSTd2 (0.08 ± 0.01 µmol/min/mg)[33]. Peroxidase activities of the TuGSTd10 and TuGSTd14 are comparable to the other delta GSTs (<0.13 µmol/min/mg for *A. gambiae* GSTd1-5, 0.98 ± 0.06 µmol/min/mg for *A. gambiae* GSTd1-6, 0.65 ± 0.06 µmol/min/mg for *A. dirus* GSTd1[33] .

The specific activity of the TuGSTm09 for CDNB and DCNB is lower compared to other mu class GSTs from the cattle tick, *Boophilus annulatus* (121 µmol/min/mg for CDNB and 29.3 µmol/min/mg for DCNB, respectively[34]), but similar compared to mu class GSTs from humans[35]. The peroxidase activity of the TuGSTm09 is lower than the respective activity of the mu tick GST (62.4 µmol/min/mg[34]), but higher compared to human mu class GSTs (1.3 and 0.63 µmol/min/mg[35].

The steady-state kinetics was subsequently determined for all three GSTs (**Table 4.4**, **Figure 4.5**). The K_m values of TuGSTd10 and TuGSTd14 and TuGSTm09 for GSH are comparable with the corresponding affinities of GSTs from another mite species, *i.e. Sarcoptes scabiei* (0.50 ± 0.10 mM for ScGSTD1-1, 0.70 ± 0.20 mM for ScGSTD2-2, 0.3 mM for ScGSTD3-3; 0.30 mM for ScGSTM1-1 and 0.40 mM for GSTM2-2[36]). The K_m values for CDNB are also comparable to the ScGSTs (0.30 mM for ScGSTD1-1, 0.40 ± 0.10 mM for ScGSTD2-2, 1.2 mM for ScGSTD3-3, 0.30 mM for ScGSTM1-1 and 4.20 ± 0.10 Mm for GSTM2-2[36]). The k_{cat} values of TuGSTd10 and TuGSTd14 as well as their k_{cat}/k_m ratio values are higher for both GSH and CDNB in comparison with the delta ScGSTs[36]. TuGSTm09 exhibits a notably higher catalytic activity, for both GSH and CDNB, compared with the two mu class ScGSTs previously characterized (k_{cat} values for GSH: 0.15 min−1 for ScGSTM1-1, 0.06 min−1 for ScGSTM2- 2, and k_{cat} values for CDNB: 0.17 ± 0.01 min−1 for ScGSTM1-1, 0.10 min−1 for ScGSTM2-2[36]). The catalytic

effectiveness (k_{cat}/k_m) of TuGSTm09 was also remarkably higher, for both GSH and CDNB substrates, compared to the two mu class SsGSTs.

Table 4.4.Steady-state kinetics of TuGSTd10,TuGSTd14 and TuGSTm09.

Kinetic parameter	TuGSTd10	TuGSTd14	TuGSTm09
K_m (mM) GSH	0.33 ± 0.05	3.79 ± 0.69	2.34 ± 0.31
K_m (mM) CDNB	0.84 ± 0.13	1.69 ± 0.24	0.26 ± 0.04
k_{cat} (min^{-1}) GSH	1.59 ± 0.06	1.22 ± 0.10	23.4 ± 1.52
k_{cat} (min^{-1}) CDNB	3.06 ± 0.23	1.78 ± 0.25	14.7 ± 0.85
K_{cat}/K_m ($mM^{-1} \cdot min^{-1}$) GSH	4.72	0.32	10
K_{cat}/K_m ($mM^{-1} \cdot min^{-1}$) CDNB	3.64	1.08	56.53

All values are means ± S.D. of three independent experiments. Results were determined by varying the concentration of GSH (0.075-15 mM) and CDNB (0.03-3 mM) at fixed concentrations of CDNB (0.99 mM) and GSH (2.47 mM) respectively.

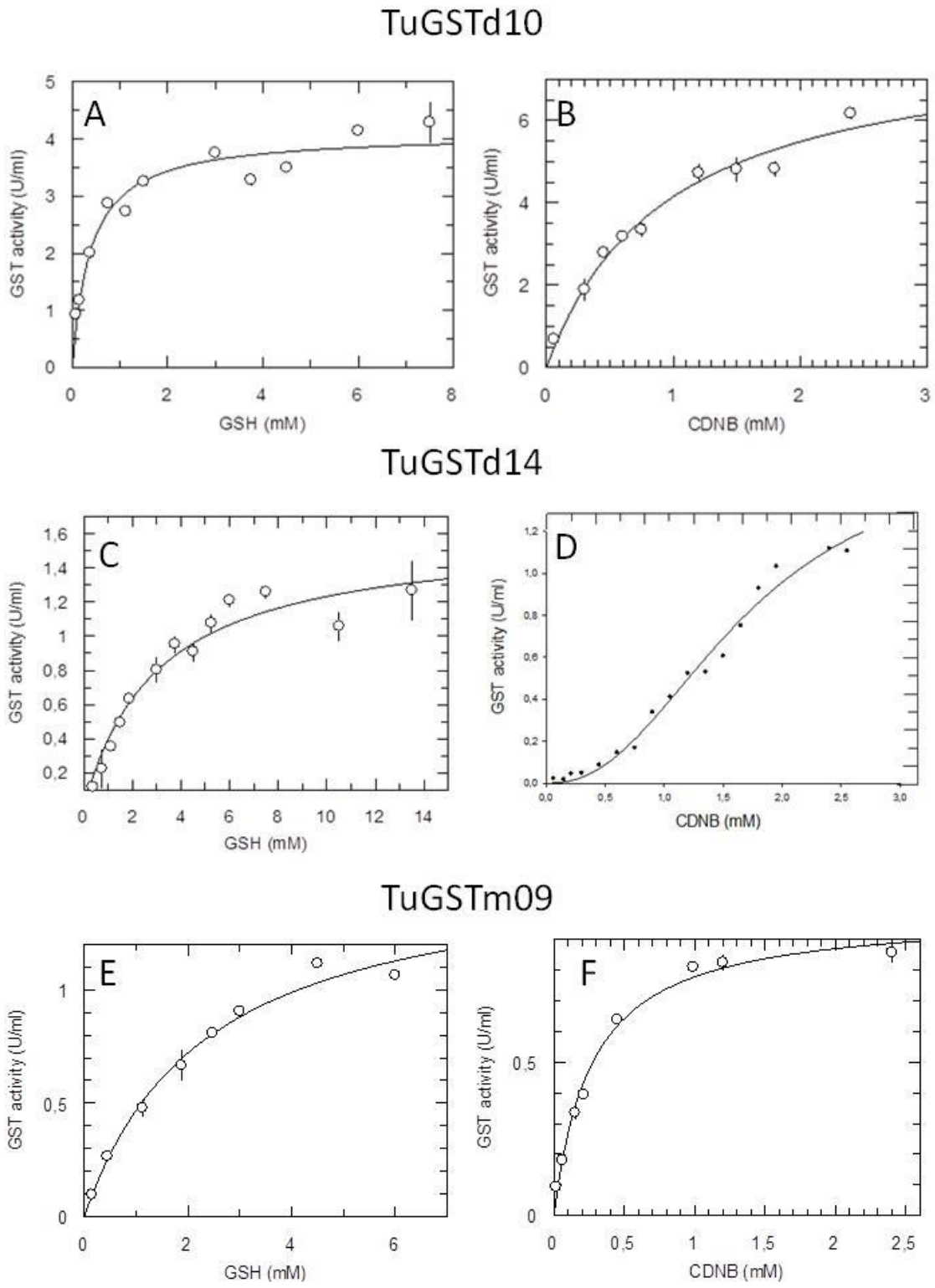
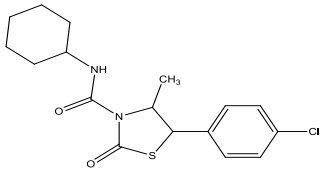
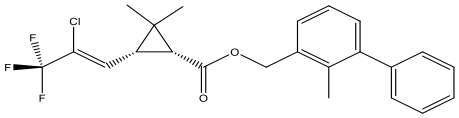
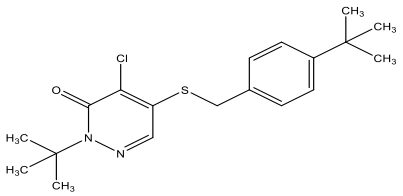
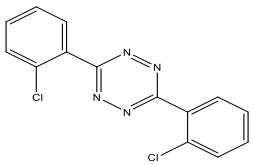
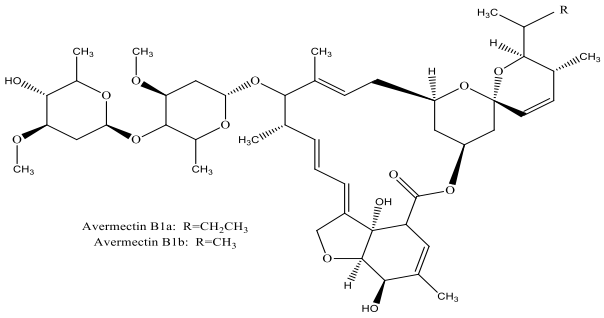


Figure 4.5. Kinetic analysis of TuGSTd10 (A, B), TuGSTd14 (C, D) and TuGSTm09 (E,F). A, C, E: kinetic analysis using the GSH as a variable substrate (0.075-15 mM) and a fixed concentration of CDNB (0.99 Mm). **B, D, F**: kinetic analysis using CDNB as a variable substrate (0.03-3 mM) and GSH at a fixed concentration (2.47 mM).

4.1.3.3 Enzyme–acaricide/insecticide interaction studies

Inhibition assays were performed, in order to investigate the possible interaction of the three recombinant enzymes with the acaricides/insecticides that show reduced toxicity against Mar-ab strain (*i.e.* hexythiazox, pyridaben, bifenthrin, clofentezine and abamectin)[25]. All acaricides/insecticides were used in a 0.05 mM final concentration and the percentage inhibitions in the activity of TuGSTs toward CDNB are presented in **Table 4.5**. With the exception of bifenthrin that caused 7.27 ± 1.70% inhibition, none of the acaricides/insecticides exhibited significant levels of inhibition of the TuGSTd10 activity under assay conditions, indicating that TuGSTd10 may not strongly interact with any of these active ingredients. Similarly, bifenthrin and abamectin did not cause any inhibitory effect in TuGSTm09 under assay conditions but hexythiazox, pyridaben and bifenthrin caused a low inhibition of 12.94 ± 3.74%, 11.21 ± 3.00% and 28.09 ± 4.84%, respectively. Pyridaben and bifenthrin did not inhibit the TuGSTd14 CDNB conjugating activity, while hexythiazox caused 11.21 ± 3.00% and clofentezine 28.08 ± 4.84% inhibition. However, the strongest interaction– inhibition among the active ingredients and the recombinant TuGSTs included in this study was recorded for abamectin against TuGSTd14. Based on these data as well as the strong association of GSTs with abamectin resistance in several studies in the past[26], we focused our subsequent analysis on the TuGSTd14–abamectin interaction.

