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THE XENOBIOTIC TRANSCRIPTION FACTOR CAP N COLLAR C REGULATES EXPRESSION OF MULTIPLE INSECTICIDE RESISTANT GENES

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THE XENOBIOTIC TRANSCRIPTION FACTOR CAP N COLLAR C REGULATES EXPRESSION OF MULTIPLE INSECTICIDE RESISTANT GENES

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture, Food and Environment at the University of Kentucky

By

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ABSTRACT OF DISSERTATION

THE XENOBIOTIC TRANSCRIPTION FACTOR CAP N COLLAR C REGULATES EXPRESSION OF MULTIPLE INSECTICIDE RESISTANT GENES

Insecticide resistance is a global problem. Insecticide resistance management is very important, considering the time, effort, and cost of discovering and developing a new insecticide. There are diverse resistance mechanisms, but enhanced detoxification through overexpression of cytochrome P450s and target site insensitivity through mutation in insecticide binding site are the two most common mechanisms. The xenobiotic detoxification is divided into three successive phases (I, II and III), which ensures the metabolism and excretion of the detrimental toxins. Each phase comprises of a specific group of metabolizing enzymes such as P450s (phase I), GSTs (phase II) and ABC transporters (phase III). The major goal of my research was to understand the molecular mechanism of insecticide resistance in two economically important coleopteran pests, *Leptinotarsa decemlineata* and *Tribolium castaneum*. The transcriptional regulation of the P450 genes mediating insecticide resistance in *L. decemlineata* (imidacloprid-resistant) and *T. castaneum* (deltamethrin-resistant) were studied and the xenobiotic trans and cis-elements identified. RNA interference (RNAi), and reporter assays revealed that the cytochrome P450 genes involved in insecticide resistance are regulated by transcription factor Cap n Collar 'CncC' and muscle aponeurosis fibromatosis 'Maf' belonging to the b-ZIP transcription factor family. Site-directed mutagenesis was employed to identify the binding site for CncC and Maf. Sequencing of RNA isolated from CncC knockdown *T. castaneum* identified genes regulated by CncC and involved in insecticide detoxification. RNAi and insecticide bioassays, confirmed the function of select phase II (glutathione-S-transferases) and phase III (ABC transporters) identified by RNA sequencing. Overall, these data revealed that the xenobiotic transcription factor CncC is the master regulator of multiple genes that are involved in insecticide resistance.

KEYWORDS: *L. decemlineata*, *T. castaneum*, insecticide resistance, metabolic detoxification

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I would like to dedicate this dissertation to my father, Kuldip Singh Kalsi who supported me for my education at every single step.

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Insecticide resistance

Insecticide resistance is an expanding problem and posses a great challenge in agriculture and health fields. Rene Feyereisen referred insecticide resistance as a manmade example of large-scale natural selection (Feyereisen 1995). The formal definition of insecticide resistance by the Insecticide Resistance Arthropod Committee (IRAC), states, 'A heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species' (Sparks and Nauen 2015). The cases of insecticide resistance are spread across 168 countries with about 600 species of insects and mites resistant to insecticide/acaricides making insecticide resistance a global problem (Whalon et al. 2012). According to the National Research Council (NRC) 2000, an insect develops resistance within a decade of the introduction of a new insecticide (McDougall 2010). Moreover, during severe resistance, the application of a whole class of insecticide can be ineffective (Scott 2001). It takes approximately 8 to 10 years and costs approximately $256 million to discover, develop and register a new insecticide (McDougall 2010).

I will review insecticide resistance to provide a background on insecticide resistance mechanisms, insecticide detoxification enzymes and insecticide resistance incidences focusing on beetles, the insects used in my research.

1. Insecticide resistance mechanism

There are several mechanisms by which an insect can develop resistance: metabolic resistance, target site insensitivity, reduced penetration and behavioral resistance.

1.1 Metabolic resistance

In metabolic resistance, the resistant organism shows enhanced insecticide detoxification compared to the susceptible organism through alterations in quantity or quality of detoxification enzymes (Hemingway et al. 2004). It can be achieved by up-regulation, induction in expression or amplification of the genes coding for insecticide detoxification enzymes such as cytochrome P450s, carboxylesterases, glutathione –S-transferses (GSTs) (Li et al. 2007).

1.2 Target site resistance

The target site resistance occurs due to the conformational change in the proteins that serves as an insecticide-binding site or due to the reduced number of insecticide binding sites (Berenbaum 1986; Russell et al. 2004). Common examples of target insensitivities include the nerve insensitivity, insensitive acetylcholinesterase (AChE) and reduction in midgut target binding site (Simon 2014). Nerve insensitivity confers resistance to pyrethroids, organochlorines, phenylpyrazoles and neonicitinoids. *Drosophila melanogaster* were resistant to cyclodienes because of a mutation of alanine to serine in the λ-aminobutyric acid (GABA) receptor (Ffrench-Constant et al. 1993). In *Musca domestica* knockdown resistant (kdr) was observed due to a mutation in the para gene of sodium channel leading to nerve insensitivity to DDT and pyrethroid insecticides (Williamson et al. 1996).

Acetylcholinesterase (AChE) insensitivity confers resistance to organophosphates and carbamates in many insects. The AChE point mutations have been the source of resistance in many insects such as *D. melanogaster* (Menozzi et al. 2004; Mutero et al. 1994), *Culex pipiens, Anopheles gambiae, Anopheles albimanus* (Weill et al. 2003; Weill et al. 2004), *Musca domestica* (Walsh et al. 2001), *Leptinotarsa decemlineata* (Zhu et al. 1996), *Aphis gossypii* (Andrews et al. 2004; Li and Han 2004; Toda et al. 2004) *and Myzus persicae* (Nabeshima et al. 2003).

Reduction in midgut target site binding confers resistance to *Bacillus thuringiensis* toxins (Bt toxins) that are targeted to kill specific insects by either using the transgenic crops or sprays (Ballester et al. 1999; Bauer 1995; Hokkanen and Wearing 1994; Simon 2014). Bt toxins acts by binding to specific receptors on the insect's midgut epithelial layer and insects have evolved to escape binding of these toxins by reducing the number of toxin receptor sites (Hofmann et al. 1988; Tabashnik et al. 1997). Diamondback moth, *Plutella xylostela* was first to evolve resistance to Cry protein (Bt toxins) in the field (Tabashnik 1994). Reduction in the affinity of Cry protein binding to the brush border membrane vesicles in midgut epithelial cells is the major contributing factor for this resistance (Ballester et al. 1999).

1.3 Penetration resistance

Penetration resistance is caused by the ability of an insect to develop a thicker exoskeleton to reduce the absorption of insecticide and often occurs in conjunction with metabolic resistance. For example, the resistant strain of *Musca domestica* showed reduced penetration to diflubenzuron, the inhibitor of chitin synthesis as compared to the susceptible strain (Pimprikar and Georghiou 1979). DDT resistant tobacco budworm,

Heliothis virescens exhibits reduced insecticide penetration due to variability in its cuticular composition (higher levels of proteins and lipids) as compared to the susceptible strain (Vinson and Law 1971). In another example, the cotton budworm, *Helicoverpa armigera* resistant strain showed delayed deltamethrin absorption (6 h) as compared to the susceptible strain that took only an hour for absorption (Ahmad et al. 2006)

1.4 Behavioral resistance

Behavioral resistance is caused by stimulus dependent ability of an organism to avoid a lethal dose of natural or synthetic toxins due to hypersensitivity or hyperirritability (Berenbaum and Zangerl 1992; Simon 2014). The classic example for a behavioral resistance is the avoidance of mosquitoes vectoring malarial parasite to the DDT insecticide where the mosquitoes avoid exposure to DDT sprayed area in the house or insecticide treated bed nets (Roberts and Andre 1994; Thomsen et al. 2016).

2. Xenobiotic detoxification system

In its life cycle, insects are exposed to many xenobiotic compounds that are either produced naturally (allelochemicals) or synthetically (insecticides) (Feyereisen 1999). To survive these toxins, insects employ a variety of metabolizing enzymes belonging to different levels of detoxification system (Simon 2014). A general overview of the xenobiotic detoxification system is shown in Figure 1.1. The function of a detoxification system or biotransformation is to reduce the concentration of hydrophobic or lipophilic compounds by transforming them to more polar compounds that are easier to excrete from the body (Chahine and O'Donnell 2011). The detoxification mechanism can be divided into three phases i.e. Phase I, Phase II and Phase III (Xu et al. 2005).

2.1. Phase I:

The phase I enzymes are responsible for adding the polar or reactive group to the substrate (insecticide) by oxidation, reduction or hydrolysis reactions (Bernhardt 2006; Xu et al. 2005). The metabolizing enzymes working during this phase are cytochrome P450 monooxygenases, and these enzymes are prevalent in metabolic insecticide resistance via upregulation or amplification of their genes (Feyereisen 1999b; Feyereisen 2006; Ranson et al. 2002). Four cytochrome P450 families (CYP4, CYP6, CYP9, and CYP12) are often associated with insecticide resistance (Feyereisen 1999; Li et al. 2007)

2.2. Phase II:

During this phase, the metabolizing enzymes (glutathione-s-transferase, GSTs; UDP-glucuronosyltransferase; UGTs, esterases) conjugate the Phase I substrate with endogenous molecules such as amino acids, sugars, sulfate, glutathione, and phosphate (Simon 2014). One of the most important metabolizing enzymes in this phase is GST and it is known to be involved in detoxification of all major classes of insecticides (Chahine and O'Donnell 2011). An elevated level of GST expression is related with the resistance to a wide range of insecticides including organophosphates, pyrethroids and organocholrines. (Lumjuan et al. 2011; Ranson et al. 2002; Reidy et al. 1990; Riveron et al. 2014).

2.3. Phase III:

When the cells accumulate toxins/waste products, the elimination of these toxins becomes critical. Phase III is the final step, comprising of the cell membrane bound proteins belonging to ATP-binding cassette (ABC) transporter protein family. The ABC-transporters facilitates the efflux of the modified molecules/toxins by hydrolyzing ATP

molecule as a source of energy (Dermauw and Van Leeuwen 2014). The ABC transporters are also capable of eliminating toxins directly without the need of any enzymatic modification, and this step is referred as Phase 0 (Doring and Petzinger 2014).

In general, a functional ABC protein consists of four domains (1) two transmembrane domains for ligand specificity and (2) two nucleotide binding domain for binding and hydrolyzing ATP (Linton and Higgins 2007). The ABC-transporters families, ABCC and ABCG are known to function in extrusion of toxins from a cell across the plasma membrane (Chahine and O'Donnell 2011; Epis et al. 2014)

The role of the ABCA family is the least explored in both humans and insects. So far, only the study done in *T. castaneum* reveals knockdown of ABCA-9A and ABCA-9B resulted in developmental defects such as wing development during pupal-adult metamorphosis (Broehan et al. 2013)**.**

3. Model insects for insecticide resistance study

For decades insects have been used as a model to perform many biochemical and genetics studies, for example, *Drosophila* (Adams et al. 2000; Denholm et al. 2002; Wilson 1988). These model insects have an added advantage over other organisms such as short generation time, easy rearing, genome availability, genetic manipulation, etc. The fruit fly, *D. melanogaster* is the most common model used to study different aspects of insecticide resistance (Wilson 1988). Other models insects used for resistance studies are the red flour beetle, (*Tribolium castaneum)*, German cockroaches (*Blatella germanica*) mosquitoes (*Culex pipiens)* and houseflies (*Musca domestica*) (Beeman 1983; Mouches et al. 1986; Plapp 1984; Ross and Cochran 1989). Further, *T. castaneum*

is not only an important agricultural pest but also a powerful insect model that exhibits systemic RNAi making gene functional studies feasible (Richards et al. 2008). It can be used in identification of target genes for insecticide development that might be essential for control strategies for not only *T. castaneum* but for other coleopteran pests as well (Hemingway et al. 2004; Kalsi and Palli, 2015).

4. Insecticide resistance in beetles

Currently, an arthropod pesticide resistance database (APRD, Whalon et al. 2012) documents resistance in about 594 species of insects, mites, and ticks. Within the insect orders, the highest number of resistance species have been found in Diptera (195 species) followed Lepidoptera (92 species) and Coleoptera (79 species) (Table 1). The order Coleoptera is the richest and most diverse with about 350,000 species and contributes 25% of all eukaryotes (Mise et al. 2008). This order includes some agriculturally important pests that are often associated with insecticide resistance including *Leptinotarsa decemlineata,* the Colorado potato beetle (CPB). This species has shown resistance to 56 active ingredients (APRD) and 300 cases of resistance have been reported for this insect. There have been 132 reported cases of *T. castaneum* resistance, with resistance to 33 active ingredients. The highest number of resistance cases (355) has been reported for pollen beetle *Meligethes aeneus* and with resistance to 27 active ingredients.

4.1 Resistance in *Leptinotarsa decemlineata*

Leptinotarsa decemlineata is distributed in North America, Europe and parts of Asia (Alyokhin et al. 2008). It's a major defoliator of potato leaf as well as a member of

another Solanaceae family such as tomato and eggplant (Alyokhin et al. 2008; Casagrande 1987; Hare 1990). The insecticide control is the major management tool to control CPB, but it has known to gain resistance to almost all the available insecticides (Alyokhin et al. 2008; Palli 2014). The success of CPB as an agricultural pest has been attributed to several factors including (a) its co-evolution with the host plant (developing resistance to wider range of plant toxins), (b) a narrow host range (generalist herbivory), (c) diapause (migration in space and time), (d) high fecundity (300-800 eggs per generation) and (e) high mobility (both walking and flight) (Alyokhin et al. 2008). The CPB has been reported to gain resistance via all four-resistance mechanisms i.e. metabolic resistance using P450s, target site mutation, reduced insecticide penetration and behavioral resistance (Palli 2014). The cases of insecticide resistance in *L. decemlineata* are listed in Table 2 (Ahammadsahib et al. 1994; Alyokhin et al. 2008; Alyokhin and Ferro 1999; Clements et al. 2016; Harris and Turnbull 1986; Hoy and Head 1995; Ioannidis et al. 1991; Kalsi and Palli, 2017; Kim and Clark 2002; Kim et al. 2006; Kim et al. 2005; Lee et al. 1999; Mota-Sanchez 2002; Rose and Brindley 1985; Silcox et al. 1985; Soderlund et al. 1987; Stanković et al. 2004; Wierenga and Hollingworth 1993; Yoon et al. 2002; Zhu et al. 2016).

4.2 Resistance in *Tribolium castaneum*

Tribolium castaneum is a cosmopolitan pest that has a broad range of host causing a major loss to dried, stored grain products such flour, cereals, maize, beans, spices, dried pet food, dried museum specimen, etc. (Weston and Rattlingourd 2000). *T. castaneum* is a pest of corn and wheat in the USA leading to about $1 billion worth damage each year

(Jones and Sattelle 2007; Throne et al. 2003). Chemical control has been important, but *T. castaneum* has gained resistance to many of the insecticides (Andreev et al. 1999; Champ and Campbell-Brown 1970; Dyte and Blackman 1970; Zettler 1991; Zettler and Cuperus 1990). *T. castaneum* has been reported to gain resistance via behavioral resistance, target site insensitivity and metabolic resistance (Boyer et al. 2012).

Metabolic resistance: three major enzyme families contributing to insecticide resistance in *T. castaneum* are esterases, GSTs, and monooxygenases. The esterases mediated resistance has been reported for organophosphorous and malathion insecticides (Dyte and Rowlands 1968; Navarro et al. 1986; White and Bell 1988; Wool and Front 2003). In *T.castaneum* an expansion of GSTs (40 genes) has been reported with three delta GSTs involved in insecticide resistance (Chen et al. 2016b; Shi et al. 2012). The monooxygenase supergene family is expanded in *T. castaneum* with about 143 genes (Richards et al. 2008; Zhu et al. 2013b). A large number of P450s have been reported to mediate resistance to different pyrethroid insecticide in these beetles. Several genes in CYP6BQ cluster were found to be responsible for deltamethrin resistance (Kalsi and Palli, 2015; Zhu et al. 2010).

Target site insensitivity: Cyclodiene insecticide resistance in *T. castaneum* was mediated by single point mutation in Rdl (Resistance to dieldrin) gene that codes for a subunit of the GABA receptor (Andreev et al. 1999; Miyazaki et al. 1995). A mutation has also been reported in acetylcholinesterase genes in *T. castaneum* reducing sensitivities to organophosphate and carbamate insecticides (Lu et al. 2012).

5. The xenobiotic transcription factors:

Transcription factors acting as xenobiotic sensors and regulating genes involved in xenobiotic metabolism are referred as xenobiotic transcription factors (Nakata et al., 2006). The xenobiotic transcription factors consist of three different superfamilies including basic-helix-loop-helix / Per-ARNT-Sim (bHLH-PAS), nuclear receptors and basic leucin zipper (bZIP) proteins have been reported as xenobiotic sensors (Nakata et al., 2006). The bZIP proteins are named so as they contain a highly conserved bipartite bZIP domain (60 to 80 base pairs) organized into DNA binding N-terminus and a leucin zipper that facilitates the hetero/homo dimerization between bZIP proteins (Jindrich and Degnan 2016). The bZIP superfamily includes the 'Cap n Collar' (Cnc) transcription factor subfamily (Sykiotis and Bohmann, 2010). The Cnc protein was first identified in *Drosophila* and named after the expression of this protein during embryogenesis in the anteriormost labral segment (Cap) and the mandibular segment (Collar) (Mohler et al., 1991). Further, the developmental role of Cnc was discovered and it was reported that Cnc deletion-mutant embryos of *Drosophila* losses their mandibular and labral structure (Mohler et al., 1995). The Cnc proteins are present in vertebrates, invertebrates and metazoans. These proteins are defined by the presence of a 43 amino acid Cnc domain on the N-terminus of the DNA binding domain and these proteins function during the development and environmental/oxidative stress (Sykiotis and Bohmann, 2010). The *Drosophila* cnc locus codes for three different Cnc proteins including cap n collar isoform A (CncA) , cap n collar isoform B (CncB) and cap n collar isoform C (CncC) and all these proteins have a common DNA binding C- terminus but variable N-terminus (Sykiotis and Bohmann, 2008). The CncC is distinct from its isoforms (CncA and CncB

) as its N-terminus is largest and has a unique Keap 1 (kelch-like ECH associated protein-1) binding ETGE motif (Sykiotis and Bohmann, 2008). The CncC transcription factors identified in the fruit fly, *Drosophila melanogaster* and are orthologous to vertebrate protein nuclear factor erythroid-derived 2 (Nrf2) and nematode protein skinhead member-1 (SKN-1) (Sykiotis and Bohmann, 2010).

During normal conditions (mammalian cells and mice) the Nrf2 is retained in the cytoplasm sequestered by its negative regulator protein, Keap1 an actin binding protein with E3 ubiquitin ligase activity (Baird and Dinkova-Kostova, 2011; Misra et al., 2011). Under environmental/oxidative stress , Keap 1 undergoes destruction via ubiquitination and releasing Nrf2. The released Nrf2 translocates to the nucleus heterodimerizes with another bZIP transcription factor called small muscle aponeurosis fibromatosis (Maf) protein. The Nrf2-Maf heterodimer binds to the xenobiotic response elements (XRE) present in the upstream promoter regions of the genes coding for enzymes involved in xenobiotic detoxification (Baird and Dinkova-Kostova, 2011; Misra et al., 2011; Hirotsu et al., 2012; Nioi et al., 2003). Similarly the CncC-Maf heterodimer activates transcription of the detoxification genes in invertebrates. For instance, in *D. melanogaster*, CncC, Keap1 and Maf proteins controls the cellular response to xenobiotic compounds (Deng and Kerppola, 2013; Sykiotis and Bohmann, 2010). The CncC and Maf proteins function through XRE, may have differential sequence requirements (Nioi et al., 2003). The CncB and small Maf proteins are involved in the cephalic development of *Drosophila* embryogenesis (Veraksa et al. 2000).

6. Project Goal:

Deltamethrin resistant *T. castaneum* and imidacloprid resistant *L. decemlineata* were identified previously. Both these insects exhibited metabolic detoxification by upregulation of cytochrome P450 genes (Zhu et al. 2010). Therefore, the overall goal of my research was to understand the molecular mechanism of metabolic insecticide resistance mediated by cytochrome P450s in two different insect models, *T. castaneum* and *L. decemlineata*.

Goal 1: To understand the molecular mechanisms of pyrethroid resistance in *T. castaneum* (QTC279).

Objectives:

1) To identify the xenobiotic transcription factors that regulates cytochrome P450 (CYP6BQ cluster) genes involved in pyrethroid resistance.
2) To identify the cis-regulatory elements (xenobiotic response elements) that facilitates the binding of these xenobiotic transcription factors.
3) To identify pyrethroid-metabolizing genes (phase I, phase II and phase III) regulated by these transcription factor/s.

Goal 2: To understand the molecular mechanisms of imidacloprid resistance in *Leptinotarsa decemlineata* (NY strain).

Objectives:

1) To identify the xenobiotic transcription factors that regulate cytochrome P450 genes involved in imidacloprid and potato plant allelochemicals resistance.

2) To identify the cis regulatory elements (xenobiotic response elements) that facilitates the binding of these xenobiotic transcription factors.
3) To identify imidacloprid-metabolizing genes (Phase I, Phase II and Phase III) regulated by these transcription factor/s.

7. Impact and outcomes

Since current chemical insecticides can be considered a non-renewable source, scientists are developing novel molecular targets for future insecticides. Insecticide resistance due to enhanced metabolic detoxification (P450s, GSTs, etc.) is very common. Although, several studies are available documenting the plethora of genes involved in insecticide or phytotoxin resistance, the regulation of these detoxifying genes is not thoroughly understood in the invertebrate system. My research was focused on understanding the regulation of the insecticide or phytotoxin detoxification genes in beetles.

I determined the regulation of the beetle genes involved in insecticide and phytotoxin resistance, revealing that both trans-regulatory (CncC and Maf) and cis (CncC-Maf binding sites) elements regulate the detoxification genes. Additionally, I found that the xenobiotic transcription factor CncC controls the expression of many detoxification genes involved in all three phases of detoxification. Therefore, rather than developing the inhibitors for each gene involved in detoxification, it is advantageous to develop an inhibitor for the master regulator (CncC) controlling such genes. My research provides a scientific basis for considering CncC as a valuable molecular target, but more future work is needed in testing the CncC's potential targets.

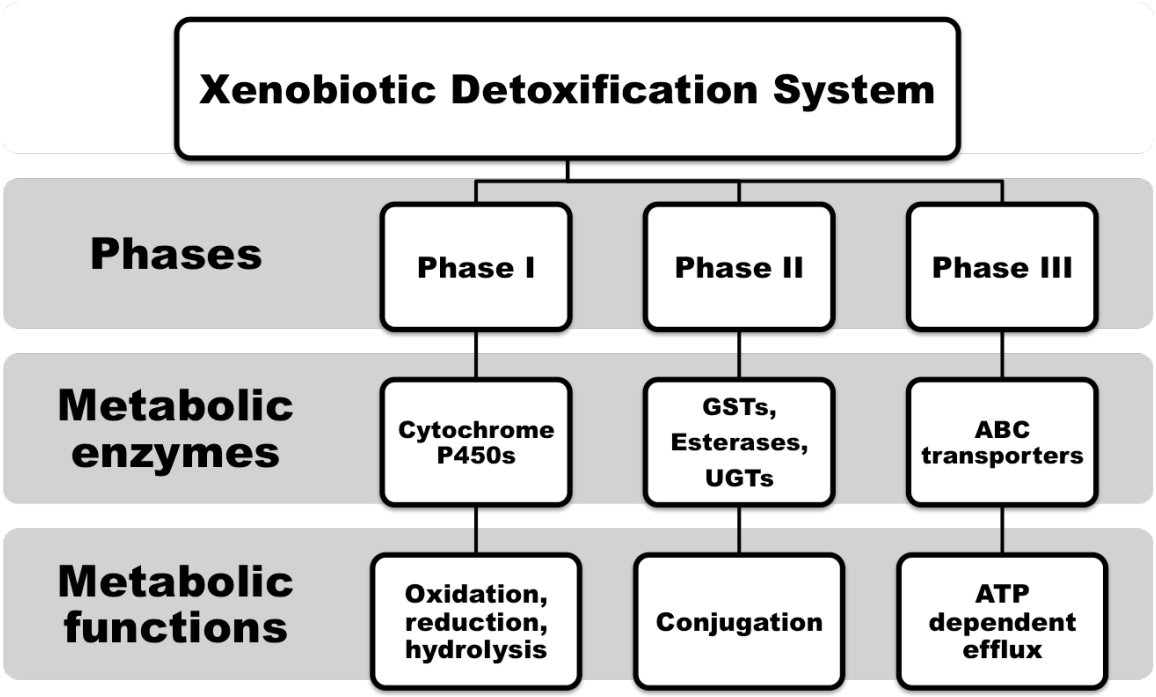
Table 1.1. The number of resistant species of insects in each order according to Arthropod Pesticide Resistance Database

Order	Number of resistant species
Coleoptera	79
Dermaptera	4
Diptera	195
Ephemeroptera	2
Hemiptera	28
Homoptera	61
Hymenoptera	18
Lepidoptera	92
Neuroptera	1
Phthireptera	10
Thysanoptera	8
Siphonaptera	9

Table 1.2. Resistance cases reported in *L. decemlineata*.

Type of resistance	Mechanism	Insecticide
Metabolic	Enhanced cytochrome P450 metabolism	Azinphosmethyl, Carbaryl, Carbofuran, Fenvalerate, Permethrin, Imidacloprid
Target site insensitivity	Acetylcholine esterase mutation	Organophosphates, Carbamates, Azinphosmethyl, Carbofuran
	Sodium channel (α-subunit mutation)	Permethrin
Reduced penetration & increase in excretion	Exist in combination with other kinds of resistance such as enhanced metabolism and target site insensitivity	Azinphosmethyl, Carbaryl
Behavioral	Avoidance to ingest Bt potato leaves, Increased flight activity	*Bacillus thuringiensis* (Bt; Cry toxins)

Figure 1.1.



The general overview of the xenobiotic detoxification system. (GSTs, glutathione-S-transferases; UGTs, UDP-glucuronosyltransferase; ABC, ATP binding cassettes).

Chapter 2: Transcription factors, CncC and Maf, regulate expression of CYP6BQ genes responsible for deltamethrin resistance in Tribolium castaneum

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Summary

Insecticide resistance is a global problem that presents an ongoing challenge to control insects that destroy crops, trees and transmit diseases. Dramatic progress has been made during the last decade on the identification of insecticide resistance-associated genes. In one of the most common resistance mechanisms, insects acquire resistance by increasing the levels of their detoxification enzymes especially the cytochrome P450 monooxygenases (P450's). Previous studies in our laboratory showed that the pyrethroid resistance in the QTC279 strain of Tribolium castaneum is achieved through constitutive overexpression of the P450 gene CYP6BQ9 by 200-fold higher in the resistant strain as compared to that in the susceptible strain. RNAi-aided knockdown in the expression of probable genes that regulate P450 gene expression in QTC279 identified cap 'n' collar C (CncC) and muscle aponeurosis fibromatosis (Maf) family transcription factors as the key regulator of these genes, CncC and Maf regulate expression of multiple genes in the CYP6BQ cluster. Studies on the promoters of these genes using reporter assays identified binding sites that mediate CncC and Maf regulation of CYP6BQ gene expression.

Keywords: Insecticide resistance, Tribolium, P450, Xenobiotic transcription factors

Introduction

Transcription factors that sense xenobiotic stress are known as xenobiotic sensors (Nakata et al., 2006). Three superfamilies of transcription factors including basic-helix-loop-helix / Per-ARNT-Sim (bHLH-PAS), nuclear receptors and basic leucin zipper (bZIP) proteins have been reported as xenobiotic sensors (Nakata et al., 2006). The transcription factors belonging to the bZIP Cap'n'collar (Cnc) subfamily proteins are conserved in different organisms including invertebrates, vertebrates and metazoans except in plants and fungi (Sykiotis and Bohmann, 2010). The cap n collar isoform C (CncC) transcription factors have been identified in the fruit fly, *Drosophila melanogaster* and are orthologous to vertebrate protein Nrf2 and nematode protein SKN-1. These proteins play an important role in defending an organism against electrophilic or oxidative stress (Misra et al., 2011; Sykiotis and Bohmann, 2010). In mammalian cells and mice, the Nrf2 is activated during the redox changes in a cell and this activated protein induces expression of xenobiotic-metabolizing, antioxidant, and other cytoprotective enzymes to maintain the homeostasis (Baird and Dinkova-Kostova, 2011). Under normal conditions, the Nrf2 is retained in the cytoplasm sequestered by its negative regulator protein, Keap1 (kelch-like ECH associated protein-1) an actin binding protein with E3 ubiquitin ligase activity (Baird and Dinkova-Kostova, 2011; Misra et al., 2011). During stress, Keap 1 undergoes destruction releasing Nrf2 that translocates to the nucleus. Consequently, Nrf2 heterodimerizes with small muscle aponeurosis fibromatosis (Maf) protein and binds to the xenobiotic response elements (XRE) present in the upstream regulatory regions of the stress-response genes leading to the expression of these genes coding for enzymes involved in detoxification (Baird and Dinkova-

Kostova, 2011; Misra et al., 2011; Hirotsu et al., 2012; Nioi et al., 2003). Similar to CncC, Maf is also a bZIP transcription factor belonging to the sMaf family (Hirotsu et al., 2012). The CncC-Maf heterodimer activates transcription of the detoxification genes. In contrast, the Maf-Maf homodimer represses the transcription of the same genes (Hirotsu et al., 2012; Nakata et al., 2006; Nioi et al., 2003). In *D. melanogaster*, the cellular response to xenobiotic compounds is controlled by CncC, Keap1 and Maf (Deng and Kerppola, 2013; Sykiotis and Bohmann, 2010). Recent reports showed nuclear localization of both CncC and Keap1 proteins in *D. melanogaster* salivary gland cells. These proteins function through XRE, also known as antioxidant response element (ARE), which is not a well-conserved sequence and may have differential sequence requirements (Nioi et al., 2003).

The Nrf2-Keap1 complex plays a vital role in cancer prevention (Sykiotis and Bohmann, 2008, 2010; Taguchi et al., 2011). The Nrf2 deficient mice when exposed to chemical carcinogen, leads to increased susceptibility to the carcinogen due to defective activation of cytoprotective genes (Deng and Kerppola, 2013; Slocum and Kensler, 2011). The mutations that disrupt the Nrf2-Keap1 complex have been found in many human cancers and hence they are being investigated as potential therapeutic targets (Deng and Kerppola, 2013; Padmanabhan et al., 2006). Similarly, in adult *D. melanogaster*, when there is xenobiotic stress; the overexpression of CncC and depletion of Keap1 gene lead to the upregulation of genes responsible for cell protection. In contrast, the Keap1 overexpression in the flies down regulates the expression of these cytoprotective genes (Sykiotis and Bohmann, 2008, 2010).

Even though a lot of research has been conducted on the xenobiotic detoxification system in vertebrates, information about the same is sparse in insects. Studies on the xenobiotic detoxification pathway are very important to understand the molecular basis of insecticide resistance. The insecticide resistance and action committee (IRAC) described four different mechanisms of insecticide resistance including the target site insensitivity, metabolic detoxification, reduced penetration and behavioral resistance. At the biochemical level, metabolic resistances due to enhanced capabilities of detoxifying enzymes as well as a decrease in target site insensitivity are the two major mechanisms of insect resistance to synthetic (insecticide) and natural (allelochemicals) xenobiotics (Nakata et al., 2006). Enhanced enzymatic detoxification is one of the most common methods of achieving insecticide resistance (Feyereisen, 2012).

