RNA-INTERFERENCE AND CONTROL OF THE GLASSY-WINGED SHARPSHOOTER AND OTHER LEAFHOPPER VECTORS OF PIERCE'S DISEASE

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ABSTRACT

We initiated a collaborative biotechnological effort to develop an RNA interference (RNAi) strategy to target and control sharpshooter vectors of *Xylella fastidiosa*, the causal agent of Pierce's disease. We have constructed cDNAs corresponding to specific genes of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, and for two genes, actin and SAR1, have generated dsRNAs *in vitro*. We will evaluate these dsRNAs for their ability to induce RNAi effects against GWSS using cells as well as whole leafhoppers. We have established GWSS cells in culture (with help from Drs. George Kamita and Bruce Hammock) and have established reproducing colonies of the GWSS insects (with help from Dr. R. Almeida) in the UC Davis Contained Research Facility. Initial experiments have shown that we can deliver and express genes in GWSS cells.

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 and http://orsted.nap.edu/openbook.php?record_id=11060&page=21). Although PD has been recognized in California for at least a century, recent events have shown the destructive potential of this disease in the California grape industry. For example, an epidemic of PD devastated commercial grapes in the Temecula region of Southern California beginning in 1997 (Blua et al., 1999). This epidemic was found to be associated with the introduction into California of the 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. One of the preferred hosts in Southern California and other areas is citrus (Adlerz 1980; Blua et al., 1999). 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 *Homalodisca vitripennis*, *Xf* is transmitted to plants by several other species of xylem-feeding leafhoppers (see Redak et al., 2004). When sharpshooter vectors acquire *Xf* from the xylem of *Xf*-infected plants, bacterial cells form a biofilm and attach in a polar fashion in the foregut (Newman et al., 2004). There, *Xf* cells multiply within the leafhopper foregut, establishing essentially a lifelong infection within the leafhopper vector. It is interesting to note that as opposed to phloem-feeding hemipterans, xylem feeders must ingest much greater volumes of plant sap. This is because xylem sap is much less nutrient rich than is phloem sap, and thus greater volumes are required to yield necessary amounts of nutrients (Milanez et al., 2003; Redak et al. 2004). However, because such large volumes are ingested, this offers the potential to deliver toxic molecules to leafhoppers, even if they are produced in low concentration in xylem sap. This is an important component of our strategy.

The complex interactions between host plants, leafhopper vectors and *Xf* make controlling PD in grapes difficult. Genetic resistance to *Xf* or its leafhopper vectors is not yet generally or universally effective in commercial grape production. However, several new strategies are being investigated for developing new resistance approaches. Some of these include several biotechnological approaches that may directly affect *Xf* or its leafhopper vectors. Here we propose a new approach, one based on RNA interference (RNAi) directed towards *Homalodisca vitripennis*.

OBJECTIVES

1. To identify and develop RNAi-inducers capable of killing or reducing the survival and/or fecundity of *Homalodisca vitripennis.*
2. To generate transgenic plants capable of expressing and delivering *Homalodisca vitripennis* deleterious RNAi molecules within their xylem.
3. To evaluate transgenic plants for their ability to generate RNAs capable of inducing RNAi vs. *Homalodisca vitripennis*.

RESULTS

Objective 1 - To identify and develop RNAi-inducers capable of killing or reducing the survival and/or fecundity of *Homalodisca vitripennis*

For this effort we will utilize *in vitro* and *in vivo* delivery systems. We will assess RNAi effects in cultured *Homalodisca vitripennis* cells as well as in whole leafhoppers. Drs. George Kamita and Bruce Hammock, UC Davis, Entomology, generously supplied these cells (see Kamita et al., 2005, GWSS cell line Z15) and have greatly assisted us in learning how to maintain and manipulate them. We have also established reproducing colonies of GWSS in the UC Davis Contained Research Facility and will perform experiments using cultured cells as well as whole insects. *Homalodisca vitripennis* insects were collected from southern California and donated to us by Dr. R. Almeida (UC Berkeley). The GWSS were transferred into the CRF facility at UC Davis and have been maintained there for more than two and a half months. So far, we have been able to establish four colonies, but also to rear new generations of GWSS. The young colonies appear to be parasitoid free.

