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

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ABSTRACT
This report presents the progress obtained in the development and application of an RNA interference (RNAi) based system aimed to target genes of the vector of *Xylella fastidiosa(Xf)*, the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*). After demonstrating that RNAi induction in GWSS cells and insects is achievable, in the year 2008-2009 we started screening a large pool of candidate genes to find the best target to control the survival of the insect vector. These data will be used to develop transgenic plants expressing dsRNAs of the target genes in their xylem tissues via EgCAD2, a xylem-specific promoter. Transgenic plants will be evaluated for their ability to induce RNAi effects on GWSS and other sharpshooter vectors of *Xf*.

LAYPERSON SUMMARY
This work presents fundamental efforts towards long term application of using RNA interference, RNAi, to help combat a plant disease of great economic importance. The disease, Pierce's disease (PD) of grapevines, is a significant threat to grape production in California and other parts of the U.S., and the causal agent of the disease, *Xylella fastidiosa (Xf)*, a xylem-limited bacterium, also causes several other extremely important plant diseases worldwide. Our effort here does not directly target *Xf*, but instead targets one of its most significant insect vectors, the glassy-winged sharpshooter (GWSS), and we combine the use of an *in vivo* system (GWSS whole insects) with an *in vitro* GWSS cell based system and demonstrate genetic and phenotypic RNAi effects. RNAi is an extremely important and broadly studied area in contemporary biology, and terms such as "magic bullet" for human medicine, and "genetic insecticide" for targeting insects have been used in the literature. Our work represents the first demonstrated RNAi effort in GWSS and our data will help to expand the possibilities to study plant-associated insects and at the same time to target the sharpshooter vectors of *Xf*, the causal agent of PD.

INTRODUCTION
The glassy-winged sharpshooter (GWSS) is among the most robust and thus most threatening vectors of *Xylella fastidiosa (Xf)*, the bacterium that causes Pierce's disease (PD) (Davis, Purcell et al. 1982), a devastating disease occurring in wine grapes from California to Texas to Florida (Myers, Sutton et al. 2007). New strategies that will lead to environmentally sound approaches to control GWSS and other insect vectors are needed. RNA interference (RNAi) has been suggested as a strategy to develop "insect-proof plants" (Gordon and Waterhouse 2007) and even referred to as a "genetic insecticide" (Scharf 2008). RNAi is a eukaryotic gene regulation/defense mechanism in which small RNA segments, small interfering RNAs (siRNAs) (21-25 nt), generated by processing of dsRNA molecules often of viral origin, specifically down-regulate complementary RNA sequences (Meister and Tuschl 2004). Recent efforts demonstrate that RNAi is inducible in many insects. Intrathoracic injection of dsRNAs has been shown to be the most effective way to induce RNAi in whole insects of many species including *Anopheles gambiae* (Blandin, Moita et al. 2002; Blair, Sanchez-Vargas et al. 2006), *Blattella germanica* (Ciudad, Piulachs et al. 2006), *Drosophila melanogaster* (Dzitoyeva, Dimitrijevic et al. 2001), *Spodoptera litura* (Rajagopal, Sivakumar et al. 2002), *Culex pipiens* (Sim and Denlinger 2009), *Lutzomyia longipalpis* (Mauricio R.V. Sant'Anna), *Cecropia pupae* (Bettencourt, Terenius et al. 2002), *Acyrthosiphon pisum* (Mutti, Louis et al.), *Rhodnius prolixus* (Araujo, Soares et al. 2009), *Aedes aegypti* (Cooper, Chamberlain et al. 2009), *Bemisia tabaci* (Murad Ghanim), *Dermacentor variabilis* (Mitchell Iii, Ross et al. 2007) and *Tribolium castaneum* (Arakane, Dixit et al.). Oral induction has also been demonstrated in several of these same species. Our effort demonstrates for the first time that RNAi activity can be induced in a leafhopper species, but also is inducible in GWSS cell lines. In the long term, RNAi can be used as an effective fundamental tool to better understand the dynamics of plant: pathogen: vector interactions as well as GWSS physiology and of course we hope as a strategy to complement overall efforts for PD control.

OBJECTIVES

The specific objectives of our effort are:

1. To identify RNAi-inducers capable of killing or reducing the survival and/or fecundity of GWSS and other sharpshooter vectors of *Xf*.
2. To generate transgenic plants capable of expressing GWSS deleterious interfering RNA molecules within their xylem.
3. To evaluate transgenic plants for their ability to induce RNAi effects vs. GWSS and other sharpshooter vectors of *Xf*.

