DEFINING THE ROLE OF SECRETED VIRULENCE PROTEINS LesA AND PrtA IN THE PATHOBIOLOGY OF XYLELLA AND IN THE DEVELOPMENT OF PIERCE'S DISEASE

"Final Progress Report for CDFA Agreement Number 14-0136-SA"

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Reporting Period: The results reported here are for work conducted on this project between July 2014 and June 2016.

ABSTRACT

The goal of this research was to understand the relationship between the expression of secreted virulence proteins by *Xylella fastidiosa* (Xf) and the leaf scorching symptoms observed during the development of Pierce's Disease (PD) and to exploit this information to develop new strategies to control PD in grapevines. The analysis of Xf Temecula 1 secreted proteins has enabled us to focus on two previously uncharacterized proteins LesA and PrtA that appear to be causal to the leaf scorching phenotype observed in PD. We generated mutant Xf that are defective in their capacity to secrete either of these two proteins and individually these mutants display unique alterations in growth and disease phenotype. The mutant lesA1 that does not make LesA protein is less virulent, therefore we conclude that LesA is a 'virulence factor', while prtA1 that does not make PrtA protein is more virulent, therefore PrtA is a 'anti-virulence factor'. LesA protein displays lipase/esterase activities and is the most abundant secreted protein with structural similarity to two less abundant secreted proteins LesB and LesC. LesA, B and C proteins produced individually in *E.coli* were infiltrated into grapevine and walnut leaf tissues and were able to induce scorching symptoms in leaves. These symptoms appear to be related to the lipase/esterase activity present in these proteins. We have analyzed the microbiome of Xf infected plants and compared these with uninfected and we observe a striking variation in the alpha diversity of the microbial community. Different parts of the plant along the axis of infection differ in their alpha diversity we compared bottom and top stems and roots. Infection with Xf leads to a loss of diversity indicating that Xf is able to colonize and displace or outcompete existing microbial communities in the xylem. More virulent strains like prtA1 are able to more quickly colonize as compared to lesA1 that was slower than the wild type strains. Since PrtA was an anti-virulence factor and possibly has a role to play in the biofilm we expressed PrtA as a transgene in transgenic tobacco. Two of the 8 transgenic plants display some anti-virulence activity and show a reduction of symptoms when infected with Xf. A deeper understanding of how these two secreted proteins LesA and PrtA function and their associated pathobiology have provided new insights into this disease and provided a new avenue for therapy against PD.

LAYPERSON SUMMARY

Pierce's disease (PD) of grapevines is caused by the bacterium *Xylella fastidiosa* (Xf), a xylem-limited bacterium that is responsible for several economically important diseases in many plants. A characteristic symptom of PD is leaf scorching, with marginal regions of leaves developing chlorosis progressing to necrosis. Blockage of xylem elements by growth of Xf biofilm leading to an interference with in planta water transport have been posited to be

the main cause of PD symptom development. This research has developed an alternative hypothesis for disease symptom development. Our analysis of Xf secreted proteins has enabled us to focus on two previously uncharacterized proteins, LesA and PrtA that play a role in the development of PD symptoms. We generated mutant *Xf* that are defective in the secretion of either of these two proteins that show alterations in bacterial physiology and plant disease phenotype. Mutant bacteria defective in secreting LesA were less virulent and displayed a biofilm behavior in culture, while the bacteria defective in the secretion of PrtA were the opposite, they were highly virulent and correspondingly displayed a planktonic growth in culture. Our experiments show that these two proteins play a role in disease progression. We have also examining the role of these secreted proteins with respect to colonization of the xylem in different plants parts grapevine and investigated the influence that these bacteria have on the resident microbial communities that inhabit these locations. We observe that more virulent strains are able to more rapidly colonize the grapevine and change the diversity of the microbial community. It is possible that these changes in community are influenced also by these secreted virulence factors specifically LesA. One of these factors PrtA that we have proposed as an anti-virulence factor is able to control symptoms when expressed in tobacco plants. An understanding of how these two proteins work has provided new insights into this disease and indicate new avenues of therapy.

