FINAL REPORT FOR CDFA AGREEMENT NUMBER 15-0218-SA: CHARACTERIZATION OF THE LIPOPOLYSACCHARIDE-MEDIATED RESPONSE TO *XYLELLA FASTIDIOSA* INFECTION IN GRAPEVINE.

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ABSTRACT

Xylella fastidiosa (*Xf*) is a gram-negative, fastidious xylem-limited bacterium that causes scorching diseases in many economically important plant species like Pierce's disease (PD) of grapevine. Lipopolysaccharide (LPS) covers most the cell surface of Gram-negative bacteria and is a well-described pathogen-associated molecular pattern (PAMP) that elicits host basal defense responses plants. To understand how LPS mediates the host-pathogen interaction for *Xf*, we performed transcriptome profiling and histological analysis of grapevines inoculated with either *Xf*, containing a wild type LPS molecule, or a *wzy* mutant, containing a truncated LPS with no O-antigen. Histological analysis indicate that the grapevine defense system can recognize a truncated LPS molecule, resulting in a strong oxidative burst that is localized to the xylem tissue. RNA-Seq analyses indicate that grapevines have differential perception of the *wzy* mutant as compare to the wild type *Xf*. Specifically, during the initial stages of pathogen invasion, grapevines perceive the *wzy* mutant as a biotic stress and mount an effective defense response to the bacterium. In contrast, in the same time frame, grapevines perceive wild type as an abiotic stress and fail to mount an effective defense response. Thus, we conclude that the O-antigen serves as an effective shield for the bacterium and allows it to skirt elicitation of initial plant defense responses to the bacterium resulting in a successful infection. In addition, we investigated grapevine tolerance to PD by initially priming plants with LPS and then challenging with *Xf*. PD symptoms are attenuated when grapevines are initially treated with purified LPS and then challenged with *Xf* 4 hrs and 24 hrs after LPS treatment, indicating that the LPS molecule can prime defenses against *Xf*. Finally, we present the first evidence that the major polysaccharide present in *Xf* wild type O antigen is a linear α1-2 linked rhamnan polymer.

LAYPERSON SUMMARY

Successful plant pathogens must overcome plant immune responses to establish themselves and cause disease. Unlike many prominent bacterial phytopathogens, *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 O antigen surface carbohydrate to shield bacterial cell surface elicitors from the grapevine immune system, effectively delaying immune recognition. By altering O-antigen structure, we identified unique grapevine transcriptional and phenotypic responses activated during *Xf* infection. These results provide novel insight into the molecular mechanisms underlying this host-pathogen interaction.

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). *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 effective control measures are limited.

Our 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

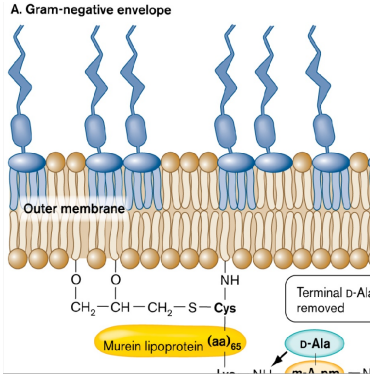

Figure 1. Schematic of a single LPS molecule containing lipid A, core polysaccharide and the O-antigen (O-polysaccharide). Adapted from Microbiology, An Evolving Science.

these studies with quantification of the electrostatic properties of the sharpshooter foregut to better understand the interface between the *Xf* cell and the insect. We then sought to test our additional hypothesis that the *Xf* LPS molecule acts as a Pathogen-Associated Molecular Pattern (PAMP), 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*., 2018).

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 PAMP. PAMPs, also known as Microbe-Associated Molecular Patterns (MAMPs), are conserved molecular signatures that are often structural components of the pathogen (i.e. LPS, flagellin, fungal chitin, etc.). 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, we observe profound changes that occur in the xylem that are linked to presence of *Xf* These include an oxidative burst and suberin deposition (**Figs. 2 and 8**) (Rapicavoli et al, 2018), as well as tylose production (**Fig. 7**). Interestingly, we also observe a significant defense-related response to *Xf* in the phloem tissue, a tissue historically overlooked in the context of this xylem dwelling pathogen that manifests in the form of callose deposition (**Fig. 8**). 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. We tested our hypothesis that 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 (Rapicavoli *et al.*, 2018).

