Pierce's Disease Control Program

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Section 1: Vector Biology and Ecology

SUPPORT FOR THE SALIVATION-EGESTION HYPOTHESIS FOR *XYLELLA FASTIDIOSA* INOCULATION: BACTERIAL CELLS CAN PENETRATE VECTOR SALIVA IN XYLEM

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ABSTRACT
Research is underway to develop varieties of grape that are resistant to Pierce's disease (PD) caused by *Xylella fastidiosa* (*Xf*). PD has become economically important since introduction of an exotic vector, glassy-winged sharpshooter (GWSS). However, achieving resistance to vector acquisition and inoculation is hampered by lack of knowledge of the *Xf* inoculation mechanism, despite nearly 70 years of research on this topic. Recent work suggests that bacterial cells spread from the cibarium (the retention reservoir) into the precibarium, from which inoculation occurs. Backus's recently published salivation-egestion hypothesis for inoculation proposes that saliva is brought up into the precibarium, where it is mechanically swept across taste organs by the precibarial valve. This mechanical action, combined with the enzymatic action of the saliva, dislodges bacteria in the precibarium, which are then forcefully egested (expelled) out of the stylets in a mixture of plant fluid and saliva that enters a xylem cell. The present preliminary study tested whether *Xf* bacteria can penetrate existing, hardened GWSS salivary sheaths. GWSS were allowed to feed for 24 hours on a grape stem, depositing salivary sheaths. After the insects were removed, the stem was needle-inoculated with *Xf* in the feeding area, then immunoprobed for *Xf* and examined by confocal laser scanning microscopy. Results showed *Xf*-lined needle punctures into xylem cells. *Xf* bacteria were drawn into xylem vessels and traveled varying distances from the punctures. Bacteria encountered and penetrated into undamaged, existing sheath saliva in xylem cells. The apparent ability of bacteria to penetrate into hardened sheaths suggests that *Xf* should be able to migrate out of newly-secreted, soft saliva, thereby providing indirect support for the egestion-salivation hypothesis.

LAYPERSON SUMMARY
Although the inoculation mechanism of *Xylella fastidiosa* (*Xf*) has been sought by scientists for nearly 70 years, it is still not known exactly how *Xf* is injected into a healthy plant by feeding sharpshooter vectors. This lack of knowledge hampers development of a novel means of host plant resistance to Pierce's disease, i.e. selection of plant traits that reduce the likelihood of inoculation by the glassy-winged sharpshooter (GWSS). Similar resistance to vector inoculation has been successfully developed in several crops with insect-vectored plant pathogens. Backus's recently published salivation-egestion hypothesis for inoculation proposes that *Xf* bacterial cells are forcefully egested (expelled) out of the vector's mouth parts in a mixture of plant fluid and saliva secreted into xylem cells. To determine whether bacteria could move into or out of saliva, GWSS were allowed to feed for 24 hours on a grape stem, depositing saliva in xylem cells. After the insects were removed, the stem was needle-inoculated with *Xf* near the feeding sites, then antibody-stained for *Xf* and examined by confocal microscopy. Results showed that *Xf* entered xylem cells and penetrated into sheath saliva already present in xylem. Bacterial penetration into such hardened saliva suggests that *Xf* should be able to penetrate out of newly-secreted, soft saliva. This finding provides indirect support for the egestion-salivation hypothesis.

INTRODUCTION
Despite nearly 70 years of research, it is still not known exactly how *Xylella fastidiosa (Xf)* is inoculated by feeding sharpshooter vectors. This lack of knowledge hampers development of a novel means of host plant resistance to Pierce's disease (PD), i.e. selection of plant traits that reduce the likelihood of inoculation by the glassy-winged sharpshooter (GWSS). Recent research (Backus and Morgan *ms. submitted*) demonstrates that bacterial cells spread/grow from part of the foregut (the cibarium, the retention reservoir) into another part (the precibarium) from which inoculation occurs. The egestion-salivation hypothesis for inoculation (Backus et al. 2009) proposes that a mixture of plant fluid plus newly secreted saliva is brought up into the precibarium, where it is mechanically swept across taste organs (Backus and McLean 1983, 1985) by the precibarial valve. This mechanical action, combined with the putative enzymatic action of β-1,4-glucanase in the saliva (Backus and Labavitch 2007), dislodges bacteria in the precibarium, which are then forcefully egested (expelled) out the stylets in saliva that is injected into a xylem cell just before fluid sucking (ingestion) commences. Research is underway to definitively test the egestion-salivation hypothesis. The present study was designed to finalize protocols for spectral separation of *Xf* and GWSS saliva, by studying the interaction between GWSS saliva and *Xf in planta*.

OBJECTIVES

1. Microscopically determine whether *Xf* cells can penetrate into existing hardened salivary sheaths in xylem.

RESULTS AND DISCUSSION

Eight GWSS were restricted to a marked, 5 cm-long area of grape (*V. vinifera* cv. 'Chardonnay') stem for 24 hrs. Approximately one hour after removal of cage and insects, a total of 200 µl of *Xf* culture (strain 'Temecula') were needle-inoculated within the marked, insect-probed area of stem. Within 30 – 60 minutes after inoculation, 15 ca. 3-mm blocks of stem tissue were excised. Blocks were microwave-fixed in paraformaldehyde, embedded in paraffin, sectioned at 10 µm, and mounted on glass slides. Wax sections were examined using epifluoresent light and a GFP filter cube using a Leica DM5000 compound light microscope (Deerfield, IL), which caused needle punctures, hardened salivary sheaths, and xylem cells to fluoresce against a dark background. Accumulations inside xylem cells that resembled bacterial biofilm also were slightly visible. Ten salivary sheaths from all over the stem were identified as lying close to needle punctures, so that *Xf* likely would be nearby. Eight of the ten sheaths were undamaged and intact; two (described below) had been punctured by the needle and were slightly damaged. Slides with sections containing these sheaths were immunoprobed for *Xf* using rabbit primary antibody (Agdia, Elkhart, IN) and goat secondary antibody with an Alexa Fluor 647 flourochrome (Invitrogen, Carlsbad, CA). BSA and secondary antibody controls were run with the test samples to determine levels of non-specific binding. Tissues were examined and imaged using a Leica SP2 AOBS confocal laser scanning microscope, and later edited in Adobe PhotoShop v. CS2 (Mountain View, CA) and Microsoft PowerPoint (Edmonds, WA).

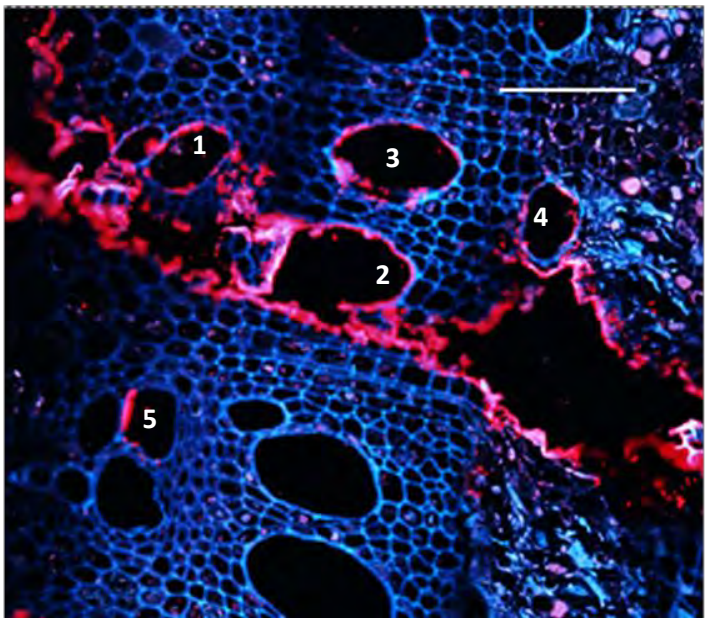

Figure 1. Needle puncture into grape stem. Red is immunostained Xf, pink is non-specific binding to certain vesicles in parenchyma cells, blue is autofluorescence of cell walls. Needle puncture directly into xylem cells (1 and 2) caused extensive cell wall damage, and introduced bacteria into xylem. Nearby, undamaged xylem cells (3, 4 and 5) were inoculated with *Xf* by other needle punctures, above or below this section. Scale bar 25 µm.

Needle punctures into grape stem caused extensive cellular damage, leaving large gaps that were lined with red-stained *Xf* deposits (**Figure 1**). Xylem cells in the direct path of needle-punctures had moderate to severe rupture of cell walls (**Figure 1,** 1 and 2). This process introduced bacteria into the xylem transpiration stream. Either an air embolism caused by cavitation or the intact transpiration stream would rapidly pull bacteria away from the site of puncture. Thus, intact xylem cells not continuous with broken xylem cells within one section often contained bacteria. Inoculation in this manner is demonstrated in cells 3, 4, and 5 (**Figure 1**), which are separate from the needle-punctured gap, yet lined with bacteria. These cells were likely inoculated by other needle punctures many sections above or below the imaged section.

GWSS salivary sheaths resembled those seen in other studies (**Figure 2a**). A salivary flange on the surface of the stem was confluent with the salivary sheath inside the stem. The hollow lumen of the

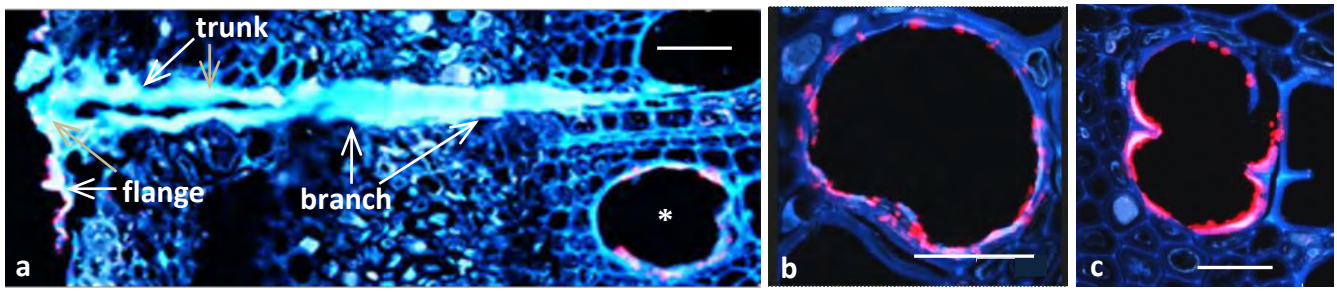

Figure 2. a. GWSS salivary sheath whose tip entered a xylem cell that did not contain bacteria (upper right, with scale bar). Sheath saliva is bright bluish-white due to stronger autofluorescence than cell walls. All other colors same as Figure 1. Intact, inoculated xylem cell (*, also imaged in part b) near the sheath. **b.** Close-up of same inoculated xylem cell (*) from part a, but in an adjoining section. **c.** Close-up of two intact, inoculated, immature metaxylem cells near the sheath. Scale bars 25 µm.

sheath trunk was visible as the sheath narrowed, indicating the furthest extension of the mandibular stylets. One or more branches extended from the trunk into the stem interior (**Figure 2a**) (Backus et al. 2005). Pictured is one of the four salivary sheaths that were immunoprobed but did not penetrate xylem cells into which *Xf* bacteria were later inoculated. However, inoculated xylem cells were nearby (**Figure 2a, b, c**).

Close-ups of intact, inoculated xylem cells showed that *Xf* bacteria not only lined the walls of the cells, but also penetrated the cell walls to varying depths (**Figure 2b**), sometimes the full width of the wall (**Figure 2c**). Such bacterial penetration strongly suggests that *Xf* can loosen the microfibrils of cellulose and other polysaccharides that form the secondary cell walls of xylem cells. It is known that the *Xf* genome codes for several cell wall-loosening enzymes such as β-1,4 endoglucanases, β-endoxylanases and β-xylosidases (three types of cellulases) and polygalacturonase (a pectinase) (Roper et al. 2007). It is also known that bacterial movement between xylem cells, establishing a systemic infection, occurs primarily through so-called pit membranes (actually primary cell wall containing pectin and cellulose, inside pits in the secondary cell wall) (Thorne et al. 2006). The pectin polymer lattice determines the pore size in pit membranes, explaining why the polygalacturonase gene has been found to be critical for establishment of systemic infection (Kirkpatrick et al. 2006, Roper et al. 2007). Nonetheless, our images suggest that (given enough time) individual or small numbers of bacteria also might be able to penetrate directly through the secondary cell walls of xylem, perhaps via action their secreted cellulases.

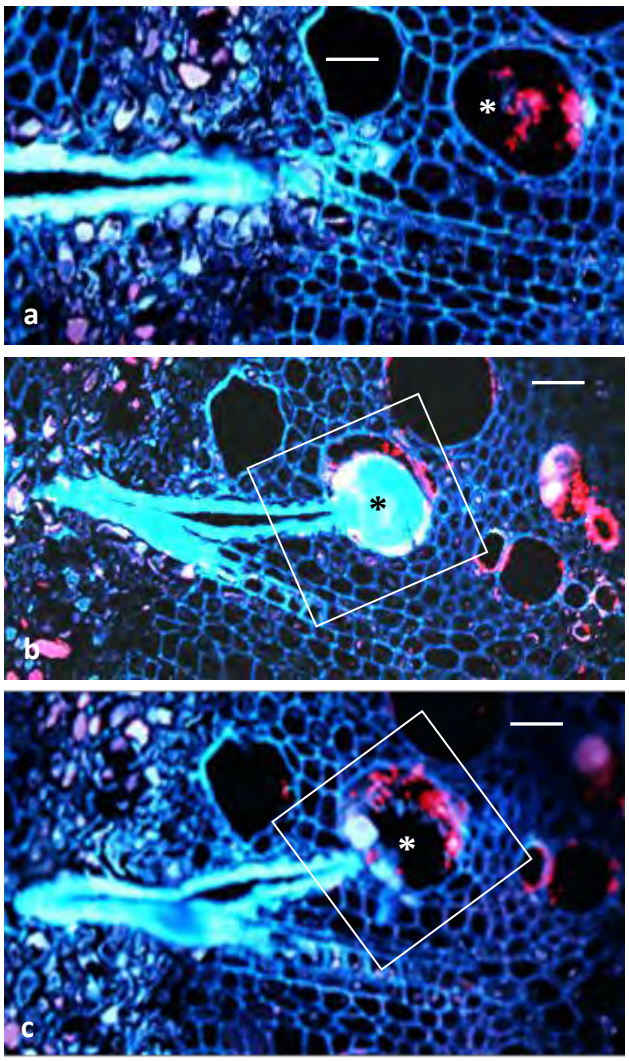

Figure 3. Three contiguous sections containing a salivary sheath with two branches. **a.** Side section of all but the tip of main branch, approaching a xylem cell with large amount of red-stained *Xf* (*). **b.** Section of the central part of main branch, showing saliva bolus inside same xylem cell (*). **c.** Other side section of main branch, showing smaller amount of saliva, more Xf. Colors same as Figure 2. Scale bars 25 µm.

Six of the ten salivary sheaths entered xylem cells that were later inoculated with *Xf*. Four of these were not close to needle punctures, so the intersected xylem cells were inoculated from distant needle punctures. Images from one representative sheath are displayed in **Figure 3**. This sheath was notable because it had a bolus of sheath saliva that was injected into its terminal xylem cell, probably after a bout of ingestion (sucking) (Backus et al. 2005) (**Figure 3b)**. It is common for GWSS sheaths to terminate in a bolus of saliva, but usually the bolus has been pulled some distance away from the site of stylet penetration into the xylem cell (Backus and Labavitch 2007). The sheath's terminal xylem cell has a large accumulation of red-staining *Xf* bacteria (**Figure 3a, c**) as do several other, nearby xylem cells (**Figure 3b**, right side). In the cell with the saliva bolus, most bacteria are clearly outside the sheath, in fact, located directly surrounding the saliva bolus. However, a small number are inside the saliva near the edge of the bolus (**Figure 4a)**.

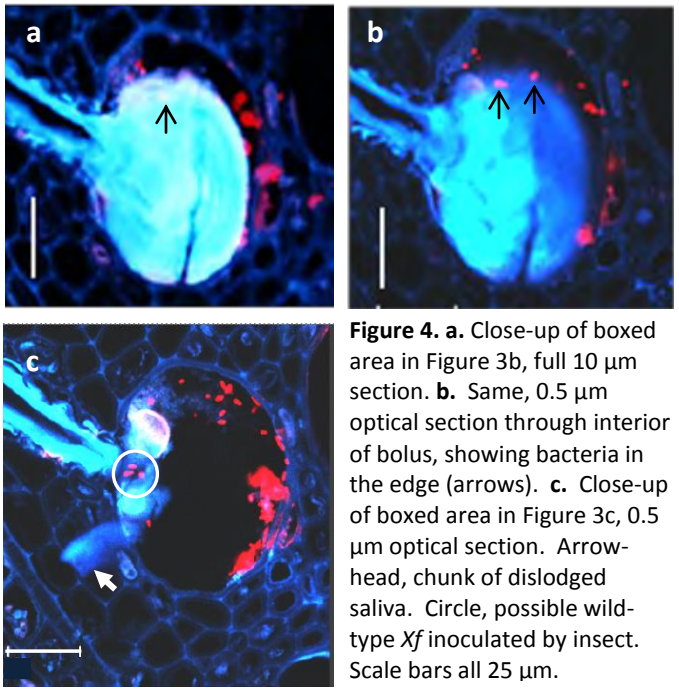

Figure 4. a. Close-up of boxed area in Figure 3b, full 10 µm section. **b.** Same, 0.5 µm optical section through interior of bolus, showing bacteria in the edge (arrows). **c.** Close-up of boxed area in Figure 3c, 0.5 µm optical section. Arrowhead, chunk of dislodged saliva. Circle, possible wild-type *Xf* inoculated by insect. Scale bars all 25 µm.

The large saliva bolus in the latter images has a crack running through its center (**Figure 4a, b**, previous page). This disruption of the hardened saliva may have occurred from pressure on surrounding tissues during needle puncturing, even though there was no apparent damage to cell walls. Although large accumulations of bacteria were transported into this xylem cell, none were found near or inside the crack in the salivary bolus. If bacteria had entered the saliva by mechanical damage during puncturing, we would expect them to be near or in this crack. This suggests that the few bacteria inside the saliva entered by some other means, perhaps via enzyme-mediated dissolving and penetrating. Because salivary sheaths of sharpshooters and other hemipterans are primarily lipoproteinaceous (Miles 1999, Alhaddad et al. *ms. submitted*), this suggests action of a protease.

The salivary sheath in **Figures 3** and **4** was also notable because three discrete bacterial cells were located at the apparent entry point of the stylet tips, on one side of the saliva bolus (**Figure 4c**, previous page, circle). It is possible that these bacteria could have penetrated into the saliva from outside. However, it is also possible, given their position compared with that of other bacteria (surrounding the salivary bolus, on the periphery of the cell) that these bacteria were wild-type *Xf* from the insect, inoculated during probing of the xylem cell. The immunostain would bind equally to any *Xf*, i.e. both naturally insect-inoculated as well as needle-inoculated GFP *Xf*. The innoculativity status of the colony GWSS used for this test was not known, however, some *Xf* contamination is likely. Clearly, however, the vast majority of bacteria found in xylem were needle-inoculated, because bacterial accumulations were only found in regions of the stem that had been punctured.

Of the six sheaths that entered xylem cells later inoculated with *Xf*, two were directly intersected by needle punctures at some point along their length. Yet other portions of both sheaths also entered xylem cells inoculated by a distant puncture. One of these two salivary sheaths is pictured in **Figures 5** and **6**. **Figure 5** shows a section that nicked the side of this two-branched sheath, thus only portions of sheath material are visible. But they clearly show the trajectory of the two branches (**Figure 5**, line 1), and how they were both intersected by a needle puncture (**Figure 5**, line 2) at nearly a right angle to the sheath trunk.

The structure of the left-hand sheath branch (as viewed in the image, **Figure 6, next page**, which shows the main section that contains most of the salivary sheath) supports the following interpretation. During feeding, the insect's stylets entered and salivated into a xylem cell (labeled by an asterisk in **Figure 5**). Saliva is indicated by the blue-white ring in the xylem cell (**Figure 6, next page**). However, the insect apparently abandoned that cell, because the sheath branch was extended further. This behavior occurs commonly during sharpshooter feeding (Backus et al. 2005, Backus et al. 2009). The upper (as viewed, **Figure 5** and **6a**) extension of the left branch was later intersected by a needle puncture and associated *Xf* bacteria (red).

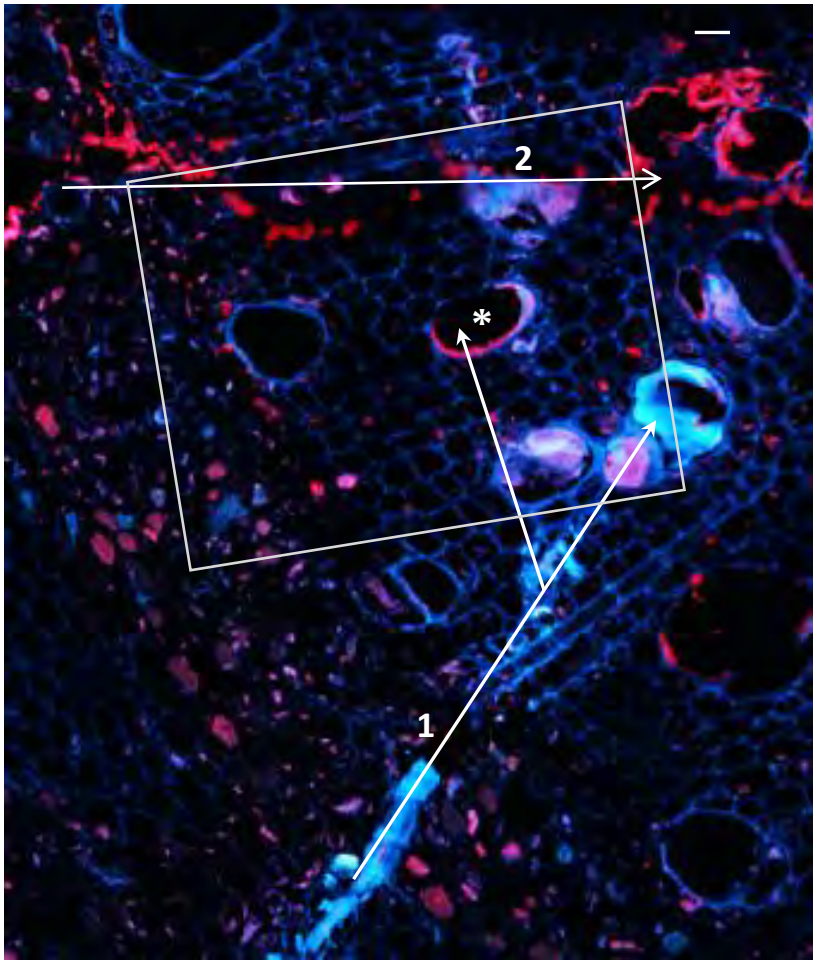

Figure 5. Side-section, showing portions of a large, two-branched salivary sheath (line 1) that was intersected by an *Xf* inoculation puncture (line 2). Box shows outline of similar image in Figure 6. Cell with asterisk is same cell as the one boxed in Figure 6. Colors as in Figure 2. Scale bar 25 µm.

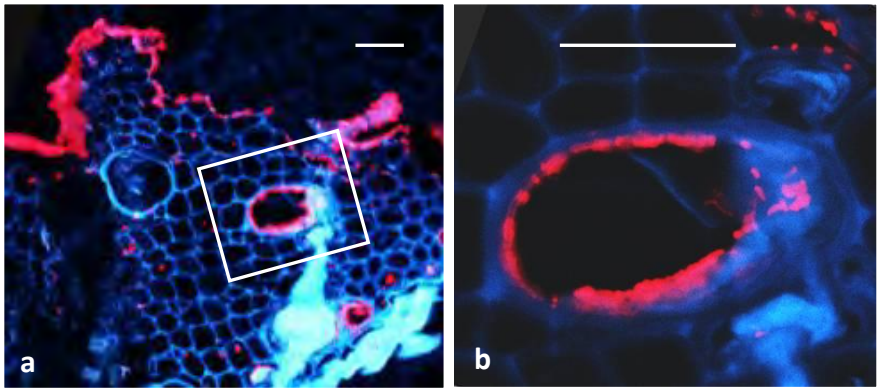

Figure 6. a. Two branches of the salivary sheath in Fig. 5, the left branch of which was intersected by a needle puncture and associated *Xf* bacteria (red). Colors as in Figure 2. Full 10 µm-thick section. **b.** 0.5 µm optical section through interior of physical section boxed in part a. Scale bars 25 µm.

In contrast, the lower portion of the left branch that had entered the abandoned xylem cell was undamaged by the needle puncture (**Figure 6a, b**). This xylem cell was inoculated by a distant puncture, not the nearby one, because its cell wall was unbroken and bacteria were not connected to the puncture in this or any adjoining section. A thick bacterial biofilm developed in this cell during the 30 – 60 minutes after needle-inoculation. It formed a circular, near-continuous lining of the cell wall that was also seen in adjoining sections (**Figure 5**). Optical sectioning through the sheath material in the xylem cell shows bacteria inside the saliva, in the same, near-continuous configuration as bacteria outside the saliva (**Figure 6b**).

In summary, the following evidence supports that *Xf* bacteria can penetrate into the portion of hardened saliva that is left by feeding GWSS inside xylem cells. 1) All six of the existing, hardened salivary sheaths that were later encountered by *Xf* needle-inoculated into xylem were found to contain small to large amounts of bacteria. 2) The *Xf* cells were embedded only in the portion of the sheath found inside the inoculated xylem cell. Therefore, the hardened saliva was not a conduit through which later-inoculated *Xf* could migrate into any plant cell. 3) *Xf* probably was not forced into salivary sheaths by the mechanical action of needle-puncturing, because: a) *Xf* was often found in undamaged xylem cells or sheaths, and b) in one sheath that was slightly torn, bacteria did not enter the sheath through the tear, but instead were found elsewhere in the sheath.

It is not known whether *Xf* penetration of GWSS sheath saliva occurs via: 1) hydrostatic pressure from transpiration or 2) cavitation propelling the bacteria forcefully into the saliva, or 3) secretion of enzymes, allowing bacteria to dissolve their way into the saliva. In any case, our findings suggest that even hardened saliva is not perfectly solid, but may be composed of a network of microfibrils with some porosity, similar to sclerotized, proteinaceous insect cuticle. Bacterial penetration into hardened saliva also suggests that *Xf* should be able to either actively or passively move out of newly-secreted, soft saliva. This finding provides indirect support for the egestion-salivation hypothesis for vector inoculation of *Xf*.

CONCLUSIONS

Results from this study show that *Xf* bacteria in grape xylem cells can penetrate GWSS saliva deposited therein, even when the saliva is 1 – 24 hours old and completely hardened. This supports that sheath saliva is a porous network of microfibrils similar to insect cuticle that, at minimum, does not present an impediment to passive bacterial movement via the transpiration flow. Additionally, it is possible that *Xf* bacteria can actively penetrate into or out of GWSS saliva, perhaps via secretion of bacterial proteases. Results of this test support the egestion-salivation hypothesis for the *Xf* inoculation mechanism, which proposes that bacteria are carried into a xylem cell by a mixture of plant fluid and saliva that is ejected into the xylem before sucking (ingestion) commences. Bacteria encased within secreted saliva can probably penetrate out into the xylem transpiration flow. Tests to definitively prove this inoculation mechanism are underway. If proven, this mechanism will support use of salivary antagonists for novel genetic approaches to PD management. Also, because both egestion and salivation into xylem have been correlated with a unique Electrical Penetration Graph (EPG) waveform (Backus et al. 2009), definitive proof of the egestion-salivation hypothesis will facilitate rapid testing of transgenic or classically bred grape plants, or plants inoculated with a benign strain of *Xf*, to determine their effects on vector inoculation behavior.

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CELL CULTURE BASED PRODUCTION OF *HOMALODISCA COAGULATA VIRUS 01* (HOCV-01): TOWARDS A GLASSY-WINGED SHARPSHOOTER BIOLOGICAL CONTROL SYSTEM

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Reporting Period: The results reported here are from work conducted November 2008 through October 2010.

ABSTRACT
The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is an invasive pest and important vector of *Xylella fastidiosa (Xf)*, xylem-limited bacteria that causes Pierce's disease in grapevine as well as other agricultural diseases. The primary method of managing the spread of *Xf* is controlling its insect vector populations. Methods such as chemical control are not insect specific and lead to problems such as residue contamination, injury to non-target organisms, and insecticide resistance. Identifying agents that can impact vector populations is the goal of a biological control strategy. In this study, we have extracted *HoCV-01* from populations of GWSS collected in Texas. GWSS primary cell cultures were produced then inoculated with the viral extract. The introduced virus killed all treated cell cultures within 5 days. Increase in virus titer in treated cell culture was monitored over time by virus-specific PCR. Increased amounts of *HoCV* infection may lead to weakened populations of GWSS.

LAYPERSON SUMMARY
The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is the most economically important insect with respect to the transmission of *Xylella fastidiosa*, the causal agent of Pierce's disease. Therefore, control of this insect is of paramount importance to the management of the disease. While insecticides have been used successfully to reduce the economic impact of this disease system, alternate methods of population insect control are needed to continue management in the future. Biological control offers alternatives to chemical control that can be effective in negatively impacting insect population without harmful environmental effects or concern for insecticide resistance. In this work, we introduced the extracted virus to GWSS cell cultures as a tool for biological control. While this virus, at natural doses, does not shown to cause significant acute mortality in live insects, it may reduce the fitness of insects to a point where other control methods would be more effective.

INTRODUCTION
The glassy-winged sharpshooter (GWSS) is the major vector of *Xylella fastidiosa (Xf)* in the southern USA (Adlerz 1980; Blua et al., 1999). The plant pathogenic bacterium, *Xf*, has caused economic losses to several agricultural industries in North America and is associated with many plant diseases such as Pierce's disease (PD), and oleander leaf scorch. PD of grapevine has become a well recognized *Xylella*-related disease; the vector profile is well known and the epidemiology of the disease is well documented (Hopkins et al., 2002). The introduction of GWSS into new areas is directly related to increased occurrence of PD in vineyards (Perring et al., 2001). Therefore, the management of PD depends heavily on the ability to control its vectors, especially GWSS. Methods of vector manipulation such as chemical control with the use of insecticides are not target specific and lead to problems such as residue contamination, injury to non-target organisms, and insecticide resistance. The search for more benevolent pest management strategies has led to the use of biocontrol agents such as fungi and parasitoids. However, by utilizing viruses that currently reside in GWSS populations, a viral bio-control that is even more precise may be developed (Hunnicutt et al., 2006).

Homalodisca coagulata virus 01 (HoCV-01) is a member of the genus *Cripavirus* and family *Dicistroviridae (*Hunnicutt et al., 2006). It is a novel virus that harbors pathogenic potential with regard to GWSS. The focus of this study was to extract whole *HoCV-01* from GWSS then inoculate GWSS cell cultures with the extracted *HoCV-01* in hopes that the cell culture would act as a medium for amplification of the virus.

OBJECTIVES
1. Inoculate the cell culture with *HoCV-01*.
2. Use cell culture as a medium to produce large volumes of *HoCV-01* for extraction.

RESULTS AND DISCUSSION
Cell Culture of GWSS. The *Hv* cells were cultured from eggs which were ready to hatch, eyespots were visible. In a sterile hood, eggs were surface sterilized with 70% Ethanol for 15 min. The eggs were rinsed twice with syringe filtered (0.22 um) sterilized water. Then ~10 eggs were placed onto a sterile watch glass, along with 20 uL of medium. The eggs were then crushed with a single tap from a sterile, rounded tip, glass rod. More medium was added to suspend cells and tissues, which were then dispensed into a 24 well, multi-well tissue culture plate (Costar®, Corning, NY). Culture media containing

antibiotics Gentamicin (10,000 U/mL) (Invitrogen, Carlsbad, CA) was added to 1.5 ml total volume in each well and incubated at 23°C.

The medium is classified as modified Wayne Hunter-2, WH2, Honey bee cell culture medium (Hunter 2010). Schneider's Insect Medium (Sigma), 0.06 Histidine solution, Hanks' Salts, Medium CMRL 1066 (Invitrogen), Gentamicin, Insect medium supplement (Cat. No. 17267, 10X, 500 ml, Sigma), Fetal Bovine Serum (Invitrogen).

Within 48 hours cells were observed attached to the substrate. Both fibroblast and monolayers developed.

Whole Virus Extraction. Infected GWSS bodies were placed in a mortar and pestle and homogenized in 100mL of phosphate buffer containing 0.02mg DETCA. The homogenate was then transferred to 50mL centrifuge tubes and centrifuged at 1600rpm for 20 minutes in an Eppendorf 5804R Centrifuge (Eppendorf, Hamburg, Germany). The resulting supernatant was split into two ultra-centrifuge tubes, combined with more phosphate buffer with DETCA, vortexed, and ultra-centrifuged at 22,000rpm for 16 hours in a Sorvall® RC-5B Refrigerated Superspeed Centrifuge (DuPont Instruments, Wilmington, DE). Following ultra-centrifugation, the supernatant was discarded, and the pellet was dissolved with 5mL phosphate buffer with 0.4% Na-deoxycholic acid and 4% Brij 52. The resulting solution was centrifuged and 1600rpm for 15 minutes, passed through a 0.45µm filter, and collected into large Eppindorf tubes. The unrefined *HoCV-01* solution was placed in a dialysis membrane, placed in a large beaker containing a stir-bar and ddH20, and placed in a refrigerator at 4°C. The ddH20 was changed out ever five-six hours until a white precipitate could be seen in the dialysis membrane. The purified *HoCV-01* solution was collected into micro-centrifuge tubes and stored at -80°C.

Cell Culture Inoculation with HoCV-01. The *Hv* culture plates were labeled V (Virus), and C (Control). Plate V was inoculated with virus (5.0 uL/well). For the next five days samples (100 uL/day) were taken from each well of plates (C,V). After virus inoculation, the plates were examined under an inverted microscope (Olympus DP30BW, IX2-SP, IX71) at 40X everyday for five days (**Figure 2**).

RNA Isolation and PCR. Total RNA was isolated from the samples collected over the five day experiment (Plates C,V) Qiagen RNeasy kit (Qiagen™, Germantown, MD); according to manufacturer's protocol. The samples (2.0 uL) were analyzed on a ND-1000 Spectrophotometer (**Figure 1**).

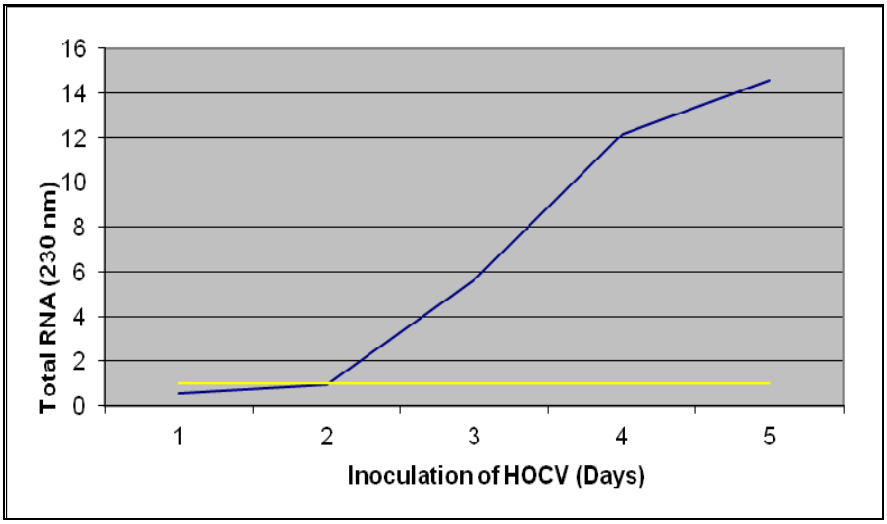

Figure 1. Total RNA increased over five days of viral inoculation (Blue). Total RNA remained the same over five days in the control plate (yellow).

Each 2µL *Hv* RNA sample was combined with 12.5µL 2X Reaction Mix (Invitrogen Molecular Probes™, Eugene, OR), 0.5µL forward (*HoCV-01* specific) primer, 0.5µL reverse (*HoCV-01* specific) primer, 0.5µL Platinum® Taq DNA Polymerase (Invitrogen Molecular Probes™, Eugene, OR), and 9.0µL DEPC H2O in solution. Samples were then subjected to a Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The thermal profile used was: (A)pre-denaturation: 50ºC for 25 min, 94 ºC for 2 min. (B)PCR amplification: 40 cycles 94 ºC for 15 sec, 52 ºC for 30 sec, 72 ºC for 30 sec. (C) Final extension: .72 ºC for 7 min. After the PCR, 7.0µL of each *Hv* DNA sample was subjected to gel electrophoresis using 7.0µL ladder, 2µL loading dye per sample, and a 1% agarose gel containing 100mL TAE buffer and 1g agarose gel. Gels were subjected to 100V and 400A for 50 minutes and observed under Ultraviolet light in a Bio Doc-It Imaging System (Cole-Parmer™, Hanwell, London) (**Figure 2**).

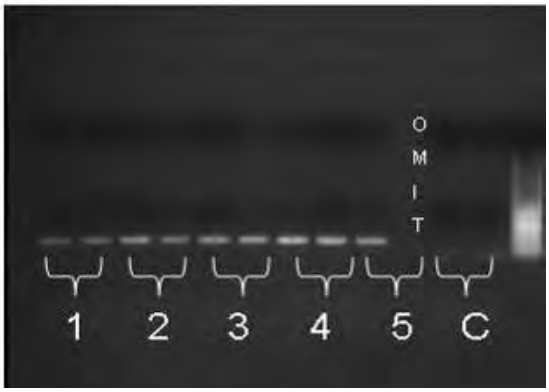

Figure 2. Increased concentration of *HoCV-01* produced from Hv cell cultures over five days.

The presence of *HoCV-01* was confirmed in the extract; since this is a general virus extraction protocol, it is possible that other known or unknown viral particles were extracted during the process. Previous studies found a new photoreovirus (Stenger et al. 2009) and phytoreovirus-like sequences (Katsar et al. 2007) present in GWSS. We hope to confirm all viral constituents that may be in our extract. We have successfully replicated *HoCV-01* through *Hv* cell culture. The concentration of *HoCV-01* increased throughout the experiment (**Figures 1 and 2**). In all trials, cell culture death was observed within five days **(Figure 3**). With these results we plan to scale up production of *HoCV-01*.

CONCLUSIONS

The presence of *HoCV-01* in populations of GWSS collected in Texas is vital in developing an ideal viral biocontrol and pest management strategy. We now have a method for mass *HoCV-01* production through the successful use of cell culture. In the future, we plan to extract the virus from the cell culture plates in hopes that in combination with other insecticides will increase the mortality rate of GWSS populations. The results of this experiment are crucial in further understanding the insect vector,GWSS. The management of PD depends heavily upon the ability to control its insect vectors.

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FUNDING AGENCIES

Funding for this project was provided by the Texas Pierce's Disease Research and Education Program, and the USDA Animal and Plant Health Inspection Service.

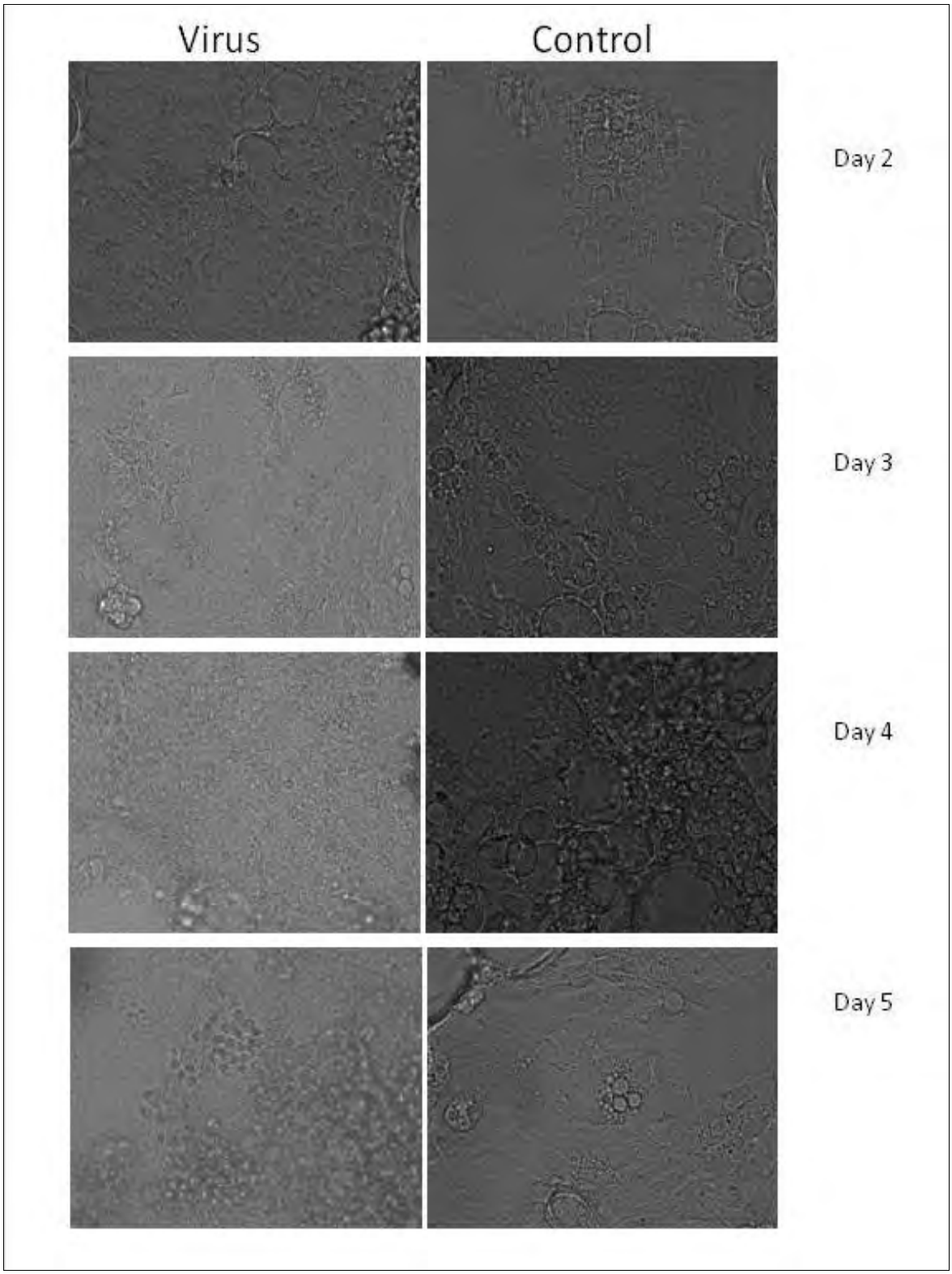

Figure 3. Shows the comparison of cells over the five day experiment assuming that day 1 was similar to day 2 of the control. Day 1 – all cells look healthy after initial inoculation (C,V). Day 2 – both plates look fairly similar, healthy (C,V). Day 3 – Low amounts of movement in the medium and within cells (V). Cells are healthy (C). Day 4 – Medium has turned a light shade of pink, high movement throughout the medium and cells, cells are looking unhealthy in appearance (V). Cells are healthy (C). Day 5 – Medium has tuned bright pink, cells are very unhealthy in appearance, assumed dead (V). Cells are healthy (C).
Note: Gentamicin was used in the medium allowing for little to no bacterial contamination.

HEMOLYMPH-ASSOCIATED SYMBIONTS: IDENTIFICATION OF *DELFTIA* SP. IN GLASSY-WINGED SHARPSHOOTERS AND INVESTIGATION INTO THEIR PUTATIVE FUNCTION

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ABSTRACT

The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) feeds on a wide variety of host plants including grapes, oleander, and citrus and is the primary vector of *Xylella fastidiosa (Xf)*, the causal agent of Pierce's disease of grapevine, oleander leaf scorch, and citrus variegated chlorosis. Additional bacterial species have been identified within GWSS which may contribute to the insect's survival and ability to adapt to the environment. *Delftia sp*., a gram negative bacterium which belongs to rRNA superfamily III or the β subclass of the *Proteobacteria*, was detected only in the insect's hemolymph. Therefore, in this study, *Delftia sp*. associated with GWSS hemolymph was further identified through direct sequencing, and the relationship between this symbiont and its host was investigated. *Delftia* is a D-amino acid amidase-producing bacterium. D-amino amidases are increasingly being recognized to be important catalysts in the stereospecific production of D-amino acids. *Delftia* may be found in the hemocoel of the GWSS to hydrolyze D-amino acid amides to yield D-amino acid and ammonia which can perform as the insect's chiral building blocks.

LAYPERSON SUMMARY

The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is a xylem feeding insect pest that has the ability to transmit the bacterium, *Xylella fastidiosa (Xf)*. This bacterium is the causal agent of Pierce's disease in the grapevine which has had a detrimental impact on the wine and grape industries. Among extensive studies done on *Xf* , many other bacteria that reside in GWSS have been identified. In a recent survey where bacterial extractions were taken from different parts of the insect's body, *Delftia* sp. was the only symbiont found only in the hemolymph (Bextine et al., 2009). Identification and isolation of *Delftia* sp. from GWSS hemolymph preludes investigative techniques that will describe the symbiotic role of this particular bacterium with its host.

INTRODUCTION

The bacterium, *Xylella fastidiosa (Xf)*, is the causal agent of several economically important plant diseases. This insect transmitted bacterium has a well understood relationship with its insect vector, the glassy-winged sharpshooter (GWSS). Many other bacteria that survive inside GWSS have been identified through next generation high throughput sequencing and these symbiotic relationships have not been elucidated (Bextine et al., 2009). *Delftia* sp. was found exclusively in the sharpshooter hemolymph and not in the insect's hemolymph. To confirm *Delftia* sp. presence in the insect's hemolymph, the bacterium was isolated in culture media and a genus-specific primer set [Delf63F (5' TAACAGGTCTTCGGACGC 3') and Delf440R (5' CCCCTGTATTAGAAGAAGCT 3')] was used to confirm presence.

Delftia sp. is a known D-amino acid amidase-producing bacterium. Investigating the symbiotic role of this bacterium in the hemolymph can be hypothesized based on this known amidase formation. In one study on amidase production, soil samples were used and based on morphology, physiological traits and 16S rRNA sequence analysis, *Delftia tsuruhatensis* was identified as the only bacterium capable R-enantioselective degradation of 2, 2-dimethylcyclopropanecarboxamide. Two other strains in the genus Delftia have been reported to produce R-stereospecific amidase: *Comamonas acidovorans* and *Delftia* acidovorans (Zheng et al., 2007). In another soil sample study, bacteria were monitored for amidase production and the strain exhibiting the strongest activity was identified as *Delftia acidovorans* strain 16. This strain produced intracellular D-amino acid amidase constitutively (Hongpattarakere et al., 2005). In order to investigate characteristics of the enzyme, amidase, was produced intracellularly, Hongpattarakere et al. (2005) analyzed its activity which was only seen in the supernatant of sonicated cell-free extract and no activity was seen from in the supernatant of culture broth. Obviously, amidase is a major enzyme produced by *Delftia* sp. The relationship between *Delftia* sp. and GWSS hemolymph is unknown Additionally, the possible role of amidase in the insects hemolymph warrents further investigation.

OBJECTIVES

1. Identify *Delftia* sp. in hemolymph.
2. Investigate relationship between *Delftia* sp. and its host.

RESULTS AND DISCUSSION

16S pyrosequencing based upon the bTEFAP methodology (Dowd et al., 2008a; Dowd et al., 2008b) optimized for theTitanium pyrosequencing platform (Roche, Indianapolis, IN) was used to identify bacterial species in GWSS whole

bodies, hemolymph, and alimentary canal. Seventeen orders (**Figure 1**) and at least 38 genera (**Figure 2**) of bacteria were detected in the insect's hemolymph. In a replicated study; sequences were taken from separately prepared extracts of hemolymph suspended in 1X PBS. The sequences were approximately 500 bp (370-820 bp) and were compared to NCBI's basic local alignment search tool (BLAST) for homologies. Some of the shorter sequences aligned to multiple genera and were placed in a separate category called "Other" because it was not clear which identification was appropriate.

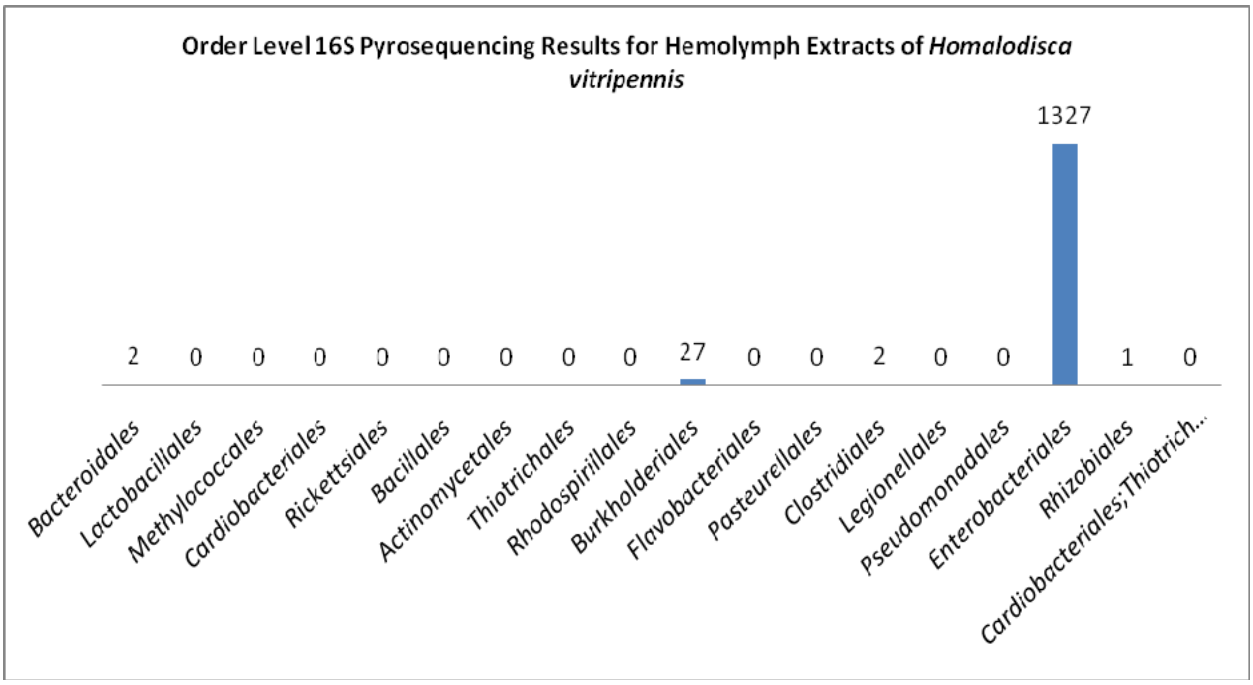

Figure 1. Order level sequencing results for the hemolymph of the GWSS. Larger numbers of sequences related to Enterobacteriales were recovered as well as Bacteroidales and Burkholderiales.

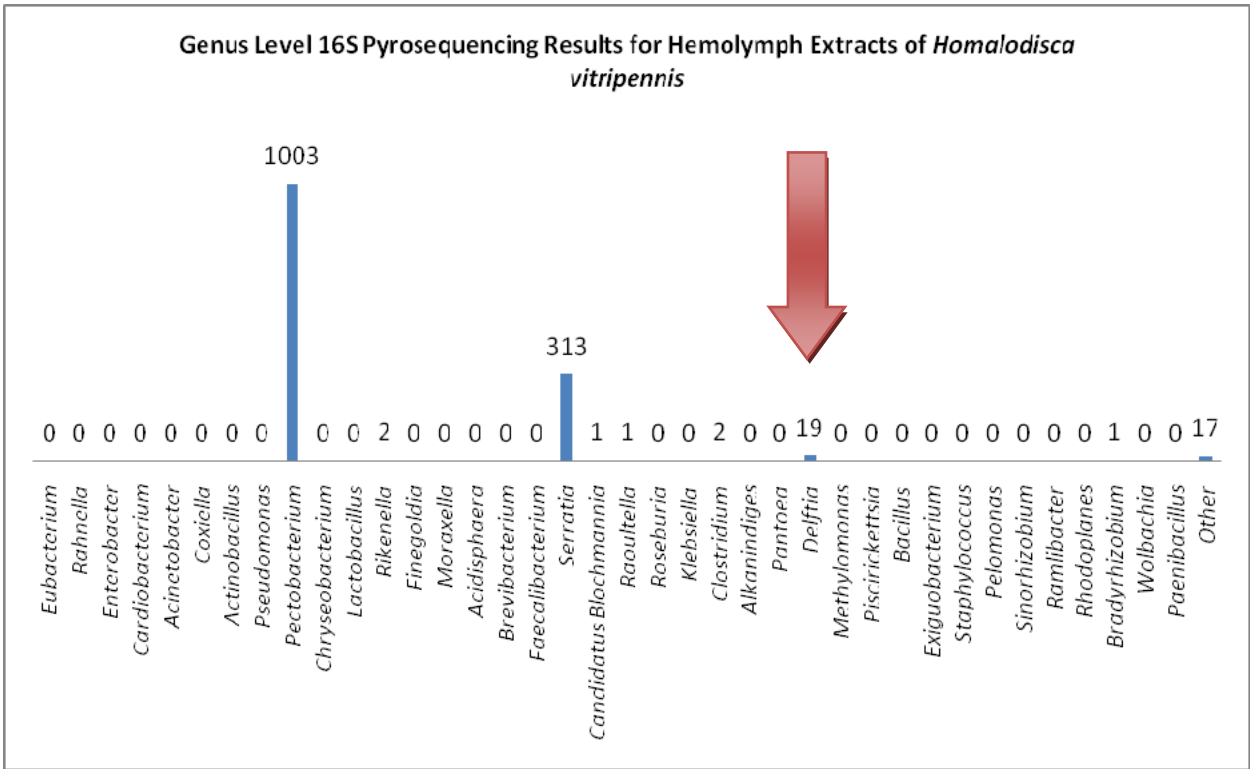

Figure 2. Genus level sequencing results for the hemolymph of the GWSS. Large numbers of *Pectobacterium* and *Serratia* were recovered. Some *Delftia* (formerly *Pseudomonas*) and other non-specific identifications were made. Note that no sequences from *Wolbachia*, an intracellular symbiont, were recovered.

Delftia sp. sequences were recovered in all GWSS hemolymph samples and in whole insect preparations. This genus was never detected in the insect's alimentary canal. *Delftia* sp. is known to be D-amino acid amidase-producing bacteria. An interesting finding was that in contrast to other amidases inhibited by low concentrations (0.5e10 mM) of urea, amidase from *D. tsuruhatensis* ZJB-05174 was not significantly inhibited by urea even at a high concentration (500 mM). (Zheng et al., 2007). Because inhibition of amidase activity was reported to involve binding of urea to the active site of the enzyme, these results suggested that this amidase from *D. tsuruhatensis* ZJB-05174 might have a different active site structure. This specific *D. tsuruhatensis* amidase stability could be a reason *Delftia* has a permanent residence in the hemocoel of GWSS. Even more excitingly, studies done on *Drosophila* have demonstrated that flies exhibit strong hemolymph amidase activity that hydrolyzes peptidoglycan into nonstimulatory fragments and that peptidoglycan recognition protein (PGRP-LB) contributes to this activity in vivo (Zaidman et al., 2006). This information could signify the presence of *Delftia* amidase as an immune defender. However, there was a study that somewhat disproves this hypothesis. In that study, the *D. acidovorans* amidase enzyme was active preferentially toward D-amino acid amides rather than their L-counterparts (Hongpattarakere et al., 2005). It exhibited strong amino acid amidase activity toward aromatic amino acid amides including D-phenylalanine amide, D-tryptophan amide and D-tyrosine amide, yet it was not specifically active toward low-molecular-weight D-amino acid amides such as D-alanine amide, L-alanine amide and L-serine amide. Moreover, it was not specifically active toward oligopeptides. Because the bacterial peptidoglycan layer is made up in part by a short (4- to 5-residue) amino acid chain, containing D-alanine, D-glutamic acid, and *meso*-diaminopimelic acid in the case of a gram negative bacteria or L-alanine, D-glutamine, L-lysine, and D-alanine in the case of a gram positive bacteria, amidase activity would not be seen hydrolyzing these low-molecular weight amino acids. Further studies must be done to prove the importance of *Delftia* sp., its amidase production, and its occurrence in the hemolymph of GWSS.

CONCLUSIONS

Delftia sp. was identified in all hemocoel samples collected from GWSS and not from the alimentary canal. This symbiont's known prominent D-amino acid amidase activity has lead to the hypothesis that this enzyme is playing a key role on the insect's survival. In particular, this amidase could contribute as a means of immune protection by its ability to hydrolyze the peptidoglycan layer of invasive pathogenic organisms. More studies must be done to describe the relationship between this symbiont and its host, but its presence in the GWSS definitely provides another avenue to study when finding an answer for GWSS managment.

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FUNDING AGENCIES

Funding for this project was provided by the Texas Pierce's Disease Research and Education Program, and the USDA Animal and Plant Health Inspection Service.

ARE NATURAL ENEMIES CONTROLLING GLASSY-WINGED SHARPSHOOTER POPULATIONS IN SOUTHERN CALIFORNIA?

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ABSTRACT
Considerable effort and expense has been invested in the classical biological control of glassy-winged sharpshooter (GWSS) with natural enemies, in particular, mymarid parasitoids that attack the eggs of GWSS. However, no comprehensive long term studies have been under taken to ascertain why GWSS populations have declined by ~93% over the last 8.5 years. The most probable cause for GWSS population declines are natural enemies, in particular egg parasitoids, with *G. ashmeadi* being the major contributor to GWSS suppression at UCR Ag. Ops. We have documented that natural enemies provide on average year round around egg parasitism of ~25%. A critical question to ask: "Is this sufficient mortality to cause GWSS populations to decline?" Preliminary investigations using very simple models suggest that this consistent level of egg mortality, irrespective of GWSS population density and other mortality factors, may have been sufficient to cause the declines observed in this study.

LAYPERSON SUMMARY
Glassy-winged sharpshooter (GWSS) populations have declined dramatically in southern California over the last 8.5 years. In 2010, average peak population densities of GWSS were only ~7% of what was measured in 2002, indicating that pest populations have declined by around 93%. The major question that needs to be answered from this study is **WHY** has this population decline occurred? If we can figure out the underlying mechanism causing GWSS populations to decrease then we will better understand how stable GWSS populations are likely to be in southern California over the long-term, and perhaps, be able to predict factors and conditions that could lead to GWSS outbreaks. Consequently, the results from these simple surveys could be of immense value to managing GWSS in southern California, especially understanding the contributions of parasitoids that attack the eggs of this pest. This understanding could greatly help agricultural producers that experience problems with this pest (e.g., grape growers) and assist with the development of sustainable management plans.

INTRODUCTION
Homalodisca vitripennis (Hemiptera: Cicadellidae), the glassy-winged sharpshooter (GWSS), and *Xylella fastidiosa (Xf)* have been the target of a major long-term research effort in California because *Xf*, a xylem-limited bacteria which is vectored by GWSS, causes a lethal malady of grapes which is known as Pierce's disease. In an effort to reduce the population densities of GWSS in California, a classical biocontrol program against GWSS was undertaken against the backdrop of an established and self-introduced parasitoid of GWSS eggs, *Gonatocerus ashmeadi* (Hymenoptera: Mymaridae). To measure the impacts of the self-established *G. ashmeadi*, a number of native Californian mymarid and trichogrammatid parasitoids that attacked GWSS eggs, and two deliberately introduced species of *Gonatocerus* from the home range of GWSS, a long-term monitoring study of GWSS and impact these parasitoids attacking GWSS eggs were having was set up and run in organic lemons at the University of California, Riverside. The results of these monitoring studies from the last 8.5 years are presented here.

OBJECTIVES
1. To conduct long-term monitoring of GWSS populations and associated egg parasitoids in organic citrus at the University of California Riverside Agricultural Operations (Ag. Ops) facility. The purpose of these long-term surveys was to document the long-term population trends of GWSS and percentage parasitism of GWSS eggs at this study site. These data will be used to determine which factors are responsible for the year to year variations observed in GWSS densities.

RESULTS AND DISCUSSION
Data collected from bi-weekly monitoring over the last 8.5 years from organic commercially-managed lemons at Ag. Ops. UC Riverside indicates that GWSS populations have declined steadily since this project was initiated in March 2002 (**Figure 1**). Peak population densities in August 2010 were only 7% of those observed in August 2002. In this 8.5 year period, GWSS populations have declined by 93% at the study site (**Figure 1**). It is uncertain whether parasitism of GWSS eggs by mymarid parasitoids is completely responsible for this downward population trend (**Figure 2**), but his possibility seems extremely likely. In California, there is a guild of natural enemies attacking GWSS eggs. The dominant parasitoid attacking GWSS in California is *G. ashmeadi* which was self-introduced into California from the home range of GWSS. Other *Gonatocerus* parasitoids associated with GWSS eggs are *G. morrilli*, *G. walkerjonesi*, *G. novofasciatus*, *G. triguttatus* and *G. fasciatus.* The latter two, *G. triguttatus* and *G. fasciatus*, were imported from Texas and Louisiana, respectively, for

the classical biological control of GWSS. Widespread establishment of these two parasitoids appears doubtful and their impact on GWSS has been negligible. Trichogrammatid parasitoid species include, *Ufens* sp., and *Zagella* sp. parasitize GWSS eggs infrequently in organic lemons at UCR Ag. Ops. *G. ashmeadi* is the dominant parasitoid at this study site, and it has provided an average of ~25% parasitism of GWSS eggs over the entire ~8.5 yrs that this study site has been monitored (**Figure 2**). It is possible that this consistent year to year level of mortality inflicted upon GWSS eggs (in addition to other mortality factors [e.g., predation and accidental death of nymphs]) by *G. ashmeadi* has caused sufficient population-level mortality that GWSS densities have steadily declined in each year of this study. Rigorous statistical analyses are now required to provide a deeper understanding of the trends in the data that have been recorded and for elucidating mechanisms most likely to be responsible (e.g., parasitism vs. weather patterns). This analysis process has begun and preliminary results from simple modeling efforts suggests that a 25% parasitism rate each year by *G. ashmeadi* could be sufficient to cause GWSS populations to decline at the rate that has been observed at the study site used for field observations.

CONCLUSIONS

GWSS populations have declined by 93% in organic lemons at UC Riverside AG. Ops over the last ~8.5 years. Preliminary analysis of this long term data set suggest that parasitism of GWSS eggs at a rate of 25% each year by the mymarid egg parasitoid *G. ashmeadi* could have been sufficient enough to have caused this decline. More detailed statistical analyses are currently underway to more thoroughly explore the trends observed in this data set.

FUNDING AGENCIES

Funding for this project was provided, in part, by the CDFA Pierce's Disesase and Glassy-winged Sharpshooter Board, and the USDA-funded University of California Pierce's Disease Research Grants Program.

Figure 1. Phenology of adult GWSS in organic Eureka lemons. Data are total counts from timed five minute surveys made every two weeks of 10 mature lemon trees at Ag. Ops. University of California, Riverside.

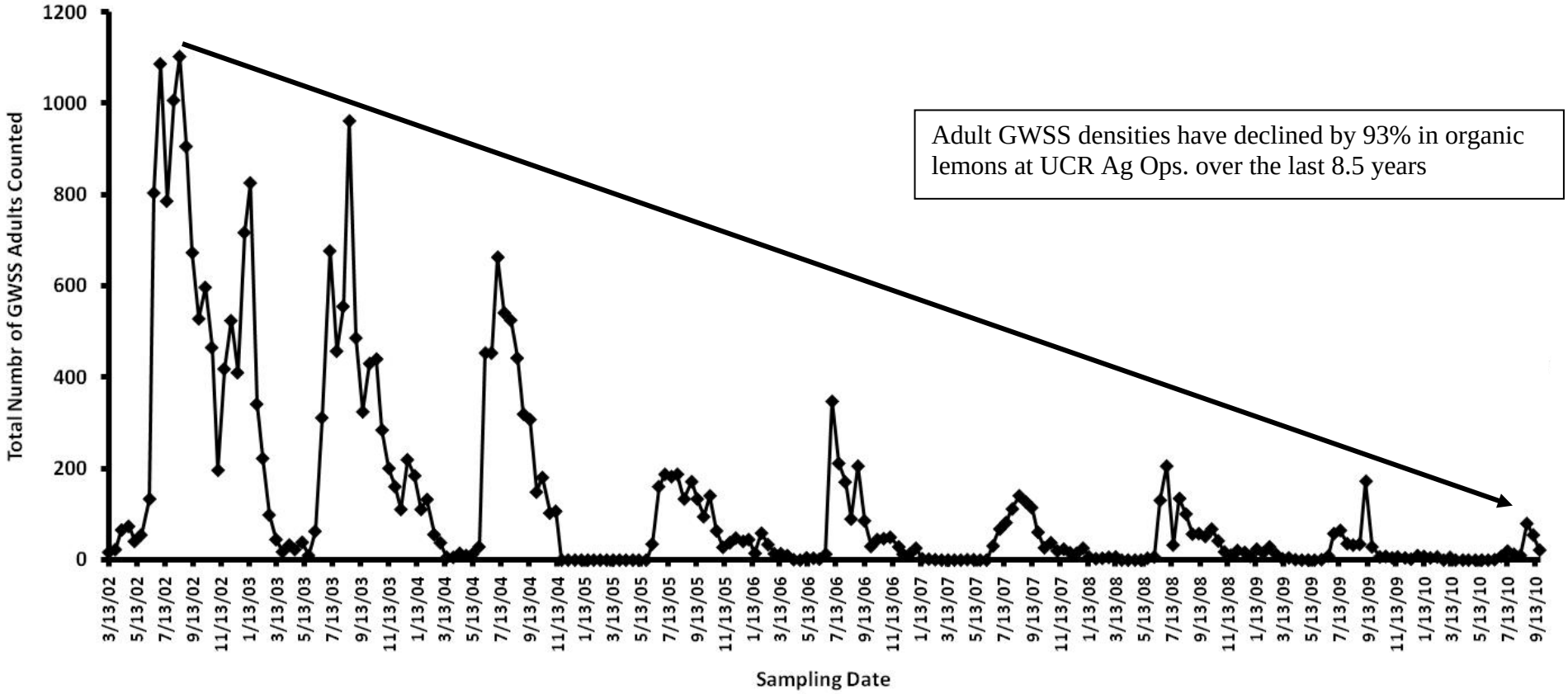
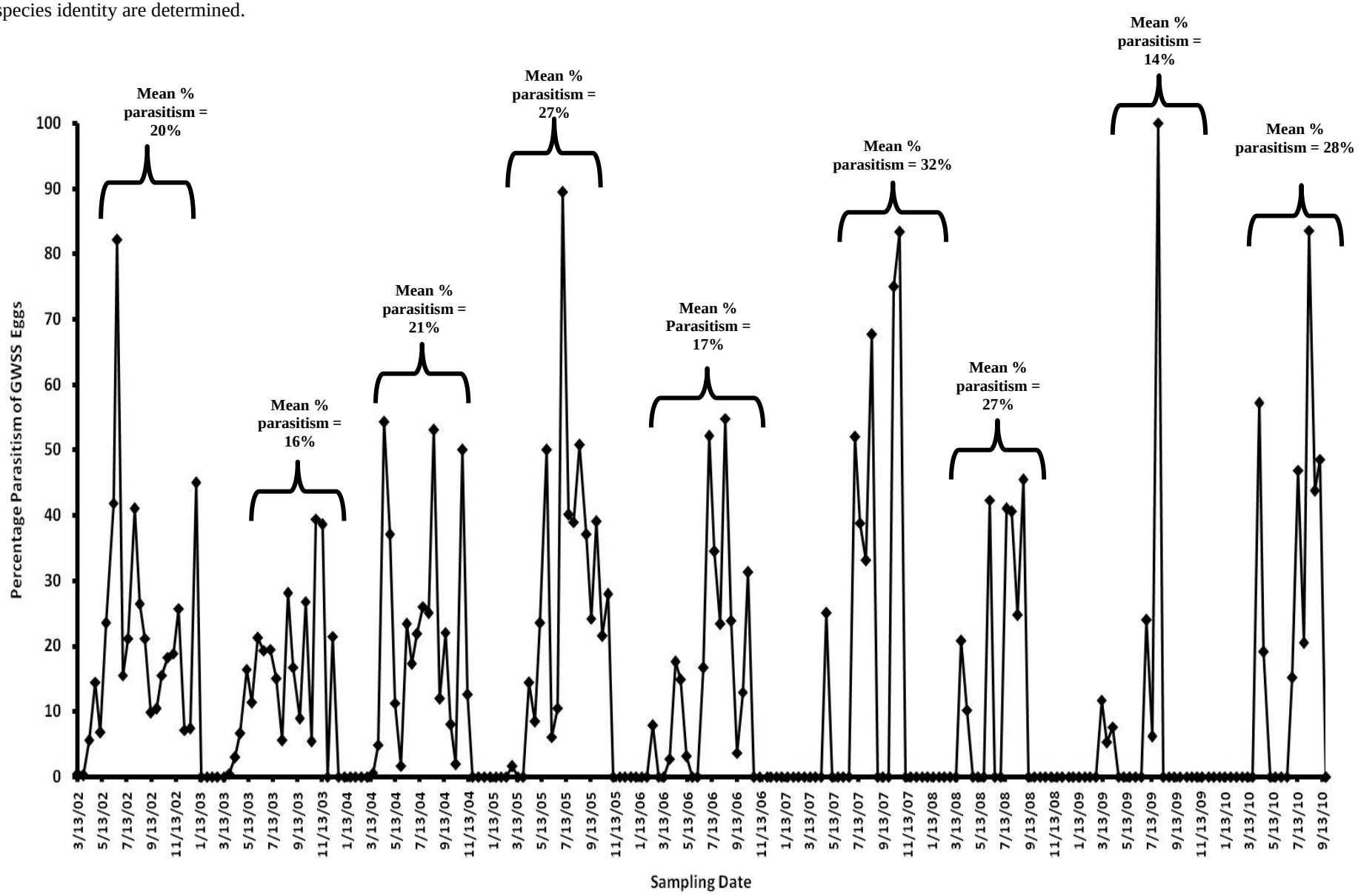


Figure 2. Percentage parasitism estimates of GWSS eggs in Eureka lemons. GWSS egg masses are collected from timed five minute surveys made every two weeks of 10 mature lemon trees at Ag. Ops. University of California, Riverside. Harvested leaves are returned to the laboratory, the number of eggs per egg mass are counted and parasitoid emergence and species identity are determined.



Percentage parasitism of GWSS eggs across all years has averaged ~ 25%

Δ9 DESATURASE FROM SHARPSHOOTERS

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ABSTRACT
Genomics approaches permit the identification of gene functions within insect pests which can then be targeted to reduce fitness. Genomic analyses of several leafhoppers identified the leafhopper delta-9 desaturase motif. Disruption of desaturase by RNAi or other strategies should reduce the biological fitness of leafhoppers, thus aiding efforts to stop the spread of Pierce's disease (PD), and other leafhopper transmitted diseases. The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) and other leafhoppers are vectors of *Xylella fastidiosa),* a xylem-limited bacteria that causes PD in grapevine as well as 'Scorch-like' pathology of other woody agricultural fruit, nut, and ornamentals. Management of PD currently depends heavily upon insecticides to suppress these vector populations. Unfortunately chemical controls are not insect specific, can lead to resistance, and often reduce non-target beneficial organisms. To extend current IPM programs alternatives need to be developed which can work within these programs. Thus, we provide genetic sequences identified with links to food utilization and oviposition, these may serve as RNAi targets to reduce leafhopper fitness.

LAYPERSON SUMMARY
The identification of genes which are critical for the survival and reproduction of insect pests opens the door for applications of natural insect management based on gene disruption, such as RNA interference (RNAi). We identified the desaturase enzymes in leafhoppers which they need to digest food, store fats, and produce eggs. This is the first step in the development of safe, advanced, management based upon the specific genetics of leafhopper pests. The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) causes economic losses to growers by spreading the plant infecting bacterium, *Xylella fastidiosa*. This bacterium causes Pierce's disease of grapevine, which can stop grape production and kill vines. Therefore, development of effective population management strategies are needed. Insecticides although successful to reduce the economic impact of these insects, ultimately will result in resistance and/or resurgence of insect pests. Thus alternative methods of population suppression are continually being developed. Here we propose the use of gene targeting (desaturases) to reduce the ability of the leafhoppers to grow and reproduce, and describe the desaturase enzymes from leafhoppers.

INTRODUCTION
Genomic approaches, these data are providing new information on the genetic basis of biology, behavior, and refinement of their phylogenetic classification (Hunter et al. 2003). Three leafhopper species, referred to as sharpshooters (Hemiptera: Cicadellidae) were examined by comparison of the available expressed sequence tags, ESTs, (Hunter datasets, NCBI). The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is the major vector of *Xylella fastidiosa (Xf)* in the southern USA. The plant pathogenic bacterium, *Xf*, has caused economic losses to several agricultural industries in North America and is associated with many plant diseases such as Pierce's disease (PD) of grapevine. The introduction of GWSS into new areas is directly related to increased occurrence of PD in vineyards (Perring et al., 2001). Therefore, the management of PD depends heavily on the ability to reduce the sharpshooter vectors, especially GWSS. Methods of vector manipulation such as chemical control with the use of insecticides are not target specific and lead to problems such as resistance, water contamination, and injury to non-target organisms. Current efforts to develop more benevolent pest management strategies has led to the use of biocontrol agents such as fungi and parasitoids. One of the most exciting developments on the horizon however is in the applications of gene disruption, RNA interference, RNAi. But before RNAi can be implemented gene targets within the sharpshooters need to be identified. Thus, we have started to characterize genes within these sharpshooter species.

OBJECTIVES
1. Use *in silco* analyses to characterize delta-9 desaturase in sharpshooters.
2. Ultimately develop RNAi to disrupt desaturases in sharpshooters.

RESULTS AND DISCUSSION

The identified sharpshooter Δ-9 desaturases were submitted to NCBI database [gb|AAU95195.1| *O. nigricans*; gb|AAT01079.1| *H. vitripennis*]. Desaturase enzymes have been shown to be highly conserved throughout Eukarya (Fungi, Protists, Plants, and Animals) and function in the processing of lipids by placing double bonds between the adjacent carbons of fatty acids. The Δ-9 desaturase also occurs embedded in the membrane of endoplasmic reticulum, ER, and functions as either palmitoyl or stearoyl Δ- 9 desaturase.

Delta-9 desaturase-1 identified as a palmitoyl desaturase within *H. vitripennis* (Hunter, 2004), and similar Δ-9 desaturase were identified within *O. nigricans* and *G. atropunctata* with the specificity of the later two unknown. The sequence analysis of sharpshooter desaturase provides evidence as to the degree of homology between these species.

Sequence analysis - Base calling was performed using TraceTuner™ (Paracel, Pasadena, CA) and low-quality bases (quality score <20) were stripped from both ends of each EST. Quality trimming, vector trimming, and sequence fragment alignments were executed using Sequencher™ software (Gene Codes, Ann Arbor, MI). Sequencher contig assembly parameters were set using a minimum overlap of 50 bp and 90% identity. Contigs joined by vector sequence were flagged for possible miss-assembly and manually edited. The Δ-9 desaturase sequences obtained from each of the three species were then aligned using Bioedit and conserved domains identified. Further sequence identity was determined based on BLAST similarity searches using the NCBI BLAST server (www.ncbi.nlm.nih.gov) with comparisons made to both non-redundant nucleic acid and protein databases using BLASTN and BLASTX, respectively. Matches with an E-value ≤-10 were considered significant and were classified according to the Gene Ontology (GO) classification system. Translated proteins were analyzed with National Center for Biotechnology Information's BLAST*p*, Pfam (www.pfam.org), InterProScan (www.ebi.ac.uk), and Expert Protein Analysis System (www.expasy.org) (**Figure 1**).

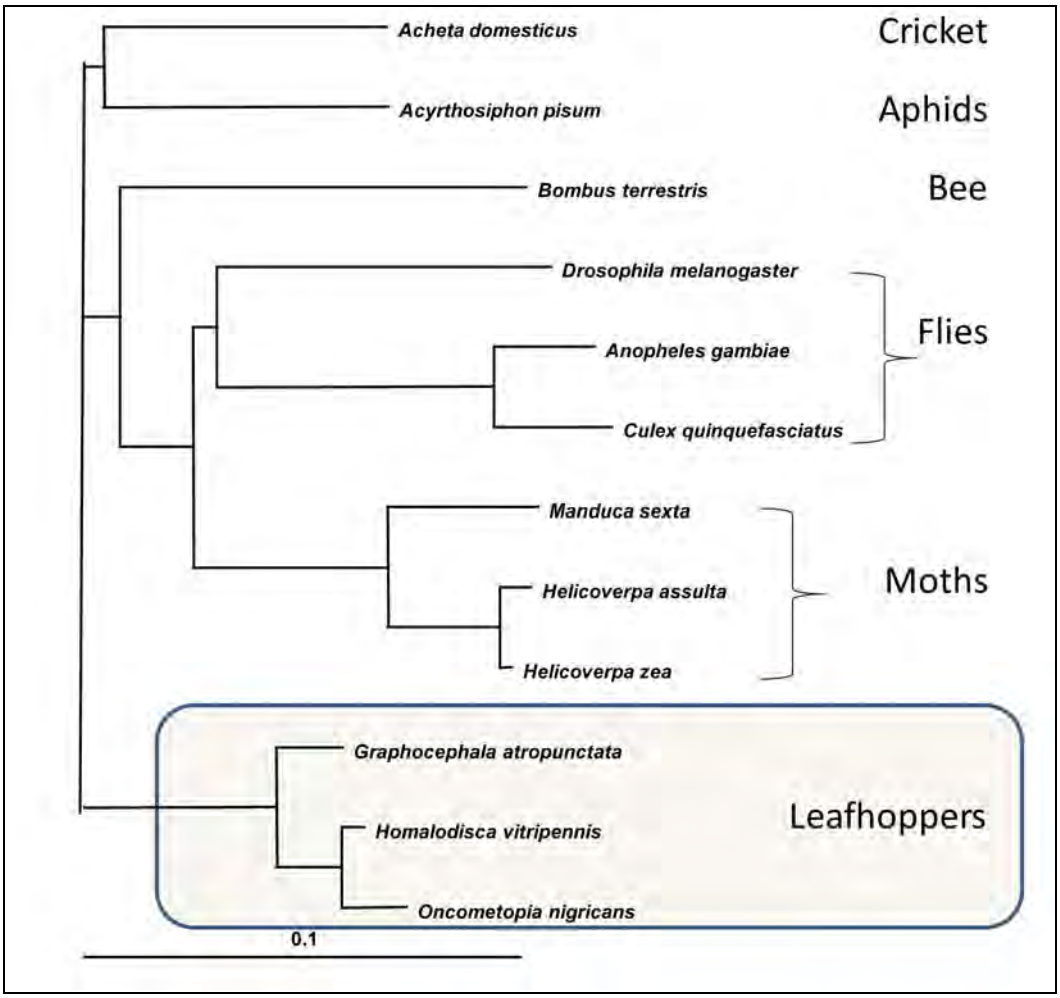

Figure 1. Tree based on Δ9 desaturase protein sequences of various insect species. Three leafhopper species are shown circled below and accession numbers as follows: *Homalodisca vitripennis* (gi|46561748|gb|AAT01079.1|.) and *Oncometopia nigricans* (gi|53830704|gb|AAU95195.1|.) based on BLAST alignments performed through NCBI (http://blast.ncbi.nlm.nih.gov/ Blast.cgi).

Another partial protein sequence that was identified as *Graphocephala atropunctata* showed 70% coverage and a $9e^{-109}$ e-value when aligned with *H. vitripennis.* Multiple sequence alignments of predicted desaturase amino acid sequences were performed using ClustalX version 1.81 algorithms (Thompson *et al.,* 1997).

The fact that the largest percentage of ESTs from these leafhoppers, code for lipid metabolism or related processes which should not be surprising considering that lipids and lipid transport play vital roles in insect physiology. The insect cuticle, often accounts for up to 50% of the dry weight of an insect, contains a number of layers containing lipid mixtures with lipid transport systems used for movement into these layers (Lockey 1984). Significant levels of lipids are also deposited into the oocyte during oogenesis to be used as energy for the embryo (Downer and Matthews, 1976) with approximately 30-40% of the dry weight of an egg being lipids (Ziegler and Antwerpen, 2006).

Expression analyses by real-time rtPCR across all life stages resulted in lack of detection of desaturase expression in eggs. However, all other instars 1^{st}-5^{th} expressed Δ9 desaturase showing a significant increase in the 5th instar and adults which correlated to increased feeding on a widening host plant range to complete development, and the formation of new organs (ie. Ovaries). There was no significant difference in expression levels of Δ9 desaturase among 1^{st}- 4^{th} instars. The 5^{th} instars showed significantly increased levels of desaturase compared to the previous instars, while adults expressed significantly greater levels from all other life stages. The expression of the GWSS Δ9 desaturase for pheromone production is unlikely as there is agreement between the absence of reports of pheromone production in adult GWSS females or males, and the lack of a significant difference in the levels of gene expression between the adult females and males.

CONCLUSIONS

We isolated sharpshooter sequences with homology to Δ9 desaturase 1. Desaturase plays crucial roles in lipid biosynthesis and their levels have been linked to the developmental age of organisms (Aguilar and Mendoza 2006). Desaturases are essential components of a variety of cellular tasks such as catalysis, transmembrane transport and signaling, and are intimately associated with cellular membranes. The majority of analyses performed on insect fatty acyl-CoA desaturases thus far have been focused on Dipteran and Lepidopteran insects leaving scant information from Hemipterans. The deduced amino acid sequence from the GWSS Δ9 desaturase shares 77% similarity with the *Acheta domesticus* desaturase and has significant (>50%) similarity with other insect delta-9 desaturases plus having many features of acyl-CoA desaturase to provide evidence towards its function and importance in sharpshooter biology.

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RNA INTERFERENCE TO REDUCE SHARPSHOOTERS, THE GLASSY-WINGED SHARPSHOOTER, AND THE ASIAN CITRUS PSYLLID

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Reporting Period: The results reported here are from work conducted November 2008 through October 2010.

ABSTRACT
Short interfering RNAs which can be used to reduce gene expression, or 'silence' this expression in a sequence specific manner is call RNA interference, RNAi. In the case of hemipteran pests, RNAi has only recently been shown to disrupt their biology. We propose that RNAi has the potential to be applied in an area wide management strategy, thereby suppressing the pest populations to reduce disease spread. Previously we generated leafhopper genetic datasets from three species of known vectors of *Xylella fastidiosa (Xf)*, the plant infecting bacterium which causes Pierce's disease (PD) of grapevine. Using genomic analyses we identified a subset of potential genetic targets which may be used to suppress these sharpshooter leafhoppers. The primary disease vector, the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) vectors the *Xf* xylem-limited bacteria that causes PD in grapevine as well as 'Scorch-like' pathogens of other woody agricultural fruit, nut, and ornamental crops. But many other species of sharpshooters are also capable of transmitting *Xf*. Therefore, we chose to produce two constructs of dsRNA to Arginine kinase, one from the GWSS leafhopper, and one for the Asian citrus psyllid. Both insects feed on citrus and transmit different bacterial pathogens. We treated several host plants (Grapevine, Citrus, Okra, and Chrysanthemum), using cut flush, to seedlings, to mature grapevines and citrus trees for this study. The dsRNA treated plants caused an increase in mortality for both the GWSS and ACP. However, when each insect fed upon the dsRNA specific to the other insect no increase in mortality was observed. RNAi due to this specificity may be an excellent treatment to reduce insect pests while protecting the non-target beneficial organisms.

LAYPERSON SUMMARY
We demonstrate that a naturally occurring molecule, double-stranded RNA, dsRNA, can induce an RNA interference, RNAi, response in leafhoppers and psyllids. This means that insect specific genes can be down regulated such that the insects' fitness is reduced (ie. lays fewer eggs or dies). The specificity of the dsRNA's showed that each was only functional within the designed species, so the leafhopper –dsRNA worked only in leafhoppers but not in the psyllid, and *vice versa*. RNAi has become one of the most studied systems and is being developed for human health, as well as to solve problems in agriculture. The RNAi strategy uses the natural systems already in place within animals, insects and plants. We propose that RNAi strategies should be pursued for area wide insect pests suppression programs, and may further provide new management approaches to other pest species.

INTRODUCTION
RNA interference, RNAi, refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire *et al*., 1998) and is a phenomenon that may be useful in many avenues of disease and pest management, including , we hypothesize, management of hemipteran pests. These approaches, such as the implementation of RNAi in insect pest management are rapidly being developed on many fronts, such as to improve the health of beneficial insects (Hunter et al, 2010). RNAi applications have been proposed and evaluated for their use in many biological problems from human health to agricultural pests. Thus, we propose that RNAi has the potential to be applied in an area wide management strategy, thereby suppressing leafhopper and psyllid populations to reduce disease spread.

To identify the mRNA's, which would be targeted by RNAi, we previously generated expression libraries from sharpshooter, based on expressed sequence tags, EST's, from three species of known vectors of *Xylella fastidiosa (Xf)*, which causes Pierce's disease (PD) of grapevine (Hunter et al,2003-2010, NCBI). Using genomic analyses we identified a subset of potential genetic targets which may be used to suppress these sharpshooter leafhoppers.

One of the major breakthroughs which launched RNAi approaches into the realm of practical applications has been the development of the ability to produce kilogram quantities of dsRNA (www.beeologics.com). An example of this is the power of an RNAi product which has been shown to increase the health of honey bees in the presence of bee viruses,

demonstrating the first study of its kind (Hunter et al, 2010). With the development of similar RNAi products it is predicted that soon these products will be approved and commercially available for agricultural use. Once there is a pathway for the evaluation and approval of RNAi products it is possible to envision many such solutions being developed to address PD and other insect transmitted diseases.

OBJECTIVES

1. Get dsRNA into grapevines and citrus trees for delivery of RNAi to leafhoppers and psyllids.
2. Demonstrate functionality of RNAi in leafhoppers and psyllids.
3. Ultimately develop RNAi for leafhopper and psyllid area wide suppression and disease management.

RESULTS AND DISCUSSION

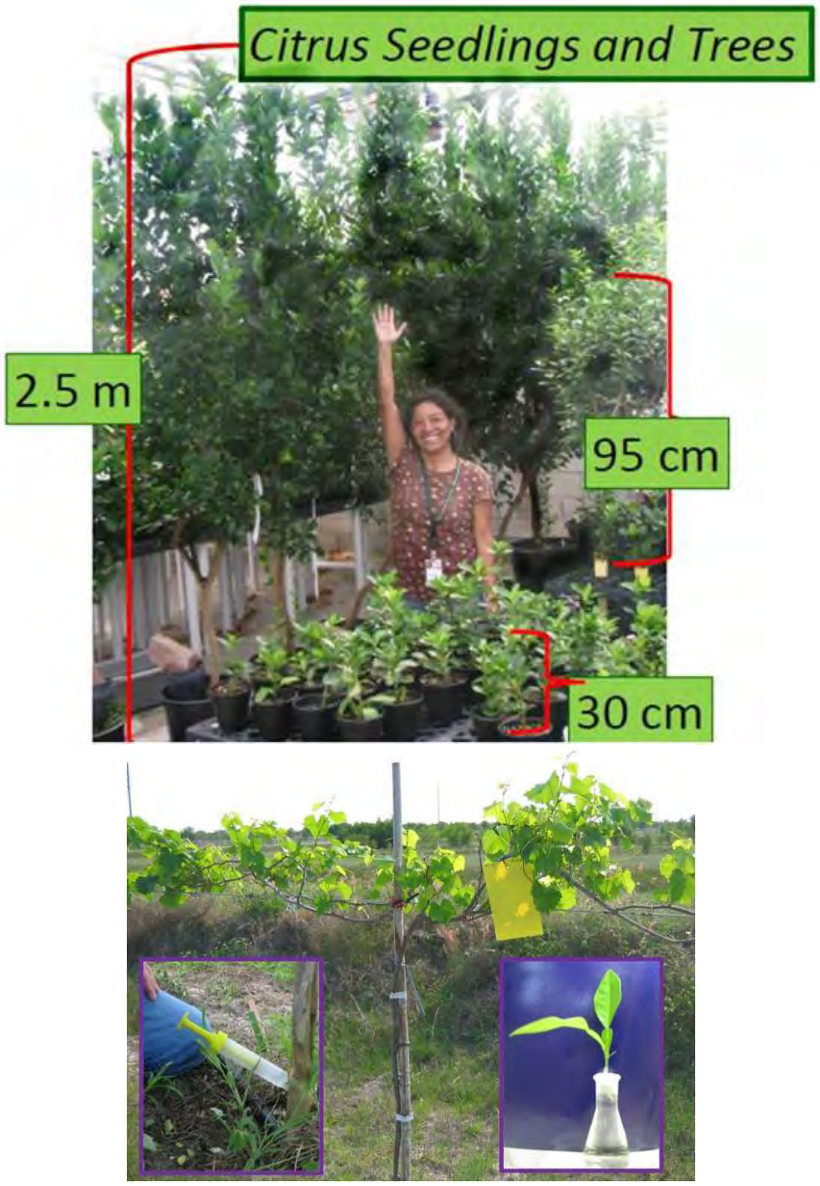

Figure 1. Plants from seedlings to mature citrus trees and grapevine were successfully treated with dsRNA. When the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis)*, and the Asian citrus psyllid were fed on these treated plants they were both shown to have ingested the dsRNA. This is an important finding as the leafhoppers are feeding from the xylem and the psyllids are feeding from the phloem, thus the dsRNA moved throughout the trees vascular system.

Preliminary results: Successfully show that we can get dsRNA uptake in trees (Key Limes) as tall as 2.5 meters {~8 ft} within four days, at a dosage of 250 ml (10 mg/ml) dsRNA in 18.93 L {5 gal US} water. Injection trials were also used on citrus as well as into grapevines. Grapevines and/or citrus trees had root mass rinsed to remove some of the soil, and then set into a plastic barrel where they soaked in the solution. Insects were placed on either controls treated with water or dsRNA treated grapevines or citrus trees for three days post treatments.

Soil interaction study: Citrus seedlings (Valencia, 95 cm {~3 ft}) tall in octagon pots, either rinsed of soil from roots, or left fully intact in pots were also permitted to soak for five d in solution, water level roughly 1/3 up the container. Soil did not deter uptake nor detection of the dsRNA.

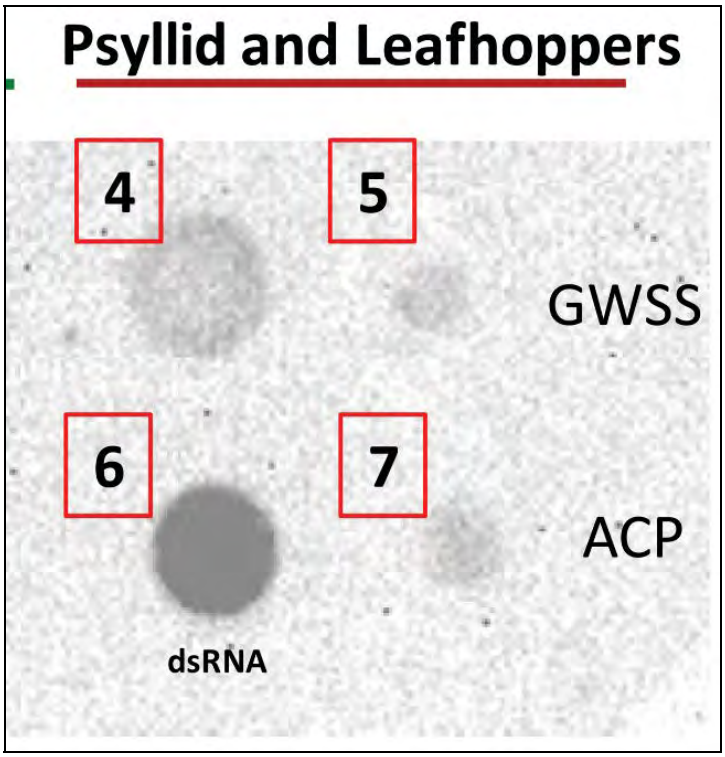

Figure 2. Detection of *ingested dsRNA* in both glassy-winged sharpshooter (GWSS), and the Asian Citrus Psyllid after feeding for 3 days on either grapevine or citrus treated with dsRNA.

RNAi increases GWSS mortality: In two separate studies using the same dsRNA for Arginine kinase (dsRNA-AK) [Hunter, ARS, FL using citrus, and grapevine] and [Bextine and Hail, using Chrysanthemum stems], both determined that when GWSS fed upon plant seedlings or flush which had absorbed the dsRNA-AK, the sharpshooters died earlier and at a higher rate than the controls within a five day period.

Future studies: Currently in Florida we have eight citrus trees (sweet orange) (>2.5 m) and 4 grapevine (Noble) being treated by injection to determine dsRNA movement and persistence under field conditions.

CONCLUSIONS

RNAi was successful in increasing the mortality of GWSS and the Asian citrus psyllids. Sharpshooters and Psyllids were shown to ingest dsRNA from cuttings, seedlings, mature grapevine and/or citrus trees. This is the first demonstration of an RNAi effect for these insects, and the ability to induce RNAi in these insects from feeding on plants containing dsRNA. The results support the proposal that a RNAi host delivery strategy may be an efficient area wide approach to population suppression against these hemipteran insects {ie. The GWSS and the Asian citrus psyllid}.

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TRANSMISSION EFFICIENCIES OF TWO STRAINS OF *XYLELLA FASTIDIOSA* FROM CULTURE THROUGH GLASSY-WINGED SHARPSHOOTERS

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ABSTRACT
Xylella fastidiosa (Wells) (*Xf*) is a gram-negative, xylem-limited bacterium that causes diseases in various crops by blocking xylem or water vessels of plants. It can induce Pierce's disease (PD) of grapevine, almond leaf scorch, oleander leaf scorch (OLS), and citrus variegated chlorosis. The xylem sap-feeding insects generally serve as the biological vectors in the process of *Xf* transmission, which occurs when the insect inserts its mouthparts into the plant's xylem vessels. The glassy-winged sharpshooter (GWSS), plays an important role in *Xf* transmission for it has an extremely broad host plant range and spreads across the southern and western states. Because it was reported that GWSS previously inoculated with OLS strain was not able to transmit the subsequently acquired PD strain to grapevine, the competition for space and nutrition among different strains of *Xf* might impact the ability of an insect to be a proficient vector. In order to evaluate if biofilm formation by the PD strain is negatively impacted by the presence of a non-PD strain, we inoculated GWSS with various combinations of *Xf* strains. The competition between two strains of *Xf* was evaluated via the melting curve analysis and a novel molecular beacon system. When introduced first, the antecedent non-PD *Xf* strain was likely to become dominant in the foregut of GWSS and significantly suppress of the biofilm formation of PD strains that colonized later.

EXPERIMENTAL ANALYSIS OF BIOLOGICAL PARAMETERS AND VECTOR ABILITY OF GLASSY-WINGED SHARPSHOOTERS FROM ALLOPATRIC POPULATIONS IN CALIFORNIA

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ABSTRACT
The glassy-winged sharpshooter [GWSS; *Homalodisca vitripennis* (Germar)], is native to the southeastern United States and northeastern Mexico. It was detected in southern California in the late 1980s and in the San Joaquin Valley in 1999, where it transmits the bacterium *Xylella fastidiosa* (*Xf*) to grapevines and other crops. The transmission efficiency of *Xf* to grapevines and the reproductive success of hybrid and pure line GWSS from two allopatric populations in California (Riverside (RIV) and Bakersfield (BAK)) were evaluated under identical controlled conditions. To tests the effects of GWSS origin (RIV versus BAK), gender, and age on transmission, insects were given a 96h acquisition access period on infected grapevines and a 72h inoculation access period on healthy grapevines. At conclusion of the test, ~33% of test plants were infected, with no effect of GWSS origin, gender, or age on transmission, confirming that these factors do not affect transmission. Comparison of reproductive success based on origin found that the preoviposition period in both female generations was significantly shorter for RIV (F_0 = 28.2 days and F_1 = 62.3 days) than BAK females (F_0 = 46.1 days and F_1 = 170.4 days). There were no significant differences in fecundity and longevity among the F_0 and F_1 mating pair treatments. There was a gradual decrease in the number of viable eggs deposited by GWSS females, suggesting that females exhausted sperm reserves and that re-mating may be necessary to produce viable progeny. From a management perspective, delayed reproductive maturity and polyandry are weak links in GWSS's biology that may be exploited through mating disruption or insect sterilization strategies to reduce population growth and augment pressure by natural enemies.

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SURVEY OF GREEN SHARPSHOOTER POPULATIONS IN AND NEAR VINEYARDS IN THE SAN JOAQUIN VALLEY

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ABSTRACT
Pierce's disease and almond leaf scorch disease have an episodic history in California that predates arrival of the glassy-winged sharpshooter. Within California's Central Valley, the green sharpshooter (*Draeculacephala minerva*) is the most abundant and widely distributed vector of *Xylella fastidiosa*. Previous reports indicate that grape and almond are occasional hosts of *D. minerva*, whereas grassy weeds present in pastures and irrigated alfalfa fields are preferred hosts. To better understand movement of *D. minerva* into vineyards, eight vineyards in the San Joaquin Valley were sampled. At each vineyard, potential source habitats for *D. minerva* were identified: pastures, alfalfa fields, and grassy ditches. Abundance of *D. minerva* in source habitats was assessed using sticky traps and/or sweeps. To document movement of *D. minerva* into vineyards, 16-20 sticky traps were placed around each vineyard and changed biweekly. Finally, weed ground cover in each vineyard was evaluated and if present swept on a monthly basis. Abundance of *D. minerva* was greatest in permanent pastures followed by alfalfa fields. Populations of *D. minerva* were largely absent from grassy ditches. Catches of *D. minvera* on traps surrounding vineyards was rare, but occurred at seven of eight vineyards during the study. As a blunder trap was used and traps covered only a small fraction of vineyard perimeter, low trap catches were unsurprising and the fact that catches occurred at nearly all sites suggests regular movement of *D. minerva* into vineyards. However, *D. minerva* was observed in weedy ground cover at only two vineyard sites and was never observed on the vines themselves, suggesting that movement into vineyards was transient. Lack of establishment of *D. minerva* populations in vineyards may be due to the ephemeral nature of vineyard weed populations. The results reinforce previous reports that grape is not a preferred host of *D. minerva* and that habitats outside of vineyards are likely to play a key role in *D. minerva* population dynamics, particularly locations with permanent irrigated grasses.

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Section 2: Vector Management

UNDERSTANDING THE DYNAMICS OF NEONICOTINOID INSECTICIDAL ACTIVITY AGAINST THE GLASSY-WINGED SHARPSHOOTER: DEVELOPMENT OF TARGET THRESHOLDS IN GRAPEVINES

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ABSTRACT
Systemic neonicotinoid insecticides are effective against adult and immature stages of the glassy-winged sharpshooter. One reason for the use of systemic treatments is that they exploit the xylophagous feeding behavior of the insect. Our current data show that neonicotinoid treatments have an additional contact activity on emerging first instars before they begin feeding. Egg masses that were exposed to systemic treatments of dinotefuran were unaffected by the treatments and the first instars developed fully within the eggs. However, upon emergence, the insects became susceptible to the effects of insecticide residues that were present in the surrounding tissues. In a series of bioassays, we were able to show that there was a dose-response between insecticide residues and first instar mortality. From these data, and data currently being generated from additional bioassays, a target threshold for dinotefuran will be derived. This target value can then be used as an indicator of the efficacy of treatments and the level of protection vines are receiving.

LAYPERSON SUMMARY
The systemic neonicotinoids imidacloprid and dinotefuran are effective insecticides that growers can use for long-term management of glassy-winged sharpshooter (GWSS) populations. Because of the contrasting chemical properties of these insecticides (e.g. dinotefuran is about 80-fold more water soluble than imidacloprid), growers can now choose the most suitable product to meet their pest management needs. In this study, we are determining the concentration of dinotefuran that is needed within the xylem of plants to kill a feeding GWSS. We have demonstrated that the toxicity of dinotefuran against GWSS adults is close to that of imidacloprid, and we have also shown that nymphs emerging from an egg mass are susceptible to the contact activity of dinotefuran before they commence feeding.

INTRODUCTION
Our research program focuses on the use of chemical insecticides for the management of glassy-winged sharpshooter (GWSS). We are dedicated to formulating safe and effective treatment programs for California growers, given the almost complete reliance by the grape industry on this method of control. We have conducted extensive trials in Coachella, Napa and Temecula valley vineyards to evaluate the uptake and persistence of three neonicotinoids – imidacloprid, thiamethoxam, and dinotefuran – under the diverse range of climatic, soil, and agronomic conditions associated with these regions. We have an understanding about how the different chemical properties, particularly water solubility, of these neonicotinoids can be exploited to achieve optimum uptake into vines, and we have developed sensitive techniques that allow us to monitor the levels of insecticide present within the vines. To exploit this knowledge further for the benefit of California grape production, we need to ensure that the concentrations of insecticide present within the vines are reaching levels that are effective at rapidly killing GWSS before they can infect vines with Pierce's disease. We also need to understand whether there is a sub-lethal impact of these insecticides on GWSS, since anti-feedant activity may not necessarily eliminate the threat that an infective sharpshooter poses to a vine. Our past and current research projects have established the threshold levels of imidacloprid needed to kill a GWSS at 10 ng/ml xylem fluid, and optimized treatment regimes for growers that will ensure these thresholds are attained following applications via different irrigation methods (drip, sprinkler). In 2007, a new systemic neonicotinoid, Venom (active ingredient dinotefuran), received full registration for use on grapes. Our work in this area has demonstrated the excellent uptake of these new insecticides following systemic application to vines (Toscano et al., 2007). This is good news for vineyard operators who have experienced problems with imidacloprid. Imidacloprid has been the predominant neonicotinoid in use in vineyards, but our research has shown that its uptake and persistence within vines varies dramatically between regions (Coachella Valley, Napa Valley, Temecula Valley). Despite its apparent poor uptake, growers continue to rely on imidacloprid in many areas. The perception is that the insecticide will work well in all areas given its successful implementation in Temecula vineyards (Byrne and Toscano, 2006). Dinotefuran offers a potential solution to overcoming the problems encountered with imidacloprid use – its rate of uptake is faster and it can reach higher concentrations at peak uptake than imidacloprid under the more challenging situations. It also exhibits favorable persistence. Having established that the uptake and persistence of dinotefuran is superior to imidacloprid in terms of insecticidal titers reached in the xylem, it is important to ensure that the levels attained in the xylem are active against sharpshooters. Comparative data on the efficacy of systemic dinotefuran against GWSS is not available. This study addresses the gap in our knowledge of dinotefuran thresholds needed for effective control of adult and immature GWSS.

OBJECTIVE

1. Determine target thresholds for systemic neonicotinoids against GWSS in grapevines.

RESULTS AND DISCUSSION

Update on Adult Bioassays

In our previous report, we showed that the concentrations of dinotefuran in the xylem of cotton plants used for bioassays of adult GWSS could be effectively controlled (Byrne and Toscano, 2009). To further validate this finding, we have conducted additional tests that have incorporated lower dose rates. The inclusion of these lower rates will ensure that a full range of dose-response data will be available for the probit analysis.

Dinotefuran levels within the xylem are quantified by ELISA. Although the lower limit of detection of the ELISA is 1.5 ppb, the limit of detection for grape xylem is 10 ppb because of the need for a dilution step to eliminate matrix effects. In bioassays, we are detecting mortality when insects feed on xylem that has levels of insecticide below the detection range of the ELISA. For this reason, we need to better understand the correlation between treatment rate and xylem concentration. With a better understanding of the relationship, we hope to develop a simple model from which the concentrations in xylem from plants treated at the lowest rates (and which are undetectable by ELISA because they are out of range) can be determined by extrapolation.

Update on Bioassays of Egg Masses

We evaluated the effect of dinotefuran against the eggs of the GWSS. Adult GWSS were confined in cages with cotton, which is an excellent host for GWSS oviposition. Leaves with egg masses (not older than 24 hours) were cut from the plants and the petioles inserted into vials containing a range of insecticide concentrations. The uptake of insecticide into each leaf was allowed to proceed for 24 hours and the leaves were then transferred to leaf boxes. The leaf boxes were maintained under lights until the normal period of embryonic development was completed (untreated controls were used to indicate the normal period of development). Mortality of nymphs was assessed for up to five days after the beginning of emergence.

As with imidacloprid (Byrne and Toscano, 2007), the nymphs developed fully within the egg mass and only succumbed to the effects of contact with dinotefuran during emergence. In contrast to our previous data for imidacloprid, where we observed an LC_{50} of 39 ng/cm^2 leaf, the indications from our current data set show that dinotefuran is slightly more toxic to the first instars than imidacloprid (**Figure 1**). Also, the slope of the dose-response curve is extremely steep, as was observed for imidacloprid.

CONCLUSIONS

At current label recommendations, the rate of uptake of dinotefuran into grapevines is faster than imidacloprid and concentrations of dinotefuran at peak uptake are higher (Toscano et al., 2007). These two properties make dinotefuran a strong candidate for inclusion in a sharpshooter management strategy, provided that effective concentrations are reached within the xylem. Dinotefuran is highly toxic to emerging first instars, and our data suggest that the insecticide is slightly more toxic than imidacloprid. As with imidacloprid, the toxic effect is not manifested until the nymphs emerge from the egg mass, suggesting that dinotefuran and imidacloprid act as contact insecticides.

The systemic neonicotinoids imidacloprid and dinotefuran are effective insecticides that growers can use for long-term management of GWSS populations. Because of the contrasting chemical properties of these insecticides, growers can now choose the most suitable product to meet their pest management needs. One of the interesting observations from this study has been that the concentrations of insecticide present within the xylem can be managed by choosing the appropriate application rate. This is a very powerful tool that could be used to optimize insecticide applications and manage insecticide use more effectively.

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FUNDING AGENCIES

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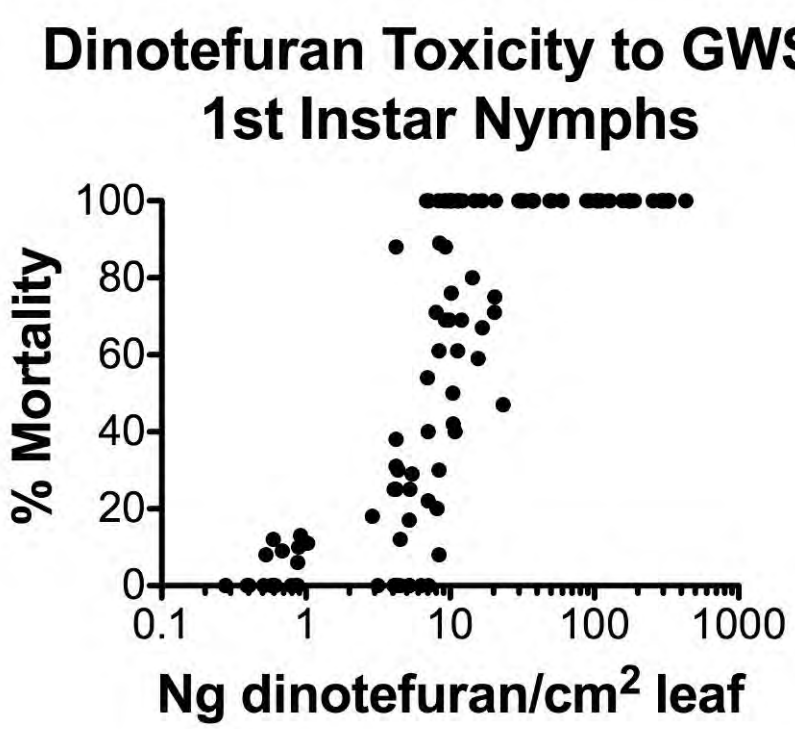

Figure 1. Toxicity of dinotefuran to emerging 1st instar GWSS. The petioles of leaves containing egg masses were placed in vials containing different concentrations of insecticide for 24 h systemic uptake. Leaves were then transferred to leaf boxes where the egg masses were allowed to continue their development. The survivorship of nymphs was determined for 5 days after the first indications of emergence.

DEVELOPMENT OF EFFECTIVE MONITORING TECHNIQUES FOR SHARPSHOOTERS AND THEIR PARASITOIDS.

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ABSTRACT
Assessing the efficacy of the ongoing sharpshooter egg parasitoid biocontrol program is the focus of this project. Since 2000 *Gonatocerus morgani*, *G. morrilli*, and *G. triguttatus* have been reared and released by the program at sites throughout Southern California and the southern Central Valley. While data presented in the most recent CDFA report (2009) demonstrates the effectiveness of the release program, more data concerning the extent of released species populations, the effects of parasitism by native competitors, and the host preferences of the parasitoids involved is needed. D. Cooksey has developed a multiplex PCR system for the simultaneous identification of *Xylella fastidiosa (Xf)* strains (Hernandez-Martinez *et al.*, 2006), in conjunction with his research in comparative and functional genomics of *Xf*. As Supervisor of the release program, D. Morgan is an expert in the biology, ecology, systematics, and identification of the host (Son *et al.*, 2009) as well as the parasitoid species targeted in this study. C. LeVesque has developed a high throughput testing program for citrus Huanglongbing disease that employs high resolution melting curve analysis. The development of the proposed multiplex high resolution melting real-time PCR system will greatly enhance the data acquisition of the CDFA parasitoid release biocontrol program.

LAYPERSON SUMMARY
Glassy-winged sharpshooter (GWSS) populations are partially controlled by biological control agents. It is essential to have the capacity to identify the parasitoid species, host species and the extent of parasitism in order to evaluate the effectiveness of the control strategy. The current methods rely on identification of eclosed parasitoids after long incubations under artificial conditions which many do not survive. An accurate and rapid method for identification of the eggs of sharpshooter species, determining whether eggs are parasitized, and by which parasitoid species, would greatly facilitate the development of the release program. A single-step multiplex real-time PCR assay for sharpshooters and their parasitoids is such a method, and its development will significantly enhance the reporting of GWSS parasitism.

INTRODUCTION
The only methodology currently available for post-release monitoring of glassy-winged sharpshooter (GWSS) parasitoids involves the collection and incubation of field-collected GWSS eggs. Since the eggs are removed from the field before development has been completed, the possibility of further parasitism is eliminated. Therefore, parasitism rates are underestimated. Because optimal incubation conditions vary for each parasitoid species, significant developmental mortality can occur during the two-week or longer incubation period needed for wasps and GWSS to eclose, resulting in some species being significantly underreported. No economical method for identifying whether eggs are from GWSS or the native smoke tree sharpshooter (STSS) is currently available. Therefore, a more efficient method for monitoring biological control activity is essential if we are to have more accurate, timely, and economic reporting of GWSS parasitism.

Development of a single-step multiplex high resolution melting (HRM) curve real-time PCR assay for sharpshooters and their parasitoids will allow for accurate reporting of GWSS parasitism. This method can identify the species of host, GWSS or STSS, and its parasitoids simultaneously within half a day of collection, rather than two weeks. In addition, old egg masses should be able to be used after wasp eclosion, as the pupal and sharpshooter egg casing can be analyzed. Determining the effectiveness of the different parasitoid species in the various environments encompassed in the current range of GWSS will facilitate the refinement of control strategies and lead to better suppression of GWSS.

OBJECTIVES
1. Develop primer pairs that can be used in a multiplex high resolution melting curve analysis real-time PCR system for each species of sharpshooter and parasitoid.
2. Through the use of degenerate primers, clone the target genes from those species of parasitoid for which there is no sequence data available.
3. Determine the limits of detection of each species of sharpshooter and parasitoid. Based on other studies, we are confident we will be able to detect developing parasitoid embryos in sharpshooter eggs. We hope to be able to determine the both the host and parasitoid species from sharpshooter egg cases from which the parasitoids have eclosed by amplifying the layer of cells which remain in the parasitoid egg (Oda and Akiyama-Oda, 2008).

RESULTS AND DISCUSSION

Because the available sequences proved unsuitable for the development of satisfactory HRM primers we have used the iCODEHOP program of the University of Washington (Rose *et al.*, 1998), in conjunction with the Lasergene suite of sequence analysis programs, to design degenerate primers targeting a number of genes characterized in various insect species (**Table 1**). Amplification parameters are being developed using the Epicentre Biotechnologies (Madison, WI) FailSafe™ PCR PreMix Selection Kit. PCR products are being cloned using the pGem T-Easy (Promega Corporation, Madison, WI) and the TOPO TA (Invitrogen Corporation, Carlsbad, CA). Cloned PCR products are being sequenced by the Genomics Core sequencing service of the Institute for Integrative Genome Biology on the U.C. Riverside campus.

Table 1. All sequences are 5' to3'. The consensus portions of primers are in uppercase letters while the degenerate portions are in lower case. R = A,G; Y = C,T; M = A,C; K = G,T; S = G,C; W = A,T; H = A,C,T; B = G,T,C; V = G,C,A; D = G,A,T; and N = A,C,G,T.

Gene	Primer	Sequence	Reference
Antennapedia	AP-1CHBF	TGCCATACCCAAGATTCccnccntayga	Hoskins *et al.*, 2007
	AP-1CHCR	CAGGAGGCGTACACAACTGgytgytgytgyt	
	AP-2CHDF	AAGTGCATCAAAACCATCATCAyatggsnatgt	
	AP-2CHER	TTCCAGGGTTTGGTATCTGgtrtangtytg	
Cryptochrome	CC-1CHCF	GGAATAAATAAAATATGTTTCGAACAAgaytgygarcc	Yuan *et al.*, 2007
	CC-1CHDR	TCCACGGTATGCAGGAACatytgrtangt	
KAAT1/CAATCH1	KCAT-1CHAF	GGGCCTGGGCAACGTntggmgnttyc	Castagna *et al.* ,1998
	KCAT-1CHER	CCACGTAGGGGAAGATGgcnarraarta	
	KCAT-2CHEF	GCCTACTTCCTGGCCATCttyccntayrt	
	KCAT-2CHGR	AGAAGTCGAACTTGGCGATGryrtcnggrta	
Maltase	MAL-1CHAF	TGGCAGCACGGAAACTTCtaycarrtnta	Russell *et al.*, 2009
	MAL-1CHDR	CCGGGTTCCGGTAGTTCarrtcngsytg	
V-ATPase c	VATPc-1CHAF	TGTCCGACGACCTGggnaarytnga	Merzendorfer *et al.*, 2000
	VATPc-1CHCR	GTGGTCAGGTATTCGGAGTCCwrdatraartg	
	VATPc-2CHCF	GCTTCCAGTGGGATATGgcnaartaycc	
	VATPc-2CHER	CATCAGCTTGGTCATCTCGttyttnccngc	
Vitellogenin	VIT-1CHDF	CCAAGAACAAAAACAAGGTCTGTTCmgnaaratgga	Meng *et al.*, 2006
	VIT-1CHFR	CTGGTTTATGGGCGGTAccngtccaytc	
	VIT-2CHGF	CTGGTAAACAAGGTCCAATTTACaargcngarac	
	VIT-2CHJR	TCGGATTTTGGCATGTTGttnggrttytg	
	VIT-3CHNF	CGCCCATAACTTCTACCCAACncayatgtayg	
	VIT-3CHQR	GCCACTCTCACTTCGTATGGTtcngcngtrtt	

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RNA-INTERFERENCE AND CONTROL OF THE GLASSY-WINGED SHARPSHOOTER AND OTHER LEAFHOPPER VECTORS OF *XYLELLA FASTIDIOSA*.

