RNA interference of three up-regulated transcripts associated with insecticide resistance in an imidacloprid resistant population of *Leptinotarsa decemlineata*

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d Article info

1. Introduction

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is a major agricultural pest of potatoes in the Central Sands production region of Wisconsin. Previous studies have shown that populations of *L. decemlineata* have become resistant to many classes of insecticides, including the neonicotinoid insecticide, imidacloprid. Furthermore, *L. decemlineata* has multiple mechanisms of resistance to deal with a pesticide insult, including enhanced metabolic detoxification by cytochrome p450s and glutathione S-transferases. With recent advances in the transcriptomic analysis of imidacloprid susceptible and resistant *L. decemlineata* populations, it is possible to investigate the role of candidate genes involved in imidacloprid resistance. A recently annotated transcriptome analysis of *L. decemlineata* was obtained from select populations of *L. decemlineata* collected in the Central Sands potato production region, which revealed a subset of mRNA transcripts constitutively up-regulated in resistant populations. We hypothesize that a portion of the up-regulated transcripts encoding for genes within the resistant populations also encode for pesticide resistance and can be suppressed to re-establish a susceptible phenotype. In this study, a discrete set of three up-regulated targets were selected for RNA interference experiments using a resistant *L. decemlineata* population. Following the successful suppression of transcripts encoding for a cytochrome p450, a cuticular protein, and a glutathione synthetase protein in a select *L. decemlineata* population, we observed reductions in measured resistance to imidacloprid that strongly suggest these genes control essential steps in imidacloprid metabolism in these field populations.

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1. Introduction

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is a major agricultural pest of potatoes (*Solanum tuberosum*) encompassing the United States, Mexico, Europe, and Asia, inclusive of over 16 million km² [1]. Studies have shown that many populations of *L. decemlineata* obtained from agricultural fields with a prior history of imidacloprid use have measurable levels of resistance [2–6]. Imidacloprid, a neonicotinoid insecticide (IRAC MoA 4A), became commercially available in 1995 when it was registered in potatoes [6–8], and quickly gained popularity with pest managers because of its flexibility in application patterns (at-plant, foliar spray, side-dress, etc.). As an at-plant application, neonicotinoids have a long duration residual activity against many pest species and are relatively safe for humans and other mammals [9]. Relatively soon after its initial registration, studies indicated that the effectiveness of imidacloprid against target populations of *L. decemlineata* started to decline, and field populations of beetles developed resistance in select locations of the United States [2,4–6]. Even as populations of *L. decemlineata* continue to increase in their relative levels of insensitivity to imidacloprid, it remains as one of the most widely used insecticides for control of problematic populations of *L. decemlineata*. In the Central Sands region of Wisconsin specifically, most potato producers rely on an at-plant (in-furrow or seed treatment) application of a neonicotinoid (e.g. imidacloprid, thiamethoxam) for control of *L. decemlineata*, as well as other key pests of the potato crop. As levels of product sensitivity continue to decline in *L. decemlineata*, producers and pest managers are facing new and emerging challenges to control this pest, resulting in repeated foliar applications of alternate mode of action insecticides, reduced grower profit, and a larger environmental footprint of potato pest management [10,11].

The pathways in which neonicotinoids are detoxified in insects have been studied in-depth [12,13], highlighting the role of cytochrome p450 and glutathione S-transferase enzymes as principle agents in neonicotinoid metabolism. Recently, three studies described transcriptomic resources that enhance our ability to classify modes of resistance in *L. decemlineata* [3,14,15]. Using transcriptomic data as a
template to measure genetic responses to imidacloprid, experimental assays of mRNA transcript level regulation in *L. decemlineata* [3] found multiple, up-regulated mRNA transcripts (statistically significant increases in mRNA transcript abundance levels) that were constitutively active in beetles from two resistant field populations in Wisconsin when compared to a reference susceptible strain. In the present study, we attempt to assess the role of these up-regulated mRNA transcripts that encode for genes responsible for insecticide resistance by knock-down (suppression of transcripts that encode for targeted genes) experiment using RNA interference (RNAi). We hypothesize that after reduction of transcript abundance (gene knock-down) utilizing RNAi of key genes involved in imidacloprid resistance, we can re-establish a susceptible phenotype in a resistant population of beetles. Successful re-establishment of a susceptible phenotype from a resistant *L. decemlineata* population would strongly suggest which genes encode for essential steps in imidacloprid metabolism in these field populations.

