Final Report for CDFA Contract Number 06-0225

Project Title: "The pit membrane barrier to *X. fastidiosa* movement in grapevines: Biochemical and physiological analysis"

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The results reported here are for research performed between July 1, 2006 and June 30, 2010.

Project objectives, activities, progress and findings

Objective 1. To characterize the biochemical action of *Xf* EGase, *in vitro* and *in planta* and determine if it is inhibited by plant proteins that have been identified as xyloglucan-specific endoglucanase (EGase)-inhibiting proteins (XGase-IPs).

We had reported that the introduction of PG and EGase to the xylem of explanted grapevine stems causes breakdown of pit membrane (PM) structure (Labavitch, 2006) while increasing pit membrane porosity (Labavitch et al. 2005). With colleagues (Agüero et al., 2005) we have reported that the expression of the gene encoding the pear fruit PG-inhibiting protein (PGIP) in transgenic grapevines slows the development of PD. We now show that the *Xf* EGase digests xyloglucan (XyG), a cell wall polysaccharide that is likely to be the PM target of the EGase (**Figure 1**).

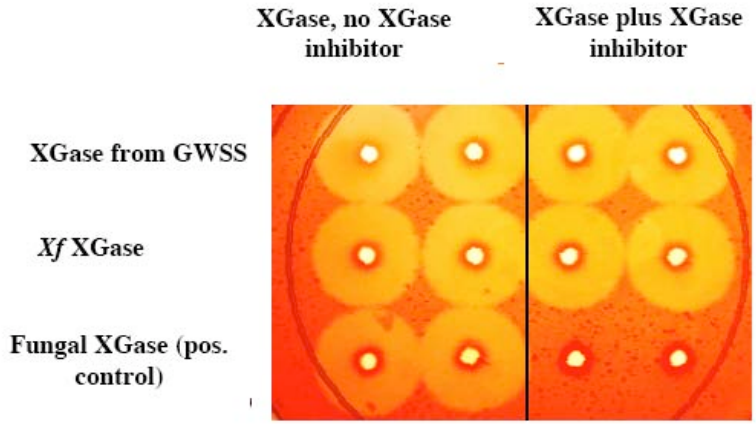

Figure 1. Shown is a radial diffusion assay of XGase activity. The xyloglucan (XG) substrate is dissolved in buffer and then mixed with melted agar. The agar is poured into a Petri dish and hardens. Wells are cut in the agar and then samples of the GWSS, *Xf* or fungal XGases are placed in the wells (left half). As the enzyme diffuses into the substrate-containing agar it digests it. The agar is stained with the dye Congo red to reveal the presence of undigested XG. The bigger the clear zones (shown above in yellow) the greater the XGase activity. The XGases were mixed with the tomato XGase-IP (right hand half) and the XGase activity was determined as described above. If the addition of the XGase-IP has caused inhibition of the XGase (i.e., reduces the size of the clear zone, as for the positive control) then it is an effective inhibitor. However, neither the GWSS nor the *Xf* XGase was inhibited (i.e., the clear zones are the same size whether the XGase-IP is present or not).

The data in Figure 1 indicate that the *Xf* EGase can be considered to be a xyloglucanase (XGase). Therefore, if the tomato protein that has been identified as an XGase-inhibiting protein (XGase-IP) is able to inhibit the *Xf*-XGase, then expressing it in combination with the pear PGIP in transgenic grapevines could provide substantially enhanced PD tolerance.

