Final Progress Report for CDFA Agreement Number 11-0146-SA

Title: Development and use of recombinant *Homalodisca coagulata Virus-1* for controlling *Homalodisca vitripennis*, the Glassy-winged sharpshooter.

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Time Period of Report: July1, 2011 to June 30, 2014.

Introduction: We received two, one year grants, plus a one year no-cost extension for this effort. We focused our collaborative efforts on attempting to develop *Homalodisca vitripennis*-infecting viruses for use as agents to help manage *H. vitripennis*, and then indirectly help manage Pierce's disease of grapevines. Viruses are the most abundant microbes on earth, with estimates as high as 10^{31} [1], and although viruses are often identified as pathogens, their roles in nature are not always associated with disease. Viruses often exist without causing disease in specific hosts, and in many instances viruses have proven to be useful for a variety of beneficial applications including use as biological control agents for insect pests [2]. If we could identify viruses that caused disease, or those that did not, both would be useful for our goals. Initially in year one we attempted to use the naturally-occurring *Homalodisca coagulata virus – 1* (HoCV-1), and *Flock House virus* (FHV), a model system virus for our work. We envisioned that both could assist our efforts and allow for more rapid progress. We used *H. vitripennis* cells (GWSS Z-15) and whole insects for our virus transmission assays. In year two we focused our efforts only on HoCV-1. This report presents our data from the past two years.

Objectives:

Our long term objectives were to develop and utilize the naturally occurring virus, HoCV-1, and engineer it to be useful for GWSS control either by modifying HoCV-1 to express toxic peptides or to induce systemic RNA interference (RNAi) in recipient, recombinant HoCV-1-infected GWSS. Our specific objectives are:

Objective 1. Development of HoCV-1 infectious cloned cDNAs;

Objective 2. Expression of GFP or other stable sequences in GWSS-Z15 cells or whole H. coagulata insects by using HoCV-1.