The metabolic resistance is a process that involves the biochemical transformation of a xenobiotic compound ultimately reducing its affinity for the target site or increase in excretion of insecticide (Li et al., 2007). This occurs due to constitutive overexpression, induced expression, amplification or variation in coding sequences in the major detoxification enzymes, cytochrome P450 monooxygenases (P450's), esterases (hydrolases) and glutathione-S-transferases (GST's) (Li et al., 2007; Feyereisen, 2012). Insects most frequently employ the cytochrome P450-mediated mechanism to become insecticide resistant (Feyereisen, 2012). P450's are the hemoproteins that are present in virtually all-aerobic and diverse organisms i.e., mammals, plants, insects, bacteria etc. Most commonly, insects acquire resistance by increasing the metabolic capabilities of their detoxification enzymes especially the cytochrome P450 monooxygenases (P450's) (Feyereisen, 2012; Georghiou, 1972; Li et al., 2007; Simon, 2008).

Previous research from our laboratory showed that a cytochrome P450 gene (CYP6BQ9) expressed predominantly in the brain is responsible for the majority of deltamethrin resistance in the red flour beetle, *Tribolium* castaneum QTC279 strain. The QTC279 beetles can detoxify deltamethrin at a much faster rate due to >200 fold higher expression of CYP6BQ9 when compared to its expression in the susceptible strain, Lab-S (Zhu et al., 2010). The work presented in this chapter is the next step in this research to understand the molecular mechanisms underlying the regulation of the CYP6BQ genes present in the cluster. In this chapter, we delineated major xenobiotic transcription factor/s involved in transcriptional regulation of CYP6BQ genes and identified the putative cis-element that facilitates transcription factor binding.

Additionally, *T. castaneum* makes an ideal model to study the regulation of insecticide resistance genes because of availability of genome sequence and well functioning RNAi in all tissues and developmental stages. Nonetheless, it's a worldwide notorious pest of stored grains that is ranked among top 20 arthropods that have been reported to develop insecticide resistance to all five major classes of insecticides (Zhu et al., 2010).

Materials and Methods

T. castaneum rearing, RNAi, RNA isolation, cDNA preparation and qRT-PCR.

QTC279 strain was reared on organic wheat flour containing 10% yeast maintained in the dark at 32°C and 55 ± 2% relative humidity. Gene-specific primers containing the T7 promoter (Table 2.1 and 2.2) and the genomic DNA were used to amplify fragments of transcription factor genes and the PCR products were used for

dsRNA synthesis. Genomic DNA was extracted from *T. castaneum* adults (GA strain, ten adults) using the DNeasy Tissue Kit (QIAGEN). dsRNAs were synthesized using the MEGAscript T7 RNAi Kit (Ambion, Inc.). For microinjections, one- to two- week-old adults were anesthetized for 4-5 min with ether vapor and were temporarily fixed on a glass slide using double sided tape. At least 40 beetles were injected with 0.1-µl dsRNA (0.8–1 µg) into the dorsal side of their first or second abdominal segment. Injection needles were prepared from the capillary tube using needle puller (Idaho Technology). For control 40 beetles were injected with *malE* dsRNA targeting a bacterial gene and used as a control. Injected beetles were removed from the glass slide and were maintained for 5-8 days depending on their gene knockdown efficiency. The total RNA was isolated from five individual beetles using the TRI reagent (Molecular Research Center Inc., Cincinnati, OH). The DNase1 was used to remove the contaminating DNA from the total RNA (Ambion Inc., Austin, TX) and 1 µg of total RNA for each sample was used for cDNA synthesis. QRT-PCR reactions were performed using a common program as follows: initial incubation of 95° C for 3 min was followed by 40 cycles of 95°C for 10 s, 55°C for 1 min, relative levels of mRNAs were quantified in triplicates and normalized using an internal control (ribosomal protein 49, RP49, mRNA).

Deltamethrin bioassay

For the bioassays 25-30 injected beetles were used. Beetles were exposed to a filter paper surface treated with serial dilutions of technical grade deltamethrin (Bayer Environmental Science) prepared in ethanol. The concentration of deltamethrin varied ranging from 0.5 µg/µl to 5 µg/µl. Beetle mortality was scored after 24 hr exposure in the dark at 32°C and 55 ± 2% relative humidity. Mean and standard errors for each time

point was obtained from at least three independent bioassays. All data was analyzed using MINITAB statistical software (MINITAB 14; Minitab, Inc).

Reporter and promoter constructs

To investigate the binding site for the transcription factors CncC and Maf, the selected CYP6BQ genes promoters were cloned into the pGL3 vector containing a minimal promoter (TGGAAACATATACGCAGTAAACATATATAAAGCGCGGCGGGCGGCGCGCGG CGACAGTGTCGGCCGG). Promoters regions of CYP6BQ6, CYP6BQ7, CYP6BQ9 and CYP6BQ12 were amplified using genomic DNA extracted from the QTC279 strain of *Tribolium* (10 adults) using DNAesy tissue kit (QIAGEN). Various DNA fragment for CYP6BQ12 promoter truncations and mutated promoter truncations were amplified using the CYP6BQ12 promoter region DNA as a template. The open reading frame for CncC and Maf were cloned into the expression vector, pIEX-4. The primers used for amplification of promoter fragments are shown in Table 2.1 and 2.2.

Reporter assays

The BCIRL-TcA-CLG1 (TcA) cells and Bm-12 cells were routinely maintained at 26°C in Excel 420 medium with 10% heat inactivated fetal bovine serum FBS and antibiotics. The transfection experiments were performed in 48-well cell culture plates. Initial experiments on the promoter analysis and the promoter truncation analysis were performed in the Bm-12 cells. Upon availability of TcA cells, the binding site analysis for CncC and Maf was performed in these cells. The cells were seeded at a density of 2 $x10^5$ cells per ml. 24 h after seeding, the medium was removed and the cells were washed with fresh medium containing no FBS and antibiotic. The cells were co-transfected with the

transfection mixture containing 0.5 µg of the promoter construct, 0.5 µg of CncC plasmid vector and 0.5 µg of Maf plasmid per well using 0.5 µl of cellfectin transfection reagent in 80 µl of medium without FBS. For *T. castaneum* cell line, Xtreme-HP-fugene was used as transfection reagent. After incubation for 6 h, 170 µl of medium with 10%FBS and antibiotics were added to the cells. Cells were harvested at 48 hr after transfection. Media was removed followed by addition of 100 µl of lysis buffer and the cell plate was placed on a shaker for 30 minute to facilitate the cell lysis. 20 µl of cell lysate was used for luciferase activity and 20 µl was used to determine the protein concentration using Bradford's assay. The luciferase activity was measured using Fluoroskan Ascent Fl machine (ThermoLabsystems). Three independent transfections of three replicates for each construct were performed. Differences in the promoter activity among the full-length promoter constructs and their promoter truncations were tested by Tukey's HSD test for multiple comparisons.

Results

Xenobiotic transcription factors CncC and Maf regulate expression of CYP6BQ genes

To understand the regulation of constitutively overexpressed CYP6BQ genes in the QTC279 strain of *T. castaneum*, the first step was to find the xenobiotic transcription factors that regulate expression of these genes. We hypothesized that xenobiotic sensors belonging to one of the xenobiotic transcription factor superfamilies might be involved in regulation of CYP6BQ genes. Seven transcription factors (Table 2.1) that likely regulate the expression of CYP6BQ cluster were selected based on the literature review (Barouki et al., 2007; Emmons et al., 1999; Godlewski et al., 2006; Xie and Chiang, 2013). To

identify the transcription factor that regulates expression of CYP6BQ genes, the expression of each the transcription was knockdown in QTC279 beetles using the RNAi. Bioassays were performed to determine deltamethrin toxicity in RNAi beetles. The CYP6BQ mRNA levels were determined in RNAi and control beetles using qRT-PCR. Injection of dsRNA caused an efficient knockdown of all seven-transcription factors tested (Fig. 2.1 A , Fig. 2.2 A , and Fig. 2.3). Out of the seven transcription factors tested, knockdown of the three transcription factors, CncC, Maf and Met caused a significant increase in the mortality caused by deltamethrin (98-100%) as compared to the mortality (52%) observed in the control beetles injected with *malE* dsRNA (Fig. 2.4.). Knockdown of JH receptor, methoprene tolerant (Met), caused an increase in deltamethrin induced mortality but compared to the control beetles no significant change in the CYP6BQ9 expression was observed in these RNAi beetles (Fig. 2.5). The CncC knockdown beetles showed 26.6, 2.4, 27, 2.4, 3.7, 212.6, 263.6, 1.5, 2.4 and 13.7-fold decrease in the mRNA levels of CYP6BQ2, CYP6BQ3, CYP6BQ4, CYP6BQ5, CYP6BQ6, CYP6BQ7, CYP6BQ9, CYP6BQ10, CYP6BQ11 and CYP6BQ12 respectively (Fig. 2.1 B). Also, knockdown in the expression of Maf caused a decrease in the expression of the same CYP6BQ genes (Fig. 2.2 B). In the Maf knockdown beetles the expression of CYP6BQ2, CYP6BQ3, CYP6BQ4, CYP6BQ6, CYP6BQ7, CYP6BQ9, CYP6BQ10, CYP6BQ11 and CYP6BQ12 was decreased by 41.4, 34.2, 2.9, 3.9, 4, 20.4, 33.9, 2, 2.1 and 10.4-fold respectively. The expression of CYP6BQ1 and CYP6BQ8 was not affected by either CncC or Maf knockdown (Fig. 2. 1 B and Fig. 2.2 B). Interestingly, the relative mRNA levels of both CncC and CYP6BQ9 are higher by 22 and 875-fold respectively in the resistant strain (QTC279) as compared to their levels in the susceptible strain (LBS). The

relative mRNA levels of both CncC and CYP6BQ9 in the resistant strain are also higher than the general lab strain by 3-fold and 175 -old respectively (Fig. 2.6). These data suggest that the xenobiotic transcription factors, CncC and Maf play important roles in regulation of the CYP6BQ genes.

Analysis of CYP6BQ gene promoters

The luciferase reporter assays were conducted to determine the activity of CYP6BQ promoters. Four promoter sequences (CYP6BQ6, 649 bp; CYP6BQ7, 481 bp; CYP6BQ9, 287 bp and CYP6BQ12, 1650 bp) were cloned into the pGL3 vector. CncC and Maf transcription factor were cloned into pIEX-4 expression vector. The transcription factor and the promoter constructs were transfected in to Bm-12 cells. As shown in Figure 2.7 A, co-transfection of constructs expressing CncC and Maf increased the activity of the luciferase reporter gene regulated by CYP6BQ12 promoter by 917-fold as compare to the luciferase gene expression in the cells transfected with the reporter construct alone. In the cells co-transfected with a construct expressing CncC or Maf alone increased the luciferase reporter gene regulated by CYP6BQ12 promoter 25 and 8.5-fold respectively. A similar increase in the activity of the luciferase reporter gene regulated by CYP6BQ6, CYP6BQ7 and CYP6BQ9 was observed in the presence of both CncC and Maf (Figs. 2.7 B-D). Co-transfection of constructs expressing CncC and Maf showed 12.5, 65.6, and a 4-fold increase in the reporter gene regulated by CYP6BQ6, CYP6BQ7 and CYP6BQ9 respectively. When co-transfected with construct expressing Maf alone two and 3.9-fold increase in the reporter gene regulated by CYP6BQ6 and CYP6BQ7 respectively was detected. Similarly, when co-transfected with a construct expressing CncC alone 7, 2.6 and 2.5-fold increase in the reporter gene regulated by CYP6BQ6,

CYP6BQ7 and CYP6BQ9 respectively was detected. These data suggest that binding of CncC and Maf may enhance the expression of CYP6BQ genes.

Identification of CncC-Maf binding site

Promoter truncation assays were used to identify CncC-Maf binding sites. To find the CncC and Maf binding site, we decided to concentrate on CYP6BQ12 promoter because of its highest activity among the promoters tested. The promoter region of the CYP6BQ12 gene was truncated into different size fragments and the fragments were cloned into pGL3 vector. Each construct containing truncation of the CYP6BQ12 promoter was co-transfected with CncC and Maf constructs. After performing the luciferase assays, the truncations that showed significantly (at *P* value <0.05) higher luciferase activity were selected for generation of the next set of truncations. Comparison of four truncations of the CYP6BQ12 promoter showed that the binding site might be located between -1563 and -463 (Fig. 2.8 A). Two additional truncations were produced and assaying of these two truncations showed that the binding site might be located in the region between -1563 to -1097 (Fig. 2.8 B). Three additional truncations (-1563 to -1373, -1392 to -1232 and -1251 to -1097) were produced and assaying of these truncations showed that the binding site is located in a fragment containing -1392 to -1232 region of the promoter (Fig. 2.8 C). The online software ALGGEN-PROMO was used to predict the Nrf2-Maf binding site (GCAGTAC) in the 160-nucleotide fragment. The putative binding site was further tested by making various point mutations and by the deletion of the entire binding site from the CYP6BQ12 promoter fragment (Fig. 2.9 A). Reporter assays with mutant constructs after transfection of the reporter, CncC and Maf constructs into TcA cells (at this time the cell line developed from *T. castaneum* became available

and we tested these constructs in TcA cells) showed that changing GCAGTAC to GaAaTAC eliminated CncC-Maf induced reporter activity (Fig. 2.9 B). In addition, deleting entire binding site (Truncation 14 containing -1473 to -1382 fragment), mutating second nucleotide C to A, third nucleotide A to G or fourth nucleotide G to A caused a significant decrease (only two fold increase in the reporter activity in the presence of CncC and Maf) in the reporter activity when compared to the control (Truncation 13, Fig. 2.9 B). Taken together, these data suggest that C, A and G at 2^{nd}, 3^{rd} and 4^{th} positions in the binding site play critical roles in CncC-Maf binding. This binding site is also present in the promoters of other CYP6BQ genes (Fig. 2.10).

Discussion

Previous studies in our laboratory showed that the overexpression of brain specific CYP6BQ9 gene is the key mediator of the deltamethrin resistance observed in the QTC279 strain of *T. castaneum*, but the mechanism of the overexpression for this gene is not known. The focus of the current study was to determine mechanisms of overexpression of CYP6BQ genes in QTC279 beetles. RNAi-aided knockdown of possible xenobiotic transcription factors showed that CncC and its heterodimer partner Maf control the expression of not only CYP6BQ9 but also other genes in the CYP6BQ cluster. Studies in mammalian cell lines and tissues identified Nrf2 as the transcription factor responsible for the activation of genes coding for enzymes that metabolize xenobiotic compounds (Deng and Kerppola, 2013; Nakata et al., 2006). Similarly, in insects, the role of Nrf2 homolog, CncC, has been identified as a key regulator of the expression of xenobiotic response genes (CYP6A2, and glutathione S-transferase D1) induced by phenobarbital and paraquat in adult *D.melanogaster* (Misra et al., 2011; Misra

et al., 2013; Sykiotis and Bohmann, 2008). These studies are consistent with our data included in this chapter as we found that knockdown in the expression of CncC in the deltamethrin resistant beetles showed a significant decrease in CYP6BQ9 mRNA levels resulting in 100% mortality when the RNAi beetles were exposed to the deltamethrin. The knockdown of Maf also showed a significant decrease in CYP6BQ9 mRNA levels with about 98% mortality of beetles was observed upon deltamethrin treatment. Injection of dsRNA for both the transcription factors also showed a significant decrease in CYP6BQ9 mRNA levels resulting in 100% mortality of beetles after deltamethrin treatment. Met knockdown beetles also showed 99% mortality upon treatment with deltamethrin but the change in CYP6BQ9 mRNA levels was insignificant. This may be due to the fact that Met knockdown compromises the health of beetles causing death within ten days after injection (Parthasarathy et al., 2008b).

The metabolism mediated by cytochrome P450-dependent monooxygenases is one of the most common methods that insects employ to detoxify insecticides and develop resistance against them (Feyereisen, 2012). Many genes belonging to four different CYP families i.e. CYP4, CYP6, CYP9 and CYP12 have been documented to be upregulated and cause increased metabolism of insecticides leading to resistance against them (Li et al., 2007). Numerous resistant strains of insects have been identified that showed the elevated expression of multiple P450 genes resulting in the development of resistance against different insecticides (Zhu et al., 2010). A few such examples are pyrethroid resistant *Anopheles gmabiae* (CYP6P3 and CYP6M2), DDT resistant *D. melanogaster* (CYP6G1 and CYP12D1), pyrethroid resistant *Culex quinquefasciatus* (CYP9M10 and CYP4H34) and pyrethroid resistant *Helicoverpa armigera* (CYP9A12

and CYP9A14) (Komagata et al., 2010;Yang et al., 2008). Since CYP6BQ9 is a member of the twelve-gene cluster, after the knockdown of each xenobiotic transcription factor, we also checked the expression of all other members of the cluster. We found that CncC and Maf RNAi beetles not only showed a significant decline in CYP6BQ9 expression but the mRNA levels of other CYP6BQ genes (except CYP6BQ1 and CYP6BQ8) were also lower in RNAi beetles when compared to their expression in control beetles. Zhu et al 2012 showed that CYP6BQ9 played a major role in deltamethrin resistance in QTC279 strain of *T. castaneum* but based on their microarray data they found two other CYP6BQ genes are upregulated in QTC279. Further, the authors suggested the involvement of more than one CYP gene in resistance, as the knockdown of CYP6BQ9 gene was insufficient in providing a complete block of deltamethrin resistance. Based on our current results it is possible the QTC279 strain is employing multiple members of CYP6BQ genes to achieve resistance. The fact that CncC and Maf RNAi beetles exhibit 100 % mortality upon deltamethrin treatment suggests that CncC and Maf may regulate most if not all of the CYP genes responsible for deltamethrin resistance in this beetle.

The partnership between the Nrf2 and Maf is indispensable for binding to XRE and activating xenobiotic genes (Hirotsu et al., 2012; Misra et al., 2011; Misra et al., 2013; Nakata et al., 2006). These previous studies support our results that showed the requirement of CncC and Maf for expression of CYP6BQ6 genes. We investigated the importance of both CncC and Maf in regulating the promoter region of CYP6BQ genes (CYP6BQ12, CYP6BQ7, CYP6BQ9, and CYP6BQ6) and found that the presence of both the transcription factors is required as the luciferase activity was reduced in the absence of these transcription factors. It is possible that CncC alone is binding to the Nrf2

binding site. But it has also been reported that CncC homolog Nrf2 unlike SKN-1 is not able to bind as a monomer and does require a heterodimer partner (Tullet et al., 2008). Nrf2 can also heterodimerize with other bZIP-transcription factors (Jun, Fos and ATF4) and can bind to the ARE sequence but the importance of these heterodimer partners in controlling the downstream genes is unknown (Venugopal and Jaiswal, 1998; He et al., 2001). Maf proteins are capable of forming homodimers that bind to the Maf binding site called MARE but the Maf homodimers are negative regulators as they lack the canonical activation domain (Hirotsu et al., 2012; Motohashi et al., 2002). Since, we did not observe any significant changes in the luciferase activity when the promoters were co-transfected with the Maf construct alone, the involvement of Maf homodimers in the regulation of CYP genes in *T. castaneum* is unlikely.

The Nrf2 and Maf heterodimers are known to bind to the XRE sequence called Nrf2-Maf binding site present in the promoter region of detoxifying genes (Hirotsu et al., 2012; Misra et al., 2011). Maf homodimers bind to the special sequence called MARE (TGCTGACTCAGCA) that is rich in GC and is known to be embedded in the Nrf2-Maf site (He et al., 2001; Motohashi et al., 2006). The Maf homodimers are known to compete with the Nrf2-maf heterodimer to bind to the XRE site (He et al., 2001; Motohashi et al., 2006). Further, GC box present on the 3' end of the binding site has been suggested to be crucial for binding of Nrf2-Maf complex (Yamamoto et al., 2006). Based on the luciferase assay and using the promo software we were able to find a seven nucleotide, GCAGTAC, CncC-Maf binding site in the promoter region of CYP6BQ12 promoter. By creating the point mutations in the putative binding site, we found that the three nucleotides CAG (GCAGTAC) are crucial for CncC and Maf regulated expression of the

reporter gene. Interestingly, the GC sequence is present at the 5' prime end of the GCAGTAC binding site present in CYP6BQ12 promoter. Also, the C in the GC of this binding site is crucial for CncC-Maf binding similar to the requirement of C in Nrf2-Maf binding site. Taken together, the experiments described in this chapter identified transcription factors and their binding site that mediate expression of CYP6BQ cluster genes responsible for deltamethrin resistance in *T. castaneum*. Further studies on the presence and functioning of the identified transcription factors and binding sites in insects that develop resistance against different classes of insecticides will help to understand mechanisms involved in regulation of genes coding for proteins involved in detoxification of insecticides.

Table 2.1. List of various xenobiotic sensors in insect and their vertebrate orthologs.

	Transcription Factor Superfamily	**Xenobiotic sensors in insects**	**Vertebrate ortholog**
1	**Basic-helix-loop-helix / Per-ARNT-Sim (bHLH/PAS) proteins**	Spineless- arestapedia (Ss)	Aryl hydrocarbon receptor (Ahr)
		Methoprene tolerant (Met)	
2	**Basic and leucin zipper (bZIP)**	Cap n Collar c (CncC)	NF-E2 related factor (Nrf2)
		muscle aponeurosis fibromatosis (Maf)	muscle aponeurosis fibromatosis (Maf)
3	**Nuclear receptor (NR)**	Hepatocyte Nuclear Factor 4 (HNF4)	HNF4α & HNF4λ
		Hormone Receptor 96 (HR96)	SXR/PXR & CAR
		Ultra spiracle (USP)	Retinoid X receptor (RXR)

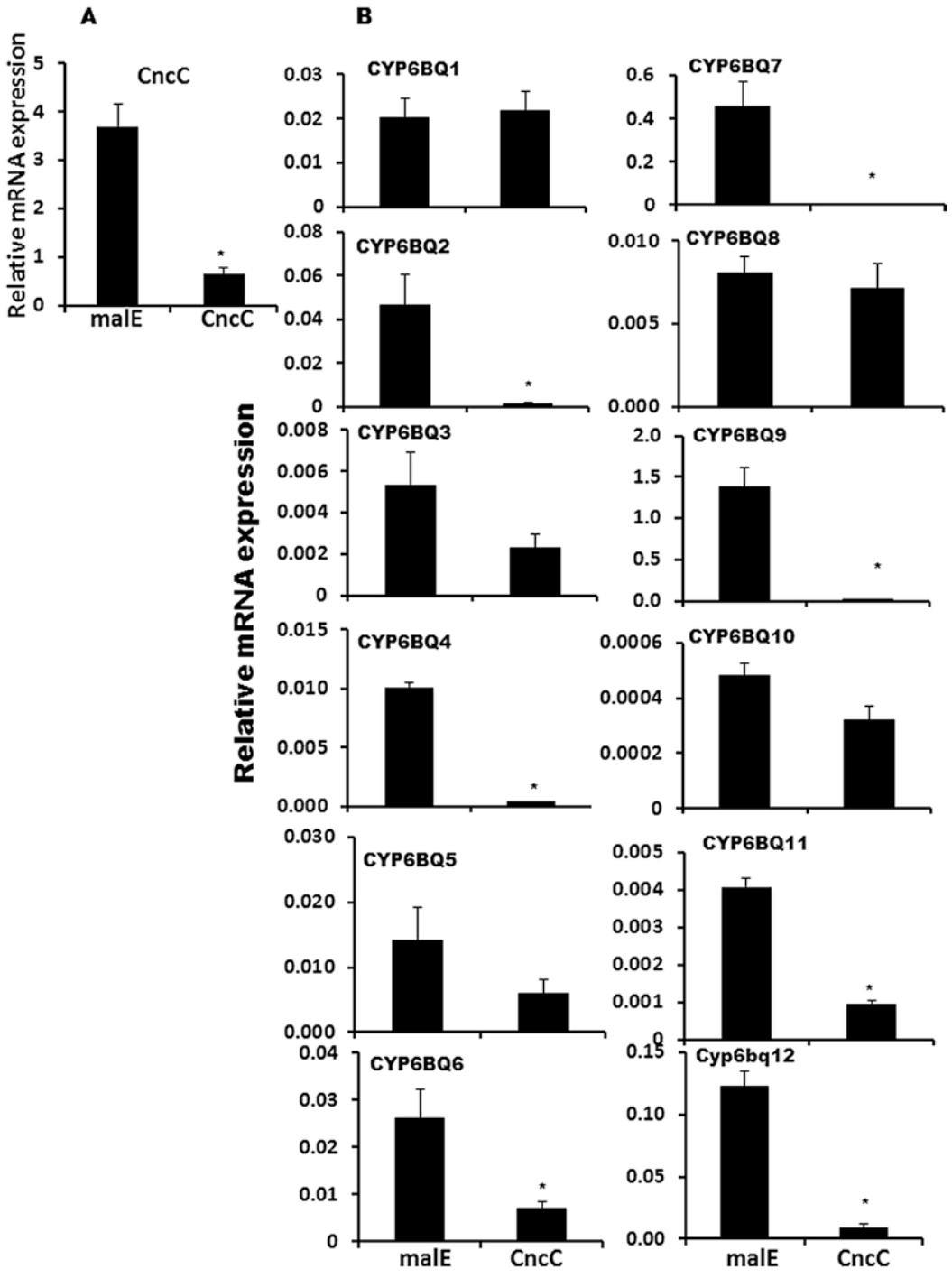
Table 2.2. Primers used to prepare the promoter constructs.

Truncation	Position	Size (bp)	Forward Primer	Reverse Primers
CYP6BQ12	-1563 to +87	1651	ctagctagcCTGGACTAGGGAGAAAATCCTTG	ccgctcgagACTCTCACTTGATTTGACACTGA
CYP6BQ6	-603 to -45	649	cggggtaccTAATAGTCTCGAGTGAACTTATCC	ccgctcgagGATCAACATGTTAGAAT GGGTTT
CYP6BQ7	-361 to +119	481	cggggtaccGGCATGTGGCTAAATGTCG	ccgctcgagGCACGGTTGTCTTACATTTAATGAG
CYP6BQ9	-383 to -96	288	cggggtaccTCTAATAAAGCAATCCCTCCAACT	ccgctcgagCGTTGCGTTATTATACTCACC G
Cyp6BQ12	-1563 to -463	1101	cggggtaccCTGGACTAGGGAGAAAATCCTTG	ccgctcgagGAATCTGTACTTTCTCCCACT
Cyp6BQ12	-463 to + 87	505	cggggtaccGTTTTATCATAGTCACGCTGGAA	ccgctcgagACTCTCACTTGATTTGACACTGA
Cyp6BQ12	-444 to +87	532	cggggtaccTATTCTCAATACCACAATCAT	ccgctcgagACTCTCACTTGATTTGACACTGA
Cyp6BQ12	-1563 to -438	1126	cggggtaccCTTTCAACGAACAACTTGCA	ccgctcgagACTCTCACTTGATTTGACACTGA
Cyp6BQ12	-1563 to -1097	467	cggggtaccCTGGACTAGGGAGAAAATCCTTG	ccgctcgagAGGATACTTTCCAAATTAGAGC
Cyp6BQ12	-1251 to -1097	155	cggggtaccAGTTACCTAGTTGATTGGTA	ccgctcgagAGGATACTTTCCAAATTAGAGC
Cyp6BQ12	-1392 to -1232	160	cggggtaccCTTATATCCTTTTACTATTA	cgctcgagTACCAATCAACTAGGTAACT
Cyp6BQ12	-1563 to - 1373	191	cggggtaccTAATAGTAAAAGGATATAAG	ccgctcgagAGGATACTTTCCAAATTAGAGC
Cyp6BQ12	-1392 to -1280	113	cggggtaccCTTATATCCTTTTACTATTA	ccgctcgagTGAAAATAATCTCGTACTGCA
Cyp6BQ12	-1392 to -1302	91	cggggtaccCTTATATCCTTTTACTATTA	ccgctcgagTCATACCTCAATTATGTTTAT
Cyp6BQ12-mutation-1	-1392 to -1293	100	cggggtaccCTTATATCCTTTTACTATTA	ccgctcgagCGT ATTTCAATCATACCTCAAT
Cyp6BQ12-mutation-2	-1392 to -1280	113	cggggtaccCTTATATCCTTTTACTATTA	ccgctcgagGTACTGTAATCATACCTCAAT
Cyp6BQ12-mutation-3	-1392 to -1280	113	cggggtaccCTTATATCCTTTTACTATTA	ccgctcgagGTACTCCAATCATACCTCAAT
Cyp6BQ12-mutation-4	-1392 to -1280	113	cggggtaccCTTATATCCTTTTACTATTA	ccgctcgagGTACCGCAATCATACCTCAAT
Cyp6BQ12-mutation-5	-1392 to -1280	113	cggggtaccCTTATATCCTTTTACTATTA	ccgctcgagGTATTGCAATCATACCTCAAT
Cyp6BQ12-mutation-6	-1392 to -1280	113	cggggtaccCTTATATCCTTTTACTATTA	ccgctcgagGTTCTGCAATCATACCTCAAT

Table 2.3. Primers used in protein expression, RNAi and qRT-PCR.

