Cell Wall-Degrading Enzymes Enlarge the Pore Size of Intervessel Pit Membranes in Healthy and *Xylella fastidiosa*-Infected Grapevines[1][C][W][OA]

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The pit membrane (PM) is a primary cell wall barrier that separates adjacent xylem water conduits, limiting the spread of xylem-localized pathogens and air embolisms from one conduit to the next. This paper provides a characterization of the size of the pores in the PMs of grapevine (*Vitis vinifera*). The PM porosity (PMP) of stems infected with the bacterium *Xylella fastidiosa* was compared with the PMP of healthy stems. Stems were infused with pressurized water and flow rates were determined; gold particles of known size were introduced with the water to assist in determining the size of PM pores. The effect of introducing trans-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA), oligogalacturonides, and polygalacturonic acid into stems on water flux via the xylem was also measured. The possibility that cell wall-degrading enzymes could alter the pore sizes, thus facilitating the ability of *X. fastidiosa* to cross the PMs, was tested. Two cell wall-degrading enzymes likely to be produced by *X. fastidiosa* (polygalactuoronase and endo-1,4-β-glucanase) were infused into stems, and particle passage tests were performed to check for changes in PMP. Scanning electron microscopy of control and enzyme-infused stem segments revealed that the combination of enzymes opened holes in PMs, probably explaining enzyme impacts on PMP and how a small *X. fastidiosa* population, introduced into grapevines by insect vectors, can multiply and spread throughout the vine and cause Pierce's disease.

In grapevine (*Vitis vinifera*) stems, water moves with the transpiration stream from one vessel to the next, traversing the scalariform bordered pits in the secondary wall (Mullins et al., 1992; Stevenson et al., 2004b). Water must also pass through the pit membrane (PM) that occurs between a pit pair (i.e. opposing pits in adjacent vessels; Mauseth, 1988; Dickison, 2000). A PM is the shared primary cell walls and middle lamella of vessels that were not covered with the secondary wall of neighboring vessel elements as the development of the water conducting system progressed. PMs are composed of cellulose microfibrils embedded in a polysaccharide matrix of hemicellulose and pectins (Mauseth, 1988; Fisher, 2000; Tyree and Zimmermann, 2002). The fine mesh-like polysaccharide structure of PMs provides minute openings (pores) through which water can move with minimal restriction to other vessels or neighboring parenchyma cells. In angiosperm trees, the diameters of most of these pores have been described to vary between 5 and 20 nm (Choat et al., 2003, 2004), but pores of up to several hundred nanometers have occasionally been observed (Sperry et al., 1991; Sano, 2005; Wheeler et al., 2005). The small size of the PM pores is a safety mechanism that limits the expansion of gas bubbles from one cavitated vessel to its neighbors and the movement of pathogens from one infected, water-filled vessel to its neighbors as water moves through the xylem system (Sperry and Tyree, 1988; Nakaho et al., 2000; Tyree and Zimmermann, 2002). It is thought that pore size is largely controlled by the physical arrangement of hydrated pectins and the cross-links that they establish with themselves and other polysaccharides in the PM (Fleischer et al., 1999; Zwieniecki et al., 2001). The infiltration of vessels with solutions having modifications in ion content or pH causes changes in hydraulic resistance that are consistent with swelling or shrinking of pectins and the consequent changes in PM pore size (Zwieniecki et al., 2001).

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The xylem-limited bacterium *Xylella fastidiosa* is the causal agent of Pierce's disease (PD) of grapevines. *X. fastidiosa* is vectored by sharpshooter (Cicadellidae) and spittlebug (Cercopidae) insects that feed on xylem sap and introduce the bacteria into xylem vessels (Varela et al., 2001). In order for the bacterial population to become systemic, individual bacterial cells must cross the PMs that separate two adjoining vessels. *X. fastidiosa* is a rod-shaped bacterium with dimensions ranging from 250 to 500 × 1,000 to 4,000 nm (Mollenhauer and Hopkins, 1974), making them too large to pass freely through the majority of the PM pores that have been described in angiosperms. Nevertheless, evidence of *X. fastidiosa* cells traversing PMs and gaining access to an adjacent vessel has been reported (Newman et al., 2003). This intervessel movement of *X. fastidiosa* cells was observed too frequently by Newman et al. (2003) to be considered the result of random encounters with damaged PMs; thus, they proposed that *X. fastidiosa* is able to degrade the grapevine PM. The involvement of cell wall-degrading enzymes during PD first had been proposed based on indirect evidence from the development of internal symptoms and the location of bacteria in *X. fastidiosa*-infected shoots, but the absence of evidence for bacterial enzyme production limited wide acceptance of this idea (Hopkins, 1989; Fry and Milholland, 1990a; Purcell and Hopkins, 1996). However, the observation of intervessel *X. fastidiosa* movement described above and reports that the *X. fastidiosa* genome contains several genes similar to those encoding cell wall-degrading polygalacturonase (PG) and endo-1,4-β-glucanase (EGase) in other bacteria (Simpson et al., 2000; Wulff et al., 2003) suggested the contrary. Indeed, a *X. fastidiosa* mutant disrupted in the *pglA* gene, which encodes an endo-PG, was restricted to the point of inoculation and unable to move systemically in grapevine, indicating that PG plays a major role in vessel-to-vessel movement (Roper et al., 2007). Furthermore, recombinant *X. fastidiosa* PG (Roper et al., 2007) and EGase (this study) are capable of digesting polygalacturonic acid (PGA) and β-1,4 linked glucans, respectively. The recent detection of PG in the xylem sap of infected vines and less severe symptom development in transgenic grapevines expressing a pear (*Pyrus communis*) PG-inhibiting protein (pear PGIP [pPGIP]) also suggests that *X. fastidiosa* uses cell wall-degrading enzymes to open up PM pores to facilitate vessel-to-vessel movement (Agüero et al., 2005).

We have reported that during early stages of *X. fastidiosa* infection, some stems presented exceptionally high hydraulic conductivities (higher than comparable healthy stems), which was attributed to enzymatic digestion of the PMs (Perez-Donoso et al., 2007). In this study, the size of PM pores in healthy and *X. fastidiosa*-infected stems was approximated by flushing the xylem of grapevine stem explants from the base with water containing particles of known size and determining if the particles could be recovered in the water collected from the distal ends of the explants. We also tested the ability of the PG and EGase activities that have been reported in *X. fastidiosa* (Roper, 2006; Roper et al., 2007) for digesting the intervessel PM and increasing the size of the PM pores and report that the pPGIP inhibits *X. fastidiosa*'s PG, presumably explaining why expression of the pPGIP-encoding gene in transgenic grapevines suppresses PD development (Agüero et al., 2005). Finally, the effects of stem infiltration with the chelator trans-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA), PGA (a model homogalacturonan pectin), or oligogalacturonides (OGAs; potential signal molecules generated when PGA is digested by PG) on PM pore size were also evaluated.

