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Multi-gene resistance to neonicotinoids in the Colorado potato beetle, Leptinotarsa decemlineata

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Abstract

The Colorado potato beetle, *Leptinotarsa decemlineata*, is a significant pest of potato, and its impact on agriculture is measured on a global scale. The beetle is mainly controlled by neonicotinoid insecticides, however, resistance development is a growing concern. Resistance to neonicotinoids is thought to involve elevated activity of detoxifying enzymes and xenobiotic transporters that break-down and excrete insecticide molecules. Here, using mRNA sequencing, I identified multiple detoxifying enzyme and xenobiotic transporter genes transcriptionally up-regulated in a neonicotinoid resistant strain of beetles. I then used RNA interference to knock down the transcript levels of the ten most promising genes in resistant beetles to test their possible roles in resistance. The silencing of two detoxifying enzyme genes, a cytochrome P450 (*CYP4Q3*) and a uridine 5'-diphospho-glycosyltransferase (*UGT 2B5*), significantly increased susceptibility of resistant beetles to the neonicotinoid insecticide imidacloprid. My results indicate that over-expression of these two genes contributes to neonicotinoid resistance.

Keywords

Colorado potato beetle, metabolic resistance, neonicotinoid resistance, cytochrome P450, esterase, uridine 5'-diphospho-glycosyltransferases, glutathione S-transferase, ATP-binding cassette transporter, mRNA sequencing, RNA interference

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List of Abbreviations

AAFC	Agriculture and Agri-Food Canada
ABC	Adenosine triphosphate binding cassette
ACh	Acetylcholine
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bp	Base pair
BWA	Burrows-Wheeler Alignment
cDNA	Complementary Deoxyribonucleic acid
CYP	Cytochrome P450
DDT	Dichlorodiphenyltrichloroethane
DEPC	Diethylpyrocarbonate
DESeq	Differentially expressed sequence
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetraacetic acid
FDR	False discovery rate
gDNA	Genomic Deoxyribonucleic acid
GFP	Green fluorescent protein
GST	Glutathione S-transferases
HSD	Honest significant difference
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani
LD_x	Lethal dose required to kill x% of test subjects
LoRDC	London Research and Development Centre
MCS	Multi cloning site

MDR	Multidrug resistance
mRNA	Messenger RNA
MRP	Multidrug resistance-associated proteins
nAChR	Nicotinic acetylcholine receptors
NADH	Nicotinamide adenine dinucleotide hydride
nt	Nucleotide
OD	Optical density
*P*adj	*P* adjusted value
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-seq	RNA sequenced
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RS	Resistant strain
SAM	Sequence Alignment/Map
SEM	Standard error of the mean
siRNA	Small interfering RNA
SS	Sensitive strain
TAE	Tris acetate EDTA
UGT	Uridine 5'-diphospho-glycosyltransferases
w/v	Weight/volume

Chapter 1. Introduction

1.1 Insecticides and insecticide resistance

Insecticides are natural or synthetic compounds that are deployed to kill insects deemed a menace to agriculture and public-health. Properly applied, insecticides can help increase crop yield or quality, or both, and do so by protecting crops from defoliation and other damages (Saravi and Shokrzadeh, 2011). It is estimated that without insecticide use, up to 70% of crop yield could be lost to pests (Gianessi, 2009). In addition, insecticides serve to prevent transmission of vector-borne diseases, such as malaria, yellow fewer, and dengue. Currently, insecticide-treated bed nets remain an important facet of vector-borne disease control around the world (Raghavendra *et al*., 2011). According to recent estimates, the number of deaths from malaria has halved in Africa since 2000, and nearly 70% of this reduction is attributed to insecticide use (WHO, 2015).

The contributions of insecticides to crop protection and to the control of vector-borne diseases are, however, accompanied by major risks. For example, improper use of insecticides causes water, soil, and air contamination that transfers insecticide residues along food chains (Saravi and Shokrzadeh, 2011). This can ultimately result in destruction of wildlife and death of beneficial and non-target organisms such as honeybees and parasites of pests. Further, exposure to insecticides is associated with various long-term health effects in humans, ranging from neurological dysfunctions, respiratory and reproductive effects, to cancer (Pimentel, 2005). Additionally, evolution of resistance to insecticides by pests has become a serious impediment to control of agriculturally and medically important pests (IRAC, 2011).

Evolution of insecticide resistance is a response of insects to selective pressure by insecticides, leading to failure of an insecticide to achieve the intended level of control (IRAC, 2011). Resistance develops when some rare individuals in a population carry alleles that confer resistance, which in turn are selected by repeated use of the same insecticide favouring the same alleles (Liu, 2015). In time, selection for resistant alleles can render the insecticide less effective. The rate at which the insecticide resistance

evolves depends on several factors, including frequency and intensity of insecticide application (Ffrench-Constant, 1995), frequency and effect of resistance alleles (Ffrench-Constant, 2013), and relative fitness of resistant strains compared to sensitive strains (Kliot and Ghanim, 2012). To date, more than 500 arthropod species have evolved some level of resistance to at least one class of insecticide (Bourguet *et al*., 2013), and the number is expected to rise.

Insecticide resistance causes significant crop losses, has an impact on public health, for instance in malaria control, and costs billions of dollars annually (Pimentel, 2005). Crop loss due to insecticide resistance is estimated to be $1.5 billion dollars per year in the United States alone (Pimentel, 2005). This cost mainly arises from the need to apply insecticides in higher quantities and increased frequencies to achieve a satisfactory control of target pests showing decreased sensitivity to insecticides. Also, evolution of insecticide resistance in insect vectors of human diseases threatens progress made in the global fight against malaria. Since 2010, resistance to at least one insecticide in a malaria vector population has been reported in 60 countries (WHO, 2015).

1.2 Insecticide resistance mechanisms

Insecticides work by interacting with protein targets and interfering with essential biological mechanisms in insects (Casida and Durkin, 2013). Insecticide resistance occurs in all orders of insects and can result from several different mechanisms. These mechanisms include target site insensitivity, decreased penetration, increased excretion, or metabolic detoxification of insecticides. Target site insensitivity results from mutations in amino acid sequence of a target protein to which an insecticide molecule would normally bind and exert its effect (Liu, 2015). Mutations alter target protein structure such that it no longer interacts with insecticide molecules efficiently. This mechanism has been shown to be an important factor in resistance to pyrethroid (Rinkevich *et al*., 2013) and organophosphate (Malekmohammadi and Galehdari, 2016) class insecticides. Decreased penetration of insecticides occurs due to resistant insects possessing a thicker cuticle. This is caused by higher protein and lipid content in the cuticle and/or altered cuticular sclerotization which slows the penetration of insecticide molecules through the

insect cuticle or digestive tract lining (Ahmad *et al*., 2006), resulting in resistance. Increased excretion of insecticides is the mechanism whereby unaltered insecticide molecules are actively transported out of cells before they can reach their target sites (Dermauw and Van Leeuwen, 2014). Decreased penetration and increased excretion are usually found in combination with other resistance mechanisms rather than being a single mechanism of resistance on their own. Finally, metabolic detoxification results from increased metabolism or detoxification of insecticide molecules (Li *et al*., 2007), and is the most common mechanism of insecticide resistance that occurs in the majority of insect pests (IRAC, 2011).

1.3 Metabolic resistance

Metabolic resistance is the best understood mechanism contributing to insecticide resistance. This mechanism is believed to be derived from an ancestral ability to neutralize dietary toxins (Brattsten, 1988). To deter feeding by herbivorous insects, plants produce a broad range of toxic defence compounds known as allelochemicals (Mithöfer and Boland, 2012). To counter the toxic effects of the plant defence compounds, herbivorous insects in return utilize an array of detoxifying enzymes and xenobiotic transporters that break-down and excrete toxins from the insect body (Wybouw *et al*., 2015). Hence, abilities of herbivorous insects to metabolize plant allelochemicals ultimately serve as pre-adaptations to the capability to detoxify insecticides.

Metabolic resistance to insecticides is caused by increased break-down and excretion of insecticide molecules as a result of qualitative (Li *et al*., 2007) or quantitative (Zhu *et al*., 2013a) changes in proteins involved in detoxification and excretion. A qualitative change in a protein results in expression of a structurally altered protein with increased catalytic activity and substrate affinity while quantitative change occurs as a result of increased production of a protein (Li *et al*., 2007). Such changes are usually caused by coding sequence variation (Bass *et al*., 2014), mutations in regulatory elements (Feyereisen, 2012), or gene amplification (Bass and Field, 2011). Hence, by possessing more efficient forms or higher quantities of detoxifying enzymes and xenobiotic transporters, resistant insects are able to metabolize and excrete insecticides faster than their sensitive

counterparts. This effectively reduces the concentration of insecticide molecules in the insect body before they can reach their target sites, resulting in resistance.

Metabolic detoxification of toxic compounds, including insecticides, is a three-stage process. The phases are: I- direct metabolism, II- conjugation, and III- excretion (Xu *et al*., 2005) (Figure 1.1). During phase I and II metabolism, toxic molecules are converted to metabolites that are often less toxic and more water soluble than the original molecules. This is accomplished by unmasking, or *de novo* introduction of, reactive and polar groups (e.g. -OH, $-NH_2$, or –SH) in the toxic molecules through several reactions. The phase I reactions responsible include oxidation, hydrolysis, or reduction (Dawkar *et al*., 2013). These reactions increase the reactivity of the molecules and further facilitate downstream modifications in phase II, whereby conjugation of a polar compound, such as the tripeptide glutathione or uridine diphosphate (UDP) sugars, to phase I metabolites takes place (Sheehan *et al*., 2001; Ahn *et al*., 2012). Finally, in phase III, excretion of phase I and II metabolites from cells is performed by xenobiotic transporters (Dermauw and Van Leeuwen, 2014). The most important detoxifying enzymes and xenobiotic transporters involved in the abovementioned processes are cytochrome P450s (CYPs) and esterases in phase I (Feyereisen, 2012; Li *et al*., 2007), glutathione S-transferases (GSTs) and uridine 5'-diphospho-glycosyltransferases (UGTs) in phase II (Bock, 2016; Enayati *et al*., 2005), and ATP-binding cassette (ABC) transporters in phase III (Merzendorfer, 2014).

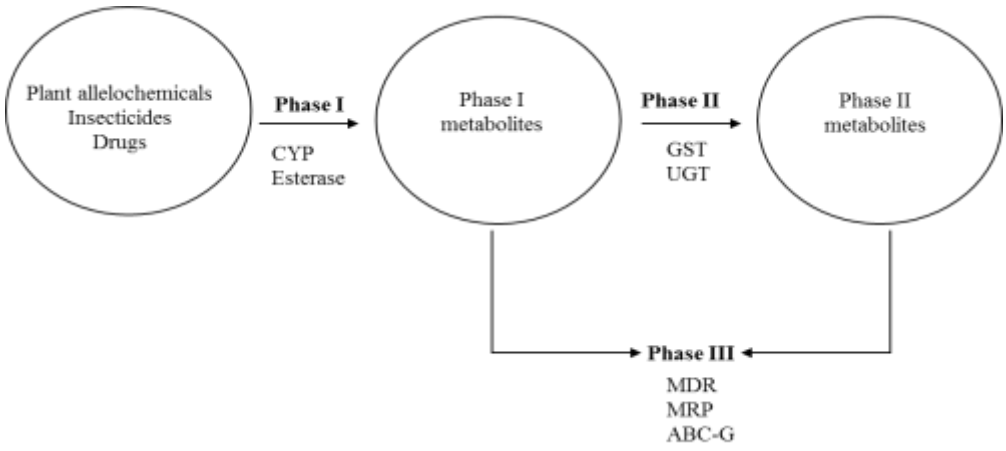


Figure 1. 1. Schematic representation of metabolic detoxification of toxic compounds. CYP= Cytochrome P450, GST= Glutathione S-transferase, UGT= Uridine 5'-diphospho-glycosyltransferases, MDR= Multidrug resistance protein, MRP= Multidrug resistance-associated protein, ABC-G= ATP Binding Cassette G subfamily. Adapted from Brattsten (1988).

1.3.1 Phase I metabolism: Cytochrome P450s

An important group of metabolic enzymes involved in phase I metabolism is the cytochrome P450s (CYPs), which comprise a superfamily of hemoproteins (Feyereisen, 2012). CYPs are found in all kingdoms of life, and have a broad range of functions, including hormone biosynthesis and metabolism of endogenous and exogenous compounds (Scott, 1999). CYP enzymes are capable of metabolizing a diverse range of chemical molecules such as plant allelochemicals, microbial toxins, and insecticides, and carrying out different catalytic reactions including mono-oxygenation and reduction (Feyereisen, 2012). These reactions make toxic molecules more reactive, hence more amenable to further metabolism in phase II (Scott and Wen, 2001). Such capabilities are attributed to the presence of multiple CYP enzymes, some of which can metabolize more than 20 different substrates (Scott, 1999). For instance, 57 functional CYPs are found in humans (Sim and Ingelman-Sundberg, 2010), while the red flour beetle, *Tribolium castaneum*, and the fly *Drosophila melanogaster* possess a total of 133 and 85 functional CYP genes, respectively (Zhu *et al*., 2013b; Adams *et al*., 2000).

Given their genetic diversity and abilities to metabolize a wide range of chemical molecules, CYPs are proposed to be the only enzyme family capable of conferring resistance to all classes of insecticides (Li *et al*., 2007). Indeed, CYP-mediated resistance to many classes of insecticides, including pyrethroids (Gao *et al*., 2012) and neonicotinoids (Markussen and Kristensen, 2010) has been identified in several orders of insects, including Lepidoptera, Coleoptera, and Diptera (Feyereisen, 2012). Generally, overexpression of one or more CYP enzymes, due to changes in the *cis*-or-*trans*-acting regulatory loci, is the primary mechanism for CYP-mediated resistance (Hemingway *et al*., 2004; Li *et al*., 2007). In some cases, however, gene amplification or qualitative changes can also result in overproduction and enhanced activities of CYP enzymes, resulting in resistance (Puinean *et al*., 2010; Amichot *et al*., 2004).

1.3.2 Phase I metabolism: Esterases

The second group of enzymes involved in phase I metabolism is the esterase family. These enzymes are found ubiquitously in both eukaryotes and prokaryotes and have critical functions in development, reproduction, digestion, and xenobiotic detoxification (Montella *et al*., 2012). The majority of insect esterases characterized thus far belong to the carboxyl/ cholinesterase (CCE) superfamily, which plays an important role in insecticide metabolism (Teese *et al*., 2010). These enzymes catalyze the hydrolysis of insecticides that contain an ester bond to generate acid and alcohol as metabolites that can be excreted from cells more easily, thus resulting in reduced sensitivity. For instance, hydrolysis of ester bonds of carbamate, pyrethroid, and organophosphate insecticides by esterases plays an important role in the detoxification of these compounds in many insects (Bass and Field, 2011). In addition, esterases can confer resistance by sequestering insecticides away from their target site (Karunaratne *et al*., 1993). Similar to CYP enzymes, esterase-mediated resistance is primarily caused by overexpression due to gene amplification and up-regulation as well as coding sequence variation (Li *et al*., 2007).

1.3.3 Phase II metabolism: Glutathione S-transferases

Glutathione S-transferases (GSTs) are a diverse family of phase II metabolic enzymes that catalyze the conjugation of reduced tripeptide glutathione to a variety of substrates, including insecticides. By doing so, they increase the water solubility of the insecticides and facilitate their excretion from cells, hence contributing to resistance (Enayati *et al*., 2005). GSTs often act on the metabolites from phase I reactions, but they can also operate independent of phase I metabolism, depending on the chemical structure of the molecules being metabolized. In addition, GSTs can confer resistance by binding insecticide molecules via a sequestration mechanism (Kostaropoulos *et al*., 2001). Similar to phase I metabolic enzymes, overexpression of GSTs often positively correlates with resistance to insecticides, including neonicotinoids and pyrethroids (Yang *et al*., 2013b; Lumjuan *et al*., 2011). Apart from their function in insecticide detoxification, GSTs also have roles in intracellular transport, biosynthesis of hormones, and protection against oxidative stress generated by reactive oxygen species (Hayes *et al*., 2004).

1.3.4 Phase II metabolism: Uridine 5'-diphospho-glycosyltransferases

Uridine 5'-diphospho-glycosyltransferases (UGTs) are another class of phase II metabolic enzymes found in all kingdoms of life. These enzymes also comprise a large multigene family and their function is to catalyze the conjugation of UDP sugars to a broad range of substrates (Bock, 2016). In vertebrates, UGT enzymes are considered to be the most important phase II metabolic enzymes and play important roles in metabolism of endogenous and exogenous compounds (Jancova *et al*., 2010). For instance, the human genome contains 22 UGT encoding genes, which, alongside the phase I metabolic enzymes, are responsible for detoxifying the majority of clinical drugs (Rowland *et al*., 2013).

In insects, considerably higher numbers of UGT encoding genes have been identified. For example, the cotton bollworm *Helicoverpa armigera* and *T. castaneum*, possess 42 and 43 UGT genes, respectively (Ahn *et al*., 2012). In insects, UGTs have several other crucial functions including olfaction, pigmentation, sequestration, and metabolism of plant secondary metabolites (Krempl *et al*., 2016). Although there is only limited

information, a potential role of UGTs in insecticide metabolism has been also implied by several studies which showed that expression of a number of UGT enzymes is increased in insecticide resistant strains compared to sensitive strains (Yang *et al*., 2013b; Riaz *et al*., 2013). The contribution of these upregulated genes to resistance remains to be elucidated.

1.3.5 Phase III metabolism: ATP-binding cassette (ABC) transporters

The final stage of metabolic detoxification of toxic molecules involves excretion of the phase I and II metabolites from the cells (Xu *et al*., 2005). This function is performed by several xenobiotic transporters, among which ABC transporters are the most prominent (Dermauw and Van Leeuwen, 2014). ABC transporters, which use energy in the form of ATP to drive substrates across the cellular membranes, are considered to be one of the largest transporter families in all living organisms (Dean *et al*., 2001).

In animals, ABC transporters are divided into eight subfamilies (denoted as ABC-A through ABC-H) based on their sequence similarities (Merzendorfer, 2014). They can transport an array of substrates such as sugars, peptides, metals, lipids, inorganic ions, xenobiotics, and drugs out of cells (Buss and Callaghan, 2008). Because of their abilities to transport many substrates, ABC transporters are often associated with the multidrug resistance (MDR) phenotype whereby cells display decreased sensitivity to a wide range of toxic molecules, mainly as a result of increased efflux of the molecules (Buss and Callaghan, 2008). This phenomenon is often observed in human tumor cells resistant to multiple chemotherapeutics, and is mainly caused by overexpression of one or more ABC transporters (Schinkel and Jonker, 2012). Likewise, increased expression of ABC transporters is also linked to insecticide resistance in insects (Dermauw and Van Leeuwen, 2014).

The majority of the ABC transporters involved in resistance belong to three subfamilies: ABC-B, ABC-C, and ABC-G (Leslie *et al*., 2005). The proteins of the ABC-B subfamily are also known as MDR proteins or permeability glycoproteins, and were the first ones to be linked to the MDR phenotype (Riordan *et al*., 1985). These transporters have been thoroughly studied in many species, and have been shown to transport a wide range of

substrates, including insecticides (Buss and Callaghan, 2008). Of note, although the chemical structures of molecules transported by MDRs are different, they tend to be moderately lipophilic and have molecular weight of over 300 Da (Hofsli and Nissen-Meyer, 1990; Schinkel and Jonker, 2012).

The second ABC transporter subfamily linked to insecticide resistance is the ABC-C subfamily, whose members are also known as multidrug resistance-associated proteins or MRPs. MRPs and MDRs share similar structures; yet, they do not share high amino acid sequence identities (Cole *et al*., 1992). Further, MRPs differ in their substrate specificity and are mainly involved in efflux of toxic compounds conjugated with polar groups such as glutathione and UDP sugars (Liu *et al*., 2012; Leslie, 2012). Hence, MRPs work in concert with the phase II metabolic enzymes such as GSTs and UGTs to confer resistance to insecticides (Dermauw and Van Leeuwen, 2014). In addition to MDRs and MRPs, members of the ABC-G subfamily have been also implicated in the MDR phenotype. For instance, in humans, overexpression of ABC-G2 plays a role in efflux of drugs used for breast cancer treatment (Kerr *et al*., 2011). In insects, insecticide resistant strains also show increased transcription of ABC-G transporters (Yang *et al*., 2013a,b; You *et al*., 2013). However, the possible contribution of this subfamily to insecticide resistance has been only recently recognized (Merzendorfer, 2014) and functional studies are needed to ascertain their role in resistance.

1.4 Constitutive and induced metabolic resistance to insecticides

An important feature of metabolic resistance to insecticides is the transcriptional up-regulation of detoxifying enzyme and ABC transporter genes in insecticide resistant insects, which usually results in their constitutive overexpression of the aforementioned proteins (Liu, 2015; Li *et al*., 2007). In fact, constitutive overexpression of these genes is arguably the most common mechanism seen in many insecticide resistant insects (Feyereisen, 2012; Li *et al*., 2007). For instance, constitutive overexpression of multiple CYPs, GSTs, esterases, UGTs, and ABC transporters is associated with neonicotinoid resistance in the whitefly, *Bemisia tabaci* (Yang *et al*., 2013b), in the tarnished plant bug, *Lygus lineolaris* (Zhu and Luttrell, 2015), and in the cotton aphid, *Aphis gossypii* (Pan *et*

al., 2015) as well as pyrethroid resistance in the house fly, *Musca domestica* (Gao *et al*., 2012). However, in some insects, constitutive overexpression of the resistance-related genes may come with a fitness cost due to changes in resource and energy reallocation to produce these proteins in high quantities continuously (Kliot and Ghanim, 2012).

In fact, insecticide resistant strains of *B. tabaci* and the brown planthopper, *Nilaparvata lugens,* have lower fecundity, longevity, larval survival rate, and adult emergence rate than those of the susceptible strains (Feng *et al*., 2009; Liu and Han, 2006). Therefore, in some cases, rather than maintaining high levels of proteins all the time, resistance-related genes are induced only upon insecticide exposure. For instance, several studies have shown that exposure to different insecticides induces different detoxifying enzymes and ABC transporter genes in several insects (Liang *et al*., 2015; Le Goff *et al*., 2006; Han *et al*., 2016; Epis *et al*., 2014). This phenomenon has been observed frequently and has been postulated to allow resistant insects to conserve energy in the absence of the chemical stimulus (Terriere, 1984). As with constitutive overexpression, induction of the detoxifying enzymes and ABC transporters also leads to enhanced metabolic detoxification, hence resistance, but it also allows resistant insects to have an adaptive plasticity between conserving energy and survival in the presence of toxic chemicals (Terriere, 1984).

1.5 Sites of metabolic detoxification in insects

In insects, metabolic detoxification of insecticides and plant allelochemicals mainly occurs in three tissues: midgut, fat body, and Malpighian tubules (Yang *et al*., 2007). The midgut, which is one of the largest tissues in insects, is generally likened to mammalian intestine, and it has important roles in digestion and absorption of nutrients from the ingested food (Shen *et al*., 2013). It is also the first tissue where the ingested xenobiotics undergo detoxification (Yang *et al*., 2007). The fat body, on the other hand, is often equated to mammalian liver and plays essential roles in storage of fats, proteins, carbohydrates, and metabolism of foreign compounds (Mittapalli *et al*., 2010). Insect fat bodies form loose lobes or sheets of cells that freely bath in the insect hemolymph. Hence, they can easily take up the toxic molecules from the hemolymph circulating in the

insect body cavity. The third organ involved in insecticide metabolism is the Malpighian tubules which are analogous to mammalian kidneys. These are long, thin, blind-ended tubules lying freely in the insect body cavity, and function in osmoregulation and excretion of waste from the insect body (Dow and Davies, 2006). All these three tissues are able to actively metabolize and excrete insecticides, having enriched expression of xenobiotic detoxifying enzymes and ABC transporters relative to the rest of the insect body (Dow and Davies, 2006; Yang *et al*., 2007).

1.6 Identification of detoxifying enzymes and ABC transporters involved in insecticide resistance

A common feature of all detoxifying enzymes and ABC transporters is their diversification in insects. For instance, the *D. melanogaster* genome contains 85 CYP (Zhou *et al*., 2015), 35 esterase, 38 GST, 34 UGT (Ahn *et al*., 2012), and 56 ABC transporter encoding genes (Dermauw and Van Leeuwen, 2014). Also, many studies have shown that multiple detoxifying enzymes and ABC transporters are probably involved in detoxification of different insecticides (Gao *et al*., 2012; Liu *et al*., 2011). Therefore, identification of the specific genes involved in insecticide resistance can be extremely difficult. One way to overcome such difficulty is to generate transcriptomic data and analyze genome-wide expression profiles. For instance, by comparing the mRNA expression profiles of resistant and sensitive insects, it is possible to identify differentially expressed genes, either constitutively or upon insecticide exposure, between the two strains. From the differentially expressed genes, target genes can be selected and their potential role in resistance can be further investigated.

1.6.1 RNA sequencing (RNA-seq) to identify differentially expressed genes

One of the most commonly used methods to generate transcriptomic data for measuring gene expression is RNA-seq (Wang *et al*., 2009). This method typically involves conversion of a sample RNA (total or mRNA) into cDNA libraries. These libraries are subsequently sequenced on a high throughput sequencing platform such as Illumina's Genome Analyzer. This generates millions of short sequences (30-400 bp), or namely the 'reads', that are then mapped to a reference genome or transcriptome. Then, the number

of reads mapping to each gene or contig from the transcriptome are counted to measure gene expression levels (Wang *et al*., 2009). Finally, a software, such as DESeq (Anders and Huber, 2012), is commonly used to detect the genes whose expression levels differ between experimental and control groups (e.g. resistant and sensitive strains of insects). Once the differentially expressed genes between resistant and sensitive insects are identified, the potential contribution of these differentially expressed genes to insecticide resistance can be investigated using a functional gene analysis tool such as RNA interference (Perrimon *et al*., 2010).

1.7 RNA interference

RNA interference (RNAi) is a eukaryotic cellular response whereby the presence of a double-stranded RNA (dsRNA) molecule in the cells triggers the post-transcriptional degradation of an endogenous mRNA molecule that has similar nucleotide sequences to that of the dsRNA (Fire *et al*., 1998). The RNAi pathway is conserved in a wide range of organisms, including plants (Baulcombe, 2004), insects (Misquitta and Paterson, 1999), nematodes (Fire *et al*., 1998), and fungi (Chang *et al*., 2012), and provides a defence mechanism against virus infection and transposable elements (Cullen, 2014). The pathway is also involved in regulation of gene expression levels (Bartel, 2004).
Briefly, the RNAi pathway involves cleavage of a precursor dsRNA molecule of exogenous or endogenous origin into small dsRNA molecules of approximately 21 to 23 nucleotides by a cytosolic enzyme called Dicer (Bernstein *et al*., 2001). These small dsRNA molecules later interact with a multi-enzyme complex called RNA-induced silencing complex (RISC) (Hammond *et al*., 2000). dsRNA molecules get unwound and one of the strands, named the guide strand, is incorporated into the RISC. The guide strand then directs the RISC to bind to a specific mRNA molecule having nucleotide sequences complementary to the guide strand sequence. Complementary base pairing between the guide strand and target mRNA results in degradation of the mRNA or translational arrest, depending on the degree of complementarity (Liu *et al*., 2004), hence the protein for which the mRNA encodes is no longer produced (Figure 1.2).

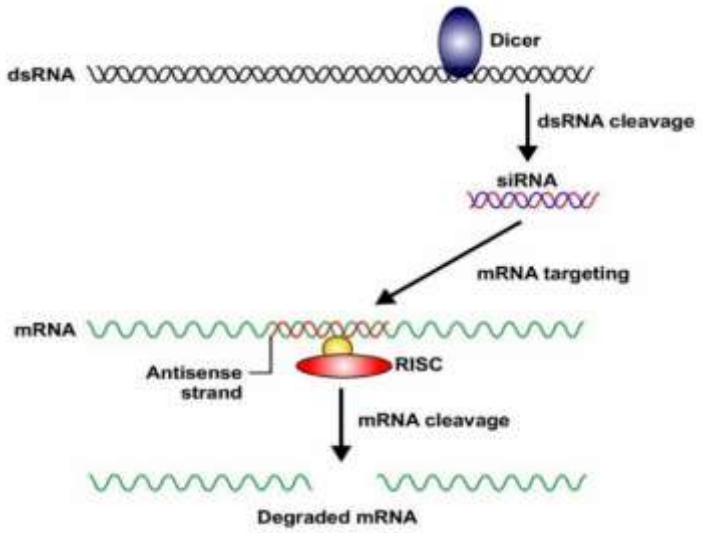


Figure 1. 2. A simplified schematic representation of RNAi-mediated post-transcriptional gene silencing in eukaryotic cells. Long double-stranded RNA (dsRNA) is processed by Dicer enzyme to generate small interfering RNA (siRNA). The antisense strand of siRNA is later used by RNA-induced silencing complex (RISC) to guide mRNA cleavage. Modified with permission from Macmillan Publishing Ltd. [Nature Rev. Genet.] (McManus and Sharp, 2002) copyright (2002).

To date, three major RNAi pathways have been described in eukaryotes: microRNA (miRNA), PIWI-interacting RNA (piRNA), and small interfering RNA (siRNA) (Carthew and Sontheimer, 2009; Thomson and Lin, 2009). These pathways are triggered differently and have various functions. For instance, both piRNA and miRNA precursors are encoded in the genome itself while siRNA is exogenous in origin, derived from dsRNA viruses or dsRNA molecules introduced to the cells experimentally (Carthew and Sontheimer, 2009). In terms of function, both siRNAs and miRNAs regulate gene expression in a post-transcriptional manner. However, the main difference between the two is that the former are highly specific with one mRNA target, while the latter have multiple mRNA targets (Hashimoto *et al*., 2013). The piRNAs, on the other hand, exert their functions mostly in the germline, and play a role in transposon silencing (Holoch and Moazed, 2015). Among the three, the siRNA pathway is of interest in terms of

studying gene function since dsRNA for any gene can be theoretically introduced into cells to induce gene silencing.

1.7.1 RNAi as a tool to study gene function

RNAi can be used as a tool for studying gene function as it allows silencing of a gene without having to mutate the endogenous copy. Since its first discovery in the nematode *Caenorhabditis elegans,* by Fire *et al*. (1998), RNAi has become the method of choice for studying gene function in plants and invertebrates (Katoch *et al*., 2013; Senthil-Kumar and Mysore, 2010). In their ground-breaking work on RNAi, Fire *et al*. (1998) showed that injection of dsRNA molecules corresponding to a target gene in *C. elegans* effectively suppressed the accumulation of endogenous mRNA transcripts *in vivo*. It was also shown that dsRNA-induced gene silencing was systemic and heritable (Burton *et al*., 2011; Fire *et al*., 1998; Kennerdell and Carthew, 2000). Ever since, gene silencing by introduction of an exogenous dsRNA into cells has been widely used in many organisms, including insects, to study the functions of genes involved in many processes such as embryonic development (Angelini *et al*., 2005), behaviour (Nelson *et al*., 2007), and insecticide resistance (Revuelta *et al*., 2009).

1.7.2 dsRNA delivery methods in insects

To date, three major methods have been developed to deliver exogenous dsRNA to the insects. These include injection of dsRNAs into the hemocoel, soaking, and feeding (Katoch *et al*., 2013; Xiong *et al*., 2013). Injection of dsRNA is one of the first methods developed and used by Fire *et al*. (1998) to silence genes in *C. elegans*. This method is highly efficient in supressing gene expression because it allows delivery of dsRNA directly into the target tissue (Yu *et al*., 2013). Another advantage is that the dose of dsRNA can be controlled. However, injections can be invasive and often cause serious mechanical damage, which may influence the experimental outcome (Scott *et al*., 2013; Liu *et al*., 2010). Soaking the insects in a solution containing dsRNA is a less-invasive and more convenient way to deliver dsRNA to insects, but it has limited applications as only certain life stages of insects are amenable to absorbing dsRNA through their cuticle (Katoch *et al*., 2013). Feeding is another efficient and convenient way to deliver dsRNA,

but it requires dsRNA to be taken up by the gut cells. For this method, dsRNA can be produced *in vitro* (Yu *et al*., 2013), in bacteria (Timmons *et al*., 2001), or in transgenic plants (Xiong *et al*., 2013) and fed to the insects. Because of its natural route of entry, RNAi through feeding can also offer a promising pest control strategy as it can be used to suppress expression of essential genes to reduce insect survival or fitness (Burand and Hunter, 2013).

1.7.3 dsRNA uptake by cells

A prerequisite for silencing genes through the siRNA pathway is the uptake of exogenous dsRNA molecules by target cells. This is what is known as environmental RNAi whereby cells take up dsRNA from their environment (Whangbo and Hunter, 2008). This process is mainly accomplished by two uptake mechanisms in invertebrates: endocytosis-mediated and transmembrane channel-mediated uptake (Jose, 2015). The role of endocytosis in dsRNA uptake was initially inferred by studies demonstrating that blocking of the endocytosis pathway impairs RNAi in *D. melanogaster* S2 cell lines (Saleh *et al*., 2006; Ulvila *et al*., 2006). Later, this was also shown to be the main mechanism in several insects including *T. castaneum* (Xiao *et al*., 2015), the Colorado potato beetle, *Leptinotarsa decemlineata* (Cappelle *et al*., 2016), and the desert locust, *Schistocerca gregaria* (Wynant *et al*., 2014).

