DO CELL WALL STRUCTURES LIMIT *XYLELLA FASTIDIOSA* DISTRIBUTION IN INOCULATED, PIERCE'S DISEASE SUSCEPTIBLE AND RESISTANT GRAPEVINES?

Principal Investigator:
John Labavitch
Dept. of Plant Sciences
University of California
Davis, CA 95616
jmlabavitch@ucdavis.edu

Co-Principal Investigator:
Qiang Sun
Dept. of Biology
University of Wisconsin
Stevens Point, WI 54481
qsun@uwsp.edu

Cooperators:
Steven Lindow
Dept. Plant & Microbial Biology
University of California
Berkeley, CA 94720

Andrew Walker
Dept. of Viticulture & Enology
University of California
Davis, CA 95616

Hong Lin
SJVASC
USDA, ARS
Parlier, CA 93648

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ABSTRACT
Symptom development of grapevine Pierce's disease (PD) is determined largely by the extent to which the bacterial pathogen *Xylella fastidiosa (Xf)* spreads in infected vines via their water-conducting system. Thin primary cell wall regions called pit membranes (PMs) separating the adjacent vessels of the water-conducting system are the barriers to the systemic spread that *Xf* must clear by using its cell wall degrading enzymes (CWDEs). Therefore, the presence/absence of the CWDEs' target polysaccharides in the intervessel PMs may determine the movement of *Xf* in the water-conducting system, contributing to PD susceptibility/resistance of the host grapevine. This report introduces our recent development of a new method for detecting the presence, relative concentration and distribution of potential target polysaccharides of *Xf*'s CWDEs in grapevine PMs. This method combines scanning electron microscopy and immunohistochemical techniques and is effective in visualizing some polysaccharides in intervessel and vessel-parenchyma PMs. Compared with the technique we developed previously, this method provides a way to visualize PM structure at a much higher resolution while revealing the PM's polysaccharide composition. Our technique has the potential to identify cell wall polysaccharides exposed at the PM surface and changes in the PM polysaccharide components and quantities that occur during the degradation of intervessel PMs. This should provide some essential information for a better understanding of the PD resistance mechanisms of grapevines.

LAYPERSON SUMMARY
Understanding of grapevine Pierce's disease (PD) resistance/susceptibility mechanisms is essential to development of new PD-resistant grapevine germplasm to be deployed in vineyards. Our study focused on the structural factors of grapevines which limit the systemic spread of *Xylella fastidiosa (Xf)* and thus contribute to the PD resistance of grapevines. This report deals with the cell wall structural barrier to the bacterial spread, the so-called pit membrane (PM), describes a new method to detect the PM's polysaccharide compositions *in situ*, and reveals the spatial distributions and quantities of a group of polysaccharides which might affect *Xf* systemic spread. The further development of this method should contribute to a comprehensive understanding of the PD resistance of grapevines and help identify PD-resistant grapevine germplasm obtained through genetic improvement programs.

INTRODUCTION
As a vascular disease, Pierce's disease (PD) is causing a severe threat to the wine industry in the United States and some other countries. The causal pathogen is the xylem-limited bacterium, *Xylella fastidiosa (Xf)*, which is introduced to the vessel system of a grapevine by xylem sap-feeding insect vectors and spreads, multiplies and establishes its population only via the vessel systems of an infected vine (Purcell and Hopkins, 1996). It is clear that PD symptom development depends largely on whether the systemic spread of the initially introduced *Xf* throughout the vine can occur (Krivanek and Walker, 2005; Labavitch, 2007; Lin, 2005; Lindow, 2006a, b, 2007a, b; Rost and Matthews, 2007). *Xf* cells are initially introduced by an insect vector to few vessels and must carry out successive movement across vessels for the bacterial population to become systemic in the grapevine. The neighboring vessels are separated from one another by lignified cell walls; however, pits provide a possible communication pathway through the lignified cell walls because the only barrier presented by a pit is the thin primary wall regions called pit membranes(PMs) which contain the primary cell walls of the two adjacent vessels and a middle lamella (Esau, 1977). While the mesh of wall polysaccharides in a PM does not impede the passage of water, its pores are too small for *Xf* cells to pass through (Labavitch et al., 2004). Thus, it has been proposed that *Xf* cells secrete cell wall-degrading enzymes (CWDEs) to remove some wall polysaccharides, thus enlarging the PM porosity to permit systemic spread of *Xf*.

