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

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ABSTRACT
Here are presented the progress on a collaborative biotechnological work aimed to develop an RNA interference (RNAi) strategy designed to control sharpshooter vectors of *Xylella fastidiosa*, the causal agent of Pierce's disease. In the year 2007-2008 we constructed cDNAs corresponding to specific genes of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, and evaluated dsRNAs for their ability to induce RNAi effects against GWSS. We performed injection experiments in GWSS insects and evaluated effects by assessing target RNA degradation. Identified genes will be used to develop transgenic basil plants such that dsRNAs are expressed in xylem tissues via EgCAD2, a xylem-specific promoter. Transgenic plants will be evaluated for their ability to induce RNAi effects on GWSS.

INTRODUCTION
Pierce's disease (PD), caused by the xylem-limited bacterium, *Xylella fastidiosa* (*Xf*), is an important threat to the California grape industry (http://www.aphis.usda.gov/lpa/pubs/fsheet_faq_notice/fs_phglassy.html http://orsted.nap.edu/openbook.php?record_id=11060&page=21). The most important recent epidemic of PD in California was found to be associated with the introduction of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, an invasive sharpshooter leafhopper known to be indigenous to parts of the Southeastern United States (Blua et al., 1999). The GWSS is a large, robust leafhopper with a broad host range including many native, ornamental and crop plants. The combination of this new PD vector species, its wide host range, abundance of host plants, its affiliation for citrus as a host for reproduction, and its ability for long-distance dispersal (Blua and Morgan, 2003) has raised concerns that PD and GWSS are important threats to the California grape industry beyond the Temecula region.

In addition to being transmitted by GWSS, *Xf* is transmitted to plants by several other species of xylem-feeding leafhoppers (Redak et al., 2004). It is interesting to note that as opposed to phloem-feeding hemipterans, xylem feeders must ingest much greater volumes of plant sap. And it is the ingestion of large volumes of plant sap that offers the potential to deliver toxic molecules to leafhoppers, even if these molecules are produced in low concentration in xylem sap. This is an important component of our strategy.

We propose a new approach, one based on RNA interference (RNAi) directed towards GWSS. RNAi leads to sequence specific degradation of target RNA molecules within the cell cytoplasm, resulting in eliminating or reducing gene expression (mRNA degradation) or antiviral immunity (degradation of viral genomic RNAs) (Lu et al., 2004; Brodersen and Voinnet 2006). There are already several examples of practical implementation of RNAi-based technologies for agriculture. For example, RNAi-based strategies for conferring plant resistance to bacterial, nematode and virus induced plant diseases have been demonstrated, and some have been even used in commercial agriculture (Escobar et al., 2001; Huang et al., 2006; Gonsalves 1998). Recently, particular attention was devoted to RNAi efforts targeting insects. For example in the November 2007 issue of *Nature/biotechnology,* the "news and views" article was entitled "RNAi for insect-proof plants" (Gordon, K.H. and P.M. Waterhouse, 2007) and two research articles in that issue presented current RNAi efforts towards insect pests of plants (Baum, J.A., et al., 2007; Mao, Y.B., et al., 2007). In 2008, in *Trends in Biotechnology* another article was titled "RNAi-mediated crop protection against insects" (Price, D.R. and J.A. Gatehouse, 2008). Of relevance to this proposal is that RNAi offers opportunities for targeting *H. vitripennis* via RNAi-based disruption of essential GWSS genes, thereby resulting in insect deleterious effects.

OBJECTIVES
The objectives of our research effort are to develop new and effective, environmentally sound strategies for controlling the GWSS and other leafhopper vectors of *Xf*. Our goal is to develop strategies that are effective and will provide control for PD of grapes, but also have flexibility for use in other important California crops.

The specific objectives of our effort are:
1. To identify and develop RNAi-inducers capable of killing or reducing the survival and/or fecundity of GWSS.
2. To generate transgenic plants capable of expressing and delivering GWSS deleterious RNAi molecules within their xylem.
3. To evaluate transgenic plants for their ability to generate inducers capable of inducing RNAi vs. GWSS.

RESULTS AND DISCUSSION

For this effort we utilized in vitro and in vivo delivery systems. We assessed RNAi effects in cultured GWSS cells. We also screened dsRNAs in whole leafhoppers by injection, and we are optimizing dsRNA insect delivery by feeding in vitro.

