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PROJECT TITLE: Characterization of the lipopolysaccharide-mediated response to *Xylella fastidiosa* infection in grapevine.

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REPORTING PERIOD: The results reported here are from work conducted July 2015 to present (February 2016).

INTRODUCTION: *Xylella fastidiosa (Xf),* a gram-negative fastidious bacterium, is the causal agent of Pierce's disease (PD) of grapevine (*Vitis vinifera*) and several other economically important diseases (Chatterjee *et al*., 2008; Varela, 2001). *Xf* is limited to the xylem tissue of the plant host and is transmitted by xylem-feeding insects, mainly sharpshooters. Extensive xylem vessel blockage occurs in infected vines (Sun *et al*., 2013), and symptoms include leaf scorch, raisining of berries, stunting, and vine death. PD has devastated some viticulture areas in California and there are currently no effective control measures available to growers besides rogueing of infected vines and severe pruning.

Our ongoing study confirms that lipopolysaccharide (LPS) is a major virulence factor for *Xf.* LPS comprises approximately 75% of the Gram-negative bacterial cell surface, making it the most dominant macromolecule displayed on the cell surface (Caroff & Karibian, 2003; Foppen *et al*., 2010; Madigan, 2012). LPS is a tripartite glycolipid that is generally comprised of a highly conserved lipid A, a polysaccharide core, and a variable O-antigen polysaccharide (Whitfield, 1995) (**Fig. 1**). We demonstrated that compositional alterations to the outermost portion of the LPS, the O-antigen, significantly affected the adhesive properties of *Xf,* consequently affecting biofilm formation and virulence (Clifford *et al*., 2013). Depletion of the 2-linked rhamnose in the O-antigen locks *Xf* in the initial surface attachment phase and prevents biofilm maturation (Clifford *et al*., 2013). In addition, we demonstrated that truncation of the LPS molecule severely compromises insect acquisition of *Xf* (Rapicavoli *et al.* 2015). We coupled these studies with quantification of the electrostatic properties of the sharpshooter foregut to better understand the interface between the *Xf* cell and the insect. Our recently funded project tested our additional

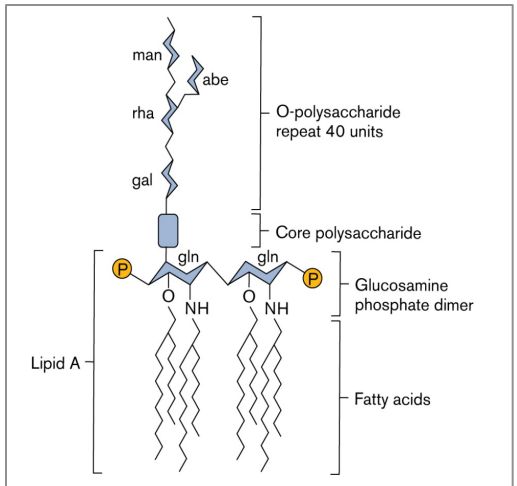

Figure 1. Schematic of a single LPS molecule containing lipid A, core polysaccharide and the O-antigen (O-polysaccharide). Adapted from Microbiology, An Evolving Science.

hypothesis that the *Xf* LPS molecule acts as a Pathogen-Associated Molecular Pattern, and the long chain O-antigen serves to shield *Xf* from host recognition, thereby modulating the host's perception of *Xf* infection (Rapicavoli *et al*. *in preparation*).

Contrary to the role of LPS in promoting bacterial survival *in planta*, the immune systems of plants have also evolved to recognize the LPS structure and mount a basal defense response to counteract bacterial invasion (Dow *et al.*, 2000; Newman *et al.*, 2000). *Xf* is introduced by its insect vector directly into the xylem; a non-living tissue, which cannot mount a defense response on its own. However, in other systems, profound changes do occur in the adjacent living parenchyma cells upon infection, suggesting that these cells communicate with the xylem and are capable of recognizing the presence of a pathogen (Hilaire *et al.*, 2001). The plant immune system can recognize several regions of the LPS structure, including the conserved lipid A and core polysaccharide components (Newman *et al.*, 2007; Silipo *et al.*, 2005). Bacteria can also circumvent the host's immune system by altering the structure of their LPS molecule. Specifically, bacteria can display different O-antigen profiles by varying the extent of polymerization or by completely abolishing synthesis of the O-antigen depending on the environment and developmental phase of the cell (Bergman *et al.*, 2006; Guerry *et al.*, 2002; Lerouge and Vanderleyden, 2002). Our working hypothesis is that during the interaction between *Xf* and a susceptible grapevine host, the bacterium's long chain, rhamnose-rich O-antigen shields the conserved lipid A and core-oligosaccharide regions of the LPS molecule from being recognized by the grapevine immune system, providing an opportunity for it to subvert the basal defense response and establish itself in the host. A similar scenario occurs in *E. coli*, where truncation of the O-antigen caused an increased sensitivity to host serum suggesting the full length O-antigen provides a masking effect towards the host immune system (Duerr *et al.*, 2009; Guo *et al.*, 2005). *Salmonella enterica* subsp. *enterica* sv. (*S.*) Typhimurium also possesses an O-antigen that aids in evasion of the murine immune system (Duerr *et al.*, 2009).

