REPORT TYPE: Interim Progress Report for CDFA agreement number 15-0218-SA

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 February 2016 to present (July 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 roguing 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, an oligosaccharide 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 hypothesis that the *Xf* LPS molecule acts as a Pathogen-Associated Molecular Pattern, and the

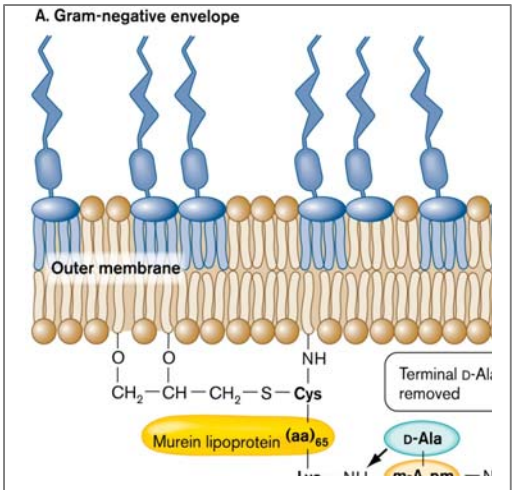

Figure 1. Schematic of a single LPS molecule containing lipid A, core polysaccharide and the O-antigen (O-polysaccharide). Adapted from Microbiology, An Evolving Science.

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). LPS is considered a Pathogen-Associated Molecular Pattern (PAMP). PAMPs, also known as Microbe-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 basal defense responses. 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 *Xf* LPS PAMP. *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. Clearly, *Xf* has evolved a mechanism to circumvent the host basal defense response as it successfully colonizes and causes serious disease in grapevine. Our working hypothesis is that during the compatible 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 basal defense responses and establish itself in the host. A similar scenario occurs in *Salmonella enterica* subsp. *enterica* sv. (*S.*) Typhimurium, where O antigen aids in the evasion of the murine immune system (Duerr *et al.*, 2009).

To explore the role of LPS as a shield against basal defense responses in grapevine, we investigated elicitation of an oxidative burst, an early marker of basal defense responses, *ex vivo* in *V. vinifera* 'Cabernet Sauvignon' leaf disks exposed to either wild type *Xf* or *wzy* mutant cells. When we examined ROS production in response to whole cells, *wzy* mutant cells (in which lipid A-core is exposed) induced a stronger and more prolonged oxidative burst in 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 (in which lipid A-core would be shielded by O antigen) failed to produce a sharp peak as compared with the *wzy* mutant, and ROS production plateaued much sooner (around 60 minutes) (data not shown). To determine where ROS production was localized *in situ*, we performed DAB (3,3′-diaminobenzidine)-mediated tissue printing of grapevine petioles that were inoculated with wild type *X. fastidiosa*, *wzy* mutant, or 1X PBS buffer as a control. DAB reacts with H_2O_2, which is the major ROS associated with the oxidative burst in plants, to produce a reddish-brown color. Grapevines inoculated with the *wzy* mutant exhibited more intense H_2O_2 production prominently localized in the xylem vessels **(Fig. 2A)**, indicating that the *wzy* mutant elicits a more robust oxidative burst than wild type *Xf*. Further quantitative comparison of staining intensity among the treatments, using ImageJ, indicated that, indeed, *wzy* elicits significantly more ROS in the xylem than does

