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Vector Biology and Ecology

GLASSY-WINGED SHARPSHOOTER FEEDING DOES NOT CAUSE AIR EMBOLISMS IN THE XYLEM OF WELL-WATERED PLANTS

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ABSTRACT
Plant xylem vessels are under negative hydrostatic pressure (tension) as evapotranspiration of water from the leaf surface pulls the column of water in xylem upwards. When xylem fluid flux is under extreme tension, any puncture or breakage of the xylem vessel wall can cause formation of air embolisms that instantaneously empty the length of the xylem vessel (cavitation), disrupting xylem flow. Xylem fluid-ingesting hemipteran insects like the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar), penetrate their stylets into xylem cells and imbibe many times their body weight in xylem fluid each day. It has not been known whether GWSS stylet penetration embolizes xylem, however, embolisms from vector feeding have been suggested as one mechanism of xylem blockage in Pierce's disease symptoms. To date, no method has been successful in visualizing xylem during real-time stylet penetration, to determine whether or not air embolism occurs. The present study used videography of live, feeding GWSS under X-ray phase contrast microscopy at the Argonne National Laboratory, to determine whether air was present in stylets or xylem before, during, or after xylem penetration. Air is an excellent contrast agent for X-ray microscopy, and is readily visible in both plant cells and insect stylets. Insects were monitored via electrical penetration graph, to identify when their stylets had penetrated xylem in cowpea stems. After feeding was terminated, stems were cut; subsequent entry of air into xylem was visible in stems under X-ray. X-ray videographs before, during, and after stylet penetration to and inside xylem showed no air present in stylets, and only in cut, not intact, xylem cells. It is hypothesized that salivary sheaths secreted by GWSS during stylet penetration prevent cavitation.

LAYPERSON SUMMARY
Xylem-feeding insects like the glassy-winged sharpshooter (GWSS) penetrate their needle-like mouth parts (stylets) into xylem (water-conducting) cells in plants, and swallow many times their body weight in xylem fluid each day. This is in spite of the fact that plant xylem cells are under extreme hydrostatic suction (tension). Any puncture of the xylem vessel wall can cause formation of explosive air bubbles, disrupting xylem flow. It has not been known whether GWSS stylet penetration causes air bubbles in xylem, however, it has been suggested that air bubbles induced by GWSS feeding might contribute to xylem blockage causing Pierce's disease (PD) symptoms. To date, no method has been successful in directly visualizing xylem real-time during GWSS feeding, to determine whether or not air bubbles develop. The present study video-recorded live, feeding GWSS under an X-ray microscope at the Argonne National Laboratory, to determine whether air was present in stylets or xylem before, during, or after GWSS feeding. Air is an excellent contrast agent for X-ray microscopy, and is visible in both plant cells and insect stylets. X-ray images before, during, and after GWSS feeding inside xylem showed no air present in either stylets or xylem cells. After feeding ended, stems were cut; subsequent air bubble formation in xylem was visible in stems. Thus, air bubble formation probably does not contribute to PD symptoms. Hardened saliva that is secreted by GWSS during feeding may prevent formation of bubbles.

INTRODUCTION
Xylem vessels are under extreme negative hydrostatic pressure (i.e. tension) as evapotranspiration from the leaf surface draws the column of water in xylem upwards. This metastable column of water is highly prone to physical rupture (cavitation) leading to an air embolism that can completely halt xylem flux (i.e. hydraulic failure). Evidence is mounting that Pierce's disease (PD) symptoms in grapevine are the result of a cascade of plant responses (Chatelet et al. 2011) that include xylem embolism in its earliest stages, well before onset of symptoms (Pérez-Donoso et al. 2007, McElrone et al. 2008). Xylem embolism occurs more often in *Xf*-infected than control plants and precedes significant hydraulic failure (McElrone et al. 2008). Therefore, it is likely that air embolism plays an important, albeit incompletely understood, role in *Xylella fastidiosa* (*Xf)* pathogenesis.

Three hypotheses have been proposed for induction of embolisms in *Xf* pathogenesis. First, the earliest embolisms might result from sharpshooter stylet penetration of xylem vessels (Backus, J.M. Labavitch, A. McElrone, A. Pérez-Donoso, pers. comm.; Crews et al. 1998). Second, early embolisms could be directly induced by *Xf* bacteria in xylem vessels, either by reducing water surface tension or by damaging pit membranes (McElrone et al. 2008). Third, xylem plugging by bacteria, gums, and tyloses could exacerbate hydraulic failure via additional cavitation (McElrone et al. 2003). This would not explain air embolisms early in pathogenesis, but could explain the interaction of water stress and air embolisms in *Xf* pathogenesis (McElrone et al. 2003). The present project tested the first of these three hypotheses.

OBJECTIVE

1. Determine whether air occurs in GWSS stylets or xylem before, during, or after vector feeding on a well-watered sunflower plant using real-time videography of live insects, feeding under X-ray phase contrast microscopy.

RESULTS AND DISCUSSION

GWSS, sunflower plants, and electrical penetration graph (EPG) equipment were shipped to the Advanced Photon Source at the Argonne National Laboratory under appropriate USDA APHIS PPQ permits. GWSS's feeding on sunflower stems were simultaneously subjected to X-ray phase contrast microscopy and recorded via EPG, using the following experimental protocol. Insects were tethered to gold wire, then held on sunflower for 1 – 10 hrs, then were starved for 1 – 3 hrs prior to recording. A sunflower plant was placed in a holder in the X-ray room, in the path of the beam. A wired insect was plugged into the head stage amplifier of the EPG monitor, positioned on the vertical stem, and EPG monitoring begun. Lights were turned out and the room was vacated and sealed in anticipation of beam-on; however, to minimize deleterious effects on the insect from X-rays, the beam was not immediately turned on. When the insect's stylets probed the plant, feeding waveforms were digitally recorded and displayed on a Dell Latitude laptop computer, as previously described (Dugravot et al. 2008, Backus et al. 2009). The GWSS was allowed to probe without X-ray exposure until its stylets reached xylem (as indicated by X waves) (Backus et al. 2009) and ingestion had ensued for 15 to 40 min. The X-ray beam was then turned on and video-recording of the plant and insect's proboscis begun. Using The Observer® video acquisition software, EPG waveforms and video output from the X-ray microscope were synchronized and simultaneously displayed on the computer. After a few minutes of recording, the X-ray was turned off, lights turned on, and the room was re-opened and entered. The site of probing was marked and the insect was gently disturbed to artificially terminate the probe. The plant stem was then cut 1 – 3 cm below the mark, allowing air to begin to enter the xylem. The lights were turned off, room vacated, sealed, and the X-ray turned back on, to video-record the progression up the stem of air entering xylem cells (requiring about 10 – 30 min, due to slow evapotranspiration in the cool, dark room). Each experiment lasted 2 – 3 hrs, and was repeated six times, although one insect did not achieve xylem ingestion (see below).

To visualize air inside stylets, three additional sharpshooters were X-rayed and video-recorded while attempting to feed on empty, air-filled Parafilm® sachets, although no EPG was possible due to lack of electrical conductivity of air. Four intact sunflower stems were also X-ray video-recorded for 5 min without sharpshooter feeding, as controls, including two stems that were used several minutes later for experiments, described above. Thus, plants were X-ray imaged before, during and after sharpshooter feeding and stem cutting, and air was clearly visualized both inside insect and plant controls.

X-ray microscopy reveals the interior of biological specimens in unaccustomed ways. First, X-rays have infinite depth of field, so X-ray images are completely flattened. Thus, all visible structures in the interior of a specimen that lie in the cross-sectional area of the beam are superimposed on one another in the same focal plane, regardless of the thickness of the specimen. Second, structures are variably visible based on the strength of contrast agents present in the specimen. Heavy metals such as iodine are excellent contrast agents, but difficult to use with live insects. On the other hand, air is an excellent contrast agent for X-rays, and visible inside both insects and plants. However, degree of visibility depends upon brightness/amount of air relative to the thickness of the subject. We used air as the sole contrast agent in this study.

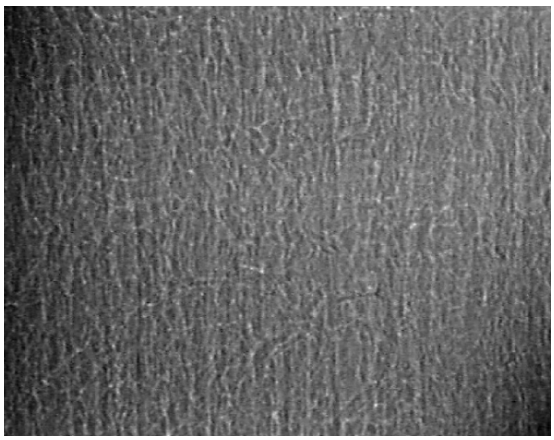

Figure 1. X-ray image of the vascular region of a sunflower stem 10 min after the start of GWSS probing and 5 cm above the probing area. Note bright, reticulated pattern of air in intercellular spaces around parenchyma cell, but absence of xylem cell striation.

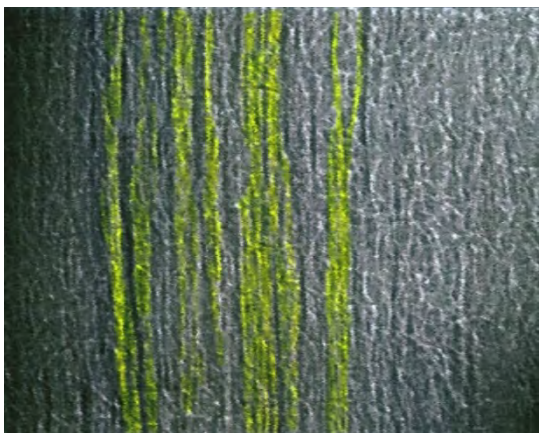

Figure 2. False-colored X-ray image of the vascular region of a sunflower stem 80 min after the start of GWSS probing and 1 cm below the probing area, showing air-filled xylem vessels with superimposed intercellular spaces. Yellow color, here and elsewhere denotes air.

Prior to cutting, sunflower stems were mostly opaque to X-rays, with a tight pattern of reticulation that was probably caused by intercellular air spaces between cells in the ground tissue on the stem periphery (**Figure 1**). No vertical striations were visible. In contrast, obvious, white vertical striations interpreted to be air-filled xylem vessels (**Figure 2**; yellow highlighting) became visible 3 – 5 min after stems were cut, slowly spreading from the cut end of the stem upwards. This occurred in every cut stem, regardless of whether or not the stem had been probed by sharpshooters. The stem in **Figure 2** was imaged 39 min after cutting; air filling of the full length of xylem vessels generally occurred within 90 min of cutting.

Further evidence that white striations represent air-filled xylem is shown in **Figure 3**, displaying a series of X-ray images taken within one minute of each another. We observed two air bubbles in a single xylem vessel, with a narrow strip of fluid between the two bubbles. As we watched, the shape and position of the fluid strip changed (**Figures 3a – c**), and eventually disappeared completely as the two air bubbles suddenly merged (**Figure 3d**). Air bubble movement in this shape and manner could only be explained by air filling of a xylem vessel.

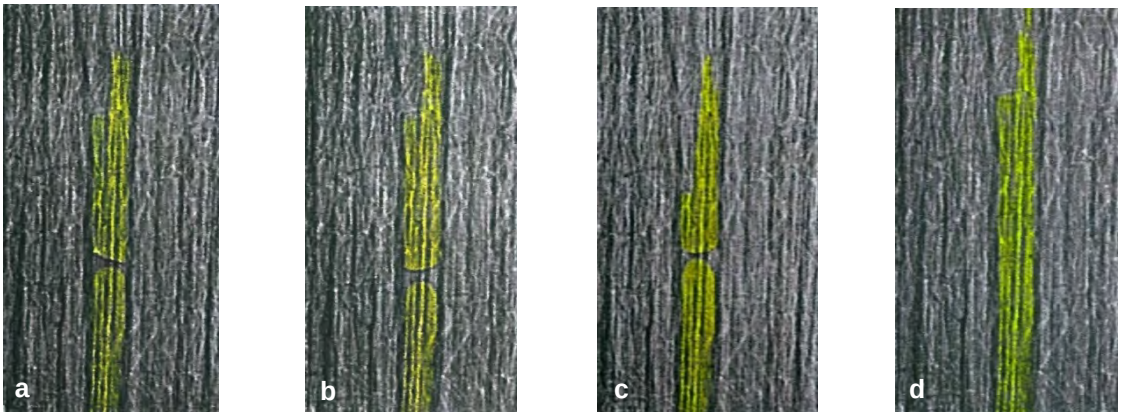

Figure 3. False-colored X-ray images of two air bubbles in a xylem cell, moving and merging. **a.** Two air bubbles in a xylem vessel, 70 min 43 sec after start of stylet probing. **b.** Same vessel, 11 sec later. **c.** Same vessel, 5 sec later. **d.** Same vessel, 43 sec later, after bubbles merged.

GWSS attempting to ingest from an air-filled Parafilm® sachet briefly sucked air into their stylet food canals (**Figure 4**; yellow highlighting), proving that air would be readily visible inside the proboscis (external to the feeding substrate) and stylets (internal to the substrate) using X-ray microscopy. In contrast, sharpshooter stylets inserted into sunflower stems, both before and during xylem ingestion, did not contain air (**Figure 5**). Only a faint outline of the stylets was visible shallowly inserted into the stem, and no air-filled xylem vessels were visible during or after xylem ingestion. The image in **Figure 5** was taken 38 min after the start of stylet probing, during xylem ingestion. Two min later, this insect (no. 1) pulled

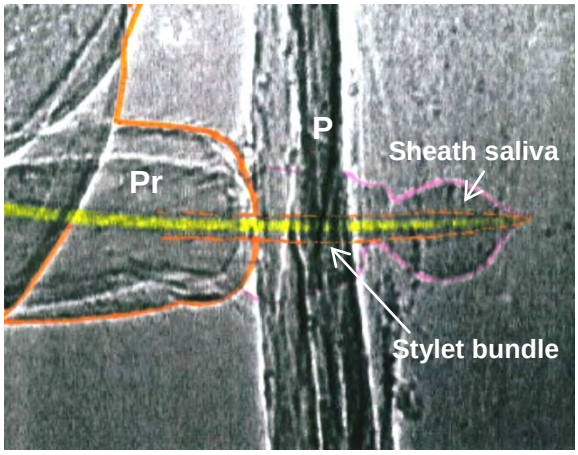

Figure 4. False-colored X-ray image of a GWSS proboscis (Pr) pressed to a Parafilm® (P) feeding sachet, with stylet bundle extended into a recently secreted bead of sheath saliva. Orange, outlined insect structures; pink, outlined saliva.

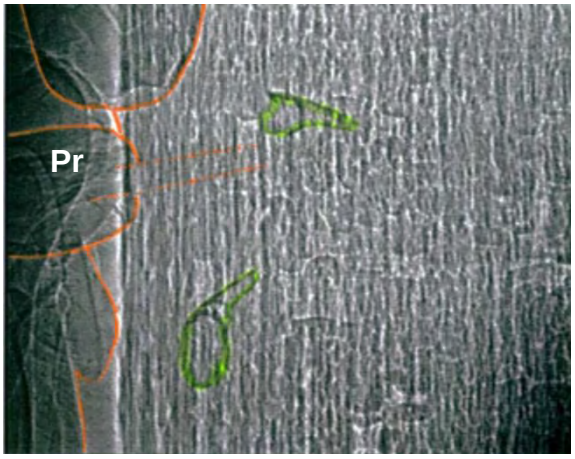

Figure 5. False-colored X-ray micrograph of insect no. 1's proboscis (Pr) pressed to a sunflower stem, during xylem ingestion recorded via EPG. Orange, outlined insect structures; green, outlined trichomes on stem surface. Note intercellular reticulation.

out its stylets and the stem was cut. At no time before, during, or after xylem contact and ingestion was air observed either inside the proboscis, stylets, or xylem vessels. The only time air could be seen inside xylem vessels was after air embolisms were artificially introduced via stem cutting.

After air had completely filled the xylem vessels and intercellular spaces of the first sunflower stem observed (for insect no. 1), an X-ray survey of the entire stem revealed a structure closely resembling a GWSS salivary sheath (**Figure 6a**) inside the stem, in the same location as the marked probe. Thereafter, a whole-stem X-ray survey was performed at the end of each repetition. Similar structures were seen in X-ray images for three out of the six repetitions (**Figure 6b**). One-mm blocks of sunflower tissue were excised from the marked areas of all probed stems, fixed and later prepared for paraffin-sectioning, saffranin-fast green counterstaining, and examination via light microscopy, using previously described methods (Backus et al. 2009). In all six cases, typical GWSS salivary sheaths were later found in probed tissues (**Figure 6c**), demonstrating that the structures seen in X-ray images were sheaths. However, they were only visible when air had filled the stem, and not in all cases. Likelihood of observing salivary sheaths in stems was related to stem thickness; very thick stems did not allow observation, even though salivary sheaths were present.

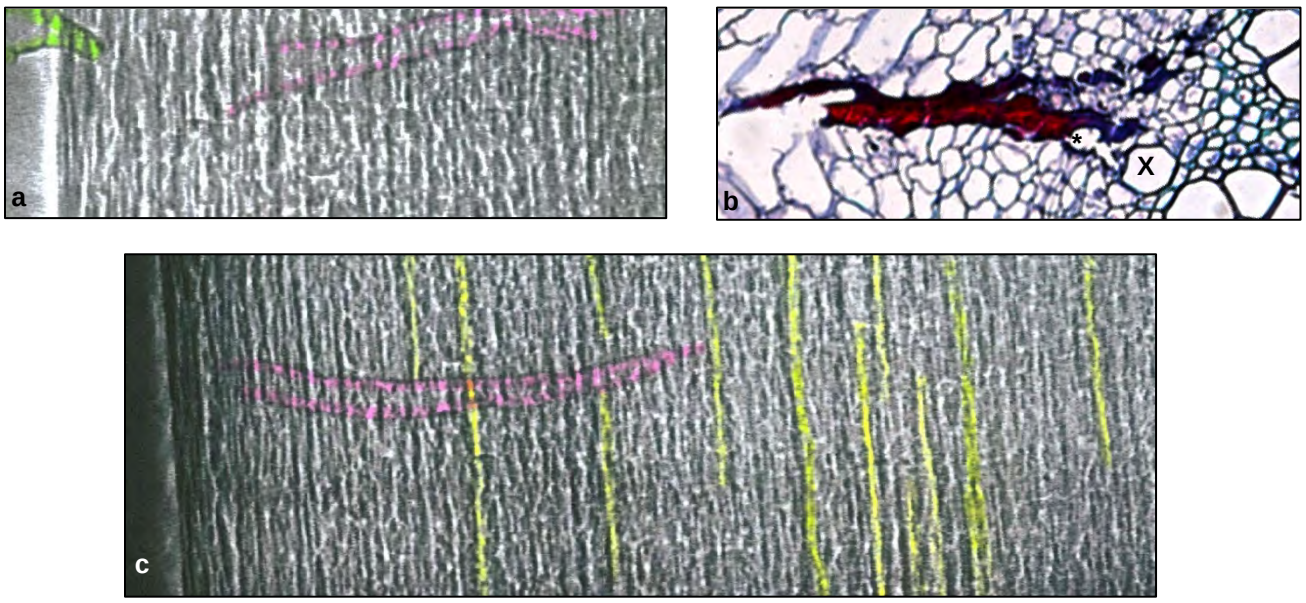

Figure 6. X-ray and light micrographs of GWSS salivary sheaths in probed sunflower stems. **a.** X-ray micrograph (side view, from transversely viewed stem) of sheath from insect no. 1; image was taken before the sheath was observed, hence only part is visible. **b.** Light micrograph of same salivary sheath from insect no. 1 (viewed from above, from cross-sectioned stem). Note thin-walled, hollow, lower sheath branch (*) to large xylem cell (X). The last sheath branch made is always hollow when the probe is artificially terminated. X-ray sheath image in **Figure 6a** corresponds to the red-stained saliva; blue-stained saliva is not visible in X-ray. **c.** X-ray micrograph of salivary sheath from insect no. 2, more clearly showing the full size of the sheath in relation to xylem vessels from multiple, superimposed bundles. Pink, outlined saliva; green, outlined trichome.

Another observation of GWSS saliva is noteworthy, although not related to air embolisms in xylem. **Figure 7** shows a large deposit of presumed watery (digestive enzyme-containing) saliva on the periphery of the stem, immediately below the salivary flange marking the entry point of the saliva. This deposit was left by insect no. 6, the only insect that died from X-ray exposure before its stylets could reach xylem. EPG waveforms indicated that this insect performed pathway activities (formation of the salivary sheath) for 20 min (a very long time) without beginning xylem ingestion. Indeed, later paraffin-sectioning showed that the weakened insect made a large, multi-branched salivary sheath that never arrived at a xylem cell. The sheath was not visible under X-ray, probably due to the thickness of that particular stem. Nonetheless, this insect's probe is remarkable because, in its weakened condition, the insect apparently left a large accumulation of watery saliva that loosened cell walls sufficiently to cause more air-entry than for the surrounding cells. Watery saliva, though hypothesized to be produced by GWSS (Alhaddad et al. 2011, Backus et al. ms. submitted), has never been visualized *in planta*.

Embolism of xylem vessels may be one of the first steps in the cascade of plant responses underlying symptom development in Pierce's disease (PD) (McElrone et al. 2008). However, the present work demonstrates that air embolism cannot be caused by GWSS stylet penetration into a xylem vessel, at least for a well-watered plant, for which these results are most applicable. Water stress due to extreme light, temperature, and/or lack of soil moisture interacts with *Xf* infection in an additive manner to worsen hydraulic failure and PD symptoms (McElrone et al. 2003). It is possible that GWSS feeding under those circumstances might trigger air embolisms. This possibility will need to be examined using X-ray microscopy in future

Figure 7. X-ray micrograph of sharpshooter watery saliva deposit from insect no. 6. Round deposit of saliva on the left-outside of stem (F) is the salivary flange, marking the entry point of the stylets. Watery saliva mixed with sheath saliva was injected at this point, flowing downward with gravity. Pink, outlined saliva.

studies. It is interesting to note, however, that once a stem had been cut and xylem cells artificially embolized, GWSS refused to initiate stylet penetration, let alone ingestion. Insects also were very reluctant to probe air-filled Parafilm® sachets. It is possible that presence of too much air in plant tissues, including xylem, could be a deterrent to GWSS feeding.

CONCLUSIONS

Real-time videography of GWSS feeding under X-ray microscopy revealed no air in the proboscis, stylets, or xylem vessels inside well-watered sunflower stems. In contrast, air was visible inside the stylet food canal of insects that had probed an air-filled Parafilm® sachet and briefly sucked up air, as well as in xylem vessels that had been artificially embolized via severing of the stem. The latter controls demonstrate that it would have been possible to detect air in the stylets or xylem if GWSS feeding had triggered air embolisms. We conclude that stylet penetration of a xylem vessel during GWSS feeding does not cause air embolisms in well-watered plants. Continued research will be necessary to determine whether the same is true for water-stressed or *Xf*-infected plants.

Development of novel strategies for PD management depend upon understanding *Xf* pathogenesis. Eliminating the role of vector feeding in onset of xylem embolism will allow researchers to concentrate on the impact of bacterial colonization of pit membranes, production of gums, and plant responses such as tyloses. Because plant responses are under strong genetic control, it is possible that new transgenic mechanisms of resistance to *Xf* could result from such studies.

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CELL CULTURE BASED PROPAGATION OF THE GLASSY-WINGED SHARPSHOOTER AS A METHOD TO REPLICATE *HOMALODISCA COAGULATA VIRUS-01* FOR VECTOR MANAGEMENT

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ABSTRACT
Invasive species management is crucial in agricultural production. The glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*) is the primary vector for *Xylella fastidiosa*, a xylem-limited bacterium. A dicistroviridae virus known as *Homalodisca coagulata virus-01* (HoCV-01*)* has been identified that infects and increases mortality rates in GWSS when used alongside chemical insecticides. The virus requires an insect host for propagation, thus making a cell culture approach a logistical and economical method towards producing a biological control agent. In this study, we have developed a system for large-scale propagation of GWSS cell cultures from primary cell cultures. Mass production of GWSS cells via culturing techniques will allow us to produce HoCV-01 virus in large enough quantities to be utilized as a method of insect control.

LAYPERSON SUMMARY
The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is an invasive leafhopper species that has been the source of great economic loss in agriculture throughout California and the southwest. GWSS are the primary mode of transmission of *Xylella fastidiosa,* the causal agent of Pierce's disease. Developing a biological control agent for this disease has become increasingly vital towards controlling insect populations and overcoming the problems of harmful environmental effects and insect resistance to chemical insecticides. Utilizing a virus that has been shown to increase mortality in GWSS colonies, this work focuses on a cell culture based approach to propagate GWSS cell lines that act as vectors to grow live virus, which can then be extracted. Since the target virus cannot grow outside of the insect, successful cultivation of GWSS cells is critical to the development of a viral based biological control agent

INTRODUCTION
The xylem-limited bacterium *Xylella fastidiosa (Xf)* is the causal agent in Pierce's disease (PD) and is principally transmitted by the glassy-winged sharp shooter (GWSS, *Homalodisca vitripennis),* an invasive leafhopper species. GWSS are indigenous to the southern United States and northeastern Mexico but have successfully invaded new territory including California and the Hawaiian island of Oahu (Hunnicutt, 2008). PD has been a source of great economic loss in many agricultural arenas throughout North America, particularly in grape vineyards. Introduction of GWSS into new vineyards has been linked directly with an increase in PD (Perring et al., 2001). GWSS are voracious xylem feeders, enabling them to rapidly spread the plant pathogen and making control of this invasive pest crucial to disease management. The ability of GWSS to cover large ranges presents a great risk to agriculture within the United States as well as internationally if this invasive species is not managed.

Use of other vector management techniques such as insecticides that are non-specific has lead to additional problems including insecticide resistance and residue contamination, whereas utilizing a virus that is already present within GWSS populations to develop a viral biological control presents a target specific option for pest management (Hunnicutt et al., 2006). A dicistroviridae virus known as *Homalodisca coagulata virus-01* (HoCV-01*)* has been identified that infects and increases mortality rates in GWSS. The focus of this study was to expand on previously unsuccessful attempts to rear infected glassy-winged sharpshooters to adulthood by utilizing cell culture techniques versus live insect colonies.

OBJECTIVES
1. Successfully propagate primary cell lines of glassy-winged sharp shooters via tissue cell culture techniques.
2. Establish production of the target virus HoCV-01 in vitro.
3. Amplify extracted HoCV-01 to develop biological control agent.

RESULTS AND DISCUSSION
The GWSS cell cultures of established cell lines were obtained from the USDA Agricultural Research Service and centrifuged at 350rpm for six minutes in a 15 amp 5804 R centrifuge (Eppendorf™, Hamburg, Germany). A loose pellet was observed in each tube **(Figure 1)**. The supernatant was drawn off without disturbing the pellet and then 8mL of fresh medium, classified as modified Wayne Hunter-2, WH2, Honey bee cell culture medium (Hunter, 2010), was added to disturb each pellet. The suspended cells were transferred to 25cm^2 tissue culture flasks (4mL per flask) that have been treated to promote cell attachment (Corning®, Lowell, MA), and kept in an incubator at an approximate temperature of 24 degrees

Celsius and 53% humidity. All handing of cell cultures took place inside a Purifier Class II Biosafety Cabinet (LABCONCO™, Kansas City, MO) culture hood.

Culture flasks were examined using an inverted microscope (Olympus DP30BW, IX2-SP, IX71) at 10X magnification. Cells were observed to attach to the substrate within *48 hours* of initial transfer. Over a 10-day period the flasks were examined every-other day, and at the end of the period fibroblasts had developed and were observed across the flask surface **(Figure 2)**.

With successful cultivation in flasks, the cells were transferred to 48-well sterile tissue culture plates, with lids, that have been treated to promote cell attachment (GREINER CELLSTAR®, Monroe, NC). Transfer of cells was completed using 0.25% Trypsin EDTA (Invitrogen™, Carlsbad, CA) to detach cells from the culture surface, centrifugation in a 15 amp 5804 R centrifuge (Eppendorf™, Hamburg, Germany) at 350rpm for six minutes, and introduction of 0.5mL of fresh medium per well.

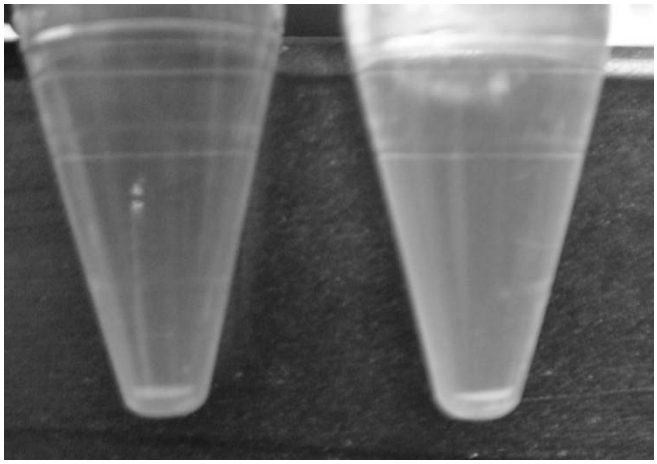

Figure 1. Initial GWSS cells received formed loose pellets upon centrifugation in preparation for transfer to cultures.

CONCLUSIONS

Successful cultivation of GWSS cells is critical to the development of a viral based biological control agent. We now have a system for mass production of cells. In this future, we plan to inoculate cell culture plates with HoCV-01 and extract viral titers until optimal viral extraction time frames are determined and use this method of cell culturing to mass produce the novel HoCV-01 virus as an alternative method for disease control. The results of this work are essential in furthering methods of management of the insect vector for PD, reducing the prevalence of the disease and lessening its economic impacts.

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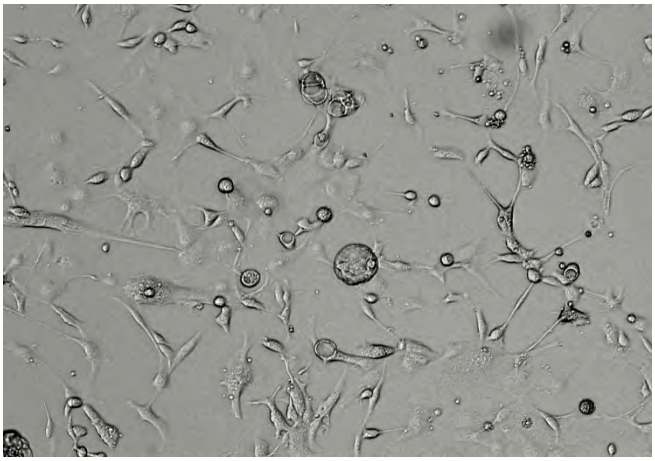
FUNDING AGENCIES

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.



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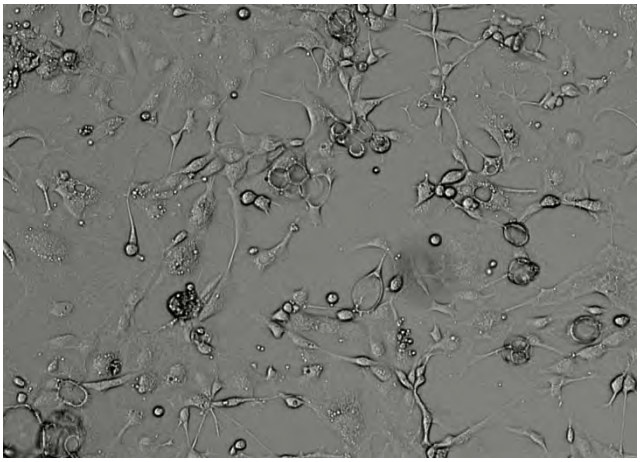

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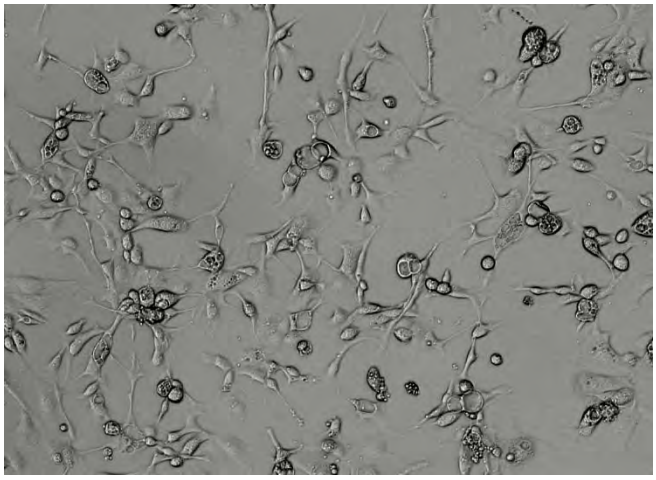

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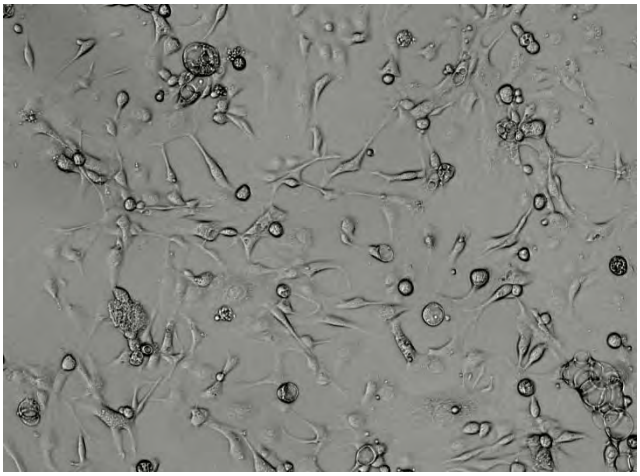

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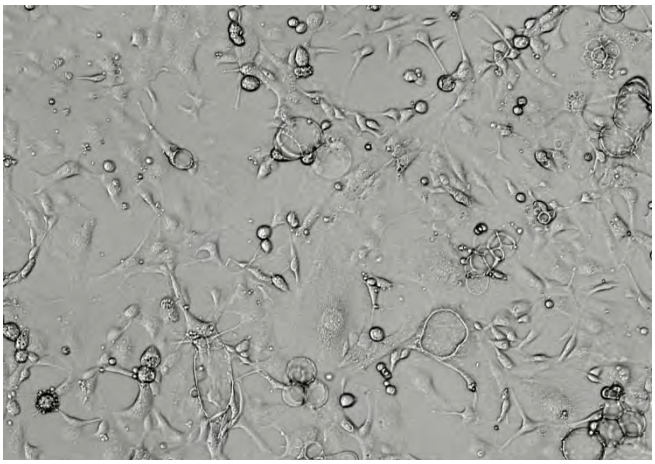

DAY 10

Figure 2. Timeline of growth of GWSS cell cultures over the 10-day incubation period. Substrate attachment and formation of fibroblasts was observed on day 2, 48-hours after initial culture introduction. Continued growth was observed over the 10-days until both fibroblast and monolayer development was observed across the growth surface.

A METAPOPULATION ANALYSIS OF GLASSY-WINGED SHARPSHOOTER IN TEXAS

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Reporting Period: The results reported here are from work conducted November 2010 to October 2011.

ABSTRACT
An understanding of the metapopulation dynamics of the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) and its interaction with associated bacteria, most notably *Xylella fastidiosa (Xf)*, is essential for detecting and predicting shifts in Pierce's disease (PD) dynamics and the development of long term and effective management strategies for PD. With this study we attempt to understand how GWSS populations are laid out across Texas vineyards and what factors affect how those populations interact with associated bacteria, most importantly *Xf*. GWSS were collected on sticky traps from nine vineyards across Texas. Wings collected from the insects were use to determine relative ages based on red pigmentation in the wings. In future work, the presence and relative quantities of PD strain *Xf* contained in the insects will be determined and analyzed along with relative age data to attempt to identify any correlation between these two factors. For 3-4 insects from each vineyard, total genomic DNA was extracted, and used for amplified fragment length polymorphism analysis (AFLP) and 454 pyrosequencing of DNA fragments generated. The AFLP/sequencing data will be used to determine if there are genetically distinct populations of GWSS across Texas vineyards and the structure of those populations.

LAYPERSON SUMMARY
Because of its ability to cause Pierce's disease (PD) by transporting *Xylella fastidiosa* to grapevine, the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is, economically, one of most important insect pests to the wine and table grape industries in Texas, California, and Florida. By looking at samples of GWSS from vineyards across Texas, an effort is being made to understand where distinct populations of GWSS exist in the state, how those populations interact with another, and how they change over time. The information gained from this study may help to provide a forecast for the future of PD in vineyards across Texas, as well as, provide information that may prove useful in the development of management strategies to combat PD. In this work, relative age was determined for GWSS collected from vineyards across Texas and genetic analysis was performed to identify distinct populations of GWSS in Texas.

INTRODUCTION
The glassy-winged sharpshooter (GWSS) is a potential vector of a number of plant pathogens including those that affect oleander, almond, and grapevine. This insect has been identified as the primary vector of *Xylella fastidiosa (Xf)*, the causal agent of Pierce's Disease (PD) in grapevine (Davis et al. 1978). Within *Xf*, a single strain group has been identified to contain all PD causing strains of the species (Chen et al. 2002). With new techniques being developed in the study of the both the GWSS and *Xf* it is important to utilize those techniques to track the population dynamics the insect vector and how the organisms interact with one another at the population and metapopulation level.

In 2010, Hail et al. identified three GWSS carrying PD strain *Xf* in vineyards that had no previous history of *Xf* positive GWSS. A state-wide population study incorporating this information could help answer how likely an event like this would be to occur again and if it is already occurring. Molecular markers have been utilized in the past to study GWSS populations. In 2004, using inter-simple sequence repeat (ISSR) analysis, de León et al. identified genetically distinct populations across the United States and divided these populations into the western (California) and southwestern (Texas and Florida) groups. This study was; however, was unable to resolve specific details of population structure within Texas. It also suggested that distinct biotypes may be present within GWSS (deLeon et al. 2004). Additional population analysis specific to Texas may be able to resolve population structure within the state as well as provide clues to the origin(s) of Texas populations and the presence of distinct biotypes within those populations. Amplified fragment length polymorphism (AFLP) analysis which, in many studies, has outperformed other molecular markers (Meudt and Clarke 2006), may provide the information needed to answer these questions. Direct sequencing of AFLP fragments may increase the information provided by these markers and allow for more robust analysis.

The age structure of a GWSS population may prove an important factor in the population's ability to spread PD. Once an adult GWSS has been colonized by *Xf*, infectivity is sustained throughout the lifetime of the insect as the bacteria multiply within the foregut of the sharpshooter. This gives GWSS the ability to infect a host at any time after colonization during its adult life (Severin 1949; Hill and Purcell 1995). Also, the age of GWSS has been shown to be correlated with the number of sensilla of mouthparts of the insect. This may have some effect on the vectoring ability of the sharpshooter (Leopold et al. 2003). Timmons et al. 2011 showed that it is possible to accurately estimate the age of a GWSS by using digital photography to measure the amount of red pigmentation in the wings of the sharpshooter. If the ages of glassy-winged sharpshooters

could the correlated with the quantity of *Xf* housed within those insects, this could provide information regarding the risk of PD infection. Along with population structure analysis, this could provide valuable insight on the probability of PD becoming more prevalent and spreading to new areas in the near future.

OBJECTIVES

1. Successfully determine relative ages of GWSS collected from vineyards across Texas for use in additional analysis incorporating insect associated *Xf* data.
2. Complete amplified fragment length polymorphism (AFLP) and direct sequencing of AFLP fragments for use in population analysis.

RESULTS AND DISCUSSION

The average relative ages from GWSS collected from seven vineyards were shown in **Figures 1 and 2**. It can be seen from **Figure 2** that the results suggest that both Post Oak Vineyard and Oak Creek Vineyard show significantly but slightly higher predicted population mean of GWSS relative ages than Delaney Grapevine Vineyard. Despite these noted differences, the sample sizes used for this portion of the study, especially for Oak Creek Vineyard and relatively small variation seen from this dataset, it is difficult to state any conclusions concerning differences average ages of sharpshooters across Texas vineyards. Future work will focus on the collection of larger sample sizes of GWSS from vineyards for aging at later dates and the estimation of actual ages of GWSS collected. This will allow for comparisons between vineyards based on a more informative data set as well as information regarding the change in age structure of populations of GWSS over large segments of time (seasons and years). Additional future work will attempt to detect any correlation between GWSS age and presence and quantity of PD strain *Xf* present in the foregut of the insect.

The AFLP and 454 pyrosequencing analysis generated a total of 31,593 sequence fragments from 25 GWSS genomic DNA samples from collected from seven vineyards (**Table 1**) with sequence sizes ranging from to 124 to 467 bp. **Figure 3** shows a subset of sequences from sample 10D from Post Oak Vineyard. Future analysis of the AFLP sequencing data will focus on clustering the data into identical sequences within samples and performing metapopulation structure population structure analysis using a variety of techniques.

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

ACKNOWLEDGEMENTS

We thank the vineyard owners and managers for participating in the Texas Pierce's Disease Program

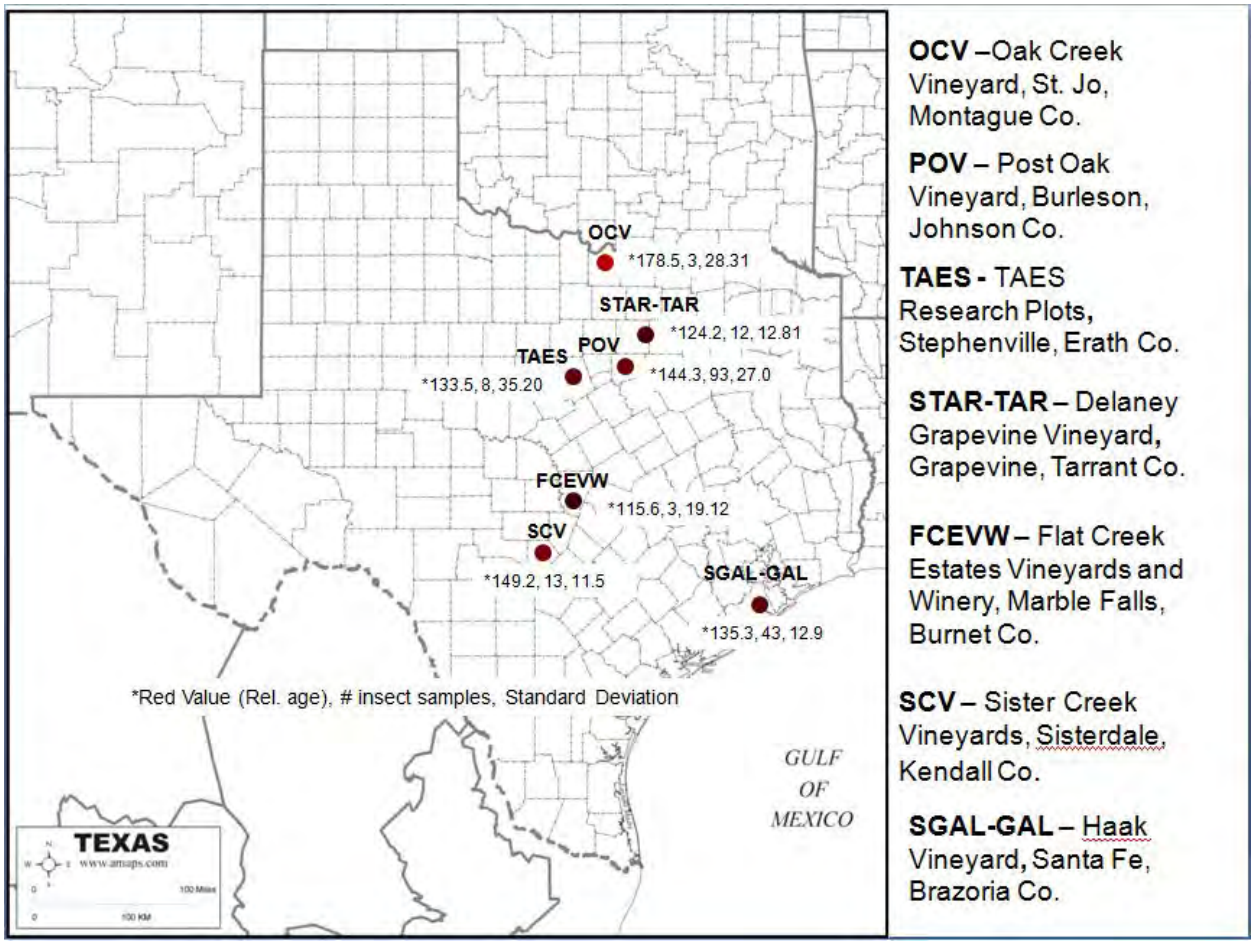

Figure 1. The average relative ages of GWSS from seven vineyards across Texas. Relative ages of GWSS collected from Texas vineyards between the dates of 07/31/2007 and 09/04/2007 were determined by measuring red pigmentation in the wings, averaged for each vineyard, and representing those ages as circle with amounts of red proportional to the average amount of red pigment found in the wings. Lower red pigmentation values represent greater age and vice versa.

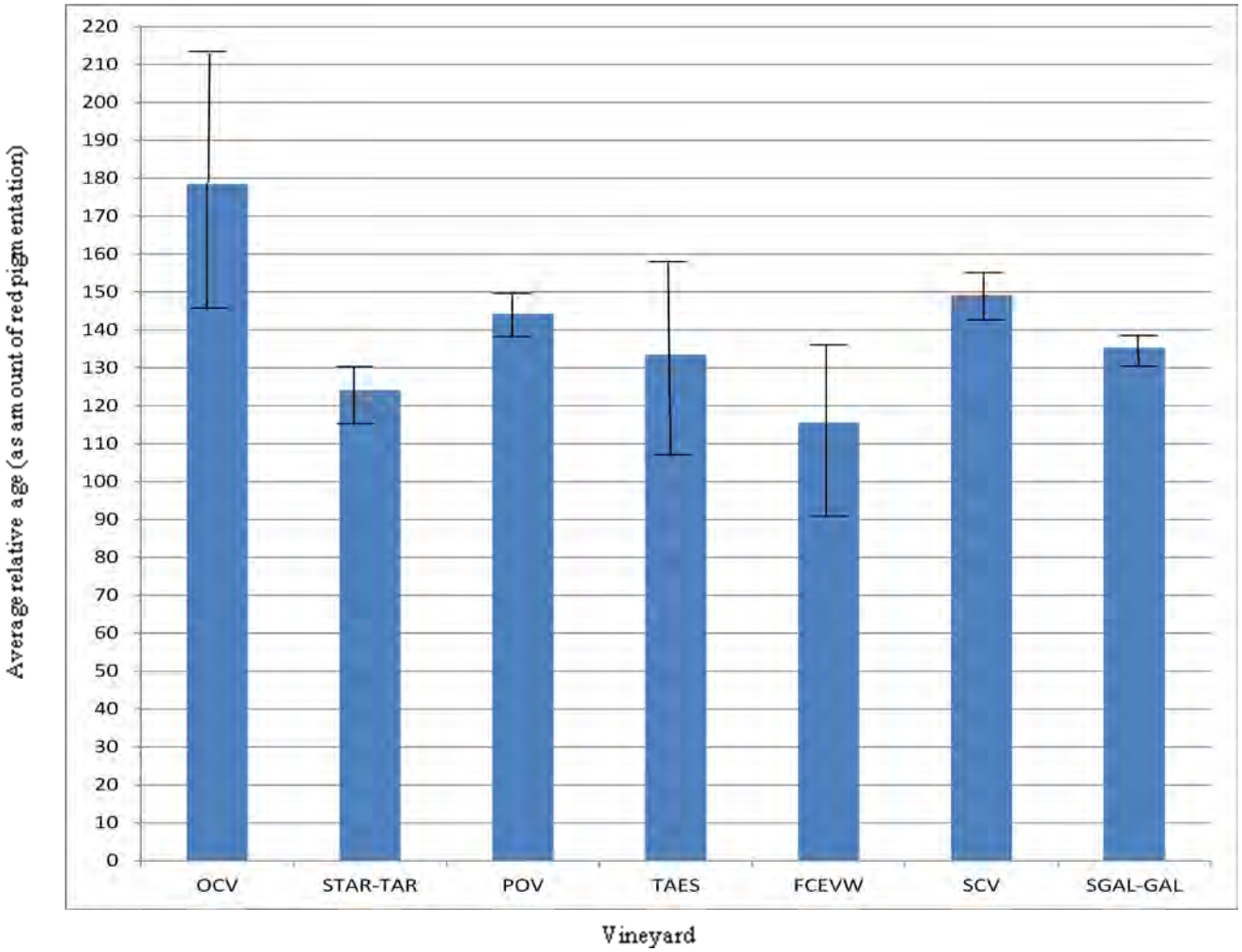

Figure 2. The average relative ages of GWSS from seven vineyards across Texas. Relative ages of GWSS collected from Texas vineyards between the dates of 07/31/2007 and 09/04/2007 were determined by measuring red pigmentation in the wings and averaged for each vineyard. Lower red pigmentation values represent greater age and vice versa. Error bars represent confidence intervals of 95% for the population mean.