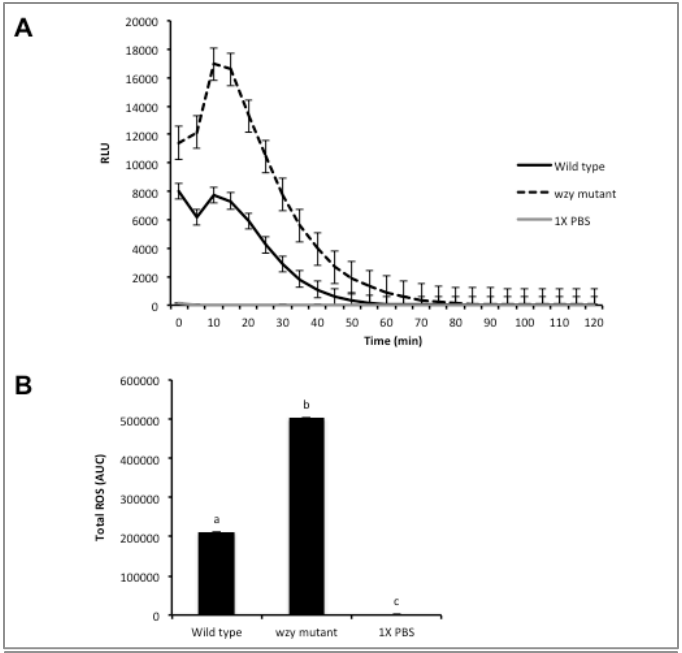

Figure 2. O antigen-modulated ROS production *ex vivo*. Discs of *V. vinifera* 'Cabernet Sauvignon' leaves were treated with 20µL of a 10^8 CFU/mL suspension of *X. fastidiosa* wild type or *wzy* mutant cells, or a 1X PBS inoculated control. **(A)** *wzy* mutant cells (containing a severely truncated O antigen) induced a significantly stronger oxidative burst that persisted nearly 20 minutes longer than leaves inoculated with wild type bacteria (which contained a fully polymerized O antigen). Graph represents the mean of 24 replicates per treatment ± standard error of the mean. **(B)** Total ROS production is reported as area under the curve (AUC) for plot of luminescence intensity over time. Treatments with different letters over the bars were statistically different ($P < 0.05$).

LPS is considered a Pathogen Associated Molecular Pattern (PAMP). PAMPs, also known as Microbial Associated Molecular Patterns (MAMPs), are conserved molecular signatures that are often structural components of the pathogen (ie. LPS, flagellin, fungal chitin, etc.). These PAMPs are recognized by the host as "non-self" and can be potent elicitors of the basal defense response. This line of defense against invading pathogens is referred to as PAMP triggered immunity (PTI) and represents the initial layer of defense against pathogen ingress (Nicaise *et al.*, 2009). PTI is well studied in both mammalian and plant hosts. However, little is known about the mechanisms involved in perception of LPS in grapevine, particularly the

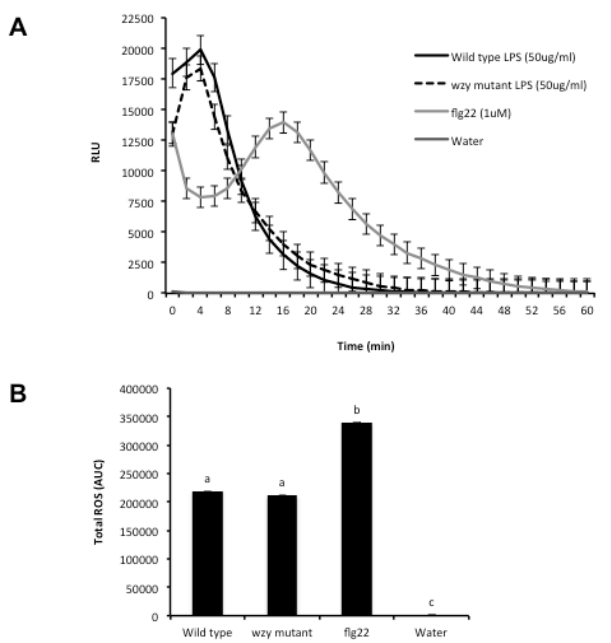

Figure 3. LPS-induced ROS production *ex vivo*. Discs of *V. vinifera* 'Cabernet Sauvignon' leaves were treated with 20µL of purified LPS from *X. fastidiosa* wild type or *wzy* mutant cells, or a water negative control. **(A)** The amplitude of ROS production remained similar for both wild type and *wzy* mutant LPS, reaching max production at approximately 4 minutes and plateaued starting around 30 minutes. **(B)** Total ROS production is reported as area under the curve (AUC) for plot of luminescence intensity over time. Treatments with different letters over the bars were statistically different ($P < 0.05$).

