Resistance to double-stranded RNA (dsRNA) in Colorado potato beetle (*Leptinotarsa decemlineata* Say): Genetic and molecular insights

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Swati Mishra
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Just like moons and like suns,
With the certainty of tides,
Just like hopes springing high,
Still I'll rise.

-Maya Angelou

To all the women before me
and the ones that will follow,
who dare to exist in spaces they were told they don't belong.

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ABSTRACT

Double-stranded RNA (dsRNA) insecticides delivered through transgenic and non-transgenic means have been approved for commercialization in the US. As with other insecticides development of insect resistance is expected, necessitating the implementation of an Insecticide Resistance Management (IRM) framework for their long-term use. Laboratory-selected insect resistance strains can provide useful insights for an effective IRM plan. The Colorado potato beetle (CPB) is the target pest for the first commercial dsRNA foliar spray, making it an ideal model to study dsRNA resistance. In this dissertation, we studied the previously described CEAS 300 strain of CPB that exhibits >11,100-fold resistance against V-ATPase subunit A dsRNA. We demonstrate that resistance in CEAS 300 is inherited as a recessive autosomal trait supporting the potential of the refuge strategy in delaying resistance. Resistance in CEAS 300 is s polygenic trait which suggests the presence of multiple resistance mechanisms. Bioassays with chemical pesticides indicate a lack of cross-resistance, supporting their combined use with dsRNA insecticides in reducing selection pressure. While no differences in dsRNA stability in digestive fluids were observed, uptake of dsRNA in CEAS 300 midgut cells was reduced compared to susceptible insects in fluorescence microscopy and small RNA sequencing. Transcriptomic analyses indicate suppression of clathrin-mediated endocytosis (CME), a key dsRNA uptake pathway, in CEAS 300. At the same time, several immune and stress-related pathways are activated in CEAS 300, suggesting their potential role in dsRNA resistance. Candidate resistance-associated genes in CEAS 300 were also identified using bulked segregant analysis (BSA). Following linkage analysis, these can be used to monitor resistance alleles in field populations and estimate the risk of resistance. Taken together, this dissertation provides critical information regarding resistance characteristics and mechanisms in a strain of CPB. This information can guide strategies to potentially overcome resistance as well as create a resistance management framework to delay resistance.

TABLE OF CONTENTS

Chapter One: Double-stranded RNA insecticides: Activity, applications and resistance challenges....1

1.1 Introduction 2

1.2 Parameters affecting insecticidal activity of dsRNA 4

1.3 Delivery of dsRNA to potato pests 6

1.4 Safety of insecticidal dsRNA 8

1.5 Use of dsRNA against potato pests 10
1.5.1 Potato psyllid (Bactericera cockerelli) 10
1.5.2 28-spotted potato ladybird (Henosepilachna vigintioctopunctata) 11
1.5.3 Colorado potato beetle (Leptinotarsa decemlineata) 12
1.5.4 Peach-potato aphid (Myzus persicae) 13
1.5.5 Potato tuber moth (Phthorimaea operculella) 14

1.6 Potential mechanisms of resistance to insecticidal dsRNA 14
1.6.1 Behavioral avoidance 15
1.6.2 Reduced penetration 15
1.6.3 Metabolic resistance: detoxification, degradation, sequestration and excretion 16
1.6.4 Reduced dsRNA uptake 17
1.6.5 Target site insensitivity 18

1.7 Current mechanistic dsRNA resistance data 19
1.7.1 Evidence for resistance potential 19
1.7.2 Selection of resistance against dsRNA produced in transgenic plant 19
1.7.3 Selection of resistance against dsRNA delivered by non-transgenic method 20

1.8 Fitness costs of resistance to dsRNA 21

1.9 Resistance management for dsRNA 22

1.10 Concluding remarks and future prospects 24

References 26

Chapter Two: Characterization of resistance to topically delivered double-stranded RNA (dsRNA) in Colorado potato beetle (Leptinotarsa decemlineata) 40

Abstract 41

2.1 Introduction 43

2.2 Materials and methods 45
2.2.1 Insect colonies 45
2.2.2 Bioassays with chemical pesticides 46
2.2.3 Bioassays to determine inheritance of dsRNA resistance 47

2.3 Results 49

2.3.1 Cross-resistance to chemical pesticides 49
2.3.2 Genetics of dsRNA resistance in CEAS 300 51
2.4 Discussion 52
References 54
Chapter Three: Mechanistic description of resistance to topically applied double-stranded RNA (dsRNA) in a Colorado potato beetle (Leptinotarsa decemlineata Say) population 61
Abstract 62
3.1 Introduction 63
3.2 Materials and Methods 65
3.2.1 Insect colonies 65
3.2.2 Nuclease assays 66
3.2.3 Confocal microscopy 66
3.2.4 Small RNA (sRNA) sequencing and analysis 67
3.2.5 Targeted RNA sequencing 68
3.2.6 Quantitative PCR (qPCR) 69
3.2.7 Bioassays with piperonyl butoxide (PBO) and dsRNA 69
3.3 Results 70
3.3.1 Double-stranded RNA (dsRNA) stability 70
3.3.2 Monitoring uptake of dsRNA inside the larval midgut cells 70
3.3.3 Accumulation of small RNAs (sRNAs) inside gut cells 71
3.3.4 Differentially expressed genes identified from targeted RNA sequencing analysis 71
3.3.5 Bioassays with piperonyl butoxide (PBO) and dsRNA 73
3.4 Discussion 74
3.5 Conclusions 78
References 80
Chapter Four: Transcriptomic and genomic analysis of dsRNA resistance in Colorado potato beetle (Leptinotarsa decemlineata, Say) 96
Abstract 97
4.1 Introduction 98
4.2 Materials and methods 100
4.2.1 Insect colonies 100
4.2.2 Sample preparation for RNA-seq and analysis 100
4.2.3 Sample preparation for bulked segregant sequencing and analysis 101
4.3 Results 102
4.3.1 Differentially expressed genes (DEGs) between untreated susceptible and resistant insects 102
4.3.2 Differentially expressed genes (DEGs) upon dsRNA treatment 103

4.3.3 Genomic regions associated with dsRNA resistance 105
4.4 Discussion 106
References 111
Chapter Five: Conclusions 130
Genetics of resistance in CEAS 300 132
Cross-resistance to other control methods 133
Mechanism of dsRNA resistance in CEAS 300 134
Genomic loci associated with dsRNA resistance 137
Final remarks 139
References 140
Vita 146

LIST OF TABLES

Table 2.1- Concentration-response bioassays of three Colorado potato beetle colonies (GC, METT and CEAS 300) against small molecule pesticides in potato leaf bioassays. Shown are the lethal concentrations (amount of active ingredient expressed in ppm) killing 50% of the individuals in the population (LC_{50}) and corresponding 95% fiducial limits from probit estimates. Also shown are probit fitting parameters (slope and χ^2 values) and susceptibility ratios **56**

Table 2.2- Percentage mortality observed in progenies from different crosses when exposed to 800 µg/ml of V-ATPase subunit A dsRNA in 20-day leaf-feeding bioassays. These mortalities were used to determine the genetics of resistance in CEAS 300 .....**57**

Table 3.1- Total number of sRNA reads mapping to the full-length V-ATPase subunit A transcript (expressed in reads per million, rpm) in dsRNA susceptible (METT or GC) and resistant (CEAS 300) untreated or treated samples **83**

Table 3.2- List of dsRNA uptake associated genes in Colorado potato beetle sequenced using targeted sequencing.....**83**

Table 3.3- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between untreated dsRNA resistant (CEAS 300) and susceptible (METT) insects, as identified from targeted RNA-sequencing analysis. Highlighted genes are common in comparisons between untreated CEAS 300 and untreated METT and GC strains.....**83**

Table 3.4- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between untreated dsRNA resistant (CEAS 300) and susceptible (GC) CPB insects, as identified from targeted RNA-sequencing analysis. Highlighted genes are common in comparison between untreated CEAS 300 and untreated METT and GC strains.....**85**

Table 3.5- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between treated dsRNA resistant (CEAS 300) and susceptible (METT) CPB populations, as identified from targeted RNA-sequencing analysis. Highlighted genes are common in comparison between treated CEAS 300 and untreated GC and METT strains.....**85**

Table 3.6- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between treated dsRNA resistant (CEAS 300) and susceptible (GC) CPB populations, as identified from targeted RNA-sequencing analysis. Highlighted genes are common in comparison between treated CEAS 300 and untreated METT and GC strains.....**86**

Table 3.7- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between untreated and treated dsRNA resistant CPB population, CEAS 300, as identified from targeted RNA-sequencing analysis.....**87**

Table 3.8- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between untreated and treated larvae from the dsRNA susceptible CPB GC population as identified from targeted RNA-sequencing analysis.....**87**

Table 3.9- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between untreated and treated larvae from the dsRNA susceptible CPB METT population, as identified from targeted RNA-sequencing analysis.....**88**

Table 3.10- Change in transcript level (mean fold-change ± SE) of target genes in larvae from dsRNA susceptible (GC) and dsRNA resistant (CEAS 300) strains using targeted sequencing or qPCR analysis.....**89**

Table 4.1- Number of differentially expressed genes (DEGs) between different treatments among susceptible (METT and GC) and dsRNA-resistant (CEAS) strains of Colorado potato beetle.....**114**

Table 4.2- Differentially expressed genes with |Log2FC| ≥2.5 and adjusted p-value ≤0.05 in untreated larvae from the dsRNA-resistant CEAS 300 strain of Colorado potato beetle compared to two susceptible strains (METT and GC) **114**

Table 4.3- Gene set enrichment analysis (GSEA) between untreated larvae from susceptible (METT) and the dsRNA-resistant (CEAS 300) strains of Colorado potato beetle.....**115**

Table 4.4- Pathways significantly over-represented (p-adjusted <0.05) among differentially expressed genes in untreated larvae from dsRNA susceptible CPB strain METT insects when compared to dsRNA resistant CEAS 300 insects.....**116**

Table 4.5- Gene set enrichment analysis (GSEA) between untreated larvae from susceptible (GC) and the dsRNA-resistant (CEAS 300) strains of Colorado potato beetle.....**117**

Table 4.6- Pathways significantly over-represented (p-adjusted <0.05) among differentially expressed genes in untreated larvae from dsRNA susceptible CPB strain GC insects when compared to dsRNA resistant CEAS 300 insects.....**117**

Table 4.7- Differentially expressed RNAi-related genes (adjusted p-value ≤0.05) in dsRNA resistant CPB strain CEAS 300 when compared to two susceptible strains METT and GC.....**118**

Table 4.8- Number of differentially expressed genes (DEGs) between untreated and treated samples of dsRNA susceptible (METT and GC) and resistant (CEAS 300) strains of Colorado potato beetle.....**119**

Table 4.9- Pathways significantly over-represented (p-adjusted <0.05) among differentially expressed genes in untreated larvae from dsRNA susceptible CPB strain METT when compared to dsRNA-treated larvae.....**119**

Table 4.10- Pathways significantly over-represented (p-adjusted <0.05) among differentially expressed genes in untreated larvae from dsRNA susceptible CPB strain GC when compared to dsRNA-treated larvae.....**120**

Table 4.11- Pathways significantly over-represented (p-adjusted <0.05) among differentially expressed genes in untreated larvae from dsRNA resistant CPB strain CEAS 300 when compared to dsRNA-treated larvae.....**121**

Table 4.12- Differentially expressed (p<0.05) genes in the RNAi pathway in response to treatment with dsRNA in susceptible (METT, GC) and dsRNA-resistant (CEAS 300) strains of Colorado potato beetle.....**122**

Table 4.13- Differentially expressed genes with Log2FC ≥2.5 and adjusted p-value ≤0.05 in treated larvae from the dsRNA-resistant CEAS 300 strain of Colorado potato beetle compared to two susceptible strains (METT and GC) **123**

Table 4.14- Loci located on genomic regions within peaks of polymorphism significantly associated with the dsRNA resistance phenotype in the CEAS 300 strain. Only genes expressed in the midgut of Colorado potato beetle larvae (as established from RNA-seq data) are shown.....**124**

LIST OF FIGURES

Figure 1.1- Schematic diagram of the A) mode of action of insecticidal dsRNA and B) various potential mechanisms of resistance against insecticidal dsRNA.....**37**

Figure 1.2- Larvae from susceptible or a dsRNA-resistant population of Colorado potato beetle (*Leptinotarsa decemlineata*) when fed on untreated potato leaves (A) or potato leaves dipped in a solution of 400 µg/ml of dsRNA (B) for 7 days.....**38**

Figure 2.1- Concentration-response curves of GC, METT and CEAS larvae when exposed to increasing amount of active ingredient pyrethrin+ PBO (ppm).....**58**

Figure 2.2- Concentration-response curves of GC, METT and CEAS larvae when exposed to increasing amount of active ingredient spinosad (ppm).....**58**

Figure 2.3- Concentration-response curves of GC, METT and CEAS larvae when exposed to increasing amount of active ingredient abamectin (ppm).....**59**

Figure 2.4- Concentration-response curves of GC, METT and CEAS larvae when exposed to increasing amount of active ingredient imidacloprid (ppm).....**59**

Figure 3.1- Agarose gel showing relative stability of a dsRNA when incubated with stock (1mg/ml), 10X and 50X dilutions of METT, GC, and CEAS 300 gut fluids collected as regurgitate or by dissection, for 1 hour at room temperature.....**90**

Figure 3.2- Histological gut sections of CPB larvae from susceptible (METT) and resistant (CEAS 300) colonies, treated with dsRNA or water as a control, observed under a confocal microscope. Mouse anti-dsRNA J2 antibody was used as the primary antibody to detect dsRNA in tissue samples and Mouse Alexa 594 antibody was used as the secondary antibody.....**91**

Figure 3.3- Fold increase in small RNAs (sRNAs) mapping to the target transcript V-ATPase subunit A upon dsRNA treatment in susceptible (METT and GC) and resistant (CEAS 300) CPB larvae. Asterisks denote significant differences (Kruskall-Wallis ANOVA on Ranks followed by a Dunnet's pairwise multiple comparison to the control group METT), p-value < 0.05) **92**

Figure 3.4- Percentage mortality in CPB larvae from dsRNA susceptible (METT and GC) and resistant (CEAS 300) colonies in response to different treatments. Asterisks denote significant differences within each population (Kruskall-Wallis ANOVA on Ranks followed by a Dunnet's pairwise multiple comparison to the control group (Tween), p-value < 0.05) **93**

Figure 4.1- Principal component analysis (PCA) plot showing the distribution of untreated (circles) or dsRNA treated (triangles) samples from susceptible (METT- blue, GC- green) and dsRNA-resistant (CEAS 300- peach) strains of Colorado potato beetle.....**125**

Figure 4.2- Bulked segregant analysis (BSA) comparing SNP frequency between susceptible (METT and GC) and dsRNA-resistant (CEAS 300) strains of Colorado potato beetle. Peaks above the red line are considered significantly associated with the resistance phenotype ($p<0.05$). Peaks with red arrows (chromosomes 5, 10, and 11) were found to be common when comparing METT vs CEAS 300 and GC vs CEAS 300 separately...**126**

Chapter One

Double-stranded RNA insecticides: Activity, applications and resistance challenges

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1.1 Introduction

Initially observed as a defensive mechanism against tobacco ringspot virus (Wingard 1928), RNA interference (RNAi) was first formally described in the nematode *Caenorhabditis elegans* (Fire et al. 1991) and subsequently described across Eukaryota. The RNAi process in the cell is initiated by the presence of a double-stranded RNA (dsRNA), a class of non-coding RNA comprised of two complementary strands that can be endogenous or delivered exogenously. The result of the RNAi process is the degradation of the messenger RNA (mRNA) complementary to the dsRNA sequence, effectively suppressing gene expression (i.e., the protein encoded by the gene is not made) (Fig. 1.1). Applications of dsRNA-based technology in agriculture range from protecting plants and beneficials (Vogel et al. 2019) to managing herbicide resistance in weeds (Perotti et al. 2020).

Two different types of RNAi response have been described based on whether reduction in gene expression is limited to the cells in which the dsRNA is introduced or expressed (cell-autonomous RNAi), or the response is also observed in cells or tissues beyond the site of dsRNA application (non-cell autonomous RNAi). Within non-cell autonomous RNAi, silencing may involve dsRNA taken up from the environment (environmental RNAi) or transferred from other cells and tissues (systemic RNAi). As an example of non-cell autonomous RNAi, injection of dsRNA into the head or tail of *C. elegans* produced silencing of the target gene throughout the individual and transgenerational silencing was also observed in its progeny (Fire et al. 1998).

Insects belonging to different taxonomic orders display systemic RNAi (Aronstein and Saldivar 2005; Zhou, Oi, and Scharf 2006; Zhang, Zhang, and Zhu 2010; Bolognesi et al. 2012), and there are also examples of knockdown in eggs from dsRNA-treated females, a process called parental RNAi. However, unlike in plants and nematodes where systemic RNAi requires a mechanism termed transitive RNAi involving an RNA-dependent RNA polymerase (RdRP) producing secondary small interfering RNAs (siRNAs), insect genomes seem to lack RdRP genes (Gordon and Waterhouse 2007) and production of secondary siRNAs (Li et al. 2018). Thus, systemic RNAi in insects is not transitive and may involve different mechanisms for the spread of silencing. One possibility is that siRNAs resulting from dsRNA processing are incorporated in extracellular vesicles (EVs), which are capable of inducing an RNAi response in recipient cells (Mingels et al. 2020).

The Colorado potato beetle (*Leptinotarsa decemlineata*) is an important pest of potatoes that has been used as a model species to characterize the steps involved and their relevance in insecticidal RNAi. Uptake of dsRNA in gut cells of *L. decemlineata* involves channel proteins with homology to the systemic RNA interference-deficiency (SID) proteins of *C. elegans* as well as clathrin-mediated dsRNA endocytosis (Cappelle et al. 2016). Upon uptake into the cytoplasm, the dsRNA is cleaved into 21-25 bp long small interfering RNAs (siRNAs) by the action of a multidomain ribonuclease (RNase) type III enzyme called Dicer. Processing of dsRNA to siRNAs in *L. decemlineata* seems to require the dsRNA-binding protein StaufenC and reduced StaufenC expression is associated with resistance to dsRNA in cultured cells (Yoon et al. 2018). The generated siRNAs are loaded into the RNA-induced silencing complex (RISC) and unwound, yielding a guide strand complementary to the target mRNA and a passenger strand that is degraded. The guide strand directs the binding of the RISC complex to the complimentary mRNA, which is

followed by its degradation by the RNAase protein Argonaute. Degradation of a target mRNA encoding an essential insect protein can result in growth inhibition (Mao et al. 2007; Zhu et al. 2012), developmental abnormalities (Xiong et al. 2013), and/or mortality (Baum et al. 2007).

1.2 Parameters affecting insecticidal activity of dsRNA

Efficiency of dsRNA varies depending on several factors, including insect species, target gene, dsRNA design, method of delivery, and dsRNA dosage. In general, species in Coleoptera are the most sensitive to dsRNA, while lepidopterans and hemipterans are largely recalcitrant (Cooper et al. 2019b). Critical but not sufficient to insecticidal RNAi is the existence of a complete core RNAi machinery, including Argonaute (Ago), Dicer (Dcr), and RNA binding protein (R2D2) genes in the targeted pest (Dowling et al. 2016). For example, distinct susceptibility to dsRNA among *L. decemlineata* instars correlates with expression levels of core RNAi machinery genes (Guo et al. 2015), and relatively high susceptibility to dsRNA in this insect may be associated with duplicated core components of the siRNA pathway (Schoville et al. 2018).

Lack of susceptibility to dsRNA may also be dictated by physiological adaptations. For instance, gut nucleases degrading dsRNA and entrapment of dsRNA in early and late endosomes after uptake explain the insensitivity of lepidopteran insects to dsRNA (Arimatsu et al. 2007) (Yoon, Gurusamy, and Palli 2017). Gut nucleases also affect dsRNA activity in other insects, including *L. decemlineata* (Spit et al. 2017). Consequently, efforts have focused on improving insecticidal RNAi by increasing dsRNA stability and/or uptake using nuclease inhibitors (Castellanos et al. 2019), formulating dsRNA with synthetic polymers (Christiaens et al. 2018; Parsons et al. 2018), and encapsulating with transfection reagents (Whyard, Singh, and Wong 2009) or clay nanosheets (Mitter et al. 2017). Recent evidence suggests that the uptake of dsRNA

with a “paperclip” structure by an undescribed mechanism may overcome insect recalcitrance to RNAi when is related to reduced dsRNA uptake (Abbasi et al. 2020b).

The choice of target gene also contributes to the probability of successful insecticidal RNAi. The optimal target gene has stable, high level expression and encodes a vital protein that performs essential functions that cannot be rescued by other gene products. Large scale RNAi screens in model insects, such as *Tribolium castaneum* (Ulrich et al. 2015) or *Drosophila melanogaster* (Danielsen et al. 2016; Bingsohn et al. 2017) have helped identify genes involved in immunity and the proteasome as prime targets. Orthologues in crop pests to targets identified in these model organisms are reported as effective targets (Knorr et al. 2018). Mechanisms explaining distinct target efficacy include feedback mechanisms increasing rates of target gene transcription, target transcripts being short-lived and not allowing completion of the RNAi process, and sequence-specific stability of certain dsRNA molecules (Bellés 2010).

For any specific target gene, the dsRNA sequence, length, and dosage affect its ability to elicit an insecticidal RNAi response. Recent evidence suggests that in insects dsRNA cleavage may have sequence preference (Guan, Hu, et al. 2018). Longer dsRNA molecules allow production of a higher number of distinct siRNAs during cleavage by Dicer, potentially increasing RNAi efficiency. On the other hand, longer dsRNAs also have higher probability of producing siRNAs matching non-target mRNAs in the same or related insect species, potentially reducing specificity. In addition, there is evidence that dsRNA length may influence uptake by the cell (Bolognesi et al. 2012). Typically, dsRNAs in the 200-400 bp range are used for effective and specific activity, with a single 21 bp fragment matching the target mRNA being sufficient to elicit a lethal RNAi response. While increasing dsRNA dose is expected to augment RNAi efficacy, high dsRNA dosage or treatment with multiple dsRNAs can lead to oversaturation of the RNAi machinery,

resulting in an inefficient RNAi response (Miller et al. 2012). Determining the optimal length, sequence, and dosage for a particular dsRNA is important when considering the cost of dsRNA production, especially for application in large crop field extensions.

1.3 Delivery of dsRNA to potato pests

Commercial development of dsRNA-based insecticidal products depends on the feasibility of a dsRNA delivery method for the target pest considered. The dsRNA should be available in parts of the plant involved in the feeding habits of the target pest (leaf chewing, sap-sucking, or root feeding). Typically, dsRNA delivery approaches are broadly classified into transgenic (or transformative) when they involve production of the dsRNA in the host (also called host-induced gene silencing, HIGS), and non-transgenic (or non-transformative) mostly involving formulations incorporating dsRNA. An example of HIGS against a potato pest is the use of transgenic potato plants producing dsRNA to control *L. decemlineata*. Nuclear dsRNA expression in this case can result in processing by plant cell nucleases to small RNAs which may be ineffective against the targeted insect. Alternatively, production of the dsRNA in the nuclease-free chloroplast environment overcomes this limitation and allows for stable accumulation of high levels of dsRNA. Therefore, transplastomic potato expressing dsRNA is more effective in *L. decemlineata* control compared to potato plants expressing the dsRNA in the nucleus (Zhang et al. 2015). An additional advantage of the transplastomic approach for regulatory purposes is that dsRNA expression is limited to photosynthetic (mainly leaves) tissues where the insect feeds, while potato tubers and pollen are essentially dsRNA-free and safe for animal and human consumption. In both nuclear and plastid expression, dsRNA may be combined with other plant-incorporated protectants (PIPs) with distinct modes of action, such as insecticidal proteins from bacteria, to broaden the range of activity and extend durability.

Non-transgenic dsRNA delivery against potato pests includes topical dsRNA sprays and root drenches. Both methods involve dsRNA products with lower developmental costs and a more streamlined regulatory pathway compared to transgenic technologies and are also not affected by still low acceptability of genetically modified plants by consumers. While sprays and drenches allow better control over the dsRNA dosage, they are also susceptible to dsRNA degradation by nucleases in the environment and wash-off, which may add extra labor and costs because of the need for repeated application. Effective use of foliar dsRNA sprays, also known as spray-induced gene silencing (SIGS), has been demonstrated for *L*. *decemlineata* in greenhouse (San Miguel and Scott 2016) and field tests (Bramlett, Plaetinck, and Maienfisch 2020; Petek et al. 2020).

Although foliar dsRNA sprays provide an efficient control strategy for leaf-chewing insects, they are probably ineffective against insects feeding on phloem sap or plant stems. While not yet tested against potato pests, immersion, irrigation, and root drenching with a dsRNA solution followed by absorption of the dsRNA by plant roots and tissues can induce insecticidal RNAi in insects feeding on the plant (Ghosh et al. 2018), providing effective control of pierce-sucking and stem-borer insects.

To have practical application, non-transgenic dsRNA delivery methods require production of large quantities of dsRNA in a cost-effective manner, competitive with production costs for synthetic chemical insecticides. One possible approach is engineering microbes (bacteria, fungi, and viruses) to produce the dsRNA. For instance, in virus-induced gene silencing (VIGS) the dsRNA target is introduced in the genome of a virus specific to the target insect species so that viral replication produces dsRNA inside the host insect cell ((Taning et al. 2018). This technique adds specificity of virus-host interactions to the dsRNA sequence specificity of insecticidal RNAi technology. Similarly, an RNAase III-deficient strain of the bacterium *Escherichia coli* was

engineered to produce dsRNAs targeting essential genes in *L. decemlineata*, and feeding on the bacteria caused a significant reduction in target transcript levels and larval growth and survival (Zhu et al. 2011).

1.4 Safety of insecticidal dsRNA

As for any new insecticidal technology, it is important to consider the potential effects of dsRNA insecticides on non-target organisms, the environment, as well as safety of treated food products. Risks related to both transgenic (Bachman et al. 2016) and non-transgenic (Romeis and Widmer 2020) dsRNA delivery approaches are examined as part of the registration process before approval for commercialization. The high specificity dictated by dsRNA sequence complementarity provides one of the most specific insecticidal modes of action available. Experimental evidence supports that dsRNA toxicity depends on the number of contiguous random 21 bp matches between the dsRNA and a transcript (Bachman et al. 2013). For instance, dsRNA targeting the V-ATPase subunit A gene of *D. v. virgifera* displayed >10-fold lower activity in *L. decemlineata* compared to *D. v. virgifera*, even though the gene displays 83% sequence identity between the two species (Baum et al. 2007). Thus, careful consideration of the dsRNA sequence is important to ensure little to no identity to any gene in a non-target organism. Availability of insect genomic and transcriptomic resources allows *in silico* prediction of potential risks to non-target species due to sequence identity (Mogren and Lundgren 2017). However, any potential effects predicted *in silico* need to be experimentally tested because eliciting a toxic RNAi response depends on additional parameters beyond sequence identity. For instance, dsRNA targeting the V-ATPase subunit A gene in honeybee (*Apis mellifera*) had no impact on adult survival (Vélez et al. 2016) demonstrating that sequence identity alone is insufficient to observe a toxic RNAi effect and highlighting the importance of bioassays in assessing potential off-target effects.

