Evaluating different double-stranded RNA structures for their ability to control pest flea beetles.

by

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Abstract

Canola is an economically important Canadian crop that suffers significant annual losses by damage from feeding flea beetles (*Phyllotreta cruciferae* and *P. striolata).* Increasing incidences of resistance and concerns about off-target effects with current insecticides demands new methods of control. Recently, environmentally safer approaches to pest control have been investigated using RNA interference (RNAi), a sequence-specific gene silencing mechanism triggered by exogenous double-stranded RNA (dsRNA), to selectively induce the mortality of targeted species.

This study examined three different dsRNA structures for their ability to kill *P. striolata* flea beetles and thereby reduce feeding damage on canola leaves. Long linear dsRNAs (212-214 bp), short hairpin RNAs (hpRNAs) (21-24 bp), and short paperclip RNAs (pcRNAs) (21-24 bp) targeting mRNAs of three essential genes, *Ras opposite (Rop), Sec23,* and *Snf7* in *P. striolata* were investigated. *P. striolata* adults were fed dsRNA-treated canola leaf disks, and impacts on insect survivorship and leaf material consumption were recorded over an eight-day period.

pcRNAs targeting *Sec23* and *Snf7,* and long dsRNAs targeting *Sec23* and *Rop* effectively killed flea beetles, resulting in the reduced consumption of treated canola leaf tissues. Consumption of both the *Sec23*-specific long dsRNA and pcRNAs caused similar levels of flea beetle mortality (68% and 76%, respectively), whereas only the *Rop*-specific long dsRNA and the *Snf7* pcRNA were effective at killing the beetles (76% and 84%, respectively). hpRNAs proved the least effective across all gene targets, killing at most, between 40-52% of flea beetles, depending on the gene target, and in general, these values were not significantly different relative to the negative controls. While this study examined dsRNAs specific for only three target genes, it provides evidence that RNAi-

based pesticides have the potential to control these economically important pests and that short pcRNAs can be as effective as conventional long linear dsRNAs.

Acknowledgements

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I am also grateful to my parents, Sheila and Mike Verhaeghe, for their support throughout this project. Lastly, I would like to thank Andrea Guarino for her encouragement and assistance while editing our drafts and practicing our Honours Thesis defences.

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Introduction

Since its discovery three decades ago, a molecular process capable of sequence-specific gene regulation and pathogen defence, described as RNA interference (RNAi), has attracted considerable interest throughout the scientific community (Fire et al. 1991). RNAi is a conserved biological response that is either naturally mediated or induced by the introduction of exogenous double-stranded RNA (dsRNA) molecules to down-regulate the expression of protein-coding genes (Hannon 2002). RNAi has numerous applications, including revolutionizing the discovery and understanding of gene functions as a reverse genetics tool (Sen and Blau 2006), uses in clinical therapeutics (Bumcrot et al. 2006; Lin et al. 2008), and the potential to improve pest control strategies with the development of transgenic plants and species-specific pesticides (Zhu et al. 2011; Kunte et al. 2020; Chen et al. 2022; Dong et al. 2022).

RNAi processing begins with introducing exogenous dsRNA into an organism through ingestion, injection, or soaking, which is subsequently taken up into the cytosol of cells (Fire et al. 1998; Singh et al. 2013; Clark and Pazdernik 2016). The endonuclease Dicer then cleaves the long dsRNA into small interfering RNAs (siRNAs) that are 21 to 23 nucleotides in length (Zamore et al. 2000; Moore et al. 2010). The antisense strand of the siRNA duplex binds to the RNA-induced silencing complex (RISC) with the assistance of Argonaute proteins and double-stranded RNA-binding proteins (Moore et al. 2010). RISC enables the siRNA to bind a complementary mRNA molecule, which is subsequently cut by Argonaute and degraded by other endogenous nucleases (Moore et al. 2010). If enough of the target mRNA is degraded, the amount of specific protein will be reduced, which at the very least, can cause phenotypic changes in the affected cell, but

may also impact development or induce mortality of the entire organism (Singh et al. 2013; Vatanparast et al. 2017).

Due to its sequence-specific nature, RNAi has been extensively used as a reverse genetics tool to knock down gene expression and examine a gene's function (Hannon 2002; Mohr et al. 2010). Its specificity has also attracted researchers interested in applying RNAi to suppress cancer-associated genes (Berns et al. 2004; Sachse and Echeverri 2004; Lin et al. 2008). Clinical therapeutics using RNAi might also be explored as a potential treatment for infectious diseases, disorders, and viral infections (Bumcrot et al. 2006; Lin et al. 2008). Additionally, RNAi is emerging as a promising method of pest control to protect agricultural crops and control disease-vectoring insects due to its ability to induce selective mortality of the targeted species (Abbasi et al. 2020; Kunte et al. 2020).

RNAi technologies serve as a possible alternative to chemical pesticides in agriculture. RNAi-based pesticides are designed to silence essential, sequence-specific insect genes by either developing transgenic plants that express dsRNAs or using foliar dsRNA sprays for crop protection (Zhu et al. 2011; Yan et al. 2020; Chen et al. 2022; Dong et al. 2022). The choice of using transgenic plants versus foliar sprays depends on the feeding strategies of the targeted pest. For instance, applying foliar sprays onto plants is effective against insects that damage leaf tissues by chewing, as they can easily ingest large amounts of topically applied dsRNA (Hoang et al. 2022). In contrast, targeting sap-sucking insects requires dsRNA uptake and movement through the plant's vascular tissues, which is generally achieved with the development of transgenic plants (Dong et al. 2022). When developing RNAi crop protection tools, it is necessary to conduct moderate to high throughput bioassays that assess the impacts of dsRNAs on the insects

following (i) the direct application of dsRNA molecules onto the target insect, with penetration likely occurring through inter-segmental membranes or through their spiracles, (ii) feeding on dsRNAs that were topically applied to plant tissues, or (iii) microinjections into the hemolymph of target pests (Yu et al. 2013; Killiny et al. 2014; Kunte et al. 2020). To ensure sufficient dsRNA is delivered to the target pest to induce RNAi knockdown, optimization of dsRNA efficacy is also required. dsRNA optimization considers multiple factors, including improving the dsRNA delivery method, the choice of gene and the selected region and length within the target gene, the dsRNA concentration, and the dsRNA stability (Miller et al. 2012; Fan et al. 2022; Hoang et al. 2022).

A crop that is particularly threatened by damage from insect populations is canola. In Canada, canola is the number one source of farm crop revenue and has increased from approximately 3 billion to 10 billion dollars in just twenty years (LMC International 2020). Furthermore, when considering direct and indirect impacts on the Canadian economy and employment sector, the total annual economic impact of the canola value chain in Canada is averaged at nearly 30 billion dollars (LMC International 2020). Although canola crop revenue is on the rise, severe economic losses have occurred due to yield reductions caused by two species of flea beetles, *Phyllotreta cruciferae* and *P. striolata*, which are considered the most severe insect pests of canola (Knodel et al. 2017). Flea beetles attack canola seedlings by creating small, highly dispersed feeding pits on the cotyledons and defoliating apical meristems (Gavloski and Lamb 2000). At this stage of development, seedlings are extremely vulnerable to physical deterioration due to their inability to compensate for physical damage once the apical meristems have been defoliated (Gavloski and Lamb 2000). As a result, even moderate feeding can impede plant development and decrease crop yields (Lamb 1984). Specifically, up to 10% of

canola crop losses can be attributed to flea beetle damage annually, resulting in over 300 million dollars' worth of lost farm income across North America (Lamb and Turnock 1982; Knodel et al. 2017).

To reduce damages caused by flea beetles, chemical pesticides, including neonicotinoids, are regularly applied to canola crops (Knodel et al. 2017). However, neonicotinoids are a chemical class that cause acute insecticide poisoning and lethal effects on off-target species (Pistorius et al. 2010; Bartling et al. 2019). Additionally, sub-lethal doses can contribute to impaired biosensory abilities of other species in the ecosystem, which may reduce individual fitness due to increased vulnerability to environmental stressors (Bartling et al. 2019). Although chemical control is the primary method used to protect canola crops, severe yield losses still occur since flea beetle populations are not adequately suppressed (Lamb and Turnock 1982). To better protect canola plants and mitigate environmental consequences, RNAi technologies are being explored as a safer alternative to traditional pesticides by developing foliar dsRNA sprays and transgenic plants expressing dsRNA (Zhu et al. 2011; Khajuria et al. 2018).

The similarity of siRNA fragments for targeted gene orthologues among other species must be considered to ensure that RNAi pesticides are species-specific. Determining whether off-target species might be affected by flea beetle-specific dsRNAs can be evaluated by either using bioassays that target other species or by conducting phylogenetic analyses. Furthermore, when conducting bioassays to test the effects of dsRNAs on flea beetles, only *P. striolata* colonies can be maintained and used for experimentation over the winter months. Rearing of *P. cruciferae* is a labour-intensive process with little success in establishing colonies due to a required hibernation period and 16:8 light-dark photoperiod (Kinoshita et al. 1979). Furthermore, any attempts to rear

P. cruciferae with an artificial diapause in our lab have been unsuccessful (Whyard, unpublished data). However, since a high degree of sequence similarity between *P. cruciferae* and *P. striolata* exists between targeted genes, the development of dsRNAs for *P. striolata* can theoretically extend to target *P. cruciferae*, which can be confirmed with phylogenetic analyses.

RNAi has the potential to be an alternative to conventional pesticides, largely due to the sequence-specific nature of dsRNA. However, limitations in using dsRNAs can mainly be attributed to highly variable insect responses due to different mechanisms that occur throughout the RNAi response (Silver et al. 2021). For instance, variations in the sensitivity of target genes have been observed in coleopterans, with gene knockdown ranging between 61% to 93% (Zhu et al. 2011). Additionally, target insect populations may develop resistance to long dsRNAs, which severely impacts the effectiveness of RNAi applications. Mechanisms for developing resistance to dsRNAs include mutations to dsRNA target genes or other processes in the RNAi pathway (Palli 2014). Alternative design strategies have been suggested to overcome these possible resistance mechanisms, including targeting multiple transcripts using a single dsRNA construct or mixing different dsRNAs in a combined treatment (Silver et al. 2021).

Furthermore, the resistance to ingested dsRNA due to reduced uptake of long dsRNA by the gut cells has also been observed in insects (Khajuria et al. 2018). After only 11 generations of increasing dosage selection in the western corn rootworm (*Diabrotica virgifera virgifera)*, impaired luminal uptake was observed, rendering them resistant to any dsRNA (Khajuria et al. 2018). To overcome this form of resistance, nanoparticles (Das et al. 2015) or liposomes (Tayler et al. 2019) may be required for improved entry into gut cells. However, using microcarriers would increase the cost of

dsRNA-based pesticides (Tayler et al. 2019). Moreover, further research would need to be conducted, since microcarriers are not yet appropriate for large-scale applications of dsRNA as an insect control strategy (Tayler et al. 2019).

Overcoming RNAi refractoriness or resistance due to reduced uptake of dsRNA molecules may also be achieved by using an alternative to conventional dsRNA constructs, such as a paperclip RNA (pcRNA) (Abbasi et al. 2020). pcRNAs (~23 bp) are shorter in length than conventional linear dsRNA molecules (>200 bp) and have two non-covalently sealed ends. Interestingly, pcRNAs reduced the transcripts of targeted genes to the same degree as long dsRNA in a study evaluating pharmacological inhibitors of clathrin-mediated endocytosis in the mosquito *Aedes aegypti* (Abbasi et al. 2020). pcRNAs are predicted to be more resistant to nuclease degradation in the gut due to their protected ends, resulting in a higher level of gene knockdown. Additionally, unlike the uptake of siRNA (21 bp), short hairpin RNA (hpRNAs) (23 bp), and long dsRNA molecules (~210 bp), which utilize clathrin-mediated endocytosis, the uptake of pcRNA in mosquito cells uses a different, yet to be determined pathway (Abbasi et al. 2020). Due to the different uptake mechanism of pcRNA, resistance due to reduced uptake may be overcome. Designing dsRNA constructs that exploit different uptake pathways may serve as a plausible solution to induce RNAi knockdown with a higher level of efficacy and circumvent RNAi-resistant pest populations (Abbasi et al. 2020).