Name	Purpose	Forward Primer	Reverse primers
Maf	Expression	CGCGGATCCGCCTCAGGAGCCCCGAAAATC	ATAAGAATGCGGCCGCCAAATTCTCTAATTCGGTTGG
CncC	Expression	CGGGGTACCGTGGATATGGATCTGATTG	GCCAAGCTTATCTTTGTGACCTTGAGGTG
CncC	dsRNA	AACGCTTGTCCAAGTACGACCTGA	GTTTGAGCAACGTTTGCCGTGAGA
CncC	QRT	CGAGTGACTGACGTTATTGGTGCT	GTACTACAGTTCCAAGAAGTACGG
Maf	dsRNA	TGCGAGACTTGAACCGACAACTCA	CTGGTGCGAATGCTGCTCATTTCA
Maf	QRT	GGACATTTCGGACGATGAATTG	GCGCCTTCTTTGCTTCATAC
CYP6BQ1	QRT	ATTTCGGAGCATCACCCCTTTACG	GGTTGAATAAATGCCCACTGAGAGGG
CYP6BQ2	QRT	CGGCAACTGTGCTAGTTTATTTAAAGC	AAACATAAACACGCCACCATGTCTG
CYP6BQ4	QRT	TTCGGCAACTGTGCTAGTTTATTG	ACCATGTCTAAGATTTTTCTCCCG
CYP6BQ5	QRT	TCAAACCTTGGCTGATTGTGCCTC	TTCGAGGCCAAATGCAACTGAACC
CYP6BQ6	QRT	CGACCGACCCTGAAATTATCAAGC	ACTTAATGGATCGGCCTCTTCGTC
CYP6BQ7	QRT	5'-TATGAACGAATGCGCAACAAGCGG	TTAAGGCTGTTGCATTCAAGCCCG
CYP6BQ11	QRT	GTACCAAGCCTAAACCCTGTG	GAAAATGCCACCGTGTTTGTAG
CYP6BQ12	QRT	ACACCGGGTTTGAAGGACATCATG	GCACTCAATTCCAAAGCCGACAG

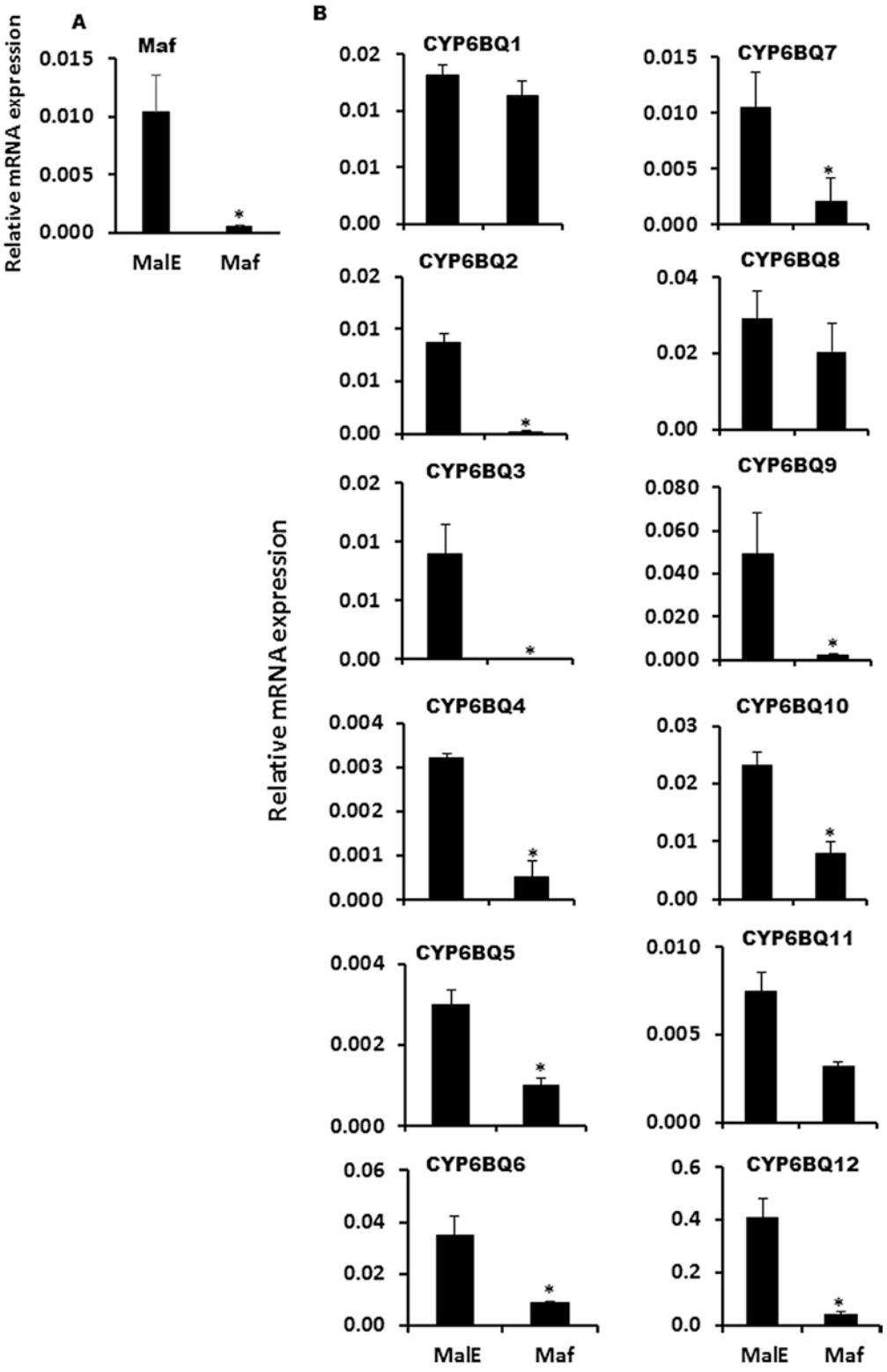
Figure 2.1.



Knockdown in the expression of CncC in *T. castaneum* QTC279 strain causes reduction in the expression of CYP6BQ genes. A. Knockdown efficiency of CncC in QTC279.

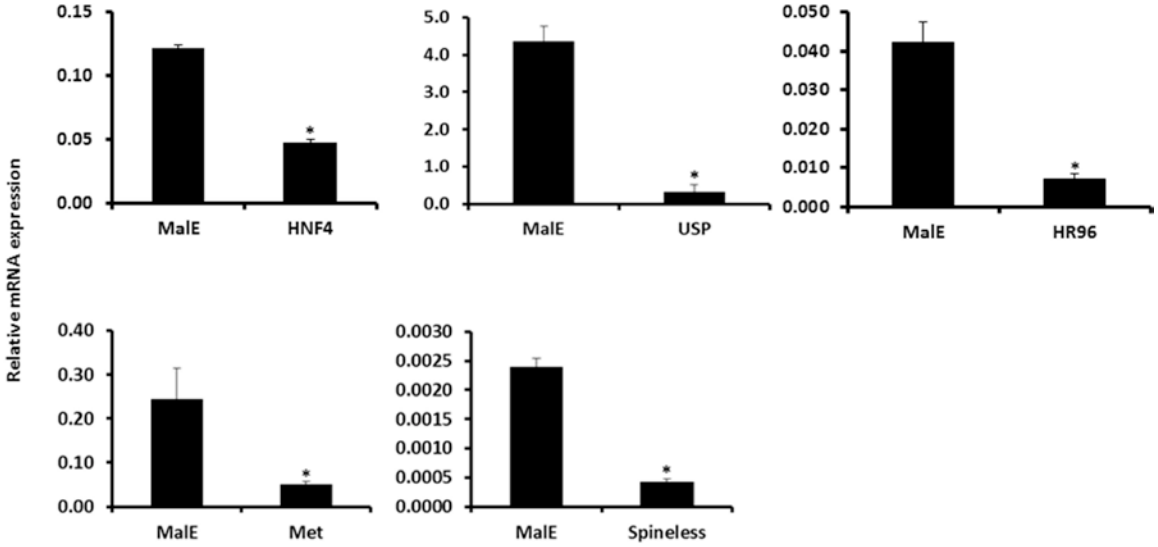
Total RNA was isolated from CncC or malE dsRNA injected adults on the 7th day after injection. The RNA was converted to cDNA and used to quantify expression of CncC mRNA levels. RP49 mRNA levels measured in the same samples at the same time were used for normalization. Mean + S.E. (n = 3) are shown. The data were analyzed using Student t-Test. *, significantly different at $P < 0.05$. B. The relative mRNA levels of CYP6BQ cluster genes were determined in beetles injected with CncC or malE dsRNA. RP49 mRNA levels measured in the same samples at the same time were used for normalization. Mean + S.E. (n = 3) are shown. The data were analyzed using Student t-Test. *, significantly different at $P < 0.05$.

Figure 2.2.


A
Maf
Relative mRNA expression
0.015
0.010
0.005
0.000
MalE
Maf
B
Relative mRNA expression
CYP6BQ1
CYP6BQ2
CYP6BQ3
CYP6BQ4
CYP6BQ5
CYP6BQ6
CYP6BQ7
CYP6BQ8
CYP6BQ9
CYP6BQ10
CYP6BQ11
CYP6BQ12
MalE
Maf

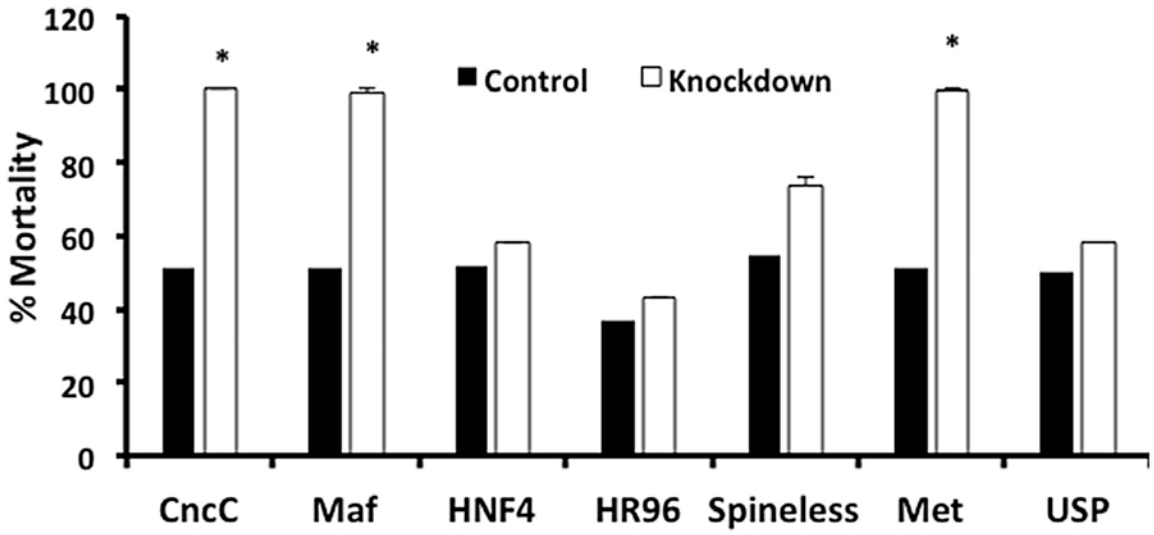
Knockdown in the expression of Maf in *T. castaneum* QTC279 strain causes reduction in the expression of CYP6BQ genes. A. Knockdown efficiency of Maf in QTC279. Total RNA was isolated from Maf or malE dsRNA injected adults on the 7th day after injection. The RNA was converted to cDNA and used to quantify expression of Maf mRNA levels. RP49 mRNA levels measured in the same samples at the same time were used for normalization. Mean + S.E. (n = 3) are shown. The data were analyzed using Student t-Test. *, significantly different at $P < 0.05$. B. Relative mRNA levels of CYP6BQ cluster genes in Maf or malE dsRNA injected beetles were determined. RP49 mRNA levels measured in the same samples at the same time were used for normalization. Mean + S.E. (n = 3) are shown. The data were analyzed using Student t-Test. *, significantly different at $P < 0.05$.

Figure 2.3.



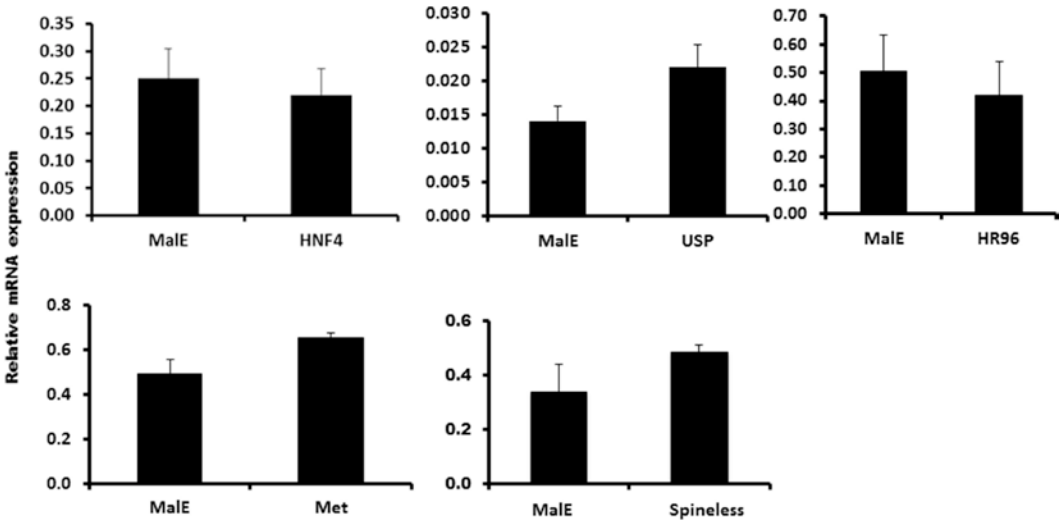
Knockdown efficiency of select transcription factors. Total RNA was isolated from beetles injected with *malE*, Met, USP, Spineless, HR96 and HNF4 dsRNA target genes. RP49 mRNA levels measured in the same samples at the same time were used for normalization. Mean + S.E (n = 3) are shown.

Figure 2.4.



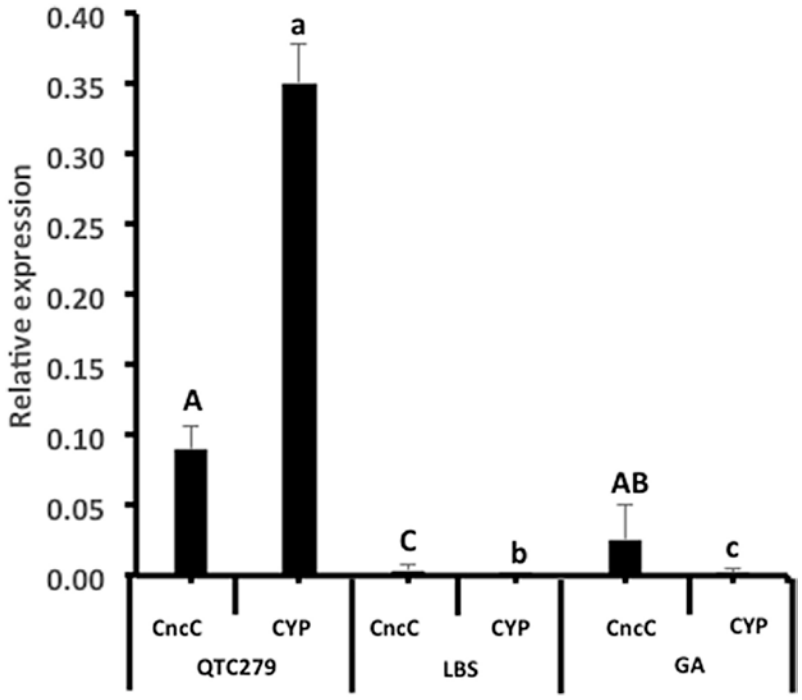
Knockdown in the expression of CncC, Maf and Met increases deltamethrin induced mortality. *T. castaneum* adults were injected with CncC, Maf, HNF4, HR96, spineless, USP and Met dsRNAs. At eight days after injection, the beetles were exposed to deltamethrin and mortality was recorded at 24 h after deltamethrin treatment. Control beetles were injected with malE dsRNA. Mean + S.E. (n = 25–30) are shown. The data were analyzed using Student t-Test. *, significantly different at $P < 0.05$.

Figure 2.5.



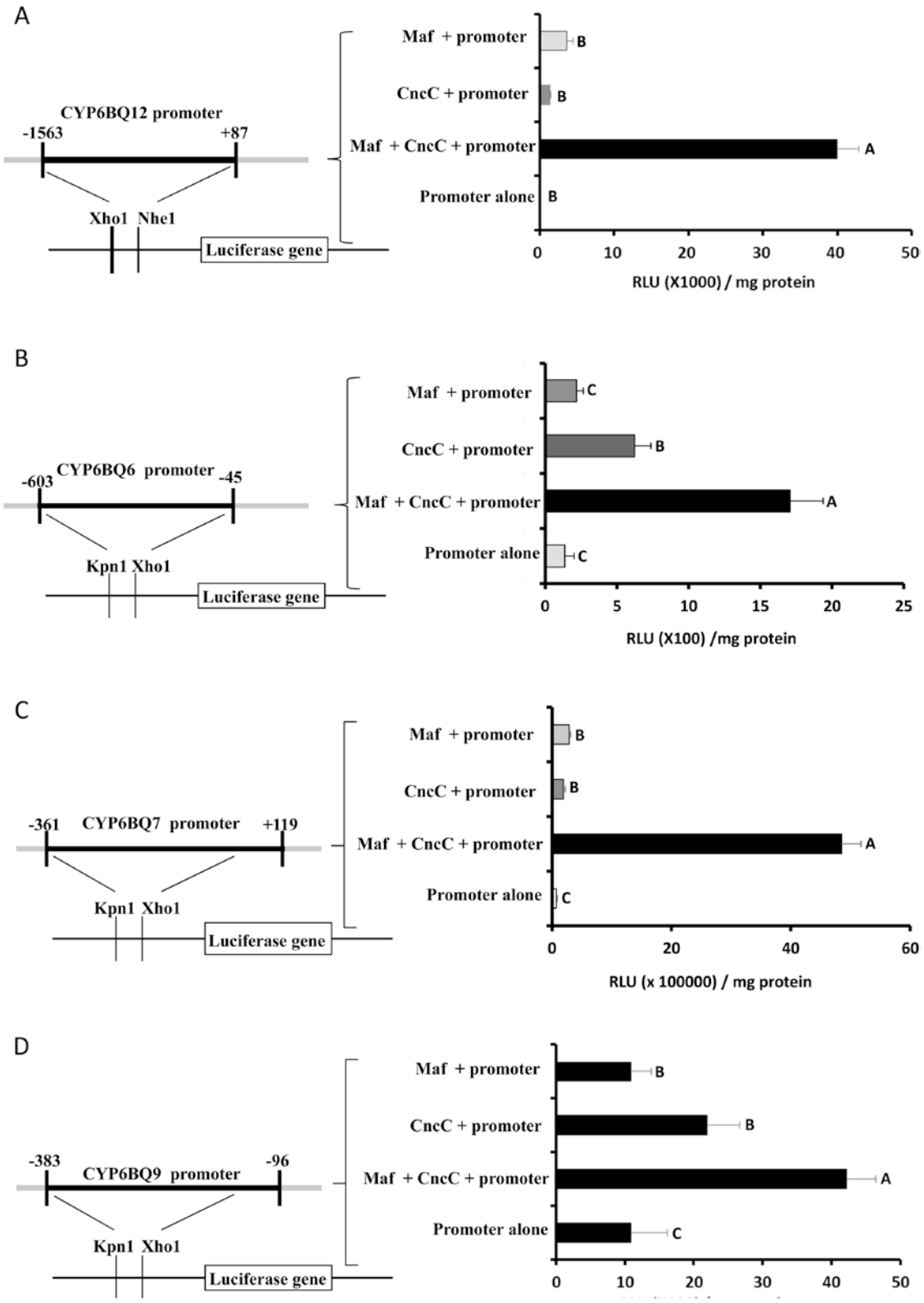
Relative mRNA levels of CYP6BQ9 in beetles injected with *malE*, Met, USP, Spineless, HR96 and HNF4 dsRNA and CncC in different strains of *T. castaneum*. Total RNA isolated from RNAi beetles was converted to cDNA and used to determine relative mRNA levels of CYP6BQ9. RP49 mRNA levels measured in the same samples at the same time were used for normalization. Mean + S.E (n = 3) are shown.

Figure 2.6.



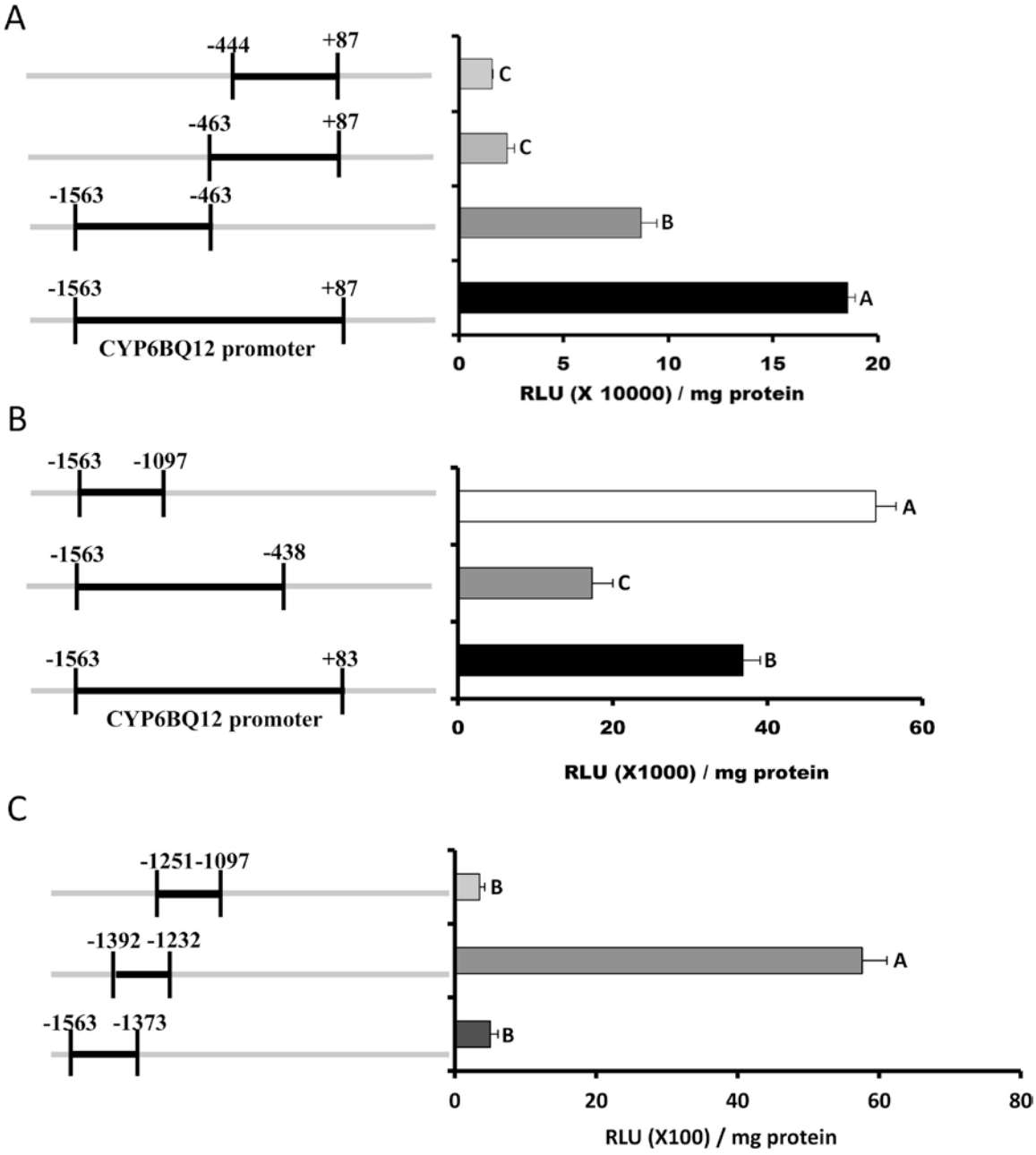
Comparison of relative mRNA levels of CYP6BQ9 and CncC in different strains of *T. castaneum*. Total RNA isolated from deltamethrin resistant (QTC279), deltamethrin susceptible (Lab S) and general lab strain (GA) was converted to cDNA and used to determine relative mRNA levels of CncC and CYP6BQ9 genes. RP49 mRNA levels measured in the same samples at the same time were used for normalization. Mean + S.E (n = 3) are shown. The data were analyzed using Tukey's HSD. A, B and C (CncC) and a, b and c (CYP6BQ9) denotes significant difference at $P < 0.05$.

Figure 2.7.


A
CYP6BQ12 promoter
-1563
+87
Xho1 Nhe1
Luciferase gene
Maf + promoter
CncC + promoter
Maf + CncC + promoter
Promoter alone
B
C
A
0 10 20 30 40 50
RLU (X1000) / mg protein
B
CYP6BQ6 promoter
-603
-45
Kpn1 Xho1
Luciferase gene
Maf + promoter
CncC + promoter
Maf + CncC + promoter
Promoter alone
C
B
A
C
0 5 10 15 20 25
RLU (X100) /mg protein
C
-361
CYP6BQ7 promoter
+119
Kpn1 Xho1
Luciferase gene
Maf + promoter
CncC + promoter
Maf + CncC + promoter
Promoter alone
B
B
A
C
0 20 40 60
RLU (x 100000) / mg protein
D
-383
CYP6BQ9 promoter
-96
Kpn1 Xho1
Luciferase gene
Maf + promoter
CncC + promoter
Maf + CncC + promoter
Promoter alone
B
B
A
C
0 10 20 30 40 50

The luciferase levels in cells transfected with CYP6BQ promoters, CncC, and Maf expression constructs. Each promoter activity was determined in the presence of both the proteins (CncC and Maf), in the presence of either protein (CncC + empty pIEx-4 vector or Maf + empty pIEx-4 vector) or no protein (promoter + empty pIEx-4 vector). The cells were harvested at 72 h after transfection and the luciferase activity was quantified. Mean + S.E. (n = 3) are shown. The data were analyzed using Tukey's HSD, ($P < 0.05$). A. CYP6BQ12 (1651 bp) promoter in pGL3 basic vector −1563 to +87 bp. B. CYP6BQ6 (649 bp) promoter in pGL3-TATAA – vector −603 to −45 bp. C. CYP6BQ7 (481 bp) promoter in pGL3 –TATAA-vector −361 to +119 bp. D. CYP6BQ9 (287 bp) partial promoter pGL3 –TATAA-vector −383 to −96 bp.

Figure 2.8.



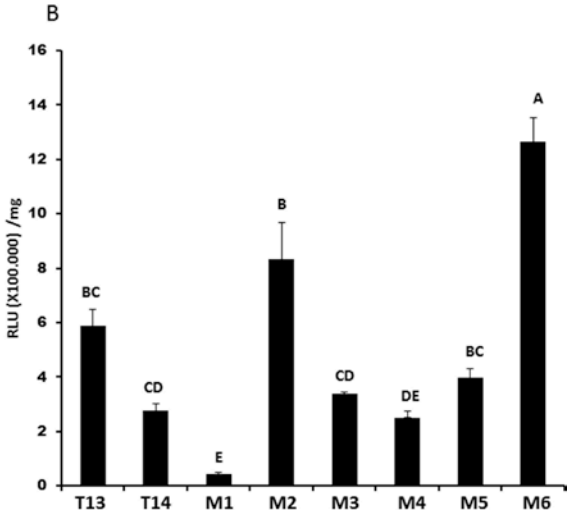
Analysis of CYP6BQ12 promoter truncations. Bm-5 cells were co-transfected with Maf, and CncC and the reporter vector containing truncated fragments of CYP6BQ12 promoter. The cells were harvested at 72 h after transfection and the luciferase activity was quantified. Protein was estimated using Bradford's assay to normalize the luciferase

readings. Mean + S.E. (n = 3) are shown. The data were analyzed using Tukeys HSD, ($P < 0.05$). The solid black line represents the regions that are cloned into the reporter vector. A. First three truncations tested. B. Three additional truncations tested. C. Three truncations of −1563 to −1097 fragment tested.

Figure 2.9.

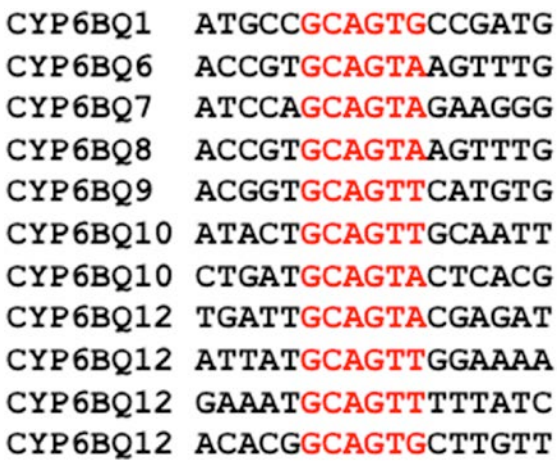
A

Wild Type (T13)	GCAGTAC
M1	GaAaTAC
M2	aCAGTAC
M3	GaAGTAC
M4	GCgGTAC
M5	GCAaTAC
M6	GCAGaAC
Binding site deletion (T14)	No binding site



The relative contribution of nucleotides in the predicted Maf and CncC binding site analyzed using point mutants. A. Point mutations used in the experiment. B. Mutated constructs, CncC and Maf, were transfected into TcA cells in a 48-well plate. Following transfection, cells were lysed after 48 h. Protein was estimated using Bradford's assay to normalize the luciferase readings. Mean + S.E. (n = 3) are shown. The data were analyzed using Tukey's HSD, ($P < 0.05$).

Figure 2.10.



The conserved CncC-Maf binding sites in the promoter region of CYP6BQ genes are shown.

Chapter 3: Transcription factor cap n collar C regulates multiple cytochrome P450 genes conferring adaptation to potato plant allelochemicals and resistance to imidacloprid in *Leptinotarsa decemlineata* (Say)

This chapter has been published as Kalsi, M. and Palli, S.R., 2017. Transcription factor cap n collar C regulates multiple cytochrome P450 genes conferring adaptation to potato plant allelochemicals and resistance to imidacloprid in *Leptinotarsa decemlineata* (Say). *Insect Biochemistry and Molecular Biology*, *83*, pp.1-12.

Summary

Colorado potato beetle (CPB), *L. decemlineata* is a notorious pest of potato. Co-evolution with Solanaceae plants containing high levels of toxins (glycoalkaloids) helped this insect to develop an efficient detoxification system and resist almost every chemical insecticide introduced for its control. Even though the cross-resistance between plant allelochemicals and insecticides is well acknowledged, the underlying molecular mechanisms are not understood. Here, we investigated the molecular mechanisms involved in detoxification of potato plant allelochemicals and imidacloprid resistance in the field-collected CPB. Our results showed that the imidacloprid-resistant beetles employ metabolic detoxification of both potato plant allelochemicals and imidacloprid by upregulation of common cytochrome P450 genes. RNAi aided knockdown identified four cytochromes P450 genes (CYP6BJ$^{a/b}$, CYP6BJ1v1, CYP9Z25, and CYP9Z29) that are required for defense against both natural and synthetic chemicals. These P450 genes are regulated by the xenobiotic transcription factors Cap n Collar C, CncC and muscle aponeurosis fibromatosis, Maf. Studies on the CYP9Z25 promoter using the luciferase

reporter assay identified two binding sites (i.e. GCAGAAT and GTACTGA) for CncC and Maf. Overall, these data showed that CPB employs the metabolic resistance mediated through xenobiotic transcription factors CncC and Maf to regulate multiple P450 genes and detoxify both imidacloprid and potato plant allelochemicals.

Keywords: Insecticide, Resistance, Colorado potato beetle, Metabolic resistance, Detoxification.

Introduction

Leptinotarsa decemlineata (Say), commonly known as Colorado potato beetle (CPB) is a serious threat to potato as both larvae and adults can cause severe losses to potato production depending on the plant stage attacked (Alyokhin et al. 2013b). Colorado potato beetle has kept scientists intrigued for several decades owing to its quick adaptation to insecticides. According to the Arthropod Pesticide Resistance Database (APRD), CPB has gained resistance to 55 active ingredients (Whalon et al. 2012). Colorado potato beetle is an actively microevolving species whose biology provides an additional advantage against insecticides such as their ability to distribute well in space (high fecundity and dispersal) and time (diapause) (Alyokhin et al. 2013a; Alyokhin et al. 2013b). For instance, the resistance to neonicotinoid, imidacloprid, was developed after only two years of its initial commercialization in 1995 (Bass et al. 2015; Whalon et al. 2012). Populations of CPB are widely distributed in North America, Europe, and Asia therefore, insecticide resistance in this insect could become a global phenomenon (Alyokhin et al. 2008).

