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, Professor, Department of Plant Pathology, University of California, One Shields Avenue, Davis, CA. 95616, 530-752-0302, bwfalk@ucdavis.edu

Cooperators: Dr. Raja Sekhar Nandety, Postdoctoral Researcher, Department of Plant Pathology, Univ. of California, One Shields Avenue, Davis, CA. 95616, 530-752-5218, nandety@ucdavis.edu

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. 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 and demonstrate RNA interference (RNAi) activity against *Homalodisca vitripennis* or the Glassy-winged sharpshooter (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. We have made considerable progress during the past year and are in excellent position to complete remaining of our objectives during the remainder of the grant period. We have published three new refereed journal articles (Nandety et al., 2013a; Nandety et al., 2013b; and Kamita et al., 2013) and are working on two manuscripts, one on RNAi on glassy-winged sharpshooter (Pitman et al., 2014) and a manuscript on GWSS microRNA identification (Nandety et al., 2014). We have also presented an oral talk at International conference Plant and Animal Genome in San Diego, (Nandety et al., 2014).

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. We showed expression of the GUS gene *in vivo* in the transgenic potato plants and were able to further test the localized xylem expression of the GUS marker gene. We have demonstrated the ability of stable transgenic plants to display gene expression through the use of RT-PCR and small RNA northern blots. We were thus far able to generate and evaluate potato plants transgenic for constitutive expression of GWSS-actin and GWSS-chitin deacetylase to produce dsRNAs (siRNAs) and the corresponding down regulation of their host specific mRNA targets in GWSS adult insects. Encouraged by the results of GUS transgene expression in the xylem tissues of potato transgenic plants (spatial restriction of the transgene), we have also developed the transgene

constructs for GWSS-chitin deacetylase and GWSS-actin driven by xylem expressing, EgCAD promoter to generate small RNAs specific for GWSS mRNA in hopes of expressing these mostly in the xylem and generated stable transgenic lines in potatoes. We were successfully able to develop transgenic plants that generate small RNAs for GWSS-chitin deacetylase but GWSS-actin transgenic plants could not be recovered due to toxicity. Further, we are in the process to test the efficacy of these promoters (constitutive 35S and EgCAD) in the delivery of small RNAs (dsRNAs) into specific plant tissues that might streamline the delivery process.

OBJECTIVES:

Our primary and sub-objectives are:

1. *To assess the effectiveness of GWSS hairpin RNA transgenic plants against GWSS mRNA accumulation and insect fecundity, survival and development.*

   A. Temporal and spatial analysis of GWSS mRNA targeting.

   B. Assessing RNAi effects on GWSS fecundity, development and survival.

Objective 1A. Generation of potato transgenic lines was done in the potato cultivar, Desiree. In order to generate dsRNAs (siRNAs) against GWSS target sequences (actin, chitin deacetylase) corresponding cDNAs were cloned into a Gateway-compatible binary vector pCB2004B under the EgCAD promoter. Stable plant transformation with EgCAD-GWSS-chitin deacetylase and EgCAD-GWSS-actin was performed via recharge at the UC Davis Ralph M. Parsons plant transformation facility (http://ucdptf.ucdavis.edu/). We screened these transgenic potato plants for insert composition and demonstrated the presence of the transgenes (Fig 1). We previously showed the presence of GWSS-chitin deacetylase and GWSS- actin transgene-associated small RNAs in potato plants, expressing these anti-GWSS transgenes driven by the 35S constitutive promoter. Table 1 shows the number of potato lines that we now have for the chitin deacetylase sequence expressed from the EgCAD promoter.

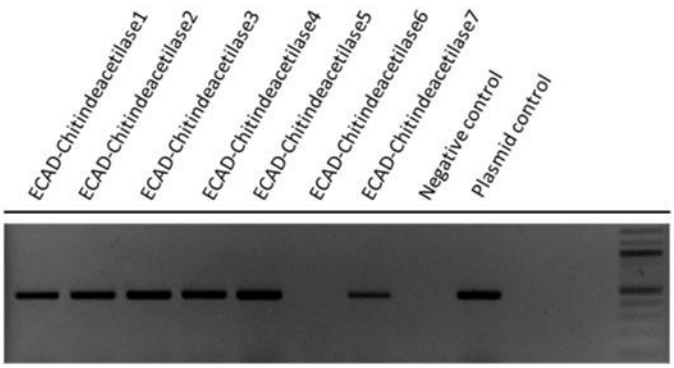

Fig 1: ECAD-GWSS chitin deacetylase transgenic potato plants were tested for gene expression.

