Interim progress report for CDFA Agreement # 12-0216-SA

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

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Time period covered by the report: 7/1/12 – 3/15/13

List of objectives and description of activities conducted to accomplish each objective:
The specific objectives of our effort are:

1. To generate and evaluate transgenic potato plants for their ability to generate small RNAs capable of inducing RNAi effects in *Homalodisca vitripennis*.

2. To identify GWSS interfering RNAs for practical application.
   a) To utilize transgenic potato plants as efficient alternatives for identifying, delivering, and evaluating efficacious interfering RNAs.
   b) To enhance production of interfering RNAs *in planta*.

Description of activities, accomplishments and results for each objective:

We have made significant progress during the past few years and are in excellent position to complete most of our objectives during the upcoming year. We have published two refereed journal articles (Rosa et al., 2010) and (Rosa et al., 2011) submitted a new manuscript (Nandety et al., 2012) and have presented six symposium reports (Falk et al., 2010), (Rosa et al., 2010), (Falk et al., 2011), (Nandety et al., 2011), (Nandety et al., 2012) (Nandety et al., 2013) (see sections V and VI). In addition we presented our work at various national and international meetings during this past eighteen months (see section VI). RNA interference applications are at the forefront for controlling insect pests and vectors, and our work here is very timely. Here we present our progress towards the development and application of an RNA interference (RNAi) based system aimed to target genes of the vector of *Xylella fastidiosa*, *Homalodisca vitripennis* or the Glassy-winged sharpshooter (GWSS). After demonstrating that RNAi induction in GWSS cells and insects is achievable, we began screening a large pool of candidate genes to find the best targets to control the survival of GWSS. These data were used to develop transgenic *Arabidopsis* and potato plants that express double-stranded RNAs (dsRNAs) for the insect targets. We also made stable *Arabidopsis* and potato transgenic plants that express GUS marker genes using 35S and a *Eucalyptus gunii* minimal xylem-specific promoter to control the spatial expression of the genes. While we were able to show expression of the GUS gene *in vivo* in the T2 transgenic *Arabidopsis* plants in the previous report, within the past few months we were able to further test this concept in potato transgenic plants and were able to show a localized xylem expression. We also were able to show the ability of potato plants transgenic to GWSS-Actin and GWSS-cuticle to produce dsRNAs and

down regulate specific mRNA targets in GWSS adult insects. Encouraged by the results of GUS gene expression in the xylem (spatial restriction of the transgene) we then developed the transgene constructs to generate GWSS mRNA specific targets that are driven by xylem expressing ECAD promoter. Since our update in October of 2012, we found effective targets from the large scale GWSS transcriptome sequencing project that we adopted. We have a well-built transcriptome data set for GWSS insects that covers 35Mb of the genome which we are planning to publish shortly.

RNAi in *H. vitripennis* cells and insects. Initially, we used 14 GWSS Genbank cDNA sequences corresponding to known proteins in order to synthesize RNAi inducer molecules, dsRNAs. We then tested whether RNAi was inducible in GWSS cells and insects, and we showed that RNAi activity is inducible in GWSS (1). Quantitative RT-PCR, semi quantitative RT-PCR, and Northern blot of small and large RNA fractions showed that RNAi was achieved in cells and insects injected with dsRNA where target mRNAs were partially degraded and specific siRNAs (short-interfering RNAs), hallmarks of RNAi, were detected (1). We presently were able to show the success of our transgenic potato plants in their ability to down regulate the specific mRNA targets when they were fed upon by GWSS (see Figure 5). The inducibility of RNAi in the GWSS cells helped us design the following set of experiments.