Xf LPS PAMP. Clearly, *Xf* has evolved a mechanism to circumvent the host basal defense response as it successfully colonizes and causes serious disease in grapevine. To explore the role of LPS as an elicitor of basal defense responses in grapevine, we investigated elicitation of an oxidative burst, an early marker of the basal defense response, in *V. vinifera* 'Cabernet Sauvignon' leaf disks exposed to either wild type *Xf* or the *wzy* mutant. ROS production was quantified *ex vivo* through the chemiluminescence of luminol and monitored over 60 minutes. Interestingly, whole *wzy* mutant cells induced a stronger and more prolonged oxidative burst response from grapevine leaf disks than did wild type *Xf.* Specifically, ROS production peaked at around 12 minutes and lasted nearly 90 minutes. Wild type *Xf* cells failed to produce a sharp peak as compared with the *wzy* mutant, and ROS production plateaued much sooner (around 60 minutes) (**Fig. 2**). Pre-treatment of plants with LPS can prime the defense system resulting in an enhanced response to subsequent pathogen attack. This defense-related memory is called "priming" and stimulates the plant to initiate a faster and/or stronger response against future invading pathogens (Conrath, 2011). Pepper leaves pre-treated with LPS isolated from incompatible (non-virulent) xanthomonads had enhanced expression of several pathogenesis-related (PR) proteins after being challenged with virulent *X. campestris* pv. vesicatoria (Newman *et al.*, 2000). We hypothesized that pre-treatment with LPS isolated from *Xf* would result in an increase in the grapevine's tolerance to *Xf* by stimulating the host basal defense response. Our *ex vivo* data showing that both wild type *and wzy* mutant LPS elicit an oxidative burst, an early marker of defense that can potentiate into systemic resistance, in grapevine leaf disks support this hypothesis (**Fig. 3**). To determine if the primed state affects the development of PD symptoms, we documented disease progress in plants that were pre-treated with either wild type or *wzy* LPS and then challenged with *Xf* either 4 or 24 hours later. **Notably, we observed a decrease in PD severity in vines pre-treated with *Xf* LPS and then challenged with *Xf* (Fig. 4)**.

Specifically, we inoculated 24 vines/treatment/time point. Plants were rated 10 weeks later on a disease index scale of 0-5, with 0 being healthy and 5 being dead or dying (Guilhabert & Kirkpatrick, 2005). As shown in **Fig. 4**, plants pre-treated with either wild type or *wzy* LPS were delayed in PD symptom development when challenged with *Xf* 4 hours later, compared to those plants that received no LPS pre-treatment. This indicates that treatment with either form of LPS (wild type or *wzy*) does elicit a defense response against *Xf* within a 4-hour time window. We reason that the purified forms of wild type LPS and *wzy* LPS both elicit a priming response because all portions of the LPS molecule, including the conserved lipid A + core LPS, which are most often associated with activity of the LPS as a PAMP are exposed and available for recognition by the grapevine immune system. Whereas, in intact cells, the majority of the LPS molecule (lipid A and some of the core LPS) is embedded in the bacterial outer membrane and

shielded from perception by the host immune system. Interestingly, two-way ANOVA analysis indicates that there is a significant interaction between time of inoculation and type of LPS applied, supporting our hypothesis that we will see large differences in the long term defense responses elicited by *wzy* LPS vs. wild type LPS at later time points than what are in the process of testing.

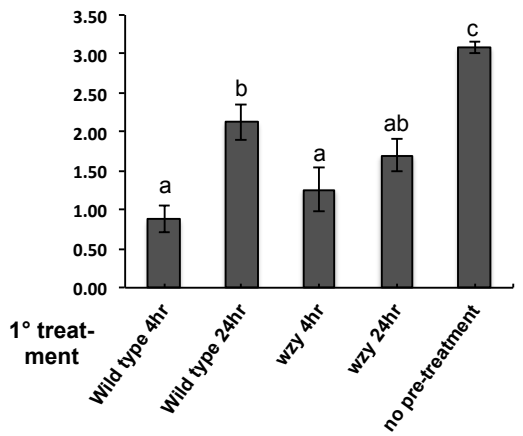

Figure 4. Pierce's Disease ratings of LPS pre-treated plants. Mean disease rating of Cabernet Sauvignon grapevines pre-treated with wild type or *wzy* mutant LPS (50µg/mL), then challenged with *Xf* at either 4 or 24hr post LPS treatment. We inoculated 24 vines/treatment/time point. Disease ratings were taken at 10 weeks post-challenge. **The LPS pre-treated plants are significantly delayed in symptom development, compared with plants that did not receive pre-treatment**. Bars represent standard error of the mean.

