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

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III. List of objectives and description of activities conducted to accomplish each objective:
Homalodisca. vitripennis (GWSS) is one of the most economically significant insect vectors of *Xylella fastidiosa*, the causal agent of Pierce's disease. Our longterm goal is to develop and use RNA interference (RNAi) as a means to help manage GWSS and other leafhopper vectors of *X. fastidiosa* and thereby contribute to efforts for controlling Pierce's disease. We have taken a directed fundamental effort to meet our long term goal and during the past two years we have made very good progress especially on the first two objectives, and are still working on objective 3. Specific results are given below. We used *H. vitripennis* (glassy-winged sharpshooter [GWSS]) cell and whole insect systems for our efforts. Experiments in *H. vitripennis* cells were concluded in Fall 2009. With the data derived from this study, we submitted a manuscript to the *Journal of RNAi and gene silencing* (http://www.libpubmedia.co.uk/RNAiJ/AimsAndScope.htm), and it has been "accepted pending revision". Our studies in *H. vitripennis* cells demonstrated that RNAi can be achieved in these cells, providing evidence that *H. vitripennis* cultured cells retain RNAi key components. Furthermore, we provided researchers with reverse genetic tools for gene function studies in sharpshooter cells. Based on our success so far, we feel confident that using RNAi as one component of efforts to control *H. vitripennis* vectors constitutes an important research effort. The specific objectives of our effort here are:

1. To identify RNAi-inducers capable of killing or reducing the survival and/or fecundity of *Homalodisca vitripennis* (*H. vitripennis*) and other sharpshooter vectors of *Xylella fastidiosa* (*X. fastidiosa*).
2. To generate transgenic plants capable of expressing *Homalodisca vitripennis* deleterious interfering RNA molecules within their xylem.
3. To evaluate transgenic plants for their ability to induce RNAi effects vs. *Homalodisca vitripennis* and other sharpshooter vectors of *X. fastidiosa*.

Initially, our efforts were somewhat limited due to a lack of identified GWSS sequences which could serve as both effectors and targets for RNAi studies. Nevertheless, we used this limited sequence information and were successful in utilizing standard cell culture methods for evaluating RNAi in cells. With the knowledge gained there and the same sequence information, we were then able to induce RNAi in whole insects via injection, and design systems for oral and topical delivery of RNAi inducers. Most important for our own progress is that we have shown that RNAi-induced negative phenotypes can be demonstrated in both cultured cells and whole GWSS. During the past year we were also able to greatly expand the list of potential sequences for anit-GWSS RNAi efforts. We analyzed three EST libraries available in GenBank. One thousand seventy three (1073) contigs and 2057 singlets returned significant hits from GenBank, for a total of more than 3100 sequences. As expected, the great majority of these sequences correspond to structural and housekeeping genes, but a great number corresponded to genes of potential interest as RNAi targets, including genes for cuticle formation, larval development, juvenile hormones, central nervous system development, eye morphogenesis and development, lipid and carbohydrate metabolism expressed in gut tissues and genes expressed specifically in salivary glands. Experiments are underway to assess these potential RNAi targets. These sequences provide a very valuable resource for efforts to understand and control GWSS and other sharpshooter vectors of *X. fastidiosa*.

We are currently attempting to demonstrate that the sequences identified by us in objective 1 can be used as RNAi effectors to GWSS via *in planta* delivery, as part of objectives 2 and 3. To test this we have already submitted binary constructs designed to express the GWSS actin hairpin RNA (identified in the previous year as best RNAi inducer) in the xylem of woody plants to the U C Davis Ralph M. Parsons Plant Transformation Facility. Transgenic grape and citrus plants will be generated, and we will evaluate them for the ability to induce RNAi in GWSS.

IV. Summary of major research accomplishments and results for each objective: Objective 1. Identification of RNAi inducers in *H. vitripennis* cells and insects. We used a previously developed GWSS cell line, and whole GWSS insects for our studies. All whole insect GWSS studies were done in the UC Davis Biosafety 3P Contained Research Facility. Cell culture experiments were done in the lab on campus as cultured GWSS cells are not quarantined. We were able to effectively induce RNAi effects by using several sequences in both GWSS cell and whole insect systems, as shown by decrease of target mRNAs (determined by real time RT-PCR analyses, see Table 1 and Fig.1).

Table 1. List of sequences identified as GWSS proteins in GenBank. Sequences in the top row were cloned by us, while we were unable to obtain sequences in bottom row.