The tomato XGase-IP (Qin et al., 2003; York et al., 2004) was provided by our colleague, Dr. Will York, at the Complex Carbohydrate Research Center at the University of Georgia. *Xf* EGase/XGase was isolated from *E. coli* transformed by Dr. Caroline Roper to express one of the pathogen's ß-1,4-glucanase-encoding genes (Roper et al., 2007). We also tested the ability of the tomato XGase-IP to block the activity of a purified GWSS ß-1,4-glucanase and a fungal XGase provided by colleagues at Novozymes (positive control) (**Figure 1***).* While the absence of inhibition of the *Xf* EGase/XGase indicates that the tomato XGase-IP will not be useful for enhancing tolerance of PD, this result does not eliminate the idea from consideration. We have studied the PG-inhibiting proteins (PGIPs) of plants for many years. They are very selective in the PGs that they inhibit (Stotz et al., 2000). Some PGs are strongly inhibited by a given PGIP while other PGs are not inhibited at all. It is reasonable to think that XGase-IPs display the same selectivity. However, we are not engaged in studies to discover new sources of XGase-IPs. However, as additional inhibitors are reported we will attempt to obtain them in order test their action against the *Xf* XGase.

Our presumption that the PM target of the *Xf* EGase is xyloglucan (XyG) but to our knowledge no studies had provided direct biochemical data localizing any polysaccharides to the PMs of any plant species. Thus, if we are to understand the *in vivo* action of the *Xf* EGase, PG or any other pathogen cell wall modifying enzyme in PD development we must first identify the polymers present in the PM. Direct isolation of PMs is not possible. However, there are now a number of sources of antibodies that specifically bind to cell wall polysaccharides, thus permitting characterization of cell wall components using immunolocalization techniques. Dr. Qiang Sun, formerly a postdoctoral researcher with UCD researchers Mark Matthews and Tom Rost, began work in our program in early October, 2006 and made remarkable progress in characterization of grapevine PMs using antibodies that bind to homogalacturonan pectins and XyGs, the two polysaccharides that we feel are the most likely targets of the *Xf* PG and EGase/XGase.

These studies were carried out with by using monoclonal antibodies that recognize (1) homogalacturonan (HG) with a low level of methyl-esterification (antibody JIM 5), (2) HG with a high level of methyl-esterification (antibody JIM 7), (3) HGs that are cross-linked via Ca^{2+}-cross bridges (antibody 2F4) and (4) fucosylated XyG (antibody CCRC-M1). After reaction with the primary antibody (i.e., the antibody that recognizes specific wall polysaccharide structures), the bound primary antibody is revealed by using a secondary antibody labeled with green fluorescent FTIC that can be observed using a confocal or fluorescence microscope (**Figs. 2 & 3**). In some of the images we have also used these antibodies to ask what the cell walls surrounding tyloses are composed of (**Figs. 2 & 3**, right images). This is an important question related to vascular system obstructions in PD-infected grapevines. Tyloses form early in inoculated vines and numerous tyloses have been

reported to accumulate in grape vessels, often completely obstructing them. Tyloses develop from parenchyma cells that are adjacent to vessels and the primary walls of these parenchyma cells share the vessel-parenchyma PMs illustrated in some of the images below.