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 shown in Rapicavoli *et al*, 2018). 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, the *wzy* mutant, or a 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 wild type *Xf* **(Fig. 2B)**. To determine if the intensity of the *wzy*-induced ROS burst in the xylem had direct antimicrobial activity against *Xf*, we performed a H_2O_2 survival assay. 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)**.

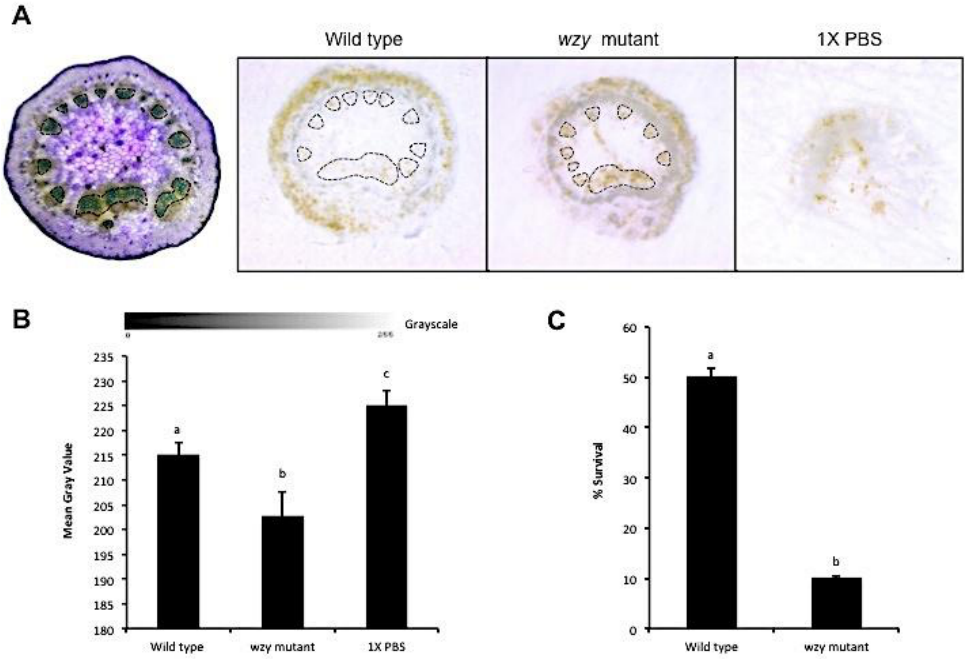

Figure 2. *In situ* localization of O antigen-modulated ROS production in the xylem. (A) DAB-mediated tissue printing at 15 min. post-inoculation revealed a strong production of H_2O_2 in the xylem vessels of grapevines inoculated with *wzy* mutant cells. **(B)** Mean gray value of DAB-stained images, 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 more intense stain, and thus higher amounts of H_2O_2 ($P < 0.05$). **(C) H_2O_2 survival assay.** *Xf* 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 *Xf* 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.

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, Newman *et al.*, 2000). We demonstrate 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 dise3ase 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) (**Rapicavoli et al, 2018).