Rearing GWSS and other PD vectors. A colony of GWSS insects was collected from Riverside and donated to us by Dr. R. Almeida (UC Berkeley). The colony was transferred into the Controlled Research Facility (CRF) at UC Davis and insects were reared there for more than one year. We were able not only to keep the original colony in good condition, but also to rear continuously new generations of GWSS. Dr. Almeida also provided us with a colony of *Draeculacephala minerva (D. minerva)*. We decided to rear these insects because they are not quarantined in northern California, they are easy to rear and they are California native vectors of PD. As for GWSS, we were able to maintain and rear colonies of *D. minerva*.

GWSS cells. Since GWSS insects are quarantined in Northern California, we decided to test the effect of RNAi in the GWSS cells. We received these cells from the Dr. Bruce Hammock laboratory (Kamita et al., 2005, GWSS cell line Z15). We were able to optimize a cell transfection protocol with an efficiency higher than 50%, as measured using a siRNA fluorescein labeled control. Cell viability was measured with trypan blue staining (data not shown). Alexa-Fluor 488 phalloidin (Invitrogen) was used to label F- actin of GWSS cells grown and fixed on glass slides. This labeling procedure allowed a first evaluation of actin integrity and structural appearance in GWSS cells transfected with actin dsRNA (**Figure 1).**

Choice of dsRNA inducers: Fourteen GWSS nucleotide sequences, derived from EST based nucleotide sequences available in GenBank and translatable in putative proteins, were used to design gene specific primers and to generate cDNAs from GWSS cell line Z15. The same approach was used for *D. minerva*. Corresponding sequences were amplified by RT-PCR, cloned and sequenced to confirm their identity.

cDNAs of actin, arginin kinase, lian 2 (a non-LTR retrotransposon) and sar1 mRNAs expressed in the GWSS cell line and *D. minerva* insects were cloned in pGMTeasy vetor in both orientations relative to the T7 RNA polymerase promoter, and sequenced. The vectors were used for T7 RNA polymerase-mediated in vitro transcription to generate specific dsRNAs (Ambion, dsRNA MegaScript). These dsRNA were delivered via transfection in GWSS cells, and two of them (sar1 and actin) via injection in GWSS insects.

Evaluation of RNAi effects in GWSS cells and whole insects. Realtime RT-PCR primer/probe sets were designed and tested via real time RT-PCR assays of GWSS cell derived RNA.

RNAi in GWSS cells. Real time RT-PCR was used to measure the amount of target mRNA in GWSS cells.
Actin mRNA was first used as the RNAi target in GWSS cells. Upon cell transfection with actin dsRNA, a reduction of the corresponding mRNA was observed, indicating effective RNAi in cells (data not shown). In a time course experiment, actin dsRNA and actin hairpin loop (cloned in the Gateway pMT-Dest 48 plasmid, Invitrogen) were used in cell transfection. The strongest RNAi effects were observed upon dsRNA delivery at 72 hours post transfection (hpt**) (Figure 2**).

When cells were transfected with actin dsRNA, siRNA and the actin hairpin loop plasmid to identify the best effector for RNAi, the most efficient RNAi inducer proved to be actin dsRNA (data not shown). SiRNA was found to be a good alternative RNAi inducer.

Actin and sar1 mRNAs were compared as targets for RNAi, via transfection of GWSS cells with actin and sar1 dsRNA respectively. In these experiments, sets of GWSS cells were also transfected with GWSS arginin kinase and lian2 dsRNAs as control of genes expressed in GWSS; and gfp dsRNA as exogenous control. RNAi was evaluated 72 hpt. Experiments were repeated three times. Of these, twice the cell transfection was performed in three replicates and once in two duplicates. Real time RT-PCR samples were always loaded in duplicates. As result, actin dsRNA seems to be a better RNAi inducer than sar1 dsRNA (**Figure 3**).

RNAi in GWSS insects. RNA interference was experimentally assessed in GWSS insects. Sets of 15 nymphs were injected with 1µg of dsRNAs (gfp, actin or sar1) or buffer, and groups of 5 insects were sacrificed 1 and 3 dpi. Total RNA was extracted from the insects and the amount of the mRNA was measured by two methods, real time RT-PCR and semi-quantitative RT-PCR. Experiments were repeated three times. Semi-quantitative RT-PCR and real time RT-PCR results were comparable, and both confirmed that injection of dsRNA of corresponding endogenous genes in insects produced a reduction of the mRNA, indicating RNAi in insects (**Figures 4 and 5).**

Xylem specific promoter cloning. We cloned the full length EgCAD2 xylem specific promoter from *Eucalyptus gunii* in pGEMTeasy, and we are subcloning this promoter into the binary vector AKK 1431, obtained from Govindarajulu Manjula, (C.G. Tylor lab Donald Danforth Plant Science Center, St. Louis). The plasmid contains the GUS gene. Our contructs will be evaluated in transgenic basil plants and then the GUS sequence will be replaced by an RNAi inducer as identified above.

