California Melon Research Board
2018 Annual Report

I. Project title:

Evaluation of RNAi strategies for reducing whitefly populations on melon.

II. Principal investigator:

William M. Wintermantel, Ph.D., Research Plant Pathologist
U.S. Dept. of Agriculture, Agricultural Research Service
1636 East Alisal Street, Salinas, CA 93905
Phone: (831) 755-2824; FAX: (831) 755-2814
Email: bill.wintermantel@ars.usda.gov

III. Co-PIs:

Navneet Kaur, Ph.D., Postdoctoral Research Molecular Biologist
U.S. Dept. of Agriculture, Agricultural Research Service
1636 East Alisal Street, Salinas, CA 93905
Phone: (831) 755-2826; FAX: (831) 755-2814
Email: navneet.kaur@ars.usda.gov

IV. Cooperating personnel

Wayne Hunter, Ph.D., Research Entomologist, USDA-ARS, Ft. Pierce, FL.

V. Location(s) where work was performed

USDA-ARS, U.S. Agricultural Research Station, Salinas, California,

VI. Objectives

1. Evaluate dsRNA constructs for their ability to induce whitefly mortality on treated melon.

2. Evaluate induced whitefly mortality on melon following treatment with cloned DNA constructs based on previously evaluated dsRNA.

3. Determine duration of RNAi-based control of whitefly in treated melon plants.

VII. Results and Analysis (This is a summary report as we did not request renewal because some issues need to be worked out before revisiting this approach with melon).

Objectives 1 &2:

Confirmation of "Best Constructs" for Advanced Evaluations on Melon and Other Crops
Prior to the beginning of this project we evaluated 35 double stranded RNA (dsRNA) constructs from our laboratory using an *in vitro* system in which sweet potato whiteflies (*Bemisia tabaci* MEAM1; aka. Biotype B) feed on a sugar solution containing each dsRNA construct individually. Our colleagues in Charleston, SC did the same with their 35 constructs for a total of 70 evaluated using the *in vitro* system. From those experiments, the ten most effective dsRNA constructs were selected for further evaluation and advanced testing based on the results of those preliminary tests, including six constructs from Salinas were selected, along with four from Charleston. From late spring through mid-summer, 2017, both the Salinas and Charleston Labs evaluated mortality of *B. tabaci* MEAM1 (biotype B common in California melon production areas) and collaborators in Dar es Salaam, Tanzania evaluated mortality of *B. tabaci* SSA1-SG3 (a whitefly that affects cassava and other plants in East Africa) in identical tests to validate initial results (ring test). Results of comparative studies by the Wintermantel Lab (Salinas) and the African Lab (Dar es Salaam) were highly consistent, with both labs confirming the best performance by the same three constructs (WK1, WK2 and WH9) with the two different *B. tabaci* variants (MEAM1 and SSA1-SG3), although results varied slightly in which was best depending on the whitefly type being targeted. Genes targeted by constructs are intentionally not mentioned here and are replaced by coded acronyms in this report. Results demonstrated that two constructs, both developed and initially tested by the Wintermantel Lab (the second in conjunction with a collaborator) performed the best against both types of whiteflies; whereas a third was also very effective but to a lesser degree against both whiteflies (**Fig. 1, Fig. 2**). Therefore, we had great confidence that these two constructs should be developed further for whitefly control through experiments on both melon (this project), and for cassava and tomato.