Arginine kinase	Mitochondrial porin	tropomyosin	Ubiquitin conjugating enzyme	Rab11	Kinase c receptor	Sar1	Histone H3	Muscle and cytoplasmic actin	Ferritin GF2	Fructose 1,6-biphosphate aldolase
rhodopsin	Delta-9 desaturase 1	Activated protein kinase c receprot	vitellogenin							

Of the sequences above we used arginine kinase, tropomysosin, Histone H3, Fructose 1,6 biphosphate aldolase, Sar1, muscle actin, Ferritin GF2 and ubiquitin conjugating enzyme for RNAi experiments. We assessed effects by real time RT-PCR for whole insects injected with dsRNAs corresponding to the GWSS target mRNAs (see Fig 1 for Fructose 1,6-biphosphate aldolase results).

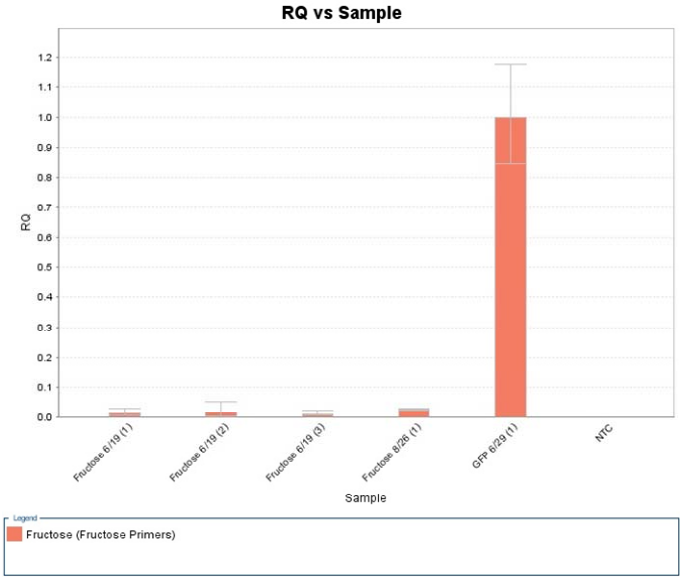


Figure1. Example of reduction of mRNA expression as measured by real time RT-PCR. Twenty insects were inthratoracically injected with 1 µg of fructose 1,6-biphosphate aldolase dsRNA, and 20 with GFPdsRNA. Five days after injection, survival insects devided in pools of 3 to 5 insects each were subjected to RNA extraction and their fructose mRNA level was measure by semiquantitative real time RT-PCR (columns 1 to 4). Data were compared to the
fructose 1,6-biphosphate aldolase mRNA level of GFP dsRNA injected insects (column 5).

Our longterm goal is to orally deliver RNAi effectors and to induce RNAi effects in GWSS, and this work is ongoing. However, because injection is an efficient and rapid, albeit artificial means to induce RNAi, we still used whole insect injection as a means to screen candidate sequences for phenotypic effects on GWSS. We monitored the survival rate for the injected insects (Fig.2 shows results for one experiment), after injecting different age nymphs in groups of 15 insects each with the above mentioned dsRNAs, and recording their survival 5 days post injection.