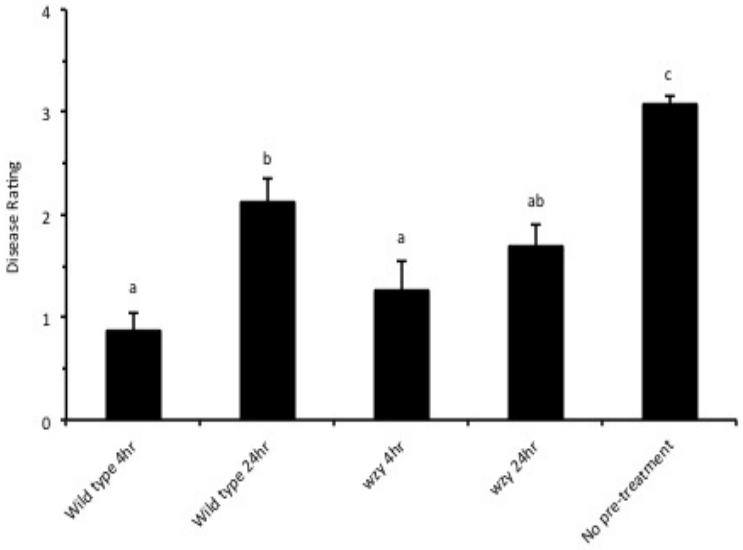

Figure 3. Pierce's Disease symptom severity in grapevines primed with purified *Xf* LPS. Average disease ratings of *V. vinifera* 'Cabernet Sauvignon' grapevines pre-treated with wild type or *wzy* mutant LPS (50µg/mL), then challenged at 4 h or 24 h post-LPS treatment with live *Xf* cells. Disease ratings 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 ($P < 0.05$). Graph represents the mean of 24 samples per treatment. Bars indicate standard error of the mean.

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 (Rapicavoli et al, 2018). The goal was to identify genes that are differentially expressed when plants are inoculated with either wild type or the *wzy* mutant while using mock-inoculated plants as the controls. 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 in this time point 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 **(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*., 2018). Complete RNAseq data can be found in the supplementary information in Rapicavoli et al, 2018. Nature Communications, 9 (1): 390.