In recent years, RNAi has come into widespread use for in vivo studies of insects, to specifically target a gene of interest and assay phenotypic impacts [16]. This technique is tractable in *L. decemlineata*. Zhu et al. [17] used double-stranded RNA (dsRNA) produced in bacteria to knock-down housekeeping genes that encode for essential biological functions in *L. decemlineata*. Following ingestion of bacteria producing an RNAi trigger, knock-down of the target genes was confirmed; therefore, per os RNAi is effective in *L. decemlineata*. Additionally, RNAi triggers (e.g., β-actin) can be produced in *pianta* to effectively deliver dsRNA and induce lethality in early instars of *L. decemlineata* [18]. Successful RNA interference can be influenced by several factors including delivery of the RNA, size of the target transcript and associated dsRNA, and the ability to observe or accurately measure the phenotypic effects of the specific knock-down on the target. In the current study, we used a micro-injection approach to deliver dsRNA directly to the hemocoel of adult *L. decemlineata*, to develop proof of concept in the role of three candidate genes in imidacloprid resistance. More specifically, the current investigations focused on individuals from a specific resistant field population (Systemic-3) of *L. decemlineata* which has an estimated resistance ratio of 11.16 (imidacloprid resistance when compared to a reference, susceptible field population) measured in the 2014 crop year [3], and is a population which has been monitored for imidacloprid resistance since 2007 [4]. Moreover, this population possessed elevated transcript levels for genes, including 13 cytochrome p450s and 23 cuticular proteins, which were involved in several potential modes of insecticide resistance when compared to a susceptible population [3].

2. Experimental methods

2.1. Confirmation of mRNA transcripts encoding genes of interest

Among a set of 562 up-regulated, mRNA transcripts found to be significantly more abundant in an imidacloprid resistant population (Systemic-3) [3] compared to an imidacloprid susceptible population, three transcripts encoding for genes were chosen as candidate targets for dsRNA-based, RNA interference; a cytochrome p450 (NCBI accession number GEEF01131148), a cuticular protein (NCBI accession number GEEF01064138), and a glutathione synthetase (NCBI accession number GEEF0119768). A 2.77, 3.96, and 2.90 fold change in constitutively regulated transcript abundance was measured in this resistant *L. decemlineata* population and was determined to be statistically significant in a whole-transcriptome assay. Their potential role in insecticide resistance, and their abundant transcript levels, highlights them as candidates for functional validation. These transcripts were subjected to RACE PCR to generate full-length sequences, using the Smarter Rapid Amplification of cDNA Ends (RACE) cDNA amplification kit (Clontech, Mountain View, CA) and primers designed from the transcriptome sequences. The resulting products from RACE were inserted into pCR™-Blunt II-TOPO® vector and cloned into One Shot® E. coli Chemically Competent cells (Life Technology, Grand Island, NY) and delivered for sequencing (University of Wisconsin-Madison Biotech Center, Madison, WI). Once obtained, sequence data was combined with transcriptomic data to obtain full length sequences (Supplementary Table S1). RACE primers are illustrated in Table 1.