RESULTS

PM Pore Size in Healthy and *X. fastidiosa*-Infected Vines

In independent experiments performed on healthy grapevines during spring 2005, fluorescent polystyrene beads with average diameters of 1,000, 300, or 29 nm were introduced with water into the proximal ends of several 0.5-m-long stem explants (Fig. 1). The beads were not recovered at the distal end of the stems (they were not visible with the microscope in the collected eluting fluid), indicating that the size of the pores in the intervessel PMs was smaller than the smallest (29 nm) beads used in the experiments. Xylem conduits of healthy stems were also infused with colloidal gold microspheres of 20 or 5 nm in diameter. The 5-nm microspheres moved through these stem segments (dark color developed when silver enhancer was added to the eluting fluid; Fig. 2), whereas the 20-nm microspheres did not. This suggests that the porosity of vessel PMs in healthy vines is between 5 and 20 nm, much too small to permit free vessel-to-vessel passage of *X. fastidiosa*. However, when this experiment was repeated using nonsymptomatic stem segments from *X. fastidiosa*-inoculated shoots or the noninoculated shoot from a two-shooted vine that had been inoculated in its second shoot, the 20-nm microspheres moved through the xylem and were collected at the distal end. These results indicate that increased PM pore size, perhaps due to decreased PM polysaccharide integrity, is one consequence of *X. fastidiosa* presence, even when visible external symptoms of PD are not evident.

Cell Wall-Degrading Enzymes Increased PM Pore Size

Two pure, recombinant, hydrolytic enzymes, an EGase from *X. fastidiosa* and a PG from *Aspergillus niger*, both expressed in *Escherichia coli*, were flushed in pulses through healthy grapevine stems (Fig. 1). Flushing a mixture of EGase and PG, but not the use of either alone, allowed the passage of the 20-nm gold microspheres (Fig. 2), suggesting that the combined action of enzymes was needed to increase the average diameter of the PM pores. Serial fractions of the eluting fluid were collected at the distal end of the

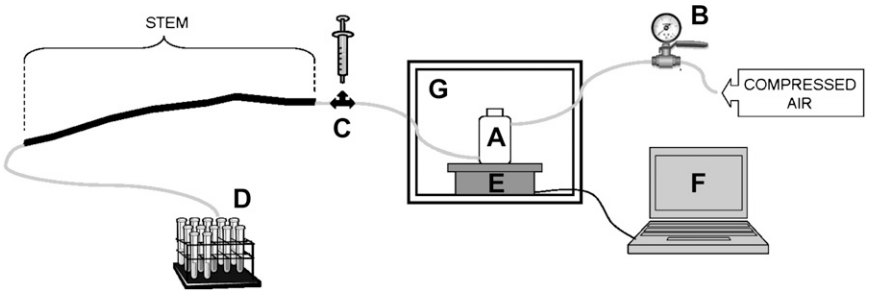

Figure 1. Schematic representation of the system designed to (1) infuse different solutions into grapevine stems without interrupting the water flow and (2) measure water flow rate through the stems. Compressed air pressurizes the water container (A); two pressure gauges (B) in series are used to keep a constant pressure during the experiments. The basal end of the stem is attached to plastic tubing and to a set of three-way valves and loops (C) used to introduce particles, enzymes, chelator, or carbohydrates into the water flow. The distal end of the stem explant (actually its more apical end) is fitted into plastic tubing to facilitate the collection of eluent using a fraction collector (D). The water container is on top of an electronic balance (E) that is connected to a computer (F) programmed to automatically record the weight change of the container every 15 s. The water reservoir and the electronic balance were placed inside a cardboard box (G) fixed to the bench surface, providing a shield from air currents and limiting vibrations.

stem for the period in which water, gold microspheres, and enzymes were being flushed through the explants. Colorimetric assays revealed the presence of uronic acid, a breakdown product from pectic polymers, only in the fractions collected after the enzyme combination was introduced (Fig. 3). Similarly, assays to detect the presence of PG and EGase activities in the collected fractions were positive only in the fractions collected after both enzymes were introduced together into the stems. The introduction of the gold microspheres into the stem did not have a major impact or only gradually decreased water flow rate (Fig. 3). On the other hand, the addition of the hydrolytic enzymes quickly reduced the water flow rate in most experiments. Nevertheless, in some stems in which only PG was introduced, the water flow rate was not considerably reduced; sometimes it was increased (Fig. 3B). Furthermore, the reduction in water flow rate observed after stem infiltration with the PG and EGase mix was sometimes followed by a partial recovery of the flow rate (as seen in Fig. 3A about 0.5 h after the injection of PG and EGase). The inconsistent changes in water flow rate observed in the stems upon the introduction of hydrolytic enzymes could be explained by the enzymatic release of polysaccharides or oligosaccharides from PMs, which may have two contrasting consequences for water movement within individual vessels. First, the removal of some of the uronide component (detected colorimetrically in the fractions collected after the enzymes were added) from the polysaccharide matrix of the PMs would enlarge the size of the pores, decreasing resistance to water flow. Second, the released polysaccharides or oligosaccharides may occlude the pores at vessel PMs located downstream from the PMs initially impacted by enzymatic digestion, which could explain the reduction in water flow rate observed in most of the enzyme-introduction experiments.

The data for the impacts of PG and EGase on water flux reflect the combined changes in resistance along the entire conduit path through stem explants. The complexity of the data for the time course of changes in water movement following treatment, therefore, could reflect changes in a series of PMs as well as the development of vessel obstructions along the path. However, the impact of enzyme action at the level of individual PMs is much easier to interpret. Scanning electron microscopy examination of PMs in water- (control) and enzyme-infused stem segments makes clear that PM polysaccharides are disrupted by enzyme treatment (Fig. 4). Images of both intervessel and

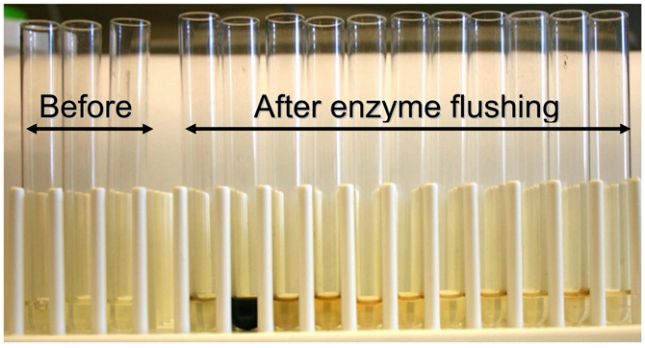

Figure 2. Serial fractions of the eluent collected at the distal end of an explanted stem before and after flushing a pulse of PG + EGase through the stem. Colloidal gold microspheres (20 nm) were infused into the stem with water before adding the enzymes. Normally, 20-nm gold particles cannot cross the PMs; however, after the addition of enzymes, the particles passed through. The presence of gold particles in the eluting fluid is revealed by the formation of a black precipitate when colloidal gold reacts with the "silver enhancer" solution. There was no open xylem vessel from the base to the tip of the stem, based on the inability of air under low pressures to pass through the stem explant; thus, the gold particles had to move through at least one PM. [See online article for color version of this figure.]