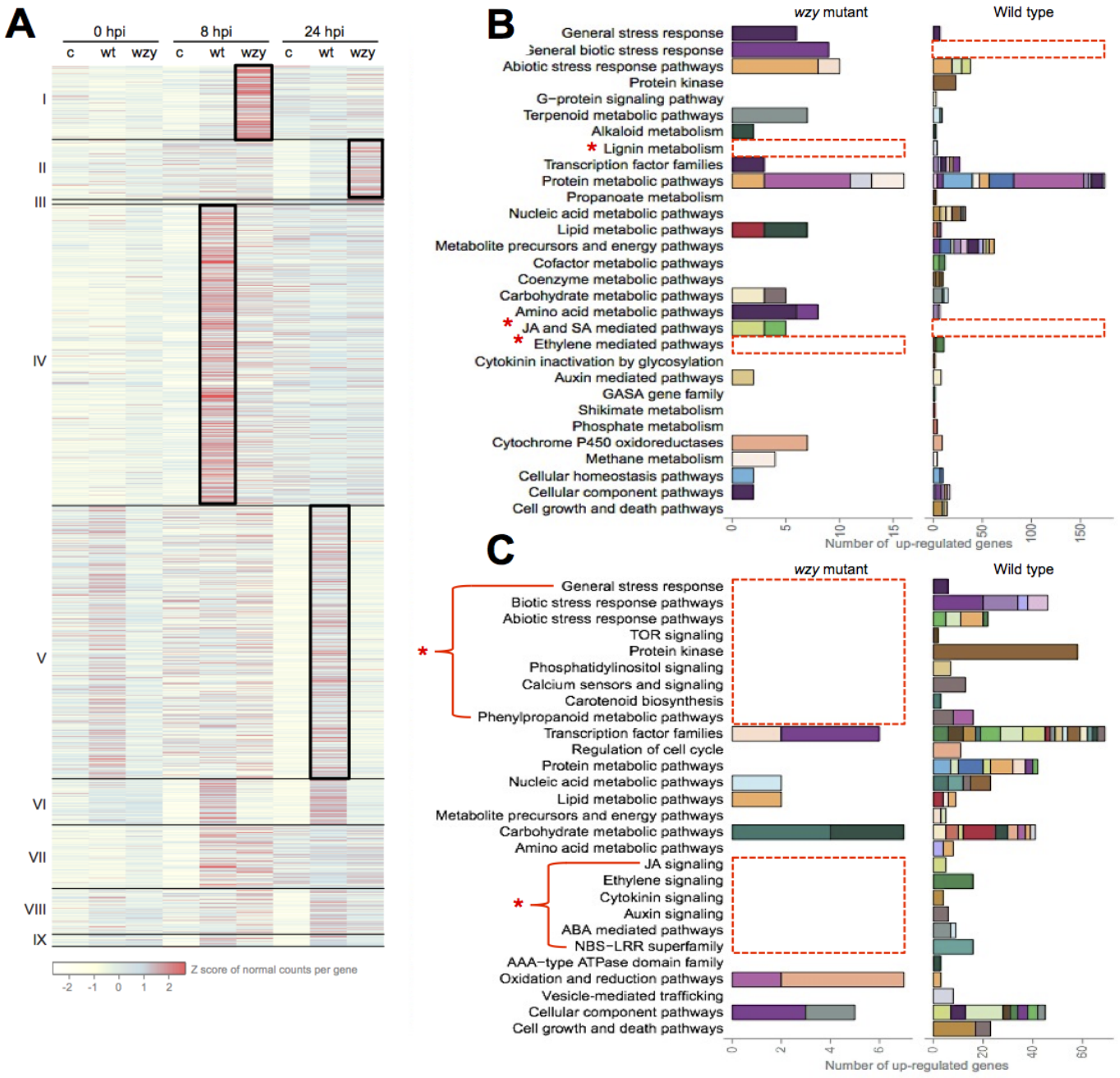

Figure 4. Grapevine responses to early infections by *wzy* mutant and wild type *Xf*. (A) Up-regulated grape genes ($P < 0.05$) in response to *wzy* mutant or wild type bacteria at 8 and 24 hours post-inoculation (hpi) when compared to the wounded control (c). Genes are classified into nine groups (I - 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.

OBJECTIVES

1. Examination of the temporal response to *Xf* lipopolysaccharide
2. Examination of *Xf* lipopolysaccharide-mediated defense priming in grapevine
3. Linking *Xf* lipopolysaccharide structure to function

RESULTS AND DISCUSSION

Objective 1: **Examination of the temporal response to *Xf* lipopolysaccharide.**

In addition to initiating PTI, PAMPs are known to induce systemic resistance (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 to elicit PD symptoms. In fact, mutants that stay localized at the original point of infection do not cause disease (Roper *et al*., 2007), 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 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.

Objective 1a. Relative expression of early response genes in LPS treated plants.

To validate and further support our findings in our RNA-seq data from grapevine responses to early infections by the *wzy* mutant and wild type *Xf* cells (**Fig. 4**), we examined expression fold-changes (log2) of early response genes observed in grapevines treated with 50µg/mL wild type or *wzy* mutant LPS (lipid A-core exposed in both types of LPS) or diH_2O at 24 post-inoculation. We chose nine genes that were enriched during early infection in grapevines treated with the *wzy* mutant and wild type cells to perform qRT-PCR on grapevines treated with wild type or *wzy* mutant LPS at 24 h post-inoculation. Eight of nine genes were up-regulated in both wild type and *wzy* mutant LPS treatments. Interestingly, grapevines responded similarly to wild type and *wzy* LPS. Our results validate our previous RNA-seq data and support our hypothesis that the highly-conserved lipid A and the oligosaccharide core but not the O antigen act as a PAMP to elicit early plant immunity (**Fig. 5**) (Rapicavoli et al, 2018).