Environmental risk assessment for transgenic maize plants producing dsRNA targeting the vacuolar sorting protein Snf7 transcript in *D. v. virgifera* did not detect deleterious effects even at the maximum expected environmental exposure in any of the non-target arthropods, beneficial soil microbes, and aquatic and terrestrial vertebrates tested (Bachman et al. 2016).

Another important consideration in assessing risks of dsRNA is its availability and persistence in the environment after application. Persistence tests in soil and sediment-water support that topical dsRNA degrades within 2 days of application (Dubelman et al. 2014; Fischer et al. 2017), reducing environmental risks of runoff to water sources, bioaccumulation, and biomagnification. However, dsRNA formulation to increase efficacy in field application may involve enhanced stability and/or persistence. Therefore, it is advisable to conduct stability and off-target impact tests for finalized dsRNA products in a case-by-case manner.

The vertebrate system presents physiological barriers to consumed dsRNAs, including nucleases in saliva or blood and the gastrointestinal tract, along with the highly acidic environment in the stomach (Petrick et al. 2013). Consequently, significant systemic absorption of intact dsRNAs after ingestion of materials treated with dsRNA-based biopesticides is highly improbable in humans and other vertebrates (Rodrigues and Petrick 2020). Additionally, studies have also shown that the bioavailability of diet-derived dsRNAs is considerably lower in magnitude compared to that required for biological activity (Cottrill and Chan 2014; Dickinson et al. 2013; Snow et al. 2013). The long history of safe consumption of naturally occurring dsRNAs in plants and other foods (Zeng et al. 2019), even when significant identity between dsRNAs and human genes exists (Jensen et al. 2013; Ivashuta et al. 2009), further supports the safety of dsRNA pesticides for food production.

1.5 Use of dsRNA against potato pests

Insecticidal dsRNA technology has demonstrated promising results in a considerable number of studies testing diverse dsRNA gene targets against potato insect pests (Table 1), as discussed in the following sections. This technology represents a safer alternative to current control strategies for pests of cultivated potato, which mostly focus on the use of small molecule insecticides with broader specificity.

1.5.1 Potato psyllid (*Bactericera cockerelli*)

The potato psyllid is a serious pest feeding on the phloem of solanaceous plants and can also vector the zebra chip disease of potatoes caused by the endosymbiont *Candidatus* Liberibacter psyllaurous (solanacearum) (Munyaneza, Crosslin, and Upton 2007). Typically, hemipteran insects such as *B. cockerelli* are not as susceptible to dsRNA when compared to the vigorous response observed in coleopterans. Though not fully understood, factors such as lack of core RNAi machinery components, degradation of dsRNA in insect guts, and inefficient uptake of dsRNA by insect gut cells are some plausible explanations for this lack of robust RNAi response in hemipteran insects, as reviewed elsewhere (Christiaens, Swevers, and Smagghe 2014). Accordingly, different dsRNA delivery methods tested to induce an RNAi response in *B. cockerelli*, resulted in modest reductions in target transcript levels and insect mortality. An initial study showed mortality doubled when compared to controls in treatments with injection or oral delivery of dsRNAs or siRNAs targeting *Actin*, *ATPase*, *Hsp70*, or *CLIC* genes in adult *B. cockerelli* (Wuriyanghan, Rosa, and Falk 2011). An alternative study used VIGS to deliver siRNAs targeting *B. cockerelli Actin* and *ATPase* genes through a recombinant tobacco mosaic virus, resulting in reduced psyllid fecundity (Wuriyanghan and Falk 2013). Transgenic tomato plants expressing dsRNA against the *aquaporin* gene caused ~40% mortality in female *B. cockerelli*

(Tzin et al. 2015). These results suggest preliminary evidence that further optimization of delivery may lead to levels of activity needed for commercial use of insecticidal dsRNA against *B. cockerelli*.

1.5.2 28-spotted potato ladybird *(Henosepilachna vigintioctopunctata)*

The 28-spotted potato ladybird or Hadda beetle is a pest of solanaceous and cucurbit plants native to southeastern Asia, but it has also spread to Brazil, Argentina, Australia, and New Zealand. Feeding damage on leaves by *H. vigintioctopunctata* results in plant growth retardation and subsequent yield losses.

The amenability of coleopteran insects to a potent RNAi response suggested *H. vigintioctopunctata* as a potential candidate for control with dsRNA technology. Feeding 3rd instar *H. vigintioctopunctata* larvae with dsRNA targeting the death-associated inhibitor of apoptosis protein 1 (*diap1*) gene resulted in rapid feeding cessation, but this was not observed with dsRNA targeting the *v-ATPase A* and *E* genes (Chikami et al. 2019). Similar results were reported when the dsRNA targeting *diap 1* was encapsulated in the bacterium *Corynebacterium glutamicum* (Hashiro et al. 2019). Similarly, feeding on eggplant leaf discs coated with *E. coli* cultures expressing dsRNA targeting the *v-ATPase B* (Lü, Guo, et al. 2020) and vacuolar sorting protein SNF7 (Lü, Liu, et al. 2020) genes increased *H. vigintioctopunctata* mortality by 15- and 21-fold, respectively. Greenhouse assays using spraying of potato plants with bacteria expressing dsRNA targeting the *ecdysone receptor* gene inhibited the *H. vigintioctopunctata* larva-pupa transition and reduced leaf consumption (Wu et al. 2020). These observations confirm the potential for developing dsRNA-based products against *H. vigintioctopunctata*.

1.5.3 Colorado potato beetle *(Leptinotarsa decemlineata)*

Larvae of *L. decemlineata* can completely skeletonize potato plants and result in complete crop losses, and this pest is known to have an impressive ability to develop resistance against almost all known classes of pesticides (Alyokhin et al. 2008). These features, together with the high susceptibility of *L. decemlineata* to dsRNA (Baum et al. 2007) identify this pest as an optimal model for the development of insecticidal RNAi products. In consequence, several target genes and various dsRNA delivery methods, including foliar sprays, bacterially expressed dsRNA, and transgenic potato plants, have been shown effective against *L. decemlineata*.

Diet feeding tests identified dsRNAs targeting the *V-ATPase subunit A* and *subunit E* genes as highly effective against *L. decemlineata* larvae (Baum et al. 2007). In greenhouse tests, dsRNA targeting the *L. decemlineata Actin* gene protected the plants for up to 28 days and was not readily washed off from the leaves once dried (San Miguel and Scott 2016). Heat-killed *E. coli* cells expressing dsRNAs targeting the *Actin*, transport protein SEC23 (*Sec23*), *vATPase B*, and coatomer subunit beta (*COPβ*) genes produced ~100% mortality when fed to *L. decemlineata* larvae (Zhu et al. 2011). In the same study, dsRNA targeting the *vATPase E* gene resulted in ~50% mortality, highlighting the importance of target gene selection for efficient insect control.

Prototype products based on sprayable dsRNA solutions targeting *L. decemlineata* genes have demonstrated promising results and are undergoing regulatory review for commercialization. In addition, transgenic potato lines expressing dsRNA targeting the juvenile hormone acid methyltransferase (*JHAMT*) gene were shown to affect fecundity and reduce *L. decemlineata* populations in field trials (Guo et al. 2018). High levels of toxicity to *L. decemlineata* were achieved by expressing dsRNA targeting the *actin* gene in the chloroplast versus the nucleus

(Zhang et al. 2015), supporting the future use of these transplastomic potato plants for *L. decemlineata* control.

1.5.4 Peach-potato aphid *(Myzus persicae)*

The major damage caused by *M. persicae* is not due to direct feeding on its host plants but due to the transmission of a variety of pathogens, such as Potato Y and Potato leafroll viruses, making it one of the most destructive agricultural pests in the world (Hogenhout et al. 2008). Furthermore, the development of resistance against pesticides has fueled interest in developing RNAi-based strategies to control this pest.

As evidence for the potential use of RNAi in addressing the vectoring capacity of *M. persicae*, silencing the cuticular protein *MCPCP2* gene reduced Potato virus Y transmission by 47% compared to controls (Bahrami Kamangar et al. 2019). However, the efficacy of dietary dsRNA in this insect appears greatly challenged by the production of midgut nucleases (Ghodke et al. 2019).

Experiments with artificial diet containing dsRNA targeting the voltage-gated sodium channels (*VGSC*) gene resulted in up to 65% mortality in 3rd instar *M. persicae* nymphs, along with significantly lower fecundity and longevity in adults (Tariq et al. 2019). Delivery of dsRNA targeting the cuticle protein 19 (*CP19*) to *M. persicae* adults through a Brassica leaf resulted in <50% increased mortality (Shang, Ding et al. 2020). Transgenic tobacco plants expressing dsRNA targeting the *M. persicae hunchback* gene induced approximately a 20% reduction in fecundity and biomass (Mao and Zeng 2014). These observations of limited toxicity with distinct delivery methods suggest the need for research identifying better gene targets and optimizing dsRNA delivery to overcome midgut nucleases and elicit a robust insecticidal response.

1.5.5 Potato tuber moth *(Phthorimaea operculella)*

The potato tuber worm is probably the most damaging lepidopteran pest of cultivated potato (Rondon 2010). The risk of potato infestation by this pest prevails throughout the growing season as the larvae feeding on foliage are capable of tunneling into the tubers, thus making them unmarketable. Thus, in addition to overcoming commonly described refractoriness to insecticidal RNAi in Lepidoptera, delivery of dsRNA through transgenic potato plants would be required to effectively prevent tuber damage by this pest. The high specificity of dsRNA compared to other plant-incorporated protectants would be critical when assessing the safety of transgenic potato tubers. There is currently only one published study reporting successful insecticidal RNAi by injection of dsRNA silencing the chitin synthase A (*CHSA*) gene in *P. operculella* larvae (Mohammed et al. 2017). Evidence of efficacy through feeding would be needed in identifying effective dsRNA targets as candidates for expression in transgenic potato plants against *P. operculella*.

1.6 Potential mechanisms of resistance to insecticidal dsRNA

As with any other pesticide, intense use of the new insecticidal dsRNA products is expected to increase selection for the evolution of resistance (Gao et al. 2024). Theoretically, alterations in any of the steps involved in the RNAi process could result in resistance, including behavioral avoidance, degradation of dsRNA by gut nucleases, reduced dsRNA uptake, mutations yielding anon-functional RNAi machinery, and mutations in the target gene preventing recognition of the complementary mRNA by the siRNA guide strand in the RISC complex (Fig. 1.1). Following sections provide a critical assessment and summary of currently available information characterizing resistance to insecticidal RNAi, and the genes and mechanisms that may be involved.

1.6.1 Behavioral avoidance

There are very few documented cases of behavioral changes associated with insect resistance and are mostly found in addition to other resistance mechanisms (Sparks et al. 1989). For example, Cry3Aa-resistant Colorado potato beetle (*Leptinotarsa decemlineata*) displayed increased flight activity upon ingestion of Cry3A (Alyokhin and Ferro 1999). Since the biochemical and physiological mechanisms controlling behavioral avoidance are not well understood, the role that these behaviors may play in resistance to RNAi pesticides remains mostly unexplored. Aversion or avoidance behaviors may increase levels of resistance in insects already carrying a major resistance allele. Moreover, behaviors such as increased flight or movement away from treated areas can result in an expanded flow of resistance alleles and should be carefully considered while designing resistance management strategies.

1.6.2 Reduced penetration

The first physical barrier encountered by insecticides is the insect cuticle. Biochemical changes in the insect cuticular structure can give rise to penetration resistance where either a lower amount of insecticide reaches its target, or slower absorption allows more time for detoxification enzymes to act on the insecticide (Panini et al. 2016). For instance, the thickening of the cuticle was associated with resistance to pyrethroids in strains of the common bed bug (*Cimex lectularius*) (Lilly et al. 2016) and the malaria vector *Anopheles funestus* (Wood et al. 2010). Up-regulation of cuticular protein genes was detected in *L. decemlineata* populations exposed to imidacloprid and chlorothalonil (Clements et al. 2018). Even though reduced penetration typically confers low levels of resistance and is mostly observed along with other major resistance mechanisms, it could still be an important factor leading to cross-resistance.

Passage through the cuticle is not the primary route of entry for dsRNA molecules inducing an insecticidal RNAi response in phytophagous insects. However, there is evidence for insecticidal RNAi by direct spraying of a dsRNA solution onto larvae of the Asian corn borer (*Ostrinia furnacalis*) (Wang et al. 2011). Soaking in a dsRNA solution has also been reported efficient in inducing gene silencing in *Diaphorina citri* nymphs (Yu et al. 2017) and *Aedes aegypti* pupae (Arshad et al. 2021). Even though dsRNAs are considerably larger than small molecule insecticides such as pyrethroids, these observations suggest possible passage of dsRNA through the insect cuticle. Increased penetration of dsRNA formulated with a polymer/detergent increased RNAi-induced mortality in the soybean aphid (*Aphis glycines*) (Zheng et al. 2019). Consequently, reduced penetration could be a potential mechanism of resistance to a dsRNA delivered by direct spraying or soaking.

1.6.3 Metabolic resistance: detoxification, degradation, sequestration and excretion

Insect metabolic resistance is the capability of individuals to display reduced susceptibility by detoxifying, degrading, sequestering, and/or excreting toxic compounds at a faster rate than susceptible counterparts. It is one of the most common mechanisms of resistance observed against chemical insecticides (Li, Schuler, and Berenbaum 2006; Hemingway 2000; Panini et al. 2016; Ranganathan, Narayanan, and Kumarasamy 2022; Vontas, Katsavou, and Mavridis 2020), and often hardest to manage due to cross-resistance stemming from the low specificity typically displayed by detoxification enzymes.

Degradation or faster processing has been associated with resistance against insecticidal proteins (Jurat-Fuentes, Heckel, and Ferré 2021). In the case of RNAi, nucleases in the digestive system and hemolymph of insects can degrade dsRNAs and modulate the success of the RNAi response (Singh et al. 2017; (Allen and Walker 2012). The extent of this dsRNA degradation by

nucleases is commonly associated with the distinct efficacy of RNAi insecticides among insect species (Garbutt et al. 2013; Almeida Garcia et al. 2017; Spit et al. 2017; Christiaens, Swevers, and Smagghe 2014). Moreover, the knockdown of nucleases improves RNAi efficiency (Guan, Li, et al. 2018; Tayler et al. 2019; Prentice et al. 2019; Ahmad et al. 2024; Dalaisón-Fuentes et al. 2023). In overcoming this potential resistance mechanism, the protection of dsRNA by encapsulation or complexation with polymers (Christiaens et al. 2018; Parsons et al. 2018), transfection agents (Cooper et al. 2021; Gurusamy et al. 2020), and various nanocarriers (Das et al. 2015; Mitter et al. 2017) results in increased RNAi efficiency.

Four major detoxification enzyme families have been identified as playing a major role in insect metabolic resistance pathways, including cytochrome P450 (cytP450), ABC transporter, glutathione-S-transferase (GST), and esterase superfamilies (Panini et al. 2016). Molecular studies have demonstrated that increased detoxification results from gene duplications or mutations in gene promoter regions resulting in overexpression of one or more of these metabolic enzymes. On the other hand, mutations in coding regions can also result in more efficient enzyme isoforms (Torres et al. 2018). However, dsRNAs are structurally and metabolically very different from active compounds in chemical pesticides. Consequently, the aforementioned enzymes are not expected to metabolize dsRNAs or cause resistance to insecticidal RNAi.

1.6.4 Reduced dsRNA uptake

Successful uptake of dsRNAs by target cells is necessary to initiate the RNAi process, thus reduced or lack of dsRNA uptake can result in resistance to RNAi. The importance of dsRNA uptake for insecticidal RNAi efficacy is highlighted by an increased RNAi response when dsRNA uptake is promoted in insects that are otherwise insensitive to RNAi (Whyard, Singh, and Wong 2009). The potential of reduced dsRNA uptake in resistance to RNAi was first demonstrated in the

oriental fruit fly (*Bactrocera dorsalis*), where initial exposure to a dsRNA targeting an endogenous gene resulted in refractoriness to a second dsRNA treatment (Li et al. 2015). This refractoriness was independent of the targeted gene, but depended on dsRNA concentration and involved a decrease in endocytosis, a mechanism mediating dsRNA uptake in insects (Vélez and Fishilevich 2018). Results from *in vitro* studies testing different dsRNA lengths and structures support that reduced uptake explains the inability of shorter RNAs to trigger the RNAi response (Koo et al. 2023). Importantly, reduced dsRNA uptake would result in resistance independent of the dsRNA sequence.

1.6.5 Target site insensitivity

Mutations in target sites for active ingredients in pesticides result in high levels of resistance and potential cross-resistance to pesticides targeting the same site. For instance, mutations in acetylcholine esterase result in resistance to carbamates and organophosphates (Alyokhin et al. 2008; Elfekih et al. 2014; Menozzi et al. 2004), mutations in the voltage-gated sodium channel result in resistance to DDT and pyrethroids (Martinez-Torres et al. 1999; Araújo et al. 2011; Silva, Santos, and Martins 2014; Valmorbida et al. 2022), and disrupting mutations in receptor genes result in resistance to multiple insecticidal Cry proteins (Jurat-Fuentes, Heckel, and Ferré 2021).

In the case of RNAi, the complementary mRNA may be considered as the target site for the dsRNA. Identical 15-21 bp sequence matches to the mRNA are usually necessary for recognition by the RISC complex and an effective RNAi response (Bolognesi et al. 2012). Mutations in the target gene that is transcribed to the mRNA could result in a lack of mRNA recognition, preventing silencing and resulting in resistance. However, a mismatch rate of <3% between the dsRNA and targeted transcripts did not affect silencing or dsRNA toxicity in *L. decemlineata* (He et al. 2020). Target mutations would be of special relevance as a potential resistance mechanism when using

short dsRNA sequences or siRNAs with a very limited mRNA target region. On the other hand, long dsRNAs (200-400 bp) reduce the effects of target site mutations by generating multiple siRNAs and increasing the probability of sequence complementarity to the target mRNA.

1.7 Current mechanistic dsRNA resistance data

1.7.1 Evidence for resistance potential

The efficiency of insecticidal RNAi follows a broad spectrum, from relatively low dsRNA doses inducing 90% or higher knockdown efficiency in some Coleopterans to Lepidopterans and hemipterans displaying high refractoriness (Terenius et al. 2011; Wang et al. 2016). Even within the Coleoptera Order, not all families are equally sensitive to insecticidal RNAi (Willow and Veromann 2021). Additionally, even within the same species, there is variation in susceptibility to insecticidal RNAi. For instance, susceptibility to dsRNA varied up to 7.5-fold in European populations of *L. decemlineata* (Mehlhorn et al. 2020). Tests with Ledprona in populations of *L. decemlineata* from the U.S. detected significant differences in susceptibility based on location (Pallis et al. 2022). Variation in RNAi efficiency was also documented in populations of *Locusta migratoria* based on geographical location and phylogenetic clade of origin (Sugahara et al. 2017) and even among 83 different genotypes of *A. pisum* from a single naturally occurring population (Yoon et al. 2020). This natural variation suggests that candidate resistance alleles and the potential for resistance exist in natural populations. The relatively quick selection of resistance against dsRNAs delivered by transgenic and non-transgenic methods (as discussed in the following sections) supports a high frequency of resistance alleles in some field populations.

1.7.2 Selection of resistance against dsRNA produced in transgenic plant

The first experimental evidence for resistance against insecticidal RNAi involved selecting Western corn rootworm (*Diabrotica virgifera virgifera*) in the field on transgenic maize expressing

the *DvSnf7* dsRNA (Khajuria et al. 2018). Selection for eleven generations on the transgenic maize resulted in a *D. v. virgifera* population displaying about 130-fold resistance to the purified dsRNA. Resistance was transmitted as an autosomal recessive trait and was linked to a single locus. Gut cells from resistant rootworms displayed impaired dsRNA uptake, explaining the failure to induce the RNAi process even when using alternative dsRNA targets (Khajuria et al. 2018). The exact molecular mechanism behind this phenotype remains unknown though the resistance locus was determined to be in linkage group 4 (LG4). The RNAi-resistant rootworms remained susceptible to insecticidal Bt proteins, as expected from the distinct mode of action of the two insecticides.

1.7.3 Selection of resistance against dsRNA delivered by non-transgenic method

Selection of resistance to a foliar application of a dsRNA targeting the V-ATPase subunit A gene was performed on a population of *L. decemlineata* generated from collections in 9 states in the USA (Mishra et al. 2021). The stringent selection protocol relied on increasing the dsRNA selection dose between generations and using adult emergence as the endpoint. After nine generations of selection, resistance to the dsRNA reached >11,100-fold compared to the unselected parental population. Feeding on potato leaves dipped in a lethal concentration of dsRNA for susceptible *L. decemlineata* does not result in negative effects in larvae from the resistant strain (Fig. 1.2). Although resistance to a foliar application of dsRNA in *L. decemlineata* is polygenic, it shares autosomal and recessive inheritance with rootworm resistance to transgenic maize producing dsRNA (Mishra et al. 2021). Also as observed in resistant rootworms, no cross-resistance against the Cry3Aa Bt insecticidal protein was observed in dsRNA-resistant *L. decemlineata* (Mishra et al. 2021). Moreover, no changes in susceptibility to small molecule insecticides commonly used for control, such as pyrethroids and neonicotinoids, were detected in dsRNA-resistant *L. decemlineata* (Mishra, Moar, and Jurat-Fuentes 2024). The mechanism of

resistance to dsRNA in this population of *L. decemlineata* remains to be elucidated, yet cross-resistance observed to a dsRNA targeting a different gene suggests target-site mutations are not involved in resistance (Mishra et al. 2021).

More recently, selection with a foliar overlay using increasing concentrations of a dsRNA targeting the signal recognition particle 54 k (*Srp54k*) gene in the willow leaf beetle (*Plagiodera versicolora*) resulted in >4,110-fold resistance after seven generations of selection (Liao et al. 2024). Cross-resistance to alternative dsRNAs and maintained susceptibility to the Cry3Bb insecticidal Bt protein in the resistant *P. versicolora* matched the dsRNA resistance phenotype observed for *D. v. virgifera* and *L. decemlineata*. Also, in agreement with *D. v. virgifera* and *L. decemlineata*, resistance in *P. versicolora* was an autosomal recessive trait. While no significant differences were observed in dsRNA stability in midgut fluids from susceptible and resistant *P. versicolora*, reduced uptake of fluorescein-labeled dsRNA was detected in midgut cells of resistant compared to susceptible larvae (Liao, Zhang, and Zhang 2024).

Available data support reduced dsRNA uptake as a common mechanism of resistance to dsRNAs delivered through transgenic plants and foliar application, providing a pathway for improvement of RNAi insecticides and overcoming resistance. For example, a “paperclip” dsRNA (pcRNA) structure may use uptake mechanisms distinct from more commonly used dsRNAs (Abbasi et al. 2020a). However, the efficacy of pcRNA in overcoming resistance to dsRNA has not been reported. Diverse formulations and/or carriers may also enhance dsRNA uptake and overcome resistance (Silver, Cooper, and Zhu 2021).

1.8 Fitness costs of resistance to dsRNA

Information on the mechanisms and genes involved in resistance is vital to guide the design and improvement of the insect resistance management (IRM) framework for delaying the onset of

resistance to RNAi. Another critical aspect of IRM is the potential existence of fitness costs associated with resistance to RNAi. These fitness costs of resistance negatively impact survival rate, growth, fecundity, and/or adult longevity in resistant compared to susceptible insects in the absence of the active ingredient (Kliot and Ghanim 2012). Fitness costs can lead to a reduction in resistance allele frequency when the insecticide pressure is removed (Gassmann, Carrière, and Tabashnik 2009; Thiago Affonso and Ademir Jesus 2016). Depending on the mechanism of resistance, some fitness costs may be predicted, although pleiotropic effects can result in unanticipated effects. For instance, an altered cellular RNAi machinery in resistant insects could result in higher susceptibility to viruses, which are considered the original target of the RNAi response (Gammon and Mello 2015), or other significant fitness costs given the role of the RNAi machinery in several endogenous processes. In agreement with this prediction, the silencing of a single gene in the RNAi machinery (*Ago1*) resulted in reduced survival and oviposition rates in adult *D. v. virgifera* (Wu et al. 2017). Unfortunately, information on fitness costs in insects that are resistant to RNAi is limited to a study in a dsRNA-resistant *L. decemlineata* population when compared to two susceptible populations under laboratory conditions (Pinto et al. 2023). In that study, no differences between susceptible and RNAi-resistant strains were found in life tables and multiple biological parameters, including fecundity, fertility, larval developmental time, larval mortality, and adult longevity. This lack of relevant fitness costs suggests that once resistance alleles are selected, they may be maintained at a high frequency in field populations, even in the absence of insecticidal RNAi pressure. Consequently, appropriate IRM strategies are critically needed to effectively delay the onset of resistance.

1.9 Resistance management for dsRNA

Sustainable long-term efficacious use of any insecticidal technology depends on an efficient

IRM plan. Strategies to delay resistance against RNAi insecticides are guided by available information from dsRNA-resistant insects and lessons learned from successful integrated pest management (IPM) programs. One of the major drivers for resistance evolution in field populations is constant selection pressure with a single or multiple pesticides sharing mode of action. Thus, since so far cases of resistance to dsRNA are independent of the target gene, combinations of multiple dsRNAs targeting distinct genes would not be an effective IRM strategy. In contrast, the lack of cross-resistance in dsRNA-resistant insects to insecticidal Bt proteins (Khajuria et al. 2018; Mishra et al. 2021) and chemical (small molecule) insecticides (Mishra, Moar, and Jurat-Fuentes 2024) supports their distinct mode of action and potential use in combination or rotation to delay resistance emergence. Accordingly, Ledprona applications will be incorporated in programmed pesticide rotations as part of a sound IPM program for *L. decemlineata* control while minimizing resistance evolution (Huseth et al. 2014).

Another important consideration is the delivery of a sufficiently high dose of dsRNA in RNAi insecticides to ensure complete target insect mortality. Exposure to sub-lethal doses can promote increased frequency of minor resistance alleles and thus lead to rapid evolution of resistance. This issue can be challenging in the case of transgenic RNAi delivery, where dsRNA expression levels may be limited by crop biology or genetics. On the other hand, sublethal effects can also contribute to population control when they significantly affect viability or reproduction. For instance, exposure of fourth-instar *L. decemlineata* larvae to potato leaves treated with a low dose of Ledprona interfered with pupation and resulted in adults with reduced mobility, fertility, and fecundity (Pallis et al. 2023). In addition, the definition of a ‘high’ dose is dependent on the target insect. For instance, while coleopteran mortality can be achieved with as low as 0.1 µg of dsRNA, hemipterans, and lepidopterans require much higher doses (Cooper et al. 2019a; Terenius

et al. 2011). Thus, IRM plans need to consider both the dsRNA delivery method and target insect species.