While these findings indicate that pcRNAs can effectively mediate RNAi in mosquitoes as an alternative structure to long dsRNA, further research is required to observe whether persistent knockdown of transcripts will occur in flea beetles. Phylogenetic analyses that examine whether dsRNA targeting flea beetle genes can potentially induce an RNAi response in other organisms must also be investigated. My

objective is to compare which dsRNA structures and gene targets are the most effective in causing the mortality of *P. striolata* and thereby reduce feeding damages that occur to canola leaf tissues. It is hypothesized that different dsRNA structures will have varying efficacies in killing flea beetles. It is also hypothesized that using different gene targets will have varying degrees of efficiency in inducing the mortality of flea beetles. To investigate gene knockdown in *P. striolata*, three protein-coding mRNA sequences with essential functions will be targeted: *Ras opposite (Rop), Sec23* and *Snf7*. *Rop* and *Sec23* were selected for this study, as previous research in our lab demonstrated that long dsRNAs targeting these two genes effectively killed mosquito larvae (Whyard, unpublished data). A positive control dsRNA targeting the *Snf7* gene was also used in this assay, as this dsRNA effectively killed *P. cruciferae* flea beetles in past assays (Whyard, unpublished data). Bioassays were conducted by monitoring the survival and leaf disk consumption of *P. striolata* after consuming the *Rop, Sec23,* and *Snf7* gene targets for the long dsRNA, short hpRNA, and short pcRNA structures.

Methods

Phylogenetic analysis

To confirm the identity of the two target genes, *Rop* and *Sec23,* the predicted translated coding sequences of the *P. striolata* dsRNA-targeted sequences were compared to predicted orthologous sequences (found within the NBCI database) of ten different insect species of interest. The ten chosen species included closely related insects in the order Coleoptera, insects of economic and ecological importance, and model insect species. The predicted orthologous protein sequences were trimmed and then aligned using the Multiple Alignment using Fast Fourier Transform (MAFFT) (Madeira et al.

2022). The aligned protein sequences were subsequently used to generate Maximum Likelihood phylogenetic trees with MEGAX software with a bootstrap value of 1000 replications and default parameters (Fig. 1).

To evaluate whether off-target species might be affected by *P. striolata*-specific dsRNAs, the *P. striolata Rop* or *Sec23* dsRNA sequences were individually aligned to each of the ten chosen species using Clustal Omega (Madeira et al. 2022). Each pairwise alignment was visually inspected to ensure no conserved sequences of 21 or more nucleotides occurred, as it has been proposed that 21-23 nucleotide fragments are used as guide RNAs for target recognition in RNAi, which cleave target mRNA in 21-23 nucleotide intervals (Zamore et al. 2000; Elbashir et al. 2001 Figs. 2, 3). Multiple sequence alignments were performed for each gene target to list all species comparisons for both gene targets.

Flea beetle maintenance

Approximately 1000 lab-bred *P. striolata* were shipped once a month from BASF (Charlotte, NC, United States). The flea beetles were reared in an incubator or insectary at 27°C, 50% humidity, and maintained on canola plants in mesh cages (modified from Zhao et al. 2008).

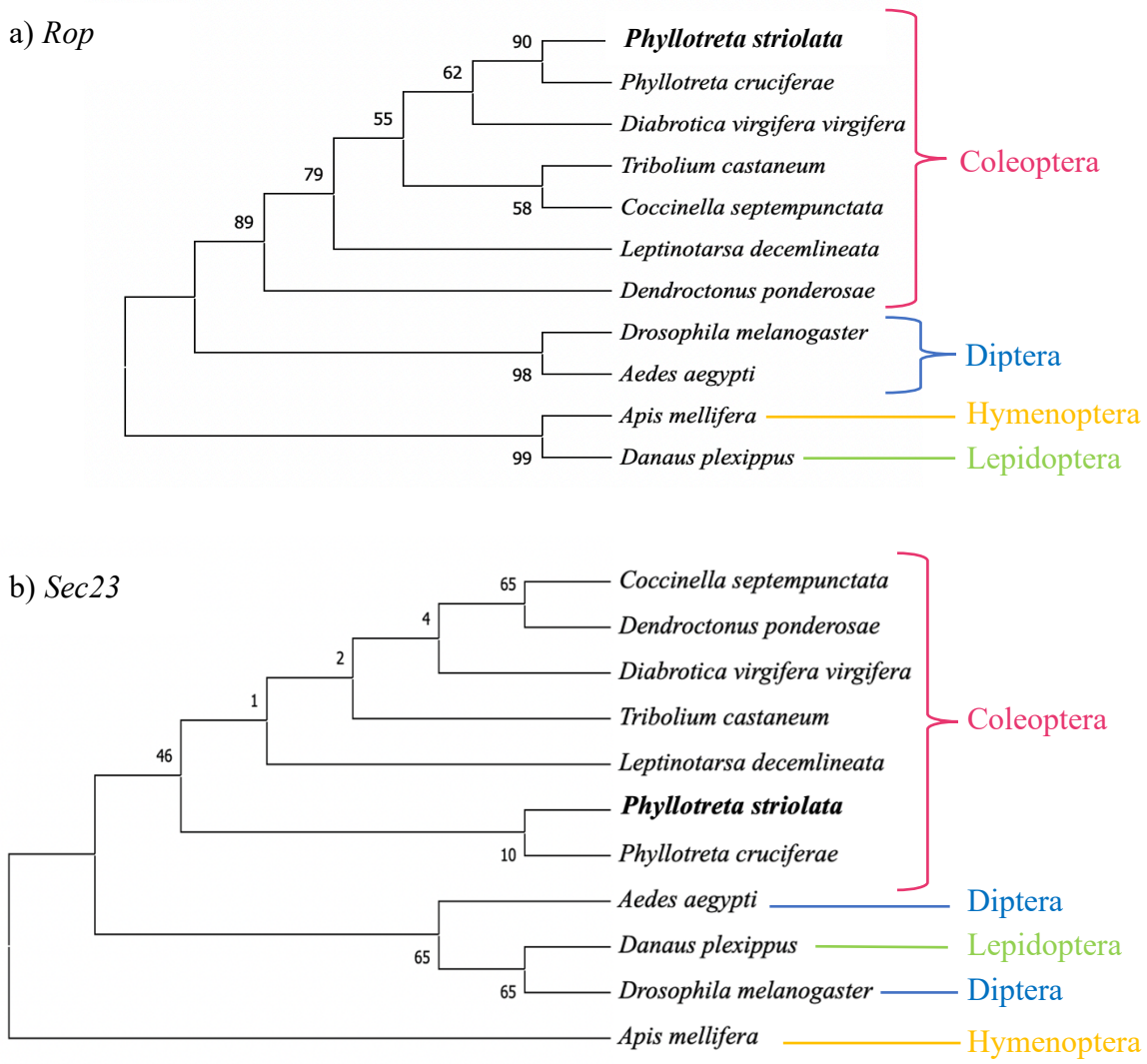

Figure 1. Phylogenetic analysis of the two target flea beetle amino acid sequences to predicted orthologues obtained from NCBI. Phylogenetic relationships for **a)** *Rop* and **b)** *Sec23* were inferred using the Maximum Likelihood method and JTT matrix-based model in MEGAX. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were collected using Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model and then selecting the topology with superior log likelihood value. The phylogenetic analyses were conducted in MEGA X and involved 11 amino acid sequences with a total of 77 positions in the final dataset.

(Stecher et al. 2020)

```
A. mellifera        ACATCATTTAGACAAATTACGAATTATTGCATTATATGTTATTAGTAAAAATGGTATTTC      60
D. melanogaster     CTCGAACTACGACAAGGTTCGCATCATTGCCCTGTACGTCATGATCAAGAACGGAATTTC      60
A. aegypti          GTCCAACTACGATAAGGTTCGCATTATCGCTCTGTACGTCATGATTAAAAATGGCATTTC      60
D. plexippus        CAAGAACTGTGACAAGATGCGTATCATCGCGCTGTACATAATGTCCAAGAACGGTATATC      60
T. castaneum        AAATGAATACGATAAAATGCGCATTATCGCACTATACGCAATGACAAAAAATGGAATCAC      60
L. decemlineata     CAATGAATATGACAAAATGCGGATCATAGCCTTATACGCAATGACAAAAAATGGTATAAC      60
P. striolata        GAACGAATACGATAAGATGCGCATCATAGCTTTATACGCTATGACCAAAAACGGTATAAC      60
P. cruciferae       GAACGAATACGATAAGATGCGCATCATAGCTTTGTACGCTATGACCAAAAATGGAATAAC      60
D. virgifera        CAATGAATACGATAAGATGCGTATTATAGCATTGTACGCCATGACGAAAAACGGCATCAC      60
C. septempunctata   CAACGAGTACGACAAGATGAGGATCATCGCGCTGTACGCGATGACCAAGAACGGCATAAC      60
D. ponderosae       TGAGCAGTACGACAAGATGCGCATCATAGCGCTGTATGCGATGATCAAGAACGGGATCAC      60
                         * *  ** **  *  * ** ** **  * **    **    ** ** ** **  *

A. mellifera        TGAAGAAAATCTTAATCGTCTTGTTCATCATGCTCAAATATCTCCTGATGATAAACAAAC      120
D. melanogaster     CGAGGAGAACCTGACCAAGCTGTTTACGCATGCCCAGCTGTCGCCGAAGGATCAGGATAT      120
A. aegypti          CGAAGAGAACTTGACCAAACTGGTCACGCACGCCCAGATCGAACCAAAGGAAAGAGAAAT      120
D. plexippus        GGAGGAGAACCTCAACCGCCTGGTGACGCACGCCCAGCTAGAACCATCCGACAAACAGGC      120
T. castaneum        TGAAGAAAACTTATCCAAGTTGGCCACGCACGCCCAAATCAAGGACAAACAAACTATTGC      120
L. decemlineata     AGAAGAAAACTTGTCAAAGCTTGCCACTCACGCCCAAATTAAGGATAAGCAGACAATTGC      120
P. striolata        CGAGGAAAATCTTACGAAGCTAGCTACACACGCGCAGATTAAAGACAAACAAACTATCGC      120
P. cruciferae       CGAGGAAAATCTTACAAAGCTAGCAACGCACGCCCAAATTAAAGATAAACAAACTATTGC      120
D. virgifera        AGATGAAAATCTCTCCAAATTGGCTACCCATGCCCAAATCAAAGACAAACAGACCATCGC      120
C. septempunctata   GGAGGAGAACCTGTCCAAACTGGCGACGCACGCGCAGATCAAGGACAAGCAGACCATAGC      120
D. ponderosae       CGAGGAAAACCTGCAGAAGCTGGCCACCCATGCCCAGATCGCCCAGAAGCAGACCATCTC      120
                     ** ** **  *        *       ** ** **  *           *

A. mellifera        TATAGTAAACATGGCTAATCTTGGTATTAATATAGTTGTAGATGGTGGTAATCGTA---A      177
D. melanogaster     GGTGCGTAACCTTAGTTGCTTGGGCATCAATGTCATTGCAGATTCGCGCAAGA------A      174
A. aegypti          GATCACCAATTTGAGCTACCTGGGAATCAACGTCATTGCTGATGGCAACCGTA------A      174
D. plexippus        GCTGCTCAACCTCGCCAACCTTGGACTCAATGTTGTCGTTGAT------GGCAATAGGAA      174
T. castaneum        C------AATTTACAACTCTTGGGTGTGAACGTCATCAGTGATGGTGGTAACCGTA---A      171
L. decemlineata     T------AATTTGCAATATCTAGGCGTCAATGTCATCAACGATGGTGGCAAGAGAA---A      171
P. striolata        T------AACCTGCAGCTCTTGGGAGTTAACGTCATTAACGATGGTGGTCCTAGAA---A      171
P. cruciferae       T------AACCTTCAGCTCTTGGGAGTTAATGTCATTAATGATGGTGGCCCTAGAA---A      171
D. virgifera        C------AACCTTCAGTTACTTGGAGTCAACGTTATTAATGATGGAGGACCAAGAA---A      171
C. septempunctata   C------AATCTCCAGCTTCTGGGCGTGAACGTCCTGAGCGACGTGGGCGTCAGGAAGCC      174
D. ponderosae       C------AACATTCAGTACCTGGGGGTGAACGTGGTGAACGATGGCGGCAACCGCA---A      171
                           **  *        * **  * **  *  *    **

A. mellifera        AAAATTATACACCGTACAACGTAAAGAAAGAATTACAGAAC      218
D. melanogaster     GCAG---TACTCGGTGCCGCGCAAGGAGCGCACCACAGAAA      212
A. aegypti          AAAAGGATATTCGGTACCAAGGAAGGAGCGTATAAATGAAC      215
D. plexippus        GAAGCAGTACCAAGTACCGCGGAAGGAGAGAATCACTGAAC      215
T. castaneum        AAAACCGTACACGGTGCCGCGTAAAGAACGCATAACTGAAC      212
L. decemlineata     CAAAGGTTACACCATACCGAGAAAAGAAAGAATCACAGAGC      212
P. striolata        AAAGCTGTACACCGTACCGCGCAAAGAGCGAATTACAGAAC      212
P. cruciferae       AAAGATCTACACCGTACCGCGCAAAGAGCGAATTACAGAAC      212
D. virgifera        AAAACAATATACAGTACCGCGCAAAGAAAGAATTACAGAAC      212
C. septempunctata   CAAACAGTACACGGTGCCGCGCAAGGAGAGGATCACCGAAC      215
D. ponderosae       GAAGCAGTACACAGTGCCGCGCAAGGAGCGAATCACCGAGC      212
                      *    **     * *   * ** **  * *  *  **
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Figure 2. Multiple sequence nucleotide alignments for predicted *Rop* orthologues. *P. striolata Rop* long dsRNA sequence was compared against ten other species using Clustal Omega. The selected *P. striolata* siRNA for hpRNA and pcRNA design was highlighted in yellow. Conserved sequences of 21 or more nucleotides shared with *P. striolata* were highlighted in blue Accession numbers are listed as follows: (*Apis mellifera*, XM_396375.7), (*Drosophila melanogaster*, CP023337.1), (*Aedes aegypti*, XM_021854571.1), (*Danaus plexippus*, XM_032661972.1), (*Tribolium castaneum*, NM_001170684.1), (*Leptinotarsa decemlineata*, KY285050.1), (*Diabrotica virgifera virgifera*, XM_028277042.2), (*Coccinella septempunctata*, MW452565.1, (*Dendroctonus ponderosae*, XM_019910394.2).

(Madeira et al. 2022)

