REPORT TYPE: Renewal Progress Report for CDFA agreement 14-0144-SA

PROJECT TITLE: Characterization and inhibition of *Xylella fastidiosa* proteins secreted by the Type II secretion system and their secretion machinery.

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Reporting Period: The results reported here are from work conducted July 2014 to Present.

INTRODUCTION

Xylella fastidiosa (*Xf*) is a xylem-limited bacterial pathogen that is the causal agent of Pierce's Disease (PD) of grapevine (Hopkins and Purcell, 2002, Chatterjee et al., 2008, Purcell and Hopkins, 1996). In order to systemically colonize the xylem, *Xf* must be able to move efficiently from one xylem vessel element to adjacent vessels. These xylem vessels are connected by pit membranes, which are porous primary cell wall interfaces that are composed of cellulose microfibrils embedded in a meshwork of pectin and hemicellulose (Buchanan, 2000, Sun et al., 2011). The pore sizes of these pit membranes range from 5 to 20 nm, and serve to prevent the movement of air embolisms and pathogens within the xylem (Mollenhauer & Hopkins, 1974, Buchanan, 2000). Indeed, these small pore sizes do prevent the passive movement of *Xf* between xylem vessels given that the size of the bacterium is 250-500 x 1,000-4,000 nm (Perez-Donoso et al., 2010, Mollenhauer & Hopkins, 1974). In order to move from one vessel to another, it has been shown through genomic and experimental evidence that *Xf* utilizes Cell Wall-Degrading Enzymes (CWDEs), including a polygalacturonase (PG) and at least one β-1,4 Endoglucanase (EGase), to break down the pit membrane's network (Roper et al., 2007, Perez-Donoso et al., 2010). Furthermore, PG is necessary for pathogenicity in grape and has become a primary target for *Xf* inhibition studies (Roper et al, 2007). However, PG alone is not sufficient for pathogenicity in grape and *Xf* requires both PG and an EGase for pit membrane degradation (Perez-Donoso et al., 2010). Therefore, elucidating the role of EGases in pit membrane degradation is critical for understanding systemic movement within the xylem. The *Xf* genome contains three genes that encode canonical EGases: *egl* (PD2061) *rlpA* (PD1236) and *engXCA2* (PD1851). A fourth annotated EGase, *engXCA1* (PD 1856), putatively encodes a modular hybrid protein that contains both an EGase domain and an expansin domain (Simpson et al., 2000). Expansins are primarily plant proteins that function to non-enzymatically loosen the cell wall during development (e.g., cell elongation, fruit ripening). Recently, expansins have been found in several plant-associated bacteria, most of which have a significant xylem-dwelling phase in their lifestyle (Nikolaidis et al., 2014). It is predicted that these EGases and PG are delivered into the xylem by the Type II Secretion System (T2SS). Preliminary data demonstrate that *X. fastidiosa* with a deficient T2SS display a non-pathogenic phenotype similar to that of the *Xf pglA* mutant that is deficient in production of PG, suggesting that the T2SS is essential for *Xf* pathogenicity. Therefore, our central hypothesis is that *Xf* utilizes other CWDEs and an endoglucanase/expansin hybrid protein in concert with PG to breach the pit membranes and that the majority of these are secreted by the Type II Secretion System. We are determining the role that each of these components plays in pit membrane degradation and systemic movement, and subsequently if they are good candidates for potential inhibition to limit Pierce's Disease development.

OBJECTIVES

1. Characterization of *Xf* host cell wall degrading enzymes and an endoglucanase/expansin protein.
2. Inhibition of *Xf* endoglucanases and the endoglucanase/expansin using endoglucanase-inhibiting proteins.
3. Characterization of the *Xf* Type II secretion system.
4. Inhibition of the *Xf* Type II secretion system.

RESULTS AND DISCUSSION

Objective 1: Characterization of *Xf* host cell wall degrading enzymes and an endoglucanase/expansin.