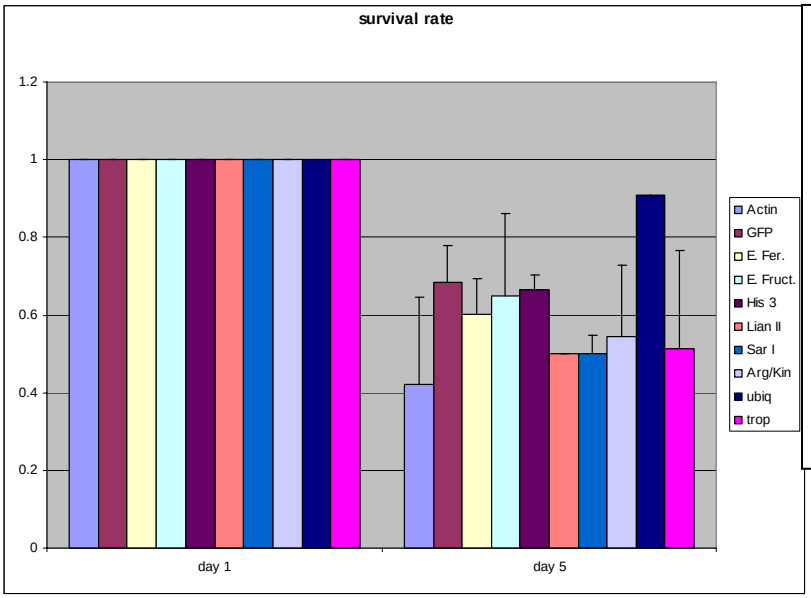


Figure 2. Fifteen nymphs were injected with 500-1000 ng dsRNAs. Injections were repeated three times, for a total of 45 nymphs per treatment and 10 treatments. Columns on the right represent the survival rate of the injected insects 5 days post injection, compared to day 1 (left columns).

The resulting data were variable, but in all experiments, the mRNA for actin, proved to be a very good target. Actin is essential for the normal development of GWSS insects and cultured cells, and we were able to obtain significant knockdowns in actin mRNAs in both cells and insects (Fig. 3), and a corresponding production of siRNAs (small interfering RNAs, hallmarks of RNAi activity). When actin dsRNAs were injected into whole insects the reduction in actin mRNA was obvious as seen by northern hybridization analyses (Fig. 3). Furthermore, immunoblot analysis showed that the actin protein was similarly reduced (Fig. 4). Taken together these data were very encouraging in demonstrating specific target knockdown.

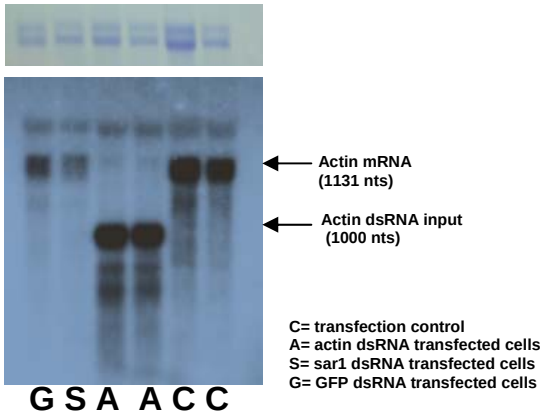


Figure 3. Transfection of actin dsRNA in *H. vitripennis* -Z15 cells results in a reduction in the level of actin mRNA. Cells were transfected with transfection reagent (C), 2 µg of actin dsRNA (A), sar1 dsRNA (S), GFP dsRNA (G) and harvested 72 hpt. Large and small RNA fractions were extracted (Ambion , MirVana PARIS) and 0.5 µg of the large fractions were electrophoresed on a denaturing 1.5% agarose gel. RNA was transferred to a positively charged nylon membrane (NitroBind, Cast, Pure Nitrocellulose, GE) and UV crosslinked. 32P-UTP labeled negative sense actin RNA transcripts were generated *in vitro* using T7 RNA Polymerase (T7 MAXIscript, Ambion), and used as probe. Hybridization was performed using standard procedures (PerfectHyb Plus, Hybridization Buffer, SIGMA/ALDRICH).
Reduction of the actin RNA was visible in actin dsRNA treated cells (A), compared to the controls (C, S and G). Upper arrow indicates the position of actin mRNA, and lower arrow the position of the input actin dsRNA (1000nts).

