RNA-INTERFERENCE AND CONTROL OF THE GLASSY-WINGED SHARPSHOOTER AND OTHER LEAFHOPPER VECTORS OF *XYLELLA FASTIDIOSA*.

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ABSTRACT
Here we present our progress obtained in the development and application of an RNA interference (RNAi) based system aimed to target genes of sharpshooter vectors of *Xylella fastidiosa*, the causal agent of Pierce's disease (PD). We have demonstrated RNAi effects in glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) whole insects after intrathoracic injection of dsRNAs, by monitoring insect phenotype and molecular markers. Phenotypic effects vary from none observed to death, but the latter has been observed so far only when specific instars were injected with actin dsRNA inducers. We have also developed tools for RNAi, including constructs designed for expressing dsRNAs as RNAi inducers to be employed in generation of transgenic plants. The constructs were inserted in a user-friendly Gateway binary vector that facilitated simple cloning steps. This vector has two forms, one including the 35S promoter and a second version containing the *Eucalyptus gunii* minimal xylem-specific promoter. Some of these constructs have been already employed and transgenic plants are being developed by the Ralph Parsons UC Davis Plant transformation facility, while other constructs have been developed to use in 'in house' generation of transgenic *Arabidopsis thaliana* via flower dipping. We broadened the choice of target GWSS genes including genes expressed in the insect midgut, since our results showed that ingested dsRNA is concentrated mainly in that specific insect body part. We believe that our efforts are well spent, and that results in the use of RNAi to assist in efforts to control the PD vectors are well on their way.

LAYPERSON SUMMARY
Pierce's disease (PD) of grapevines is one of the plant diseases caused by the Gram-negative bacterium *Xylella fastidiosa (Xf)*. This bacterium, upon inoculation in the plant host, travels within and attaches to the plant xylem vessels where, after multiplication, produces biofilms which interfere with the water-flow in the infected plants. Resulting infected plants can die between one and five years after inoculation. *Xf* is vectored by many leafhoppers, but one of the most important vectors in many areas is the glassy-winged sharpshooter (GWSS). The importance of GWSS can be attributed to its ability to colonize more than 100 species of plants, its propensity for long distance dispersal and its capacity for ingesting large volumes of fluids from colonized plants. We are taking a contemporary molecular targeting approach to disrupt normal gene expression in GWSS and other sharpshooter vectors of *Xf* as a strategy to help control these important insect vectors of *Xf*. We are attempting to develop RNA-interference (RNAi) as a tool to target and kill GWSS and other sharpshooter vectors of *Xf*. RNAi is normally used by plants, arthropods, some fungi and even nematodes to degrade specific mRNAs, including those of the host during normal organismal development, but also those of pathogenic viruses. We have clearly demonstrated that introducing specific double-stranded RNAs (dsRNAs) into GWSS cells, and by intrathoracic injection into whole insects we can inititate the RNAi-mediated degradation of homologous mRNAs, and in some cases death. We are now attempting to identify genes necessary for the fitness and survival of GWSS, and we are generating transgenic plants (grapevines but also potential trap plants such as citrus and potatoes) expressing dsRNAs corresponding to the identified GWSS mRNAs. Our hope is that ingestion of these dsRNA molecules by GWSS will trigger RNAi activity in the recipient insects, resulting in the subsequent degradation of the targeted mRNAs and corresponding debilitating effects on sharpshooters, thereby contributing to strategies for PD control.

INTRODUCTION
During the work supported by this research program, we developed tools to induce RNA interference (RNAi) in the insect vectors of Pierce's disease, and in particular in glassy-winged sharpshooter (GWSS). We were able to induce RNAi for specific genes *in vitro* in a GWSS cell line developed at UC Davis (Kamita et al., 2005; Rosa et al., 2010) and in whole insects (Rosa et al., 2011). We also optimized protein, small and large RNA hybridization and real time PCR techniques to detect the extent of RNAi induced in the different systems. We were able to generate phenotypes in both cells and 5th GWSS instars affected by RNAi. While our previous efforts were limited by the scarce amount of nucleotide sequences available in GenBank, our present research is based on the sequence analyses of extensive GWSS EST data released via GenBank. We cloned a series of genes predicted to be expressed during insect digestion and during molting, and we inserted sequences for these genes into an easy to manipulate binary vector (Lei et al., 2007) set up for hairpin RNA transcription in plants. We also modified this vector substituting the 35S promoter with a minimal xylem specific promoter cloned by us from *Eucalyptus gunii*. The potential of these series of vectors is great, in fact we are using these plasmids now to generate transgenic plants of different species (grape, citrus and potatoes) via the Ralph M. Parson Foundation facility of UCD and at the same time to generate in a fast and convenient way *Arabidopsis thaliana* plants via flower dipping Clough (1998).