The second mechanism involves the uptake of dsRNA molecules through two transmembrane proteins, so called systemic RNA interference deficient 1 and 2 (SID-1 and SID-2) proteins. These proteins are required for both environmental as well as systemic RNAi responses. The systemic RNAi occurs when silencing signals spread from one cell to another or from one tissue type to another (e.g. from midgut to fat body in insects), resulting in silencing of the target gene throughout the organism (Whangbo and Hunter, 2008). SID-2 is a gut-specific transmembrane protein in *C. elegans* and is required for initial internalization of dsRNA molecules into gut cells (Winston *et al*., 2007). Hence, it plays a role in uptake of dsRNA from the environment, similar to the endocytosis pathway. However, no insect homologs of SID-2 have been identified so far (Cappelle *et al*., 2016). SID-1, on the other hand, is found in many but not all insects, and

is expressed in all cell types. SID-1 acts as a channel that allows diffusion of dsRNA molecules from one cell to another (Winston *et al*., 2002). Therefore, it plays an important role in systemic RNAi.

1.8 The Colorado potato beetle: an agricultural pest

The Colorado potato beetle, *L. decemlineata* (Say) (Coleoptera: Chrysomelidae), is an oligophagous pest of solanaceous crops which include potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), and eggplant (*Solanum melongena*). The beetle is thought to have originated in central Mexico and the southern United States, where its primary host is buffalobur, *Solanum rostratuand,* and several other species in the Solanaceae (Jacques, 1988). After the start of potato cultivation in the southern United States more than 170 years ago, the beetle rapidly adapted to this new host and became the most important and destructive insect pest of potato (Hare, 1990); hence, earning its common name. The beetle reached Canada more than 130 years ago (Radcliffe and Lagnaoui, 2007) and is now established in most provinces, including Prince Edward Island, Manitoba, Alberta, New Brunswick, Québec, and Ontario where the majority of Canadian potato cultivation occurs (AAFC, 2013). During World War I, the beetle was also introduced to Western Europe, and from there, it rapidly spread to Asia and Western China. Currently, the beetle's range covers about 16 million km^2 and continues to expand (Weber, 2003).

The Colorado potato beetle is an holometabolous insect, meaning that it undergoes complete metamorphosis, with its life cycle including four distinct stages: egg, larva, pupa, and adult (Figure 1.3). The beetle overwinters in the soil as an adult, which then emerges from the soil in the spring (Jacques, 1988). After locating their host plant through walking or flying, the adult beetles start to feed immediately and oviposit within 5-6 days (Alyokhin *et al*., 2008). The females are highly fecund, capable of laying up to 800 eggs during their life span. The eggs are deposited underneath the leaves of the host plant in masses of 20 or more. All eggs within a mass hatch simultaneously within several days and the larvae begin feeding on the host plant. The larva passes through 4 instars within 10-20 days, after which the mature fourth instar larva burrows into the soil

to become a quiescent pre-pupa, and then pupates (Hare, 1990). New adults emerge from the soil in 10-20 days and become reproductively active within 5-7 days. In a year, the beetle can go through one to three overlapping generations depending on the climate (Alyokhin *et al*., 2008). In the fall, the adults enter diapause for overwintering. Entering diapause is facultative, induced by short-day photoperiod. When reared under optimal conditions (25°C, 50% relative humidity, and 16:8 h light:dark photoperiod), the laboratory populations complete their life cycle every four to six weeks.

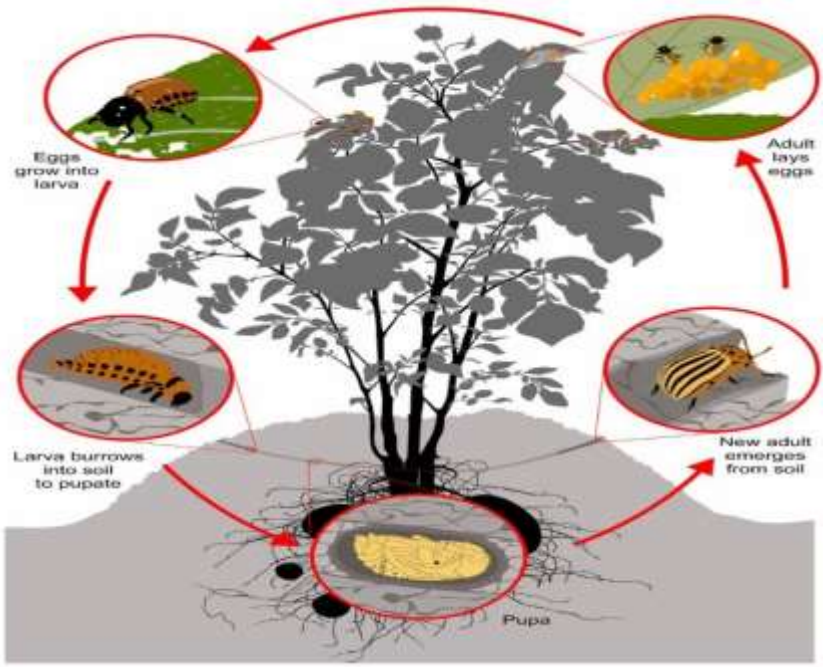


Figure 1. 3. Life cycle of the Colorado potato beetle. Adult beetle emerges from soil and deposits eggs underneath the leaves of the host plant. The eggs hatch and larvae feed on the host plant. Full-grown larva burrows into soil to pupate and new adult emerges.

The Colorado potato beetle is a leaf chewing beetle, and both larva and adult voraciously feed on the leaves of the same host until they are completely defoliated. The fourth instar larva causes the most damage with daily consumption of about 40 cm^2 of foliage, followed by adults consuming about 10 cm^2 of foliage daily (Weber, 2003). Defoliation caused by the beetle decreases the ability of plants to produce nutrients which leads to significant yield losses, reaching up to 64% (Hare, 1980). Although the beetle prefers

potato over other hosts (Hitchner *et al*., 2008), it also causes substantial damage to tomato and eggplant (Alyokhin *et al*., 2008).

1.8.1 Control strategies

To date, many methods have been used to control Colorado potato beetle populations, including cultural control, biological control, and chemical control (Hare, 1990). Cultural control strategies, such as crop rotation, can effectively delay the colonization of potato fields in the spring and reduce the population densities (Sexson and Wyman, 2005). However, due to high beetle mobility, there is a need for considerable separation between the rotated fields to maximize efficiency of this method, which is often not compatible with large scale monoculture practices (Hough-Goldstein and Whalen, 1996). There are also a number of arthropod species that can be used as biological control agents to manage the beetle. These include the predaceous stink bug, *Perillus bioculatus*, the ground beetle, *Lebia grandis*, and the parasitic wasp, *Edovum puttleri* (Olle *et al*., 2015). These natural enemies prey on the eggs or the small larvae and can have a negative impact on the beetle populations. Yet, due to high fecundity of the Colorado potato beetle and difficulty in rearing mass amounts of the natural enemies, this strategy fails to provide economically acceptable levels of control (Alyokhin *et al*., 2008).

Currently, chemical control remains the most efficient and practical way of managing the Colorado potato beetle in the field. In fact, without the use of chemical control, the beetle can completely destroy the plants and reduce the total yield by 40 to 64% (Noronha *et al*., 2002; Hare, 1980). The use of chemical control against the Colorado potato beetle dates back to the 1860s (Alyokhin *et al*., 2008) when farmers used inorganic compounds such as Paris green and lead arsenate to manage beetle infestations. This beetle was also one of the first target pests for the first synthetic insecticide, dichlorodiphenyltrichloroethane (DDT), and currently, more than 30 active ingredients are registered in the United States to control it (Alyokhin *et al*., 2008). These include carbamate, pyrethroid, organophosphate, and neonicotinoid class insecticides. Of these, neonicotinoids represent the most commonly used insecticides around the world, and they are the most effective

insecticides to date for controlling many pests, including the Colorado potato beetle (Jeschke *et al*., 2013).

1.8.1.1 Neonicotinoid insecticides

Neonicotinoid insecticides are nicotine analogues. These molecules mimic the neurotransmitter acetylcholine (ACh) that binds to post-synaptic nicotinic acetylcholine receptors (nAChRs) in the central nervous system. Neonicotinoids exert their effects by blocking the normal functioning of the nervous system, causing paralysis and death (Goulson, 2013). In addition, neonicotinoids have good water solubility, so they can be absorbed by the plant roots or leaves and then transported throughout the plant tissues (Jeschke *et al*., 2011). This feature makes these compounds highly effective against herbivorous insects, especially when they are used in seed treatment and through soil drench applications. Currently, there are seven commercially available neonicotinoid insecticides: imidacloprid, thiamethoxam, clothianidin, acetamiprid, thiacloprid, dinotefuran, and nitenpyram (Simon-Delso *et al*., 2015). Of these, imidacloprid accounts for the greatest proportion of neonicotinoid insecticides sold around the world (Jeschke *et al*., 2013). Neonicotinoids have been an essential component of Colorado potato beetle control since their introduction in the 1990s. For instance, in the United States alone, more than 60% of potato fields were treated with neonicotinoids in 2005 (Szendrei *et al*., 2012).

1.8.2 Insecticide resistance in the Colorado potato beetle

The Colorado potato beetle is notorious for its unprecedented capacity to develop insecticide resistance. The success of the beetle in overcoming man-made toxins is primarily attributed to the fact that it has coevolved with its hosts plants in the family Solanaceae (Alyokhin *et al*., 2015) which produce extremely toxic compounds known as glycoalkaloids (Mithöfer and Boland, 2012). Hence, having a natural ability to detoxify plant toxins probably enhances the beetle's ability to develop resistance to insecticides. In parallel with this notion, the Colorado potato beetle has been shown to have an impressive record of insecticide resistance development from the beginning of the modern insecticide era (Alyokhin *et al*., 2015). Since initial reports of DDT resistance in

the 1950s (Cutkomp *et al*., 1958), the beetle has developed resistance to 56 different insecticides, including neonicotinoids (Whalon *et al*., 2016). In fact, the beetle ranks among the top ten most insecticide resistant arthropod species in the world (Whalon *et al*., 2008).

Currently, insecticide resistant populations are found throughout the beetle's range, with new cases being reported continuously (Alyokhin *et al*., 2015). Obviously, not all populations are resistant to all available insecticides, but some populations do show resistance to multiple insecticides (Olson *et al*., 2000). In particular, populations from the northeast United States have a long history of insecticide resistance (Alyokhin *et al*., 2007; Alyokhin *et al*., 2008), mostly because of extensive use of insecticides in the region. For example, populations from Long Island, New York, have proved to be the most resistant to all classes of insecticides, including neonicotinoids (Alyokhin *et al*., 2015). In fact, the Long Island populations developed resistance to imidacloprid only 2 years after it was registered for the beetle's control (Zhao *et al*., 2000) and showed reduced sensitivity to several other insecticides at the same time (Alyokhin *et al*., 2007; Olson *et al*., 2000). Similarly, in Canada, a survey by Scott *et al*. (2015) detected some populations with resistance and reduced sensitivity to neonicotinoids. Unfortunately, reduced sensitivity often coincides with increased frequency of neonicotinoid applications to obtain effective control of the beetle, which further exacerbates the resistance problem.

1.8.3 Insecticide resistance mechanisms in the Colorado potato beetle

Similar to other insecticide resistant pests, the Colorado potato beetle employs several resistance mechanisms to cope with insecticides; however, metabolic resistance represents the most common mechanism reported (Alyokhin *et al*., 2008). Many studies have shown that metabolic resistance is involved in resistance to carbamate (Rose and Brindley, 1985), pyrethroid (Argentine *et al*., 1989), organophosphate (Ahammad-Sahib *et al*., 1994; Stanković *et al*., 2004), and abamectin (Gouamene-Lamine *et al*., 2003) classes of insecticides. Furthermore, there is accumulating evidence that metabolic resistance is also responsible for neonicotinoid resistance in the beetle (Mota-Sanchez *et*

al., 2006; Zhao *et al*., 2000). However, support for the role of metabolic resistance to many insecticides mainly comes from studies using insecticide synergists that inhibit the activity of the detoxifying enzymes *in vivo* and increase the potency of the insecticides (Metcalf, 1967). For example, use of the GST enzyme inhibitor diethyl malonate increases the toxicity of pyrethroid and organophosphate class insecticides in the resistant beetles (Zamojska *et al*., 2011; Argentine *et al*., 1989). Similarly, using piperonyl butoxide, and S,S,S-tributylphosphorotrithioate, which inhibit CYP and esterase enzymes, respectively, Zhao *et al*. (2000) and Mota-Sanchez *et al*. (2006) showed that imidacloprid resistance can be reduced significantly in the beetle. Furthermore, studies by Zhu *et al*. (2016) and Clements *et al*. (2016a) demonstrated that several genes coding for CYPs, esterases, GSTs, and ABC transporters are overexpressed constitutively or upon imidacloprid exposure in imidacloprid resistant beetle populations compared to sensitive populations. Another study identified a specific CYP gene (*CYP9Z26*) whose constitutive overexpression was associated with imidacloprid resistance in the beetle (Clements *et al*., 2016b).

1.9 Rationale and objectives of the study

Presently, neonicotinoids remain the mainstay for controlling many pests, including the Colorado potato beetle. The emergence of resistance is, however, reducing their efficacy and there is a need to modify control strategies based on a sound understanding of mechanisms governing such resistance. Although there is considerable evidence that neonicotinoid resistance in the Colorado potato beetle is conferred by metabolic resistance, we still have limited information about which genes are involved. Thus, the main goal of my thesis was to gain more knowledge on the genes involved in neonicotinoid resistance in the Colorado potato beetle. To accomplish this, I took a three-step approach. First, I used RNA-seq and qPCR analyses to identify genes encoding detoxifying enzymes and ABC transporters with increased transcription (either constitutively or upon neonicotinoid exposure) in a neonicotinoid resistant strain of the Colorado potato beetle. Second, I used RNAi to knock-down the expression of those genes in the resistant beetles. Finally, I evaluated the phenotypic effects of silencing resistance-related genes on neonicotinoid resistance using toxicity bioassays.

Chapter 2. Materials and Methods

2.1 Beetle strains and rearing conditions

I obtained Colorado potato beetles from colonies maintained at the London Research and Development Centre (LoRDC), Agriculture and Agri-Food Canada (AAFC), London, Ontario. The imidacloprid-susceptible strain, SS, was originally collected around 1990 from an AAFC research farm in London, Ontario (Dr. I. Scott, AAFC, personal communication). This strain has been in continuous culture over 160 generations without pesticide exposure and was used as a susceptible reference strain. The imidacloprid-resistant strain, RS, was originally collected in 1997 from a potato field in Long Island, NY, USA, and was maintained for 51 generations under selection for imidacloprid-resistance at the Department of Entomology, Michigan State University, East Lansing, MI, USA (Wang *et al*., 2016). The strain was obtained by AAFC in 2013 and has been reared for more than 10 generations without insecticide exposure since then. I maintained the beetles on potato plants (*S. tuberosum* var. Kennebec) at 25°C, 50% relative humidity (RH), and 16:8 h light : dark photoperiod following the previously described methods (Harris and Svec, 1976). For all experiments, I used 1-3 day-old mixed-sex adult beetles.

2.2 Topical bioassays to determine LD_{50} of imidacloprid

Previously, using topical exposure methodology, the LD_{50} of imidacloprid for the SS and the RS beetles was determined (Scott *et al*., 2015). To confirm published values, I repeated the topical exposure bioassays with technical-grade imidacloprid (Bayer CropScience Canada, Guelph, Ontario) dissolved in acetone, following the methodology described by Scott *et al*. (2015). Using the experimentally confirmed LD_{50} values, I calculated the resistance ratio between the two strains as LD_{50} for RS/LD_{50} for SS. Also, I calculated sub-lethal doses of imidacloprid for both strains from LD_{50} values so that I could use them in subsequent induction experiments. The sub-lethal doses used in the induction experiments corresponded to 10% of the LD_{50} (0.019 μg beetle^{-1} for the SS and 0.48 μg beetle^{-1} for the RS) as suggested by de Almeida Rossi *et al*. (2013).

2.3 Next-generation sequencing of mRNA (mRNA-seq)

2.3.1 Beetle treatment and dissections

To determine whether expression of detoxifying genes and ABC transporters is induced in the Colorado potato beetle upon imidacloprid exposure, I exposed the beetles topically to either sub-lethal doses of imidacloprid dissolved in acetone or to an acetone-only sham control. I performed the exposures by chilling the beetles on ice for 15 min and then by placing 1 µl of acetone (controls) or 1 µl of 480 µg mL^{-1} imidacloprid for the RS and 1 µl of 19 µg mL^{-1} imidacloprid for the SS (treatments) on the ventral thoracic segments of the beetles, using a 5 µl Hamilton syringe. After the beetles recovered from chilling, I placed five treated beetles per 5 oz cup (Dixie, Georgia-Pacific) and provided them with potato leaves for 36 h. I used a 36 h time point for the induction experiments as previous studies indicated that insecticide-induced expression of detoxifying enzymes are detected after 36 h exposure in other insects (Huang *et al*., 2013). After 36 h of exposure to imidacloprid, I dissected the beetles in Calpode's insect saline (pH = 7.2, 10.7 mM NaCl, 25.8 mM KCl, 90 mM glucose, 29 mM $CaCl_2$, 20 mM $MgCl_2$ and 5 mM HEPES) and isolated midgut, Malpighian tubule, and fat body tissues. I pooled the three tissues, immediately suspended in RNAlater buffer (Ambion, Fisher Scientific), and stored at -80°C until RNA extraction. I combined tissues from five beetles to form a biological replicate and did three biological replicates for each condition per strain, yielding a total of 12 samples.

2.3.2 Total RNA extraction and mRNA-seq

I removed the tissues from RNAlater buffer and homogenized them 10-15 sec in RLT buffer (Qiagen) with 1% β-mercaptoethanol using a Brinkmann Polytron Homogenizer (Rexdale, Ontario, Canada). I extracted total RNA using an RNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions for RNA extraction from animal cells. Then, I assessed the quality and quantity of the total RNA samples using a 2100 Bioanalyzer (Agilent) and a Qubit RNA HS (High Sensitivity) Assay kit (Fisher Scientific), respectively. I diluted the RNA samples to 300 ng μL^{-1} using DEPC-treated water and shipped 15 µL from each sample on dry ice to Génome Québec (Montréal,

Québec) for mRNA sequencing. mRNA library construction, using a TruSeq stranded mRNA kit (Illumina), and sequencing of libraries on the Illumina HiSeq 2000 platform were performed at Génome Québec, using the 100 bp single-end protocol (Illumina).

2.3.3 mRNA-seq data processing and differentially expressed sequence (DESeq) analysis

mRNA-seq reads were mapped to the reference Colorado potato beetle transcriptome downloaded from http://www.bio.unipd.it/~grapputo/CPB-Web page. The reference transcriptome was previously assembled *de novo* from transcriptomic sequence reads by Kumar *et al*. (2014) and contains a total of 121,912 contigs, which are available online in annotated versions. mRNA read processing and mapping were performed by Patrick Chapman, bioinformatics technician at LoRDC, London, Ontario. Prior to mapping, adapter sequences were trimmed using Scythe adapter trimmer version 0.991 (https://github.com/vsbuffa lo/scythe) and the reads having < 20 nt after trimming were discarded. The reads were then mapped to the reference transcriptome using Burrows-Wheeler Alignment (BWA, q=30) (Li and Durbin, 2009) version 0.7.10 to generate Sequence alignment maps (SAMs). SAM-tools version 0.1.19 was then used to remove identical reads (PCR duplicates) and the reads mapping to more than one contig in the reference transcriptome. Finally, uniquely mapping reads were imported to the DESeq package version 1.18.0 (Anders and Huber, 2012) to identify differentially expressed sequences.

To do so, I performed four different comparisons. First, I compared mRNA read counts between treated and control groups in both strains to identify differentially expressed sequences upon imidacloprid exposure. Second, I compared counts between the SS beetles and the RS beetles in control and treated groups to identify differentially expressed sequences between the two strains. Then, I imported the results to Excel files for further analysis. The output from DESeq analysis included normalized mean number of reads assigned to a contig, $\log_2$Fold change, and the statistical significance of the fold change. I defined a contig as differentially expressed if the absolute value of $\log_2$Fold change was ≥ 1 and adjusted *P*-value (*P*adj) was ≤ 0.001 after the Benjamini-Hochberg

false discovery rate (FDR) correction (Benjamini and Hochberg, 1995). Finally, I manually screened the differentially expressed contigs in all comparisons to find the ones encoding detoxifying enzymes and ABC transporters.

2.4 Validation of DESeq results using quantitative PCR (qPCR)

2.4.1 Primer design

After I identified differentially expressed sequences encoding target genes, I validated mRNA-seq results using qPCR. For this purpose, I selected 32 differentially expressed genes and used their sequences from the reference transcriptome (Kumar *et al*., 2014) to design primers. To design primers, I used several online resources, including IDT (http://www.idtdna.com/Primerquest/Home/Index), Eurofins Operon (https://www.operon.com/tools/oligoanalysis-tool.aspx), and Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). I tested primer efficiencies by generating standard curves following the guidelines described by Taylor *et al*. (2010). Also, I had the PCR products sequenced to confirm amplification of correct sequences. Sequencing was performed at the DNA sequencing facility of LoRDC, London, Ontario. The primers used in qPCR analysis are listed in Table 2.1.

Table 2. 1. List of primers used in qPCR analysis.

Contig ID[1]	Gene	Forward and reverse primers (5'-3')	Primer Efficiency (%)	Amplicon size (bp)
Ld_c756	*CYP412A2*	CAGCAAGCAAAGAGCAGAAC CTCAGTAAGCCTTCACCAATAG	97.7	170
Ld_c17267	*CYP9V1*	ATAGTGTCATCCACCAACTCA CAACCCAATAACGAGTTCTATC	92	181
Ld_rep_c33314	*CYP4Q3*	TACCCTGGTGTGAACATTAC AATGAAAGGCTGGTGTCAAG	97.1	178
Ld_rep_c34031	*CYP6BQ15*	TAGGCTGACCCCAACATTCA AATGGAATGGTCCGTGAGGA	93.4	103
Ld_rep_c34168	*CYP4Q7*	CAGCCTAAGACTTCCTTGATG GTTCGAGGGATTTGACACTAC	96.1	193
Ld_c20712	*CYP4G57*	GGGAAATGTGAAACGAGAATGTC TGAACCAGTAGTGATAAGCTGTC	92	133
Ld_rep_c51084	*CYP6K1*	CTTCACCATCCATATCCCTCAT ATGTGCTGTAACTCCTGATCC	93.5	111
Ld_rep_c41850	*CYP6BJ1*	GAACACCAGTGGATTAGGATAG ATTACATCCACCAGCCCATAC	91.4	154
Ld_c571	*MRP-4-2*	TTATCTGAACCAAACCGCTACT ACTTCTTTCACCTCGTTACTC	91	166

Table 2. 1. List of primers used in qPCR analysis.

Contig ID[1]	Gene	Forward and reverse primers (5'-3')	Primer Efficiency (%)	Amplicon size (bp)
Ld_rep_c34742	*MRP-4-3*	ATCGTCACTTTCTCTTTCGTAG GGTTGATAATCTGGAGTGAG	96	197
Ld_rep_c91275	*MRP-4-4*	CAGATAAAGTGCTCGTGATG CTCTTGCTATTCTCCTCAAATG	91	147
Ld_rep_c28427	*ABC-G*	TCACCTCCACTACAGTCAAC GCTCTGGTGGAAAGTCTAAC	99.6	158
Ld_rep_c41594	*UGT 1*	AGCACCACATTTGAGGGTAG GGTGAGTGAAGATGAGATCC	95.9	207
Ld_c190	*UGT 2B5*	TCTCCGAAGAACGGCATAG GAGTCATCTCTTCCCTTGAATGT	95.2	176
Ld_c269	*UGT 4*	CGAAGGTTGATTGGGCAGAA TCTGGACAACGCCAAGAATG	97	166
Ld_rep_c30928	*UGT 2C1*	GCTTCAGAACTGGAATGGTATC TGATGAATCTCAGACGGACAG	94.2	182
Ld_rep_c83152	*UGT 2*	TAGCATCAAACTGGCAACACA AGCACCTACATTTGAGGGTA	98.5	104
Ld_rep_c24170	*GST 1*	CCTTGCTGGTCCTTCTTATC CACAGCCAGCAAGAGGATTA	100.1	183

Table 2. 1. List of primers used in qPCR analysis.

Contig ID[1]	Gene	Forward and reverse primers (5'-3')	Primer Efficiency (%)	Amplicon size (bp)
Ld_rep_c24256	*GST Delta 1*	CTAAAGCCAAATCCGCAAGA TACTCCAGATTTGCTGACTAC	106.2	166
Ld_rep_c41971	*GST Sigma 3*	CATTGGGTTTGTTGGTCGTTTC CAGGATTTCGTGGGCTGATA	107.3	169
Ld_rep_c43735	*GST 5*	CTCCCTTACTGGTCATCCT CACAGCCAGCAAGAATGTTATC	95.3	176
Ld_rep_c40253	*GST Sigma 2*	TGGACTGGTGAGCTACTTTG CCTACTTCAATGTTACCGCTCT	95.7	174
Ld_rep_c48065	*GST Delta 2*	TTGGCGATAACGACTATTGTAG TAGCTTTGACCTTGGCATAC	101.7	141
Ld_rep_c33018	*GST Sigma 1*	TTCACTGGAAGAGCGGAAC CGGCAATGTTGGTTTGAGTT	97.9	111
Ld_rep_c44006	*GST 2*	ACCTGGAATGCTGGACTACA CTTCCACTTTCTGAGTCGG	93	121
Ld_rep_c24217	*Esterase 6*	GAATACATCGCCCACTCTTG CATCTGAGGAAGAAGGCTGA	99	175
Ld_rep_c34698	*Esterase 5*	CCCTTTCGTTGGATTCAGATAC GAAGGAGTCGCTCATCAAGA	97.6	152

Table 2. 1. List of primers used in qPCR analysis.

Contig ID[1]	Gene	Forward and reverse primers (5'-3')	Primer Efficiency (%)	Amplicon size (bp)
Ld_rep_c71421	*Esterase 4*	TATCCACCTCTGCCATCATC GACAGATGAAGGCTGGTAAC	95.7	198
Ld_rep_c77075	*Esterase Beta*	TTACTCGGGTGCAGTGAAATC CAACCATAATATTGCTCATCATTCG	95.6	197
Ld_rep_c53802	*Esterase FE4*	TTACCGTTATGGGCGAAAGT CTAAAGGTGACAAAGGCGA	99.4	119
Ld_rep_c35399	*Esterase 2*	CGTCATCTGCCGTTGTAAGA TACACGCTGGTGGGTATTTC	94.6	138
Ld_c2942	*Esterase 1*	ACCCTGCCACTTTTCCACTT ACTGACACAATCGGTGACG	94.4	177
-	*L8E*	GGTAACCATCAACACATTGG TCTTGGCATCCACTTTACC	97.4	124
-	*ARF1*	GACTGCAAGTAGGAGAAGTTG TCGGCAGAGTCTACCACAT	94.1	181
-	*EF1A*	CAGGGCAAGGTTTGAAAGATAA CCATCAGCACAGTTCCCAT	99.6	168

[1] Contig ID from Kumar *et al*. (2014).

2.4.2 cDNA synthesis and qPCR analysis

I treated total RNA samples with Turbo DNA-free DNase (Ambion, Fisher Scientific) according to the manufacturer's protocol to eliminate contaminating genomic DNA (gDNA). I verified the quality of DNase-treated RNA samples using the 2100 Bioanalyzer (Agilent). Then, I confirmed the absence of gDNA contamination by testing of no-reverse transcriptase (NRT) controls. I synthesized cDNA using 6 µg of RNA from each sample in a 120 µL reaction volume using a Superscript III First-Strand Supermix Kit (Invitrogen) as directed by the manufacturer.

I performed qPCR reactions using a SensiFAST SYBR No-ROX Mix Kit (Bioline), forward and reverse primers at 500 nM each and 2.5 µL of a 1:2 dilution of cDNA template in 10 µL reactions. I used a two-step cycling profile recommended by the kit (95°C for 2 min for one cycle, and 95°C for 5 s and 60°C for 30 s for 40 cycles) for quantitation of targets using a CFX96 Real-Time Detection System (Bio-Rad). I also performed melt-curve analysis (65-95°C for 5 s, with a 0.5°C increment) for each qPCR run to confirm amplification of a single product as well as no template controls (NTCs) to ensure reagents were free of contaminants. Three biological replicates were done for each condition and all samples were run in technical triplicate.

I normalized transcript abundance of target genes to the geometric mean of three endogenously expressed reference genes: *ribosomal protein* (*L8E*) (Zhang *et al*., 2008), *ADP-ribosylation factor 1* (*ARF1*), and *translation elongation factor 1α* (*EF1A*) (Shi *et al*., 2013). I checked the stability of reference genes using CFX Manager Software (Bio-Rad) following the guidelines described by Taylor *et al*. (2010). I estimated the relative transcript difference for target genes in the RS beetles and the SS beetles using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). All real-time qPCR data were expressed as the mean ± standard error of mean (SEM). Also, I performed *t*-tests using statistical computing language R (R Development Core Team, 2015) to determine statistical significance of changes in the expression levels of the target genes.

I validated mRNA-seq data using qPCR in two different ways. First, I used the RNA samples sent for mRNA-seq to verify the expression of 32 differentially expressed genes. I selected these genes based on their fold changes and normalized mean number of reads according to the mRNA-seq results. Of the 32 genes tested, I selected 10 genes, which showed the greatest increase in the mRNA levels in the RS beetles compared to the SS beetles according to qPCR results. Second, I repeated the RNA extraction and cDNA synthesis as described previously and re-analyzed the expression of the 10 genes to confirm the results on independent biological samples. After qPCR re-confirmation, I chose these 10 genes for RNAi silencing experiments.

2.5 Silencing of target genes using RNAi

2.5.1 Bacterial strain and growth conditions

I used *Escherichia coli* HT115 (DE3) for production of dsRNA for RNAi. This strain has the genotype F^-, *mcrA, mcrB, IN*(*rrnD-rrnE*)*1, lambda*$^-$*, rnc14::Tn10*(DE3 lysogen:*lavUV5* promoter-T7 polymerase) (Timmons *et al*., 2001). The gene encoding the enzyme RNase III, which plays a role in dsRNA degradation in bacteria, is disrupted in this strain by a Tn10 transposon carrying a tetracycline-resistance marker. I grew *E. coli* HT115 at 37°C in Luria Bertani (LB) medium, pH = 7.0, supplemented with appropriate antibiotics. I obtained solid media by the addition of 1% (w/v) Agar (EMD chemicals) to LB. I transformed *E. coli* HT115 with plasmid constructs using standard heat shock transformation methods (Froger and Hall, 2007). I used antibiotics at the following concentrations for selection of *E. coli* HT115: tetracycline (12.5 μg mL^{-1}) and ampicillin (100 μg mL^{-1}).

2.5.2 RNAi vector L4440

I used the L4440 plasmid for cloning of fragments from target genes to produce dsRNA in *E. coli* HT115. This plasmid contains two T7 polymerase promoters in opposite orientation, separated by a multi-cloning site (MCS) where gene fragments can be cloned (Timmons *et al*., 2001). The plasmid also carries ampicillin resistance gene *β-lactamase* (amp^R) for selection and was a gift from Andrew Fire (available from Addgene, plasmid # 1654). The basic structure of the plasmid is shown in Figure 2.1.

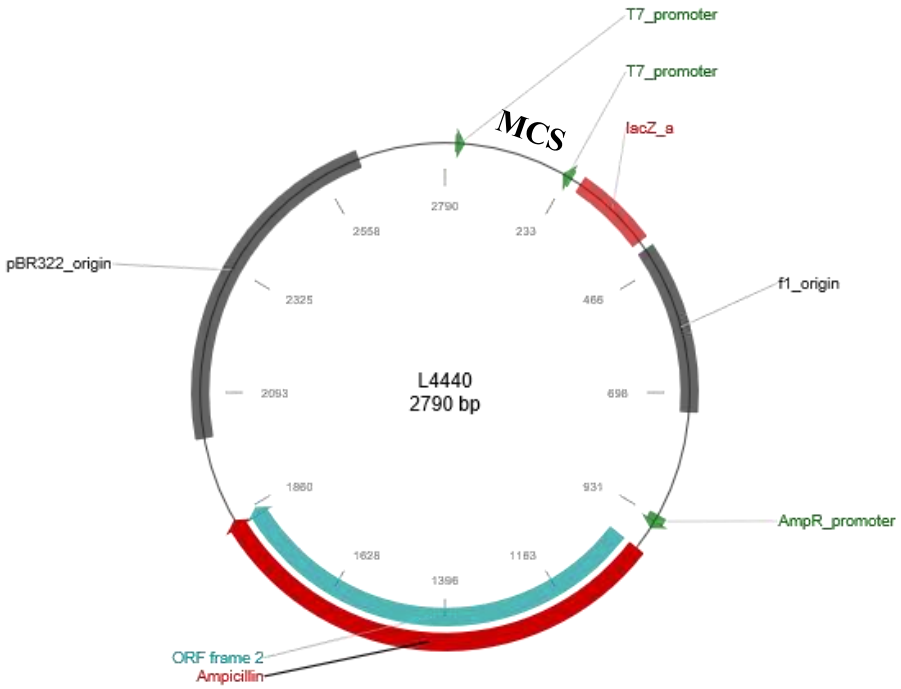


Figure 2. 1. Basic structure of L4440 used for cloning of gene fragments for dsRNA production in *E. coli* HT115. The plasmid contains Multi-cloning site (MSC) which is flanked by two T7 promoters, allowing transcription of cloned fragments from both ends for dsRNA production. Vector map was adapted and modified from: http://www.addgene.org/1654/.