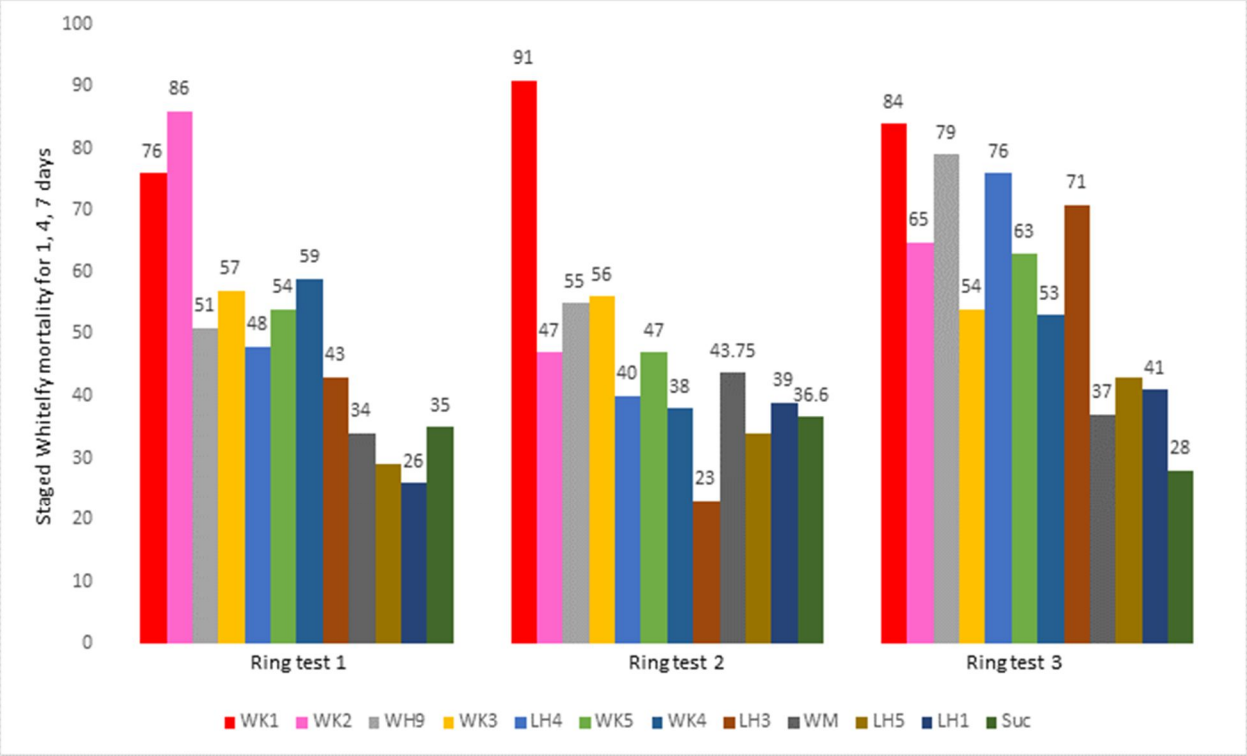

Figure 1: Sweet potato whitefly (*B. tabaci*, MEAM1) mortality induced by ten novel dsRNA constructs with feeding on 20% sucrose containing each dsRNA construct.