Table 1. List of stable transgenic lines containing hairpin constructs for GWSS chitin deacetylase.

S.No:	Transgenic line/potato	Parent line
1	ECAD-GWSS chitin Deacetylase	132052-002
2	ECAD-GWSS chitin Deacetylase	132052-003
3	ECAD-GWSS chitin Deacetylase	132052-004
4	ECAD-GWSS chitin Deacetylase	132052-005
5	ECAD-GWSS chitin Deacetylase	132052-006
6	ECAD-GWSS chitin Deacetylase	132052-007
7	35S-GFP line	Control line

We previously performed feeding assays and assessed for RNAi effects on *H. vitripennis* (GWSS) using our transgenic potato plants expressing GWSS transgenes GWSS-actin and GWSS chitin deacetylase under the 35S promoter, and the stem infusion dsRNA assays. Transgenic potato feeding assays were done using 3rd- 4th instar nymphs (previous reports, and Pitman et al., 2014, unpublished). All the experiments were carried at controlled research facility (CRF) at UC Davis campus. Briefly, we placed cuttings of the potato plants in individual cages in a growth chamber, released five nymphs per cutting, and observed mortality for two weeks (Fig. 2). Higher mortality and target mRNA reduction was observed in nymphs that fed on cuticle and actin transgenic potato cuttings as compared to the controls. Quantitative Real-Time RT-PCR (RT-qPCR) was used to quantify relative expression of the mRNAs targeted for down regulation, and was normalized with ubiquitin.

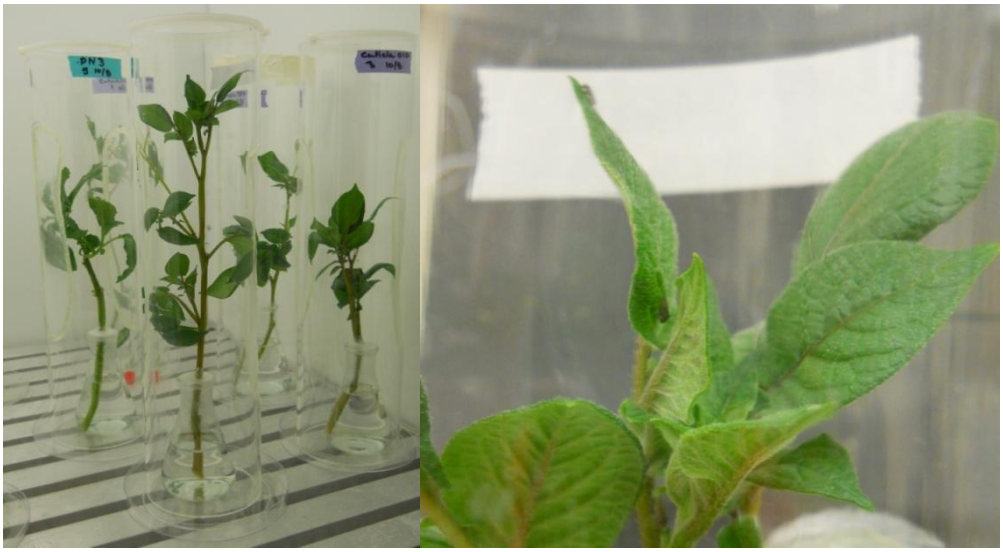

Figure 2. GWSS RNAi feeding assays on transgenic potato cuttings. At left shows stems in cylindrical cages, each containing 5 nymphs times 5 replications per treatment. Right shows a close up photo of a GWSS nymph feeding on upper potato foliage.

We are presently characterizing and evaluating the EgCAD promoter GWSS-chitin deacetylase transgenic plants. The feeding assays will be performed on these transgenic potato plants using the same approaches as used previously by us. RNAi effects on *H. vitripennis* (GWSS) will be assessed along with the mortality effects on the GWSS insects (3rd

and 4th instar nymphs). Quantitative Real-Time RT-PCR (RT-qPCR) will be used to quantify relative expression of the mRNAs targeted for down regulation as previously described.

