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 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 two years and have completed most of our objectives during 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 three manuscripts. 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 CaMV 35S promoter, and a *Eucalyptus gunii* minimal xylem-specific promoter (Ecad) to control the spatial expression of candidate interfering RNAs. We have demonstrated through RT-PCR analysis the ability of stable transgenic plants to express the transgene, and through small RNA northern blots showed their ability to generate small RNAs. In our previous reports we have shown the GWSS mortality and target mRNA level reduction in constitutively expressing GWSS-actin and GWSS-chitin deacetylase potato transgenic plants. Spatial gene expression of the Ecad promoter was verified through the GUS gene expression *in vivo* in the transgenic potato plants. 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, Ecad 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 successful in generation of transgenic plants that generate small RNAs for GWSS-chitin deacetylase but GWSS-actin transgenic plants could not be recovered due to toxicity. In the present reporting period, we were able to test the efficiency of tissue specific promoter (Ecad) in the delivery of GWSS chitin deacetylase small RNAs into plants and their effect on survival and development of GWSS insects.

OBJECTIVES:

Our primary and sub-objectives are:

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 A. The temporal and spatial analysis of delivering the small RNAs in plants and thus testing on GWSS insects was aimed at generation of potato transgenic lines with 35S and spatial expressing promoters in potato 'Desiree' background. In order to generate dsRNAs (siRNAs) against GWSS -chitin deacetylase and GWSS-actin, corresponding cDNAs were cloned into a Gateway-compatible binary vector pCB2004B under 35S and Ecad promoters respectively (Table 1). Stable plant transformation with binary vectors for 35S-GWSS-chitin deacetylase, 35S-GWSS actin, Ecad-GUS, Ecad-GWSS-chitin deacetylase and 35S-GFP was done via recharge at the UC Davis Ralph M. Parsons plant transformation facility (http://ucdptf.ucdavis.edu/). Wild type Desiree plants were used as the control plants for all experimental purposes.

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	Ecad-GUS	122099-001
8	Ecad-GUS	122099-002
9	Ecad-GUS	122099-003
10	Ecad-GUS	122099-004
11	Ecad-GUS	122099-005
12	Ecad-GUS	122099-006
13	35S-GWSS actin	112064-004
14	35S-GWSS actin	112064-008
15	35S- GWSS chitin deacetylase	102203-001
16	35S- GWSS chitin deacetylase	102203-002
17	35S- GWSS chitin deacetylase	102203-003
18	35S-GFP	122009-004
19	35S-GFP	122009-005

Table 1. List of 35S and Ecad promoter fusion GWSS transgenic potato plants along with their pedigree number.

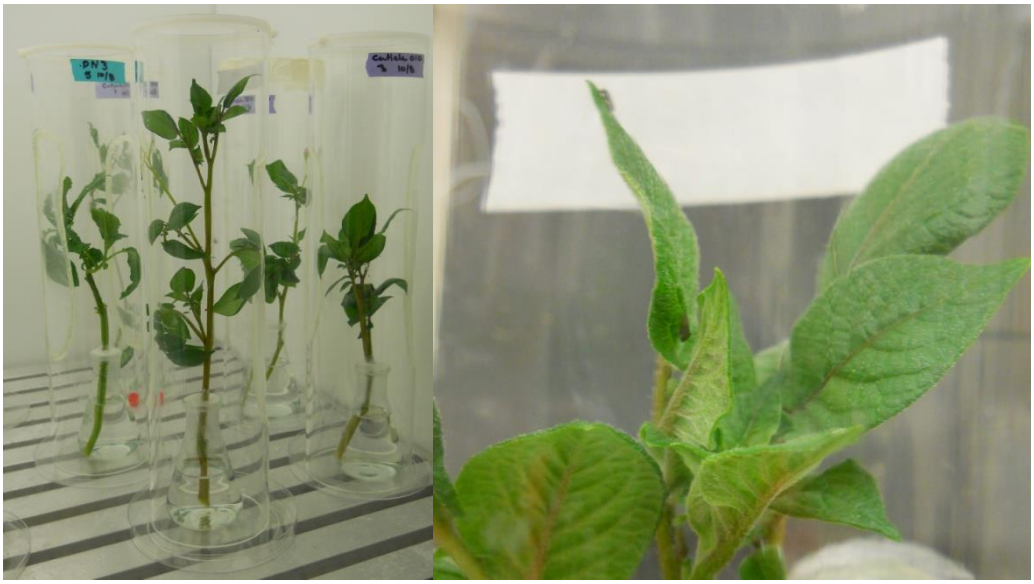

Figure 1. GWSS RNAi feeding assays on transgenic potato cuttings. At left shows stems in cylindrical cages, each containing 5 nymphs times 5 replications per treatment. Right shows a close up photo of a GWSS nymph feeding on upper potato foliage.

We screened the transgenic potato plants for insert composition and showed the presence of the transgenes respectively. We previously reported the expression of GWSS-chitin deacetylase and GWSS-actin transgene-associated small RNAs in potato plants, expressing these anti-GWSS transgenes constitutively. The number of potato lines that we now have for the GWSS- chitin deacetylase and GWSS actin expressed from 35S and Ecad promoters were presented in the table (Table 1).

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 (Fig. 1). Transgenic potato feeding assays were done using 2nd- 3rd instar nymphs (previous reports, and Pitman et al., 2014, unpublished). All experiments were done at the UC Davis Contained Research Facility (CRF). Higher mortality and target mRNA reduction were observed in nymphs that fed on chitin deacetylase 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 (previous report).