It was previously determined that *Xf* EngXCA2, which is one of the four putative *X. fastidiosa* EGase-encoding genes is a major contributor to the pit membrane dissolution and the synergistic effects of the PG and the EngXCA2 were sufficient to increase pit membrane pore size (Perez-Donoso et al., 2010). Indeed, recombinant EngXCA2 was capable of digesting carboxymethyl cellulose (CMC) and xyloglucan (XyG) polymers, which both contain β-1,4-linked glucan backbones and are representative of substrates *Xf* would likely encounter in grapevine primary cell walls (Roper, 2006; Perez-Donoso et al., 2010). Given the role EngXCA2 plays in pit membrane degradation, we hypothesize that other predicted EGases produced by *Xf* may impact pit membrane integrity as well. The *egl* gene is predicted to encode a β-1,4 EGase belonging to the glycoside hydrolase family 5 as indicated in the CAZy (Carbohydrate Active Enzyme) database. Glycoside hydrolase family 5 proteins hydrolyze glycosidic bonds between two carbohydrates or a carbohydrate and non-carbohydrate moiety and have activities ranging from EGases to mannanases. *rlpA* putatively encodes a lipoprotein containing a Barwin-related EGase domain belonging to the glycoside hydrolase family 45. This family contains proteins with only EGase activity. The last gene annotated as an EGase is *engXCA1*, which encodes an EGase/expansin hybrid putatively involved in plant cell wall disassembly. This is of particular interest because expansins are primarily found in the plant kingdom and are non-enzymatic proteins that function to loosen the cell wall during plant growth without enzymatic digestion of the wall (Cosgrove, 2000). Expansins facilitate cell wall loosening by binding to their target polysaccharide and disrupting the weak bonds between the glycans and the cellulose microfibrils, allowing turgor pressure from within the cell to expand the cell wall (Cosgrove, 2000). Expansin-like proteins with similar structure and function were later found in a few bacterial species that associate with plants likely as a result of cross-kingdom horizontal gene transfer (Nikolaidis et al., 2014). These bacterial expansins are thought to enhance the activity of bacterial CWDEs by loosening the cell wall, thereby promoting wall breakdown, colonization and virulence. Interestingly, orthologs of at least one bacterial expansin (EXLX1) are found in several plant pathogens, including *Xylella, Xanthomonas*, *Ralstonia* and *Erwinia* species (Kerff et al., 2008, Georgelis et al., 2014). While these are phylogenetically diverse bacteria, they all share the commonality that they spend the majority of their lives in the xylem tissue of plants. It is hypothesized that they are involved in host colonization (Kerff et al., 2008). In the *Xf* pathosystem, they could potentially weaken the wall and more readily expose carbohydrate targets for digestion by the suite of other *Xf* CWDEs.

Characterization of the *Xf* EGase/Expansin hybrid protein.

The gene *engXCA1* was cloned from the *X. fastidiosa* Temecula 1 genome into the pET200 Directional TOPO expression vector (Fig. 1A). The plasmid construct (pET200::*engXCA1*) was then transformed into the *E. coli* strain BL21 Star, and recombinant protein expression was induced with 1 mM IPTG for six hours at 37°C. The bacterial cells were lysed using the B-PER lysis reagent containing lysozyme and DNaseI (ThermoFisher) and the lysate was run on an SDS-polyacrylamide gel (Fig. 1B). The lysate was analyzed by Western Blot using a monoclonal α-His-tag primary antibody and a polyclonal alkaline phosphatase (AP) secondary antibody (Fig. 1C). The Western Blot was developed using an AP development kit (Bio Rad), and the protein sequence was confirmed by Mass Spectrometry. Analysis of the soluble and insoluble lysate fractions determined that expression at 37°C did not favor soluble recombinant protein, so conditions were optimized to facilitate the presence of recombinant protein in the soluble fraction. The samples were incubated for four hours at either 25°C or 18°C in the presence of 0.1 mM IPTG, and GelQuant.NET software provided by biochemlabsolutions.com was used to calculate the band intensity for the soluble fraction relative to the insoluble fraction (Fig. 1D). After

incubation at 25°C, the soluble fraction contained 7.3% of the total recombinant protein, while incubation at 18°C