Given the recessive mode of inheritance reported in the available cases of insect resistance to dsRNA, the refuge paradigm used for insecticidal Bt proteins in transgenic crops (Bourguet, Desquilbet, and Lemarié 2005) may also be useful to reduce the frequency of resistant alleles to dsRNA in field populations. This paradigm requires maintaining an untreated refuge of plants near treated plants to promote mating between the mostly susceptible individuals emerging from untreated plants with resistant individuals emerging from fields after exposure to dsRNA. If recessive resistance remains the case, resulting heterozygous individuals would be susceptible to the RNAi insecticide. However, low adoption rates due to economic disadvantages to farmers maintaining an effective refuge population would promote the evolution of resistance, as reported for Bt crops (Reisig 2017).

Even after an IRM plan is formed and applied, it needs continuous consideration for its effectiveness, as target field populations change over time. Having baseline susceptibility data for RNAi insecticides for different target insects would help track the evolution of resistance and allow proactive decisions to be made as the field susceptibility levels start to drop. Having information on resistance alleles and their frequency would also help estimate risks of resistance evolution and make informed decisions towards sustainable use of RNAi insecticides in different geographical locations.

1.10 Concluding remarks and prospects

Biopesticides and transgenic crops based on dsRNA as active ingredient to generate an insecticidal RNAi response are an effective and environmentally sound tool for insect control. With the expected dramatic increase in the use of these RNAi pest control technologies comes the

increased risk for the evolution of resistance. Successful laboratory selection of resistance in field-collected individuals of three insect models supports that the genetic potential for resistance exists. Further studies are urgently needed to better understand how and what type of resistance may emerge from the commercialization of RNAi insecticides. Characterization and mechanistic description of resistance provide critical information guiding IRM decisions and optimization of RNAi insecticides. Based on the currently available information, combining RNAi with other modes of action, and possibly application of the refuge paradigm should help delay the onset of resistance in field populations. Knowledge of resistance alleles and their frequency in field populations will also help direct the sustainable use of RNAi insecticides in varied geographies.

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Appendix

Figures

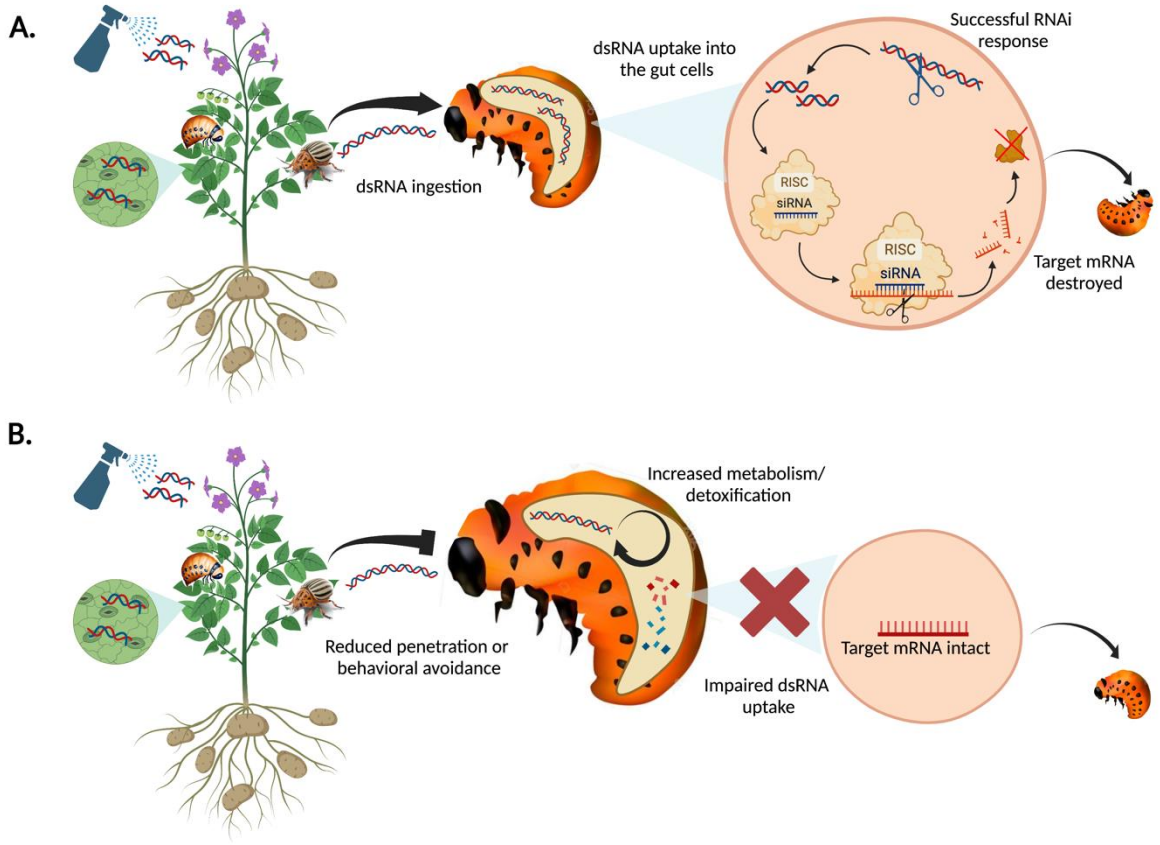


Figure 1.1- Schematic diagram of the A) mode of action of insecticidal dsRNA and B) various potential mechanisms of resistance against insecticidal dsRNA.

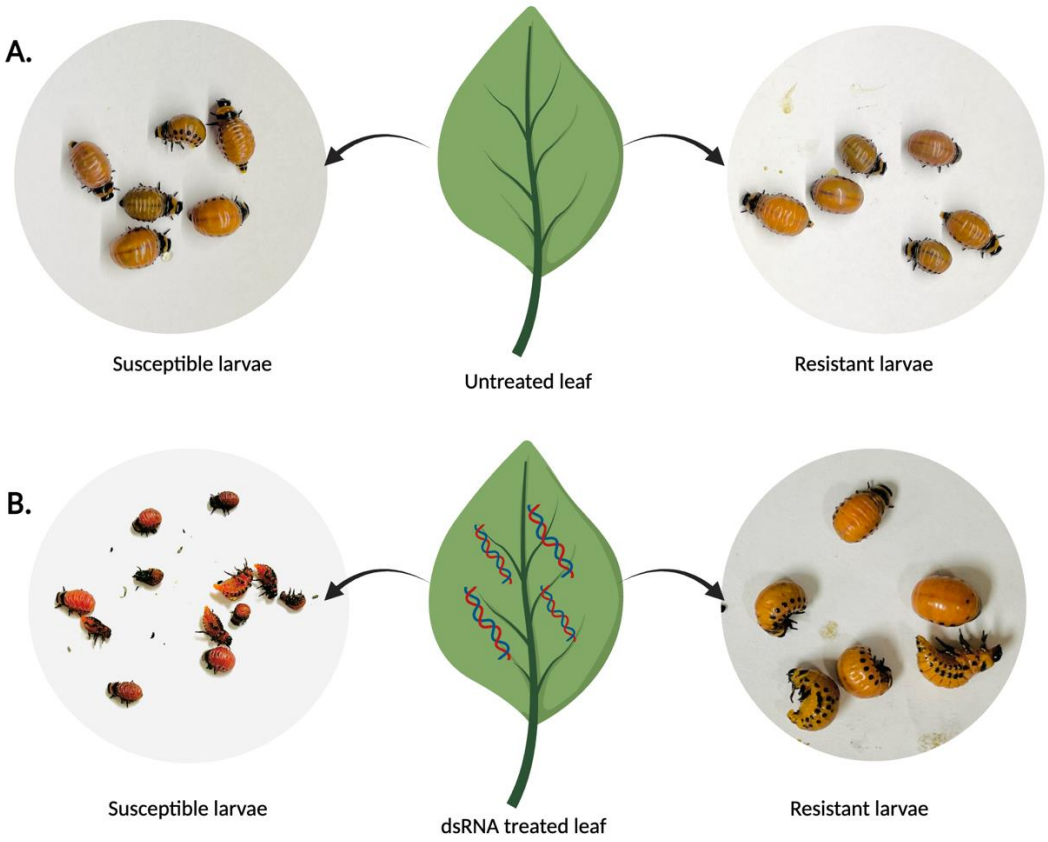


Figure 1.2- Larvae from susceptible or a dsRNA-resistant population of Colorado potato beetle (*Leptinotarsa decemlineata*) when fed on untreated potato leaves (A) or potato leaves dipped in a solution of 400 µg/ml of dsRNA (B) for 7 days.

Chapter Two

Characterization of resistance to topically delivered double-stranded RNA (dsRNA) in Colorado potato beetle (*Leptinotarsa decemlineata*)

Parts of this chapter were originally published in the following articles:

Mishra, S., W. Moar, and J. L. Jurat-Fuentes. 2024. 'Larvae of Colorado potato beetle (Leptinotarsa decemlineata Say) resistant to double-stranded RNA (dsRNA) remain susceptible to small-molecule pesticides', *Pest Manag Sci*, 80: 905-09.

Contributions:
S.M., W.M. and J.J.-F. conceived the study, S.M. performed the experiments, collected and analyzed the data, and wrote the manuscript draft; all authors contributed to data discussion and writing the final version of the article

Mishra, Swati, James Dee, William Moar, Jodi Dufner-Beattie, James Baum, Naymã Pinto Dias, Andrei Alyokhin, Aaron Buzza, Silvia I. Rondon, Mark Clough, Sandy Menasha, Russell Groves, Justin Clements, Ken Ostlie, Gary Felton, Tim Waters, William E. Snyder, and Juan Luis Jurat-Fuentes. 2021. 'Selection for high levels of resistance to double-stranded RNA (dsRNA) in Colorado potato beetle (Leptinotarsa decemlineata Say) using non-transgenic foliar delivery', *Scientific Reports*, 11: 6523.

Contributions:
S.M., J.D., W.M, J.D.-B., J.B. and J.J.-F. conceived the study; S.M., J.D., and N.P.D. performed the experiments; S.M., and J.D. collected and archived the data; S.M., J.D., W.M., and J.J.-F. analyzed the data; A.A., A.B., S.R., M.C., S.M., R.G., J.C., K.O., G.F., T.W., and W.S. collected Colorado potato beetle individuals; S.M., J.J.-F., and W.M. wrote the manuscript draft; all authors contributed to data discussion and writing the final version of the article.

Abstract

Implementation of resistance management tools is crucial for the continued efficacy of insect control technologies. An important aspect of insect resistance management (IRM) is the combined or sequential use of different modes of action to reduce selection pressure and delay the evolution of resistance. This is especially important for insect pests with an established ability to develop resistance to insecticides, such as the Colorado potato beetle (*Leptinotarsa decemlineata*, CPB). A new class of insecticides based on double-stranded RNA (dsRNA) activating the gene silencing RNA-interference (RNAi) pathway has been approved for commercialization in the US against CPB. However, there is no information available on the potential for cross-resistance between dsRNA insecticides and other classes of insecticides used against CPB. Information on the inheritance of resistance is also important in designing an IRM framework. Capitalizing on the

availability of a CPB strain highly resistant to dsRNAs, we performed bioassays testing susceptibility to diverse chemical insecticide classes, along with bioassays to determine the genetics of dsRNA resistance. Our results demonstrate a lack of cross-resistance to four chemical insecticides in the dsRNA-resistant strain of CPB. We also demonstrate that resistance is inherited as an autosomal recessive trait and is polygenic. These results support the use of these different insecticide classes along with dsRNA-based insecticides and the implementation of refuge strategy as part of an effective IRM framework aimed at delaying resistance in CPB.

2.1 Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is the most economically important defoliator of potato crops globally, also affecting other solanaceous plants such as eggplants, pepper, and tomatoes. If left unmanaged, CPB alone can cause estimated losses up to $2.5 billion (USD) (Skryabin 2010). In fact, CPB is believed to be the instigator behind the development of the modern-day pesticide industry with diverse chemical, biological, and cultural approaches developed to control this aggressive pest (Alyokhin, Rondon, and Gao 2022). Nonetheless, CPB has shown a remarkable ability to develop pesticide resistance through diverse mechanisms, including enhanced detoxification, target site insensitivity, reduced penetration, and increased excretion (Alyokhin et al. 2008). The Insecticide Resistance Action Committee (IRAC) database documents a total of 306 cases of CPB resistance to 56 commercial insecticides, including all modes of action targeting this pest (Whalon 2019). These reports exemplify the difficulty of controlling CPB and highlight the need to develop novel effective insecticides against this pest.

Insecticidal RNA interference (RNAi) is based on using a double-stranded RNA (dsRNA) molecule complementary to a portion of a vital insect gene so that silencing gene expression through the RNAi pathway results in morphological or reproductive abnormalities and/or lethality (Baum et al. 2007; Zhu et al. 2012; Xiong et al. 2013). This novel pest control technology provides important safety and environmental benefits, primarily high specificity due to sequence dependency (Bachman et al. 2016), and short environmental life of the active ingredient i.e. dsRNA molecule (Dubelman et al. 2014; Fischer et al. 2017). Consequently, there is great interest in the commercial development of RNAi-based insecticides, although high efficacy is currently limited mostly to coleopterans (Baum et al. 2007; Cooper et al. 2019; Terenius et al. 2011). The first sprayable dsRNA active Ledprona (GreenLight Biosciences) targets CPB and was approved

for commercialization by the U.S. Environmental Protection Agency (EPA) (Pallis et al. 2022; EPA 2023). Despite the high natural susceptibility of CPB to insecticidal RNAi (Zhu et al. 2011; Baum et al. 2007), laboratory selection experiments have provided evidence for the ability of CPB to develop high levels of resistance to a dsRNA delivered as a foliar application (Mishra et al. 2021). These observations highlight the high risk of resistance to dsRNA biopesticides in CPB and underline the need for effective insect resistance management (IRM) tools.

An important tool in IRM is the use of different modes of action (MOA) to reduce selection pressure from a single MOA, thus delaying resistance development. Consequently, it is important to determine cross-resistance to other commonly used MOAs against CPB. Using previously described dsRNA-resistant strain CEAS 300 (chronically exposed adults surviving 300 ug/ml concentration), we demonstrated a lack of cross-resistance to Cry3Aa insecticidal protein from *Bacillus thuringiensis* (Bt). Similar to our results, no cross-resistance was observed to Cry3Bb Bt insecticidal proteins in other dsRNA-resistant strains of western corn rootworm (*Diabrotica virgifera virgifera*) and willow leaf beetle (*Plagiodera versicolora*). These observations support the combined use of Bt insecticidal proteins with dsRNA insecticides to delay resistance onset. However, there is no available information on how resistance to dsRNA affects susceptibility to small molecule insecticides, the most commonly used control tool against CPB. In this study, we tested representative actives from four of the major insecticide classes used against CPB, which affect nerve/muscle action (Sparks et al. 2020). Insecticide mode of action groups represented among the tested actives included pyrethrins (sodium ion channel modulators), spinosyns (nicotinic acetylcholine receptor allosteric modulators), avermectins (glutamate-gated chloride channel allosteric modulators) and neonicotinoids (nicotinic acetylcholine receptor competitive modulators). It is important to consider that even though these insecticides represent different

modes of action there have been reports of cross-resistance between these pesticide classes in CPB (Mota-Sanchez et al. 2006), further highlighting the importance of testing the potential of their cross-resistance with dsRNA pesticides. In this study, we performed bioassays with four commonly used chemical insecticides against CPB including pyrethrins, neonicotinoids, spinosyns, and avermectins (Alyokhin, Rondon, and Gao 2022).

Another important aspect of IRM is the use of a refuge strategy to dilute resistance alleles in field populations in order to delay the onset of resistance. When implemented appropriately, refuge has proven very successful in delaying resistance for example against Bt crops in the US (Tabashnik, Brévault, and Carrière 2013; Tabashnik et al. 2012). However, information on the inheritance pattern of resistance is needed to determine the potential success of the refuge strategy. Additionally, whether resistance is monogenic or polygenic can provide useful insights into the resistance mechanism. In both western corn rootworm (WCR) and willow leaf beetle dsRNA-resistant strains, the inheritance of resistance was recessive and autosomal (Khajuria et al. 2018; Liao, Zhang, and Zhang 2024). Resistance in WCR linked to a single locus. Herein, we performed bioassays with larvae from F1 crosses and backcrosses to determine the inheritance of dsRNA resistance in CEAS 300. Results from this study provide critical information for the design of an IRM framework for the effective and sustainable use of dsRNA insecticides in controlling CPB.

2.2 Materials and methods

2.2.1 Insect colonies

A geographically diverse population of Colorado potato beetle (CPB, *Leptinotarsa decemlineata*), named GC for "general colony", was used for selection for resistance against a foliar application of a double-stranded RNA (dsRNA) (Mishra et al. 2021). The resulting resistant population was named CEAS 300 for "chronically exposed adult surviving 300 ug/ml of dsRNA).

Another colony generated from adults received from the University of Maine, named METT, was included as a dsRNA-susceptible reference in some experiments. All three colonies were maintained in aluminum screened cages (61 cm tall and wide, 122 cm depth) in a greenhouse bay maintained at 27-30°C, >70% RH, 18L:6D photoperiod, and fed potato plants (*Solanum tuberosum* var. desiree) *ad libitum* in 11.3-liter (3 gallons) containers. Larvae and adults of all three colonies were reared separately to avoid competition for resources between the two stages. Eggs from adult cages were transferred to larval cages and allowed to hatch and feed on potted potato plants *ad libitum.* The larvae were allowed to pupate inside the potting soil from which they emerged as adults, which were then collected and moved to the adult cages for mating and oviposition.

2.2.2 Bioassays with chemical pesticides

Commercial formulations of four different classes of insecticides were tested using a leaf dip bioassay against the three CPB colonies. Testing products included Bonide Pyrethrin garden insect spray (ai pyrethrins: 1%; PBO: 10%), Bonide Colorado potato beetle beater (ai spinosad: 0.5%), Syngenta Avid 0.15EC (ai abamectin: 2%) and Bayer Admire Pro (ai imidacloprid: 42.8%). It is important to note that pyrethrin insecticides against CPB, including the Bonide Pyrethrin product tested in this work, commonly contain piperonyl butoxide (PBO), an oxygenase inhibitor and known synergist that increases susceptibility and overcomes resistance to pyrethrin associated with enhanced detoxification (Wang et al. 2016).

Second instar CPB larvae were offered potato leaves dipped in an insecticide solution prepared with 0.1% Tween-20 as a wetting agent. Following preliminary bioassays, 5-6 insecticide solution concentrations were selected to produce a range of mortality from 10% to 100% for each insecticide for accurate LC_{50} values. Leaves dipped in a 0.1% Tween-20 solution were used as a control. A single bioassay container containing 15 larvae randomly selected from different egg

clutches was considered a biological replicate. For each insecticide concentration, five biological replicates were tested for a total of 75 larvae per concentration, a minimum of 375 larvae tested per pesticide. Larvae were allowed to feed on treated leaves for 48 hours (Dively et al. 2020) before being transferred to untreated potato leaves. Mortality was recorded on the third day since the initiation of the bioassay. Probit analysis was performed to determine LC_{50} values and LC_{50} ratios using the CEAS as the reference population and corresponding 95% confidence limits using POLO-PLUS software (LeOra Software). The probit regression lines were also tested for equality and parallelism using the POLO-PLUS software. Significant differences in LC_{50} values were estimated based on non-overlapping 95% confidence limits, as described elsewhere (Robertson JL 2007). For all the bioassays, control mortality was <15%.

2.2.3 Bioassays to determine inheritance of dsRNA resistance

Crosses were set up between adults from susceptible (GC and METT) and dsRNA-resistant (CEAS 300) strains of CPB to determine the dominance of inheritance of resistance to dsRNA. Pupae from each strain were sexed (Pelletier 1993) and kept separated by gender in 2-liter plastic containers. When adults emerged, they were fed potato leaves until they became sexually mature (approximately two weeks). Crosses used ten adults of each sex from each parental strain, and they were placed in a bug dorm (BioQuip Products Inc., Compton, CA) with a single potato plant in a 11-liter pot for feeding and oviposition. Plants were changed as needed after 3 to 5 days. Eggs were harvested after three days and used for bioassays as described above but utilizing a single discriminatory dsRNA concentration of 800 µg/ml that killed 100% of susceptible larvae but did not affect larvae from the CEAS 300 strain. The bioassay protocols were as described above.

Abbott's formula was used to calculate corrected mortality when control mortality was greater than 5% (Abbott 1925). Results from these bioassays (Table 2.2) were used to estimate the

degree of dominance (*h*), using the single-concentration method, as described elsewhere(Liu and Tabashnik 1997). According to this method, dominance (*h*) is estimated as $h = (w_{12} - w_{22})/ (w_{11} - w_{22})$, where w_{11}, w_{12}, and w_{22} are the fitness of resistant homozygotes, heterozygotes, and susceptible homozygotes, respectively, at the tested dsRNA concentration. The value of *h* ranges from 0 for completely recessive to 1 for completely dominant. The fitness of treated resistant homozygotes was considered as 1, while the fitness of susceptible homozygotes and heterozygotes was estimated by dividing their survival rate by the survival rate of resistant homozygotes. The survival rate was estimated as (100% - mortality %) at each dsRNA concentration.

To determine the number of genes involved with dsRNA resistance, two sets of backcrosses were conducted by mating adults from the F1 generation resulting from CEAS 300 x METT crosses with male or female beetles from the susceptible (METT, BC1 backcross), or female beetles from resistant (CEAS 300, BC2 backcross) parental strains. Larvae from both backcrosses were exposed to 800 µg/ml of dsRNA targeting the *V-ATPase subunit A transcript*, using the bioassay protocols described above. Results of these bioassays (Table 2.2) were used to estimate the number of genes associated with resistance. A chi-square (χ^2) goodness of fit was performed to test the null hypothesis of monogenic resistance, following the methods of Sokal and Rohlf (Sokal RR 1981). The theoretical value of $\sum\chi^2$ was calculated as $\chi^2 = (N_i - pn_i)^2 / pqn_i$, where N_i is the observed number of deaths in the backcross generation at the given dsRNA concentration, *p* is the expected mortality in the backcross generation estimated as described by Georghiou (Georghiou 1969), and $q = 1-p$. The null hypothesis (monogenic resistance) was rejected if the calculated value of $\sum\chi^2$ was greater than the table value of $\sum\chi^2$ at the 0.05 significance level and 1 degree of freedom.

2.3 Results

2.3.1 Cross-resistance to chemical pesticides

All the chemical pesticides tested were highly active against both dsRNA susceptible (GC and METT) and resistant CPB strains, though the level of activity among different groups varied. Of all, abamectin demonstrated highest activity against the CPB larvae from all three colonies while pyrethrin+PBO activity was the lowest as evident from their LC_{50} values (Table 2.1). Though not statistically significant, dsRNA susceptible METT larvae demonstrated lowest susceptibility to these chemical pesticides. Importantly, no significant differences were observed in the LC_{50} estimates among the dsRNA susceptible and dsRNA resistant CPB colonies for any of the pesticide group tested as apparent by overlapping 95% fiducial limits as well as LC_{50} ratios. Additionally, the probit regression lines for abamectin, spinosad and pyrethrin for all three populations had no significant differences in slope or intercept suggesting no qualitative or quantitative differences in detoxification enzymes. However, for imidacloprid, the probit regression lines for the three populations were parallel but not equal, evidence of equal slope but significantly different intercept

For pyrethrin+PBO, the lowest concentration produced 36.1% mortality in GC and METT, and 38.3% mortality in CEAS 300 larvae respectively, while the mortality due to highest concentration was 100% for all three strains (Fig. 2.1). Based on these results, the LC_{50} was estimated to be 1.8 ppm (1.2- 2.5) and 1.9 ppm (1- 2.6) for dsRNA susceptible colonies GC and METT respectively, while it was 1.7 ppm (1.1- 2.3) for CEAS 300. The overlapping LC_{50} fiducial limits suggest no significant differences in larval susceptibility to pyrethrin+PBO in the three CPB strains.

Similar to pyrethrin+PBO, the highest spinosad concentration caused 100% mortality for all three CPB strains while the mortality due to the lowest concertation was 21.7%, 20% and 14.7% in GC, METT and CEAS 300 larvae (Fig. 2.2). Subsequently, the LC_{50} was estimated to be 0.003% (0.002- 0.004) for GC, 0.004% (0.003- 0.005) for METT and 0.003% (0.002- 0.004) for CEAS 300 colony (Table 2.1). Again, no significant differences were observed in the larval response to any of the spinosad concentrations tested or the LC_{50} estimates among the three colonies (One-way ANOVA, p=0.9).

Abamectin was found to be the most active pesticide against CPB larvae with the lowest LC_{50} estimates among all pesticide groups tested (Table 2.1). The range of concentrations tested induced mortality covering 32% to 100% (Fig. 2.3). For dsRNA susceptible colonies GC and METT, the LC_{50} was estimated to be 0.03 ppm (0.02- 0.05) and 0.04 (0.02-0.07) ppm respectively while it was 0.03 ppm (0.02- 0.06) for the dsRNA resistant colony CEAS 300, though it was not significantly different from that of GC or METT (Table 2.1). Furthermore, no statistical differences in mortality were observed at any of the concentrations tested (One-way ANOVA, p=0.9).

As with other pesticides, the highest concentration of Imidacloprid tested also caused 100% larval mortality for all three strains (Fig. 2.4). The LC_{50} estimates were 0.76 ppm (0.49- 0.98), 1.49 ppm (1.09- 1.88), and 0.96 ppm (0.39- 1.49) for GC, METT, and CEAS 300 respectively. As evident from overlapping 95% fiducial limits, no significant differences were found in larval susceptibility from the dsRNA susceptible and dsRNA resistant colonies (One-way ANOVA, p=0.8).

2.3.2 Genetics of dsRNA resistance in CEAS 300

Individuals from the F1 generation from crossing GC x CEAS 300 were used in bioassays testing the transmission of the resistance trait. There were no significant differences in susceptibility depending on the gender of the resistant parent in the cross (P = 0.208, t-test), supporting that resistance was not sex-linked or maternally inherited (Table 2.2). Consequently, in further analyses, data from crosses in which male or female CEAS 300 beetles were used as parents, were pooled. Notably, we observed lower (56.6%) than expected (100%) susceptibility in replicated tests with offspring from GC x GC crosses (Table 2.2), which we interpreted as potential contamination of the GC strain with CEAS 300 individuals. Therefore, we included tests with METT as a reference susceptible strain and confirmed that as observed for GC, resistance was not dependent on the gender of the resistant parent in the cross (P = 0.177, t-test), supporting autosomal resistance.

The value of dominance (*h*) was calculated to determine if resistance was transmitted as a recessive (*h* = 0), incompletely recessive (*h* between 0 and 0.5), or dominant (*h* = 1) trait. The estimated degree of dominance for offspring from the GC x CEAS 300 crosses was *h* = 0.37, while for the METT x CEAS 300 it was *h* = 0.06. The *h* value from GC x CEAS 300 was probably influenced by the lower susceptibility to the dsRNA detected in the GC strain, which could be due to contamination with CEAS 300 individuals. However, results from METT x CEAS 300 agreed with those from GC X CEAS 300 crosses and supported the conclusion that resistance is transmitted as an incompletely recessive trait. Based on these observations, only the METT strain was used in subsequent backcrossing experiments to determine the number of loci involved.

