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*

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INTRODUCTION: Our primary objectives are to evaluate and demonstrate RNA interference (RNAi) activity against *Homalodisca vitripennis* or the Glassy-winged sharpshooter (GWSS). We have previously demonstrated induction of RNAi effects in GWSS and evaluated different strategies to induce RNAi effects in GWSS. We envision that RNAi approaches can be part of long term strategies to help control GWSS and other sharpshooter vectors of *Xylella fastidiosa*, the causal agent of Pierce's Disease of grapevines, but only if we understand how they work and optimize the delivery of RNAi inducers. We are focused now on answering the latter questions, we have built upon our previous progress and initiated some new directions in our GWSS RNAi efforts. We also continue to work closely with Dr. S. George Kamita.

We continue to use 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, but we are still optimizing how to evaluate RNAi effects from transgenic potatoes. It is also now our intent to directly use grapevines, but instead of using transgenic grapevine plants, we are now attempting to develop grapevine-infecting viruses 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 for these experiments, the 35S constitutive promoter and the EgCAD promoter from *Eucalyptus gunii* which has shown the ability to target developing xylem tissues. A table showing our plants, and their analysis by small RNA hybridization analysis was presented in our Dec 2014 report published in the Pierce's Disease control Program Symposium Proceedings (pp. 16 – 22), and are not included here. These assays showed that all transgenic plants produced specific small interfering RNAs; we were also able to induce 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, and we are repeating feeding experiments in a different format as described below.

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. For our most recent experiment we used small rooted cuttings in soil, as opposed to the dilute nutrient solution, with caged 4th and 5th instar GWSS nymphs. The GWSS nymphs were allowed to feed for 5 days and we are currently analyzing the data from this experiment. It is possible the rooted plants may produce better RNAi effects in GWSS than the nutrient solution cutting experiments. 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 a little problematic as they like to move among plants and feed on different species, in fact in order to have sufficient reproduction we rear them in cages containing basil, cotton and cowpea plants. Our current, ongoing studies are now using small rooted cuttings in soil. We are starting experiments using 2nd and 3rd instar nymphs and maintaining the test times as long as possible, or until nymphs molt into adults. After optimizing the RNAi assays we will move on to assessing additional targets and even using artificial microRNAs (objective II).

Objective II. We have begun evaluating three approaches for expressing artificial microRNAs (amiRNAs) in plants, which will be described later. Our intent here is two-fold: one is to use specific amiRNAs to target GWSS mRNAs and reduce the possibilities for potential RNAi off-target effects which are more possible with longer, dsRNA RNAi inducers (Nunes, 2013); and second, we have identified several GWSS-novel miRNAs by Illumina-based sequencing and bioinformatics analysis (see Fig. 1). 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 have used agroinfiltration of *N. benthamiana* plants, followed by 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 are considering this at least as an experimental approach (see Objective III).

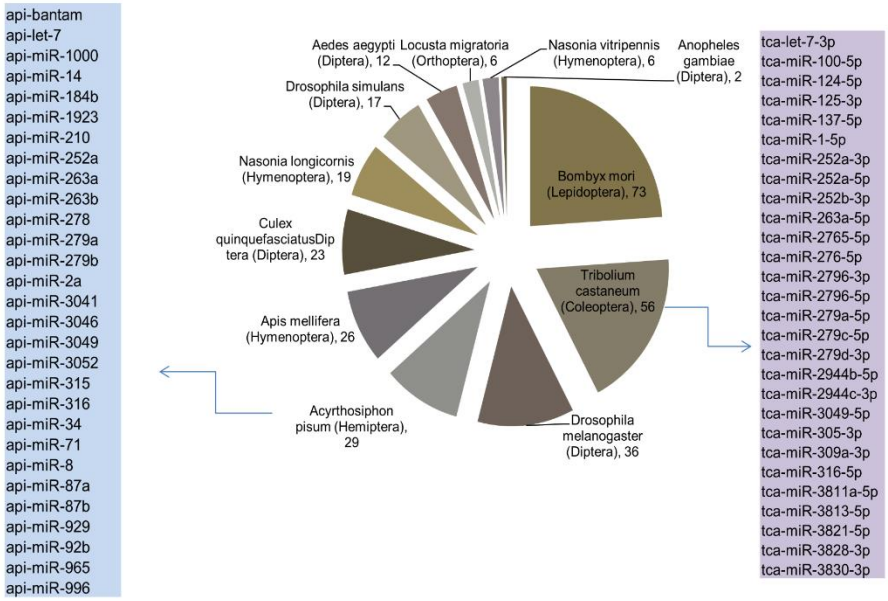

Figure 1. The microRNA profile analysis of GWSS adult insects revealed the presence of microRNAs that are conserved between different insects. GWSS adults also share some microRNA

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. 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; Hajeri et al., 2014), and GLRaV-2 in grapevines (Dolja and Koonin, 2013). We have GLRaV-7 in culture and have designed primers that allow for us to successfully amplify a region of the GLRaV-7 genomic RNA. Efforts are underway to clone the entire genome as a cDNA and then engineer it for use in grapevines.

Based on our success with expressing amiRNAs in plants (see Objective II above) and comments from the grant review panel last year, we are now considering using GRBaV also as at least an experimental tool to express amiRNAs in grapevines. We have established cultures of GRBaV and have cloned the genomic ssDNA. We are attempting to generate an infectious cDNA version for use in grapevines.

Publications and presentations:

1. Nandety, R. S., Kuo, Y.-W., Nouri, S., and Falk, B. W. 2014. Emerging strategies for RNA interference (RNAi) applications in insects. Bioengineered, DOI: 10.4161/21655979.2014.979701

2. Pitman, T. L., Nandety, R. S., Warren, J. G., and Falk, B. W. RNAi effects in *Homalodisca vitripennis*, a xylem feeding leafhopper vector of *Xylella fastidiosa*, induced by transgenic plants. In Preparation.

3. Nandety, R. S., Sharif, A., Kamita, S. G., Ramasamy, A., and Falk, B. W. Identification of novel and conserved microRNAs in Homalodisca vitripennis, the glassy-winged sharpshooter by expression profiling. Submitted to PLoS One.

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 to help control the glassy winged sharpshooter and other leafhopper vectors of *Xylella fastidiosa*. RNAi is already used in commercial agriculture for plant virus control, and the many new publications demonstrating experimental successes with various plant-feeding insects suggest that RNAi could have a role in helping to manage Pierce's Disease of grapevines.

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. 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. Our effort here does not directly target *Xylella fastidiosa*, but instead targets one of its most significant insect vectors, the Glassy-winged sharpshooter, *Homalodisca vitripennis*, and other sharpshooter vectors of *X. fastidiosa*.

We focused our recent efforts on evaluating transgenic potato plants to evaluate their potential for inducing RNAi effects in *H. vitripennis*, and for identifying optimal RNAi inducer delivery systems. Potatoes are easier and faster to transform and regenerate than grapes, and the glassy-winged sharpshooter feeds readily on these plants. We also generated large scale genomic data along with small RNA datasets, which will help us for future genetic/genomic efforts against *H. vitripennis*.

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. Because we had approximately $58,000 in carryover funds and were granted a no-cost extension on those funds, here we requested $26,085 for year 1 and $86,014 for year 2 ($112,099 total new funds). 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.

Literature Cited

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