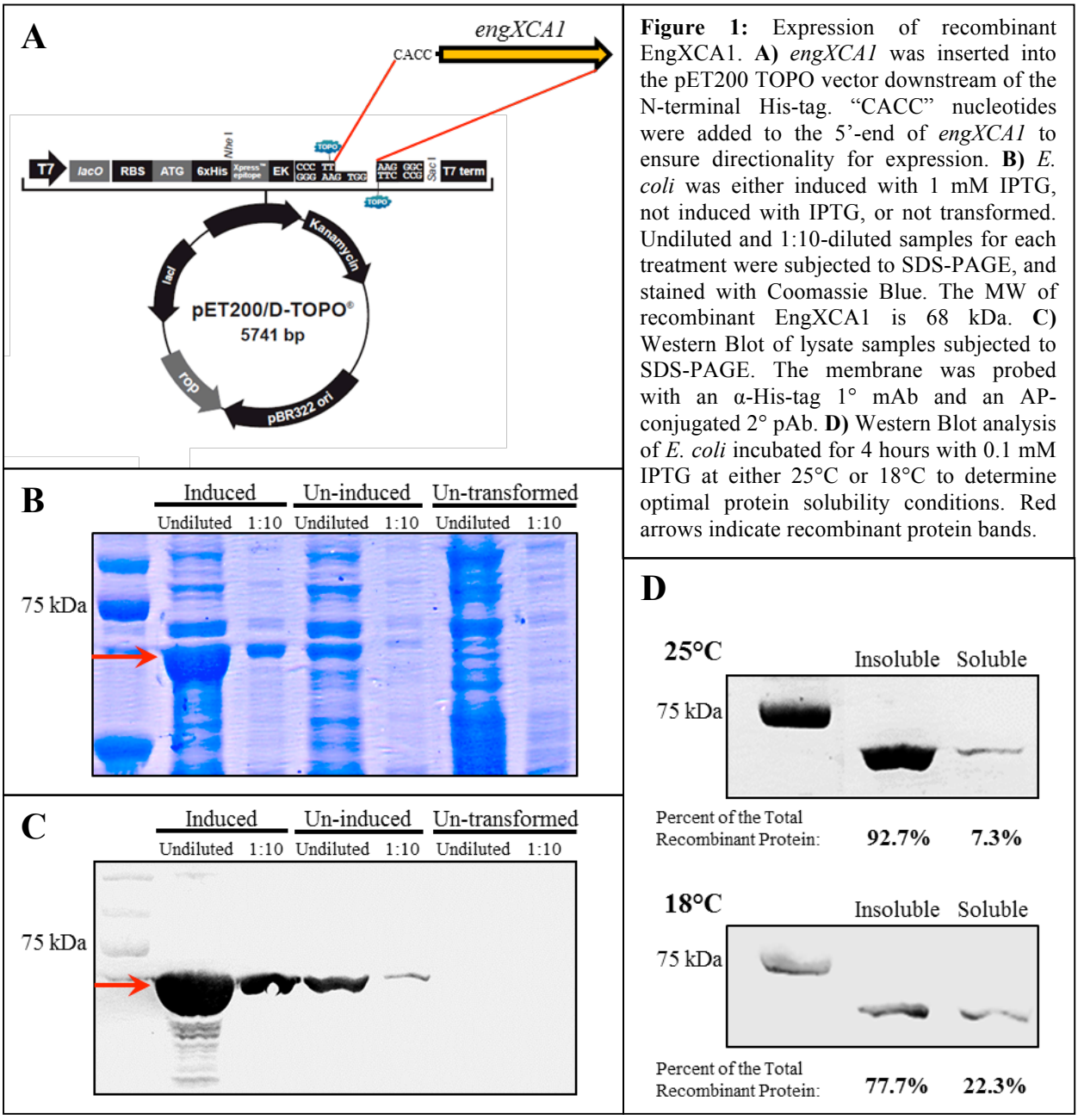

Figure 1: Expression of recombinant EngXCA1. **A)** *engXCA1* was inserted into the pET200 TOPO vector downstream of the N-terminal His-tag. "CACC" nucleotides were added to the 5'-end of *engXCA1* to ensure directionality for expression. **B)** *E. coli* was either induced with 1 mM IPTG, not induced with IPTG, or not transformed. Undiluted and 1:10-diluted samples for each treatment were subjected to SDS-PAGE, and stained with Coomassie Blue. The MW of recombinant EngXCA1 is 68 kDa. **C)** Western Blot of lysate samples subjected to SDS-PAGE. The membrane was probed with an α-His-tag 1° mAb and an AP-conjugated 2° pAb. **D)** Western Blot analysis of *E. coli* incubated for 4 hours with 0.1 mM IPTG at either 25°C or 18°C to determine optimal protein solubility conditions. Red arrows indicate recombinant protein bands.

yielded 22.7% of the total recombinant protein in the soluble fraction. Using the optimized induction conditions (18°C, 0.1 mM IPTG, 4 hours) and increasing the total volume of bacterial cells from 10 ml to 40 ml allowed for the expression of a sufficient quantity of soluble recombinant EngXCA1 protein to proceed with protein purification. The recombinant protein was purified via column chromatography using Ni-NTA resin (ThermoFisher), following the product instructions. Elution of the protein was monitored by absorbance readings at 280 nm. A sufficient quantity of recombinant protein was eluted from the column and subsequently dialyzed using a Slide-A-Lyzer cassette (ThermoFisher) in PBS. After concentrating the protein, an absorbance reading at 280 nm was taken to determine the concentration, only to discover that no protein remained after dialysis and subsequent concentration. Dialysis membranes are made of regenerated cellulose, thus, it is likely that the recombinant EGase may be binding to the membrane. Currently, alternative purification and dialysis methods are being pursued.