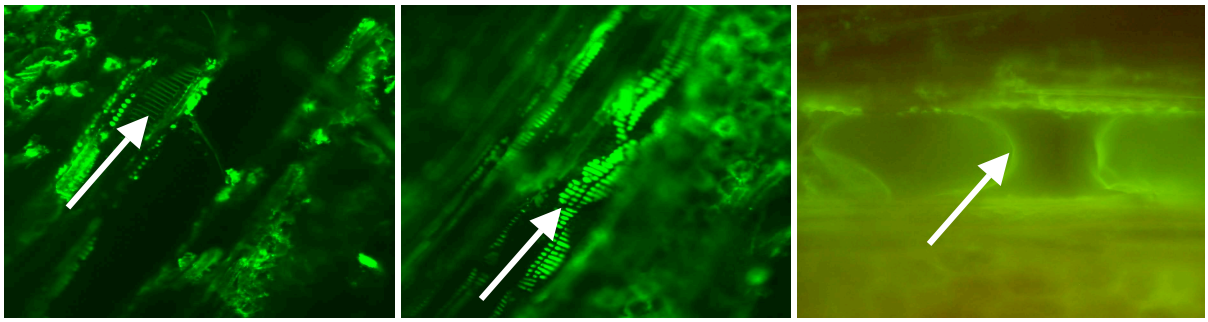

Figure 2. The use of JIM 5 reveals the presence of weakly Me-esterified HGs in the walls of grapevine intervessel PMs (left image), in vessel-parenchyma pit membranes (middle image) and in developing tyloses (right image). The arrows in the left and middle images point to the rows of PMs that are aligned in the vertical orientation along the length of a vessel. In the right image, the arrow points to the wall of one tylose in a vessel that contains at least two tyloses. The left and center images were made using the confocal microscope. The right image is from the fluorescence microscope.

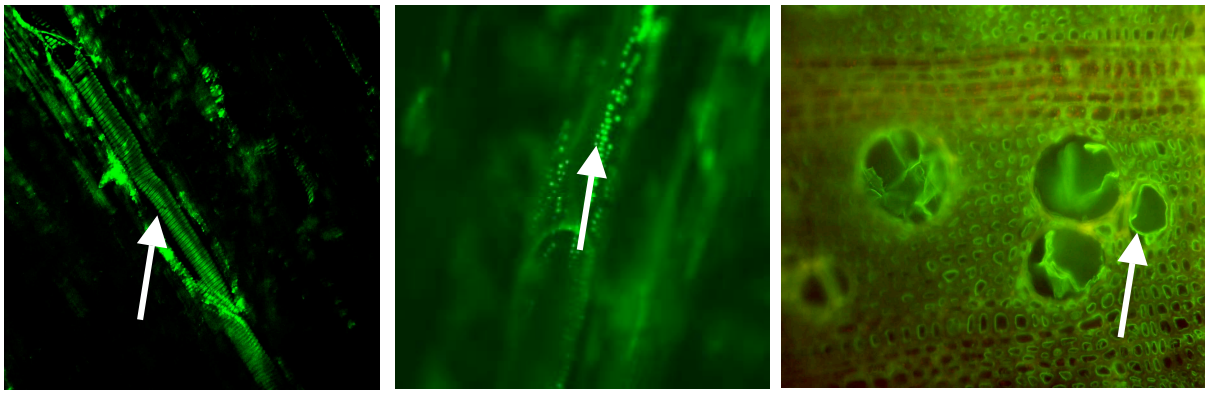

Figure 3. The use of CCRC-M1 reveals the presence of XyG in the walls of grapevine intervessel pit membranes (left image), and the use of JIM7 reveals the presence of extensively Me-esterified HG in vessel- parenchyma pit membranes (middle image) and in developing tyloses (right image). The arrows in the left and middle images point to the rows of PMs that are aligned in the vertical orientation along the length of a vessel. In the right image, the arrow points to the wall of one tylose in a vessel that contains at least two tyloses. The left and center images were made using the confocal microscope. The right image is from the fluorescence microscope.

Note: After our success in demonstrating the presence of homogalacturonans and XyGs in the PMs of PD-susceptible 'Chardonnay' grapevines (above), we (investigators Q. Sun and J. Labavitch) obtained additional funding from CDFA (08-0174 and 10-0266) to determine whether the PMs of PD-susceptible and PD-resistant grape germplasm differed in the polysaccharide compositions of their PMs. These studies developed a great deal more detail about relative polysaccharide compositions of PMs in different grape germplasm and about the degradation of PM

polysaccharides as inoculation with *X. fastidiosa* led to the development of PD symptoms in susceptible grape germplasm.

Objective 2. To examine the full range of effects on grapevine pit membrane porosity that result from introduction of cell wall-degrading polygalacturonase (PG) and EGase.

When this proposal was submitted in Winter, 2006 we anticipated that graduate student Alonso Perez would continue with us as a postdoc in the work of this project. However, his good fortune in getting a university position in Santiago, Chile made it impossible for us to start these studies in the summer of 2006, when the project was approved. However, Dr. Sun worked with Dr. Carl Greve through late Fall, 2006 and in the first part of 2007 to learn the techniques that Perez developed for testing pit membrane porosity (tests involve the passage of colloidal gold particles of defined sizes through stems, Labavitch et al, 2005).