Xf's genome contains genes whose sequences suggest that they encode two types of CWDEs: polygalacturonase (PG) and endo-1,4-β-glucanase (EGase) (Simpson et al., 2000) and heterologous expression of the putative *Xf* PG and EGase genes (Agüero et al., 2005; Labavitch et al., 2006; Roper et al., 2007; Pérez-Donoso et al., 2010) produces proteins capable of

digesting homogalacturonan pectin and xyloglucan, respectively, polysaccharides that are often found in dicot cell walls (Carpita and Gibeaut, 1993). Furthermore, the introduction of PG and EGase to explanted grapevine stems causes breaks in the PM polysaccharide network and permits *Xf* cells to pass through intervessel PMs (Pérez-Donoso et al., 2010). These studies also demonstrate the presence of these potential polysaccharide targets of the bacterial CWDEs in grapevine intervessel PMs.

In our previous studies, we developed a technique which combines immunohistochemistry and confocal laser scanning microscopy (CLSM) to detect polysaccharide compositions of PMs (Labavitch and Sun, 2008). By comparing several candidate pectic and hemicellulosic polysaccharides among grape genotypes with different PD resistance, we have identified in grapevine PMs the kinds of polysaccharides which are the potential substrates of *Xf*'s PG and EGase (Labavitch and Sun, 2008, 2009). Our previous investigation on intervessel PM integrity in infected PD-susceptible grape genotypes has also clarified the process of PM degradation (Labavitch and Sun, 2009). The CLSM combined with the immunohistochemical method is excellent in identifying the PM cell wall compositions without obvious tissue damage and artifacts, but it poses some limitations in resolution when detailed structure and structural changes of intervessel PMs must be investigated. In this report, we describe our recent exploration of combining of the immunohistochemical method and scanning electron microscopy (SEM) to investigate PM polysaccharides. This technique provides a substantial resolution improvement suitable for the investigation of delicate structures and structural changes while it is still useful in identifying polysaccharide compositions. We expect this new technique can reveal PM polysaccharide compositions and dynamic changes in PMs during the process of intervessel PM degradation simultaneously. This combination of benefits may provide an efficient approach to evaluate the PD resistance/susceptibility of grapevine germplasm.

OBJECTIVES (**Note**: Only Objectives 1 and 2 in the proposal were approved for funding.)

1. Determine if the development of xylem obstructions (tyloses and pectin-rich gels) and the polysaccharide structure and integrity of PMs are affected by *Xf* inoculation of grapevines transformed to express the PGIP from pear and other plant species in rootstocks and in scions.
2. Determine whether there are differences in PM porosity or polysaccharide structure between resistant and susceptible grapevines. To what extent are these PM characteristics and the production of tyloses and gels modified by introduction of *Xf* to PD-resistant and -susceptible genotypes?
3. Determine the extent to which changes in pathogen virulence resulting from altered production of diffusible signal factor (DSF) correlate with the appearance of tyloses, gels and damaged PMs in inoculated vines.
4. Determine whether the impacts of inoculation on PM integrity and the production of vascular system occlusions identified in tested greenhouse-cultured vines also occur in infected vines growing in the field.

RESULTS AND DISCUSSION

Xf-infected vines of *Vitis vinifera* var. Chardonnay were used in this investigation. Each grapevine was grown in a greenhouse from a grafted root stock and two robust buds from each scion were left at the base, subsequently leading to the development of two shoots. At week four, vines were inoculated with *Xf* at the 6th internode from the base of one shoot for each vine. The two shoots of each vine were maintained with 20-25 internodes by pruning the tops off.

Samples were collected from all the internodes of each vine at week 12 after inoculation and were fixed in 4% paraformaldehyde. Internode samples were then trimmed into thin xylem segments exposing the transverse, radial and tangential surfaces. After being washed in PIPES buffer and in 3% MP (milk powder)/phosphate-buffered saline (PBS), some segments were used for control and the others for treatment. For treatment, xylem segments were incubated first with JIM5 (a rat monoclonal antibody recognizing weakly methyl-esterified homogalacturonans—Me-HGs) diluted in 3% MP/PBS and then with anti-rat IgG antibody conjugated with colloidal gold particles of 10 nm. After removing the excess antibody with PBS and distilled water washes, the samples were treated with a silver enhancement kit in a darkroom. This process causes silver particles to associate the gold particles, increasing the sizes of the silver-enhanced gold particles so that they are visible under SEM. Then, the samples were dehydrated via an ethanol series, critical point dried, and coated with Au-Pt. The coated samples were observed and photographed with a scanning electron microscope at 3.0kV under the modes of secondary electron and backscatter electron, respectively. As an experimental control, samples were processed in the same way except that they were incubated with 3% MP/PBS instead of JIM 5.