INTRODUCTION

Xylella fastidiosa (Xf) is a fastidious, xylem-limited gamma-proteobacterium that causes several economically important diseases in many crops including grapevine, citrus, periwinkle, almond, oleander, and coffee (Davis et al. 1978, Chatterjee et al. 2008). In the field, Xf is vector-transmitted by various xylem sap-feeding sharpshooter insects (Purcell and Hopkins 1996, Redak et al. 2004). The *Xf* subspecies *fastidiosa* (Xff), as exemplified by the California strain Temecula 1, causes Pierce's disease (PD) in grapevine. The Xf life cycle and virulence mechanism are not entirely understood (Chatterjee et al. 2008). This research focused on understanding the pathobiology of *Xf* that leads to disease; specifically, the underlying mechanism that leads to leaf scorching symptoms. Understanding the pathobiology could lead to the development of new strategies to control PD in grapevines in California. The secretion of virulence factors by pathogens are an important tool that are used to trigger plant disease. Unlike closely related pathogens from genus *Xanthomonas*, Xf does not possess the type III secretion system (T3SS) that is used to inject effector proteins into plant cells (Van Sluys et al. 2002). However, *Xanthomonas* and *Xf* have in common a similar type II secretion system (T2SS) that is used to secrete a battery of important extracellular enzymes that are responsible for virulence (Ray et al. 2000). In Xf, genes have been identified that code for plant cell wall degrading enzymes (CWDEs) such as polygalacturonase, cellulase, lipase/esterase and several proteases (Simpson et al. 2000). These enzymes may aid Xf migration inside xylem vessels by degrading the pit membrane and also help release the carbohydrates necessary for bacterial nutrition and survival. One T2SS virulence factors, a polygalacturonase encoded by pglA, lost pathogenicity when it was mutated and resulted in Xf that was unable to colonize grapevine (Roper et al. 2007). This confirmed an earlier finding that expression of a polygalacturonase inhibitory protein that possibly blocked the action of the virulence factor PglA provided resistance to PD (Aguero et al. 2005). Cell wall degradation by CWDEs also releases oligosaccharides as products, which can induce potent innate immune responses from plants. The plant immune responses include production of phytoalexins, fortification of cell walls through deposition of callose, oxidative burst, and induction of programmed cell death (Darvill and Albersheim 1984, Ryan and Farmer 1991, Braun and Rodrigues 1993).

OBJECTIVES

The goal of this proposal is to define the role that *Xylella* secreted protein LesA and PrtA play in the Pierce's disease phenotype of grapevine

Objective 1: Define the mechanism of action of LesA and PrtA gene products

Activity 1: Express LesA, B, C and PrtA individually and examine their role in the virulence response of *Xylella* cultures
Activity 2: Metagenome analysis of xylem tissues infected by strains mutated for Les A, B, C and PrtA
Activity 3: Develop transgenic SR1 tobacco expressing PrtA and evaluate protection against *Xylella* virulence

RESULTS AND DISCUSSION

Objective 1: To define the mechanism of action of LesA and PrtA gene products.

Our previous analysis revealed 24 secreted proteins in cultures of *Xf* Temecula 1. Of these, we have characterized two proteins, LesA and PrtA. A proteomic analysis of infected leaf tissues revealed five of the 24 secreted *Xf* proteins, the most abundant of which is LesA. To further characterize the role of these proteins, we used insertional mutagenesis of *Xf* cultures and expressed the respective proteins in *E. coli* to identify their function in PD.

Activity 1: Express LesA, B, C and PrtA individually and examine their role in the virulence response of *Xylella* cultures.

The most abundant Xf protein in infected grapevine leaves displaying PD symptoms was an uncharacterized Xf protein that we have designated LesA. It has a 35 amino acid secretion peptide consistent with it being secreted. Immunogold localization of LesA in fixed cells using antibodies against LesA revealed that most of the protein was embedded within the secreted matrix surrounding Xf cells, confirming that LesA is a secreted protein (Fig 1). We compared the structure of lesA to proteins in the PDB database and found a close structural similarity to a *Xanthomonas oryzae* pv. oryzae (*Xoo*) LipA that has lipase and esterase activity (Fig 2; Aparna et al. 2009). Lipase activity was confirmed by growing Xf cultures on plates containing tributryn, a triacylglyceride of butyrate, zones of clearance were clearly visible surrounding the colonies indicating lipase activity (Fig 1).

