IMMUNOHISTOCHEMISTRY OF β -1,4-GLUCANASE, THE MAJOR ENZYMATIC COMPONENT OF GLASSY-WINGED SHARPSHOOTER SALIVA, IN PROBED GRAPE PETIOLES

Principal Investigator:
Elaine A. Backus
Crop Diseases, Pests, & Genetics
USDA, ARS, PWA
Parlier, CA 93648
elaine.backus@ars.usda.gov

Co-Principal Investigators:
John M. Labavitch
Department of Plant Sciences
University of California
Davis, CA 95616
jmlabavitch@ucdavis.edu

Kim Kingston*
Department of Plant Sciences
University of California
Davis, CA 95616
kim.kingston@dpi.vic.gov.au

Researchers:
Holly Shugart
Crop Diseases, Pests, & Genetics
USDA, ARS, PWA
Parlier, CA 93648

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

David Morgan
Calif. Dept. of Food & Agriculture
Mt. Rubidoux Field Station
Riverside, CA 92501

*Present address: Department of Primary Industries, RMB 1145 Chiltern Valley Rd., Rutherglen, VIC 4685, Australia.

Reporting Period: The results reported here are from work conducted October 1, 2007 to September 30, 2008.

ABSTRACT
The overall goal of our project is to determine whether β-1,4 glucanase (EGase), the major enzymatic protein in the saliva of glassy-winged sharpshooter (GWSS), co-localizes via immunocytochemistry with the few 'pioneer' *Xylella fastidiosa* (*Xf*) cells that are inoculated by this vector's probing (stylet penetration) behaviors. If it does, then this suggests that the enzymatic portion of GWSS saliva is a carrier of the bacteria during inoculation. This year, we acquired commercially-produced, polyclonal antibody serum to purified EGase from GWSS salivary glands, and developed methods for separate immunolocalization of: 1) this purified EGase, and 2) Green fluorescent protein (GFP) *Xf* in grape petioles, using commercial *Xf* antibody, to complement the GFP *Xf* detection method devised last year. We also performed three experiments to monitor feeding of inoculative GWSS, via Electrical Penetration Graph (EPG) technology. Feeding was recorded on healthy grape petiole, followed by histological processing of the fed-upon grape tissues. To date, results show that glucanase is found throughout the solid salivary sheath that encases stylets (the piecing-sucking mouthparts of GWSS that penetrate the plant). However, glucanase was not found diffused into adjoining plant cells along the stylet pathway, as was hypothesized. Glucanase was the major constituent of the deep, narrow sheath branches that enter the xylem, indicating that glucanase is injected into xylem during feeding. If our hypothesis on the role of saliva in inoculation is supported, it suggests that future development of salivary antagonists could enable interference with *Xf* inoculation of grape.

INTRODUCTION
As introduced in detail in Backus and Labavitch (2006, 2007), this goal of this project is to determine whether the major enzyme in glassy-winged sharpshooter (GWSS) saliva histologically co-localizes with *Xylella fastidiosa* (*Xf).* Sheath saliva of hemipterans in fed-upon plants can be routinely imaged histologically (e.g. Leopold et al. 2003, Backus et al. 2005). However, no researcher studying hemipteran feeding has ever *directly* visualized enzymatic watery saliva in plants, due to its usually fluid and dispersive nature. This project will also partially test Backus's hypothesis that enzymatic salivary secretions of GWSS aid in the cell-to-cell movement of newly inoculated *Xf* cells. Salivary enzymes may break down pit membranes, allowing the few pioneer bacterial cells inoculated during feeding to move between adjoining xylem cells. Carbohydrase enzymes with very high activity for cell wall polymer-degradation, especially β-1,4 glucanase (EGase, often identified as cellulase in the literature), have been found in GWSS salivary gland fractions (Labavitch 2006, unpub. data). We use immunohistological methods combined with Electrical Penetration Graph (EPG) monitoring of GWSS feeding, to determine whether *Xf* co-localizes with EGase in saliva.

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 by immunocytochemistry.

RESULTS
Objective 1 – Purify and characterize β-1,4-glucanase and develop antibodies.
Study a: β-1,4-glucanase isolation and purification
EGase was isolated and purified last year in the Labavitch lab, from GWSS salivary glands previously dissected in the Backus lab (Backus & Labavitch 2007).

Study b: Determination of cell-wall degrading properties of β-1,4-glucanase
The glucanase enzyme was purified based on its ability to digest carboxymethyl cellulose. However, tests showed that it could also digest xyloglucan (XyG), a major cell wall hemicellulosic polysaccharide that is present in grape tissues, including leaf petioles. At least one of the β-1,4-glucanase enzymes of *Xf* also digests XyG. Therefore, the ability of the tomato xyloglucanase-inhibiting protein to block the action of the GWSS glucanase was tested. Unfortunately, as was found for the *Xf* glucanase, no inhibition was detected (Labavitch, 2006).