2.5.3 Cloning of gene fragments into L4440

I used standard restriction enzyme cloning techniques (https://www.addgene.org/plasmid-protocols /subcloning/) to clone fragments from the 10 selected genes and a *green fluorescent protein* (*GFP*) gene into L4440 plasmid. First, I selected target regions from the 10 genes and *GFP* using the E-RNAi web tool (Horn and Boutros, 2010) which allows design of optimized dsRNA constructs for efficient silencing. Then, I designed primers with restriction enzyme cut sites to amplify 380 - 430 bp PCR products. All primer pairs contained a *Not*I cut site on the forward primer (5'-GCGGCCGC-3') and a *Sal*I cut site on the reverse primer (5'-GTCGAC-3'), at the 5' end. The primers used in cloning are listed in Table 2.2.

I used the cDNA extracted from the RS beetles or GFP::L4440 plasmid (containing the full-length *GFP* gene sequence; available from Addgene, plasmid # 11335) as template to amplify target regions. I performed PCR reactions in 200 µL volume using 2 U Platinum Taq DNA polymerase (Invitrogen), 1× PCR buffer, forward and reverse primers at 400 nM each, 200 nM dNTP mix, 1.5 mM $MgCl_2$, and 4 µL 1:10 dilution of cDNA or GFP::L4440 as template. I used a Veriti Thermal Cycler (Applied Biosystems) with the following temperature profile: 94°C for 3 min initial denaturation, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, then 72°C for 7 min final extension and held at 4ºC. Then, I analyzed the PCR products by electrophoresis through 1.5% agarose (w/v) gels in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA). I stained the gels with GelGreen (Biotium) and visualized bands using a Gel Doc XR+ System (BioRad). I extracted the PCR products from the gels using a QIAquick Gel Extraction Kit (Qiagen) and quantified them using a NanoDrop spectrophotometer (Thermo Scientific).

Then, I performed double digestions of L4440 plasmid and purified PCR products with restriction enzymes *Not*I and *Sal*I (New England Biolabs). I carried out the reactions in 50 µL volumes containing 6 µg plasmid or PCR product, 40 U of *Sal*I and *Not*I, and 1× NEBuffer 3.1 at 37°C overnight. The reactions were stopped by incubating tubes at 65°C for 20 min. I subsequently separated the digested DNA on 1.5% agarose (w/v) gels and extracted the bands using the QIAquick Gel Extraction Kit (Qiagen). I ligated the digested PCR fragments into linearized plasmid in a 10 µL reaction volume using a molar ratio of 3:1 insert to vector at 4°C overnight using the T4 DNA ligase Kit (Invitrogen) in accordance with the manufacturer's instructions. I transformed *E. coli* HT115 with 3 µL of the resulting ligation reaction and grew the cells on LB agar plates containing ampicillin and tetracycline. I identified positive colonies by PCR, using the same primers as those used in cloning.

I grew the positive colonies overnight in 5 mL of LB with ampicillin and tetracycline at 37°C in a shaker incubator and extracted plasmid from 4 mL culture using a QIAprep Spin Miniprep Kit (Qiagen). I confirmed cloning of the correct sequences into L4440 through sequencing of the inserts using plasmid-specific forward and reverse primers

(Table 2). After sequence verification, I prepared *E. coli* HT115 glycerol stocks from the positive colonies by mixing of 500 µL of overnight culture with 500 µL of 50% sterile glycerol. The glycerol stocks were stored at -80°C for future use. The plasmid constructs used to produce dsRNA for the target genes are listed in Table 2.3.

Table 2. 2. List of primers used in cloning and sequencing of plasmid constructs.

Contig ID[1]	Gene	Forward and reverse primers (5'-3')[2]	Product size (bp)
Ld_rep_c34031	*CYP6BQ15*	TAGCGGCCGCAACATCCTCACGGACCATTC ACAGGTCGACGGGTGCCTTAATTTCGATTTC	420
Ld_c17267	*CYP9V1*	TAGCGGCCGCGCATTTTGCTGCTTGTGAAG ACAGGTCGACATAGTGGCCGCCCTGTATTA	400
Ld_rep_c34168	*CYP4Q7*	TAGCGGCCGCCATCTCCTGACGTCCGAATC ACAGGTCGACTGAATCGCTGGCTAGGAGAAG	387
Ld_rep_c33314	*CYP4Q3*	TAGCGGCCGCTTGGACCAGCAATCGCCT ACAGGTCGACTCGCACGAAAACACTTCAAA	413
Ld_c2942	*Esterase 1*	TAGCGGCCGCTCGAATCCAACAAGTGGTGA ACAGGTCGACGCCGCTGAAACCTGGTAGTA	408
Ld_rep_c35399	*Esterase 2*	TAGCGGCCGCTTCAATTCAGCGGTATGTGC ACAGGTCGACCCAGGCACCATTATTGACT	400
Ld_c571	*MRP-4-2*	TAGCGGCCGCGTGTCTGTTGGAAACCCCAT ACAGGTCGACGAATCCGTTTGGATCATCAGC	424
Ld_rep_c28427	*ABC-G*	TAGCGGCCGCTGGTGACTTTTCCACTGGG ACAGGTCGACGAAGAGTGTCCTTTGCCTC	384
Ld_rep_c41594	*UGT 1*	TAGCGGCCGCTCACTCATGGCGGTTTGTTG ACAGGTCGACGACGCTAGCATCAAACTGGC	409

Table 2. 2. List of primers used in cloning and sequencing of plasmid constructs.

Contig ID[1]	Gene	Forward and reverse primers (5'-3')[2]	Product size (bp)
Ld_c190	*UGT 2B5*	TAGCGGCCGCTTTCCATCTCCGCATGAAAT ACAGGTCGACTAGCTATGCCGTTCTTCG	404
-	*GFP*	TAGCGGCCGCCCATGCCCGAAGGTTATGTA ACAGGTCGACGGACAGGTAATGGTTGTCTGG	449
-	L4440 sequencing primers	GACCGGCAGATCTGATATCATC CTCACTGGCCGTCGTTTTAC	-

[1]Contig ID from Kumar *et al*. (2014); [2]Restriction enzyme cut sites are underlined.

Table 2. 3. Plasmid constructs used in this study.

Plasmid[1]	Description	Source
L4440, Amp^R	RNAi feeding vector, empty backbone	Addgene plasmid # 1654
GFP::L4440, Amp^R	Contains full length *GFP* sequence	Addgene plasmid # 11335
GFP-RNAi::L4440, Amp^R	dsRNA production for *GFP* control	This study
CYP4Q3::L4440, Amp^R	dsRNA production for *CYP4Q3*	This study
CYP4Q7::L4440, Amp^R	dsRNA production for *CYP4Q7*	This study
CYP9V1::L4440, Amp^R	dsRNA production for *CYP9V1*	This study
CYP6BQ15::L4440, Amp^R	dsRNA production for C*YP6BQ15*	This study
MRP-4-2::L4440, Amp^R	dsRNA production of *MRP-4-2*	This study
ABC-G::L4440, Amp^R	dsRNA production for *ABC-G*	This study
UGT 1::L4440, Amp^R	dsRNA production for *UGT 1*	This study
UGT 2B5::L4440, Amp^R	dsRNA production for *UGT 2B5*	This study
Esterase 1::L4440, Amp^R	dsRNA production for *Esterase 1*	This study
Esterase 2::L4440, Amp^R	dsRNA production for *Esterase 2*	This study

[1]Amp^R is resistance to ampicillin (β-lactamase)

2.5.4 Induction of *E. coli* HT115 to produce dsRNA

I scraped *E. coli* HT115 cells from the glycerol stocks using a sterile pipette tip and inoculated cells into 15 mL of LB containing ampicillin and tetracycline. I incubated the cultures overnight at 37°C with shaking at 220 rpm. Then, I inoculated 10 mL of the overnight culture into 1 L of LB containing antibiotics and grew the cells at 37°C with shaking at 220 rpm until optical density at 600 nm (OD_{600}) was 0.4 - 0.6. At this point, I transferred 5 mL of the culture into a 15 mL culture tube and incubated further for 6 h to have an un-induced control. To the remaining culture, I added isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM to induce production of dsRNA in the *E. coli* HT115 cells. I grew the cells for 6 h after addition of IPTG. At the end of 6 h incubation, I removed 1 mL of induced and un-induced cultures and centrifuged at 10,000 g for 5 min. Then, I re-suspended the pellets in 150 µL RNAlater buffer (Ambion, Fisher Scientific) and stored at 4°C for total nucleic acid extraction to confirm production of dsRNA. I centrifuged the remaining induced culture at 10,000 g for 10 min at 4°C and washed the pellet once with 20 mL of 1× PBS (pH = 7.4, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Then, I re-suspended the pellet in 100 mL of 1× PBS buffer to concentrate the culture 10×. I aliquoted the concentrated cultures in 10 mL volumes and stored them at -80°C until dsRNA feeding assays were done.

2.5.5 Confirmation of dsRNA in *E. coli* HT115

I confirmed the production of dsRNA in *E. coli* HT115 by extracting total nucleic acid from the bacteria using a MasterPure Complete DNA and RNA Purification Kit (Illumina). I treated the total nucleic acids with Turbo DNA-free DNase (Ambion, Fisher Scientific) to eliminate contaminating DNA. Finally, I separated the samples on 1.5% agarose (w/v) gels, stained the gels with GelGreen (Biotium), and visualized the bands corresponding to dsRNA fragments using a Gel Doc XR+ System (BioRad).

2.5.6 dsRNA feeding assays to silence expression of target genes in the RS beetles

I performed dsRNA feeding assays using the protocol described by Wan *et al*. (2014), with minor modifications. First, I thawed the 10 mL *E. coli* HT115 aliquots at room temperature. Then, I dipped potato leaves into the *E. coli* HT115 suspensions, containing dsRNA for the selected genes or *GFP* (negative control), or into 1× PBS buffer (negative control). Next, I dried the leaves under airflow on a metal mesh for 1 h and placed one leaf per Petri dish (50 × 9 mm) lined with moist filter paper (Whatman qualitative no. 5). Then, I starved the RS beetles for 2 h and placed one beetle per Petri dish. I allowed the beetles to feed *ad libitum* on the treated leaves for four days. I replaced the leaves with new treated ones every day to ensure the beetles received a continuous supply of dsRNA. After four days, I dissected the beetles and collected midgut, Malpighian tubules, and fat body tissues as described previously. I pooled tissues from three beetles to form a biological replicate and I repeated each treatment three times. I performed the total RNA extraction, cDNA synthesis and qPCR reactions as described previously to confirm silencing of target genes in the RS beetles. I analysed the mRNA transcript levels of target genes in the RS beetles fed on 1× PBS, dsRNA for *GFP*, or dsRNA for the target genes using one-way ANOVA followed by Tukey's HSD *post hoc* analysis in R (R Development Core Team, 2015). Then, I selected the genes that were silenced significantly for further analysis to investigate their potential role in imidacloprid resistance.

To silence two genes simultaneously, I grew two strains of *E. coli* HT115 to produce dsRNA for two target genes in separate flasks as described previously. After the 6 h induction, I concentrated the cultures 10× and then mixed them at a 1:1 ratio prior to feeding as described previously.

2.6 Phenotypic effects of silencing selected genes on imidacloprid resistance

I repeated the dsRNA feeding bioassays on a second group of RS beetles as described previously, with minor modifications, to determine if silencing of the targeted genes increased the susceptibility of the RS beetles to imidacloprid. In this set of experiments, I fed the RS beetles with potato leaves dipped in a suspension of *E. coli* HT115 (control) or

in *E. coli* HT115 producing dsRNA for *GFP* or for the target genes. This time, I placed 13 treated leaves in a large Petri dish (90 × 15 mm) lined with a moist filter paper (Whatman qualitative no. 5). Then, I starved the beetles for 2 h and then placed 13 beetles per Petri dish. As before, the beetles fed on the treated leaves *ad libitum* for four days. After 4 days, I randomly selected 3 out of 13 beetles for dissection to isolate midgut, Malpighian tubules, and fat body tissues, and then processed them as before for RNA extraction, cDNA synthesis, and qPCR analysis to confirm gene silencing. I topically exposed the remaining 10 beetles to 2.7 µg beetle^{-1} of imidacloprid (LD_{20} for RS beetles) as described previously. I provided the beetles with fresh treated leaves daily after imidacloprid exposure and monitored survivorship daily for seven days. I repeated each experiment three times. I counted the beetles that were moribund and dead at the end of seven days using the criteria described by Zhao *et al*. (2000) and performed Kaplan-Meier survival analysis and Log-rank tests in R (R Development Core Team, 2015) to determine whether differences existed in survival between the control and treatment groups. I anticipated a 20% mortality in the control groups and attributed any significant increase from 20% in mortality in the experimental groups to the fact that expression of the target gene was silenced. I chose the LD_{20} for the bioassays as this dose causes an observable toxicity to the beetles, but it is low enough that a wide range of increased mortality can be measured. I determined the LD_{20} of imidacloprid experimentally by exposing beetles to several doses of imidacloprid (4.8 µg beetle^{-1}, 3 µg beetle^{-1}, and 2.7 µg beetle^{-1}) using topical exposures and by monitoring survival for seven days.

Chapter 3. Results

3.1 Topical bioassays to determine LD_{50} of imidacloprid

I experimentally determined the LD_{50} of imidacloprid for the SS and RS beetles to be 0.19 μg beetle^{-1} and 4.8 μg beetle^{-1}, respectively. The LD_{50} of the RS beetles was the same as the published values (Scott *et al*., 2015) whereas the LD_{50} value for the SS beetles was higher (0.19 μg beetle^{-1} versus 0.052 μg beetle^{-1}) than the published value (Scott *et al*., 2015). From the LD_{50} values, I calculated the resistance ratio of the RS to be 25.3.

3.2 mRNA sequencing

3.2.1 Read processing and quality control

In total, mRNA sequencing of 12 libraries yielded 733,519,988 reads. The number of raw reads per library ranged from 53.96 to 71.62 million, with a mean of 61.13 million (Table 3.1). The proportion of reads per sample mapping to the reference transcriptome ranged from 85.5% to 88.9%. The number of uniquely mapping reads per library ranged from 20.12 to 25.75 million, with a mean of 22.67 million (Table 3.1). A total of 65.92 million reads from RS control, 65.75 million reads from RS treated, 64.40 million reads from SS control, and 75.83 million reads from SS treated were used for DESeq analysis.

Table 3. 1. Summary of RNA-seq data before and after mapping.

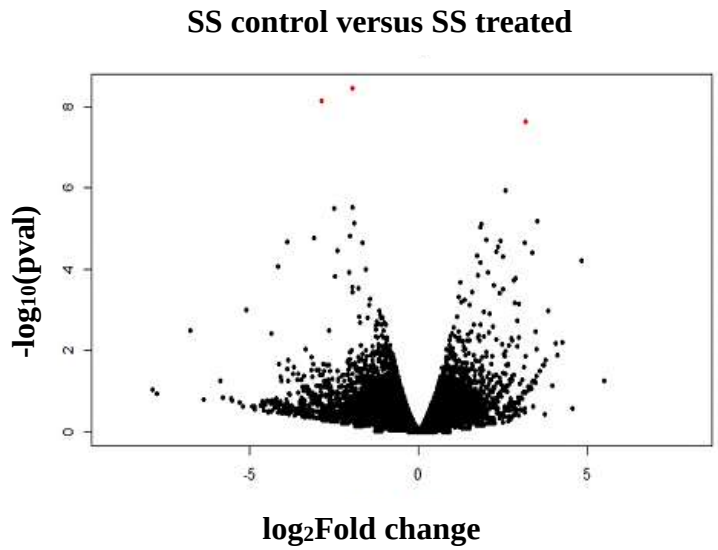
Sample	Total raw reads	Mapped reads	% of mapped reads	Uniquely mapped reads	% of uniquely mapped reads
RS control biorep1	58,892,932	51,250,746	87.0	22,586,506	44.1
RS control biorep2	62,045,496	53,781,131	86.7	22,718,902	42.2
RS control biorep3	55,059,038	48,065489	87.3	20,613,349	42.9
RS treated biorep1	56,314,953	49,728,565	88.3	20,880,310	42.0
RS treated biorep2	59,390,168	52,781,462	88.9	21,679,808	41.1
RS treated biorep3	61,402,233	54,166,043	88.2	23,186,761	42.8
SS control biorep1	59,130,951	51,889,523	87.7	21,596,640	41.6
SS control biorep2	61,911,228	53,098,114	85.8	22,677,904	42.7
SS control biorep3	53,963,077	46,221,323	85.6	20,124,899	43.5
SS treated biorep1	67,965,501	59,293,557	87.4	24,536,335	41.4
SS treated biorep2	71,622,431	62,820,839	87.7	25,748,613	41.0
SS treated biorep3	65,821,980	56,273,024	85.5	25,551,793	45.4

3.3 Imidacloprid-induced transcriptomic responses in two strains of Colorado potato beetle

I monitored the transcriptomic changes in the SS and the RS beetles in response to imidacloprid exposure. I identified three differentially expressed contigs in the SS beetles 36 h after exposure to imidacloprid (Figure 3.1A). Of these, one encoding a long-chain specific Acyl-CoA dehydrogenase was up-regulated while another encoding a 40S ribosomal protein was downregulated (Table 3.2). The third contig was down-regulated and was an unknown sequence.

In the RS beetles, however, I identified a total of 56 differentially expressed contigs 36 h after exposure to imidacloprid. Of these, 17 were up-regulated and 39 were down-regulated (Figure 3.1.B). mRNA transcript levels of several genes such as pancreatic triacylglycerol lipase, tetrahydrofolate ligase, alcohol dehydrogenase, sphingolipid delta 4 desaturase, and mono-carboxylate transporter were upregulated. Conversely, a number of contigs encoding detoxifying enzymes (CYP4G57 and UGT 2A2) and several protease inhibitors (serpin and trypsin inhibitors) were downregulated upon imidacloprid exposure (Table 3.2).

A)



B)

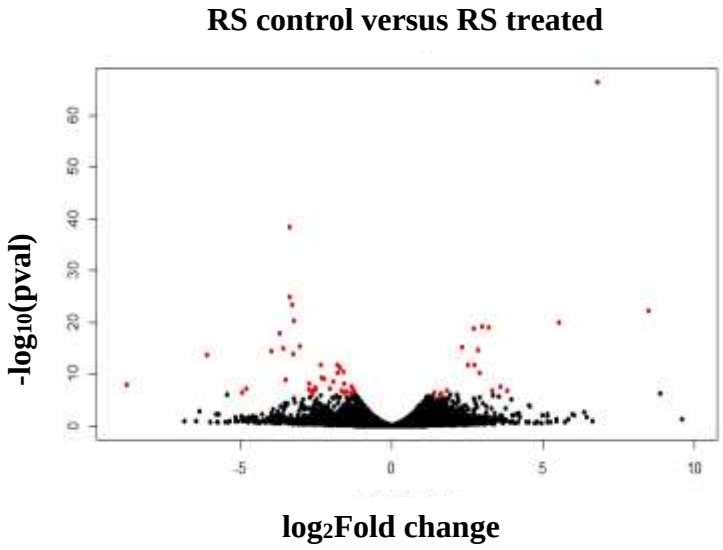


Figure 3. 1. Volcano plots reporting differentially expressed contigs in two strains of Colorado potato beetle after 36 h exposure to sub-lethal doses of imidacloprid.
A) Comparison between SS control and SS treated groups. **B)** Comparison between RS control and RS treated groups. Contigs that are differentially expressed at FDR of ≤ 0.001 and fold change of $\left|\log_2\right| \geq 1$ are coloured red.

Table 3. 2. Differentially expressed contigs in the SS and RS beetles 36 h after exposure to sub-lethal doses of imidacloprid.

Contig ID[1]	Read count Control[2]	Read count Treated[2]	Log_2Fold Change	*P*-adj[3]	Sequence description	Regulation
SS control versus SS treated comparison						
Ld_rep_c41674	426.00	108.42	-1.97	3.00E-04	40S ribosomal protein S23	down
Ld_c118603	50.25	6.77	-2.89	3.00E-04	NA	down
Ld_rep_c81084	12.15	108.92	3.16	6.00E-04	Long-chain specific Acyl-CoA dehydrogenase	up
RS control versus RS treated comparison						
Ld_c3106	64.55	421.54	2.71	1.50E-15	Pancreatic triacylglycerol lipase	up
Ld_c5123	27.02	211.67	2.97	8.34E-16	NA	up
Ld_c14428	101.57	271.08	1.42	6.57E-04	Hypothetical alcohol dehydrogenase	up
Ld_c18286	7.85	58.50	2.90	2.23E-07	NA	up
Ld_rep_c28987	77.06	236.88	1.62	8.70E-04	Sphingolipid delta 4 desaturase	up
Ld_rep_c39453	3.79	53.66	3.82	3.41E-04	NA	up
Ld_rep_c39694	0.32	112.48	8.48	1.11E-18	Hypothetical protein	up
Ld_rep_c42473	16.83	120.06	2.83	1.72E-11	Pancreatic triacylglycerol lipase-like	up
Ld_rep_c46724	175.54	1175.70	2.74	7.46E-09	Cysteine rich protein	up
Ld_rep_c54636	1.39	64.38	5.53	1.21E-16	Signal recognition particle receptor Beta subunit	up

Table 3. 2. Differentially expressed contigs in the SS and RS beetles 36 h after exposure to sub-lethal doses of imidacloprid.

Contig ID[1]	Read count Control[2]	Read count Treated[2]	Log_2Fold Change	*P*-adj[3]	Sequence description	Regulation
RS control versus RS treated comparison						
Ld_c63048	4.91	48.81	3.31	2.98E-04	Golgin subfamily A member 4 like protein	up
Ld_c65715	18.51	105.81	2.52	7.06E-09	Pancreatic triacylglycerol lipase	up
Ld_rep_c70589	4.72	523.87	6.80	3.63E-62	Tetrahydrofolate ligase	up
Ld_c86547	31.74	112.74	1.83	3.38E-04	Laccase 2	up
Ld_rep_c86591	68.39	340.23	2.31	5.45E-12	Tetrahydrofolate ligase	up
Ld_rep_c87745	2.34	28.47	3.60	6.35E-05	Uncharacterized AB hydrolase domain-containing protein	up
Ld_rep_c115399	16.21	150.60	3.22	1.25E-15	Mono-carboxylate transporter	up
Ld_c6999	68.77	16.58	-2.05	1.46E-04	NA	down
Ld_c13969	5260.31	501.98	-3.39	3.39E-21	Serpin B3 predicted	down
Ld_c15254	335.58	49.85	-2.75	2.02E-04	Diapause-associated transcript-2	down
Ld_c15570	415778.30	164283.94	-1.34	6.50E-05	Galactose specific C type lectin	down
Ld_c20196	520.11	208.45	-1.32	6.67E-04	Delta-1-pyrroline-5-carboxylate synthase	down
Ld_c20531	137.77	23.35	-2.56	8.68E-05	Diapause-associated transcript-2	down
Ld_c20712	109.07	21.93	-2.31	1.83E-06	CYP4G57	down
Ld_c22416	6559.04	2068.45	-1.66	3.04E-04	NA	down

Table 3. 2. Differentially expressed contigs in the SS and RS beetles 36 h after exposure to sub-lethal doses of imidacloprid.

Contig ID[1]	Read count Control[2]	Read count Treated[2]	Log_2Fold Change	*P*-adj[3]	Sequence description	Regulation
RS control versus RS treated comparison						
Ld_c22787	8308.68	842.48	-3.30	1.03E-19	Serpin peptidase inhibitor	down
Ld_c23576	54.86	3.45	-3.99	2.61E-11	Chitin synthase 2	down
Ld_rep_c25416	130.67	25.57	-2.35	8.54E-09	Uncharacterized protein	down
Ld_rep_c25784	1904.89	579.04	-1.72	2.82E-08	Protease inhibitor	down
Ld_rep_c28188	1318.66	377.82	-1.80	7.06E-09	PR-5-like protein	down
Ld_rep_c30928	461.78	180.49	-1.36	8.21E-04	UGT 2A2-like isoform X1	down
Ld_rep_c33837	117.50	30.39	-1.95	9.20E-06	Chitinase 4 isoform	down
Ld_rep_c35072	271.94	0.62	-8.77	2.74E-05	Cysteine proteinase	down
Ld_rep_c35635	721.44	87.98	-3.04	3.18E-12	Long-chain-fatty-acid-CoA	down
Ld_rep_c35761	36.11	3.17	-3.51	3.33E-06	Odorant binding protein	down
Ld_rep_c37170	45.00	7.92	-2.51	2.42E-04	Glutathione synthetase-like	down
Ld_rep_c38258	1592.46	535.20	-1.57	7.12E-04	Chitotriosidase-1 predicted	down
Ld_rep_c38340	113.16	18.30	-2.63	3.82E-04	Eukaryotic translation initiation factor 4E-1A	down
Ld_rep_c41749	513.12	203.84	-1.33	7.12E-04	Pyruvate dehydrogenase E1 component subunit alpha	down
Ld_rep_c43253	3667.19	1530.68	-1.26	3.81E-04	Serine protease inhibitor like	down
Ld_c43259	336.75	95.81	-1.81	1.87E-07	Bovine pancreatic trypsin inhibitor domain protein	down

Table 3. 2. Differentially expressed contigs in the SS and RS beetles 36 h after exposure to sub-lethal doses of imidacloprid.

Contig ID[1]	Read count Control[2]	Read count Treated[2]	Log_2Fold Change	*P*-adj[3]	Sequence description	Regulation
RS control versus RS treated comparison						
Ld_rep_c45255	194.67	41.41	-2.23	2.68E-06	Ubiquitin isoform X22	down
Ld_rep_c49805	31.49	1.02	-4.95	7.59E-04	Putative serine/threonine-protein kinase	down
Ld_rep_c50326	887.39	132.38	-2.74	2.22E-05	Cysteine knot toxin	down
Ld_rep_c55354	1146.11	94.30	-3.60	7.92E-12	Serpin B3 predicted	down
Ld_rep_c59515	337.14	35.05	-3.27	8.08E-11	Serpin 1	down
Ld_c70373	19.43	0.69	-4.81	1.37E-04	NA	down
Ld_c77462	14.31	0.00	NA	3.21E-04	NA	down
Ld_c81761	8448.02	808.21	-3.39	1.73E-34	Serpin	down
Ld_rep_c85571	209.09	74.10	-1.50	4.57E-04	Hypothetical protein	down
Ld_c86940	33563.34	11017.56	-1.61	1.29E-07	Macrophage mannose receptor	down
Ld_c87632	150320.21	49979.27	-1.59	1.79E-05	Myb-like protein P-like	down
Ld_rep_c89615	34.04	5.52	-2.62	9.06E-04	Glucose dehydrogenase	down
Ld_c106517	89.16	6.86	-3.70	1.16E-14	NA	down
Ld_rep_c112150	47.65	0.69	-6.10	1.27E-10	NA	down
Ld_rep_c117290	1362.39	143.47	-3.25	7.88E-17	Serpin peptidase inhibitor 18	down

[1]Contig ID from Kumar *et al*. (2014); [2] Read counts represent mean normalized counts from three biological replicates; [3]Adjusted P-value corrected for false discovery rate; NA = not available.

3.4 Differentially expressed sequences between the SS and the RS beetles

I analysed differentially expressed sequences between the SS and the RS beetles to identify transcriptomic differences between the two strains. For this purpose, I compared the mRNA-seq reads from the two strains using the reads from the control and the treatment groups separately. When I compared the reads from the SS control and the RS control groups, I identified 7572 differentially expressed contigs, of which, 4220 showed increased and 3352 showed decreased transcript levels in the RS beetles (Figure 3.2A). Intriguingly, of the 7572 differentially expressed contigs, only 55.2% (4180 contigs) encoded known proteins. The 100 most significantly over-transcribed contigs, of which 19 were unknown sequences, had $\log_2$Fold changes of 13.63 to 10.03 in the RS beetles and included sequences for glucose dehydrogenases, fatty acid binding proteins, cytochrome c oxidases, digestive proteases, and heat shock proteins (Appendix A). Whereas, the 100 most significantly under-transcribed contigs, of which 25 were unknown sequences, had $\log_2$Fold changes of -14.15 to -8.83 in the RS beetles and included sequences for ribosomal proteins, members of cathepsin family, aldo-keto reductases, and endopolygalacturonases (Appendix B).

When I compared the reads from the SS treated and the RS treated groups, I obtained similar results. In this comparison, however, I identified 6632 differentially expressed contigs of which 3923 showed increased and 2709 showed decreased expression levels (Figure 3.2B). Among the 6632 differentially expressed contigs, only 54.64% (3624 contigs) encoded proteins with known functions. The 100 most significantly over/under-transcribed contigs had similar $\log_2$Fold changes and identities as in the control group comparison (Appendix A and B). Overall, I found a considerable overlap among the differentially expressed contigs between the two comparisons. Of the 7572 and 6632 differentially expressed contigs in the control and in the treatment groups, respectively, 5424 contigs were common to both (Figure 3.3).

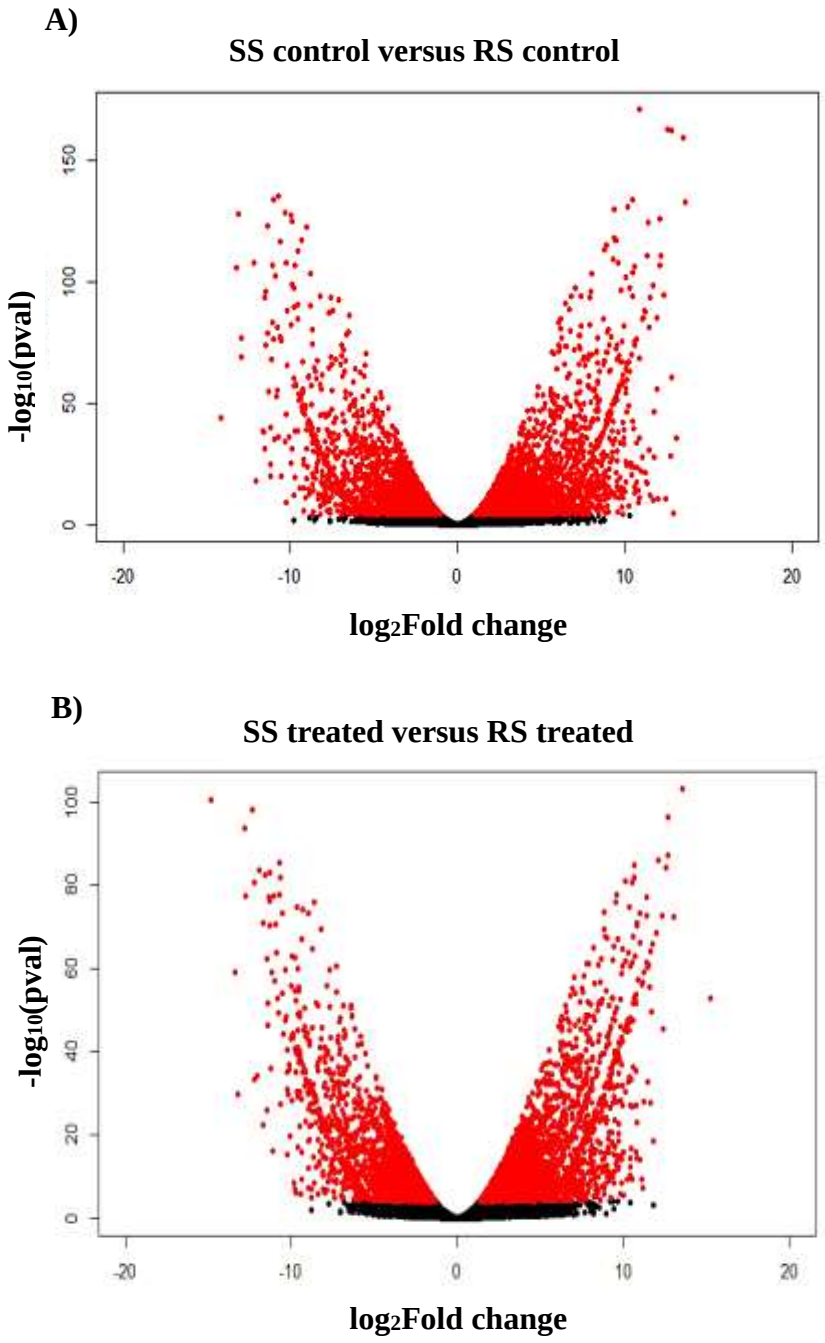


Figure 3. 2. Volcano plots showing differentially expressed contigs between two strains of Colorado potato beetle. A) Comparison between SS control versus RS control groups. **B)** Comparison between SS treated versus RS treated groups. Contigs that are differentially expressed at FDR of ≤ 0.001 and fold change of $\left| \log_2 \right| \geq 1$ are coloured red.

