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‘Functional expression and characterization of detoxification enzymes in insects and mites’

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Ευχαριστίες

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ABSTRACT

Studying the enzymes by which agricultural pests develop resistance to insecticides, aiming to develop new means of managing and overcoming this resistance, a crucial task for crop protection. I studied detoxification genes and enzymes, which are associated within the resistance phenotype in two major pests:

The two spotted spider mite *Tetranychus urticae* is one of the most damaging pests in agriculture. *T. urticae* is extremely polyphagous; it can feed on hundreds of plants including important agricultural crops. Genome wide gene expression analysis (micaroarray) of a multiresistant strain of *T.urticae* (Marathonas) showed, among others, the association of two Mu class Glutathione S tranferases (GSTs), TuGSTm07 and TuGSTm09, with the resistant phenotype. In this study, both were functionally expressed and kinetically characterized and their potential to interact with insecticides/acaricides were examined. TuGSTm07 found to interact with bifenthrin causing 70% inhibition of its CDNB coguncating activity. The IC50 value was determined at 7,7103 ± 0,956 μM, showing strong interaction. The three dimensional structure of TuGSTm07 was predicted based on X-ray of other Mammals' Mu GST. Docking with bifenthrin was performed and revealed that bifenthrin most likely binds to the active site of the enzyme.

Bactocera oleae (Gmelin) (Diptera: Tephritidae) is the most important insect pest of the olive tree, causing 30 % loss of the olive crop in Mediterranean countries including Greece. The pyrethroid α-cypermethrin is currently used against *B. oleae*, however high levels of resistance have been recently observed. Recently, after examination of the expression changes in resistant populations compared to susceptible, fourteen genes were found to be commonly up-regulated. Among them, contig00436, putative member of the *CYP6* cytochrome P450 family, was identified as the most striking hit. In this study, the overexpression of contig00436 was validated in resistant populations by real time quantitative PCR, cloned into pcW-ompA expression vector and functionally expressed, aiming to investigate if it is capable of metabolizing α-cypermethrin *in vitro*.

ΠΕΡΙΛΗΨΗ

Η μελέτη των ενζύμων μέσω των οποίων γεωργικά παράσιτα αναπτύσσουν ανθεκτικότητα σε εντομοκτόνα έχει ως στόχο την ανάπτυξη νέων μέσων καταπολέμησης και διαχείρισης του προβλήματος και είναι εξαιρετικής σημασίας για την προστασία των καλλιεργειών. Στη διάρκεια της μεταπτυχιακής μου διατριβής μελέτησα γονίδια και ένζυμα αποτοξικοποίησης σε δύο πολύ σημαντικούς εχθρούς καλλιεργειών:

Το άκαρι *Tetranychus urticae* είναι ένα από τα πιο καταστροφικά παράσιτα των καλλιεργειών. Είναι εξαιρετικά πολυφάγο αφού μπορεί να τραφεί με εκατοντάδες φυτών μεταξύ αυτών και φυτά τεράστιας γεωργικής σημασίας. Ανάλυση της γονιδιακής έκφρασης ενός στελέχους τετρανύχου (Μαραθώνας), ανθεκτικό σε πολλά εντομοκτόνα/ακαρεοκτόνα, έδειξε, μεταξύ άλλων, και δύο GSTs (TuGSTm07, TuGSTm09) να σχετίζονται με τον ανθεκτικό αυτό φαινότυπο. Στην παρούσα μελέτη, εκφράστηκαν λειτουργικά τα δύο αυτά ένζυμα και χαρακτηρίστηκαν κινητικά. Ακόμη, ελέγχθηκε η ικανότητα τους να αλληλεπιδρούν με εντομοκτόνα/ακαραιοκτόνα. Το ένζυμο TuGSTm07 έδειξε να αλληλεπιδρά με το bifenthrin, το οποίο και είχε ως αποτέλεσμα την αναστολή της ενεργότητας του ενζύμου σε πρότυπο υπόστρωμα (CDNB) κατά 70%. Η τρισδιάστατη δομή του ενζύμου TuGSTm07 προβλέφθηκε βασιζόμενη σε κρυσταλλογραφικά δεδομένα άλλων GST ενζύμων Mu κλάσης από θηλαστικά. Πειράματα docking του ενζύμου με το bifenthrin έδειξαν ότι πιθανότατα το bifenthrin προσδένεται στο ενεργό κέντρο του ενζύμου.

Το έντομο *Bactocera Oleae* (Gmelin) (Diptera: Tephritidae) είναι το πιο σημαντικό παράσιτο της ελιάς και προκαλεί ζημιές που αγγίζουν το 30% στις μεσογειακές χώρες συμπεριλαμβανομένης και της Ελλάδας. Το πυρεθροειδές α-cypermethrin έχει χρησιμοποιηθεί πρόσφατα ενάντια στον δάκο αλλά έχουν παρατηρηθεί αυξημένα επίπεδα ανθεκτικότητας. Πρόσφατα, μετά από εξέταση των αλλαγών στην έκφραση γονιδίων σε ανθεκτικά στελέχη δάκου σε σχέση με ευαίσθητα, δεκατέσσερα γονίδια βρέθηκαν να υπερεκφράζονται από κοινού στα ανθεκτικά στελέχη. Μεταξύ αυτών το contig00436 πιθανό μέλος της οικογένειας *CYP6* των

κυτοχρωμικών οξειδασών P450s, ταυτοποιήθηκε ως το πιο πιθανό υποψήφιο να σχετίζεται με τον φαινότυπο. Σε αυτήν την μελέτη, ποσοτική PCR πραγματικού χρόνου επιβεβαίωσε την υπερέκφραση του contig00436 στους ανθεκτικούς πληθυσμούς, κλωνοποιήθηκε σε φορέα έκφρασης PCW-ompA και εκφράστηκε λειτουργικά, ώστε να διερευνηθεί η ικανότητα του να μεταβολίζει το α-cypermethrin *in vitro*.

ABBREVIATIONS

ALA	Aminolevulinic acid
bp	Base pairs
BSA	Bovine Serum Albumin
CCE	Carboxylesterase
cDNA	Complementary Deoxyribonucleic acid
CDNB	1-chloro-2,4-dinitrochlorobenzene
CO	Carbon monoxide
CPR	Cytochrome P450 reductase
CuOOH	Cumene Hydroperoxide
DNA	Deoxyribonucleic acid
DDT	1,1'-(2,2,2-trichloroethane-1,1-diyl)bis(4-chlorobenzene
EDTA	Ethylenediaminetetraacetic acid
FC	Fold Change
GPx	Glutathione peroxidase
GSH	g-L-glutamyl-L-cysteinylglycine
GSSG	Oxidized Glutathione Disulfide
GST	Glutathione S-transferase
HPLC	High Performance Liquid Chromatography
IC50	The half maximal Inhibitory Concentration
ICIPE	International Centre of Insect Physiology and Ecology
IPTG	Isopropyl-beta-D-thiogalactopyranoside
Kcat	Catalytic Constant
kD	Killodalton
Km	Michaelis Constant
LC50	Lethal Concentration 50
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
Ni-NTA	Nickel- Nitrilotriacetic acid
OD	Optical Density
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
RR	Resistance Ratio
RT	Room Temperature
S. D.	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
U	Enzyme Unit

1. General introduction

1.1 Problems caused by pests

Insects and mites, of the most adaptable forms of life, are the most diverse species of animals living on earth. While less than 0,5 % of the total known number of them can be considered as pests [1], this number is still large enough to cause great damage to public health, farm animals and crops. Not only are they the most common vectors of human diseases, causing more than a million deaths annually according to the World Health Organization [2] but they are also responsible for destroying one fifth of the world's total crop production annually (International Centre of Insect Physiology and Ecology ICIPE, www.icipe.org). Today, the consequences of the latest are of great challenge both in the tropics and sub-tropics, where the climate provides a highly favorable environment for a wide range of insects, and in the developing countries where the problem of competition from pests is further complicated with a rapid annual increase in the human population (2.5-3.0 %) in comparison to a 1,0 % increase in food production [1, 2].

The spider mite *Teytranychus urticae* (Koch) (**Figure 1.1A**) is one of the most important and damaging species because of its ability to feed and develop on 1500 host plant species , including several economically important agricultural crops [3]. The olive fruit fly *Bactocera oleae* (Gmelin) (Diptera: Tephritidae) (**Figure 1.1B**) is the most important insect pest of the olive tree. The larvae are monophagous, and feed exclusively on olive fruits. The damage caused by tunneling of larvae in the fruit results in about 30 percent loss of the olive crop in Mediterranean countries including Greece [4], where large commercial production occurs.

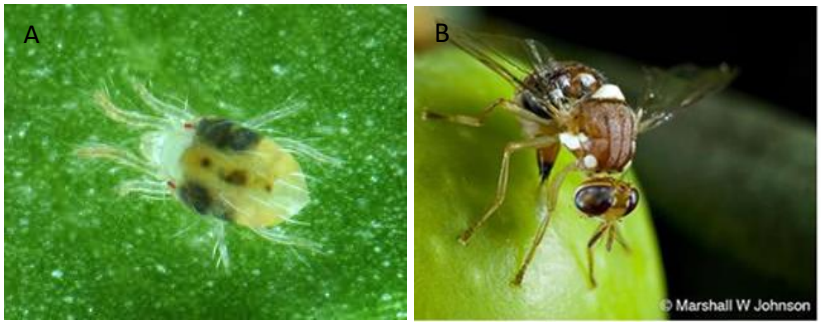


Figure 1.1 The spotted mite *Tetranychus urticae* (Koch) (A) (image form: www.promip.agr) **and the olive fruit fly *B. oleae* (B)** (image from ref. [3]). Both pests cause great damage to agricultural crops

1.2 Pest management strategies

Several ways for the sustainable control of pests' populations have been recorded till today, including: physical, biological, genetic and chemical control. Physical control includes methods that physically keep insect pests from reaching their hosts. Barriers and various types of traps are the most common examples [5]. Biological is the conscious use of living beneficial organisms called natural enemies for the control of the pests. Biological control includes the application of available predators, parasitoids , parasites and pathogens as natural enemies [5]. For example *Phytoseiulus persimilis* have been used for spider mite control and management [6]. Genetic control includes the release of genetically modified insects into the wild, aiming to reduce the population levels [7]. For example, a line of *Aedes aegypti* which carry dominant lethal genes was generated [8].

Still the most common and effective method is the use of insecticides/acaricides, which cause rapid death. Different chemical compounds, mainly targeting the central nervous system of the pest are used, including: organochlorides, organophosphates carbamates, pyrethroids, neonicotinoids etc. The best known organochloride, DDT, functions by opening sodium channels in the insect's nerve cells [9]. Organophosphates and carbamates also target the insect's nervous system. Organophosphates interfere with the enzyme acetylcholinesterase, disrupting nerve impulses and killing or disabling the insect [10]. Carbamates have similar mechanisms, but a much shorter duration of action and they are generally considered as less toxic compounds [10]. Neonicotinoids are synthetic analogues of the natural insecticide nicotine [11]. Compared to organophosphate and carbamate insecticides neonicotinoids cause less toxicity in birds and mammals than insects. These chemicals are acetylcholine receptor agonists [11]. Treated insects exhibit paralysis and death [12]. Pyrethroid insecticides, from natural pyrethrins to photostable analogues, represent important weapons against insect pests of both economic and medical importance. They target the sodium channels of the peripheral and central nervous systems [13]. Some of these compounds have been effectively used against *T. urticae* and *B.oleae* but the intense use has led to the development of resistance [14-17].

