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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Reporting Period: The results reported here are from work conducted October 2004 to October 2005.

ABSTRACT

INTRODUCTION

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 that was 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**

Although some aspects of the model are still being tested (the current project), our hypotheses have proven to be quite accurate. We have shown that xylem vessel obstruction (tyloses, plant cell wall component-derived gels, and, perhaps, 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; Labavitch and Matthews, 2003) and, more recently, at disciplinary scientific society meetings (Perez et al., 2004; Roper et al., 2004) and in referred reports (Stevenson et al., 2004).

This research has drawn together an assortment of UC Davis (UCD) researchers, each bringing a different disciplinary research orientation to the study. In addition, through regular discussions at UCD and with other researchers who have become colleagues as a result of meetings at the annual PD Symposia, we have begun to see how important connections can be made between our studies and those of other PD researchers. In this progress report, we discuss the successes we have had in filling the gap in the portion of the model that proposes the links:

***Xf* population increase => production of enzymes by *Xf* => cell wall digestion => oligosaccharide signals => ethylene synthesis rise**

These successes include the demonstration that the putative *Xf* polygalacturonase (PG) gene actually encodes a PG and the fact that this PG contributes to symptom development in inoculated grapevines. We also discuss work designed to determine whether xylem vessels become non-functional when the glassy-winged sharpshooter (GWSS) feeds. Our project also attempts to link the grapevine PD-related gene expression studies of Doug Cook et al. (UCD Plant Pathology) with the developmental, biochemical and physiological characterization of PD development that is part of our continuing work. That effort will be discussed briefly.

OBJECTIVES

1. Complete testing of our model of PD development in grapevines.
2. Determine whether 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 three aspects of the model not previously tested. The first is the idea that cell wall breakdown caused by the action of bacterial enzymes like the pectin-degrading enzyme PG that was putatively encoded by an identified *Xf* open reading frame did, in fact, encode a PG that was important in PD development. 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.

Does the *Xf* "PG" gene encode PG and what role does the gene product play in infection?

The progress report for one of our companion proposals (PIs Labavitch, Backus and Morgan) provides a detailed description of the experiments and data that are relevant to this topic. In short:

1. The PG sequence was cloned and then expressed in transgenic *E. coli*. Protein was isolated from the transformed *E. coli* and shown to have PG activity. Because we want to use the isolated *Xf* PG in tests of its effects on grapevine xylem integrity and we have been able to isolate only a small amount of the PG thus far, we will continue to work to obtain more of the enzyme.
2. The PG gene of *Xf* was functionally knocked out by insertion of an interrupting DNA sequence in it. The resulting PG-minus *Xf* bacteria were still viable, both *in vitro* and in grapevines, but they were not able to induce PD symptoms when inoculated into vines. This provides important proof that some process involving PG action that occurs in grapevines is crucial for PD development! We will continue to work to determine what the PG is doing.

The data suggesting that *Xf* PG plays an important role in disease development is consistent with the report of our PD research colleagues (Aguero et al., 2005) showing that the expression in *V. vinifera* of the pear fruit PG-inhibiting protein (a gene cloned in our labs several years ago) leads to decreased PD symptom development in inoculated vines.

Are *Xf* cell wall-digesting enzymes found in the vessels of infected vines?

At the 2003 PD Symposium, we reported on the efforts of Ph.D. candidate Caroline Roper which led to the cloning of one of the putative *Xf* endo-β-1,4-glucanase (BGase)-encoding genes, expression of that sequence in transgenic *E. coli*, and demonstration that the expressed gene did code for a BGase activity (Labavitch and Matthews, 2003). We are interested in factors that may open the cell wall meshwork of pit membranes to permit passage of *Xf* in diseased vines. However, as with the PG discussed above, we do not know the role (if any) of the BGase in grapevine xylem. This question is being addressed in this year's work from the biochemical and anatomical perspectives. However, an important adjunct to those direct tests of *in vivo* enzyme function would be the demonstration that the proteins are present in infected vines. We have developed some of the immuno-histochemical tools and expertise needed to address this important question.