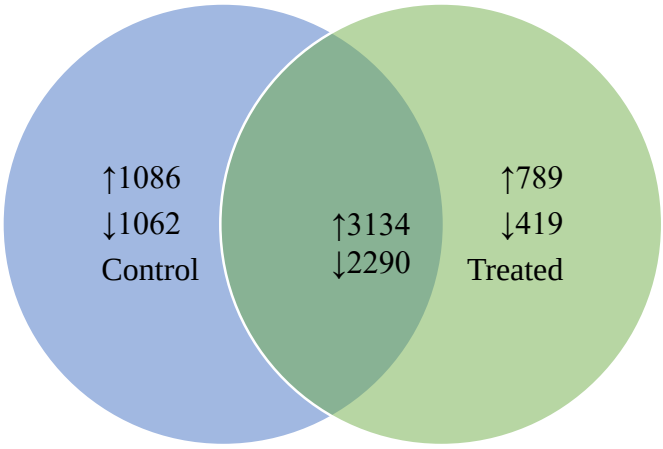


Figure 3. 3. Total number of contigs that are significantly differentially expressed in two strains of Colorado potato beetles from two comparisons. ↑ = number of contigs showing increased mRNA transcript levels in the RS compared to the SS; ↓ = number of contigs showing decreased mRNA transcript levels in the RS compared to the SS.

3.5 Identification of significantly differentially expressed detoxifying enzyme and ABC transporter transcripts between the RS and the SS beetles

I manually screened differentially expressed sequences between the two strains to identify transcripts encoding detoxifying enzymes and ABC transporters in the two comparisons. In the control group comparison, I identified 102 contigs, of which 74 showed increased, while 28 showed decreased, transcript levels in the RS beetles. Of the 74 contigs showing increased transcript levels, 24 corresponded to CYP, 13 to UGT, 14 to esterase, 15 to GST, and 8 to ABC transporter encoding transcripts (Table 3.3). Whereas, among the 28 contigs showing decreased transcript levels, there were 6 CYP, 2 UGT, 9 esterase, 4 GST, and 7 ABC transporter encoding transcripts (Table 3.3).

In the treatment group comparison, I found 106 contigs, of which 84 had increased while 22 had decreased transcript levels. Among the 84 contigs that were increased, I identified a total of 42 CYP, 11 UGT, 13 esterase, 10 GST and 8 ABC transporter encoding transcripts (Table 3.3). Interestingly, more CYP encoding contigs were present in the

treatment groups comparison than of the control groups. Among the 22 contigs showing decreased transcript levels, there were 5 CYP, 1 UGT, 9 esterases, 4 GSTs and 3 ABC transporters (Table 3.3). Overall, there was considerable similarity in the differentially expressed contigs in the treatment group and the control group comparisons. Of the 102 and 106 contigs encoding detoxifying enzymes and ABC transporters in control and treated groups, respectively, 76 were common to both (Figure 3.4).

Amongst the contigs showing increased transcript levels for CYP enzymes, CYP9, CYP6, and CYP4 families showed significant enrichment in the RS beetles. In the GST family, sigma class was enriched the most, followed by delta and epsilon classes. With regards to the ABC transporter gene family, transcripts from MRP family were enriched the most. Among the esterases enriched in the RS, most were not categorised into a specific family while several were in the acetylcholine esterase and carboxylesterase families. Within the UGT enzyme family encoding contigs, most were not assigned to a family, while several were in the UGT 2 and antennal enriched UGT families. Table 3.4 shows all contigs encoding detoxifying enzymes and ABC transporters differentially expressed in the RS beetles compared to the SS beetles. Also shown in the table are the normalized mean number of reads aligning to each contig from both strain in control and treatment group comparisons, their associated log2Fold changes, and the adjusted *P*-values.

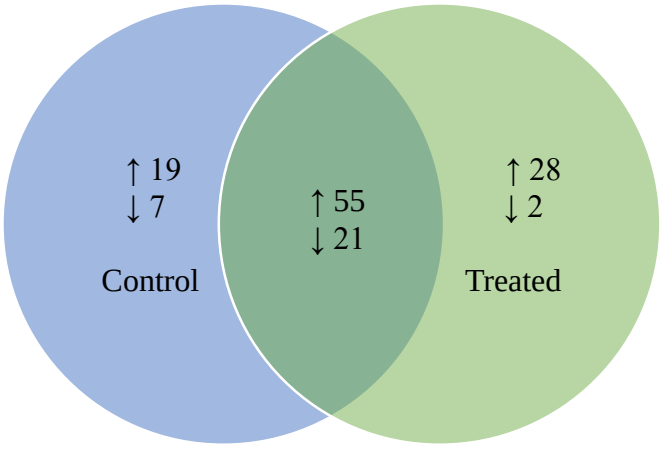


Figure 3. 4. Total number of contigs corresponding to detoxifying enzyme and ABC transporter genes that are differentially expressed in the RS beetles compared to the SS beetles in two comparisons. ↑ = number of contigs showing increased mRNA transcript levels in the RS compared to the SS; ↓ = number of contigs showing decreased mRNA transcript levels in the RS compared to the SS.

Table 3. 3. Total number of significantly differentially expressed contigs encoding detoxifying enzymes and ABC transporters in the RS beetles compared to the SS beetles in two comparisons.

Gene Family	SS control versus RS control comparison			SS treated versus RS treated comparison		
	Number of contigs	Increased mRNA transcript	Decreased mRNA transcript	Number of contigs	Increased mRNA transcript	Decreased mRNA transcript
Cytochrome p450	30	24	6	47	42	5
GST	19	15	4	14	10	4
UGT	15	13	2	12	11	1
Esterase	23	14	9	22	13	9
ABC transporter	15	8	7	11	8	3
Total number of contigs	102	74	28	106	84	22

Table 3. 4. List of significantly differentially expressed contigs encoding detoxifying enzymes and ABC transporters in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description[2]	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]	Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]
CYPs									
Ld_rep_c34031	***CYP6BQ15***	**943.26**	**8.33**	**6.82**	**1.04E-32**	**1163.67**	**7.15**	**7.35**	**9.74E-30**
Ld_rep_c51084	***CYP6K1***	**25.86**	**0.32**	**6.32**	**1.21E-09**	**NA**	**NA**	**NA**	**NA**
Ld_c20712	***CYP4G57***	**108.33**	**1.61**	**6.07**	**8.18E-28**	**NA**	**NA**	**NA**	**NA**
Ld_rep_c41850	***CYP6BJ1***	**40.4**	**0.65**	**5.97**	**8.94E-05**	**84.28**	**0**	**NA**	**9.98E-04**
Ld_rep_c61559	*CYP6EF1*	16.29	0.33	5.61	7.99E-06	32.17	0.29	6.8	6.88E-10
Ld_rep_c91876	*CYP9Z12V1*	10.78	0.32	5.06	7.62E-04	12.87	0	NA	3.34E-05
Ld_rep_c33314	***CYP4Q3***	**960.36**	**61.04**	**3.98**	**2.77E-38**	**845.53**	**58.8**	**3.85**	**3.79E-21**
Ld_rep_c48733	predicted *CYP*	73.43	5.07	3.86	1.45E-12	43.18	7.79	2.47	7.49E-05
Ld_rep_c25417	*CYP4Q7*	1592.4	113.41	3.81	7.08E-22	1504.4	107.76	3.8	6.76E-16
Ld_rep_c36308	*CYP6BU1*	34.48	2.92	3.56	6.57E-08	26.29	2.05	3.68	1.06E-05
Ld_rep_c27085	*CYP412A2*	210.53	31.1	2.76	1.40E-15	NA	NA	NA	NA

Table 3. 4. List of significantly differentially expressed contigs encoding detoxifying enzymes and ABC transporters in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description[2]	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]	Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]
Ld_c756	***CYP412A2***	**354.61**	**59.35**	**2.58**	**3.02E-16**	**291.72**	**58.8**	**2.31**	**5.17E-06**
Ld_rep_c45335	*CYP9Z14V3*	81.61	14.79	2.46	1.59E-05	95.37	0	NA	2.69E-23
Ld_rep_c63019	*CYP413A1*	67.8	13.15	2.37	1.48E-07	90.96	1.73	5.72	1.58E-18
Ld_c981	*CYP12A4*	2435.68	504.67	2.27	2.83E-16	3093.83	526.09	2.56	3.03E-06
Ld_c259	*CYP6BQ15*	7776.35	1995.9	1.96	5.78E-06	2630.12	584.94	2.17	1.51E-08
Ld_rep_c30474	*CYP301B1*	240.41	62.9	1.93	4.53E-06	NA	NA	NA	NA
Ld_c20506	*CYP6EH1*	3049.34	823.72	1.89	2.55E-04	2721.08	961.85	1.5	1.48E-04
Ld_c72702	*CYP12H2*	910.62	261.14	1.8	3.68E-10	1074	310.82	1.79	7.85E-05
Ld_rep_c75503	*CYP6BQ16*	341.18	104.44	1.71	3.90E-04	570.19	68.72	3.05	1.46E-04
Ld_rep_c24490	*CYP6BQ15*	2476.07	802.12	1.63	4.88E-09	7307.52	2236.98	1.71	3.95E-05
Ld_c22309	*CYP314A1*	445.14	161.76	1.46	7.85E-06	NA	NA	NA	NA
Ld_c55986	*CYP314A1*	617.71	240.19	1.36	5.60E-05	NA	NA	NA	NA

Table 3. 4. List of significantly differentially expressed contigs encoding detoxifying enzymes and ABC transporters in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description[2]	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]	Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]
Ld_rep_c68743	*CYP4G57*	228.6	0	NA	9.88E-34	265.73	0	NA	1.03E-40
Ld_rep_c77588	*CYP4Q2*	NA	NA	NA	NA	16	0.32	5.63	1.36E-05
Ld_rep_c75331	*CYP4Q3*	NA	NA	NA	NA	57.97	0	NA	3.72E-18
Ld_rep_c27801	*CYP6BQ15*	NA	NA	NA	NA	1882.53	546.02	1.79	1.90E-04
Ld_rep_c27273	*CYP6BQ16*	NA	NA	NA	NA	398.14	148.05	1.43	7.96E-04
Ld_c62736	*CYP6BQ4*	NA	NA	NA	NA	129.52	20.44	2.66	6.81E-07
Ld_rep_c25628	*CYP6K1*	NA	NA	NA	NA	663.75	106.88	2.63	5.95E-11
Ld_c21643	*CYP6K1-like*	NA	NA	NA	NA	1127.36	117.29	3.26	7.77E-17
Ld_c54867	*CYP6K1-like*	NA	NA	NA	NA	1596	171.83	3.22	1.31E-12
Ld_rep_c30807	*CYP9V1*	NA	NA	NA	NA	587.82	123	2.26	1.08E-04
Ld_rep_c75102	*CYP9V1*	NA	NA	NA	NA	1593.76	346.61	2.2	3.60E-06
Ld_c2908	*CYP9V1*	NA	NA	NA	NA	4348.67	172.64	4.65	3.18E-07

Table 3. 4. List of significantly differentially expressed contigs encoding detoxifying enzymes and ABC transporters in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description[2]	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]	Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]
Ld_rep_c36819	*CYP9V1*	NA	NA	NA	NA	16427.8	2853.9	2.53	3.25E-08
Ld_c2974	*CYP9V1*	NA	NA	NA	NA	784.7	252.45	1.64	9.26E-04
Ld_rep_c25506	*CYP9V1*	NA	NA	NA	NA	882.76	327.69	1.43	6.43E-04
Ld_rep_c26493	*CYP9V1*	NA	NA	NA	NA	1048.64	406.26	1.37	9.80E-04
Ld_c17267	***CYP9V1***	**NA**	**NA**	**NA**	**NA**	**2946.92**	**87.91**	**5.07**	**5.74E-07**
Ld_c85173	*CYP9V1*	NA	NA	NA	NA	236.21	69.1	1.77	4.60E-05
Ld_c8495	*CYP9Z14V2*	NA	NA	NA	NA	3390.8	708.3	2.26	1.74E-06
Ld_rep_c62610	*CYP9Z18*	NA	NA	NA	NA	35.24	0	NA	1.60E-12
Ld_c22715	*CYP9Z20V1*	NA	NA	NA	NA	358.1	79.24	2.18	9.74E-08
Ld_rep_c71725	*CYP9Z4*	NA	NA	NA	NA	82.79	11.74	2.82	7.03E-08
Ld_rep_c49324	*CYP9Z4*	NA	NA	NA	NA	1695.88	314.08	2.43	3.29E-10
Ld_rep_c26346	*CYP9Z4*	NA	NA	NA	NA	274.1	55.01	2.32	8.30E-05

Table 3. 4. List of significantly differentially expressed contigs encoding detoxifying enzymes and ABC transporters in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description[2]	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]	Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]
Ld_rep_c61980	*CYP9Z4*	NA	NA	NA	NA	91.87	25.26	1.86	2.44E-04
Ld_rep_c34317	*CYP6BQ15*	30.33	145.65	-2.26	3.65E-10	34.34	148.64	-2.11	4.06E-06
Ld_rep_c75371	*CYP412A1*	9.77	52.29	-2.42	1.29E-06	10.75	70.46	-2.71	7.68E-07
Ld_c20095	*CYP412A2*	7.6	41.63	-2.45	1.15E-05	NA	NA	NA	NA
Ld_rep_c34168	***CYP4Q7***	**15.34**	**100.01**	**-2.71**	**4.76E-11**	**9.88**	**81.2**	**-3.04**	**1.08E-07**
Ld_rep_c60423	*CYP4C1*	2.39	107.94	-5.5	2.42E-19	11.47	117.76	-3.36	1.52E-11
Ld_rep_c48659	*CYP6BK17*	0.66	43.89	-6.06	2.11E-14	0	62.1	NA	6.89E-18
Esterases									
Ld_rep_c71421	***Esterase 4***	**194.83**	**0.31**	**9.28**	**3.74E-32**	**244.63**	**0.32**	**9.56**	**5.59E-38**
Ld_rep_c36657	*Carboxyl-Esterase 1*	82.38	0.32	7.99	8.41E-19	102.95	0.61	7.41	5.63E-10
Ld_rep_c34698	***Esterase 5***	**880.32**	**3.53**	**7.96**	**2.97E-31**	**1067.45**	**4.98**	**7.74**	**6.92E-47**
Ld_rep_c77075	***Esterase Beta***	**51.99**	**0.32**	**7.33**	**2.29E-05**	**66.03**	**0.32**	**7.7**	**2.34E-18**

Table 3. 4. List of significantly differentially expressed contigs encoding detoxifying enzymes and ABC transporters in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description[2]	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]	Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]
Ld_rep_c35289	*Acetly cholinesterase 1*	292.39	5.15	5.83	7.48E-42	362.14	7.42	5.61	7.73E-31
Ld_rep_c53802	***Esterase FE4***	**65.66**	**1.92**	**5.1**	**5.85E-16**	**53.01**	**0.32**	**7.36**	**9.12E-07**
Ld_rep_c35399	***Esterase 2***	**389.18**	**14.51**	**4.75**	**1.44E-22**	**481.4**	**9.29**	**5.7**	**3.92E-33**
Ld_rep_c46562	*Esterase 3*	121.55	5.26	4.53	5.34E-15	131.62	5.61	4.55	5.65E-18
Ld_c2942	***Esterase 1***	**4078.42**	**299.34**	**3.77**	**7.68E-39**	**3480.81**	**622.92**	**2.48**	**4.21E-10**
Ld_rep_c24217	***Esterase 6***	**2934.8**	**730.27**	**2.01**	**3.25E-10**	**3576.25**	**931.15**	**1.94**	**3.12E-07**
Ld_c2931	*Carboxyl-Esterase 2*	530.97	134.3	1.98	2.49E-09	436.62	102.6	2.09	2.59E-07
Ld_rep_c25830	*Acetyl cholinesterase 2*	3374.43	1409.01	1.26	5.83E-04	NA	NA	NA	NA
Ld_c5150	*Esterase 7*	2203.66	1084.67	1.02	7.14E-04	NA	NA	NA	NA
Ld_rep_c28597	*Esterase 8*	181.41	0	NA	5.25E-46	269.05	0	NA	5.70E-41
Ld_rep_c29016	*Esterase 9*	NA	NA	NA	NA	5169.89	1472.28	1.81	2.61E-06
Ld_rep_c27045	*Carboxyl-esterase 3*	NA	NA	NA	NA	35.93	161.03	-2.16	3.83E-05

Table 3. 4. List of significantly differentially expressed contigs encoding detoxifying enzymes and ABC transporters in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description[2]	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]	Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]
Ld_rep_c36550	*Esterase FE4*	0	60.88	NA	1.27E-21	0.32	52.44	-7.35	1.14E-14
Ld_rep_c34853	*Venom carboxyl Esterase-6-like*	0	73.02	NA	7.20E-25	0	61.49	NA	2.83E-07
Ld_rep_c68979	*Carboxyl-esterase 4*	107.2	373.43	-1.8	5.46E-06	NA	NA	NA	NA
Ld_rep_c33690	*Esterase 10*	47.19	245.23	-2.38	1.58E-11	100.9	290.99	-1.53	7.00E-04
Ld_rep_c33908	*Esterase FE4*	51.71	353.71	-2.77	6.86E-09	31.99	372.96	-3.54	3.64E-08
Ld_rep_c36417	*Esterase FE4*	74.76	528.18	-2.82	2.35E-09	47.6	611.83	-3.68	2.87E-19
Ld_rep_c24505	*Acetyl cholinesterase 3*	185.92	1924.85	-3.37	7.24E-06	301.48	1385	-2.2	6.55E-09
Ld_rep_c24395	*Esterase 11*	1.01	47.12	-5.55	1.59E-14	0.32	23.69	-6.2	1.45E-07
Ld_rep_c26610	*Alpha-Esterase*	1.73	469.03	-8.08	4.67E-21	0.74	492.83	-9.39	1.17E-08
GSTs									
Ld_rep_c33018	***GST Sigma 1***	**2034.78**	**3.24**	**9.3**	**1.0E-106**	**3193.14**	**4.67**	**9.42**	**7.80E-63**
Ld_rep_c40253	***GST Sigma 2***	**91.86**	**1.58**	**5.86**	**1.99E-13**	**101.25**	**0.63**	**7.32**	**3.89E-23**

Table 3. 4. List of significantly differentially expressed contigs encoding detoxifying enzymes and ABC transporters in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description[2]	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]	Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]
Ld_rep_c24170	***GST 1***	**2058.82**	**38.51**	**5.74**	**1.88E-68**	**2815.36**	**35.51**	**6.31**	**5.68E-46**
Ld_rep_c41971	***GST Sigma 3***	**105.26**	**4.2**	**4.65**	**2.79E-22**	**160.07**	**5.06**	**4.98**	**1.46E-21**
Ld_rep_c24256	***GST Delta 1***	**1275.78**	**232.16**	**2.46**	**4.29E-18**	**1726.07**	**277.96**	**2.63**	**6.04E-12**
Ld_rep_c26032	*GST Epsilon 6*	449.15	96.75	2.21	2.92E-13	NA	NA	NA	NA
Ld_rep_c44006	***GST 2***	**122.07**	**27.08**	**2.17**	**6.89E-09**	**155.54**	**17.52**	**3.15**	**2.08E-10**
Ld_c19072	*GST 2C1-like*	50.78	12.51	2.02	4.86E-05	NA	NA	NA	NA
Ld_rep_c24751	*GST Sigma 4*	7397.42	2715.74	1.45	1.02E-04	7786.49	2590.57	1.59	4.82E-05
Ld_rep_c38387	*GST Epsilon*	1438.38	574.55	1.32	5.21E-06	NA	NA	NA	NA
Ld_rep_c33334	*GST Omega-1*	892.55	389.81	1.2	6.62E-05	NA	NA	NA	NA
Ld_rep_c46479	*GST Theta*	359.43	158.96	1.18	2.62E-04	NA	NA	NA	NA
Ld_rep_c25066	*GST Epsilon 3*	1757.33	799.76	1.14	1.25E-04	NA	NA	NA	NA
Ld_rep_c50771	*GST 3*	36.27	0	NA	1.38E-04	11.86	0	NA	6.78E-05

Table 3. 4. List of significantly differentially expressed contigs encoding detoxifying enzymes and ABC transporters in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description[2]	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]	Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]
Ld_rep_c33605	*GST 4*	NA	NA	NA	NA	1082.3	4835.01	-2.16	1.75E-08
Ld_rep_c48065	***GST Delta 2***	**107.98**	**0**	**NA**	**2.25E-33**	**164.63**	**1.86**	**6.47**	**3.46E-27**
Ld_rep_c54053	*GST Epsilon 7*	171.67	474.9	-1.47	1.18E-06	129.74	578.04	-2.16	5.67E-05
Ld_rep_c43735	***GST 5***	**148.33**	**939.47**	**-2.66**	**2.79E-20**	**171.28**	**1002.53**	**-2.55**	**1.02E-10**
Ld_rep_c34301	*GST 6*	38.28	249.18	-2.7	2.72E-13	41.5	309.17	-2.9	4.86E-12
Ld_rep_c38198	*GST 7*	0.69	815.64	-10.2	7.68E-86	0	1018.35	NA	1.85E-61
UGTs									
Ld_rep_c84840	*UGT 2C1*	356.14	0.33	10.06	1.31E-52	541.38	0.32	10.71	2.50E-51
Ld_rep_c41594	***UGT 1***	**192.9**	**0.65**	**8.22**	**7.10E-30**	**150.01**	**0.29**	**9.02**	**3.92E-30**
Ld_rep_c83152	***UGT 2***	**124.34**	**0.64**	**7.61**	**5.06E-20**	**99.73**	**0.32**	**8.3**	**1.60E-15**
Ld_rep_c45975	*Antennal-enriched UGT*	70.11	2.61	4.75	1.46E-17	152.99	0.96	7.32	2.39E-28
Ld_rep_c28339	*UGT 2B15*	95.58	23.96	2.00	6.09E-07	325.53	22.81	3.84	1.93E-07

Table 3. 4. List of significantly differentially expressed contigs encoding detoxifying enzymes and ABC transporters in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description[2]	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]	Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]
Ld_rep_c58571	*UGT 3*	63.67	16.15	1.98	1.37E-05	129.87	21.11	2.62	1.69E-08
Ld_c190	***UGT 2B5***	**522.5**	**147.93**	**1.82**	**9.03E-10**	**706.64**	**90.68**	**2.96**	**9.26E-14**
Ld_c269	***UGT 4***	**1174.08**	**409.05**	**1.52**	**3.64E-04**	NA	NA	NA	NA
Ld_rep_c39043	*UGT 2B23*	144.31	54.94	1.39	1.23E-04	231.8	48.42	2.26	1.21E-07
Ld_rep_c33389	*UGT 5*	1281.78	576.6	1.15	1.08E-04	NA	NA	NA	NA
Ld_rep_c38005	*Antennal-enriched UGT*	143.76	0	NA	1.18E-34	276.16	0	NA	1.77E-41
Ld_rep_c35232	*UGT 6*	56.36	0	NA	1.96E-10	79.17	0	NA	1.23E-21
Ld_rep_c30928	***UGT 2C1***	**458.59**	**0**	**NA**	**1.77E-72**	**196.28**	**0**	**NA**	**9.46E-14**
Ld_rep_c28388	UGT 7	0.35	543.8	-10.62	5.63E-75	32.71	605.18	-4.21	4.01E-23
Ld_rep_c84951	*UGT 2C1-like*	21.57	71.06	-1.72	8.60E-05	NA	NA	NA	NA
ABC transporters									
Ld_rep_c28427	***ABC-G***	**22.29**	**0.66**	**5.09**	**5.20E-05**	**NA**	**NA**	**NA**	**NA**

Table 3. 4. List of significantly differentially expressed contigs encoding detoxifying enzymes and ABC transporters in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description[2]	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]	Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]
Ld_rep_c27116	*MRP 4-1*	12.63	0.66	4.27	4.95E-04	29.86	1.26	4.57	7.00E-08
Ld_c11003	*ABC-B6*	61.31	3.88	3.98	1.53E-07	NA	NA	NA	NA
Ld_c571	***MRP 4-2***	**285.89**	**37.77**	**2.92**	**5.86E-08**	**663.94**	**17.43**	**5.25**	**6.63E-32**
Ld_rep_c26545	*ABC-B6 mitochondrial*	615.56	241.68	1.35	6.66E-06	NA	NA	NA	NA
Ld_c62808	*MRP-2*	80.57	0	NA	2.90E-27	147.01	0	NA	3.10E-31
Ld_c60098	*MRP 1-like*	NA	NA	NA	NA	65.09	7.86	3.05	3.39E-07
Ld_c17819	*MRP 4-like1*	NA	NA	NA	NA	549.63	169.99	1.69	4.66E-05
Ld_c20956	*MRP 4-like2*	NA	NA	NA	NA	20.53	0	NA	5.24E-06
Ld_rep_c34742	***MRP-4-3***	**291.03**	**0**	**NA**	**4.25E-59**	**467.12**	**0**	**NA**	**2.74E-50**
Ld_c24118	*MRP-4 like4*	14.12	0	NA	5.25E-06	27.5	0	NA	2.94E-10
Ld_c7947	*MRP like protein*	47.61	123.94	-1.38	2.40E-04	NA	NA	NA	NA
Ld_c12043	*MRP 4-like5*	91.46	275.31	-1.59	7.75E-07	NA	NA	NA	NA

Table 3. 4. List of significantly differentially expressed contigs encoding detoxifying enzymes and ABC transporters in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description[2]	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]	Read count RS[3]	Read count SS[3]	Log_2 Fold change	*P*-adj[4]
Ld_c73069	*MRP like protein*	121.75	399.13	-1.71	2.47E-08	NA	NA	NA	NA
Ld_c24114	*MRP 4-like 5*	117.69	416.18	-1.82	2.03E-09	NA	NA	NA	NA
Ld_c56678	*MRP*	38.75	334.54	-3.11	3.47E-07	77.85	359.7	-2.21	9.57E-08
Ld_c6433	*MRP-1*	11.59	368.52	-4.99	2.73E-41	15.34	220.04	-3.84	1.13E-16
Ld_rep_c91275	***MRP 4-4***	**0.35**	**619.43**	**-10.81**	**6.23E-79**	**1.02**	**547.07**	**-9.06**	**8.00E-43**

[1]Contig ID from Kumar *et al*. (2014); [2]Genes selected for qPCR validation of mRNA-seq data are bolded; [3]Read counts represent mean normalized counts from three biological replicates; [4]Adjusted *P*-value corrected for false discovery rates; NA = not available.

3.6 Other major significantly differentially expressed genes between the SS and the RS beetles

In addition to genes encoding detoxifying enzymes and ABC transporters, genes whose products are involved in oxidative stress response, immune response, general stress response, energy production, carbohydrate metabolism, and anti-oxidative stress response were also enriched in the RS beetles compared to the SS beetles (Appendix C). I identified several contigs encoding genes involved in oxidative stress response, including xanthine dehydrogenase oxidase, S-adenosyl-l-homocysteine hydrolase, monothiol glutaredoxin, peroxiredoxin, and Acyl-CoA dehydrogenases. There were also quite a few contigs encoding for immunity related proteins such as apolipoprotein binding protein, acidic mammalian chitinase, cathepsin L, chitinase 5 and 6, and attacin-like immune protein. In terms of general stress response proteins, contigs encoding heat shock proteins, chaperones, and members of the ubiquitin family showed increased transcript levels. Contigs encoding products involved in energy metabolism were represented by malate dehydrogenases, ATP synthases, cytochrome b5, isocitrate dehydrogenases, succinate dehydrogenase, NADH dehydrogenase iron-sulfur protein, succinyl-CoA synthase, and NADH dehydrogenase ubiquinone proteins. In terms of carbohydrate metabolism, genes encoding proteins involved in glycolysis such as phosphofructokinase, aldolases, pyruvate kinase, alcohol dehydrogenase, were all over-transcribed in the RS beetles. In addition, contigs encoding proteins involved in electron transport chain were exemplified by cytochrome reductase c and NADH dehydrogenase ubiquinone. Finally, genes encoding products involved in anti-oxidative stress response were represented by glutathione peroxidase and superoxide dismutase enzymes.

3.7 Validation of DESeq results using qPCR

To validate results from DESeq analysis, I tested transcript levels of 32 genes using qPCR. Among these, eight encoded CYPs, seven esterases, eight GSTs, five UGTs, and four ABC transporters. Overall, approximately 65% of the genes had a similar trend in mRNA transcript levels as in the DESeq analysis. However, estimated fold change differences obtained by the two methods differed substantially (Table 3.5).

Based on qPCR results, the highest fold change increase for CYPs was for *CYP6BQ15* gene (~ 81 fold), followed by *CYP4Q7* (~ 18 fold), *CYP9V1* (~ 14 fold), and *CYP4Q3* (~ 10 fold) (Figure 3.5A). Interestingly, *CYP4Q7* showed decreased transcript levels in the RS beetles in DESeq analysis (Table 3.5). Among the esterase genes, *Esterase 2* had the highest fold increase (~ 85 fold), followed by *Esterase 1* (~ 9 fold) (Figure 3.5B). The highest fold increase from ABC transporters was for *ABC-G* (~ 51 fold), followed by *MRP-4-2* (~ 6 fold) gene (Figure 3.5C). With regards to UGT genes, *UGT 1* had a fold change increase of ~ 6995 – the highest fold increase among all the genes tested. This was followed by *UGT 2B5*, which had a fold increase of ~ five (Figure 3.5D). Finally, among the eight GSTs, three genes showed slightly increased transcript levels in the RS; yet, none of them had a fold change of ≥ two (Figure 3.5E).

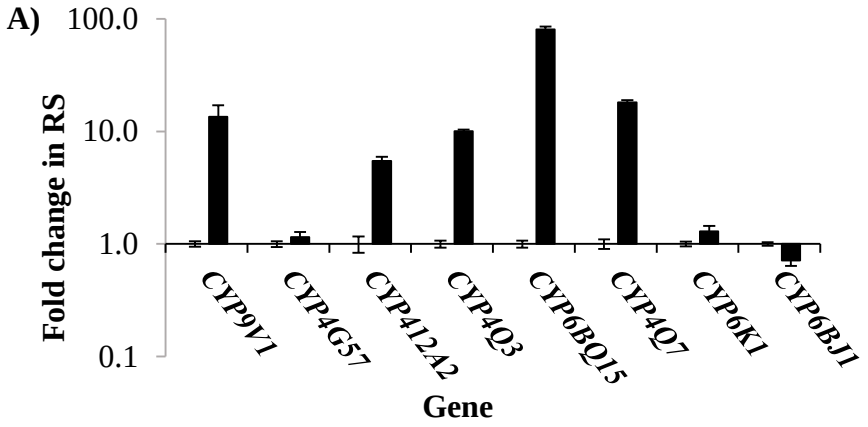
Table 3. 5. Estimated fold change differences for 32 genes in the RS beetles compared to the SS beetles from DESeq and qPCR analyses.

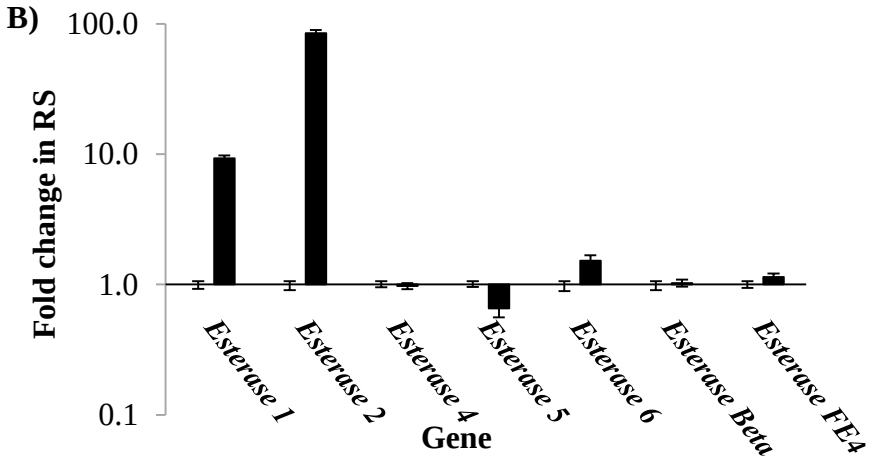
Gene[1]	Fold change in qPCR	Fold change in DESeq	Trend[2]
CYP9V1	13.53	33.52	S
CYP4G57	1.15	67.12	S
CYP412A2	5.47	5.98	S
CYP4Q3	10.03	15.73	S
CYP6BQ15	80.79	113.21	S
CYP4Q7	18.15	0.15	O
CYP6K1	1.30	80.07	S
CYP6BJ1	0.71	62.57	O
Esterase 1	9.26	13.62	S
Esterase 2	84.81	26.83	S
Esterase 5	0.65	249.34	O
Esterase 4	0.97	623.54	O
Esterase 6	1.52	4.02	S
Esterase Beta	1.03	160.98	O
Esterase FE4	1.14	34.26	S
UGT 2B5	4.52	3.53	S
UGT 4	2.69	2.87	S

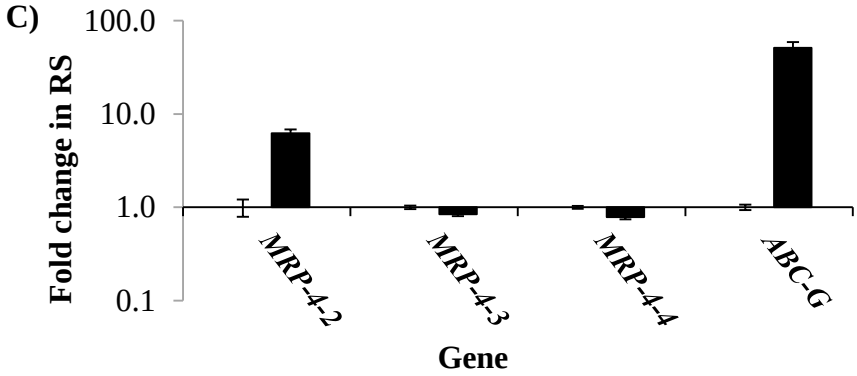
Table 3. 5. Estimated fold change differences for 32 genes in the RS beetles compared to the SS beetles from DESeq and qPCR analyses.

