2020-2021 CALIFORNIA LEAFY GREENS RESEARCH PROGRAM
RESEARCH PROPOSAL

Project Title: Efficacy of RNA interference (RNAi) technology to manage thrips and viruses in lettuce

Principal Investigators:
Daniel K. Hasegawa
Research Entomologist
USDA-ARS, Crop Improvement and Protection Research Unit
1636 East Alisal Street, Salinas CA 93905 USA
Tel. 831-755-2826
daniel.hasegawa@usda.gov

William M. Wintermantel
Research Plant Pathologist
USDA-ARS Crop Improvement and Protection Research Unit
1636 East Alisal Street, Salinas CA 93905 USA
Tel. 831-755-2824
bill.wintermantel@usda.gov

Abstract:
Western flower thrips is the primary insect vector for impatiens necrotic spot virus (INSV), a virus that has become an increasing problem in lettuce production in the Salinas Valley. Only limited strategies exist for thrips management due to the lack of efficacious chemistries and restrictions on maximum residue limits. Furthermore, thrips are problematic as they create cosmetic issues in lettuce based on the standards set by customers. There are also no direct methods for managing INSV. This has created a need for new management strategies. RNA interference (RNAi) technology is an emerging strategy that has massive potential for the agriculture sector and has been demonstrated to be effective as a biopesticide for crop protection against insect pests and pathogens. RNAi is a natural process that results in gene silencing in insects, plants, and humans, and its use as a biopesticide can be applied using methods that avoid genetic engineering of plants. Furthermore, RNAi-based biopesticides can be tailored to be highly specific and are completely degradable in the environment, providing unique advantages over many conventional insecticides. Here, we successfully designed and synthesized double-stranded RNA (dsRNA) molecules targeting western flower thrips and INSV. We demonstrate successful uptake of dsRNAs through the roots of young lettuce plants, systemic movement into the leaves, and show its stability for at least 14 days. Furthermore, preliminary data suggests there may be an effect during the onset of INSV symptom development, but further studies are required. This research is equivalent to a proof-of-concept phase of product development but do show promise for an RNAi application in lettuce. Ongoing studies are further characterizing the utility of RNAi for managing thrips and INSV in lettuce.

Objectives:

1. Identify and synthesize active RNAi triggers to target thrips and INSV. This objective is to identify genetic sequences that are unique to western flower thrips and INSV and synthesize numerous active RNAi triggers, known as double-stranded RNA (dsRNA). The synthesized dsRNA can then be tested for their potential to manage thrips and INSV.
   Deliverables: Small-scale generation of active dsRNA that can be tested for their potential to induce mortality of western flower thrips and prevent or reduce INSV in lettuce.
2. Efficacy of RNAi technology using non-GMO strategies to manage thrips and INSV. This objective involves small-scale laboratory and greenhouse experiments to optimize the delivery and characterize the stability of dsRNA that is transiently delivered to lettuce. The objective will also test the potential for dsRNAs to kill western flower thrips and prevent or reduce INSV severity in lettuce.
   Deliverables: This objective works towards developing a proof-of-concept for using RNAi technology as a strategy for managing thrips and INSV and is considered early Phase 1 of the product development pipeline.

Procedures:

Objective 1: Identify and synthesize active RNAi triggers to target thrips and INSV. Genes from western flower thrips were selected for RNAi targeting. Genetic alignments using the NCBI database, https://blast.ncbi.nlm.nih.gov/Blast.cgi was performed to select sequence regions that are unique to western flower thrips. Selected sequences were then aligned to other organisms, including other insects, plants, and humans to check for specificity to western flower thrips. The three sequences targeting three different genes were then synthesized as a single concatemer, which was PCR amplified, followed by *in vitro* transcription of double stranded RNAs (dsRNAs) using opposing T7 promoters (Ambion). DsRNAs were purified and checked for quality and the same strategy was implemented for designing RNAi targets to the INSV genome.