CONCLUSIONS

RNAi effects have been demonstrated in GWSS cells and insects after delivery of dsRNA, and GWSS cells can be used to screen candidate gene silencing targets. This study provides the evidence that RNAi might be useful as part of the overall strategy to control *Xf* leafhopper species and to break the cycle between bacterial diseases and their hosts. Future work includes the identification of suitable RNAi targets, the production of transgenic plants expressing dsRNAs in their xylem and the study of the fate of ds/siRNA delivery in insects after feeding.

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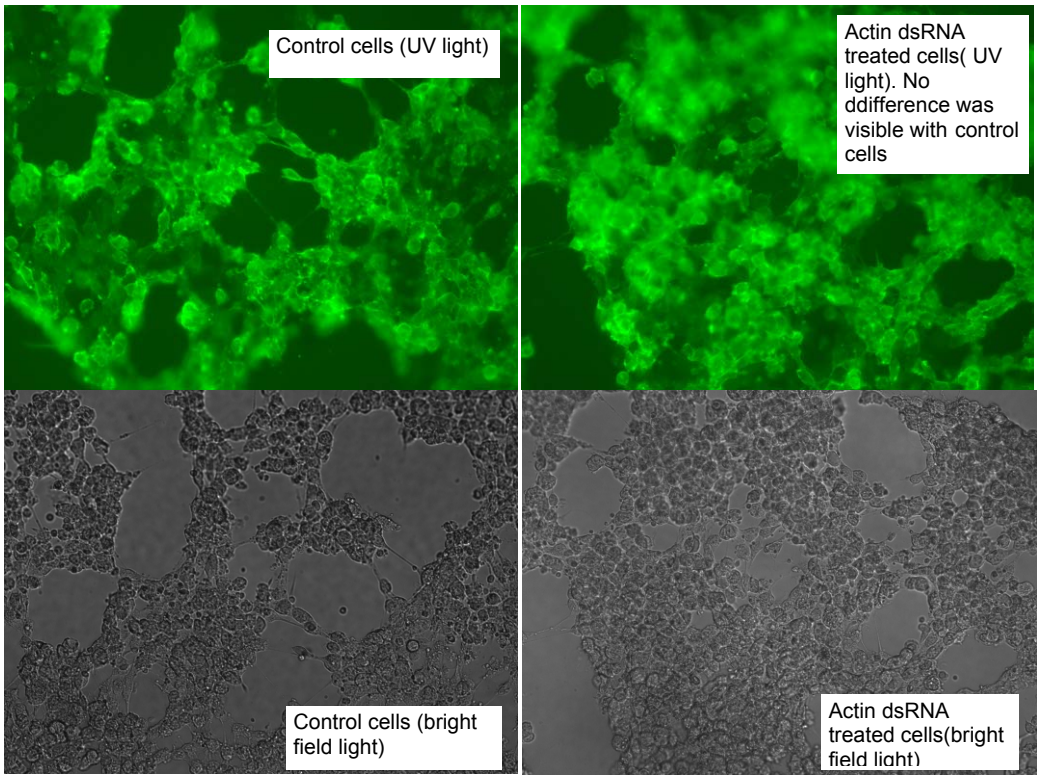

Figure 1. Microscope observations at 24 hpt. GWSS cells were grown on a glass slide and treated with Alexa-Fluor 488 phalloidin (Invitrogen).

