

WHERE, WHEN AND HOW DO INGESTION AND OTHER FEEDING BEHAVIORS OF THE GLASSY-WINGED SHARPSHOOTER ALLOW INOCULATION OF *XYLELLA FASTIDIOSA*?

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ABSTRACT

This year, we completed the laboratory portion of work that identified an aspect of electrical penetration graph (EPG) ingestion waveform (C) of glassy-winged sharpshooter (GWSS) that definitively represents xylem ingestion. We also demonstrated that extravasation is correlated with the B1 waveform. Both B1 and C waveforms may play a role in the behavior that facilitates inoculation of *Xylella fastidiosa* (*Xf*). Results this year support that both amount and location of *Xf* binding in the foregut of GWSS ("vector load") are critical for success of subsequent inoculation. The precibarium is the first area colonized during acquisition, and also may be the most important location for subsequent inoculation. If insects become "maximally loaded" with *Xf*, preliminary results suggest that a single probe by a single vector can cause a lethal infection. These results will help solve the PD/GWSS problem by providing powerful new tools for future studies, answers to numerous questions about how vector transmission works, and new potential targets for host plant resistance.

INTRODUCTION

The behaviors comprising within-plant feeding (a.k.a. stylet penetration) of hemipteran vectors are intricate and complex, and vary enormously among species. Yet, a deep understanding of stylet penetration is particularly important for sharpshooter vectors because behavior plays a crucial role in transmission of non-circulatively transmitted pathogens like *Xylella fastidiosa* (*Xf*). Thanks to EPG monitoring, sharpshooter stylet penetration can now be observed in detail, in real-time. Two stylet penetration behaviors emphasized in this project likely control *Xf* inoculation. They are uptake of plant fluids into the gut (ingestion) and expulsion of bacteria-laden fluids (egestion or extravasation).

OBJECTIVES

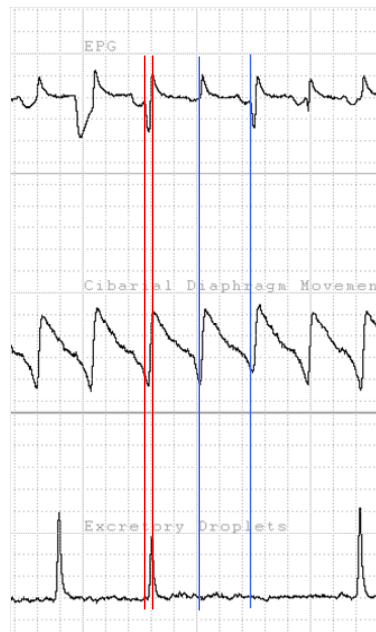
1. Characterize ingestion behavior, especially to: (a) identify in which cell types various durations of ingestion (C) are occurring, and (b) how to recognize that by EPG alone.
2. Characterize extravasation behavior, especially to: (a) correlate the B1 waveform with fluid flow in and out of the stylets, and (b) determine in which plant cells this behavior occurs.
3. Characterize behavior-*Xf* interactions that permit inoculation, especially to (a) identify the behaviors (i.e. ingestion, extravasation or both) during which bacteria are expelled, and (b) whether bacterial expulsion is into xylem, or any plant cell type penetrated, or both.

RESULTS

Insect Availability

Last year's progress report announced that we had solved all problems with availability of experimental plants, insects and bacteria. Unfortunately, one month after writing that report, our source for clean glassy-winged sharpshooters (GWSS), D. Morgan of CDFA, told us he could no longer provide insects due to unforeseen colony issues. For most of this year, we have attempted to use GWSS lab-reared from egg masses laid by adults that we field-collected from ornamental plants in Bakersfield (for *Xf* inoculation studies) or we have substituted smoketree sharpshooter (STSS) (for waveform correlation studies without *Xf*). These attempts were partially successful, but consequences were severe for one experiment. As of this writing (late September 2006), we have been promised a few clean insects from Morgan, to re-do key experiments.

Figure 1. Three simultaneously recorded voltage signals from EPG (top), cibarial diaphragm movements (middle) and production of excretory droplets (bottom). Light-detecting diodes were placed on the video screen to generate voltage signals for diaphragm movements (increased voltage = more uplifted diaphragm) and excretory droplet expulsion (peaks).