RESULTS AND DISCUSSION

RNAi in GWSS cells and insects. Initially, we used 14 GWSS GenBank cDNA sequences corresponding to known proteins in order to synthesize RNAi inducer molecules, dsRNAs. We then tested whether RNAi was inducible in GWSS cells and insects, and we were able to show that RNAi activity is inducible in GWSS. Sets of dsRNA molecules were delivered to GWSS cells via lipid-based transfection, and to GWSS nymphs via intrathoracic injection or by feeding on cuttings immersed in a solution containing dsRNAs. Real time RT-PCR, semi quantitative RT-PCR, Northern blot of small and large RNA fractions showed that RNAi was achieved in cells and insects injected with dsRNA, where target mRNAs were partially degraded and specific siRNA, hallmarks of RNAi, were detected.

Because there are several potential sharpshooter vectors of *Xf*, the sequences isolated from GWSS were also amplified from the blue-green sharpshooter (BGSS; *Graphocephala atropunctata*) and from the green sharpshooter (*Draeculacephala minerva*) and cloned. This demonstrated a high degree of sequence conservation among these distinct sharpshooters, and the resulting sequences could be used to develop a general RNAi strategy to control multiple *Xf* vectors.

The above results showed anticipated reductions in target mRNAs. Therefore we evaluated if corresponding encoded proteins were reduced and if visible phenotypic results were induced. Western Blot analysis also showed a reduction of actin protein in GWSS nymphs injected with actin dsRNA (**Figure 1**). In addition, some of the injected nymphs did not complete ecdysis, demonstrating a striking phenotypic effect in whole insects vs. those injected with control gfp dsRNAs (**Figure 2**). A visible phenotypic effect was also obtained in GWSS cells transfected with actin dsRNA, where aberrations of the actin filaments occurred starting 72 hours post transfection. (**Figure 3**).

Because our results so far were dependent on a limited number of GWSS sequences so far available in Genbank, we analyzed three EST libraries deposited in GenBank. Twenty thousand thirty (20,030) EST sequences were analyzed using the Arthropod EST analysis pipeline at Kansas State University. One thousand nine hundred seventeen (1917) contigs were assembled and 6561 input reads were retained. The average length of the assembled contigs was 570 bp. NCB BLASTX was used to find sequence similarities in GenBank for the assembled contigs and singletons. One thousand seventy three (1073) contigs and 2057 singlets returned significant hits from GenBank, for a total of more than 3100 sequences. As expected, the great majority of these sequences correspond to structural and housekeeping genes, but a great number correspond to genes of potential interest as RNAi targets, including genes for cuticle formation, larval development, juvenile hormones, central nervous system development, eye morphogenesis and development, lipid and carbohydrate metabolism expressed in gut tissues and genes expressed specifically in salivary glands. Experiments are underway to begin assessing these potential RNAi targets.

Xylem specific promoter cloning.

The specific xylem promoter EgCAD2 was cloned from *Eucalyptus gunii*. The sequence was fused to the GUS reporter gene in the binary PCB 301 vector. Then, GUS expression driven by the xylem specific promoter was accessed in a transient *Agrobacterium tumefaciens* assay in *N. benthamiana* plants. Upon staining for GUS activity, results showed that blue product was restricted to the main vascular tissues. This gives confidence in this promoter, which will now be used to attempt to express specific interfering RNAs in the xylem of transgenic plants. Choosing which plant to use initially is difficult. However, citrus has been implicated as an important GWSS host plant in southern California, and Carrizo citrange is one of the plants easily transformed and manipulated at UC Davis. It also is a host of GWSS in our studies (**Figure 4**), thus it will allow us to rapidly test our hypothesis for xylem delivery of RNAi molecules.

CONCLUSIONS

Xf is an important bacterial pathogen of economically important crops such as grape, but also citrus and almond. The ability to minimize the economic impact of this bacterium depends on the presence and abundance of its biological vectors and GWSS is the most effective vector of *Xf* transmission in some agricultural areas. RNAi-based efforts directed toward the control of insect plant pests are now becoming more feasible, and RNAi for insects as GWSS has great potential application.