Table 1. Quantity of AFLP sequence fragments generated from AFLP PCR and sequencing analysis. Total genomic DNA was extracted from 25 GWSS from seven different vineyards and amplified with amplified fragment length polymorphism (AFLP) PCR. Fragments generated from AFLP were sequenced with 454 pyrosequencing.

Vineyard	Sample #	# of AFLP sequence fragments
Flat Creek Estates Vineyards and Winery, Marble Falls, Burnet Co.	32A	1037
Flat Creek Estates Vineyards and Winery, Marble Falls, Burnet Co.	33A	496
Flat Creek Estates Vineyards and Winery, Marble Falls, Burnet Co.	34A	923
Haak Vineyard**,** Santa Fe, Brazoria Co.	53A	1049
Haak Vineyard**,** Santa Fe, Brazoria Co.	57G	1353
Haak Vineyard**,** Santa Fe, Brazoria Co.	57J	1913
Haak Vineyard**,** Santa Fe, Brazoria Co.	57V	1463
Oak Creek Vineyard, St. Jo, Montague Co.	23A	1136
Oak Creek Vineyard, St. Jo, Montague Co.	23B	1286
Oak Creek Vineyard, St. Jo, Montague Co.	24A	1327
Post Oak Vineyard, Burleson, Johnson Co.	10D	1095
Post Oak Vineyard, Burleson, Johnson Co.	12E	1638
Post Oak Vineyard, Burleson, Johnson Co.	40I	1661
Post Oak Vineyard, Burleson, Johnson Co.	43D	1153
Sister Creek Vineyards, Sisterdale, Kendall Co.	26B	1095
Sister Creek Vineyards, Sisterdale, Kendall Co.	29A	1903
Sister Creek Vineyards, Sisterdale, Kendall Co.	30B	1539
Sister Creek Vineyards, Sisterdale, Kendall Co.	31A	1026
TAES Research Plots**,** Stephenville, Erath Co.	17A	1169
TAES Research Plots**,** Stephenville, Erath Co.	18A	1063
TAES Research Plots**,** Stephenville, Erath Co.	21B	1214
TAES Research Plots**,** Stephenville, Erath Co.	22A	1102
TJ	27C	1071
TJ	27E	976
TJ	27JK	1905
Total		31593

Figure 3. A subset of sequences generated from amplified fragment length polymorphism (AFLP) and sequencing analysis from GWSS DNA sample 10D from Post Oak Vineyard, Burleson, Johnson Co. TX. Total genomic DNA was extracted from 25 GWSS from seven different vineyards and amplified with amplified fragment length polymorphism (AFLP) PCR. Fragments generated from AFLP were sequenced by 454 pyrosequencing.

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VOUCHERING SPECIMENS OF EGG PARASITOIDS OF THE GLASSY-WINGED SHARPSHOOTER COLLECTED BY THE CDFA PIERCE'S DISEASE BIOLOGICAL CONTROL PROGRAM IN CALIFORNIA AND TEXAS A&M IN TEXAS

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Reporting Period: The results reported here are from work conducted July 2011 to October 2011.

ABSTRACT
This is a new project which has just started; its main objectives are to label (using archival paper and unique plastic database numbers), identify, database (including georeferencing), preserve, and partially dry from ethanol and point-mount about 17,000 voucher specimens of mymarid and trichogrammatid egg parasitoids of the glassy-winged sharpshooter (GWSS). These were either collected (reared) by the CDFA GWSS Biological Control Program personnel in California since 2001 in the course of pre- and post-release surveys, are irreplaceable vouchers of the colonies of the biological control agents that were released in California (both exotic and native), or were collected by staff of Texas A&M in Texas since 2007. The specimens from Texas will be transferred into leak-proof vials with good stoppers to prevent alcohol leakage, labeled properly using acid-free archival paper, and databased using UC Riverside Entomology Research Museum numbering system which then can be made available online if desired. Valuable representatives of each species (at least 10% of all the specimens) will be dried from ethanol using a critical point dryer and point-mounted as museum quality vouchers, and also will be labeled and databased. Taxonomic identifications will be checked and, when necessary, corrected by the PI. Most voucher specimens will be eventually transferred for storage to the California State Collection of Arthropods (CDFA) in Sacramento, its permanent depository; some duplicate representatives and a few taxonomically important specimens will be also deposited in the UC Riverside Entomology Research Museum.

LAYPERSON SUMMARY
Important, irreplaceable, and numerous voucher specimens of the glassy-winged sharpshooter egg parasitoids from California and Texas will be curated in the course of this one-year project. Museum-quality specimens will be prepared and preserved.

INTRODUCTION
Recently (since 1997), major efforts have been undertaken by the CDFA/glassy-winged sharpshooter (GWSS) Biological Control Program to survey for egg parasitoids of GWSS in California and to release several egg parasitoid species (*Anagrus epos* Girault, *Gonatocerus* spp.) from other states in the USA and also northeastern Mexico as part of the classical biological control effort (CDFA 2011).

It is well known that the taxonomic impediment in identification of natural enemies may adversely affect the biological control efforts against agricultural pests. In the case of the GWSS, early misidentifications (due to objective reasons, such as partially inaccurate existing keys) of one of the species of the California native egg parasitoids of GWSS (as *Gonatocerus morrilli* (Howard)) resulted in the inability of biological control practitioners to distinguish them from the introduced "real" *G. morrilli* from Texas and northwestern Mexico. Therefore, contamination of the colonies in the mass-rearing program was noticed only after the molecular methods distinguished them as two genetically different entities. The "California *G. morrilli*" was later described taxonomically as a new species, *Gonatocerus walkerjonesi* Triapitsyn, based on the combination of molecular evidence and some morphological differences that are difficult to observe without special preparation of the specimens (Triapitsyn 2006). Another native species, *Gonatocerus morgani* Triapitsyn, was also described from Orange Co., CA (Triapitsyn 2006); it is now being mass-produced and released in other parts of California infested with GWSS (Son et al. 2011). As proper part of the ongoing biological control program against GWSS, the CDFA GWSS Biological Control Program has conducted extensive pre- and post-surveys of the egg parasitoids of GWSS in California from 2001. These surveys, which also included egg parasitoids of the native sharpshooter in California, the smoke-tree sharpshooter *Homalodisca liturata* Ball, have resulted in collection of about 10,000 specimens of egg parasitoids (Mymaridae and Trichogrammatidae) which are stored, along with voucher specimens of the numerous colonies of GWSS egg parasitoids maintained by the CDFA, in several thousand vials at the CDFA Mt. Rubidoux Field Station in Riverside, CA. Also, Dr. Forrest L. Mitchell has kindly donated to the CDFA GWSS Biological Control Program 900 vials of GWSS parasitoids collected in Fredricksberg Co., TX, by Texas A&M staff. These insects were collected since 2007, each vial containing parasitoids that have emerged from a single egg mass (ca. 8 individuals per vial). Date and location from which each mass emerged have been recorded.

OBJECTIVES

1. Check the taxonomic identities of all the specimens of GWSS egg parasitoids from California; pull out specimens of taxonomic and voucher interest.
2. Transfer of the bulk of the voucher specimens from Texas into leak-proof vials for long-term storage; label properly (using archival, acid-free paper) and database all the vials using barcodes with unique numbers. Fully identify and catalog approximately 7,000 GWSS parasitoids collected by Texas A&M in the native range of GWSS.
3. Prepare valuable representatives of each species (at least 10% of all the specimens) that will be dried from ethanol using a critical point dryer, point-mounted as museum quality vouchers, labeled, and databased.

RESULTS AND DISCUSSION

This is a new project, currently the PI is working on the identifications of the thousands of specimens from California and on pulling out the voucher specimens of taxonomic interest for drying from alcohol and point-mounting, followed by labeling and databasing. Particularly, all the specimens of *Ufens* (Trichogrammatidae) are being identified to species: two are known in North America as GWSS egg parasitoids (Triapitsyn 2003; Al-Wahaibi et al. 2005). As soon as specimens from Texas are received (they are now being prepared to be shipped), work will begin on them as well.

CONCLUSIONS

We are curating these collections to preserve the invaluable voucher specimens for further analyses (including molecular, distributional, taxonomic, biological, etc.), thus making them available. The specimens and information on them will be useful for the California Department of Agriculture GWSS/PD Biological Control Program and biological control research practitioners in this state and beyond.

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ACKNOWLEDGMENTS

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EFFECTS OF PLANT WATER STRESS AND TEMPERATURE ON GLASSY-WINGED SHARPSHOOTER FEEDING IN RELATION TO TRANSMISSION OF *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted October 1, 2004 to September 30, 2011.

ABSTRACT
The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar), is an economically important pest of grapevine, stone fruits, nursery trees, and ornamental plants in California because it transmits *Xylella fastidiosa* (*Xf*). Two related studies examined whether GWSS feeding behaviors that control *Xf* acquisition and inoculation are affected by environmental stresses such as plant water stress, cold ambient temperature, and diel light conditions. Both studies monitored feeding via electrical penetration graph (EPG). Effects of ambient air temperatures and light intensities on GWSS feeding on *Euonymus japonica* plants were studied outdoors (in Bakersfield, CA; a certified infested, non-agricultural area) during early spring. Effects of plant water stress were studied indoors, comparing feeding on well-watered vs. water-stressed citrus under high-pressure sodium vapor lamps. For both studies, EPG waveforms representing pathway phase (searching for xylem), X waves (xylem contact, likely to control *Xf* inoculation), and waveform C (ingestion of xylem fluid, *Xf* acquisition) were analyzed. Results showed no significant differences in feeding duration on *Euonymus japonica* for daylight vs. nighttime light intensities. However, xylem-sap ingestion occurred for significantly longer duration when ambient temperatures were higher than 10°C, and only occurred at temperatures below 10°C when ingestion was continued from a preceding, warmer period. Xylem-sap ingestion also was longer on well-watered than water-stressed citrus plants. Frequencies of X waves were higher, both at high temperatures and when plants were well-watered. Thus, both acquisition and inoculation behaviors are increased during warm air temperatures and when plants are well-watered.

FUNDING AGENCIES
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We thank Theresa de la Torre and Holly Shugart, ARS Parlier, for technical assistance.

IDENTIFICATION OF APPROPRIATE SYMBIONTS FOR PARATRANSGENIC-BASED CONTROL OF THE GLASSY-WINGED SHARPSHOOTER

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Reporting Period: The results reported here are from work conducted April 2010 through October 2011.

ABSTRACT
Glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis* (Germar, 1821)) is a highly polyphagous Hemipteran pest of wine and table grapevines and transmits the xylem limited bacterium *Xylella fastidiosa*, the causal agent of Pierce's disease in grapevines (*Vitis*, L.). In a previous study which surveyed the GWSS microbiome, sequences homologous to *Delftia sp*. were identified exclusively in the insect's hemolymph. Based on the results of the survey, this bacterium was selected as the most likely candidate for future paratransgenesis-based control studies. In this study, *Delftia* was isolated from the hemolymph of GWSS and identified by PCR.

PHENOLOGY OF *XYLELLA FASTIDIOSA* AND *DRAECULOCEPHALA MINERVA* IN CALIFORNIA ALMOND NURSERIES: AN ASSESSMENT OF PLANT VULNERABILITY TO ALMOND LEAF SCORCH DISEASE

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ABSTRACT
Almond leaf scorch disease is caused by the xylem-limited bacterium *Xylella fastidiosa (Xf)*, which is transmitted by several species of leafhoppers. The objectives of this research were to elucidate the fate of bacterial inoculations in almond nursery plants; to elucidate patterns of insect vector population dynamics and movement relative to host plant assemblages in habitats surrounding commercial nurseries; and to investigate the temporal distribution of *Xf*-infected plants in those habitats. In an experimental nursery, disease incidence was markedly affected by rootstock type. Prior to budding, nursery plants were immune from bacterial infection if using Nemaguard roostock. After budding with a susceptible scion, plants were vulnerable to infection regardless of the rootstock type. Surveys in commercial nurseries revealed that only habitats with permanent grass cover sustained vector populations throughout the season. A total 87 plant samples tested positive for *Xf* (6.3%) using ELISA, with a higher number of *Xf*-infected plants found in weedy alfalfa fields than in other habitat types. Among *Xf*-positive plants, 33% were winter annuals, 45% were biannuals or perennials, 22% were summer annuals. Collectively, these findings identified an infection pathway other than primary spread that may occur in established orchards.

FUNDING AGENCIES
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We thank Alessandra Rung and Raymond Gill for identifying the leafhoppers; Bradley D. Hanson, Ellen Dean, and Joseph M. DiTomaso for their help identifying the plants; Theresa De La Torre, Greg Phillips, Mario Venegas, and Aaron J. Salyers for technical assistance; and the anonymous almond nurseries and their neighbors for providing research sites.

GLASSY-WINGED SHARPSHOOTER EXCRETA PRODUCTION AND EGG MATURATION ON GRAPEVINES

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ABSTRACT
To better understand glassy-winged sharpshooter (GWSS) movement and reproduction in vineyards, studies evaluating GWSS feeding (as measured by excreta production) and egg maturation on grapevines were conducted. In 2010, studies compared excreta production and egg maturation of female GWSSs on self-rooted grapevines (cv. Chardonnay) that were inoculated with *Xylella fastidiosa (Xf)* or water. For reference, excreta production and egg maturation was simultaneously evaluated on cowpea. The mean number of eggs matured per female on each test plant was determined by confining females to plant stems, allowing females to feed but discouraging them from ovipositing. After five days, each female was dissected to determine egg load (number of mature eggs carried in the abdomen). A subset of females was dissected prior to the start of the experiment (referred to as baseline), so that the mean number of eggs matured during the test could be determined. Females held on cowpea produced 3.6 times more excreta and carried 3.6 times more eggs than females held on grapevines, regardless of whether the grapevines were inoculated with *Xf*. Further, egg loads of females held on grapevines for five days were not significantly greater than egg loads of females that were dissected prior to the start of the experiment, suggesting that females on grapevines did not mature any eggs during the test. Two studies were completed in 2011, both using grapevines (cv. Chardonnay) grafted on a 101-14mg rootstock. The first study in 2011 compared excreta production and female preference for *Xf* inoculated versus water inoculated grapevines. While females on water inoculated grapevines produced more excreta than females on *Xf* inoculated grapevines the difference was not significant. Nonetheless, in choice tests females were most frequently observed on water inoculated versus *Xf* inoculated grapevines. The second study in 2011 compared excreta production and egg maturation of females on non-inoculated grapevines and cowpea. Similar to tests in 2010, egg maturation was evaluated by comparing egg loads of females after one week of feeding on test plants to egg loads of females dissected prior to the start of the test. While females on cowpea produced 1.5 times more excreta and 1.4 times more eggs than females on grapevine, the relative differences in excreta production and egg maturation between cowpea and grapevine were smaller in 2011 than in 2010. Further, whereas females on grapevine in 2010 did not produce significantly more eggs than baseline females, females on grapevine during tests in 2011 carried 2.3 times more eggs than baseline females. Accordingly, excreta production and egg maturation of females on grapevines in tests in 2011 appeared to be greater than that of females on grapevines in 2010. One possible explanation for this observation is that tests in 2010 used self-rooted Chardonnay whereas tests in 2011 used Chardonnay grafted on a rootstock. Tests in 2012 will directly compare GWSS performance on self-rooted Chardonnay versus Chardonnay grafted on a rootstock.

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Section 2: Vector Management

DEVELOPMENT OF EFFECTIVE MONITORING TECHNIQUES FOR SHARPSHOOTERS AND THEIR PARASITOIDS.

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ABSTRACT

Gonatocerus morgani, *Gonatocerus morrilli*, and *Gonatocerus triguttatus* have been reared and released by the Pierce's Disease program at sites throughout Southern California and the southern Central Valley since 2000. However, data concerning the extent of released species populations, the effects of parasitism by native competitors, and the host preferences of the parasitoids involved is still needed, even though data presented in the most recent CDFA report (2010) demonstrates the effectiveness of the release program. In conjunction with his research in comparative and functional genomics of *Xylella fastidiosa (Xf)*, D. Cooksey has developed a multiplex PCR system for the simultaneous identification of *Xf* strains (Hernandez-Martinez *et al.*, 2006). D. Morgan, 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, is the supervisor of the release program. C. LeVesque directs a high throughput testing program for citrus Huanglongbing disease that employs high resolution melting curve analysis as developed by Lin *et al*., 2011. The development of the proposed high resolution melting (HRM) real-time PCR system will greatly enhance the data acquisition of the CDFA parasitoid release biocontrol program which will assist in assessing the efficacy of the ongoing sharpshooter egg parasitoid strategy.

LAYPERSON SUMMARY

In order to efficiently use biological control agents it is essential to have the capacity to identify the parasitoid species, host species and the extent of parasitism. These parameters must be known in order to evaluate the effectiveness of the control strategy. The current method used in the glassy-winged sharpshooter (GWSS) biological control program relies on identification of eclosed parasitoids after long incubations under artificial conditions. Often the parasitoids do not survive. It would greatly facilitate the development of the release program if an accurate and rapid method for identification of the eggs of sharpshooter species, determining whether eggs are parasitized, and by which parasitoid species, were available. The proposed single-step real-time HRM PCR assay for sharpshooters and their parasitoids will provide such a tool and will significantly enhance the reporting of GWSS parasitism.

INTRODUCTION

The post-release collection and incubation of field-collected glassy-winged sharpshooter (GWSS) eggs is currently the only methodology available for monitoring the GWSS biocontrol program. Since the eggs are removed from the field before development has been completed, the possibility of further parasitism is eliminated and, therefore, parasitism rates are underestimated. In addition, 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. This results in some species being significantly underreported. If there is no sharpshooter emergence, there currently is no economical method for identifying whether eggs are from GWSS or the native smoke tree sharpshooter (STSS). Therefore, it is essential that a more efficient method for monitoring biological control activity be developed if we are to have more accurate, timely, and economic reporting of GWSS parasitism.

Accurate reporting of GWSS parasitism will be accomplished with the development of a single-step HRM real-time PCR assay for sharpshooters and their parasitoids. The identity of the species of host, GWSS or STSS, and its parasitoids can simultaneously be determined with this method within half a day of collection, rather than two weeks. In addition, because the wasp pupal and sharpshooter egg casing can be analyzed, old egg masses should be able to be used after wasp eclosion. The refinement of control strategies by determining the effectiveness of the different parasitoid species in the various environments encompassed in the current range of GWSS will lead to better suppression of GWSS populations.

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

Degenerate primers were designed to target the COI gene in each of the wasp species, while a separate pair of primers were designed to target the STSS and GWSS COI genes (**Table 1**). These were used to clone ~150 bp fragments from each species to be used as control plasmids for HRM (**Figure 1**).

While HRM primers and reaction conditions are being optimized, GWSS eggs are being collected which have been parasitized by the different wasp species included in the study. These eggs are obtained by setting up fresh egg masses with recently eclosed female wasps. The GWSS eggs are dissected from the leaf tissue at specific intervals after the wasps have been observed ovipositioning.

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Table 1. Primer sequences where R = A,G; K = G,T; and W = A,T.

Primer Name	Sequence
WP-COI-F	5'-CATAGAGGWCCWTCWGTAGATTTATC-3'
WP-COI-R	5'-GATCAACAAAATAARGTWAKTTTTTC-3'
SS-COI-F	5'-GCAACTATAATTATTGCAGTACC-3'
SS-COI-R	5'-GATCATATTATTGAAATTGATATA-3'

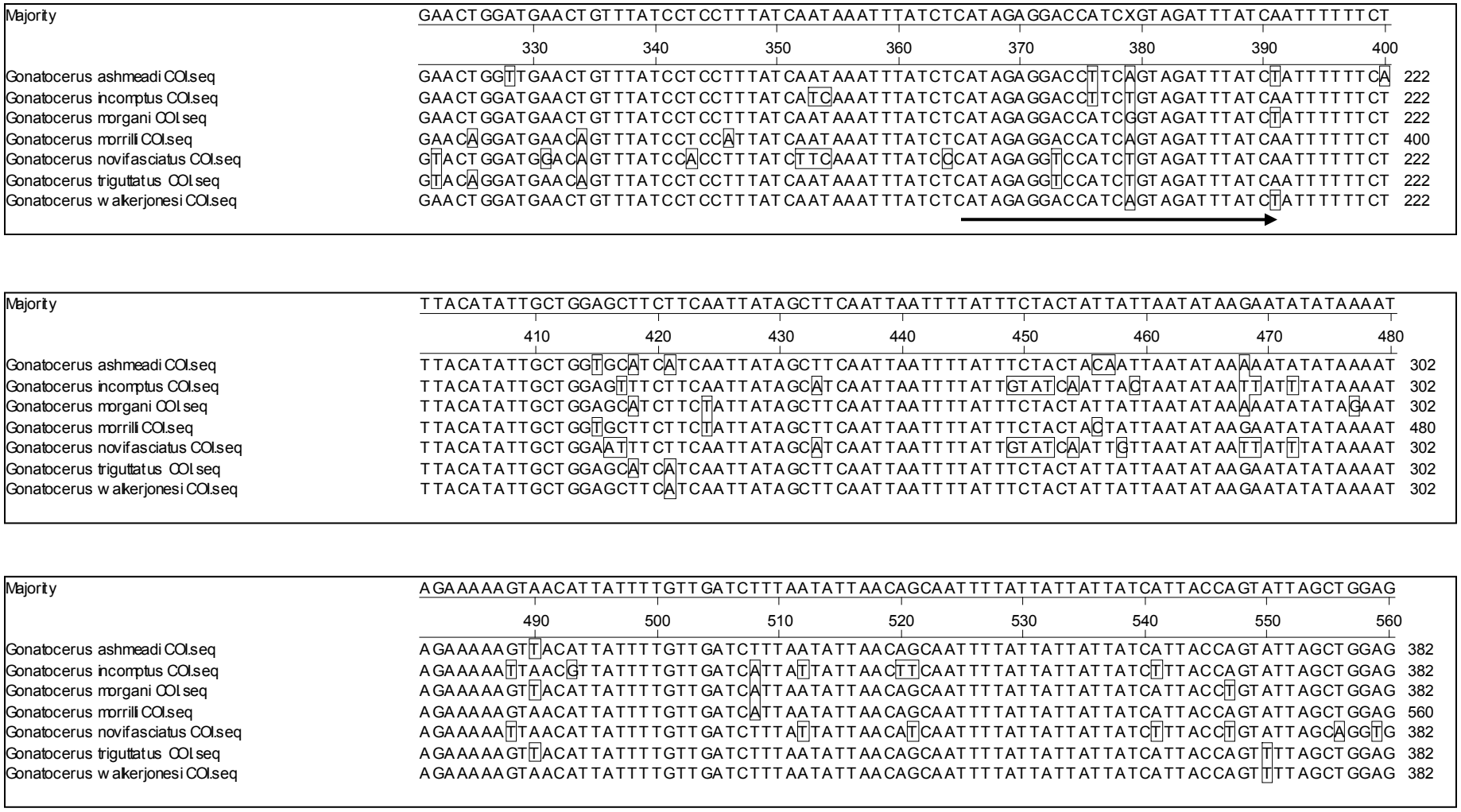

Figure 1. Alignment of *Gonatocerus* COI sequences using ClustalW (Slow/Accurate, IUB) with sequence differences boxed and primers indicated by arrows:

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. In each of the past two seasons we have surveyed 34 vineyards in the Temecula Valley that differ in their use of systemic insecticides. In 2011, as in 2010, vineyards that either consistently or irregularly applied imidacloprid to vines had similarly low PD prevalence. Prevalence was more variable in those vineyards that have not treated with systemic insecticides, with several vineyards having nearly no diseased vines but also two vineyards that showed more than 5% disease. These surveys will continue for a final season, after which we will use changes in prevalence among years and estimates of GWSS abundance to estimate rates of pathogen 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 sharpshooter 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 sharpshooter 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 sharpshooters spreading Pierce's disease (PD) 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 thus far indicate that on average vineyards that employ systemic insecticides tend to have low PD prevalence. Yet, given that some of the untreated or intermittently treated fields had extremely low prevalence, it is plausible that systemic insecticides may not need to be applied every year – at least for sites or years with low vector pressure. We will continue these disease surveys for a final year, and then calculate overall estimates of disease spread in the region.

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 glassy-winged sharpshooter. 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 glassy-winged sharpshooter 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 sharpshooter 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 sharpshooters 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 glassy-winged sharpshooter abundance and, ultimately, PD severity.

OBJECTIVES

1. Understand if within-vineyard sharpshooter chemical control reduces vector pressure and PD spread.

We are currently in the middle of the second of three seasons of fall disease surveys. Next year we will conduct the final season of surveys, then use the three years of disease prevalence estimates, vector abundance, imidacloprid concentrations, and *Xf* genotype distribution to draw inferences regarding the relative rates of pathogen spread in fields differing in management tactics.

RESULTS AND DISCUSSION

Last summer we interviewed several vineyard owners and vineyard managers in the Temecula region to identify vineyards with a range of imidacloprid treatment histories. This season we collected leaf samples from all 34 vineyards included in the study (**Figure 1**), based on those interviews, to verify that our information regarding imidacloprid treatment history was accurate. Those samples are currently being processed.

Beginning in February 2011 we deployed yellow-sticky traps in each of the vineyards to monitor GWSS populations. The traps were collected monthly, GWSS and generalist predators were counted, then new traps were deployed. This monitoring data is still being collated. June sharpshooter counts up through June were very low, with no obvious difference in catches between vineyards differing in chemical control strategy.

Like last year, we again surveyed PD prevalence in the 34 vineyards. This work is ongoing. Thus far the estimates based only on visual symptoms have been completed (**Figure 2**). The results suggest that there is substantial variability in disease prevalence among sites. Sites treated every year or irregularly with systemic insecticide had similarly low apparent disease prevalence based on visual scores (1.1% and 1.5%, respectively). On average, sites that were not treated with imidacloprid

for at least several years had higher prevalence (2.6%), but with a large range from just 0.5% to nearly 7%. Given the potential for misidentification of disease status based on visual surveys we collected up to 50 putatively symptomatic samples from each vineyard to be cultured for the presence of *Xf*. In addition we collected 100 asymptomatic samples from each vineyard to be tested for latent infection using ELISA. These asymptomatic and symptomatic samples are currently being processed, the results of which will be used to refine the estimate of disease prevalence based on symptoms alone.

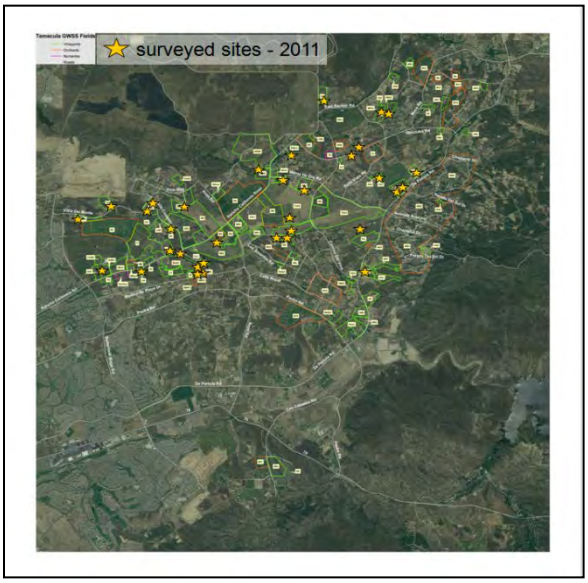

Figure 1. Field sites included in the 2010 and 2011 Pierce's disease surveys. Collectively the sites represent 34 separate vineyard blocks that have been untreated (n=9), intermittently treated (n=12), or regularly treated (n=13) with imidacloprid.

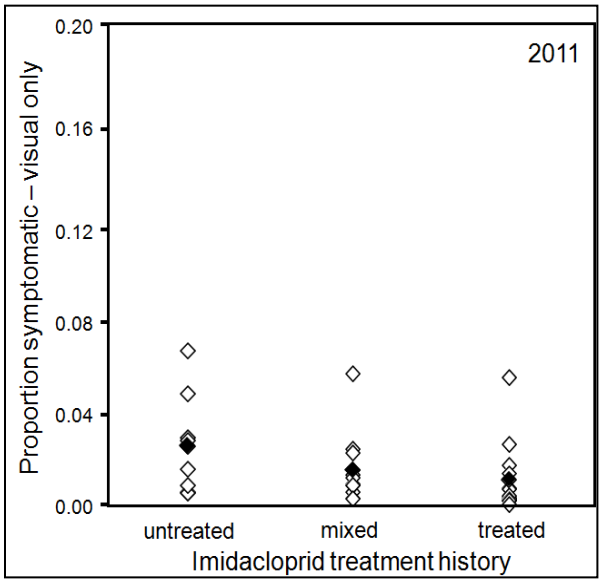

Figure 2. Proportion of vines with Pierce's disease symptoms in fields with different insecticide treatment histories. Observations from preliminary 2011 surveys in Temecula Valley based on putative symptoms alone. Some points overlap (n=9, 12, 13 for Untreated, Mixed and Treated respectively). Filled symbols represent means.

CONCLUSIONS

Results so far suggest that current PD prevalence throughout the Temecula Valley region is low. There is a trend for differences in prevalence based on chemical control strategies, with untreated vineyards having the highest average prevalence, but whether those differences are due to recent management, historical artifacts, or differences in vector pressure remains unclear. Ultimately estimates of year-to-year changes in prevalence are needed (i.e. incidence), which are ongoing, to determine the precise impact of within-vineyard systemic insecticides on disease spread.

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DEVELOPMENT AND USE OF RECOMBINANT *HOMALODISCA COAGULATA VIRUS-1* FOR CONTROLLING THE GLASSY-WINGED SHARPSHOOTER.

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ABSTRACT
The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is an efficient vector of *Xylella fastidiosa (Xf)*, causal agent of Pierce's disease of grapevines. We are investigating using recombinant insect viruses to deliver toxic peptides and/or induce systemic RNA interference (RNAi) and thereby induce mortality in recipient GWSS. We are attempting to develop recombinant *Homalodisca coagulata virus-1* (HoCV-1) so as to specifically infect GWSS. We will engineer HoCV-1 to deliver and express toxic peptides and/or effector RNAs only upon infection of recipient GWSS. We are taking two approaches to develop recombinant HoCV-1, one a straightforward cDNA cloning approach and the second involves amplification using a baculovirus. We are also using a second virus, *Flock house virus*, for which recombinant clones are already available. It is our hope that the results obtained from these studies will be used for developing an effective biological-based control strategy to help control GWSS and other sharpshooter vectors of *Xf*.

LAYPERSON SUMMARY
The glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*) transmits the bacterium, *Xylella fastidiosa (Xf)*, which causes Pierce's disease of grapevines. We are attempting to use natural, GWSS-infecting viruses as part of a strategy to kill GWSS. We are attempting to engineer viruses to deliver toxic peptides, and or deliver GWSS RNAs that will activate the RNA interference (RNAi)-based immune system. We hope that one or both approaches will result in GWSS mortality thereby preventing the spread of *Xf*. RNAi can be triggered by effector RNAs, and results in degradation of the specific RNAs that have sequence homology with the effector RNA. It is known that in plants and some insects, RNAi occurring in localized tissues can trigger RNAi in the whole organism. For example, actin is a protein that is indispensible in normal cell function, and if a double-stranded RNA containing actin mRNA sequence is introduced to a localized tissue of an organism, the organism will not have enough actin produced locally in the inoculated tissue or in whole system depending on whether a systemic RNAi occurs in the organism or not. We are attempting to determine whether systemic RNAi can be induced in GWSS by using recombinant *Homalodisca coagulata virus-1* (HoCV-1), a naturally-occurring virus that specifically infects GWSS. If successful, our studies may lead to new and effective methods to help control the GWSS population.

INTRODUCTION
The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is a highly efficient vector of *Xylella fastidiosa (Xf)*, the causal agent of Pierce's disease (PD) of grapevines. We are investigating new approaches based on using a GWSS-infecting virus, *Homalodisca coagulata virus-1* (HoCV-1), as a tool to help manage GWSS. We will engineer HoCV-1 to express toxic peptides which can kill infected GWSS upon virus replicaion. As a second approach we are attempting to use HoCV-1 to deliver GWSS RNAs so as to induce RNA interference (RNAi) in recipient GWSS.

RNAi is a gene silencing mechanism that is initiated by a presence of double stranded RNA (dsRNA). The resulting RNAi activates the cell machineries for identification and degradation of the target RNA, which is the RNA having homologous sequence to the dsRNA that has triggered RNAi. This mechanism is known to occur in many eukaryotes such as plants, animals and insects as an indispensible part of normal developmental processes and innate immunity. In plants, locally triggered RNAi induced by viral RNA replication or an experimental inoculation of dsRNA sends a warning signal to the whole plant so that the cells not invaded by the dsRNA are prepared to silence the foreign RNA that may invade them. This phenomenon is called systemic RNA interference, and the signal responsible for triggering the systemic silencing is a small interfering RNA (siRNA) that results from the RNAi pathway. The resulting siRNA is presumed to be amplified by a plant RNA dependent RNA polymerase (RdRp). Although insects are not known to have RdRp, systemic silencing occurs in some insects through some unknown mechanisms (Huvenne and Smagghe). It is unknown whether feeding of dsRNA or short-hairpin RNA (shRNA) can cause systemic silencing in GWSS or not.

We are now attempting to develop an infectious clone of *Homolodisca coagulata virus-1* (HoCV-1). HoCV-1 is a virus isolated from field-collected GWSS. It is a single-stranded, positive-sense RNA virus belonging to the family *Dicistroviridae*. It has a monopartite genome composed of uncapped 5'-end and polyadenylated 3'-end. Although its sequence has been published, no infectious clone is yet available. We will use the GWSS embryonic cell-line, Z-15 (Kamita et al., 2005), as well as whole GWSS as tools for our work. Once the infectious clone is developed, we will modify the HoCV-1 genome sequence to produce a highly-specific, recombinant virus either delivering toxic peptides or anti-GWSS RNA effectors with the ultimate goal of helping to control GWSS populations.

OBJECTIVES

1. Development of HoCV-1 infectious cloned cDNAs;
2. Expression of GFP or other stable sequences in GWSS-Z15 cells or whole GWSS insects by using HoCV-1.

RESULTS AND DISCUSSION

We are only three months into this project. Our primary focus so far has been to develop a virus, preferably HoCV-1, that we can use for our peptide/RNA expression studies. While our main effort has been with HoCV-1, we have also added a second virus that should be easier to manipulate at least initially. This is *Flock house virus* (FHV). FHV belongs to the family *Nodaviridae*, and is a non-enveloped, positive-sense RNA virus originally isolated from the grass grub (*Costelytra zealandica*) in New Zealand. It has a bipartite genome and has been shown to multiply in insects from four different orders (Hemiptera, Coleoptera, Lepidoptera, Diptera). In adult mosquitoes, FHV has been used successfully to systemically express GFP (Dasgupta et al., 2003), and thus we are attempting to use the FHV system (Dasgupta et al., 2003) to systemically express GFP in GWSS. This will serve as a surrogate virus system until our HoCV-1 system is optimized. FHV will be used to express peptides, and to perform initial systemic RNAi experiments using GWSS.

Testing for Systemic Silencing in GWSS

We have FHV cDNA clones that can produce FHV genomic RNAs and make infectious FHV particles in *Drosophila* S2 cells. In order to confirm the biological activity of the constructs, we co-transfected S2 cells with the copper-inducible *Drosophila* metallothionein promoter driven FHV RNA1 cDNA and FHV RNA2 cDNA, and collected the transfected cells and the medium 4 days post transfection. Also, in parallel, we co-transfected another batch of S2 cells with FHV RNA1 cDNA fused to GFP sequence and FHV RNA2 cDNA. As expected, the S2 cells transfected with these constructs produced GFP signal (**Figure 1**)

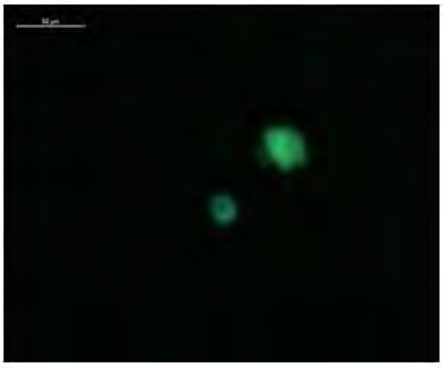

Figure 1. GFP signal produced by FHV in transfected Drosophila S2 cells.

In adult mosquitoes, FHV has been engineered to systemically express GFP (Dasgupta et al., 2003). We hope to adapt this system to test for systemic silencing in GWSS. GFP will be systemically expressed in GWSS with FHV DIeGFP (**Figure 2**). A defective interfering (DI) RNA of FHV originating from the genomic RNA 2 interferes with FHV genomic RNA replication through greater template efficiency with the viral replicase. The construct DIeGFP that contains the viral replication sites, packaging signal and expresses GFP is available, which we will use to produce FHV virions containing DIeGFP. This will be accomplished by transfecting *Drosophila* Schneider 2 (S2) cells with the pMT vectors expressing FHV RNA1, RNA2, and DIeGFP.

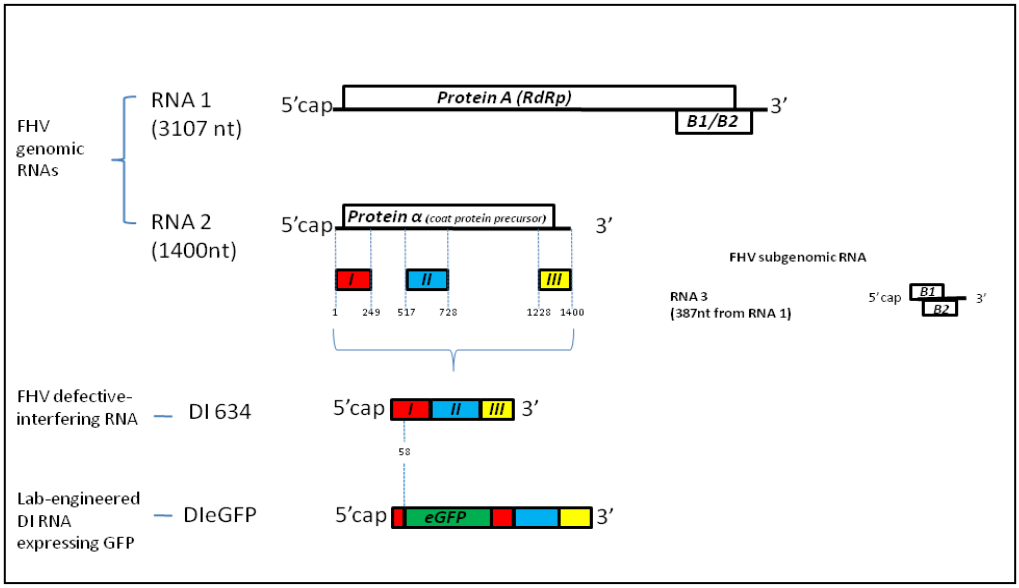

Figure 2. Lab-engineered DI RNA expressing GFP (Dasgupta, et al., 2003**.)**

Once infectious virions have been produced in the cell system we will begin whole insect experiments. The purified virions will be injected into GWSS for GFP expression, which we will confirm with fluorescent microscopy. To then test for systemic silencing of GFP we will feed GFP-expressing GWSS GFP dsRNA or short-interfering RNA and analyze insect tissues using fluorescent microscopy.

Making an Infectious Clone of *Homalodisca coagulata Virus-1*

We are using two approaches to develop recombinant, infectious HoCV-1. First, we will use a binary expression system involving expression of bacteriophage T7 polymerase and a double-stranded DNA with a T7 promoter driven HoCV-1 sequence in GWSS embryonic cell-line, Z-15. Such a binary expression system has been used in animal cells to produce infectious viruses including poliovirus. We have constructs expressing T7 polymerase with or without nuclear localization signal (NLS) that has been tested in yeasts, plants, and mammalian cells (Dunn et al., 1988). We have subcloned the T7 polymerase gene under an insect promoter (IE) and will co-transfect Z-15 cells with T7 driven HoCV-1 construct (**Figure 3**).

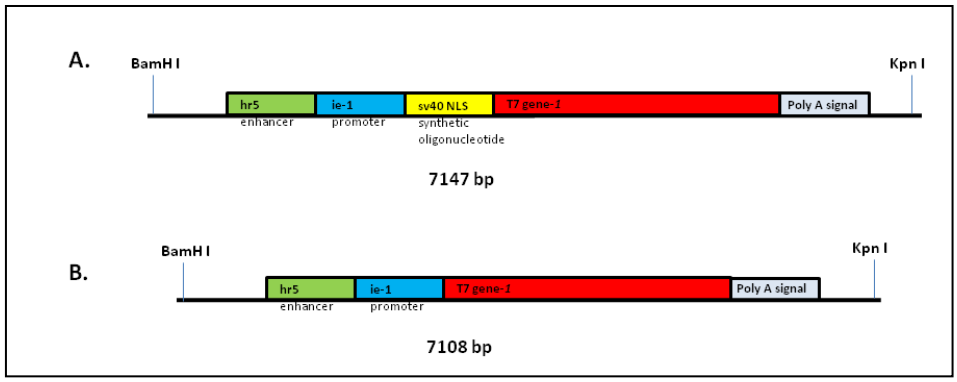

Figure 3. Maps of pIE T7 gene-1 with (A) or without (B) nuclear localization signal (thin line indicates pUC8 sequences.

We will test for the virion formation with electron microscopy and Northern blot analysis, then test for GWSS infectivity in whole insects. If infectious HoCV-1 virions are produced from this binary expression system, we will modify the HoCV-1sequence to carry a dsRNA or shRNA for VIGS experiments with the ultimate goal of using the virus as a tool to help control the GWSS population.

Our second approach uses an insect baculovirus-based approach. The Bac-to-Bac Baculovirus Expression System (Invitrogen) was used to generate recombinant baculoviruses. The first step of this process involves cloning of the target

sequence, in this case the complete genome of the HoCV-1, into the shuttle vector pFASTBac1. Because of the large size of the HoCV-1 genome, the cloning was performed in three steps (**Figure 4**). In Step I, the 5'-end of the HoCV-1 genome was amplified by RT-PCR from total RNA isolated from GWSS adults using tailed PCR primers. The tailed PCR primers were designed to incorporate *Bam*HI restriction endonucleases sites at the ends of the amplicon. The amplicon was digested with *Bam*HI and inserted at the *Bam*HI site of pFastBac1. The insert of the resulting plasmid, pFB-HoCV1-5'end, was checked for orientation and sequenced. In Step II, the 3'-end of the HoCV-1 genome was RT-PCR amplified as in Step II using a tailed 3'-PCR primer that incorporated a *Hin*dIII site at the 3'-end of the amplicon and a HoCV-1-specific 5'-primer. The resulting amplicon was digested with *Sal*I and *Hin*dIII and inserted at the *Sal*I and *Hin*dIII sites of pFB-HoCV1-5'end in order to generate the plasmid pFB-HoCV1-5'/3' ends. Finally, in Step III, the *Afl*II-*Xba*I internal fragment of HoCV-1 from pRzHoCV1 was excised and ligated into the corresponding sites of pFB-HoCV1-5'/3' ends to generate pFB-HoCV1.

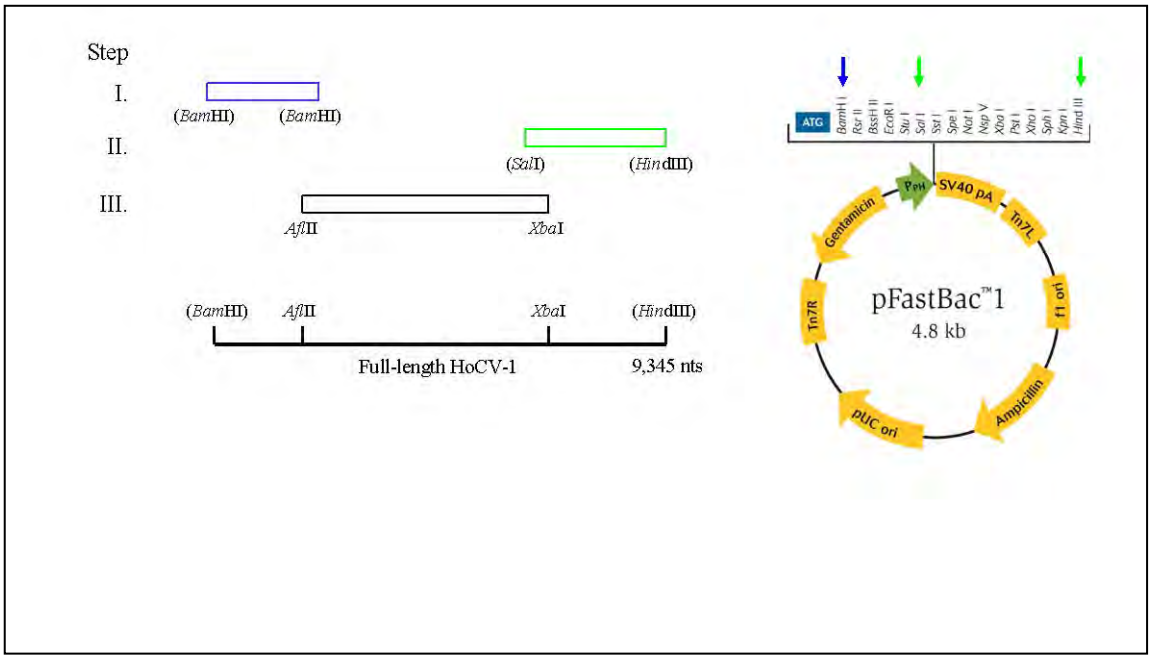

Figure 4. Cloning HoCV-1 cDNA into pFastBac1.

Recombinant baculoviruses carrying the complete HoCV-1 genome were constructed in two steps as shown in **Figure 5**. In the first step, the recombinant baculovirus shuttle vector pFB-HoCV1 was transformed into competent DH10Bac *E. coli* cells. White colonies that formed on the selection medium (i.e., colonies formed by *E. coli* carrying recombinant bacmid DNAs) were re-isolated by streaking on the same selection medium and individual colonies were amplified in liquid medium containing selection antibiotics. Bacmid DNAs were isolated from the liquid medium cultures and confirmed by PCR to carry the HoCV-1 genome. In the second step, recombinant baculoviruses were generated in Sf-9 cells by transfection of Sf-9 cells with bacmid DNAs carrying the HoCV-1 genome using Cellfectin reagent. Following transfection the recombinant baculoviruses were subject to one round of amplification in Sf-9 cells.

