DIFFERENTIAL EXPRESSION OF GENES OF *XYLELLA FASTIDIOSA* IN XYLEM FLUID OF CITRUS AND GRAPES

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Reporting Period: The results reported here are from work conducted October 2007 to September 2008.

ABSTRACT
Understanding regulatory pathways controlling microbial pathogenesis can lead to a better understanding of virulence factors as potential targets for disease control strategies. Preliminary research has shown that citrus is tolerant or resistant to Pierce's disease (PD) strains of *Xylella fastidiosa* (*Xf*) but may serve as a reservoir for the bacterium. Commercial citrus (lemon, orange and grapefruit) orchards in proximity to vineyards in Temecula Valley were selected to determine the effect of xylem fluid on *Xf*. Current results revealed that pure xylem fluid of grapefruit, orange, and lemon caused the bacterial cells to form aggregations of large whitish clumps, whereas the xylem fluid of grape caused them to form a visible thick biofilm. Macroarray analysis was conducted with 111 genes, predicted by the *Xf* genome sequence to have roles in virulence, as well as nucleic acid and protein metabolism, cellular transport and stress tolerance. There were 28 genes with greater expression in xylem fluid of grape, vs. that of citrus. The virulence regulator *xrvA*, transcriptional regulators *algU, agmR, gcvR*, two-component regulators *gacA* and *colS*, and posttranscriptional regulator *hsq*, were expressed at higher levels in grape xylem fluid. Other genes that were over expressed in grape xylem included *hsf*, *xadA*, *fimT, pilI, pilT, pilU,*and *pilY1*, related to attachment, biofilm formation, and twitching motility of *Xf* within xylem. The data indicated that grape xylem fluid stimulates the expression of virulence genes, likely contributing to PD in grapevine. *Xf* may use gene regulatory mechanisms to respond to changing environments in the xylem of plants, and host range may in part be determined by differential regulation of virulence genes in different host xylem conditions.

INTRODUCTION
Xylella fastidiosa (*Xf*) is mainly vectored by the glassy-winged sharpshooter (GWSS) in southern California (Raju et al, 1983; Sorensen and Gill, 1996). Previous studies in California have identified 94 plant species in more than 28 plant families as hosts of *Xf* (Costa et al., 2004). Most of them are symptomless but may serve as inoculum sources for vector acquisition of *Xf*. Studies in the Temecula Valley showed that the proximity of citrus groves to vineyards influenced the incidence and severity of Pierce's disease (PD) in grapevine (Perring et al., 2001). PD infection is most severe when the grapevines are adjacent to citrus, and the damage declines as one moves away from citrus. Although the GWSS feeds on and moves back and forth between citrus and grape plants, there is no *Xf*-caused disease symptom in citrus in the area. This implies that citrus plants are resistant or tolerant to *Xf* but may be a reservoir, harboring the pathogen for GWSS acquisition while grape plants are susceptible (Bi et al., 2007b). Transmission of PD by leafhoppers from citrus to grapevines has indeed been documented (Hopkins et al., 1978). Little is known about the biochemical mechanisms involved in host plant resistance/susceptibility to *Xf* in this citrus and grape system.

It was recently reported that certain amino acids are essential for *Xf* growth, and that glucose stimulates growth, while fructose and sucrose have an inhibitory effect (Leite et al., 2004). Our preliminary data indicated that there were large differences in xylem fluid amino acid and sugar contents between grapes and citrus currently growing in the Temecula Valley (Bi et al., 2007a). Xylem fluid of citrus significantly inhibits biofilm formation by PD strains of *Xf* compared to xylem fluid of grape (Bi et al., 2007b). However, the xylem fluid chemical components in citrus and grape, and their role in *Xf* gene expression and host plant resistance and susceptibility to *Xf*, are not well known. Further research is needed to determine the effect of host plant xylem fluid on expression of *Xf* virulence factors and to elucidate the mechanisms that are involved. Host plant resistance has been recognized as the most cost effective and environmentally safe method for controlling many major microbial pathogens of economic plants. Understanding the biochemical mechanisms involved may lead to the development of resistant varieties or anti-*Xf* chemicals for existing grapevines.

OBJECTIVES
1. Investigate the effect of host plant xylem fluid on *Xf* aggregation, biofilm formation, and gene expression *in vitro*.
2. Determine the role of specific chemical components in citrus xylem fluid in *Xf* resistance.

