2021-2022 CALIFORNIA LEAFY GREENS RESEARCH PROGRAM
RESEARCH PROPOSAL

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

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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, depending on the buyer and the market. 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 it's use as a biopesticide can be applied using methods that avoids genetic engineering of plants. Furthermore, RNAi-based biopesticides can be tailored to be species-specific and are completely degradable in the environment, providing unique advantages over many conventional insecticides. With previous support from the California Leafy Greens Research Program, we have successfully designed and synthesized double-stranded RNAs (dsRNAs; the active triggers of RNAi) to target western flower thrips and INSV. We are currently in the process of conducting small-scale efficacy experiments using these dsRNAs. This proposal has one objective that will focus on continuing these efficacy experiments to better understand the potential, as well as identify limitations for using RNAi technology as a crop protection strategy for managing thrips and INSV in lettuce. If successful, the data will set a foundation for continuing efforts to establish RNAi technology as a method to manage thrips and INSV in commercial lettuce production, and can serve as a model

for developing these technologies for managing other insect pests and pathogens that are relevant to leafy greens production in CA.

Objective:

1. Efficacy of RNAi technology using non-GMO strategies to manage thrips and INSV. *Deliverables*: Work towards a proof-of-concept for using RNAi technology as a strategy for managing thrips and INSV in lettuce.

Procedures:

Objective 1: 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 was assessed by performing RT-PCR using primers specific to the dsRNA. To test the efficacy of dsRNAs targeting INSV, dsRNA (INSV-1) was delivered to lettuce plants in a similar fashion, followed by mechanical inoculation of INSV after 6 days. Water treatment was used as the negative control for the inoculations and a total of 7-10 plants were used for each treatment. Plants were evaluated 9, 14, and 21 days following inoculation and scored for visual symptoms of INSV infection. A visual INSV severity score of 0-4 (0 = no symptoms, 4 = severe yellowing and necrosis) was used for the evaluations (**Figure 1**).

Results:

Three genes were selected for RNAi targeting in the western flower thrips and 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 INSV. 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. INSV-1 was confirmed via RT-PCR to be present in the leaf tissue for at least 21 days after absorption, while INSV-2 was present in leaf tissue for at least 14 days. Studies are ongoing to determine the longevity of WFT-1 in leaf tissues, and if INSV-1 and INSV-2 can persist in leaves for longer than 21 and 14 days, respectively (**Table 1, Figure 2**).

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 showed a reduction in INSV symptoms 9, 14, and 21 days after virus inoculation (**Figure 3**). These studies are ongoing to assess potential effects of dsRNAs on INSV infection beyond the 21 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 4**).

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, like 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 21 days after application. We also provide evidence that dsRNA targeting INSV may influence the onset of symptom development of INSV infection. However, further studies are required to fully understand if the dsRNAs are influencing 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 continue to become cheaper, the implementation of RNAi technologies for integrated pest management will arrive, if a product can pass through the rigor of regulatory protocols.

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**	**3 genes**	**491**	✓	✓	✓	✓	**TBD**	**TBD**	**TBD**
INSV-1	**INSV**	**1 gene**	**196**	✓	✓	✓	✓	✓	✓	✓
INSV-2	**INSV**	**1 gene**	**524**	✓	✓	✓	✓	✓	✓	**TBD**

Table 1: Absorption and stability of double stranded RNA into lettuce plants via root drenching. Three dsRNAs were synthesized and allowed to be absorbed via roots of lettuce plants at the first true leaf stage, followed by reverse-transcription PCR (RT-PCR) on extracted leaf RNA to evaluate the presence of dsRNA 3, 7, 14, and 21 days after absorption.