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ABSTRACT
Here we present our progress obtained in the development and application of an RNA interference (RNAi) based system aimed to target genes of sharpshooter vectors of *Xylella fastidiosa*, the causal agent of Pierce's disease (PD). We have demonstrated RNAi effects in glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) whole insects after intrathoracic injection of dsRNAs, by monitoring insect phenotype and molecular markers. Phenotypic effects vary from none observed to death, but the latter has been observed so far only when specific instars were injected with actin dsRNA inducers. We have also developed tools for RNAi, including constructs designed for expressing dsRNAs as RNAi inducers to be employed in generation of transgenic plants. The constructs were inserted in a user-friendly Gateway binary vector that facilitated simple cloning steps. This vector has two forms, one including the 35S promoter and a second version containing the *Eucalyptus gunii* minimal xylem-specific promoter. Some of these constructs have been already employed and transgenic plants are being developed by the Ralph Parsons UC Davis Plant transformation facility, while other constructs have been developed to use in 'in house' generation of transgenic *Arabidopsis thaliana* via flower dipping. We broadened the choice of target GWSS genes including genes expressed in the insect midgut, since our results showed that ingested dsRNA is concentrated mainly in that specific insect body part. We believe that our efforts are well spent, and that results in the use of RNAi to assist in efforts to control the PD vectors are well on their way.

LAYPERSON SUMMARY
Pierce's disease (PD) of grapevines is one of the plant diseases caused by the Gram-negative bacterium *Xylella fastidiosa (Xf)*. This bacterium, upon inoculation in the plant host, travels within and attaches to the plant xylem vessels where, after multiplication, produces biofilms which interfere with the water-flow in the infected plants. Resulting infected plants can die between one and five years after inoculation. *Xf* is vectored by many leafhoppers, but one of the most important vectors in many areas is the glassy-winged sharpshooter (GWSS). The importance of GWSS can be attributed to its ability to colonize more than 100 species of plants, its propensity for long distance dispersal and its capacity for ingesting large volumes of fluids from colonized plants. We are taking a contemporary molecular targeting approach to disrupt normal gene expression in GWSS and other sharpshooter vectors of *Xf* as a strategy to help control these important insect vectors of *Xf*. We are attempting to develop RNA-interference (RNAi) as a tool to target and kill GWSS and other sharpshooter vectors of *Xf*. RNAi is normally used by plants, arthropods, some fungi and even nematodes to degrade specific mRNAs, including those of the host during normal organismal development, but also those of pathogenic viruses. We have clearly demonstrated that introducing specific double-stranded RNAs (dsRNAs) into GWSS cells, and by intrathoracic injection into whole insects we can inititate the RNAi-mediated degradation of homologous mRNAs, and in some cases death. We are now attempting to identify genes necessary for the fitness and survival of GWSS, and we are generating transgenic plants (grapevines but also potential trap plants such as citrus and potatoes) expressing dsRNAs corresponding to the identified GWSS mRNAs. Our hope is that ingestion of these dsRNA molecules by GWSS will trigger RNAi activity in the recipient insects, resulting in the subsequent degradation of the targeted mRNAs and corresponding debilitating effects on sharpshooters, thereby contributing to strategies for PD control.

INTRODUCTION
During the work supported by this research program, we developed tools to induce RNA interference (RNAi) in the insect vectors of Pierce's disease, and in particular in glassy-winged sharpshooter (GWSS). We were able to induce RNAi for specific genes *in vitro* in a GWSS cell line developed at UC Davis (Kamita et al., 2005; Rosa et al., 2010) and in whole insects (Rosa et al., 2011). We also optimized protein, small and large RNA hybridization and real time PCR techniques to detect the extent of RNAi induced in the different systems. We were able to generate phenotypes in both cells and 5th GWSS instars affected by RNAi. While our previous efforts were limited by the scarce amount of nucleotide sequences available in GenBank, our present research is based on the sequence analyses of extensive GWSS EST data released via GenBank. We cloned a series of genes predicted to be expressed during insect digestion and during molting, and we inserted sequences for these genes into an easy to manipulate binary vector (Lei et al., 2007) set up for hairpin RNA transcription in plants. We also modified this vector substituting the 35S promoter with a minimal xylem specific promoter cloned by us from *Eucalyptus gunii*. The potential of these series of vectors is great, in fact we are using these plasmids now to generate transgenic plants of different species (grape, citrus and potatoes) via the Ralph M. Parson Foundation facility of UCD and at the same time to generate in a fast and convenient way *Arabidopsis thaliana* plants via flower dipping Clough (1998).

OBJECTIVES

1. To evaluate existing transgenic plants for their ability to generate RNAs capable of inducing RNAi effects vs. GWSS.
2. To identify GWSS interfering RNAs for practical application.
   2.a. To utilize transgenic *Arabidopsis thaliana* plants as efficient alternatives for identifying, delivering, and evaluating efficacious interfering RNAs.
   2.b. To enhance production of interfering RNAs *in planta*.
   2.c. To evaluate alternative strategies to deliver and screen high number of RNAi inducers to GWSS.

RESULTS AND DISCUSSION

To evaluate existing transgenic plants for their ability to generate RNAs capable of inducing RNAi effects vs. GWSS .
Grapevines *Thompson seedless* and Carrizo citrange were transformed and regenerated at the Ralph M. Parsons Foundation Plant Transformation Facility. So far, we have received seven grape transformants engineered to express the GUS reporter gene under control of the 35S promoter. These plants, which will be used as controls in our experiments, are already in our greenhouse and will be tested for GUS expression once they reach appropriate size (in the next month). Unfortunately, transformation and regeneration of grape and citrus plants has not been successful using our initial xylem promoter constructs. We have modified this promoter to now be a minimal, easier to use promoter. In order to make more rapid progress we investigated using herbaceous GWSS host plants for transformation/regeneration. Potato is a good host for GWSS (**Figure 1**) and is readily transformed and regenerated. Therefore, transformation and regeneration of potato plants is proceeding, and we expect to obtain the first explants containing specific GWSS genes before the summer 2011. These will be vegetatively propagated, analyzed for the desired transgene and RNAi effectors, and used for GWSS-RNAi experiments in the UC Davis CRF.

Figure 1. GWSS adults and nymphs colonizing potato plant.

To identify GWSS interfering RNAs for practical application.
To generate binary vectors for simple cloning and which can express hairpin dsRNAs in plants, we used the Gateway modified pCB2004B vector (**Figure 2**). This vector has a 35S promoter cloned between DraIII directional restriction enzyme sites, and 4 attR sequences in a head to tail orientation, flanked by a spacer region. The 4 attR sequences are efficient sites for recombination, and any insert cloned in the compatible pCR8-TOPO entry clone can be moved in both pCB2004B recombination sites in one simple clonase reaction, resulting in a fully functional plasmid expressing a dsRNA hairpin loop. The pCB2004B plasmid has a glufosinate resistance gene for plant selection and we will use it for transformation of the species indicated here. The 35S promoter can be digested and replaced by any other promoter flanked by DraIII restriction sites.

We have already cloned 10 novel cDNA sequences of GWSS genes involved in digestion processes and cuticle formation. These genes encode for the following proteins:

> a hydrolase, a glucosyltransferase, a peptidase, two trypsins, a transladolase, a sugar transporter a serine-type endopeptidase inhibitor, a transketolase, a cuticle regeneration and a structural cuticle proteins.

All the constructs are now in a head to tail orientation to generate hairpin RNA in the binary vector under the control of the 35S promoter. We are now in the process of moving the same constructs so hairpin dsRNAs will be expressed via the minimal xylem specific promoter cloned previously by us from *Eucalyptus gunii*.
We also identified and are cloning GWSS genes expressing the following proteins:

a triosephosphate isomerase involved in glycolysis, gluconeogenesis and fatty acid biosynthesis, a sugar transporter, three yellow proteins involved in male courtship and cuticle pigmentation, two additional lipid transporters, an additional serine-type endopeptidase.

We will assess these candidate sequences for RNAi effects during the upcoming year.

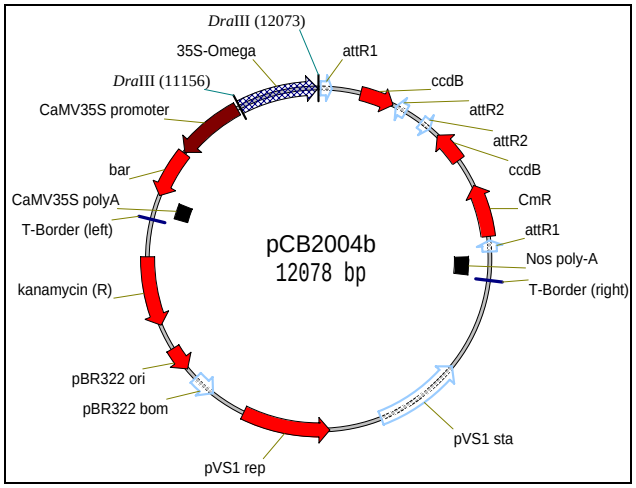

Figure 2. Map of pCB2004b, binary plasmid, Gateway compatible and designed for generating hairpin dsRNAs in transgenic plants

2.a. To utilize transgenic *Arabidopsis thaliana* plants as efficient alternatives for identifying, delivering, and evaluating efficacious interfering RNAs.

We selected and propagated the *Arabidopsis thaliana* ecotype Cape Verdi Islands (CVi-0). This ecotype has larger leaves and presents a more robust growth, and will be more appropriate in supporting insects of large size such as GWSS (**Figure 3**). Every ten days, groups of *A. thaliana* plants are flower-dipped in solutions of *Agrobacterium tumefaciens* transformed with the binary plasmids made as described above. To enhance the percentage of transgenic seeds at collection, set seeds are removed before dipping. After the dipping, plants are returned for continued growth. When seeds show signs of maturity, the dipped plants are set aside to dry down before seeds are collected. The collected seeds are air-dried and then planted at the CRF. Selection for transgenic seedlings is done by 2-3 applications of glufosinate ammonium (200-300 µM), as transgenic *A. thaliana* carries bacterial bialophos resistance gene (*BAR*) encoding the enzyme phosphinotricin acetyl transferase (PAT) and thus, plants with this gene are resistant to glufosinate ammonium. The surviving seedlings will be used to perform RNAi experiments. GWSS nymphs will be caged in groups of five on individual transgenic seedlings. Non-transgenic *A. thaliana* plants will be used as controls. GWSS fitness and survival will be monitored for 10 days, at that time insects as well as plant tissues will be collected. The amount of target mRNAs will be measured in insects by real time PCR or northern blot, and the presence of specific siRNAs will be assessed. Transgenic plants will be also tested for the presence of transgenes by PCR. The use of transgenic *A. thaliana* plants as alternative to identify efficacious interfering RNAs has shown to be a feasible method in our hands, and we are planning to complete the first experiments on insect survival in the next three months.

2.b. To enhance production of interfering RNAs *in planta*.

We used a transient *Agrobacterium tumefaciens* assay to generate an exogenous hairpin mRNAs accompanied by linear mRNA (with the same sequence of one of the two polarities present in the hairpin mRNA) in *Nicotiana benthamiana* plants, and we estimated by northern blot analysis, the amount of specific siRNA generated compared to plants infiltrated only with the exogenous hairpin mRNA. Our results suggest that there were not differences between the two treatments, and that amount of siRNAs generated by dicing of the dsRNA produced by the hairpin construct should be sufficient to induce RNAi in the insects feeding on the plants. This suggests that we will use the more simple approach to generate transgenes only expressing the hairpin dsRNAs.

Figure 3. GWSS nymphs colonizing *Arabidopsis thaliana* plant.

2.c. To evaluate alternative strategies to deliver and screen high number of RNAi inducers to GWSS.
We are considering the use of viral vectors based on *Cucumber mosaic virus* (CMV), to deliver RNAi inducers to GWSS. We already performed preliminary experiments and were able to infect both basil and *Arabidopsis thaliana* plants with different strains of CMV, belonging to subgroup I and II. We also have determined that we can detect by RT-PCR the virus-specific RNAs in GWSS nymphs fed on the virus-infected melon plants. CMV is a virus that invades most tissues, except it is not believed to invade the xylem of the plant host. Our results here raise interesting questions as to whether GWSS acquires RNAs (and other compounds) from other plant tissues than just xylem, or if CMV may also invade xylem tissues. In any case our results also suggest that we can possibly viruses to deliver RNAi inducers molecules GWSS via virus-infected plants. We are proceeding with that now as a strategy to complement the above efforts, and to quantify the amount of specific RNAs ingested by GWSS from CMV-infected vs. transgenic plants.

CONCLUSIONS
We are moving forward with development and potential application of RNAi as a tool to assist in efforts to control sharpshooter vectors of *Xf*. Efforts are underway by many groups to assess RNAi strategies for controlling insect vectors of plant pathogens and thus we are likely to learn more not only from our efforts, but also from those of the many labs working in this new research area. We have demonstrated RNAi effects, including death of GWSS, after injection of dsRNAs corresponding to specific mRNA targets. Other dsRNAs directed towards different targets showed no effects. Taken together these data show that identifying the correct target will be critical. So far effects have come from intrathoracic injection of dsRNAs, we are now focused on dsRNA oral delivery and comparison with results obtained from injection. We are in good position to continue to make progress and the upcoming year should be even more informative for RNAi-based studies on insect vectors of plant pathogens.

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THE ENDOCRINE SYSTEM OF THE GLASSY-WINGED SHARPSHOOTER, A VIABLE INSECTICIDE TARGET

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Reporting Period: The results reported here are from work conducted August 2010 to October 2010.

ABSTRACT
Minor disruption of the endocrine system can result in dramatic changes in insect development. Juvenile hormone analog (JHA) insecticides are green compounds that selectively disrupt the insect endocrine system. In this project we are testing the efficacy of JHAs against glassy-winged sharpshooter (GWSS) eggs, nymphs, and adults. We are also evaluating the potential of juvenile hormone esterase (JHE)- and juvenile hormone epoxide hydrolase (JHEH)-encoding genes as a target for gene silencing-based control of GWSS. In terms of mode of action, the effects of JHA application, and JHE and/or JHEH knockdown are similar in that both approaches can enhance "JH action" during periods of developmental when endogenous levels of JH are exceptionally low.

LAYPERSON SUMMARY
The overall goal of this project is to study and exploit targets within the endocrine system of the glassy-winged sharpshooter (GWSS) that can be used to reduce the spread of Pierce's disease. We are taking two complementary approaches in this project. The first is a direct approach in which the efficacy of juvenile hormone analog (JHA) insecticides such as fenoxycarb and pyriproxyfen are being tested against GWSS eggs, nymphs, and adults. The objective of this direct approach is to identify JHAs that can efficiently (*i*) reduce the emergence of nymphs from eggs, (*ii*) keep nymphal insects in the nymphal stage, and/or (*iii*) reduce egg viability prior to oviposition. The results of the JHA efficacy trials will have near-term applicability since the JHAs that we are testing are US-EPA registered and commercially available. The second approach involves the identification and characterization of GWSS genes that metabolize a key hormone in insects called JH. The objective of this approach is to evaluate these genes as a potential target for gene silencing.

INTRODUCTION
Insect development is precisely regulated by the relative titers of juvenile hormone (JH) and molting hormones (i.e., ecdysteroids). JHs form a family of sesquiterpenoids (**Figure 1A**) that regulate key biological events in insects including reproduction, behavior, polyphenisms, and development (reviewed in (Riddiford, 2008). Minor disruption of an insect's hemolymph JH levels can result in dramatic alterations its development. Juvenile hormone analog (JHA) insecticides are green compounds that selectively target the insect endocrine system (reviewed in (Dhadialla et al., 2005; Henrick, 2007). JHAs such as methoprene, fenoxycarb, and pyriproxyfen (**Figure 1B**) are US EPA-registered compounds that are commonly used to control mosquitoes, fleas, whiteflies, ants, and other insect pests. JHAs function as mimics (both structural and biological) of juvenile hormone (JH), a key insect developmental hormone. When pest insects are exposed to JHAs at a time during development when JH titer is normally undetectable, abnormal nymphal-pupal development and/or death is induced. Similar abnormal development morphology can also be induced by the inhibition of a JH-selective esterase (JHE) with a chemical inhibitor such as OTFP (Abdel-Aal and Hammock, 1985). Inhibition of JHE putatively results in JH titers that are not below the threshold required for normal development. In this project we are attempting to identify and clone the *jhe* gene and related JH epoxide hydrolase, *jheh*, gene. The potential of these genes as a target for gene silencing (so that glassy-winged sharpshooter (GWSS) development can be disrupted) will also be evaluated.

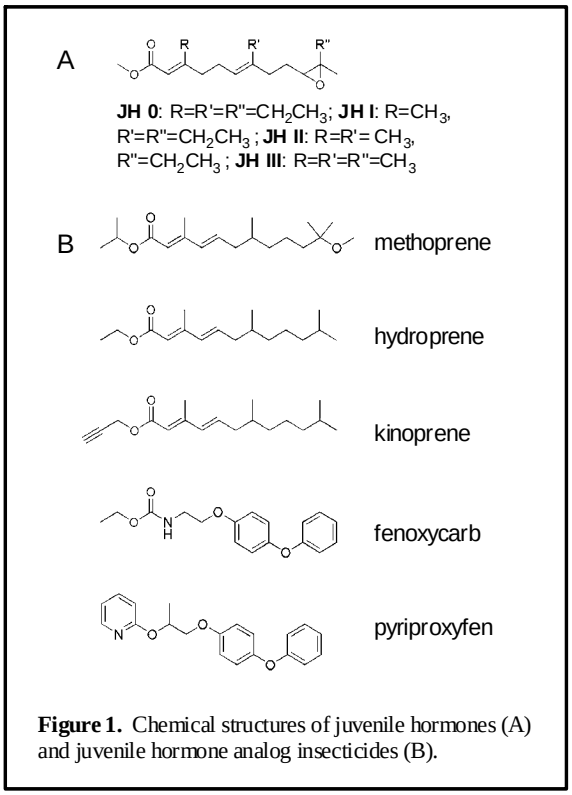

Figure 1. Chemical structures of juvenile hormones (A) and juvenile hormone analog insecticides (B).

OBJECTIVES

1. Evaluate the efficacy of JHA insecticides
    A. Determine median lethal dose and effective time in nymphs
    B. Determine median lethal dose in eggs
    C. Evaluate ovicidal effects following treatment of adult females
2. Characterize authentic JH esterase (JHE) activity in GWSS
    A. Quantify authentic JHE activity in 5th instar nymphs
    B. Evaluate the ability of OTFP to inhibit JHE activity in nymphs
3. Isolate *jhe* gene and characterize recombinant JHE protein
    A. Isolate JHE coding sequence from GWSS
    B. Express and biochemically characterize recombinant GWSS JHE
    C. Test the efficacy of an RNAi approach to silence GWSS *jhe*

RESULTS AND DISCUSSION

I. Evaluate the efficacy of JHA insecticides

A laboratory colony of GWSS has been established at the UC Davis Contained Research Facility with the help of our cooperator Bryce W. Falk. The GWSS colony is reared in an environmental growth chamber (Percival Scientific, Perry, IA) set for a 14 h:10 h light:dark cycle at 24ºC and 70% relative humidity. Within the chamber the GWSS are grown in Bug Dorm insect cages each containing two cowpea, two cotton, and two basil plants in five-inch pots. The colony appears to be very robust with a full range of nymphal instars and adults. Oviposition of eggs occurs primarily on cotton and cowpea. Experiments to determine the development time of GWSS reared on basil are currently in progress. In these experiments, newly emerged nymphs are placed on a single basil plant (ca. 10 cm in height) that is placed in a cylindrical (10 x 24 cm) acrylic cage that is capped at one end with nylon mesh. These cages are also placed in the environmental growth chamber described above. The development of insects is scored daily. The current data indicate that the development times of each nymphal instar on basil (i.e., first instar: 5.3±0.5 days; second instar: 4.3±0.5 days; third instar: 5.0 days) are very similar to those found on sweet potato by Lauziere and Setamou (2009). In preliminary experiments in which first instar nymphs were exposed to the JHA methoprene, no mortality or delays in development (approximate 30 day long development time to adult eclosion) were found. These nymphs were exposed to the methoprene (0.2 ml of a 0.5 or 5.0 ppm solution applied to the surface of a 20- ml glass vial (32 cm^2 surface area)) for only one hour. As soon as we determine the exact development times of GWSS under our rearing conditions, a full complement of dose- and time-respnse experiments with methoprene and other JHAs will be performed.

II. Characterize authentic JH esterase (JHE) activity in GWSS

Experiments to characterize JHE activity in GWSS will be performed using precisely staged nymphs. Experiments to determine the developmental times of GWSS under our rearing conditions are still in progress (see Section I above).

III. Isolate *jhe* gene and characterize recombinant JHE protein

In order to clone potential JHE- and JHEH-encoding genes of GWSS, total RNAs were collected from 5th instar nymphs. The total RNAs (1.2 µg) were used for first strand cDNA synthesis using the poly-T primer CDS III/3' PCR (Clontech, Mountain View, CA). Attempts to PCR-amplify the 3'-end and 3'-UTR of the putative *jhe* and *jheh* genes of GWSS were made using numerous degenerate primers that recognized conserved sequences in known JHE and JHEH gene (reviewed in (Kamita and Hammock, 2010). Candidate amplicons following the PCRs are now being cloned into plasmid vectors and will be sequenced.

CONCLUSION

A little over two months have passed since we started this project, during this time we have established our laboratory colony of GWSS and are in the process of determining precise developmental times under our rearing conditions. Determination of precise developmental times is critical for quantifying the efficacy and effects of the JHAs, effects of JHE-inhibitors, and JHE/JHEH levels in GWSS. We expect to complete determining GWSS developmental times during the next month.

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RIVERSIDE COUNTY GLASSY-WINGED SHARPSHOOTER AREA-WIDE MANAGEMENT PROGRAM IN THE COACHELLA AND TEMECULA VALLEYS

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Reporting Period: The results reported here are from work conducted October 2009 to September 2010.

ABSTRACT
Riverside County has two general areas where citrus groves interface with vineyards, the Coachella and Temecula Valleys. The Coachella Valley with 10,438 acres of table grapes in proximity to 12,000 acres of citrus and the Temecula Valley with 2,000 acres of wine grapes in proximity to 1,000 acres of citrus are vulnerable to Pierce's disease (PD), caused by the bacterium *Xylella fastidiosa.* The grapes in the Coachella and Temecula areas of Riverside County are in jeopardy because of the glassy-winged sharpshooter (GWSS), the vector of the PD bacterium, build up in adjacent citrus groves. Citrus is an important year around reproductive host of GWSS in Riverside County, but also one that concentrates GWSS populations over the winter months during the time that grapes and many ornamental hosts are dormant. GWSS weekly monitoring in citrus in grapes began in March 2000 in Temecula Valley and 2003 in Coachella Valley by trapping and visual inspections. Temecula valley GWSS populations in 2008 reached levels not seen prior to the initiation of the area wide GWSS program in 2000. Coachella Valley GWSS populations have decreased dramatically since the treatment program was initiated in 2003.

INTRODUCTION
The glassy-winged sharpshooter (GWSS) vectors the bacterium that causes Pierce's disease (PD). This insect and bacterium are a severe threat to California's 890,000 acres of vineyards and $61 billion dollar industry. An area-wide GWSS management program was initiated in Temecula in 2000 to prevent this vector's spread into other California grape growing regions. In Temecula valley itself, the wine grape industry and its connecting tourist industry generate $100 million of revenue for the economy of the area. GWSS/PD caused a 40% vineyard loss and almost destroyed the connecting tourist industry. The area wide GWSS management program initiated in the spring of 2000 saved the industry from a 100% loss. Only a continuation of an area-wide GWSS management program will keep the vineyards viable in Temecula. The table grape industry in the Coachella Valley is represented by 7,797 acres of producing vines, which generate fresh market grapes valued at an average of $117 plus million annually (Coachella Valley Acreage and Agricultural Report 2009). The GWSS was identified in the Coachella Valley in the early 1990's. Population increases of this insect in Coachella Valley in the last three years have increased the danger of PD occurrence in this area, as has occurred in similar situations in the Temecula and San Joaquin Valleys. In July 2002, the occurrence of *Xylella. fastidiosa*, the PD bacterium, was found in 13 vines from two adjacent vineyards in the southeastern part of Coachella Valley. With this discovery, and the increasing GWSS populations, there was and is a real need to continue an area-wide GWSS/PD management program. The GWSS area wide management program is needed to prevent an economic disaster to the work forces and connecting small businesses of Mecca, Thermal, Coachella, Indio, etc. that depend upon the vineyards for a big portion of their incomes. Only a continuation of an area wide GWSS/PD management program will keep the vineyards viable in Coachella. At present there are no apparent biological or climatological factors that will limit the spread of GWSS or PD. GWSS has the potential to develop high population densities in citrus. Insecticide treatments in citrus groves preceded and followed by trapping and visual inspections to determine the effectiveness of these treatments are needed to manage this devastating insect vector and bacterium. Approximately 1,600 acres of citrus in Riverside County were treated for the GWSS in April through September, 2010 between a cooperative agreement with USDA-APHIS and the Riverside Agricultural Commissioner's Office under the "Area-Wide Management of the Glassy-Winged Sharpshooter in the Coachella and Temecula Valleys."

OBJECTIVES
1. Delineate the areas to be targeted for follow-up treatments to suppress GWSS populations in the Temecula and Coachella Valleys for 2010 and 2011.
2. Determine the impact of the GWSS area-wide treatments to suppress GWSS populations in citrus groves and adjacent vineyards.

METHODS, RESULTS AND CONCLUSIONS
The programs in Coachella and Temecula were dependent upon grower, pest management consultants, citrus and vineyard manager's participation. The areas encompass approximately 28,000 acres. Representatives of various agencies were involved in the program, they were as follows: USDA-ARS, USDA-APHIS, CDFA, Riverside County Agricultural Commissioner, UC-Riverside, UC Cooperative Extension, and grower consultants. Representatives of these agencies meet to review the program. Newsletters are sent to growers, managers, wineries, and agencies with information on GWSS

populations and insecticide treatments via e-mail. The information from Temecula is sent weekly, while information from Coachella goes to the various parties monthly.

The GWSS/PD citrus groves and vineyards within the Temecula GWSS/PD management areas were monitored weekly to determine the need and effect of insecticide treatments on GWSS populations. In August, 2008, because of the lack of GWSS trap catches in Coachella valley, a bi-weekly schedule was initiated. Yellow sticky traps (7 x 9 inches) were used help determine GWSS population densities and dispersal/movement within groves and into vineyards (**Figures 1 and 2**). Approximately 1,400 GWSS yellow sticky traps are monitored in the Riverside county area wide program. Based on trap counts and visual inspection, approximately 1,000 acres of citrus were treated in Temecula valley for GWSS in 2010. In 2010, a total of 596 acres of citrus were treated to manage GWSS in Coachella valley. Three hundred acres of citrus of the citrus were treated with Admire Pro (imidacloprid) and 296 acres were treated with Nuprid (imidacloprid) in Coachella Valley for GWSS area wide management. Admire Pro and Nuprid were both applied at the rate of 14 oz/acre. Because of high Temecula GWSS trap catches in the late summer and early autumn of 2008 and 2009, imidacloprid (Admire Pro) applications in citrus were initiated in April, 2010 (**Figure 3**). Admire Pro was applied at the rate of 14 oz/acre. Of the 1,000 acres of treated citrus, 46 acres of organically farmed citrus were treated with PyGanic (1.4% Pyrethrins) at 18 oz/acre. Because of the low residual of the organic insecticides the organic citrus was treated three times during the season. PyGanic treatments were applied to the 46 citrus acres in early June, late July and September.

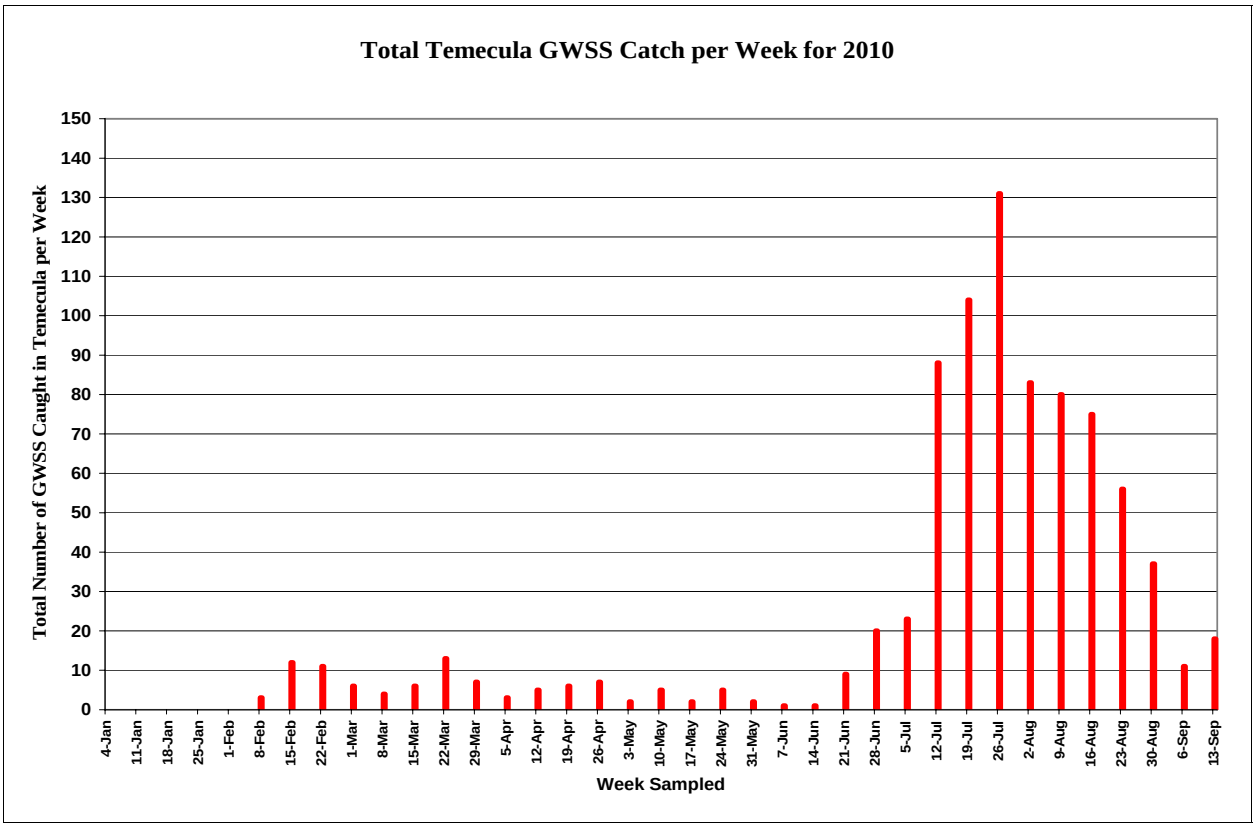

Figure 1. In 2010, the highest numbers of adult GWSS were trapped in July, reaching a total of 131.

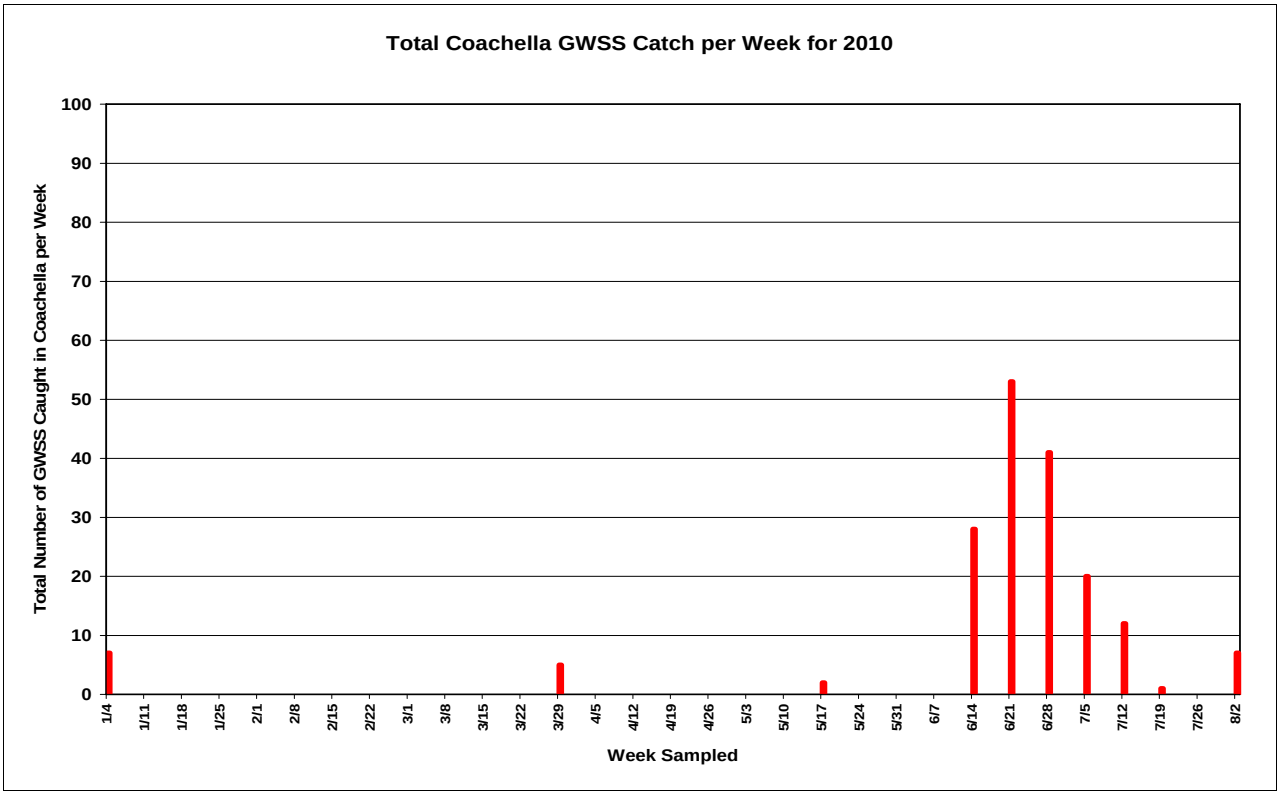

Figure 2. GWSS populations in Coachella Valley peaked in June with a high of 53 trapped

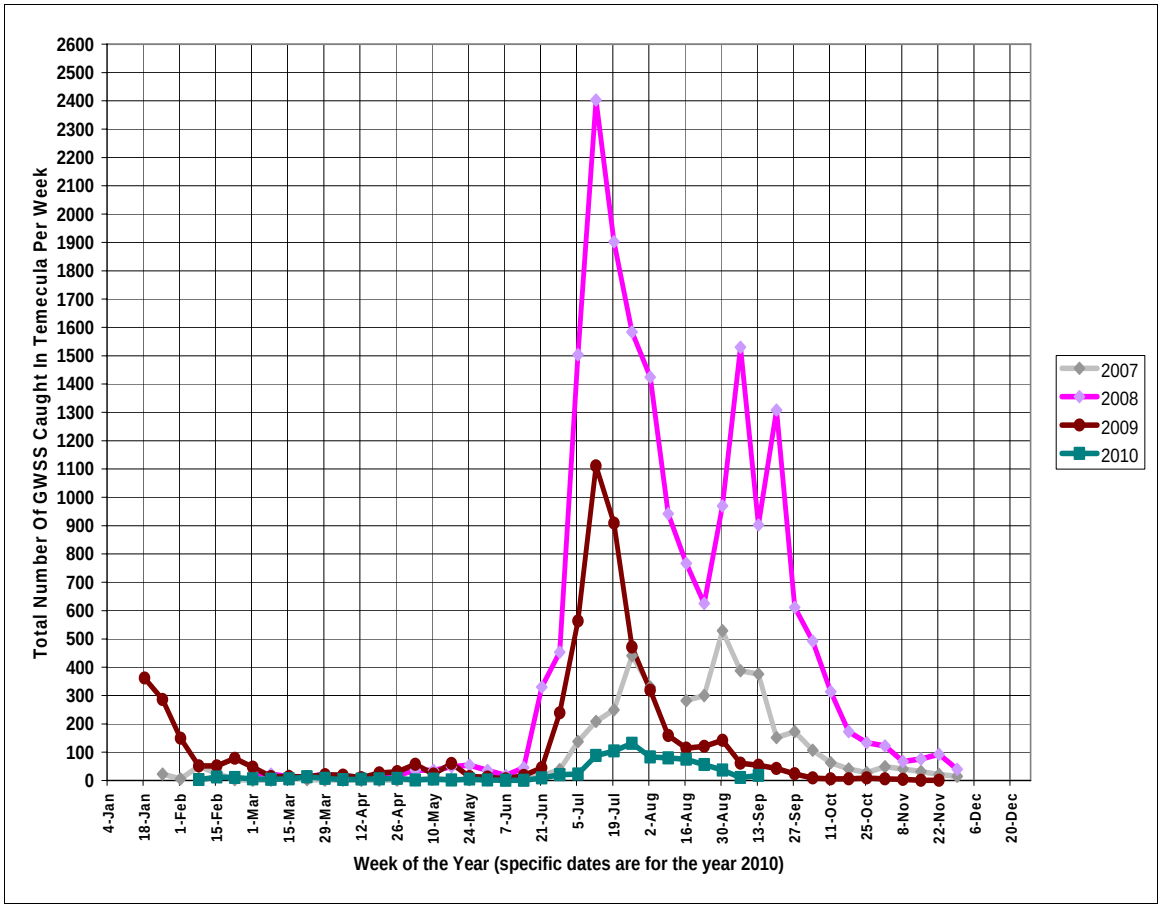

Figure 3. Temecula GWSS population comparisons from 2007-10.

For a successful area-wide GWSS management program with large acreages of citrus, a management program has to be maintained. Organic insecticides are not as effective as the neonicotinoid insecticides such as imidacloprid for controlling GWSS. Therefore, organic insecticides will have to be applied more frequently than its synthetic counterpart. In our Riverside County GWSS area wide program organic citrus groves pose challenges to area-wide GWSS management programs.

For more information on the management of GWSS visit the web site http://ucbugdr.ucr.edu.

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We would like to especially thank Ben Drake of Drake Enterprises for his input and counsel and the grape and citrus grower, managers and pest control advisors for their needed cooperation to make the Riverside County GWSS area wide management program successful. We want to thank Heavenly Clegg for her development of the Temecula GWSS newsletter and Gevin Kenny for managing the Temecula GWSS monitoring and data analysis.

GLASSY-WINGED SHARPSHOOTER INFESTATIONS INDUCE GRAPE HOSTS TO PRODUCE VOLATILE COMPOUNDS WHICH MAY ATTRACT EGG PARASITOIDS

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Reporting Period: The results reported here are from work conducted June 2010 to October 2010.

ABSTRACT
Natural predators or parasitoids may be attracted to prey by specific volatiles emitted from prey-infested host plants. Glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar), is a pest of particular concern as a vector of the Pierce's disease pathogen, *Xylella fastidiosa*. GWSS egg parasitoids (e.g. *Gonatocerus ashmeadi* Girault and related species) are attracted to GWSS-infested plants via olfactory cues (Krugner et al. 2008). This project evaluated infested grape hosts for production of novel or increased quantities of volatiles that may attract egg parasitoids. In a closed-chambered system, volatile compounds were captured on PorpakQ sorbent over an eight-hour period from grapes that were either infested or not infested with GWSS eggs. Volatile compounds were desorbed into methyl tert-butyl ether and analyzed by gas chromatography. Early results suggest at least one compound (putatively identified as 1,8-cineole) was present in higher amounts around infested plants than non-infested plants, and was positively associated with number of egg masses per plant. This compound subsequently will be examined for ability to attract GWSS egg parasitoids. Discovery of a host-produced compound that attracts parasitoids to GWSS egg masses could affect pest management programs. For instance, breeders could screen for host cultivars that produce higher levels of the parasitoid-attracting compound when infested. The attractant compound also could act as a lure for egg parasitoids, aiding studies that monitor parasitoid numbers.

LAYPERSON SUMMARY
Glassy-winged sharpshooter (GWSS) populations are being controlled, in part, by egg parasitoids, which apparently detect one or more volatile chemicals to find hosts with egg masses. This study examined both GWSS-infested and non-infested grapes to discover which volatile compounds, if any, may be produced by the host as a result of being infested. Early results suggest that at least one compound (putatively identified as 1,8-cineole) was present at higher concentrations around infested plants, and concentration was significantly correlated with number of egg masses on infested grapes. Following the confirmation of these results, these compounds will be tested for ability to attract parasitoids of GWSS. These results should allow breeders to select cultivars which naturally produce more parasitoid-attracting compounds when infested by GWSS (increasing efficacy of egg parasitism as a management strategy), and to provide a lure to attract egg parasitoids.

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FUNDING AGENCIES
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Section 3: Pathogen Biology and Ecology

BLOCKING *XYLELLA FASTIDIOSA* TRANSMISSION

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ABSTRACT
The insect-transmitted bacterium *Xylella fastidiosa (Xf)* colonizes the foregut of its insect vectors. We provide evidence suggesting that chitin in the cuticle of leafhopper vectors of *Xf* may serve as a carbon source for this bacterium. Chitin-enhanced media resulted in *Xf* growth to larger populations. In addition, chitin induced phenotypic changes such as increased adhesiveness. Furthermore, transcriptional changes in the presence of chitin were observed. We demonstrated chitinolytic activity by *Xf* and identified an ortholog of chitinase A (*chiA*) in the *Xf* genome. *chiA* encodes a protein of 351 amino acids with an expected molecular mass of 40 kDa. *chiA* is in a locus that consists of genes implicated in polysaccharide degradation; ChiA was determined to be secreted by cells. We cloned *chiA* into *Escherichia coli* and endochitinase activity was detected in transformants. These findings show that *Xf* has chitinolytic activity and that chitin utilization may be important for vector colonization. Moreover, chitin induces *Xf's* gene regulation and biofilm formation. The data presented here were recently published (Killiny *et al* 2010)

INTRODUCTION
Xylella fastidiosa (Xf) has a complex life history since it has to interact with host plants and insect vectors. In our previous work we characterized the molecular interactions of *Xf* with the surface of its vector's foregut. We showed that the initial attachment occurs through carbohydrate binding proteins including hemagglutinins (Killiny & Almeida 2009b). Moreover, we found that pectin from the host plant induced the transmissibility of *Xf* by regulating gene expression and making cells stickier to surfaces. This adhesive state is essential for the initial attachment to foregut and eventually transmission to plants (Killiny & Almeida 2009a). In this report we focus on the characterization of a chitinase we found in *Xf*. Similarly to pectin, chitin induces strong phenotypic changes in this bacterium. Chitin can also be used as the sole carbon source by *Xf*. The utilization of chitin as a signal resulting in phenotypic changes to cells, including adhesiveness, may be essential for sharpshooter colonization.

OBJECTIVES
1. Molecular characterization of the *Xf*-vector interface.
2. Identification of new transmission-blocking chitin-binding proteins.

RESULTS
I- Chitin enhances *Xf* growth and induces the adhesiveness: *Xf* has chitinase activity.
Our previous work revealed the importance of plant polysaccharides in inducing *Xf* cells into a transmissible (adhesive) state (Killiny & Almeida 2009a). Additionally, we demonstrated that *Xf* cells have carbohydrate binding proteins, including hemagglutinin-like proteins and fimbrial adhesins, which are required for the efficient transmission (Killiny et al 2009b). These proteins bind to the cuticle surface of the vector foregut in the initial step of mouthpart colonization. We showed that these proteins are up-regulated in a defined medium (XFM) supplemented with pectin. Once the cells are acquired by the insect vector they attach to the surface of foregut, multiply, and form a biofilm. Adult vectors then can transmit *Xf* throughout its life. Here we provide evidence that chitin affects gene expression and maintains *Xf* cells, possibly in a 'permanent' adhesive state. **Figure 1** describes the effect of chitin in *Xf* growth, phenotype, and the gene expression of genes implicated in the adhesions to surfaces. We also provide the first evidence of the chitinase activity presence in *Xf* cells.

II- *Xylella fastidiosa* uses chitin as a sole carbon source.
The *in silico* analyses revealed the presence of an ortholog of chitinase A (PD1826) in the genome of *Xf* (**Figure 2**). We confirmed the chitinase activity of PD1826 by the expression of gene in *E. coli* and then detected the activity using ([4-MU(GlcNAc)$_3$] (**Figure 3D**). The growth of *Xf* in XFM, which contains colloidal chitin as a sole carbon source (**Figure 3A**), and on Chitin-yeast plates (**Figure 3C**) suggest that *Xf* uses chitin as a carbon source. Using quantitative PCR, we showed that the expression of *chiA* (PD1826) is correlated by bacterial population growth through time (**Figure 3A**). By using [4-MU(GlcNAc)$_3$**],** we also detected an increase in the chitinase activity (**Figure 3B**)**.** In **Figure 4** we used the hind wings of the glassy-winged sharpshooters to mimic the foregut surface. GFP-*Xf* cells were initially suspended on XFM medium with no carbon source and then this suspension was placed on the wing tissue. The increase of fluorescence over time reflected the growth of cells and confirms that *Xf* can utilize chitin from the wing surface as a carbon source. GFP-*rpfF*-*Xf* cells (Newman *et al* 2003) did not grow as much as the wildtype strain. It is known that *rpfF* mutant is not transmissible due to lack of the diffusible signaling factor (DSF) (Newman *et al* 2004). DSF regulates the expression of important genes

such as the fimbrial and afimbrial adhesions and also ChiA. We predicted genes implicated in the chitin utilization machinery similar to those in other chitinolytic bacteria. Here, we present a Hypothetical model for this machinery in *Xf* (**Figure 5A**). **Figure 5B** provides evidence for degradation of blue-green sharpshooter foregut surface by *Xf*.

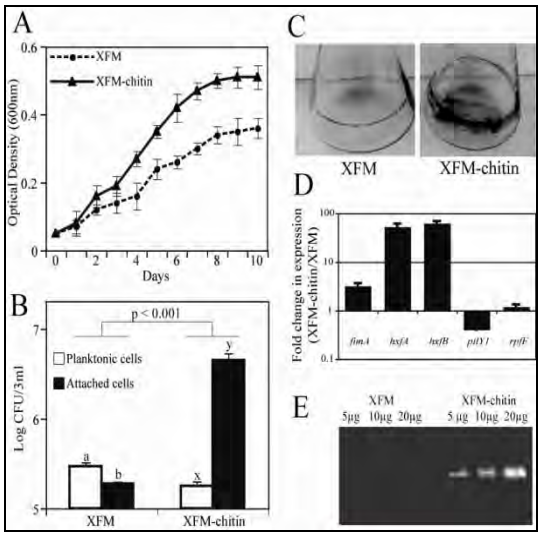

Figure 1. Effect of chitin on *Xf*. A) Growth curve of *Xf* in XFM in presence or absence of colloidal chitin. B) Log-transformed cell numbers present as planktonic or attached as a biofilm on glass in liquid XFM. Different letters on bars indicate statistically different treatments. C) Biofilm formation of *Xf* in a shaking culture; the biofilm is stained at the broth-air interface. D) Chitin induced transcriptional changes in *Xf*. E) In-gel chitinase activity ([4-MU(GlcNAc)$_3$] cleavage) of *Xf* whole-cell culture extracts.

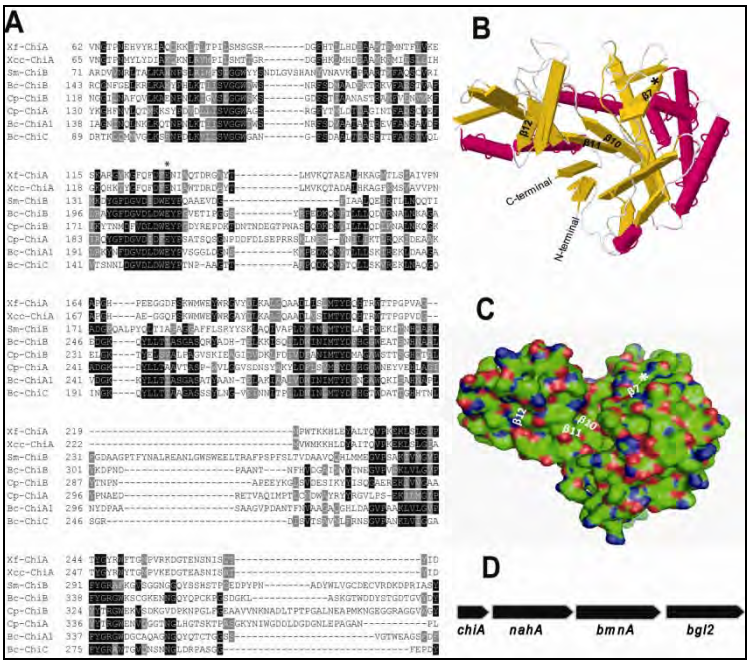

Figure 2. *In silico* analyses of *chiA* and its translated protein. A) Alignment of putative catalytic region in bacterial chitinases family 18 of *Xylella fastidiosa* (*Xf*), *Xanthomonas campestris campestris* (Xcc), *Serratia marcescens* (Sm), *Bacillus cereus* (Bc), *Clostridium paraputrificum* (Cp), and *Bacillus circulans* (Bc). Conserved amino acids are indicated with black shading and those with high similarity score are in gray. The glutamic acid residue identified as a proton donor necessary for activity is marked with an asterisk. B) Predicted three-dimensional structure and C) molecular surface of *Xf* ChiA; regions with similarity to the glycoside.

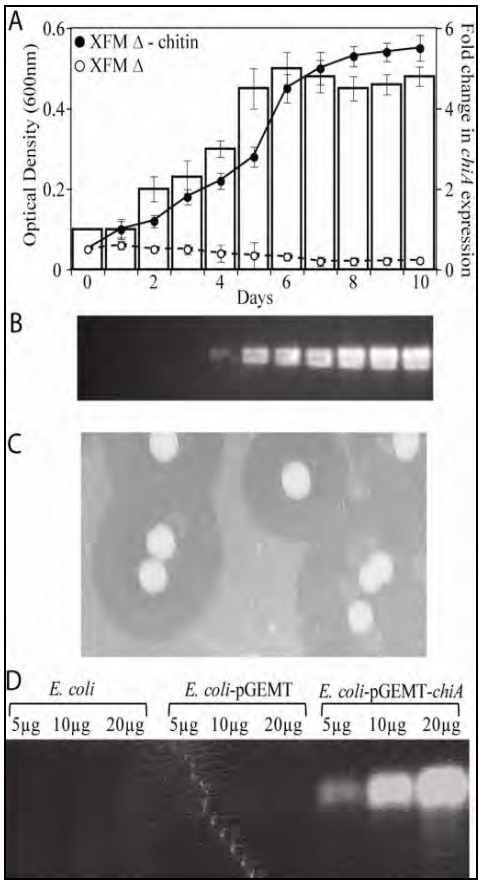

Figure 3. Chitin utilization by *Xf*. A) Bacterial growth curve in XFM medium without carbon sources (XFM Δ) or with chitin added as the only carbon source (XFM Δ-chitin); bars (second Y-axis) show the progression of *chiA* expression over time. B) In-gel chitinase activity ([4-MU$(GlcNAc)_3$] cleavage) over time for *Xf* culture filtrates in liquid XFM Δ-chitin, demonstrating ChiA secretion. C) *Xf* colonies grown on chitin-agar medium; the clear zones around colonies indicate chitin degradation. D) In-gel chitinase activity ([4-MU$(GlcNAc)_3$] cleavage) for *Escherichia coli* strains grown in LB medium.

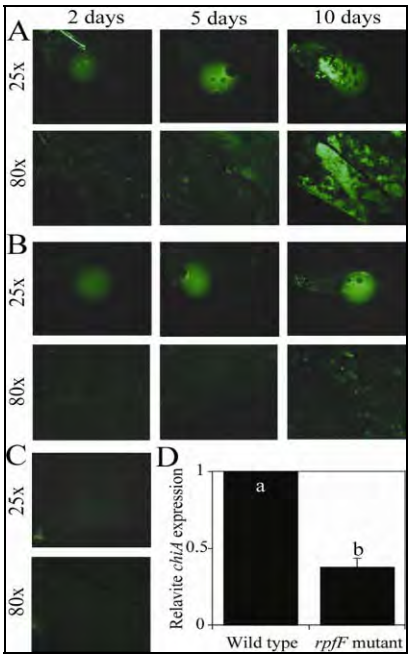

Figure 4. *Xf* growth and biofilm formation on hindwings of leafhopper vectors. Cells were suspended in XFM Δ and drops were placed on wings. A) Wild-type and B) cell-cell signaling *rpfF* mutant cells were incubated up to 10 days on wings. C) Control, medium XFM Δ without cells. The upper pictures were taken at 25x magnification and are suspension droplets; the lower pictures were taken at 80X magnification after removing the drop of medium and rinsing the wings with water. D) *chiA* expression in the cell-cell signaling mutant *rpfF* compared to the wild-type; different letters on bars represent statistically different treatments.

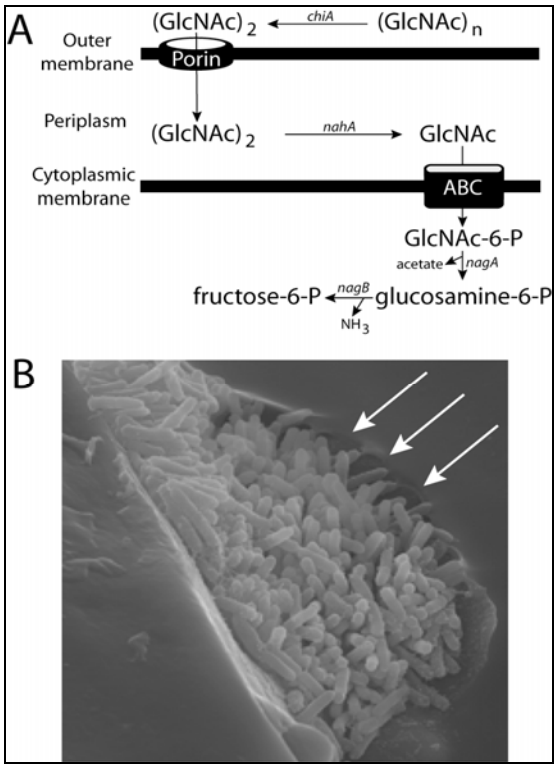

Figure 5. A) Hypothetical model for chitin utilization in *Xf*. Chitin is hydrolyzed to chitobiose outside the cell by *chiA* and passively transported into the periplasmic space as a dimer. *nahA* converts the substrate into *N*-acetylglucosamine, which is phosphorylated and transported into the cytoplasm via an ABC transporter. B) Scanning electron microscopy micrograph of *Xf* cells colonizing the mouthparts of a leafhopper vectors. The arrows indicate potential degradation of the chitinous surface at the fringe of microcolony.

CONCLUSIONS

The aim of this project is to learn more about molecular interactions of *Xf* with its insect vector and to identify moleculess that can be used to disrupt such interactions, and subsequently block the transmission. We found that *Xf* uses chitin as a carbon source. We also showed that fimbrial and afimbrial adhesions are up regulated in response to chitin utilization. These findings suggest that the chitin is second to pectin in its importance as an environmental factor regulating gene expression in *Xf*. While pectin is essential in introducing the adhesive state of the bacterial cells, chitin functions to maintain this state. Both components are required for the successful transmission of *Xf* by its leafhopper vectors to other host plants.

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FUNDING AGENCIES

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EVALUATING VARIATIONS IN RESISTANCE AND GLASSY-WINGED SHARPSHOOTER TRANSMISSION RATE AMONG GRAPEVINE VARIETALS

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Reporting Period: The results reported here are from work conducted March 2010 to October 2010.

ABSTRACT
We evaluated the susceptibility of different *Vitis vinifera* varieties to *Xylella fastidiosa (Xf)* infection under greenhouse conditions. We further compared *Xf* transmission efficiency by the glassy-winged sharpshooter in no choice trials for the tested varieties. Our results indicated that there is a great degree of variability in symptom development among the tested varieties. Furthermore, a significant variation in bacterial populations in leaf petioles was also detected among experimental varietals. While Crimson seedless and Grenache Noir had the lowest bacterial populations, Flame seedless had the highest bacterial population. Transmission efficiency was not influenced by grape variety or by the bacterial population in leaf petioles.

LAYPERSON SUMMARY
The degree of susceptibility to Pierce's disease is under evaluation for 18 commonly used grapevine varieties. Our results indicate that symptom severity and pathogen populations in leaf petioles vary among varietals. For example, while Crimson Seedless and Grenache Noir had the lowest pathogenic bacterial populations within petiole tissue, Flame Seedless possessed the highest bacterial population. The transmission efficiency of the plant pathogen by the glassy-winged sharpshooter did not differ among tested varieties. Vector transmission rate was also not affected by the pathogen's population in petioles. Establishing an objective categorization of the degree of susceptibility of grape varieties is currently ongoing.

INTRODUCTION
The degree of plant susceptibility to infectious pathogens is a measure, understanding of which is important for managing disease spread in agricultural systems (e.g. Kolmer 1996; Leung et al. 2003). Genetic variability among host plants may influence the level of plant resistance and/or tolerance to infections (Kover & Schaal 2002). Here, we consider a plant tolerant if it shows limited or no visual disease symptoms despite being infected by a large pathogen population. We refer to a host as resistant if the pathogen population (hereafter, 'infection level') remains low in the infected host. 'Resistance' and 'tolerance' are used as relative terms.

The xylem-limited bacterium *Xylella fastidiosa (Xf)* is the etiological agent of the epidemic Pierce's disease (PD) in grapevines (Purcell 1997, Hopkins and Purcell 2002). PD symptoms include leaf scorch, irregular maturation of the cane, and dieback of the apex of the plant (Krivanek et al. 2005). Although *Vitis vinifera* cultivars are generally susceptible to *Xf* infection (Krivanek and Walker 2005), anecdotal field observations (A.H. Purcell and J. Hashim-Buckey, personal communication) and a few experimental studies (e.g. Raju and Goheen 1981; Fry and Milholland 1990; Krivanek et al. 2005) indicate that differences exist in symptom severity among varietals. Indeed, symptom severity is correlated with the infection level of the host (Fry and Milholland 1990; Alves et al 2004; Krivanek and Walker 2004). In addition to variation in symptom severity, bacterial populations may also affect vector transmission efficiency among varietals as it has been shown that *Xf* transmission efficiency depends on the level of infection in the source plants (Hill & Purcell 1997). A greater exposure to bacteria can increase acquisition efficiency and, subsequently, the inoculation rate. This report includes data on evaluations of bacterial population growth and the transmission efficiency of glassy-winged sharpshooter (GWSS) among several commonly used grape varietals.

OBJECTIVES
1. Evaluating the degree of varietal susceptibility to *Xf* infections
2. Comparing *Xf* transmission efficiency by the glassy-winged sharpshooter among grape varietals
3. Measuring overwinter recovery from infection for different grape varietals

RESULTS AND DISCUSSION
Objective 1.
Variation in *Xf* populations colonizing different host plant species have been documented previously (e.g. Alves et al. 2004; Krivanek et al 2005). Our objective was designed to evaluate the extent of host colonization and symptom severity among eighteen varieties of *Vitis vinifera* that are commonly used in California. In March 2009 grape cuttings were needle-inoculated with the STL strain of *Xf* at the base of the main shoot (n=22 per variety). We reported the results of symptom development among several commonly used grapevine varieties in a previous report. We also quantified bacterial

populations in the petioles of respected varieties using quantitative PCR (**Figure 1a, b**). Our ANOVA results (repeated-measures) showed significant differences among varieties in bacterial populations within petioles ($F_{13, 166} = 2.4$, P = 0.005). There was no significant effect of sampling week 8 (**Figure 1a**) or 12 (**Figure 1b**) on the bacterial populations ($F_{1, 166} = 0.13$, P = 0.7). No interaction between variety and sampling date was detected ($F_{13, 166} = 1.55$, P = 0.10). Grenache Noir and Crimson seedless formed a statistically homogeneous subset with the lowest bacterial populations. Rubired, Merlot, French Colombard, Syrah, Pinot Noir, Cabernet Sauvignon, Thompson Seedless, Barbera, Ruby seedless, Red Globe, and Chardonnay were the 11 varieties forming a statistically homogeneous intermediate subset (varieties are arranged in an ascending order of infection level). Flame seedless was the single variety with the greatest bacterial population in petioles, which did not fall into any of the above subsets (**Figure 1a, b**). By comparing bacterial population growth and symptom severity it can be safely concluded that Rubired represents one of the least susceptible varieties tested in this study.

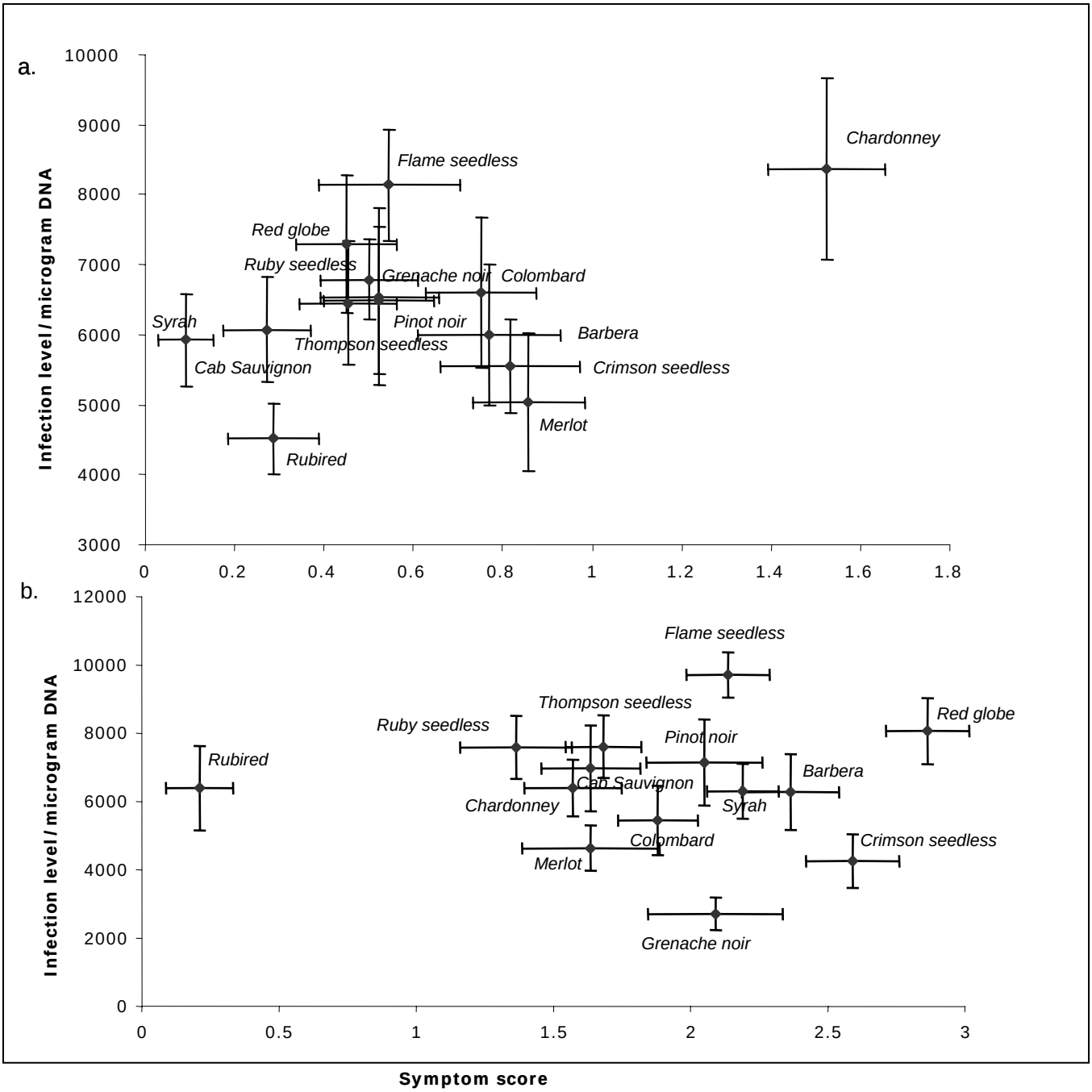

Figure 1. Scatter plots of mean infection levels (qPCR) versus mean symptom development scores (0 to 5 scale) for each of the 14 tested grape varieties (N=20 for most varieties); a) week 8, b) week12. Error bars represent ±1se.

We are currently in the process of analyzing petioles samples of another time block of the similar experiment with a subset of 10 varieties. We chose to perform a second time block as we thought some of the variations in symptom development during our summer experiment (**Figure 1a**) could have been affected by random environmental factors. Symptoms have been scored for the latest time block on weeks 8, 12, 16 and 20 (results not presented here) and following the completion of our quantitative PCR analysis our goal would be to establish an objective measure to categorize grape varietals base on their degree of susceptibility to *Xf* infection. This will be done by contrasting bacterial population level against symptom severity score in every single variety (currently in progress).

Objective 2:
Three hundred and ninety six GWSS were caged individually on 22 mechanically inoculated plants of each of the 18 varieties for a 48-hour acquisition access period. Insects were moved individually to a healthy host of the same variety and were allowed to feed for six days (inoculation access period). After four months petioles of the test plants were cultured on PWG medium to detect successful transmission events. Data from source plants, which tested negative for *Xf* presence based on the quantitative PCR data from 'objective 1', were not included in the transmission rate analyses. A binary logistic regression model with variety as a category, date as a repeated category, and infection level as a covariate (continuous) showed that transmission success of the glassy-winged sharpshooter was independent from plant genotype (Wald X_{13}^2= 8.13, P= 0.83), transmission date (Wald X_1^2= 0.89, P= 0.35) and infection level of the source plant (Wald X_1^2= 0.16, P= 0.68; **Figure 2**) (mean infection level (± SE): Successful transmissions, 6382.5 (700.6); failed transmissions, 6395.5 (212)). Our finding is also supported by Lopes et al (2009), who detected no association between host plant species (with different infection levels) and GWSS transmission. In contrast, Hill and Purcell (1997) showed that a relationship between the infection level of the source plant and the probability of a successful transmission is expected. Our failure to find a relationship may be the result of an overall low successful transmission incidence or the relatively low variations in bacterial populations among varieties. Indeed, with the exception of Flame Seedless, Crimson Seedless and Grenache noir, the rest of the tested varieties formed a statistically homogeneous group with respect to bacterial populations. In addition, the GWSS tends to prefer stem tissue rather than leaf petioles for feeding. Testing of stems to determine *Xf* populations in that tissue is more challenging and such large experiments would not have been possible. Ongoing studies are addressing this question in more detail.

Figure 2: An illustration of the overall relationship between the transmission rate and the bacterial infection level of the source plant.

Objective 3 (in progress):
Twenty cuttings of 11 commonly used grapevine varieties have been inoculated with STL strain of *Xf* in July 2010. All inoculated plants will be tested to confirm successful inoculations. Infected plants are scheduled to be transferred to an outdoor facility in November 2010. Starting April 2011, petioles of the experimental plants will be tested for *Xf* presence by PWG culturing. The goal of these assays is to determine overwinter recovery of varieties from *Xf* infections during the previous year.

In addition to the objectives listed in this report, our original proposal includes questions addressing GWSS feeding behavior and its role in transmission and pathogen spread. In particular, we are investigating host-choice as well as within-host feeding site preference of the GWSS. In previous reports we presented our findings on feeding site selection and its link to bacterial acquisition efficiency. Briefly, we showed that in spite their preference to feed on stem tissue, possibly due to

background matching behavior, GWSS acquire more bacterial cells from petioles and leaves (although statistically non-significant). This part of the study is currently under review for publication. The GWSS's response to bacterial presence as well as visual PD symptoms is also under investigation.

CONCLUSIONS

This study follows a recommendation by the PD advisory panel and aims to objectively quantify *Xf*-resistant and *Xf*-tolerant varietals and the role of GWSS in spreading *Xf*. We showed the variability of symptom development and bacterial population growth among several grape varieties. Although our final results is pending upon completion of our second time block analysis, so far we showed that Flame seedless has the greatest bacterial population growth compared to the other tested varieties. The varieties Grenache Noir and Crimson Seedless had the lowest bacterial population growth. The transmission efficiency of the GWSS did not differ among our tested varietals. Likewise, the transmission efficiency was not affected by the variations in the bacterial population levels. Experiments are ongoing and our final results can be used to evaluate the feasibility of using existing *Vitis vinifera* cultivars to control PD spread by quantifying resistance, tolerance, and GWSS behavior for several important table and wine grape varietals. This work will provide recommendations to growers in affected areas on which varietals to use in order to minimize and contain the pathogen spread.

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GENETIC ANALYSIS OF THE *ZONULA OCCLUDENS TOXIN* (*ZOT*) GENE IN TEXAS ISOLATES OF *XYLELLA FASTIDIOSA*

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ABSTRACT
Multiple subspecies of the phytopathogenic bacterium *Xylella fastidiosa (Xf)* exist which are pathogenic to distinct plant hosts, such as grapes, oleander, almonds, and citrus. Previously, DNA sequence analysis of the *mopB* and *gyr*B genes has been used to separate *Xf* strains into their subspecies groups. In this study, DNA sequence analysis of the *Zonula occludens toxin (Zot)* gene was used to corroborate the genetic variation found between 44 Texas strains of *Xf*. This approach provided variable gene sequences that allow for categorization of *Xf* at both the subspecies and population level. *In silico* translation of the *Zot* gene sequence, and subsequent protein model predictions showed conserved secondary structure, transmembrane regions, and signal cleavage sites despite differences in amino acid code. Results of the analysis of this gene were consistent with the phylogeny found using the more conserved *mop*B and *gyr*B genes at the subspecies level and can be used to differentiate populations within subspecies. The analysis of these variable genes and gene regions provide additional opportunities for new diagnostic and disease management techniques.

LAYPERSON SUMMARY
In this study, we sequenced one of the genes, the *Zot* gene, in the *Xylella fastidiosa* (*Xf*) genome. *Xf* has been implicated as the cause of several plant diseases that cause plant death and crop loss, including Pierce's disease (PD), almond leaf scorch, and citrus variegated chlorosis. By identifying and comparing the sequences of *Zot*, which has been implicated as a disease causing gene, we are able to determine the relationship of the different *Xf* subspecies, and may also be able to identify different populations of *Xf*, such as those from California versus those from Texas. This will allow researchers to track the spread of PD, among others, and may be useful in detailing the mechanisms by which *Xf* causes disease.

INTRODUCTION
Xylella fastidiosa (Xf) is a Gram-negative, xylem-limited, fastidious, insect-transmitted, gammaproteobacteria (Wells et al. 1987). Five subspecies of *Xf* exist, including *Xf fastidiosa* which causes Pierce's disease (PD), *Xf sandyi* which cases oleander leaf scorch (OLS), *Xf multiplex* which causes almond leaf scorch (ALS), *Xf pauca* which causes citrus variegated chlorosis (CVC), and *Xf tashke* (Purcell 1997, Schaad et al. 2004, da Silva et al. 2007, Randall et al. 2009). *Xf* has distinctly different host ranges; though some strains of *Xf* are only pathogenic in a single host species, others cause disease in a variety of hosts (Hopkins and Purcell 2002, Almeida et al. 2003). As much as 30% of the *Xf* genome is prophage in origin(Simpson et al. 2000, Van Sluys et al. 2003, Monteiro-Vitorello et al. 2005). Other research has shown that most of the sequence variation in *Xf* subspecies occurs in coding regions derived from bacteriophages (de Mello Varani et al. 2008).

The *Zonula occludens toxin* (*Zot*) in *Xf* strains has been suggested as a new potential virulence factor in CVC caused by *Xf 9a5c*, a member of subspecies *pauca* (da Silva et al. 2007). *Zot* genes are also found in the genomes of several other gammaproteobacteria, including *Vibrio cholera*, *Xanthomonas campestris*, *Stenotrophomonas maltophilia* and *Ralstonia solanacearum* (Koonin 1992, Johnson 1993, Chang et al. 1998, Hagemann et al. 2006). The *Zot* gene found in *V. cholerae* has great sequence similarity to the protein product I (pI) of the filamentous phage Pf1, and is most likely derived from a Pf1-like phage (Koonin 1992). The pI protein, which shares similarity to many *Zot* proteins in *Xf*, has both an extracellular and intracellular region, and is necessary for phage packing and transport across the cell membrane in many filamentous phages (Koonin 1992, Di Pierro et al. 2001, Schmidt et al. 2007). A homologous protein of the *Zot* family is found in many *Vibrio cholerae* strains and has been linked to disruption of tight junctions (Johnson 1993), and diarrheagenicity in *V. cholerae* that lack the cholerae toxin (Di Pierro et al. 2001). A *Zot* gene can also be found in strains of pathogens that, like *Xf*, are found in the Xanthomonadaceae family, namely *Xanthomonas campestris*, which causes lesions and loss of water in plant tissue (Block et al. 2005), and a *Stenotrophomonas maltophilia* strain, which can cause severe health problems such as endocarditis and bacteremia (Hagemann et al. 2006). A search of available *Xf* genomes in NCBI reveals that each *Xf* strain possesses multiple copies of *Zot* genes (Schreiber et al. 2010). Three distinct subgroups exist amongst these *Zot* genes. Most abundant are the members of the *Zot1* subgroup, which are found in PD strains *Temecula1*, *M23*, *GB 514*, and *Ann-1* (Schreiber et al. 2010).

OBJECTIVES
1. Sequence the *Zot1* gene in Texas strains of *Xylella fastidiosa*
2. Translate the *Zot1* nucleotide sequence *in silico* to identify amino acid changes
3. Identify changes in predicted protein structure resulting from changes in amino acid sequence
4. Analyze the phylogeny of the *Zot1* gene in Texas strains of *Xylella fastidiosa*

RESULTS AND DISCUSSION

Subspecies identification was performed using *gyrB* and *mopB* (Morano et al. 2008) (**Table 1**). Direct sequencing yielded 41 *Zot1* sequences useful for phylogenetic analysis. Quality trimming yieldegd 861bp sequences that shared 96.0% sequence identity. The sequences were fairly divergent, with XX synonymous substitutions and XX nonsynonymous substitutions. The Texas strains identified as subspecies *fastidiosa* differed from California *fastidiosa* strains in six fixed, synonymous substitutions. These substitutions were found in the middle region of *Zot1*, and may prove to be useful in identifying populations of subspecies *fastidiosa* in the future. Subspecies *fastidiosa* sequences shared 98.9% identity, subspecies *sandyi* sequences shared 99.7% identity, and subspecies *multiplex* sequences shared 96.1% identity. The increased divergence between subspecies *multiplex Zot1* sequences may be a result of the larger number of hosts that the *multiplex* strains were collected from.

Table 1. Sample ID, Collection Site, County of Collection, Host Plant, Scientific Name of Host Plant, and Subspecies ID.

Sample ID	Collection ID	County	Host Plant	Scientific Name	Subspecies ID
A	MCC CER 040	McCulloch	Vigonier grape	*Vitis vinifera*	*fastidiosa*
B	VAL VAL 041	Val Verde	Herbemont grape	*Vitis sp. cross*	*fastidiosa*
C	LLA FAL 747	Llano	Chardonnay grape	*Vitis vinifera*	*fastidiosa*
D	XFJK 13.87	Erath	Glassy-winged sharpshooter	*Homalodisca vitripennis*	*fastidiosa*
E	LLA FAL 634	Llano	SO4 Rootstock for grape	*Vitis sp. cross*	*fastidiosa*
F	XFJK 12.57	Erath	Cabernet Sauvignon grape	*Vitis vinifera*	*fastidiosa*
G	XFJK 12.69	Erath	Zinfandel grape	*Vitis vinifera*	*fastidiosa*
H	XFJK 14.11	Erath	Ruby Cabernet grape	*Vitis vinifera*	*fastidiosa*
I	GIL GRA 315	Gillespie	Wine grape	*Vitis vinifera*	*fastidiosa*
J	2018 GIL 007	Gillespie	innoc. Chardonnay, reisolated	*Plantanus sp. (Vitis sp.)*	*fastidiosa*
K	BAN POL 055	Bandera	Black Spanish grape	*Vitis sp. cross*	*fastidiosa*
L	HEN GRA 038	Henderson	Blanc du Bois grape	*Vitis sp. cross*	*fastidiosa*
N	LLA FAL 738	Llano	SO4 Rootstock for grape	*Vitis sp. cross*	*fastidiosa*
O	LLA FAL 745	Llano	SO4 Rootstock for grape	*Vitis sp. cross*	*fastidiosa*
1	GIL BEC 514	Gillespie	Wine grape	*Vitis vinifera*	*fastidiosa*
2	GIL BEC 519	Gillespie	Wine grape	*Vitis vinifera*	*fastidiosa*
3	GIL BEC 528	Gillespie	Wine grape	*Vitis vinifera*	*fastidiosa*
4	GIL GRA 316	Gillespie	Wine grape	*Vitis vinifera*	*fastidiosa*
5	MCC CER 011-1	McCulloch	Cabernet Sauvignon grape	*Vitis vinifera*	*fastidiosa*
7	TRA FLA 338	Travis	Muscat Blanc grape	*Vitis vinifera*	*fastidiosa*
8	TRA FLA 380	Travis	Tinta Madiera	*Vitis vinifera*	*fastidiosa*
9	VAL VAL 033	Val Verde	Black Spanish grape	*Vitis sp. cross*	*fastidiosa*
10	XFJK 21.4	Erath	Ruby Seedless grape	*Vitis vinifera*	*fastidiosa*
11	MED PRI 023	Medina	Oleander	*Nerium oleander*	*sandyi*
12	MED PRI0 45-1	Medina	Oleander	*Nerium oleander*	*sandyi*
13	MED PRI 047	Medina	Oleander	*Nerium oleander*	*sandyi*
14	MED PRI 049-2	Medina	Oleander	*Nerium oleander*	*sandyi*
15	MED PRI 054	Medina	Oleander	*Nerium oleander*	*sandyi*
18	BAN POL 039	Bandera	Golden Rod	*Solidago sp.*	*multiplex*
20	GIL BEC 626B	Gillespie	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*
21	GIL BEC 627	Gillespie	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*
22	GIL BEC 628A	Gillespie	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*
24	GIL GRA 281	Gillespie	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*
27	KIM 001	Kimble	Redbud	*Cercis canadensis*	*multiplex*
28	KIM 004	Kimble	Redbud	*Cercis canadensis*	*multiplex*
30	LLA FAL 651	Llano	Heart leaf Peppervine	*Ampelopsis cordata*	*multiplex*
31	LLA FAL 718A	Llano	Narrow leaf Sumpweed	*Iva texensis*	*multiplex*
33	LLA FAL 752	Lamar	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*
34	MCC CER 044	McCulloch	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*
36	UVA 122A	Uvalde	Sycamore	*Plantanus sp.*	*multiplex*
37	UVA 521-2B	Uvalde	Red Bud	*Cercis sp.*	*multiplex*
38	UVA TAM 115	Uvalde	Western Soapberry	*Sapindus saponaria L. var. drummondii*	*multiplex*
39	VAL VAL 072A	Val Verde	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*

The *Zot1* nucleotide sequences were then translated *in silico* to identify amino acid differences. This produced sequences of 281 amino acids long that shared 96.4% identity. These sequences were submitted to Pfam for identification (Finn et al. 2008). Each sequence was noted as belonging to the Zot protein family with an error value greater than $5e^{-10}$. Tertiary structure homology was predicted using the PHYRE program (Kelly and Sternberg 2009). The PHYRE search identified the N-Terminus of the Zot protein of *Neisseria meningitidis* (PDB code 2R2A) (data not shown) as the most similar structure to the sequences submitted, with a minimum error value of $2e^{-10}$. This, too, points to a conserved structure in the Zot1 protein in *Xf*. The Zot1 protein sequences were then subjected to secondary-structure prediction, transmembrane region prediction, and signal sequence cleavage site prediction (**Figure 1**) (Drummond et al. 2009). The predicted secondary structures, signal sequence cleavage sites, and transmembrane regions were very similar, indicating a degree of conservation amongst the structure of Zot1 proteins, despite the divergent nucleotide sequences. Particularly promising, the signal cleavage site predictions indicate two areas where cleavage may occur. The Zot protein is known to have a cleaved, exotoxic C-Terminus in *Vibrio cholerae* (Di Pierro et al. 2001). The predicted cleavage sites closest to the C-Terminus in the Zot1sequences from *Xf* correspond to the expected cleavage site of the Zot protein in other bacteria. Additionally, these regions of the Zot1 protein exhibits 98.7% sequence identity, higher than the average for the entire sequence. Further research to determine the exact cleavage site of the Zot1 protein.

Figure 1. Protein structure prediction produced using EMBOSS tool plug-in for Geneiousv. 5.1. A representative sequence from each phylogenetic cluster is represented to display structure homology across groups. Cellular region is indicated by pink arrows, transmembrane regions are indicated by red arrows, signal cleavage sites are indicated by green arrows. Secondary structure prediction is above the amino acid sequence. Blue arrows indicate turns, yellow arrows indicate alpha helices, and purple cylinders indicate beta sheets.

Phylogenetic analysis yielded a tree topology that was predicted by the *gyrB* and *mopB* identification (**Figure 2**). The Texas samples of this study were supplemented with sequences retrieved from NCBI's GenBank, including two sequences from both *Xf* M23 and Temecula1, one sequence from *Xf* Ann-1, and three *Zot1* sequences previously sequenced in house. Though recombination blurred distinctions between groups, sequences separated into complexes according to their subspecies. These complexes are highly supported, as evidenced by their high bootstrap values. Additionally, the California *fastidiosa* strains (*Xf M23 and Temecula1*) separated from the Texas *fastidiosa* strains (*Xf Texas 1* and Samples 10, K, N, and O). The group composition of the complexes was not reflective of geographic proximity or host plant identity (data not shown). The high degree of similarity between sequences despite geographic range, and the high levels recombination indicate a large amount of mixing between different strains of *Xf*.

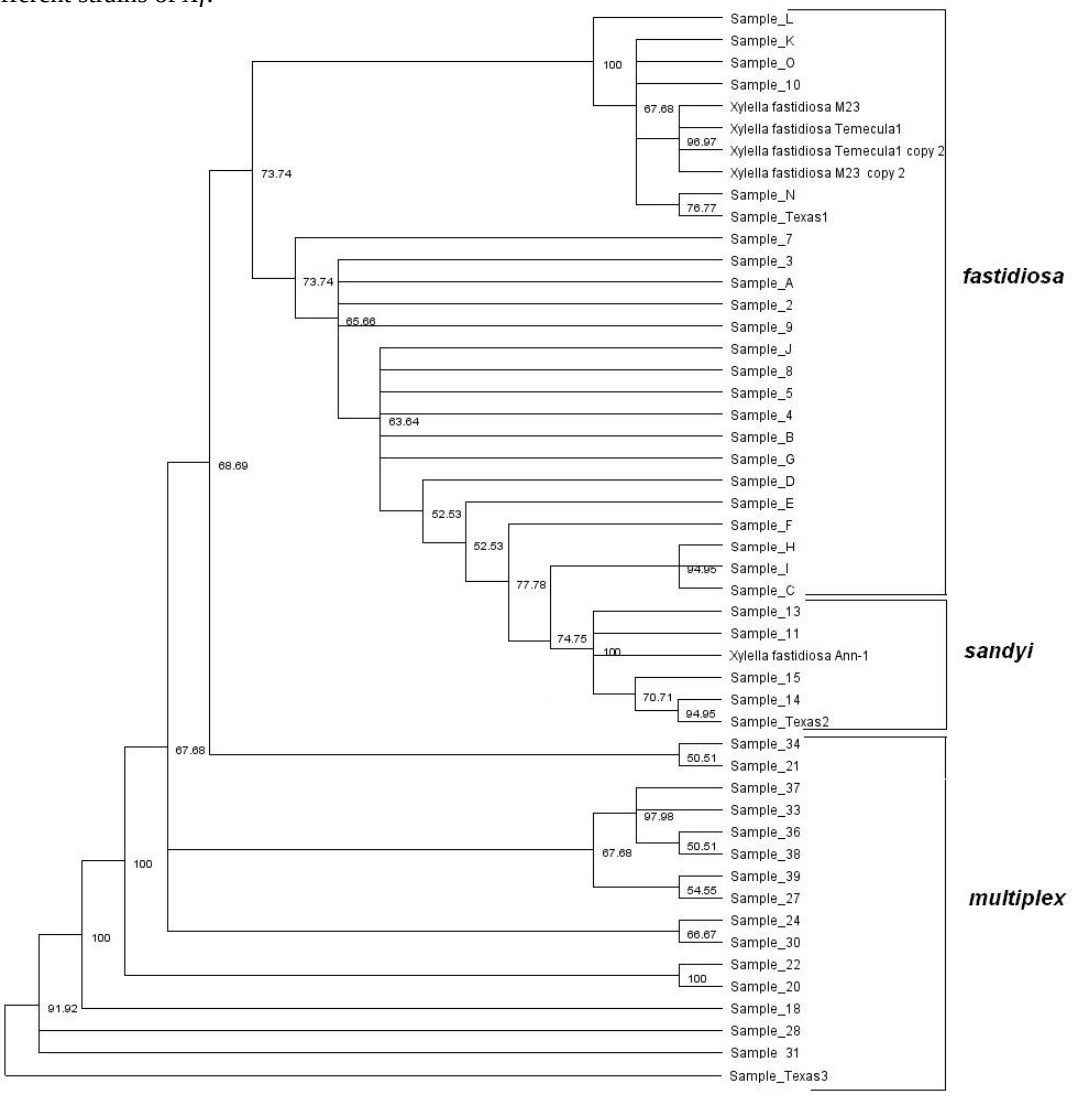

Figure 2. Phylogenetic tree produced using the Maximum-Likelyhood method and the HKY evolutionary model with a ratio of 0.774 invariable sites, four categories of substitution, and a gamma shape of 0.931.

Little is known about the *Zot* protein's structure due to the complexity of isolating *Zot* for analysis as a result of its transmembrane region (Koonin 1992, Hagemann et al. 2006, Schmidt et al. 2007). *In silico* translations and structure predictions offer great insights into protein function and classification and has been found to be accurate and sensitive. By using *in silico* analysis, the sequence variation found in *Zot1* genes between subspecies has been shown to yield differences in protein structure. Predictive modeling shows that the variation found in the amino acid code does not translate to altered protein structure. This similarity in structure, despite changes in both the nucleotide and amino acid code, indicates that the *Zot1* gene may not be evolving due to host-pathogen interactions.

The first step in determining host range in differentially pathogenic bacteria is placing the bacteria into clades (Morano et al. 2008). Many techniques for identification and classification exist; however, the complexity of *Xf* pathogen makes categorization based on morphology or pathogenicity difficult (Almeida et al. 2003). Additionally, *Xf* has been shown to

have limited genomic variability within clades and region, and that the majority of strain specific genes occur in prophage regions, though they contain genomic islands which enable rapid evolution (Van Sluys et al. 2003). Techniques that focus on classification based on well conserved regions shared by all *Xf* subspecies might then miss putative evolutionary growth and adaptation. The *Zot1* gene insertion is an excellent target for QRT-PCR, and the small sequence differences can be targeted by restriction enzyme digestion analysis for quick and accurate identification and classification of *Xf* subspecies and populations.

CONCLUSIONS

Comparative analysis *Zot1*genes and proteins provide accurate, population level differentiation therefore allows researchers greater ability to track the spread of economically important phytopathogens. Additionally, *in silico* translation and analysis of *Zot1* describes in greater detail differences between strains, and describes possible conformation changes that result from sequence changes between strains. Taken together, these results show that *Zot1* is a useful target for differential sequence analysis and can be used to elucidate the phylogenetic history of *Xf*, and its spread through the U.S.

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THE RECOMBINATION RATE OF THE *ZOT* AND *GYRASE B* GENES OF *XYLELLA FASTIDIOSA*

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ABSTRACT
Xylella fastidiosa (Xf) is a Gram-negative gamma proteobacteria that is responsible for several economically important plant diseases. The *Zonula occludens toxin* (*Zot*) is an exotoxin produced and secreted by *Xf* that has been suggested as a potential virulence factor in other research. This report is a description of the recombination rates, nucleotide diversity, and rates of linkage disequilibrium of both the *Zot* gene and the housekeeping gene *gyraseB* (*gyrB*). The *Zot* gene has a much higher degree of nucleotide diversity, recombination rate, and less intragene linkage disequilibrium. This indicates that the *Zot* gene is undergoing more selection pressure than the *gyrB* gene. Additionally, this report suggests that *Xf* has higher than reported rates of recombination, but that this recombination is masked by similar sequence identity.

LAYPERSON SUMMARY
In this study, we examined the rates of recombination in two genes in the *Xylella fastidiosa (Xf)* genome. Recombination occurs when strands of DNA interact, and sometimes switch. These instances occur in bacteria when bacteriophages, viruses that infect bacteria, insert their genetic material into a bacterial cell, when bacteria undergo a form of mating, called conjugation, or when bacteria uptake foreign DNA from outside of their cell. Recombination plays an important role in evolution, by rearranging chromosomes, inserting new genetic sequences, or exchanging bits of genes from one strand of DNA to another. It is an important source of mutation in bacteria, and little work has been done to study recombination in *Xf*.

INTRODUCTION
Xylella fastidiosa (Xf) is a Gram-negative, xylem-limited, fastidious, insect-transmitted, gamma proteobacteria (Wells et al. 1987). Five subspecies of *Xf* exist, including *Xf fastidiosa* which causes Pierce's disease (PD), *Xf sandyi* which cases oleander leaf scorch , *Xf multiplex* which causes almond leaf scorch, *Xf pauca* which causes citrus variegated chlorosis (CVC), and *Xf tashke* (Purcell 1997, Schaad et al. 2004, da Silva et al. 2007, Randall et al. 2009). *Xf* has distinctly different host ranges; though some strains of *Xf* are only pathogenic in a single host species, others cause disease in a variety of hosts (Hopkins and Purcell 2002, Almeida et al. 2003). As much as 30% of the *Xf* genome is prophage in origin (Simpson et al. 2000, Van Sluys et al. 2003, Monteiro-Vitorello et al. 2005). Research has shown that most of the sequence variation in *Xf* subspecies occurs in coding regions derived from bacteriophages (de Mello Varani et al. 2008). High rates of chromosomal rearrangements, recombinations, and gene loss has been detected in the prophage regions of *Xf* (Monteiro-Vitorello 2005). The *Zonula occludens toxin* (*Zot*) has been suggested as a new potential virulence factor in CVC caused by *Xf 9a5c*, a member of subspecies *pauca* (da Silva et al. 2007). *Zot* genes are also found in the genomes of many other pathogenic bacteria, including *Vibrio cholera*, *Xanthomonas campestris*, *Stenotrophomonas maltophilia*and *Ralstonia solanacearum* (Koonin 1992, Johnson 1993, Chang et al. 1998, Hagemann et al. 2006). The *Zot* genes in *Xf* appear in prophage regions of the genome (Monteiro-Vitorello et al. 2005, de Mello Varani et al. 2008). Several of these *Zot*genes share sequence homology with the *Zot*gene found in *Vibrio cholera*, which is derived from the pI protein of a bacteriophage (Johnson 1993). The prophagic pI protein is integral to proper virus packaging and export (Change et al. 1998). A search of available *Xf* genomes in NCBI reveals that each *Xf* strain possesses multiple copies of *Zot* genes (Schreiber et al. 2010). Three distinct subgroups exist amongst these *Zot*genes. Most abundant are the members of the *Zot1* subgroup, which are found in PD strains *Temecula1, M23, GB 514*, and *Ann-1* (Schreiber et al. 2010).

Recombination events can affect bacterial evolution (Maynard Smith et al. 1994), but little work on the recombination of prophage regions of *Xf* has been done. Recombination rates have been shown to affect clonal complex composition and influence the phylogenetic structure of *Xf*(Scally et al. 2005). However, short divergence times, and a low rate of mutation has led to a high degree of clonality amongst *Xf* strains (Schuenzel et al. 2005). This high degree of similarity means reduces the chances of accurately identifying recombination rates, as recombination events between identical sequences are undectable via sequence analysis (Posada et al. 2002). As such, only a small fraction of recombination events are accurately identified in sequences with high degrees of similarity (>99%), resulting in underestimates of recombination rates (Hudson and Kaplan 1985).

This study is a presentation of materials describing the differences in recombination between a housekeeping gene, *gyrB*, and a prophage gene with significant sequence divergence, *Zot1*. The *gyrB*gene was chosen because of its use in phylogenetic analysis and its conserved nature (Morano et al. 2008), while the *Zot1* gene was chosen because it is most prevalent amongst the PD strains of *Xf*, is significantly divergent, and is prophage in origin.

OBJECTIVES

1. Sequence the *Zot1*and *gyrB*genes in Texas strains of *Xf.*
2. Identify areas of recombination using visual inspection methods as well as *in silico* analysis.
3. Compare rates of recombination between a prophage gene, *Zot1* and a housekeeping gene, *gyrB.*

RESULTS AND DISCUSSION

Subspecies identification was performed using *gyrB* and *mopB* (Morano et al.2008) (**Table 1**). Quality trimming of the *Zot1* sequences yielded 861bp sequences that shared 96.0% sequence identity. The sequences were fairly divergent, with 69 synonymous substitutions and 35nonsynonymous substitutions. Quality trimming of the *gyrB* sequences yielded 631bp sequences that shared 99.0% sequence identity. The *gyrB* sequences contained 15 synonymous substitutions and 3nonsynonymous substitutions. This indicates that the *Zot1* gene is more divergent than the *gyrB* gene.

Table 1. Sample ID, Collection Site, County of Collection, Host Plant, Scientific Name of Host Plant, and Subspecies ID.

Sample ID	Collection ID	County	Host Plant	Scientific Name	Subspecies ID
A	MCC CER 040	McCulloch	Vigonier grape	*Vitisvinifera*	*fastidiosa*
B	VAL VAL 041	Val Verde	Herbemont grape	*Vitis sp. cross*	*fastidiosa*
C	LLA FAL 747	Llano	Chardonnay grape	*Vitisvinifera*	*fastidiosa*
D	XFJK 13.87	Erath	Glassy-winged sharpshooter	*Homalodiscavitripennis*	*fastidiosa*
E	LLA FAL 634	Llano	SO4 Rootstock for grape	*Vitis sp. cross*	*fastidiosa*
F	XFJK 12.57	Erath	Cabernet Sauvignon grape	*Vitisvinifera*	*fastidiosa*
G	XFJK 12.69	Erath	Zinfandel grape	*Vitisvinifera*	*fastidiosa*
H	XFJK 14.11	Erath	Ruby Cabernet grape	*Vitisvinifera*	*fastidiosa*
I	GIL GRA 315	Gillespie	Wine grape	*Vitisvinifera*	*fastidiosa*
J	2018 GIL 007	Gillespie	innoc. Chardonnay, reisolated	*Plantanus sp. (Vitis sp.)*	*fastidiosa*
K	BAN POL 055	Bandera	Black Spanish grape	*Vitis sp. cross*	*fastidiosa*
L	HEN GRA 038	Henderson	Blanc du Bois grape	*Vitis sp. cross*	*fastidiosa*
N	LLA FAL 738	Llano	SO4 Rootstock for grape	*Vitis sp. cross*	*fastidiosa*
O	LLA FAL 745	Llano	SO4 Rootstock for grape	*Vitis sp. cross*	*fastidiosa*
1	GIL BEC 514	Gillespie	Wine grape	*Vitisvinifera*	*fastidiosa*
2	GIL BEC 519	Gillespie	Wine grape	*Vitisvinifera*	*fastidiosa*
3	GIL BEC 528	Gillespie	Wine grape	*Vitisvinifera*	*fastidiosa*
4	GIL GRA 316	Gillespie	Wine grape	*Vitisvinifera*	*fastidiosa*
5	MCC CER 011-1	McCulloch	Cabernet Sauvignon grape	*Vitisvinifera*	*fastidiosa*
7	TRA FLA 338	Travis	Muscat Blanc grape	*Vitisvinifera*	*fastidiosa*
8	TRA FLA 380	Travis	TintaMadiera	*Vitisvinifera*	*fastidiosa*
9	VAL VAL 033	Val Verde	Black Spanish grape	*Vitis sp. cross*	*fastidiosa*
10	XFJK 21.4	Erath	Ruby Seedless grape	*Vitisvinifera*	*fastidiosa*
11	MED PRI 023	Medina	Oleander	*Nerium oleander*	*sandyi*
12	MED PRI0 45-1	Medina	Oleander	*Nerium oleander*	*sandyi*
13	MED PRI 047	Medina	Oleander	*Nerium oleander*	*sandyi*
14	MED PRI 049-2	Medina	Oleander	*Nerium oleander*	*sandyi*
15	MED PRI 054	Medina	Oleander	*Nerium oleander*	*sandyi*
18	BAN POL 039	Bandera	Golden Rod	*Solidago sp.*	*multiplex*
20	GIL BEC 626B	Gillespie	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*
21	GIL BEC 627	Gillespie	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*
22	GIL BEC 628A	Gillespie	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*
24	GIL GRA 281	Gillespie	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*
27	KIM 001	Kimble	Redbud	*Cerciscanadensis*	*multiplex*
28	KIM 004	Kimble	Redbud	*Cerciscanadensis*	*multiplex*
30	LLA FAL 651	Llano	Heart leaf Peppervine	*Ampelopsis cordata*	*multiplex*
31	LLA FAL 718A	Llano	Narrow leaf Sumpweed	*Iva texensis*	*multiplex*
33	LLA FAL 752	Lamar	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*
34	MCC CER 044	McCulloch	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*
36	UVA 122A	Uvalde	Sycamore	*Plantanus sp.*	*multiplex*
37	UVA 521-2B	Uvalde	Red Bud	*Cercis sp.*	*multiplex*
38	UVA TAM 115	Uvalde	Western Soapberry	*Sapindussaponaria L. var. drummondii*	*multiplex*
39	VAL VAL 072A	Val Verde	Giant Ragweed	*Ambrosia trifida L. var. Texana Scheele*	*multiplex*

The sequenced genes were aligned using Geneious v. 5.1. Analysis of the sequences was performed using DnaSP to identify sequence characteristics (Rozas et al. 2003).Nucleotide Diversity, per nucleotide (π) and average number of nucleotide changes per gene (θ) were calculated to identify the differences in nucleotide variability between the *Zot1* and *gyrB* genes (**Table 2**). These numbers show that the *Zot1* gene has a much higher rate of variability than the *gyrB* gene. The rate of recombination per gene, R, was calculated using the minimum number of recombinations statistic (Rm) following the protocol of Hudson and Kaplan (1987), (**Table 2**). Although the minimum number of recombinations was much higher for the *Zot1* gene, the *gyrB*gene displayed a higher rate of recombination overall. Finally, the rate of linkage disequilibrium of each gene was calculated, as an indirect measure of recombination (**Table 3**). The ZZ statistic of Rozas et al. (2001) was used in place of the Z_{nS} proposed by Kelly (1999) for greater accuracy in determining the rates of recombination. The higher

ZZ value of the *Zot1* gene indicates a greater amount of recombination is apparent in the *Zot1* gene than in the *gyrB*genes. The much larger number of informative sites, corrected using the Bonferroni calculations, for the *Zot1* gene supports the conclusion derived from the ZZ statistic.

Table 2. Results of sequence analysis using DnaSP v. 5.1.

Sequence Characteristics	Test	*Zot gene*	*gyrB gene*
Nucleotide Diversity	**Nucleotide Diversity, per nucleotide (π)**	**0.0355**	**0.0112**
	Average number of nucleotide changes per gene (θ)	**29.31**	**2.394**
Recombination	**Minimum number of recombinations, Rm**	**20**	**0**
	Estimate of recombination per gene, R	**4.1**	**6.3**
Linkage Disequilibrium	**Fisher's Exact Test**	**1444**	**12**
	with Bonferroni correction	**129**	**11**
	Chi squared Test	**2408**	**12**
	with Bonferroni correction	**545**	**11**
	ZZ Value	**0.2371**	**0.2004**

Coalescent simulations were performed using DnaSP v. 5.1 based on the θ statistic and the observed rate of recombination for each gene to predict the Rm statistic and ZZ statistic in a hypothetical population (Rozas et al 2003). Simulations were run 1000 times in order to obtain a predicted average, and a 95% confidence interval. This average was then compared to the observed value to identify the probability that the observed value lies outside the predicted bell curve. The coalescent simulations show that the observed values of the *Zot1* gene lie outside the predicted bell curve for both the Rm and ZZ statistic. This indicates either relaxed negative selection or positive selection pressures are working on the *Zot1* gene to increase genetic diversity by overcoming negative selection sweeps that are common to gene recombination.

Table 3. Coalescent simulations performed using DnaSP v. 5.1. Simulations were run using observed values of Rm and the θ statistic.

Rm	Observed Value	Simulated Average	95% confidence interval	p-value of observed Rm statistic
Zot gene	20.00	3.49	1.00 to 7.00	0.000***
gyrB gene	2.00	2.20	0.00 to 3.00	0.681(ns)

ZZ Statistic	Observed Value	Simulated Average	95% confidence interval	p-value of observed ZZ statistic
Zot gene	0.237	0.053	-0.015 to 0.159	0.001 **
gyrB gene	0.200	0.041	-0.077 to 0.213	0.07(ns)

CONCLUSIONS

The results of the experiments performed in this project suggest that the *Zot1* gene is evolving rapidly and is prone to recombination events. As Zot proteins have been identified as potential virulence factors, this phenomenon deserves greater scrutiny. Previous reports have identified relatively low rates of recombination in *Xf*. The high sequence similarity between strains of *Xf*, as much as 98% between subspecies, may be masking high rates of recombination that leave no genetic trace when they occur between highly similar strains. Further research into divergent regions of the *Xf* genome to determine actual rates of recombination is warranted, given the rate of cohabitation common to *Xf* strains.

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ROBUST ONE-HOUR GENOTYPING OF *XYLELLA FASTIDIOSA* STRAINS USING FRET PROBES

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ABSTRACT
Epidemiological studies of Pierce's disease (PD) can be confounded by a lack of genetic information on the bacterial causative agent, *Xylella fastidiosa* (*Xf*). PD in grape is caused by genetically distinct strains of *Xf* subsp. *fastidiosa* (*Xff*), but is not caused by numerous other strains or subspecies of *Xf* that typically colonize plants other than grape. Detection assays such as ELISA and qPCR are effective at detecting and quantifying *Xf* presence or absence, but offer no information on *Xf* subspecies or strain identity. Surveying insects or host plants for *Xf* by current ELISA or qPCR methods provides only presence/absence and quantity information for any and all *Xf* subspecies, potentially leading to false assessments of disease threat. This study provides a series of adjacent-binding fluorescence resonance energy transfer (FRET) DNA melt analysis probes (Cardullo et al. 1988) that are capable of efficiently discriminating *Xf* subspecies and strain relationships in one hour real-time PCR reactions.

LAYPERSON SUMMARY
Pierce's disease (PD) of grape is the single greatest factor limiting grape production in Texas. PD outbreaks have caused major economic loss to the grape industry in California as well. The disease is caused by a particular grape strain of a bacterium that is spread between plants by insects that feed on grapevines. Diagnostic tests to detect the bacterium will detect the grape strain as well as numerous other closely related strains of the bacterium that do not cause PD. Lack of an efficient means to distinguish the grape strain of the bacterium from other closely related strains that cause no harm to grape is hindering an understanding of the disease cycle. This project provides efficient DNA-based tests to distinguish the closely related bacterial strains from one another.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a xylem-limited bacterium that causes leaf scorch diseases in a wide array of plant species, and it is vectored by a number of xylophagous insects. Several subspecies of the bacterium have been named, including *Xf* subsp. *fastidiosa* (*Xff*) that causes PD in grape; *Xf* subspecies *sandyi* (*Xfs*) (Schuenzel et al. 2005) that causes oleander leaf scorch; *Xf* subsp. *pauca* (*Xfp*) that causes citrus variegated chlorosis, *Xf* subsp. *taschke* (*Xft*) that causes leaf scorch in chitalpa (Randall et al. 2007); and a genetically diverse subspecies, *Xf* subsp. *multiplex* (*Xfm*) that causes leaf scorch diseases in a large number of tree species (Schaad et al. 2004; Schaad et al. 2004). Many of the subspecies may occupy multiple hosts, but cause disease symptoms in only a select subset of potential plant hosts (Hopkins et al. 2002). While methods for efficient detection of the bacterium exist, such as ELISA and qPCR, epidemiological studies can be hindered because the detection assays commonly used detect all subspecies, but do not provide subspecies or strain identification. Isolating and culturing strains is a laborious and time-consuming process due to the fastidious nutritional requirements and slow growth habit of the bacterium. A multilocus sequence typing (MLST) system for *Xf* has been developed that is capable of generating sufficient genetic information to easily discriminate subspecies and strains (Scally et al. 2005), and although the method has been streamlined (Yuan et al. 2010), it remains a time-consuming process. In order to complement more informative and more time-consuming assays such as MLST, we have developed several real-time PCR probe sets capable of rapid and robust subspecies and strain identification by DNA melt analysis. The probe sets target many of the same genes utilized in the established MSLT assay, so that a rapid preview of important strain differences can be generated. These probe sets are shown to be capable of identifying *Xf* DNA polymorphisms even when the *Xf* DNA is a small proportion of a mixed DNA isolation containing plant, insect, and microbial DNA from other species.

OBJECTIVES
1. Develop rapid genotyping methods capable of distinguishing *Xf* subspecies and strains using plant and insect DNA extractions where the proportion of *Xf* DNA may be very small in relation to contaminating DNA.

RESULTS AND DISCUSSION
Ten genes that have been previously identified as highly conserved among *Xf* strains in previous work to develop a MLST assay (Yuan, Morano et al. 2010) were selected and aligned using VectonNTI (Invitrogen, Carlsbad CA) to visually select informative single nucleotide polymorphisms (SNPs). Alignments included the 26 stains used in the MLST project. AlleleID software (Premier Biosoft International, Palo Alto, CA) was used to align sequences and indicate SNPs that could potentially discriminate between the fully sequenced type strains for each *Xf* subspecies. AlleleID was then used to design adjacent-binding FRET probes for DNA melt assays to discriminate the SNP differences. Real-time PCR was carried out in a

384-well Roche LightCycler 480 real-time PCR instrument. Immediately following amplification, a DNA melt assay was conducted by annealing probe and amplicon, then ramping up sample temperature while continuously collecting fluorescence data. Melt curves and negative first derivative plots of DNA melt data were generated with the melt curve analysis module in the LightCycler software suite. An example of two SNPs used for *Xf* probe design are shown in **Figure 1**.

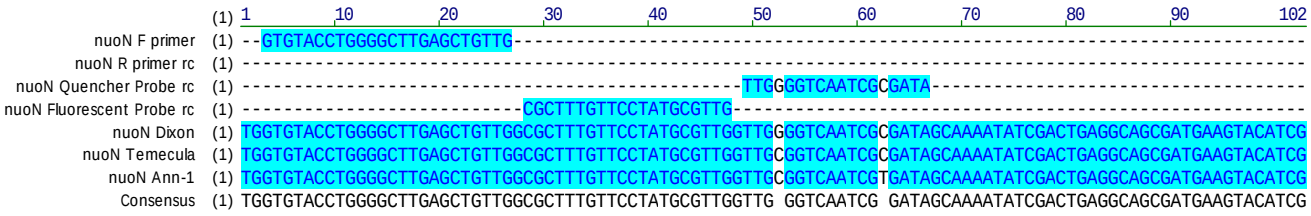

Figure 1. Partial DNA sequence alignment of the *Xf nuoN* gene. Forward primer, fluorescent probe, and quencher probe binding sites are shown above the type strains for *Xfm* (Dixon), *Xff* (Temecula), and *Xfs* (Ann-1). Identical sequence has a blue background while SNP positions have a white background.

For the *nuoN* gene, two variable sites are present within the sequence of the quencher probe. The probe is designed to have 100% identity to the *Xfm* strains described in the MLST work. The quencher probe has 1 G/C mismatch with the *Xff nouN* amplicon at the 15th nucleotide. The quencher probe has two mismatches with the *Xfs nuoN* amplicon, a C/T mismatch at the 5th nucleotide and the same G/C mismatch as with Temecula at the 15th nucleotide from 5' to 3' (quencher probe reverse complement is shown in **Figure 1**).

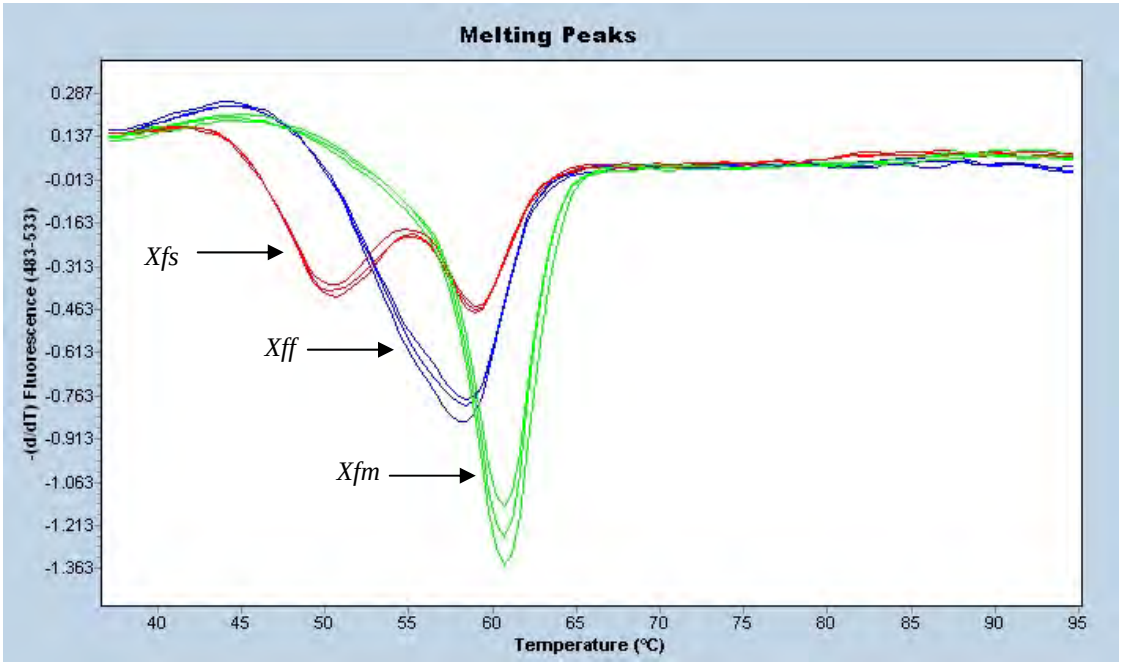

Figure 2. Melting analysis of *Xf* strains using *nuoN* FRET probes. The quencher probe has 100% sequence identity to the Dixon strain of *Xfm*, and the melt is indicated in green. The Temecula strain of *Xff* has a single base mismatch with the probe and its melt is indicated in blue. The Ann-1 strain of *Xfs* has two mismatches with the probe, and its melt is indicated in red.

The melting curves for the *nuoN* gene allow easy discrimination between the *Xfm*, *Xff*, and *Xfs* type strains by using only 2 adjacent-binding DNA FRET probes (**Figure 2**). As expected, the *Xfm* melt has the highest melting temperature (Tm) at 60.7° C, since the probe has 100% sequence identity to the Dixon *Xfm* strain. The Temecula *Xff* Tm is slightly lower at 59.07° C due to the 1 bp mismatch between the probe and *Xff* amplicon. The Ann-1 *Xfs* melt displays two Tm's, one at 59.07° C, equivalent to the *Xff* melt, and due to the same mismatch that occurs in *Xff*. The second *Xfs* Tm is at 50.53° C, and gives the *Xfs* melt profile two distinctive and characteristic troughs, allowing extreme ease of interpretation in scoring melt differences. Additionally, the *nuoN* probes function equally well when used on pure DNA from axenically-grown cultures, or when used on plant or insect DNA extractions where the proportion of *Xf* DNA is a very small part of a complex DNA sample.

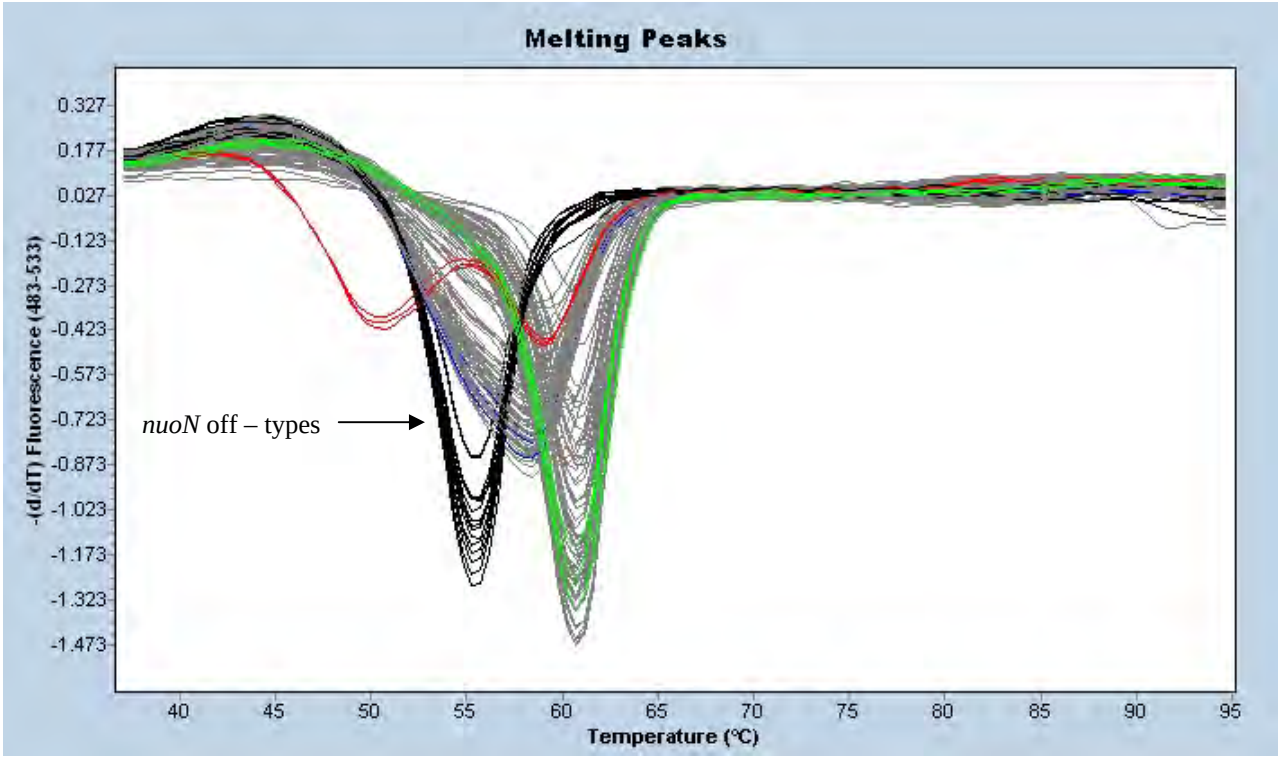

Figure 3. Melt analysis of 93 insect DNA extractions using the *nuoN* probe set. Melt standard curves included in the analysis are Dixon *Xfm* in green, Temecula *Xff* in blue, and Ann-1 *Xfs* in red. Insect sample melts are gray, except for several with unusual melt characteristics that are black and indicated by the arrow.

As a step in developing these assays, non-specific DNA binding dyes such as sybr green I and LCgreen plus were explored as an inexpensive option in developing a multilocus melt typing assay for *Xf*. These assays were unsatisfactory due to close and variable melting temperatures when using pure DNA from cultured type strains. The assays were simply unusable when amplifying *Xf* DNA from plant and insect DNA extractions, presumably due to melt variation caused by novel alleles not encountered in sequence databases when designing assays, as well as by inherent variation in melting PCR amplicons with non-specific DNA binding dyes. This probe-based genotyping method is robust in the face of unexpected genetic variation, and highly consistent due to short probe length. For example, **Figure 3** shows *nuoN* melting curves from a number of insect DNA extractions. A group of samples, all from Gillespie County Texas do not show the characteristic melt of *Xfm*, *Xff*, or *Xfs*. Instead, these samples all melt at 55.44° C. The *nuoN* assay alone would not be sufficient to group these samples with any of the identified subspecies. Additional probe melt assays with genes such as *lacF*, *petC*, *pilU*, and *gyrB* have identified these *nuoN* off-types as *Xfm* (**Figure 4)**. Thus, a novel *nuoN* allele has been identified for *Xfm* in insect samples from Gillespie County Texas. Probe-based melt analysis may be used in this way to quickly and efficiently screen for strains that should be given a more thorough genetic evaluation by additional methods.