The ability of the recombinant baculoviruses (i.e., AcHoCV1) to express HoCV-1-specific RNA (i.e., positive-sense HoCV-1 RNA) was investigated by RT-PCR (**Figure 5**, step 2). Total RNA was first isolated from the cells of recombinant baculovirus-infected Sf-9 cells, then treated with DNAse I. First strand cDNA synthesis for the RT-PCR reaction was done using strand-specific primers that were specific for either the positive- or negative-sense RNA of HoCV-1. The positive signal using the positive-sense RNA specific primer indicated that the HoCV-1 genomic RNA is expressed by the recombinant baculovirus. The positive signal using the negative-sense RNA specific primer indicated that RNA dependent RNA polymerase (RdRP) is translated from the baculovirus expressed HoCV-1 genomic RNA. And, furthermore, that this RdRP is capable of producing the intermediate negative-sense RNA that is required for the replication of HoCV-1.

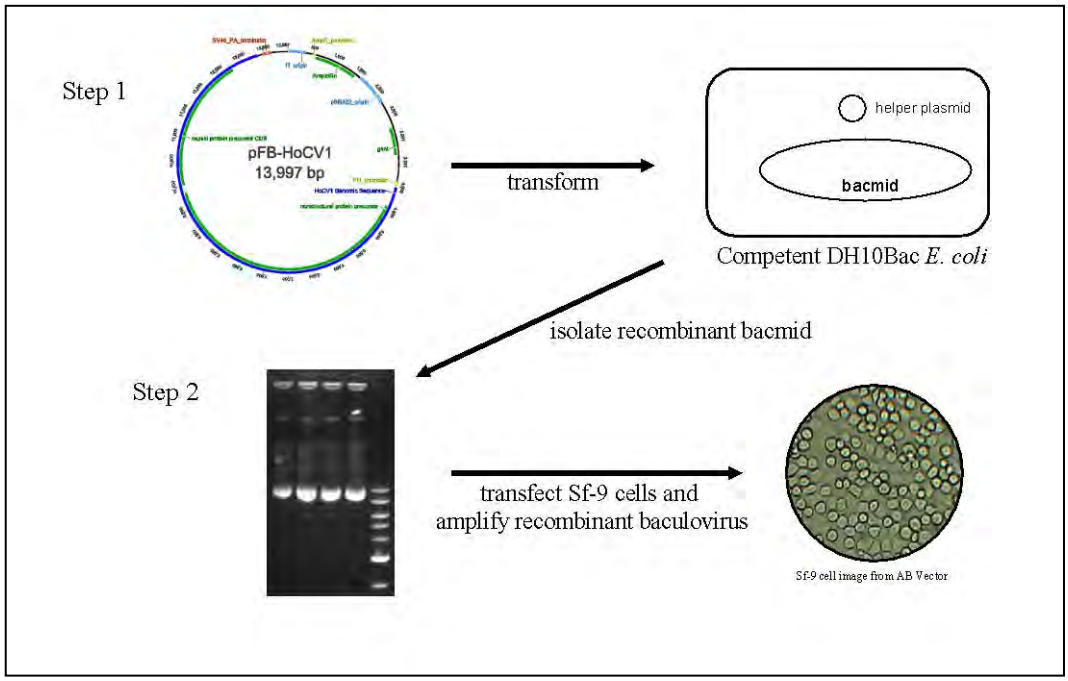

Figure 5. Generation of recombinant bacmid and baculovirus.

CONCLUSIONS

We have only been working on this effort for three months so far. During the reporting period, we began working with two viruses so as to ensure success. We have conceived the experimental strategies, tested the FHV constructs for its biological activity, and made the plasmid constructs that can express T7 polymerases in insects. The experiments described above are on-going and rapidly progressing. FHV is easy to work with and our HoCV effort looks very promising so far. The results of the experiments using FHV system will be critical in designing RNAi strategies for controlling GWSS population, and the infectious HoCV-1 cDNA clones will be invaluable in assessing the feasibility of using virus infecting GWSS as a biopesticide.

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

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Reporting Period: The results reported here are from work conducted July 2011 to October 2011.

ABSTRACT
Here we present our progress towards the development and application of an RNA interference (RNAi) based system aimed to target genes of the vector of *Xylella fastidiosa*, the glassy-winged sharpshooter (GWSS). After demonstrating that RNAi induction in GWSS cells and insects is achievable, we began screening a large pool of candidate genes to find the best targets to control the survival of GWSS. These data were used to develop transgenic *Arabidopsis* and potato plants that express dsRNAs of the targets. We also made stable *Arabidopsis* transgenic plants that express GUS marker genes using 35S and a *Eucalyptus gunii* minimal xylem-specific promoter. Transgenic plants are being evaluated for their ability to produce dsRNAs and will be tested against GWSS adult insects. Encouraged by our efforts to find effective targets, we adopted large scale sequencing of the GWSS transcriptome as well as the small RNA complement from GWSS adult insects. We were able to generate 35 million reads and nine million reads of the short read sequence data for transcriptomic and small RNA sequences in our initial run.

LAYPERSON SUMMARY
This work presents fundamental efforts towards long term application of using RNA interference, RNAi, to help combat a plant disease of great economic importance. The disease, Pierce's disease (PD) of grapevines, is a significant threat to grape production in California and other parts of the U.S. The causal agent of the disease, *Xylella fastidiosa (Xf)*, is a xylem-limited bacterium that also causes several other extremely important plant diseases worldwide. Our effort here does not directly target *Xf*, but instead targets one of its most significant insect vectors, the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, and we combine the use of transgenic and next generation sequence based methods to effectively target GWSS RNAs. RNAi is an extremely important and broadly studied area in contemporary biology, and terms such as "magic bullet" for human medicine, and "genetic insecticide" for targeting insects have been used in the literature. Our work represents the first demonstrated RNAi effort towards GWSS and our data will help to expand the possibilities to study plant-associated insects and at the same time to target the sharpshooter vectors of *Xf*, the causal agent of PD.

INTRODUCTION
The leafhopper *Homalodisca vitripennis* (Germar) (formerly *H. coagulata*), also known as the glassy-winged sharpshooter (GWSS), is among the most robust and thus most threatening vectors of *Xylella fastidiosa* (*Xf*), the bacterium that causes Pierce's disease (PD) [1], a devastating disease occurring in winegrapes [2]. New strategies that will lead to environmentally sound approaches to control GWSS and other insect vectors of plant pathogens are needed. RNA interference (RNAi) has been suggested as a strategy to develop "insect-proof plants" [3] and even referred to as a "genetic insecticide" [4]. RNAi is a eukaryotic gene regulation/defense mechanism in which small RNA segments, small interfering RNAs (siRNAs) (21-25 nt), generated by processing of dsRNA molecules often of viral origin, specifically down-regulate complementary RNA sequences [5]. Recent efforts demonstrate that RNAi is inducible in many insects. Intrathoracic injection of dsRNAs has been shown to be the most effective way to induce RNAi in whole insects of many species including *Anopheles gambiae* [6-7], *Blattella germanica* [8], *Drosophila melanogaster* [9], *Spodoptera litura* [10], *Culex pipiens* [11], *Lutzomyia longipalpis* [12], *Cecropia pupae* [13], *Acyrthosiphon pisum* [14], *Rhodnius prolixus* [15], *Aedes aegypti* [16], *Bemisia tabaci* [17], *Dermacentor variabilis* [18] and *Tribolium castaneum* [19]. Oral induction has also been demonstrated in several of these same species. Our present and previous efforts demonstrate for the first time that RNAi activity can be induced in a leafhopper species, but also is inducible in GWSS cell lines [20]. In the long term, RNAi can be used as an effective fundamental tool to better understand the dynamics of plant: pathogen: vector interactions as well as GWSS physiology and we hope as a strategy to complement overall efforts for Pierce's disease control.

OBJECTIVES

The specific objectives of our effort are:

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

RESULTS AND DISCUSSION

RNAi in GWSS cells and insects

Initially, we used 14 GWSS Genbank cDNA sequences corresponding to known proteins in order to synthesize RNAi inducer molecules, dsRNAs. We then tested whether RNAi was inducible in GWSS cells and insects, and we were able to show that RNAi activity is inducible in GWSS [20]. Real time RT-PCR, semi quantitative RT-PCR, and Northern blot of small and large RNA fractions showed that RNAi was achieved in cells and insects injected with dsRNA where target mRNAs were partially degraded and specific siRNA, hallmarks of RNAi, were detected [20]. The inducibility of RNAi in the GWSS cells helped us design the following set of experiments.

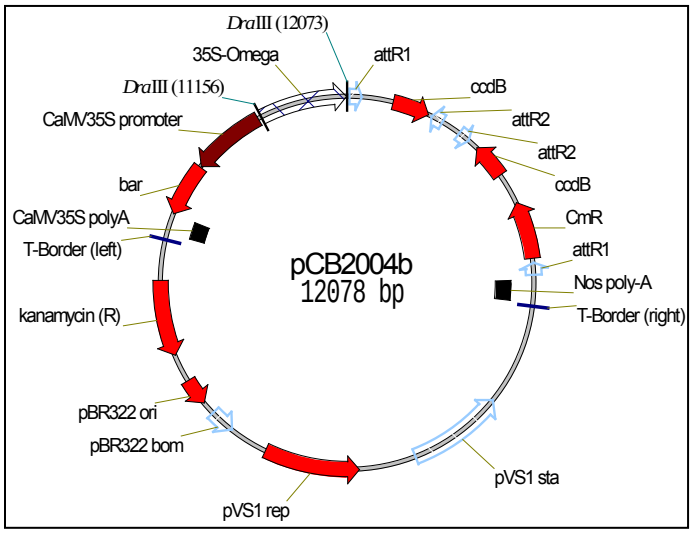

Figure 1. Diagrammatic representation of the vector pCB2004B used for generation of GWSS transgene constructs. The binary construct is designed to produce short hairpin between the sense and antisense target genes that will result in the production of small RNAs in the transgenic plants (*Arabidopsis* and potato plants).

Generation of transgenic lines

For the purpose of generating the *Arabidopsis* transgenic lines we used a different ecotype, Cape Verdi (Cvi). Compared to Columbia (Col-0) it has larger leaves and presents more robust growth, and will be more appropriate in supporting insects of large size such as GWSS. In order to generate dsRNAs that can target the insect, GWSS target sequences (**Table 1**) were cloned into a gateway-compatible binary vector pCB2004B (**Figure 1**). The target sequences were cloned in head to tail direction in the gateway vector with a non-homologous sequence between them. Upon transcription in transgenic plants, these constructs will yield double-stranded, hairpin RNAs of the desired sequence. The expression vectors carrying the insect target sequences of interest were first cloned into *E.coli* and *Agrobacterium tumefaciens* and they have been sequence verified. *A. tumefaciens* cultures carrying the sequences of interest were used to transform *A. thaliana Cvi* plant ecotypes through the floral dip process. Arabidopsis T_0 plants were screened for resistance against the selectable marker *BAR* gene, and we were able to confirm T_1 transgenics. Further sets of transformation of *Arabidopsis* plants were underway to generate more independent transgenic lines for the GWSS target genes that had less than three independent transgenic lines. Also, efforts are underway to generate more transgenic lines for other target genes of GWSS that were not previously described. We are in the process of obtaining the homozygous transgenic *Arabidopsis* lines that will be used for screening against GWSS.

We have used three of the constructs (**Table 2**) to transform potato plants. Transformation/regeneration was performed via recharge at the UC Davis Ralph M. Parsons plant transformation facility (http://ucdptf.ucdavis.edu/) and approximately ten

independent transgenic lines were obtained for each of the constructs. We have performed screening of these transgenic potato plants for insert composition and have established the presence of a transgene similar to the procedure as described for *Arabidopsis* transgenic lines. The presence of chitin deacetilase transgene in the potatoes resulted in the production of small RNAs in those transgenic plants. In contrast to the approach with *A. thaliana*, we will vegetatively propagate the T_0 plants and use them for RNAi experiments with GWSS. Potatoes are an excellent host plant for GWSS so we expect them to be very useful for our efforts here.

Table 1. GWSS insect sequences used for cloning and generation of *Arabidopsis* transgenic lines.

Construct Name*	Protein Encoded	Length of PCR Product (bp)	*E. coli* DH5-α Sequence Verified	*A. tumefaciens* EHA105 PCR Verified	Number of *Arabidopsis* transgenic lines generated
GWSS 965	Zinc Metalloproteinase	443	Yes	Yes	None
GWSS 989	Glucosyltransferase	576	Yes	Yes	3 independent lines
GWSS 1591	Sugar Transporter	668	Yes	Yes	One independent line
GWSS 1377	Serine Proteaseserpin	645	Yes	Yes	2 independent lines
GWSS 364	Trypsin	605	Yes	Yes	2 independent lines
GWSS 975	Transaldolase	800	Yes	Yes	3 independent lines
GWSS 366	Sugar Transporter	888	Yes	Yes	None
GWSS 500	Serpin	418	Yes	Yes	4 independent lines
GWSS 745	Trypsin	756	Yes	Yes	None
GWSS 512	Transketolase	1435	Yes	Yes	None

In addition to the promoter effects of the GWSS target genes under the 35S promoter, we have started generating the constructs under a specific xylem promoter EgCAD2 was cloned from *Eucalyptus gunii*. The sequence was fused to the GUS reporter gene in the binary pCB301 vector. Then, GUS expression driven by the xylem specific promoter was accessed in a transient *Agrobacterium tumefaciens* assay in *N. benthamiana* plants. Upon staining for GUS activity, results showed that blue product was restricted to the main vascular tissues. This gives confidence in this promoter, which will now be used to attempt to express specific interfering RNAs in the xylem of transgenic plants. We have generated our initial set of transgenic plants in *Arabidopsis* which expresses the *GUS* gene under the xylem specific promoter, but we have not yet tested the T2 generation.

Table 2. GWSS insect sequences used for cloning and generation of potato transgenic lines in the variety Desiree.

Name (pCB2004B)	pedigree	variety	selection	Small RNA northerns	GSP F/R
Chitin deacitilase	102203	Kennebee\ Desiree	BAR	Produce small RNAs	primers giving multiple bands
GWSS actin	112064	Desiree	BAR	Not done	9 out of 11 tested are positive
GWSS cuticle	112073	Desiree	BAR	Not done	9 out of 9 are positive

Feeding assays

In addition to the transgenic plant approaches, based on recent reports in the literature (Killiny and Almeida, 2009, PNAS 106:22416) and personal communications from other scientists, we have evaluated *in vitro* feeding approaches for GWSS (**Figure 2**). If successful this will allow for much more rapid screening of candidate sequences for their abilities to induce RNAi effects via oral acquisition. We have a number of candidate sequences which we are testing for RNAi (**Table 3**). These are used for *in vitro* transcription and known quantities of dsRNAs are used for *in vitro* acquisition, followed by using three different *in vitro* oral acquisition approaches. These included using a modified membrane feeding approach based on Killiny and Almeida, a modified tygon tubing delivery system, and using basil infusion (basil stems directly inserted into dsRNA solutions). We have used the basil infusion in the past and it offers some advantages as well as disadvantages. Together, these approaches can supplement the transgenic plant approaches, and if we can make any consistent, this will allow us to rapidly screen target sequences without having to develop transgenic plants, thereby saving time and effort towards our ultimate goal.

Table 3. GWSS insect sequences used for cloning and generation of potato transgenic lines in the variety Desiree.

S.No:	List of GWSS target genes	Cloned into plasmid	dsRNA prepped
1	Actin	pGEMT-easy	Yes
2	Ferritin	pGEMT-easy	Yes
3	Ubiquitin	pGEMT-easy	Yes
4	Lian2	pGEMT-easy	Yes
5	SAR1	pGEMT-easy	Yes
6	Fructose1,6 aldolase	pGEMT-easy	Yes
7	RAB1	pGEMT-easy	Yes
8	Tropomyosin	pGEMT-easy	Yes
9	Delta 9 desaturase	pGEMT-easy	Yes
10	Mitochondrial porin	pGEMT-easy	Yes
11	GFP	pJL24	XX

Next Generation Sequencing of GWSS Adult Insects

The developmental regulation of insects through the use of small RNAs has been well studied. In our efforts to study the regulation of GWSS insect genes and identify RNAi targets, we took an alternate approach using high throughput parallel sequencing to identify the small RNAs from the GWSS insects. For our work, we noticed GWSS transcriptome data is lacking information for the identification of small RNA reads. To address this and identify the loci of the small RNAs that were originated from the short read sequencing, we sequenced the transcriptome of GWSS through the use of mRNA sequence methods as described in **Figure 2**. The sequencing of GWSS mRNA transcriptome was done through paired end sequencing on Illumina GA-II Platform. Both the mRNAseq library data and the small RNAseq library data were generated from the GWSS adult insects (**Figure 2**).

The sequencing reads from the transcriptomic data were assembled into scaffolds with a minimum size of 200 bases using Oases transcriptome assembler. We were able to assemble approximately 32.9Mb of the transcriptome across 47,265 loci and 52,708 transcripts. The average transcript length assembled was 624 nucleotides. Roughly 15 million of the total reads were found to be unique for the genome (**Table 4**) and 51% of the reads were incorporated into the assembly. The sequencing reads were then mapped back to the assembled transcripts with up to one mismatch. The reads that could not be mapped back to the reference assembly are being analyzed for the possible discovery of new viruses that may be infecting the GWSS insects. With the help of these sequencing reads, we aim to study the GWSS insect target genes and we hope to identify the small RNAs that target the GWSS target genes in a highly specific manner.

Table 4. Sequencing summary of the GWSS adult insect reads after the quality control.

Samples	No: of reads	Unique reads	%unique	%GC
mRNA seq-left	32,947,747	14,891,609	45.20%	46.56%
mRNA seq-Right	32,948,747	15,112,284	45.87%	46.68%
Small RNA seq	22,133,363	4,081,113	18.44%	55.59%

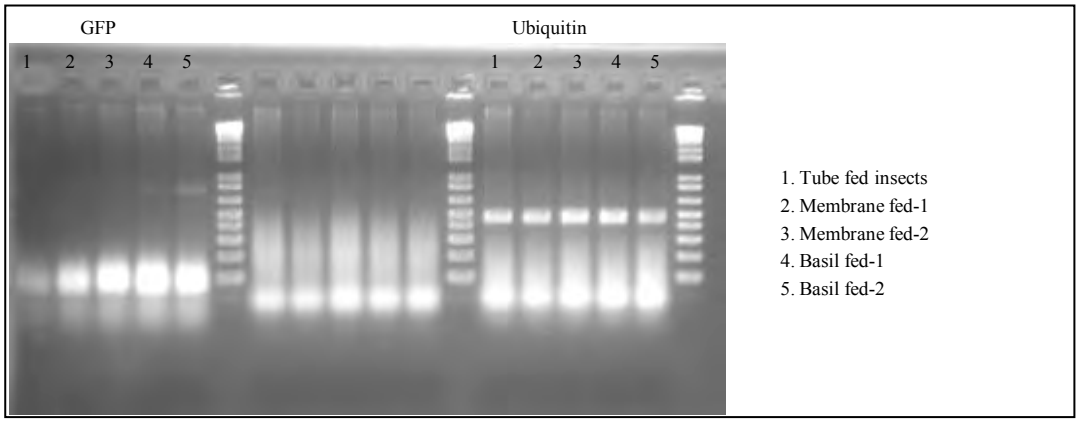

Figure 2. Comparison of three different feeding assays on GWSS insects. PCR amplification of GFP PCR product from the adult GWSS insects after they are fed with the GFP PCR product in either of the following forms: Tube feeding, Membrane feeding and Basil feeding.

CONCLUSIONS

Xf is an important bacterial pathogen of economically important crops such as grape, citrus and almond. The ability to minimize the economic impact of this bacterium depends on the presence and abundance of its biological vectors and GWSS is the most effective vector of *Xf* transmission in some agricultural areas. RNAi-based efforts directed toward the control of insect plant pests are now becoming more feasible, and RNAi for insects such as GWSS has great potential applications. The results presented here show that RNAi can be induced both *in vitro* (GWSS -Z15 derived cell line) and *in vivo* in GWSS nymphs. We showed that GWSS -Z15 cells can be used to screen candidate gene silencing targets, and that since RNAi is active in cells, it could also be used to study GWSS gene function via mRNA knockdown. More notably, the employment of RNA silencing in whole GWSS insects could offer help towards a potential solution for control of the vector. Future work includes the screening of more RNAi targets, the production of transgenic plants expressing dsRNAs in their xylem and the study of GWSS insects grown on the transgenic plants.

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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 2011.

ABSTRACT
Death or dramatic changes in normal insect development can result from minor disruption of the insect endocrine system. Juvenile hormone (JH) is a key insect developmental hormone that has biological effects at low nanomolar levels in the hemolymph. JH analog (JHA) insecticides are green compounds that can mimic the action of JH and 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 JH 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 JH levels are exceptionally low.

LAYPERSON SUMMARY
The insect endocrine system is a highly selective and highly sensitive target for insect control and for reducing vector competence. The overall goal of our project is to study and exploit targets within the endocrine system of glassy-winged sharpshooter (GWSS) that can be used to control GWSS or reduce its ability to spread Pierce's disease. We are taking two complementary approaches. Our direct approach is to determine the efficacy of various concentrations of juvenile hormone analog (JHA) insecticides against GWSS eggs, nymphs, and adults. A key objective of this approach is to quantify the minimum level of JHA insecticide that can efficiently reduce the emergence of nymphs from eggs and keep nymphal insects in the nymphal stage. The results of this direct approach will have near-term applicability since the JHA insecticides that we are testing are US-EPA registered and commercially available. Our indirect approach involves the identification and characterization of genes that are unique to GWSS endocrine system that metabolize a key insect hormone called JH. The objective of this approach is to evaluate these genes as potential targets for gene knockdown.

INTRODUCTION
Insect development is precisely regulated by the relative titers of juvenile hormone (JH) and molting hormones (i.e., ecdysteroids) (**Figure 1**). JHs form a family of sesquiterpenoids (**Figure 2A**) 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 insect death or dramatic alterations in insect development. Juvenile hormone analog (JHA) insecticides are green compounds that selectively target the insect endocrine system by mimicking the biological action of JH (reviewed in Dhadialla et al., 2005; Henrick, 2007). JHAs such as methoprene, fenoxycarb, and pyriproxyfen (**Figure 2B**) are US EPA-registered compounds that are commonly used to control mosquitoes, fleas, whiteflies, ants, and other insect pests. JHAs function as structural and/or biological mimics of JH. 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.

Abnormal developmental morphologies, similar to those induced by JHAs are also 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 clone and characterize the *jhe* gene and related JH epoxide hydrolase, *jheh*, gene as potential target genes for an RNAi-based strategy for the control of glassy-winged sharpshooter (GWSS).

OBJECTIVES
The current project is a continuation of our previous UC Pierce's Disease Research Grants Program (UC PDRGP)-funded project (#2010-259, 8/1/10-7/31/11). Objectives I, II, and III are from our previous project. Objectives IV and V are from our current UC PDRGP-funded grant. We are continuing work on all five of these objectives.

1. Evaluate the efficacy of JHA insecticides.
2. Characterize authentic JH esterase (JHE) activity in GWSS.
3. Isolate *jhe* gene and characterize recombinant JHE protein.
4. Investigate delayed effects of low dose JHA insecticide exposure.
5. Characterize recombinant JH epoxide hydrolase (JHEH) from GWSS.

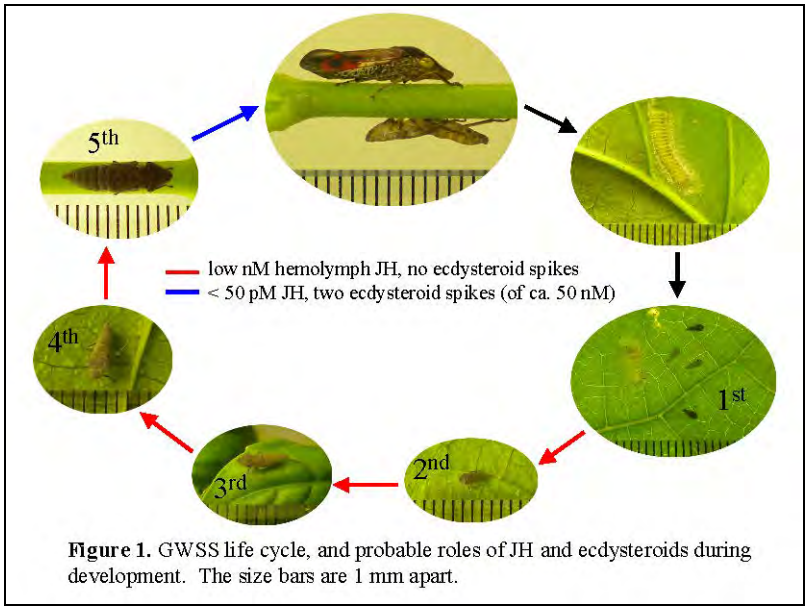

Figure 1. GWSS life cycle, and probable roles of JH and ecdysteroids during development. The size bars are 1 mm apart.

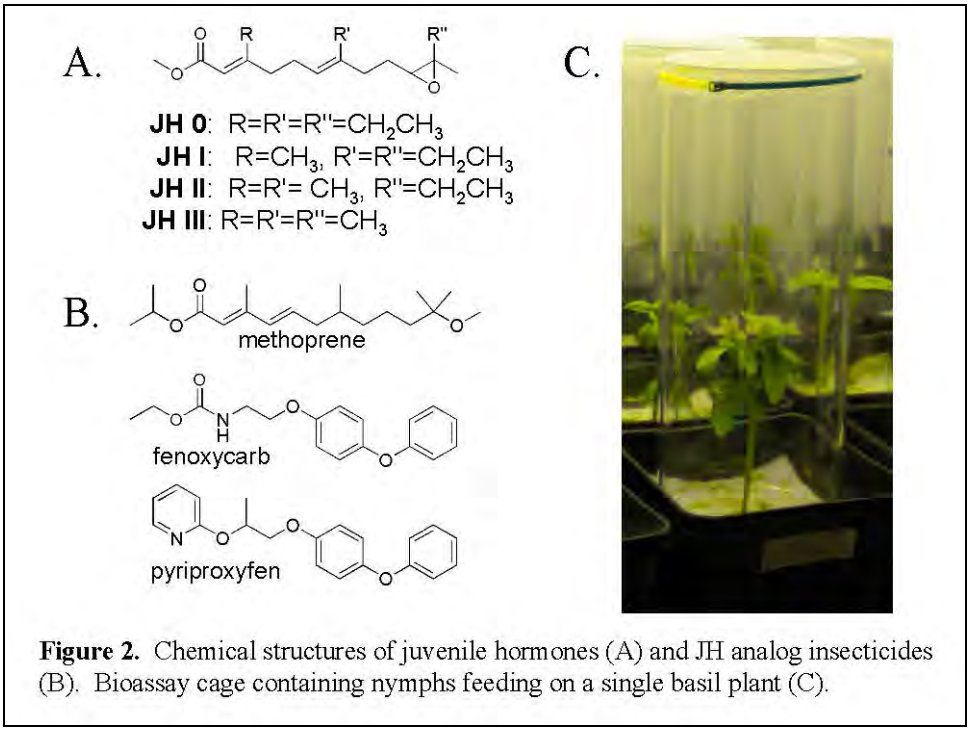

Figure 2. Chemical structures of juvenile hormones (A) and JH analog insecticides (B). Bioassay cage containing nymphs feeding on a single basil plant (C).

MATERIALS AND METHODS

Insect Rearing. Laboratory colonies of GWSS are maintained at the UC Davis Contained Research Facility in an environmental growth chambers set at 24ºC, 70% relative humidity, and a light:dark cycle of 14 h:10 h. Within the chambers the GWSS are grown in Bug Dorm insect cages each containing at least two cowpea, two cotton, and two basil plants.

Bioassays. The life cycle of GWSS was determined using freshly emerged nymphs placed on a single, caged, basil plant (ca. 10-15 cm in height) where the lower stems were removed (**Figure 1C**). The insects were observed daily for signs of molting (i.e., the presence of exuviae and increased head size).

Bioassays. First instar 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 1 hour. Following exposure groups of five nymphs were placed on a single basil plant and observed daily as described above.

Gene cloning. Total RNAs were isolated from 5th instar nymphs at days 6, 7, 8, 9, and 10 post ecdysis. First strand cDNAs were generated from total RNA using a Creator SMART cDNA library construction kit (Clontech). Random amplification of the 3'-cDNA ends (3'RACE) was performed using an anchor primer and degenerate primers that recognized conserved sequences (reviewed in Kamita and Hammock, 2010) in known JHEs (e.g., GQSAG) and JHEHs (e.g., KPDTIG).

RESULTS AND DISCUSSION

A simple bioassay was developed to measure the development time of GWSS. The bioassay uses a single host basil plant for rearing the insects (**Figure 2C**). Using this bioassay, the duration of 1st, 2nd, 3rd, 4th, and 5th instar nymphs was 6.2±0.8, 4.9±0.8, 5.5±0.8, 7.0±1.1, and 10.7±1.0 days, respectively. The combined length of all of the nymphal stages was about 34 days. The developmental times were the same regardless of oviposition substrate (cotton or cowpea) on which the eggs were laid (**Table 1**).

Table 1. Mean development time of GWSS reared on basil.

Nymphal instar	Development time (days ± s.d.)[1]			
	Eggs from cotton	No. of insects	Eggs from cowpea	No. of Insects
1st	6.2 ± 0.9	110	6.1 ± 0.7	103
2nd	4.9 ± 0.6	68	4.9 ± 0.8	76
3rd	5.4 ± 0.8	57	5.5 ± 0.7	61
4th	7.0 ± 1.3	47	7.0 ± 0.9	56
5th	10.5 ± 0.9	43	10.7 ± 1.0	49
total days	34.0		34.2	

[1]No statistical differences were found in development times based on egg source (i.e., cotton or cowpea)

When first instar nymphs were exposed to the JHA methoprene at a dose 0.5 ppm for 1 h, the duration of the 1st instar was shorter by about one day, whereas that of 2nd, 3rd, 4th, and 5th instars was similar to nymphs that were not exposed to methoprene (**Table 2**). When the methoprene dose was increased 10-fold to 5.0 ppm, the length of the 5th instar was longer by about two days (**Table 2**). However, unlike insects that were not exposed to methoprene, there was tremendous variation in the length of this instar with some insects remaining as 5th instars for more than 20 days. We hypothesize that methoprene, a highly non-polar compound that easily penetrates the cuticle, is retained within the tissues of some insects and exerts a biological effect even after multiple molts. These finding suggest that exposure even at a dose as low as 5 ppm will have significant effects on the life history of GWSS. We are currently repeating these experiments with the JHA pyriproxyfen.

Table 2. Mean development time of GWSS following methoprene exposure.

Nymphal instar	Development time (days ± s.d.)/concentration	
	0.5 ppm	5.0 ppm
1st	4.8 ± 1.3	4.7 ± 0.8
2nd	5.7 ± 2.2	5.8 ± 0.9
3rd	4.7 ± 0.6	5.4 ± 0.9
4th	6.8 ± 0.5	7.2 ± 0.8
5th	10.7 ± 1.4	12.7 ± 8.8
total days	32.7	35.8

Both JHE and JHEH activities were found in the hemolymph of 5th instar GWSS nymphs. JHE activity was highest at days six,seven, and eight of the 5th instar with values of 24.4 ± 3.5, 21.8 ± 1.9, 22.7 ± 2.4 pmol of JH acid formed per min per ml of hemolymph. JHEH activity was consistently lower than JHE activity and showed a peak of 9.3 ± 1.7 pmol of JH diol formed per min per ml of hemolymph at day six of the 5th instar. Semiquantitative PCR analyses were consistent with the enzymatic activity assays, and identified putative *jhe* and *jheh* gene expression at days eight, nine, and 10 of the 5th nymphal instar (**Figure 3**). We are currently attempting to clone JHE- and JHEH-encoding sequences by 3'- and 5'-RACE approaches.

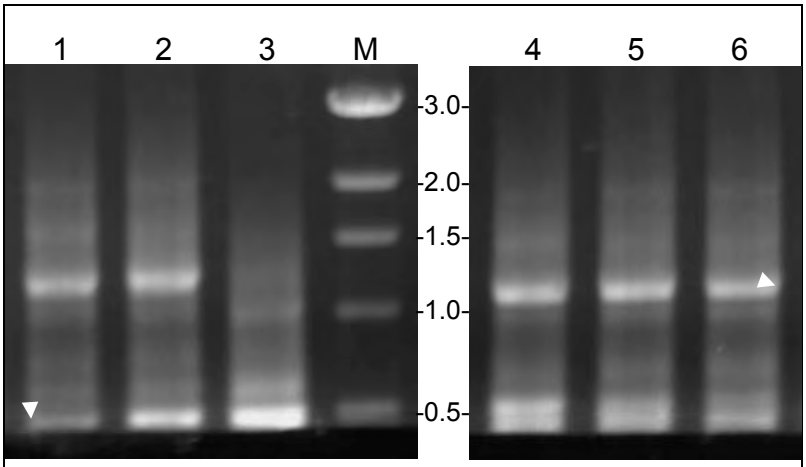

Figure 3. Expression of the putative *jheh* (left arrowhead) and *jhe* (right arrowhead) genes in 5th instar GWSS nymphs at day 8 (lanes 1 and 4), day 9 (lanes 2 and 5), and day 10 (lanes 3 and 6) of the instar. The size of molecular weight standards (lane M) is shown in kDa.

CONCLUSIONS

We have established a robust and easy to use bioassay for GWSS. Using this bioassay we have determined precise developmental times under standard rearing conditions. Knowledge of precise developmental times are critical for quantifying the efficacy and effects of the JHAs, effects of JHE-inhibitors, and enzyme activity levels of JHE/JHEH in GWSS. We have also used our bioassay to determine baseline lethal dose values with the JHA standard methoprene. We are continuing our bioassays at low doses and are in the process of isolating and characterizing JHE and JHEH encoding sequences as possible targets of RNAi.

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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 2010 to September 2011.

ABSTRACT
Riverside County has two general areas where citrus groves interface with vineyards, the Coachella and Temecula Valleys. The Coachella Valley with 8,270 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 a 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 10,465 acres of producing vines, which generate fresh market grapes valued at an average of $110 plus million annually. 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 2,469 acres of citrus in Riverside County were treated for the GWSS in April through June 2011 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 glassy-winged sharpshooter (GWSS) populations in the Temecula and Coachella Valleys for 2011 and 2012.
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/Pierce's disease (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 & 2**). Approximately 1,400 GWSS yellow sticky traps are monitored in the Riverside county area wide program. Based on trap counts and visual inspection, 775.25 acres of citrus were treated in Temecula valley for GWSS in 2011. In 2011, a total of 1,694 acres of citrus were treated to manage GWSS in Coachella valley. All the citrus were treated with Admire Pro (imidacloprid), with the exception of 13 acres in Temecula valley. Admire Pro was applied at the rate of 14 oz/acre. The thirteen acres were treated with PyGanic (1.4% pyrethrins) at 18 oz/acre. Because of Temecula GWSS trap catches in the late summer and early autumn of 2008, 2009, and 2010, imidacloprid (Admire Pro) applications in citrus were initiated in April, 2011 (**Figure 3**).

For a successful area-wide GWSS management program with large acreages of citrus, a management program has to be maintained. For more information on the management of GWSS visit the web site http://ucbugdr.ucr.edu.

FUNDING AGENCIES

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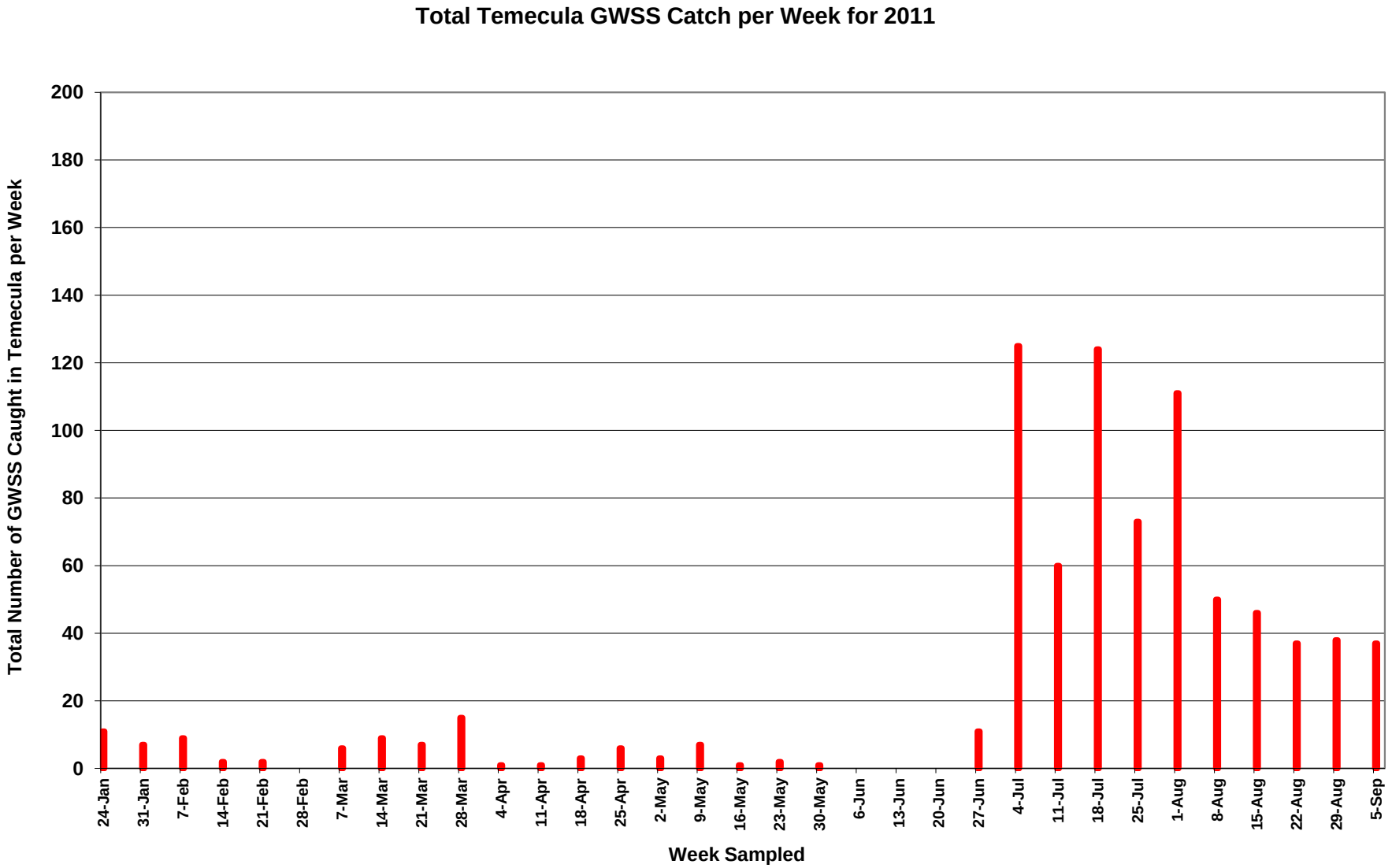

Figure 1. In 2011, the highest numbers of adult GWSS were trapped in July, reaching a total of 125.

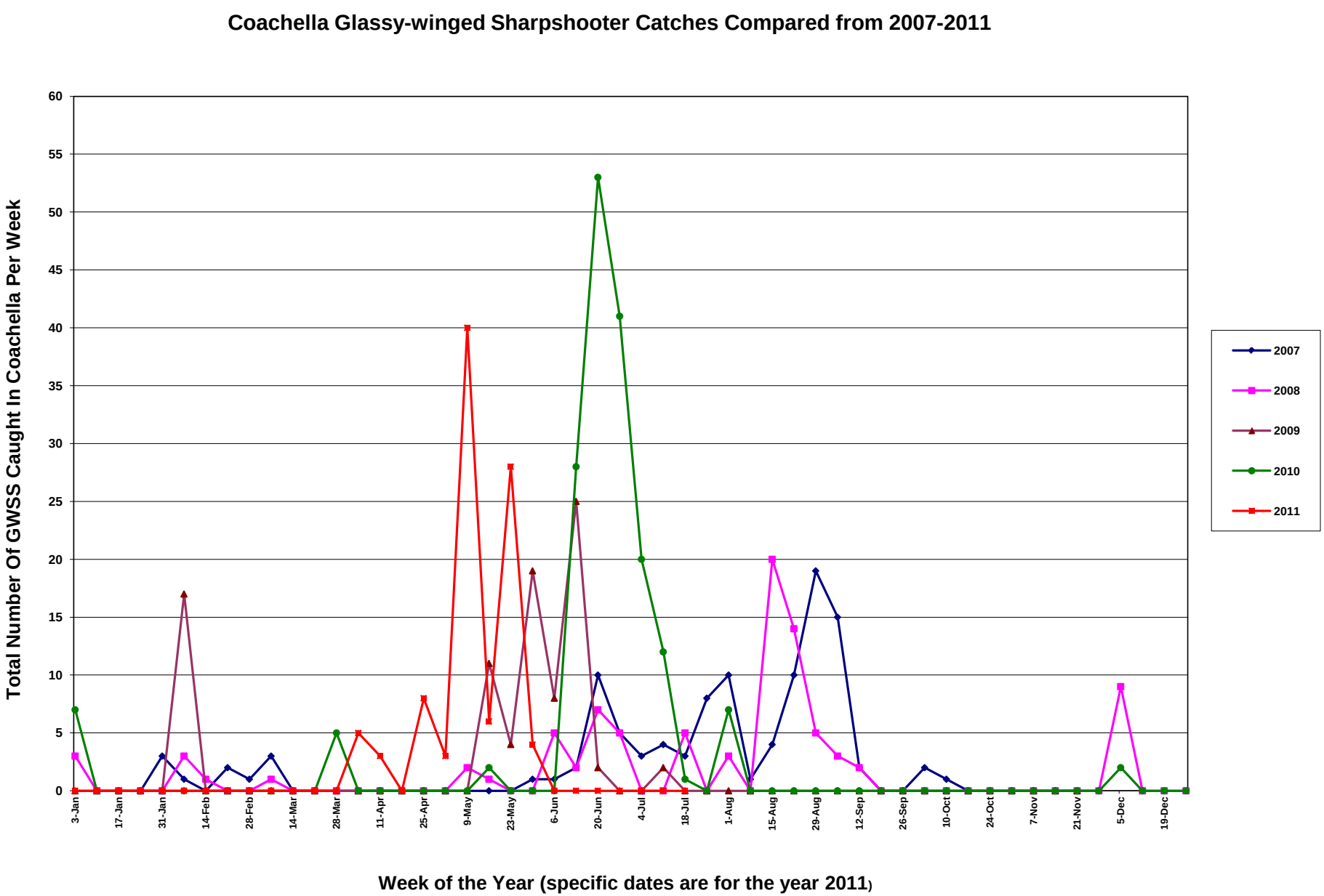

Figure 2. Coachella GWSS population comparisons from 2007-11.

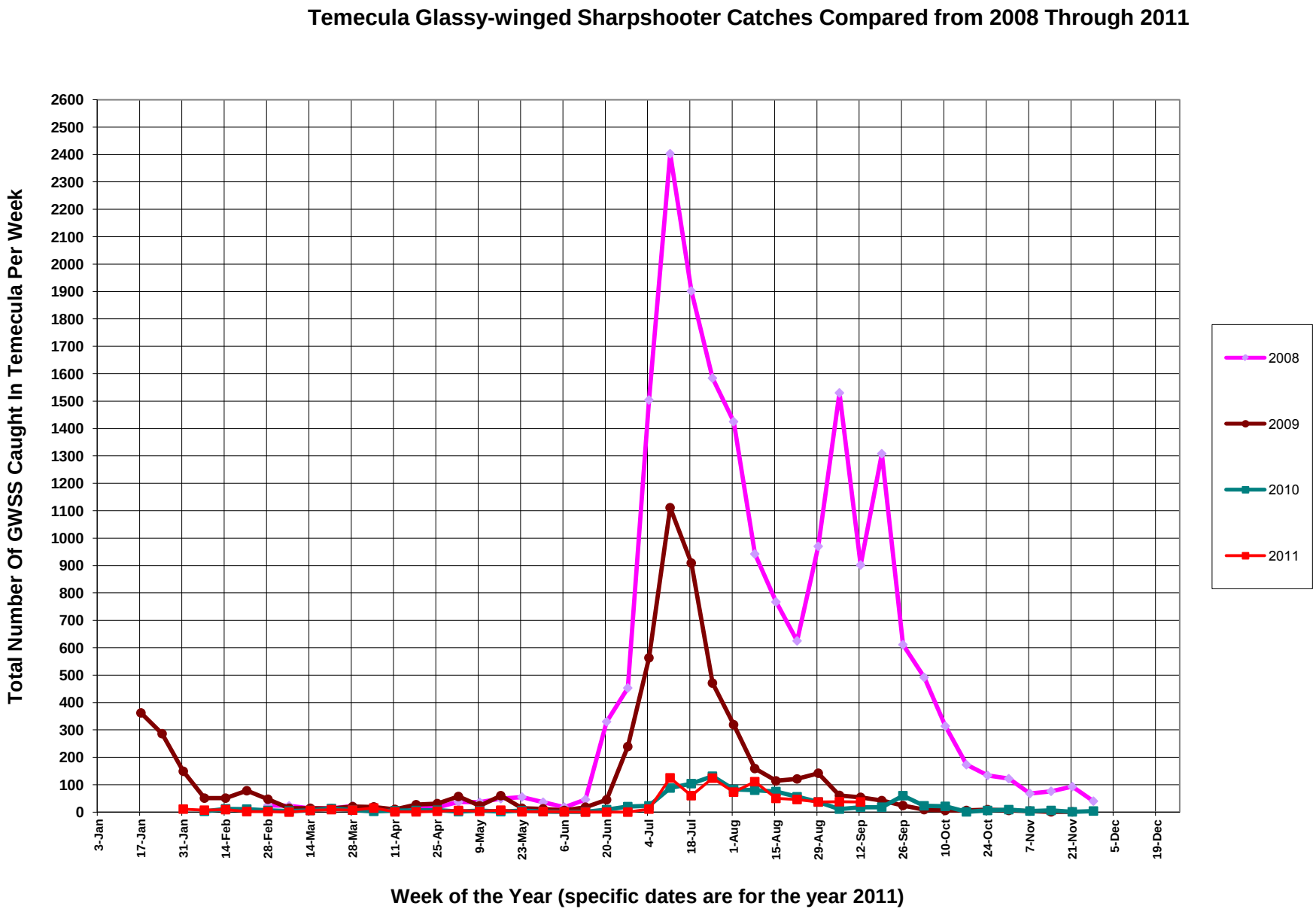

Figure 3. Temecula GWSS population comparisons from 2008-11

Section 3:

Pathogen Biology and Ecology

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*. In the reporting period, we cultured Temecula-1 (a PD strain) into grapevine, mulberry and citrus in vitro, with inoculated PD3 medium and non-inoculated xylem fluid as controls, to detect differential growth and expression patterns. Temecula-1 grew similarly in pure xylem fluid of grapevine, mulberry and citrus. Transcriptional profiles based on macroarray analysis of 110 pathogeneticity-related genes showed that 27 genes had higher expression, and three lower, in grape xylem fluid compared with that of mulberry and citrus. Expression of these genes in mulberry vs. citrus was not significantly different. Although the PD strain grew similarly in xylem fluid from all three hosts, the increased expression of pathogenicity genes likely contributes to disease development in grape by Temecula-1, whereas there were no symptoms are produced by this strain in mulberry and citrus.

LAYPERSON SUMMARY
We have previously shown different gene expression profiles of Pierce's disease (PD) strain A05 in the xylem fluid of grapevine (PD-susceptible) vs. citrus (PD-tolerant). This raised the possibility that the differential host range of *Xylella fastidiosa (Xf)* strains may to some extent be related to their genetic response to the chemical composition of xylem fluid from the different hosts. In this report we used grape, mulberry and citrus fluid to compare the expression patterns of another PD strain, Temecula-1. With its fully annotated genomic information, we hope to more accurately explain the influence of host xylem chemistry on the growth and pathogenesis of *Xf*, and to use this information to develop strategies to interfere with disease development in susceptible plants.