We have shown that the introduction of PG and EGase to explanted stem segments will rapidly degrade PMs of PD-susceptible 'Chardonnay" (Pérez-Donoso et al., 2010), allowing the subsequent passage of *X. fastidiosa* cells (i.e., the enzymes acted to increase PM porosity in a way that would facilitate the systemic spread of a locally introduced, small *X. fastidiosa* population, presumably leading to fully developed PD). Given that we now feel that the PMs of grape germplasm that is not susceptible to PD have cell wall compositions that differ from that of 'Chardonnay', we should determine if enzyme addition degrades the PMs of the PD-resistant grape lines. However, we have not been able to identify a graded impact on 'Chardonnay' PM integrity by treatments with reduced amounts of PG and EGase. This may reflect the very local secretion of the enzymes in the vicinity of PM surfaces, potentially reflecting the mechanisms that control *X. fastidiosa*'s ability to modify PMs (Lindow and Chatterjee, 2008; Lindow et al., 2008). Images that show the progressive breakdown of PMs in infected 'Chardonnay' vines indicate that there is substantial erosion of PM structure before gaps in the PM that are sufficiently large to permit the passage of *X. fastidiosa* cells (see the note, above and Sun and Labavitch, 2010). It would be important for experiments testing the relative PG and EGase susceptibility of PMs from susceptible and resistant grape lines to use the minimum amount of enzymes required to digest the PMs of PD-susceptible grape germplasm.

Objective 3: To repeat our 2005 observations of a late Spring, dramatic increase in the porosity of grapevine pit membranes.

Tests run over the course of two Spring/Summer seasons did not show a convincing pattern of increasing PM pore sizes in 'Chardonnay' vines as we got later into the growing season. This does not mean that there is no such pattern. It may just mean that vine management variables that we were not aware of in the 2005 season were responsible for our 2005 measurements and these were not adequately matched in the 2008 and 2009 seasons.

Objective 4: Determine whether the plant hormone ethylene is important in determining whether PD develops in inoculated PD-susceptible 'Chardonnay' vines (**Note**: This objective was added after project 06-0225 was funded.)

Very early in our group's research on Pierce's Disease, my colleague Mark Matthews (UCD Viticulture and Enology Department) and I proposed a model of disease development (**Figure 4**) that was used to test a number of ideas about how PD developed after *X. fastidiosa* had been introduced to grapevines. This model included a role for the natural plant "stress" hormone, ethylene.

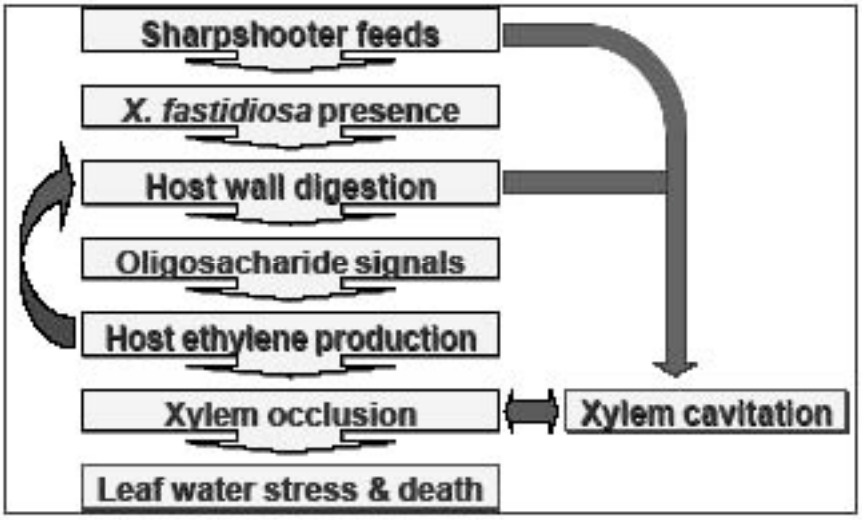

Figure 4. Early model of the factors resulting from *X. fastidiosa* introduction to a grapevine, which ultimately lead to the development of visible Pierce's Disease symptoms.