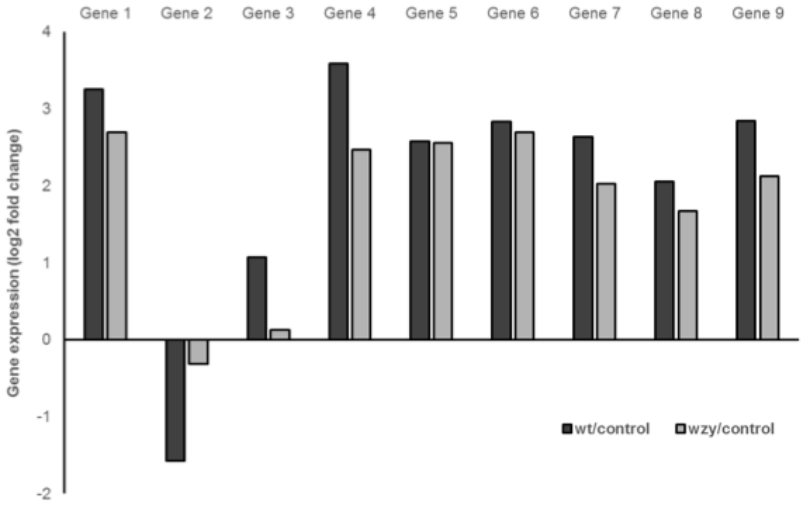

Figure 5. Expression fold-changes of early response genes in LPS treated plants. Expression fold-changes of early response genes observed in *V. vinifera* 'Cabernet Sauvignon' grapevines treated with wild type or *wzy* mutant LPS (50µg/mL) or dH_2O. Genes 1-9 correspond to: *VIT_11s0052g01780* (1-deoxy-D-xylulose-5-phosphate synthase), *VIT_00s0253g00040* (monocopper oxidase), *VIT_08s0040g02200* (peroxidase ATP2a), *VIT_01s0127g00400* (polygalacturonase), *VIT_14s0060g00480* (S-adenosylmethionine synthetase 1), *VIT_13s0067g02360* (peroxidase, class III), *VIT_11s0052g01650* (pathogenesis-related protein 1 precursor), *VIT_04s0008g00420* (clavata1 receptor kinase), and *VIT_11s0052g01150* (nicotianamine synthase), respectively.

1b. 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 tested 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.

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. 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. 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.*, 2011). Grape genes with significant differential expression were grouped into 26 clusters according to their patterns of expression across time points **(Fig. 6).** Local tissue of *wzy*-infected plants induced genes enriched in cell wall metabolism pathways, specifically pectin modification, at 4 weeks post-inoculation **(Fig. 6A)**. 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. 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. 6A)**.

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. 6B)**. 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. 6B)**. 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 *Xf* 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 (**Figs. 7 and 8**).