```
A. mellifera            ATTTCTACAAGTATTTAATAATTCTCCCGATGAAACTAGTTTTTATAGGCATATGCTCAT   60
D. ponderosae           GTTCTTGCAGGTGTTCAATAATTCACCAGATGAAACTTCATTTTACAGGCACATGCTGAT   60
T. castaneum            ATTCCTCCAAGTTTTCAACAACTCCCCAGACGAGACCTCGTTCTACCGCCACATGCTGAT   60
D. virgifera            ATTTCTTCAAGTATTCAATAATTCTCCGGACGAGACTTCATTCTACAGACACATGTTGAT   60
C. septempunctata       ATTCTTGCAAGTTTTCAACAATTCCCCGGATGAAACGTCCTTCTACAGACACATGCTGAT   60
P. striolata            GTTCCTGCAAGTTTTCAATAATTCTCCTGATGAAACATCCTTCTATCGGCATATGTTGAT   60
P. cruciferae           GTTCCTGCAAGTTTTCAATAATTCTCCTGATGAAACGTCGTTCTATAGACATATGTTGAT   60
D. plexippus            GTTCCTTCAAGTATTCAATAATTCACCCGATGAAACTACTTTCTACAGACACATGTTAAT   60
D. melanogaster         GTTCCTGCAGGTCTTCAACAACTCGCCCGACGAGACCACATTCTATCGACACATGCTAAT   60
L. decemlineata         GTTCCTACAGGTGTTCAACAATTCTCCCGACGAAACGTCCTTCTACAGGCACATGCTTAT   60
A. aegypti              ATTCCTTCAAGTTTTCAACAATTCACCAGACGAAACGACCTTCTACAGGCACATGCTGAT   60
                         **  * ** ** ** ** ** ** ** ** ** **    ** **  * ** *** * **

A. mellifera            GCGAGAAGATTTAACAAATTCTTTGATAATGGTACAGCCAATTTTGTACAGTTATGGATT   120
D. ponderosae           GAGGGAGGATTTGACCCAATCTCTCATTATGATTCAGCCTCTTTTGTATAGCTACAGCTT   120
T. castaneum            GCGGGAGGACCTCACCCAAAGTCTCATTATGATCCAGCCGATTTTATACAGTTATAGTTT   120
D. virgifera            GAGGGAAGATCTTACTCAATCTTTGATAATGATTCAACCTATTTTGTATAGTTATAGTTT   120
C. septempunctata       GAGGGAAGATCTCACCCAGTCGTTGATAATGATACAACCGATATTGTACAGTTATAGTTT   120
P. striolata            GAGAGAAGACCTTACTCAATCACTGATCATGATTCAACCAATTTTGTACAGTTACAGTTT   120
P. cruciferae           GAGAGAAGATCTTACTCAATCATTGATCATGATTCAACCAATTTTGTACAGTTACAGTTT   120
D. plexippus            GCGTGAGGACCTCACCCAGTCCCTCATCATGATCCAGCCCATACTGTACTCCTACAGTTT   120
D. melanogaster         GCGCGAGGACCTCACCCAATCGCTGATCATGATCCAGCCTATTCTCTACAGCTACTCGTT   120
L. decemlineata         GCGTGAAGACCTCACGCAGTCGCTGATCATGATCCAGCCGATACTCTACAGCTACAGTTT   120
A. aegypti              GCGGGAAGACCTCACCCAATCGCTGATCATGATTCAGCCCATTCTCTACTCGTACTCCTT   120
                        * * ** **  * **  *     * ** *** * ** **  *  * **    **    **

A. mellifera            TAGTGGTCCTCCAGAACCAGTTTTATTAGATACATCGTCTATTCAGCCAGATAGAATTTT   180
D. ponderosae           CAATGGGCCTCCAGAACCAGTTCTTTTGGACACCAGCTCAATTCAGCCCGACAAAATTTT   180
T. castaneum            CAACGGCCCCCCTGAACCCGTCCTCCTCGACACTAGTTCCATTCAACCCGATCGAATCCT   180
D. virgifera            CAATGGTCCACCAGAGCCTGTATTACTAGATACTAGCTCCATTCAACCTGACAGAATATT   180
C. septempunctata       CAACGGACCTCCGGAACCTGTTCTTCTTGATACCAGTTCCATTCAACCCGATAAGATCCT   180
P. striolata            CAACGGCCCCCCTGAGCCTGTACTATTAGATACGAGTTCCATACAGCCCGATAGAATATT   180
P. cruciferae           CAACGGCCCTCCGGAGCCTGTTCTATTAGATACGAGTTCCATACAGCCCGATAGAATACT   180
D. plexippus            CGGAGGTCCCCCAGAGCCTGTTCTGCTGGACACTTCCTCCATACAGCCTGACAGGATACT   180
D. melanogaster         CAACGGCCCTCCAGAACCGGTCCTCCTCGATACGGCTTCCATCCAGGCGGATCGCATCCT   180
L. decemlineata         CAATGGACCACCGGAACCTGTGCTTTTGGATACGAGTTCCATCCAGCCCGATAGAATTCT   180
A. aegypti              CAACGGACCGCCGGAACCCGTACTCCTGGACACGTCCTCAATCCAACCGGATCGCATCCT   180
                            ** ** ** ** ** **  *  * ** **    ** ** **  * **    **  *

A. mellifera            ATTGATGGATACTTTCTTCCAAATCCTTATATTC 214
D. ponderosae           GCTCATGGATACCTTCTTCCAAATTTTGATTTTC 214
T. castaneum            TCTCATGGACACATTTTTCCAAATTTTGATTTTT 214
D. virgifera            ACTTATGGATACTTTCTTCCAAATATTAATTTTC 214
C. septempunctata       GCTCATGGACACCTTCTTCCAAATTTTGATTTTC 214
P. striolata            GCTTATGGACACGTTTTTCCAAATTTTGATATTC 214
P. cruciferae           GCTTATGGACACGTTTTTCCAAATTTTGATATTC 214
D. plexippus            GCTTATGGATACTTTCTTCCAGATCTTGATATAC 214
D. melanogaster         CCTGATGGACACGTTCTTTCAGATTCTGATTTAC 214
L. decemlineata         GCTCATGGACACTTTCTTCCAGATTCTGATATTC 214
A. aegypti              GCTTATGGACACCTTCTTCCAAATTCTGATATTC 214
                          * ***** ** ** ** ** **  * ** *
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Figure 3. Multiple sequence nucleotide alignments for predicted *Sec23* orthologues. *P. striolata Sec23* long dsRNA sequence was compared against ten other species using Clustal Omega. The selected *P. striolata* siRNA for hpRNA and pcRNA design was highlighted in yellow. Conserved sequences of 21 or more nucleotides shared with *P. striolata* were highlighted in blue. Accession numbers are listed as follows: (*Apis mellifera*, XM_006567377.3), (*Dendroctonus ponderosae*, XM_019903277.2), (*Tribolium castaneum*, XM_015981342.1), (*Diabrotica virgifera virgifera*, MK474471.1), (*Coccinella septempunctata*, XM_044889985.1), (*Danaus plexippus*, XM_032665261.1), (*Drosophila melanogaster*, CP023337.1), (*Leptinotarsa decemlineata*, KY285048.1), (*Aedes aegypti*, XM_001652733.2).

(Madeira et al. 2022)

Identification of target sequences

Two protein-coding mRNA sequences with essential functions were targeted for this study: *Rop* and *Sec23* (Table 1). *Rop* is involved in intracellular vesicle trafficking, and *Sec23* is the core component of the coat protein complex II, which regulates the transport of newly synthesized proteins and lipids from the endoplasmic reticulum to the Golgi apparatus (Salzberg et al. 1993; Jing et al. 2019). *Rop* has been identified as a highly potent gene in RNAi studies targeting the western corn rootworm, red flour beetle (*Tribolium castaneum*), and the rape pollen beetle (*Meligethes aeneus*) (Knorr et al. 2018). *Sec23* is another essential, highly lethal RNAi target to the western corn rootworm (Vélez et al. 2020). Interestingly, Vélez et al. (2020) demonstrated that 85% mRNA transcript knockdown resulted in a 40% reduction of Sec23 protein levels, which indicates that complete protein knockdown is not necessary to achieve *Sec23* RNAi-mediated mortality.

Using a *P. striolata* transcriptome previously developed in our lab (Whyard, unpublished data), the *Rop* and *Sec23* genes were identified using a BLAST search based on their sequence similarities (76 and 84% amino acid identities, respectively) to predicted orthologues in two beetle species, *T. castaneum* and *Leptinotarsa decemlineata* (Colorado potato beetle).

Table 1. Sequences of *P. striolata Rop* and Sec23 gene targets.

Gene	Sequence
Rop	GGATCACATGCGCAACATCGTTCCGATTCTTCTAGACACCAA AATAACGAACGAATACGATAAGATGCGCATCATAGCTTTAT ACGCTATGACCAAAAACGGTATAACCGAGGAAAATCTTACG AAGCTAGCTACACACGCGCAGATTAAAGACAAACAAACTAT CGCTAACCTGCAGCTCTTGGGAGTTAACGTCATTAACGATGG TGGTCCTAGAAAAAAGCTGTACACCGTACCGCGCAAAGAGC GAATTACAGAACAAACTTACCAGATGTCGAGATGGACACCG GTTATTAAAGA
Sec23	GCCGGTTCATTCAGGTTGGGCCAAAACTTCAGCCTCTATCCG CAATTCATGTATCACCTAAGGAGATCTCAGTTCCTGCAAGTT TTCAATAATTCTCCTGATGAAACATCCTTCTATCGGCATATG TTGATGAGAGAAGACCTTACTCAATCACTGATCATGATTCAA CCAATTTTGTACAGTTACAGTTTCAACGGCCCCCCTGAGCCT GTACTATTAGATACGAGTTCCATACAGCCCGATAGAATATTG CTTATGGACACGTTTTTCCAAATTTTGATATTC

Preparation of dsRNA

DNA templates for long dsRNA (212 bp for *Rop,* 214 bp for *Sec23*), hpRNA (24 bp for *Rop*, 21 bp for *Sec23*), and short pcRNA (24 bp for *Rop*, 21 bp for *Sec23*) targeting the *Rop* and *Sec23* genes in *P. striolata* were obtained from Integrated DNA Technologies (Tables 2 and 3). DNA templates for the *β-glucuronidase (gus)* negative controls and *Snf7* positive controls were also obtained from Integrated DNA Technologies (Table 4). To anneal the hpRNA and pcRNA templates, the two ultramer DNA oligonucleotides were heated at 95°C for 2 minutes, followed by incrementally decreasing the temperature to 22°C (Fig. 4). All DNA templates were flanked by T7 promoter sequences, which enabled *in-vitro* transcription using the T7 RNA polymerase in the MEGAscript RNAi kit (Invitrogen). The resulting sense and antisense RNAs were incrementally cooled from 75°C to 22°C to anneal the segments into the desired double-stranded structures. Long

dsRNA was purified by centrifugation with spin columns from the MEGAscript RNAi kit (Invitrogen), while ethanol precipitation was used to purify hpRNA and pcRNA. Following purification, both constructs' concentration in solution was measured using a BioTek Take3 spectrophotometer. The quality of structures was determined by resolving a small aliquot on an ethidium bromide-stained 1.5% agarose gel and checking for the presence of a single distinct band.

Table 2. Sequences of *P. striolata* long dsRNA gene targets.