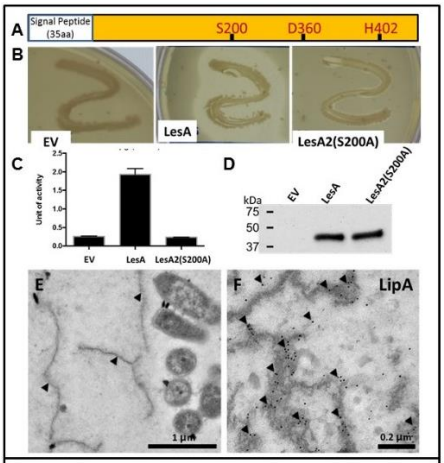

Fig 1: LesA encoded by the Xf tem1 locus PD1703 is a secreted virulence factor with lipase/esterase activity

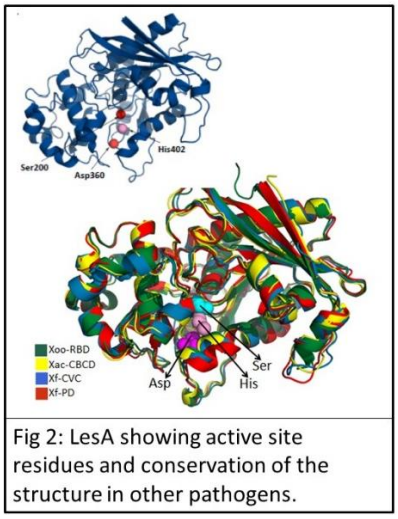

Fig 2: LesA showing active site residues and conservation of the structure in other pathogens.

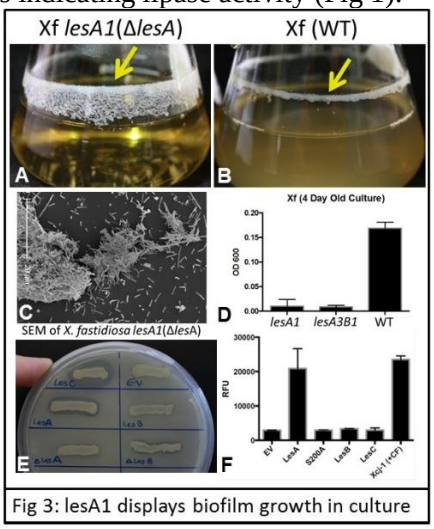

Fig 3: lesA1 displays biofilm growth in culture

Based on this structural prediction, we aligned the active site residues S200, D360 and H402 of LesA with LipA from *Xoo*. We then threaded LesA with the known structure of the Xoo LipA and there was an excellent alignment of active site residues (Fig 2). Additionally, LesA was found to be highly conserved among both *Xylella* and *Xanthomonas* strains (Fig 2). To determine whether LesA had both lipase and esterase activities, we expressed LesA in *E. coli* and made a mutant version, LesA2, in which the S200 serine in the protein was substituted with an alanine residue. We then analyzed the activity by growing/harvesting *E. coli* strains they displayed both lipase activity evaluated on agar plates containing tributryn as well as esterase activity by assaying the *E. coli* extracts using the substrate 4-methyl umbelliferone butyrate and measuring the formation of 4-methyl umbelliferone (4MU) the product of the reaction (Fig 1 and 3). Esterase activity was clearly seen in strains expressing LesA and to a lesser extent in those expressing LesA2 or the empty vector (EV). *E.coli* expressing LesA showed zones of clearance but not those expressing LesA2 (Fig 1 and 3). In addition, LesA protein was detected on western blots from cultures expressing both LesA and LesA2 (Fig 1). To investigate the function of the LesA protein we created a functional knockout strain via homologous recombination where the genomic region encoding LesA was disrupted via the insertion of a gene encoding resistance to the antibiotic kanamycin, this mutant strain was called lesA1. Among the 24 proteins secreted by *Xf* cultures, LesA was the most abundant, but we also identified LesB and LesC, proteins with strong homology to LesA. An alignment of the protein sequences revealed a conservation of the active site residues of Les A in LesB and C. Les B is located adjacent to the lesA on the Xf genome, but LesC is located at some distance away. Since lesA and B were located together, we created a double knock-out using kanamycin, this particular strain is designated lesA3B1. We confirmed by PCR comparison of wild type *Xf* with lesA1 and LesA3B1 that we had knocked out one and both genes. LesA1 and LesA3B1 show less lipase and esterase activities (Fig 3). We expressed LesA,B and C in *E.coli* so we could study the lipase./esterase activities they possessed. We observed some differences among the activities of LesA, B and C. Les A has both lipase and esterase activities, LesB has neither lipase nor esterase activity for the substrates that we tested and LesC has lipase activity similar to LesA but no corresponding esterase activity directed to butyrate substrates (Fig 3). LesA1 and lesA3B1 cultures displayed increased aggregation, in contrast to wild type Xf strains. When grown in flasks, a clear biofilm ring was visible for the wild type, but a much larger and more