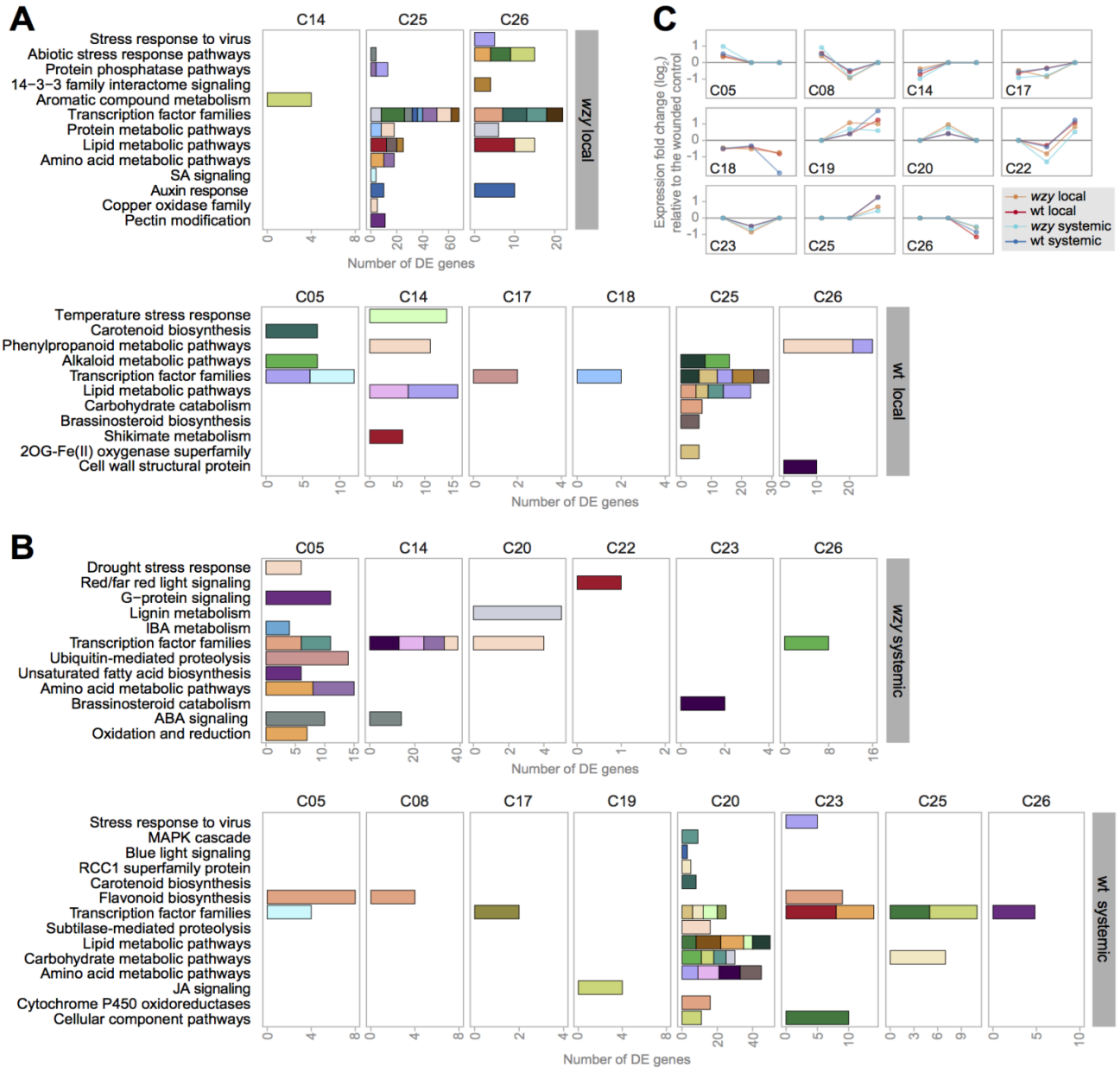

Figure 6. Transcriptomic analysis of late grapevine responses to *Xf* wild type and *wzy* mutant strains in local and systemic tissue. Enriched grape functional pathways ($P < 0.05$) in differentially expressed (DE) gene clusters representing local **(A)** or systemic **(B)** responses to *Xf* inoculation. 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. **(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.

Objective 1c. 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 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. 7 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. 7 panel B)**, and vessels were often completely occluded with multiple tyloses **(Fig. 7 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. 8, arrow)**, suggesting communication between the xylem and phloem regarding the presence of *Xf*. 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. 8*)**. 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 (Rapicavoli et al, 2018).

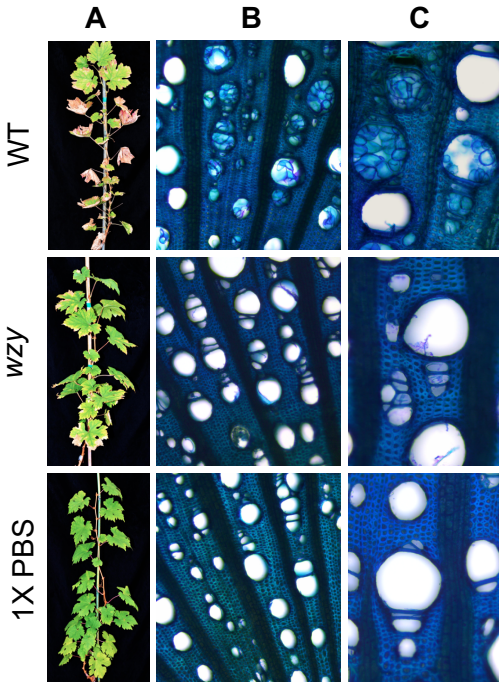

Figure 7. Tylose development in PD-infected grapevines. Images represent grapevines at 18 weeks post-inoculation, treated with wild type *Xf* cells, *wzy* mutant cells, or 1X PBS buffer. **(A)** Representative images of PD progress prior to histological examination. **(B)** Micrograph showing tylose 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.