1.3 Insecticide resistance and insecticide resistance mechanisms

Insecticide resistance is a reduction in the sensitivity of a population to an insecticide. It is an inherited trait that makes the population to survive at doses in which normally would die [18]. The development of insecticide resistance is

influenced by many factors, including genetics, biology/ecology and control operations [19]. The way insects and mites can overcome such effects have been extensively studied and usually is a result of changes in its physiological or ecological properties. Mechanisms of decreased response to the pesticides (interaction of a pesticide with its target site), or mechanisms of decreased exposure (penetration, distribution, metabolism and excretion) can contribute to development of resistance [20, 21] (**Figure 1.2**). The majority of cases involve changes in the sensitivity of the target site due to point mutations, and/or sequestration/metabolism of the insecticide before it reaches the target site due to quantitative or qualitative changes in major detoxification enzymes (**metabolic-biochemical resistance**)[22].

Metabolic resistance is caused by the action of detoxification enzymes, i.e cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs) and carboxylesterases (CCEs), which metabolize insecticides/ acaricides into non-toxic derivatives [22]. Metabolic resistance usually appears either as a result of over-expression of enzymes capable of detoxifying insecticides or by amino acid substitutions within the genes encoding for these enzymes that increases the affinity of the enzyme for the insecticide and can result in high levels of resistance (**Figure 1.2**) [22]. These large enzyme families may contain multiple enzymes with broad overlapping substrate specificities, and there is a high probability that at least one member of the family will be capable of metabolizing one or more insecticides [23].

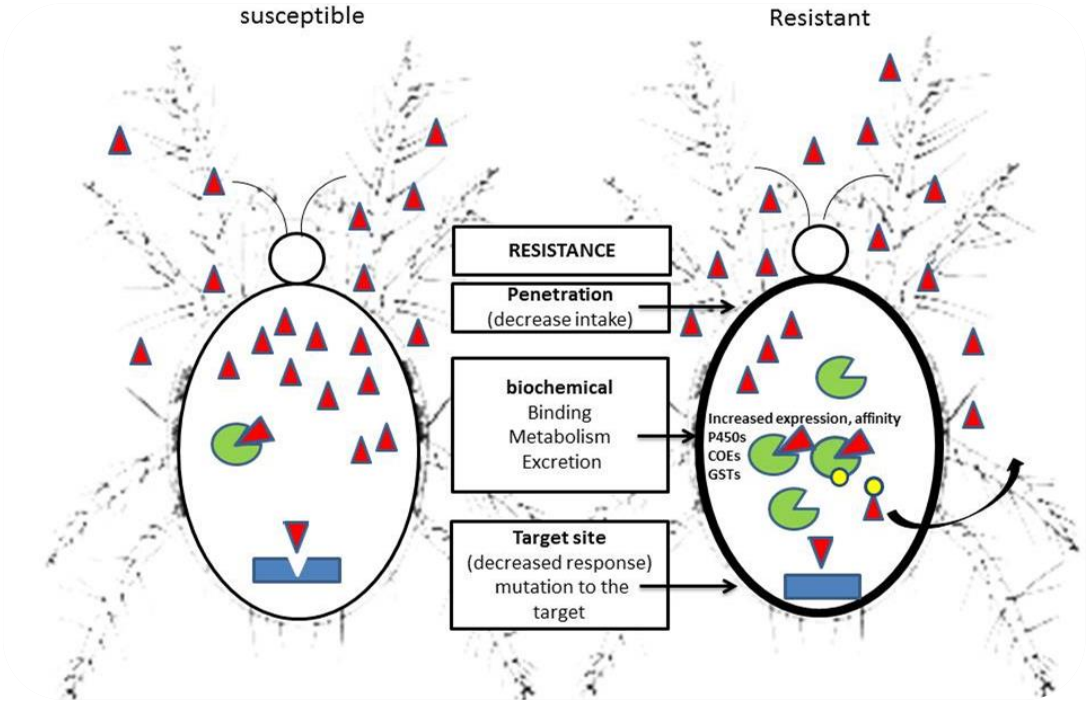


Figure 1.2 ***Graphical summary of the mechanisms by which pests develop resistance****. Red: insecticide, green: detoxification enzyme, blue: the target of the insecticide, yellow: a functional group (e.g hydroxyl group, epoxide) added to the insecticide*

1.4 Enzymes involved in metabolic resistance

1.4.1 Esterases based resistance

Insecticides such as organophosphates (OPs), carbamates and pyrethroids which contain ester linkages are susceptible to hydrolysis. An important group of metabolic enzymes involved in ester-hydrolysis- based resistance are esterases. Esterases are frequently implicated in the resistance of insects through gene amplification, up-regulation, coding sequence mutations, or a combination of these mechanisms [22]. Esterases are hydrolases that split ester containing compounds by the addition of water to produce acid and alcohol [24]. In detoxification based resistance mechanisms two types of esterases play an important role : carboxylesterases and phosphatases. The first type belongs to the A-esterase family and plays a significant role in degrading organophosphates, carbamates and pyrethroids [25]. Phosphatases are A-esterases which detoxify organophosphorus insecticides especially phosphates in insects. An example is the hydrolysis of paraoxon in house flies as well as the hydrolysis of alkyl-groups of OPs [25]. Esterase-based resistance has been studied extensively at biochemical and molecular level in *Culex* mosquitoes. Broad-spectrum organophosphate resistance is conferred by the elevated esterases of *Culex*. All these esterases act by rapidly binding and slowly turning over the insecticide. In other words they sequester rather than metabolize the pesticide as reviewed by Hemingway [23].

1.4.2 Glutathione S tranferases (GSTs) mediated resistance

The glutathione S-transferases (GSTs) are members of a large family of multifunctional intracellular enzymes ubiquitously distributed in nature. They are involved in the detoxification of both endogenous and xenobiotic compounds via glutathione conjugation, dehydrochlorination, glutathione peroxidase (GPx) activity [26]. These enzymes also have non catalytic functions related to sequestration of carcinogens, intracellular transport of a variety of hydrophobic compounds and modulation of signal transduction pathways [27]. This diversity of enzymatic and nonenzymatic functions is related to the genetic capacity to encode different GST isoforms by most organisms [26]

GSTs were originally grouped into three classes (I, II, and III) in insects, while mammalian GSTs grouped into one microsomal and eight cytosolic classes (alpha ,

mu, pi, theta, sigma, zeta, kappa and omega) [22]. Recent advances in insects' genome projects revealed an increased diversity of GSTs. Thus, a unified nomenclature system (corresponding to the mammalian GST classes) has been proposed [28]. According to that, Class I and III insect GST are designated Delta and Epsilon classes respectively, while, the class II insect GST is included in several mammalian classes (delta, epsilon, omega, sigma, theta, zeta). Recently, within the genome of *T. urticae*, members of Mu class were identified [29]. GST classification is based on the identity of the amino acid sequence (> 40%) and other properties such as phylogenetic relationships, immunological properties, tertiary structure and their ability to form heterodimers [22].

The metabolism of xenobiotics is often divided into three phases: modification (phase I), conjugation (phase II), and excretion (phase III). GSTs play a vital role in detoxification and antioxidant defense in phase II of drug metabolism. In this phase GST follows the phase I of drug-metabolism which is often catalyzed by cytochrome P450 (CYP) superfamily. The CYP enzymes introduce a functional group such as an epoxide into the xenobiotic [23]. That, offers an electrophilic center that is attacked by reduce glutathione (GSH), in a reaction catalyzed by GSTs [22, 30]. Then the compound conjugated with GSH can be removed from the cell during phase III of drug metabolism, a process which requires the participation of transporters such as multi-drug resistance associated protein (MRP) [31]. The GSTs have been involved also in phase I (increased insecticide metabolism - decreased target exposure) of insecticide detoxification in some cases or mechanisms by mediating the O-dealkylation or O-dearylation of organophosphorus insecticides [32] and in the dehydrochlorination of organochlorines [33]. GSTs may also contribute to insecticide resistance, by detoxifying primary insecticide metabolism or lipid peroxidation byproducts [34], and by binding insecticide molecules (such as pyrethroids) via a sequestration mechanism [35]. Epoxide containing compounds, alkyl- and aryl-halides, isothiocyanates, *α,β* unsaturated carbonyls and quiones are compounds that can be catalyzed by GSTs (**Figure 1.3**) [27].

GSTs can mediate resistance to organophosphate (OP), organochlorines, and pyrethroids by gene amplification or overexpression [22]. One or more GSTs have often been implicated in the resistance to organophosphates (OPs) in the house-fly, *Musca domestica*, organochlorine (OC) 1,1,1-trichloro-2,2-bis-(p-chlorophenyl) ethane (DDT) in the fruit fly, *Drosophila melanogaster* and more recently also reported in pyrethroid (PYR) resistance strains of planthopper, *Nilaparvata lugens* [26]. In mosquitoes, the GST based resistance is the major mechanism of DDT-resistance. In *A. aegypti* at least two GSTs are elevated in DDT-resistant insects while in *Anopheles gambiae* a large number of different GSTs are elevated, some of which are class I GSTs [23]. Four GST isoenzymes have been purified from *Plutella*

xylostella, two of which, GST3 and the closely related GST4, degrade the OPs insecticides parathion, methylparathion, and paraoxon [22].