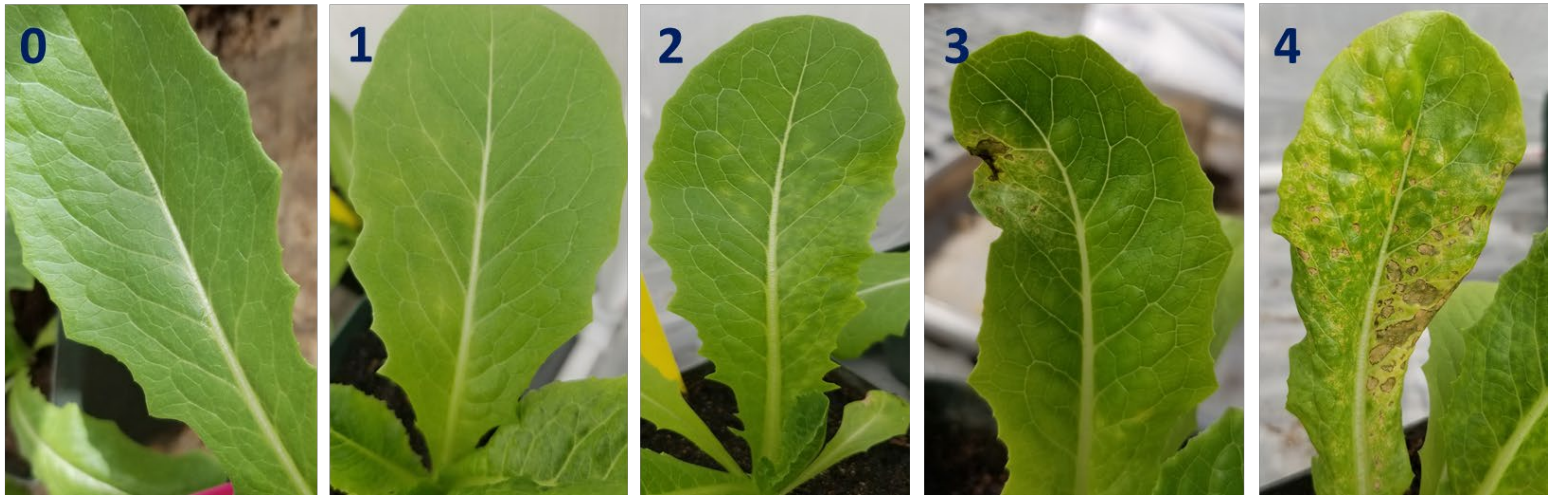

Figure 1: INSV symptom severity scoring system used in the current study. 0 = no symptoms; 1 = minor yellowing and onset of symptoms; 2 = extensive yellowing of leaves, but lack of necrosis; 3 = yellowing of leaves and onset of necrosis; 4 = severe yellowing and necrosis of leaves.

Figure 2: 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.

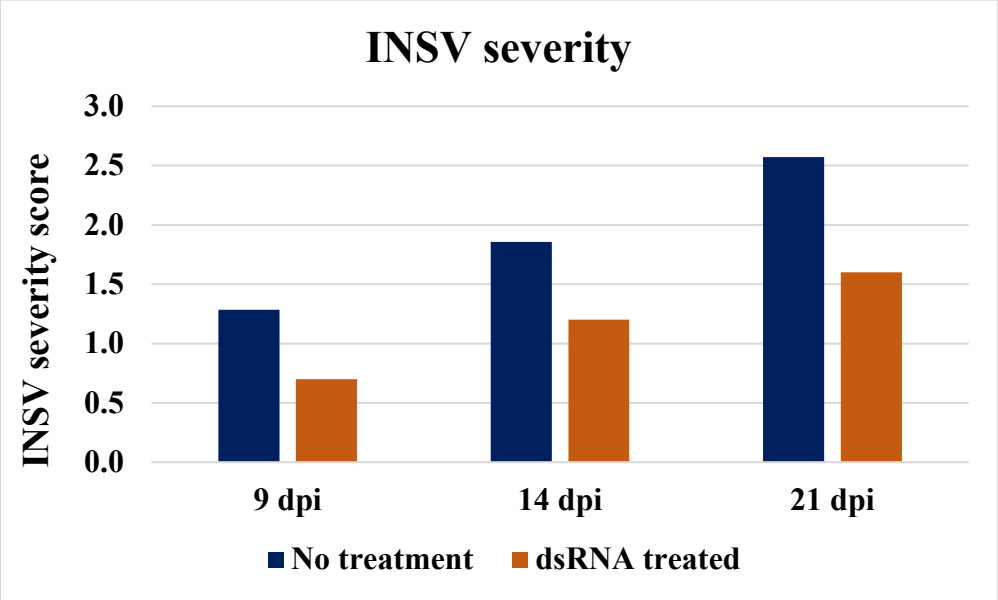

Figure 3: Mechanical inoculations with INSV in romaine plants that absorbed dsRNA targeting INSV. Romaine plants at the two true leaf stage, were allowed to absorb 1 ug of dsRNA (INSV-1), followed by mechanical inoculation with INSV 6 days later. Plants were evaluated for symptom development 9, 14, and 21 days post inoculation (dpi).

Figure 4: 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.