Once the recombinant protein is purified, it will be used to test for endoglucanase activity in radial diffusion assays and reducing sugar assays (Kasana, 2008; Johnsen and Krause, 2014; Gross, 1982). However, bacterial cell lysate can also be used in radial diffusion assays if the recombinant endoglucanase is the only cellulose-degrading enzyme present. As recombinant EngXCA1 is expressed in *E. coli*, which does not produce cellulose-degrading enzymes, the bacterial cell lysate can be used to assess the endoglucanase activity of EngXCA1. We conducted a preliminary experiment where after protein induction and bacterial cell lysis, 20 µl of the whole cell lysate, the insoluble fraction, and the soluble fraction were spot-plated on CMC-Agar plates (0.2% CMC, 1.7% Agar) and incubated at 28°C for 24 hours. 20 µg of cellulase from *Trichoderma reesei* was used as a positive control, and the B-PER lysis reagent containing lysozyme and DNaseI was used as the negative control. After 24 hours, the plates were stained with Gram's Iodine for five minutes and then washed with de-ionized water. The positive control produced a clear zone of hydrolysis while the negative control did not induce hydrolysis and the whole cell lysate and the soluble fraction produced zones of hydrolysis comparable to that of the positive control, while the insoluble fraction only produced a smaller, weaker zone of hydrolysis (Fig. 2). This indicates that EngXCA1 has endoglucanase activity, though a more robust radial diffusion assay with additional negative controls including the cell lysates of both untransformed *E. coli* and *E. coli* transformed with the empty vector will be performed.

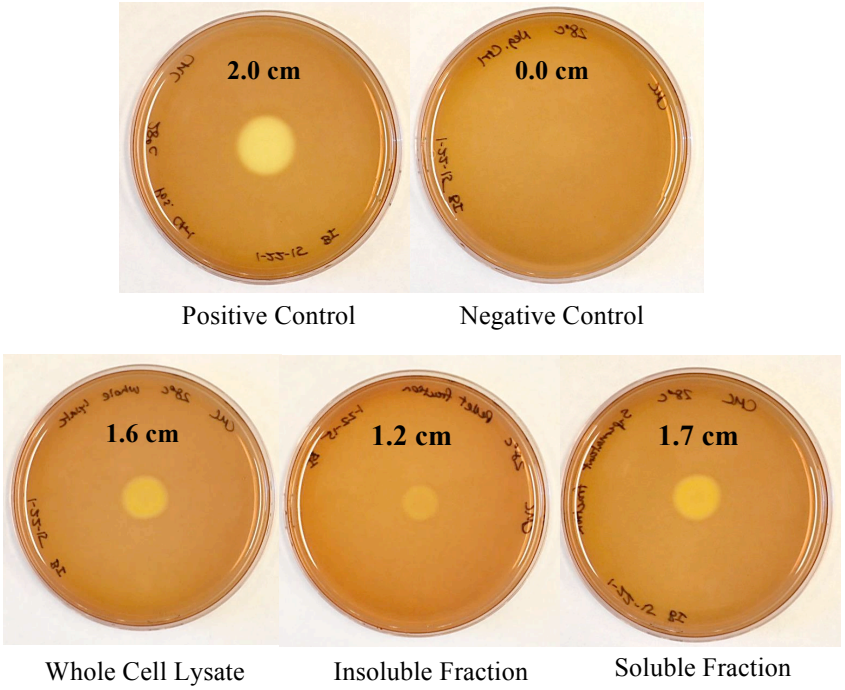

Figure 2: Radial diffusion assay to determine the endoglucanase activity of recombinant EngXCA1. Whole bacterial cell lysate was tested, as well as the insoluble and soluble lysate fractions. 20 µg of cellulase from T. reesei served as the positive control while the B-PER cell lysis reagent containing lysozyme and DNaseI (ThermoFisher) served as the negative control. Measurements correspond to the diameter of the zone of hydrolysis.

We will also assess expansin activity of the recombinant protein (i.e., its ability to promote the extension of plant tissues that are subjected to stress) in close collaboration with the Cosgrove Laboratory (Penn State University). The cell wall elongation assay will then be performed using an extensometer apparatus as described by Cosgrove, D. J. (1989), and expansin activity will be determined by measuring the extension of wall specimens over a 2-h period.