Most importantly, now that we have established that we can directly elicit an LPS-mediated defense response and also induce the primed state in grapevine, we propose to assess how long the temporal window of the heightened defense response and primed state lasts by increasing the amount of time between the inoculation with the LPS and the challenge with live *Xf* cells. In our currently funded project, we are testing our working hypothesis that the grapevine is recognizing the conserved core/lipid A portions of the *Xf* LPS molecule and that the long chain O-antigen serves to camouflage the rest of the LPS PAMP (the core-lipid A complex) from being recognized by the host innate immune system, allowing *Xf* to circumvent the innate immune response and successfully colonize the host. We have completed the global RNA-seq-based transcriptome profiling facet of this project where we sequenced the transcriptomes of grapevines treated with wild type, *wzy* mutant cells, or 1XPBS buffer. PTI usually causes major transcriptional reprogramming of the plant cells within hours after perception (Dow *et al.*, 2000; Tao *et al.*, 2003), so our initial experiments were targeted toward early time points during the infection process (0, 8, and 24 hours post-inoculation). Thus far, the RNA-seq data demonstrate that the grapevine is activating defense responses that are distinct to each treatment and time point. For example, enrichment analysis of early *wzy*-responsive genes identified predominant biological processes associated with cellular responses to biotic stimulus and oxidative stress. This included a significant increase in the production of thioredoxins, glutaredoxins, and other ROS-scavenging enzymes involved in antioxidant defense. In addition, there was high expression of genes involved in the production of phytoalexins (e.g. stilbene synthase), antimicrobial peptides (e.g. thaumatin), and PR genes. In contrast, early wild type-responsive genes were enriched primarily in responses to abiotic or general stresses (i.e., drought, oxidative, temperature, and wounding stresses) and were not directly related to immune responses. This indicates that the O-antigen does, indeed, serve to shield the cells from host recognition, allowing them to establish an infection (Rapicavoli *et al*. *in preparation*).

Plants also modulate small RNA (sRNA) pathways based on recognition of PAMPs or pathogen effectors (Weiberg *et al*., 2014). ssRNAs and RNA interference pathways are another important layer to the plant immune response and play a major role in the regulation of the host immune response. These sRNAs induce silencing of their target genes both at the transcriptional and posttranscriptional levels (Weiberg *et al*., 2014). High throughput sRNA profiling has been used to show that expression of endogenous host sRNAs are differentially regulated upon pathogen invasion in model and non-model plant systems (Weiberg *et al*., 2014, Katiyar-Agarwal & Jin, 2010, Seo *et al*., 2013). Specifically, an endogenous citrus microRNA was significantly up-regulated in trees infected with *Candidatus* Liberibacter asiaticus, causal

agent of Citrus Huanglongbing. This sRNA was specifically found to be involved in the host phosphorus uptake pathway and exogenous application of phosphorus reduced HLB severity (Zhao *et al*., 2013; Sagaram *et al*., 2009). sRNAs have been shown to be long range signals involved in plant defense against pathogens (Sarkies & Miska, 2014) and, in fact, can cross graft unions (Goldschmidt, 2014).

Our objectives are geared toward further characterizing the *Xf* O-antigen portion of the LPS molecule and linking its structure to its function as a virulence factor and potent elicitor of the host defense response in grapevine. We plan an interdisciplinary approach that includes looking at molecular mechanisms of infection from both the bacterial and host plant perspective. Our approach will include assessing the biological impact of alterations in the LPS structure to the grapevine response to *Xf* using transcriptional and sRNA profiling. To achieve this, we will integrate biochemical LPS structural information with information about the grapevine transcriptome and sRNAs garnered from next generation sequencing technology (RNA-Seq). Ultimately, our goals are to decipher how the abundant cell surface molecule, LPS, mediates the host-pathogen interaction in grapevine and to develop a preventative treatment for PD based on this information.

OBJECTIVES OF PROPOSED RESEARCH

Objective I: Examination of the temporal response to *Xf* lipopolysaccharide
Objective II: Examination of *Xf* lipopolysaccharide-mediated defense priming in grapevine
Objective III: Linking *Xf* lipopolysaccharide structure to function