The results presented here show that RNAi can be induced both *in vitro* (GWSS -Z15 derived cell line) and *in vivo* in GWSS nymphs. We showed that GWSS -Z15 cells can be used to screen candidate gene silencing targets, and that since RNAi is active in cells, it could also be used to study GWSS gene function via mRNA knockdown. The mRNAs targeted for RNAi in this study were chosen from a limited number of sequences currently available for GWSS, but the same approach can be applied to the other genes identified in the analysis of the GenBank GWSS EST libraries. More notably, the employment of RNA silencing in whole GWSS insects could offer help towards a potential solution for control of the vector. Future work

includes the screening of more RNAi targets, the production of transgenic plants expressing dsRNAs in their xylem and the study of GWSS insects grown on the transgenic plants.

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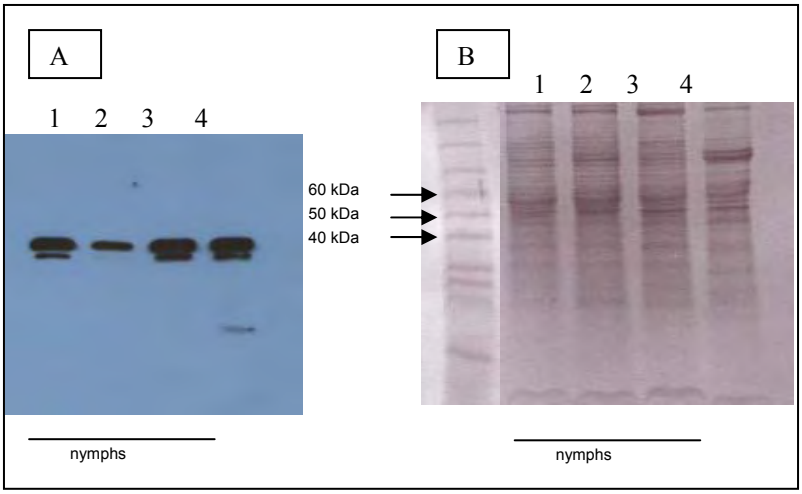

Figure 1. GWSS nymph injected with actin dsRNA shows decreased actin protein level. Fifteen third and fourth instar GWSS nymphs were injected with 1µg actin dsRNA in 1µl volume, with 1 µg GFP dsRNA or with 1 µl injection buffer and left on basil plants for five days. Then, proteins were extracted from three living and one dead insect and subjeted to Western blot analysis, using actin antibodies specific for *Drosophila melanogaster* . Results show a decrease in actin protein in the nymph injected with actin dsRNA and alive five days post injection (gel lane 2 panel A), compared to the other insects (gel lanes 1, 3 and 4 in panel A). Coomassie staining shows equal amounts of proteins loaded for each sample (panel B), 15 µg total proteins were loaded in each lane. Treatments: Lane 1: Actin dsRNA injected nymph, collected dead one day after injection. Lane 2: Actin dsRNA injected nymph collected dead five days after injection. Lane 3: GFP dsRNA injected nymph collected dead five day after injection. Lane 4: Uninjected adult.

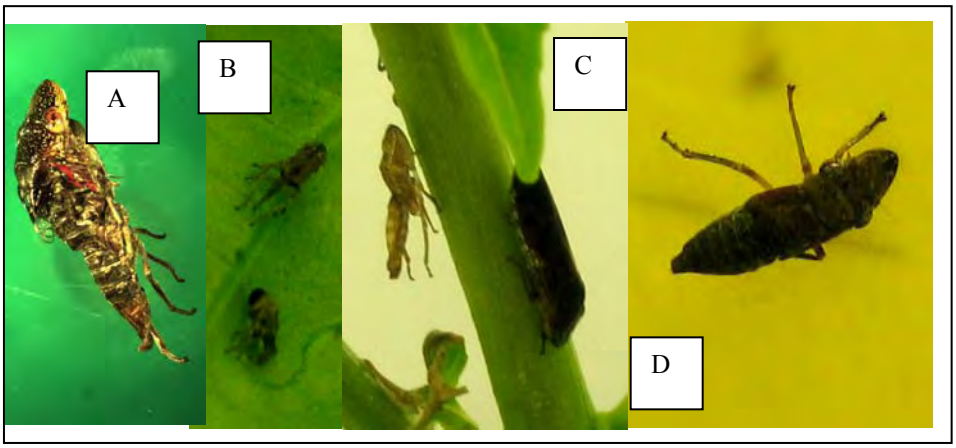

Figure 2. GWSS nymphs injected with actin dsRNA died during molting. Fifteen third and fourth instar GWSS nymphs were injected with 1ug actin dsRNA in 1ul, or with 1 ul injection buffer and left on basil plants for five days. During this period, two of the actin dsRNA injected insects couldn't complete molting and died. In panel A, one of the nymphs with incomplete molting is shown. In other panels the presence of exoskeletons on a basil leaf indicate the completion of molting in the observed group of nymphs (picture B). Shot of an exoskelton close to an adult that succesfully completed molting (picture C). Injected nymph showing a normal phenotype (picture D). Experiment was repeated three times with similar outcome.