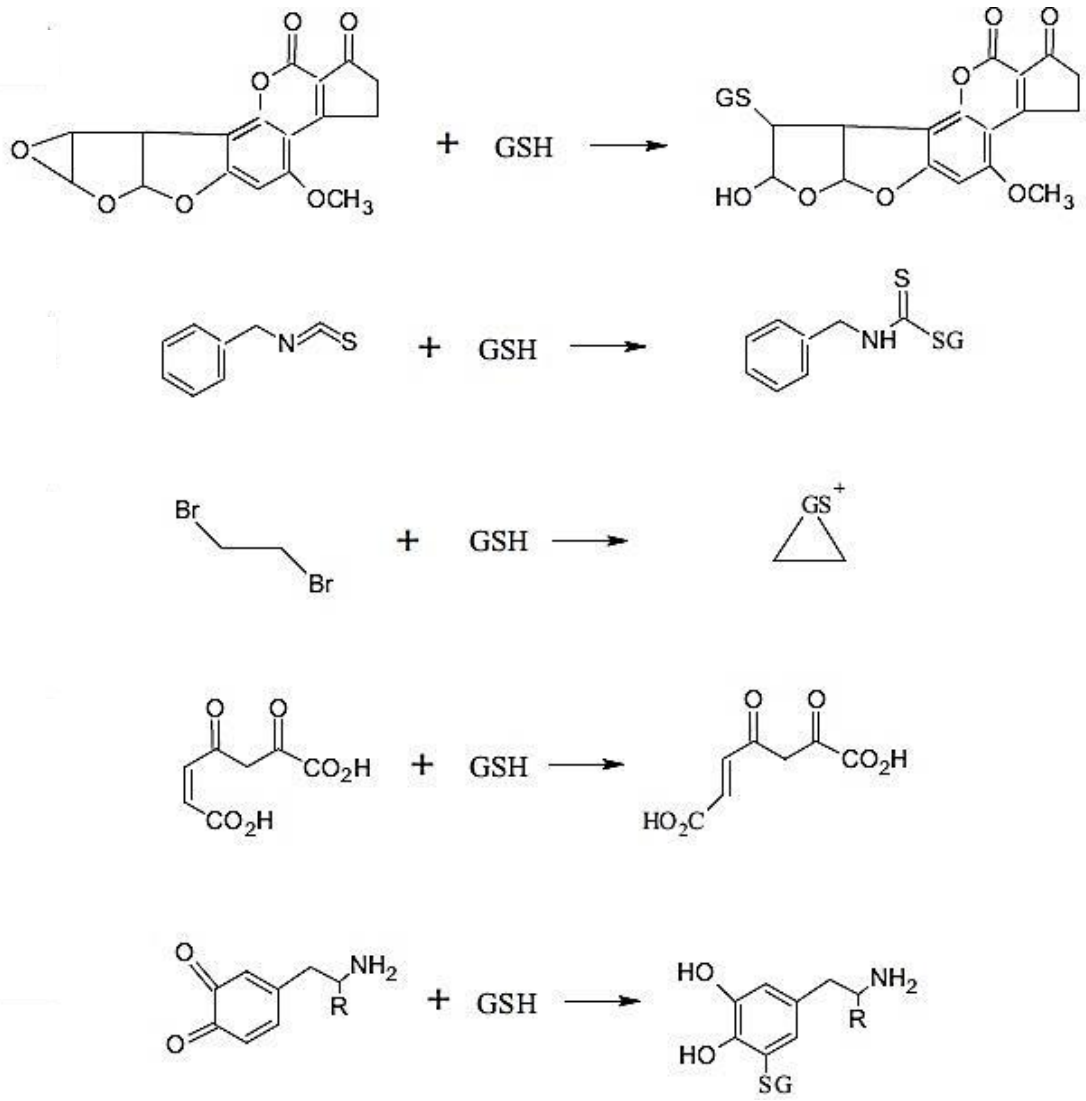


Figure 1.3.*Examples of GST catalysed reactions: aflatoxin, B1-8,9-epoxide, benzylisothiocyanate, dibromoethane, maleylacetoacetate, a model o-quinones*

It is well established that cytosolic GSTs are homo- or heterodimeric proteins, consisting of two monomers of approximately 25 kDa in size each **(Figure1.4)** [36]. Each subunit folds into two domains, the N-terminal (extreme 5´) and C-terminal (extreme 3´) joined by a variable linker region. The N-terminal domain (~ 80 residues) is arranged in a βαβαββα motif (**Figure 1.4**) and contains the majority of the residues for the binding of GSH. It is the most conserved domain among the different classes [36]. The larger C-terminal domain consists of a variable number of

alpha helices (**Figure 1.4**), and includes largely the residues of hydrophobic H-site or substrate binding site. The high level of diversity in this region confers in part the specificity of the GSTs for a broad range of electrophilic substrates [37].

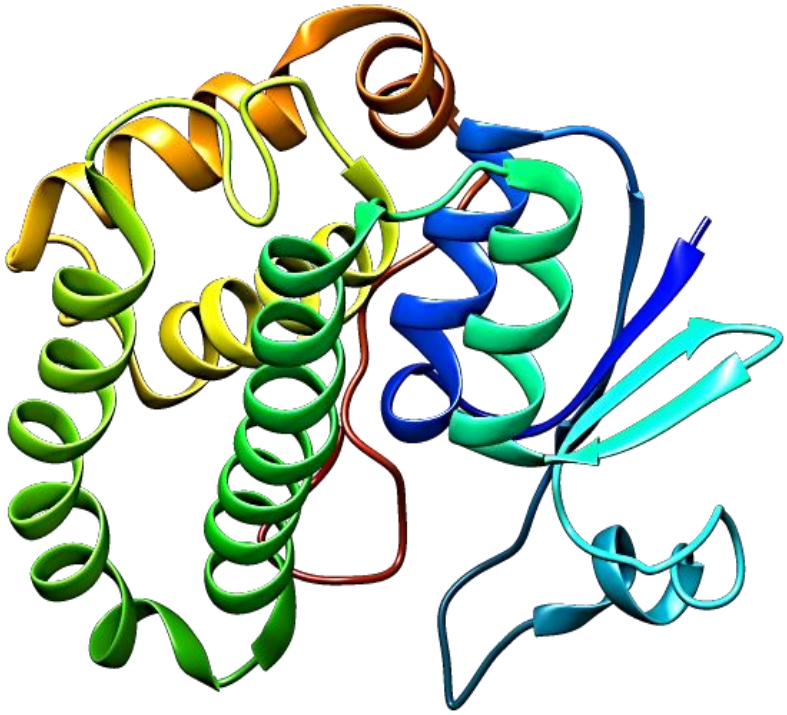


Figure 1.4 ***The Crystal structure of hGSTM2-2*** *(The structure was created with chimera 1.10.1 graphics system, using coordinates from the pdb, file: 2UGT, downloaded from http://www.rcsb.org/). The hGSTM2-2 is a homodimer, however only the monomer is presented here. The G-site (deep blue- cyan) and H-site (green- deep red) are also shown.*

1.4.3 P450-Mediated Resistance

The P450 monooxygenases are a complex family of enzymes found in most organisms, including insects. Most commonly, P450 enzymes bind molecular oxygen and receive electrons from NADPH to introduce an oxygen molecule into the substrate [23]. Because of their genetic diversity, broad substrate specificity, and catalytic versatility, P450s and their associated P450 reductases comprise a system that can catalyze reactions which involve almost all classes of insecticides [22]. P450 monooxygenases are involved in the metabolism of virtually all insecticides, via activation of the molecule in the case of organophosphorus insecticides, or more generally through detoxification.

Based on amino acid sequence homologies, a nomenclature system was proposed. According to that, all families have the CYP prefix followed by a numeral for the family, a letter for the subfamily, and a numeral for the individual gene. To date insect P450s have been assigned to six families: five are insect-specific and one, CYP4, has sequence homologies with families in other organisms[38]

Resistance caused by cytochrome P450s in many cases appear as a result of up-regulation either by mutations in trans regulatory loci or via indels (or mutations) in cis acting elements [22]. In *Musca domestica* for example the overexpression of *CYP6A1* and *CYP6D1* alleles is due at least in part to loss-of-function mutations in negative regulatory loci on chromosome 2 of the Rutgers and LPR (Learn pyrethroid resistant) strains [39]. Also multiple mutations and short indels are detected in the promoter sequences of *CYP6D1* (43 mutations, one 15-bp insertion located downstream of a putative silencer element for the Gfi-1-like repressor MdGfi-1) and in *CYP6D3* (16 nucleotide substitutions) in the house fly as reviewed by Li *et al.*2007 [22]. Resistance can also be caused by coding sequence changes. In *D. melanogaster* an increasing amount of evidence, including the incomplete loss of resistance when P450s are not overexpressed, suggests that most probably point mutations play a secondary role in P450-mediated resistance[40].

Elevated monooxygenase activity is associated with pyrethroid resistance in *Anopheles stephensi, Anopheles subpictus, An. gambiae* and *Cullex quinquefasciatus* [23]. In the house fly CYP6D1 can catalyze hydroxylation of the pyrethroid insecticides permethrin, deltamethrin, and cypermethrin at the 4’ position of the pyrethroid phenoxybenzyl moiety in the presence of cytochrome b5 [41]. Abamectin, a macrocyclic lactone has been recently shown to be metabolized by the CYP392A16, a cytochrome P450 associated with high levels of acaricide resistance in *T. urticae*. CYP392A16 is the first enzyme from an agricultural pest that is shown to be capable of metabolizing an active ingredient of the very important insecticide family of the avermectins [42].

1.5 Aims of the project

A multiresistant strain (Marathonas) isolated from a greenhouse near Athens exhibited extremely high levels to a variety of insecticides. Microarray studies showed the association of GSTs with this resistance phenotype. Although there is a strong association of some GSTs with the resistance in the literature, the putative role of GSTs had not been studied at protein level as yet. In the first part, of my master, studied the putative role of two GSTs (Mu class) in resistance at the protein level.

The second part of the master aims to understand the molecular mechanisms (identify, validate and functionally express the responsible enzymes) underlying the resistance of the olive fruit fly *Bactrocera oleae* to the pyrethroid insencticide α-cypermethrin aiming to use them in metabolic assays with α-cypermethrin.

Indentifying and studying the detoxication enzymes, by which agricultural pests develop resistance to insecticides, can facilitate the development of new means of managing and overcoming this resistance, a crucial task for crop protection.

2. Functional expression and characterization of two Mu-class Glutathione S Transferases associated with insecticide resistance in *Tetranychus urticae*

2.1 Introduction

Within the genome of *T. urticae*, 32 glutathione S transferases (GSTs) were identified belonging to the classes: delta (16 GSTs), mu (12 GSTs), omega (2 GSTs) and theta (1 GST) [29]. Two of the Mu class GSTs (TuGSTm07 and TuGSTm09), , were found up-regulated in a multi-resistant strain (Marathonas) isolated from Athens and exhibited high levels of resistance to a variety of pesticides (i.e. abamectin, hexythiazox, clofentezine, bifenthrin and pyridaben) [43] (**Table 2.1** data were taken from ref [42]).

Table 2.1 Toxicity of various insecticides in the Abamectin-resistant line (Mar-ab) compared to the susceptible line London of *Tetranychus urticae*

Insexticide/ strain	Regression Parameters			RR
	LC50 (mg/L) (95% CI)	Slope±SE	$\chi 2^{b}$	
Abamectin	512.2 (430.8-578.7)	4.3± 0.76	14	1642
Hexythiazox	>10000[a]			>1500
Clofentezin	>10000[a]			>1000
Bifenthrin	3571 (2861-4331)	2.2± 0.3	14	426

[a]Mortality at 10,000 mg/L was below 5% at 72 h post-treatment. [b]A χ^2 (Chi-squared) test was used to assess how well the individual LC50 values observed in the bioassays agreed with the calculated linear regression lines (LeOra Software 1987).

In mammals, Mu-class GSTs have been found to be involved in catalyzing the reaction of glutathione with endo- and xenobiotics, and in a variety of cellular processes like detoxification of endogenous and exogenous compounds in the liver, brain, and testis [44]. Although, the abundance of mu-class GSTs in mites like: *Sarcoptes scabiei* [45], *Ixodes scapularis* [46], *Varroa destructor* [47] and *Panonychus citri* [48] has been reported, there are no reports till today to demonstrate the interaction of Mu class GSTs with insecticides/acaricides in mites. Recently, it was suggested that Mu class GSTs could be possibly involved in detoxification of pyridaben in field populations in *P. citri* [48]. However, there is no available

information about the interaction between mu GSTs and acaricides/insecticides at a molecular level in *P. citri*.

Here we report the molecular cloning, expression and kinetic characterization of two mu-class GSTs associated with a multiresistant phenotype in the two spotted spider mite *T. urticae*, as well as their potential to interact with a variety of insecticides/ acricides.