Antibodies were generated to recombinant *Xf* PG and BGase. The open reading frames encoding these enzymes were cloned, over-expressed in *E. coli* and then purified by nickel column chromatography. The purified recombinant proteins were separately injected into rabbits to generate antibodies. The resulting antisera were tested by ELISA against the respective purified *Xf* BGase or PG to confirm the rabbits' production of anti-BGase or anti-PG antibodies.

Western blots using the anti-BGase antibodies as a probe detected the production of BGase by cultured *Xf* cells. This is the first demonstration that *Xf* makes a BGase in culture. Western blots using the anti-PG antibodies as a probe did not detect the production of PG by cultured *Xf*. We infer from our results with the PG-knockout *Xf* line (above) that PG is made in grapevines when infective *Xf* (i.e., the wild-type "Fetzer" strain) are growing in vines. Thus we conclude that factors present in the vine, but not in culture, are required for induction of the expression of the *Xf* PG gene. Experiments are currently underway to use both of these antibodies to test for the presence of the *Xf* wall polysaccharide-degrading enzymes in the tissues of infected vines. In order to optimize this line of investigation, Caroline Roper participated in an immuno-histochemistry techniques course at Woods Hole Oceanographic Institute in late Fall, 2004.

Does *Xf* presence in grapevines affect pit membrane porosity?

In previous reports, we have described tests that indicate the porosity (i.e., the space between the polysaccharides) of vessel pit membranes is between 5 and 29 nm, much too small to permit passage of *Xf*. We have refined those tests by using colloidal gold particles having diameters of 5 and 20 nm. While the particles are very difficult to see under the microscope, their presence can be readily detected chemically by reacting samples containing the particles with Sigma Chemical Company's "silver enhancer". A segment of grapevine stem is fitted into a tube attached to a valve device that permits introduction of a small volume of water containing colloidal gold particles to the stem while maintaining pressure on a water line that drives water through the vessels of the segment. Introduction of food coloring, whose movement through the stem is not impeded by pit membranes; to the system and collection of the water + dye exiting the stem at the distal end indicates that the volume of water needed to move from one end of a 50 cm stem segment is less than 200 µl. Colloidal gold particles with a 5 nm can move through healthy stem segments, particles of 20 nm diameter cannot (Figure 1). However, when we used a vine that was showing the initial visible symptoms of PD at its base (i.e., its older internodes) and tested the movement of colloidal gold particles through a stem segment cut from the younger portion of the stem that had not yet begun to show PD symptoms, particles of 20 nm diameter moved through the xylem and were collected at the distal end. These results suggest that decreased pit membrane polymer integrity, hence increased pit membrane porosity, occurs in healthy-appearing stems on infected vines. This suggests that pit membranes are being opened up in infected vines, perhaps to permit the systemic movement of *Xf*.

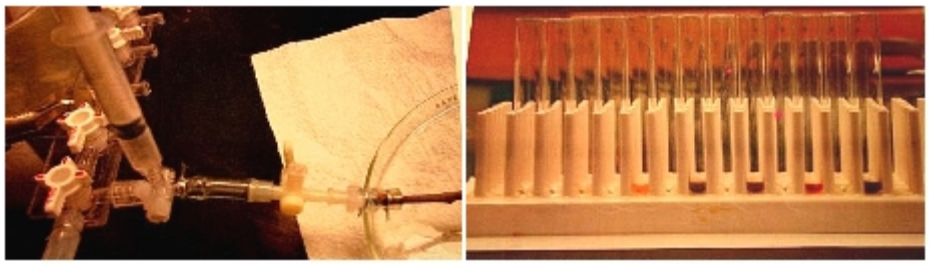

Figure 1. The photo on the left shows the valve system that permits the introduction of a small volume of colloidal gold particle-containing water to a pressurized stream that moves water, particles, and, if added, food coloring through grapevine stem segments. The segment is at the lower right in the picture. The photo on the right shows a series of test tubes, each containing 100mL of the water (+ gold particles) flushed from the distal end of the segment shown in the left-hand photo. The first 4 tubes from the left in the tube rack show no color. The emergence of gold particles, beginning with tube 5 from the left, is revealed by the addition of silver enhancer which gives a colored reaction product in the presence of gold.