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 in a 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 xylem fluid 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 xylem fluid compared with citrus xylem fluid. However, some genes had greater expression in citrus xylem fluid (Shi et al. 2010). We have also shown differential growth and expression patterns in xylem fluid 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 xylem fluid 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 from February to August 2011 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.

Bacteria Growth *in Vitro*

Cells of *Xf* strain Temecula-1 were collected from seven-day-cultured PD3 agar and adjusted to an optical density of 0.05 at 600 nm in xylem fluid from grapevine, mulberry and citrus in borosilicate glass culture tubes, which were placed on a rotary shaker under constant agitation at 28°C for 20 days as previously described (Shi *et al*., 2010). Xylem fluid without the bacteria, and bacteria inoculated in PD3 broth were used as controls. Bacterial cells were dispersed by repeated pipetting and vortexing. Bacterial cell concentration in the tubes was determined by measuring the OD_{600} at 10 and 20 days after culture. Cells from the tubes were cultured on plates containing PD3 medium , and then confirmed to be *Xf* using specific primer pair RST31/RST33 (Minsavage *et al.* 1994).

The Temecula-1 strain of *Xf* could grow in pure xylem fluid of grapevine, mulberry and citrus with low densities compared to those in PD3 medium. *Xf* cell densities in grape xylem fluid were not significantly different than in mulberry and citrus fluid, and the cell densities increased by 20 days in all xylem fluids (**Figure 1**). *Xf* cells have been reported to grow in xylem fluid of grape (Andersen et al, 2007; Zaini et al, 2009, Shi et al, 2010) and citrus (Shi et al, 2010). In this study, mulberry was used as another symptomless host for the PD strain, and its xylem fluid could also support the growth of *Xf* stain Temecula-1.

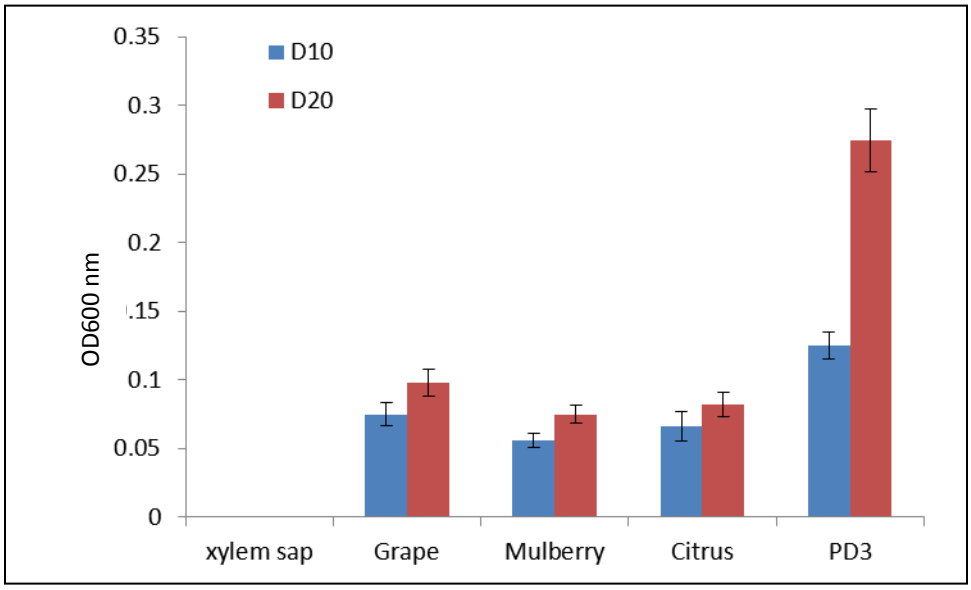

Figure 1. One *Xf* stain Temecula-1 was inoculated into xylem fluid of grape, mulberry, and citrus at OD600 nm of 0.05 in borosilicate glass culture tubes, which were placed on a rotary shaker at 28°C. PD3 medium and non-inoculated xylem fluids were used as controls. The cell concentrations in xylem fluid of grape, mulberry and citrus were measured at OD600 nm at 10 and 20 days after inoculation. All tubes were covered with a black cardboard box to protect the culture from light.

RNA Isolation and Macroarray Analysis

The extraction of total RNA from the cultures of *Xf* isolated grown in xylem fluid, the determination of qualities of isolated prokaryotic RNA, the synthesis of cDNA, and cDNA digoxigenin (DIG)-labeled by reverse transcription (RT), were all done using previously described methods (Shi et al., 2010). DNA macroarray nylon membranes were hybridized with DIG-labeled cDNA following the manufacturer's instructions (Roche Applied Science, IN, USA). Signal intensities of spots on the membranes were analyzed using Quantity one® software (Bio-Rad, CA) per the methods of Shi et al. (2010). Briefly, one-way analysis of variance of the expression values was used to select differentially expressed genes among mRNA samples. Genes with expression levels significantly different among combinations were identified using the student's t-test (α=0.05).

Of 110 selected genes (Hernandez-Martinez, 2005) from *Xf* tested in the DNA macroarray, 30 genes were differentially expressed in grape xylem fluid vs. mulberry and citrus fluid, but there is no significant difference between the expression of these genes in mulberry and citrus fluids (**Table 1**). Three genes had lower expression in grape fluid compared with citrus and mulberry, and 27 genes had higher expression in grape fluid, which included some virulence factors and virulence

regulatory genes, such as *rsmA*, *algU* and *gacA*, In addition, some genes involved in the biogenesis of pili and twitching motility were also highly expressed in grape xylem fluid compared with xylem fluid from other the non-host plants.

Table 1. Genes of *Xf* strain Temecula-1 differentially expressed in cells growing in xylem fluid of grape, mulberry, and citrus.

Gene ID [1]	Name	Hypothetical function	Index [2,3]		P value [4]	
			G/M	G/C	G/M	G/C
XF2228	*algH*	Transcriptional regulator	3.73/3.17		2.3E-04/1.9E-03	
XF2466	*pglA*	polygalacruronase precursor	2.93/2.65		6.0E-03/7.0E-03	
XF0125	*rsmA*	Carbon storage regulator	2.87/2.60		1.1E-03/1.0E-04	
XF2625	*htpX*	Heat shock protein	2.86/2.62		1.1E-04/9.9E-03	
XF2538	*pilC*	Fimbrial assembly protein	2.86/2.50		6.5E-03/5.9E-03	
XF2239	*algU or algT*	RNA polymerase sigma-H factor	2.85/2.37		6.1E-03/5.5E-03	
XF0478	*pilY1*	Fimbrial assembly protein	2.80/2.56		1.6E-03/1.0E-04	
XF1940	*msrA or pms*	Peptide methionine sulfoxide reductase	2.77/2.50		7.7E-03/6.9E-03	
XF2420	*mviN*	Virulence factor	2.68/2.18		5.2E-03/4.6E-03	
XF2608	*gacA*	Transcriptional regulator (luxr/uhpa family)	2.65/2.25		2.0E-04/1.7E-04	
XF2397	*hlyB*	Toxin secretion ABC transporter ATP-binding protein	2.62/2.34		1.7E-03/2.8E-03	
XF0432	*brk*	Brkb protein	2.61/2.43		8.0E-03/9.3E-03	
XF0028	*fimT*	Pre-pilin like leader sequence	2.58/2.19		1.7E-04/1.6E-04	
XF1804	*sphIM*	Site-specific DNA-methyltransferase	2.52/2.16		6.6E-03/5.6E-03	
XF0619	*cutA, cycY*	periplasmic divalent cation tolerance protein	2.45/2.53		1.3E-04/1.0E-04	
XF0132	*copA*	Copper resistance protein A precursor	2.42/2.22		6.8E-03/5.9E-03	
XF0506	*vapE*	Virulence-associated protein E	2.14/1.96		1.1E-04/2.9E-03	
XF0285	*hrA*	Heat shock protein	2.13/1.95		2.6E-03/3.3E-03	
XF1954	*pilIalgH*	pilus biogenesis protein	2.08/1.88		4.2E-03/3.7E-03	
XF1858	*exsb*	Transcriptional factor	2.07/1.93		7.1E-03/6.8E-03	
XF0962	*gcvR*	Transcriptional regulator	2.06/1.79		3.0E-04/2.4E-03	
XF1379	*HI1201*	Luciferase	2.05/1.84		1.0E-04/7.7E-03	
XF0591	-	Virulence factor	2.04/1.86		5.1E-03/4.4E-03	
XF1182	*act*	lipase modulator	1.98/1.66		4.2E-03/3.2E-03	
XF2539	-	Fimbrial protein	1.98/1.61		1.0E-02/1.1E-02	
XF0677	*pilZ*	Type 4 fimbriae assembly protein	1.93/1.77		5.2E-03/4.7E-03	
XF2545	*pilR*	Two-component system, regulatory protein	1.83/1.58		1.3E-04/1.0E-04	
XF0122	*lexa*	Lexa repressor	0.50/0.45		2.0E-04/1.0E-04	
XF0081	*fimD*	outer membrane usher protein	0.10/0.08		1.0E-04/8.7E-03	
XF0858	*surE*	Survival protein	0.07/0.06		3.2E-04/1.0E-04	

[1]Genes ID were determined on the basis of 9a5c genomic sequences at the NCBI website

[2]Hybridization signal intensity (mean of three hybridization replicates) obtained with grape was divided by that obtained with mulberry(G/M) and citrus (G/C) respectively to obtain the ratio

[3]Normalized hybridization signals for grape, mulberry and citrus for indicated genes were significantly different based on ANOVA and t- test ($P<0.05$)

[4]Genes having > 1.5 or < 0.6 ratios were designated as having a higher or a lower expression in grape respectively. There is no transcriptional difference of all loci between Temecula-1 inoculated into mulberry and citrus.

CONCLUSIONS

Xylem fluid is nutrient-poor compared with culture media, but it is still feasible for culturing *Xf* in vitro (Andersen et al. 2007; Zaini et al. 2009; Shi et al.2010). In this study, we compared the influence of grape, mulberry and citrus xylem fluids on one PD strain Temecula-1 in vitro. Results showed that the PD strain grew similarly in the xylem fluid from host and non-host plants, but different gene expression profiles were observed with Temecula-1 inoculated into grape xylem fluid vs. that from mulberry and citrus. We will also examine specific chemical components of xylem fluid that influence virulence gene expression, with the goal of discovering components that could be used to reduce virulence gene expression for practical disease control.

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THREE NEW PATHOGENICITY EFFECTORS OF PIERCE'S DISEASE NOT FOUND IN BIOCONTROL STRAIN EB92-1

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ABSTRACT

Xylella fastidiosa (*Xf*) infects a wide range of plant hosts and causes economically serious diseases, including Pierce's disease (PD) of grapevines. *Xf* biocontrol strain EB92-1 is infectious to grapevines but does not cause symptoms. The draft genome of EB92-1 revealed: 1) that it was nearly identical in gene order and sequence to Temecula; 2) no unique or additional genes were found in EB92-1 that were not previously identified in Temecula, and 3) EB92-1 appeared to be missing genes encoding 10 potential pathogenicity effectors found in Temecula (Zhang et al 2011). The latter included a type II secreted lipase (LipA; PD1703), two identical genes from a duplicated prophage region encoding proteins similar to zonula occludens toxin (Zot; PD0915 and PD0928) and all six predicted hemagglutinin-like proteins (PD0986, PD1792, PD2108, PD2110, PD2116 and PD2118). PCR analyses and subsequent sequencing of the PCR products confirmed that all 10 genes were missing, at least from their expected locations, in EB92-1. Leaves of tobacco and citrus inoculated with crude protein extracts of the Temecula PD1703 gene over-expressed in *Escherichia coli* exhibited hypersensitive cell collapse in less than 24 hrs. PD1703, driven by its native promoter, was cloned in shuttle vector pBBR1-MCS5 downstream from the *lacZ* promoter; this construct conferred strong secreted lipase activity to *Xanthomonas citri*, *E. coli,* and EB92-1 in plate assays. Although pathogenicity of the *X. citri* exconjugant expressing PD1703 was not evidently increased on citrus, pathogenicity of the EB92-1 exconjugant with PD1703 showed significantly increased symptoms on grapes as compared with an EB92-1 exconjugant carrying the empty vector. Similarly, Temecula PD0928 (Zot) and PD0986 (hemagglutinin) were also cloned in shuttle vector pBBR1-MCS5 and downstream from the *lacZ* promoter; when moved into EB92-1, both exconjugants also showed significantly increased symptoms on grape in comparison to EB92-1 with the empty vector. These results suggest that the Temecula PD1703 lipase, the two redundant PD0915 and PD0928 Zot proteins and at least one hemagglutinin all contribute to the pathogenic symptoms elicited by *Xf* on grapes.

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 determined the genomic DNA sequence of EB92-1 to 98% completion, allowing comparisons of this strain to strain Temecula, which causes PD. Most of the EB92-1 genes were nearly identical in gene order and protein sequence with those found in Temecula. No unique or additional genes were found in EB92-1 that were not previously identified in Temecula. However, 11 genes found in Temecula were not found in EB92-1; 10 of these encoded predicted secreted pathogenicity effectors that had not previously been associated with PD. Four of these missing genes were functionally tested to determine if they actually contributed to disease or not, and all four were found to enhance PD symptoms. This data identifies new molecular targets with potential to suppress disease symptoms.

INTRODUCTION

Symptoms of Pierce's disease (PD) include concentric rings of leaf scorch, almost reminiscent of a toxin. Symptoms of citrus variegated chlorosis (CVC) include brown, gummy lesions with necrotic centers. How these symptoms are elicited has been an open question for years. Hopkins (2005) discovered an effective PD biocontrol strain, *Xylella fastidiosa (Xf)* EB92-1, which infects grapevine and survives for many years, yet without causing any symptoms of PD. EB92-1 can be inoculated in a single location in a *Vitis 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 different? We reasoned that comparisons of the genomic sequence of EB92-1 with Temecula might reveal genetic differences enabling discovery of important PD pathogenicity factors and/or factors present in EB92-1 that may be triggers of host defense.

OBJECTIVES

The objectives of the two-year proposal are listed below.

1. Obtain nearly the complete EB92-1 genome DNA sequence. This objective was completed and is now published (Zhang et al., 2011);
2. Compare EB92-1 with Temecula and identify all unique open reading frames (ORFs) and differences, ranking the top 40 candidate ORFs for evaluation as elicitors. This objective was completed and is now published (Zhang et al., 2011).

3, 4, & 5. Evaluate two defense response assays designed to test the hypothesis that EB92-1 produces an elicitor that Temecula does not, to identify such elicitors and perform defense response assay screens. These objectives were not pursued because it became apparent from the results of sequence comparisons in Objective 2 that Temecula produced

pathogenicity effectors that EB92-1 did not. No genes were identified in EB92-1 that were not already present in Temecula. EB92-1 appeared to be a more highly evolved PD strain than Temecula, having lost specific pathogenicity factors---some redundant---that cause disease. It is not yet clear if EB92-1 is more likely to survive than Temecula, as some of the pathogenicity factors may enhance parasitic survival.

RESULTS AND DISCUSSION

The *Xf* biocontrol strain EB92-1 genome is very similar and syntenic to Pierce's disease (PD) strains. By far the majority of the primary BLAST hits were to Temecula; 92% of predicted EB92-1 proteins had more than 99% identity with Temecula proteins. Genome comparisons of EB92-1 revealed a high level of synteny with Temecula1. A plasmid sequence similar to the Temecula plasmid (pXFPD1.3) was also found in EB92-1. No unique or additional genes were found in EB92-1 that were not previously identified in Temecula1. However, 11 genes found in Temecula were not found in EB92-1; 10 of these encoded predicted secreted pathogenicity effectors (Zhang et al., 2011). Two predicted Temecula type II secreted enzymes, a serine protease (PD0956) and a lipase (PD1703), appeared to be missing from EB92-1. Two of three predicted zonula occludens toxin (Zot) genes, PD0928 and PD0915, also appeared to be missing in EB92-1. Zot is an important secreted virulence factor for *Vibrio cholerae* (Uzzau et al., 1999) and has been suggested as a potential virulence factor in CVC strain 9a5c (da Silva et al., 2007). Surprisingly, all six predicted hemagglutinin-like proteins (PD0986, PD1792, PD2108, PD2110, PD2116, and PD2118) appeared to be missing in EB92-1. Two of these (PD1792 and PD2118) carry a type V two-partner secretion (TPS) domain (Clantin et al., 2004); the remaining four have no TPS domain. Independent knockout mutations of PD1792 and PD2118 in Temecula1 caused an increase in virulence (Guilhabert et al., 2005). In contrast, a knockout of the only functional hemagglutinin-like protein with a TPS domain in *Xanthomonas axonopodis* pv. *citri* caused a loss of virulence (Gottig et al., 2009). Reported below are the results of experiments designed to functionally test the roles of four representatives of these 10 potential secreted pathogenicity factors.

A predicted type II secreted esterase, LipA, (PD1703), was entirely missing from EB92-1, evidently as a result of a deletion. This conclusion was confirmed by PCR analysis (**Figure 1**) and subsequent sequencing. PD1703 is an apparent *lipA* ortholog of *Xanthomonas oryzae*; *lipA* is known to directly contribute to pathogenic symptoms of *X. oryzae* by degradation of host cell walls, eliciting programmed cell death (Rajeshwari et al, 2005; Jha et al, 2007; Aparna et al 2009).

PD1703 exhibited strong esterase activity in *Xanthomonas* citri, *E.coli,* and EB92-1. PD1703 was cloned with its native promoter (690bp) and predicted secretion leader peptide in pBBR1MCS-5 (downstream from the *lacZ* promoter), creating pSZ26. 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).

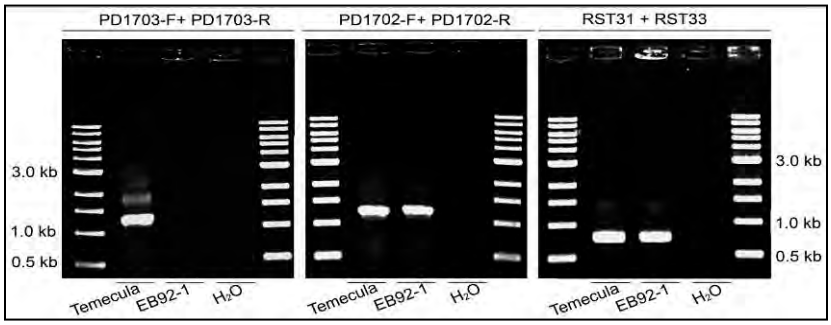

Figure 1. PCR confirmation of PD1703 in Temecula and EB92-1.

Agar plates containing the substrate and indicator were poured and wells created by removal of agar with a cork borer. Culture supernatants from centrifuged cells grown to late mid-log phase (ca. OD = 0.7) were added to the wells as indicated in **Figure 2**. The third row in **Figures 2A and 2B** shows the reaction of the indicator to supernatants from *X. citri* B21.2 and *E. coli* Mach1-T1 exconjugants, respectively, carrying cloned PD1703 (pSZ26), respectively, and the fourth row in **Figure 2C**, shows the reaction to the supernatant from the EB92-1 exconjugant carrying cloned PD1703 (pSZ26), demonstrating relatively strong amounts of secreted lipase in these culture supernatants (the crude supernatants were not concentrated or purified). These levels of lipase activity were not present in the supernatants of wild type Temecula or EB92-1, nor in *X. citri* B21.2, *E. coli* Mach1-T1, *X. citri* B21.2 and *E. coli* Mach1-T1 transconjugant with another lipase (PD1702, with its native promoter) cloned from Temecula1, or these same strains carrying the empty vector pBBR1MCS-5.

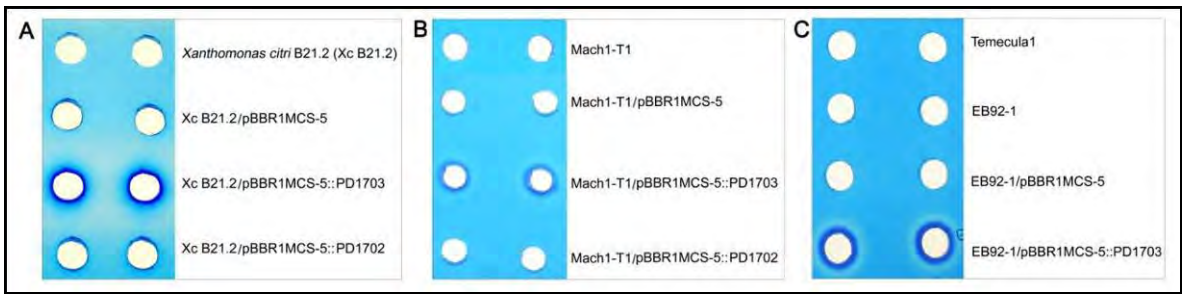

Figure 2. *In vitro* secreted esterase assay of culture supernatants from *X. citri* B21.2, *E. coli*, and *Xf* strains Temecula and EB92-1.

Crude protein extracts of PD1703 overexpressed in BL21 (DE3)/pET-27b induced hypersensitive cell collapse in tobacco and citrus. Crude protein was extracted from *E. coli* BL2 (DE3) carrying pET27b and expressing PD1703 from *lacZ* using a Qiagen protein extraction kit. Protein was resuspended in 200 ul of supplied buffer; some protein remained insoluble but was applied in a suspension and labeled "total protein" in **Figure 3** below. Total protein was centrifuged at high speed to pellet the insoluble protein, and the remaining soluble protein is labeled "supernatant" in **Figure 3** below. A total of 50 ul of total and 50 ul of soluble protein was added to the wells created in the agar plate shown in **Figure 3A**, below. Approximately 10 ul of crude protein suspension ("total protein") was injected into the tobacco and citrus leaves for each inoculation zone circled in **Figure 3B**.

An amount of crude protein suspension that measured 15-18 mg/ml elicited a rapid cell collapse that was visible starting 14 hrs post inoculation in tobacco. The reaction became stronger by two days. Photos of reacting tobacco and citrus leaves at 48 hrs post inoculation are shown in **Figure 3B**. By comparison, 4-5 ug/ml of purified *X. oryzae* LipA were required to elicit browning of rice in infiltrated zones by degradation of cell walls (Jha et al. 2007).

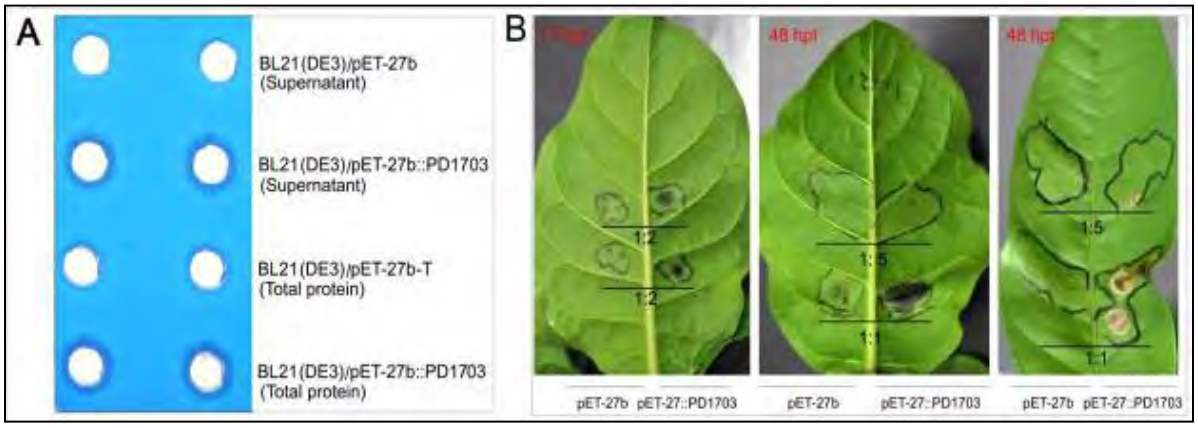

Figure 3 PD1703 crude protein exhibited esterase activity in agar plate assays (A), and induced hypersensitive cell collapse in SR1 tobacco (B, left and center) and citrus (B, right). Equivalent amounts of crude protein from empty vector (pET-27b) or expressing PD1703 (pET-27b::PD1703) were inoculated. A dilution range of 1:1, 1:2, and 1:5 was applied to these leaves; the 1:2 dilution represents 15 – 18 mg/ml of suspended crude ("total protein").

Temecula PD1703 lipase (in pSZ26), PD0928 Zot (in pSZ41), and PD0986 (in pPC3.1) hemagglutinin all contribute to the pathogenic symptoms elicited by *Xf* on grapes. All *Xf* strains were grown in PD3 medium for 36-48 hours at 28° C. As mentioned above, PD1703 was cloned with its native promoter (690bp) and predicted secretion leader peptide in pBBR1MCS-5 (downstream from the *lacZ* promoter), creating pSZ26. For pathogenicity assays, 4-6 week old *V. vinifera* cv. Carignane were inoculated with 10 ul cultures of each strain (OD_{600} = 0.245). Bacterial cultures were diluted in SCP buffer and were inoculated by stem puncture at two points in each of the three internodes, starting with the second internode. Each experiment was repeated at least three times with 3-4 replications in each independent experiment. Plants were maintained under green house and carefully observed for the appearance of symptoms. Observations were recorded from the time the first visible symptoms appeared (6-7 weeks post inoculation). Inferences were drawn from at least three independent experiments.

Pathogenic symptoms elicited by Temecula began to appear by the 5th week after inoculation, and continued to develop up to 12 weeks. Most of the inoculated plants were killed after 12 weeks. Plants inoculated with EB92-1/pSZ26 (Lipase;

Figure 4) and EB92-1/pSZ41 (ZOT; **Figure 5**) showed slightly delayed pathogenesis with visible symptoms becoming evident by the end of six weeks. The infection progressed slowly and remained restricted to 9-10 internodes in case of strains pSZ26 and pSZ41. At the end of three months, plants inoculated with EB92/pSZ26 reached a total of 30% infection and EB92/pSZ41 reached a total of 22 %. EB92-1/ pBBRMCS5 (empty vector) never reached higher than 12% during the period (**Table 1**). Plants inoculated with EB92-1/pPC3.1 (hemagglutinin; **Figure 6**) exhibited symptoms almost as rapidly as the wild type (by the 5th week after inoculation) but with reduced severity (40% infection for EB92-1/pPC3.1 vs. 62% for Temecula by 48 days post inoculation).

Plants inoculated with EB92-1/pSZ26 produced necrosis delimited with pinkish areas. As the infection progressed the entire lamina underwent necrosis leading to defoliation. However the infection progressed slowly compared to wild type strains and remained restricted to 9-10 internodes above the lowest point of inoculation. By contrast, wild type Temecula infections advanced to the tip of the vine (refer **Figure 4**).

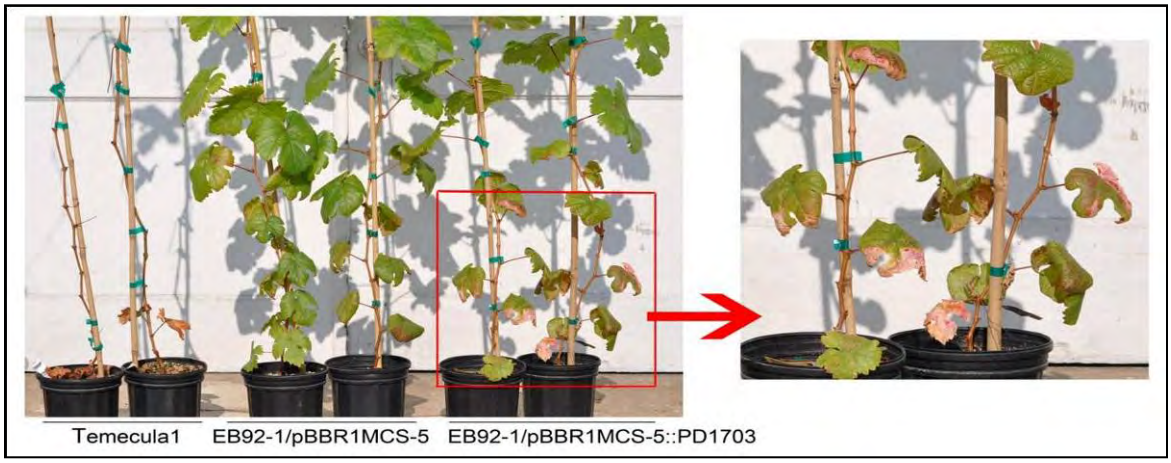

Figure 4. Pathogenic symptoms elicited by Temecula, EB92-1/ pBBRMCS5 (empty vector), and pSZ26 (PD1703 lipase), respectively. (Enlargement shows boxed area on right). Photos taken 90 days post-inoculation.

The infection of EB92-1/pSZ41 (ZOT) was also comparatively slow with visible symptoms becoming evident only by the end of six weeks. The inoculated plants showed prominent anthocyanosis with green veinal areas. As the infection advanced the infected leaves underwent necrosis resulting in defoliation (**Figure 5**).

Figure 5. Pathogenic symptoms elicited by pSZ41 (ZOT; PD0928). These lower leaves dropped off 10 days later. Photo taken 90 days post-inoculation.

The infection of EB92-1/pPC3.1 (hemagglutinin; PD0986) was surprisingly fast by comparison with the other two transconjugants, with visible symptoms elicited by the transconjugants becoming evident by the beginning of the 5th week in

two independent experiments (15 grape plants inoculated with transconjugants total). All plants inoculated with transconjugants showed prominent necrotic areas very similar to those observed with the wild type (**Figure 6**).

Figure 6 Pathogenic symptoms elicited by EB92-1/pPC3.1 (hemagglutinin; PD0986). Photo taken 48 days post-inoculation.

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FUNDING AGENCIES

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Table 1. Results of 3 independent experiments comparing pathogenicity of Temecula & EB92-1 exconjugants carrying pBBRMCS5 (empty vector), pSZ41(PD0928; Zot) or pSZ26 (PD1703; lipase).

	# Total leaves	# Asympto-matic leaves	# Sympto-matic leaves	# Bare petioles	# Bare nodes	# Total leaf production	# Total diseased leaves	Diseased leaves/ total	Avg. diseased leaves/ total
Temecula (WT)	9	1	8	3	23	35	34	0.97	0.70
	11	0	11	4	23	38	38	1.00	
	7	0	7	4	20	31	31	1.00	
	4	0	4	6	23	33	33	1.00	
	38	17	21	3	4	45	28	0.62	
	39	28	10	0	0	38	10	0.26	
	53	42	11	1	10	64	22	0.34	
	61	38	23	0	2	63	25	0.40	
	33	17	16	0	2	35	18	0.51	
	25	13	12	0	8	33	20	0.61	
	0	0	0	0	20	20	20	1.00	
	23	12	11	0	20	43	31	0.72	
EB92/ pBBRMCS5	31	30	1	0	0	31	1	0.03	0.12
	40	35	5	0	2	42	7	0.17	
	40	37	3	0	2	42	5	0.12	
	40	39	1	0	1	41	2	0.05	
	38	34	4	0	3	41	7	0.17	
	30	29	1	0	4	34	5	0.15	
	24	23	1	0	4	28	5	0.18	
	37	36	1	0	0	37	1	0.03	
	76	71	5	0	0	76	5	0.07	
	46	41	5	0	3	49	8	0.16	
	43	40	3	0	3	46	6	0.13	
	40	37	3	0	4	44	7	0.16	
	43	39	4	0	4	47	8	0.17	
EB92/ pSZ41 (Zot)	41	35	6	0	0	41	6	0.15	0.22
	33	29	4	0	3	36	7	0.19	
	30	27	3	0	2	32	5	0.16	
	32	30	2	0	4	36	6	0.17	
	41	36	5	0	1	42	6	0.14	
	33	28	5	1	0	34	6	0.18	
	29	23	6	0	0	29	6	0.21	
	13	10	3	0	5	18	8	0.44	
	41	34	7	0	0	41	7	0.17	
	57	47	10	0	6	63	16	0.25	
	18	12	6	1	3	22	10	0.45	
EB92/ pSZ26 (lipase)	34	28	6	0	3	37	9	0.24	
	38	36	2	0	3	41	5	0.12	
	30	22	8	0	5	35	13	0.37	
	41	37	4	0	3	44	7	0.16	
	32	23	9	0	1	33	10	0.30	
	34	28	6	0	7	41	13	0.32	0.30
	23	18	5	1	4	28	10	0.36	
	36	28	8	0	0	36	8	0.22	
	33	20	13	0	0	33	13	0.39	
	7	4	3	1	2	10	6	0.60	
	35	24	11	0	0	35	11	0.31	

CONTINUED ASSESSMENT OF *XYLELLA FASTIDIOSA* FIMBRIALADHESINS 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 September 2010 to September 2011.

ABSTRACT
Specific biological characteristics of *Xylella fastidiosa* (*Xf*) Temecula were investigated in microfluidic flow chambers *in vitro* by examining the influence of xylem saps from Pierce's disease (PD) susceptible *Vitis vinifera* and resistant *V. champinii* and *V. smalliana* grapevines on *Xf* motility. Compared to that observed in *V. vinifera* sap, type IV pili mediated twitching motility of *Xf* was significantly reduced in saps from both resistant grapevines and is associated with down-regulation of type IV pili associated genes. The *Xf* response in resistant sap mirrors observations in citrus sap: down-regulation of type IV pili associated genes, lack of twitching motility, and resistance to pathogenesis by *Xf* Temecula. Overall the findings suggest that PD resistance in certain grape cultivars is associated with sap that blocks motility through gene regulation, limits aggregation, and lessens production of biofilms by *Xf.*

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 observed reduced motility in sap from a resistant grapevines as well as a 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, cell aggregation, 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, as well as 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 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 *Vitis 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 bacteria were 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 observations reported that both type I and type IV pili are involved in aggregation and biofilm development (Li, 2007), and that 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 (reported in 2010 PD symposium).
2. Assess pili-associated functions in grapevine sap from *Vitis vinifera* cultivars and *Vitis* species expressing distinct PD resistance and susceptibly. (preliminary report in 2010 PD symposium).
3. Assess pil and fim gene expression for conditions in Objective 2 that exhibit significant differences in functional *Xf* activities.
4. Compare pili-associated functions in grapevine vs. citrus sap

RESULTS

***Xf* motility in various grapevine saps**

Xf cells were assessed for motility in microfluidic flow chambers. Most *Xf* cells exhibited twitching motility in PD2 broth (**Figure 1**). After a period of adjustment from growth on PD media to sap *Xf* cells in *V.vinifera* sap from NY also showed nearly complete motility (96%) (**Figure 1**). In *V. champinii* and *V. smalliana* saps from NY, relatively few *Xf* cells attached to the chamber surface; furthermore, few (3% and 1%, respectively) *Xf* cells exhibited twitching motility (**Figure 1**). Similar responses were observed for saps obtained from grapevines grown in Davis, CA (**Figure 1**). These results suggest that twitching is either upregulated in PD-susceptible sap or suppressed in PD-resistant sap.

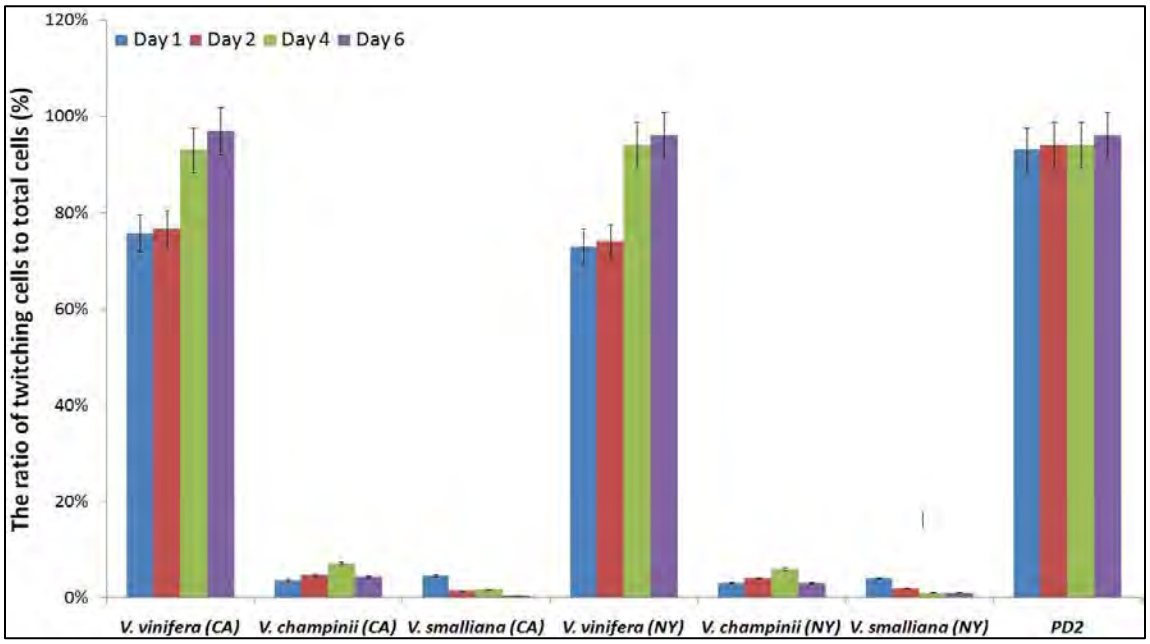

Figure 1. Twitching motility of *Xf* in various saps. The ratio of twitching cells to total cells in PD2 broth and saps of *V. vinifera*, *V. champinii*, and *V. smalliana* in microfluidic flow chambers was assessed over 6 days.

***Xf* response following exchange of sap types**

To determine if *Xf* behavior in saps could be modified under different sap conditions, we acclimated cells in microfluidic chambers to PD-susceptible *V. vinifera* sap and then exchanged it with a PD-resistant sap. *Xf* cells in PD-susceptible *V. vinifera* sap were motile and developed large cell aggregates [**Figures 2A(a) and (g)**]. After four days growth in *V. vinifera* susceptible sap. the sap was replaced with PD-resistant *V. smalliana* sap. After eight hours, the cell aggregates began to disperse and twitching was reduced [**Figure 2A(b)**]. In an adjacent *V. vinifera* perfused sap control chamber the cells continued to exhibit motility and form aggregates [**Figure 2A(h)**]. By day five *Xf* cells in *V. smalliana* sap were uniformly distributed within the chamber and only 5% were motile [**Figure 2A(c)**], while ca. 85% of the *Xf* cells in *V. vinifera* sap

continued to twitch and form aggregates [**Figure 2A(i)**] (**Figure 4**). From day five to eight, cells in *V. smalliana* sap formed a thin biofilm layer within the chamber [**Figures 2A(c)-(f)]** whereas cells in the control chamber developed a robust biofilm [**Figures 2A(i)-(l)**].

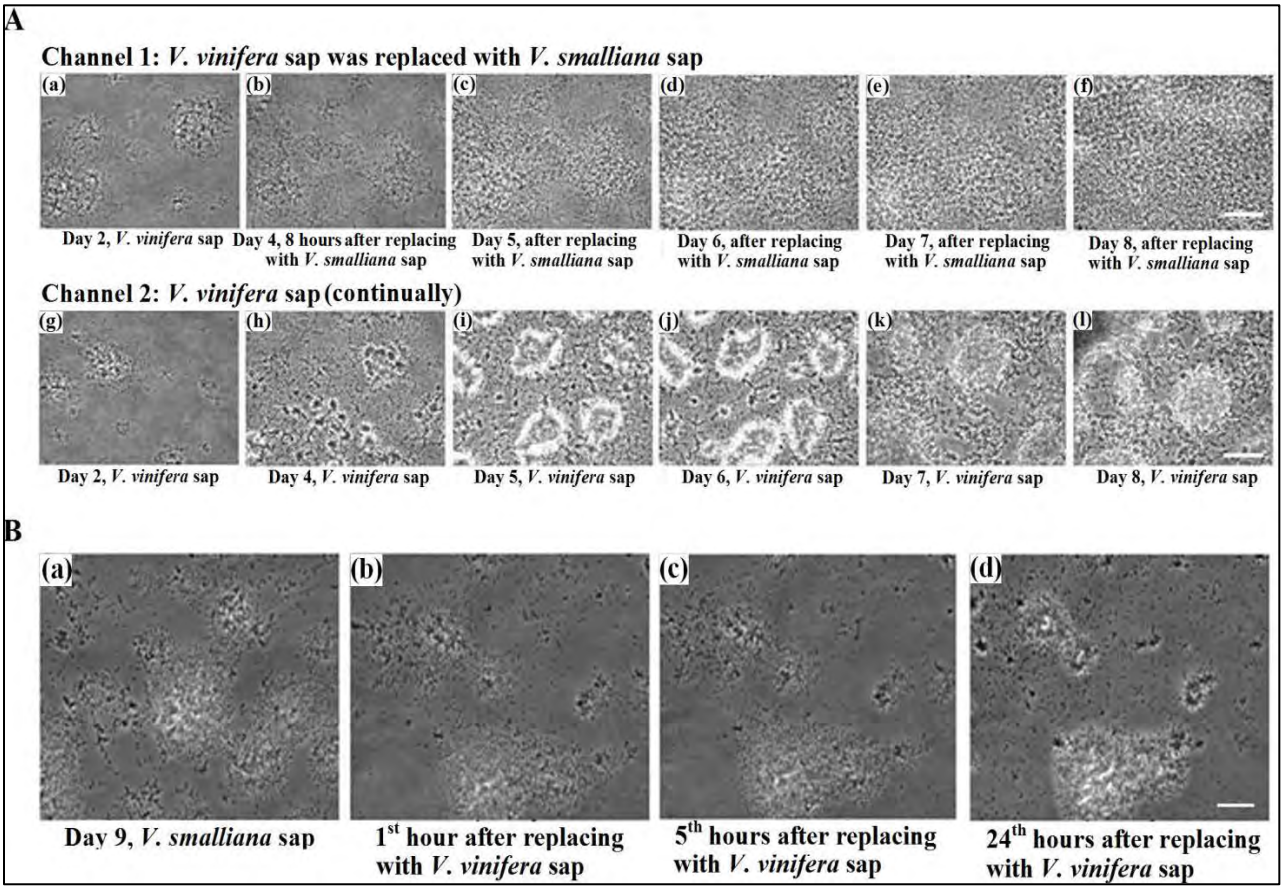

Figure 2. Twitching motility of *Xf* in PD-susceptible and PD-resistant saps. **(A)** Images taken before and after *V. vinifera* sap was exchanged with *V. smalliana* sap. In one microfluidic chamber *Xf*cells were continually exposed to PD-susceptible *V. vinifera* sap for eight days l (g-l). In another chamber *Xf* cells the*V. Vinifera* sap was changed to PD-resistant *V. smalliana* after day four (a-f). **(B)** *Xf* cells that had been grown in *V. vinifera* and changed to *V. smalliana* sap were exchanged back to *V. vinifera* sap on day nine (a-d). Scale bar equals 50 µm.

To determine if the *Xf* cells could return to the twitching phenotype, the PD-resistant *V. smalliana* sap in the chamber was replaced with *V. vinifera* sap on day nine [**Figure 2B(a)**]. After one to five hours the cells began to twitch and aggregates began to form [**Figures 2B(b) and (c)**], and by 24 hours large aggregate appeared [**Figure 2B(d)**]. When quantified, 3% of *Xf* cells expressed twitching motility in PD-resistant *V. smalliana* sap, whereas, 75% of the *Xf* cells were motile after *V. smalliana* sap replaced the PD-susceptible *V. vinifera* sap (**Figure 3**). Similar observations were made when *Xf* cells were grown in microfluidic chambers in *V.vinifera* and then replaced with *V. champinii* sap and back to *V. vinifera* sap (data not shown). These data suggest that either a) PD-susceptible saps induce the function or expression of type IV pili, resulting in twitching motility or b) PD-resistant saps inactivate the function or expression of type IV pili, resulting in the loss of twitching motility. The results of reduced motility are consistent with previous reports that *Xf* spreads faster in xylem vessels of PD-susceptible compared to PD-resistant or tolerant grapevines (Hopkins, 1984; Fry and Milholland, 1990).

Affect of sap on type IV pili-related

A number of mechanisms could be envisioned as to how a component in sap activates or inhibits motility such as altering gene expression of pili-associated genes. To explore gene expression, we analyzed the mRNA levels of representative genes *pilI*, *pilJ*, *pilG, pilA*, *pilQ*, and *pilR* involved in type IV pili biogenesis and regulation (**Figure 4**). The *pilI, pilJ,* and *pilG* gene encode proteins in the chemotaxis-like operon, Pil-Chp (Cursino, 2011). Chemosensory-like proteins have been implicated in motility, pili formation, transcriptional regulation, and exopolysaccharide production (Kirby, 2009). While the exact function of Pil-Chp is unknown, we previously found that disruption of the operon blocks twitching motility without inhibiting type IV pili biogenesis (Cursino, 2011). The PilQ protein is predicted to be a multimeric protein that forms the pore through which the type IV pili thread. We previously found that mutations in *pilQ* prevented type IV pili formation and motility (Meng, 2005). The PilR protein is predicted to belong to a two-component regulatory system, PilR/PilS, in which

PilS activates PilR, which in turn regulates *pilA* transcription; PilA is the major pilin protein of the type IV pilus (Winther-Larsen and Koomey 2002). PilA mutants of *P. aeruginosa* have reduced virulence (Comolli, 1999), and *pilA* mutants of *R. solanacearum* cause less severe wilting symptoms in tomato plants (Kang, 2002). Disruption of *pilR* leads to *Xf* without type IV pili and incapable of twitching motility (Li, 2007). In this study we found that *Xf* grown in *V. smalliana* and *V. champinii* saps, but not *V. vinifera* sap, fail to express *pilI*, *pilQ*, and *pilR*. These findings suggest that sap from resistant plants fail to support type IV pili production, block twitching motility, and therefore limit the pathogen spread within the plant. Given that multiple genes are down regulated in the resistant saps (or upregulated in susceptible sap), a key chemical component in sap may target an upstream regulator gene for type IV pili.

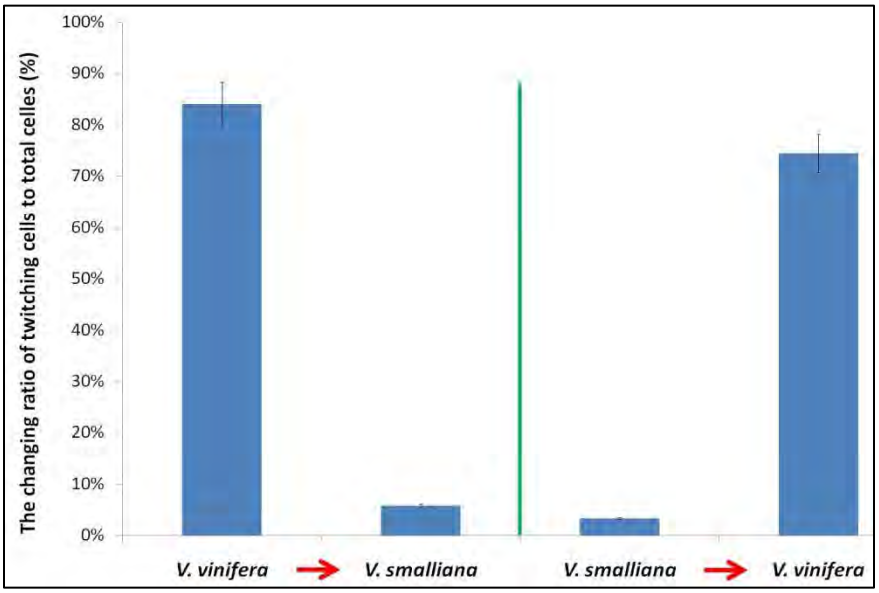

Figure 3. The changing ratio of twitching cells to total *Xf* cells after *V. vinifera* sap were replaced with *V. smalliana* sap and back to *V. vinifera* sap.