2.2 Materials and methods

2.2.1 Cloning, functional expression and purification of recombinant GSTs

Total RNA of adult individuals of *T. urticae* was extracted using RNeasy mini kit (Qiagen), treated with Turbo DNAse to ensure that no genomic contamination existed and reversed transcribed with superscript III reverse transcriptase (Invitrogen Life Technologies) using oligo-dT primer. The obtained cDNAs was used in PCR reactions for the amplification of the sequences encoding for TuGSTs, using the same set of primers: 5-CACCATGGCACCAGTTATCGGTTATTGG (forward) and 5-TCAATATGGCTTTTGAATTGTGTCATTTCC (reverse) and DNA polymerase (Thermo Scientific). PCR conditions were 95^{o}C for 3 min, followed by 35 cycles of 95^{o}C for 30 sec, 61^{o}C for 30 sec and 60^{o}C for 30 sec. The pET100/D-TOPO vector (**Figure 2.1**), (Invitrogen Life Technologies) was used for the cloning of the PCR products and Nucleospin Plasmid (Macherey-Nagel) was used for plasmid extraction. In total, six different clones were send for sequencing in order to ensure that the cDNA sequences inserted correctly and to separate the two GSTs (97% amino acid sequence identity) .

E.coli BL21(DE3) competent cells were used for heterologous expression of TuGSTm07 and TuGSTm09, containing the corresponding plasmids. TuGSTm07 was grown in 2Lt LB at 37^{o}C in the presence of 100μg/ml ampicillin, while for the production of TuGSTm09, 5 x 400ml LB were used because of problems in large scale production. When the absorbance at 590 nm was at 0,7-1, the induction of TuGSTm07 and TuGSTm09 was carried out by the addition of 1mM and 0.5mM isopropyl b-D-thiogalactoside (IPTG) respectively. Cells were harvested by centrifugation at 5.000g for 20min after four hours of induction, and re-suspended in sodium phosphate buffer (20mM sodium phosphate buffer, 40mM imidazole, 500mM NaCl, pH 7.4).The sample was then sonicated and centrifuged in 10.000g for 30min at 4^{o}C. The supernatant was collected and the GSTs were purified employing Ni-NTA chromatography (Qiagen) following manufacturer's instructions.

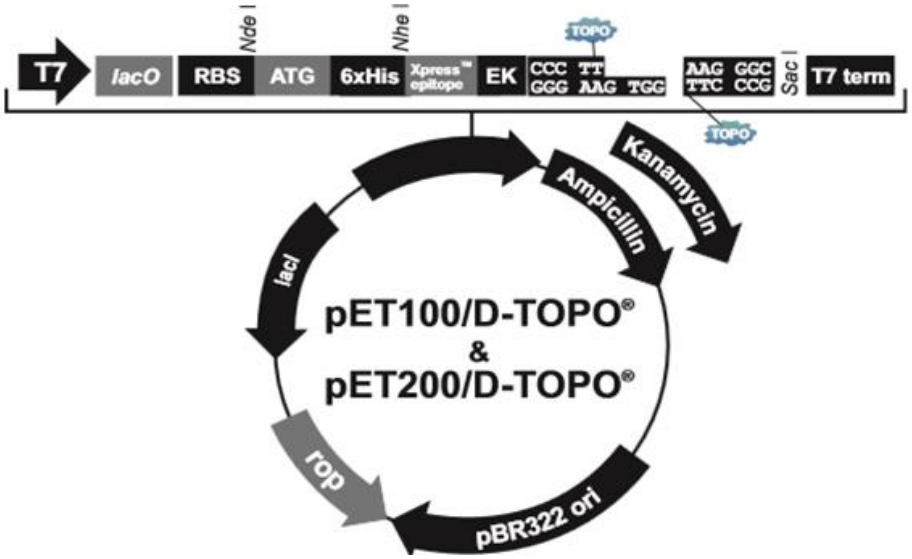


Figure 2.1 ***Expression vector used for molecular cloning of TuGSTs****. Some features of pET100 are: T7 promoter, lac operator (lacO), Ribosome binding site (RBS), Polyhistidine (6xHis) region, Xpres epitope, EK recognition site, TOPO recognition sites ROP, ORF, lacI OR*

Bradford assay (Bradford, 1976) [49] was used for the determination of the concentration of the proteins, while the purity of the recombinant enzymes was observed via SDS-PAGE gel.

2.2.2 Determination of substrate specificities for model substrates and kinetic studies

The activity of the GSTs was determined by measuring the initial rate of the enzyme-catalyzed conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB, Sigma-Aldrich) and 1,2- dicloro-4-nitrobenzene (DCNB, Sigma-Aldrich) at 25 °C and pH 6.5 as described by Habig *et al*. (1974) [50]. This initial rate was determined spectrophotometrically by monitoring the increase in absorbance at 340 nm over time. Correction was made for the nonenzymic conjugation by recording the increase in absorbance at 340nm without the addition of enzyme. Glutathione peroxidase activity was determined by coupling the reduction of cumene hydroperoxide (CuOOH, Sigma Aldrich) by GSH to the oxidation of NADPH by oxidated glutathione disulfide (GSSG) with glutathione reductase according to the method described in Simmons *et al.* (1989) [51]. The activity is expressed as µmol/min per mg of protein. For kinetic studies, initial velocities were determined at

pH 6.5 using the spectrophotometric assay described above at 25°C in 0,1M potassium phosphate buffer. GSH concentrations were varied from 0,075 to 15 mM at fixed concentrations of CDNB (0,99mM). Initial velocities were also determined at a constant concentration of GSH (2,475 mM), while CDNB was used in the concentration range of 0,03 to 3 mM. All the measurements were carried out in 96-well plates (NuncMaxiSorp) using a SpectraMaxM2e multimode microplate reader (Molecular Devices, Berkshire, UK). The kinetic parameters kcat and Km were determined by fitting the steady-state data to the Michaelis- Menten equation using GraFit3 software (Ericathus Software Ltd., Version 3.06).

2.2.3 Enzyme – acaricides/insecticides interaction studies

Abamectin, hexythiazox, clofentezine, bifenthrin and pyridaben (Sigma-Aldrich) were used in 10% methanol (of the final volume of the reaction) for their possible interaction with Mu GSTs. The inhibition of activity of the GSTs towards CDNB, was observed spectrophotometrically by monitoring the decrease in absorbance at 340 nm over time, in the presence of 0,05mM of the insecticide/acaricide with the concentrations of 0,99mM and 2,47mM for the CDNB and GSH respectively. For the IC_{50} calculation, the percentage inhibition of the TuGSTm07 activity was measured in different concentrations of bifenthrin (in a range of 1 to 100μM) in the presence of 0,99mM CDNB. The Grafit3 software (Ericathus Software Ltd., Version 3.06) was used for the sigmoidal curve. Plates of 96 wells (NuncMaxiSorp) were used for all the measurements as well as SpectraMaxM2e multimode microplate reader (Molecular Devices, Berkshire, UK).All the experiments were carried out at 25oCaccording to the method described [50].

2.2.4 Molecular modeling, and docking with bifenthrin

The isoenzyme hGST2-2 of Mu-class was selected as the most similar protein to TuGSTm07 (PDB entry 2C4J). Molecular modeling was started by submission of TuGST sequence to the Swiss-model server using the human GST coordinates as a crystallographic structure template. The global model quality score was estimated 0.9443 suggesting high quality of the model. For inspection of models and crystal structures the program PyMOL (http://www.pymol.org/) was used. Blind docking was performed using the Swiss-Dock server and the dimer model of TuGSTm07 structure was used

2.3 Results and discussion

2.3.1 Cloning, heterologous expression and purification of TuGSTs

cDNA sequences were successfully cloned into the vector pET100/DTOPO. *E.coli* BL21 (DE3) competent cells that used for heterologous expression of both GSTs resulted in large amount of TuGSTm07 production when induced with 1mM IPTG at 37oC for 4 h. However under the same conditions, TuGSTm09 was mainly obtained as inclusion bodies. Except from 37^{o}C, two other temperatures (25^{o}C and 28^{o}C) and three different final concentrations of IPTG (1mM, 0,5mM and 0.1mM) for each temperature were tested for the induction of TuGSTm09 for 4h. In addition, at 28^{0}C, the two concentrations of IPTG (0,5mM and 0,1mM) were used, overnight. All conditions were then tested in SDS-PAGE for the amount of the enzyme that contain in the soluble fraction **(figure 2.2**). The decrease in the concentration of IPTG from 1mM to 0,5mM with decreasing the temperature from 37^{o}C to 28^{o}C resulted in sufficient enough amount of TuGSTm09 in the soluble fraction for downstream analysis.

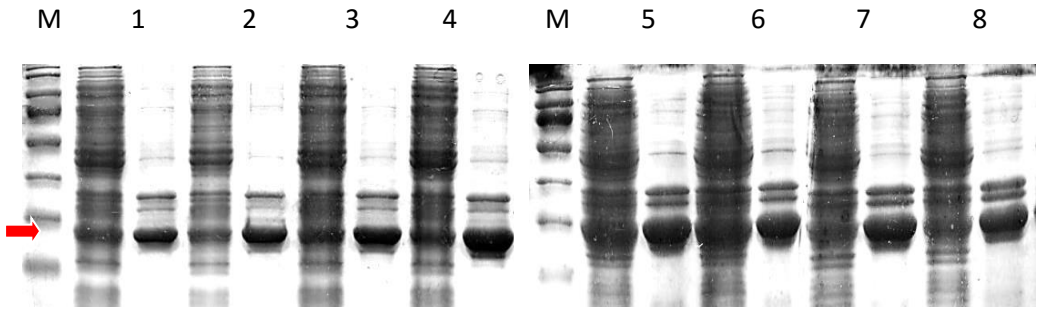


Figure 2.2 ***Supernatants and pellets in 10% SDS-PAGE after induction in different conditions***. *The induction was performed: (1-2) at 28^{0}C, overnight with 0,5 and 0,1mM IPTG respectively. (3-5): at 25^{o}C for 4h with 1mM, 0,5mM and 0,1mM IPTG respectively. (6-8): at 28^{o}C for 4h with 0,5mM, 1mM and 0,1mM IPTG respectively. (M): refers to the Marker. The red arrow indicates the molecular weight of the GST m09 monomer (25kDa)*

The purification of both GSTs was carried out employing metal affinity chromatography (Ni-NTA columns) taking advantage of the high affinity of the nickel for the His aminoacid (HIS-tag) and the proteins were obtained in purity and in high amounts (**Figure 2.3**) when eluted with high concentration (500mM) of imidazole. They were also found to be catalytically active.