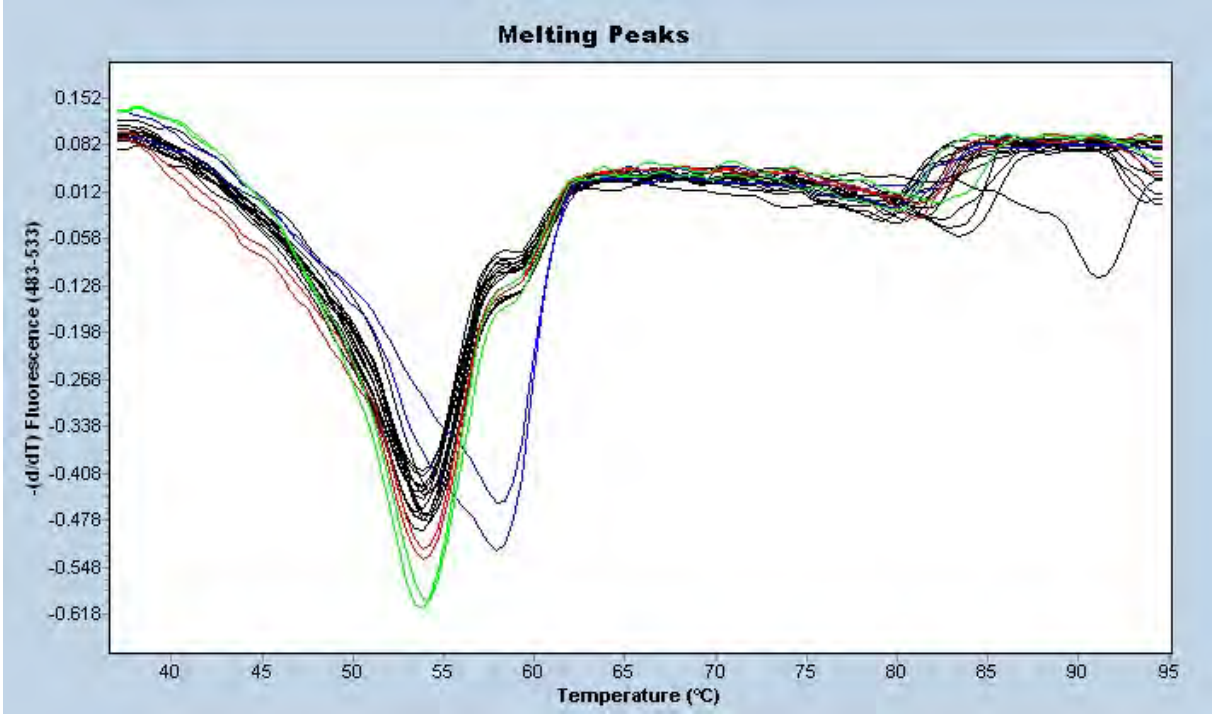

Figure 4. Melt analysis of *nuoN* off-types using the *gyrB* probe set. Melt standard curves included in the analysis are Dixon *Xfm* in green, Temecula *Xff* in blue, and Ann-1 *Xfs* in red. Off-types from the *nuoN* probe melt analysis are black. *Xfm* and *Xfs* have identical melts with the *gyrB* probe set, and all *nuoN* off-types show a non-*Xff* melt profile.

CONCLUSIONS

Probe-based DNA melt assays for several *Xf* genes have been developed that allow rapid discrimination of subspecies and strain relationships. The assays are efficient, requiring little DNA template, so that *Xf* DNA can be genotyped even if it is a small proportion of the DNA in a sample originating from a plant on insect DNA extraction. Many of the genes in the assay are already part of a MLST assay for *Xf*, so that the probe-melt assay may complement the MLST assay as an indicator of samples that require genotyping of greater depth.

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MULTIPLEX PCR MARKERS: STEPS TOWARDS SATURATING THE *XYLELLA FASTIDIOSA* GENOME

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ABSTRACT
Xylella fastidiosa (*Xf*) is a bacterium that can cause leaf scorch disease in many plant species, is an asymptomatic colonizer of many plant species, and cannot successfully colonize other plants (Hopkins et al. 2002). Although some molecular determinants of successful colonization have been discovered (Chatterjee et al. 2008), much work remains in order to unravel the genetics of host specificity in various *Xf* strains. Additionally, the same can be said of virulence determinants. Many questions remain about why certain strains cause disease in one plant species but not in other species (Almeida et al. 2008). This project is an attempt to develop a high-density DNA marker system that can quickly and efficiently screen for variation in the *Xf* genome at hundreds of loci. Hundreds of highly conserved *Xf* genes have been aligned using the seven fully sequenced *Xf* genomes currently available, and the alignments have been screened for informative DNA polymorphisms. A multiplex PCR strategy using amplification tags has been employed (Boutin-Ganache et al. 2001), and electrophoresis in capillary sequencing instruments is being utilized to accurately assess DNA amplicon size differences. A 50 marker proof-of-concept test has been conducted and marker number is being increased towards a goal of 400 total. An evaluation of *Xf* strain collections with a high-density marker system should facilitate the identification of additional genetic factors influencing host specificity and virulence, and should provide additional information about recombination frequency among *Xf* strains.

LAYPERSON SUMMARY
Different strains of the bacterium *Xylella fastidiosa (Xf)* cause many different plant diseases of economic significance, such as Pierce's disease of grape and citrus variegated chlorosis. Different strains of *Xf* are specific to certain host plants and cause disease in a small subset of potential host plants. Many of the genetic factors that provide host specificity and development of disease remain unknown. This project is an effort to develop enough molecular markers in the *Xf* genome so that additional genetic determinants of host specificity and disease can be mapped using *Xf* strain collections.

INTRODUCTION
Genetic tests for *Xylella fastidiosa (Xf)* range from single gene tests that only detect presence or absence of any strain of the bacterium, either by conventional PCR (Minsavage et al. 1994) or qPCR (Schaad et al. 2002), to complete genome sequencing projects that sequence every base pair (Simpson et al. 2000). At one end of the spectrum, the single gene tests produce almost no genetic information except that the target gene is conserved and present. Either conventional PCR or qPCR are rapid, inexpensive, and simple to analyze. At the other end of the spectrum, complete genome sequencing is expensive, though cost is decreasing rapidly, and time-consuming. Although automated annotation of a genome sequence can be accomplished with a limited number of keystrokes, the important biological data can be difficult to discern due to the sheer volume of information to contend with. Indeed, each of the seven completely sequenced *Xf* genomes contain important answers to *Xf* biology that are now unexplained, even though many of the sequences have been available for years, and these answers will be explained in months and years to come as proper questions are framed. Genetic tests that are intermediate in information content include multiplex PCR assays (Hernandez-Martinez et al. 2006) and multilocus sequence typing (MLST) (Scally et al. 2005). Multiplex PCR is as rapid and inexpensive as conventional PCR and provides information about both presence/absence and also produces a limited amount of genetic information to distinguish subspecies as well. Multiplex PCR lacks the sensitivity of detection that single gene assays can achieve, and thus far has been used to amplify a limited number of loci. MLST involves sequencing seven housekeeping genes and it produces a few kilobases of sequence data. The method is more time-consuming, more laborious, and more expensive than multiplex PCR, but it produces much more genetic information than any method other than complete genome sequencing. The amount of data produced by MLST is easily analyzed, will easily discriminate between subspecies and strains, and can even detect genetic recombination.

While each of the genotyping methods described above may be appropriately used to answer different questions related to *Xf* genetics, no method currently available can rapidly survey a large number of genetic loci. This project is an effort to create a

large number of genetic markers to saturate the *Xf* genome so that a genetic fingerprint can be rapidly generated at minimal expense. Presently the goal is to generate 400 informative DNA markers. Each individual marker will have low information content, but together, due the number of markers, a highly informative genetic fingerprint will be generated. Roughly assuming an average *Xf* genome size of 2.5 Mb, and roughly assuming an average of 2,500 genes per *Xf* genome, 400 markers would give average genome coverage of one marker every 6250 bp, or one marker every 6.25 genes among *Xf* strains.

A densely-saturated marker system can be utilized in several ways. There are a number of questions concerning *Xf* host specificity determinants and *Xf* virulence determinants that could use additional tools such as these to help identify important genomic regions in given strains that may be involved. Very little is known about the genetics of host specificity in *Xf*. Any new information would be beneficial. While there are a number of genes that have been identified as generally important in the colonization of plants (Reddy et al. 2007; Chatterjee, Wistrom et al. 2008), few are plant host specific. The possibility exists that the genetic components of disease response are entirely in the plant genome. Given the highly interconnected nature of other plant-microbe interactions, this seems unlikely, and a high-density marker system may help identify important genomic regions common to most *Xf* infecting grape, for example. Additionally, this marker system should provide a substantive assessment of the importance of horizontal gene transfer in *Xf* strains. In other gamma proteobacteria such as *Escherichia coli*, horizontal gene transfer has been shown to be much more prevalent among virulent strains than among commensal strains (Wirth et al. 2006). Virulent *E. coli* often possess a mosaic genome composed of genetic segments from several other *E. coli* groups, and additionally show higher levels of recombination and mutation than do non-pathogenic groups. The genes responsible for horizontal gene transfer exist in the *Xf* genome (Monteiro-Vitorello et al. 2005), and recombination is detected in MLST studies using only seven genes and a limited number of isolates. This project should, on a broader scale, complement previous studies documenting genetic recombination in *Xf* strains, and could potentially help identify additional genomic regions and genes associated with pathogenicity.

OBJECTIVES

1. Identify and align 450 informative sequence polymorphisms using all seven fully sequenced *Xf* genomes.
2. Design 200 insertion/deletion (indel) and 200 single nucleotide polymorphism (SNP) assays for subspecies and strain identification.
3. Use multiplex PCR amplification to efficiently create highly informative genetic fingerprints in a single day.

RESULTS AND DISCUSSION

Complete genomes for the seven fully sequenced *Xf* strains have been downloaded and are being used to create gene alignments for conserved genes. Previous bioinformatics projects have identified conserved genes among all the sequenced genomes (Doddapaneni et al. 2006). The conserved genes are being aligned and screened visually for informative polymorphisms using VectorNTI software (Invitrogen, Carlsbad CA).

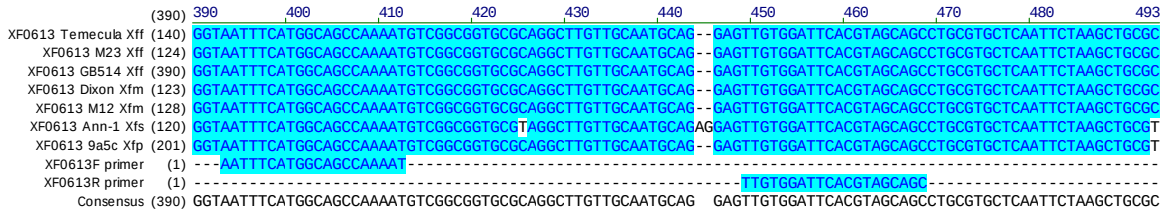

Figure 1. DNA sequence alignment of the 7 available *Xf* genomes at the XF0613 locus. A representative 2 bp indel polymorphism that would allow discrimination of *Xfs* from other subspecies is included in this portion of the alignment. Forward and reverse primer binding sites are also indicated.

In the first iteration of the project 200 indel markers are being developed. A typical alignment is shown in **Figure 1**, with the indel in the center of the sequence. Primers are designed that flank the indel marker in areas of conserved sequence using the primer3 website. Primers are designed to fit in size groups so that PCR amplicons are between 100 bp and 400 bp in size. The 200 indel markers are being amplified in multiplex PCR reactions with approximately 10 loci per reaction. The entire 200 gene indel set will require 20 PCR reactions for marker generation. Forward primers are labeled with an amplification tag as previously described (Boutin-Ganache, Raposo et al. 2001). Depending on the capillary electrophoresis instrument, several color channels can be used for efficient data collection. We are currently using ABI 3130 sequencing instruments capable of three colors for samples and one color for size markers. Amplification reactions labeled with FAM, HEX, and NED can be pooled into the same well following PCR and data can be collected simultaneously for three different bacterial strains. Gene designations follow those of the *Xfp* genome sequencing project (Simpson and ONSA 2000). A 10-plex multiplex grouping with expected amplicon sizes for the seven sequenced strains is shown in **Table 1**. A representative electropherogram from a 11-plex PCR reaction is shown in **Figure 2**. A 50 marker proof-of-concept experiment has already been carried out with both local isolates and fully sequenced type strains.

Table 1. Expected amplicon sizes for 10 loci amplified by multiplex PCR.

Locus	Temecula *Xff*	M23 *Xff*	GB514 *Xff*	Ann-1 *Xfs*	Dixon *Xfm*	M12 *Xfm*	9a5c *Xfp*
XF0053	88	88	88	95	95	95	88
XF1419	112	112	112	109	109	109	109
XF0294	129	129	129	127	127	127	127
XF1470	158	158	158	159	154	154	154
XF2759	179	179	179	197	197	197	197
XF2316	237	237	237	230	230	230	230
XF0388	267	267	267	267	269	269	269
XF1972	292	292	292	292	293	293	286
XF1267	345	345	345	345	355	355	345
XF0454	371	371	371	380	380	380	381

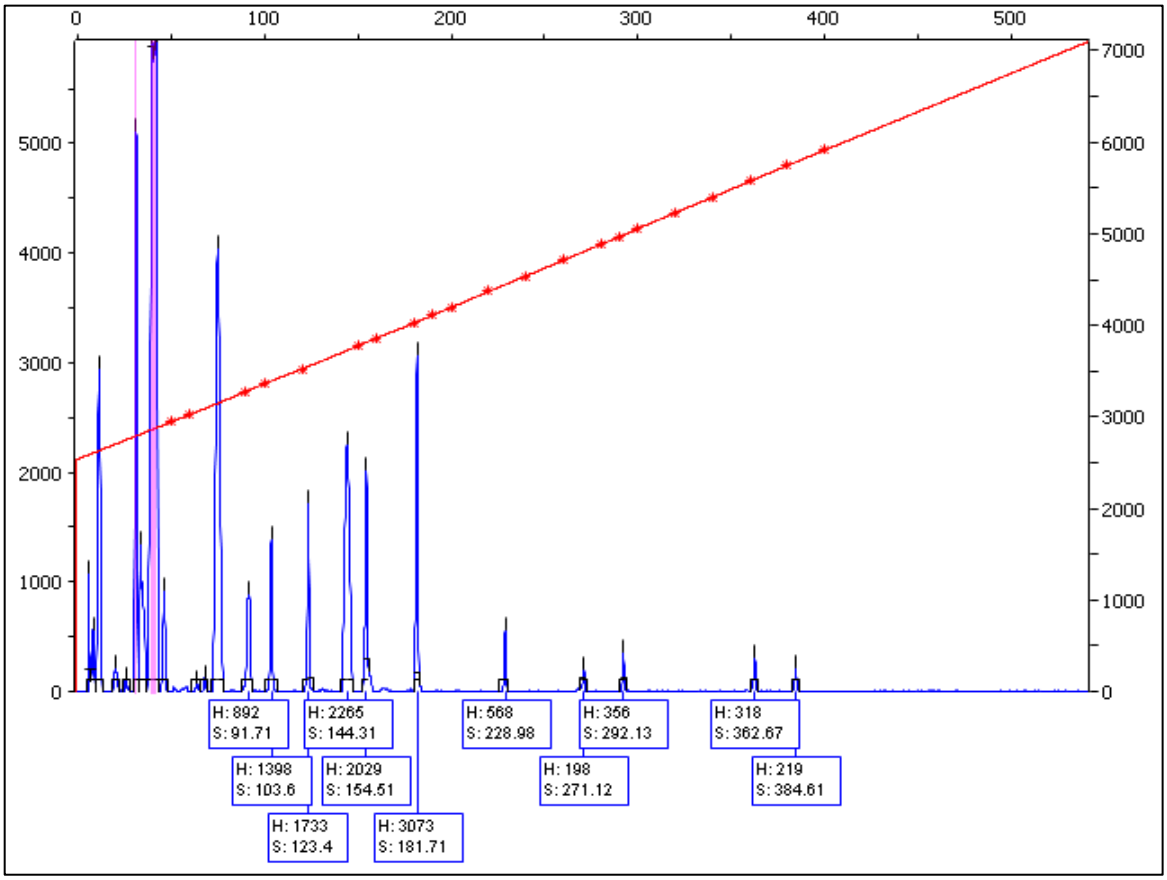

Figure 2. A representative 11-plex amplification of *Xf* indel loci. Peak sizes in the electropherogram are determined by comparison to internal size standards contained in every sample. Size standards are indicated by asterisks on the red line above the samples.

The second iteration of the project involves the development of 200 SNP markers. SNP markers are ubiquitous in *Xf* gene comparisons, so that in cases where no suitable indel exists for amplification in a given *Xf* conserved gene, there are invariably several SNPs in the gene that can be used for marker development. The level of multiplexing possible with SNP markers should be higher than is possible with indel markers. By using a similar amplification tag strategy to that used for the indel markers with the addition of size adapters (Lindblad-Toh et al. 2000), an even greater number of SNPs can be assayed per well than is possible with indel multiplex reactions, because the single base extension amplification products are precisely known sizes *a priori*. SNP markers can thus be designed in closer size ranges so that more can be analyzed per lane in single capillary gel runs.

CONCLUSIONS

In this project we are developing a large number of molecular markers to screen the *Xf* genome for important traits, to detect recombination levels, and to create a rapid and informative genetic fingerprinting system. Numerous markers have been

developed to date, and proof-of-concept experiments indicate that the system will perform as expected. At this time, marker development is approaching the halfway point.

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INFLUENCE OF HOST XYLEM CHEMISTRY ON REGULATION OF *XYLELLA FASTIDIOSA* VIRULENCE GENES AND HOST SPECIFICITY

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ABSTRACT
Xylella fastidiosa (Xf), a xylem-limited plant pathogen, causes leaf scorch diseases in many plant hosts, but individual strains may exhibit considerable host specificity. In previous work, we began to look at the effect of different host xylem fluids on expression of virulence genes. In a Pierce's disease (PD) strain of *Xf*, several virulence genes were more highly expressed in xylem fluid of grapevine vs. xylem fluid of citrus, a non-host plant for the PD strain (Shi et al., 2010). This finding suggested that host range of *Xf* may be influenced by differential expression of virulence genes in response to different host xylem chemistry. This project is to further explore that hypothesis with several strain/host combinations and to investigate components of xylem fluid that are responsible for either inducing or repressing virulence in *Xf*. We have inoculated strains of *Xf* from grapevine, almond, mulberry (Temecula-1, Ann1, M12 and Mul024) into xylem fluid extracted from each of those hosts and citrus to detect differential growth patterns and compare the gene expression using DNA macroarray and microarray techniques. Our progress in this work is described here.

LAYPERSON SUMMARY
We have shown that genes involved in disease induction by a Pierce's disease strain of *Xylella fastidiosa (Xf)* were expressed differently in sap of a susceptible plant (grape) vs. a resistant plant (citrus). This raises the possibility that the host range of different strains of *Xf* is in part due to differential regulation of bacterial genes in response to differences in chemical components of plant sap. We are further testing this idea by examining gene expression in *Xf* grown in sap with several different strain/host combinations for which we have already defined whether the particular combinations result in susceptibility, resistance, or tolerance. We will also examine specific chemical components of plant sap that influence bacterial gene expression, with the goal of discovering components that could be used for practical disease control by reducing expression of genes necessary for disease induction.

INTRODUCTION
Xylella fastidiosa (Xf) is a gram-negative gamma-proteobacterium limited to the xylem system of plants (Wells et al. 1987) and is transmitted by xylem-feeding insects (Purcell, 1990). It has been known to cause disease in a wide range of economically important plants in America, such as grapevine, citrus, mulberry, almond, peach, plum, coffee, oleander, and etc (Hopkins, 1989). *Xf* has been divided into four different subspecies (Schaad et al. 2004; Schuenzel. Et al. 2005): i) subsp. *fastidiosa*, ii) subsp. *sandyi*, iii) subsp. *multiplex*, and iv) subsp. *pauca*. The subspecies of *Xf* differ in host range, and strains within some of the subspecies can also differ widely in their host specificity. We are interested in the possible contribution of differences in host xylem fluid chemistry in determining the host specificity of specific strains.

Xf not only causes diseases at variety of host plants, but it can grow in symptomless hosts that can serve as sources of inoculum (Costa et al., 2004). Our previous study reported differential growth and expression profiles of a Pierce's disease (PD) strain inoculated into pure sap from grapevine (a symptomatic host for PD) and citrus (symptomless with PD). A number of virulence-related genes were expressed at a greater level in grapevine sap compared with citrus sap. However, some genes had greater expression in citrus sap (Shi et al. 2010). We have also shown differential growth and expression patterns in sap from different genotypes of grapevines (PD-tolerant vs. PD-susceptible) with a PD strain (Shi et al., Unpublished data). Understanding which specific chemical components of plant sap influence virulence gene expression could lead to strategies for practical disease control.

OBJECTIVES
1. Assess virulence gene expression of several different host-range strains of *Xf* in the xylem fluid of a common set of plant hosts.
2. Assess the influence of specific components of plant xylem fluids on the expression of virulence genes of *Xf*.

RESULTS AND DISCUSSION
Preparation of xylem fluid
Xylem fluid of grape, citrus, almond and mulberry was collected in August and September, 2010 in Riverside using a pressure chamber apparatus as previously described (Anderson *et al*., 1992; Bi *et al*., 2007). After sterilized using 0.22 um filters, all the xylem fluids were stored at −80°C until use.

In total, at least 40ml fluid was needed from each plant host: four (isolates) x three (replicates) x three ml (minimum volume for *Xf* growth in vitro). So far, we have collected the following amounts: grapevine (42ml), almond (12ml), mulberry (21ml) and citrus (50ml) and are continuing to collect xylem fluid this fall.

DNA macroarray preparation

DNA macroarray membranes were prepared with 110 selected genes with putative roles in *Xf* virulence, as well as others involved in the metabolism of nucleic acids and proteins, and cellular transport and stress tolerance, based on the genome sequences of *Xf* 9a5c (a CVC strain) (Simpson et al., 2000) and *Xf* Temecula-1(a PD strain) (Van Sluys et al., 2003). DNA fragments (average 600 bp) of the ORFs of the 110 genes were individually amplified by specific PCR from the genomic DNA of *Xf* Temecula-1, purified, and spotted onto nylon membranes (Hybond, Amersham Pharmacia Biotech Inc., NJ) using a manual 384-pin replicator (V&P Scientific Inc., CA). Spotted DNA was denatured with 0.4M NaOH, neutralized with standard saline phosphate EDTA, UV cross-linked, and boiled in 0.1% sodium dodecyl sulfate (Hernandez-Martinez, 2005). The primers for specific regions of genes XF0077, XF0889 and XF1968 were modified, because the original primers gave no amplification. The updated primer sequences are XF0077 (for-CGGCCTAGTGTGATAGCTT-, rev-CCAAGTTGAACTGATCAAGAC-); XF0889 (for-GGCAAGAAACATCACCATC-, rev-CCGATTTGAAAGGTGCTC-); and XF1968 (for-GCAAATATTGGGGAATCG-, rev-AAACTCAACGCCGAAGAT-). In total, 74 membranes were prepared for macroarray hybridization. Total RNA extracted for Temecula-1 cells grown on PD3 was used to verify that the hybridization protocol as described previously (Shi et al., 2010) was working well, and we are now growing *Xf* strains in the different xylem fluids for extraction of RNA to conduct our macroarray studies.

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GENOMIC SEQUENCING OF BIOCONTROL STRAIN EB92-1 AND IDENTIFICATION OF ELICITOR(S) OF EFFECTIVE DEFENSE IN *VITIS VINIFERA* AGAINST PIERCE'S DISEASE

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ABSTRACT

A draft genome of infectious but asymptomatic *Xylella fastidiosa* (*Xf*) strain EB92.1 that currently contains 2,478,730 bps of genomic sequence in 191 contigs has been created. By far the majority of the primary Blast hits were to *Xf* strain Temecula; based on the size of the Temecula genome (2,519,802 nt); based on the size of the Temecula genome (2,519,802 nt), this draft EB92.1 genome is ca. 98% complete. Comparative analyses of Temecula vs. EB92.1 revealed that EB92.1 is highly similar to Temecula in both gene order and synteny.

Type I, II and V secretion system effectors are all found in Temecula and were comparatively examined in EB92.1. No additional Type I effectors (hemolysins or colicins) were found to date in the draft EB92.1 genome that were not found in Temecula and the complete repertoire of known Temecula Type I effectors were found. This might indicate that Pierce's disease (PD) symptoms *per se* are not likely caused by these effectors, although an essential role in host colonization or adaptation is still indicated for these effectors. However, two EB92.1 type V subtilisin-like serine protease autotransporters were found to be only 71% and 69% identical, respectively, to their Temecula homologs, PD0218 and PD0313. It is possible that these variant serine proteases affect maturation of one or more Type I effectors. This idea is under experimental investigation.

A type II secreted Temecula lipase, PD1703, appeared to be completely missing from EB92.1. An *in vitro* lipase assay confirmed that EB92.1 showed no secreted lipase activity, while Temecula exhibited strong secreted lipase activity in the same assay. This lipase is an apparent *lipA* homolog of *Xanthomonas oryzae*; *lipA* is known to directly contribute to pathogenic symptoms of *X. oryzae*. A DNA clone carrying PD1703 conferred strong secreted lipase activity to *Xanthomonas*; similar tests are underway to transform EB92.1 with this lipase.

LAYPERSON SUMMARY

Xylella fastidiosa strain EB92.1 is infectious to grapevines but causes no symptoms and has been used for biological control of Pierce's disease (PD). We have determined the genomic DNA sequence of EB92.1 to 98% completion, allowing comparisons of most genes of this strain to strain Temecula, which causes PD. What are the PD-determining genes in Temecula that are not found in EB92.1? Most of the EB92.1 genes are nearly identical in gene order and protein coding with those found in Temecula. However, notable exceptions were found in three genes that are suspected to be involved either in creating the symptoms of PD or in combating competitive bacterial strains in the same xylem niche. If any of these three genes can be proven to contribute to the disease, it will become a new molecular target with potential to control the disease.

INTRODUCTION

Type I multidrug resistance (MDR) efflux system of *Xylella fastidiosa* (*Xf*) is absolutely required for both pathogenicity, and more importantly, survival of the Pierce's disease (PD) pathogen in grape (Reddy et al., 2007). Knockout mutations of the single *tolC* gene in strain Temecula are extremely sensitive to compounds produced by susceptible *Vitis vinifera* grapes, including phytoalexins, and mutants such as strain M1 were not recovered from *Vitis vinifera* grapes after inoculation (Reddy et al., 2007). As a part of the work involving defense compound and phytoalexin sensitivity tests, we discovered that the wild type strain Temecula, with its lone multidrug resistance (MDR) efflux system, is much more sensitive to plant-derived antimicrobial chemicals than most other bacterial plant pathogens, which all carry multiple MDR efflux systems. This may mean that the opportunistic *Xf* is living at the quantitative edge of survival, and ***Xf* may only be capable of surviving in the xylem vessels of hosts in which a defense response is not triggered.**

Hopkins (2005) discovered an effective PD biocontrol strain, *Xf* EB92-1, which infects grapevine and survives for many years. EB92-1 can be inoculated in a single location in a *V. vinifera* grapevine and the entire plant is protected from PD for years (Hopkins, 2005). How does this strain infect grape, and yet not cause disease? What factors are present in Temecula that may be triggers of host defense? What factors are different?

OBJECTIVES

This is the first year of a two year project. The first year's objectives were:

1. Obtain nearly the complete EB92-1 genome DNA sequence.
2. Compare EB92-1 with Temecula and identify all unique ORFs and differences, ranking the top 40 candidate ORFs for evaluation as elicitors.

RESULTS AND DISCUSSION

A draft genome of infectious but asymptomatic *Xf* strain EB92.1 that currently contains 2,478,730 bps of genomic sequence in 191 contigs has been created. The average contig length is currently 12,977 bps (Min: 100 bps, Max: 149,098 bps). By far the majority of the primary Blast hits were to *Xf* strain Temecula; based on the size of the Temecula genome (2,519,802 nt); based on the size of the Temecula genome (2,519,802 nt), this draft EB92.1 genome is ca. 98% complete. Comparative analyses of Temecula vs. EB92.1 revealed that EB92.1 is highly similar to Temecula and the gene order exhibits a very high level of synteny. The average GC Content is 53% (Min: 30%, Max: 70%). The gene coding percentage is 81%.

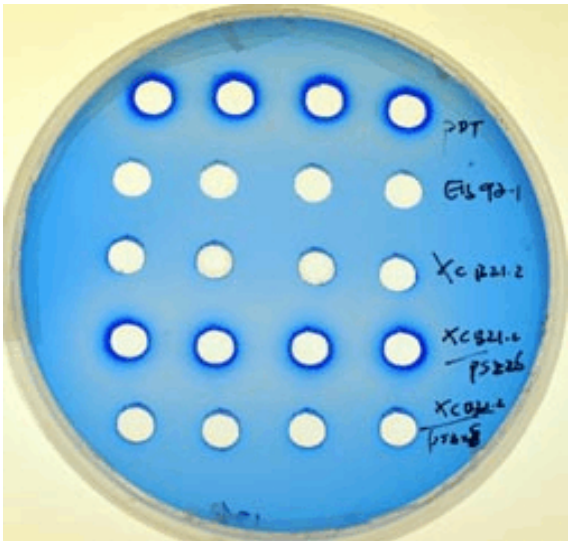
Predicted gene elements:

* 2387 genes coding for mRNA (protein coding ORFs)
* 48 genes coding for tRNA
* 3 genes coding for rRNA
* 0 repetitive elements

No additional hemolysins or colicins were found to date in the draft EB92.1 genome that were not found in Temecula. More importantly, no additional hemolysins or colicins were present in Temecula that were not found in EB92.1. The fact that the complete repertoir of known Temecula Type I effectors were found, with 100% identity, in EB92.1 (which does not cause PD), means that PD symptoms per se are not likely caused by these effectors, although an essential role in host colonization or adaptation is still indicated (Flores-Cruz et al. 2009).

Type I, II and V secretion system effectors are all found in Temecula and were comparatively examined in EB92.1. No additional Type I effectors (hemolysins or colicins) were found to date in the draft EB92.1 genome that were not found in Temecula and the complete repertoire of known Temecula Type I effectors were found, with 100% identity, in EB92.1. This might indicate that PD symptoms *per se* are not likely caused by these effectors, although an essential role in host colonization or adaptation is still indicated for these effectors. However, two EB92.1 type V subtilisin-like serine protease autotransporters were found to be only 71% and 69% identical, respectively, to their Temecula homologs, PD0218 and PD0313. Guilhabert and Kirkpatrick (2005) reported a Tn*5* mutation in PD0218 as mildly reduced in pathogenicity. Since a mutation in PD0218 causes failure of Temecula to secrete a Type I bacteriocin, PD1427 (Igo, 2009 PD Symposium Proceedings, p97-101), it is possible that these variant serine proteases affect maturation of one or more Type I effectors. A modified, but functional PD0218 may secrete a modified bacteriocin that is toxic to Temecula and help explain the biological control properties of EB92.1. This idea is under experimental investigation.

A potentially significant discovery is that a type II secreted Temecula lipase, PD1703, was completely missing within an assembled contig of EB92.1, evidently as a result of a genomic deletion. This result was confirmed by multiple PCR analyses of the evidently deleted region. An *in vitro* lipase assay was conducted using Tween 20 as the substrate and 0.01% Victoria Blue B as indicator, exactly as described by Samad et al. (1989)



In the figure at left, each row of four wells was filled with 50 ul of cell culture supernatant in the following order (top row to bottom row):

<--Temecula (labeled PDT)

<--EB92.1 (labeled EB92-1)

<--*Xanthomonas citri* B21.2 (labeled Xc B21.2)

<--*X. citri* B21.2 transconjugant plus cloned PD1703, driven by native promoter and cloned in pBBR1-MCS5 (downstream from *lacZ* promoter).

<--*X. citri* B21.2 transconjugant plus cloned PD1702, driven by native promoter and cloned in pBBR1-MCS5 (downstream from *lacZ* promoter).

The top row (Temecula supernatant) and fourth row (*X. citri* B21.2 transconjugant plus cloned PD1703 supernatant), clearly demonstrate strong amounts of secreted lipase in the culture supernatants (not concentrated or purified). This level of lipase activity is not present in the supernatants of EB92.1, *X. citri* B21.2, or *X. citri* B21.2 transconjugant with another lipase (PD1702) cloned from Temecula, but also present (with one amino acid substitution) in EB92.1. These assays were repeated twice, and including as a control *X. citri* / pBBR1-MCS5 (not shown).

The PD1703 lipase is a homolog of *lipA* from Xanthomonas oryzae (GenBank Accession XOO0526). The *X. oryzae lipA* is known to be a cell wall degrading esterase/lipase that elicits host defenses, including programmed cell death, in rice (Jha et al. 2007; Aparna et al. 2009). Mutations of *lipA* in *X. oryzae* lead to a partial loss of pathogenicity on rice (Rajeshwari et al. 2005; Jha et al. 2007).

Finally, a unique hemagglutin-like protein in Temecula, PD0986, appeared to be missing in the EB92.1 genome. Since mutations of hemagluttinins in *Xf* show increased virulence are thought to be important in impairing *Xf* movement in the plant xylem (Guilhabert & Kirkpatrick, 2005), loss of PD0986 may well assist the asymptomatic EB92.1 in increasing its movement in grapevine and make it more effective as a biocontrol agent. Although the EB92.1 genome is incomplete, it deserves mention that the Temecula region in which this locus is found appears to be missing from the middle of a large EB92.1 contig. There were no good hits of PD0986 to any small contigs, making it appear unlikely that further sequencing will uncover this locus in EB92.1.

CONCLUSIONS

The identification of two genes that encode comparatively significantly different modified serine protease autotransporters that in turn may affect Type I effectors provides potential additional support for the idea that Type I effectors, such as colicins and hemolysins, can affect pathogenicity. However, even knockout mutations of these autotransporters do not explain the major differences between the biocontrol strain EB92.1 and PD-causing Temecula. The discovery of a *lipA* homolog in Temecula that is missing in EB92.1, and which is known in the literature as a pathogenicity factor and defense elicitor may help explain, at least in part, why EB92.1 fails to elicit PD symptoms. This must be confirmed, but this potential lead would not have been made without the genomic sequence of both Temecula and EB92.1. If pathogenicity genes are found in Temecula that are not found in biological control strain EB92-1, and if these genes can be demonstrated to be primarily responsible for generating PD symptoms, then methods to interfere with the mechanism of action of such genes may be found that would not be otherwise considered.

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CONTINUED ASSESSMENT OF *XYLELLA FASTIDIOSA* FIMBRIAL ADHESINS AS IMPORTANT VIRULENCE FACTORS IN PIERCE'S DISEASE: INFLUENCE OF XYLEM SAP

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Reporting Period: The results reported here are from work conducted March 2010 to October 2010.

ABSTRACT

Specific biological characteristics of *Xylella fastidiosa* (*Xf*) Temecula were investigated in microfluidic flow chambers *in vitro* by examining the effect of xylem sap from Pierce's disease (PD) susceptible *V. vinifera* and resistant *V. smalliana* grapevines on *Xf* cell growth, aggregation, biofilm formation, and motility. Growth of *Xf* was observed in both *V. smalliana and V. vinifera* xylem saps in microfluidic flow chambers. While *Xf* cell density increased in *V. smalliana* sap, the cells exhibited a reduction in aggregation and biofilm formation compared to that observed in *V. vinifera* sap. In addition, motility via pilus twitching activity was reduced in *V. smalliana* sap when compared to similar activities in *V. vinifera* sap, indicating *V. smalliana* sap may inhibit the function of type IV pili. Normal twitching motility of *Xf* was restored once *V. smalliana* sap was exchanged with *V. vinifera* sap, indicating that chemical components of *V. vinifera* sap possibly influence the function of type IV pili of *Xf* cells, that in turn may affect aggregation.

LAYPERSON SUMMARY

Cells of *Xylella fastidiosa* (*Xf*) aggregate, form biofilms, and occlude the host's vascular (xylem) system, resulting in Pierce's disease symptoms in grapevine. Colonization of grapevine xylem by *Xf* involves migration of individual cells through a process of twitching motility by which hair-like type IV pili are repeatedly extended from the cell, attach to the xylem surface, and are retracted, pulling the cell forward. Using microfluidic 'artificial' chambers through which xylem sap from highly susceptible and resistant grapevines is flowing, the biological behavior of *Xf* in these saps was assessed. Toward this we have observed reduced motility in sap from a resistant grapevine, *V. smalliana*. Also reduction in formation of cell aggregates and biofilms.

INTRODUCTION

This project continues efforts toward understanding the biological relationship between *Xylella fastidiosa (Xf)* cells and the xylem environment, and specifically the roles of fimbrial adhesins (type I and type IV pili, and associated proteins) in *Xf* virulence, motility, aggregation and autoaggregation, and biofilm development. The research targets the functional biology of *Xf* in xylem sap. It tests and explores traits of sap and xylem vessels from resistant and susceptible grapevines, and eventually that of citrus, that may inhibit or promote *Xf* cell activities associated with *pil* and *fim* gene products.

Previous observations describing roles for fimbrial adhesins (type I and type IV pili) in *Xf* virulence, motility, aggregation, and biofilm development have provided insight into their genetic mechanisms and regulation (De La Fuente, 2007; 2008). Studies on *Xf* motility and biofilm formation under natural conditions viz., *in planta,* have been hindered in part by the optical inaccessibility of vascular tissue. Recent studies have shown the importance of xylem sap chemistry on growth, aggregation, and attachment of *Xf* cells, highlighting the establishment of stable cultures in 100% xylem sap (Andersen, 2007; Zaini, 2009). Studies with *V. riparia* and *V. vinifera* cv. Chardonnay sap (100%) in either microfluidic chambers or in culture tubes have shown that the pathogen responds to this more natural chemical environment differently than it does in rich artificial media such as PD2 (Zaini, 2009). Aggregation and biofilm development are enhanced (Zaini, 2009), and early indication is that twitching motility is also greater—in both the number of *Xf* cells and in rate of movement. It was reported that xylem sap from Pierce's disease (PD) resistant *V. rotundifolia* maintained *Xf* in a planktonic state, whereas the bacterium was more likely to form aggregates when incubated in xylem sap from susceptible *V. vinifera* cultivars (Liete, 2004). Those directed the attention to a more natural environmental system for *Xf*—one that will greatly enhance the value and significance of information generated in studying *Xf* in an *in vitro* system: the inclusion of xylem sap and xylem vessel tissue.

Previous work that both type I and type IV pili are involved in aggregation and biofilm development (Li, 2007), type IV pili of *Xf* are involved in twitching motility within the xylem vessels of grapevine (Meng, 2005). Citrus is often grown adjacent to vineyards in California and may be considered a potential reservoir for PD *Xf* (Bi, 2007). Xylem sap from commercial citrus plantings in Temecula (grapefruit, orange, lemon) did not support *Xf* biofilm development while at the same time

grapevine xylem sap obtained from adjacent vineyards supported thick biofilms (Shi, 2010). Citrus xylem sap did not support the induction of a number of *pil* and *fim* genes, such as *pilT*, a gene that encodes for type IV pilus retraction (necessary for twitching motility), *pilY1*, a gene encoding a type IV pilus tip adhesion protein, *pilI*, *pilU*, and *fimA* that encodes the type I pilus subunit (Shi, 2010). The significant reduction in *pil* and *fim* gene expression in citrus sap is notable for at least two reasons: i) it may explain, in part, why the PD strain of *Xf* is not symptomatically expressed in citrus i.e. it does not move from the sites of introduction (no twitching motility), nor does it form biofilms, and ii) it may provide valuable clues into what chemical factors from citrus sap may be exploited in grape to reduce or inhibit similar gene product expression. Based on those data, it may be that in xylem sap from Pierce's disease resistant grapevines that pili function is suppressed.

OBJECTIVES

Objectives covered in this report include:

1. Establish a baseline of *Xf* activity *in vitro* for grapevine sap. This will include temporal and spatial activities for pili-associated functions—motility, cell aggregation, and biofilm formation.
2. Assess pili-associated functions in grapevine sap from *Vitis vinifera* cultivars and *Vitis* species expressing distinct PD resistance and susceptibly.

RESULTS

Xf survives in *V. vinifera* and *V. smalliana* sap

We used grapevine xylem sap from bleeding *V. vinifera* and *V. smalliana* vines collected in Geneva, NY during the Spring of 2009 and 2010. *V. vinifera* is known to be susceptible to PD and *V. smalliana* is resistant (Fritschi, 2007; Lu, 2008). To verify whether *V. smalliana* sap was inhibitory to *Xf*, growth of *Xf* was assessed in *V. vinifera* and *V. smalliana* sap as well as in PD2 broth. The *Xf* cell density in *V. smalliana* sap was lower than in *V. vinifera* sap. However, overtime the cell density in *V. smalliana* sap increased (**Figure 1**). At 5, 8, 24, 72 and 120 hours after introducing *Xf* cells into PD2 broth, *V. vinifera*, and *V. smalliana* saps in glass tubes described as above, 100µl xylem sap or PD2 broth containing *Xf* cells was removed from the tubes, serial diluted, and plated onto PD2 agar, and incubated at 28ºC for 7 to 10 days. The resulting bacterial colonies were counted for each dilution, and were described as colony forming unit (CFU)/ml^{-1}. CFU/ml^{-1} in *V. smalliana* sap increased after introducing *Xf* cells into xylem sap. *Xf* cells grew well in *V. vinifera* sap, which is consistent with previous observations for growth of *Xf* in xylem sap (Andersen, 2007; Zaini, 2009; Shi, 2010). The increase in the number of *Xf* cells in *V. smalliana* sap (**Figure 1**) suggest *V. smalliana* sap is not lethal to *Xf* and supports growth, but at a reduced rate.

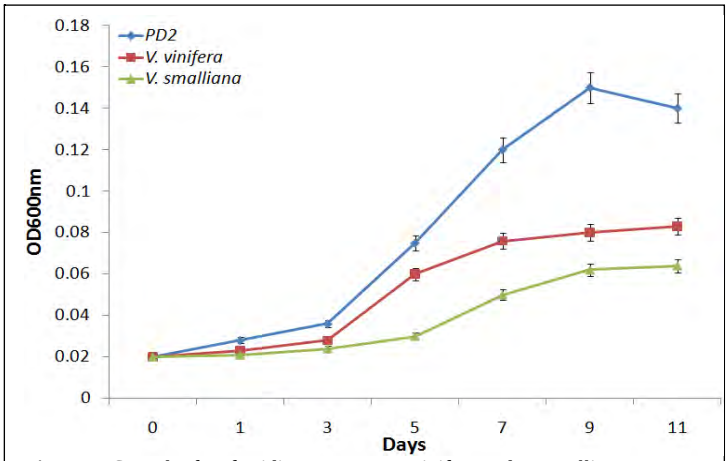

Figure 1. Growth of *X. fastidiosa* in PD2, *V. vinifera* and *V. smalliana* sap. The cell concentration in the xylem sap and PD2 was measured at OD_{600} at every 2 days after inoculation.

Xf aggregation and biofilm formation in *V. vinifera* and *V. smalliana* sap

The relative percentage of *Xf* cell aggregation in PD2 broth, *V. vinifera* and *V. smalliana* sap was measured as previously described (Burdman, 2000). Aggregation of *Xf* cells, overtime, was lower in *V. smalliana* sap than in *V. vinifera* sap and PD2 broth. Biofilm formation of *Xf* in PD2 broth, *V. vinifera* and *V. smalliana* sap was determined by a crystal violet staining method (Leite, 2004). *Xf* had a reduced biofilm development in *V. smalliana* sap compared to *V. vinifera* sap and PD2 broth (**Figure 2**). This indicates that *Xf* cells were able to survive and multiply in *V. smalliana* sap, but with reduced aggregation and biofilm formation.

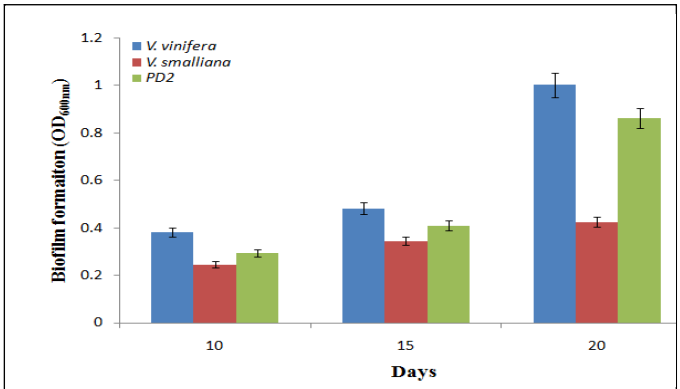

Figure 2. Quantitative assessment of biofilm formation of *Xf* in various media. Data represent three replicates for each experiment.

Aggregation of *Xf* cells in *V. vinifera* and *V. smalliana* sap in microfluidic flow chambers

A new four-channeled chamber was developed and used to facilitate experimental observations of several parameters at the same time (**Figure 3**). The width and depth of the individual channels was designed so that in subsequent experiments thin sections of grapevine xylem wood can be placed in the channels for direct observation of *Xf* on xylem vessel walls—in xylem fluid.

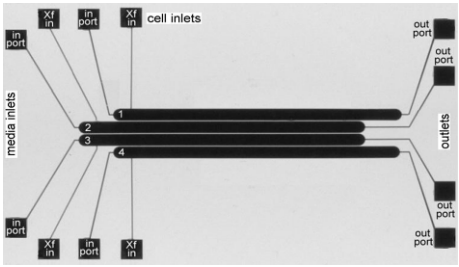

Figure 3. Four-channeled microfluidic device with each channel having media in and out ports, and one access port for introduction of *Xf*.

In PD2, *Xf* cells were observed to aggregate and form large clumps at 2-7 days after introduction of the *Xf* cells into the microfluidic flow chamber (**Figure 4**). The aggregates eventually developed a thick biofilm and slowed flow. *Xf* cells in *V. vinifera* sap formed many small clumps throughout the chamber. However, *Xf* cells in *V. smalliana* sap aggregated to form smaller clumps distributed throughout the chamber. This result indicates *V. smalliana* sap may inhibit aggregation of *Xf* cells.

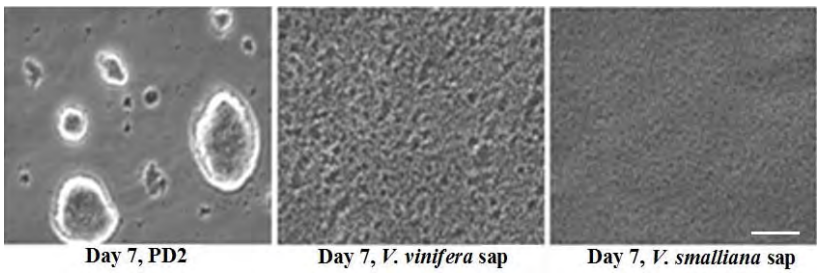

Figure 4. Images taken after 7 days of introducing *Xf* in PD2 and xylem sap. Large aggregates are formed in PD2, small aggregates in *V. vinifera* sap; and smaller clumps in *V. smalliana* sap. Scale bar equals 50 µm.

Twitching of *Xf* in *V. vinifera* and *V. smalliana* sap in microfluidic flow chamber

Microfluidic flow chambers provide a constant supply of 'new' sap and represent a condition more akin to that *in planta* as opposed to culturing in test tubes. *Xf* cells twitched in PD2 broth and *V. vinifera* sap at 2-8 days after introducing of the *Xf* cells into microfluidic flow chamber. The cells aggregated and formed relatively homogenous biofilms after nine days in the chamber. In *V. smalliana* sap, relatively few *Xf* cells attached to the glass surface; furthermore, few *Xf* cells exhibited

twitching motility. Long non-separated cell profiles with clear division points were observed, and confirmed that *Xf* cells were able to grow in *V. smalliana* sap. The fact that *Xf* cells in *V. smalliana* sap exhibit greatly reduced twitching activity may explain the observation that *Xf* spreads faster in xylem vessels of PD-susceptible grapevines compared to PD-resistant or tolerant grapevines (Hopkins, 1984; Fry & Milholland, 1990).

Effect of changing PD2 to *V. smalliana* sap on the twitching motility of *Xf* cells

Xf cells were observed to twitch and to form large aggregates in PD2 broth, but not in *V. smalliana* sap during the 1st-2nd day after introducing the *Xf* cells into the chamber (**Figure 5**). On day three, PD2 was exchanged with *V. smalliana* sap for 3-5 additional days. Interestingly, after about eight hours, the aggregated clumps disaggregated, and *Xf* cells dramatically reduced twitching motility (**Figure 4**). Finally, *Xf* cells became uniformly distributed within the chamber. The data suggest that either the presence or absence of a chemical component of *V. smalliana* sap prevents efficient aggregation of *Xf* cells, and prevents *Xf* cells from twitching.

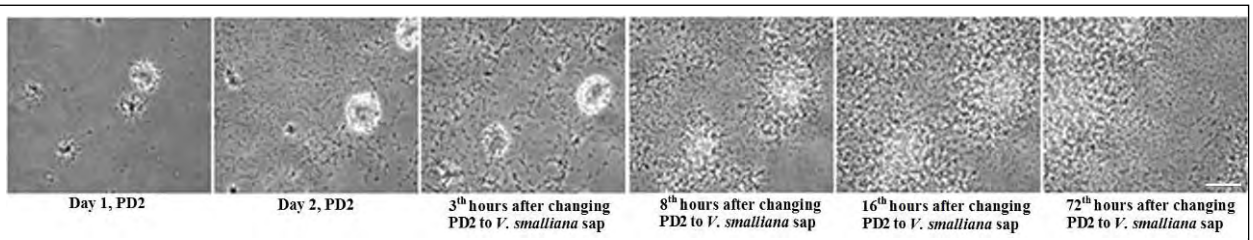

Figure 5. Images taken before and after PD2 was changed to *V. smalliana* sap. Aggregates in PD2 disaggregated in *V. smalliana* sap. Scale bar equals 50 µm.

Effect of changing *V. vinifera* to *V. smalliana* sap on the twitching motility of *Xf* cells

Xf cells were observed to readily twitch and to form aggregates in *V. vinifera* sap during the 1st-2nd day after introducing the *Xf* cells (**Figure 6**). On day three, *V. vinifera* sap was exchanged with *V. smalliana* sap for three additional days. After eight hours, aggregated clumps disaggregated. *Xf* cells dramatically reduced twitching motility and appeared to attach to chamber surface at one cell pole (presumably, the pilus pole). Five days after exchanging sap, the *Xf* cells were uniformly distributed within the chamber and without the formation of large aggregates. As a control, *Xf* cells in *V. vinifera* sap in an adjacent channel continued to twitch and form aggregates, eventually forming a robust biofilm. These results indicate that *V. smalliana* sap affect the function of type IV pili of *Xf* cells, and thus prevents *Xf* cells from twitching and aggregating.

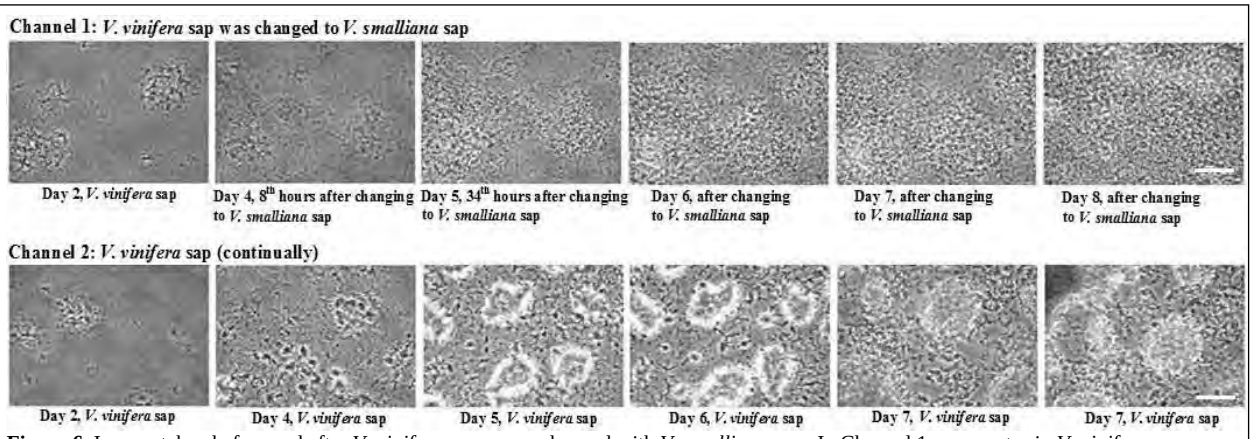

Figure 6. Images taken before and after *V. vinifera* sap was exchanged with *V. smalliana* sap. In Channel 1, aggregates in *V. vinifera* sap were disaggregated in *V. smalliana* sap. In Channel 2, *Xf* aggregated and twitched to form biofilm in continuously *V. vinifera* sap. Scale bar equals 50 µm.

Effect of changing *V. smalliana* to *V. vinifera* sap on the twitching motility of *Xf* cells

In *V. smalliana* sap during the 1st-9th day after introducing the *Xf* cells into the chamber, *Xf* cells were observed to not be twitching, but were attached at one pole to the chamber surface (**Figure 7**). On day nine, *V. smalliana* sap was exchanged with *V. vinifera* sap (**Figure 7**). After one hour, few *Xf* cells were twitching; however, after five hours cells within the aggregates dispersed, and many initiated twitching activity; after twenty-four hours, many more cells were actively twitching. The loss of twitching motility of *Xf* in *V. smallaina* sap was recovered once the sap was exchanged with *V. vinifera* sap (**Figure 7**), suggesting the function of type IV pili was inhibited by a components in *V. smallaina* sap. The chemical components of *V. vinifera* sap may activate/induce the function of type IV pili of *Xf* cells.

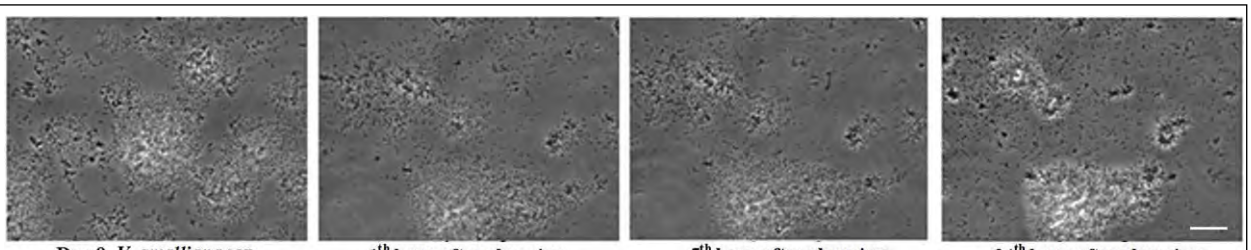

Figure 7. Images taken before and after *V. smalliana* sap was changed *V. vinifera* to sap. Aggregates in *V. smalliana* sap were disaggregated and free *Xf* cells started twitching in continuously *V. vinifera* sap. Scale bar equals 50 µm.

CONCLUSIONS

The symptomatic development of PD in grapevine is related to biological features of the *Xf* pathogen and how it interacts with its host. By establishing more natural features of xylem vessels environment to study the motility and aggregation of *Xf*, we hope to provide a better understanding of the biological features of the *Xf* in natural xylem sap. The long-distance directional upstream migration of *Xf* might enhance intraplant spread of the bacteria and colonize grape xylem vessels from the initial site of infection. The present results might suggest that the inhibition of twitching motility of *Xf* by the chemical components in *V. smallaina* sap may limit the spread of *Xf* in xylem vessels in PD-resistant grapevines, resulting in the restriction of *Xf* to fewer xylem vessels and less proportion of *Xf* colonized vessels, which results in a limitation of systemic infection and no PD development in resistant grapevine.

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EXPLOITING A CHEMOSENSORY SIGNAL TRANSDUCTION SYSTEM THAT CONTROLS TWITCHING MOTILITY AND VIRULENCE IN *XYLELLA FASTIDIOSA*

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ABSTRACT

Previously we demonstrated that twitching motility in *Xylella fastidiosa* is dependent on an operon encoding signal transduction pathway proteins (*pilG, pilI, pilJ, pilL, chpB* and *chpC*), which is related to the system that controls flagella movement in *Escherichia coli*. We report three advances in examining this system. First, we have examined the operon genes more closely. We have discovered that the operon is essential for the twitching phenotype, determined the importance of each gene individually, and discovered that the *chpB* gene is non-functional. Further characterization of these genes is underway. Second, we have found that the chemoreceptor, PilJ, is localized to one pole of the cell. We are currently determining if the chemoreceptors and pili are found at the same pole of the cell. Third, we have continued our examination of *chpY*, a gene similar to *pilG*, which plays a role in Pierce's disease development.

LAYPERSON SUMMARY

This project involves studying the chemical sensing pathway by which the plant pathogen *Xylella fastidiosa* is able to control its movement within the plant environment. We examined a gene cluster essential for cell movement (twitching motility), we identified where the initial protein regulating the signaling response is located in the cell, and we determined that chemical sensing is important for developing disease symptoms. These results give insight into targets for preventing Pierce's disease.

INTRODUCTION

Bacteria sense and respond to changes in their environment, integrating the signals to produce a directed response. *Xylella fastidiosa* (*Xf*) is a non-flagellated, xylem-restricted Gram-negative bacterium that moves within grapevines via twitching motility that employs type I and type IV pili (Meng et al. 2005). Movement appears to be controlled by a chemosensory system similar to that first reported in *E. coli* in which a group of *che* genes regulates the rotational movement of flagella. Transmembrane chemoreceptors bind chemical stimuli in the periplasmic domain and activate a signaling cascade in their cytoplasmic portion to ultimately control the direction of flagella rotation (Hazelbauer et al. 2008). We previously found that the chemosensing gene cluster is an operon (named Pil-Chp) that regulates type IV pili, and that disruption of the operon leads to a decrease in Pierce's disease (PD) symptoms (**Figure 1**). Herein, we further characterize the genes in the Pil-Chp operon and describe our advances in understanding the role of *pilJ* signaling in *Xf*.

OBJECTIVES

1. Complete characterization of the single chemosensory regulatory system of *Xf* and its function in PD. In particular, we will focus on its role in mediating bacterial movement and biofilm formation. Toward this end we will:
   a. Obtain *Xf* mutants in the *pilJ* gene that encodes the single methyl-accepting chemotaxis protein in *Xf*.
   b. Assess virulence and motility of *pilJ* mutants in grapevines, as well as previously created mutants deficient in related chemosensory genes, *pilL* and *chpY*.
2. Identify environmental signals that bind PilJ and activate chemosensory regulation. Toward this end we will:
   a. Express PilJ or a chimeric form of PilJ in a strain of *E. coli* previously deleted of all methyl-accepting chemotaxis protein genes.
   b. Subsequently, candidate signals will be screened using the above *E. coli* system for activation of motility.

RESULTS AND DISCUSSION

Construction of the *Xf* chemosensory operon null mutant strains. The construction of a non-polar, allelic exchange mutant of *pilJ* gene in *Xf* was performed according to Chatterjee *et al*. 2008 with slight modifications. We also disrupted the other genes in the Pil-Chp operon: *pilG*, *pilI*, *chpB*, and *chpC*. The disruption of each gene in marker-exchange mutants was confirmed by PCR (not shown). The *pilL* gene was disrupted previously with polar transposon mutations (Hoch et al. 2008a).

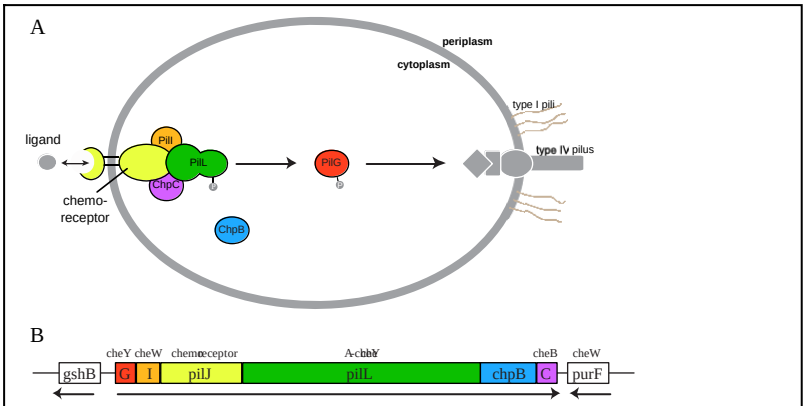


Figure 1. **The *Xf* chemotaxis system.** A) Model for chemosensory regulation of twitching motility in *Xf*. The chemoreceptor PilJ senses environmental signal(s). ChpC/PilI couples PilL to PilJ. PilL phosphorylates its hybrid CheY-like receiver domain and PilG. ChpB is homologous to ligand adaptation proteins but appears non-functional. B) The Pil-Chp chemosensory operon genes with *E. coli* homologous genes shown above and direction of transcription shown below.

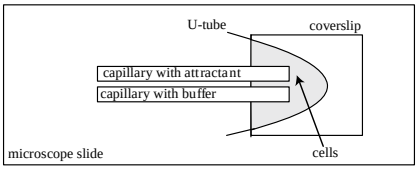
Construction of plasmids to complement the *Xf* chemosensory operon null mutant strains. To complement the non-polar *pilJ, pilG, pilI, chpB,* and *chpC* gene disruptions, we needed to construct plasmids containing the chemotaxis operon promoter region. The Pil-Chp operon lies 256 bp downstream of the unrelated gene *gshB*, which is transcribed in the opposite direction (**Figure 1**). Based on computer programs and the spacing between *gshB* and the Pil-Chp operon, we cloned two potential Pil-Chp operon promoters into *Xf*-compatible plasmids. We then cloned the various Pil-Chp genes into these constructs. We are currently preparing to transform the constructs into the null mutants in order to perform complementation studies.

Twitching motility of the *Xf* chemosensory operon null mutant strains. Previously we observed that the *pilL* mutant was twitching minus on both PW agar surfaces and in microfluidic chambers (Hoch et al. 2008a). Examination of the *pilJ* mutant on PW agar surfaces revealed colony morphologies with smooth margins consistent with loss of type IV pili twitching motility function (data not shown) (Meng et al. 2005). These findings were confirmed by measuring twitching motility of cells in microfluidic chambers. Similarly, the *pilG* and *pilI* mutants exhibited no movement, whereas the *chpB* mutant performed twitching motility like wild-type cells. We are currently characterizing the *chpC* mutant.

Identifying the chemosensory attractant. To examine what sap component results in a motility response, the putative *Xf* chemoreceptor was expressed in *E. coli*. The *pilJ* gene failed to complement the *E. coli* chemotaxis system (Hoch et al. 2009). To facilitate PilJ functioning in an *E. coli* system, we constructed chimeric chemoreceptors that contained the periplasmic ligand binding domain of the *Xf* PilJ fused to the cytoplasmic signaling domain of the *E. coli* serine chemoreceptor, Tsr. The chimera successfully activated the *E. coli* CheA kinase as the CheA binding site is maintained in the Tsr cytoplasmic portion (Hoch et al. 2009).

To confirm that PilJ binds to a molecule in grape sap, we tested the chimeras using a modified capillary assay (Grimm and Harwood, 1997). In this assay, a chamber is created between a microscope slide and coverslip separated by a capillary tube bent into a U-shape (**Figure 2**). Capillaries containing potential attractant or buffer in 2% agar are placed in the chamber. Cells suspended in aerated buffer are then added. Cells expressing the ligand adaptation system "sense" ligand via the chemoreceptors and migrate to the tip of the attractant-containing capillary creating a visible dense swarm of cells within 10-15 minutes. Cells expressing the chimera construct failed to respond to saps known to support twitching motility of *Xf*. Numerous reasons could explain this result. The attractant may have been at a concentration too low for assay detection, the attractant may require a *Xf* transporter protein to enter the periplasmic space, or the attractant may bind a *Xf* periplasmic binding protein before docking to the chemoreceptor. Alternative approaches are being explored.

A



B

	wt E. coli	chimera
LB media	+	nd
10mM serine	+	nd
PW media	nd	-
V. riparia	nd	-
V. viniferia	nd	-

Figure 2. Modified capillary assay and results. A) Cells are placed in a chamber with capillaries filled with agar and the appropriate attractant. If a capillary contains an appropriate chemical stimulus, the cells will swarm around the capillary tip. B) Cells were grown in T broth at 30°C and induced at log phase. At OD_{600}=0.4 they were washed 3 times in 10mM phosphate buffer pH 7.0 with 0.1mM EDTA and resuspended to OD_{600}=0.01. Capillary tubes were filled with 2% agar and appropriate attractant (LB media or serine for wt cells; PW media or sap for chimera-expressing cells) or buffer. Cells either swarmed (+) or did not swarm (-) at the tips as observed by 40X magnification. nd = not determined.

Localization of the chemoreceptor. Chemoreceptor localization has been studied in only a handful of organisms and found to be polar or cytoplasmic (Maddock and Shapiro 1993; Harrison et al. 1999; Bardy and Maddock 2005; DeLange et al. 2007). In *E. coli*, chemoreceptors cluster into a polar lattice that presumably allows the receptors to work in concert and amplify the signal (Parkinson et al. 2005). As a result, the chemoreceptors are physically at a distance from the flagella. In *Pseudomonas aeruginosa* the chemoreceptor PilJ is polar localized, however it was found to be at both poles and its location relative to the type IV pili was not identified (DeLange et al. 2007). We wish to learn if the *Xf* PilJ is a) polar localized, b) at one or both poles, and c) if it co-localizes with the pili. To answer this question, we expressed PilJ protein for antibody production and used it to label the chemoreceptor. We discovered that PilJ is expressed at a single cell pole (**Figure 3**). Immunocytochemical localization of an antibody that highlights an array of *Xf* proteins, including pili, revealed a distinct labeling at one end of the *Xf* cells consistent with the single pole location of type I and type IV pili (Meng et al. 2005; Hoch et al 2008b). We will next perform co-localization experiments to determine if the chemoreceptors and pili are at the same or opposite pole.

Figure 3. Localization of PilJ. Microscope images of wt *Xf* cells with anti-PilJ antibody (left) or anti-*Xf* antibody (right) that highlights the pili.

Pil-Chp operon and ChpY. The *chpY* gene lies downstream of the Pil-Chp operon and has homology to the *Xf pilG* gene. In a similarly organized chemosensing-like system, the *P. aeruginosa* Pil-Chp operon has downstream genes that produce proteins that associate with the operon protein products (Whitchurch et al. 2004). In addition to the *pilG*-like domain, the *chpY* has GGDEF and EAL related regions indicating its involvement in biofilm formation (Burr et al 2008). Deletion of *chpY* results in reduced cellular motility, unaltered pili biogenesis, and increased biofilm formation (Burr et al 2008).
To determine if there was a regulatory relationship between the Pil-Chp operon and *chpY*, we performed RT-PCR on the pill gene in a *chpY* null strain. When *chpY* is deleted, *Xf* shows no changes in the expression of *pilL* (**Figure 4a**). However, *chpY*

does have an effect on PD. Grapevines inoculated with the *chpY* mutant had increased PD progression compared to a wt *Xf* infection (**Figure 4b**), which may stem from increased biofilm formation or reduced twitching motility.

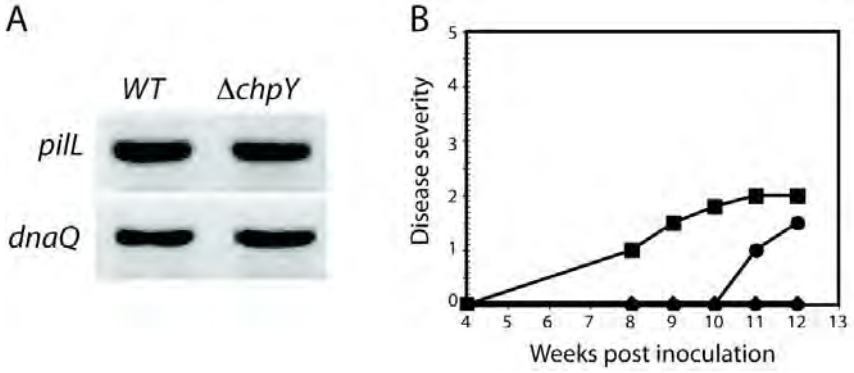


Figure 4. **Pil-Chp operon and the importance of ChpY for Pierce's disease.** A) The expression of the *pilL* gene from the Pil-Chp operon is unaffected by the presence of *chpY*. B) Grapevines (n=6) inoculated with wt *Xf* (circle), *chpY* mutant cells (square), or buffer (triangle) were assessed for disease progression over 12 weeks.

CONCLUSIONS

Our results with the Pil-Chp mutants show that the operon is required for twitching motility in *Xf*. Interestingly, some of the genes in the operon may not be functional. Currently we are studying additional phenotypes of the mutants including growth and biofilm formation. We have determined that PilJ is polar localized in *Xf* and will learn if it is co-localized with the pili. We are attempting new approaches to find the chemosensing stimuli in grape sap. Additionally, we are exploring *chpY*, which does not appear to be directly involved in chemosensing, but plays a role in biofilm formation and twitching motility.

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THE ROLE OF TYPE V SECRETION AUTOTRANSPORTERS IN THE VIRULENCE OF *XYLELLA FASTIDIOSA*

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ABSTRACT
The *Xylella fastidiosa* Temecula1 genome encodes six proteins that have been classified as AT-1 autotransporters. Autotransporters are multi-domain proteins that are responsible for secreting a single specific polypeptide (passenger domain) across the outer membrane of Gram-negative bacteria. Here, we describe our genetic characterization of PD0218, PD0313, and PD0950, the three autotransporters predicted to have proteolytic activity. In the laboratory, a strain carrying a mutation in all three protease genes has properties similar to wild type in terms of its growth rate and its ability to aggregate and form a biofilm. When infected into grapevines, the triple mutant shows very few PD symptoms in spite of the fact that its ability to migrate and colonize the xylem is indistinguishable from wild type. We have also compared of the properties of strains missing only one protease versus strains missing two proteases. These studies suggest that PD0313 and PD0950 are both virulence factors and that PD0218 is involved for reducing virulence, possibly through its interaction with PD0950. Finally, comparisons of the secreted proteins from the three single mutants suggest that each protease has a different set of target proteins. Experiments are currently underway to examine the secreted protein profiles for the double and triple mutants.

LAYPERSON SUMMARY
Bacteria have developed numerous strategies for infecting and colonizing host organisms; many involve proteins that are either secreted to the cell surface or released into the external environment. Some of these proteins are virulence factors that allow the bacteria to adhere to the host tissue, utilize nutrients available within this tissue, and counteract any defense response launched by the host. However, other proteins are involved in minimizing the damage caused by these virulence factors. Here, we describe our work on three proteins secreted by *Xylella fastidiosa* Temecula1 that are classified as serine protease autotransporters. Mutational analysis indicates that two of the proteins act as virulence factors, whereas the third protein appears to decrease virulence. Understanding the mode of action for each of the proteases will allow us to develop methods for interfering or enhancing their function, thereby reducing the damage caused by this important plant pathogen.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a Gram-negative, endophytic bacterium, which is responsible for a number of economically important plant diseases [reviewed in (1, 7)]. *Xf* is spread from infected plants to uninfected plants by xylem-feeding insects, such as sharpshooters and spittle bugs. The ability of *Xf* to colonize the plant and to incite disease is dependent upon the capacity of the bacterium to produce a diverse set of virulence factors. Comparison of the *Xf* genome to other bacterial pathogens has resulted in the identification and characterization of a number of genes that are potential virulence factors. Many of these virulence determinants are proteins that are either secreted to the bacterial cell surface or released into the external environment (4, 6). Another important category of proteins are "anti-virulence factors" or host-protective virulence factors (13). These proteins are not as easy to identify using genomic methods. These pathogen-encoded "anti-virulence" proteins are thought to be important in modulating the activity of the host-damaging virulence factors. It has been suggested that protecting the host cell from too much damage may be an integral part of the strategy adopted by bacterial pathogens to survive and reproduce in their host organisms.

The focus of this project is the Type V secretion AT-1 autotransporters of *Xf* strains that cause Pierce's disease (PD) of grapevines (*Xf*-PD). AT-1 autotransporter proteins have been identified as rational targets for the design of novel vaccines directed against Gram-negative pathogens (15). AT-1 systems are dedicated to the secretion of a single specific polypeptide, the passenger domain, across the outer membrane. What happens to the passenger domain after it reaches the cell surface is dependent upon the specific autotransporter. Some passenger domains are not cleaved and protrude from the cell surface. This is a common feature of the adhesin autotransporters. Other passenger domains are cleaved from their membrane anchor (the β-barrel domain) and either remains loosely associated with the cell surface or are released into the environment. Not surprising, the ultimate location of each passenger domain appears to be integrally associated with its physiological function.

Based on genomic analysis, there are six members of the AT-1 autotransporter family in *Xf*-PD. Functional sequence predictions indicate that three of these secreted proteins have proteolytic activity (PD218, PD0313, PD0950), one protein has lipase/esterase activity (PD1879), and two proteins are thought to act as adhesins (PD0528, PD1379). The goal of this

project is to determine the role of the AT-1 autotransporters in cell physiology and virulence and to examine how we might exploit these proteins to control PD.

OBJECTIVES

1. Identify the proteins/molecules that interact with the two adhesin autotransporters.
2. Identify proteins and virulence factors requiring one of the three serine protease autotransporters for their maturation.
3. Identify peptides/small molecules that interfere with the function of the *Xf-PD* autotransporters.
4. Examine the feasibility of exploiting the unique properties of the autotransporters to develop strategies for controlling PD.

RESULTS AND DISCUSSION

Overview of the serine protease autotransporters

Functional sequence predictions indicate that three *Xf*-PD proteins (PD0218, PD0313, and PD0950) are members of the phylogenetic clade containing the S8 subtilisin-like serine protease autotransporters (14). Members of this family have been implicated in defense, growth on proteinaceous compounds, and the proteolytic maturation of virulence factors. Although many serine proteases have broad specificities, some are very specialized. One of the best studied members of this clade is the SphB1 autotransporter protein of *Bordetella pertussis* (3). SphB1 serves as a specialized maturation protease, responsible for the timely maturation and extracellular release of the filamentous haemagglutinin FHA. One of the goals of this project is to determine the specificity and targets of PD0218, PD0313 and PD0950. As a first step in this analysis, we have generated strains containing mutations in one, two, or all three of the AT-1 serine proteases. A list of these mutants and some of their phenotypic properties is presented in **Table 1**.

Table 1. The AT-1 serine protease mutants.

Strain	AT-1 Mutation(s)	Biofilm formation	Disease Severity*
Temecula1	Wildtype	100%	3.3
TAM147	PD0218::Cm^R	78%	4.5
TAM152	PD0313::Gm^R	36%	1.2
TAM146	PD0950::Em^R	68%	4.2
TAM148	PD0218::Cm^R, PD0950::Em^R	77%	4.5
TAM150	PD0218::Cm^R, PD0313::Gm^R	70%	4.3
TAM151	PD0313::Gm^R, PD0950::Em^R	84%	1.2
TAM153	PD0218::Cm^R, PD0313::Gm^R, PD0950::Em^R	92%	2.0

* Three plants were inoculated for each mutant. Disease severity was assessed weekly using the visual scale (0-5) described by Guilhabert and Kirkpatrick (5).

Our examination of the properties of these mutants under laboratory conditions and in grapevines has provided some insights into the roles of the individual proteases. These studies have also revealed that the interactions between these proteases are complex.

Properties of the triple mutant, TAM153

Based on comparison of the growth properties of TAM153 to *Xf* Temecula1, the absence of the three AT-1 serine proteases does not impact the growth rate and has only a modest impact on biofilm formation (92% wild type levels). However, their absence has a profound impact on the properties of TAM153 in grapevines. For this analysis, Thompson seedless grapevines were infected by the pinprick method and disease severity was assessed weekly using the visual scale (0 to 5) described by Guilhabert and Kirkpatrick (5). As shown in **Figure 1A**, by week 21, grapevines infected with wild type were exhibiting marginal leaf scorching and matchsticks characteristic of PD. In contrast, the grapevines infected with TAM153 display very few symptoms and appear similar to the uninfected control plants. We also assessed the bacterial population and movement at week 26 following inoculation. In this experiment, petiole tissues were harvested at different distances above the point of inoculation. The bacterial population within each sample was then determined by plating onto PD3 agar (5). As shown in **Figure 1B**, high populations of TAM153 were recovered within the petiole tissue at the different locations and the colony-forming units per gram (CFU/g) were typically greater than wild type. Based on these results, we conclude that the lack of PD symptom development in TAM153 is not due to the impact of the three mutations on the ability of *Xf* to migrate or colonize the xylem.

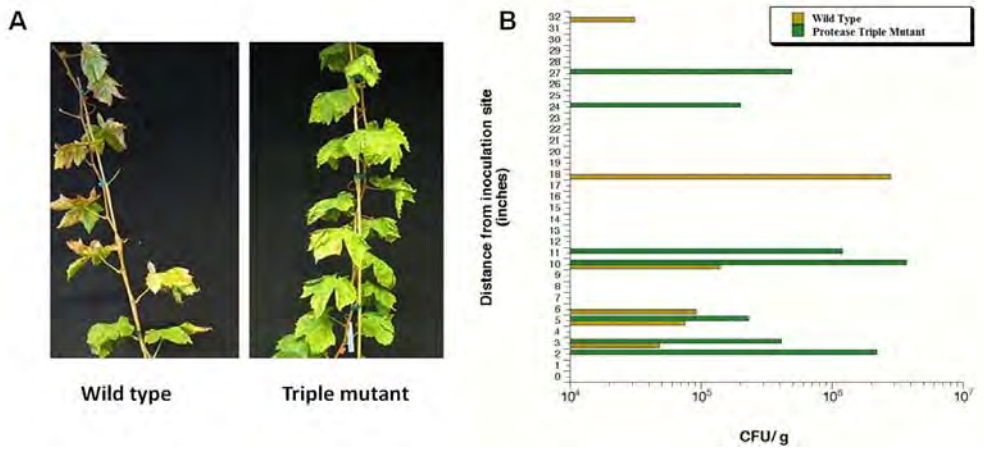

Figure 1. The impact of the triple mutant on *Xf* virulence *in planta*. An *Xf* suspension of wild type or the triple mutant TAM153 was used to inoculate Thompson seedless grapevines using the standard pinprick method. (A)Photographs show a representative vine 21 weeks after infection. (B) Petiole tissues were harvested at 26 weeks and the bacterial populations were determined at different locations above the site of inoculation.

What can we learn about the function of the individual proteases from the single mutants?

The PD0218 mutant TAM147: A strain carrying a mutation in PD0218 (TAM147) was only slightly defective in biofilm formation (80% wild-type levels); its major impact was on virulence in grapevines. Grapevines infected with TAM147 showed earlier symptom development, higher disease scores over a period of 26 weeks, and earlier vine death than wild-type infected plants. Hypervirulent phenotypes have been reported for numerous *Xf* mutants, such as the hemagglutinin HxfA mutant (5) and the cell-cell signaling-deficient RpfF mutant (11). One hypothesis is that proteins like HxfA and RpfF facilitate the attenuation of *Xf* pathogenicity by limiting colonization, thereby reducing the rate of xylem vessel occlusion (1, 5). It is possible that the PD0218 protease plays a similar role, perhaps through the maturation of a target protein that is important for grapevine colonization.

The PD0313 mutant TAM152: The strain carrying a mutation in the PD0313 locus (TAM152) had a number of characteristics that distinguished it from wild-type *Xf*. First, TAM152 cells did not aggregate (clump) when grown in PD3 broth. Second, there was a significant decrease in biofilm formation under laboratory conditions (36% wild-type levels; **Table 1**). Finally, grapevines infected with TAM152 exhibited very few PD symptoms and we were unable to recover TAM152 from petiole tissue at 26 weeks. This would suggest that PD0313 is required for *Xf* to survive in grapevines, a phenotype previously reported for mutations in *tolC* (12). Interestingly, both double and triple mutants containing the PD0313 mutation can be recovered from infected grapevines. This suggests that the PD0313 protease may only be important for grapevine survival when one of the other AT-1 proteases is present.

The PD0950 mutant TAM146: Strains carrying a mutation in PD0950 (TAM146) exhibited a phenotype very similar to strains carrying a mutation in PD0218 (TAM147). Both mutants were only slightly defective in biofilm formation and exhibited a hypervirulent phenotype in grapevines. Based on these properties, we initially assumed that PD0950 would facilitate the attenuation of *Xf* pathogenicity. However, as described below, studies of the double mutants revealed a more complex role for PD0950 in *Xf* virulence.

What can we learn about the function of the individual proteases and their interactions from the double mutants?

Comparison of triple mutant TAM153 to the three double mutants allowed us to examine how the presence of a single AT-1 protease impacted *Xf* cell physiology and virulence. The data concerning the contributions of the three proteases are presented in **Figure 2**.

PD0218: The double mutant TAM151, which is missing both PD0313 and PD0950, exhibited very few PD symptoms and its ability to colonize and migrate within the xylem was similar to wild type. The reduction of virulence in the presence of PD0218 is consistent with the hypervirulent phenotype observed for strains missing PD0218. Together, these results imply that the PD0218 protease actually reduces the virulence of *Xf*, making it an "anti-virulence" factor.

PD0313: The double mutant TAM148, which is missing both PD0218 and PD0950, exhibited a hypervirulent phenotype. Since PD0313 is the only AT-1 protease present in the strain, this would suggest that PD0313 is responsible for the observed increase in PD symptoms. This result, together with the results obtained for the PD0313 single mutant (TAM152), support the hypothesis that PD0313 is a virulence factor.

PD0950: Based on the hypervirulent phenotype exhibited by strains missing PD0950, we initially classified PD09050 as an "anti-virulence" factor. However, the double mutant TAM150, which has only the PD0950 protease, also exhibited a hypervirulent phenotype. How might the presence and absence of PD0950 produce the same phenotype in grapevines? One possible explanation was revealed by TAM152, which carries the wild-type genes for both PD0950 and PD0218. The low level of disease in TAM152 infected grapevines suggests that the virulence-reducing PD0218 protease is masking the activity of PD0950.

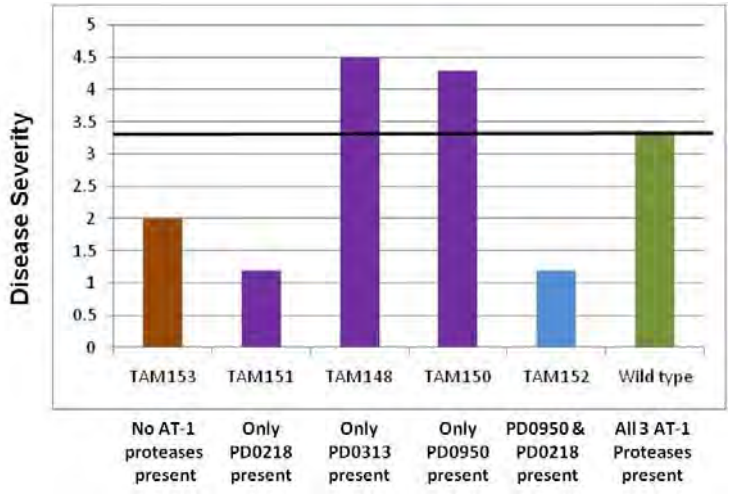

Figure 2. Contributions of the AT-1 proteases to disease severity. Grapevines were inoculated with different *Xf* strains and their impact on disease severity was assessed at week 21. The colors of the bars indicate the number of mutations present in the strain: gold (three), purple (two), blue (one), and green (none).

What are the potential targets of the AT-1 serine proteases?

To address this question, we compared the protein composition of the outer membrane, the membrane vesicles, and the secretome of the various mutants to wild type on different percentage SDS-PAGE gels stained with Syphro Ruby. Comparisons of the banding patterns of the various samples allowed us to identify proteins that are affected by the presence or absence of a specific protease. Some of the most interesting results came from our analysis of the secretome of the single mutants.

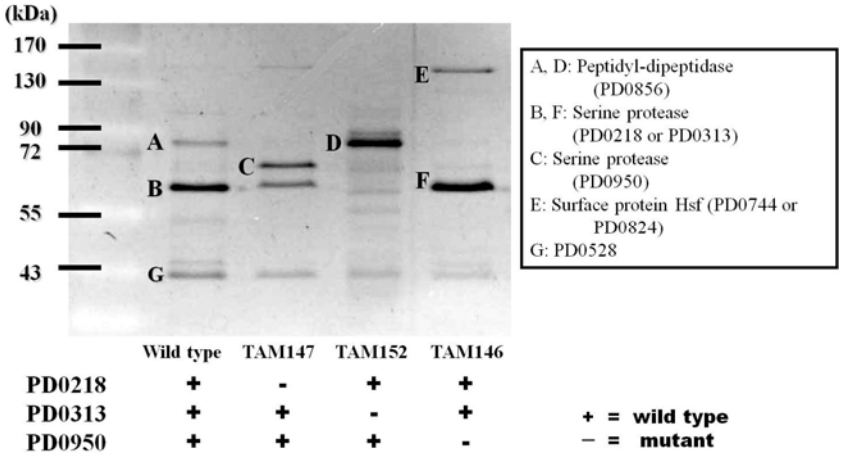

Figure 3. Comparison of the proteins secreted by the protease mutants and wild type. The secreted proteins were concentrated using an Amicon centricon filter and separated on a 8% SDS-PAGE gel. The gels were stained with Syphro Ruby and the indicated bands were excised and analyzed by MALDI-TOF-MS at the UC Davis Molecular Structure Facility.

As shown in **Figure 3**, each mutant exhibits a unique protein profile. In some cases, a specific protein is missing, such as the absence of peptidyl-dipeptidase in TAM147 and TAM 146. In other cases, a protein appears in the secretome of a specific mutant that is missing from the supernatant fraction prepared from wild-type cells. One example is the *Xf* ortholog to the cell surface protein Hsf, which is found in the secretome of the PD0950 mutant, but not wild type. In *Haemophilus influenza*, Hsf has been shown to mediate adherence to host cells and plays an important role in its pathogenicity (2). Hsf is a large protein that has been classified as a trimeric autotransporter adhesin (TAA) protein [for a review, see (9)]. TAA proteins, which belong to AT-2 autotransporter family, form fibers that are attached to the bacterial cell surface through their C-terminal β-barrel domain. Unlike AT-1 autotransporters, the passenger domains of TAA proteins remain covalently linked to β-barrel domain and are not normally released into the extracellular milieu. The presence of the Hsf passenger domain in the supernatant of the PD0950 mutant suggests that the PD0950 protease is involved either directly or indirectly in controlling whether or not the Hsf passenger domain is released from the bacterial cell surface.

What can we learn from the heterologous expression studies in E. coli?

Another way in which we have studied the function of the individual AT-1 proteases is to express each of these proteins on the surface of the *E. coli* strain UT5600. UT5600, which is deficient in the outer membrane protease OmpT, is commonly used for autodisplay (also known as live-cell surface display) (8). These studies support the hypothesis that each protease has a different set of target proteins. For example, when plated on a solid medium containing Tween 20 and $CaCl_2$ (10), *E. coli* strains expressing the PD0218 protein are surrounded by a halo, which is indicative of lipase activity (**Figure 4**). Halos are not found around strains expressing either PD0313 or PD0950 (data not shown). The simplest explanation for this result is that PD0218 acts on one or more of the inactive lipases of *E. coli* and the resulting maturation of a lipase is responsible for the observed phenotype. We are currently trying to determine the identity of this lipase(s) in order to help identify PD0218 substrates in *Xf*.

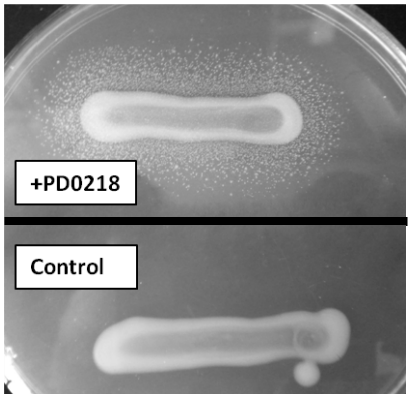

Figure 4. Phenotype of an *E. coli* strain expressing PD0218.
E. coli strain UT5600 containing either the vector pBBR1MCS-5 (control) or plasmid pAM216 (+PD0218) were grown on solid medium containing Tween 20 and $CaCl_2$. The presence of a halo on this medium is indicative of lipase activity.

CONCLUSIONS

Autotransporters have been identified as rational targets for the design of novel vaccines and control strategies. The goal of this project is to characterize the six autotransporters of *Xf*. During the period under review, we have been examining how the absence of one or more autotransporters affects *Xf* cell physiology and virulence. Here, we described our characterization of strains carrying mutations in the three autotransporters predicted to have proteolytic activity. Specifically, we compared the phenotypic properties of strains carrying mutations in one, two, or all three serine protease autotransporters. These studies suggest that PD0313 and PD0950 are both virulence factors and that PD0218 is involved for reducing virulence, possibly through its interaction with PD0950. Furthermore, comparisons of the secreted proteins from the three single mutants suggest that each protease has a different set of target proteins. Experiments designed to probe the interactions between the individual proteases and to investigate the mechanism underlying their different role in *Xf* virulence are currently underway.

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THE OXIDATIVE STRESS RESPONSE: IDENTIFYING PROTEINS CRITICAL FOR *XYLELLA FASTIDIOSA* SURVIVAL IN GRAPEVINES

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Reporting Period: This project has just been funded. Therefore, we are only reporting preliminary results.

ABSTRACT
A key component of the initial plant response to bacterial infection is the rapid production and accumulation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anions. These elevated levels ROS are highly toxic to the bacteria and can disrupt many cellular processes through their oxidation of lipids, modification of proteins and damage to DNA. Therefore, most pathogens have evolved a variety of enzymes capable of detoxifying ROS. The goal of this project is to understand how *Xylella fastidiosa* (*Xf*) responds to different types of ROS and to characterize the enzymes and regulatory proteins induced in this response. Our initial studies have focused on OxyR, a regulatory protein involved in the response to hydrogen peroxide. Strains carrying a null mutation in *oxyR* are more sensitive to killing by hydrogen peroxide and are impaired in their ability to attach to solid surfaces. Experiments are underway to determine the impact of the *oxyR* null mutation on survival and symptom development in grapevines.

LAYPERSON SUMMARY
One of the immediate responses of plants to invading microorganisms is the release reactive oxygen species (ROS), such as hydrogen peroxide. ROS are thought to serve as antimicrobial agents and as signals to activate further plant defense reactions. This project is designed to uncover the vulnerabilities of *Xylella fastidiosa* (*Xf*) to reactive oxygen species (ROS) during the initial stages of infection and during the later stages when the bacteria are protected by a biofilm. These vulnerabilities could be exploited for disease control. Another goal is to genetically engineer a bioluminescent *Xf* strain that will allow researchers to monitor *Xf* 's response to specific prophylactic or curative measures for Pierce's disease (PD) in living tissues. This strain would facilitate many different types of research, thereby expediting the development of treatments for mitigating PD.

INTRODUCTION
An important stress for plant pathogens are reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{\bullet-}$). ROS are a key component of the initial plant defense response (often termed the "oxidative burst") and are produced by the plant at the point of invasion during the first five minutes after exposure to a potential pathogen (Apel and Hirt, 2004; Bolwell and Daudi, 2009). This initial response is often followed by a second response, which involves more prolonged production of ROS (1.5-3 hours after invasion) (Apel and Hirt, 2004). The plant defense response typically requires contact between the pathogen and metabolically active cells. Since xylem tissue, for the most part, is not living, the introduction of a pathogen into the xylem might not result in significant ROS production by the xylem tissue itself. However, pathogens in the xylem make contact with the adjacent living parenchyma cells via the pit membranes, which could result in higher ROS levels in the xylem. Another source of ROS comes from differentiating thin-walled xylem cells and particular non-lignifying xylem parenchyma cells, which are capable of sustained H_2O_2 production (Barcelo, 2005). This H_2O_2 is important for the cross-linking that occurs during the lignification process of developing xylem elements. Since the H_2O_2 produced by the xylem parenchyma can diffuse widely between neighboring xylem cells, bacteria present in the xylem are likely exposed to H_2O_2, especially when introduced into the tips of growing shoots where the majority of xylem lignification is occurring. Therefore, it seems likely that the immediate detoxification of ROS is critical for bacterial survival in the plant xylem.

The immediate detoxification of ROS is accomplished, in part, by scavenging enzymes designed to cope with specific oxidative stresses. Comparative genomics suggests that many of these enzymes are present in *Xylella fastidiosa (Xf)* (**Table 1**).

Table 1. Predicted *Xf* enzymes.

Enzyme	***Xf* gene(s)**	**Oxidative signal**	**Regulator**
Alkyl hydroperoxide reductase	*ahpC, ahpF*	H_2O_2, organic peroxides	OxyR
Catalase	*cpeB*	H_2O_2.	OxyR
Superoxide dismutase	*sodA, sodM*	superoxides	unknown
Thiol-dependent peroxidase	*ohr*	organic peroxides	unknown

One predicted *Xf* scavenger enzyme is the alkyl hydroperoxide reductase AhpCF, a two-component NADH peroxidase. This enzyme, which is required for optimal resistance to both hydrogen and organic peroxides, is the predominant scavenger at low concentrations of H_2O_2 (Imlay, 2008). In contrast, at high H_2O_2 concentrations, catalases are induced and become the primary scavenging enzymes. We have already established that *Xf* catalase is encoded by the *cpeB* gene (Matsumoto et al., 2009). The primary scavenger enzymes of superoxides are the superoxide dismutases (SODs), which convert $O_2^{\bullet-}$ to H_2O_2. *Xf* is predicted to encode two members of the iron/manganese superoxide dismutase family (SodA and SodM). Finally, studies of *Xf*-CVC identified a thiol-dependent peroxidase, encoded by the *ohr* gene (Cussiol et al., 2003). Unlike the other scavenging enzymes, Ohr belongs to a family of proteins that are only present in bacteria. This property led Cussiol et al. (Cussiol et al., 2003) to suggest that Ohr might be a promising target for drug development in medicine and agriculture. The goal of Objective 2 is to characterize these key scavenger enzymes.

Not surprisingly, since peroxide and superoxide stresses do not always occur simultaneously, many bacteria have evolved distinct sensing mechanisms to detect different forms of oxidative stress and to induce the synthesis of a particular set of scavenging enzymes. In most bacteria, the response to hydrogen peroxide stress is regulated by the transcription factor OxyR. The *Xf* ortholog of this protein is encoded by PD0747. Our screening of the *Xf* genome for potential OxyR binding sites suggests that both catalase (CpeB) and alkyl hydroperoxide reductase (AhpCF) are controlled at the transcriptional level by OxyR. OxyR-like systems are widespread among bacteria (Imlay, 2008), including many plant pathogens. The resulting transcription regulatory network allows differential expression of H_2O_2-induced genes in terms of growth phase, cell density, and biofilm formation (Imlay, 2008; Shanks et al., 2007). Therefore, the OxyR-mediated oxidative stress response pathway helps bacteria survive the initial exposure to ROS through induction of scavenger enzymes and later exposure from ROS that may be present during biofilm formation. Based on this link between oxidative stress and biofilm formation, we hypothesize that OxyR participates in the signaling that triggers *Xf* to enter and maintain the biofilm state within the plant. The resulting biofilm, in turn, affects plant colonization, virulence and acquisition by the insect. We will test this hypothesis in the series of experiments in Objective 1, 3, and 4.

The goal of objective 5 is to develop a sensitive reporter system for detecting the response of *Xf* to ROS with the plant xylem. Our strategy will be to generate luciferase fusions using the Lux reporter system from *Photorhabdus luminescens* (Meighen, 1993). The bioluminescent reaction is catalyzed by bacterial luciferase (LuxAB) that oxidizes $FMNH_2$ and a long-chain fatty-acid aldehyde the presence of molecular oxygen. The fatty-acid aldehyde is synthesized by LuxC, LuxD, and LuxE. The advantage of this system over other luciferase reporter systems is that it does not require an exogenous substrate. The development of a LUX reporter system will allow Pierce's disease (PD) researchers to monitor the response of their gene of interest in a noninvasive manner in the host insect or plant. It might also be used to monitor the early responses of *Xf* to various treatments, including treatments involving transgenic plants and for some high throughput screening applications.

OBJECTIVES

1. Determine the key components in the response of *Xf* to ROS and the contribution of OxyR to this regulation.
2. Determine the role of the scavenging enzymes designed to cope with specific oxidative stresses in *Xf* cell physiology and virulence.
3. Determine the role of the transcription factor OxyR in oxidative stress sensing, biofilm formation, and virulence.
4. Test mutants generated in Objectives 2 and 3 for virulence in grapevines and for sharpshooter transmission.
5. Develop a bioluminescent (Lux) reporter system for *Xf*.

PRELIMINARY RESULTS

The Igo laboratory has already described their characterization of a *Xf* strain containing a null mutation *cpeB*, the gene encoding catalase (Matsumoto et al., 2009). This mutant is more sensitive to hydrogen peroxide than a wild-type strain. Moreover, our preliminary experiments suggest that a functional catalase is required for virulence in grapevines.

The Roper laboratory has generated a null mutation in the *oxyR* gene by site-directed mutagenesis and has begun to examine how this mutant responds to oxidative stress. The results from the hydrogen peroxide degradation assay are shown in **Figure 1**.

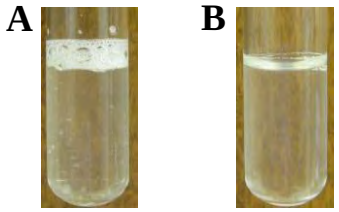

Figure 1. Hydrogen peroxide degradation assay.
Bubbles indicate the presence of gaseous oxygen resulting from the breakdown of H_2O_2.
A *Xf* wild type. (**B**) *Xf oxyR* null mutant.

In this assay, a 3% H_2O_2 solution was added to *Xf* wild type and *oxyR* null mutant liquid cultures. Within 30 seconds, bubbles formed in the tube containing the wild-type culture. In contrast, few bubbles were present in the *oxyR* null mutant culture, indicating that this mutant is significantly impaired in its ability to degrade H_2O_2 (**Figure 1**). Furthermore, the *oxyR* null mutant exhibited greater sensitivity to H_2O_2 than wild type in a disk diffusion assay. For this analysis, *Xf* wild type and the *oxyR* null mutant was grown in liquid medium to an OD_{600}=0.1. Inocula from these cultures (300 µL) were added to 3 mls of PD3 top agar, which was poured onto PD3 plates. A paper disk treated with 10 µL of 100 mM H_2O_2 was then placed on the center of the top agar and zones of growth inhibition were measured after seven days of incubation. Three replications were performed. The inhibition zone diameter of the *oxyR* null mutant was higher than wild type indicating that the *oxyR* null mutant was significantly more sensitive to H_2O_2 than wild type (**Figure 2**). Taken together, these results suggest that the *oxyR* null mutant is likely compromised in its ability to mount an effective oxidative stress response.

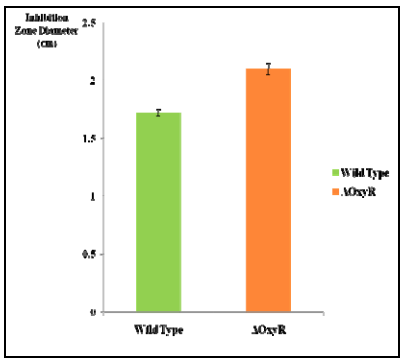

Figure 2. Measurements of the diameter of the zone of inhibition surrounding a paper disc containing 10 µL of 100 mM H_2O_2. Results are shown for both *Xf* wild type and the *oxyR* null mutant. The larger zone of inhibition correlates to an increase in sensitivity to H_2O_2. The error bars indicate the standard deviation.

The OxyR protein may also play an important role in regulating gene products (at the transcriptional level) involved in the attachment of *Xf* to surfaces and biofilm formation. **Figure 3** shows the results from experiments examining the impact of the *oxyR* null mutation on surface attachment to both polystyrene and glass tubes, which was assessed based on a protocol described in (Espinosa-Urgel et al., 2000). Cultures of wild type *Xf* or the *oxyR* null mutant were grown in polystyrene tubes and glass tubes at 28°C, upright without agitation. After seven days, 100 µL of 0.1% crystal violet was added to the culture medium and incubated for 20 min. The presence of attached cells was visualized as a purple ring on the tube side wall. Cultures containing *Xf* wild type had clear purple rings at the air-medium interface in both polystyrene and glass tubes. Notably, there was no purple ring observed for the *oxyR* null mutant, indicating the absence of attachment by the Δ*oxyR* mutant. Cell attachment was then quantified by measuring the absorbance of the eluted crystal violet solution. These data support the conclusion that the *Xf oxyR* null mutant is significantly reduced in its ability to attach to the solid surfaces (**Figure 3**).

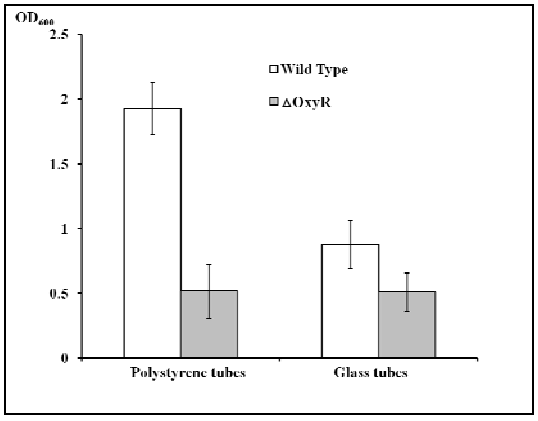

Figure 3. Quantification of surface attached cells for *Xf* wild type and *oxyR* null mutant for polystyrene and glass tubes surfaces. Attached cells were stained with crystal violet, and then eluted with ethanol; the OD_{600} of the eluted solution was measured. The error bars indicate the standard deviation.

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GENOMIC CHARACTERIZATION OF A LYSOGENIC PHAGE FROM *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted January 15, 2010 to December 1, 2010.

ABSTRACT
Xylella fastidiosa (Xf) is an important pathogen causing diseases on several economically important crops, such as grape and almond, in California. Despite recent intensive efforts to study this nutritionally fastidious pathogen, many biological traits of the bacterium, such as bacteriophages, remain unclear and deserve more research attention. We have consistently observed the presence of phage particles from supernatants of *Xf* culture. Further study on phage requires genome characterization. In this study, we conducted an experiment to induce the release of lysogenic phage particles from bacterial hosts in PD3 broth. Phage particles were enriched from culture supernatant. Examination by electron microscopy showed particles with morphology similar to those in Family Podovirideae. Strain Dixon showed a relatively higher phage titer and therefore was used for phage genome characterization. Bacterial chromosomal DNA in the phage preparation was removed by DNAse digestion. Phage DNA was amplified by PCR using random primers, cloned, and sequenced. A BLAST search identified a cloned sequence matching to a prophage region in the whole genome sequence of several *Xf* strains. This sequence also shares similarity (but was not identical) with a phage sequence from a Texas strain of *Xf*. Using the published whole genome sequences as a guide, we are trying to identify the whole phage genome. Several challenges need to be overcome: 1) Phage titer is still not sufficiently high to generate large quantity of phage DNA for analysis; 2) DNAse inhibitor are present in phage preparations; and 3) The genome sequence of strain Dixon is not enclosed and sequence quality remains to be evaluated.

LAYPERSON SUMMARY
Xylella fastidiosa (Xf) is a bacterium causing many plant diseases including grape Pierce's disease and almond leaf scorch disease in California. This research is to study a virus or bacteriophage that could infect the pathogenic bacterium. Current efforts are to identify the genetic make-up of the bacterial virus, which has received little studies by far. Five genes have been detected and more studies are underway. Our research goal is to learn more about this bacterial virus so that more information will be available for future use to control xylellae diseases.

INTRODUCTION
Xylella fastidiosa (Xf) is an important pathogen causing disease on several economically important crops, such as grape and almond, in California. Despite recent intensive efforts to study this nutritionally fastidious pathogen, many biological traits of the bacterium, such as bacteriophages, remain unclear and deserve more research attention. Bacteriophages play important roles in bacterial biology including nutrient cycling, horizontal gene transfer, environmental adaptation and pathogenicity. Characterization and understanding the bacteriophage could lead to the identification of critical genes or biological processes useful for disease control.

Typically, bacteriophages are observed and enumerated using a plaque assay. The host bacterial cells are exposed to some environmental stress such as heat, UV radiation or chemical compound such as mitomycin C. These stresses trigger the shift for bacteriophage from lysogenic to lytic phase and form phage particles. Bacterial cells are plated as a lawn and bacterial phage plaques can be observed as clearings in the lawn. However, *Xf* is nutritionally fastidious and grows very slowly on agar plates of the available media. Chen and Civerolo (2008) developed a technique to collect phage particles from supernatants of prolong broth culture. Phage particles were morphologically characterized and most of them belong to Family Podovirideae. Summer et al (2010) reported the use of a Texas strain of *Xf* that formed bacterial lawn to isolate a phage through plaque assay. Although we have not tested the Texas strain, our attempts to develop culture lawn with our *Xf* collection have not been successful.

OBJECTIVE
In this study, we continued the protocol of Chen and Civerolo (2008) to collect *Xf* phage particles and explored the use of whole genome sequence information to characterize a lysogenic phage from *Xf*. Strain Dixon was selected because it generated relatively high titer of phage particles under our experimental condition, and a shot-gun whole genome sequence for strain Dixon is also available for reference.

MATERIALS AND METHODS
Bacterial cultivation and phage isolation. *Xf* strain Dixon was maintained in PW (Periwinkle wilt) broth culture at room temperature on a shaker table. From a two week old culture, five ml were used to inoculate 250 ml of either PW broth or PD3 broth. The cultures were grown at 25 ºC for four weeks. Broth cultures were then centrifuged to pellet cells and

supernatant was retained. Supernatant was filtered through Centricon Plus-70 100K MWCO filters (Millipore, Carrigtwohill, Co Cork. Ireland). The presence of phage particles in the phage prep were confirmed by electron microscopy examination after negative stain.

For phage genomic analysis, 17µl of phage prep was treated with Baseline-ZERO DNase (Epicentre, Madison WI) at the concentration of 1 MBU for 0.5 hours at 37ºC. This step was to digest any remaining bacterial chromosomal DNA, leaving only phage DNA contained within the phage capsid. The removal of chromosomal DNA was confirmed by the lack of PCR amplification with primers (four Primers, namely) for the detection of *Xf* chromosomal DNA (**Table 1**).

Ampification and confirmation of phage DNA. Phage prep treated with DNase was then used for direct PCR. Phage DNA was amplified using random primers OPA02, OPA3, OPA09, OPA11 and OPA18 with the following PCR procedure; PCR master mix per reaction contained: 16.8 µl molecular biology grade water, 2.5 µl dNTPs, 2.5 µl 10x PCR buffer, 0.2 µl TaKaRa Taq (5U/µl, TaKaRa Taq™ Hot Start Version, TaKaRa BIO INC. Otsu, Shiga, Japan), 0.5 µl of each primer (5 µM) and 2 µl of phage prep. PCR was run on a PTC-200 Peltier Thermal Cycler (MJ Research). RAPD PCR program was set as 96 ºC denature one min, 35 ºC annealing two min, 72 ºC extension two min for each cycle and a total of 40 cycles with a final 72 º extension of six minutes. Amplified DNA was visualized after agarose gel electrophoresis and staining.

RAPD PCR amplicons were cloned in pGEM-T plasmid vector. Cloned plasmids were sequenced using BigDye Turminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Austin TX). Excess dye was removed using BigDyeXTerminator Purification kit (Applied Biosystems, Austin TX). Sequencing reaction was run on a 16 capillary 3130xl Genetic Analyzer (Applied Biosystems, Austin TX). DNA sequences were used as query for BLAST search against GenBank DNA sequence database that included seven whole genome sequences of *Xf* strains. Among them, the sequences of strain 9a5c, Temecula1, M12, M23 and GB514 were enclosed and the sequences of strain Ann-1 and Dixon were not enclosed.

Genomic characterization of phage genome. BLAST search with cloned RAPD identified a prophage region in the genome of Temecula1, GB514, M23 and 9a5c. Sequences in the open reading frame (ORF) up- and down- streaming this genomic locus were then used to design PCR primer sets using Primer 3 program (http://frodo.wi.mit.edu/primer3/). These primer sets were used for PCR amplifications in the attempt to identify more genes in the phage genome. Initial primers used were designed using the genome sequence of strain Temecula1 as a reference. These primers were not specific enough to amplify prophage genes of strain Dixon. Primers were then redesigned by finding the homologous sequence in Dixon chromosome using NCBI's BLAST analysis and Primer3 program. The following PCR procedure was used: PCR master mix included 16.8 µl molecular biology grade water, 2.5 µl dNTPs, 2.5 µl 10x PCR buffer, 0.2 µl TaKaRa Taq (TaKaRa Taq™ Hot Start Version, TaKaRa BIO INC. Otsu, Shiga, Japan) 0.5 µl of each primers and 2 µl phage prep. Setting for PCR program was: 96 ºC denature for one min., 55 ºC annealing for 30 sec, 72 ºC extension for 30 sec per cycle with a total of 30 cycles and a final extension of 72 ºC for onr min. Amplified DNA was visualized after agarose gel electrophoresis and staining.

RESULTS AND DISCUSSION

A total of 250 µl of phage preparation (phage prep) was obtained from 250 ml of bacterial culture. **Figure 1** shows phage particles from electron microscopy. Phage preps from PD3 broth showed more particles than those from PW broth culture. Previously, strain Dixon was considered not capable of growing on PD3 medium. In this study, strain Dixon was able to grow on PD3. However, initial inoculum was grown in PW medium and may have provided limited amount of essential nutrients. Nevertheless, strain Dixon was growing under stress. This explains why more phage particles were observed from PD3 medium, rather than PW medium. Morphology of phage particles is consistent with Podovirideae type phage particles (**Figure 1**).

Among the five RAPD primers, only OPA03 amplified a DNA band of ~1,200 bp from the phage prep (**Figure 2**). Sequence from this PCR amplicon matched with prophage regions in the whole chromosomal sequence of *Xf* strains GB514, M23, Temecula1, and 9a5c (**Figure 3**). Shorter sequence with high similarity were also found from strain M23 and phage Xfas53, isolated from a Texas strain of *Xf*. All these evidence indicates that the observed particles were indeed true phages. Interestingly, the amplicon from phage particles was slightly larger than the strain Dixon chromosomal DNA.

Since phage particles were induced from pure *Xf* culture, it is assumed that the phage genome was a direct excision of a prophage sequence from the bacterial genome. Both phage and prophage should share the same set of genes. Data obtained so far seem to support this assumption (**Table 2**). The 4Primer used in this study targets the 16S rDNA locus of the bacterial chromosome and does not amplify DNA from phage prep if chromosomal DNA was completely digested by DNase. This is, however, not always true in our experiment. This may be due to the incomplete removal of chromosomal DNA in some batches of the phage preps. Repeating PCR experiment helped to correct the problem. The whole genome sequence of *Xf* strain Dixon is currently available in public domain. Yet the genome has not been enclosed. The five ORFs tested in this study are located on contig 89. Using the whole genome sequence as a guide, we will continue to characterize the whole genome of the phage isolated from this bacterial strain.

CONCLUSIONS

This research provides genomic evidence, in addition to our previous morphological observation, that *Xf* strains harbor bacteriophage(s). One practical perspective of phage research is to review the mechanisms of phage-bacteria interaction, or how a phage lyses a bacterial cell. Phage research has a potential for disease control.

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Summer, E. J., Enderle, C. J., Ahern, S. J., Gill, J. J., Torres, C. P., Appel, D. N., Black, M. C., Young, R., and Gonzalez, C. F. 2010. Genomic and biological analysis of phage Xfas53 and related prophages of *Xylella fastidiosa*. Journal of Bacteriology 192:179–190

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We thank Greg Phillips for his technical assistance.

Table 1. List of primers for phage genomic characterization from *Xf* strain Dixon.

Primer Name	Primer sequence	Size (bp)
Dix1091_564f	TAGCGATACCGCTCAGACCT	564
Dix1091_564r	GTGCACAGACATTGGATTGG	
Dix1092_572f	GCTGTTTTGCTTGACGTTGA	572
Dix1092_572r	TCCACCGTTGAGTATGACGA	
Dix1093_800f	GACGCGCTTTCTGTTACCTC	800
Dix1093_800r	ATTTTTGTCCGCAAAATTCG	
Dix1094_518f	GCAAAGTCGAAGCCCTACTG	518
Dix1094_518r	GAACGATCCGTAACCACCAC	
Dix1095_448f	TCCCAAACCTCGTTCATACC	448
Dix1095_448r	GCGACAGATTGAGGGGTAAA	
OPA 03	AGTCAGCCAC	variable

Table 2. Summary of phage genomic characterization from *Xf* strain Dixon .

Template	Primer	PCR			
		1	2	3	4
Phage prep	DIX1091	+	+	+	+
Dixon DNA	DIX1091	+	+	+	+
Phage prep	4Primer	+	+	-	-
Dixon DNA	4Primer	+	-	+	+
Phage prep	DIX1092	+	+	+	+
Dixon DNA	DIX1092	+	+	+	+
Phage prep	4Primer	-	-	-	+
Dixon DNA	4Primer	+	+	+	+
Phage prep	DIX1093	+	+	+	+
Dixon DNA	DIX1093	+	+	+	+
Phage prep	4Primer	-	-	-	-
Dixon DNA	4Primer	-	-	+	+
Phage prep	DIX1094	+	+	+	+
Dixon DNA	DIX1094	+	+	+	+
Phage prep	4Primer	-	-	-	-
Dixon DNA	4Primer	-	-	+	+
Phage prep	DIX1095	+	+	+	+
Dixon DNA	DIX1095	+	+	+	+
Phage prep	4Primer	-	-	-	+
Dixon DNA	4Primer	-	-	+	+

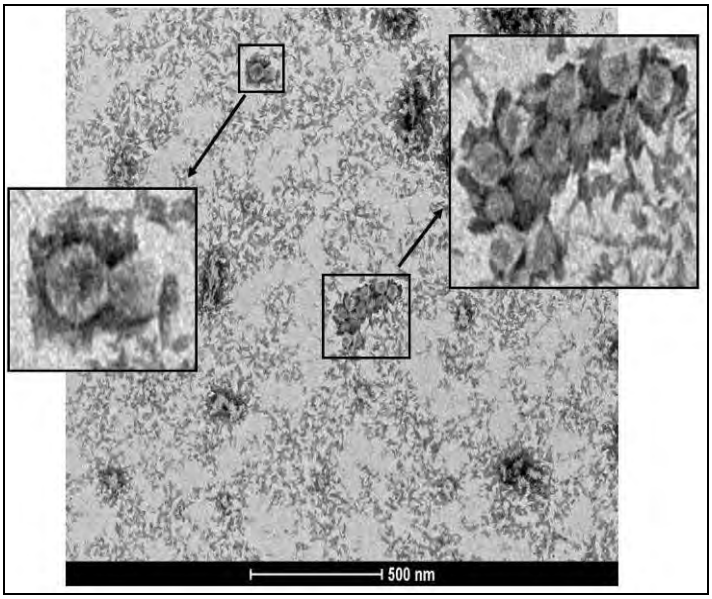

Figure 1. Electron micrograph of phage preparation from prolong (four weeks) culture of *Xf* strain Dixon.

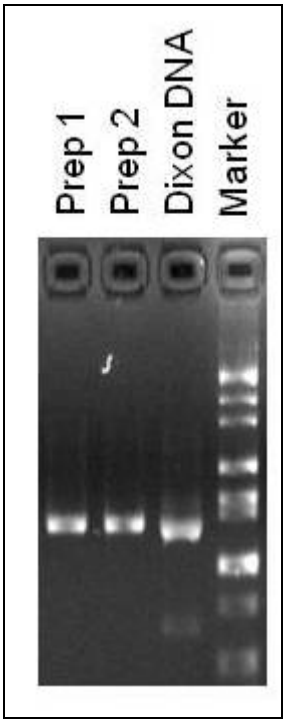

Figure 2. RAPD PCR from phage preparation of *Xf* strain Dixon.