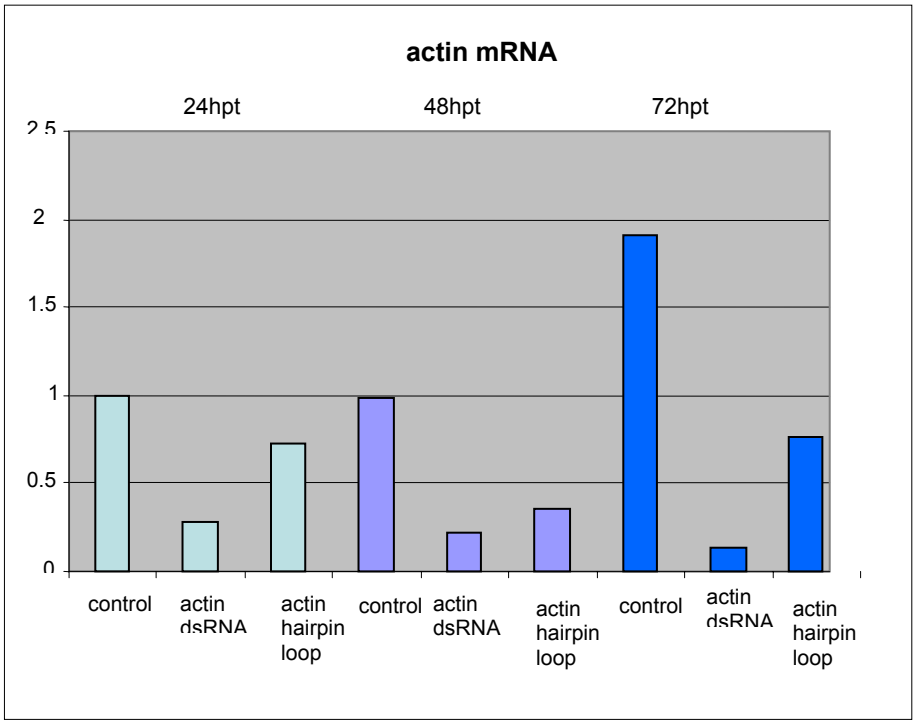

Figure 2. Cells transfected with dsRNA actin, or the actin hairpin loop plasmid were harvested 24, 48 and 72 hpt and their actin mRNA levels were quantified by real time RT-PCR. Actin mRNA was reduced the most at 72hpt.