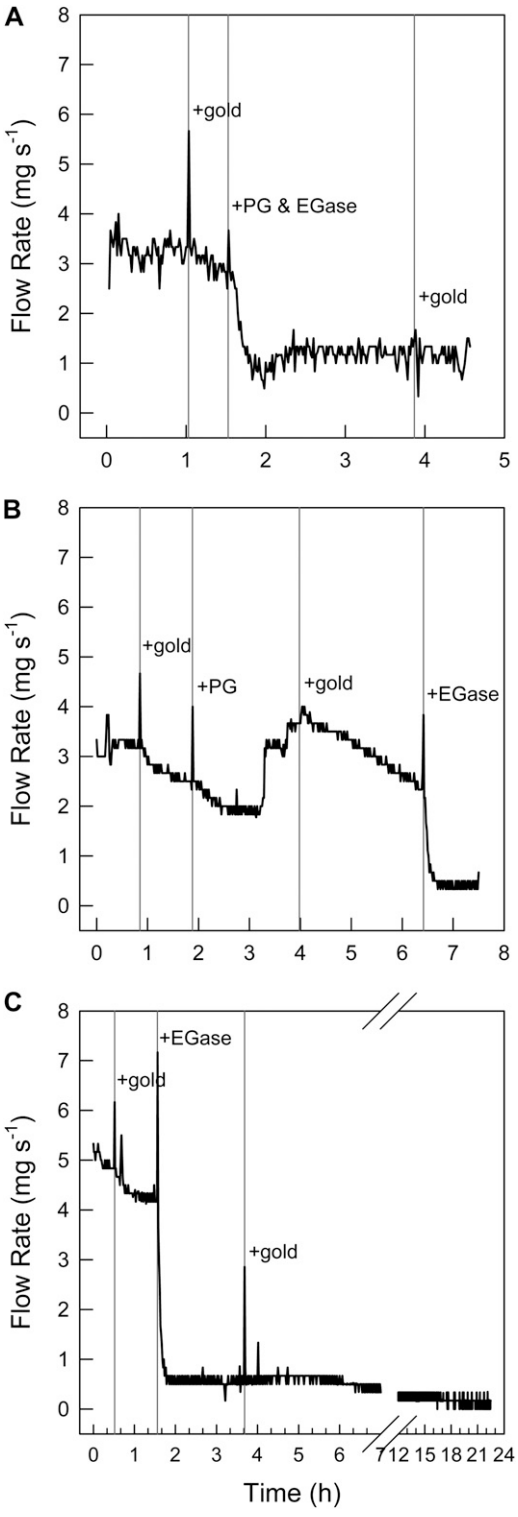

Figure 3. Water flow rates over the course of several perfusion experiments. The plotted rates correspond to the average of four data points per minute. The vertical lines show the times of introduction of colloidal gold and enzymes. The presence of gold particles in the eluting fluid was assayed in the collected fractions throughout the experiments (see Fig. 2). A, Example of a gold and enzyme infiltration experiment. Gold particles added the first time were detected in the eluent only after PG + EGase had also been introduced into the stem, whereas the second pulse of colloidal gold was detected immediately after its introduction. Sharp rises or drops in flow rate between consecutive treatments reflect temporary pressure changes associated with switching between solutions. B, Example of an experiment in which gold particles, PG, and EGase were injected sequentially into the stem. C, Example of an experiment in which gold particles and EGase alone were infiltrated into the stem. In B and C, gold particles were not detected after the introduction of PG or EGase alone.

vessel-parenchyma pits and PMs are shown because the much larger pit apertures of the vessel-parenchyma pits make the impact of PG and EGase easier to see. However, the enzyme-caused breaks in the intervessel PMs can be seen through the narrow apertures in the secondary wall that arches over these PMs.

pPGIP Inhibits *X. fastidiosa* PG

The facts that (1) a PG-deficient strain of *X. fastidiosa* neither spreads systemically in grapevines nor causes PD (Roper et al., 2007) and (2) expression of the pPGIP in grapevines suppresses PD development in inoculated vines (Agüero et al., 2005) suggested that pPGIP inhibited the pathogen's PG, but inhibition has not been specifically reported. In fact, some early reports have suggested that PGIPs will not inhibit bacterial PGs (Cervone et al., 1990; Johnston et al., 1993). However, incubation of pear fruit protein extracts containing PGIP activity (based on inhibition of *Botrytis cinerea* PG; data not shown) inhibited the PG in extracts of *E. coli* that had been transformed to express the *X. fastidiosa* PG-encoding sequence *pglA* (Fig. 5). The increase in reducing sugars over time was roughly linear over the course of both incubations, with the slope of the increase in the sample incubated in the presence of pear proteins being roughly 50% of the slope in the absence of pear extract.

CDTA Released Pectins from the PMs and Increased Pore Size

CDTA is a chelating agent that can bind and remove Ca^{2+} from cell walls. For this reason, CDTA-containing solutions are used to extract the so-called ionically bound pectins from cell walls (Selvendran and O'Neil, 1987). When a CDTA solution was continuously fed into grapevine stems, it solubilized uronic acid and neutral sugars that were subsequently detected in the collected fractions. CDTA also allowed the passage through the stems of the 20-nm gold microspheres (Fig. 6). These results strongly suggest that pectins are involved in determining the size of the PM pores. They also imply that the pectin-degrading PG described for *X. fastidiosa* could indeed act to increase the PMP, potentially facilitating the systemic spreading of the bacteria.

OGA and PGA Introduction into Stems Reduced Water Flow Rate

The introduction of OGAs (average molecular mass approximately 0.9 kD) or PGA (average molecular

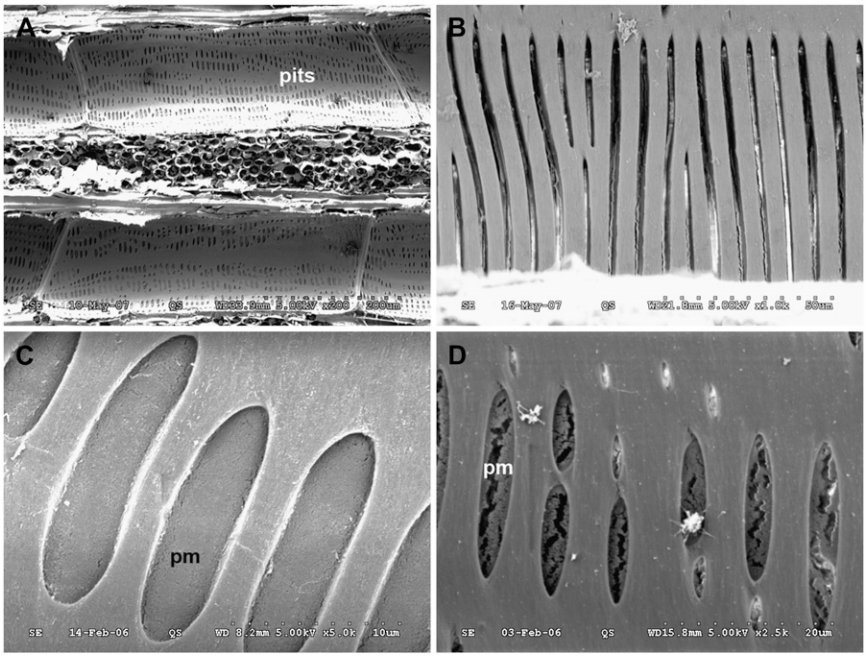

Figure 4. Scanning electron microscopy images of tangential longitudinal sections of secondary xylem vessels depicting the effect of cell wall-degrading enzymes on the integrity of PMs. A, Two vessels separated by a ray of parenchyma cells, showing abundant vessel-parenchyma pits. B, Bordered intervessel pits are arranged in a scalariform pattern, and broken intervessel PMs can be seen through the narrow aperture of each pit in the xylem of a stem explant that was treated with PG and EGase. C, Intact vessel-parenchyma PMs (pm) in the xylem without enzyme treatment. D, Broken vessel-parenchyma PMs in the xylem treated with PG and EGase.

mass approximately 30 kD) into grapevine stems partially or completely stopped the water flow (Fig. 7). The OGA preparation contained carbohydrate chains substantially shorter than those present in the PGA (determined by HPLC; Melotto et al., 1994; data not shown), but the OGA preparation contained some oligomers with a degree of polymerization that likely made them much larger than the average diameter of the pores at the PMs. In a stem flushed with OGAs, the average size of the OGAs recovered in the eluting fluid at the distal stem end was substantially reduced (HPLC analysis), suggesting that the larger oligomers in the OGA preparation had been retained (i.e. filtered out) by the PMs (data not shown). By the time the experiment was terminated, the introduced OGAs had stopped water flow through the treated stem (Fig. 7A).