Choice of dsRNA inducers

Fourteen *Homalodisca vitripennis* 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. Corresponding sequences were amplified by RT-PCR (Figure 1). Two of the above mentioned sequences, corresponding to the vitellogenin and rhodopsin genes, could not be amplified from the *Homalodisca vitripennis* cell line mRNA pool. This may be because these genes are expressed only in whole insects. cDNAs of three actin mRNAs and SAR1 mRNA expressed in the *Homalodisca vitripennis* cell line were cloned in pGMTeasy vector in both orientations downstream of the T7 RNA polymerase promoter, and sequenced. The vectors were directly used for T7 RNA polymerase-mediated *in vitro* transcription to generate specific dsRNAs (Ambion, dsRNA MaxiScript; see Figure 2). These dsRNA will be delivered via transfection into GWSS cells, and via injection into *Homalodisca vitripennis* whole insects.

Cell transfection system

GWSS line Z15 cells were transfected with a plasmid expressing GFP under the control of an inducible insect promoter (kindly provided by Dr. Shou-wei Ding, UC Riverside). "DOTAP," "FuGene HD" transfection reagents from Roche and "Cellfectin" transfection reagent from Invitrogen were compared for their ability to assist in transfecting GWSS cells. Manufacturer protocols were followed in all the transfection experiments. The maximum transfection efficiency (equal to 5%) was obtained using the Cellfectin transfection system (Figure 3).

Realtime RT-PCR primers/ probe sets were designed and tested using real time RT-PCR assays of GWSS cell derived RNA. The resulting amplifications plots were specific (Figure 4). This system will be used to measure the amount of SAR1 and actin mRNAs in transfected cells and whole *Homalodisca vitripennis* insects, following RNAi delivery. Since the assays will be performed using only one gene per experiment, the second gene will be used as an endogenous control.

We are attempting to develop a microscopic means to assess RNAi effects in GWSS cells also, and complement our real time RT-PCR efforts. This is based on actin development/staining assays. Figure 5 shows light microscopic, fluorescent analysis of actin when GWSS cells are grown on glass cover slips.

CONCLUSIONS

During the first three months of this project, we were able to successfully rear *Homalodisca vitripennis* insects, to amplify and clone a pool of GWSS specific gene sequences, to produce dsRNAs for two genes and to develop different assays to test the outcome of RNAi experiments in *Homalodisca vitripennis* cell lines and whole insects. We believe that we are in an excellent position to move on to experiments within objective 1, and evaluate GWSS cells and whole insects for RNAi effects.

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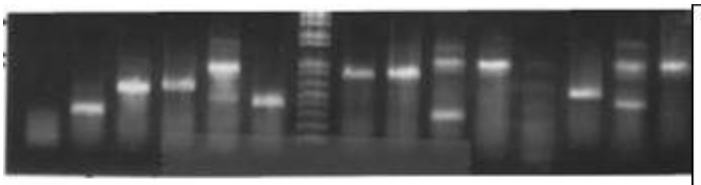


Fig. 1. 1.5% agarose gel: one tube RT-PCR products using gene specific primers designed based on GenBank available sequences.From left to right, products represent vitellogenin, Histone 3, SAR 1, RAB1 1, Kinase creceptor, Ubiquitin conjugatine enzyme, 1 Kb marker, tropomyosin, Micotchondrial porin, Delta 9 saturase 1, Fructose 1,6 biphosphate aldolase, rhodopsin, ferritin, Arginine kinase, Actin.