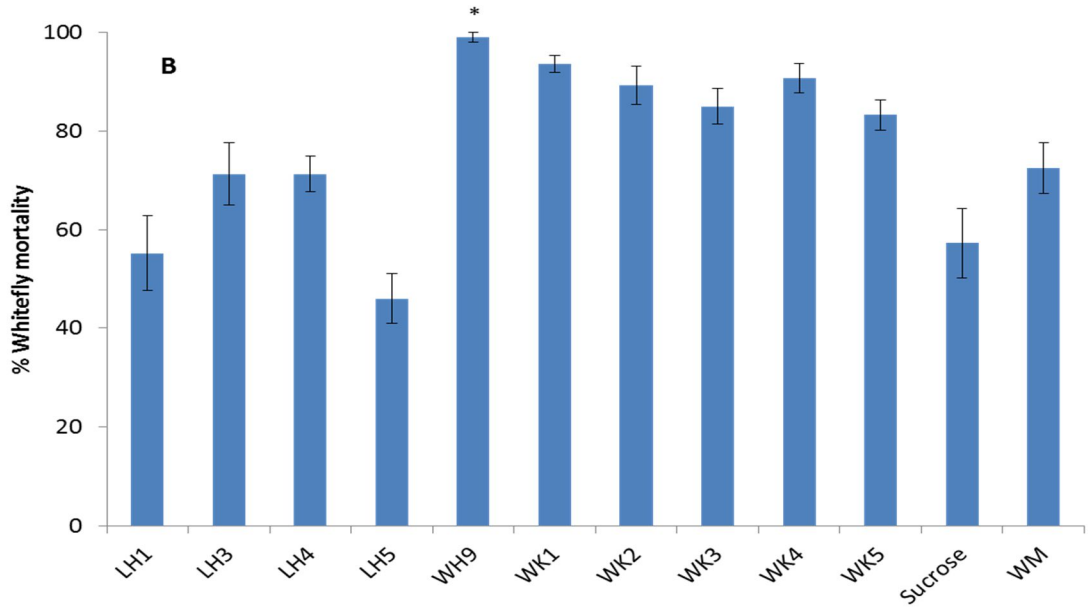

Figure 2: Average percentage of cassava *B. tabaci* (SSA1-SG3) mortality at 7 days after experiment initiation for ten dsRNAs and non-target controls (WM and sucrose only). DsRNAs with an asterisk (*) were significantly different from controls (WM and sucrose) (Tukey-Kramer test, P = 0.05).

Four new constructs were developed during summer 2018 focused on interference with virus transmission, and these were based on results of comparative gene expression studies using whiteflies feeding on melon plants infected with CYSDV and healthy melon, as well as two additional virus-host systems (Kaur & Wintermantel, unpublished). Initial tests simply evaluated the rate of mortality induced by these constructs to establish a baseline, with results ranging from 47 to 66% mortality, although with higher than desired rates for a control construct as well (33%). Subsequent tests will evaluate impact on reducing virus transmission rates, but due to concerns with efficiency of performance in melon, most of these early tests may be performed over the winter in tomato until limitations with melon can be overcome.

Leaf Uptake Experiments

The three most effective constructs were all from the Wintermantel Lab and were evaluated on melon using two methods during the 2017 project year: leaf uptake of dsRNA followed by exposure to whiteflies, and spray treatment of leaves with dsRNA followed by exposure to whiteflies. The first approach involved leaf uptake of dsRNA in an effort to determine appropriate concentrations for experiments. In this experiment, leaves of young, 2 true leaf stage melon plants were detached using a razor blade and petioles were placed in microcentrifuge tubes containing three different concentrations of dsRNA suspended in water. Leaves were allowed to absorb solution, after which petioles were placed in a sealed tube of water, allowed to set overnight, then 20 whiteflies were added to each leaf. This method was used for evaluation of each of the constructs. Numbers of dead whiteflies were recorded each day for 15 days. Although some whitefly mortality did occur, the method was not effective for inducing whitefly mortality on melon (results not shown) whereas similar experiments in tomato had been quite successful. We suspect melon may take longer periods of time to distribute the dsRNA throughout the leaf than tomato, perhaps due to structural differences in connections between the different components of the vascular system between these types of plants, meaning that translocation of the dsRNA or its induced signal that causes gene silencing is not as efficient in melon as it is in tomato.

Foliar Spray Experiments

A different approach to evaluate application of dsRNA for whitefly control in melon production involved direct treatment of leaves with dsRNA for induction of RNAi. This is an approach adapted for nursery or perhaps even field application to seedling melon plants. In these experiments conducted predominantly during year 1 of the project, each dsRNA construct to be tested was diluted in treatment buffer to a specific concentration and placed in a 10 ml spray bottle. Four leaf-stage melon seedlings were treated with the suspension, but prior to treatment all but the topmost leaf was removed, to limit surface area on which whiteflies can feed (easier to evaluate). The solution was then sprayed on the remaining leaf and allowed to dry overnight. The next morning, each seedling was covered with a 4" diameter plexiglass vial containing a mesh on top with a cotton-plugged port through which whiteflies can be delivered to the vial (**Figure 3**), and 30 whiteflies are added to each vial. Whiteflies were allowed to feed on plants for 14 days (**Figure 4**), after which live and dead whiteflies are counted to determine mortality rate for each construct. Experiments were performed with five of the most effective constructs, and each construct was replicated five times per experiment. Results are shown in **Table 1**.