Gene	**Sequence**
Rop	GAACGAATACGATAAGATGCGCATCATAGCTTTATACGCTA TGACCAAAAACGGTATAACCGAGGAAAATCTTACGAAGCT AGCTACACACGCGCAGATTAAAGACAAACAAACTATCGCT AACCTGCAGCTCTTGGGAGTTAACGTCATTAACGATGGTGG TCCTAGAAAAAAGCTGTACACCGTACCGCGCAAAGAGCGA ATTACAGAAC
Sec23	GTTCCTGCAAGTTTTCAATAATTCTCCTGATGAAACATCCTT CTATCGGCATATGTTGATGAGAGAAGACCTTACTCAATCAC TGATCATGATTCAACCAATTTTGTACAGTTACAGTTTCAACG GCCCCCCTGAGCCTGTACTATTAGATACGAGTTCCATACAG CCCGATAGAATATTGCTTATGGACACGTTTTTCCAAATTTTG ATATTC
Snf7	GGATGACATAGCTGAACAGCACGATATTGCCAATGAGATTA CTACTGCTATTAGCCAGCCGGTTGGCTTCAATGATGATCTG GATGAGGATGAATTAGAAAGTGAACTAGAAAAGCTTGAAC AAGAAGGATTAGAGGAAGATTTGCTGAAAGTGCCCGGTCCT ACTGAATTACCTGCTGTACCTACAGGAGCTATCGCTAAGCC AGTTAAAC
gus	GCTGAAGAGATGCTCGACTGGGCAGATGAACATGGCATCGT GGTGATTGATGAAACTGCTGCTGTCGGCTTTAACCTCTCTTT AGGCATTGGTTTCGAAGCGGGCAACAAGCCGAAAGAACTG TACAGCGAAGAGGCAGTCAACGGGGAAACTCAGCAAGCGC ACTTACAGGCGATTAAAGAGCTGATAGCGCGTGACAAAAA CCACCC

Table 3. Sequences of *P. striolata* siRNAs for *Rop* and *Sec23* gene targets.

Gene	siRNA structure	Sequence
Rop		
	siRNA	GCTTTATACGCTATGACCAAAAAC
	hpRNA top strand	ACCTCATAATACGACTCACTATAGCTTTATACGCT ATGACCAAAAACAAAAAAAAAGTTTTTGGTCATA GCGTATAAAGCTA
	hpRNA bottom strand	TAGCTTTATACGCTATGACCAAAAACTTTTTTTTT GTTTTTGGTCATAGCGTATAAAGCTATAGTGAGT CGTATTATGAGGT
	pcRNA top strand	ACCTCATAATACGACTCACTATAGTTTTTGGTCAT AGCGTATAAAGCAAAAAAAAAAGCTTTATACGCTA TGACCAAAAACGGCATGAAAAAAAAAACATG
	pcRNA bottom strand	CATGTTTTTTTTTCATGCCGTTTTTGGTCATAGCG TATAAAGCTTTTTTTTTTGCTTTATACGCTATGACC AAAAACTATAGTGAGTCGTATTATGAGGT
New *Rop*		
	siRNA	GGAAAATCTTACGAAGCTAGCTAC
	hpRNA top strand	ACCTCATAATACGACTCACTATAGGAAAATCTTA CGAAGCTAGCTACAAAAAAAAAAGTAGCTAGCTTC GTAAGATTTTCCTC
	hpRNA bottom strand	GAGGAAAATCTTACGAAGCTAGCTACTTTTTTTTT GTAGCTAGCTTCGTAAGATTTTCCTATAGTGAGTC GTATTATGAGGT
	pcRNA top strand	ACCTCATAATACGACTCACTATAGTAGCTAGCTT CGTAAGATTTTCCAAAAAAAAAGGAAAATCTTAC GAAGCTAGCTACACCATGAAAAAAAAACATG
	pcRNA bottom strand	CATGTTTTTTTTTCATGGTGTAGCTAGCTTCGTAA GATTTTCCTTTTTTTTTGGAAAATCTTACGAAGCT AGCTACTATAGTGAGTCGTATTATGAGGT
Sec23		
	siRNA	GAGAAGACCTTACTCAATCAC
	hpRNA top strand	ACCTCATAATACGACTCACTATAGAGAAGACCTT ACTCAATCACAAAAAAAAAGTGATTGAGTAAGG TCTTCTCTC
	hpRNA bottom strand	GAGAGAAGACCTTACTCAATCACTTTTTTTTTGTG ATTGAGTAAGGTCTTCTCTATAGTGAGTCGTATTA TGAGGT
	pcRNA top strand	ACCTCATAATACGACTCACTATAGGTGATTGAGT AAGGTCTTCTCAAAAAAAAAGAGAAGACCTTACT CAATCACTGCATGAAAAAAAAACATG
	pcRNA bottom strand	CATGTTTTTTTTTCATGCAGTGATTGAGTAAGGTC TTCTCTTTTTTTTTGAGAAGACCTTACTCAATCAC CTATAGTGAGTCGTATTATGAGGT

Table 4. Sequences of *P. striolata* siRNAs for *gus* and *Snf7* controls.

Gene	siRNA structure	Sequence
gus		
	siRNA	GATGCTCGACTGGGCAGATGAAC
	hpRNA top strand	ACCTCATAATACGACTCACTATAGATGC TCGACTGGGCAGATGAACAAAAAAAAA GTTCATCTGCCCAGTCGAGCATC
	hpRNA bottom strand	GATGCTCGACTGGGCAGATGAACTTTTT TTTTGTTCATCTGCCCAGTCGAGCATCT ATAGTGAGTCGTATTATGAGGT
	pcRNA top strand	ACCTCATAATACGACTCACTATAGATGC TCGACTGGGCAGATGAACAAAAAAAAA GTTCATCTGCCCAGTCGAGCATCTCCAT GAAAAAAAAACATG
	pcRNA bottom strand	CATGTTTTTTTTTCATGGAGATGCTCGA CTGGGCAGATGAACTTTTTTTTTGTTCAT CTGCCCAGTCGAGCATCTATAGTGAGTC GTATTATGAGGT
Snf7		
	siRNA	GATTACTACTGCTATTAGCCAGC
	hpRNA top strand	ACCTCATAATACGACTCACTATAGATTA CTACTGCTATTAGCCAGCAAAAAAAAA GCTGGCTAATAGCAGTAGTAATCTC
	hpRNA bottom strand	GAGATTACTACTGCTATTAGCCAGCTTT TTTTTTGCTGGCTAATAGCAGTAGTAAT CTATAGTGAGTCGTATTATGAGGT
	pcRNA top strand	ACCTCATAATACGACTCACTATAGCTGG CTAATAGCAGTAGTAATCAAAAAAAAA GATTACTACTGCTATTAGCCAGCTCCAT GAAAAAAAAACATG
	pcRNA bottom strand	CATGTTTTTTTTTCATGGAGCTGGCTAAT AGCAGTAGTAATCTTTTTTTTTGATTACT ACTGCTATTAGCCAGCTATAGTGAGTCG TATTATGAGGT

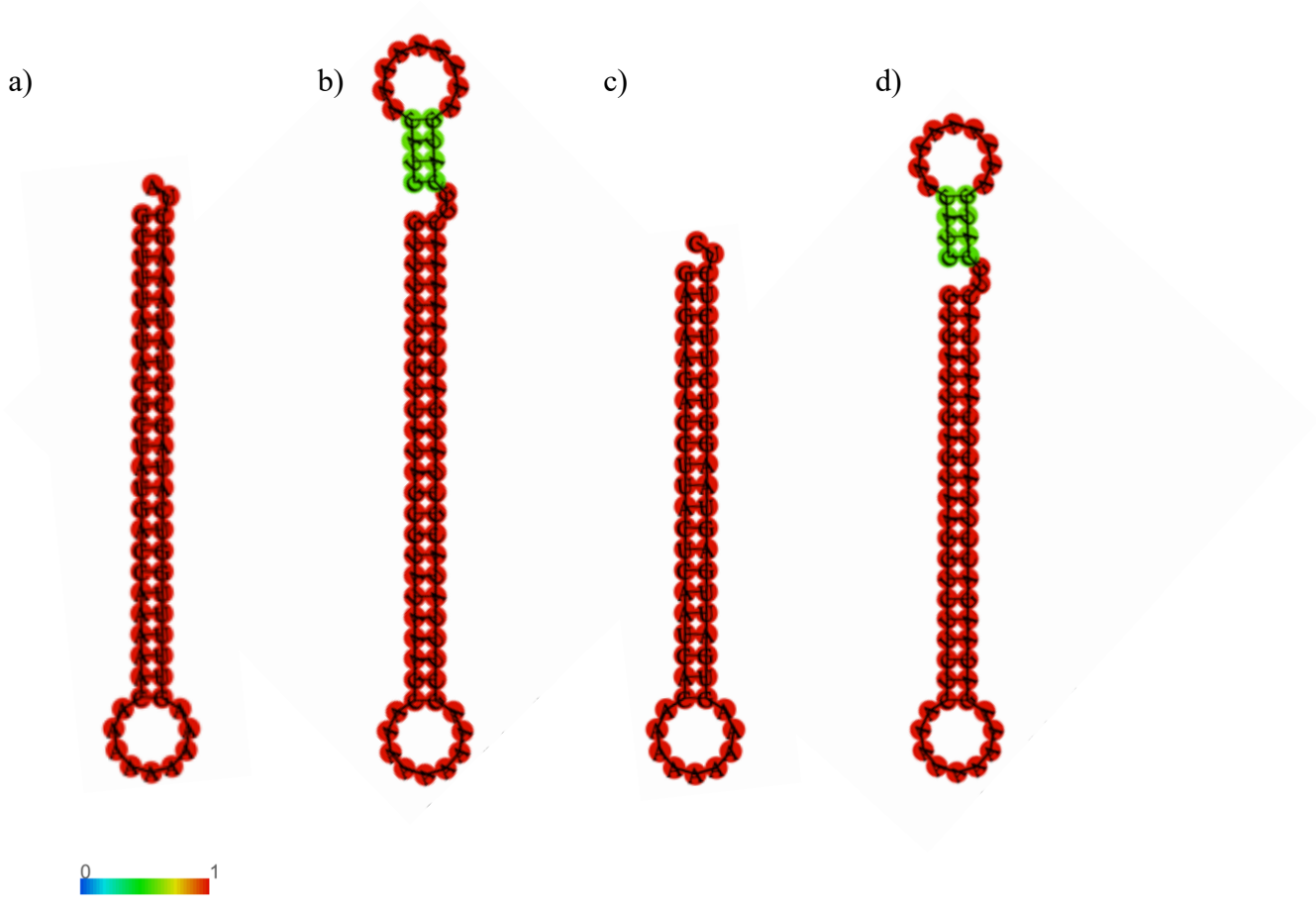

Figure 4. Predicted minimum free energy secondary structure of hpRNA and pcRNA. **a)** *Rop* hpRNA, **b)** *Rop* pcRNA, **c)** *Sec23* hpRNA, and **d)** *Sec23* pcRNA structures were predicted with the RNAfold server. The probability of the predicted structure is colour-coded, with the highest likelihood of structure denoted in red. The green portions of the molecules indicate that the short (4 bp) region had a lower likelihood of confirmed structure relative to the rest of the molecule.
(Gruber et al. 2008).

In-vitro transcription of Rop pcRNA

Rop pcRNA synthesis was repeated on three separate occasions since *in-vitro* transcription failed to produce a structure that would resolve on the agarose gel (Fig. 5). Despite the lack of a visible band on the agarose gel, the BioTek Take3 spectrophotometer detected concentrations of the *Rop* pcRNA that were similar to concentrations of *Rop* hpRNA, *Sec23* hpRNA and *Sec23* pcRNA, indicating that RNA was present, but not in the paperclip structure (Table 5).