Table 4.5. Percentage inhibition of TuGSTd10, TuGSTd14 and TuGSTm09 activity by selected acaricides/insecticides.

Acaricide/ Insecticide	Structure	Inhibition of enzyme activity (%) TuGSTd10	TuGSTd14	TuGSTm09
Hexythiazox		n.d.	11.21 ± 3.00	12.94 ± 4.55
Pyridaben		n.d.	n.d.	25.84 ± 14.00
Bifenthrin		7.27 ± 2.70	n.d.	n.d.
Clofentezine		n.d.	28.05 ± 4.84	27.68 ± 2.99
Abamectin		n.d.	38.46 ± 3.79	n.d.

All values are means ± S.D. of three independent experiments. Enzymes were assayed using GSH and CDNB as substrates and acaricides/insecticides were used in a 0.05 mM final concentration.n.d.: not detected (under assay conditions).

To evaluate the inhibitory potential, the concentration of abamectin needed to inhibit the CDNB conjugating activity by half (IC50) was calculated by a dose–response curve (**Figure 4.6 A**) and was determined at 88.47 ± 7.55 μM, showing significant inhibition. Dixon plot analysis with varying CDNB or abamectin concentrations was performed in order to define the type of inhibition (**Figure 4.6 B**). The resulting three linear curves intersect above the x axis proving that the inhibition is competitive and the inhibition constant (K_i) was determined at 34.06 ± 0.68 μM. The Ki is almost 50-fold lower than Km for CDNB (**Table 4.4**), indicating that most likely abamectin is a strong inhibitor. Interestingly, the competitive type of inhibition implies that abamectin competes with CDNB for the same site, thus binds adjacent to the H-site of the enzyme.

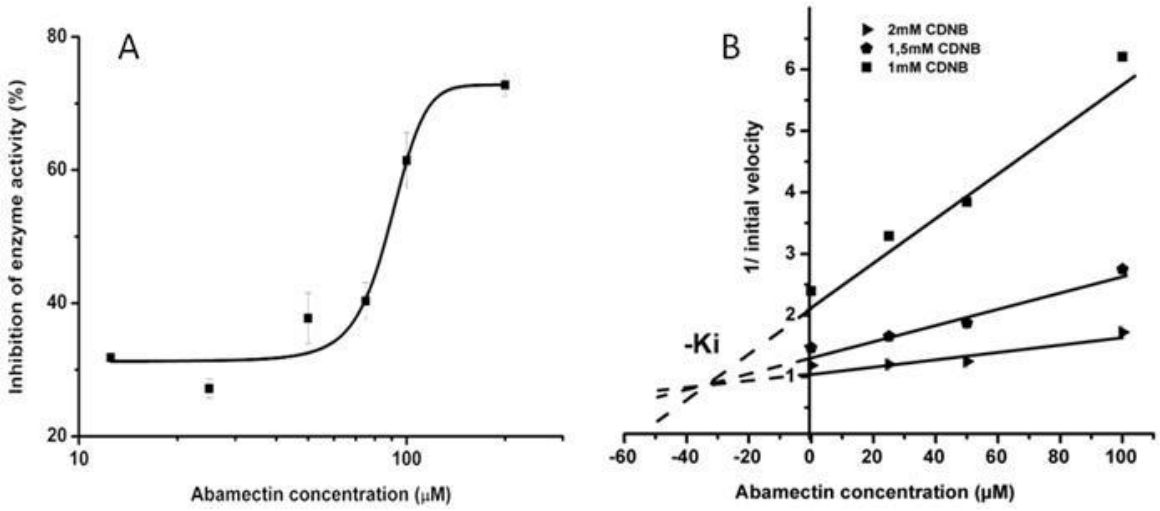


Figure 4.6. A: Dose-response curve for the inhibition of CDNB conjugating activity of TuGSTd14 by different abamectin concentrations. Six different concentrations of abamectin (12.5-200 μM) and 0.99 mM CDNB were used and data are mean of three replicates ± S.D. **B: Dixon plot analysis for the inhibition of CDNB conjugating activity of TuGSTd14 by different abamectin concentrations.** Three different concentrations of CDNB (0.1, 1 and 2mM) and 4 different concentrations of abamectin (0, 25, 50, 100μM) were used and data are mean of three replicates ± S.D. Analysis denoted a competitive type of inhibition and the K_i was determined at 34.06 ± 0.68 μM.

4.1.4 Conclusions

This study provided further evidence and supported earlier work that GSTs are likely to play a role in abamectin resistance in *T. urticae*[26, 27]. However further studies on the metabolic fate of abamectin in resistant and susceptible spider mites are needed in order to provide functional evidence for a catalytic interaction of abamectin with arthropod GSTs, resulting in conjugated metabolites, which seem to be elusive so far. We cannot exclude that the observed competitive inhibition of CDNB conjugation by abamectin is due to its binding to regions adjacent the active site, *i.e.* not directly interfering with the substrate recognition site.

4.2. Functional characterization of TuGSTd05 associated with resistance to cyflumetofen

4.2.1 Introduction

4.2.1.1 Study of cyflumetofen resistance in *T.urticae*

Cyflumetofen (molecular formula: $C_{24}H_{24}F_3NO_4$) is a novel benzoyl acetonitrile acaricide developed by Otsuka AgriTechno Co., Ltd.[37] (**Figure 4.7**). It was first applied in the field in 2007. It shows activity against a number of *Tetranychus* and *Panonychus* mites such as *T. urticae* and *Panonychus citri* and displays no- effects in non target beneficialal organisms such as predators and honey bees[37], making it suitable for Integrated Pest Management (IPM). Cyflumetofen represents an acaricide with a novel mode of action and it has been shown that acts as an inhibitor of the complex II in the mitochondrial electron transport chain[37, 38].

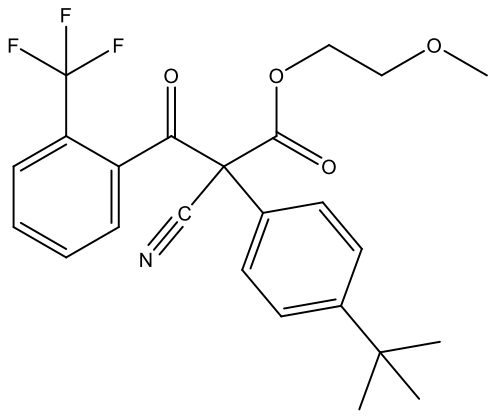


Figure 4.7. The chemical structure of cyflumetofen.

Cyflumetofen is a very new acaricide and has not been registered in Europe yet. Therefore, with the exception of cross-resistance[39], resistance cases have not been recorded as yet in the field. Recently, a mite strain was selected for cyenopyrafen

(complex II inhibitor) in the laboratory using a field collected parental strain which was susceptible to inhibitors of complex II, and selection also conferred cross-resistance to cyflumetofen[40]. Resistance ratio (RR) for cyflumetofen was calculated equal to 21.79 compared to the susceptible strain[40]. Diethyl maleate (DEM), a synergist which specifically inhibits the GSTs, strongly synergized the majority of cyflumetofen resistance, which suggests the involvement of GSTs in the resistance phenotype[40]. Subsequent gene expression analysis, by microarrays as well as real-time qPCR, identified the gene Tu*GSTd05* as one of the most over-expressed genes.

4.2.1.2 Aim of the study

In this study, the functional expression of the Tu*GSTd05* in *E. coli* and the examination of catalytic properties against model substrates as well as the study of its interaction with cyflumetofen was conducted, aiming to investigate the capability of TuGSTd05 to metabolize cyflumetofen *in vitro*.

4.2.2 Materials and methods

4.1.2.1 Cloning, functional expression and purification of recombinant TuGSTd05

A Pfu DNA polymerase (Thermo scientific, USA) was used to PCR amplify the Tu*GSTd05* gene derived from the resistant strain, using the primers 5'-CACCATGGTCATCGAACTGTAC-3' (forward) and 5'-TTAACCAAGCCTGGCCCGAA-3' (reverse). PCR conditions were 94^{o}C for 2 min, followed by 34 cycles of 94^{o}C , 55^{o}C for 30 sec, 72^{o}C for 1 min. PCR product was purified using NucleoSpin Extraxt II kit (Macherey-Nagel) and directly cloned into pET100/D-TOPO (Invitrogen Life Technologies, **Figure 4.2**/ Chapter 4.1). The expression and purification of recombinant protein was performed according to the procedure described in 4.2.1.1.

4.1.2.2 Site- directed mutagenesis of TuGSTd05

The TuGSTd05-pET100/D-TOPO construct was used as the template for amplification reactions with QuikChange II XL Site- Directed Mutagenesis Kit (Agilent Life Technologies) according to manufacturer's instructions. For the generation of Tyr107 to Phe (TuGSTd05 Y107F mutant) the following primers were used (mismatches are underlined): 5'-CCAAGAGAGGCGAAGAGTGATCCATTATCCCAGTAA-3' (forward) and 5'-TTACTGGGATAATGGATCACTCTTCGCCTCTCTTGG-3' (reverse). PCR reactions were treated with DpnI (provided by QuikChange II XL Site- Directed Mutagenesis Kit) to digest the paternal methylated constructs and the remaining plasmids were transformed in TOP10 competent cells. Plasmid were extracted from several colonies using NucleoSpin Plasmid (Macherey-Nagel, Germany) and sent for sequencing (StarSeq, Germany) to verify the presence of the desired mutations and the absence of spontaneous ones. A clone of the correct DNA sequence for the mutant was selected for downstream experiments. Functional expression and purification of TuGSTd05 Y107 was performed as described in section 4.2.1.1.

4.2.2.3 Determination of substrate specificities for model substrates and kinetic studies

Substrate specificities and kinetics were determined as described in section 4.2.1.2. For kinetic measurements of TuGSTd05, initial velocities were determined in the presence of 2.475 mM GSH and CDNB was used in the concentration range of 0.03-0.75 mM, while when CDNB was used at a fixed concentration (0.99 mM), GSH was used in the concentration range 0.075-1.5 mM.