profound ring of biofilm was visible for lesA1 (Fig 3). We further confirmed this observation using scanning EM, where lesA1 showed marked aggregation of cells (Fig 3). To investigate the role of lesA in the virulence response and PD development, we infected grapevine plants as described earlier (Dandekar et al. 2012) inserting ~10 million bacteria at the bottom of grapevines about 10 cm above the soil. Plants were scored at 10 weeks. Pierce's disease symptoms were clearly visible starting at 10 weeks for the wild type Tem1 strains, but neither the lesA1 nor the lesA3B1 strains showed any symptoms at this time point (Fig 4). Infiltrating the LesA protein into grapevine leaves led to scorching, but infiltrating the LesA2 mutant protein that lacks both lipase and esterase activities did not (Fig 4). These results clearly show that the presence of the secreted LesA protein is related to leaf scorching and that the activity of the lipase/esterase is necessary for the observed symptoms. The *in planta* testing in grapevine leaves via syringe infiltration is difficult, however, we were also able to use walnut leaves and obtain cell death symptoms. In Walnut leaves, LesA, LesB and LesC were capable of causing lesion while just PBS or LesA2 (functional mutant: S200A) protein and empty vector (data not shown) displayed no symptoms.

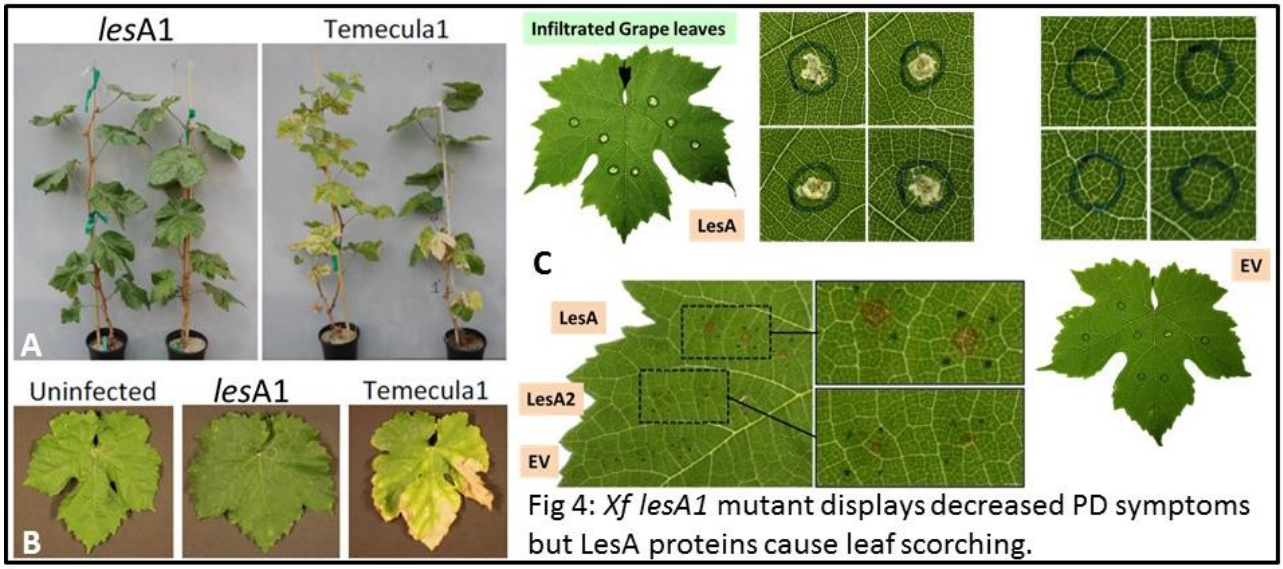

Fig 4: *Xf lesA1* mutant displays decreased PD symptoms but LesA proteins cause leaf scorching.

The secreted protein PrtA was previously annotated also as an uncharacterized protein. We analyzed the structure of PrtA, comparing it with proteins in the PDB database, and found a close structural match to an extracellular alkaline serine protease. Based on this structural prediction, we were able to align the amino acid residues in the active site showing a perfect alignment of these active site residues (Fig 5). Also, prtA is highly conserved among various *Xylella* strains, but interestingly not among Xanthomonas strains like LesA is.