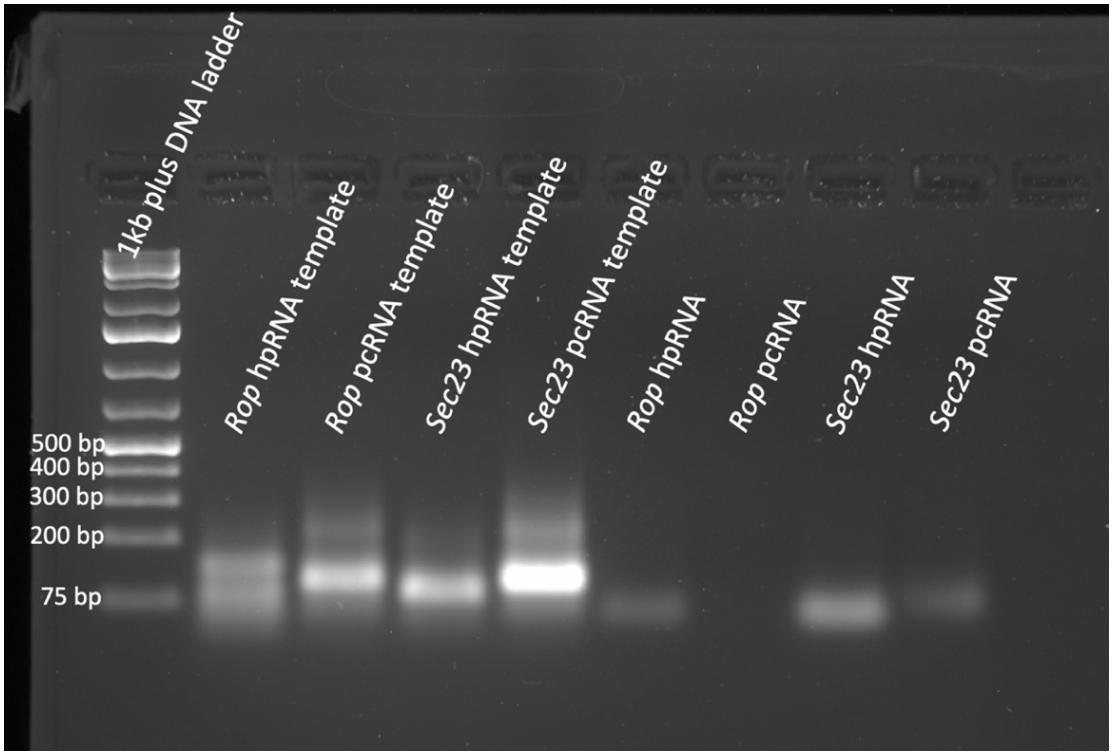

Figure 5. Resolution of template DNA and dsRNA on an agarose gel. 1.5% ethidium bromide-stained agarose gel was submerged in 1X TRIS Borate EDTA (TBE) buffer and resolved at 90V for 40 minutes. In-vitro synthesis of *Rop* pcRNA failed to produce a visible band in lane 7. 1Kb plus DNA ladder was loaded in the first lane, template DNA was loaded in lanes 2 to 5, and purified dsRNA was loaded in lanes 6 to 9.

Table 5. Concentrations of *Rop* and *Sec23* dsRNA.

dsRNA structure	**Concentration (ng/µL)**
Rop hpRNA	2439.568
Rop pcRNA	3176.447
Sec23 hpRNA	3437.865
Sec23 pcRNA	742.354

To verify that the oligonucleotides from Integrated DNA Technologies were indeed the correct sequence, the *Rop* annealed DNA was ligated into the pJET1.2/blunt vector and was sequenced. 100% sequence identity between the sequenced plasmid and *Rop* pcRNA sequence was obtained. Failure to produce a structure that resolved on the agarose gel may be due to secondary or tertiary structure folding that was not predicted by the RNAfold algorithm (Smith 2006). To correct the *Rop* pcRNA structure, an alternative siRNA was designed for *Rop*, and new hpRNA and pcRNA ultramers were ordered (Table 3). The new *Rop* hpRNA and pcRNA were synthesized and tested in the feeding bioassay.

Flea beetle bioassays

Feeding bioassays were conducted to determine the efficacy of three different dsRNA structures for both *Rop* and *Sec23* gene targets. Small (0.5 cm) circular disks of canola leaf obtained from lab-grown plants were cut using a hole punch. Each leaf disk was lightly scratched on the surface with a thumbtack to ensure the dsRNA was evenly

distributed. dsRNA was diluted with nuclease-free water to a final concentration of 125 ng/μL. Each leaf disk was coated in 500 ng of either *P. striolata Rop* dsRNA, *P. striolata Sec23* dsRNA, *P. striolata Snf7* dsRNA as a positive control, *gus* as a negative control, or without any dsRNA treatment as an additional negative control. Elution buffer (10 mM Tris-HCl pH 7, 1 mM EDTA) from the MEGAscript RNAi kit was also tested by coating some leaf disks with 4 μL of the solution. Elution buffer was tested since non-specific mortality occurred in previous bioassays and was predicted to be a result of using the elution buffer, which contains EDTA, a chelating agent.

A positive control dsRNA, targeting the *Snf7* gene, was also used in these assays, as this dsRNA killed at least 50% of field-caught *P. cruciferae* in previous field seasons (Whyard, unpublished data). The *Snf7* gene encodes an essential protein that has a role in intracellular trafficking and was also the first commercial RNAi product, as developed by Bayer CropScience (Bolognesi et al. 2012). Ingestion of *Snf7* dsRNA results in an RNAi response, including stunted larval growth and mortality of coleopteran species, such as the western corn rootworm (Bolognesi et al. 2012). A negative control dsRNA, specific for *gus,* was also used in the bioassays; *gus* is a bacterial gene specific to *Escherichia coli* (Chen et al. 2019). BLAST analyses confirmed that the *gus* sequence was not present in the partial *P. striolata* transcriptome.

Once dry, two coated leaf disks were placed into one-ounce plastic cups along with five adult flea beetles and covered by a lid with three small holes to permit airflow. A total of 1000 ng of dsRNA (two leaf disks, each with 500 ng) was delivered to each cup of beetles. The cups were placed onto a layer of moistened paper towels in 6.4 L containers and stored in an insectary in the animal holding facility to help maintain humidity. Ten replicate cups were used for each dsRNA treatment or control, totalling 50

beetles per treatment. Five replicate cups from each treatment were used in the 8-day feeding assay, while five replicate cups from each treatment were set aside to extract RNA and perform qRT-PCR later. For replicates used in the feeding assay, mortality and leaf disk consumption were recorded every two days, and two new leaf disks with a new application of dsRNA or buffer were placed in the cup throughout the 8 days. In terms of the replicates collected for RNA extractions and qRT-PCR, five replicates of each treatment were collected on day 5. Then, five flea beetles from a cup were transferred to a 1.5 mL Safe-Lock microcentrifuge tube (Eppendorf), and 300 µL lysis buffer (supplemented with 6 µL β-mercaptoethanol) was added to each microcentrifuge tube. A total of 70 samples (n = 5) were frozen and preserved at -80°C. Flea beetles will be homogenized for RNA extraction to carry out qRT-PCR at a later time to assess whether RNAi-mediated knockdown can be detected by comparing the transcripts of two reference genes, ribosomal protein L19 (RPL19) and actin 5C (Act5C), to normalize the target genes' transcript levels and to calculate the degree of knockdown.

Statistical analysis

Data analysis was conducted with a one-way ANOVA to determine the differences in *P. striolata* survival and differences in leaf disk consumption at day 8 for each treatment (GraphPad Prism 4, GraphPad Software). Log-rank (Mantel-Cox) or Gehan-Breslow-Wilcoxon tests were used to determine the significance for all treatments compared to the no-treatment condition and *gus* (n = 5). Mann-Whitney U-tests were used to determine the significance in leaf disk consumption for all treatments relative to the no-

treatment condition and *gus* (n = 5). For all statistical tests, a P value ≤ 0.05 indicated significance.

Results

Phylogenetic analysis

To find putative orthologues of the *P. striolata Rop* and *Sec23* genes, a BLAST search of each long dsRNA nucleotide sequence was used to obtain the translated protein coding sequence for ten species of interest. Then, Maximum Likelihood phylogenetic trees were constructed (Stecher et al. 2020; Fig. 1). The phylogenetic tree for the *Rop* gene target confirmed that the *P. striolata* gene most closely resembled that of its sibling species, *P. cruciferae*, and also fell within an apparent monophyletic clade of *Rop* genes from other predicted beetle species, supported by high bootstrap values (Fig. 1a). In comparison, the phylogenetic tree generated for *Sec23* was weakly supported, with low bootstrap values observed throughout the tree (Fig. 1b). Furthermore, a lack of resolution occurred within the order Diptera, with *Drosophila melanogaster* being classified as more closely related to the monarch butterfly (*Danaus plexippus*) than to another dipteran, *A. aegypti* (Fig. 1b).

Additionally, to determine whether the *Rop* and *Sec23 P. striolata* dsRNAs might affect any off-target species, a visual inspection of the pairwise alignments of the long dsRNAs was conducted to assess whether there were conserved sequences of 21 or more nucleotides in other species (Zamore et al. 2000; Elbashir et al. 2001; Figs. 2, 3). The only conserved sequences long enough to form siRNAs potentially capable of inducing RNAi occurred in the sibling species, *P. cruciferae*. Between *P. striolata* and *P. cruciferae*, a

92.9% pairwise identity with two separate 33 and 34 conserved nucleotide sequences was identified in *Rop* (Fig. 2). In *Sec23*, a 96.3% pairwise identity with 35, two 36, and 46 nucleotide sequences were conserved between *P. striolata* and *P. cruciferae.* (Fig. 3).

Mortality of P. striolata

Mortality of *P. striolata* was assessed every two days throughout the 8-day bioassay, and significance in survival was evaluated using Log-rank (Mantel-Cox) tests or Gehan-Breslow-Wilcoxon tests (GraphPad Prism 4, GraphPad Software). Significant differences between the survival of *P. striolata* that did not receive any dsRNA ("no-treatment condition") were observed between *P. striolata* treated with elution buffer, *Rop* long dsRNA, *Rop* pcRNA, all *Sec23* treatments, and all *Snf7* positive controls (Fig. 6). Unexpectedly, significant differences between the "no-treatment" and *gus* pcRNA were also observed, which suggests that the *gus* pcRNA may have some off-target effects in this beetle.

The pcRNAs induced the highest levels of mortality for *Sec23* and *Snf7* (Fig. 6). Significant differences were observed between both the *Sec23* and *Snf7* pcRNAs ($P \leq 0.05$ for both the Log-rank test and the Gehan-Breslow-Wilcoxon test) relative to either the no-treatment condition or the *gus* pcRNA negative control. By day 8 of the bioassay, the pcRNA targeting *Sec23* resulted in 76% mortality of *P. striolata*, while the *Snf7* pcRNA positive control caused 84% mortality. In comparison, no significant differences were observed when comparing the mortality of *P. striolata* treated with *Rop* pcRNA to *gus* pcRNA and resulted in only 44% mortality.

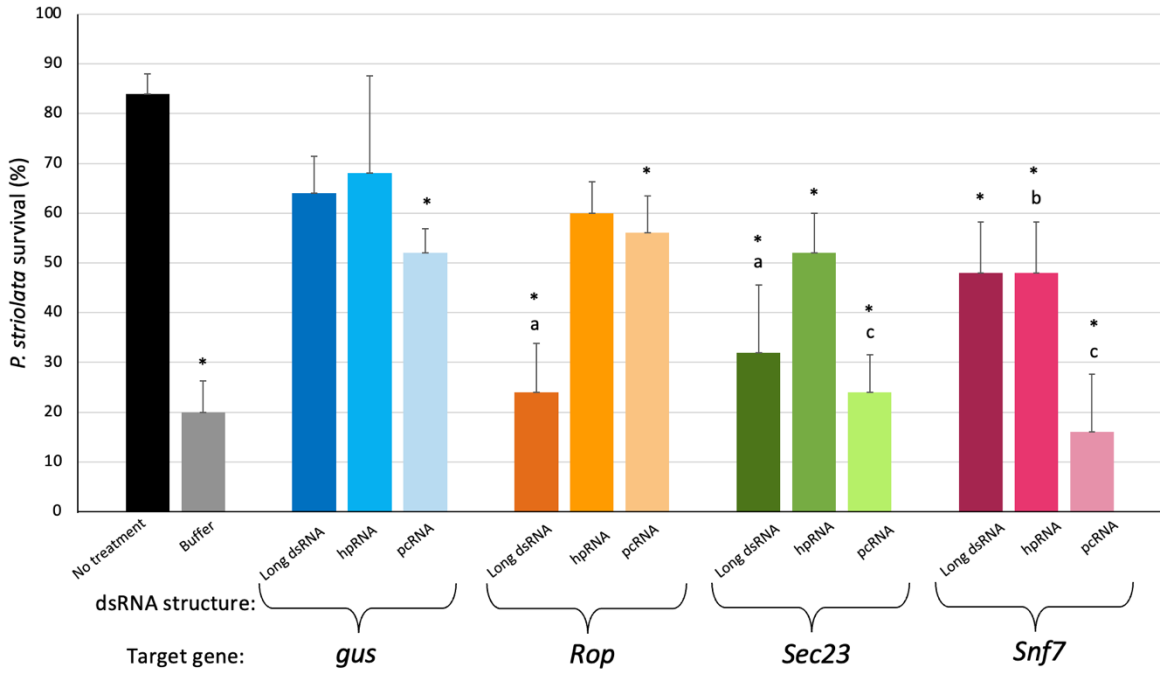

Figure 6. Percentage of *P. striolata* survival at day 8 of the feeding bioassay. Data are presented as the mean + SEM of n = 5 replicate treatments using a one-way ANOVA. Log-rank (Mantel-Cox) or Gehan-Breslow-Wilcoxon tests were conducted to determine significance. The following symbols denote significance when $P \leq 0.05$: (*) significant difference relative to the no-treatment condition; (a) significant difference relative to *gus* long dsRNA; (b) significant difference relative to *gus* hpRNA; (c) significant difference relative to *gus* pcRNA.