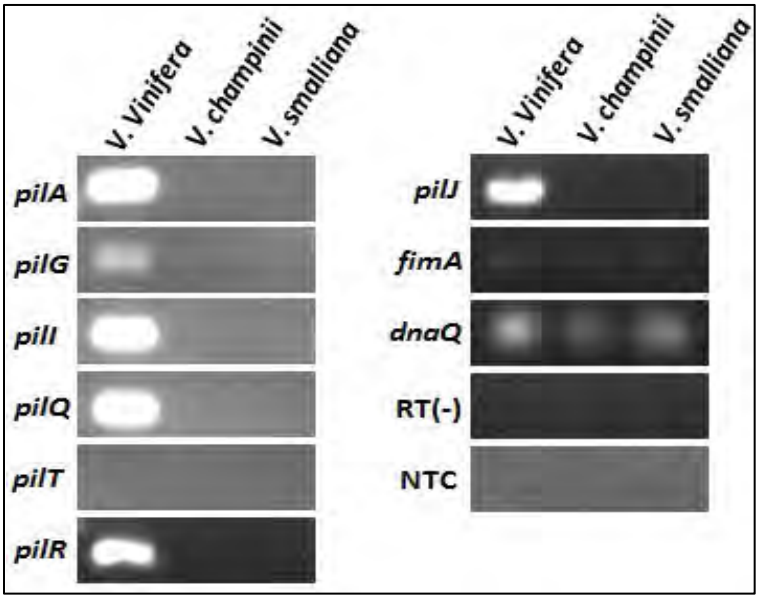

Figure 4. The detection of differential expression of type IV pili related genes *Xf* in saps of *V. vinifera*, *V. champinii*, and *V. smalliana* by reverse transcription polymerase chain reaction (RT-PCR). Positive control was DanQ expression. Negative control wasRT (-) negative control: all RT reactioncomponents but no RT reverse enzyme, and NTC: all RT reaction components but no RNA template.

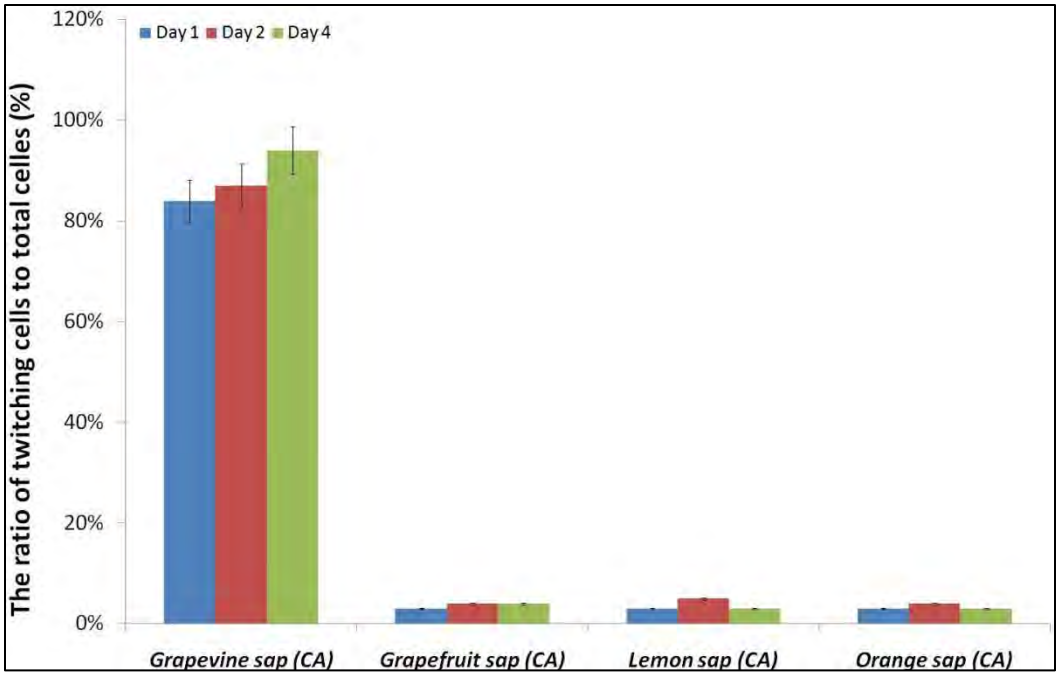

Figure 5. Twitching motility of *Xf* in various saps. The ratio of twitching cells to total cells in saps of grapevine (*V. vinifera*) and citrus (grapefruit, lemon, and orange) from CA in microfluidic flow chambers was assessed.

***Xf* motility in citrus sap**

Xf gene expression observed in PD-resistant saps mirrors findings when *Xf* is grown in citrus sap (Shi, 2010), and *Xf* Temecula does not produce disease in citrus plants (Perring, 2001). Therefore we wanted to determine if *Xf* is also non-motile in sap from citrus plants. *Xf* was found to be twitching impaired in saps of grapefruit, lemon, and orange (**Figure 5**). *Xf* cells in sap of grapevine (*V. vinifera*) from CA showed high levels of motility (94%+/-4). In saps of grapefruit, lemon, and orange from CA, relatively few (4%+/-2, 3%+/-2, and 3%+/-2, respectively) cells exhibited twitching motility (**Figure 5**). These results suggest that twitching of *Xf* cells is suppressed in citrus sap.

Comparison of responses in PD-resistant and citrus sap

The finding that grape sap composition alters gene expression of type IV pili associated genes is particularly interesting in light of resistance in non-grape plants. Citrus plants infected with *Xf* strain 9a5c exhibit a PD-like response known as citrus variegated chlorosis (CVC) (Chang, 1993; Hartung, 1994; Purcell & Hopkins, 1996). However, the *Xf* Temecula strain does not exhibit CVC even when PD-infected plants are found next to citrus orchards (Perring, 2001; Bi, 2007). Why Temecula does not produce disease is unknown. When examining twitching motility, we found that both PD-resistant and citrus saps fail to support mobility. Additionally, just as we found with PD-resistant sap, *Xf* grown in citrus sap is known to result in down regulation of type IV pili biogenesis and regulatory genes (Shi, 2010). While additional resistant mechanisms unique to each plant may play key roles in preventing *Xf*-induced disease, our findings suggest potential universal mechanisms for disease regulation.

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 chemical differences among saps of susceptible and resistant grapevine are highly desired to understand the biochemical mechanisms of host resistance to *Xf*. The present results suggest that the inhibition of twitching motility of *Xf* by 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, named Pil-Chp, 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 chemotaxis system. First, we have examined the operon genes more closely. We have discovered that the operon is essential for the twitching phenotype, biofilm formation, and Pierce's disease (PD) development. Examining each Pil-Chp gene individually we learned that the first four genes are critical for twitching and that all genes play a role in biofilim formation and PD symptoms. Second, we have tentatively found a twitching minus medium to which we can add known sap components, examine for twitching recovery, and determine the component driving motility. Third, we have continued our examination of *chpY*, a gene similar to *pilG*, which plays a role in PD 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 are determining what chemical signals are 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 system with similarities 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 (see review Hazelbauer et al. 2008). We previously found that the homologous 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.

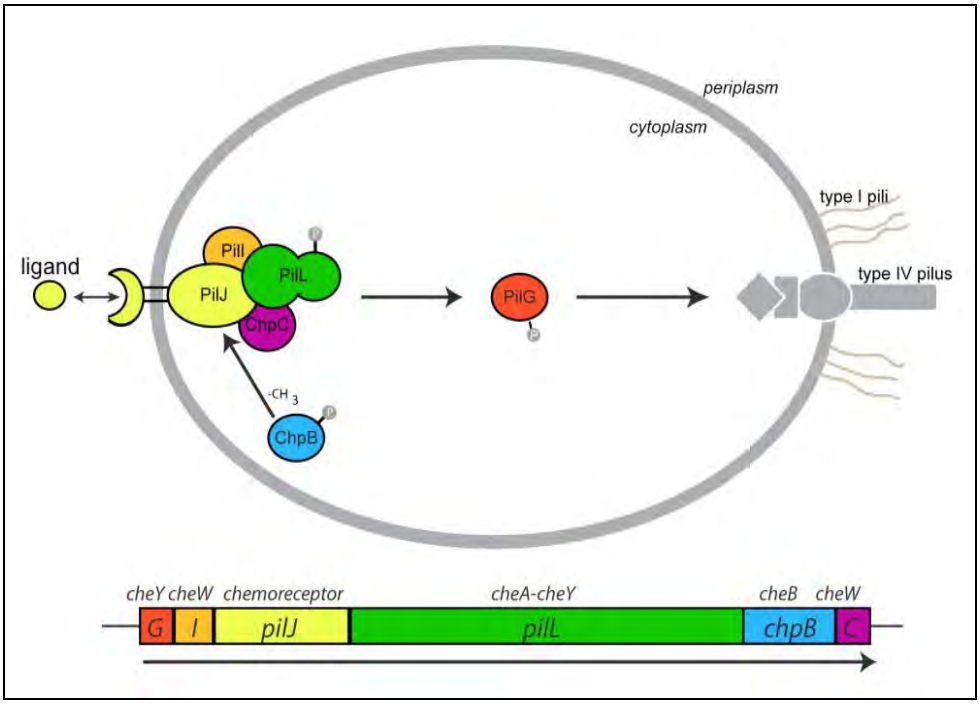

Figure 1. **The *Xf* Pil-Chp operon.** A) Model for operon protein products regulating twitching motility in *Xf*. The chemoreceptor PilJ senses environmental signal(s). ChpC/PilI couples PilL to PilJ. PilL phosphorylates its hybrid CheY-like receiver domain and PilG. ChpB is homologous to ligand adaptation proteins and may act as a phospho-transfer protein or ligand adaptor modulator. B) The Pil-Chp operon genes with *E. coli* homologous genes shown above and direction of transcription shown below.

RESULTS AND DISCUSSION

Construction of the *Xf* Pil-Chp operon null mutant strains. The Pil-Chp operon was disrupted previously with a polar transposon mutation in the *pilL* gene (Cursino et al. 2011). We subsequently constructed non-polar, allelic exchange mutants of all Pil-Chp operon genes, including *pilL*, according to Chatterjee *et al*. 2008 with slight modifications. The disruption of each gene in marker-exchange mutants was confirmed by PCR (not shown).

Construction of plasmids to complement the *Xf* Pil-Chp operon null mutant strains. To complement the non-polar gene disruptions, we constructed *Xf*-compatible plasmids containing the chemotaxis operon promoter region (Hoch et al. 2010). We then cloned the various Pil-Chp genes into these constructs and transformed the constructs into the null mutants. Successful transformation was confirmed by PCR (data not shown).

Twitching motility of the *Xf* Pil-Chp operon null mutant strains. We observed that the Pil-Chp transposon polar mutant was twitching minus on both PW agar surfaces and in microfluidic chambers (Cursino et al. 2011). PW agar surfaces revealed colony morphologies with smooth margins consistent with loss of type IV pili twitching motility function (Meng et al. 2005). This mutant retained type IV pili (Cursino et al. 2011), indicating that the Pil-Chp operon regulates twitching and not pili formation. Results were confirmed in microfluidic chambers (Cursino et al. 2011). Examination of the non-polar Pil-Chp operon gene mutants on PW agar and in chambers revealed that *pilG*, *pilI*, *pilJ*, *pilL* mutants are twitching minus (data not shown). Complementation of these genes recovered twitching (data not shown). Interestingly, the *chpB* and *chpC* mutants performed twitching motility like wild-type cells (data not shown).

Biofilm formation by the *Xf* Pil-Chp operon null mutant strains. Using a crystal violet assay (Zaini et al. 2009), we found that the Pil-Chp transposon polar mutant produced less biofilm compared to wild-type *Xf* cells (Cursino et al. 2011). Additionally, all of the non-polar Pil-Chp operon gene mutants produced less biofilm, even twitching plus mutants *chpB* and *chpC* (**Figure 2**).

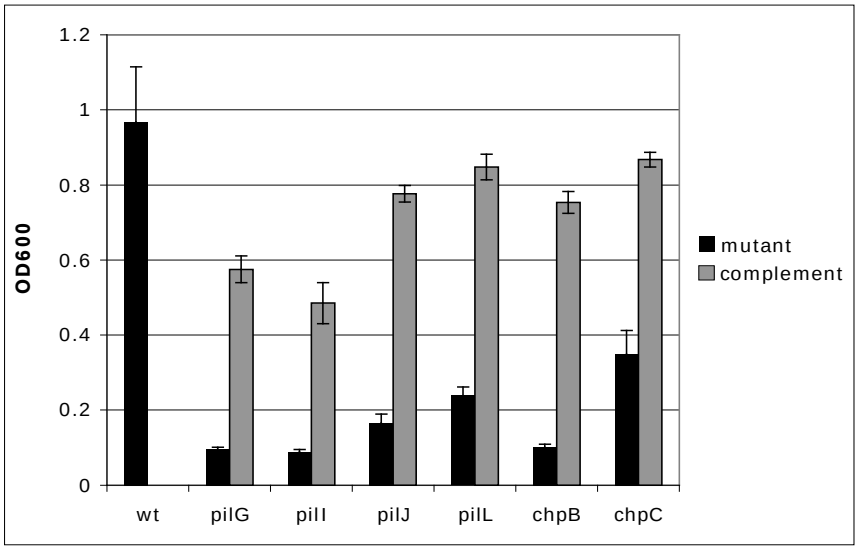

Figure 2. Biofilm formation with Pil-Chp operon mutants. Biofilm formation by wild-type, Pil-Chp non-polar gene mutants, and complemented mutants in *Xf* cells grown in culture flasks following 10 days of growth with agitation. Quantitation of biofilm formation was performed by the crystal violet assay (Zaini et al. 2009).

***In planta* results with the *Xf* Pil-Chp operon null mutant strains.** We discovered that the Pil-Chp transposon polar mutant cells induced less PD symptoms *in planta* compared to inoculation with wild-type cells (Cursino et al. 2011). Twitching minus non-polar Pil-Chp operon mutants *pilG*, *pilI*, and *pilJ* induced less PD disease *in planta* and symptoms plateaued after 20 weeks (**Figure 3**). Conversely, the Pil-Chp operon twitching plus *chpB* and *chpC* mutants induced full PD symptoms, however the disease was delayed compared to wild-type cells. We are currently testing the non-polar *pilL* mutant *in planta*.

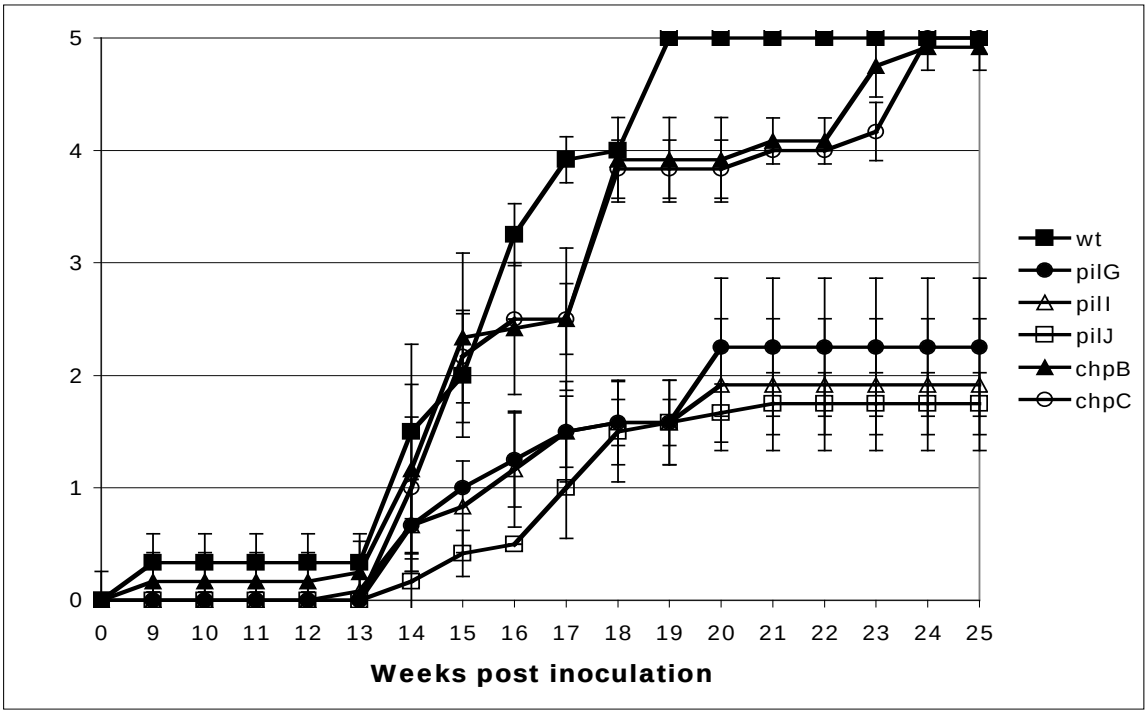

Figure 3. PD development in Pil-Chp operon gene mutants. *Vitis vinifera* L. cv. Cabernet Sauvignon vines were inoculated with wild-type or Pil-Chp non-polar gene mutants and PD disease was assessed over 25 weeks. Plants inoculated with the *pilL* mutant are currently being analyzed. PD rating on a scale of 0-5 (Guilhabert and Kirkpatrick 2005).

Identifying the chemosensory attractant. To examine what component(s) in sap drives the motility response, numerous assays were attempted (Hoch et al. 2009, Hoch et al. 2010). We desired a medium that supports *Xf* growth at wild-type levels but on which colonies do not develop fringe. We could identify the motility stimuli by adding sap components to the medium and testing for fringe recovery. We have found that *Xf* cells grow at wild-type levels in PW medium without soytone but fail to produce a fringe on this medium (data not shown). We are currently developing this method and will begin testing sap components.

Pil-Chp operon and *chpY* gene. The *chpY* gene lies downstream of the Pil-Chp operon and has homology to the *Xf pilG* gene that codes for a phospho-shuttle protein. In the similarly organized P*seudomonas aeruginosa* Pil-Chp operon, downstream genes produce proteins that are proposed to associate with the Pil-Chp operon protein products (Whitchurch et al. 2004). In addition to the *pilG*-like domain, *chpY* has GGDEF and EAL regions that is known to regulate cyclic di-GMP (Ryan et al. 2006). However the putative ChpY GGDEF and EAL regions appear to be non-functional, as they lack the expected enzymatic amino acid residues (data not shown). 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 Pil-Chp *pilL* gene in the *chpY* null strain. When *chpY* is deleted, *Xf* shows no changes in *pilL* expression (data not shown). However, *chpY* does have an effect on PD. Grapevines inoculated with the *chpY* mutant had increased PD progression compared to a wt *Xf* infection (data not shown), which may stem from the *chpY*-induced increased biofilm formation or reduced twitching motility. To test whether increased biofilm formation was related to increased expression of biofilm forming *gum* genes (Roper et al. 2007), we performed RT-PCR (**Table 1**). The *gumD and gumJ* were found to have a three-fold increased expression in the *chpY* mutant as compared to wild-type cells. Similarly, extracellular polymeric substance (EPS) production was three-fold higher in the *chpY* mutant strain as compared to wild-type cells. We also examined the production of extracellular enzymes in the *chpY* null strain, as these enzymes are known pathogenic factors (Thowthampitak et al. 2008). For the enzymes tested (carboxymethylcellulose, polygaracturonase, protease, and pectin methylesterase) no differences were found between the wild-type and the *chpY* mutant strains (data not shown).

Table 1. Effect of *chpY* on the expression of gum genes. The levels of gene expression in *Vitis vinifera* sap were tested by RT-PCR and normalized to *dnaQ* expression. The experiments were performed three times, with three replicates each. The standard deviations of the normalized means are shown.

Strains	**Genes**		
	chpY	*gumJ*	*gumD*
Wild-type	1.09±0.5	1.36±0.1	1.18±0.2
chpY mutant	ND	2.61±0.4	2.83±0.3
Complemented *chpY*	2.17±0.3	1.25±0.2	1.02±0.1

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 play a role in twitching motility but all play a role in biofilm formation and PD development. We appear to have found an assay to determine the chemical stimuli in grape sap driving motility. Additionally, we report that although *chpY* gene protein product does not regulate the Pil-Chp operon, it does contribute to PD development by upregulating the *gum* genes, which leads to an increased biofilm phenotype.

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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 of grapevine (Wells et al., 1981). *Xf* is closely related to *Xanthomonas oryzae* pv. *oryzae* (*Xoo)*. 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 Pierce's disease of grapevine. *Xf* causes disease by colonizing the xylem vessels, blocking the flow of water in the grapevine. In many plant pathogenic bacteria, 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 compound 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 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 in *Xanthomonas* and *Xf* the molecules are referred to as diffusible signal factors (DSF). The auto-inducer is a means by which bacteria recognize bacterial 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 homologs 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. We will repeat the pathogenicity assays again next year and 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 an Ax21 activity assay using our 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 controls, 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*. This result suggests that *Xf* Ax21 is unable to trigger Ax21-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 of Ax21. Further research will be conducted to better understand the role of Ax21 in *Xf* pathogenicity and cell-cell communication.

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 cell growth assays, *Xf*Δax21 grows to a lower population density than wild type *Xf* (**Figure 2**). There was no significant difference (95% CI) in biofilm formation or cell aggregation between *Xf* Fetzer (wt) and *Xf* Δ*ax21* (**Figures 3, 4**). Pathogenicity assays on Thompson seedless grapevines were conducted in the greenhouses this summer. We found no significant differences in colonies isolated from the point of inoculation or 25cm above the point of inoculation (**Figure 7**). Furthermore, we found similar levels of disease severity in *Xf* Δ*ax21* and wt *Xf* 18 weeks post-inoculation (**Figure 8**).

Both *Xf*Δ*phoP* and *Xf*Δ*phoQ* were found to have significantly (95% CI) less biofilm formation than wt *Xf* after ten days static incubation using the crystal violet assay (**Figure 5**). Furthermore, we also found that *Xf*Δ*phoP* and *Xf*Δ*phoQ* had significantly (95% CI) less cell-aggregation (**Figure 6**). We also found that there was no significant difference in biofilm formation or cell-cell aggregation between *Xf*Δ*phoP* and *Xf*Δ*phoQ*. This result would be expected, since PhoP and PhoQ collectively make up a two-component regulatory system (TCS). A mutant deficient in one gene should exhibit the same phenotype as a mutant deficient in the second gene of the TCS. Pathogenicity assays on Thompson seedless grapevines were conducted in the greenhouses this summer. We found no colonies isolated from the point of inoculation or 25cm above the point of inoculation (**Figure 7**). Furthermore, we found significantly reduced levels of disease severity compared to grapevines inoculated with wt *Xf* 18 weeks post-inoculation (**Figure 8**).

Although we isolated no live colonies from grapevines inoculated with *Xf* Δ*phoP*, and *Xf* Δ*phoQ*, it appeared that the vines exhibited mild PD-related symptoms. These mild symptoms are most likely due to a variety of non-PD issues that were stressing the grapevines including nutrient deficiency, insect damage and scorching from the greenhouse lamps. Therefore, we think the observed PD symptoms were difficult to differentiate from the other problems we encountered in the greenhouse. We plan to do PCR on the inoculated grapevines to track the movement of the mutants within the grapevine as well as to double check that all grapevines were successfully inoculated.

CONCLUSIONS

We have made good progress on determining the functional role of Ax21 and the PhoP/Q TCS in *Xf,* although further comparison of wild-type *Xf* and *Xf*Δax21 needs to be done. We are currently awaiting final results from microarray analysis of *Xf*Δ*ax21, Xf*Δ*phoP*, and *Xf* Δ*phoQ*. We are currently conducting microarray analysis and once complete, we will continue to look into genes regulated by PhoP/Q. Furthermore, we are confirming whether or not a metabolically active Ax21 peptide is secreted by *Xf*. We will begin work on Objective 3 this fall.

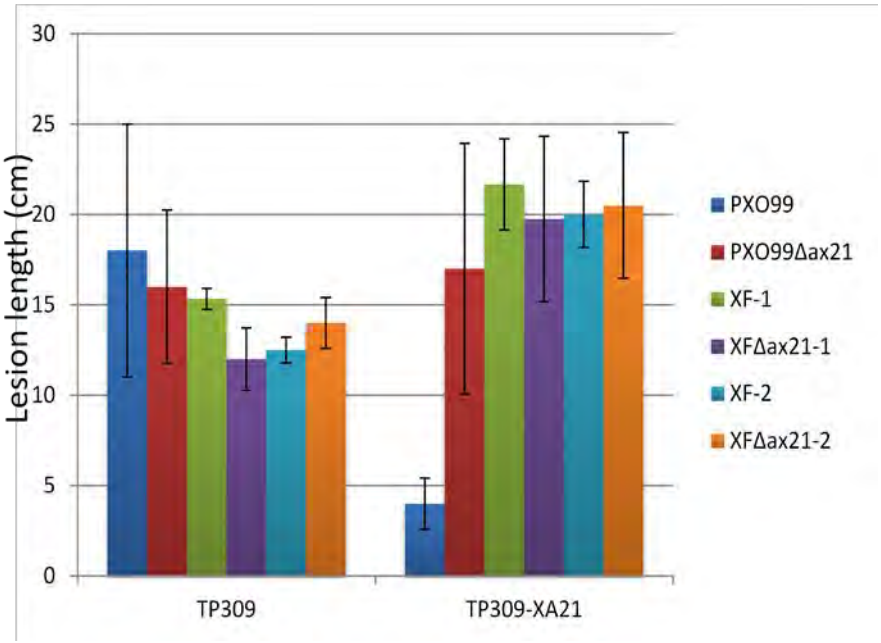

Figure 1. Lesion length on 6 week old TP309, susceptible to *Xoo* PXO99 strain, and TP309-XA21, resistant to PXO99 rice plants inoculated with PXO99Δ*raxST* strain five hours after pretreatments of supernatants. PXO99 and *Xf* indicate wild type of *Xoo* and *Xf* strains, respectively. PXO99Δ*ax21* and *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.

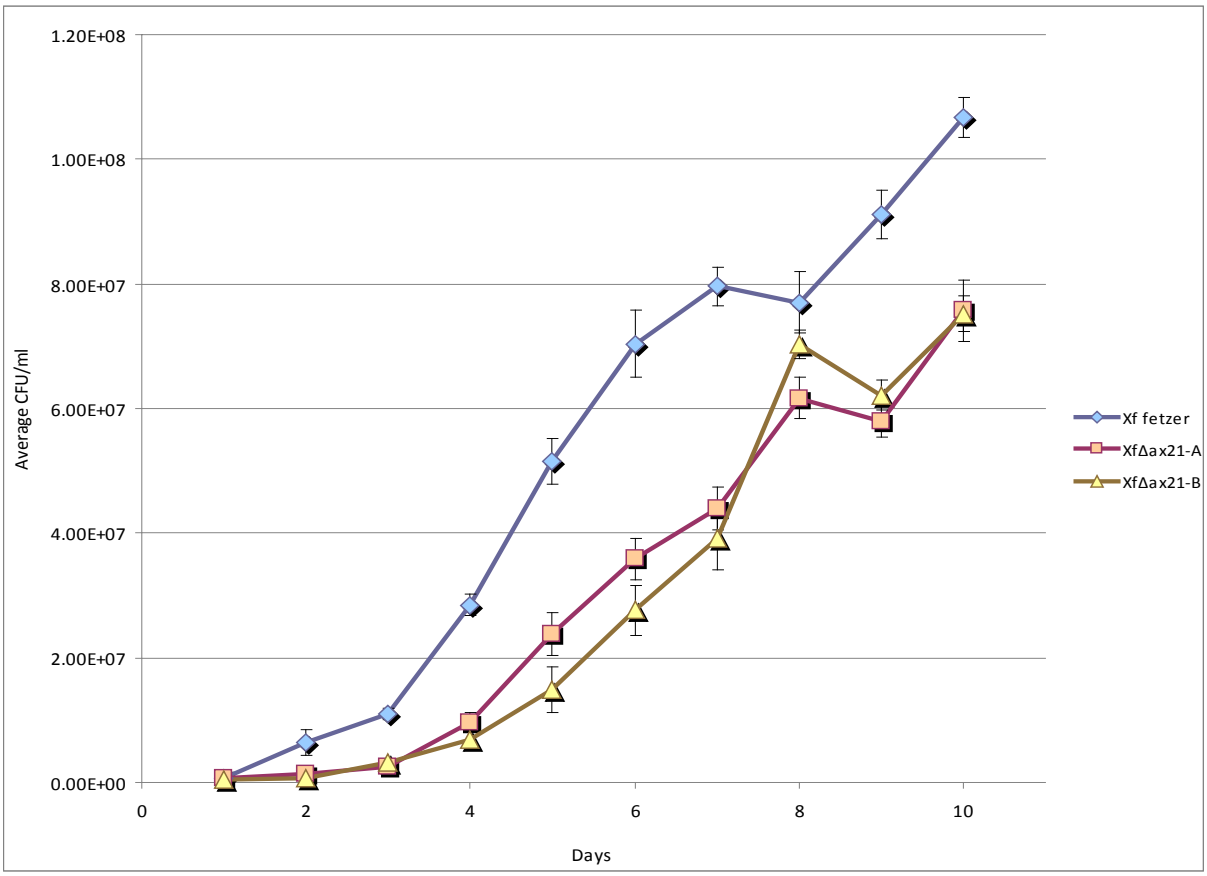

Figure 2. Bacterial growth of wild type *Xf* fetzer, *Xf*Δax21-A, and *Xf*Δax21-B. Values shown are the means of 5 samples +/- error.

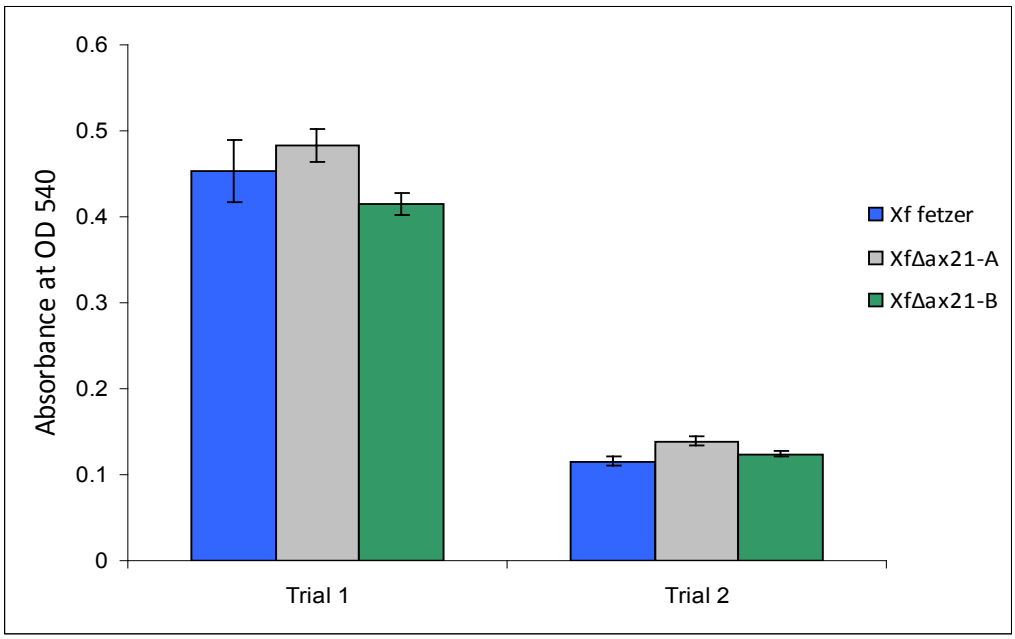

Figure 3. Comparison of biofilm formation in wild-type *Xf* fetzer, *XfΔ*ax21-A, and *XfΔ*ax21-B in stationary cultures as determined by the crystal violet staining method. Values shown are the means of 10 samples +/- error.

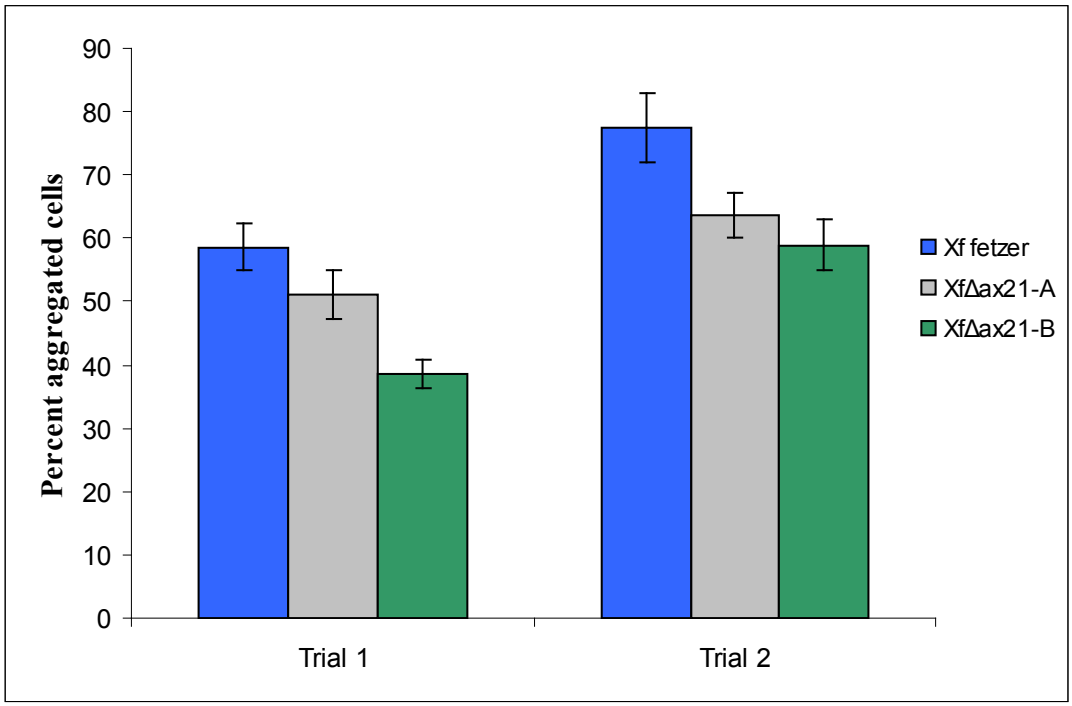

Figure 4. Comparison of percent aggregated cells in wild-type *Xf* fetzer, *XfΔ*ax21-A, and *XfΔ*ax21-B. Percentage of aggregated cells was determined as described by Guilhabert and Kirkpatrick, 2005. Values shown are the means of 10 samples +/- error.

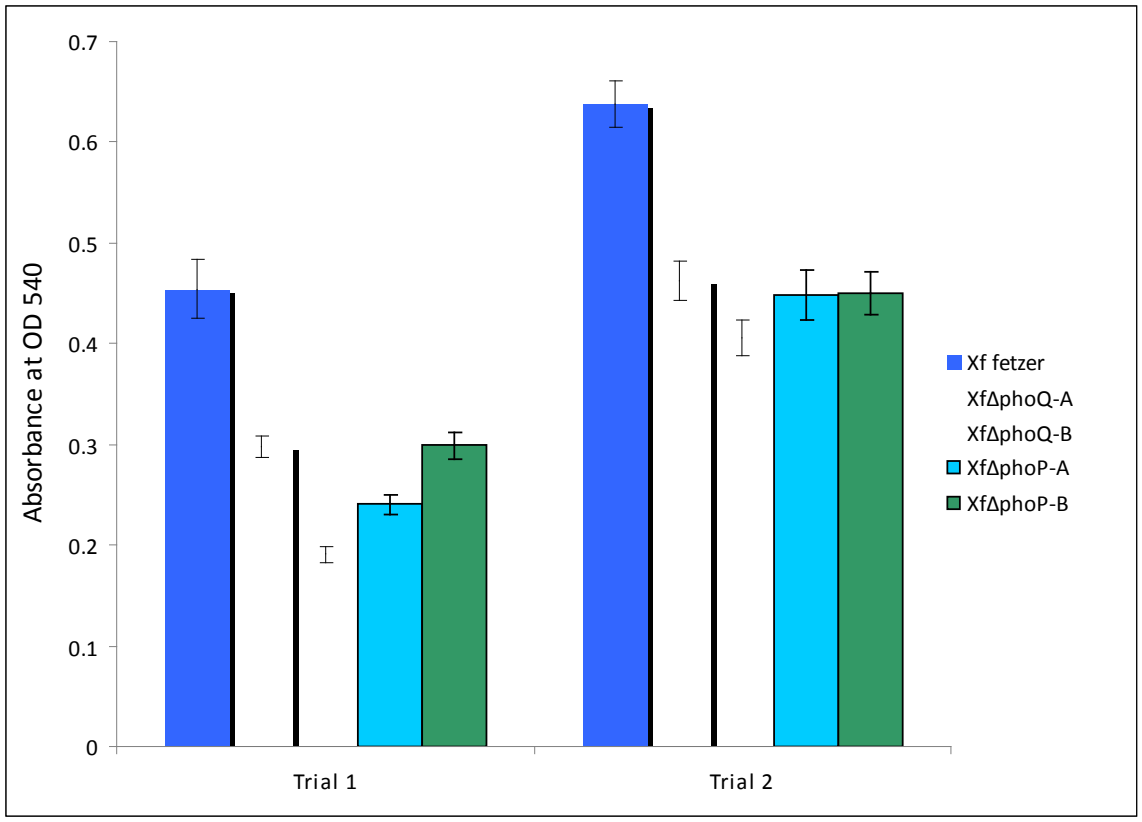

Figure 5. Comparison of biofilm formation by wild type *Xf* Fetzer, *XfΔphoP*, and *XfΔphoQ* after 10 days growth in static culture. Values shown are mean +/- standard error.

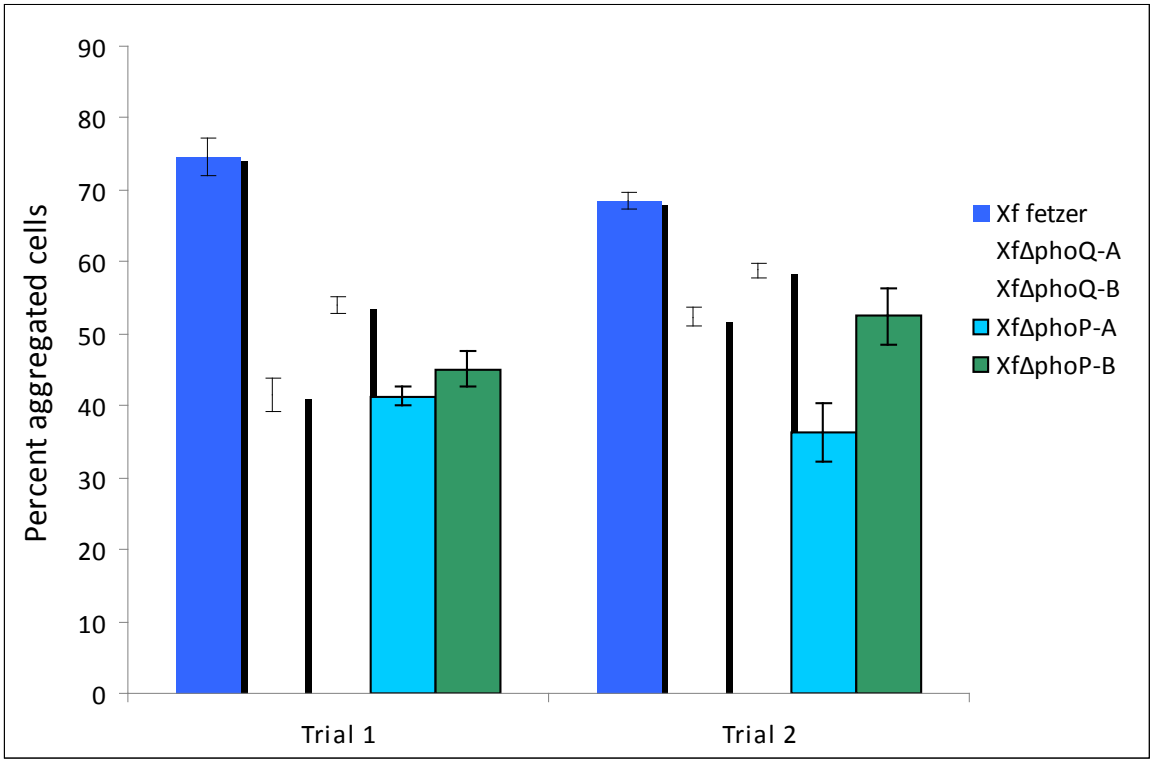

Figure 6. Comparison of percent aggregated cells by wild type *Xf* Fetzer, *Xf ΔphoP*, and *Xf ΔphoQ* after 10 days growth in static culture. Values shown are mean +/- standard error.

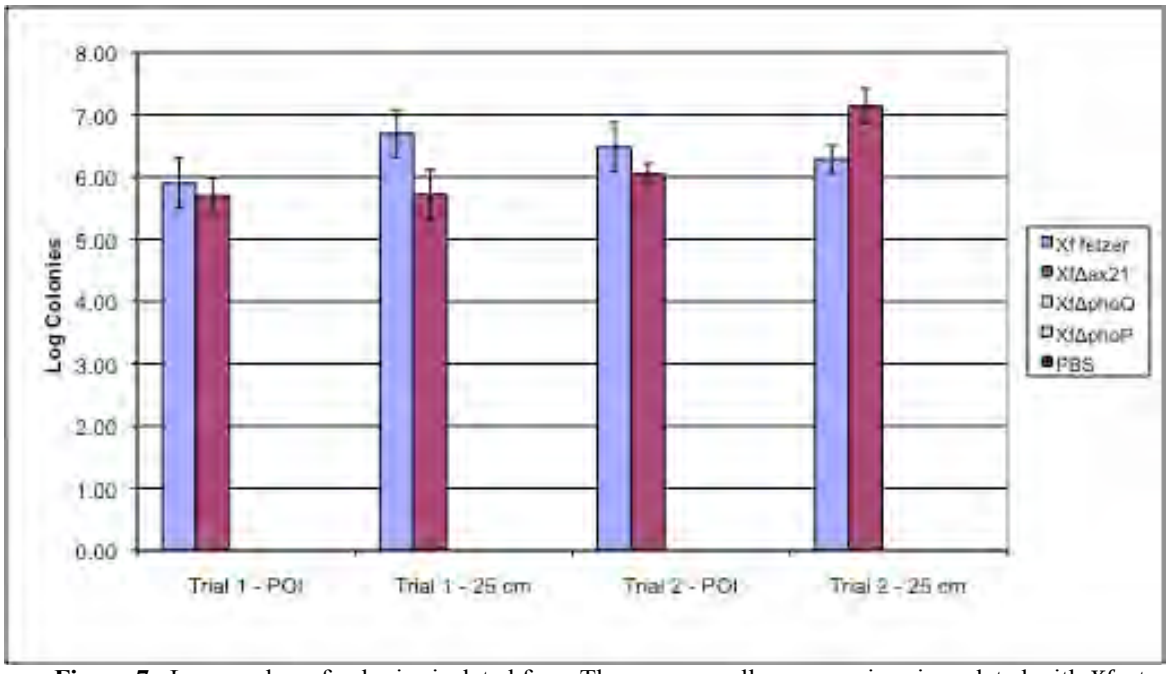

Figure 7. Log number of colonies isolated from Thompson seedless grapevines inoculated with *Xf* wt, mutants or PBS (negative control) 18 weeks post-inoculation. Isolations were taken from a petiole at the point of inoculation and a petiole 25 cm from the point of inoculation. Values shown are mean +/- standard error.

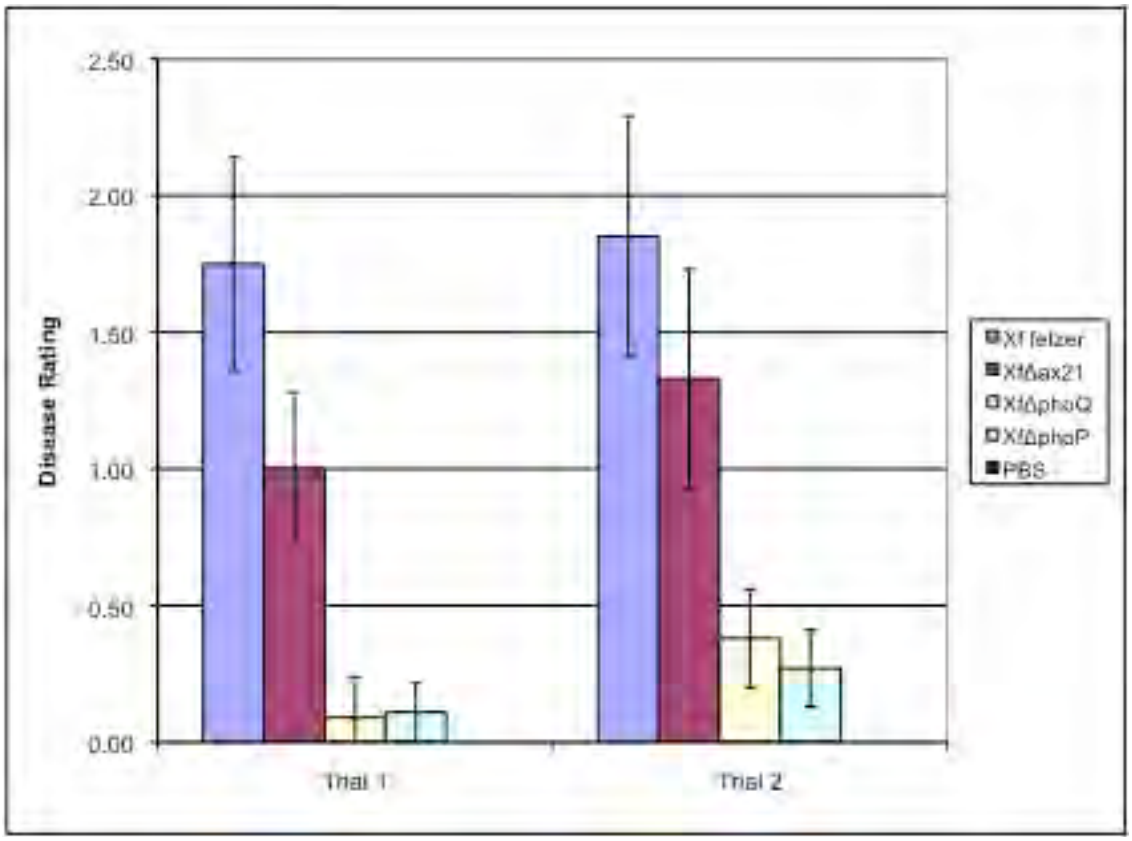

Figure 8: Disease ratings of Thompson seedless grapevines inoculated with *Xf* wt, mutants or PBS (negative control) 18 weeks post-inoculation. Values shown are mean +/- standard error.

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CHARACTERIZATION OF *XYLELLA FASTIDIOSA* LIPOPOLYSACCHARIDE AND ITS ROLE IN KEY STEPS OF THE DISEASE CYCLE IN GRAPEVINE.

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Reporting Period: The results reported here are from work conducted July 2011 to October 2011.