4.2.2.4 Study of the interaction with cyflumetofen

The potential interaction of TuGSTd05 and TuGSTd05 Y107F with cyflumetofen (94.9% purity, Sigma-Aldrich, UK) was determined by analyzing the inhibition of activity toward 1-chloro-2, 4 dinitrobenzene (CDNB) in the presence of 0.05mM cyflumetofen (in 10% final concentration of methanol). For IC50 calculation, percentage inhibition of activity was determined at different concentrations of cyflumetofen, in the presence of 10% methanol and 0.99 mM CDNB. Cyflumetofen was used in a range of 0.01- 50 µM for TuGSTd05, 6.25- 300 µM for TuGSTd05 Y107F. Measurements and data analysis were conducted as described in section 4.1.2.3.

4.2.2.5 HPLC-MS analysis

For the identification of possible cyflumetofen-GSH conjugates 0.04 mg of TuGSTd05 were incubated with 100 µM of cyflumetofen and 2.5 mM of GSH at 30^{o}C. Samples were incubated for 0 and 1 hours. As negative controls reaction mixtures without enzyme and reaction mixtures without cyflumetofen were incubated for 0 and 1 hour. Experiment was performed in 3 independent replicates.

Prior to the HPLC-MS analysis, the samples were desalted with solid phase extraction (Bond Elut LRC-C18, 200 mg cartridges, Agilent, USA) as follows: initially the cartridges were pre-conditioned with 2 ml acetonitrile, followed by 3 ml 2.5% acetonitrile in water and then samples were diluted to 15 ml with water and loaded to the cartridges; cartridges were subsequently washed with 1 ml water and samples were eluted with 1.3 ml acetonitrile. Eluents were transferred to HPLC autosampler vials and 0.7 ml water was added. Sample injections (20 µl loop) were performed via a Surveyor Autosampler (Thermo Finnigan, USA). The chromatographic separation was achieved using a Surveyor LC system (Thermo Finnigan, USA), equipped with a Gemini C18 (3 µm, 100 mm × 2 mm) analytical column (Phenomenex, USA). Gradient elution was applied with 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in nanopure water (B) for 18.5 min (0-1 min 10% A, 15-16 min 100% A, 16.1-18 min 10% A). Flow rate was set at 200 µl/min. The mass detection was achieved with a TSQ

Quantum triple quadrupole (Thermo Finnigan, USA) with positive electrospray ionisation (ESI) as ionisation source. Mass spectrometer was operated in single ion monitoring, full scan, neutral loss and product ion scan modes. The system was controlled by the Xcalibur software, which also used for the data acquisition and analysis. The optimum mass spectrometer parameters were set as follows: spray voltage at 5000 V, sheath gas pressure at 35 arbitrary units (au), auxiliary gas pressure at 10 au, capillary temperature at 300 °C, source collision induced dissociation at 26 eV, collision gas pressure at 2 mTorr and collision energy at 25 eV. Sheath/auxiliary gas was high purity nitrogen and collision gas was high purity argon.

HPLC-MS experiments were conducted in collaboration with Environmental Chemical Processes Laboratory, Chemistry Department, University of Crete (Dr. Antonios Myridakis and Prof. E.G. Stephanou).

4.2.2.5 Molecular modeling of TuGSTd05 and docking of cyflumetofen in to TuGSTd05

The protein sequence of TuGSTd05 was modeled using the Swiss-model server[41] and the 3vk9.1.A (UniProt) was used as the template for model construction. The global model quality score (GMQS) was estimated 0.75. Molecular docking was carried out using the Swiss-Dock server[42]. Blind docking was conducted using default parameter settings. The model and docking were viewed using the program PyMOL[43].

4.2.3 Results and discussion

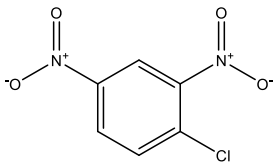
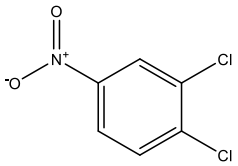
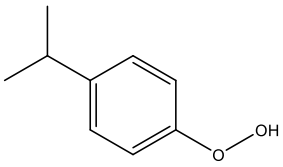
4.2.3.1 Cloning, heterologous expression and purification of TuGSTd05

The coding sequence of Tu*GSTd05* was successfully amplified and cloned into pET100/D-TOPO vector (Invitrogen Life Technologies, USA). Protein expression was induced with 1 mM IPTG for 4 hours at 37^{o}C in *E. coli* BL21(DE3) cells, resulting in good yields of protein production. The majority of the recombinant TuGSTd05 was found in the soluble fraction from which it was successfully purified using Ni-NTA chromatography. The recombinant TuGSTd05 was found to be catalytically active against the model substrate CDNB.

4.2.3.2 Substrate specificities and kinetic properties of recombinant TuGSTd05

TuGSTd05 was assayed against the model substrates CDNB and DCNb in order to examine if it exhibits glutathione transferase activity as well as against the model substrate CuOOH in order to examine if exhibits peroxidase activity (**Table 4.6**). TuGSTd05 displays glutathione transferase activity as it is capable of conjugating CDNB and DCNB to GSH. The specific activity for CDNB was remarkably higher compared to the other *T. urticae* delta GSTs previously characterized, TuGSTd10 (1.10 ± 0.25 µmol/min/mg) and TuGSTd14 (0.69 ± 0.05 µmol/min/mg) (Chapter 4.1), and similar to other delta GSTs from insects, such as the mosquitoes *A. gambiae* and *A. dirus*[33]. The specific activity for DCNB was also higher than the respective activities of TuGSTd10 (0.01 ± 0.00 µmol/min/mg) and TuGSTd14 (0.08 ± 0.01 µmol/min/mg) (Chapter 4.1) and comparable with the mosquitoes delta GSTs[33]. TuGSTd05 displayed activity against CuOOH which was also higher than that of TuGSTd10 (0.34 ± 0.16 µmol/min/mg) and TuGSTd14 (0.10 ± 0.01 µmol/min/mg) (Chapter 4.1) as well as the *A. gambiae* and *A. dirus* delta GSTs[33].

Table 4.6. Substrate specificities of TuGSTd10, TuGSTd14 and TuGSTm07.

		Specific activity[a] (Unit[b]/mg)
Substrate	**Structure**	**TuGSTd05**
1-Chloro-2,4dinitrobenzene (CDNB)		103.18 ± 8.03
1,2-Dichloro-4-nitrobenzene (DCNB)		0.56 ± 0.12
Cumene hydroperoxide (CuOOH)		1.00 ± 0.49

The values presented in table are means of three independent experiments ± S.D. [a]Refers to the amount of product produced per minute per mg of the total enzyme at 25oC. [b]One unit (U) is the amount of enzyme that catalyzes the reaction of 1 µmol of substrate per minute at 25^{o}C.

The steady-state kinetic parameters of TuGSTd05 for the CDNB conjugating reaction were also determined and results are shown in **Table 4.7/ Figure 4.8**. TuGSTd05 displayed higher affinity for both GSH and CDNB compared to the other *T. urticae* delta GSTs. K_m value for GSH is significantly lower than that of TuGSTd10 (0.33 ± 0.05 mM) and TuGSTd14 (3.79 ± 0.69 mM) (Chapter 4.1) as well as lower compared to the K_m values determined for delta GSTs of the mite species *S. scabiei* [36]. Similarly TuGSTd05 displayed a lower K_m value for CDNB compared to TuGSTd10 (0.84 ± 0.13 mM) and TuGSTd14 (1.69 ± 0.24 mM) and *S. scabiei* GSTs[36]. TuGSTd05 exhibits a notably higher catalytic activity (k_{cat} value), for both GSH and CDNB, compared with the 2 delta TuGSTs and ScGSTs[36] (see also chapter 4.1). The catalytic effectiveness

(K_{cat}/k_m) of TuGSTd05 was also remarkably higher, for both GSH and CDNB, compared to the other mite delta GSTs previously characterized[36] (see also chapter 4.1).

Table 4.7.Steady-state kinetics of TuGSTd05.

Kinetic parameter	TuGSTd05
K_m (mM) GSH	0.03 ± 0.00
K_m (mM) CDNB	0.15 ± 0.06
k_{cat} (min^{-1}) GSH	32.71 ± 2.63
k_{cat} (min^{-1}) CDNB	95.79 ± 16.25
K_{cat}/K_m ($mM^{-1} \cdot min^{-1}$) GSH	654.2
K_{cat}/K_m ($mM^{-1} \cdot min^{-1}$) CDNB	638.6

All values are means ± S.D. of three independent experiments. Results were determined by varying the concentration of GSH (0.075- 1.5 mM) and CDNB (0.03-0.75 mM) at fixed concentrations of CDNB (0.99 mM) and GSH (2.47 mM) respectively.

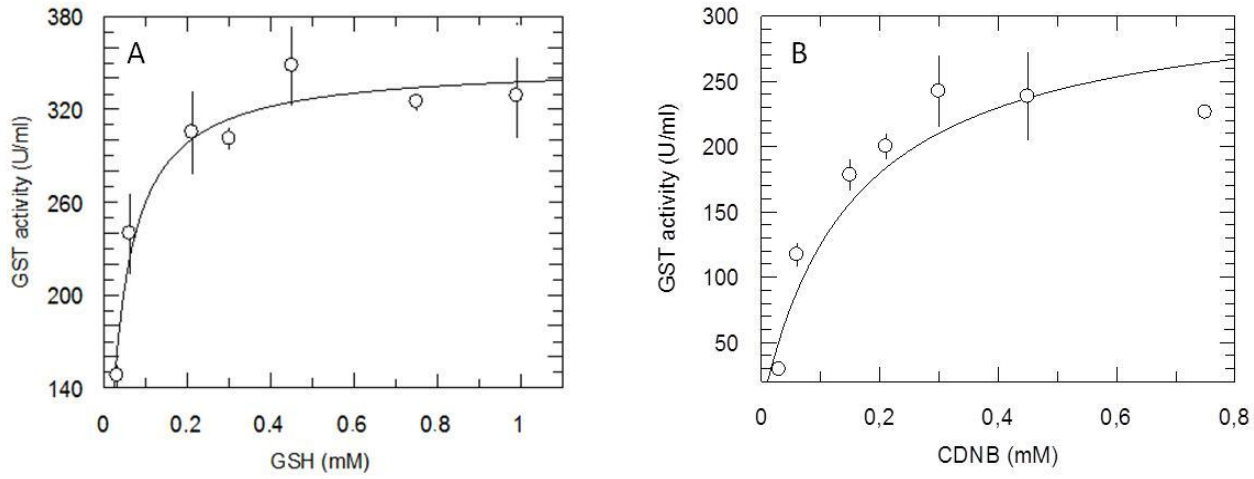


Figure 4. 8. Kinetic analysis of TuGSTd05. A: kinetic analysis using the GSH as a variable substrate (0.075-1.5 mM) and a fixed concentration of CDNB (0.99 Mm). **B**: kinetic analysis

using CDNB as a variable substrate (0.03- 1.5 mM) and GSH at a fixed concentration (2.47 mM).