Gene[1]	Fold change in qPCR	Fold change in DESeq	Trend[2]
UGT 1	6994.70	298.79	S
UGT 2C1	1.03	458.59	O
UGT 2	1.27	195.70	S
MRP-4-3	0.84	291.03	O
MRP-4-2	6.22	7.57	**S**
MRP-4-4	0.79	5.60E-4	S
ABC-G	51.06	33.98	**S**
GST Sigma 2	0.87	58.02	O
GST Sigma 3	1.09	25.08	O
GST 5	0.14	0.16	S
GST 2	0.93	4.51	O
GST Delta 2	1.32	107.98	S
GST Delta 1	1.37	5.50	S
GST Sigma 1	1.30	628.21	S
GST 1	0.73	53.46	O

[1]Genes selected for RNAi analysis are bolded; [2]S = same trend and O = opposite trend.


A)
100.0
10.0
1.0
0.1
Fold change in RS
CYP9V1
CYP4G57
CYP412A2
CYP4Q3
CYP6BQ15
CYP4Q7
CYP6K1
CYP6BJ1
Gene


B)
100.0
10.0
1.0
0.1
Fold change in RS
Esterase 1
Esterase 2
Esterase 4
Esterase 5
Esterase 6
Esterase Beta
Esterase FE4
Gene


C)
100.0
10.0
1.0
0.1
Fold change in RS
MRP-4-2
MRP-4-3
MRP-4-4
ABC-G
Gene

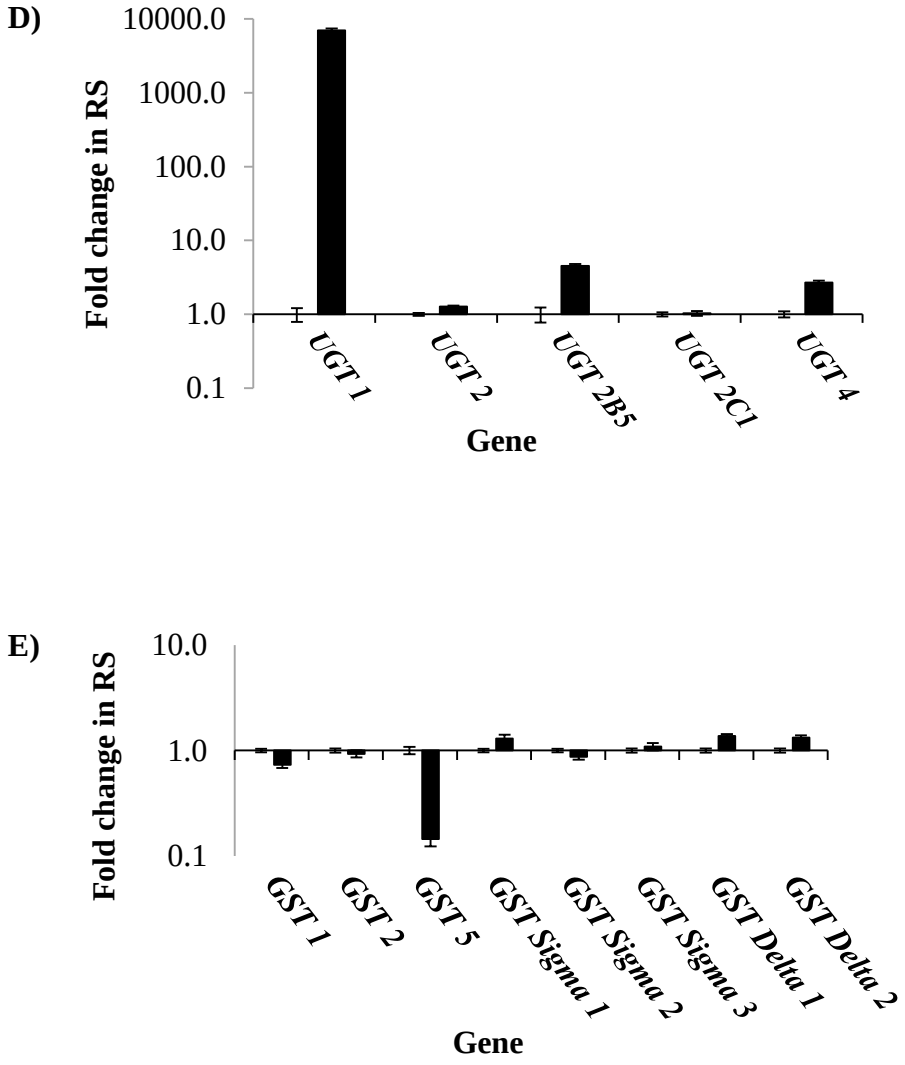


Figure 3. 5. qPCR validation of 32 differentially expressed genes in the RS beetles compared to the SS beetles. The normalized quantity of mRNA in the RS beetles was calculated using the ΔΔCt method relative to normalized mRNA levels in the SS beetles (set to 1 in graphs). **A)** Genes encoding CYPs; **B)** Genes encoding esterases; **C)** Genes encoding ABC transporters; **D)** Genes encoding UGTs; **E)** Genes encoding GSTs. Data are expressed as mean relative quantity ± SEM (n = 3).

3.7.1 Expression of ten selected genes in independent samples

I verified expression of 10 genes showing the highest fold increase in the RS beetles on independent biological samples. These genes included four CYPs, two esterases, two ABC transporters, and two UGTs. qPCR analysis confirmed that the 10 selected genes were significantly over-transcribed in the RS beetles compared to the SS beetles (Figure 3.6). The gene showing the most pronounced overexpression was *UGT 1*, which was expressed at a fold increase of ~ 1566, followed by *Esterase 2* (~ 113 fold) and *CYP6BQ15* (~ 79 fold) (Table 3.6). I selected all 10 genes for RNAi knockdown experiments.

Table 3. 6. **Statistical analysis of ten genes over-expressed in the RS beetles compared to the SS beetles**. Asterisks represent significant changes in the mRNA transcript levels in *t*-tests (**$P \leq 0.01$, ***$P \leq 0.001$), n=3.

Gene	T test value	*P*-value	Fold change
CYP6BQ15	45.62	6.9E-07***	79.38
CYP9V1	4.23	0.006**	9.82
CYP4Q7	12.32	0.0005***	7.24
CYP4Q3	11.63	0.0001***	5.79
Esterase 1	12.78	0.0001***	6.22
Esterase 2	23.63	0.0008***	112.89
MRP-4-2	3.75	0.01**	2.97
ABC-G	13.89	8.0E-4***	35.67
UGT 1	55.50	3.2E-07***	1565.56
UGT 2B5	12.78	0.0001***	3.91

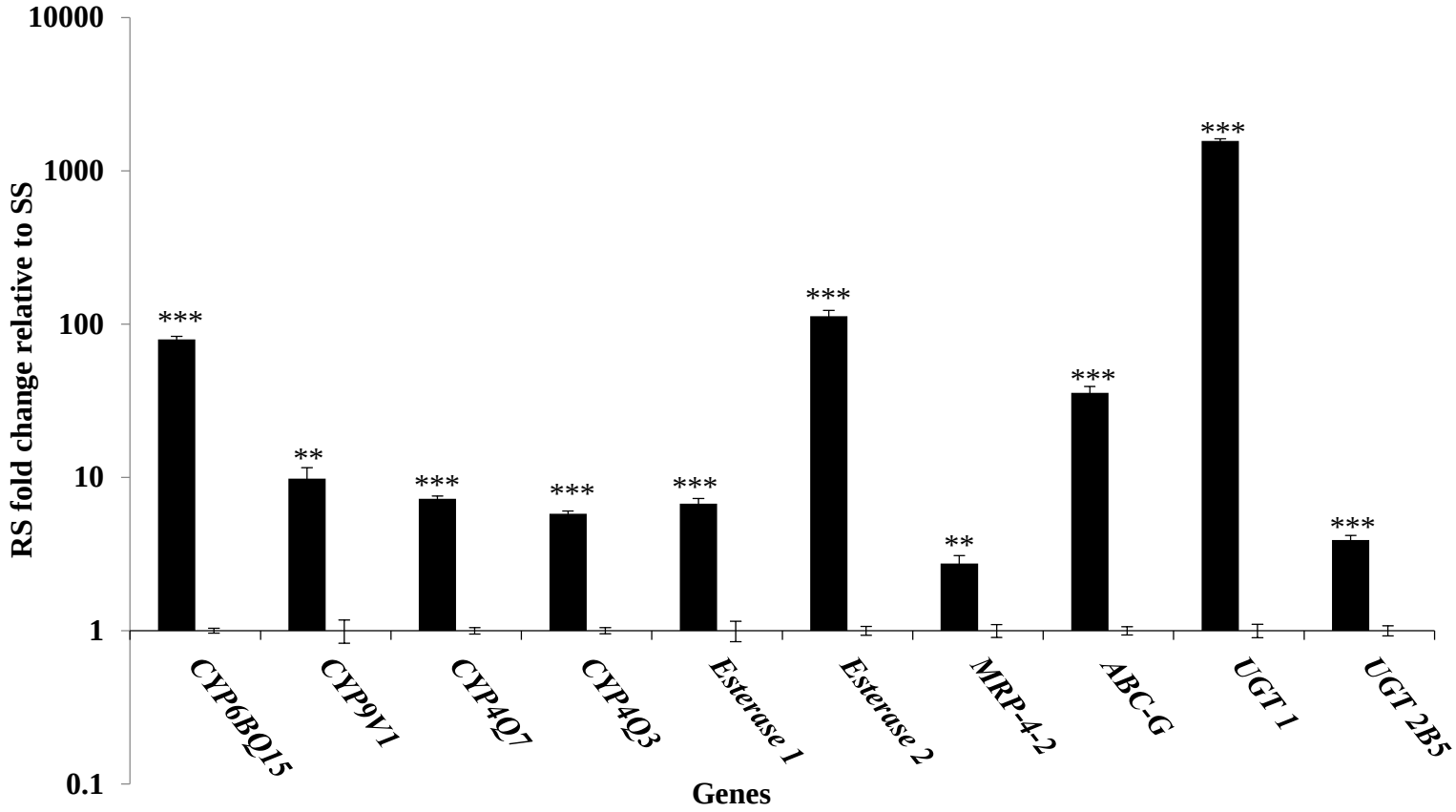


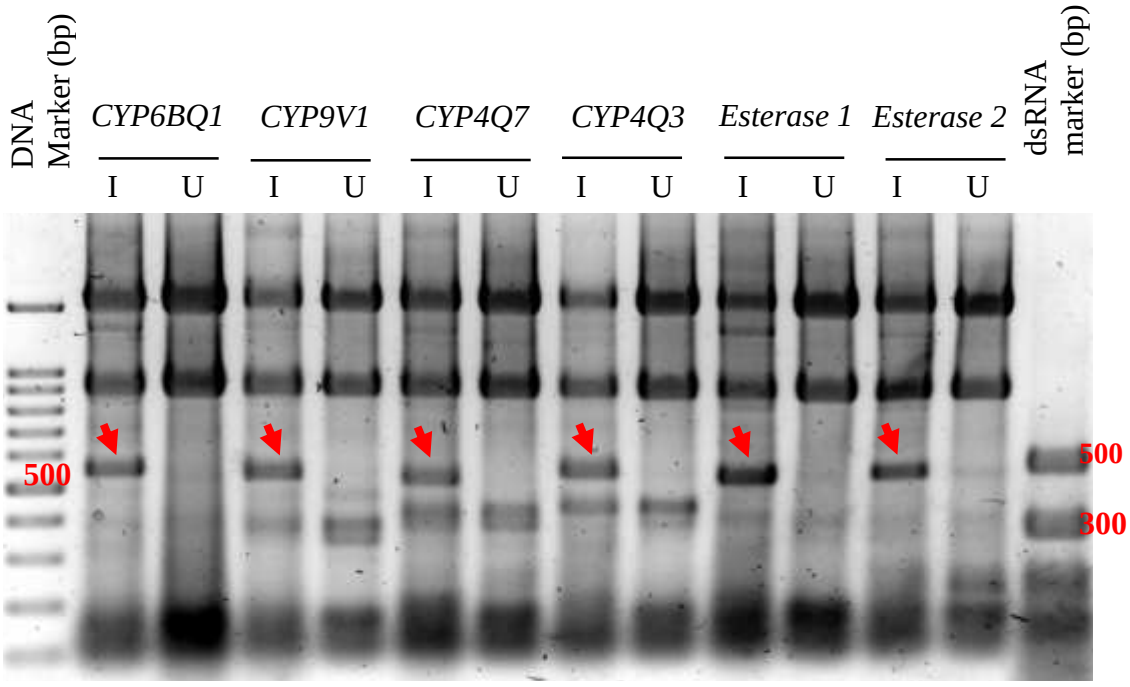
Figure 3. 6. Fold increase in normalized mRNA expression levels of 10 genes in the RS beetles relative to normalized expression levels in the SS beetles from qPCR analysis on independent biological samples. Data are expressed as mean relative quantity ± SEM. Asterisks represent significant changes in the mRNA transcript levels in *t*-tests (**$P \leq 0.01$, ***$P \leq 0.001$), n = 3.

3.8 Silencing of ten target genes using RNAi

3.8.1 Confirmation of dsRNA production in *E. coli* HT115

I used *E. coli* HT115 to produce dsRNA for 10 target genes as well as the *GFP* gene. I confirmed successful dsRNA production in each bacterial strain by visualizing unique dsRNA bands on an agarose gel. dsRNA bands were present only after *E. coli* HT115 strains were induced with IPTG (Figure 3.7A and B). Also, a dsRNA marker was included to confirm the expected sizes of dsRNA species on the gels.

A)



B)

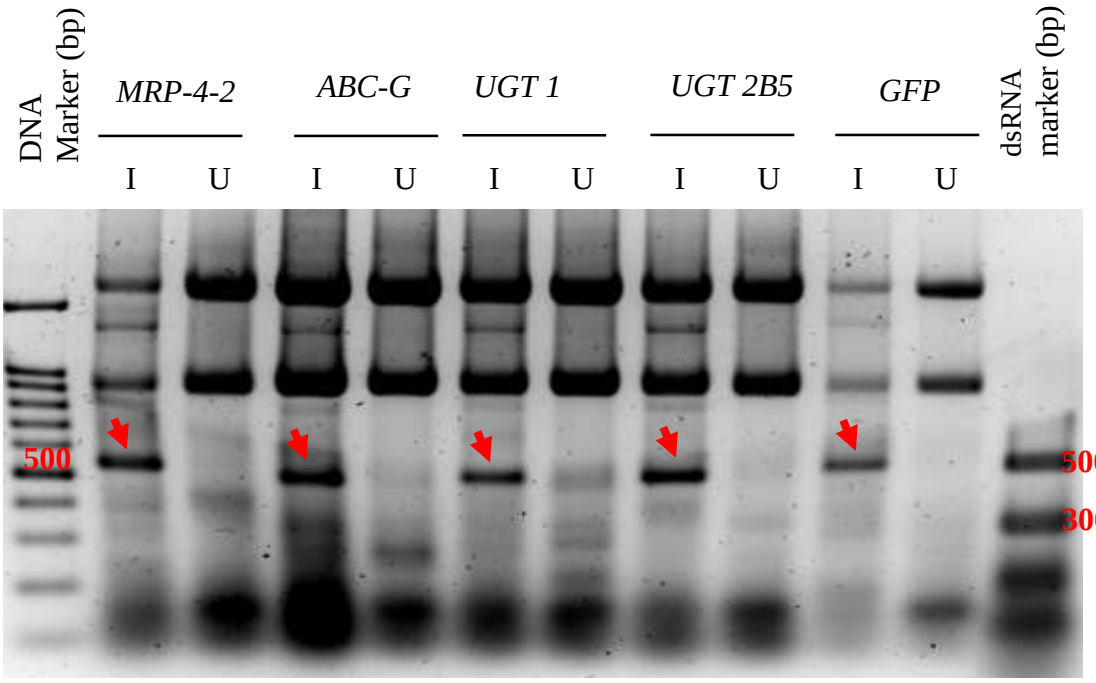


Figure 3. 7. Successful production of dsRNA in *E. coli* for target genes. I and U indicate lanes loaded with a total RNA sample extracted from bacteria that were induced or not induced with IPTG, respectively. **A)** Shows confirmation of dsRNA production for *CYP6BQ15*, *CYP9V1*, *CYP4Q7*, *CYP4Q3*, *Esterase 1*, and *Esterase 2*. **B)** Shows confirmation of dsRNA production for *MRP-4-2*, *ABC-G*, *UGT 1*, *UGT 2B5*, and *GFP*. The positions of dsRNA species are marked with red arrows.

3.8.2 Detection of RNAi knockdown of the ten target genes using qPCR

Feeding of dsRNA resulted in a significant reduction in the mRNA levels of 7 out of 10 genes (Figure 3.8). Results from one-way ANOVA tests showed that effects of dsRNA feeding on the mRNA transcript levels were significant for the genes *CYP6BQ15* ($F_{2,6}$ = 16.7, P = 0.0035), *CYP4Q7* ($F_{2,6}$ = 10.9, P = 0.010), *CYP4Q3* ($F_{2,6}$ = 19.21, P = 0.0024), *Esterase 1* ($F_{2,6}$ =120.5, P = 1.43E-05), *ABC-G*, ($F_{2,6}$ = 18.03, P = 0.0029), *UGT 1* ($F_{2,6}$ = 26.4, P = 0.0010), and *UGT 2B5* ($F_{2,6}$ = 102.8, P = 2.28e-05). The Tukey's HST showed that mRNA levels of all seven genes did not differ significantly between feeding with dsRNA-*GFP* and PBS controls ($P > 0.05$).

The mRNA levels of *MRP-4-2* gene did not differ significantly between the dsRNA fed beetle and two controls ($F_{2,6}$ = 3.1, P = 0.11). For the *CYP9V1* gene, ANOVA results yielded significant variation among the mRNA transcript levels from the three conditions ($F_{2,6}$ = 8.89, P = 0.016). However, the Tukey's HST showed that although the difference in the mRNA transcript levels of *CYP9V1* in the dsRNA group was significantly different than the PBS control (P = 0.014), it was not significantly different than the *GFP* control (P = 0.072). Also, for *Esterase 2* gene, ANOVA results showed that there was a significant variation in the mRNA transcript levels from the three conditions ($F_{2,6}$ = 26.4, P = 0.0010). The Tukey's HST showed that the mRNA transcript for *Esterase 1* gene in dsRNA fed beetles was significantly different than that of PBS and *GFP* control groups (P = 0.00084 and P = 0.023, respectively). However, the difference between mRNA transcript levels between PBS and *GFP* controls was also significant for this gene (P = 0.027).

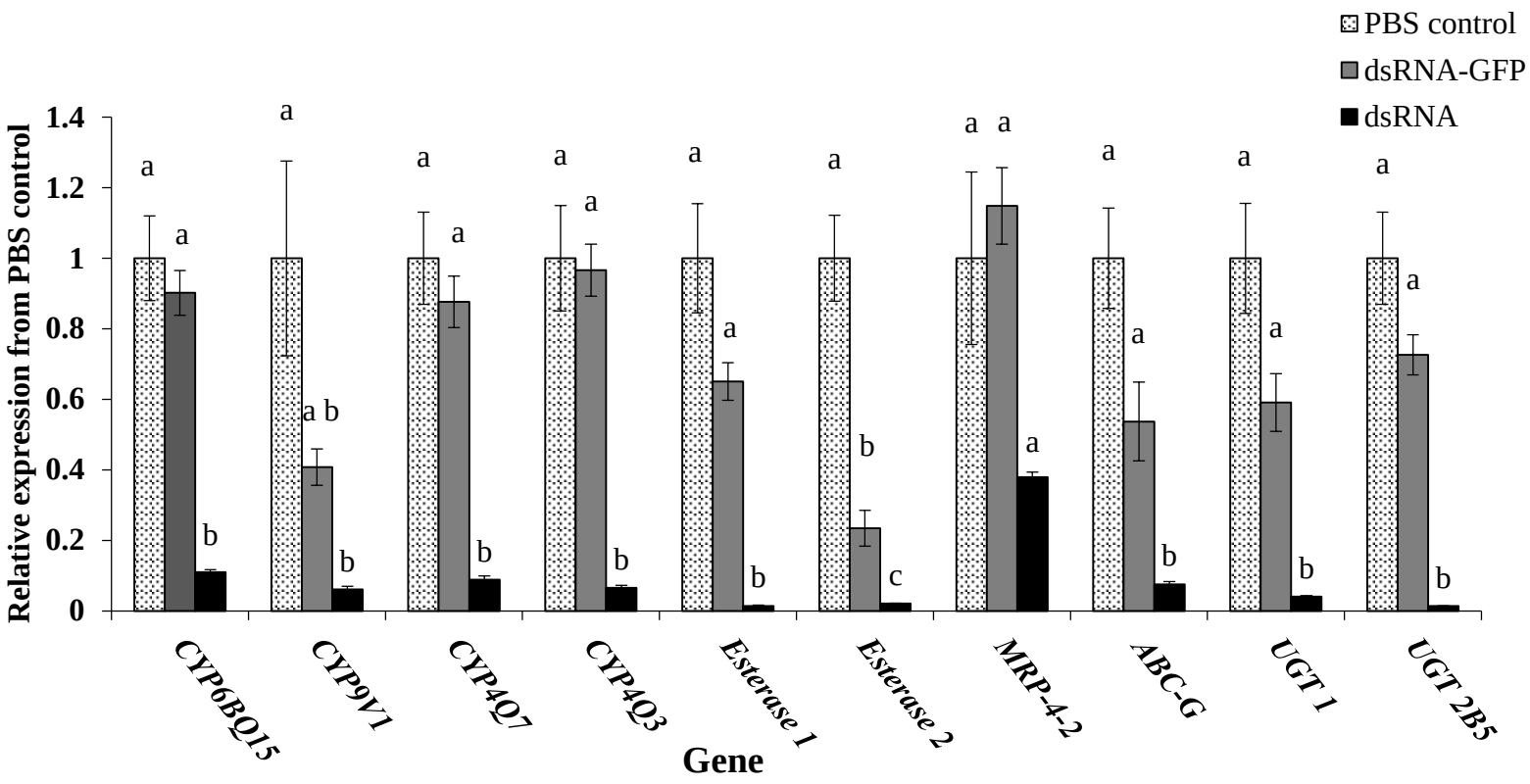
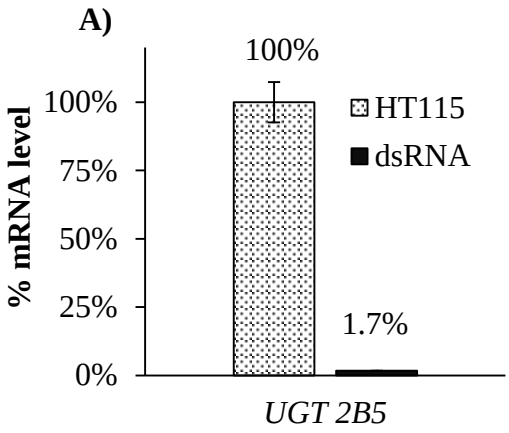


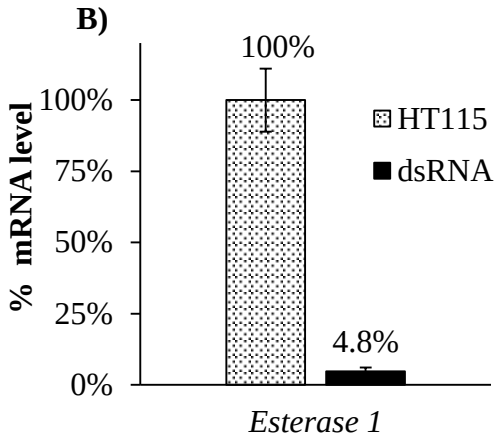
Figure 3. 8. Effects of ingesting bacterially produced dsRNA for 10 target genes on the normalized relative mRNA levels. Normalised relative expression of target genes in the RS beetles fed with potato leaves treated with *E. coli* producing dsRNA for ten genes and *GFP* relative to the RS beetles fed with potato leaves treated with PBS (control). Data are expressed as mean relative quantity ± SEM, n=3. Letters placed above bars denote significant differences in mRNA levels for each gene. Means with the same letter are not significantly different ($P > 0.05$) according to Tukey's HST (one-way ANOVA).

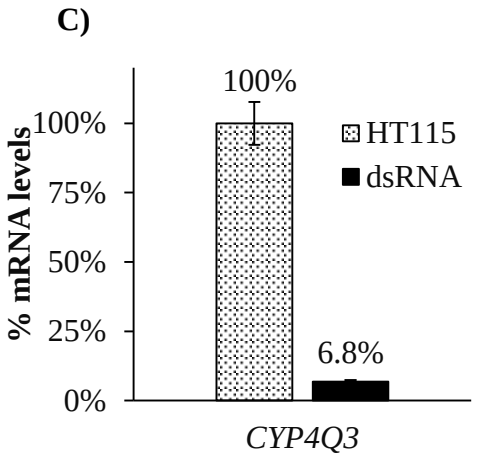
3.9 Phenotypic effects of silencing genes on imidacloprid resistance

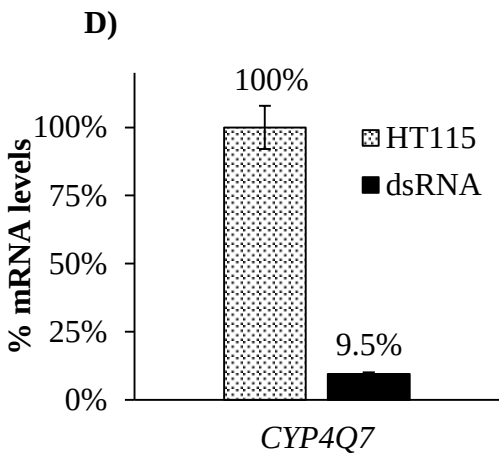
I evaluated the phenotypic effects of knocking down transcript levels of seven genes (*CYP6BQ15*, *CYP4Q3*, *CYP4Q7*, *ABC-G*, *Esterase 1*, *UGT 1*, and *UGT 2B5*) on imidacloprid resistance using bioassays. First, I repeated the qPCR analyses for the seven genes to confirm gene silencing in dsRNA fed beetles compared to the *E. coli* HT115 fed (no dsRNA, control) beetles and calculated the percent relative expression of genes after dsRNA feeding relative to control (Figure 3.9). I found that mRNA levels were reduced the most in beetles fed on dsRNA-*UGT 2B5* (98.3% reduction, Figure 3.9A), followed by dsRNA-*Esterase 1* (95.2% reduction, Figure 3.9B) and dsRNA-*CYP4Q3* (93.2% reduction, Figure 3.9C) compared to the control. The least pronounced silencing was seen in dsRNA-*ABC-G* fed beetles which had a 65% reduction in mRNA levels.

Moreover, the results showed that silencing of *CYP4Q3* and *UGT 2B5* significantly increases the toxicity of imidacloprid in the RS beetles. Mortality of the RS beetles increased 26.7% from the control when insects were fed with dsRNA-*CYP4Q3* (log rank $\chi^2 = 4.3$, df = 1, $P = 0.037$, n = 30, Figure 3.10A) and then exposed to LD_{20} of imidacloprid. Similarly, knocking down the mRNA levels of U*GT 2B5* gene increased beetle mortality by 23.3% from the control group (log rank $\chi^2 = 4.3$, df = 1, $P = 0.038$, n = 30, Figure 3.10B). Although statistically not significant, silencing of *Esterase 1* and *CYP4Q7* also resulted in a slight increase in mortality: 13.3% and 10%, respectively (Figure 3.10C and D). In contrast, feeding insects with dsRNA-*GFP* did not increase the toxicity of imidacloprid significantly (Figure 3.10E). Further, I found no significant differences in survival between control and dsRNA-*CYP6BQ15*, dsRNA-*UGT 1*, or dsRNA-*ABC-G* fed beetles (Figure 3.10F, G, and H).


A)
100%
100%
75%
50%
25%
0%
% mRNA level
HT115
dsRNA
1.7%
UGT 2B5


B)
100%
100%
75%
50%
25%
0%
% mRNA level
HT115
dsRNA
4.8%
Esterase 1


C)
100%
100%
75%
50%
25%
0%
% mRNA levels
HT115
dsRNA
6.8%
CYP4Q3


D)
100%
100%
75%
50%
25%
0%
% mRNA levels
HT115
dsRNA
9.5%
CYP4Q7

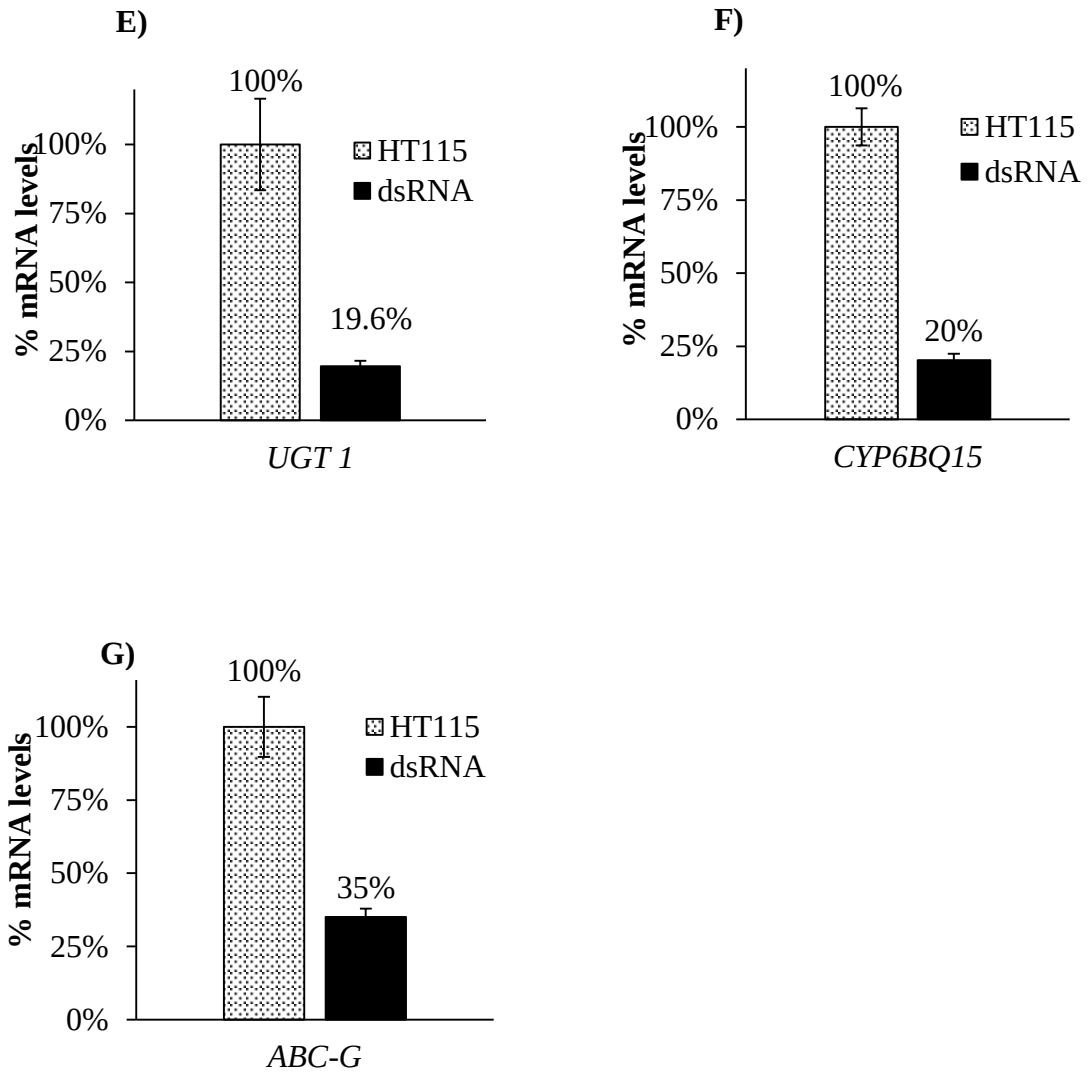
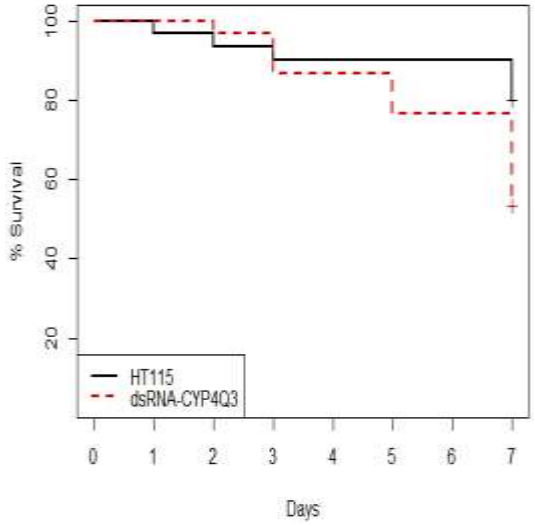
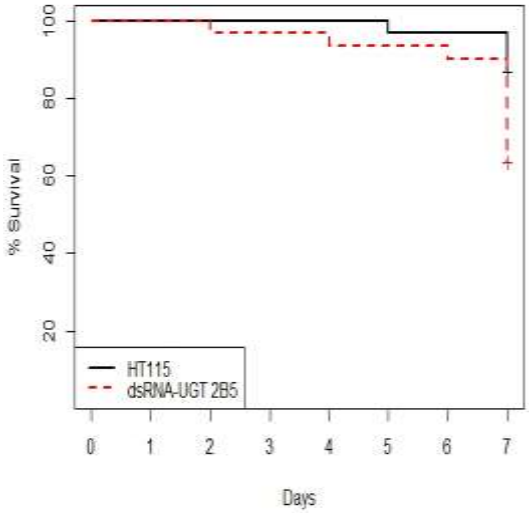


Figure 3. 9. Confirmation of RNAi knockdown for 7 target genes. Normalized mRNA quantities are set to 100% in the control group (HT115) and the % mRNA levels in the dsRNA fed beetles were calculated relative to the control. **A)** *UGT 2B5*; **B)** *Esterase 1*; **C)** *CYP4Q3*; **D)** *CYP4Q7*; **E)** *UGT 1*; **F)** *CYP6BQ15*; **G)** *ABC-G*. Data are expressed as mean relative quantity ± SEM, n=3.