ABSTRACT
This project aims to elucidate the molecular mechanisms that *Xylella fastidiosa* (*Xf*) uses in its interaction with host plants. We are focusing on the lipopolysaccharide (LPS) component of the outer membrane. LPS consists of lipid A, core oligosaccharides, and a variable O-antigen moiety. We are specifically investigating the role of O-antigen as it has been implicated as a virulence factor in several other Gram-negative bacterial species. We hypothesize that O-antigen is also involved in virulence of *Xf* on grapevine. Moreover, we are investigating the function of LPS in surface attachment and cell-cell aggregation, two important steps in biofilm formation, a trait necessary for successful colonization of host xylem. We are also determining the role that LPS plays in host specificity observed for this pathogen.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) is a bacterium capable of colonizing many different plant hosts. It is the causal agent of Pierce's disease of grapevine, which has caused major losses to the California grape industry. *Xf* also causes disease in other economically important crops, such as almond, citrus, and oleander. While all identified *Xf* strains belong to the same species, some isolates can cause disease in one host, but not the other. For example, oleander isolates do not incite symptom development in grapevine and vice versa. One major goal of this project is to understand the bacterial characteristics that dictate host specificity. This research is particularly focused on elucidating the role of the *Xf* cell surface component, lipopolysaccharide (LPS), in the pathogenic interaction between the grapevine, almond, and oleander hosts. Because LPS plays an important role in several host-pathogen interactions, we are investigating the contribution of LPS in allowing *Xf* to colonize its host. LPS imparts traits that may contribute to pathogenesis such as the ability to attach to host cell walls. Should LPS prove to be important during *Xf* interaction with its host, its abundance on the bacterium's cell surface makes it a logical target for disease control. Antimicrobial compounds that disrupt or retard LPS biosynthesis exist which make bacteria more susceptible to other stresses. Potentially, these compounds could be used in combination with other anti-*Xf* compounds to control disease.

INTRODUCTION
Xylella fastidiosa (Xf) is a Gram-negative bacterium comprised of strains that cause disease on several economically important crops, such as grapevine, almond, and oleander. We are currently exploring the role of lipopolysaccharide (LPS) as both a virulence factor and host specificity determinant for this pathogen.

LPS is primarily displayed on the outer surface of Gram-negative bacteria, thereby mediating interactions between the bacterial cell wall and its environment. LPS is composed of three parts: 1) lipid A, 2) oligosaccharide core, and 3) O-antigen polysaccharide (**Figure 1**) (10). We are focusing on the O-antigen portion of LPS in three *Xf* isolates that colonize different hosts: Temecula1, a causal agent of Pierce's disease (PD); M12, a causal agent of almond leaf scorch (ALS); and Ann-1, the causal agent of oleander leaf scorch (OLS). Recognition, attachment, and biofilm formation are important stages in the interactions between *Xf*, the sharpshooter vector and its plant hosts. We aim to elucidate the role of LPS in the formation of biofilms, the development of PD on grapevine and in host specificity. Based on previously reported investigations of LPS in host-pathogen interactions (5, 9), we have identified two genes, *waaL* (PD0077) and *wzy* (PD0814), that are predicted to encode proteins required for a fully functional O-antigen moiety in *Xf*. Wzy is a polymerase that plays a role in chain length determination of the O-antigen, prior to the O-antigen ligation onto the oligosaccharide core component by WaaL (**Figures 1 and 2**).

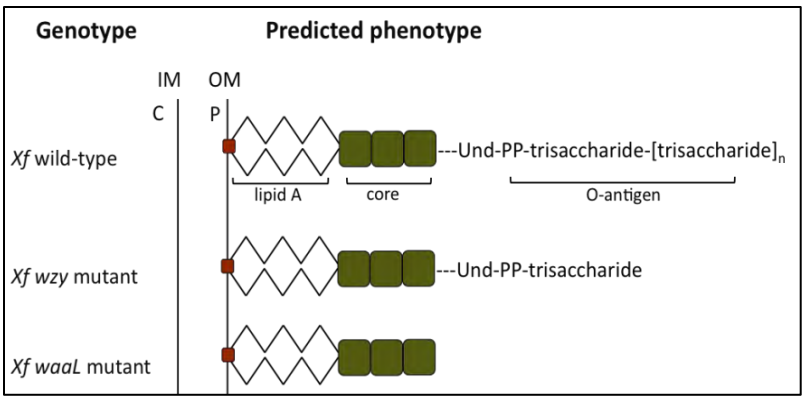

Figure 1. *Xf* strains lacking a functional Wzy (polymerase) are predicted to have truncated O-antigen with only the initiating polysaccharide unit. Strains lacking a functional WaaL (ligase) are predicted to lack the O-antigen. Und-PP = undecaprenylphosphate; IM = inner membrane; OM = outer membrane; C = cytoplasm; P = periplasm.

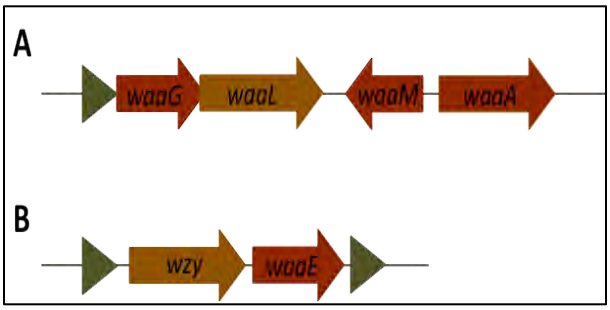

Figure 2. The genomic context of *waaL* **(A)** and *wzy* **(B)** in *Xf* provides evidence for prediction of genes that encode proteins involved in LPS biosynthesis. *waaG* = glycosyl transferase, *waaM* = lipid A biosynthesis lauroyl acyltransferase, *waaA* = 3-deoxy-D-octulosonic acid (KDO) transferase, *waaE* = beta 1,4 glycosyl transferase. Further, WaaL and Wzy are predicted to belong to the Wzy_C protein family of O-antigen ligases. Green triangles represent hypothetical proteins.

OBJECTIVES

1. To determine the contribution of *Xf* Temecula1 WaaL and Wzy in:
   a. LPS biosynthesis
   b surface attachment and biofilm formation
   c. virulence on grapevine
   d. host specificity
2. To determine LPS profiles of wild-type PD, ALS, and OLS strains.

RESULTS AND DISCUSSION

Objective 1a. To determine the contribution of *Xf* Temecula1 WaaL and Wzy in LPS biosynthesis

We identified two genes in the *Xf* LPS biosynthetic pathway, *waaL* and *wzy*, that encode proteins predicted to be important for production of a complete O-antigen component (see above). Mutations of these loci in *Xf* Temecula1 reveal a significant reduction in O-antigen in the *waaL* mutant strain and a lack of O-antigen in the *wzy* mutant strain. Both mutant phenotypes were restored by introducing *waaL* or *wzy* into their respective mutant genomic backgrounds using the chromosomal complementation vector, pAX1Cm (7) (**Figure 3**).

Objective 1b. To determine the role of O-antigen in biofilm formation

Biofilm formation is an important component of the plant-microbe interaction. To test the role of LPS in *Xf* biofilm formation, we are quantifying the ability of the Temecula1 *waaL* and *wzy* mutant strains in surface attachment and cell-cell aggregation. Interestingly, when grown in glass tubes, the Temecula1 *waaL* mutant attached to a greater extent than wild-type, which is contrary to attachment of this strain when grown in polypropylene or polystyrene where it attached less than wild-type (**Figure 4A**). Preliminary results suggest that *wzy* plays little role in *in vitro* surface attachment (**Figure 4B**). We

are currently testing the complemented strains in surface attachment to these same surfaces. As an extension of this work, we are investigating the role of WaaL and Wzy in attachment to biologically relevant surfaces such as chitin and cellulose. The ability of Temecula1 *waaL* mutant to aggregate was diminished in all tested materials, while the Temecula1 *wzy* mutant is reduced in aggregative capability only in polypropylene and polystyrene (**Figure 5**).

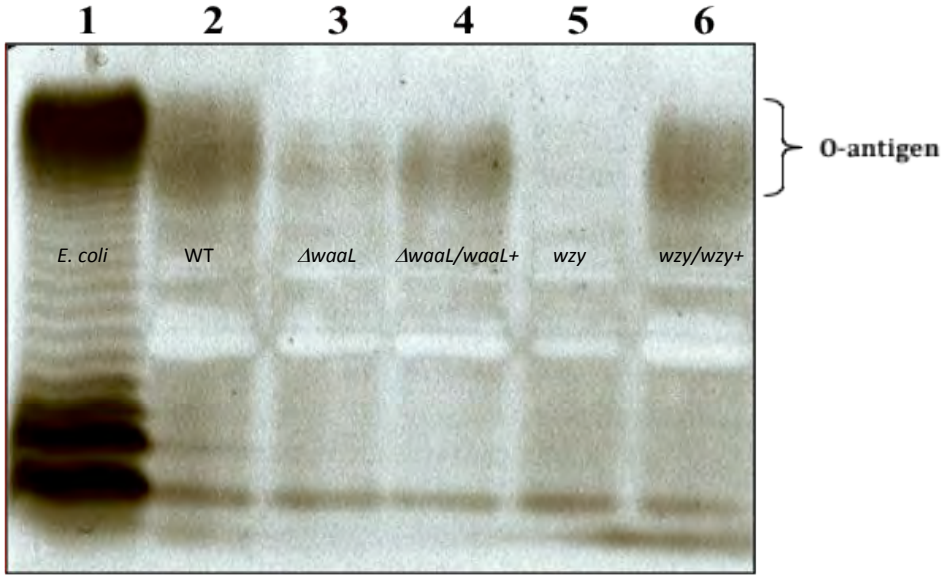

Figure 3. Mutations in key enzymes of the *Xf* LPS biosynthetic pathway result in reduction or abolishment of O-antigen. Temecula1 lacking a functional WaaL (lane 3) had less O-antigen than the wild-type (lane 2) while Temecula1 lacking Wzy (lane 5) appeared to have no distinguishable O-antigen moiety. Complementation of these mutants restored O-antigen quantity to near that of the wild-type parent (lanes 4 and 6). LPS was extracted from cells normalized to cell density using a modified hot phenol method (6). Samples were analyzed on a 4, 12% discontinous Tricine-PAGE gel and silver stained (8). (1) *E. coli* standard, (2) Temecula1 wild-type, (3) Temecula1*waaL*, (4) Temecula1*waaL*/*waaL*+, (5) Temecula1*wzy*, (6) Temecula1*wzy*/*wzy*+.

Objective 1c. To evaluate the contribution of O-antigen in development of Pierce's Disease
Temecula1 O-antigen mutant strains were inoculated into grapevine, cv. "Thompson Seedless' according to the method of Hill and Purcell (1995) (4). Plates were rated weekly on a disease index scale of 0-5 based on PD symptom development (3). PD symptoms were detected four weeks after inoculation, similar to other PD virulence studies conducted in Riverside County. There is no difference in disease progress between the Temecula1 *waaL* mutant and the wild-type strains. In contrast, the Temecula1 *wzy* mutant was delayed in causing PD symptoms on grapevine and, after 11 weeks of incubation, did not cause the extensive PD development observed in plants inoculated with wild-type (**Figure 6**). Plants inoculated with 1X PBS buffer control did not develop any PD symptoms.

At 39 days post-inoculation (5th week), a population study of grapevine petioles was performed in order to quantify any colonization differences between Temecula1 wild-type and O-antigen mutant strains that may be occurring *in planta*. Fifty-three percent of petioles tested from plants inoculated with Temecula1 wild-type were colonized, while 67% of petioles tested from plants inoculated with Temecula1*waaL* were colonized and preliminary statistical analysis indicates that Temecula1 *waaL* strain is colonizing to a slightly higher degree than the wild-type (**Table 1**). No bacteria were recovered from *wzy*-inoculated plants at this early time point. However, a second population study done 95 days post-inoculation (13th week) is currently underway to determine at what level the *wzy* and Δ*waaL* mutants colonize the plants at a later stage of infection. We hypothesize that because PD symptom development has progressed in *wzy*-inoculated plants, albeit at a much slower rate than WT-inoculated plants, the bacterial titer has increased since the first sampling time and we will be able to recover viable *wzy* cells during the second sampling. The colonization studies have been performed and we are awaiting the results of that experiment.

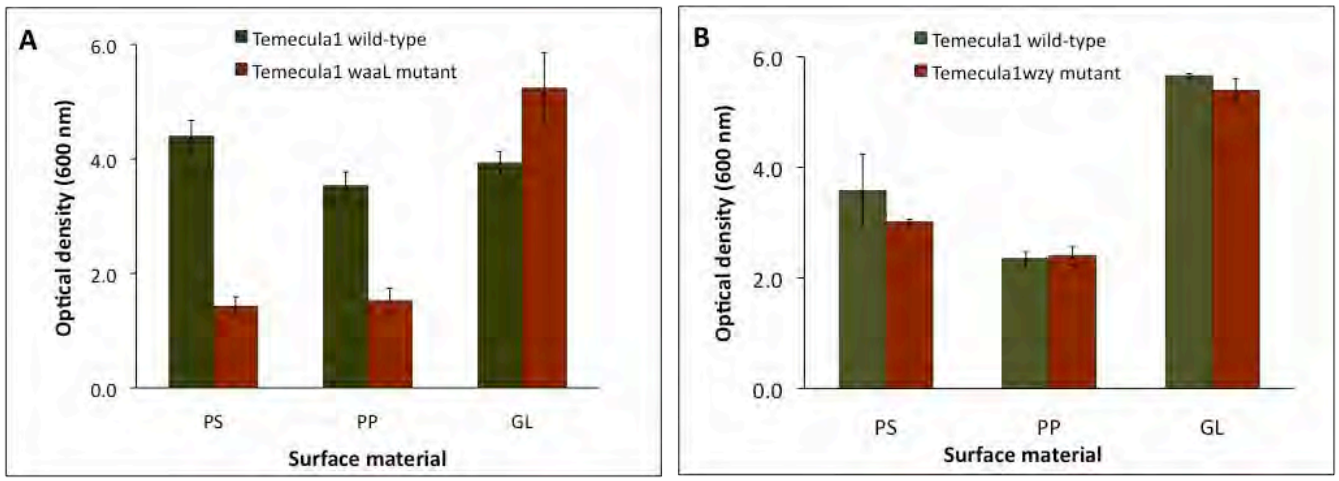

Figure 4. Preliminary results suggest that WaaL plays a role in the ability of *Xf* to attach to various surfaces. **A)** the Temecula1 strain lacking WaaL is reduced in attachment to polystyrene (PS) and polypropylene (PP), but increased in attachment to glass (GL) when compared to wild type. **B)** Wzy appears to play little role in surface attachment on any surface tested. Strains were grown in PD3 medium at 28 °C at 100 rpm for 7 days. Biofilms formed on the surface of the medium/air interface were stained with 1% crystal violet and collected in 95% ethanol (2). Bars represent standard error.

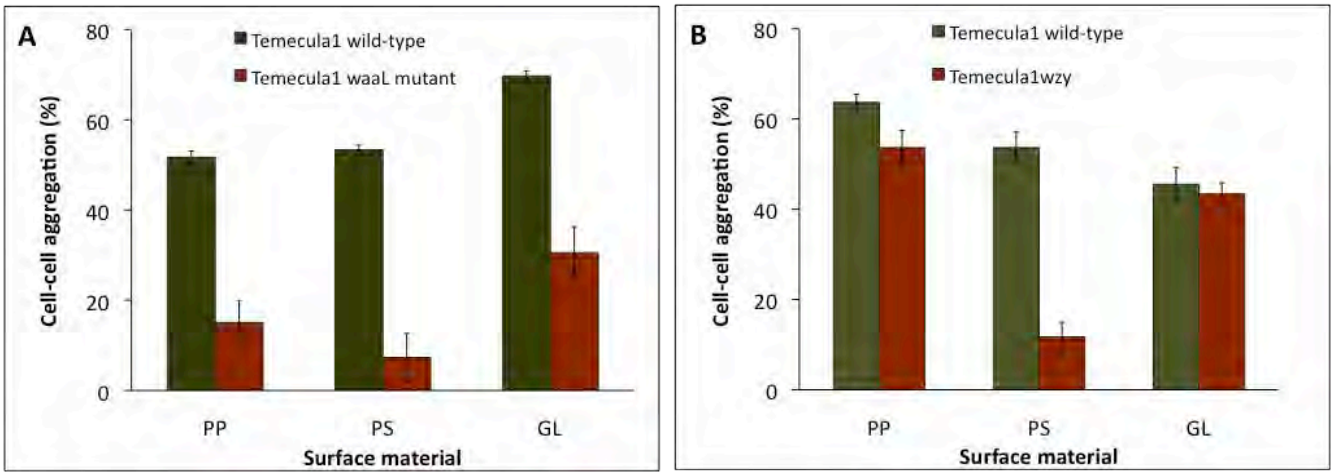

Figure 5. O-antigen plays a role in the ability of *Xf* cells to aggregate. **A)** the Temecula1*waaL* mutant cells are reduced in the ability to adhere to one another when grown in polypropylene (PP), polystyrene (PS), and glass (GL). **B)** the Temecula1*wzy* mutant cells are reduced in cell-cell aggregation in PP and PS, but not GL. Strains were grown in PD3 medium at 28 °C without agitation for 10 days. Percent aggregation is the ratio of unsettled (ie, aggregating) cells in the upper portion of the test tube to the total number of cells (3). Bars represent standard error.

Objective 1d. To investigate the role of O-antigen in host specificity

While there are likely several factors that contribute to host specificity of *Xf*, we would like to investigate if O-antigen is involved. *Xf* Temecula1 can colonize and cause disease in grape and, to some extent, in almond. *Xf* M12, an ALS strain, causes disease in almond and elicits some symptoms in grape (1). Moreover, *Xf* Ann-1 cannot cause disease in grape or almond and neither Temecula 1 nor M12 can cause disease in oleander. We speculated that removal or truncation of the O-antigen would affect the ability of Temecula1 to infect (and elicit scorch symptoms) on grape, almond, and oleander. Further, perhaps changes in O-antigen will allow Ann-1 to become a pathogen of grape or almond while M12 and Temecula1 strains become pathogens of oleander.

We currently have *Xf* Temecula1 and the Temecula O-antigen mutant strains inoculated into almond and oleander. Preliminary results demonstrate that the Temecula1*waaL* mutant strain is more virulent in almond than its Temecula1 wild type parent and is similarly virulent to the ALS wild-type strain M12. This suggests that alterations in the O-antigen moiety of the LPS molecule do affect host specificity. We are currently awaiting the results from host specificity studies conducted in oleander. These plants take longer (approx. 10 months) for OLS symptoms to develop. We are also constructing the corresponding *waaL* and *wzy* mutants in the M12 and Ann-1 strains of *Xf*. Loss of Wzy function (Temecula1 *wzy* mutant)

does not seem to alter the host specificity of the Temecula1 strain. As expected, *Xf* Ann-1, the OLS strain, does not cause scorch symptoms on almond (**Figure 7**).

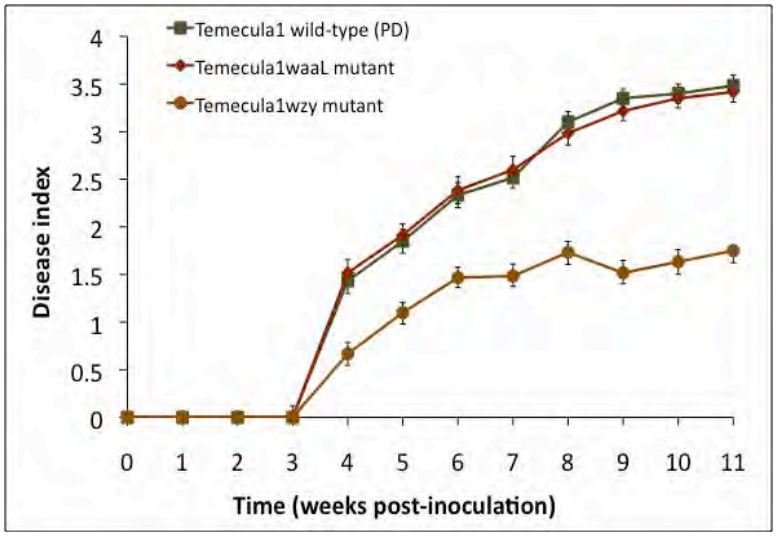

Figure 6. Disease progress of *Xf* Temecula1 wild-type and O-antigen mutant strains on grapevine cv. 'Thompson Seedless'. The *wzy* mutant strain lags behind the wild-type in causing scorching symptoms and does not cause wild-type levels of PD. The *waaL* mutant appears to be as virulent as wild-type. Data are means of three independent assays with 10 replications each. Bars represent standard error.

Table 1. Mean populations in tissue of colonized grapevine at 39 days post-inoculation.

Strain	**Xylem population (per g tissue)[a]**
Temecula1 wild-type	5.09 (± .21)
Temecula1*waaL*	5.69 (± .16)
Temecula1*wzy*	Not detected[b]

[a] mean population values are log transformed; parenthetical values represent standard error
[b] populations below detection threshold (<500 CFU)

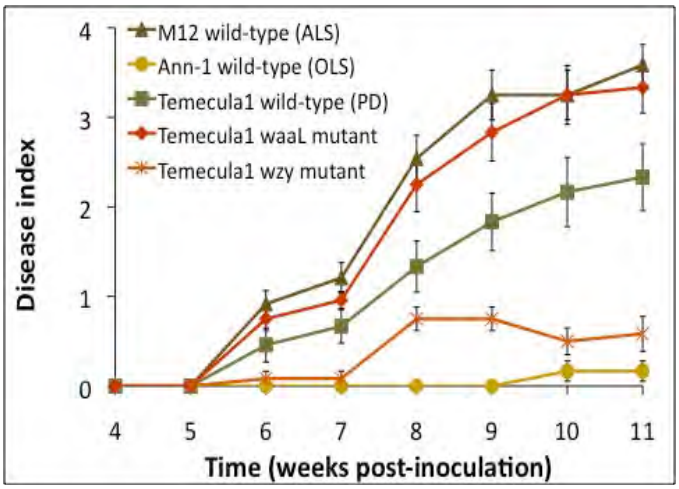

Figure 7. Disease progress of ALS strain M12, OLS strain Ann-1, PD strain Temecula1, and Temecula1 O-antigen mutants on almond cv. 'Sonora.' Loss of WaaL may render a PD strain as virulent on almond as the ALS wild-type strain M12. Data are means of two independent assays with 6 replications each. Bars represent standard error.

Objective 2. LPS profile of *Xf* Temecula1 (grape), M12 (almond), and Ann-1 (oleander)

Variation in LPS can be classified as "smooth" (those with O-antigen) and "rough" (those without O-antigen). Bacteria can possess both variants simultaneously which can be readily visualized by electrophoretic analysis. The three isolates used in this study were grown on PD3 (for Temecula1 and Ann-1 strains) or PW (for M12 strain) solid medium. Cells were harvested and normalized to cell density. LPS was extracted as described above. There are slight differences among the strains with regard to the high MW O-antigen and it appears that the O-antigen population of the Ann-1 strain may be comprised of trisaccharide repeats of slightly greater length (**Figure 8**). We are further characterizing the LPS molecule for all three strains using more sensitive biochemical techniques to identify the carbohydrate composition and linkage of the sugars in the *Xf* LPS molecule.

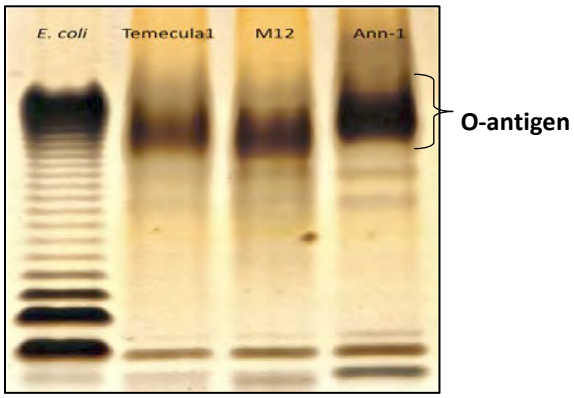

Figure 8. There are minor differences between *Xf* wild-type strains used in this study (Temecula 1, M12 and Ann-1). These differences are primarily in the polysaccharide length that comprises the majority of the O-antigen. LPS was extracted from cells normalized to cell density, run on a discontinuous 14% Tricine-SDS PAGE gel, and silver stained. LPS extracted from *Escherichia coli* (Sigma) was used as the standard.

CONCLUSIONS

The main focus of this project is to further understand the molecular mechanisms governing *Xf* virulence. We are working toward understanding the role of the O-antigen component of LPS in contributing toward virulence and those behaviors associated with xylem colonization, such as cell wall attachment and cell-cell aggregation required for biofilm formation. The broad host range, but stringent host specificity, of *Xf* provides an opportunity to study the molecular mechanisms underlying the essential traits that lead to host specificity observed for this pathogen. There are likely numerous bacterial traits that contribute to host specificity of *Xf*, and we hypothesize that LPS plays a role. Because of its abundance in the outer membrane, LPS may provide a target for disease control, as it appears to be implicated as an important factor in disease development.

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SMALL RNAS IN *XYLELLA FASTIDIOSA* AND THEIR EPIDEMIOLOGICAL APPLICATIONS

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Reporting Period: The results reported here are from work conducted from March 2011 to September 2011.

ABSTRACT
Bacterial non-coding small RNAs (sRNAs) have attracted considerable attention due to their roles in regulating numerous cellular processes including survival, adaptation and pathogenesis. Sequence variation in sRNA genes reflect a previously unrecognized source of genomic diversity in bacteria. *Xylella fastidiosa (Xf)* is an important bacterial pathogen causing many economically important diseases such as almond leaf scorch, citrus variegated chlorosis and Pierce's disease of grapevine. Little is known about sRNAs in this bacterium. Therefore, a research project was initiated to search for sRNAs in *Xf*. The complete genome sequences of four *Xf* strains (9a5c, M12, M23, and Temecula1) representing three *Xf* subspecies were selected and scanned for sRNA genes with established computer programs. Candidate sRNA genes were identified in all of the four *Xf* strains (46 in strain 9a5c, 50 in strain M12, 49 in strain M23, and 47 in strain Temecula1). Candidate sRNA genes ranged in size from 40 to 350 bp. Expression of sRNA genes was proved using a procedure involving quantitative reverse transcriptase PCR (qRT-PCR) and confirmed with negative detection with primers from regions flanking the predicted sRNA genes. BLAST analysis showed that 34 sRNA genes were shared by all three *Xf* subspecies. To test for epidemiological application of variable sRNA genes, four sRNA genes in strain M23 were selected to design PCR primers. A total of 22 different bacterial strains were cultured in PW broth at 28 ºC for 14 days. DNA was extracted and used as templates for RT-PCR with the four sRNA primer sets. Both inter- and intra- subspecies variation of *Xf* strains was observed.

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POPULATION STRUCTURE OF *XYLELLA FASTIDIOSA* OF CITRUS VARIEGATED CHLOROSIS IN SAO PAULO STATE: FROM A SMALL BRANCH TO THE WHOLE STATE

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ABSTRACT

The economic and social importance of citrus production in Brazil and the high economic loss caused by citrus variegated chlorosis (CVC) in Brazilian orchards have resulted in an extensive research program involving the sequencing of the genome of *Xylella fastidiosa (Xf)* subsp. *pauca* (*Xf*-pa, strain 9a5c), the causal agent of CVC. Other genomes of *Xf*-pa are currently being sequenced. Despite the great interest in genomics of *Xf*-pa, relatively little information is available about the population genetics of this pathogen. We previously showed that different *Xf*-pa haplotypes were found in CVC-diseased plant and hypothesized that it was a consequence of multiple inoculation events originating from different sources (Coletta-Filho and Machado, 2003, Geographical genetic structure of *Xylella fastidiosa* from citrus in São Paulo State, Brazil Phytopathology, 93:28-34). Based on those findings we studied the role of spatial scale on the genetic diversity of *Xf*-pa in sweet orange plants affected by CVC from various geographical regions of Sao Paulo state. We used analyses of molecular variance (AMOVA) to test hypothesis that genetic differentiation among populations increased from central (branch) to peripheral sites (geographic regions). Spatial scale ranged from 4–6 cm long branches to hundreds of kilometers, including within and among citrus plant comparisons. At the smallest spatial scale (single branch) five isolates were obtained, 10 to 15 isolates were obtained from each plant, and 40 isolates total from each orchard (geographic regions), in total 160 isolates from four geographical regions were typed. The genetic structure of *Xf*-pa was determined using 16 simple sequence repeats (SSR) loci. AMOVA analysis using the Euclidean squared distances between pairs of isolate) revealed significant genetic differentiation among all population levels: i) within branches (F_{ST} = 0.856, Var. = 14.0%); ii) among branches within plants (F_{SC} = 0.374, Var. = 8.58%); iii) among plants within regions (F_{SC} = 0.77, Var. = 40.75%); and iv) among regions (F_{CT} = 0.44,Var. = 36.71%). These results indicate that spread of CVC in Sao Paulo state occurred in a wave-like fashion, but a significant percentage of genetic variability among isolates was observed at the smallest scale sampled (small branches), which could be attributed to natural recombination events among isolates rather than different sources of multiple infections, as previously hypothesized. On the other hand, it is possible that the loci used here evolve quickly, which would also be a possible interpretation for these data. Future work is needed to address these competing hypotheses.

FUNDING AGENCIES

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Section 4:

Pathogen and Disease Management

VINE CONDITION AND *XYLELLA FASTIDIOSA* SEROLOGY FOR THREE NATIVE GRAPE SPECIES, SELECTED *VITIS VINIFERA* ON ROOTSTOCKS, AND SELECTED UNGRAFTED ROOTSTOCKS

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ABSTRACT
Three native *Vitis* species in southwest Texas rarely have even minor Pierce's disease (PD) symptoms or react to *Xylella fastidiosa (Xf)* ELISA tests. When planted next to highly susceptible *Vitis vinifera* cultivars with severe PD and a large glassy-winged sharpshooter population, *V. cinerea* var. *helleri*, *V. monticola*, and *V. mustangensis* developed PD symptoms and reacted to ELISA tests. *V. cinerea* var. *helleri* and *V. monticola* had very little iron deficiency (chlorosis). *V. mustangensis* had the lowest *Xf*-ELISA OD values. *V. cinerea* var. *helleri* and *V. monticola* had delayed leaf senescence, which may indicate delayed root senescence, a trait thought to hinder cotton root rot disease (caused by *Phymatotrichopsis omnivora*) of winegrape. *V. monticola* crosses should be included in PD and CRR breeding line tandem screening efforts.

LAYPERSON SUMMARY
In addition to Pierce's disease (PD) resistance and other pest and soil problems, we considered which native Texas grapes may have traits indicating potential parents for rootstock improvement efforts to address cotton root rot disease. Cotton root rot occurs in a large area of the PD geographical range, from Texarkana to southern Utah to central Mexico. Delayed leaf senescence in *V. monticola* suggest it may also contribute rootstock traits to help control CRR.

INTRODUCTION
Pierce's disease (PD) in the warmer regions of Texas and southwestern U.S. has caused early death of high susceptible *Vitis vinifera* cultivars for at least 300 years. Native *Vitis* species in the same area usually have no PD symptoms or serological reactions for *Xylella fastidiosa (Xf)*. These wild species continue to be used in cultivar improvement efforts for fruiting and rootstock cultivars (Covert, 2008) including PD resistance. Among the problems previously addressed with crosses involving wild grape species found in Texas and the southwestern U.S. are root pests (grape phylloxera insect, plant parasitic nematodes) and soil problems (high pH calcareous soils, poor drainage, droughty soils). As progress on PD control has increased vine longevity, incidence of cotton root rot disease has increased. The soil borne fungus *Phymatotrichopsis omnivora* causes cotton root rot disease (CRR) in most of Texas and in five other southwestern states and Mexico. High incidence of CRR has long been linked to high pH soils. Native *Vitis* species in Texas may be useful in developing rootstocks with resistance to both PD and CRR. Early publications note resistance of several Vitis sp. to CRR, including *Vitis monticola, berlandieri* and *candicans* (*mustangensis*). Plants classified as resistant developed new roots and survived in spite of the fungal infection (Taubenhaus and Ezekiel, 1936).

OBJECTIVE
1. Evaluate chlorosis, PD, and leaf condition in *V. cinerea* var. *helleri* (*V. berlandieri*), *V. monticola*, and *V. mustangensis (V. candicans)* at an irrigated high pH soil site in southwest Texas with intense PD.

RESULTS AND DISCUSSION
Vitis cinerea var. *helleri* and *V. monticola* growing in furrow-irrigated calcareous high pH soil had less iron deficiency than *V. mustangensis*. Under very intense PD, *V. mustangensis* trended toward lower mean *Xf*-ELISA optical densities than *Vitis cinerea* var. *helleri* and *V. monticola* in second leaf and third leaf, possibly indicating that mustang grape limits populations of *Xf* more than the other two species. Both *V. monticola* entries, two of six *V. mustangensis* entries, and Salt Creek rootstock always had less than 15% total leaf necrosis.

CONCLUSIONS
Three native grapes species found in southwest Texas have been used by plant breeders for many decades to address various soil insect, nematode, PD, and soil problems. In this preliminary trial, leaf chlorosis indicated less iron uptake from high pH soil, ELISA OD indicated *Xf* cell numbers, and leaf necrosis indicated cumulative effects of iron uptake, PD, and senescence. Cotton root rot disease (CRR), caused by *Phymatotrichopsis omnivora*, occurs within a large part of the PD geographic range and both pathogens have high optimal temperatures. *P. omnivora* apparently becomes more aggressive on senescing plants, and our data suggest that senescence varies among grape genotypes. In grain sorghum, plant breeders selected 'stay green' stalk and root traits separately from grain maturity date to help solve late season root and stalk diseases (Thomas and Howarth, 2000). Perhaps native *Vitis* species may be useful parents for rootstocks improvement efforts that address both PD and CRR. Entry numbers in this preliminary trial were not adequate for drawing firm conclusions. However, these data

suggest that *V. monticola* crosses should be included in CRR screening efforts. Genetic resistance alone will probably never eliminate PD or CRR risk.

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Table 1. Plant conditions and *Xylella fastidiosa*-serology reactions of three *Vitis* species, selected rootstocks, and selected *V. vinifera* on rootstocks at Uvalde, TX. Green cells had the least chlorosis (rating >2.5). Blue cells had OD<0.5 (<0.3 is negative) with ELISA. Yellow cells had lowest leaf necrosis ratings (≤10%).

		Chlorosis[a]						Optical Density, *Xylella fastidiosa*-ELISA						Leaf necrosis, percent[b]					
		14Sep10		12Nov10		7Sep11		14Sep10		12Nov10		7Sep11		14Sep10		14Nov10		7Sep11	
Entry	N[c]	$\bar{x}$	SD[d]	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Vitis cinerea var. *helleri* Population 8[e]	14	3.0	0.0	2.2	0.4	2.8	0.4	1.3	0.5	0.7	0.4	0.8	0.5	0.2	0.3	33	22	6	5
V. monticola pooled	7	3.0	0.0	2.9	0.4	3.0	0.0	0.4	0.5	0.9	0.4	0.8	0.4	0.0	0.0	4	4	2	2
Population 5[e]	4	3.0	0.0	3.0	0.0	3.0	0.0	0.2	0.2	1.0	0.5	1.1	0.4	0.0	0.0	1	0	2	2
Population 7[e]	3	3.0	0.0	2.7	0.6	3.0	0.0	0.7	0.5	0.8	0.3	0.5	0.2	0.0	0.0	7	5	2	2
V. mustangensis pooled	20	1.7	0.7	1.8	0.4	1.3[f]	0.5	0.4	0.4	0.3	0.4	0.4[f]	0.3	0.7	1.6	20	26	10[f]	13
Rio Medina[g]	5	1.8	0.4	1.6	0.5	1.2	0.4	0.5	0.4	0.6	0.6	0.6	0.3	0.1	0.2	2	2	10	10
Marble Falls[g]	2	2.0	1.4	2.0	0.0	1.0	0.0	0.6	0.5	0.0	0.0	0.4	0.6	0.1	0.1	8	4	13	17
Stonewall[g]	1	1.0	.	2.0	.	1.0	.	1.1	.	0.1	.	0.0	.	0.0	.	50	.	50	.
Tow[g]	6	2.2	0.8	1.8	0.4	1.8	0.4	0.4	0.2	0.2	0.2	0.5	0.3	0.0	0.1	10	6	2	4
Uvalde[g]	1	1.0	.	1.0	.	.	.	1.0	.	0.3	.	.	.	0.0	.	99	.	.	.
Uvalde South Getty[g]	5	1.0	0.0	1.8	0.4	1.0	0.0	0.1	0.1	0.0	0.0	0.3	0.2	2.4	2.5	35	20	10	9
Champanel[h]	5	2.0	0.0	2.0	0.0	2.0	0.0	1.2	0.8	1.0	0.6	1.1	0.7	0.4	0.4	44	26	3	3
Couderc 1613 (1613C)[i]	4	1.0	0.0	2.3	0.5	1.5	0.6	1.7	0.2	1.8	0.0	1.5	0.3	37.5	11.9	83	13	89	10
Dog Ridge[j]	5	3.0	0.0	2.4	0.5	2.3[k]	1.0	0.9	0.4	0.4	0.2	0.6	0.5	0.1	0.1	8	5	14[k]	18
Harmony[l]	1	1.0	.	2.0	.	.	.	1.1	.	0.2	.	0.8	.	20.0	.	99	.	100	.
Kober 5BB[m]	2	3.0	0.0	2.0	0.0	2.5	0.7	1.1	0.5	0.7	0.0	1.1	0.4	0.4	0.2	45	0	33	39
Lenoir (Black Spanish)[n]	1	2.0	.	2.0	.	1.0	.	1.3	.	1.1	.	1.3	.	15.0	.	15	.	20	.
Salt Creek (Ramsey)[o]	4	3.0	0.0	2.5	0.6	2.0	0.0	0.3	0.2	0.4	0.2	0.4	0.1	0.4	0.4	4	1	4	1
S04[p]	5	2.6	0.5	2.2	0.4	2.0	0.0	1.1	0.7	0.7	0.3	1.2	0.2	0.3	0.4	23	12	21	2
Chardonnay/Couderc 1613	1	3.0	.	2.0	.	1.0	.	1.6	.	1.5	.	1.6	.	25.0	.	85	.	95	.
Chardonnay/Salt Creek	1	2.0	.	2.0	.	2.0	.	1.5	.	1.4	.	1.7	.	10.0	.	75	.	99	.
Chardonnay/Teleki 5C[q]	2	3.0	0.0	2.0	0.0	2.5	0.7	1.0	0.0	0.9	0.7	1.2	0.1	27.5	3.5	73	4	80	14
Merlot/Harmony	1	2.0	.	2.0	.	2.0	.	1.8	.	.	.	2.0	.	25.0	.	85	.	80	.
Merlot/Kober 5BB	3	3.0	0.0	3.0	0.0	2.7	0.6	1.4	0.1	1.3	0.2	.	0.2	11.0	3.6	78	8	67	14
Merlot/Teleki 5C	1	3.0	.	3.0	.	2.0	.	1.3	.	0.6	.	1.8	.	50.0	.	80	.	85	.

[a]Chlorosis rated as 1=chlorotic, 2=intermediate chlorosis, 3=green.

[b]Leaf necrosis due to severe iron deficiency induced by high pH soil, PD, and senescence.

[c]N=number of plants evaluated unless indicated otherwise.
[d]SD=standard deviation.
[e]Open pollinated seedlings from one parent.
[f]N=19.
[g]Rooted cuttings from one plant.
[h]*V. champinii* x *V. labrusca*.
[i]Includes *V. labrusca*, *V. riparia*, *V. vinifera*.
[j]*V.* x *champinii* (*V. candicans* and *V. rupestris*).
[k]N=4 plants.
[l]Includes *V. labrusca*, *V. riparia*, *V.* x *champinii*, *V. vinifera*.
[m]*V. berlandieri* x *V. riparia.*
[n]*V. aestivalis*, *V. cinerea*, *V. vinifera* (50%).
[o]*V.* x *champinii*.
[p]*V. berlandieri* Resseguier x *V. riparia.*
[q]*V. berlandieri* x *V. riparia.*

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

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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.

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, with pruning weights more than 200% that of the next largest vines.

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 Pierce's disease 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.

OBJECTIVE
To evaluate the impact of rootstock variety on expression of PD symptoms and vine growth 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.

Dormant pruning was conducted in January 2011, and the weight of prunings of each vine, head trained and spur pruned, was collected.

Table 1.

Rootstock	Number of vines	Weight of dormant prunings, Jan. 2011, kg	Weight of dormant prunings, Jan. 2010, kg
Florilush	10	0.71	0.73
Freedom	6	0.71	0.82
Lenoir	10	0.81	0.65
Tampa	10	0.97	0.88
Dog Ridge	9	2.32	1.70

Vines grafted on Dog Ridge had the highest pruning weights in both 2011 and 2010. In 2011, pruning weights of vines on Dog Ridge were more than twice the mass of vines on Tampa. In both 2010 and 2011, vines grafted on Tampa were second in pruning weight to vines grafted on Dog Ridge.

Preliminary results indicate that Chardonnay vines grafted on Dog Ridge were the largest and had the lowest expression of PD symptoms. Dog Ridge is a candidate rootstock for the Lower Rio Grande Valley to increase the vine size of PD susceptible varieties and may be suitable for PD resistant or tolerant varieties as well.

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

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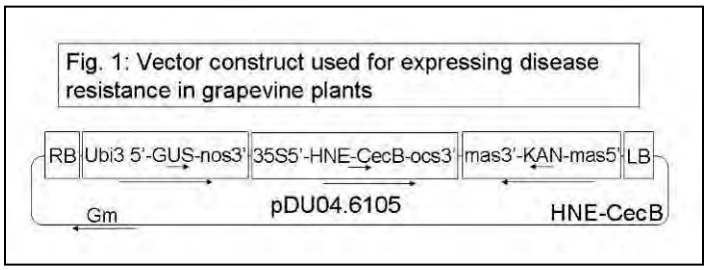
ABSTRACT
We have successfully established two field plantings to investigate two greenhouse-tested strategies to control the movement and clear *Xylella fastidiosa* (*Xf*), a xylem-limited, Gram-negative bacterium that is the causative agent of Pierce's disease (PD) in grapevine. A key virulence feature of *Xf* resides in its ability to digest pectin-rich pit pore membranes that connect adjoining xylem elements, enhancing long distance movement and vector transmission. The first strategy tests the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) from pear to inhibit *Xf* polygalacturonase activity necessary for long distance movement. Our second strategy enhances clearance of bacteria from *Xf*-infected xylem tissues via the expression of a chimeric antimicrobial protein, CAP. The expectation is that expressing these two proteins will prevent *Xf* movement and reduce *Xf* inoculum, curbing the spread of PD in California vineyards. Transgenic grapevine plants expressing either PGIP or CAP have been planted in two locations, one in Riverside County and the other in Solano County. These transgenic grapevines are being evaluated both as plants on their own roots and as rootstocks grafted with untransformed Thompson Seedless (TS) scions. At the Riverside County site, the plants have been naturally infected. At the Solano County site, plants on their own root were mechanically infected with *Xf* on 06/27/2011 to validate resistance to PD under field conditions. Two hundred and twenty four transgenic or untransformed control vines, own-rooted or grafted with untransformed TS, were planted in Riverside County on 05/08/2010. In Solano County, 112 own-rooted transgenic and untransformed control vines were planted on 08/02/2010 and 112 untransformed TS scions grafted onto transgenic or untransformed rootstocks were planted on 06/27/2011. At the Riverside County site, *Xf* infection has been confirmed and PD symptoms will be scored as they become apparent to validate resistance to PD under field conditions. At the Solano County site, non-grafted plants have been mechanically inoculated with the *Xf* type strain (Temecula 1), but no *Xf* infection or PD symptoms have been detected to date. CAP- and PGIP-expressing transgenic grapevine lines in Solano County have been evaluated phenotypically; no differences were found between transgenic and untransformed. The DNA of CAP- and PGIP-expressing transgenic grapevine lines in Solano County has been checked to confirm the presence of the transgene.

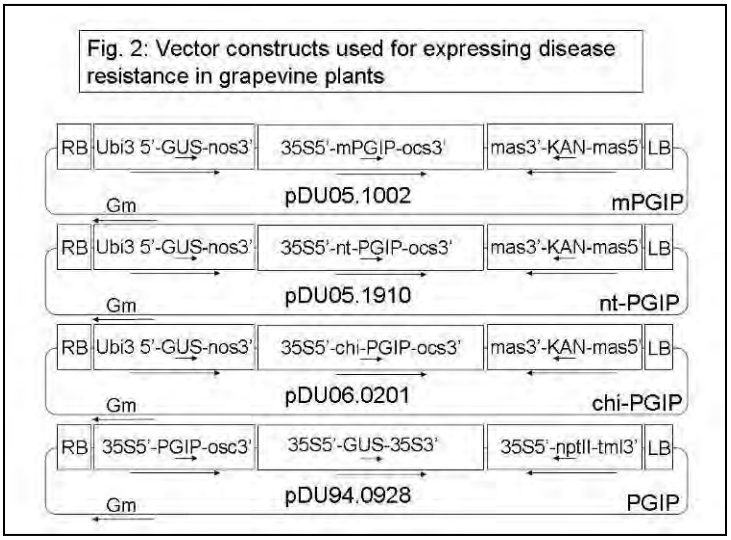
LAYPERSON SUMMARY
Transgenic grapevines are being evaluated as rootstocks to demonstrate the field efficacy of two strategies to control Pierce's disease (PD) in California grapevines. The first strategy uses transgenic rootstocks to control the movement of the bacterium *Xylella fastidiosa (Xf)* in 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 a chimeric antimicrobial protein. At the Riverside County site, *Xf* infection has been confirmed and PD symptoms will be scored as symptoms become more apparent to validate resistance to PD under field conditions. At the Solano County site, non-grafted plants have been mechanically inoculated with *Xf* type strain (Temecula 1), but no *Xf* infection or PD symptoms have been detected to date. CAP- and PGIP-expressing transgenic grapevine lines in Solano County have been evaluated phenotypically; no visible differences were seen between transgenic and untransformed vines. CAP- and PGIP-expressing transgenic grapevine lines in Solano County have been also been tested to confirm the presence of the transgene.

INTRODUCTION
Xylella fastidiosa (Xf), a xylem-limited Gram-negative bacterium, is the causative agent of Pierce's disease (PD). A key feature of *Xf* virulence is its ability to digest pectin-rich pit pore membranes that connect individual xylem elements (Roper et al., 2007), enhancing long distance movement and vector transmission. In this project, we are examining the ability of xylem-targeted polygalacturonase inhibiting protein (PGIP, Aguero et al., 2005, 2006) and a chimeric antimicrobial protein (CAP, Kunkel et al., 2007) to restrict bacterial movement and 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 antimicrobial 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 untransformed 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 *Xf* spread and thus disease virulence. 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, will serve as controls. 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. Vector pDU05.1002 (event 31-25) eliminates the endogenous signal peptide; the expressed PGIP cannot be secreted and should not limit *Xf* spread.





The objective described here directly addresses the first 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

1. Validate the efficacy of *in planta*-expressed CAP and PGIP containing different signal peptides to inhibit and clear *Xf* infection in xylem tissue and to pass through the graft union under field conditions.

The goals of this project are to field-test four CAP- and four PGIP-expressing transgenic Thompson Seedless grapevine lines to evaluate their horticultural characteristics and resistance to PD. Transgenic grapevines are being evaluated at two field

locations as own-rooted plants and as transgenic rootstocks grafted with untransformed TS scions. One field location has PD pressure and plants have been naturally infected with *Xf*. In the location with no PD pressure, grapevines have been mechanically inoculated with *Xf*.

RESULTS AND DISCUSSION

Propagation, field planting, and grafting of CAP and PGIP transgenic grapevines.

Four selected transgenic grapevine lines expressing CAP and four expressing different PGIP constructs were propagated from cuttings in the greenhouse to obtain 48 clones of each line. After the root system developed, cuttings were transferred to 5.5-inch pots to develop into plants. Twenty-four clones were grafted with untransformed TS scions. Well-established plants were transferred to the lath house to acclimatize and then planted in two experimental fields. Two hundred and ten transgenic or untransformed vines, own-rooted or grafted with untransformed TS scions, were planted in Riverside County on 5/8/10 and the remaining 10 were planted on 3/6/11, completing the planting at this location **(Figure 3, Table 1)**. We also planted 110 transgenic and untransformed vines on their own roots on 8/2/10 and 110 vines grafted with untransformed TS scions on 6/27/11 in Solano County, completing the planting at this location (**Figure 3, Table 2**).