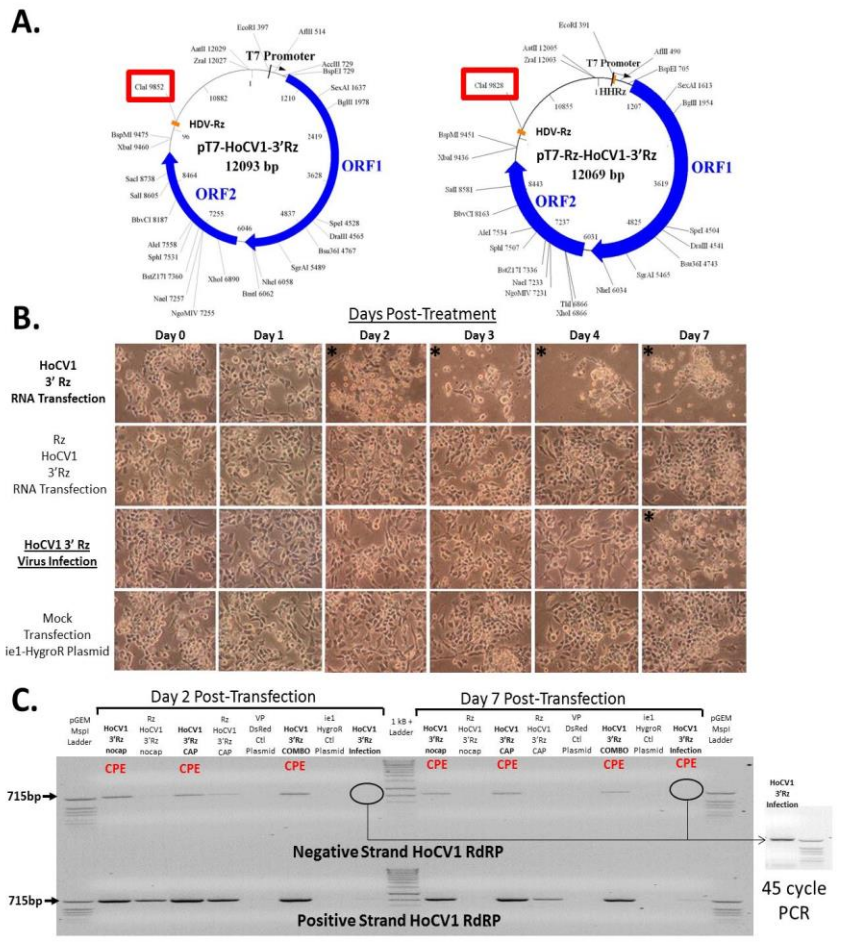

Figure 1. A. Vector diagram of pT7-HoCV1-3'Rz and pT7-Rz-HoCV1-3'Rz. The T7 promoter is indicated by the black bar and arrow. Hammerhead (HHRz) and Hepatitis Delta Virus (HDFV-Rz) ribozymes are indicated as orange boxes. pT7-HoCV1-3'Rz lacks the HHRz. HoCV-1 open reading frames (ORF) 1 and 2 are indicated as blue arrows. The *ClaI* restriction site (red box) is used to linearize plasmid for *in vitro* transcription. **B.** Cytopathic effects (black asterisks) were induced in GWSS Z-15 cells after transfection using HoCV-1 RNA transcripts and with HoCV-1 virus generated from the pT7-HoCV1-3'Rz plasmid. **C.** Negative and positive RNA strands for the HoCV-1 RNA-dependent RNA-polymerase (RdRP) were detected by 30 cycle RT-PCR for HoCV-1 RNA transfected from both plasmids, although only transfections with pT7-HoCV1-3'Rz RNA caused strong cytopathic effects (indicated by red CPE) in GWSS-Z15 cells. Weak signals for positive (30 cycles) and negative (45 cycles-indicated) strand RdRP RNA were also detected in virus overlayed from the HoCV-3'Rz transfection onto new GWSS-Z15 cells.

Results:

Objective 1. HoCV-1 does not cause obvious disease in GWSS, and although it is most commonly found in GWSS its natural host range includes other sharpshooters [3]. Thus, our idea was to engineer this virus to be an effective and specific pathogen. We had previous success with a virus similar to HoCV-1 (the aphid-infecting *Dicistrovirus*, *Rhopalosiphum padi virus* (RhPV) [4], where we developed an infectious clone of RhPV. Here we initially took the same approach with HoCV-1.

We successfully cloned full length HoCV-1 cDNAs (Fig. 1A). We generated a series of different constructs to help increase the probability of success. *In vitro* transcription was performed using these constructs to generate HoCV-1 transcripts which were delivered to GWSS Z-15 cells (Fig. 1B and C). After transfection with HoCV-1 transcripts with extended or un-extended 5'-ends, Z-15 cells showed severe cytopathic effects (CPE; Fig. 1B). Control cells did not, thus these results suggested that we most likely had generated infectious HoCV-1 cloned cDNAs. In order to support the cytopathology data, we also performed reverse-transcription polymerase chain reaction (RT-PCR) assays to identify specific RNAs resulting from HoCV-1 replication. Both the HoCV-1 genomic-sense strand (positive-strand) and its complementary strand (negative-strand) RNAs were amplified by RT-PCR analysis following the transfection indicating that the virus was replicating (Fig. 1C). pT7-Rz-HoCV1-3'Rz generated transcripts were less efficient

