Interim progress report for CDFA Agreement # 11-0146-SA

Project Title: Development and use of recombinant *Homalodisca coagulata* virus-1 for controlling GWSS

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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. **Development and evaluation of recombinant HoCV-1 for inducing systemic RNAi in GWSS;**
2. **Development of cell culture and baculovirus-based systems for recombinant virus production.**

Description of activities, accomplishments and results for each objective:

The following description is relative to objective 1, objective 2 is underway.

In vivo feeding assays Studies are underway to test the infectivity of HoCV-1 virus generated *in vitro* through feeding assays performed against a virus-free GWSS colony. GWSS Z-15 cells were transfected with RNA generated *in vitro* from pT7-mutRz-HoCV1-3'Rz, Rz-HoCV1-3'Rz, and HoCV1-3'Rz clones. Twenty micrograms of RNA for each clone was transfected into individual T_{25} flasks and cells were harvested on Day 3 post-transfection when predominant CPE were observed in the HoCV1-3'Rz flask (**Figure 1**). Supernatants were collected and harvested cells resuspended in 250 □L 1X PBS prior to freezing and sending to the University of California, Davis. GWSS feeding assays to detect HoCV1 in virus-free GWSS are being conducted at UC Davis.

TEM of virions An identical transfection experiment was performed to generate infected GWSS-Z15 cells for analysis by transmission electron microscopy (TEM) at Iowa State University. On day 3 post-transfection of RNA, GWSS cells in T_{25} flasks were fixed by flooding with a 1X PBS-2% paraformaldehyde-3% glutaraldehyde solution and submitted to the TEM facility at ISU for processing and analysis of HoCV-1 particle production in the cells. We are currently awaiting results and images from this study following repair of the ISU TEM that was down over the winter.

HoCV antibody production The HoCV-1 ORF 2 (capsid polyprotein) was expressed in *E. coli* to generate a fusion protein for polyclonal antibody production against the HoCV-1 capsid. The HoCV-1 ORF 2 was cloned as a fusion with maltose-binding protein (MBP) into pMal C2X (New England Biolabs, Ipswich, MA). Trial expression studies indicated co-purification of the full length fusion protein (~145 kDa) as well as several possible derivatives from autocleavage of the polyprotein at internal sites in purified samples (**Figures 2, 3**). As many products were suspected sizes of MBP-bound and unbound fragments generated from cleavage of the polyprotein at internal sites (**Figures 2, 3**), we utilized the purification product mixture for polyclonal antibody generation against all capsid species. Using this method, antibodies against different specific capsid components can be affinity purified from the polyclonal antiserum if necessary in the future. A large scale purification procedure was performed to generate sufficient protein for injection into rabbits to raise polyclonal antibodies. Injections were initiated on 2-11-13, and bleeds are expected for testing antibody specificity to the HoCV1 capsid proteins within 2 months.