SUMMARY OF ACCOMPLISHMENTS AND RESULTS

Objective I: **Examination of the temporal response to *Xf* lipopolysaccharide.**
In addition to initiating PTI, PAMPs are known to induce systemic resistance (i.e. resistance in distal plants organs) (Erbs & Newman, 2003; Mishina & Zeier, 2007). Moreover, when used as a pre-treatment, LPS can systemically elevate resistance to bacterial pathogens in *A. thaliana* (Mishina & Zeier, 2007), a phenomenon known as defense priming. It has been documented that a pathogen does not necessarily have to cause HR to elicit systemic resistance in the form of systemic acquired resistance Mishina & Zeier, 2007). There is substantial experimental evidence indicating that *Xf* must achieve systemic colonization in the xylem in order to elicit PD symptoms. In fact, mutants that stay localized at the original point of infection do not cause disease (Roper *et al*., 2005), and those that can move more rapidly throughout the xylem are hypervirulent (Newman *et al*., 2004, Guilhabert & Kirkpatrick, 2005). Because we have observed a decrease in PD symptom severity following exposure to *Xf* LPS (**Fig. 4**), we hypothesize that LPS may be involved in eliciting a downstream systemic defense response that prevents movement of *Xf* within the xylem network. In this objective, we will test this hypothesis and further explore the spatial persistence of the observed tolerance to PD in grapevines exposed to wild type vs. *wzy* mutant cells using transcriptional and sRNA profiling of petioles distal to the initial inoculation site. In addition, we will test the temporal persistence of the elicited defense response by testing later time points in the infection process than in our initial study. This will provide much sought after information about which defense pathways, and possibly defense-related hormones, are induced by the *Xf* LPS PAMP in grapevine and, most importantly, may identify facets of those pathways that can be manipulated for PD control.

1a. Transcriptome profiling. The application of transcriptome profiling approaches using next generation RNA sequencing (RNA-seq) allows us to profile the expression of nearly all genes in a tissue simultaneously and monitor the activation or suppression of specific defense pathways at the genome scale. In this Objective, we will shift our focus to characterize the grapevine transcriptional response at systemic locations distal to the POI and at longer time points than our previous study where we looked at early time points of 0, 8, and 24 hours post-inoculation. This will test our hypotheses (i) that truncated *Xf* O-antigen is more readily perceived by the grapevine immune system, allowing the plant to mount an effective defense response to *Xf* and (ii) that the initial perception of the truncated LPS, belonging to the *wzy* mutant, is propagated into a prolonged and systemic response.

In the summer of 2015, individual vines were inoculated with either wild type *Xf*, the *wzy* mutant, or with 1X PBS buffer (Clifford *et al*., 2013). We inoculated three vines for each treatment. The cells were delivered mechanically by inoculating a 40µl drop of a 10^8 CFU/ml bacterial cell suspension into the main stem near the base of the plant. Petioles were harvested at two different locations on the plant: at the POI (local) and 5 nodes above the POI (systemic). We harvested at 4 different time points post-inoculation: time 0 = petiole harvested just before pre-treatment, 48 hours, 1 week, and 1 month post-inoculation). All harvested petioles were immediately frozen in liquid nitrogen, prior to RNA extraction. We are aware of the differences in host colonization between wild type and *wzy* mutant *Xf* strains. In addition to the direct responses arising from the different LPS compositions, we will likely observe differences in the transcriptional or sRNA profiles of plants treated with one or the other *Xf* genotype. In particular, consequences of the development of disease upon inoculation with the virulent wild type strain, such as drought stress symptoms, should be expected in the experimental time frame (Choi *et al*., 2013). However, we expect that comparisons with mock-inoculated plants will allow us to identify specific expression markers associated with systemic spread of resistance responses that result from *wzy* mutant LPS recognition.

RNA was extracted from the harvested petioles and sequencing libraries were generated from the polyadenylated plant messenger RNA and sequenced using the Illumina HiSeq 2000 platform. Transcript expression levels were determined by alignment of the sequencing reads using the STAR aligner (Dobin *et al*., 2013) onto the PN40024 grape genome reference. Unmapped reads were *de novo* assembled using Trinity (Grabherr *et al*., 2011) to identify transcripts that were not present in the reference genome. Statistical inference using DESeq2 (Anders & Huber, 2010) was applied to determine with confidence the subset of genes that were up- or down-regulated by LPS treatment (Cantu *et al.*, 2011b). Based on our experimental design, we had a total of 72 samples for RNA-Seq analysis (3 grapevines x 3 treatments x 4 time points x 2 petioles/plant=72 samples). We are currently analyzing this data, and validation of the RNA-Seq findings using quantitative PCR (to monitor expression of genes we found to be differentially regulated from the RNA-Seq analysis) is currently underway. The entire experiment will be repeated during summer 2016.

1b. Global sRNA profiling. This portion of the study is being conducted in close collaboration with Dr. Hailing Jin (UCR), a renowned expert in the field of plant sRNAs and their role in plant defense against pathogen attack. We propose to characterize the endogenous grapevine sRNAs that are elicited by *Xf* invasion in an LPS-mediated fashion. Our goal is to identify sRNAs in grapevines that are up-regulated during *Xf* invasion. More specifically, we are focusing our study on sRNAs that are a part of propagating the defense response elicited by the *Xf* LPS PAMP. sRNAs have been shown to be long range signals involved in plant defense against pathogens (Sarkies & Miska, 2014) and can cross graft unions (Goldschmidt, 2014). We envision that, in a future study, the identified sRNA(s) could potentially be exploited for disease control by transforming rootstocks to produce the sRNA for delivery into the scion.

