DEVELOPMENT AND USE OF RECOMBINANT *HOMALODISCA COAGULATA VIRUS-1* FOR CONTROLLING THE GLASSY-WINGED SHARPSHOOTER.

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ABSTRACT
The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is an efficient vector of *Xylella fastidiosa (Xf)*, causal agent of Pierce's disease of grapevines. We are investigating using recombinant insect viruses to deliver toxic peptides and/or induce systemic RNA interference (RNAi) and thereby induce mortality in recipient GWSS. We are attempting to develop recombinant *Homalodisca coagulata virus-1* (HoCV-1) so as to specifically infect GWSS. We will engineer HoCV-1 to deliver and express toxic peptides and/or effector RNAs only upon infection of recipient GWSS. We are taking two approaches to develop recombinant HoCV-1, one a straightforward cDNA cloning approach and the second involves amplification using a baculovirus. We are also using a second virus, *Flock house virus*, for which recombinant clones are already available. It is our hope that the results obtained from these studies will be used for developing an effective biological-based control strategy to help control GWSS and other sharpshooter vectors of *Xf*.

LAYPERSON SUMMARY
The glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*) transmits the bacterium, *Xylella fastidiosa (Xf)*, which causes Pierce's disease of grapevines. We are attempting to use natural, GWSS-infecting viruses as part of a strategy to kill GWSS. 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 *Xf*. RNAi can be triggered by effector RNAs, and results in degradation of the specific RNAs that have sequence homology with the effector RNA. It is known that in plants and some insects, RNAi occurring in localized tissues can trigger RNAi in the whole organism. For example, actin is a protein that is indispensible in normal cell function, and if a double-stranded RNA containing actin mRNA sequence is introduced to a localized tissue of an organism, the organism will not have enough actin produced locally in the inoculated tissue or in whole system depending on whether a systemic RNAi occurs in the organism or not. We are attempting to determine whether systemic RNAi can be induced in GWSS by using recombinant *Homalodisca coagulata virus-1* (HoCV-1), a naturally-occurring virus that specifically infects GWSS. If successful, our studies may lead to new and effective methods to help control the GWSS population.

INTRODUCTION
The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is a highly efficient vector of *Xylella fastidiosa (Xf)*, the causal agent of Pierce's disease (PD) of grapevines. We are investigating new approaches based on using a GWSS-infecting virus, *Homalodisca coagulata virus-1* (HoCV-1), as a tool to help manage GWSS. We will engineer HoCV-1 to express toxic peptides which can kill infected GWSS upon virus replicaion. As a second approach we are attempting to use HoCV-1 to deliver GWSS RNAs so as to induce RNA interference (RNAi) in recipient GWSS.

RNAi is a gene silencing mechanism that is initiated by a presence of double stranded RNA (dsRNA). The resulting RNAi activates the cell machineries for identification and degradation of the target RNA, which is the RNA having homologous sequence to the dsRNA that has triggered RNAi. This mechanism is known to occur in many eukaryotes such as plants, animals and insects as an indispensible part of normal developmental processes and innate immunity. In plants, locally triggered RNAi induced by viral RNA replication or an experimental inoculation of dsRNA sends a warning signal to the whole plant so that the cells not invaded by the dsRNA are prepared to silence the foreign RNA that may invade them. This phenomenon is called systemic RNA interference, and the signal responsible for triggering the systemic silencing is a small interfering RNA (siRNA) that results from the RNAi pathway. The resulting siRNA is presumed to be amplified by a plant RNA dependent RNA polymerase (RdRp). Although insects are not known to have RdRp, systemic silencing occurs in some insects through some unknown mechanisms (Huvenne and Smagghe). It is unknown whether feeding of dsRNA or short-hairpin RNA (shRNA) can cause systemic silencing in GWSS or not.

We are now attempting to develop an infectious clone of *Homolodisca coagulata virus-1* (HoCV-1). HoCV-1 is a virus isolated from field-collected GWSS. It is a single-stranded, positive-sense RNA virus belonging to the family *Dicistroviridae*. It has a monopartite genome composed of uncapped 5'-end and polyadenylated 3'-end. Although its sequence has been published, no infectious clone is yet available. We will use the GWSS embryonic cell-line, Z-15 (Kamita et al., 2005), as well as whole GWSS as tools for our work. Once the infectious clone is developed, we will modify the HoCV-1 genome sequence to produce a highly-specific, recombinant virus either delivering toxic peptides or anti-GWSS RNA effectors with the ultimate goal of helping to control GWSS populations.