Backcrosses of the F1 generation from METT x CEAS 300 crosses ($F1_{METTxCEAS300}$) to the susceptible METT (BC1 backcross) or resistant CEAS 300 (BC2 backcross) strains were used in

testing for monogenic versus polygenic resistance. Survival of larvae after exposure to dsRNA was used in the Chi-square goodness-of-fit model with monogenic resistance as the null hypothesis. Significant differences ($P < 0.05$) were detected between the observed and expected mortalities of both backcrosses. The estimated $\sum\chi^2$ value for BC1 was 415.6, while for BC2 it was 9.09, both of which are significantly higher than the tabulated χ^2 value of 3.84 at the 0.05 significance level and 1 degree of freedom. These results support that resistance to dsRNA in CEAS 300 involves multiple loci.

2.4 Discussion

Commercial development of effective and sustainable pesticides requires a relevant investment of labor and funds; therefore, the efficacy of new control methods such as dsRNA pesticides should be protected by rigorous implementation of IRM frameworks that rely heavily on integrated pest management (IPM) principles. One of the main principles in delaying resistance evolution is the combined use of different modes of action. Although the mode of action of dsRNA pesticides differs from chemical insecticides tested in this study, some common immune or stress-related responses activated upon exposure to noxious compounds could affect all of them. In CPB, these stress and enhanced detoxification responses are commonly observed in resistance to insecticides (Clements et al. 2018). While the mechanisms involved in resistance to dsRNA in CEAS 300 remain unknown, enhanced immunity and/or stress responses could potentially affect susceptibility to chemical insecticides. These mechanisms can be especially consequential in IPM situations where different modes of action are used with the expectation of no cross-resistance. In addition, survival provided by these mechanisms can also result in accelerated evolution of resistance, which needs consideration when designing IRM frameworks. Previous publications provided data supporting the combined use of dsRNA pesticides and Bt insecticidal proteins (Liao

2024) (Mishra et al. 2021; Khajuria et al. 2018). When comparing susceptibility to tested chemical insecticides between susceptible and dsRNA-resistant CPB strains, we found no significant differences in slope or intercept, clearly supporting the lack of cross-resistance. Although the relevance for field control and frequency of the resistance mechanisms observed in CEAS 300 in field CPB populations needs further testing, data from this study supports the combined use of the tested chemical insecticide products with insecticidal dsRNA in controlling and reducing risks of resistance in CPB.

The use of refuge strategy is another highly effective component of an IRM framework. Similar to WCR and willow leaf beetle dsRNA resistant strains, resistance in CEAS 300 is inherited as a recessive and autosomal trait. Thus, the use of refuge is expected to be effective in delaying resistance against dsRNA pesticides in field insect populations. While dsRNA resistance in the WCR strain is linked to a single locus, resistance in CEAS 300 was determined to be polygenic. This observation is expected from a laboratory-selected strain exposed to moderate (usually LC_{50}) and subsequently increasing doses of insecticide. This usually results in the selection of multiple mechanisms each with a small effect. Even though resistance selected in fields is expected to be controlled by a single major gene due to high selection pressure (more than LC_{99}), population genomic studies in CPB support polygenic nature of insecticide resistance possibly due to the long history of insecticide control and resistance development. The possibility of polygenic resistance against dsRNA insecticides arising in field CPB populations further highlights the importance of the design and implementation of a sound IRM framework. Through this study, we provide crucial information that would inform strategies to delay resistance and ensure effective long-term use of dsRNA pesticides.

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Appendix

Tables and Figures

Table 2.1- Concentration-response bioassays of three Colorado potato beetle colonies (GC, METT and CEAS 300) against small molecule pesticides in potato leaf bioassays. Shown are the lethal concentrations (amount of active ingredient expressed in ppm) killing 50% of the individuals in the population (LC_{50}) and corresponding 95% fiducial limits from probit estimates. Also shown are probit fitting parameters (slope and χ^2 values) and susceptibility ratios.

Chemical pesticide	**Colony**	**LC_{50} (95% FL)**	**Slope ± SE**	**χ^2 (df)**	**Ratios (95% FL)**
Pyrethrin + PBO	GC	1.8 (1.2- 2.5)	2.37 ± 0.31	4.91 (3)	0.97 (0.74- 1.28)
	METT	1.9 (1- 2.6)	2.175 ± 0.26	8.17 (3)	0.95 (0.7- 1.29)
	CEAS 300	1.7 (1.1- 2.3)	2.46 ± 0.32	3.87 (3)	1
Spinosad	GC	0.15 (0.1- 0.2)	1.91 ± 0.22	3.57 (3)	1.07 (0.82- 1.41)
	METT	0.2 (0.15- 0.25)	2.07 ± 0.26	2.54 (3)	0.91 (0.70- 1.18)
	CEAS 300	0.15 (0.1- 0.2)	2.39 ± 0.28	0.34 (3)	1
Abamectin	GC	0.0006 (0.0004- 0.001)	1.01 ± 0.15	1.18 (3)	1.01 (0.58- 1.76)
	METT	0.0008 (0.0004- 0.0014)	0.86 ± 0.14	1.96 (3)	0.79 (0.45- 1.39)
	CEAS 300	0.0006 (0.0004- 0.0012)	0.76 ± 0.14	1.50 (3)	1
Imidacloprid	GC	0.33 (0.21- 0.42)	2.74 ± 0.45	1.32 (3)	1.27 (0.81- 1.98)
	METT	0.64 (0.47- 0.80)	2.46 ± 0.29	2.49 (3)	0.65 (0.44- 0.96)
	CEAS 300	0.41 (0.17- 0.64)	2.18 ± 0.28	3.64 (3)	1

Table 2.2- Percentage mortality observed in progenies from different crosses when exposed to 800 µg/ml of V-ATPase subunit A dsRNA in 20-day leaf-feeding bioassays. These mortalities were used to determine the genetics of resistance in CEAS 300.

Test	N[a]	Corrected mortality (%) ± SE
F1 bioassay 1 - GC and CEAS 300		
GC (♀) X GC (♂)	4	56.6 ± 10.0
GC (♀) X CEAS 300 (♂)	4	31.7 ± 5.7
CEAS 300 (♀) X GC (♂)	4	40.0 ± 7.7
CEAS 300 (♀) X CEAS 300 (♂)	4	1.7 ± 1.7
F1 bioassay 2 – METT and CEAS 300		
METT (♀) X METT (♂)	8	100.0 ± 0.0
METT (♀) X CEAS 300 (♂)	4	95.6 ± 3.3
CEAS 300 (♀) X METT (♂)	8	94.0 ± 2.1
CEAS 300 (♀) X CEAS 300 (♂)	8	7.7 ± 1.7
Backcross bioassay – METT, CEAS 300 and F1		
METT (♀) X METT (♂)	8	98.0 ± 1.7
METT (♀) X F1[b] (♂)	8	73.7 ± 7.2
F1 (♀) X METT (♂)	4	96.4 ± 1.9
CEAS 300 (♀) X F1 (♂)	4	36.6 ± 4.3
CEAS 300 (♀) X CEAS 300 (♂)	4	13.3 ± 9.4

[a]Number of biological replicates performed, each consisting of 15 randomly selected larvae.
[b]F1 generation resulting from crossing METT and CEAS 300.

Figures

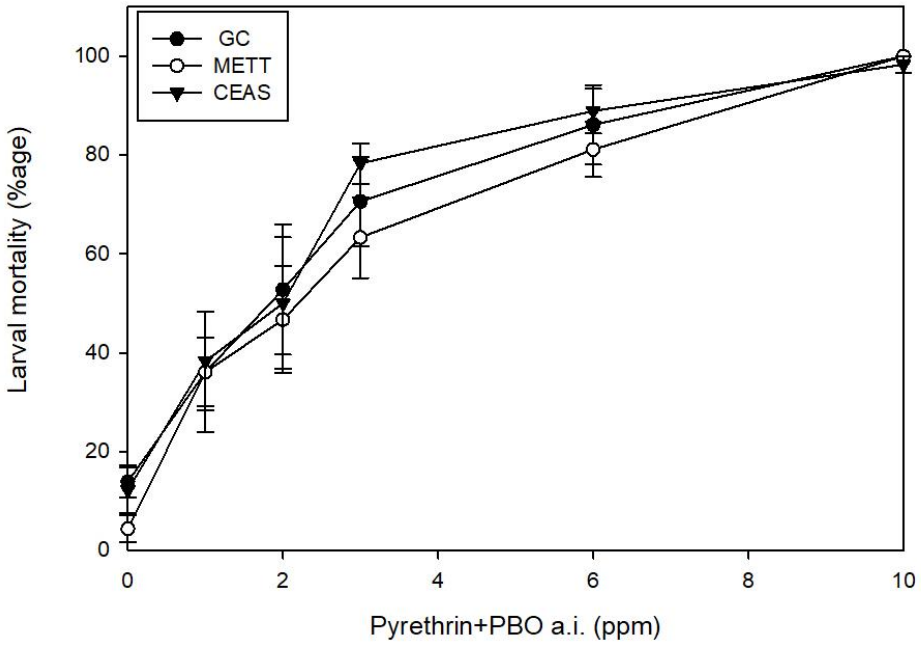


Figure 2.1- Concentration-response curves of GC, METT, and CEAS 300 larvae when exposed to increasing amounts of active ingredient pyrethrin+ PBO (ppm)

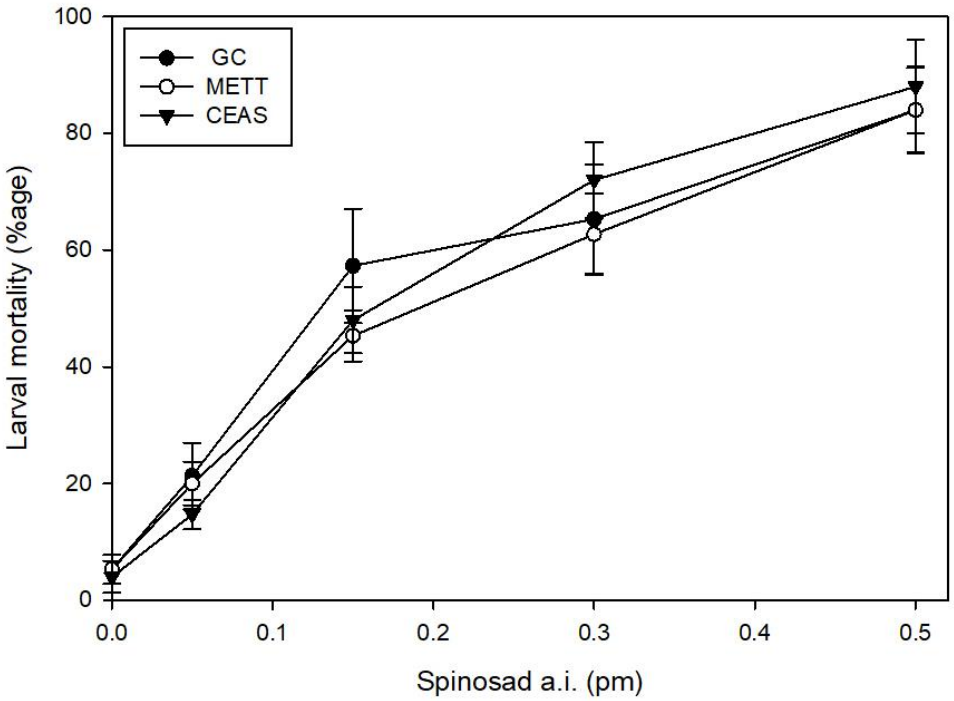


Figure 2.2- Concentration-response curves of GC, METT, and CEAS 300 larvae when exposed to increasing amounts of active ingredient spinosad (ppm).

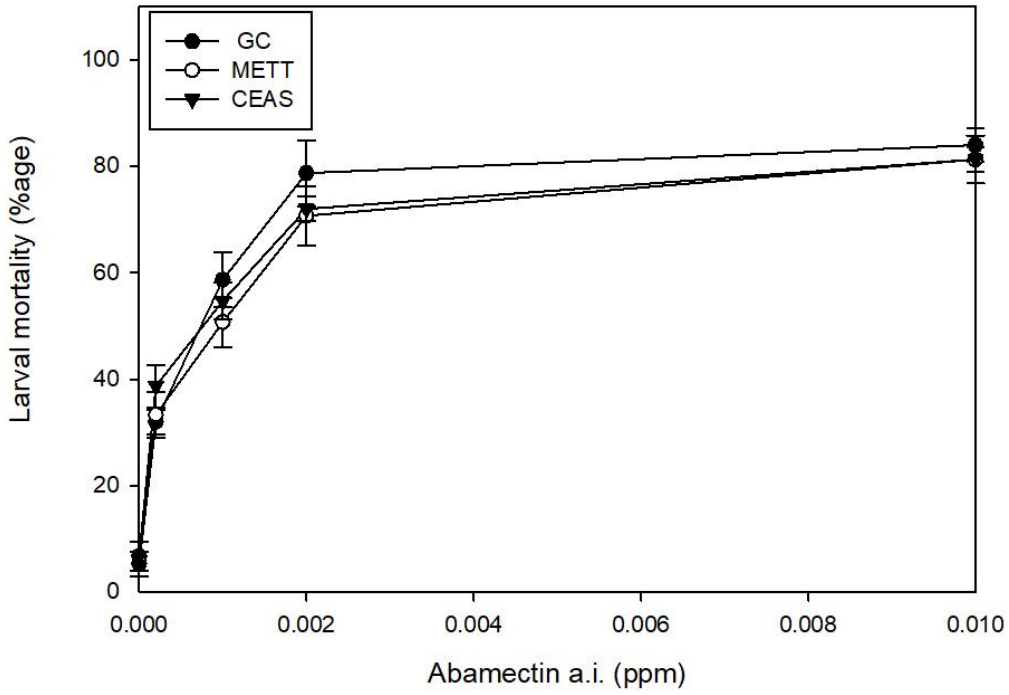


Figure 2.3- Concentration-response curves of GC, METT, and CEAS 300 larvae when exposed to increasing amounts of active ingredient abamectin (ppm).

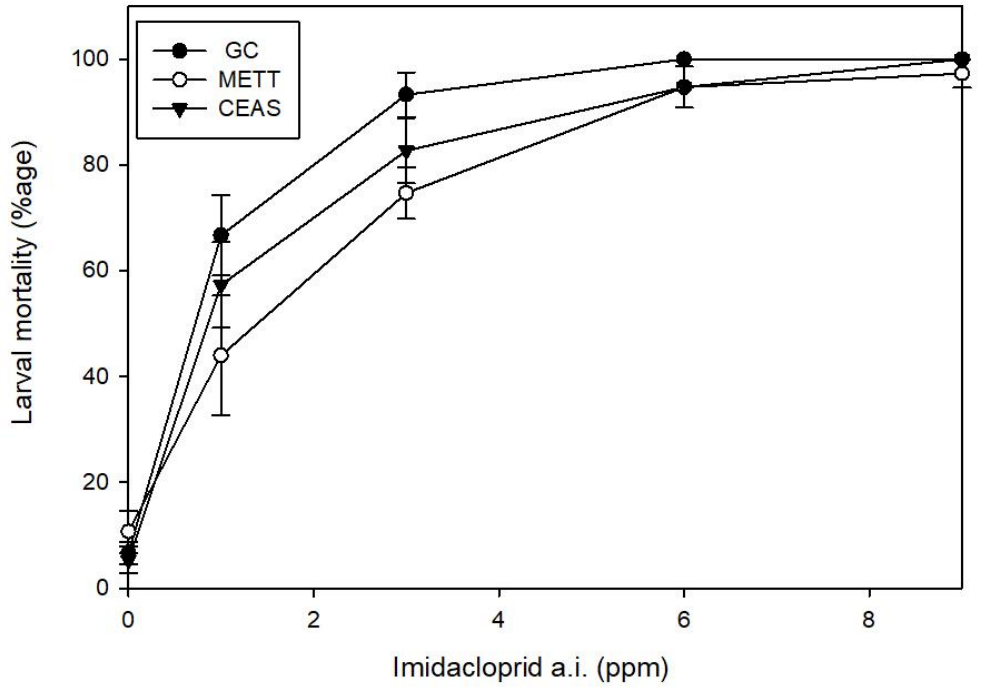


Figure 2.4- Concentration-response curves of GC, METT, and CEAS 300 larvae when exposed to increasing amounts of active ingredient imidacloprid (ppm).

Chapter Three

Mechanistic description of resistance to topically applied double-stranded RNA (dsRNA) in a Colorado potato beetle (*Leptinotarsa decemlineata* Say*)* population

Abstract

Double-stranded RNA (dsRNA) based insecticides are the latest tools for insect control, functioning by sequence-specific silencing of target insect genes via the RNA-interference (RNAi) pathway. Understanding resistance mechanisms that insects might develop against commercial dsRNA-based insecticides is crucial to ensure their long-term efficacy. Colorado potato beetle (CPB, *Leptinotarsa decemlineata* Say) is the target of the first commercial dsRNA foliar spray making it an important model to study resistance. We previously described selection from a field-derived population of a highly resistant population of CPB, CEAS 300, using foliar delivery of a dsRNA targeting the *V-ATPase subunit A* gene. The current study aims to elucidate the mechanism of resistance in this dsRNA-resistant CPB population. We demonstrate no differences in dsRNA stability in larval gut fluids from two susceptible and the CEAS 300 population. Reduced uptake of dsRNA in the CEAS 300 strain was detected when monitoring the uptake of dsRNA by midgut cells using confocal microscopy and by reduced accumulation of small RNAs (sRNAs), as well as differential expression of genes involved in the uptake of dsRNA between susceptible and resistant larvae. Additionally, genes previously implicated in resistance to chemical pesticides in CPB were differentially expressed in CEAS 300. Taken together, these results support reduced uptake of dsRNA as a potential resistance mechanism, as also observed for two other chrysomelid species, but also introduce a potential additional mechanism specific to CPB. These results further increase our understanding of dsRNA resistance mechanisms and will allow the development of strategies that can overcome resistance. Identification of resistance-associated genes can also be used to monitor resistance alleles in field populations of CPB.

3.1 Introduction

Insect control using double-stranded RNAs (dsRNAs) has gained considerable popularity over the last decade (Baum et al. 2007; Vélez et al. 2023; Mishra and Jurat-Fuentes 2022). These dsRNAs activate the RNA interference (RNAi) pathway resulting in the target messenger RNA (mRNA) complementary to the dsRNA being degraded, thereby silencing target gene expression (Fire et al. 1998). These pesticides provide a novel mode of action for insect control and have the potential to overcome resistance issues with current insect control technologies such as chemical pesticides and insecticidal Cry and Vip proteins derived from *Bacillus thuringiensis* (Bt). Since the activity of dsRNAs requires sequence complementarity with the target mRNA, these insecticides are one of the most specific modes of action, thus ensuring no harmful effects to non-target organisms and beneficial insects (Bachman et al. 2016; Bachman et al. 2013). The short half-life of dsRNA molecules also reduces the environmental risks of runoff to water sources, bioaccumulation, and biomagnification, making them one of the most sustainable control methods against destructive agricultural pests (Fischer et al. 2017; Dubelman et al. 2014).

The effectiveness of these dsRNA-based pesticides is evident from the high interest in the development of commercial products based on this technology (Head et al. 2017; Rodrigues et al. 2021), although efficacy varies among insect orders and is high in Coleoptera (Cooper et al. 2019). Nevertheless, both transgenic and foliar spray-based commercial products have been developed. The SmartStax Pro transgenic maize targeting rootworms (*Diabrotica* sp) expresses a dsRNA targeting the *sucrose non-fermenting 7* (*Snf7)* gene in addition to two cry proteins from Bt and was approved for commercialization by the Environmental Protection Agency (EPA) (Head et al. 2017). A foliar spray containing Ledprona, a dsRNA targeting the *Proteasome Subunit Beta Type-5* (*PSMB5*) gene in Colorado potato beetle, has also been approved by the EPA in early December

2023 (EPA 2023). These dsRNA pesticides provide a new tool at a time when resistance cases against current control technologies in agricultural pests are increasing. However, dsRNA-based insecticidal technologies are not impervious to resistance themselves. Importantly, our knowledge of the resistance mechanisms that different insect species may develop against these pesticides is limited.

Multiple mechanisms that could lead to resistance against dsRNA insecticides have been postulated, yet there are only a few empirical examples of resistance development. In the corn rootworm (*Diabrotica virgifera virgifera*), selection on transgenic corn plants expressing a dsRNA resulted in resistance (≥130-fold) that was recessive, autosomal, independent of the dsRNA sequence, and linked to a single genetic locus (Khajuria et al. 2018). Importantly, resistance was associated with impaired dsRNA uptake inside the gut cells, an important step for the successful initiation of the RNA interference (RNAi) pathway. To compare how a different insect species and a different dsRNA delivery method might affect resistance development, in 2021 our group described the selection of high levels of resistance (>11,100-fold) in a strain of CPB named CEAS 300 (Chronically Exposed Adult Survivors from exposure to 300 ug/ml of dsRNA) using non-transgenic foliar-based delivery of dsRNA (Mishra et al. 2021). Resistance in this case was also recessive, autosomal, and independent of the dsRNA sequence. Additionally, we found evidence for polygenic inheritance of resistance, suggesting plausible multiple resistance mechanisms within the same population. Recently, another study reported the selection of resistance to a dsRNA in willow leaf beetle (*Plagiodera versicolora*) using foliar dsRNA delivery, with resistance being recessive, autosomal, and independent of the dsRNA sequence (Liao, Zhang, and Zhang 2024). Whether resistance is monogenic or polygenic in this case is not known. Confocal microscopy images support the lack of dsRNA uptake associated with resistance in that population.

These results provide important insights into the similarities and differences in resistance against dsRNA insecticides based on the method of dsRNA delivery and the insect model used for selection.

In this chapter, we perform a mechanistic investigation into dsRNA resistance in the CEAS 300 population. Since resistance is independent of the dsRNA sequence (Mishra et al. 2021), target site mutations were not explored as a mechanism involved in resistance. We found no differences in the stability of a dsRNA in the gut fluids from susceptible and CEAS 300 larvae, suggesting no role of gut nucleases in the resistance mechanism. Upon testing the uptake of dsRNAs by microscopy and sequencing of small RNAs, we observed reduced uptake inside the midgut cells of resistant compared to susceptible larvae. Sequencing of selected RNA transcripts for genes implicated in dsRNA uptake and transport, and resistance to chemical insecticides in CPB. The differentially expressed genes were further validated by performing quantitative PCR (qPCR). Results provide insights into the resistance mechanisms that CPB populations might develop in response to the use of dsRNA-based insecticides.

3.2 Materials and Methods

3.2.1 Insect colonies

Insects from three previously described CPB populations were used (Mishra et al. 2021). General colony (GC) and Maine tween tolerant (METT) strains are susceptible to dsRNA and are used as references. The dsRNA-resistant population CEAS 300 (chronically exposed adult survivors) was selected using dsRNA targeting the *V-ATPase subunit A* gene and exhibits >11,100-fold resistance compared to the parental colony (GC). All three colonies were maintained as described elsewhere (Mishra et al. 2021).

3.2.2 Nuclease assays

Eggs from susceptible (METT and GC) and resistant (CEAS 300) CPB colonies were collected from the greenhouse. Hatched larvae were reared on untreated potato leaves till 3rd instar. Digestive fluids were obtained from five 3rd instar larvae by dissecting the gut or by gently pressing the sides of the abdomen using blunt forceps to obtain regurgitate. Guts were homogenized in 20 µl of Ringer's solution (150 mM NaCl, 1 mM $CaCl_2$, 10 mM KCl, 40 mM $MgCl_2$, 40 mM $NaHCO_3$, 90 mM sucrose, pH 7.2). Gut debris was separated from fluid contents by centrifugation at 15,000 rpm for 5 min. Digestive fluid samples were quantified using the Qubit fluorometer (Invitrogen) and diluted at 1 mg/ml (stock) in Ringer's solution. The 1 mg/ml stock was used to prepare 10X and 50X dilutions using Ringer's solution.

Digestive fluid samples were incubated with 500 ng of a dsRNA targeting the V-ATPase subunit A gene (a generous gift from Greenlight Biosciences). After 1h at room temperature (25ºC) digestion of the dsRNA was monitored by 1% agarose gel electrophoresis.

3.2.3 Confocal microscopy

Confocal microscopy was used to visualize dsRNA inside midgut cells. Mouse anti-dsRNA monoclonal antibody J2 (SCICONS™) was used to detect dsRNA in midgut histological sections. Larvae from susceptible (METT) and resistant (CEAS 300) CPB colonies were reared on untreated potato leaves until 3rd instar. Larvae were starved for 24 hours followed by drop feeding 25 µg of V-ATPase subunit A dsRNA prepared in 1 mg/ml sucrose to promote consumption. Each larva was closely monitored to ensure ingestion. After 6 hours, guts were dissected and fixed in 4% paraformaldehyde overnight and then sent for sectioning at the Histology Department at the University of Tennessee College of Veterinary Medicine (Knoxville, TN). Prepared sections (4 µm thick) were first rehydrated following a series of washes (10 min in xylol, then 5 min each in

100% ethanol, 70% ethanol, and water). Sections were then permeabilized in 0.1% Triton X-100 in 1X PBS for 5 mins, followed by heat-induced antigen retrieval in 1mM EDTA at 95°C for 20 min (Dhir et al. 2018). After cooling for 20 mins sections were incubated in blocking buffer (8% BSA in 1X PBS) at 4°C overnight. Subsequently, sections were probed with mouse anti-dsRNA J2 antibody (SCICONS^{TM}) diluted 1:200 in blocking buffer, for 2 h at room temperature. After washing 5 times for 10 mins each in 0.1% Triton X-100 in 1X PBS, sections were incubated with goat anti-mouse Alexa Fluor^{TM} 594 antibody (ThermoFisher) for 1 h at room temperature. Sections were then washed as before mounting with Fluoromount-G^{TM} Mounting medium with DAPI (Invitrogen). Some slides were prepared in the absence of primary antibody to ensure the specificity of secondary antibody. Sections were observed under a confocal microscope (Leica SP8 White Light Laser Confocal System) at the Advanced Microscopy and Imaging Center (University of Tennessee, Knoxville).

3.2.4 Small RNA (sRNA) sequencing and analysis

Third instar susceptible (GC, METT) and resistant (CEAS 300) larvae were fed potato leaves treated with 800 µg/ml V-ATPase A dsRNA solution prepared in 0.1% Tween-20. Larvae fed on leaves dipped in 0.1% Tween-20 were used as an untreated control. After 48 hours, insect guts were dissected, snap-frozen in liquid nitrogen, and stored at -80ºC until further use. Each biological replicate consisted of a pool of 3 guts, 3 biological replicates were collected for untreated samples, and 5 replicates for dsRNA-treated samples. RNA was extracted using the RNAeasy Mini kit (Qiagen) and sent for small RNA (sRNA) library preparation and sequencing at Novogene (Sacramento, CA).

Pre-processed reads (trimmed and adapter removed) received from Novogene were mapped to the full-length CPB *V-ATPase subunit A* transcript (XM_023156517) using the

ShortStack software (Axtell 2013). The number of 20-25 bp long sRNAs expressed as reads per million (rpm) were compared for each sample. In the untreated samples of GC and CEAS a high amount of sRNAs mapped to the *V-ATPase subunit A* transcript, possibly due to housekeeping regulation of gene expression. To account for that background, a fold increase in the abundance of mapped sRNAs was calculated for each population by dividing the number of mapped sRNAs in treated samples by that in untreated samples.