Early work demonstrated that ethylene treatment of grapevines caused the formation of vascular system occlusions (gel and tyloses) and an increase in stem resistance to water flux (Pérez-Donoso et al., 2007), symptoms that were typical for grapevines with PD. These observations seemed to support the inclusion of a role for ethylene in the model. However, this had not been directly tested until now. In this final report for CDFA contract number 06-0225, we report on our direct test of a role for ethylene in PD symptom development.

The best test of a role for ethylene in in PD development would be one in which grapevines inoculated with *X. fastidiosa* are unable to respond to ethylene even if they produce the hormone in response to sensing of the pathogen or some activity of the pathogen. 1-methylcyclopropene-1-carboxylic acid (1-MCP) is a gaseous molecule that blocks plant responses to ethylene because it binds to (thus, inactivates) ethylene receptors. 1-MCP-treated plants remain blind to ethylene until time has passed and the plant has produced new ethylene receptors. Several formulations of 1-MCP have been commercially produced. However, we were uncertain whether internal tissues in woody grapevine stems would be sufficiently accessible or responsive to 1-MCP. A subsidiary of Rohm and Haas (Agrofresh) had developed a sprayable version of 1-MCP and a preliminary test suggested that it would be effective in blocking response to ethylene. We reported on this preliminary trial in an earlier progress report for 06-0225. The trial was based on the observation that late winter pruning of grapevine stems stimulated the blockage of the xylem in the pruned stems because tyloses formed quickly. This had been

reported to be an ethylene response (Sun et al., 2007) and in our trial, 1-MCP applications to pruned grapevines prevented pruning-induced tylose formation. Thus we had confidence that we could use the sprayable 1-MCP formulation to eliminate ethylene responses in *X. fastidiosa*-inoculated grapevines. However, we noted that in this trial the rapid formation of tyloses (less than a week after pruning) meant that only one or two 1-MCP applications would be needed, whereas the much slower development of PD symptoms following needle inoculation of vines with *X. fastidosa* cell suspensions was likley to take more than 2 months (i.e., 8+ spray applications of 1-MCP).

Our growing awareness about the development of PD following *Xylella* inoculation of vines prevented us from confidently predicting whether blocking an inoculated vine's ability to respond to ethylene would slow the development of PD symptoms or accelerate them. Our experiment, therefore, was simply to spray test vines with an effective dose of 1-MCP (based on the trial described) at weekly intervals, manage the treated vines in the greenhouse, and pay attention to what happened.

Treatments began on May 24, 2010. A 20-vine test group (Chardonnay 23 on 101 rootstock, vines in 1 gallon pots) were sprayed with 2 liters of 0.05% Silwet L-77 surfactant and 0.0026% AfxRD-038 (1-MCP formulation) in water. The material was donated to us by Dr. Deirdre Holcroft of the AgroFresh company. The AfxRD-038 (Rohm & Haas) when mixed with water releases 1-MCP. All vines were needle inoculated at 4 points low on the stem with 20 ml droplets of water containing 2×10^4 *X. fastidiosa* cells. At weekly intervals the 1-MCP treated vines were removed from the greenhouse and sprayed with freshly mixed 1-MCP preparation, as above.

Observations for the first 7 weeks revealed no differences between the 1-MCP-treated and control vines, all vines looking healthy. At week 8 the control grapevines all displayed typical symptoms of PD (leaf scorch, green islands, etc.). However, while the 1-MCP-treated vines showed no PD symptoms, they all showed extreme phtytoxicity symptoms (leaves turned brown and dried out). We surmise that this might have been a response to the Silwet wetting agent followed by heat exposure in the greenhouse. Treatments of vines were discontinued at this point. The PD-infected control vines continued to deteriorate, all of these vines showed matchstick petioles and some died. The 1-MCP-treated vines began to show local recovery, with vigorous growth of green tissue at the stem apices in all 20 treated vines.