2.2. dsRNA generation

A set of 17 tagged primers (Table 2) were designed to amplify –250–500 bp lengths of the genes of interest to generate PCR products for in vitro transcription and production of dsRNA (Supplementary Table S2). Primer specificity was checked against the transcriptome using standalone BLAST [19]. Specific gene segments were amplified using RT-PCR from RNA converted to cDNA extracted from adult, female *L. decemlineata* collected from the Systemic-3 population [3]. A heterologous control, used to induce RNAi machinery, was also generated for the gene that encodes enhanced green fluorescent protein (EGFP). The amplified segments were run on a 1.2% agarose gel, extracted and purified with a Gel/PCR DNA Fragment Extraction Kit (IBI scientific, Peosta, IA). The resulting fragments were inserted into pCR™-Blunt II-TOPO® vector and cloned into One Shot® E. coli Chemically Competent cells (Life Technology, Grand Island, NY). Confirmation of the correct insert was evaluated by sequencing plasmids for the gene segments (Biotech center, Madison, WI). To generate large quantities of template, plasmids with the correct inserts were used for Phusion high fidelity amplification RT-PCR (New England Biolabs, Ipswich, MA) with T7 tagged primers. The amplification products were cleaned with a Gel/PCR DNA Fragment Extraction Kit (IBI scientific, Peosta, IA) and used in T7 RiboMax Express Large Scale RNA Production System (Promega, Madison, WI). The single stranded RNA products were cleaned using an Illustra™ MicroSpin™ C-25 Column (GE Healthcare, Piscataway, NJ, USA). Single stranded RNA were annealed to the complimentary strand by incubation at 94 °C/1 min followed by 26 °C/10 min. The concentration of dsRNA was measured using a Nanodrop (Thermo Fisher Scientific, Waltham, MA) and all samples were brought to a concentration of 500 ng/µl prior to injection into the hemocoel.

2.3. RNAi though dsRNA knock-down

In late July and early August of 2014, approximately 1500 2nd generation, adult *L. decemlineata* (Systemic-3) with a documented history of imidacloprid insensitivity [3] were collected from an agricultural field in the Central Sands region of Wisconsin. All adult beetles were caged for at least 72 h in insect-proof cages in a greenhouse and fed fresh, untreated potato foliage before any studies were conducted. A preliminary diagnostic assay was initiated to first determine an appropriate concentration of dsRNA that would result in suppression of target gene transcript abundance in *L. decemlineata* using dsRNA for the Comp115309 cytochrome p450 gene. Specifically, dsRNA was diluted to produce a variety of concentrations from (100 ng/µl–500 ng/µl) and injected into adult female *L. decemlineata* between the 1st and 2nd abdominal sternite using a 10 µ Hamilton syringe (Hamilton Company, Reno, NV). At 24 and 48 h after injection, RNA was extracted from whole insect preparations using Trizol (Life Technology, Grand Island, NY) and cDNA was generated with a high capacity, cDNA reverse transcription kit (Applied Biosciences, Foster City, CA). A preliminary RT-PCR was conducted to observe knock-down of transcript abundance at 24 and 48 h after exposure to different concentrations of dsRNA. The reaction was performed at 98 °C for 30 s followed by 98 °C for 10 s, 63 °C for 30 s, and 72 °C for 30 s, totaling 18 cycles, followed by a final extension step of 72 °C for 10 min. PCR product was visualized on a 1.2% agarose gel. The preliminary results demonstrated reduced transcript abundance levels at a concentration as low as 190 ng/µl and 24 h after injection. However, using quantitative PCR diagnostic assays, the greatest knock-down was not observed until the concentration of dsRNA had reached, or surpassed, 500 ng/µl and 48 h had passed after injection (Supplementary Fig. S3). Based on this data, approximately
100 adult beetles were similarly injected per group for each gene target. As previously described, individual beetles were injected with 1 μl of the 500 ng/μl dsRNA concentrate using a 10 μl Hamilton syringe (Hamilton Company, Reno, NV). All adult beetles were injected on the ventral surface of the abdomen between the 1st and 2nd sternites (Fig. 1). Beetles were placed back into their respective cages (based upon gene target) for a period of 48 h to accommodate the RNAi process.