Insecticide resistance is an example of rapid microevolution (Ffrench-Constant et al. 2004; Lenormand et al. 1999; Silva et al. 2012) that can occur due to the upregulation of gene expression, amplification or alteration in gene structure (Feyereisen et al. 2015; van Straalen et al. 2011). Up-regulation expression of a gene can also be ascribed to the change in the gene structure i.e. changes in the cis-regulatory elements or the trans-regulatory elements (Bass and Field 2011; Feyereisen et al. 2015; van Straalen et al. 2011). For example, in *Drosophila*, the mutation in the trans-acting repressor lead to the constitutive overexpression of CYP6A8 gene. In house flies the trans-acting factor

controlled the overexpression of CYP6A1 gene (Cohen et al. 1994) while the changes in both cis and trans regulatory element resulted in of overexpression of CYP6D1 gene (Liu and Scott 1997). In a recent study, the upregulation of CYP6BQ genes in the deltamethrin resistant strain of *T. castaneum* was shown to be controlled by both cis- and trans-regulatory factors (Kalsi and Palli, 2015). Previous studies in CPB have documented four different resistance mechanisms; enhanced detoxification enzymes production (e.g. cytochrome P450's, esterases and carboxylesterases), target site insensitivity (e.g. acetylcholine receptors, sodium channel receptor), reduced penetration or increase in insecticide excretion and behavioral resistance (Alyokhin et al. 2008). Recent transcriptomics studies have shown the involvement of many P450 genes in CPB resistance to imidacloprid (Zhu et. al. 2016). Imidacloprid is a systemic neonicotinoid that acts by binding to the nicotinic acetylcholine receptor in the nervous system and interfering with the neuronal transmission resulting in paralysis or death of an insect (Stanneck et al. 2012).

Detoxification of synthetic or natural xenobiotics such as an insecticide or plant toxins involves three phases consisting several metabolizing enzymes (Liska 1998; Nakata et al. 2006; Reddy et al. 2012). Phase I mainly consists of the enzymes from cytochrome P450 supergene family, where the P450 enzymes oxidize the parent molecule in the presence of NADPH as a cofactor to add a reactive group such as hydroxyl group (Feyereisen and Lawrence 2012). Phase II involves metabolizing or conjugating enzymes such as glutathione-S-transferases (GSTs), N-acetyltransferase, sulfotransferases (SULT) etc. (Kostaropoulos et al. 2001; Ranson et al. 2011). These enzymes make phase I intermediates into more water-soluble compounds through conjugation. Phase III

involves transporters such as p-glycoprotein, the multi-drug resistance associated protein (MDR) or ATP binding cassette (ABC) transporters and these transporters are responsible for the elimination of the xenobiotic detoxification byproducts from the cells (Broehan et al. 2013; Glavinas et al. 2004).

The detoxification genes are regulated by a common mechanism of transcriptional activation and the transcription factors involved in activation of such genes are grouped into three xenobiotic superfamilies including nuclear receptor (NR), basic-helix-loop-helix/per-ARNT-SIM (bHLH-PAS) and basic lucine zipper (bZIP) (Nakata et al. 2006). In mammals, the heterodimer bZIP transcription factors Nrf2-Keap1 (NF-E2-related factor 2 and Kelch-like ECH Associated Protein 1) represents a signaling pathway that plays a crucial role in regulation of a battery of cytoprotective genes involved in xenobiotic or oxidative responses in many human diseases (Slocum and Kensler 2011; Sykiotis and Bohmann 2010). At basal conditions, Keap1 represses Nrf2 by promoting its proteasomal degradation using ubiquitin ligase in the cytoplasm. Whereas, under oxidative stress, Keap1 act as a stress sensor releasing Nrf2 which translocates to the nucleus (Zhang 2006). Nrf2 heterodimerizes with small Maf (muscle aponeurosis fibromatosis; bZIP transcription factor) proteins and binds the specific sequences in the promoter region often referred to as antioxidant or xenobiotic response elements (ARE or XRE), initiating the transcription of several antioxidant/detoxification genes (Hirotsu et al. 2012). The Nrf2 ortholog in invertebrates is known as cap 'n' collar C (CncC), these proteins regulate the expression of a plethora of P450s that are involved in metabolism and detoxification (Deng 2014; Karim et al. 2015; Malhotra et al. 2010). In *Drosophila melanogaster*, three different Cnc proteins have been identified (i.e. CncA, CncB and

CncC) but only CncC contains Keap1 binding ETGE motif (Sykiotis and Bohmann 2008). The xenobiotic transcription factors CncC and Maf have been shown to play an important role in insecticide resistance in invertebrates including beetles and fruit flies (Kalsi and Palli, 2015; Wan et al. 2014).

Since chemical control remains the primary management tool for CPB, understanding the molecular mechanisms of regulation of resistance genes is very important. To understand molecular mechanisms of imidacloprid resistance in CPB, we have identified four P450 genes responsible for imidacloprid detoxification that are induced by both potato leaf extract and imidacloprid. These genes are regulated by the xenobiotic transcription factors cap n collar C (CncC) and muscle aponeurosis fibromatosis (Maf) binding to (GCAGAAT and GTACTGA) cis-element present in their promoter. Finding the cis and trans-regulatory elements controlling the multiple imidacloprid resistance genes could be crucial for fighting insecticide resistance in CPB, as these proteins and sequences could serve as novel targets to screen for inhibitors of detoxification genes (P450s).

Material and Methods

Insects

The imidacloprid-resistant strain of CPB was collected on a farm in Long Island, NY and the susceptible strain was obtained from the Department of Agriculture, New Jersey. Both the strains were reared on potato plants (Dark Red Norland potatoes) in the greenhouse in separate cages at 25±5 °C under a light: dark regime of 16:8 h. Adults and larvae were reared in different cages (BugDorm-2120 Insect Tent, MegaView Science

Co., Ltd). Egg masses were collected daily and transferred to a separate cage with a fresh potato plant to avoid cannibalism.

Cell culture

CPB cell line (Lepd-SL1) established from the pupal tissues was obtained from Dr. Goodman at USDA-ARS, Columbia, MO (Long et al. 2002). The cells were cultured in EX-CELL® 420 medium (Sigma-Aldrich) containing 10% FBS (Seradigm, VWR) and 1µg/ml antibiotic (Penicillin) in a 5 ml sterile flasks at 26°C. The *Spodoptera frugiperda* (Sf9) cells were routinely maintained in Sf-900 II SFM (Life Technologies) medium at 26°C.

Double-stranded RNA preparation and gene knockdown

The gene knockdown studies in CPB were conducted by feeding RNAi method. Two different dsRNA preparation methods were used (i) the dsRNA kit and (ii) HT115 bacterial system. The use of different dsRNA preparation methods (kit or bacterial) does not cause variability in the gene knockdown experiments (Zhu et al. 2011). The dsRNA (GFP, CYP9Z29 and CYP6BJ1v1) were synthesized using the MEGAscript dsRNA kit (Ambion™). The dsRNA (GFP, CncC, CYP9Z25, and CYP96BJ$^{a/b}$) were synthesized using HT115 bacteria following the procedure described by Zhu et al**.** 2011. The bacterially synthesized dsRNA (200 µl) or the in vitro-synthesized dsRNA (20 µg/µl diluted in 200 µl water) was sprayed onto the freshly plucked potato leaves and the treated leaves were dried for an hour. The control beetles were fed with bacterial synthesized or in vitro synthesized dsRNA depending on the target gene dsRNA used for each treatment. The beetles were starved for 24 h before feeding. The starved beetles were fed on the dsRNA-treated leaves for three days continuously by replacing old leaves

with freshly treated leaves on each day. After three days, beetles were fed on untreated fresh potato leaves for additional two days. On day six, total RNA extracted was used to determine knockdown efficiency using qRT-PCR.

RNA isolation, cDNA preparation, and qRT-PCR

For the CncC differential expression and CncC knockdown studies, the total RNA was isolated from different organs such as the brain, fat body, midgut, Malpighian tubules and the rest from three individual beetles together serving as one biological replicate using the TRI reagent (Molecular Research Center Inc., Cincinnati, OH). Three to four biological replicates were used for each experiment. For the P450 knockdown studies in adults, three beetles were used for total RNA extraction, each beetle serving as a biological replicate. In the case of insect cells, each well served as a biological replicate and a minimum of three replicates were used for each treatment. The DNAse 1 treated total RNA (TURBO™ DNAse, Ambion™) was eluted into the nuclease-free water and stored at -20° C. Three micrograms of total RNA was used for each sample to prepare cDNA using M-MLV reverse transcriptase kit (Invitrogen™). QRT-PCR was performed using the sample cDNA and specific primers either to check the knockdown efficiency or the mRNA expression levels of CncC and P450 genes (CYP6BJ$^{a/b}$, CYP6BJ1v1, CYP9Z25, and CYP9Z29). The relative levels of mRNAs were quantified using three biological replicates and normalized using ribosomal protein as an internal control (ribosomal protein 4, ribosomal protein 3, and ribosomal protein 18, mRNA). For each reaction, 2 µl cDNA, 5 µl FastStart SYBR Green Master (Roche Diagnostics, Indianapolis, IN), 2.6 µl nuclease free water, and 0.2 µl each of forward and reverse gene

specific primers were used (stock 10 µM). Each experiment was repeated at least three times.

Imidacloprid bioassay in adults

Adult beetles (1-2 week old and irrespective of sex) were treated with 0.5 to 1 µg of imidacloprid (technical grade, Chem Service) based on the preliminary LD_{50} imidacloprid bioassays. Since imidacloprid can also be toxic by contact, 1 µl of the droplet was applied to the abdomen on the ventral surface using a 50 µl Hamilton® microsyringe (Hamilton Company, Reno, NV). Treated beetles were kept in an incubator for 24 h (26±1◦C, RH=70%, L:D=16:8), after which the mortality was recorded.

Promoter constructs and reporter assays

In the preliminary experiment, the Lepd-SL1 cells showed poor transfection efficiency. Therefore, Sf9 cells were used to perform the reporter assays. The full-length promoter, promoter truncations and mutant of truncations of the CYP9Z25 promoter region were cloned into the pGL3 basic vector (Promega) containing a minimal promoter (Kalsi and Palli, 2015). The genes coding for *T. castaneum* CncC and Maf proteins were cloned into the pIEx-4™ expression vector (Novagen, EMD Millipore). Cells were counted using the hemocytometer and seeded at a density of 1 X 10^5 cells per well in a 48-well plate at 24 h prior to the experiment. The transfection and the luciferase assays were performed as described in Kalsi and Palli, 2015.

Potato leaf extract (PLE) preparation

Four grams of freshly plucked potato leaves (2-3 days old; washed and dried) were crushed into a fine powder using liquid nitrogen with the help of pestle and mortar.

The crushed powder was transferred to a 15 ml Falcon tube and 10 ml of 100% ethanol was added. The contents of the tube were mixed thoroughly for a minute using a vortex mixer, and then the tube was kept on ice for 30 minutes. The tube containing the potato leaf contents was centrifuged at 3700 rpm for 15 min at 4°C using a tabletop centrifuge (Eppendorf, Centrifuge 5810 R). The supernatant was transferred to a fresh glass vial and stored at -80°C until use (stock potato leaf extract, PLE). One ml of stock solution was added to a well in a 6-well plate under the laminar hood, and the extract was allowed to dry (4-6 h) until the ethanol was completely evaporated. After that 1 ml of the medium (EX-CELL 420 + 10% FBS + Antibiotic) was added and left for 15-20 minutes. The contents of the well were mixed by continuous pipetting and then transferred to a fresh 15 ml Falcon tube. The contents of the tube were diluted 10X with the fresh medium and stored at -20° C until use. This stock was further diluted with fresh medium to prepare five serial dilutions. To select the particular dilution for the experiment, the prepared dilutions were added to the Lepd-SL1 cells that were seeded 24 h before the addition of extract. The dilution of the extract that did not affect the health of cells was selected for the further experiment. For control, a well in 6-well plate was added with 100% ethanol and upon evaporation, fresh medium was added and mixed with continuous pipetting.

Lepd-SL1 cells treatments

The in-vitro studies were conducted in the Lepd-SL1 cells to provide additional evidence to verify the conclusions from the in vivo studies (i.e. resistance beetles). Because of simplicity and lack of influence from the endogenous factors, the cell lines are routinely used to obtain additional confirmatory data.

Treatment of Lepd-SL1 cells with potato leaf extract

The Lepd-SL1 cells were seeded into a 6 well plate (1 X 10^6 cells /well in 2 ml medium) at 24 h prior to the treatment. The following day, the old medium was removed and the cells were treated with 200 µl serial dilutions of potato leaf extract in triplicates. The control cells were treated with the equal amount of fresh control medium. After the 24 h treatment, the medium was removed from wells; cells were washed with 1% PBS and used for extracting RNA.

Treatment of Lepd-SL1 cells with Imidacloprid

Imidacloprid was prepared in DMSO in order to treat the cells. The Lepd-SL1 cells were seeded into a 6-well plate (1 X 10^6 cells /well in 2 ml medium) for 24 h. On the following day, old medium was removed, and the cells were treated with 1µg/µl of Imidacloprid for 6, 24, 48 and 36 h in triplicates. Control cells were added with the equal amount of the DMSO. After the allotted time, the medium was removed, and the cells were washed with 1% PBS and used for extracting RNA.

Treatment of CncC knockdown Lepd-SL1 cells with imidacloprid

The gene knockdown studies were performed in Lepd-SL1 cells using dsRNAs that were synthesized using the MEGAscript T7 RNAi Kit (Ambion, Inc.). Twenty-four hours prior to the treatment, the Lepd-SL1 cells were seeded into a 6-well plate (1 X 10^6 cells / well in 2 ml medium). The serum free medium (1 ml) mixed with 6 µg of CncC or GFP (control) dsRNA was added to the cells. After 4 h of incubation, 1 ml of medium containing 20% serum was added to each well. Cells were then cultured for 48 h, followed by addition of imidacloprid to the cells and bringing the final concentration to 1µg/µl. Control cells were treated with the equal amount of DMSO alone. After 24 h of

treatment (Imidacloprid/DMSO), the cells were washed with 1XPBS, followed by total RNA extraction as described earlier.

Statistical Analysis

For statistical analysis, JMP 11.0 software (SAS, Cary, NC) was used. A significant difference between two groups (control and treatment) was analyzed using a Student's t-test for the value of $P < 0.05$. To compare the significant differences between more than two groups Tukey HSD was used for the value of $P < 0.05$.

Results

Expression of CncC in Imidacloprid resistant beetles

Previous studies in *T. castaneum* (deltamethrin resistant strain) showed that the xenobiotic transcription factor CncC is constitutively overexpressed and served as a key regulator of multiple P450 genes (CYP6BQ genes) responsible for deltamethrin resistance (Kalsi and Palli, 2015). We wanted to test if CncC plays a similar role in the regulation of imidacloprid resistance genes in CPB. The CncC mRNA levels were compared in the resistant and susceptible strains of CPB during larval, pupal and adult stages. The highest levels of CncC mRNA were detected during the pupal (42-fold) and the adult (14-fold) stages in the imidacloprid-resistant strain when compared to its levels in the susceptible strain (Fig. 3.1 A). The CncC mRNA levels were not significantly different among the developmental stages of the susceptible strain tested. Further, CncC mRNA levels were compared among different tissues dissected from both susceptible and resistant adults (Fig. 3.1 B). Brain, midgut, Malpighian tubules, and the fat body from the

resistant beetles showed the higher CncC mRNA levels as compared to the levels in the tissues dissected from the susceptible strain.

Transcription factor CncC regulates the expression of cytochrome P450 genes

CncC knockdown in adults: Since the expression levels of xenobiotic transcription factor CncC were higher in the resistant strain; we hypothesized that CncC might be involved in the regulation of P450 genes responsible for imidacloprid resistance. A recent study done in our lab showed the potential role of 21 P450 genes in detoxification of potato leaf allelochemicals and imidacloprid resistance in CPB (Zhu. et. al. 2016). From these 21 genes, we selected eight P450 genes (CYP9Z25, CYP9Z26, CYP9Z29, CYP9Z^{d} , CYP9Z^{g} , CYP6BJ$^{a/b}$ and CYP6BJ1v1) that not only expressed at higher levels in the resistant strain (> 2 to 5-fold) but also showed induction when beetles were either fed on potato leaves or treated with imidacloprid. The CncC knockdown was performed in the 1-2 week-old imidacloprid-resistant adult beetles, and the expression levels of the eight candidate P450 genes were determined in the midgut tissue. Fig. 3.2 A shows the systemic knockdown of CncC gene in all the tissues tested. The expression level of four P450 genes (CYP6BJ$^{a/b}$, CYP6BJ1v1, CYP9Z25, and CYP9Z29) was significantly reduced in the midgut tissue following CncC knockdown (Fig. 3.2 B).

Knockdown of insecticide resistance genes and the imidacloprid bioassay: Since the CncC knockdown resulted in the lower expression of the cytochrome P450 genes, we tested to determine if CncC knockdown causes an increase in imidacloprid toxicity. The CncC or GFP dsRNA was fed to resistant beetles and these knockdown beetles were exposed to LD_{50} dose of imidacloprid. The CncC-knockdown beetles treated with the imidacloprid (LD_{50}), showed only 5% survival as compared to the control beetles that

showed 54% survival (Fig. 3.3 A). These data suggest that CncC is required for imidacloprid resistance. To determine if the four P450s that are affected by CncC knockdown are also required for imidacloprid resistance, the resistant beetles were fed with dsRNA targeting CYP6BJ$^{a/b}$, CYP6BJ1v1, CYP9Z25 or CYP9Z29 gene and the dsRNA-treated beetles were further tested for their resistance in imidacloprid bioassays. The survival rates for the beetles treated with CYP6BJ$^{a/b}$, CYP6BJ1v1, CYP9Z25 or CYP9Z29 dsRNA were 21, 12.5, 30 and 25% respectively when compared 54% in the control beetles fed with GFP dsRNA (Fig. 3.3 A and B). These data suggest that P450 genes that require the presence of CncC for their expression play an important role in imidacloprid resistance.

Imidacloprid induction of P450 genes in Lepd-SL1 cells: The results from the CncC knockdown and imidacloprid bioassay in adults suggested that CncC is an important regulator of the four P450 genes (i.e. CYP6BJ$^{a/b}$, CYP6BJ1v1, CYP9Z25 or CYP9Z29) that were responsible for imidacloprid resistance. Therefore, in our follow-up experiments, we only tested these four P450 genes. Experiments were conducted using the CPB cell line, Lepd-SL1, to determine whether or not imidacloprid induces the expression of the four P450 genes and if CncC is required for imidacloprid induction of these genes. Lepd-SL1 cells were treated with CncC dsRNA for three days followed by Imidacloprid (1µg/µl) exposure for 24 h. The mRNA levels of the four genes (CYP6BJ$^{a/b}$, CYP6BJ1v1, CYP9Z25 and CYP9Z29) were measured using the total RNA isolated from the treated and control cells. The expression of all four genes was induced by imidacloprid (Fig. 3.4). Interestingly, the knockdown of CncC reduced imidacloprid

induction of the same four genes (Fig. 3.5 A and B) suggesting that CncC is required for imidacloprid induction of P450 genes.

Potato leaf extract induction of P450 genes in Lepd-SL1 cells: Next we wanted to check whether potato plant allelochemicals also induce the expression of the same four genes. The cells were exposed to potato leaf extract (PLE) for 24 h and the mRNA levels of CYP6BJ$^{a/b}$, CYP6BJ1v1, CYP9Z25, and CYP9Z29 were determined using qRT-PCR. An increase in the mRNA levels of all four genes tested was detected in cells exposed to PLE when compared to their levels in control cells (Fig. 3.6 A). Further, CncC knockdown followed by PLE treatment resulted in a significant reduction in the expression in the same P450 genes that were induced by PLE (Fig. 3.6 B and C).

Identification and characterization of CYP gene promoters

As we found that the heterodimer partners CncC-Maf are required for the expression of four P450 genes, we wanted to identify the binding site for these proteins in the promoter regions of these P450 genes using the luciferase reporter assays. The preliminary experiments performed in Lepd-SL1 cells showed an inconsistent luciferase activity. To overcome this problem, we selected lepidopteran cell line (Sf9 developed from *Spodoptera frugiperda*) to perform the reporter assays because of its superior performance compared to Lepd-SL1 cells in respect to both transfection and repetition of results. We first searched CPB genome, to locate the promoter regions for four genes coding for CYP6BJ$^{a/b}$, CYP6BJ1v1, CYP9Z25, and CYP9Z29. We were able to identify promoters of genes coding for CYP9Z25 and CYP6BJ$^{a/b}$. The promoter regions of the other two genes were absent in the genome due to gaps in sequencing. Preliminary experiments showed higher luciferase activity for CYP9Z25 promoter when compared to

the activity of CYP6BJ$^{a/b}$ promoter. Therefore CYP9Z25 was chosen for further studies. The CYP925 promoter was cloned into pGL3 TATA vector containing a minimal promoter (Kalsi and Palli, 2015). The transfection of this construct by itself or along with CncC or Maf or CncC and Maf expression constructs into the Sf9 cells showed that this promoter is most active in the presence of both CncC and Maf proteins (Fig. 3.7). Because of the high sequence similarity between the CPB and *T. castaneum* CncC (88.06%) and Maf (80.03%) protein, the *T. castaneum* CncC and Maf expression constructs were used for co-transfection with the promoter. Promoter truncation assays were used to identify CncC-Maf binding sites in the CYP9Z25 promoter. The CYP9Z25 promoter was divided into five fragments, and each fragment was cloned into a pGL3 vector containing a minimal promoter (Fig. 3.8 A). Each construct containing truncation of the CYP9Z25 promoter was co-transfected with *T. castaneum* CncC and Maf expression constructs or pIEx-4 empty vector and the luciferase assay was performed. The luciferase activity was higher for the full-length promoter as well as for all the truncations in the presence of both CncC and Maf when compared to their activity in the absence of these proteins (Fig. 3.8 B). In the presence of CncC and Maf, the luciferase activity increased by 20, 22.3, 15.4, 59.4 and 2.3-fold for full-length promoter (Full), truncation -305 to -827 bp (T-1), truncation -13 to -325 bp (T-2), truncation -657 to -827 bp (T-3), truncation -674 to -13 bp (T-4) and truncation -444 to -827 bp (T-5) respectively. Further, comparing the activity of the full promoter and all the truncations in the presence of CncC and Maf, it is evident that truncations T-1 and T-4 showed significantly higher luciferase activity similar to the full-length promoter. Whereas, the promoter truncations T-2, T-3 and T-5 showed significantly lower luciferase activity.

These data suggest that T-1 and T-4 might have the binding sites for CncC and Maf. The online software ALGGEN-PROMO (Messeguer et al. 2002) was used to predict the CncC-Maf (Nrf2-MafK) binding site in the T-1 and T-4. The software predicted two putative binding sites (GCAGAAT and GTACTGA) that were common in both the T-1 and T-4 truncation. To confirm these putative sites for CncC-Maf binding, we introduced mutations in the binding site with the help of PCR site-directed mutagenesis.

Figure. 3.9 A shows the schematic of the mutant and wild-type constructs, Mutant-1 (M-1), Wild type (WT-1), Mutant-2 (M-2), Wild type-2 (WT-2), Mutant-3 (M-3) and Wild type-3 (WT-3). The construct WT-1 contains one binding site (i.e., GCAGAAT) that was mutated to GaAaAAT in the construct M-1. The construct WT-2 contains two binding sites (GCAGAAT and GTACTGA) including the binding site present in WT-1. In the construct M-2, the second binding site GTACTGA was mutated to aTACTaA, leaving the first binding site intact. The construct WT-3 contains only second binding site (GTACTGA) and that was mutated in construct M-3 (aTACTaA). All the constructs were co-transfected with CncC and Maf expression vectors into Sf9 cells and the luciferase activity between the mutated constructs and the corresponding wild-type constructs were compared. The reporter assay results showed that both the predicted binding sites are important for the CncC and Maf binding (Fig. 3.9 B). The luciferase activity of the reporter construct M-1 was reduced by 2.5-fold, as compared to its wild type WT-1. Interestingly, the luciferase activity for M-2 was same as WT-2 suggesting that the second binding site mediates CncC and Maf binding. The luciferase activity of the reporter construct M-3 was reduced by 2.5-fold, as compared to its wild type WT-3. These data on the mutations of the predicted biding sites confirm the function of two

CncC and Maf binding site in the promoter region of CYP9Z25 gene. The point mutations revealed that the second (C) and the fourth (G) nucleotide in the CncC and Maf-1 binding site are important. The nucleotides first (G) and sixth (G) seem to be important for the CncC and Maf-2 binding.

Discussion

The transcriptomic repertoire of CPB CYPome was analyzed in imidacloprid-resistant, and susceptible beetles and 21 differentially expressed P450 genes were identified (Zhu et al. 2016). These 21 P450 genes are overexpressed in the resistant strain as well as induced by the both the potato leaf extract and imidacloprid. These data suggest a potential role of these P450 genes in detoxification of both the potato allelochemicals and imidacloprid. Our current studies revealed that CPB develops defense against both potato plant allelochemicals and imidacloprid through four common P450 genes (CYP6BJ$^{a/b}$, CYP6BJ1v1, CYP9Z25, and CYP9Z29) that are regulated by the xenobiotic transcription factor 'CncC' and its heterodimer partner Maf. The luciferase reporter assay for the CYP9Z25 genes depicts the requirement of both the proteins, CncC and Maf (Fig. 3.7, 3.8 B and 3.9 B). The knockdown of CncC in the adult beetles lead to downregulation of four P450 genes in the midgut tissue (Fig. 3.2 A and 3.2 B). Similarly, the CncC knockdown in the cells blocked imidacloprid induction of the same four P450 genes (Fig. 3.5 A and B). Treating the CPB cells with PLE, induced the same P450 genes and the PLE induction of these genes was reduced after CncC knockdown (Fig. 3.6 A, B and C). Further, the knockdown of CncC as well as the four target P450 genes enhanced the susceptibility of resistant beetles to imidacloprid (Fig. 3.3). Higher levels of constitutive expression of CncC were detected in the resistant strain when compared to its

levels in the resistant strain (Fig. 3.1 A and B). Taken together, these data suggest that CncC regulates genes coding for P450s that play important roles in imidacloprid resistance.

Predominantly, insects gain the resistance to the natural and synthetic xenobiotics by evolving their detoxification system by increasing the metabolic capabilities or/and reducing the xenobiotic target site sensitivity. Hitherto, the cross-resistance mechanisms between phytotoxins and insecticides are attributed to the detoxification enzymes such as cytochrome P450s and GSTs (Després et al. 2007). In brown planthopper, *Nilaparvata lugens* overexpression of two P450 genes (i.e. CYP6ER1 and CYP6AY1) leads to imidacloprid resistance (Bao et al. 2016). The black swallowtail, *Papilio polyxene* is a specialist herbivore that detoxifies the furanocoumarins (phytotoxin) by overexpression of P450 genes in the midgut and fat body (Cohen et al. 1992). Després et al., 2007 suggested that insects that show phytochemicals adaptation and insecticide resistance usually adopt a single mechanism of resistance (e.g. metabolic resistance) rather than multiple resistance mechanisms. Similarly, the metabolic detoxification role of multiple P450s in our studies is supported by many other studies that have documented the involvement of more than one cytochrome P450 in insecticide resistance (Kalsi and Palli, 2015; Liu et al. 2011) or adaptation to plant toxins (Li et al. 2002; Schuler 1996).

Our data showed that resistant beetles exhibit constitutive expression of CncC as well as significantly higher expression of CncC in the midgut, fat body, and Malpighian tubules when compared to that in susceptible insects (Fig. 3.1 A and B). However, the expression of CncC was observed in all the tissues of both the resistant and susceptible strains. Similar constitutive overexpression of CncC was reported in the deltamethrin

resistant strain of *T. castaneum* (Kalsi and Palli, 2015). CncC protein is known to regulate the basal and the inducible expression of antioxidant and detoxification genes (Adachi et al. 2007). Our data revealed that the CncC is expressed in both susceptible and resistant strains. In the susceptible strain, the CncC protein may be important for maintenance of the homeostatic functions of an insect and under the normal conditions, and the expression levels are controlled by the oxidative state of the cell (Sykiotis and Bohmann 2010). However, during the xenobiotic attack of an insecticide, the Keap1-CncC detoxification pathway gets activated for instance in the deltamethrin resistant strain of *T. castaneum* (QTC279), DDT-resistant strains of *D. melanogaster* (91R and RDDTR) or oltipraz treated strain of *D. melanogaster* (Kalsi and Palli, 2015, Misra et al. 2013 , Sykiotis et al. 2008).

The midgut, fat body, and Malpighian tubules are the major organs of detoxification in insect with abundant expression of P450 genes (Feyereisen 1999; Scott and Lee 1993). Therefore, the increase in the expression of CncC in these tissues (midgut, fat body and Malpighian tubules) of resistant CPB beetles suggests that CncC facilitates detoxification of toxins (Fig. 3.1 B). In *D. melanogaster* the CncC expression was detected in the midgut and epidermis but not in the fat body (Sykiotis and Bohmann 2008). The integument has also been implicated in detoxification, because of its direct exposure to insecticides (Zhu et al. 2013a). Hence, the expression of CncC in the remaining organs including epidermis is supported by these studies.

The CncC knockdown in resistant CPB beetles followed by treatment with imidacloprid caused a significantly reduced survival but also a significant decrease in the expression of identified cytochrome P450s (Fig. 3.2 A and B). In another beetle system

(i.e. *T. castaneum*), the CncC knockdown leads to 100% mortality after deltamethrin treatment, and there was a significant reduction in the expression of multiple P450 genes in the whole body.