Generation of plant transgenic lines: For the purpose of generating the *Arabidopsis* transgenic lines we used a different ecotype, Cape Verdi (Cvi). Compared to Columbia (Col-0) it has larger leaves and presents more robust growth, and will be more appropriate in supporting insects of large size such as *H. vitripennis*. In order to generate dsRNAs that can target the insect, GWSS target sequences (Table 1) were cloned into a gateway-compatible binary vector pCB2004B (Figure 1). The target sequences were cloned in head to tail direction in the gateway vector with a non-homologous sequence between them. Upon transcription in transgenic plants, these constructs will yield double-stranded, hairpin RNAs of the desired sequence. The expression vectors carrying the insect target sequences of interest were first cloned into *E.coli* and *Agrobacterium tumefaciens* and they have been sequence verified. *A. tumefaciens* cultures carrying the sequences of interest were used to transform *A. thaliana Cvi* plant ecotypes through the floral dip process. *Arabidopsis* T_0 plants were screened for resistance against the selectable marker *BAR* gene, and we were able to confirm T_1 transgenics. We were also able to obtain homozygous transgenic *Arabidopsis* lines that are ready to screen against GWSS insects.

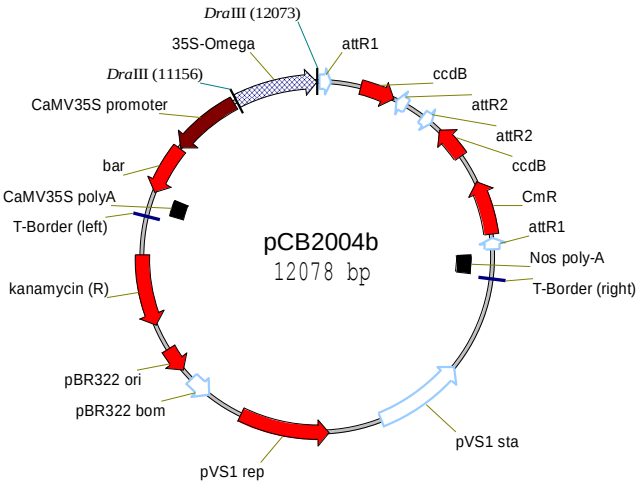

Figure 1. Gateway binary expression vector used for designing AntiGWSS transgene constructs

We were also able to develop transgenic potato plants(Kennebec and Desiree varieties) against GWSS target genes Actin, Cuticle and Chitin Deacitilase (Table 2). Transformation / regeneration were performed via recharge at the UC Davis Ralph M. Parsons plant transformation facility (http://ucdptf.ucdavis.edu/) and approximately ten independent transgenic lines were obtained for each of the constructs. We have performed screening of these transgenic potato plants for insert composition and have established the presence of a transgene similar to the procedure as described for

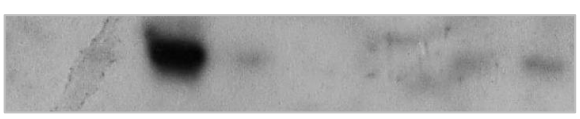

Figure 2. Small RNA northern hybridization analysis of transgenic potato plants expressing GWSS cuticle. The small RNA blot here shows the presence of anti-cuticle siRNAs in the transgenic plants (T1 to T4). The transient expression (positive control) and wild type plants (negative control).

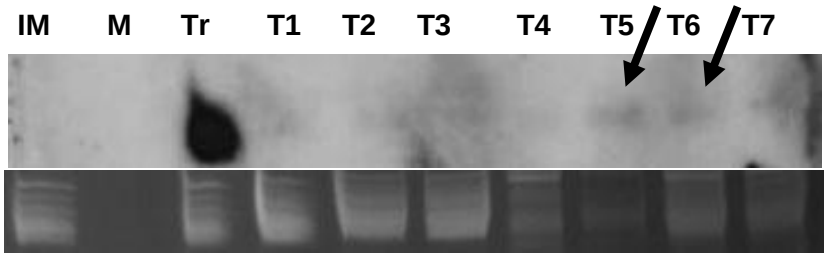

Figure 3. Small RNA northern hybridization analysis of GWSS-Actin transgenic potato plants. Arrows indicate positions of GWSS anti-actin siRNAs. Lower intensity siRNA signals are present in many of the other lines.

