Interim Progress Report for CDFA Agreement Number 11-0146-SA**.**

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

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Time period covered by the report: March 1, 2012 to June 30, 2012.

Introduction

The glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*) transmits the bacterium, *Xylella fastidiosa*, which causes Pierce's disease of grapevines. We are attempting to use GWSS-infecting viruses as part of a strategy to control GWSS population. We are attempting to engineer these viruses to deliver toxic peptides to kill GWSS, 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 two viruses for our work. These are: *Homalodisca coagulata virus-1* (HoCV-1), a naturally occurring virus that specifically infects GWSS, and *Flock house virus* (FHV), an insect virus that has a very wide insect host range and also is easy to manipulate in the lab. If

successful, our studies may lead to new and effective methods to help control the GWSS population.

List of objectives

1. Development of *Homalodisca coagulata virus-1* (HoCV-1) infectious cloned cDNAs;
2. Expression of GFP or other stable sequences in GWSS-Z15 cells or whole *H. coagulata* insects by using HoCV-1.

Description of activities conducted to accomplish each objective, and summary of accomplishments and results for each objective

Objective 1. Development of *Homalodisca coagulata virus-1* (HoCV-1) infectious cloned cDNAs

We have successfully cloned full length HoCV-1 cDNA. *In vitro* transcription was performed using the constructs HoCV1-3'RZ (produces HoCV-1 RNA) and mutRZ-HoCV1-3'RZ (produces HoCV-1 RNA that can not produce functional proteins) and delivered to GWSS-Z15 cells. After transfection with HoCV-1 transcripts produced with HoCV1-3'RZ, GWSS-Z15 cells showed severe cytopathic effects that were not observed in the GWSS-Z15 control or in cells transfected with mutRZ-HoCV1-3'RZ (Fig. 1). Both the genomic-sense strand (positive-strand) and the complementary strand (negative-strand) of HoCV-1 RNAs were amplified by RT-PCR

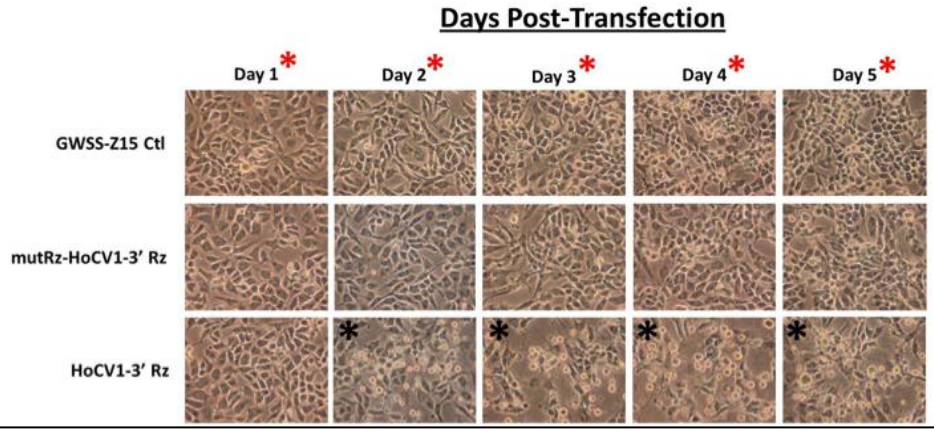

Figure 1: Time course of infection in GWSS-Z15 cells following transfection of *in vitro* transcribed HoCV1-3'Rz, or mutRz-HoCV1-3'Rz RNA. Cells (5 wells/construct) were transfected with 8 µg of HoCV1-3'Rz RNA, or 8 µg of mutRzHoCV1-3'Rz *in vitro* transcribed HoCV-1 viral RNA. Images were taken at day 1, day 2, day 3, day 4, and day 5 post-transfection. CPE were observed in GWSS cells transfected with HoCV1-3'Rz RNA on days 2, 3, 4, and 5 (black asterisks) that were not observed in the other wells. Samples of cells (1 mL) were removed from the wells on each day for quantitative real-time PCR analyses of negative and positive sense viral RNA (red asterisks). Images were taken at 40X objective magnification.

analysis following the transfection indicating that the virus was replicating (data not shown).