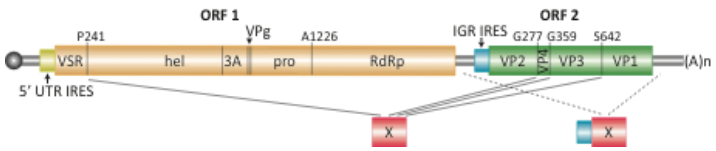

Figure 2. Potential insertions sites for expression of foreign proteins and RNAs from the RhPV and HoCV-1 genomes. Genome organization shows ORF 1 (orange) and ORF 2 (green) which encode proteins separated by cleavage sites indicated at estimated positions by vertical lines. Precise cleavage sites, estimated by alignment are labeled by the first amino acid downstream of the cleavage (e.g. P241 = proline at amino acid 241 in ORF 1). A1226 and S642 cleavages are from Nakashima and Nakamura (2008). We predict Q219 using alignment in Nayak et al. (2010), and G277, G359 using VP structures in Tate et al. (1999). Sequence encoding the protein of interest (e.g. toxin or fluorescent protein) indicated by red box (protein X) may be inserted at as a separate ORF, preceded by the IGR IRES (blue) at noncoding regions (gray bars) at sites indicated by dashed lines. Host RNA sequences can also be inserted at these sites for the RNAi strategy (objective 3). Alternatively, protein X coding region can be inserted within ORFs, at cleavage sites indicated by solid lines. Predicted functions of polyprotein cleavage products are viral suppressor of RNAi (VSR), helicase (hel), picornavirus-like protease 3A (3A), genome-linked protein (VPg), 3C-like protease (pro), RNA-dependent RNA polymerase (RdRp), virion proteins (VP1-4). Non-coding features include the VPg protein (sphere), 5' untranslated region IRES (5' UTR IRES), intergenic region IRES (IGR IRES), and poly(A) tail [(A)n)].

HoCV-1 and GWSS possibly due to enhanced RNA degradation following ribozyme cleavage at the 5' end. Thus, both the cell cytopathology and the RT-PCR analyses suggested that our cloned HoCV-1 cDNAs were infectious to GWSS Z-15 cells and offered an opportunity for us to move forward.

We next attempted to engineer the HoCV-1 infectious clones to express YFP (yellow fluorescent protein) and mCherry (modified red fluorescent protein) reporters as part of the transcribed viral sequence (refer to Fig. 2). This would allow for simple, efficient testing of our constructs in both Z-15 cells and whole GWSS insects. We used sites that were predicted to tolerate insertion of foreign sequences. If this was successful, these sites could also be used for future efforts to insert foreign sequences coding for toxic peptides or interfering RNAs. Unfortunately, we failed in this approach.

Objective 2. In year one, in addition to utilizing HoCV-1, we explored the possibility of using a second virus, *Flock house virus* (FHV). FHV belongs to the family *Nodaviridae*, and is a non-enveloped, positive-sense RNA virus that has a bipartite genome. This virus been shown to multiply in insects from four different orders (Hemiptera, Coleoptera, Lepidoptera, Diptera) and even plants. We felt that if FHV infected GWSS or GWSS Z-15 cells, we could use it to more rapidly evaluate candidate peptides and/or RNA sequences. We obtained infectious, recombinant constructs producing FHV genomic RNAs 1 and 2 (pMT FHV RNA1 and pMT FHV RNA2; gift from Dr. Shou-wei Ding, UC Riverside). The plasmid backbone (pMT) of the constructs contains a copper-inducible *Drosophila* metallothionein promoter that drives an efficient transcription of FHV genomic RNA. We showed that FHV could infect *Drosophila* (S2) and Z-15 cells (Fig. 3) in our initial experiments. We also engineered FHV to express GFP as a marker to use in our RNAi studies. This recombinant was useful in S2 cells (Fig. 4), but not in Z-15 cells, thus FHV proved to not be useful for our longer term strategies and thus we terminated efforts with FHV and focused exclusively onHoCV-1.

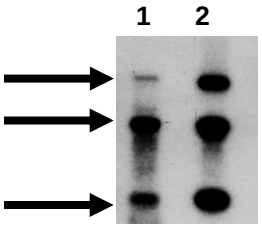

Figure 3. Lanes contain 1; FHV-inoculated S2 cells, 2; FHV-inoculated GWSS Z-15 cells. Arrows at left indicate FHV RNAs 1, 2 and 3, from top to bottom, respectively. RNA 3 is a subgenomic RNA from

Transfection assays in whole insects

In year two we focused efforts on HoCV-1 and attempted to efficiently infect GWSS Z-15 cells and whole insects using both wild-type virus (from naturally-infected GWSS) and our HoCV-1 clones. We established HoCV-1 GWSS colonies at the UC Davis CRF. We attempted to infect healthy GWSS with the GWSS-Z15 transfected cell extracts both by injection and oral acquisition. RNA from five infectious clones of