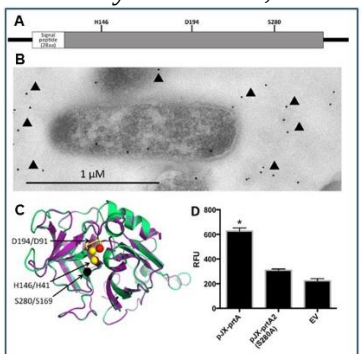

Fig 5: PrtA encoded by the Xf tem1 locus PD0956 is a secreted protein virulence factor with protease activity

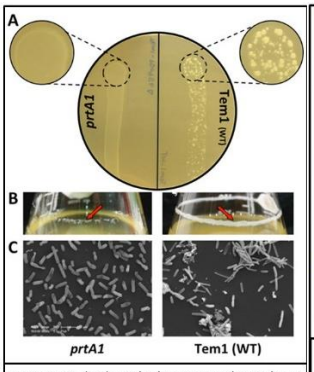

Fig 6: prtA1 displays planktonic growth in culture

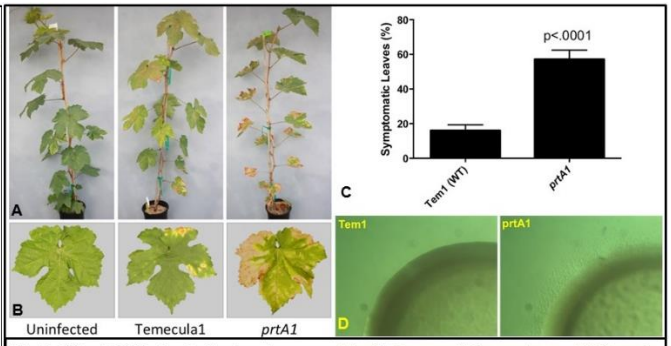

Fig 7: *Xf prtA1* Mutant displays increased twitching motility and a rapid Pierce's Disease phenotype earlier than WT *Xf* strains.

The protease activity of prtA was confirmed by expressing the encoded protein, PrtA in *E. coli* and we were able to demonstrate a lack/lesser activity in a mutant (prtA2) where one of the active site residues (S280 mutated to A280) was mutated as compared to the wild type PrtA enzyme using fluorescent labelled casein as the substrate (Fig 5). To investigate the function of this protein we created a functional knockout strain via homologous recombination where the genomic region encoding PrtA was disrupted via the insertion of a gene encoding resistance to the antibiotic gentamycin, this mutant strain was called prtA1. We confirmed via PCR analysis that the coding region of prtA was disrupted in the prtA1 mutant. Expectedly, the mutant strain displayed less protease activity and PrtA was not detectable in secreted proteins from prtA1 cultures, while PrtA was detectable in wild type cultures. Growth characteristics of prtA1 cultures revealed that it was markedly more planktonic than the

wild type Xf Tem1 strain (Fig 6). Growth on plates showed less aggregation and when grown in flasks, a clear biofilm ring was formed by wild type but not prtA1 cultures (Fig 6). We used scanning EM to confirm that wild type cultures showed marked aggregation whereas prtA1 appeared to be exclusively planktonic (Fig 6). Since planktonic forms have reportedly displayed more virulence (Newman et al., 2004; Chatterjee et al., 2008), we infected grapevine plants as described earlier (Dandekar et al. 2012) in the main stem, 10 cm above the soil. Plants were scored at 10 weeks. Pierce's disease symptoms were clearly visible with prtA1 infections starting at six to eight weeks much earlier to that observed with the wild type (Fig 7). A comparison of prtA1 and wild type colonies shows twitching motility at the margins of prtA1 colonies, confirming enhanced movement consistent with the observed enhanced virulence (Fig 7).

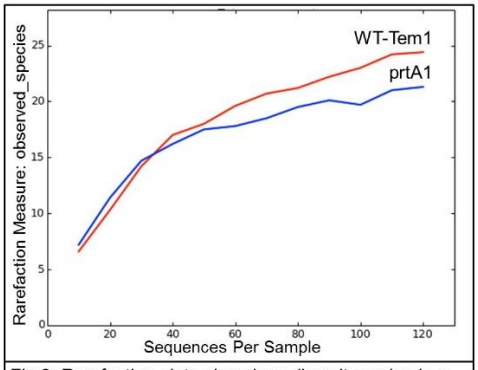

Fig 8: Rarefaction plots show how diversity varies in a sample with the number of sequences sampled.