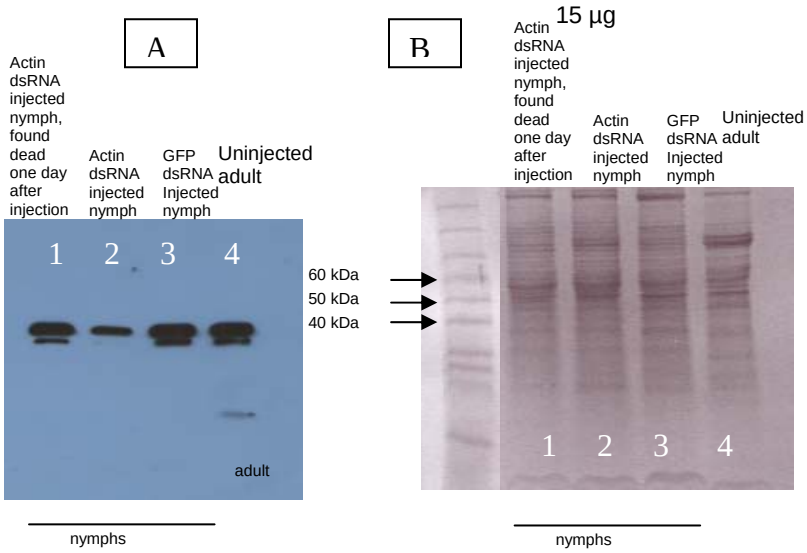
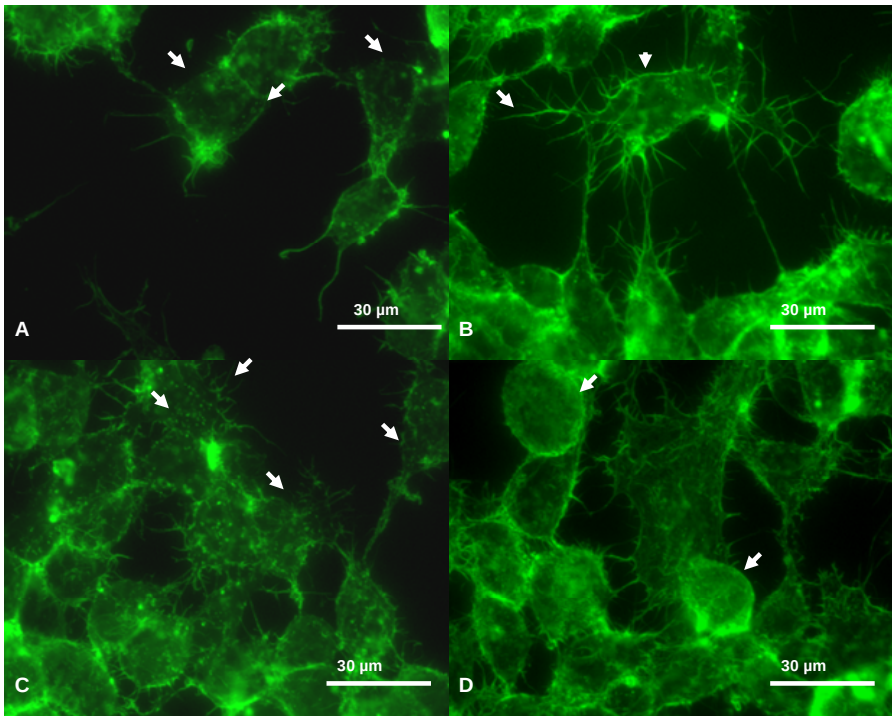


Fig. 4. GWSS nymphs injected with actin dsRNA showed decreased actin levels. Fifteen 3rd and 4th instar GWSS nymphs were injected with 1ug actin dsRNA in 1ul, with 1 ug GFP dsRNA or with 1 ul injection buffer, and left on basil plants for 5 days. Then, proteins were extracted from 3 living and 1 dead insect and analyzed by Western blot analysis, using actin antibodies specific for *Drosophila melanogaster* . Results show a decrease in actin in the nymph injected with actin dsRNA and alive 5 days post injection (gel lane 2 panel A), compared to the other insects (gel lanes 1, 3 and 4 in panel A). Coomassie staining shows equal amounts of proteins loaded for each sample (panel B).

The above data were all based on molecular analyses to detect RNAi effects, but they also suggest that if actin is an important component of cells and whole insects, its knockdown by RNAi should allow us to see a detectable phenotype. Therefore, we treated GWSS cells and injected 5th instar GWSS nymphs with 2 and 1 µg of actin dsRNA respectively, to attempt to induce a negative phenotype. Both cells and insects showed clear and repeatable RNAi effects (See Figs. 5 and 6). Cells showed decreased elongation of actin filaments and accumulation of granules in the cytoplasm of actin dsRNA-transfected but not control cells (Fig. 5). Phenotypic effects were more evident and obvious in whole insects. When GWSS nymphs were injected with actin dsRNAs, 5th instar nymphs failed to develop into adults and died (Fig. 6). These effects were seen only in actin-treated GWSS suggesting that RNAi phenotypes and even death can be induced in whole insects.