4.2.3.3 Study of the interaction of TuGSTd05 with cyflumetofen

To investigate whether GSTd05 interacts with cyflumetofen and thus may play a role in resistance to cyflumetofen, in vitro inhibition of the enzyme in the presence absence of cyflumetofen was performed. GSTd05 was assayed using GSH (2.475 mM) and CDNB (0.99mM) as substrates and cyflumetofen was used in a 0.05mM final concentration (**Figure 4.9 A**). The presence of cyflumetofen caused a strong inhibition (98.24 ± 1.24%). To evaluate the inhibitory potential, the concentration of cyflumetofen needed to inhibit the CDNB conjugating activity by half (IC50) was calculated by a dose-response curve (**Figure 4.9 B**) and was determined at 0.94 ± 0.05 µM, showing strong levels of inhibition.

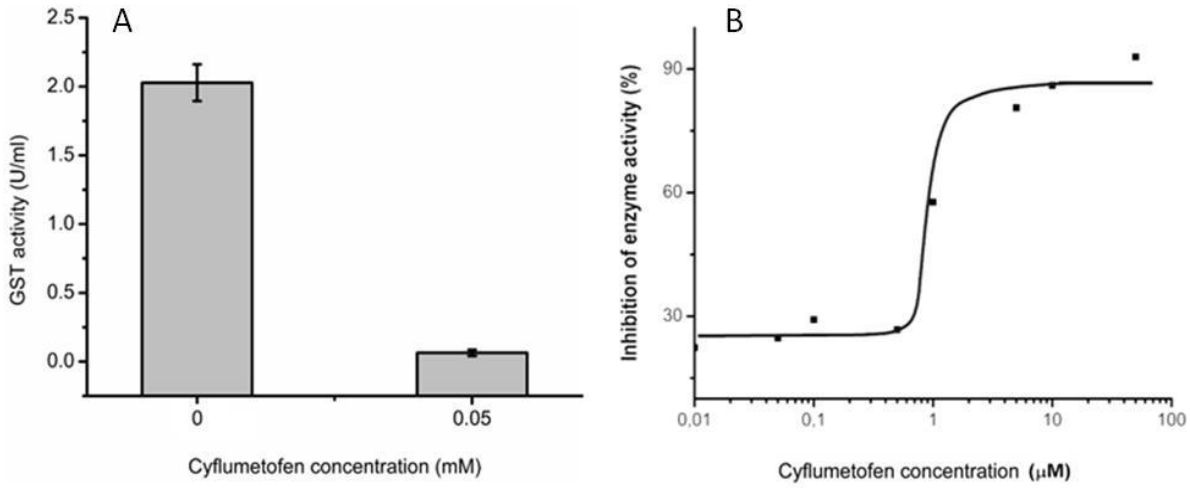


Figure 4.9. Interaction of TuGSTd05 with cyflumetofen. A: GST activity (U/ml) against the model substrate CDNB in the absence (0 mM) and the presence (0.05 mM) of cyflumetofen. Values and ± are means of three independent experiments. **B**: Dose-response curve for the inhibition of CDNB conjugating activity of TuGSTd05 by different cyflumetofen concentrations. Eight different concentrations of cyflumetofen (0.01-100 µM) and 0.99 mM CDNB were used and data are mean of three replicates ± S.D.

4.2.3.4 Evaluation of the capability of TuGSTd05 to catalyze the conjugation of cyflumetofen with GSH

HPLC-MS experiments were performed in order to investigate the possible conjugation of cyflumetofen with GSH, catalyzed by TuGSTd05 *in vitro*. Full scan mass spectrometric analysis did not provide enough sensitivity in order to detect any metabolites, under our experimental conditions. Therefore, cyflumetofen molecular peak $[MH]^+$ at +448 m/z, was followed with single ion monitoring and the possible conjugate of cyflumetofen with GSH was monitored with neutral loss scan at 129 amu. The 129 amu loss represents a neutral fragment of GSH (loss of glutamate) which is characteristic for GSH conjugates and can be used for the detection of metabolite formation by mass spectrometric techniques (Baillie and Davis, 1993). Cyflumetofen was greatly degraded during the incubation at 37^{o}C, so it was not possible to conduct strong evidence about possible metabolism by monitoring its abundance. However, neutral loss scan analysis (129 amu) revealed two peaks at ~1.6 min and ~6.5 min both at +480.3 m/z only present in the presence of TuGSTd05, in both 0 and 1 h experiments (**Figure 4.10 A**). The 2 peaks were not detected in any of the control samples (GSH and cyflumetofen, cyflumetofen and TuGSTd05, GSH and TuGSTd05 at 0 and 1h). The abundancies of these two conjugate peaks were in similar levels for 0 and 1 h assays. Both metabolite peaks showed the same m/z (+480.3) meaning that they have the same molecular weight (isobaric isomers). The results provide strong indication that TuGSTd05 catalyzes the conjugation of GSH to cyflumetofen *in vitro.*

The +480.3 m/z was analyzed with product ion scan in order to obtain structural information for the two peaks. MS/MS spectra were identical for the two peaks (**Figure 4.10 B, C**). The fragmentation pattern showed, except from neutral loss 129, another three indicative for GSH conjugates neutral losses: 79, 147 and 307 (e.g. neutral loss of 307 amu is indicative of the abduction of a whole GSH molecule). Based on that, the +480.3 m/z could consist of GSH (307 amu), a proton (1 amu) and a part of cyflumetofen with 172 amu. Fragments lower than +173 were not observed so we cannot obtain further information about the metabolites structure from MS/MS spectra. Further fragmentation (with higher collision energies) of +480.3 was not

possible since the obtained signal was inadequate. Based on the +173 m/z, the cyflumetofen structure and the clear indications that a GSH conjugate is formed, we suggest that the carbonylic carbon (Figure 8, red cycled) could be attached by a GSH molecule forming one of the detected metabolites (**Figure 4.11**).

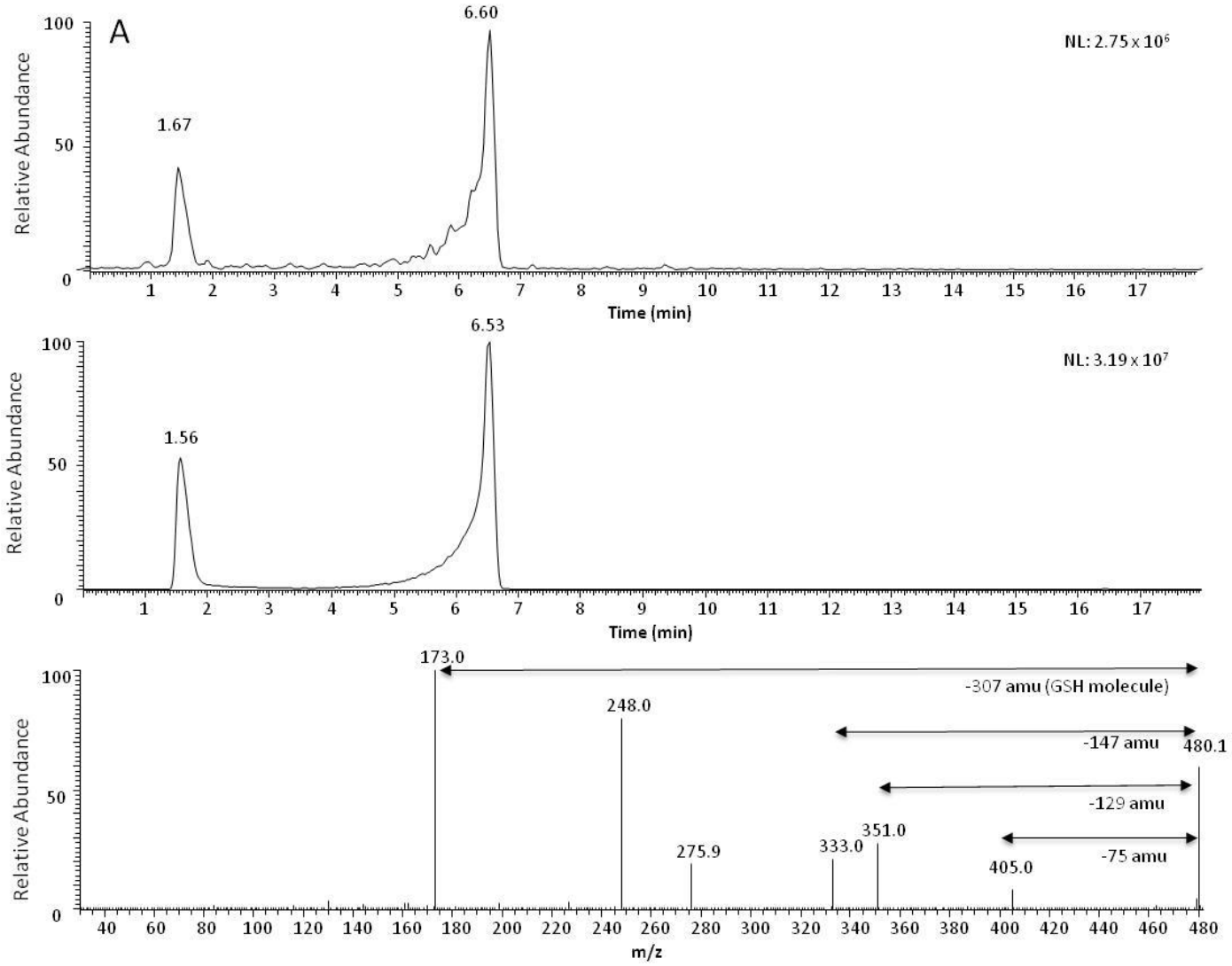


Figure 4.10. HPCL-MS analysis cyflumetofen-GSH conjugates, catalyzed by TuGSTd05 *in vitro*. A: **Neutral loss scan chromatogram in the presence of TuGSTd05**. Two peaks were detected only when TuGSTd05 was added in the reaction mixture. **B. Product ion scan chromatogram of +480.3 m/z. C: Mass spectrum of +480.3 m/z.** The indicative for GSH conjugates neutral losses of 75, 129, 147 and 307 amu are highlighted.