Construction and sequencing of sRNA libaries: We have isolated sRNAs from the petioles harvested from the same plants that were inoculated in Obj. 1a, using an optimized Trizole extraction protocol that allows for isolation of mRNA as well as of sRNAs, for RNA-seq and small RNA-seq analyses, respectively (Cantu *et al*., 2010).

sRNA libraries were produced using the TruSeq Small RNA Sample Preparation Kit and subjected to multiplex sequencing using an Illumina HiSeq2500 platform. Adapters were trimmed using CLC Genomics Workbench. Approximately 116 million RNA reads with length ranging from 18 to 26nt were obtained. In all samples, reads showed a similar and expected pattern of size distribution with peaks at 21 and 24 nt. These reads corresponded to an average of 1 million of unique small RNA sequences per sample. Protein coding gene targets in the *V. vinifera* PN40024 genome could be identified unambiguously for 20% of the small RNA sequences. An average of 4,557 gene targets per sample were identified. The small RNA sequences included 134 of the known *Vitis* microRNAs. As recently reported by Kullan et al. (2015 http://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-015-1610-5), the vvi-miR166 family was the most abundant representing about 94% of the total expression counts. These results show that we can successfully extract, sequence and annotate small RNAs from grape petioles. Further work will be carried out to identify small RNAs that accumulate differentially in plants inoculated with the different *Xf* strains.

1c. Evaluation of the persistence of LPS-mediated response to *Xf* through dormancy. As an additional, and important, facet to this Objective, we will determine if the grapevine responses elicited by *Xf* LPS persist through dormancy. During the upcoming summer months, we will replicate the experimental design described in Obj. 1a, except instead of using live *Xf* cells, we will inoculate plants with gently killed wild type or *wzy* mutant cells. These cells will be gently killed with 0.1% glutaraldehyde, then washed and re-suspended in 1XPBS. The reason for using killed cells is that that plants inoculated with live wild type cells have poor leaf emergence the following spring after dormancy and would be unusable for this experiment. Once the grapevines push new growth in the spring, we will harvest petioles from a uniform location in the plant. RNA will be extracted from these petioles and for cost-effectiveness, we will use qPCR (rather than RNA-Seq) to monitor expression of specific marker genes or sRNAs that we found to be differentially regulated in Obj. 1a. This will allow us to determine if any change at the transcriptome level or sRNA level persists through dormancy.

Objective 2: Examination of *Xf* lipopolysaccharide-mediated defense priming in grapevine. Pre-treatment of plants with LPS can prime the defense system resulting in an enhanced response to subsequent pathogen attack. This phenomenon is referred to as priming and stimulates the plant to initiate a more rapid and robust response against future invading pathogens (Conrath, 2011). In this objective, we hypothesize that pre-treatment with LPS isolated from *Xf* O-antigen mutants results in a difference in the grapevine's tolerance to *Xf* by stimulating the host basal defense response. This hypothesis is supported by our preliminary data shown in **Figs. 3** and **4**.

2a. Temporal persistence of LPS-mediated defense priming. We pre-treated grapevines with LPS isolated and purified from either wild type *Xf*, the *wzy* mutant, or diH_20 (Clifford *et al*., 2013). Specifically, we inoculated 20 grapevines/treatment/time point with 50 µg/ml of either LPS re-suspended in diH_20. Vines inoculated with diH_20 alone served as the negative controls for the experiment. In our current study, we have found that 50 µg/ml is a suitable concentration to elicit an oxidative burst and to potentiate defense priming in grapevines (**Figs. 3** and **4**). This is also in agreement with studies performed in *A. thaliana* (Zeidler *et al.* 2004). Thus, we will use this same LPS concentration for this Objective. The LPS was delivered by needle-inoculating a

40µl drop of the LPS preparation into the main stem at the base of the plant. We then challenged 15 of the vines for each treatment by inoculating a 40µl of a 10^8 CFU/ml suspension of live wild type *Xf* cells in 1X PBS at either 4 hours, 24 hours, 48 hours, 1 week, or 1 month post-LPS treatment. The remaining 5 vines/treatment/time point were inoculated with 1X PBS to serve as negative controls. These time points include additional later time points than the ones we previously tested because we also want to establish the duration of the priming effect following treatment with LPS. These inoculations were performed using the pin-prick method as previously described (Hill & Purcell, 1995). The live wild type cells were inoculated near the point of the original LPS inoculation. Plants were visually examined for PD symptom development throughout the infection process and rated on an arbitrary disease rating scale of 0-5 where 0=healthy and 5-dead or dying (Guilhabert & Kirkpatrick, 2005). Thus far, data are consistent with the previous year, further supporting that pre-treatment of plants with LPS results in attenuated PD symptoms.