Assessment of the biological contribution of the *Xf* EGase/Expansin to pathogenicity and host colonization. To test the role of the *Xf* EGase/expansin *in planta*, we constructed a deletion mutant (Δ*engXCA1*) in the *Xf* Temecula 1 strain using established mutagenesis techniques and confirmed the mutant via PCR (Matsumoto et al., 2009). We mechanically inoculated the Temecula 1 wild-type and the Δ*engXCA1* mutant into grapevine (Cabernet Sauvignon variety) using the pin-prick method (Hill and Purcell, 1995). Grapevines inoculated with 1X phosphate buffered saline (PBS) were used as negative

controls. Both the wild-type and the Δ*engXCA1* mutant were inoculated into 10 plants each and the experiment was repeated three times (30 plants/treatment). This entire experiment was replicated in two grape varieties, Chardonnay and Cabernet Sauvignon. We quantified *Xf* populations in the plants by isolating *Xf* from the petioles at the point of inoculation (POI) (11 weeks post-inoculation) and ≥ 37 cm above the POI (12 weeks post-inoculation) to determine the ability of the EGase mutants to systemically colonize the host (Fig. 3). The statistical differences between wild-type and Δ*engXCA1* mutant populations at both POI and ≥ 37 cm above the POI were determined by ANOVA. While there was a significant difference in colonization at the POI (P = 0.027), the difference in colonization at ≥ 37 cm above the POI was statistically insignificant (P = 0.155), indicating that the Δ*engXCA1*mutant is not impaired in movement 37 cm above the POI. We will test distances further from the POI this summer. A similar trend was also observed in experiments conducted with the Chardonnay variety (*data not shown*). In these experiments, we inoculated at the base of the plants. However, in a natural scenario, sharpshooters prefer to feed on new green growth and the bacteria migrate in a basipetal direction against the flow of sap. We speculate that EngXCA1 may play a role in this basipetal movement and in future experiments we plan to inoculate plants closer to the shoot apex rather than at the base to assess for basipetal movement.

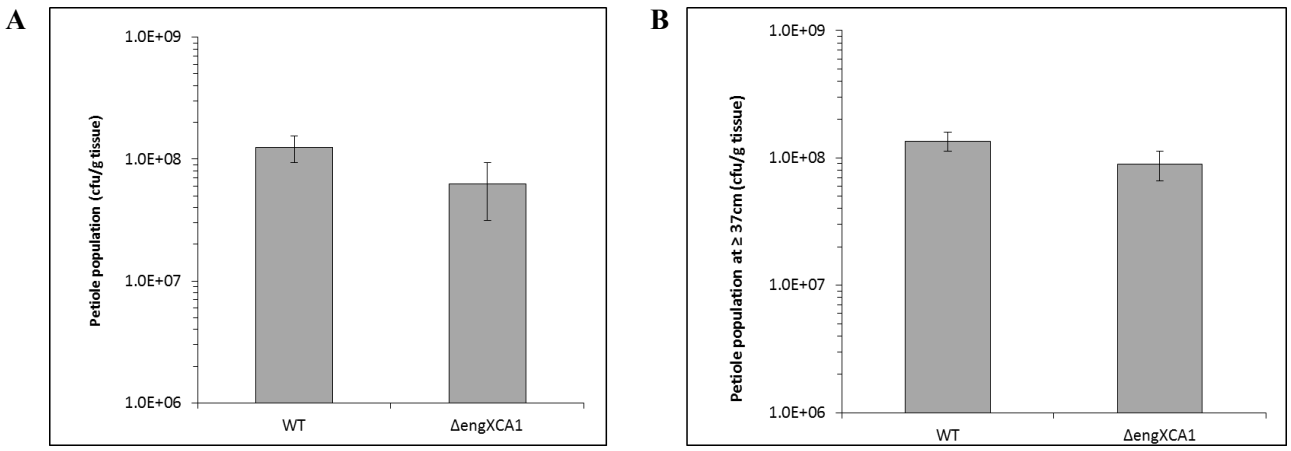

Figure 3: *In planta* populations of the Δ*engXCA1* mutant relative to the 1 wild-type strain. **A)** *In planta* populations of the Δ*engXCA1* mutant are significantly lower than those of the wild-type strain at the point of inoculation (POI) quantified at 11 weeks post-inoculation . **B)** *In planta* populations of the Δ*engXCA1* mutant are not significantly different from those of the wild-type strain at ≥ 37 cm above the point of inoculation at 12 weeks post-inoculation. Data are the means of three independent assays with ten replications each. Bars represent the standard error of the mean.

Disease ratings for all plants were taken, using a scale of 0-5 with 0=healthy and 5=dead as described by Guilhabert and Kirkpatrick (2005). Interestingly, the Δ*engXCA1* mutant strain is less virulent than the wild-type parent strain (Fig. 4). Furthermore, the percentage of plants inoculated with the Δ*engXCA1* mutant strain rating 2 or higher on the disease index was significantly less than the percentage of plants inoculated with wild-type *Xf* rating 2 or higher over a 14-week period (Fig. 5). This indicates that the onset of disease in plants inoculated with the Δ*engXCA1* mutant is significantly delayed relative to plants inoculated with wild-type *Xf*. This experiment was also repeated in Chardonnay and a similar disease development trend was also observed (*data not shown*). It is very interesting that despite colonizing the plants to the same levels as wild type *Xf*, the onset and severity of PD symptoms is delayed in plants inoculated with the Δ*engXCA1* mutant.