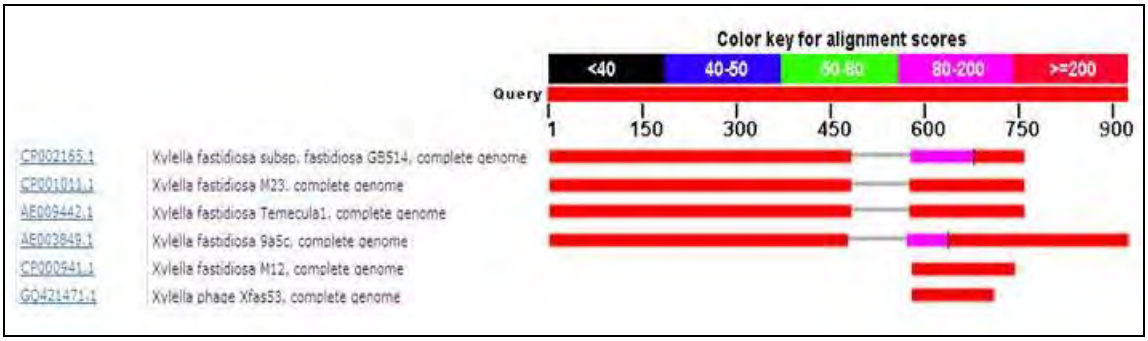

Figure 3. BLAST results of a phage sequence from *Xf* strain Dixon against GenBank DNA database.

GENETIC STRUCTURE OF *XYLELLA FASTIDIOSA* WITHIN TWO IMPORTANT GRAPE GROWING REGIONS IN THE UNITED STATES: CALIFORNIA AND TEXAS

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ABSTRACT
Xylella fastidiosa (*Xf*) causes Pierce's disease (PD) in grapevine. Here, we report genetic diversity and population genetic structure of grape *Xf* strains between two important grape growing regions in the United States, California and Texas. Using multilocus microsatellite (also known as simple sequence repeat) markers, genetic diversity of *Xf* was measured in California and Texas populations with a grand mean haploid genetic diversity of 0.427. Partitioning of genetic diversity (heterozygosity) across 13 microsatellites (SSR) found high values within the two different grape growing regions with 0.460 within Californian isolates, and 0.452 within Texas isolates, respectively. Cluster analysis of Nei's genetic distances and hierarchical analysis of molecular variance separated Californian isolates from Texas regardless of host, and also showed significant genetic differentiations between the isolates collected from these two broad geographic regions. Pairwise (F_{ST}) comparisons of local level geographical structure within Californian populations found significant genetic differentiations among the isolates collected from Mendocino, Sonoma, Napa Kern and Riverside counties. However, some populations from the most genetically diverse Napa County differed from each other, and shared genetic similarities with Kern and Riverside separately. On the other hand, in Texas no geographic association was observed with grape or non-grape strains although host-associated structure was observed with these strains there. Bayesian modeling using the STRUCTURE software indicated that *Xf* in California and Texas may be derived from different origins regardless of host. However, the observation that some California counties had stains with up to 17% Texas origin leading us to hypothesize the introduction of Texas origin into California. The introduction of Texas *Xf* strains seems to have initiated in southern California (Temecula region) followed by range expansion throughout different regions in California.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) causes Pierce's disease in grapevine. In this study, we report genetic diversity and population genetic structure of grape *Xf* strains between two improtant grape growing regions in the United States, California and Texas. Using multiple sets of molecular markers, analysis showed genetic differentiations in both in California and Texas' populations. Further genetic analyses indicates local level geographical structure within Californian populations where significant genetic differentiations were found among the isolates collected from Mendocino, Sonoma, Napa, Kern and Riverside counties. However, some isolates from Napa County differed from each other and showed some genetic similarity with Kern and Riverside separately. On the other hand, no geographic association was observed in grape or non-grape strains in Texas. Sharing and distribution of 17% Texas origin in different regions (counties) in Californian implied the possibility of recent introduction of Texas strains into California.

INTRODUCTION
California and Texas are productive agricultural ecosystems, and are both important American viticultural regions in United States. California accounts for nearly 90 percent of the entire American wine production, and the Texas wine industry is continuing its steady pace of expansion and has gained a reputation as an established wine growing region in the United States. The winegrowers of Texas have dealt with Pierce's disease (PD) for over a century. PD has also been a serious but intermittent threat in the California grape-growing regions for more than 100 years (Purcell 1997). Limited genetic variations among crop cultivars and oftentimes mono-culture practices may impose directional selection on the pathogen (*Xylella fastidiosa (Xf)* populations. Changes in pathogen population structure or virulence can lead to resistance breakdown. Therefore, to understand about the epidemiology of the PD disease caused by *Xf*, it is critical to understand the genetic diversity, gene flow and genetic structure of this pathogen. Until now, no detailed genetic information at the population level is available for the *Xf* grape strains within the United States from Florida to California or outside the USA in Central and South America. Previous molecular genetics studies mostly by conserved genes were unable determine population differentiations of grape strains of *Xf* at the local level even at the wider geographical ranges of the United States (Hendson et al. 2001; Schuenzel et al. 2005; Yuan et al. 2010). PD strains have been present in the United States since at least since the

1880s (Pierce 1892), and the evolutionary process associated with the genetic variation was expected to be considerably more rapid at non-coding loci (e.g. microsatellite) than conserved gene regions (Yuan et al. 2010), therefore, here we have investiagated *Xf* grape strains using highly variable microsatellite markers. We also incorporated some non-grape strains (based on the availabily of samples), and comapreted their genetic diversity and structure with grape strains. In this study, we have employed the 13 most informative microsatellite markers (Lin et al. 2005), and used them to analyze *Xf* grape populations at the local as well as wider geographic level i.e., within and between two important grape growing regions in the United States i.e., California and Texas.

OBJECTIVES

1. To investigate genetic structure of *Xf* grape strains between two important grpae growing regions in the United States (California and Texas).
2. To analyze the genetic diversity and population structure of *Xf* grape populations at the local geographic areas within California and within Texas.
3. To investigate some non-grape strains (based on the availabily of samples) and compare their structre with grape strains.

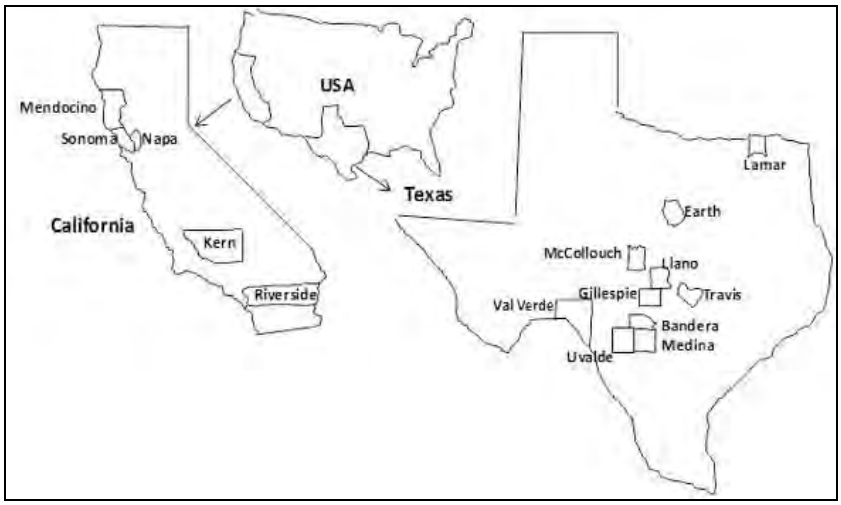

Figure 1. Sampling locations of *Xylella fastidiosa* populations across California and Texas.

RESULTS AND DISCUSSION

Within population genetic diversity in California and Texas

We investigated 12 *Xf* populations across five areas (counties) from California including some sub-populations from Sonoma and Napa countries based on the location of vineyard or type of cultivar. In Texas, 10 populations (both grape and non-grape strains) were investigated from ten different areas (counties) (**Figure 1**). Overall, strain diversity of *Xf* throughout California and Texas was found to be high in microsatellite DNA polymorphism analyses. The allelic diversity at the microsatellite loci analyzed where 7-13 alleles were detected per locus (data not shown). Grand mean genetic diversity (0.427) for overall *Xf* isolates at California and Texas was found to be high. Highly variable microsatellite markers were capable of distinguishing genetic diversities across the populations in different regions in California and Texas. Partitioning of genetic diversity in California showed that Napa county is the most diversified regions for *Xf* where heterozygosities ranged from 0.246 (Oakville) to 0.620 (north of the city of Napa). In Texas, the lowest diversity was found in non-grape population i.e, Medina (within oleander strains) (**Table 1**). Although the overall diversity was high, there were relatively lower levels of allelic diversities at individual population levels in California and Texas separately (**Table 1**). Np, or number of private alleles (alleles unique to a single population in the data set), were low overall and only slightly higher at a single locus in a single population of St. Helena Napa (variety: Cabernet Sauvignon) in California. This Np of 0.077 did not indicate strong distinction of this population from others. However, higher frequencies of rare alleles at two Texas populations (Medina at 0.308 and Val Verde at 0.231) indicate private distinction of these populations from others (**Table 1**). This distinction likely resulted from the strains variations based on the specific host plants as the respective private alleles were found within all Oleander strains in Medina and giant ragweed strains in Val Verde.

Table 1 Population information and descriptive statistics of microsatellite loci across different populations of *Xylella fastidiosa* in California and Texas, USA

Population ID	Counites	Vineyear or Location	Host	Cultivar	N	Na	Ne	Np	H
California									
CA-1	Mendocino	Hopland, southern	Grape	Sauvignon Musque	5	1.9	1.8	0.0	0.404
CA-2	Sonoma	Russian River, Healdsburg	Grape	Sauvignon blanc	10	2.4	2.0	0.0	0.461
CA-3	Sonoma	Northern Santa Rosa,	Grape	Pinot noir	9	2.6	1.8	0.0	0.356
CA-4	Sonoma	Dry Creek Valley,	Grape	Cabernet franc	3	2.2	2.0	0.0	0.466
CA-5	Sonoma	Alexander Valley,	Grape	Merlot	5	1.5	1.3	0.0	0.172
CA-6	Napa	Rutherford	Grape	Chardonnay	7	2.2	1.8	0.0	0.317
CA-7	Napa	St. Helena(1)	Grape	Chardonnay	6	2.1	1.6	0.0	0.325
CA-8	Napa	Oakville	Grape	Cabernet Sauvignon	5	1.8	1.6	0.0	0.246
CA-9	Napa	St. Helena(2)	Grape	Cabernet Sauvignon	8	2.5	2.0	0.1	0.438
CA-10	Napa	North of the city of Napa	Grape	Chardonnay	10	4.0	3.1	0.0	0.620
CA-11	Kern	Eastern Bakersfield	Grape	Colombard	10	3.8	2.6	0.0	0.517
CA-12	Riverside	Temecula	Grape	Chardonnay	5	2.8	2.5	0.0	0.550
Average					6.9	2.5	2.0	0.0	0.406
Texas									
TX-1	Gillespie		7 Grape ; 2 Ragweed		9	4.2	2.8	0.0	0.625
TX-2	Bandera		1 Grape; 1 Ragweed		2	1.7	1.7	0.0	0.346
TX-3	Earth		3 Grape, 2 Ragweed		5	4.0	3.8	0.0	0.695
TX-4	Lamar		Grape		3	1.9	1.8	0.0	0.380
TX-5	McCollouch		1 Grape; 1 Ragweed		2	1.5	1.5	0.0	0.231
TX-6	Llano		5 Grpae; 1 Ragweed		6	3.1	2.5	0.0	0.560
TX-7	Medina		Oleander		4	1.5	1.4	0.3	0.198
TX-8	Travis		Grape		4	2.2	2.0	0.1	0.424
TX-9	Uvalde		1 Grape, 2 ragweed		3	2.3	2.2	0.0	0.509
TX-10	Val Verde		4 Grape ; 1 Ragweed		5	2.7	2.5	0.2	0.549
Average					4.3	2.5	2.2	0.1	0.452

N, Number of individuals; Na, number of alleles per locus; Ne, Number of effective alleles per locus; Np, Number of private alleles per locus, H, Gene diversity.

Genetic differentiation and population genetic structure at the wider and local geographic level

Cluster analysis of Nei's genetic distances (Nei 1978) with microsatellite loci separated Californian isolates from Texas isolates (**Figure 1A**). This was expected based on their geographical range due to geographic isolation or their sources of origin.

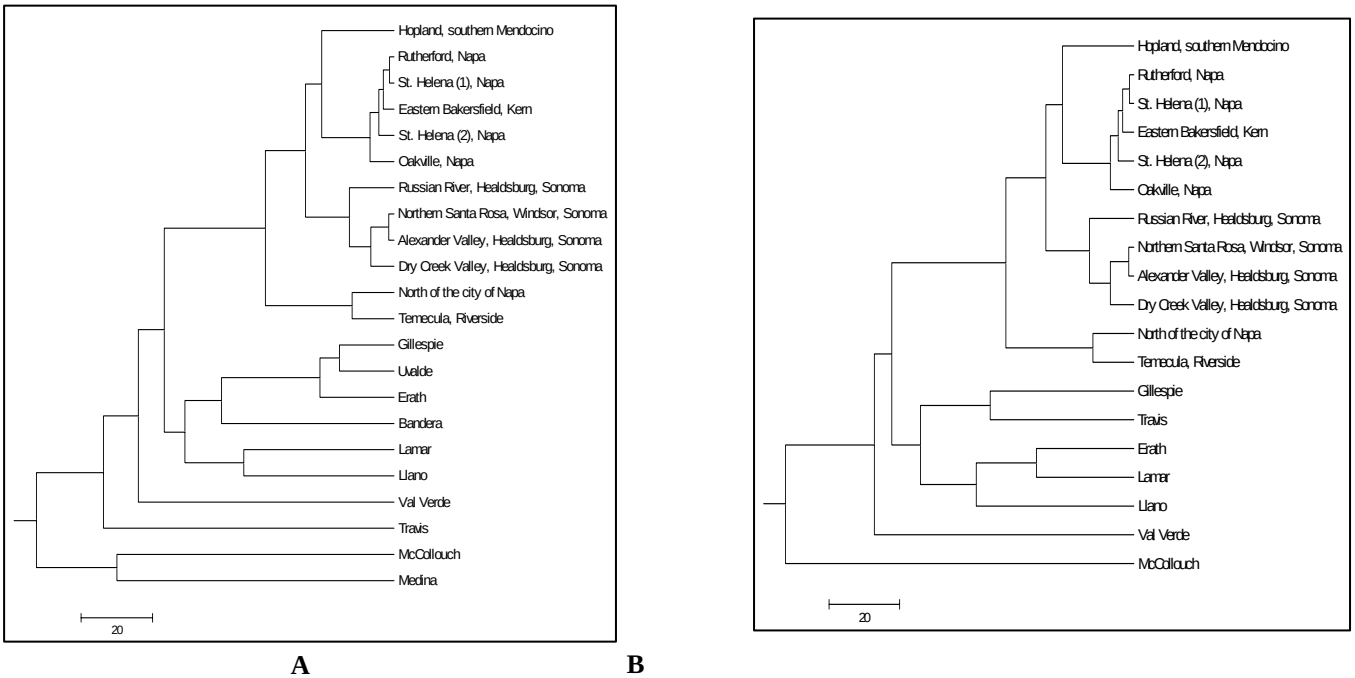

Figure 1 A. Dendrogram of genetic similarity of *Xf* within between grape growing regions in California (five regions with 12 grape populations) and Texas (10 regions with 10 populations contains grape and non- grape). **B.** Only grapes strains in California and Texas. We excluded Bandera and Uvalde from this analysis as they contained only one isolates when we excluded non-grape strains from these two populations.

Within California some local level population differentiations were identified. Different sub-populations at the most diversified wine grape producing regions in California i.e., Napa County produced two major groups. Each of the major genetic group of Napa mixed with the populations of two geographically distant counties i.e., Kern, Riverside (Temecula). However, in a broad sense, clear separation was observed among the isolates of Mendocino, Sonoma, Napa (except those isolates shared genetic similarity with Kern and Riverside), Kern and Riverside (**Figure 1 A**). Similar observations were found in the previous study though there was some relation between Kern and Riverside (Lin et al. 2005). No grouping was observed according to grape cultivar in California. In Texas, three smaller groupings were also formed but the remainder of the populations were found to be independent i.e., did not group with other populations. Though the genetic similarity of Gillespie-Uvalde–Earth is not recognized based on the geography, the host is likely influencing these on groupings, as these populations contain both grape and with few non-grape strains. When we excluded non-grape populations from those populations, Gillespie-Earth did not fall in one group (**Figure 1B**). Therefore, it can be inferred that in Texas genetic differentiations between the populations is influenced by the host i.e. grape and ragweed (subsp. *multiplex*) as described by (Morano et al. 2008).

Comparisons of pairwise F_{ST} values between the populations also evaluated the genetic differentiations among populations for short divergence time. Local level genetic variation within two important grape growing regions in the United States i.e., California and Texas are presented in **Table 2**.

Table 2 Pairwise F_{ST} comparison between *Xylella fastidiosa* grape populations in Californa and Texas

Californai-All Host	Hopland, southern Mendocino	Russian River, Healdsburg, Sonoma	Northern Santa Rosa, Windsor, Sonoma	Dry Creek Valley, Healdsburg, Sonoma	Alexander Valley, Healdsburg, Sonoma	Rutherford, Napa	St. Helena (1), Napa	Oakville, Napa	St. Helena (2), Napa	North of the city of Napa	Eastern Bakersfield, Kern
Russian River, Healdsburg, Sonoma	0.199										
Northern Santa Rosa, Windsor, Sonoma	0.352	0.172									
Dry Creek Valley, Healdsburg, Sonoma	0.113	0.029	0.010								
Alexander Valley, Healdsburg, Sonoma	0.458	0.259	-0.046	0.098							
Rutherford, Napa	0.331	0.196	0.373	0.295	0.527						
St. Helena (1), Napa	0.286	0.202	0.383	0.255	0.540	-0.045					
Oakville, Napa	0.333	0.224	0.422	0.327	0.597	0.114	0.020				
St. Helena (2), Napa	0.183	0.170	0.335	0.151	0.438	0.041	-0.003	0.074			
North of the city of Napa	0.205	0.177	0.306	0.122	0.378	0.168	0.155	0.178	0.149		
Eastern Bakersfield, Kern	0.205	0.158	0.286	0.115	0.384	0.019	0.032	0.163	0.051	0.102	
Temecula, Riverside	0.284	0.308	0.444	0.201	0.537	0.347	0.316	0.398	0.273	0.017	0.160

Texas-All Host	Gillespie	Bandera	Erath	Lamar	McCollouch	Llano	Medina	Travis	Uvalde
Bandera	0.112								
Erath	0.031	-0.109							
Lamar	0.286	0.203	0.095						
McCollouch	0.058	0.071	-0.215	0.229					
Llano	0.212	0.121	0.056	0.215	0.020				
Medina	0.450	0.493	0.325	0.576	0.503	0.472			
Travis	0.159	0.280	0.119	0.334	0.228	0.252	0.576		
Uvalde	-0.019	-0.025	-0.027	0.304	0.039	0.190	0.464	0.216	
Val Verde	0.236	0.036	0.071	0.242	0.034	0.162	0.417	0.247	0.198

TX-Grape Only in Texas	Gillespie	Erath	Lamar	McCollouch	Llano	Travis
Erath	0.086					
Lamar	0.262	0.054				
McCollouch	0.066	-0.150	0.205			
Llano	0.250	0.156	0.245	0.141		
Travis	0.153	0.085	0.315	0.205	0.304	
Val Verde	0.245	0.099	0.253	0.072	0.235	0.274

Gray highlighted pairs showed no significant genetic differentiation

Our microsatellite marker analysis showed significant genetic differentiations among the local geographic areas (Mendocino, Sonoma-Napa-Kern-Riverside) or even at the very local level i.e., within the counties (between some isolates of Napa and Sonoma County) in California. However, some populations of Napa County did not show significant genetic differentiations with geographically distant countries Kern and Riverside separately. These results suggest the evidence of the genetic similarity or the possibility of gene flow between Napa and Kern, and Napa and Riverside. In Texas, most of the populations (combining grape and non-grape strains) strains were not well differentiated according to the geography. Geographic structures were not observed as well from the few number of genetically differentiated population pairs when we excluded non-grape populations (**Table 2**). However, significant genetic differentiations were observed between the all populations pairs that were compared with Medina (all oleander strains), which indicates host associated genetic differentiations. Some clues about the influences of host on differentiating population were also noticed from the pairs compared with Lamer i.e., when we excluded non-grape (ragweed strains), genetic differentiations was not significant with Llano and Val Verde (**Table 2**).

In individual-based clustering analysis, Bayesian modeling approach within STRUCTURE predicted that *Xf* strains throughout the grape growing regions in California (grape), and Texas (grape and non-grape) derived from two different clusters i.e., two different origins/ancestors (**Figure 2**). Majority of the Californian strains (83%) fall into its own distinct

origin i.e., cluster1 (Red). In Texas 98% individuals (both grape and non-grape strains) fall into another distinct cluster II (Yellow) with a pure ancestry. Seventeen % strains in California showed genetic similarity to Texas strains, and which are more or less distributed every region in California we studied. Though a very small percentage of Californian origin is found admixed within one population of Texas (Gillespie County, 11% individuals admixed with 27% Californian ancestry), it certainly indicates the evidence of appearing Californian origin in Texas. The diversity of Texas isolates from grape and non-grape hosts is consistent with the idea that there have been at least two different types of ancestor/ strains since at least the 1880s (Pierce 1892), a non-grape and a grape strain. This work also suggests that populations of the grapevines have evolved uniformly as a unique genetic structure over the past 100 plus years in their own geographical areas with strong selection pressure, and with perhaps one episode of gene flow between these two grape growing regions.

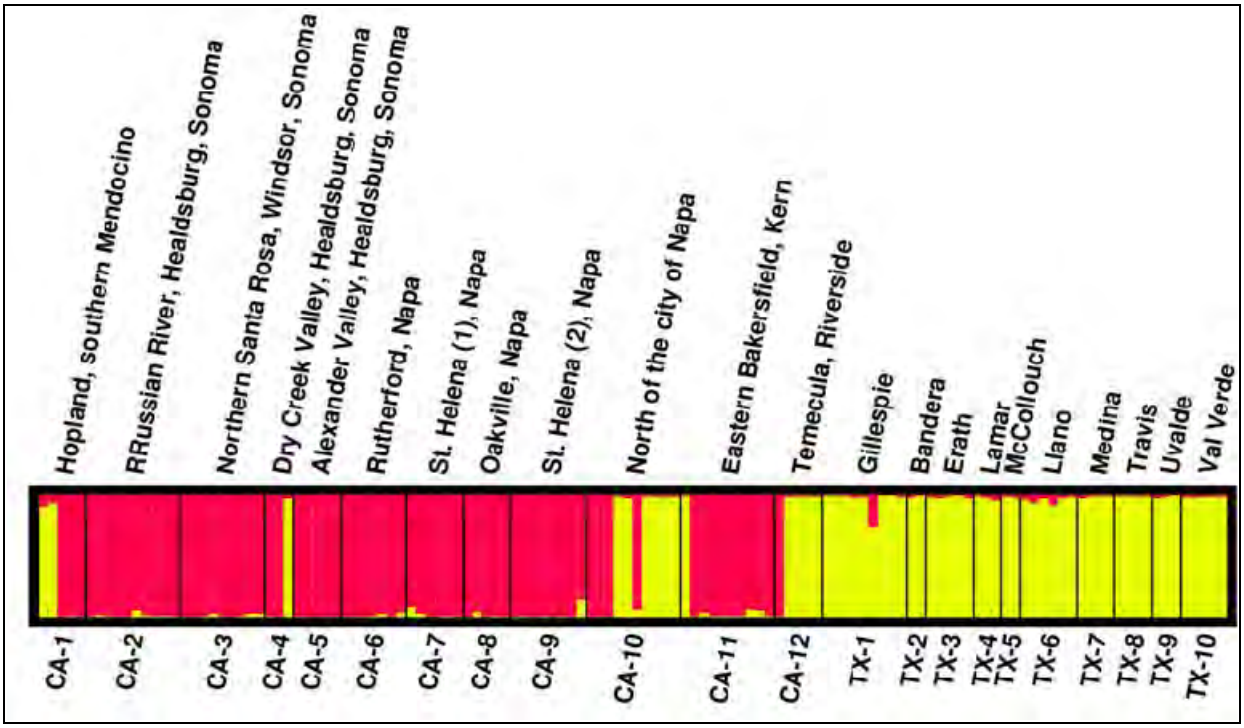

Figure 2 Individual assignment by STRUCTURE analysis; there were two clusters (K). Black lines within the squares distinguish populations.

However, sharing and distribution of 17% Texas origin in different regions (counties) in Californian let us propose a hypothesis of recent introduction of Texas strains into California (or less likely California strains back to Texas). This close genetic similarity of Texas and California grape strain populations could be explained by the introduction of the insects vectors, the glassy-winged sharpshooter (GWSS) (*Homalodisca vitripennis*), into the Southern California (Temecula region) in the early time, and its subsequent range expansion throughout California (De Leon et al. 2004; Morano et al. 2008). A phylogeny of GWSS has revealed that populations of this insect pest introduced into California likely originated from Texas (De Leon et al. 2004).

CONCLUSIONS

Xf genetic analysis clearly identified two different genetic structures of *Xf* grape strains in California and Texas, and are consistent with the introduction of Texas origin to California though the insects vectors, the GWSS. This multi-locus marker system is able to distinguish local level genetic differentiations based on the geography in California, and identified some clues on host associated genetic differentiations in Texas. These findings may provide necessary information to better understanding genetic diversity and evolutionary potential of *Xf* populations in California and Texas.

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CONTROL OF PIERCE'S DISEASE USING PATHOGEN SIGNAL MOLECULES

A Combined Report of the Following Projects:

-- Control of Pierce's Disease by Methods of Pathogen Confusion --

-- Exploiting Pathogen Signal Molecules for Control of Pierce's Disease --

-- Enhancing Control of Pierce's Disease by Augmenting Pathogen Signal Molecules --

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ABSTRACT

Xylella fastidiosa (Xf) produces an unsaturated fatty acid signal molecule called diffusible signal factor (DSF) that modulates gene expression in cells as they reach high numbers in plants. By increasing the expression of a variety of afimbrial adhesins while decreasing the expression of pili involved in twitching motility as well as extracellular enzymes involved in degrading pit membranes and hense movement between vessels, DSF accumulation suppresses virulence of *Xf* in grape. We thus are exploring different ways to elevate DSF levels in plants to achieve disease control via "pathogen confusion." Plants expressing *rpfF* from *Xf* produce low levels of DSF and are highly resistant to Pierce's disease (PD). Chloroplast targeting of RpfF substantially increased DSF production. *Xf* moved much less rapidly in *rpfF*-transformed grape, colonized many fewer xylem vessels, and achieved a much lower population size indicating that elevated DSF levels suppressed movement within the plant. As exogenous sources of DSF applied in various ways to grape suppressed pathogen mobility and hence virulence we have further studied the chemical identity of DSF. Preliminary evidence suggests that DSF is comprised of three closely related fatty acid molecules. One component is 2-Z-tetradecenoic acid (hereafter called C14-cis) while a second compound termed C12-cis is apparently also produced. The chemical identity of a third component is as yet undetermined. We are currently determining the relative activity of these forms of DSF and if such molecules cooperate in regulating gene expression in *Xf*. Preliminary evidence suggests that C12-cis may preferentially participate in processes related to insect colonization compared to plant virulence, hinting that the various forms of DSF may preferentially affect different behaviors of *Xf*. Some reduction in disease severity was observed in grape scions grafted to DSF-producing rootstocks suggesting that DSF produced by rootstocks can somewhat move to scions and confer disease control' the control of disease was substantially less than that seen in transformed scions however. Naturally-occurring endophytic bacteria within grape are being assessed for DSF production; only about 1% of the endophytic bacteria in grape produce DSF and these are being tested for their ability to move within plants after inoculation. As studies of pathogen confusion will be greatly facilitated by having an improved bioassay for the DSF produced by *Xf*, we have been developing several immunological and biochemical means to assay for the presence of DSF using *Xf* itself as a bioindicator. Bioassays based on immunological detection of the cell surface adhesion XadA and EPS as well as by quantifying mRNA associated with these genes in *Xf* have been developed. Gene expression in *Xf* exposed to various levels of DSF can also be directly assessed using *phoA* reporter gene fusions. RpfF- mutants of *Xf* that do not produce DSF adhere much less tenaciously to grape vessels than do WT strains and we thus are developing assays to more rapidly screen transgenic plants for their resistance to PD as well the efficacy of chemical analogs of DSF to induce resistance

LAYPERSON SUMMARY

Xylella fastidiosa (Xf) produces an unsaturated fatty acid signal molecule called diffusile signal factor (DSF). Accumulation of DSF in *Xf* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in *Xf*, but the overall effect is to suppress the virulence of *Xf* in plants. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption (pathogen confusion) as a means of controlling Pierce's disease (PD). We have investigated both the role of DSF-production by *Xf* on its behavior within plants, the manner in which other bacterial strains affect such cell signaling, the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, performed genetic transformation of grape to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control. Elevating DSF levels in plants should reduce movement of *Xf* in the plant. We have found naturally-occurring bacterial endophyte strains that can produce large amounts of DSF; we are testing them for their ability to move within plants and to alter the abundance of DSF sufficiently to reduce the virulence of *Xf*. Given that DSF overabundance appears to mediate an attenuation of virulence in *Xf*

we have transformed grape with the *rpfF* gene of *Xf* to enable DSF production in plants; such grape plants produce at least some DSF and are much less susceptible to disease. Higher levels of expression of DSF have been obtained in plants by targeting the biosynthetic enzymes to the chloroplast. Some reduction in disease severity was observed in grape scions grafted to DSF-producing rootstocks suggesting that DSF produced by rootstocks can somewhat move to scions and confer disease control. The chemical composition of DSF itself is being determined so that synthetic forms of this signal molecule can be made and applied to plants in various ways. We have found that the adherence of *Xf* to grape tissue is much more tenacious in the presence of DSF, and we thus are developing assays to more rapidly screen transgenic plants for their resistance to PD as well the efficacy of chemical analogs of DSF to induce resistance.

INTRODUCTION

We have found that the virulence of *Xylella fastidiosa (Xf)* is strongly regulated in a cell density-dependent fashion by accumulation of a signal molecule called diffusible signal factor (DSF) encoded by *rpfF* and involving signal transduction that requires other *rpf* genes. We now have shown that *Xf* makes a DSF molecule that is recognized by *Xanthomonas campestris pv. campestris (Xcc*) but slightly different than the DSF of *Xcc* (**Figure 1**).

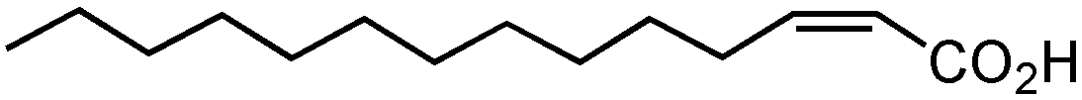

Figure 1.

Our on-going work suggests that it also makes other, closely related signal molecules as well. In striking contrast to that of Xcc, *rpfF*- mutants of *Xf* blocked in production of DSF, exhibit dramatically increased virulence to plants, however, they are unable to be spread from plant to plant by their insect vectors since they do not form a biofilm within the insect. These observations of increased virulence of DFS-deficient mutants of *Xf* are consistent with the role of this density-dependent signaling system as suppressing virulence of *Xf* at high cell densities. Our observations of colonization of grapevines by *gfp*-tagged *Xf* are consistent with such a model. We found that *Xf* normally colonizes grapevine xylem extensively (many vessels colonized but with only a few cells in each vessel), and only a minority of vessels are blocked by *Xf.* Importantly, rpfF- mutants of *Xf* plug many more vessels than the wild-type strain. We thus believe that *Xf* has evolved as an endophyte that colonizes the xylem; blockage of xylem would reduce its ability to multiply since xylem sap flow would cease and thus the DSF-mediated virulence system in *Xf* constrains virulence. That is, *Xf* would benefit from extensive movement throughout the plant where it would partially colonize xylem vessels but would have evolved not to grow too excessively within a vessel, thereby plugging it and hence blocking the flow of necessary nutrients in the xylem sap. Given that the DSF signal molecule greatly influences the behavior of Xf we are investigating various ways by which this pathogen can be "confused" by altering the local concentration of the signal molecule in plants to disrupt disease and/or transmission. We thus are further exploring how DSF-mediated signaling occurs in the bacterium as well as ways to alter DSF levels in the plant. Our work has shown that the targets of Rpf regulation are genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel as well as adhesins that modulate movement. Our earlier work revealed that several other bacterial species can both positively and negatively interact with the DSF-mediated cell-cell signaling in *Xf.* In this period we have extensively investigated both the role of DFS-production by *Xf* on its behavior within plants, the patterns of gene regulation mediated by DSF, the frequency with which other endophytes can produce signal molecules perceived by *Xf*, have further characvterized the behavior of *Xf* in grape genetically transformed to produce DSF, and explored other means to alter DSF abundance in plants to achieve Pierce's disease (PD) control. We have particularly emphasized the development of various methods by which DSF abundance in plants can be assessed so that we can make more rapid progress in testing various ways to modulate DSF levels in plants, and have also developed more rapid means by which the behavior of *Xf* in plants can be assessed that does not require the multi-month PD assay. Lastly, we have developed better methods to assess DSF-mediated changes in phenotypes in *Xf.*

COMBINED OBJECTIVES

1. Evaluate plants with enhanced production of DSF for disease control
2. Determine if DSF is transferable within plants – eg. whether DSF production in rootstocks can confer resistance to PD in the scion
3. Evaluate enhanced DSF-producing endophytic bacteria for control of PD
4. Investigate DSF-overproducing strains of *Xf* as biocontrol agents for PD and whether *Xf* strains previously identified with biocontrol potential exhibit an elevated production of DSF
5. Determine if resistance to PD is associated with low rates of degradation of DSF by plants
6. Determine those plant factors that confer induction of virulence genes in *Xf* and whether susceptibility to PD is due to differences in induction of virulence factors in the pathogen by the plant
7. Identification and characterization of low molecular weight signaling molecule (DSF) central to behavior of *X. fastidiosa*
8. Design and synthesize low molecular weight compounds capable of interfering with signal molecule function in *Xf*

9. Using novel, improved biosensors for the DSF produced by *Xf*, identify naturally-occurring endophytic bacteria which produce Xf DSF, and evaluate them for biological control of PD after inoculation into plants in various ways.
10. Evaluate plants with enhanced production of DSF conferred by co-expressing RpfB, an ancillary protein to DSF biosynthesis, along with the DSF synthase RpfF for disease control as both scions and as rootstocks.
11. Optimize the ability of DSF-producing in rootstocks to confer resistance to PD in the scion.
12. Determine the movement and stability of synthetic DSF and chemical analogs of DSF applied to plants in various ways to improve disease control.

RESULTS AND DISCUSSION

Production of DSF in transgenic plants for disease control.

We have expressed the *rpfF* gene in several different plant species to investigate whether DSF excess can lead to reduced disease caused by *Xf*. Grape (Freedom) has been transformed at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis with a non-targetted *rpfF* construct. These plants produced only very low levels of DSF but are MUCH less susceptible to *PD* (**Figure 2**). While *Xf* spread throughout non-transformed plants causing disease on petioles located great distances from the point of inoculation, disease was observed only very close to the point of inoculation in *rpfF*-expressing plants. We measured the movement of *Xf* in these plants by measuring both the population size of *Xf* in stems and petioles at different distances from the point of inoculation, as well as to observe the fraction of vessels to which a gfp-marked strain of *Xf* had moved using fluorescence microscopy (**Figure 3**). *Xf* was greatly limited in its movement in plants producing DSF as evidenced by both a lower population size at sites distal to the point of inoculation and a lower incidence of vessel colonization at all points; both would contribute to low disease severity.

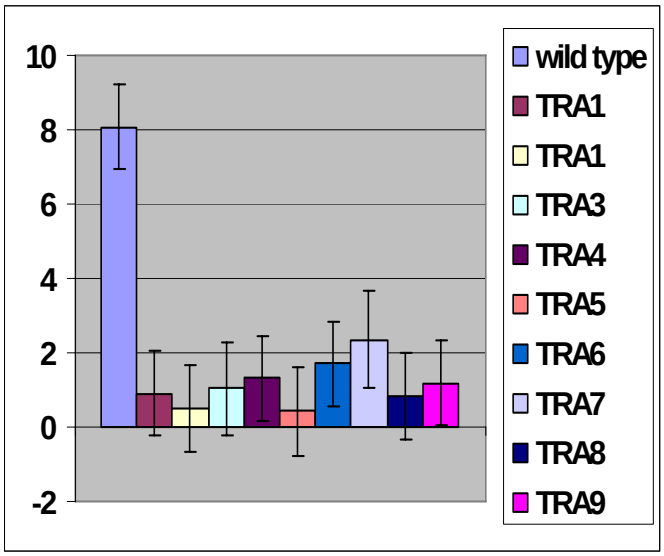

Figure 2. Disease severity (# symptomatic leaves/plant) on Freedom grape transformed with the r*pfF* gene encoding DSF production and inoculated with *Xf*.

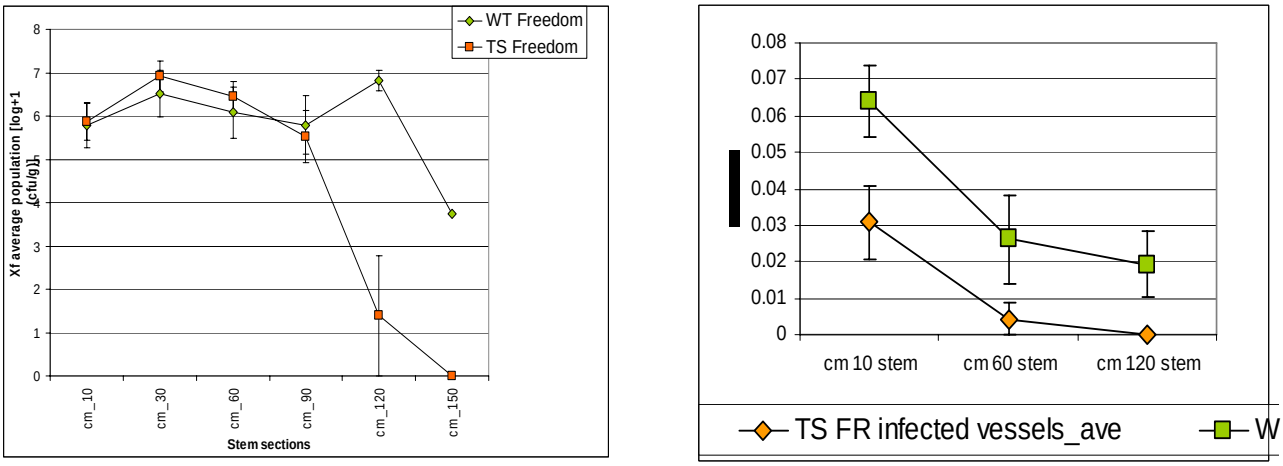

Figure 3. (Left) Population size of *Xf* in one cm stem segments at different distances from the point of inoculation on non-transformed Freedom grape (green) and in *rpfF*-transformed Freedom (red). The vertical bars represent the standard error of mean log population size. (Right). Proportion of xylem vessels in stems of transformed Freedom grape (green) and in *rpfF*-transformed Freedom (orange) colonized with a gfp-marked strain of *Xf* at different distances from the point of inoculation.

Figure 4. Detection of DSF, visualized as green gfp fluorescence from macerates of leaves from wild-type Thompson seedless (left panel) or different *rpfF*-transformed plants (right panel). In each panel the left-most column is from samples taken near the point of inoculation while the center and right column are from stem segments taken 60 cm and 120 cm from the point of inoculation, respectively.

We have recently transformed grape (Thompson seedless) with an *rpfF* gene that has been modified to direct the protein product to the chloroplast where fatty acid synthesis (and DSF synthesis) should be much enhanced compared to its production in the cytosol, the presumed location of RpfF in the current transformed plants. Our preliminary results with an improved DSF bioindicator (described below) revealed that DSF could be detected in several of the transgenic targeted RpfF lines (**Figure 4**) – this is in contrast to the non-targeted plants were levels of DSF are apparently too low to detect with this biosensor.

We also have inoculated the chloroplast-targeted rpfF plants with a gfp-marked strain of *Xf* to assess differences in its movement within the DSF-producing and normal Thompson seedless grape using fluorescence microscopy. Ten Transgenic Chloroplast-targeted rpfF Thompson seedless plants, (which was a mix of several trnasformed lines) and ten non-transformed Thompson seedless were stem inoculate with a gfp-marked wild type *Xf*. At eight weeks after inoculation one cm stem segments were sampled at 10,60,120, cm distal from the point of inoculation, and *Xf* populations were determined by culturing and CFU/gr populations were estimated via dilution plating. While the population size of *Xf* in the rpfF-transformed lines were similar to that in untransformed lines near the point of inoculation, population sizes were about 10-

fold lower in the rpfF-transformed lines at more distal sites on the vine such as 120 cm from the point of inoculation (**Figure 5**).

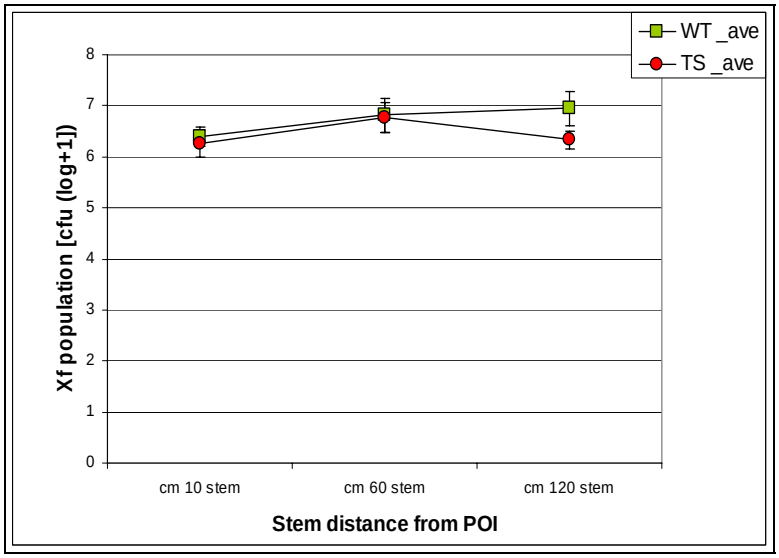

Figure 5. Population size of *Xf* in wild type Thompson seedless grape (squares) or transgenic rpfF-expressing grape (circles) at different distances from the point of inoculation.

Microscopy was also carrying out at the same sampling sites to assess the frequency with which xylem vessels were colonized by *Xf*. We recorded as positive any vessel harboring *Xf* irrespective of whether they harbored few cells or many cells. An average of five stem cross sections were examined for each sampling distance from the point of inoculation on each plant (**Figure 6**). The proportion of vessels of the *rpfF*-transformed grape that were colonized by *Xf* was only about 50% that in non-transgenic lines, suggesting that the movement of the pathogen through the plant was inhibited by expression of *rpfF* and thus production of DSF in the plant. It was also noteworthy that the incidence of vessel colonization varied greatly between transgenic lines evaluated, with some lines having a similar incidence of colonization as the wild-type line while others having very little colonization.

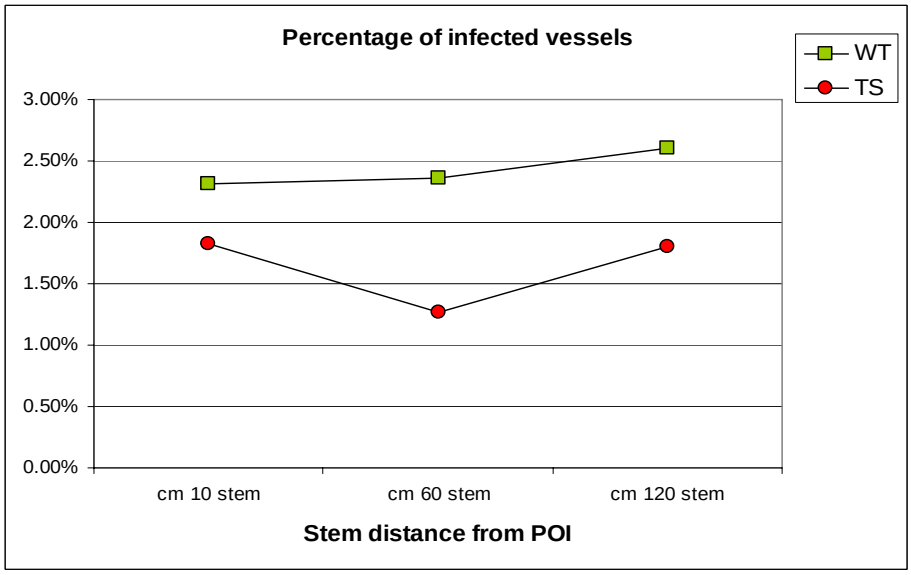

Figure 6: Average of *Xf*- infected vessels per grape stem cross section in transgenic rpfF-transformed Thompson grape (red circle) and in non-transformed Thompson seedless in (green squares).

Further support for the possibility that more than one fatty acid signal molecule is made by RpfF was obtained by the use of a Thin Layer Chromatography (TLC) method to assess the fatty acids produced by *Xf*. In this method, acidified ethyl acetate extracts of culture supernatants of a wild-type *Xf* strain and an RpfF- mutant and a RpfB mutant were subjected to TLC and fatty acids visualized by iodine vapors. Interestingly, three different fatty acids were visualized in the wild type strain, while these were largely missing in an RpfF- mutant, with only very small amounts of two other putative fatty acids present (**Figure 7**). It also was of interest to see that the RpfB mutant produced an altered pattern of putative fatty acids, with the major chemical species produced by the WT strain missing, and much larger amounts of one of the other species produced. The top-most spot observed in extracts of a WT strain of *Xf* co-migrates with C14-cis, a chemical form of DSF that we have previously characterized. Interestingly, the middle band found in the WT strain, which migrated similarly to the topmost, and most abundant band seen in the rpfB mutant, co-migrates with C12-cis. It is noteworthy, that a re-examination of the DSF species produced by *Xanthomonas campestris* pv. campestris using different methods have revealed that this species also produces C12-cis. The most prominent, lower, band seen in the WT strain is not observed in the *rpfB* mutant. This supports the model that RpfB, a putative long-chain fatty acyl CoA ligase, serves to produce suitable substrates for RpfF, the DFS synthase. Although RpfB is not required for DSF synthesis in *Xf*, it presumably aids in DSF synthesis by encoding long chain fatty acyl CoA ligase which might increase availably of the appropriate substrates for DSF synthesis by RpfF. It is interesting to note that *rpfB* mutants have an altered behavior compared to *rpfF* mutants and WT strains of *Xf*. While rpfF mutants are hypervirulent in grape, *rpfB* mutants were nearly as virulent as WT strain. In contrast, while rpfF mutants are non-transmittiable by sharpshooters, the rpfB mutants exhibit only a slight decrease in their transmission (**Figure 8**) suggesting that they are retained by sharpshooters more efficiently than the rpfF mutant. Given that the *rpfB* mutant appears to make C12-cis but not C14-cis, this suggests that C12-cis is sufficient to enable signaling that leads to insect transmission but does not greatly affect virulence. We expect that co-expression of RpfB and RpfF in the chloroplast will further enhance the DSF levels in plants. We have produced transgenic *Arabidopsis* plants with such a construct and find evidence of high levels of DSF production. Pathogenicity assays with the *rpfF* mutant of *Xcc* indicated that the transgenic plants expressing both *rpfB* and *rpfF* transgenic plants can better complement the virulence of the non pathogenic *rpfF* mutant of *Xcc*. Based on this and other data, we are expressing both RpfF and RpfB simultaneously in transgenic for optimum production of suitable DSF molecules. We thus are preparing genetic constructs to transform grape with these two genes to further enhance DSF production.

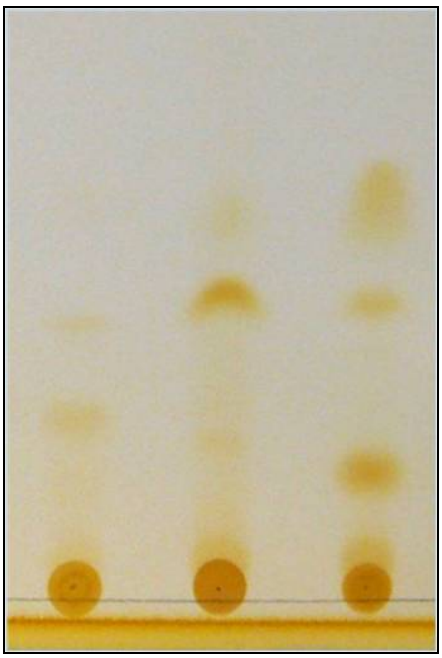

Figure 7. Fatty acids resolved by TLC from a RpfF mutant of *Xf* (left lane) a RpfB mutant (center lane) and a wild type strain of *Xf* (right lane). Fatty acids were visualized after exposure to iodine vapor.

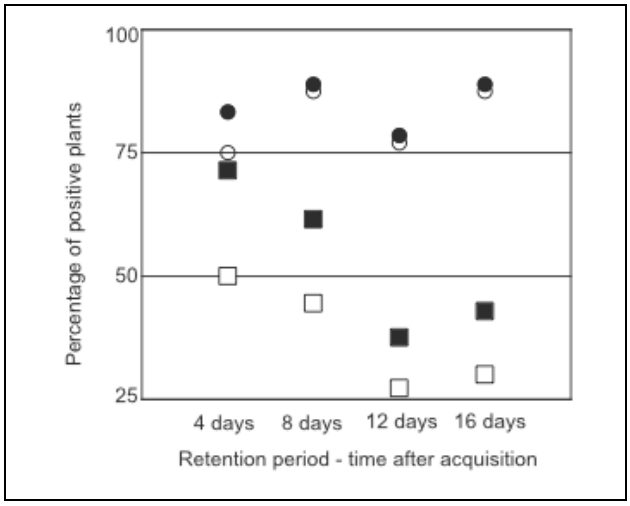

Figure 8. Retention of wild type and *rpfB* mutant strains of *Xf* by leafhopper vectors as indicated by their transmission rates on successive plant transfers. Individual leafhoppers were transferred to new grapevines every 4 days after acquiring the bacteria on source vines infected with *rpfB* mutant or the wild type. Circles represent the wild type transmission rates, squares the *rpfB* mutant; solid symbols include rates only for insects that transmitted to at least one plant in the serial transfer, empty symbols represent total proportion of plants infected.

Studies of movement of *Xf* in plants

Our studies have suggested strongly that adhesion of *Xf* to plant tissues inhibits movement of the pahtoen through the plant, and hence tends to reduce the virulence of the pathogen. RpfF- mutants of *Xf* that do not produce DSF adhere to glass surfaces and to each other much less effectively than WT strain that produce DSF. This is consistenty with the apparent rpfF-regulation of adhesins such as HxfA, HxfB and XadA etc. To better correlate levels of DSF in the plant and the stickiness of the *Xf* cells we have developed a practical assay to measure and compare stickiness of *Xf* cells in grapes infected with *Xf gfp-Wt* and *Xf* mutants. In this assay, the release of cells of *Xf* from stems and petioles tissue from grape infected with *Xf* wild type Tem and gfp-rpfF mutant were compared. Tissues from infected Thompson seedless grapes were surface sterilized with 70% alcohol, and 30 % bleach. From the sterile tissues, 5mm stem or petiole segments were cut and placed individually in sterile buffer and shaken gently for 20 minute. After 20 minutes the number of cells released from the cut end of the segment were estimated by dilution plating on PWG. To determine the total number of cells in a given sample (the number of cells that potentially could have been released by washing) the washed segment was macerated and *Xf* populations again evaluated by dilution plating. Total cell populations were calculated by summing the cells removed by washing and those retained in the segment. The ratio of easily released cells to the total cells recovered in the samples was termed the release efficiency. In both stems and petioles the release efficiency of the *rpfF* mutant was much higher than that of the WT strain (**Figure 9**). This very striking difference in the adhesiveness of the *Xf* cells experiencing different levels of DSF in the plant suggest that this release efficiency assay will be valuable for rapidly assessing the susceptibility of grapes treated in various ways. For example, the adhesion of cells could be measured within a couple of weeks after inoculation of WT *Xf* cells into transgenic plants harboring various constructs designed to confer DSF production in plants, or in plants treated with DSF producing bacteria or topical application of chemicals with DSF-like activity. Such an assay would be far more quick than assays in which disease symptoms must be scored after several months of incubation, and could be employed during those times of the year such as the fall and winter when disease symptoms are difficult to produce in the greenhouse.

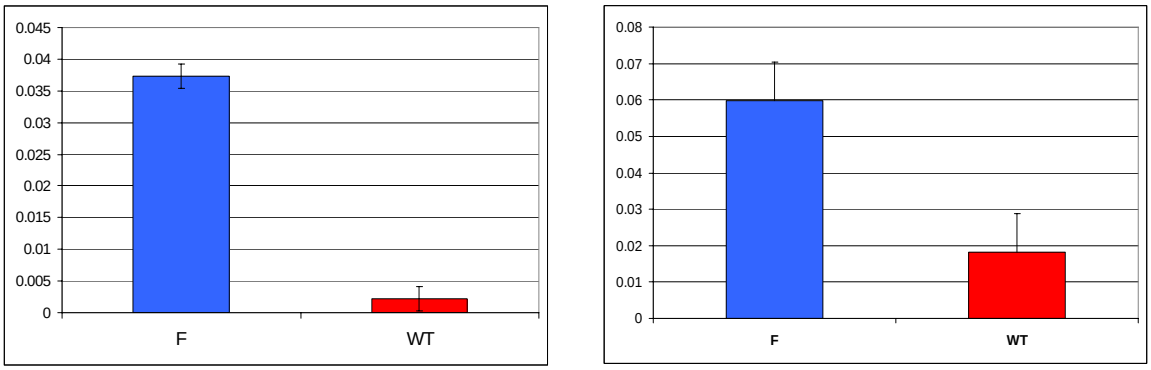

Figure 9. Proportion of total cells of a gfp-marked WT strain of *Xf* (red) and a gfp-marked rpfF mutant of *Xf* (blue) in petioles (left) or stems of Thompson seedless grape (right) that were released during gentle washing of the segments in buffer. The vertical bars represent the standard error of estimates of the proportion of released cells for a given treatment.

While the movement of *Xf* has been recognized as an important trait necessary for disease, the process is still poorly understood. Other studies we have performed in our lab are strongly supportive of a model of progressive and sequential colonization of a large number of xylem vessels by *Xf* after inoculation of a single vessel. Furthermore, we believe that the process of movement of *Xf* through plants is a stochastic one which is characterized by growth in a given xylem vessel into which it is introduced followed by "active escape" of at most a few cells into adjacent uncolonized vessels, and then further multiplication of the cells which starts the process anew. We thus are exploiting the use of mixtures of phenotypically identical strains of *Xf* differing by only one or two genes to better understand the process of progressive movement of *Xf* through plants. We hypothesize that anatomical features of plants (nature of pit membranes and other barriers to vessel to vessel movement in the stem) limit the number of *Xf* cells that can transit from one vessel to another and are major factors conferring resistance in plants. It would be expected that the stochastic (random) processes that would tend to segregate cells of one strain from another in the process of progressive movement would increase the degree of segregation with distance from the point of inoculation (with increasing numbers of vessels the cells had to traverse to get from one part of the stem to another given that each vessel in grape is an average of only about 10 cm long). That is, *Xf* must move from one xylem vessel to another dozens or hundreds of times to be able to move longitudinally down a vine as well as laterally across the vine to achieve the extensive colonization of the stem that are typical of diseased vines. If, at each step in this movement process only a few cells are transferred then, by chance, xylem cells distal to the inoculation point will receive by chance only one of the two genotypes of the pathogen. Thus, for a given plant inoculated with a mixture of cells, the proportion of one strain compared to the other would either increase or decrease along a predictable trajectory given the stringency of the "bottleneck" that it faced while moving from one vessel to another. Our efforts to test this model of movement of *Xf* through grape tissue has been hindered by the fact that isogenic strains of this pathogen that differ even slightly in virulence (ability to move within the plant) result in one strain predominating at distal parts of the plant, presumably due to a slightly higher likelihood of successful movement to adjacent xylem vessels at each step in the colonization process. For example, WT cells of *Xf* always moved further and at higher frequency than *rpfB* mutants. Likewise, random Tn5-mutants of *Xf* generated by the Kirkpatrick lab also were inferior to the WT strain and were not recovered when mixtures were inoculated into grape. We now have identified *phoA* mutants as having identical behavior in grape as the WT strain, enabling us to examine the process of spatial segregation of cells of *Xf* during colonization of grape. The population size of the WT strain of *Xf* was similar to that of the *phoA* mutant whether inoculated singly or in a mixture (**Figure 10**). More importantly, the proportion of cells of the two strains that were recovered from different locations within a given plant differed greatly between plants (**Figure 11**). For example, eight plants were inoculated with an equal mixture of the WT and *phoA* mutant, yet in some plants all of the cells recovered from locations either 10 cm or 120 cm from the point of inoculation were either one strain or the other; seldom was a mixture of both strain found, and a similar fraction of the plants harbored one strain or the other, suggesting that the two strains had an equal likelihood to move within the pant, but that stochastic processes determined the movement. We hypothesize that resistant grape varieties harbor anatomical differences from susceptible varieties that limit the movement of *Xf* from vessel to vessel. Such plant would thus present a more extreme "bottleneck" to *Xf* at each movement event and hence we would expect a more rapid segregation of mixtures of *Xf* at a given point away from inoculation. We thus are currently further exploring the spatial dependence of this segregation process in different grape varieties that differ in resistance to PD. Not only should this provide considerable insight into the process of movement which, while central to the disease process, remains very poorly understood, but it should also provide new tools for screening grape germplasm for resistance to *Xf*.

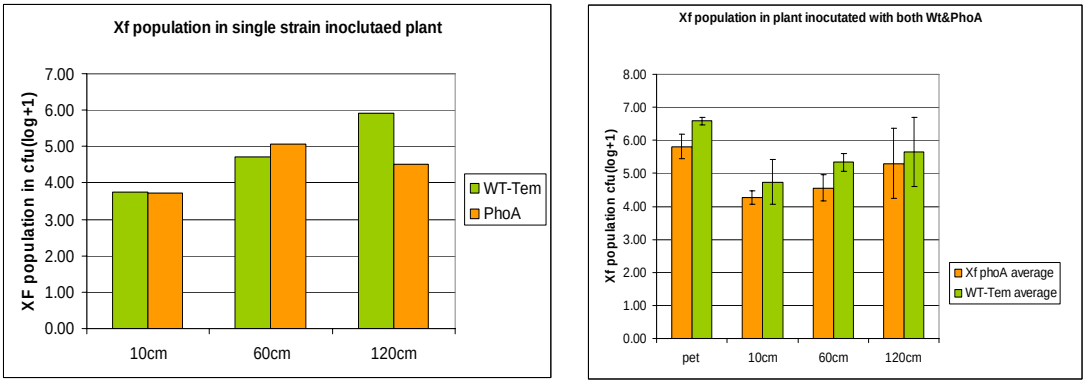

Figure 10. Population size of a WT strain of *Xf* (green) or of a PhoA mutant (orange) at various distance from the point of inoculation when inoculated single (left panel) or in equal mixtures (right panel).

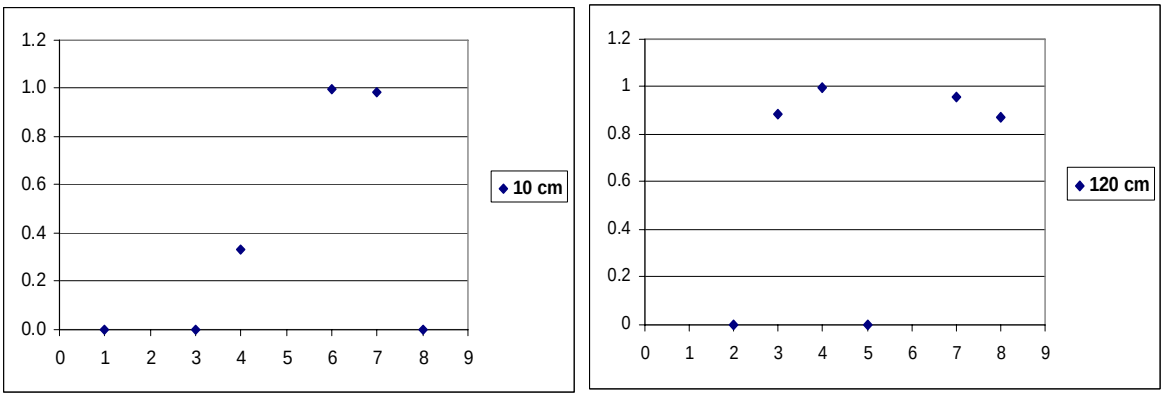

Figure 11. Proportion of cells recovered at 10 cm (left panel) or 120 cm (right panel) from the point of inoculation from individual plants that were inoculated with an equal mixture of a WT strain of *Xf* and a PhoA mutant (abscissa) that were the WT strain.

Graft transmissibility of DSF. To test whether DSF is mobile within the plant we are performing grafting experiments in which DSF-producing Freedom grape transformed with the rpfF of *Xf* are used as rootstocks to which normal Cabernet Sauvignon grape were green-grafted as a scion. As a control, normal Freedom was also used as a rootstock. These plants were inoculated with *Xf* to test whether normal scions on DSF-producing rootstocks have a lower susceptibility to *Xf* colonizationed as a rootstock scions. Initial estimates of disease severity indicate that there were about 30% less symptomatic leaves of the normal Cabernet scion when grafted onto a *rpfF*-expressing rootstock compared with plants on a normal Freedom rootstock (**Figure 12**). Thus, like in the studies of the *rpf*F-expressing tobacco, it appears that DSF production in the scion is more efficacious for disease control than is the expression of *rpfF* in the rootstock. We are repeating these experiments and will be inoculating the plants in a variety of ways to determine the efficiency of disease control from rootstock modification. We are repeating these grafting experiment both with the non-targeted *rpfF* Freedom as a rootstock as well as the chloroplast-targeted *rpfF* Thomson seedless as a rootstock. Pesticide injury suffered by these plants in our greenhouses during the summer of 2010 have delayed the completion of these studies since the plants were severely stunted and the grafts had to be redone.

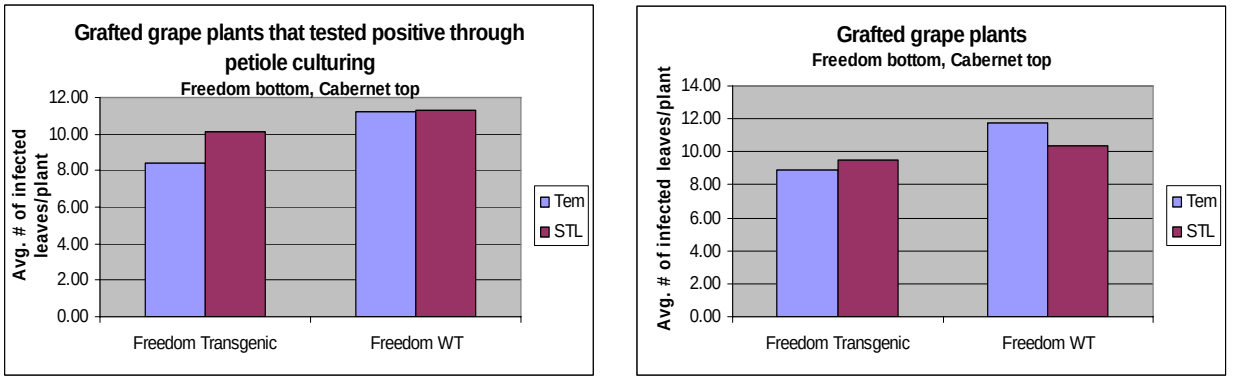

Figure 12. Severity of PD (left box) or number of leaves per vine infested with *Xf* (right box) of Freedom grape having a Cabernet sauvignon grape scion grafted onto the rootstock noted on the abscissa. Plants were inoculated with either *Xf* strain Temecula (blue) or strain STL.

Disease control with endophytic bacteria**.** The severity of PD is reduced when DSF-producing bacteria such as rpfF-expressing *E. coli* and *E. herbicola* and certain *Xanthomonas* strains are co-inoculated with *Xf* into grape. Importantly, the control of disease by DSF-producing bacteria was associated with their ability to produce DSF since strains that were blocked in ability to produce DSF were greatly reduced in their ability to suppress disease, indicating that elevated DSF in the plants was the cause of disease suppression. We have been successful in producing large quantities of DSF in endophytes such as *Erwinia herbicola* and also in lab strains of *E. coli*. Unfortunately, these bacterial strains do not move within grape after inoculation, thereby restricting their ability to interact with *Xf* except at sires of co-inoculation. Presumably to achieve control of PD by endophytic bacteria where *Xf* might be inoculated at any point in the plant by insect vectors it will be important to utilize endophytic bacteria that can colonize much of the plant in order that DSF be present at all locations within the plant. Naturally-occurring endophytic bacteria that produced the DSF sensed by *Xf* and which might move extensively within the plant would presumably be particularly effective as such biological control agents, but until now it was impossible to identify such strains. We recently were able to transform a putative efficient endophyte of plants, *Rizobium etili* G12 with both the *Xcc* and *Xf rpfF* (DSF biosynthetic gene) and have obtained some production of DSF in this strain. This DSF-producing endophyte has been inoculated into grape to determine both its ability to move and multiply within grape as well as its ability to interfere with the disease process. The *R. etli* strain G12 was found to move within grape tissue after inoculation into either the stem or the leaves. When measured four weeks after inoculation by puncture inoculation into one site in the stem measurable populations of *R. etli* were seen as far as 50 cm away from the point of inoculation. While the population size away from the point of inoculation were relatively low in this short time interval since inoculation, this strain clearly has the ability to move within grape. The evidence we have obtained so far, however, suggests that this bacterium moves relatively slowly in grape, and thus such a strain would have to be inoculated into grape substantially in advance of the pathogen in order to achieve high levels of disease control. In addition, the DSF-producing R. etli strain is recombinant, and hence may face regulatory scrutiny before it might be used in production viticulture. For that reason we have initiated a study of naturally-occuring bacterial endophytes for their ability to produce DSF. This objective was possible since much effort devoted in the last two years has resulted in the development of better biosensors for the DSF produced by *Xf* (C14-cis and related molecules) (discussed below). We now have several highly sensitive assays for *Xf* DSF. The DSF produced by *Xcc* and that made by *Xf* are slightly different and the *Xcc*-based biosensor for DSF is MUCH more responsive to the *Xcc* DSF than to the C14-cis produced by Xf; while the *Xcc*-biosensor can detect as little as about 100 nM of *Xcc* DSF it can detect C14-cis only in concentrations above about 1 mM. We thus previously could not easily the production of molecules in bacterial endophytes that were similar to the DSF made by *Xf*. Our new biosensor, however has allowed us to screen large numbers of bacteria for *Xf* DSF production.

Both the older *Xcc* and new *Xf*-DSF specific biosensors developed in our lab were used to screen natural bacterial endophytes recovered from bluegree sharpshooter(BGSS) insect heads and grape plants. We are executing this part of the project using two approaches; the first approach is building our own endophytes library using mainly endophytes isolated from insect head and wild grapes. The second approach involved screening an existing large grape endophyte library which was kindly made available to us by Dr. B. Kirkpatrick.

BGSS adults and nymphs were surface sterilized, macerated, and diluted onto 10% TSA. The emerging colonies were harvested and transferred to KB plates where they were over-sprayed with either of the two biosensors and GFP fluorescence monitored.

Our initial results reveal that 0.9% of the endophytes recovered from either insect mouthparts or from grape xylem produce either a DSF detected by the *Xcc*-specific DSF biosensor, or the *Xf* DSF-specific biosensor or both. More that twice as many

strain produce a DSF detected by the *Xcc*-specific DSF biosensor, suggesting that the DSF produced by *Xf* is not as common as that produced by other bacteria such as environmental *Xanthomonas* strains etc. Many more strains remain to be tested and a relatively large library of DS-producing bacteria is anticipated. This collection of DSF-producing bacteria will then be assessed for their ability to grow and move within grape plants as well as their ability to reduce symptoms of PD when co-inoculated and pre-inoculated into grape before *Xf*.

Development of an *Xcc* biosensor efficient in detecting *Xf* DSF. For many of the objectives of this project, an improved bioindicator for DSF would be very valuable. Such a biosensor will be needed to accurately screen transgenic plants for DSF production as well as to screen to endophytic bacteria capable of DSF produciotn. Likewise, the direct application of DSF to plants as well as the application of analogs of DSF for disease control will be greatly expedited by use of direct assays for DSF abundance in treated tissues. Until now, we have been using an *Xcc*-based biosensor in which the *Xcc* endoglucanase gene is linked to a GFP reporter gene. Previous studies have shown that this biosensor is able to detect the DFS made by *Xf* but that it detects *Xf* DSF with a much lower efficiency then the *Xanthomonas* DSF since the two molecules apparently differ slightly. In this reporting period we have made considerable progress in the construction and characterization of *Xanthomonas campestris* -based *Xf*-DSF specific biosensors. In order to be able to sense *Xf* DSF we constructed two independent *Xcc* -based DSF biosensors specific to the DSF produced by *Xf* RpfF and which is sensed by *Xf* RpfC. In the first sensor, we replaced the Rpf-DSF detection system of *Xcc* with that of homologous components from *Xf*. An *Xcc* mutant in which both *rpfF* and *rpfC* was deleted was transformed with a pBBR1MCS-2 based plasmid harboring *Xf rpfC* and *rpfF* genes. In a previous study, *Xf rpfC* was introduced into an *Xcc rpfC* mutant resulting in the repression of DSF production (the *Xcc rpfC* mutant itself is a DSF hyper-producer) but not of EPS production and protease activity. Based on that observation it was concluded that the *Xf* RpfC is capable of interacting with the *Xcc* RpfF to control its DSF production activity, but that the *Xf* rpfC was not capable of interacting with the *Xcc* RpfG to initiate down-stream signal transduction. In that sensor the *Xcc rpfG* was left intact. *Xf* RpfG, RpfC and RpfF are presumed to function in concert to mediate signal transduction in the following manner: *Xf* RpfF synthesize DSF, and we have shown that it produces DSF molecules recognizable by *Xcc* RpfC and in other bacterial hosts (*E. coli*, *E. herbicola R. etli etc.*). RpfC, upon interaction with DSF, is thought to phosphorelay to RpfG. RpfG is a cyclic di-GMP phosphodiesterase. Since cyclic di-GMP plays a regulatory role in many bacteria species, it is expected that the *Xf rpfGCF* system, capable of reducing the levels of cyclic di-GMP, will affect the expression of genes regulated by cyclic di-GMP in any host which its gene expression relays upon cyclic di-GMP. In *Xcc*, DSF and cyclic di-GMP were shown to regulate the expression of many virulence genes including the cellulase gene *engXCA*. Therefore, the functionality of the *Xf rpfGCF* system was determined in *Xcc* based on the activity of an *engXCA'::gfp* transcriptional fusion. Introduction of *Xf rpfG* to the *Xcc rpfCF* mutant, did not affect the GFP fluorescence signal produced by the *engXCA'::gfp* fusion suggesting that *Xf rpfG* was in its inactive state. Introduction of *Xf rpfG* and *rpfC* together however, resulted in a two-fold increase in the *engXCA* promoter activity. This strain, although it harbors *Xf rpfC*, exhibited a slight increase in *gfp* fluorescence in response to only high levels (100 µM) of 2-*Z*- tetradecenoic acid, the putative DSF molecule produced by *Xf*. In order to address this puzzling observation, *Xf rpfF* was subsequently introduced to this strain. Upon addition of *Xf rpfF* the *engXCA* promoter activity decreased to background level and was similar to that of the strain harboring *Xf rpfG* only, suggesting that it represses the RpfC-RpfG interaction. Addition of 0.1-100 µM 2-*Z*-tetradecenoic acid, de-repressed RpfC-RpfG interaction allowing *gfp* to be expressed in a dose-response manner (**Figure 13**).

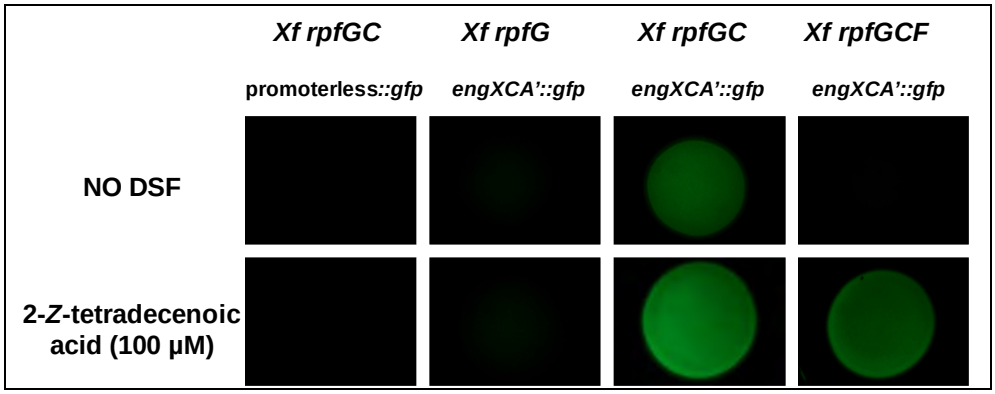

Figure 13. GFP fluorescence exhibited by a mutant strain of *Xcc* lacking its own *rpf C* and *rpfF* genes but into which the various *rpf* genes from *Xf* noted on the top of each column were added. GFP fluorescence in the presence or absence of added DSF were then visualized. Note that this biosensor yields gfp fluorescence specifically in the presence of DSF when the *XF rpfG, rpfC* and *rpfF* genes have been added.

The DSF production activity of *Xf* RpfF in this strain was tested - yielding no observed DSF production. Assuming that *Xf* RpfC, like *Xcc* RpfC, represses DSF production, the DSF production activity of *Xf* RpfF was further tested in the absence of *Xf* RpfC or both *Xf* RpfG and RpfC. In both cases, activity was restored suggesting that *Xf* RpfC interact with RpfF and controls its DSF production activity.

A second *Xcc*-based *Xf* DSF sensor was constructed that is composed of an *Xcc rpfF* and *rpfC* double mutant into which *Xf rpfF* and *rpfG* and a hybrid *rpfC* allele composed of the predicted trans-membrane domain of *Xf* RpfC and the cytoplasm domain of the *Xcc* RpfC has been added. The *eng'::gfp* transcriptional fusion in *Xcc* that was described above was inactive in the absence of DSF but was strongly induced by ≥0.1 µM 2-*Z*- tetradecenoic acid. We compared the activity of these two *Xf*-DSF specific biosensors to the activity of our original *Xcc*-DSF specific biosensor (*Xcc rpfF* mutant harboring an *eng'::gfp* reporting fusion) in response to diluted DSF extracts obtained from *Xcc* and from *Xf*. We named the three different sensors based on their DSF sensing element, *Xcc*-rpfC, *Xf*-RpfC and *Xf-Xcc* chimeric RpfC. While both of the *Xf*-DSF specific biosensors (*Xf*-RpfC and *Xf-Xcc*-RpfC) did not respond at all to the diluted DSF extract obtained from *Xcc* culture, the *Xcc*-DSF specific strain (*Xcc*-RpfC) exhibited a clear dose-dependent behavior to an elevated extract strength (**Figure 14** left). When exposed to DSF extract obtained from *Xf* cultures, *Xf*-RpfC and *Xf-Xcc*-RpfC clearly responded to increased levels of *Xf* DSF while the *Xcc*-RpfC biosensor barely responded to the lowest dilution tested (**Figure 14** right).

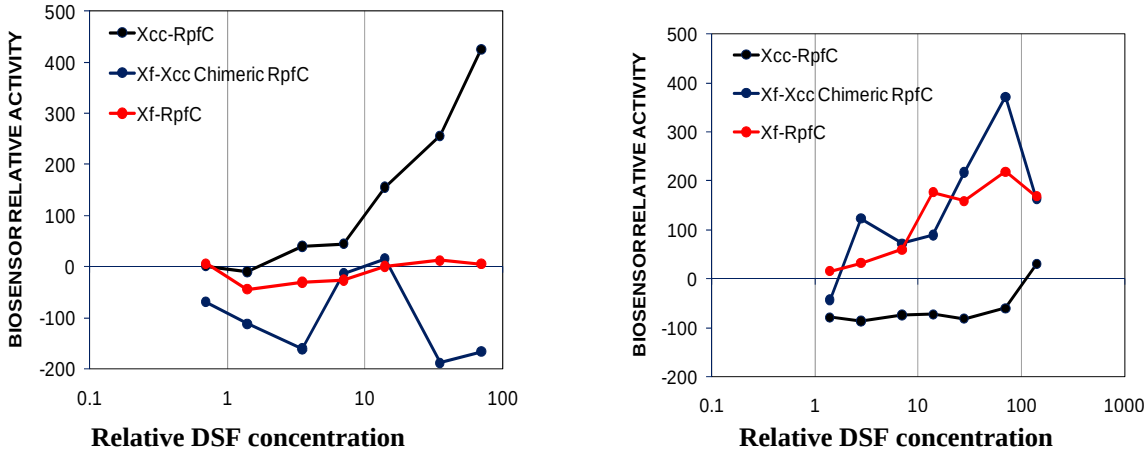

Figure 14. GFP fluorescence of the *Xf* DSF specific biosensors (red and dark blue) of the *Xcc*-specific DSF biosensor (black) to increasing concentrations of DSF from culture extracts of *Xcc* (left panel) or from *Xf* (right panel).

To test the specificity of the DSF biosensors we compared the activity of those sensors to a panel of pure DSF analogues. Inspection of the intensity of the respond (GFP level) by fluorescence microscopy revealed that *Xcc*-RpfC is activated in a stronger manner by DSF-like molecules with shorter acyl chain lengths (C11-*cis*, C12-*cis* and C13-*cis*) than by DSF homologs with either shorter or longer acyl chain lengths (**Figure 15**). In contrast, the *Xf* DSF-specific biosensors *Xf*-RpfC and *Xf*-Xcc-RpfC responded more strongly to C13-*cis* and C14-*cis* then to shorter chain fatty acids (**Figure 17**). Given that C14-cis is produced by *Xf* it appears that the RpfC-based biosensors respond more strongly to the DSF molecule to which the cell has evolved to respond. Thus the two *Xf* DSF specific DSF biosensors, particularly the *Xf-Xcc* RpfC biosensor is much more responsive to C14-cis, and hence will be far more useful that the original *Xcc* RpfC biosensor for assessing DSF levels in plants and bacterial cultures.

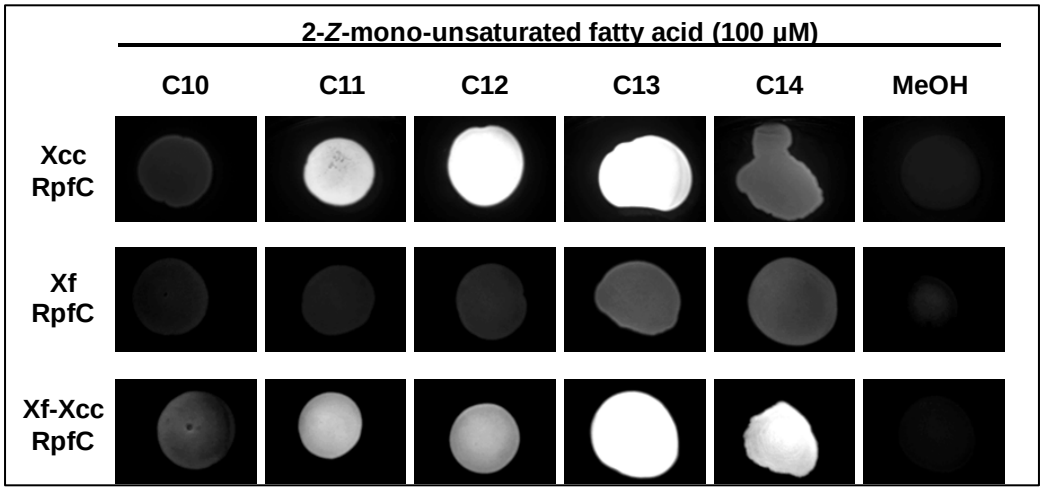

Figure 15. GFP fluorescence exhibited by the *Xcc*-based biosensors listed on each row when grown in culture media to which 100 uM of the DSF-like compounds with different acyl chain lengths shown in each column.

Identification of additional DSF molecules. Since we were successful in developing a DSF biosensor that is much more responsive to the DSF produced by *Xf* (compared to the original DSF biosensor that was much more responsive to *Xcc* DSF than to *Xf* DSF) we have continued studies to determine the structures of the various molecules made by *Xf* that are involved in rpfF-mediated signaling. It is clear that *Xf* produces C14-cis and that this molecule is active in regulating the behavior of *Xf*. As shown in **Figure 9** preliminary evidence has been obtained, that like *Xcc*, *Xf* may produce more than one related signal molecule. That is, DSF may not be a single molecule, but instead may be a family of closely related molecules. We expect that the molecules will be closely related to each other structurally, but they might have different effects on the cell. The *Xf*-RpfC sensor was used to quantify biologically active fractions of crude extracts of *Xf* cultures of a wild type strain (500-series of fractions) and from an *Xf rpfC* mutant (900-series of fractions) that were separated on the basis of polarity by HPLC. Fractions No. 504 and 901 (not assayed here) had previously been shown to contain C14-cis and thus perhaps other fatty acids and therefore, were sub-fractionated here. In both extracts, a more polar fraction (fractions 507 and 905) was found to contain an active compound as assayed by the *Xf-Xcc* RpfC biosensor. Fraction 507 was further fractionated and its sub-fraction 2.2 was found to be active (**Figure 16**). Thus more than one molecule besides C14-cis with biological activity appears to be active in both extracts. Work is underway to chemically characterize these additional biologically active compounds.

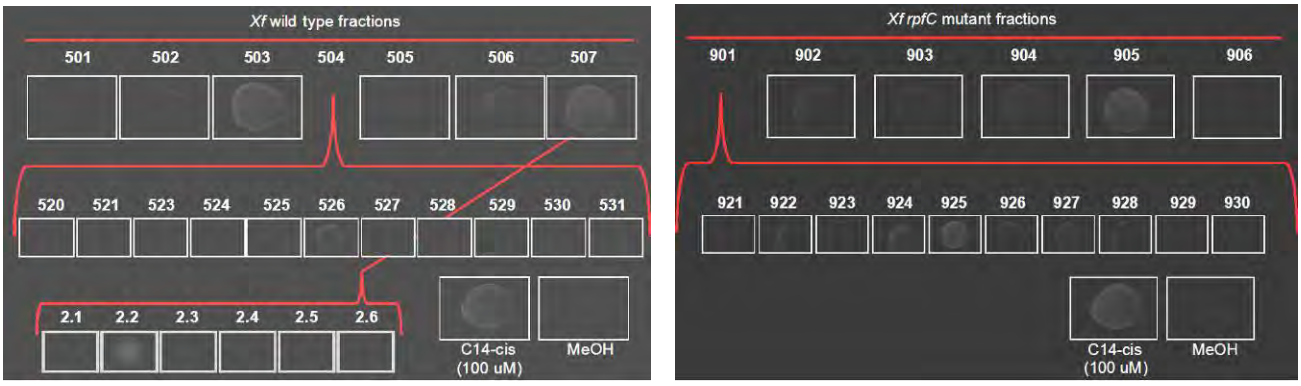

Figure 16. GFP fluorescence detected in various fractions of an ethyl acetate extract of a WT *Xf* culture (left panel) or of a culture of an RpfC mutant (right panel). Sub-fractionation of active fractions is noted with the brackets.

Development of reporter gene systems for us in *Xf*. The study of the response of *Xf* to DSF as well as other studies of its response to other plant compounds etc. would be greatly enhanced by the ability to easily monitor gene expression via the use of reporter genes. Previous attempts to establish *gfp* or *inaZ*-based transcriptional fusions in *Xf* failed, presumably due to its incapability to express foreign genes properly (a phenomena though to be related to *Xf* peculiar genetic codon usage). We have now successfully developed a reporter gene system by utilizing *Xf* endogenous *phoA* gene (encoding alkaline phosphatase) as a reporter gene. *Xf phoA* was cloned under the control of *E. coli lacZ* promoter and its activity was

confirmed in *E. coli*. Six different promoter::*phoA* transcriptional fusions were established, each harboring an *Xf* promoter of a gene associated with virulence (*hxfA*, *hxfB*, *pglA*, *pilB* and *rpfF*) and as a control, the 16S-rRNA gene promoter (**Figure 17**). Three of these constructs, cloned in the broad host range vector pBBR1MCS-5 were transformed to an *Xf phoA* mutant along with the promoterless *phoA* vector that serves here as a negative control. *Xf* PhoA relative level in these four strains (*Xf* Δ*phoA* harboring *hxfA'::phoA*, *hxfB'::phoA*, 16S-rRNA'*::phoA* or promoterless*::phoA*) which reflects promoter activity, was determined following four days of growth on PWG plates supplemented with 15 ug/ml gentamicin (to force maintenance of the plasmids). Activity of the promoterless*::phoA* construct was subtracted from the activity of the promoter*::phoA* fusions. As expected, induction of the 16S-rRNA promoter was always the strongest; *hxfA* promoter induction was ca. 20-fold higher than that of *hxfB* but ca. 10-folds lower than that of the 16S-rRNA promoter (**Figure 18**). These results suggest that the *phoA* reporter gene system will be quite valuable for assessing gene expression in vitro.

Figure 17. Expression vector harboring *phoA* from *Xf* that is fused to promoters from several virulence genes in *Xf*.

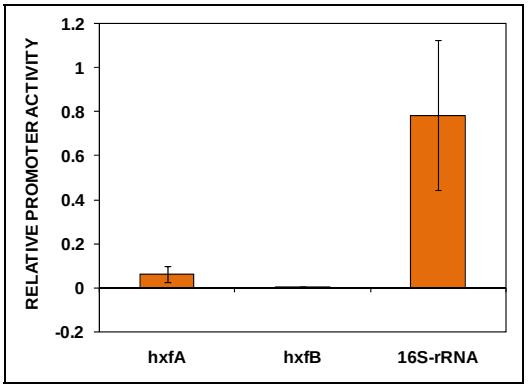

Figure 18. PhoA activity measured in *Xf* harboring fusions of a promoterless *phoA* reporter gene with the promoters of the genes noted on the abscissa.

We have made much effort in this reporting period to developing methods to use *Xf* itself to detect DSF**.** Among the several genes that we know to be most strongly regulated by DSF include *pil* genes involved in twitching motility, several genes such as *fimA* and *hxfA* and *HxfB* which are involved in cell-surface adhesion, and gum genes involved in production of EPS. We thus have examined the phenotypes of an *rpfF*- mutant and WT strain of *Xf* exposed to different amounts of DSF to determine if it can be used to bioassay for the presence of DSF. Initial results are encouraging. For example, cells of the *rpfF*- mutant which are not adherent, and thus which do not form cell-cell aggregations became much more adherent to each other when DSF was added to shaken broth cultures. The increased adherence is readily visualized as an enhanced ring of cell-cell aggregates that forms at the liquid-air interface of shaken cultures (**Figure 19**). Thus it appears that we may be able to assess the concentration of DSF in samples using a cell adhesion assay using *Xf* cells, although both assays are time consuming and somewhat qualitative.

In addition to estimating the transcriptional expression of genes known to be regulated in response to accumulation of DSF, we are also exploring ways of measuring the amounts of gene products (proteins) or EPS that are made in response to the presence of DSF. For example, we are exploring whether we can detect EPS production by *Xf* both in culture and in plants

by use of antibodies that recognize the EPS of *Xf*. Such antibodies have recently been described by the group of Bruce Kirkpatrick. Our initial results suggest that DSF-deficient RpfF- mutants of *Xf* exhibit little or no EPS production as monitored by use of fluorescently-labeled antibodies directed against EPS. A gfp-marked RpfF- strain of *Xf* could be used as a DSF detector both in culture and *in planta* by examining co-localization of constitutive GFP fluorescence and red fluorescence when a red-fluorophore-labeled anti-EPS antibody is applied to a sample; GFP fluorescent cells that were not also labeled with the antibody stain would indicate lack of DSF availability while cells that were both GFP and red fluorescent would indicate the presence of DSF.

We have also explored the use of immunofluorescence to detect other DSF-regulated proteins in *Xf*. Antibodies to XadA were provided by Dr. Alessandra Souza who had developed this tool to detect a homologous protein in CVC strains of *Xf*. The antibodies cross-reacted strongly to the XadA from grape strains of *Xf*. In preliminary experiments we find that cells of an *rpfF* mutant of *Xf* harbor very little XadA when grown on XFM minimal medium without added DSF, but that significant amounts of XadA is detected with the antibody when DFS-containing extracts from an rpfC mutant of *Xf* were added (**Figure 19**). These are very exciting results in that it suggests that such a biosensor would be very useful within plants to monitor the temporal and spatial patterns of DSF production within plants, as well as allow us to monitor the dispersal and stability of DSF that has been applied to plants, or of DSF which has been produced by transgenic plants themselves. We also observed a very curious effect of DSF on XadA; while some of this protein is cell-associated, a much lower proportion of this protein is released to the outside of the cell when cells are exposed to DSF (**Figure 20**). Quite importantly, the proportion of XadA that is retained by the cell is strongly influenced by the amount of DSF that the cell is exposed to. Addition of DSF from an extract of a DSF-producing strain of *Xf* reduced its secreted portion in both the wild type and the *rpfF* mutant (**Figure 20**). The DSF-dependent retention of XadA is being exploited as a measure of DSF content of samples in further analysis of DSF analogs as well as further reexamination of related DSF molecules that are being made by *Xf* and other bacteria.

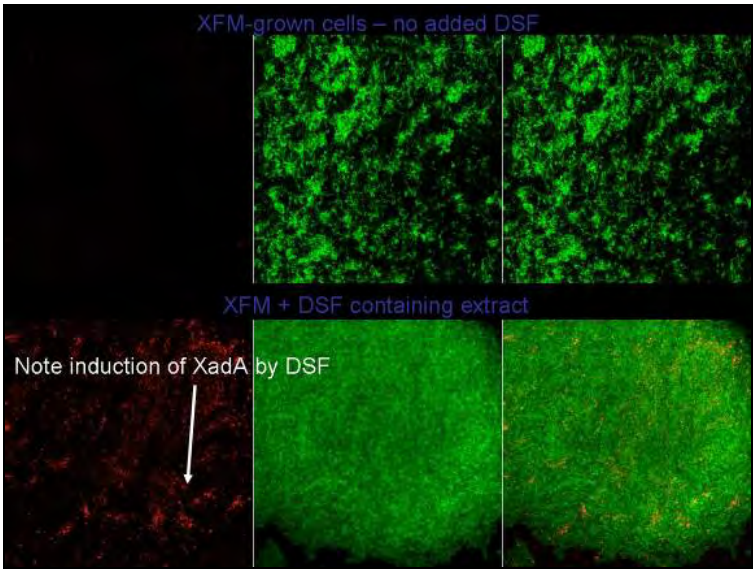

Figure 19 Cells of rpfF- mutant of *Xf* grown on a minimal medium and then stained with Syto-9 (green) and probed with rhodamine-labeled anti-XadA antibody (red). Cells were grown in XFM minimal medium without added DSF extract (top) or with added DSF-containing cell culture extract (Bottom).

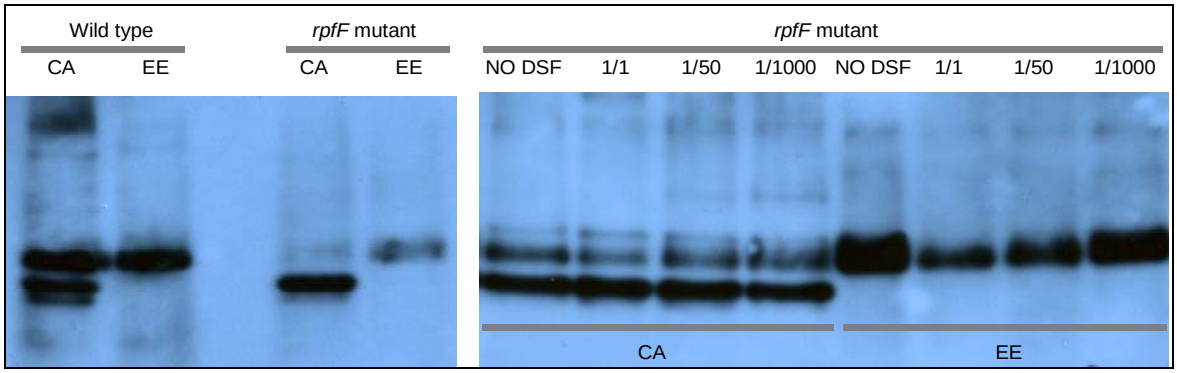

Figure 20: Left panel: Expression of XadA in wild type and rpfF mutant (CA = Cell-Associated; EE = Extracellular Environment). Right panel: Effect of DSF-crude extract (1/1 = 1 % strength [200 µl / 20 ml], 1/50 = diluted by 50 and 1/1000 = diluted by 1000) on XadA localization to the extracellular environment in *Xf rpfF* mutant. A non-specific band appears under the XadA band and can be seen only in the CA samples.

CONCLUSIONS

Since we have shown that DSF accumulation within plants is a major signal used by *Xf* to change its gene expression patterns and since DFS-mediated changes all lead to a reduction in virulence in this pathogen we have shown proof of principle that disease control can be achieved by a process of "pathogen confusion". Several methods of altering DSF levels in plants, including direct introduction of DSF producing bacteria into plants, and direct application of DSF itself to plants appear promising as means to reduce PD. Transgenic DSF-producing plants appear particularly promising and studies indicate that such plants provide at least partial protection when serving as a rootstock instead of a scion. Based on work done on other plant species in which a chloroplast-targeted DFS synthase has provided much higher levels of DSF production, we are hopeful that such a construct in grape will provide even higher levels of PD control in our current studies. While the principle of disease control by altering DSF levels has been demonstrated, this work addresses the feasibility of how achieve this goal, and what are the most practical means to achieve disease control by pathogen confusion. Our continuing work will address which method is both most practical and efficacious. The tools we have developed to better detect the specific DSF molecules made by *Xf* will be very useful in our on-going research to test the most efficacious and practical means to alter DSF levels in plants to achieve disease control. These tools are being used to screen for more efficacious naturally-occurring DSF producing bacterial endophytes, as well as to identify chemical compounds that more strongly induce changes in behavior of *Xf*.

FUNDING AGENCIES:

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NEW *XYLELLA FASTIDIOSA* GENOMES FROM TEXAS

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ABSTRACT
In this report we present the final completion of the first *Xylella fastidiosa* (*Xf*) genome from Texas, *Xf* grape strain GB514. Preliminary report of the genome was presented at the Southwestern Entomology meetings in spring 2010, but the genome has now been uploaded to NCBI. We present the accession numbers and initial findings as we begin to compare this genome to two California *Xf* grape strains. Not surprisingly, GB514 shows strong homology to the California grape strain of Xf (M23 and Temecula). GB514 also includes a plasmid which contains genes for a completely functional Type IV secretion system and shows 94% query coverage with a plasmid recently published from a *Xf* mulberry strain. The mulberry strain plasmid (and ours) shows alignment to a plasmid from the earthworm symbiont *Verminephrobacter eiseniae*. We report differences between the California *Xf* grape genomes and this Texas grape strain for the *zonula occludens toxin* (*zot*) gene (a possible virulence gene). In addition to analyzing this genome, we report on our planned sequence of a second Texas *Xf* isolate called MM067-2 from Pecan. According to preliminary genome comparison (MLST) this isolate is from an "oak" type of the *Xf multiplex subsp*. This "oak" type has been isolated from multiple states and no oak isolates of *Xf* have yet been sequenced.

LAYPERSON SUMMARY
Comparison of *Xylella fastidiosa* (*Xf*) genomes is a powerful tool to search for virulence genes and to map the evolutionary history of an organism. Instead of comparing strains one gene at a time, genome comparisons allow scientists to quickly identify where strains are different. These differences likely hold the key as to why some isolates are pathogenic in some plants and not others. Genome studies also allow us to search for better sequences to use in detection (making detection more efficient or strain specific). We have sequenced a *Xf* grape strain from Texas and uploaded the genome (and plasmid) to the NCBI (national genomic database) for all to use. This is the first *Xf* genome from outside California (with the exception of the first *Xf* genome which was done in Brazil). We have uncovered a few distinctions between this Texas grape strain and those in California. We have also begun sequencing a second genome from Texas. This second genome is from pecan and preliminarily looks like an 'oak' type. This will be the first 'oak' type of *Xf* sequenced and will likely present useful information about the natural history of *Xf* in North America.

INTRODUCTION
Xylella fastidiosa (*Xf*) Wells *et al.* is the gram-negative plant pathogen which causes Pierce's disease in grapevines, as well as diseases in other plants such as almonds, citrus and oleander. Over the last decade a total of six *Xf* genomes have been sequenced including the citrus variegated chlorosis strain 9a5c from Brazil (Simpson et al. 2000), Temecula grape strain (Van Sluys et al. 2003), the California oleander strain Ann-1 and almond leaf scorch strain Dixon (Copeland et al. 2002a, b) and two additional *Xf* isolates form California, a grape strain M23 and an almond strain M12 (Chen et al. 2007). The analysis of genomes of *Xf* outside of California is essential for us to address questions about the natural history of *Xf* in North America. Preliminary analysis suggested that the grape strains in Texas and California were highly similar (Yuan et al. 2007), but the whole genome comparison is required to search for small but potentially significant differences in the genomes. We have also begun sequencing an additional *Xf* isolate from pecan.

OBJECTIVES
1. Sequence and upload the genome from a Texas grape strain of *Xf* (*Xylella fastidiosa subsp. fastidiosa*).
2. Compare the Texas grape strains to the two grape *Xf* genomes which have been done in California.
3. Report on the new Texas *Xf* genome sequencing project underway.

RESULTS AND DISCUSSION

We have sequenced aTexas grape strain of Xf (GB514) using whole genome shotgun titanium pyrosequencing with 25X coverage. The project revealed two primary contigs, a main chromosome of 2.49 megabases and a possible low copy plasmid. Accession numbers for this project are **CP002165** for the main chromosome and **CP002166** for the plasmid. Not surprisingly, the main chromosome of GB514 shows strong homology to the California grape strain of *Xf* (M23 and Temecula). However, comparison of grape *Xf* genomes in Texas and California has and will likely continue to uncover small differences which can help us address questions about the natural history of *Xf* in North America. For example, the California grape strains each have two versions of a potential virulence factor called the *zonula occludens toxin* (*zot*) gene (da Silva et al. 2007). There are two copies of *zot1* and one copy of *zot2* in California grape strains whereas GB514 contains only one version (one copy zot2 and no zot1). Did GB514 lose its copy of zot1 or did zot1 evolve only among the California *Xf* grape strains?

The plasmid of *Xf* GB514 codes for 39 functional genes of which 11 are for proteins used in the Type IV secretion system of gram negative bacteria, virulence factors involved in the injection of DNA or substrates into host plants (Cascales and Christie 2003). According to Cascales and Chirsite (2003) and Christie (personal communication) these genes are sufficient for a completely functional Type IV secretion system. What role might this plasmid play in virulence of this particular isolate? Of additional interest, the plasmid from grape strain of Xf GB514 shows 94% query coverage with a plasmid recently published from a *Xf* mulberry strain (Stenger et al. 2010). This plasmid (and our plasmid) shows strong homology with a plasmid from a bacterial symbiont *Verminephrobacter eiseniae* of earthworms. We thought this alignment unusual (and perhaps unlikely) until we read about the published finding of Stenger and colleagues (2010). Does this plasmid may have some broad host range within environmental bacteria? Is this plasmid frequent in *Xf* isolates regardless of strain or host?

In addition to analyzing the GB514 genome, we are currently underway with our second Texas *Xf* genome sequencing project. We plan to sequence the *Xf* isolated called MM067-2. This isolate was isolated from the host of pecan in Texas. This "oak" type has been isolated from multiple states and no oak isolates of *Xf* have yet been sequenced. Preliminary analysis of this isolate using MLST (Schuenzel et al. 2005), suggests it is a multiplex isolate (*Xf subs. multiplex*) of the "oak" type.

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TOOLS TO IDENTIFY POLYGALACTURONASE-INHIBITING PROTEINS TRANSMITTED ACROSS GRAPEVINE GRAFTS

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ABSTRACT
The CDFA Pierce's Disease and Glassy-winged Sharpshooter Board's Research Scientific Advisory Panel review in 2007 and subsequent RFPs have given top priority to delivery of Pierce's disease (PD) control candidates, including polygalacturonase-inhibiting proteins (PGIPs), from grafted rootstocks. Four currently funded projects – two scientific research projects and two field trials of transgenic PD control lines – use PGIPs as a control strategy to limit the spread of *Xylella fastidiosa (Xf)* in the xylem network and thereby limit PD symptom progression in infected vines. A monoclonal antibody to the pear fruit PGIP, the protein expressed by the aforementioned grape lines, is being developed to detect, quantify, and observe the localization of the pear PGIP in transformed grapevines and grafted vines with transformed rootstocks. Pear PGIP is being isolated from previously transformed *Arabidopsis thaliana* plants and from mature green 'Bartlett' pear fruit tissue. Monoclonal antibody production by Antibodies, Inc. will begin once sufficient quantities of properly glycosylated, active PGIP have been purified.

LAYPERSON SUMMARY
Xylella fastidiosa (Xf) utilizes a key enzyme, polygalacturonase (*Xf*PG), to spread from one xylem vessel to the next, eventually leading to the development of Pierce's disease (PD) symptoms in infected vines. Plant proteins called PG-inhibiting proteins (PGIPs) selectively inhibit PGs from bacteria, fungi, and insets. Our collective work has identified a PGIP from pear fruits as partially inhibiting PD symptom development in grapevines expressing the pear fruit PGIP. Current projects, including field trial evaluations, require a monoclonal antibody specifically recognizing the pear fruit PGIP to detect, quantify, and characterize the protein's role in *Xf*PG inhibition in transformed grapevines. We are currently purifying active pear PGIP from two plant sources for commercial antibody production.

INTRODUCTION
Pierce's disease (PD) incidence has been associated in several studies with the spread of the causal agent, *Xylella fastidiosa* (*Xf*), throughout the xylem vasculature of infected grapevines. The spread of bacteria from one vessel to the next utilizes bacterial cell wall modifying enzymes to degrade the pit membranes separating adjacent vessels (Pérez-Donoso et al., 2010). One such enzyme, a polygalacturonase (XfPG), has been well characterized and is a PD virulence factor (Roper et al., 2007). Several previous projects have analyzed the effectiveness of PG-inhibiting proteins (PGIPs) in minimizing the detrimental effects of pathogen and pest attack on various plants. Two currently funded projects both use pear fruit PGIP (pPGIP) to restrict Xf movement: "Optimizing grape rootstock production and export of inhibitors of *X. fastidiosa* PG activity" (PI Labavitch) and "*In planta* testing of signal peptides and antimicrobial proteins for rapid clearance of *Xylella*" (PI Dandekar).

This project was developed to generate a monoclonal antibody that selectively recognizes the pear fruit pPGIP protein. The monoclonal antibody is a necessary tool for both aforementioned research projects and the related project "Field evaluation of grafted grape lines expressing PGIPs" (PI Powell) and will allow for detection and quantification of pPGIP without cross-reactive interference from the native PGIP. Plants can therefore be more efficiently screened for the presence of the pPGIP protein, whether directly produced in, or transported to the plant tissue of interest.

OBJECTIVES
1. Using existing plants expressing histidine-tagged pPGIP and fresh pear flesh, prepare pPGIP protein and provide it to Antibodies, Inc. to develop mouse hybridoma lines expressing monoclonal antibodies against the pear PGIP.
2. Calibrate the antibodies produced by the hybridoma clones to determine effective dilutions for use in detecting the pPGIP protein.
3. Use the antibody to detect transgenic pear PGIP in xylem sap of own-rooted and grafted grapevines.

RESULTS AND DISCUSSION
Objective 1: Purification of pear PGIP from transgenic Arabidopsis leaves and pear fruit.
The generation of a monoclonal antibody requires purified protein to be used as the antigen. PGIPs are heavily glycosylated plant cell wall proteins that require certain glycosylation levels for activity (Powell et al., 2000). The project "Optimizing grape rootstock production of and export of inhibitors of *X. fastidiosa* PG activity" (Labavitch, 2008) generated transgenic *Arabidopsis thaliana* plants expressing the pPGIP protein fused to a C-terminal histidine tag for purification. Rosette leaves

from these transgenic plants were frozen in liquid nitrogen and ground with a mortar and pestle. The resulting powder was mixed with a binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) and centrifuged at 500 x g to remove the leaf debris. The supernatant was mixed with an equal volume of immobilized nickel-sepharose matrix for separation and purification of the histidine tagged pPGIP from the crude extract. The matrix-pPGIP slurry was washed with several volumes of binding buffer and bound protein was eluted with several volumes of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). A minimal amount of total protein was found in the eluate after centrifugation and removal of the matrix, as determined by Bradford assays. Work to refine the transgenic protein purification process is ongoing.

In addition to obtaining pPGIP from the *Arabidopsis* protein expression system, pPGIP extraction from fresh pear fruit flesh is underway. pPGIP was purified from mature green 'Bartlett' pears according to Stotz et al. (1993) with modifications. Peeled, cored, and sliced pears (2 kg) were homogenized in 2 L of extraction buffer (1 M sodium acetate, pH 6, 1 M NaCl, 1 % [w/v] polyvinylpolypyrrolidone, 0.2% [w/v] sodium bisulfite). The homogenate was stirred at 4°C for 1 hour then filtered through three layers of Miracloth with manual pressure. The liquid fraction was centrifuged (10,000 rpm, 20 min, 4°C) and the supernatant collected. The pellet fraction and Miracloth rententate were combined and resuspended in 1 L extraction buffer, stirring 1 hour at 4°C. In some aliquots, the initial homogenate was degassed prior to centrifugation, eliminating the need for the Miracloth filtration. After centrifugation, the supernatants were combined. Total protein precipitating between 50% and 100% ammonium sulfate saturation was collected and resuspended in 100 mM sodium acetate, pH 6, and extensively dialyzed at 4°C against 10 mM sodium acetate, pH 6, using 6000-8000 molecular weight cut-off membranes.

The dialyzed ammonium sulfate fraction was mixed with an equal volume of 2x ConA buffer (200 mM sodium acetate, pH 6, 2 M NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM $MnCl_2$). A small volume was applied to a Concanavalin A-sepharose column which was then washed with several volumes of 1x ConA buffer. Bound protein was eluted with 250 mM methyl-α-D-mannopyranoside, followed by 1 M methyl-α-D-mannopyranoside. The initial column chromatography purification has not separated the glycosylated pPGIP protein from the dialyzed fraction, potentially due to improper binding conditions. Current efforts center on concentrating pPGIP protein and optimizing the column chromatography protocol.

pPGIP activity was measured throughout purification by radial diffusion assays (Taylor and Secor, 1988). Samples of the pear homogenate were able to fully inhibit a PG (BcPG) mixture from *Botrytis cinerea* (B05.10) culture filtrates. For BcPG preparation, fungal cultures were grown for 12 days in total darkness in 250 mL modified Pratt's medium, supplemented with 1 g/L Difco yeast extract and 3 g/L citrus pectin, inoculated with $1x10^5$ spores (Fergus, 1952). The fungus and media were filtered through 11 µm cellulose filters then 1.2 µm glass microfiber filters to remove any residual fungus and concentrated by dialysis against polyethylene glycol through 6000-8000 molecular weight cut-off membranes. The pPGIP purification preparations resulted in 66% and 59% reductions in BcPG activity after ammonium sulfate precipitation and subsequent dialysis, respectively.

CONCLUSIONS

Tagged pPGIP protein is being isolated from *Arabidopsis thaliana*. Concurrently, native pPGIP is being purified from 'Bartlett' pear fruits. pPGIP activity against *B. cinerea* PG has been maintained in all purification steps. Purified pPGIP protein will be delivered to Antibodies, Inc. for monoclonal antibody production.

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THE ROLE OF LIPOPOLYSACCHARIDE IN VIRULENCE, BIOFILM FORMATION, AND HOST SPECIFICITY OF *XYLELLA FASTIDIOSA*

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ABSTRACT
This project aims to further elucidate the molecular mechanisms *Xylella fastidiosa* (*Xf*) employs during the infection of its host. We are focusing on the lipopolysaccharide (LPS) component of the outer membrane of *Xf*. LPS contains a conserved lipid A and core portion and a variable O-antigen portion. In particular, we are examining the variable portion of the LPS molecule, the O-antigen. O-antigen as been implicated in virulence in many bacterial species and we hypothesize it may also be involved in *Xf* virulence. More specifically, we are investigating if this particular portion of the LPS molecule contributes to *Xf* surface attachment and biofilm formation, two critical steps for successful infection of the xylem of the host. Additionally, we will determine if LPS contributes to the high level of host specificity observed for this pathogen.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) is a bacterium that has the ability to infect and colonize many different plant species, causing significant damage in some. In grapevine, this disease is known as Pierce's disease (PD), which has caused major losses to the California grape industry. *Xf* also infects other economically important crops such as almond, oleander and citrus. Interestingly, while all *Xf* isolates belong to the same group or species, some isolates can cause disease in one host but not another. For example, oleander isolates cannot cause disease in grapevine and vice versa. One major goal of this project is to understand the bacterial mechanisms that dictate this high level of host specificity. This research project is specifically focused on understanding the role of the *Xf* cell surface component lipopolysaccharide (LPS) in the pathogenic interaction between the grapevine, almond, and oleander hosts. LPS plays an important role in virulence for many bacterial pathogens. We are investigating the involvement of LPS in *Xf* colonization of its host and other key aspects of the disease process, like attachment to the plant cell wall. Most importantly, should LPS prove to be an important factor during *Xf* plant infection, its abundance on the bacterial cell surface makes it a logical target for disease control. Furthermore, antimicrobial compounds exist that disrupt LPS synthesis or weaken the LPS layer, making the bacterium more sensitive to other stresses. Therefore, compounds targeted towards LPS synthesis could increase the efficacy of other anti-*Xf* compounds currently being developed by other researchers when both are used in conjunction.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a gram negative, xylem-limited bacterium with a broad host range. *Xf* causes disease in economically important hosts such as grape, almond, citrus, coffee, peach, plum and alfalfa as well as several tree and ornamental hosts such as oleander. Additionally, *Xf* colonizes many plant species that never develop any visible symptoms or stresses. (Hopkins and Purcell, 2002). The molecular mechanisms that determine this host specificity are poorly understood. We are currently exploring the role of lipopolysaccharides (LPS) as both a virulence factor and host specificity determinant of *Xf*. We are focusing on the O-antigen portion of the LPS molecule in 3 isolates of *Xf* that colonize different hosts: Fetzer, a Pierce's Disease (PD) isolate; Dixon, an almond leaf scorch (ALS) isolate; and Ann-1, an oleander leaf scorch (OLS) isolate. While the grape and almond isolates are considered to be separate subspecies or pathovars, both ALS and PD isolates can cause disease in grapevine (Almeida and Purcell, 2003). However, *Xf* isolated from grapevines cannot cause disease in almonds indicating a fundamental difference between ALS and PD isolates. Furthermore, the oleander strain cannot infect grape or almond and both the almond and grape strains cannot infect oleander. This provides an opportunity to study the role of the O-antigen moiety of the LPS molecule as a potential host specificity determinant for *Xf*.

LPS is primarily displayed on the cell surface, thereby mediating interactions between the bacterial cell and its environment by way of initial adhesion of the bacterial cell to a surface or host cell (Genevaux *et al*. 1999, Nesper *et al*. 2001). LPS (sometimes called "endotoxin") has also been implicated as a major virulence factor in animal and plant and pathogens, such as *Escherichia coli*, *Xanthomonas campestris* pv. *campestris*, and *Ralstonia solanacearum* (Muhldhorfer and Hacker 1994, Dow *et al*. 1995; Hendrick *et al*. 1984). Additionally, host perception of LPS is well documented and occurs in both plants and animals (Newman *et al*. 2000). The immune system can recognize several regions of the LPS structure and can mount a defense response against bacterial invasion based on this recognition. Bacteria can also circumvent the host's immune system by altering the structure of their LPS molecule or by masking it with capsular or exopolysaccharides.

LPS is composed of 3 parts: 1) lipid A, 2) core oligosaccharide and 3) O-antigen polysaccharide (see **Figure 1**). Lipid A is anchored in the membrane and the core oligosaccharides are attached on the preformed lipid A molecule. O-antigen is

assembled in the cytoplasm and subsequently ligated onto the core oligosaccharide-lipid A complex. Both lipid A and core oligosaccharide are relatively conserved among bacterial species while the O-antigen is highly variable even amongst strains of the same species, thus contributing to the serotype designation of different strains within the same species. To our knowledge, O-antigen is not required for bacterial viability but is often implicated in virulence and host specificity where even small changes in the type and order of the sugars comprising the O-antigen can result in major changes in virulence.

The lifestyle of *Xf* requires attachment to diverse carbohydrate-based substrates such as the plant xylem wall and chitin in the mouthparts and foregut of the sharpshooter insect vector. In both environments, *Xf* forms biofilms or biofilm-like structures. Previous studies show that *Xf* produces an extracellular exopolysaccharide (EPS) that is present in small quantities during initial surface attachment and early biofilm formation. However, in mature biofilms this EPS (termed fastidian gum) is a major component of the 3-dimensional *Xf* biofilm both *in vitro* and *in planta* (Roper *et al*. 2008). Other studies have demonstrated that proteinacous adhesins such as type I pili and hemagluttinins contribute to surface adhesion and cell-cell aggregation (Li *et al*. 2007, Guilhabert and Kirkpatrick, 2005). Because of the location and abundance of LPS in the outer membrane we hypothesize that LPS also plays a key role in mediating initial attachment to the carbohydrate substrates *Xf* encounters in the plant and insect as well as the development of a mature biofilm. To accomplish our objectives, we are utilizing mutants that are disrupted in the O-antigen biosynthesis pathway to better understand the role of LPS in virulence, host-specificity, and biofilm formation.

OBJECTIVES

1a. Characterization and comparison of the LPS profiles from the grape, almond and oleander strains of *Xf* .
1b. Investigate the possibility of phase variation in *Xf* LPS.
2. Construct *Xf* mutants in O-antigen biosynthetic genes.
3. Test virulence and host specificity of the O-antigen mutants *in planta.*
4. Test attachment and biofilm formation phenotypes of *Xf* O-antigen mutants.

RESULTS AND DISCUSSION

Objective 1a. Characterization and comparison of the LPS profiles from the grape, almond and oleander strains of Xf. LPS is a tripartite molecule consisting of lipid A, an oligosaccharide core, and O-antigen. LPS variants can be classified as "rough" (those lacking the O-antigen) and "smooth" (those with all three components). A bacterial species can possess both rough and smooth variants and this difference is easily discerned by electrophoretic analysis. Three experimental isolates: *Xf* Fetzer (grape), Dixon (almond), and Ann-1 (oleander) were grown in PW broth or on solid PW medium. Cells were harvested and LPS was extracted using the hot-phenol method described by Westphal and Jann (1965) followed by electrophoresis on sodium deoxycholate-PAGE and Tricine-SDS PAGE gels. Preliminary results suggest that LPS extracted from the grape strain Temecula 1 grown on solid media contains both rough and smooth forms. Sodium deoxycholate gels is used to visualize the general forms of LPS (i.e., rough vs. smooth), while Tricine-SDS PAGE gels provide enhanced resolution of individual LPS bands, allowing the visualization of subtle differences in LPS profiles between the three isolates, a method we are also using when characterizing the LPS mutants (Δ*waaL* and Δ*wzy*) generated in this study.
In other systems, it has been shown that the rate of LPS biosynthesis differs with respect to growth phase and that, generally, LPS is synthesized in greater quantities when bacterial populations have reached stationary phase. Therefore, we are currently determining detailed growth curves for the three *Xf* isolates to ensure that we harvest LPS from similar points in their growth phase (mid-log and stationary) before comparing LPS profiles. We have conducted detailed growth curves by enumerating cell density (OD_{600nm}) coupled with dilution plating and colony counts and found that for the Fetzer and Dixon isolates, exponential growth occurs between ~2-5 days of incubation and stationary phase is reached at ~7-8 days of incubation. Growth curves are still in progress for the slower growing Ann-1 isolate. At this time, both solid and liquid cultures are being utilized for the hot phenol LPS extraction and subsequent visualization by sodium deoxycholate-PAGE and Tricine-SDS PAGE analyses.