Arabidopsis transgenic lines. The presence of GWSS-cuticle (Figure 2) and
GWSS- Actin (Figure 3) transgenes in the potatoes resulted in the production of small RNAs in those transgenic plants. In contrast to the approach with *A. thaliana*, we are vegetatively propagating the T_0 plants and are in the process of testing the same for RNAi experiments with GWSS. Potatoes are an excellent host plant for GWSS so we expect them to be very useful for our efforts here. We have characterized these plants to ensure that they contain the desired transgene(s) and for some, that they generate the desired siRNAs (Figure 2 and Figure 3). In addition to the transgenic plants expressing GWSS target genes under control of the 35S promoter we have started generating constructs under a specific xylem promoter EgCAD2, which was cloned from *Eucalyptus gunii*. In our initial experiments, the sequence was fused to the GUS reporter gene in the binary vector pCB301.

Table 1: GWSS insect sequences used for cloning and generation of *Arabidopsis* transgenic lines.

Construct Name*	Protein Encoded	Length of PCR Product (bp)	*E. coli* DH5-α Sequence Verified	*A. tumefaciens* EHA105 PCR Verified	Number of *Arabidopsis* transgenic lines generated
GWSS 965	Zinc Metalloproteinase	443	Yes	Yes	None
GWSS 989	Glucosyltransferase	576	Yes	Yes	3 independent lines
GWSS 1591	Sugar Transporter	668	Yes	Yes	One independent line
GWSS 1377	Serine Proteaseserpin	645	Yes	Yes	2 independent lines
GWSS 364	Trypsin	605	Yes	Yes	2 independent lines
GWSS 975	Transaldolase	800	Yes	Yes	3 independent lines
GWSS 366	Sugar Transporter	888	Yes	Yes	None
GWSS 500	Serpin	418	Yes	Yes	4 independent lines
GWSS 745	Trypsin	756	Yes	Yes	None
GWSS 512	Transketolase	1435	Yes	Yes	None

Table 2: GWSS insect sequences used for cloning and generation of potato transgenic lines.

GWSS Targets	Potato Pedigree	Potato Variety	Selection Method	Small RNA Production
Chitin Deacetylase	102203	Kennebec	Basta	yes (shown previously)
Chitin Deacetylase	102203	Kennebec	Basta	yes (shown previously)
Actin	112064	Desiree	Basta	yes (Figure 3)
Cuticle	102203	Desiree	Basta	yes (Figure 2)

Histochemical staining showed that GUS expression was restricted to the main vascular tissues. We previously generated transgenic *Arabidopsis* that expressed the *GUS* gene under the xylem specific promoter, which we

have tested in the T2 generation for the presence of transgene and we were able to show the expression of GUS under a dissecting microscope (previous report, October 2012). As discussed and proposed in our previous report, we were recently able to generate the transgenic potato plants for the GUS transgene under control of the ECAD promoter. These plants were also generated via recharge at the UC Davis Ralph M. Parsons plant transformation facility (http://ucdptf.ucdavis.edu/) and approximately ten independent transgenic lines were obtained. In the figure below (Figure 4), we have shown