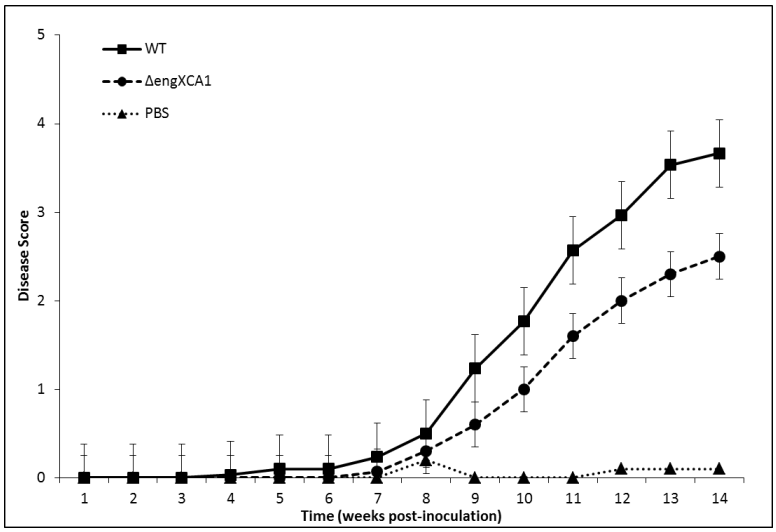

Figure 4: Disease progress of the Δ*engXCA1* mutant and the wild-type strains over 14 weeks. The Δ*engXCA1* mutant strain lags behind the wild-type strain in Pierce's Disease symptom development. 1X PBS served as the negative control. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

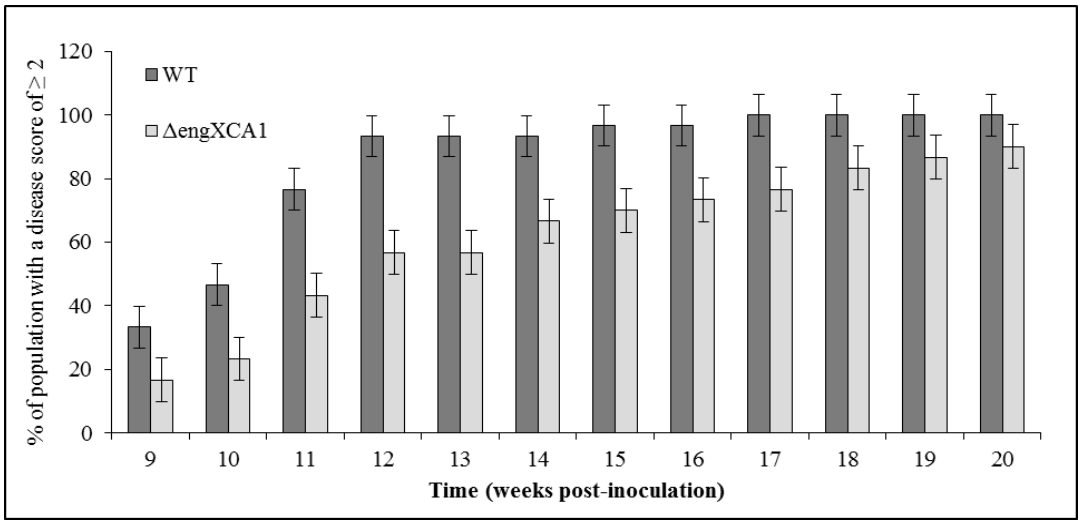

Figure 5: Percentage of plants rating a 2 or higher on the Pierce's Disease scale. The percentage of plants inoculated with the Δ*engXCA1* mutant strain that rated 2 or higher was consistently less than the percentage of plants inoculated with the wild-type strain over a period of 14 weeks. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

Objective 2: Inhibition of *Xf* endoglucanases and the endoglucanase/expansin using endoglucanase inhibiting proteins. Because the combined action of a PG and an *Xf* EGase was required to digest pit membranes, both are logical targets for inhibition. PG is a major pathogenicity factor for *Xf* and grapevines expressing a pear PGIP were more tolerant to *Xf* infection (Aguero et al., 2005). Several plant proteins have also been identified and characterized as xyloglucan-specific EGase inhibiting proteins (XEGIPs) that could potentially inhibit *Xf* EGases. These include XEGIPs from tomato and tobacco (Naqvi et al., 2005, Qin et al., 2003). We propose to assess the ability of the tobacco and tomato XEGIPs to inhibit the degradative ability of the *Xf* EGases and the EGase/expansin.