The Nrf2 in vertebrates is homologous to CncC in invertebrates (Sykiotis and Bohmann 2010) and the partnership between CncC (or Nrf2) and Maf have been documented to be indispensible for regulation of the xenobiotic detoxification genes in both vertebrate or invertebrate systems (Hirotsu et al. 2012b; Itoh et al. 1997; Kalsi and Palli, 2015; Nguyen et al. 2005). In mammals, the widely recognized core XRE sequence for binding of Nrf2-Maf complex is mostly degenerate and the Nrf2-Maf binding site is not well conserved among different species (Nioi et al. 2003). This has been true for the invertebrate system as well for instance the CncC-Maf binding site in *D. melanogaster* is 15 bp (i.e. TAGTCATGGTGATAG) (Misra et al. 2011) while in *T. castaneum*, it is 7 bp (i.e. GCAGTAC) (Kalsi and Palli, 2015). Some other studies (vertebrate system) emphasized that the Nrf2-Maf heterodimer requires an Activator Protein-1(AP-1) like element (TGAC) and a GC box (i.e. GCA sequence) on the 3' end of the XRE (Hirotsu et al. 2012; Zhang et al. 2003). On the contrary, in the invertebrate system such as *T. castaneum*, the GC box was found on the 5' end of the XRE (i.e. GCAGTAC) and apparently no AP-1 like element is required (Kalsi and Palli, 2015). In the current study, we characterized two different CncC and Maf binding sites (i.e. GCAGAAT and GTACTGA) in the promoter region of CYP9Z25 gene in CPB with the help of reporter and point mutation assays (Fig. 3.9 A and B). Kalsi and Palli, 2015 reported the presence of GC box on the 3' end of CncC and Maf binding site (GCAGAAT) located in the

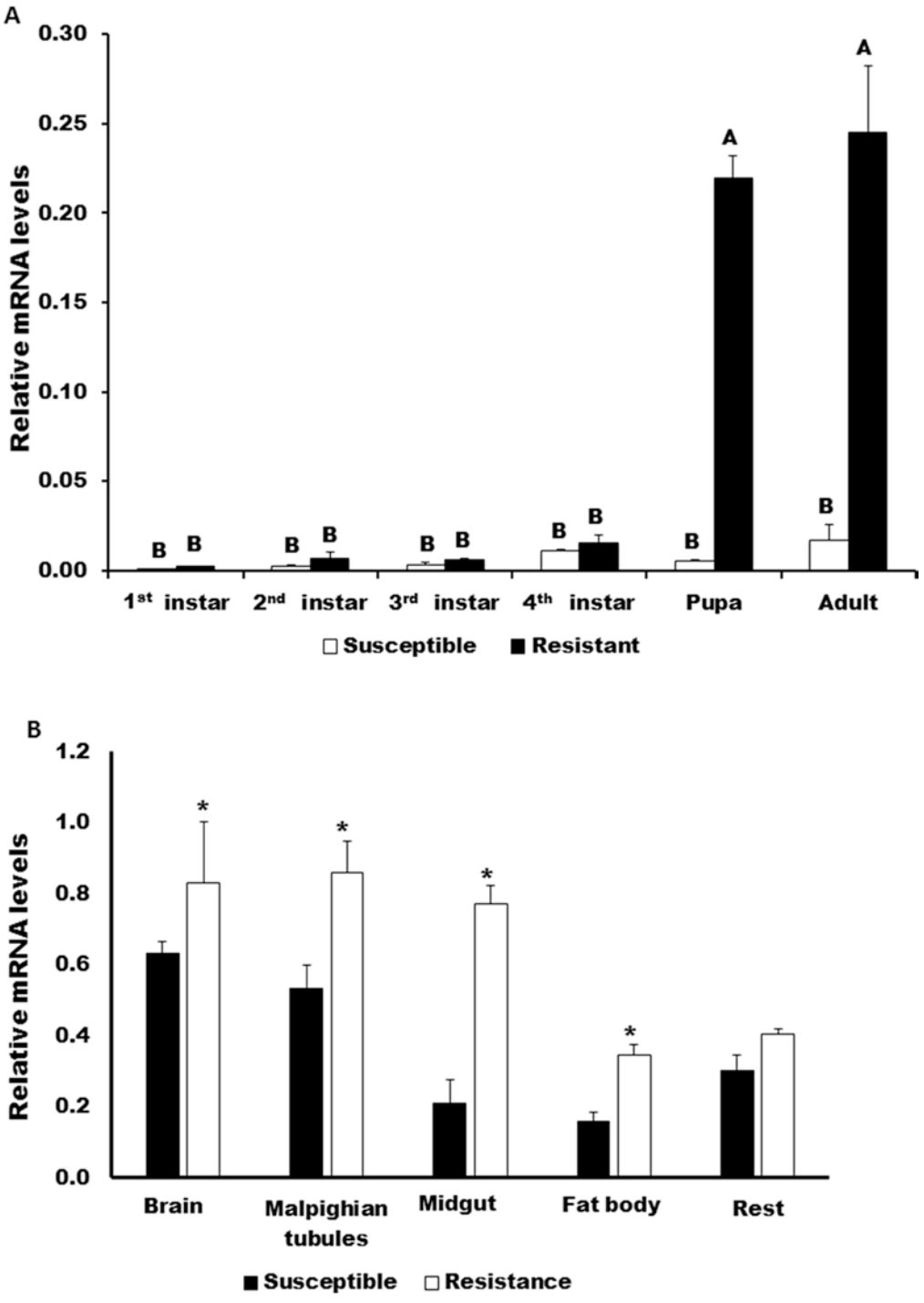
CYP9Z25 promoter. However, the second binding site of the CYP9Z25 promoter (i.e. GTACTGA) contains T instead of C at the 2nd nucleotide position.

In conclusion, from a cohort of highly expressed cytochrome P450 genes in the resistant CPB (Zhu et al. 2016), the current study identified four cytochrome P450s that are involved in the metabolism of both the potato plant allelochemicals and imidacloprid. We further identified the transcription factors (CncC and Maf) and their binding sites that regulate these cytochrome P450s. Further studies on the genome wide transcriptome profiling and functional genomics studies are underway to identify other detoxification genes (Phase 1, II and III) responsible for the metabolism of potato allelochemicals and imidacloprid, and regulated by xenobiotic transcription factor CncC. It remains to be seen, if and how the constitutive expression of CncC results in fitness cost to the beetle. But we hypothesize that utilizing the same set of cytochrome P450 genes regulated by the same transcription factors to metabolize both plant allelochemicals and insecticide may provide advantages to develop mechanisms for defense against new toxins encountered by the insects hence providing an evolutional advantage.

Table 3.1. List of primers used in experiments.

Construct name	Position	Forward Primer	Reverse Primers
CYP9Z25 FULL	-13 to -827	5'-cggggtaccTTGTGAATTTGAACGTCTGTTC-3'	5'-ccgctcgagCTGAAAAATGTTATTATACA-3'
CYP9Z25 T-1	-305 to -827	5'-cggggtaccTTGTGAATTTGAACGTCTGTTC-3'	5'-ccgctcgagATCACCACTCCCAAACTTTC -3
CYP9Z25 T-2	-13 to -325	5'-cggggtaccGAAAGTTTGGGAGTGGTGAT-3'	5'-ccgctcgagCTGAAAAATGTTATTATACA-3'
CYP9Z25 T-3	-675 to -827	5'-cggggtaccTTGTGAATTTGAACGTCTGTTC-3'	5'-ccgctcgag ATG TCC TCC CAC TCC ACC -3'
CYP9Z25 T-4	-674 to -13	5'-cggggtaccGGTGGAGTGGGAGGACAT-3'	5'-ccgctcgagCTGAAAAATGTTATTATACA-3'
CYP9Z25 T-5	-444 to -827	5'-cggggtaccCTGTGAAAGCGGTCCAA-3'	5'-ccgctcgagCTGAAAAATGTTATTATACA-3'
Wild type-1	-578 to -472	5'-cggggtaccGCAGAATGACATAGGTTGA-3'	5'- ccgctcgagCAATATATGAAGTTGATGA -3'
Mutant-1	-578 to -472	5'-cggggtaccGAAAAATGACATAGGTTGACTTCATA-3'	5'- ccgctcgagCAATATATGAAGTTGATGA -3'
Wild type-2	-578 to -465	5'-cggggtaccGCAGAATGACATAGGTTGA-3'	5'- ccgctcgagTCAGTACCAATATATGAAG -3'
Mutant-2	-578 to -465	5'-cggggtaccGCAGAATGACATAGGTTGA-3'	5'- ccgctcgagTTAGTATCAATATATGAAGTTGATG -3'
Wild type-3		5'-cggggtaccGACATAGGTTGACTTCATACCA -3'	5'- ccgctcgagTCAGTACCAATATATGAAG -3'
Mutant-3		5'-ccgctcgagTCAGTACCAATATATGAAGTTGATG -3'	5'- ccgctcgagTTAGTATCAATATATGAAGTTGATG -3'
Gene Name	Type	Forward Primer	Reverse Primers
qLd-CncC	qRT Primer	5'-GTTGGATGAAGCTTTGCAACTCG-3'	5'-GTACCGTCAACGTCCAAAGAGATG-3'
dsLd-CncC	dsRNA Primer	5'-CCGATTTCCAGGGATAAG-3'	5'-TGGTTTCCGTCTGCATCTCG-3'
dsLd-CYP9Z25	dsRNA Primer	5'-ATGCTTCAACTCACTTTGGGGGTG-3'	5'-AAAGACCTATGGTCGGTGAAGTGG-3'
dsLd-CYP9Z29	dsRNA Primer	5'-TTTCTTCGAGGCGCTTTAGAT-3'	5'-CTAAGAGTTCGGCCCTTGATT-3'
dsLd-CYP6BJ[a-b]	dsRNA Primer	5'-GTCTTTCGTTCCCATTGTTCC-3'	5'-GAAGGTTGGACAACCAGATTTAC-3'

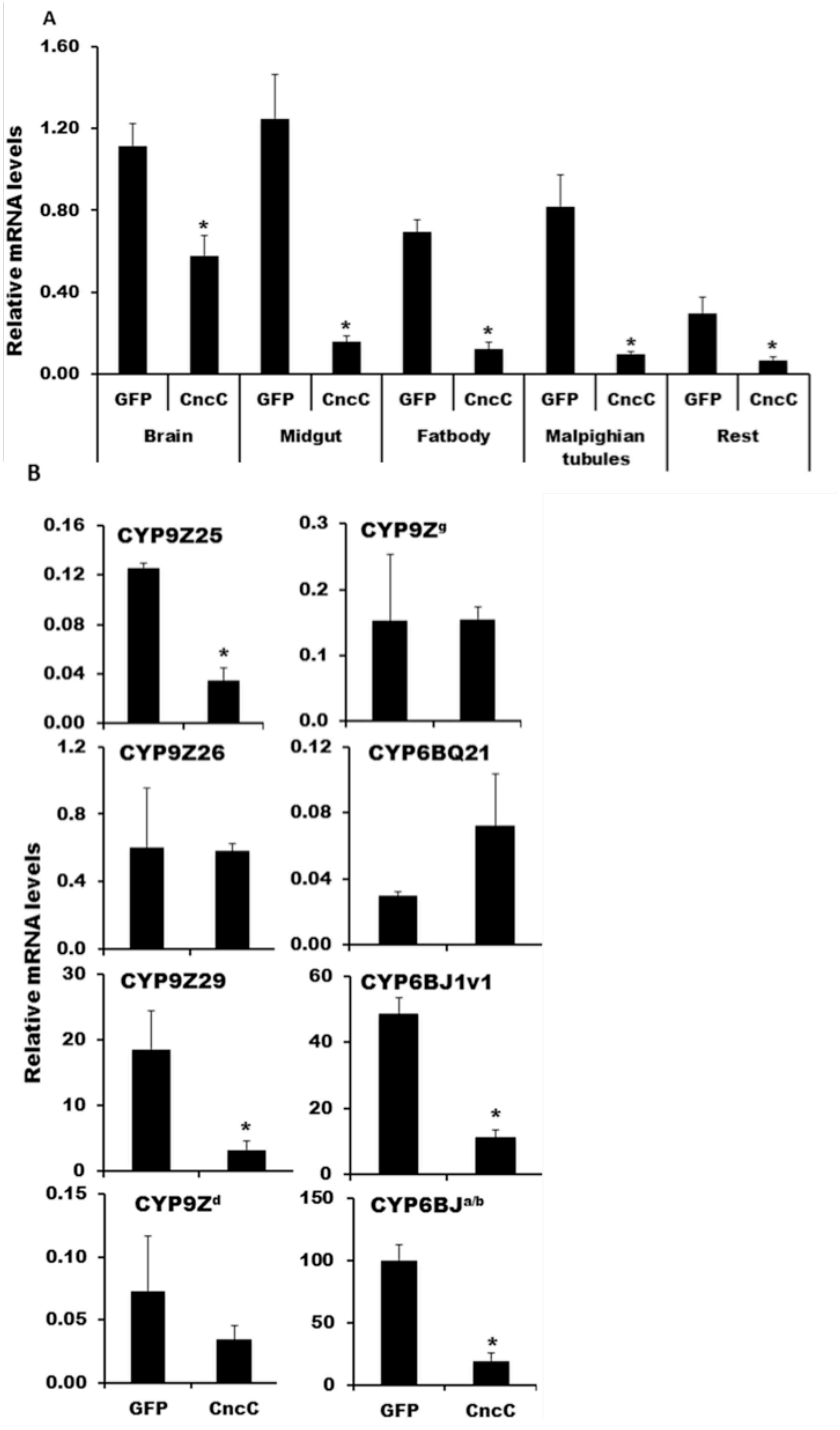
Figure 3.1.



Expression of CncC gene in imidacloprid-resistant and susceptible strains of CPB. Total RNA was extracted from staged insects (A) or tissues dissected from staged insects (B). Total RNA was extracted from three different beetles serving as three independent

biological replicates. Total RNA was isolated from four tissues (i.e. brain, Malpighian tubules, midgut, fat body, and the remaining tissues) dissected from three individual beetles serving as the three independent biological replicates. Three micrograms of total RNA was converted to cDNA and used in qRT-PCR to determine the mRNA levels for CncC. For normalization, the mRNA levels of ribosomal protein (RP4) was used as an internal control. Mean + S.E. (n = 3) are shown. The data were analyzed using Student *t*-Test. * denotes significant difference at $P < 0.05$.

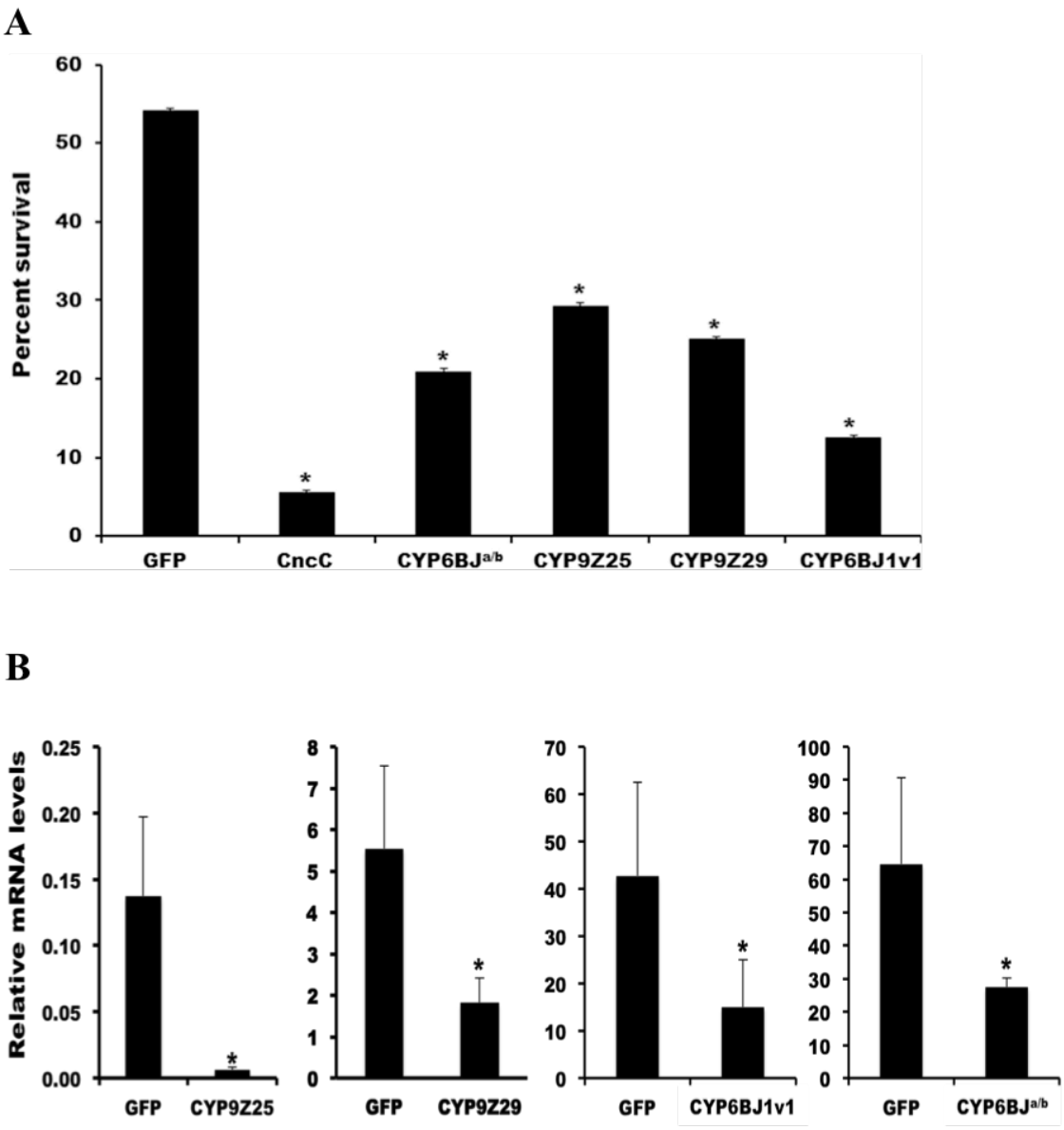
Figure 3.2.



A. Systemic knockdown of CncC in the adult CPB. Adult beetles were fed on CncC, and GFP dsRNA spread on fresh potato leaves for three days. Then the beetles were fed on

fresh potato leaves. On the sixth day, total RNA was isolated from the brain, midgut, fat body, Malphigian tubules, and the remaining tissues. Isolation of RNA and determination of CncC mRNA levels were performed as described in Figure 3.1. B. Expression levels of P450 genes in the midgut dissected from CncC knockdown beetles. For determination of P450 mRNA levels, the total RNA extraction and cDNA preparation from the midgut dissected from CncC knockdown beetles that were fed on CncC dsRNA were performed as described in Figure 3.1.

Figure 3.3.

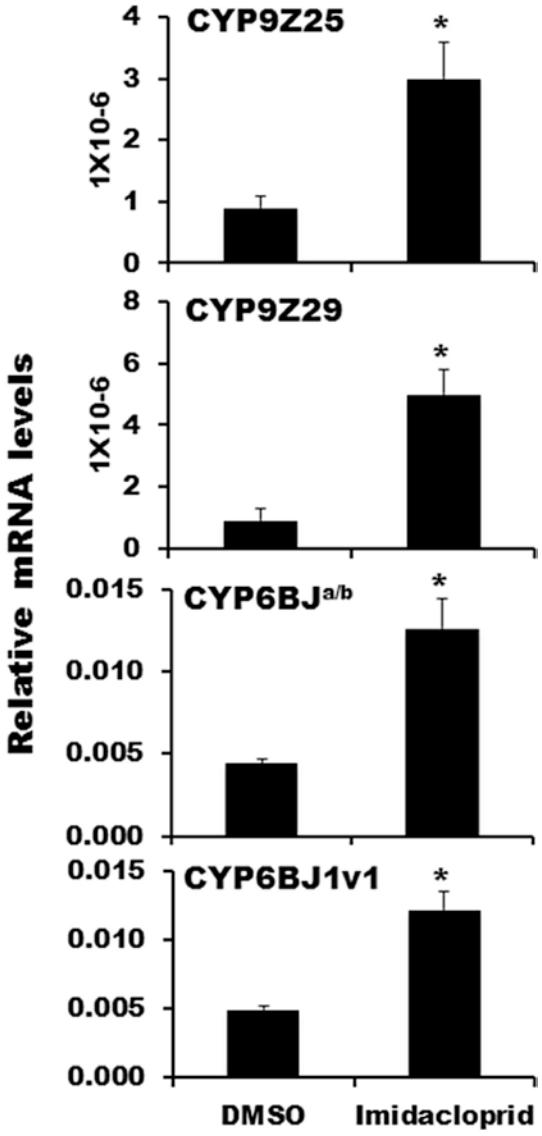


A. Knockdown in the expression of CncC, CYP6BJ[a], CYP9Z29, CYP9Z25 and CYP6B1v1 increases imidacloprid induced mortality in the resistant strain. The adults (1–2-weeks old) were fed on CncC, CYP6BJ[a], CYP9Z29, CYP9Z25 and CYP6B1v1 dsRNAs. Control beetles were fed on GFP dsRNA. On the sixth day after feeding dsRNA, the beetles were exposed to imidacloprid (LD_{50} = 0.5–1 μg/μl), and mortality

was recorded at 24 h after imidacloprid treatment. Mean + S.E. (n = 25–30) are shown. The data were analyzed using Student *t*-Test. *, denotes significant difference at $P < 0.05$.

B. Knockdown efficiency of cytochrome P450s.

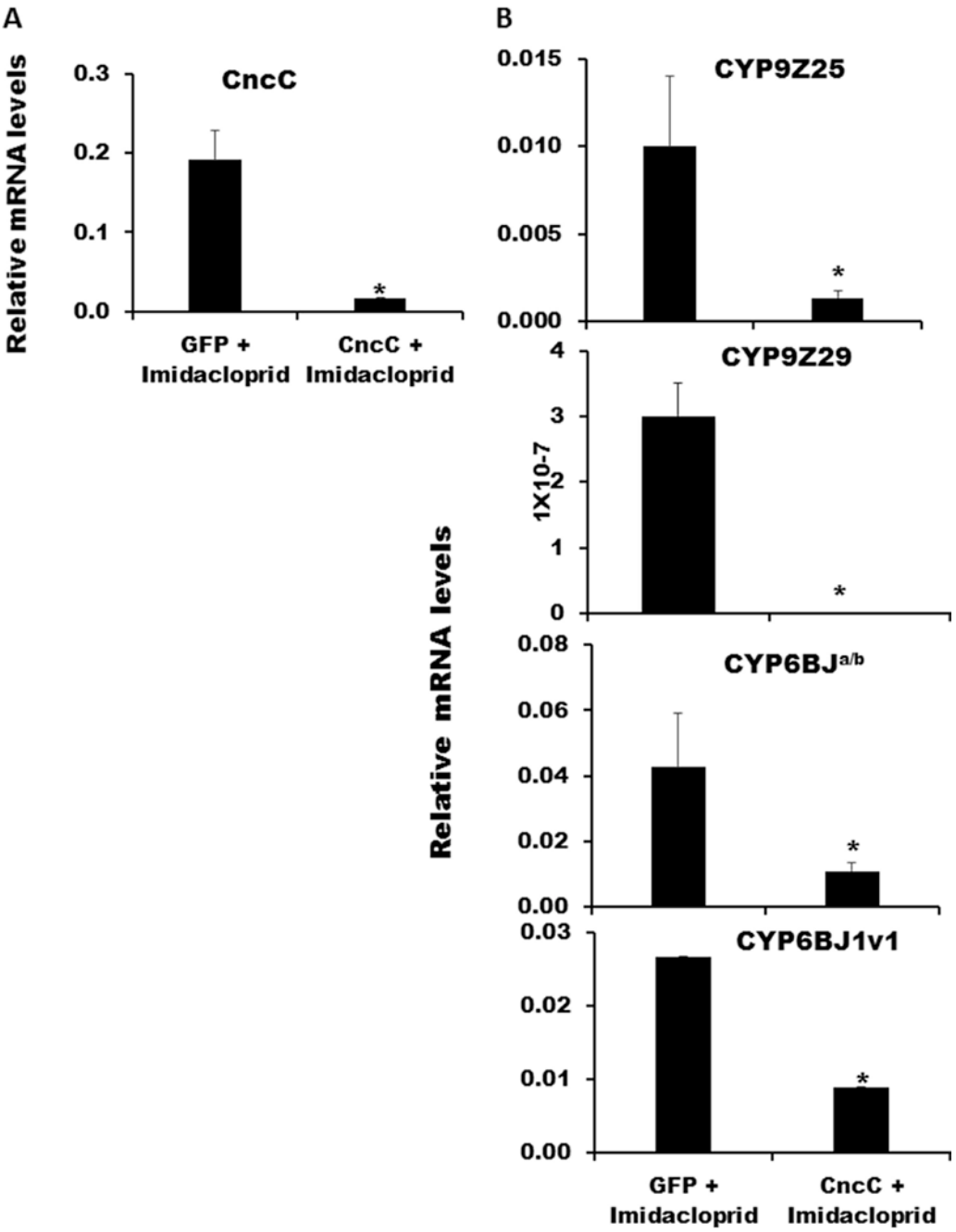
Figure 3.4.



Imidacloprid induction of P450 genes in Lepd-SL1 cells. The Lepd-SL1 cells seeded at a density of 1×10^6 cells/well were exposed to imidacloprid (1 μg/well) for 24 h. The control cells were treated with the same amount of DMSO. Three μg of total RNA isolated from the cells was used to prepare the cDNA to quantify the mRNA levels of P450 genes. RP4 and RP18 mRNA levels quantified at the same time in the same

samples were used for normalization. Means + S.E (n = 3) are shown. The data were analyzed using Student's t-test. * Significantly different at $P < 0.05$.

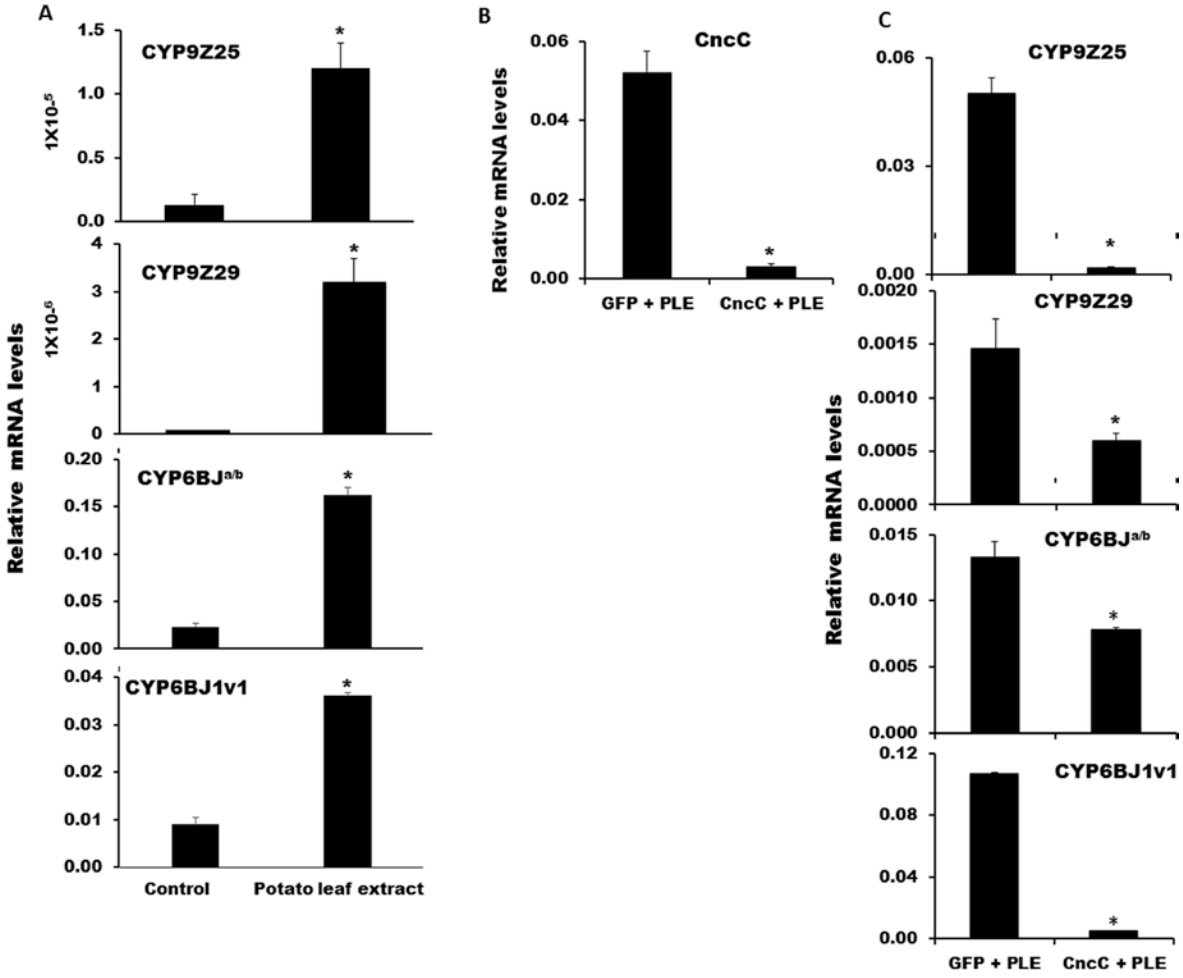
Figure 3.5.



A. CncC knockdown in the Lepd-SL1 cells. The Lepd-SL1 cells seeded at a density of 1×10^6 cells/well were treated with CncC or GFP dsRNA (6 µg/well) for 48 h followed by imidacloprid induction (1 µg/well) for 24 h. Total RNA isolation, determination of

mRNA levels and statistical analysis were performed as described in Figure 3.1. The expression levels of P450 genes in the CncC knockdown cells exposed to imidacloprid. The Lepd-SL1 cells seeded at a density of 1×10^6 cells/well were exposed to CncC dsRNA (6 μg/well) for 48 h followed by imidacloprid induction (1 μg/well) for 24 h. Total RNA was collected from the cells in each well, and each well was considered as one biological replicate. cDNA was prepared using three micrograms of total RNA to quantify the mRNA levels of each P450 gene. RP4 and RP18 mRNA levels quantified at the same time in the same samples were used for normalization. Means + S.E (n = 3) are shown. The data were analyzed using Student's t-test. * Significantly different at $P < 0.05$.

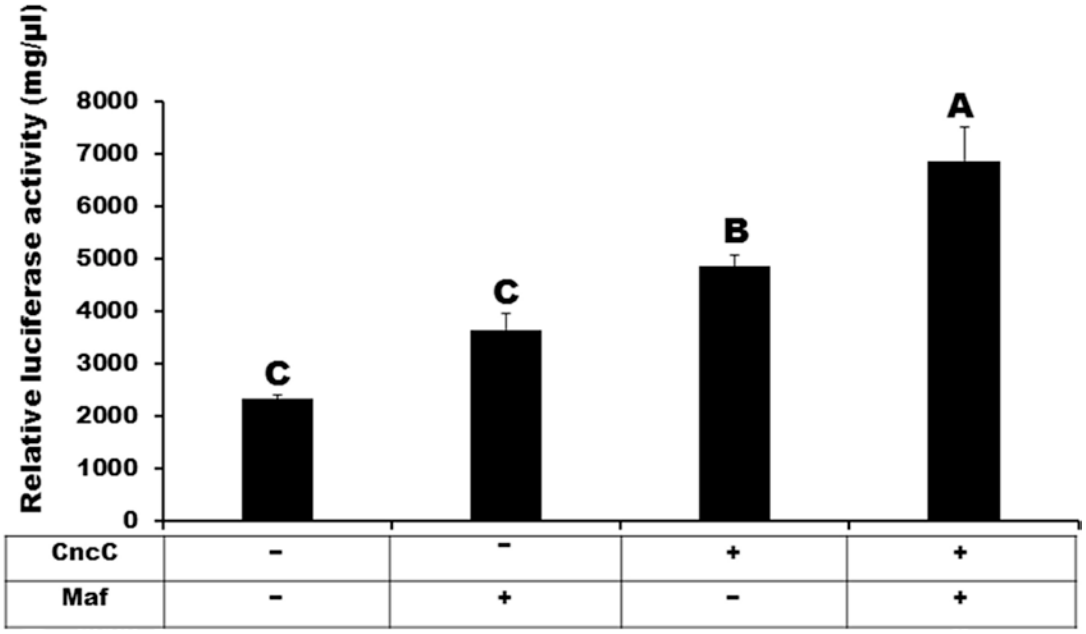
Figure 3.6.