2.4. Phenotypic assays of dsRNA knock-down

Assays were preformed to observed median lethal concentration (LC50) on knock-downs for each gene to determine if the transcript knock-down resulted in a phenotypic change in the imidacloprid resistant population. Forty hours post-injection, beetles were bioassayed with a 1 μl solution of imidacloprid (Technical grade 98.80%) carried in acetone ranging in concentrations between 0 and 1000 ppm applied the dorsal surface between the first and second sternites. Specifically, beetles that survived dsRNA injections were divided into 5 equal groups ranging from approximately 9 to 15 individuals and treated with a range of imidacloprid concentrations. All adult L. decemlineata were held in an incubator at 26 °C, 70% humidity, a phosphomannose isomerase (PMI) transcript variant X1 (accession XM 008196868.1) with an estimated identity of 64% as of March 2016. Finally, Comp 114026 (glutathione synthetase) had a query coverage of 79% to T. castaneum (cytochrome) had a query coverage of 79% to T. castaneum (cytochrome p450 (cyp9Z26, accession KJ476503.1) and with an estimated identity of 97%. Comp 105889 (cuticular protein) had the highest query coverage of 41% to Tribolium castaneum cuticle protein 18.7 (accession XM 964708.2) and an estimated identity of 66%. Comp 459 (glutathione synthetase) had a query coverage of 79% to T. castaneum glutathione synthetase-like transcript variant X1 (accession XM 008196868.1) with an estimated identity of 64% as of March 2016.

An appropriate concentration of dsRNA required for successful gene knock-down was determined to be 500 ng/μl (Supplementary Fig. S3). Mortality from injection ranged from 18% in the cytochrome p450 knock-down to 49% in the cuticular protein knock-down. Forty-eight hours after the beetles were injected with dsRNA, expression levels of the transcripts were measured with qPCR; the outcome of which resulted in successful gene knock-down. β-actin primers were used as a reference gene.

| Table 2 |
| T7 tagged primers used to generate dsRNA for RNAi in adult Leptinotarsa decemlineata. T7 sequence is provided in uppercase letters. |

<table>
<thead>
<tr>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′-TAAATAGCTACATATAGGGGCAAATGATTGATGCTA-3′</td>
<td>5′-TAATAGCTACATATAGGGGCAAATGATTGATGCTA-3′</td>
</tr>
<tr>
<td>5′-TAATAGCTACATATAGGGGCAAATGATTGATGCTA-3′</td>
<td>5′-TAATAGCTACATATAGGGGCAAATGATTGATGCTA-3′</td>
</tr>
<tr>
<td>5′-TAATAGCTACATATAGGGGCAAATGATTGATGCTA-3′</td>
<td>5′-TAATAGCTACATATAGGGGCAAATGATTGATGCTA-3′</td>
</tr>
<tr>
<td>5′-TAATAGCTACATATAGGGGCAAATGATTGATGCTA-3′</td>
<td>5′-TAATAGCTACATATAGGGGCAAATGATTGATGCTA-3′</td>
</tr>
</tbody>
</table>

3. Results

The RACE amplification produced full transcript sequences as illustrated in Supplementary Table S1. Following a BLAST search using elongated sequence, Comp 115309 (cytochrome p450) had the highest query coverage of 97% to L. decemlineata cytochrome p450 (cyp9Z26, accession KJ476503.1) and with an estimated identity of 97%. Comp 105889 (cuticular protein) had the highest query coverage of 41% to Tribolium castaneum cuticle protein 18.7 (accession XM 964708.2) and an estimated identity of 66%. Finally, Comp 114026 (glutathione synthetase) had a query coverage of 79% to T. castaneum glutathione synthetase-like transcript variant X1 (accession XM 008196868.1) with an estimated identity of 64% as of March 2016.

An appropriate concentration of dsRNA required for successful gene knock-down was determined to be 500 ng/μl (Supplementary Fig. S3). Mortality from injection ranged from 18% in the cytochrome p450 knock-down to 49% in the cuticular protein knock-down. Forty-eight hours after the beetles were injected with dsRNA, expression levels of the transcripts were measured with qPCR; the outcome of which resulted in successful gene knock-down. β-actin was used as a reference gene.
in the knock-downs to ensure that the injections themselves did not affect the levels of transcript expression. Knock-down efficiency varied among the target genes, with glutathione synthetase resulting in 27.56× less expression than the control, the cytochrome p450 resulting in 14.97× less expression, and the cuticular protein resulting in 5.83× less expression Table 4.