3.2.5 Targeted RNA sequencing

To prepare samples for targeted RNA sequencing, larvae from susceptible (GC or METT) and resistant (CEAS 300) CPB populations were reared on untreated potato leaves till third instar. Early fourth instar larvae were starved overnight, and the following day fed a 5 µl droplet of a 1 mg/ml sucrose solution containing 25 µg of dsRNA targeting the *V-ATPase subunit A* gene. Larvae were closely monitored to ensure they completely consumed the droplet, and then they were fed on untreated potato leaves until gut dissection at 48h post-drop-feeding. A pool of 3 dissected guts was considered one biological replicate and there were 3 biological replicates per treatment. Total RNA was isolated from guts using the RNeasy Mini kit (Qiagen) according to the manufacturer's specifications. The PrimeScript 1st strand cDNA Synthesis Kit (Takara) was used to prepare cDNA from 2 µg of total RNA, following the manufacturer's instructions. Samples were then submitted to Floodlight Genomics LLC (Knoxville, TN) for targeted sequencing (Clements et al. 2021). The panel of sequencing targets included genes involved in the uptake of dsRNA in CPB (Schoville et al. 2018) and genes with overexpression in CPB populations resistant to chemical pesticides (Clements et al. 2021). Raw reads received were aligned to targeted sequences using CLC Genomics Workbench version 12 (QIAGEN) to get the number of mapped reads. Count data was used for expression analysis using DESeq2 software (Love, Huber, and Anders 2014). Genes

with an adjusted p-value <0.05 were considered significantly differentially expressed within each comparison.

3.2.6 Quantitative PCR (qPCR)

A different set of gut samples from larvae treated the same way as described for targeted sequencing were used for extracting RNA and preparing cDNA as described above. Quantitative PCR reactions (total volume of 10 µl) included 2 µl of 10 × diluted cDNA, 5 µl of SYBR green master mix (Thermofisher), 0.4 µM of each primer, and 3 µl of nuclease-free water. The thermocycler conditions included one denaturation cycle at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 s, and annealing/extension at 60°C for 30s. All the reactions were performed in technical triplicates. Three housekeeping genes *RP10*, *Arf1,* and *Arf4* were used for normalization to estimate the relative transcript levels (Mehlhorn et al. 2020). Primer efficiencies were calculated using a four-point ten-fold dilution series. The geometric mean of transcript levels of the three housekeeping genes was used as an internal control to compare the transcript levels of genes of interest. Transcript levels in CEAS 300 were expressed relative to untreated METT or GC. Mean transcript levels and corresponding standard errors were calculated from three independent biological replicates.

3.2.7 Bioassays with piperonyl butoxide (PBO) and dsRNA

Piperonyl butoxide (PBO) (Exponent Insecticide Synergist, MGK) was used as an inhibitor of cytochrome P450 oxygenase (Silcox, Ghidiu, and Forgash 1985) in topical leaf bioassays. A pyrethroid pesticide active against CPB, Black Flag Extreme Home Insect Control concentrate (a.i. deltamethrin 0.32%), was included as a control for PBO activity. For susceptible insects (GC and METT populations) V-ATPase subunit A dsRNA was used at the concentration of 0.18 µg/ml

(LC_{50} for GC population) while for CEAS 300 larvae, a concentration of 2000 µg/ml was used. All solutions were prepared in 0.1% Tween-20 to ensure uniform coating of potato leaves.

Second instar larvae, selected from different egg batches to ensure genetic variability, were allowed to continuously feed on treated leaves until pupation, with fresh leaves added every 24 hours. Mortality in treatments with deltamethrin was assessed after 2 days. Four biological replicates each consisting of 15 larvae were included for each treatment. Control mortality consistently remained below 15%. Significant differences among treatments within each population were determined using Kruskall-Wallis ANOVA on Ranks followed by Dunnett's pairwise multiple comparisons to the control group (p-value <0.05).

3.3 Results

3.3.1 Double-stranded RNA (dsRNA) stability

Upon incubation of CPB regurgitate or gut fluids with dsRNA for 1 hour, degradation of dsRNA was observed in stock concentrations (1 mg/ml) of regurgitate but not in gut fluid samples (Fig. 3.1). The dsRNA was stable at 10X and 50X dilutions of both regurgitate and dissected gut fluids (Fig.3.1). No differences in dsRNA stability were observed in samples from the susceptible (METT and GC) and resistant (CEAS 300) populations tested.

3.3.2 Monitoring uptake of dsRNA inside the larval midgut cells

Uptake of dsRNA was assessed using fluorescence immunohistochemistry. The midgut cell nuclei were localized by the blue signal from DAPI, whereas the dsRNA was detected by a red signal from the secondary antibody (Fig. 3.2). In the gut sections from larvae treated only with sucrose solution, no dsRNA signal was observed. Gut sections from the METT population had dsRNA localized around the midgut cell nuclei, supporting the uptake of dsRNA inside these cells.

In contrast, gut samples from the CEAS 300 larvae showed very low dsRNA signal inside midgut cells.

3.3.3 Accumulation of small RNAs (sRNAs) inside gut cells

Small RNA (sRNA) sequencing in untreated samples detected a lower number of 20-25 bp long sRNAs mapping to the *V-ATPase subunit A* transcript when compared to treated samples. In GC and CEAS 300 mapped reads in untreated samples (157.5 and 93.53 reads per million respectively) were significantly higher than in METT (5 reads per million) (Table 3.1). Considering this variation, a fold increase in the abundance of mapped sRNAs after treatment for each population was estimated. Treated samples from METT and GC colonies had a significant increase in mapped sRNAs when compared to their respective untreated samples (Fig. 3.3). Specifically, METT-treated samples showed an 89.4-fold (± 31.6) increase, while GC-treated samples displayed an increase of 31.1-fold (± 6). The treated CEAS 300 samples showed the lowest increase in accumulation of these sRNAs with an 8.8-fold (± 1.9) when compared to untreated samples.

3.3.4 Differentially expressed genes identified from targeted RNA sequencing analysis

Targeted RNA sequencing was performed to identify genes differentially expressed in the dsRNA-resistant CPB. Genes included in the analysis were either those responsible for the uptake (Table 3.2) and transport of dsRNA or those previously implicated to be differentially expressed in response to chemical pesticides in CPB (Clements et al. 2021). Using untreated larvae from the GC and METT populations as reference, a total of 15 genes were found to be differentially expressed (p-value <0.05) in the untreated samples from CEAS 300 (Tables 3.3 and 3.4). The qualitative pattern of downregulation or upregulation of genes remained the same irrespective of the susceptible population used for reference, though the differences in expression were higher

when comparing CEAS 300 and METT. Of the 15 differentially expressed genes, 13 genes are associated with response to chemical pesticides, out of which 11 were upregulated in CEAS 300 while 2 were downregulated (Tables 3.3 and 3.4). Two dsRNA uptake-related genes, *SRC-2* and *Sil-A*, were up-regulated in CEAS 300 samples when compared to both METT and GC untreated samples. Though *clathrin heavy chain (chc)* was not differentially expressed between CEAS 300 and GC untreated samples, it was downregulated (0.07 fold-change) in CEAS 300 compared to METT untreated samples. Comparing METT and CEAS 300 untreated samples revealed 6 differentially expressed dsRNA uptake-associated genes (Table 3.3), while only 2 of these 6 genes were found to be differentially expressed when comparing untreated CEAS 300 and GC samples (Table 3.4).

Next, we compared dsRNA-treated samples from GC and METT with CEAS 300. Interestingly, the list of differentially expressed genes in these comparisons was quite different from that of the comparison between untreated samples (Tables 3.5 and 3.6). Only 5 genes related to pesticide resistance were commonly differentially expressed in the comparisons between treated METT or GC and CEAS 300 samples. All of these genes are different from those identified in the comparison between untreated samples. Of these, *cholinesterase-like* was downregulated in the CEAS 300 treated samples, while the other 4 genes were upregulated (Tables 3.5 and 3.6). In the case of dsRNA uptake-related genes, *SRC-2* and *Sil-C* genes were still up-regulated in the CEAS 300 samples compared to both METT and GC-treated samples. In contrast, *clathrin heavy chain (chc)* was found to be downregulated (3.39 Log2FC) in CEAS 300-treated samples when compared to treated METT samples but not when compared to GC-treated samples. Comparisons of untreated and treated samples among individual populations reveal a strong response to dsRNA treatment in susceptible insects from GC and METT populations (Tables 3.7 and 3.8) while only

3 genes were differentially expressed between untreated and treated CEAS 300 (Table 3.9). These genes were *cholinesterase-like, cytochrome P450 4d2-like,* and the dsRNA uptake-associated gene *ARF-1*. The detoxification and metabolic resistance-related genes found to be upregulated in METT and GC upon treatment with dsRNA (Tables 3.8 and 3.9) were constitutively upregulated in CEAS 300.

Differential expression patterns identified from targeted RNA sequencing were validated by testing five target genes with qPCR analysis. Table 3.10 shows the fold change in target genes as estimated from targeted RNA-seq and qPCR analysis when comparing METT untreated or GC untreated to CEAS 300 untreated samples. For three out of the five genes, the trend of differential expression (i.e. upregulation or downregulation) agreed between the two approaches (Table 3.10). For example, based on targeted RNA sequencing *clathrin heavy chain (chc)* was 14-fold downregulated in CEAS 300 untreated samples when compared to METT untreated samples, while it was 10-fold downregulated based on the qPCR analysis. For two other target genes, *probable multidrug resistance-associated protein lethal* and *SRC-2*, results from targeted and qPCR were in agreement. However, transcript levels for *cytochrome P450 4d2-like* were found to be 19.02-fold upregulated in CEAS 300 untreated samples in targeted RNA-seq analysis but 2-fold downregulated in qPCR analysis. Similarly, *NADH-quinone oxidoreductase subunit B-like* showed an opposite trend in the two techniques.

3.3.5 Bioassays with piperonyl butoxide (PBO) and dsRNA

Bioassays testing susceptibility to dsRNA targeting the V-ATPase subunit A gene that included piperonyl butoxide (PBO) as an inhibitor of cytochrome P450 enzymes (Silcox, Ghidiu, and Forgash 1985) were performed with the susceptible and dsRNA-resistant CPB colonies. Control mortality (0.1% Tween-20) was less than 15% in all bioassays. Treatment with 0.1% PBO alone did not cause mortality significantly different from controls for any of the three CPB

populations (Fig. 3.4). Mortality in deltamethrin treatments reached 53.3% for GC, 55% for METT, and 66.7% for CEAS 300. The addition of PBO to deltamethrin increased mortality to 100% in GC and CEAS 300 and 98.7% in METT. Treatment of GC and METT with 0.18 µg/ml of dsRNA caused 51.7% and 40% mortality, respectively. In CEAS 300, mortality observed when fed a dsRNA concentration of 2000 µg/ml was not significantly different from control mortality. The addition of PBO to bioassays with dsRNA caused no significant change in mortality for any of the three populations (Fig. 3.4).

3.4 Discussion

Larvae of CPB are the target of Ledprona, the first sprayable dsRNA-based active recently approved for commercialization (Rodrigues et al. 2021; EPA 2023). The use of dsRNA-based sprays against CPB coupled with the known history of this pest to rapidly evolve resistance to numerous insecticides (Alyokhin et al. 2008) highlights the importance of studying the evolution of resistance against dsRNA-based insecticides in CPB. Currently, available data on resistance mechanisms to dsRNA is limited to populations of two other chrysomelids: corn rootworm and willow leaf beetle, and in both cases, impaired uptake of dsRNA was found to be associated with resistance (Khajuria et al. 2018; Liao, Zhang, and Zhang 2024). In this study, we report on potential mechanisms in >11,000-fold resistance to dsRNA targeting the *V-ATPase subunit A* gene in the CEAS 300 population of CPB (Mishra et al. 2021).

Increased nuclease activity resulting in dsRNA degradation is a potential resistance mechanism that has been associated with RNAi in recalcitrant insects (Shukla et al. 2016; Allen and Walker 2012; Christiaens, Swevers, and Smagghe 2014). Knockdown of gut specific nucleases in CPB, fall armyworm (*Spodoptera frugiperda*), and cotton boll weevil (*Anthonomus grandis*), increased susceptibility to orally delivered dsRNA (Spit et al. 2017; Almeida Garcia et al. 2017;

Koo, Zhu, and Palli 2024). The stability of dsRNA in regurgitate or gut fluids did not differ between the susceptible and dsRNA-resistant CPB strains in this study, suggesting that dsRNA degradation is not associated with resistance. Similar results were also reported for corn rootworm and willow leaf beetle dsRNA-resistant populations (Khajuria et al. 2018; Liao, Zhang, and Zhang 2024), suggesting that nuclease-based resistance is probably not an expected mechanism to evolve in field populations.

Reduced dsRNA uptake by midgut cells in CEAS 300 larvae was observed in microscopic examination and inferred from small RNA (sRNA) sequencing. Surprisingly, in both GC and CEAS populations, untreated samples had a high proportion of sRNAs mapping to the target V-ATPase subunit A transcript, possibly due to endogenous regulation via the RNAi pathway. This level of regulation may help explain why V-ATPases are some of the most susceptible targets for RNAi-induced mortality in insects (Baum et al. 2007; Mishra et al. 2021; Zeng et al. 2022). The lack of mapped sRNAs in METT untreated samples may be due to geographical differences between populations. Since GC was created by pooling insects from 13 different locations, it is possible that CPB populations at one of these locations have endogenous regulation of V-ATPase in response to stress, and CEAS 300 could have inherited this from GC while being selected. It would be interesting to map sRNAs from untreated insects to transcripts of other successful gene targets in CPB and see if the susceptibility correlates to sRNAs mapping to these transcripts. The highest increase in the proportion of mapped sRNAs after treatment with dsRNA was observed for METT, correlating with the relatively higher sensitivity to dsRNAs in this population compared to GC. A lower increase in mapped sRNA abundance was observed in GC, which may help explain the loss of susceptibility previously reported for this population, possibly due to contamination with resistant insects (Mishra et al. 2021). As expected, dsRNA-resistant insects demonstrated the

lowest increase in sRNAs mapping to the target transcript upon dsRNA treatment, thus supporting microscopy results that reduced dsRNA uptake in midgut cells is associated with resistance. Since reduced dsRNA uptake by midgut cells was also reported in other dsRNA-resistant beetle populations (Khajuria et al. 2018; Liao, Zhang, and Zhang 2024), this may be a common mechanism by which insects develop resistance to dsRNA.

Targeted sequencing revealed dsRNA uptake-associated genes to be differentially expressed in the CEAS 300 population. Specifically, genes associated with clathrin-mediated endocytosis (CME) such as *clathrin heavy chain (chc)* and *epsin-like* were downregulated while *SRC-2* was upregulated when compared to METT. In comparison, only *SRC-2* was differentially expressed between untreated GC and CEAS 300 samples. CME is a major dsRNA uptake pathway in insects, particularly in CPB (Cappelle et al. 2016) and inhibition of this pathway reduces RNAi-induced gene silencing (Cappelle et al. 2016; Pinheiro et al. 2018; Xiao et al. 2015; Wynant et al. 2014; Ulvila et al. 2006). For example in CPB, using an inhibitor of CME, chlorpromazine hydrochloride, resulted in a reduction of target gene silencing up to 88% (Cappelle et al. 2016). Loss of dsRNA uptake due to disruption of the CME pathway was also reported in oriental fruit flies (*Bactrocera dorsalis*) refractory to RNAi (Li et al. 2015). In this study, a primary dsRNA treatment targeting an endogenous gene prevented silencing upon secondary exposure and was associated with lower expression of genes involved in CME. Genes related to an alternate dsRNA uptake pathway in CPB mediated by SID-1-like transmembrane proteins (Cappelle et al. 2016), *Sil-A* and *Sil-C*, were upregulated in CEAS 300 insects (Tables 1 and 2). This may indicate a compensatory mechanism in CEAS 300 in response to the disruption of the endocytic pathway.

Results from targeted sequencing and qPCR analyses differed in some cases. For instance, qPCR showed that *chc* was downregulated in untreated CEAS 300 when compared to untreated

GC samples, but this was not detected in targeted sequencing. Since samples for targeted RNA sequencing were prepared in 2019 and those for qPCR analysis in 2022, disparities between targeted sequencing and qPCR results could be due to changes in populations over time. In our publication from 2021, we discussed reduced susceptibility in the parental population GC over time, possibly due to contamination with some CEAS 300 individuals. This could explain why targeted sequencing did not detect differences in *chc* expression between GC and CEAS 300. However, over the years the frequency of putative resistance alleles may have decreased in GC since the population is not kept under selection. In contrast, CEAS 300 is regularly selected using 800 µg/ml V-ATPase subunit A dsRNA, which could have contributed to the detected difference in the more recent samples by qPCR. Alternatively, the observed differences in results from targeted RNA-sequencing and qPCR analysis could be due to the inherent technical differences between the two techniques. It is not uncommon to obtain variable results when using these two methods. For example, in one study using qPCR to validate transcriptome analysis results, 16% of the genes exhibited inconsistent results between the two techniques.

Several insecticide resistance and stress-related genes were found to be differentially expressed in the CEAS 300 insects. Generally, a greater number of differentially expressed genes and to a higher extent were identified when comparing METT and CEAS 300 samples. This is expected since METT is expected to be more genetically distinct from CEAS 300 than GC, which is the parent population for CEAS 300. Since the presence of dsRNA molecules mimics viral infections, upregulation of stress and viral defense-related genes may have a potential role in dsRNA resistance. Some of the up-regulated genes in CEAS 300, such as cytochrome P450s have been reported to be upregulated in response to cucumber mosaic virus infection in green peach aphid (*Myzus persicae*) (Liang et al. 2021). Upregulation of detoxification enzymes associated

with insecticide resistance was also observed in *Aedes aegypti* in response to Zika virus infection, suggesting their potential role in viral defense (Zhao et al. 2018). Polygenic resistance in CEAS 300 supports the combination of reduced dsRNA uptake and enhanced stress response in resistance and may help explain the high levels of dsRNA resistance observed in this population. However, bioassays using PBO to inhibit the activity of cytochrome P450 enzymes did not result in differences in susceptibility to dsRNA in susceptible or resistant CPB insects. Lack of cross-resistance to chemical pesticides in CEAS 300 also supports this observation (Mishra, Moar, and Jurat-Fuentes 2024) even though increased cytochrome P450 activity is commonly associated with insecticide resistance (Scott 1999; Ye et al. 2022). Therefore, it is important to recognize that correlation does not imply causation. Increased expression of cytochrome P450s and other metabolic enzymes may not directly contribute to dsRNA resistance in CEAS 300 but can be part of a broader stress or immune response that ultimately provides some level of resistance.

3.5 Conclusions

The purpose of this study was to elucidate the mechanism(s) of dsRNA resistance in a highly resistant population of CPB (CEAS 300), selected using foliar delivery of dsRNA targeting the *V-ATPase subunit A* gene. We report reduced uptake of dsRNA and enhanced stress and/or immune response to be associated with dsRNA resistance in CEAS 300. Impaired uptake of dsRNA has also been reported in dsRNA-resistant populations of *D. v. virgifera* (Western corn rootworm) and *P. versicolora* (willow leaf beetle). However, this study represents the first report of downregulation of a major dsRNA uptake pathway-related gene, *clathrin heavy chain (chc)*, as being associated with reduced uptake in a resistant population. These results can inform strategies to overcome dsRNA resistance in the field. For example, developing delivery methods or RNA structures that allow by-passing clathrin-mediated endocytosis can potentially overcome resistance. One example is paper clip RNA (pcRNA), which seems to utilize a clathrin-

independent mechanism for uptake (Abbasi et al. 2020). We also report for the first time a potential association of an enhanced stress/immune response with dsRNA resistance. Whether a similar response is present in other dsRNA-resistant beetle species is not known. This secondary mechanism may be specific to CPB given its long and complex history of insecticide resistance (Chen et al. 2023). Our results provide valuable information supporting reduced uptake as a common mechanism of dsRNA resistance observed in three different chrysomelid species and also present a potential mechanism that may be specific to CPB. It is important to consider this secondary mechanism of dsRNA resistance when designing a resistance management framework for the use of dsRNA foliar sprays to control CPB.

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Appendix

Tables and Figures

Table 3.1- Total number of sRNA reads mapping to the full-length V-ATPase subunit A transcript (expressed in reads per million, rpm) in dsRNA susceptible (METT or GC) and resistant (CEAS 300) untreated or treated samples.

Sample	Reads mapped (rpm)	Fold increase (± se)
Untreated METT	5.11	89.3 (± 31.6)
Treated METT	456.63	
Untreated GC	157.45	31.1 (± 6)
Treated GC	4897.13	
Untreated CEAS	93.54	8.8 (± 1.9)
Treated CEAS	708.73	

Table 3.2- List of dsRNA uptake-associated genes in Colorado potato beetle sequenced using targeted sequencing.

Uptake pathway	Gene name
Clathrin Endocytosis	*Clathrin hc*
	AP 50
Systemic RNAi – dsRNA uptake	*SID1-related C (Sil C)*
	SID1-related A (Sil A)
Systemic RNAi- dsRNA export	*Epsin-like*
	Epn-1
Endosome transport	*Arf72A*
dsRNA uptake	*FBX011*
	HPS4
	SRC-2

Table 3.3- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between untreated dsRNA resistant (CEAS 300) and susceptible (METT) insects, as identified from targeted RNA-sequencing analysis. Highlighted genes are common in comparisons between untreated CEAS 300 and untreated METT and GC strains.

Process	Gene	FC	Log2FC
	Pupal cuticle protein G1A-like	17.39	4.12
	Probable cytochrome P450 305a1	17.39	4.12

Table 3.3- Continued

Insecticide resistance	***Cytochrome P450 4d2-like***	19.03	4.25
	NADH-quinone oxidoreductase subunit B-like	38.32	5.26
	Acetylcholine receptor subunit alpha-like	31.78	4.99
	Heat shock 70 kDa protein-like	4.11	2.04
	Probable cytochrome P450 49a1	0.76	-0.40
	Pupal cuticle protein 20-like	0.83	-0.27
	Heat shock protein 68-like	0.73	-0.45
	Cuticle protein 19-like	14.42	3.85
	Cuticle protein 8-like	3.73	1.90
	Probable cytochrome P450 301a1, mitochondrial	4.20	2.07
	Cytochrome P450 6k1-like	2.01	1.01
	Probable multidrug resistance-associated protein lethal (2)03659	0.81	-0.31
	Multidrug resistance-associated 1 isoform X3	0.85	-0.24
	Major heat shock 70 kDa protein Ab-like	0.69	-0.54
	Cholinesterase-like	0.85	-0.23
dsRNA uptake	*Epsin-like*	0.10	-3.36
	Clathrin	0.07	-3.90
	SRC-2	0.05	-4.45
	FBX011	0.59	-0.75
	SID-1 related A	4.44	2.15
	SID-1 related C	1.59	0.67

Table 3.4- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between untreated dsRNA resistant (CEAS 300) and susceptible (GC) CPB insects, as identified from targeted RNA-sequencing analysis. Highlighted genes are common in comparison between untreated CEAS 300 and untreated METT and GC strains.

Process	Gene	FC	Log2FC
Insecticide resistance	***NADH-quinone oxidoreductase subunit B-like***	40.50	5.34
	Cytochrome P450 4d2-like	15.45	3.95
	Probable cytochrome P450 301a1, mitochondrial	4.89	2.29
	Acetylcholine receptor subunit alpha-like	22.63	4.50
	Cuticle protein 19-like	5.82	2.54
	Pupal cuticle protein 36-like	10.70	3.42
	Probable multidrug resistance-associated protein lethal (2)03659	9.13	3.19
	Heat shock 70 kDa protein-like	3.36	1.75
	(2R)-3-sulfolactate dehydrogenase (NADP (+))-like	3.89	1.96
	Cytochrome P450 6k1-like	1.97	0.98
	Pupal cuticle protein G1A-like	3.94	1.98
	Probable cytochrome P450 305a1	3.97	1.99
dsRNA uptake	***SRC-2***	3.43	1.78
	SID-1 related A	3.76	1.91

Table 3.5- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between treated dsRNA resistant (CEAS 300) and susceptible (METT) CPB populations, as identified from targeted RNA-sequencing analysis. Highlighted genes are common in comparison between treated CEAS 300 and untreated GC and METT strains.

Process	Gene	FC	Log2FC
	NADH-quinone oxidoreductase subunit B-like	12.38	3.63
	Cholinesterase-like	0.81	-0.30

Table 3.5- Continued

Insecticide resistance	*Endocuticle structural glycoprotein SgAbd-8- like*	9.38	3.23
	2R)-3-sulfolactate dehydrogenase (NADP (+))-like	1.97	0.98
	Heat shock protein 68-like	0.82	-0.29
	Pupal cuticle protein 20-like	0.90	-0.15
	Pupal cuticle protein 36-like	3.32	1.73
dsRNA uptake	***SID-1 related C***	2.83	1.50
	ARF-1	0.52	-0.94
	Clathrin	0.09	-3.40
	SRC-2	3.86	1.95

Table 3.6- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between treated dsRNA resistant (CEAS 300) and susceptible (GC) CPB populations, as identified from targeted RNA-sequencing analysis. Highlighted genes are common in comparison between treated CEAS 300 and untreated METT and GC strains.

Process	Gene	FC	Log2FC
Insecticide resistance	***Cholinesterase-like***	0.59	-0.77
	(2R)-3-sulfolactate dehydrogenase (NADP(+))-like	1.79	0.84
	UDP-glucuronosyltransferase 2B10-like	1.28	0.36
	Cuticle protein 19-like	13.93	3.80
	Probable cytochrome P450 49a1	0.53	-0.91
	Multidrug resistance-associated 1 isoform X3	0.85	-0.23
	Cytochrome P450 6k1-like, transcript variant X1	1.23	0.30
	Probable multidrug resistance-associated protein lethal (2)03659	1.27	0.35
	Cuticle protein 8-like	0.80	-0.32
	Pupal cuticle protein 36-like	4.29	2.10
	Cytochrome P450 6k1-like	0.80	-0.33
	Pupal cuticle protein 20-like	1.13	0.17
	Major heat shock 70 kDa protein Ab-like	1.24	0.31
dsRNA uptake	***SID-1 related C***	1.40	0.49
	SID-1 related A	3.76	1.91
	SRC-2	2.35	1.23

Table 3.7- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between untreated and treated dsRNA resistant CPB population, CEAS 300, as identified from targeted RNA-sequencing analysis.

Mechanism	Target transcript	FC	Log2FC
Potential insecticide resistance	*Cholinesterase-like*	0.72	-0.47
	Cytochrome P450 4d2-like	0.64	-0.64
dsRNA uptake	*ARF-1*	0.55	-0.86

Table 3.8- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between untreated and treated larvae from the dsRNA susceptible CPB GC population as identified from targeted RNA-sequencing analysis.

