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 towards the development and application of an RNA interference (RNAi) based system aimed to target genes of the vector of *Xylella fastidiosa*, the glassy-winged sharpshooter (GWSS). After demonstrating that RNAi induction in GWSS cells and insects is achievable, we began screening a large pool of candidate genes to find the best targets to control the survival of GWSS. These data were used to develop transgenic *Arabidopsis* and potato plants that express dsRNAs of the targets. We also made stable *Arabidopsis* transgenic plants that express GUS marker genes using 35S and a *Eucalyptus gunii* minimal xylem-specific promoter. Transgenic plants are being evaluated for their ability to produce dsRNAs and will be tested against GWSS adult insects. Encouraged by our efforts to find effective targets, we adopted large scale sequencing of the GWSS transcriptome as well as the small RNA complement from GWSS adult insects. We were able to generate 35 million reads and nine million reads of the short read sequence data for transcriptomic and small RNA sequences in our initial run.

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. The causal agent of the disease, *Xylella fastidiosa (Xf)*, is a xylem-limited bacterium that 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), *Homalodisca vitripennis*, and we combine the use of transgenic and next generation sequence based methods to effectively target GWSS RNAs. 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 towards 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 leafhopper *Homalodisca vitripennis* (Germar) (formerly *H. coagulata*), also known as 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) [1], a devastating disease occurring in winegrapes [2]. New strategies that will lead to environmentally sound approaches to control GWSS and other insect vectors of plant pathogens are needed. RNA interference (RNAi) has been suggested as a strategy to develop "insect-proof plants" [3] and even referred to as a "genetic insecticide" [4]. 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 [5]. 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* [6-7], *Blattella germanica* [8], *Drosophila melanogaster* [9], *Spodoptera litura* [10], *Culex pipiens* [11], *Lutzomyia longipalpis* [12], *Cecropia pupae* [13], *Acyrthosiphon pisum* [14], *Rhodnius prolixus* [15], *Aedes aegypti* [16], *Bemisia tabaci* [17], *Dermacentor variabilis* [18] and *Tribolium castaneum* [19]. Oral induction has also been demonstrated in several of these same species. Our present and previous efforts demonstrate for the first time that RNAi activity can be induced in a leafhopper species, but also is inducible in GWSS cell lines [20]. 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 we hope as a strategy to complement overall efforts for Pierce's disease control.

OBJECTIVES

The specific objectives of our effort are:

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

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 [20]. Real time RT-PCR, semi quantitative RT-PCR, and 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 [20]. The inducibility of RNAi in the GWSS cells helped us design the following set of experiments.

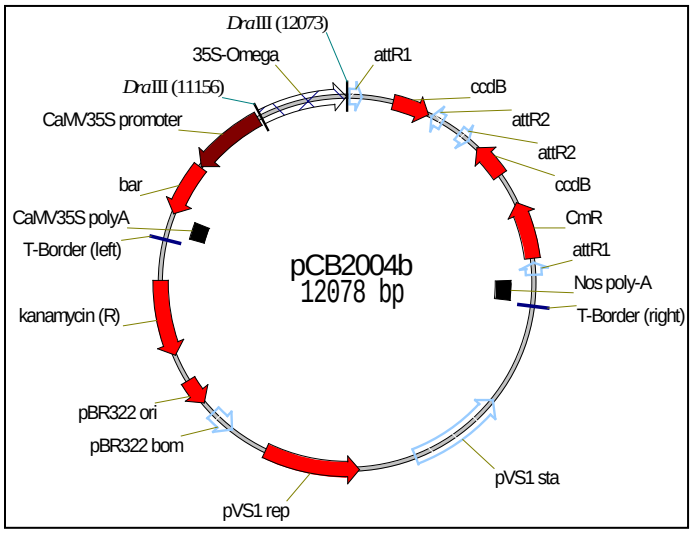

Figure 1. Diagrammatic representation of the vector pCB2004B used for generation of GWSS transgene constructs. The binary construct is designed to produce short hairpin between the sense and antisense target genes that will result in the production of small RNAs in the transgenic plants (*Arabidopsis* and potato plants).