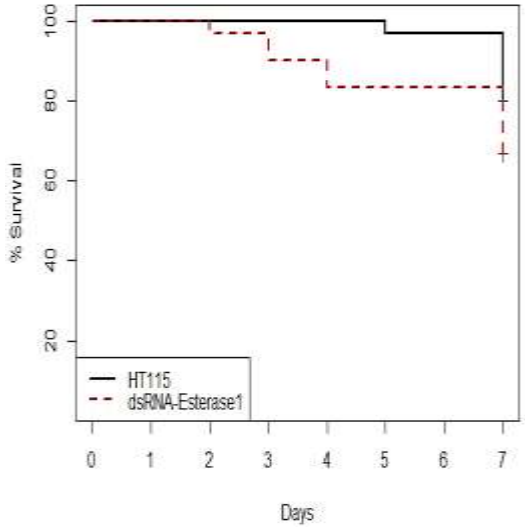
A)


% Survival
100
80
60
40
20
0
1
2
3
4
5
6
7
Days
HT115
dsRNA-CYP4Q3

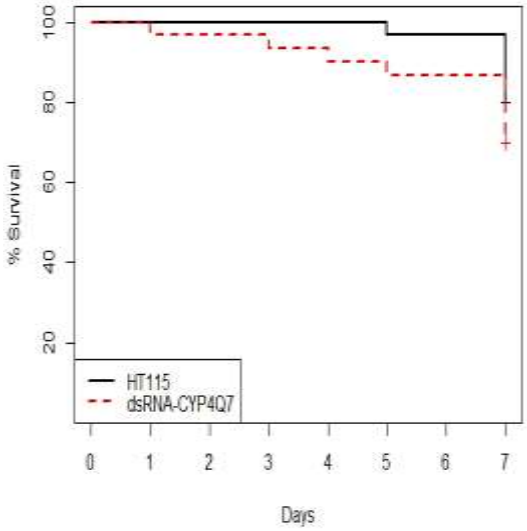
B)


% Survival
100
80
60
40
20
0
1
2
3
4
5
6
7
Days
HT115
dsRNA-UGT 2B5

C)


% Survival
100
80
60
40
20
0
1
2
3
4
5
6
7
Days
HT115
dsRNA-Esterase1

D)


% Survival
100
80
60
40
20
0
1
2
3
4
5
6
7
Days
HT115
dsRNA-CYP4Q7

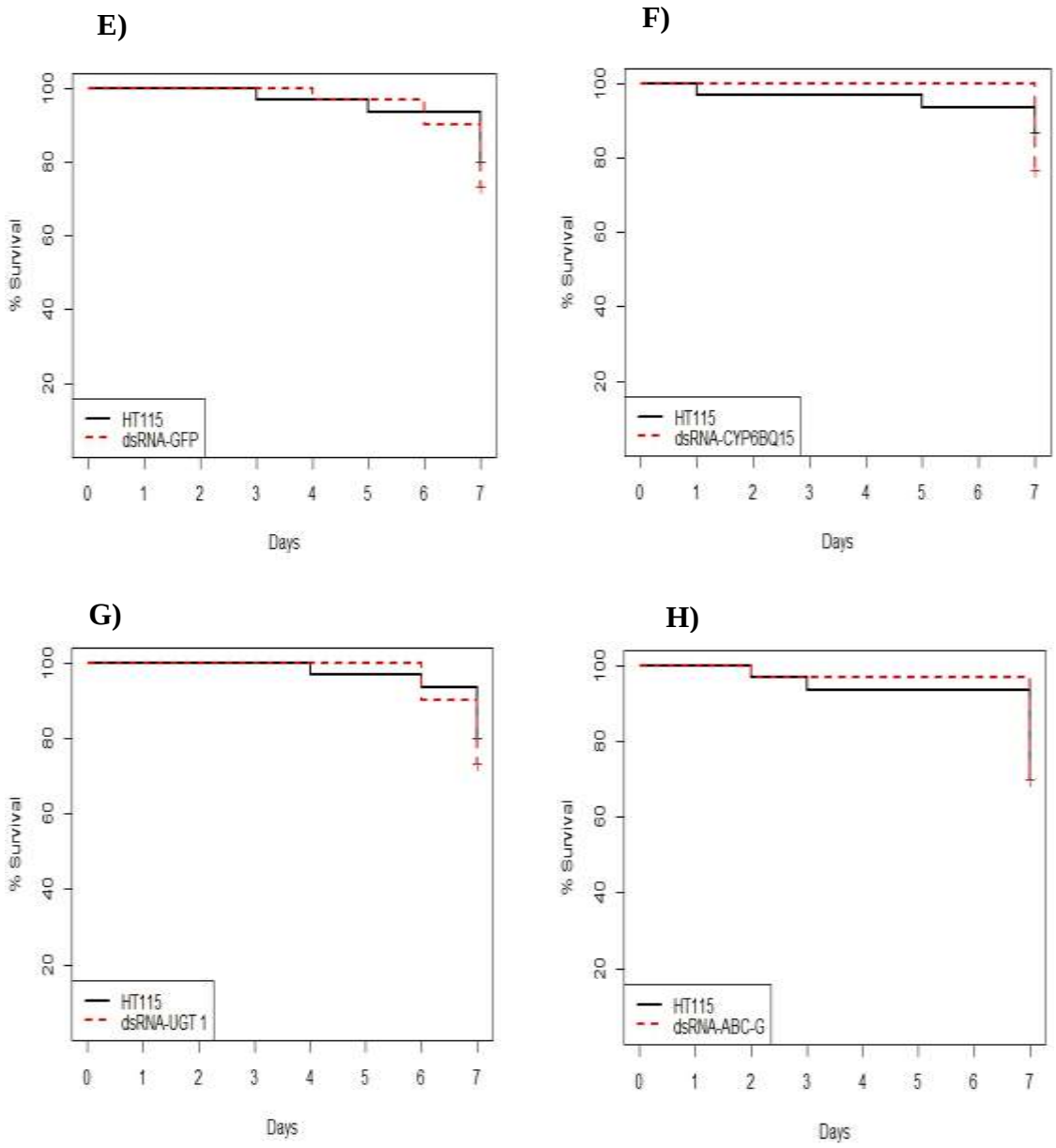


Figure 3. 10. Kaplan-Meier survival curves illustrating the percent survival of the RS beetles exposed to LD_{20} of imidacloprid after ingesting dsRNA. Beetles either ingested *E. coli* HT115 (control) or *E. coli* HT115 producing dsRNA for **A)** *CYP4Q3*, **B)** *UGT 2B5*, **C)** *Esterase 1*, **D)** *CYP4Q7*, **E)** *GFP*, **F)** *CYP6BQ15*, **G)** *UGT 1*, and **H)** *ABC-G* genes.

3.10 Simultaneous knockdown of two genes and its phenotypic effect on imidacloprid resistance

To test for a possible synergistic action of two genes on imidacloprid resistance, transcript levels of two genes were knocked-down simultaneously. For this, two genes, *CYP4Q3* and *UGT 2B5*, were selected based on the fact that when these two genes were silenced individually, the toxicity of imidacloprid in the RS beetles increased significantly. qPCR results confirmed that mRNA transcript levels of *CYP4Q3* and *UGT 2B5* were knocked down by 89.5% and 98.5%, respectively, when the insects were fed with a 1:1 mixture of two *E. coli* HT115 strains producing dsRNA for the two genes (Figure 3.11).

Simultaneous silencing of two genes increased the mortality of the RS beetles by 13.4% compared to the control after exposure to LD_{20} of imidacloprid. The result was not statistically significant (log rank $\chi^2 = 1.4$, df = 1, $P > 0.05$, n = 30) (Figure 3.12).

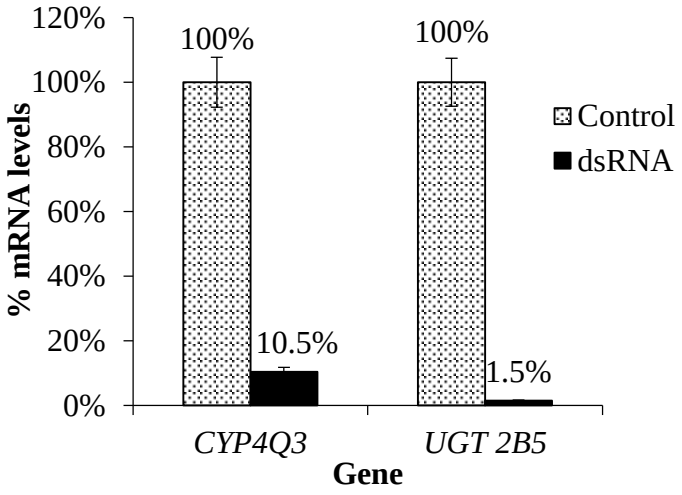


Figure 3. 11. qPCR confirmation of simultaneous RNAi knockdown of *CYP4Q3* and *UGT 2B5*. Normalized mRNA quantities are set to 100% in the control group (*E. coli* HT115 fed) and the % mRNA levels in the dsRNA fed beetles were calculated relative to the control. Data are expressed as mean relative quantity ± SEM, n=3.

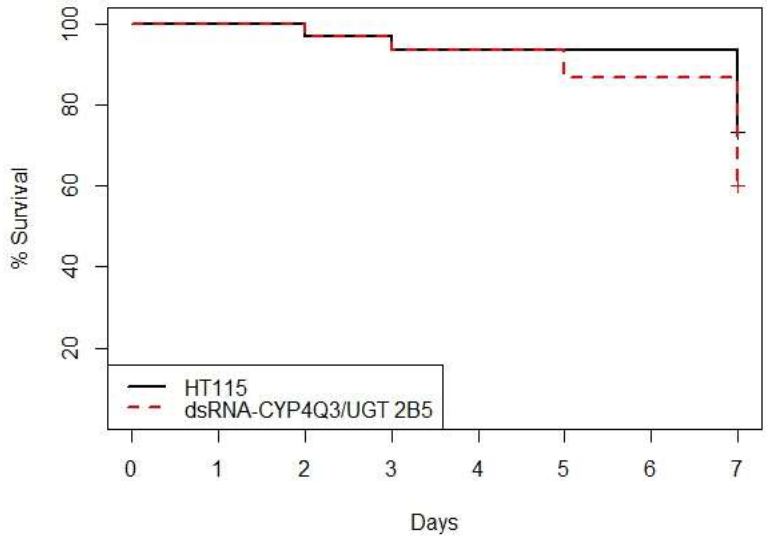


Figure 3. 12. Kaplan-Meier survival curve illustrating the percent survival of the RS beetles exposed to LD_{20} of imidacloprid after ingesting *E. coli* HT115 or dsRNA for *CYP4Q3* and *UGT 2B5* simultaneously.

Chapter 4. Discussion

Insecticide resistance is a global problem presenting a great challenge for control of economically important insect pests. In recent years, development of resistance to neonicotinoid insecticides by many pests has become a serious threat to pest control. This resistance, alongside the lack of alternative compounds to manage resistant populations, makes the effective control of pests very challenging. Therefore, elucidating the mechanisms governing insecticide resistance is crucial. The overall objective of my thesis was to gain more knowledge of mechanisms involved in neonicotinoid resistance in the Colorado potato beetle, a pest notorious for its propensity to develop insecticide resistance. In particular, I focused on the potential contribution of metabolic resistance and sought to identify detoxifying enzyme and ABC transporter genes associated with neonicotinoid resistance in this beetle.

Overall, I identified multiple detoxifying enzyme and ABC transporter genes that were transcriptionally upregulated in the imidacloprid resistant strain of the Colorado potato beetle. The upregulation of these genes was constitutive and exposure to sub-lethal doses of imidacloprid did not induce an increase in their transcript levels, at least to a detectable level. Further, I successfully knocked down the expression of seven upregulated genes using RNAi and evaluated their contribution to imidacloprid resistance in the resistant beetles using a bioassay. I found that RNAi knock-down of transcription for a cytochrome P450 (*CYP4Q3*) and a UGT enzyme (*UGT 2B5*) gene resulted in a significant increase in susceptibility of resistant insects to imidacloprid, indicating possible involvement of these enzymes in neonicotinoid resistance. However, although significant, individual silencing of these two genes only accounted for a fraction of the resistance exhibited by the RS beetles. Therefore, I sought to determine if the two genes I identified had synergistic action on neonicotinoid resistance by silencing them simultaneously. However, I found no evidence of such synergist action by the two genes. I conclude that metabolic resistance plays a significant role in imidacloprid resistance in the Colorado potato beetle, and there are multiple genes with functional redundancies involved in the process.

4.1 Stability of neonicotinoid resistance in the Colorado potato beetle

Previous studies demonstrated that in the absence of insecticide pressure, resistant insects can revert towards susceptibility (Ferguson, 2004; Shah *et al*., 2015), probably due to fitness costs associated with maintaining resistance. In support of this notion, Alyokhin *et al*. (2015) showed that resistance to imidacloprid was reduced by ten-fold in field populations of the Colorado potato beetle from southern Maine, in the United States, over a five-year period. However, my results from topical bioassays showed that the LD_{50} of imidacloprid in the resistant strain I used has remained stable after 10 generations in the absence of insecticide pressure. This suggests that there are differences between the field and laboratory pressured populations in terms of stability of resistance. This outcome is not surprising, as in the field, gene flow between resistant and sensitive beetles prevents emergence of strains homozygous for resistance (Alyokhin *et al*., 2008) whereas the resistant strain I used was previously pressured continuously with LD_{90} over 50 generations (Wang *et al*., 2016), which probably gave rise to a homozygous resistant population.

Surprisingly, I found that the LD_{50} for the sensitive strain was slightly higher than the published values (Scott *et al*., 2015). Such variation in the results of bioassay repeats is not uncommon (Skovmand *et al*., 1997), and can be caused by many factors including the age of the insects used, incubation temperature and humidity levels during bioassays, the mode of exposure to insecticide as well as other technical variations (Thiery and Hamon, 1998). Regardless, the resistance ratio between the two strains was more than 25, which is considered high, based on previous investigations (Clements *et al*., 2016a; Mota-Sanchez *et al*., 2006).

4.2 Induction of genes upon neonicotinoid exposure in the Colorado potato beetle

mRNA-seq results showed that exposure to sub-lethal doses of imidacloprid equal to 10% of LD_{50} did not result in upregulation of any detoxifying enzyme or ABC transporter genes in either the neonicotinoid resistant or sensitive strain of the Colorado potato beetle. This might imply that induction of these genes is not the primary mechanism for neonicotinoid resistance in the beetle. Contrary to my findings, a recent study by Zhu *et*

al. (2016) identified multiple CYP genes upregulated upon imidacloprid exposure in neonicotinoid resistant beetles. However, in their induction experiment, Zhu *et al*. (2016) used LD_{50} of imidacloprid. Similarly, in *B. tabaci*, exposure to LD_{80} of thiamethoxam led to induction of several detoxifying enzyme genes (Yang *et al*., 2013b). This pattern suggests that induction of detoxifying enzyme and ABC transporter genes by neonicotinoids may be dose-dependent. In support of this notion, Yang Y. *et al*. (2016) showed that more detoxifying enzyme genes were upregulated when the whitebacked planthopper, *Sogatella furcifera*, was exposed to a higher dose (LD_{85}) of the neonicotinoid insecticide cycloxaprid (a new generation neonicotinoid) than a lower dose (LD_{15}). Hence, it is possible that the sub-lethal doses I used failed to exert enough pressure on the beetles to result in upregulation of the resistance-related genes. That being said, upregulation of detoxifying enzyme and ABC transporter genes by exposure to an insecticide does not necessarily mean they have roles in insecticide detoxification. In fact, a *CYP6B1* gene in *D. melanogaster* plays a role in imidacloprid resistance (Kalajdzic *et al*., 2012), but is not induced by exposure to imidacloprid (Kalajdzic *et al*., 2013).

I also found that exposure to sub-lethal doses of imidacloprid elicited stronger transcriptomic responses in the resistant beetles than the sensitive beetles. Transcript levels of several enzymes involved in lipid and carbon catabolism were upregulated in the resistant beetles whereas only one enzyme involved in lipid metabolism was upregulated in the sensitive beetles. In addition, several protease inhibitor genes were downregulated in the resistant beetles. Interestingly, one function of protease inhibitors in insects is to block activity of digestive protease enzymes (Gubb *et al*., 2010). Therefore, I suggest that the up/down-regulation of these genes probably allows resistant beetles to meet higher energy demands during stress. In addition, I found an alcohol dehydrogenase gene to be upregulated in the resistant beetles. Alcohol dehydrogenases are often upregulated in insecticide resistant insects (Zhu and Luttrell, 2015; David *et al*., 2014), and are linked to detoxification of xenobiotics and to protection against oxidative stress generated during metabolism (Hayes *et al*., 2004). Finally, two detoxifying genes, *CYP4G57* and *UGT 2A2*, were downregulated in the resistant beetles. This is not unusual

as downregulation of some detoxifying enzyme genes is also observed in other insects exposed to insecticides (Yang *et al*., 2013b; do Nascimento *et al*., 2015). In humans, UGT 2A2 enzymes are mainly expressed in nasal mucosa tissue and are thought to aid in odourant signal termination (Sneitz *et al*., 2009). Therefore, it is possible that in insects they have similar functions. The role of CYP4G57 in insecticide resistance is not known, but it has also shown slight downregulation in Colorado potato beetles exposed to the pyrethroid insecticide, cyhalothrin (Wan *et al*., 2013).

4.3 Constitutive differences in the mRNA levels between the neonicotinoid resistant and sensitive strains of the Colorado potato beetle

Using mRNA-seq reads, I performed two DESeq analyses to identify differentially expressed sequences between the neonicotinoid sensitive and resistant beetles. Because I found a considerable overlap between the two comparisons, I limit my discussion to general trends.

4.3.1 Overall differences between resistant and sensitive beetles

Overall, I found striking differences between the transcriptome profiles of the two beetle strains. On average, there were more than 7000 differentially expressed sequences. However, I expected such differences given that the two strains I used have originated from two different geographic regions and have experienced unique environmental pressures throughout their life history. In fact, Clements *et al*. (2016a) showed that even distinct populations of beetles collected from similar geographic and agricultural regions can have big differences in their transcriptomic profiles.

Another intriguing finding was that around 45% of the differentially expressed sequences were unknown sequences. Furthermore, some of these sequences had the highest fold change increases in the resistant beetles. However, this phenomenon is not unique to my study. Similar observations were also made by Dermauw *et al*. (2012) who studied the transcriptomic changes in the spider mite, *Tetranychus urticae*, caused by changes in host plants. Interestingly, when I manually analyzed some of these most differentially expressed unknown sequences, I found that several lacked open reading frames, which implies that they might be sequences from long non-coding RNA species. Generally,

these RNA species have important roles in development, epigenetics, and regulation of transcription (Mercer *et al*., 2009). Further genetic analyses are needed to determine the identity and significance of these differentially expressed sequences. Also, using *in silico* analyses, the protein products of those unknown sequences possessing open reading frames can be predicted.

I also found that a large number of genes associated with energy metabolism had elevated transcript levels in the resistant beetles. For instance, genes involved in digestion, carbohydrate metabolism, and energy production were all significantly over-transcribed. These findings are consistent with the assumption that insecticide resistant insects require higher energy production to maintain their resistance mechanisms in addition to their basic physiological processes (Araújo *et al*., 2008a). Consequently, increased expression of genes, directly or indirectly associated with energy production, is expected. Similar observations have been also made in many other insecticide resistant insects, including the green peach aphid, *Myzus persicae* (Silva *et al*., 2012) and the mosquito, *Culex pipiens pallens* (Lv *et al*., 2016). Furthermore, studies on the maize weevil, *Sitophilus zeamais*, confirmed enhanced activities of enzymes involved in digestion and energy metabolism in the insecticide resistant strains (Guedes *et al*., 2006; Araújo *et al*., 2008a,b; Lopes *et al*., 2010).

Elevated metabolic activity to generate more energy often results in increased production of reactive oxygen species (ROS), such as superoxide anions, hydroxyl radicals, and hydrogen peroxide. Increased ROS levels can result in extreme stress in cells and cause damage to key biomolecules (Finkel and Holbrook, 2000). Some of the best known antioxidant enzymes capable of detoxifying ROS are superoxide dismutases and glutathione peroxidases. I found that sequences corresponding to these genes were also over-transcribed in the resistant beetles. In addition, sequences encoding heat shock proteins and chaperones were over-transcribed in the resistant insects. These proteins protect insects against a wide range of biotic and abiotic stresses and help with the correct folding of proteins (Zhao and Jones, 2012). Expression of these genes is also frequently upregulated in other insecticide resistant insects, too (Lv *et al*., 2016; Yang *et al*., 2013b).

Furthermore, several genes involved in general oxidative stress and immune responses were also over-transcribed in the RS beetles. Taken together, my findings imply that maintaining resistance is energetically demanding, and resistant beetles have evolved many complex responses that allow them to coordinate expression of different sets of genes. This in turn enables them to enhance their energy production while avoiding any significant oxidative damage that may occur during the process. However, these are all assumptions based on incomplete data, and more studies are required to develop a thorough understanding of the function of these differentially expressed genes in the resistant insects. In particular, further proteomic studies are needed to correlate mRNA levels with protein levels, which would allow more informed conclusions about the potential role of these genes in resistance.

I also analyzed the 100 most significantly down-regulated contigs, and found ribosomal protein genes (S18, L15, L36, and S40), members of the cathepsin family (Cathepsin D, B, and I), and several reductases to be downregulated in the resistant beetles. Although ribosomal proteins were previously considered to be stably expressed, recent studies have demonstrated that they do show variation in their expression levels in animals exposed to pesticides (Tanguy *et al*., 2005; Alon *et al*., 2012). Similarly, variation in the expression levels of members of the cathepsin family and several reductases have also been observed in other insecticide resistant insects (Zhu and Luttrell, 2015). Cathepsins are lysosomal proteases involved in normal cellular protein degradation and turnover (Turk *et al*., 2012), and they have roles in insect development and metamorphosis (Gui *et al*., 2006). However, the significance of their downregulation in insecticide resistant insects is yet to be determined.

4.3.2 Differentially expressed detoxifying enzyme and ABC transporter genes

Because my induction experiments did not reveal any detoxifying enzyme and ABC transporter genes upregulated upon neonicotinoid exposure, I focused on the constitutively differentially expressed genes in the resistant beetles. I identified multiple differentially expressed sequences encoding CYPs, esterases, GSTs, UGTs, and ABC transporters. My results showed that there were more of these genes in the resistant

beetles with increased transcript levels than decreased transcript levels, which was expected.

Among these five protein superfamilies, CYPs have been studied the most extensively, and there is considerable evidence for their involvement in insecticide resistance. For instance, RNAi knockdown of *CYP6BG1* reduces resistance of the diamondback moth, *Plutella xylostella*, to the pyrethroid insecticide permethrin (Bautista *et al*., 2009). Similarly, silencing of *CYP353D1V2* increases susceptibility of the small brown plant hopper, *Laodelphax striatellus*, to imidacloprid (Elzaki *et al*., 2016). In my analysis, CYPs showed the highest number of differentially expressed sequences. In particular, I found that the members of CYP9, CYP6 and CYP4 families had the most enrichment in the resistant beetles. Interestingly, all three families are often overexpressed and associated with insecticide resistance in insects (Feyereisen, 2012). For example, constitutive overexpression of *CYP6M1* and *CYP4C64* in *B. tabaci* (Karunker *et al*., 2008; Yang X. *et al*., 2013), *CYP6CY3* in *M. persicae* (Puinean *et al*., 2010), and *CYP6ER1* in *N. lugens* (Bass *et al*., 2011) are all associated with neonicotinoid resistance. Similarly, overexpression of *CYP9M10* in the mosquito, *Culex quinquefasciatus* (Itokawa *et al*., 2010) and *CYP6BQ23* in the pollen beetle, *Meligethes aeneus* (Zimmer *et al*., 2014) is linked to pyrethroid resistance.

Evidence for a role for esterases in insecticide resistance is vast (Montella *et al*., 2012), and using RNAi, several studies have identified specific esterase genes involved in organophosphate resistance in *A. gossypii* (Gong *et al*., 2014) and acaricide resistance in the carmine spider mite, *Tetranychus cinnabarinus* (Shi *et al*., 2016). The contribution of esterases to neonicotinoid resistance, however, has been mostly inferred from synergistic studies (Mota-Sanchez *et al*., 2006; Zhao *et al*., 2000) and transcriptome profiles of neonicotinoid resistant insects (Ilias *et al*., 2015; Zhu and Luttrell, 2015). Until recently, the same was true for the GST enzymes. However, a recent study by Yang X. *et al*. (2016) used RNAi to demonstrate involvement of a specific GST gene in neonicotinoid resistance in *B. tabaci*. My results from DESeq analyses showed that multiple esterase and GST genes are also upregulated in a neonicotinoid resistant strain of the Colorado potato beetle. Interestingly, most of the GST sequences belonged to sigma, delta, and

epsilon classes, which are frequently associated with insecticide resistance in other insects (Lumjuan *et al*., 2011; Qin *et al*., 2012; Yang X. *et al*., 2016).

Although the role of UGT enzymes and ABC transporters in drug resistance is well established in humans, studies analyzing their role in insecticide resistance are still in their infancy (Dermauw and Van Leeuwen, 2014; Ahn *et al*., 2012). Of the five protein superfamilies I focused on in my analysis, these two are probably the least well described in the Colorado potato beetle, as no studies have been done to identify or analyze the expression patterns of these genes in the beetle. In the reference transcriptome by Kumar *et al*. (2014), most of the transcripts corresponding to these two protein superfamilies are predicted based on the sequence similarities to genes in other insects. Nonetheless, in line with other transcriptomic studies, I uncovered multiple transcripts annotated as UGT and ABC transporter genes overexpressed in the resistant beetles, implying a potential role in neonicotinoid resistance. Although roles for ABC transporters in resistance to several insecticides have been demonstrated through RNAi in some insects (Yoon *et al*., 2011; Figueira-Mansur *et al*., 2013), it is still unclear what role, if any, they have in neonicotinoid resistance. Similarly, to date, no studies have been undertaken to determine the potential role of the UGT enzymes in neonicotinoid resistance. Overall, I found that DESeq analysis provided a comprehensive list of candidate detoxifying enzyme and ABC transporters genes that are potentially involved in neonicotinoid resistance in the Colorado potato beetle. However, this analysis, although useful, is incomplete, because the false positivity rate of DESeq analysis can be high (Rajkumar *et al*., 2015), and differential expression of individual transcripts needs to be validated. Also, further functional studies are needed to determine the possible role of each of these candidate genes in the neonicotinoid resistance in the beetle. Therefore, I employed RNAi to study the function of some of these genes in neonicotinoid resistance in the beetle.

4.3.3 qPCR validation of DESeq results for detoxifying enzyme and ABC transporter genes

Metabolic resistance to insecticides is mainly caused by upregulation of detoxifying enzymes and ABC transporter in the resistant insects. Hence, I wanted to ensure that the

genes I selected for further analysis were truly over-transcribed in the resistant beetles. I selected 32 genes based on my DESeq analysis and verified their expression using qPCR. Unfortunately, none of the GST transcripts I selected showed significant upregulation in the resistant insects as revealed by qPCR analysis. The same was also true for several other transcripts from the other gene families. It is possible that DESeq analysis mis-identified those non-confirming transcripts as differentially expressed due to poor alignment of the mRNA-seq reads to the reference transcriptome. This could be caused by sequencing errors or polymorphisms in individual RNA samples (Degner *et al*., 2009). Also, poor annotation and assembly in the reference transcriptome could also contribute to poor alignment of reads. Additionally, I observed differences in the magnitude of fold changes between qPCR and mRNA-seq based methods. However, this is expected as qPCR and RNA-seq analyses use different approaches to measure gene expression. I suggest that using digital droplet PCR could produce more comparable data to mRNA-seq as it measures absolute number of transcripts, similar to mRNA-seq (Hindson *et al*., 2013).

4.4 RNAi knockdown of targeted genes in the Colorado potato beetle

Previous studies have shown that RNAi works efficiently in the Colorado potato beetle, making it possible to perform functional gene analyses (Zhu *et al*., 2011; Palli, 2014). Here, I also demonstrate efficient silencing of multiple genes in the beetle through RNAi. For this set of experiments, I selected 10 detoxifying enzyme and ABC transporter genes whose mRNA levels were most over-transcribed (based on qPCR analysis of 32 targets) in the neonicotinoid resistant beetles. I also included *GFP* dsRNA as a control in my experiments to detect off target effects of RNAi, which occurs when RNAi causes degradation of untargeted mRNAs. I showed that dietary ingestion of specific dsRNA produced in bacteria significantly reduced the mRNA transcripts of *CYP6BQ15*, *CYP4Q7*, *CYP4Q3*, *Esterase 1*, *ABC-G*, *UGT 1*, and *UGT 2B5* genes in the experimental groups compared with 1× PBS and *GFP* dsRNA ingested beetles. Because the mRNA levels for these genes were not reduced in the *GFP* dsRNA fed insects, I assumed that the silencing of the aforementioned genes was specifically triggered by the presence of specific dsRNA molecules. Hence, I decided to carry my experiments one step further

and to perform bioassays to determine if the silencing of these seven genes would have any effects on the neonicotinoid resistance.

I also found that although the *CYP9V1* and *Esterase 2* genes were silenced in the experimental groups compared with 1× PBS control, mRNA levels for these two genes were reduced in the *GFP* control, too. There are two possibilities as to why this happened. First, it is possible that these genes were downregulated in response to the presence of bacteria in the diet. Second, it might have been caused by off target effects of dsRNA for the *GFP* gene. In fact, off target effects caused by dsRNA representing a segment of the *GFP* gene has been also shown to occur in other insects such as the honey bee, *Apis mellifera* (Nunes *et al*., 2013). Due to this uncertainly, I did not pursue further experiments involving these two genes.

Of the 10 genes I selected for RNAi, I observed the least reduction in mRNA levels for the *MRP-4-2* gene. Previous studies suggested that RNAi efficacy varies depending on the genes targeted (Terenius *et al.*, 2011). Factors, including secondary structures of mRNA (Fakhr *et al*., 2016), complementarity of siRNAs to unrelated mRNAs (Jackson *et al.*, 2003), and stability of the dsRNA molecule (Yu *et al*., 2013) can influence the efficacy of RNAi for a given gene. Therefore, inefficient silencing of the *MRP-4-2* might have been caused by any of the above mentioned and/or by other factors. One way to increase the probability of silencing a gene is to design multiple dsRNA molecules for different positons on the gene, which can increase the likelihood of finding an optimal spot for efficient RNAi. Future studies could employ this approach to accomplish significant silencing of *MRP-4-2,* so that its potential role in neonicotinoid resistance can be studied.

