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

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Abstract

Colorado potato beetle (CPB), *Leptinotarsa 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

1. 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 (ARPD), 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 overexpression of CYP6D1 gene (Liu and Scott, 1997). In a recent study, the upregulation of CYP6BQ genes in the deltamethrin resistant strain of *Tribolium 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

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

99 The detoxification genes are regulated by a common mechanism of transcriptional
100 activation and the transcription factors involved in activation of such genes are grouped
101 into three xenobiotic superfamilies including nuclear receptor (NR), basic-helix-loop-
102 helix/per-ARNT-SIM (bHLH-PAS) and basic lucine zipper (bZIP) (Nakata et al. 2006).
103 In mammals, the heterodimer bZIP transcription factors Nrf2-Keap1 (NF-E2-related
104 factor 2 and Kelch-like ECH Associated Protein 1) represents a signaling pathway that
105 plays a crucial role in regulation of a battery of cytoprotective genes involved in
106 xenobiotic or oxidative responses in many human diseases (Slocum and Kensler, 2011;
107 Sykiotis and Bohmann, 2010). At basal conditions,
108 Keap1 represses Nrf2 by promoting its proteasomal degradation using ubiquitin ligase in
109 the cytoplasm. Whereas, under oxidative stress, Keap1 act as a stress sensor releasing
110 Nrf2 which translocates to the nucleus (Zhang, 2006). Nrf2 heterodimerizes with small
111 Maf (muscle aponeurosis fibromatosis; bZIP transcription factor) proteins and binds the
112 specific sequences in the promoter region often referred to as antioxidant or xenobiotic
113 response elements (ARE or XRE), initiating the transcription of several
114 antioxidant/detoxification genes (Hirotsu et al., 2012). The Nrf2 ortholog in invertebrates
115 is known as cap 'n' collar C (CncC), these proteins regulate the expression of a plethora of
116 P450s that are involved in metabolism and detoxification (Deng, 2014; Karim et al.,
117 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).

2. Material and Methods

2.1 Insects: 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.

2.2. 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.

2.3. 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 prior to 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.

2.4. 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 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 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.

2.5. 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 LD₅₀ 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.

2.6. 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-4TM expression vector (Novagen, EMD Millipore). Cells were counted using the hemocytometer and seeded at a density of 1 X 10⁵ 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.

2.7. 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.

2.8. 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.

2.8.1. Treatment of Lepd-SL1 cells with potato leaf extract: The Lepd-SL1 cells were seeded into a 6 well plate (1×10^6 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 μ l 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.

2.8.2. 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 (1×10^6 cells / well in 2 ml medium) for 24 h. On the following day, old medium was removed and the cells were treated with 1 μ g/ μ l of Imidacloprid for 6, 24, 48 and 36 h in triplicates. Control cells were added with 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.

2.8.3. 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 (1×10^6 cells / well in 2 ml medium). The serum free medium (1 ml) mixed with 6 μ g 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 1 μ g/ μ l. 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.

2.9. Statistical Analysis

For statistical analysis, JMP 11.0 software (SAS, Cary, NC) was used. 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 $P < 0.05$.

3. Results

3.1. 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 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. 1A). 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. 1B). 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.

3.2. Transcription factor CncC regulates the expression of cytochrome P450 genes

3.2.1 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. 2A 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. 2B).

3.2.2. 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 LD₅₀ dose of imidacloprid. The CncC-knockdown beetles treated with the imidacloprid (LD₅₀), showed only 5% survival as compared to the control

beetles that showed 54% survival (Fig.3). 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. S1 and Fig. 3). These data suggest that P450 genes that require the presence of CncC for their expression play an important role in imidacloprid resistance.

3.2.3. 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.4). Interestingly, the knockdown of CncC reduced imidacloprid

induction of the same four genes (Fig. 5A and B) suggesting that CncC is required for imidacloprid induction of P450 genes.

3.2.3 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. 6A). 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. 6B and 6C).