Fig. 3. Vials containing treated melon plants

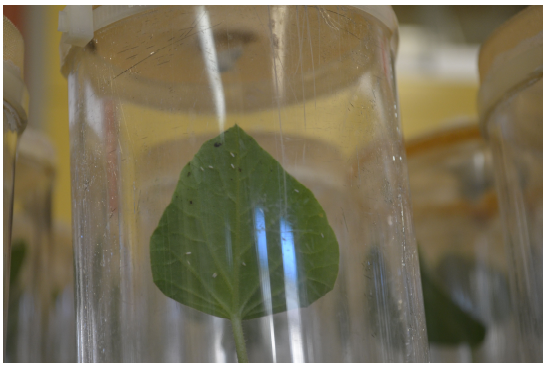

Fig. 4. Whiteflies feeding on melon leaf

Table 1. Melon leaf-spray assays

dsRNA construct	Rep1	Rep2	Rep3	Mortality (%)
WK1	23	28	20	78.9
WK3	24	24	23	78.9
WK2	24	23	23	77.8
WK4	28	29	23	88.9
WK5	22	30	20	80.0
WM	19	14	8	45.6
TNT2 only	24	16	26	73.3

Method: 1 leaf attached from 2-3 weeks old Top Mark melon was used for spray.
10ug of dsRNA per plant in 1 ml of 0.05% TNT2 spray solution
Dead whiteflies were counted after 14 days

Results of the spray experiments shown in Table 1, did not show significant differences in whitefly mortality between test constructs and controls. The TNT2 control, in which leaves were sprayed only with the solution used to suspend dsRNA, resulted in about the same rate of mortality as test constructs, although a lower rate of mortality was observed with the construct targeting a gene unique to watermelon (negative control gene used because this gene is not present in the whitefly genome). **Following further testing showing similar results it was determined there is further work to do to optimize performance of topical dsRNA treatments in melon. Therefore our focus shifted to examine ways to improve whitefly mortality using topical delivery systems.**

Objective 3: Determine duration of RNAi-based control of whitefly in treated melon plants.

Nuclease Treatment

One concern with oral ingestion of dsRNA in hemipteran insects such as whiteflies, is the potential for rapid degradation of dsRNA following ingestion, or simply breakdown before ingestion can occur. The Wintermantel Lab (USDA-ARS, Salinas, CA) and collaborator, W.

Hunter (USDA-ARS, Ft. Pierce, FL) have been evaluating approaches to improve delivery methods and optimize performance in plants treated with dsRNA for induction of whitefly mortality.

Natural nucleases rapidly degrade exogenously applied dsRNA (Dubleman, et al 2014, PLoS One 9:1–7), such as topically applied sprays. DsRNA constructs targeting nucleases encoded by the whitefly genome were developed to enhance the performance of mortality-inducing dsRNAs. This is based on a recent publication by Luo et al. (Insect Biochem. Mol. Biol. 88 [2017]: 21-29) in which the authors demonstrated increased performance of mortality-inducing constructs when dsRNA targeting nucleases were combined with those targeting other specific functions. Similar results have been found with other studies. Two of the nucleases we are targeting were based on those used by Luo et al. (2017), whereas another was developed by the Wintermantel Lab at ARS-Salinas based on the results of the RNA-seq analysis conducted in our laboratory (Kaur et al., 2017; BMC Genomics (2017) 18: 370 and Kaur et al., BMC Genomics, submitted). The nuclease genes targeted were found to be expressed in whiteflies with FPKM ranges from 0.43 to 4.83 with a mean near 3.0 based on RNA-Seq data generated from whiteflies in experiments comparing gene expression in whiteflies fed on ToCV-infected or healthy tomato (Kaur et al., 2017) as well as those fed on CYSDV-infected or healthy melon (Kaur et al., BMC Genomics, submitted). DsRNA constructs against the nuclease genes were synthesized commercially and all experiments were conducted at USDA-ARS in Salinas. All three nuclease-targeting dsRNAs are being used together along with individual dsRNAs targeting a specific whitefly gene or genes, with the goal of suppressing nuclease genes that could interfere with RNAi performance. Optimization of timing of nuclease targeting dsRNA is continuing, and preliminary studies suggest it is important that dsRNA targeting nucleases must be active in plants prior to delivery of whitefly mortality-inducing dsRNA constructs for performance enhancement to occur (data not shown). These experiments should result in improved performance of mortality-inducing dsRNAs compared to untreated controls and should enhance effectiveness and longevity of dsRNA inducing constructs.