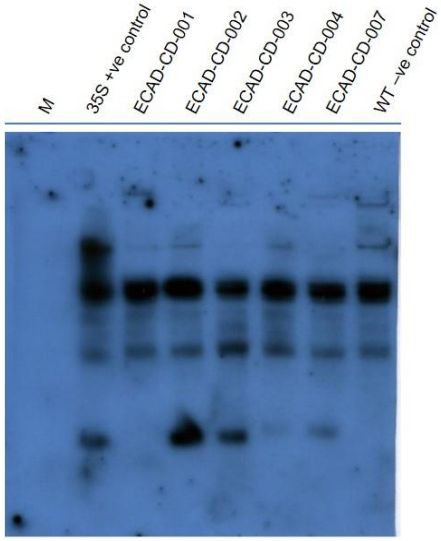

Figure 2. ECAD-GWSS chitin deacetylase transgenic potato plants were tested for small RNA expression

Objective 1B. Assessing RNAi effects on GWSS fecundity, development and survival. In order for us to study the effects of RNAi activity 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. We performed gene expression studies on the Ecad-GWSS-chitin deacetylase plants generated as described above (Table 1). Plant gene expression studies were performed using small RNA northern blots to assess for the expression of the desired anti-chitin deacetylase small RNAs in these plants (Fig. 2). The small RNAs extracted from the above transgenic plants were analyzed using poly acrylamide- urea gels (PAGE-urea) and were probed with a 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. 2). We next evaluated the Ecad-GWSS-chitin deacetylase transgenic plants against GWSS nymphs. All the feeding assays on GWSS insects (2nd- 3rd instar nymphs) were performed at the CRF. The potato transgenic lines (#2 and #3) for feeding assays were selected based on their ability to express small RNAs against GWSS-chitin deacetylase. Original cultivars of potatoes (non-transgenic) used to generate the transgenic lines were used as control plants.

Figure 3. Mortality Assay measured as percent survival of GWSS 2nd and 3rd instars against wild type and Ecad-CD transgenic potato plants. **E2**/ **E3** = potato transgenic lines

In our first set of mortality experiments, the sample size was 15 insects (2nd and 3rd instar nymphs) per event tested. From the results, we have not identified a significant percentage (11%) of survival difference between the insects fed on wild type and transgenic potato plants

that (Fig. 3). Based on our first experimental assay, we have expanded the trial experiment to accommodate higher sample size for each replication. In our second replication experiment we have increased the sample size to 34 insects (2nd and 3rd instar nymphs) per each event tested (Table 2). The experiment was designed to sufficiently include five replications per each event. Further, the mortality (% survival) assay was performed in the exact similar conditions at the CRF. The observable survival % difference was found to be replicated at 7 days post treatment in both of the replicated trials. The survival percent difference was found to be at 11% after 7-8 days after treatment under the conditions tested by us (Fig. 3 and Table 2).

Date	**WT-1**	**WT-2**	**WT-3**	**WT-4**	**WT-5**	**ECAD #2-1**	**ECAD #2-2**	**ECAD #2-3**	**ECAD #2-4**	**ECAD #2-5**
06/25	7-7	6-6	7-7	7-7	7-7	7-7	7-7	7-7	7-7	6-6
06/26	7-7	6-6	7-7	7-7	7-7	7-7	7-7	7-7	7-7	6-6
06/29	7-5	6-3	7-6	7-4	7-3	7-3	7-4	7-5	7-3	6-3
07/01	7-4	6-2	7-2	7-2	7-2	7-1	7-2	7-2	7-2	6-2

Date	**ECAD #3-1**	**ECAD #3-2**	**ECAD #3-3**	**ECAD #3-4**	**ECAD #3-5**
06/25	7-7	6-6	8-8	7-7	6-6
06/26	7-7	6-6	8-8	7-7	6-6
06/29	7-3	6-3	8-4	7-5	6-3
07/01	7-2	6-2	8-3	7-2	6-0

Table 2: The second replicated trial for mortality test of ECAD-chitin deacetylase potato transgenics against GWSS 2nd-3rd instar nymphs. **Annotation: WT** –Wild type potato plants; Ecad- represents the Ecad-CD transgenic potato plants; # **2** and **3**: Known transgenic potato lines expressing small RNAs. Data is represented in **a-b** format. **a:** total number of nymphs and **b:** total nymphs survived.

In addition to the above mentioned approaches for assessing RNAi effects on GWSS survival, we will compare the effects of transgenic plants (Ecad-chitin deacetylase and 35S-actin and 35S-chitin deacetylase) derived siRNAs against the GWSS fecundity and development. As described above, the insect mortality and development will also be observed through the *in vivo* based approaches. In order to fully understand the effective role of small RNAs generated in the transgenic potato plants and their effect on the GWSS mRNAs, Quantitative Real-Time RT-PCR (RT-qPCR) will be used to quantify relative expression of the mRNAs targeted for down regulation as previously described. 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.

In our approach thus far, we have not observed significant difference in the percent survival between the controls and transgenic events involving Ecad-GWSS-chitin deacetylase. This could be possible due to ineffective gene targets chosen or gene copy issues. These and other problems with the study of development, survival and gene expression can be circumvented by

stacking GWSS anti-mRNA target sequences into the same cultivar of potato. For example, using GWSS-actin in addition to GWSS dicer gene would provide for a better RNAi effects on these insects.

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).

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 ($89,931) to support one postdoctoral scientist and other support help, 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 new proposal and part of the remaining funds will roll over into the next funding period.

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.