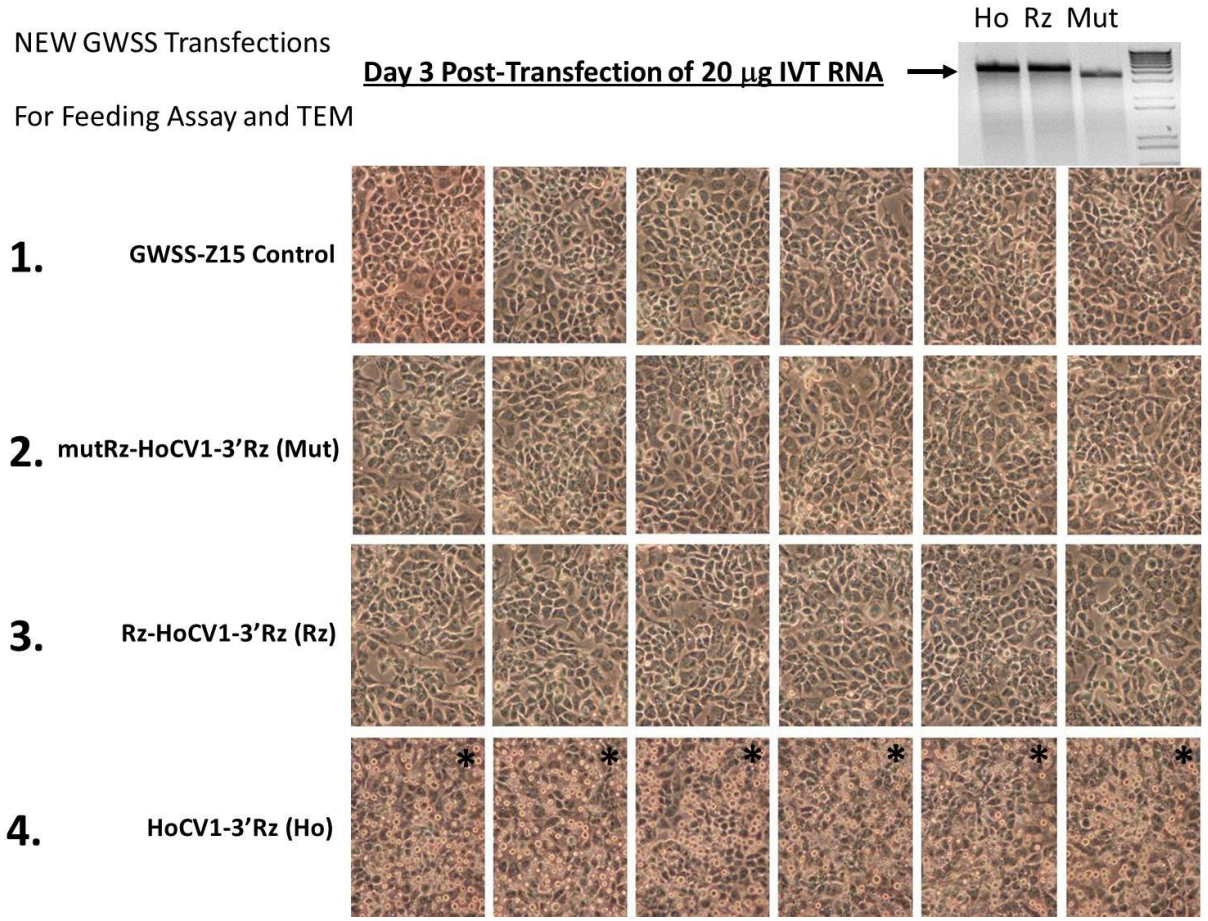

Figure 1: 3-Day Infection of GWSS-Z15 cells in T_{25} following transfection of *in vitro* transcribed HoCV1-3'Rz, Rz-HoCV1-3'Rz or mutRz-HoCV1-3'Rz RNA. Cells were transfected with 20 μg of HoCV1-3'Rz RNA, 20 μg of RzHoCV1-3'Rz or 20 μg of mutRzHoCV1-3'Rz *in vitro* transcribed HoCV-1 viral RNA (**top right**). Images were taken at day 3 post-transfection. CPE were observed in GWSS cells transfected with HoCV1-3'Rz RNA (**black asterisks**) that were not observed in the other wells. Images were taken at 40X objective magnification.

Silencing constructs Plasmids are under construction to generate hairpin dsRNA targeting *GFP* (negative control), *H. vitripennis actin*, and *H. vitripennis chitin deacetylase*. Plasmid clones supplying fragments of all target genes were developed at UC Davis and the GWSS sequences have been shown to be effective in RNAi assays (1, 2). Sense and anti-sense strands were amplified for all targets (**Figure 4**) and are being cloned with a 30 base pair spacer into a pGEM-13 Zf + vehicle vector carrying an extended multi-cloning site. Following completion of the hairpin RNAi constructs in pGem-13 Zf +, they will be directly digested out and cloned into the Xba I site of the functional pT7-HoCV1-3'Rz viral infectious clone immediately upstream of the synthetic polyadenylation signal. Tests of functionality for the RNAi-encoding HoCV-1 variants will be performed against GWSS Z-15 and virus-free GWSS insects using quantitative real-time PCR to detect knockdown of target genes. We will also monitor for increased virus-induced mortality in the live GWSS insects.