DISCUSSION

The small size of PM pores (less than 20 nm) should prevent the systemic spread of *X. fastidiosa* throughout the grapevine via the xylem. Nevertheless, passage through PMs and systemic *X. fastidiosa* movement occur in infected grapevines. Based on sequence analysis, the genome of *X. fastidiosa* possesses the potential to encode cell wall-degrading enzymes, including one PG-encoding gene and multiple genes encoding EGase activities. Indeed, the putative PG gene and one of the putative EGase genes have been cloned and expressed in *E. coli*, and the heterologously expressed proteins were shown to have the predicted activities (Roper et al., 2007). PMs are thought to represent the portions of the primary cell walls of procambial cells that had differentiated into water-conducting vessels or tracheids but were not covered with lignified secondary walls as pit fields were created during xylem differentiation (Esau, 1977). Grapes are dicots, so their primary walls should contain homogalacturonan pectins and xyloglucans (Carpita and Gibeaut, 1993). These should be the primary determinants of PMP and also are likely substrates for *X. fastidiosa*'s PG and EGase. In fact, the proteins expressed from the cloned and expressed *X. fastidiosa* PG and EGase digest PGA and fucosylated xyloglucan, respectively (Roper et al., 2007; Supplemental Figs. S2 and S3). Immunolocalization studies have shown these polysaccharides to be present in grapevine PMs (Q. Sun and J.M. Labavitch,

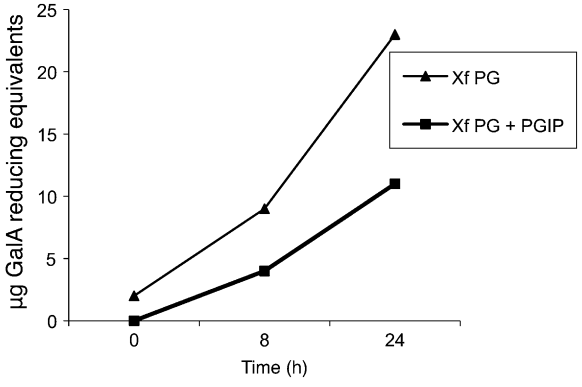

Figure 5. pPGIP inhibits *X. fastidiosa* PG. The *X. fastidiosa* (Xf) PG-encoding gene was expressed in *E. coli*, and protein was isolated from cells that were induced to express the PG. Protein was isolated from mature green Bartlett pear fruits using a high-ionic-strength buffer. The *X. fastidiosa* PG was incubated with PGA in the absence and presence of the dialyzed pear fruit protein extract, and aliquots of the reaction mixtures were tested for reducing sugars at intervals.

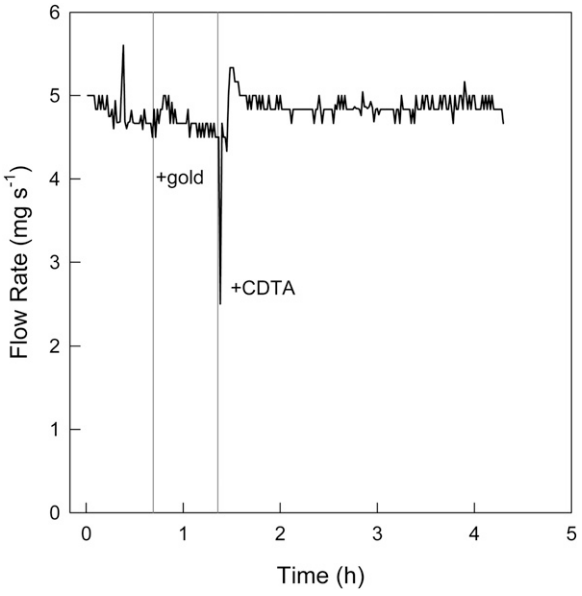

Figure 6. Flow rate through a stem explant flushed with deionized water. A 2-mL pulse of gold particle suspension was added 0.7 h after the beginning of the experiment. Water flushing was then continued for an additional 0.67 h, and then the water in the reservoir was replaced with 0.05 M CDTA solution, buffered at pH 6.0. The addition of CDTA did not change significantly the flow rate through the stem. Uronic acids were found in the eluent collected immediately after the CDTA addition. Colloidal gold was found in the eluting fluid fractions collected subsequent to the CDTA introduction and the detection of uronic acids in the eluent.

unpublished data). Thus, it is not surprising that introduction of EGase and PG into the vascular system of grapevine stem explants would act on these exposed primary wall polymers to increase the size of the pores, allowing *X. fastidiosa* passage.

The enlargement of PM pore size should reduce resistance to water flow in the xylem but, simultaneously, also could increase the risk of air seeding into the vessels. VanderMolen et al. (1983) reported that when PG was introduced into castor bean (*Ricinus communis*) petioles, it caused a rapid vessel obstruction by pectin-rich gels and that this was mediated by a rapid PG effect on ethylene production. Perez-Donoso et al. (2007) reported that grapevines respond to ethylene exposure, at least in the longer term, by producing vessel obstructions (tyloses and gels), and Sun et al. (2007) have confirmed that tylose formation in grape stems is triggered by ethylene. Whether such obstructions might occur in the time frame of the experiments in our study is unknown. Similar vascular occlusions have been observed in stems and petioles of *X. fastidiosa*-infected vines (Fry and Milholland, 1990b; Stevenson et al., 2004a). Thus, while enzyme introduction should open a path for *X. fastidiosa* and increase water flow, the maintenance of this increased rate of water flow is not certain because the interactions between enzymes and PM cell walls, cell wall digestion products, the grapevine vascular system, and, potentially, ethylene could also lead to vessel occlusion.

Furthermore, it is not clear how these vascular system-occluding structures form in planta. Cell wall-degrading enzymes should digest polysaccharides that are components of the PM into smaller molecules (polymers and oligomers) that are likely to