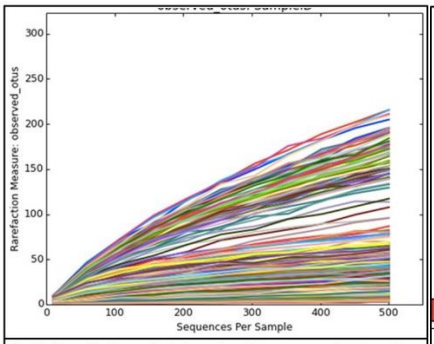

Fig 9: Rarefaction plot showing the sequencing depth of samples as it pertains to new microbial OTU discovery in the Xf. infection experiments.

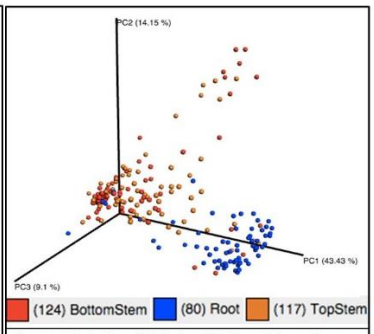

Fig. 10: PCoA plot of Weighted Unifrac Values. Data shows a difference between root tissue (blue) and stem(red and orange). Bottom Stem tissue is not highly differentiable from Top Stem tissue.

Activity 2: Metagenome analysis of xylem tissues infected by strains mutated for Les A, B, C and PrtA

The secreted proteins may influence the grapevine microbiota and indirectly that interaction could influence the disease outcome, we investigated the microbial communities in the xylem of grapevine. Since there is not much information available on the microbial communities in grapevine we investigated a comparison of Thompson Seedless (TS) samples infected with different Xf strains; one unable to make PrtA (prtA1), wild type *Xf* (Tem1), and uninfected tissue. Grapevine stem and root tissues were investigated to determine the alpha-diversity of the xylem microbial communities along the axis of infection. DNA was extracted and the V4 region of the 16S rRNA gene was amplified using region-specific primers and sequenced using standard protocols as agreed upon in the Earth Microbiome Project (http://www.earthmicrobiome.org/emp-standard-protocols/) to reveal the composition of resident microbial communities (Caporaso et al. 2012).

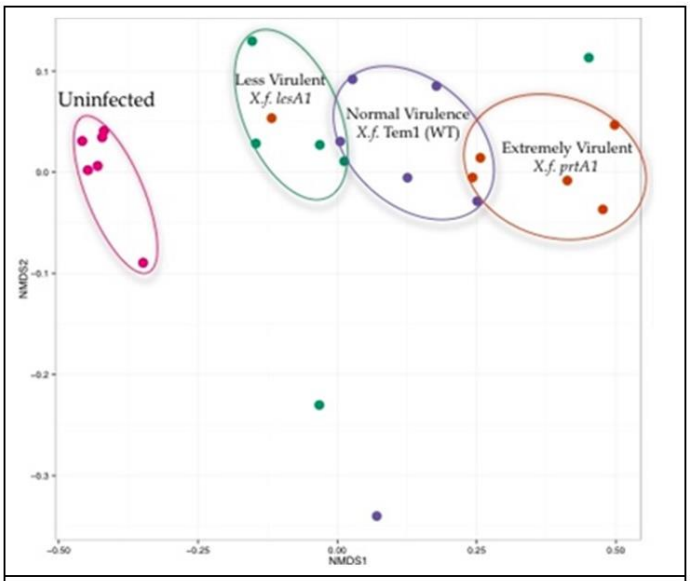

Figure 11: NMDS of non-leaf tissue between nodes 10 and 11 above infection point. Separation between uninfected and those infected with wild type compared to mutants differing in virulence