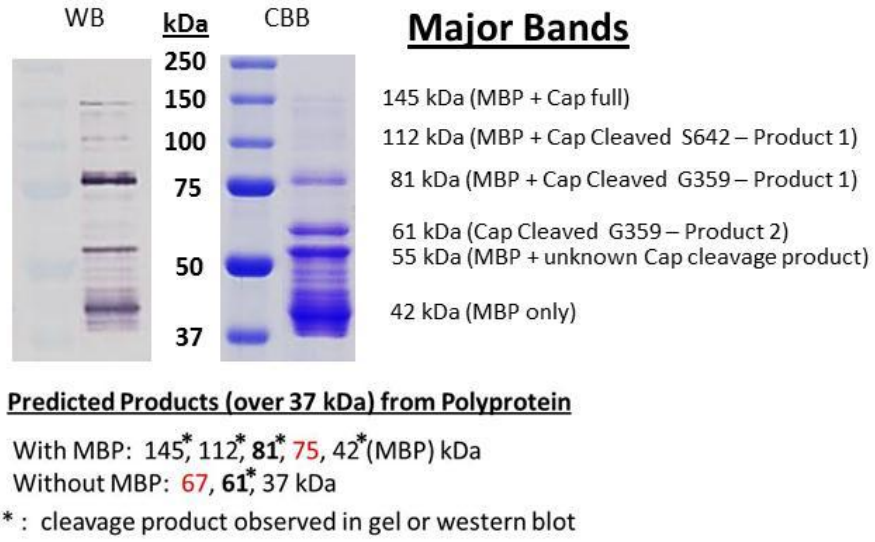

Figure 2: Expression and purification of HoCV-1 ORF 2 in *E. coli* as a fusion with maltose binding protein (MBP). The HoCV1 capsid polyprotein (ORF 2) was inserted downstream and in-frame with MBP in the pMal-C2X expression vector (NEB, Ipswich, MA). Expression and purification of the fusion protein was performed in *E. coli* BL21 cells. The predicted full-length fusion protein (145 kDa) as well as several derivative products from possible autocleavage of the polyprotein were observed in anti-MBP western blot (WB-left) and Coomassie Blue (CBB-right) analyses. Sizes of predicted autocleavage products that could possibly be seen on the 10% SDS-PAGE gel are indicated with actual observed fragments denoted with asterisks. Black bold and red letters indicate corresponding products with and without MBP following cleavage at a single site in the polyprotein. One fragment observed in both Coomassie and anti-MBP analyses at 55 kDa could not be explained by current prediction for cleavage sites in the HoCV-1 ORF 2.

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ATSQQIHDT                                                    9
METHSHEPINTNIDGETSENTFEEKREITHFTEDDRVLTDAVTEITSLPL 59
SLLQYGDEPREHSVISFLQRPEKIATVTWTTAQTKTTNLVSLPIPSSVLT 109
TMYREKLRGFGQLRADIVFKLQFNSRPFQAGRLIATYIPVPAYLLQRTRM 159
ARASLTRLTSLPNVIIDISKQTECNITLPYVSSFTHYDLTSGGGDWGLFD 209
LWVYSPLSSASSQTINISIRAYLDNVRLGAPTQQSLVTAEKMLKANVQTR 259
DLSRGTSSCGSISVRAQGGKQTAGSGDGSFGSLLNKGTKSVTMQEQSAGT 309      G277
ISRVGHSIREGLVRGLNVVGEFIPGLSEITDTANSVAMGVLNTLAAFGLG 359      G359
KPKNLDKIAPRTLHAFSDFAQATGVDNGHILSLHGDNKVTVLPGFAGSNT 409
DELSMTYLMQTLQYYDTHTISTTTAVGTQIAAYRVTPFRFDLDLAKTAQS 459
FVSGSPLINFGQPNLQWYIGSNFKYWRGDIVMHLALVKTDYHSVRLKIVY 509
DPMAQSAAAVTYDASEYCYSIVVDFRDKTDIYVRLPFISATPWKLVPPST 559
YTGYTPPPVNQQEGLSTYSGYVAVFVDNILQASSAVVSQSIEMVSEFCAA 609
SNLDMGFPHGGQNWIPISTVLNPGDPIQADVQSTFAADGILKTRTNMQEN 659      S642
TLDIKNITGMAPRPLYDNITSYTTGEEVYSLRMLMKRFNWIASVPSGQAS 709
IALPNTVKTIDAAAPVSNPNQIVDIRTGPPYANNTVSDCALVDVVGALFA 759
FRAGGFRWKAWDSGSELISAYLVPFGPYNTYGIPSSTFTNLISNTSVYEL 809
DSRQVKGSAEFATPFYHPCYTQVNSNFSYFTEGGEPDLYFHFTQPQTVTV 859
VSRSNPGSEMNIAKSAGDDLNFGFLLGVPDCLPSQIVAGLLSRPSSQPNL 909
PNSTPIS*
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Figure 3: Polyprotein product of HoCV-1 ORF 2 and predicted internal autocleavage sites. Autocleave sites (G277, G359, and S642) predicted for the capsid polyprotein are highlighted in yellow.