Objective 2: Efficacy of RNAi technology using non-GMO strategies to manage thrips and INSV. Synthesized dsRNAs were delivered to romaine lettuce plants (v. Abilene) by root absorption at the first to second true leaf stage. Approximately 1.0 ug of dsRNA was delivered per plant, followed by transplanting back into soil. Leaf samples were collected 3, 7, and 14 days later and RNA was extracted to determine the absorption, systemic movement, and stability of the dsRNA in the lettuce plant. Absorption, movement, and stability of dsRNA in the plant were assessed by performing RT-PCR using primers specific to the dsRNA. To test the efficacy of dsRNAs targeting INSV, dsRNA was delivered to lettuce plants in a similar fashion, followed by mechanical inoculation of INSV after 6 days. Plants were monitored for 9 days following inoculation and scored for visual symptoms of INSV infection. These studies are ongoing, as well as research to test the efficacy of dsRNAs targeting western flower thrips.

Results:

Three genes were selected for RNAi targeting in the western flower thrips, referred to as *GeneHH1-3,* and these were synthesized as a single concatemer (referred to as WFT-1) with a total length of 491 nucleotides (**Table 1**). A similar strategy was implemented for designing

RNAi targets to the INSV genome. A single gene was selected to be targeted twice using two different lengths of dsRNAs. The first, INSV-1 had a length of 196 nucleotides, while the second, INSV-2, had a length of 524 nucleotides (**Table 1**). The reason for selecting two different lengths of the same target gene was to assess whether the length of the dsRNA would affect the absorption efficiency or stability of the dsRNA in lettuce.

All three dsRNAs, WFT-1, INSV-1, and INSV-2 were successfully absorbed through the roots of lettuce seedlings, with INSV-1 confirmed via RT-PCR to be present in the leaf tissue for at least 14 days after absorption. WFT-1 and INSV-2 are currently being assessed for their stability in lettuce plants, while INSV-1 is being tested to assess the longevity of the dsRNA in the plant (**Figure 1**).

Lettuce plants were then challenged with INSV via mechanical inoculation in the laboratory to assess the effects of absorbed dsRNA targeting the virus. Plants that had absorbed either INSV-1 or INSV-2 dsRNA appeared to either show a reduction or at least a delay in the onset of INSV symptoms 9 days after virus inoculation (preliminary data; **Figure 2**). These studies are ongoing to assess potential effects of dsRNAs on INSV infection beyond the 9-day time point. Current studies are also ongoing to assess the efficacy of dsRNAs targeting western flower thrips. Several non-choice RNAi feeding bioassays have been developed throughout this project to carry out these studies and we hope to report on this data later (**Figure 3**).

Name	Target Organism	Target gene(s)	Length (nt)	Designed	dsRNA synthesis	Root drench	Detect in leaves: 3 days	Detect in leaves: 7 days	Detect in leaves: 14 days	Detect in leaves: 21 days
WFT-1	Western flower thrips	*GeneHH1* *GeneHH2* *GeneHH3*	491	✓	✓	✓	✓	TBD	TBD	TBD
INSV-1	INSV	*GeneHH4*	196	✓	✓	✓	✓	✓	✓	TBD
INSV-2	INSV	*GeneHH5*	524	✓	✓	✓	✓	TBD	TBD	TBD

Table 1: Double stranded RNAs generated for testing potential for root absorption, stability, and efficacy against western flower thrips and INSV.

Figure 1: Root absorption assays in romaine lettuce. Double-stranded RNA absorbed into the roots of lettuce seedlings at the first true leaf stage and allowed to grow for 21 days to assess absorption efficiency and stability of the dsRNA.

dsRNA name	Number of plants showing symptoms of INSV at 9 dpi
Untreated	4/5
INSV-1	2/5
INSV-2	2/5

Figure 2: Mechanical inoculations with INSV in romaine plants possessing dsRNA targeting the virus. Romaine plants at the two true leaf stage, 6 days after root absorption of dsRNA INSV-1, INSV-2, or untreated control. Plants were mechanically inoculated with INSV and reevaluated for symptom development 9 days post inoculation (dpi). The number of plants exhibiting INSV symptoms (out of 5 plants per treatment) at this time point are presented here.