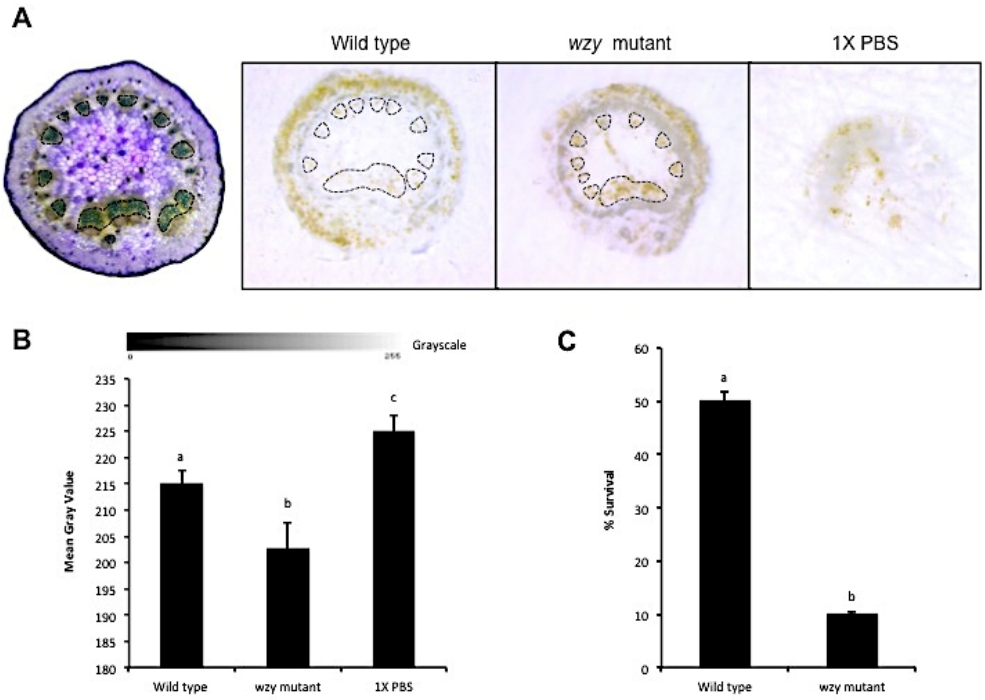

Figure 2. *In situ* localization of O antigen-modulated ROS production in the xylem. (A) DAB-mediated tissue printing at 15 minutes post-inoculation revealed a strong production of H_2O_2 specifically in the xylem vessels of grapevines inoculated with *wzy* mutant cells. **(B)** Mean gray value of DAB-stained images, representing differences in staining intensity. Grayscale intensities vary from 0 to 255; 0 = black, 255 = white, and the values in between make up the shades of gray. The mean gray value of DAB-stained images from *wzy* mutant-inoculated plants is significantly lower than wild type or 1X PBS-inoculated plants, indicating a darker, or more intense stain, and thus higher amounts of H_2O_2. Treatments with different letters over the bars were statistically different ($P < 0.05$). **(C) Hydrogen peroxide survival assay.** Suspensions of *X. fastidiosa* wild type or *wzy* mutant cells were incubated with 100μM H_2O_2 for 10 min, followed by dilution plating and enumeration. Survival percentages of *wzy* mutant cells were significantly lower than *X. fastidiosa* wild type cells ($P < 0.0001$). Following treatment with H_2O_2, only 10.06% of *wzy* mutant cells survived compared with 50.21% of wild type cells. Data are means of three biological replications.

wild type *X. fastidiosa* **(Fig. 2B)**. To determine if the intensity of the *wzy*-induced ROS burst in the xylem had direct antimicrobial activity against *X. fastidiosa*, we performed an H_2O_2 survival assay. Previously, we demonstrated that the *wzy* mutant was more sensitive to H_2O_2 stress, but survival rates in an oxidative environment were not quantified in that study. We chose a final concentration of 100μM H_2O_2 based on the lower threshold of ROS detected by the DAB staining method (DAB staining detects H_2O_2 in the range of 100μM – 10mM). In addition, to mirror the kinetics of peak ROS production seen *in vivo*, we exposed the cells to H_2O_2 for ten minutes. Due to the increased sensitivity of the mutant cells to H_2O_2, we hypothesized that the *wzy* mutant-induced oxidative burst is lethal to *wzy* mutant cells. Indeed, only 10.06% of *wzy* mutant cells survived, compared with 50.21% of wild type cells **(Fig. 2C)**.

In addition to the role of LPS in promoting bacterial infection, 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. 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. 3)**.

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 **(Fig. 4A)**. For example, enrichment analysis of *wzy*-responsive genes at 8 hpi identified predominant biological processes associated with cellular responses to biotic stimulus and oxidative stress **(Fig. 4B)**. 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, wild type-responsive genes at this time point were enriched primarily

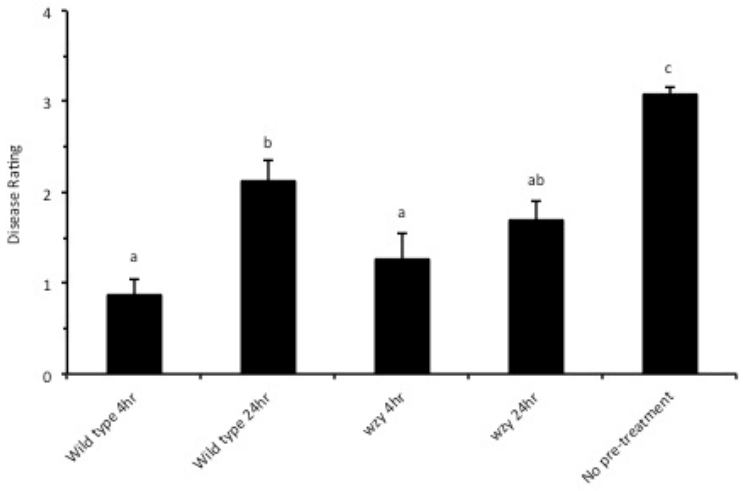

Figure 3. Pierce's Disease symptom severity in grapevines primed with purified *X. fastidiosa* LPS. Average disease ratings of *V. vinifera* 'Cabernet Sauvignon' grapevines pre-treated with wild type or *wzy* mutant LPS (50µg/mL), then challenged at 4 h or 24 h post-LPS treatment with live *X. fastidiosa* cells. Disease ratings were taken at 12 weeks post-challenge. The LPS pre-treated plants are significantly attenuated in symptom development, compared with plants that did not receive pre-treatment. Graph represents the mean of 24 samples per treatment. Bars indicate standard error of the mean. Treatments with different letters over the bars were statistically different ($P < 0.05$).