Objective 1. Correlation of ingestion with EPG waveforms

Study a: Ingestion-activity correlations and cell types in which it occurs

An electromyographic (EMG) study of cibarial dilator muscle activities was completed by post-doc S. Dugravot (Dugravot et al., ms. in prep.). The project temporally correlated EMG signals of muscle potentials with video images of cibarial diaphragm movements (visible when backlit) and production of excretory droplets (Figure 1). The C waveform was 100% correlated with cibarial dilator activity. The valley portion of C represented the rapid, muscular uplift of the diaphragm (i.e. sucking; Figure 1, red lines) and the plateau represented the slower, non-muscular drop of the diaphragm (i.e. swallowing; Figure 1, blue lines). In addition, once sustained ingestion was underway, excretory droplets were also correlated with cibarial pumping; 2 – 4 pumps produced a single droplet (Figure 1). Droplets ceased when cibarial pumping ceased, especially during interruption (N) waveforms corresponding to salivation into xylem (Backus and Labavitch 2006) (data not shown). However, the synchrony of droplets and cibarial pumping did not hold for the beginning and end of ingestion. Each insect varied greatly in the onset of droplet production in relation to onset of pumping, and likewise, the end of pumping did not immediately signal the end of droplet production. Thus, we confirm that production of excretory droplets is an excellent correlate of sustained ingestion. However, we caution that droplet production cannot be used to time onset or cessation of ingestion, which differed by up to 40 min.

Data from this EMG study were complemented by a second study performed by Backus at the Argonne National Lab. EPG waveforms were recorded from wired GWSS that were subjected to high-energy X-ray imaging during feeding, which allowed the cibarial muscle movements to be directly viewed. Video images and waveforms are being analyzed, but preliminary results support the above findings. Significantly, it was also observed that atypical C waveform shapes, hypothesized to correlate with ingestion from non-xylem cells, were actually caused by unusual cibarial muscle contractions. In fact, all aspects of C waveform fine structure were correlatable with cibarial muscle movements alone. Other correlations will be possible as data are analyzed.

A third project completed this year was a histological study to determine which ingestion events were performed in xylem, and whether that ingestion tissue can be identified by waveform appearance alone. We examined salivary sheath branches of artificially terminated probes made by STSS feeding on cowpea stem (see sample waveform in Backus and Labavitch 2006). Preliminary results strongly support that waveform C is virtually always correlated with xylem, but can occur in any of several xylem cell types. Very early, especially short-duration, C events usually occurred in primary protoxylem cells or small, unlignified secondary xylem cells. In contrast, somewhat later, especially longer-duration, C events occurred in large, lignified secondary xylem cells.

Study b: Recognizing ingestion from waveforms alone

Results from Study a (to date) support that waveform C represents ingestion, but its fine structure is not correlated with ingestion tissue type. Yet, C predominantly occurs in xylem, and the type of xylem cell (and perhaps functionality) appears to be correlated with the C event order and/or the event duration.

Objective 2. Correlation of extravasation with EPG waveforms

Study a: Correlate B1 waveform with muscle movements and fluid flow in and out of stylets

The EMG study described under Objective 1 above also examined muscle potentials from the precibarial valve muscles, which are hypothesized to control extravasation. Results conclusively showed that the precibarial valve is voluntarily moved

only during pathway waveforms, not during C. The valve muscle potentials occurred throughout pathway, and strongly resembled waveform B1 spikelet bursts (B1s) (data not shown). Because B1 is the only waveform that is ubiquitous throughout pathway, this supports that B1s represents valve fluttering. The directionality of streaming potentials implies that fluid moves in and out of the precibarium during this behavior. This work supports earlier findings correlating B1 with *Xf* inoculation. B1 apparently represents a combination of fluid uptake and expulsion (extravasation) (via precibarial valve fluttering) for tasting, plus small amounts of salivation.