Study c: Development of antibody to β-1,4-glucanase
Polyclonal antibodies were raised in guinea pig by Antibodies, Inc. (Davis, CA) in late November 2007, then were further purified in the Labavitch lab in December 2007. This objective was completed when antibody serum was delivered to the Backus lab, in January 2008.

Objective 2 – Determine whether GWSS salivary proteins (from EPG-controlled insect feeding) affect the presence/distribution of inoculated *Xf*.
*Studies a and b: Immunocytochemistry of probes by a) clean and b) GFP-*Xf *inoculative GWSS*
Our ultimate goal for this objective is to combine five challenging procedures into one large experiment with the following steps (first described in Backus & Labavitch 2007):

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 three – six min, as described in Backus & Labavitch (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/saliva.

In this way, we hoped 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.

Postdoctoral Research Associate Kim Kingston worked in the Backus lab from 1st July 2007 until 30th June 2008, under joint supervision by Backus and Labavitch. Prior to her arrival, preliminary attempts were made to achieve steps 1 – 3, above, which partially failed. We attempted to view salivary sheaths containing GFP *Xf* left by inoculative GWSS, using the fluorescence-retaining protocols developed last year (Backus & Labavitch 2007). To our dismay, sheath saliva was so strongly autofluorescent (at all excitation wavelengths) that its brightness overwhelmed and overlaid the lesser brightness of the GFP *Xf* (except in a few rare cases wherein the sheath was very diffuse, confirming presence of bacteria in the sheath; Backus 2007). Also, *Xf* were not seen outside the salivary sheath, only embedded within sheath saliva. These results confounded the rest of our tests because we could not be sure that all bacteria injected by every insect would consistently be visible.

Consequently, we spent the first six mo. of Kingston's tenure (including the first four mo. of the reporting period) successfully developing a protocol for a more reliable means of histologically detecting *Xf*, i. e. immunolocalization. We used commercially-prepared *Xf* primary antibody from rabbit (Agdia, Elkhart, IN) and secondary, anti-rabbit conjugated with Alexa Fluor 647 (Invitrogen, Carlsbad, CA) with mechanically inoculated vs. control grape petioles (**Figure 1**). We anticipate that this protocol will enable visualization of GFP *Xf* inoculated into the plant by GWSS, even if bacteria are embedded in the salivary sheath.

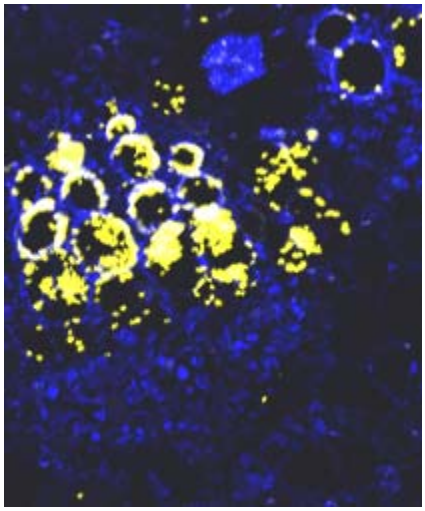

Figure 1. Immunohistological confocal image of grape petiole xylem (autofluorescent cell walls, blue) mechanically inoculated with *Xf* (yellow). White coloration is overlay of yellow and blue.

Also in Kingston's first six mo., we performed two major experiments to EPG-record feeding of putatively inoculative GWSS on healthy grape petioles, following the procedure outlined in step 2, above. Putatively clean GWSS were provided by David Morgan (CDFA). Recordings were performed in the dark, using a petiole whose leaf was masked by wet tissue paper and plastic wrap (according to a procedure developed with advice of Andrew McElrone, ARS Davis, CA). This was to reduce the likelihood that bacteria injected into the xylem would be rapidly pulled out of the confocal field of view by xylem tension. Following each recording, the insect head was fixed in 4% paraformaldehyde, for separate examination by confocal microscopy to verify the presence of GFP *Xf* in the cibarium and

precibarium. Petiole tissue was excised and fixed under dim light, then further prepared for sectioning and later confocal microscopy.

Once the EGase antibody serum arrived, we spent Kingston's last six mo. developing a successful protocol for its use in immunoprobing and -localizing EGase in GWSS salivary sheaths. The protocol visualizes the EGase primary antibody (from Objective 1) with goat-anti-guinea pig secondary antibody conjugated with Alexa Fluor 647 or 568 (Invitrogen, Carlsbad, CA) (**Figures 2a and b**).