Relative quantitative real time PCR reactions were performed to detect infectivity and relative quantities of negative- and positive-sense HoCV-1 viral RNA (Fig. 2). A significant increase in the amount of HoCV-1 negative and positive sense RNA was detected between days

1 and 2 post-transfection in GWSS-Z15 cells transfected with HoCV1-3'Rz with a gradual decrease documented thereafter through day 5 post transfection. Positive-sense RNA appeared at levels up to 10X higher than negative sense viral transcript in duplicate qRT-PCR studies targeting the HoCV-1 IGR region. Taken together, these data strongly suggest that we have developed an full length HoCV-1 genomic RNA cDNAs, and that the transcripts generated *in vitro* are infectious at least to GWSS cells. More work must be done to optimize these cloned cDNAs and to evaluate them in whole GWSS insects.

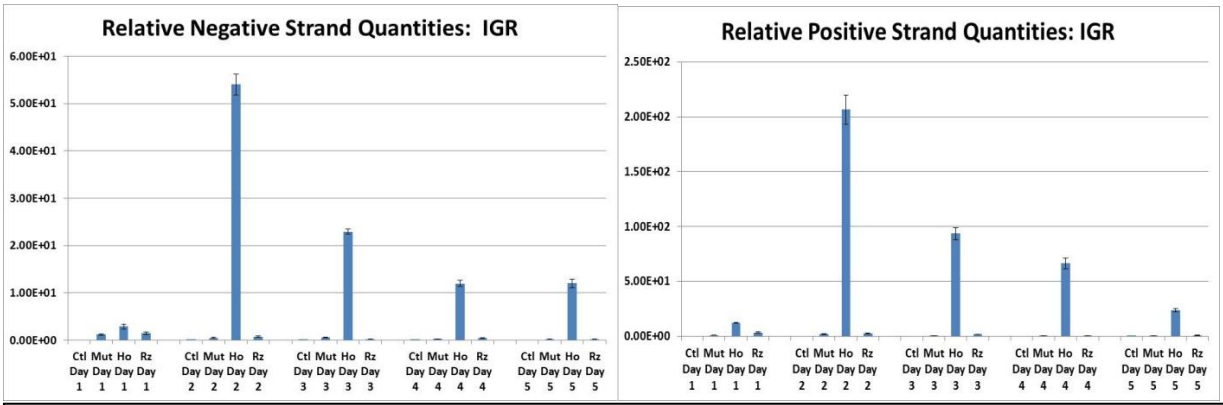

Figure 2: Relative quantitative real-time PCR following the time course of infection (Rep-3) in GWSS-Z15 cells after transfection of *in vitro* transcribed HoCV1-3'Rz, Rz-HoCV1-3'Rz (HoCV1 RNA with no 5'-cap) or mutRz-HoCV1-3'Rz RNA. An active infection cycle was observed following transfection of HoCV1-3'Rz RNA with negative and positive strand viral RNAs increasing significantly by day 2 post-transfection followed by gradual decreases in quantities thereafter. Relative quantities of positive sense viral RNA were approximately 10X higher than negative sense viral RNA when at peak levels (day 2 post-transfection) in GWSS-Z15 cells. Active RNA replication was not apparent following transfection of Rz-HoCV1-3'Rz or mutRz-HoCV1-3'Rz RNA. *H. vitripennis actin* was used as a reference for standards and RNA starting quantity normalization. Error bars represent +/- one standard deviation from the mean relative starting quantity.