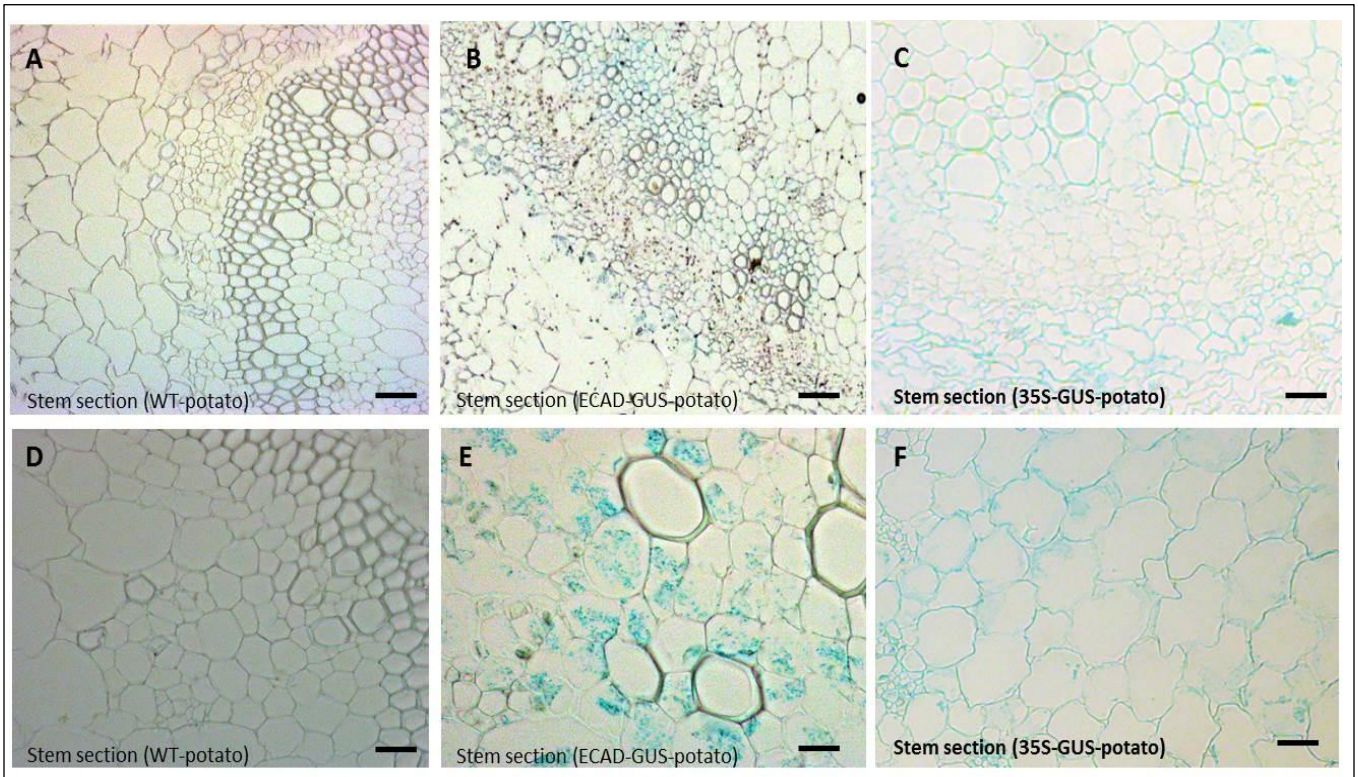

Figure 4. Cross sections of potato stems of non-transgenic (A & D), and GUS transgenic with the Ecad2 xylem-specific promoter (B & E) or 35S promoter (C & F). GUS expression is noted by the blue coloring. All images are taken under Zeiss bright field microscope.

specific expression of the GUS marker gene in xylem (Figure 4B and 4E) in comparison to wild type tissues (Figures 4A and 4D) or a constitutively driven GUS marker gene expressed across all tissues (Figures 4C and 4F). Thus, we are able to show the specificity of GUS marker gene expression to xylem tissues, which when coupled with insect target dsRNA expression can help us generate the much anticipated siRNAs in the xylem. Encouraged by our xylem expression studies, we have generated xylem promoter driven GWSS-mRNA targets (Table 2) in a gateway based binary plasmid and have further validated them to contain GWSS targets in fusion with ECAD promoter. We have recently initiated the generation of the same transgenic plants which should be available to us very shortly to screen for the effectiveness of the spatial distribution of siRNAs against all GWSS life stages.

Feeding assays: In addition to the transgenic plant approaches we have evaluated *in vitro* feeding approaches for GWSS based on recent reports in the literature (Killiny and Almeida, 2009, PNAS 106:22416) and personal communications from other scientists, and have concluded that basil infusion method was effective in testing for artificial diet feeding experiments (previous report, October 2012). We took advantage of the same method and have successfully and rapidly screened candidate sequences for their abilities to induce

RNAi effects via oral acquisition (Figure 5). We have a number of candidate sequences which we are testing for RNAi (previous report). The candidate sequence targets are cloned into vectors suitable for *in vitro* transcription and the dsRNAs that are made as a result of *in vitro* transcription will be used through the basil infusion method. We have used the basil infusion in the past and it offers some advantages as well as disadvantages.