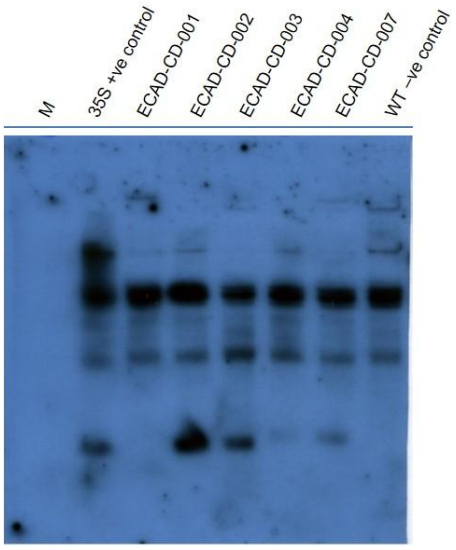

Fig 3: ECAD-GWSS chitin deacetylase transgenic potato plants were tested for small RNA expression.

We performed gene expression studies on the initial EgCAD-GWSS-chitin deacetylase plants generated as described above (Table 1). Plant gene expression studies were performed by using small RNA northern blots to assess for the expression of the desired anti-chitin deacetylase small RNAs in these plants. The small RNAs were extracted from the above transgenic plants were analyzed on 15 % poly acrylamide- urea gels (PAGE-urea) and were probed with chitin deacetylase small RNA probe synthesized *in vitro*. Based on the small RNA hybridization blot experiments, we were able to see the expression of small RNAs specific for GWSS chitin deacetylase (Fig 3). This gives confidence regarding our use of the EgCAD xylem promoter. Original cultivars of potatoes (non-transgenic) used to generate the transgenic lines were used as control plants.

Future work includes the possibility of using a stacked gene approach towards generation of small RNAs for multiple targets of GWSS and other sharpshooters from the same transgenic plants (proposed under Falk/Nandety proposal, 2014). This kind of approach can be achieved by stacking GWSS anti-mRNA target sequences into the same cultivar. For example, using GWSS-actin in addition to GWSS dicer gene or combination of GWSS dicer with GWSS argonaute gene would provide for a better RNAi effects on these insects.

Objective Ib. In order for us to study the effect of RNAi on growth and development of GWSS insects, we have taken *in vitro* and *in vivo* approaches to identify optimal interfering RNAs for use in RNAi experiments. Since our last update in October of 2013, we have identified additional effective RNA targets from our large scale GWSS transcriptome sequencing project. We have a well-built transcriptome dataset for GWSS and covers 35 Mb of the genome (Nandety et al., 2013, PLoS One). In addition we have generated a profile map of transcriptome with the available small RNA and micro RNA data. The latter will help identify optimal interfering RNA forms for future targeting of GWSS and likely other sharpshooter vectors of *X. fastidiosa*. We have previously reported the transcriptome data and small RNA data in detail through publications (Nandety et al., 2013a and Nandety et al., 2013b). In addition to the above datasets we were also able to identify several conserved and novel micro RNAs from GWSS adult insects through bioinformatics analysis based on their size and folding patterns. In our analysis thus far, we have identified conserved as well as sixteen novel micro RNAs (Fig 4 and Nandety et al., Unpublished).