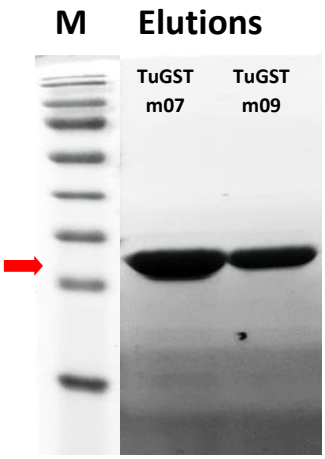


Figure 2.3 ***10% SDS-Page for the purification of TuGSTs****. The red arrow indicates the 25kDa which is the molecular weight of the monomer.*

2.3.2 Substrate specificities and kinetic properties of recombinant TuGSTs

The specific activities, or the amount of product produced per minute per mg of the total protein at 25^{o}C, of TuGSTs were tested against model substrates. To investigate if TuGSTs exhibit gloutathione transferase activity as well as gloutathione peroxidase activity. CDNB, DCNB and CuOOH were selected as model substrates and **Table 2.2** summarizes the results. Both GSTs are capable of transferring GSH in model substrates (CDNB and DCNB) as well of exhibiting glutathione peroxidase activity (towards CuOOH). The specific activity of the Mu class GSTs from *Tetranychus urticae* (GSTm07 and GSTm09) for CDNB and DCNB is lower compared to other Mu class GSTs from the cattle tick, *Boophilusannulatus* (121 µmol/min/mg for CDNB and 29,3 µmol/min/mg for DCNB, respectively)[47], but similar compared to Mu class GSTs from human[44]. The peroxidase activity of the both TuGSTs is lower than the respective activity of the Mu GST isolated from cattle tick (62,4µmol/min/mg) [47], but higher compared to human Mu class GSTs (1,3 and 0,63 µmol/min/mg,)[44].

Table 2.2 **Substrate specificities of Mu class *T. urticae* (Marathonas strain) GSTs for CDNB, DCNB and CuOOH.**

Substrate	Specific activity[a] (Unit[b] mg^{-1})	
	GSTm07	GSTm09
1-Chloro-2,4-dinitrobenzene (CDNB)	17,914 ± 0,935	15,94 ± 0,70
1,2-Dichloro-4-nitrobenzene (DCNB)	0,480 ± 0,214	0,09 ± 0,00
Cumene hydroperoxide, (CuOOH)	2,830 ± 0,035	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 25oC. [b] One unit (U) is the amount of enzyme that catalyzes the reaction of 1 µmol of substrate per minute at 25oC

The basic kinetic parameters for CDNB and GSH were also determined (**Table 2.3**) based on the method described in materials and methods. The Km values, in other words the amount of substrate needed for the enzyme to obtain half of its maximum rate of reaction, of TuGSTs for GSH are comparable with the values of Mu GSTs from *S. scabiei* (0.30 mM for ScGSTM1-1 and 0.40 mM for GSTM2-2). TuGSTm07 and TuGSTm09, exhibit also higher catalytic activities, for both substrates, compared with the two Mu class ScGSTs previously characterized (kcat values for GSH: 0,15 min^{-1} for ScGSTM1-1, 0,06 min^{-1} for ScGSTM2-2, and kcat values for CDNB: 0,17 ± 0,01 min^{-1} for ScGSTM1-1, 0,10 min^{-1} for ScGSTM2-2,[45]). The catalytic "effectiveness" (kcat/km) of TuGSTs was also remarkably higher, for both GSH and CDNB substrates, compared to the two Mu class SsGSTs [45].Especially in the case of TuGSTm07 the kcat/km value is for the CDNB conjugation reaction is notably high suggesting that the enzyme converts the substrate into product much faster.

Table 2.3 Steady-state kinetic analysis of TuGSTs for the CDNB conjugation reaction

Kinetic parameter	**TuGSTm07**	**TuGSTm09**
Km(mM) GSH	0,73 ± 0,07	2,34 ± 0,31
Km(mM)CDNB	0,20 ± 0,05	0,26 ± 0,04
k*cat*(min^{-1}) GSH	35,43 ± 1,5	23,4 ± 1,52
k*cat*(min^{-1}) CDNB	32,85 ± 2,96	14,7 ± 0,85

K*cat*/Km ($mM^{-1}\cdot min^{-1}$) GSH	48,53	10
K*cat*/Km ($mM^{-1}\cdot min^{-1}$) CDNB	164,25	56,53

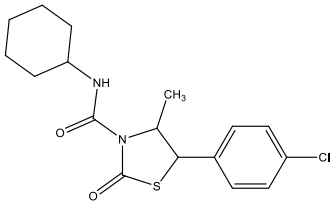
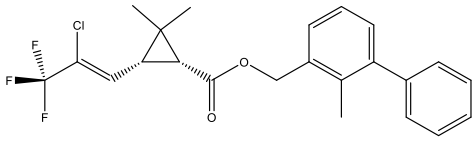
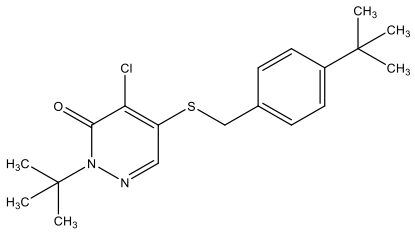
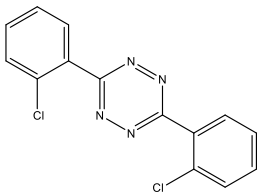
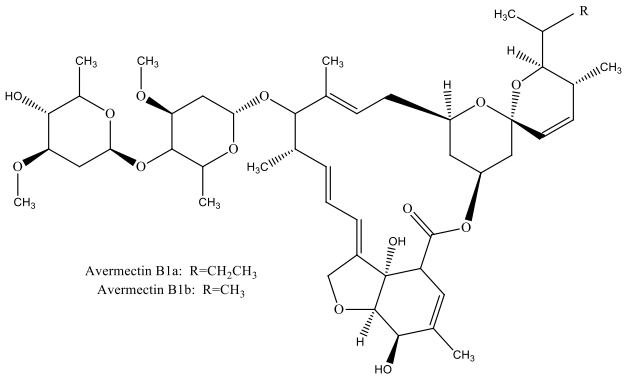
All values are means ± S.D. of three independent experiments. Results were determined by varying the concentration of GSH (0,075-15Mm) and CDNB (0,03-3mM) at fixed concentrations of CDNB(0,99Mm) and GSH (2,47Mm) respectively.

2.3.3 Enzyme – acaricides/insecticide interaction studies

In order to investigate the potential interaction of TuGSTm07 and TuGSTm09 with the insecticides/acaricides that showed reduced toxicity in Marathonas strain, inhibition assays were performed. The insecticides/acaricides abamectin, bifenthrin, clofentezine, hexythiazox and pyridaben were tested for their ability to inhibit the activity of the Mu class GSTs to conjugate CDNB with GSH. The percentage inhibitions in the activity of TuGSTs towards CDNB are presented in **Table 2.4**. Pyridaben and clofentezine caused ~25 % inhibition in the conjugating activity of TuGSTm09 and hexythiazox caused ~13%. While, bifenthrin and abamectin did not cause any inhibitory effect in TuGSTm09 under assay conditions. Remarkably, bifenthrin caused 75% inhibition in the activity of TuGSTm07. With the exception of clofentezine that caused a 15% inhibitory effect in the TuGSTm07 activity no other chemical compound tested appear to have any effect under assay conditions.

To investigate further the inhibition/ interaction of TuGSTm07 with bifenthrin, the concentration of bifenthrin needed to inhibit the CDNB conjugating activity by half (IC_{50}) was calculated by a dose–response curve (**Figure 2.4**). IC_{50} is a measure of the effectiveness of the inhibitor and in this case indicates the concentration of bifenthrin is needed to inhibit the CDNB conjugating activity, catalyzed by TuGSTm07, by half and was determined at 7,7103 ± 0,956 μM, showing significant inhibition.

Table 2.4 Percentage inhibition of activity (U/ml) of *T. urticae* GSTs derived from the effect of acaricides/insecticides

		Percentage inhibition of activity[a] (U[b]/ml)	
Acaricide/ Insecticide	**Structure**	**TuGSTm07**	**TuGSTm09**
Hexythiazox		n.d.	12,94 ± 4,55
Pyridaben		n.d	25,84 ± 14
Bifenthrin		72.2±6.1	n.d
Clofentezine		14.07±4	27,68 ± 2,99
Abamectin		n.d	n.d

All values are means ± S.D. of three independent experiments .[a] Refers to the amount of product produced per minute per ml of the total enzyme at 25°C.[b] One unit (U) is the amount of enzyme that catalyses the reaction of 1 µmol of substrate per minute at 25°C. Enzymes were assayed using GSH and CDNB as substrates and acaricides/insecticides were used in a 0,05mM final concentration

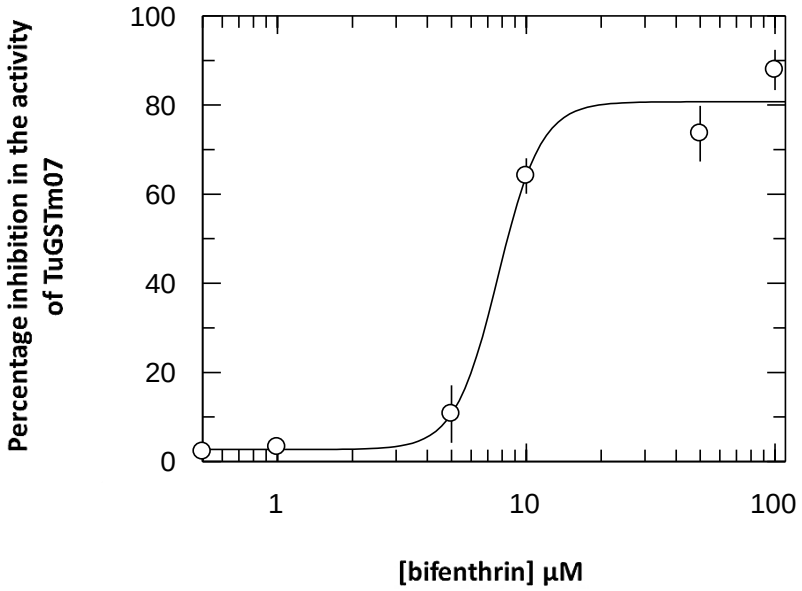


Figure 2.4 ***Dose response curve for IC50 calculation of bifenthrin***. *Four different concentrations of bifenthrin were used: 1, 5, 10, 50, 100 µM. All values are means ± S.D. of three independent experiments. The IC50 was determined at 7,7103 ± 0,956 µM.*

2.3.4 Molecular modeling of TuGSTm07 and docking with bifenthrin

In order to further study the molecular interaction between TuGSTm07 and bifenthrin, molecular modeling and docking studies were performed. Based on X-ray data derived from human GSTM2-2 (PDB: 1HNA) the TuGSTm07 structure was predicted. TuGSTm07 monomer consists of two domains the N- and C- terminal domain **(Figure 2.5A**). The N terminal domain contains the majority of the residues for the binding of GSH and referred as G-site [30]. The G-Site has the folding topology βαβαββα arranged in the order β2, β1, β3 and β4 with β3 anti-parallel to the others, forming a regular β-sheet with a right-handed twist surrounded by three α-helices **(Figure 2.5A)**. A key characteristic found in the structure of GSTm07 is the catalytic amino acid Tyr 7 (**Figure 2.5B,D**). This residue contains a hydroxyl group that may acts as a hydrogen bond donor to the thiol group of GSH, contributing to stabilization of reactive thiolate anion (GS−) which is a nucleophile group for an electrophilic substrate. This domain is the most conserved domain among members of different classes of GSTs [30]. The C-terminus domain is consisted of five a-helices **(Figure 2.5A)**. This site is responsible for the binding of the electrophilic substrate

(hydrophobic ligand binding site ,H-site) [52]. The H-site of GSTm07 is located next to the G-site (GSH binding site), exposed to the bulk solvent and is formed by hydrophobic residues such as: Phe 105, Trp 112, Leu 10, Leu 74 mainly from the C-terminal domain **(Figure 2.5D)**. The H-site exhibits a low degree of sequence identity between different members giving the enzyme unique biochemical characteristics (e.g. different substrate and inhibitor specificities) [52] **(Figure2.5B)**. Within the structure of Mu-class GSTs, the residue Tyr 116 is known to provide electrophilic assistance in addition of GSH to xenobiotics. Tyr 116 was also identified in TuGSTm07. The two domains are connected by a short linker that begins at the end of helix H3 **(Figure 2.5A)**. Another important structural characteristic usually the class Mu possess, is the mu-loop **(Figure 2.5A)** [53]. The "mu loop," an 11-residue loop spanning amino acid residues 33–43, is a characteristic structural feature of the Mu class of glutathione S transferases. This is a consequence of an insertion in the nucleotide sequence and the loop. It represents one of the three structural elements that create the active site of the enzyme. Studies that deleted the mu-loop in rat GSTM1-1 suggested that the mu loop is not essential for the enzyme to maintain its structure nor is it required for the enzyme to retain some catalytic activity. However, it is important for the enzyme's affinity for its substrates and make the enzyme more thermostable [53].