OBJECTIVES

1. Development of HoCV-1 infectious cloned cDNAs;
2. Expression of GFP or other stable sequences in GWSS-Z15 cells or whole GWSS insects by using HoCV-1.

RESULTS AND DISCUSSION

We are only three months into this project. Our primary focus so far has been to develop a virus, preferably HoCV-1, that we can use for our peptide/RNA expression studies. While our main effort has been with HoCV-1, we have also added a second virus that should be easier to manipulate at least initially. This is *Flock house virus* (FHV). FHV belongs to the family *Nodaviridae*, and is a non-enveloped, positive-sense RNA virus originally isolated from the grass grub (*Costelytra zealandica*) in New Zealand. It has a bipartite genome and has been shown to multiply in insects from four different orders (Hemiptera, Coleoptera, Lepidoptera, Diptera). In adult mosquitoes, FHV has been used successfully to systemically express GFP (Dasgupta et al., 2003), and thus we are attempting to use the FHV system (Dasgupta et al., 2003) to systemically express GFP in GWSS. This will serve as a surrogate virus system until our HoCV-1 system is optimized. FHV will be used to express peptides, and to perform initial systemic RNAi experiments using GWSS.

Testing for Systemic Silencing in GWSS

We have FHV cDNA clones that can produce FHV genomic RNAs and make infectious FHV particles in *Drosophila* S2 cells. In order to confirm the biological activity of the constructs, we co-transfected S2 cells with the copper-inducible *Drosophila* metallothionein promoter driven FHV RNA1 cDNA and FHV RNA2 cDNA, and collected the transfected cells and the medium 4 days post transfection. Also, in parallel, we co-transfected another batch of S2 cells with FHV RNA1 cDNA fused to GFP sequence and FHV RNA2 cDNA. As expected, the S2 cells transfected with these constructs produced GFP signal (**Figure 1**)

Figure 1. GFP signal produced by FHV in transfected Drosophila S2 cells.

In adult mosquitoes, FHV has been engineered to systemically express GFP (Dasgupta et al., 2003). We hope to adapt this system to test for systemic silencing in GWSS. GFP will be systemically expressed in GWSS with FHV DIeGFP (**Figure 2**). A defective interfering (DI) RNA of FHV originating from the genomic RNA 2 interferes with FHV genomic RNA replication through greater template efficiency with the viral replicase. The construct DIeGFP that contains the viral replication sites, packaging signal and expresses GFP is available, which we will use to produce FHV virions containing DIeGFP. This will be accomplished by transfecting *Drosophila* Schneider 2 (S2) cells with the pMT vectors expressing FHV RNA1, RNA2, and DIeGFP.

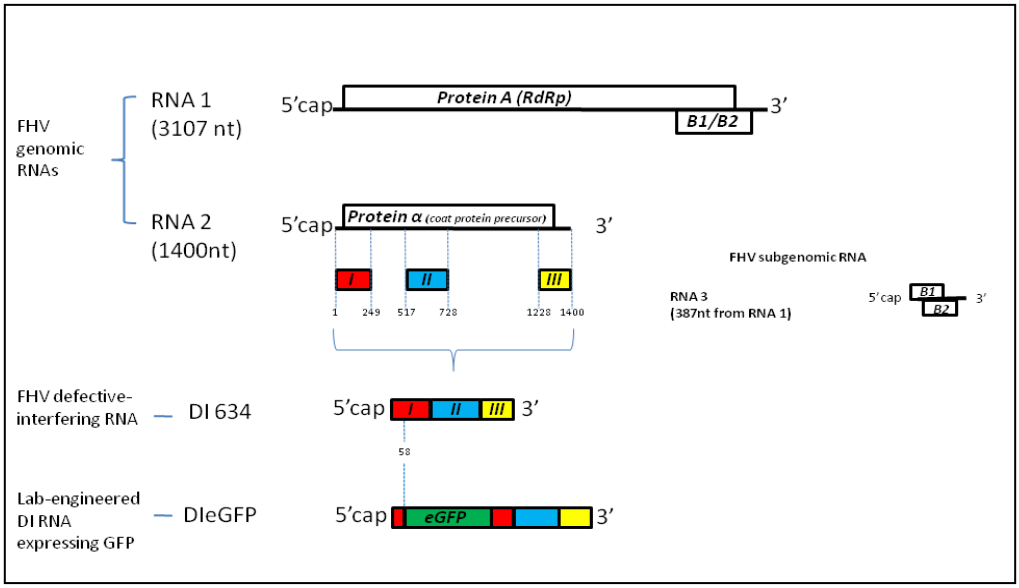

Figure 2. Lab-engineered DI RNA expressing GFP (Dasgupta, et al., 2003**.)**

Once infectious virions have been produced in the cell system we will begin whole insect experiments. The purified virions will be injected into GWSS for GFP expression, which we will confirm with fluorescent microscopy. To then test for systemic silencing of GFP we will feed GFP-expressing GWSS GFP dsRNA or short-interfering RNA and analyze insect tissues using fluorescent microscopy.