A pulse (2 mL) of two pure cell wall-degrading enzymes, a β-1,4-glucanase (EGase) extracted from *Xf*, and a recombinant polyglacturonase (PG) from *A. niger*, have been flushed through stems using the same device described in Figure 1. The combination of EGase + PG, but not the use of either, alone, allowed the passage of the 20 nm gold particles, indicating that these enzymes in conjunction were able to increase the size of the pit membrane pores (Figure 2) and the water flow rate through the stem (Figure 3). Also, the analysis of serial fractions collected at the end of the stem has revealed the presence of polysaccharides containing uronic acid in the eluding fluid after the enzymes were added. This is further evidence supporting the possibility that *Xf* uses cell wall-degrading enzymes to cross the intervessel pit membranes and to move systemically in grapevine shoots.

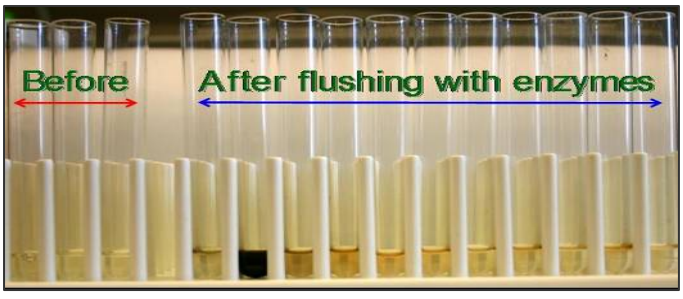

Figure 2. After a pulse of pure PG+EGase, the 20 nm gold particles pass through. The gold can be seen immediately after adding both enzymes, although it concentrates in the second fraction collected after the addition.

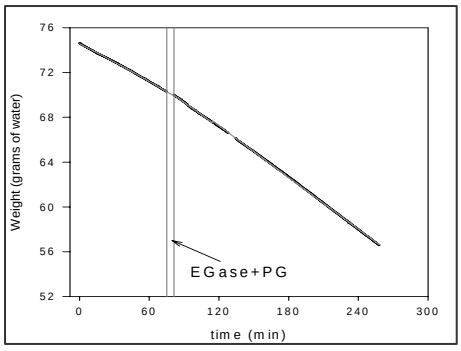

Figure 3. The reduction in weight of the reservoir that delivers water to the system depicted in Figure 1 is used to monitor water flow rate through the stem. The flow rate is faster after the enzymes' addition, as noted by the change in the slope.

CDTA is a chelating agent that can release charged uronides (pectins) from the pit membranes. When CDTA was introduced into the stems it also allowed the passage of the 20 nm gold particles. This is a confirmation that pectins are involved in regulating the size of the pit membrane pores. Thus the "putative" pectinase activity described for *Xf* could indeed result in an increase of the porosity size. On the other hand, after oligogalacturonides (G12) or polygalacturonic acid (PGA) were introduced into grapevine stems they partially or completely stopped the water flow (Figure 4). The possibility that pectic materials may participate in the occlusion of vessels of *Xf*-infected vines is being currently assessed.

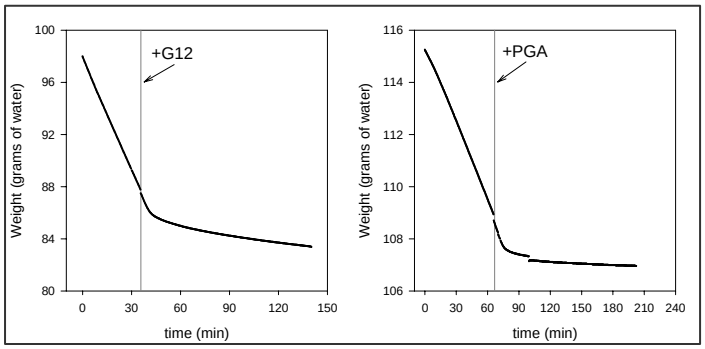

Figure 4. The addition of G12 reduced greatly the flow rate through the stem, whereas PGA completely stopped the flow about half an hour after its addition. G12 contains shorter chains than PGA; but both have a degree of polymerization that likely makes them much larger than the pores in the pit membranes. The addition times of G12 and PGA are indicated by the vertical bars. PG action on PGA would convert it into oligomers smaller than those in G12.