Our data indicate that the technique described above will detect the presence and distribution of weakly Me-HGs recognized by JIM5 in the vessel PMs (**Figures 1-3**). Silver-enhanced particles were observable under the secondary electron and backscatter electron modes (**Figures 1-3**). In experimental controls (samples not incubated with JIM5), silver-enhanced particles were very few and were randomly distributed over lateral vessel walls, indicating the background noises with this technique could be efficiently suppressed in both backscatter electron (**Figure 1A, B**) and secondary electron (**Figure 1C, D**) modes. In all the samples treated with the primary and secondary antibody followed by silver enhancement, enhanced gold particles were found to be mostly restricted in the PM parts with intact structure, including both vessel-parenchyma PMs (**Figure 2A-C**) and intervessel PMs (**Figure 2D**). This has indicated the presence of weakly Me-HGs in the PMs, which is consistent with the result we obtained previously using the combined CLSM and immunohistochemistry method (Labavitch and Sun, 2008, 2009). However, the silver-enhanced particles were randomly distributed through each type of PM,

suggesting the random distribution of weakly Me-HGs. This information cannot be obtained using our initial method due to its relatively low resolution.

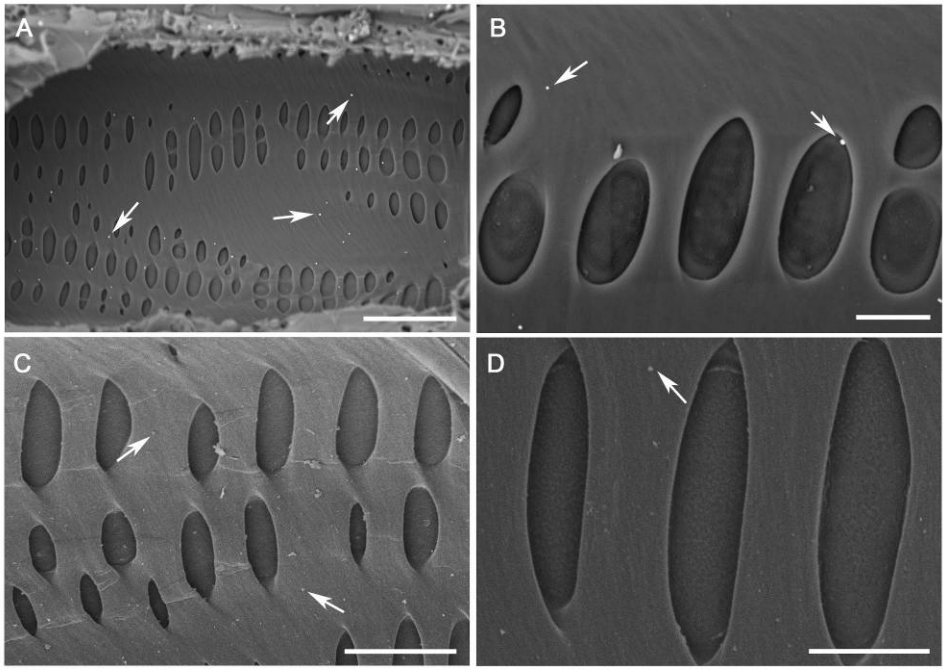

Figure 1. Xylem tissue incubated without JIM 5 but with colloidal gold-conjugated secondary antibody followed by silver enhancement in experimental controls. Silver enhanced gold particles are visible even at a low magnification under SEM and are randomly distributed in small amounts. A-B. Scanning electron micrographs taken under backscattering electron mode, showing white, easy-to-distinguish silver-enhanced gold particles (arrows). C-D. Images taken under secondary electron mode, showing silver-enhanced gold particles (arrows) are not easy to distinguish from their background. Bars in A, B, C and D are equal to 25, 5, 10 and 5µm, respectively.