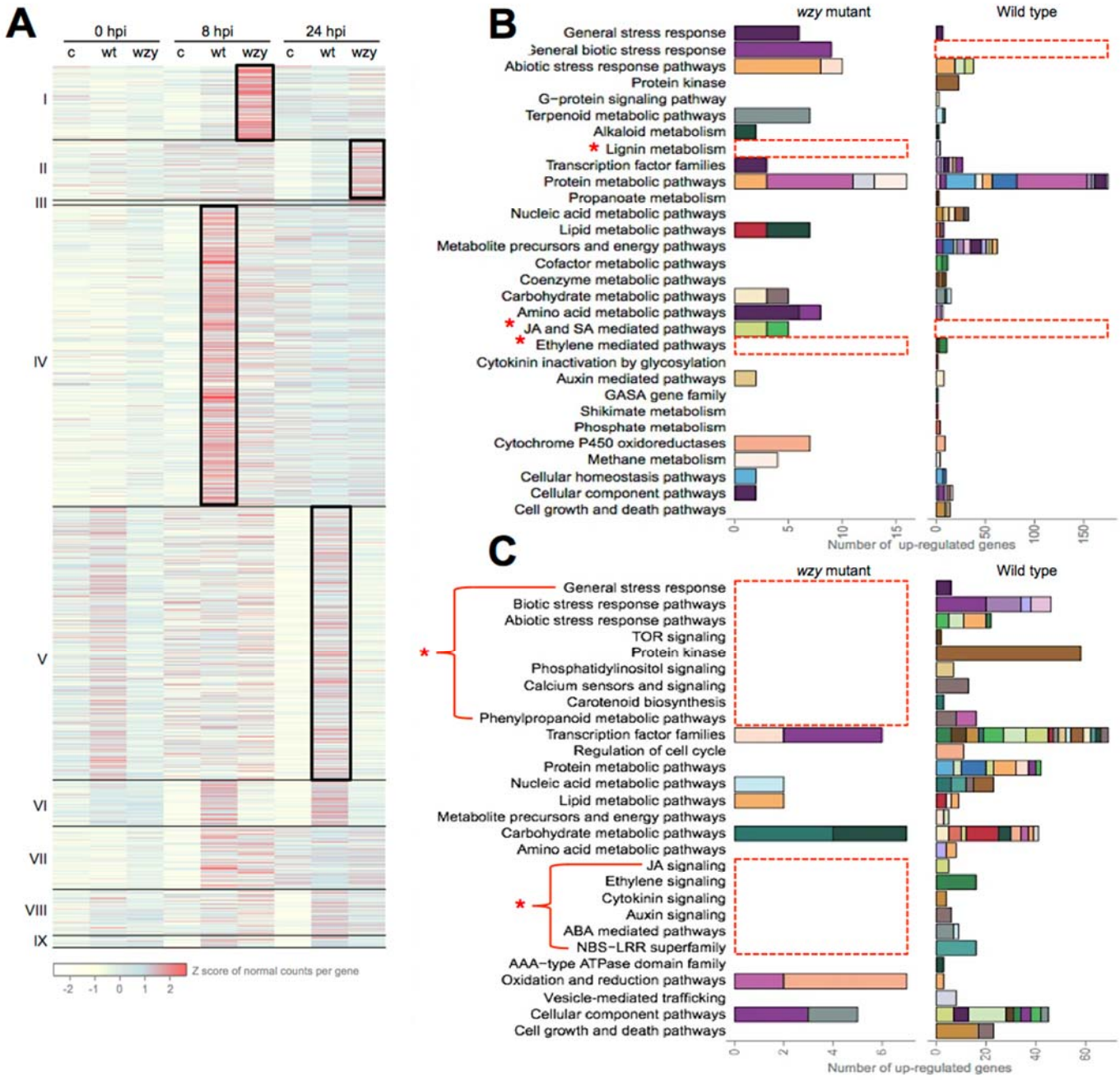

Figure 4. Grapevine responses to early infections by *wzy* mutant and wild type *X. fastidiosa*. (A) Up-regulated grape genes ($P < 0.05$) in response to *wzy* mutant (wzy) or wild type (wt) bacteria at 8 and 24 hours post-inoculation (hpi) when compared to the wounded control (c). Genes are classified into nine groups (I - IX) based on their expression pattern. The colors in the heat map represent the Z score of the normal counts per gene, and black boxes represent gene groups in each treatment that exhibited the most pronounced differences in expression at each time point. **(B)** Enriched grape functional pathways ($P < 0.05$) among genes up-regulated during wzy (Group I) or wt (Group IV) infections at 8 hpi. **(C)** Enriched grape functional subcategories ($P < 0.05$) among genes up-regulated during wzy (Group II) or wt (Group V) infections at 24 hpi. Colored stacked bars represent individual pathways. Red boxes highlight functions of interest (*) that are enriched in one treatment, but not enriched in the other at each time point.

in responses to abiotic or general stresses (i.e., drought, oxidative, temperature, and wounding stresses) and were not directly related to immune responses **(Fig. 4B)**. Notably, by 24 h post-inoculation, overall transcriptional profiles of both *wzy* and wild type-inoculated vines shifted dramatically. Grape genes in *wzy* mutant-inoculated vines were no longer enriched for immune-specific responses, and we speculate that this is due to the effective O antigen-modulated oxidative burst. In contrast, genes of wild type-inoculated plants were strongly enriched for immune responses **(Fig. 4C)**. We hypothesize that at 8 h, the high molecular weight O antigen is still effectively shielding wild type cells, therefore causing a delay in plant immune recognition. However, by 24 h post-inoculation, the production of ethylene-induced plant cell wall modifications, compounded by progressing bacterial colonization and the potential release of DAMPs via bacterial enzymatic degradation of plant cell walls, has triggered grapevine immune responses, and the plant is now fighting an active infection. 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). sRNAs and RNA interference pathways are another important layer to the plant immune response and play a major role in the regulation of host immune responses. 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 modulator of host defense responses 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, we hypothesize that LPS may be involved in eliciting a downstream systemic defense response that prevents movement of *Xf* within the xylem network. This objective tests this hypothesis and further explores 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 examined 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 shifted 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 tests our hypotheses that (i) 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 4 weeks post-inoculation. All harvested petioles were immediately frozen in liquid nitrogen, prior to RNA extraction. NA 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). Grape genes with significant differential expression were grouped into 26 clusters according to their patterns of expression across time points **(Fig. 5).**

Local tissue of *wzy*-infected plants induced genes enriched in cell wall metabolism pathways, specifically pectin modification, at 4 weeks post-inoculation **(Fig. 5A)**. This is a stark contrast with wild type-inoculated vines, in which these pathways were up-regulated as early as 8 h post-inoculation. This likely explains why this pathway is not enriched in local tissue of wild type-inoculated vines at these later time points. The induction of SA-mediated signaling pathways in *wzy*-inoculated vines was further supported by the presence of 4 genes, including two Enhanced disease susceptibility 1 (EDS1) genes, *VIT_17s0000g07370* and *VIT_17s0000g07420*. EDS genes are known defense genes associated with the SA pathway and have been implicated in grapevine defenses against powdery mildew. The consistent enrichment and up-regulation of SA-associated genes (and thus, the maintenance of the signal), including the presence of PR-1 and other salicylic acid-responsive genes at 8 h post-inoculation, strongly suggests that the plant is preventing the development of infections by *wzy* cells via an SA-dependent pathway. In wild type vines, consistent enrichment of JA-associated genes was further supported by the presence of 9 genes functioning in the metabolism of alpha-linolenic acid, which serves as an important precursor in the biosynthesis of JA **(Fig. 5A)**.