Contrary to our initial hypothesis, there was no evidence that glucanase diffused into plant tissues adjoining the salivary sheath **(Figure 2**). At no concentration of EGase antibody was a diffuse 'halo' seen only around the salivary sheath, as has been enzymatically detected surrounding aphid salivary sheaths (Ma et al. 1990). Salivary glucanase was strictly localized to the sheath, though along its entire length. The polyclonal EGase antibody also bound non-specifically to small plastids and certain vacuole contents (**Figure 2a, *'s**). Nonetheless, these were easily distinguished from saliva by their paler coloration and their widespread distribution in all parenchy-mous tissues (**Figure 2a**). For most of the sheath length, EGase was co-localized with the hardening (autofluorescent) constituents of the sheath. However, the narrowest, deepest branches of the sheath (which ultimately enter the target xylem cell), were composed almost exclusively of EGase. They did not autofluoresce like the rest of the sheath. Also, EGase both bound to and evidently infiltrated the cell walls of the xylem, probably due to cell wall loosening caused by the enzyme (**Figure 2b**). This would produce a very strong seal of the stylets into the cell, as hypothesized by Backus et al. (2005). In addition, glucanase-labeled saliva was found in xylem cells distant from the sheath, indicating that it traveled from the site of injection (**Figure 3**).

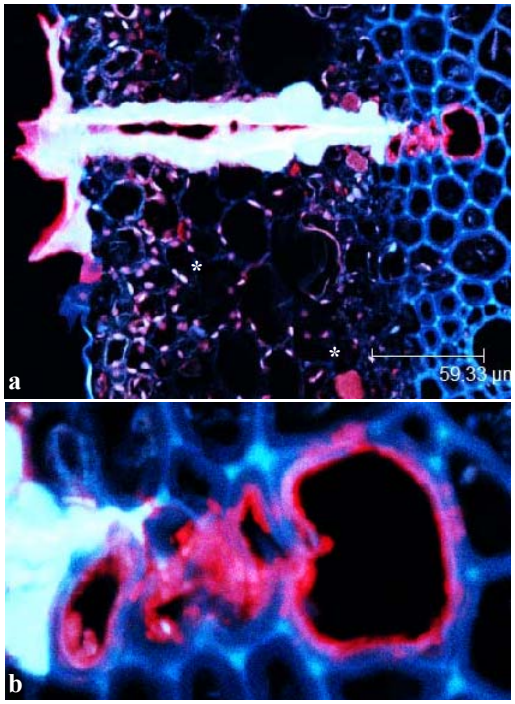

Figure 2. a. Immunohistological confocal image of a GWSS salivary sheath in grape petiole. Single-branched sheath penetrates to a large, lignified xylem cell (autofluorescent cell walls, blue). Sheath is brightly autofluorescent in blue and green wavelengths, while the EGase fluorochrome is red. White coloration is overlay of all wavelengths. * plastids and vacuole contents also binding polyclonal EGase. **b.** Higher magnification view of the terminal sheath branch and xylem cell.

Toward the end of Kingston's tenure, we performed one more EPG experiment to generate GWSS-inoculated grape petioles, this time with young, newly eclosed adults from a putatively clean GWSS colony in Fresno, CA. Thus, a total of about 160 GWSS probes were EPG-recorded on grape petiole, producing salivary sheaths for immunohistology work.

Although proposal funding has ended, work on this project will continue to completion using ARS in-house funds. Steps 4 and 5 (above) will be performed during the coming year, with the addition of immunolocalization of *Xf* from probed grape petioles. We will attempt to co-localize both *Xf* and EGase in the GWSS saliva within probed grape petioles (step 5, above).

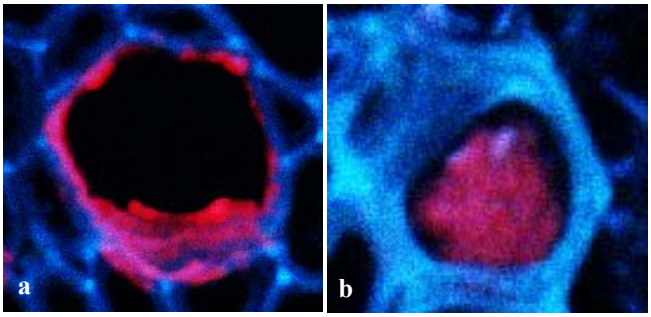

Figure 3. a. EGase-containing sheath saliva lining a xylem cell several sections away from a salivary sheath. Note infiltration of walls. **b.** A large blob of EGase-containing sheath saliva inside a xylem cell, many sections away from, but in the same terminal xylem cell as, a salivary sheath.

CONCLUSIONS

The described findings continue to support the following hypotheses: 1) cell wall-degrading salivary enzymes are 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 stylet penetration, and become embedded in the salivary sheath, as well as injected directly into xylem cells, and 3) EGase-containing saliva infiltrates xylem cell walls at its site of injection and further distant; it could potentially interact with pit membranes at any site. 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 other vector species, could be related to salivary enzyme composition. Biochemical analysis of saliva in other vectors (e.g. Brazilian vectors of Citrus Variegated Chlorosis, or vectors of Oleander and Almond Leaf Scorches) could aid understanding of the epidemiology of all xylellae diseases.

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FUNDING AGENCIES

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