ABSTRACT

*Xylella fastidiosa* (*Xf*), 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 effectors 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.
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. 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 H₂O₂, 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$_2$O$_2$ 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$_2$O$_2$ survival assay. We chose a final concentration of 100µM H$_2$O$_2$ based on the lower threshold of ROS detected by the DAB staining method (DAB staining detects H$_2$O$_2$ 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$_2$O$_2$ for ten minutes. Due to the increased sensitivity of the mutant cells to H$_2$O$_2$, 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, 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 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) (Rapicavoli et al, 2018).
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 dH2O 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 dH2O. 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.
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 Xf cells, wzy mutant cells, or 1X PBS re-suspended in diH2O. Vines inoculated with diH2O 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 glycossides-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 α-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.

REFERENCES CITED

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