For the long dsRNAs, the highest mortality was observed when targeting *Rop*, followed by the dsRNA targeting *Sec23* (Fig. 6). Significant differences were observed between both *Rop* and *Sec23* long dsRNAs ($P \leq 0.05$ for both the Log-rank test and the Gehan-Breslow-Wilcoxon test) compared to either the no-treatment condition or the *gus* long dsRNA treatment. Consuming long dsRNA resulted in the mortality of 68% and 76% of *P. striolata* in *Sec23* and *Rop,* respectively. In contrast, *Snf7* long dsRNA was not significantly different from that of *gus* long dsRNA and only resulted in 52% mortality of *P. striolata*.

The hpRNA dsRNA structures resulted in the lowest levels of mortality for all *P. striolata* gene targets (Fig. 6). Significant differences were observed between *Sec23* and *Snf7* hpRNA ($P \leq 0.05$ for both the Log-rank test and the Gehan-Breslow-Wilcoxon test) compared to the no-treatment condition. In contrast, only the *Snf7* hpRNA and not the *Sec23* hpRNA was significantly different from that of the *gus* hpRNA ($P \leq 0.05$ for the Gehan-Breslow-Wilcoxon test). The *Rop* hpRNA was not significantly different from the no-treatment condition or to *gus* hpRNA. The *Snf7* hpRNA produced the highest percentage of mortality in *P. striolata,* with a mortality rate of 52% (Fig. 6). In comparison, the consumption of hpRNA induced mortalities of 40% and 48% of *P. striolata* in *Rop* and *Sec23* treatments, respectively.

Relative to the no-treatment condition, a significant difference between flea beetles that consumed undiluted elution buffer from the MEGAscript RNAi was observed (Fig. 6). The use of elution buffer alone resulted in 80% mortality of *P. striolata.* For this reason, all bioassays reported in Figure 6 are based on the dilution of dsRNAs in nuclease-free water, and all data based on earlier bioassays using dsRNAs diluted in elution buffer are not presented.

Leaf disk consumption

To assess whether the topically applied dsRNAs reduced the flea beetles' feeding activity, the leaf disk consumption percentage was scored every two days by visual estimation. Significant differences in average leaf disk consumption were observed between all treatments, *gus* controls, and *Snf7* controls relative to the no-treatment condition (Fig. 7). The average leaf disk consumption in *gus* long dsRNA was also significantly higher compared to *Sec23* long dsRNA ($P \leq 0.05$ for both the Log-rank test and the Gehan-Breslow-Wilcoxon test), while the average leaf consumption of *Rop* pcRNA was significantly higher than *gus* pcRNA ($P \leq 0.05$ for both the Log-rank test and the Gehan-Breslow-Wilcoxon test). Overall, high and variable feeding rates were observed between treatments, with the average leaf disk consumption ranging between 45% and 87% throughout the assay (Fig. 7).

A trend that emerged across different treatments was that a slight increase in leaf disk consumption occurred on day 4 (Fig. S1a). After day 4, treatments that had the highest percentage of *P. striolata* mortality, notably *Sec23* long dsRNA*, Sec23* pcRNA, *Snf7* pcRNA, and elution buffer, experienced declines in leaf disk consumption (Fig. 6; S1a). Furthermore, treatment groups that had the highest survival of *P. striolata,* including *Rop* hpRNA, *Rop* pcRNA, *Sec23* hpRNA, generally had a higher average consumption of leaf disks (Figs. 6, S1a).

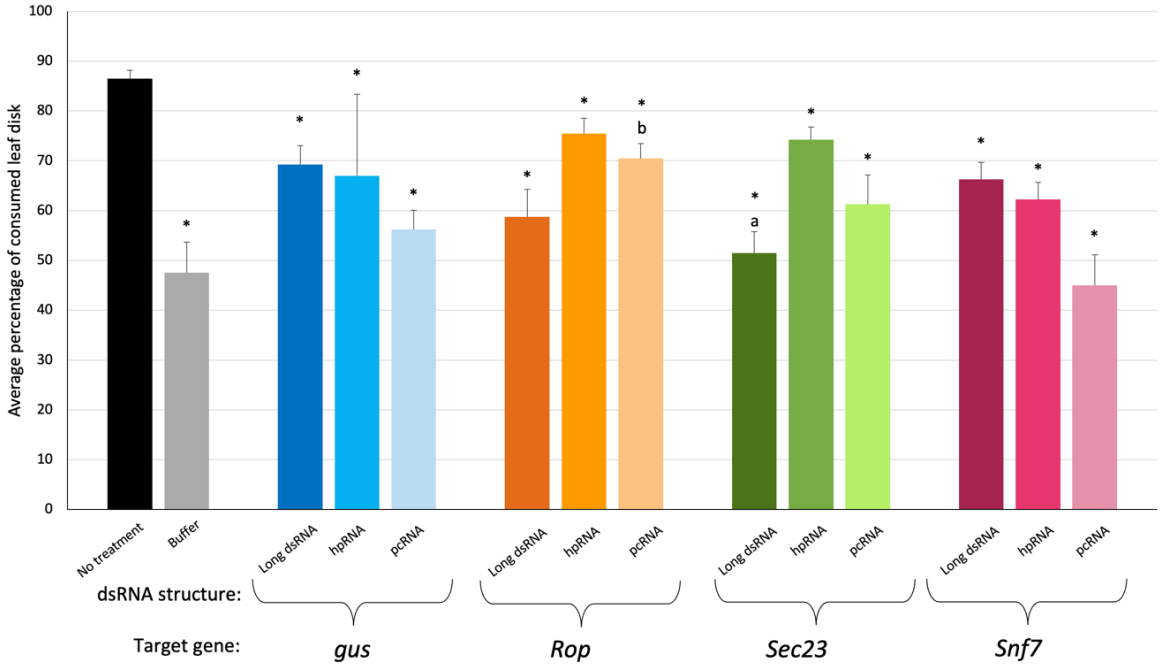

Figure 7. Average percentage of *P. striolata* leaf disk consumption. *P. striolata* leaf disk consumption for all replicates was averaged across the entire 8-day assay (n = 20). The average leaf disk consumption per day was plotted. Mann-Whitney tests were conducted to determine significance. The following symbols denote significance when $P \leq 0.05$: (*) significant difference relative to the no-treatment condition; (a) significant difference relative to *gus* long dsRNA; (b) significant difference relative to *gus* pcRNA.

Discussion

Effective control of flea beetles is critical for the sustainable production of canola. With growing concerns about the adverse environmental effects along with developing resistance due to continuous applications of current chemical insecticides, new pest management strategies are required (Pistorius et al. 2010; Knodel et al. 2017; Bartling et al. 2019). RNAi-based pesticides are being developed to control a variety of crop pests, as their species-specific nature offers the promise of an environmentally safer approach to protecting our valued crops (Killiny et al. 2014; Yan et al. 2020; Chen et al. 2022). In this study, different dsRNA structures were examined for their ability to kill *P. striolata* flea beetles and thereby reduce feeding damage on canola leaves. Of the three different dsRNA structures examined, long linear dsRNAs and short pcRNAs proved effective at killing flea beetles, which also resulted in reduced consumption of the treated canola leaf tissues. However, the different gene targets had varying abilities to kill flea beetles for both the long dsRNA and pcRNA structures. The third dsRNA structure, the hpRNAs, proved the least effective, and in most cases, largely ineffective, relative to the negative control treatments, in controlling flea beetles. While this study examined dsRNAs specific for only three target genes, it provides us with some clear evidence that RNAi-based pesticides have the potential to control these economically important pests.

Selection of target genes

In this study, two new genes, and one gene previously examined (*Snf7*), were targeted in *P. striolata* based on previous observations that these dsRNAs were effective at killing another insect, the mosquito *A. aegypti* (Whyard, unpublished data). Using

BLAST searches of a partial *P. striolata* transcriptome, putative orthologues of *Rop* and *Sec23* were identified based on their closest similarity to those genes in other insects' genome databases. Maximum likelihood phylogenetic analyses provided evidence that the Rop partial amino acid sequence shares considerable similarity to predicted Rop proteins from other insects (Stecher et al. 2020; Fig. 1). As expected, the *P. striolata* Rop sequence was more similar to Rop in other beetles and showed an increasing degree of sequence divergence from predicted Rop proteins of insect clades that are more distantly related to beetles. These data suggest that the putative *P. striolata* Rop protein is indeed orthologous to other insect Rop proteins (Chen et al. 2022; Fig. 1a).

In contrast, the phylogenetic tree generated for the Sec23 protein was weakly supported, with low bootstrap values observed at most branch points (Fig. 1b). While each of the genes were annotated as *Sec23* within NCBI, they lack much sequence similarity. This suggests that either the *Sec23* gene is highly mutable, resulting in divergent proteins across different species, or that the gene we evaluated is not a bona fide Sec23 orthologue. Of note, a conserved Sec23 protein domain has been identified; the gelsolin-like protein domain (Gramates et al. 2022). The gelsolin-like protein domain, identified at the C-terminal of *Sec23* in *Drosophila* (IPR007123), shares a 73.75% conserved nucleotide identity with *Sec23* in *P. striolata*, as verified by conducting a BLAST against the *Drosophila* gelsolin-sequence with *P. striolata* (Gramates et al. 2022; Paysan-Lafosse et al. 2022; Fig. S2). This portion of the protein's structure, which has a key role in the regulation of actin polymerization, is conserved across species and is currently the only evidence available that the *P. striolata* sequence encodes this protein (Weeds et al. 1986).

It should also be noted that only the last 84 nucleotides of the selected *Sec23* long dsRNA are a part of this gelsolin-like domain, which is 261 nucleotides in length (Fig.

S3). If the selected long dsRNA was shifted to include more of the gelsolin-like domain, a higher conservation of nucleotides between species would have occurred and perhaps generated a more clearly resolved phylogenetic tree. Nevertheless, the overall lack of conservation of the *P. striolata Sec23* sequence to that of any other insects suggests that the dsRNA used in this study would be specific for the flea beetles, as the selected dsRNA should not generate siRNAs that can produce an RNAi response in off-target insect species.

To further explore this idea, pairwise alignments of *Rop* and *Sec23 P. striolata* dsRNAs with the putative orthologues in other insects were visually inspected. The only conserved sequences that were 21 or more nucleotides in length, which are long enough to form siRNAs capable of inducing RNAi, occurred in the sibling species, *P. cruciferae* (Zamore et al. 2000 Fig. 2, 3). Between the *Rop* nucleotide sequences for *P. striolata* and *P. cruciferae*, a 92.9% pairwise identity and two separate conserved sequences that were 33 and 34 nucleotides in length were observed (Fig. 2). Similarly, the *Sec23* nucleotide sequences for *P. striolata* and *P. cruciferae* had a 96.3% pairwise identity, along with four conserved sequences that ranged from 35 to 46 nucleotides in length (Fig. 3). The high degree of similarity between the *P. striolata* and *P. cruciferae* nucleotide sequences for both genes of interest indicates that multiple siRNAs can theoretically be designed to specifically target both species of flea beetles.

In the other species of interest, the largest number of conserved contiguous nucleotide sequences with *P. striolata* ranged from 8-17 nucleotides or 11-19 nucleotides in *Rop* and *Sec23,* respectively. As these sequences are shorter than the required 21 nucleotide sequence for a functional siRNA, no predicted off-target effects should occur, at least in the species examined here, when using these dsRNAs as pesticides. However,

to confirm the specificity of the selected *P. striolata* dsRNAs, it will be necessary to carry out feeding bioassays on non-target insects to ensure that there are no lethal or sublethal impacts (Bachman et al. 2013).

Effect of dsRNA structure on P. striolata mortality

The dsRNA structure and gene targets that induced the highest levels of mortality in *P. striolata* were *Sec23* and *Snf7* pcRNAs (Fig. 6). By day 8 of the assay, pcRNA targeting *Sec23* resulted in the mortality of 76% of *P. striolata*, which is comparable to 84% mortality observed in the *Snf7* pcRNA positive control. Given that *Sec23* pcRNA had a similar effect in inducing mortality of *P. striolata* compared to the previously tested *Snf7* dsRNA, this *Sec23* pcRNA should be considered in the development of alternative dsRNA-based insecticides. However, quantifying changes in gene expression after ingestion of these pcRNAs via qRT-PCR is an essential next step to verify that RNAi knockdown is indeed occurring.

Although *Sec23* and *Snf7* pcRNAs efficiently increased the mortality of *P. striolata*, no significant differences were observed when comparing the mortality of *P. striolata* treated with *Rop* pcRNA to the no-treatment condition or to *gus* pcRNA (Fig. 6). The selected *Rop* pcRNA might not have been as effective as the pcRNAs used for other gene targets due to variation in the secondary or tertiary structure of the targeted mRNAs, which cannot be accounted for with the RNA structure algorithms used in this study (Smith 2006). To elaborate, siRNAs were chosen from Integrated DNA Technologies design software tool, which predicts effective siRNA based on an algorithm that searches a target gene sequence, calculates scores of sequence candidates, and ranks optimal

siRNAs (Owczarzy et al. 2008). Although the design algorithm is regularly updated as more models are published, permitting more effective siRNAs to be predicted, we cannot be certain that the resulting siRNA will produce an efficient RNAi response (Smith 2006; Owczarzy et al. 2008). This is because not all siRNAs targeting an mRNA will have equal access to the RNA due to the variation in stable folding of the secondary and tertiary structure of each mRNA (Smith 2006).