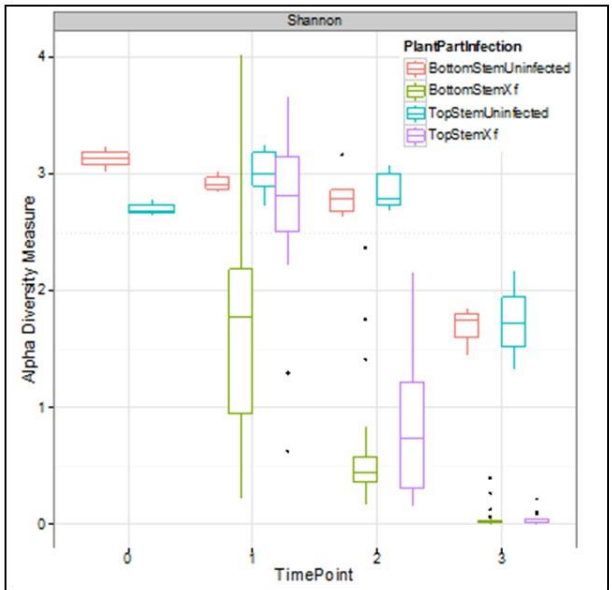

Fig. 12: Shannon Alpha Diversity measure showing "Top" and "Bottom" aerial tissue. Uninfected and Xf infected compared at four time points. Key: 0=Pre-infection, 1= 2 WPI, 2=6 WPI 3=10 WPI.

Initial extractions of DNA from grapevine tissues revealed a high proportion of host chloroplast DNA that was abundantly extracted and that greatly diminished the sequence depth needed to analyze the composition of resident microbial communities. We employed the use of specific PCR blockers to selectively inhibit the amplification of grapevine chloroplast sequences (Orum 2000). This was successful and rarefaction plots of the samples extracted in our infection study show that novel OTU's are indeed extracted and that they plateau upon

increasing sequence depth, indicating that we sampled a majority of the resident microbial community (Fig. 8 & 9). An analysis of the alpha diversity in the different tissue samples revealed that the top and bottom stem tissues clearly separate from root tissues (Fig 10). This observed differences in alpha diversity shown in Fig 10, allowed us to compare the divesity of the resident microbial communities after challenge with wild type Xf and also lesA1 and prtA1 mutant bacteria. We chose to compare just the stem segments and the results show not only a clear seperation based on infected and non infected stem segments but also we observed a clustering based on the degree of virulence (Fig 11). The mutant lesA1 that we show in activity 1 to be less virulent clusters more closely to uninfected whereas those sample tissues obtained from plants infected with the highly virulent prtA1 strain show the least similiarity (Fig 11). Next we investigated what was driving the separation between infected and uninfected samples. To do so, we began by looking at the alpha diversity (within sample diversity) of each sample and the variance of these samples. In both "Top" and "Bottom" tissue where we observe a clear loss of diversity over time, which was clearly visible with both Shannon (Fig. 12) and Observed OTU Alpha Diversity measures (data not shown). At pre-infection the alpha-diversity of top and bottom tissue was similar, though bottom tissue was significantly more diverse. This was so as the bottom tissue is older and closer to the soil. Alpha diversity is lost initially near the point of infection (bottom tissue) seen at two weeks' post-infection (2WPI; Fig 12). As the infection spreads over time we observe changes in microbial diversity near the top part of the vine which was first noted at six weeks post infection (6WPI; Fig 12). Both top and bottom tissues show significant decreases in alpha diversity when compared to uninfected tissue.

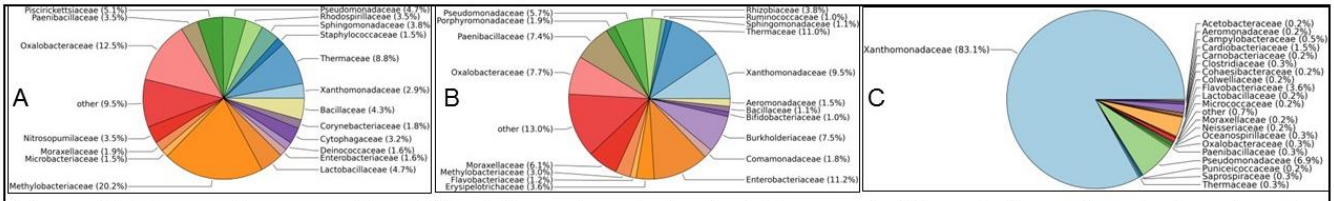

Figure 13: Taxonomic composition of "Top" tissue (non-leaf material from node 2 to node 3 over time broken down by family. A. Pre-Infection B. 2 weeks post-infection C. Six weeks post-infection. Average composition of a minimum of 5 samples per time point

From pre-infection to six weeks' post-infection we also see the composition of the microbiome change dramatically. Figure 13 shows the taxonomic composition of the microbiome broken down by family for vines infected with Xf Tem1 (Wt) over time. After Xf infection, the composition of the microbiome quickly becomes completely dominated by the family *Xanthomadaceae*. This corresponds to the decrease in alpha diversity observed in Figure 12.