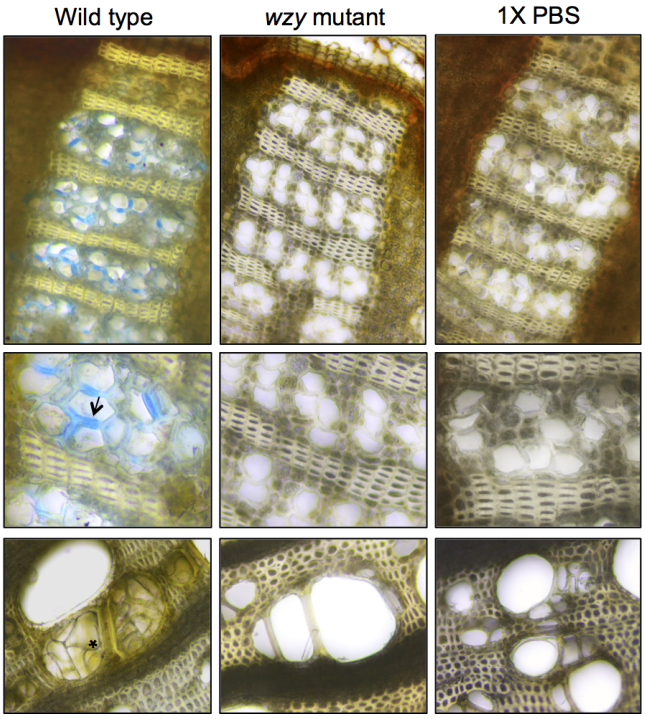

Figure 8. Callose and suberin deposition in PD-infected grapevines. Images represent grapevines at 18 weeks post-inoculation, treated with wild type *Xf* cells, *wzy* mutant cells, or 1X PBS buffer. Wild type-inoculated plants exhibited widespread callose deposition in the phloem tissue (appears as blue 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.

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* and *wzy* mutant results in a difference in the grapevine's tolerance to *Xf* by stimulating the host basal defense response.

Objective 2a. Temporal persistence of LPS-mediated defense priming.
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 (**Fig. 3**), but we did not observe 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. 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.

Objective 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 most of *Xf* inoculum.

Objective 3: Linking *Xf* lipopolysaccharide structure to function.
We have obtained 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 (Rapicavoli *et al*, 2018). 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. 9A)**. 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).

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, most of the 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. 9B)**. In contrast, most of the *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. 9C1)**. 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 ⟨-L-rhamnan backbone substituted with either two or one β-d-Xyl residues **(Fig. 9C2,3)**. 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.

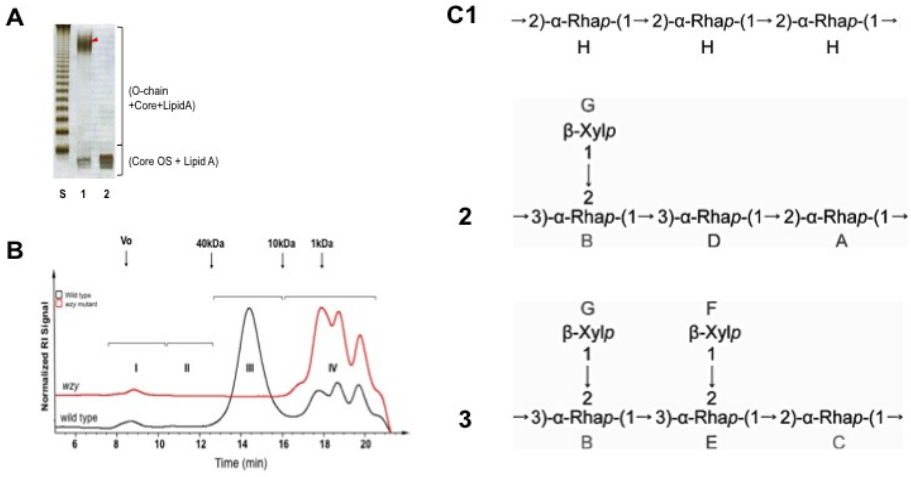

Figure 9. LPS composition and structure analysis. (A) DOC-PAGE analysis of LPS isolated from *Xf* wild type and *wzy* mutant. Lane S = *Salmonella enterica* s. Typhimurium, S-type LPS; Lane 1 = Wild type; Lane 2 = *wzy* mutant. Red arrow indicates the presence of high molecular weight O antigen that is not observed in the *wzy* mutant LPS. **(B)** SEC chromatograms of polysaccharides liberated from LPS of *Xf* 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 *Xf* wild type O antigen polymer is composed primarily of a linear α1-2 linked rhamnan. A repeat unit of the second polymer consists of --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.

CONCLUSIONS

RNA-seq and histological analysis show the grapevine defense system can recognize a truncated LPS molecule, resulting in a strong oxidative burst and a small production of tyloses. Grapevines produce many tyloses, phytoalexins and other antimicrobial compounds when inoculated with *Xf* wild type. In addition, PD symptoms are attenuated when grapevines are challenged with *Xf* 4 hrs. and 24 hrs. after LPS treatment, showing that the LPS molecule can prime defenses against *Xf*. Finally, we present the first evidence that the major polysaccharide present in *Xf* wild type O antigen is a linear α1-2 linked rhamnan. We show *Xf* high molecular O antigen is a critical virulence factor in PD. Our results provide unprecedented insight into the molecular mechanisms underlying host-pathogen interaction in Pierce's Disease.

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