Process	Target transcript	FC	Log2FC
Insecticide resistance	*Probable cytochrome P450 301a1, mitochondrial*	5.7	2.51
	Cytochrome P450 4d2-like	13.93	3.80
	NADH-quinone oxidoreductase subunit B-like	20.53	4.36
	Probable multidrug resistance-associated protein lethal (2)03659	9.71	3.28
	Cytochrome P450 6k1-like	1.66	0.73
	Acetylcholine receptor subunit alpha-like	19.56	4.29
	Pupal cuticle protein 36-like	3.66	1.87
	Probable multidrug resistance-associated protein lethal (2)03659	0.49	-1.03
	Pupal cuticle protein 20-like	0.68	-0.56
	Cholinesterase-like	0.68	-0.56
	Cytochrome P450 4d2-like	0.62	-0.69
	Heat shock protein 70 B2-like	0.56	-0.83
	Heat shock protein 68-like	0.62	-0.70

Table 3.8- Continued

	Multidrug resistance-associated 1 isoform X3	0.55	-0.86
	Heat shock protein 68-like	0.59	-0.77
	(2R)-3-sulfolactate dehydrogenase (NADP (+))-like	2.57	1.36
dsRNA uptake	*SRC-2*	2.68	1.42
	SID-1 related A	2.46	1.30
	AP50	0.78	-0.36

Table 3.9- Differentially expressed genes (expressed in fold change (FC) or Log2FC, p-value <0.05) between untreated and treated larvae from the dsRNA susceptible CPB METT population, as identified from targeted RNA-sequencing analysis.

Process	**Target transcript**	**FC**	**Log2FC**
Resistance to chemical pesticides	*Pupal cuticle protein G1A-like*	16.91	4.08
	Probable cytochrome P450 305a1	16.91	4.08
	Cytochrome P450 4d2-like	16.45	4.04
	Acetylcholine receptor subunit alpha-like	10.85	3.44
	NADH-quinone oxidoreductase subunit B-like	1.66	0.73
	Acetylcholine receptor subunit alpha-like	4.11	2.04
	Probable cytochrome P450 301a1, mitochondrial	4.72	2.24
	Cuticle protein 8-like	3.66	1.87
	Cuticle protein 19-like	6.82	2.77
	Probable cytochrome P450 49a1	0.68	-0.56
	Probable multidrug resistance-associated protein lethal (2)03659	0.76	-0.39
	Cholinesterase-like	0.77	-0.37
	Heat shock 70 kDa protein-like	1.91	0.93
	SID-1 related C	0.55	-0.85

Table 3.9- Continued

dsRNA uptake	*Clathrin*	1.87	0.90
	FBX011	0.66	-0.60

Table 3.10- Change in transcript level (mean fold-change ± SE) of target genes in larvae from dsRNA susceptible (GC) and dsRNA resistant (CEAS 300) strains using targeted sequencing or qPCR analysis.

Target gene	**CEAS untreated relative to METT untreated using targeted sequencing**	**CEAS untreated relative to METT untreated using qPCR**	**CEAS untreated relative to GC untreated using targeted sequencing**	**CEAS untreated relative to GC untreated using qPCR**
Cytochrome P450 4d2-like	19.02	0.1 (±0.04)	15.48	3.52 (±1.91)
Probable multidrug resistance-associated protein lethal (2)03659	3.24	2.47 (±0.91)	9.14	1.24 (±0.42)
NADH-quinone oxidoreductase subunit B-like	38.31	0.55 (±0.34)	40.53	1.12 (±0.44)
SRC-2	21.85	1.37 (±0.33)	1.7	1.13 (±0.32)
Clathrin	0.07	0.09 (±0.04)	NA	0.40 (±0.13)

Figures

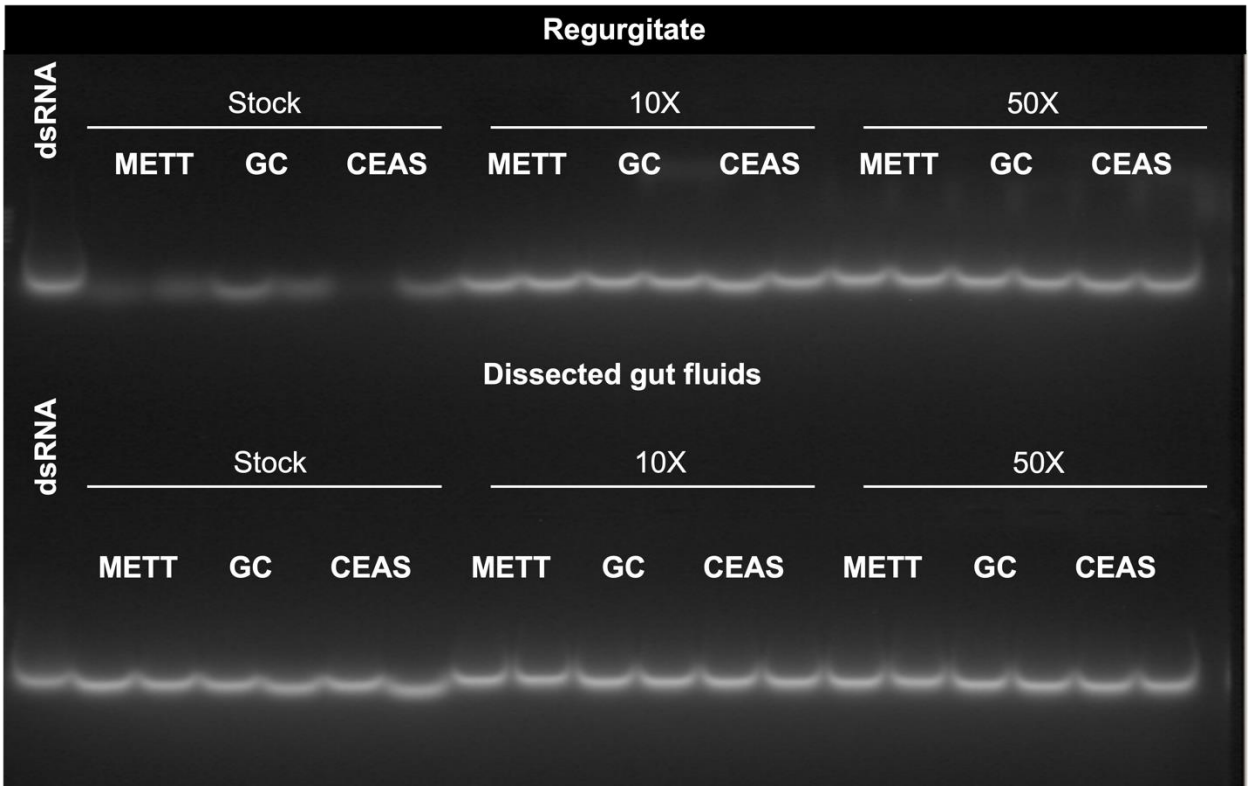


Figure 3.1- Agarose gel showing relative stability of a dsRNA when incubated with stock (1mg/ml), 10X and 50X dilutions of METT, GC, and CEAS 300 gut fluids collected as regurgitate or by dissection, for 1 hour at room temperature.

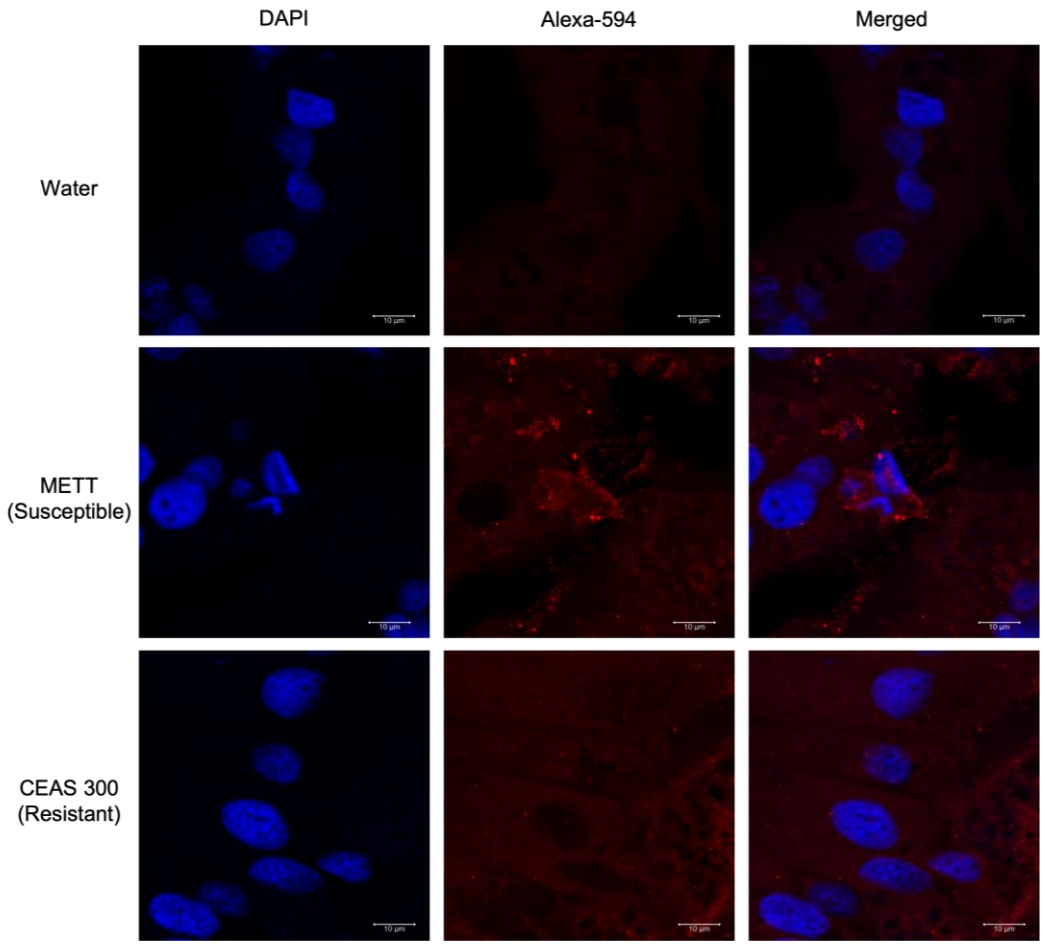


Figure 3.2- Histological gut sections of CPB larvae from susceptible (METT) and resistant (CEAS 300) colonies, treated with dsRNA or water as a control, observed under a confocal microscope. Mouse anti-dsRNA J2 antibody was used as the primary antibody to detect dsRNA in tissue samples and Mouse Alexa 594 antibody was used as the secondary antibody.

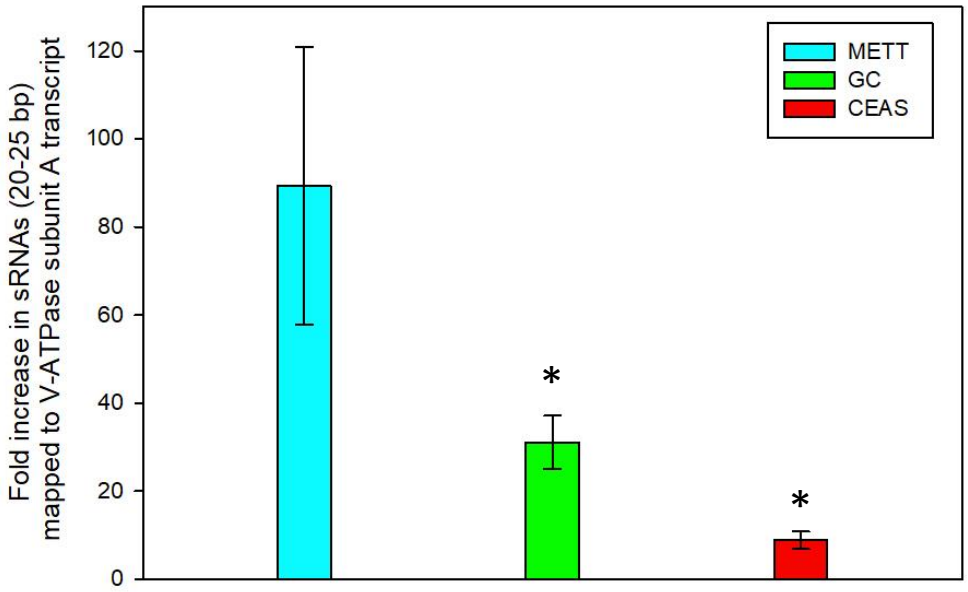


Figure 3.3- Fold increase in small RNAs (sRNAs) mapping to the target transcript V-ATPase subunit A upon dsRNA treatment in susceptible (METT and GC) and resistant (CEAS 300) CPB larvae. Asterisks denote significant differences (Kruskall-Wallis ANOVA on Ranks followed by a Dunnet's pairwise multiple comparison to the control group METT), p-value < 0.05).

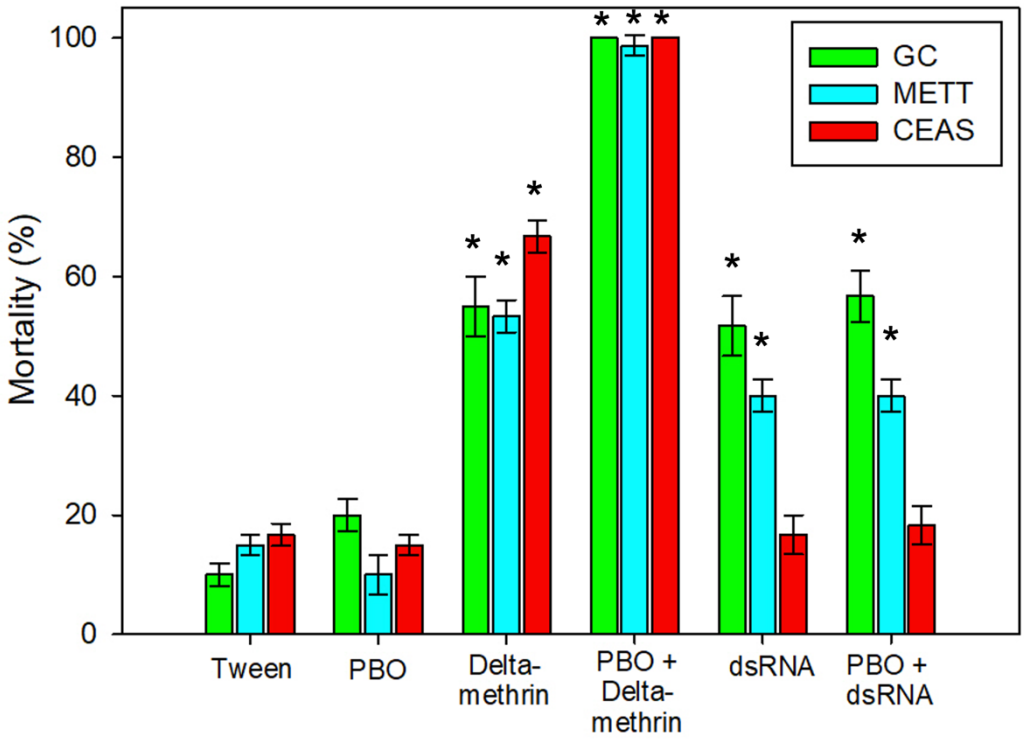


Figure 3.4- Percentage mortality in CPB larvae from dsRNA susceptible (METT and GC) and resistant (CEAS 300) colonies in response to different treatments. Asterisks denote significant differences within each population (Kruskall-Wallis ANOVA on Ranks followed by a Dunnet's pairwise multiple comparison to the control group (Tween), p-value < 0.05).

Chapter Four

Transcriptomic and genomic analysis of dsRNA resistance in Colorado potato beetle (*Leptinotarsa decemlineata*, Say)

Abstract

Double-stranded RNA (dsRNA) insecticides represent a novel mode of action for insect control, thus safeguarding them against rapid resistance development in target insects is crucial. The Colorado potato beetle (CPB, *Leptinotarsa decemlineata* Say) is the target pest of the first dsRNA sprayable insecticide and is unfortunately also notorious for quickly developing insecticide resistance. This study used a previously described laboratory-selected dsRNA-resistant strain of CPB to investigate transcriptomic and genomic changes associated with resistance. Comparisons of whole transcriptome sequencing revealed genes associated with one of the dsRNA uptake pathways, clathrin-mediated endocytosis to be downregulated in resistant CPB. Bulked segregant analysis identified genomic regions significantly associated with dsRNA resistance, providing a short list of candidate genes for elucidation of the resistance mechanism. This information guides the development of tools to delay and overcome resistance. Candidate resistance alleles identified can be validated and utilized for DNA-based monitoring of CPB populations in different geographic regions as part of a comprehensive resistance management framework.

4.1 Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), is renowned as one of the most problematic pests in agriculture due to its extensive track record of developing resistance to insecticides (Huseth et al. 2014; Alyokhin et al. 2008). Recognized as the most serious defoliator of potato (*Solanum tuberosum*) worldwide, it can also feed on other Solanum species including tomato (*S. lycopersicum*), pepper (*S. annum*), and eggplant (*S. melogena*) (Hare 1990). Aside from having a broad host range, CPB also demonstrates the ability to thrive in diverse climates, as seen in its continuous expansion across North America, Europe, and Asia (Weber 2003). High genome plasticity is thought to have aided its ability to adapt to a wide host range and stressors such as varied climates and insecticides (Schoville et al. 2018). Different populations of CPB have developed documented resistance to 56 different active ingredients (Whalon 2019) including the latest control chemical, Imidacloprid (Scott, Tolman, and MacArthur 2015). Without a suitable control plan, CPB can cause up to 100% defoliation on potato plants, leading to substantial yield losses for farmers (Alyokhin et al. 2008). Consequently, there is an immediate need to develop novel control tools against this pest and also understand potential resistance mechanisms to develop effective resistance management strategies.

RNA interference (RNAi) is a multi-step pathway that results in the degradation of a messenger RNA (mRNA) complementary to a double-stranded RNA (dsRNA) that activates the RNAi pathway (Fire et al. 1998). The use of RNAi to selectively silence vital insect genes and cause mortality has been demonstrated as an efficient insect control strategy (Baum et al. 2007; Rodrigues et al. 2021; Pallis et al. 2022; Vélez et al. 2023). These insecticides trigger the RNA-interference (RNAi) pathway in the insect cells upon dsRNA ingestion, resulting in failure to produce a protein or production of an incomplete protein, ultimately leading to insect mortality.

The first dsRNA-based active ingredient targeting CPB (Ledprona; GreenLight Biosciences, Medford, MA, USA) has been approved for commercialization by the US Environmental Protection Agency (EPA) (EPA 2023).

Though dsRNA insecticides present a highly effective tool against CPB (Pallis et al. 2022; Ma et al. 2020; Petek et al. 2020; Hussain et al. 2019), it is important to acknowledge that resistance against these insecticides is inevitable. Previous work from our group demonstrated the successful selection from field-collected beetles of a strain of CPB (CEAS 300) with >11,000-fold resistance to a dsRNA delivered on the surface of potato leaves (Mishra et al. 2021). Resistance also resulted in cross-resistance to a dsRNA targeting a different gene, but not to the Cry3Aa insecticidal protein from *Bacillus thuringiensis* (Bt) nor to small molecule chemical insecticides commonly used to control CPB (Mishra, Moar, and Jurat-Fuentes 2024; Mishra et al. 2021). The inheritance of resistance in the CEAS 300 strain was determined to be autosomal, recessive, and polygenic. Additionally, no fitness cost was observed in the dsRNA-resistant compared to the susceptible insects (Pinto et al. 2023).

Reduced uptake of dsRNA by midgut cells was observed as a potential mechanism of resistance in the CEAS 300 strain (Chapter 3). Building on these results, the main objective of the current chapter was to identify gene(s) related to dsRNA uptake that may exhibit differential expression in the CEAS 300 compared to a reference susceptible strain using RNA sequencing (RNA-Seq) analysis. This comprehensive transcriptome approach also helps identify additional genes or pathways linked to dsRNA resistance considering the polygenic nature of dsRNA resistance in the CEAS 300 strain. To identify genomic loci associated with resistance, bulked segregant analysis (BSA) comparing allele frequencies between the susceptible and CEAS 300

populations was performed. Results from this study offer deeper insights into the mechanism of dsRNA resistance and aid in the identification of genes likely to contribute to resistance.

4.2 Materials and methods

4.2.1 Insect colonies

Insects from three previously described CPB populations were used for both RNA-Seq and Bulked segregant analysis (BSA) (Mishra et al. 2021). General colony (GC) and Maine tween tolerant (METT) strains are susceptible to dsRNA and are used as references. The dsRNA-resistant population CEAS 300 (chronically exposed adult survivors) was selected using dsRNA targeting the V-ATPase subunit A gene and exhibits >11,100-fold resistance compared to the parental colony (GC). All three colonies were maintained as described elsewhere (Mishra et al. 2021).

4.2.2 Sample preparation for RNA-seq and analysis

Guts dissected from early third instar CPB larvae from each strain were used for RNA sequencing. For each of the three colonies (GC, METT, and CEAS 300) both untreated and samples from individuals drop-fed 25 µg of V-ATPase subunit A dsRNA were prepared. Treatment with this dose induces a significant reduction in target transcript expression in GC and METT, indicating a successful RNAi response, but not in CEAS 300 (Mishra et al. 2021).

Total RNA was extracted from dissected guts using the Qiagen RNeasy Mini kit, following manufacturer's instructions. Three and five biological replicates, a pool of 3 individual guts each, were included for untreated and dsRNA-treated samples, respectively, for a total of 24 samples. Samples were sent to Novogene (Sacramento, CA) for mRNA library preparation and sequencing. For each sample, 40 M raw read pairs (150 bp) were obtained from sequencing using the Illumina Novaseq 6000 platform.

Raw reads were first trimmed and adapters removed using Cutadapt (Martin 2011). Processed reads were then mapped to the *Leptinotarsa decemlineata* reference genome assembly GCF_000500325.1 using STAR (Dobin et al. 2013). The number of mapped reads was calculated using HTSeq-count (Anders, Pyl, and Huber 2015) and the R package DESeq2 was used to identify differentially expressed genes (DEGs) among different comparisons with an adjusted p-value cut-off of 0.05 (Love, Huber, and Anders 2014). To get further insights into the physiological differences between different groups, ClusterProfiler was used to perform over-representation (OR) analysis and gene set enrichment analysis (GSEA) using the KEGG database (Wu et al. 2021). Furthermore, raw read counts of known RNAi-related genes (Schoville et al. 2018) were extracted and tested for significant differential gene expression using DESeq2 (adjusted p-value <0.05).

4.2.3 Sample preparation for bulked segregant sequencing and analysis

The carcasses of 60 individual larvae from each colony (GC, METT, and CEAS 300) were used for DNA extraction. The CEAS 300 larvae were fed on leaves treated with 800 µg/ml dsRNA to ensure the selection of larvae with a strong resistance phenotype. DNA was extracted using the Qiacube HT and QIAmp 96 DNA kit (Qiagen) and quantified using the Qubit dsDNA HS Assay kit (Invitrogen). A pool combining 100 ng of DNA from 20 individual samples was prepared for a biological replicate, and three pools were sequenced per colony. The pools were then used for library preparation and sequencing on an Illumina NovaSEQ device using the S4 cell and a 2x150 bp paired-end configuration with dual indexing.

Processed DNA reads were mapped to the GCF_000500325.1 reference genome assembly using BWA-MEM (Li 2013), followed by variant calling using mpileup, and SAMtools (Danecek et al. 2021). Single nucleotide polymorphisms (SNPs) were then filtered and analyzed based on

the ΔSNP index and G' method using QTLseqR with default settings and a 1 Mb sliding window size (Mansfeld and Grumet 2018). Samples from both susceptible colonies (GC and METT) were individually compared with the dsRNA-resistant strain (CEAS 300) samples using the same approach. Regions with false discovery rates less than 0.01 were considered to be associated with the resistant phenotype and genes within these regions identified.

4.3 Results

4.3.1 Differentially expressed genes (DEGs) between untreated susceptible and resistant insects

To understand gene expression changes associated with dsRNA resistance, untreated samples from dsRNA susceptible colonies (METT and GC) were compared with those of the dsRNA-resistant colony (CEAS 300). When comparing METT and CEAS 300 samples, a total of 399 DEGs (115 up and 284 down) were identified (Table 4.1), while in the comparison between untreated GC and CEAS 300 samples, 310 genes (219 up and 91 down) exhibited differential expression (Table 4.1). When comparing CEAS 300 with both the susceptible (GC and METT) strains, 61 genes were differentially expressed (Table 4.2). Intriguingly, a notable number of these genes were annotated as 'uncharacterized' suggesting the existence of potentially undiscovered components crucial to RNAi in these insects. Some of these uncharacterized genes were found to be long non-coding RNAs (lncRNAs) on the NCBI database.

To understand the physiological pathways associated with the DEGs, gene set enrichment analysis (GSEA) using the KEGG pathway database was conducted. Results from GSEA demonstrate that untreated METT and CEAS 300 insects exhibited distinct patterns in metabolic pathway regulation. Specifically, pathways such as Carbon metabolism, Oxidative phosphorylation, Citrate cycle (TCA cycle), and motor proteins were suppressed in METT but activated in CEAS 300 (Table 4.3). Conversely, degradation pathways such as lysosomes,

mitophagy, and autophagy were found to be activated in METT and suppressed in CEAS 300 (Table 4.3). Over-representation (OR) analysis also revealed similar results where genes upregulated in METT relative to CEAS 300 belonged to pathways such as lysosome, apoptosis, efferocytosis, and endocytosis (Table 4.4). When comparing untreated GC and CEAS 300 using GSEA, a total of 26 pathways were found to be differentially regulated, with 23 of these being activated in CEAS 300 (Table 4.5). Over-representation analysis also revealed lysosome, efferocytosis, and endocytosis pathways to be up-regulated in GC relative to CEAS 300 (Table 4.6).

None of the known RNAi-related genes exhibited |Log2FC| of ≥2.5 when comparing untreated CEAS 300 samples with either METT or GC. A separate differential expression analysis of these genes using DESeq2 revealed that dsRNA uptake-associated genes *Clathrin heavy chain (chc)* and *FBX011* were significantly down-regulated in CEAS 300 compared to the two susceptible strains (Table 4.7).

4.3.2 Differentially expressed genes (DEGs) upon dsRNA treatment

Differential gene expression analysis comparing untreated and dsRNA-treated samples was performed to understand the response to dsRNA treatment in susceptible and resistant CPB colonies. Among the three colonies, METT exhibited the lowest number of DEGs in response to dsRNA treatment. Specifically, 84 genes were differentially expressed in treated METT samples, with 23 up-regulated and 61 down-regulated (Table 4.8). The other susceptible colony (GC) had 172 DEGs, with 115 up-regulated and 57 down-regulated genes (Table 4.8). Lastly, the dsRNA-resistant colony CEAS 300 displayed the most significant response, with 462 differentially expressed genes, including 138 up-regulated and 324 down-regulated (Table 4.8).

Over-representation (OR) analysis of the differentially expressed genes between untreated and treated METT insects revealed that the genes with significantly reduced expression upon dsRNA treatment mostly belonged to metabolic and genetic information processing pathways, specifically biosynthesis of cofactors, lipoic acid metabolism and protein export (Table 4.9). Conversely, genes with significantly increased expression upon dsRNA treatment were associated with signal transduction pathways, such as TGF-beta signaling and Hippo signaling, as well as Cytoskeleton in muscle cells (Table 4.9).