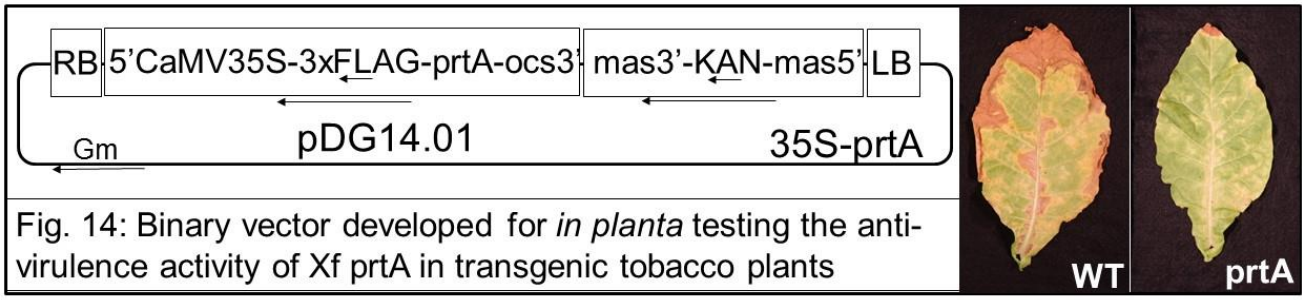

Fig. 14: Binary vector developed for *in planta* testing the anti-virulence activity of Xf prtA in transgenic tobacco plants

Activity 3: Develop transgenic SR1 tobacco expressing PrtA and evaluate protection against *Xylella* virulence

To test the anti-virulence phenotype of PrtA, we cloned the prtA coding region into a binary vector under the CaMV35S promoter after codon optimization for expression in tobacco (Fig 14). The binary vector construct was introduced into a disarmed strain of *Agrobacterium* (EHA105) via electroporation to create a functional system for plant transformation. Thirteen transgenic SR1 tobacco lines have been generated at the UC Davis Parson Transformation Facility. We have screened 8 of these plants and they are all positive for the presence of the kan genes and express PrtA as detected using an anti-FLAG antibody. We did not detect the protein using an anti-PrtA antibody. The resulting plants were tested for their susceptibility to Xf infection using a previously established technique and 2 of the 8 independent transgenic tobacco lines tested showed some level of tolerance (Fig 14; Francis et al., 2008). These results show that PrtA holds some promise as an anti-virulence factor this observation needs to be confirmed in transgenic grapevines.

CONCLUSIONS

The goal is to understand the virulence mechanisms of *Xylella fastidiosa* (Xf) that lead to leaf scorching symptoms observed in Pierce's Disease (PD) and to exploit this information to develop new strategies to control

PD in grapevines. The blockage of xylem elements and the interference with water transport by *Xf* is regarded to be the main cause of PD symptom development. The analysis of Xf Temecula 1 secreted proteins has enabled us to focus on two previously uncharacterized proteins LesA and PrtA. We generated mutant Xf that are defective for each of these two genes and they show alterations in disease phenotype, lesA1 is less virulent while prtA1 is more virulent. LesA displays lipase/esterase activities and is the most abundant but is very similar to two additional less abundant proteins LesB and LesC also secreted by Xf. Expression of LesA,B and C individually in *E.coli* indicate that these proteins can induce scorching symptoms in grapevine and walnut leaves. These symptoms appear to be related to the lipase/esterase activity present in these proteins. The PrtA protein has protease activity and Xf-prtA1 mutants are highly virulent suggesting that this protein may somehow block disease. We have investigated the role these particular proteins have on the composition and distribution of the microbiome. A fairly large dataset has been generated and is being currently analyzed to evaluate the differences in the composition of the microbiome in different tissues and at different stages of infection. We have built vectors to test the anti-virulence activity of PrtA by expressing it in transgenic SR1 tobacco plants. The analysis reveals that two of the eight line tested show evidence of tolerance/resistance to infection. The understanding of how these two proteins work has shown that; LesA is a good diagnostic for PD infection and that expression of PrtA in tobacco shows promising results PrtA needs to be tested in grapevines to determine if it can provide a new avenue of therapy against PD.

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FUNDING AGENCY
Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.