LINKING THE MODEL OF THE DEVELOPMENT OF PIERCE'S DISEASE IN GRAPEVINES TO AN UNDERSTANDING OF THE DYNAMICS OF GLASSY-WINGED SHARPSHOOTER TRANSMISSION OF *XYLELLA FASTIDIOSA* TO GRAPEVINES AND GRAPEVINE GENE EXPRESSION MARKERS OF PIERCE'S DISEASE

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ABSTRACT

For several years we have been studying the development of Pierce's disease (PD) in grapevines. Our studies have been guided by a model of PD development proposed with our initial application for funding. The Model proposed several "steps" in disease development following introduction of the PD causal agent, the bacterium *Xylella fastidiosa* (*Xf*):

***Xf* introduction to vessels =>vessel cavitation =>initial water deficit => *Xf* population increase => production of enzymes by *Xf* =>cell wall digestion => oligosaccharide signals => ethylene synthesis rise => a "wave" of vessel occlusion beyond the infection site => collapse of vine water transport => leaf abscission => vine death**

Our hypotheses have proven quite accurate, although aspects of the model are still being tested. We have shown that xylem vessel obstruction (tyloses, plant cell wall component-derived gels, and bacterial extracellular polysaccharides) and consequent reductions in stem water transport capacity are early consequences of infection with *Xf*, before bacterial populations are substantial and have spread far from the inoculation point. We have shown that ethylene treatment of vines also triggers vessel obstruction development and reduced water movement and that ethylene emanation from vines may increase following infection. We have also developed data for xylem vessel length distributions in grapevines and shown that *Xf* must pass through vessel pit membranes if the bacterial population is to develop systemically, thus suggesting that digestion of cell wall polymers in the pit membranes is likely to be important to disease spread. These findings are reported in several reports at the annual PD Symposium (Labavitch et al., 2001, 2002, 2004, 2005; Labavitch and Matthews, 2003) and, more recently, at disciplinary scientific society meetings (Perez et al., 2004; Roper et al., 2004) and in refereed reports (Stevenson et al., 2004). We describe herein the continuing studies that have made clear that the *Xf* genome contains genes that encode cell wall-degrading polygalacturonase (PG) and endo-β-1,4-glucanase (BGase) and that these two enzymes are sufficient to open the pit membrane network, suggesting that this is the mechanism used by the pathogen to permit systemic development in infected grapevines.

INTRODUCTION

Overall, many of the investigators listed above are involved in three CDFA-supported projects that are centered in the Labavitch lab. Two of these projects are outgrowths of our earlier project that was designed to test our proposed model for Pierce's disease (PD) development. Thus, it is difficult to avoid discussing some of the work in our other two projects in this report for the third project, which is an expansion of the primary model to link it to the studies of other PD researchers.

OBJECTIVES

1. Complete testing of our model of PD development in grapevines.
2. Determine whether glassy-winged sharpshooter (GWSS) feeding on grapevines is accompanied by xylem vessel cavitation.
3. Determine whether the grapevine "regulators" that we have identified as important to development of PD affect the expression of grapevine genes that have been shown to be important markers of *Xf* presence/PD infection.

RESULTS

Objective 1. Complete testing of our model of PD development in grapevines.

Efforts in this research year have examined four aspects of the model not previously tested. The first is the hypothesis that cell wall breakdown caused by the action of bacterial enzymes, like the pectin-degrading enzyme, PG contributes to the ability of *Xf* to systemically colonize the grapevine xylem which ultimately leads to disease. We demonstrated that the open reading frame encoding a putative PG did, in fact, encode a functional PG. Furthermore, *Xf* mutants lacking PG did not move from the point of inoculation and did not cause PD when inoculated into grapevine. The second is related to work designed to show whether *Xf* wall-digesting enzymes are present in the xylem of infected vines. The third pertains to descriptions of the porosity of the pit membranes that separate one vessel from its neighbors. The fourth pertains to the idea, discussed by many but never actually demonstrated, that *Xf* produces an exopolysaccharide (EPS) and that this EPS is associated with the pathogen in infected grapevines. While the first three of these questions are clearly relevant to the examination of the "*Xf* enzymes and cell wall digestion" section of our model (above) they are also addressed in a second project that grew out of our model testing efforts. The title of that project is "The contribution of the pectin-degrading enzyme polygalacturonase (PG) in transmission of *Xf* to grape and use of PG-inhibitor proteins for transgenic resistance to Pierce's Disease" and the specific data that pertain to *Xf*'s PG and the pathogen's β-glucanase and their role in Pierce's disease development, specifically the opening of pit membranes, can be found in the report for that project in these *Proceedings*. The final question in this section, related to the production of EPS by *Xf*, is addressed below.