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

We are in the process of engineering infectious clones to express YFP and mCherry reporters as part of the transcribed viral sequence (refer to Fig. 3). We next will convert HoCV-1 into a highly specific, lethal virus by engineering it to express a peptide that is toxic only when expressed intracellularly within target sharpshooters. This includes primarily GWSS, but as HoCV-1 has a host range that is reported to include other sharpshooters (Hunnicutt, 2008), our success here has broader applicability for *X. fastidiosa* vector control. The toxic peptides to be employed are lethal only to insects, and only when expressed intracellularly. Thus, only sharpshooters infected by the modified virus will be negatively affected by the peptides.

In addition, we have been working with *Flock house virus* (FHV) in our studies of using viruses to express toxic peptides and/or induce RNAi in GWSS. 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). We have 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. Northern blot RNA hybridization analysis suggested that FHV infects GWSS cells (Data not shown; Fig 3 of our previous report), however this has proven to be inconsistent.

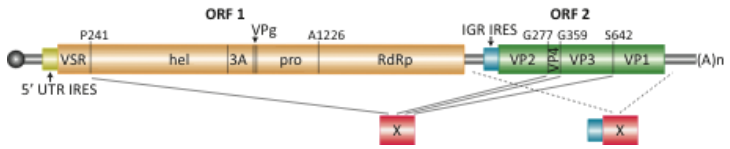

Figure 3. Potential insertions sites for expression of foreign proteins and RNAs from the RhPV and HoCV-1 genomes. Genome organization shows ORF 1 (orange) and ORF 2 (green) which encode proteins separated by cleavage sites indicated at estimated positions by vertical lines. Precise cleavage sites, estimated 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)].

In adult mosquitoes, FHV has been engineered to systemically express GFP (Dasgupta et al., 2003). A defective interfering (DI) RNA of FHV originating from the genomic RNA 2 retains the RNA replication sites and packaging signal. We have subcloned the DI RNA into pMT plasmid vector, and verified its infectivity in S2 cells (data not shown).
We then subcloned the GFP sequence into the FHV DI RNA in the pMT construct in a way that it will not disrupt the replication sites and the packaging signal as described (Dasgupta et al., 2003). We transfected *Drosophila* Schneider 2 (S2) cells with the pMT vectors expressing FHV RNA1, RNA2, and DIeGFP to produce FHV virions containing DI RNA that express GFP. The transfected S2 cells were observed under fluorescence microscope and some cells showed GFP expression (Fig. 4). We then collected the transfected cells and lysed them by freezing and thawing. The cell lysate was filtered and used for inoculating the healthy S2 and GWSS Z-15cells. Several days after the inoculation, the S2 and GWSS Z-15 cells were observed under a fluorescence microscope. Very few S2 cells were infected with the FHV expressing GFP. However, we did not detect the green fluorescence in Z-15 cells. It is probably because of the inefficiency of producing the DI-eGFP carrying-FHV due to the co-transfection using 3 different plasmids. Constructing one plasmid that expresses all 3 RNAs may solve this problem.

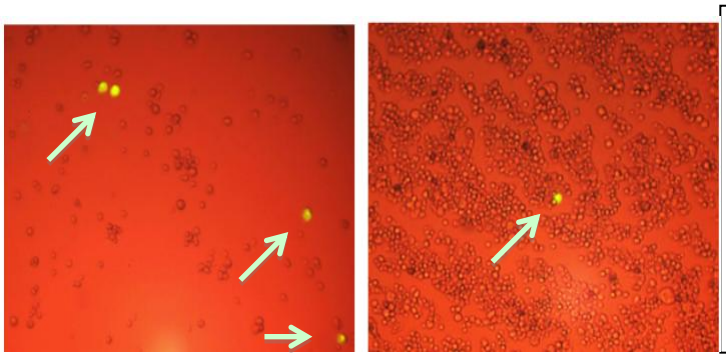

Figure 4. FHV infected S2 cells under fluorescence microscope. S2 cells on the left panel were transfected with the plasmids that express FHV RNA1, FHV RNA2 and FHV DI RNA that contains GFP sequence. The transfected cells were collected, frozen and thawed 3 times, and filtered through a filter with 0.22um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

Once a high efficiency in GFP-expressing FHV production is achieved, we will inoculate GWSS Z-15 cells with the FHV harboring DIeGFP RNA again. The expression will be verified by the confocal microscopy and Northern blot RNA hybridization analysis. Then the same region on the DI RNA that was used for inserting the eGFP sequence will be used for subcloning various DNA sequences. Since this FHV system is easier to work with than the HoCV-1 system, it can be used to perform initial experiments screening for suitable peptides for expression and RNAs for inducing RNAi in GWSS.