4.5 Effects of silencing resistance-related genes on neonicotinoid resistance in the Colorado potato beetle

My results from the bioassays showed that RNAi knock-down of transcription for *CYP4Q3* and *UGT 2B5* genes results in a significant reduction in imidacloprid resistance in the Colorado potato beetle. When *CYP4Q3* and *UGT 2B5* mRNA levels were reduced by 93.2% and 98.3%, respectively, compared with the control, mortality of the resistant

beetles exposed to LD_{20} of imidacloprid increased significantly, by more than two fold for each gene. In comparison, the mortality of the beetles fed on the dsRNA for *GFP* did not change significantly. This strongly suggests that enhanced toxicity of the imidacloprid was due to RNAi of the two genes. Based on my results, I suggest that overexpression of these two genes in the resistant beetles plays a part in imidacloprid detoxification. My finding provides further evidence for the role of CYP enzymes in neonicotinoid resistance. Recently, a CYP enzyme gene, *CYP9Z26*, was shown to contribute to imidacloprid resistance in a Colorado potato beetle population from the Central Sands region of Wisconsin (Clements *et al*., 2016b), and *CYP4Q3* gene represents the second gene from the CYP superfamily shown to contribute to imidacloprid resistance in the beetle. In addition, in this study, for the first time, I showed that RNAi of a UGT gene also increases toxicity of imidacloprid in the beetle. Further, this is also the first study to infer a role for a UGT enzyme in neonicotinoid resistance in insects.

Although RNAi of *CYP4Q3* and *UGT 2B5* resulted in a significant increase in beetle mortality upon imidacloprid exposure, individual silencing of the genes did not completely block imidacloprid resistance. This finding prompted me to test if *CYP4Q3* and *UGT 2B5* had a synergistic effect in resistance. For this purpose, I fed the resistant beetles with a mixture of two strains of *E. coli* HT115, each producing dsRNA for one gene, to suppress mRNA of the two genes simultaneously. Although I accomplished efficient knock down of both genes (a reduction of 89.5% for *CYP4Q3* and 98.5% for *UGT 2B5* in mRNA levels), simultaneous silencing of the two genes did not increase the mortality of the beetles significantly after imidacloprid exposure. Although disappointing, my results are actually consistent with previously published data. Lack of and/or reduced phenotype due to simultaneous silencing of two genes has been also observed in previous studies. For instance, Zhang *et al*. (2015) demonstrated that simultaneous silencing of two essential genes, *B-ACTIN* and *SHRUB*, resulted in reduced mortality of Colorado potato beetle larvae compared with the mortality rate when the two genes were silenced individually. Similar observations were made in *C. elegans*, as well (Kamath *et al*., 2000). In addition, studies conducted in *T. castaneum* and *H. armigera*,

found no indication of synergism when multiple genes were targeted simultaneously (Ulrich *et al*., 2015; Mao *et al*., 2015). Taken together, these results suggest that targeting two resistance-related genes at the same time may not enhance insecticide toxicity. However, different gene combinations may give different results as shown by Min *et al*. (2010), and more studies are needed to determine the mechanisms involved in this interesting observation.

Bioassay results for the remaining five genes, *CYP6BQ15*, *CYP4Q7*, *Esterase 1*, *ABC-G*, and *UGT 1*, implied that silencing of these genes does not affect the toxicity of imidacloprid significantly in the Colorado potato beetle. However, I did observe a slight increase in the mortality of the beetles when *Esterase 1* and *CYP4Q7* were knocked down, 13.3% and 10%, respectively. Although not statistically significant, I suggest that these two genes probably have minor roles in imidacloprid resistance. However, I cannot confidently make a clear conclusion about the role of *CYP6BQ15*, *ABC-G*, and *UGT 1* for the following reasons. First, mRNA levels of these three genes had the highest fold increases in the resistant beetles compared to sensitive beetles. Second, although RNAi knock-down of these genes was significant, the degree of expression reduction was not as efficient as with the other four genes. While I achieved more than 90% reduction in mRNA levels for *CYP4Q3*, *UGT 2B5*, *Esterase 1* and *CYP4Q7*, the mRNA reduction remained at 80.4%, 80%, and 65% for *CYP6BQ15*, *UGT 1*, and *ABC-G*, respectively. Therefore, it is possible that RNAi failed to reduce the mRNA levels enough to cause a significant reduction in protein levels; hence, no effects in imidacloprid toxicity were observed. On the other hand, the highest fold increase in mRNA levels does not necessarily imply that the gene would have the greatest contribution to resistance. In fact, a study by Bao *et al*. (2016) showed that two CYP genes, *CYP6AY1* and *CYP6ER1*, had equal contribution to imidacloprid resistance in *N. lugens* despite the fact that the latter had much higher fold increase in mRNA levels in some resistant populations. In addition, overexpression of a particular gene may serve functions other than enhanced insecticide metabolism. Furthermore, most of the time, mRNA levels do not accurately reflect functional protein levels (Nie *et al*., 2006). Indeed, Yang *et al*. (2013b) demonstrated only a moderate correlation between mRNA and protein levels in a thiamethoxam

resistant strain of *B. tabaci*. Hence, whether *CYP6BQ15*, *ABC-G*, and *UGT 1* play a role in neonicotinoid resistance in the Colorado potato beetle is not clear from my results. Future studies can attempt to achieve a more efficient RNAi of these genes to rule out their contribution. Further, transcriptomic data can be complemented with proteomic data to investigate the correlation between mRNA and protein levels.

4.6 Future directions

While this research provided important insights into the neonicotinoid resistance in the Colorado potato beetle, there are still many questions remaining to be addressed. In fact, there are several possible avenues of study that can further our knowledge of neonicotinoid resistance in this beetle. For example, although my results provided strong evidence for involvement of *CYP4Q3* and *UGT 2B5* in neonicotinoid resistance, more studies could be conducted to determine whether the protein products of these two genes can indeed metabolize imidacloprid. To accomplish this objective, full length cDNAs of these genes must first be cloned. Then, the proteins can be expressed in insect cell lines for further metabolic studies as described by Zhu *et al*. (2010). This kind of study can provide more direct evidence for a role of *CYP4Q3* and *UGT 2B5* in neonicotinoid metabolism in the Colorado potato beetle. Additionally, expression of these two genes could be analyzed in other neonicotinoid resistant populations to determine if the molecular basis of neonicotinoid resistance is shared among different resistant populations. Furthermore, because RNAi of *CYP4Q3* and *UGT 2B5* did not completely block imidacloprid resistance in the resistant beetles, I suggest that one or more of the other over-transcribed genes in the resistant beetles play additional roles in resistance. Unfortunately, I was not able to achieve an efficient knock-down of *MRP-4-2*, so its potential contribution to resistance remains to be elucidated. Moreover, I was not able to confirm any GST enzymes with up-regulated transcript levels. However, a recent study has shown that up-regulation of GSTs does contribute to neonicotinoid resistance in *B. tabaci* (Yang X. *et al*., 2016). Therefore, another course of study would be to analyze potential roles of the remaining over-transcribed detoxifying enzyme and ABC transporter genes in resistance through RNAi. Furthermore, as I alluded to previously, complementing transcriptomic data with proteomic data can provide a more

comprehensive view of mechanisms involved in neonicotinoid resistance in the Colorado potato beetle.

In addition, my results do not exclude the possibility that other mechanisms also contribute to neonicotinoid resistance in the beetle. Previously, Mota-Sanchez *et al*. (2006) suggested that decreased penetration and increased excretion were not major factors in imidacloprid resistance. However, a potential role of target site insensitivity to imidacloprid was implied by Tan *et al*. (2008) who demonstrated that central nervous system preparations from imidacloprid resistant beetles had reduced sensitivity to inhibition by imidacloprid. Although no mutation has been reported in the target nAChR so far, further studies could investigate the role of target site insensitivity in neonicotinoid resistance in the beetle.

4.7 Conclusions and significance of the study

My results provide evidence for metabolic resistance as the mechanism for neonicotinoid resistance in the Colorado potato beetle. The most important finding of this work was the identification of two detoxifying enzymes that play roles in imidacloprid resistance. The constitutive overexpression of these genes probably allows resistant beetles to metabolize insecticide molecules more efficiently, resulting in resistance. My results also imply that neonicotinoid resistance in the beetle is controlled by multiple genes, some of which remain to be identified. It appears that in addition to having an enhanced detoxification system, resistant beetles also have improved energy metabolism, which may help them mitigate the potential cost of resistance. The knowledge gained from this study is important as it gives us new opportunities to develop novel pest control strategies that can exploit the mechanisms mediating resistance. For instance, RNAi knock-down of resistance-related genes, in combination with chemical insecticides, can offer a new pest control strategy. This could significantly reduce chemical insecticide use and lessen the possibility of resistance development by the target pests.

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Appendices

Appendix A: List of the 100 most significantly over-transcribed contigs in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[2]	Read count SS[2]	Log_2 Fold change	*P*-adj[3]	Read count RS[2]	Read count SS[2]	Log_2 Fold change	*P*-adj[3]
Ld_rep_c43798	predicted , uncharacterized protein	8204.21	0.65	13.63	1.26E-129	8165.7	1.2	12.7	1.14E-92
Ld_rep_c26748	NA	7766.87	0.67	13.51	1.07E-155	11198.8	0.3	15.2	8.74E-51
Ld_rep_c51562	cathepsin L	10886.46	0.00	NA	1.08E-27	8167.4	1.3	12.7	8.83E-84
Ld_rep_c44316	lysosomal aspartic protease	2766.24	0.32	13.06	2.42E-34	2460.1	0.9	11.4	2.38E-74
Ld_c70856	gulucose dehydrogenase	2426.66	0.31	12.92	3.97E-04	1734.9	0.0	NA	2.34E-72
Ld_rep_c33964	NA	9290.59	1.30	12.80	2.12E-158	10903.2	5.0	11.1	5.99E-54
Ld_rep_c27340	endopolygalacturonase	2348.12	0.33	12.78	4.84E-59	1769.6	0.0	NA	5.64E-62
Ld_rep_c63457	NA	2251.11	0.33	12.72	4.83E-27	2123.6	0.0	NA	1.33E-69
Ld_rep_c34696	fatty acid binding protein	9945.55	1.64	12.57	5.23E-159	11536.2	1.0	13.6	7.19E-99
Ld_c10927	putative nonstructural polyprotein	3644.66	0.65	12.46	5.53E-10	5566.8	0.0	NA	1.81E-30
Ld_rep_c45055	NA	3278.29	0.65	12.31	2.79E-92	3267.0	0.0	NA	9.95E-83
Ld_rep_c34729	translocator protein	1546.88	0.33	12.18	5.24E-108	1781.8	0.6	11.4	4.62E-70
Ld_rep_c41824	NA	2798.09	0.64	12.10	3.42E-123	2065.7	1.2	10.7	6.59E-34
Ld_rep_c37815	fatty acid binding protein	1372.62	0.31	12.10	2.31E-104	1218.2	0.6	11.0	9.82E-64
Ld_rep_c28953	putative nonstructural polyprotein	1288.99	0.31	12.01	8.64E-10	1927.2	0.0	NA	9.14E-24
Ld_rep_c35778	uncharacterized protein	1298.94	0.33	11.93	8.49E-83	1473.2	1.3	10.2	1.36E-44
Ld_rep_c32285	C-1-tetrahydrofolate synthase, cytoplasmic	2403.30	0.62	11.91	7.75E-54	4068.0	2.8	10.5	9.83E-78

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Ld_rep_c50623	ATP synthase B chain	3452.91	0.00	NA	5.54E-34	3107.8	0.0	NA	4.99E-20
Ld_rep_c62154	glycoside hydrolase family 1	2197.79	0.65	11.73	7.02E-27	1362.9	1.0	10.5	5.48E-32
Ld_rep_c41136	NADH dehydrogenase	1054.63	0.31	11.72	7.67E-45	1007.2	0.9	10.1	1.90E-59
Ld_c8981	putative nonstructural polyprotein	1109.76	0.33	11.70	2.76E-09	1581.1	0.0	NA	1.50E-19
Ld_c10518	Fatty acid-binding protein	2949.68	0.00	NA	4.43E-27	3009.9	0.0	NA	2.51E-67
Ld_rep_c58273	NA	2931.09	0.00	NA	1.92E-129	2932.1	0.0	NA	7.34E-81
Ld_c38552	PREDICTED: cell wall protein DAN4	898.41	0.31	11.49	2.31E-91	933.8	0.6	10.5	3.52E-59
Ld_rep_c112888	NA	945.79	0.33	11.47	6.67E-79	1658.5	0.9	10.8	9.63E-68
Ld_rep_c25271	NA	2836.42	0.00	NA	2.52E-06	9454.8	0.0	NA	8.23E-65
Ld_c11	ankyrin 2,3/unc44	2640.77	0.97	11.41	8.81E-122	3480.5	0.6	12.6	3.90E-81
Ld_rep_c43234	ribosomal protein L35	2552.94	0.99	11.33	3.93E-16	3444.0	0.6	12.4	1.10E-43
Ld_rep_c44555	activating transcription factor of chaperone	3252.48	1.31	11.28	1.61E-16	3649.7	2.1	10.7	8.41E-26
Ld_rep_c41496	fk506-binding protein	744.64	0.31	11.22	1.27E-20	949.3	0.3	11.7	1.13E-47
Ld_rep_c46616	digestive cysteine protease intestain	743.09	0.31	11.22	1.85E-85	436.9	0.3	10.4	1.60E-09
Ld_rep_c38424	aminopeptidase n	2094.52	0.00	NA	5.65E-77	2520.5	1.9	10.4	6.23E-72
Ld_rep_c43642	endopolygalacturonase	2092.84	0.00	NA	2.90E-119	1960.0	0.0	NA	5.43E-65
Ld_rep_c33133	juvenile hormone binding protein 5p2	5252.39	2.61	10.97	1.19E-20	15302.7	4.3	11.8	2.17E-17
Ld_c119494	NA	1865.29	0.00	NA	8.57E-12	2832.0	0.0	NA	2.82E-32
Ld_rep_c43982	PREDICTED: 40S ribosomal protein S20	1836.85	0.99	10.86	2.87E-34	4155.5	1.0	12.1	1.43E-82

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Ld_rep_c41492	NA	1777.79	0.96	10.86	1.76E-66	1457.1	0.6	11.2	8.57E-07
Ld_rep_c33956	NA	572.55	0.32	10.79	2.22E-08	568.2	0.3	10.8	2.21E-13
Ld_rep_c38768	gamma-interferon-inducible lysosomal thiol reductase-like	1468.65	0.94	10.61	1.40E-103	1707.9	1.0	10.8	2.58E-68
Ld_rep_c45344	mitochondrial NADH-ubiquinone oxidoreductase	1561.40	0.00	NA	1.11E-48	1603.8	0.0	NA	1.78E-61
Ld_rep_c27051	PREDICTED: salivary glue protein Sgs-3-like	499.66	0.32	10.60	3.15E-73	361.8	0.3	10.1	4.59E-29
Ld_rep_c107864	15-hydroxyprostaglandin dehydrogenase [NAD(+)]	473.53	0.31	10.57	1.21E-14	566.6	0.3	10.8	6.49E-15
Ld_rep_c39603	lysosomal thiol reductase ip30 precursor	1487.69	0.00	NA	1.56E-108	1298.5	0.3	12.0	5.06E-66
Ld_rep_c34022	heat shock 90 kDa protein	4515.89	3.23	10.45	1.23E-130	6092.9	3.8	10.6	1.07E-81
Ld_rep_c38335	fatty acid binding protein	1369.12	0.98	10.45	1.85E-101	1661.7	0.3	12.4	3.74E-70
Ld_rep_c40314	serpin peptidase inhibitor 21	1358.90	0.00	NA	8.70E-106	1115.0	0.0	NA	7.33E-65
Ld_rep_c60237	alpha subunit of glucosidase	436.74	0.32	10.40	1.15E-22	369.2	0.3	10.3	1.94E-43
Ld_rep_c39625	digestive cysteine proteinase intestain	1342.23	0.00	NA	3.14E-41	1142.3	0.6	10.9	3.80E-55
Ld_rep_c46263	PREDICTED: NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	1197.22	0.96	10.29	3.95E-95	1012.8	0.3	11.6	7.50E-62
Ld_rep_c62899	charged multivesicular body protein 1b	382.77	0.31	10.26	2.11E-65	351.1	0.3	10.3	1.56E-08

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Ld_rep_c33223	PREDICTED: beta-galactosidase-1-like protein 2	760.93	0.62	10.25	1.89E-47	1078.6	0.6	10.7	1.70E-60
Ld_rep_c38121	NADH dehydrogenase	1203.90	0.00	NA	5.33E-77	1110.9	0.3	11.8	2.17E-63
Ld_rep_c47469	glyceraldehyde-3-phosphate dehydrogenase	1188.64	0.00	NA	9.89E-102	1154.2	0.0	NA	1.41E-65
Ld_rep_c68350	PREDICTED: mucin-22-like	4194.41	3.57	10.20	1.08E-127	5253.1	4.6	10.1	3.67E-78
Ld_rep_c24443	PREDICTED: probable isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	1082.50	0.96	10.14	4.07E-23	882.4	0.6	10.4	2.01E-58
Ld_rep_c38712	PREDICTED: myosin heavy chain, muscle isoform X10	1078.82	0.97	10.12	4.98E-62	1525.3	0.6	11.2	7.99E-27
Ld_rep_c26668	PREDICTED: mitochondrial amidoxime reducing component 2	347.56	0.31	10.12	9.22E-58	369.0	0.3	10.2	7.90E-45
Ld_rep_c84840	hypothetical / UDP-glucuronosyltransferase 2C1	356.14	0.33	10.06	1.31E-52	541.4	0.3	10.7	2.50E-51
Ld_rep_c25054	hypothetical protein	1066.75	0.00	NA	7.63E-40	815.0	0.6	10.5	8.37E-16
Ld_c76967	NA	1053.25	0.31	11.72	2.82E-96	853.7	0.0	0.0	1.12E-60
Ld_rep_c71546	NA	976.83	0.33	11.52	3.15E-11	868.6	0.0	NA	1.50E-34
Ld_rep_c43121	cytochrome c mitochondrial	894.22	0.32	11.44	9.11E-30	834.3	1.9	8.8	2.58E-05
Ld_rep_c47594	uncharacterized protein	1638.60	0.62	11.36	2.89E-108	1701.5	2.1	9.6	9.42E-65
Ld_rep_c81703	PREDICTED: transketolase-like protein 2-like isoform X1	758.40	0.33	11.15	5.11E-86	935.5	0.9	10.0	3.60E-58

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Ld_rep_c82363	NA	683.61	0.31	11.10	8.40E-83	877.7	0.0	NA	4.22E-17
Ld_rep_c43646	hypothetical protein	671.13	0.31	11.07	1.18E-26	747.2	1.0	9.6	3.72E-14
Ld_rep_c27673	PREDICTED: succinyl-CoA:3-ketoacid coenzyme A transferase	675.37	0.32	11.03	1.63E-82	569.7	0.0	NA	4.52E-52
Ld_c86940	PREDICTED: macrophage mannose receptor 1-like	33331.21	17.77	10.87	7.22E-167	12011.6	16.7	9.5	1.46E-38
Ld_rep_c57651	membrane alanyl aminopeptidase 2	576.92	0.31	10.85	2.59E-10	NA	NA	NA	NA
Ld_rep_c48478	PREDICTED: myophilin	1116.24	0.62	10.80	3.64E-13	892.6	0.9	9.9	2.28E-41
Ld_rep_c27121	PREDICTED: succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial	580.28	0.33	10.77	1.81E-22	828.7	0.0	NA	6.71E-50
Ld_rep_c37703	PREDICTED: 28S ribosomal protein S30, mitochondrial	559.44	0.33	10.71	1.91E-32	507.6	0.6	9.6	1.42E-30
Ld_rep_c120208	serpin	516.89	0.31	10.69	2.05E-74	407.3	0.0	NA	7.06E-48
Ld_rep_c39155	PREDICTED: V-type proton ATPase catalytic subunit A	530.70	0.32	10.68	1.97E-16	366.1	0.6	9.2	2.48E-04
Ld_rep_c52143	NA	505.08	0.32	10.61	3.53E-12	470.4	0.0	NA	1.06E-23
Ld_rep_c43605	PREDICTED: succinate dehydrogenase cytochrome b560 subunit, mitochondrial isoform X2	963.26	0.64	10.57	7.45E-72	789.5	1.5	9.0	1.03E-11
Ld_rep_c45422	ATP-dependent RNA helicase p62	498.19	0.33	10.55	2.61E-38	929.3	1.2	9.6	1.04E-26

Appendix A: List of the 100 most significantly over-transcribed contigs in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[2]	Read count SS[2]	Log_2 Fold change	*P*-adj[3]	Read count RS[2]	Read count SS[2]	Log_2 Fold change	*P*-adj[3]
Ld_rep_c35194	NA	489.88	0.33	10.52	9.84E-23	455.5	0.0	NA	7.98E-37
Ld_rep_c35173	Basigin	460.26	0.32	10.48	3.22E-41	339.5	1.2	8.2	2.59E-40
Ld_c17405	NA	948.33	0.67	10.47	1.42E-91	1085.7	0.0	NA	9.52E-65
Ld_rep_c50585	PREDICTED: ATP synthase subunit b, mitochondrial	1280.99	0.94	10.42	5.31E-68	1209.5	1.9	9.3	7.27E-60
Ld_rep_c102487	PREDICTED: glycine N-methyltransferase	449.60	0.33	10.40	1.70E-19	334.1	0.6	9.1	1.69E-08
Ld_rep_c109647	AMP dependent coa ligase	839.83	0.65	10.35	4.86E-24	628.0	0.0	NA	2.50E-55
Ld_rep_c55149	reactive oxygen species modulator 1-like protein	408.99	0.32	10.31	2.07E-61	467.7	2.2	7.7	3.15E-43
Ld_c101664	PREDICTED: glucose dehydrogenase [FAD, quinone]-like	1262.60	0.00	NA	0.000545	904.5	0.0	NA	1.78E-61
Ld_rep_c32720	NA	783.77	0.65	10.25	4.91E-07	702.6	0.0	NA	9.51E-15
Ld_rep_c29157	ankyrin repeat domain protein	376.55	0.31	10.23	5.35E-65	341.5	0.3	10.0	1.68E-43
Ld_rep_c45536	PREDICTED: ester hydrolase C11orf54 homolog isoform X2	376.82	0.33	10.14	8.73E-65	326.1	0.3	10.0	4.54E-05
Ld_rep_c38370	alpha amylase isoform 1	1112.77	0.00	NA	2.29E-37	1030.1	0.0	NA	8.12E-28
Ld_rep_c24864	PREDICTED: pathogenesis-related protein 5	370.14	0.33	10.12	2.81E-64	378.2	0.0	NA	2.78E-15
Ld_rep_c59454	Natterin-4	344.17	0.31	10.11	6.00E-45	328.7	0.9	8.5	9.57E-41
Ld_rep_c50321	NA	1088.96	0.00	NA	7.97E-09	791.9	0.0	NA	7.44E-04
Ld_rep_c33762	PREDICTED: OCIA domain-containing	359.06	0.33	10.07	1.40E-54	527.0	0.0	NA	2.38E-52

Appendix A: List of the 100 most significantly over-transcribed contigs in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description	SS control vs RS control comparison				SS treated vs RS treated comparison			
		Read count RS[2]	Read count SS[2]	Log_2 Fold change	*P*-adj[3]	Read count RS[2]	Read count SS[2]	Log_2 Fold change	*P*-adj[3]
	protein 1								
Ld_rep_c42314	PREDICTED: pyruvate kinase isoform X2	1412.20	1.31	10.07	3.09E-57	1261.5	1.3	10.0	2.77E-62
Ld_rep_c69002	counting factor associated protein d-like	1072.68	0.00	NA	1.63E-98	1073.2	0.0	NA	7.74E-52
Ld_rep_c33885	digestive cysteine protease intestain	1068.40	0.00	NA	7.74E-42	817.1	1.0	9.7	1.97E-33
Ld_rep_c92666	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B	354.64	0.33	10.06	1.89E-16	174.0	0.3	9.1	3.22E-15
Ld_rep_c34224	PREDICTED: D-2-hydroxyglutarate dehydrogenase, mitochondrial-like	683.38	0.65	10.05	2.60E-62	719.6	4.4	7.3	1.49E-45
Ld_rep_c27228	glycoside hydrolase family 1	1324.26	1.26	10.04	2.50E-99	1281.9	5.6	7.8	1.85E-12
Ld_rep_c48055	PREDICTED: GDP-mannose 4,6 dehydratase	349.84	0.33	10.04	8.52E-25	409.3	0.0	NA	5.96E-48

[1]Contig ID from Kumar *et al*. (2014); [2]Read counts represent mean normalized counts from three biological replicates; [3]Adjusted *P*-value corrected for false discovery rates; NA = not available

Appendix B: List of the 100 most significantly under-transcribed contigs in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description	SS control vs RS control comparison				SS treated vs RS treated comparison			
		RS read count[2]	SS read count[2]	Log_2 Fold change	*P*-adj[3]	RS read count[2]	SS read count[2]	Log_2 Fold change	*P*-adj[3]
Ld_c115122	dbj	0.66	12057.29	-14.16	1.66E-42	0.32	9649.73	-14.87	2.02E-96
Ld_rep_c33112	lipid storage droplets surface-binding protein 1	0.31	2982.94	-13.22	1.91E-103	0.38	3588.52	-13.21	1.79E-28
Ld_rep_c24183	tetraspanin 29fb	0.31	2782.34	-13.11	6.99E-125	0.00	2852.83	NA	2.09E-78
Ld_rep_c39566	cathepsin d	1.63	13027.22	-12.96	5.42E-75	2.33	12206.20	-12.36	1.9E-94
Ld_rep_c41520	nadh dehydrogenase	0.35	2709.94	-12.93	7.16E-67	0.68	3222.00	-12.21	1.13E-77
Ld_rep_c121145	NA	0.31	1450.10	-12.17	4.94E-105	0.38	1275.58	-11.72	3.8E-21
Ld_rep_c54768	cathepsin b	0.69	2942.39	-12.05	5.99E-17	0.00	2849.54	NA	1.08E-10
Ld_rep_c43651	lysosomal aspartic protease	21.40	70495.01	-11.69	2.97E-37	147.36	57766.76	-8.61	4.47E-73
Ld_rep_c46424	endo-beta- -glucanase	0.69	2182.19	-11.62	7.24E-37	0.36	2483.03	-12.76	1.27E-74
Ld_rep_c39694	NA	0.31	923.72	-11.52	3.01E-91	122.64	956.90	-2.96	3.34E-14
Ld_rep_c120490	NA	0.35	1009.81	-11.51	3.18E-30	0.68	976.16	-10.49	1.5E-42
Ld_rep_c41818	cathepsin l	1.73	5015.06	-11.50	6.44E-72	3.89	6709.68	-10.75	3.66E-82
Ld_rep_c35446	ribosomal protein s18	0.35	1000.67	-11.50	1.41E-93	0.64	1205.98	-10.87	1.48E-61
Ld_rep_c40879	aconitate mitochondrial	0.35	922.24	-11.38	7.88E-77	0.36	992.26	-11.44	1.43E-49
Ld_rep_c33297	ribosomal protein l15	1.01	2601.96	-11.34	4.53E-120	1.40	3012.00	-11.08	1.4E-74
Ld_rep_c24624	endopolygalacturonase	0.35	886.84	-11.32	3.94E-53	0.00	1248.72	NA	8.07E-65
Ld_rep_c46001	mitochondrial enolase superfamily member 1-like	0.35	810.61	-11.19	4.44E-19	0.72	887.91	-10.28	1.2E-29
Ld_rep_c40639	aldo-keto reductase	1.01	2327.51	-11.18	9.18E-39	1.04	2674.83	-11.33	1.71E-73
Ld_rep_c38269	cg7630 cg7630-pa	0.35	800.93	-11.18	5.69E-24	0.72	949.98	-10.37	1.68E-57

Appendix B: List of the 100 most significantly under-transcribed contigs in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description	SS control vs RS control comparison				SS treated vs RS treated comparison			
		RS read count[2]	SS read count[2]	Log_2 Fold change	*P*-adj[3]	RS read count[2]	SS read count[2]	Log_2 Fold change	*P*-adj[3]
Ld_rep_c32934	alkyl hydroperoxide reductase thiol specific antioxidant	0.35	768.73	-11.12	2.40E-66	0.68	493.56	-9.50	8.04E-21
Ld_rep_c42152	NA	0.69	1494.40	-11.08	1.86E-104	0.00	1584.89	NA	8.17E-69
Ld_rep_c33587	3-hydroxybutyrate dehydrogenase type 2	0.31	667.25	-11.05	3.53E-81	0.36	749.53	-11.03	8.53E-55
Ld_rep_c118801	gb	1.98	4121.26	-11.02	1.89E-130	3.11	4998.83	-10.65	7.23E-79
Ld_rep_c38103	cg8844	0.35	717.49	-11.02	1.67E-74	1.42	635.31	-8.81	0.000166
Ld_rep_c32243	long form-like	0.63	1234.00	-10.94	4.90E-34	0.74	1981.90	-11.40	2.01E-44
Ld_rep_c43679	NA	0.66	1281.05	-10.92	8.45E-100	0.70	1738.91	-11.28	1.19E-67
Ld_rep_c48224	NA	1.04	1908.27	-10.84	2.12E-51	1.72	2034.39	-10.21	2.27E-28
Ld_rep_c118845	NA	0.35	621.76	-10.81	1.18E-53	0.72	611.39	-9.74	3.82E-50
Ld_rep_c91275	multidrug resistance-associated protein 4-like	0.35	619.43	-10.81	6.23E-79	1.02	547.07	-9.06	8E-43
Ld_rep_c34409	imaginal disc growth factor 4	2.67	4590.55	-10.75	9.67E-132	1.74	4456.89	-11.32	5.37E-80
Ld_rep_c33154	allergen aca s 13	0.35	580.33	-10.71	6.09E-35	0.72	368.00	-9.01	1.72E-28
Ld_rep_c39060	46 kda fk506-binding nuclear protein	0.35	549.77	-10.63	2.64E-75	2.08	662.92	-8.32	1.48E-12
Ld_rep_c34112	nucleoside diphosphate kinase	1.38	2196.21	-10.63	9.44E-114	1.67	2518.59	-10.56	9.27E-71
Ld_rep_c28388	udp-glucose:glycoprotein glucosyltransferase	0.35	543.80	-10.62	5.63E-75	32.71	605.18	-4.21	4.01E-23
Ld_rep_c72903	endopolygalacturonase	0.35	526.82	-10.57	5.62E-74	3.22	490.16	-7.25	9.14E-41
Ld_rep_c26880	apolipophorins	0.63	932.30	-10.54	8.16E-19	1.06	523.88	-8.95	2.47E-11
Ld_rep_c29126	glycoside hydrolase family protein 48	3.02	3802.59	-10.30	3.27E-125	3.23	4848.45	-10.55	7.22E-52

Appendix B: List of the 100 most significantly under-transcribed contigs in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description	SS control vs RS control comparison				SS treated vs RS treated comparison			
		RS read count[2]	SS read count[2]	Log_2 Fold change	*P*-adj[3]	RS read count[2]	SS read count[2]	Log_2 Fold change	*P*-adj[3]
Ld_rep_c47956	NA	1.32	1657.68	-10.30	6.05E-33	1.70	2098.38	-10.27	8.4E-39
Ld_rep_c45291	luciferin-regenerating enzyme	0.35	433.01	-10.29	1.34E-08	0.00	443.18	NA	1.52E-07
Ld_rep_c51152	ribosomal protein l36	1.35	1659.42	-10.26	4.28E-105	0.97	1901.92	-10.94	5.93E-68
Ld_rep_c35243	dihydrolipoamide dehydrogenase e3 subunit	3.68	4422.53	-10.23	3.38E-44	3.19	5308.71	-10.70	9.17E-75
Ld_rep_c36106	NA	0.31	374.33	-10.22	6.79E-64	0.32	431.82	-10.39	1.32E-45
Ld_rep_c38198	glutathione s-transferase	0.69	815.64	-10.20	7.68E-86	0.00	1018.35	NA	1.85E-61
Ld_rep_c47320	odorant binding protein 4	2.29	2462.98	-10.07	5.10E-35	3.70	2545.01	-9.43	2.83E-05
Ld_rep_c40467	dorsal switch protein isoform d	0.66	671.22	-9.99	7.50E-80	1.06	789.19	-9.54	2.18E-53
Ld_rep_c38474	proteasome subunit beta type-3-like	0.35	349.37	-9.98	2.04E-24	0.00	569.73	NA	9.8E-52
Ld_rep_c27242	juvenile hormone-inducible protein	4.06	4043.80	-9.96	1.32E-124	7.08	4576.64	-9.34	1.66E-71
Ld_c103774	NA	0.31	302.66	-9.91	3.54E-30	1.04	340.09	-8.36	6.78E-24
Ld_rep_c24801	cellular retinoic acid binding protein	3.78	3631.26	-9.91	2.89E-122	4.62	3851.25	-9.70	3.63E-72
Ld_rep_c26372	lola	4.09	3907.32	-9.90	1.65E-96	5.90	4643.56	-9.62	1.64E-43
Ld_rep_c26369	transferrin	0.35	310.43	-9.81	2.14E-58	0.70	345.08	-8.95	9.78E-41
Ld_rep_c55780	NA	0.35	310.24	-9.81	1.95E-58	0.70	352.65	-8.98	3.54E-41
Ld_rep_c39730	protein kinase shaggy-like	0.35	309.61	-9.80	2.35E-58	0.00	356.65	NA	5.91E-44
Ld_rep_c35491	protein phosphatase 1b-like	0.31	279.25	-9.80	1.85E-55	0.64	404.13	-9.29	2.11E-43
Ld_rep_c45300	atpase membrane sector associated protein	1.35	1203.68	-9.80	2.70E-95	1.42	1275.95	-9.82	2.33E-60
Ld_rep_c48067	proactivator polypeptide	1.01	888.92	-9.79	3.49E-87	4.10	778.21	-7.57	2.36E-21