Enrichment analyses of *wzy*-responsive genes in systemic tissue included drought stress response pathways, namely genes enriched in ABA signaling (seen at 48 h post-inoculation) **(Fig. 5B**). Subsequently at 1 week post-inoculation, the enrichment of lignin metabolism genes is likely part of the vine's stepwise response to this abiotic stress. This is in contrast with wild type-inoculated vines in which these pathways were enriched at 8 h post-inoculation. Enrichment analysis of wild type-responsive genes in systemic tissue included regulation and signaling pathways, including MAPK and G protein signaling **(Fig. 5B)**. Furthermore, genes enriched in ERF transcription factors were up-regulated at 4 weeks post-inoculation, demonstrating that activation of ethylene-mediating signaling is perpetuated during the infection process. Notably, beginning at 1 week, genes enriched in JA-mediated signaling pathways were up-regulated in systemic tissue, and expression continued to increase at 4 weeks post-inoculation. This consistent enrichment and up-regulation provides further support for the role of JA in grapevine responses to wild type *Xf*. Our findings establish that this phytohormone pathway is initiated within the first 24 h post-inoculation, and the signal is consistently maintained in both local and systemic tissue. A total of 7 genes enriched in callose biosynthesis were up-regulated at 4 weeks post-inoculation, in response to wild type cells, which is over half of the total callose-related genes in the genome. The consistent up-regulation of these genes (beginning at 24 h post-inoculation) establishes this structural barrier as an important plant defense response to *Xf* infection.

Overall, the RNAseq data strongly indicate that during the days and weeks post-inoculation with *wzy* mutant cells, grapevines are no longer fighting an active infection. We hypothesize that the intense *wzy*-induced oxidative burst during the first 24 h post-inoculation, in combination with other pathogenesis-related responses, had a profound antimicrobial effect on invading *wzy* cells. These responses likely eliminated a large majority of *wzy* mutant populations, and the plant no longer sensed these cells as a biotic threat. In contrast, following recognition of wild type *X. fastidiosa* cells at 24 h post-inoculation, grapevines began responding to an active threat and initiated defense responses, such as the production of phytoalexins and other antimicrobial compounds. Furthermore, these vines were actively trying to prevent

systemic spread of the pathogen through the production of structural barriers, such as tyloses and callose.

1b. Histological examination of grapevines inoculated with *Xf* wild type or the O antigen mutant. To corroborate the enrichment of plant cell wall metabolic pathways seen in the transcriptomic data, we performed histological examination of stem tissue in grapevines inoculated with *Xf* wild type or *wzy* mutant or 1XPBS control. Vascular occlusions are commonly produced by plants in response to infection with vascular pathogens. Tyloses are outgrowths of the xylem parenchyma cell into the vessel lumen and are abundant in PD-susceptible grapevines. In fact, in susceptible grape genotypes, tyloses can occur in over 60% of the vessels in a transverse section of vascular tissue (Sun *et al*., 2013). Tylose formation is considered a late response to *Xf.* Thus, we examined tylose formation in grapevines at 18 weeks post-inoculation with wild type or *wzy* mutant *X. fastidiosa* cells, compared with 1X PBS control vines. *Wzy* mutant-inoculated vines rated a 2 or below, representing a few leaves exhibiting marginal necrosis; Wild type-inoculated vines rated over 3, representing over half of the vine exhibiting foliar necrosis; and 1X PBS controls rated 0, showing no PD symptoms **(Fig. 6 panel A)**. We observed pronounced differences in the abundance of tyloses in response to wild type vs *wzy* mutant-inoculated plants. In wild type-inoculated vines, tyloses were present in nearly all xylem vessels **(Fig. 6 panel B)**, and vessels were often completely occluded with multiple tyloses **(Fig. 6 panel C)**. In contrast, *wzy* mutant-inoculated vines contained very few tyloses. In the case where a tylose was present, it was often one large tylose that only partially occluded the vessel. All control vines, inoculated with 1X PBS, were free of occlusions. In addition to tyloses, the plant vascular tissue can initiate additional reinforcement of the cell walls to limit bacterial growth in infected plants. This includes callose and suberin deposition. Light microscopy of infected stems revealed widespread deposition of callose in the phloem tissue of *Xf* wild type-infected plants **(Fig. 7, arrow)**, suggesting communication between the xylem and phloem regarding the presence of *X. fastidiosa*. This is the first evidence of callose production in grapevine in response to *Xf*. In addition, we also provide the first evidence of a pronounced deposition of suberin, associated specifically with tylose-occluded vessels **(Fig. 7*)**. In contrast, *wzy* mutant-infected plants showed little to no evidence of either callose or suberin in the vascular tissue, and these plants looked similar to 1X PBS control plants.

1c. 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.

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.