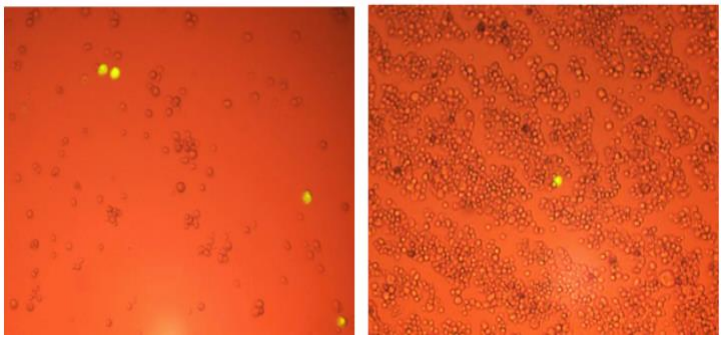

Figure 4. FHV infected S2 cells examined by fluorescence microscopy. S2 cells on the left panel were transfected with the plasmids that express FHV RNA1, FHV RNA2 and FHV DI RNA that contains GFP sequence. The transfected cells were collected, frozen and thawed 3 times, and filtered through a filter with 0.22um pores. The S2 cells on the right panel were inoculated with the cell lysate. The arrows indicate the cells expressing GFP from FHV

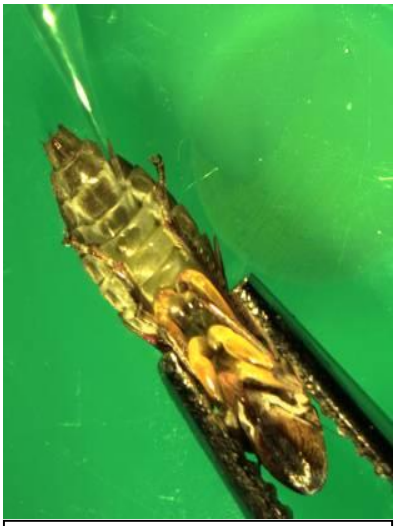

Figure 5. Injection of adult GWSS.

HoCV1 and two controls were used to transfect GWSS-Z15 cells: HoCV-3'Rz, HoCV-3'Rz old, Rz-HoCV-3'Rz, Rz-HoCV-3'Rz old, mutant Rz-HoCV-3'Rz, elongation factor RNA, GWSS and transfection buffer. An additional negative control for the infection experiments was untreated GWSS-Z15 cell suspension. For the injections, 1µL of needle homogenized cell suspension in injection buffer (10 mM Tris-HCl, pH 7.0,1 mM EDTA) was injected into adult GWSS between tergites 3 and 4 of the ventral aspect using a 33 gauge needle (Fig. 5). Three insects were injected per HoCV-3'Rz construct. After injection the insects were put in cages with a basil plant for one week, and then RNA collected as a treatment group. For feeding assays we used basil cuttings approximately 5cm in length and submerged the cut end in a suspension of cell pellet and supernatant of approximately 1.5mL volume. Three insects per treatment were given an acquisition period of three days on the basil cuttings, then moved to basil plants for four days, after which RNA was extracted from each group. One-tube RT-PCR was used to detect infection with primer pairs specific to inter-genomic region 1 and coat protein of HoCV-1. The positive control was GWSS RNA from a naturally infected insect. All treatment groups tested negative for HoCV-3'Rz (Fig. 6). Unfortunately, these data showed no evidence for replication of our HoCV-1 in adult GWSS.