Objective 1b. Investigate the possibility of phase variation in Xf LPS. Phase variation is the process by which Gram-negative bacteria undergo changes in antigenic properties in response to shifts in environmental conditions. Such changes are documented to occur in the extracellular and membrane-bound polysaccharide portions of the cell surface, including exopolysaccharide, capsular polysaccharide, and LPS (Bergman *et al*. 2006; Lerouge and Vanderleyden, 2002). We expect that the O-antigen moiety of LPS may differ depending on the types of carbohydrate available. We are investigating the possibility of phase variation in *Xf* Fetzer LPS by examining if there is a change in the LPS profile in cells grown in different culture conditions: i) PW, ii) PW amended with grapevine xylem sap, and iii) grapevine xylem sap alone.
Xylem sap (springtime bleeding sap) from 15 year-old 'Cabernet Sauvignon' grapevines was kindly provided by Dr. Philippe Rolshausen (Dept. of Plant Pathology & Microbiology, UCR). The collected fluid was filter-sterilized and assayed for carbohydrate content. Analysis of carbohydrate content (Dygert *et al*. 1965) indicated a reducing sugar content of ~1mg per mL of xylem sap. This will provide us with a means of normalizing the xylem sap based on carbohydrate content before adding it to the PW growth medium and ensure that equivalent concentrations of xylem sap constituents are added to each culture tube. Characterized xylem sap was aliquoted into10 mL volumes and stored at -80 C. *Xf* cells were successfully grown in PD3 amended with either 50% or 90% xylem sap using a protocol previously described by Zaini *et al*. 2009. LPS has been isolated from these cultures and is awaiting electrophoretic characterization.

Objective 2. Construct mutants in Xf with altered LPS profiles.
Genes targeted for mutation in the LPS biosynthetic pathway encode proteins necessary for the completion of a fully functional O-antigen moiety. We have identified two genes (*waaL* and *wzy*) putatively involved in *Xf* O-antigen biosynthesis. Both genes occur in single copy in all three *Xf* genomes used in this study. *waaL* (PD0077) encodes an O-antigen ligase that is responsible for attaching the completed O-antigen onto the assembled lipid A/core component of the LPS. Mutations in the *waaL* homologue of *Escherichia coli* prevent the ligation of O-antigen (Perez *et al*. 2008). Therefore, we predict that mutation of *waaL* will result in *Xf* strains that lack O-antigen ("rough" LPS mutants) (**Figure 1**). The second gene of interest is *wzy* (PD0814). *wzy* encodes an O-antigen polymerase protein that plays a role in chain length determination of the O-antigen, prior to its ligation onto the core component of LPS. Mutations in *wzy* in *E. coli* and *Shigella flexneri* resulted in severely truncated O-antigen (Carter *et al*. 2007; Cheng *et al*. 2007; Daniels *et al*. 1998). We predict that a *wzy* deletion will result in *Xf* strains that carry a truncated O-antigen ("semi-rough" mutants) (**Figure 1**).

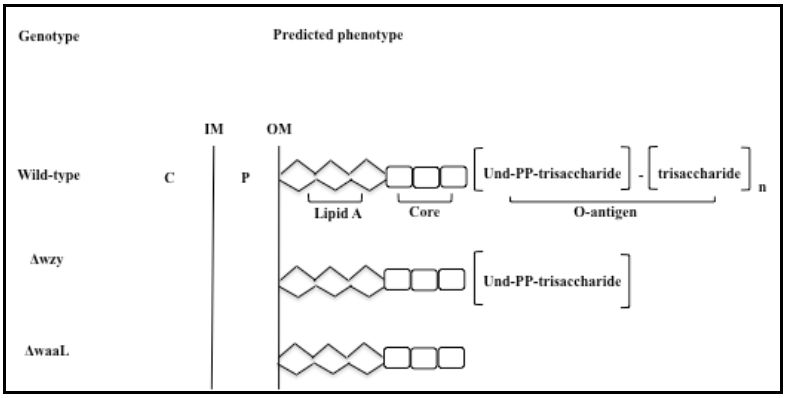

Figure 1. Models of a wild type *Xf* LPS molecule containing all three components (lipid A, core polysaccharide, and O-antigen), a hypothetical Δ*wzy Xf* LPS molecule with truncated O-antigen (i.e., consists only of undecaprenyl pyrophosphate linked to a single trisaccharide unit), and a hypothetical Δ*waaL Xf* LPS molecule completely devoid of O-antigen.
IM=Inner membrane; OM= Outer membrane; C= cytoplasm; P=periplasm

Prior to designing our mutant construction strategies, we conducted protein alignment and domain analyses using NCBI (www.ncbi.nlm.nih.gov), Wellcome Trust SangerInstitute Pfam (pfam.sanger.ac.uk), and Integrated Microbial Genomes (www.img.jgi.doe.gov/cgi-bin/pub/main.cgi) software. These analyses ensure that when creating the *waaL* and *wzy* mutants, we delete the proper catalytic domains of both the WaaL and Wzy proteins rendering them non-functional. The protein alignment results indicate that WaaL and Wzy are highly conserved among all the *Xf* strains used in this study (**Figure 2**). Additionally, protein domain analysis identified a Wzy_C catalytic domain in both WaaL and Wzy, providing further evidence that these proteins are involved in LPS biosynthesis (Wzy_C domain is enclosed in the box in **Figure 2**A and 2B). Wzy_C domains are found in the family of proteins containing O-antigen ligase (including the well characterized *E. coli* O-antigen ligase, RfaL). This domain contains the necessary amino acid residues for O-antigen ligase activity (Perez *et al*. 2008). There is considerable variation in primary amino acid sequence among O-antigen ligases and O-antigen polymerases, even between closely related bacterial species (Raetz *et al*. 2007; Schnaitman and Klena, 1993). When analyzed by blastx, WaaL homologues in *Xf* Dixon and Ann-1 have 80 and 81% identity, respectively, compared to Temecula 1 PD0077. Similarly, Wzy homologues in *Xf* Dixon and Ann-1 have 80 and 79% identity compared to PD0814.

Furthermore, we analyzed the gene neighborhood surrounding the *waaL* and *wzy* genes in each of the 3 *Xf* genomes using the *Xf* Comparative Genome Project site (www.xylella.lncc.br) as well as Integrated Microbial Genomes (www.img.jgi.doe.gov/cgibin/ pub/main.cgi). Understanding the genomic context within which *waaL* and *wzy* lie provides further insight into the *Xf* LPS biosynthetic pathway. Not unexpectedly, these analyses demonstrated that both *waaL* and *wzy* are located in similar gene neighborhoods in all three strains. Function prediction for proteins encoded by genes neighboring *waaL* and *wzy* include those known to be important in LPS biosynthesis in enteric bacteria (Schnaitman and Klena, 1993). Representation of the genomic context of *waaL* and *wzy* is shown in **Figure 3**.

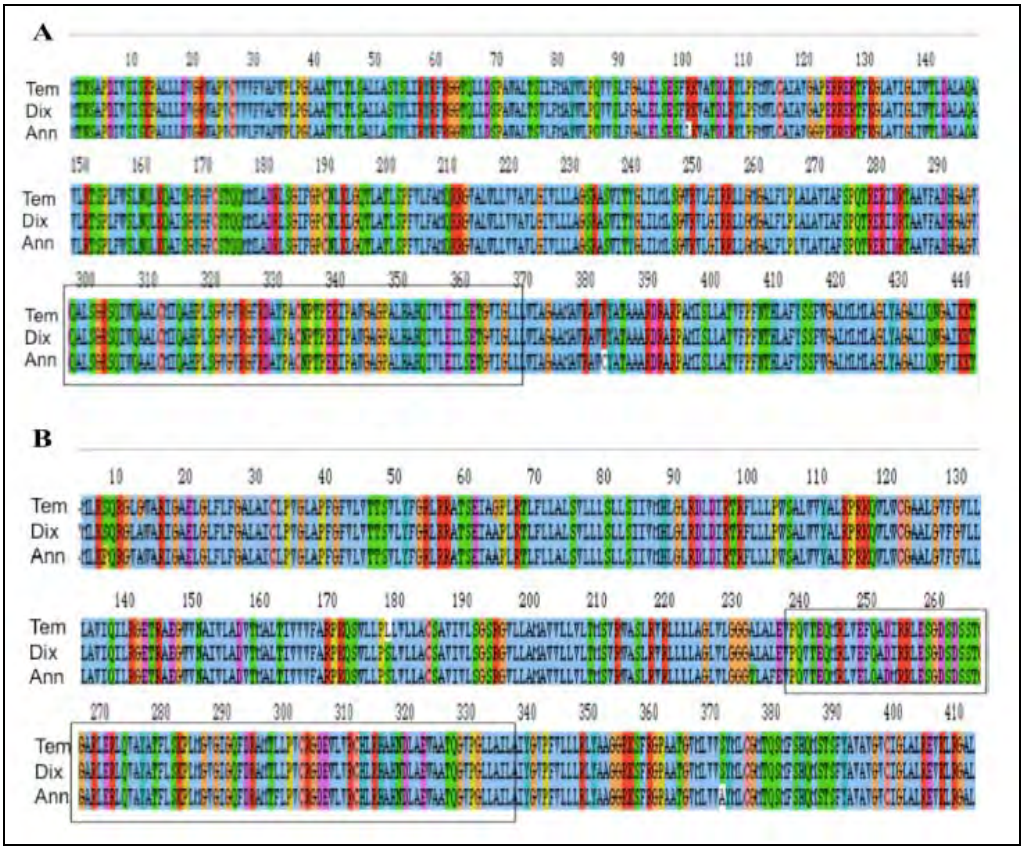

Figure 2. Protein Alignment and evidence for a Wzy_C domain in **A,** WaaL and **B,** Wzy of *Xf* strains Temecula1 (grape), Dixon (almond), and Ann-1 (oleander). The Wzy_C domain belongs to Pfam 04932, a family of proteins that includes the O- antigen ligases. The predicted Wzy-C domain is enclosed in the box in both A and B.

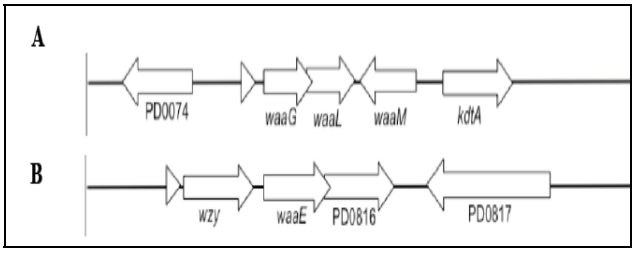

Figure 3. Representation of the genomic context of *waaL* and *wzy* in *Xf*. The gene arrangement within all 3 genomes of interest is highly similar. **A,** *waaL* lies within a cluster of several genes involved in LPS biosynthesis: *waaG* encodes glycerol transferase which transfers sugars to the core component; *waaM* encodes lauroyl acyltransferase, an enzyme responsible for transferring activated myristate or laurate to the lipid A moiety; *kdtA* encodes 3-deoxy-D-manno-octulosonic acid transferase, the enzyme that initiates the synthesis of core oligosaccharides to lipid A. **B,** *wzy* is located directly upstream of *waaE*, the gene that encodes a glycosyl transferase. PD0816 and PD0817 are currently annotated as genes belonging to protein families involved in teichoic acid and riboflavin biosynthesis, respectively.

A mutagenesis construct to make the *waaL* mutants in all three strains (due to high nucleotide sequence similarity between strains, the same construct can be used for all three strains) has been completed (**Figure 4**). Briefly, Fetzer *waaL* was PCR amplified and cloned into pCR8/GW/TOPO (Invitrogen). Restriction digest with *Age*I and *Eco*NI removed a 1383 bp fragment from the *waaL* amplified region and was replaced by the 1239 bp *Eco*RI Kan-2 (kanamycin resistance) fragment from pUC18 (Guilhabert *et al*, 2001) by blunt-end cloning, resulting in pJC3 (pJC*waaL*::*kan-2*). pJC3 was electroporated into *Xf* Fetzer, Dixon, and Ann-1 competent cells as previously described (Matsumoto *et al*, 2009) where a double recombination event would replace the full length *waaL* ORF with the selective marker, kan-2. The genomic context of the

waaL deletion mutant is shown in **Figure 5**.

A *waaL* deletion mutant in Ann-1 has been confirmed by PCR amplification of the *waaL* region with *waaL* primers and kan-2 primers as well as being resistant to 5µg/mL kanamycin (**Figure 4**). Further confirmation using primers designed to the flanking region at the point of the Kan-2 cassette insertion is underway. Candidate mutant strains of *waaL* deletion mutants in Fetzer and Dixon are currently being evaluated. All strains are confirmed at the genus-species as well as strain level by PCR and restriction digest analysis (Minisavage *et al*. 1994, Chen *et al*. 2005, Huang, 2009). A similar strategy is currently underway to create pJC4 (pJC*wzy*::*kan-2*). Preliminary data indicate that the Ann-1 Δ*waaL* mutant is devoid of smooth LPS (ie. O-antigen) as predicted. We are currently analyzing the LPS of this mutant more closely and have begun characterization of the Fetzer Δ*waaL* mutant.

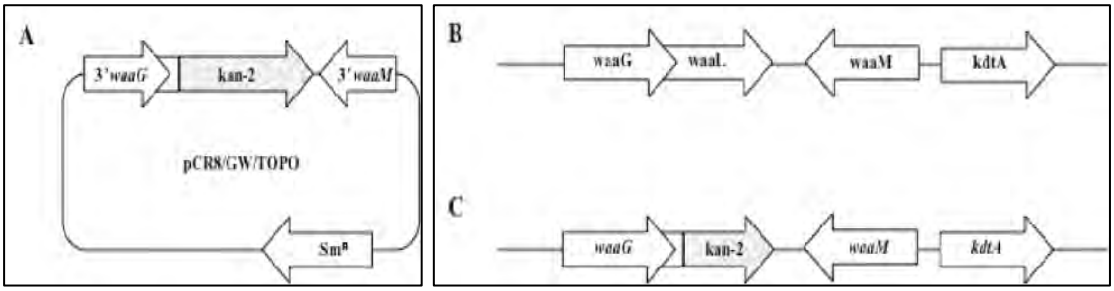

Figure 4. The construction of a Δ*waaL* mutant. **A,** mutagenesis construct pJC3 (pJC*waaL*::*kan-2*). After cloning the wild-type fragment into pCR8/GW/TOPO, 1384 bp of the *waaL* ORF was replaced by *kan-2*, a gene encoding for kanamycin resistance. The left flanking region consists of the 3[1] end of the PD0076 ORF (*waaG*), which has partial overlap with *waaL*, the 5[1]end of which remains intact in this construct. The stippled arrow represents the kan-2 cassette. The white box to the right of kan-2 represents the right flanking region (3' *waaM*). Note, figure not drawn to scale. **B,** *waaL* in the wild-type genomic context and **C,** *waaL* in the deletion mutant where *waaL* is replaced with kan-2. The 5' region of *waaL* remains intact to ensure a fully functional WaaG**.**

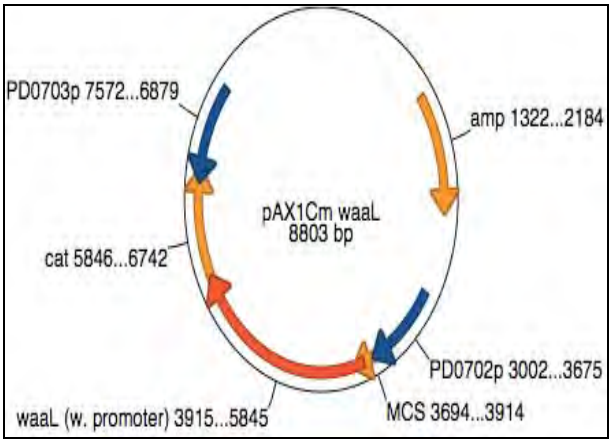

Figure 5. The *waaL* ORF with its promoter was amplified from *Xf* strains Fetzer, Dixon, and Ann-1 using primers containing *Xba*I ends and was cloned into the *Xba*I site of the multiple cloning region upstream of the chloramphenicol resistance gene (cat) in pAX1Cm. pAX1Cm plasmid courtesy of Dr. Michelle Igo (UCD).

Using the method of Matsumoto *et al* (2009), we have constructed complementation vectors with amplified Δ*waaL* and its promoter from each of the *Xf* strains of interest (Fetzer, Dixon, and Ann-1) (represented in **Figure 5**). Because of the divergence in amino acid sequence, we are interested to see if *waaL* from one strain can complement the Δ*waaL* mutant phenotype in other strains, as well as its own. For example, we will introduce the wild-type *waaL* from Fetzer back into the Fetzer Δ*waaL* mutant strain and also into the Dixon and Ann-1 Δ*waaL* mutant strains (**Table 1**). We will analyze the LPS isolated from the cross complemented mutants by running electrophoretic gels as described above. The same course of action will be taken with *wzy* mutants and complemented strains.

Objective 3. Test virulence and host specificity of the O-antigen mutants in planta.
As stated in Objective 2, mutant strains complemented with the wild-type locus will also be used for *in planta* studies. This approach will provide insight into whether LPS plays a role in virulence as well as host specificity observed in these three isolates. This can be determined by inoculating plants with each wild type, mutant, and complemented strain into their hosts as well as their non-hosts (**Table 1**). Inoculating with the complemented mutant strains will confirm any role of O-antigen in the ability to promote disease on grapevine, almond, and oleander.

Plant experiments are currently underway to evaluate the *Xf* Ann-1Δ*waaL* and *Xf* wild-type in the oleander host as well as almond and grapevine. The plants are six weeks post-inoculation and we are awaiting symptom development (ie. usually occurs 12 weeks post-inoculation).

Table 1. Virulence and host specificity assays.

	Host inoculation – for LPS profile and disease progress		
***Xf* strain**[a]	**Grape**	**Almond**	**Oleander**
WT Fetzer	host	+	+
Δ*waaL* Fetzer	host	+	+
Δ*waaL*/*waaL*+. Fetzer	host	+	+
Δ*wzy* Fetzer	host	+	+
Δ*wzy*/*waaL*+. Fetzer	host	+	+
WT Dixon	+	host	+
Δ*waaL* Dixon	+	host	+
Δ*waaL*/*waaL*+	+	host	+
Δ*wzy* Dixon	+	host	+
Δ*wzy*/*wzy*+ Dixon	+	host	+
WT Ann-1	+	+	host
Δ*waaL* Ann-1	+	+	host
Δ*waaL*/*waaL* Ann-1	+	+	host
Δ*wzy* Ann-1	+	+	host
Δ*wzy*/*wzy*+ Ann-1	+	+	host

[a] WT=wild-type, *waaL* and *wzy* mutant strains are or will be constructed as deletions, complemented strains are mutant strains with a wild-type copy of the gene of interest introduced into the genome via homologous recombination from the pAX1Cm vector (provided courtesy of Dr. Michele Igo; UCD). + represents inoculation of a non-host.

Objective 4. Test attachment and biofilm formation phenotypes of Xf O-antigen mutants.
No activity for this reporting period.

CONCLUSIONS
The primary goal of this project is to further understand the molecular mechanisms of *Xf* virulence and host specificity *in planta*. We are investigating if the O-antigen component of the LPS molecule plays a key factor in behaviors associated with xylem colonization, such as attachment to the plant cell wall and biofilm formation. While there are likely several factors that contribute to host specificity of *Xf*, we hypothesize that O-antigen presence and composition plays a major role in host specificity. We feel that the wide host range but stringent host specificity of different *Xf* isolates affords a unique opportunity to study the molecular mechanisms underlying the host specificity observed for this pathogen. Should LPS prove to be an important factor during *Xf* plant infection, its abundance in the bacterial outer membrane makes it a rational target for disease control.

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COMPARATIVE GENOMICS OF *XYLELLA FASTIDIOSA* SOUTH AMERICAN STRAINS

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ABSTRACT

Using DNA microarray hybridization we have previously identified significant differences in gene composition between *Xylella fastidiosa (Xf)* CVC (Citrus Variegated Chlorosis) strains 9a5c and J1a12[1]. Strain 9a5c exhibit virulent phenotype both in citrus and in hosts used as models of infection while strain J1a12 displays less virulent phenotype in citrus and tobacco. The genomic differences identified include the absence or high divergence of 14 coding sequences in the genome of J1a12, which correlates with its less virulent phenotype. To deepen the comparison between virulent and less virulent CVC strains, as well as between strains isolated from infected plants in South America, we are conducting a project aiming the complete genome sequencing and comparison of several *Xf* strains. Through the comparison of these new genomic sequences with known genomes of other *Xf* strains as well as other phytopathogens we hope to enlarge the knowledge of the repertoire of genes potentially associated with evolution, adaptation to hosts, pathogenicity and virulence. Currently, we have sequenced the genomes of four strains (J1a12, U24d, Fb7 and 3124). Strains and U24d and Fb7 were isolated from citrus plants with symptoms of CVC, respectively in the State of São Paulo (Brazil) and Argentina. Strain 3124 was isolated from a coffee plant with leaf scald symptoms. Initial analysis of these four new genomes confirm the previously observed differences between the genomes of J1a12 and 9a5c and allowed identification of additional differences between the genomes of J1a12 and 9a5c, including sequences that are apparently unique J1a12. Among these differences is the presence of an additional 27,258 bp plasmid that has not been described in other citrus isolates and shows extensive identity with the recently described plasmid from *Xf* mulberry-infecting strains [2]. Moreover phylogenetic trees based on multiple loci, separate, as expected, the strains from North and South America into distinct groups and suggest the occurrence of a higher frequency of genetic recombination between South American strains.

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XYLELLA FASTIDIOSA GENES AND PHENOTYPES RELATED TO HOST SPECIFICITY

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ABSTRACT
Xylella fastidiosa (*Xf*) strains differ in host specificity, although the molecular basis of the specific plant-bacteria interactions has not been identified. There is also evidence of variability in host range among isolates collected from within the same host species. Due to the lack of knowledge about the relationship between host specificity and genetic variability among *Xf* strains, this project was implemented to identify traits involved in *Xf* host preference. A collection of *Xf* isolates was screened for genetic and phenotypic characteristics that may be related to the interaction with the host. The preliminary collection consists of 24 *Xf* isolates collected from diverse plant hosts and multiple geographic locations in the US. For the genetic characterization, Multi Locus Sequence Analysis (MLSA) was performed using 10 candidate environmentally-mediated genes. Selected genes are hypothesized to be influenced by environmental factors and include genes related to surface attachment, motility, virulence, chemotaxis, and membrane transport functions. Primers for PCR amplification of these genes were designed and validated, and phylogenetic analyses were conducted on sequence information obtained from the amplified genes. For phenotypic characterization, traits related to interaction with the plant hosts and insect vectors, such as twitching motility and attachment, were analyzed *in vitro*. Preliminary genotypic and phenotypic results indicate that differences in environmentally-mediated gene sequences and differences in the motility and attachment of *Xf* isolates are related to the host plant from which the isolate was collected. Future expansion of this work will include adding more strains to the culture collection and including new genes and phenotypic traits in the analysis.

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A STABLE SHUTTLE VECTOR FOR *XYLELLA FASTIDIOSA* BASED ON AN ENDOGENOUS INCP-1 PLASMID

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ABSTRACT
Xylella fastidiosa (*Xf*) strain RIV11 harbors a 25 kbp plasmid (pXF-RIV11) belonging to the incP1 incompatibility group. Replication and stability factors of pXF-RIV11 were identified and used to construct plasmids able to propagate in both *Xf* and *Escherichia coli*. Sequences required for replication in *E. coli* and conferring antibiotic resistance were derived from the cloning vector pCR2.1. Replication in *Xf* required a 1.4 kbp region from pXFRIV11 containing a replication initiation gene (*trfA*) and the adjacent origin of DNA replication (*ori*V). This region also conferred plasmid replication in *Agrobacterium tumefaciens*, *Xanthomonas campestris*, and *Pseudomonas syringae*. Constructs containing the *trfA* gene and *ori*V derived from pVEIS01, a similar 31 kbp incP1 plasmid of the earthworm symbiont *Verminephropbacter eiseniae*, also were competent for replication in *Xf*. As expected, constructs bearing only *trfA* or *ori*V from either incP1 plasmid were unable to replicate in *Xf*. Although these incP1 replicons could be maintained in *Xf* under antibiotic selection, removal of selection resulted in loss of the plasmid. A novel toxin/antitoxin (*pem*I/*pem*K) addiction system of pXFRIV11 conferred stability of incP1 replicons in *Xf* in the absence of antibiotic selection. The resulting 6 kbp *Xf* shuttle vector (pXF20-PEMIK) also contains 10 unique endonuclease recognition sites for insertion of foreign DNA.

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Section 4: Pathogen and Disease Management

BIOLOGICAL CONTROL TRIALS WITH EB92-1 IN TEXAS

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LAYPERSON SUMMARY

There are no direct control recommendations for the management of Pierce's disease (PD) of grapes caused by *Xylella fastidiosa (Xf)*. As a result, losses in productivity to this recalcitrant bacterium are heavy in Texas with sometimes disastrous consequences for winegrape growers. Disease control still depends largely on suppressing insect vector populations, eliminating competing weeds and supplemental hosts, and the use of less desirable, resistant grape cultivars. A new biological control option has been successfully tested in other states for protecting high risk, susceptible vines with a "benign" strain of the pathogen. This strain, designated EB92-1, was originally isolated from an elderberry plant and shown to cause only mild, or no symptoms when inoculated into grape. Several experimental approaches previously have been used to demonstrate the protective properties of the benign strain. These approaches include both greenhouse and vineyard tests where plants were inoculated with EB92-1 prior to artificial inoculation with the pathogen. The use of a benign strain of a pathogen to protect plants from a more virulent strain of the same pathogen has been demonstrated in numerous other plant diseases. There are unique reasons why such a biocontrol agent might be realistic for the PD problem on grapes. Should the use of EB92-1 successfully protect grapevines in Texas from colonization by *Xf*, this will be the first and only direct control method designed to increase vine productivity growing in high risk PD regions.

INTRODUCTION

This project is designed to test a potential biological control agent for Pierce's disease (PD) of grapes, caused by *Xylella fastidiosa (Xf)* subsp. *Piercei*. Biological control agents of plant diseases come in many forms (Agrios 2005). Their success not only depends on properties of the pathogen and the host, but also on the economics involved in production of the affected crop. PD of grapes has the attributes for which biological control would be a particularly attractive approach for management (Hopkins 1989, Purcell and Hopkins 1996, Raju and Wells 1986). The crop is very high in value, is intensively managed and the pathogen threatens grapevines over a wide range of the area where they are grown. Should a biological control be proven effective, there will be a huge demand for its use. Also, *Xf* has a complex population structure in which there is a wide variety of strain specificity occurring throughout a very large host range. The cross infectivity among these hosts makes it possible for the bacterium to successfully colonize different hosts while eliciting a variety of responses, some of which may be minimal or non-existent.

Xf is a native, endemic pathogen in Texas. PD is a limiting factor for growth of *Vitis vinifera* varieties in many of the winegrape regions in the state. Current recommendations for PD control can be expensive and inconsistent. As a result, growers face a great deal of anxiety over sustained production in existing vineyards, as well as a lack of confidence in selecting varieties for replanting and establishing new vineyards. These circumstances present the ideal environment in which to test a potential biocontrol agent to reduce losses from PD.

One approach for biocontrol of a plant disease is the introduction of a unique strain of the relevant pathogen that can mitigate the properties of the virulent, problematic strain of the same pathogen. Tests have shown that a strain of *Xf* from elderberry can be inoculated into grape and reduce the ability of the virulent, native grape strain to cause disease (Hopkins 2005, Hopkins et al. 2007). This strain, designated EB92-1 has been applied to grapevines and successfully reduces disease severity when the vines were subsequently challenged by a virulent strain of *Xf* (Hopkins 2005).
This project would be a new line of investigation under the Texas Pierce's Disease Research and Education Program. The use of EB92-1 is already being tested in the California Program (Hopkins et al. 2007). This project would compliment the California research and add valuable information to the use of this potential biocontrol under conditions found in Texas. The proposed project would be one of the few projects in the Texas Program aimed at directly controlling the pathogen in the vine. Although a management program for PD exists for Texas growers (Kamas et al., 2004), losses continue to mount throughout all winegrape regions in the State. If successful, it would represent a valuable addition to our ability to prolong vine productivity and relieve the current losses being sustained by Texas grape producers.

The following Objectives are proposed to thoroughly test the use of EB92-1 under a variety of conditions as a potential biocontrol for PD of grapes in Texas. Each is needed to investigate promising conditions for which the potential biological control agent might be applied.

OBJECTIVES

1. Treat grapevines in greenhouses with EB92-1 prior to inoculation with a native virulent grape strain of *Xf* subsp. *fastidiosa.*
2. Treat grapevine cuttings with EB92-1 prior to planting into vineyards at high risk to *Xf.*
3. Treat grapevines in infected vineyards with EB92-1 to test for preventive and therapeutic properties of the potential biocontrol agent.

RESULTS AND DISCUSSION

Objective 1 Greenhouse tests. The elderberry strain EB92-1 was inoculated into potted grapevines either alone or four weeks prior to inoculation with a fresh grapevine-strain of the pathogen. There were 10 vines of four varieties (Merlot, Viognier, Cabernet Sauvignon, and Blanc du Bois) in each of the treatments. A proven needle-inoculation technique was used to directly introduce the bacterium (adjusted for concentration of 10^5 to 10^6 CFU/ml) into the vascular system of the vine (Hopkins and Adlerz 1988, Hopkins 2005). Two additional treatments consisted of the grapevine strain alone and a buffer inoculated control. The greenhouse trials were initiated in June, 2009 and monitored weekly for symptom development and survival for two growing seasons. Typical symptoms were quantified and verified utilizing ELISA (enzyme linked immunosorbent assay) and/or QRT-CPR (quantitative real time – polymerase chain reaction). Symptoms, or a disease rating scale of 1 - 6 was used, where 1 = healthy, 2 = 5-40% scorch, 3 = 50 - 90% scorch, 4 = 100% scorch with no dieback, 5 = 100% scorch with dieback, and 6 = dead.

At the end of the first growing season, some trends in the responses of vines to the treatments were evident (**Figure 1**). Merlot and Viogner exhibited the greatest level of symptom development when inoculated only with the grapevine strain of the pathogen. The Cabernet sauvignon and Blanc du Bois did not react negatively in any of the treatments. The EB92-1 inoculations resulted in some symptom development, but also appeared to suppress symptoms in the combination treatment relative to the grape strain inoculations. At the end of the second growing season (**Figure 1, B**.), the trends were very similar to the first. One notable difference was the development of symptoms in the Cabernet sauvignon in the grape strain inoculations, but again, the combined treatment appeared to suppress symptoms in that variety.

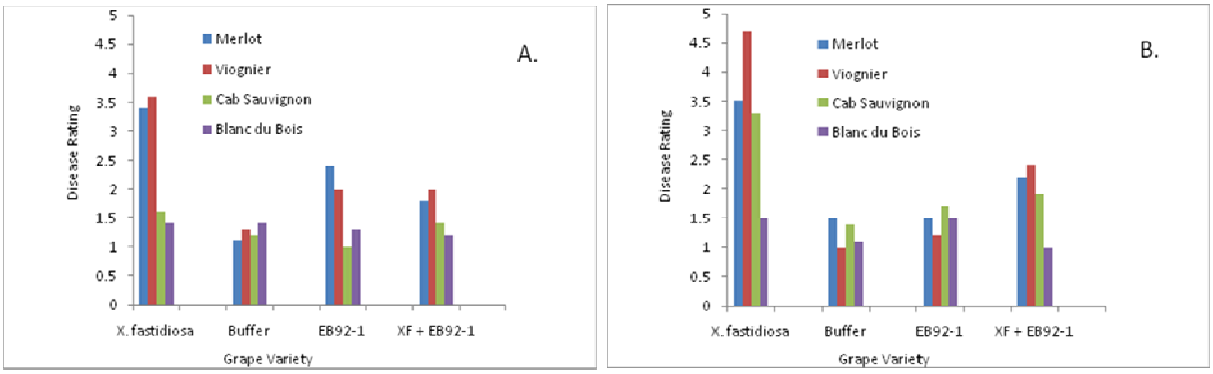

Figure 1. Average disease ratings (A. September, 2009 and B. September, 2010) for grapevines injected with 4 treatments consisting of a grape strain of *Xf*, a benign strain (EB92-1), both, or a buffer control. Vines were treated in June 2009.

Objective 2. Treated cuttings. Cuttings of the same four varieties used in Objective 1were grown in greenhouses prior to treatment and transplanting to vineyards in June – July, 2009. Ten vines of each variety were treated by inoculating the lower stem in the same manner described in Objective 1. Treatments consisted of a bacterial suspension of *Xf* strain EB92-1 (n = 10), a buffer "inoculation" (n = 10), and 10 untreated vines. Four weeks after inoculation, the vines were planted in Palacios Vineyard, a production vineyard near Brenham, TX. This location is considered to be a high risk vineyard for natural infection by native *Xf* subsp. *piercei* and has been the focus of numerous PD research projects. The vines were monitored monthly for development of typical PD symptoms, rated, and analyzed as described for Objective 1.

There were no symptoms of natural infection during the first growing season in any of the treatments, nor have any symptoms developed thus far during the 2010. The levels of PD in Palacios vineyard have been much lower during the 2010 growing season, probably due to frequent rainfall, massive roguing efforts, weed control and vector management. This plot

will continue to be monitored and will be sampled for analyses with ELISA and QRT-PCR to assess the level of infections in the vines.

Objective 3. Mature vine treatments in vineyards. This objective was accomplished by inoculating mature, high risk vines in two vineyards with the potential biocontrol *Xf* strain EB92-1. The first was Palacios Vineyard where Objective 2 was completed. Four varieties were treated, including Merlot, Shiraz, Cabernet sauvignon, and Blanc du Bois. Vines for treatment were selected in blocks according to records of vine health and current observations regarding status of infection by *Xf*. There were two treatments consisting of an injection with EB92-1 (n = 5), a buffer injection (n = 5), and 5 vines were left untreated in the block. The two treatments and untreated checks were repeated 4 times for each variety, bringing the total number of vines for each variety in the experiment to 60. A set (n = 15 vines) of the three groups were arranged in sequence in 3 – 4 adjacent rows. Bacterial suspensions were prepared as in the first two objectives. However, 4 – 6 ml of bacterial suspension or buffer was injected into the stems of the vines with a 50 ml syringe. Vines were be monitored in the same manner as in Objective 2. Mean disease severity ratings for the treatments will be compared separately.

As in the case of Objective 2, the low level of disease development in Palacios vineyard during the 2010 growing season has obscured any obvious potential treatment effects to date. None of the Blanc du Bois vines show any signs of scorching. Only two Merlot vines have developed symptoms, each in the untreated set of vines. In the Cabernet sauvignon, 2 untreated vines and 3 vines treated with EB92-1 have developed symptoms. One vine injected with buffer has developed symptoms in the Shiraz.

The treatment of mature vines was repeated at the Fredericksburg Experimental Vineyard in Fredericksburg, TX. The treatments were similar to those at Palacios but with more varieties. An analysis of the Fredericksburg plots was underway at the time of submission of this report.

CONCLUSIONS

These experiments are testing the ability of the elderberry strain of *Xf* (EB92-1) to protect grapevines from the effects of the more aggressive grape strain of the pathogen. Of the three experiments, the greenhouse inoculations gave the most convincing case for suppression of disease development in vines inoculated with a preventive dose of the biocontrol strain prior to inoculating with the virulent strain. The effect was seen best in two varieties, Merlot and Viognier, but further analyses are needed to determine the significance of these results. These are probably the most susceptible of the four varieties. Blanc du Bois is considered to be tolerant to *Xf*, and this was again confirmed in the greenhouse inoculations. Although Cabernet sauvignon exhibited some resistance in the first year of treatment with the *Xf* grape strain, the vines may be succumbing in the second year. This experiment will be maintained and analyzed for at least one more year to determine if the biocontrol is lasting, and whether there is a varietal influence on the ability of EB92-1 to protect vines. However, these preliminary results are encouraging.

Results of the field trials will also need more time to become sufficient for proper analyses. During the interim, we will be sampling vines for ELISA and QRT-PCR to determine the presence of the pathogen and assist in evaluating their health in the absence of symptoms. A few of the vines inoculated with EB92-1 have developed classic PD symptoms in both greenhouse studies and field trials. This was also noted to occur in previous studies, but was shown to have no lasting detrimental impact on vine health. At this point it should not be considered problematic for the potential use of EB92-1 in prolonging the productivity of grapevines at high risk to infection by the grape strain of *Xf*.

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PROGRESS OF PIERCE'S DISEASE IN THREE *VITIS VINIFERA* SCIONS GRAFTED ON THREE ROOTSTOCKS

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ABSTRACT
We are documenting Pierce's disease (PD) progress, vigor, and bud break dates in selected *Vitis vinifera* scion x rootstock combinations. Own-rooted 'Chardonnay,' 'Merlot,' and 'Cabernet Sauvignon' (checks) and these scions grafted in all combinations on Freedom, Paulsen 1103, and Dog Ridge were established at Uvalde and Stonewall, TX in 2009. Selected border plants at Uvalde were inoculated with the PD bacterium (*Xylella fastidiosa* subsp. *fastidiosa*) 10-11May2010 and insecticide was not used. There was no inoculation or insecticide in plots at Stonewall but imidacloprid was injected through drip irrigation on adjacent established vines for control of glassy-winged sharpshooter (GWSS) and other vectors. Incidence of early-stage PD was 81% by 10Sep2010 at Uvalde but near 0% at Stonewall 26Aug2010. Scion accounted for more or similar proportions of variability of variables compared to rootstock. There were several significant low level scion x rootstock interactions. We expected scions to hold up the best on Dog Ridge, but Paulsen 1103 looked better for PD and vigor in September of the second-leaf vines at Uvalde. We attribute the higher-than-anticipated PD incidence to inoculation of border Chardonnay plants and high GWSS populations which facilitating vector acquisition. GWSS was the predominant vector species, and adult and nymph numbers were high for several weeks. Rootstocks most delayed bud break on Chardonnay. Evaluations of both plantings will continue, with fruiting in 2011.

LAYPERSON SUMMARY
Two ongoing vineyard trials explore rootstock effects on Pierce's disease (PD) in Gillespie and Uvalde Counties in Texas. While all *Vitis vinifera* scions are considered susceptible to *Xylella fastidiosa* subsp. *fastidiosa*, the bacterium that causes PD, there are noticeable differences in timing of symptom expression. On 10Sep2010, 'Cabernet Sauvignon' had less severe symptoms but the greatest incidence and ELISA optical density (OD) reading in positive plants compared to 'Merlot' and 'Chardonnay.' In Texas where late spring freezes often occur, Chardonnay is unsuited due to early bud break, severe PD symptoms soon after infection, and ultimate death. At the high vigor Uvalde site, all three rootstocks somewhat delayed bud break date and increased vigor, vine diameter, and leaf color (before Fe supplementation) compared to own-rooted scions. To date, Paulsen 1103 rootstock provided more PD and vigor benefits than Dog Ridge. Freedom has inefficient Fe uptake in the high pH soil and requires aggressive Fe supplementation for green leaf color. At the moderate vigor Stonewall site, essentially no PD has occurred and Fe uptake is not limiting.

INTRODUCTION
There is anecdotal evidence in Texas that progress of Pierce's disease (PD), caused by *Xylella fastidiosa* (*Xf*) subsp. *fastidiosa*, differs among *Vitis vinifera* scions (e.g., 'Chardonnay'>'Merlot'>'Cabernet Sauvignon'). PD symptom severity and vine mortality are apparently greater under environmental stresses, including over-cropping. Rootstocks are used to overcome environmental stresses and can affect vine vigor and fruit load. In a previous 3-year study we found that 12 ungrafted rootstocks commonly used in Texas had PD reactions ranging from vine mortality to mild leaf scorch symptoms. More information is needed on rootstock effects on scion PD symptom development (3), vine growth, and productivity. This study compares PD progression in *Vitis vinifera* scions (representing a limited range of PD progress rates) grafted on rootstocks previously shown to exhibit severe, intermediate, and mild PD reactions. Both vineyards will be allowed to fruit in 2011 and crop load potential will be adjusted according to pruning weights. Evaluations will continue as vines at both sites experience winter cold, spring pruning, fruit load stress, and PD vector activity.

OBJECTIVES
1. Document scion x rootstock effects on progress of PD, vigor, and bud break date.

RESULTS AND DISCUSSION

Chardonnay, Merlot, and Cabernet Sauvignon were omega-grafted, callused, and rooted in spring 2008 in all combinations with rootstocks we previously showed had severe (Freedom, interspecific cross includes *V. labrusca*, *V. riparia*, *V. champinii*, *V. vinifera*) (1), intermediate (Paulsen 1103, *V. berlandieri* x *V. rupestris*), and mild (Dog Ridge, *V. champinii*) PD symptoms. Dormant virus-tested scion and rootstock wood was procured for propagation (Foundation Plant Services, University of California, Davis).

Treatments (3 scions x 3 rootstocks) and controls (own-rooted scions) were established in 2009. Sites near Stonewall, TX (Gillespie County, pH 6.5, PD history, imidacloprid use in adjacent vineyard block, Malbec/Paulsen 1103 border) and Uvalde (Uvalde County; pH 8.3; no vineyard nearby; own-rooted Chardonnay in a mixed border; adjacent to citrus, piñon pine and other ornamentals used by GWSS) had five replications and five plants per plot. Plants in first-leaf (2009) had no definite symptoms, and rare ELISA positive reactions (optical density, OD≥0.300) in leaf samples biased towards PD symptoms (Stonewall 1% 24Aug09, 0% 27Oct09; Uvalde 0% 31Aug09, 1% 16Nov09). Borders at Stonewall were not inoculated and GWSS feeding was probable but not observed. Stonewall plots and borders had no definite symptoms on 26Aug2010 and symptom-biased leaf samples had 0% ELISA positives. Own-rooted Chardonnay new growth in the Uvalde border was twice inoculated 10-11May2010 with *Xf* winegrape isolate GIL BEC 625 and glassy-winged sharpshooter (GWSS) feeding activity occurred for >5 months. PD symptoms at Uvalde developed first in borders then in many plots. All Uvalde plants received soil drenches and foliar sprays of Fe and other minor element supplements with extra applications for own-rooted and Freedom treatments.

We emphasize Uvalde main factor effects in this report (**Tables 1, 3**), although five parameters had low level significant scion x rootstock interactions. Scion choice accounted for the most variability (compare MS values) of six parameters in 2010 (**Table 2**). Growth stage and pruning weights at Stonewall had similar responses (data not presented).

Late summer 2010 ELISA tests were all negative at Stonewall but 81% positive for *Xf* at Uvalde where PD symptoms overall mean (including own-rooted checks) was 6% leaves (Table 1). Consistent with previous observations in young plantings, Cabernet Sauvignon had less severe PD symptoms than Merlot and Chardonnay, but Cabernet Sauvignon had the most positive plants and highest mean OD values (Tables 1,3). Perhaps *Xf* numbers peak and collapse sooner in infected Chardonnay than in Merlot and Cabernet Sauvignon.

Table 1. Responses in 2010 of three *V. vinifera* cultivars own-rooted and grafted on three rootstocks at Uvalde, TX adjacent to GWSS refuges where selected border plants were inoculated with *Xf* and imidacloprid insecticide was not used for sharpshooter control.

Scion	Rootstock	Pruning wt., g[a]		Vine dia., mm[b]		Canopy color[c]		PD symptoms[d]		Positive, %[e]		Positive plants OD[f]	
'Chardonnay'	Own	119	f[g]	9	g	1.5	g	9.4	de	49	a	1.291	bcd
'Chardonnay'	Dog Ridge	382	ab	14	cd	2.7	ab	11.4	e	81	cde	1.157	abc
'Chardonnay'	Freedom	293	c	13	def	2.7	ab	4.8	ab	66	abc	1.035	a
'Chardonnay'	Paulsen 1103	434	a	15	c	2.9	a	5.8	abc	83	cde	1.162	abc
'Merlot'	Own	52	h	8	g	2.4	cd	7.0	bcd	61	ab	1.214	abcd
'Merlot'	Dog Ridge	156	e	12	f	2.4	cde	7.8	cde	97	de	1.106	ab
'Merlot'	Freedom	114	f	11	f	2.4	cde	7.4	bcd	93	de	1.071	ab
'Merlot'	Paulsen 1103	198	d	13	cde	3.0	a	6.2	bcd	69	bc	1.013	a
'Cabernet Sauvignon'	Own	80	g	12	ef	2.3	de	5.0	abc	98	e	1.349	cd
'Cabernet Sauvignon'	Dog Ridge	397	ab	19	a	1.9	f	4.6	ab	97	de	1.264	bcd
'Cabernet Sauvignon'	Freedom	179	de	16	bc	2.1	e	3.5	a	100	e	1.369	cd
'Cabernet Sauvignon'	Paulsen 1103	333	bc	17	b	2.5	bc	3.5	a	77	bcd	1.401	d

[a]Pruning weights 2April2010. Days-after-planting was significant covariate. Data were transformed ($\log_{10}$ y) before analysis. De-transformed least squares means (10^x) are geometric means.

[b]25Feb2010 at 10 cm above graft union or equivalent for own-rooted.

[c]1=chlorotic, 2=intermediate, 3=green on 10Sep2010; wind damage was significant covariate; least squares means.

[d]Percent leaves with PD symptoms including scorched and defoliated ,10Sep10. Data were transformed (square root (y+0.5)) before analysis. De-transformed least squares means (x^2 + error mean square) are presented here.

[e]Percent plants *Xf*-positive with ELISA (optical density ≥0.300) from 10Sep2010 leaf samples biased towards PD symptoms.

[f]OD means for *Xf*-positive plants only.

[g]Means followed by the same lower case letter were not significantly different (P≤0.05); least squares means, PDIFF option.

More scion PD symptoms on Dog Ridge than Freedom and Paulsen 1103 rootstocks was unexpected, as was the high PD incidence in scions on Dog Ridge (90%, based on ELISA tests) (**Table 3**). Once infections crosses the graft union, Freedom is expected to succumb to PD. Dog Ridge rootstock sustained new scion growth into late summer on several plants, and infected GWSS may have preferred feeding on that new growth, allowing *Xf* to increase in the new scion growth.

Cabernet Sauvignon and Chardonnay had greater pruning weight than Merlot (**Table 3**). Cabernet Sauvignon had greater vine diameter than Chardonnay, and Merlot had less vine diameter than the other two scions. Freedom had less pruning weight and vine diameter than Dog Ridge and Paulsen 1103. Rootstock explained more variability in vine diameter than in pruning weight (**Table 2**).

Spring freezes in April are a frequent problem in Texas and growers need information on practices that affect bud break date. Pruning after bud break is used for some scions and locations. At Uvalde, Chardonnay began growth very early, as expected (E-L growth stage 3 woolly bud 23-27Feb10) (**Figure 1**). Merlot and Cabernet Sauvignon had similar growth stage at 22Mar10, then Merlot accelerated (E-L3 22-24Mar10) compared to Cabernet Sauvignon (E-L3 28Mar-10Apr10). Rootstocks somewhat delayed growth stage compared to own-rooted vines of all scions, but scion had far more effect on growth stage date than rootstock. Paulsen 1103 delayed Chardonnay E-L5 about 1 week. It is widely recognized that this rootstock effect is highly specific to scion.

Own rooted plants at Uvalde mostly had low vigor, pruning weight, and vine diameter, and poor leaf color (even with additional Fe supplements) as expected due to high soil pH (**Table 1**). September chlorotic canopy in Cabernet Sauvignon and Dog Ridge may be related to propensity for prolonged vegetative growth after the last Fe application during summer stress which can reduce Fe uptake. On 10Sep10, Paulsen 1103 had the best green canopy color (**Table 3**). Poor plant condition probably reduced GWSS adult feeding, and this may explain less PD in Chardonnay. Freedom rootstock had more early-season chlorosis in 2009 and 2010 than Dog Ridge and Paulsen 1103, but Freedom eventually responded to extra Fe supplements.

CONCLUSIONS

Benefits from rootstocks are apparent in second-leaf at both Uvalde and Stonewall including vigor, a slight delay in bud break, and tolerance of high pH limestone-based soils (**Tables 1,3**). Rootstocks had some effects on PD in second-leaf at Uvalde. ELISA OD was quite high even for mild PD symptoms in Cabernet Sauvignon, reminiscent of reports for native American hybrid scions. Own-rooted *V. vinifera* cultivars are clearly not adapted at Uvalde (**Table 1**), where own-rooted and Freedom treatments received more iron supplements from late winter through early summer than other treatments. Vigor and chlorosis differences may have influenced PD insect vector usage among plants at Uvalde. Significant GWSS refuges at the Uvalde site where PD increased dramatically highlight the importance of site selection, vegetation management near vineyards, and sharpshooter control. Nearby sources of *Xf* are very dangerous for vineyards with *V. vinifera* scions, and rapid PD development at Uvalde points to the need for early detection and vigorous roguing in highly susceptible cultivars. Imidacloprid used adjacent to the Stonewall site suppressed GWSS and other xylem-feeding vectors in 2010, providing impetus for trap crop experiments. We will continue evaluations in late fall 2010 and beyond.

Table 2. Sources of variance in 2010 of three *V. vinifera* cultivars grafted on three rootstocks (own-rooted checks data deleted) at Uvalde, TX adjacent to GWSS refuges where selected border plants were inoculated with *Xf* and imidacloprid insecticide was not used for GWSS control.

		Pruning wt, g[a]		**Vine dia., mm**[b]		**Canopy color**[c]		**PD sympt.**[d]		**Positive**[e]		**Pos. plants OD**[f]	
Source of variance[g]	df	MS[h]	Pr > F	MS	Pr > F	MS	Pr > F	MS	Pr > F	MS	Pr > F	MS	Pr > F
Replication	4	0.07	0.0093	52.1	0.0003	0.69	0.0139	1.4	0.2781	25	0.0643	0.34	0.0211
Scion	2	2.74	0.0001	303.5	0.0001	7.55	0.0001	16.6	0.0001	49	0.013	1.41	0.0001
Rootstock	2	0.14	0.0009	163.7	0.0001	5.13	0.0001	8.6	0.0004	46	0.0161	0.01	0.8854
Scion x rootstock	4	0.06	0.0208	44.3	0.0013	0.55	0.0406	2.8	0.0371	40	0.0074	0.12	0.4101
Days-after-planting	1	0.11	0.0207							208	0.0001		
Wind damage	1					8.09	0.0001					0.57	0.028
Total df		206		207		224		224		224		118	

[a]Pruning weights 2April2010. Data were transformed ($\log_{10}$ y) before analysis.
[b]25Feb2010 at 10 cm above graft union or equivalent for own-rooted.
[c]1=chlorotic, 2=intermediate, 3=green 10Sep2010.
[d]Percent leaves with PD symptoms (scorching, defoliation) 10Sep2010; data transformed (square root (y+0.5)) before analysis.
[e]Percent plants positive for *Xf* with ELISA (OD ≥0.300) from 10Sep2010 leaf samples biased towards PD symptoms.
[f]Negative plants were omitted for optical density analysis.
[g]Single df indicate significant covariates used to adjust least squares means.
[h]Mean square from PROC GLM.

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Table 3. Responses in 2010 of three *V. vinifera* cultivars grafted on three rootstocks (own-rooted checks data deleted) at Uvalde, TX adjacent to GWSS refuges where selected border plants were inoculated with *Xf* and imidacloprid insecticide was not used for GWSS control. Scion x rootstock interactions were significant (P<0.05) for all variables (data not presented).

Factor	Pruning wt, g[a]		Vine dia, mm[b]		Canopy color[c]		PD symptoms[d]		Positive, %[e]		Positives OD[f]	
						Scion						
'Chardonnay'	354	a[g]	14	b	2.8	a	6.7	b	75	a	1.113	a
'Merlot'	147	b	12	c	2.6	b	6.7	b	85	ab	1.061	a
'Cabernet Sauvignon'	336	a	16	a	2.2	c	3.5	a	92	b	1.340	b
						Rootstock						
Dog Ridge	277	a	15	a	2.3	b	7.3	b	90	b	1.173	
Freedom	211	b	12	b	2.4	b	4.7	a	87	ab	1.155	
Paulsen 1103	299	a	15	a	2.8	a	4.7	a	75	a	1.186	

[a]Pruning weights 2April2010; analysis on transformed ($\log_{10}$ y) data; de-transformed least squares means (10^x) are geometric means.
[b]25Feb2010 at 10 cm above graft union or equivalent for own-rooted.
[c]1=chlorotic, 2=intermediate, 3=green on 10Sep2010.
[d]Percent leaves with PD symptoms (scorching, defoliation) 10Sep2010; analysis on transformed (square root (y+0.5)) data; de-transformed least squares means (x^2 + error mean square) are presented here.
[e]Percent plants *Xf*-positive with ELISA (OD ≥0.300) from 10Sep2010 leaf samples biased towards PD symptoms.
[f]Negative plants deleted from analysis.
[g]Means followed by the same lower case letter were not significantly different (P≤0.05); least squares means, PDIFF option.

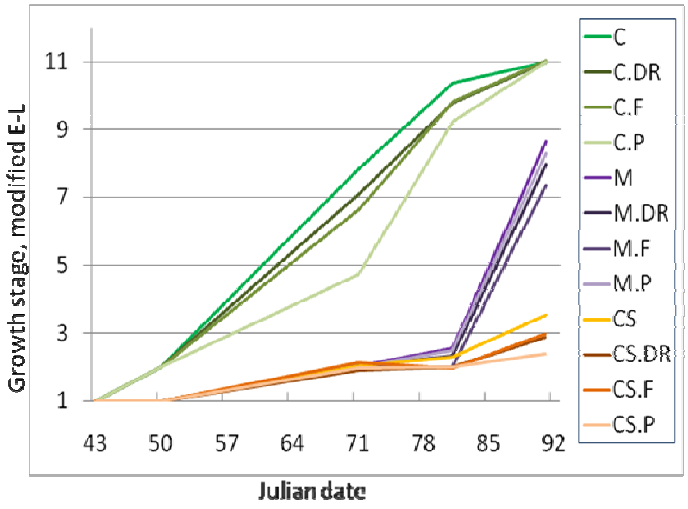

Figure 1. Second leaf early season growth stages at Uvalde, TX for 'Chardonnay' (C), 'Merlot' (M), and 'Cabernet Sauvignon' (CS) own-rooted and grafted on Dog Ridge (DR), Freedom (F), and Paulsen 1103 (P). Data are the greatest growth stage (modified E-L system, 5=rosette of leaf tips visible) anywhere on a plant. Observations Julian (and calendar) dates were 43(12Feb), 50 (19Feb), 71 (12Mar), 81 (22Mar), and 91 (1Apr2010).

GRAPE ROOTSTOCK VARIETY INFLUENCE ON PIERCE'S DISEASE SYMPTOMS IN CHARDONNAY

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Reporting Period: The results reported here are for work conducted October 1, 2009 through September 30, 2010.

ABSTRACT
Chardonnay is a *Vitis vinifera* scion variety that is susceptible to Pierce's disease (PD). We are evaluating the effect of rootstock variety on PD symptom expression in Chardonnay grown in an experimental vineyard at Weslaco, Texas with high natural PD pressure and abundant vectors, including glassy-winged sharpshooter. The rootstocks under evaluation are Dog Ridge, Florilush, Tampa, Lenoir, and Freedom. Natural *Xylella fastidiosa* infection is permitted to test the effect of rootstock variety on PD in the Chardonnay scions. Vines grafted on Dog Ridge are much larger than on other rootstocks, which are similar in size; PD symptoms in vines grafted on Dog Ridge were the lowest observed amongst the rootstocks.

LAYPERSON SUMMARY:
Rootstocks that reduce Pierce's disease (PD) symptoms of the scion could be one way to reduce damage from PD without changing scion varieties or clones. Rootstocks have been reported to reduce PD symptoms, but have not definitely been shown to provide or confer sufficient protection against PD to be recommended as a PD management tool. We are evaluating several rootstocks in a PD prone area, the Lower Rio Grande Valley of Texas. Chardonnay (PD susceptible) vines grafted on Dog Ridge rootstock were the largest in the trial and had the fewest PD symptoms.

INTRODUCTION
Rootstocks are widely in use in viticulture to manage damage from soil-borne pests and provide adaptation to soils. In citrus (He et al. 2000) and peach (Gould et al. 1991), rootstock variety has been reported to impact expression of *Xylella fastidiosa* (*Xf*) diseases in scions. Pierce (1905) reported that rootstock variety affected expression of "California vine disease" in grape. Grape rootstock trials in Mississippi showed a large effect of rootstock trial on vine longevity in a region recognized for high Pierce's disease (PD) pressure (Loomis 1952, 1965, Magoon and Magness 1937). If grape rootstocks could contribute PD resistance or tolerance to their scions, this would be a major benefit to viticulture in PD prone areas. Elite wine, juice, and table grape varieties could be grown in areas where viticulture is currently restricted to PD resistant and tolerant varieties whose consumer appeal is low.

The Rio Grande Valley is an excellent location for the field evaluation of PD resistant plant germplasm and PD management techniques. Many insect vectors of *Xf* are native to the region, including the glassy-winged sharpshooter. Susceptible grapevine varieties are infected naturally with *Xf* in the vineyard and demonstrate characteristic PD symptoms and decline. The Rio Grande Valley is similar to many viticultural regions in California; the region is flat, irrigated, and supports multiple types of crops (citrus, grains, vegetables) in close proximity. The Rio Grande Valley is an ideal test environment due to heavy PD pressure, with abundant vectors and inoculum, in contrast to many other locations, especially California, which demonstrate substantial cycling of PD incidence. The USDA Agricultural Research Service Kika de la Garza Subtropical Agricultural Research Center in Weslaco, Texas is located in the heart of the Rio Grande Valley and provides an ideal experimental location for the evaluation of PD management practices, including rootstock evaluation.

Five rootstocks are being evaluated in this project. Freedom is a complex interspecific hybrid developed as a root-knot nematode resistant rootstock by the USDA ARS, Fresno, California (Clark 1997); its parentage includes *Vitis vinifera, V. labrusca, V. x champinii, V. solonis,* and *V. riparia* (Garris et al. 2009). Freedom is widely used in California viticulture. Dog Ridge is a *V. x champinii* selection recognized for its nematode resistance and resistance to PD, but it is rarely used as a rootstock. Lenoir, most probably a *V. aestivalis*/*V. vinifera* hybrid, was used historically as a rootstock and presently is cultivated as a wine grape in PD prone regions (including some parts of Texas) (Galet 1988). Tampa (Mortensen and Stover 1982) includes a *V. aestivalis* selection and the juice grape Niagara (a *V. labrusca* hybrid) in its parentage. Florilush (Mortensen et al. 1994) is a selection from the cross Dog Ridge x Tampa. Both Florilush and Tampa were selected by the University of Florida as PD resistant rootstocks for bunch grapes. PD resistance is necessary for rootstock mothervines to thrive in Florida, so the PD resistance of Florilush and Tampa should not be construed necessarily as contributing to the PD response of the scions.

OBJECTIVES

1. To evaluate the impact of rootstock variety on expression of PD symptoms in naturally infected PD susceptible *Vitis vinifera* scion varieties Chardonnay.

RESULTS AND CONCLUSIONS

Grafted vines of Chardonnay on five rootstocks (Freedom, Tampa, Dog Ridge, Florilush, and Lenoir) were planted at the Kika de la Garza Subtropical Agricultural Research Center in Weslaco, Texas in July, 2006. Evaluation of PD response of the vines began in 2007. Experimental vineyard establishment was good and several vines flowered and fruited in 2007 and 2008.

Symptoms on leaves were assessed in October 2009. Percent leaves with marginal necrosis symptom of PD were determined for each vine. Dormant pruning was conducted in January, 2010, and the weight of prunings of each vine, head trained and spur pruned, was collected.

Rootstock	Number of vines	Mean % leaves with marginal necrosis, Oct. 2009	Weight of dormant prunings, Jan. 2010, kg
Florilush	10	98	0.73
Freedom	9	98	0.82
Lenoir	10	82	0.65
Tampa	10	73	0.88
Dog Ridge	9	57	1.70

Preliminary results indicate that Chardonnay vines grafted on Dog Ridge were the largest and had the least PD symptoms. Additional years of vineyard observations will be necessary before making rootstock recommendations based on vineyard performance. In the Lower Rio Grande Valley at the USDA ARS research vineyard, vines are not demonstrating downy mildew or black rot or more than slight powdery mildew infection; PD remains the chief disease in the research vineyard. Marginal necrosis symptoms are increasing from year to year.

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HNE-CecB CHIMERIC ANTIMICROBIAL PROTEIN AND POLYGALACTURONASE-INHIBITING PROTEIN TRANSGENIC GRAPEVINES FIELD TRIAL

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Reporting Period: The results reported here are from work conducted March 2010 to September 2010.

ABSTRACT
We have successfully initiated two field trials to investigate two greenhouse-tested strategies to control the movement of and to clear *Xylella fastidiosa* (*Xf*), a xylem-limited, Gram-negative bacterium that is the causative agent of Pierce's disease (PD). A key virulence feature of *Xf* is its ability to digest pectin-rich pit pore membranes inside the host plant's xylem elements, permitting long distance movement and potentially enhancing vector transmission. The first strategy being evaluated tests the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) from pear as an effector protein to counter virulence associated with bacterial PG activity. Our second strategy enhances clearance of bacteria from *Xf*-infected xylem tissues via the expression of a chimeric antimicrobial protein, HNE-CecB. The expectation is that expressing these proteins will prevent *Xf* movement and reduce its inoculum, curbing the spread of PD in California vineyards. Transgenic grapevine plants expressing either PGIP or the HNE-CecB chimeric antimicrobial protein have been planted in two locations, one in Riverside County and the other in Solano County. These transgenic grapevines will be evaluated as plants on their own roots and as rootstocks grafted with wild type Thompson Seedless (TS) scions. At the Riverside County site, the plants will be naturally infected. At the Solano County site, plants will be mechanically infected with *Xf* to validate resistance to PD under field conditions. Two hundred and thirteen transgenic and wild type control vines, own rooted and grafted with wild type TS, were planted in Riverside County on 5/8/10. In Solano County, 112 transgenic and wild type controls on their own roots vines were planted on 8/2/2010. HNE-CecB- and PGIP-grafted plants for the Solano County site are being generated for planting in November 2010.

LAYPERSON SUMMARY
Transgenic grapevines are being evaluated as rootstocks to demonstrate the field efficacy of two strategies to control Pierce's disease in California grapevines. The first strategy uses transgenic rootstocks to control the movement of the bacterium *Xylella fastidiosa (Xf)* in the water-conducting xylem of the vine through the expression of polygalacturonase-inhibiting protein. The second strategy tests whether transgenic rootstocks can clear *Xf* infections in xylem tissues through the expression of an HNE-CecB chimeric antimicrobial protein.

INTRODUCTION
Xylella fastidiosa (Xf), a xylem-limited Gram-negative bacterium, is the causative agent of Pierce's disease (PD). A key feature of *Xf* resides in its ability to digest pectin-rich pit pore membranes inside the xylem element (Roper et al., 2007), permitting its long distance movement and enhancing its virulence and vector transmission. In this project, we are examining the ability of the xylem-targeted effector proteins polygalacturonase inhibiting protein (PGIP, Aguero et al., 2005, 2006) and a chimeric antimicrobial protein (HNE-CecB, Kunkel et al., 2007) to restrict bacterial movement and to clear *Xf* under field conditions (Dandekar et al., 2009). The expectation is that expression of these proteins will prevent *Xf* movement and reduce its inoculum, reducing spread of PD.

We are field testing four independent transgenic lines (40-41, 40-89, 40-92, and 41-151) resulting from transforming grapevine plants with the vector pDU04.6105 expressing the chimeric HNE-CecB protein (**Figure 1**). In each location, 24 plants are being field tested: 12 replicates of each line as non-grafted plants and 12 as transgenic rootstocks grafted with wild type Thompson seedless scions.

We have also planted vines carrying four different constructs of PGIP (**Figure 2**). The four different modifications allow us to better understand how to control/restrict disease spread and virulence of *Xf*. Two versions have different signal peptide sequences to identify which most efficiently localizes PGIP to xylem tissues and which provides the best distribution through the graft union into untransformed scion tissues. In vector pDU05.1910 (event 52-08), the pear PGIP signal peptide was

replaced with a signal peptide from a grapevine xylem-secreted protein that is similar to the PRp27-like protein from *Nicotiana tobacum*. In vector pDU06.0201 (event 45-77), the pear PGIP protein was linked to a signal peptide from the Ch1b chitinase protein found in the xylem of grapevine (*Vitis vinifera*). The remaining two vectors, with and without the endogenous signal peptide, serve as controls. Vector pDU05.1002 (event 31-25) eliminates the endogenous signal peptide so the expressed PGIP cannot be secreted and should not limit *Xf* spread. The construct pDU94.0928 (event TS50), which uses the pear PGIP's own endogenous peptide, will serve as a control to evaluate the efficiency of exogenous signal peptides in targeting PGIP to the xylem tissue.

Fig. 1: Vector construct used for expressing disease resistance in grapevine plants

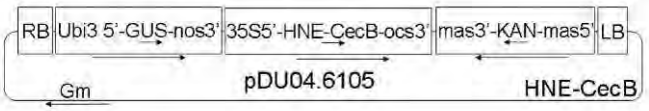
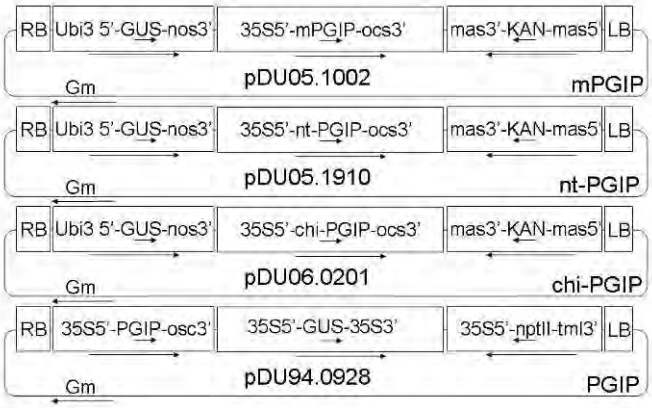


Fig. 2: Vector constructs used for expressing disease resistance in grapevine plants



The objective described in this report directly address the number 1 RSAP priority outlined in the "Top 5 to 10 Project Objectives to Accelerate Research to Practice" handout released at the December 2009 Pierce's Disease Research symposium: "Accelerate regulatory process: Establish and facilitate field trials of current PD control candidate vines / endophytes / compounds in multiple locations". This document updates the priority research recommendations provided in the report "PD/GWSS Research Scientific Review: Final Report" released in August 2007 by the CDFA's Pierce's Disease Research Scientific Advisory Panel.

OBJECTIVES

The goals of this project are to field test four HNEC-CecB and four PGIP transgenic grapevine lines to evaluate their horticultural characteristics and their resistance to PD. Transgenic grapevines will be tested in two field locations as non-grafted plants and as transgenic rootstocks grafted with wild type scions. One field location has PD pressure and plants will be naturally infected with *Xf*. In the location with no PD pressure, grapevines will be mechanically inoculated with *Xf*.

Objective 1. Validate the efficacy of *in planta* expressed chimeric HNE-CecB and PGIP proteins containing different signal peptides to inhibit and clear *Xf* infection in xylem tissue and through the graft union in grapevines under field conditions.

RESULTS AND DISCUSSION

Propagation, field planting, and grafting of HNE-CecB and PGIP transgenic grapevines.

Four selected transgenic grapevine lines expressing HNE-CecB and four expressing different PGIP constructs were propagated from cuttings in the greenhouse to obtain 48 clones of each line. These lines showed resistance to PD under greenhouse conditions after mechanical *Xf* inoculation. After the root system was developed, the plants were transferred to 5.5-inch pots. Twenty-four clones were grafted with wild type TS scions **(Figure 3**). Once acclimatized, they were transferred to the lath house and finally planted in two experimental fields. Two hundred and thirteen transgenic and wild type controls, own rooted or grafted with wild type TS scions, were planted in Riverside County on 5/8/10, where they are exposed to glassy-winged sharpshooters (GWSS), the insect vector for *Xf*. We also planted 112 transgenic and wild type controls on their own roots in Solano County on 8/2/2010 (**Figure 4**). Additional HNE-CecB and PGIP grafted plants for Solano County field are being generated for planting in November 2010.

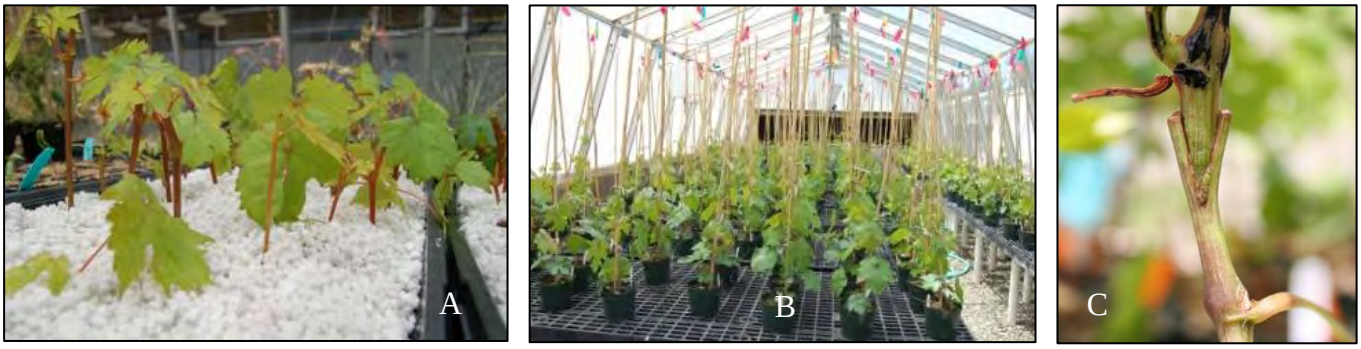


Figure 3. A) Propagated plants from vegetative tissue; B) Rooted plants and C) Grafted plant

Figure 4. Riverside and Solano County transgenic grape fields.

Evaluate preservation of varietal characteristics in transgenic grapevines grown as plants on their own roots or used as rootstocks.

HNE-CecB- and PGIP-expressing lines will be evaluated in the field in 2011. Grafted and non-grafted transgenic grapevine lines will be evaluated phenotypically for shoot growth and leaf shape to see if they are normal and have maintained the horticultural and varietal characteristics of the parental genotype TS. This examination will use the variables proposed by the International Organization of Vine and Wine (OIV, 1983).

Evaluate PD resistance of HNE-CecB and PGIP grapevines after inoculation with *Xf*.
Grafted and non-grafted transgenic and control grapevines planted in Riverside County will be naturally infected with *Xf* to validate resistance to PD under field conditions in 2011. Grafted and non-grafted grapevines planted in Solano County will be mechanically inoculated with *Xf* (Almeida and Purcell, 2003). PD symptoms will be scored on each infected plant using a standardized score based on percentage of leaf area scorching, a characteristic of PD (Krivanek et al., 2005a, 2005b). Expression of *hne* and *pgip* genes will be analyzed using Quantitative Real-Time PCR (qRT-PCR). PGIP movement from transformed rootstocks into wild type scions will be monitored using antibody detection and a radial diffusion assay that evaluates PGIP activity.

CONCLUSIONS
Selected HNE-CecB- and PGIP-expressing grapevines and wild type controls were successfully planted as non-grafted plants and as transgenic rootstocks in Riverside County. Presence of the GWSS at the Riverside County field is very promising, since natural insect inoculation of these plants is one of the main objectives of this trial. Non-grafted HNE-CecB- and PGIP-expressing grapevines and wild type controls were also successfully planted in the Solano County field. Grafted plants for the Solano County field are being generated to be planted in November 2010.

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IN PLANTA TESTING OF SIGNAL PEPTIDES AND ANTI-MICROBIAL PROTEINS FOR RAPID CLEARANCE OF *XYLELLA*

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ABSTRACT

Xylella fastidiosa (*Xf*), a xylem-limited, Gram-negative bacterium, is the causative agent of Pierces's disease (PD). A key feature of *Xf* is its ability to digest pectin containing pit pore membranes inside the host plant's xylem elements, permitting long distance movement and enhancing virulence and vector transmission. In this project, we are evaluating the ability of xylem-targeted effector proteins polygalacturonase inhibiting protein (PGIP) and a chimeric antimicrobial protein to restrict the movement of *Xf* and clear infection. The expectation is that expressing these proteins will prevent *Xf* movement and reduce its inoculum, curbing the spread of PD. Transgenic grapevine plants expressing either PGIP (six versions) or the human neutrophil elastase-cecropin B (HNE-CecB) chimeric antimicrobial protein (two versions), have been obtained and the first plants have been tested to validate their susceptibility to PD.

Plants expressing pear PGIP have six different modified sequences to target gene expression. Four constructs contain different signal peptide sequences to identify which most efficiently localizes PGIP to xylem tissues and which provides the best distribution through the graft union into untransformed scion tissues. A fifth construct has the original pear signal peptide and the sixth lacks a signal peptide as a control. Forty-four PGIP *in vitro* transgenic lines have been transferred to the greenhouse. Sixteen were manually inoculated with *Xf* and scored for tolerance to PD and movement of PGIP protein. Quantitative Real-Time PCR (qRT-PCR) analyses to compare *pgip* gene expression among different transgenic lines and untransformed control lines have been initiated. The remaining lines are being multiplied for future *Xylella* inoculation experiments.

Transgenic Thompson Seedless grapevines expressing the chimeric anti-microbial protein HNE-CecB expressed with its own signal peptide or with that from pear PGIP (pgip-HNE-CecB) have been obtained. The expressed chimeric anti-microbial protein has two functional domains. The surface recognition domain, SRD, specifically binds to the *Xf* outer-membrane protein MopB. The other domain inserts into the membrane, forming pores that lyse and kill *Xf*. Twenty-one of 47 HNE-CecB transgenic grapevine lines have been manually inoculated with *Xf* in the greenhouse. Preliminary observations show that most transgenic HNE-CecB-expressing lines showed limited or delayed disease symptoms compared to the severe PD symptoms of untransformed control plants. Five lines were substantially more resistant than the rest. Magnetic resonance imaging (MRI) of stem sections revealed fewer clogged vessels in these transgenic lines. Xylem sap from HNE-CecB transgenic lines with the resistant phenotype killed *Xf* more efficiently than that from untransformed controls. Interestingly, DNA extracted from the same HNE-CecB transgenic lines showed lower pathogen load than control plants. HNE protein expressed in transgenic plants was detected using enzyme-linked immunosorbent assay (ELISA). The remaining HNE-CecB and pgip-HNE-CecB lines are in the process of greenhouse propagation to conduct future *Xf* infection tests.

In addition to ongoing mechanical inoculation, we are in the initial preparation steps of a new and more precise experiment which is using blue-green sharpshooters (BGSS) (transmitting insect vector) to infect our more promising transgenic lines determined from previous results (**Figure 1**).

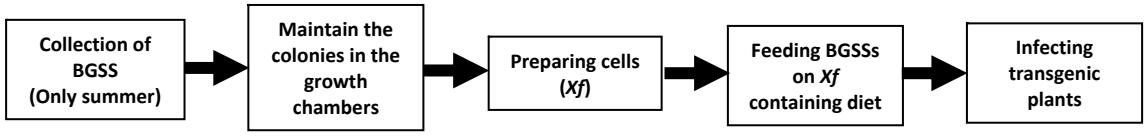

Figure 1: Steps involved in infection experiment by BGSS.

LAYPERSON SUMMARY

Transgenic grapevines are being evaluated as rootstocks that produce two types of effector proteins that may control Pierce's disease (PD) in untransformed scion cultivars grafted to such rootstocks. PD is caused by growth and movement of the bacterium *Xylella fastidiosa* (*Xf*) in the water-conducting xylem of the vine. As individual xylem elements are invaded and blocked, the vine is stressed, reducing vigor and productivity and eventually killing the vine. In this project, we are examining the ability of the xylem-targeted effector proteins polygalacturonase inhibiting protein (PGIP) to reduce disease severity by restricting the movement *Xf* across xylem elements and of a chimeric antimicrobial protein (HNE-CecB) to clear *Xf*, preventing its ability to colonize. We have created 44 vines expressing six different modifications of PGIP to better understand its ability to restrict disease spread. Sixteen of these plants are being evaluated in the greenhouse for resistance to PD and five lines show increased tolerance to PD over untransformed controls. Untransformed scions have been grafted movement of PGIP across the graft union. We have also evaluated 21of 47 HNE-CecB/pgipHNE-CecB lines in the greenhouse for clearance of *Xf*. At least five of the 21 evaluated lines show good tolerance to *Xf* infection and magnetic resonance imaging (MRI) of infected stem sections revealed fewer clogged vessels in the transgenic vines than in the controls, indicating clearance of the bacteria. Xylem sap from HNE-CecB transgenic lines inhibited growth of *Xf* more than sap from control vines. Interestingly, DNA extracted from the same HNE-CecB lines showed lower pathogen load than control plants. Further experiments with these transgenic lines will confirm the efficacy of these two effector proteins in controlling this important disease of grapevines.

OBJECTIVES

1. Evaluate the efficiency of different signal sequences in targeting PGIP to grapevine xylem tissue, through the graft union, and inhibiting infection with *Xf*.
2. Validate expression of chimeric antimicrobial proteins in transgenic grapevines, test for anti-*Xf* activity *in planta*, and test for graft transmissibility.

RESULTS AND DISCUSSION

1. <u>Evaluate the efficiency of different signal sequences in targeting PGIP to grapevine xylem tissue, through the graft union, and inhibiting infection with *Xf*</u>

Transgenic vines were created that express pear PGIP with six different signal peptide options (pDU06.0201 used chiPGIP; pDU05.1910, ntPGIP; pDA05.XSP, xspPGIP; pDU05.0401, ramyPGIP; pDU05.1002, mPGIP; and pDU94.0928, pPGIP). Four constructs contained different signal peptide sequences to identify which most efficiently localizes PGIP to xylem tissues and which provides the best distribution through the graft union into untransformed scion tissues. One construct used the pear signal peptide and the sixth was a control with no signal peptide. PGIP transgenic tissue extracts were assayed to validate transgene expression, which was highly variable. Based on polygalacturonase inhibiting activity, 44 PGIP *in vitro* transgenic lines (1 pPGIP, 6 ntPGIP, 8 chiPGIP, 12 mPGIP, 8 ramyPGIP, and 9 xspPGIP) have been transferred to the greenhouse.

Each acclimated transgenic line was propagated to obtain four to six mother plants that were further propagated to provide cuttings for *Xylella fastidiosa (Xf)* infection and grafting experiments. From each line, 25 to 35 plants were propagated from cuttings at the same time. *Xf infection* experiments were done in multiple rounds. Each round consisted of five to six transgenic lines with wild type Thompson Seedles s(TS) and TS50 as negative and positive control, respectively. Each round of experiments included 30 plants from each transgenic line. Fifteen were inoculated (Almeida and Purcell 2003) and the remaining 15 were non-inoculated controls. The positive control, TS50, is a transgenic PGIP-expressing grapevine previously described (Aguero et al. 2005).

Transgenic and control plants were inoculated with 20µL of a GFP-expressing *Xf* 3A2 culture (Newman et al. 2003) containing ~20,000,000 cells. The plants were inoculated with 10 µL the first day and re-inoculated with 10µL the second day; for each inoculation an independently grown *Xf* culture was used. The *Xf* was introduced into each plant three to four inches above the soil using an insect pin. Plants were pruned regularly and kept approximately 90-100cm tall until Pierce's Disease (PD) symptoms appeared. The time required to conduct each round of *Xf* challenge was 33 to 37 weeks, starting from *in vitro* plants transferred to greenhouse until the appearance of the first PD symptoms.

Twenty-two out of 44 PGIP transgenic lines are currently being evaluated for resistance to PD. Three rounds of infection have been completed on 16 PGIP transgenic lines manually inoculated with *Xf*. PD symptoms were scored on each infected plant using a standardized score based on percentage of leaf area scorching, a characteristic of PD (Krivanek et al. 2005a, 2005b). TS50 and five PGIP transgenic lines showed more tolerance to PD than the untransformed control (**Table 1**). Quantitative Real-Time PCR (qRT-PCR) analyses to compare *pgip* gene expression in PGIP transgenic lines and the untransformed control have been initiated. RNA was isolated from bottom and top stem sections of each infected and uninfected grapevine. Preliminary qRT-PCR data (Ct value, **Figure 2**) confirm that transgenic lines with more tolerance to PD had higher *pgip* gene expression. The remaining ntPGIP, chiPGIP, mPGIP, ramyPGIP, and xspPGIP lines are being propagated for future *Xf* challenge in the greenhouse. Those lines with low or moderate PD symptoms after manual inoculation will be tested by insect inoculation of *Xf*.

Table 1. PGIP transgenic lines status and results

Lines with yellow highlight are included in the field trial at Riverside and Solano counties. Siganl peptides: Chitinase (chi) and pathogen related protein 27 (PRp27)-like protein from *Nicotiana tobacum* (nt) from *Vitis vinifera*, polygalacturonase inhibiting protein from pear (pPGIP), rice amylase from *Oryza sativa* (Ramy), xylem sap protein from *Cucumis sativus* (XSP)

Line ID #	Gene	Construct	Status	Result
061045-015	chiPGIP	pDU06.0201	Tested-Round3	No Resistance
061045-056	chiPGIP	pDU06.0201	Tested-Round3	No Resistance
061045-070	chiPGIP	pDU06.0201	Tested-Round3	No Resistance
061045-077	chiPGIP	pDU06.0201	Tested-Round3	Low Resistance
061045-078	chiPGIP	pDU06.0201	Tested-Round3	No Resistance
061045-083	chiPGIP	pDU06.0201	Tested-Round3	No Resistance
061034-007	ntPGIP	pDU05.1910	Tested-Round4	No Resistance
061034-010	ntPGIP	pDU05.1910	Tested-Round4	No Resistance
061052-008	ntPGIP	pDU05.1910	Tested-Round4	High Resistance
061052-016	ntPGIP	pDU05.1910	Tested-Round4	High Resistance
061036-27	ntPGIP	pDU05.1910	Tested-Round5	No Resistance
061131-22	mPGIP	pDU05.1002	Tested-Round5	High resistance
061131-25	mPGIP	pDU05.1002	Tested-Round5	Low Resistant
061131-29	mPGIP	pDU05.1002	Tested-Round5	Low Resistance
061131-32	mPGIP	pDU05.1002	Tested-Round5	No Resistance
TS50	pPGIP	pDU94.0928	Tested-Round3-4	High resistance
061131-17	mPGIP	pDU05.1002	Tested-Round9	Exp. in progress
061131-35	mPGIP	pDU05.1002	Tested-Round9	Exp. in progress
061132-30	Ramy	pDU05.0401	Tested-Round9	Exp. in progress
061132-32	Ramy	pDU05.0401	Tested-Round9	Exp. in progress
061133-21	XSP	pDA05.XSP	Tested-Round9	Exp. in progress
061133-24	XSP	pDA05.XSP	Tested-Round9	Exp. in progress
061036-30	ntPGIP	pDU05.1910	To be tested	
061045-032	chiPGIP	pDU06.0201	To be tested	
061045-035	chiPGIP	pDU06.0201	To be tested	
061131-19	mPGIP	pDU05.1002	To be tested	
061131-21	mPGIP	pDU05.1002	To be tested	
061131-24	mPGIP	pDU05.1002	To be tested	
061131-27	mPGIP	pDU05.1002	To be tested	
061131-28	mPGIP	pDU05.1002	To be tested	
061131-31	mPGIP	pDU05.1002	To be tested	
061132-19	Ramy	pDU05.0401	To be tested	
061132-24	Ramy	pDU05.0401	To be tested	
061132-28	Ramy	pDU05.0401	To be tested	
071073-33	Ramy	pDU05.0401	To be tested	
071073-40	Ramy	pDU05.0401	To be tested	
071073-83	Ramy	pDU05.0401	To be tested	
061133-2	XSP	pDA05.XSP	To be tested	
061133-22	XSP	pDA05.XSP	To be tested	
061133-23	XSP	pDA05.XSP	To be tested	
061133-25	XSP	pDA05.XSP	To be tested	
061133-27	XSP	pDA05.XSP	To be tested	
061133-28	XSP	pDA05.XSP	To be tested	
061133-29	XSP	pDA05.XSP	To be tested	
061133-32	XSP	pDA05.XSP	To be tested	

To evaluate the efficiency of secretion, each transgenic line expressing each of the signal sequences fused to PGIP will be used as transgenic rootstocks grafted to wild type scions. After growth xylem sap will be extracted from the stem and leaves of the wild type scion to evaluate the amount of PGIP that is translocated via the xylem into the wild type tissues. The movement of the PGIP protein from the rootstock up into the xylem of the wild type scion was evaluated using a radial diffusion assay (Aguero et al. 2005). Preliminary testing of PGIP activity using leaf extracts and xylem sap from non-grafted TS50 (positive control), ChiPGIP 45-35 and ChiPGIP 45-83 showed PG inhibiting activity. The same lines when grafted

also showed inhibiting activity from leaf extract and xylem sap. TS50 showed the highest activity in grafted and non-grafted leaf and non-grafted xylem sap. Interestingly, xylem sap from Chi45-35 and Chi45-83 showed a greater inhibition when they were grafted with wild type TS as compared to non-grafted, indicating that the PGIP was moving quite efficiently from the rootstock to the scion with these particular signal peptides.

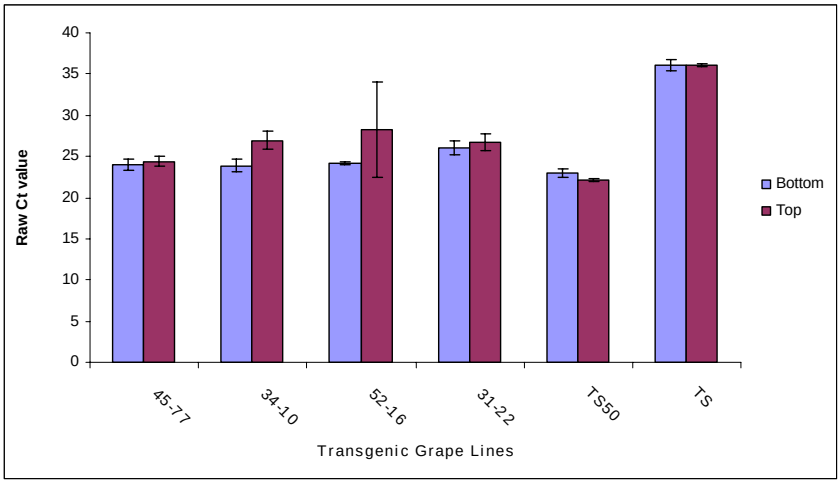

Figure 2: Expression of *pgip* gene on top and bottom stem section of transgenic grapevines inoculated with *Xf*. Lower Ct values represent higher pgip expression.

2. Validate expression of chimeric antimicrobial proteins in transgenic grapevines, test for anti-*Xf* activity *in planta*, and test for graft transmissibility

Transgenic grapevine plants were obtained with the two constructs pDU04.6105 (Elastase-Cecropin = HNE-CecB) and pDA05.0525 (pgipSP-Elastase-Cecropin= pgipHNE-CecB) as described in earlier reports. Twenty-six of 47 HNE-CecB lines are currently being evaluated for resistance/tolerance to PD. The first four rounds of infection have been completed for 21 transgenic lines. Within six to seven weeks post inoculation, the first PD-associated leaf scorch symptoms appeared: formation of green islands on the cane and scorching around outer edges of the lower leaves were visible on control grapevines (**Figure 3**). Most transgenic HNE-CecB-expressing lines showed less or delayed disease symptoms than controls and five lines were substantially more resistant than the rest. PD symptoms on each infected plant were numerically scored based on percentage leaf area scorch (**Table 2**).

Figure 3: Transgenic line (40-60) in the right showing less disease symptom compared to non-transgenic TS on the left.

Table 2. HNE-CecB and pgipHNE-CecB Transgenic Lines Status and results
Lines with yellow highlight are included in the field trial at Riverside and Solano counties.

Line ID Num.	Gene	Construct	Status	Result
0999040-39	HNE-CecB	pDU04.6105	Tested-Round1	Low Resistance
0999040-41	HNE-CecB	pDU04.6105	Tested-Round1	Moderately Resistance
0999041-151	HNE-CecB	pDU04.6105	Tested-Round1	High Resistance
0999041-166	HNE-CecB	pDU04.6105	Tested-Round1	Moderately Resistance
0999041-179	HNE-CecB	pDU04.6105	Tested-Round1	Low Resistance
0999040-36	HNE-CecB	pDU04.6105	Tested-Round2	Moderately Resistance
0999040-74	HNE-CecB	pDU04.6105	Tested-Round2	Moderately Resistance
0999040-89	HNE-CecB	pDU04.6105	Tested-Round2	High Resistance
0999040-92	HNE-CecB	pDU04.6105	Tested-Round2	High Resistance
0999041-146	HNE-CecB	pDU04.6105	Tested-Round2	Highly Resistance
0999041-157	HNE-CecB	pDU04.6105	Tested-Round2	Moderately Resistance
0999040-87	HNE-CecB	pDU04.6105	Tested-Round6	Not Resistance
0999040-96	HNE-CecB	pDU04.6105	Tested-Round6	Not Resistance
0999040-97	HNE-CecB	pDU04.6105	Tested-Round6	Not Resistance
0999041-164	HNE-CecB	pDU04.6105	Tested-Round6	Moderately Resistance
0999041-181	HNE-CecB	pDU04.6105	Tested-Round6	Low Resistance
0999040-60	HNE-CecB	pDU04.6105	Tested-Round7	Moderately Resistance
0999040-80	HNE-CecB	pDU04.6105	Tested-Round7	Not Resistance
0999040-85	HNE-CecB	pDU04.6105	Tested-Round7	Not Resistance
0999041-180	HNE-CecB	pDU04.6105	Tested-Round7	Not Resistance
099946-019	pgip HNE-CecB	pDA05.0525	Tested-Round7	Not Resistance
099944-007	pgip HNE-CecB	pDA05.0525	Tested-Round8	Exp. in progress
099946-002	pgip HNE-CecB	pDA05.0525	Tested-Round8	Exp. in progress
099946-014	pgip HNE-CecB	pDA05.0525	Tested-Round8	Exp. in progress
0999040-4	HNE-CecB	pDU04.6105	Tested-Round8	Exp. in progress
099944-026	pgip HNE-CecB	pDA05.0525	To be tested	
099946-018	pgip HNE-CecB	pDA05.0525	To be tested	
099946-020	pgip HNE-CecB	pDA05.0525	To be tested	
061073-016	pgip HNE-CecB	pDA05.0525	To be tested	
061073-028	pgip HNE-CecB	pDA05.0525	To be tested	
061073-036	pgip HNE-CecB	pDA05.0525	To be tested	
0999040-11	HNE-CecB	pDU04.6105	To be tested	
0999040-42	HNE-CecB	pDU04.6105	To be tested	
0999040-51	HNE-CecB	pDU04.6105	To be tested	
0999040-62	HNE-CecB	pDU04.6105	To be tested	
0999040-69	HNE-CecB	pDU04.6105	To be tested	
0999040-78	HNE-CecB	pDU04.6105	To be tested	
0999040-79	HNE-CecB	pDU04.6105	To be tested	
0999040-81	HNE-CecB	pDU04.6105	To be tested	
0999040-106	HNE-CecB	pDU04.6105	To be tested	
0999040-112	HNE-CecB	pDU04.6105	To be tested	
0999041-125	HNE-CecB	pDU04.6105	To be tested	
0999041-132	HNE-CecB	pDU04.6105	To be tested	
0999041-155	HNE-CecB	pDU04.6105	To be tested	
0999041-168	HNE-CecB	pDU04.6105	To be tested	
0999041-169	HNE-CecB	pDU04.6105	To be tested	
0999041-171	HNE-CecB	pDU04.6105	To be tested	
0999041-174	HNE-CecB	pDU04.6105	To be tested	

MRI images from stem sections approximately 15 to 20 cm above the inoculation point revealed that clearance of bacterial inoculums in transgenic lines expressing fewer PD symptoms correlated to fewer clogged vessels than in the control lines. To obtain MRI xylem vessel cross section images, an Avance 400 instrument was used. Instrument settings were: TR: 110.7, TE: 4.5ms, FA: 30.0deg, TA: 1:25NEx4, FOV: 1.2cm, MTX 256/192, Pos-0.80mmF.

Xylem sap extracted from grape plants expressing HNE-CecB kills bacteria. *Xf* was incubated with xylem sap extracted from transgenic lines at 28°C on a shaker. For each sample, three different dilutions of the *Xf*-xylem sap mixture were plated on PD3 media each hour for five hours. This experiment reveals the antimicrobial activity of sap from transgenic lines expressing HNE-CecB antimicrobial protein. Transgenic lines expressing high phenotypic resistance (**Table 2**) also displayed higher *Xf* mortality rates than untransformed and buffer controls.

Semi-Quantitative PCR analysis of Xf DNA accumulation was performed on groups of three stems sections collected approximately 10 to15 cm above the point of inoculation. For each transgenic line, DNA was extracted from groups of three stem samples representing three individual plants. Plants expressing HNE-CecB contained less bacterial DNA, and thus a lower pathogen load in the plant tissue, indicating more clearance.

We detected the HNE protein in transgenic plants using enzyme-linked immunosorbent assay (ELISA). Commercially available polyclonal antibody generated against elastase was used to create an ELISA sandwich assay; however, for greater precision we must generate a more specific monoclonal antibody against the chimeric protein (**Figure 4**). Total protein was extracted from transgenic plant tissue using a sodium bicarbonate extraction buffer (0.1M NaHCO3, pH 8.6). A 96-well MaxisorpTM microtiter plate (NUNC, NY, USA) was coated with 100 µL of the transgenic or control crude protein extracts overnight at 4ºC. The wells were washed one time with PBS-T 0.1% and blocked for one hour at 37ºC with PBS-BSA 3%. After blocking, wells were washed twice with PBS-T 0.1% followed by incubation with anti-elastase antibody (1:1000) at 37ºC for 1 hour. The plate was washed three times with PBS-T 0.1% followed by incubation with AP-conjugated anti-rabbit diluted (1:5000) in PBS-BSA 3% for 1 h at 37ºC. The plate was washed four times in PBS-T 0.1%, developed at 405 nm.

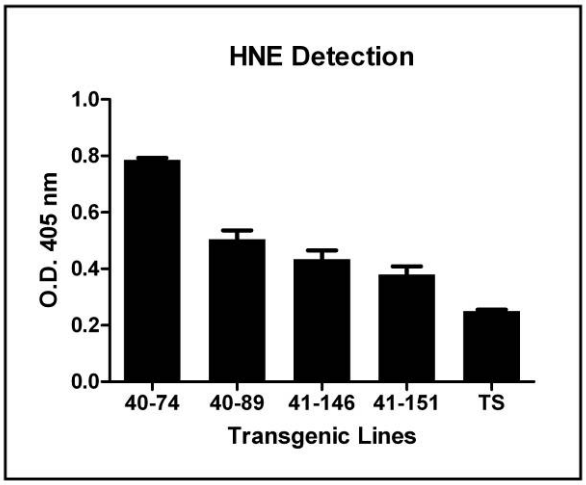

Figure 4. HNE detection by ELISA

CONCLUSIONS

The main objective of this project is to validate the efficacy of the xylem targeted effector proteins polygalacturonase inhibiting protein (PGIP) to limit movement and of a chimeric antimicrobial protein HNE-CecB to clear *Xf*.

Transgenic grapevines lines expressing PGIP with four different signal peptides are being evaluated for their improved ability to secrete PGIP long distance through the graft union. Sixteen of these plants are being evaluated in the greenhouse for resistance to PD and five lines show increased tolerance to PD over untransformed controls. Untransformed scions have been grafted onto PGIP transgenic lines to evaluate the long distance movement of PGIP. Quantitative Real-Time PCR (qRT-PCR) analyses to compare *pgip* gene expression among different transgenic lines and untransformed control lines have been initiated.

Twenty one HNE-CECB transgenic grapevine lines have been evaluated for PD resistance by inoculating with *Xf*. Several promising transgenic lines showed low or moderate symptoms of PD. MRI stem sectioned images revealed a variation in number of vessels clogged between negative control and HNE-CECB transgenic lines, indicating that clearance of the bacteria may be occurring in some of the transgenic lines (Dandekar et al. 2009). Further protein assays are required to detect and quantify the expression of transgenic protein.

Insect inoculation of transgenic plants via bluegreen sharpshooter has been initiated to test a more natural mode of disease transmission and better predict field resistance levels, since these vines will be inoculated with a more realistic number of bacteria.

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GRAPE RECOGNITION OF *XYLELLA* SURFACE PROTEINS AND THEIR RELATIONSHIP TO PIERCE'S DISEASE SYMPTOM DEVELOPMENT

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ABSTRACT
The aim of this project is to understand the role played by surface proteins of *Xylella fastidiosa* (*Xf*), especially the translation elongation factor "temperature unstable" (EF-Tu), in disease induction by this bacterium, the causative agent of Pierce's disease (PD) of grapevine, and to use this information to interfere with disease induction. Previously we demonstrated that *Xf* EF-Tu induces chlorosis when pressure infiltrated into leaves of *Chenopodium quinoa*, suggesting that EF-Tu may be a protein recognized by plants as a signal of *Xf* infection. Although the primary function of EF-Tu in eubacteria is in protein synthesis, certain bacterial species have evolved to use EF-Tu for other applications, including binding the bacterium to host cells. EF-Tu is associated an insoluble fraction of *Xf* cells and hence cannot be purified by conventional methods. The use of *Agrobacterium* to program plant cells to synthesize and target this protein to the plant apoplast has allowed the testing of EF-Tu's ability to cause PD-like symptoms in both *Nicotiana tabacum* cv. SR-1 and Thompson seedless grapes without purifying the EF-Tu protein. For both plant species, infiltration of *Agrobacterium* carrying an engineered binary vector allowed in planta expression and apoplast secretion of EF-Tu, resulting in PD-like symptoms in the absence of *Xf* cells or other *Xf* proteins. To introduce Agrobacterium into the difficult-to-infiltrate grapevine leaf, we developed a new infiltration procedure. To locate EF-Tu within the *Xf* cell, we treated *Xf* cells with detergent and lysozyme and examined the resulting spheroplast-like structures, which were found to retain EF-Tu. The EF-Tu that is present in these spheroplast-like structures, compared to EF-Tu in intact cells, is far more accessible to staining by fluorescent anti-EF-Tu antibody. Intact *Xf* cells were examined by immunogold electron microscopy of thin sections from centrifuged *Xf* cell pellets. EF-Tu and MopB, a major outer membrane protein of *Xf*, were very similarly distributed, primarily near the cell surface. These results support our earlier assertion that a fraction of EF-Tu is associated with the outer or inner membrane of the *Xf* cell surface.

LAYPERSON SUMMARY
The elongation factor "temperature unstable," EF-Tu, is an abundant soluble protein in most bacteria but in *Xylella fastidiosa* (*Xf*) is a protein found mainly at or just under the bacterial cell surface and in an insoluble fraction when the cells are disrupted. Insolubility prevents purification of EF-Tu by conventional means and makes determining the functions of *Xf* EF-Tu difficult. *Agrobacterium tumefaciens* is a bacterium capable of delivering genes to plant cells. We engineered *A. tumefaciens* to induce, when infiltrated into leaves, plant cells to generate *Xf* EF-Tu and to secrete the protein into intercellular spaces, bypassing the need to purify EF-Tu. Infiltrated leaves of both tobacco and grapevine developed symptoms characteristic of Pierce's disease (PD). That *Xf* EF-Tu, in the absence of *Xf* cells or any other *Xf* protein, appears to induce PD-like symptoms suggests that host plant reaction to EF-Tu released from *Xf* cells may be important in PD and may explain the observation of leaf-margin scorching in natural *Xf* infections even though *Xf* bacteria may be present only more centrally in the leaf or petiole. Understanding how this protein is recognized and how recognition leads to symptoms may lead to new ways of ameliorating PD symptoms.

INTRODUCTION
Long term, economical and sustainable control of Pierce's disease (PD) is likely to be achieved most effectively by deploying grapevine cultivars resistant to or tolerant of *Xylella fastidiosa (Xf).* Interference with symptom development (i.e., creation of tolerance) is conceivable by preventing the full functioning of *Xf* virulence factors. The mechanisms by which *Xf* induces symptoms in infected grapevine have not been established. *Xf* surface proteins are candidates for symptom-inducing factors. Examples of *Xf* surface proteins are a major outer membrane protein MopB (Bruening and Civerolo 2004), the hemagglutinin-like minor outer membrane proteins H*Xf*A and H*Xf*B (Guilhabert and Kirkpatrick 2005), a protein that is recognized by a single chain, monoclonal antibody (Bruening et al. 2008), and possibly a form of the protein synthesis elongation factor "temperature-unstable" (EF-Tu) (Bruening et al. 2008). We reported earlier that EF-Tu was the major component of a minor trailing band observed after electrophoresis of partially purified MopB through sodium dodecyl sulfate- (SDS-) permeated polyacrylamide gel. *Xf* EF-Tu was recovered by elution from excised gel pieces from the trailing band and was shown to induce chlorosis in *Chenopodium quinoa* (Bruening et al. 2007), whereas *Xf* MopB produced in transformed *E. coli* failed to induce chlorosis in *C. quinoa*. These observations suggest that the chlorosis-inducing factor in our MopB preparations may be *Xf* EF-Tu and not MopB, formerly the candidate chlorosis-inducing factor.

EF-Tu is one of a small number of highly conserved eubacterial macromolecules that have been categorized as "microbe-associated molecular patterns" (MAMPs) because of their ability to induce defense responses in specific plants (Jones and

Dangl 2006). Flagellin, chitin, certain lipopolysaccharides, and a few other molecules are other MAMPs. EF-Tu is the most abundant soluble protein of rapidly growing *E. coli* cells, so it is reasonable for it to serve as a signal for the presence of bacteria. The MAMP activity of *E. coli* EF-Tu is illustrated by alkalization of the medium of cultured *Arabidopsis thaliana* cells on exposure to subnanomolar concentrations of EF-Tu. When introduced at 1 µM concentration by pressure-infiltration into Arabidopsis leaves, *E. coli* EF-Tu induced resistance to *Pseudomonas syringae* and caused Arabidopsis to accumulate defense gene mRNAs (Kunze et al. 2004). *E. coli* EF-Tu and *Xf* EF-Tu gene sequences show 77% identity and 88% similarity in amino acid sequence, and both proteins induce chlorosis when pressure infiltrated into C. chenopodium leaves (Bruening et al. 2007). Those regions that show identity between the *E. coli* and *Xf* EF-Tu gene sequences also showed >90% identity with >100 eubacterial EF-Tu sequences (Kunze et al. 2004). Some bacteria have evolved an EF-Tu protein with at least one additional function, beyond participating in polypeptide chain elongation or acting as an elicitor. *Mycoplasma pneumoniae* and *Lactobacillus johnsonii* appear to use EF-Tu as an adhesin that is responsible for the binding of these bacteria to human cells, and, in the case of *M. pneumoniae*, antibody to EF-Tu was demonstrated to interfere with attachment to human cells (Dallo et al. 2002, Granato et al. 2004). Therefore, it will not be surprising if *Xf* EF-Tu is found to be capable of inducing reactions in grapevine, including reactions that lead to symptom development. This work is an extension of our previous project entitled "Exploiting *Xylella fastidiosa* Proteins For Pierce's Disease Control"**.** The objectives for the current project are given below.

OBJECTIVES

1. Test *Xf* EF-Tu for its ability to induce scorching in grapevine
2. Identify a grapevine receptor for *Xf* EF-Tu
3. Interfere with *Xf* EF-Tu-induction of scorching using RNAi or by expression of alternative receptor
4. Characterization of *Xf* EF-Tu and its immobilization and localization

RESULTS AND DISCUSSION

Xf EF-Tu expression from Agrobacterium induces PD-like symptoms in SR-1 Nicotiana tabacum

Results reported here advance Objective 1. The *Xf* cell surface location and known MAMP activity of EF-Tu suggest that accumulation of this protein in the intercellular spaces of leaves could induce scorching symptoms at locations not reached by *Xf* cells. The occurrence of *Xf* EF-Tu in an insoluble fraction after disruption of *Xf* cells prevents EF-Tu purification by conventional liquid phase separation methods. Therefore, we took the alternative approach of inducing plant cells to synthesize *Xf* EF-Tu and to secrete the protein into the (extracellular) apoplast. The apoplastic targeting sequence P14 from the plant pathogenesis-related protein PR-1 (Vera et al. 1989) has been shown by Jim Lincoln of the David Gilchrist laboratory to direct green fluorescent protein (SGFP) to intercellular spaces (data not shown).

The state of the expressed EF-Tu amino end could be significant to its biological activity because the known receptor for *E. coli* and other EF-Tu's in brassicas, designated elongation factor receptor (EFR), appears to recognize the EF-Tu amino end (Kunze et al. 2004). Therefore, the *Cowpea mosaic virus* 24K protease (CPMV 24KPro, a protease that includes cleavage of Q/M bonds in its range of specificity and capable of releasing itself from the interior region of a polyprotein) was interposed between P14 and *Xf* EF-Tu (**Figure 1**, construction E1) to create the native methionine amino end of the *Xf* EF-Tu protein. The *Xf* EF-Tu construct, P14-CPMV24KPro-*Xf* Ef-Tu, that replaces the SGFP with *Xf* EF-Tu, construct was difficult to make. Creation of construction E1, but not G1, required strong selection accomplished by use of a chloroamphenicol resistance gene cassette, suggesting a sickening effect of *Xf* EF-Tu on E. coli even without intentional expression of the protein.

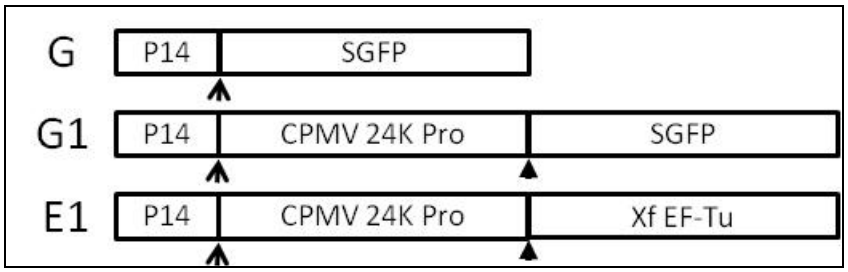

Figure 1. Coding portions of constructs used to export SGFP or *Xf* EF-Tu to the apoplast after Agrobacterium infiltration. P14 and SGFP are defined above. CPMV 24K Pro is the protease of *Cowpea mosaic virus*. The open arrowheads locate the cleavage site of the P14 apoplastic targeting sequence removing protease and the closed arrowheads locate the cleavage site of the CPMV 24K Pro, intended to release *Xf* EF-Tu with the authentic amino end methionine.

The presence of the CPMV 24KPro in construction E1 could interfere with apoplast targeting of *Xf* EF-Tu. Therefore, agroinfiltration into *N. benthamiana* of constructions G (gift from the Gilchrist laboratory) and G1 were compared (**Figure 2**). As expected, construction G targeted GFP to the intercellular spaces. Construction G1 produce little or no GFP signal but apparently induced a spongiform appearance suggesting loss of cells in the stomatal cavities. Similar results were observed for agroinfiltration of *N. tabacum* line SR-1 (not shown). Although these results, and our observation of only limited accumulation of 24KPro (not shown), both suggest that construction E1-mediated accumulation of *Xf* EF-Tu will be limited, the loss of plant cells could result in greater release of EF-Tu into intercellular spaces.

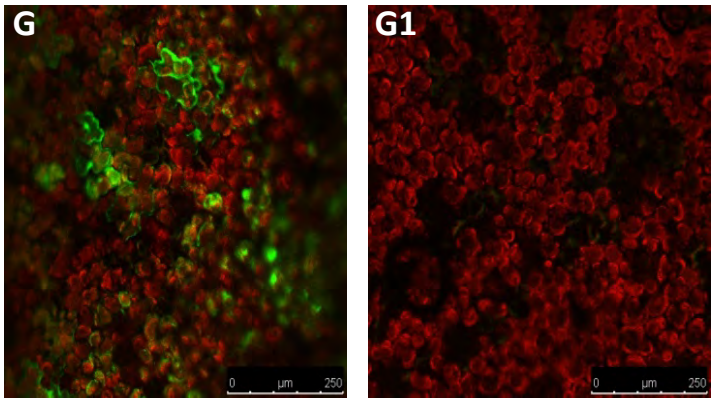

Figure 2. Laser confocal microscope images of *N. benthamiana* infiltrated with Agrobacterium strain GV2260 containing the binary vector pCB4NN with inserts G or G1 (**Figure 1**).

Agroinfiltration of constructs G (image not shown), G1 and E1 resulted in development of chlorosis within two days. However, the leaf areas infiltrated with construct E1 (P14-CPMV24KPro-EF-Tu) SGFP (**Figure 3**, panel A, right leaf panel) showed an incipient necrosis at two days post infiltration and, at five days, substantial necrosis (**Figure 3**). Areas infiltrated with G or G1 constructions remained chlorotic at five days post infiltration. These necrotic symptoms are similar to those seen for SR-1 tobacco inoculated with *Xf* (Francis et al. 2008).

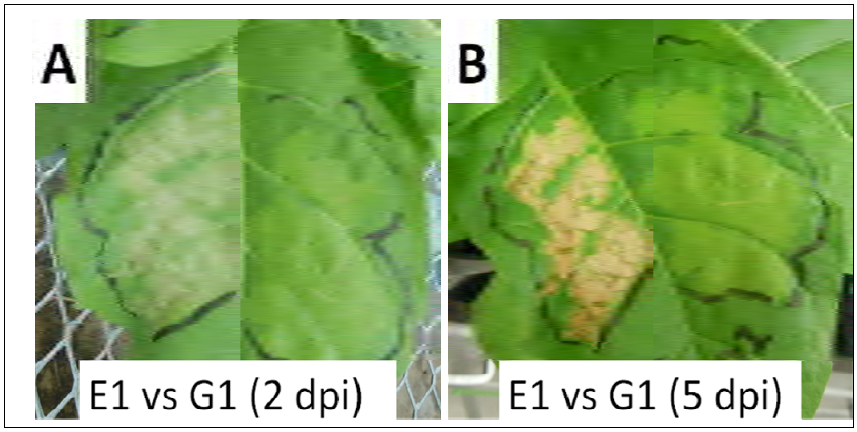

Figure 3. Opposite half-leaf agroinfiltrations of SR-1 tobacco with constructs E1 and G1 (**Figure 1**) for expression of P14-CPMV24KPro-SGFP and P14-CPMV24KPro-*Xf* EF-Tu, respectively. Photographs were taken at two and five days post infiltration.

The obvious interpretation of the **Figure 3** result is that EF-Tu is recognized by SR-1 tobacco, resulting in the development of symptoms characteristic of *Xf* infection of SR-1 tobacco but without an actual *Xf* infection or the presence of *Xf* cells or even the presence of any other *Xf* protein. There is, however, a reservation about this interpretation. Although similar binary vectors were used for the E1 and G1 constructions, the 3' and 5' untranslated regions flanking the fusion protein open reading frames (ORFs) of **Figure 1** are different. The E1 construct has CPMV RNA2 5' and 3' untranslated regions to increase translation efficiency (pEAQ-HT vector from George Lomonosoff , Sainsbury Laboratory, Norwich, UK) (Sainsbury and Lomonossoff 2008). The G and G1 constructs use the TMV omega 5' untranslated region (Gallie et al.

1987). Therefore, it is conceivable that the untranslated regions contribute to the differential seen in **Figure 3**. Constructs with the same 3' and 5' regions are complete and will be tested shortly. EF-Tu and GFP constructs without CPMV 24KPro also are being prepared. Another complicating factor is the possibly sickening effects of the constructs on *E. coli* and *A. tumefaciens* itself. We will be preparing constructs with introns inserted into either the CPMV24KPro, or *Xf* EF-Tu coding regions, or both, to allow the bacteria to escape the putative toxic effects of these constructions.

Xf EF-Tu expression from Agrobacterium induces PD-like symptoms in Thompson seedless grapevine

Results reported in this section advance Objective 1. The leaves of tobacco plants are readily pressure infiltrated with *Agrobacterium* cell suspensions by simply pressing a syringe (no needle) against the lower leaf epidermis, supporting the leaf at the opposite upper epidermis, and gradually pressing the syringe piston. The infiltrated leaf area is demarcated by the appearance of water logging. Grapevine leaves resist pressure infiltration so effectively that only a region corresponding to the bore of the syringe end shows water logging or, subsequently, reporter gene (typically GFP) activity. For most infiltration sites, no reporter activity is observed under the microscope (J. E. Lincoln, personal communication).

To test the constructions for symptom induction by agroinfiltration into grapevine, the infiltration process must be improved. In the new method developed here, grapevine leaves, detached or still on the excised cane or the intact potted plant, were very lightly abraded on the lower epidermis by dusting with 600 grit carborundum, wetting with water, and then rubbing very lightly with a gloved finger for 15 -20 seconds. The leaf was rinsed with distilled water and placed, lower epidermis up, on a glass frit filter support connected to house vacuum. The weak vacuum holds the leaf in place. 200ul of Agrobacterium suspension in dilute wetting agent was applied to the lower epidermis. Substantial entry of the liquid was evidenced by apparent water soaking in a number of small areas spread over carborundum-rubbed area of the leaf. At one day post infiltration (dpi), there was no evidence of macroscopic damage or any negative effects on the leaf from the infiltration process.

GFP fluorescence was observed in areas infiltrated with either the G (**Figure 4**.) or G1 (data not shown) constructions. A distinction between the results for the *Nicotiana* species and grapevine is that the 24KPro of the G1 construction did not seem to cause cell death in grapevine.

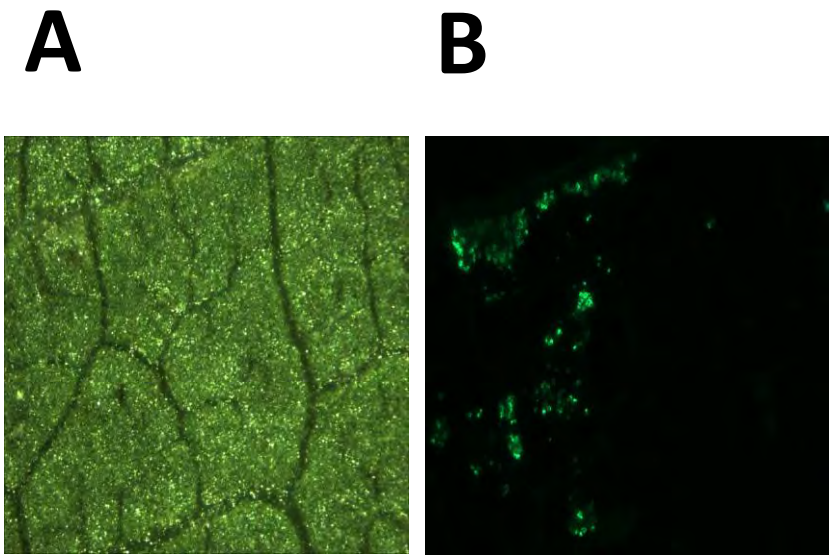

Figure 4. Images of grape leaf infiltrated with *Agrobacterium* containing the G (P14-GFP) construct. A. White light image. B. 36 second exposure using 488nm light and GFP expression filters. Note the SGFP expression is directly below the gouge seen in the upper left of panel A.

Figure 5 shows results from an experiment in which the E1 construction, designed to express EF-Tu, was agroinfiltrated into leaves on a detached cane of Thompson Seedless grapevine. A necrosis was visible at one dpi, and the necrotic area expanded with time. These symptoms were similar to those seen for E1-infiltrated SR-1 tobacco (**Figure 3**). Infiltration with the GFP-expressing G1 construction did not induce necrosis (image not shown).