Making an Infectious Clone of *Homalodisca coagulata Virus-1*

We are using two approaches to develop recombinant, infectious HoCV-1. First, we will use a binary expression system involving expression of bacteriophage T7 polymerase and a double-stranded DNA with a T7 promoter driven HoCV-1 sequence in GWSS embryonic cell-line, Z-15. Such a binary expression system has been used in animal cells to produce infectious viruses including poliovirus. We have constructs expressing T7 polymerase with or without nuclear localization signal (NLS) that has been tested in yeasts, plants, and mammalian cells (Dunn et al., 1988). We have subcloned the T7 polymerase gene under an insect promoter (IE) and will co-transfect Z-15 cells with T7 driven HoCV-1 construct (**Figure 3**).

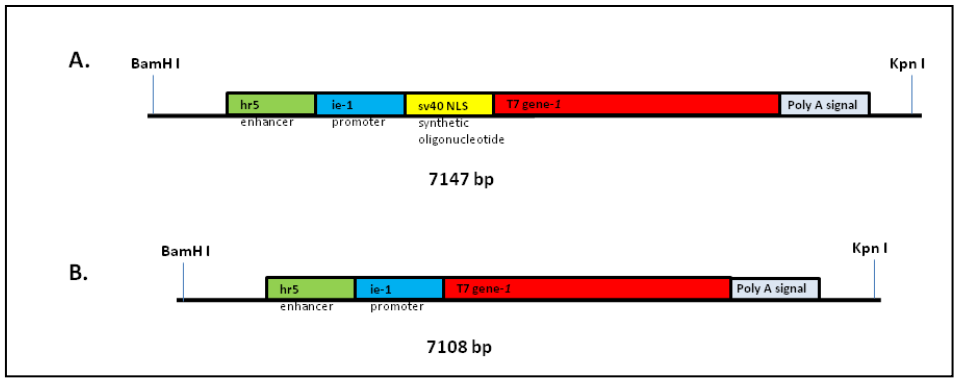

Figure 3. Maps of pIE T7 gene-1 with (A) or without (B) nuclear localization signal (thin line indicates pUC8 sequences.

We will test for the virion formation with electron microscopy and Northern blot analysis, then test for GWSS infectivity in whole insects. If infectious HoCV-1 virions are produced from this binary expression system, we will modify the HoCV-1sequence to carry a dsRNA or shRNA for VIGS experiments with the ultimate goal of using the virus as a tool to help control the GWSS population.

Our second approach uses an insect baculovirus-based approach. The Bac-to-Bac Baculovirus Expression System (Invitrogen) was used to generate recombinant baculoviruses. The first step of this process involves cloning of the target

sequence, in this case the complete genome of the HoCV-1, into the shuttle vector pFASTBac1. Because of the large size of the HoCV-1 genome, the cloning was performed in three steps (**Figure 4**). In Step I, the 5'-end of the HoCV-1 genome was amplified by RT-PCR from total RNA isolated from GWSS adults using tailed PCR primers. The tailed PCR primers were designed to incorporate *Bam*HI restriction endonucleases sites at the ends of the amplicon. The amplicon was digested with *Bam*HI and inserted at the *Bam*HI site of pFastBac1. The insert of the resulting plasmid, pFB-HoCV1-5'end, was checked for orientation and sequenced. In Step II, the 3'-end of the HoCV-1 genome was RT-PCR amplified as in Step II using a tailed 3'-PCR primer that incorporated a *Hin*dIII site at the 3'-end of the amplicon and a HoCV-1-specific 5'-primer. The resulting amplicon was digested with *Sal*I and *Hin*dIII and inserted at the *Sal*I and *Hin*dIII sites of pFB-HoCV1-5'end in order to generate the plasmid pFB-HoCV1-5'/3' ends. Finally, in Step III, the *Afl*II-*Xba*I internal fragment of HoCV-1 from pRzHoCV1 was excised and ligated into the corresponding sites of pFB-HoCV1-5'/3' ends to generate pFB-HoCV1.