Figure 3. Riverside (left) and Solano County (right) transgenic grapevine plantings.

Table 1. Riverside Field Evaluation planted on May 18, 2010 and March 6th 2011.

Non-grafted		Grafted	
Event ID	**# Planted**	**Event ID**	**# Planted**
CAP lines			
40-41	12	40-41G	12
40-89	12	40-89G	12
40-92	12	40-92G	12
41-151	12	41-151G	12
PGIP Lines			
31-25	12	31-25G	12
45-77	12	45-77G	12
52-08	12	52-08G	12
TS50	12	TS50G	12
Control lines			
TS	16	TS-G	12

Table 2. Solano County field evaluation planted on July 6th 2010 and July 27th 2011.

Non-grafted		Grafted	
Event ID	**# Planted**	**Event ID**	**# Planted**
CAP lines			
40-41	12	40-41G	12
40-89	12	40-89G	12
40-92	12	40-92G	12
41-151	12	41-151G	12
PGIP Lines			
31-25	12	31-25G	12
45-77	12	45-77G	12
52-08	12	52-08G	12
TS50	12	TS50G	12
Control lines			
TS	16	TS-G	12

CAP- and PGIP-expressing transgenic and untransformed grapevine lines in Solano County were randomly sampled and tested for the transgenes by PCR (**Table 3**). DNA was isolated from young leaves collected from the field using the Qiagen DNeasy Plant Mini kit according to manufacturer's instructions. DNA was PCRed using ActinF (TACAATGAGCTTCGGGTTGC) and ActinR (GCTCTTTGCAGTTTCCAGCT) to determine DNA quality. Elastase primers were HNE5' (GCAGTTCAGAGGATCTTCGAGGATGG) and HNE3'(TTACTAGAGTGCTTTTGCTTCTCCCAG). Primers for PGIP determination were CaMV 35S-2 (GACGTAAGGGATGACGCACAAT) and MPGIP-4 (CGGATCCTTACTTGCAGCTTGGGAGTGGAGCACCG).

Table 3. PCR genotyping of Solano County transgenic grapevine lines.

Event ID	Inserted Gene	ActinF/R	HNE3/5	CaMV35S/mPGIP4
CAP lines				
40-41	HNE	Positive	Positive	Negative
40-89	HNE	Positive	Positive	Negative
40-92	HNE	Positive	Positive	Negative
41-151	HNE	Positive	Positive	Negative
PGIP Lines				
31-25	PGIP	Positive	Negative	Positive
45-77	PGIP	Positive	Negative	Positive
52-08	PGIP	Positive	Negative	Positive
TS50	PGIP	Positive	Negative	Positive
Control				
TS	None	Positive	Negative	Negative

Evaluate preservation of varietal characteristics in transgenic grapevines grown as whole plants or used as rootstocks. CAP- and PGIP-expressing transgenic grapevine lines in Solano County were evaluated phenotypically in September 2011 to verify that horticultural and varietal characteristics of the parental genotype TS were unchanged. This examination was accomplished using the first 12 descriptors from the "Primary descriptor priority list" proposed by the International Organization of Vine and Wine (OIV, 1983). The descriptors used were 2) density of prostrate hairs on young shoot tips, 3) number of consecutive shoot tendrils, 4) color of upper side of blade on young 4th leaves, 5) shape of mature leaf blades, 6) number of lobes on mature leaves, 7) area of anthocyanin coloration on main veins on upper side of mature leaf blades, 8) shape of teeth on mature leaves, 9) degree of opening of mature leaves/overlapping of petiole sinuses, 10) mature leaf petiole sinus bases limited by veins, 11) density of prostrate hairs between main veins on lower side of mature leaf blades, and 12) density of erect hairs on main veins on lower sides of mature leaf blades. Riverside County CAP- and PGIP-expressing transgenic grapevines lines will also be phenotypically evaluated this year.

Evaluate PD resistance of HNE-CecB and PGIP transgenic grapevines after inoculation with *Xf*.
Thirty-four pooled (Row samples A-I, Lines 1-25) petiole samples from grafted and non-grafted transgenic and control grapevines planted in Riverside County, a positive infected control TS, and *Xf* were evaluated using a commercial ELISA kit for *Xf* detection (Agdia, Elkhart, IN). The assay is based on a mixture of *Xf* antibodies against eight grape *Xf* isolates.

Sample extracts were also plated on PD3 medium and *Xf* growth was verified by PCR using the EFTU and 16s primers. The ELISA (**Figure 4**) and PCR assay (**Figure 5**) results confirmed *Xf* infection in Riversida County. *Xf* cell counts will be done when we get enough *Xf*-infected vines. Since infection was confirmed in pooled samples at Riverside, individual grapevine lines will be sampled and tested for *Xf* infection. PD symptoms in Riverside County will be scored on each infected plant as they appear using a standardized score based on percentage of leaf area scorching, a characteristic of PD (Krivanek et al., 2005a, 2005b). As PD symptoms develop in Riverside County, we can evaluate PD resistance in CAP and PGIP grapevines. Non-grafted petiole samples planted in Solano County that were mechanically inoculated with *Xf* (Almeida and Purcell, 2003) in July of 2011 show no *Xf* infection to date.

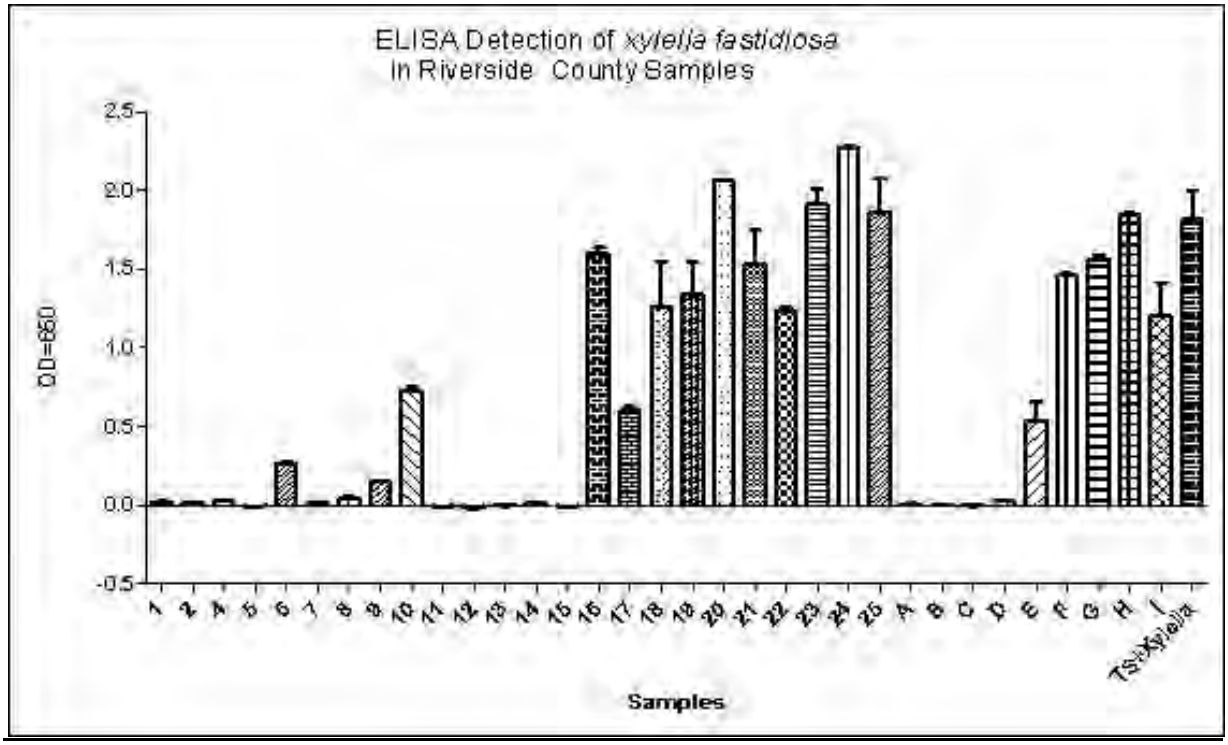


Figure 4. *Xf* detection in Riverside County pooled samples using ELISA.

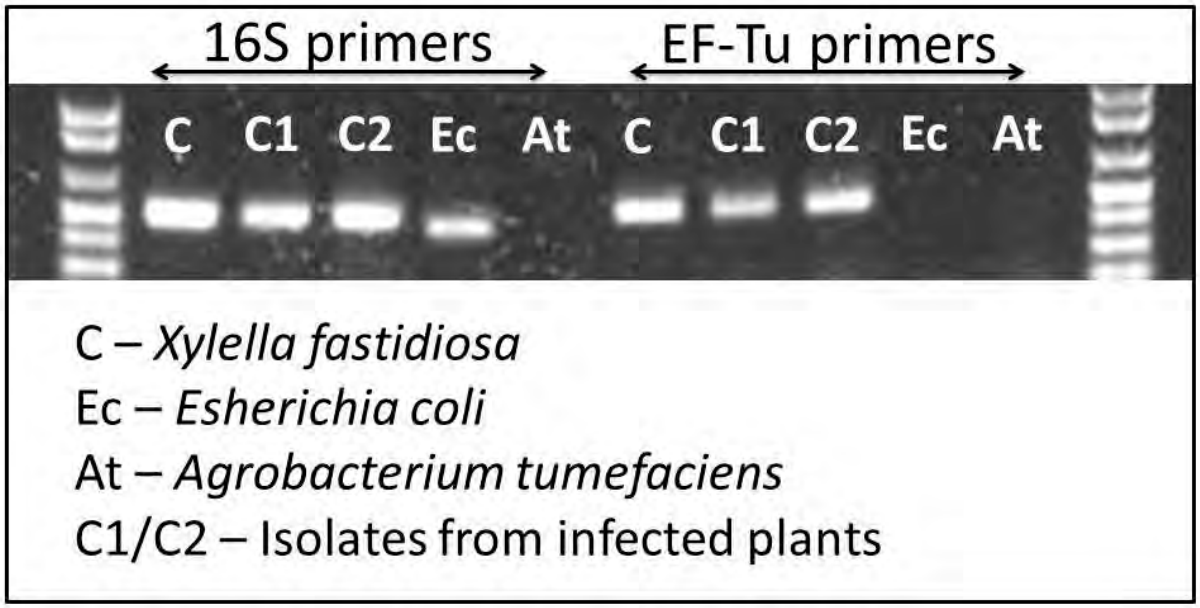


Figure 5. *Xf* detection in Riverside County samples using PCR.

CONCLUSIONS

We have successfully initiated two field trials to validate two greenhouse-tested strategies to control the movement and clearance of *Xf*, a xylem-limited, Gram-negative bacterium that is the causative agent of PD. A key virulence feature of *Xf* resides in its ability to digest pectin-rich pit pore membranes that interconnect the host plant's xylem elements, enhancing long distance movement and vector transmission. The first strategy being evaluated tests the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) from pear to counter virulence associated with *Xf* PG activity. Our second strategy enhances clearance of bacteria from *Xf*-infected xylem tissues using a chimeric antimicrobial protein, CAP. The expectation is that expressing these proteins will prevent *Xf* movement and reduce its inoculum size, curbing the spread of PD in California vineyards. Transgenic grapevine plants expressing either PGIP or CAP along with untransformed controls have been successfully planted in two locations. In Riverside County, planting is now complete with all 220 vines in the ground: 210 planted on 05/08/2010 with the remaining 10 planted on 03/06/2011. In Solano County, where planting is also completed with all 220 vines in the ground, 112 were planted on 08/02/2010 and the remaining 108 on 06/27/2011. These transgenic grapevines will be evaluated as plants on their own roots and as rootstocks grafted with untransformed Thompson Seedless (TS) scions. At the Riverside County site, the plants have been naturally infected by wild glassy-winged sharpshooter and *Xf* presence was confirmed by ELISA and PCR assays. PD symptoms will be scored as they appear to validate resistance to PD under field conditions. At the Solano County site, non-grafted vines have been mechanically inoculated with the *Xf* type strain (Temecula 1), but no *Xf* infection or PD symptoms have been detected to date. CAP- and PGIP-expressing transgenic grapevine lines in Solano County have been evaluated phenotypically using the first 12 descriptors from the "Primary descriptor priority list" proposed by the International Organization of Vine and Wine (OIV). No phenotypical/horticultural differences were observed between transgenic and untransformed TS vines. CAP- and PGIP-expressing transgenic grapevine lines in Solano County have been also been genotyped, confirming the presence of the inserted transgene in all lines.

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ENGINEERING MULTI-COMPONENT RESISTANCE TO PIERCE'S DISEASE IN CALIFORNIA GRAPEVINE ROOTSTOCKS

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Reporting Period: The results reported here are from work conducted July 2011 to September 2011.

ABSTRACT
We propose to develop transgenic grapevine rootstocks resistant to Pierce's disease (PD) using new transformation protocols for the commercially relevant grapevine rootstocks '101-14' and '1103-P.' Our genetic approach involves developing transgenic rootstocks that deliver therapeutic proteins like NE-CB that can protect an untransformed scion from PD. Our approach is to improve grapevine transformation, exploring the possibility of *in planta* transformation of shoot apical meristems (SAM). We have successfully isolated SAM tissues from grapevine rootstocks '101-14' and '1103-P' and from 'Thompson Seedless' as a control. These SAM tissues are being tested for regeneration potential using different media and hormone concentrations that have worked in other grapevine cultivars. The outcome of this research would be the successful development of a more efficient and rapid transformation system for commercially relevant grapevine rootstock and scion varieties using SAM.

LAYPERSON SUMMARY
Current strategies for Pierce's disease (PD) control emphasize deploying transgenic rootstocks that deliver PD control to the untransformed scion. Present technology must be improved to effectively generate transgenic, PD-resistant versions of preferred commercial rootstocks like '101-14' and '1103-P.' We propose to refine different components of the current transformation protocol for greater efficiency, allowing more rapid production and testing of plants. We will transform rootstock '110-14' with an existing vector that incorporates a chimeric protein (NE-CB) to provide enhanced resistance to PD in the scion. We have already initiated development of a more efficient and rapid transformation system for grape rootstocks.

INTRODUCTION
Several presentations at the 2010 Pierce's Disease Symposium highlighted transgenic strategies using various promising transgenes with potential for conferring resistance to *Xylella fastidiosa* (*Xf*), the causal agent of Pierce's disease (PD). However, most such projects have not yet demonstrated such control in commercially significant rootstocks (Dandekar, 2010; Gilchrist, 2009; Labavitch, 2010; and Lindow, 2009). This is partly because the current grapevine transformation and regeneration system was developed at UC Davis a decade ago in rootstocks and scion varieties like *Vitis vinifera* 'Thompson Seedless' (Aguero et al., 2005b, 2006). This system is cumbersome and slow because it uses embryogenic callus developed from young anthers, a tissue available for one brief period during each growing season. It takes around ~6-8 months to generate transgenic somatic embryos from callus lines derived from anther tissue. Additionally, somatic embryogenic callus lines are not available for some widely used commercial rootstocks such as '101-14.' To overcome this hurdle, we are developing a transformation system using meristematic stem cells present in the shoot apical meristem (SAM). In plants like grape, all aboveground plant parts are generated from a cluster of stem cells present in the central dome of the SAM (Sablowski, 2007). Genetic factors regulated through cytokinin signaling determine and control the number of stem cells (Gordon et al., 2009). Several research- and commercial-scale transformations use meristem tissue from different crops. Use of SAM for transformation has occurred with a limited number of grapevine varieties. Mullins et al. (1990) co-cultivated adventitious buds of *Vitis rupestris* 'St. George' rootstock with *Agrobacterium* and produced transgenic plants. However, the methodology was never repeated. Mezzetti et al. (2002) transformed *V. vinifera* 'Silcora' and 'Thompson Seedless', cultivars with a strong capacity to differentiate adventitious shoots, using a meristematic tissue culture system. The culture type was unique and the overall application to other cultivars is unclear. Levenko and Rubtsova (2000) used *in vitro* internode explants to transform three *V. vinifera* scions and a rootstock, but did not provide sufficient details for the technique to be repeated. Dutt et al. (2007) described a simple transformation system for 'Thompson Seedless' using explants from readily obtainable micropropagation cultures. Tissues from etiolated cultures and meristem wounding using fragmented meristems gave the best results. This latter system has not been tested in many rootstocks. Taken together, these studies indicate that SAM is an interesting tissue to investigate, particularly since it is available all year. Additionally, much is now known about

various developmentally regulated genes in plants like *Arabidopsis* that suggests how hormone input can be used to manipulate the developmental patterns of SAM (Galinha et al., 2009).

OBJECTIVES

The goal of this project is to develop a shoot apical meristem-based regeneration system to produce transgenic grapevine rootstocks to control PD. This goal will be accomplished by two activities:

Activity 1: Develop a SAM-based regeneration system for important rootstocks.

Activity 2: Transform, select, regenerate, and confirm transgenic grapevine plants using this new system.

We have focused on activity one, the development of a regeneration system using stem cells present in the shoot apical meristem.

RESULTS AND DISCUSSION

Activity 1: Develop a shoot apical meristem regeneration system for grapevines that provides faster, more efficient production of transgenic plants.

Meristematic domes contain stem cells that make all aboveground parts of the plant (Sablowski, 2007; Gordon et al., 2009). Our first step has involved developing expertise and proficiency at dissecting and excising the meristematic dome from field-grown '101-14' and '1103-P' rootstocks, using 'Thompson Seedless' as a control. A similar technique is used routinely for pathogen elimination at UC Davis Foundation Plant Services. We have worked closely with Adib Rowhani and his colleagues to learn the best technique for excising a SAM. A pictorial outline of the process with '101-14' is shown (**Figure 1**). The sterile meristematic tissues are then cultured to examine their potential for organogenesis or somatic embryogenesis using different hormone concentrations and combinations. We are currently investigating various hormone and media compositions to identify those that lead to proliferation of the SAM; some are described below.

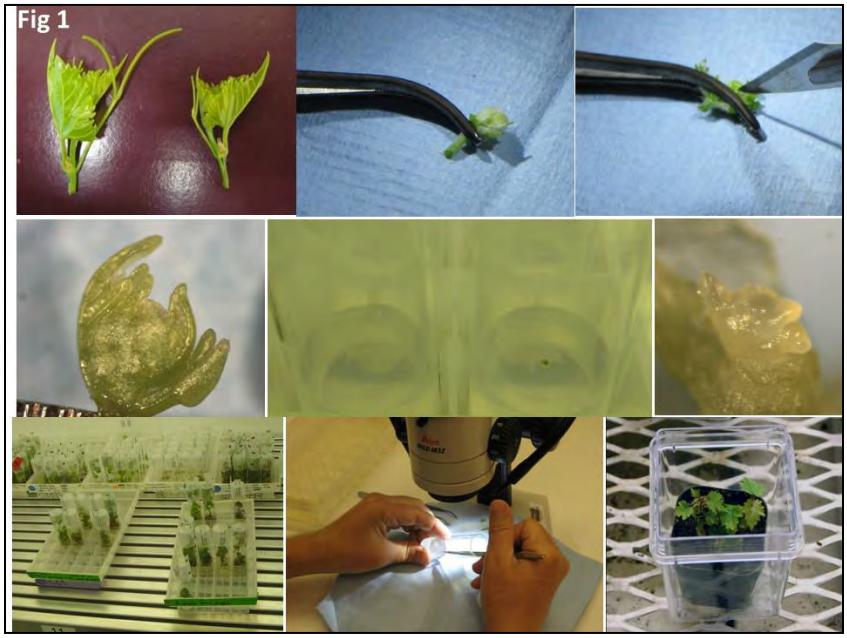

Figure 1. Process for the excision of shoot apical meristem (SAM) from '101-14' grapevine shoot tips.

We have been following published protocols that use different hormone concentrations and combinations to proliferate SAMs and induce a "meristematic bulk" (MB) (Mezzetti at al. 2002, Dhekney et al 2011). This is done using benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), and naphthaleneacetic acid (NAA). '101-14' shoot tips were harvested and immediately dissected to produce explants composed of the apical meristem plus microscopic leaf primordia. Explants were immediately placed in Petri dishes containing induction media composed of Murashige and Skoog (MS) salts and vitamins, 0.1 g L^{-1} myo-inositol, 30 g L^{-1}sucrose and hormones 2,4-D and BA at four and five different concentrations, respectively (**Table 1**). There were three explants per treatment, replicated three times (nine explants total per treatment). The explants are transferred to fresh medium every three weeks and incubated in the dark at 25ºC.

Table 1. Effect of BA and 2,4-D levels on somatic embryogenesis of '101-14' grape rootstock.

BA mg L^{-1}	**0.1**	**0.2**	**0.5**	**1.0**	**2.0**
2,4-D mg L^{-1}					
0	B1	B2	B3	B4	B5
0.5	B6	B7	B8	B9	B10
1	B11	B12	B13	B14	B15
2	B16	B17	B18	B19	B20

At this point, the domes have neither swelled nor proliferated in treatments B1 to B5, B7 to B10, B12 to B15, and B18 to B20. Treatments B6, B11, B16, and B17 show interesting morphogenesis: they are actively multiplying and possibly regenerating. The type of morphogenesis appears to be somatic embryogenesis but further time is required to confirm this.

We have also investigated using different media, using 'Thompson Seedless' (TS) as a control with which we have previous experience in culture and '101-14' and '1103-P' rootstocks. Shoot tips were harvested from field-grown material and immediately dissected to produce explants composed of the apical meristem plus microscopic leaf primordia. Explants were immediately placed in Petri dishes containing five different induction media, N1 to N5, composed of Nitsch and Nitsch (NN) salts and vitamins, 0.1 g L^{-1} myo-inositol, 20 g L^{-1} sucrose, 0.5 µM BA, and 0 µM (N1), 1 µM (N2), 5 µM (N3), 10 µM (N4), or 50 µM (N5) 2,4-D (**Table 2**). Here we are testing the ability of 2,4-D to induce somatic embryogenesis. There were 15 explants per treatment, replicated three times, for 45 explants per treatment. The explants have been transferred to fresh medium every three weeks and incubated in the dark at 25ºC. Resulting callus will be transferred to MS salts and vitamins medium with 1 µM BA and 11 µM Naphthaleneacetic acid (NAA) for development of somatic embryos.

Table 2. Effect of BA and 2,4-D levels on somatic embryogenesis of TS, '101-14'and '1103-P' grape rootstock.

Medium	**N1**	**N2**	**N3**	**N4**	**N5**
BA µM	5	5	5	5	5
2,4-D µM	0	1	5	10	50

In addition to solid medium, we will try liquid medium to stimulate growth and differentiation. This has proved useful in both regeneration and transformation (Humberto Prieto's personal communication, La Platina Research Station, National Agriculture Institute, Santiago de Chile). For the liquid phase, we will use "temporary immersion" (TI) in medium that is identical to the solid medium except for the absence of solidifying agent. This phase will be done in collaboration with David Tricoli at the UC Davis Parsons Plant Transformation Facility.

CONCLUSIONS

We are currently developing a shoot apical meristem-based grapevine regeneration system for faster, more efficient production of transgenic plants. We have initiated experiments with solid medium to study the effect of the cytokinin benzyladenine (BA) and the auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) on somatic embryogenesis of TS and '101-14', and '1103-P' rootstocks. Transgenic rootstocks have been proposed as the best strategy to develop PD-resistant grapevines. However, current transformation protocols use embryogenic callus lines developed from anther culture for transformation and available cultures do not include rootstock genotypes currently used by growers in California. Also, current callus lines have been in culture for a long time and fresh new cultures must be selected, a task limited to a single season each year. This research seeks to overcome this seasonal limitation by developing a transformation system using a SAM, a starting material that is available year-round.

The results of this research will benefit other research groups working on transgenic strategies to control PD and has the potential to benefit research in other crops where transgenic approaches are sought to create pathogen resistance in rootstocks. The objective described in this proposal directly addresses the research priorities outlined in 'attachment A' of the 2010 PD/GWSS proposal RFA. It also addresses the top RSAP priority in the "Enabling tools- Development of grape regeneration and transformation systems for commercially important rootstocks" handout released in December 2009. This document outlines the "Top 5 to 10 Project Objectives to Accelerate Research to Practice" and 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.

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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 of *Xylella fastidiosa* (*Xf*) surface proteins, especially the translation elongation factor "temperature unstable" (EF-Tu) in disease development induced by this bacterium, the causitive agent of Pierce's disease (PD) of grapevine, and to use this understanding to interfere with disease development. Our earlier work showed that the infiltration of preparations enriched in *Xf* EF-Tu protein into *Chenopodium quinoa* leaves induced a chlorosis reaction, suggesting that EF-Tu may contribute to symptom development in *Xf* infections. EF-Tu of certain eubacteria has been recognized as a MAMP, a microbe-associated molecular pattern, i.e., a macromolecule that is characteristic of a class of microorganisms and thus well suited to signaling the invasion of a member of that class. Unlike the EF-Tu of most eubacteria, *Xf* EF-Tu is associated with an insoluble fraction, which makes its purification by conventional methods difficult. Previously, we demonstrated that using *Agrobacterium tumefaciens* to program plant cells to produce apoplast-targeted *Xf* Ef-Tu resulted in the development of scorching symptoms characteristic of PD in *Nicotiana tabacum* cultivar SR-1 and Thompson seedless grapevines. The application of transgenic Agrobacterium obviated the need to purify EF-Tu. We report here that the use of *A. tumefaciens* carrying new binary constructs with intron inserts in *Xf* EF-Tu gene, both with and without apoplastic targeting sequences, also induced PD-like symptoms in SR-1 tobacco. Infiltrations of these same *Agrobacterium* strains into *Nicotiana benthamiana* did not lead to symptom development, consistent with the known lack of an EF-Tu receptor in *N. benthamiana*. We expected that apoplastic targeting would be required for symptom induction because, in the infected plant, *Xf* resides in the xylem rather than interacting with living cells. Interpreting the similar response of SR-1 tobacco to EF-Tu targeted and not targeted to the apoplast is confounded by the much greater accumulation of EF-Tu for the non-apoplast-targeted, compared to the untargeted, construct. We employed transposon mutagenesis to map regions of EF-Tu needed for symptom induction. Our results suggest that the symptom-inducing region lies between amino acids 91 and 291. Previously, we found that digestion of *Xf* cells with lysozyme in a detergent solution generated a membrane- and EF-Tu-containing insoluble fraction in which EF-Tu is accessible to antibody, whereas the EF-Tu of intact *Xf* cells is not accessible. Here we demonstrate that infiltration of the insoluble, EF-Tu-containing fraction into Thompson seedless grapevine leaves stimulated expression from a grapevine promoter known to be specifically activated in *Xf* infection.

LAYPERSON SUMMARY
The elongation factor "temperature unstable", EF-Tu, is one of the most abundant proteins in most bacteria. In *Xylella fastidiosa (Xf)* it is found at or just below the cell surface and is associated with an insoluble fraction which makes purification difficult. To avoid the need to purify the *Xf* EF-Tu, we have used *Agrobacterium*, a bacterium that can program plant cells to produce exogenous proteins, to produce *Xf* EF-Tu either with or without targeting EF-Tu to the outside of the plant cell. Production of either targeted or untargeted EF-Tu in *Nicotiana tabacum* cv SR-1 led to development of Pierce's disease(PD)-like symptoms. We have shown that only part of the *Xf* EF-Tu protein is necessary to produce these symptoms. Also, the insoluble fraction, which has much more accessible EF-Tu protein than intact cells, is capable of stimulating grapevine responses in the same way that *Xf* infection can. The apparent ability of *Xf* EF-Tu protein to stimulate grape and the production PD-like symptoms in the absence of intact *Xf* cells or other *Xf* proteins supports a role for the *Xf* EF-Tu protein recognition in PD and could lead to novel methods for reducing PD symptom development.

INTRODUCTION
Effective long term control of Pierce's disease(PD) will likely require the development of resistant or tolerant grapevine cultivars. Resistance or tolerance could be achieved by interfering with symptom development, which presumably requires at least partial interference with the functioning of *Xylella fastidiosa (Xf)* virulence factors. While the mechanism of *Xf* virulence factor function has not been established, *Xf* surface proteins, such as the 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) and a form of the translation elongation factor "temperature unstable" (EF-Tu), are possible candidates. We reported earlier that partially purified MopB was capable of inducing chlorosis in *Chenopodium quinoa* (Bruening et al. 2007); however, *Xf* MopB produced in and purified from *E. coli* did not induce this chlorosis. Eluted protein from a minor trailing band from electrophoresis of this partially purified MopB preparation through a sodium dodecyl sulfate-permeated polyacrylamide gel induced chlorosis in *C. quinoa*. The major component of this trailing band was EF-Tu suggesting that it, and not MopB, is the chlorosis inducing factor.

EF-Tu is one of several highly conserved eubacterial macromolecules known as "microbe-associated molecular patterns" (MAMPs) because of their ability to induce innate immunity in both plants (Jones and Dangl 2006) and animals. Flagellin, chitin and certain lipopolysaccharides are other examples of MAMPs. EF-Tu is the most abundant protein (up to 700,000 molecules per cell) in most bacterial cells making it a reasonable signal for the presence of bacterial infection. The MAMP activity of *E. coli* EF-Tu is illustrated by its ability to induce alkalization of the medium of cultured *Arabidopsis thaliana* cells at subnanomolar concentrations. Pressure infiltration of *E. coli* EF-Tu at 1μM into *Arabidopsis* leaves leads to both resistance to *Pseudomonas syringae* and the accumulation of defense gene mRNAs (Kunze et al. 2004). The *E. coli* EF-Tu and *Xf* EF-Tu are highly similar (77% identity and 88% similarity in their amino acid sequence), and they both induce chlorosis when pressure infiltrated into *C. quinoa* leaves. The regions that show identity between the *E. coli* and *Xf* EF-Tu protein sequence also show >90% identity with >100 eubacterial Ef-Tu sequences (Kunze et al. 2004). In some bacteria, the EF-Tu protein has at least one additional activity--that of an adhesin. *Mycoplasma pneumoniae* and *Lactobacillus johnsonii* appear to use EF-Tu 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 would not be surprising that the apparently surface associated *Xf* Ef-Tu would be capable of inducing reactions in grapevine, including those 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

Transient expression of Xf EF-Tu in* Nicotiana tabacum *induces Pierce's disease-like symptoms

Our earlier agro-infiltration constructs were designed to express *Xf* EF-Tu for accumulation in the plant cell cytoplasm or in the intercellular spaces (apoplast). A protease-encoding sequence was incorporated to generate a near-wildtype amino-end to EF-Tu. These constructs apparently slowed the growth of *Agrobacterium tumefaciens*, and the protease provoked a reaction when it was transiently expressed in plants. **Figure 1** diagrams new constructs from which the protease was eliminated and into which an intron was inserted to prevent expression in *A. tumefaciens*. Extracellular targeting was through the P14 apoplastic targeting sequence (Vera et al. 1989), which has been shown by Jim Lincoln of the David Gilchrist laboratory to effectively cause secretion of the green fluorescent protein (GFP) into the apoplast. For convenience, in this report apoplast-targeted construct is designated P, and the cytoplasm-targeted construct is designated I. The positioning of the intron after codon 3 should prevent synthesis of EF-Tu in *A. tumefaciens* and thus any toxic effects from premature expression. Due to the uncertainty in the site of signal protease cleavage to release P14, the amino end produced by expression from these new constructs may not be the same as the authentic EF-Tu amino end.

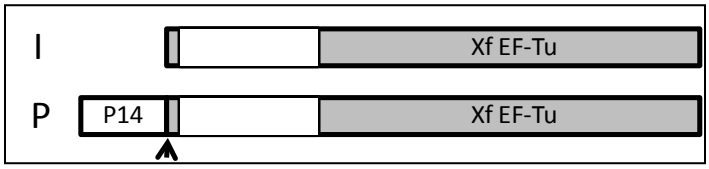

Figure 1. Coding sequence for I and P constructs. The I construct is intended to generate EF-Tu in the cytoplasm of agro-infiltrated leaves. The P construct is intended to direct intercellular accumulation of EF-Tu. In the P diagram, the arrow represents the site of cleavage that releases the P14 peptide. The grey boxes represent *Xf* EF-Tu coding regions. The gus intron is preceded in the construct by a sequence encoding only the first three amino acids of *Xf* EF-Tu. The intron was derived from the intron in the gus-encoding sequence found in the pCAMBIA vectors and was originally derived from the castor bean catalase gene. These constructs were cloned into pEAQ-HT, an expression vector that gives high levels of translation (Sainsbury and Lomonosoff, 2008).

The two strains of *A. tumefaciens* used here, in order of increasing aggressiveness, are LBA4404 and GV2260. Each was transformed with the P and with the I constructs, which were compared with untransformed *A. tumefaciens*. In later experiments, with each of the three *A. tumefaciens* strains, the untransformed strain and the same strain bearing pEAQ-HT vector without insert, induced very similar reactions on SR-1 tobacco (not shown), supporting the appropriateness of the untransformed *A. tumefaciens* control. LBA4404, bearing either the I or the P construct, induced mild scorching symptoms in agro-infiltrated SR-1 tobacco (*Nicotiana tabacum*), whereas untransformed *A. tumefaciens* induced, at most, only a mild chlorosis (compare the three panels on the left half of **Figure 2**). As expected, agro-infiltration with strain GV2260

containing the I and P constructs induced stronger scorching symptoms compared to LBA4404 (compare the left and right halves of **Figure 2**).

Figure 2. Comparison of scorch symptoms produced on SR-1 tobacco by infiltration with *Agrobacterium* alone (C) and *Agrobacterium* containing the I and P constructs. Two *Agrobacterium* strains were tested, LBA4404 and GV2260. Cultures were grown overnight at 28°C then diluted 1/10 and growth was allowed to continue for several hours until the cultures reached mid-log phase (A_{600} = ~0.6). The cells were washed once with water and their A_{600} were adjusted to 0.5 A_{600} before infiltration. Pictures were taken at five days post infiltration (dpi), although symptoms were usually easily seen between two and three dpi.

The I construct lacks the apoplast-targeting P14 sequence and therefore should direct synthesis of cell-confined EF-Tu. The observed strong scorching symptoms is inconsistent with recognition by an EFR homolog because the ligand-binding portion of the EFR protein is known to be extracellular (Albert et al. 2010) and *Xf* is not expected to be capable of delivering *Xf* proteins to the plant cell interior (Meidanis et al. 2002). The observed scorching symptoms after intracellular accumulation of *Xf* EF-Tu could be explained if there is (i) a functional intracellular receptor of EF-Tu, (ii) escape of some EF-Tu from the cell or (iii) toxicity of the EF-Tu protein not related to its MAMP activity. To address this last possibility, we infiltrated *N. benthamiana*, which is known not to have an EFR homologue, with GV2260 containing the I and P constructs. **Figure 3** shows *N. benthamiana* leaves infiltrated at the same time as the SR-1 tobacco leaves shown in **Figure 2**. No symptoms were present in the *N. benthamiana* compared with the strong scorching symptoms seen in the SR-1 tobacco at 5 dpi. By nine dpi, a mild yellow chlorosis was visible in the I construct infiltrated *N. benthamiana* leaves compared to the C and P construct infiltrated leaves. This lack of symptoms is consistent with the absence of an EFR homologue in *N. benthamiana*. It is also consistent with the EF-Tu not being generally toxic to plant cells, at least for *N. benthamiana*. This non-toxicity in *N. benthamiana* and is also supported by studies of GFP expression from the pEAQ-HT vector in GV2260, which show much greater accumulation of GFP in *N. benthamiana* than in SR-1 tobacco (results not shown).

Protein was extracted from the *N. benthamiana* leaves at nine dpi, subjected to denaturing gel electrophoresis and immunoblotted using anti-*Xf* EF-Tu antibodies. As shown in **Figure 4**, a small amount of protein, visible between the 37.5KDa and 50KDa standards, was detected only in the extract from the I sample while no signal was observed for the lane receiving the P sample, which is consistent with previous experiments where P14-containing, apoplast targeted GFP produced far less GFP fluorescence and GFP protein than was observed for the GFP construct lacking P14. GFP expressed from the pEAQ-HT vector represents a significant fraction of the *N. benthamiana* cell protein (Sainsbury and Lomonosoff, 2008). Therefore, the small amount of *Xf* EF-Tu produced in the otherwise superior protein-producing *N. benthamiana* supports the notion that a specific reaction with a receptor, rather than an inherent toxicity, is responsible for the scorching symptoms seen in SR-1 tobacco. The low level of protein produced may be due to codon bias differences between the plants used and the *Xf* bacteria, for example the preference for CGC codons for arginine in prokaryotes while it is rarely used in plants, or may be due to poor splicing. In either case, the amount of EF-Tu produced is sufficient to elicit a strong response.

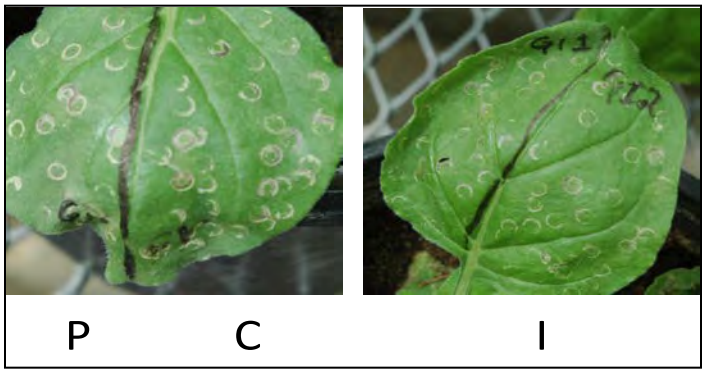

Figure 3. Test of symptom development in N. Benthamiana after infiltration with GV2260 containing I and P constructs. Left panel, opposite half leaves were infiltrated with GV2260 alone (C) or containing the P construct (P). Right panel, leaf infiltrated with GV2260 containing the I construct. The cultures used with the same as used in the experiment shown in **Figure 2** and were infiltrated at the same time. Images taken at five dpi.

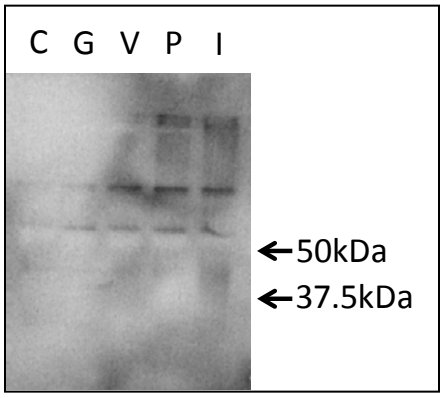

Figure 4. Immunoblot with anti-*Xf* EF-Tu antibody of protein extracts of *N. benthamiana* leaves extracted nine dpi. Extracts are from uninfiltrated leaves (C), leaves infiltrated with GV2260 only (G), GV2260 containing empty pEAQ-HT (V), GV2260 containing the P construct (P) and GV2260 containing the I construct (I).

Since the I construct produced scorching symptoms in SR-1 tobacco, we undertook experiments aimed at defining the portion of the EF-Tu protein that is recognized to initiate development of these symptoms. A EZ-Tn5 <Tet-1> transposon from Epicentre Technologies was inserted randomly into the I construct plasmid DNA in vitro. Transformation into *E. coli* followed by selection on kanamycin and tetracycline plates allowed only those plasmids that had at least one inserted transposon to be recovered. The promoter that transcribes the tetracycline resistance gene could lead to production of *Xf* EF-Tu protein fragments through internal initiation if the transposon is inserted in the appropriate orientation. Because this could lead to toxic effects during cloning, only those plasmids where the transposon is in the opposite orientation to the *Xf* EF-Tu were chosen. These clones were selected by PCR using a primer outside the *Xf* EF-Tu coding sequence and a primer within the transposon such that only the desired plasmids would give a PCR product. The size of this PCR product was used to roughly map the insertion sites and appropriate clones were sequenced to determine the exact insertion site. Two clones were transformed into *A. tumefaciens* strain GV2260 and infiltrated into SR-1 tobacco. **Figure 5** shows the locations of these two clones and shows that in clone I-10, the deletion of the C-terminal 105 amino acids did not destroy the ability of the produced *Xf* EF-Tu protein fragment to induce scorching. However, in clone I-12, leaving only the 91 N-terminal amino acids did destroy said ability. A peptide representing the 18 N-terminal amino acids is sufficient to bind and activate the *Arabidopsis* EFR protein (Kunze et al. 2004). When this peptide was infiltrated into *Chenopodium quinoa* leaves, the chlorosis that characteristically develops after infiltration of preparations enriched in *Xf* EF-Tu (Bruening et al. 2007) was not seen. These results suggest that the receptors for EF-Tu in *Chenopodium* and SR-1 tobacco may be recognizing different epitopes than the epitope recognized by the *Arabidopsis* EFR protein. Nevertheless, the receptor proteins in *Chenopodium* and SR-1 tobacco could be EFR homologs.

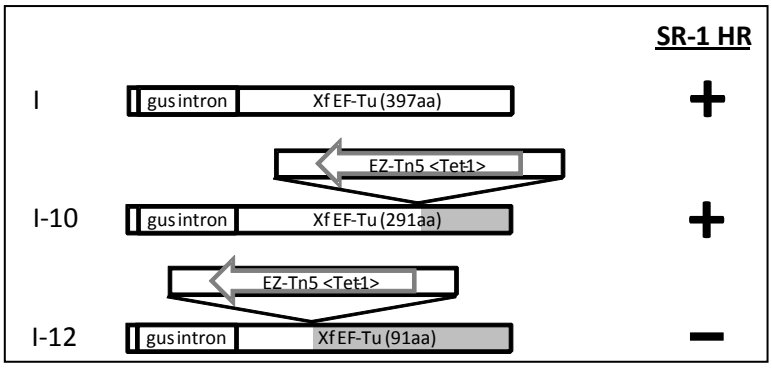

Figure 5. Transposon tagged construct I in *A. tumefaciens* strain GV2260 and tested in SR-1 tobacco. I-10 and I-12 are the designation for the tested clones. The number of amino acids of *Xf* EF-Tu before the insertion site for each clone tested is shown in parenthesis. The grey colored area corresponds to sequence 3' of the first stop codon resulting from the transposon insert.

The EF-Tu protein is considered to consist of three domains. Clone I-10 has enough of the carboxyl end sequence of EF-Tu deleted to completely remove domain 3 (Song et al. 1999). The spontaneous polymerization of E. coli EF-Tu maps to domain 3. Polymerized EF-Tu is speculated to be a component of the primative prokaryotic cytoskeleton (Soufo et al. 2010). One possible mechanism by which *Xf* EF-Tu could be inherently toxic is by polymerizing. The fact that SR-1 tobacco recognition of *Xf* EF-Tu does not require the polymerization domain suggests that *Xf* EF-Tu is not inherently toxic, as we have already tentatively concluded from other evidence presented and interpreted above.

Stimulation of an EF-Tu responsive grape promoter by a Xf cell-derived sub-cellular fraction

Previously, we described material that is released from *Xf* cells after incubation with Bugbuster, a commercial bacterial lysis solution, and lysozyme. A fraction, designated fraction P, was recovered by centrifugation and gradient centrifugation of the partially digested *Xf* cell material (Bruening et al. 2008). Electron microscopy of fraction P revealed spheres of approximately the same surface area of the original *Xf* cells, suggesting that components of fraction P are at or very near the surface of the intact cells (Feldstein et al. 2010). Immunogold electron microscopy with anti-*Xf* EF-Tu antibody showed that fraction P retained *Xf* EF-Tu (Feldstein et al. 2010). Separate incubations of fraction P and intact *Xf* cells with Alexa 488-labeled anti-*Xf* EF-Tu antibody, followed by washing and centrifugation, resulted in labeling of fraction P but not of the intact cells (**Figure 6A**). These results suggest that the *Xf* EF-Tu, like the EF-Tu proteins of a few other bacteria, is associated with the outer surface of the bacterial cell but is not accessible in the intact cell. For certain pneumococci and meningococci bacteria, the surface associated EF-Tu is not accessible to antibody and is made accessible only after treatment of the cells with heat or ethanol (Kolberg et al. 2008).

Viable or heat-inactivated *Xf* cells induce a chlorotic reaction when pressure infiltrated into *Chenopodium quinoa* leaves. This activity was traced to the EF-Tu of *Xf* cells (Bruening et al. 2007). Not surprisingly, fraction P stimulated a similar reaction after pressure infiltration into *C. quinoa* leaves. Transcription from a grapevine promoter, 9353, which was discovered in the laboratory of Douglas Cook and exploited in the laboratory of David Gilchrist, is activated by *Xf* infection of grapevine (Gilchrist and Lincoln 2008). To test the ability of fraction P to activate, in grapevine leaves, transcription from promoter 9353, *A. tumefaciens* bearing a 9353-driven GFP construct was co-infiltrated with fraction P. At five days after infiltration, leaf lamella were examined under a laser confocal microscope. **Figure 6B** shows that only when the fraction P material was infiltrated was a GFP signal seen in the infiltrated areas, suggesting that a component of fraction P, presumably EF-Tu, is sufficient to activate *Xf*-infection-specific promoter 9353.

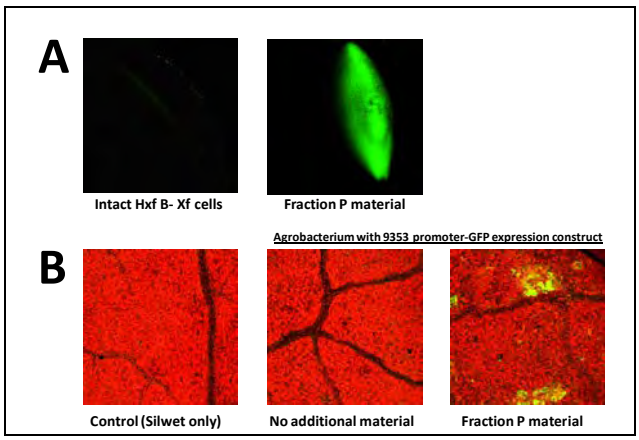

Figure 6. *Xf* cell-derived fraction P has antibody-accessible EF-Tu and activates transcription from a *Xf*-infection-specific grapevine promoter. (**A)** Recovery of fluorescent anti-*Xf* EF-Tu antibody in the insoluble fraction after incubation with intact *Xf*-H*Xf*B cells, **left panel,** and with fraction P material, **right panel**. Fraction P material or cells were incubated with Alexa 488-labeled anti-*Xf* EF-Tu and then washed with buffer and collected by centrifugation. The centrifugation pellet, collected in the bottom of an Eppendorf tube, was viewed under a fluorescent microscope. (**B**) Laser confocal microscope images of Thompson Seedless grape leaves infiltrated with Silwet only, **left panel**, with Silwet and *A. tumefaciens* strain bearing the *Xf* infection-specific 9353 promoter-GFP expression construct, **middle panel,** or with Silwet, the 9353 promoter-GFP expression construct and fraction P material, **right panel**. The Silwet wetting agent increases the entry of the *A. tumefaciens* cells in the infiltration method used.

CONCLUSIONS

Xf EF-Tu has been shown to be associated with or near the outer surface of the *Xf* cell. This peripheral location could allow recognition by the plant of the *Xf* EF-Tu protein. Due to the difficulties of purifying the *Xf* EF-Tu protein itself, we have used *Agrobacterium* to induce plant cells to produce the *Xf* EF-Tu protein themselves. This production leads to PD-like symptoms. While targeting of this protein to the intercellular spaces of the leaf does not appear necessary in these experiments, the difference in the amount of protein produced may allow some of the more highly produced intracellular protein to reach the presumably extracellular receptor. We have also found that removal of the C-terminal domain 3 of the EF-Tu protein does not interfere with symptom development, but that larger deletions did, suggesting that the recognized region of EF-Tu is between amino acids 91 and 291. This is inconsistent with the EF-Tu region known to bind to the *Arabidopsis* EFR receptor, which consists of the amino-end 18 amino acids. This suggests that the *Arabidopsis* receptor EFR and the presumed grapevine and SR-1 tobacco receptors may be recognizing different regions of the EF-Tu protein.