Docking analysis of bifenthrin into TuGSTm07 was performed (**Figures 2.5C, D**). It was revealed that most preferably, bifenthrin binds to the active site of the enzyme (**Figure 2.5B,D**). It has been reported that contacts between the GSTs and ligands are mainly Van der Waals interactions [54]. Such interactions with bifenthrin could be possible through hydrophobic amino acids: Phe 41, Phe 105, Trp 8, Trp 46, Trp 112 and Leu 35, Leu 60 (**Figure 2.5D**). Interestingly, residue Tyr 116, a residue proved to be involved in the protonation of the oxygen of equilateral triangle ring containing substrates [55], is involved in the interaction with bifenthrin, which also contains a equilateral triangle ring.

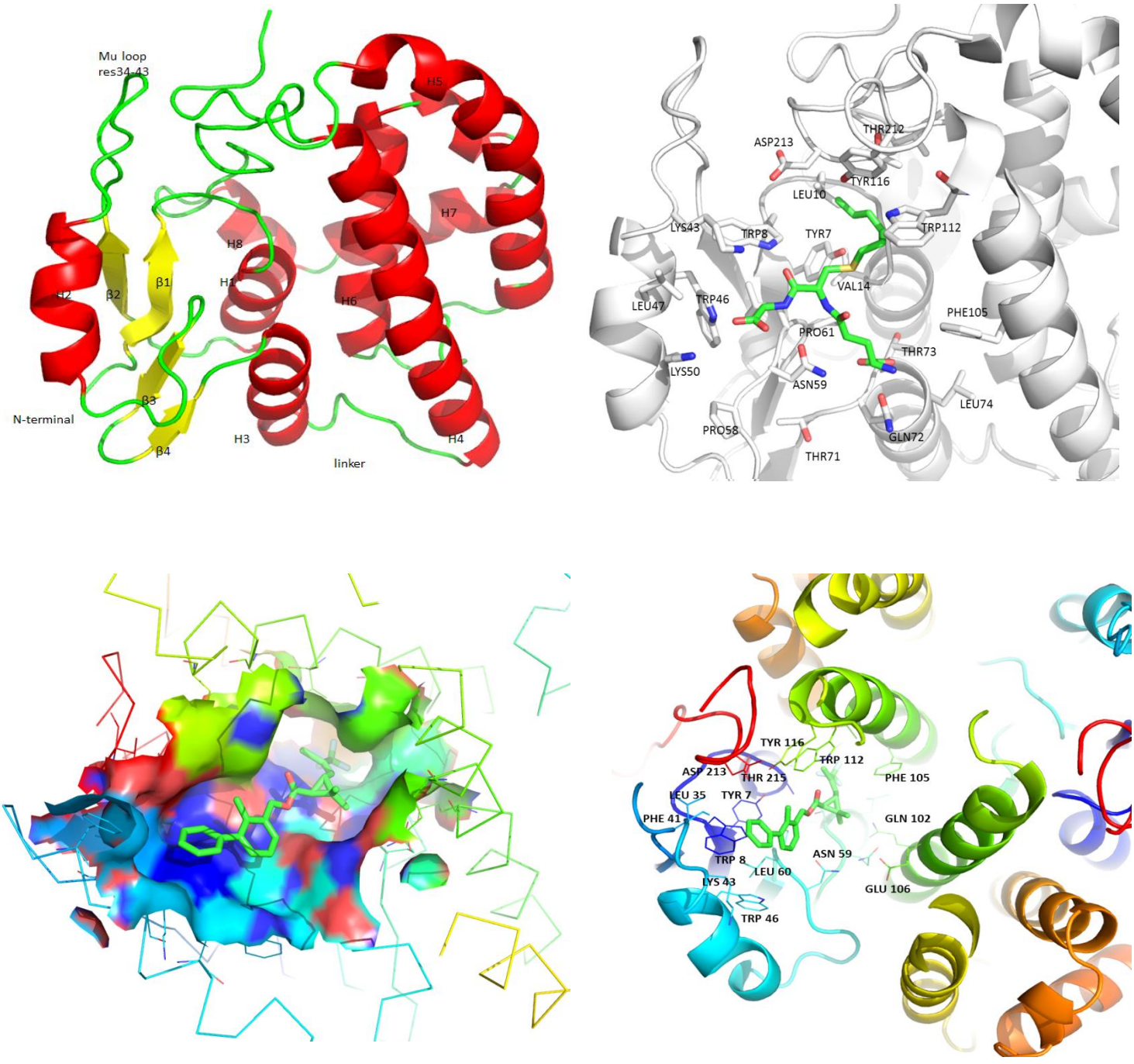


Figure 2.5 ***Modeling of TuGSTm07 and blind docking with bifenthrin*** *(A) Ribbon diagram of the TuGSTm07 monomer model. Helices (H) are in red, β-strands in yellow. The C- and N terminals the linker and the Mu-loop are labeled. The molecular figure was created using PyMOL [56]. (B) Important residues that contribute to G- and H-site formation and the catalytic aminoacid TYR7 are highlighted here. (C) Blind docking of bifenthrin with TuGSTm07 dimer model, using Swiss-Dock server. Bifenthrin binds to the active site of the enzyme. (D) Residues of TuGSTm07 potentially interact with bifenthrin.*

3. Molecular characterization of pyrethroid resistance in the olive fruit fly *Bactrocera oleae*

3.1 Introduction

B. oleae (Gmelin) (Diptera: Tephritidae) is the most important insect pest of the olive tree. The fly lays its eggs in the olive fruit and the larvae feed and grow in the mesocarp. The larvae are extremely monophagous, and feed exclusively on olive fruits. The infestation leads to direct (table olives are unsuitable for consumption) or indirect (reduction in oil quality) damage. In Greece, 30–35% economic losses due to *B. oleae* have been recorded [4].

The olive fruit fly is highly attracted to the yellow color, which has been incorporated into the design of several types of traps used for monitoring and mass trapping. However, concern for the high numbers of beneficial insects caught on yellow traps has led to the abandonment of this tactic in some regions [57]. Over the last four decades, the management of olive fruit fly has been based on the use of organophosphate insecticides in cover sprays and bait sprays (e.g., dimethoate and fenthion) [16, 58] but their intense use has led to the development of resistance [16]. Pyrethroids such as α-cypermethrin are particularly important and now widely applied due to several advantages over other insecticides in term of low cost, safety (less toxic to mammals) and duration of residual action. During the last several years there has been an increase in the use of pyrethroids for the control of *B.oleae*as an alternative to OPs [57].

Unfortunately, even short-term intensive and widespread use of pyrethroids can led to the development of pyrethroid resistance. Despite recent progresses, molecular mechanisms underlying pyrethroid resistance remain to be poorly understood. Pyrethroid resistance has been associated with target-site mutations within the domain II of the para type sodium channel gene in some cases and more frequently It has been proved that increased P450-mediated detoxification is a major mechanism of pyrethroid resistance in insects [59, 60]. To date, more than 2,000 insect P450s have been assigned to 67 families based on the identity at the amino-acid sequence level and the members of *CYP6* family have been verified to be involved xenobiotic metabolism [60]

The possible occurrence of target-site mutations within the domain II region of the *B. oleae* paratype sodium channel gene, which could be responsible for the pyrethroid resistance, was recently investigated [16, 61]. Analysis of domain II sequences in individuals from the most resistant populations, which survived the highest

insecticide dosages, did not reveal resistance-associated aminoacid substitutions. These data indicate no association between pyrethroid resistant and mutations on domain II (IIS4–IIS6) [16, 61]. However the implication of P450 monooxygenases in pyrethroid resistance of other Tephritidae, such as *B. dorsalis* [62] and and *Dacus ciliates Loew* [63]*,* is in line with the recent observations.

A recent study in our lab, used transcriptomic approaches to investigate the possible implication of metabolic enzymes in resistant populations of *B. oleae* to α-cypermethrin [61]. After examination of the expression changes in two resistant strains (AN14, ANik12) in relation to three susceptible (LAB-L, HYB FOKIDA), 14 genes were found to be commonly up-regulated in all comparisons [61]. Contig00436 was the most striking hit. Although the genome of *B. oleae* remains unknown, recent analysis of the *B. oleae* transcriptome and phylogenetic classification of the major detoxification gene families [64] revealed that contig00436 share high amino acid sequence identity with members of *CYP6* family and is a putative homologue of *Cyp6a23* of *Drosophila ananassae*.

Belonging to *CYP* family, Contig00436 is potentially of interest because they have been linked to insecticide resistance in several species [57, 65]and the physiological mechanism by which CYP enzymes act to reduce insecticide susceptibility is well studied in many cases[22]. There are many reports demonstrating the relationship between pyrethroid resistance and elevated activity of CYP6 family members in different mosquito species [60]. For example, *CYP6Z1*, *CYP6Z2*, *CYP6M2* and *CYP6P3* gene were found overexpressed in pyrethroid-resistant strains of *Anopheles gambiae* [66], *CYP6M2* and *CYP6P3* in *Anopheles arabiensis* [67], *CYP6Z6*, *CYP6M6* and *CYP6M11* gene in *Aedes aegypti*[68], *CYP6AA7* gene in *Cullex quinquefasciatus* [69]and *CYP6F1* gene in *Cullex pipiens pallens* [70]. *In vitro* metabolism assays have demonstrated that CYP6D1 can catalyze hydroxylation of the pyrethroid insecticides permethrin, deltamethrin, and cypermethrin [22]. Especially members of CYP6 family, such as CYP6P3, CYP6M2 and CYP6P9b, were proved to directly metabolize pyrethroids [60] .These findings in combination with our data indicate, most probably, P450 based detoxification resistant mechanism in *B. oleae*.