RESULTS AND DISCUSSION
Bacteria cell aggregation in xylem fluid. Aggregation and attachment of *Xf* was observed after culturing a PD strain in pure xylem fluid of grapefruit, orange, lemon, and grape. The bacterial cells aggregated to form large clumps in grapefruit, orange, and lemon fluid (**Figure 1**). This may cause the bacteria to remain in only a few xylem vessels after introduction to

the citrus by a sharpshooter, without much mobility within the xylem of citrus. In contrast, the bacterium formed less aggregation in grape fluid, consistent with the known ability of PD strains to move easily within the xylem of grapevines.

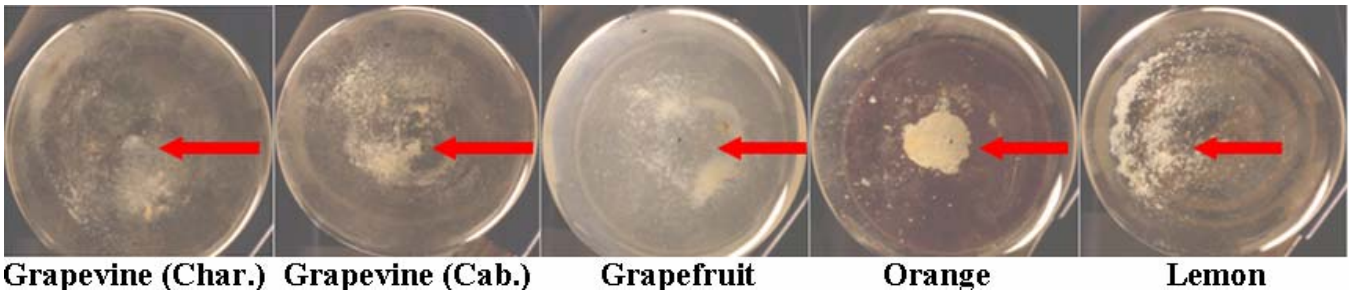

Figure 1. A fresh *Xf* Temecula A05 culture (Costa et al., 2004) was inoculated into xylem fluid of grape, grapefruit, orange, and lemon at OD_{600}=0.02 in borosilicate glass culture tubes on a rotary shaker at room temperature (around 24°C). All tubes were covered with a black box to prevent the xylem fluid from light during the shaking culture. Photographs were taken after 10-12 weeks. The red arrow indicates the aggregated cells in xylem fluid.

DNA macroarray analysis of gene expression in citrus and grape xylem fluid. DNA macroarray membranes were prepared with 111 selected genes with putative roles in virulence, as well as others involved in nucleic acid and protein metabolism, cellular transport and stress tolerance, based on the genome sequences of *Xf* 9a5c (a CVC strain) (Simpson, et al, 2000) and Temecula1 (a PD strain) (Van Sluys, et al., 2003). Total RNAs were extracted from *Xf* Temecula1 cultured in the pure xylem fluid of grapefruit, orange, lemon and grape using a Quiagen RNAeasy mini kit (Quiage, CA). The purified mRNA was separated from total RNA using an mRNA-ONLY™ Prokaryotic mRNA Isolation Kit according to the manufacturer's protocol (Epicentre, WI), and used for synthesizing cDNA probes for array hybridization by reverse transcription (RT). DNA macroarray nylon membranes were hybridized with DIG-labeled cDNA probes following the manufacturer instructions (Roche, Molecular Biochemical, Indianapolis, IN). The signal intensities of spots on the membranes were analyzed using the Quantity one® software (Biorad). Thirty genes were differentially expressed in grape fluid compared to citrus fluid (**Table 1**).