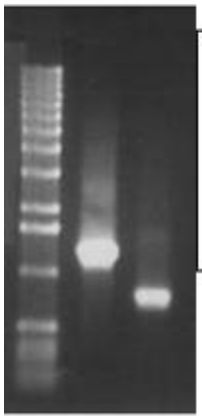
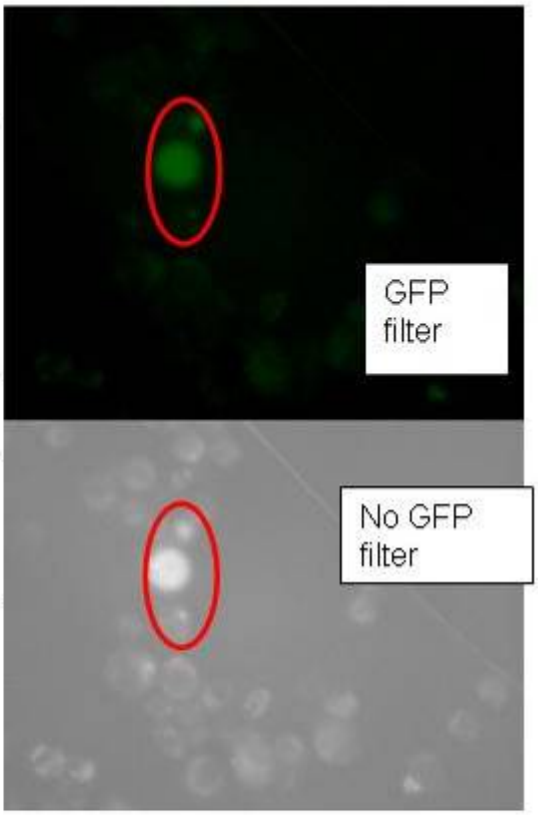


Fig. 2. In vitro transcrtipts were annealed to dsRNA and analyzed by agarose gel electrophoresis. Lane 1 = markers, 2 = actin dsRNA and 3 = SAR1 dsRNA.

Fig. 3. GWSS cells at right were electroporated with a GFP-expressing plasmis. Top shows fluorescent cells, bottom shows light visualization. Maximum efficiency so far is 5%.



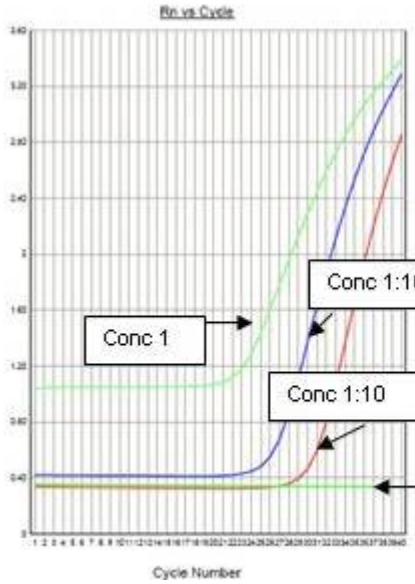


Fig. 4. Real time PCR using SYBRGreen and Actin primers.

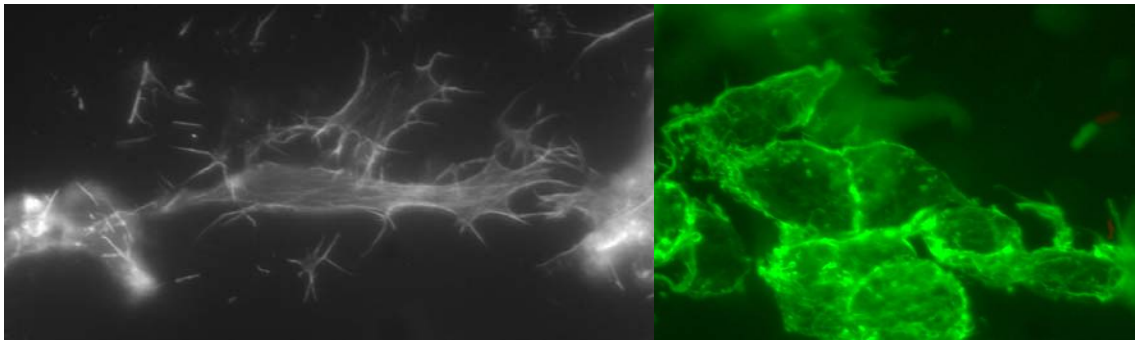


Figure 5. Image of GWSS cells grown on a glass cover and stained with phalloidin. Using transmitted light, actin filaments are white. However when examined with a UV filter, actin filaments are green. Fibroblast like cell is visible in the middle.