Appendix B: List of the 100 most significantly under-transcribed contigs in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description	SS control vs RS control comparison				SS treated vs RS treated comparison			
		RS read count[2]	SS read count[2]	Log_2 Fold change	*P*-adj[3]	RS read count[2]	SS read count[2]	Log_2 Fold change	*P*-adj[3]
Ld_rep_c34857	GM22606 [Drosophila sechellia]	0.35	302.62	-9.77	3.56E-11	0.00	664.01	NA	8.22E-23
Ld_rep_c47257	endopolygalacturonase	2.42	2099.39	-9.76	2.92E-37	140.28	2911.52	-4.38	6.92E-13
Ld_rep_c37563	40s ribosomal protein s3a-like	2.01	1727.09	-9.75	3.52E-104	3.19	2098.78	-9.36	9.08E-65
Ld_rep_c42755	ornithine aminotransferase	0.63	526.89	-9.71	1.63E-18	0.32	720.98	-11.13	4.19E-15
Ld_rep_c39044	grpe protein mitochondrial-like	0.35	284.00	-9.68	6.43E-56	0.36	338.98	-9.89	1.11E-41
Ld_rep_c33529	NA	0.35	281.90	-9.67	1.13E-55	0.00	316.66	NA	5.15E-16
Ld_c18497	ubiquitin protein ligase	1.01	765.55	-9.57	1.35E-82	0.00	814.53	NA	9.84E-58
Ld_rep_c45303	cathepsin d	3.02	2290.85	-9.57	6.14E-110	1.07	2825.35	-11.36	1.78E-74
Ld_s114049	NA	0.35	258.64	-9.54	3.09E-37	0.00	305.90	NA	2.84E-41
Ld_rep_c36957	aldo-keto reductase	1.29	958.20	-9.54	2.92E-88	1.38	1100.04	-9.64	7.28E-58
Ld_rep_c47123	probable signal peptidase complex subunit 2-like	0.31	228.47	-9.51	6.76E-50	0.36	229.89	-9.33	2.14E-35
Ld_s82845	rad23-like b	0.35	246.08	-9.47	6.62E-52	0.38	273.99	-9.50	4.08E-38
Ld_c98800	ref	0.35	238.62	-9.43	5.17E-51	0.00	273.42	NA	4.79E-38
Ld_rep_c48776	NA	0.35	237.64	-9.42	3.81E-14	1.09	266.46	-7.93	4.85E-34
Ld_rep_c44938	methylthioadenosine phosphorylase	0.35	235.26	-9.41	1.09E-50	0.36	263.38	-9.52	1.84E-37
Ld_rep_c26011	cation transport regulator-like protein 2	0.35	229.91	-9.37	5.35E-50	0.36	288.76	-9.66	5.75E-39
Ld_rep_c38692	cg12948 cg12948-pa	0.31	204.74	-9.35	7.24E-47	1.38	187.69	-7.09	3.41E-29
Ld_rep_c25456	aminopeptidase n	4.90	3102.77	-9.31	2.01E-114	10.45	2677.84	-8.00	2.52E-37
Ld_rep_c37063	glucosyl glucuronosyl transferases	0.66	411.40	-9.28	3.27E-65	25.80	450.44	-4.13	2.75E-21

Appendix B: List of the 100 most significantly under-transcribed contigs in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description	SS control vs RS control comparison				SS treated vs RS treated comparison			
		RS read count[2]	SS read count[2]	Log_2 Fold change	*P*-adj[3]	RS read count[2]	SS read count[2]	Log_2 Fold change	*P*-adj[3]
Ld_rep_c29804	gtp-binding protein 1-like	0.35	212.47	-9.26	6.36E-48	1.11	233.05	-7.71	2.19E-33
Ld_rep_c33130	NA	1.29	784.86	-9.25	1.86E-52	0.36	848.79	-11.21	7.65E-57
Ld_c20037	NA	0.31	190.95	-9.25	4.74E-45	1.47	216.25	-7.20	2.45E-31
Ld_rep_c28224	40s ribosomal protein sa-like	5.06	3065.22	-9.24	7.24E-54	4.27	4778.55	-10.13	3.64E-34
Ld_rep_c38279	ejaculatory bulb-specific protein 3	0.31	189.01	-9.24	3.80E-05	0.36	177.48	-8.95	1.24E-06
Ld_rep_c40769	NA	1.01	603.44	-9.23	1.52E-23	1.00	747.63	-9.54	1.54E-52
Ld_rep_c38498	fatty acyl- reductase cg5065-like	0.66	389.90	-9.21	2.02E-14	0.70	462.45	-9.37	2.07E-27
Ld_rep_c28452	lethal isoform a	0.35	204.65	-9.21	1.56E-25	0.32	217.83	-9.40	2.87E-27
Ld_rep_c53822	monocarboxylate transporter 14	0.35	198.40	-9.16	4.42E-46	0.68	226.41	-8.38	5.67E-34
Ld_rep_c115984	NA	0.35	196.61	-9.15	6.50E-44	0.36	242.85	-9.41	5.88E-15
Ld_rep_c35377	NA	0.35	191.84	-9.11	3.06E-10	0.00	281.61	NA	4.95E-05
Ld_rep_c42079	venom serine protease 34-like	0.63	338.78	-9.08	2.05E-47	2.08	270.39	-7.02	4.26E-05
Ld_rep_c41474	hypothetical protein TcasGA2_TC007084 [Tribolium castaneum]	8.52	4545.62	-9.06	1.08E-119	18.57	5890.98	-8.31	4.32E-12
Ld_c115307	NA	0.31	165.16	-9.04	4.00E-30	1.07	206.97	-7.59	2.81E-13
Ld_rep_c33730	glutaredoxin-related protein mitochondrial-like	1.01	528.11	-9.04	9.02E-38	0.00	691.05	NA	3.62E-14
Ld_rep_c35023	NA	1.32	685.71	-9.02	8.32E-18	12.80	1277.61	-6.64	3.8E-16
Ld_rep_c35968	abhydrolase domain-containing protein 16a-like	0.31	156.93	-8.97	4.20E-40	0.00	186.05	NA	1.93E-33
Ld_rep_c24532	cyt-b5-pb	1.01	499.25	-8.96	6.53E-23	1.74	584.76	-8.39	1.13E-17

Appendix B: List of the 100 most significantly under-transcribed contigs in the RS beetles compared to the SS beetles in two comparisons.

Contig ID[1]	Sequence description	SS control vs RS control comparison				SS treated vs RS treated comparison			
		RS read count[2]	SS read count[2]	Log_2 Fold change	*P*-adj[3]	RS read count[2]	SS read count[2]	Log_2 Fold change	*P*-adj[3]
Ld_c72810	NA	0.69	335.88	-8.92	2.62E-59	0.72	425.19	-9.21	3.87E-44
Ld_rep_c74285	ribonucleic acid binding protein s1	0.35	167.85	-8.92	8.65E-42	0.00	230.23	NA	7.72E-27
Ld_rep_c41061	globin 1	0.69	335.68	-8.92	8.43E-14	0.00	384.02	NA	2.21E-29
Ld_rep_c44022	grpe protein mitochondrial-like	0.69	329.06	-8.89	9.57E-59	0.38	360.10	-9.90	1.25E-42
Ld_s47599	NA	0.35	164.55	-8.89	2.70E-41	0.00	125.47	NA	1.77E-27
Ld_rep_c119525	NA	0.35	163.73	-8.89	3.41E-41	1.76	224.45	-7.00	3.96E-31
Ld_c10864	NA	0.69	316.84	-8.84	5.05E-12	0.72	646.55	-9.82	2.69E-06

[1]Contig ID from Kumar *et al*. (2014); [2]Read counts represent mean normalized counts from three biological replicates; [3]Adjusted P-value corrected for false discovery rates; NA = not available.

Appendix C: Contigs over-transcribed in the RS beetle from two comparisons and are encoding genes involved in oxidative stress response, immune response, general stress response, energy production, carbohydrate metabolism, and anti-oxidative stress response.

Contig ID[1]	Sequence description	SS control vs RS control				SS treated vs RS control			
		Read count RS[2]	Read count SS[2]	Log2 Fold change	*P*-adj[3]	Read count RS[2]	Read count SS[2]	Log2 Fold change	*P*-adj[3]
Ld_rep_c43844	NADH:ubiquinone oxidoreductase	16.63	0.65	4.69	2.07E-05	21.26	0.92	4.53	4.67E-06
Ld_rep_c45344	NADH-ubiquinone oxidoreductase aggg	1561.40	0.00	NA	1.11E-48	1603.79	0.00	NA	1.78E-61
Ld_rep_c29111	xanthine dehydrogenase oxidase	285.32	55.27	2.37	4.78E-08	313.27	66.85	2.23	5.55E-08
Ld_rep_c34893	s-adenosyl-l-homocysteine hydrolase	76.58	0.33	7.84	1.06E-08	54.67	0.00	NA	1.88E-14
Ld_rep_c91134	peroxiredoxin 1-like	57.22	7.20	2.99	2.94E-09	57.69	7.78	2.89	6.85E-07
Ld_rep_c38195	peroxiredoxin 3	334.46	60.63	2.46	1.39E-04	297.58	91.07	1.71	4.84E-05
Ld_rep_c34726	peroxiredoxin 4	24.95	0.00	NA	2.33E-10	26.04	0.61	5.41	5.20E-08
Ld_rep_c73681	peroxiredoxin 6	62.72	0.66	6.58	4.02E-20	56.52	0.96	5.88	5.29E-04
Ld_rep_c46309	peroxiredoxin prdx5	55.09	0.00	NA	2.20E-20	56.44	0.00	NA	9.82E-18
Ld_rep_c48555	peroxiredoxin-like protein	298.70	2.29	7.03	7.96E-17	594.24	0.96	9.28	3.17E-17
Ld_rep_c32942	thioredoxin domain-containing protein 1	159.79	0.00	NA	1.13E-42	181.90	0.63	8.16	1.08E-14
Ld_rep_c38919	thioredoxin domain-containing protein 1	1026.48	245.69	2.06	1.18E-09	1091.24	266.35	2.03	2.33E-07
Ld_rep_c37450	thioredoxin domain-containing protein 17-like	32.38	0.00	NA	5.57E-13	36.69	0.00	NA	6.56E-13
Ld_s66181	thioredoxin domain-containing protein 9	19.80	0.00	NA	8.06E-06	33.97	0.00	NA	3.88E-12
Ld_rep_c33205	thioredoxin peroxidase 2	283.34	2.94	6.59	1.84E-48	332.30	3.19	6.70	1.10E-35

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Contig ID[1]	Sequence description	SS control vs RS control				SS treated vs RS control			
		Read count RS[2]	Read count SS[2]	Log2 Fold change	*P*-adj[3]	Read count RS[2]	Read count SS[2]	Log2 Fold change	*P*-adj[3]
Ld_rep_c30698	thioredoxin reductase mitochondrial-like	496.50	0.00	NA	7.22E-75	540.40	0.95	9.15	4.42E-49
Ld_rep_c91153	catalase	2082.94	6.46	8.33	2.35E-51	2439.64	10.07	7.92	8.23E-59
Ld_rep_c51914	acyl-CoA dehydrogenase	274.22	0.32	9.73	2.51E-18	289.68	0.32	9.81	5.13E-27
Ld_rep_c35290	aldehyde dehydrogenase	8315.43	2402.62	1.79	5.70E-11	11766.68	2691.20	2.13	2.09E-08
Ld_rep_c38092	superoxide dismutase	66.96	14.36	2.22	2.01E-06	103.94	14.73	2.82	9.04E-06
Ld_rep_c42823	glutathione peroxidase	314.57	0.00	NA	2.87E-61	368.89	0.00	NA	3.58E-36
Ld_c20348	immune-related hdd11	30.89	0.97	5.00	7.84E-10	26.02	0.00	NA	3.94E-08
Ld_c14138	variable lymphocyte receptor c	302.08	85.78	1.82	8.56E-09	412.93	90.80	2.19	6.51E-08
Ld_rep_c38662	cathepsin l	374.88	1.27	8.20	1.47E-06	163.04	1.58	6.69	9.96E-18
Ld_rep_c24206	cathepsin l	69.49	0.00	NA	1.22E-11	90.35	0.00	NA	3.03E-07
Ld_rep_c51562	cathepsin L	10886.46	0.00	NA	1.08E-27	8167.40	1.26	12.66	8.83E-84
Ld_rep_c32849	cathepsin l precursor	10231.05	431.09	4.57	8.93E-10	10183.12	513.52	4.31	1.55E-27
Ld_rep_c25159	cathepsin l-like protein cysteine proteinase	30.94	0.00	NA	5.36E-09	23.72	0.00	NA	3.85E-07
Ld_rep_c44958	cathepsin l-like proteinase	38.82	0.33	6.86	1.71E-13	49.70	0.32	7.26	4.34E-15
Ld_rep_c37824	apolipophorins-partial	80.40	0.00	NA	3.88E-27	108.23	0.00	NA	1.22E-26
Ld_c85564	apolipoprotein a-i-binding protein	553.39	181.89	1.61	7.34E-08	626.55	211.38	1.57	1.51E-04

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Ld_rep_c33281	apolipoprotein d	101.45	2.22	5.52	5.82E-25	130.94	4.44	4.88	1.63E-19
Ld_rep_c32181	apolipoprotein d	166.18	43.89	1.92	2.97E-06	190.29	45.27	2.07	2.42E-04
Ld_rep_c24735	apolipoprotein d-like	1152.36	388.04	1.57	1.72E-05	1301.01	450.47	1.53	1.32E-04
Ld_rep_c39198	low quality protein: tetraspanin-9	42.40	0.00	NA	2.40E-09	48.17	0.32	7.22	1.84E-14
Ld_s47728	chitinase 3	77.47	1.27	5.93	7.51E-14	120.14	3.46	5.12	5.98E-13
Ld_rep_c27305	chitinase 5 precursor	195.16	7.13	4.77	3.05E-31	189.11	11.43	4.05	1.48E-17
Ld_c3215	chitinase 5 precursor	286.83	10.67	4.75	2.87E-36	252.42	8.73	4.85	5.50E-24
Ld_rep_c25744	chitinase 6	978.47	67.26	3.86	2.59E-28	954.84	66.08	3.85	8.39E-08
Ld_rep_c38596	chitinase 6	25.52	3.22	2.99	4.09E-05	46.06	1.92	4.59	2.60E-09
Ld_c2889	chitinase-3-like protein 1-like	701.13	61.20	3.52	2.87E-15	699.04	53.52	3.71	7.30E-07
Ld_rep_c38830	hsp90 co-chaperone cdc37	41.51	0.00	NA	3.22E-16	68.48	0.65	6.72	1.03E-08
Ld_rep_c82783	hsp90 co-chaperone cdc37	30.93	1.27	4.61	3.88E-09	NA	NA	NA	NA
Ld_rep_c45478	hsp90 cochaperone cdc37 homologue	289.12	0.32	9.81	2.63E-57	432.45	0.32	10.41	1.35E-11
Ld_rep_c38218	10 kda heat shock mitochondrial-like	1600.76	4.52	8.47	9.89E-16	2274.75	4.41	9.01	5.24E-65
Ld_rep_c25736	heat shock 70 kda protein cognate 5-like	1193.09	144.58	3.04	8.55E-26	1276.62	176.82	2.85	2.09E-13
Ld_c5844	heat shock 70 kda protein cognate 5-like	4644.06	2061.10	1.17	5.53E-04	NA	NA	NA	NA
Ld_rep_c34022	heat shock 90 kDa protein	4515.89	3.23	10.45	1.23E-130	6092.87	3.84	10.63	1.07E-81

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Ld_rep_c33458	heat shock protein 67b2-like	203.51	24.65	3.05	6.89E-18	183.98	31.74	2.54	8.96E-09
Ld_rep_c37460	heat shock protein 70 b2	55.54	1.00	5.80	3.21E-17	58.03	0.32	7.49	7.45E-17
Ld_c17599	heat shock protein 70 b2	52.40	5.90	3.15	7.70E-05	51.13	4.05	3.66	5.59E-09
Ld_rep_c28189	heat shock protein 75 mitochondrial-like	336.39	0.00	NA	3.96E-51	246.79	0.32	9.60	9.63E-12
Ld_rep_c57900	heat shock protein 83	276.47	0.66	8.72	9.11E-12	738.79	1.48	8.97	2.16E-52
Ld_rep_c38303	heat shock protein 90	896.90	2.90	8.28	1.62E-50	1416.60	0.93	10.57	2.91E-65
Ld_rep_c26158	heat-responsive protein 12	313.77	0.00	NA	7.75E-54	151.21	0.63	7.90	2.32E-10
Ld_rep_c56265	heat-responsive protein 12	102.93	0.33	8.27	5.86E-20	42.65	0.61	6.12	5.29E-08
Ld_c6311	ubiquitin carboxyl-terminal	736.07	42.94	4.10	1.02E-33	791.32	50.60	3.97	6.90E-22
Ld_rep_c28322	ubiquitin carboxyl-terminal hydrolase 2	48.15	1.28	5.23	2.22E-14	42.78	4.01	3.41	2.55E-07
Ld_c18773	ubiquitin carboxyl-terminal hydrolase 2	342.45	28.75	3.57	1.27E-26	379.30	66.73	2.51	1.57E-06
Ld_c17882	ubiquitin carboxyl-terminal hydrolase 2	64.97	16.89	1.94	1.85E-05	54.13	12.97	2.06	3.15E-04
Ld_rep_c32579	ubiquitin carboxyl-terminal hydrolase isozyme l5	22.16	1.30	4.09	2.62E-06	NA	NA	NA	NA
Ld_rep_c35262	ubiquitin fusion degradation protein 1 homolog	246.84	38.49	2.68	1.11E-15	237.21	38.55	2.62	8.15E-10

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Ld_rep_c45724	ubiquitin isoform cra_a	5704.79	2112.42	1.43	2.39E-07	NA	NA	NA	NA
Ld_rep_c33491	ubiquitin ribosomal protein s27ae fusion protein	454.36	12.64	5.17	3.47E-46	563.41	19.83	4.83	5.29E-28
Ld_rep_c47641	ubiquitin-conjugating enzyme e2	523.56	154.34	1.76	8.92E-07	621.30	155.67	2.00	8.14E-07
Ld_rep_c26177	ubiquitin-conjugating enzyme e2 g2	87.41	24.26	1.85	5.90E-06	NA	NA	NA	NA
Ld_rep_c61178	ubiquitin-conjugating enzyme rad6	186.02	0.31	9.22	8.74E-33	281.47	0.58	8.93	6.41E-23
Ld_rep_c34071	ubiquitin-fold modifier-conjugating enzyme 1	298.41	60.87	2.29	1.26E-07	288.60	22.58	3.68	1.53E-09
Ld_c1295	ubiquitin-like domain-containing ctd phosphatase 1	475.71	98.99	2.26	3.93E-04	556.17	115.15	2.27	9.72E-09
Ld_rep_c48468	ubiquitin-like protein atg12-like	41.55	0.00	NA	8.26E-07	48.27	0.00	NA	6.52E-16
Ld_rep_c30848	ubiquitin-like-conjugating enzyme atg3-like	370.20	172.70	1.10	7.98E-04	NA	NA	NA	NA
Ld_c57318	ubiquitin-protein ligase	232.19	0.64	8.51	1.17E-49	260.65	0.61	8.73	7.52E-38
Ld_rep_c27279	ubiquitin-protein ligase e3a	124.67	0.00	NA	1.01E-36	135.67	0.00	NA	5.28E-30
Ld_rep_c49904	ubiquitin-protein ligase e3c-like	58.69	1.58	5.21	1.77E-16	43.38	0.32	7.07	1.54E-13
Ld_rep_c43126	isocitrate dehydrogenase	265.98	53.68	2.31	7.38E-10	221.34	72.97	1.60	3.86E-04

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Ld_rep_c24443	probable isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	1082.50	0.96	10.14	4.07E-23	882.40	0.64	10.43	2.01E-58
Ld_rep_c47429	malate dehydrogenase	497.66	0.00	NA	5.42E-75	549.45	0.00	NA	1.05E-26
Ld_rep_c32861	mitochondrial 2-oxoglutarate malate carrier protein	1965.84	221.49	3.15	3.71E-28	2241.77	474.32	2.24	2.46E-07
Ld_rep_c45401	cytosolic malate dehydrogenase	725.61	0.98	9.53	6.34E-50	605.41	0.61	9.95	7.13E-26
Ld_rep_c57147	kynurenine--oxoglutarate transaminase 3-like	973.53	238.46	2.03	4.97E-04	670.72	159.27	2.07	1.83E-04
Ld_rep_c32861	mitochondrial 2-oxoglutarate malate carrier protein	1965.84	221.49	3.15	3.71E-28	NA	NA	NA	NA
Ld_c20366	procollagen- -oxoglutarate 5-dioxygenase 3	79.14	10.86	2.86	1.38E-10	79.97	5.82	3.78	8.03E-09
Ld_c21790	procollagen- -oxoglutarate 5-dioxygenase 3	173.79	66.82	1.38	8.80E-05	NA	NA	NA	NA
Ld_rep_c24443	probable isocitrate dehydrogenase [	1082.50	0.96	10.14	4.07E-23	NA	NA	NA	NA
Ld_rep_c43126	isocitrate dehydrogenase	265.98	53.68	2.31	7.38E-10	NA	NA	NA	NA
Ld_rep_c38398	succinyl-coa synthetase beta chain	43.57	0.00	NA	2.42E-11	54.16	0.29	7.55	5.66E-16
Ld_rep_c43605	succinate dehydrogenase	963.26	0.64	10.57	7.45E-72	789.52	1.53	9.01	1.03E-11

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Ld_c21828	cytochrome b560 subunit, succinate dehydrogenase	3342.50	1254.88	1.41	2.02E-05	NA	NA	NA	NA
Ld_rep_c38121	nadh dehydrogenase	1203.90	0.00	NA	5.33E-77	1110.89	0.32	11.77	2.17E-63
Ld_rep_c24832	nadh dehydrogenase	83.19	0.32	8.01	7.50E-08	128.96	0.93	7.12	1.03E-25
Ld_rep_c41511	nadh dehydrogenase	141.93	0.00	NA	4.64E-33	120.46	0.00	NA	3.39E-28
Ld_c15521	nadh dehydrogenase	136.66	0.97	7.14	1.10E-11	128.42	0.63	7.66	1.17E-26
Ld_rep_c47356	nadh dehydrogenase	124.31	0.00	NA	1.29E-36	128.00	0.64	7.64	4.78E-15
Ld_rep_c41592	nadh dehydrogenase	299.09	5.09	5.88	2.47E-07	NA	NA	NA	NA
Ld_rep_c50821	nadh dehydrogenase	44.27	0.00	NA	6.05E-17	38.59	0.00	NA	1.22E-09
Ld_rep_c49635	nadh dehydrogenase	42.03	0.00	NA	2.84E-16	30.86	0.00	NA	2.64E-11
Ld_rep_c39319	nadh dehydrogenase	27.87	0.00	NA	4.73E-08	31.16	0.00	NA	7.39E-06
Ld_rep_c45274	nadh dehydrogenase	30.62	1.61	4.25	1.72E-08	NA	NA	NA	NA
Ld_rep_c50004	nadh dehydrogenase	17.25	0.00	NA	3.03E-07	18.81	0.00	NA	2.20E-07
Ld_rep_c49966	nadh dehydrogenase	915.94	77.48	3.56	2.33E-23	996.03	92.50	3.43	6.10E-18
Ld_rep_c47239	nadh dehydrogenase	15.18	1.30	3.54	5.38E-04	NA	NA	NA	NA
Ld_rep_c33732	nadh dehydrogenase	783.27	176.66	2.15	1.34E-13	743.23	214.82	1.79	8.94E-06
Ld_rep_c48271	nadh dehydrogenase	363.38	163.86	1.15	9.79E-04	NA	NA	NA	NA
Ld_rep_c41136	NADH dehydrogenase	1054.63	0.31	11.72	7.67E-45	1007.16	0.92	10.09	1.90E-59
Ld_rep_c104675	NADH dehydrogenase	213.24	0.62	8.41	2.08E-10	369.32	0.00	NA	1.83E-46

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	[ubiquinone] 1 alpha subcomplex subunit 13								
Ld_rep_c51011	nadh dehydrogenase 1 alpha subcomplex subunit 5	766.63	217.87	1.82	3.10E-08	642.50	166.69	1.95	1.82E-06
Ld_rep_c48451	nadh dehydrogenase iron-sulfur protein mitochondrial	525.33	0.66	9.65	1.15E-67	596.22	0.63	9.88	1.83E-35
Ld_rep_c45514	nadh dehydrogenase iron-sulfur protein mitochondrial	293.46	0.00	NA	1.88E-37	316.38	0.00	NA	1.20E-43
Ld_rep_c39377	nadh dehydrogenase iron-sulfur protein mitochondrial	148.17	0.00	NA	6.19E-41	176.51	0.00	NA	4.91E-34
Ld_rep_c33720	nadh dehydrogenase iron-sulfur protein	20.99	0.00	NA	7.98E-09	21.75	0.00	NA	1.58E-07
Ld_rep_c38049	nadh dehydrogenase subunit 1	34.83	4.84	2.85	4.26E-05	29.76	5.25	2.50	6.02E-04
Ld_rep_c75093	nadh dehydrogenase-like protein	24.46	2.57	3.25	1.67E-04	NA	NA	NA	NA
Ld_rep_c85656	nadh dehydrogenase-ubiquinone fe-s protein 2 precursor	457.48	2.59	7.46	3.50E-19	357.69	3.77	6.57	1.09E-35
Ld_rep_c43605	succinate dehydrogenase cytochrome b560 subunit	963.26	0.64	10.57	7.45E-72	NA	NA	NA	NA
Ld_c21828	succinate dehydrogenase	3342.50	1254.88	1.41	2.02E-05	NA	NA	NA	NA

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Ld_rep_c41846	Cytochrome b-c1 complex subunit 2,	315.17	0.32	9.93	2.87E-34	258.90	1.18	7.78	8.10E-36
Ld_rep_c37845	cytochrome c oxidase assembly protein COX11, mitochondrial	312.66	0.31	9.97	5.32E-32	255.50	0.00	NA	1.59E-37
Ld_rep_c25269	synthesis of cytochrome c oxidase	316.15	128.03	1.30	5.43E-05	NA	NA	NA	NA
Ld_rep_c43121	cytochrome c mitochondrial	894.22	0.32	11.44	9.11E-30	834.26	1.85	8.82	2.58E-05
Ld_rep_c28266	cytochrome c oxidase subunit i	41.54	0.95	5.45	2.79E-05	30.34	1.60	4.25	1.73E-07
Ld_rep_c39475	cytochrome c oxidase subunit va	388.98	1.91	7.67	5.48E-14	365.30	2.80	7.03	4.47E-06
Ld_rep_c47606	cytochrome c oxidase subunit va	10.53	0.00	NA	1.79E-04	NA	NA	NA	NA
Ld_rep_c44216	cytochrome c oxidase subunit va	1028.15	157.31	2.71	1.66E-16	1054.19	170.33	2.63	1.03E-11
Ld_rep_c25141	cytochrome c oxidase subunit viia polypeptide 2	619.61	0.00	NA	2.22E-38	530.12	0.32	10.71	6.13E-51
Ld_rep_c38475	cytochrome c1	1902.50	549.42	1.79	1.55E-06	1698.51	641.35	1.41	5.25E-04
Ld_rep_c41525	cytochrome oxidase biogenesis protein	65.76	0.00	NA	2.41E-23	57.78	0.00	NA	5.67E-18
Ld_rep_c44856	cytochrome oxidase subunit partial	130.71	51.34	1.35	3.36E-04	NA	NA	NA	NA
Ld_rep_c24903	cysteine-rich venom	709.52	50.87	3.80	6.06E-21	1184.84	54.04	4.45	1.14E-17
Ld_c10333	cytochrome b5 type b-like	407.29	185.85	1.13	3.47E-04	NA	NA	NA	NA

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Contig ID[1]	Sequence description	SS control vs RS control				SS treated vs RS control			
		Read count RS[2]	Read count SS[2]	Log2 Fold change	*P*-adj[3]	Read count RS[2]	Read count SS[2]	Log2 Fold change	*P*-adj[3]
Ld_rep_c33846	cytochrome b561	320.72	0.96	8.39	1.34E-57	312.96	1.92	7.35	2.55E-37
Ld_rep_c24862	cytochrome b-561 domain containing 2	170.80	44.86	1.93	3.00E-08	194.51	40.92	2.25	2.71E-07
Ld_rep_c39812	h+ transporting atp synthase subunit e	151.82	0.00	NA	2.26E-41	139.98	0.00	NA	2.07E-30
Ld_rep_c42307	h+ transporting atp synthase subunit e	213.75	59.44	1.85	3.32E-08	166.59	58.28	1.52	8.14E-04
Ld_rep_c26527	h+ transporting atp synthase subunit g	374.55	12.21	4.94	7.05E-08	442.20	18.66	4.57	4.91E-25
Ld_rep_c88298	mitochondrial atp synthase coupling factor 6	591.13	1.93	8.26	3.65E-21	481.44	2.15	7.81	5.17E-21
Ld_rep_c34394	mitochondrial f0 atp synthase d	4872.83	119.76	5.35	5.76E-32	3546.07	165.31	4.42	2.85E-09
Ld_rep_c24705	mitochondrial f0 atp synthase d	1265.32	299.44	2.08	3.45E-06	NA	NA	NA	NA
Ld_rep_c50585	ATP synthase subunit b,	1280.99	0.94	10.42	5.31E-68	1209.50	1.86	9.34	7.27E-60
Ld_rep_c24464	vacuolar atp synthase subunit e	1223.81	2.28	9.07	5.62E-22	1435.91	2.49	9.17	1.68E-34
Ld_rep_c38687	vacuolar atp synthase subunit s1	373.68	2.54	7.20	3.59E-57	540.04	3.09	7.45	1.25E-43
Ld_rep_c32802	vacuolar atpase subunit d	456.18	20.66	4.46	1.02E-15	387.55	16.20	4.58	1.12E-24
Ld_rep_c50623	ATP synthase B chain	3452.91	0.00	NA	5.54E-34	3107.78	0.00	NA	4.99E-20
Ld_rep_c33537	atp synthase beta	2477.77	8.63	8.16	6.30E-12	1965.94	12.39	7.31	2.06E-29
Ld_rep_c36014	atp synthase delta	612.54	23.58	4.70	1.32E-10	450.85	38.47	3.55	7.65E-11

Appendix C: Contigs over-transcribed in the RS beetle from two comparisons and are encoding genes involved in oxidative stress response, immune response, general stress response, energy production, carbohydrate metabolism, and anti-oxidative stress response.

Contig ID[1]	Sequence description	SS control vs RS control				SS treated vs RS control			
		Read count RS[2]	Read count SS[2]	Log2 Fold change	*P*-adj[3]	Read count RS[2]	Read count SS[2]	Log2 Fold change	*P*-adj[3]
Ld_rep_c62812	atp synthase gamma mitochondrial	89.81	7.78	3.53	7.21E-08	69.31	10.54	2.72	1.21E-04
Ld_c16198	atp synthase mitochondrial f1 complex assembly	184.84	49.93	1.89	4.53E-08	NA	NA	NA	NA
Ld_c13257	atp synthase subunit mitochondrial	39.12	9.89	1.98	3.76E-04	NA	NA	NA	NA
Ld_rep_c48493	atp synthase-coupling factor mitochondrial	30.22	0.00	NA	6.85E-08	28.33	0.00	NA	1.75E-10
Ld_rep_c66793	atpase family aaa domain-containing protein 1	144.15	50.97	1.50	2.41E-04	NA	NA	NA	NA
Ld_rep_c25024	atpase family aaa domain-containing protein 1-a-like	208.31	85.73	1.28	1.90E-04	NA	NA	NA	NA
Ld_rep_c42467	atpase inhibitor-like protein	1990.81	304.69	2.71	1.01E-12	NA	NA	NA	NA
Ld_c4278	atpase n2b-like	1190.74	302.48	1.98	2.46E-09	NA	NA	NA	NA

[1]Contig ID from Kumar *et al*. (2014); [2]Read counts represent mean normalized counts from three biological replicates; [3]Adjusted P-value corrected for false discovery rates; NA = not available.

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