Validation of macroarray data. Several potential virulence-related genes were chosen to validate the differential expression levels of genes in xylem fluid of grape and citrus using RT-PCR (**Figure 2**). rRNA was detectable in all xylem fluid in this RT-PCR condition. No RNA was detectable in a water control. Expression of the genes *xrvA, hsq, gacA, algU,* PD0062*, pilI, pilU, PD0312, hsf, pcp, secG, hspA, clpP, msrA* and *tapB* RNA was increased in grape fluid, and *pglA* and PD0143 RNA were increased in grapefruit, orange and lemon fluids (**Figure 2**). Genes predicted to be involved in virulence regulation, such as the virulence regulator *xrvA*, transcriptional regulators *algU, agmR, gcvR*, two-component regulators *gacA* and *colS*, and posttranscriptional regulator *hsq*, were expressed at higher levels in grape fluid. Inside the plant's xylem, *Xf* is exposed to a range of variable stress factors, such as changes in osmolarity, availability of nutrients, and agents generating reactive oxygen intermediates (Alves et al., 2004). To ensure survival, *Xf* may respond to these stress situations via regulatory mechanisms involving specific regulatory genes. The regulatory genes *algU* (Shi et al, 2007) and *gacA* (Cooksey, 2007) were previously shown to have roles in regulating many potential virulence factors in *Xf.* Hfq, an abundant RNA-binding protein, may indirectly affect biofilm formation in *Xf* through a complex *hfq*/*rsmB*/*rsmA*-mediated system (Shi et al, 2007). Genes involved in surface structures and attachment components, such as PD0312, *hsf,* and *xadA*, were expressed more highly in grape fluid than citrus. *hsf* (PD0744) has a high similarity to the *hsf* adhesin gene of *Haemophilus influenza* (St Geme et al., 1996), and *xadA* encodes a putative afimbrial outer membrane protein adhesion (Simpson et al., 2000). The expression of *hsf* and *XadA* was increased in grape fluid, likely contributing to an enhanced ability to adhere to xylem cell walls. It was reported that *hsq* and *hspA* were regulated by *algU* (Shi et al, 2007) and *xadA* and *hsf* were regulated by *gacA* (Cooksey, 2007). Genes involved in the biogenesis and twitching motility of type I pili and type IV pili in *Xf*, such as PD0062*, fimT, pilI, pilT, pilU, pilY1* (Simpson et al., 2000), were shown to have higher expression in grape fluid. It is reported that type I pili function in cell-cell aggregation and biofilm formation, and type IV pili are involved in twitching motility within the xylem of host plants (Meng et al., 2005). The expression of genes encoding type I and type IV pili was increased in grape fluid, likely contributing to an enhanced ability to aggregate, form biofilm, and move within the xylem, contributing to PD symptoms in grapevines. Since the expression of *secD* and *secG* was increased in grape fluid, the secretion of proteins by the type II, *sec*-dependent secretion system may enhance bacterial survival in grape. Genes involved in physiological metabolism under stress, such as heat shock protein genes *hspA* and *cplP,* and sulfoxide reductase gene *msrA*, cation tolerance protein *cutA*, and hypothetical protein PD0008, PD1741 and PD2031, were also more highly expressed in grape fluid. In contrast, the polygalacturonase gene, *pglA*, and hemolysin, had increased expression in citrus fluid. The data indicate that the chemical compounds or elements in xylem fluid of different plants differentially affect the regulation of virulence and the survival of *Xf* within xylem.

Table 1. Differential expressed genes of *Xf* in grape fluid comparing to citrus fluid