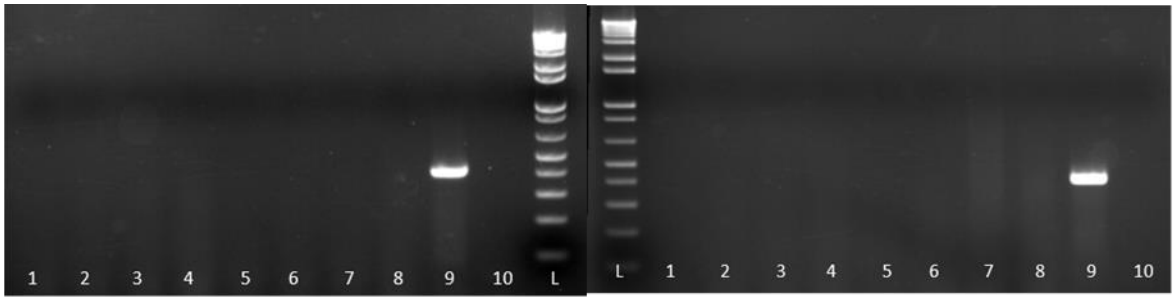

Figure 6. One-tube RT-PCR amplifying a segment of HoCV1 IGR (left) or coat protein (right) from insects that were fed transfected Z15 cell suspensions. Results from injection were identical. Treatments were Z15 cell transfected with constructs of HoCV1 infectious clones. **1.** HoCV-3'Rz **2.** HoCV-3'Rz old **3.** Rz-HoCV-3'Rz **4.** Rz-HoCV-3'Rz old **5.** Mutant Rz-HoCV-3'Rz **6.** Elongation factor RNA **7.** Transfection buffer Z15 cells **8.** No treatment Z15 cells **9.** Naturally infected HoCV1 GWSS **10.** No template control.

Our failure to transmit HoCV-1 from initially infected Z-15 cells suggested that something was wrong with our virus construct. We used transmission electron microscopy to assess HoCV-1 transfected Z-15 cells and failed to find virus particles in cells, even in those cells that were RT-PCR positive. Thus, although our data suggested HoCV-1 replication in Z-15 cells, HoCV-1 virus particles were not formed. This could explain our inability to transfer HoCV-1 from Z-15 cells to whole insects.

We attempted to engineer HoCV-1 cDNAs to contain and express GWSS cDNA sequences that could be used at least for RNA silencing studies in GWSS Z-15 cells. Hairpin RNAi cassettes against *GFP* (control*), H. vitripennis actin*, and *H. vitripennis chitin deacetylase* have been completed in the pGEM-13Zf+ vehicle (Fig. 7). Sequences were verified by linearizing with an enzyme in the hairpin region (Xho I, Sac I, Sac II, Nru I, or Nar I) and sequencing linear templates. Hairpin RNAi cassettes can be transferred directly from the pGEM-13Zf+ vehicle to the HoCV1-3' Rz infective clone utilizing the enzyme XbaI to complete infective HoCV1-3'Rz clones carrying the RNAi cassettes. Due to time and funding constraints, we failed to complete this part of the project.

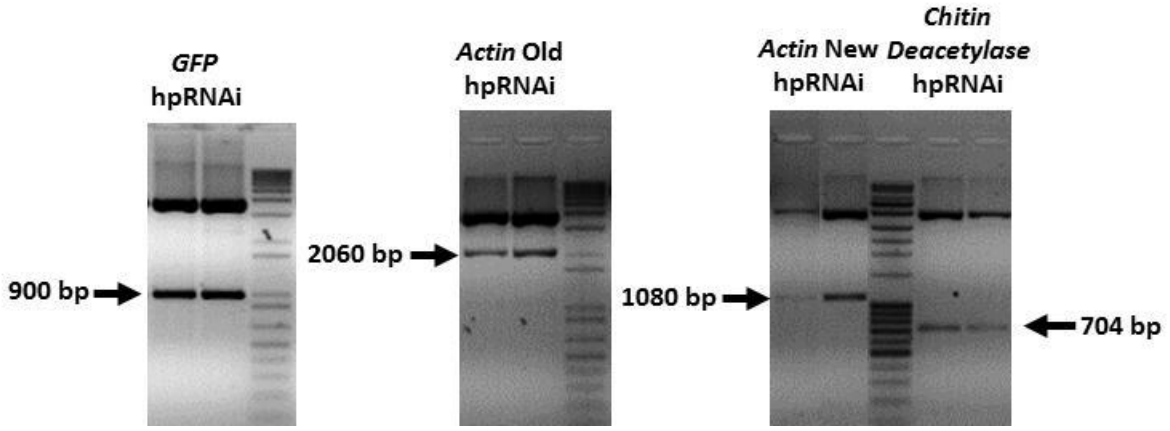

Figure 7. Positive pGEM-13 Zf+ clones obtained carrying hpRNAi cassettes against *GFP*, *H. vitripennis actin*, and *H. vitripennis chitin deacetylase*. Approximate sizes of hpRNAi when released from the vector are: *GFP*-900 bp; *Actin Old*-2060 bp; *Actin New*-1080 bp; *Chitin Deacetylase*-704 bp.