Currently, we are working on expressing and purifying these *Xf* EGases and assessing their activity as stated in Objective 1. Once these studies have been completed, we will test for inhibition using a radial diffusion assay performed in agarose containing either CMC or XyG as a substrate with increasing

concentrations of each XEGIP. In addition, we will quantify the generation of reducing sugars produced by the *Xf* EGases or EGase/expansin alone or in combination with each of the XEGIPs (Naqvi et al., 2005)

Objective 3: Characterization of the *Xf* Type II secretion system. The T2SS is composed of twelve-fifteen different proteins depending on the species that is involved either structurally or mechanistically involved in the function of the T2SS. These proteins are encoded in a single operon and the *Xf* genome contains a similar operon similar strongly suggesting a functional T2SS (Jha et al., 2005). The T2SS can be divided into four different subassemblies that are 1) the pseudopilus; 2) the Out membrane complex; 3) the inner membrane platform and 4) the secretion ATPase. The pseudopilus is composed primarily of the major pseudopilin protein, G (XpsG), and also contains the minor pseudopilins, S, H, I, J and K (XpsH, I, J and K). The XpsE ATPase harnesses the energy that drives secretion through the T2SS via hydrolysis of ATP. Proteins destined for secretion by the T2SS are first delivered to the periplasm via the Sec or Tat-dependent secretion pathway where they are folded (Slonczewski, 2014). The T2SS then uses a pilus-like piston to push proteins through the T2 channel. This piston action is a function of the cyclic assembly and disassembly of pilin subunits (primarily XpsG).

We have created a mutation in the *xpsE* gene, encoding the putative ATPase that powers the T2SS. Grapevines inoculated with the *xpsE* mutant never developed PD symptoms and remained healthy, a phenotype similar to the *Xf pglA* mutant (Fig. 6). Thus, we have compelling preliminary data indicating that *Xf* has a functional T2SS system and the proteins secreted by T2SS are critical for the infection process.

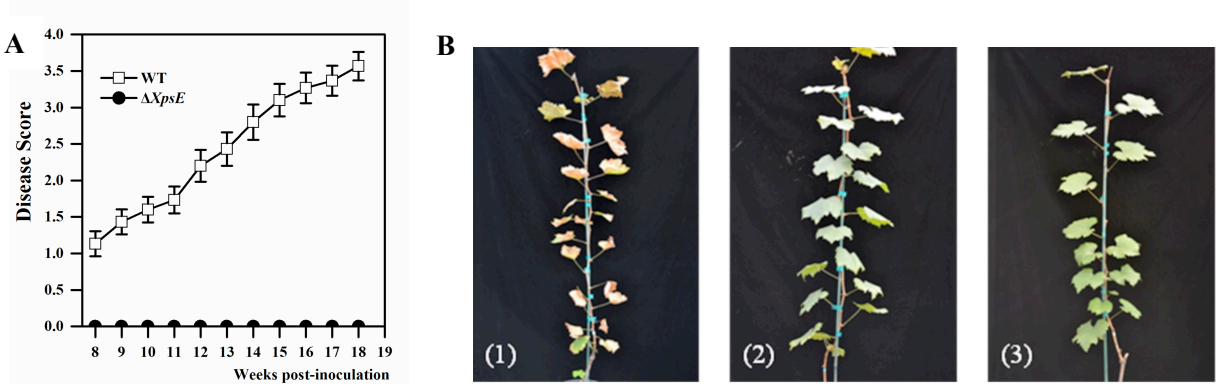

Figure 6: The *Xf* T2SS is necessary for PD development in grapevine. **A)** the Δ*xpsE* mutant does not incite PD symptoms in *V. vinifera* cv. Chardonnay grapevines. Disease severity was based on a visual disease scale from 0 (no disease) to 5 (dead). **B)** Representative images of plants from the virulence assay are shown here, 1=Wild type-inoculated, 2=Δ*xpsE*-inoculated, 3= 1X PBS buffer-inoculated. Plants shown are 11 weeks post-inoculation.

We hypothesize that the non-pathogenic phenotype of the *ΔxpsE* mutant is due largely to the inability to secrete host CWDEs. Indeed, we have indirect experimental evidence that *Xf* utilizes the T2SS to secrete PG. This is based on an assay performed on the defined growth medium, XFM. When XFM is supplemented with pectin as the sole carbon source, this induces production of copious amounts of the carbohydrate-based exopolysaccharide (EPS) (Killiny & Almeida, 2009). Pectin is a complex carbohydrate comprised in its simplest form of repeating galacturonic acid residues. Therefore, when grown on XFM with pectin as the sole carbon source, we hypothesize that *Xf* must first digest the pectin source utilizing its endo-polygalacturonase (Roper et al., 2007) and likely other pectin-digesting enzymes that eventually disassemble the pectin polymer into individual galacturonic acid residues that can then feed into various metabolic processes within the bacterium, such as EPS production.

