BETA-1,4 GLUCANASE IN GLASSY-WINGED SHARPSHOOTER SALIVA AND ITS POSSIBLE ROLE IN INFECTION AND MOVEMENT OF *XYLELLA FASTIDIOSA*

Project Leaders:

Elaine A. Backus
USDA, ARS, PWA
SJVASC
Parlier, CA 93648
elaine.backus@ars.usda.gov

John M. Labavitch
Department of Plant Sciences
University of California
Davis, CA 95616
jmlabavitch@ucdavis.edu

Researchers:

Kim Kingston
Department of Plant Sciences
University of California
Davis, CA 95616

Holly Shugart
USDA, ARS, PWA
SJVASC
Parlier, CA 93648

L. Carl Greve
Department of Plant Sciences
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted October 1, 2006 to September 30, 2007.

ABSTRACT

The purpose of this project is to determine whether β-1,4 glucanase (EGase), the major enzymatic protein in the watery saliva of glassy-winged sharpshooter (GWSS), co-localizes via immunocytochemistry with the few 'pioneer' *Xylella fastidiosa* (*Xf*) cells that are inoculated into a plant by this vector. If it does, then this suggests that watery, enzymatic saliva of the vector is a carrier of the bacteria during inoculation, and that therefore the saliva might somehow aid in this process. This year, we: 1) completed and tested the development of a method to retain fluorescence of *Xf* cells that are transformed to express green fluorescent protein (GFP) through the paraffin-sectioning process, 2) dissected an additional 1500 pairs of salivary glands for protein extraction, 3) extracted and purified EGase, and 4) contracted with a commercial company to produce antibodies to it. Work in the coming year will complete the objectives by using secondary antibodies to the EGase and confocal laser scanning microscopy to co-localize the EGase with GFP *Xf*.

INTRODUCTION

Many researchers, including ourselves, are investigating how *Xf* moves from cell to cell**,** because it is a crucial mechanism for the earliest stages of infection. It has been hypothesized that *Xf* can cause Pierce's disease (PD) only if bacteria can 'break out' of the initial, imprisoning inoculation xylem vessel(s) to produce a systemically increasing population (Hopkins 1989). Thus, *Xf* lateral movement through adjacent vessels is one determinant of initial infection success. Recent evidence by Labavitch and colleagues (Roper et al. 2007) studying *Xf* movement through stems supports the idea that pit membranes limit bacterial movement. A sufficient quantity of cell wall-degrading enzymes can break down parts of the primary cell wall network of the pit membrane, allowing bacteria to pass. Mature *Xf* populations produce the enzymes polygalacturonase (PG) and β-1,4 glucanase (EGase, often identified as cellulase in the literature) (Roper et al. 2007). Thus, in later stages of infection, those enzymes may function to facilitate systemic movement. However, fewer than 200 cells are typically inoculated by sharpshooters (Hill and Purcell 1995). It seems to us, therefore, that these few pioneer bacteria first inoculated by a vector are unlikely at first to produce a sufficient titer of enzymes to digest through the pit membrane.

It is routine to histologically image sheath saliva in fed-upon plants (e.g. Leopold et al, 2003, Backus et al 2005). However, no researcher has ever *directly* visualized watery saliva in plants, due to its fluid and dispersive nature. Yet Backus and colleagues have defined electrical penetration graph (EPG) waveforms that represent salivation and ingestion (Joost et al. 2006; Dugravot et al. 2008). Histology of salivary sheaths in probed plant tissues, correlated with EPG waveforms, revealed the cell types into which saliva is injected (Backus et al. 2005). Watery saliva is mixed with and spreads out from the salivary sheath, in all plant cells penetrated by the stylets, including xylem. Labavitch and colleagues recently have found very high activity of cell wall polymer-degrading enzymes, especially EGase, in GWSS salivary gland fractions (unpublished data). Thus, cell wall-degrading salivary enzymes are injected into xylem along with very few bacterial cells during *Xf* inoculation by GWSS. This finding led Backus to hypothesize that the small number of pioneer bacteria initially inoculated are aided in their cell-to-cell movement (therefore their ultimate infection) by the enzymatic salivary secretions of their vector.