At the start of week 9, 5 vines were selected form each group and shoot cuttings from several internodes and leaves distal to the inoculation site were assayed for *X. fastidiosa* by PCR. The results were mixed with some control and some treated plants being positive for the *X. fastidiosa.* A repeat of the PCR tests was made in the following week and *Xylella* was present in 80% of the tissues tested from the control and 1-MCP-treated vines. By week 10, 60% of the control plants were dead or nearly so. However, all of the 1-MCP-treated plants remained alive and displayed vigorously growing, healthy green tissues at their apices.

Conclusion: This experiment is not easy to interpret. The phytotoxicity symptoms displayed by the 1-MCP-treated tissue make clear that more of the aspects of the 1-MCP treatments must be tested before repeating the experiment. Our earlier test of

1-MCP impacts on pruning-induced tylose formation had required only one application of the ethylene antagonist and had not triggered phytotoxic responses. However the fact that *X. fastidiosa* cells were present in both the 1-MCP-treated and control vines while only the control vines with (presumably) an active ethylene response system succumbed to PD is very intriguing and probably deserves additional examination. A repetition of this experiment is not in our plans at present.

Summary of major research accomplishments and results for each objective

Objective 1: We have tested the ability of the tomato XGase-inhibiting protein to inhibit the XGase from *X. fastidiosa*. Even though the test showed no inhibition, we now have a useful assay for testing other potential XGase inhibitors. Our immunolocalization images provide the first data for PM polysaccharide compositions for any plant species. New studies based on this preliminary grape PM work have led us to believe that the PM polysaccharide distributions of PD-resistant grape germplasm are different from the PM polysaccharide organization in PD-susceptible grapes. Thus, we are now in a good position to provide a specific description of biochemical changes occurring in grapevine pit membranes after *X. fastidiosa* infection and several specific chemical treatments that may affect pit membrane porosity. Results from our new PM composition-related work could lead to a rapid way to screen newly developed grapevine germplasm for relative PD resistance/susceptibility. Confirmation of this will require considerable testing.

Until additional EGase-inhibiting proteins are identified, whether the use of EGase inhibitors effective against *X. fastidiosa*'s EGase might be useful in enhancing grapevine resistance to PD remains an open and untested question.

Objective 2: We have not been able to define PG and EGase "dosages" that lead to minimum enzyme concentrations required to open PM polysaccharide networks, thus allowing *X. fastidiosa* passage.

Objective 3: Follow up tests did not reveal seasonal patterns of increasing grape stem PM porosity.

Objective IV. The test using 1-MCP to block grapevine responses to ethylene appeared to influence PD development in treated vines. However, this conclusion should not be viewed s crtain because of the phytotoxic response of 1-MCP-treted vines to the spray applications.

Intellectual Property:

No new intellectual property will be developed by this work. It is conceivable that the (1) use of PM polysaccharide screening for relative PD susceptibility or resistance and/or (2) manipulation of the grapevine's ethylene response sensitivity could have a beneficial impact on the vine's response to *X. fastidiosa* introduction. Both of these issues would require additional testing. Thus our results could shape "grapevine manipulation" efforts that eventually could lead to the development of intellectual property.

Appropriate References:

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The Relationship of the Potential Results from this Project and Solutions to the PD Problem in CA:

The ability of pit membranes to withstand the impacts of *Xf* and its cell wall-degrading enzymes and prevent the systemic spread of the pathogen appears to be a key to grapevine resistance to PD. Continuing studies based on the ideas that were explored for the first time in project 06-0225 are already underway. Whether this continuing work (projects 08-0174 and 10-0266) identifies additional opportunities for grapevine protection is not certain at this time.