The phenotypic effects of a reduction in transcript abundance (gene knock-down) was assessed in L. decemlineata using an LC50 topical bioassay. Gene knock-downs were shown to re-establish a level of susceptibility, as shown in the resulting LC50 experiments, and effective knock-down was confirmed by qPCR. Results of the LC50 topical bioassays are presented in Table 5 and measured 48 and 168 h after topical application. The no injection control had an estimated LC50 of 732.24 ppm after 48 h and 495.33 ppm after 168 h, representing the mean LC50 of the resistant Systemic-3 population. The EGFP control had a significantly lower LC50 compared to the controls (4.62 ppm after 48 h and 1.62 ppm after 168 h), indicating the no injection control had a lower toxicity than the EGFP control. The cytochrome p450 and glutathione synthetase knock-downs were both lower than controls after 48 h, while the cytochrome p450 and glutathione synthetase protein knock-downs were both lower than controls after 168 h.

4. Discussion

Select populations of L. decemlineata throughout the United States have recently developed resistance to many of the major classes of insecticides, including the Group 4A neonicotinoid class, in several potato production regions [2,4–6,11]. One hypothesis to explain L. decemlineata’s capability to rapidly develop resistance to imidacloprid relies on the pre-formed molecular mechanisms used to metabolize plant secondary metabolites (alkaloids), including cytochrome p450s and glutathione-s-transferases [1,15]. The purpose of the current study was to determine whether three sets of transcripts encoding for genes which are up-regulated in a resistant population are at least partially responsible for increased imidacloprid resistance and whether RNAi could be used to knock-down putative resistance genes with the goal of re-establishing a susceptible phenotype. Clements [3] identified candidate, resistance genes that had mRNA transcripts highly upregulated when comparing imidacloprid susceptible and resistant populations in Wisconsin. In the current study, we knocked-down one gene including an up-regulated glutathione synthetase (partially responsible for the production of glutathione used by glutathione S-transferase to carry out phase 2 metabolisms of neonicotinoids), the most up-regulated cuticular protein, and one of the most up-regulated cytochrome p450s in the resistant population, compared to a susceptible population. Furthermore, after the RACE generation of full-length sequences, the cytochrome P450 had the highest query coverage of 97% to a L. decemlineata cytochrome p450 (cyp9Z26, accession KJ476503.1). Zhu demonstrated that cyp9Z26 was also significantly up-regulated in a Long Island, NY imidacloprid resistant population [15], which suggests that in both a Wisconsin and New York population, the up-regulation of this important detoxification mechanism could be responsible for imidacloprid resistance.

A principle goal of the current study was to re-establish susceptibility to the insecticide imidacloprid within a resistant population of L. decemlineata as a possible component of pest management. Although RNAi possesses considerable potential for studying gene function, RNAi may also possess some drawbacks, including potential off-target effects on genes that have similar homology, and it is possible to knock-down non target genes and genes of other non-target species [22]. We took care when designing primers and creating unique dsRNA for each gene. Confirmation of RNAi knock-down was measured 48 h after injection and qPCR. To establish that no general knock-down took place, we used an internal control of β-actin to compare transcript expression. A successful knock-down of genes of interest was observed to varying degrees; the expression of glutathione synthetase was knocked down 27.56× compared to the control, the cytochrome p450 was knocked down 14.97×, and the cuticular protein had the smallest knockdown of only 5.83× when compared to the control. A wide range of variation among gene targets was observed in the current study using LC50 bioassays are presented in Table 5 and measured 48 and 168 h after topical application. The no injection control had an estimated LC50 of 732.24 ppm after 48 h and 495.33 ppm after 168 h, representing the mean LC50 of the resistant Systemic-3 population. The EGFP control had a significantly lower LC50 compared to the controls (4.62 ppm after 48 h and 1.62 ppm after 168 h), indicating the no injection control had a lower toxicity than the EGFP control. The cytochrome p450 and glutathione synthetase knock-downs were both lower than controls after 48 h, while the cytochrome p450 and glutathione synthetase protein knock-downs were both lower than controls after 168 h.