Figure 5. GWSS feeding on basil stem which is submerged in a solution of double-stranded RNA.

We have generated a series of transgenic plants (potatoes and *A. thaliana*) for our RNAi studies so far (see Tables 1 and 2). We have used several GWSS mRNAs as targets and characterized these plants to ensure that they contain the desired transgene(s) and for some, that they generate the (Figures 2 and 3) desired siRNAs The plants are engineered with hairpin constructs to generate dsRNAs (see previous reports) but we have used different promoters to express the dsRNAs in plants. These are the *Cauliflower mosaic virus* 35S promoter which gives general constitutive gene expression across many tissues, and the EgCad2 xylem-specific promoter from *Eucalyptus gunii* (see Figure 4 for the difference in GUS expression for these two promoters). With the generation of EgCad2 promoter driven hairpin RNAi transgenics against GWSS mRNA targets, we hope to significantly alter the composition of xylem tissue RNAi effectors thus rendering them to provide significant amounts of small RNAs that are specific to GWSS adult and juvenile insects. Initially we examined mortality of 3rd and 4th instar nymphs in no-choice feeding assays on the 35S transgenic potatoes expressing: actin, chitin deacetylase, or cuticle dsRNAs; or the wild-type variety Kennebec (Figure 6). Based on these results we looked for differences in mRNA levels between 4th instar nymphs feeding on Kennebec or cuticle transgenic potatoes. We allowed the insects to feed for 5 days then collected samples from the remaining live insects, as the bodies degrade very quickly after death. The majority of evidence so far suggests that RNAi effects in insects are not systemic, that changes only occur in the cells in contact with the RNAi effector RNAs. Thus, we examined whether there were knockdown effects by dissecting out the digestive tracts of the experimental insects, extracting RNA from the tissue, and performing quantitative RT-PCR. Real time assays were developed using Primer Express® software by Invitrogen (Grand Island, NY) with 18S endogenous controls for eukaryotic organisms. Our preliminary efforts with transgenic potatoes expressing GWSS-cuticle when screened against GWSS insects showed a significant ten-fold reduction in the GWSS cuticle mRNA target expression in the guts (Figure 7).

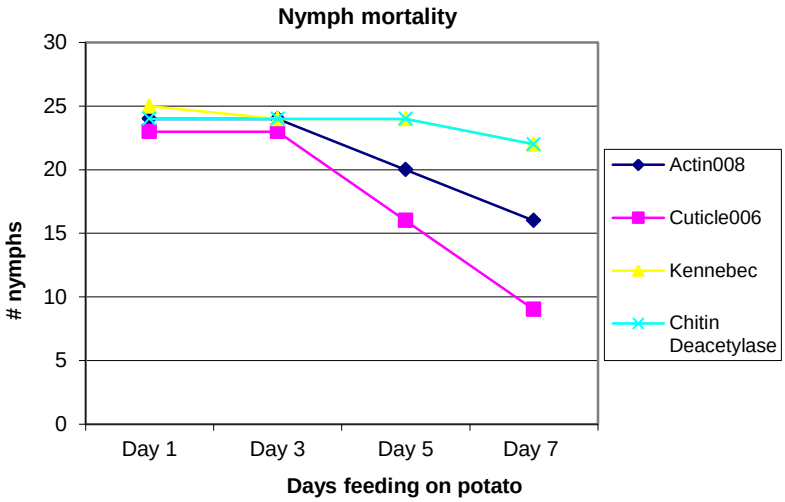

Figure 6. Nymph mortality on 35S promoter driven dsRNA transgenic potato plants or wild-type Kennebec. Each treatment consists of five replicated potato cuttings with five 3rd to 4th instar nymphs feeding on them for one week.