2a. Temporal persistence of LPS-mediated defense priming. In the summer of 2015, we inoculated 20 grapevines/treatment/time point with 50 µg/ml of either wild type or *wzy* mutant LPS re-suspended in diH_20. Vines inoculated with diH_20 alone served as the negative controls for the experiment. Based on our previous greenhouse trials, we have found that 50 µg/ml is a suitable concentration to elicit an oxidative burst and to potentiate defense priming in grapevines. This is also in agreement with studies performed in *A. thaliana* (Zeidler *et al.* 2004). Thus, we used the 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 4 weeks post-LPS treatment. The remaining 5 vines/treatment/time point were inoculated with 1X PBS to serve as negative controls. We included the additional later time points (48 h, 1 wk, and 4 wk) because we also wanted 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). Data was consistent with the previous year for the 4 and 24 h time points, but we did not see significant attenuation of PD symptoms in the remaining later points. This indicates that the primed state may be transient, and it is possible that these plants may need repeated applications of LPS throughout the trial to help maintain the primed state. We plan to conduct a future experiment examining the efficacy of repeated applications of LPS on the development of PD. Furthermore, enumeration of bacterial populations in both local (POI) and systemic (5 nodes above POI) tissue at 4 weeks post-challenge with *Xf* cells was consistent with the previous year, in which titer was not significantly different between treatments. Because we do see a difference in disease progress in the earlier time points, 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, and in addition to evaluating titer and PD symptom development, we will perform additional histochemical examination of tissue.

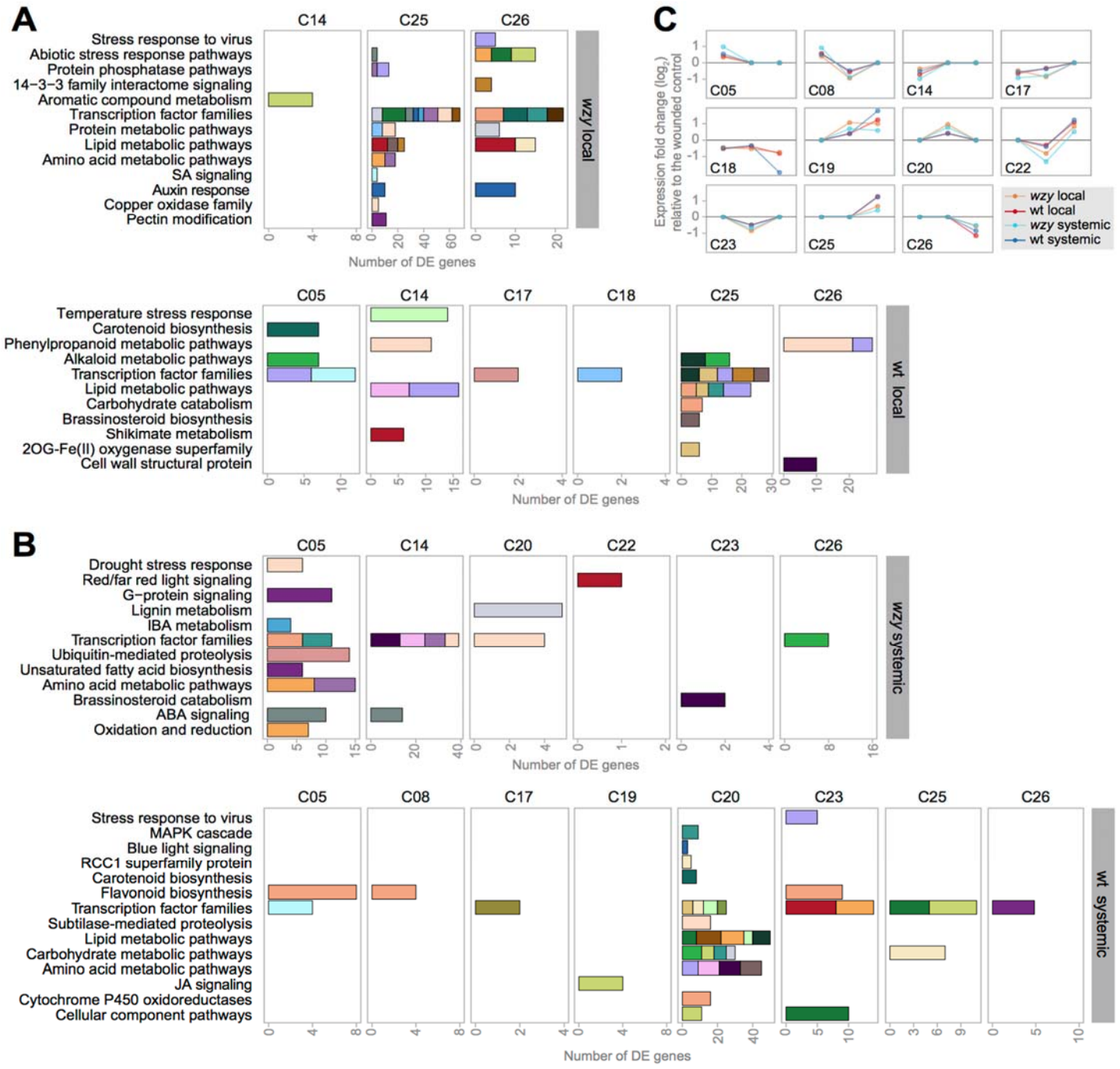

Figure 5. Transcriptomic analysis of late grapevine responses to *X. fastidiosa* wild type and *wzy* mutant strains in local and systemic tissue. Enriched grape functional pathways ($P < 0.05$) in differentially expressed (DE) gene clusters representing local **(A)** or systemic **(B)** responses to *X. fastidiosa* inoculation. Only enriched pathways related to grapevine immune responses and that were unique to wild type (wt) or *wzy* mutant inoculations are depicted. Colored stacked bars represent individual pathways. The complete dataset including the color legend for each pathway is available in Table S6. **(C)** Patterns of expression of gene clusters enriched in functional pathways with biological relevance. Lines represent the medoids for each cluster. Dots represent expression fold changes of each medoid (log2) at a given time point post-inoculation (in order: 48 h, 1 week, and 4 weeks) when compared to the wounded control.