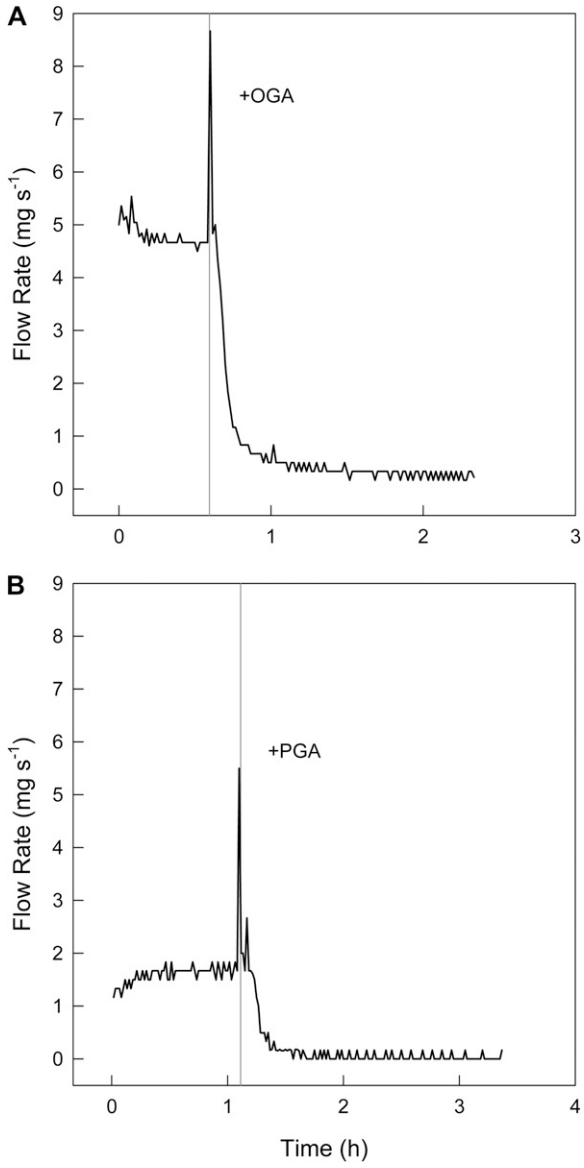

Figure 7. A, Water flow rate through the stem was greatly reduced soon after the addition of OGAs (average degree of polymerization [DP] = 8.56), and it practically stopped about 1.7 h after OGA introduction. Some uronic acid was detected in the two fractions of the eluent collected after the addition of OGAs (the first of these fractions was completed 24 min after the introduction of OGAs). The analyses of total uronic acid and uronic acid reducing ends in the collected eluent fractions indicated an average DP of less than 2, which suggests that the larger oligomers in the OGA preparation were retained within the stem, presumably at the PMs. B, PGA (average DP = 100) completely stopped the flow through the explant about 0.5 h after its introduction. The addition times of OGAs and PGA are indicated by the vertical bars.

be relatively mobile within individual water conduits. These enzyme products would be free to interact with one another, perhaps forming aggregates that could plug vessels and reduce water flow. It is not possible to predict whether continuous enzyme action would ultimately dismantle these aggregates, resulting in long-term increased water flow. Furthermore, the introduction of enzymes, as we have done in this report, presumably mimics *X. fastidiosa*'s enzyme production during its colonization of and spread through the grapevine xylem system (Chatterjee et al., 2008). This enzyme action could weaken the exposed primary cell walls of the PMs between living xylem parenchyma cells and water conduits, allowing them to be pushed into the vessel lumens by the parenchyma cell's turgor, initiating tylose formation. Our experiments have only begun to address these hypotheses and uncertainties within the context of the dynamic PM structure and physiology that influence water transport in the xylem.

Our results provide evidence supporting the possibility that cell wall-degrading enzymes produced by *X. fastidiosa* might be used to increase PM pore size, allowing intervessel movement of bacteria. However, grapevines present relatively rare, large openings (200 nm or less) in the PMs, like the ones described to occur in most angiosperm trees (Sperry et al., 1991; Sano, 2005; Wheeler et al., 2005). These might facilitate the passive movement of *X. fastidiosa* between vessels. Furthermore, recent reports have shown the presence of open pathways (conduits) within the primary xylem of grapevine and described how these may be exploited by *X. fastidiosa* during infection (Chatelet et al., 2006; Thorne et al., 2006). Their data show that *X. fastidiosa* could potentially move passively through the primary xylem (up to three nodes in the stem and into the leaf secondary vein system) quite rapidly but that PMs would have to be breached for movement in the secondary xylem of the shoot. Also, the notably long xylem vessels of grapevine should facilitate the rapid movement of *X. fastidiosa*. However, as in most dicots, the vessel length distribution of grapevines is skewed toward shorter vessels in a negative exponential fashion (Sperry et al., 2005; Chatelet et al., 2006). Therefore, *X. fastidiosa* would be much more likely to be introduced by an insect vector into a short vessel than a long vessel by random chance at the time of inoculation and to encounter numerous short vessels during systemic migration through the vascular system. Thus, it seems unlikely that systemic infection of grapevine by *X. fastidiosa* could be accomplished by means of a purely passive process.

The use of PG or EGase alone did not sufficiently alter the structure of the PM polysaccharide matrix to permit the movement of the gold particles. However, the introduction of a combination of PG and EGase into grapevine stems resulted in the passage of the 20-nm gold particles and the release of uronic acid into the eluting fluid. The studies of the primary cell walls of suspension cultured sycamore (*Acer pseudoplatanus*) cells that led to the first widely accepted primary cell wall model (Bauer et al., 1973; Keegstra et al., 1973) demonstrated that PG digestion of pectins enhanced the ability of exogenously added fungal EGase to digest xyloglucan. Thus, our observation that the combined action of PG and EGase is required for increasing PMP implies that PG must remove pectins from PMs to expose xyloglucans, which then are cleaved by EGase, altering the PM matrix and allowing the 20-nm particles to pass through.

The majority of the stems into which the PG and EGase were introduced showed a considerable reduction in the water flow rate soon after enzyme introduction, but in a few stems in which PG alone was introduced, higher water flow rates were detected (Fig. 3). This observation may be in agreement with studies in which substantial reductions in hydraulic resistance were reported after the digestion of the PMs of vessel-less species following incubation of stems with commercial cellulase (Schulte et al., 1987; Schulte and Gibson, 1988). These studies also described microscope observations that revealed that stem enzyme incubation frequently led to complete elimination of the PMs. However, data on the variety of wall polymer-digesting activities of the enzyme preparation were not provided by the authors, and because commercial enzymes often contain diverse polysaccharide-digesting activities (Labavitch and Greve, 1983), it is difficult to draw conclusions from their observations about the specific PM polysaccharides affected. The reduction in the water flow rate that occurred in most stems after the introduction of the enzymes suggests that the digestion of the PMs (i.e. their pectic and hemicellulosic constituents) may have been only partial. The reduction in water flow rate that we observed could be attributed to enzymatic release of relatively high M_r polysaccharides that clogged the xylem conduits located in the digested PMs or farther downstream (toward the explants' distal ends [i.e. toward the apex]). This idea is supported by the detection of uronic acid in the eluting fluid fractions collected after the addition of the enzymes in these stems. Also, at least in the longer lasting perfusion experiments, the decline in water flow rate may have resulted from our use of deionized water, rather than dilute KCl solution, as a perfusate (Zwieniecki et al., 2001; Tyree and Zimmermann, 2002). In general, the water flow rate partially recovered approximately 0.5 h after the initial reduction, perhaps due to the continued action of the enzymes that released polysaccharides from the occluded PMs or digestion of PM polymers previously released. Also, following the introduction of enzymes, a common observation was a pattern of uronic acid and gold particle elution in collected fractions that cycled from a relatively high content to a relatively low content, suggesting that the enzymes were digesting PM polysaccharides sequentially downstream. This interpretation would suggest that the first pulse of particles or uronic acid represented flow through vessels that contained only one PM (e.g. through a

single vessel) for the length of the flushed explant, the second represented vessels with only flow through two PMs, and so on. By this reasoning, we propose the model that in serial vessels the flow of gold particles or enzymes between successive PMs would be relatively rapid, slowing at PMs while enzymes acted to enlarge PM pores, which would increase the flow and particle movement rates until the next PM was encountered. This model of how PM barriers and enzyme action on PM integrity interact to explain the flux patterns in our artificial infusion system could reflect how the *X. fastidiosa* population spreads in grapevines infected by insect vector introduction of the bacterium into xylem vessels. Chatterjee et al. (2008) have identified a diffusible signal factor (DSF) that influences the expression of several *X. fastidiosa* genes; this includes suppression of the expression of the PG- and EGase-encoding genes at high *X. fastidiosa* density. Thus, PM-to-PM movement of *X. fastidiosa* when the pathogen population is relatively low (thus, low DSF concentration) early in disease development could be relatively rapid due to the expression of PG and EGase, with delays upon reaching an intact PM. Then, very local production of the wall-digesting enzymes would remove the barrier and permit more rapid movement until the next PM is encountered. When the bacterial cell density increases, this *X. fastidiosa* systemic spread would slow as PG and EGase expression is suppressed due to increasing local concentrations of DSF.