Here the overexpression of contig00436 (GAKB01000438.1), found overexpressed in α-cypermethrin resistant populations of *B. oleae*, by microarray experiment, was validated with RT-qPCR. The molecular cloning and the expression of the contig00436 are also reported, aiming to investigate if it is capable of metabolizing α-cypermethrin *in vitro.*

3.2 Materials and methods

3.2.1 RNA extraction and cDNA synthesis

For each one of the five populations used in the microarray experiments, two resistant: AgNik12, AN14 and three susceptible LAB-L, HYB, FOKIDA, total RNA was extracted from 4 biological replicates of ten, one to three days old, females using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNAs were treated with DNase I (RNase- Free DNase Set Qiagen) to remove any contaminating DNA. RNA quantity was measured using NanodropND-1000 spectrophotometer (NanoDrop Technologies)and RNA quality was judged in a 1.5% agarose gel. Using Superscript III reverse transcriptase (Invitrogen Life Technologies), Oligo-dT 20 primers (Invitrogen Life Technologies) and 2μg of the RNA as template, cDNA synthesis was performed according to the manufacturer's instructions.

3.2.2 Validation of the overexpression of Contig00436 (GAKB01000438.1) with RT-qPCR

For quantitative reverse transcription PCR (RT-qPCR), primers were designed using Primer – BLAST online analysis software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), based on cDNA sequences retrieved from previous work[64], and are listed in **Table3.1**. PCR reactions of 25 μl performed on a MiniOpticon two-color Real-Time PCR detection system (BioRad), using 0.20 μM primers and KapaSYBR FAST qPCR master mix (Kapa- Biosystems) . A 5-fold dilution series of pooled cDNA was used assess the effieciency of the qPCR reaction for each gene-specific primer pair. A no template control (NTC) was also included to detect possible contamination. Melt curve analysis was performed to test the specificity of amplicons. Experiments were performed using 3 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). Relative expression levels were calculated according to Pfaffl[71].

3.2.3 Cloning and co-expression of contig00436 (GAKB01000438.1) with CPR, and preparation of membranes

A KapaTaq DNA polymerase (KapaBiosystems) was used to PCR amplify sequence corresponding to the full-length open reading frame of the P450 gene encoded by

contig00436, using 1µl of cDNA derived from AN14 population cDNA as template and the primers listed in **Table 3.1**. PCR conditions were 95 ^{o}C for 2 min, followed by 30 cycles of 95 ^{o}C for 30 sec, 55 ^{o}C for 30 sec and 72 ^{o}C for 1 min and 40 sec. PCR product was purified using NucleoSpin Extract II kit (Macherey- Nagel) and inserted into the pCW-OmpA2 vector (**Figure 3.1**) [72] pre-digested with the same restriction enzymes.

For functional expression, *Escherichia coli* JM109 competent cells were co-transformed with the pCW-OmpA2-contig00436 plasmid and the expression vector pACYC-AgCPR containing cytochrome P450 reductase from *Anopheles gambiae* (AgCPR, GenBank accession number: AY183375) and peIB signal sequence [72]. Transformed cells were grown in Terrific Broth (Sigma–Aldrich) containing 100 µg/ml ampicillin and 34 µg/ ml chloramphenicol until the optical density at 595 nm reached an OD = 0,9-1. Then, the heme precursor d-aminolevulinic acid (ALA) was added to a final concentration of 1 mM. Expression was induced by the addition of 1 mM isopropyl-1-thio-β-d-galactopyranoside (IPTG).

Spheroplasts were prepared by adding TSE buffer (0.1 M Tris–acetate, pH 7.6, 0.5 M sucrose, 0.5 mM EDTA) containing 0,25 mg/ml lysozyme to the cell pellet and gentle mixing for 60 min at 4 °C. The solution was centrifuged at 2800 × *g* for 25 min at 40 °C and the spheroplast pellet was resuspended in spheroplast re-suspension buffer (0.1 M potassium phosphate buffer, pH 7,6, 6 mM magnesium acetate, 20% glycerol) containing 0,1 mM dithiothreitol, 1 mM phenyl-methane-sulfonyl-fluoride (PMSF), 1 mg/ml aprotinin and 1 mg/ml leupeptin. The suspension was sonicated and the membrane fraction was pelleted by ultracentrifugation at 180,000 × *g* for 1 h, at 40 °C. The resulting membranes were diluted in TSE buffer, total protein concentration was determined by Bradford assay [49]. P450 concentration and CPR activity were estimated by monitoring cytochrome C reduction [73, 74].

Table 3.1 .**Primers used for RT-qPCR validation and Cloning**

Gene/Sequence Name	GenBank ID*	Primer (q, c)[a]	Sequence (5'-3')[b]	Tm	Product size
Contig00436	GAKB01000438.1	F(c)	GAGCTCATGAGCTTGTTCTTGAACTTGTTGG	55	
		R(c)	TCTAGATTACAAGCTCTCCACTCGCAG	55	
		F(q)	GAAAGCGAATACCGAACGGC	60	225
		R(q)	CCATCCTTTCCGTCCTTGGT	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

** http://www.ncbi.nlm.nih.gov/nuccore/?term=Bactocera%20Oleae%20, **q**: primers used for real time qPCR, **c**: primers used for cloning, b: Underlying sequence denotes the introduction of restriction sites to facilitate cloning.*

3.3 Results and discussion

3.3.1 RNA extraction and cDNA synthesis

RNA was successfully extracted from resistant (ANik12, AN14) and susceptible populations of *B.oleae* (LAB-L, FOKIDA, HYB) Prior to cDNA synthesis the quality of RNAs were checked in a 1% agarose gel. The concentrations were determined with nanodrop. The samples were then used for cDNA synthesis. Using as template 2μg of each RNA sample cDNA was created for all samples for RT-qPCR. In order to check the success of the synthesis, PCR using primers of beta- actin (GAKB01001968.1) and the newly produced cDNAs as the template was performed . After PCR, samples were run in an 1% agarose gel and a sharp band at the correct product size was observed (**Figure 3.2**) revealing that cDNAs were successfully created for all samples.

Figure 3.2 ***PCR products tested in agarose gel 1%.*** *1μl of each RNA sample loaded in each well. The 100 bp DNA ladder labeled as (M were used. The expected size (~300bp) indicated by a red arrow. A positive (+) and a negative control (-) were also included. The name above the cDNAs indicates the strain from which total RNA extracted; cDNAs were synthetized in three replicates for each strain.*

3.3.2 Validation of the overexpression of Contig00436 (GAKB01000438.1) with RT-qPCR

In order to validate the folds of up-regulation as derived from microarray [61], RT-qPCR was performed using the primers summarized in **Table 3.1** and the expression levels of the two resistant populations were compared with each one of the three susceptible. Quantitative PCR confirmed the constitutive over-expression of contig00436 in all resistant versus susceptible comparisons showing that this gene most probably associated with α-cypermethrin resistance phenotype in ANik12 and AN14 populations (**Figure 3.3** the microarray data, taken from Doctoral thesis of Pavlidi, N. [61] are also presented here for comparison purposes)**.**

Expressed as Log_2 (FC) ,in ANik12 resistant strain the contig00436 were found to be 5,58 (± 0,46), 3,73 (±0,47), 2,19 (± 0,44) folds overtranscribed with qPCR in relation to the LAB-L, HYB and FOKIDA respectively, which are in agreement with the microarray values which were determined at 4,27, 3,51, 1,47 in relation to the same susceptible strains respectively. Similarly, for the other resistant strain, AN14, the transcript levels of contig00436 were also significantly elevated. With qPCR 4,94 (±0,30), 3,11 (± 0,30), 1,52 (± 0,30) folds and with microarray 4,02, 3,23, 1,22 folds higher levels in relation to LAB-L, HYB, FOKIDA were determined, respectively.

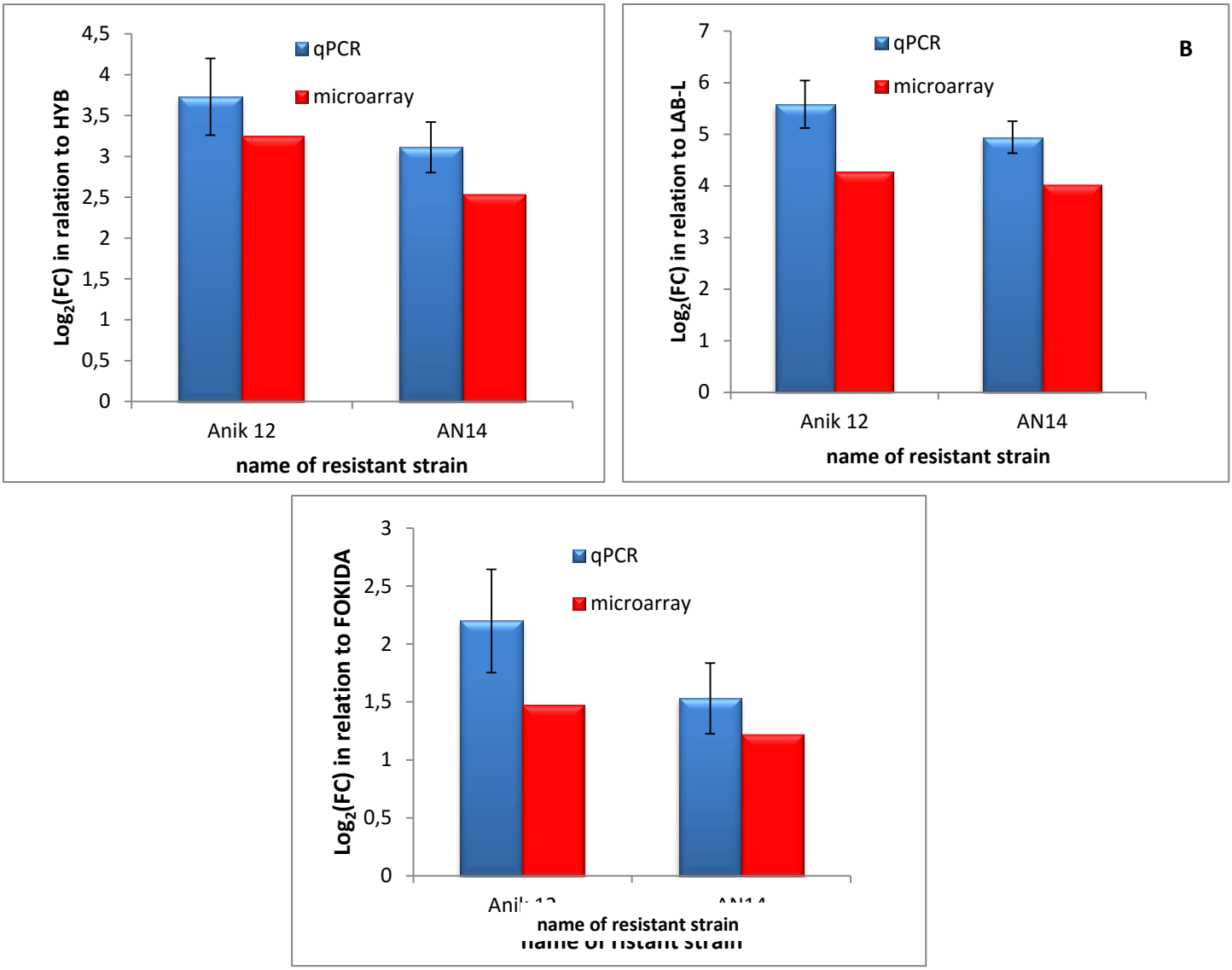


Figure 3.3 ***Validation of the overexpression of CYP enzyme (Contig00436) by RT-qPCR****. The resistant strains: AN14, ANik12 in relation to: (A) HYB susceptible strain, (B): LAB-L susceptible strain, (C):FOKIDA susceptible strain. Microarray data by Pavlidi N. (Doctoral Thesis) ref 61 also presented here for comparison. Data are means of four biological replicates ± S.D.*