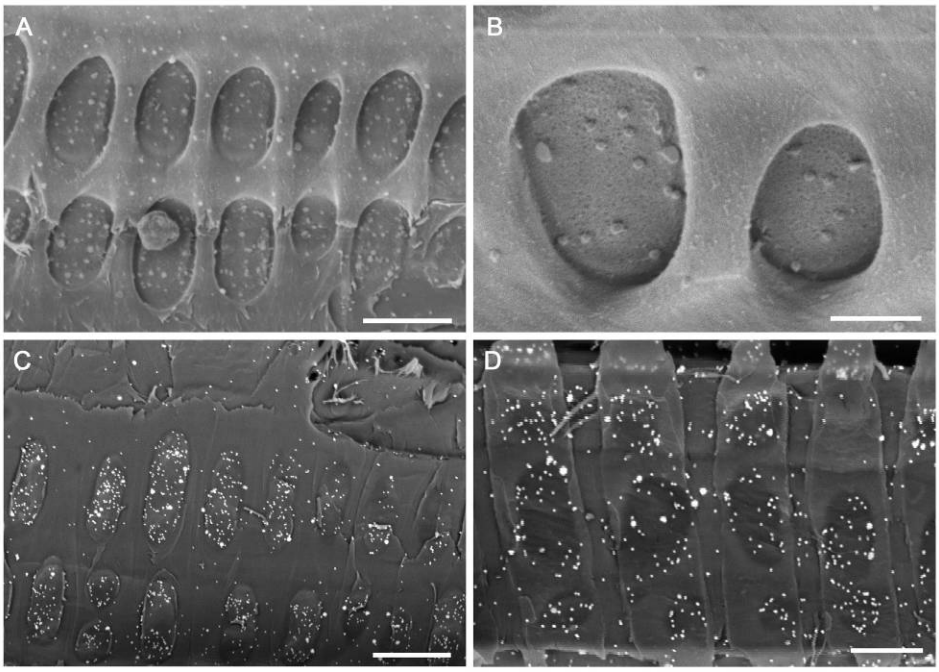

Figure 2. Images of xylem tissues treated with both JIM 5 and gold-conjugated secondary antibodies followed by silver enhancement, taken under secondary electron mode (A-B) and backscatter electron mode (C-D). A-C. Silver-enhanced particles are present on vessel-parenchyma PMs, showing the rich presence of weakly Me-HGs. D. Abundant presence of weakly Me-HGs on intervessel PMs is indicated by silver enhanced particles. Bars in A, B, C and D are equal to 5, 2.5, 10 and 5µm, respectively.

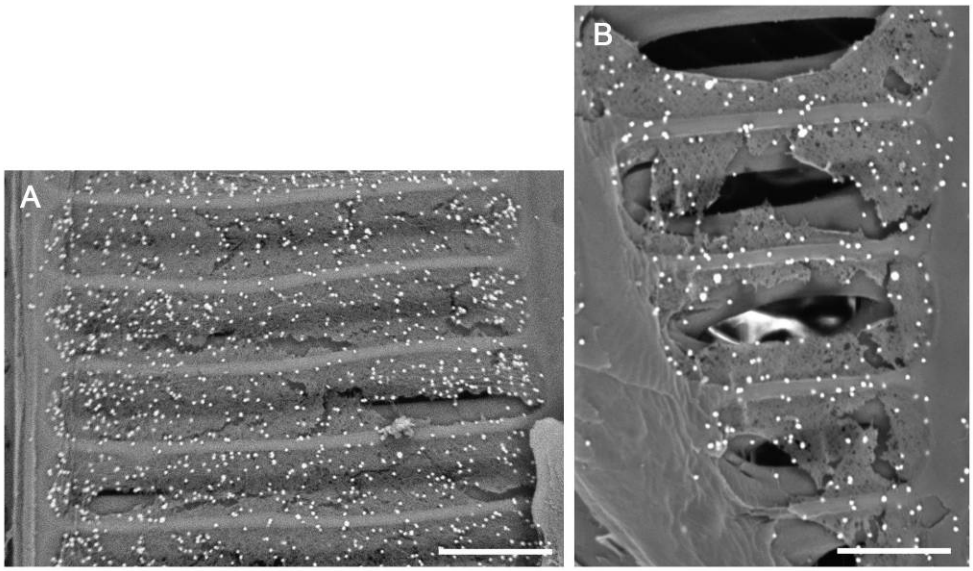

Figure 3. Distribution and quantity of weakly Me-HGs in intervessel PMs at two different stages of the PM degradation process. A. Dense silver enhanced gold particles on less degraded intervessel PMs, indicating more Me-HGs exposed at the PM surface. B. Some weakly Me-HGs are still left in highly degraded intervessel PMs. Bars in A and B are equal to 10 and 5μm, respectively.

Degrading intervessel PMs were compared with intact PMs to reveal any possible differences that are due to the PM degradation process. As in the intact PMs, weakly Me-HGs were detected in both slightly and more completely degraded intervessel PMs and were randomly distributed throughout the PMs (**Figure 3**). There was no obvious difference in the density of silver-enhanced particles between intact PMs (**Figure 2D**) and slightly degraded PMs (**Figure 3A**), but the density of the particles decreased in the more completely degraded PMs. It seems reasonable to believe that *Xf*'s CWDEs should first attack the polysaccharides exposed at the PM surface because the relatively small pore size of the PMs (5 to 20 nm) would prevent diffusion of the relatively large CWDE molecules into the PM. However, removal of the superficial polysaccharides should subsequently loosen the PM's polysaccharide meshwork and make the underlying target polysaccharides accessible to the enzymes. That here is no difference in the distribution and quantity of silver-enhanced particles between intact and slightly degraded PMs suggests that weakly Me-HGs might be present at some depth into the PM as well as being present on the PM surface. If so, our enhanced method should also be suitable for the investigation of spatial distribution and quantity of a polysaccharide type both throughout the PM thickness as well as across its exposed face.

CONCLUSIONS

1. The technique we developed is effective for visualizing pectic polysaccharides in both intervessel and vessel-parenchyma PMs under conventional SEM.
2. The technique has revealed the distribution and quantity of weakly Me-HGs in both intact and degrading PMs of Chardonnay vines.

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