The introduction of CDTA into the stems released uronic acids and allowed the passage of the 20-nm gold particles (Fig. 6). This is further evidence supporting the idea that altering the PM polysaccharide complement results in changes in the pore size of the PMs. Specifically, we present strong evidence indicating that the removal of the Ca^{2+} cross-linked pectic component of PMs is enough to increase the size of the pores. CDTA is expected to have a more general impact on PM pectin solubilization than PG. This is because CDTA has a much smaller M_r than the enzyme, which facilitates its passage through PM pores as well as its access to the pectins embedded in the PM polysaccharide matrix. Also, while the efficiency of the enzymes depends on their specific glycosidic cleavage activity and the structure and accessibility of the pectin substrates in the PM, CDTA's chelating action would solubilize pectins in a less specific and restricted way. Thus, it is not surprising that stem xylem infusion with CDTA, but not with PG alone, resulted in particle movement. However, the fact that the combined use of PG and EGase also resulted in larger pores supports the idea that *X. fastidiosa* uses the combination of its cell wall-degrading enzymes to cross intervessel PMs and move systemically in grapevine shoots.

The introduction of OGAs and PGA into the stems demonstrated that polysaccharides of medium to high M_r can occlude the xylem conduits and reduce, or completely stop, the water flow through the stems within 0.5 h after their addition (Fig. 7). In contrast to the reduction in flow rate caused by enzyme action, the water flow rate did not recover after the introduction of OGAs or PGA into the stems. Additionally, in one of the OGA-introduction experiments, an HPLC comparison of the size distribution of the OGAs introduced into the stem and the OGAs that were collected in fractions of stem effluent indicated that only the smaller oligomers were able to pass the PM barriers. The larger oligosaccharides in the OGA preparation were not recovered in the eluting fluid, suggesting that they were retained at the PMs of the vessels. The OGAs were not introduced along with chelator, raising the possibility that the larger OGAs, like the tagged OGAs used by Vreeland et al. (1989) as histochemical probes for wall-localized homogalacturonans, may have bound to the PMs via the Ca^{2+} associated with their pectin network.

The hydrolytic action of PG on PM polysaccharides would probably generate oligosaccharides that are not normally present in undamaged cells. These oligosaccharides, particularly OGAs, have been reported to possess biological activity. Pectin-derived oligomers like the OGAs used here have been reported to have impacts on growth, fruit ripening, and several aspects of plant responses to pathogens, including responses that are triggered by increases in plant ethylene synthesis (Melotto et al., 1994; Ridley et al., 2001). Both a mixture of *Fusarium oxysporum* PG and other cell wall-degrading enzymes and ethylene exposure induced vascular gel formation in castor bean leaves 24 to 48 h after treatment (VanderMolen et al., 1983). In grapevines, exposure to ethylene triggered the rapid (within 10 d) formation of tyloses and pectin gels that occlude water conduits, as observed in PD-infected grapevines (Pérez-Donoso, 2007).

The average size of *X. fastidiosa* cells (250–500 × 1,000–4,000 nm; Mollenhauer and Hopkins, 1974) is much too large to pass through the pores of intact PMs. If the introduction of the bacterium into a few grapevine vessels is to lead to PD, the bacterial population must increase and spread throughout the vine. The data presented herein suggest that *X. fastidiosa*'s PG and EGase act together to digest PM polysaccharides, presumably facilitating the pathogen's systemic spread and PD development. These results make clear why PG is a *X. fastidiosa* virulence factor (Roper et al., 2007), and the demonstration that the pPGIP inhibits the *X. fastidiosa* PG in vitro (Fig. 5) explains why PD symptom development is suppressed in inoculated transgenic grapevines that express the pPGIP-encoding gene (Agüero et al., 2005). Data also suggest that pectin digestion products, particularly large oligosaccharides, might disperse from the site of PM degradation and plug PM pores "downstream" from the site of enzyme (and presumably pathogen) action. To our knowledge, this issue has not been raised previously in the context of diseases caused by *X. fastidiosa*, and it offers a possible explanation for the intriguing fact that PD symptoms are often found in leaves somewhat distal to where the bulk of the vessel colonization has occurred in the petiole or stem.

MATERIALS AND METHODS

Plant Material

Rooted cuttings of cv Chardonnay grapevines (*Vitis vinifera*) were potted in 7.6-L containers in March 2005. The vines were grown and kept in greenhouse conditions (25°C, 45% relative humidity, 16-h photoperiod, and drip irrigation) until March 2006. The plants were pruned regularly to maintain two shoots of up to a maximum length of 2.5 m. The age of the shoots used for the first infiltration experiments (colloidal gold particles and enzymes) ranged from 1 to 2 months. The combined water flow rate and infiltration experiments were performed using shoots from different plants that had been growing for 3 to 6 months. The total length of the stem segments used in the different experiments varied from 0.5 m (May 2005) to 1.4 m (December 2005). Nonsymptomatic shoots from *Xylella fastidiosa*-infected plants that had been needle inoculated the previous season (Perez-Donoso et al., 2007) were used for the PMP size measurements performed in April and May 2005; healthy control plants of the same age were also utilized for these measurements.

Determination of PM Pore Size

In order to determine the size of the pores in the PMs of grapevine stem vessels, particles of known size were introduced at the base of the stems as described below and in Figure 1. If particles were larger than the size of the pores, the PMs should act as sieves and retain the particles. However, if the particles are smaller than the pores, they should be able to cross the PMs and be recovered with the eluting fluid at the distal end of the stem. Fluorescent polystyrene beads with average diameters of 1,000, 300, and 29 nm (Polysciences) and colloidal gold microspheres with diameters of 20 and 5 nm (Sigma-Aldrich) were infused at the base of the stems. The presence or absence of the polystyrene beads in the collected fractions was confirmed by observing aliquots with the compound microscope and UV light. The presence of colloidal gold was visualized using a silver enhancer kit (Sigma-Aldrich). Preliminary tests using equal parts of products S5020 and S5145 (Sigma-Aldrich) to prepare the silver enhancer indicated that a positive reaction developed (turned dark color) within 2 min when the silver enhancer was mixed with 1:10, 1:50, and 1:100 dilutions of the gold colloid suspension in water (0.01%, w/v), whereas a control developer solution (1:1 mix of water and silver enhancer) remained clear. A positive reaction occurred within 10 min when the silver enhancer was mixed with a 1:1,000 dilution of the gold suspension, whereas the control developer solution remained clear. When the silver enhancer was mixed with a 1:10,000 dilution of the gold suspension, the mix started to change color after 30 min but the control developer solution also started to develop a dark color, although less intense than the sample containing colloidal gold. Thus, in the gold microsphere-introduction experiments, it was considered that the gold did not cross the PMs if a positive reaction between the silver enhancer and eluting fluid collected in the fractions was not apparent within 15 min. The color change of a control developer solution of silver enhancer and water was also monitored for all the experiments.