Does *Xf* produce an extracellular polysaccharide (EPS) and is this associated with bacteria that have colonized grapevine xylem water conduits? The sequence information for the *Xf* genome suggests that the pathogen should produce an EPS like that produced by *Xanthomonas campestris.* Because the *X. campestris* EPS is important for development of diseases caused by the pathogen it seemed reasonable to determine if the predicted similar EPS of *Xf* contributes to PD development. Thus answers to the questions posed above are potentially crucial to understanding how PD develops. Caroline Roper contacted Prof. L. Ielpi of the University of Buenos Aires who had reported on his studies of EPSs produced by a number of *X. campestris* strains with mutations in the genes encoding proteins involved in synthesis of its EPS. One of these was predicted to produce an EPS with a structure like that predicted for the putative *Xf* EPS. Prof. Ielpi kindly provided a sample of this mutant EPS variant and it was used to raise polyclonal antibodies. These were used to produce an immunoaffinity chromatography column that was used to purify cross-reactive polysaccharide from gel-like material that accumulated on the walls of flasks used for liquid culture of *Xf*. The structural analysis of this affinity-purified polysaccharide is now underway. The anti-*Xf* EPS antibodies were also used in a Protein A double sandwich ELISA assay now being used to quantify EPS production by the pathogen. These results are not presented in this report but are described in Caroline's dissertation (Roper, 2006) and in a manuscript that is now in rough draft form.

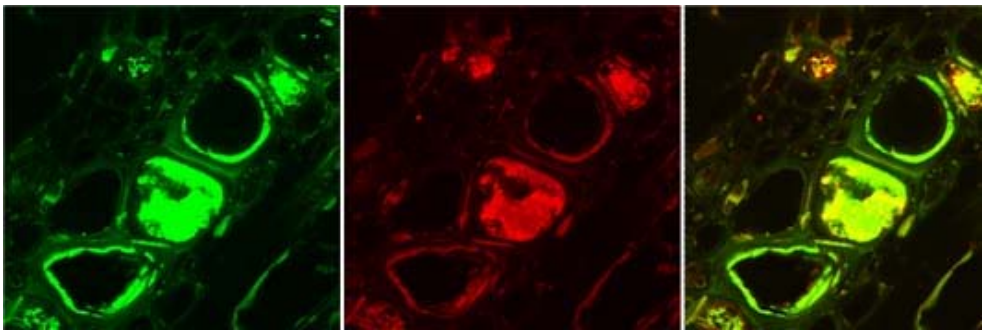

Figure 1. The co-localization of *Xf* cells and EPS. In the left panel a confocal laser-scanning microscope was used to show the presence of GFP-tagged *Xf* in the petiole xylem of a leaf from an infected grapevine. In the center panel the rabbit anti-*Xf* EPS antibodies were used to bind the EPS and then a red fluorescing dye (AlexaFluor 568)-tagged anti-rabbit serum to show the EPS based on the red fluorescent signal. In the right panel the two images are merged to give a yellow to orange signal indicating the co-localization of *Xf* cells and EPS.

The anti-*Xf* EPS antibodies were also used to demonstrate the presence of cross-reactive material, presumably *Xf* EPS, in *Xf* biofilms formed *in vitro* and *in planta*. The EPS often co-localized with *Xf* cells and appears to contribute to xylem vessel occlusion although the exact role of EPS in virulence is unclear (Roper 2006).

Objective 2. Determine whether GWSS feeding on grapevines is accompanied by xylem vessel cavitation.

This Objective is addressed by a combination of researchers with expertise in (1) electrical penetration graph (EPG) monitoring of sharpshooter feeding on grapevine xylem (Backus and colleagues, USDA), and (2) water-moving capacity of

xylem vessels (Shackel, Matthews and Labavitch, UCD). Progress was made this year in developing the protocols needed for both the insect and plant portions of the project. However, these tests have proven to be more challenging than was foreseen at the time the proposal was written.