Similarly in GC, dsRNA treatment resulted in decreased expression of genes belonging to a high number of carbohydrate metabolism and xenobiotics degradation pathways, while genes with increased expression belonged to membrane transport and signal transduction pathways along with some metabolic pathways (Table 4.10). In CEAS 300, a higher number of pathways were significantly regulated, as expected by the high number of DEGs observed for CEAS 300 untreated vs treated insects (Table 4.11). However, like GC and METT, genes down-regulated upon dsRNA treatment in CEAS 300 belonged to metabolic and catabolic pathways while those involved in signal transduction and transport were up-regulated (Table 4.11).

Again, no RNAi pathway-related genes with |log2FC| ≥2.5 were identified among the DEGs in METT and GC. In contrast, treated CEAS 300 insects showed up-regulation of *Argonaute-1* (*Ago1*, LOC111515957) and *Aubergine* (*Aug/Piwi*, LOC111504772), which are part of the micro RNA (miRNA) and piwi-interacting RNA (piRNA) pathways, respectively, rather than the small interfering RNA (siRNA) pathway typically associated with RNAi of exogenous dsRNA (Bonning and Saleh 2021). Analyzing the differential expression of these genes separately (without any |Log2FC| cut-off) revealed that in all three strains, core RNAi machinery genes such as *Ago2a, Dicer2a, FBX011, LDL,* and *Gawky* were significantly upregulated (adjusted p-value

≤0.05) upon dsRNA treatment (Table 4.12). Surprisingly, some dsRNA uptake-associated genes such as *SC-R2, AP50,* and *R2D2* were down-regulated in all three strains (Table 4.12).

A comparison of treated METT or GC and CEAS 300 samples demonstrated a lower number of differentially expressed genes than the comparison between untreated samples (Table 4.1). Furthermore, in the principal component analysis (PCA) plot (Fig. 4.1), untreated samples from the three colonies formed distinct clusters, indicating highly variable expression profiles. In contrast, dsRNA-treated samples clustered closely together independently of strain, suggesting similar expression profiles in response to dsRNA treatment. A comparison of treated METT and CEAS 300 samples displayed 166 DEGs (58 up and 108 down) while that of treated GC and CEAS 300 samples displayed 69 DEGs (49 up and 20 down) (Table 4.1). Only 23 genes were commonly differentially expressed when comparing treated samples from the two susceptible strains to the CEAS 300 (Table 4.13).

Gene set enrichment analysis of DEGs between treated METT and CEAS 300 samples exhibited only one pathway, Ribosome (involved in genetic information processing and translation) to be activated in CEAS 300, likely due to the limited number of DEGs between the two groups. Similarly, no specific pathway was found to be changed between treated GC and CEAS samples.

4.3.3 Genomic regions associated with dsRNA resistance

Bulked segregant analysis (BSA) was performed to identify genomic regions with genetic variability associated with the dsRNA-resistant phenotype. Allele frequency was compared between dsRNA-resistant CEAS and both susceptible (GC and METT) strains individually. Based on the ΔSNP and G' prime values, peaks significantly associated with CEAS in both comparisons were chosen for further investigation. Three peaks located on chromosomes 5, 10, and 11 were

detected (Fig. 4.2). Within the chromosome 5 peak, only the *MOXD1 homolog 2* (LOC111517289) gene was found (Table 4.14). This gene also exhibited down-regulation with a log2FC of -1.6 (p-adjusted= 0.03) in untreated CEAS 300 relative to METT samples. However, it was not significantly differentially expressed in treated METT and CEAS 300 samples. *MOXD1 homolog 2* is predicted to comprise a copper-dependent monooxygenase-like domain, suggesting its likely involvement in metabolic and detoxification processes.

The BSA peak on chromosome 10 comprised nine genes, while the peak on chromosome 11 contained 33 genes (Table 4.14). Among all these, 23 genes were expressed in the midgut. Although none of the known RNAi pathway genes were present on this list, it included several transcription factors and kinases. LOC111507146 located on chromosome 11 is an uncharacterized gene in the current Colorado potato beetle assembly. However, an Insecta BLAST search revealed it to be 71% identical (E- value 1e-55) to the microtubule-associated protein *Jupiter-like* in *Anoplophora glabripennis* (Asian long-horned beetle). Additionally, two genes predicted to contain zinc domains (*BTB/POZ domain-containing protein kctd15 and zinc finger protein 608-like)* were also identified on chromosomes 10 and 11 respectively. The *TIMELESS-interacting protein* gene located on chromosome 11 is predicted to enable DNA binding activity. Lastly, several genes involved in immune or stress response such as *cuticle protein-like*, *prostaglandin reductase 1-like*, pathogenesis-related protein 5-like, etc. were also identified (Table 4.14).

4.4 Discussion

Double-stranded RNA (dsRNA) insecticides, both transgenic and foliar sprays, have been approved for commercialization by the US Environmental Protection Agency (Head et al. 2017; EPA 2023). Consequently, it is imperative to study potential resistance mechanisms against these insecticides. Impaired uptake of dsRNA was associated with resistance in both corn rootworm

(Khajuria et al. 2018) and willow leaf beetle (Liao, Zhang, and Zhang 2024) dsRNA resistant populations. However, there is no information regarding what dsRNA uptake pathways or genes might be responsible for this phenotype. Reduced dsRNA uptake was also observed in the CPB dsRNA-resistant strain CEAS 300 (chapter 3). Here, whole transcriptomic and genomic analyses were performed to gain a comprehensive view of differential gene expression and genomic loci associated with dsRNA resistance in the CEAS 300 strain.

Whole transcriptome analysis revealed that the endocytosis pathway was suppressed in dsRNA-resistant insects. Particularly, dsRNA uptake genes *Clathrin heavy chain (chc)* and *FBX011* showed significantly reduced expression in CEAS 300 compared to both susceptible strains. Clathrin-mediated endocytosis (CME) is the primary mechanism of dsRNA uptake in insect cells (Pinheiro et al. 2018; Cappelle et al. 2016; Xiao et al. 2015; Wynant et al. 2014; Ulvila et al. 2006). Suppression of CME using its inhibitor chlorpromazine hydrochloride resulted in failure of target gene silencing in CPB (Cappelle et al. 2016). It is important to note that in some insects (including CPB), an additional uptake pathway based on SID-1-like transmembrane proteins can allow some level of dsRNA internalization (Cappelle et al. 2016). Nevertheless, suppression of the endocytic pathway presents a potential resistance mechanism in CEAS 300. Similar results were also observed in the oriental fruit fly *Bactrocera dorsalis* where refractoriness to a second dsRNA treatment was associated with disruption of the endocytic pathway, following a primary dsRNA treatment (Li et al. 2015). The refractory flies also demonstrated reduced expression of endocytosis genes such as *clathrin heavy chain (chc).* The potential involvement of clathrin-mediated endocytosis in dsRNA resistance highlights the potential of utilizing different uptake strategies capable of circumventing this pathway, thereby facilitating dsRNA internalization. For example, the uptake of a novel dsRNA structure called paper-clip RNA

(pcRNA) is independent of clathrin-mediated endocytosis (Abbasi et al. 2020) and can thus potentially overcome uptake-mediated resistance to dsRNA. Other uptake strategies based on diverse formulations or complexing dsRNA with nanocarriers may also improve dsRNA internalization and overcome resistance (Silver, Cooper, and Zhu 2021).

Treatment with dsRNA resulted in alterations in several metabolic and immune-related pathways in all three CPB colonies. As dsRNA typically originates from viral molecules and triggers innate immune response pathways like RNAi, Toll, Imd, and Jak-STAT, such a reaction to dsRNA treatment is expected (Ferreira et al. 2014; Choi and Hyun 2012; Dostert et al. 2005; Saleh et al. 2009). However, the magnitude of this metabolic and immune reaction was most pronounced in dsRNA-resistant insects. This intensified immune response could offer an additional level of tolerance to dsRNA in resistant CPB. Enhanced immunity has been implicated to be involved in resistance against insecticidal proteins from Bt in lepidopteran pests (Xiao et al. 2023; Lawrie et al. 2020; Rahman et al. 2004). Several long non-coding RNAs (lncRNAs) were also found to be differentially expressed in CEAS 300. Many recent studies have highlighted the involvement of lncRNAs in modulating resistance to various insecticides (Shi et al. 2022; Meng et al. 2021; Liu et al. 2017). Identifying potential gene targets of these lncRNAs either by expression correlation analysis or structural analysis can provide further insights into the interplay between lncRNAs and genes associated with resistance to dsRNA.

Resistance to dsRNA in CEAS 300 was shown to be polygenic (Mishra et al. 2021). As expected, multiple genomic loci were found to be significantly associated with dsRNA resistance in BSA analyses. Using both susceptible colonies as references, polymorphisms in three genomic regions were found to be significantly linked with resistance. Of the 24 genes identified in these regions, several protein kinases and transcription factors were present. The phosphorylation

activity of protein kinases and action by transcription factors regulate signal transduction and several fundamental cellular processes. Regulation of insecticide resistance genes involved in detoxification, such as cytochrome P450, via signaling pathways involving protein kinases, has been demonstrated in several insects (Yang et al. 2020; Guo et al. 2022; Li and Liu 2017). Additionally, transcription factors such as *Maf-S* have been demonstrated to regulate insecticide resistance in *Anopheles gambiae* (Ingham et al. 2017). The protein kinases and/or transcription factors located on genomic regions associated with dsRNA resistance may also modulate dsRNA uptake directly or indirectly. Additionally, genes with nucleotide binding capacity such as zinc finger-like domains (LOC111512156 and LOC111508436) and *TIMELESS-interacting protein* may regulate transcription of resistance of associated genes.

Some metabolic stress and immunity-related genes were also detected in the BSA peaks. For example, *MOXD1 homolog 2* is located on the peak in chromosome 5, *pathogenesis-related protein 5-like* and *prostaglandin reductase 1-like* is located on the peak in chromosome 10, and *cuticle protein-like* is located on the peak in chromosome 11. These may contribute to the robust metabolic and immune response observed in CEAS 300 upon dsRNA treatment compared to METT and GC individuals. An uncharacterized gene (LOC111507146) located on chromosome 11 exhibited similarity to the *microtubule-associated protein Jupiter-like* in *Anoplophora glabripennis.* Microtubule-like structures have been implicated in the transport of the RNAi machinery in *Drosophila* cells as part of a systemic antiviral response (Karlikow et al. 2016). *Clavesin-2-like* protein located on the peak in chromosome 11 has been implicated in endocytosis and secretion (Katoh et al. 2009), and it exhibits phosphatidylinositol bisphosphate (PIP) binding activity. PIPs regulate clathrin-mediated endocytosis (Yeung et al. 2006; Cernikova, Faso, and Hehl 2020; McMahon and Boucrot 2011), and thus *clavesin-2-like* may be involved in the

disruption of clathrin-mediated endocytosis detected in CEAS. *Rhophilin-2-A* is also present in the peak on chromosome 11. This protein regulates actin organization (Peck et al. 2002) which is an important element of endocytosis (Galletta and Cooper 2009).

This study provides a short list of candidate resistance genes in the CEAS 300 strain of CPB. Functional validation of these genes would be required to confirm their role in resistance. Alternatively, linkage analysis could be performed to narrow down the list of candidate genes or identify alleles strongly associated with resistance. Identifying resistance alleles will allow the development of DNA-based tools to screen and estimate resistance risks in field CPB populations to extend the utility of sprayable dsRNA pesticides against this pest.

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Appendix

Tables and Figures

Table 4.1- Number of differentially expressed genes (DEGs) between different treatments among susceptible (METT and GC) and dsRNA-resistant (CEAS 300) strains of Colorado potato beetle.

Comparison	Total DEGs[a]	Up-regulated	Down-regulated
METT untreated vs CEAS untreated	399	115	284
GC untreated vs CEAS untreated	310	219	91
METT treated vs CEAS treated	166	58	108
GC treated vs CEAS treated	69	49	20

a- Differentially expressed genes with |Log2FC| ≥2.5 and adjusted p-value ≤0.05

Table 4.2- Differentially expressed genes with |Log2FC| ≥2.5 and adjusted p-value ≤0.05 in untreated larvae from the dsRNA-resistant CEAS 300 strain of Colorado potato beetle compared to two susceptible strains (METT and GC).

Accession number	**Gene description**
Up-regulated in CEAS 300	
LOC111510965	*glycine-rich cell wall structural protein 1-like*
LOC111505758	*piggyBac transposable element-derived protein 4-like*
LOC111514670	*cytochrome P450 4C1-like*
LOC111511172	*cyclin-A2-like*
LOC111513235	*uncharacterized*
LOC111509387	*uncharacterized (lncRNA)*
LOC111506573	*retinoid-inducible serine carboxypeptidase-like*
LOC111511678	*venom carboxylesterase-6-like*
LOC111508638	*dynein heavy chain 8, axonemal*
LOC111517908	*osmotic avoidance abnormal protein 3-like*
LOC111509267	*esterase FE4-like*
LOC111513389	*glycine-rich protein DOT1-like*
LOC111510333	*cuticle protein 64-like*
LOC111507061	*ETV5-related protein Ets96B-like*
LOC111516026	*uncharacterized*
LOC111507509	*glucose dehydrogenase [FAD, quinone]-like*

Table 4.2- Continued

Down-regulated in CEAS 300	
LOC111504449	*uncharacterized (lncRNA)*
LOC111504808	*carboxylesterase 1D-like*
LOC111509486	*serine-aspartate repeat-containing protein F-like*
LOC111509129	*protein atonal-like*
LOC111514683	*uncharacterized (lncRNA)*
LOC111513047	*uncharacterized*
LOC111504811	*uncharacterized*
LOC111506886	*uncharacterized*
LOC111513687	*uncharacterized*
LOC111516976	*hillarin-like*
LOC111511815	*uncharacterized*
LOC111515675	*membrane-associated guanylate kinase*
LOC111511935	*teneurin-a*
LOC111503575	*protein dopey-1 homolog*
LOC111516573	*E3 ubiquitin-protein ligase HUWE1-like*
LOC111516244	*coiled-coil domain-containing protein 141-like*
LOC111511764	*voltage-dependent calcium channel subunit alpha-2/delta-3*
LOC111508175	*forkhead box protein F1-like*
LOC111513727	*cathepsin L-like proteinase*
LOC111509005	*cathepsin L1-like*
LOC111513904	*uncharacterized*
LOC111511108	*cathepsin L1-like*
LOC111506650	*uncharacterized*
LOC111516853	*cathepsin L1-like*
LOC111515207	*uncharacterized (lncRNA)*
LOC111504029	*sodium/potassium/calcium exchanger 4*
LOC111503375	*uncharacterized*
LOC111513539	*transmembrane protein 127-like*
LOC111515399	*uncharacterized*
LOC111517731	*uncharacterized*
LOC111517687	*cathepsin L-like proteinase*
LOC111515980	*uncharacterized*
LOC111510532	*trypsin-7-like*
LOC111516874	*uncharacterized*
LOC111506760	*uncharacterized*
LOC111504797	*uncharacterized (lncRNA)*
LOC111512228	*gamma-aminobutyric acid receptor subunit beta-like*

Table 4.2- Continued

LOC111503313	*para-nitrobenzyl esterase-like*
LOC111517077	*uncharacterized (lncRNA)*
LOC111502143	*insulin-like growth factor-binding protein complex acid labile subunit*
LOC111504729	*neuronal acetylcholine receptor subunit alpha-7*
LOC111509971	*autophagy-related protein 2 homolog B-like*
LOC111514076	*uncharacterized*
LOC111513879	*lipase 3-like*
LOC111507111	*uncharacterized*

Table 4.3- Gene set enrichment analysis (GSEA) between untreated larvae from susceptible (METT) and the dsRNA-resistant (CEAS 300) strains of Colorado potato beetle.

KEGG id	Pathway description	Enrichment Score (ES)	Normalized ES	Adjusted p-value
ldc01200	Carbon metabolism	-0.64	-1.94	1.1E-05
ldc00190	Oxidative phosphorylation	-0.65	-1.94	1.1E-05
ldc04140	Autophagy - animal	0.46	1.69	1.1E-03
ldc04142	Lysosome	0.45	1.68	1.1E-03
ldc00020	Citrate cycle (TCA cycle)	-0.69	-1.83	3.7E-03
ldc00270	Cysteine and methionine metabolism	-0.64	-1.71	9.2E-03
ldc01230	Biosynthesis of amino acids	-0.59	-1.65	1.3E-02
ldc04814	Motor proteins	-0.54	-1.60	2.0E-02
ldc04137	Mitophagy - animal	0.44	1.52	3.9E-02

Table 4.4- Pathways significantly over-represented (p-adjusted <0.05) among differentially expressed genes in untreated larvae from dsRNA susceptible CPB strain METT insects when compared to dsRNA resistant CEAS 300 insects.

Category	Sub-category	KEGG id	Pathway description
UP			
Environmental Information Processing	Signal transduction	ldc04350	TGF-beta signaling pathway
Environmental Information Processing	Signal transduction	ldc04310	Wnt signaling pathway
Environmental Information Processing	Signal transduction	ldc04068	FoxO signaling pathway
Environmental Information Processing	Signal transduction	ldc04013	MAPK signaling pathway - fly
Cellular Processes	Transport and catabolism	ldc04137	Mitophagy - animal

Table 4.4- Continued

Cellular Processes	Transport and catabolism	ldc04140	Autophagy - animal
Environmental Information Processing	Membrane transport	ldc02010	ABC transporters
Organismal Systems	Aging	ldc04213	Longevity regulating pathway - multiple species
NA	NA	ldc04148	Efferocytosis
Environmental Information Processing	Signal transduction	ldc04392	Hippo signaling pathway - multiple species
Cellular Processes	Transport and catabolism	ldc04142	Lysosome
Cellular Processes	Cell growth and death	ldc04215	Apoptosis - multiple species
Cellular Processes	Transport and catabolism	ldc04144	Endocytosis
Cellular Processes	Cell growth and death	ldc04214	Apoptosis - fly
Environmental Information Processing	Signal transduction	ldc04341	Hedgehog signaling pathway - fly
DOWN			
Cellular Processes	Cell motility	ldc04814	Motor proteins

Table 4.5- Gene set enrichment analysis (GSEA) between untreated larvae from susceptible (GC) and the dsRNA-resistant (CEAS 300) strains of Colorado potato beetle.

ID	Description	Enrichment Score (ES)	Normalized ES	Adjusted p-value
ldc00190	Oxidative phosphorylation	-0.65	-2.77	6.30E-09
ldc03010	Ribosome	-0.54	-2.33	6.30E-09
ldc01200	Carbon metabolism	-0.44	-1.93	0.00062
ldc00510	N-Glycan biosynthesis	-0.61	-2.06	0.00113
ldc00040	Pentose and glucuronate interconversions	-0.51	-1.93	0.00113
ldc03040	Spliceosome	-0.37	-1.63	0.00260

Table 4.6- Pathways significantly over-represented (p-adjusted <0.05) among differentially expressed genes in untreated larvae from dsRNA susceptible CPB strain GC insects when compared to dsRNA resistant CEAS 300 insects.

Category	Sub-category	KEGG id	Pathway description
UP			
Organismal Systems	Aging	ldc04213	Longevity regulating pathway - multiple species

Table 4.6- Continued

Environmental Information Processing	Signal transduction	ldc04350	TGF-beta signaling pathway
Cellular Processes	Transport and catabolism	ldc04140	Autophagy - animal
Cellular Processes	Transport and catabolism	ldc04142	Lysosome
Cellular Processes	Transport and catabolism	ldc04144	Endocytosis
Organismal Systems	Immune system	ldc04624	Toll and Imd signaling pathway
NA	NA	ldc04148	Efferocytosis
Metabolism	Lipid metabolism	ldc00564	Glycerophospholipid metabolism
Environmental Information Processing	Signal transduction	ldc04391	Hippo signaling pathway - fly
DOWN			
Metabolism	Carbohydrate metabolism	ldc00040	Pentose and glucuronate interconversions
Genetic Information Processing	Folding, sorting and degradation	ldc04141	Protein processing in endoplasmic reticulum
Metabolism	Global and overview maps	ldc01200	Carbon metabolism
Metabolism	Carbohydrate metabolism	ldc00053	Ascorbate and aldarate metabolism
Metabolism	Glycan biosynthesis and metabolism	ldc00510	N-Glycan biosynthesis
Metabolism	Energy metabolism	ldc00190	Oxidative phosphorylation
Metabolism	Glycan biosynthesis and metabolism	ldc00513	Various types of N-glycan biosynthesis
Metabolism	Carbohydrate metabolism	ldc00052	Galactose metabolism
Metabolism	Amino acid metabolism	ldc00380	Tryptophan metabolism
Metabolism	Global and overview maps	ldc01240	Biosynthesis of cofactors
Metabolism	Amino acid metabolism	ldc00280	Valine, leucine and isoleucine degradation

Table 4.7- Differentially expressed RNAi-related genes (adjusted p-value ≤0.05) in dsRNA-resistant CPB strain CEAS 300 when compared to two susceptible strains METT and GC.

Gene	Log2FC	Gene	Log2FC
Untreated METT vs CEAS 300			
Chc	-0.89	*FBX011*	-0.68
SC-R2	1.73	*Maeistrom*	0.46

Table 4.7- Continued

Rsd-3	-0.71		
Untreated GC vs CEAS 300			
Epn-1	-1.64	*TudorN*	0.53
Loqs	1.39	*Chc*	-0.51
LDL	-1.59	*VhaSFD*	0.50
FBX011	-0.97	*Dicer2a*	-0.85
Arf72	0.71	*Gawky*	-0.72
Sil-A	0.76	*SLR*	-0.47
Vha16	0.69		

Table 4.8- Number of differentially expressed genes (DEGs) between untreated and treated samples of dsRNA susceptible (METT and GC) and resistant (CEAS 300) strains of Colorado potato beetle.

Comparison	Total DEGs[a]	Up-regulated	Down-regulated
METT untreated vs treated	84	23	61
GC untreated vs treated	172	115	57
CEAS untreated vs treated	462	138	324

a- Differentially expressed genes with Log2FC ≥2.5 and adjusted p-value ≤0.05

Table 4.9- Pathways significantly over-represented (p-adjusted <0.05) among differentially expressed genes in untreated larvae from dsRNA susceptible CPB strain METT when compared to dsRNA-treated larvae.

Category	**Sub-category**	**KEGG id**	**Pathway description**
UP			
Metabolism	Global and overview maps	ldc01240	Biosynthesis of cofactors
Metabolism	Metabolism of cofactors and vitamins	ldc00785	Lipoic acid metabolism
Genetic Information Processing	Folding, sorting and degradation	ldc03060	Protein export
DOWN			
Environmental Information Processing	Signal transduction	ldc04350	TGF-beta signaling pathway
NA	NA	ldc04820	Cytoskeleton in muscle cells
Environmental Information Processing	Signal transduction	ldc04391	Hippo signaling pathway
Environmental Information Processing	Signal transduction	ldc04392	Hippo signaling pathway

Table 4.10- Pathways significantly over-represented (p-adjusted <0.05) among differentially expressed genes in untreated larvae from dsRNA susceptible CPB strain GC when compared to dsRNA-treated larvae.

Category	**Sub-category**	**KEGG id**	**Pathway description**
UP			
Organismal Systems	Immune system	ldc04624	Toll and Imd signaling pathway
Metabolism	Metabolism of cofactors and vitamins	ldc00830	Retinol metabolism
Metabolism	Xenobiotics biodegradation and metabolism	ldc00980	Metabolism of xenobiotics by cytochrome P450
Metabolism	Global and overview maps	ldc01240	Biosynthesis of cofactors
Metabolism	Carbohydrate metabolism	ldc00053	Ascorbate and aldarate metabolism
Metabolism	Xenobiotics biodegradation and metabolism	ldc00982	Drug metabolism cytochrome P450
Metabolism	Metabolism of cofactors and vitamins	ldc00790	Folate biosynthesis
Metabolism	Xenobiotics biodegradation and metabolism	ldc00983	Drug metabolism
Metabolism	Carbohydrate metabolism	ldc00040	Pentose and glucuronate interconversions
Metabolism	Metabolism of other amino acids	ldc00430	Taurine and hypotaurine metabolism
Metabolism	Metabolism of cofactors and vitamins	ldc00860	Porphyrin metabolism
Metabolism	Amino acid metabolism	ldc00260	Glycine, serine and threonine metabolism
DOWN			
Environmental Information Processing	Membrane transport	ldc02010	ABC transporters
Metabolism	Carbohydrate metabolism	ldc00040	Pentose and glucuronate interconversions
Metabolism	Carbohydrate metabolism	ldc00520	Amino sugar and nucleotide sugar metabolism
Metabolism	Global and overview maps	ldc01250	Biosynthesis of nucleotide sugars

Table 4.10- Continued

Environmental Information Processing	Signal transduction	ldc04392	Hippo signaling pathway multiple species

Table 4.11- Pathways significantly over-represented (p-adjusted <0.05) among differentially expressed genes in untreated larvae from dsRNA-resistant CPB strain CEAS 300 when compared to dsRNA-treated larvae.

Category	**Sub-category**	**KEGG id**	**Pathway description**
UP			
Metabolism	Carbohydrate metabolism	ldc00630	Glyoxylate and dicarboxylate metabolism
Metabolism	Amino acid metabolism	ldc00260	Glycine, serine and threonine metabolism
Metabolism	Amino acid metabolism	ldc00330	Arginine and proline metabolism
Metabolism	Amino acid metabolism	ldc00270	Cysteine and methionine metabolism
Metabolism	Metabolism of cofactors and vitamins	ldc00790	Folate biosynthesis
Metabolism	Global and overview maps	ldc01230	Biosynthesis of amino acids
Metabolism	Carbohydrate metabolism	ldc00030	Pentose phosphate pathway
Metabolism	Global and overview maps	ldc01200	Carbon metabolism
Cellular Processes	Transport and catabolism	ldc04146	Peroxisome
Metabolism	Metabolism of other amino acids	ldc00480	Glutathione metabolism
Metabolism	Metabolism of other amino acids	ldc00430	Taurine and hypotaurine metabolism
Metabolism	Amino acid metabolism	ldc00350	Tyrosine metabolism
Metabolism	Global and overview maps	ldc01250	Biosynthesis of nucleotide sugars
Metabolism	Carbohydrate metabolism	ldc00500	Starch and sucrose metabolism
Organismal Systems	Immune system	ldc04624	Toll and Imd signaling pathway
DOWN			
Environmental Information Processing	Signal transduction	ldc04391	Hippo signaling pathway - fly
Cellular Processes	Transport and catabolism	ldc04144	Endocytosis
Environmental Information Processing	Signal transduction	ldc04350	TGF-beta signaling pathway
Cellular Processes	Transport and catabolism	ldc04148	Efferocytosis

Table 4.11-Continued

Organismal Systems	Aging	ldc04213	Longevity regulating pathway - multiple species
Environmental Information Processing	Signal transduction	ldc04068	FoxO signaling pathway
Cellular Processes	Transport and catabolism	ldc04140	Autophagy - animal
NA	NA	ldc04820	Cytoskeleton in muscle cells
Environmental Information Processing	Signal transduction	ldc04150	mTOR signaling pathway
Environmental Information Processing	Signal transduction	ldc04392	Hippo signaling pathway - multiple species
Environmental Information Processing	Signal transduction	ldc04013	MAPK signaling pathway - fly

Table 4.12- Differentially expressed ($p<0.05$) genes in the RNAi pathway in response to treatment with dsRNA in susceptible (METT, GC) and dsRNA-resistant (CEAS 300) strains of Colorado potato beetle.