Fig. 5. Actin representative morphology in *H. vitripennis* -Z15 cells after transfection with actin dsRNA.. Cells were transfected with 2 µg of actin dsRNA (A and C), or GFP dsRNA (B and D) and harvested 72 hpt. Actin filaments in the cell membrane and cytoplasmic area were largely disturbed (arrows in A and C). (A) *H. vitripennis* cells showing partial disruption of the actin organization at the cell plasma membrane. Some filaments began to break and the cells failed to branch out. (B) *H. vitripennis* cells showing no changes in actin filament distribution and polymerization. Healthy isolated cells were connected through a densely branched actin filament network. (C)



H. vitripennis cells showing severe disruption of actin filaments. The short fragments of actin filaments were scattered throughout the cytoplasm. Some actin fragments tended to aggregate into clusters below the plasma membrane and obvious twisted actin cables could be observed. (D) Actin filaments were found primarily in the cell cytoplasm as a continuous and organized net in control cells. All observations were at 72 hpt.

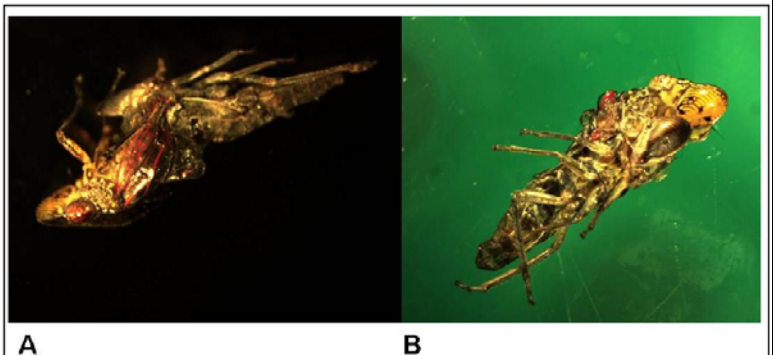


Figure 6. Fifth instar GWSS nymphs were injected with actin dsRNA. A: GWSS molting into adult but died before completing ecdysis. B: GWSS dead while molting.

Actin as a target proved to be an important proof of concept supporting our longterm goal. We are moving to test it now in transgenic grape and Carrizo plants (see bleow). However, actin may not be the most desirable target in the longterm, and other sequences may be much better. Therefore, by mining public EST databases we were able to greatly increase the number of GWSS sequence targets for our RNAi studies. Twenty thousand thirty (20,030) GWSS EST sequences were analyzed using the Arthropod EST analysis pipeline at Kansas State University. One thousand seventy three (1073) contigs and 2057 singlets returned significant hits from GenBank, for a total of more than 3100 sequences. One thousand nine hundred seventeen (1917) contigs were assembled and 6561 input reads were retained. The average length of the assembled contigs was 570 bp. NCB BLASTX was used to identify sequence similarities in GenBank for the assembled contigs and singletons. In this way, we identified several potential gene targets including some which are deleterious to insect development. We cloned several of these GWSS mRNA cDNA sequences including some related to cuticle and chitin formation, neural development and juvenile hormone esterase homologs, and used them in injection studies. We

generated dsRNAs corresponding to the identified sequences by *in vitro* transcription and are presently assessing these potential RNAi targets.

We also began experiments designed to assess oral delivery of RNAi effectors to GWSS. For this we would like to utilize a rapid way to accurately test RNAi effectors without having to use transgenic grape or other typical GWSS host plants, which is a time-consuming process just to obtain transgenic materials. We have relied on the ability of cut stems of herbaceous GWSS hosts (e.g. basil) to rapidly absorb intact dsRNAs into their xylem by natural transpiration. We showed in last years report that dsRNAs are rapidly and universally spread throughout such cut stems and that the dsRNAs introduced into the xylem are essentially intact, and not degraded into smaller fragments. Here we performed artificial feeding experiments by infusing basil cuttings with solutions spiked with candidate dsRNAs. Pools of 5 to 20 GWSS nymphs were left on the infused cuttings for 24 hours, and then moved to caged rooted plants. Insect survival was recorded until all nymphs on the cutting infused with the control GFP dsRNA molted into adult insects (Fig. 7).