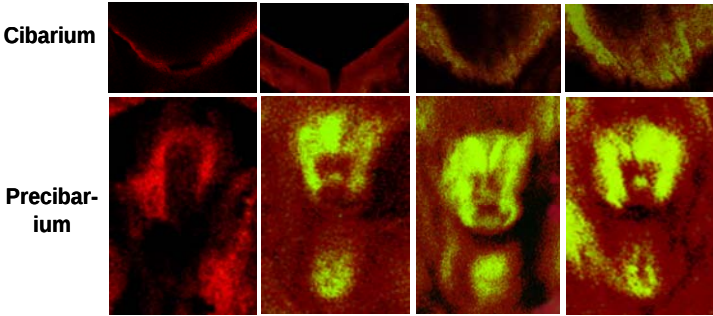
Study b: Determine in which plant cells B1 occurs

A combination of the GWSS EMG study and STSS sheath histology studies above, plus results from earlier studies, continues to support that B1 occurs throughout pathway, in all cell types penetrated by the stylets. Work detailed in Backus and Labavitch 2006 shows that B1 can occur in xylem cells, just prior to or interrupting ingestion events.

Objective 3. Characterize behavior-*Xf* interactions that permit inoculation

Study a: Identify the behaviors (ingestion, extravasation or both) during which bacteria are expelled

Last year's progress report detailed findings by the previous post-doc, P.H. Joost, that green fluorescent protein (GFP)-expressing *Xf* were seen embedded in salivary sheaths and floating freely in artificial diet on which putatively inoculative GWSS fed. Yet, only 1 out of 40 EPG-recorded probes revealed GFP. In the year since that work, we have demonstrated that the low success rate of Joost's experiment was due to not enough GFP-*Xf* available in infected plants, so that his ultra-clean GWSS from Morgan could not acquire enough *Xf*. We perfected our acquisition protocol, and produced confocal laser scanning microscope (CLSM) images that demonstrated that GFP-*Xf* are acquired first into the precibarium and then into the cibarium over an 8 day acquisition access period (AAP) (Figure 2). These images were showcased in our poster at the 2005 PD Symposium (Backus et al. 2005). We term these insects "clean, maximally loaded." This year, now more confident of our acquisition protocols, a second post-doc (S. Dugravot) attempted anew to perform this diet-inoculation experiment.



	2 day AAP (N = 2)		3 day AAP (N = 8)		6 day AAP (N = 6)		8 day AAP (N = 3)	
Location	<i>Xf</i> conc.	% of insects	<i>Xf</i> conc.	% of insects	<i>Xf</i> conc.	% of insects	<i>Xf</i> conc.	% of insects
Cibarium	0 or light	50: 50%	light	100%	med.- heavy	100%	heavy	100%
Precibarium	0 or light	50: 50%	medium	12.5%	heavy	100%	heavy	100%

FIG 2. CLS micrographs of representative epipharyngeal halves of cibaria (top) and precibaria (bottom; more magnified) at 2, 3, 6 or 8 days AAP.

After four months of efforts this year, we concluded that the attempt was again unsuccessful. Like last year, however, we learned much from the problems encountered. Our efforts to rear clean (non-inoculative) GWSS from egg masses in the absence of Morgan's insects were apparently unsuccessful. None of the nearly 60 probes that were observed in artificial diets showed signs of GFP-*Xf*, even though the insects were given 8 – 13 d AAP's on infected acquisition grape plants. Indeed, when we dissected the GWSS heads and examined their foreguts via CLSM, we invariably found much less GFP-*Xf* than last year's maximally loaded insects (data not shown). Large, flocculent aggregations of autofluorescing (non-GFP) microbes were visible in both areas, but primarily in the cibarium; GFP-*Xf* were merely tucked in amongst these, in small to medium aggregations. We concluded that the insects had already acquired competing microbes into the prime acquisition (and inoculation) sites, and again there was too little GFP-*Xf* present to inoculate into the diet. We term these insects "dirty, topped-off." These findings support the hypothesis that competition for binding sites occurs in the insect's foregut. A third post-doc, B. Reardon, will attempt this diet-inoculation project again, after we have received (hopefully) ultra-clean GWSS from Morgan.

Study b: Determine into which plant cells bacteria are expelled

In November 2005 we performed a plant inoculation experiment to complement the diet inoculation study described above. For this test, we were able to use the last of the ultra-clean insects provided to us last year by Morgan. Recent CLSM of their heads has shown that the precibaria of these insects acquired GFP-*Xf* (Figure 3, next page) during their 5 – 8 day AAP's, prior to use in the inoculation test. GFP-*Xf*-inoculative insects were each EPG-recorded for a single, standardized probe composed of pathway plus 3-6 min of ingestion on a healthy 'Cabernet Sauvignon' grapevine petiole. Each plant was then held in an insect-exclusion cage in the greenhouse for one of 4 time periods: 0, 10, 20 or 40 d, whereupon the petiole tissue in the immediate vicinity of the marked probe site was prepared, sectioned, and examined using epifluorescence light microscopy (ELM) with a GFP filter cube. The 0 d tissue actually was excised 2 – 4 min after the end of the probe. In addition, when plants became obviously symptomatic for PD (at 3 months), 8 – 10 leaves above the fed-upon petiole were assayed for *Xf* using PCR.