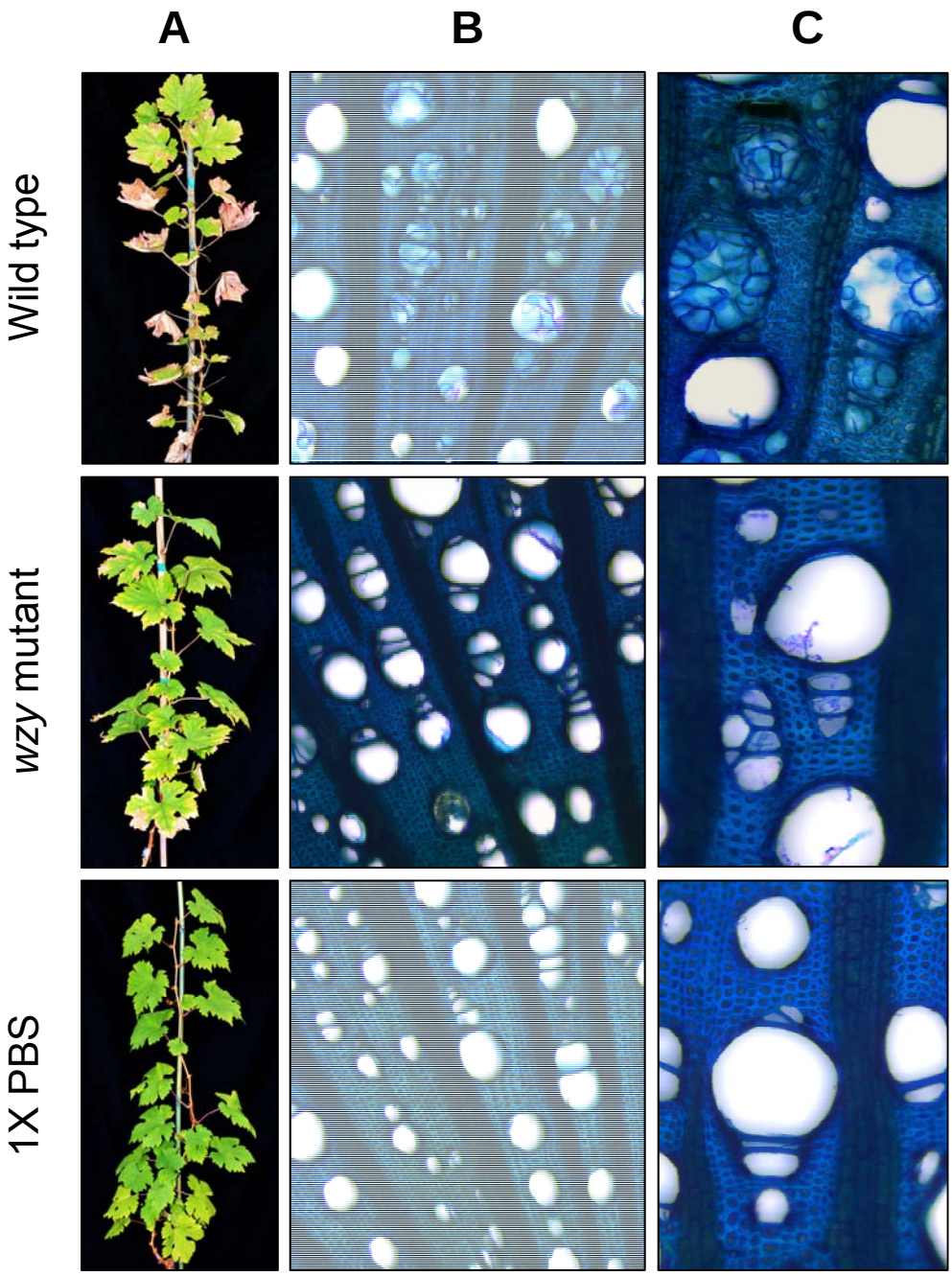

Figure 6. Tylose development in PD-infected grapevines. Images represent grapevines at 18 weeks post-inoculation, treated with wild type *X. fastidiosa* cells, *wzy* mutant cells, or 1X PBS buffer. **(A)** Representative images of PD progress prior to histological examination. **(B)** Micrograph showing tylose production in cross sections of grapevine xylem (brightfield Toluidine Blue O). **(C)** Close-up of xylem vessels showing complete occlusion with multiple tyloses (*) in wild type-inoculated vines. A few small tyloses also occurred in these vines (closed arrowheads). Tyloses were largely absent in the xylem vessels of *wzy* mutant-inoculated vines. No tyloses were present in the stems of 1X PBS-inoculated vines.