A. Potato leaf extract induction of P450 genes in Lepd-SL1 cells. The Lepd-SL1 cells seeded at a density of 1×10^6 cells/well were treated with 100X diluted potato leaf extract for 24 h. Isolation of RNA and determination of P450 mRNA levels were performed as described in Fig. 3.1.B. CncC knockdown in the Lepd-SL1 cells. The Lepd-SL1 cells seeded at a density of 1×10^6 cells/well were treated with CncC or GFP dsRNA (6 μg/well) for 48 h followed by 100X diluted potato leaf extract for 24 h. Total RNA isolation, determination of mRNA levels and statistical analysis were performed as

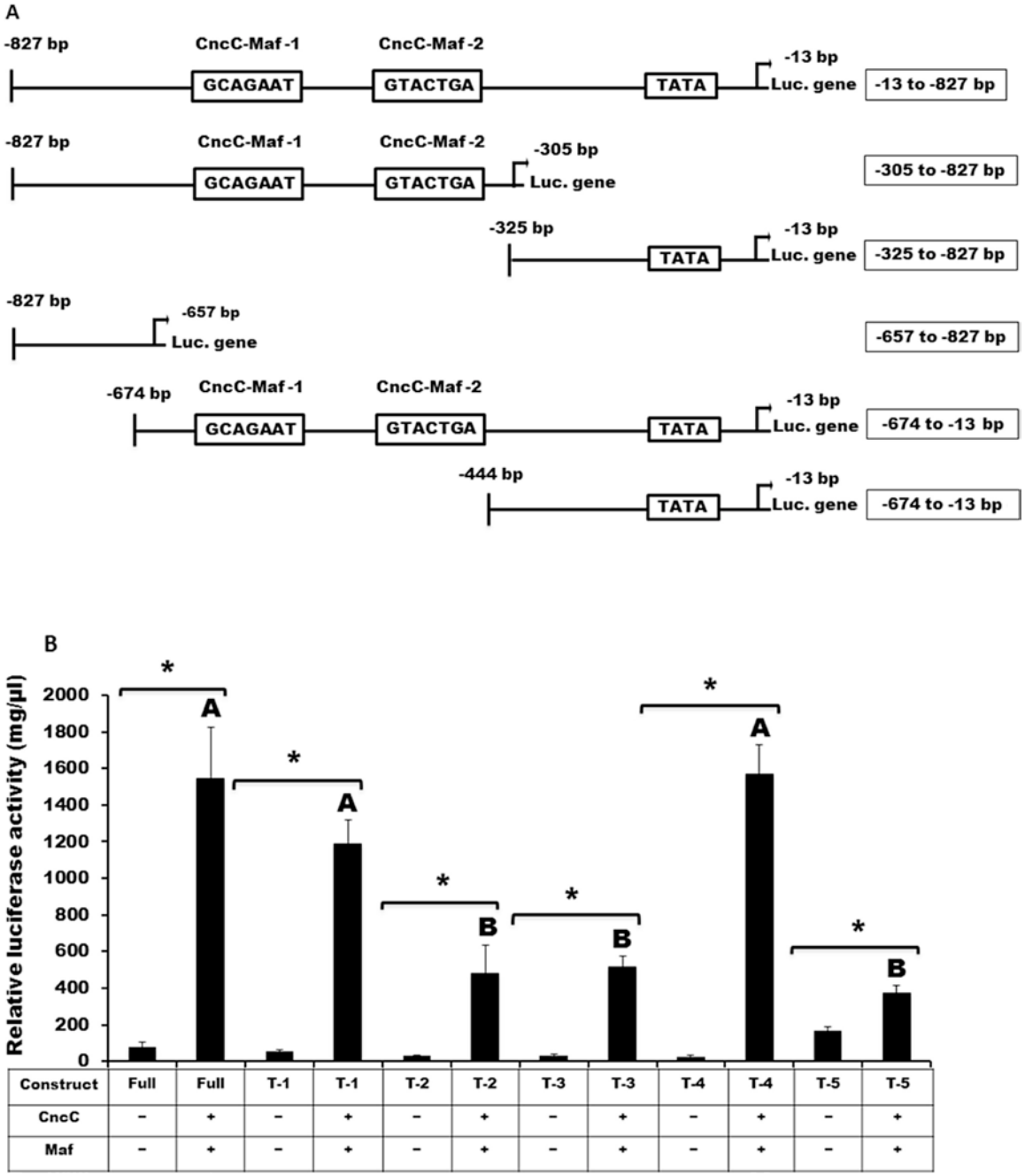
described in Figure 3.1. The expression levels of P450 genes in the CncC knockdown cells exposed to potato leaf extract. The Lepd-SL1 cells seeded at a density of 1×10^6 cells/well were exposed to CncC dsRNA (6 μg/well) for 48 h followed by 100X diluted potato leaf extract for 24 h. Total RNA was collected from the cells in each well, and each well was considered as one biological replicate. cDNA was prepared using three micrograms of total RNA to quantify the mRNA levels of each P450 gene. RP4 and RP18 mRNA levels quantified at the same time in the same samples were used for normalization. Means + S.E (n = 3) are shown. The data were analyzed using Student's t-test. * Significantly different at $P < 0.05$.

Figure 3.7.



The heterodimer partners CncC and Maf increase the luciferase activity of the CYP9Z25 promoter. The pGL3 CYP9Z25 promoter constructs by itself or along with CncC or Maf or both CncC and Maf expression constructs were transfected into Sf9 cells. The cells were harvested at 48 h after transfection, and the luciferase activity was quantified. Means + S.E (n = 3) are shown. The data were analyzed using Tukey's HSD. Letters A, B and C denote significant difference at $P < 0.05$.

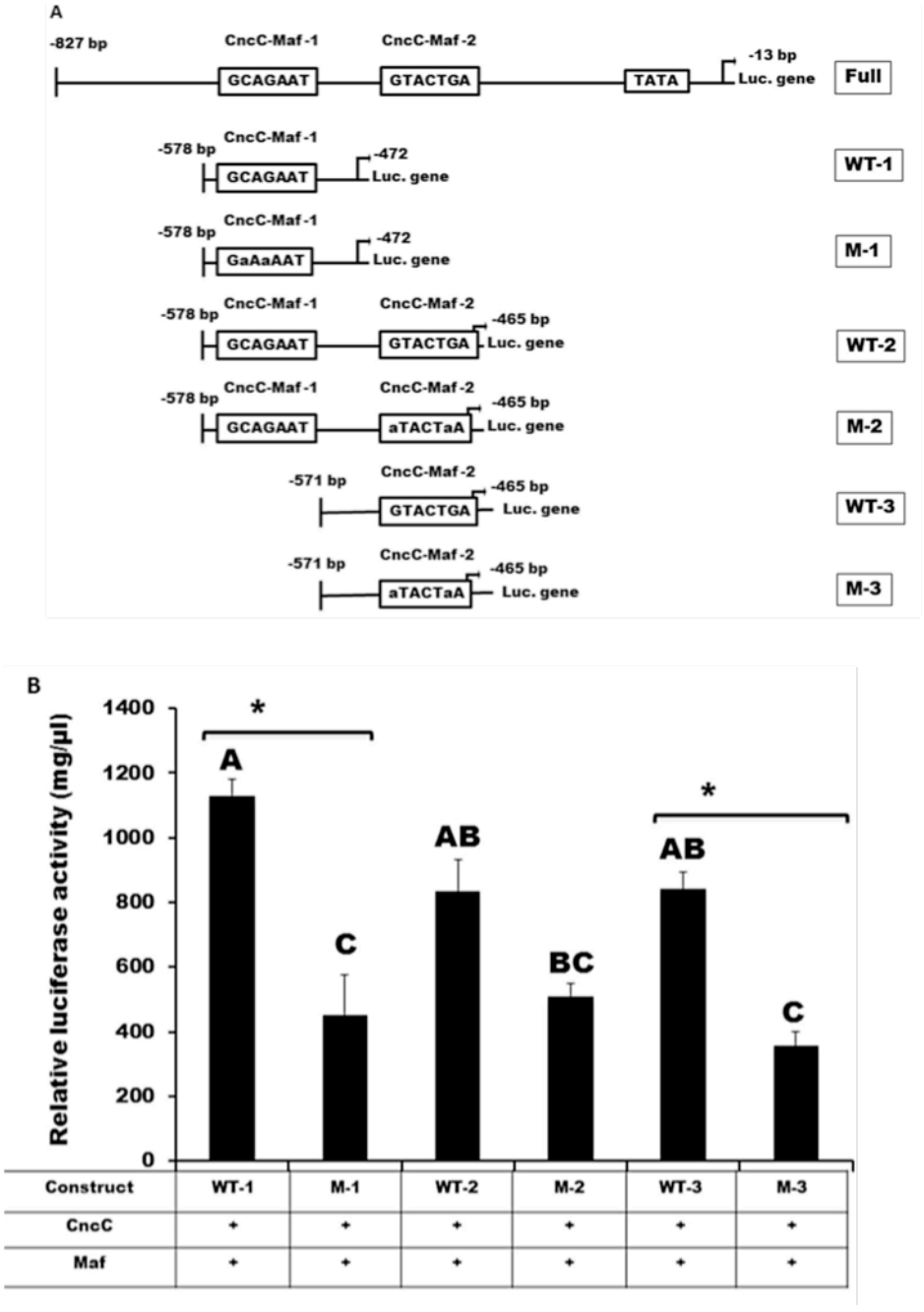
Figure 3.8.



Analysis of CYP9Z25 promoter truncations. To determine the luciferase activity of CYP9Z25 promoter truncation constructs in the presence or absence of CncC and Maf, the reporter and expression constructs were transiently transfected into the Sf9 cells. The

cells were harvested at 48 h after transfection and luciferase activity was quantified. Protein concentrations were determined using Bradford's assay and used to normalize the luciferase readings. Mean + SE (n = 3) are shown. The luciferase activity for the truncations co-transfected with both proteins (CncC and Maf) were analyzed using Tukey's HSD and letters A, and B denotes significant difference at $P < 0.05$. The full-length promoter and each truncation with both the proteins and no proteins were compared using Student's t-test. *, significantly different at $P < 0.05$.

Figure 3.9.



A. Schematic representation of PCR-directed mutagenesis constructs prepared. B. The relative contribution of nucleotides in the predicted Maf and CncC binding sites analyzed using point mutants. PCR-directed mutagenesis was performed to introduce mutations

into CncC and Maf binding sites −1 and 2 present in the CYP9Z25 promoter. The full-length promoter sequences containing two predicted CncC and Maf binding sites along with the wild-type (WT-1, WT-2 and WT-3) and mutant (M1, M2 and M3) promoter regions were cloned into pGL3 vector and evaluated in Sf9 cells as described in Figure 3.9. Mean + SE (n = 4) are shown. The luciferase activity for all the truncations co-transfected with both proteins (CncC and Maf) were analyzed using Tukey's HSD and letters A, B and C denote significant difference at $P < 0.05$. The wild type and their corresponding mutated constructs were compared using Student's t-test. *, significantly different at $P < 0.05$.

Chapter 4: CncC regulated insecticide resistance genes in *Tribolium castaneum*

Summary

In invertebrates, a heterodimer of xenobiotic transcription factors, cap n collar C isoform (CncC) and muscle aponeurosis fibromatosis (Maf) mediate cellular defense. In insects, these proteins regulate expression of genes involved in insecticide detoxification. In the current study, we performed sequencing of RNA isolated from *Tribolium castaneum* pyrethriod resistant strain (QTC279) beetles injected with CncC or green fluroscence protein (GFP, control) dsRNA. Differential expression analysis of RNA sequences identified 662 genes that showed a decrease and 91 genes that showed an increase in expression (at a p value $\leq$0.01 and log2 fold change of $\geq$1.5) in CncC knockdown insects when compared to their expression in control insects. We focused on the downregulated genes and selected a subset of 27 genes (21 with a predicted function in xenobiotic detoxification and six randomly picked) and verified their differential expression using qRT-PCR. RNAi and insecticide bioassays were employed to study the function of six of these genes coding for CYP4G7, CYP4G14, GST-1 and four ABC transporters, ABCA-UB, ABCA-A1 and ABCA-A1L and ABCA-9B involved in all three phases of insecticide detoxification. These data suggest that CncC is a major regulator of genes coding for proteins involved in detoxification of insecticides.

Keywords: Red flour beetle, RNA-seq, insecticide bioassay, P450s, GSTs, ABC transporters

Introduction

Tribolium castaneum (Herbst) is a notorious pest of stored products (Andrić et al., 2010). It serves as a great model organism because of availability of genome sequence as well functioning systemic and parental RNA interference (Bucher et al., 2002; Miller et al., 2012; Tomoyasu et al., 2008). This insect has been used in studies on development (Brown et al., 2009; Dreyer et al., 2010; Parthasarathy et al., 2008a; Parthasarathy et al., 2008b; Tomoyasu and Denell, 2004), immunity (Altincicek et al., 2013; Eggert et al., 2015; Roth et al., 2009; Zou et al., 2007), reproduction (Parthasarathy et al., 2010a; Parthasarathy et al., 2010b; Sheng et al., 2011) and insecticide resistance (Julio et al., 2017; Kalsi and Palli, 2015; Zhu et al., 2010). *Tribolium castaneum* has developed resistance to 33 active ingredients (Whalon et al., 2012). Not surprisingly, the *T. castaneum* genome sequence revealed an expansion of gene families belonging to the detoxification enzymes such as P450s (Richards et al., 2008).

Natural and synthetic xenobiotics are metabolized by xenobiotic detoxification system comprising of three different phases, each phase with a distinctive role in metabolism (Li et al., 2007; Liska, 1998). The phase I system largely consists of cytochrome P450 enzymes that catalyze the oxidation process to reduce the biological activities of many xenobiotics and drugs (Bernhardt, 2006; Le Goff et al., 2003). These enzymes provide an initial protection for insects against insecticides (Bernhardt, 2006; Daborn et al., 2007; Le Goff et al., 2003; Werck-Reichhart and Feyereisen, 2000). The phase II system consists of several different enzymes including glutathione-S-transferases (GSTs), carboxylesterases and UDP-glucouronosyl transferases (UGTs) that are involved in the conjugation of oxidized phase I byproducts into more hydrophilic products (Liska,

1998; Nho and Jeffery, 2001). The insect GSTs based on the cellular location are classified into two groups, cytosolic and microsomal. The cytosolic group is further subdivided into six classes (Delta, Epsilon, Omega, Sigma, Theta and Zeta) where Delta and Epsilon are the unique subclasses present only in insects (Ketterman et al., 2011; Ranson et al., 2002; Sheehan et al., 2001).

The phase III proteins, ATP-binding cassette transporter (ABC transporters) proteins belong to one of the largest genes families of transporters and are primarily active in the elimination of phase II generated hydrophilic products across the plasma membrane by hydrolyzing ATP molecule as an energy source (Dermauw and Van Leeuwen, 2014). The ABC transporters in the ABCB and ABCG gene family have the capacity to directly eliminate the toxins out of the cells without any enzymatic modification often referred as phase 0 (Doring and Petzinger, 2014; Sarkadi et al., 2006).

Xenobiotic transcription factors (XTFs) play important roles in the regulation of expression of genes coding for proteins involved in phase I, II and III xenobiotic metabolism system. One of the XTF superfamilies contains Cap n Collar (Cnc) proteins that are conserved across all organisms (mammals, metazoans, invertebrates) except in plants and fungi (Nakata et al., 2006; Sykiotis and Bohmann, 2010). The basic leucin zipper (bZIP) transcription factor known as CncC in invertebrate (*Drosophila melanogaster*, *T. castaneum* and *Leptinotarsa decemlineata*), the SKN-1 (skinhead family member 1) in metazoans (*Caenorhabditis elegans*) and Nrf2 (nuclear factor erythroid-2 related factor-2) in vertebrates (mouse and human) are homologous proteins that play important roles in oxidative stress resistance (An and Blackwell, 2003; Kalsi and Palli, 2015; Sykiotis and Bohmann, 2010). The CncC transcription factors are

characterized by the presence of a 43 amino acid Cnc domain at the N-terminus of the DNA binding domain; they also contain keap1 binding ETGF motif (Sykiotis and Bohmann, 2008). The activation of cellular defense system, the Keap1-Nrf2 pathway, by oxidative stress (reactive oxygen species, ROS) or electrophilic attack has been extensively studied (Ma, 2013; Sykiotis and Bohmann, 2008, 2010). Under normal conditions, cytoskeleton tethered Keap1 (Kelch-like ECH associated protein 1) protein inhibits the activity of CncC/Nrf2 passively by forming heterodimers and actively by the destruction of polyubiquitinated CncC protein through proteasome (Itoh et al., 1999; Kang et al., 2004). However, under xenobiotic attack the Keap1 protein act as a sensor releasing the CncC. The CncC then binds to its heterodimer partner Maf (muscle aponeurosis fibromatosis) and the complex binds to the antioxidant-response elements (ARE) present in the promoters of the target genes involved in detoxification. Many studies in vertebrates (mice and human) have reported Nrf-2 regulation of expression of genes coding for proteins involved in phase II and phase III detoxification. Studies in invertebrates (flies and beetles) to date also showed the involvement of CncC in the regulation of phase I (P450s) and phase II (GSTs) enzymes in response to the toxins/insecticide exposure (Kalsi and Palli, 2015; Misra et al., 2011; Sykiotis and Bohmann, 2008). For example, in deltamethrin resistance strain of *T. castaneum*, CncC and Maf regulate multiple cytochrome P450s (8 CYP6BQ cluster genes) (Kalsi and Palli, 2015). CncC and Maf also regulate four P450s (CYP9Z25, CYP9Z29, CYP6BJ$^{a/b}$, and CYP6BJIv1) in another important coleopteran insect, *Leptinotarsa decemlineata* (Kalsi and Palli, 2017). These studies showed the role of CncC and Maf in the regulation of genes coding for phase I detoxification enzymes (P450s). We still do not know if CncC

and Maf regulate expression of phase II and phase III genes. Therefore, the main goal of the current study is to identify genes that are regulated by transcription factor CncC and verify their function in insecticide resistance. The RNA-seq data identified 622 genes that were downregulated (>1.5 fold, $P \leq 0.01$) in CncC knockdown beetles. These include genes coding for P450s, GST and ABC transporters. The differential expression pattern derived from RNA-seq results for 25 out of 27 select genes was confirmed by qRT PCR. RNAi and insecticide bioassays were employed to determine the function of selected genes (i.e. P450s, GSTs, ABC transporters) in insecticide resistance. These studies confirmed the contribution of five CncC regulated genes (*CYP4G14, GST-1, ABCA-UB, ABCA-1A, ABCA-1AL*) to insecticide resistance.

Materials and Methods

Insects

The resistant strain of *T. castaneum*, QTC279 was reared on organic wheat flour containing 10% yeast. The beetles were maintained in an incubator in the dark at 32 °C and 55 ± 2% relative humidity.

Double stranded RNA preparation, gene knockdown, total RNA extraction, and qRT-PCR

Double stranded RNA preparation, gene knockdown, total RNA extraction, and qRT-PCR were performed as described previously (Kalsi and Palli, 2015). Briefly, the dsRNA was synthesized using the *T. castaneum* cDNA template and MEGAscript RNAI kit (Ambion™). For dsRNA knockdown, the injection needles were prepared with the capillary tubes (Drummond Scientific Co.) pulled by micropipette puller (Sutter

Instrument Co., USA). Beetles of *T. castaneum* were injected with 100 nl (5-7 µg/µl) dsRNA. Total RNA was extracted from individual beetle using the TRI reagent (Molecular Research Center Inc., Cincinnati, OH). Two micrograms of DNAse I (TURBO ™ DNAse, Ambion ™) treated total RNA was used for making the cDNA using M-MLV reverse transcriptase. The cDNA was used as the template to quantify the relative mRNA levels using qRT-PCR. RP49 (ribosomal protein 49) was used as an internal control for normalization. The primers used in qRT-PCR and dsRNA preparation have been published previously (Kalsi and Palli, 2015; Zhu et al., 2013; Zhu et al., 2010) or shown in Table 4.1.

RNA Sequencing Library preparation and Illumina sequencing

Three micrograms of total RNA was used for preparing RNA sequencing (RNA-seq) libraries using the total RNA extracted from three dsGFP and dsCncC injected beetles. The RNA-seq library preparation methods used were previously described (Hunt, 2015). In brief, the total RNA enriched for Poly (A) was separated using oligo DT beads (Ingelman-Sundberg et al., 2000) followed by RNA fragmentation. Fragmented RNA was used for cDNA preparation in a two-step method using SMARTScribe reverse transcriptase enzyme (Clontech, Laboratories, Inc.) and reverse transcriptase primers (Integrated DNA Technologies) containing a unique 3-base pair barcode, a sequencing adaptor for Illumina platform followed by a random hexamer on the 3' end (Table. 4.1). The prepared cDNA was used in a strand-switching step with strand-switching primer, SMART 7.5 (SMART technology, Clontech, Laboratories, Inc.). The cDNA size (300-600 bp) selection was performed using the HighPrep™ PCR beads (MAGBIO). The size selected cDNA was used as a template for PCR amplification with Phire Hot Start II Taq

DNA polymerase (Thermo Scientific) followed by purification using post PCR clean up system (HighPrep™ PCR, MAGBIO). The quality and quantity assessment of libraries were done using Bioanalyzer with the Agilent high-sensitivity DNA chips (Agilent Technologies, USA). The NanoDrop 2000 spectrophotometer (Thermo Scientific Inc., Delaware, USA) was used to determine the concentration of samples. The libraries were pooled and sent for sequencing at The Sequencing and Genomics Technologies Center of Duke University. The libraries were sequenced using the Illumina HiSeq4000.

Quality control and RNA-seq data analysis

Quality control including demultiplexing, trimming, adaptor removal was done using the default parameters in CLC Genomics Workbench (Version 9.5.1, Qiagen Bioinformatics, USA). The reads were mapped to the reference genome, the *Tribolium castaneum* genome using CLC Genomic Workbench. The default settings used for mapping include mismatch cost =2, insertion cost =3, deletion Cost =3, length fraction = 0.8, similarity fraction = 0.8. The read counts were Log10 transformed followed by normalization of the transformed data using the scaling method. The empirical analysis of differential gene expression (EDGE) was performed using the parameters including estimated total count filter cutoff 5, estimate tagwise dispersion, Bonferroni correction and false discovery rate (FDR) corrected *P* values. To identify the genes that are significantly downregulated by CncC knockdown, a *P*-value of ≤ 0.01 and ≥ 1.5-fold change were used as filters.

Functional Annotation

The Blast2Go Pro software was used for functional annotation of the transcripts by comparing the sequence with nr_alias_arthropod database with blast expectation value

(e-value) 1.0E-6. The annotated transcripts were assigned with GOSlim (Gene Ontology multi level) terms under the categories such as biological processes, molecular functions, and cellular components.

Tefluthrin bioassay

Tefluthrin was used in bioassays. Tefluthrin is a pyrethroid insecticide that acts by modulating sodium channels (Wu et al., 2009). For the insecticide bioassay, 50-60 beetles were injected with 100 nl dsRNA (5-7 µg/µl). Beetles were fed on organic wheat flour containing 10% yeast and were placed in an incubator in the dark at 32°C and 55 ± 2% relative humidity for five days. Beetles were checked daily for injection or dsRNA specific mortality. On the sixth day after injection, beetles (40 per treatment, 5x8 replicates) were exposed to the filter paper surface treated with tefluthrin prepared in acetone (LD50, 0.625 µg/µl) in a 24 well plate. Insecticide induced beetle mortality was scored at 24 h after exposure to the insecticide.

Statistical Analysis

The student's t-test and one-way ANOVA was used to analyze the statistically significant difference between the control and knockdown samples for the value of $P < 0.05$ (JMP 11.0 software, SAS, Cary, NC). The heatmaps were generated using the CLC Genomics Workbench.

Results

Transcriptome profiling and quality control

The total RNAs isolated from QTC279 strain beetles injected with dsCncC or dsGFP (control) were converted to cDNAs and used in qRT-PCR to determine the mRNA levels of CncC,

CYP6BQ2, CYP6BQ6, CYP6BQ7 and CYP6BQ9. The qRT-PCR data showed that CncC gene was knockdown by more than 90% and mRNA levels of all four CYP genes decreased by more than 90% in CncC knockdown beetles when compared to their expression in control beetles injected with GFP dsRNA (Fig. 4.1). These data confirmed results reported in our recent paper (Kalsi and Palli, 2015) and showed that CncC regulates these P450 genes. To identify other genes regulated by CncC, the RNA isolated from beetles injected with CncC or GFP dsRNA was used to make libraries and the libraries were sequenced. The dataset yielded a total of 114,872,874 reads and 86,528,263 (75.3%) of those reads were mapped to *T. castaneum* reference genome. Out of the 14,503 genes previously in the *T. castaneum* genome, 14,483 were included in the six libraries sequenced (Table 4.2).

The principal component analysis (PCA) plot of sequences obtained from six libraries prepared using RNA isolated from dsGFP and dsCncC injected insects showed grouping of the sequences from three replicates for the treatment and the control (Fig. 4.2). These data suggest similarity of sequences among replicates for treatment and control differences between the treatment and control.

Differential gene expression analysis

The differential gene expression patterns among six replicates (three dsCncC and three dsGFP treated) constructed as a heat map confirmed PCA analysis results and revealed similarity in the expression levels of multiple genes among three replicates from the treatment (dsCncC) and control (dsGFP) as well as the differences between the treatment and control (Fig. 4.3). We used ≥1.5-fold change with an FDR corrected value of $P \leq 0.01$ as a filter to select genes differentially expressed between dsCncC treatment and control. Based on this criterion, 91 genes showed an increase and 622 genes showed a decrease in their expression in dsCncC injected beetles when compared to their

expression in dsGFP injected control beetles (Fig. 4.4). The heat maps of 91 upregulated and 622 downregulated genes are shown in Figures 4.5 and 4.6 respectively. Since the goal of these studies is to identify CncC regulated genes that contribute to insecticide resistance, we focused our further studies on 622 genes that showed a decrease in their expression in CncC knockdown beetles.

Annotation of genes that require CncC for their expression

The sequences of 622 genes identified as those that require CncC for their expression were analyzed for gene ontology using Blastx searches in NCBI non-redundant protein database using Bast2GO Program. Out of the 622 genes, 575 (92 %) showed positive Blast hits with an e value > 1 X 10^{-6}. Enzymes with oxidoreductase (7 genes), transferases (10 genes), hydrolases (24 genes) are among the major enzyme groups coded by 622 genes that require CncC for their expression (Fig. 4.7). The BGI WEGO plot showed enrichment of GO terms for the molecular function group, oxidoreductase as well as for locomotor and reproduction in the biological functional groups for the proteins coded by genes that showed a decrease in expression in CncC knockdown beetles when compared to their expression in control beetles (Fig. 4.8). Some of the genes that showed a decrease in their expression in CncC knockdown beetles code for phase I (P450), phase II (i.e. GSTs, Esterase-B1 and two UGTs) and phase III detoxification proteins (ABC transporters, ABCA-UB, ABCA-1A, ABCA-1AL and ABCA-9B (Fig. 4.8).

Validation of CncC regulated genes using qRT-PCR

To verify the gene expression patterns predicted by RNA-seq data, we performed qRT-PCR for 27 genes selected from 622 CncC regulated genes. Twenty-one of the 27 genes were selected based on their potential role in insecticide detoxification as revealed by gene ontology data (Table 4.3). Six genes (Tektin-1, 26S proteasome non-ATPase regulatory subunit, Zinc finger-91, Odorant binding protein-15, Heat shock protein-70 and COP-9 singlasome) were picked randomly. Twenty-five out of 27 genes tested by qRT-PCR showed a decrease in their expression in dsCncC-injected beetles when compared to their expression in control beetles injected with dsGFP (Fig. 4.9, Fig. 4.10). Two P450 genes (CYP4BN1, CYP4C1) which showed a decrease in expression in dsCncC injected beetles when compared to their expression in control beetles injected with dsGFP in RNA-seq data did not show significant differences in their mRNA levels between dsCncC knockdown and control by qRT-PCR analysis (Fig. 4.9, Fig. 4.10). Despite some difference in the fold changes in the mRNA levels of 25 genes between treatment and control determined by RNA-seq and qRT-PCR methods, the gene expression patterns (i.e. decrease in CncC knockdown beetles) of 25 out of 27 genes predicted by RNA-seq data were confirmed by qRT-PCR suggesting that most of the gene expression pattern predictions from the RNA-seq data are reliable.

Functional validation of select detoxification genes involved in insecticide resistance

Six candidate genes coding for proteins belonging to different detoxification phases were selected to determine their function in insecticide resistance. QTC279 beetles were injected with dsRNA targeting GFP (control), CYP4G7, CYP4G14, GST-1, ABCA-UB, ABCA-1A and ABCA-1AL. Six days after injection of dsRNA, knockdown efficiency

and tefluthrin toxicity were determined. As shown in Figure 4.11, injection of dsRNA targeting each of the six select detoxification genes caused a significant reduction in their mRNA levels. Knockdown of all the genes tested, except CYP4G7, showed significantly lower survival rate as compared to the control beetles treated with an LD50 dose of tefluthrin (Fig. 4.12). These data suggest that CYP4G7, CYP4G14, GST-1, ABCA-UB, ABCA-1A and ABCA-1AL contribute to pyrethroid resistance in *T. castaneum*.

Discussion

The identification of CncC regulated genes that code for proteins involved in all three phases of insecticide detoxification resulting in resistance development against insecticides is an important finding. We sequenced RNA isolated from pyrethroid resistant QTC279 beetles injected with dsCncC or dsGFP (as a control). Differential expression analysis of RNA sequences from treatment and control identified 622 genes that sowed a decrease in their expression in CncC knockdown beetles. The decrease in the expression of 25 out of 27 genes (selected from the 622 genes) was confirmed by qRT-PCR adding credibility to the predictions based on RNA-seq data. We then employed RNAi and insecticide bioassays to show the contribution of six select CncC regulated genes to insecticide resistance. These six genes (CYP4G7, CYP4G14, GST-1, ABCA-UB, ABCA-1A and ABCA-1AL) represent proteins that are involved in all three phases of insecticide detoxification.

The phase I enzyme, the cytochrome P450 mediated resistance through upregulation of their expression in resistance strains or induction by insecticide itself is the most prevalent detoxification mechanism observed in insecticide resistant insects

(Scott, 1999). The *T. castaneum* genome contains a total 143 P450 genes (10 pseudogenes and 133 functional genes) (Zhu et al., 2013). The RNA-seq data identified 16 P450s belonging to CYP3, CYP4, CYP6 and CYP9 gene families that are regulated by CncC (Table 2). Further, the knockdown of CYP4G14 followed by insecticide bioassay confirmed its role in resistance. CYP4G7 knockdown also reduced the survival of beetles after insecticide treatment. However, the CYP4G7 knockdown beetles survival is not significantly different from the control mortality. Four main families of P450s genes, CYP4, CYP6, CYP9, and CYP12 have been implicated in conferring resistance to a wide range of insecticides and are known to be expressed in insect detoxification organs including midgut, Malpighian tubules and fat body (Feyereisen, 1999; Li et al., 2007; Liang et al., 2015). In *T. castaneum* the genes coding for CYP345A1, and CYP4G7 are induced by pyrethroid insecticide (cypermethrin, permethrin) (Liang et al., 2015). In phosphine resistant strain of *T. castaneum,* an overexpression of CYP4Q4 gene in the midgut has been reported (Yujie Lu (2015). Many P450 genes including CYP6A (CYP6A1, CYP6A2), CYP9A (CYP9A1 and CYP9A2) have been shown to be involved in xenobiotic detoxification in insects (Feyereisen, 2012). In the CYP6 family, the CYP6BQ genes (CYP6BQ2, CYP6BQ7, CYP6BQ9, CYP6BQ11 and CYP6BQ12) are regulated by both CncC-Maf in a pyrethroid resistant strain of *T. castaneum* (QTC279 strain). The same study also identified a CncC-Maf binding site in the promoter region of these CYP6BQ genes (Kalsi and Palli, 2015). These genes are also expressed at higher levels in the QTC279 strain as compared to their levels in susceptible strain (Zhu et al., 2013).