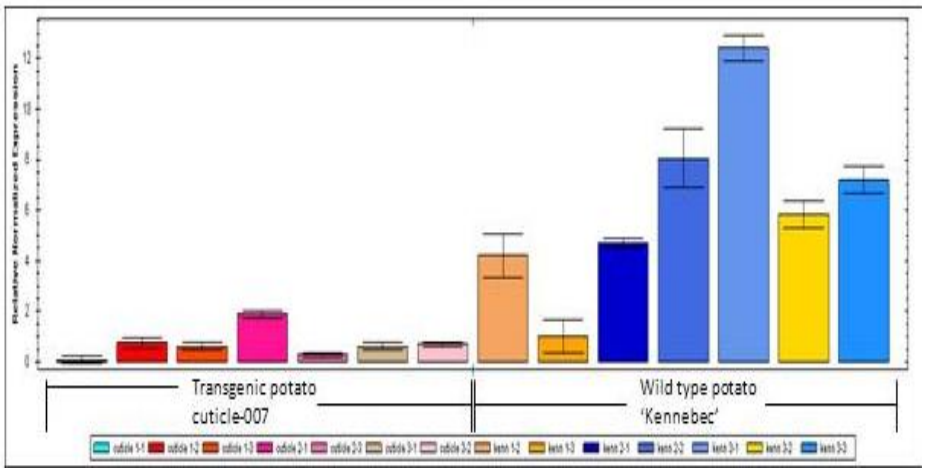

Figure 7. RNAi effects on GWSS after feeding on potato stems transgenic for a GWSS cuticle hairpin RNA. Upper histogram shows real-time qRT-PCR data for the GWSS cuticle mRNA after feeding on transgenic potato stems (bars at left as indicated) or on non-transgenic potato stems (bars at right).

Next Generation Sequencing of GWSS adult insects:

The developmental regulation of insects through the use of small RNAs has been well studied. In our efforts to study the regulation of GWSS insect genes and identify RNAi targets, we took an alternate approach using high throughput parallel sequencing to identify the small RNAs from the GWSS insects. For our work, we noticed GWSS transcriptome data is lacking information for the identification of small RNA reads. To address this and identify the loci of the small RNAs that were originated from the short read sequencing, we sequenced the transcriptome of GWSS through the use of mRNA sequence methods as described before (previous report). The sequencing of GWSS mRNA transcriptome was done through paired end sequencing on Illumina GA-II Platform. Both the mRNAseq library data and the small RNAseq library data were generated from the GWSS adult insects.