Water Flow and Stem Infiltration Apparatus

The vines were taken to the laboratory, leaves were excised at the lamina-petiole junction, and petioles were sealed with hot plastic glue. The stems were excised underwater and connected to the water flow meter apparatus (Fig. 1). Deionized, filtered (0.22 μm), degassed water was infused into the base of the stems at constant pressure: 100 kPa was used for the measurements made between June 3 and July 12, 2005, as well as for the experiments performed in March 2006. Between July 28 and December 12, 2005, the basal flow became too fast for consistent measurement of flow rates, and collection of effluent from stem explants and infusion experiments were carried out at 28 kPa, after an initial 5-min explant water flush at 100 kPa. Deionized water has long been known to cause a slow decline in xylem flow rates, probably as a result from the swelling of pectins in the PMs, leading to a reduction in pore size (Zwieniecki et al., 2001). Nevertheless, deionized water and not a dilute KCl solution (1–10 mM) was used as purfusate in these experiments in order to avoid confounding effects or altering the activity of enzymes and the CDTA chelator on PMs. Water flow rate was determined by recording the weight change of the pressurized water container (W) over time (t), and data points were retrieved automatically with a computer every 15 s (Fig. 1). Instantaneous rates (dW/dt) were averaged for 1-min periods, and the resulting water flow rates were plotted. Then, solutions containing particles of known size or agents that were expected to modify the integrity or porosity of the PMs (e.g. hydrolytic enzymes, chelators, pectins, and pectin oligomers) were injected into the system without interrupting the flow. Eluent flow rate was also continuously monitored after these injections, and serial fractions of the eluting fluid were collected at the distal end of the stem throughout the experiments (Fig. 1). Twenty-eight experiments in which a pulse of different solutions was introduced into the stems were performed between April 2005 and March 2006. PG alone was introduced in five experiments, EGase alone in three experiments, EGase followed by PG was assayed one time, PG followed by EGase two times, PG + EGase were assayed in 11 experiments, PGA in one experiment, OGAs two times, and CDTA in three experiments. Previous examinations of gold particle movement in stem explants demonstrated that 5-nm particles passed freely through explants but 20-nm particles did not, establishing grapevine PMP at between 5 and 20 nm. Thus, in our tests to determine factors causing increased PMP, a pulse of colloidal gold particles with an average diameter of 20 nm was injected into the stems before and after introducing the solutions.

Enzyme, CDTA, OGA, and PGA Introduction into Stems

The hydrolytic enzymes EGase and PG, a CDTA solution, OGAs, and PGA were also added into the system, and their effects on PM pore size and water flow rate were tested. The EGase was obtained from inclusion bodies purified from *Escherichia coli* expressing a recombinant *X. fastidiosa* EGase encoded by the gene (PD1851) showing homology to known EGase genes (Simpson et al., 2000; Wulff et al., 2003; Xia et al., 2004). The PG was cloned and purified from *Aspergillus niger* (Armand et al., 2000). It was kindly provided by Dr. Carl Bergmann (Complex Carbohydrate Research Center, University of Georgia). Assays of EGase and PG activity were based on increases in reducing sugar level in incubation mixtures, as described (Gross, 1982). The enzyme preparations introduced into stems contained 1.0 unit mL^{-1} EGase and PG (1 unit equals the amount of enzyme that produces 1.0 μmol of reducing equivalents in 1 h). The CDTA was introduced as a 0.05 M solution prepared in 0.1 M sodium acetate at pH 6.0. Mixtures of citrus pectin oligomers (OGA) were prepared as described by Campbell and Labavitch (1991). PGA (Sigma Chemical) and OGA were dissolved in water at 0.4% (w/v). Unless stated otherwise, all the solutions were introduced in a single 2-mL pulse. After some of the experiments, fractions collected both before and after adding the recombinant enzymes were tested for PG and EGase activities using radial diffusion assays (Taylor and Secor, 1988) with PGA and tamarind (*Tamarindus indica*) xyloglucan (XyG; Megazyme International) used as substrates, respectively. Postincubation detection of areas of polysaccharide substrate digestion in the radial diffusion assays utilized ruthenium red or Congo red staining for PG or EGase assays, respectively. In some experiments, these fractions also were tested for uronic acid content using the *m*-phenylphenol reagent (Blumenkrantz and Asboe-Hansen, 1973). Determination of the sieving effects caused by the PMs at the xylem conduits was done by comparing the chromatographic profiles of citrus pectin oligomers before and after being introduced into a vine shoot. Analysis of the eluted oligomer size distributions was carried out using a Dionex HPLC system (model BioLC) equipped with a pulsed amperiometric detector and a 4- × 250-mm PA-1 anion-exchange column (Dionex), as described by Melotto et al. (1994).

Isolation of *X. fastidiosa* EGase

The EGase introduced to the xylem system of grapevine stem explants was obtained by cloning one of several putative *X. fastidiosa* EGase-encoding genes, expression of the cloned sequence in *E. coli*, and recovery of active protein from protein inclusion bodies. EGase activity was confirmed in vitro using carboxymethyl cellulose (CMC) and fucosylated XyG as substrates.

PCR Amplification and Cloning of the PD1851 Open Reading Frame (*engXCA*) Encoding EGase

PCR was used to amplify the PD1851 (*engXCA*) open reading frame from genomic DNA of the *X. fastidiosa* Temecula strain (Hendson et al., 2001) using Platinum Taq DNA Polymerase High Fidelity (Invitrogen). Primers were designed to amplify, beginning at the ATG start codon and ending immediately before the (TAG) stop codon, to allow the addition of a vector-encoded hexa-His tag to the C terminus of the protein. The primers were generated

containing restriction sites *Nco*I and *Hin*dIII (Roper, 2006). For all PCRs, the following parameters were used: denaturation at 95°C for 1 min (5 min for the first cycle) and annealing for 1 min at 56°C. The extension step was 72°C for 2 min (10-min final cycle). All PCRs were carried out for 35 cycles. A 1,845-bp PCR product containing the entire *engXCA* open reading frame was amplified from *X. fastidiosa* genomic DNA using the primers indicated (Roper, 2006). The PCR product was cloned into the pCR2.1-TOPO vector to create pMCR6. pMCR6 was digested with *Nco*I and *Hin*dIII (New England Biolabs). The *Nco*I-*Hin*dIII fragment obtained was cloned into the pET-20b(+) expression vector to create pMCR7 and transformed into *E. coli* Novablue (Novagen, EMD Biosciences). Plasmids were extracted from transformants and screened by restriction analysis for the correct insertion of the *engXCA* amplicon. A plasmid with the correct insert was sequenced using the same primers to confirm that the insertion was in frame.