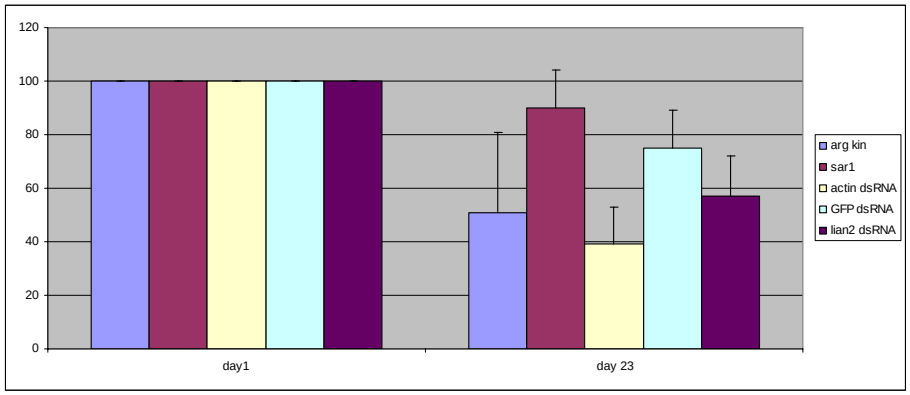


Figure 7. Groups of 5, 10, 15 and 20 nymphs were fed on cuttings dipped in dsRNA solutions for one day, and then moved on potted plants for 23 days. Their survival was observed (right columns) compared to the survival observed one day post injection (left columns).

Initial experiments showed high variation in insect survival. This could be due to a number of factors including that we can only deliver dsRNA effectors by this approach for one to two days, at which time the stems show greatly reduced ability to uptake and transport materials. Therefore to help better understand cut stem delivery, we are now quantifying the amount of targets acquired by GWSS individuals after feeding on cut stems for defined time periods. We added known amounts of marker dsDNA (dsDNA is easier to quantify than is dsRNA) into the test solutions and measured by quantitative real time PCR the amount of DNA acquired by individual insects. In one experiment, 10 adult GWSS were left feeding for 24 hours on a basil cutting that absorbed 32 µg of dsDNA (size 500 bp). After 24 hours, individual insects retained between 16 ng and 2.6 µg of dsDNA (the amplified real time PCR product size is 78 bp). The average of the amount of DNA found in the insects was 500 ng. This result shows the variability of this approach, but also offers the means to quantify the amounts of xylem fluids acquired by GWSS. This will also be important in the evaluation of transgenic plants expressing RNAi inducers against feeding GWSS.

Objective 2. To generate transgenic plants capable of expressing *Homalodisca vitripennis* deleterious interfering RNA molecules within their xylem. **Xylem specific promoter cloning and generation of transgenic plants expressing RNAi inducers.** The specific xylem promoter EgCAD2 was cloned from *Eucalyptus gunii* (see last years report). The

sequence was fused to the GUS reporter gene in the binary pCB301 vector, as control to verify the expression of mRNA in xylem tissues. Actin hairpins were also cloned under the control of the xylem promoter, as well as separately under the constitutive 35S promoter (Fig. 8). Transgenic Citrus, *Poncirus trifoliata* (L.) Raf. x *C. sinensis* Carrizo, and Grape, *Vitis vinifera* Thompson Seedless, are currently being produced for us now by the UC Davis Ralph M. Parsons Plant Transformation Facility. The first sets of plants should be available in the next few months for evaluation (**Objective 3).**

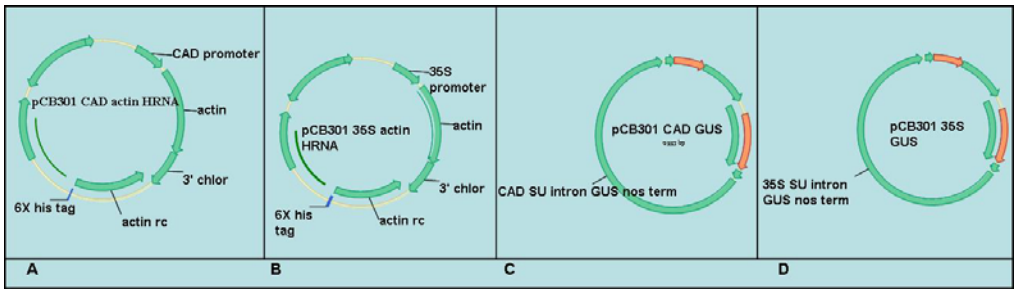


Fig.8. Diagrams of binary vectors used to generate transgenic plants. A: pCB301 with actin hairpin loop sequence (actin HRNA) driven by the xylem specific promoter CAD (EgCAD2). B: same sequence driven by the constitutive promoter 35S. C: beta-glucuronidase (GUS) reporter gene driven by the CAD promoter in pCB301. D: GUS driven by the 35S promoter.

V. Publications or reports resulting from the project:

- RNAi effects on actin mRNAs in *Homalodisaca vitripennis* cells. Rosa C., K.S.G., Dequine H., Wuriyanghan H., Lindbo J.A., Falk B.W., to Journal of RNAi and gene silencing. Accepted pending revision.