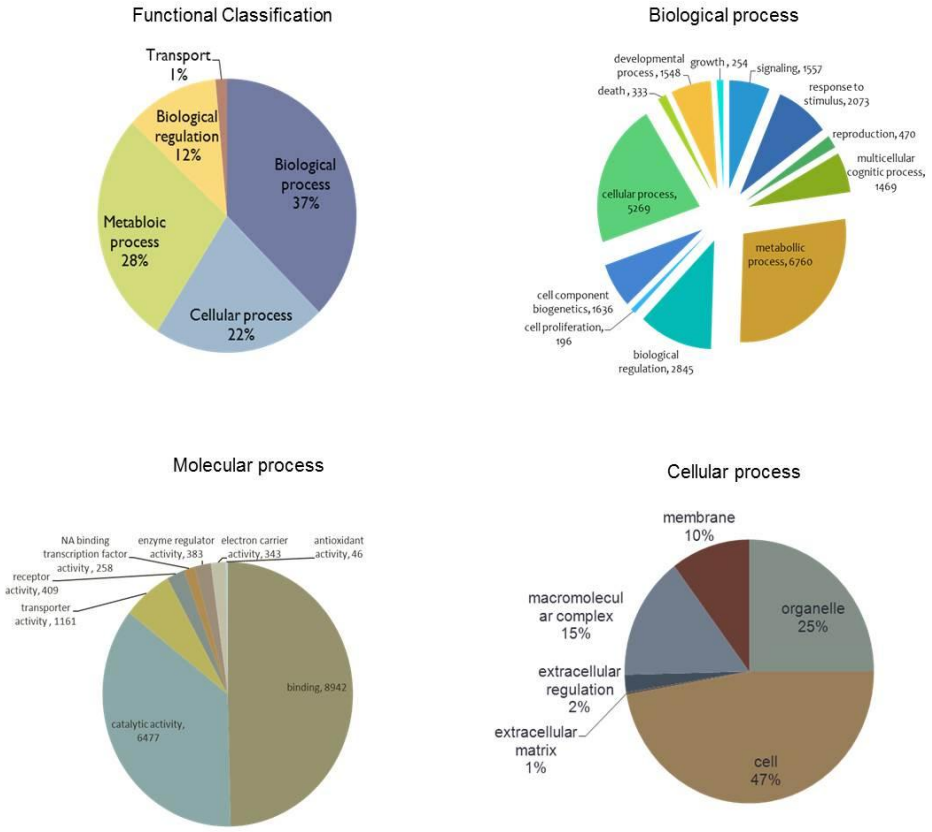

Figure 8. Functional classification of the proteins that were identified from the transcriptome of adult GWSS insects. The classification of the GWSS proteins was further classified with their functional processes which were subdivided into Biological, Molecular and Cellular process.

The sequencing reads from the transcriptomic data were assembled into scaffolds with a minimum size of 200 bases using Oases transcriptome assembler. We were able to assemble approximately 32.9Mb of the transcriptome across 47,265 loci and 52,708 transcripts. The average transcript length assembled was 624 nucleotides. Roughly 15 million of the total reads were found to be unique for the genome and 51% of the reads were incorporated into the assembly. Homologous protein domains from translated *H. vitripennis* transcriptomic sequences were identified by searching against the Pfam database using HMMER. Blast2GO was used to assign putative functionalities, GO terms, and KEGG (Kyoto Encyclopedia of

Genes and Genomes) based metabolic pathways. Final GO assignments were defined based on a 10% filter for all three processes profiled at level 2. GWSS proteins were classified based on the function as biological, molecular and cellular as shown in Figure 8. The sequencing reads were then mapped back to the assembled transcripts with up to one mismatch. The reads that could not be mapped back to the reference assembly were analyzed for the virus discovery that resulted in the identification of *Homalodisca Coagulata virus* (HoCV-1) and *Homalodisca reovirus* (HoVRV) that infect the GWSS insects. With the help of these sequencing reads, we aim to study the GWSS insect target genes and we hope to identify the small RNAs that target the GWSS target genes in a highly specific manner.

Publications or reports resulting from the project:

1. Rosa, C., Kamita, S. G., Dequine, H., Wuriyanghan, H., Lindbo, J. A., and Falk, B. W. 2010. RNAi effects on actin mRNAs in *Homalodisca vitripennis* cells. J. RNAi Gene Silencing 6:361 – 366.

2. Falk, B. W. and Rosa, C. RNA-Interference and control of the glassy-winged sharpshooter and other leafhopper vectors of *Xylella fastidiosa*. 2010. Pp 39 – 43, Symposium Proceedings, Pierce's Disease Research Symposium. Dec. 15 – 17, 2010. Manchester Grand Hyatt, San Diego, CA.

3. Rosa C, Kamita, S. G., and Falk, B. W. 2012. RNA-interference is induced in the glassy-winged sharpshooter Homalodisca vitripennis by actin dsRNA. *Pest management science* Jul; 68 (7):995-1002.

4. Raja Sekhar Nandety, Viacheslav Y. Fofanov, Heather Koshinsky, Drake C. Stenger and Bryce W. 2012. Small RNA populations for two unrelated viruses exhibit different biases in strand polarity and proximity to terminal sequences in the insect host *Homalodisca vitripennis*. (Manuscript submitted).