In the current reporting period we have been working on optimizing transfection assays in whole insects and the GWSS cell line Z15. We received cell pellets and cell culture supernatant from Iowa State to use in insect experiments. We attempted to inoculate healthy GWSS with the GWSS-Z15 transfected cells both by injection and oral acquisition. RNA from five infectious clones of HoCV1 and two controls were used to transfect GWSS-Z15 cells: HoCV-3'Rz, HoCV-3'Rz old, Rz-HoCV-3'Rz, Rz-HoCV-3'Rz old, mutant Rz-HoCV-3'Rz, elongation factor RNA, and transfection buffer. An additional negative control for the GWSS infection experiments was untreated GWSS-Z15 cell suspension. For the injections, 1µL of needle homogenized cell suspension in injection buffer (10 mM Tris-HCl, pH 7.0,1 mM EDTA) was injected into adult GWSS between tergites 3 and 4 of the ventral aspect using a 33 gauge needle. Three insects were injected per HoCV-3'Rz construct. After injection the insects were put in cages with a basil plant for one week, and then RNA collected as a treatment group. For feeding assays we used basil cuttings approximately 5cm in length and submerged the cut end in a suspension of cell pellet and supernatant of approximately 1.5mL volume. Three insects per treatment were given an acquisition period of three days on the basil cuttings, then moved to basil plants for four days, after which RNA was extracted from each group. One-tube RT-PCR was used to detect infection with primer pairs specific to inter-genomic

region 1 and coat protein of HoCV-1. The positive control was GWSS RNA from a naturally infected insect. All treatment groups tested negative for HoCV-3'Rz.

We worked to optimize cell line transfection to generate infectious virus, however we did not have positive results. We used the initial protocol and plasmids developed at Iowa State University, in which single stranded RNA is generated from plasmid template for transfection experiments. We used *in vitro* transcription kits that generated both capped and uncapped messenger RNA (Fig. 8). This was followed by cell transfection and analysis at 3 days post transfection. Using two-step RT-PCR we were unable to find both positive and negative sense HoCV1 RNA from these experiments, indicating that virus replication was not occurring. We tried using different transfection reagents, but were unable to generate positive results. To see if the problem was RNA degradation during transfection, we then tried to generate a plasmid with an insect derived promoter in front of the HoCV1 sequence, which would then be expressed in the Z15cells.

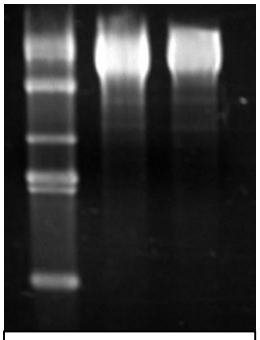

Figure 8. RNA gel of capped (left) and uncapped (right) HoCV1 from in vitro transcription that was used for transfection of Z15 cells.

We used a Baculovirus promoter, *ie1*, and the promoter to heat shock 70 gene in *Drosophila melanogaster*, *hsp70*, to use plasmid template for the transfection experiments instead of single stranded RNA. DNA is a more robust template to work with, and we already have a vector system that works with SF9 cells using this promoter. Previous research has shown the *ie1* system to work in *Bombyx moorei* and *Drosophila melanogaster*, so we designed experiments to see if they would be effective for the Z15 cell line (1). Initially, we used the plasmid pAcp+IE1lacZ, a plasmid with the promoter *ie1* and *lac-z* gene to determine if it can drive production of messenger RNA and protein in Z15 cells. We also used the plasmid pAcDZ1, which contains the heat shock 70 promoter, *hsp70*, with the *lacz* gene. We were able to transfect the Z15 cell line to express the *lac-z* gene with both constructs. Unfortunately, we found we would not be able to use *lac-z* as a reporter gene in whole insects because reporter substrates, such as x-gal, were hydrolyzed by naturally occurring enzymes in whole insects, causing no differentiation between treatments and controls. We then redesigned the constructs to have the *ie1* or *hsp70* promoters drive GFP synthesis, creating plasmids pAcP+IE1GFP and pAcDGFP. At the same time we used restriction digestion to take both promoters and insert them upstream of the HoCV1 sequence flanked by ribozymes, creating the plasmids pIE1-RzHoCV1Rz and pHSP70-RzHoCV1Rz. Unfortunately, this was as far as we got before June.