DsRNA Movement and Stability in Plants: Using fluorescently labelled dsRNA, research in a collaborator's lab (W. Hunter, USDA-ARS, Ft. Pierce, FL) found that the dsRNA can be detected in new growth on treated plants as early as three days following soil treatment. This work was done on citrus as part of a parallel project but has direct implications for research on melon. Results demonstrated that dsRNA remains detectable in plant tissue for at least 45 days following uptake through the roots after a 10-100 µg/ml dsRNA solution was poured over the roots and taken up by plants (**Fig. 5**). There is no reason to believe results would differ substantially in other hosts, although we are currently evaluating durability of control in tomato (this has not yet been tested in melon due to difficulty in obtaining uniform movement of the dsRNA molecules in melon). Information is presented here as it demonstrates that if we can establish effective movement of the dsRNA through melon, there is great potential for long-term control of whitefly using this technique.

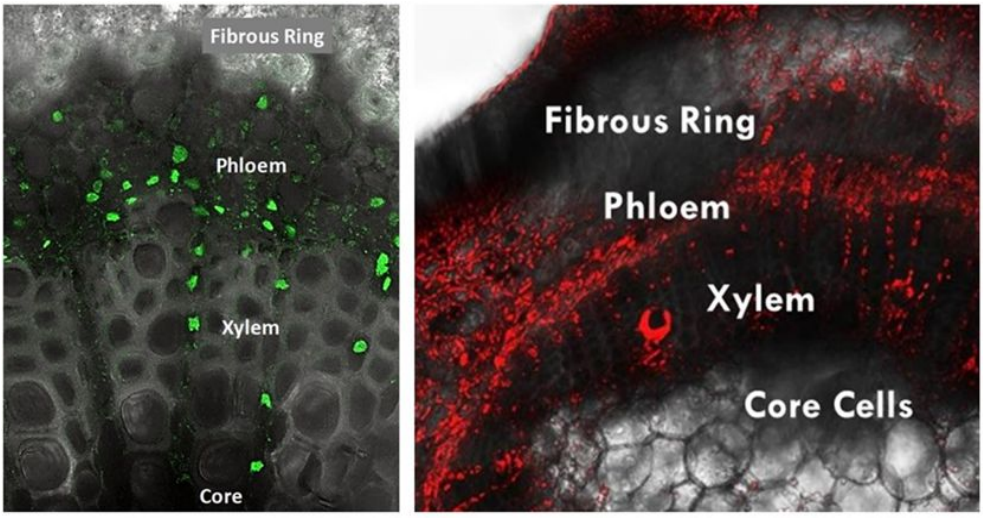

FIG. 5. Fluorescent labeled dsRNA shows systemic movement in citrus trees and leaves, sweet orange, *Madam vinous* and *Citrus sinensis*. *Soil-applied and root absorbed labeled dsRNA was detected in the xylem and phloem of new growth leaves on dsRNA-treated citrus seedlings (two year old potted sweet orange) at 3 days post soil applied treatments. The results supported previous results on larger citrus trees and grapevines in the field which detected dsRNA for over 45 days post soil applications to the root zones (Hunter et al, 2012;* Southwestern Entomologist 37(1):85-87*).* **Leaf tests**: the control leaf (*left*) was a check for autofluorescence, leaf counterstained with *NucGreen*® Dead 488 reagent, which is suitable for staining nuclei in fixed-cell preparations and tissue sections (Thermo Fisher Scientific). Treated citrus leaf (*right*). Mid rib cross section, at 72 hrs post absorption with Fluoro-tagged dsRNA (ULYSIS® Nucleic Acid Labeling *Kit [Thermo* Fisher Scientific*]:* Molecular Probes cat#U21650. (Hunter, W.B., Lopez, S.P., Holland, C.S, Boyle, MJ. 2018, in prep).