Gene	Log2FC	Gene	Log2FC
ME Untreated vs treated			
Arf72	-1.32	*Vha16*	-0.80
LDL	1.72	*FBX011*	0.73
Auv/Piwi	1.57	*Loqs*	0.49
VhaSFD	-0.64	*AP50*	-0.59
Dicer2a	0.98	*Rab7*	-0.77
R2D2	-0.94	*Gawky*	0.89
CG4562	-0.89	*Ago1*	1.29
Transline	-0.97	*Sil-C*	-0.47
Light	0.70		
GC Untreated vs treated			
Ago2b	-0.93	*Drosha*	-0.44
Sil-A	0.63	*RdRp elp-1*	0.78
Rab7	-0.69	*AP50*	-0.47
Ago1	1.30	*SC-R2*	-0.71
Gawky	0.58	*Ago2a*	0.47
Dicer2a	0.76	*SDN-1*	-0.38
Auv/Piwi	1.19	*CG4562*	-0.56
Epn-1	-0.63	*NSS*	-0.32
Rsd-3	0.57	*Staufen*	-0.29
CEAS 300 Untreated vs treated			
Dicer2a	1.95	*SC-R2*	-1.28

Table 4.12 - Continued

Maeistrom	-0.69	*VhaSFD*	-0.70
Gawky	1.35	*R2D2*	-1.00
Ago1	2.69	*Rsd-3*	0.73
LDL	2.04	*PRMT*	-0.70
FBX011	1.07	*Light*	1.32
Epn-1	1.34	*TudorN*	-0.50
Auv/Piwi	2.50	*RdRp elp-1*	0.87
SLR	0.85	*Transline*	-0.85
Clp-1	-0.83	*AP50*	-0.37
Chc	0.65	*Ago2a*	0.70

Table 4.13- Differentially expressed genes with Log2FC ≥2.5 and adjusted p-value ≤0.05 in treated larvae from the dsRNA-resistant CEAS 300 strain of Colorado potato beetle compared to two susceptible strains (METT and GC).

Accession number	**Gene description**
Up-regulated in CEAS 300	
LOC111514940	*Uncharacterized*
LOC111515263	*cilia- and flagella-associated protein 57-like*
LOC111516385	*cilia- and flagella-associated protein 57-like*
LOC111512606	*uncharacterized*
LOC111511172	*cyclin-A2-like*
LOC111510629	*eukaryotic translation initiation factor 3 subunit J-like*
Down-regulated in CEAS 300	
LOC111501986	*Uncharacterized (lncRNA)*
LOC111506760	*Uncharacterized*
LOC111509342	*uncharacterized (lncRNA)*
LOC111517077	*uncharacterized (lncRNA)*
LOC111514683	*uncharacterized (lncRNA)*
LOC111513047	*uncharacterized*
LOC111507055	*cathepsin L1-like*
LOC111516391	*uncharacterized*
LOC111515553	*heat shock 70 kDa protein-like*
LOC111506811	*arylphorin subunit alpha-like*
LOC111509005	*cathepsin L1-like*
LOC111517687	*cathepsin L-like proteinase*
LOC111515980	*uncharacterized (lncRNA)*
LOC111510896	*zinc finger protein 271-like*
LOC111502760	*odorant receptor 46a-like odorant receptor 46a-like*

Table 4.13- Continued

LOC111515502	*uncharacterized (lncRNA)*
LOC111514365	*protein toll-like*

Table 4.14- Loci located on genomic regions within peaks of polymorphism significantly associated with the dsRNA resistance phenotype in the CEAS 300 strain. Only genes expressed in the midgut of Colorado potato beetle larvae (as established from RNA-seq data) are shown.

Chromosome	**Accession number**	**Name**
5	LOC111517289	*MOXD1 homolog 2*
10	LOC111512156	*BTB/POZ domain-containing protein kctd15*
	LOC111506216	*pathogenesis-related protein 5-like*
	LOC111510462	*prostaglandin reductase 1-like*
	LOC111509661	*transcription initiation factor TFIID subunit 4-like*
	LOC111509662	*U3 small nucleolar RNA-associated protein 18 homolog*
11	LOC111508989	*clavesin-2-like*
	LOC111508995	*homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 2 protein*
	LOC111508996	*TIMELESS-interacting protein*
	LOC111508997	*translation initiation factor IF-2, mitochondrial*
	LOC111508998	*HD domain-containing protein 2*
	LOC111510949	*uncharacterized*
	LOC111512582	*cuticle protein-like*
	LOC111512585	*casein kinase I*
	LOC111507146	*uncharacterized in CPB (*BLAST results reveal similarity to *microtubule-associated protein Jupiter-like* in *Anoplophora glabripennis)*
	LOC111507147	*oxysterol-binding protein-related protein 6-like*
	LOC111507148	*E3 ubiquitin-protein ligase AMFR-like*
	LOC111507149	*cyclin-dependent-like kinase 5*
	LOC111507150	*dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1*
	LOC111513355	*V-type proton ATPase subunit C*
	LOC111513357	*non-lysosomal glucosylceramidase-like*
	LOC111513067	*rhophilin-2-A*
	LOC111506570	*alpha-protein kinase 1-like*
	LOC111508436	*zinc finger protein 608-like*

Figures

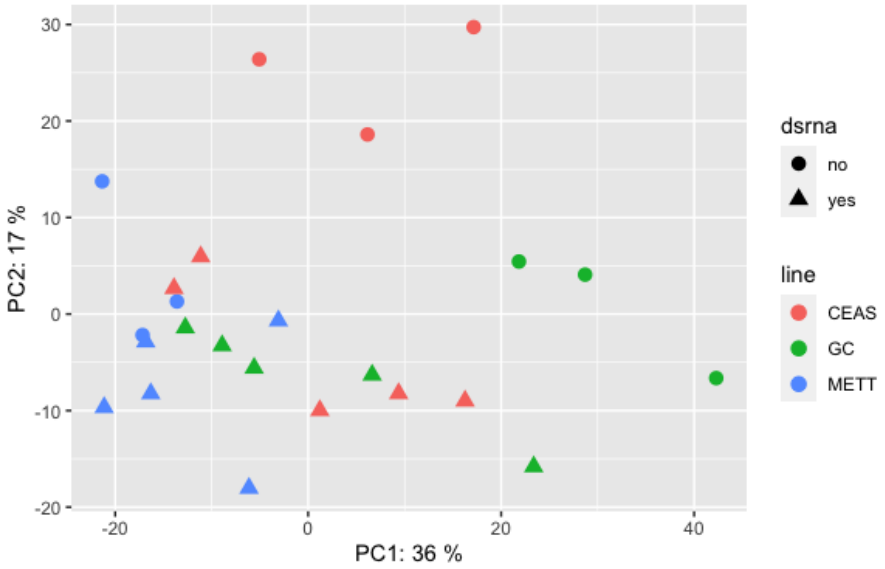


Figure 4.1- Principal component analysis (PCA) plot showing the distribution of untreated (circles) or dsRNA treated (triangles) samples from susceptible (METT- blue, GC- green) and dsRNA-resistant (CEAS 300- peach) strains of Colorado potato beetle.

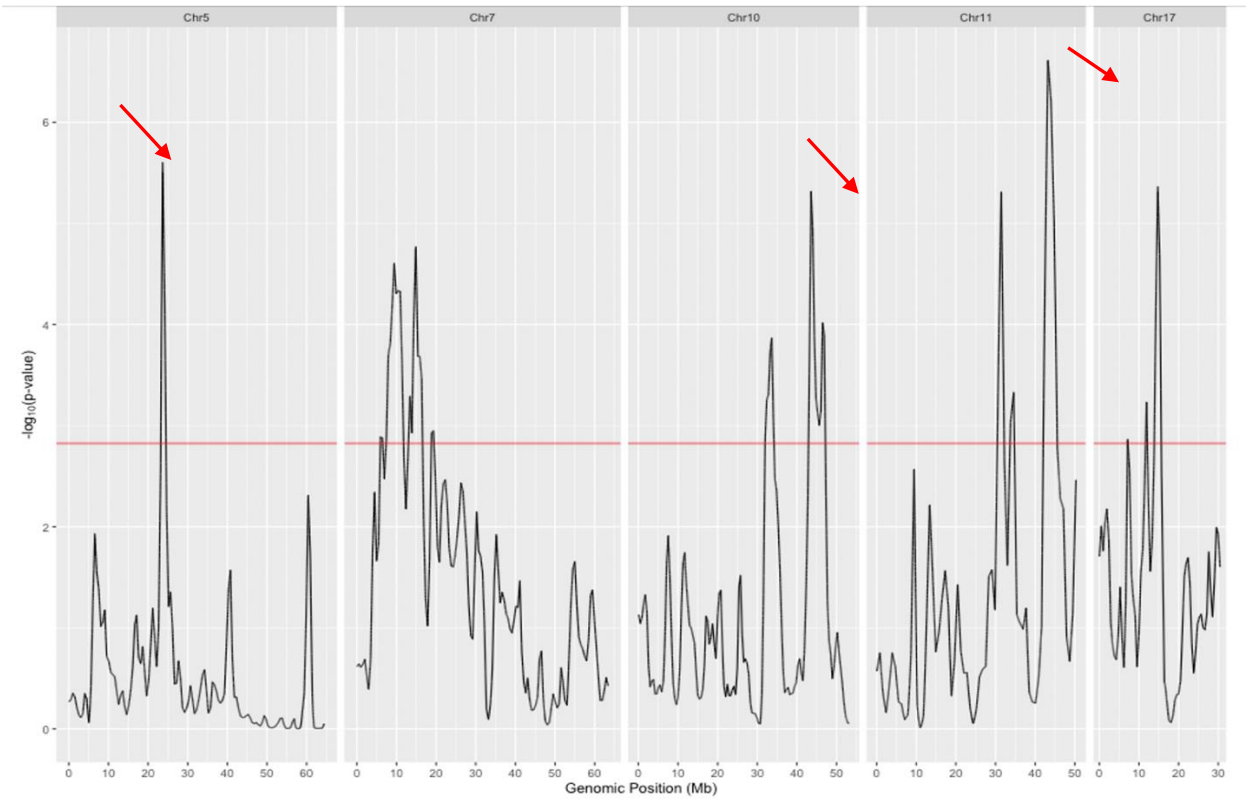


Figure 4.2- Bulked segregant analysis (BSA) comparing SNP frequency between susceptible (METT and GC) and dsRNA-resistant (CEAS 300) strains of Colorado potato beetle. Peaks above the red line are considered significantly associated with the resistance phenotype ($p<0.05$). Peaks with red arrows (chromosomes 5, 10, and 11) were found to be common when comparing METT vs CEAS 300 and GC vs CEAS 300 separately.

Chapter Five

Conclusions

Double-stranded RNA (dsRNA) insecticides inhibit the expression of target genes by initiating the RNA-interference (RNAi) pathway. Chapter 1 of this dissertation offers a comprehensive review of RNAi technology in insect control, its safety, various aspects that affect efficacy, and current knowledge of resistance mechanisms. Two RNAi-based products differing in the dsRNA delivery method are approved for commercialization by the US EPA. SmartStax Pro is a maize event providing, in addition to three insecticidal proteins from *Bacillus thuringiensis* (Bt), transgenic delivery of a dsRNA targeting the *sucrose non-fermenting 7* gene (*Snf7)* in *Diabrotica virgifera virgifera* (western corn rootworm, CRW) (Head et al. 2017). Calantha is a foliar spray containing Ledprona, a dsRNA targeting the *proteasome subunit beta type-5* (*PSMB5*) gene in *Leptinotarsa decemlineata* (Colorado potato beetle, CPB) (Pallis et al. 2022; EPA 2023).

Insects are expected to ultimately develop resistance against dsRNA-based insecticides. However, we can influence the rate at which resistance develops through an effective insecticide resistance management (IRM) framework. An effective proactive IRM plan includes three key components: reducing selection pressure through different MOAs, diluting resistance alleles using refuges, and regularly monitoring for resistance in field insect populations. These components are effective under specific assumptions. For instance, alternate MOAs can reduce selection pressure only if there is no cross-resistance. Similarly, the success of the refuge strategy depends on resistance being a recessive trait such that heterogenous individuals remain susceptible. Selection and characterization of resistance colonies is important to inform the IRM framework for new insecticides. Identifying resistance alleles also facilitates the development of DNA-based screening methods as quicker and highly sensitive tools to complement bioassay-based methods in monitoring for resistance which are agnostic of the resistance gene.

This dissertation aimed to characterize and elucidate the resistance mechanism in a CPB population with ≥11,100-fold resistance (CEAS 300) against foliar delivery of a dsRNA targeting the *V-ATPase subunit A* gene (Mishra et al. 2021). We also compare our results to the only two other known dsRNA-resistant insect populations; a colony of CRW with ≥130-fold resistance to maize expressing DvSnf7 dsRNA (Khajuria et al. 2018), and a willow leaf beetle (*Plagiodera versicolora*) colony displaying ≥4110-fold resistance to foliar delivery of a dsRNA targeting the *Srp54k* gene (Liao, Zhang, and Zhang 2024).

Genetics of resistance in CEAS 300

The second chapter of this dissertation aimed to determine the inheritance pattern of resistance in CEAS 300. Understanding the inheritance pattern and genetics of resistance is important for the successful implementation of an effective IRM plan. Refuge areas are parts of the field where the insecticide is not applied, allowing the survival of a high number of susceptible individuals. Mating of rare survivors emerging from treated areas with refuge individuals dilutes the frequency of resistance alleles by generating heterozygous individuals, which are controlled when resistance is a recessive trait. This refuge strategy, when implemented appropriately, has played an important role in delaying resistance against Bt crops in the US as part of the IRM framework (Tabashnik et al. 2012; Tabashnik, Brévault, and Carrière 2013). Whether resistance is autosomal or sex-linked also affects the spread of resistance alleles within a population, with sex-linked alleles generally expected to spread faster (Argentine, Clark, and Ferro 1994). Lastly, determining if resistance is monogenic or polygenic can provide useful insights into the resistance mechanism.

We report that resistance in CEAS 300 is a recessive autosomal trait, as also documented in dsRNA-resistant CRW and willow leaf beetle populations (Liao, Zhang, and Zhang 2024;

Khajuria et al. 2018). Insecticide resistance is typically a recessive trait, though some cases of dominant inheritance exist (Campagne et al. 2013). These results support the use and expected effectiveness of the refuge strategy in delaying dsRNA resistance in field insect populations.

In CRW dsRNA resistance is linked to a single locus (Khajuria et al. 2018), while in willow leaf beetle this has not been reported. Our findings indicate that resistance in CEAS 300 is polygenic, suggesting the involvement of multiple genes with cumulative effects on resistance. Laboratory selection of insect populations with moderate (LC_{50} for example) and increasing doses of insecticide often results in polygenic selection (McKenzie and Batterham 1994). In the field, however, selection would occur with a high dose (more than LC_{99}), which should select for resistance controlled by a single major gene (McKenzie and Batterham 1994). In CPB, population genomic studies have revealed that insecticide resistance has a polygenic architecture across the US, suggesting polygenic resistance may occur in these field populations (Chen et al. 2023; Yang, Crossley, et al. 2022). Studying the mechanisms of resistance in a polygenic population such as CEAS 300 allows the identification of candidate resistance mechanisms that may eventually get selected in field populations.

Cross-resistance to other control methods

Using multiple MOAs either in combination or in rotation decreases the selection pressure for resistance to a single MOA. This is crucial since high selection pressure is the primary driver for the evolution of resistance and by reducing it the development of resistance can be slowed. Additionally, combining multiple MOAs increases effectiveness through ‘redundant killing’, meaning insects tolerant to one MOA can be eliminated by the second.

In all three available cases of dsRNA-resistant insect populations, selection of resistance with one dsRNA sequence led to cross-resistance to alternate dsRNAs as well (Mishra et al. 2021;

Khajuria et al. 2018; Liao, Zhang, and Zhang 2024). Therefore, combining multiple dsRNA sequences is not likely to delay resistance. This also suggests that resistance is due to the failure of a general step along the RNAi pathway and is not dependent on the target sequence. However, combining multiple dsRNA sequences can still be useful in achieving a 'high dose' effect in insects where RNAi is less efficient or where a single gene target is not effective in causing mortality. By targeting multiple genes simultaneously, even if each gene is not highly silenced, the combined effect may still result in mortality through 'additional killing'. In red spider mite (*Tetranychus urticae)* a chimeric dsRNA targeting two genes simultaneously demonstrated higher toxicity than each of the dsRNAs alone (Kwon et al. 2016).

Resistance to dsRNAs did not result in cross-resistance to insecticidal Bt proteins (Mishra et al. 2021; Khajuria et al. 2018; Liao, Zhang, and Zhang 2024). In the second chapter of this dissertation, the dsRNA-resistant CEAS 300 population was shown not to display cross-resistance to chemical pesticides commonly used to control CPB. The lack of cross-resistance to Bt proteins or any of the tested chemical pesticides suggests that combining these multiple MOAs can be an effective strategy to delay dsRNA resistance in CPB.

Mechanism of dsRNA resistance in CEAS 300

Information on the mechanism of resistance can allow the development of strategies to overcome resistance. Identification of resistance alleles can also allow DNA-based monitoring of resistance in estimating resistance risks in field populations. The third chapter of this dissertation aimed at identifying which step in the RNAi pathway is altered in the dsRNA-resistant CEAS 300 population. Comparison of dsRNA stability in midgut fluids and regurgitate revealed no differences between susceptible and resistant CPB. Even though gut nuclease activity impacts the success of RNAi response in many insects, current information from three dsRNA-resistant strains

does not support increased nuclease activity as a resistance mechanism against dsRNA insecticides. Immunohistochemistry of gut sections revealed reduced uptake of dsRNA inside midgut cells of CEAS 300 larvae. Quantification of small RNAs (sRNAs) by sequencing also demonstrates a significantly lower accumulation of sRNAs in resistant insects arising from V-ATPase subunit A dsRNA upon treatment. Impaired uptake of dsRNA was also associated with resistance in CRW and willow leaf beetle (Liao, Zhang, and Zhang 2024; Khajuria et al. 2018), though no further information on the exact mechanism of impaired uptake or related genes was provided. Targeted sequencing of genes associated with dsRNA uptake in CPB demonstrated reduced expression of *clathrin heavy chain (chc)* and *epsin-like* genes, involved with clathrin-mediated endocytosis (CME), in CEAS 300 compared to susceptible larvae. Uptake of dsRNA is mediated by CME in many insects (Pinheiro et al. 2018; Xiao et al. 2015; Wynant et al. 2014; Ulvila et al. 2006), including CPB (Cappelle et al. 2016). However, another dsRNA uptake pathway involving SID-related genes is also present in CPB (Cappelle et al. 2016). This may explain why dsRNA uptake is not completely absent in CEAS 300 insects. Moreover, two SID-related genes, *Sil-A* and *Sil-C*, were upregulated in CEAS 300, suggesting a compensatory uptake mechanism in response to disruption of the endocytic pathway.

In Chapter 4, a whole transcriptome sequencing approach was used to identify global differential pathways between susceptible and resistant CPB. This approach also revealed endocytosis to be suppressed in CEAS 300. In the oriental fruit fly (*Bactrocera dorsalis*), refractoriness to dsRNA was due to disruption of the endocytic pathway and involved downregulation of several genes involved in CME in refractory flies (Li et al. 2015). Treatment with hydrogen peroxide (H_2O_2) to enhance actin assembly, an essential element of CME, resulted in reversion to susceptibility. *Phosphatase and actin regulator 1-like* (LOC111503509) is down-

regulated in CEAS 300 compared to both METT (2.2-fold) and GC (1.8-fold). Testing these insects with H_2O_2 in addition to dsRNA may further confirm the role of CME in dsRNA resistance. Paper-clip RNA (pcRNA), a dsRNA structure that folds onto itself, is taken up by a clathrin-independent pathway in *Aedes aegypti* and causes target gene silencing comparable to long dsRNAs (Abbasi et al. 2020). If pcRNAs can cause significant silencing in susceptible CPB, testing them with CEAS 300 insects can also confirm the association of CME with dsRNA resistance. Additionally, pcRNAs may be used to overcome dsRNA resistance. Several dsRNA carrier systems are also being explored to improve dsRNA uptake in insects refractory to RNAi. These systems may potentially be used to overcome dsRNA resistance by increasing uptake when resistance arises in the field.

Interestingly, genes implicated in metabolic resistance to insecticides in CPB, such as cytochrome p450s, are constitutively up-regulated in CEAS 300 insects. Bioassays with PBO, an inhibitor of cytochrome p450 enzymes (Silcox, Ghidiu, and Forgash 1985), did not affect resistance to dsRNA in CEAS 300. Whole transcriptome sequencing demonstrated that several immune and stress-related pathways are also up-regulated in CEAS 300 insects. These pathways and genes, including cytochrome p450, are up-regulated in response to virus infection in *Aedes aegypti* and *Myzus persicae* (Liang et al. 2021; Zhao et al. 2018). A constitutive enhanced immune response may be an additional mechanism contributing to dsRNA resistance in CEAS 300. Suppressing immune-related genes by RNAi has been shown to enhance susceptibility to Bt insecticidal proteins in *Bombyx Mori* (Yang, Sun, et al. 2022) and *Spodoptera littoralis* (Caccia et al. 2020). Feeding *Citrobacter freundii*, a bacterium naturally present in the CPB gut microbiome, increased susceptibility to *Bacillus thuringiensis* var. *tenebrionis* due to changes in immunity (Polenogova et al. 2022). Whether an enhanced immune response is associated with dsRNA

resistance in CEAS 300 can be tested by comparing susceptibility to pathogens, especially viral pathogens. Though no significant difference in mortality between susceptible and dsRNA-resistant CPB was observed at the highest Cry3Aa protoxin concentration tested, a slight increase in tolerance was observed at intermediate concentrations. It would be interesting to test if increased tolerance is observed when using a bacterial Bt culture instead of a purified toxin. If the role of enhanced immune response in dsRNA resistance is confirmed, combining dsRNA insecticides with immune suppressors or targeting immunity-related genes may allow for better control and delay resistance evolution.

It is important to highlight that several DEGs in CEAS 300 compared to both METT and GC (19 out of 45 in untreated insects and 9 out of 17 in treated insects) are annotated as 'uncharacterized' in the CPB assembly. Some of these are long non-coding RNAs (lncRNAs) and may be involved in the epigenetic regulation of dsRNA resistance associated genes. The involvement of lncRNAs in insecticide resistance has been demonstrated in several insects (Shi et al. 2022; Meng et al. 2021; Liu et al. 2017). However, several of the 'uncharacterized' genes are predicted to be protein-coding. This may suggest that many genes with potential involvement in dsRNA resistance are yet unknown.

Genomic loci associated with dsRNA resistance

Identification of genomic loci associated with resistance can further the understanding of resistance mechanisms and allow monitoring of resistance alleles in field populations. Regular monitoring of resistance levels to new insecticides allows for keeping track of resistance levels and identifying regions at high risk of resistance. In Chapter 4 of this dissertation, bulk segreant analysis (BSA) was performed to identify genomic regions associated with resistance in CEAS 300. As expected from the polygenic nature of resistance, multiple peaks representing significantly

different loci between susceptible (GC and METT) and resistant (CEAS 300) were detected. These peaks are located on chromosomes 5, 10, and 11, and contain 1, 5, and 18 genes, respectively. Two genes with nucleotide-binding domains were identified on chromosomes 10 and 11. Though the direct role of these genes in dsRNA binding is not known, the presence of RNA binding domains indicates the possibility of interaction with dsRNA or siRNAs generated by the RNAi pathway. The mechanism of systemic spread of the RNAi signal in insects is largely unknown. It is possible that these nucleotide binding proteins are involved in the systemic spread of siRNAs and thus, mutations in these genes can result in resistance due to lack of systemic spread. Located on chromosome 11, an uncharacterized CPB gene showed similarity to *microtubule-associated protein Jupiter-like* from *Anoplophora glabripennis* (Asian long-horned beetle). Microtubule-like structures have been suggested to play a role in systemic antiviral response in Drosophila cells (Karlikow et al. 2016). Alternatively, the nucleotide binding proteins may function as transcription factors and modulate the expression of dsRNA uptake or immune/stress-related genes. One transcription factor on chromosome 10 and three kinase genes on chromosome 11 were also identified. Both transcription factors and kinases are involved in the regulation of gene expression and have been associated with insecticide resistance (Yang et al. 2020; Guo et al. 2022; Li and Liu 2017, Ingham et al. 2017).

Clavesin-2-like was located on the peak of chromosome 11, contains a phosphatidylinositol bisphosphate (PIP) binding domain, and is involved in clathrin-mediated endocytosis (Katoh et al. 2009). The role of *clavesin-2-like* in the disruption of CME and dsRNA resistance in CEAS 300 can be confirmed using CRISPR-cas9. Additionally, linkage tests can be used to further narrow down the list of candidate loci and develop DNA-based screening tests for resistance monitoring.

Final remarks

This dissertation provides valuable information that can guide the development of an IRM framework for dsRNA insecticides, particularly foliar dsRNA sprays targeting the Colorado potato beetle. Our results support the use of a refuge strategy as well as the use of alternative control methods such as Bt insecticidal proteins and chemical insecticides to delay resistance. It is however important to acknowledge IRM as a social science issue rather than a purely scientific problem. Resistance management frameworks are only effective when followed appropriately. Thus, importance should be given to proper education and compliance with IRM by the farmers. An important aspect of RNAi-based insecticides is their slow mode of action compared to chemical insecticides. This expectation should be carefully relayed to farmers to limit unnecessary treatment when quick insect mortality is not observed. The association of reduced dsRNA uptake and disruption of clathrin-mediated endocytosis pathway provides an opportunity to develop delivery methods to overcome resistance. A list of candidate genes associated with resistance is also identified, and upon further validation can be used for monitoring resistance alleles in field CPB populations.

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VITA

Swati Mishra received her bachelor's in biotechnology from Punjab Agricultural University, India. She moved to the US in 2017 to pursue Masters in Entomology and Plant pathology at the University of Tennessee, Knoxville under the guidance of Dr. Juan Luis Jurat-Fuentes. After completing her masters in 2019, she started her PhD journey in the same lab. Her research focused on characterizing double-stranded RNA (dsRNA) resistance mechanisms in the Colorado potato beetle. During this time, she gained significant technical expertise in dsRNA insecticides, insect rearing, bioassay design, microscopy, and transcriptomic and genomic data analyses.

Throughout her academic journey, Swati has been recognized for her excellence and dedication. She has received several prestigious awards, including the Graduate Student Senate Award for Excellence in Graduate Research and the Outstanding Research Publication award from her department. Her work has been published in leading scientific journals and she has been an active presenter at various international and national scientific conferences. In addition to her research accomplishments, Swati has demonstrated strong leadership and mentoring skills, organizing seminars and symposia, advocating for graduate student needs, and guiding undergraduate and visiting scholars in their research endeavors.