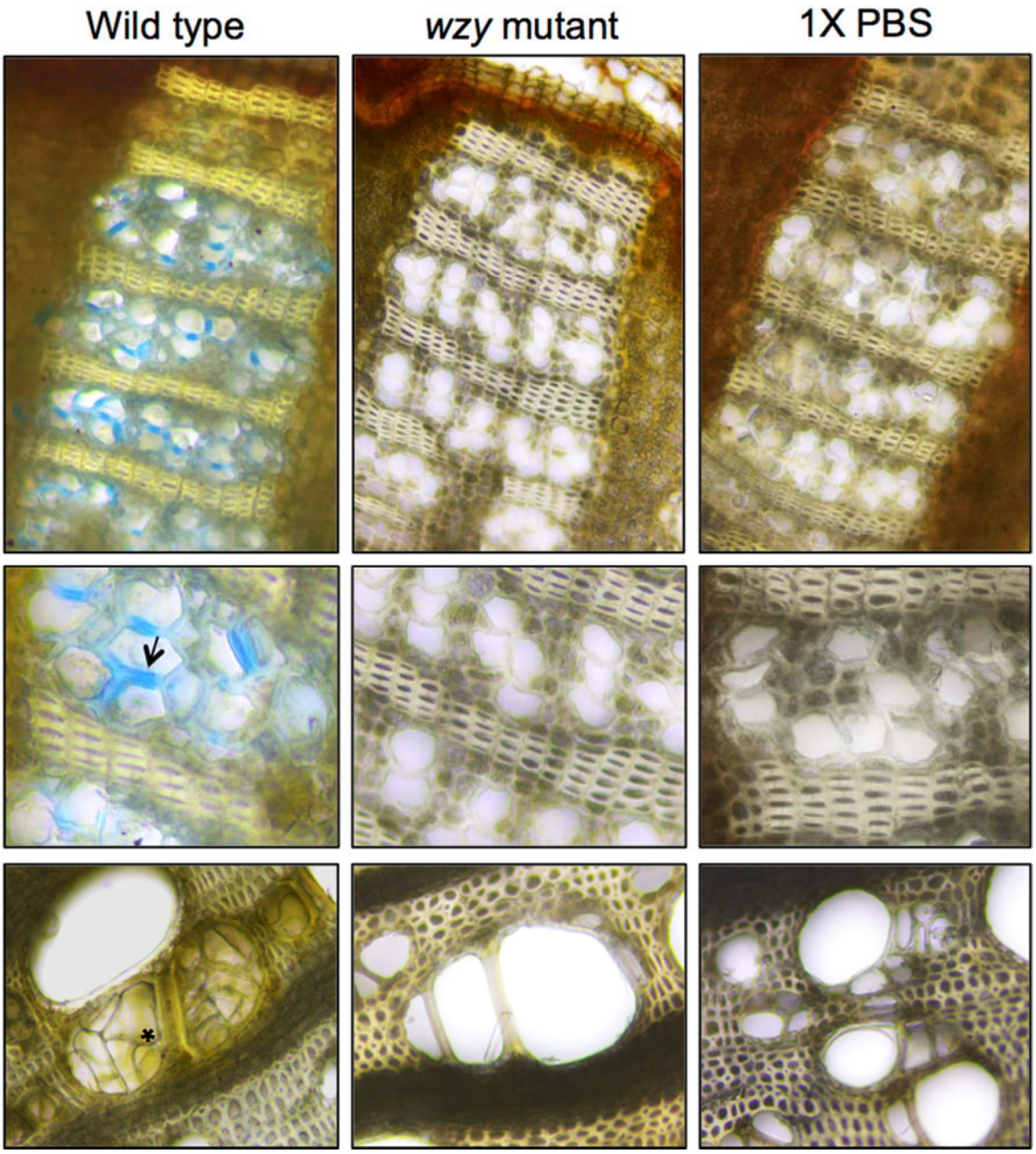

Figure 7. Callose and suberin deposition in PD-infected grapevines. Images represent grapevines at 18 weeks post-inoculation, treated with wild type *X. fastidiosa* cells, *wzy* mutant cells, or 1X PBS buffer. Wild type-inoculated plants exhibited widespread callose deposition in the phloem tissue (appears as blue color, indicated by arrow). In addition, there was pronounced deposition of suberin in xylem vessels (indicated by gold color), especially in vessels with multiple tyloses (*). No callose or suberin was present in the stems of 1X PBS-inoculated vines.

2b. Examination of persistence of defense priming through dormancy. In the fall of 2015, we pruned back all the grapevines inoculated in this Objective and allowed them to go dormant. We examined the temporal phenology of these grapevines throughout the winter months and into the spring of 2016. The premise of this part of the objective relates to the normal phenology of a grapevine, which is 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 hypothesized that the grapevines that did not receive LPS pre-treatment would have poor leaf emergence and be severely stunted. Conversely, we hypothesized that grapevines pre-treated or "primed" with LPS would have better growth and vigor as compared to those that did not receive pre-treatment. We had originally planned to 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) had passed the phenological stage of leaf emergence and exhibited PD symptoms, we had planned to revert to rating them on the established PD symptom (described in Obj. 2a). While the vines produced new shoots following the winter months, we did not observe the difference in leaf emergence or vigor between the treatments. It is possible that the vines were pruned too severely, removing a majority of *X. fastidiosa* inoculum.

Objective 3: Linking *Xf* lipopolysaccharide structure to function. 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 were 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 **(Fig. 8A)**. The CCRC has recently completed 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 carbohydrate portion of LPS (core+O-chain) was released from lipid A by mild acid hydrolysis, and the O-chain was purified by size exclusion and other chromatography techniques. A structure of the polymer was determined via NMR spectroscopy and mass spectrometry, and absolute configuration of sugars (D-, L-) in the polymer was determined by GC-MS (Gerwig *et al*., 1978).

In order to describe structural properties of O antigen in wild type and *wzy* mutant LPS, the polysaccharide moiety (O antigen + core) was liberated from LPS (lipid A) and resolved based on molecular size. Comparative analysis of SEC profiles indicated different distributions of polysaccharides in both strains. In the wild type strain, a majority of polysaccharide (40.8% total column load) was eluted in Fraction III (average molecular mass of approximately 10-20kD) and a remainder (24.8% of total column load) in Fraction IV **(Fig. 8B)**. In contrast, a majority of *wzy* polysaccharide (55.0% total PS column load) was eluted in Fraction IV (average molecular mass below 10kDa), which was only present in low quantity in the wild type parent. This fraction likely represented different molecular size forms of core oligosaccharide or truncated core-O antigen polysaccharide. Fraction I that was eluted in void (Vo) column was due to traces of unhydrolyzed, intact LPS. Monosaccharide analysis, including the determination of absolute configurations of O antigen polysaccharides from the wild type strain (SEC fraction III),

confirmed the presence of L-rhamnose and D-xylose in an 8:1 molar ratio. Based on methylation analysis and 1D/2D NMR data, we present the first evidence that the major polysaccharide present in *Xf* wild type O antigen is a linear α1-2 linked rhamnan **(Fig. 8C1)**. We also have evidence that *Xf* wild type cells maintain a heterogeneous population of O polysaccharides. Combining all analytical data, a repeat unit of the second polymer consists of α-L-rhamnan backbone substituted with either two or one β-d-Xyl residues **(Fig. 8C2,3)**. Additional analysis will need to be conducted to determine if these substitutions are autonomous LPS molecules on the cell surface or if they are linked to the same core oligosaccharide as the primary linear α1-2 linked rhamnan structure.