Presentations on research:

1. Falk, B W. RNAi approaches for helping to control insect vectors of plant pathogens. Invited Lecture. XVIII Conference of the IOCV, Campinas/SP/Brazil, November 7 – 12, 2010.

2. Rosa, C., Kamita, S. G., Dequine, H., Ethier, K., and Falk, B. W. RNA interference (RNAi) in *Homalodisca vitripennis*. Abstract 1147. 58th Annual Meeting of the Entomological Society of America. Dec 12 – 15, 2010. Town and Country Convention Center, San Diego, CA.

3. Falk, B. W. RNA interference (RNAi) efforts against insect vectors of plant pathogens. Abstract 0779. 58th Annual Meeting of the Entomological Society of America. Dec 12 – 15, 2010. Town and Country Convention Center, San Diego, CA

4. Falk, B. W. RNAi strategies for insect vectors of plant pathogens. Keynote lecture 1. 2nd International Research conference on Huanglongbing. Jan 10 – 14, 2011. Caribe Royale Hotel and Convention Center, Orlando, Florida.

5. Falk, B.W. Keynote address. RNA interference (RNAi) strategies for insect vectors of plant pathogens. 3rd Biennial ISU aphid research symposium. Seed Science Center, Iowa State University, Feb 15, 2011.

6. Falk, B.W. RNA interference (RNAi) strategies for management of insect vectors of plant pathogens. PD symposium, Sacramento, CA, December 15, 2011.

7. Raja Sekhar Nandety, Tera L. Pitman, Mandy Lin, Sophie Kiss, Kim Song and Falk, B.W. Next Generation sequencing and RNAi approaches for the control of Glassy winged Sharpshooters, PD symposium, Sacramento, CA, December 15, 2011.

8. Raja Sekhar Nandety, Tera L. Pitman and Falk, B.W. Genome wide RNAi approaches and mapping to two specific viruses that inhabit the host *Homalodisca vitripennis*, Bay Area Symposium on virus research, UC Berkley, CA, September 15, 2012.

9. Raja Sekhar Nandety. Next Generation Sequencing : Integrative host-microbe genomics on two different viruses associated with the glassy-winged sharpshooter. Invited Lecture. Granlibakken Symposium on Host Microbe Interactions, Granlibakken, CA, October 19, 2012.

10. Raja Sekhar Nandety, Tera L. Pitman, Almas S and Falk, B.W. Next Generation sequencing and RNAi approaches for the control of Glassy winged Sharpshooters, Plant and Animal Genome Conference XXI, San Diego, CA, January 11-17th, 2013.

Research relevance statement:

RNAi is a natural biological activity for controlling gene expression and anti-viral defense in a majority of eukaryotic organisms, including insects. The application of RNAi directed toward the control of different types of insect plant pests is becoming more feasible and promising. In our efforts, we were able to induce RNAi in *H. vitripennis* cells lines and whole insects, and are evaluating using 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 persons summary of current year's 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 efforts this year on generating transgenic potato plants to evaluate their potential temporally and spatially for inducing RNAi effects in *H. vitripennis*, and for developing and identifying optimal RNAi inducer delivery systems. Potatoes are easier and faster to transform and regenerate than are grapes, and the glassy-winged sharpshooter feeds readily on these plants. We were also able to show the expression of the reporter genes in specific plant tissues (xylem, water conducting tissue) compared to our previous expression in the entire plant tissue. Apart from the above mentioned accomplishments, we have generated large scale genomic data that was further analyzed for the identification of GWSS targets which will help us gear towards the control.

Status of funds:

We were awarded one year funding to support one postdoctoral scientist (Raj Nandety is the lead postdoc on this project), 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 are on track, spending wise, to use the funds as proposed in our original proposal budget, and anticipate that the funds requested for next year will allow us to complete this project.

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.

Literature cited.

1. Rosa C*, et al*. (2010) RNAi effects on actin mRNAs in Homalodisca vitripennis cells. *J RNAi Gene Silencing* 6(1):361-366.