Publications produced and pending, and presentations made that relate to the funded project

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.* Dec 13, 2011 (online print available; DOI 10.1002/ps.3253).

Falk, B. W., Bonning, B., Miller, W. A., Stenger, D., Choi, S. H., and Kamita, S. G. Development and use of recombinant Homalodisca coagulate 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.

Falk, B.W., Choi, S. H., Pitman, T. L., Nandety, R. S., Kamita, S. G., Bonning, B., Miller, W. A., Kroemer, J., Stenger, D., and Spear, A. Hemipteran-infecting viruses as tools for vector management. December 13-15, 2011. Pierce's Disease symposium, Sacramento, CA.

Nandety, R. S., Pitman, T. L., Lin, M., Kiss, S., Song, K., and Falk, B.W. Next Generation sequencing and RNAi approaches for the control of Glassy winged Sharpshooters. December 13-15, 2011. Pierce's Disease symposium, Sacramento, CA.

Choi, S. H., Kamita, S. G., Pitman, T. L., and Falk, B. W. Development and use of recombinant *Homalodisca coagulata virus-1* for controlling *Homalodisca vitripennis,* the glassy-winged sharpshooter: Testing for FHV infection in Glassy-winged sharpshooter. December 13-15, 2011. Pierce's Disease symposium, Sacramento, CA.

Research relevance statement

We have made excellent progress during the past year. We have published one peer-reviewed article (Rosa et al., 2011) and one symposium report (Falk et al., 2011), in addition to giving several presentations of our work at various meetings during the past year (see above). In addition to verifying the infectivity of the lab-generated HoCV-1, we have in our lab another virus system using FHV for gene delivery and RNAi induction in GWSS. This FHV system will complement our HoCV-1 system to expedite our work in controlling GWSS population to prevent spread of *Xylella fastidiosa* and expand our knowledge in virus-mediated pest management.

Layperson summary of project accomplishments

During the past year, we successfully engineered infectious clones of HoCV-1 and verified the biological activity in 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 efforts to control GWSS population in California to prevent the spread of *Xylella fastidiosa*. We are currently modifying our virus constructs for delivering RNAs that can express toxic peptides or induce RNAi in GWSS insects and Z-15 cell-line. The knowledge gained from this study will be used further to develop a virus system to control the population of *Xylella fastidiosa* residing inside GWSS.

Status of funds

We were awarded one years funding ($112,427) to support research efforts at UC Davis, Iowa State University and the USDA Parlier Research Center. This included personnel at all three locations and funds for supplies, travel, etc., plus limited travel. We are on track, spending wise, to use the funds as proposed in our original proposal budget, and anticipate that the funds awarded to us will be spent by the end of this project year.

Summary and status of intellectual property produced during this research project

We will work with UC for managing any intellectual property or technologies that may arise from this effort.

Literature cited

Dasgupta, R., Cheng, L.L., Bartholomay, L.C., Christensen, B.M., 2003. Flock house virus replicates and expresses green fluorescent protein in mosquitoes. The Journal of general virology 84, 1789-1797.

Hunnicutt, L.E., Mozoruk, J., Hunter, W. B., Crosslin, J. M., Cave, R. D., and Powell, C. A., 2008. Prevalence and natural host range of Homalodisca coagulata virus-1 (HoCV-1). Arch Virol 153, 61-67.

Rosa, C., Kamita, S.G., Falk, B.W., 2011. RNA interference is induced in the glassy winged sharpshooter Homalodisca vitripennis by actin dsRNA. Pest management science.