OBJECTIVES

1. Purify and characterize β-1,4 glucanase (EGase), a putatively cell wall-degrading salivary enzyme of GWSS, and develop antibodies for *in planta* localization of saliva.
2. Determine whether GWSS salivary proteins (injected into grape during EPG-controlled insect feeding) affect the distribution of recently inoculated *Xf*, as detected systemically by PCR and locally by immunocytochemistry.

RESULTS

Objective 1 – Purify and characterize β-1,4-glucanase and develop antibodies

Study a: Enzyme purification and characterization

Over 1500 paired salivary glands were successfully dissected by Backus and colleagues from wild GWSS field-collected on ornamental shrubs in Bakersfield, CA, during June and July, 2007. Glands were frozen in extraction buffer at -20 ^{o}C and hand-carried from Fresno to Davis. Protein extraction, purification, and assaying of EGase were performed in the lab of Labavitch by Greve in August to September, 2007, and purified EGase was delivered to Antibodies, Inc., in Davis, CA. Polyclonal antibody serum will be raised in guinea pig by late November, then purified in the Labavitch lab, for later Objective 2 work by Kingston planned for January 2008. At present, enzyme characterization is scheduled for spring, 2008.

Objective 2 – Determine whether GWSS salivary proteins affect the presence/distribution of inoculated *Xf*

Studies a and b: Immunocytochemistry of probes by clean vs. GFP-Xf inoculative GWSS

Our ultimate goal for this objective is to combine five challenging procedures into one experiment with the following steps:

1) Allow one group of GWSS to acquire *Xf* expressing green fluorescent protein (hereafter, GFP-*Xf*) *(*Study a) and another (control) group to remain non-inoculative (Study b), then…
2) EPG-record a single, standardized probe consisting of pathway followed by ingestion lasting no more than 3 – 6 min, as described in Backus (2006), then…
3) Excise, histologically prepare, and section the fed-upon grape tissue, using methods that retain fluorescence of GFP, then…
4) Probe the sectioned tissue with primary antibody to EGase (from Objective 1) then secondary, fluorescently conjugated antibody, and finally…
5) Use confocal laser scanning microscopy (CLSM) to simultaneously locate and image autofluorescent salivary sheaths and cell walls, GFP-*Xf*, and fluorescently-stained EGase/watery saliva.

Time permitting, these studies may also include a time-course in which fed-upon plants are held for varying time periods before excision and preparation for microscopy. In this way, we hope to visualize the location of both watery saliva (i.e. EGase) and sheath saliva in relation to presence, location and movement of *Xf* bacterial cells, during certain EPG waveforms.

This year, Backus and Shugart made further progress developing each of the individual protocols to be combined in the larger test. In particular, we completed development of an all-new method for retaining the fluorescence of GFP *Xf* throughout the entire procedure for classical histology fixation, embedding in paraffin, sectioning and examination. An example of GFP-*Xf* in grapevine is shown in Figure 1. Such a procedure has never been accomplished before, by any research group using GFP, anywhere.