The CYP4 family is considered to be the most ancient and abundant among CYP families (Bradfield et al., 1991). In *T. castaneum,* the CYP4 family consists of 28 genes constituting 19.6 % of total P450 genes identified in the genome. The TcCYP4 family contains three subfamilies including CY4G, CYP4Q and CYP4B where the CYP4G family is the smallest with only two members (i.e. CYP4G7 and CYP4G14) (Zhu et al., 2013). In insects, the CYP4 P450s perform a wide range of functions including (i) insecticide resistance as reported in *Culex pipiens* (Shen et al., 2003) *, Blatella germanica* (Pridgeon et al., 2003), *Diabrotica vergifera vergifera* (Scharf et al., 2001), *Chironomous tentans* (Londoño et al., 2004) and *Diaphorina citri* (Tiwari et al., 2011), (ii) detoxification of plant toxins such as monoterpenes in *Dendroctonus armandi* (Dai et al., 2014), (iii) caste differentiation in *Reticulitermis flavipes* (Zhou et al., 2006) , (iv) diapause in *Antheraea yamami* (Yang et al., 2008), (v) odorant/pheromone metabolism in *Mamestra brassicae* (Maïbèche-Coisne et al., 2005) and *Phylloppertha diversa* (Maïbèche-Coisne et al., 2004) (vi) hydrocarbon synthesis in different insects including *Drosophila melanogaster* (CYP4G1), *Musca domestica* (CYP4G2), and *Acyrthosiphon pisum* (CYP4G51) (Chen et al., 2016; Qiu et al., 2012). Interestingly, our RNA seq data showed a decrease in the expression of CYP4G7 and CYP4G14 genes in CncC knockdown beetles and qRT-PCR confirmed these data. RNAi and insecticide bioassays showed that both these P450s could contribute to pyrethroid resistance in QTC279 beetles. How do these P450s contribute to resistance in QTC270 beetles? Since the CYP4G subfamily genes have been reported to be involved cuticular hydrocarbon synthesis, it is possible that the *T. castaneum* CYP4G7 and CYP4G14 genes might play a role in insecticide penetration resistance in this beetle. Further studies are required to

compare the cuticles in the susceptible and resistance strains as well as in CncC, CYP4G7 and CYP4G14 knockdown beetles. The previous studies in *Drosophila melanogaster* have shown CncC regulation of P450s are involved in insecticide resistance (Misra et al., 2011; Sykiotis and Bohmann, 2008). We also documented CncC regulation of P450 genes involved in insecticide resistance in *T. castaneum* and *L. decemlineata* (Kalsi and Palli, 2015; Kalsi and Palli, 2017).

The phase II enzyme, GST contributes to resistantce to different classes of insecticides including organophosphates, chlorinated hydrocabons and pyrethroids (Clark et al., 1986; Enayati et al., 2005; Hemingway et al., 1991; Tang and Tu, 1994; Vontas et al., 2001; Yamamoto et al., 2009), The RNA-seq data predicted four genes coding for phase II enzymes that are downregulated in CncC knockdown beetles. Among them, the expression GST-1 is significantly reduced in the CncC knockdown beetles. Also, GST-1 knockdown in the resistant beetles increased their mortality after insecticide treatment. Similarly, in *D. melanogaster*, CncC regulates GSTD1 and GSTE1 expression as the expression of these genes is significantly reduced in CncC knockdown or mutant flies (Deng and Kerppola, 2013; Sykiotis and Bohmann, 2008).

The second major finding of this chapter is the discovery that CncC transcription factor regulates expression of ABC transporters, the phase III detoxification proteins. The function of the phase III proteins, ABC transporters, in insecticide/acaricides resistance has been reported for 27 different insecticides involving three ABC families (i.e. ABCBs, ABCCs and ABCGs) mainly based on the observation of their upregulation in resistant strains (Buss and Callaghan, 2008; Dermauw and Van Leeuwen, 2014; Epis et al., 2014). In the current study, we identified four ABC transporters genes (ABCA-UB, ABCA-1A,

ABCA-1AL andABCA-9B) that showed a significant decrease in their expression in CncC knockdown beetles suggesting that these ABC transporters are regulated by the transcription factor, CncC. The RNAi and insecticide bioassays confirmed the function of ABCA-UB, ABCA-1A, and ABCA-1AL in pyrethroid resistance. To date, no CncC regulated ABC transporter-mediated insecticide resistance has been reported in invertebrates. Further, the ABCA gene family function in insecticide resistance has not also been reported previously. However, the role of Nrf-2 in the induction of ABC transporters from ABCC and ABCG family has been reported in human liver cells (HepG2) in response to oxidative stress (Adachi et al., 2007). In another study performed in the vertebrate system (mice, *in vivo* or *in vitro*), the oxidative stress caused by the chemical sulforaphane led to Nrf-2 activation resulting in upregulation of members of three different ABC transporter families (ABCB1, ABCC2, and ABCG2) (Wang et al., 2014). Several other RNA sequencing experiments in vertebrates have documented oxidative stress/xenobiotics-Nrf-2 induction of ABC transporter genes and the presence of Nrf-2-Maf binding sites in the promoter region of these ABC transporter genes (Hirotsu et al., 2012; Ji et al., 2013; Maher et al., 2007; Malhotra et al., 2010). In conclusion, our study identified xenobiotic transcription factor CncC as a major regulator of multiple detoxification genes representing all three phases of detoxification. Manipulation of CncC function by RNAi or by using small molecule inhibitors could help in developing methods for fighting insecticide resistance.

Table 4.1. List of primers used in the experiments.

qRT PCR Primers	Forward	Reverse
qTc-proteasome	AAAGATGACTCGGACGATGAC	ACATGTCGAGGGACACATTC
qTc-Tektin1	GCGAGTTGAGGCTGAATGT	TGTCCTCCTCCAGCTGTATT
qTc-COP9-singlasome	CGAAGAATCACGCCGAGAAA	TTCGAGGGTCGTTTCGTAGA
qTc-Odorant binding	AACCGTCGTCGTTCTAATCC	CTTTGATTCTTTGTCCACCTAGC
qTc-Zinc finger-1	CAGCACGTGACCTCATCAA	CAACTCCCGCTCGTGATAAA
qTc-ZincFinger91	CCACCAAATACCAGTACCAGAG	CGCTTGCCCTTGTCTATGT
qTc-HSP70-F	CCAAGGCAAAGAACTCAACAAA	CACGGACGGCTTCTGAATTA
qTc-ABCA-UA	GACTCGCCCATAACTGTTAGA	CAGCGAAACTGCGTGATAAG
qTc-ABCA-3A	AGGGAAATGAAAGCTCGTACC	GGTGTCAGGTAGCAAGTCATC
qTc-ABCA-1	GCCGTCAACTGTCTTTGTTTAG	TCATCGCCAGTCAGCATTT
dsRNA primers	**Forward**	**Reverse**
dsTc-CYP4G14	CACCGGACAGAAGTGGAAAG	GGCGTATTTCGTTTGGTTGAAG
ds-Tc-CYP4G7	CAATAACGCGGTCAAGGAAAC	GGTCTGTCACTGTCTTGGAAA
dsTc-ABCA-UA	ACACTGGTTAGCATGGTTTCT	CCTTGTGGTGGTACGTCATT
ds-ABCA-3A	GCTCATCGGTTCTAGGGATTT	CTTTGAGCTGTGCTTCTTTCTC
ds-ABCA-1	TCAACGCAGACGACGATTT	TGGATGGAAAGGCTGGTATTC
dsTc-GST	CCAGTTCGGGCTGTTCTAAT	CGGATAATCGGCCATGAAATTG
Barcode primers	**Primer sequence**	
Barcode AGG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNAGGNNNNNN	
Barcode AAC	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNAACNNNNNN	
Barcode ACG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNACGNNNNNN	
Barcode AGA	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNAGANNNNNN	
Barcode CAA	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNCAANNNNNN	
Barcode ACG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNACGNNNNNN	

Table 4.2. Summary statistics for *T.castaneum* transcriptome sequencing obtained using Illumina HiSeq4000.

	(GFP + CncC) knockdown	GFP knockdown	CncC knockdown
Total reads	114872874	84704154	30168720
Mapped reads	86528263;(75.3%)	65300734; (77.1%)	21227529; (70.4%)
Unique mapped reads	79882591; (92.3%)	60672799; (92.9%)	19209792; (90.5%)
Coverage	117.5	86.6	30.9

Table 4.3. Detoxification genes identified in the RNA-seq data that require CncC for their expression. The heatmap shows the Log_{10} transformed dsGFP and dsCncC RPKM.

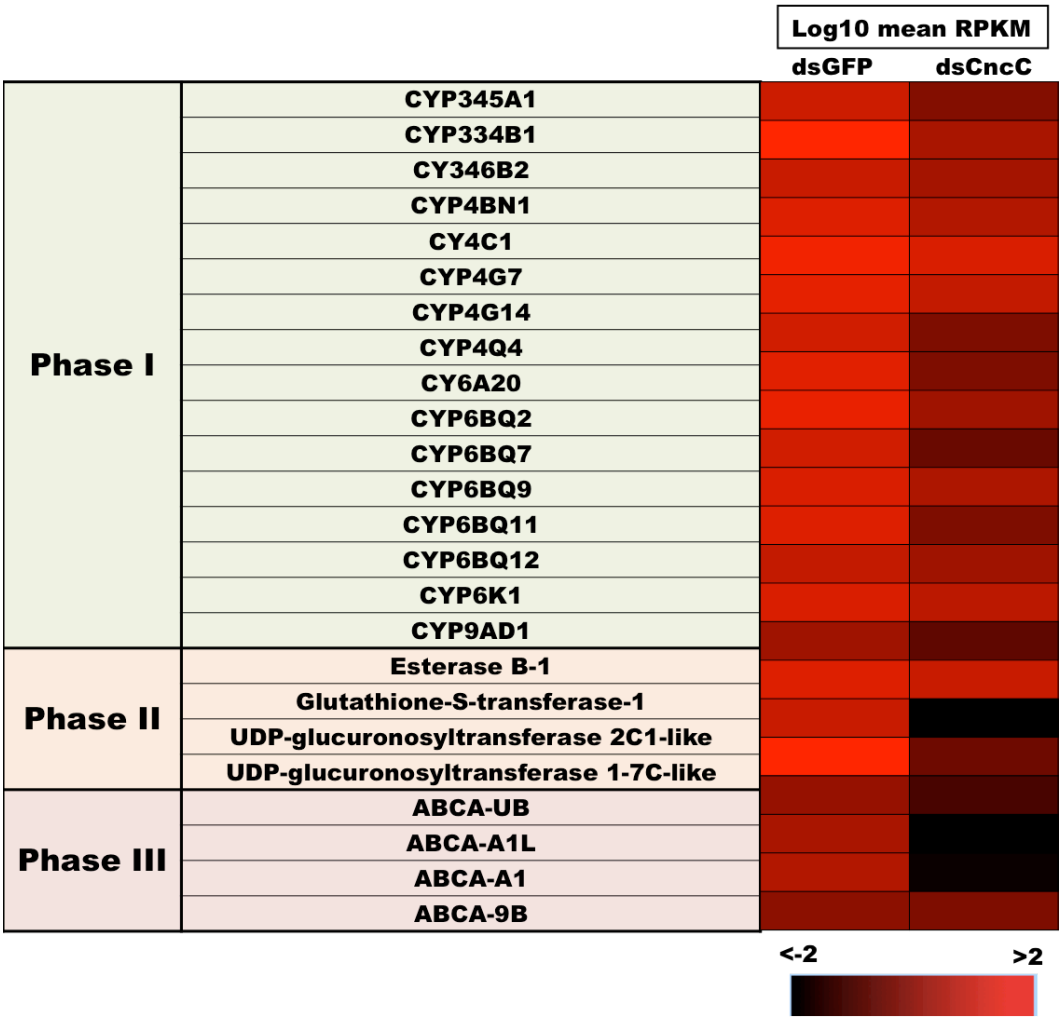
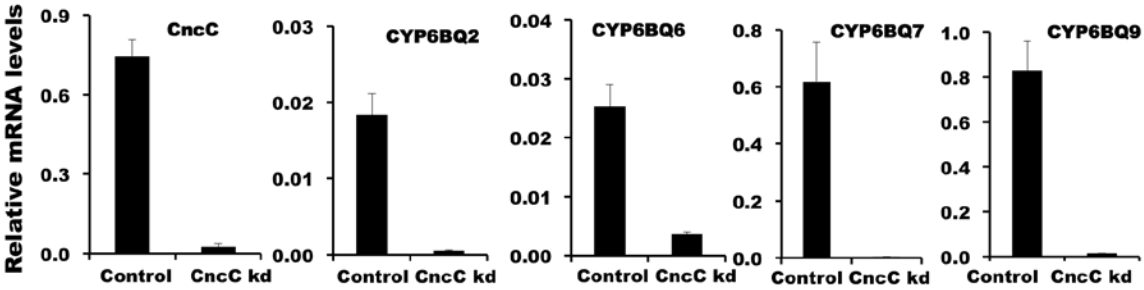
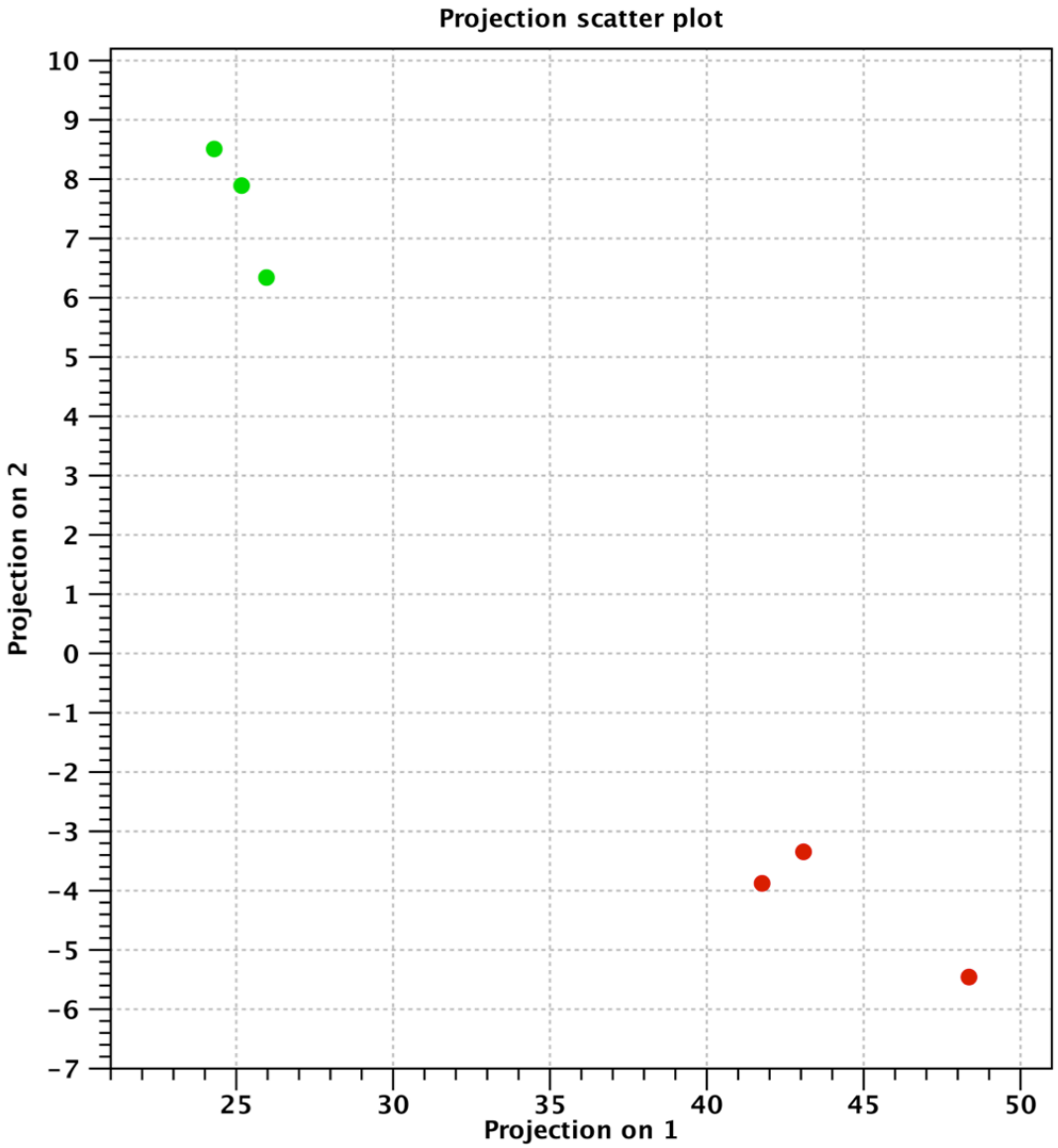


Figure 4.1.



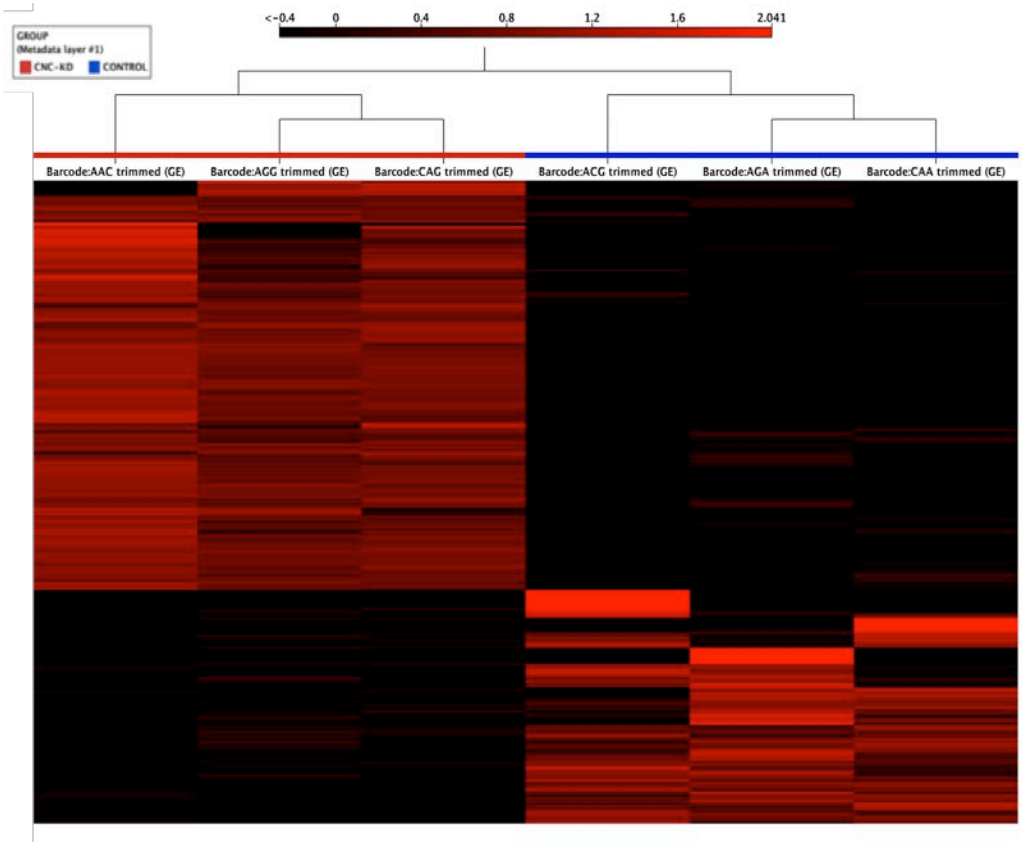
The significant knockdown in CncC dsRNA injected beetles used in preparation of RNA-seq libraries and the expression CncC regulated P450 genes (CYP6BQ2, CYP6BQ6, CYP6BQ7 and CYP6BQ9) were checked in the same beetles. Control beetles were injected with dsGFP. One-week-old beetles were injected with 100 nl of dsRNA (5-7 µg/µl). Total RNA was extracted from beetles on the 6th day after injection, and two µg of total RNA was used to make cDNA. The housekeeping gene RP49 was used as an internal control. Mean + S.E (n=3) are shown, and the data were analyzed using Student *t*-Test with *, denotes the significant different at the value of $P \leq 0.05$.

Figure 4.2.



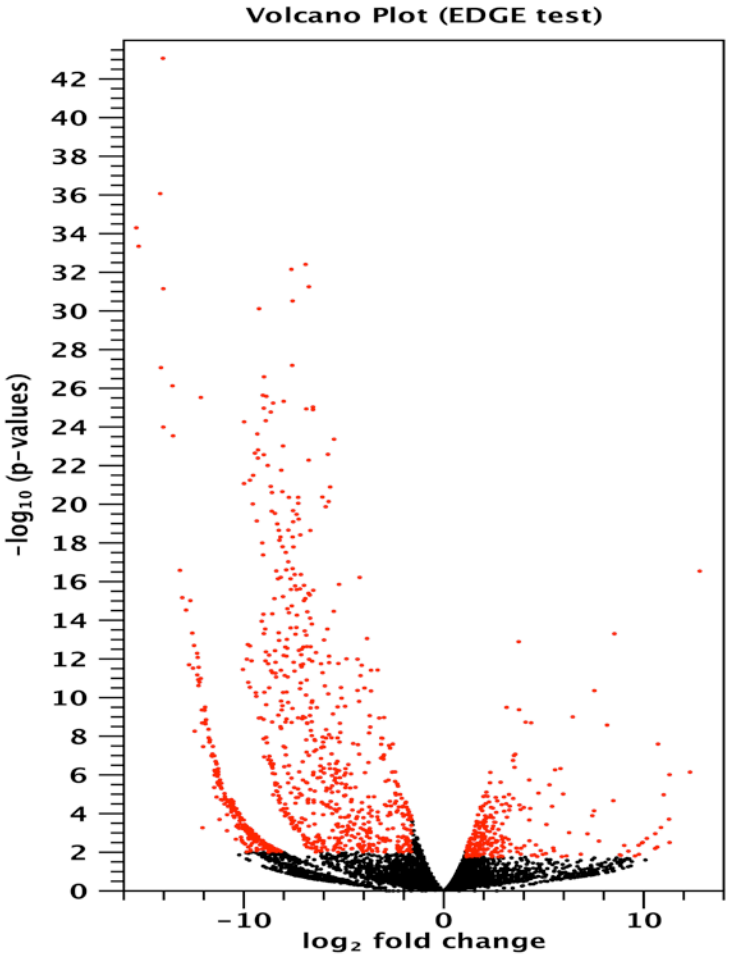
Principal component analysis plot (PCA) for the RNA-seq libraries of three replicates each from dsGFP (control), and dsCncC (experimental) injected insects. Green dot represent dsCncC replicates and red dot represents dsGFP replicates.

Figure 4.3.



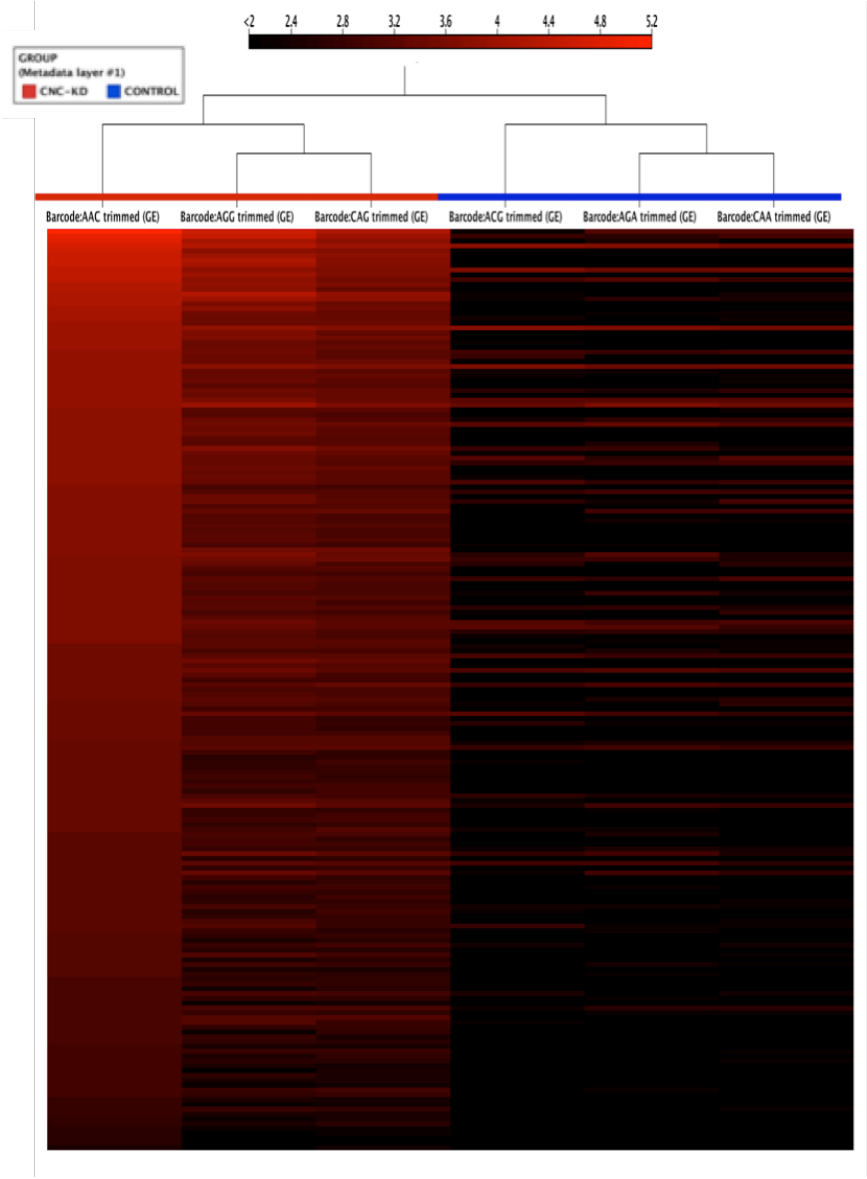
The heatmap for RNA-seq dataset based differential expression profile of the transcripts regulated by Cap n collar isoform C (CncC) protein in *T. castaneum*. The heatmap was generated by the CLC Genomics software version 9.5.1 (Qiagen Bioinformatics, USA), using the normalized mean expression values derived form RNA-seq data from the GFP and CncC knockdown *T. castaneum* beetles. The color key above represents the relative expression value.

Figure 4.4.



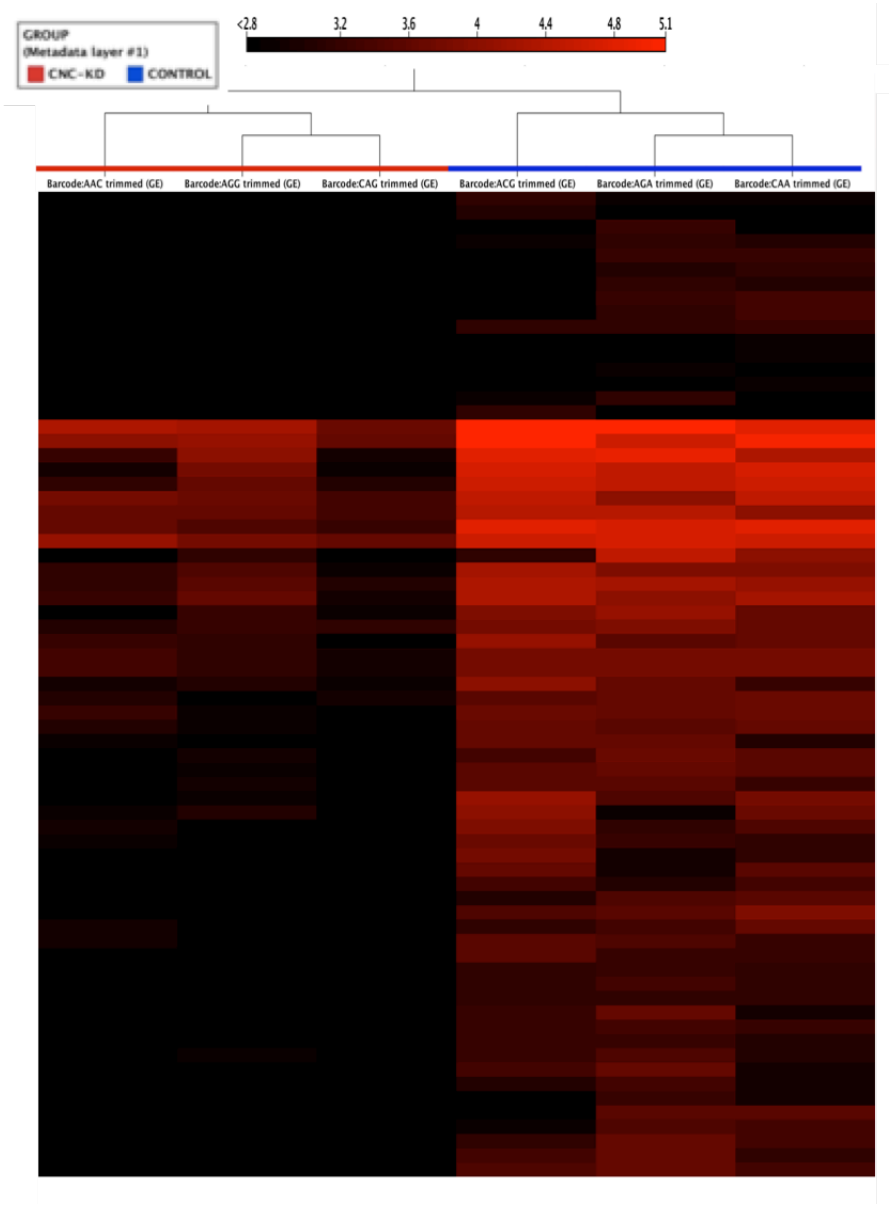
A volcano plot showing differentially expressed genes in the CncC knockdown beetles at the value of $P \leq 0.01$ and $\leq$ or ≥ 1.5 fold change (red dots). The X and Y-axis represent the - $\log_{10}$ *P*-value and $\log_2$ fold change of mean normalized values respectively.

Figure 4.5.



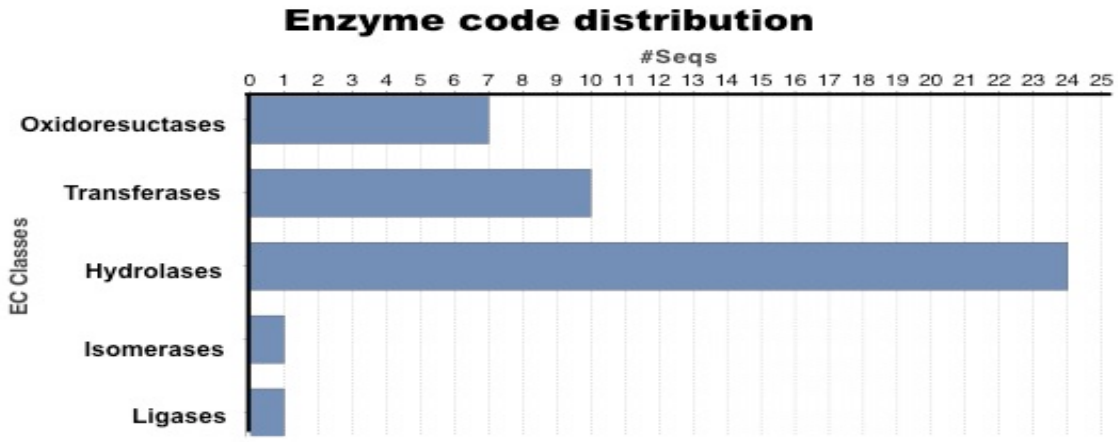
A heat map showing the upregulated transcripts (91 genes) in the dsCncC group as compare to the dsGFP group (each group with three replicates) at the value of $P \leq 0.01$ and ≥ 1.5 fold change in transcript expression. The heatmap was prepared as described in in the Figure 4.3.

Figure 4.6.