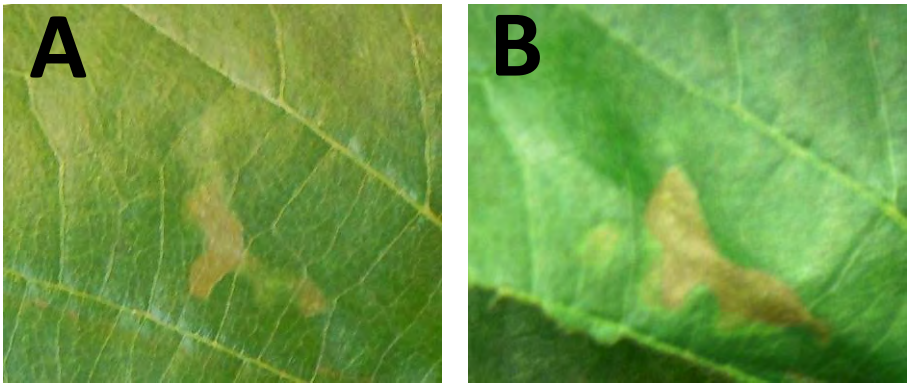

Figure 5. Symptoms induced on Thompson seedless grapevine leaves after infiltration with *Agrobacterium* containing the E1 construct (**Figure 1**). A. Symptoms at one dpi. B. Symptoms at two dpi. The yellowing that appears below the brown area in panel B is a photographic artifact resulting from removal of an infiltrated panel for microscopy, which allowed a leaf below to be observed through the opening.

EF-Tu and mopB localization in Xf cells by electron microscopy and immunogold labeling

This section is concerned with Objective 4. *Xf* strains HxfA- and HxfB- were used in these experiments. These *Xf* strains bear transposon-inactivated genes for hemagglutinin-like proteins A and B (Guilhabert and Kirkpatrick, 2005) and were selected because of their rapid growth in culture. We have previously shown that treatment of *Xf* cells with Bugbuster (EMD BioSciences), a proprietary detergent solution, and recombinant egg white lysozyme, followed by low speed centrifugation leads to recovery of roughly two thirds, by volume, of the original cell pellet material (Bruening et al. 2008). In contrast, similar treatment of *E. coli* cells or *Xanthomonas campestris* cells resulted in complete clearing of the solution with insignificant recovery of material after low speed centrifugation. The *Xf*-derived material that survived Bugbuster and lysozyme treatment was layered on a 50 to 80% sucrose gradient and centrifuged, resulting in a substantial band of intermediate density (Bruening et al. 2008). Immunoblots showed that most of the EF-Tu protein is present in this sucrose-buoyant band. Ms. Darlene Hoffmann of USDA-ARS Parlier examined these samples by electron microscopy, comparing untreated and Bugbuster- and lysozyme-treated *Xf* cells. A rough estimate of the surface area of intact HxfA- *Xf* cells cells (**Figure 6A**), which are approximately 2 µ long and 0.5 µ diameter, is comparable to an estimate for the surface area of the apparently spherical entities, of average diameter 0.7 µ, that survived treatment of HxfA- *Xf* cells with Bugbuster and lysozyme (**Figure 6B**). The spherical particles were not seen in electron micrographs of *Xf* cell treated with Bugbuster alone.

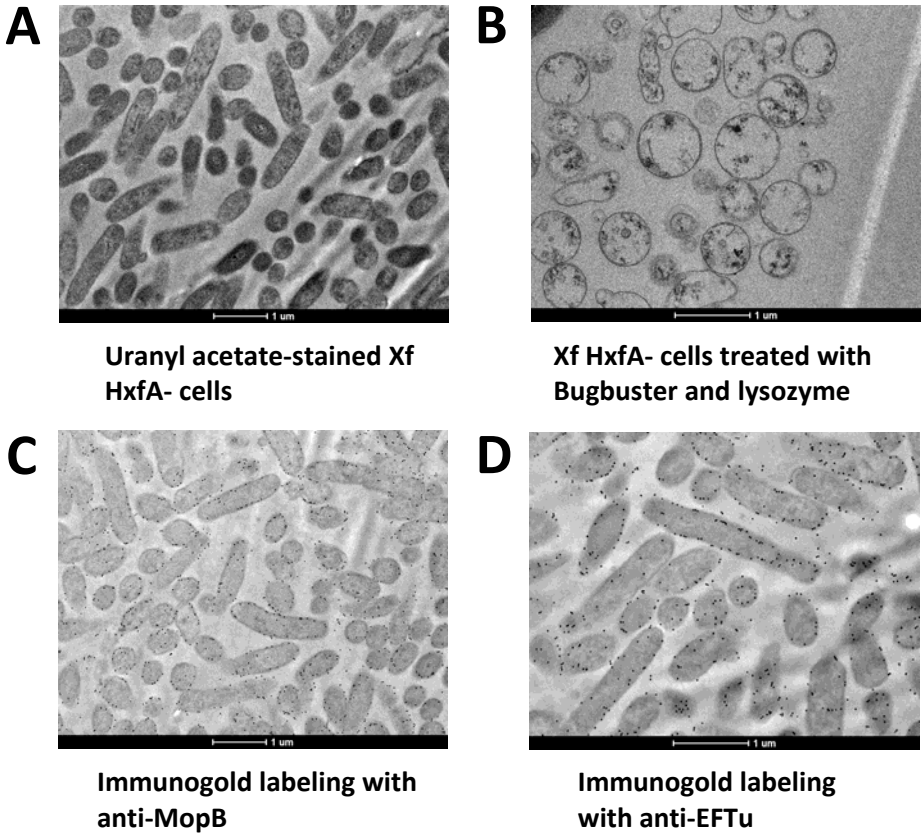

Figure 6. Thin section transmission electron micrographs of *Xf* cells and material released from *Xf* cells by treatment with detergent and lysozyme. **A**. intact HxfA- *Xf* cells. **B**. HxfA- *Xf* cells treated with Bugbuster detergent and recombinant egg white lysozyme. Material was centrifuged, and the resulting pellet was sectioned. Sections were stained with uranyl acetate to prepare the sample for microscopy. For C and D, sections were not stained but were exposed to rabbit polyclonal antibody raised against (**C**) partially purified *Xf* MopB (abundant outer membrane protein) or against (**D**) a synthetic peptide from the *Xf* EF-Tu amino acid sequence coupled to keyhole limpet hemocyanin, each antibody at a dilution of 1:250. The secondary was immunogold-labeled goat anti-rabbit antibody.

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As was observed previously, intact *Xf* cells bind antibody to *Xf* MopB but did not bind Alexa-488-labeled anti-EF-Tu antibody sufficiently to show localized fluorescence. However, the material that survived treatment with Bugbuster detergent and lysozyme was found to bind the fluorescent anti-EF-Tu antibody tightly enough to survive centrifugal washing. As is shown by **Figure 7**, detergent- and lysozyme-treated *Xf* cell material that was partially purified by sucrose gradient centrifugation bound the fluorescent antibody. However, much more of the fluorescent antibody bound to the pellet material

recovered after conventional centrifugation, suggesting that the EF-Tu, though immobilized in the treated *Xf* cell material, can be released by the multiple washing steps that occur as the treated *Xf* cell material passed from the top of the gradient to its buoyant position.

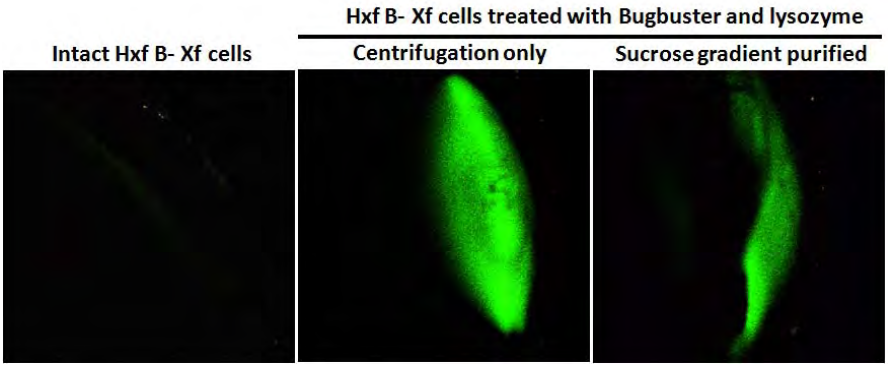

Figure 7. Binding of Alexa-488-labeled anti-EF-Tu antibody to HxfB- *Xf* cells that had been treated with Bugbuster and lysozyme. Intact HxfB- *Xf* cells and HxfB- *Xf* cells incubated with detergent and lysozyme were compared. The latter were treated in two ways, either recovered after incubation by conventional centrifugation or by conventional centrifugation followed by centrifugation through a 50% to 80% sucrose gradient and then recovery from sucrose solution by another centrifugation step. Preparations were exposed to Alexa-488-labeled anti-EF-Tu antibody for one hour, and the materials were washed three times by centrifugation. After the final centrifugation, the bottoms of the Eppendorf tubes were examined under 488 nm illumination using a stereo microscope.

Figure 6, panels C and D, provide evidence about the distribution of MopB and EF-Tu within intact HxfA- cells, using immunogold labeling with antibodies against these two proteins. As expected, MopB, which is presumed to be the major outer membrane protein of *Xf*, appears to localized primarily at the periphery of the cells. As EF-Tu is a component of the cellular translational machinery, it would be expected that it would be localized to the interior of the cells. However, the majority of *Xf* EF-Tu appeared to be distributed similarly to the distribution for MopB, also apparently localized to the cellular periphery, consistent with a possible role for *Xf* EF-Tu in a cytoskeleton-like structure. That exposure of XxfA- cells to detergent and lysozyme makes EF-Tu available for binding fluorescent antibody, whereas MopB antibody can react directly of HxfA- cells suggests that EF-Tu may not be as close to the cell surface as MopB is. A preliminary analysis of the spatial relationship between the immunogold particles bound by antibody to MopB and the immunogold particles bound by antibody to EF-Tu suggests that they are essentially coincident, but more sophisticated analyses are in progress.

CONCLUSIONS

Xf EF-Tu has been demonstrated to be present in a peripheral location of the *Xf* cell, similar to that of the outer membrane protein MopB, using fluorescent antibody and immunogold labeling. A peripheral location could allow for the release and recognition by the plant of EF-Tu, consistent with our earlier observation that *E. coli* EF-Tu protein, introduced as the purified protein into leaf intercellular spaces, is capable of activating a *Xf*-responsive grapevine promoter. Here we used transient expression and constructions designed to cause EF-Tu to be synthesized in leaf cells and be secreted into leaf intercellular spaces. This construct induced PD-like symptoms in tobacco and grapevine, without the presence of *Xf* cells or other *Xf* proteins. Very likely, grapevine has a receptor, possibly similar to the EFR receptor of *Arabidopsis*, that can detect *Xf* EF-Tu. The system developed here should allow us to identify regions of the *Xf* EF-Tu protein molecule that are recognized by the grapevine receptor. With this information in hand, we can attempt to reduce PD symptom development by interfering with this recognition.

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PIERCE'S DISEASE CONTROL AND BACTERIAL POPULATION DYNAMICS IN WINEGRAPE VARIETIES GRAFTED TO ROOTSTOCKS EXPRESSING ANTI-APOPTOTIC SEQUENCES.

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ABSTRACT
Previous and ongoing research in our lab: 1) established a determinative role for apoptosis, a genetically regulated process of programmed cell death (PCD), in the leaf scorch and cane death symptoms in Pierce's disease (PD), 2) developed a functional cDNA screen for PCD-suppressing plant genes and isolated six candidate DNA sequences capable of suppressing PCD when expressed as transgenes. Subsequent experiments confirmed that two different anti-PCD DNA sequences (VvP14 and UT456), when introduced into the fruited PD-susceptible cultivar Thompson Seedless and the commercial rootstock Freedom, protected against PD symptoms and limited bacterial titer four to six orders of magnitude below that reached in untransformed control vines. All untransformed control plants died within 2-3 months after inoculation while the transgenic plants were asymptomatic for 12 months, after which they were pruned, and cuttings made for a second inoculation. Results from the second inoculation showed a positive relationship between message level of UT456, a reduction in PD symptoms, a several fold reduction in bacteria titre in the inoculated plants and that the bacteria were uniformly distributed in the stem over the 20 cm sampled length at 10^3-10^4 cells per 0.1 gm stem tissue. The net effect of these transgenes is to limit bacterial titer but not distribution of bacteria in the asymptomatic plants. From the perspective of the grape-bacterial interaction, it appears that the anti-PCD genes suppress PD symptoms and functionally confine *Xylella fastidiosa* (*Xf*) to an endophytic ecology in the xylem equivalent to that seen in the related asymptomatic host *Vitis californica*. Although the data is preliminary, results from grafting experiments indicate the protective effect of these genes may be transferred across a graft union to protect a susceptible untransformed scion. Eight commercial wine varieties are being evaluated under controlled greenhouse conditions for susceptibility to PD. First sampling data indicates considerable variation among the varieties exists in degree of symptom severity and bacterial titer. Lastly, under an APHIS permit secured by PIPRA, the first set of transgenic plants expressing VvP14 and UT456 have been planted in the field.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) induces Pierce's disease (PD) symptoms that are the result of the activation of a genetically regulated process of programmed cell death (PCD). We identified six novel anti-PCD genes from a grape cDNA library functional screen for ability to suppress PCD. Two of these grape sequences, VvP14 and UT456, when expressed as transgenes in the PD susceptible Thompson Seedless plants, suppressed PD symptoms and dramatically reduced bacterial levelS in inoculated plants. The remaining four genes were tested this year, along with VvP14 and UT456; each of the four provided substantial suppression of both PD symptoms and bacterial titer. However, none were as effective as VvP14 and UT456. Currently in progress are a series of experiments designed to evaluate whether the protective effect of these two sequences can protect untransformed susceptible winegrape scions across a graft union. Preliminary data suggest that 50% or more of the susceptible scions grafted to either VvP14 or UT456 showed less PD symptoms and had lower bacterial titers than the unprotected control plants. While these results are encouraging, they are not complete or definitive and the experiment is continuing. Currently, comparable grafted plants are being prepared for field planting in the coming year and a suite of commercial winegrape varieties are being grafted to the transgenic rootstocks for inoculation experiments similar to those used in these preliminary tests. The relative susceptibility of the suite of eight commercial winegrape varieties is being tested under controlled conditions prior to field testing these varieties as scions on the transgenic rootstocks. Mechanism of action experiments initiated recently suggests a genetically conserved basis for suppression of PCD and the protection against PD. This project is now moving the proof-of-concept to potential application and characterization of these plants under field conditions with appropriate APHIS permits: initial field plantings were begun in July 2010.

INTRODUCTION
Susceptibility in most plant-microbe interactions depends on the ability of the pathogen to directly or indirectly regulate genetically determined pathways leading to apoptosis or programmed cell death (PCD). The induction of PCD results in an orderly dismantling of cells while maintaining integrity of the plasma membrane until internal organelles and potentially harmful contents including phenolics, reactive oxygen and hydrolytic enzymes have been rendered harmless to contiguous cells. However, when the cell contents are released in this manner they can serve as nutrients for microbial cells when they are present in the immediate environment (2). In the case of *Xylella fastidiosa (Xf)* and many other plant pathogenic bacteria, the bacteria live predominantly as endophytes or epiphytes but occasionally as pathogens. The relative susceptibility of the individual plant species is determined by unknown genetic factors. Presumably, sensitivity to the presence of the bacteria expressed as cell death-dependent symptoms is the result of signals expressed by the bacteria that lead to activation of PCD

as appears to be the case with Pierce's disease (PD). Hence, bacteria, like *Xf* ,could receive nutrients from cells adjacent to the xylem that are triggered to undergo PCD and gradually releasing contents of the grape cell into the apoplastic space surrounding the xylem. Subsequent experiments indicated that two different anti-PCD DNA sequences (P14 and UT456), when introduced into the fruited PD-susceptible cultivar Thompson Seedless and the commercial rootstock Freedom, protected both against PD symptoms and limited bacterial titer four to six orders of magnitude below that reached in untransformed control vines. While protection against PD appears to be feasible with this transgenic approach, the next step is to determine whether transfer of this protection can occur across a graft union. Our current experiments involve transformed rootstocks (Freedom and Thompson Seedless) expressing P14 or UT456 grafted to untransformed winegrape scions to be tested through greenhouse inoculation with analysis of PD symptoms, quantitative assessment of message level, message movement, and bacterial titer in untransformed grafted scions. Initial testing will evaluate these parameters with untransformed Thompson Seedless scions. Concurrently, controlled greenhouse testing of relative susceptibility is being done on eight commercial winegrape varieties (Chardonnay, Pinot Gris, Sauvignon Blanc, Cabernet Sauvignon, Pinot Noir, Zinfandel, Syrah and Merlot) to establish quantitative and qualitative base line data on these commercial varieties before any field evaluation is undertaken. In addition, these experiments will provide quantitative data on bacterial population dynamics and PD symptoms on the entire suite of winegrape varieties. This data set addresses one of the stated needs in the 2010 RFP, namely, that much anecdotal but little quantitative data exists on the relative susceptibility of commercial winegrape varieties. In summary, experimental results to date confirm progress in identifying DNA transcripts of grape which, if regulation of the natural transcripts is altered in transgenic plants, result in the suppression of symptoms of PD with an associated limitation in bacterial titer to levels generally associated with a benign endophytic association.

OBJECTIVES 2010-2012

1. Complete the evaluation of the additional four candidate anti-apoptotic genes now successfully transformed into PD susceptible Thompson Seedless plants. **Table 1** includes the four genes plus VvP14, UT456 and baculovirus p35.
2. Evaluate the relative susceptibility of eight commercial winegrape varieties to PD and titer of Xf in the tissues. Varieties: Chardonnay, Pinot Gris, Sauvignon Blanc, Cabernet Sauvignon, Pinot Noir, Zinfandel, Syrah and Merlot. Untransformed Thompson Seedless, VvP14 and UT456 are reference lines used in previous experiments. This objective addresses the research priority in the RFP regarding short term collection of quantitative data of the relative resistance (susceptibility) of commercial winegrape varieties.
3. Initiate experiments to assess the potential for protection against PD across a graft union by VvPR1 and UT456, first with Thompson Seedless as the untransformed scion.
4. Perform inoculations the eight winegrape varieties, initially on their own rootstocks and subsequently on Freedom and Thompson Seedless rootstocks expressing VvPR1 and UT456.
5. Investigate the mechanism underlying the protection against PD by VvP14 and UT456. (2010-2012).
6. Determine presence and movement of the mRNA and/or protein of VvP14 and UT456 across the graft union into the untransformed Thompson Seedless O2A scion. (2010-2012)
7. Collaborate with PIPRA to obtain permits to enable field evaluation of transgenic VvPR1 and UT456 in a location providing for controlled inoculation.
8. Secure patent protection as intellectual property for those genes that prove to be capable of blocking PD in grape.

RESULTS AND DISCUSSION

Evaluation of the additional four candidate anti-apoptotic genes to suppress PD symptoms in susceptible Thompson Seedless plants. The protective genes or DNA sequences, isolated by a functional anti-PCD screen (1), have been described in earlier reports to this symposium in 2008 and 2009 (3,4). **Table 1** summarizes the results of the final series of inoculations of remaining four potential anti-apoptotic genes designated WG71, WG23, Y390, and Y376. DNA sequence analysis of these genes indicates the presence of orthologs in other plants including potato and tomato. These sequence relationships are presented in **Table 1**. Inoculation of individual canes by the needle prick method delivered 10-20 µl at of the Temecula strain *Xf* at a concentration of 10^5 cfu/ml (2,000 cells or less) (10). Presence of bacteria in the inoculated tissue is determined by qPCR and reported as the number of cells per 0.1 gm of stem tissue (**Table 1**). All four candidate genes suppressed PD symptoms and reduced bacterial titer in the inoculated canes but were not superior to VvP14 or UT456 in either case. These genes will be maintained in clonally propagated plants and patent protection sought but will not be tested further. Ongoing greenhouse and field experiments will focus on VvP14 and UT456.

Relative susceptibility of eight commercial winegrape varieties to PD and measurement of *Xf* titer in the tissues under controlled greenhouse inoculation conditions.

We initiated experiments to obtain quantitative data on bacterial population dynamics and relative PD susceptibility of a suite of commercial winegrape varieties under controlled greenhouse inoculation conditions and is designed to avoid any vagaries associated with natural infection and glassy-winged sharpshooter (GWSS) preferences. This objective addresses one of the stated needs in the 2009-2010 RFP, namely, that much anecdotal but little quantitative data exists on the relative susceptibility of commercial winegrape varieties. The varieties tested include Chardonnay, Pinot Gris, Sauvignon Blanc, Cabernet Sauvignon, Pinot Noir, Zinfandel, Syrah and Merlot with untransformed Thompson Seedless, VvP14 and UT456 as reference lines. These experiments also provide baseline disease information for 2011-2012 experiments to test potential protection of these varieties when grafted to rootstocks expressingVvP14 and UT456. Data collected will include bacterial

titer, movement and disease symptoms. Selected clones of each variety were inoculated by the needle prick method with Temecula strain of *Xf* delivering 10-20 µl at bacterial concentration of 10^5 cfu/ml (2,000 cells or less). Results of the first series of evaluations are shown in **Figure 2**. All varieties were susceptible PD in terms of symptom expression and exhibited 1-3 orders of magnitude higher bacterial titers four months after inoculation than the asymptomatic *Vitis californica* or transgenic Vv P14 or UT456 comparison plants. Pinot Gris had the highest bacterial titer and exhibited the most severe symptoms while Syrah was the most tolerant with symptoms and bacterial titer nearly as low as *V. californica*. The symptom level and bacterial titers appeared to be well correlated as seen in the photos of representative plants of each variety.

Table 1. List of potential plant anti-apoptotic genes derived from functional cDNA screen. Each is then evaluated as transgenes in the PD susceptible grape clone, Thompson Seedless O2A. Disease rating is a 1-5 scale with 1 = asymptomatic and 5 = defoliated. Bacterial titers are expressed as bacterial cells per 0.1gm of stem tissue. Evaluations were done at 4 months post inoculation. See Figure for representative pictures

Designation	Gene Ortholog Link	Results (rating/*Xf* titer)
O2A	Untransformed Thompson Seedless control plant	R=5; 10^7
WG71	cytokine-like protein	R= 2; 10^4
WG23	Cupin-like protein	R= 2; 10^4
Y390	Metallothionein	R= 2; 10^4
Y376	Mycorrhizal up regulated gene	R= 2; 10^3
I35	Baculovirus P35, caspase inhibitor	R= 2; 10^4
UT456	3'UTR of grape ortholog of the p23 gene from potato and tomato	R=1; 10^3
Vv P14	Pathogenesis related protein	R=1; 10^3

Initiate experiments to assess the potential for protection against PD across a graft union by VvPR1 and UT456
Experiments were undertaken to determine if the protective effect of these genes is capable of being transferred across a graft union to protect a susceptible scion. PD susceptible untransformed Thompson Seedless was grafted onto Freedom and Thompson Seedless transgenic for VvP14 and UT456. The preliminary data suggest that 50% or more of the susceptible scions showed less PD symptoms and had reduced bacterial titer (**Table 2** and **Figure 3**). While these results are encouraging, they are far from complete or definitive. Currently, comparable grafted plants are being prepared for field planting in the coming year and the entire suite of commercial winegrape varieties are being grafted to the transgenic rootstocks for inoculation experiments similar to those shown in these preliminary tests (see objective 4).

Initiate mode of action studies for VvP14 and UT456
These studies are just beginning but to date we have found two novel and possibly linked mechanisms for VvP14 and UT456 action. First, the transgenic P14 coding sequence is translationally blocked in healthy cells but is readily translated when the tobacco, tomato or grape cells are under chemical or pathogenic (death) stress. Secondly, the noncoding UT456 sequence contains small RNA hairpin structures that show a high degree of sequence conservation with the P14 3'UTR. Initial *in vitro* protein translation studies indicate that the UT456 contains a signal that activates translation. There is precedent for translational blockage by the 3'UTR in plant systems and for RNA movement from roots to tubers (5). Expression of the UT456 activated the translation of the P14 protein in transgenic tobacco leaves. In addition P14 antibodies, used in immunoprecipitation assays to detect potential P14 interacting factors, were successful in identifying 3 P14-interacting proteins, HP70, HP90 and RACK1 from plant extracts. Interestingly, these three proteins have previously been reported to interact directly with each other and occur in a membrane associated complex involved in innate immunity in rice plants. Work has also begun to assess a role for the potential small RNA hairpin loops within UT456 to activate P14 translation using RNA protection assays.

Figure 1. Summer 2010 results of greenhouse Pierce's disease assay of transgenic grapes expressing PCD blocking genes. Photos taken and *Xf* titers were measured by qPCR at 4 months after inoculation. White inset is the name of the transgenic line and blue inset numbers indicate the titer of *Xf* bacteria in 0.1g of stem tissue.

Figure 2. Relative sensitivity of wine grapes to Pierce's Disease**.** Eight commercial wine grape cultivars including Cabernet Sauvignon, Chardonnay, Sauvignon Blanc , Pinot Gris, Pinot Noir, Merlot, Syrah and Zinfandel were mechanically inoculated with *Xf* and compared to inoculated controls *Vitis californica* and Thompson seedless (see figure 1). Photos taken and *Xf* titers (red inset numbers) in 0.1g of stem tissue were measured by qPCR at 4 months after inoculation.

Table 2. Rootstock expressing transgenes grafted to untransformed Thompson Seedless scions

Transgenic notation	Relevant genotype (transgenic rootstocks grafted to untransformed Thompson seedless scions)	Percent of transgenic graft-protected plants with Xf titers less than or equal to *Vitis californica*	Range of bacterial load per 0.1 gm of stem in at 4 months post inoculation
TS02A FD456-15	CaMV 35S-driven 456 Freedom rootstock	50%	10^3 - 10^4
TS02A FDP14-13	CaMV 35S-driven P14 Freedom rootstock	50%	10^3 - 10^4
TS02A TS456-8	CaMV 35S-driven 456 Thompson seedless rootstock	66%	10^3 - 10^4
TS02A TSP14-9	CaMV 35S-driven P14 Thompson seedless rootstock	100%	10^4 - 10^5
TS02A Control	Untransformed Thompson Seedless scion	none	10^6 - 10^7
Vitis californica	Asymptomatic wild type untransformed host.	no death after 12 months post inoculation	10^4
Transgenic notation	**Relevant genotype (transgenic rootstocks grafted to untransformed Thompson seedless scions)**	**Percent of transgenic graft-protected plants with Xf titers less than or equal to *Vitis californica***	**Range of bacterial load per 0.1 gm of stem in at 4 months post inoculation**
TS02A FD456-15	CaMV 35S-driven 456 Freedom rootstock	50%	10^3 - 10^4
TS02A FDP14-13	CaMV 35S-driven P14 Freedom rootstock	50%	10^3 - 10^4
TS02A TS456-8	CaMV 35S-driven 456 Thompson seedless rootstock	66%	10^3 - 10^4
TS02A TSP14-9	CaMV 35S-driven P14 Thompson seedless rootstock	100%	10^4 - 10^5
TS02A Control	Untransformed Thompson Seedless scion	none	10^6 - 10^7
Vitis californica	Asymptomatic wild type untransformed host.	no death after 12 months post inoculation	10^4

Develop methods for and determine presence and movement of the mRNA and/or protein of P14 and UT456 across the graft union into the untransformed Thompson Seedless O2A scion. (2010-2012)

We have developed protocols for amplifying, cloning and sequencing microRNAs generated from transgenic expression of P14 and UT456. RNA was isolated from grape tissue (MirVana miRNA isolation kit; Applied Biosystems); poly(A) tails were added and cDNA synthesized (nCODE VILO miRNA cDNA synthesis; Invitrogen); cDNA was amplified by PCR and cloned into plasmids for sequencing. These protocols will be used to look for mobile microRNA in extracts from untransformed scions grafted to transgenic rootstocks. P14 antibodies will also be used to test directly for the presence of transgenic P14 protein in the grafted scions.

Figure 3. Potential protection across a graft union**.** Representative control and transgenic plants expressing the genes indicated in Table 2. All grafts have untransformed Thompson seedless "02A" scions. FD is untransformed Freedom rootstock control. All plants photographed and *Xf* titers taken 4 months after inoculation with *Xf*. Age of plants at the time of inoculation was approximately 22 months. Samples and photos were taken at four months after inoculation.

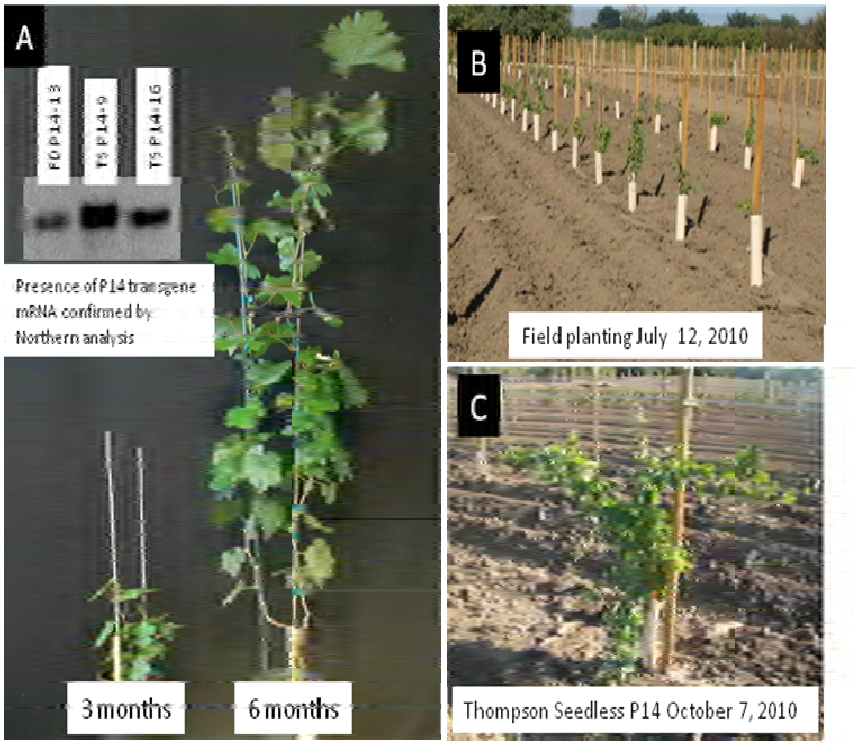

Figure 4. Field Trial. Panel A illustrates the time –course development of transgene expressing rootstocks grafted to untransformed PD susceptible Thompson Seedless scions. The inset shows northern blot analysis of the P14 transgenic lines currently planted in the field trial confirming the presence of the introduced P14 message. Panels B and C are field views of the transgenic plants and controls in the APHIS approved location: (A) at the time of planting and (B) 3 months after planting.

Collaborate with PIPRA to obtain permits to enable field evaluation of transgenic PR1 and CB456 in a location providing for controlled inoculation. APHIS permit was obtained, secure field located, and planting initiated in July, 2010 (**Figure 4**).

Secure patent protection as intellectual property for those genes that prove to be capable of blocking PD in grape. The grape plants containing the anti-PCD genes and the grafted rootstocks will require the use of several patented enabling technologies. Record of invention disclosures have been submitted to the UC Office of Technology Transfer. The research proposed reported herein will provide data on the activity and mechanism of action of the protective transgenes in grape relative to the presence, amount and movement of *Xf* in the transformed and untransformed grape plants

CONCLUSIONS

Xf induces PD symptoms that result from activation of a genetically regulated process of programmed cell death in susceptible grapes. We identified six novel anti-PCD genes from cDNA libraries of grape. Two of these grape sequences expressed as transgenes in grape, suppressed PD symptoms and dramatically reduced bacterial titer in inoculated plants. Preliminary data suggest that protective sequences may function across a graft union. This project has identified a basis for PD symptoms and a genetic mechanism to suppress symptoms and bacterial growth with an infected plant. If needed in the future, a transgenic strategy exists to address PD. The next two years are committed to assessing this strategy in eight commercial winegrape varieties under field conditions and evaluating the mechanism of action to develop data for patent protection of the DNA sequences.

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BIOLOGICAL CONTROL OF PIERCE'S DISEASE OF GRAPEVINE WITH BENIGN STRAINS OF *XYLELLA FASTIDIOSA*

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ABSTRACT
In the Bella Vista Vineyard in Temecula, loss of plants to extreme water stress and nutritional problems have forced abandonment of the trial on Orange Muscat and, probably, will also eliminate the Cabernet Sauvignon trial. In the Orange Muscat test, 35-40% of the vines had died after two years from something other than Pierce's disease (PD), probably water stress. In Preston Vineyards in Sonoma, EB92-1 was reducing the incidence of PD in Viognier when compared to untreated vines in this first year of characteristic PD symptom development in the trial. The test on Barbera has been discontinued due to lack of PD. With adequate irrigation, both the Chardonnay and Reisling test vines in the Beringer Vineyard in Napa were vigorous and grew well in this second growing season. PD began to develop in the test vines of both varieties and trends were for reduced PD in the EB92-1 treatments compared to the untreated. In comparisons of methods of treatment with EB92-1 in three-year-old Merlot vines, EB92-1 was controlling PD equally well after injection into the rootstock, scion, or rootstock and scion. Vines developed using scion wood from mother vines of Chardonnay infected with EB92-1 had less PD than vines developed with uninfected scion wood or even from clean plants injected with EB92-1. Development of plants with scion wood from infected mother vines could eliminate the need to inject every vine by pin pricking.

LAYPERSON SUMMARY
In trial plantings of Orange Muscat and Cabernet Sauvignon in Bella Vista Vineyard in Temecula, almost half of the Orange Muscat, treated or untreated vines have died from something other than Pierce's disease (PD), probably water stress. Many of the surviving vines were severely stunted and barely reached the trellis wire after more than two years and three growing seasons. This makes it impossible to obtain good data so the trial has been abandoned. Approximately a fourth of the Cabernet Sauvignon also have died and this test will be abandoned unless plant survival and vigor improve. Since there was no PD in the Barbera in Preston Vineyards in Sonoma, no data can be obtained from this test either. The other three tests are going well. In Preston Vineyards in Sonoma, EB92-1 was controlling PD in Viognier when compared to untreated vines. In Beringer Vineyard in Napa, PD began to develop in the tests on Chardonnay and Reisling and trends were for reduced PD in the EB92-1 treated compared to the untreated. In both the Sonoma and Napa trials, symptoms are just beginning to develop so it is early to draw definitive conclusions on the control trends. We are evaluating the use of mother vines infected with the biocontrol strain EB92-1 as propagation material for scion wood. In 2010, vines developed using scion wood from mother vines of Chardonnay infected with EB92-1 had less PD than vines developed with uninfected scion wood. This indicates that there could be transfer of the biological control from the mother plant through scion wood. Development of plants with scion wood from infected mother vines could eliminate the need to inject every vine by pin pricking.

INTRODUCTION
Pierce's disease (PD) of grapevine is a chronic problem for the California grape industry and has become more of a threat to the industry with the introduction of the glassy-winged sharpshooter (GWSS) (Hopkins and Purcell, 2002). PD is especially damaging in the southeastern USA where it is endemic and is the primary factor limiting the development of a grape industry based on the high-quality European grapes (*Vitis vinifera* L.). The only feasible control for PD is resistance. The results of our 10 years of research on the biological control of PD of grapevine by cross protection with weakly virulent strains of *X. fastidiosa (Xf)* have demonstrated that this is a potential means of controlling this disease (Hopkins, 2005). We have identified at least one strain that was able to control PD in *V. vinifera* for 14 years in Central Florida. We are testing this strain in commercial vineyards on a limited basis and, if these tests are successful, the strain will be ready for commercial use. The overall goal of this project is to develop a biological control system for PD of grapevine that would allow the production of *V. vinifera* in California and other areas where PD and the GWSS are endemic.

In previous research with the biocontrol strain, the bacteria were injected into the grapevines after they were transplanted into the vineyard. This is a labor-intensive procedure. Three methods in order of increasing desirability are vineyard injection, nursery injection, and propagating wood from mother vines that are infected with the biocontrol strain. We are currently

evaluating injection of the biocontrol strains into the vines in the nursery, prior to transplanting into the vineyard. The use of scion or rootstock propagating wood from mother vines that are already infected with the biocontrol strain would make this technology less labor-intensive, less costly, and more consistent. It would eliminate any variability in the relative effectiveness of injections into different plants.

OBJECTIVES

1. To evaluate strain EB92-1 of *Xf* for the biological control of PD of grapevine in new plantings in the vineyard in California.
2. To evaluate strain EB92-1 of *Xf* for the protection of older established grapevines against PD in California vineyards.
3. To develop a PCR based assay that can quickly differentiate the PD biocontrol strain EB 92-1 from pathogenic, wild type *Xf* strains.
4. To evaluate rapid, efficient methods of treatment with strain EB92-1 of *Xf* for the biocontrol of PD in *V. vinifera* in the vineyard.

RESULTS AND DISCUSSION

Field trials evaluating strain EB92-1 for biological control of PD in vineyards in California

All plants for the vineyard tests in the Bella Vista Vineyard in Temecula, in the Beringer vineyard in the Napa Valley, and in Preston Vineyards in the Sonoma Valley were planted in April, 2008 in greenhouses at UC Davis. For transplanting into the Bella Vista Vineyard in Temecula, 50 Orange Muscat were inoculated with the biocontrol strain, EB92-1, on June 26, and 50 were left untreated as controls. Fifty Cabernet Sauvignon/110R were treated and 50 were untreated controls. These plants were transported to Temecula and transplanted into plots in the Bella Vista Vineyard on July 21-22.

In late fall 2008, PD-like symptoms were observed in most of the vines at Bella Vista, treated or untreated (Observation by Barry Hill). However, it was very hot and dry in 2008 and some of these symptoms may have been due to the weather. In the summer of 2009, PD symptoms were still extensive in the Bella Vista Vineyard, but were observed in only about half of the vines that had symptoms in 2008. All of the vines, treated and untreated, were under severe water stress and this may have caused some of the PD-like symptoms. Differences in the incidence of leaf scorch between the treated and untreated vines were not significant. The Orange Muscat planting was interspersed with mature vines that were nearly 100% infected with PD. This entire planting, except our experimental vines were removed during the winter of 2009, leaving only our young plants scattered in the vacant vineyard.

In September 2010, all the young plants in the Bella Vista vineyard appeared to have severe water and nutritional stress. PD-like symptoms were extensive in the plants that were still alive, treated and untreated. Many plants died without ever having any PD symptoms, probably due to the lack of water and poor nutrition. It is difficult to discern whether the PD-like symptoms are due to water stress or whether water stress increases PD. In the Orange Muscat test, 35-40% of the vines had died after two years from something other than PD, probably lack of water. Twenty-two percent of the Cabernet Sauvignon also had died, probably from water stress. In both the Cabernet Sauvignon and Orange Muscat, many of the vines were severely stunted and barely reached the trellis wire after three seasons and more than two years. Therefore, the Orange Muscat test is definitely lost. The Cabernet Sauvignon test will probably have to be abandoned also, but better irrigation and fertilization could salvage it. We will re-evaluate it in 2011.

For Preston Vineyards in Sonoma, 50 Barbera/110R and Viognier/110R from were inoculated with EB92-1 and 50 vines of each were left as untreated controls. These plants were transported to Sonoma and transplanted as replants for missing vines in a mature vineyard the last week of July, 2008. On August 26, 2009, these vines were mapped for symptoms. All of the Barbera vines appeared to be healthy with no PD symptoms. The block of Barbera did not appear to have any PD symptoms, even in the older vines. The disease pressure appears to be very low in this Barbera block. In September 2010, there were still no symptoms in the Barbera block, either in the new test vines or the older vines. This test will not be evaluated in 2011, because of the lack of disease.

In the Viognier test, there were a few vines that had minor yellow and/or necrotic leaf margins on the basal leaves in 2009, but there were no definitive symptoms. The Viognier block has significant PD incidence in the mature vines and these new test vines began to develop PD symptoms in 2010 (**Table 1**). Symptoms were not very severe, but there were more symptomatic vines in the untreated vines than in the EB92-1 vines. This trend indicated that EB92-1 was reducing the incidence of PD in the Viognier. With the amount of symptoms in the mature Viognier vines, PD should continue to develop in the young test vines.

For transplanting into the Beringer Vineyard in Napa, 50 Reisling/3309 and 50 Chardonnay/3309 were treated with EB92-1 on June 25, 2008 and 50 vines of each were left untreated as controls. The vines were transplanted as replants for missing vines in Beringer Vineyard in early April 2009. On August 26, 2009, these vines had not started to develop PD symptoms. Many of the vines were exhibiting drought stress.

Table 1. Biocontrol of PD in two-year-old grapevines in Northern California vineyards, 9/8/10.				
	EB92-1 treated vines:		**Untreated vines:**	
Cultivar	**#PD vines/total**	**Disease rating[1]**	**#PD vines/total**	**Disease rating[1]**
Beringer Vineyard, Napa				
Chardonnay	3/45 (7%)	0.1	4/48 (8%)	0.1
Reisling	4/47 (9%)	0.1	6/51 (12%)	0.1
Preston Vineyard, Sonoma				
Viognier	8/48 (17%)	0.2	13/48 (27%)	0.3
UTotalU	15/140 (11%)	0.1	23/147 (16%)	0.2
[1]Disease rating was an average per vine on a scale of: 0 = no symptoms; 1 = any symptom of PD, such as marginal necrosis (MN) on a basal leaf; 2 = definite, moderate symptoms on <50% of vine; 3 = severe symptoms on >50% of vine; 4 = dead plant.				

With better irrigation, both the Chardonnay and Reisling test vines in the Beringer Vineyard were vigorous and grew well in this second growing season. PD began to develop in the test vines of both varieties (**Table 1**). The trends were for less PD in the EB92-1 treatments and more PD in Reisling than Chardonnay. However, these are very early results as the plants are only in their second season and less than two years old. With extensive PD in the mature Chardonnay and Reisling vines, disease should continue to develop in the young test vines.

Field trial evaluating EB92-1 for the protection of older established grapevines against PD in California vineyards
Since PD is rapidly developing in the mature Chardonnay block at Beringer Vineyard in Napa, it was chosen for an evaluation of EB92-1 for the prevention of PD development in mature, producing grapevines. Randomly, forty vines were inoculated with EB92-1 and 40 vines were chosen as controls. On September 8, 2010, the vines were inoculated with strain EB92-1 in the main trunk, approximately equidistant from the graft and the trellis wire Vines were injected by boring a small hole into the trunk with an electric drill. Two ml of the bacterial suspension will be injected into each hole using a nail-injector syringe.

Comparison of treatment methods with strain EB92-1 for biocontrol of PD
On May 29, 2007, Merlot/101-1 plants were injected with EB92-1 in the greenhouse. Treatments were (1) EB92-1 in scion only, (2) EB92-1 in rootstock only, (3) EB92-1 in both rootstock and scion, and (4) Nontreated. On June 21, vines were transplanted into the vineyard in 3 replications of three plants per treatment. In 2009, PD began to occur in these vines, but there were no obvious differences between treatments.

In 2010, the EB92-1 treated vines had less PD than the untreated (**Table 2**). PD biocontrol was obtained whether EB92-1 was injected into the scion, the rootstock, or both. The treatments appeared to be equally effective.

Table 2. Effect of methods of treatment of grape plants with *Xf* strain EB92-1 on biological control of PD.

	% PD incidence in August 2010 in:[1]
Treatment	**Merlot/101-14**
Scion injection	13
Rootstock injection	11
Scion & Rootstock injection	14
Scion field injection	-
Untreated	38

[1]%PD is the number of plants with symptoms divided by total number of plants x 100.

Plants of Chardonnay/Salt Creek were obtained by grafting green cuttings from Chardonnay plants from the vineyard onto rooted cuttings of Salt Creek. The grafting was done between May and July in 2007. Grafted plants were transplanted into the vineyard on August 14, 2007. Treatments included (1) Cuttings from Chardonnay not infected with EB92-1 on Salt Creek, (2) Cuttings from EB92-1 inoculated Chardonnay on Salt Creek, and (3) Cuttings from Chardonnay not infected with EB92-1 on Salt Creek, but injected in the vineyard with EB92-1 on August 29. In the first year, there were no significant differences among the Chardonnay/Salt Creek treatments.

In 2009, plants developed by injecting clean scion with EB92-1 in the field or by using scion wood from mother vines of Chardonnay infected with EB92-1 had less PD than plants developed with uninfected scion wood. In 2010, injection with EB92-1 and the use of scion wood from mother vines infected with EB92-1 had less PD than untreated vines (**Table 3**). Surprisingly, there was less PD in plants developed from scion wood from EB92-1 infected mother plants than in plants that

were injected directly with EB92-1. While it may be too early to draw conclusions, this indicates that there could be transfer of the biological control from the mother plant through scion wood. Further development of the symptoms will be observed. This evaluation of scion from treated mother vines is especially significant, because scion wood from infected mother vines could be an efficient treatment method that does not require a lot of additional hand labor over normal production practices.

Table 3. Transmission of biocontrol in scion from infected Chardonnay mother plant grafted onto Salt Creek rootstock.

Treatment	**% PD incidence in August 2010:**
Scion from clean Chardonnay	40
Scion from clean Chardonnay injected with EB92-1 in the field	27
Scion from EB92-1 Chardonnay mother plant	9

CONCLUSIONS

In three-year-old Merlot vines in Apopka, FL, EB92-1 was controlling PD equally well after injection into the rootstock, scion, or rootstock and scion. Vines developed using scion wood from mother vines of Chardonnay infected with EB92-1 had less PD than vines developed with uninfected scion wood or from uninfected scion wood injected with EB92-1. Development of plants with scion wood from infected mother vines could eliminate the need to inject every vine by pin pricking.

In the Bella Vista Vineyard in Temecula, loss of plants to extreme water stress and nutritional problems have forced abandonment of the test on Orange Muscat and, probably, will also destroy the Cabernet Sauvignon test. If vigor can be restored to the plants, it may be possible to salvage the Cabernet Sauvignon test. This will be determined in 2011. In Preston Vineyards in Sonoma, the Barbera test was discontinued because there was no PD, but the Viognier block was doing very well. In this first year of clear symptom development, EB92-1 was reducing the incidence of PD in Viognier. In this second growing season for tests in Beringer Vineyard in Napa, both the Chardonnay and Reisling tests were growing very well and PD was developing in both tests. There were no significant differences yet, but there was a trend toward less PD in the EB92-1 treated vines. Of the six tests of biocontrol of PD in new plantings established in 2008-2009, two tests will probably have to be discontinued due to plant death from water stress and nutritional problems and one test discontinued due to the lack of PD. Early in the other three tests, EB92-1 appears to be reducing PD incidence. The successful completion of the biocontrol tests in Temecula, Sonoma, and Napa would lead to an effective control of PD that is environmentally friendly. This project should yield results within the next two years and if the control is successful, there should be a biological control for PD available for commercial use in vineyards in California.

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IDENTIFICATION AND UTILIZATION OF COLD TEMPERATURE INDUCED GRAPEVINE METABOLITES TO MANAGE PIERCE'S DISEASE.

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ABSTRACT
This work builds on discoveries made in the past six years of research on better understanding the mechanism(s) responsible for the Pierce's disease (PD)-cold curing phenomenon. A thaumatin-like (TLP) grape protein was found in elevated levels in the xylem sap from cold-exposed vines. We have cloned and expressed TLP in *E .coli* and our preliminary findings show that crude TLP protein extract possesses anti-*Xylella fastidiosa* (*Xf*) activity *in vitro*. Greater amounts of total phenolics were measured in xylem sap extracted from cold-exposed vines. We are beginning to characterize these phenolic compounds, and assess their potential anti-*Xf* activity *in vitro*. Previously, greenhouse grown Pinot Noir and Cabernet Sauvignon vines treated with commercial abscisic acid (ABA) were shown to have higher levels of recovery from PD than non-treated vines as well as producing higher levels of polyphenolic compounds. This fall applied ABA, both soil drench and foliar sprays, to PD-affected field grown Riesling vines in the Napa Valley.

LAYPERSON SUMMARY
Previous work on "cold curing" of Pierce's disease (PD) affected grapevines led to the identification of thaumatin-like protein (TLP) in grapevine xylem sap. TLP is expressed in greater amounts in vines that have been exposed to cold temperatures and may be associated with the cold curing phenomenon. Currently we have cloned and expressed TLP in *E. coli*. Producing TLP in *E. coli* should allow us to produce enough protein to better evaluate the role of TLP in curing of PD. Crude extracts of *E. coli* expressing TLP were applied to PD3 medium plates and initial results showed adding TLP to PD3 growth media greatly inhibited the growth of one strain of *Xylella fastidiosa*. We are currently preparing to make transgenic grapevines that express this protein at higher levels. Previous work also identified polyphenolic compounds as a possible mediator of the "cold curing" phenomenon. Here, we show that a specific polyphenolic compound, resveratrol, is produced in vines that experience cold curing, while it is absent from grapevines grown in warmer environments. In a previous project, we were able to induce "cold curing" of PD in Davis, CA (an area that traditionally does not show extensive PD "cold curing") by applying the plant hormone ABA to the vines. This fall we applied ABA to PD-affected, field-grown vines in Napa.

INTRODUCTION
In our previous work we characterized many biological parameters of xylem sap from cold-exposed (freezing temperatures) and "warm," (non-freezing) temperatures in both field grown and cold chamber exposed grapevines. We found that *Xylella fastidiosa (Xf)*-infected potted grapevines that were exposed to freezing temperatures at several sites in Northern California and vines exposed to -5C in cold chamber emerged pathogen free the following summer (Meyer and Kirkpatrick, 2004-2008). We measured many different biological parameters, such as pH, organic acid, sugar and ion concentrations, and osomolarity in Pierce's disease (PD)-susceptible *Vitis vinifera* 'Pinot Noir' and PD-less susceptible *V. vinifera* 'Cabernet Sauvignon' grapevines over three winters.

One of the parameters determined in these previous studies was the protein profiles of cold- and warm-treated xylem sap. One of these proteins, a thaumatin-like protein (TLP), was significantly up regulated in cold exposed vines. We have cloned

and expressed the *V. vinifera* TLP protein and showed some inhibition of *Xf* growth when crude protein extracts from TLP-expressing *E. coli* were applied to PD3 medium plates.
We have also been assessing the potential role that xylem sap phenolic compounds may play in the "cold curing" process. In collaboration with the Waterhouse lab at UC Davis, we are characterizing the phenolic compounds in cold and warm xylem sap by HPLC/MS, and have identified that a major polyphenol in cold-exposed xylem sap is trans-resveratrol.

OBJECTIVES

1. Over-express the grapevine thaumatin-like protein (TLP) in transgenic grapevines. Prepare anti-TLP antibodies to quantify TLP in transgenic xylem sap using ELISA and western blot analyses.
2. Inoculate TLP-expressing grapevines with Xf and determine the incidence and severity of PD in TLP-transgenic versus non-transgenic *V. vinifera*.
   a. Fractionate and chemically characterize the phenolic compounds that are present in xylem sap from cold-exposed grapevines.
   b. Compare the phenolic compound composition and concentration in xylem sap extracted from cold- and warm-exposed *V.vinifera* grapevines as well as grapevines treated with ABA under non-freezing conditions.
   c. Determine if these compounds affect Xf growth/survival *in vitro.*
3. Determine if foliar and drench applications of ABA can increase PD-curing rates in field-grown vines under non-freezingconditions.

RESULTS AND DISCUSSION

We have cloned and expressed *Vitis vinifera* thaumatin-like protein using the pet-30b (Invitrogen) protein expression vector in Novagen BL21(DE3) *E. coli* (**Figure 1**). To obtain recombinant TLP for anti *Xf* experiments, BL21 cells were grown overnight and harvested by centrifugation. The supernatant was discarded and the pellet was resuspended in 5 ml of 20 mM phosphate buffer pH 7.0. Lysozyme (2 mg/mL) was added and incubated for 30 minutes at room temperature on a shaker. The solution was then sonicated eight times at 10 second bursts. This suspension was centrifuged at 5000rpm for 20 minutes at 4°C. The supernatant was discarded and the pellet was purified in a modified protocol originally described by Daniell et al. (2000). The pellet was resuspended in 5ml of solubilisation buffer (100 mM Tris/Acetate, pH 8.6, 6 M guanidine-HCl, and 0.5mM EDTA). The solution was then passed through a 0.22 um filter.

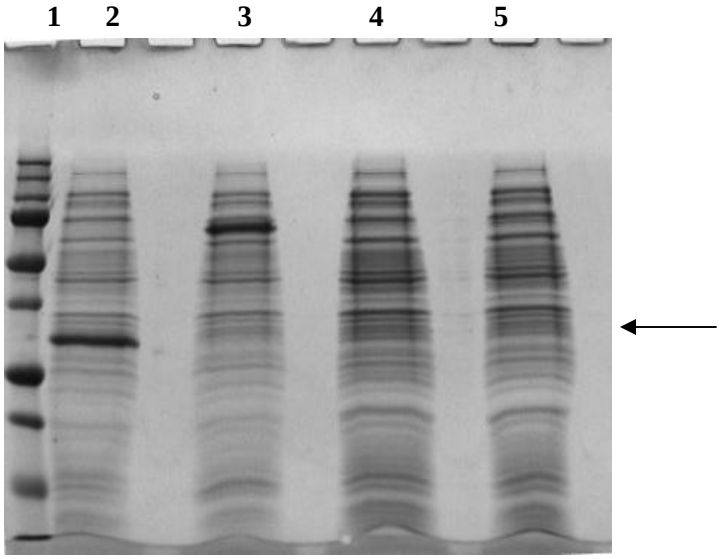

Figure 1. Thaumatin-like Protein (TLP) that was cloned and expressed using a *E.coli* expression vector. Note that arrow points to the correct size of grapevine TLP protein (~35 kD).

Lane 1: Dual color SDS ladder.
Lane 2: Cell lysate from IPTG induced *E.coli* with TLP construct.
Lane 3: Cell lysate from IPTG induced *E.coli* with a polygalacturonase (PG) construct (positive control).
Lane 4: Cell lysate from *E.coli* with TLP construct, not induced.
Lane 5: Cell lysate from *E.coli* with PG construct, not induced.

The filtered solution was then dialyzed for 16 hours at 4°C in 1 L of stirred phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.2), with 0.306 g/l oxidized glutathiones and 0.307g/l reduced glutathiones. The solution in the dialysis tubing was collected and centrifuged at 5000rpm for 30 minutes. The pellet and supernatant were separated. The pellet was resuspended in 5ml of phosphate buffer and passed through a sterile 0.22 um filter. The supernatant was also filter sterilized.

Sterile dialysis prepared protein suspensions and potassium phosphate buffer solutions were combined at a 1:1 ratio with 10-14 day old liquid grown *Xf*. *Xf* and deionized water combined at a 1:1 ratio, applied at 200ul/plate, provided baseline control for treatment comparisons. *Xf* was added at a ratio of 1:1 with the following suspensions: supernatant from the dialysis product for both the empty vector (Pet30b with no TLP insert) and the Cabernet TLP protein (CS3), pellet from the dialysis product resuspended in 20mM phosphate buffer (pH 7.0) for both the empty vector and the CS3 protein, and 20mM phosphate buffer (pH 7.0) only.

Survival of *Xf* was measured at by plating the bacterial suspensions at the following five time points: immediately after combining the buffer or protein suspension with the bacterial cells, 16 hours after the first sampling, 24 hours after the first sampling, 40 hours after the first sampling, and 48 hours after the first sampling. The plates were incubated at 28°C for 11-14 days, photographed, and the effectiveness of the treatments was determined visually (**Figure 3**).

Xylem sap total phenolics from ABA-treated and non-treated controls have been measured. Trends showing that total phenolics were found in higher concentrations in cold-exposed vines were also seen in the ABA-treated vines (Meyer and Kirkpatrick, 2008). In addition, the phenolic content in ABA-treated vines was higher than non-treated vines (**Figure 2**).

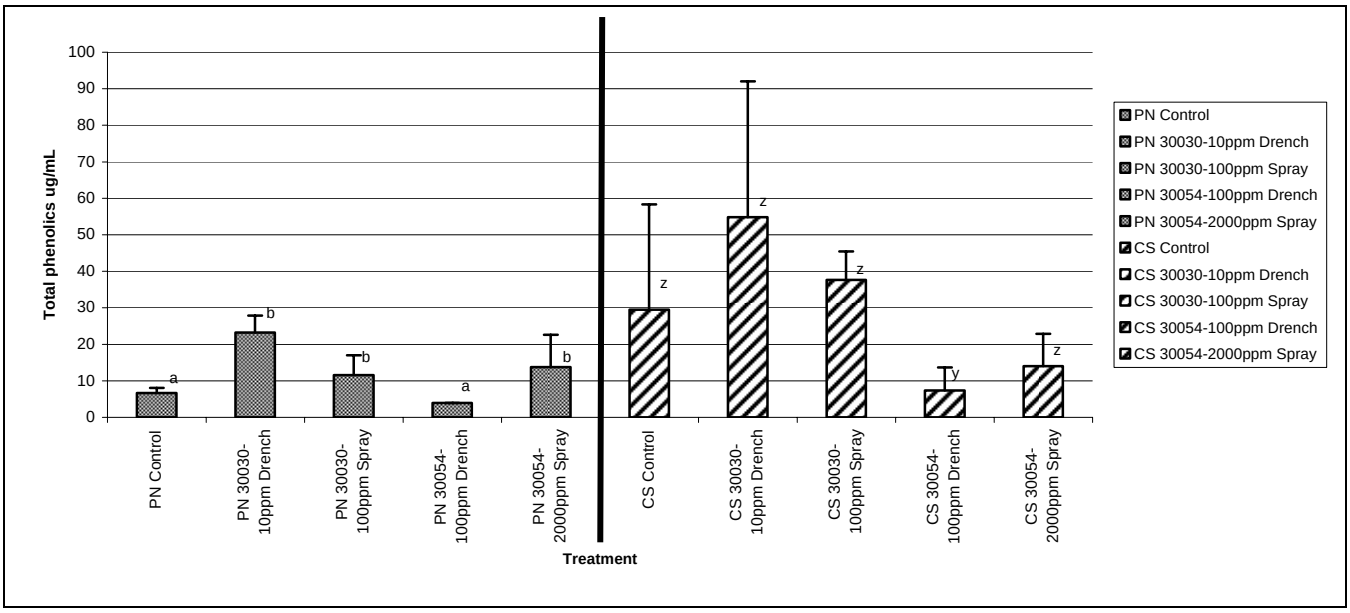

Figure 2. Total phenolic content of xylem sap from ABA treated vines as measured by a gallic acid colorometric assay. PN = Pinot noir xylem; CS = Cabernet sauvignon xylem sap. 30054 = a natural ABA, 30030 a chemically modified ABA. Different letters are significantly different by unpaired t-test with a 2-tailed p-value<=0.05

Xylem sap was expressed from dormant 'Cabernet Sauvignon' and 'Pinot Noir' grape vine canes obtained from the Chateau Leidigh Estate Winery located in Placer County in February, 2009. Previous work has shown that grape vines infected with *Xf* show significant recovery when exposed to cold winter temperatures, such as those experienced in this Placer county vineyard. As a control, we also collected sap from Davis grown vines where curing, due to warmer temperatures, is significantly less than that observed in Placerville. Sap was expressed by placing canes in a "pressure bomb", allowing one end of the cane to protrude from the cylinder, and then pressurizing the chamber with air to pressures between 300 and 400 psi, to collect the xylem sap exudate. These samples were kept frozen at -80C until they were analyzed by High Performance Liquid Chromatography/Mass Spectrometry by Mauri Anderson of the Waterhouse Lab.

The xylem sap samples were chromatographed using reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with electrospray ionization (ESI) mass spectrometry (MS), which produced good resolution. Phenolic compounds were identified based on retention time, UV spectra from diode array detection, and MS using commercially available reference standards. In the Placerville (cold) Pinot Noir samples, a number of phenolic compounds were identified: B procyanidins, catechin, epicatechin, trans-resveratrol, caftaric acid, and a resveratrol tetramer. Cabernet Sauvignon samples

produced an identical polyphenol profile except that the resveratrol tetramer was not present. Interestingly, the warm Pinot Noir sap lacked characteristic peaks for trans-resveratrol as well as the resveratrol tetramer. The fact that resveratrol is present in vines that experience "cold curing" while it is absent in vines that do no undergo "cold curing" suggests that resveratrol may play a role in the curing process. Over the next two winters we will continue to make sap collections and hope to make a clear profile of what polyphenolic conmpounds are present in cold sap, as well as when in the year these polyphenolics appear. Last year we reported on the anti-*Xf* activity of trans-resveratrol *in vitro*. These results have been subsequently supported by Maddox et al. (2009).

In 2010 we began an effort to identify and quantify different polyphenolic compounds in xylem sap over the winter months. We collected sap from Pinot Noir and Cabernet Sauvingon vines (not infected with PD) in Winters, Ca (warm environment) and Placervlle, Ca (cold environment) during the months of January, February, March, and April. As of this writing, we have only been able to analyze the January Pinot Noir samples. In the cold samples we found B procyanidins (flavanoids), Catechin, Epicatechin, Caftaric acid, Coutaric acid, and Quercetin 3-glucuronide. The warm sap had the same polyphenolic profile. The the amount of each phenolic compound in these sap xylem samples have yet to be quantified, but based on relative signal strength, quercetin 3-glucuronide is present at concentrations seven fold higher in cold sap than in warm sap. We are currently working on analyzing the rest of the samples in order to gain a clearer understanding of what phenolic compounds are present in xylem sap, and when exactly they appear. We are also in the process of studying the effect on viability of *Xf* grown in these sap samples *in vitro*.

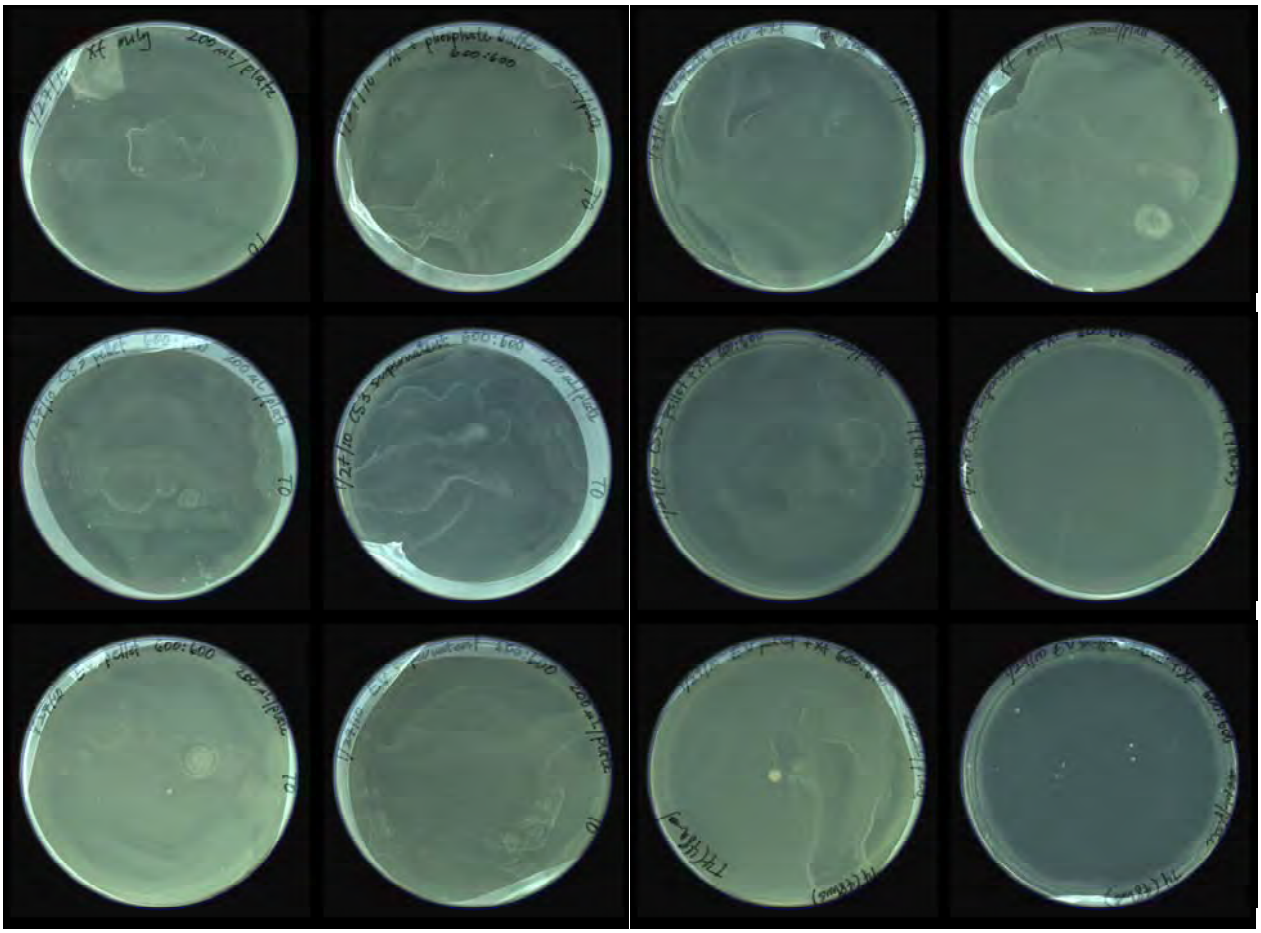

Figure 3. Dialyzed protein product plate assay results. Time 0=buffer/protein combined initially; 48 hours=48 hours after time 0. Treatments from the top left corner: column 1: *Xf* and water 10^8 CFU- time 0; CS3 pellet and *Xf*- time 0; Empty vector pellet-time 0; column 2: *Xf* and 0.2M phosphate buffer- time 0; CS3 supernatent and *Xf*- time 0; Empty vector supernatent and *Xf*- time 0; column 3: *Xf* and water- 48 hours; CS3 pellet and *Xf*- 48 hours; Empty vector pellet- 48 hours; column 4: *Xf* and 0.2M phosphate buffer-48 hours; CS3 supernatent and *Xf*- 48 hours; Empty vector supernatent and *Xf*- 48 hours.

CONCLUSIONS

The expression of recombinant *Vitis vinifera* TLP, and the subsequent plating experiments suggest that this protein may play a role in the cold curing process. We are currently preparing the appropriate constructs in order to make transgenic grapevines that over express TLP under non-freezing temperatures.

Phenolic compounds, specifically trans-resveratrol, show promise as agents that are harmful to the growth of *Xf*. The results of our plate assays are supported both in the literature and by the fact that we detected no resveratrol in warm winter sap collected in Davis, where we observe significantly less overwinter curing than in Placerville. It has been previously reported that resveratrol production in *Vitis vinifera* can be up-regulated by several diverse factors such as plant injury, UV light exposure, and pathogen invasion (Pryce et al. 1976, and Gautheron et al. 1991). It is possible that cold temperatures may serve as an external stress that increases the production of trans-resveratrol. Based on our preliminary 2010 data, in which higher concentrations of xylem sap phenolics were found in areas that experience cold curing suggest that elevated levels of xylem sap phenolic compounds may play a role in the cold curing process.

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INHIBITION *OF XYLELLA FASTIDIOSA* POLYGALACTURONASE TO PRODUCE PIERCE'S DISEASE RESISTANT GRAPEVINES

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ABSTRACT
Polygalacturonases (PG) (EC 3.2.1.15), catalyze the random hydrolysis of 1, 4-alpha-D-galactosiduronic linkages in pectate and other galacturonans. *Xylella fastidiosa* (*Xf*) possesses a single PG gene, *pglA* (PD1485) and *Xf* mutants deficient in the production of PG lose pathogenicity and are compromised in their ability to systemically infect grapevines. We have cloned the *Xf pglA* gene into a number of protein expression vectors and a small amount of active recombinant PG has been recovered, unfortunately most of the protein expressed is found in inclusion bodies in an inactive form. The goal of this project is to use phage panning to identify peptides or single chain fragment variable antibody (scFv) libraries that can bind to and inhibit *Xf* PG. Once peptides or scFvs are discovered that can inhibit PG activity in vitro these peptides will be expressed in grapevine root stock to determine if the peptides can protect the plant against PD.

LAYPERSON SUMMARY
This period we have made significant progress on Objectives 1-3. Most importantly we seem to have made progress on what has been the biggest obstacle thus far in this project, which is creating enough enzymatically active *Xf* PG to pan and test our putative inhibitory phage against. Initial *Xf* PG over-expression experiments in *Xylella fastidiosa* (*Xf)* have been encouraging. Once we confirm the results by repeating our activity assay we should have the ability to produce a large quantity of enzymatically active *Xf* PG. We can then test the efficacy of the inhibitory phage we have obtained from panning against the peptides representing the active site of *Xf* PG.

INTRODUCTION
Polygalacturonases (PG) have been shown to be virulence factors of a number of plant pathogenic bacteria including *Ralstonia solanacearum*, *Xanthomonas campestris*, and *Erwinia carotova* (Huang and Allen 2000; Dow et al. 1989; Lei et al. 1985). *Xylella fastidiosa* (*Xf*) possesses a single PG gene *pglA* (PD1485), and mutation of this gene results in lost pathogenicity and reduced ability to systemically infect grapevines (Roper et al. 2007). In order for *Xf* to systemically infect a grapevine it must break down the pit membranes that separate individual xylem elements. Pectic polymers determine the porosity of the pit membrane (Baron-Epel, et al. 1988; Buchanan et al. 2000) and *Xf* PG allows the bacterium to breakdown the pectin in these membranes. The premise of this research is to identify a peptide that can be expressed in the xylem of a grapevine that can suppress *Xf* PG activity thus limiting the ability of *Xf* to spread systemically through grapevines and cause Pierce's disease (PD).

To accomplish this we will use phage display of a random dodecapeptide library and a scFv antibody library attached to the coat protein gp38 of M13 phage in a phage panning experiment using active recombinant *Xf* PG as the target. After three rounds of panning, phage that show a high binding affinity for *Xf* PG will be screened for their ability to inactivate PG activity in vitro in reducing sugar assays. Once a suitable inhibitory peptide is discovered it will be cloned into an agrobacterium binary vector and used to transform tobacco and grapevines by the UCD Plant Transformation Facility. These transgenic plants will then be inoculated with *Xf* and compared to non transgenic plants in PD symptom progression. If significant disease inhibition is shown we will use these transgenic grapevines as rootstock and see if they can also provide resistance to grafted scions.

OBJECTIVES
1. Isolate a sufficient amount of biologically active *Xf* polygalacturonase (PG) enzyme to conduct phage panning and PG-inhibition assays.
2. Isolate M13 phage that possess high binding affinities to *Xf* and/or AA PG, or synthetic peptides specific for the active sites of several PGs from a M13 random peptide and scFv library.
3. Sequence candidate binding phage and determine if selected M13 phage and the gp38 M13 protein
4. which mediates phage binding to *Xf* PG can inactivate PG activity in vitro.
5. Clone anti-*Xf* PG gp38 protein into an Agrobacterium binary vector and provide this construct to the
6. UCD Plant Transformation facility to produce transgenic SR1 tobacco and Thompson Seedless
7. grapevines.
8. Determine if anti-*Xf* PG gp38 protein is present in xylem sap of transgenic plants.

9. Mechanically inoculate transgenic plants with *Xf* and compare PD development with inoculated, non-transgenic control plants.

RESULTS

Objective 1: Isolate a sufficient amount of biologically active *Xf* polygalacturonase (PG) enzyme to conduct phage panning and PG-inhibition assays.

Our previous work in trying to express *Xf* PG using commercially available *E. coli* and *P. pastoris* expression systems, as well as, an agroinfection-compatible Tobacco mosaic virus protein expression system (Lindbo 2007) did not provide us with a sufficient amount of active enzyme. An inducible expression system has not yet been developed for *Xf*; however previous work has shown that it is possible to over-express proteins in *Xf* (Newman et al 2003). Newman et al. were able to stably over-express the green fluorescent protein (GFP) in *Xf*; because of this we decided it may be a viable option to over-express *Xf* PG in a similar manner.

Modifying the same vectors used to construct GFP over-expressing *Xf* with the *Xf* PG gene and using the transformation protocols of Matsumoto et al. (2009) we have generated 22 putative *Xf* PG over expressing transformants. Transformants were tested by colony PCR to confirm that they contained the correct size insert. Fourteen of the 22 transformants were grown in PD3 Kan for 10 days at 28C. The cultures were centifuged at 10,000g for 10 minutes and the media fraction was collected. One ml of media fraction was concentrated using TCA precipitation resulting in a 20X concentration of the media fraction, these samples were then analyzed by polyacrylamide gel electrophoresis and Western blot analysis. Media fractions from two of the transformants were concentrated 20X using centrifugal concentrators with a 9 Kd molecular weight cut off. These concentrated samples were used in a PG cup plate assay and one of the transformants *XF*-6 appears to be enzymatically active (**Figure 1**) (Taylor and Secor 1988). We have repeated this experiment with similar results; however, the amount of PG produced is still rather small. In order to create a larger amount of active enzyme we are currently moving the constructs onto plasmids known to replicate in *Xylella*, e.g., pBBR1MCS-5 and p*XF*20-PemIK (Kovach et al. 1995, Reddy et al. 2007, Stenger in press). If we can show these results to be repeatable we are confident that using these transformants we can easily produce a large amount of enzymatically active *Xf* PG for use in this experiment as well as to others who need this enzyme for use in their work.

Additionally, we are using the pProbe vector series to characterize the *Xf* PG promoter region (Miller et al., 2000). When a promoter on the plasmid is active or activated *gfp* will be produced, giving a visual indication of gene expression from the promoter. The promoter region of *Xf* PG was PCR amplified and inserted into pProbe *gfp* tagless and we are in the process of testing them. These constructs should allow us to determine if there is a compound that can induce the production of *Xf* PG from the native *Xf* PG promoter.

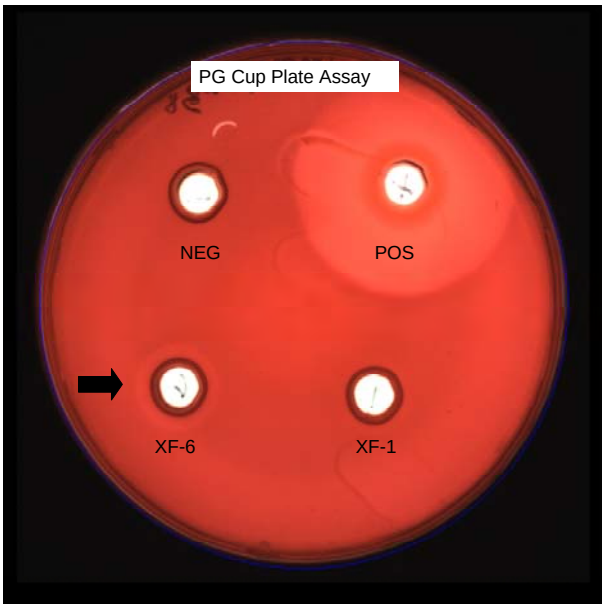

Figure 1. PG cup plate assay, a clear halo representing enzyme activity is evident in the positive control (POS) from *A. acleatus* and PG over-expressing transformant *XF*-6.

Objective 2: Isolate M13 phages that possess high binding affinities to *Xf* PG from a M13 random peptide library.
We have done extensive *in silico* analyses of the enzymatically active sites of several phytopathogenic bacterial and fungal PGs such as *Pectobacterium carotovora ssp. carotovora* and *Aspergillus aculeatus* (Pickersgill et al 1998, Cho et al 2001). The PGs from these other microbes have been well studied and structural studies have shown that the active site amino acids, consisting of roughly eight amino acids and the tertiary structure of the PGs are highly conserved across all fungal and bacterial PGs (Pickersgill et al 1998, Cho et al 2001, Shimizu et al 2002, Abbott and Boraston 2007). Furthermore, previous research using phage display technologies showed that many of the phage that bound to a variety of enzymes also bound to and inactivated the enzyme (Hyde-deRuyscher et al, 1999).

Having a very good idea of where the *Xf* PG active site may be located on the protein, and which amino acids are involved in catalysis and substrate binding, we had synthesized two 14mer peptides derived from the *Xf* PG sequence, one which will target the active site directly and a second that will target an area providing entry into the active site (**Figure 2**).

Peptide	Sequence
Peptide 1	DSPNSNGLQMKSDAC
Peptide 2	STGDDHVAIKARGKC

Figure 2. Sequences of synthesized peptides.

Additionally, these peptides were injected into rabbits to create polyclonal antibodies. These polyclonal antibodies were used in a western blot that confirmed that the antibodies created against each 14-mer peptide could also recognize full length *Xf* PG (data not shown).

We have completed the phage panning procedure for peptide 2 using the Tomlinson I and J scFv libraries. At the end of the third round of selection a polyclonal ELISA with BSA conjugated peptide 2 as the target was run which showed that each library (I and J) of scFvs showed a higher binding affinity to BSA conjugated peptide 2 than to BSA alone, or to the wells of the plate. With this knowledge 90 individual colonies from each library were picked from the third round phage pool and used in a monoclonal ELISA to determine which monoclonal scFvs had the highest binding efficiencies. The eight clones from each library (I and J) providing the highest ELISA absorbance readings were chosen for sequencing. We have currently only sequenced the heavy chain variable portion of the scFv and although none of the eight clones from each of libraries shared the exact same sequence they did have similarities to each other. The eight monoclonal phages from each library (I and J) were then used individually as the primary antibodies in a western blot to confirm that monoclonal phage raised against the 14-mer peptide 2 conjugated to BSA would also be able to identify full length recombinant PG (**Figure 3**) (Tanaka et al 2002).

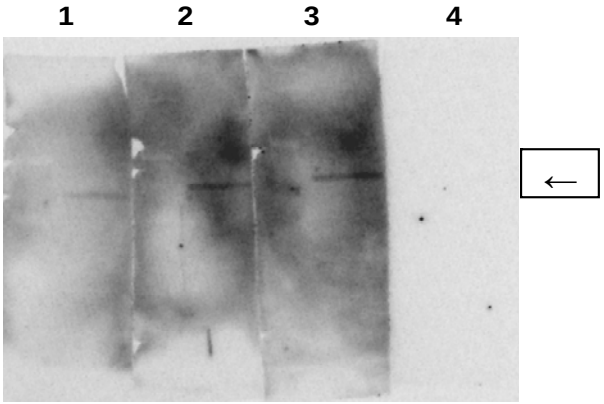

Figure 3. Western blot analysis of 3 representative monoclonal scFv phages (J-library).Lanes 1-3 are *E. coli* lysate containing recombinant *Xf* PG each membrane strip was reacted with a single monoclonal phage from the third round of panning . Arrow represents location of *Xf* PG band. Molecular weight markers are on the left side of each gel strip. Lane 4 is a conjugate control that was not reacted with any monoclonal phage.

Objective 3: Determine if selected M13 phage and the gp38 M13 protein which mediates phage binding to *Xf* PG can inactivate PG activity in vitro.
We have sequenced the heavy chain variable regions of the 16 candidate monoclonal phage and although none of the eight clones from each of libraries shared the exact same sequence they did have similarities to each other. Once we obtain enough active *Xf* PG we will use the monoclonal phages in *Xf* PG inhibition assays. Once a candidate phage is found that can inhibit *Xf* PG *in vitro* we will then express the scFv protein alone and determine if the protein itself can also inhibit *Xf* PG activity *in vitro*. We will then be able to clone the anti-*Xf* PG protein into an Agrobacterium binary vector and provide this construct to the UCD Plant Transformation facility to produce transgenic SR1 tobacco and Thompson Seedless grapevines. Once we have transgenic plants we will be able to complete objectives 5 and 6.

Objective 4: Clone anti-*Xf* PG gp38 protein into an Agrobacterium binary vector and provide this construct to the UCD Plant Transformation facility to produce transgenic SR1 tobacco and Thompson Seedless grapevine.
Once suitable inhibitory phage peptides are discovered in objective 3 we can begin objective 4.

Objective 5: Determine if anti-*Xf* PG gp38 protein is present in xylem sap of transgenic plants.
Objective 4 needs to be completed before work on objective 5 can begin.

Objective 6: Mechanically inoculate transgenic plants with *Xf* and compare PD development with inoculated, non-transgenic control plants.
All previous objectives must be completed before we can start objective 6.

CONCLUSIONS
We have made good progress thus far in finding suitable PG activity assays to use in the PG-inhibition assays. We are currently exploring different expression systems to generate more active PG to use in phage panning and activity assays. We have acquired 16 candidate scFv phage, by panning against peptide 2 conjugated to BSA that are capable of indentifying full length *Xf* PG that we will be using in *Xf* PG inhibition assays, as we have described previously. If one of the candidate phage can inhibit *Xf* PG activity *in vitro* then we can transform grapevines with the peptide and determine if they provide plants with resistance to PD.

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ISOLATION, CHARACTERIZATION, AND GENETIC MANIPULATION OF *XYLELLA FASTIDIOSA* HEMAGGLUTININ GENES

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ABSTRACT AND INTRODUCTION

Xylella fastidiosa (Xf) cell-cell attachment is an important virulence determinate in Pierce's disease (PD). Our previous research has shown that if two hemagglutinin (HA) genes which we named HxfA and HxfB are mutated *Xf* cells no longer clump in liquid medium and the mutants form dispersed "lawns" when plated on solid PD3 medium (Guilhabert and Kirkpatrick, 2005). Both of these mutants are hypervirulent when mechanically inoculated into grapevines, i.e. they colonize faster, cause more severe disease symptoms and kill vines faster than wild-type (wt) *Xf*. If either HxfA or HxfB is individually knocked out there is no cell-cell attachment, which suggests that BOTH HA genes are needed for cell-cell attachment. It is clear that these proteins are very important determinants of pathogenicity and attachment in *Xf*/plant interactions. The *Xf* HAs essentially act as a "molecular glue" that is essential for cell-cell attachment and likely plays a role in *Xf* attachment to xylem cell walls and contributes to the formation of *Xf* biofilms. Recent work reported by the Almeida lab has also shown the importance of HAs in vector transmission (Killiny and Almeida, 2009). The knowledge we gained here about the basic biology of *Xf* HA proteins provided the foundation for completing the last step of this project, where we transformed tobacco and grapevine plants with HA genes. Hopefully, these genes will be expressed in the xylem of transgenic plants and potentially act as a 'molecular glue' to retard systemic movement of inoculated *Xf* cells through grapevine xylem. If successful, this approach might provide a novel form of resistance against PD.

LAYPERSON SUMMARY

Hemagglutinin (HA) proteins play an important role in adhesion and biofilm formation of *Xylella fastidiosa (Xf).* Previous studies by Guilhabert and Kirkpatrick, 2005 showed that mutants in the identified HA genes no longer formed clumps in liquid medium like wild-type (wt) *Xf* cells. Clearly the HA proteins play an important role in mediating cell-cell interactions. Research in the Almeida lab has also shown that HA proteins play important roles in attachment processes during vector transmission (Killiny and Almeida 2009). Research conducted in our lab has shown that HA proteins are present in the outer membranes of *Xf* cells and that these proteins are also secreted into culture medium at low concentrations. Interestingly, we also showed that HAs are embedded in vesicles as it has been reported for some pathogenic Gram-negative bacteria (Kuehn and Kesty, 2005). The 10.5 kb HA genes should theoretically encode a protein of approximately 360 kD, however we have shown that the native size of the HA proteins in the outer membranes, culture supernatants and membrane of vesicles is approximately 220 kD. To identify the cleavage site where the processing occurs, we isolated native secreted HA proteins from culture supernatant. These proteins were analyzed by mass spectrometry and we determined that the cleavage site lies 2300aa downstream from the N-terminus of the gene such that approximately one third of the C-terminal part is cleaved off forming the full-length native HA . We used this information to create binary plasmids containing the identified portions of the HA proteins that mediate attachment and used them to generate putatively HA-expressing transgenic tobacco and grapevines. Transgenic tobacco were evaluated by PCR, RT-PCR and serological assays to detect expressed *Xf* HA. We verified that the transformed tobaccos produced HA mRNA but were unable to detect HA in the xylem sap by ELISA and western blot analysis. Ten transgenic grapevines lines were produced using a HA construct that should contain the cell-cell binding domains and 13 independent grapevine lines have been produced using the full-length native 220kD HA protein. The transgenic grapevines are now being grown and propagated to produce plants that will be further evaluated using molecular and *Xf* pathogenicity assays.

OBJECTIVES

1. a. Use antibodies we have prepared against a conserved, putative adhesion domain (AD2) that is present in both *Xf* hemagglutinins (HA) to determine the native size and location of *Xf* HA in cultured *Xf* cells and PD-affected grapevines.
   b. Determine if these antibodies (Fab fragments) can prevent cell-cell clumping in liquid *Xf* cultures.
   c. Prepare an affinity column using HA domain antibodies and isolate native *Xf* HAs from culture cells. Establish the identity of affinity purified, putative HAs by N-terminal sequencing.
   d. Determine if native HAs and HA domain fusion proteins can bind to *Xf* cells.
   e. Inject affinity purified HA proteins into rabbits and obtain HxfA and B specific-antibodies. Determine if HxfA and B specific antibodies can block cell-cell clumping of *Xf* grown in liquid medium.
2. a. PCR-amplify, clone and express as fusion proteins, additional hypothetical adhesion domains of HxfA and B.

b. Prepare rabbit polyclonal antibodies against each HxfA/B domain fusion protein. Determine the native size and location of *Xf* HA in *Xf* cultured cells using AD1-3 and AD4 antibodies.
c. Determine if antibodies against various HxfA/B domain fusions can block cell-cell clumping of *Xf* grown in liquid medium.

3. a. Transform grapevines and tobacco, an experimental host of Xf and an easily transformable plant (Francis et al., 2008), with *Xf* HA binding domains. Use antibodies prepared in Objective 2 to determine if *Xf* HA proteins can be found in tobacco xylem fluid.
b. Mechanically inoculate HA-transgenic grapevines and tobacco with wild type (wt) *X*f cells. Compare disease progression and severity in transgenic tobacco and grapevines with non-protected controls.

RESULTS

Objectives 1 and **2** are completed and results were reported previously (Pierce's Disease Research Symposium Proceedings 2007-2009 and in the manuscript: Voegel, et al., 2010. Localization and characterization of *Xylella fastidiosa* hemagglutinin adhesins, Microbiology 156: 2172 – 2179.

Objective 3.

To overcome problems that might occur trying to transform plants with a full-length hemagglutinin (HA) gene, we first determined the size of native *Xf* HA proteins. We found that both native HxfA and HxfB were processed from their potential size of 360 kD to 220 kD native size. Furthermore, we identified the cleavage site in the HA proteins. We also identified domains in the N-terminal portion of the HA proteins that mediate cell-cell attachment and showed that the active protein is secreted (Voegel, et al, 2010). Based on these results we prepared 2 different constructs for transformating grapevines and tobacco; one construct contained the N-terminal hemagglutination domains (AD1-3) and one contained the entire native 220 kD protein (220).

AD1-3 and 220 were PCR amplified from the gene HxfB (PD1792) using proof reading polymerase and wt Temecula genomic DNA as template. The resulting 4000 bp and 6300 bp PCR products were cloned into pCR-2.1-TOPO and fully sequenced using primers generated every 600 bp along the cloned HA fragments. The obtained sequences were aligned into a contig using the program Sequencher to verify amplification of the correct sequences. To enable secretion of the bacterial HAs outside the eukaryotic cells of tobacco and grape, a signal peptide pGIP (Aguero et al., 2005) was synthesized by DNA2.0 (Menlo Park, CA) and fused N-terminally to the cloned HA products. The codon usage of the synthesized signal peptide was optimized for expression in eukaryotes. The pGIP-HA fusions were cloned into vector pDE00.0113 (Dandekar lab) containing the 35S promoter and ocs3' terminator creating pDE00.0113-pGIP-AD1-3 and pDE00.0113-pGIP-220. After verifying its integrity by sequencing, pDE00.0113-pGIP-AD1-3 was digested with AscI and the resulting cassette cloned into the binary vector pDU97.1005 (Dandekar lab) creating pDU08.2407. The plasmid was transformed into *Agrobacterium tumefaciens* strain EHA101 and the culture given to the Ralph M. Parsons foundation transformation facility on the UC Davis campus who performed the transformation of SR-1 tobacco.

Unfortunately, the pDU97.1005 marker gene nptII confers resistance to kanamycin. In transformation experiments with grapes using binary plasmids containing the nptII gene many escapes were observed by the transformation facility which prolonged the time needed to identify and generate transformed grapevines. Therefore we digested pDE00.0113-pGIP-AD1-3 and pDE00.0113-pGIP-220 with EcoRI and ligated the resulting cassette into the binary plasmid pCAMBIA1300 (Canberra, Australia) which confers resistance to hygromycin. This marker gene is more suitable for transformation of grapevines than nptII and is functional in grapevines as well as is tobacco. Binary plasmids pCAMBIA-pGIP-AD1-3 and pCAMBIA-pGIP-220 were transformed into *Agrobacterium tumefaciens* strain LBA4404 and the cultures given to the Ralph M. Parsons foundation transformation facility who will transform Thompson seedless grapevines and SR-1 tobacco with pCAMBIA-pGIP-220 and pCAMBIA-PGIP-AD1-3.

We received our first AD1-3 transformed tobacco plants in 2009. DNA was extracted from the T0 tobacco plants and tested by PCR for the presence of T-DNA and 9/11 and 10/11 tested positively as shown in **Figure 1**. The PCR positive lines were tested by RT-PCR to determine whether the constructs were expressed; all of the T-DNA positive lines tested positively by RT-PCR indicating the HA constructs were being transcribed. Attempts were made to detect hemagglutinin in both tobacco xylem sap and in total leaf proteins using both ELISA and Western blot analysis. Unfortunately we were not able to detect measurable amount of HAs using either serological technique.

Six transgenic tobacco plants of lines 1 and 5, which were transformed with the AD1-3 construct and 6 plants from lines G and J, which were transformed with the 220kD construct were mechanically inoculated with wild type Fetzer *Xf*. Eight weeks after inoculation *Xf* was isolated from leaves that were 25cm above the point of inoculation. The results are shown in **Table 1** below.

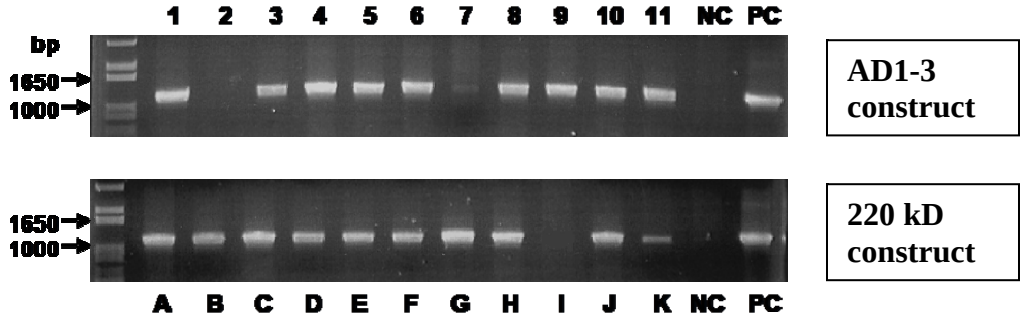

Figure 1. PCR analysis to confirm T-DNA insertion in 11 tobacco transformed lines T0 for AD1-3 and 220.

Table 1.

Tobacco Plant inoculated with *Xf*	**CFU/g of tissue 25 cm above P.O.I.**
Wild type SR1 tobacco	9.9 (+/- 7.3) x 10^5
Line 1	9.1 (+/- 16.2) x 10^2
Line 5	1.3 (+/- 1.5) x 10^6
Line G	3.3 (+/- 8) x 10^2
Line J	2.5 (+/- 3.8) x 10^3

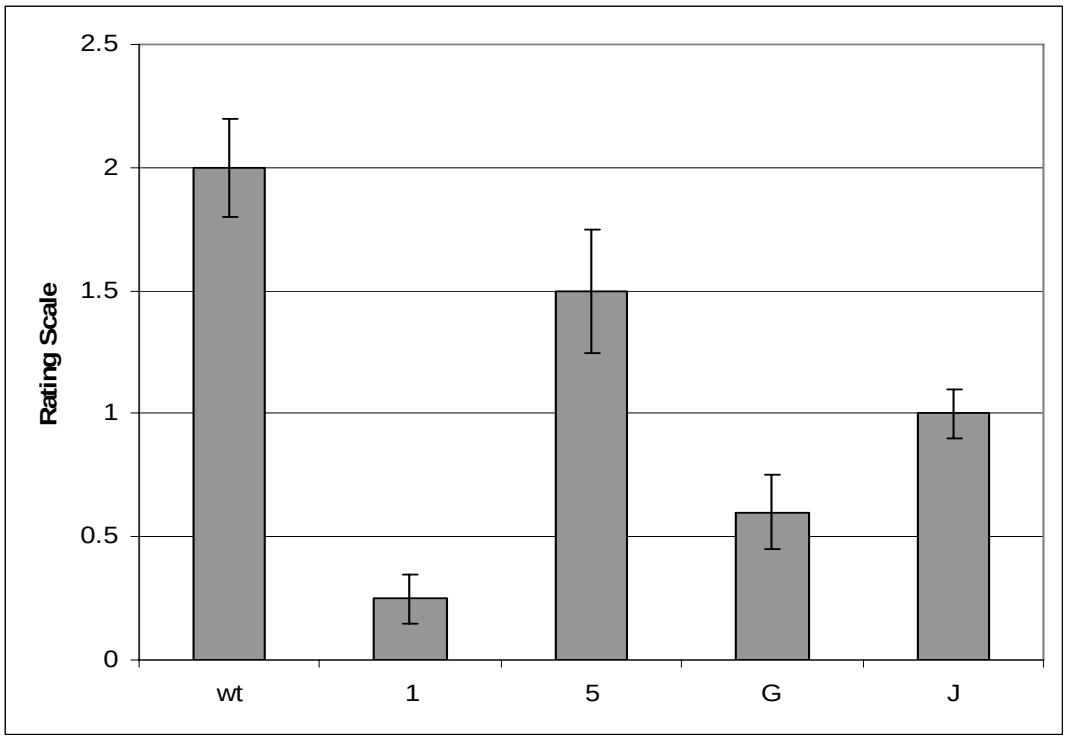

Figure 2. Disease severity ratings (0=healthy to 5 = severe symptoms) showed Lines 1, G and J developed significantly less severe disease symptoms that the non-transformed Sr1 tobacco 8 weeks following inoculation with *Xf*.

There was a good correlation between the number of *Xf* CFUs that could be isolated 25cm above the point of inoculation and the severity of disease symptoms 8 weeks after inoculation with *Xf* (**Figure 2**). Lines 1, G and J yielded fewer *Xf* bacteria upon isolation and the severity of symptoms in those lines was less than *Xf*-inoculated non-transformed SR1 tobacco.

Unfortunately similar analyses of T2 generation transgenic tobacco were not as encouraging because the amount of variation in the plant reps rendered no statistically significant differences in disease severity between the T2 lines and the wild type SR1 tobacco (**Figure 3**).

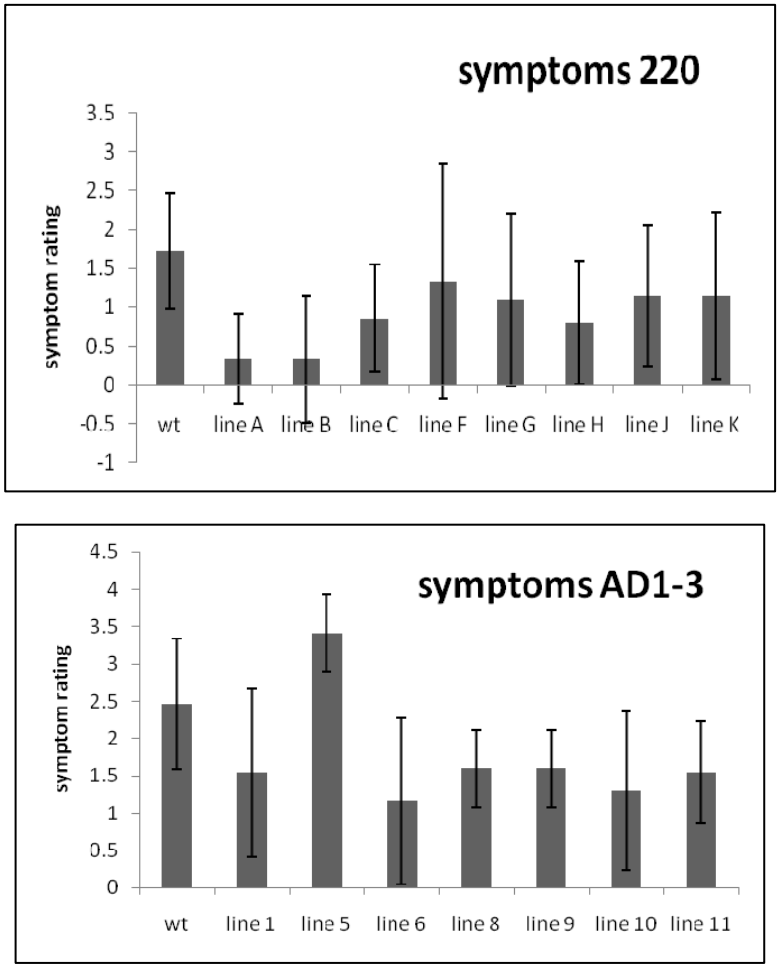

Figure 3. Disease severity in T2 HA-transgenic SR1 tobacco based on a 0 (healthy) to 5 (dead) 8 weeks after inoculation with wild type Fetzer *Xf*.

Table 2. Percentage of HA-transgenic and wild type SR1 tobacco plants in which NO *Xf* was isolated at 25cm above the point of inoculation. Lines 1, 5, 6, 8, 9, 10 and 11 are SR1 tobacco transformed with the AD1-3 hemagglutinin construct while lines A-K were transformed with the 220kD hemagglutinin construct.

line	wt	1	5	6	8	9	10	11	
%	14	36	16	25	10	40	60	18	
line	wt	A	B	C	F	G	H	J	K
%	33	33	60	42	33	60	50	28	14

While there was no statistically significant differences in disease severity of transformed versus non-transformed SR1 tobacco, we did observe differences in the number of inoculated plants in which *Xf* could be isolated 25cm from the point of inoculation (**Table 2**). For example, for the AD1-3 construct *Xf* was not isolated in only 145 of the wild type SR1 tobacco, while 60% of the Line 10 transgenic plants were devoid of *Xf* at 25cm above the POI, suggesting that the HA transgenic line maybe slowing the systemic movement of *Xf* compared to the movement in non-transformed tobacco. There 2 lines (B and G) whose numbers of *Xf*-free plant reps were less than non-transformed tobacco but a larger number of the control plants

were *Xf*-free at 25cm compared to the Ad1-3 experiment. Because we now have transgenic grapevines in hand to evaluate we felt that it wasn't worth additional effort to repeat testing of the T2 tobacco lines.

In mid-August 2010 we began receiving Thompson seedless grapevines from the UCD Plant transformation facility. In mid-October we received the final transgenic lines: 10 lines transformed with the AD1-3 HA construct and 13 lines transformed with the 220kD construct. These were grown in a growth chamber until they were hardened off enough to be moved into a greenhouse where they are being raised under supplemental lighting. We anticipate that the vines will have grown sufficiently by the end of November that cutting can be taken and rooted to generate more plants to work with. These vines will be subjected to the same PCR, RT-PCR, ELISA and western blot analyses that we used on the transgenic tobaccos. Reps of each line will be mechanically, and hopefully with the assistance of the Almeida lab at UC Berkley, sharpshooter inoculated with *Xf* in the spring. To date the production of the transgenic grapevines has taken 14 months and likely another 4 to 5 months will be required to assess their potential resistance or susceptibility to PD.

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OPTIMIZING GRAPE ROOTSTOCK PRODUCTION AND EXPORT OF INHIBITORS OF *XYLELLA FASTIDIOSA* POLYGALACTURONASE ACTIVITY

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Reporting Period: The results reported here are from work conducted October 2009 to October 2010.

ABSTRACT
The CDFA Pierce's Disease (PD) and Glassy-winged Sharpshooter Board's Research Scientific Advisory Panel review in 2007 and subsequent RFPs have given top priority to delivery from grafted rootstocks of PD control candidates, including polygalacturonase-inhibiting proteins (PGIPs). Optimal PGIPs for inhibition of *Xylella fastidiosa* (*Xf*) polygalacturonase (PG) are being selected from several plant sources. Fourteen candidate PGIPs have been chosen and homology models were generated to predict interactions with and potential inhibition of *Xf*PG. PGIPs from pear, rice, and orange were determined to be the most likely inhibitory proteins for *Xf*PG. Recombinant protein expression systems have been developed for *Xf*PG and each candidate PGIP. Initial inhibition assays have shown that the pear fruit PGIP is a more effective inhibitor of *Xf*PG than the PGIP from tomato, however both PGIPs limit XfPG symptom development in tobacco leaf infiltration assays. Expression of additional PGIPs to test is underway and other non-vinifera *Vitis* PGIPs are being pursued.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) uses a key enzyme, polygalacturonase (PG), to spread from the initial point of inoculation throughout the grapevine; this spread leads to Pierce's disease (PD) symptom development. Plant proteins called PG-inhibiting proteins (PGIPs) are produced by many plants and selectively inhibit PGs from bacteria, fungi, and insects. Pear fruit PGIP is known to inhibit *Xf*PG and to limit PD development in inoculated grapevines which have been transformed to express the pear protein. PGIPs are graft transmissible so we are interested to determine which PGIP best inhibits *Xf*PG and how well, when expressed in transgenic rootstocks, this PGIP prevents PD development in *Xf* inoculated wild-type scions. We have modeled 14 candidate PGIPs to predict how they physically interact with *Xf*PG and to combine this knowledge with *in vitro* and *in planta* assay results measuring the ability of each candidate PGIP to inhibit *Xf*PG. For these inhibition assays we are developing separate systems to generate high levels of active *Xf*PG and PGIPs. The best inhibiting PGIPs will be expressed in test grape rootstock germplasm and, after grafting, their ability to limit PD development in non-transgenic scions will be determined.

INTRODUCTION
Xylella fastidiosa (*Xf*), the causative agent of Pierce's disease (PD) in grapevines, has been detected in infected portions of vines. Several lines of evidence support the hypothesis that *Xf* uses cell wall-degrading enzymes to digest the polysaccharides of plant pit membranes separating the elements of the water-conducting vessel system, the xylem, of the vines. *Xf*'s cell wall degrading enzymes break down these primary cell wall barriers between cells in the xylem, facilitating the systemic spread of the pathogen. Recombinantly expressed *Xf* polygalacturonase (PG) and β-1,4-endo-glucanase (EGase), cell wall degrading enzymes that are known to digest cell wall pectin and xyloglucan polymers, respectively, have been shown to degrade grapevine xylem pit membranes and increase pit membrane porosity enough to allow passage of the bacteria from one vessel to the next (Pérez-Donoso *et al.*, 2010). *Xf* cells have been observed passing through similarly degraded pit membranes without the addition of exogenous cell wall degrading enzymes, supporting the conclusion that the enzymes are expressed by *Xf* and allow its movement within the xylem (Labavitch and Sun, 2009). Roper *et al.* (2007) developed a PG-deficient strain of *Xf* and showed that the mutant bacterial strain was unable to cause PD symptoms; thus, the *Xf*PG is a virulence factor of the bacteria that contributes to the development and spread of PD.
PG-inhibiting proteins (PGIPs) produced by plants are selective inhibitors of PGs and limit damage caused by fungal pathogens (*B. cinerea*; Powell *et al.*, 2000) as well as by insects (*Lygus hesperus*; Shackel *et al.*, 2005). Agüero *et al.* (2005) demonstrated that by introducing a pear fruit PGIP (*pPGIP*) gene (Stotz *et al.*, 1993) into transformed grapevines, the susceptibility to both fungal (*B. cinerea)* and bacterial (*Xf)* pathogens decreased. This result implied that the pPGIP provided protection against PD by inhibiting the *Xf* PG, reducing its efficiency as a virulence factor. In fact, recombinant *Xf*PG is inhibited *in vitro* by pPGIP-containing extracts from pear fruit (Pérez-Donoso *et al.*, 2010). In a key preliminary observation for the PD control approach investigated in this project, Agüero *et al.* (2005) demonstrated that transgenic pPGIP protein

could be transported from transformed grapevine rootstocks, across a graft junction and into wild-type scions. pPGIP also has been shown, this year, to be transported from rootstocks across grafts into the aerial portions of tomato plants.
The overall goal of the project is to develop transgenic grape rootstock lines that express PGIPs that effectively reduce the virulence of *Xf*. The project is designed to identify specific PGIPs that optimally inhibit the virulence factor, *Xf*PG, and to express these PGIPs in grape rootstocks to provide PD protection in scions. The expression of PGIPs will utilize transformation components with defined intellectual property (IP) and regulatory characteristics, as well as expression regulating sequences that result in the maximal production of PGIPs in rootstocks and efficient transport of the proteins through the graft junctions to the aerial portions of vines so that *Xf* movement is limited in infected scion tissues.

OBJECTIVES

1. Define a path to commercialization of a PD control strategy using PGIPs, focusing on IP and regulatory issues associated with the use of PGIPs in grape rootstocks.
2. Identify plant PGIPs that maximally inhibit *Xf*PG.
3. Assemble transcription regulatory elements, *Xf*-inducible promoters, and signal sequences that maximize PGIP expression in and transport from roots.
4. Create PGIP-expressing rootstocks and evaluate their PD resistance.

RESULTS AND DISCUSSION

Objective 1: A path to commercialization of transgenic rootstocks

PIPRA IP analyst, Gabriel Paulino, has served as the main liaison for issues associated with the potential commercialization of transgenic grapevine rootstocks for several CDFA PD/GWSS Board funded projects. He has obtained the necessary APHIS-USDA authorizations to begin testing these PD control strategies in field locations. 'Thompson Seedless' and 'Chardonnay' grapevines expressing the pear fruit PGIP (pPGIP) gene were planted in a jointly operated field trial in Solano County during July, 2010. More details can be found in the report "Field evaluation of grafted grape lines expressing PGIPs" (PI Powell).

Objective 2A: Propagation and grafting of grape lines expressing and exporting pPGIP

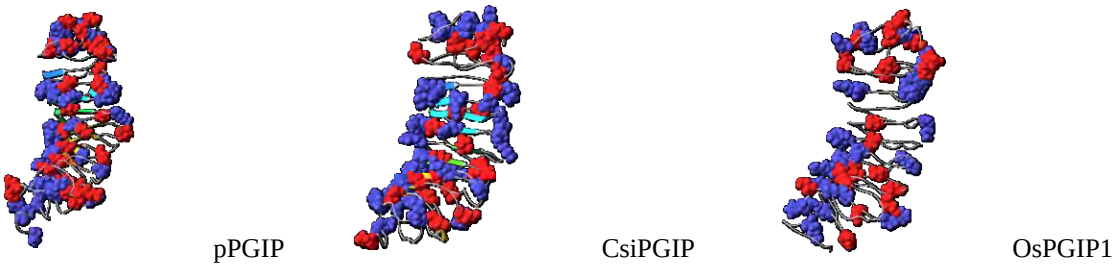
The transgenic 'Thompson Seedless' and 'Chardonnay' grapevines expressing the pPGIP described in Aguero *et al.* (2005) have been maintained in the greenhouse. Previous attempts at vegetative propagation for grafting studies proved inconsistent. Total plant numbers for both cultivars and control plants not expressing pPGIP were increased during this reporting period with the help of an aeroponic cloner (EZ-Clone, Inc., Sacramento, CA). Non-lignified stem segments, three nodes in length, were transferred to individual sites within the cloner. Roots began forming on dark-grown, constantly misted basal regions in 1-2 weeks. The application of 1000 ppm IBA to basal regions immediately after cutting did not result in increased rooting time or yield.

Grafted plants are being generated to verify the transport of pPGIP protein from transgenic rootstocks, across the graft junction, into scion tissue not expressing any foreign PGIP. Grafting has been attempted with both green and semi-lignified stem segments for all graft combinations. Grafts of six 'Thompson Seedless'plants and one 'Chardonnay' plant have been formed by a modified wedge grafting technique whereby scion sections of 1 to 2 nodes were stripped of foliage and cut with perpendicular apical ends and wedge basal ends. These sections were fitted into notched rootstock stems of equal maturity. The grafts were secured with Parafilm M, a clothespin, and a translucent bag to prevent desiccation. Other green grafting techniques, such as chip budding, have been attempted with limited success.

Work in the project "Tools to identify PGIPs transmitted across grapevine grafts" (PI Powell) is developing a monoclonal antibody to recognize pPGIP, but not the native grape PGIP, in these own-rooted and grafted grapevines. UC Davis Biochemistry and Molecular Biology Ph.D. candidate, Victor Haroldsen, has shown pPGIP crossing graft junctions from transgenic tomato rootstocks into wild-type tomato scion leaf tissue. For these experiments, he used existing stocks of polyclonal pPGIP antibodies after concentrating leaf extract samples 30-fold. Similar experiments using the aforementioned grafted grapevines will probe isolated xylem sap from scion tissues. Combined with the monoclonal antibody, the increased reactivity will allow for quantification of pPGIP crossing the graft junction into wild-type tissues.

Objective 2B: Selection of PGIPs as PD defense candidates and PGIP-XfPG modeling

The 14 candidate PGIPs were previously selected for *in vitro* and *in vivo Xf*PG inhibition assays based on predicted protein charge and phylogenetic analyses. The homology models created for *Xf*PG, the polygalacturonic acid substrate for PG, and each of the candidate PGIPs have provided unique predictive tools to interpret the inhibition mechanisms and physical interactions between XfPG and the PGIPs (Labavitch, 2009). Dynamic reaction simulations predicted that two clusters of amino acids, #63-74 and #223-226, must be unblocked for *Xf*PG to cleave its substrate *in silico*. The long columns of electronegative residues on the concave faces of the PGIP's leucine rich repeat structure bind to these critical regions (**Figure 1**). This information coupled with surface chemistry mapping predicts pPGIP, CsiPGIP, and OsPGIP1 to be the best inhibitors of *Xf*PG.



pPGIP CsiPGIP OsPGIP1

Figure 1. Homology models of 3 prime candidate PGIPs (CsiPGIP-orange;,OsPGIP-rice). The column of electronegative residues (red) on the concave faces of each protein may align with critical residues on XfPG important for inhibition.

A closer look at the dynamic reaction simulations highlighted other specific residues that may also influence PG-PGIP binding. Strong hydrogen bonding occurs between residues on PPGIP and Tyr303 of *Xf*PG, bringing them together in a potentially inhibitory manner (**Figure 2**). Electrostatic repulsions between VvPGIP residues and *Xf*PG Tyr303 prevent a similar alignment and may predict a failure to inhibit *Xf*PG. Combining modeling predictions and future inhibition data will allow us to score the results of predicted interactions and infer other potentially useful interactions between the candidate PGIPs and other PGs.

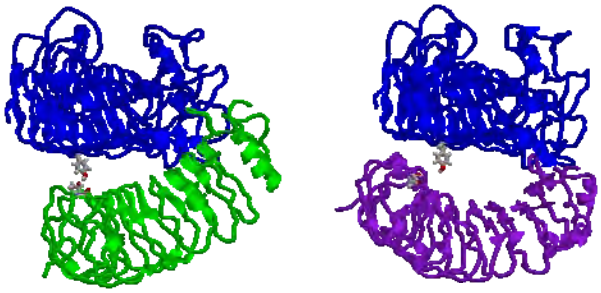


Figure 2. PG-PGIP complexes. Tyr303 of *Xf*PG (blue) binds strongly with a region of pPGIP (green) which is not possible with VvPGIP (purple). Interactions such as this might influence PG-PGIP interaction and inhibition.

Adding to the information gained from the 14 candidate PGIP homology models, other unpublished PGIP sequences from non-vinifera *Vitis* varieties will be modeled in the future. These sequences will be obtained as part of a collaboration, currently in negotiation, with a research group at Stellenbosch University, South Africa. The sequences are the property of an industry board associated with the Institute for Wine Biotechnology at Stellenbosch University. It will be of interest to note how the models of these non-vinifera PGIPs compare to the modeled structure of VvPGIP from *Vitis vinifera* cv. 'Pinotage.'

Objective 2C: XfPG expression and purification

The *Xf*PG expression system utilizing Drosophila S2 cells is being developed to yield large amounts of active, stable *Xf*PG protein for *in vitro* inhibition assays. The cloning strategy fused the coding sequence of *Xf*PG to a C-terminal histidine tag for purification and an N-terminal targeting sequence for protein secretion (Labavitch, 2009). Media from transiently transfected cells induced to express *Xf*PG has a small amount of PG activity, as shown by radial diffusion assay (**Figure 3**; Taylor and Secor, 1988). *Xf*PG was purified from the medium and pelleted Drosophila cell lysate by immobilized nickel column chromatography. The medium and resuspended cell lysate were separately loaded onto the gravity flow column and each was eluted with several volumes of EB (50 mM sodium phosphate, 0.3 M NaCl, 250 mM imidazole). The eluate was analyzed by Western blotting and Coomassie staining SDS-PAGE. Proposed *Xf*PG bands, cross-reacting with a tagged antibody recognition site on the recombinant protein, were visualized at 78 kDa in Western blots for cell lysate preparations (**Figure 4**). The protein bands in the cell medium preparation eluant were visualized at 68 kDa by Coomassie staining (**Figure 5**). Each of these preparations showed slight PG activity, as measured by reducing sugar analysis (Gross, 1982). These activities, however, diminished over time. Current work is focused on generating stably transfected recombinant cell lines to provide more consistent stocks of *Xf*PG. The methods for purifying and storing the protein are also being analyzed to reduce possible causes of the loss of PG activity.

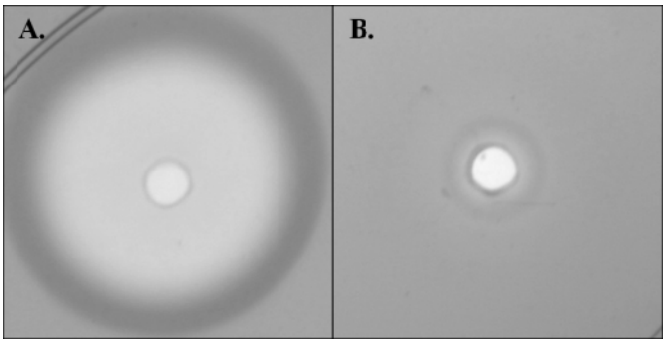


Figure 3. Radial diffusion assay of concentrated PG from *Botrytis cinerea* (A) or culture media from induced *Xf*PG-expressing Drosophila cells (B). The clearing zone diameter is related to amount of PG activity.

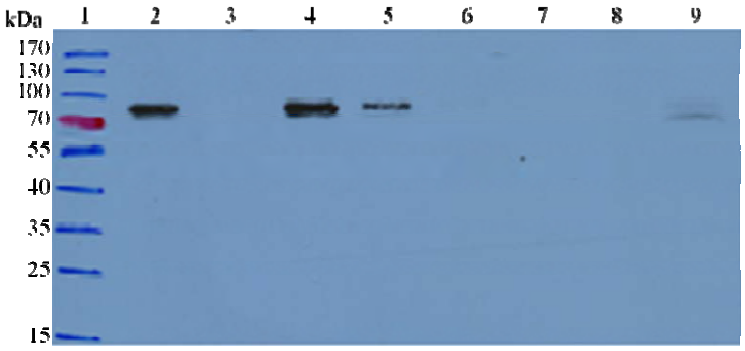


Figure 4. Western blot analysis of partially purified cell lysate after XfPG protein expression. 15 mL crude XfPG lysate was purified by column chromatography and selected fractions were analyzed by Western blotting. Lane 1 = pre-stained ladder, lane 2 = flow-through #4, lane 3 = wash #10, lanes 4-7 = elution fractions #1-4, lane 8 and 9 = cellular medium. Recombinant XfPG protein was eluted with 250 mM imidazole and probed with the anti-V5 primary antibody and anti-mouse HRP secondary antibody.