Expression of the Recombinant EngXCA Protein in *E. coli*

E. coli BL21 (DE3) plysS was transformed with either pMCR7 or the empty pET-20b(+) vector (Novagen, EMD Biosciences) by heat shock and plated on Luria-Bertani (LB)-Glc medium + 100 μg mL^{-1} carbenicillin and 34 μg mL^{-1} chloramphenicol. Single colonies were grown overnight at 37°C in 3 mL of LB-Glc broth + 100 μg mL^{-1} carbenicillin and 34 μg mL^{-1} chloramphenicol. The overnight cultures were pelleted by centrifugation (8,000 rpm for 5 min) and resuspended in 250 mL of LB-Glc + 100 μg mL^{-1} carbenicillin and 34 μg mL^{-1} chloramphenicol. This culture was grown to an optical density at 600 nm of 0.4, and the cells were harvested by centrifugation and resuspended in 250 mL of fresh LB-Glc + 100 μg mL^{-1} carbenicillin and 34 μg mL^{-1} chloramphenicol containing 0.4 mM isopropyl β-D-1-thiogalactopyranoside for protein induction. Cultures were shaken at room temperature overnight. Aliquots were removed before and after induction, normalized to an optical density at 600 nm of 2.5, and pelleted by centrifugation. Fifty microliters of 1× Laemmli sample buffer (Laemmli, 1970) was added to each sample. Samples were boiled for 5 min and subjected to SDS-PAGE (Sambrook and Russell, 2001) to confirm the presence of the recombinant protein. *E. coli* BL21 (DE3) plysS transformed with the empty pET-20b (+) vector served as the negative control. Molecular weight was estimated by comparing the recombinant protein band with the standard (Precision Plus Dual Color standards; Bio-Rad Laboratories; Supplemental Fig. S1). The size of the stained, recombinant protein is consistent with that predicted for the putative EGase (64.8 kD).

Purification and Enzymatic Analysis of the Recombinant *X. fastidiosa* EngXCA Protein

The recombinant EngXCA protein was purified as inclusion bodies, denatured, and refolded as described by Xia et al. (2004). Following renaturation, the protein preparation was purified on a 36-cm × 2.8-cm Sephacryl S-200 column (Pharmacia, GE Healthcare) equilibrated in 50 mM sodium acetate, pH 5.0, and eluted at a rate of 1 mL min^{-1}. Twenty-five microliters of the purified EngXCA protein was subjected to SDS-PAGE to confirm the M_r and purity of the protein.

EGase activity was assayed by a radial diffusion assay in agarose and 0.1 M sodium acetate buffer, pH 5.0, based on the technique of Taylor and Secor (1988). A lysate (50 μL) of *E. coli* transformed with pMCR7 was placed in the well of a radial diffusion plate containing either CMC (Sigma-Aldrich) or XyG derived from tamarind (Megazyme International). Fifty microliters of a lysate of *E. coli* BL21 (DE3) plysS transformed with the empty pET-20b(+) vector served as a negative control. Each sample was tested in duplicate, and the radial diffusion assays were repeated three times. To visualize zones of hydrolysis, radial diffusion plates were stained with 0.1% Congo red in distilled, deionized water for 1 h and developed for 2 h with 1 M NaCl based on the technique of Teather and Wood (1982; Supplemental Fig. S2). A distinct clearing zone on the substrate-containing agarose gel was observed around wells that had been loaded with protein from the pMCR7-transformed *E. coli*; there were no clear zones when protein from empty vector-transformed *E. coli* was used. In addition, the enzymatic activity detected by the radial diffusion plate assay was confirmed by 2-cyanoacetamide-based reducing sugar assays (Gross, 1982) using either 0.4% CMC or XyG as substrate. Fifty microliters of the purified *X. fastidiosa* EngXCA protein was added to 950 μL of 0.4% CMC or XyG in 100 mM sodium acetate, pH 5.0. Aliquots (200 μL) were removed immediately (time 0), 30 min, 1 h, and 2 h and placed in 1 mL of 1 M sodium borate, pH 9.0, to stop the reaction. Reducing sugar content was measured in a spectrophotometer at 276 nm, and data were evaluated in relationship to a Glc standard curve. The assays were done in duplicate, and each experiment was repeated twice (Supplemental Fig. S3). When protein from pMCR7-transformed *E. coli* was used, a steady increase in reducing sugars was observed for incubations with CMC and XyG; no increase in reducing sugar content was observed when protein from empty vector-transformed *E. coli* was used.

pPGIP Inhibition of *X. fastidiosa PG*

The *pglA* open reading frame encoding the *X. fastidiosa* PG was cloned and expressed in *E. coli*. Active enzyme was isolated from induced cultures as described by Roper et al. (2007) and used in tests of the ability of PGIP-containing proteins extracted from cv Bartlett pear (*Pyrus communis*) fruits to inhibit the PD pathogen's PG. Protein was extracted from mature, green pear fruit in 0.1 M sodium acetate buffer (pH 6.0) containing 1 M NaCl, as described by Abu-Goukh et al. (1983). The PGIP-enriched fraction was the protein soluble in 50% saturated ammonium sulfate but insoluble in 100% saturated ammonium sulfate. Precipitated protein was collected by centrifugation, dialyzed against 50 mM sodium acetate (pH 5.5), and used in assays. Recombinant *X. fastidiosa* PG (200 μL) was combined with 800 μL of pH 5.5 sodium acetate buffer and 1 mL of 0.4% PGA and incubated at 37°C. The reaction mixtures used for testing pPGIP inhibition of the *X. fastidiosa* PG contained 200 μL each of pear fruit protein extract and recombinant PG, 600 μL of sodium acetate buffer, and 1 mL of PGA. At the start of incubation (0 time) and at intervals during the course of incubation, duplicate 200-μL aliquots from each of the two reaction mixes were tested for reducing end generation (Gross, 1982); a solution of GalUA was used for a reducing sugar standard curve.

Visualization of the Impact of EGase and PG on Grapevine PM Structure

Grapevine stem segments (approximately 120 cm in length) were used to evaluate the effect of the two enzymes on PM integrity. The stem segments were cut from 12-week-old vines in degassed water without exposing the cut ends to air. Segments were inserted into the flow apparatus (Fig. 1), and PG and EGase were infused together as a pulse, as described above. The water flow system remained running for 20 min after the introduction of the enzyme mixture, and flow was then stopped for 10 to 12 h. As a control, a pulse of water, instead of the enzyme mixture, was introduced to the system. After these treatments, several 1-cm segments were obtained from the third internode from the cut end into which water flow was introduced. These were immediately fixed in formalin-acetic acid-alcohol (Ruzin, 1999) for 24 h. The fixed treated and control lengths were trimmed with a razor blade in 50% ethanol into 3-mm-thick stem sections exposing the tangential surface of the xylem tissue. The sections were dehydrated through an ethanol series (70%, 80%, 90%, 95%, and 100%; 30 min at each step). The ethanol-dehydrated samples were processed with critical point drying (Samdri-780A; Tousimis Research) followed by gold coating (Desk II Cold Sputter-Etch Unit; Denton Vacuum). The coated xylem samples were observed with a scanning electron microscope at an accelerating voltage of 5 kV (S-3500 N; Hitachi).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AAO29683, AAO29329, and XM_001397030.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. SDS electrophoresis of the protein recovered from *E. coli* expressing the *X. fastidiosa* PD1851 (*engXCA*; putative EGase-encoding) open reading frame.

Supplemental Figure S2. Radial diffusion assays showing the activity of the expressed, putative EGase against carboxymethylcellulose and fucosylated tamarind xyloglucan.

Supplemental Figure S3. Reducing sugar assays showing the activity of the expressed, putative EGase against carboxymethylcellulose and fucosylated tamarind xyloglucan.

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