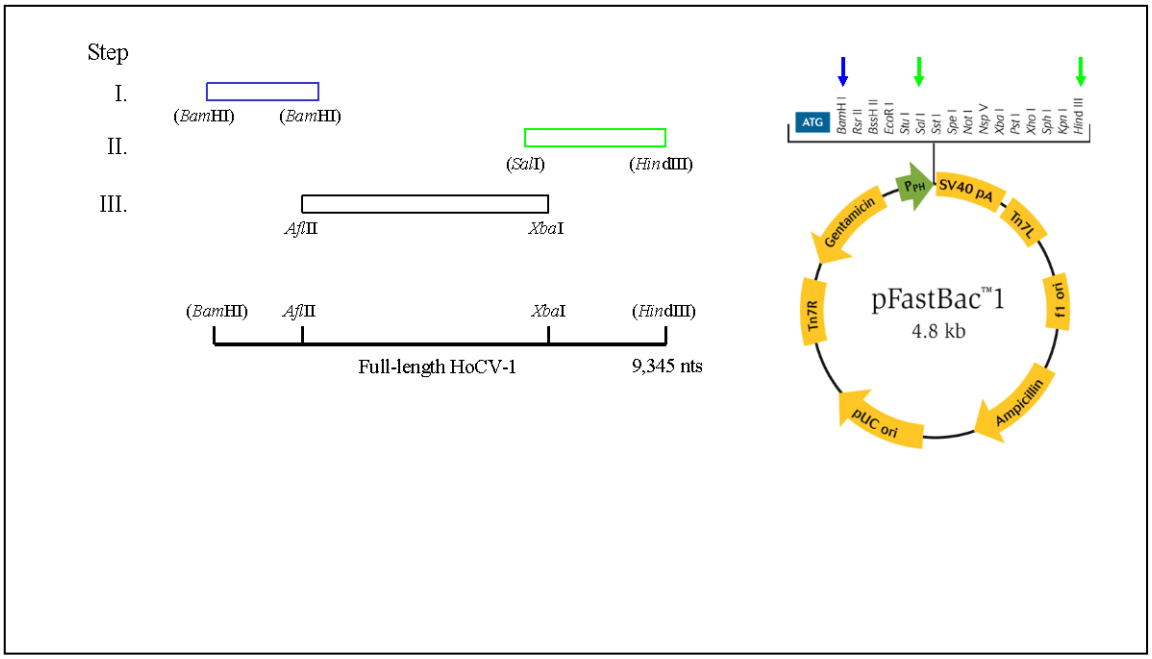

Figure 4. Cloning HoCV-1 cDNA into pFastBac1.

Recombinant baculoviruses carrying the complete HoCV-1 genome were constructed in two steps as shown in **Figure 5**. In the first step, the recombinant baculovirus shuttle vector pFB-HoCV1 was transformed into competent DH10Bac *E. coli* cells. White colonies that formed on the selection medium (i.e., colonies formed by *E. coli* carrying recombinant bacmid DNAs) were re-isolated by streaking on the same selection medium and individual colonies were amplified in liquid medium containing selection antibiotics. Bacmid DNAs were isolated from the liquid medium cultures and confirmed by PCR to carry the HoCV-1 genome. In the second step, recombinant baculoviruses were generated in Sf-9 cells by transfection of Sf-9 cells with bacmid DNAs carrying the HoCV-1 genome using Cellfectin reagent. Following transfection the recombinant baculoviruses were subject to one round of amplification in Sf-9 cells.

The ability of the recombinant baculoviruses (i.e., AcHoCV1) to express HoCV-1-specific RNA (i.e., positive-sense HoCV-1 RNA) was investigated by RT-PCR (**Figure 5**, step 2). Total RNA was first isolated from the cells of recombinant baculovirus-infected Sf-9 cells, then treated with DNAse I. First strand cDNA synthesis for the RT-PCR reaction was done using strand-specific primers that were specific for either the positive- or negative-sense RNA of HoCV-1. The positive signal using the positive-sense RNA specific primer indicated that the HoCV-1 genomic RNA is expressed by the recombinant baculovirus. The positive signal using the negative-sense RNA specific primer indicated that RNA dependent RNA polymerase (RdRP) is translated from the baculovirus expressed HoCV-1 genomic RNA. And, furthermore, that this RdRP is capable of producing the intermediate negative-sense RNA that is required for the replication of HoCV-1.