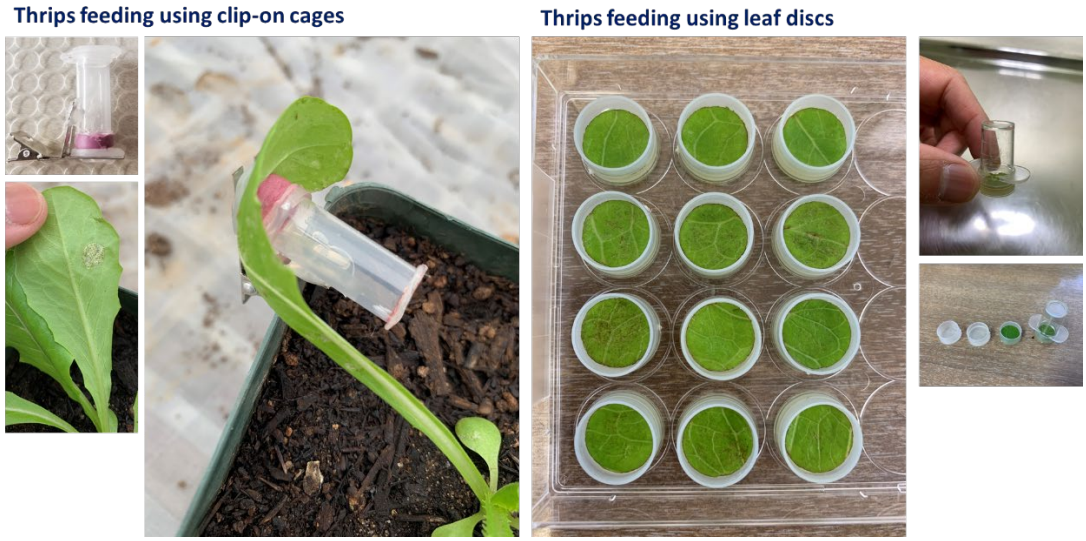

Figure 3: Thrips feeding bioassays to test efficacy of dsRNAs in lettuce plants. Several non-choice bioassays have been developed to assess the effects of RNAi on thrips survival when fed on plants that have absorbed the dsRNA that has been generated in this study. Clip-on cages allow for containment and recovery of thrips after feeding on a restricted location, whereas leaf-disc assays require the detachment of leaf tissue following dsRNA uptake. Leaf discs are placed in agar chambers to keep the tissue hydrated, followed by the introduction of a fixed number of thrips. Thrips mortality can be scored through the clear walls of the chamber.

Discussion:

RNA interference technology (RNAi) has numerous applications for crop protection and improvement. RNAi is a natural process in plants, insects, and humans that results in gene silencing. In this process, the formation of double stranded RNAs (dsRNAs) leads to the degradation of complementary mRNA, resulting in reduced expression for that gene. Identifying regions within a gene that are unique to a particular organism can be used to design specific dsRNAs. Using this knowledge, we can design dsRNAs to target genes that are critical to thrips survival and INSV replication, similar to what has been demonstrated as a tool for managing other insect pests and to provide host plant resistance to several plant pathogens, including viruses and fungi. This proposal explored the development of RNAi technology for managing thrips and INSV affecting lettuce.

Here, we demonstrated that dsRNAs can be successfully absorbed into the root systems of lettuce plants, and furthermore, moves systemically throughout the plant and can be recovered from leaf tissue for at least 14 days after application. We also provide preliminary evidence that dsRNA targeting INSV may have an effect on the onset of symptom development of INSV infection. However, further studies are required to fully understand if the dsRNAs are having an effect on virus replication, pathogenicity, and/or movement within the plant. If there is a level of protection that is conferred by the introduction of dsRNA, it would be important to understand how long protection against INSV lasts. It is expected that this may be limited by the stability

and longevity of the introduced dsRNAs. Future studies will explore these questions, as well as pursue efficacy studies to determine whether dsRNAs targeting western flower thrips is a viable management tool for protection of lettuce from thrips and thrips-transmitted viruses such as INSV.

The long-term goal of this project is to establish an RNAi-based tool that can effectively manage thrips and INSV using non-GMO methods and fits into the model of lettuce production practices in the Salinas Valley. While this proposal specifically focuses on the goals that are equivalent to early Phase 1 development of a new product, there are subsequent objectives that will need to be addressed. If efficacy studies continue to show promise, one future objective will be to optimize a delivery strategy for the dsRNAs at the field level – a topic that is highly popular and constantly changing as new technologies are emerging to enhance the stability and delivery of dsRNAs. As these technologies continue to advance and the cost to produce dsRNAs at field-scale levels continues to become cheaper, the implementation of RNAi technologies for integrated pest management will arrive, as long as a product can pass through the rigor of regulatory protocols.