Results are still being fully analyzed, but preliminary findings are startling. First, 100% of the 36 plants (9 reps per treatment) became unambiguously symptomatic for PD and died within 6 months after the inoculation probe. Control,

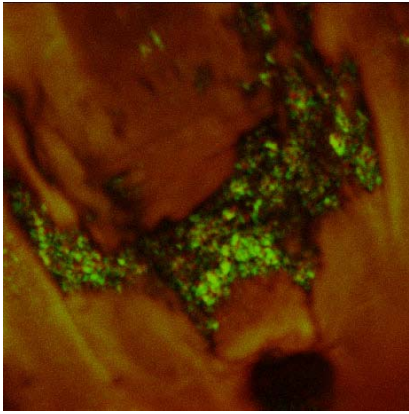


Figure 3. Close-up view of the precibarial valve and the distal half of the epipharyngeal basin in a GWSS allowed a 5 day AAP, showing green GFP-*Xf*. The plant was PCR-positive for *Xf*. Note better resolution than for Fig. 2, due to improved CLSM protocols. 320x

tissue, we must be able to prepare, embed and section the tissue. However, standard, alcohol-based histological preparations quench the fluorescence of GFP; hence, all researchers using confocal visualization of GFP-*Xf* use unsectioned tissues. Our GFP-*Xf* samples fluoresced slightly, but at the same (yellow) wavelength as lignified xylem cell walls (as in Figure. 4). After much effort and with the help of S. Ruzin, we have devised the first-ever protocols for classical histological preparation of GFP in plant tissues. Although improvements still will be made in the coming weeks, we now can definitively identify GFP-*Xf* in longitudinally-sectioned xylem cells and spectrally separate bacterial cells from cell walls (Figure 4).

CONCLUSIONS

Our findings will help solve the PD/GWSS problem by providing: 1) A powerful tool in EPG for studies of host plant resistance, including a natural, insect-inoculation bioassay and eventual development of a resistance index for genotype screening (the Stylet Penetration Index); 2) Insights into the mechanism of *Xf* transmission (acquisition and inoculation); 3) Numerous spin-offs from such basic findings, such as information for risk assessment models, with implications for all levels of the *Xylella*-sharpshooter-grape pathosystem, including ecological, epidemiological and management; and 4) Knowledge of new potential targets for grape breeding and transgenic resistance.

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FUNDING AGENCIES

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healthy plants subjected to all the same treatments (except inoculation) thrived, and none died. All plants were kept on an automatic irrigation regime in the greenhouse, and none became dessicated. PCR tests were encouraging, but require further optimization in a November repeat of the experiment. A majority of plants in each treatment were positive for *Xf*: 100% of the 0 d plants, and 56% each for the 10, 20 and 40 d plants. We suspect that the 44% negative were false negatives, due to either concentration of template below the detection limit, or problems with inhibitors. The strong symptom results and adequate PCR results suggest that 100% of these grape plants were inoculated with *Xf* by a single GWSS probe, which led to a lethal, systemic PD infection. If an upcoming repetition of this experiment verifies our findings, it will be the first time that GWSS has been experimentally shown to exhibit 100% vector efficiency per individual insect, let alone from a single probe. Future refinement of the procedure could provide a bioassay for reliable, natural insect-inoculation that could be useful for comparative host plant resistance studies.