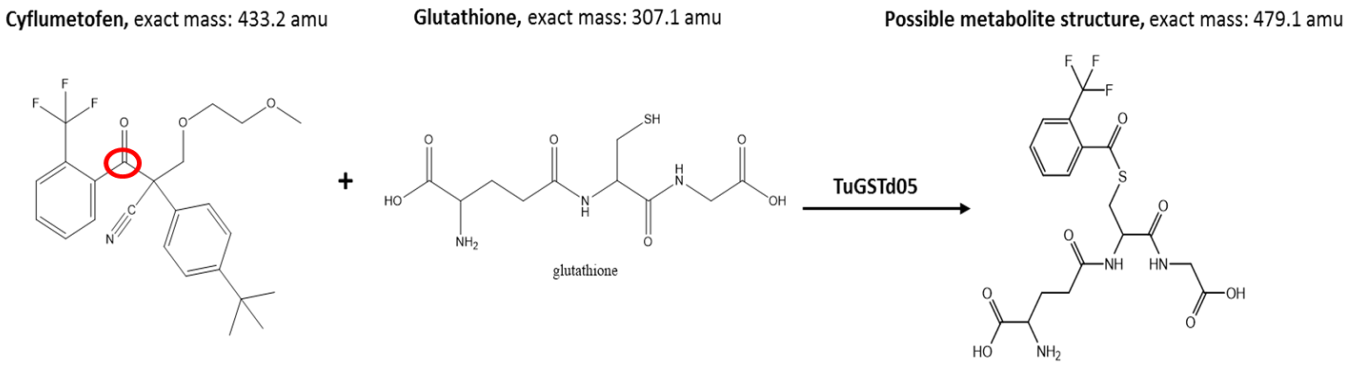


Figure 4.11. Structures of cyflumetofen, GSH and possible conjugate. The proposed site of the attack of cyflumetofen by ionized glutathione is encycled.

4.2.3.5 Overall inspection of TuGSTd05 predicted structure

In order to better understand the catalyitcal properties of TuGSTd05 and the molecular interactions between TuGSTd05-cyflumetofen, the three-dimentional structure of TuGSTd05 was predicted using the Swiss-model server[41].

The monomer consists of two domains, an N-terminal domain (residues 1-78) with thioredoxin-like βαβαββα secondary structure topology and an alpha-helical C-terminal domain (residues 89- 215) (**Figure 4.12 A**). The βαβαββα topology is arranged in the order β2, β1, β3 and β4, with β4 anti-parallel to the others, forming a β sheet which is surrounded by 3 α-helices (H1, H2, H3). After H3 there is a short linker (residues 79-85) that links the N- and C- terminal domains. The C-terminal domain consists of 5 α-helices (H4H5H6H7H8). The G-site (GSH binding site) is formed from helices H1, H2 and H3 in the N-terminal domain (**Figure 4.12 A, B)**. Several amino acids which contribute to the G-site formation are shown in **Figure 4.12 C**. Ser11 was identified as the potential catalytic residue (**Figure 4.12 A, C**). It is known that a Ser residue plays the role of the catalytic residue in delta class GSTs[44]. Ser11 could serve as the hydrogen bond donor to the thiol group of GSH, facilitating the stabilization of the anion form (GS^-) which attacks a nucleophilic center forming a GSH conjugate. The H-site (hydrophobic ligand binding site) is located next to the G-site and mainly formed by aromatic hydrophobic residues *i.e*. Tyr107, Phe115, Phe207 from H4 and H8 helices (**Figure 4.12 D**). Interestingly, the hydroxyl group of the polar residue Tyr107 points to

the H-site and may play an important role to the intramolecular interaction with the substrates of TuGSTd05.

Coulombic surface analysis revealed that the G-site and the H-site posses a positive electrostatic potential, which may facilitate the formation of GSH anion (GS^-) and to the binding of polar ligands (**Figure 4.12 E**). The electron-sharing network, a structural characteristic of delta GSTs that contributes catalysis[45, 46] was also identified in the 3D model structure of TuGSTd05. The residues Gln16, Glu66, Ser67 and Asp102 seem to form the electron-sharing network (**Figure 4.12 F**).

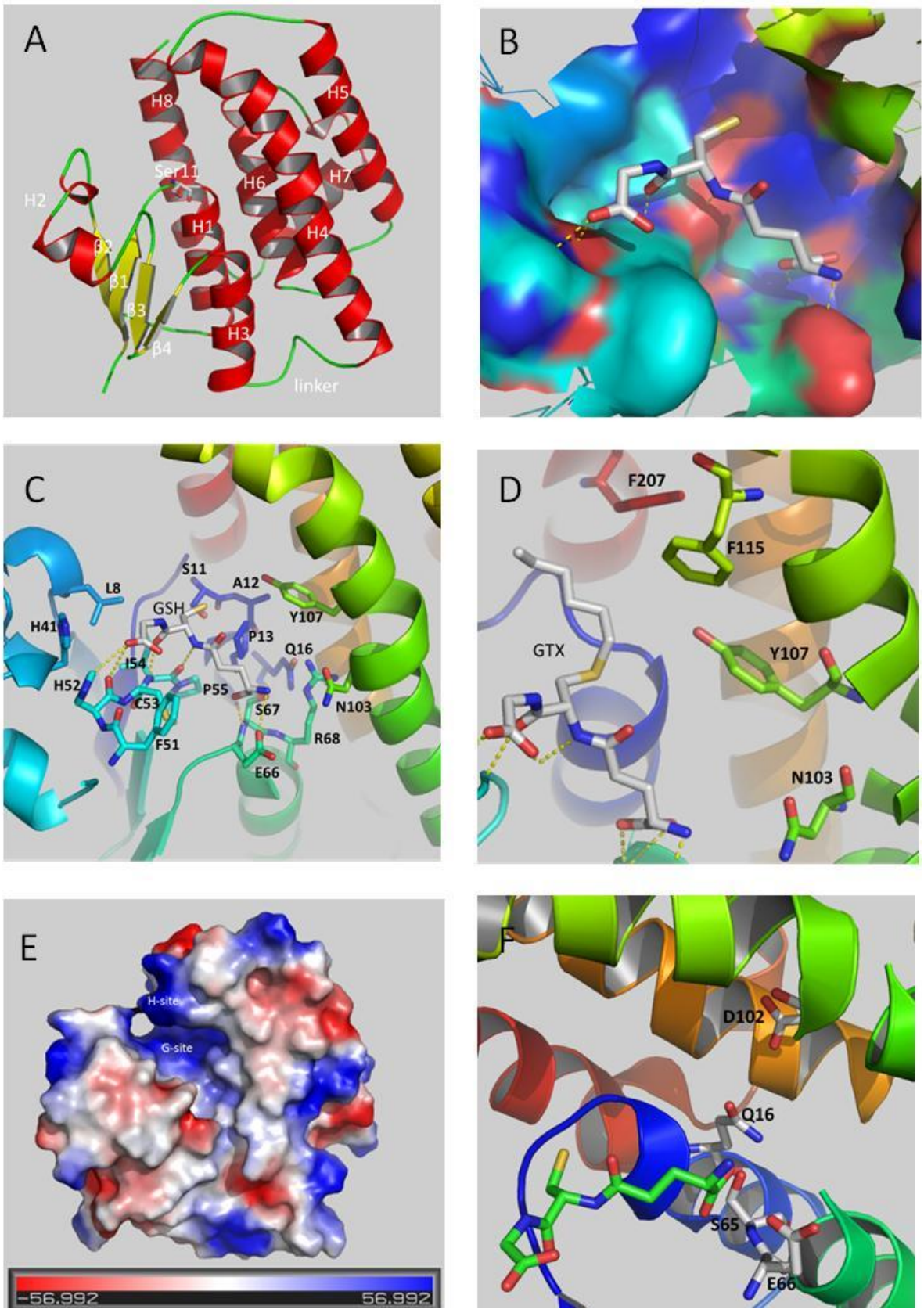


Figure 4.12. Structural features of TuGSTd05 protein model. A: Ribbon representation of the TuGSTd05 monomer. The N-terminal domain, with a βαβαββα secondary structure and the C-terminal domain consisting of 5 helices (H4-H8) is shown. The potential catalytic residue Ser11

is highlighted. B: Surface analysis of GSH binding in the TuGSTd05 protein model. GSH binds to the G-site formed by H1, H2 and H3 helices. C: Residues potentially involved in the G-site formation. D: Residues potentially involed in H-site formation. E. Coulombic surface analysis. Neutral surface is in grey, positively charged in blue and negatively charged in red. The G-site and the H-site possess a positive electrostatic potential. F: The electron-sharing network of TuGSTd05, formed by the residues Gln16, Glu66, Ser67 and Asp102. All figures were created using PyMOL[43].

4.2.3.6 Docking of cyflumetofen into TuGSTd05 and identification of key residues, potentially involved in the interaction with cyflumetofen

To understand the interaction of TuGSTd05 with cyflumetofen more in depth at the molecular level, docking studies with cyflumetofen were conducted. Blind docking was performed using the Swiss-Dock server[42] and the dimer model of TuGSTd05 structure was used (**Figure 4.7**) It was revealed that the most favorable binding mode is in the active site of the enzyme (**Figure 4.13 A**), with a ΔG of -7.93 kcal/mol. Docking results are in line with the metabolic studies (section 4.2.3.4) where binding of cyflumetofen into the active site of the enzyme is required for the catalytically formation of a GSH conjugate.

Analysis of the binding site suggests that the binding of cyflumetofen is mediated by hydrophobic interactions with hydrophobic residues such as Pro10, Pro13, Ala12, Ala15, Phe115, Phe207 and Pro210 (**Figure 4.13 B**). It is also likely that π-aromatic interactions between the aromatic ring of cyflumetofen and residues may contribute in the proper conformation of cyflumetofen in the active site as for example, a π-aromatic interaction may occur between the aromatic ring of P115 with the aromatic rings of cyflumetofen (face to edge interactions) (**Figure 4.13 B).** Interestingly, a hydrogen bond is formed between the –OH group of Tyr107 and the -O atom of cyflumetofen, which may play a significant role in the binding of TuGSTd05 with cyflumetofen (**Figure 4.13 E**). This hydrogen bond could also play a role in the GSH conjugation of cyflumetofen as potentially leads to the formation of the keto

tautomeric form of cyflumetofen and the increament of the electroniophilicity of the carbonylic carbon, facilitating its attack by ionized glutathione (GS^-).