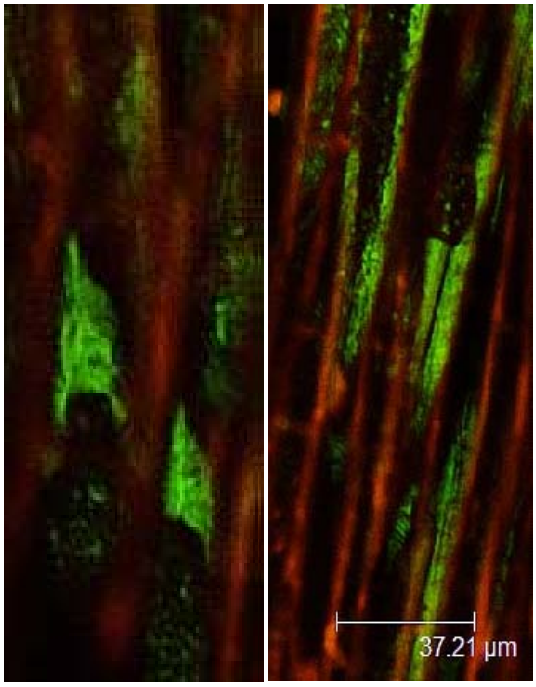

Figure 1. Green *Xf* revealed in paraffin-embedded and longitudinally-sectioned stems from mechanically inoculated grape, at the site of inoculation. **Left:** High magnification view of two xylem vessel elements completely occluded with green *Xf*. **Right:** Lower magnification view of multiple vessels with bacterial biofilm.

CONCLUSIONS

The described findings support the following hypotheses, which will be further tested this year: 1) watery saliva is injected during the earliest stages of stylet penetration, as well as further along the pathway and into a xylem ingestion cell, 2) GFP-*Xf* exit the stylets during all parts of the probe, and become embedded in the salivary sheath, as well as injected directly into xylem cells, and 3) the bacteria move into areas first traversed by the watery saliva. Findings from this study will help solve the PD/GWSS problem by opening up all-new avenues for transgenic host plant resistance. Novel transgenes could be developed by engineering an inhibitor of the salivary components that aid inoculation. In addition, differences in vector efficiency among GWSS populations, or numerous other vector species, could be related to salivary enzyme composition. Testing of other vector species (e.g. Brazilian vectors of Citrus Variegated Chlorosis, or vectors of Oleander and Almond Leaf Scorches) could aid understanding of the epidemiology of all *Xylella* diseases.

REFERENCES

Backus, E. A., J. Habibi, F. Yan, M. R. Ellersieck. 2005. Stylet penetration by adult *Homalodisca coagulata* on grape: Electrical Penetration Graph waveform characterization, tissue correlation, and possible implications for transmission of *Xylella fastidiosa*. Ann Entomol Soc. Am. 98: 787-813.

Backus, E. A. 2006. Where, when and how do ingestion and other feeding behaviors of the glassy-winged sharpshooter allow inoculation of *Xylella fastidiosa*? Proc. 2006 Pierce's Disease Research Symposium.

Dugravot, S., E. A. Backus, B. Reardon, and T. A. Miller. 2008. Correlations of cibarial muscle activities with EPG waveforms and excretion in sharpshooter (Hemiptera: Cicadellidae: Cicadellinae) vectors of *Xylella fastidiosa*. J. Insect Physiol. *In press.*

Hill, B. L., A. H. Purcell. 1995. Acquisition and retention of *Xylella fastidiosa* by an efficient vector, *Graphocephala atropunctata*. Phytopathol. 85: 209-212.

Hopkins, D. L. 1989. *Xylella fastidiosa*: Xylem-limited bacterial pathogen of plants. Ann. Rev. Phytopathol. 27: 271-290.

Joost, P. H., E. A. Backus, D. J. W. Morgan, F. Yan. 2006. Specific stylet activities by the glassy-winged sharpshooter, *Homalodisca coagulata* (Say), are correlated with AC EPG waveforms. J. Insect Physiol. 52: 327-337.

Leopold, R. A., T. P. Freeman, J. S. Buckner, D. R. Nelson. 2003. Mouthpart morphology and stylet penetration of host plants by the glassy-winged sharpshooter, *Homalodisca coagulata.* Arth. Struc. Func.32: 189-199.

Roper, M. C., L. C. Greve, J. G. Warren, J. M. Labavitch, and B. C. Kirkpatrick. 2007. *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in *Vitis vinifera* grapevines. Molecular Plant Microbe Interactions 20: 411-419.

FUNDING AGENCIES

Funding for this project was provided by the University of California Pierce's Disease Grant Program.