Second, unfortunately, our histological preparations were not successful, but we have determined the cause and are developing new protocols to solve the problem. In order to locate salivary sheaths in plant

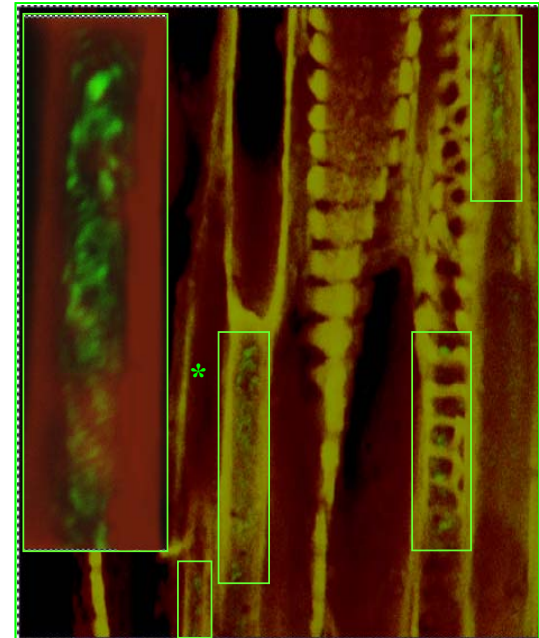


Figure 4. GFP-*Xf* revealed (small green boxes) in paraffin-embedded and longitudinally-sectioned stems from mechanically inoculated grape. Inset: a section adjoining the box with the *, magnified. This represents ~70% of the green signal that will be visible using the final protocol. 300x.

THE ROLE OF GLASSY-WINGED SHARPSHOOTER SALIVARY ENZYMES IN INFECTION AND MOVEMENT OF *XYLELLA FASTIDIOSA*

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ABSTRACT

The purpose of this project is to determine whether a vector's enzymatic saliva aids the establishment of the few 'pioneer' *Xylella fastidiosa* (*Xf*) cells that are inoculated into a plant; thus the bacteria would co-localize with the saliva. Previous work showed that watery saliva of glassy-winged sharpshooter (GWSS) degrades cell walls, and is injected into xylem during feeding. Saliva contains cell wall-degrading enzymes, notably β -1,4-glucanase (EGase), which can degrade pit membranes that impede cell-to-cell movement of bacteria. We plan to immunoprobe for salivary EGase, in grape stems fed-upon by GWSS that have acquired green fluorescent protein (GFP)-transformed *Xf*, to co-localize *Xf* with both sheath and watery saliva. This year, we: 1) improved protocols for histology of salivary sheaths using epifluorescence light microscopy, 2) dissected 500 pairs of salivary glands to extract EGase and raise antibodies, 3) showed histologically that the salivary sheath dissolves over time, resulting in cellular abnormalities typical of cell-wall loosening, and 4) showed that sheath and watery saliva are directly injected into and travel through xylem cells.

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. *Xf* can cause PD only if bacteria can 'break out' of the initial, imprisoning inoculation xylem vessel(s) to produce an increasing population that becomes systemic. Thus, initial infection success is dependent, in part, on *Xf* lateral movement through adjacent vessels. Recent evidence by Labavitch and colleagues studying *Xf* movement through stems supports the idea that pit membranes limit bacterial movement. Sufficient quantity of cell wall-degrading enzymes can digest parts of the primary cell wall network of the pit membrane, allowing bacteria to pass. The enzymes polygalacturonase (PG) and β -1,4-glucanase (EGase, often identified as cellulase in the literature) are produced by mature *Xf* populations, typical of the biofilms in xylem that are seen in later stages of infection, and may function in this way. It seems to us, however, that the few pioneer bacteria inoculated by a vector are unlikely to produce a sufficient titer of enzymes to digest through the pit membrane. Fewer than 200 cells are typically inoculated by sharpshooters (Hill and Purcell 1995).

Although it is routine to histologically image sheath saliva in fed-upon plants (e.g. Leopold et al, 2003, Backus et al 2005b), no researcher has ever directly visualized watery saliva in plants, for any hemipteran. Due to its fluid and dispersive nature, it is unstainable by conventional means. However, Backus and colleagues have defined electrical penetration graph (EPG) waveforms that represent salivation and ingestion (Joost et al. 2006). Correlating EPG with histology of probed plant tissues revealed the cell types into which saliva is injected (Backus et al. 2005). Watery saliva becomes mixed with, and spreads out from, the salivary sheath and enters the xylem. Labavitch and colleagues recently have found very high activity of EGase and other cell wall polymer-degrading enzymes in GWSS salivary gland fractions. The evidence that copious quantities of cell wall-degrading saliva are injected along with very few bacterial cells 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

Award notification for this new grant was received in June 2006. However, as usual, the official start of the project will be delayed until spring 2007, due to budgeting and paperwork circumstances beyond our control. Nevertheless, we made progress in these four months analyzing pertinent previous research, using Backus's in-house ARS funds.