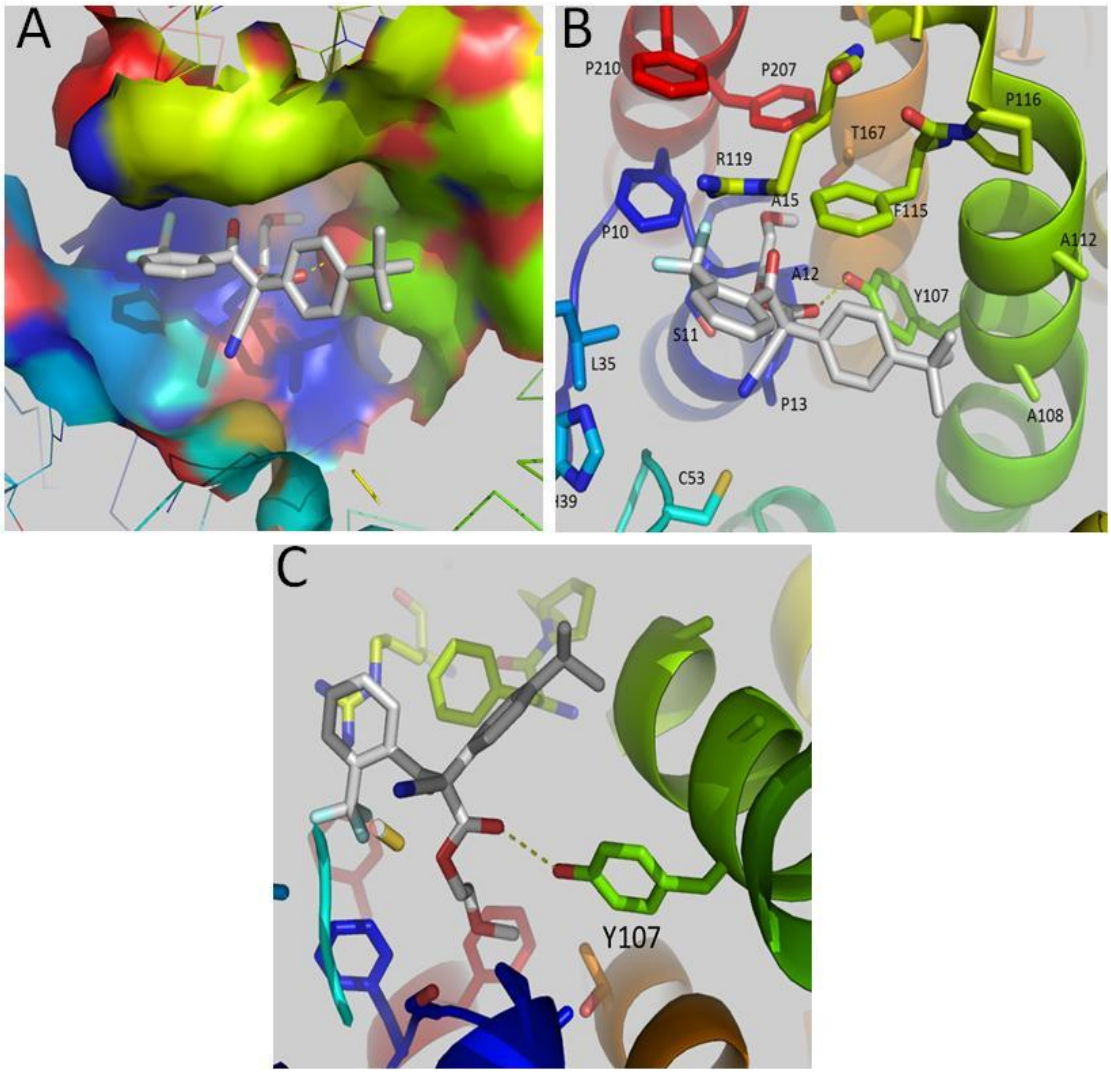


Figure 4.13. The predicted molecular interaction between TuGSTd05 and cyflumetofen. A: The preferable mode of interaction of TuGSTd05 with cyflumetofen. Cyflumetofen binds intro the active site of the enzyme, **B**: Residues that may contribute in the interaction of TuGSTd05 with cyflumetofen, **C**: The molecular interaction between the residue Tyr107 and cyflumetofen. All figures were created using PyMOL[43].

4.2.3.7 Functional investigation of the role of Tyr107 in the interaction with cyflumetofen

Docking analysis pointed out the residue Tyr107 as one of the potential key active residues for the interaction of TuGSTd05 with cyflumetofen. To assess its possible role, I changed the amino acid Tyr107 by site-directed mutagenesis and determined the cyflumetofen binding capability as well as the metabolism capacity of the mutant protein compared to the wild type TuGSTd05. The amino acid Tyr107 was changed to Phe, an amino acid with almost identical chemical structure with Tyr that lacks the -OH group of the phenolic ring, which was pointed out from docking analysis to play a vital role in the interaction with cyflumetofen. The Tu*GSTd05* coding sequence cloned in pET100/D-TOPO (Invitrogen Life Technologies, USA) was successfully mutagenized and the mutant protein (referred as TuGSTd05 Y107F) was induced with 1 mM IPTG for 4 hours at 37^{o}C in *E. coli* BL21(DE3) cells. Recombinant TuGSTd05 was highly produced, purified via chelate affinity chromatography and was found to be catalytically active against the model substrate CDNB.

Inhibition assays were performed in order to investigate the interaction of TuGSTd05 Y107F with cyflumetofen in comparison with the wild type TuGSTd05. Cyflumetofen was used in 0.01 and 0.05 mM final concentrations and the effect in the initial velocities of the two enzymes are shown in **Figure 4.14 A.** Cyflumetofen caused a 39.06 ± 0.97 % inhibition of the wild type TuGSTd05 when 0.01 mM final concentration was used and 96.05 ± 5.04% when 0.05 mM were used. The activity of TuGSTd05 Y107F was not affected while 0.01 mM cyflumetofen were used while decreased by 25.98 ± 7.89% in the presence of 0.05 mM. Results showed that TuGSTd05 Y107F mutant displayed weaker interaction compared to the wild type enzyme. To evaluate the effect of the Tyr107 to Phe mutation in the affinity of TuGSTd05 with cyflumetofen, the concentration of cyflumetofen needed to inhibit the CDNB conjugating activity by half (IC50) was calculated by a dose-response curve (**Figure 4.14 B**) and was found equal to 51.08 ± 9.51 μM. The IC50 is 55-fold lower than that of the wild type enzyme (0.09 ± 0.05) showing that the Tyr107 to Phe mutation negatively affects the affinity of cyflumetofen for the enzyme, pointing out a potential

crucial role of the amino acid Tyr107 in the binding of cyflumetofen into TuGSTd05. To examine if the Tyr107 to Phe mutation affects the metabolism (conjugation of GSH with cyflumetofen) capacity of the enzyme, HPLC-MS experiments with wild type TuGSTd05 and TuGSTd05 Y107F were conducted. Metabolite/Conjugate molecular peak (+480 m/z) was followed with single ion monitoring (data not shown) and no significant differences in metabolite abundance were observed, under experimental conditions. Taken all data together, indicate that the substitution of Tyr107 with Phe did not change the catalytic activity of TuGSTd05 against cyflumetofen, however the amino acid Tyr107 most likely plays a key role in the binding of cyflumetofen into TuGSTd05.

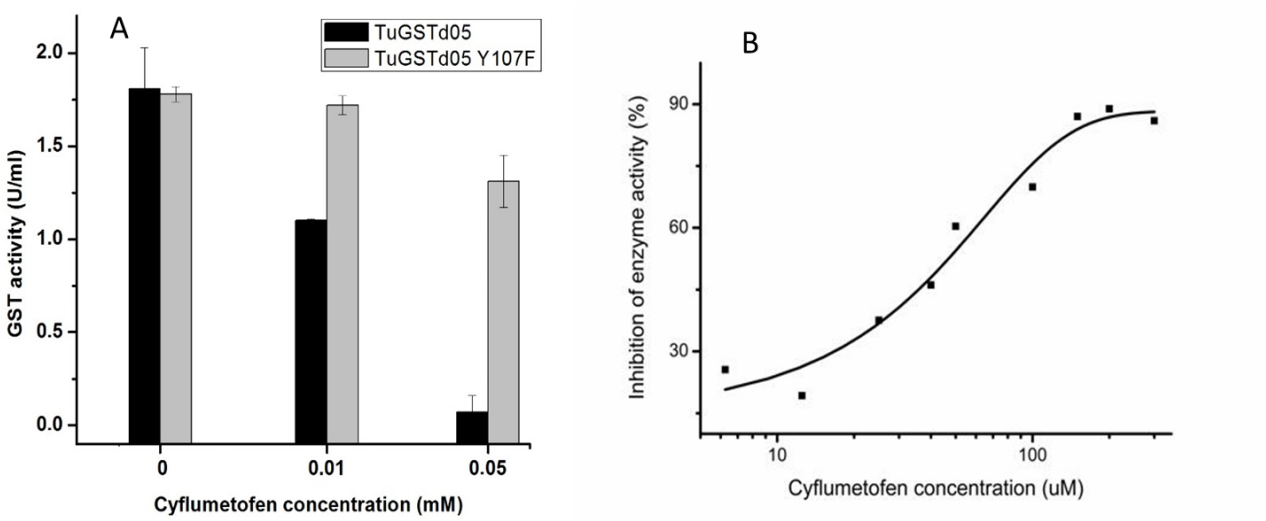


Figure 4.14. A: Interaction of TuGSTd05 Y107F mutant with cyflumetofen. GST activity (U/ml) against the model substrate CDNB in the absence (0 mM) and the presence (0.01 and 0.05 mM) of cyflumetofen. Values are means of three independent experiments ± S.D. Values for wild type TuGSTd05 are also given. **B**: **Dose-response curve for the inhibition of CDNB conjugating activity of TuGSTd05 Y107F by different cyflumetofen concentrations**. Nine different concentrations of cyflumetofen (6.25-300 μM) and 0.99 mM CDNB were used and data are mean of three replicates ± S.D.

4.2.4 Conclusions

In this study, I functionally expressed TuGSTd05, a GST previously shown to be involved in resistance of *T. urticae* to the novel acaricide cyflumetofen. The catalytic properties of the enzyme were examined as well as its interaction with cyflumetofen, at the protein level. It was shown that TuGSTsd05 is likely to play an important role in the cyflumetofen resistance in *T. urticae.* Strong evidence were provided that TuGSTd05 catalyzes the conjugation of GSH to cyflumetofen *in vitro* and the possible site of attack as well as key amino acids (such as Tyr107) possibly implicated in the interaction were identified. This study represents the first convincing report, at the functional-protein level, for the implication of an acari GST in resistance, and it enhances our understanding for the resistance mechanisms in *T. urticae*.