2b. Quantification of *Xf* titer in grapevines following LPS pre-treatment- Bacterial populations were quantified in both local and systemic petioles 4 weeks after challenge with wild type *Xf* or when the positive control grapevines begin rating a 1 or 2 on the disease scale. In our experience, grapevines begin showing symptoms and rating a 1-2 on the disease scale beginning at 4 weeks post-inoculation in the greenhouses housed on the UCR campus. We harvested one petiole at the POI (local) and one from 5 nodes above the POI (systemic) and isolated and quantified bacteria as previously described (Roper *et al*., 2005, Clifford *et al*., 2013)). In brief, petioles were surface sterilized, then ground in sterile 1X PBS. The resulting slurry was serial diluted to a $1/10^8$ dilution. 20µl of each 1:10 dilution was plated onto individual plates of solid PD3 medium and then incubated at 28ºC. The resulting colonies were enumerated and normalized to tissue fresh weight of the petioles. We kept record of whether the harvested petiole was symptomatic or not and factored that into our disease ratings for Obj. 2a. Interestingly, data are consistent with the previous year in which titer at the POI is not significantly different between treatments (Figure 5). However, we do see a difference in disease progress, indicating that there may be differences in other host defense responses, such as the production of tyloses and other host-derived vascular obstructions. We will repeat this experiment during the upcoming summer months, and in addition to evaluating titer and PD symptom development, we will perform additional histochemical examination of tissue. We were only able to isolate bacteria from systemic tissue in three plants, and therefore, this data is not included. Due to the fastidious, slow-growing nature of *Xf*, we speculate that 4 weeks may be too early to isolate bacteria from such a distal location.

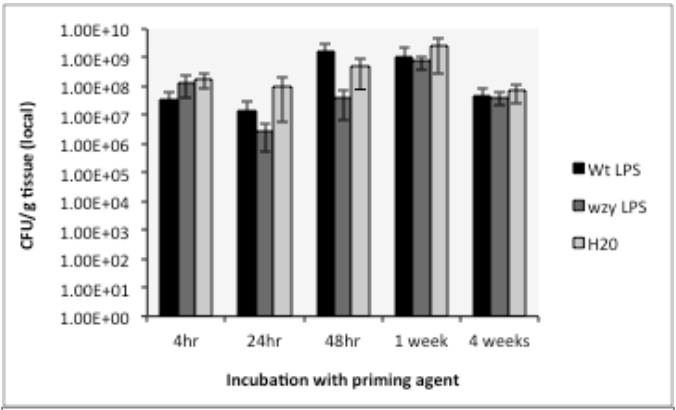

Figure 5. Bacterial titer in LPS-primed plants. Average CFU/g in local tissue. Plants were pre-treated for 4hr, 24hr, 48hr, 1 week, or 4 weeks with either wild type or *wzy* mutant LPS or a H2O control. Incubation with the priming agent was followed by a challenge with wild type *Xf* whole cells. Isolations were performed 4 weeks post-challenge. There is no significant difference in bacterial titer in LPS pre-treated plants, compared with the H2O control plants. Bars represent standard error of the mean.

2c. Examination of persistence of defense priming through dormancy. We have pruned back all the grapevines inoculated in this Objective and allowed them to go dormant (Figure 6). We have been visually examining the temporal phenology of these grapevines throughout the winter

months and will continue to document their progress during the spring and summer. The timing of the steps of the normal phenology of a grapevine are known to be impacted by infection with pathogens. Typically, grapevines severely infected with PD will have abnormal leaf emergence the following spring and will remain stunted throughout the growing season. We hypothesize that the grapevines that did not received LPS pre-treatment will have poor leaf emergence and will be severely stunted. Whereas, we hypothesize that grapevines pre-treated or "primed" with LPS will have better growth and vigor as compared to those that did not receive pre-treatment. We will score the grapevines in the spring on a scale of 1-3 where 1=vigorous leaf emergence; 2= delayed leaf emergence and 3=poor/no leaf emergence. Once the negative control plants (those challenged with only 1X PBS) have passed the phenological stage of leaf emergence, and once plants begin to show PD symptoms, we will then revert to rating them on the established PD symptom rating scale that is described in Obj. 2a.

Figure 6. Pruned LPS-primed plants. Plants primed with LPS have been cut back to just above the POI and allowed to go dormant. Leaf emergence and PD symptom development will be documented over the following months.