As our tests of the ability of 5 and 20nm beads to pass through stem segments continued into the summer months (2005) we noted a transition in the apparent porosity of pit membranes. Beginning in mid-July we noted a substantially increased stem water conductivity (tested as shown in the "flow rate" studies described by Figure 4) and the ability of both 5 and 20 nm particles to pass the length of the stem, with no prior enzyme treatments required. Work by Eleanor Thorne in a Rost and Matthews project showed that bacteria were able to travel from stem segment explants a relatively long distance, including passage through petioles and into 2° and 3° leaf veins. It is not clear exactly when in the season these studies were done. Thus it becomes important to determine if seasonal changes in pit membrane porosity, perhaps reflecting a change in the pit membrane porosity of more recently developed vessels, is a factor contributing to a (perhaps) "seasonal" change in the ability of *Xf* to move systemically through a grapevine. Although we are at the end of this season, we are hoping to determine if this apparent "opening" of pit membrane pores is sufficient to permit passage of bacteria without enzyme "assistance." This is a question that requires further study.

Objective 2. To determine whether GWSS feeding on grapevines is accompanied by xylem vessel cavitation.
This Objective will be addressed by a combination of researchers (details in the second-year proposal submitted) who will combine expertise in monitoring of the electrical signals produced by sharpshooters as they feed on grapevine xylem (Backus and colleagues), and characterizing the water-moving capacity of xylem vessels (Shackel, Matthews and Labavitch). Our time in the first year was spent primarily in establishing the techniques and infrastructure required to bring an assortment of techniques together.

Progress was made this year in developing the research infrastructure and protocols needed for the insect portion of the cavitation project. However, this proved to be more challenging than was foreseen at the time the proposal was written. Co-PI Backus's lab renovation in Parlier was delayed due to unforeseen problems acquiring building materials. The lab became fully functional in August 2004, whereupon work began immediately to perfect EPG protocols with the smoke tree sharpshooter (STSS), *Homalodisca liturata*. These protocols were successfully developed.

At the UC Davis end, Ph.D. candidate Alonso Pérez has developed the MRI techniques that will be used to determine whether vessels that the insect has been feeding become air-filled (i.e., cavitated) following the end of feeding (see the progress report for Shackel and Labavitch). Alonso Pérez also attended Dr. Backus' EPG Workshop at the State University of California, Fresno in July, 2005. At the workshop he was trained specifically in the recording and analysis of STSS probing waveforms. We have been also tuning and testing a device for monitoring the acoustic emissions produced at the time of a vessel cavitation. Now that the acoustic monitoring instrumentation is ready for our use and tested, we will combine its use with Backus' EPG techniques and Pérez' MRI approaches to obtain the answer to the question about feeding-related vessel cavitation. The first attempts at these experiments are scheduled for October, 2005. The information we obtain should be of substantial value for understanding how GWSS transmission to grapevines occurs.

Objective 3.: To 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 demonstration that the *Xf* PG gene actually encodes a pectin-degrading enzyme is important. Adding to that importance is our observation that *Xf* that lacks a functional PG gene is unable to induce PD symptoms in grapevines places *Xf* cell wall degradation capacity in a key position in PD development, consistent with the suggestion of the model that has served as the central thread of our research. However, we have not actually shown that the PG or, for that matter, the *Xf* BGase actively digests grapevine pit membrane-localized polysaccharides. That will be a focus of our studies in the coming year. In fact, Alonso Perez has recently been developing the histochemical analytical skills needed to answer some of these questions. Interaction with the projects of Tom Rost and Mark Matthews will add additional power to the analysis.

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. It is our view that 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 reported here and in a companion progress report 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*.

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FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and the USDA Animal and Plant Health Inspection Service.