Interim Progress Report for CDFA Agreement Number 12-0216-SA

Title of project: RNA-interference and control of the glassy-winged sharpshooter (*Homalodisca vitripennis*) and other leafhopper vectors of *Xylella fastidiosa*

Principal Investigator (PI): Dr. Bryce W. Falk, Distinguished Professor, Department of Plant Pathology, University of California, One Shields Avenue, Davis, CA. 95616, 530-752-0302, bwfalk@ucdavis.edu

Cooperators: Tera Pitman, Staff Research Associate, Department of Plant Pathology, Univ. of California, One Shields Avenue, Davis, CA. 95616, 530-752-5218, tlpitman@ucdavis.edu

Dr. Jeremy Warren, Postdoctoral Scientist, Department of Plant Pathology, Univ. of California, One Shields Avenue, Davis, CA. 95616, 530-752-5218, jgwarren@ucdavis.edu

Dr. S. G. Kamita, Project Scientist, Department of Entomology, Univ. of California, One Shields Avenue, Davis, CA 95616, sgkamita@ucdavis.edu

Dr. Kris Godfrey, Project Scientist and Director, UC Davis Biosafety 3P Contained Research Facility. U C Davis, Davis, CA 95616, 530-754-2104, kegodfrey@ucdavis.edu

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INTRODUCTION: Our primary objectives are to evaluate the potential of RNA interference (RNAi) strategies for use against *Homalodisca vitripennis,* the Glassy-winged sharpshooter (GWSS), and other sharpshooter vectors of *Xylella fastidiosa*, the causal agent of Pierce's Disease of grapevines. We have previously demonstrated RNAi effects in GWSS and evaluated different strategies to induce RNAi effects in GWSS. Our goals are to evaluate RNAi approaches which could be part of long term strategies to help control GWSS and other sharpshooter vectors of *X. fastidiosa*.

We have used potato plants as surrogates for transgenic RNAi-based approaches for GWSS. We have generated stable transgenic potato plants using the constitutive, non-tissue-specific 35S promoter, and a *Eucalyptus gunii* minimal xylem-specific promoter (EgCAD) to control the spatial expression of candidate interfering RNAs. GWSS feed on potatoes for our experiments, and we have demonstrated target mRNA knockdown but no obvious phenotypes in GWSS. We are now evaluating using bacteria and grapevine-infecting viruses as surrogates to express interfering RNAs in grapevines.

OBJECTIVES:

I. **Generate transgenic plants for novel effective targets of GWSS and other sharpshooters.**

II. **Generate and use microRNAs from different developmental stages of GWSS insects.**

III. **Assess the potential of using plant viruses for delivery of small RNA effectors.**

Description of Activities Conducted

Objective I. We compared transgenic potato plants engineered to express interfering RNAs to target GWSS. We used two different promoters to drive interfering RNAs in plants for these experiments, the 35S constitutive promoter and the EgCAD promoter from *Eucalyptus gunii* which has shown the ability to target developing xylem tissues. We performed molecular biological analysis on plants, and did feeding assays with GWSS on these plants. The assays showed that all transgenic plants produced specific small interfering RNAs; we were also able to induce detectable RNAi effects in GWSS as determined by RT-qPCR analysis of target mRNAs, but we failed to generate a detectable phenotype. We now believe that this may be due at least in part because of how we performed our assays.

Our first experiments used potato cuttings with caged 4th and 5th instar GWSS nymphs. The cuttings were placed in dilute nutrient solution and GWSS remained on cuttings for ~7 days. Our ongoing efforts with phloem-feeding hemipterans have shown similar results, but we have been able to see negative phenotypes only when we allow target insects to develop on test plants, they must go through nymphal instar stages and molt. For GWSS this is problematic as these insects like to move among plants and feed on different plant species, in fact in order to have sufficient GWSS reproduction we rear them in cages containing basil, cotton and cowpea plants. Thus, we have stopped doing these experiments with transgenic plants but are now using engineered bacteria in attempts to produce interfering RNAs in the xylem and deliver them to GWSS. For our initial attempts with this strategy we engineered compromised *E. coli* to express the green fluorescent protein (GFP). When we inserted potato cuttings into solutions of these bacterial we observed GFP fluorescence in the xylem tissues and in GWSS that fed on these plants. We are now engineering *E. coli* to express interfering RNAs and will assess their efficacy by stem infusion feeding experiments.

Objective II. We identified several GWSS-novel miRNAs by Illumina-based sequencing and bioinformatics analysis (Nandety et al., 2015). We have so far only identified miRNAs in adult GWSS, but our goals are to identify potential miRNAs that may be GWSS instar-stage specific and evaluate their potential for use in RNAi towards GWSS.