Gene ID[a]	NAME	HYPOTHETICAL FUNCTION	Grape/Citrus[b,c]	*P* Value[d]	Expression in Grape
PD1905	*xrvA*	Virulence regulator	1.8	1.2E-03	Higher
PD2040	*acvB*	Virulence protein	1.9	3.4E-03	Higher
PD0066	*hsq*	RNA-binding protein	2.7	1.5E-03	Higher
PD2068	*gacA*	Two-component regulator	1.8	3.0E-04	Higher
PD1276	*algU*	Transcriptional regulator	1.6	1.5E-03	Higher
PD0268	*agmR*	Transcriptional regulator (luxr/uhpa family)	1.9	5.2E-03	Higher
PD1738	*gcvR*	Transcriptional regulator	1.6	2.4E-03	Higher
PD1920	*colS*	Two-component system, sensor protein	1.6	1.4E-03	Higher
PD0019	*fim*T	Pre-pilin like leader sequence	2.0	1.8E-03	Higher
PD0062	-	Fimbrial subunit precursor	2.6	1.1E-03	Higher
PD0846	*pilI*	Pilus biogenesis protein	1.8	4.5E-03	Higher
PD1147	*pilT*	Twitching motility protein	1.6	3.2E-03	Higher
PD1148	*pilU*	Twitching motility protein	1.8	1.5E-03	Higher
PD1611	*pilY1*	Fimbrial assembly protein	2.3	2.1E-04	Higher
PD0312	-	Outer membrane protein precursor	2.5	1.2E-03	Higher
PD0731	*xadA*	Outer membrane protein	2.3	4.5E-03	Higher
PD0744	*hsf*	Surface protein	1.7	3.4E-03	Higher
PD0757	*pcp*	Peptidoglycan-associated outer membrane lipoprotein precursor	1.6	1.7E-03	Higher
PD0182	*secD*	Protein-export membrane protein	2.1	1.4E-03	Higher
PD0246	*secG*	Protein-export membrane protein	2.4	1.2E-03	Higher
PD1280	*hspA*	Low molecular weight heat shock protein	2.3	1.8E-03	Higher
PD0472	*clpP*	ATP-dependent Clp protease proteolytic subunit	1.6	1.1E-03	Higher
PD0859	*msrA*	Peptide methionine sulfoxide reductase	1.6	2.1E-03	Higher
PD1536	*cutA*	Periplasmic divalent cation tolerance protein	1.8	1.6E-03	Higher
PD1475	*ccmA*	Heme ABC transporter ATP-binding protein	1.8	4.1E-03	Higher
PD2031	-	Hypothetical protein	2.5	1.2E-03	Higher
PD0008	-	Hypothetical protein	1.8	1.2E-03	Higher
PD1741	-	Hypothetical protein	2.0	1.7E-04	Higher
PD1485	*pglA*	Polygalacturonase precursor	-1.7	1.8E-03	Lower
PD0143	-	Hemolysin III protein	-1.6	1.7E-03	Lower

[a]Genes were determined on the basis of *Xf* Temecula1 genomic sequences at the NCBI website (Simpson et al. 2000). [b]The hybridization signal intensity (mean of three hybridization replicates) obtained with grape was divided by that obtained with citrus to obtain grape/citrus ratio. [c]The normalized hybridization signals for those genes between grape and citrus are significantly different as analyzed by Student's *t* test ($P < 0.05$). [d]Genes having >1.5 or <0.66 final grape/citrus ratios were designated as having higher or lower expression in grape, respectively.

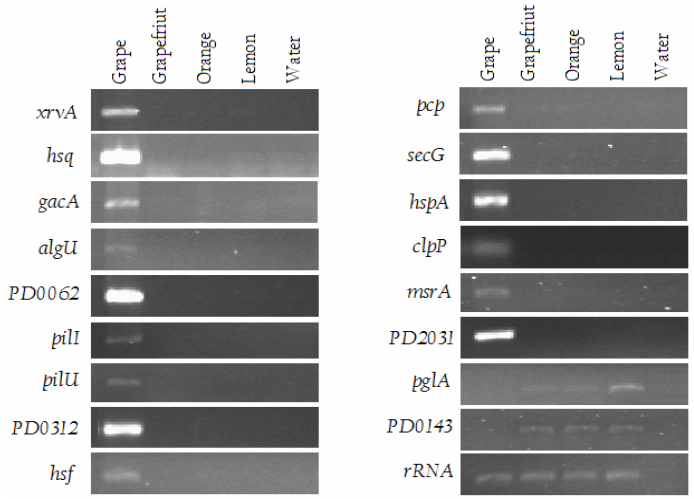

Figure 2. Reverse transcription polymerase chain reaction (RT-PCR) of differentially expressed genes of *Xf* in xylem fluid of citrus and grape. Water was the control.

CONCLUSIONS

Aggregation and biofilm formation of *Xf* were differentially influenced by the xylem fluid of citrus vs. grape. Grape fluid stimulated the expression of genes predicted to be involved in virulence, attachment, biofilm formation, and twitching motility of *Xf* within xylem, likely contributing to PD in grapevine. Citrus may be resistant or tolerant to the PD strain of *Xf*, in part, because citrus xylem fluid does not support the induction of a number of virulence genes, or has substances that repress expression. Identification of specific chemical components of citrus xylem fluid which influence expression of virulence genes in *Xf* is being assessed.

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FUNDING AGENCIES

Funding for this project was provided by the University of California Pierce's Disease Grant Program, the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and the University of California Agricultural Experiment Station.