In support of our hypothesis that PG (and other CWDEs) are secreted through the T2SS, we demonstrate that the Δ*xpsE* mutant produces visibly less EPS on XFM+pectin medium resulting in a much less mucoid phenotype. Furthermore, when wild type *Xf* and Δ*xpsE* are grown on XFM+galacturonic acid (i.e., the monomeric sugar that makes up the pectin polymer), both strains produce similar amounts of EPS (*data not shown*). We infer from this that, indeed, breakdown of the

pectin substrate is necessary to produce EPS and when the T2SS is disrupted this prevents secretion of PG and the subsequent breakdown of pectin.

Objective 4: Inhibition of the *Xf* Type II secretion system.
Proteins destined for secretion by the T2SS are first exported to the periplasm by the Sec or Tat pathways. *Xf* appears to only possess the Sec-dependent secretion pathway. Disruption of the T2SS by small molecule inhibitors was demonstrated in *Pseudomonas aeruginosa* and *Burkholderia pseudomallei*, and could be used to inhibit the *Xf* Sec-dependent pathway (Moir et al., 2011). A chemical compound library will be screened for Sec-inhibitory molecules, including those compounds used by Moir et al. (2011). Inhibition of the Sec-dependent pathway will be confirmed by monitoring the secretion of a CWDE using a polyclonal antibody raised against EngXCA2 and analyzed via Western Blot and ELISA.

PUBLICATIONS PRODUCED AND PRESENTATIONS MADE

Publications

Roper, C., Kirkpatrick, B., Labavitch, J. and Cosgrove, D. 2014. Characterization and inhibition of *Xylella fastidiosa* proteins secreted by the Type II secretion system and their secretion machinery. California Department of Food and Agriculture. p.74-81. Proceedings of the Pierce's Disease Research Symposium, Sacramento, CA.

Presentations

Roper, C. 2014. Characterization and inhibition of *Xylella fastidiosa* proteins secreted by the Type II secretion system and their secretion machinery. Presented as an Oral Presentation at the Annual Pierce's Disease Symposium, Sacramento, CA.

Ingel, B., Kirkpatrick, B., Labavitch, J., Cosgrove, D. and Roper, C. 2014. Characterization and inhibition of *Xylella fastidiosa* proteins secreted by the Type II secretion system and their secretion machinery. Presented as a Poster at the Annual Pierce's Disease Symposium, Sacramento, CA.

RESEARCH RELEVANCE STATEMENT

Our goal is to first understand the roles each of the EGases produced by *Xf* has in pit membrane degradation, as well as the role of the T2SS in secreting these CWDEs. Ultimately, we speculate that inhibition of the EGases and/or the T2SS will significantly reduce the ability of *Xf* to systemically colonize its grapevine host. Preliminary results indicate that the EGase/expansin hybrid protein (EngXCA1) maintains endoglucanase activity. These experiments will be repeated with additional negative controls, followed by reducing sugar assays to confirm these findings. EngXCA1 also appears to play a role in virulence, and could possibly be an elicitor of the host defense response. These studies will be repeated with the addition of the *engXCA1/engXCA1+* complement strain to confirm these results. In addition, an *Xf* strain with a deficient T2SS (Δ*xpsE*) displayed reduced virulence than unmodified *Xf*, lending credence to the hypothesis that the T2SS secretes CWDEs such as PG and EngXCA2 that are necessary for systemic colonization. We speculate that further characterization of these EGases and the T2SS will elucidate significant targets for controlling Pierce's Disease.

LAYPERSON SUMMARY

Xylella fastidiosa relies on degradation of the plant cell wall to move within the grapevine. This is accomplished by the cooperation of at least two classes of enzymes that target different components of the complex scaffold of the plant cell wall. A major goal of this research is to further elucidate the factors that lead to disassembly of the plant cell wall, thereby, allowing the bacteria to systemically colonize the plant. Systemic colonization is highly correlated with Pierce's Disease development and preventing movement of the bacteria is critical to devising successful control strategies. We propose that characterizing and inhibiting *Xf* enzymes that facilitate movement throughout the plant and/or the secretion machinery responsible for delivering those *Xf* enzymes into the grapevines water pipes will provide a comprehensive approach to restriction of disease development.

STATUS OF FUNDS

The funding for this project is largely going towards supporting a Ph.D. graduate student, Mr. Brian Ingel. This project is the main focus of his Ph.D. dissertation. We anticipate spending the remainder of the salary, supply, services and greenhouse recharge money associated with this project as it progresses.

INTELLECTUAL PROPERTY STATEMENT

Thus far, there is no Intellectual Property associated with this project.

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