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### Table 4

Quantitative PCR confirmation of transcripts levels that encode for genes, plus mean percent survival estimates of adult *Leptinotarsa decemlineata* 48 h post injection.

<table>
<thead>
<tr>
<th>Gene Target</th>
<th>Cytochrome p450</th>
<th>Cuticular protein</th>
<th>Glutathione synthetase</th>
<th>EGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin (control)</td>
<td>21.30 ± 0.36</td>
<td>21.30 ± 0.36</td>
<td>21.30 ± 0.36</td>
<td>NA</td>
</tr>
<tr>
<td>β-actin (knock-down)</td>
<td>21.18 ± 0.07</td>
<td>21.30 ± 0.18</td>
<td>20.24 ± 1.8</td>
<td>NA</td>
</tr>
<tr>
<td>Targeted expression in control</td>
<td>20.45 ± 1.07</td>
<td>25.30 ± 1.17</td>
<td>27.14 ± 1.70</td>
<td>NA</td>
</tr>
<tr>
<td>Targeted expression in knock-down</td>
<td>24.33 ± 1.27</td>
<td>27.90 ± 0.21</td>
<td>30.91 ± 2.17</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Table 3**

Quantitative PCR primers and primer efficiency used for quantification of transcript abundance after dsRNA injection.

<table>
<thead>
<tr>
<th>Forward Primer (5′-3′)</th>
<th>Reverse Primer (5′-3′)</th>
<th>Primer efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin (reference) Comp115309 (cytochrome p450) Comp105889 (cuticular protein) Comp14626 (glutathione synthetase)</td>
<td>CATCAGAGCTGACTCCTCTCCGTG</td>
<td>CAGCAGGCCTGAACTTTCCGTAG</td>
</tr>
<tr>
<td>CGAGAAATCCGACATTCGACAG</td>
<td>ACACAGTCTGCTCTTCGGCAG</td>
<td>1.98</td>
</tr>
<tr>
<td>CTCAGTGGTCTCCGTATACAC</td>
<td>AGCGTATCTCGAATAGTTG</td>
<td>1.94</td>
</tr>
<tr>
<td>CAGCAGGCCTGAACTTTCCGTAG</td>
<td>CAGCAGGCCTGAACTTTCCGTAG</td>
<td>1.97</td>
</tr>
</tbody>
</table>
bioassays to evaluate the effects of gene knock-down and a possible return to a susceptible phenotype. Baseline LC50 estimates for the resistant field population (Systemic-3) was estimated to be 495.33 ppm after 168 h, whereas the cytochrome p450 knock-downs had an estimated LC50 value of 145.60 ppm, the cuticular protein had an estimated LC50 value of 340.65 ppm and the glutathione synthase LC50 had an estimated LC50 value of 254.85 ppm. The estimated LC50 value of the EGFP injected control was found to be 335.53 ppm. This value suggests that the dsRNA EGFP itself may have had a potential effect on insect survivorship. Although EGFP is not present within the genome of *L. decemlineata*, it may be possible that the EGFP dsRNA had off target effects resulting in lower LC50 values. Although EGFP is commonly used as a control in RNAi studies, previous research has demonstrated that dsEGFP can have off target effects and may have substantial indirect effects on transcript levels associated with multiple biological processes [3]. For example, a study conducted in *Bactericera cockerelli* demonstrated relativity high mortality in individuals injected with dsRNA encoding for GFP. When injected with 200 nl of 100 ng/µl dsRNA, mortality of 58% was observed after 6 days [24]. Nevertheless, all of our target gene knock-downs showed a decrease in resistance in the LC50 assays. This suggests that the down-regulation of these genes can result in a more susceptible phenotype from an imidacloprid resistant population and that up-regulation may be partially responsible for increased imidacloprid resistance. The knock-down that showed the largest decrease in resistance was the cytochrome p450, further suggesting that this gene may play a significant role in imidacloprid resistance in this select population of *L. decemlineata*.