VI. Presentations on research:

- Falk, B. RNA interference and the glassy-winged sharpshooter. Research presentation to grape commodity group, Lodi, CA. March 9, 2009.
- Rosa, C. RNA interference and *Homalodisca vitripennis*, the glassy-winged sharpshooter. 4th Annual UC Davis Host-Microbe Interaction Research Retreat. Granlibakken, Tahoe City, CA, October 1st 2009.
- Rosa, C. RNA interference against the glassy-winged sharpshooter, a vector of Pierce's disease of grapevines. 2009 Pierce's Disease Research Symposium, Sacramento, CA, December 12th 2009.

VII. Research relevance statement: RNA interference (RNAi) is a relatively recently discovered fundamental component of gene regulation and antiviral defense within cells of eukaryotic organisms. RNAi leads to sequence specific degradation (or possibly translational arrest) of target mRNA molecules within the cell cytoplasm, resulting in eliminating or reducing gene expression or antiviral immunity. RNAi can be induced by double-stranded RNAs (dsRNAs), which are "recognized" by cellular surveillance systems resulting in their processing by host enzyme complexes to yield short RNAs (21 – 25 nt). One strand of the resulting short RNA associates with the argonaute proteins, the catalytic components of the RNA-induced silencing complex (RISC). The short RNA serves to guide the RISC complex to homologous target RNAs which are degraded by RNase III activity of argonaute. Thus, when host mRNAs

are targeted, their expression is affected as is that of the encoded protein. Various aspects of RNAi probably now represent the most intensely studied fields in all of biology. In addition to fundamental research in this area, there are already several examples of practical application of RNAi-based technologies for agriculture. For example, RNAi-based strategies for conferring plant resistance to bacterial, nematode and virus induced plant diseases have been demonstrated, and some even used in commercial agriculture. And recent RNAi efforts targeting insects have become more common. The objectives of our research effort are to investigate and develop new and effective, environmentally sound strategies for controlling the GWSS and other leafhopper vectors of *X. fastidiosa*. Our goal is to develop strategies that are effective and will provide control for PD of grapes, but also have flexibility for use in other important California crops. Of relevance to this proposal is that RNAi offers opportunities for targeting *H. vitripennis* via RNAi-based disruption of essential GWSS genes, thereby resulting in insect deleterious effects. Our work is relevant to the research recommendations of the PD/GWSS Scientific Summit and the National Academies' National Research Council's report including Categories 1 & 2 of the NAS PD research recommendations, specifically under recommendations 3.3, 3.5, 4.5 and 5.1.

VIII. Lay summary of current year's results: This work presents fundamental efforts towards long term application of using RNA interference, RNAi, to help combat a plant disease of great economic importance. The disease, Pierce's Disease of grapevines, is a significant threat to grape production in California and other parts of the U.S., and the causal agent of the disease, *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 we combine the use of an *in vivo* system (*H. vitripennis* whole insects) with an *in vitro H. vitripennis* cell based system. We demonstrated that RNAi effects can be induced *H. vitripennis* cell lines and insects, as shown by molecular analyses (real time RT-PCR, RNA hybridization), and more importantly by visual phenotypic effects in cultured *H. vitripennis* cells and whole insects. We also made specific DNA constructs to deliver these RNA effectors to transgenic plants and their development is ongoing. RNAi is an extremely important and broadly studied area in contemporary biology, and terms such as "magic bullet" for human medicine, and "genetic insecticide" for targeting insects have been used in the literature. Our work represents the first demonstrated RNAi effort in GWSS and our data will help to expand the possibilities to study plant-associated insects and at the same time to target the sharpshooter vectors of *X. fastidiosa*, the causal agent of Pierce's disease.

IX. Status of funds: We requested three years funding to support one postdoctoral scientist (Co-PI Rosa), a graduate student, an undergraduate intern, plus funds for standard benefits. We also requested funds for routine supplies, recharge facility recharge costs and limited travel. We were awarded two years of funding including $119,677 and $122,574 for years one and two, respectively. We are on track, spending wise, to use these funds as proposed in our original proposal budget.

X. Summary and status of intellectual property produced during the research project: We will work with UC for managing any intellectual property or technologies that may arise from this effort. We submitted an overview of our work for evaluation by the UC Davis Technology Transfer team, they declined to pursue it at this time.