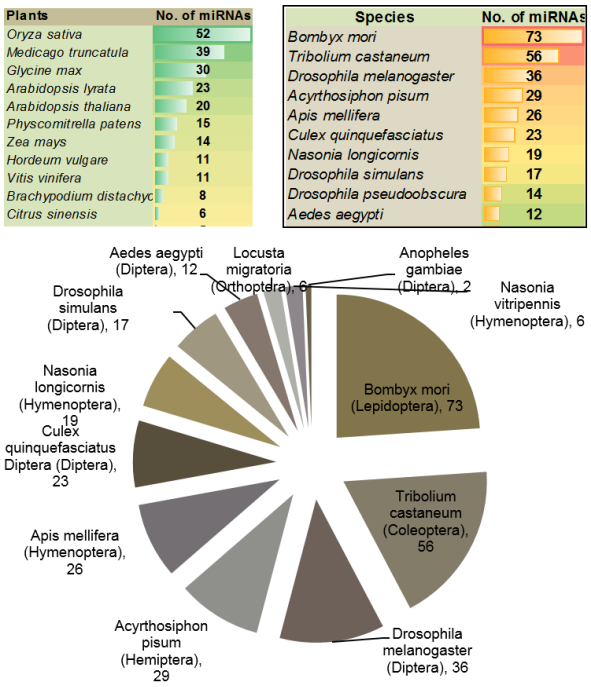

Figure 4: The microRNA profiling analysis of GWSS adult insects revealed the presence of microRNAs that are conserved between different insects. GWSS adult insects also share the microRNA conservation with plants besides insects.

Such information will be very useful to test their efficacy in the RNAi of GWSS insects. We suspect a higher role for microRNAs during the development of insects that can be used for our advantage in management of these insect pests. These micro RNAs were validated by quantitative real time PCR (stem-loop RT-PCR methods) and also through the micro RNA northern blots shown below (Figure 5). These naturally occurring host micro RNAs from GWSS adults can be valuable information for the design of RNAi experiments in GWSS insects for elucidating their roles in development. We envision that we could overexpress the precursors or the natural micro RNAs through various delivery mechanisms to test for their roles in the development of GWSS insects. The developmental stage micro RNA profiling (proposed under Falk/Nandety 2014 proposal) will also help us determine the specific micro RNAs expressed in each of the five developmental stages in GWSS, which will be invaluable information for us to design experiments at those specific stages.

In addition to the micro RNA approaches, we will compare the effects of transgenic plants (EgCAD-chitin deacetylase and 35S-actin and 35S-chitin deacetylase) derived siRNAs against the GWSS development. As previously described under objective 1, the insect mortality and development will also be observed through the in vivo based approaches. In addition, we were also testing the dsRNA based feeding assays to quickly evaluate the target gene suppression in GWSS and to assess any developmental effects on GWSS. Thus by the use of *in vitro* and *in vivo* based approaches to assess the RNAi effects on GWSS survival and development, we plan to monitor the developmental effects of RNAi on GWSS.