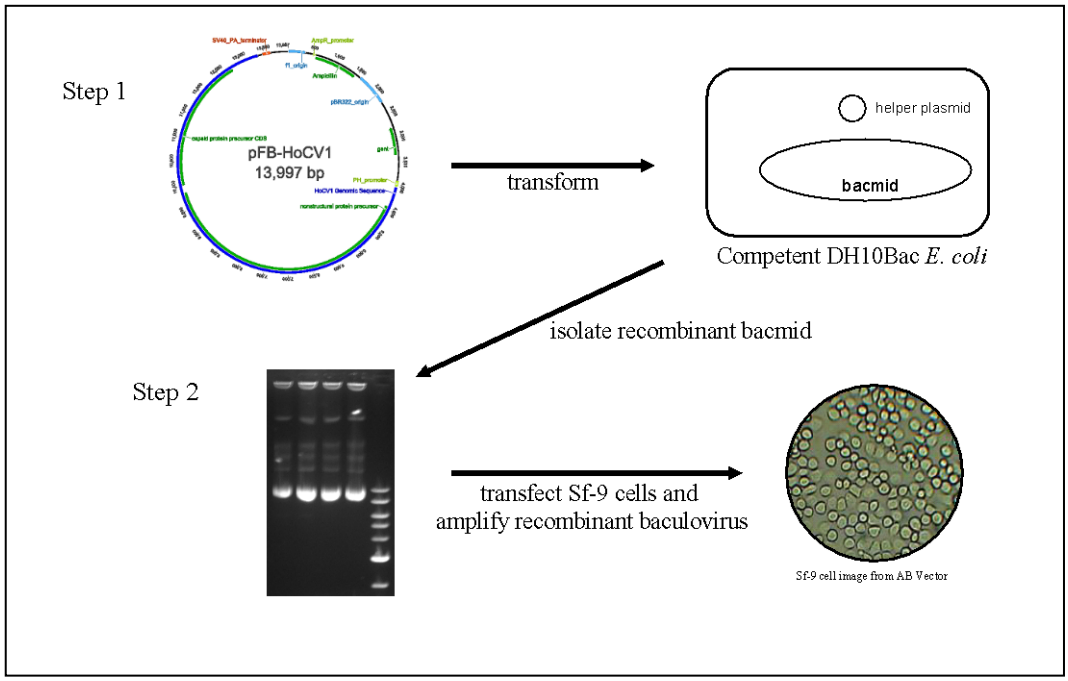

Figure 5. Generation of recombinant bacmid and baculovirus.

CONCLUSIONS

We have only been working on this effort for three months so far. During the reporting period, we began working with two viruses so as to ensure success. We have conceived the experimental strategies, tested the FHV constructs for its biological activity, and made the plasmid constructs that can express T7 polymerases in insects. The experiments described above are on-going and rapidly progressing. FHV is easy to work with and our HoCV effort looks very promising so far. The results of the experiments using FHV system will be critical in designing RNAi strategies for controlling GWSS population, and the infectious HoCV-1 cDNA clones will be invaluable in assessing the feasibility of using virus infecting GWSS as a biopesticide.

REFERENCES CITED

Dasgupta, R., Cheng, L. L., Bartholomay, L. C., and Christensen, B. M. (2003). Flock house virus replicates and expresses green fluorescent protein in mosquitoes. J Gen Virol **84**(Pt 7)**,** 1789-97.

Dunn, J. J., Krippl, B., Bernstein, K. E., Westphal, H., and Studier, F. W. (1988). Targeting bacteriophage T7 RNA polymerase to the mammalian cell nucleus. Gene **68**(2)**,** 259-66.

Huvenne, H., and Smagghe, G. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. J Insect Physiol **56**(3)**,** 227-35.

Kamita, S. G., Do, Z. N., Samra, A. I., Hagler, J. R., and Hammock, B. D. (2005). Characterization of cell lines developed from the glassy-winged sharpshooter, *Homalodisca coagulata* (Hemiptera: Cicadellidae). In Vitro Cell Dev Biol Anim **41**(5-6)**,** 149-53.

Yap, C. C., Ishii, K., Aoki, Y., Aizaki, H., Tani, H., Shimizu, H., Ueno, Y., Miyamura, T., and Matsuura, Y. (1997). A hybrid baculovirus-T7 RNA polymerase system for recovery of an infectious virus from cDNA. Virology **231**(2)**,** 192-200.

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