OBJECTIVES

1. To evaluate existing transgenic plants for their ability to generate RNAs capable of inducing RNAi effects vs. GWSS.
2. To identify GWSS interfering RNAs for practical application.
   2.a. To utilize transgenic *Arabidopsis thaliana* plants as efficient alternatives for identifying, delivering, and evaluating efficacious interfering RNAs.
   2.b. To enhance production of interfering RNAs *in planta*.
   2.c. To evaluate alternative strategies to deliver and screen high number of RNAi inducers to GWSS.

RESULTS AND DISCUSSION

To evaluate existing transgenic plants for their ability to generate RNAs capable of inducing RNAi effects vs. GWSS .
Grapevines *Thompson seedless* and Carrizo citrange were transformed and regenerated at the Ralph M. Parsons Foundation Plant Transformation Facility. So far, we have received seven grape transformants engineered to express the GUS reporter gene under control of the 35S promoter. These plants, which will be used as controls in our experiments, are already in our greenhouse and will be tested for GUS expression once they reach appropriate size (in the next month). Unfortunately, transformation and regeneration of grape and citrus plants has not been successful using our initial xylem promoter constructs. We have modified this promoter to now be a minimal, easier to use promoter. In order to make more rapid progress we investigated using herbaceous GWSS host plants for transformation/regeneration. Potato is a good host for GWSS (**Figure 1**) and is readily transformed and regenerated. Therefore, transformation and regeneration of potato plants is proceeding, and we expect to obtain the first explants containing specific GWSS genes before the summer 2011. These will be vegetatively propagated, analyzed for the desired transgene and RNAi effectors, and used for GWSS-RNAi experiments in the UC Davis CRF.

Figure 1. GWSS adults and nymphs colonizing potato plant.

To identify GWSS interfering RNAs for practical application.
To generate binary vectors for simple cloning and which can express hairpin dsRNAs in plants, we used the Gateway modified pCB2004B vector (**Figure 2**). This vector has a 35S promoter cloned between DraIII directional restriction enzyme sites, and 4 attR sequences in a head to tail orientation, flanked by a spacer region. The 4 attR sequences are efficient sites for recombination, and any insert cloned in the compatible pCR8-TOPO entry clone can be moved in both pCB2004B recombination sites in one simple clonase reaction, resulting in a fully functional plasmid expressing a dsRNA hairpin loop. The pCB2004B plasmid has a glufosinate resistance gene for plant selection and we will use it for transformation of the species indicated here. The 35S promoter can be digested and replaced by any other promoter flanked by DraIII restriction sites.

We have already cloned 10 novel cDNA sequences of GWSS genes involved in digestion processes and cuticle formation. These genes encode for the following proteins:

> a hydrolase, a glucosyltransferase, a peptidase, two trypsins, a transladolase, a sugar transporter a serine-type endopeptidase inhibitor, a transketolase, a cuticle regeneration and a structural cuticle proteins.

All the constructs are now in a head to tail orientation to generate hairpin RNA in the binary vector under the control of the 35S promoter. We are now in the process of moving the same constructs so hairpin dsRNAs will be expressed via the minimal xylem specific promoter cloned previously by us from *Eucalyptus gunii*.
We also identified and are cloning GWSS genes expressing the following proteins:

a triosephosphate isomerase involved in glycolysis, gluconeogenesis and fatty acid biosynthesis, a sugar transporter, three yellow proteins involved in male courtship and cuticle pigmentation, two additional lipid transporters, an additional serine-type endopeptidase.

We will assess these candidate sequences for RNAi effects during the upcoming year.

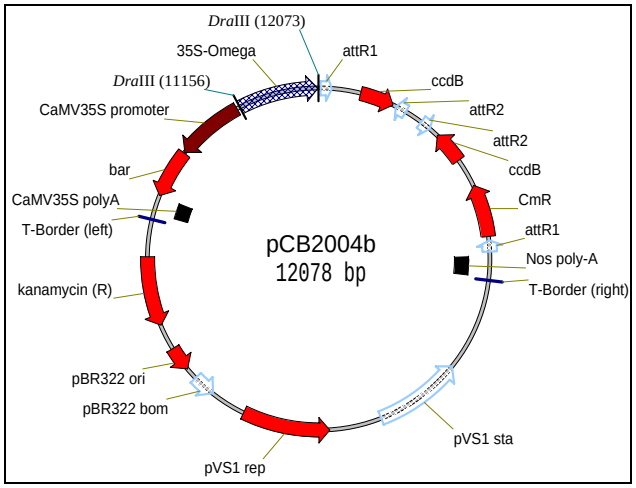

Figure 2. Map of pCB2004b, binary plasmid, Gateway compatible and designed for generating hairpin dsRNAs in transgenic plants

2.a. To utilize transgenic *Arabidopsis thaliana* plants as efficient alternatives for identifying, delivering, and evaluating efficacious interfering RNAs.