3.3. 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. 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. 8A). 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. 8B). 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. 9A 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.9B). 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 binding 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.

4. 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. 7, 8B and 9B). The knockdown of CncC in the adult beetles lead to downregulation of four P450 genes in the midgut tissue (Fig. 2A and 2B). Similarly, the CncC knockdown in the cells blocked imidacloprid induction of the same four P450 genes (Fig. 5A and 5B). Treating the CPB cells with PLE, induced the same P450 genes and the PLE induction of these genes was reduced after CncC knockdown (Fig. 6A, 6B and 6C). Further, the knockdown of CncC as well as the four target P450 genes enhanced the susceptibility of resistant beetles to imidacloprid (Fig. 3). Higher levels of constitutive expression of CncC were detected in the resistant strain when compared to its levels in the resistant strain

(Fig. 1A and 1B). 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., 2002a; Li et al., 2002b; 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. 1A and 1B). 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.1B). 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., 2013). 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, 2A and 2B). In another beetle system

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

458 The Nrf2 in vertebrates is homologous to CncC in invertebrates (Sykiotis and Bohmann,
459 2010) and the partnership between CncC (or Nrf2) and Maf have been documented to be
460 indispensable for regulation of the xenobiotic detoxification genes in both vertebrate or
461 invertebrate systems (Hirotsu et al., 2012; Itoh et al., 1997; Kalsi and Palli, 2015; Nguyen
462 et al., 2005). In mammals, the widely recognized core XRE sequence for binding of Nrf2-
463 Maf complex is mostly degenerate and the Nrf2-Maf binding site is not well conserved
464 among different species (Nioi et al., 2003). This has been true for the invertebrate system
465 as well for instance the CncC-Maf binding site in *Drosophila* is 15 bp (i.e.
466 TAGTCATGGTGATAG) (Misra et al., 2011) while in *T. castaneum*, it is 7 bp (i.e.
467 GCAGTAC) (Kalsi and Palli, 2015). Some other studies (vertebrate system) emphasized
468 that the Nrf2-Maf heterodimer requires an Activator Protein-1(AP-1) like element
469 (TGAC) and a GC box (i.e. GCA sequence) on the 3' end of the XRE (Hirotsu et al.,
470 2012; Zhang et al., 2003). On the contrary, in the invertebrate system such as *T.*
471 *castaneum*, the GC box was found on the 5' end of the XRE (i.e. GCAGTAC) and
472 apparently no AP-1 like element is required (Kalsi and Palli, 2015). In the current study,
473 we characterized two different CncC and Maf binding sites (i.e. GCAGAAT and
474 GTACTGA) in the promoter region of CYP9Z25 gene in CPB with the help of reporter
475 and point mutation assays (Fig. 9A and 9B). Kalsi and Palli 2015 reported the presence
476 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 I, 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 evolutionary advantage.

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Figure Legends

Figure 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 2A. 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, Malpighian tubules, and the remaining tissues. Isolation of RNA and determination of CncC mRNA levels were performed as described in Figure 1.

Figure 2.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 1.

Figure 3. 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 ($LD_{50} = 0.5-1 \mu\text{g}/\mu\text{l}$), 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 $P < 0.05$.

Figure 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 ($1 \mu\text{g}$ / 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 5A. 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 ($6 \mu\text{g}$ / well) for 48 h followed by imidacloprid induction ($1 \mu\text{g}$ / well) for 24 h. Total RNA isolation, determination of mRNA levels and statistical analysis were performed as described in Figure 1.

Figure 5B. 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 μ g / well) for 48 h followed by imidacloprid induction (1 μ g / 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 6A. 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 Figure 1.

Figure 6B. 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 μ g / 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 1.

Figure 6C. 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 μ g / 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 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 $P < 0.0$

Figure 8A. The schematic representation of the CYP9Z25 promoter truncations.

Figure. 8B. 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 9A. Schematic representation of PCR-directed mutagenesis constructs prepared.

Figure 9B. 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 8. 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 $P < 0.05$.

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