We are attempting to express amiRNAs in plants have used agroinfiltration of *N. benthamiana* plants and specific plasmid constructs for this. We then used small RNA hybridization and Illumina sequencing to assess production of amiRNAs. These experiments showed that we can produce specific amiRNAs in plants by two methods: one by using a binary plasmid vector to produce the specific amiRNA; and second by using a modified begomovirus A component to replicate and express higher levels of amiRNAs in plants. The latter suggests that it is worth investigating using Grapevine red blotch associated virus (GRBaV)(Krenz et al., 2014) as a means for generating specific amiRNAs in grapevines. We have expanded our efforts for experimental approach and are making progress in this regard (see Objective III).

Objective III. Our efforts here are based on our previous successes using plant-infecting viruses to express interfering RNAs in plants, which produced negative phenotypes in specific phloem-feeding target insects (Wuriyanghan and Falk, 2013; Khan et al., 2013). We are engineering *Grapevine leafroll-associated virus-7* (GLRaV-7) as our primary virus for these studies. This is based on successes by others using *Citrus tristeza virus* in citrus (Dawson and Folimonova, 2013; Folimonov et al., 2007), and GLRaV-2 in grapevines (Dolja and Koonin, 2013). We have amplified the GLRaV-7 genomic RNA as cDNA and cloned specific fragments. We are attempting to use the same plasmid vector and approach that has been used successfully for CTV (Dawson and Folimonova, 2013).

As noted in Objective II we also are cloning copies of the GRBaV DNA and will attempt to use it to express sequences in grapevine plants. The advantage of GRBaV is that it replicates within the plant cell nucleus whereas GLRaV-7 replicates in the cytoplasm. Thus GRBaV offers the potential to use the plant cell nuclear processing machinery to express other types of interfering RNAs such as amiRNAs. We have successfully amplified and cloned genomic GRBaV DNA and are currently constructing an infectious clone to use in our experiments.

The final strategy is utilizing bacteria to produce GWSS gene specific interfering RNAs directly in the plant xylem. Previous research has shown that dsRNAs targeting specific insect genes can be reliably produced in HT115 *E. coli.* Furthermore, when insects fed on intact HT115 *E. coli* expressing these dsRNAs the target genes were downregulated (li et al 2011, Tian et al 2009, Zhu et al 2010). To determine if this strategy could work with GWSS we have cloned a portion of the GWSS actin gene to be expressed in HT 115 *E. coli.* These actin dsRNA expressing bacteria will be feed to GWSS using potato cuttings submerged in a solution of the bacteria. The GWSS will then be tested to see if gene expression levels are affected. If we get positive results using the HT115 *E. coli* we will then transform these constructs into the biocontrol strain of *Agrobacterium vitis* F2/5 to see if we can achieve similar results with a bacterium that is able to colonize the grapevine xylem and thus be directly acquired by GWSS.

Publications and presentations:

1. Nandety, R. S., Sharif, A., Kamita, S. G., Ramasamy, A., Falk, B. W. 2015. Identification of novel and conserved microRNAs in *Homalodisca vitripennis*, the Glassy-Winged sharpshooter by expression profiling. PLoS One DOI:10.1371/journal.pone.0139771.

Research relevance: RNAi is a natural biological activity for controlling gene expression and for anti-viral defense in a majority of eukaryotic organisms, including insects. The application of RNAi directed toward different types of insect plant pests is becoming more feasible and promising. In our efforts, we were able to induce RNAi effects in *H. vitripennis* and evaluated initial transgenic plants as a means to initiate RNAi-specific negative phenotypes on the glassy winged sharpshooter and other leafhopper vectors of *Xylella fastidiosa*. We have been unsuccessful in this longterm practical goal but we are attempting different approaches including using microbes (bacteria and/or viruses) as tools to deliver interfering RNAs in plants.

Lay person's summary of results: This work presents fundamental efforts towards understanding the feasibility of applying RNA interference (RNAi), to help combat Pierce's Disease of grapevines by targeting the insect vector, *H. vitripennis* or GWSS. Pierce's Disease is a significant threat to grape production in California and other parts of the U.S., and the causal agent, *Xylella fastidiosa*, a xylem-limited bacterium, also causes several other extremely important plant diseases worldwide. We focused our recent efforts on evaluating transgenic plants to evaluate their potential for inducing RNAi effects in *H. vitripennis*, and for identifying optimal RNAi inducer delivery systems. We have so far had minimal success. We also generated large scale genomic data along with small RNA datasets, which will help us for future genetic/genomic efforts against *H. vitripennis*, and are currently evaluating using microbial agents (plant-infecting viruses and/or xylem inhabiting bacteria) to express interfering RNAs in plants.

STATUS OF FUNDS: We were awarded two years of funding to support one postdoctoral scientist, an undergraduate intern, plus funds for standard benefits. We also requested funds for routine supplies, research facility (Biosafety 3P Contained Research Facility) recharge costs and limited travel. We are on track to spend these funds as proposed.

FUNDING AGENCIES: Funding for this project was provided by the USDA-funded CDFA/University of California Pierce's Disease Research Grants Program.

Summary and status of intellectual property associated with the project: No intellectual property has developed so far.

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