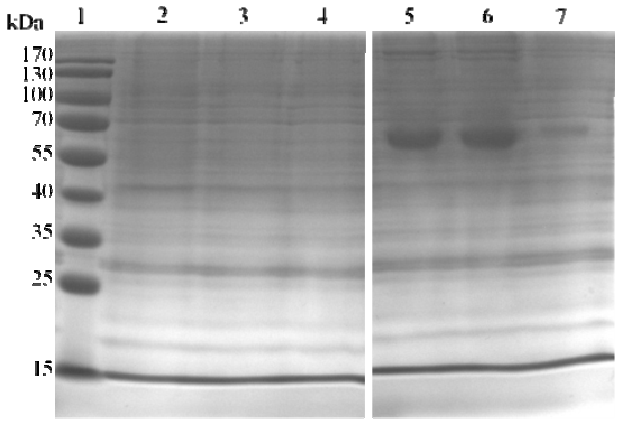


Figure 5. Partially purified XfPG protein eluted with 250 mM imidazole. Coomassie stained polyacrylamide gel electrophoresis. Lane 1 = pre-stained ladder, lanes 2-4 = cell lysate fractions #1-3, lanes 5-7 = cellular medium fractions #1-3.

Objective 2D: Expression of PGIPs in Arabidopsis and tobacco for XfPG assays
The previously reported strategies for cloning each of the 14 candidate PGIPs into pCAMBIA-1301 and transformation into *Agrobacterium tumefaciens* (EHA105 pCH32) continues (**Table 1**; Labavitch, 2009). The full-length *Xf*PG construct was successfully cloned into the transformation vector and transformed into *Agrobacterium*. This construct, and soon the pPGIP::*Xf*PG fusion construct (still in progress) provide a potential diagnostic tool to test the efficacy of each PGIP *in planta*.

Table 1. Cloning progress chart. Checkmarks indicate completed checkpoints while circles indicate work in progress.

Protein (Organism)	**Cloning Progress Checkpoints**				
	Source tissue acquired	PGIP cDNA isolated	Transformed into *E. coli*	Transformed into *Agrobacterium*	Plant transformation
AtPGIP1 (Arabidopsis)	✓	✓	✓	O	-
AtPGIP2 (Arabidopsis)	✓	✓	✓	O	-
BnPGIP1 (Rapeseed)	✓	✓	O	-	-
CaPGIP (Pepper)	✓	O	-	-	-
CsiPGIP (Orange)	✓	O	-	-	-
FaPGIP (Strawberry)	✓	✓	O	-	-
OsPGIP1 (Rice)	✓	✓	O	-	-
OsPGIP2 (Rice)	✓	✓	O	-	-
PvPGIP2 (Bean)	✓	✓	O	-	-
PpePGIP (Peach)	O	-	-	-	-
PfPGIP (Firethorn)	✓	O	-	-	-
pPGIP (Pear)	✓	✓	✓	✓	✓
LePGIP (Tomato)	✓	✓	✓	✓	O
VvPGIP (Grape)	O	-	-	-	-
XfPG (Xylella)	✓	✓	✓	✓	✓
pPGIP::XfPG	✓	✓	✓	O	-

Co-infiltration of *Agrobacterium* cultures harboring *Xf*PG and either pPGIP or LePGIP in pCAMBIA-1301 was carried out as described by Joubert *et al.* (2007). Fully formed leaves of *Nicotiana benthamiana* and *N. tabacum* were infiltrated with constant manual pressure using a needle-less syringe, forcing bacterial cultures into the abaxial leaf tissue. In most cases, initial infiltration zones were marked on the adaxial surface and had measured areas of approximately 35 mm^2. Visual symptom development was observed at 24 and 72 hours post infiltration (hpi, **Figure 6**). Infiltration with cultures harboring the *Xf*PG construct resulted in marked wilting, localized water soaking, and chlorotic lesions developing in the infiltration zone. Leaves co-infiltrated with *Xf*PG and PGIP cultures displayed attenuated symptoms while leaves infiltrated with just PGIP or empty vector cultures showed no symptom development. LePGIP was less effective than pPGIP at inhibiting wilting and lesion development when co-infiltrated with *Xf*PG. Further work to quantify the results will provide a measure of the inhibition of *Xf*PG by each cloned PGIP. We anticipate that the fusion construct pPGIP::*Xf*PG will yield more easily scored results than the native *Xf*PG construct due to the signal sequence from pPGIP predicted to target the translated *Xf*PG protein to the cell apoplastic space where it can either degrade the pectin-rich middle lamellae and cell walls or be inhibited by any co-infiltrated PGIP. PGIP is naturally targeted to the apoplast.

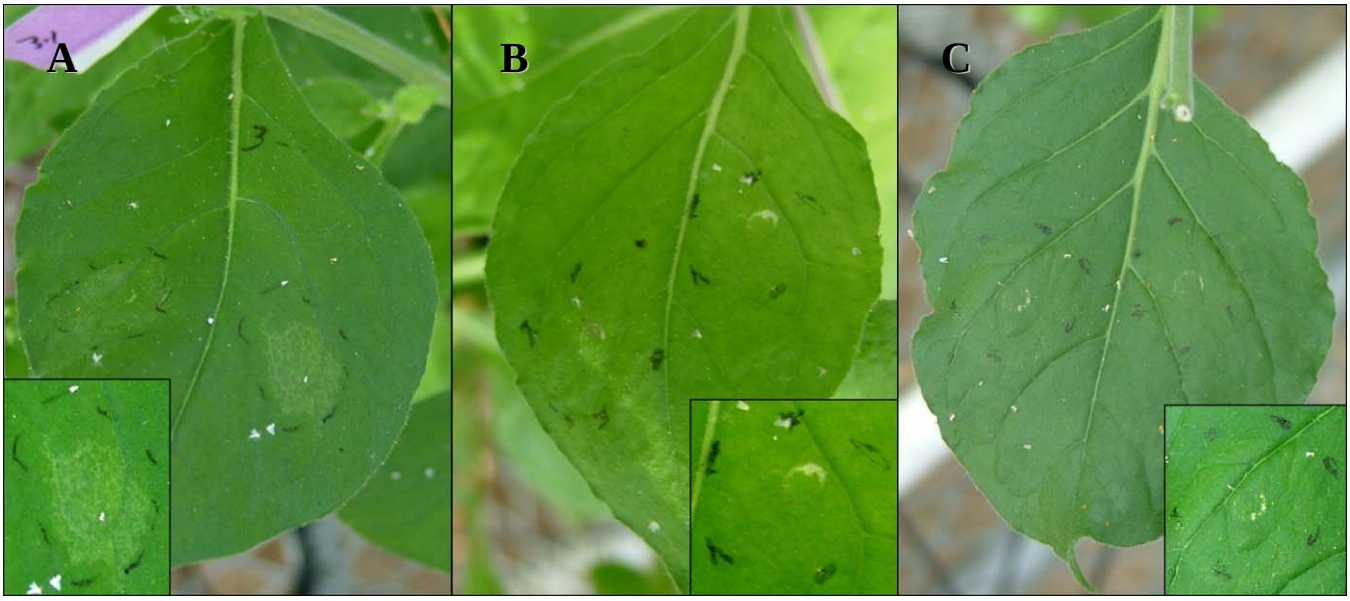


Figure 6. Transient expression of *Xf*PG, pPGIP, and LePGIP in *N. benthamiana* leaves by infiltration with *Agrobacterium* cultures. Chlorotic lesions and water soaking mark the site of agro-infiltrations with *Xf*PG (A). Symptoms are reduced when *Xf*PG is co-infiltrated with pPGIP (B) or LePGIP *Agrobacterium* (C). Inserts show details of infiltration sites. Black marks indicate the borders of the initial zone infiltrated.

Objective 3: Maximize PGIP expression in and transport from roots
Information pertaining to potential signal sequences targeting PGIPs to xylem tissues for transport to and across graft junctions into wild-type scions has been reported by the project "*In planta* testing of signal peptides and anti-microbial proteins for rapid clearance of *Xylella*" (PI: A. Dandekar).

Objective 4: No activity for this reporting period.

CONCLUSIONS
The ability of one of the candidate PGIPs discussed here, pPGIP, to provide PD resistance to wild-type scions will be determined by the recently initiated field trial. This will be a key step in advancing the use of transgenic rootstocks for PD control in commercial applications. Homology models of all 14 candidate PGIPs have been constructed and critical residues for *Xf*PG-PGIP interaction were discovered. Recombinant *Xf*PG, produced from transiently transfected Drosophila cells, was purified and shown to have a low level of PG activity. Further work to clone and express the candidate PGIPs continues. *In planta* co-infiltration assays have shown that both pPGIP and LePGIP are able to inhibit the chlorotic lesion development in tobacco leaves that is caused by *Xf*PG-harboring *Agrobacterium*.

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ENDOPHYTIC BACTERIA ASSOCIATED WITH SHARPSHOOTERS, INSECT VECTORS OF *XYLELLA FASTIDIOSA* SUBSP. *PAUCA*

Principal Investigator:
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ABSTRACT
Xylella fastidiosa (Xf) subsp. P*auca* causes citrus variegated chlorosis (CVC) disease in Brazil, resulting in significant production losses in the citrus industry. *Xf* is mainly transmitted by three species of sharpshooters (Hemiptera: Cicadellidae) in Brazil; *Dilobopterus costalimai* (Young), *Acrogonia citrina* Marucci & Cavichioli and *Oncometopia facialis* (Signoret). We identified bacterial communities associated with the heads of surface-sterilized insect vectors of *Xf* that were collected from CVC affected citrus groves in Brazil. Bacteria were isolated and analyzed by amplified ribosomal DNA restriction analysis (ARDRA) and sequencing, revealing the presence, among the most abundant genera, of the well-known citrus endophytes *Methylobacterium* spp. and *Curtobacterium* spp. Specific PCR systems for the detection of these genera indicated high frequencies of presence of these bacteria in sharpshooters. The remaining bacterial community was compared in distinct vector species and at different period of the year by denaturing gradient gel electrophoresis (DGGE), showing its responsiveness to the climate change over the year. These results represent a new basis for the knowledge about the interaction symbiotic-pathogenic bacteria inside insect vectors and provides a basis for further work on the biocontrol of phytopathogens like *Xf*.

LAYPERSON SUMMARY
We report the bacterial communities associated with the heads of surface-sterilized insect vectors of *Xylella fastidiosa (Xf)* that were collected from citrus variegated chlorosis (CVC) affected citrus groves in Brazil. Many aspects can influence the transmission of a pathogen by an insect vector such as the low concentration of *Xf* cells in the citrus plants and the low number of colonized vessels in affected plants. The interaction between different bacteria inside the insect foregut can also influence the transmission, as once inside the foregut, bacterial interaction, such as competition for nutrients, space and other complex interactions could occur. *C. flaccunfaciens* is a potential candidate for biological control of CVC because an antagonism between *C. flaccumfaciens* and *Xf* was strongly indicated *in vitro* and *in vivo* including inhibition of growth of *Xf* and reduced severity of the disease symptoms in the presence of this phytopathogen. The ability demonstrated by *C. flaccumfaciens* to colonize plant tissues in the presence of *Xf* and the reduction of disease symptoms caused by *Xf* are prerequisites for the use of this endophytic bacterium as a biocontrol agent. Since members of the genus *Curtobacterium* were consistently detected in the insect vectors of *Xf* as demonstrated in the present study, they fulfill another requirement of candidates for biological control of *Xf*.

INTRODUCTION
Brazil is the largest producer of citrus fruit in the world, also supplying most of the international market for concentrated orange juice. By 2005, the percentage had increased to 43%, and citrus variegated chlorosis (CVC) was present in all citrus growing regions of Brazil (Bové & Ayers 2008). The disease is caused by the xylem-limited gram-negative bacterial pathogen, *Xylella fastidiosa (Xf)* subsp. *Pauca* (Hartung et al. 1994). Endophytic microorganisms live within plants without causing apparent harm to the host. We have studied the possible use of endophytes as vehicles to control both phytopathogens and insects (Azevedo et al. 2000). Endophytes colonize ecological niches similar to that of phytopathogens (Gai et al 2009a), which gives them access as possible candidates as biocontrol agents. In citrus, several endophytic bacteria were isolated and *Methylobacterium* spp. and *Curtobacterium flaccumfaciens* were further determined as the main endophytic species interacting with *Xf* (Araújo et al. 2001). These species vary in population density when CVC-affected and asymptomatic plants are compared. Later, Lacava et al. (2004) reported that the growth of *Xf* was inhibited by endophytic *C. flaccumfaciens* and stimulated by *Methylobacterium* sp. A similar effect was demonstrated by the reduced severity of the *Xf* colonization in plants priory colonized by *C. flaccumfaciens* (Lacava et al. 2007a). In Brazilian citrus groves, *Dilobopterus costalimai*, *Oncometopia facialis* and *Acrogonia citrina* are the most common sharpshooters found. The transmission efficiency of bacteria is a measure of the ability to successfully acquire bacteria from an affected plant and transmit to healthy ones. The efficiency of *Xf* transmission leafhopper species ranges from 0 to 100%, however the lower values are found when

transmission among citrus plants is considered. The transmission rates for the main species associated with CVC vary from 1% to 5% (Redak et al. 2004). Many aspects can influence the transmission of a pathogen by an insect vector such as the low concentration of *Xf* cells in the citrus plants (Almeida & Purcell 2003) and the low number of colonized vessels in affected plants. The interaction between different bacteria inside the insect foregut can also influence the transmission, as once inside the foregut, bacterial interaction, such as competition for nutrients, space and other complex interactions could occur.

OBJECTIVES

The aims of this work were:

1. Access the bacterial population associated with the main sharpshooters responsible for the transmission of *Xf* in citrus.
2. Evaluate the diversity of heterotrophic bacteria by amplified ribosomal DNA restriction analysis (ARDRA).
3. Compare the bacterial community colonizing insects from distinct species and collected from citrus at distinct period of the year by denaturing gradient gel electrophoresis (DGGE).

RESULTS

A total of 17.230 bacterial colonies were counted in plating from different species and at distinct sampling time. These colonies were classified into three distinct morphological groups. The concentration of bacteria (CFU/insect head) belonging to each morphological group. The populations of group G1 (*Methylobacterium*) remained constant for *O. facialis* and *D. costalimai* in March and May, decreasing in June. From *A. citrina* the presence of G1 bacteria decreased from May. Group 2 (*Curtobacterium*) was higher in March for *O.. facialis* and *D.. costalimai*, decreased in May and was undetected in *D. costalimai* in June and group G3 other bacteria. The population of *Curtbacterium* spp. in *A. citrina* remained constant during the period of analysis.. A subsample of the total number of colonies obtained (120 colonies) was subjected to the genotypic characterization by ARDRA. In total, 16 cleavage patterns were observed, determining the ribotypes constituting the heterotrophic bacterial communities from sharpshooter heads. Among these ribotypes, the colonies from the two targeted groups, *Curtobacterium* spp and *Methylobacterium* spp., has revealed a fidelity in the pattern of the 16S rRNA gene cleavage, allowing this approach to measure the proportion of these bacteria within the sampled colonies (**Figure 1**). Other bacterial haplotypes were characterized randomly and sequences have shown the affiliation of the isolates to the genera *Bacillus*, *Brachybacterium*, *Brevibacillus*, *Brevundimonas*, *Nocardia*, *Paenibacillus*, *Pseudoclavibacter*, *Rhodococcus*, *Sphingomonas* and *Staphylococcus*.. DNA samples directly extracted from insect heads were used for specific amplification of DNA from bacteria affiliated with the genera *Curtobacterium* and *Methylobacterium*. The number of positive samples was unique to each sharpshooter species.. The detection of DNA from *Curtobacterium* spp. was positive in 89.6% of the samples from *O. facialis*, 39.1% of *D. costalimai* and 70% of *A. citrina*. In a similar analysis for *Methylobacterium* spp. the numbers of positive PCR were 51.7% for *O. facialis*, 8.7% for *D. costalimai* and 20% of the *A. citrina*. Another point found in this work was the comparative phylogeny of isolates from both targeted groups with endophytic isolates obtained in previous works. The sequences UIA-12-R, UIA-13-R, UIA-14-R and ER1.6 are *Curtobacterium flaccumfaciens* and AR1.6/11, SR1.6/2 and SR3/27 are *Methylobacterium* species isolated from citrus. Sequences from DGGE bands were also included in the clustering and it was possible to observe a clustering of some sharpshooter-associated bacteria with endophytes for both bacterial groups; *Curtobacterium* spp. (**Figure 2**) and *Methylobacterium* spp (**Figure 3**). Hence, besides the partial grouping of vector bacteria and endophytes, clusters containing only bacteria from one source were found. The DGGE analysis showed variable fingerprinting according to the period when insects were collected. The first separation was observed for samples collected in June, with a further sub-branching for samples from May and March (**Figure 4**). These variations were based on changes in the abundance of bands and also in the intensity of similar bands found in distinct samples..

CONCLUSIONS

The bacterial communities associated with vector insects and plants differ in abundance through the yearly season.. Endophytic bacteria could influence disease development by reducing the insect transmission efficiency due to competition with pathogens in host plants and also in insect foreguts. In addition the bacterial communities in the foregut of insect vectors of *Xf* changed with time, environmental conditions and in different insect species. However, members of the genus *Curtobacterium* were consistently detected in the sharpshooters foregut and are commonly isolated from the xylem of citrus plants, and because of this, they may be candidates for biological control. Since members of the genus *Curtobacterium* were consistently detected in the insect vectors of *Xf* as demonstrated in the present study, they fulfill another requirement of candidates for biological control of *Xf*, i.e. they can colonize both the insect vectors of *Xf* and citrus plants.

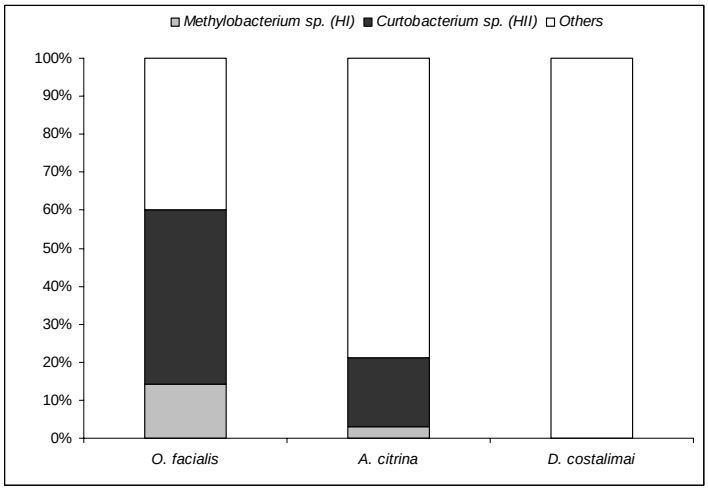

Figure 1. Proportion of the ARDRA ribotypes I and II (cleavage made with endonuclease *Alu*I), related to *Methylobacterium* spp and, *Curtobacterium* spp. respectively.

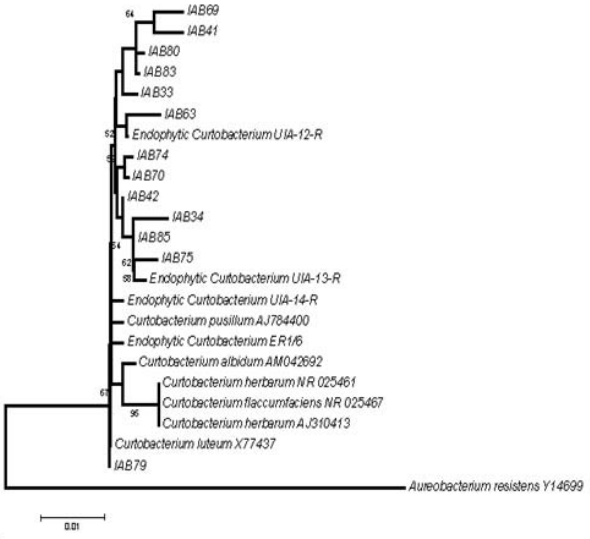

Figure 2. Neighbor-joining clustering among sharpshooters and plant associated bacteria sequences with types strains of *Curtobacterium* spp. A partial fragment of the 16SrRNA gen was used for comparisons.

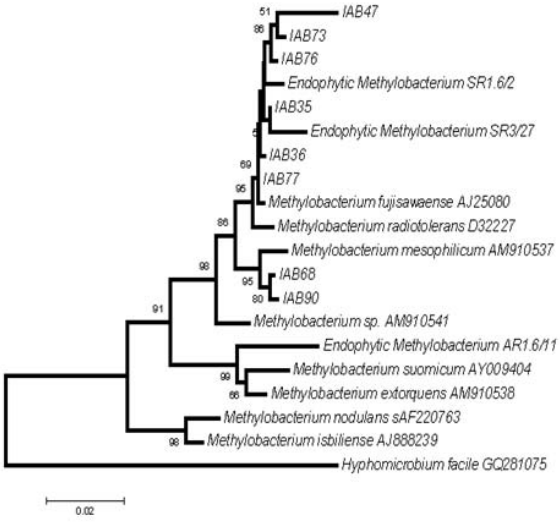

Figure 3. Neighbor-joining clustering among sharpshooters and plant associated bacteria sequences with types strains of *Methylobacterium* spp. A partial fragment of the 16SrRNA gen was used for comparisons.

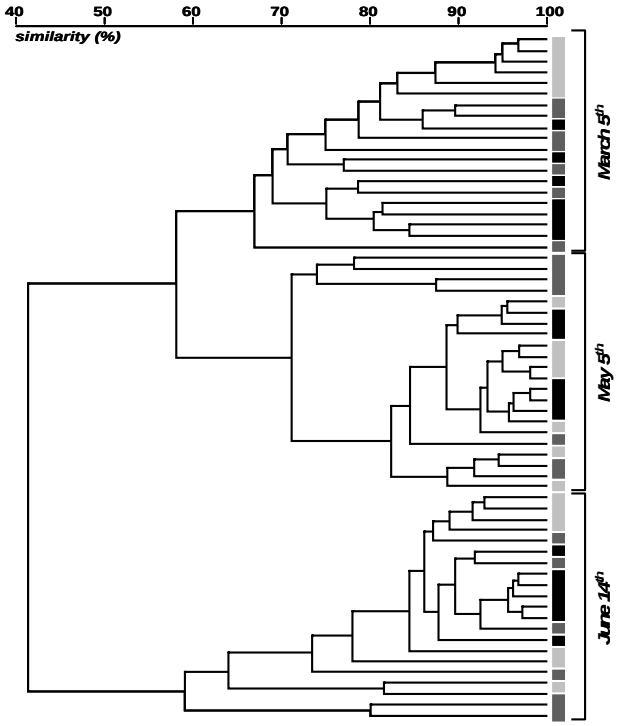

Figure 4. Clustering of DGGE fingerprinting generated by UPGMA based on the similarity calculated by the Pearson correlation. Sharpshooters species are represented by dark gray bars (*O. facialis*), light gray (*A. citrina*) and black bars (*D. costalimai*).

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FUNDING AGENCIES

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FIELD EVALUATION OF DIFFUSIBLE SIGNAL FACTOR PRODUCING GRAPE FOR CONTROL OF PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted March 2010 to October 2010.

ABSTRACT
A cell density-dependent gene expression system in *Xylella. fastidiosa* (*Xf*) mediated by a small signal molecule called diffusible signal factor (DSF) which we have now characterized as 2-Z-tetradecenoic acid (hereafter called C14-cis) controls the behavior of *Xf*. The accumulation of DSF attenuates the virulence of *Xf* by stimulating the expression of cell surface adhesins such as HxfA, HxfB, Xada, and fimA (that make cells sticky and hence suppress its movement in the plant) while down-regulating the production of secreted enzymes such as polygalacturonase and endogluconase which are required for digestion of pits and thus for movement through the plant. Artificially increasing DSF levels in plants in various ways increases the resistance of these plants to Pierce's disease (PD). Disease control in the greenhouse can be conferred by production of DSF in transgenic plants expressing the gene for the DSF synthase from *Xf*; such plants exhibit high levels of disease resistance when used as scions and confer at least partial control of disease when used as rootstocks. This project is designed to test the robustness of disease control by pathogen confusion under field conditions where plants will be exposed to realistic conditions in the field and especially under conditions of natural inoculation with insect vectors. We are testing two different lineages of DSF-producing plants both as own-rooted plants as well as rootstocks for susceptible grape varieties in two field sites. Plants were established in one field site on August 2, 2010. Disease severity and population size of the pathogen will be assessed in the plants as a means of determining their susceptibility to PD.

LAYPERSON SUMMARY
Xylella. fastidiosa coordinates its behavior in plants in a cell density-dependent fashion using a diffusible signal factor (DSF) which acts to suppress its virulence in plants. Artificially increasing DSF levels in grape by introducing the *rpfF* gene which encodes a DSF synthase reduces disease severity in greenhouse trials. We are testing two different lineages of DSF-producing plants both as own-rooted plants as well as rootstocks for susceptible grape varieties. Disease severity and population size of the pathogen will be assessed in the plants as a means of determining their susceptibility to Pierce's disease.

INTRODUCTION
Our work has shown that *Xylella fastidiosa* (*Xf)* uses diffusible signal factor (DSF) perception as a key trigger to change its behavior within plants. Under most conditions DSF levels in plants are low since cells are found in relatively small clusters, and hence cells do not express adhesins that would hinder their movement through the plant (but which are required for vector acquisition) but actively express extracellular enzymes and retractile pili needed for movement through the plant. Disease control can be conferred by elevating DSF levels in grape to "trick" the pathogen into transitioning into the non-mobile form that is normally found only in highly colonized vessels. While we have demonstrated the principles of disease control by so-called "pathogen confusion" in the greenhouse, more work is needed to understand how well this will translate into disease control under field conditions. That is, the methods of inoculation of plants in the greenhouse may be considered quite aggressive compared to the low levels of inoculum that might be delivered by insect vectors. Likewise, plants in the greenhouse have undetermined levels of stress that might contribute to Pierce's disease (PD) symptoms compared to that in the field. Thus we need to test the relative susceptibility of DSF-producing plants in the field both under conditions where they will be inoculated with the pathogen as well as received "natural" inoculation with infested sharpshooter vectors. We also have recently developed several new sensitive biosensors that enable us to measure *Xf* DSF both in culture and within plants. We could gain considerable insight into the process of disease control by assessing the levels of DSF produced by transgenic *rpfF*-transformed grape under field conditions.

OBJECTIVES
1. Determine the susceptibility of DSF-producing grape as own-rooted plants as well as rootstocks for susceptible grape varieties for Pierce's disease.
2. Determine population size of the pathogen in DSF-producing plants under field conditions.
3. Determine the levels of DSF in transgenic *rpfF*-expressing grape under field conditions as a means of determining their susceptibility to Pierce's disease.

RESULTS AND DISCUSSION

Disease susceptibility of transgenic DSF-producing grape in field trials.

Field tests are being performed with two different genetic constructs of the *rpfF* gene in grape and assessed in two different plant contexts. The *rpfF* has been introduced into Freedom (a rootstock variety) in a way that does not cause it to be directed to any subcellular location (non-targeted). The *rpfF* gene has also been modified to harbor a 5' sequence encoding the leader peptide introduced into grape (Thompson seedless) as a translational fusion protein with a small peptide sequence from RUBISCO that presumably causes this RpfF fusion gene product to be directed to the chloroplast where it presumably has more access to the fatty acid substrates that are required for DSF synthesis (chloroplast-targeted). These two transgenic grape varieties are thus being tested as both own-rooted plants as well as rootstocks to which susceptible grape varieties will be grafted. The following treatments are thus being examined in field trials:

Treatment 1	Non-targeted RpfF Freedom
Treatment 2	Chloroplast-targeted RpfF Thompson
Treatment 3	Non-targeted RpfF Freedom as rootstock with normal Thompson scion
Treatment 4	Chloroplast-targeted RpfF Thompson as rootstock with normal Thompson scion
Treatment 5	Normal Freedom rootstock with normal Thompson scion
Treatment 6	Normal Thompson rootstock with normal Thompson scion
Treatment 7	Normal Freedom
Treatment 8	Normal Thompson

Treatments 5-8 serve as appropriate control to allow direct assessment of the effect of DSF expression on disease in own rooted plants as well as to account for the effects of grafting per se on disease susceptibility of the scions grafted onto DSF-producing rootstocks.

One field trial was established in Solano County on August 2, 2010. Twelve plants of each treatment were established in randomized complete block design. Self-rooted plants were produced by rooting of cuttings (about 3 cm long) from mature vines of plants grown in the greenhouse at UC Berkeley. Cuttings were placed in a sand/perlite/peatmoss mixture and subjected to frequent misting for about four weeks, after which point roots of about 10 appeared. Plants were then be transferred to one gallon pots and propagated to a height of about 1 m before transplanting into the field. Grafted plants were produced in a similar manner. 20 cm stem segments from a susceptible grape variety were grafted onto 20 cm segments of an appropriate rootstock variety and the graft union wrapped with grafting tape. The distal end of the rootstock variety (harboring the grafted scion) was then be placed in rooting soil mix and rooted as described above. After emergence of roots, the grafted plant were then transplanted and grown to a size of about 1 m as above before transplanting into the field site.

The plants have all survived and are growing well (**Figure 1**). The plants are too small to inoculate in the 2010 growing season and hence will be inoculated in 2011 (no natural inoculum of *Xf* occurs in this plot area and so manual inoculation of the vines with the pathogen will be performed. Because researchers from both UC-Berkeley and UC-Davis will be contributing treatment to each plot, and since the controls for some researchers will be the same, some control plants will be shared between research groups. All plants in Solano County will be inoculated by needle puncture through drops of *Xf* of about 10^6 cells/ml as in previous studies. Due to severe damage suffered by some plants in the greenhouse at UC Berkeley due to pesticide applications, there were not sufficient plants available to initiate the trial at Riverside County: The plants needed for this trial are being regenerated and will be ready for planting before the 2011 growing season. The plants at the Riverside County location will not be artificially inoculated, but instead will be subjected to natural infection from infested sharpshooter vectors having access to *Xf* from surrounding infected grape vines. Disease symptoms will be measured bi weekly starting at eight weeks after inoculation at the Solano County site, or about eight weeks after transplanting into the field site at the Riverside County location. Leaves exhibiting scorching symptoms characteristic of PD will be counted on each occasion, and the number of infected leaves for each vine noted. ANOVA will be employed to determine differences in severity of disease (quantified as the number of infected leaves per vine) that are associated with treatment.

Figure 1. Overview of research plot in which DSF-producing plants are established (left). Closeup of transgenic Freedom vines in mid-September (right).

CONCLUSIONS

The transgenic plants have been successfully established in one of two field sites, with the second field site to be established in late 2010. The first disease assessments should be made in 2011. Since substantial disease control has been observed in these plants in the greenhouse, these tests should provide a direct assessment of the utility of such transgenic plants for disease control in the field.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

FIELD EVALUATION OF GRAFTED GRAPE LINES EXPRESSING POLYGALACTURONASE-INHIBITING PROTEINS

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ABSTRACT
The CDFA Pierce's Disease (PD) and Glassy-winged Sharpshooter Board's Research Scientific Advisory Panel review in 2007 and subsequent RFPs have given top priority to delivery of PD control candidates, including polygalacturonase-inhibiting proteins (PGIPs), from grafted rootstocks. Previously transformed 'Thompson Seedless' and 'Chardonnay' grapevines expressing a PGIP from pear fruit show reduced PD incidence when inoculated with *Xylella fastidiosa (Xf).* These grapevines were propagated vegetatively for PD assessment in field trial locations in Solano and Riverside Counties. Fifty-one transgenic and null-transformed control, own-rooted, grapevines were planted in Solano County on 7/6/2010. Grafted plants, utilizing the pPGIP-expressing vines as rootstocks, are being generated to be planted in 2011. PD resistance and agronomic viability will be assessed in future seasons.

LAYPERSON SUMMARY
Xylella fastidiosa (Xf) utilizes a key enzyme, polygalacturonase (PG), to spread from one xylem vessel to the next, eventually leading to the development of Pierce's disease (PD) symptoms in infected vines. Plant proteins called PGIPs selectively inhibit PGs from bacteria, fungi, and insets. Our collective work has identified a PGIP from pear fruits as partially inhibiting PD symptom development in grapevines expressing the pear PGIP. These vines are being analyzed in two field trials to measure their resistance to both mechanical *Xf* inoculations and to natural PD pressure in Solano and Riverside Counties, respectively. PGIPs have been shown to be graft transmissible so the transgenic grapevines will also be used as rootstocks to measure how much resistance will be provided to scion tissues taken from grapevines without the transgenic PGIP.

INTRODUCTION
Pierce's disease (PD) symptom development has been extensively linked to the spread of the causal bacterium, *Xylella fastidiosa* (*Xf*), throughout the xylem network of infected grapevines. Previous research has highlighted a plant cell wall modifying enzyme of *Xf*, polygalacturonase (*Xf*PG), as a PD virulence factor and therefore, a target of potential PD resistance strategies (Roper et al., 2007). *Xf* uses cell wall modifying enzymes, such as *Xf*PG, to degrade the pectin-rich pit membranes separating adjoining xylem vessels, permitting the spread of the bacterium throughout the xylem tissue. Plant PG-inhibiting proteins (PGIPs) are selective inhibitors of bacterial, fungal, and insect PGs and have provided protection from pests producing such PGs when upregulated in transgenic crop plants. Agüero et al. (2005) generated 'Chardonnay' and 'Thompson Seedless' grapevines expressing a PGIP (pPGIP) from pear fruit. These plants had reduced PD susceptibility and it was shown that the pPGIP was active across a graft junction when the transgenic lines were used as rootstocks grafted to wild-type scions.

The grapevines transformed with the pPGIP protein are part of a funded project to optimize the activity, expression, and export of PGIP proteins from transgenic rootstocks to provide PD protection in wild-type scions: "Optimizing grape rootstock production and export of inhibitors of *Xf* PG activity" (PI Labavitch). These plants have previously only been observed in greenhouse settings. The goal of this project is to verify that the transgenic grapevines expressing pPGIP (1) have increased resistance to PD and (2) maintain the appropriate agronomic traits necessary for commercial release. This will be examined in own-rooted plants and in grafted plants where the pPGIP grapevine is the rootstock.

This field trial proposal was funded jointly with proposals from D. Gilchrist, A. Dandekar and S. Lindow. The plants from these trials have been planted at the same locations and the APHIS-USDA authorizations have been handled by G. Paulino through PIPRA.

OBJECTIVES
1. Scale up the number of grafted and own-rooted pPGIP expressing lines.
2. Plant and maintain grafted and own-rooted lines in two locations with different PD pressure.
3. Evaluate relevant agronomic traits of vines in two locations.
4. Determine PD incidence in pPGIP expressing grafted and own-rooted lines. Test for *Xf* presence and, if present, determine the extent of infection.

RESULTS AND DISCUSSION

Objective 1: Generate enough grafted and own-rooted grapevines for the field trial

The pPGIP expressing 'Chardonnay' and 'Thompson Seedless' grapevines generated by Agüero et al. (2005) have been maintained in the greenhouse. Vegetative cuttings of non-lignified stem sections from transgenic and null-transformed plants of both cultivars were rooted in an aeroponic cloning manifold (EZ-Clone Inc., Sacramento, CA). Stem cuttings, three nodes in length, were basally dipped in 1000 ppm IBA solution for five minutes and transferred to individual sites in the misting chamber. Apical regions received continuous light while basal nodes received constant misting in darkness until roots began to form (**Figure 1**). Rooted cuttings were transferred to soil and maintained in the greenhouse. Grafting was attempted with both green and semi-lignified stem segments for all possible graft combinations. A modified wedge grafting technique was used whereby scion sections of 1 to 2 nodes were stripped of foliage and cut into wedge. These sections were fit into notched rootstock stems regions of equal maturity. The graft union was covered with Parafilm M, secured by a clothespin, and the entire scion piece was covered loosely by a translucent bag to prevent desiccation. Other green grafting techniques, such as saddle grafting and chip budding, were attempted with the rooted cuttings. So far, no technique has proven effective enough to generate the required number of grafted plants for either field trial location. More cuttings are being vegetatively propagated for grafting during the winter.

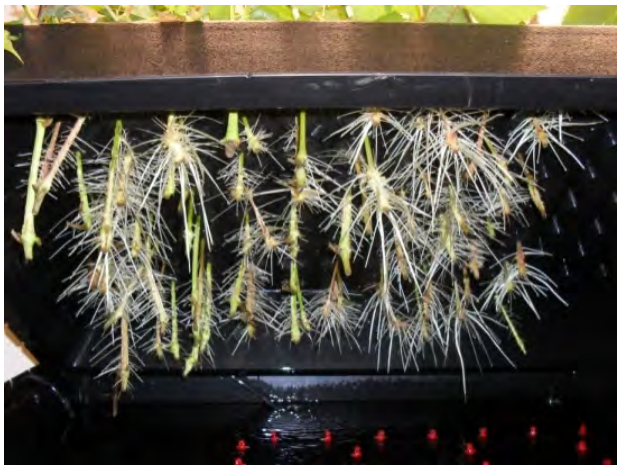

Figure 1. Grapevine cuttings rooting in the EZ-Clone aeroponic manifold.

Objective 2: Establish field trial sites

Two field trial sites were chosen to assess the PD resistance and general agronomic viability of own-rooted and grafted pPGIP expressing grapevines. The primary site in Solano County, CA has no natural PD pressure while the secondary site in Riverside County, CA has high natural PD pressure. The two locations will provide natural variation in climate and PD pressure so that trait assessments will be relevant to much of California's viticultural land.

Rooted cuttings of transgenic 'Chardonnay' and 'Thompson Seedless' grapevines were genotyped by PCR analysis for the presence of the pPGIP transgene. PCR primers were used to amplify the pPGIP gene from 0.5 mm leaf punches taken from young leaves near the growing tip (**Figure 2**). For this purpose, the Phire Plant Direct PCR Kit (Finnzymes Oy.) was used without the need for manual DNA extraction prior to PCR analysis. Grapevines with the proper gene expression were moved to lathe house facilities five weeks before field planting for hardening off. During this time, the vines were trained to one major shoot and pruned biweekly to encourage vigorous growth.

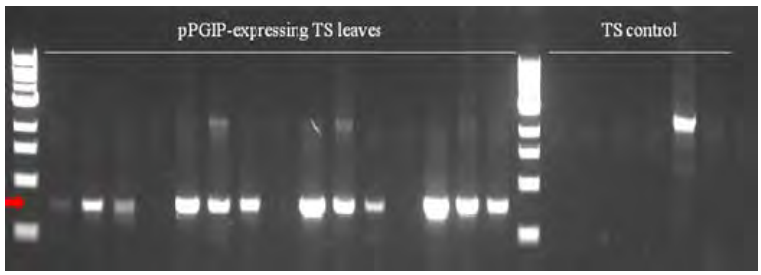

Figure 2. Representative DNA analysis. The pPGIP coding sequence was amplified from 12 cuttings taken from transgenic 'Thompson Seedless' (TS) grapevines. This band was absent in null-transformed control plants.

The field sites are shared by projects testing other transgenic PD control grapevines from PIs: D. Gilchrist, A. Dandekar, and S. Lindow. The 1.66 acre field in Solano County, CA was planted July 6, 2010. At this time, all own-rooted vines satisfying PCR analysis were hand-planted in a randomized block design with blocks consisting of two or three individuals in the same treatment (**Table 1**). The grapevines were planted approximately 8 ft. apart and tied to wooden stakes. After several weeks, trellising wires were added at 40 in. and 52 in. The vines grew vigorously throughout the summer, with most reaching at least the lower trellising wire (**Figure 3**). Flowers and fruit were not observed during year one. PD incidence recordings will begin in year two after mechanical inoculations with *Xf*. Newly generated grafted vines will be moved into the Solano County field early in year two.

Table 1. Total number of grapevines planted in Solano County. Dashed shapes represent pPGIP expressing grapevine rootstocks and/or scions; solid shapes are null-transformant controls (no pPGIP). Vines will be mechanically inoculated with *Xf* during year 2.

		Own-Rooted Plants (#)		Grafted Plants[a](#)	
Cultivar	Grafting Strategy	To Be Inoculated (year 2)	Non-Inoculated	To Be Inoculated (year 2)	Non-Inoculated
Chardonnay		8	4	9	4
		-	-	9	4
		9	4	9	4
Thompson Seedless		8	4	9	4
		-	-	9	4
		9	5	9	4

[a]Grafted plants are currently being generated and will be planted in year 2

Figure 3. The Solano County field location 15 weeks after planting. **A**, Panoramic view of the field, including PD control grapevines from other researchers. **B-C**, pPGIP-expressing Thompson Seedless and Chardonnay grapevines showing vigorous vegetative growth.

CONCLUSIONS

Fifty-one own-rooted Chardonnay and Thompson Seedless grapevines, including those expressing pPGIP, were generated by vegetative propagation, genotyped by PCR, and planted as part of a field trial in Solano County, CA. Planned grafted vines are being generated to be planted next year. Mechanical *Xf* inoculations in Solano County and field plantings in Riverside County will take place next year.

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CONTROL OF PIERCE'S DISEASE WITH FUNGAL ENDOPHYTES OF GRAPEVINES ANTAGONISTIC TO *XYLELLA FASTIDIOSA*

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ABSTRACT

The goal of this research is to identify xylem dwelling fungi that are antagonistic to *Xylella fastidiosa* (*Xf*) that could be implemented as a preventive or curative treatment for Pierce's disease. We hypothesize that some of the fungal endophytes present in PD-escaped grapevines possess anti-*Xf* properties, likely due to the production of secondary metabolites. We have identified and sampled from vineyards located in Napa and Riverside Counties that are under high disease pressure and identified both diseased and PD-escaped grapevines. We isolated fungal endophytes living in the xylem sap and in one-year-old canes and wood spurs of these vines. We identified them by PCR and sequence analysis of the ribosomal DNA. *Aureobasidium* and *Cladosporium* were predominant in both the xylem sap and wood tissue in all vineyards sampled. Several other fungi had a low incidence and were found only in certain varieties. Notably, three fungal strains have inhibitory effects on *Xf* growth *in vitro*. Furthermore, crude extracts of one of these antagonistic fungi showed similar inhibitory effects. In future work, we will test these strains and fungal products *in planta*.

LAYPERSON SUMMARY

Several management strategies for Pierce's disease (PD) are currently being deployed but as of today, successful management largely involves vector control through the use of insecticides. Here we propose to test an alternative control strategy to complement those currently in place or being developed. Our goal is to identify fungi inhabiting grapevine that are antagonistic to *Xylella fastidiosa* (*Xf*). We hypothesized that in natural field settings grapevine escape PD because the organisms residing in the vine do not allow the establishment of *Xf*. In 2009 and 2010, we have sampled from vineyards in Napa and Riverside Counties that are under high disease pressure and identified fungi living in the sap, canes and spurs of diseased and escaped grapevines. We have selected three fungi that inhibit *Xf* growth in culture. We also extracted compounds secreted by these fungi and identified one fungus able to make natural products inhibitory to the bacterium. In the future our goals are to; 1) extend our sampling in order to screen more fungi with potential for *Xf* growth inhibition; 2) pre-introduce the selected fungal candidates in PD inoculated grapevine cuttings to see if it results in prophylactic control; 3) identify the chemical nature of the natural products produced by the fungi that are antagonistic to *Xf* and determine if we can use them as a curative treatment on PD-infected grapevines.

INTRODUCTION

Current Pierce's disease (PD) management strategies largely involve vector management through the use of insecticides (Byrne and Toscano, 2009). This has contained the spread of the disease (Jetter and Morse, 2009). However, for sustained control of PD, strategies that either target the bacterium or impart resistance to the plant host are required. There are several ongoing research avenues investigating the use of transgenic grapevines and rootstocks that show resistance to PD (Aguero et al., 2005; Kirkpatrick, 2009; Lindow, 2009; Gilchrist and Lincoln, 2009). There is also a traditional breeding approach focused on introducing PD resistance into *Vitis vinifera* grapevines (Walker and Tenscher, 2009). Integrated control strategies are also being investigated in natural vineyard settings. These include the use of natural parasitoids to the glassy-winged sharpshooter (GWSS) (Cooksey, 2009) and inoculation of grapevine with mild *Xylella fastidiosa* (*Xf)* strains that may provide cross protection prior to infection with a virulent three strain of *Xf* (Hopkins, 2009). However, there are no effective curative measures that can clear an infected grapevine of *Xf* besides severe pruning, assuming that the bacteria have not colonized the trunk of the grapevine resulting in a chronic infection.

Notably, control of PD with fungi or fungal metabolites is a largely unexplored research area, although fungi are known to produce an array of secondary compounds that have antimicrobial properties (Getha et al., 2009; Mathivanan et al., 2008). Indeed, using fungi as biocontrol agents against plant disease is an active area of research. Some examples include the use of *Trichoderma* species to control avocado white root rot, the use of *Penicillium oxalicum* to control powdery mildew of strawberries, and the use of fungal endophytes to control frosty pod of cacao (Cal et al. 2008; Mejia et al, 2008; Rosa and

Herrera, 2009). In addition, bio-pesticides that are fungal spore-based are commercially available and registered on grapevine in California.

This proposal focuses on identifying endophytic fungi in grapevine and evaluating their potential as biocontrol agents against *Xf*. Our objectives are to characterize the microbiological diversity in grapevines that escaped PD in natural vineyard settings, and compare this population to PD-infected grapevines with the goal of identifying fungi that are unique to PD-escaped vines. We hypothesize that some of these fungal endophytes possess anti-*Xf* properties, likely due to the production of secondary metabolites. Once identified, we will assess the ability of these endophytes and their natural products (ie. secondary metabolites) for inhibitory activity against *Xf in vitro*. Finally we will determine in greenhouse tests if 1) endophytic fungi have potential use as prophylactic bio-control agents for control by inoculating grapevine cuttings with endophytic, *Xf*-antagonistic fungi has a prophylactic effect against PD; and 2) if injection of fungal natural products have curative properties in PD-infected grapevines cuttings. If successful, we envision that these control strategies can be implemented at the nursery level (for endophytes) or directly in the field (for natural products).

OBJECTIVES

1. Identify fungal endophytes that are present in xylem sap and xylem tissues of PD-escaped grapevines but not in PD-symptomatic grapevines.
2. Evaluate the antagonistic properties of the fungal candidates to *Xf in vitro* and conduct a preliminary characterization of the chemical nature of the inhibitory compound(s).
3. Evaluate biological control activity of the fungal candidates *in planta*.

RESULTS AND DISCUSSION

Field Sampling and identification of fungi. In August 2009, we sampled one-year-old canes from grapevines varieties Chardonnay and Cabernet Sauvignon at the research farm on the UC Riverside campus. Although apparently healthy, these grapevines were submitted to the constant disease pressure present in Riverside County, especially given that this vineyard was next to the UCR Citrus Germplasm Depository that supports a large population of the GWSS. Canes were pressure-bombed and 100µl of the sap was plated on general fungal medium, Potato Dextrose Agar (PDA), amended with tetracycline to inhibit bacterial growth. After two weeks of growth at room temperature, the fungi growing were transferred to fresh PDA medium in order to obtain pure cultures. Fungal DNA was extracted from these pure cultures with a Qiagen DNA extraction kit. Following this, the ribosomal DNA was PCR-amplified (600 base pairs) and sequenced (forward and reverse). Fungal taxa were identified after comparing the r-DNA sequence to homologous sequences posted in the GenBank database. We identified five taxa from the sap of these vines, namely *Aureobasidium, Cladosporium, Cryptococcus, Cochliobolus and Chaetomium*.

In August 2010, we sampled from four varieties in two vineyards in Napa County and one vineyard in Riverside. Grapevine varieties included Chardonnay, Merlot, Riesling and Cabernet Sauvignon. We collected one-year-old canes including the wood spur from blocks that had both diseased and PD-escaped grapevines (**Figure 1**). Samples were brought back to the lab and canes were pressure-bombed to extract the xylem sap. Following extraction, 100µl of the xylem sap was plated on general fungal medium, Potato Dextrose Agar (PDA), amended with tetracycline to inhibit bacterial growth. In addition, wood chips were excised from the one-year-old cane and spur and were also plated on PDA-tetracycline medium. Fungi were cultured and identified as described above. The list of endophytic fungi present in escaped and diseased grapevines is presented in **Table 1**. *Cladosporium* and *Aureobasidium* were present in all grapevine varieties and were also found in the xylem sap. Other fungi listed were only found in one grapevine variety of the four sampled and primarily from one-year-old canes and spurs. Additional gene sequencing and morphological identification is currently being conducted to determine the species name of the fungal taxa isolated from the escaped and PD-diseased grapevines.

***In vitro* inhibition assays**. Culturable fungal candidates were evaluated in an *in vitro* inhibition assay for antagonism against *Xf*. In brief, *Xf* liquid cultures were adjusted to OD600nm=0.1 (approx. 10^7 CFU/ml). 300 µl of the *Xf* cell suspension was added to 3 ml of PD3 medium containing 0.8% agar and briefly vortexed. This mixture was overlayed onto a petri plate containing PD3 medium. A #4 size cork borer was flame sterilized and used to cut out a circle of agar containing fungal mycelium from a petri plate containing a fungal culture. This circle was placed onto the plates previously inoculated with *Xf*. Plates were incubated at 28ºC for 10 days and then observed for an inhibition zone around the fungal colony. Measurements were taken of the inhibition zone and recorded (**Figure 2**). Fungal species with inhibition zones were considered inhibitory to *Xf*. To date, we have identified three fungal taxa that are antagonistic to *Xf in vitro*. We are currently testing the other fungi for inhibition of *Xf* using this assay.

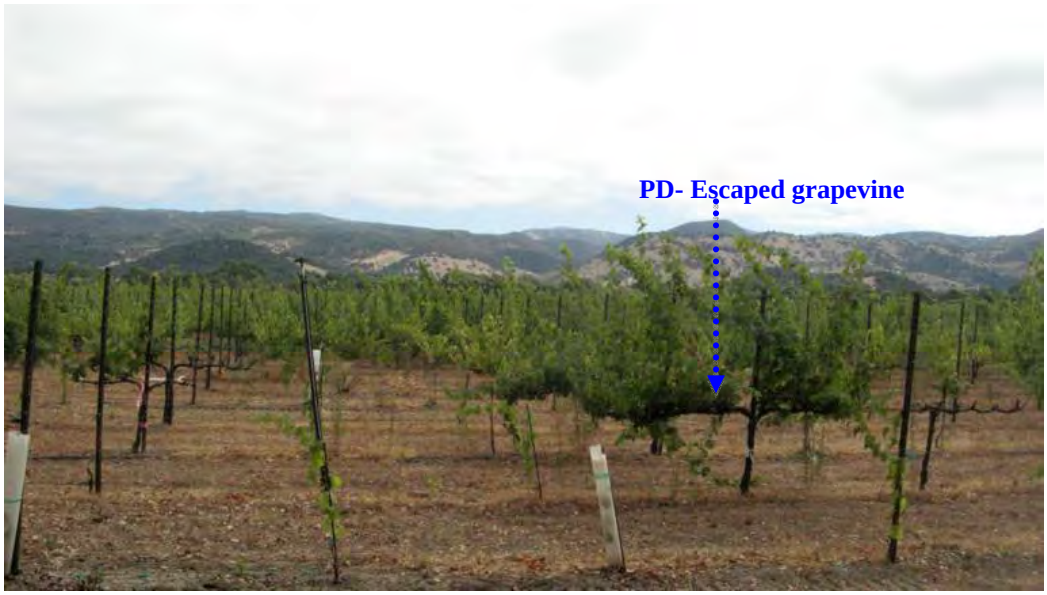

Figure 1. PD-escaped- and diseased grapevines in a Riesling block in Napa County.

Table 1: Identification and percent recovery of fungal taxa from PD-escaped and PD-infected grapevines. Results are based on sampling from three vineyards in Napa and Riverside County, and include four grapevine varieties (Merlot, Cabernet Sauvignon, Chardonnay, Riesling). Fungi were isolated from xylem sap and one-year-old cane and spur wood.

Escaped Grapevine (n=11)		Diseased Grapevine (n=11)	
Fungal Taxa	% Recovery	Fungal Taxa	% Recovery
		Fusarium	18
		Ulocladium	27
		Pezizomycete	9
		Didymella	9
		Unknown taxon 4	9
Cladosporium	82	*Cladosporium*	63
Aureobasidium	82	*Aureobasidium*	91
Alternaria	27	*Alternaria*	27
Cryptococus	9	*Cryptococus*	18
Geomyces	9	*Geomyces*	9
Penicillium	9	*Penicillium*	9
Ustilago	18		
Drechslera	9		
Discostroma	9		
Unknown taxa 1	9		
Unknown taxa 2	9		
Unknown taxa 3	9		

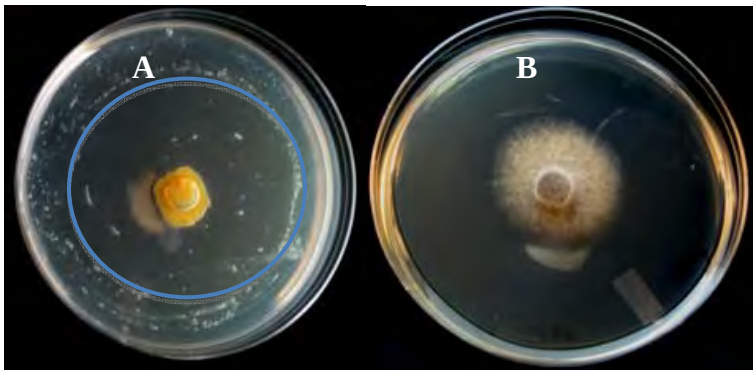

Figure 2: *In vitro* inhibition assay. Two fungal taxa were co-cultured with *Xf* on PD3 medium. Results show a halo of inhibition around the fungal growth (A) indicated by the blue circle and total inhibition of *Xf* growth (B) in comparison to the control (**Figure.3A**).

Isolation of fungal natural products inhibitory to *Xf*. Crude extracts of the three inhibitory fungi were prepared as follows. Agar plugs of 0.5 cm diameter of each fungus were used to inoculate 250 mL liquid media, and the fungi cultivated at room temperature with shaking. After seven days, each culture was extracted with three portions of 125 mL ethyl acetate, the extracts dried over sodium sulfate, and the solvent removed *in vacuo*.

***In vitro* inhibition assay using crude natural product extracts**. *Xf* cultures were prepared as described above. Crude extracts from the three different inhibitory fungi were re-suspended in sterile ethyl acetate to a concentration of 2 mg/ml. Volumes corresponding to a total extract mass of 1 mg, 0.1mg, and 0.01mg were pipetted onto sterile paper discs and allowed to dry in a laminar flow hood. Once dry, the paper discs containing the crude extracts were placed onto the *Xf* cultures and incubated at 28ºC for seven days. Following this, plates were observed for a halo of inhibition around the paper disc. To date, we have identified one fungal taxon producing natural compounds that are antagonistic to *Xf in vitro* (**Figure 3**).

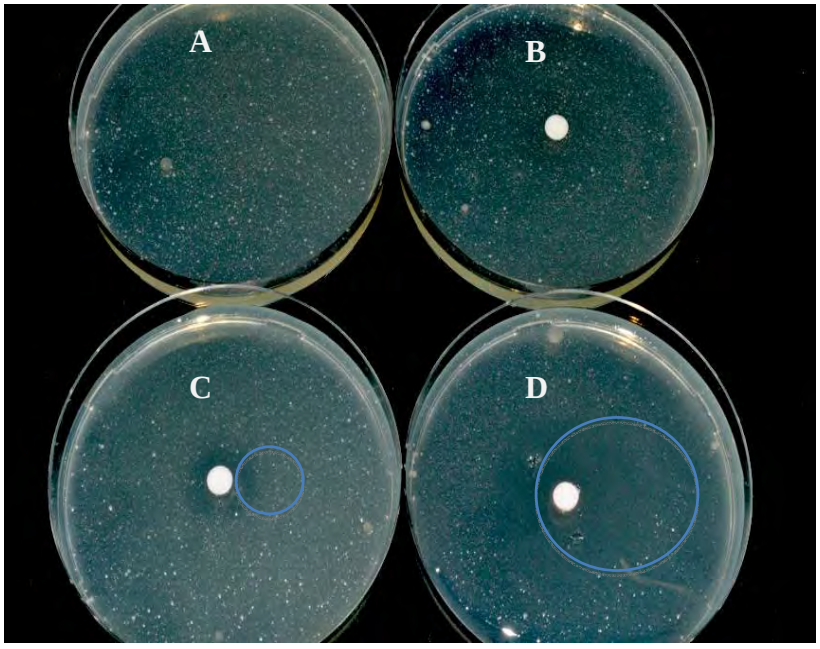

Figure 3: *In vitro* inhibition assay for natural fungal products. Crude fungal natural product extracts were re-suspended in ethyl acetate and pipetted onto paper disc to yield a range of extract masses. Solid PD3 medium was inoculated with *Xf* and overlayed with the paper discs. **A** (*Xf* only) and **B** (ethyl acetate only) show no halo of inhibition, whereas, **C** (0.1 mg crude extract) and **D** (1 mg crude extract) show a halo of inhibition that increases as the concentration of crude extract increases.

CONCLUSIONS

The goal of this research is to identify fungal strains or natural fungal products that have an antagonistic effect towards *Xf*. Thus far, we have isolated three promising fungal candidates that inhibit *Xf in vitro*. In addition, one of these fungi is producing measurable amounts of a secreted natural product that is inhibitory to *Xf*. We are currently characterizing and testing the other fungi that we have isolated from PD-escaped vines as well as the natural product extracts produced by these fungi. In future work, we will inoculate these fungal strains into grapevine cuttings to evaluate their efficacy as a prophylactic control treatment for PD that would limit the establishment of the bacterium in grapevines in natural vineyard settings. Additionally, we are further characterizing the natural products by anti-*Xf* fungi and will evaluate their efficacy as an application product that may have curative effects for grapevines already infected with PD.

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CHARACTERIZATION OF *XYLELLA FASTIDIOSA* GENES REQUIRED FOR PATHOGENICITY

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ABSTRACT
Xylella fastidiosa (Xf) is a gram-negative, xylem-limited plant pathogenic bacterium and the causal agent of Pierce's disease (PD) of grapevine (Wells et al., 1981). *Xf* is closely related to *Xanthomonas oryzae* pv. *oryzae* (*Zoo).* Recent findings indicate that the sulfated Type 1 secreted protein Ax21 is required for density-dependent gene expression and consequentially pathogenicity of *Xoo*. Two two-component regulatory systems (TCSs) are required for Ax21 mediated immunity. Orthologs for both of the TCSs and Ax21 have been found in *Xf*. In this study, we will investigate the role of Ax21 and the two TCSs that regulate Ax21 in *Xf*.

LAYPERSON SUMMARY
Xylella fastidiosa (Xf) is a plant pathogenic bacterium and the causal agent of disease in a variety of economically important crops, including PD of grapevine. *Xf* causes disease by colonizing the xylem vessels, blocking the flow of water in the grapevine. In many plant pathogenic bacterium's, biofilm formation plays a key role in virulence. A biofilm is a population of microorganisms attached to a solid or liquid interface. The production of biofilm is regulated by quorum sensing system, in which bacteria communicate with one another via small molecular weight compounds. In *Xanthomonas oryzae* pv. *oryzae (Xoo),* a bacterial species related to *Xf,* it has been shown that Ax21, a sulfated peptide, is a quorum sensing compounds that is required for biofilm formation and virulence. Furthermore, two two-component regulatory systems (TCSs) have been identified that are required for Ax21 activity in *Xoo*. In this research, we will investigate the biological function of Ax21 and the two TCSs orthologs that were identified in the *Xf* genome.

INTRODUCTION
Xylella fastidiosa (Xf) is a gram-negative, xylem-limited plant pathogenic bacterium and the causal agent of Pierce's disease (PD) of grapevine (Wells et al., 1981). *Xf* is found embedded in the plant matrix in clumps, which leads to the xylem vessel blockage. The formation of biofilms allows for bacteria to inhabit an area different from the surrounding environment, potentially protecting itself from a hostile environment. Furthermore, biofilm formation is an important factor in the virulence of bacterial pathogens. Biofilm formation is a result of density-dependent gene expression (Morris and Monier, 2003). Density-dependent biofilm formation is triggered by the process of quorum sensing (QS). In QS, bacteria are able to communicate with each other via small signal compounds, generically called "auto-inducers" and the specific case of Xanthomonas and *Xf* the molecules are referred to as diffusible signal factors (DSF). The auto-inducer is a means by which bacteria recognize population size, and mediate the expression of specific genes when bacterial populations reach a threshold concentration. (Fuqua and Winans, 1994; Fuqua et al., 1996).

In *Xanthomonas oryzae* pv. *oryzae (Xoo),* Ax21 is a sulfated, Type 1 secreted protein that is a quorum sensing compound. Ax21 was recently shown to be a requirement for induction of density-dependent gene expression, including biofilm formation (Lee et al., 2006; Lee et al., 2009). In *Xoo,* two two-component regulatory systems (TCSs) required for Ax21-mediated activity have been found and orthologs of the TCSs and Ax21 were identified in the *Xf* genome (Simpson et al., 2000). In order for an active Ax21 gene product to be produced, two TCSs are required: RaxR/H and PhoP/Q (Burdman et al., 2004; Lee et al., 2008). The goal of this research is to investigate the role of Ax21 and the associated two component regulatory genes in *Xf*.

OBJECTIVES
1. Determine the functional role of the Ax21 homolog in *Xf*.
2. Determine the functional role of the PhoP/PhoQ two-component regulatory system in *Xf*.
3. Identify GacA-regulated genes in *Xoo* through microarray analysis and compare with *Xf GacA*-regulated genes.

RESULTS AND DISCUSSION

In the few months that we have worked on this project we made deletion knockout strains of Ax21, *PhoP* and *PhoQ* in *Xf*. For the Ax21 knockout strain, we conducted a variety of assays including pathogenicity on grapevines, biofilm formation, cell-cell aggregation and growth rate. Unfortunately, the grapevine pathogenicity assay did not give us any meaningful data this year because the plants in the greenhouse inoculated with both the wild-type *Xf* and *Xf*Δax21 exhibited foliar symptoms unrelated to PD. We will repeat these pathogenicity assays again next year. We will also inoculate grapevines with *Xf*Δ*PhoP* and *Xf*Δ*PhoQ* mutants.

Xf has an ortholog of ax21 gene (Lee, et al. 2009). To test if *Xf* has Ax21 activity, we carried out Ax21 activity assay with a previous described method (Lee, et al., 2006). Rice leaves from TP309, susceptible to Xoo PXO99, and TP309-XA21, resistant to PXO99, were cut at the tip and pretreated with supernatants from wild type (*Xf*) and Ax21 knockout (*Xf*Δax21) of *Xf*. Supernatants from *Xoo* PXO99 and PXO99Δax21 were used as positive and negative control, respectively. Five hours later the pretreated leaves were inoculated with the *raxST* knockout strain (PXO99Δ*raxST*), which lacks Ax21 activity. Ax21 activity was evaluated by measuring lesion lengths three weeks after inoculation. If *Xf* had Ax21 activity, leaves of TP309-XA21 pretreated by supernatants from *Xf* would show resistance to PXO99Δ*raxST* strain, but not leaves pretreated by supernatants from *Xf*Δax21. However, both leaves pretreated by supernatants from *Xf* and *Xf*Δax21 were susceptible to PXO99Δ*raxST*. It means *Xf* does not possess Ax21 activity, indicating it is unable to trigger XA21-mediated immunity in our rice plant bioassay (**Figure 1**). A lack of secretion and/or sulfation system in *Xf* may be the cause of the lack of Ax21 activity because *Xf* does not have orthologs of *raxA*, which is required for secretion of Ax21, and *raxST*, which is required for sulfation on Ax21. Further research will be conducted to better understand the role of Ax21 in *Xf* pathogenicity and cell-cell communication.

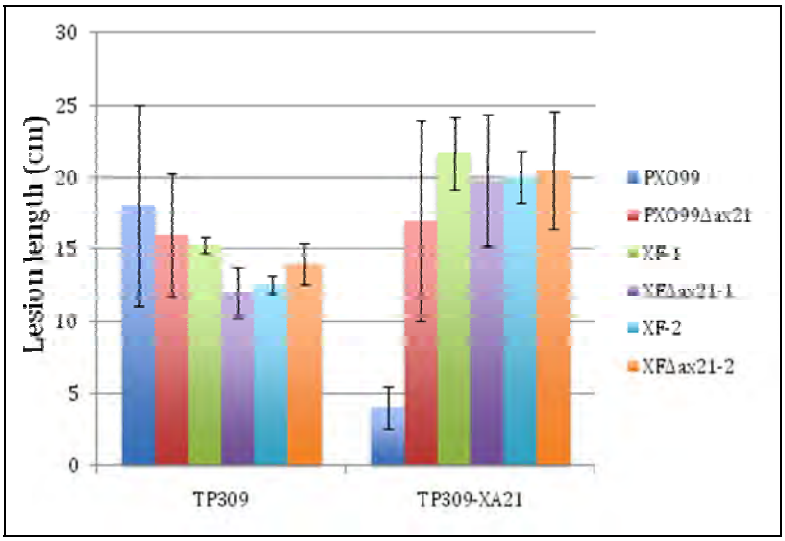

Figure 1: Lesion length on 6 week old TP309, susceptible to *Xoo* PXO99 strain, and TP309-XA21, resistant to PXO99 rice plants inoculated with PXO99Δ*raxST* strain five hours after pretreatments of supernatants. PXO99 and *Xf* indicate wild type of *Xoo* and *Xf* strains, respectively. PXO99Δ*ax21* and *Xf*Δax21 indicates ax21 deletion mutants of *Xoo* and *XF*, respectively. -1 represents supernatants from 8 days incubation culture, -2 represents supernatants from 11 days incubation culture. Each value represents the mean +/- SD.

Based on cell growth, cell-cell aggregation and biofilm production assays, we found some differences between the wild-type *Xf* and *Xf*Δax21. Based on preliminary cell growth results, it appears that the *Xf*Δax21 mutant grows to a lower population density than wild type *Xf* , although it does grow at a similar rate to the wild-type (**Figure 2**). Biofilm production of *Xf*Δax21 is slightly higher than the wild-type (**Figure 3**) when grown statically and measured by the crystal violet method. However, when the mutant and wild type strains were grown in a flask on a shaker, visual inspection showed there was considerably less biofilm formed by the mutant than the wild type strain. This observation needs to be repeated and the amount of biofilm produced will be quantified by the crystal violet method.

Based on the cell-cell aggregation assay, *Xf*Δax21 form less aggregated cells than wild type (**Figure 4**).

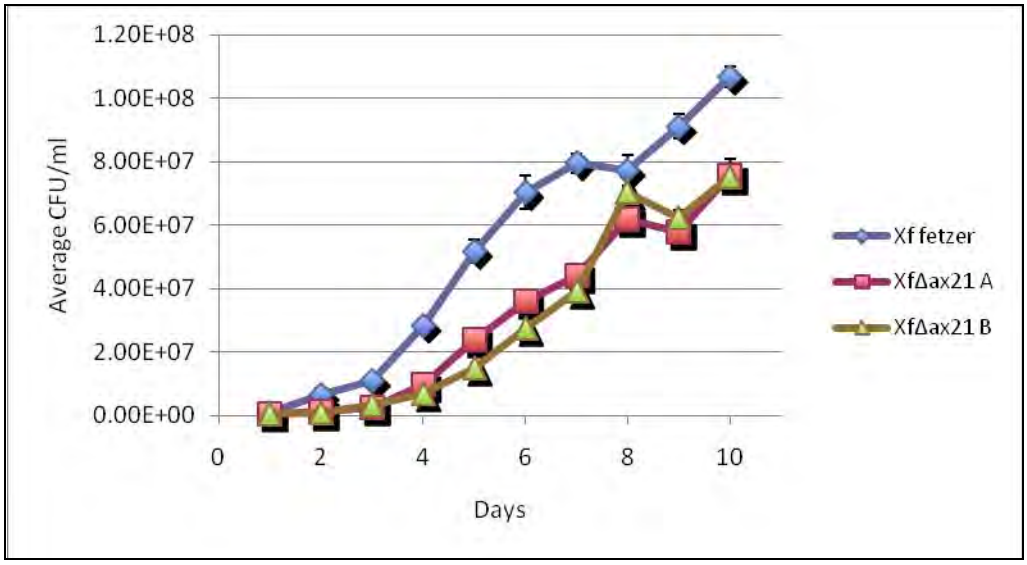

Figure 2. Bacterial growth of wild type *Xf* fetzer, *XfΔ*ax21-A, and *XfΔ*ax21-B. . Values shown are the means of 5 samples +/- error.

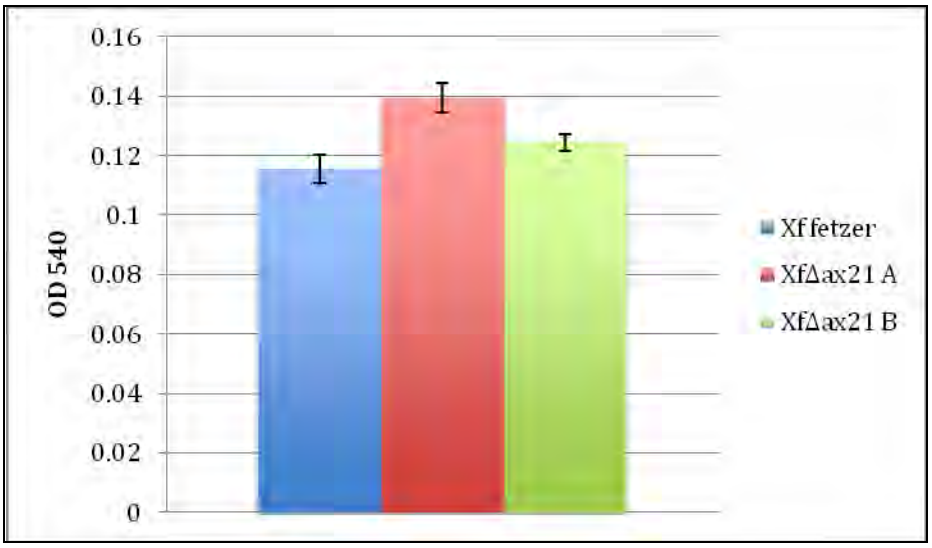

Figure 3. Comparison of biofilm formation in wild-type *Xf* fetzer, *XfΔ*ax21-A, and *XfΔ*ax21-B in stationary cultures as determined by the crystal violet staining method. Values shown are the means of 10 samples +/- error.

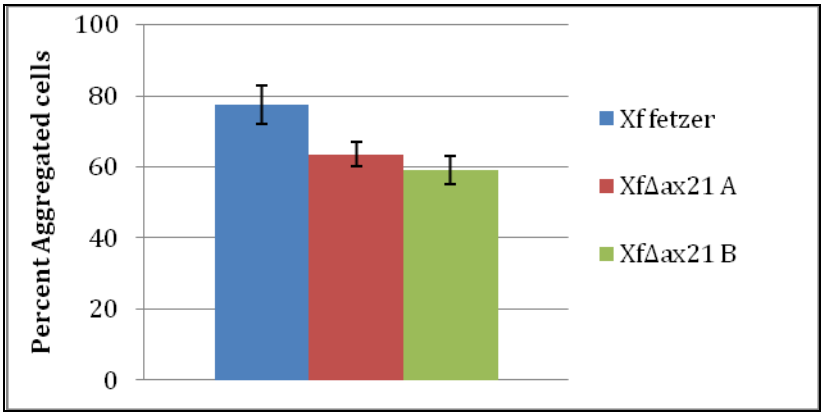

Figure 4. Comparison of percent aggregated cells in wild-type *Xf* fetzer, *XfΔ*ax21-A, and *XfΔ*ax21-B. Percentage of aggregated cells was determined as described by Guilhabert and Kirkpatrick, 2005. Values shown are the means of 10 samples +/- error.

CONCLUSIONS

We have made good initial progress on determining the functional role of Ax21 in *Xf*, although further comparison of wild-type *Xf* and *Xf*Δax21 needs to be done. We have also begun work on objective 2. We are in the process of looking at the differences in cell growth, biofilm formation and cell-cell aggregation of *XfΔPhoP* and *XfΔPhoQ*. We anticipate the combined data from objectives one and two will allow us to better understand the effects of Xa21 and the TCSs the mediate Ax21 activity in *Xf*. Next spring, pathogenicity assays on grapevines will allow us to assess the effects of both Ax21 and the PhoP/Q TCS on the virulence of *Xf*. We will begin work on objective 3 in the coming year.

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Section 5: Crop Biology and Disease Epidemiology

LINKING WITHIN-VINEYARD SHARPSHOOTER MANAGEMENT TO PIERCE'S DISEASE SPREAD

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ABSTRACT
Pierce's disease (PD) management in southern California vineyards hinges on chemical control of populations of the vector, the invasive glassy-winged sharpshooter (GWSS) residing in citrus. Growers also frequently apply systemic insecticides in vineyards, but the efficacy of these treatments for disease management is not known. We are conducting a series of surveys in treated and untreated vineyards in Temecula Valley to determine the relative economic value of within-vineyard chemical control for PD management. Thus far the results from preliminary surveys suggest that although PD prevalence varies greatly among vineyards, those that were regularly treated with systemic insecticide tend to have lower prevalence than fields that were intermittently treated or untreated. These surveys will be continued over the next few years to evaluate the relative importance of vector pressure and chemical control for disease spread. Ultimately, survey data will be used to quantify rates of secondary spread and the spatial distribution of *Xylella fastidiosa* strains, which is needed for drawing inferences regarding GWSS movement and pathogen sources.

LAYPERSON SUMMARY
One of the main tools for dealing with the glassy-winged sharpshooter (GWSS) in southern California and the southern San Joaquin vineyards is the application of insecticides. Systemic insecticides (imidacloprid) are regularly applied to citrus, which is a preferred plant type for GWSS, to reduce insect abundance before they move into vineyards. These treatment programs have been successful, reducing GWSS populations to a fraction of what they once were. Grape growers frequently use systemic insecticides in vineyards as well to reduce further the threat of GWSS spreading Pierce's disease among vines. However, no measurements have been made about whether these costly insecticide treatments are effective at curbing disease spread. We are conducting a series of disease surveys in Temecula Valley to understand whether chemical control of GWSS in vineyards is justified. Results from preliminary surveys indicate that the frequency of disease in vineyards varies greatly, but generally fields that are treated regularly tend to have less diseased vines than those that are not treated. We will continue these disease surveys over the next few years to more fully evaluate this question.

INTRODUCTION
Chemical control of insect vectors plays a crucial role in many disease mitigation programs. This is true not only for the management of mosquito-borne diseases of humans, such as malaria and dengue fever, but also for limiting disease epidemics in a wide range of agricultural crops. In southern California vineyards chemical control at both the area-wide and local scales may affect the severity of Pierce's disease (PD), by reducing the density or activity of the primary vector, the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*; Castle et al. 2005).

The bacterial pathogen *Xylella fastidiosa (Xf)* is endemic to the Americas, and is widespread throughout the western and southeastern U.S. This xylem-limited bacterium is pathogenic to a wide variety of plants, including several important crop, native, ornamental, and weedy species (Purcell 1997). In the Western U.S. the most economically significant host is grapevine, in which *Xf* causes PD. Multiplication of the bacterium in vines plugs xylem vessels, which precipitates leaf scorch symptoms and typically kills susceptible vines within a few years (Purcell 1997).

Xf can be spread by several species of xylem sap-feeding insects, the most important being the sharpshooter leafhoppers (Severin 1949). Historically PD prevalence has been moderate, with a pattern that is consistent with primary spread into vineyards from adjacent riparian habitats by the native blue-green sharpshooter (*Graphocephala atropunctata*). However, beginning in the late 1990s severe outbreaks occurred in southern California and the southern San Joaquin Valley that are attributable to the recent establishment of the GWSS. This invasive sharpshooter is not inherently more efficient at transmitting the pathogen than are native sharpshooters (Almeida and Purcell 2003). Instead its threat as a vector appears to

stem from a combination of ability to achieve extremely high densities (Blua et al. 1999) and promote vine-to-vine (i.e. secondary) disease spread (Almeida et al. 2005).

Citrus trees themselves are not susceptible to the strains of *Xf* found in the U.S. (though strains found in Brazil have caused significant economic losses to their citrus industry – Purcell 1997). None-the-less citrus plantings figure prominently in the epidemiology of *Xylella* diseases in California. Many portions of southern California and the southern San Joaquin Valley have vineyards in close proximity to citrus groves (Sisterson et al. 2008). This is important because citrus is a preferred habitat for the GWSS at key times of the year, allowing this vector to achieve very high densities (Blua et al. 2001). High vector populations then disperse seasonally out of citrus into nearby vineyards, resulting in clear gradients of PD prevalence (i.e. proportion of infected plants) as a function of proximity to citrus (Perring et al. 2001).

Given the importance of citrus in PD epidemiology, citrus groves have been the focus of area-wide chemical control programs, initiated in the Temecula and Coachella Valleys in the early 2000s and shortly afterward in Kern and Tulare Counties (Sisterson et al. 2008). The southern California programs use targeted application of systemic insecticides, such as imidacloprid, to limit GWSS populations residing within citrus. Census data in citrus show substantial year to year variation in GWSS abundance that may stem from incomplete application, the use of less effect organically-derived insecticides, or inadequate irrigation to facilitate uptake - which makes the consistent management of sharpshooter populations a challenge (Toscano and Gispert 2009). None-the-less trap counts have been, overall, much reduced compared to pre-area-wide counts. The effect of chemical control can be seen clearly in early insect surveys which found significantly fewer GWSS in treated relative to untreated citrus and in vineyards bordering treated versus untreated groves (R. Redak and N. Toscano, unpublished data). Thus, these area-wide control programs have been considered successful in southern California (Toscano and Gispert 2009), and the swift implementation of an area-wide management program in Kern County has been credited with limiting the severity of PD outbreaks (Sisterson et al. 2008).

Research into imidacloprid uptake by grape also has been initiated, and target concentrations high enough to suppress GWSS activity (approx. 10 μg/L of xylem sap) can be achieved and will endure for several weeks in mature vines (Byrne and Toscano 2006). This information coupled with the success of area-wide programs in citrus appears to have led to relatively widespread adoption by grape growers of imidacloprid application in vineyards to reduce further exposure to *Xf*. In Temecula Valley, for example, it is estimated that 70% of vineyards use imidacloprid, at an approximate cost of $150-200 per acre (N. Toscano, personal communication). Yet consistent treatment of vineyards with systemic insecticides is neither universal, nor have there been any measures of how effective these costly treatments are at reducing PD incidence.

We are studying the epidemiological significance of chemical control in vineyards, via a multi-year series of field surveys in Temecula Valley. This work will address gaps in empirically-derived observations regarding the cascading effects of vineyard imidacloprid applications on GWSS abundance and, ultimately, PD severity.

OBJECTIVES

The overall goal of this project is to understand **does within-vineyard sharpshooter chemical control reduce vector pressure and PD spread?**

This project was initiated this summer. We are currently in the middle of the first of three seasons of Fall disease surveys. Over the next two additional seasons we will continue these surveys and collect additional data on vector abundance, imidacloprid concentrations, and *Xf* genotype distribution.

RESULTS AND DISCUSSION

In the Fall of 2009 we conducted a pilot survey of some Temecula vineyards. These surveys relied on visual PD symptoms for five pairs of regularly treated and untreated vineyards, plus a 6th unpaired treated vineyard. Disease prevalence varied greatly among fields, ranging from 0 to 22%, with lower overall prevalence in treated compared to untreated fields (**Figure 1**). For all five pairs, the untreated field had equal or higher (by up to 20%) prevalence, with four of the six treated fields having no apparent infected vines.

This summer we interviewed several vineyard owners and vineyard managers in the Temecula region to identify vineyards with a range of imidacloprid treatment histories. Of the 88 distinct properties for which we acquired information 66 were treated regularly with imidacloprid, 14 were treated intermittently, and eight properties were not treated with imidacloprid for at least the last four years. These treatment histories will be verified this Fall and next Spring by sampling plant tissue and quantifying imidacloprid concentration. In September we began the first of three years of disease surveys in a collection of these Temecula vineyards. For each vineyard we noted the proportion of vines showing PD symptoms and we collected plant tissue samples which are being cultured, to verify infection in putatively symptomatic vines, and ELISA, to estimate latent (i.e. non-symptomatic) infections. Thus far, we have results of just the visual symptoms from 14 vineyards. The results from these preliminary data support the pilot study. Although PD prevalence varies greatly among vineyards, those that were treated consistently with systemic insecticide tend to have lower prevalence than fields that were intermittently treated or were untreated (**Figure 2**).

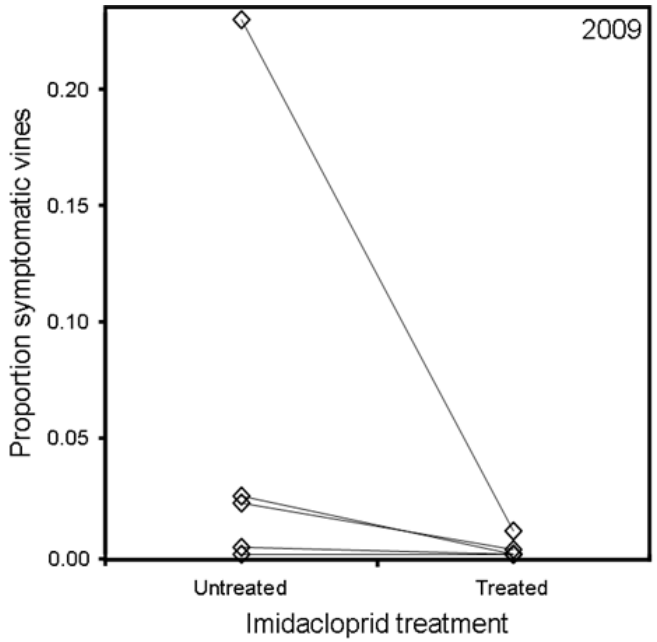

Figure 1. Proportion of plants displaying Pierce's disease symptoms in pairs of imidacloprid treated or untreated vineyards. Observations from Temecula Valley pilot surveys, Fall 2009. Lines connect untreated vineyards with a treated neighboring vineyard. Some data points for the Treated fields overlap (n=6).

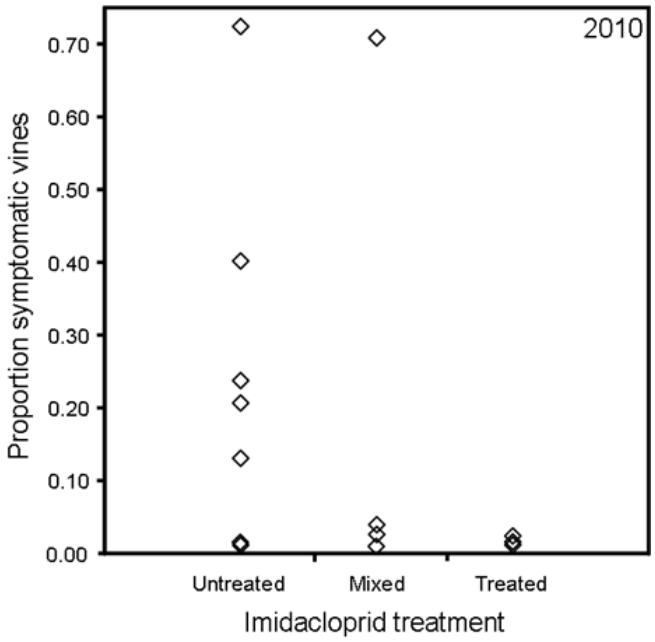

Figure 2. Proportion of plants with Pierce's disease symptoms in fields categorized as being regularly treated with imidacloprid, a mixed treatment history, or never been treated with imidacloprid. Observations from preliminary 2010 surveys in Temecula Valley. Some points overlap (n=7, 4, 3 for Untreated, Mixed and Treated respectively).

CONCLUSIONS

Results so far suggest that within-vineyard sharpshooter chemical control may be effective at reducing disease pressure. However these results should be viewed as preliminary. More complete surveys are needed to estimate the effect of chemical control on vector abundance in vineyards and year-to-year increases in disease (i.e. incidence).

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DO CELL WALL STRUCTURES LIMIT *XYLELLA FASTIDIOSA* DISTRIBUTION IN INOCULATED, PIERCE'S DISEASE SUSCEPTIBLE AND RESISTANT GRAPEVINES?

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ABSTRACT
Symptom development of grapevine Pierce's disease (PD) is determined largely by the extent to which the bacterial pathogen *Xylella fastidiosa (Xf)* spreads in infected vines via their water-conducting system. Thin primary cell wall regions called pit membranes (PMs) separating the adjacent vessels of the water-conducting system are the barriers to the systemic spread that *Xf* must clear by using its cell wall degrading enzymes (CWDEs). Therefore, the presence/absence of the CWDEs' target polysaccharides in the intervessel PMs may determine the movement of *Xf* in the water-conducting system, contributing to PD susceptibility/resistance of the host grapevine. This report introduces our recent development of a new method for detecting the presence, relative concentration and distribution of potential target polysaccharides of *Xf*'s CWDEs in grapevine PMs. This method combines scanning electron microscopy and immunohistochemical techniques and is effective in visualizing some polysaccharides in intervessel and vessel-parenchyma PMs. Compared with the technique we developed previously, this method provides a way to visualize PM structure at a much higher resolution while revealing the PM's polysaccharide composition. Our technique has the potential to identify cell wall polysaccharides exposed at the PM surface and changes in the PM polysaccharide components and quantities that occur during the degradation of intervessel PMs. This should provide some essential information for a better understanding of the PD resistance mechanisms of grapevines.

LAYPERSON SUMMARY
Understanding of grapevine Pierce's disease (PD) resistance/susceptibility mechanisms is essential to development of new PD-resistant grapevine germplasm to be deployed in vineyards. Our study focused on the structural factors of grapevines which limit the systemic spread of *Xylella fastidiosa (Xf)* and thus contribute to the PD resistance of grapevines. This report deals with the cell wall structural barrier to the bacterial spread, the so-called pit membrane (PM), describes a new method to detect the PM's polysaccharide compositions *in situ*, and reveals the spatial distributions and quantities of a group of polysaccharides which might affect *Xf* systemic spread. The further development of this method should contribute to a comprehensive understanding of the PD resistance of grapevines and help identify PD-resistant grapevine germplasm obtained through genetic improvement programs.

INTRODUCTION
As a vascular disease, Pierce's disease (PD) is causing a severe threat to the wine industry in the United States and some other countries. The causal pathogen is the xylem-limited bacterium, *Xylella fastidiosa (Xf)*, which is introduced to the vessel system of a grapevine by xylem sap-feeding insect vectors and spreads, multiplies and establishes its population only via the vessel systems of an infected vine (Purcell and Hopkins, 1996). It is clear that PD symptom development depends largely on whether the systemic spread of the initially introduced *Xf* throughout the vine can occur (Krivanek and Walker, 2005; Labavitch, 2007; Lin, 2005; Lindow, 2006a, b, 2007a, b; Rost and Matthews, 2007). *Xf* cells are initially introduced by an insect vector to few vessels and must carry out successive movement across vessels for the bacterial population to become systemic in the grapevine. The neighboring vessels are separated from one another by lignified cell walls; however, pits provide a possible communication pathway through the lignified cell walls because the only barrier presented by a pit is the thin primary wall regions called pit membranes(PMs) which contain the primary cell walls of the two adjacent vessels and a middle lamella (Esau, 1977). While the mesh of wall polysaccharides in a PM does not impede the passage of water, its pores are too small for *Xf* cells to pass through (Labavitch et al., 2004). Thus, it has been proposed that *Xf* cells secrete cell wall-degrading enzymes (CWDEs) to remove some wall polysaccharides, thus enlarging the PM porosity to permit systemic spread of *Xf*.

Xf's genome contains genes whose sequences suggest that they encode two types of CWDEs: polygalacturonase (PG) and endo-1,4-β-glucanase (EGase) (Simpson et al., 2000) and heterologous expression of the putative *Xf* PG and EGase genes (Agüero et al., 2005; Labavitch et al., 2006; Roper et al., 2007; Pérez-Donoso et al., 2010) produces proteins capable of

digesting homogalacturonan pectin and xyloglucan, respectively, polysaccharides that are often found in dicot cell walls (Carpita and Gibeaut, 1993). Furthermore, the introduction of PG and EGase to explanted grapevine stems causes breaks in the PM polysaccharide network and permits *Xf* cells to pass through intervessel PMs (Pérez-Donoso et al., 2010). These studies also demonstrate the presence of these potential polysaccharide targets of the bacterial CWDEs in grapevine intervessel PMs.

In our previous studies, we developed a technique which combines immunohistochemistry and confocal laser scanning microscopy (CLSM) to detect polysaccharide compositions of PMs (Labavitch and Sun, 2008). By comparing several candidate pectic and hemicellulosic polysaccharides among grape genotypes with different PD resistance, we have identified in grapevine PMs the kinds of polysaccharides which are the potential substrates of *Xf*'s PG and EGase (Labavitch and Sun, 2008, 2009). Our previous investigation on intervessel PM integrity in infected PD-susceptible grape genotypes has also clarified the process of PM degradation (Labavitch and Sun, 2009). The CLSM combined with the immunohistochemical method is excellent in identifying the PM cell wall compositions without obvious tissue damage and artifacts, but it poses some limitations in resolution when detailed structure and structural changes of intervessel PMs must be investigated. In this report, we describe our recent exploration of combining of the immunohistochemical method and scanning electron microscopy (SEM) to investigate PM polysaccharides. This technique provides a substantial resolution improvement suitable for the investigation of delicate structures and structural changes while it is still useful in identifying polysaccharide compositions. We expect this new technique can reveal PM polysaccharide compositions and dynamic changes in PMs during the process of intervessel PM degradation simultaneously. This combination of benefits may provide an efficient approach to evaluate the PD resistance/susceptibility of grapevine germplasm.

OBJECTIVES (**Note**: Only Objectives 1 and 2 in the proposal were approved for funding.)

1. Determine if the development of xylem obstructions (tyloses and pectin-rich gels) and the polysaccharide structure and integrity of PMs are affected by *Xf* inoculation of grapevines transformed to express the PGIP from pear and other plant species in rootstocks and in scions.
2. Determine whether there are differences in PM porosity or polysaccharide structure between resistant and susceptible grapevines. To what extent are these PM characteristics and the production of tyloses and gels modified by introduction of *Xf* to PD-resistant and -susceptible genotypes?
3. Determine the extent to which changes in pathogen virulence resulting from altered production of diffusible signal factor (DSF) correlate with the appearance of tyloses, gels and damaged PMs in inoculated vines.
4. Determine whether the impacts of inoculation on PM integrity and the production of vascular system occlusions identified in tested greenhouse-cultured vines also occur in infected vines growing in the field.

RESULTS AND DISCUSSION

Xf-infected vines of *Vitis vinifera* var. Chardonnay were used in this investigation. Each grapevine was grown in a greenhouse from a grafted root stock and two robust buds from each scion were left at the base, subsequently leading to the development of two shoots. At week four, vines were inoculated with *Xf* at the 6th internode from the base of one shoot for each vine. The two shoots of each vine were maintained with 20-25 internodes by pruning the tops off.

Samples were collected from all the internodes of each vine at week 12 after inoculation and were fixed in 4% paraformaldehyde. Internode samples were then trimmed into thin xylem segments exposing the transverse, radial and tangential surfaces. After being washed in PIPES buffer and in 3% MP (milk powder)/phosphate-buffered saline (PBS), some segments were used for control and the others for treatment. For treatment, xylem segments were incubated first with JIM5 (a rat monoclonal antibody recognizing weakly methyl-esterified homogalacturonans—Me-HGs) diluted in 3% MP/PBS and then with anti-rat IgG antibody conjugated with colloidal gold particles of 10 nm. After removing the excess antibody with PBS and distilled water washes, the samples were treated with a silver enhancement kit in a darkroom. This process causes silver particles to associate the gold particles, increasing the sizes of the silver-enhanced gold particles so that they are visible under SEM. Then, the samples were dehydrated via an ethanol series, critical point dried, and coated with Au-Pt. The coated samples were observed and photographed with a scanning electron microscope at 3.0kV under the modes of secondary electron and backscatter electron, respectively. As an experimental control, samples were processed in the same way except that they were incubated with 3% MP/PBS instead of JIM 5.

Our data indicate that the technique described above will detect the presence and distribution of weakly Me-HGs recognized by JIM5 in the vessel PMs (**Figures 1-3**). Silver-enhanced particles were observable under the secondary electron and backscatter electron modes (**Figures 1-3**). In experimental controls (samples not incubated with JIM5), silver-enhanced particles were very few and were randomly distributed over lateral vessel walls, indicating the background noises with this technique could be efficiently suppressed in both backscatter electron (**Figure 1A, B**) and secondary electron (**Figure 1C, D**) modes. In all the samples treated with the primary and secondary antibody followed by silver enhancement, enhanced gold particles were found to be mostly restricted in the PM parts with intact structure, including both vessel-parenchyma PMs (**Figure 2A-C**) and intervessel PMs (**Figure 2D**). This has indicated the presence of weakly Me-HGs in the PMs, which is consistent with the result we obtained previously using the combined CLSM and immunohistochemistry method (Labavitch and Sun, 2008, 2009). However, the silver-enhanced particles were randomly distributed through each type of PM,

suggesting the random distribution of weakly Me-HGs. This information cannot be obtained using our initial method due to its relatively low resolution.

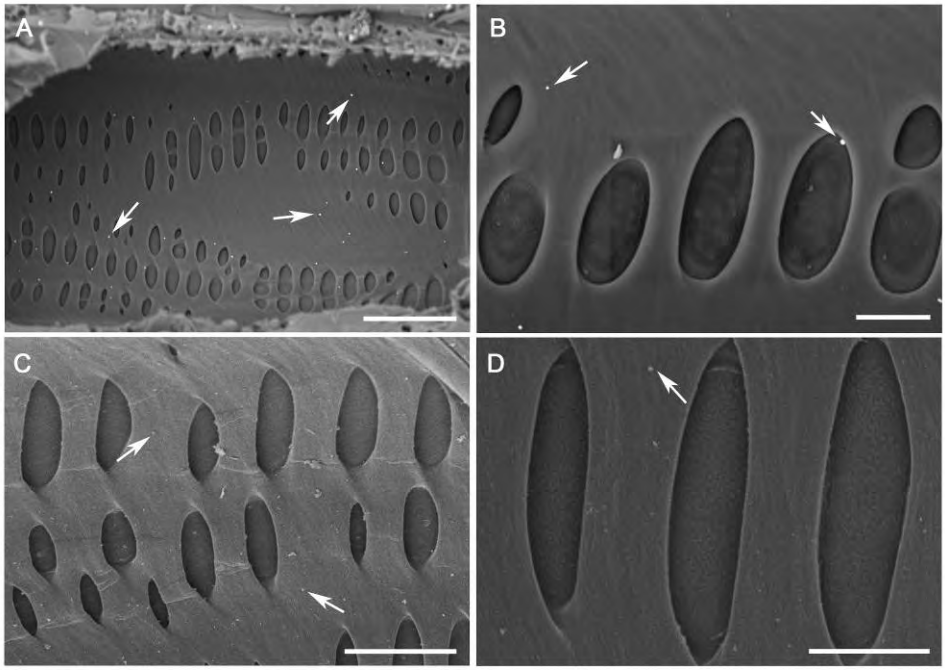

Figure 1. Xylem tissue incubated without JIM 5 but with colloidal gold-conjugated secondary antibody followed by silver enhancement in experimental controls. Silver enhanced gold particles are visible even at a low magnification under SEM and are randomly distributed in small amounts. A-B. Scanning electron micrographs taken under backscattering electron mode, showing white, easy-to-distinguish silver-enhanced gold particles (arrows). C-D. Images taken under secondary electron mode, showing silver-enhanced gold particles (arrows) are not easy to distinguish from their background. Bars in A, B, C and D are equal to 25, 5, 10 and 5µm, respectively.

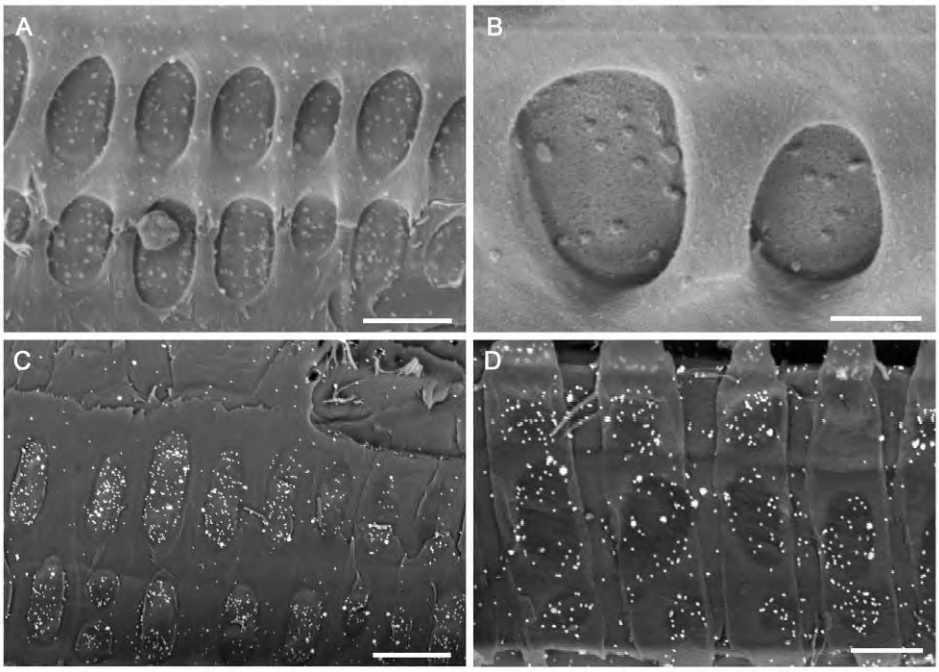

Figure 2. Images of xylem tissues treated with both JIM 5 and gold-conjugated secondary antibodies followed by silver enhancement, taken under secondary electron mode (A-B) and backscatter electron mode (C-D). A-C. Silver-enhanced particles are present on vessel-parenchyma PMs, showing the rich presence of weakly Me-HGs. D. Abundant presence of weakly Me-HGs on intervessel PMs is indicated by silver enhanced particles. Bars in A, B, C and D are equal to 5, 2.5, 10 and 5µm, respectively.

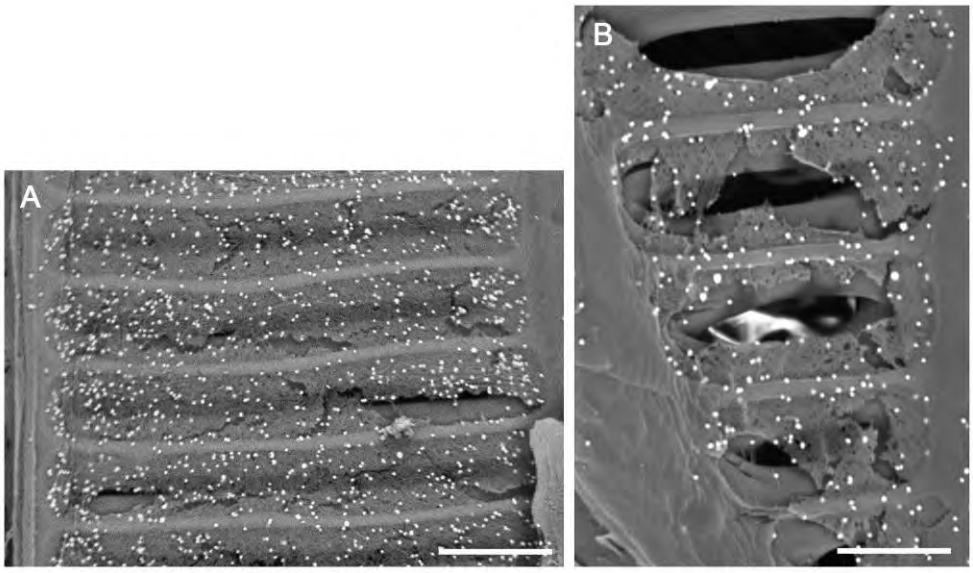

Figure 3. Distribution and quantity of weakly Me-HGs in intervessel PMs at two different stages of the PM degradation process. A. Dense silver enhanced gold particles on less degraded intervessel PMs, indicating more Me-HGs exposed at the PM surface. B. Some weakly Me-HGs are still left in highly degraded intervessel PMs. Bars in A and B are equal to 10 and 5μm, respectively.

Degrading intervessel PMs were compared with intact PMs to reveal any possible differences that are due to the PM degradation process. As in the intact PMs, weakly Me-HGs were detected in both slightly and more completely degraded intervessel PMs and were randomly distributed throughout the PMs (**Figure 3**). There was no obvious difference in the density of silver-enhanced particles between intact PMs (**Figure 2D**) and slightly degraded PMs (**Figure 3A**), but the density of the particles decreased in the more completely degraded PMs. It seems reasonable to believe that *Xf*'s CWDEs should first attack the polysaccharides exposed at the PM surface because the relatively small pore size of the PMs (5 to 20 nm) would prevent diffusion of the relatively large CWDE molecules into the PM. However, removal of the superficial polysaccharides should subsequently loosen the PM's polysaccharide meshwork and make the underlying target polysaccharides accessible to the enzymes. That here is no difference in the distribution and quantity of silver-enhanced particles between intact and slightly degraded PMs suggests that weakly Me-HGs might be present at some depth into the PM as well as being present on the PM surface. If so, our enhanced method should also be suitable for the investigation of spatial distribution and quantity of a polysaccharide type both throughout the PM thickness as well as across its exposed face.

CONCLUSIONS

1. The technique we developed is effective for visualizing pectic polysaccharides in both intervessel and vessel-parenchyma PMs under conventional SEM.
2. The technique has revealed the distribution and quantity of weakly Me-HGs in both intact and degrading PMs of Chardonnay vines.

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BREEDING PIERCE'S DISEASE RESISTANT TABLE AND RAISIN GRAPES AND THE DEVELOPMENT OF MARKERS FOR ADDITIONAL SOURCES OF RESISTANCE

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ABSTRACT
The first BC4 *Vitis arizonica* crosses made in 2009 resulted in 283 resistant seedlings with molecular markers for PdR1 that were planted in the field. These seedlings will have high fruit quality as they consist of 97% *V. vinifera*. A total of 998 seedlings from all *V. arizonica* crosses made in 2009 were screened with molecular markers for PdR1 while still in test tubes from embryo culture and 413 (47%) were resistant. The use of molecular markers to select for Pierce's disease (PD) resistance and greenhouse screening to select for powdery mildew(PM) resistance allows the pyramiding of these two types of resistance and 31 plants with both were planted in the field. In 2010, 28 of 41 crosses attempted were successful and produced 1,600 berries, 2,114 ovules and 293 (13.9%) embryos for PD resistance. An additional 13 crosses to pyramid PD and PM resistance produced 1,782 berries, 2,172 ovules and 341 (15.7%) embryos. Eight seeded crosses made in 2010 produced 415 seed for PD resistance. Evaluation of BC3 fruiting seedlings continued with the selection of 19 new raisin seedlings with PdR1 molecular markers for propagation in production trials. The fruit quality of these selections is similar to the most advanced selections in our raisin breeding program. Over 500 seedlings from the BD5-117 family, with PD resistance different than *V. arizonica*, are being evaluated for cluster size, berry size and color, and seed/aborted seed size to determine fruit quality. Two hundred and forty-seven seedlings of this family have been evaluated in the greenhouse for PD resistance. A rough molecular map is being developed from this population to determine additional molecular markers for PD resistance.

LAYPERSON SUMMARY
Although Pierce's disease (PD) has existed in California since the late 1800s, the introduction of the glassy-winged sharpshooter to California in the late 1990's significantly increased the spread and damage caused by PD. A collaborative breeding program was started in 2000 to develop PD resistant table and raisin grapes with high fruit quality comparable to that existing in markets today. Fifth generation (BC4) crosses for quality table and raisin grapes with *Vitis arizonica* source of PD resistance were made again this year. These families will have high fruit quality as they consist of 97% V. vinifera. An example of increased fruit quality is the selection this year of 19 new raisin grapes made from BC3 *V. arizonica* families which will be propagated for production trials. Three new BC2 *V. arizonica* raisin selections were made and will be propagated for production trials. The use of molecular markers has allowed the selection of PD resistant seedlings while they are still in test tubes. Four hundred thirteen resistant seedlings were selected from 998 seedlings this year, thereby making the breeding program more efficient. Advanced selections are screened in the greenhouse to verify PD resistance. The use of molecular markers and greenhouse screening has made possible the pyramiding of PD with PM resistance. Forty-five PD resistant plants were selected by markers from 98 plants and 31 of these were resistant to PM in the greenhouse screen. Over 500 seedlings from BD5-117 family, a source of resistance that is different from *V. arizonica*, are being evaluated a second time for fruit characteristics to map with resistance. To date, 247 seedlings have been tested in the greenhouse for resistance to PD. A frame-work molecular map is being made with 65 fluorescent labeled SSR markers. This collaborative research between USDA/ARS, Parlier and University of California, Davis has the unique opportunity to develop high quality PD resistant table and raisin grape cultivars for the California grape industry where PD could restrict the use of conventional table and raisin grape cultivars.

INTRODUCTION
Pierce's disease (PD) has existed in California since the late 1800s when it caused an epidemic in Anaheim. A number of vectors for PD already exist in California, and they account for the spread and occurrence of the disease. The introduction of the glassy-winged sharpshooter to California in the 1990's significantly increased the spread and damage caused by PD. Other vectors exist outside California and are always a threat. All of California's commercially grown table and raisin grape cultivars are susceptible to PD. An effective way to combat PD and its vectors is to develop PD resistant cultivars so that PD epidemics or new vectors can be easily dealt with. PD resistance exists in a number of *Vitis* species and in *Muscadinia*. PD resistance has been introgressed into grape cultivars in the southeastern United States, but fruit quality is inferior to *V. vinifera* table and raisin grape cultivars grown in California. Greenhouse screening techniques have been improved to expedite the selection of resistant individuals (Krivanek et al. 2005, Krivanek and Walker 2005). Molecular markers have also been identified that make selection of PD resistant individuals from *V. arizonica* even quicker (Krivanek et al. 2006). The USDA, ARS grape breeding program at Parlier, CA has developed elite table and raisin grape cultivars and germplasm with high fruit quality. Embryo rescue procedures for culturing seedless grapes are being used to quickly introgress the

seedless trait with PD resistance (Emershad et al. 1989). This collaborative research gives the unique opportunity to develop high quality PD resistant table and raisin grape cultivars for the California grape industry.

OBJECTIVES

1. Develop PD resistant table and raisin grape germplasm/cultivars with fruit quality equivalent to standards of present day cultivars.
2. Develop molecular markers for Xf/PD resistance in a family (SEUS) other than *V. arizonica*.

RESULTS AND DISCUSSION

Objective 1

Twenty-eight of 41 crosses using *V. arizonica* source of resistance made in 2010 were successful and produced 1,601 berries, 1,700 ovules, and 293 embryos (17% embryos/ovules) (**Table 1**). The majority of these crosses were BC4 and BC3 crosses. The seedlings obtained from these crosses should have high fruit quality as they now have 93 to 97% *V. vinifera* in their background. Berry set was very poor this year due to the late spring and poor weather conditions. An additional 13 crosses to pyramid PD (*V. arizonica)* resistance with powdery mildew (PM) (*V. romanetii*) resistance produced 1,782 berries, 2,172 ovules and 341 (15.7%) embryos. Molecular markers should be available soon to select for PM resistance. Eight seeded crosses made in 2010 produced 415 seed for PD resistance (**Table 2**).

The first BC4 crosses (97% *Vitis vinifera*) was made in 2009 and consisted of 31 crosses of which 19 produced 3,931 berries, 5,000 ovules and 1,372 embryos and 846 plants. A total of 283 BC4 PD resistant plants based on PdR1 markers were planted in the field. Leaves from all 2009 *V. arizonica* PdR1 plants were taken when seedlings were still in test tubes starting in November, 2009. They were tested for resistance with molecular markers for the PdR1 locus on chromosome 14. Results for one BC2, six BC3 and 19 BC4 seedless x seedless families (89-0908 *V.* arizonica source of resistance) is shown in **Table 3**. A total of 998 individuals were tested with SSR markers and 877 showed markers on both sides of the PdR1 region as expected. A total of 413 individuals (47% of those showing markers) were resistant and planted to soil in cups for growth in the greenhouse before planting to the field in April, 2010. Forty-six percent of the plants showing markers were susceptible. This is very similar to the ratio of resistant and susceptible plants obtained for over 1,600 F1, BC1, BC2 table and raisin seedlings reported by Riaz et al. 2009. The susceptible and recombinant individuals were discarded, making more efficient use of greenhouse and field space. One BC4 cross made in 2009 which combined PD resistance from *V. arizonica* with PM resistance from *V. romanetii*. Usually resistance from this source of PM resistance segregates in a 1:1 resistant:susceptible ratio. The seedlings that had PD resistant markers were screened in the greenhouse for PM resistance. Of the 44 PD resistant seedlings screened, 31 were PM resistant and planted to the field. Inoculation of plants with *Xylella fastidiosa (Xf)* in the Greenhouse (method of Krivanek et al. 2005, Krivanek and Walker 2005) was done to determine resistance of 63 selected individuals from *V. arizonica* (**Table 4**) of which 49 were resistant. These seedlings represent the best table and raisin selections that have been used as parents or planted in production trials. An additional 95 selections are in the greenhouse tests and will be evaluated for PD resistance before the end of this year. Greenhouse testing is absolutely necessary to make the final decision about resistance of individual selections. The highest level of resistance is being obtained from *V. arizonica* and BD5-117 and their use as parents will continue to be emphasized.

The majority of the 400 resistant BC2 and BC3 *V. arizonica* seedlings planted in 2008 have fruited and have been evaluated. From these seedlings, three BC2 and 19 BC3 tray dried raisin selections were made in 2010 which are good enough for propagation into advanced production trials in 2011. These selections range in berry size from Thompson Seedless down to Zante Currant size. The seedlings planted in 2009 had very little fruit production this year and will be evaluated in 2011. Older seedlings are being evaluated again for fruit quality to determine the best to propagate for production trials or for use as parents. In 2010, 13 raisin selections (11 BC2 and two BC3) were planted in production trials. No *Xf* infections have been found to date in the twelve advanced selections planted in a replicated plot at the USDA ARS research station, Weslaco, Texas.

Objective 2

The PD resistant grape selection BD5-117 from Florida was hybridized with the seedless table grape selection C33-30 and 500 individuals are now fruiting of the 565 seedlings. Fruit samples were taken from all seedlings for cluster weight, berry weight and seed/seed trace weight as an indication of fruit quality. Two years data for most of the seedlings has now been collected. Greenhouse testing for PD resistance has been accomplished on 210 individuals, with 109 rated clearly resistant or clearly susceptible. One hundred twenty-one of the 210 individuals were evaluated in 2010 (**Table 3**). The 70 polymorphic markers tested on 154 individuals, greenhouse PD resistance evaluations and fruit characteristics are ready to be mapped. Thirty-five additional polymorphic primers have been labeled and are being tested on the 154 seedlings.

CONCLUSIONS

Additional families for the development of PD resistant seedless table and raisin grape cultivars are being produced. Emphasis was placed on making additional BC4 *V. arizonica* PD resistant families. These families will have high fruit quality as they consist of 97% *V. vinifera*. The use of molecular markers has simplified and sped up the identification of PD resistant individuals from *V. arizonica.* Seedless table and raisin grape selections with PD resistance and improved fruit

quality have been made in both BC3 *V. arizonica* and F1 BD5-117 families. For example, 19 new raisin selections were made from BC3 *V. arizonica* families and will be propagated for production trials. Two hundred and forty-seven seedlings from the BD5-117 family to develop a frame-work map for this source of PD resistance have been evaluated in the greenhouse for PD resistance. The development of PD resistant table and raisin grape cultivars will make it possible to keep these grape industries viable in PD infested areas. Molecular markers will greatly aid the selection of PD resistant individuals from SEUS populations.

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Table 1. 2010 table and raisin grape PD resistant seedless crosses that set fruit and the number of ovules and embryos produced.

Female	Male	Type	No. Emas-culations	No. berries Opened	No. Ovules	No. Embryos
89-0908 *V. rupestris* x *V. arizonica*						
07-5052-036	Y538-58	Table BC4	1000	9	14	8
07-5052-036	Y151-58	Table BC4	500	47	54	33
07-5052-71	07-5054-12	Table BC3	1950	28	26	9
06-5501-229	Y129-176	Table BC3	1468	80	42	18
06-5501-229	Y529-4	Table BC3	1411	49	23	2
06-5501-229	Y540-193	Table BC3	1936	86	54	11
06-5501-229	Y537-32	Table BC3	1500	384	416	62
05-5502-15	Y129-161	Table BC3	687	31	38	2
06-5501-423	05-5501-53	Table BC2	2905	6	13	0
07-5052-090	Y143-39	Raisin BC4	1081	9	7	0
07-5058-016	Y124-203	Raisin BC4	2359	29	29	0
07-5058-016	Y144-135	Raisin BC4	2609	24	31	0
07-5058-029	B82-43	Raisin BC4	1400	92	121	12
07-5058-029	Y143-39	Raisin BC4	2100	155	165	39
07-5058-029	A51-60	Raisin BC4	806	27	12	1
B82-43	07-5052-109	Raisin BC4	2500	152	254	55
07-5058-029	05-5551-049	Raisin BC4	2572	79	120	17
07-5052-086	05-5551-049	Raisin BC4	2800	2	2	0
07-5052-086	06-5551-239	Raisin BC4	2910	78	50	7
A63-85	07-5052-61	Raisin BC4	3500	9	8	0
07-5052-52	07-5054-6	Raisin BC4	1600	6	5	1
07-5058-016	07-5052-61	Raisin BC4	2500	3	4	0
07-5061-134	07-5052-182	Raisin BC4	2037	125	128	12
07-5061-86	07-5052-182	Raisin BC4	2384	35	3	1
05-5551-012	05-5551-049	Raisin BC3	2500	7	10	0
05-5551-012	A51-60	Raisin BC3	1500	4	5	1
05-5551-116	Y144-157	Raisin BC3	3056	15	16	0
A49-82	06-5551-239	Raisin BC3	2800	30	50	2
Total			**56,371**	**1,601**	**1,700**	**293**
PM resistance combined with 89-0908 *V. rupestris* x *V. arizonica*						
07-5052-032	B88-69	Table BC4	550	101	2	0
07-5052-036	B88-69	Table BC4	1100	29	40	12
Y314-17--04	05-5502-05	Table BC3	2574	307	474	68
Y314-17--04	05-5501-53	Table BC3	2570	24	67	13
05-5501-06	Y308-289	Table BC3	2590	105	112	44
05-5502-15	Y308-311	Table BC3	436	54	55	2
07-5058-029	Y302-152	Raisin BC4	2568	66	50	3
Y302-178	06-5551-242	Raisin BC3	806	87	118	1
Y309-397	06-5551-242	Raisin BC3	600	13	25	4
Y520-202--08	06-5551-239	Raisin BC3	2647	667	850	115
05-5551-012	Y308-345	Raisin BC3	2500	11	11	3
Y518-112	05-5551-049	Raisin BC3	2100	32	19	0
Y309-397	07-5060-061	Raisin BC3	2306	62	50	10
Total			**23,347**	**1,558**	**1,873**	**275**

Table 2. 2010 table and raisin grape PD resistant seeded crosses and the number of seeds produced.

89-0908 *V. rupestris* x *V. arizonica*				
Female	Male	Type	No. Emas-culations	No. seed
07-5052-061	Y129-161	Table BC4	600	49
07-5052-061	07-5052-36	Table BC4	1200	68
04-5002-18	Y536-1	Table BC2	1069	115
04-5002-23	Y536-1	Table BC2	1127	80
04-5002-23	Y129-161	Table BC2	1145	13
07-5052-168	A51-60	Raisin BC4	1035	9
07-5052-168	Y144-157	Raisin BC4	1177	1
04-5002-18	Y133-206	Raisin BC2	1206	80
Total			8,559	**418**

Table 3. Determination of seedling resistance based on PdR1 molecular markers for all 89-0908 families made in 2009.