4.2.5 References

1. http://www.montpellier.inra.fr/CBGP/spmweb.
2. Tomczyk, A. & Kropczynska, D. in Spider Mites, Their Biology, Natural Enemies and Control (eds. Helle, W. & Sabelis, M.W.) 312-330 (Elsevier, Amsterdam, 1985).
3. Park, Y.L. & Lee, J.H. Leaf cell and tissue damage of cucumber caused by twospotted spider mite (Acari: Tetranychidae). *J Econ Entomol* **95**, 952-7 (2002).
4. Van Leeuwen, T., Dermauw, W., Grbic, M., Tirry, L. & Feyereisen, R. Spider mite control and resistance management: does a genome help? *Pest Manag Sci* **69**, 156-9 (2013).
5. http://blog.metmuseum.org.
6. Knowles, C.O. in Molecular Mechanisms of Resistance to Agrochemicals (ed. Sjut, V.) 57-77 (Springer, New York, 1997).
7. Dekeyser, M.A. Acaricide mode of action. *Pest Manag Sci* **61**, 103-10 (2005).
8. Van Leeuwen, T.V., Vontas, J. & Tsagkarakou, A. in Biorational Control of Arthropod Pests: Application and Resistance Management (eds. Ishaaya, I. & Horowitz, A.R.) 351-370 (Springer, 2009).
9. Croft, B.A. & Van de Been, H.E. Ecological and genetic factors influencing evolution of pesticide resistance in Tetranychid and Phytoseiid mites. *Exp Apl Acar* **4**, 277-300 (1988).
10. Van Leeuwen, T., Vontas, J., Tsagkarakou, A., Dermauw, W. & Tirry, L. Acaricide resistance mechanisms in the two-spotted spider mite Tetranychus urticae and other important Acari: a review. *Insect Biochem Mol Biol* **40**, 563-72 (2010).
11. Hayes, J.D. & Wolf, C.R. Role of glutathione transferases in drug resistance. In Glutathione Conjugation (Academinc Press, 1988).
12. Lyall, M.S., Dundas, S.R., Curran, S. & Murray, G.I. Profiling markers of prognosis in colorectal cancer. *Clin Cancer Res* **12**, 1184-91 (2006).
13. Clark, A.G. & Shamaa, N.A. Evidence that DDT-dehydrochlorinase from the house fly is a glutathione S-transferase. *Pest Biochem Physiol* **22**, 249-261 (1984).

14. Vontas, J.G., Small, G.J. & Hemingway, J. Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens. *Biochem J* **357**, 65-72 (2001).
15. Kostaropoulos, I., Papadopoulos, A.I., Metaxakis, A., Boukouvala, E. & Papadopoulou-Mourkidou, E. Glutathione S-transferase in the defence against pyrethroids in insects. *Insect Biochem Mol Biol* **31**, 313-9 (2001).
16. Grbic, M. et al. The genome of Tetranychus urticae reveals herbivorous pest adaptations. *Nature* **479**, 487-92 (2011).
17. Fisher, M.H. & H., M. Chemistry in ivermectin and abamectin (Springer, 1989).
18. Putter, J. et al. Avermectins: novel insecticides, acaricides and nematicides from a soil microorganism. *Exp* **37**, 963-964 (1981).
19. Wolstenholme, A.J. & Rogers, A.T. Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. *Parasitology* **131 Suppl**, S85-95 (2005).
20. Lynagh, T. & Lynch, J.W. Molecular mechanisms of Cys-loop ion channel receptor modulation by ivermectin. *Front Mol Neurosci* **5**, 60 (2012).
21. Lasota, J.A. & Dybas, R.A. Abamectin as a pesticide for agricultural use. *Acta Leiden* **59**, 217-25 (1990).
22. Kwon, D.H., Yoon, K.S., Clark, J.M. & Lee, S.H. A point mutation in a glutamate-gated chloride channel confers abamectin resistance in the two-spotted spider mite, Tetranychus urticae Koch. *Insect Mol Biol* **19**, 583-91 (2010).
23. Dermauw, W. et al. The cys-loop ligand-gated ion channel gene family of Tetranychus urticae: implications for acaricide toxicology and a novel mutation associated with abamectin resistance. *Insect Biochem Mol Biol* **42**, 455-65 (2012).
24. Dermauw, W. et al. A link between host plant adaptation and pesticide resistance in the polyphagous spider mite Tetranychus urticae. *Proc Natl Acad Sci U S A* **110**, E113-22 (2013).
25. Riga, M. et al. Abamectin is metabolized by CYP392A16, a cytochrome P450 associated with high levels of acaricide resistance in Tetranychus urticae. *Insect Biochem Mol Biol* **46**, 43-53 (2014).
26. Konanz, S. & Nauen, R. Purification and partial characterization of a glutathione S-transferase from the two-spotted spider mite, Tetranychus urticae. *Pestic Biochem Physiol* **79**, 49-57 (2004).
27. Stumpf, N. & Nauen, R. Biochemical markers linked to abamectin resistance in Tetranychus urticae (Acari: Tetranychidae). *Pest Biochem Physiol* **72**, 11-121 (2002).
28. Ranson, H. et al. Evolution of supergene families associated with insecticide resistance. *Science* **298**, 179-81 (2002).
29. Segura-Aguilar, J. & Lind, C. On the mechanism of the Mn3(+)-induced neurotoxicity of dopamine:prevention of quinone-derived oxygen toxicity by DT diaphorase and superoxide dismutase. *Chem Biol Interact* **72**, 309-24 (1989).
30. Baez, S., Segura-Aguilar, J., Widersten, M., Johansson, A.S. & Mannervik, B. Glutathione transferases catalyse the detoxication of oxidized metabolites (o-quinones) of catecholamines and may serve as an antioxidant system preventing degenerative cellular processes. *Biochem J* **324 (Pt 1)**, 25-8 (1997).
31. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-54 (1976).
32. Habig, W.H., Pabst, M.J. & Jakoby, W.B. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**, 7130-9 (1974).
33. Che-Mendoza, A., Penilla, R.P. & Rodriguez, A. Insecticide resistance and glutathione S-transferases in mosquitoes: a review. *Afr J Biotechnol* **8**, 1386-1397 (2009).
34. Shahein, Y.E. et al. Molecular cloning, expression and characterization of a functional GSTmu class from the cattle tick Boophilus annulatus. *Vet Parasitol* **152**, 116-26 (2008).
35. Campbell, E., Takahashi, Y., Abramovitz, M., Peretz, M. & Listowsky, I. A distinct human testis and brain mu-class glutathione S-transferase. Molecular cloning and characterization of a form present even in individuals lacking hepatic type mu isoenzymes. *J Biol Chem* **265**, 9188-93 (1990).
36. Molin, E. (, Swedish University of Agricultural Sciences, Uppsala, Sweden, 2009).
37. Takahashi, N., Nakagawa, H., Sasama, Y. & Ikemi, N. Development of a new acaricide, cyflumetofen. *J Pestic Sci* **37**, 263-264 (2012).

38. Hayashi, N., Sasama, Y., Takahashi, N. & Ikemi, N. Cyflumetofen, a novel acaricide - its mode of action and selectivity. *Pest Manag Sci* **69**, 1080-1084 (2013).
39. Khajehali, J., Van Nieuwenhuyse, P., Demaeght, P., Tirry, L. & Van Leeuwen, T. Acaricide resistance and resistance mechanisms in Tetranychus urticae populations from rose greenhouses in the Netherlands. *Pest Manag Sci* **67**, 1424-33 (2011).
40. Khalighi, M., Tirry, L. & Van Leeuwen, T. Cross-resistance risk of the novel complex II inhibitors cyenopyrafen and cyflumetofen in resistant strains of the two-spotted spider mite Tetranychus urticae. *Pest Manag Sci* **70**, 365-8 (2014).
41. http://swissmodel.expasy.org.
42. http://www.swissdock.ch/.
43. DeLano, W.L. in, (ed. Scientific, D.) (San Carlos, CA, USA, 2002).
44. Bocedi, A. et al. The impact of nitric oxide toxicity on the evolution of the glutathione transferase superfamily: a proposal for an evolutionary driving force. *J Biol Chem* **288**, 24936-47 (2013).
45. Winayanuwattikun, P. & Ketterman, A.J. An electron-sharing network involved in the catalytic mechanism is functionally conserved in different glutathione transferase classes. *J Biol Chem* **280**, 31776-82 (2005).
46. Axarli, I., Dhavala, P., Papageorgiou, A.C. & Labrou, N.E. Crystal structure of Glycine max glutathione transferase in complex with glutathione: investigation of the mechanism operating by the Tau class glutathione transferases. *Biochem J* **422**, 247-56 (2009).

Chapter 5

General discussion and future research directions

The objective of this thesis was the study of the adaptation of arthropod pests to chemical insecticides and phytotoxins, with emphasis in detoxification mechanisms.

In chapter 1, "The transgenic expression of the *A. aegypti* cytochrome P450 *CYP9J28* in *D. melanogaster*", I ectopically expressed in transgenic *Drosophila* the Aaeg*CYP9J28*, a cytochrome P450 that was previously associated with pyrethroid resistance by transcription and *in vitro* studies The GAL4/UAS system was used, in order to drive the transgenic expression of Aaeg*CYP9J28* in the appropriate tissues and it was showed that the transgenic expression of the *CYP9J28* confers significant levels of pyrethroid resistance. This study provided solid functional *in vivo* evidence for the implication of the Aaeg*CYP9J28* in pyrethroid resistance and showed for the first time that ectopic expression of mosquito genes in *D. melanogaster* can be a robust approach for validating candidate resistance genes. The use of *Drosophila* for the validation of candidate genes involved in insecticide resistance in mosquitoes, is a great addition to the already available tools (genetic mapping, recombinant protein, RNAi) for the analysis of the molecular mechanisms responsible for the phenotype and could be used for the validation of candidate genes in the future. The availability of transgenic lines over-expressing resistance genes, may also be used for the evaluation of the efficacy of novel insecticides together with the *in vitro* metabolic stability test using recombinant detoxification proteins. Finally, the ability to ectopically express detoxification genes in isogenic flies with mutations in target site resistance loci i.e. *kdr*, or multiple detoxification genes might further help the investigation of multi-factorial resistance mechanisms and improve our understanding for the strong resistance phenotypes that have recently evolved in mosquitoes. This line of research has already been initiated (V. Douris, personal communication). Successful introduction of SNPs for insecticide targets (such as in voltage-gated sodium channel) using Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) genome editing approach has been achieved and crosses have been designed with fly strains carrying transgenically over-expressed detoxification genes. Drosophila lines carrying in parallel target site mutations and detoxification enzyme will be used for better understanding of multi-factorial resistance phenotypes.