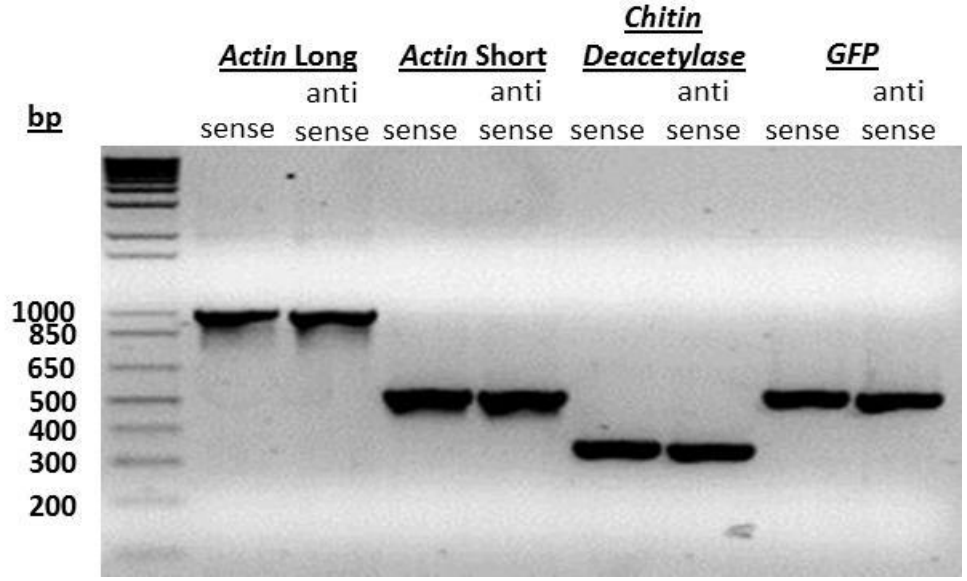

Figure 4: Sense and anti-sense PCR products produced for generation of hairpin RNAi clones against *GFP*, *H. vitripennis actin*, and *H. vitripennis chitin deacetylase*. Products were derived from pGEM-T-easy clones carrying a fragment of the open reading frame for each target gene.

Publications, reports and presentations resulting from the project:

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

2. 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.

3. 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.

4. 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.

5. Kroemer, J. A., Spear, A., Stenger, D. C., Miller, W. a., Falk, B. W., and Bonning, B. C. 2012. Dells derived from the glassy-winged shartshooter, *Homalodisca vitripennis*, support infection and replication of viral RNA from a clone of *Homalodisca coagulata virus 1* (HoCV-1). Annual meeting of the Entomological Society of America, Nov 11 – 14, Knoxville, TN.

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. Current data suggest that RNAi effects in insects are not systemic, that is the effects do not spread through the insect body as they do in plants, presumably to the lack of an RNA-dependent RNA polymerase gene. This could present a problem for effective use of RNAi for insect control. One way to initiate RNAi spread is to use a virus that spreads in the insect body. Our effort here is to engineer a naturally-occurring GWSS-infecting virus, HoCV-1, and use it to induce spreading RNAi activity in GWSS. 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) via a recombinant virus, HoCV-1, to help combat Pierce's Disease of grapevines. Our effort here does not directly target *Xylella fastidiosa*, the causal agent of Pierce's Disease, but instead targets one of its most significant insect vectors, the Glassy-winged sharpshooter, *Homalodisca vitripennis*, and other sharpshooter vectors of *X. fastidiosa*. We are attempting to develop the naturally occurring HoCV-1 for use as a biological control agent. HoCV-1 naturally occurs among GWSS populations. We have demonstrated that we can induce recombinant HoCV-1 replication in cultured GWSS cells but so far we have not been able to introduce it back into GWSS insects. Our longer term goal is to improve our existing

version of HoCV-1 so it can readily infect GWSS adults, and modify it to increase its pathogenicity in GWSS nymph and adult insects.

Status of funds:

We were awarded one year funding to support expenses for personnel, routine supplies, recharge facility (Biosafety 3P Contained Research Facility) costs and limited travel. We have spent most of the funds as proposed in our original proposal budget, but due to a postdoc leaving for another position we have ~$48,000 in the UC Davis account. I have requested a one year no-cost extension to allow us to use these funds to complete our research during the next fiscal year.

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, Kamita, S. G., Falk, B. W. (2012) RNA interference is induced in the glassy winged sharpshooter Homalodisca vitripennis by actin dsRNA. *Pest Management Science* 68:995 - 1002.
2. Rosa C*, et al.* (2010) RNAi effects on actin mRNAs in Homalodisca vitripennis cells. *J RNAi Gene Silencing* 6(1):361-366.