Generation of transgenic lines

For the purpose of generating the *Arabidopsis* transgenic lines we used a different ecotype, Cape Verdi (Cvi). Compared to Columbia (Col-0) it has larger leaves and presents more robust growth, and will be more appropriate in supporting insects of large size such as GWSS. In order to generate dsRNAs that can target the insect, GWSS target sequences (**Table 1**) were cloned into a gateway-compatible binary vector pCB2004B (**Figure 1**). The target sequences were cloned in head to tail direction in the gateway vector with a non-homologous sequence between them. Upon transcription in transgenic plants, these constructs will yield double-stranded, hairpin RNAs of the desired sequence. The expression vectors carrying the insect target sequences of interest were first cloned into *E.coli* and *Agrobacterium tumefaciens* and they have been sequence verified. *A. tumefaciens* cultures carrying the sequences of interest were used to transform *A. thaliana Cvi* plant ecotypes through the floral dip process. Arabidopsis T_0 plants were screened for resistance against the selectable marker *BAR* gene, and we were able to confirm T_1 transgenics. Further sets of transformation of *Arabidopsis* plants were underway to generate more independent transgenic lines for the GWSS target genes that had less than three independent transgenic lines. Also, efforts are underway to generate more transgenic lines for other target genes of GWSS that were not previously described. We are in the process of obtaining the homozygous transgenic *Arabidopsis* lines that will be used for screening against GWSS.

We have used three of the constructs (**Table 2**) to transform potato plants. Transformation/regeneration was performed via recharge at the UC Davis Ralph M. Parsons plant transformation facility (http://ucdptf.ucdavis.edu/) and approximately ten

independent transgenic lines were obtained for each of the constructs. We have performed screening of these transgenic potato plants for insert composition and have established the presence of a transgene similar to the procedure as described for *Arabidopsis* transgenic lines. The presence of chitin deacetilase transgene in the potatoes resulted in the production of small RNAs in those transgenic plants. In contrast to the approach with *A. thaliana*, we will vegetatively propagate the T_0 plants and use them for RNAi experiments with GWSS. Potatoes are an excellent host plant for GWSS so we expect them to be very useful for our efforts here.

Table 1. GWSS insect sequences used for cloning and generation of *Arabidopsis* transgenic lines.

Construct Name*	Protein Encoded	Length of PCR Product (bp)	*E. coli* DH5-α Sequence Verified	*A. tumefaciens* EHA105 PCR Verified	Number of *Arabidopsis* transgenic lines generated
GWSS 965	Zinc Metalloproteinase	443	Yes	Yes	None
GWSS 989	Glucosyltransferase	576	Yes	Yes	3 independent lines
GWSS 1591	Sugar Transporter	668	Yes	Yes	One independent line
GWSS 1377	Serine Proteaseserpin	645	Yes	Yes	2 independent lines
GWSS 364	Trypsin	605	Yes	Yes	2 independent lines
GWSS 975	Transaldolase	800	Yes	Yes	3 independent lines
GWSS 366	Sugar Transporter	888	Yes	Yes	None
GWSS 500	Serpin	418	Yes	Yes	4 independent lines
GWSS 745	Trypsin	756	Yes	Yes	None
GWSS 512	Transketolase	1435	Yes	Yes	None

In addition to the promoter effects of the GWSS target genes under the 35S promoter, we have started generating the constructs under a specific xylem promoter EgCAD2 was cloned from *Eucalyptus gunii*. The sequence was fused to the GUS reporter gene in the binary pCB301 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. We have generated our initial set of transgenic plants in *Arabidopsis* which expresses the *GUS* gene under the xylem specific promoter, but we have not yet tested the T2 generation.

Table 2. GWSS insect sequences used for cloning and generation of potato transgenic lines in the variety Desiree.

Name (pCB2004B)	pedigree	variety	selection	Small RNA northerns	GSP F/R
Chitin deacitilase	102203	Kennebee\ Desiree	BAR	Produce small RNAs	primers giving multiple bands
GWSS actin	112064	Desiree	BAR	Not done	9 out of 11 tested are positive
GWSS cuticle	112073	Desiree	BAR	Not done	9 out of 9 are positive

Feeding assays

In addition to the transgenic plant approaches, based on recent reports in the literature (Killiny and Almeida, 2009, PNAS 106:22416) and personal communications from other scientists, we have evaluated *in vitro* feeding approaches for GWSS (**Figure 2**). If successful this will allow for much more rapid screening of candidate sequences for their abilities to induce RNAi effects via oral acquisition. We have a number of candidate sequences which we are testing for RNAi (**Table 3**). These are used for *in vitro* transcription and known quantities of dsRNAs are used for *in vitro* acquisition, followed by using three different *in vitro* oral acquisition approaches. These included using a modified membrane feeding approach based on Killiny and Almeida, a modified tygon tubing delivery system, and using basil infusion (basil stems directly inserted into dsRNA solutions). We have used the basil infusion in the past and it offers some advantages as well as disadvantages. Together, these approaches can supplement the transgenic plant approaches, and if we can make any consistent, this will allow us to rapidly screen target sequences without having to develop transgenic plants, thereby saving time and effort towards our ultimate goal.