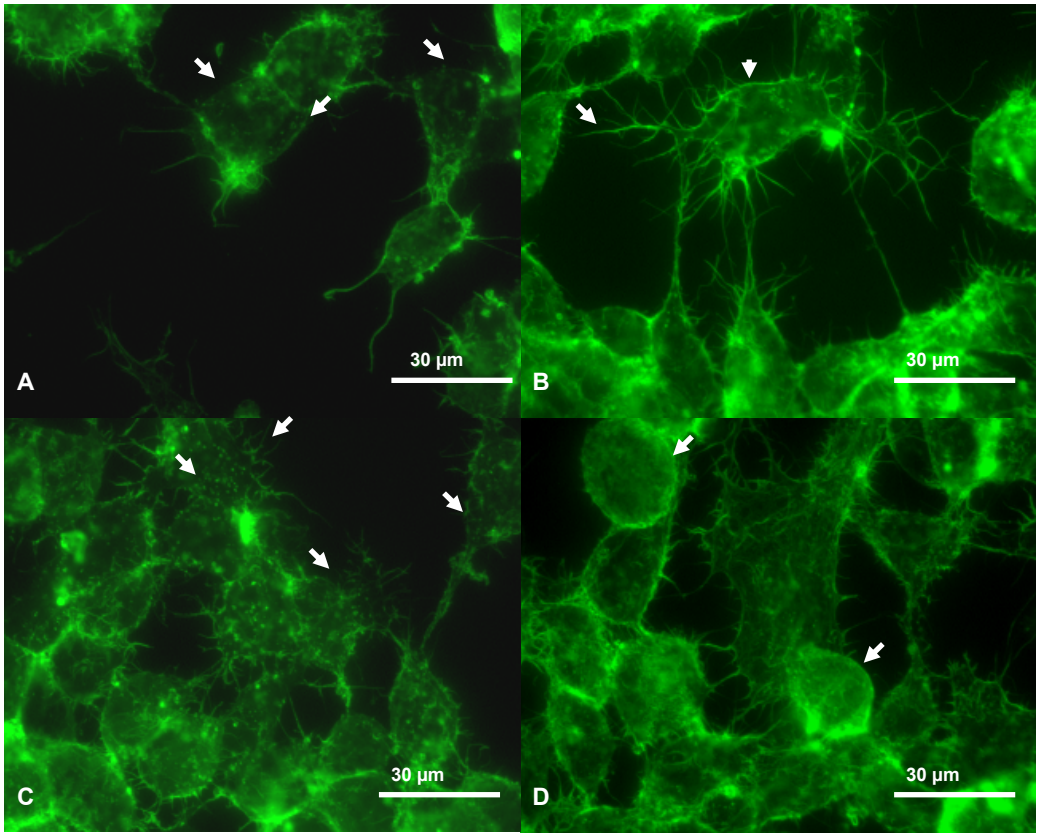

Figure 3. Actin representative morphology in GWSS -Z15 cells after transfection with actin dsRNA. Cells were transfected with 2 µg of actin dsRNA (A and C), or GFP dsRNA (B and D) and harvested 72 hpt. Actin filaments in the cell membrane and cytoplasmic area were largely disturbed (arrows in A and C). (A) GWSS cells showing partial disruption of the actin organization at the cell plasma membrane. Some filaments began to break and the cells failed to branch out. (B) GWSS cells showing no changes in actin filament distribution and polymerization. Healthy isolated cells were connected through a densely branched actin filament network. (C) GWSS cells showing severe disruption of actin filaments. The short fragments of actin filaments were scattered throughout the cytoplasm. Some actin fragments tended to aggregate into clusters below the plasma membrane and obvious twisted actin cables could be observed. (D) Actin filaments were found primarily in the cell cytoplasm as a continuous and organized net in the control cells. All observations were at 72 hours post treatment.

Figure 4. GWSS adults and nymphs feeding on Carrizo seedlings. Two GWSS adults and two nymphs were introduced in cylindrical plastic cages containing one month old Carrizo seedlings and left feeding for one week. After this period of time, all insects were alive and were feeding on the seedlings. Plants did not show any damage caused by the GWSS feeding.