Ph.D. candidate Alonso Pérez developed the MRI techniques that were used to determine whether vessels that the insect has been ingesting from become air-filled (i.e., cavitated) following the end of feeding. Pérez had previously attended Backus' EPG Workshop at California State University-Fresno in July, 2005, where he learned recording and analysis of sharpshooter EPG waveforms. Perez also tuned and tested a classical acoustic emissions (AE) monitor that has been used for over 40 years to record the ultrasonic vibrations of vessel cavitation. With this monitor, Perez visited the Backus laboratory in October 2005 to gather preliminary data using smoke tree sharpshooter (STSS) on cowpea, some of which was presented at the 2005 Pierce's Disease Research Symposium. Backus then lent her EPG equipment to Labavitch, and Perez used various combinations of EPG and AE monitors with both STSS and GWSS on grape, plus MRI imaging, during November 2005 to January 2006 at UCD. Unfortunately, the AE monitor proved unreliable for use with insects, because it was impossible to tune out vibrations made by insect movements during feeding. MRI images consistently showed no cavitation, even when many AE signals were recorded. It was therefore decided to try recording cavitations with a different, more modern approach. Perez completed his Ph.D. in March, 2006 and returned to Chile to take a position at the Catholic University in Santiago.

Backus enlisted the aid of her former colleague at the University of Missouri, Rex Cocroft, an expert in acoustic recording of insect sounds using highly sensitive, laser accelometry. Backus's head technician, Holly Shugart, a former Master's student of Cocroft's, traveled to Missouri for 3 weeks in July 2006, and attempted to use Cocroft's laser to record GWSS on both cowpea and grape. Shugart was successful in tuning the laser to detect cavitations in cut stems of cowpea, but only after drastic measures. She found that the laser detects ultrasonic vibrations as trains of extremely short pulses, each approx. 2-5 μsec in duration. The computerized recording equipment could not simultaneously record insect EPG and laser pulses, because sample rates in excess of the software maximum of 250,000 samples per sec (Hz) were required. Therefore, Backus hired colleagues at Sable Systems, Inc. to build a pulse-stretcher on rush order (over one weekend), which lengthened the pulses, allowing a more achievable sample rate of 70,000 Hz. After much tuning of the laser plus the pulse stretcher, Shugart achieved very clear, noise-free recordings of cavitation (Figure 2), which were experimentally verified by refilling cut, cavitating stems with water, observing loss of cavitations, then cutting the stems again and observing re-occurrence of cavitations (data not shown). Also, a single, successful recording was made of GWSS EPG feeding on cowpea, simultaneous with laser recording (data not shown). The laser was set at a much more sensitive level than for cut-stem cavitation controls, and did pick up trains of pulses. However, they were clearly correlated with waveforms known to represent stylet sawing, as well as walking and other insect movements. No signals as loud as cavitation were recorded during insect feeding, only during non-probing movements. These results suggest that sharpshooters do not cause cavitation during stylet penetration to the xylem, and support the MRI findings of Perez. However, this work will need to be repeated, with simultaneous recording of EPG and laser, plus before-and-after images by MRI. A laser accelerometer will need to be purchased to continue the work in California.

Objective 3: Determine whether the grapevine "regulators" that we have identified as important to development of PD affect the expression of grapevine genes that have been shown to be important markers of *Xf* presence/PD infection.

As discussed in this and previous PD research reports, we have now developed a substantial data set describing events in the development of PD in grapevines. We will work in the coming year to focus on two important PD development steps proposed by the model, but not yet fully tested. These relate to the potential roles of the plant hormone ethylene and oligosaccharides digested from grapevine cell walls in influencing the spread of the *Xf* population in vines or the vine's response to *Xf* presence. We have obtained the supplies needed to follow expression of the set of 4 grapevine genes that are expressed relatively early following *Xf* introduction into vines (Cook et al., 2003). The testing of the timing of expression will be based on real-time PCR of these 4 genes in relation to the appearance of early PD symptoms, most specifically the growth and spread of *Xf* in the weeks early after inoculation and development of vascular system occlusions as followed by MRI.

CONCLUSIONS

Our group of cooperating PD researchers feels that the best way to effectively deal with the threat caused by the disease is to fully understand its development in grapevines. A full understanding of the interaction of GWSS, *Xf* and *Vitis vinifera* should identify aspects of disease development that can be targeted by control measures that can be exploited using genetic approaches or new field management practices. We feel that the identification of a key role for *Xf* PG in PD development may provide a useful target toward which PD control measures could be directed. Should work to determine the extent to which grapevine ethylene production is a response to infection confirm our model's prediction, we will have another valuable marker of PD development as well as another potential target to exploit in terms of interrupting the systemic spread of *Xf*.