Table 3. GWSS insect sequences used for cloning and generation of potato transgenic lines in the variety Desiree.

S.No:	List of GWSS target genes	Cloned into plasmid	dsRNA prepped
1	Actin	pGEMT-easy	Yes
2	Ferritin	pGEMT-easy	Yes
3	Ubiquitin	pGEMT-easy	Yes
4	Lian2	pGEMT-easy	Yes
5	SAR1	pGEMT-easy	Yes
6	Fructose1,6 aldolase	pGEMT-easy	Yes
7	RAB1	pGEMT-easy	Yes
8	Tropomyosin	pGEMT-easy	Yes
9	Delta 9 desaturase	pGEMT-easy	Yes
10	Mitochondrial porin		Yes
11	GFP		XX

Next Generation Sequencing of GWSS Adult Insects

The developmental regulation of insects through the use of small RNAs has been well studied. In our efforts to study the regulation of GWSS insect genes and identify RNAi targets, we took an alternate approach using high throughput parallel sequencing to identify the small RNAs from the GWSS insects. For our work, we noticed GWSS transcriptome data is lacking information for the identification of small RNA reads. To address this and identify the loci of the small RNAs that were originated from the short read sequencing, we sequenced the transcriptome of GWSS through the use of mRNA sequence methods as described in **Figure 2**. The sequencing of GWSS mRNA transcriptome was done through paired end sequencing on Illumina GA-II Platform. Both the mRNAseq library data and the small RNAseq library data were generated from the GWSS adult insects (**Figure 2**).

The sequencing reads from the transcriptomic data were assembled into scaffolds with a minimum size of 200 bases using Oases transcriptome assembler. We were able to assemble approximately 32.9Mb of the transcriptome across 47,265 loci and 52,708 transcripts. The average transcript length assembled was 624 nucleotides. Roughly 15 million of the total reads were found to be unique for the genome (**Table 4**) and 51% of the reads were incorporated into the assembly. The sequencing reads were then mapped back to the assembled transcripts with up to one mismatch. The reads that could not be mapped back to the reference assembly are being analyzed for the possible discovery of new viruses that may be infecting the GWSS insects. With the help of these sequencing reads, we aim to study the GWSS insect target genes and we hope to identify the small RNAs that target the GWSS target genes in a highly specific manner.

Table 4. Sequencing summary of the GWSS adult insect reads after the quality contro**l.**

Samples	No: of reads	Unique reads	%unique	%GC
mRNA seq-left	32,947,747	14,891,609	45.20%	46.56%
mRNA seq-Right	32,948,747	15,112,284	45.87%	46.68%
Small RNA seq	22,133,363	4,081,113	18.44%	55.59%

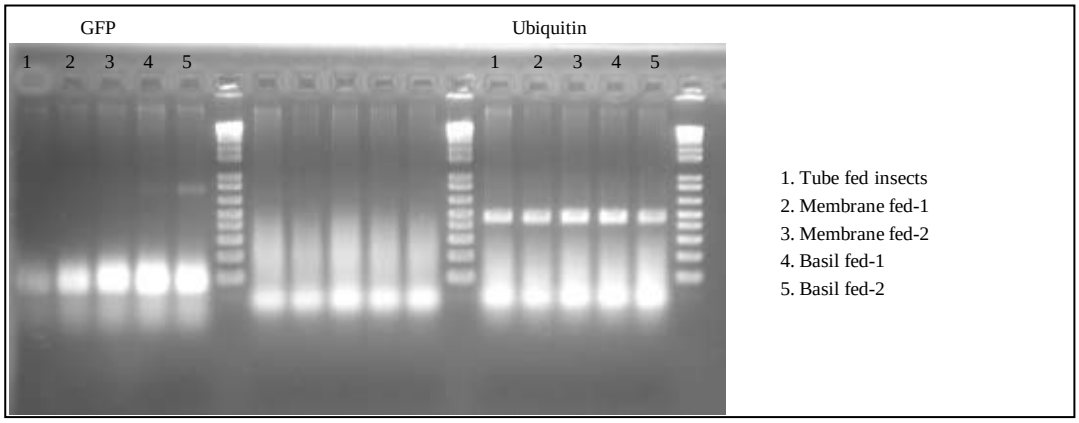

Figure 2. Comparison of three different feeding assays on GWSS insects. PCR amplification of GFP PCR product from the adult GWSS insects after they are fed with the GFP PCR product in either of the following forms: Tube feeding, Membrane feeding and Basil feeding.

CONCLUSIONS

Xf is an important bacterial pathogen of economically important crops such as grape, 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 such as GWSS has great potential applications. 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. 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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