3.3.3 Functional expression of *B.oleae CYP* (contig00436) with CPR in *E. coli*

Quantitative real time PCR analysis revealed that most probably contig00436 is associated with the pyrethroid resistance phenotype in *B.oleae* populations. This represent only a first indication for the involvement of contig00436 in pyrethroid resistance. Functional evidence is needed in order to provide insights for the metabolic fate of cypermethrin using the functional enzyme *in vitro*. The sequence

encoding for the CYP enzyme was cloned and functional expressed aiming to investigate if it is capable of metabolizing α-cypermethrin *in vitro.*

Primers introducing the restriction sites for SacI (F) and for XbaI (R) were designed for the subsequent cloning in pCW-OmpA vector. The primers presented in **Table 3.1** used in a PCR with the cDNA of the resistant strain AN14 as template using KAPA long range polymerase. The PCR product was extracted from 1% agarose gel and was cleaned with Nucleospin Gel and PCR clean-up and measured with nanodrop. It was digested with SacI ans XbaI and cloned into pCW-OmpA vector predigested with the same restriction enzymes. The positive colonies were selected with colony PCR using specific primers for the contig00436 (**Figure 3.4**). Three positive colonies (1, 5, 7) were selected, the constructs were extracted using Nucleospin plasmid kit and were sequenced in order to verify that they recruited the insert correctly. A colony of the correct sequence was selected for downstream experiments.

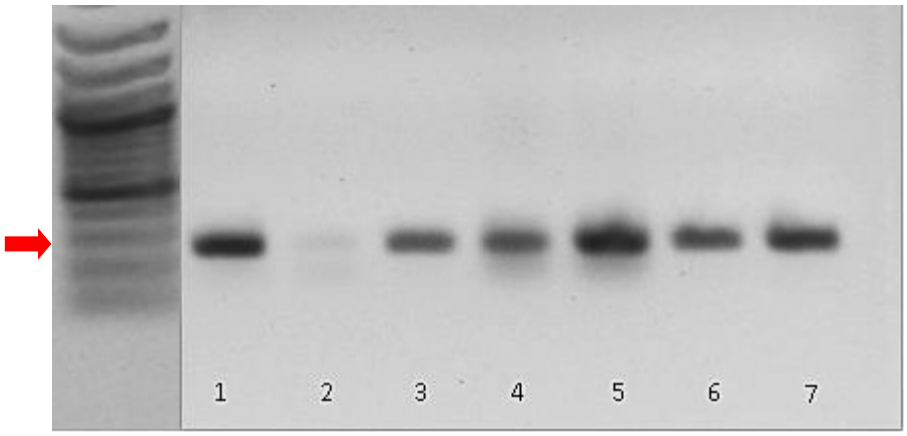


Figure 3.4 ***Agarose gel 1% for the colony PCR products.*** *All colonies except from 2 have recruited the vector with the insert. The colonies 1, 5, 7 were selected for plasmid extraction and sequencing. The red arrow indicates the expected size.*

Cytochrome P450 are membrane attached proteins and they require cytochrome P450 reductase in order to provide them electron for catalysis. Thus, in order to functionally express P450 monooxygenase (Contig00436, GAKB01000438.1) *in E. coli,* ompA and pelB signal sequences were used to direct the enzyme and CPR proteins to the inner bacterial membrane, respectively. Thus, *E. coli* JM109 competent cells were co-transformed with the pCW-OmpA2-contig00436 plasmid and the expression vector pACYC-AgCPR containing cytochrome P450 reductase. The reduced CO difference spectrum showed that the enzyme was expressed predominately in its P450 form (**Figure 3.5**), indicative of a good quality functional enzyme. The

recombinant enzyme will be tested for its metabolic efficiency against model substrates in order to verify that is catalytically active and it will be used for metabolic assays in order to investigate if it is capable of metabolizing α-cypermethrin *in vitro*.

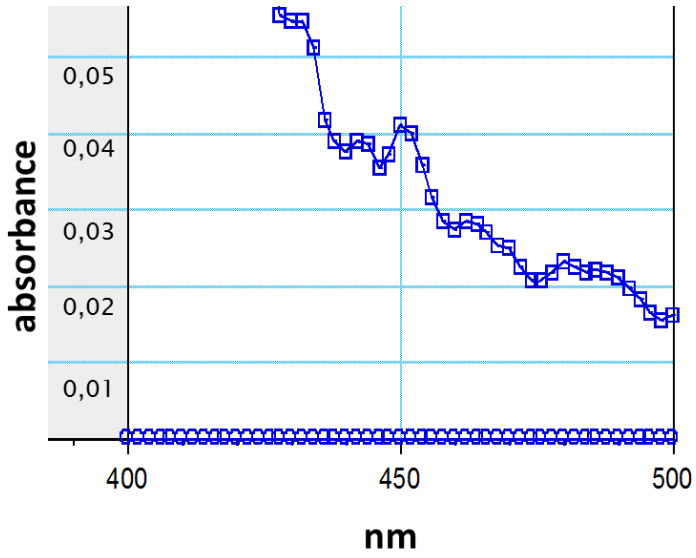


Figure 3.5 *Carbon monoxide difference spectra of bacterial membranes expressing B.oleae contig00436.*

4. General discussion and future plans

4.1 General discussion and future plans

Mu class GSTs, mostly studied in mammals, have been found to be mainly involved in drug metabolism [44]. Although the abundance of Mu-class GSTs in mites like: *S. scabie* [45]*, I. scapularis* [46] *, V. destructor* [75]*, P. citri* [76] and *T.urticae* [29] has been reported the last years, their catalytic properties as well as their physiological function have not been well studied yet. In this study, two Mu GSTs, previously found overexpressed in the multiresistant strain of *T. urticae* (Marathonas) was functionally expressed and it was shown to exhibit both glutathione tranferase and peroxidase activity. After a basic kinetic characterization, the two Mu GSTs were found to have comparable km values for GSH in relation to other GSTs from different classes [45] as well as from others of the same class [45, 47]. This is reflected by the fact that G-site is a highly conserved domain among the different classes of GSTs [44]. Usually members of Mu class show significantly lower Km values for CDNB [45], which was also confirmed in this study. This is connected to a special structural characteristic that Mu class GSTs possesses, the 'Mu-loop'. The 'Mu loop' also observed here by molecular modeling is an 11-residue loop spanning, usually created by amino acid residues 33–43. Studies deleted the 'Mu loop' revealed a decrease in the enzyme's affinity for xenobiotic substrates, demonstrating that the mu loop is a determinant of the enzyme's affinity for its substrates [53]. Finally, compared to other classes as well as with members of the same class of GSTs [45, 47], the TuGSTs characterized here, exhibited a notably higher catalytic activity (Kcat) and catalytic effectiveness (Kcat/km) for both GSH and CDNB.

The two Mu class GSTs from *T. urticae* were tested for their possible interaction with insecticides/ acaricides that showed decreased toxicity against Marathonas. TuGSTm07 was found to strongly interact with bifenthrin causing 70% inhibition of its CDNB conjugating activity. Both Mu GSTs found to interact with other insecticides pyridaben, hexythiazox and clofentezine, while abamectin does not cause any inhibitory effect on the activity of TuGSTs Mu class. Recently, abamectin was found to strongly interact with delta class GSTs in *T. urticae* [77]. Although there is a strong inhibition of TuGSTm07 activity by bifenthrin (IC50: 7,7103 ± 0,956 μM) the type of inhibition has to be determined in order to provide us valuable information if bifenthrin binds to the active site of the enzyme as observed by modeling and docking studies. The way TuGSTm07 confers resistance also should be investigated. Further studies on the metabolic fate of bifenthrin in resistant and susceptible spider

mites are needed in order to provide functional evidence for a catalytic interaction of bifenthrin with TuGSTm07, resulting in conjugated metabolites.

Since three-dimensional structure determination of proteins is a complex and time-consuming process, molecular modeling is a very good alternative to experimental structure assessment [78, 79]. The only requirement is that the target amino acid sequence is 40% or more identical to a known template protein structure [79]. Homology modeling has provided key insights into GSTs [80] . Comparative studies have shown that the three-dimensional structure of GSTs from different species and classes are remarkably similar to the glutathione binding site or domain I [30]. However, the more variable domain II plays an important role in the substrate specificity [52]. Molecular modeling complements and provides key hypothesis and insights into the future work once the amino acid sequence has been determined. In this study molecular modeling showed some of the most common structural characteristics Mu class GSTs possess and blind docking revealed that the most preferable site for the bifenthrin within the enzyme is its active site. Also it would be possible the interaction of TuGSTm07 with bifenthrin to be achieved via Van der Waals interactions through hydrophobic amino acids. However, the interaction of bifenthrin with TuGSTm07 requires a deeper investigation. A Crystallization of GSTm07 would be a study of particular interest concerning the fact that there are not crystallographic data till today from a Mu- class GST from mites and would provide insights for the catalytic properties of the enzyme.

The olive fruit fly is the major pest of commercial olives in most of the regions where olives are grown and the species exists, including Greece. Probabilities are high that *B. oleae* will continue to spread to regions where it is not established unless actions are taken to limit the movement of unprocessed olives among countries. Since classical biological control programs for olive fruit fly have not yet been successful [57], current management tactics include chemical control based mainly on pyrethroids [16, 57]. However, there is a high possibility to become ineffective if olive fruit fly populations continue to evolve resistance.

Recently, after analysis of differential gene expression in resistant olive flies (2 strains) and susceptible (3 strains) to α-cypermethrin [61], contig00436 encoding for a putative cytochrome P450 was identified to be the most striking hit among all resistant versus susceptible comparisons. The possible association between P450 activities and the resistance phenotype was further enhanced by the fact that the occurrence of target-site mutations, within the domain II region of the *B. oleae* paratype sodium channel gene which could be responsible for the pyrethroid resistance, was not be found in resistant populations of *B.oleae* [61]. In this study the overexpression of cytochrome P450 which found to be the most striking hit

(Contig 00436), possibly associated with pyrethroid resistance in *B. oleae*, were validated by RT-qPCR in all resistant populations tested.

Notably, the cytochrome P450 belongs to CYP6 family. In the literature CYP6 family enzymes were directly related with pyrethroid resistance in different species [66]. In addition, *in vitro* metabolism assays showed that members of CYP6 familly can metabolize pyrethroids in different mosquito species [66]. In order to clarify the role of contig00436, the cDNA sequence was cloned into pcW-ompA expression vector and functionally expressed. The isolated membranes will then be used for metabolic assays with α-cypermethrin. In conclusion, stronger evidence proving the association of the two cytochrome P450s and pyrethroid resistance can be obtained with *in vivo* approach (i.e. RNAi).

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