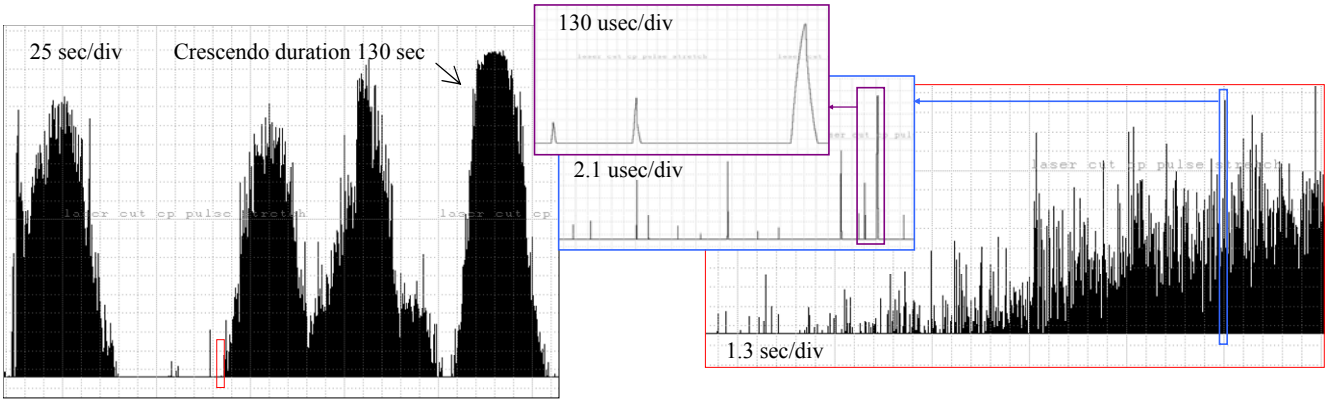

Figure 2. Control recordings of cut, cavitating cowpea stem using laser accelerometry. Rapid trains of pulse crescendos occur sequentially (black box), perhaps indicating separate groups or bundles of xylem cells cavitating. When the recording is temporally expanded, trains of thousands of individual pulses become discernible (red box in left view, expanded on right). Further expansion (blue then purple boxes) reveal individual, stretched pulses.

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THE CONTRIBUTION OF THE PECTIN-DEGRADING ENZYME POLYGALACTURONASE (PG) IN TRANSMISSION OF *XYLELLA FASTIDIOSA* TO GRAPE AND THE USE OF PG-INHIBITING PROTEINS FOR TRANSGENIC RESISTANCE TO PIERCE'S DISEASE

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ABSTRACT

Work this year has shown that the *Xylella fastidiosa (Xf) p*olygalacturonase (PG) is an endo-acting enzyme. This provides us a reference that we can now use to biochemically characterize the interaction between the pathogen's PG and PG inhibiting proteins (IPs) that is implied by the fact that transgenic expression of PGIPs protects vines against PD. We also report that PG, in combination with a bacterial β-1,4-glucanase enzyme (BGase) that has also been discussed in previous reports can act to cause tears in the pit membranes that are likely to be barriers slowing the systemic spread of the *Xf* population. We also report on a few set-backs that have slowed our examination of the roles of insect plant cell wall-degrading enzymes in PD development. Fortunately, the set-backs are now being corrected so that important questions can be addressed in work supported by a no-cost extension.

INTRODUCTION

In a companion report we have discussed continuing work that has been done to test the model we have proposed to describe the development of Pierce's disease (PD) in grapevines. That model proposes a key role for a pectin-degrading enzyme, polygalacturonase (PG). Over the past few years we have demonstrated that *Xylella fastidiosa* (*Xf*) has a PG encoding gene and that the *Xf* PG is important for disease development. This project is a spin-off from our "model" project, based on the observation that, when expressed in transgenic grapevines, plant PG-inhibiting proteins (PGIPs) provide some protection against PD.

OBJECTIVES

1. Determine whether the pectin-degrading enzyme of *Xf* contributes to the systemic spread of the bacterial population in inoculated grapevines (first priority).
2. Determine whether the pectin-degrading enzyme(s) in the salivary secretions of the glassy-winged sharpshooter (GWSS) contributes to inoculation success of *Xf* into grapevines (second priority).

RESULTS

Objective 1

Determine whether the pectin-degrading enzyme of *Xf* contributes to the systemic spread of the bacterial population in inoculated grapevines

This question is related to a major component of our model for PD development and prior results in the project focused on testing that model led to this project. We had previously shown that the *Xf* PG gene coded for some sort of PG activity. This conclusion was based on isolation of the protein from *E. coli* transformed to express the putative PG gene. This year's work involved the development of a PG assay based on identification of the products of enzyme action. If the products included a series of oligosaccharides, rather than only monosaccharide galacturonic acid, the PG would be an *endo*-acting PG (Figure 1).