In this regard, the *Rop* siRNAs that were selected for this project were not likely to have been the most effective siRNAs. The first pcRNA designed to target *Rop* failed to produce any *in-vitro* transcribed product, which might reflect an inability of that dsRNA to fold into the correct conformation predicted by the RNAfold algorithm (Fig. 4). The second pcRNA targeting Rop failed to affect flea beetle mortality, which suggests that this gene may require the design and testing of several more siRNAs before an effective one is identified (Fig. 6).

Rop long dsRNA and *Sec23* long dsRNA were both effective in killing the flea beetles, resulting in the death of 68% and 76% of *P. striolata*, respectively (Fig. 6). Since *Rop* long dsRNA was more effective compared to the siRNA designed for the hpRNA and pcRNA, this suggests that some siRNAs within that long dsRNA could initiate RNAi. Furthermore, given that the long dsRNA was ~210 bp and was diced into ~10 siRNAs that target multiple different mRNA sequences, there is a possibility that the siRNAs diced from the long dsRNA were more potent than the siRNAs that were chosen for the hpRNA and pcRNA structures for *Rop*. Considering siRNAs are not all equally as efficient, this may explain why *Rop* long dsRNA had a higher efficiency in inducing the mortality of flea beetles than *Rop* hpRNA or pcRNA. However, as mentioned previously,

monitoring these trends by quantifying gene expression with qRT-PCR is required to fully understand the efficacy of RNAi-mediated knockdown in *P. striolata*.

While the long dsRNAs for *Sec23* and *Rop* were effective at killing the flea beetles, the *Snf7* long dsRNA did not work as effectively as anticipated since previous bioassays that used *Snf7* long dsRNA to target field-caught *P. cruciferae* resulted in up to 60% mortality at day 8 (Whyard, unpublished data). However, the field-caught flea beetles were likely exposed to other environmental stressors, both abiotic (e.g. temperature and humidity fluctuations, chemical pesticides) and biotic (e.g. parasites, microbial, or viral infections). In comparison, the *P. striolata* used in this assay were likely healthier and more robust since they were lab-bred and were not exposed to many, if any, environmental stressors. It has also been noted that *P. striolata* flea beetles are more resilient to traditional pesticides, including neonicotinoid seed treatments, in comparison to *P. cruciferae* (Knodel et al. 2017). This may suggest that *P. striolata* have a better physiological tolerance than *P. cruciferae* to a broad range of chemicals, perhaps using some generalized mechanisms, and therefore require higher doses of the dsRNA to be effective.

Furthermore, the *Snf7* long dsRNA used in this bioassay might not have been as effective as previously thought since the assays targeting *Snf7* long dsRNA in *P. cruciferae* have also used 250 bp or 500 bp long dsRNA fragments to achieve higher levels of mortality (Whyard, unpublished data). In contrast, the current bioassay used *Snf7* long dsRNA that was shortened to ~210 bp to ensure that the same dosage of dsRNA was applied for every treatment. Consequently, this truncated *Snf7* long dsRNA might not have worked as effectively at killing the flea beetles since longer dsRNAs have been proven to be more effective at inducing gene silencing in other insect species (Mao et al.

2007; Kumar et al. 2012). As a result, the *Snf7* long dsRNA did not produce as high of a level of mortality in *P. striolata* as was initially expected.

For all *P. striolata* gene targets, hpRNA was the least effective dsRNA structure (Fig. 6). Short hpRNAs are considered more effective at inducing target knockdown than linear siRNAs of the same length, presumably because the hairpin structure provides some protection from nuclease degradation as one of its ends is sealed and inaccessible to an exonuclease (Bolognesi et al. 2012). Nonetheless, hpRNA may be less effective at mediating gene knockdown than pcRNA in similar RNAi studies (Abbasi et al. 2020). Presumably, pcRNA molecules mediate a higher level of gene knockdown due to their enhanced stability in which both ends are protected from nuclease degradation by forming two single-stranded loops (Abbasi et al. 2020). In a nuclease-based assay targeting *A. aegypti*, a higher degree of degradation was observed in hpRNA than in pcRNA (Whyard, unpublished data). To confirm this higher stability of pcRNA compared to hpRNA in *P. striolata*, a similar nuclease-based assay should be conducted.

Furthermore, pcRNA might mediate a higher level of transcript knockdown than hpRNA, as it enters the cells through a clathrin-independent uptake pathway (Abbasi et al. 2020). Further molecular analyses are necessary to understand whether this same clathrin-independent cellular uptake process occurs in *P. striolata* and also how this mechanism functions (Abbasi et al. 2020).

In preliminary bioassays for this study, the dsRNAs were diluted in elution buffer from the MEGAscript RNAi kit (Fig. S4b, S4c, S5). As noted earlier, the buffer alone caused high mortalities, likely because it contains EDTA, a chelating agent which sequesters divalent and trivalent metal ions that are important cofactors for many enzymes (Cooper et al. 2021; Fig. 6). EDTA is often used to improve the stability of dsRNA since

enzymes, including nucleases, require inorganic cofactors for functional catalytic activity (Cooper et al. 2021). Mg2+ is the most abundant divalent cation found within cells and is an important requirement for ligand binding and neutralizing the charge of phospholipids and nucleic acids (Maguire and Cowan 2002; Yang 2011). The sequestration of essential metal ions, including Mg2+, by EDTA may lead to detrimental effects, no longer permitting essential physiological functions to be performed. Furthermore, EDTA has been reported as toxic to certain insects in midgut tissue culture assays (Cooper et al. 2021). This toxicity might explain why elution buffer alone (i.e., without the addition of any target dsRNA) was toxic enough to kill *P. striolata*.

Some deaths were also observed in *P. striolata* that consumed *gus* dsRNA (Fig. 6). The flea beetle transcriptome is suspected to be incomplete and may therefore have some transcripts that are similar enough to the *gus* dsRNA to be knocked down by this presumed negative control dsRNA. To prevent non-specific deaths in the negative controls, alternative negative controls, such as enhanced green fluorescent protein (EGFP), should be explored (Chen et al. 2022).

Furthermore, the flea beetles might have been dying due to the non-specific effects of using high concentrations of dsRNA, which perhaps could cause a degree of osmotic shock within the gut due to increased solute (RNA) levels. To explore this possibility, lower doses of dsRNA will need to be tested to find the optimal dose of non-specific dsRNA that does not cause significant mortality. Additionally, non-specific mortality might have also occurred due to the age of the insects, as they were stored at the BASF labs and eventually shipped to the University of Manitoba as adults, and hence, they may have been close to their full lifespan upon arrival. Continuing to monitor these trends by

conducting more bioassays with larger sample sizes will be required to comprehend the effects of dsRNA consumption in *P. striolata*.

Impacts on leaf disk consumption

The current bioassay provided evidence that dsRNA treatments that killed more flea beetles reduced the feeding damage on canola leaf tissues. Specifically, the dsRNA treatments with the lowest average percentage of leaf tissue consumption, *Snf7* pcRNA, *Sec23* pcRNA, *Sec23* long dsRNA, and *Rop* long dsRNA, were also the dsRNA treatments with the highest flea beetle mortality (Figs. 6, 7). Flea beetles that consumed the elution buffer also demonstrated a lower level of leaf tissue consumption, presumably due to the high mortality observed in this treatment (Figs. 6, 7).

Significant differences in leaf tissue consumption were observed between the no-treatment condition in comparison to all other dsRNA treatments and controls, including *gus* (Fig. 7). Such differences between the feeding behaviour of *gus* relative to the no-treatment condition suggest that using dsRNA or buffer solutions to coat leaf disks may have rendered them less appealing to the flea beetles, which is a cause for concern as this may have produced-nonspecific deaths. Nonetheless, it is important to consider that a small sample size was used for the bioassay, and it will be imperative to conduct future assays using larger sample sizes to validate these findings.

Overall, the high percentage of average consumed leaf disks demonstrates that the flea beetles had a large appetite and might be consuming all the leaf material given, with possible non-specific mortality occurring due to starvation. Previous assays in which only one leaf disk per cup was used demonstrated increased mortalities of the flea beetles,

along with high levels of leaf consumption in the no-treatment and *gus* dsRNA control flea beetles (Figs. S1b, S1c, S4). In these earlier assays, such non-specific deaths suggest that non-specific deaths might have occurred due to starvation (Fig. S4). Although providing the flea beetles with two leaf disks in the current bioassay did increase the survival of the no-treatment and *gus* dsRNA control flea beetles, perhaps the beetles require a greater amount of leaf tissue to survive. For instance, similar feeding bioassays with *P. striolata* have used topically applied dsRNA onto 3 cm x 4 cm brown mustard (*Brassica juncea)* leaves (Chen et al. 2022). Chen et al. 2022 observed that the negative controls, nuclease-free water and EGFP, showed less than 10% mortality on day 8 and less than 20% mortality on day 16. In comparison, 16-48% mortality was observed in the negative controls by day 8 of the current bioassay I performed (Fig. 6). To prevent non-specific mortality due to starvation and reduce the variability of consumption rates, providing the flea beetles with larger leaf tissues should be considered in future assays.

Conclusion

In conclusion, we must develop new, sustainable pest management strategies to protect canola from the serious damages inflicted by flea beetles. Since chemical pesticides have led to increasing incidences of resistance in target pests and have caused adverse environmental effects on off-target organisms, it will be critical to investigate and develop biosafe alternatives (Pistorius et al. 2010; Bartling et al. 2019). RNAi-based pesticides show considerable promise, and in this study, the pcRNAs offer a novel version of dsRNA molecules that, in some cases, were as effective as long dsRNAs. Although this study was limited to only three target genes, these results provide valuable insights into

the potential of RNAi-based pesticides with alternative dsRNA structures for controlling insect pests that have significant economic impacts.