Publications produced and pending

Falk, B. W., Bonning, B., Miller, W. A., Stenger, D., Choi, S. H., and Kamita, S. G. Development and use of recombinant Homalodisca coagulata virus-1 for controlling the glassy-winged sharpshooter. 2011. pp 32-36, Symposium Proceedings, Pierce's Disease Research Symposium. Dec 13-15, 2011. Sheraton Grand Sacramento Hotel, Sacramento, CA.

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Nandety, R. S., Sharif, A., Pitman, T. L., and Falk, B. W. 2013. Glassy winged sharpshooter insect genome footprints through transcriptomic and small RNA sequence profiling. Abstract P0671. Plant and Animal Genome XXI. San Diego, CA.

Kamita, S. G., Oshita, G H., Wang, P., Morisseau, C., Hammock, B. D., Nandety, R. S., and Falk, B. W. 2013. Characterization of Hovi-MEH1, a microsomal epoxide hydrolase from the glassy-winged sharpshooter, *Homalodisca vitripennis*. Archives of Insect Biochemistry and Physiology. 83:171-9. doi: 10.1002/arch.21100.

Nandety, R. S., Fofanov, V. Y., Koshinsky, H., Stenger, D. C., and Falk, B. 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, Ahead of print, online May 1. **FEATURED IN *VIROLOGY HIGHLIGHTS* (http://www.elsevierblogs.com/virology/?p=16).**

Nandety, R. S., Kamita, S. G., Hammock, B. D., and Falk, B. W. Sequencing and *de novo* assembly of the transcriptome of the glassy-winged sharpshooter (*Homalodisca vitripennis*). PLoS One, under revision.

Research relevance statement

The glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*) transmits the bacterium, *Xylella fastidiosa*, which causes Pierce's disease of grapevines. We are attempting to use natural, GWSS-infecting viruses as part of a strategy to control GWSS population. We are attempting to engineer viruses to deliver toxic peptides and/or deliver GWSS RNAs that will activate the RNA interference (RNAi)-based immune system. We hope that one or both approaches will result in GWSS mortality thereby preventing the spread of *Xylella fastidiosa*. We are using recombinant HoCV-1, a naturally occurring virus that specifically infects GWSS, and FHV, an insect virus that has a wide-host range and also easy to manipulate in the lab. If successful, our studies may lead to new and effective methods to help control the GWSS population.

Lay summary of current year's results

During the project, we engineered an infectious clone of HoCV-1 and verified its biological activity in the GWSS Z-15 cell-line. We also developed another virus-system using FHV to be used in conjunction with the HoCV-1 system to expedite our progress towards developing virus-based delivery systems for GWSS. We attempted to modify our virus constructs for delivering RNAs that can express toxic peptides or induce RNAi in GWSS insects and Z-15 cell-line. Unfortunately we were unable to be successful in this effort. The knowledge gained from this study is still useful, and in the longer term might be used further to develop a virus system to target GWSS.

Status of funds

We were awarded one funding in two separate one year increments to support research efforts at UC Davis, Iowa State University and the USDA Parlier Research Center, and we requested and received a no-cost extension for some remaining funds to be spent at UC Davis. The effort was collaborative and included personnel at all three locations and funds for supplies, travel, etc., plus limited travel.

Summary and status of intellectual property produced during this research project

We filed an invention disclosure on 7/20/2012, but this has not proceeded further.

References

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2. McNeil J: **Viruses as biological control agents of insect pests**. *eOrganic; http://wwwextensionorg/article/18927* 2010.

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Funding Agencies

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