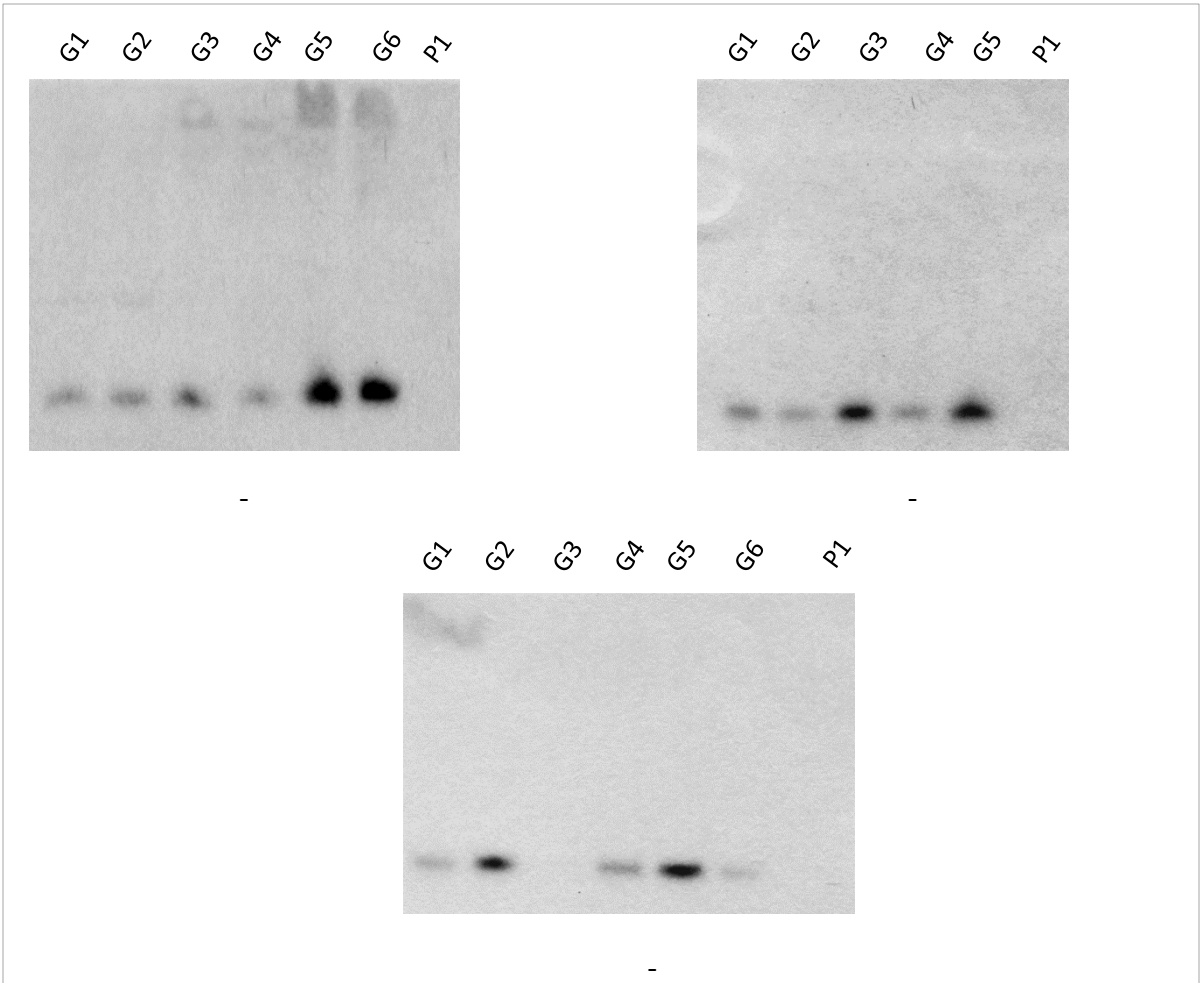

Figure 5: The microRNA profiling analysis of GWSS adult insects revealed the presence of novel and conserved microRNAs that are validated here through microRNA northern blots. GWSS small RNAs were separated on SDS-Urea PAGE gel and probed with 5' end labeled miRNA probe. The blots here validate for GWSSmiR171 (conserved microRNA), GWSSmiR276 (conserved microRNA) and a novel one (GWSSmiR29828). **Annotation:** G1 through G6-Adult GWSS insects. P1-potato small RNAs

Publications and presentations:

1. Raja Sekhar Nandety, Viacheslav Y. Fofanov, Heather Koshinsky, Drake C. Stenger and Bryce W. 2013. Small RNA populations for two unrelated viruses exhibit different biases in strand polarity and proximity to terminal sequences in the insect host *Homalodisca vitripennis*. Virology, 442:12-19.

2. Shizuo G. Kamita, Grant H. Oshita, Peng Wang, Raja Sekhar Nandety Christophe Morisseau , Bryce W. Falk and Bruce D. Hammock. 2013**.** Characterization of Hovi-

mEH1, a microsomal epoxide hydrolase from the glassy-winged sharpshooter *Homalodisca vitripennis*. Archives of Insect Biochemistry and Physiology, 83 (4):173-179.

3. Raja Sekhar Nandety, Shizuo G Kamita, Bruce D Hammock and Bryce W Falk. 2013. Sequencing and de novo assembly of the transcriptome of the glassy-winged sharpshooter (*Homalodisca vitripennis*). PLoS One 8, e81681.

4. Raja Sekhar Nandety, Shahideh Nouri, Yen-wen Kuo and Bryce W Falk. 2014. Novel strategies for target design and gene silencing in the insect vectors of plant pathogen**s. Plant and Animal Genome XXII,** San Diego CA (January 11 to January 15, 2014).

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

6. Raja Sekhar Nandety, Almas Sharif, Asokan R and Bryce W Falk. Expression profiling of microRNAs in *Homalodisca vitripennis*, the Glassy-winged sharpshooter resulted in the identification of novel microRNAs. In Preparation.

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 one year funding to support one postdoctoral scientist a graduate student/part time technician, an undergraduate intern, plus funds for standard

benefits. We also requested funds for routine supplies, recharge facility (Biosafety 3P Contained Research Facility) recharge costs and limited travel. We were awarded one year of funding $89,931. Due to some personnel changes and events beyond our control we have approximately $58,000 in carryover funds. We have submitted a new proposal which includes using these remaining funds next year.

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.