Objective 3: Linking *Xf* lipopolysaccharide structure to function. We have performed glycosyl composition and linkage analysis of LPS isolated from the *Xf* wild type and *wzy* mutant strains (Clifford *et al*., 2013). In our currently funded proposal, we endeavored to obtain structural data for both wild type and the truncated *wzy* mutant LPS, particularly the structure of O-chain by using gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectroscopy. These experiments are being conducted in close collaboration with the Complex Carbohydrate Research Center (CCRC) at the University of Georgia, Athens GA. Through glycosyl composition analysis (trimethylsilyl methyl glycosides-TMS, alditol acetates-AA) (York, 1985) of the LPS and composition and linkage analysis (Partially Methylated Alditol Acetates-PMAA (Ciucanu & Kerek, 1984)) of O-specific polysaccharide, the CCRC has confirmed that the *Xf* wild type high molecular weight O-antigen is comprised primarily of 2-linked rhamnose, verifying previously reported *Xf* LPS compositions (Clifford *et al*., 2013). They have also confirmed that the *wzy* mutant LPS is lacking the high molecular weight O-antigen present in wild type cells and appears to be capped with a single rhamnose residue. They are continuing their comprehensive study of *Xf* LPS, which will involve more extensive isolation and purification of core and O-chain polysaccharides. Knowledge of the structure of the LPS is critical to understanding which portions contain the elicitor activity. The proposed workflow will include extraction of native LPS (Westphal, 1965)(wild type and *wzy* mutant), its enzymatic purification, and size exclusion chromatography followed by DOC-PAGE analysis. The carbohydrate portion of LPS (core+O-chain) will be released from lipid A by mild acid hydrolysis, and the O-chain will be purified by size exclusion and other chromatography techniques. A fine structure of the polymer will be determined via NMR spectroscopy and mass spectrometry, and absolute configuration of sugars (D-, L-) in the polymer will be determined by GC-MS (Gerwig *et al*., 1978).

PUBLICATIONS PRODUCED AND PRESENTATIONS MADE

Publications:

Rapicavoli, J. N., Blanco-Ulate, B., Figueroa-Balderas, R., Morales-Cruz, A., Cantu, D., and Roper, M. C. O antigen-mediated evasion of plant immune recognition by *Xylella fastidiosa* during Pierce's disease. *In preparation*

Rapicavoli, J. N., Kinsinger, N., Perring, T. M., Backus, E. A., Shugart, H. J., Walker, S., & Roper, M. C. (2015). O Antigen Modulates Insect Vector Acquisition of the Bacterial Plant Pathogen *Xylella fastidiosa*. *Applied and Environmental Microbiology*, *81*(23): 8145-8154 **(AEM Spotlight and Journal Cover Photo)**

Oral Presentations:

Jeannette Rapicavoli. "Zeta potential: utilizing surface charge to explore host-pathogen interactions." Center for Plant Cell Biology Symposium, UC Riverside (December 2015) – **Tech Talk Awardee**

Poster Presentations:

Jeannette N Rapicavoli, Nichola Kinsinger, Thomas M. Perring, Crystal M. Johnston, Sharon Walker, and M. Caroline Roper. Lipopolysaccharide modulates the vector-pathogen interface of the bacterial phytopathogen, *Xylella fastidiosa*. (2015). American Phytopathological Society Annual Meeting, Pasadena, CA.

RESEARCH RELEVANCE STATEMENT

The proposed project will address a key aspect of the interaction of *Xf* with the grapevine host immune system. There is a substantial body of literature describing *Xf* virulence factors, but little is known about how the grapevine perceives *Xf* invasion over the course of the infection process. This continuing project is poised to identify key components of the grapevine defense response that are involved in host recognition of *Xf.* We will also examine if elicitation of the basal defense response leads to systemic and prolonged resistance to PD. Ultimately, we aim to identify potential PD resistance markers that we can exploit for disease control. In addition, information on potential PD resistance markers could also help guide traditional breeding programs. The overall outcome will result in a foundation of fundamental knowledge about PD at the molecular level that we will use to develop an innovative and environmentally sound deliverable approach to controlling this disease.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*), a bacterial pathogen, causes Pierce's disease (PD) of grapevine and poses a serious threat to the viticulture industry. We have demonstrated that shortening the sugar chain on the bacterial cell surface greatly affects the ability of *Xf* to be an effective

pathogen. In addition, removal of these sugars also compromises bacterial acquisition by its insect vector. Furthermore, these sugars comprise approximately 70% of the bacterial cell surface and we hypothesize that they aid in masking the cell from being effectively recognized by the grapevine immune system and, thereby, allow *Xf* to be a successful pathogen. The goal of the proposed work is to further pursue the role of these sugars in the interaction between *Xf* and the grapevine immune system and to use this information to develop and evaluate an environmentally sound, preventative treatment for PD.

STATUS OF FUNDS

The funding for this project is largely going towards supporting a Ph.D. graduate student, Mrs. Jeannette Rapicavoli in the Roper laboratory. In the Cantu laboratory, funds for this project are supporting the salary and benefits for the postdoctoral researcher Dr. Blanco-Ulate, who is conducting the bioinformatics analysis of the RNAseq and small RNA data. We anticipate spending the remainder of the salary, supply, services and greenhouse recharge money associated with this project as it progresses. This project is the main focus of Mrs. Rapicavoli's Ph.D. dissertation.

INTELLECTUAL PROPERTY

If this research leads to materials or procedures that will be subject to intellectual property restrictions, their availability and use will be subject to the policies of the University of California for managing intellectual property. (http://www.ucop.edu/ott/pdf/consult.pdf).

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