Family	Type Cross	No. Resistant[a]	No. Susceptible[b]	No. Recombinant[c]	No data[d]	Off Types	Total
09-5003	Table BC2	7	6			2	15
09-5007	Table BC3	6	9	4		3	22
09-5008	Table BC3	18	17			35	70
09-5009	Table BC3	2	1				3
09-5010	Table BC3	0	2				2
09-5011	Table BC3	12	7	1	1	7	28
09-5013	Table BC4	92	67	12	1	6	178
09-5016	Table BC4	2	1	1			4
09-5055	Raisin BC3	7	3	1		1	12
09-5056	Raisin BC4	4	8	2	2		16
09-5057	Raisin BC4	21	45	8	1	2	77
09-5063	Raisin BC4	17	16	2			35
09-5064	Raisin BC4	21	21	5	2	1	50
09-5066	Raisin BC4	82	89	11	4	1	187
09-5067	Raisin BC4	4	7	2		1	14
09-5068	Raisin BC4	30	21			1	52
09-5069	Raisin BC4	1	2			8	11
09-5070	Raisin BC4	8	6			8	22
09-5071	Raisin BC4	8	6			8	22
09-5072	Raisin BC4	6	5		1	20	32
09-5073	Raisin BC4	8	4		1		13
09-5074	Raisin BC4	6	2				8
09-5075	Raisin BC4	0	3	1		1	5
09-5076	Raisin BC4	7	6			2	15
09-5077	Raisin BC4	0	7				7
09-6054	Raisin BC4	44	46	7	1		98
Total		**413 (47% [e])**	**407 (46% [e])**	**57 (7% [e])**	**14**	**107**	**998**

[a]Resistant = marker on both sides of *PdR1* region.
[b]Susceptible = no *PdR1* markers.
[c]Recombinant= genotypes that amplified with one *PdR1* marker.
[d]No data = genotypes that failed to amplify properly.
[e]%= Number of seedlings in each category / total number of seedlings showing the proper markers.

Table 4. Results of greenhouse test for determination of PD reaction in 2010.

Population	Resistance Source	Testing Compete		In greenhouse test For evaluation by December
		No. tested	No. resistant	
BD5-117 map	BD5-117	121	44	70
Arizonica	PdR1	63	49	74
Other PD	SEUS	0	0	21
Total		**184**	**93**	**165**

CLARIFICATION OF XYLEM FEATURES LIMITING THE ENTRY AND SYSTEMIC SPREAD OF *XYLELLA FASTIDIOSA* IN GRAPEVINES

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ABSTRACT
Grapevine Pierce's disease (PD) caused by the bacterium *Xyllela fastidiosa (Xf)* is a devastating vascular disease and is jeopardizing the grape and wine industries in the United States due to the PD susceptibility of most important commercial grape genotypes. In our recent study, we compared the PD-susceptible commercial grapevine genotypes with some PD-resistant genotypes newly obtained from traditional breeding program. Comparisons included intervessel pit membrane (PM) integrity, bacterial distribution, and tylose development, which are the most important factors determining the vascular disease resistance of a host plant. Our data indicate that *Xf*-infected PD-resistant genotypes could well maintain the integrity of their intervessel PMs and were found to have a very localized distribution of *Xf* cells while infected PD-susceptible genotypes were observed to have very porous or broken intervessel PMs and a systemic distribution of *Xf* cells. This demonstrates the strong correlation of intervessel PM integrity and limited bacterial distribution in infected vines. It is also revealed that grape genotypes with different PD resistances could develop tyloses in response to *Xf* infection. However, tylose development occurred to different spatial and quantitative extents among the genotypes, with an intensive tylose development throughout a vine in PD-susceptible genotypes but only small amounts of tyloses, these close to the inoculation site in PD-resistant genotypes. This also indicates that tylose development in PD-resistant genotypes does not seriously affect the water status of infected vines and suggests that the tylose development in PD-susceptible genotypes should make the PD symptom development worse by blocking vine water transport. These results provide information for identifying the factors affecting grape resistance to PD and, most likely, have identified vine characteristics useful to efforts aimed at the development and evaluation of an efficient approach to control this terrible disease.

LAYPERSON SUMMARY
Efficient approaches in control of Pierce's disease (PD) based on the understanding of the PD-resistance mechanism of grapevine are being sought as strategies for management of PD in vineyards. This work investigated grapevine genotypes with different PD resistances by focusing on three important factors that may affect the pathogen's spread in a host grapevine and water status of an infected grapevine. Our data indicate that pit membranes (PMs; barriers of the bacterial spread through a grapevine's water conducting system) and tyloses (occluding tissues of the water-conducting tissue) may be related to the PD resistance of the host grapevine. In PD-susceptible grapevines, the causal pathogen can become systemic by breaking the PMs in the vine water conducting system and PD disease symptoms may become more extensive due to tylose blockage of the water conducting tissue in infected vines. In PD-resistant grapevines, the PMs were well maintained and restricted bacterial spread and tyloses did not significantly affect the water transport in infected vines. The information is essential for the understanding and evaluation of PD resistance of new grape germplasm obtained from traditional breeding programs.

INTRODUCTION
Pierce's disease (PD) of grapevines has caused tremendous economic losses to the wine and table grape industries in the United States. The causal *Xylella fastidiosa (Xf)* is a xylem-limited bacterium that spreads only through the vessel system of a host grapevine (Purcell and Hopkins, 1996), thus, any factors affecting the systemic expansion of the *Xf* population that has been introduced initially into one or very few vessels should be relevant to the resistance vs. susceptibility of the infected vine.

As the only avenue for the pathogen's spread, the vessel system of grapevine has attracted a lot of research attention (e.g., Chatelet et al., 2006; Sun et al., 2006, 2007; Thorne et al., 2006). Individual vessels in a grapevine's secondary xylem are relatively short (average length of 3-4 cm, Thorne et al., 2006), thus systemic movement of water, minerals or bacteria requires passage through multiple adjacently interconnected vessels. Movement from one vessel to the next requires passage through pit pairs, specialized wall structures that connect a vessel to its neighbors. In grapevines, contact with neighboring vessels occurs at multiple locations along the vessel's length and scalariform (i.e., organized in a ladder-like pattern) pit pairs

always occur in the wall regions where two adjacent vessels are in contact (Sun et al., 2006). At each pit pair, two adjacent vessels are separated only by two thin primary cell walls and one middle lamella, which are collectively called an intervessel pit membrane (PM) (Esau, 1977). Intervessel PMs of grapevines have pores with sizes varying between 5 and 20 nm (Choat et al., 2003; Pérez-Donoso et al., 2010) and thus should prevent the passage of *Xf* cells (0.25-0.5 µm x 1-4 µm in size; Mollenhauer and Hopkins, 1974) as long as the PMs remain intact.

It has been proposed that *Xf* cells use cell wall-degrading enzymes (CWDEs) to digest PM polysaccharides and achieve their systemic spread (Newman et al., 2003; Labavitch et al., 2006; Labavitch, 2007). Some microscopic examinations on xylem sections of infected grapevines have shown *Xf* cells traversing intervessel PMs (Newman et al., 2003; Ellis et al., 2010). Furthermore, the introduction of certain CWDEs to explanted grapevine stems caused breaks in the PM polysaccharide network and permitted *Xf* cells to pass through intervessel PMs (Pérez-Donoso et al., 2010). These studies have suggested that the systemic spread of the bacterial cells is achieved by disrupting the integrity of intervessel PMs.

Vascular occlusions were reported in grapevines with external PD symptoms (Stevenson et al., 2004; Lin, 2005). As the major type of vascular occlusion, tyloses (outgrowths of parenchyma cells adjacent to a vessel that expand into the vessel lumen), were found to be abundantly present in secondary xylem of the susceptible genotypes (Labavitch and Sun, 2009). Among the few studies on tylose development, most were done with PD-susceptible genotypes and the rest dealt with some susceptible and resistant grape genotypes but were focused on the vessel systems in leaf blades or petioles (Fry and Milholland,1990a,b). The lack of comprehensive information about tylose development has led to two opposed opinions about the functional roles of tyloses in grape PD; i.e., they either improve the host grapevine's PD resistance or worsen the grapevine's PD symptom development. Our previous study reported intensive tylose development in stem secondary xylem of susceptible genotypes, suggesting a possible role in enhancing the infected grapevine's PD symptoms (Labavitch and Sun, 2009). To further clarify this, studies comparing tylose development among grape genotypes with different PD resistances became important.

Although most commercial genotypes of *Vitis vinifera* are susceptible to PD, many wild *Vitis* genotypes and some hybrid *V. vinifera* crossed with wild *Vitis* genotypes have shown strong PD resistance in greenhouse evaluations (Loomis, 1958; Krivanek and Walker, 2005). In this study we used up to four grape genotypes with different PD resistances: two PD susceptible lines, *V. vinifera* var. Charodanny and *V. vinifera* var. Riesling, and two PD-resistant lines, *V. arizonica* X *rupestris* (89-0908) and *V. vinifera* X *arizonica* (U0505-01). The work in this report examined infected vines of these genotypes from the following three perspectives: integrity of intervessel PMs, distribution of *Xf* cells, and spatial distribution and quantitative character of tyloses. Our aims are to clarify the relationships between these three important factors affecting the systemic spread of the bacterial pathogen and/or plant water status and, in addition, further describe their possible relationship to the PD resistance of grapevines.

OBJECTIVES

1. Determine whether xylem structural features vary among grape genotypes with different PD resistance and clarify what structural features are related to the PD resistance of grapevines.
2. Determine whether PM polysaccharide composition and porosity and the extent of *Xf*'s spread from the inoculation site vary in grape genotypes with different PD resistance, and clarify the extent to which PM polysaccharide structure and integrity are affected by *Xf* inoculation of these genotypes.

RESULTS AND DISCUSSION

Differences in Intervessel PM Integrity among Grape Genotypes with Different PD Resistances

Grapevines of three genotypes, PD-susceptible Chardonnay and PD-resistant U0505-01 and 89-0908, were used to investigate effects of *Xf* infection on grapevine PM integrity. Vines of Chardonnay and 89-0908 were grown each from a grafted root stock in the greenhouse. Buds of each scion were removed with only two robust buds left at the base, which were allowed to develop into two shoots. When the vines of the two genotypes were four weeks-old, some were inoculated with *Xf* while others were inoculated with phosphate buffered saline (PBS) as experimental controls. The bacterial inoculation was carried out only on one shoot of each treated vine, at the 6th internode counting from the shoot base. PBS inoculation for each control vine was done in the same way except that the 0.2M PBS instead of the *Xf* inoculum was used. The two shoots of each control or *Xf*-inoculated vine were limited to about 20-25 internodes in length by pruning the tops off. U0505-01 vines were grown and inoculated in the same way except that each vine was trained to have one shoot by initially leaving only one bud at the base of the scion. That sole shoot was inoculated, either with *Xf* suspension or PBS.

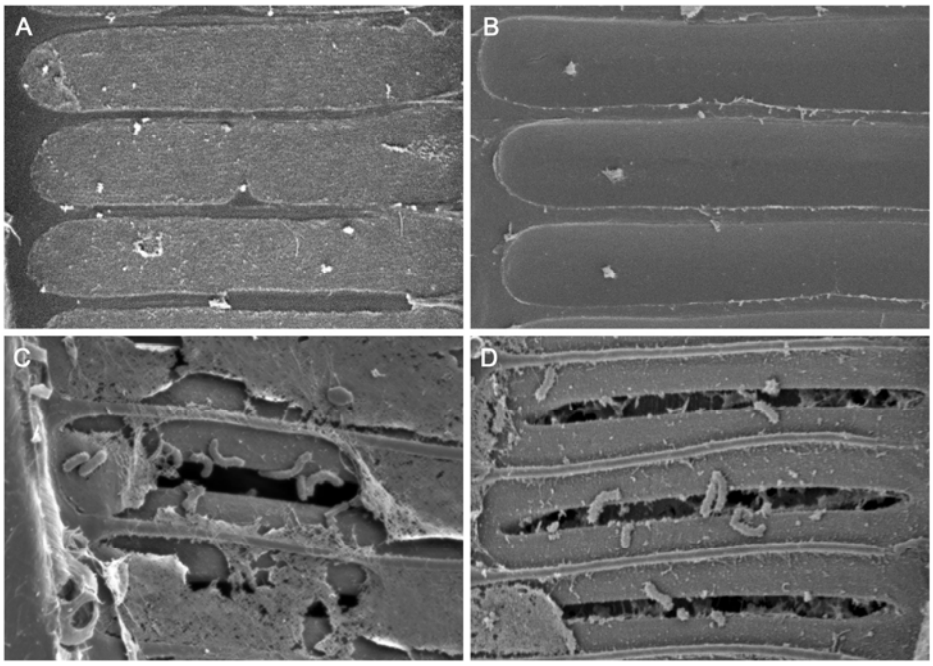

Figure 1. Effect of *Xf* infection on intervessel pit membrane (PM) integrity of grapevines with different PD resistances. Shown are inner surface views of intervessel PMs after the secondary wall borders of each pit were removed to expose the whole intervessel PM. A. Intact intervessel PMs in a vessel not associated with the bacteria in a *Xf*-infected PD-susceptible Chardonnay vine. B. Intact intervessel PMs in a *Xf*-infected PD-resistant U0505-01 vine. C-D. *Xf*-infected Chardonnay vines, showing partial (C) and complete (D) removal of intervessel PMs in vessels that also contain bacterial cells.

The grapevines of the three genotypes were examined for the integrity of their intervessel PMs 12 weeks after *Xf* inoculation, with plants inoculated with PBS used as controls. In all the genotypes, intervessel PMs were intact with no pores detectable under SEM at a magnification of 20,000 in the vessels not associated with *Xylella* cells and those of control vines (**Figure 1A**). In the *Xylella*-infected vines of the two PD resistant genotypes (U0505-01 and 89-0980), intervessel PMs remained intact in all the internodes examined, including the internode with the inoculation site (**Figure 1B**). In infected Chardonnay vines, partially broken intervessel PMs (**Figure 1C**) and/or the complete removal of PMs (**Figure 1D**) were observed in most vessels containing bacterial cells throughout the plants. Thus, *Xylella* infection and intervessel PM disruption are strongly correlated in PD-susceptible genotypes, but not in PD-resistant genotypes.

Table 1. Distributional Comparison of *Xylella fastidiosa* in Some Examplary 12-week-post-inoculation Grapevines with Different PD Resistance

internode[1]	Chardonnay (PD-susceptable)		Riesling (PD-susceptable)		89-0908 (PD-resistant)		U0505-01 (PD-resistant)
	Inoculated shoot[2]	Non-inoculated shoot	Inoculated shoot	Non-inoculated shoot	Inoculated shoot	Non-inoculated shoot	Inoculated shoot
1	+	+	-	-	-	-	-
3	+	-	+	+	+	-	-
5	+	+	+	-	+	-	+
7	+	+	+	+	-	-	-
9	+	+	-	+	-	-	-
11	+	+	+	+	-	-	-
13	+	+	+	+	-	-	-
15	+	-	+	+	-	-	N/A
17	+	+	+	+	-	N/A	N/A
19	+	+	N/A	+	N/A	N/A	N/A
21	+	N/A	N/A	-	N/A	N/A	N/A
23	+	N/A	N/A	N/A	N/A	N/A	N/A

[1]Internodes are numbered from each shoot base upwards with its first internode at the base as internode 1. [2]Inoculation site was at the 6th internode from the shoot base in Chardonnay and 89-0908 vines and at the 4th internode for the U0505-01 vine. [3]+ or - indicate that *Xylella* cells were observed or not observed in a specific internode, and N/A represents inavailability of a specific internode due to the short shoot.

Distributional Differences of *Xf* among Grapevines with Different PD Resistances

Four grape genotypes, including PD-susceptible Chardonnay and Riesling and PD resistant 89-0908 and U0505-01, were used in our experiment. Grapevines of each genotype were grown, trained and inoculated as described above. Samples were

collected from both inoculated and non-inoculated shoots (only the sole inoculated shoot for each U0505-01 vine) of each vine at week 12 after inoculation. Every other internode of each shoot was used SEM examination of the distribution of *Xylella* cells in the vines.

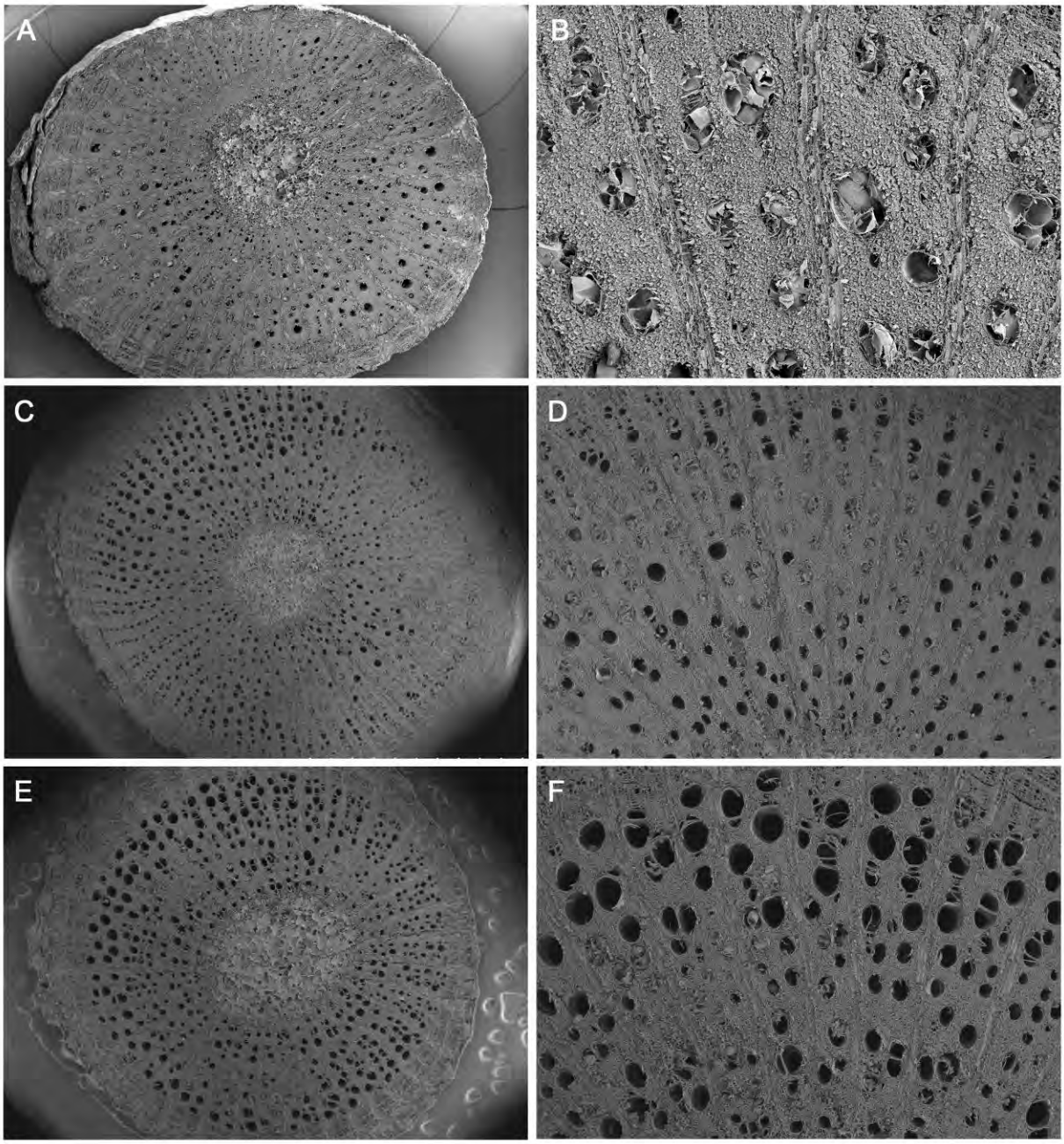

Figure 2. Effect of *Xf* infection on tylose development in grapevines with different PD resistances. Scanning electron micrographs of stem secondary xylem of a PD-susceptible Chardonnay vine (A-B) and a PD-resistant U0505-01 vine (C-F). A. A majority of the vessels were occluded with tyloses and vessels with tyloses were relatively evenly distributed in the 3rd internode above the inoculation site (internodes above the inoculation site were counted upwards with the inoculated internode designated as "zero"). B. Enlargement of part of xylem tissue in A, showing most vessels fully occluded by tyloses. C. The second internode below the inoculation site , showing presence of tyloses in the vessels of some xylem regions. D. Enlargement of part of the xylem with tylose development in C, showing a few vessels filled with tyloses. E. A majority of vessels did not contain tyloses and tylose development occurred in a few vessels in some small xylem regions in the 8th internode above the inoculation site. F. Enlargement of part of the vessels containing tyloses in E, showing a small cluster of vessels associated with tyloses.

Our data have indicated that in Chardonnay and Riesling vines, bacterial cells were observed in most or all of the internodes examined, including those in both the inoculated and non-inoculated shoots of each vine (**Table 1**). This showed not only

that the spread of *Xf* cells from the inoculation point occurred in the susceptible vines, and also that the bacterial cells moved downward from the inoculation site on an inoculated shoot to reach the non-inoculated shoot through the common trunk that the two shoots share. In 89-0908 vines, *Xf* cells were not observed in the non-inoculated shoots of all the vines that were examined. Furthermore, *Xf* cells were not always found in the inoculated shoots in some vines examined and, when pathogen cells were detected, they were seen only within a few internodes downward or upward from the inoculation sites (**Table 1**). A similar situation also was seen in the U0505-01 vines examined (**Table 1**). These observations therefore demonstrate the localized distribution of *Xf* cells in the PD-resistant genotypes and the systemic distribution in the PD-susceptible grapevines. When this is considered with the data of intervessel PM integrity, it also provides the evidence for the correlation of the systemic spread of *Xf* cells and the disruption of intervessel PM structure, which has been hypothesized in earlier reports.

Differences in Tylose Development in Grapevines with Differential PD resistance

Two grape genotypes (PD-susceptible Chardonnay and PD-resistant U0505-01) were studied for tylose development in *Xf*-infected vines at week 12 after inoculation. Growth and inoculation of the experimental vines was conducted in the same way as described above. A few internodes along the inoculated shoot, including those above and below the internode inoculated, were examined to clarify both qualitative and quantitative characteristics of tylose development.

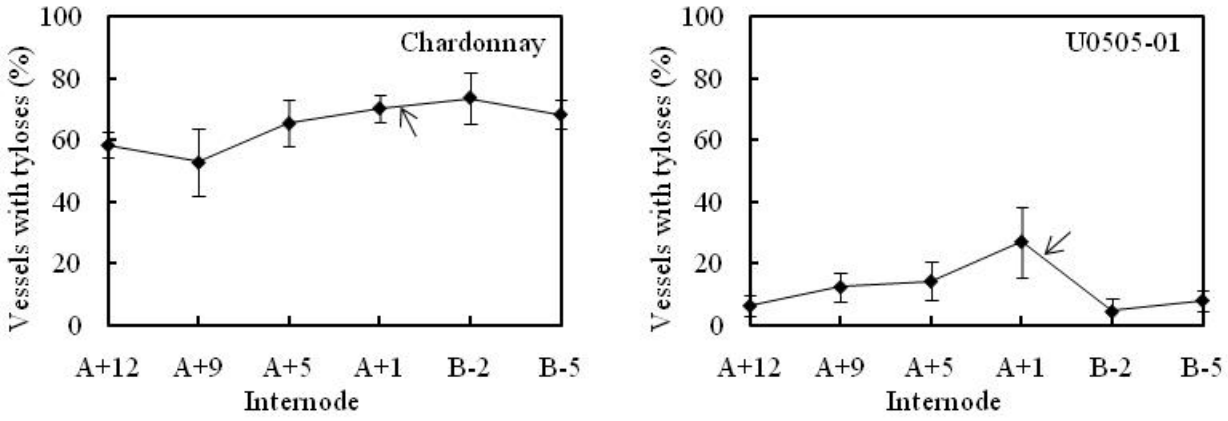

Figure 3. Quantitative comparison of tylose development in PD-susceptible Chardonnay vines and PD-resistant U0505-01 vines. Arrows indicate the internode with inoculation site. Internodes above and below the one with inoculation are indicated by "A+" and "B-", respectively and numbered upward and downward, respectively, with the internode with the inoculation site defined as zero.

Tylose development occurred in all the *Xf*-infected vines but was not observed in the buffer-inoculated control vines for the two genotypes. However, the infected vines of the two genotypes had difference in the spatial distribution of tyloses. In Chardonnay vines intensive tylose development occurred in all the internodes examined from the base to the tip of an infected vine (**Figure 2A**), and vessels with tyloses were more or less evenly distributed in the transverse sections of a stem (**Figure 2B**). In U0505-01 vines, more tyloses were observed in the internodes close to the inoculation site (**Figure 2C-D**) than in the internodes more towards the tip and base (**Figure 2E-F**), and tylose development mostly occurred in one or several secondary xylem regions in which most vessels were associated with tyloses (**Figure 2C, E**). When quantitatively compared for tylose development among the two genotypes, Chardonnay vines generally had over 60% of vessels blocked in the internodes examined throughout the vines (**Figure 3A**), but U0505-01 vines contained 5-27% tylose associated vessels (**Figure 3B**). Our previous study revealed that *Xf* cells themselves did not affect water conduction in infected Chardonnay vines because there were relatively few bacterial cells in only a few vessels in the vines even with severe PD symptoms. This led us to conclude that tyloses were the key factors influencing water transport inside sick vines (Labavitch and Sun, 2009). Furthermore, since tylose development did not always continue along individual vessels in grapevines and open vessels observed in one cross section could be occluded at other places along the vessel lengths (Sun et al., 2006, 2008). This suggests that the percentage of the vessels actually occluded by tyloses should be higher than any value based on the analysis of a single cross section. Therefore, blockage of the majority of vessels in the secondary xylem of a Chardonnay vine should inevitably affect its water supply, consequently contributing to exacerbation of PD symptom development and vine deterioration. As for the resistant genotype, vessels associated with tyloses were much fewer and were restricted to limited xylem regions. Tylose development should have limited effect on the water transport in the infected vines of resistant genotypes.

CONCLUSIONS

1. Grape genotypes with differential PD resistances show differences in intervessel PM integrity and *Xf* distribution. Large amounts of broken intervessel PMs and systemic bacterial distribution are observed in PD-susceptible genotypes while

intact intervessel PMs and restricted bacterial distribution are seen in PD-resistant genotypes. This strongly suggests the positive correlation between intervessel PM integrity and limited *Xf* distribution

2. Tyloses are present in *Xf*-infected grapevines with different PD resistances, but tylose development occurred, both spatially and quantitatively, to much lower extents in PD-resistant and –tolerant genotypes. In these genotypes, tylose development should be considered as a factor that does not lead to PD symptom development.

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BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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Reporting Period: The results reported here are from work conducted October 2009 through September 2010.

ABSTRACT
The use of marker-assisted selection (MAS) using DNA markers tightly linked with the Pierce's disease (PD) resistance gene, *PdR1* (see our companion report), and the acceleration of the seed-to-seed breeding cycle to two years have allowed very rapid progress towards the creation of PD resistant winegrapes. Seedlings from the 2009 and 2010 crosses were screened for PD resistance with MAS and only those seedlings with the markers were planted in the field. The goals of the 2010 crosses were to: 1) expand the 97% *Vitis vinifera* seedling populations with PD resistance using *PdR1* from F8909-08 (a *V. rupestris* x *V. arizonica / candicans* b43-17 hybrid); 2) create 94% *vinifera* populations with *PdR1* from b43-17 to avoid possible disruption of resistance from *V. rupestris*; 3) enlarge the 75% *vinifera* populations with PD resistance from *V. arizonica / girdiana* b42-26 to create an alternative source of PD resistance and one controlled by multiple genes; and 4) to expand the mapping population based on b42-26 to enable identification of useful markers to expedite selection of resistant progeny based on this resistance. Numerous greenhouse-based PD resistance screens were performed on breeding lines, mapping populations and new PD resistant rootstocks. Selections with *PdR1* at the 87% and 75% *vinifera* level at our Beringer, Napa County trial were inoculated and a trial with the 94% *vinifera* level was expanded. An additional field plot with 87% and 94% *vinifera* selections was planted in Healdsburg and 87% vinifera selections were sent to Alabama and Texas for evaluation. Finally, small-scale wine lots were made from five 94% *vinifera* and four 87% *vinifera PdR1* selections. The fruit and juice were evaluated of many other promising progeny at the 94% *vinifera PdR1* level.

LAYPERSON SUMMARY
Rapid progress breeding Pierce's disease (PD) resistant winegrapes continues to be made by combining the use of marker-assisted selection (MAS) for the single dominant gene *PdR1*, and aggressive vine training to produce clusters in a seedling's second season, allowing us to rapidly generate the next generation crosses of PD resistant populations. We created the first populations of 97% *vinifera* seedlings last year and many more were created in 2010. We hope to release PD resistant cultivars from the 97% *vinifera* populations. The first seedlings of the 97% *vinifera* generation will fruit in 2011. We made wines from the 94% *vinifera* selections last year and again this fall. Last year's evaluations found the 94% *vinifera* wines to be much improved over the previous 87% *vinifera* generation; color and aroma flaws associated with American species were absent in the 94% *vinifera* wines. These selections are based on *PdR1* resistance from *V. arizonica / candicans* b43-17. We are expanding populations from other resistance sources that contain multiple genes for PD resistance. It is much slower to breed with these resistance sources and fewer resistant progeny are produced from the crosses, but they provide a very valuable alternative resistance source that we can incorporate with *PdR1* to broaden PD resistance and potentially make it more durable. We expanded our Napa Valley test site with more 94% *vinifera* selections in anticipation of evaluating wines made from there. We also planted 87% and 94% *vinifera* selections at a severe PD hot spot in Healdsburg and sent 87% *vinifera* selections to Alabama and Texas for evaluation under severe PD pressure.

INTRODUCTION
The Walker lab is uniquely poised to undertake this important breeding effort, having developed rapid screening techniques for *Xylella fastidiosa* (*Xf)* resistance (Buzkan et al. 2003, Buzkan et al. 2005, Krivanek et al. 2005a 2005b, Krivanek and Walker 2005), and having unique and highly resistant *V. rupestris* x *V. arizonica* selections, as well as an extensive collection of southeastern grape hybrids, to allow the introduction of extremely high levels of *Xf* resistance into commercial grapes. We have made wine from vines that are 94% *V. vinifera*, and possess resistance from the b43-17 *V. arizonica* / *candicans* resistance source. There are two sources of *PdR1*, 8909-08 and 8909-17 – sibling progeny of b43-17. These selections have been introgressed into a wide range of winegrape backgrounds over multiple generations, and resistance from southeastern United States (SEUS) species is being advanced in other lines. However, the resistance in these later lines is complex and markers have not yet been developed to expedite breeding.

OBJECTIVES
1. Breed Pierce's disease (PD) resistant winegrapes through backcross techniques using high quality *V. vinifera* winegrape cultivars and *Xf* resistant selections and sources characterized from our previous efforts.
2. Continue the characterization of *Xf* resistance and winegrape quality traits (color, tannin, ripening dates, flavor, productivity, etc) in novel germplasm sources, in our breeding populations, and in our genetic mapping populations.

RESULTS AND DISCUSSION

Objective 1 – The breeding cycle for the development of PD resistant grapes has been reduced to two years (seed-to-seed) using MAS with the b43-17 resistance sources and their progeny. In order to take full advantage of *PdR1* we have focused our breeding efforts on backcrossing through multiple generations as quickly as possible to achieve a high percentage of *V. vinifera* parentage in hybrids containing resistance from *PdR1*. This is only possible with MAS and aggressive training practices in the vineyard to force seedlings to bloom in their second year. We produced 97% *V. vinifera* progeny last year, which were established in the vineyard this spring, and have added additional populations this year. Progeny from the 97% *V. vinifera* populations will begin fruiting in 2011. We will select individuals from these populations for wine evaluation and field-testing.

Table 1 presents the crosses made in 2010. The goals of the 2010 crosses were to: 1) Use the *PdR1* allele from F8909-08 to advance *vinifera* winegrape populations to the 97% *vinifera* (modified backcross 4 (BC)) level. These populations will be evaluated for winegrape quality and potential, and individuals will be moved to field testing followed by selection and testing for cultivar release. Seedlings from these populations will be germinated in late fall and planted in the Field in spring 2011 with the first fruit production in 2012. 2) Create populations at the 94% *vinifera* level (BC3) using resistance from *V. arizonica/candicans* b43-17. b43-17 is the source of *PdR1* and using it in crosses avoids possible confounding effects from *V. rupestris*, which was crossed with b43-17 to produce the F8909-08 and F8909-17 PD resistance selections. *Vitis rupestris* may interfere with *PdR1* expression and it is the likely source of diglucoside anthocyanins (blue purple wine color) that we detect in early generations of the backcrossing program. 3) Expand and develop BC1 populations with *V. vinifera* winegrapes using resistance from *V. arizonica/girdiana* b42-26. b42-26 has strong resistance to PD but has a complex resistance controlled by multiple genes. These crosses create 75% *vinifera* populations with an alternative PD resistance source and one controlled by multiple genes. The multigenic nature may provide a more durable resistance, but progress in selecting and backcrossing will be much slower than with *PdR1*. 4) Finally we made crosses to expand a mapping population to study the genetics of PD resistance from b42-26 and determine whether DNA makers linked to this resistance source can be found and used. We have commenced mapping in the 05347 population and this next generation (07344A) will help confirm the location and usefulness of the multiple markers we are discovering.

During this period, eight groups of plants were tested in the greenhouse for *Xf* resistance (**Table 2**). Table 2 presents the inoculation date and the "take-down" date when ELISA samples are gathered. Group A was a retest of the 94% *vinifera PdR1* parents used in the 2009 crosses. They were tested three times to ensure they had the highest resistance and to follow the extent that level of resistance (based on the level of *Xf* in a ml of macerated stem tissue) is passed through to the next generation. We run a series of bio-controls with these tests that consist of *PdR1* containing selections with consistently relatively high, medium and low levels of *Xf* in stem tissue. This process also helps judge the severity of each greenhouse screen, the results of which vary based on how well the greenhouse temperatures are regulated and the temperature's interaction with irrigation needs. Group B and D tests also focused on further assessing 94% *vinifera PdR1* selections some of which we have made wines from. Selections from these populations were also made to evaluate the impact of different *vinifera* winegrapes on the level of PD resistance. The results found that although there are cultivar differences in the levels of *Xf* in stem tissue after inoculation, the impact on resistance breeding is small if detectable (see below).

Group C was an examination of the impact of *Xf* strain selection on the severity of the greenhouse testing system. These tests were initiated after the severity of the greenhouse testing results, both in terms of plant symptoms and ELISA values, was declining when the Stag's Leap *Xf* strain was used. This strain still grew well in culture but was not being passaged back to susceptible *vinifera* hosts as often as in the past. We compared new isolates from Yountville (Beringer) and Dry Creek (Mounts) with lab cultures of Temecula and Stag's Leap strains, and Stag's Leap that was inoculated and re-extracted from Chardonnay in the greenhouse. The Beringer strain was the most aggressive while Mounts and Temecula were intermediate. Both Stag's Leap strains were less aggressive and lab cultured Stag's Leap strain was the least aggressive.

Groups E and F are the continued testing on *PdR1* containing rootstock crosses we have made. These rootstocks are being created to prevent vine death if PD resistant scions are grafted onto standard rootstocks, the majority of which are susceptible to PD. The *PdR1* winegrape selections greatly suppress *Xf* populations, but to avoid having low levels of *Xf* work their way down to the rootstock and killing it, resistant rootstocks are needed. We have done some nematode testing of these rootstocks as well. This year's tests were done to fine-tune the selection of those with the highest level of resistance.

The Group G tests are the first series of greenhouse screens for the 97% *vinifera PdR1* containing winegrapes that were planted in the spring of 2010. We planted the strongest of this group on our Y-trellis in anticipation of some fruit next year and adequate amounts for micro-scale wine making in 2011. Micro-scale winemaking will be much more possible now that the new Department winery is competed with its adjustable volume mini-fermenters with computer controlled temperatures and automated pump-overs. The final group, H, consists of 122 members of a mapping population created and tested to position the resistance genes from b42-26. We hope to link simple sequence repeat (SSR) markers to the genes controlling this resistance. These markers will be very important in efforts to combine this resistance with *PdR1* selections to broaden their PD resistance.

Objective 2 - Although resistance from other backgrounds is complex and quantitative, which results in few resistant progeny from crosses to *vinifera* cultivars, we continue to advance a number of lines. In order to better understand the limits of other PD resistance sources the following resistance sources are being studied:

V. arizonica/girdiana b42-26 – **We** have two mapping populations to explore *Xf* resistance from b42-26. A framework map of the first population, 0023, has been developed and found that resistance is controlled by multiple genes. The 0023 is a cross of (D8909-15 (*V. rupestris* x b42-26) x *V. vinifera* B90-116). Please see past reports for more information on results with the 0023 population. Because this resistance source is multigenic we need far more individuals to detect useful markers to resistance genes and to help determine which of these markers are linked to the genes responsible for the greatest extent of the resistance. We also wanted a population without *V. rupestris*, and so created the 05347 (*vinifera* F2-35 x b42-26) population. We have several hundred 05347 progeny and made crosses this year (760 seed expected) to further expand the population to allow better mapping.

We are also incorporating resistance from *V. shuttleworthii* Haines City and *V. arizonica* b40-14. Preliminary results found that b40-14 has a different form of *PdR1*. We are backcrossing to *V. vinifera* winegrapes with these resistance sources and working towards developing markers in our companion project, "Map-based identification and positional cloning of *Xf* resistance genes from different known sources of PD resistance in grapes".

Evaluating V. vinifera *cultivars and parental selections* – A previous study by Raju and Goheen (1981 Am. J. Vitic. Enol. 32:155-158) ranked 25 *V. vinifera* cultivars as sensitive to tolerant to PD based on ELISA readings from greenhouse screened plants. We wanted to retest many of our parents to determine if there was any possible contribution of varying levels of susceptibility or tolerance in our parents to the progeny. If this effect exists it does not seem to be consistent in our populations. This screening also gave us the opportunity to compare our greenhouse results with those of Raju and Goheen. **Table 3** presents these data on 34 winegrapes and bio-control standards. Our bio-control standards were included in this test and behaved as expected with *V. arizonica/candicans* b43-17 having very low values (equivalent to un-inoculated Chardonnay). The values for U0505-01 were also typically low as was Roucaneuf, a French hybrid (SV 12.309). We also use U0505-35 and -22 as bio-controls because although they contain *PdR1* they typically have moderate levels of *Xf*. Genotypes with *PdR1* generally have mean values of *Xf* (cfu/ml) lower than 500,000. We try to select parents with values below 100,000. Chenin blanc and Sylvaner were the least susceptible in the Raju and Goheen study and Sylvaner had the lowest values in our test. Its values were equivalent to Blanc du Bois, which hosts relatively high levels of *Xf* but suppresses symptom expression and survives in the southern US. Chenin blanc was intermediate in our test. Other contradictory results were Cabernet Sauvignon, which was highly susceptible in Raju and Goheen's test but moderately intermediate in our test. However, overall the groupings were similar. Although both tests were performed in the greenhouse, the inoculation techniques were different (needle inoculation vs vacuum infusion) and the greenhouse conditions, including irrigation and temperature control, which have a large influence on symptom expression and *Xf* build up but were hard to compare

Field Testing – Testing of advanced selections continues at the Beringer vineyard in Yountville, CA. In addition to natural PD pressure in this Napa Valley hot spot, we needle inoculate each spring. Eleven selections from the BC3, 94% *vinifera* crosses, grafted on our PD resistant rootstock selections, were planted at Beringer in July 2010. They are listed next followed with their last *V. vinifera* parent: 07329-01, 07329-037 (Chardonnay), 07355-042, 07355-048, 07355-057, 07355-075 (Petite Sirah), 07370-128, 07371-025, 07371-027 and 07713-051 (Carignane x Cabernet Sauvignon). We also planted a field trial at the Mounts Vineyard in Healdsburg in June 2010 with 07329-37, 07355-75 and 07713-51 (all three 94% *vinifera* with *PdR1*), and U0502-20 (87% *vinifera* with *PdR1*). These vines were planted with varying numbers of five vine replicates. The site is surrounded by PD habitat on two sides and is chronically and severely infected, they will also be needle inoculated. We sent 87% *vinifera PdR1* to Dr. Elina Coneva at Auburn University in Alabama 501-12 (50% Syrah) 30 plants, 502-01 (50% Chardonnay) 32 plants and 502-10 (50% Chardonnay) 34 plants. They were repotted there and will be planted out in spring 2011. We also sent cuttings of five 87% *vinifera PdR1* selections to Jim Kamas in Fredericksburg, TX for a trial there (U0502-10, U0502-20, U0502-26, U0502-38 and U0505-35). A trial with most of these is underway in Galveston, TX in collaboration with Lisa Morano.

Wine Making – Wines were made this fall from four 88% and five 94% *vinifera PdR1* selections growing in the evaluation block at UCD. A full description of the fruit and juice is in **Table 4**. ETS Laboratories (www.etslabs.com) of St. Helena kindly donated their fruit analysis and phenolics panel, which uses a wine-like extraction to model a larger fermentation. Wine evaluations will occur later this winter.

CONCLUSIONS

This project continues to breed PD resistant winegrapes with the primary focus on the *PdR1* resistance source so that progress can be expedited with MAS. Populations with *Xf* resistance from other sources are being maintained and expanded, but progress is slower with these sources. We continue to supply plant material, conduct greenhouse screens and develop new mapping populations for our companion project on fine-scale mapping of PD resistance to allow the characterization of the *PdR1* resistance locus. Small-scale wine making continues with advanced 94% *vinifera* selections and these selections scored very well last year. In 2011, we should make the first small wines from Napa trials with the 94% *vinifera* selections.

We plan to release PD resistant cultivars from the 97% *vinifera* populations we planted this year – they will begin fruiting in summer 2011, and continue to produce additional 97% vinifera populations with different last generation winegrape parents.

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Table 1. 2010 crosses to produce winegrapes and mapping populations with the estimated number of seeds produced.

Resistant Type	*Vinifera* Parent\grandparent of Resistant Type	*Vinifera* Types used in 2010 crosses	Estimated # of Seed
1a. Monterrey *V. arizonica/candicans* resistance source (F8909-08 = *V rupestris* x *V. arizonica/candicans* b43-17) to produce progeny with 96.875% *V. vinifera* parentage. F2-35 is 100% vinifera cross of Cabernet Sauvignon x Carignane.			
07355-020	Petite Sirah\Cabernet Sauvignon	Barbera	85
07370-028	F2-35\Chardonnay	Chardonnay, Riesling	750
07371-20	F2-35\Chardonnay	Barbera	350
1b. Monterrey *V. arizonica/candicans* (b43-17) *PdR1a* resistance source to produce progeny with 93.75% *V. vinifera* parentage, without the possible confounding effect of *V. rupestris*, which is in 8909-08 or 8909-17.			
08329-035	Tannat\Chenin blanc	Cabernet Sauvignon	85
08329-074	Tannat\Chenin blanc	Cabernet Sauvignon, Carignane	900
08329-095	Tannat\Chenin blanc	Cabernet Sauvignon	240
1c. Crosses to the b42-26 *V. arizonica/girdiana* resistance source to produce progeny that are 75% vinifera and 25% the resistance source.			
07344A-09	Grenache	Carignane	225
07344A-11	Grenache	Carignane, Cabernet Sauvignon, Chardonnay	315
07344A-12	Grenache	Carignane	180
07344A-15	Grenache	Carignane	360
07344A-25	Grenache	Carignane	360
07344A-32	Grenache	Carignane	180
07344A-33	Grenache	Carignane, Cabernet Sauvignon	180
07344A-51	Grenache	Carignane	225
07344A-54	Grenache	Carignane, Cabernet Sauvignon	270
07344A-56	Grenache	Carignane, Cabernet Sauvignon	270
07344A-61	Grenache	Carignane, Cabernet Sauvignon	360
1d. Cross to increase the 07344A *V. arizonica/girdiana* b42-26, 75% *vinifera* possible mapping population.			
05347-02	F2-35	Grenache	760

Table 2. PD resistant winegrape progeny completed or currently in greenhouse screening for PD resistance.

Group	Genotypes	# Genotypes	Inoculation Date	ELISA Date	Resistance Source(s)
A	2009 *PdR1* parents	50	11/24/09	2/25/10	F8909-08
B	94% *vinifera* parents and selections	68	12/8/09	3/9/10	F8909-08
C	*Xf* strain trial	6	3/30/10	7/6/10	F8909-08
D	94% *vinifera* parents and selections -2	145	4/13/10	7/22/10	F8909-08
E	PD rootstock test	35	6/8/10	9/30/10	F8909-08
F	08 PD stocks & recombinants	22	7/15/10	10/14/10	F8909-08
G	97% *vinifera* tests	23	7/26/10	11/22/10	F8909-08
H	05347 b42-26 mapping	122	9/23/10	12/19/10	b42-26

Table 3. Greenhouse screen results for *V. vinifera* cultivars used in our crosses and a broad range selected from a previous screen by Raju and Goheen (Amer. J. Vitic. Enol. (1981) 32:155-158)

Genotype	t-test	GH Screen Result (ref U0505-01)	Geometric mean (cfu/ml)	Mean (ln cfu/ml)	Std Error (ln cfu/ml)	Reps
Chard un-inoculated	A	R	11,959	9.4	0.1	5
b43-17	A	R	14,830	9.6	0.4	5
U0505-01	B	R	36,268	10.5	0.5	5
Roucaneuf	C	R	90,174	11.4	0.8	5
U0505-35	D	S	403,729	12.9	0.9	5
U0505-22	DE	S	695,510	13.5	0.8	4
Sylvaner	EF	S	1,099,207	13.9	0.8	4
Blanc du bois	EFG	S	1,290,448	14.1	0.4	4
Exotic	FGH	S	1,985,339	14.5	0.3	5
Zinfandel	FGHI	S	2,408,705	14.7	0.3	5
Grenache	FGHI	S	2,519,321	14.7	0.3	5
Napa Gamay	GHIJ	S	2,985,566	14.9	0.1	4
Chenin blanc	GHIJ	S	3,089,439	14.9	0.3	5
Gewurztraminer	GHIJ	S	3,104,925	14.9	0.3	5
Carnelian	GHIJ	S	3,552,279	15.1	0.2	3
Carignane	HIJ	S	3,612,462	15.1	0.2	5
Helena	HIJ	S	3,794,260	15.1	0.2	4
Green Hungarian	HIJ	S	4,171,557	15.2	0.2	5
Cabernet Franc	HIJ	S	4,654,290	15.4	0.1	5
Alicante Bouschet	HIJ	S	4,654,756	15.4	0.2	4
Mataro	IJ	S	4,753,539	15.4	0.1	5
Merlot	IJ	S	4,800,353	15.4	0.1	5
White Riesling	IJ	S	5,103,310	15.4	0.1	5
Sauvignon blanc	IJ	S	5,113,016	15.4	0.1	5
Colombard	IJ	S	5,147,903	15.5	0.1	5
F2-35	IJ	S	5,192,366	15.5	0.2	5
Chardonnay	IJ	S	5,480,462	15.5	0.2	3
Melon	IJ	S	5,554,950	15.5	0.1	5
Early Burgundy	IJ	S	5,623,698	15.5	0.1	4
Mission	IJ	S	5,991,785	15.6	0.1	4
F2-7	IJ	S	6,009,788	15.6	0.1	4
Palomino	J	S	6,036,289	15.6	0.0	5
Malbec	IJ	S	6,194,052	15.6	0.0	3
Petite Sirah	J	S	6,337,532	15.7	0.0	5
Rosa Minna	J	S	6,366,115	15.7	0.0	4
Ugni blanc	J	S	6,394,188	15.7	0.0	5
Monukka	J	S	6,496,018	15.7	0.0	5
Barbera	J	S	6,499,917	15.7	0.0	4
Cabernet Sauvignon	J	S	6,499,917	15.7	0.0	5
Flora	IJ	S	6,499,917	15.7	0.0	3
Pinot noir	J	S	6,499,917	15.7	0.0	5

Table 4a. Phenotypic observations of reference varieties and select progeny with the *PdR1* resistance source used for small lot winemaking in 2010.

Genotype	Parentage	Percent *vinifera*	2009 Bloom Date	Berry Color	Berry Size (g)	Ave Cluster Wt. (g)	Ripening Season	Prod 1=v low, 9=v high
Chardonnay	Gouais blanc x Pinot noir	100	5/22/10	W	1.0	190	early	5
07355-12	U0505-01 x Petite Sirah	94	5/25/10	B	1.2	246	early-mid	6
07355-42	U0505-01 x Petite Sirah	94	5/27/10	B	1.4	169	late	6
07355-75	U0505-01 x Petite Sirah	94	5/20/10	B	1.4	265	early	8
07713-51	F2-35 x U0502-48	94	5/19/10	W	1.4	310	early	8
07713-55	F2-35 x U0502-48	94	5/21/10	W	1.2	270	early-mid	5
U0502-10	A81-138 x Chardonnay	87	5/21/10	B	1.3	320	early	7
U0502-20	A81-138 x Chardonnay	87	5/28/10	W	1.3	150	late	8
U0502-26	A81-138 x Chardonnay	87	5/24/10	B	2.0	480	mid	7
U0505-35	A81-138 x Cab. Sauvignon	87	5/25/10	B	1.3	158	early	6
Blanc du Bois	Fla D6-148 x Cardinal	~66	5/26/10	W	2.8	175	mid-late	7
Lenoir	*V. aestivalis* hybrid	<50	6/2/10	B	1.3	157	late	6

Table 4b. Analytical evaluation of advanced selections with the *PdR1* resistance source used for small lot winemaking in 2010. Analysis courtesy of ETS Laboratories, St. Helena, CA.

Genotype	L-malic acid (g/L)	°Brix	potassium (mg/L)	pH	TA (g/100mL)	YAN (mg/L, as N)	catechin (mg/L)	tannin (mg/L)	Total antho-cyanins (mg/L)
07355-12	1.38	27.7	1990	3.25	0.90	326	82	512	2369
07355-42	1.69	26.4	1820	3.53	0.59	356	148	642	1787
07355-75	2.93	28.3	2230	3.43	0.80	275	14	555	1680
07713-51	2.59	22.6	1400	3.48	0.59	194	-	-	-
07713-55	5.87	24.3	1230	3.25	0.98	293	-	-	-
U0502-10	3.65	25.5	1850	3.40	0.80	340	78	640	1193
U0502-20	2.33	23.5	1640	3.37	0.75	357	-	-	-
U0502-26	2.71	24.6	2000	3.40	0.79	340	85	272	741
U0505-35	5.44	27.9	2010	3.41	9.60	397	118	820	1609
Lenoir	7.03	24.8	2240	3.22	1.21	183	186	268	2486

Table 4c. Sensory evaluation of reference varieties and advanced selections with the *PdR1* resistance source used for small-scale winemaking in 2010.

Genotype	Juice Hue	Juice Intensity	Juice Flavor	Skin Flavor	Skin Tannin (1=low, 4= high)	Seed Color (1=gr, 4= br)	Seed Flavor	Seed Tannin (1=high, 4= low)
Chardonnay	gold-brown	medium	apple, pear	sl fruity	1	3.5	nutty	4
07355-12	red	med-dark	red fruit	berry, fruity	2	3	spicy, hot	3
07355-42	pink-red	lt-med	fruity, honey	CS-veg	2	3	spicy	1
07355-75	pink-red	medium	plum, fruity	ripe red fruit	1	3	spicy	1
07713-51	gold-brown	medium	floral	neutral	2	4	mild spice	4
07371-55	green	pale	neutral, tart	neutral	1	4	woody	4
U0502-10	pink	light	red fruit	sl fruity, hay	2	4	nutty, spicy	2
U0502-20	pink-brown	lt-med	fruity-spicy	neutral, hay	1	3	spicy, bitter	1
U0502-26	green-brown	medium	honey, spicy	neutral	1	4	clove, spice	3
U0505-35	red-sl brown	medium	CS-veg, berry	sl vegetal	1	3	nutty	3
Blanc du Bois	brown-green	medium	floral, apple	sl vegetal	1	4	woody, bitter	3
Lenoir	red	med-dark	mildly fruity	fruity	1	4	hot	4

MAP-BASED IDENTIFICATION AND POSITIONAL CLONING OF *XYLELLA FASTIDIOSA* RESISTANCE GENES FROM KNOWN SOURCES OF PIERCE'S DISEASE RESISTANCE IN GRAPES.

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ABSTRACT
This report presents updated results on the refined genetic and physical mapping of the Pierce's disease (PD) resistance locus, *PdR1*, which originates from *Vitis arizonica/candicans* b43-17 and is flanked by the SSR markers, VVCh14-78 and VVCh14-81, within a 1cM distance. We have two BAC libraries for b43-17, each with one restriction enzyme (*Hind* III and *Mbo* I), and the screening of the *Hind* III BAC library with flanking markers was completed. The Pinot noir genome sequence was used to develop SSR markers to screen the BAC library, and these markers were used to reduce *PdR1*'s physical distance. Two screenings of the libraries identified 24 (with markers VVCh14-56 and VVCh14-10) and 17 positive BAC clones (with marker VVCh14-58). Five clones were positive with VVCh14-56 and VVCh14-58. Clone 'H69J14' (which is bigger than 200Kb) was selected for sequencing and clone spans scaffold 68 and 171 of the Pinot noir genome sequence. A total of 42,000 sequences were generated, however assembly was complicated by a large number of transposable elements in the resistance region. A Fosmid library of H69J14 is now being generated to obtain larger (35kb inserts) to help resolve assembly problems based on the repetitive regions. Clones in the region from the Pinot noir genome possess four tandem repeats of serine threonine protein kinase with leonine rich repeat domains, genes that are involved in microbial recognition and plant defense reactions. Genetic mapping is also underway in three other populations to enable a better understanding of PD resistance and of *PdR1*: the 07744 (resistance from *V. arizonica* b40-14); the 04191 (resistance from F8909-17 *PdR1a*); and the 05347 (resistance from *V. arizonica/ girdiana* b42-26). A total of 152 markers were completed for 07744 to develop the framework map. Greenhouse screening of the 07744 population is complete. Preliminary results with the 07744 indicated that PD resistance (*PdR1c*) resides on chromosome 14, in the same region where *PdR1a* (resistance from F8909-17) and *PdR1b* (resistance from F8909-08) mapped from the b43-17 background. However, b40-14's SSR alleles for resistance are very different from those of b43-17. Genetic mapping of the quantitative resistance from *V. arizonica/girdiana* b42-26 continues in the 05347 (*V. vinifera* F2-35 x b42-26) population. About 70% of the population has been greenhouse screened, the remaining 75 seedlings will be tested early next year. b42-26 is surprisingly homozygous with the SSR markers we have available, which prompted the development of 71 new markers and the acquisition of about 200 others so that genetic mapping can be improved.

LAYPERSON SUMMARY
Genetic mapping from two different forms of *Vitis arizonica* have identified a region on chromosome 14 that is responsible for Pierce's disease (PD) resistance, which we termed *PdR1*. We have mapped two forms of *PdR1* from *V. arizonica / candicans* b43-17, and have mapped a third form, *PdR1c*, that originated from *V. arizonica* b40-14. These forms are both single dominant genes for PD resistance. We are also examining another source of resistance that is controlled by multiple genes that originated from *V. arizonica / girdiana* b42-26 and have begun the fine-scale mapping necessary to determine if markers are tightly enough linked to these multiple resistance genes to be used for marker-assisted selection. We plan to combine these multiple resistance sources in our breeding program to ensure broad and durable resistance to PD. Genetic markers to these forms of resistance will make this possible and allow the confirmation and tracking of interbred progeny. These mapping efforts are also essential to physically locating and characterizing PD resistance genes. At present, the chromosome region where *PdR1* exists has been sequenced and the pieces of sequence were arranged and compared to the Pinot noir genome sequence. This comparative analysis indicates that the susceptible Pinot region carries four genes. The Pinot noir region was compared to the sequences we have from the resistant b43-17 and we identified four candidate resistant genes. We are in the process of characterizing their function and determining which are likely to be involved in PD resistance.

INTRODUCTION
Genetic mapping to identify genomic regions that carry disease resistance genes can greatly facilitate breeding and lead to the map-based positional cloning of the resistant genes. In this project, we initiated mapping of Pierce's disease (PD) resistance in different forms of *V. arizonica* (Riaz et al. 2007). These efforts are closely coupled to a breeding program focused on developing PD resistant winegrapes (see companion report). The breeding program produces and greenhouse screens the seedling populations upon which the genetic mapping program depends. While the tightly linked genetic markers generated in these mapping efforts are used to optimize and greatly accelerate the PD breeding program (Riaz et al. 2009). These markers are essential to the successful introgression of resistance from multiple sources, and thus for the production of broader and more durably resistant grapevines (Riaz et al. 2008a). Genetic maps associate DNA markers with phenotypic

traits, and allow the linking of these traits with markers positioned relative to each other on chromosomes. Fine scale mapping of given regions and careful screening of recombinant progeny (those with a given genetic marker but without resistance, or vice versa, because of a recombination event) is critical to the identification of relatively short genetic regions that can then be sequenced so the genes responsible for PD resistance can be characterized and their function studied (Riaz et al. 2008b).

OBJECTIVES

1. Completely characterize and refine the PD resistance locus on chromosome 14 by genetically mapping in four populations that derive resistance from *V. arizonica/candicans* b43-17 and its *V. rupestris* x b43-17 progeny F89090-08 (*PdR1b*) and F8909-17 (*PdR1a*): 04190 (*V. vinifera* F2-7 x F8909-08), 9621 (D8909-15 x F8909-17), 04191 (F2-7 x F8909-17), and 04373 (*V. vinifera* F2-35 x *V. arizonica/candicans* b43-17).
2. Genetically map PD resistance from other forms of *V. arizonica*: b42-26 (*V. arizonica/girdiana*) and b40-14 (*V. arizonica*).
3. Develop a BAC (bacterial artificial chromosome) library for the homozygous resistant genotype b43-17 (parent of F8909-08, and F8909-17) and screen the library with closely linked markers.
4. Complete the physical mapping of *PdR1a* and *PdR1b* and initiate the sequencing of BAC clones that carry *PdR1a* gene candidates.

RESULTS AND DISCUSSION

Objective 1. We have refined the genetic position of the *PdR1a* and *PdR1b* resistance loci between marker VVCh14-56 and VVCh14-77 in the maps of two populations 9621 and 04190. These SSR markers have unique allele sizes for resistant parents that are not found in susceptible *vinifera* parents. This characteristic makes these markers very valuable for marker-assisted selection (MAS) in our ongoing wine grape-breeding program (see companion project report). The physical distance between these markers is 200-230 Kb, and they have been used to test additional plants and identify key recombinants critical for fine-scale mapping. Currently there are three key recombinants in the 9621 population from a tested set of more than 900 plants. F8909-08 possesses the *PdR1b* resistance locus, which is being mapped in the 04190 population. The greenhouse screen was repeated for key recombinants, which also helped to refine the data. In addition, marker analysis identified 14 more recombinants from 15 different crosses (totally about 1,000 plants) based on resistance from F8909-08. An additional 11 recombinants were found from the *PdR1a* source, and greenhouse screening was completed on the 35 recombinants. This screen identified four key recombinants. In two plants the recombination event occurred between *PdR1b* and VVCh14-02, and in one plant the recombination event occurred between *PdR1b* and VVCh14-70. The greenhouse screen is being repeated for four other recombinants that had inconclusive first test results. In the most recent map, we have placed the *PdR1b* locus between markers VVCh14-81 and VVCh14-77. Both of these markers are roughly 200Kb apart based on the Pinot noir genome sequence.

The 04191 population (*V. vinifera* F2-35 x F8909-17) has 153 progeny plants and has *PdR1a*-based resistance. This population allows *PdR1* to be examined without possible confounding effects from D8909-15, the other parent of the 9621 population, since D8909-15 has a multigenic resistance from b42-26. The resistance locus *PdR1a* is mapped in the 9621 (D8909-15 x F8909-17) population, and the 04190 population mentioned above, and refined mapping focused only on chromosome 14. The 04191 population is critical for the identification of any minor genes that might contribute to PD resistance. Therefore, we expanded the framework genetic map to all 19 chromosomes. A total of 143 SSR markers representing all 19 chromosomes were added to the set of 153 seedlings, of which 141 were greenhouse screened for resistance. The greenhouse screen results of seven of the plants did not match with the marker data. These plants are being rescreened with results expected later in Fall 2010. ELISA results from remaining the 134 plants matched with the marker results. A total of 75 seedlings have low bacterial titer values and carried resistant alleles with the tightly linked flanking markers and fifty-nine seedlings had high bacterial numbers and they inherited alleles linked to the susceptibility to PD, which confirmed a single dominant gene 1:1 segregation. We are in the process of evaluating the greenhouse results, genetic mapping with 138 markers and QTL(Qualitative Trait Loci) analysis.

Objective 2. In response to recommendations from the CDFA-PD board and reviewer recommendations to broaden resistance, we are characterizing resistance from two additional sources and making good progress. The main purpose is to identify additional resistance sources, genetically map them and use tightly linked molecular markers to pyramid resistance from different backgrounds into single line. We are pursuing two other resistant *V. arizonica* forms: b42-26 *V. arizonica/girdiana* from Loreto, Baja California; and b40-14 *V. arizonica* from Chihuahua, Mexico. Although they are morphologically different than b43-17, they both posses strong resistance to PD and greatly suppress *Xylella. fastidiosa* levels in stem tissue after greenhouse screening.

Greenhouse screening data indicate that resistance from b42-26 is quantitative. A small breeding F1 population 05347 (*V. vinifera* F2-35 x b42-26) was produced in 2005, and a subset of 48 genotypes was greenhouse screened and found 35 were resistant and 13 were susceptible. A total of 337 markers were tested on small a parental data set. Results found a high level of homozygosity for b42-26 (only 113 markers were polymorphic); 184 markers were homozygous for the male parent b42-26, and 40 markers did not amplify. In the Spring of 2008 and 2009, crosses were made to increase the population size.

Currently, 239 seedlings exist in the field and greenhouse screening is complete on 63 seedlings, underway on 111, and the final set of 75 will be screened in 2011. In addition to increasing the population size, we also need more markers that are polymorphic for b42-26, a very homozygous selection. We developed 71one new SSR markers from clone sequences generated from the Vitis Microsatellite Consortium. These clones had microsatellite repeats in the beginning or end of the sequence, which left no room for primer design. A total of 238 of these clone sequences were compared to the nearly homozygous 12X Pinot noir genome in order to obtain additional flanking sequences. There were good matches for 71 of the clones and primers were designed for them; 69 of the newly designed primers amplified and 67 of them generated a clean banding pattern with *V. vinifera* DNA samples (results form this study were submitted as a research note in AJEV). We also acquired primer sequences of an additional 200 markers that have not been tested with b42-26. Marker testing on small set of parents and progeny is underway. We are now adding markers to develop a framework map of the entire population set; 50 markers have been completed and more are being added.

Resistance in *V. arizonica* b40-14 seems to be homozygous and is controlled by a single dominant gene. We mentioned in previous reports that all F1 progeny from a cross of *V. rupestris* x b40-14 (the R series) were resistant to PD except three genotypes with intermediate results. Two resistant siblings of this population were used to develop the 07388 (R8918-02 x *V. vinifera*) and 07744 (R8918-05 x *V. vinifera*) populations. A summary of the genetic mapping and QTL analysis is presented below: 227 markers were polymorphic for one of the parents; 152 were analyzed on the entire set of 122 plants; a framework map of R8918-05 was produced with MAP QTL (4.0) and the Kruskal-Wallis approach was used to complete the preliminary analysis. PD resistance mapped only on chromosome 14 – the same chromosome where *PdR1a* and *PdR1b* mapped. PD resistance from b40-14 (which we have named *PdR1c*) also maps in the same general region between flanking markers VVCh14-77 and VVIN64 and within 1.5 cM. The LOD threshold for the presence of this QTL was 33 and 82% of the phenotypic variation was explained (**Figure 1**). In 2009, crosses were made with F1 resistant selections from 07744 population.

Objective 3 and 4. Two BAC libraries were created from the homozygous resistant b43-17. Screening of the library with markers VVCh14-10, VVCh14-56 and VVCh-58 identified 41 positive clones– four of the clones were positive with VVCh14-10, VVCh14-56 markers (H23-P13, H34-B5 and H64-M16 and H45-J22) and five of them were positive with the VVCh14-56 and VVCh14-58 marker (**Figure 1a**). Two clones were selected for sequencing with an overlap of 60Kb and a spanned region of 340Kb. A shotgun library of BAC clone H64M16 was Sanger sequenced. Clone H69J14 was selected for 454 sequencing. A total of 42,000 sequences were generated and two different programs were used to assemble the sequence. However, the sequenced region was highly enriched with repetitive elements, which complicated the assembly. Newbler software as well as Lasergene program SEQMAN do not work well with sequences containing many repeated regions. In order to generate longer sequence fragments, a shot gun library was constructed for clone H69J14; 384 sequences were generated in both directions to develop paired ends in order to fill the gaps between the contigs from the 454 sequence data. We then masked the repetitive region from all the sequences (both H69J14 and H64M16 clones) to carry on the assembly with MIRA assembler program. This improved the assembly, but the contig number was still very high and not suitable for primer walking. Moreover, all the major contigs had masked repetitive regions on both ends indicating that the primer design effort would not generate sequence specific results capable of bridging the gaps. We are now in the process of developing a Fosmid library with an insert size of 35-40Kb, and the resulting 384 sequences in both directions will allow us to tag smaller contigs from the 454 and shotgun reads data. Because the fosmid clones are 35Kb inserts, it will help resolve assembly problems based on the repetitive regions.

Recently, the 12X assembly of Pinot noir (PN400204) sequence became available. It is an improved version of the 8X assembly we used previously. Detailed analyses identified four tandem repeats of serine threonine protein kinase with a leucine-rich repeat domain gene family in the resistance region (**Table 1**). All four genes have large introns indicating that they may carry transposable element like sequences. We utilized CENSOR software to screen query sequences against a reference collection of repeats to generate a report classifying all detected repeats (**Table 1**). All four genes carry DNA transposons as well as LTR(Long Terminal Repeat) retrotransposons documenting the complexity of the region. In this situation, a direct comparison of the H69J14 clone sequence to the PN40024 sequence is not advisable because it is possible that the arrangement of repetitive elements is significantly different between the resistance region of the two genomes. A comparison of a larger sequence of the region, without the resistance genes, shows up to 98% homology with the susceptible PN40024. However, the b43-17 resistance region sequences that overlap with scaffold 68 of Pinot noir matched to multiple sites and the level of similarity was reduced. These results suggest that the b43-17 genomic region with the PD resistance gene(s) is divergent from PN40024 and that transposable elements may play a major role in these sequence differences. Our main emphasis is assembling the complete region in order to make comparisons to the susceptible sequence. This will help us understand the causes of sequence divergence and the evolution of the PD resistant gene family. Previous studies have indicated that the sequence of chromosome 14 is known to carry members of 13 different families of DNA transposons and retrotransposon (Moisy et al. 2008). We identified four potential candidate genes from the partial assembly of the H69J14 clone sequence (**Table 2**). Three of the candidate genes are 94 to 98% identical to each other and they are also 50 to 70 % identical to four PN40024 genes (**Figures 2 and 3**). We have initiated cloning work with candidate gene C4000-1. In later stages, as the fosmid sequence data becomes available, more detailed analysis will be carried out.

CONCLUSIONS:

Genetic mapping efforts have identified valuable genetic markers for marker-assisted selection and enabled rapid progress towards PD resistant winegrapes (see companion report). These mapping efforts have now identified three alleles of *PdR1*: *PdR1a* and *PdR1b* derived from *V. arizonica / candi*cans b43-17; and *PdR1c* derived from *V. arizonica* b40-14. These alleles were found to map within the same general region, but suggest that although PdR1 seems to be a single gene trait, the region may be composed of a number of tightly linked genes. BAC library sequence analysis of b43-17 is resulting in candidate genes suggestions for *PdR1* and these are being compared to the PN40024 genome sequence and to similar regions in other plants. The genomic characterization of this region will help us determine how this form of PD resistance functions and which genes control it. Genetic mapping of the multigenic source of resistance, b42-26, is progressing and tightly linked markers will greatly expedite the interbreeding of this resistance with that from b43-17 to increase the durability of PD resistance.

Table 1. Details for the transposable elements, sizes of exons and introns in four genes that are present in the Pinot noir (PN40024) 12X genome sequence in the region correlated to the *PdR1* region of b43-17. The analysis of the sequence was carried out with Censor program, which compares the sequence to known repeat regions from *Vitis*, Maize, Wheat and Pine.

Gene ID (12X genome of PN40024)	Gene size (bp)	No. of exons and introns	Size without introns (bp)	Repeat class categories	Fragments	Length
GSVIVT01033116001	7,729	6, 5	2,496	DNA transposon	1	294
GSVIVT01001802001	33,894	10, 9	3,360	Transposable Element	31	13969
				DNA transposon	18	2747
				EnSpm	3	485
				Harbinger	3	305
				Helitron	3	474
				MuDR	5	640
				hAT	1	408
				LTR Retrotransposon	12	11164
				Copia	3	406
				Gypsy	8	10613
				Non-LTR Retrotransposon		
				L1	1	58
GSVIVT01001803001	11,310	10, 9	3,309	Transposable Element	8	2453
				DNA transposon	7	2182
				Harbinger	1	377
				MuDR	4	1121
				hAT	2	684
				LTR Retrotransposon	1	271
				Gypsy	1	271
GSVIVT01001804001	12,165	11, 10	2,691	Transposable Element	9	2778
				DNA transposon	8	2520
				Harbinger	1	383
				MuDR	5	1450
				hAT	2	687
				LTR Retrotransposon	1	258
				Gypsy	1	258

Table 2. Information for four candidate genes from a partial assembly of the resistance region of b43-17.

Contig ID	size (Kb)	current gene ID	Size (Kb)
Contig 4000	47.3	Contig 4000-1	3.07
Contig 4002	25.1	Contig 4002-1	2.12
Contig 3995	7.34	Contig 3995-1	2.97
Contig 3974	22.2	Contig 3974-1	2.94

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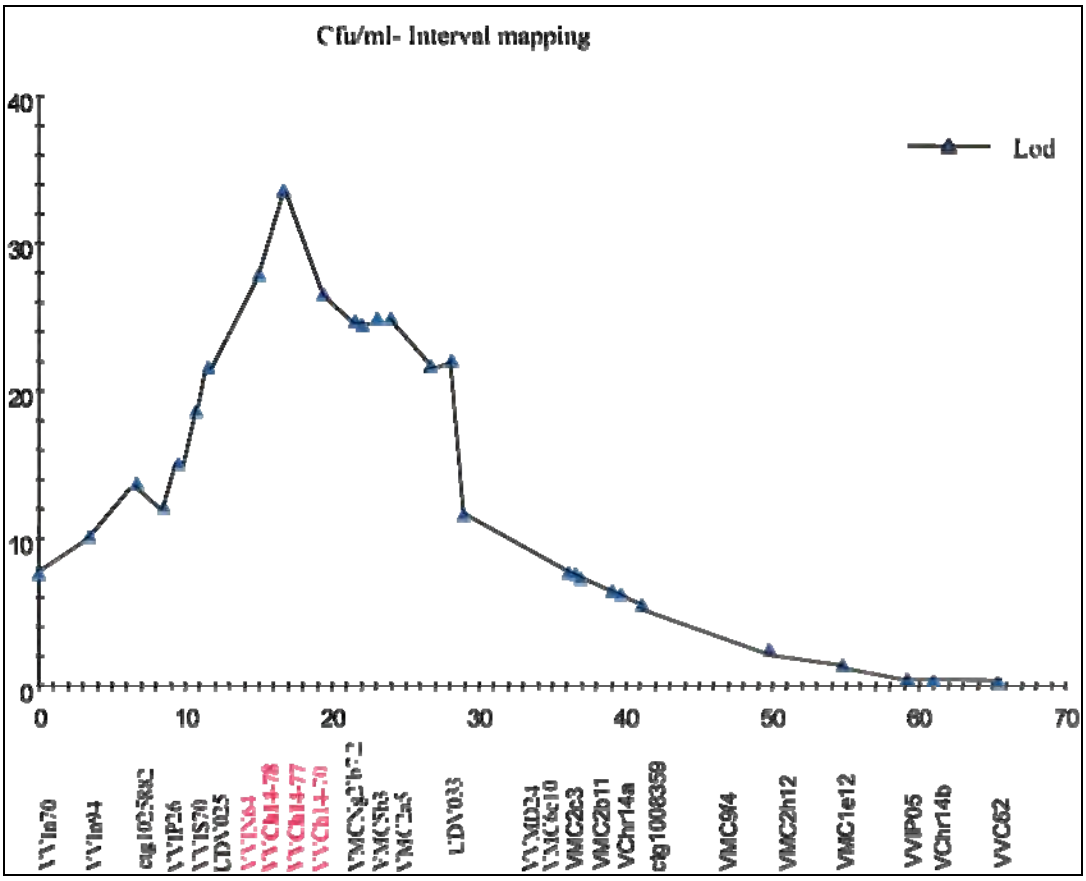

Figure 1. Interval mapping of *PdR1* indicating a peak at LDD 34.0 with the 95% confidence interval. The X-axis indicates the position of the markers; LOD values are plotted on the Y-axis.

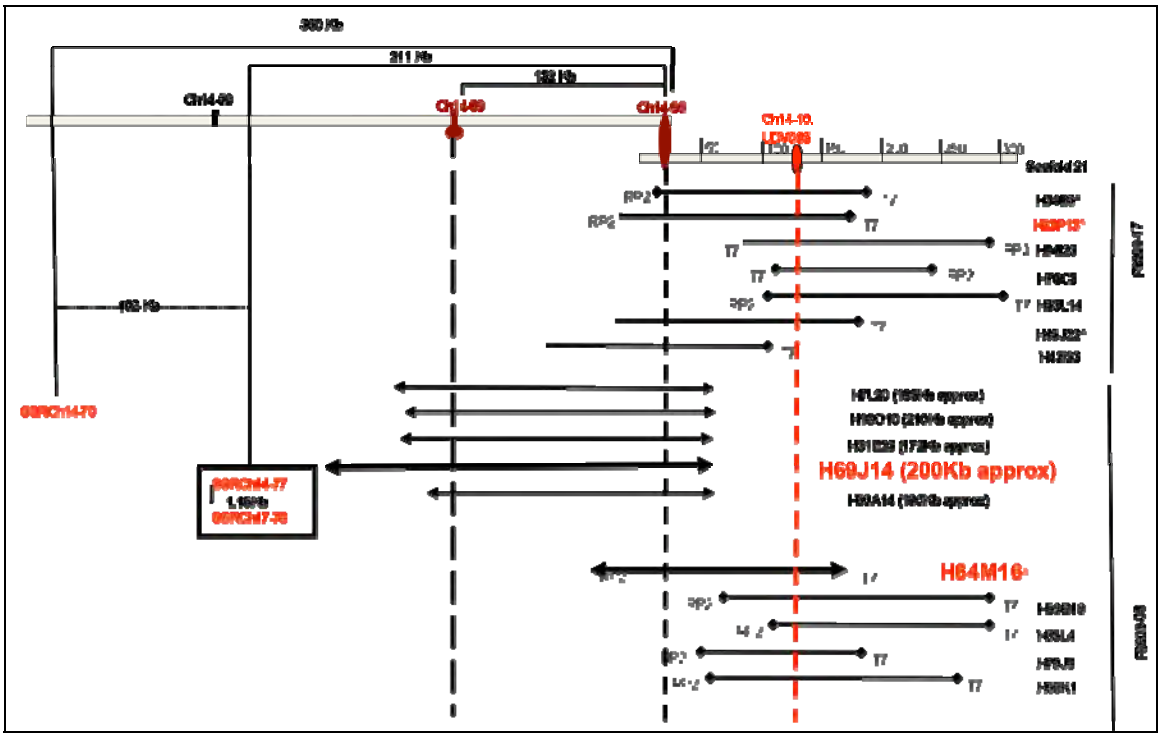

Figure 2. The arrangement of BAC clones and relative positions in comparison to the Pinot noir (PN40024) sequence. Three markers in red were used to screen the BAC library. H69J14 clone has been sequenced via 454 and sanger shotgun method. H64M16 clone has been sequenced by sanger method only. Both clones overlap for 60Kb approximately. These two clones represent the haplotype *PdR1b* region of b43-17.

Percent Identity

	1	2	3	4	5	6	7	8		
1		38.6	94.9	95.3	55.1	54.2	65.2	77.8	1	contig 4000-1.seq
2	131.9		39.3	39.3	36.9	35.8	35.4	37.4	2	contig4002-1.seq
3	5.2	128.1		98.7	57.5	56.2	66.8	78.9	3	contig3995-1.seq
4	4.9	128.1	1.1		57.9	56.5	67.3	79.2	4	contig3974-1.seq
5	69.3	143.3	63.2	62.6		66.9	39.9	60.6	5	PN-2001.seq
6	71.3	149.3	66.1	65.5	44.3		39.8	56.3	6	PN-3001.seq
7	47.0	152.9	43.9	43.1	126.0	124.3		51.3	7	PN-4001.seq
8	26.4	139.7	24.6	24.5	56.8	66.3	80.1		8	PN-6001.seq
	1	2	3	4	5	6	7	8		

Figure 3. The sequence comparisons of genes identified from the Pinot noir (PN40024) and from the partial assembly of the BAC clone of the resistant genotype b43-17

MOLECULAR CHARACTERIZATION OF THE PUTATIVE *XYLELLA FASTIDIOSA* RESISTANCE GENE(S) FROM B43-17 (*VITIS ARIZONICA / CANDICANS*)

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ABSTRACT
Significant advances in the classical and molecular breeding of Pierce's disease (PD) resistance have been made by exploiting resistance from North American *Vitis* species. The resistant species, *Vitis arizonica/candicans* b43-17, was used to study the inheritance of resistance to *Xylella fastidiosa (Xf)*, generate mapping populations and to position loci on genetic maps linked to this homozygous dominant resistance gene termed *PdR1*. BAC clones were identified with markers linked to the PD resistance locus, and they were separated into *PdR1a* and *PdR1b* categories using a polymorphic marker for b43-17 and sequencing was completed for a clone carrying the *PdR1b* locus. Preliminary data analysis found that the resistance region is enriched with repetitive transposable elements, making the sequence assembly process very challenging. From the partial assembly of the region, we identified four tandem repeats of Serine Threonine Protein Kinase with a Leucine-rich Repeat domain gene family in the resistance region. The availability of the 12X genome assembly of Pinot noir (PN40024) provided a sequence without any gaps and allowed us to make comparisons. The 12X assembly of the PD susceptible PN40024 also carried four tandem repeats of the Serine-Threonine Protein Kinase gene family and all of them carried introns. We initiated cloning of one candidate gene *PdR1b.1* for further sequence verification and to develop constructs for use in complementation experiments. We also initiated cultures of embryogenic callus of two *V. vinifera* cultivars, Chardonnay and Thompson Seedless, and one rootstock, St. George. These cultures are now embryogenic and will be used for transformation/complementation studies with *PdR1* gene candidates. To reduce the time span for generating healthy transgenic plants we also tested two different methods that employ organogenesis for *Agrobacterium*-mediated transformation. We were successful in streamlining one method that will allow us to cut down the time period needed to generate transformed plants to four to six months.

LAYPERSON SUMMARY
Our companion project "Map-based identification and positional cloning of *Xylella fastidiosa* resistance genes from known sources of Pierce's disease (PD) resistance in grapes" has identified four candidate genes coming from *V. arizonica / candicans* b43-17 that may be responsible for PD resistance. The next step in the process is to test these candidate genes by transforming them into a PD susceptible grapevine to see if one or more of the gene candidates are responsible for resistance. To do this we have to more completely sequence the PD resistance region (*PdR1*) since it contains complicating genetic factors called transposable elements. We have started this "clean-up" process. We have also developed callus tissue that is capable of developing into new plants (embryogenic) from flower tissue of Chardonnay, Thompson Seedless and St. George. The gene candidates can be inserted into these embryogenic callus tissues and if these genes are responsible for resistance the plantlets regenerated from these tissues will be PD resistant. Development of embyrogenic callus is difficult and slow and this spurred the development of an alterative technique based on meristem tissue from shoots. We now have embryogenic tissue developed from this meristematic tissue that will allow the *PdR1* gene candidates to be tested in a wider range of winegrapes and more rapidly.

INTRODUCTION
New cultivars bred to resist *Xylella fastidiosa (Xf)* infection and subsequent expression of PD symptoms will provide long-term sustainable control of PD. Disease resistant cultivars can be obtained by conventional breeding through the introgression of resistance from Native American species into elite *vinifera* wine and table grapes. Another approach is "cisgenesis" – the transformation of elite *V. vinifera* varieties with grape resistance genes with their native promoters, cloned from disease resistant American *Vitis* species. The cisgenic approach may have a more limited impact on the genome of the elite *V. vinifera* parent since very limited amounts of the *Vitis* species genome would be added to the elite parent, thus limiting the impact on its fruit and wine quality while making it PD resistant. The linkage-drag-free cisgenic approach is attractive, and also allows the opportunity to stack additional resistance genes from other *Vitis* sources, even if these genes originate from the same chromosomal position in different species or accessions (Jacobsen and Hutten 2006). We have been breeding PD resistant wine grapes, and it has been possible for us to maintain and characterize genetic populations that were by-products of the breeding program. These populations have allowed: the construction of genetic maps; identification of genomic regions associated with PD resistance and other traits of interest; the selection of markers that are tightly linked to PD resistance to greatly expedite breeding through marker-assisted selection (MAS); and the use of genetic maps to lay the foundation for the construction of physical maps to identify resistance genes (Riaz et al. 2008; Riaz et al. 2009). The physical map of the resistance region from *V. arizonica/candicans* b43-17, *PdR1*, allowed the identification of potential

candidate resistance gene(s). Preliminary comparison indicated that the *PdR1* region contains multiple tandem repeats of Serine Threonine Protein Kinase with a LRR domain (STPK-LRR) gene family. This category of genes belongs to a group involved in plant resistance. Their defense mechanism is based on compounds involved the recognition of microbe-associated molecular patterns (MAMP) like compounds, which lead to the initiation of a defense response (Bent and Mackey 2007). In order to gain insight and to verify the function of resistance gene(s), cloning and functional characterization is required. In this report, we present the cloning progress of one of the candidate resistance genes, *PdR1b.1*.

OBJECTIVES

1. Cloning, structural analysis and gene annotation via comparison of the *PdR1b* locus to the susceptible Pinot noir genome sequence using the assembled sequence of the BAC clone H64J14.
2. Expression studies of candidate genes.
3. Development of alternative protocols for genetic transformation for the validation of gene constructs
   a) *Agrobacterium*-meditation transformation of the susceptible *Vitis* cultivars (Chardonnay and Thompson Seedless, and the rootstock St. George).
   b) Transformation of tobacco.

RESULTS AND DISCUSSION

Objective 1. Cloning, structural analysis and gene annotation via comparison of the *PdR1b* locus to the susceptible Pinot noir genome sequence using the assembled sequence of the BAC clone H64J14: The preliminary assembly of the BAC clone sequence generated 8-10 contigs of significant size, but a large portion of the sequence remained unassembled. Further detailed analysis of the assembled, as well as the unassembled, sequences revealed the presence of a high number of transposable elements (TE). In fact chromosome 14 is the second largest carrier of transposable elements in the sequenced Pinot noir genome (Moisy et al. 2008). Transposable elements play key role in the diversification of disease resistance genes by allowing rapid adaptive change due to their ability to insert into regions of the genome and alter gene function; so called TE-induced gene alteration (Michelmore 1995). Considering the complexity of the *PdR1b* region, we are in the process of developing a fosmid library that would help overcome the problem of many short fragment sizes resulting from the 454 and Sanger shot gun sequencing reads. The fosmid library should produce much longer fragments and work towards a complete assembly of the region (see the companion report "Map-based identification and positional cloning of *Xf* resistance genes from known sources of PD resistance in grapes" for additional information). Meanwhile, we identified four candidate genes from the assembled contigs and have initiated cloning work with one of the candidate genes (*PdR1b.1*), which is 3.1 Kb in size.

Two pairs of primers were designed to clone the first candidate gene into a pCR4-Topo vector. The first set of primers was designed using the sequence builder program, and the second set utilized the Vector NTI program.

PD1-1F TTCTCTTTCATCCGTGAATGTAG
PD1-1R AAAAAATTCYTGGAGAGATGCT

PD1-2F GTAGGCATGATTGGGCCA
PD1-2R AAAATTCYTGGAGAGAGATGCTTATTTT

The PCR reaction was done using AccuPrime Taq Polymerase, which has improved fidelity (PCR Selection Kit- High Specificity, Invitrogen). The PCR reaction was performed at 60ºC annealing temperature and only the second set of primers amplified a fragment the size of the gene. The product of the PCR reaction was inserted into a pCR4-Topo vector. The vector was used to transform chemically competent cells of E. coli DHά5. In next step, we will sequence the DNA to verify it and the construction of binary vector will be initiated.

Objective 2. Expression studies of candidate genes by nested RT-PCR: To conduct the expression studies of the candidate genes, hardwood cuttings will be collected in November to generate plants. The stem and leaf tissue of these plants will be used for the total RNA from both resistant (b43-17, F8909-08) and susceptible genotypes (A de Serres, Chardonnay) using a cetyltrimethylammonium bromide (CTAB)-based RNA extraction protocol as described by Iandolino et al. (2004) with minor modifications. Results of this experiment will be available in the next report.

Objective 3. Development of alternative protocols for genetic transformation for the validation of gene constructs and in-vivo complementation of the candidate genes. Currently the most commonly used method for the production of transgenic or cisgenic grapes is based on *Agrobacterium* transformation followed by regeneration of plants from the embryogenic callus. We have established cultures of pre-embryogenic callus derived from anthers of *V. vinifera* 'Thompson Seedless' and 'Chardonnay' and the *V. rupestris* rootstock 'St. George'. These cultures of embryogenic calli have been used for transformation (Aguero et al, 2006).

In addition to embryogenic callus, we are testing two additional transformation methods. The first one is based on development of transgenic plants from shoot apical meristems via *Agrobacterium*-mediated transformation (Dutt et al. 2007). The ease of producing and maintaining in vitro micro-propagation cultures from a large number of cultivars makes shoot tip

based transformation a very effective system. The second method employs genetic transformation of *V. vinifera* via organogenesis (Mezzetti et al 2002). This method utilizes shoot apical meristem slices prepared from the entire meristematic bulk for *Agrobacterium*-mediated transformation of grape plants with the gene *DeH9-iaaM*. With this procedure, they were able to generate transgenic plants in much shorter time interval. We have streamlined this procedure and have already obtained transgenic shoots using Mezzetti's method in three months expressing GFP (**Figure 1**). So we expect that the time required for transformation will be shortened to approximately six months instead of one year via embryogenic callus. In later steps, we will use transformation with green fluorescent protein (GFP) to test the uniformity and effectiveness of this procedure as well as its utility with a range of cultivars; currently it is only being used with Thompson Seedless.

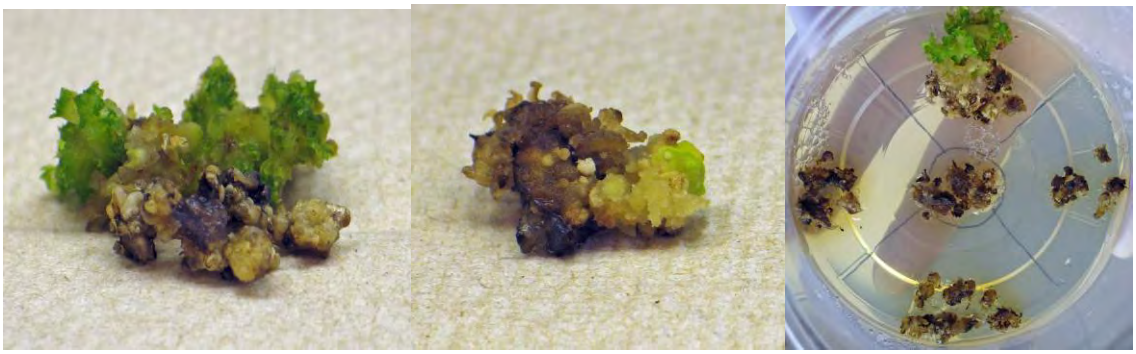

Figure 1. Regeneration and selection of meristematic transformed tissue with GFP protein (the green tissue is transformed).

CONCLUSIONS

The last step in the characterization of a resistance gene is to verify that the isolated gene functions in a host plant. This process requires that the gene be transformed into susceptible host and challenged by the disease agent. *Agrobacterium*-based transformation can be used with grape but initiating transformable and regenerable tissue is often a problem with grape. We have obtained regenerating callus of Chardonnay, Thompson Seedless and St. George for use in testing the four *PdR1* region gene candidates. We have also utilized another technique to speed the development of embryogenic tissue in this case from meristems that will allow *PdR1* gene candidates to be tested in a much broader range of genotypes. If *PdR1* gene candidates function they could be transformed into a wider range of winegrapes with this technique.

The classical methods of gene introgression have the disadvantage of potential linkage drag (inclusion of unselected genes associated with a trait) and the time required for time-consuming backcrosses and simultaneous selection steps. Cisgene micro-translocation is a single-step gene transfer without linkage drag; as well as a possible means of stacking resistance genes in existing winegrape varieties.

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GENETIC TRANSFORMATION OF *VITIS* SPP. AS A TOOL FOR THE DEVELOPMENT AND EVALUATION OF NEW TECHNICAL PLATFORMS

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ABSTRACT
Since 2002 a grapevine genetic transformation program has been carried out at La Platina Research Station of the National Agriculture Institute (INIA, Santiago de Chile) by development of a high throughput *Agrobacterium tumefaciens*-mediated transformation system using 'Thompson Seedless' somatic embryos (Reyes *et al*., 2005). Formerly focused on *Botrytis* control, the more advanced lines from this program were developed using the *chi*42 and *nag*70 genes from *Trichoderma harzianum* P1, and the *chi*33 gene from a local isolate of *T. virens*. About 3,000 candidate transgenic lines have been successfully established at greenhouse level (Hinrichsen *et al*., 2005), from which 103 lines represented by 568 GM plants were released into a biosafety field in September 2004. Results allowed the generation of a fungus tolerant population (20 top lines) based on a discriminant multivariate analysis of *B. cinerea* and *E. necator* tolerances after three seasons of assays. Scaling up of the somatic embryogenesis (SE) technology was conducted by design of a bioreactor assisted SE platform (Tapia *et al*., 2009), useful in the genetic transformation work flow of rootstock germplasms. Generation of Grapevine Fanleaf Virus resistant lines have been then started together to different approaches evaluating gene silencing in grapevines and the use of ds hairpin inducing DNA strategy. Several GM rootstock lines have been already generated and current evaluation procedures include micro-grafting assays on a population of GFLV infected Saint George plants. Lately, additional gene silencing studies are being developed as a proof of concept using undescribed *Vitis vinifera* inducible promoters conducting the expression of artificial microRNA based vectors in these species. Results and strategies will be shown in order to analyze and discuss the generation of new knowledge for grape biotechnology by networking with different research groups.

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DEVELOPMENT OF *ARABIDOPSIS THALIANA* AS A MODEL HOST FOR *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted September 2009 to September 2010.

ABSTRACT
The bacterium *Xylella fastidiosa* (*Xf*) causes Pierce's disease and a number of other plant diseases of significant economic impact. To date, progress determining mechanisms of host plant susceptibility, tolerance or resistance has been slow, due in large part to the long generation time and limited available genetic resources for grape and other known hosts of *Xf*. To overcome many of these limitations, *Arabidopsis thaliana* has been evaluated as a host for *Xf*. A pin-prick inoculation method has been developed to infect Arabidopsis with *Xf*. Following infection, *Xf* multiplies robustly and can be detected by microscopy, PCR and isolation. The ecotypes Van-0, LL-0 and Tsu-1 all allow more growth of *Xf* strain Temecula than the reference ecotype Col-0. Affymetrix ATH1 microarray analysis of inoculated vs. non-inoculated Tsu-1 reveals gene expression changes that differ greatly from changes seen after infection with apoplast colonizing bacteria. Many genes responsive to abiotic stress are differentially regulated while classic pathogenesis-related (PR) genes are not induced by *Xf* infection.

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Section 6: Economics

THE BENEFITS AND COSTS OF ALTERNATIVE POLICIES FOR THE MANAGEMENT OF PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted October 2008 to October 2010.

ABSTRACT
We propose to address economic questions related to Pierce's disease (PD) by developing and applying a quantitative model of the supply and demand for California wine and wine grapes. The economic component of this model will combine existing wine market models with some entirely new elements. The biophysical component will draw on a range of technical information and, where appropriate, evidence from crop and disease simulation modeling. To begin, we have consulted with scientists the study the disease and its vectors. Drawing on their advice, combined with information gleaned from interviews with vineyard managers, we have modeled the problem of the Blue-Green Sharpshooter (BGSS) in Northern California. By doing so, we have gained a better understanding of how to model the statewide Pierce's Disease problem. Additionally, we have begun to study the role of the Glassy-Winged Sharpshooter (GWSS) and the problems it poses in southern regions of the state. The end result will be a model that is designed specifically to evaluate the likely expected benefits from investments in alternative R&D projects related to the management of PD.

LAYPERSON SUMMARY
In the first two years of the project we have concentrated on gathering data and other information and learning about PD and the sharpshooters that spread it, creating a model of the economic impacts of the disease in the Napa Valley, and working to extend that model so that it can reflect the situation across the state. Our progress has led us to revise some aspects of the research strategy, but the work has gone generally according to plan, albeit after a delayed start. In the coming months we will work to extend that model so that it better incorporates the role of PD/GWSS in southern California and can be used to evaluate the benefits from investments in alternative R&D projects.

INTRODUCTION
It is widely accepted that Pierce's disease (PD) with its vectors, including the glassy-winged sharpshooter (GWSS, *Homalodisca coagulata*), has large current and very large potential economic consequences. However, we are not aware of other studies that have modeled and measured the economic consequences. Siebert (2001) discussed the economics and he estimated that the value of lost wine grape production—in Temecula, Riverside County, alone, in 1988 and 1989—was worth $37.9 million to California. Echoing that sense of economic importance, the National Academy of Science (2004) undertook an extensive study and published a book on California research priorities focused on PD. That book does not contain estimates of the economic consequences of PD or alternative management or control methods, but it does provide a comprehensive documentation of knowledge about the problem, as well as a useful classification of types of research and priorities for them, including economic research. Further work is needed to develop a quantitative economic understanding of PD and alternative policies to address it.

OBJECTIVES
The overall objective of this project is to develop a detailed, practical, quantitative understanding of the economic consequences of PD and alternative management strategies. More specific objectives are to quantify the current and potential economic impact of the disease, to estimate the potential economic payoff to investments in PD R&D, to evaluate alternative management strategies including alternative research investments, and to guide policy decisions, including research priorities.

Additionally, we aim to study regional differences across California in PD and its associated vectors, allowing for a better understanding of the problem and a more precise evaluation of alternative management regimes. To pursue these objectives, we propose to develop an economic model of the California wine and wine-grape sector. The model will be structured to allow us to simulate market outcomes under alternative scenarios for the prevalence of PD, and alternative technologies and policies for its management, and to assess the economic consequences of these alternatives for various stakeholder groups. The model will be designed specifically with a view to using it to evaluate the likely expected benefits from investments in alternative R&D projects related to the management of PD.

RESULTS AND DISCUSSION

Our project commenced formally on September 1, 2008. Kate Fuller has been employed as a Graduate Student Researcher to work half-time on the project. In the work to date we have emphasized investment in developing our own knowledge and information resources and creating preliminary models. One important element of this is to develop a detailed data base on the economics of wine and wine grape production in California. We have completed the data-gathering phase, and we are compiling the information into a report documenting by county and crush district for each important grape variety the area planted, yield, quantity produced (crush volume), and price over the past 30 years. We have also made some investment in learning about how to structure and use models of spatial-dynamic processes such as the spread of disease, and made significant progress in developing an understanding of the pest and disease problem, and an overview of the issues, through consulting with scientists and others and reviewing literature. Having learned that the PD/GWSS problem will be difficult to model, we opted to focus initially on studying the issues as they arise in the north coastal valleys where PD is spread by native sharpshooters (in particular, the BGSS). To guide our efforts to understand the issue in that area, we conducted interviews with vineyard managers there, utilizing a process known as "participatory mapping," in which managers were asked to sketch out PD incidence, controls, and associated costs onto aerial images of their vineyards. This approach has enabled us to develop some economic data and insight into the problem, management strategies, and costs of prevention, control, and eradication strategies, which will help us in designing approaches to study the more general problem, including the role of the GWSS. We will continue this interview strategy in the Temecula area to gain insight into the issue there.

Based on this work, Kate Fuller has written two research essays (as required by the Ph.D. program in Agricultural and Resource Economics at UC Davis). These essays entailed a review of relevant literature as well as the development of the framework for a general economic model of vector-borne disease as applicable to PD. As well as providing a useful reference document for our project, they formed the basis for Kate's oral qualifying examination and prospectus. Kate's dissertation research plan, to be conducted over the next year, entails elements related to the main objectives of the project. It begins with work on the BGSS in Northern California as a basis for work on the GWSS, ultimately providing a basis for evaluating payoffs to research.

As described above, we have been developing data and information but do not have any specific accomplishments to report beyond making progress as planned towards achieving the specified objectives for the first-year.

CONCLUSIONS

None to date.

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THE ECONOMICS OF PIERCE'S DISEASE IN CALIFORNIA

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ABSTRACT

The first goal of this research project is to estimate the medium to long-run economic impact to growers and consumers of California's grape and orange industries, and to taxpayers from the establishment of the glassy-winged sharpshooter (GWSS) in California. The public control program to date has managed to contain the GWSS in the southern part of the State. As part of the public program citrus growers are reimbursed their expenses for winter control of GWSS and a quarantine that regulates the movement of fresh citrus, fresh grapes, and nursery host crops to areas currently free of GWSS. In the southern San Joaquin County due to effective winter GWSS control in citrus, the cost for grape growers to treat GWSS using one soil application of imidacloprid a year ($50-$60 per acre) is offset by reductions in the use of other insecticides such as the foliar applications of imidacloprid and treatments for pests such as the grapeleaf skeletonizer. In the Temecula Valley; however, GWSS becomes active earlier, orchards and vineyards are generally smaller, and the orange/grape land interface is more complex causing growers in this area to incur additional costs. In addition to a soil application of imidacloprid, vineyards in the Temecula valley may also need an additional irrigation at $12.50 an acre at the time of the imidacloprid application, plus two additional spray treatments with Danitol at $35.50 an acre. Total costs for GWSS control in the Temecula Valley is $98 to $108 an acre. About $50-$60 of that cost is also offset by reductions in the use of insecticides needed to treat pests that are now controlled with the soil application of imidacloprid. The net increase in costs is about $48 an acre per year. The incidence of Pierce's disease (PD) in the Temecula Valley is also higher than before GWSS became established. Growers are replanting between 2% and 3% of vines every year due to PD for an additional cost to growers of $65 an acre. The total increase in per acre costs to control GWSS and PD is $103 a year. The average number of acres cultivated in grapes from 2005 to 2007 in the Temecula Valley is about 1,300, making the total estimated annual losses to growers in the Temecula Valley about $133,900 a year. These losses could increase substantially if the public control program were discontinued as winter GWSS treatment in citrus would cease and, without a quarantine, GWSS would spread. Costs for grape growers would increase throughout grape growing regions due to higher control costs where GWSS is currently established, and the need to implement control measures in areas currently free of GWSS.

LAYPERSON SUMMARY

The first goal of this research project is to estimate the medium to long-run economic impact to growers and consumers of California's grape and orange industries, and to taxpayers from the establishment of the GWSS in California. The public control program to date has managed to contain the GWSS in the southern part of the State. As part of the public program citrus growers are reimbursed their expenses for winter control of GWSS and a quarantine that regulates the movement of fresh citrus, fresh grapes, and nursery host crops to areas currently free of GWSS. In the southern San Joaquin County due to effective winter GWSS control in citrus, the cost for grape growers to treat GWSS using one soil application of imidacloprid a year ($50-$60 per acre) is offset by reductions in the use of other insecticides such as the foliar applications of imidacloprid and treatments for pests such as the grapeleaf skeletonizer. In the Temecula Valley; however, GWSS becomes active earlier, orchards and vineyards are generally smaller, and the orange/grape land interface is more complex causing growers in this area to incur additional costs. In addition to a soil application of imidacloprid, vineyards in the Temecula valley may also need an additional irrigation at $12.50 an acre at the time of the imidacloprid application, plus two additional spray treatments with Danitol at $35.50 an acre. Total costs for GWSS control in the Temecula Valley is $98 to $108 an acre. About $50-$60 of that cost is also offset by reductions in the use of insecticides needed to treat pests that are now controlled with the soil application of imidacloprid. The net increase in costs is about $48 an acre per year. The incidence of PD in the Temecula Valley is also higher than before GWSS became established. Growers are replanting between 2% and 3% of vines every year due to PD for an additional cost to growers of $65 an acre. The total increase in per acre costs to control GWSS and PD is $103 a year. The average number of acres cultivated in grapes from 2005 to 2007 in the Temecula Valley is about 1,300, making the total estimated annual losses to growers in the Temecula Valley about $133,900 a year. These losses could increase substantially if the public control program were discontinued as winter GWSS treatment in citrus would cease and, without a quarantine, GWSS would spread. Costs for grape growers would increase throughout grape growing regions due to higher control costs where GWSS is currently established, and the need to implement control measures in areas currently free of GWSS.

INTRODUCTION

In 1989 a pest new to California, the glassy-winged sharpshooter (GWSS), was collected in Irvine, CA. Since then it has spread throughout southern California, into the southern San Joaquin Valley including Kern County and parts of southern Tulare County, and along the coastal counties of Santa Barbara and San Luis Obispo. Detections, mainly of egg masses on nursery shipments, have been found in many counties throughout the Central Valley as far north as Tehama County, in the Napa Valley, and in the Bay Area counties (CDFA 2007; http://max.cdfa.ca.gov/pdcp-gis/pdcp-gis.asp). The main hosts for the GWSS are citrus, grapes, almonds and alfalfa. The GWSS overwinters in citrus, avocados, in riparian vegetation and some ornamentals such as crape myrtle. As grape vines and almond trees leaf out in the springtime, the GWSS moves onto those hosts.

Governmental agencies have been involved in two control programs to manage and contain the GWSS. One program involves the control of the GWSS on citrus before it can move into vineyards and transmit the Pierce's disease (PD) bacterium. This program overcomes the divide created between the citrus growers who are not typically affected by GWSS and would not typically treat for GWSS, and grape growers who are negatively affected by large populations of GWSS migrating from citrus to grapes. Currently any citrus grove within ¼ mile of a trapped vine (i.e. a trap placed in a vineyard contains a GWSS) is treated, unless the grove is located along the northern boundary of the infestation, in which case the barrier is ½ mile of a trapped vine. While some citrus growers may benefit from the control of the GWSS in their groves, chemical treatments may also disrupt IPM pest control practices, imposing additional costs on the citrus industry. All these effects are important to include in any economic analysis of PD in California.

Finally, there is a state quarantine in place to limit the spread of the GWSS into uninfested grape growing areas of California. The quarantine consists of on-site sanitation practices, inspections and surveys, and spraying plant leaves with a chemical such a methomyl (Lannate®) to treat difficult to detect egg masses not caught by inspectors. As a result, management of PD in California includes a bundle of methods that have economic impacts on the wine, table and raisin grape, citrus, and nurseries industries. These different methods to control GWSS and PD have significantly improved the situation, and damages today are not as severe as initially anticipated. Even though better methods have been developed to manage GWSS, the costs of production for each industry may not have returned to pre-GWSS infestation levels.

Due to the size of the industries affected by the control of GWSS and PD in California, even small changes in the costs of production can have a major impact on the benefits and costs to producers, consumers and taxpayers. The grape industry is a major agricultural producer in California. With average annual revenues (2004-2006) to the wine, table and raisin grape industries totaling $3 billion, grape production is the largest fruit industry in California (USDA 2006a). When revenues from the citrus and nursery industries are combined with the revenues from the grape industry, their total revenues of $20.8 billion make it the second largest agricultural sector in the U.S. behind corn ($26.8 billion) and before soybeans ($18.3 billion) (USDA 2006a; USDA 2006b; Jetter 2007).

OBJECTIVES

1) The first objective of this study is to estimate the costs and benefits to wine grape, table grape and raisin growers, consumers and taxpayers from changes in the costs of grape production due to the establishment of the GWSS. The changes in production costs will be based on current best practices and will include chemical treatments, removal of infested vines, quarantine restrictions and public control programs. The increase costs of production affect newly infested producers directly because they bear the burden of paying the increased costs of production; however, consumers and producers are also affected through the market effects due the changes in the costs of production.

Objectives 1 will be completed through the use of economic market models. Market models are used to estimate the losses to both producers and consumers when changes in the costs to grow and market a crop are significant enough to affect market prices, production and supply.

Changes in treatment costs due to the establishment of the GWSS and the public control program.

For Objective 1 data are needed on the changes in the costs of production for affected growers due to the establishment of the GWSS in California, and the amount of acreage lost to PD around the turn of the century. For Objective 1 data are also needed on grape, citrus and nursery production, prices, revenues and trade data from 1998 through 2007 (the last year for which data are available); current costs of production; and elasticities (elasticities measure the percentage change in a quantity variable for a one percent change in a price variable – for example it could measure the percentage change in production for a one percent change in the farm price.)

How the GWSS affects current production was determined through meetings held with UCCE farm advisors and growers to discuss how the establishment of the GWSS affected their pest control programs for grapes. The meetings were held in November and December 2008 in the southern San Joaquin Valley in November and December 2008. Additional information was obtained through phone interviews with Pest Control Advisors in southern California and The results of these meetings were compared to University of California Cooperative Extension Budgets to determine how all pest control

treatments changed as a result of the treatments required for GWSS (Hashim-Buckley 2007; Peacock et al. 2007; Peacock et al. 2007b; Vasquez et al. 2007). Production and price data for grapes were collected from the National Agricultural Statistics Service.

Economic Effects in the Southern San Joaquin Valley – Data to use in the market model

Objective 1. A meeting was held with grape growers, and public agencies involved with the public control program to determine how the establishment of GWSS has affected different groups in this area. Three groups are affected by control of the GWSS in the southern San Joaquin Valley, grape growers, citrus growers and taxpayers. While there is currently a low incidence of PD in Kern and Fresno counties, the incidence can rapidly increase should GWSS not be controlled.

The first line of defense against the spread of PD by the GWSS is the public control program whereby citrus is treated during the winter months to prevent the build up of GWSS populations. To control for GWSS in citrus an application of Assail is made in the fall followed by an application of imidacloprid in the spring. Imidacloprid is applied at a rate of 32 fl oz an acre (2 lb ai/gal formulation) through the irrigation system. The control program is conducted on an area-wide basis to achieve longer-term reductions in GWSS populations. The control in citrus occurs about once every three years based on monitoring of GWSS populations. However, treatments are scheduled based on monitoring and trapping. Under the public program citrus growers are reimbursed for their treatments of GWSS and participation in the public program is currently voluntary for the citrus grower. There is no economic effect on citrus growers based on changes in the costs of production.

The second line of defense against the spread of PD is to treat grape vines for GWSS. A majority of grape growers apply imidacloprid once annually to control GWSS and prevent the transmission of PD. Applications of the soil formulation of imidacloprid (such as Admire Pro) are typically at the maximum rate of 14 fl oz an acre (4.6 lb ai/gal formulation) through the irrigation system. The cost of applying imidacloprid is currently about $50-$60 an acre.

Treatments for GWSS sharpshooter control affect the control of other pests, and in turn are affected by the control of other pests. The GWSS also controls the variegated grape leafhopper and grape skeletonizer. The cost savings by growers is $62 an acre based on UCCE budgets, or about the same amount as the current costs to apply Admire Pro. GWSS control is also affected by control for the vine mealybug (VMB) (Planococcus ficus). In 1994 VMB was first found in the Coachella Valley and has since spread throughout most grape growing counties in California including the southern San Joaquin Valley. Treatment for the VMB consists of a soil application in late May or early June of imidacloprid and 2–3 spray treatments. The soil treatment is in addition to the soil treatment for GWSS in the spring.

According to the PCAs interviewed during August 2010 the incidence of PD in the southern San Joaquin Valley is at about the same, or slightly less than it was before the GWSS invaded. As a result no additional costs due to changes in the incidence of PD are being born by growers in the southern San Joaquin County. There are also no additional quarantine costs incurred by grape growers as mature fruit destined for the fresh market is hand harvested and field packed.

Total costs of production for citrus growers are also affected by the public control program and quarantines against moving citrus out of infested areas. Treatments with imidacloprid may help suppress nematodes, citrus peelminer and California red scale. Better control of these insects can be achieved by applying an additional amount of imidacloprid when treating for GWSS; however, the grower is responsible for those costs. The citrus industry is affected by the interior quarantine and fruit from infested areas needs to be inspected and treated before leaving a quarantine area. Quarantine treatments involve fumigation using EverGreen (pyrethrum + piperonyl butoxidor). Turbocide has also been mentioned as a material that can be used as a fumigant. If GWSS are found in a grower's orange shipments, the grower bears the cost of treating GWSS in his or her grove if the grower did not participate in the area wide program. This aspect of the public control program is believed to encourage greater participation by citrus growers in the control of GWSS. Because growers are reimbursed for their control costs for GWSS in citrus, there are no additional costs to the citrus industry for this program.

Economic Effects in the Temecula Valley

In the Temecula Valley there is also a public program to control GWSS. Area wide coordination of treatments has been more difficult in the Temecula Valley. Many groves are being carved up into rural homesteads and cultural procedures are completed by farm management companies instead of a grower/owner. With a lower proportion of groves being treated in the Temecula Valley than in the southern San Joaquin Valley, GWSS pest pressure is greater in the Temecula Valley.

Private treatment of GWSS in the Temecula Valley also consists of an annual treatment of Admire. However, because there is greater GWSS pest pressure, higher costs of production for grape growers in the Temecula Valley are being realized as the application of Admire is being supplemented with annual sprays of Danitol in some areas. For vineyards located near citrus groves about two applications of Danitol are needed a year. Growers in the Temecula Valley would also no longer be required to treat for the grapeleaf skeletonizer. There is no problem with leafhoppers in this area.

The Temecula Valley has a drier climate than the San Joaquin Valley. In order for growers to apply Admire when it can do the most good, a separate irrigation may be required. Farm managers with whom meetings were held estimate that half the time they need to complete a separate irrigation in order to apply Admire. The extra irrigation costs are estimated to be $12.50 on average. Danitol is applied at a rate of 11 oz per acre, with the cost per ounce equal to $1.62. With two treatments a year the cost to treat GWSS with Danitol is $35.86. The total additional cost to grape growers to treat GWSS in the Temecula Valley is about $48 a year when rounded.

In addition to GWSS treatments growers in the Temecula Valley also have a slightly higher incidence of PD than before the establishment of the GWSS. The incidence of PD is currently about 2% to 3% in the valley, up from about 1% before the GWSS established. While there are a few large plots that are infested, PD in grape vineyards tends to be localized. Growers will pull vines and replant instead of removing a whole plot. The costs to replant about 2% of vines a year is $65 an acre based on UCCE wine grape budgets for the San Joaquin Valley. The total cost to treat GWSS and manage PD is equal to $103 an acre per year in the Temecula Valley. Total grape acreage in the Temecula Valley is about 1,300 acres. Total annual costs to the grape industry in California is estimated to be about $133,900 a year.

The Temecula Valley was one of the areas hardest hit by the spread of PD by the establishment of the GWSS. Acreage lost to PD is approximately 336 acres, or 15.6% of total acreage between 1998 and 2000.

Economic Effects to the Nursery Industry

A survey was sent to 114 nursery operators to determine how the GWSS has affected their production of nursery stock and the costs associated with GWSS and PD control. A total of 35 nursery operators responded to the request. Out of the 35 nursery operators who responded eight operations had to destroy plants due to the presence of GWSS. The total wholesale value of the destroyed plants was $ 95,500 for an average loss of $10,611 per infested operation.

Questions were asked on the barrier methods used to prevent the entry of GWSS and inspections of traps installed by CDFA. Almost 30% of the operators who responded to the question used some type of barrier method. The methods used were shade cloths, an insect screen, oleander hedge or some combination of barriers. Most of the barrier methods used also provide additional protection against other pests. Oleander hedges also protect against various sucking insects, shade cloth protects against grasshoppers, and the insect screen protects against aphids and thrips. Half of the operators do some in-house monitoring of the traps installed by CDFA. Monitoring varies though from as frequently as once a week to as little as every other month.

Over 50% of the nursery operators applied pesticides to manage the GWSS. The operators used a variety of chemical treatments including Tame, Sevin, Tristar and Avid. Total application costs varied from $175/acre to $2160/acre for an average of $940 an acre.

Objectives 2 and 3

Objective 2: Estimate the costs and benefits of public policies to manage and contain the GWSS. The public control policies include public programs to treat the GWSS in citrus to prevent its spread into grape vineyards in the spring, and the associated containment program. An additional public policy to contain the spread of GWSS and, thus, the transmission of PD, is a state quarantine on the movement of nursery, citrus and other host crops out of infested regions.

The current treatment scenarios for GWSS and PD provide the baseline data for the analysis for objectives 2 and 3. For objective 2 the benefits of the public program will be estimated assuming that if no program exists individual growers will not treat the GWSS in way to prevent its spread throughout California. This will impose additional treatment costs on growers in areas currently free of the GWSS. The amount of additional treatments that will need to be completed will vary with the presence of the vine mealybug and other sharpshooters. Pest control advisors and UCCE farm advisors were surveyed by phone to determine how their costs of production would change if the public program were discontinued. In the Temecula Valley and the San Joaquin Valley where there are vine mealybug infestations if the public program were discontinued it is believed that pest pressures would increase resulting in growers spraying an additional 1-2 spray treatments a year. Growers would spray either imidacloprid or Danitol and average control costs would be about $35.00 to $50.00 a treatment, causing pest control costs to go up by $70 to $100 per acre. In counties located in the Central Valley that are not currently infested with vine mealybugs PCAs believe that growers will complete an additional 4-5 spray treatments a year for an additional cost of $140 to $200 an acre.

In the Coastal wine grower counties growers are currently treating for the blue-green sharpshooter to prevent the spread of PD in that area. The blue-green sharpshooter has one generation a year, but is usually not treated based on UCCE Sample Costs of Production. In comparison the glassy-winged sharpshooter has two generations a year and, due to its greater ability to transmit the bacterium that causes PD, it is expected that growers will treat it to prevent populations from increasing. An

additional soil application of imidacloprid and an additional two to three spray applications of Danitol or imidacloprid will be needed to keep GWSS populations suppressed. The total increase in costs is about $105 to $150 an acre per year. This data will be used in a market model to estimate the net losses to growers and consumers. The losses to growers will be estimated by the type of grape produced (i.e. table, raisin, wine) in order to complete objective 3.

3) Estimate the optimal check-off rate for the grape industries that benefit from the treatment of the GWSS on overwintering crops. The rate will take into account the costs and benefits to the grape growers in both infested areas and areas that benefit from the containment of the GWSS within infested areas, and the costs and benefits to growers of overwintering crops. The results of the first two objectives will be used as parameters in the model that estimates check-off rates.

DISCUSSION

The public control program to date has managed to contain the GWSS in the southern part of the State. Due to the effective control of GWSS in citrus in the southern San Joaquin Valley, the cost to grape growers of GWSS control has been offset by cost reductions in the use of other insecticides. The area with a net increase in the costs of production for grape production, the Temecula Valley contributes a very small share to California, and U.S. production of grapes (2.8% of U.S. production). Given the low percentage of grape production in the area with the increase in costs and no net change in costs in the areas with the greatest percentage of U.S. production, there is no shift up of the U.S. grape supply curve due to GWSS control in the southern San Joaquin Valley.

While the public GWSS control program has managed to keep the change in the costs of production to levels that do not affect market demand, the consequence for growers in the Temecula Valley is that their extra treatment costs are not partially offset by changes in market prices. The increase in changes in the costs of production then result in a decrease in profits for a grower.

If the public control program were discontinued winter GWSS treatment in citrus would cease and, without a quarantine, GWSS would spread. Costs for grape growers would increase throughout grape growing regions due to higher control costs where GWSS is currently established, and the need to implement control measures in areas currently free of GWSS. The net effect would be an increase in market prices and lower market supply, making consumers worse off in additional to producers.

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