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Appendix

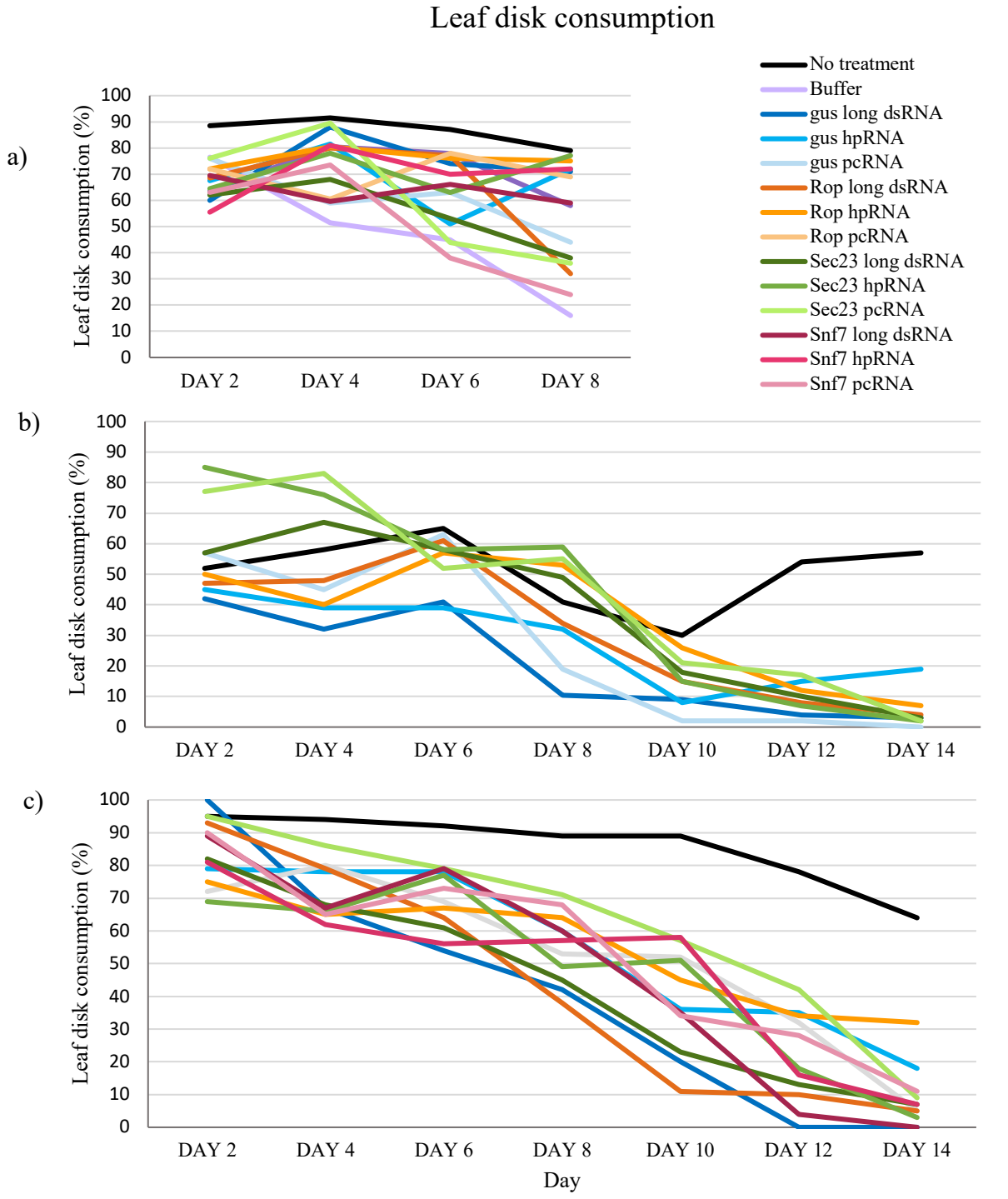


Figure S1. Percentage of *P. striolata* survival throughout three assays. *P. striolata* leaf disk consumption was observed and recorded for **a)** 8 days in the current assay discussed in this thesis, **b)** 14 days in preliminary assay #1 and **c)** 14 days in preliminary assay #2. Both preliminary assays used elution buffer to dilute all dsRNAs and only one leaf disk per cup and were therefore not presented in the results or discussion of this thesis. The average of **a)** n = 5 or **b)** n = 10 or **c)** n = 10 was used to calculate and plot each treatment.

```
P. striolata        CCTGAGCCTGTACTATTAGATACGAGTTCCATACAGCCCGATAGAATATTGCTTATGGAC      60
D. melanogaster     CCAGAACCGGTCCTCCTCGATACGGCTTCCATCCAGGCGGATCGCATCCTCCTGATGGAC      60
                    ** ** ** ** **  * ******  ****** *** * *** * **  * ** ******

P. striolata        ACGTTTTTCCAAATTTTGATATTCCACGGGGAGACTATAGCTCAATGGAGGAATTTGAAA      120
D. melanogaster     ACGTTCTTTCAGATTCTGATTTACCACGGCGAGACCATCGCCCAGTGGAGAGCCCTCAAA      120
                    ***** ** ** *** **** * ****** ***** ** ** ** *****     * ***

P. striolata        TATCAAGACATGCCCGAATACGAGAACTTTAGGCAATTGTTGCAAGCTCCTATTGATGAT      180
D. melanogaster     TACCAGGACATGCCAGAGTACGAGAACTTCAAGCAGCTGCTGCAAGCACCGGTGGACGAC      180
                    ** ** ******** ** ********** * ***  ** ******* **  * ** **

P. striolata        GCACAAGAGATATTACAAACAAGGTTCCCCATGCCGAGATATATCGACACTGAACAAGGG      240
D. melanogaster     GCGCAAGAGATTCTTCAGACGCGCTTCCCCATGCCTCGCTACATCGATACGGAGCACGGC      240
                    ** ********  * ** **  * **********  * ** ***** ** ** ** **

P. striolata        GGATCCCAGGCTAGATTTTTA       261
D. melanogaster     GGATCCCAGGCCCGCTTCCTG       261
                    **********  * **  *
```

Figure S2. Multiple sequence alignments showing the nucleotide sequence similarities between the *Sec23* gelsolin-like domain in *P. striolata* (OU900098.1) and *D. melanogaster*. The gelsolin-like domain for *D. melanogaster* was obtained from FlyBase (IPR007123).

(Gramates et al. 2022; Madeira et al. 2022)

```
Long dsRNA          GTTCCTGCAAGTTTTCAATAATTCTCCTGATGAAACATCCTTCTATCGGCATATGTTGAT      60
Gelsolin            ------------------------------------------------------------      0

Long dsRNA          GAGAGAAGACCTTACTCAATCACTGATCATGATTCAACCAATTTTGTACAGTTACAGTTT      120
Gelsolin            ------------------------------------------------------------      0

Long dsRNA          CAACGGCCCCCCTGAGCCTGTACTATTAGATACGAGTTCCATACAGCCCGATAGAATATT      180
Gelsolin            ----------CCTGAGCCTGTACTATTAGATACGAGTTCCATACAGCCCGATAGAATATT      50
                              **************************************************

Long dsRNA          GCTTATGGACACGTTTTTCCAAATTTTGATATTC--------------------------      214
Gelsolin            GCTTATGGACACGTTTTTCCAAATTTTGATATTCCACGGGGAGACTATAGCTCAATGGAG      110
                    ********************************

Long dsRNA          ------------------------------------------------------------      214
Gelsolin            GAATTTGAAATATCAAGACATGCCCGAATACGAGAACTTTAGGCAATTGTTGCAAGCTCC      170

Long dsRNA          ------------------------------------------------------------      214
Gelsolin            TATTGATGATGCACAAGAGATATTACAAACAAGGTTCCCCATGCCGAGATATATCGACAC      230

Long dsRNA          --------------------------------      214
Gelsolin            TGAACAAGGGGGATCCCAGGCTAGATTTTTA      261
```

Figure S3. Multiple sequence alignments showing the nucleotide sequence similarities between the selected *Sec23* long dsRNA and the gelsolin-like domain in *P. striolata.*

(Madeira et al. 2022)

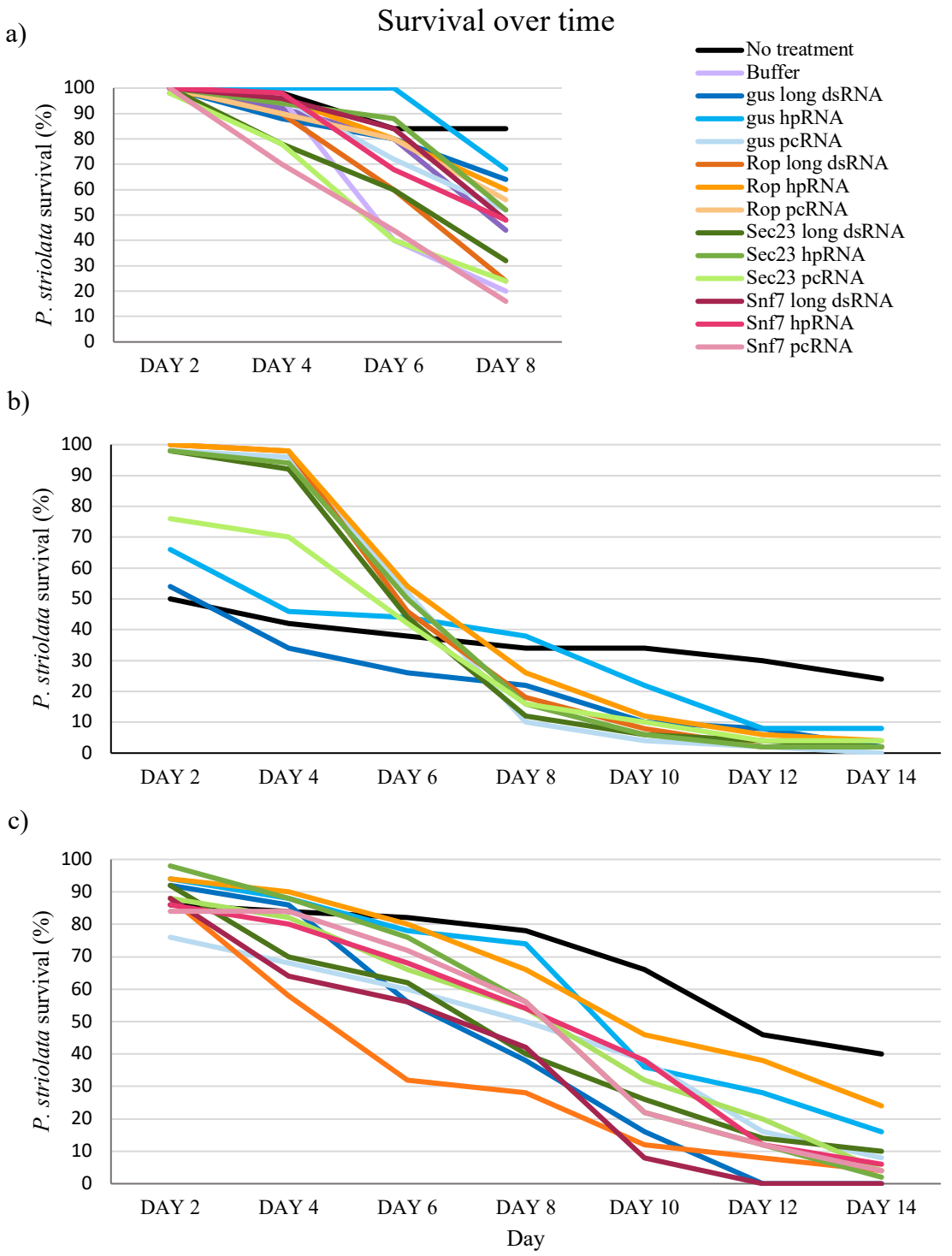
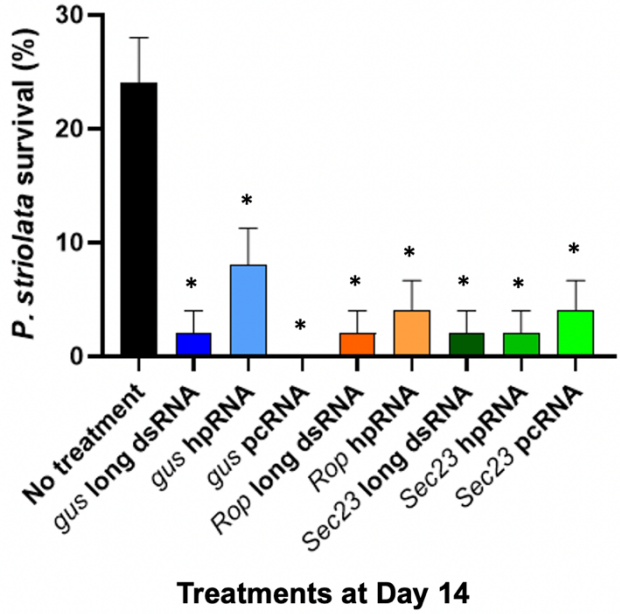


Figure S4. Percentage of *P. striolata* survival throughout three assays. *P. striolata* survival was assessed every two days during **a)** the current assay discussed in this thesis, **b)** preliminary assay #1, and **c)** preliminary assay #2. Both preliminary assays used elution buffer to dilute all dsRNAs and only one leaf disk per cup and were therefore not presented in the results or discussion of this thesis. The average of **a)** n = 5 or **b)** n = 10 and **c)** n = 10 was used to calculate and plot each treatment.

a)



b)

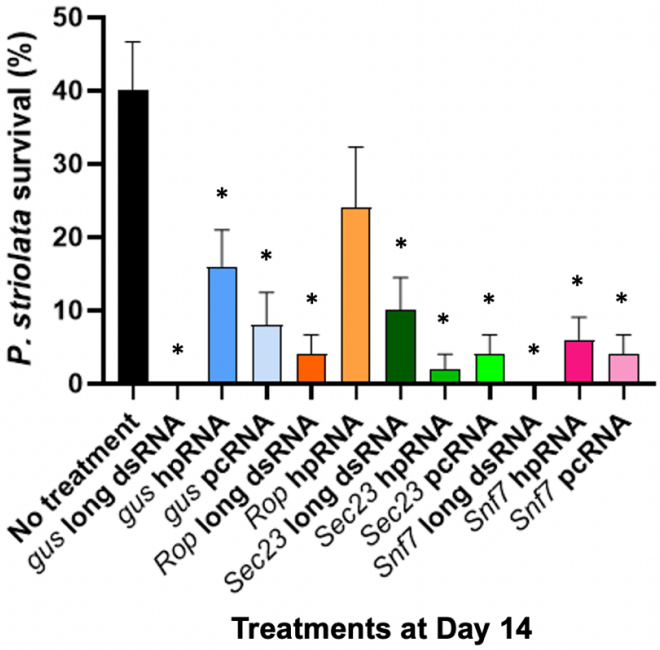


Figure S5. Percentage of *P. striolata* survival at day 14 in preliminary assays. *P. striolata* survival was assessed for **a)** preliminary assay #1 and **b)** preliminary assay #2. Data are presented as the mean + SEM of n = 10 replicate treatments using a one-way ANOVA. A Mann-Whitney U test comparing the no-treatment condition to each treatment was used to determine significance. Significance was observed between the no-treatment condition to all controls and treatments except for *Rop* hpRNA in assay #2. An asterisk denotes significance to the no-treatment condition when $P \leq 0.05$.