In chapter 2, entitled “The study of the molecular basis of insecticide resistance in *B. oleae* and its adaptation to olives, with emphasis on the role of detoxification”, a large transcriptomic dataset (more than 14,000 new sequences) was generated and a large number (132) of putative detoxification genes was identified and phylogenetically classified. This is a great addition in the sequence information for olive fruit fly, as at the time the study conducted (2013) only ~800 nucleotide and ~800 protein sequences were deposited in the NCBI database including less than 10 detoxification homologues. The new data provided will be useful for several groups studying *B. oleae*. However, there is a need to get the complete genome, as this will provide a deeper knowledge of genes, gene networks, regulatory regions etc which will allow the better understanding of the biology of this important pest. The genome sequencing is currently in progress and it is envisaged that this project will be completed in the near future. The transcriptome dataset generated in this study will be also very useful for its annotation and gene prediction validation.

Based on the obtained sequences, a microarray chip was subsequently designed and constructed. The molecular tool developed in our study is a significant input for scientific community studying *B. oleae*, will facilitate the gene expression studies and will be useful for the investigation of important aspects of the biology of the olive fruit fly.

I used the microarray chip in order to investigate the underlying mechanisms involved in pyrethroid resistance. The analysis of the differential gene expression between resistant and susceptible populations revealed the association of two P450 genes, the contig00436 (a putative homologue of *D. ananassae Cyp6a23*) and the contig02103 (a putative homologue of *D. ananassae Cyp6g2*) with the pyrethroid resistance phenotype. These findings are in agreement with previous studies, which associate P450-based detoxification with pyrethroid resistance in *B. oleae*[1] and it may contribute to the better understanding of molecular mechanisms by which *B. oleae* develops resistance to pyrethroids. However, the over-expression of these genes is only a first indication for their involvement in resistance and further validation and functional investigation is required. When other α-cypermethrin resistant populations with higher levels of resistance were tested the expression levels of contig00436 and

contig02103 were not correlated with resistance levels (A. Kabouraki 2015, unpublished data). Therefore, more gene expression analysis experiments including also additional α-cypermethrin resistant populations are necessary, in order to understand if different olive fruit fly populations over-express different P450s to cope with pyrethroids, and/or if other P450s, possibly not identified or even not included in our microarray, might have a stronger association with the pyrethroid resistance phenotype. To establish their potential role in pyrethroid resistance also further functional investigation is required. The two P450s should be functionally expressed in proper systems (i.e. *E. coli*, baculovirus) and recombinant enzymes should be tested for their ability to metabolize α-cypermethrin *in vitro*, using chromatographic and/or mass spectrometric techniques (e.g. HPLC/MS). I*n vivo* approaches could be also employed. RNAi via injections and/ or via feeding could be employed in order to down-regulate the expression of contig00436 and/ or contig02103 in resistant individuals and examine if the levels of resistance to α-cypermethrin are also increased. Alternatively, the GAL4/UAS system could be used to drive the ectopic expression of these genes in *D. melanogaster* and investigate if confers resistance. The detailed study of underlying mechanisms of pyrethroid resistance is crucial for the sustainability of efficient control for this devastating arthropod pest. This work has been already initiated for the conti00436, which has already been cloned in proper expression vector and functional enzyme was produced (V. Tseliou, M. Sc. Thesis).

The mechanisms of adaptation of the strictly monophagous olive fruit fly to olives represents an interesting aspect of evolutionary ecology and basic molecular biology. As an attempt to identify detoxification and/ or other genes which may play a role in the overcoming of plant defenses (especially phenolic compounds) and the utilization of the olive flesh I conducted transcriptomic analysis in larvae upon their development in olives and artificial diet. The over-transcription of several detoxification and digestive-related genes was confirmed upon development in olives. These detoxification genes, in particular those encoding for UPD-glucosyltransferases, may play a role in the detoxification of olive phenolic compounds. The over-expression of UDP-glucosyltransferases provides further indication and support previous work[2] for their involvement in the detoxification of plant phenols. The over-expression of

digestive genes may be implicated in the digestion of olive proteins but also may serve as a mechanism of overcoming the negative effects of trypsin inhibitors induced in the olive fruit upon *B. oleae* attack[3]. My analysis is a useful starting point for understanding the molecular mechanisms of *B. oleae* larvae adaptation to olives, however more investigation is needed for the correlation of specific genes with adaptation to olives. For example, induction experiment may be performed in the presence of phenolic compounds, such as oleuropein (the major constituent of unripe olives). Exposure in sub-lethal concentration of phenols followed by detailed gene expression analysis, by microarray and/ or real-time qPCR, could reveal genes associated with detoxification of specific phenols. Candidate genes (such as UGTs) may be functionally expressed and the interaction of recombinant enzymes with phytotoxins via inhibition assays and/ or metabolic assays may be investigated. Moreover, the study of the role of symbiotic bacteria in the adaptation is also required. In a long term, the in depth understanding of the interaction of *B. oleae* with its host would facilitate the development of more efficient, eco-friendly and species-specific strategies of control.

In chapter 3, entitled, "The functional characterization of GSTs associated with insecticide/ acaricide resistance in *T. urticae*", the interaction of specific *T. urticae* GSTs that had been associated with acaricide resistance by transcriptomic studies with insecticides/ acaricides was investigated at the protein level. TuGSTd10, TuGSTd14 and TuGSTm09 have been previously strongly associated with abamectin/ multi resistance phenotype[4], while TuGSTd05 was strongly associated with resistance to cyflumetofen (T. Van Leeuwen, unpublished data). Recombinant TuGSTd14 was found to interact with abamectin *in vitro* and Dixon plot analysis revealed that abamectin competes with CDNB for its binding, thus binds to the active site. Our study provided further evidence and supported earlier work that GSTs are likely to play a role in abamectin resistance, particularly in *T. urticae*[5, 6]. However further studies on the metabolic fate of abamectin in resistant and susceptible spider mites are required in order to provide functional evidence for a catalytic interaction of abamectin with arthropod GSTs, resulting in conjugated metabolites, which seem to be elusive so far. We cannot exclude that the observed competitive inhibition of CDNB conjugation by

abamectin is due to its binding to regions adjacent the active site, *i.e.* not directly interfering with the substrate recognition site.

Finally, the interaction of TuGSTd05 with cyflumetofen was studied. It was shown that cyflumetofen is a strong inhibitor of TuGSTd05 and targeted HPLC/ MS analysis revealed that TuGSTd05 is capable of catalyzing the conjugation of GSH to cyflumetofen. The structure of the derivative was tentatively identified and the possible site of ionized glutathione attack to cyflumetofen was proposed. To study in more depth the molecular interaction between cyflumetofen-TuGSTd05 *in silico* docking analysis was performed. It was revealed that most likely cyflumetofen binds to the active site of the enzyme and the binding is primarily mediated via hydrophobic interactions. Key amino acids potentially crucial for the interaction and/ or catalysis were identified. To assess the possible role of the most crucial amino acid, Tyr107, I changed this amino acid by site-directed mutagenesis and showed that plays a significant role in the binding of cyflumetofen into TuGSTd05. My study established the *in vitro* metabolism of cyflumetofen by TuGSTd05 and represents the first report which provides solid evidence for the implication of an acari GST in resistance. Also, it is one of the few studies which show insecticide metabolism by a GST. Other known examples are the metabolism/ dehydrochlorination of DDT by GSTe2 from *A. gambiae*[7, 8], the methyl parathion and lindane metabolism by the *M. sexta* GST-6A[9] and the metabolism of OPs by *P. xylostella* GST3 and GST4[10, 11]. In order to obtain further evidence for the role of TuGSTd05 in cyflumetofen resistance, *in vivo* validation could also be performed in the future. Moreover, it is known that a de-esterified metabolite of cyflumetofen exhibits much more toxicity than the parental compound[12]. *In vitro* inhibition and metabolism assays could be performed in the presence of the de-esterified metabolite in order to evaluate the capability of TuGSTd05 to also metabolize this compound. Crystallization of TuGSTd05 alone or in complex with cyflumetofen would provide further information regarding their interaction and this knowledge could be also used for the design of specific inhibitors in the frame of improved insecticide/acaricide resistance management strategies. Finally, for better understanding of detoxification physiology, specific antibody against TuGSTd05 will be generated (V. Balabanidou, unpublished data) to be used for the

investigation of protein localization providing more insights for the tissues/ cell types where detoxification takes place. Specific Elisa- based diagnostics could also be developed for the facilitation of resistance management in the field.

5.1 References

1. Margaritopoulos, J.T. et al. Efficacy of the pyrethroid alpha-cypermethrin against Bactrocera oleae populations from Greece, and improved diagnostic for an iAChE mutation. *Pest Manag Sci* **64**, 900-8 (2008).
2. Luque, T., Okano, K. & O'Reilly, D.R. Characterization of a novel silkworm (Bombyx mori) phenol UDP-glucosyltransferase. *Eur J Biochem* **269**, 819-25 (2002).
3. Corrado, G. et al. Molecular interactions between the olive and the fruit fly Bactrocera oleae. *BMC Plant Biol* **12**, 86 (2012).
4. Dermauw, W. et al. A link between host plant adaptation and pesticide resistance in the polyphagous spider mite Tetranychus urticae. *Proc Natl Acad Sci U S A* **110**, E113-22 (2013).
5. Konanz, S. & Nauen, R. Purification and partial characterization of a glutathione S-transferase from the two-spotted spider mite, Tetranychus urticae. *Pestic Biochem Physiol* **79**, 49-57 (2004).
6. Stumpf, N. & Nauen, R. Biochemical markers linked to abamectin resistance in Tetranychus urticae (Acari: Tetranychidae). *Pest Biochem Physiol* **72**, 11-121 (2002).
7. Ranson, H., Prapanthadara, L. & Hemingway, J. Cloning and characterization of two glutathione S-transferases from a DDT-resistant strain of Anopheles gambiae. *Biochem J* **324 (Pt 1)**, 97-102 (1997).
8. Ranson, H. et al. Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector Anopheles gambiae. *Biochem J* **359**, 295-304 (2001).
9. Wei, S.H., Clark, A.G. & Syvanen, M. Identification and cloning of a key insecticide-metabolizing glutathione S-transferase (MdGST-6A) from a hyper insecticide-resistant strain of the housefly Musca domestica. *Insect Biochem Mol Biol* **31**, 1145-53 (2001).
10. Chiang, F.M. & Sun, C.N. Glutathione transferase isozymes of diamondback moth larvae and their role in the degradation of some organophosphorus insecticides. *Pestic Biochem Physiol* **45**, 7-14 (1993).
11. Ku, C.C., Chiang, F.M., Hsin, C.Y., Yao, Y.E. & Sun, C.N. Glutathione transferase isozymes involved in insecticide resistance in diamondback moth larvae. *Pest Biochem Physiol* **50**, 191-197 (1994).
12. Hayashi, N., Sasama, Y., Takahasi, N. & Ikemi, N. Cyflumetofen, a novel acaricide- its mode of action and selectivity. *Pest Manag Sci* **69**, 1080-1084 (2013).