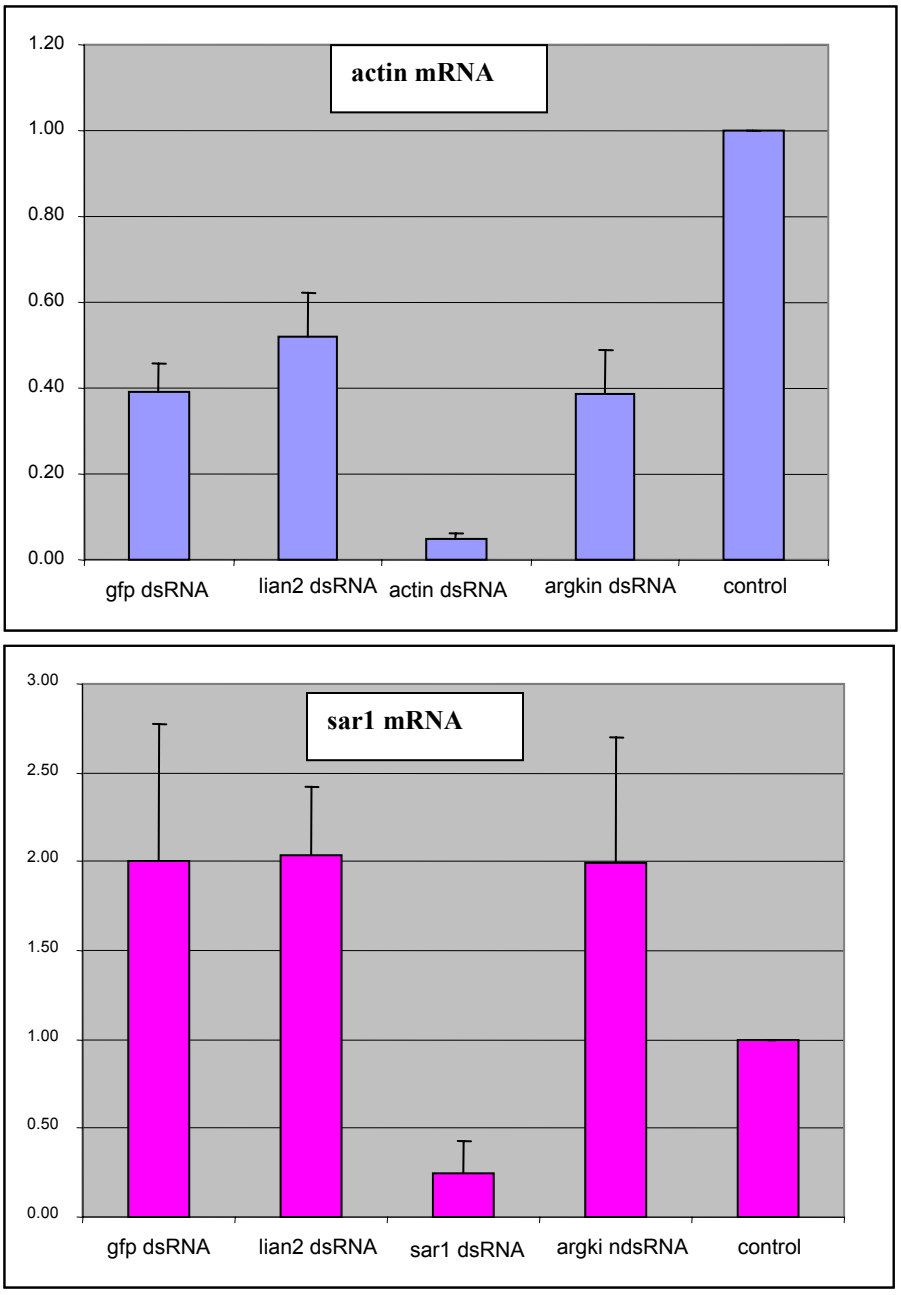

Figure 3. Actin, lian2, arginin kinase, and GFP dsRNAs (upper panel) and sar1, lian2, arginin kinase and GFP dsRNAs (lower panel) were transfected in GWSS cells. Cells were harvested 72 hpt and the level of sar1 or actin mRNAs were quantified by real time PCR. RNAi was reached as proven by the sar1 and actin mRNA reduction in sar1 and actin dsRNA transfected cells, compared to cells treated with transfection reagent only (control). Transfection of cells with actin dsRNA generated a better RNAi response compared to transfection of cells with sar1 dsRNA. Data were generated in three biological independent experiments, with 8 replicates total. Error bars above the columns indicate the standard deviation among the 8 replicates.

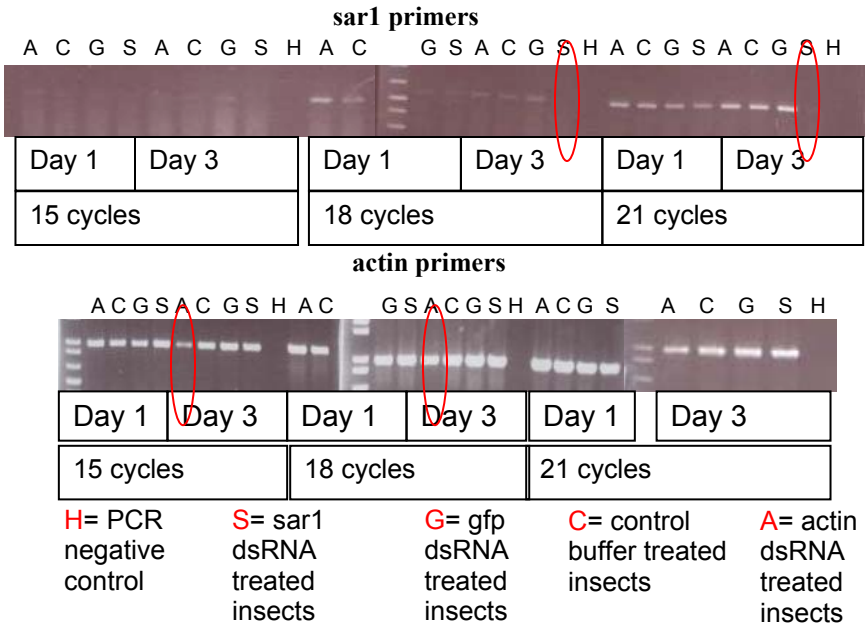

Figure 4. Gel representative of three separate experiment results. Semi-quantitative RT-PCR results, showing the RNA level in insects injected with 1µg of dsRNAs. Insects were collected 24 and 72 hour post injection. Each RT-PCR was performed using 100 ng total RNA. The PCR was stopped after 15, 18 and 21 cycles.

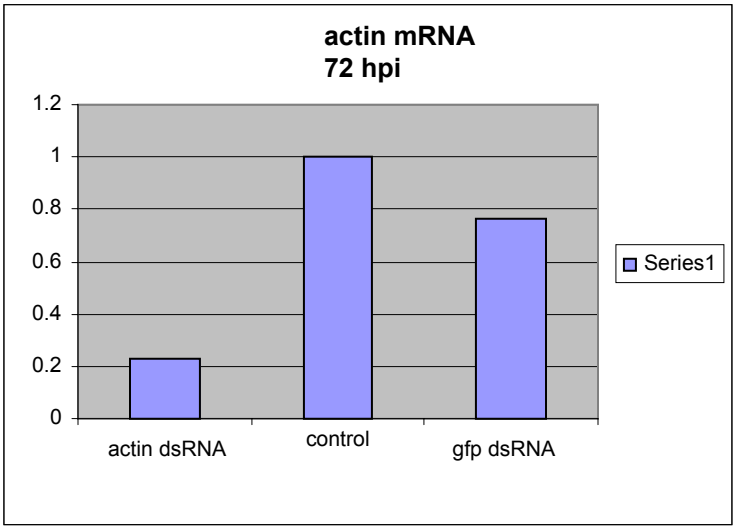

Figure 5. Real time RT-PCR on same samples as above.