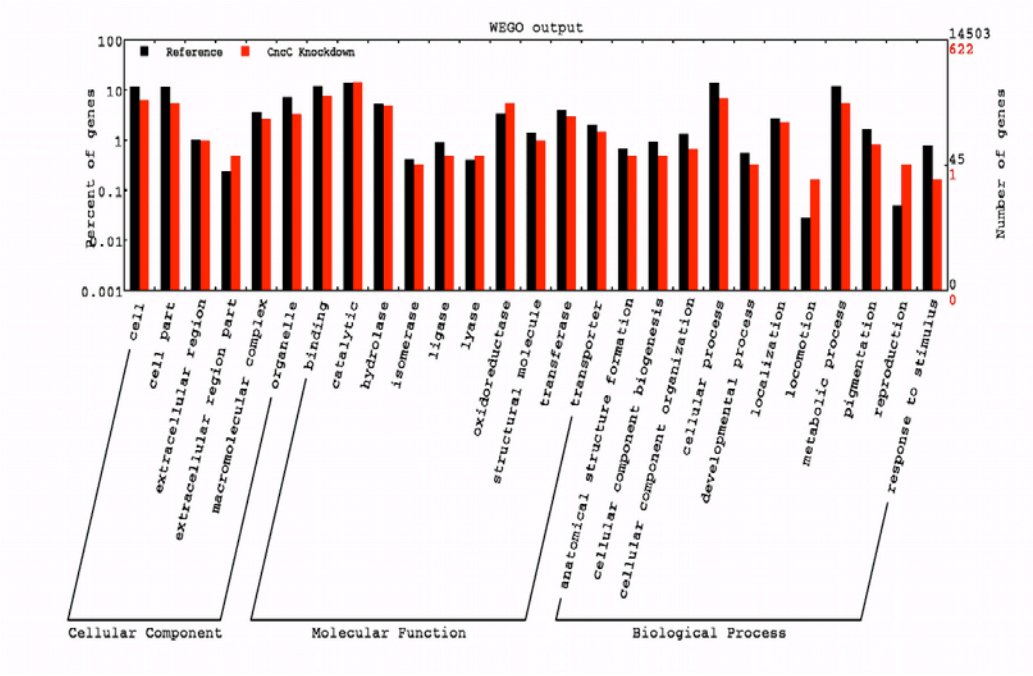
A heat map showing the downregulated transcripts (622 genes) in the dsCncC group as compare to the dsGFP group (each group with three replicates) at the value of $P \leq 0.01$ and ≤ 1.5 fold change in transcript expression. The heatmap was prepared as described in the Figure. 4.3.

Figure 4.7.



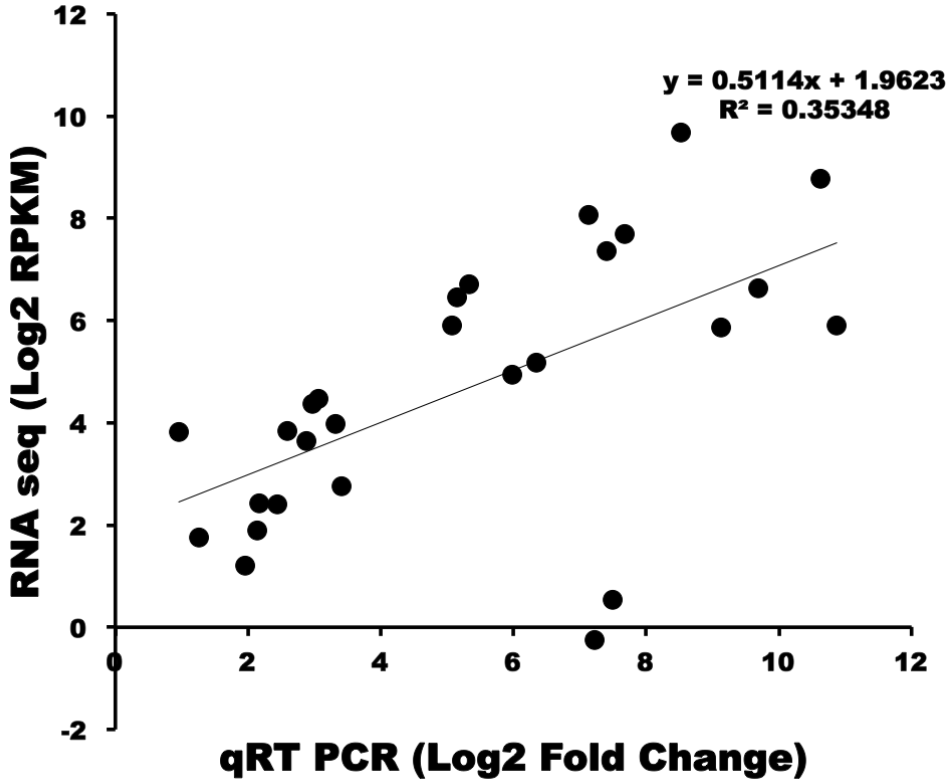
Enzyme code distribution chart. The GO assigned sequences were further assigned with enzyme code using Blast2GO. The numbers of sequences (X-axis) are categorized into five different enzyme groups (Y-axis) including the oxidoreductase (7), transferases (10), hydrolases (24), isomerases (1) and ligases (1).

Figure 4.8.



WEGO plot showing the gene ontology classification for the *Tribolium castaneum* transcriptome. The plot represents the 622 CncC downregulated genes along with the 14503 *T. castaneum* reference genes that are distributed according to the three major categories of gene ontology including; Cellular components, Molecular component and Biological function.

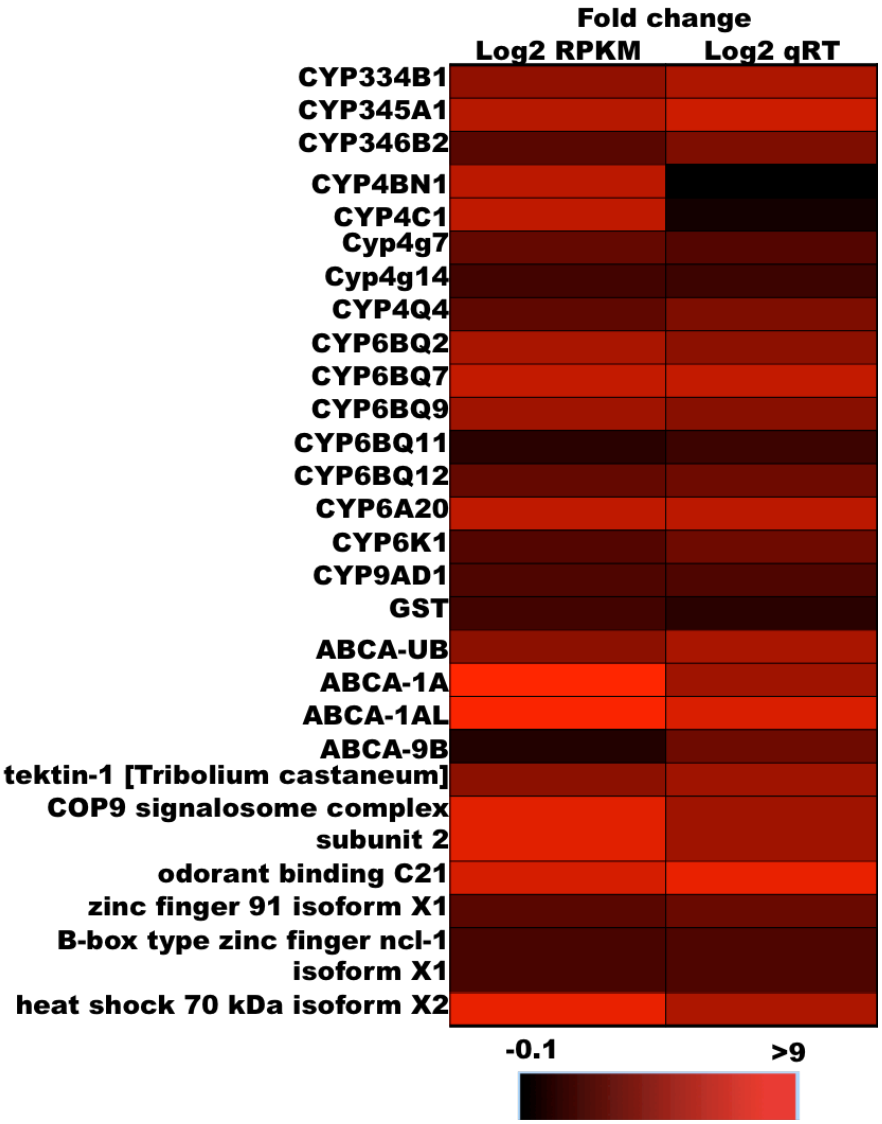
Figure 4.9.



Correlation graph between the RNA-seq (Log_2 RPKM fold change) and qRT-PCR (Log_2 fold change) in the CncC knockdown beetles. The genes that were downregulated in RNAi data were verified using qRT-PCR in CncC knockdown beetles. Out of 27 selected genes, two genes were not validated by qRT-PCR. The expression of 27 genes showed a correlation between results obtained by RNAi and qRT-PCR. R^2=0.35. One-week-old beetles were injected with dsRNA dsGFP or dsCncC (1ug/ul) and total RNA was extracted on the 6th day after injection. One microgram of total RNA was used to make cDNA, and it was used to quantify mRNA levels. The housekeeping gene RP4 was used for normalization. Mean $\pm$ S.E (n=4) are shown, and data were analyzed using student t-

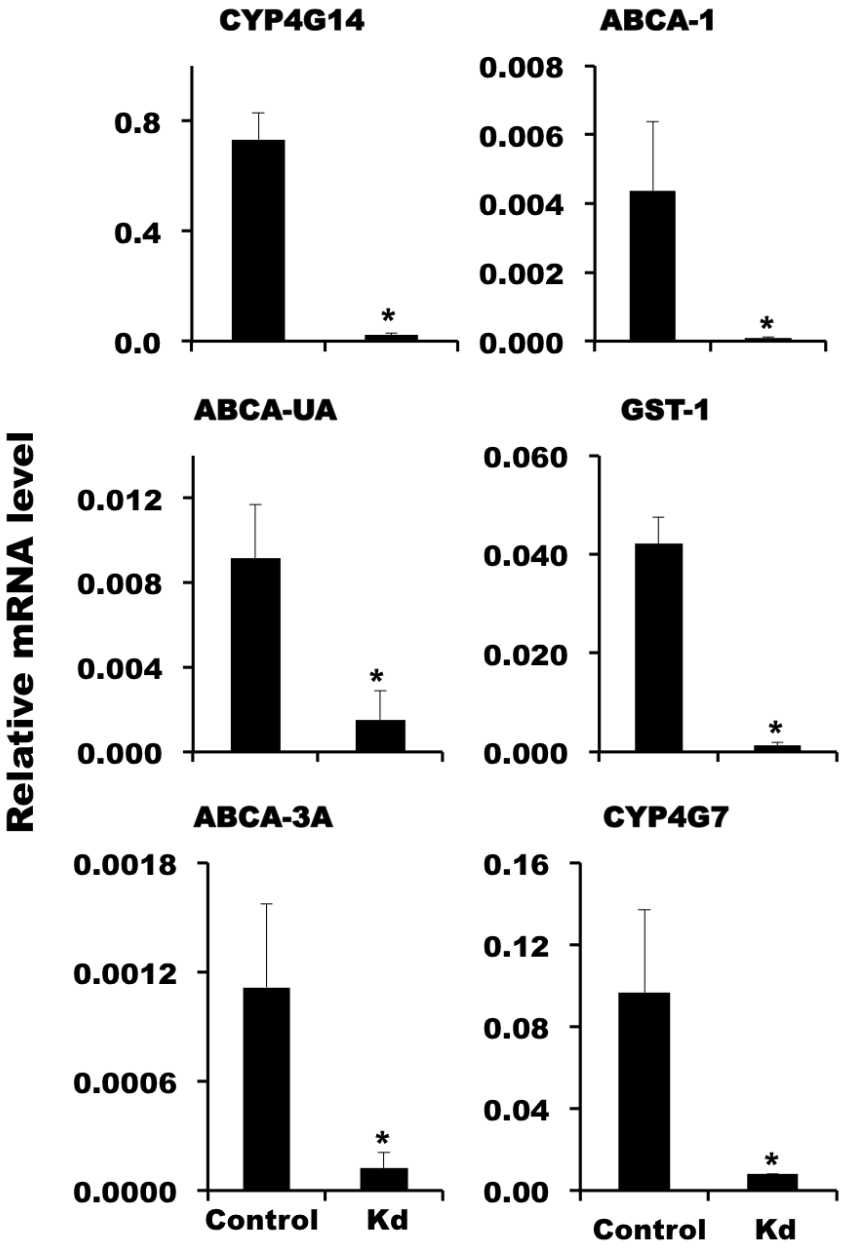
test with the significantly different values at $P \leq 0.05$. The qRT-PCR results were verified in three different sets of GFP and CncC knockdown beetles.

Figure 4.10.



The validation of RNA-seq results by RT-qPCR. The heat map showing the relative expression value for selected 27 genes by RNA seq (Log 2 fold change RPKM) on the left and qRT-PCR (Log 2 fold change Ct) on the right calculated using the mean for three biological replicates (dsGFP and dsCncC).

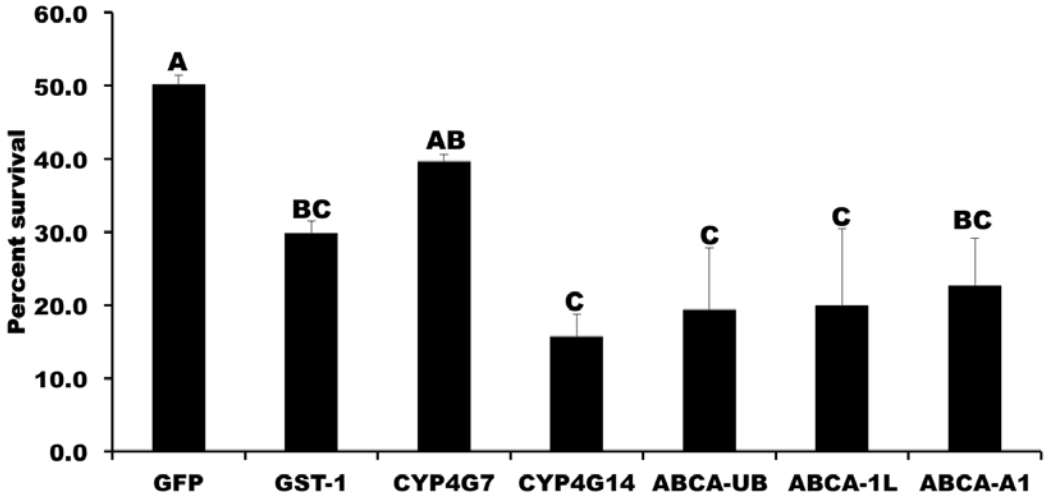
Figure 4.11.



The significant knockdown in CYP4G14, GST-1, ABCA-UA, ABCA-3A, and ABCA-1 dsRNA injected beetles. Control beetles were injected with dsGFP. One-week-old beetles were injected with 100 nl of dsRNA (5-7 µg/µl). Total RNA was extracted from beetles on the 5th day after injection, and two µg of total RNA was used to make cDNA. The

housekeeping gene RP49 was used as an internal control. Mean + S.E (n=3) are shown, and the data were analyzed using Student *t*-Test with *****, denotes the significant different at the value of $P \leq 0.05$.

Figure 4.12.



The knockdown of detoxification genes increases tefluthrin-induced mortality in the resistant QTC 279 strain of *T. castaneum*. The tefluthrin bioassay using CYP4G7, CYP4G14, GST-1, ABCA-UA, ABCA-3A, and ABCA-1 dsRNA injected beetles. The graph shows the percent survival of beetles as compared to the control (dsGFP injected). One-week-old beetles that were injected with 100 nl of dsRNA (5-7 µg/µl). On the sixth day after injection, the knockdown beetles were treated with an LD50 concentration of tefluthrin (0.625 µg/µl) treated filter paper in a 24 well plate. Mortality was recorded at 24 h after insecticide treatment. Mean + S.E. (n = 3 x 40) are shown, and the data were analyzed using one-way ANOVA where A, B, C, denotes the significantly different at the value of $P \leq 0.05$.

Chapter 5: Conclusions and future directions

Pesticide resistance is one of the major problems faced by farmers. The most common mechanism of pesticide resistance is the metabolic resistance caused by enhanced production of detoxification enzymes metabolizing insecticides at a rapid rate (Li et al. 2007). The enhanced detoxification is often attributed to the upregulation, amplification or changes in structure of genes coding for the metabolic enzymes such as cytochrome P450s, glutathione-S-transferases, esterases (Feyereisen 2012). The changes in genes structure may occur due to mutations in the cis or trans regulatory elements (Bass and Field 2011; van Straalen et al. 2011). The cis-regulatory elements could be the enhancers or the response elements present in the specific promoter region of the detoxification genes, or trans regulatory elements including the xenobiotic transcription factors regulating the detoxification genes (Feyereisen et al. 2015; Nakata et al. 2006; van Straalen et al. 2011).

My research is focused on understanding the molecular mechanism of insecticide resistance in two important coleopteran model insects (i) a stored grain pest, *T. castaneum* and (ii) an agricultural pest, *L. decemlineata*. Both these pests are known to develop resistance to insecticides in short periods of time. Broadly, my research can be divided in to two parts. Chapters 2 and 3 of this dissertation deals with understanding the regulation of insecticide resistance genes (P450s) by identifying the trans and cis-regulatory elements controlling the expression of these genes in both *T. castaneum* and *L. decemlineata*. In the next chapter (four), RNA sequencing was employed to identify insecticide resistance genes that are regulated by the xenobiotic trans-regulatory protein, Cap n collar isoform C.

The trans and cis-regulatory elements controlling the regulation of cytochrome P450 genes involved in deltamethrin resistance in *T. castaneum* were identified. Previous studies showed that deltamethrin resistant *T. castaneum* (QTC279) strain developed resistance through constitutive overexpression of a brain specific cytochrome P450 gene coding for CYP6BQ9 (Zhu et al. 2010). The expression of this gene was found to be 200-fold higher in the resistant strain as compared to its expression in the deltamehtrin susceptible strain (LBS). Further, the CYP6BQ9 gene is a part of CYP6BQ cluster comprising of 12 genes (Zhu et al. 2013b). The goal of my research was to understand the regulation of CYP6BQ genes at the molecular level (i) by finding xenobiotic transcription factors (XTFs) regulating the expression of these genes and (ii) to delineate the binding site for these transcription factors in the promoter region of CYP6BQ genes. Based on the literature review of several XTFs regulating the expression of detoxification genes, seven XTFs including spineless-aristapedia (Ss), methoprene tolerant (Met), cap n collar c (CncC), muscle aponeurosis fibromatosis (Maf), hepatocyte nuclear factor 4 (HNF4), hormone receptor-96 (HR96) and ultra spiracle (USP) were selected. For the first objective, I hypothesized that one or more of these seven XTFs would be involved in the regulation of CYP6BQ genes. After the knockdown of each gene coding for selected XTFs (a) I determined the expression of all CYP6BQ genes and (b) I conducted deltamethrin insecticide bioassays to check if there was any change in their tolerance to the insecticide. Following knockdown of XTFs, CncC and Maf, there was a significant decrease in the expression of several CYP6BQ genes including CYP6BQ9. Also, CncC, and Maf knockdown beetles showed 0% and 2% survival after deltamethrin (LD50) treatment when compared to 50% survival in control beetles. XTFs, CncC and Maf are

heterodimer partners that regulate expression of a battery of cytoprotective genes (Sykiotis and Bohmann 2008; Sykiotis and Bohmann 2010). Reporter assays showed that both CncC and Maf are required for regulation of four CYP6BQ genes CYP6BQ6, CYP6BQ7, CYP6BQ9 and CYP6BQ12.

For the second objective, I performed the promoter truncation, PCR-directed mutagenesis and luciferase assay on the CYP6BQ12 promoter. A six-nucleotide (GCAGTn) CncC and Maf binding site was identified in the promoter region of seven genes in the CYP6BQ cluster; CYP6BQ1, CYP6BQ6, CYP6BQ7, CYP6BQ8, CYP6BQ9, CYP6BQ10 and CYP6BQ12. Multiple CncC-Maf binding sites are present in CYP6BQ12 (four) and CYP6BQ10 (two) promoters.

For future studies, it would be interesting to compare the cis-regulatory element i.e. CncC-Maf binding sites between the resistant and susceptible strains of *T. castaneum*. There is a possibility that mutation in the cis-regulatory element of the resistant strain may result in constitutive overexpression of CncC and hence CYP6BQ cluster genes. The mutations in the CncC-Maf binding site can be checked, by comparing the CncC-Maf binding sequences in the resistant and susceptible *T. castaneum* beetles. Since, the CncC-Maf binding site for CYP6BQ cluster is common in the resistant strain, it is possible that these beetles have attained a common mutation when compared to the promoter region in the susceptible strain.

I characterized the cis and trans-regulatory elements that are involved in regulation of imidacloprid and potato plant allelochemicals detoxifying P450 genes in *L. decemlineata*. The transcriptomics study predicted 21 P450 genes that are overexpressed in the *L. decemlineata* resistant strain as well as these genes were found to be induced by

both imidacloprid and potato allelochemicals. The two objectives for this part of the thesis are (i) to identify the xenobiotic transcription factors that are involved in regulation of cytochrome P450 genes involved in imidacloprid and potato plant allelochemicals resistance and (ii) to identify the cis regulatory elements (xenobiotic response element) that facilitate the binding of these xenobiotic transcription factors. The data showed that the expression of CncC was highest during the pupal and adult stages of imidacloprid resistant strain of *L. decemlineata* when compared to the susceptible strain. Therefore, I hypothesized that CncC and its partner Maf could be involved in the regulation of nine-selected P450 genes (CYP9Z25, CYP9Z26, CYP9Z29, CYP9Z^{d}, CYP9Z^{g}, CYP6BJ$^{a/b}$ and CYP6BJ1v1) exhibiting both inducible and constitutive expression (>2-5 fold) in the resistant strain. The knockdown of CncC in both the adults and Lepd-SL1 cells caused a significant decrease in the expression of four P450 genes (CYP6BJ$^{a/b}$, CYP6BJ1v1, CYP9Z25, and CYP9Z29) out of nine genes tested. Further, the same four genes showed induction when Lepd-SL1 cells were exposed to potato leaf extract and imidacloprid. The beetles with knockdown of the four P450 genes and CncC, when treated with imidacloprid (LD50), exhibited an increase in susceptibility to imidacloprid insecticide. These data showed that xenobiotic transcription factor CncC regulates expression of P450 genes involved in detoxification of imidacloprid and potato allelochemicals.

For the second objective, I performed the luciferase reporter assays on the CYP9Z25 promoter. The activity of the full-length and truncated CYP9Z25 promoter was much higher in the presence of CncC and Maf proteins as compared to its expression in the absence of either or both CncC and Maf. Further with the help of PCR directed

mutagenesis and luciferase assays, I found two CncC and Maf binding sites (GCAGAAT and GTACTGA) in the CYP9Z25 promoter.

The main objectives of experiments described in my fourth chapter are (i) to identify insecticide resistant genes regulated by CncC and (ii) to identify the role of these genes in pyrethroid resistance. For this research I prepared the RNA sequencing (RNA-seq) libraries using the RNA isolated from CncC knockdown and control beetles. Sequencing of libraries from CncC knockdown and control beetle generated ca. 115 million reads and 75% of those reads were mapped back to the *T. castaneum* genome. Comparing the CncC knockdown and the control libraries (with cut off $\leq$ or $\geq$ 1.5 folds and FDR corrected $P \leq 0.01$), I found 622 genes that were downregulated while 91 genes were upregulated in the CncC knockdown animals. For further studies, I focused on the downregulated genes only. Out of 622 genes, I selected a subset of 24 genes that were predicted to be involved in insecticide resistance representing the three phases of detoxification. The subset of these 24 genes included (a) 16 phase I genes including P450s from CYP3, CYP4, CYP6 and CYP9 families (b) four phase II genes including GSTs, Esterase-B1 and two UGTs and (c) four phase III genes belonging to ABCA family (i.e. ABCA-UA, ABCA-3A and ABCA-1, ABCA-1X2). From the subset of 24 genes, six genes (i.e. CYP4G7, CYP4G14, GST-1, ABCA-UA, ABCA-3A, and ABCA-1) were selected to determine their role in insecticide resistance by injecting beetles with dsRNA targeting these genes followed by treatment with a pyrethroid insecticide. We confirmed that CYP4G14, GST-1, ABCA-UA, ABCA-3A and ABCA-1 genes were regulated by CncC and are involved in pyrethroid resistance.

Future studies in this area could investigate CncC-Maf binding sites in the promoter region of genes identified to be CncC regulated and involved in detoxification. This information might be helpful to know the direct or indirect role of CncC in regulating the detoxification genes. Further, it would be interesting to study other genes (besides detoxification) that are regulated by CncC to determine their role in the development or physiology. This can be studied by performing the functional studies (RNAi) in different stages of beetles for selected CncC regulated genes followed by observation of morphological/phenotypical changes if possible.

The research included in this thesis showed that xenobiotic transcription factor 'CncC' is a master regulator of several insecticide resistance genes belonging to different phases of detoxification. Therefore, rather than targeting the individual detoxification gene, future studies should attempt to develop CncC as a new insecticide target to control the insect populations using RNA interference. A CncC is present in most of the insects it is important to compare the CncC sequences among different insects and further designing CncC dsRNA in the non-conserved region to avoid the non-target effects. My research also showed that the CncC-Maf binding site is highly variable within species or between two species (i.e. *T. castaneum* and *L. decemlineata*) or (*L. decemlineata*) (Kalsi and Palli, 2015; Kalsi and Palli, 2017) studied. Future studies should be conducted to determine how rapidly these cis elements have evolved and the mechanism that control these changes. This can be done by continuously selecting the susceptible beetles with an insecticide (evolving resistance) and comparing the CncC-Maf binding domains in the promoter regions of genes regulated by CncC over several generation (scoring the rate of mutation).

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VITA

EDUCATIONAL BACKGROUND

Master of Science in Entomology, 2011, University of Florida

Master of Science in Biotechnology, 2006, Garhwal University, India

Bachelor of Science in Botany, 2004, University of Delhi, India

EMPLOYMENT

Graduate Research Assistant: Insect physiology and molecular biology lab, Entomology department, University of Kentucky (August 2011-). **Advisor: Dr. S.R. Palli**

Teaching assistant: Insect Physiology (Entomology 635), University of Kentucky, Spring 2012. Prepared the handouts and conducted the insect physiology labs. Scored the lab reports.

Graduate Research Assistant: Vegetable Integrated Pest Management Laboratory, TREC, University of Florida, Homestead, FL (Jan 2009- 2011). **Advisor: Dr. D.R. Seal**

Assistant Lecturer: Doon (PG) Paramedical College & Hospital, India (Aug 2006-Jan 2007).

Summer Internship: Allergy and Immunology Section, Institute of Genomics & Integrative Biology (CSIR), Delhi-7 (Feb 2006 – May, 2006). **Advisor**: **Dr. A. B. Singh**

Summer Training: Department of Biochemistry, Jamia Hamdard, Delhi (July, 2005-August, 2005) **Advisor: Dr. Salim Javed**

FELLOWSHIPS AND AWARDS

2017 **Publication scholarship**, University of Kentucky.

2016 **Graduate Student Travel Grant,** University of Kentucky.

2015 **Publication scholarship**, University of Kentucky.

2015 **Graduate Student Travel Grant,** University of Kentucky.

2012 **ESA Debate competition**, First prize.

2010 **Travel Grant**, Florida Entomological society.

2010 **MS Student paper Competition**, Second prize, Florida Entomological Society.

2010 **Travel Grant**, Graduate Student Council, University of Florida.

2009 **Travel Grant,** Graduate Student Council, University of Florida**.**

PUBLICATIONS

Peer reviewed research publications

Kalsi, M., & Palli, S. R. (2017). Transcription factor cap n collar C regulates multiple cytochrome P450 genes conferring adaptation to potato plant allelochemicals and resistance to imidacloprid in Leptinotarsa decemlineata (Say). *Insect Biochemistry and Molecular Biology, 83, 1-12.*

Kalsi, M., & Palli, S. R. (2015). Transcription factors, CncC and Maf, regulate expression of CYP6BQ genes responsible for deltamethrin resistance in Tribolium castaneum. *Insect biochemistry and molecular biology*, *65*, 47-56.

Kalsi, M., Seal, D. R., Nuessly, G. S., Capinera, J. L., & Martin, C. G. (2014). Seasonal timing, abundance, and predatory status of arthropods associated with corn infested by picture-winged flies (Diptera: Ulidiidae) in south Florida. *Florida Entomologist*, *97*(1), 168-178.

Kalsi, M., Seal, D. R., Nuessly, G. S., Capinera, J. L., & Martin, C. G. (2014). Distribution of *Zelus longipes* (Hemiptera: Reduviidae) in South Florida corn fields and its functional response to corn-infesting picture-winged flies (Diptera: Ulidiidae). *Environmental entomology*, *43*(5), 1223-1234.

Kalsi, M., Seal, D. R., Nuessly, G. S., Capinera, J. L., & Martin, C. G. (2014). Distribution of arthropod predators and their responses to *Euxesta* spp. (Diptera: Ulidiidae) in the laboratory and in corn fields in South Florida. *Florida Entomologist*, *97*(3), 911-920.

Other peer reviewed and extension publications

Shukla, J.N., **Kalsi, M.,** Sethi, A., Narva, K.E., Fishilevich, E., Singh, S., Mogilicherla, K. and Palli, S.R., 2016. Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. *RNA biology*, *13*(7), pp.656-669.

Curry, M. M, Crawley. S, **Kalsi. M**, Saeed. A, and Hunt, B. (2014). The single most promising solution to feeding the world's growing population is entomophagy: Rediscovering an ancient tradition to feed a contemporary society. In 2012 ESA Student Debate: Student Perspective on Scientific Global Issues. *American Entomologist*, *60*(4), 212-222.

Kalsi .M, and D,R. Seal., 2016. *Zelus longipes*, Online publication. Featured Creatures. Entomology and Nematology Department, Florida Department of Plant Industry, Institute of Food and Agricultural Sciences, University of Florida.

PRESENTATIONS (At national and international conferences)

Invited presentations - 2

Oral presentations - 6

Poster presentations - 2

STUDENT MEMBERSHIPS AND ACTIVITIES

- Treasurer, H Garhman Club, University of Kentucky (2015-2016)
- Career counseling at youth science summit (Lexington-2016)
- Social Committee, H Garhman Club, University of Kentucky (2014-2016)
- Student volunteer at the Entomological Society of America Annual Meeting (2012, 2015)
- Science Fair judge (grades K-12) Fayette County Science Fair, February (2012-2017)
- Outreach: Insect Night Walk at the Arboretum, September 2014
- Member of Entomological Society of America (2009-present)
- Member of Florida Entomological Society (2010-2011)
- Member H Garhman Club, University of Kentucky (2011-present)

Member of Departmental Entomology and Nematology Student Organization (ENSO), UF (2009-2011)

Member of Departmental Entomology and Nematology, Seminar committee

Student volunteer of ASHA, UF (2009-2011)

TECHNICAL SKILLS

Cloning and Transformation experiments, Transfection experiments, Isolation of plasmid, gDNA, and RNA, Protein estimation, Electrophoresis (SDS-PAGE & Agarose), Luciferase assay, Genome annotation, NGS-sequencing, dsRNA preparation using kit

and HT115 bacteria, RNAi, qRT-PCR, Insecticide bioassays, ELISA, EMSA, Cell culture, Insect microinjections, Insect rearing, Insect dissections, CLC genomics workbench, JMP, etc.