Although the results of the LC50 studies had large confidence intervals, we expect that this reflects natural variation among members of a beetle population from an agricultural field. This field population is most likely composed of a heterogeneous group of highly (homozygotic) and moderately (heterozygotic) resistant individuals. As a result, the genes that encode for detoxification are likely differentially expressed between individuals in this highly resistant population, leading to large confidence intervals. This observation has been previously described in similar field populations for 1st generation beetles in 2014, where the LC50 value was estimated to be 52.68 ppm (95% CI: 25.31–154.56) [3]. This data further suggests the possibility that not all beetles in each treatment are homozygous for the genes that encode resistance and certain beetles can be more susceptible to imidacloprid without ever knocking-down resistant genes, leading to variation in LC50 within groups. Moreover, imidacloprid resistance could be polygenic and knocking-down just a single gene may have only limited effects on *L. decemlineata* susceptibility.

RNA interference is becoming an increasingly common method to knock-down genes of interest in insects and may have important application in developing gene-targeted pesticides. Previous studies have demonstrated the ability to knock-down specific genes in larval and adult *L. decemlineata* by microinjection and feeding assays using target dsRNA [17,18,25]. Zhang [18] demonstrated a transgenic plant that could express dsRNA of *L. decemlineata* using a dsRNA homolog of β-actin which resulted in mortality of immature stages of *L. decemlineata*. Previous work by Katoch illustrates the importance of correctly choosing a delivery method for RNAi, and suggests that per os delivery of dsRNA is less effective than injecting dsRNA [22], presumably because many insect taxa do not possess the appropriate systemic interfering defective (SID) proteins necessary for dsRNA transport across midgut barriers [26]. The transcript abundance assays conducted by Clements [3] extracted RNA from whole insect preparations. To limit the effects of localized dsRNA, it was concluded that injections into the hemocoel was appropriate method to deliver dsRNA to suppress transcripts of targeted genes. Although injections were used in this study and would be impractical in an agricultural field setting, it was determined that injecting beetles with dsRNA was appropriate to demonstrate proof of concept. Successful knock-down was demonstrated by qPCR for all three genes, and was a requirement to determine whether the up-regulation of mRNA transcript levels found in the resistant population was responsible for an increase in imidacloprid resistance. Determining genes that are responsible for imidacloprid resistance through injections is one step to develop new methods for controlling beetle populations and is the building block to generate new technologies.

Injections of dsRNAs may impact insect survivorship; which is often observed within 48 h after injection as the knock-down takes place. In our investigation, mortality from the injection of dsRNA before insecticidal exposure ranged from 18 to 49%, depending on the dsRNA target, with an observed mortality of 22% in the EGFP control. The highest mortality of 49% after injection resulted from the cuticular protein. One plausible explanation may result from the fact that this cuticular protein may be multi-functional and could play important roles in other metabolic functions, inducing far greater mortality than other dsRNA injections. Work performed by Jasrapuria on *Tribolium castaneum* demonstrated the diverse functions of cuticular proteins, including the suppression of TcCPAP3-A1 by dsRNA injections leading to a lethal phenotype in mature adults [27].

### Table 5

Median lethal concentration (LC50) estimates resulting from imidacloprid bioassays at 48 and 168 h after topical application.