We selected and propagated the *Arabidopsis thaliana* ecotype Cape Verdi Islands (CVi-0). This ecotype has larger leaves and presents a more robust growth, and will be more appropriate in supporting insects of large size such as GWSS (**Figure 3**). Every ten days, groups of *A. thaliana* plants are flower-dipped in solutions of *Agrobacterium tumefaciens* transformed with the binary plasmids made as described above. To enhance the percentage of transgenic seeds at collection, set seeds are removed before dipping. After the dipping, plants are returned for continued growth. When seeds show signs of maturity, the dipped plants are set aside to dry down before seeds are collected. The collected seeds are air-dried and then planted at the CRF. Selection for transgenic seedlings is done by 2-3 applications of glufosinate ammonium (200-300 µM), as transgenic *A. thaliana* carries bacterial bialophos resistance gene (*BAR*) encoding the enzyme phosphinotricin acetyl transferase (PAT) and thus, plants with this gene are resistant to glufosinate ammonium. The surviving seedlings will be used to perform RNAi experiments. GWSS nymphs will be caged in groups of five on individual transgenic seedlings. Non-transgenic *A. thaliana* plants will be used as controls. GWSS fitness and survival will be monitored for 10 days, at that time insects as well as plant tissues will be collected. The amount of target mRNAs will be measured in insects by real time PCR or northern blot, and the presence of specific siRNAs will be assessed. Transgenic plants will be also tested for the presence of transgenes by PCR. The use of transgenic *A. thaliana* plants as alternative to identify efficacious interfering RNAs has shown to be a feasible method in our hands, and we are planning to complete the first experiments on insect survival in the next three months.

2.b. To enhance production of interfering RNAs *in planta*.

We used a transient *Agrobacterium tumefaciens* assay to generate an exogenous hairpin mRNAs accompanied by linear mRNA (with the same sequence of one of the two polarities present in the hairpin mRNA) in *Nicotiana benthamiana* plants, and we estimated by northern blot analysis, the amount of specific siRNA generated compared to plants infiltrated only with the exogenous hairpin mRNA. Our results suggest that there were not differences between the two treatments, and that amount of siRNAs generated by dicing of the dsRNA produced by the hairpin construct should be sufficient to induce RNAi in the insects feeding on the plants. This suggests that we will use the more simple approach to generate transgenes only expressing the hairpin dsRNAs.

Figure 3. GWSS nymphs colonizing *Arabidopsis thaliana* plant.

2.c. To evaluate alternative strategies to deliver and screen high number of RNAi inducers to GWSS.
We are considering the use of viral vectors based on *Cucumber mosaic virus* (CMV), to deliver RNAi inducers to GWSS. We already performed preliminary experiments and were able to infect both basil and *Arabidopsis thaliana* plants with different strains of CMV, belonging to subgroup I and II. We also have determined that we can detect by RT-PCR the virus-specific RNAs in GWSS nymphs fed on the virus-infected melon plants. CMV is a virus that invades most tissues, except it is not believed to invade the xylem of the plant host. Our results here raise interesting questions as to whether GWSS acquires RNAs (and other compounds) from other plant tissues than just xylem, or if CMV may also invade xylem tissues. In any case our results also suggest that we can possibly viruses to deliver RNAi inducers molecules GWSS via virus-infected plants. We are proceeding with that now as a strategy to complement the above efforts, and to quantify the amount of specific RNAs ingested by GWSS from CMV-infected vs. transgenic plants.

CONCLUSIONS
We are moving forward with development and potential application of RNAi as a tool to assist in efforts to control sharpshooter vectors of *Xf*. Efforts are underway by many groups to assess RNAi strategies for controlling insect vectors of plant pathogens and thus we are likely to learn more not only from our efforts, but also from those of the many labs working in this new research area. We have demonstrated RNAi effects, including death of GWSS, after injection of dsRNAs corresponding to specific mRNA targets. Other dsRNAs directed towards different targets showed no effects. Taken together these data show that identifying the correct target will be critical. So far effects have come from intrathoracic injection of dsRNAs, we are now focused on dsRNA oral delivery and comparison with results obtained from injection. We are in good position to continue to make progress and the upcoming year should be even more informative for RNAi-based studies on insect vectors of plant pathogens.

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