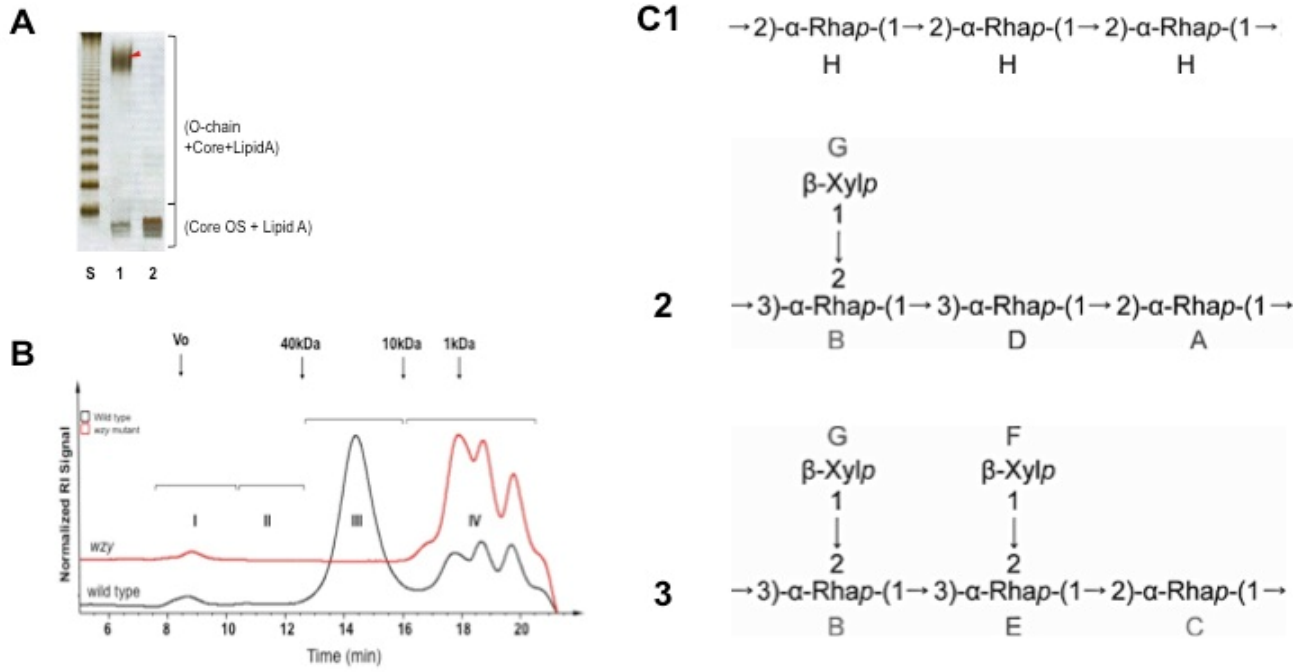

Figure 8. LPS composition and structure analysis. (A) DOC-PAGE analysis of LPS isolated from *X. fastidiosa* wild type and *wzy* mutant. Lane S = *Salmonella enterica* s. Typhimurium, S-type LPS; Lane 1 = Wild type; Lane 2 = *wzy* mutant. Red arrow indicates the presence of high molecular weight O antigen that is not observed in the *wzy* mutant LPS. **(B)** SEC chromatograms of polysaccharides liberated from LPS of *X. fastidiosa* wild type (black) and wzy mutant (red). Standard dextrans of 40,000, 10,000 and 1,000 Da were used for calibration of the Superose 12. **(C1)** The structure of *X. fastidiosa* wild type O antigen polymer is composed primarily of a linear α1-2 linked rhamnan. A repeat unit of the second polymer consists of -α-L-rhamnan backbone substituted with either one **(C2)** or two **(C3)** β-D-Xyl residues. O antigen from the *wzy* mutant is predicted to contain a single rhamnose residue.

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 acts as a shield to delay early plant immune recognition of the pathogenic bacterium, *Xylella fastidiosa*. *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. "O antigen functions as a shield during the *Xylella fastidiosa*-grapevine interaction." American Phytopathological Society Annual Meeting 2016, Tampa, FL (August 2016) – **Special Session Presentation, I.E. Melhus Award Graduate Student Symposium**

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

Poster Presentations:

Jeannette N. Rapicavoli, Barbara Blanco-Ulate, Rosa Figueroa-Balderas, Abraham Morales-Cruz, Dario Cantu, and M. Caroline Roper. Contribution of cell surface carbohydrates to the *Xylella fastidiosa*-grapevine interaction. (2016). International Society for Molecular Plant-Microbe Interactions Congress, Portland, OR – **IS-MPMI Shimamoto Travel Award**

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

Successful plant pathogens must overcome plant immune responses to establish and cause disease. Unlike many prominent bacterial phytopathogens, *Xylella fastidiosa* (*Xf*) does not

possess quintessential Type III-secreted effectors that perform this function. Although there has been extensive research identifying *Xf* virulence factors, the mechanisms utilized by this pathogen to combat plant immune responses have remained largely obscure. We demonstrate that *Xf* utilizes the prominent O antigen surface carbohydrate to shield bacterial cell surface elicitors from the grapevine immune system, effectively delaying recognition. By altering O antigen structure, we identified unique grapevine transcriptional and phenotypic responses activated during *Xf* infection. These results provide unprecedented insight into the molecular mechanisms underlying this host-pathogen interaction.

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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