<table>
<thead>
<tr>
<th>Target</th>
<th>Time point (hours)</th>
<th>N</th>
<th>Slope (SEM)</th>
<th>LC50 (ppm)</th>
<th>95% CI</th>
<th>Chi-square</th>
<th>PR &gt; Chi-square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control no injection</td>
<td>48</td>
<td>75</td>
<td>2.06 (0.60)</td>
<td>732.24</td>
<td>(486.09–1799)</td>
<td>11.61</td>
<td>0.0007</td>
</tr>
<tr>
<td>EGFP</td>
<td>48</td>
<td>65</td>
<td>2.31 (0.62)</td>
<td>274.23</td>
<td>(167.41–404.18)</td>
<td>13.79</td>
<td>0.0002</td>
</tr>
<tr>
<td>Comp115309 (cytochrome p450)</td>
<td>48</td>
<td>71</td>
<td>1.75 (0.55)</td>
<td>203.23</td>
<td>(75.45–320.23)</td>
<td>9.88</td>
<td>0.0017</td>
</tr>
<tr>
<td>Comp105889 (cuticular protein)</td>
<td>48</td>
<td>45</td>
<td>1.57 (0.69)</td>
<td>240.75</td>
<td>(25.13–530.86)</td>
<td>5.14</td>
<td>0.0234</td>
</tr>
<tr>
<td>Comp114026 (glutathione synthetase)</td>
<td>48</td>
<td>65</td>
<td>2.07 (0.60)</td>
<td>231.78</td>
<td>(121.98–351.07)</td>
<td>11.97</td>
<td>0.0005</td>
</tr>
<tr>
<td>Control no injection</td>
<td>168</td>
<td>75</td>
<td>2.45 (0.59)</td>
<td>495.33</td>
<td>(350.38–788.49)</td>
<td>11.17</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EGFP</td>
<td>168</td>
<td>65</td>
<td>3.42 (0.75)</td>
<td>335.53</td>
<td>(244.78–449.31)</td>
<td>20.78</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Comp115309 (cytochrome p450)</td>
<td>168</td>
<td>71</td>
<td>1.38 (0.53)</td>
<td>145.60</td>
<td>(9.73–262.52)</td>
<td>6.59</td>
<td>0.0103</td>
</tr>
<tr>
<td>Comp105889 (cuticular protein)</td>
<td>168</td>
<td>45</td>
<td>1.82 (0.69)</td>
<td>340.65</td>
<td>(156.93–822.69)</td>
<td>6.96</td>
<td>0.0084</td>
</tr>
<tr>
<td>Comp114026 (glutathione synthetase)</td>
<td>168</td>
<td>65</td>
<td>1.92 (0.57)</td>
<td>258.45</td>
<td>(134.92–407.55)</td>
<td>11.16</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

a Slope ± standard error of the mean (SEM) estimates of the estimated probit function regression function.

b 95% confidence interval (CI) estimates around mean LC50 estimates.

Chi-square analysis of effects of the proc probit regression.
Resistance of *L. decemlineata* to imidacloprid is now common throughout many portions of the United States [11,28]. However, neonicotinoids are still commonly used for control of this problematic pest [11]. Out of necessity, novel approaches are warranted to increase the longevity of this important class of insecticide chemistry, as well as developing an understanding of how this insect copes with insecticides and accurately determining the genes involved in resistance development. Many researchers are developing RNAi to find viable gene targets for agricultural settings and urban pests, as available insecticides are becoming less effective as pests develop resistance to insecticides [16,29–31]. The potential for RNAi to be used on up-regulated, molecular mechanisms of resistance could be a feasible option for pest management and needs to be studied in-depth. Knocking-down specific genes in insect taxa that have the ability to metabolize insecticides may open new doors in pest management practices, for example by allowing limited, short-duration pesticide applications when coupled with RNAi treatment. Many studies in the past decade have investigated possible RNAi targets, including genes such as β-actin [17,18]. Still other studies have used a variety of gene targets, including genes responsible for essential metabolic processes; one such case focuses on the disruption of metabolic processes corresponding to insect development and wing formation, resulting in the inability of insect death [32]. Some of these RNAi targets appear to require long duration exposure or repeated exposure in the field to effectively knock-out essential metabolic processes until mortality results. Further, previous studies have targeted specific receptors to determine their function in resistance [33]. Our study is unique in that we targeted metabolic detoxifying enzymes that were up-regulated in an imidacloprid resistant agricultural field population. By targeting genes that are up-regulated due to imidacloprid resistance in specific populations (classified by mRNA transcript abundance), we suggest that efficacy of conventional insecticides could be improved when used in combination with targeted RNAi technology to control resistant *L. decemlineata* populations. However, further research is needed to determine whether knock-down of one or a few key resistance genes is sufficient to increase susceptibility in an agricultural setting and improve the sustainability of *L. decemlineata* management.

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