Preliminary tests on use of Non-canonical dsRNA to Increase Persistence and RNAi Activity associated with topical treatment of plants with RNAi

In August 2018, preliminary experiments were completed by the collaborating Hunter Lab (ARS, Ft. Pierce, FL) with tomato cuttings that were treated with dsRNA of construct WH9, which has performed exceptionally well in all experiments for control of both MEAM1 and SSA1 whiteflies on tomato and cassava in both the Wintermantel Lab (USDA-ARS Salinas), and an additional collaborator's lab in Tanzania. The goal of this study was to evaluate canonical (native form) and non-canonical (altered sequence) dsRNA molecules targeting a selective area of the gene targeted by the WH9 construct. The dsRNA was delivered to tomato cuttings through traditional uptake methods as used in previous tests. In short, a 2 mL microcentrifuge tube was used to dilute the appropriate volume of dsRNA in 1 mL of sterile deionized water. The cutting is placed in the dsRNA solution and allowed complete uptake of the solution, followed by a holding period of 24-48 h to allow the dsRNA to distribute throughout the cutting before addition of whiteflies. The cutting can then be transferred to MS media for rooting, followed by transfer to soil to allow maintenance of plants for long periods of time. A negative control described above was included in experiments in which the same amounts of a dsRNA targeting Chinese sacbrood virus (CSBV; a virus not present in whiteflies or other hemipterans) was allowed to uptake into tomato.

Similar studies are in progress on tomato at USDA-ARS in Salinas and cassava at IITA in Dar es Salaam, Tanzania. Both labs received the same dsRNA (WH9) constructs from Dr Hunter's lab in September 2018 in both canonical (wild type) and noncanonical (altered to reduce nuclease degradation) forms, along with selected additional controls. In these experiments, the dsRNA is absorbed into young plant cuttings. The cuttings are rooted in plant regeneration media to allow roots to establish, and are then transferred into soil media. Leaves are harvested at designated times for each host (initially tomato and cassava) to test for the efficacy of dsRNA against the whiteflies. Studies on tomato plants are also evaluating performance of a root drench with dsRNA solution compared directly with cut tomato stems that have absorbed dsRNA and subsequently rooted. The initial experiment had to be repeated due to miscommunication on techniques, but results of new experiments should be completed by February. These experiments will allow us to evaluate efficacy of control for our best constructs, delivered transiently over a longer period of time running for several weeks to months. Furthermore, it will evaluate whether plants can retain the ability to kill whiteflies for longer periods of time when delivered as a transient application, while comparing efficacy of topical treatment of roots for absorption of dsRNA with a method by which the material is directly taken up into the stem. **Although the experiments were not conducted on melon, the results demonstrate that if we can overcome the block on movement of the RNAi signal in melon, use of topical dsRNA application to plants for control of whitefly, viruses, or other pathogens will have tremendous potential.**

Duration of whitefly control in melon

Until methods are fully established and demonstrated to be reliable for induction of RNAi in melon, it is premature to conduct studies on duration of RNAi effectiveness in melon following topical application.

During the two-year duration of this project we were unable to achieve levels of whitefly control in melon that compared to the high rates observed in tomato and cassava. This does not mean that the method will not work in melon or other cucurbits, but further work will be needed to determine how to overcome the performance issues in melon. Although we are not requesting renewal of the project, work will continue toward optimizing methods with USDA funds, and to determine ways to use this technology in melon and other cucurbits, as the approach shows tremendous promise for tomato and cassava. Given appropriate modifications we hope to achieve similar success in cucurbit crop plants as well.