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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 research in our lab first established a determinative role for a genetically regulated process of programmed cell death (PCD), in the leaf scorch and cane death symptoms in Pierce's disease (PD) and then developed a functional screen for PCD-suppressing plant genes from a cDNA library of grape genes. The functional screen identified six candidate DNA sequences potentially able to suppressing *Xylella fastidiosa* (*Xf*)- induced PCD when expressed as transgenes. Two of the sequences (VvPR1 and UT456) were selected to test for ability to suppress the PCD-dependent symptoms of PD. Greenhouse experiments over several years confirmed that these two different anti-PCD DNA sequences prevented PD symptoms in the PD-susceptible cultivar Thompson Seedless and the commercial rootstock Freedom. Furthermore, the bacterial titer in the transgenic plants was reduced 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. 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 *Xf* to an endophytic ecology in the xylem equivalent to that seen in the related asymptomatic host *Vitis californica*. Clonal copies of the transgenic and control plants were moved to two field locations under an APHIS permit secured by PIPRA and were inoculated July 21, 2011. Greenhouse data obtained from grafting experiments indicate the protective effect of these genes may be transferred across a graft union to protect a susceptible untransformed scion. Grafted plants expressing VvPR1 and UT456 in the rootstock, but not the scion, have been moved to the field site for inoculation in the spring of 2012.

LAYPERSON SUMMARY
Xylella fastidiosa 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, VvPR1 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 VvPR1 and UT456; each of the four provided substantial suppression of both PD symptoms and bacterial titer. However, none were as effective as VvPR1 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 VvPR1 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. The relative susceptibility of the suite of eight commercial winegrape varieties was tested under controlled greenhouse 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 from 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 with additional plantings to be made in Solano and Riverside Counties in 2011.

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 role of altered cell stability in disease through an evolutionarily conserved program involving programmed cell death occurs in both animals and plants. Functionally, 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. Processed in this manner, the cell contents can serve as nutrients for microbial cells when they are present in the immediate environment of the pathogen (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). Our research has focused on the effect of altering the expression of two different plant DNA sequences (PR1 and UT456). Both of these putative anti-

PCD sequences protected both against PD symptoms and limited bacterial titer four to six orders of magnitude below that reached in untransformed control vines of the susceptible cultivar Thompson Seedless and the commercial rootstock Freedom, While protection against PD appears to be feasible in plants where the transgenes are expressed constitutively throughout the plant, it remains critical to determine whether transfer of this protection can occur across a graft union to untransformed scions. Hence, we have constructed transformed rootstocks (Freedom and Thompson Seedless) expressing PR1 or UT456 grafted to untransformed Thompson Seedless and winegrape scions to be tested first by greenhouse inoculation and later in field plantings if the greenhouse tests are positive for protection. To identify possible winegrape varieties to use as differential scions we completed testing the relative susceptibility of 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 before any field evaluation is undertaken. The greenhouse assays for these eight varieties have been completed and are summarized herein. Initial greenhouse inoculation experiments indicated that the protection by PR1 and UT456 does move across the graft union. Final data on these experiments will conclude in 2011. 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. Initial data on potential for transmission of protection by these anti-PCD sequences across a graft union to protect an untransformed wild type scion is positive.

OBJECTIVES

1. Complete the evaluation of the additional four candidate anti-apoptotic genes transformed into PD susceptible Thompson Seedless plants. (2010-2011)
2. Evaluate the relative susceptibility of eight commercial winegrape varieties to PD and titer of *Xf* in the inoculated canes. (2010-2011)
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. (2010-2012)
4. Determine presence and movement of the mRNA and/or protein of VvPR1 and UT456 across the graft union into the untransformed Thompson Seedless O2A scion. (2010-2012)
5. Perform inoculations the eight winegrape varieties, initially on their own rootstocks and subsequently on Freedom and Thompson Seedless rootstocks expressing VvPR1 and UT456. (2012)
6. Investigate the mechanism underlying the protection against PD by VvPR1 and UT456. (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. (2010-2011)
8. Secure patent protection as intellectual property for those genes that prove to be capable of blocking PD in grape. (2011-2012)

RESULTS AND DISCUSSION

Complete the evaluation of the additional four candidate anti-apoptotic genes transformed into PD susceptible Thompson Seedless plants. (2010-2011)

The protective genes or DNA sequences, isolated by a functional anti-PCD screen (1), have been described in (3,4,5). Greenhouse inoculations were completed in 2010-2011 and the results summarized in **Table 1**. DNA sequence analysis of each of these genes indicates the presence of orthologs in other plants including potato and tomato (**Table 1**). Inoculation of individual canes by the needle prick method delivered 10-20 µl of the Temecula strain of *Xf* at a concentration of 10^5 cfu/ml (2,000 cells). 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 below that of the control but were not superior to VvPR1 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 are focused on VvPR1 and UT456.

Evaluate the relative susceptibility of eight commercial winegrape varieties to Pierce's disease and titer of *Xf* in the inoculated cane under controlled greenhouse inoculation conditions. (2010-2012)

Experiments were concluded on a suite of commercial winegrape varieties to obtain quantitative data on bacterial population dynamics and relative PD susceptibility. This experiment was conducted under controlled greenhouse inoculation conditions to avoid any vagaries associated with natural infection and GWSS preferences. 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, VvPR1 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 expressing VvPR1 and UT456. Data collected included bacterial titer, and "disease symptoms." Disease symptoms are herein defined as leaf defoliation, not marginal death of leaves that is generally considered to be symptoms of PD under field conditions. NOTE: it has been our consistent observation over the past seven years that marginal leaf death, often associated with PD under field conditions is meaningless and misleading under our greenhouse conditions. Uninoculated control plants frequently exhibit marginal and interveinal death reminiscent of the field PD symptoms, while leaf drop occurs only in susceptible inoculated plants. The point is that after inoculating

more than 500 plants in the greenhouse, the susceptible control plants always defoliate, show high bacterial titre, and die. In our experiments, only the transgenic protected plants retain their leaves and show low levels of bacterial titre. 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). All varieties were susceptible to PD in terms of leaf defoliation symptom expression and exhibited 1-3 orders of magnitude higher bacterial titers four months after inoculation than the asymptomatic Vitis californica or transgenic Vv PR1 or UT456 comparison plants (**Table 2**). Pinot Gris had the highest bacterial titer and exhibited the most severe defoliation 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 (**Figure 1**). The Cabernet Sauvignon and Merlot winegrape varieties have now been grafted to transgenic rootstocks expressing VvPR1 and UT456 to determine if any cross-graft protection occurs. Inoculation of these grafted plants under greenhouse conditions, comparable to the previous Thompson Seedless transgenic: scion combinations will begin in the Fall of 2011.

Conduct experiments to assess the potential for protection against PD across a graft union by VvPR1 and UT456 with Thompson Seedless as the untransformed scion. (2010-2012)

The purpose is to determine if the protective effect of these genes as observed in the primary transgenics is transferrable across a graft union to protect a susceptible scion. PD susceptible untransformed Thompson Seedless scions were grafted onto Freedom rootstocks transgenic for VvPR1 and UT456. A total of 13 untransformed control grafts were compared with 13 transformed rootstock:untransformed scions. The preliminary data suggest that all 13 of the susceptible scions showed none or far less PD symptoms and had reduced bacterial titer than the untransformed control grafted plants, all of which were dead or nearly dead by four months after inoculation with approximately 20,000 *Xf* cells per branch (**Table 3** and **Figure 2**). While these results are encouraging, the experiment is continuing with two of the winegrape varieties, (Cabernet Sauvignon and Merlot) grafted to a transgenic rootstock. Currently, comparable grafted plants are being prepared in the greenhouse for field planting in the Spring 2011 (see Objective D).

Investigate the mechanism underlying the protection against PD by VvPR1 and UT456. (2010-2012)

We have found two novel and likely linked mechanisms for VvPR1 and UT456 action. First, the transgenic PR1 protein product will suppress PCD in several plants systems we have tested. However, the PR1 coding sequence is translationally blocked in healthy cells and an in *vitro* translation system, even when the message level is high 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 hairpins that show sequence conservation with the 3'UTR of PR1 and are projected to interact with each other by RNA modeling programs. *In vitro* protein translation studies indicated that the block in translation of PR1 RNA can be relieved by the addition of UT456 RNA to wheat germ extracts. The same result was obtained by agro- infiltration assays , whereby the expression of UT456 activated the translation of the PR1 protein in tobacco leaves expressing high levels of the PR1 message that was blocked until the UT456 RNA was present and processed into a microRNA. By functional definition, microRNAs are small endogenous RNA molecules (~22-24 bases) that are processed from longer transcripts into pre-microRNA hairpin structures with final steps completed by an enzyme called Dicer. The *in vitro* activation of PR1 translation results from the release of a 22-24 bases from the native 270 base UT456 hairpin by the endogenous nuclease DICER, know to be present in the wheat germ extracts. MicroRNAs regulate gene expression by targeting by sequence homology one or more messenger RNAs (mRNAs) for translational regulation or degradation. Although the first microRNA was identified over ten years ago, it is only recently that the scope and diversity of these regulatory molecules have begun to be understood. There is precedent for translational blockage by the 3'UTR in plant systems and for RNA movement from roots to tubers (6). The presence of the sequence in UT456 with annealing ability to the 3'UTR of PR1 is the basis for the current model of PR1 and UT456 function in the *Xf*-grape system. Currently, we are searching for mobile UT456 microRNA in extracts in the transgenic grapes and as the mobile element in the untransformed scions which appear to be protected against PD symptoms when grafted to transgenic rootstocks expressing UT456. Other research in the lab has developed a highly sensitive Taqman-based assay capable of detecting specific and low abundant microRNA sequences in plant extracts. In addition, PR1 antibodies will be used to test directly for the presence of mobile PR1 protein from the rootstock into the grafted scions. In addition PR1 antibodies are being used in immunoprecipitation assays to detect potential PR1 interacting factors. To date we have been successful in identifying 3 PR1-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 to rice blast in rice plants transgenic for RACK1 (7).

Collaboration with PIPRA to obtain an APHIS permit for field planting

An APHIS permit to enable field plant of transgenic plants from the our laboratory as well as those of PIs Dandekar Lindow and Labavitch is in place. Planting of the primary transgenic plants from the respective programs was completed in July, 2010. The 2010 plantings of all four investigators survived the winter without loss. The attachment of new shoots to the trellis system, cultivation, and irrigation management progressed in a normal and effective manner. All flowers were removed before opening and extensive pruning was done to manage the plants in a fashion compatible with mechanical inoculation. All flowers and prunings were collected, bagged, and autoclaved before disposal. As of July 21, 2011, the initial planting and the second 2011 planting individuals are healthy, growing normally and all plants have a normal phenotype, true to the untransformed control plants of each parental genotype (**Figure 3**). Plants were maintained under

clean field conditions, with furrow irrigation on a regular schedule. Regular monitoring was conducted for weeds, insects, and non-PD disease. Weeds were managed by cultivation and minimal hand weeding. No significant insect or disease pressure was noted. Plants from all four laboratories were inoculated on July 21(**Figure 3**). The inoculation was by needle prick method with a delivery of ~ 20,000 *Xf* cells per inoculation site. Sampling of a limited number of inoculated canes near the inoculation site on control and VvPR1 and UT456 transgenic plants assayed by PCR confirmed the presence of the respective transgenes (**Figure 3C**) and then determined, by qPCR, to harbor a low level of *Xf* in the sampled inoculated plants. Bacterial titre ranged from undetectable in the uninoculated control plants to $1x10^{2}$ to $7.5x10^{3}$ per 0.1 g of stem tissue in the inoculated plants. Untransformed control plants were negative for the transgenes. As of October 2, 2011, no typical field symptoms or defoliation of leaves was observed on any of the inoculated plants.

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. We have identified grape DNA sequences, which when constitutively expressed in transgenic grapes suppress the death-dependent symptoms of PD and reduce the bacterial titre to a level found in PD resistant wild 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 in full plant transgenics. Initial data suggest that protective sequences may function across a graft union to protect an untransformed and susceptible wild type scion. 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 plan for the coming year is to continue the field evaluation of transgenic grapes expressing PR1 and UT456 and to test for cross graft protection by these two sequences, also under field conditions.

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FUNDING AGENCIES

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Table 1. List of potential plant anti-apoptotic genes derived from functional cDNA screen. Each has now been 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.

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, found in all plant species examined	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 and in the animal kingdom	R=1; 10^3
Vv PR1	Pathogenesis related protein found in all plant species	R=1; 10^3

Figure 1. 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. Photos taken and *Xf* titers (red inset numbers) in 0.1g of stem tissue were measured by qPCR at 4 months after inoculation compare three of the varieties with the control plants. The quantitative comparisons of all the varieties are shown in **Table 3**.

Table 2. Relative susceptibility of winegrape varietals were evaluated by qPCR following mechanical inoculations of greenhouse grown plants. Disease rating is a 1-5 scale with 1 = asymptomatic and 5 = defoliated. *Xf* bacterial titers are expressed as bacterial cells per 0.1gm of stem tissue. Evaluations were done at 4 months post inoculation. See **Figure 1** for representative pictures.

Varietal	Rating (5 is highest)	*Xf* titer
Cabernet Sauvignon	4	5×10^6
Chardonnay	3	5×10^5
Merlot	2	7×10^6
Pinot Gris	5	1×10^7
Pinot Noir	4	1×10^5
Sauvignon Blanc	3	1×10^5
Syrah	1	7×10^4
Zinfandel	3	5×10^5

Figure 2. 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. Summer 2010 results of greenhouse PD 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.

Table 3. Freedom rootstock expressing transgenes grafted to untransformed Thompson Seedless scions and mechanically inoculated with 20,000 *Xf* "Temecula" in a 20ul drop. 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 12 months post inoculation. (See **Figure 3** for a representative image of control and transgenic rootstock/ wild scion plants.)

Transgenic notation	Relevant genotype (transgenic rootstocks grafted to untransformed Thompson seedless scions)	Ratio transgenic graft-protected plants with leaf retention equal to *Vitis californica* vs those plants dead	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	8/8 healthy, none dead Rating 1-2	10^3 - 10^4
TS02A FDPR1-13	CaMV 35S-driven PR1 Freedom rootstock	5/5 healthy, none dead Rating 1-2	10^3 - 10^4
TS02A FD3 (wild type) Control	Untransformed Thompson Seedless scion	10/13 dead (R5), 3/13 barely) alive (R4	10^6 - 10^7
Vitis californica	Asymptomatic wild type untransformed host.	no death (R5) after 12 months post inoculation	10^4

Figure 3. The Gilchrist section of the Solano Field has been completely planted, as have those of all the other investigators. Currently our planting consists of 75 own-rooted and 75 grafted plants including controls (A and B). Checks of genotype identity of several plants were performed by sampling genomic DNA and using specific primers in PCR. DNA was extracted from canes of field plants 11-1, 12-8 and 11-14 and analyzed by PCR using pairs of primers for the 456 transgene, the PR1 transgene and an endogenous grape gene cytochrome c oxidase (COX2) (C). Plants of all investigators were mechanically inoculated with 20,000 cells of *Xf* "Temecula" in a 20ul drop on July 21, 2011 (D and E). Preliminary sampling and analysis by qPCR of several inoculation sites confirmed the presence of *Xf* in each of the inoculation sites tested at 1 month after inoculation.

FIELD EVALUATION OF GRAPE PLANTS EXPRESSING POTENTIAL PROTECTIVE DNA SEQUENCES EFFECTIVE AGAINST PIERCE'S DISEASE

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ABSTRACT
The objective is to evaluate transgenic grape and grape rootstocks expressing various genes from different constructs in a field site in Solano County for resistance to *Xylella fastidiosa (Xf)* (Pierce's disease strain) following mechanical injections of *Xf* into the plant stems. Over the course of the three year field evaluation, test plants in the first planting will include ungrafted conventional Thompson Seedless and Freedom plants as controls, transgenic plants from Dandekar, Labavitch, Lindow and Gilchrist projects. As additional plant material becomes available, transgenic rootstocks expressing some of the test genes grafted to untransformed Pierce's disease susceptible scions. All field operations and the handling of plant material will proceed under an APHIS permit for these materials and over a specified time period.

LAYPERSON SUMMARY
The purpose of the field planting is to evaluate transgenic grape and grape rootstocks under natural field conditions for efficiency in providing protection against Pierce's disease (PD). The site in Solano County will enable controlled inoculation and close monitoring of the host response in terms of symptoms, bacterial behavior, and plant morphology. While no fruit will be produced, assessment of the growth characteristics of the plants, inoculated and non-inoculated will be made. Over the course of the three year field evaluation, test plants in the first planting will include ungrafted conventional Thompson Seedless and Freedom plants as controls, transgenic plants from Dandekar, Labavitch, Lindow and Gilchrist projects and, as plant material from the first planting becomes available, transgenic rootstocks expressing some of the test genes will be grafted to untransformed PD susceptible scions to assess potential for disease suppression in an untransformed scion from signals in the transformed rootstocks.

INTRODUCTION
The objective is to evaluate transgenic grape and grape rootstocks expressing various genes from different constructs in a field site in Solano County for resistance to *Xylella fastidiosa (Xf)* (Pierce's disease strain) following mechanical injections of *Xf* into the plant stems. Over the course of the 3 year field evaluation, test plants will include ungrafted conventional Thompson Seedless and Freedom plants as controls, transgenic plants from Dandekar, Labavitch, Lindow and Gilchrist projects and, as plant material availability permits, transgenic rootstocks expressing some of the test genes grafted to untransformed Pierce's disease (PD)-susceptible scions will be introduced. All plants were moved as vegetative material in 2010 and 2011 to the APHIS-approved field area with no risk of pollen or seed dispersal and stored on-site in lath houses until planted. The area is adjacent to experimental grape plantings that have been infected with PD for the past two decades following mechanical inoculation in a disease nursery near this site. Over this period there has been no evidence of spread of the bacteria to uninfected susceptible grape plantings adjacent to the infected plants. In addition, there are 500 grape plants that were inoculated and infected with PD 6 years ago as part of ongoing disease research by another investigator and funded by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board. The *Xf* in this latter ongoing experiment has not spread to the uninoculated experimental controls within the experiment or to any adjacent experimental grape plants over the past 6 years. The same *Xf* strain will be used to inoculate the plants in this experiment.

OBJECTIVES
Prepare land area and manage the experimental plants with the following specifications:
1. Area will be sufficient to accommodate up to 500 plants from Lindow, Gilchrist, Labavitch, and Dandekar.
2. Row spacing is 15 feet between rows with 4 feet between plants. This spacing requires 18 rows of 28 plants each and includes a 50 foot open space around the planted area. Total area occupied by plants and buffer zones will be a minimum of 1.8 acres.

3. Each row will be staked with 7 foot grape stakes supporting 13 gauge wire in two wire trellis system with a stake at each plant site. Wires will be stretched and anchored by 7 foot pressure treated posts at the end of each row.
4. Area will be fenced to protect against rabbit invasion. The plants will be irrigated by surface furrow with several pre-plant irrigations to bring the soil to field capacity at the time of plant.
5. Field crews will be provided from these funds to assist in planting and weeding.
6. Irrigation and pest management will be provided by Cooperator Tom Kominek.
7. All flowers will be removed and all prunings will be autoclaved before disposal under conditions of the permit
8. Plants will be mechanically inoculated with *Xylella fastidiosa* in 2011 and subsequent years.

RESULTS AND DISCUSSION

All of the above objectives set out for the establishment of this field planting were completed. Land preparation, fencing, irrigation, planting and weed control were all accomplished in a timely manner to meet the initial planting date of July 12, 2010 (**Figure 1**). The second phase of the planting, including grafted transgenics was completed May 17, 2011 (**Figure 2**).

The 2010 plantings of all four investigators survived the winter without loss. The attachment of new shoots to the trellis system, cultivation, and irrigation management progressed in a normal and effective manner. All flowers were removed before opening and extensive pruning was done to manage the plants in a fashion compatible with mechanical inoculation. All flowers and prunings were collected, bagged, and autoclaved before disposal.

As of July 21, 2011, the initial planting and the second 2011 planting individuals are healthy, growing normally and all plants have a normal phenotype, true to the untransformed control plants of each parental genotype (**Figure 3**). Inoculations of the 2010 plants occurred on July 12 and July 21 (**Figures 4, 5 and 6**). The field planting will provide important data on the effectiveness of any of the transgenic strategies employed by the respective researchers.

FUNDING AGENCIES

Funding for this project was provided by CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

Figure 1. This image illustrates the field preparation, trellis and staking arrangement and a portion of the initial planting at the Solano County site.

Figure 2. This shows the Solano County site as of July 13, 2011 with newly planted grafted plants in the front rows and the Fall 2010 planting in the back rows.

Figure 3. Plants are healthy, growing normally and all plants have a normal phenotype.

Figure 4. Inoculation of grape vines with *Xf* at the Solano County site is a two or three person task.

Figure 5. Mechanical inoculation is performed by pushing a needle from the underside of the cane, placing a 20ul drop of *Xf* bacteria, and withdrawing the needle draws the drop in by negative pressure.

Figure 6. Inoculated grape vine canes were marked with orange tags at the Solano County site.

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 from extreme water stress and nutritional problems has forced abandonment of the trials on Orange Muscat and Cabernet Sauvignon. To evaluate *Xylella fastidiosa* strain EB92-1 for the biocontrol of Pierce's disease (PD) in Southern California, a new test site was established at the UC Riverside vineyard in October 2011. Fifty treated and 50 untreated Merlot and Pinot Noir vines were transplanted in mid-October. After three years in Preston Vineyards in Sonoma, there was no PD in the Barbera trial, which has been abandoned, and a very low level of PD in the Viognier trial. There were fewer vines with PD in 2011 than in 2010. In the third season in the Beringer Vineyard in Napa, there still is essentially no disease in either the Chardonnay or Reisling. Only one Chardonnay and two Reisling vines out of a total of 177 vines were considered to have the beginning of PD symptoms. In September 2010, forty mature Chardonnay vines in the Beringer Vineyard were inoculated with strain EB92-1 by boring a small hole into the trunk with an electric drill and injecting 2 ml of bacterial suspension into the hole with a nail-injector syringe. In 2011, none of the mature vines, treated or untreated, had developed any PD symptoms, further illustrating the lack of disease pressure this year. Due to the loss of trials in Temecula and the lack of PD development in trials in Sonoma and Napa, no useful information on the biological control of PD with EB92-1 in California has been obtained after three years of trials. In comparisons of methods of treatment with EB92-1 in four-year-old Merlot vines in Florida, EB92-1 was controlling PD equally well after injection into the rootstock, scion, or rootstock and scion. Control of PD in older, mature vines was evaluated by comparing pin-pricking injections of a drop of EB92-1 into a current year shoot on each of the major arms and injections by drilling a hole in the main trunk and injecting 0.5 – 1.0 ml of EB92-1 with a syringe. Drill and syringe injection of the main trunk in these mature vineyards with chronic PD was more effective than pin-pricking in reducing new cases of PD in American hybrid grapevines.

LAYPERSON SUMMARY
Trial plantings of Orange Muscat and Cabernet Sauvignon were established in Bella Vista Vineyard in Temecula for the biocontrol of Pierce's disease (PD) with a benign strain of *Xylella*. Many of the vines were stunted or have died from something other than PD, probably water stress. These trials in Temecula had to be abandoned. In 2011, the lost Temecula trials are being replaced with trials on Merlot and Pinot Noir in the UC Riverside Vineyard. The vines were transplanted in October. In Preston Vineyards in Sonoma, a trial on Barbera was abandoned because there was no PD. There is a low level of PD in the remaining Viognier trial. In Beringer Vineyards in Napa, there is a very low level of PD in the Chardonnay and Reisling trials. A trial to evaluate the effectiveness of the biocontrol strain in protecting mature, producing grapevines against infection with PD was established in Beringer Vineyard in 2010. Mature Chardonnay vines were inoculated with biocontrol strain EB92-1 by boring a small hole into the trunk with an electric drill and injecting 2 ml of bacterial suspension into the hole using a nail-injector syringe. After one season, none of the mature vines, treated or untreated , had developed any PD symptoms, further illustrating the lack of disease pressure this year. Due to the lack of PD or other cultural problems, none of the tests have produced sufficient data to tell us whether or not the biocontrol strain is effective in California. Tests are also underway in Florida to determine the most efficient and effective way to apply the biocontrol strain. The biocontrol seems equally effective when applied to the rootstock or the scion. In attempts to stop the development of PD in mature vineyards, drilling the main trunk and injecting the biocontrol strain with a syringe was more effective than pin-pricking injections of current season growth.

INTRODUCTION
Pierce's disease (PD) of grapevine is an endemic, chronic problem in the southeastern USA where it is the primary factor limiting the development of a grape industry based on the high-quality European grapes (*Vitis vinifera* L.) (Hopkins and Purcell, 2002). PD is also endemic in California and has become more of a threat to the California grape industry with the introduction of the glassy-winged sharpshooter. While vector control has been effective for PD control in some situations,

the only long-term, feasible control for PD has been resistance. Almost 20 years of research on the biological control of Pierce's disease of grapevine by cross protection with weakly virulent strains of *Xylella fastidiosa (Xf)* has demonstrated that this is a potential means of controlling this disease (Hopkins, 2005). One strain of *Xf* that was able to control PD in *V. vinifera* for 14 years in Central Florida has been identified. We are testing this strain in commercial vineyards in several states and, if these tests are successful, the strain will be ready for commercial use. In most trials with the biocontrol strain, the bacteria were injected into the grapevines either in the greenhouse or in the vineyard after transplanting. This is a labor-intensive procedure. Treatment methods that would make the technology less labor-intensive, less costly, and more consistent are being evaluated. The overall goal of this project is to develop a biological control system for PD of grapevine that would control the disease in California and other areas where PD and the glassy-winged sharpshooter (GWSS) are endemic.

OBJECTIVES

1. To evaluate strain EB92-1 of *Xf* for the biological control of Pierce's disease 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 Pierce's disease 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 *Xylella* 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

Southern California tests. 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, 2008.

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, with no significant differences in the incidence of PD between the treated and untreated vines. Symptoms did appear to be more severe in the untreated Cabernet Sauvignon vines than in the EB92-1 treated vines. The Orange Muscat planting was interspersed with mature vines that were nearly 100% infected with PD.

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 visible 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 2 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 3 seasons and more than two years. Therefore, the trials were abandoned.

To replace the lost tests in southern California, a replacement test is being established in 2011 at UC Riverside. For transplanting into the UC Riverside vineyard, 100 Merlot/1103 plants and 100 Pinot Noir/1103 plants were obtained from Sunridge Nursery in March 2011 and maintained in UC Davis greenhouse. Fifty Merlot and 50 Pinot Noir were inoculated with EB92-1 in July 2011 and fifty plants of each cultivar were kept as untreated controls. These plants were maintained in the greenhouse for six weeks and then moved outside to harden them off. These plants were transported to Riverside in mid-October and transplanted into the plots at UCR. This planting time will reduce heat stress on the transplants and, hopefully, will give them the fall season to establish a strong root system. This should result in vigorous plants in the spring of 2012 for inoculation with the PD strain of *Xf* by resident GWSS throughout the season.

Sonoma tests. 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 and this test was abandoned 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. Minor PD symptoms began to develop in a very few vines in the Viognier test in 2010. However, there were fewer vines with PD symptoms in 2011 than in 2010. There were very few new symptomatic

mature Viognier vines in the test area. After three years, the PD incidence in the test vines is very low (**Table 1**). Only two vines in the entire test had moderately severe symptoms and should normally be removed. Symptoms in the other symptomatic vines were very minor and these vines could recover.

Table 1. Biocontrol of PD in 2-year-old grapevines in Northern California vineyards on 9/6/2011.[1]

Cultivar	**Untreated vines**	**EB92-1 treated vines**
Preston Vineyard, Sonoma		
Voignier	6/48 (12%)	5/48 (10%)
Beringer Vineyard, Napa		
Chardonnay	0/42 (0%)	1/44 (2%)
Reisling	2/47 (4%)	0/44 (0%)
Total	8/137 (6%)	6/136 (4%)

[1]Disease incidence is given as number of PD symptomatic vines over total vines in treatment.

Napa tests. 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. In the third season, there still is essentially no disease in either the Chardonnay or Reisling (**Table 1**). Only one Chardonnay vine and two Reisling vines were considered to have the beginning of PD symptoms, but these were still questionable.

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.

In 2011, none of the mature vines, treated or untreated, had developed any PD symptoms, further illustrating the lack of disease pressure this year.

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 three replications of three plants per treatment.

In 2009, PD began to occur in a few of the Merlot vines. Symptoms have continued to progress in the untreated, with 43% of the vines having some symptoms (mostly minor) in 2011 (**Table 2**). All three treatments with EB92-1 were reducing symptoms. There were very few new PD infections in 2011 when compared with 2010.

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

	Merlot/101-14	
Treatment	**Aug 2010**	**June 2011**
Scion injection	13	22
Rootstock injection	11	13
Scion & Rootstock injection	14	17
Untreated	38	43

While strain EB92-1 has been shown to be effective in preventing PD in new grape plantings, there are mature vineyards that are rapidly being destroyed by PD. To evaluate control of PD in older vines, mature vines were treated either by pin-pricking injections of a drop of EB92-1 into a current year shoot on each of the major arms (branches) or by drilling a hole in the main trunk and injecting 0.5 – 1.0 ml of EB92-1 with a syringe. Drill and syringe injection of the main trunk in these mature

vineyards with chronic PD was more effective than pin-pricking in reducing new cases of PD in American hybrid grapevines during the first year after treatment (**Figure 1**).

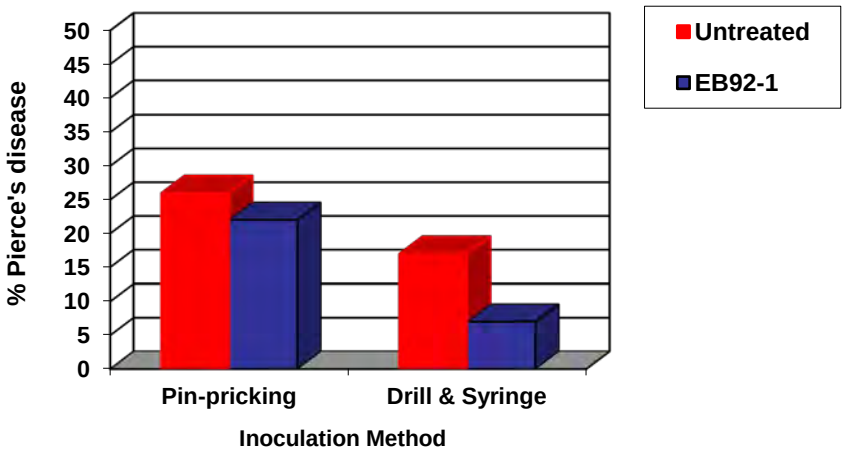

Figure 1. Effect of strain EB92-1 on PD in mature vines treated by pin-pricking compared with drill and syringe in 2009 trials**.**

CONCLUSIONS

In four-year-old Merlot vines in Florida, a trial was established to determine the most efficient and effective way to apply the biocontrol strain. EB92-1 was controlling PD equally well after injection into the rootstock, scion, or rootstock and scion. In attempts to stop the development of PD in mature vineyards, drilling the main trunk and injecting the biocontrol with a syringe was more effective than pin-pricking current season growth. Due to the loss of trials in Temecula and the lack of PD development in trials in Sonoma and Napa, no definitive information on the biological control of PD with EB92-1 in California has been obtained after three years of trials. To replace the lost tests in southern California, a test is being established in 2011 at UC Riverside. With the PD pressure in southern California, this test will yield conclusive results over the next 2-3 years. Hopefully, the Sonoma and Napa tests will develop enough PD to give us an evaluation in those areas over the same time period. If the control is successful in the current trials, along with success in other states, this project could yield results within the next 2-3 years that would provide a commercial biological control for PD for vineyards in California.

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FUNDING AGENCIES

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

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Reporting Period: The results reported here are from work conducted January 2011 to October 2011.

ABSTRACT
This work builds on discoveries made in the past seven 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 and we cloned and expressed TLP in *E.coli*. We found that TLP protein possesses higher levels of *Xylella fastidiosa (Xf)* toxicity *in vitro* compared to *E. coli* protein extracts from cells containing the cloning vector but no TLP gene. Greater amounts of total phenolics were measured in xylem sap extracted from cold-exposed vines. In collaboration with the Waterhouse lab we are characterizing these phenolic compounds and assessing their potential *anti-Xf* activity *in vitro*. One phenolic compound, trans-resveratrol, which only occurred in Pinot noir grapevines exposed to cold temperatures, and was shown by two different labs to be toxic to *Xf* cells grown *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. In fall 2010 we treated Riesling vines growing in a vineyard in Napa that had light to moderate PD symptoms vines with a foliar spray or a soil drench treatment of ABA. The severity of PD symptoms in the treated vines will be compared with the severity of symptoms in non-treated PD-affected vines in fall, 2010.

LAYPERSON SUMMARY
We have succeeded in producing recombinant thaumatiin-like grape protein (TLP) in *E.* coli. W*e* have also observed that grapevine TLP produced by *E. coli* has a deleterious effect on *Xylella fastidiosa (Xf)* when it is grown *in vitro* in the laboratory. This supports our rationale for over- expressing TLP in grapevines as a potentially promising approach to decreasing the size of *Xf* populations in Pierce's disease (PD)-affected grapevines. We have been characterizing the phenolic compounds in cold xylem sap and comparing these to warm xylem sap. We have noticed a number of differences, specifically the presence of the phenolic compound trans-resveratrol in cold sap and its absence in warm sap in some grape varieties. This suggests that trans-resveratrol may play a role in the cold curing process. In previously described research we added trans-resveratrol to solid media used to grow *Xf* and observed that the Temecula strain is inhibited at trans-resveratrol concentrations lower than those concentrations that inhibit the Fetzer strain. This further suggests that phenolic compounds play a role in the cold curing process. Ongoing field trials are examining if root or foliar applications of the plant hormone ABA could stimulate the synthesis of phenolic compounds in field grown vines infected with *Xf* and possibly decrease the severity of PD symptoms in the field vines.

INTRODUCTION
Previous research conducted in the Purcell laboratory at UC Berkeley definitively demonstrated that *Vitis vinifera* grapevines that were infected with *Xylella fastidiosa (Xf)* the bacterial pathogen that causes Pierce's disease (PD) could often be cured of the infection if exposed to freezing temperatures for some period of time. This "cold curing" phenomenon likely explains why PD is restricted to areas that have mild winter temperatures. Research conducted in our laboratory by Dr. Melody Meyer confirmed and expanded the work performed by Purcell, et. al.. She found that grapevines exposed to cold temperatures had elevated levels of a thaumatin-like protein (TLP) that has been shown to have antimicrobial properties in other plant host/pathogen interactions. We cloned and expressed the grapevine TLP in *E. coli* and showed that incubation of the cloned TLP with cultured *Xf* cells considerably decreased the viability of the *Xf* cells compared to incubating the *Xf* cells with other appropriate controls. We are now cloning the TLP gene in an Agrobacterium binary vector with the intention of over-expressing the TLP in transgenic grapevines. Once TLP-transgenic grapevines are obtained and characterized they will be inoculated with *Xf* using mechanical and insect inoculation. The vines will then be rated for symptom development and compared to non-transgenic *Xf*-inoculated vines.

Dr. Meyer's research also showed elevated levels of polyphenolic compounds in xylem sap extracted from cold exposed grapevines. In collaboration with the Waterhouse lab we have been characterizing the phenolic compounds in the xylem fluid. One phenolic compound, trans-resveratrol, only occurred in Pinot noir exposed to cold temperatures, and our lab, as well as another, showed that resveratrol was toxic to *Xf* cells *in vitro*. Dr. Meyer's research also showed elevated levels of the plant hormone abscisic acid (ABA) in xylem sap of cold exposed grapevines. She showed that exogenous application of ABA greatly increased the PD curing rates of potted grapevines exposed to the comparatively mild winter temperatures in Davis. In 2010 we applied ABA in the fall to PD-infected vines growing in a vineyard in Napa. The severity of PD symptoms in the ABA-treated vines will be compared to non-treated controls in October, 2011.

OBJECTIVES

1. Over express the grapevine TLP in transgenic grapevines. Prepare anti-TLP antibodies to quantify TLP in transgenic xylem sap using ELISA.
2. Inoculate TLP-expressing grapevines with *Xf* and determine the incidence and severity of PD in transgenic versus non-transgenic *V. vinifera*.
3. a. Fractionate and chemically characterize the phenolic compounds that are present in xylem sap from cold-exposed grapevines.
   b. Compare the phenolic content of xylem sap of grapevines treated with ABA under non-freezing conditions to phenolics in cold-exposed xylem sap.
   c. Determine if these compounds affect *Xf* growth/survival *in vitro.*
4. Determine if foliar or drench applications of ABA can increase PD-curing rates in field-grown vines under non-freezing conditions.

RESULTS AND DISCUSSION

We successfully cloned and expressed grapevine TLP in *E. coli* (**Figures 1 and 2**). We sequenced the grape TLP gene and are currently inserting it into an *Agrobacterium* binary vector system for over-expressing TLP in transgenic grapevines. Our initial plan was to use the same vector we used to generate our hemagglutinin transgenic lines, however our results using these plasmids, which use two 35S promoters to express the HA gene and uses hygromycin resistance, produced a number of lines in which crossing over by the two promoters ended up deleting all or part of the HA gene construct while maintaining the antibiotic selection. To avoid these deletion events we have decided to change the binary system to a neomycin (kanamycin) resistant plasmid driven by the nptII promoter to eliminate the chance of promoter crossing over/deletion events. We used the pUNCB5omega plasmid for initial cloning of the grape TLP gene in *E. coli*. We are now moving the TLP construct into the low copy pCB4NN plasmid. This plasmid provides the neomycin resistance and is in low copy in case there are any toxicity issues resulting from expression of TLP in Agrobacterium. We plan on submitting the appropriate TLP transformation constructs to the UC Davis plant transformation facility at the end of October, 2011. Once we get transformed plants back from the plant transformation facility we will be able to proceed with the *Xf* pathogenicity portion of this project.

We are also in the process of making a larger quantity of purified, recombinant TLP in *E. coli* which we will give to the Comparative Pathology Laboratory at UC Davis for polyclonal antibody production. The TLP-specific antibodies will be used for quantifying TLP in the transgenic grapevines.

We performed a time course experiment in which *Xf* was combined with dialyzed TLP, and then plated onto PD3 media at intervals over two days. As controls we also performed the same incubation procedure with *Xf* and water, *Xf* with empty vector supernatant (*E. coli* lysate that was not transformed with TLP), and *Xf* with a potassium buffer. We plated these suspensions directly after combining (0 hours), 16 hours, 24 hours, 40 hours and 48 hours of incubation. The plates were then incubated at 28C for 10-14 days. Our results showed that the early time course platings had no differences in *Xf* growth. However, after 48 hours post combination, the *Xf* and water control as well as the *Xf* and potassium buffer control still grew *Xf* colonies. The *Xf* combined with the empty vector supernatant showed growth, but less than the water and potassium buffer. The *Xf* that had been combined with dialyzed TLP did not show any growth.

We have analyzed sap samples collected from Placerville, CA (during the months of January and February) where cold curing occurs, as well as sap from Winters, CA where cold curing does not take place. In collaboration with the Waterhouse lab at UC Davis, we are determining accurate polyphenolic profiles for Cabernet Sauvignon clone 8 on 110R rootstock and Pinot Noir clone 2A on 101-14 rootstock. 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 polyphenolic 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 trans-resveratrol is present in vines that experience "cold curing" while it is absent in vines that do not undergo "cold curing" suggests that resveratrol may play a role in the curing process. We will analyze the MS/HPLC data after two winters of collecting sap, and should be able to provide an accurate picture of what happens with regards to polyphenolic concentrations during the winter.

We also added purified trans-resveratrol to solid media used to grow *Xf* and observed that the Temecula strain was inhibited at concentrations significantly lower than concentrations of trans-resveratrol which inhibited growth of the *Xf* Fetzer strain. While the reason for this differential sensitivity is not known, it is interesting to note that the Temecula strain was isolated in a location with comparatively mild winters while Fetzer was isolated from PD-affected vines growing in N. California. It could be possible that the Fetzer strains evolved mechanisms to detoxify low levels of phenolic compounds that were synthesized in vine exposed to the colder winter temperatures of N. California, while the Temecula strain was not subjected to elevated levels of phenolics growing in S. California.

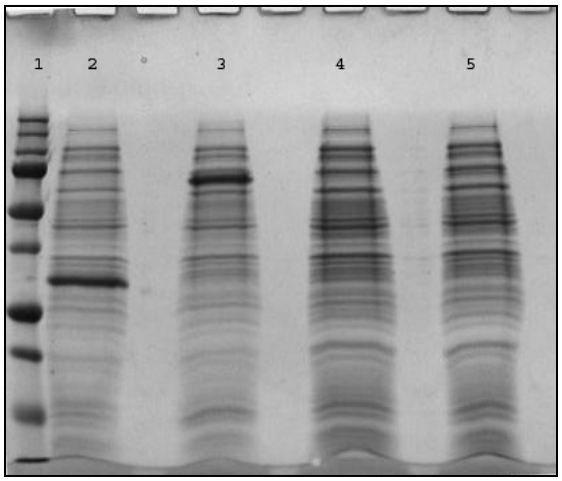

Figure 1. Recombinant *Vitis vinifera* 'Cabernet Sauvignon' TLP protein, expressed in *E. coli* and analyzed by SDS-PAGE. Lane 1: Dual Color SDS-ladder (lower band-25 kD; upper band-75 kD); Lane 2: Arrow denotes induced recombinant Cabernet Sauvignon TLP; Lane 3: Induced recombinant polygalacturonase (PG) (positive control); Lane 4: non-induced recombinant Cabernet Sauvignon TLP; Lane 5: non-induced recombinant polygalacturonase (PG).

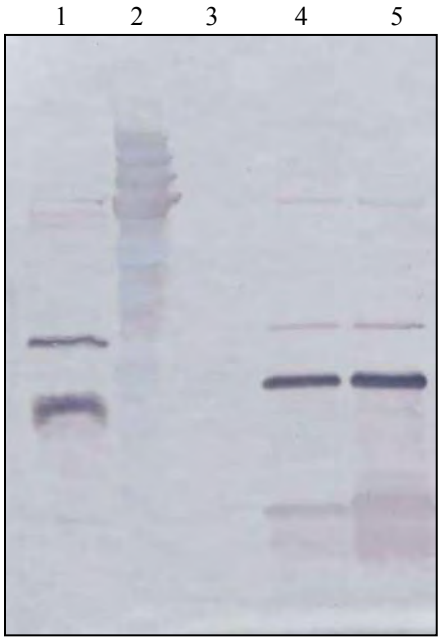

Figure 2. Western blot of SDS-PAGE of recombinant *Vitis vinifera* 'Cabernet Sauvignon' TLP protein expression using anti-His tagged antibody. Lane 1: CS3 raw lysate pellet (positive control); Lane 2: SDS Dual color ladder; Lane 3: Non-induced CS3 (negative control); Lane 4: CS3 dialysis purified pellet; Lane 5: CS3 dialysis purified supernatant.

Our previous research showed that ABA applications to greenhouse grown Cabernet Sauvignon and Pinot Noir grapevines infected with *Xf* increased overwinter curing rates in Davis, CA., a location which has relatively warm winter temperatures that induces only low rates of PD cold curing. Our previous work also showed that ABA-treated vines had higher levels of polyphenolics than vines growing in Davis which did not receive ABA applications. In October of 2010 we applied foliar sprays and root drenches of ABA to *Xf*-infected Riesling vines growing in a Napa vineyard. We will rate the severity of PD symptoms in October 2011 and compare the severity of treated versus non-treated vines.

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OPTIMIZING GRAPE ROOTSTOCK PRODUCTION AND EXPORT OF INHIBITORS OF *XYLELLA FASTIDIOSA* POLYGALACTURONASE ACTIVITY

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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 from grafted rootstocks of PD control candidates, including polygalacturonase-inhibiting proteins (PGIPs). PGIPs are plant proteins that inhibit pathogen and pest polygalacturonases (PGs). In this project, multiple PGIPs were evaluated for the efficiency of their inhibition of *Xylella fastidiosa* (*Xf*) PG. Fourteen candidate PGIPs have been chosen and predicted protein structure models were developed to identify interactions with and potential inhibition of *Xf*PG. PGIPs from pear, rice, and orange were determined to be the most likely PGIPs to effectively inhibit *Xf*PG. Recombinant protein expression systems have been developed for *Xf*PG and candidate PGIPs in grape and tobacco plants. Initial inhibition assays have shown that the pear fruit PGIP is a more effective inhibitor of *Xf*PG than the tomato PGIP, however both grape and pear PGIPs limit *Xf*PG symptom development in tobacco leaf infiltration assays. Evaluation of additional PGIPs is underway.

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. Proteins called PG-inhibiting proteins (PGIPs) are produced by many plants and these PGIPs selectively inhibit PGs from bacteria, fungi, and insects. The PGIP expressed in pear fruit is known to inhibit *Xf*PG and limit PD development in inoculated grapevines which have been engineered to express the PGIP protein normally present in pear fruit. PGIP proteinss are secreted from cells and they are can travel across graft junctions. We are interested in identifying the PGIPs that best inhibit *Xf*PG and ascertaining how well, when this PGIP is expressed in transgenic rootstocks, it prevents PD development in grafted wild-type scions inoculated with *Xf*. We have modeled the protein structures of fourteen candidate PGIPs to predict how each of them physically interacts with *Xf*PG. We will combine this knowledge with *in vitro* and *in planta* assay results measuring the ability of the candidate PGIPs to inhibit *Xf*PG in grapevines. For these inhibition assays we are developing 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 in vineyard settings.

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 that separat 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 (*Xf*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 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 by degrading the pit membranes (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 (*Botrytis 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 the grafted wild-type scions. pPGIP also has been shown 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 compare potential *Xf*PG inhibiting properties of PGIPs from a wide variety of plants in order to identify specific PGIPs that optimally inhibit the virulence factor, *Xf*PG. The goal is to express these PGIPs in grape rootstocks to provide PD protection in grafted scions. The expression of PGIPs in grape rootstocks 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 for commercialization of a PD control strategy using PGIPs, focusing on IP and regulatory issues associated with the use of PGIPs in grape rootstocks.
   a. Evaluate IP and licensing status of the plant expression construct components for the PGIP-based rootstock strategy (Year 1)
   b. Assemble grape transformation vectors utilizing PIPRA vectors with defined IP characteristics (Year 2)
2. Identify plant PGIPs that maximally inhibit *Xf* PG.
   a. Use existing pear PGIP-expressing grapes, test PD susceptibility of normal scions grafted to PGIP-expressing and -exporting roots (Years 1 and 2)
   b. Identify plant PGIPs that are efficient inhibitors of *Xf*PG (Year 1)
   c. Express PGIPs in Arabidopsis thaliana and test for optimal inhibition of *Xf* PG (Years 1 and 2)
   d. Optimally express *Xf* PG, using recombinant protein expression systems (Year 1)
   e. Model PGIP and *Xf* PG interactions to identify optimal PGIPs for PD defense (Years 1 and 2)
3. Assemble transcription regulatory elements, *Xf*-inducible promoters and signal sequences that maximize PGIP expression in and transport from roots.
   a. Make transformed grape lines using the best PGIP candidates, promoters etc. (Years 2 and 3)
4. Create PGIP-expressing rootstocks and evaluate their PD resistance.
   a. Molecular analysis of putative marker free transgenic grape plants (Year 3)
   b. Evaluate transgenic grape lines for optimal expression and export to scions of selected PGIPs (Year 3)
   c. Evaluate transgenic lines for susceptibility to *Xf* (Year 3)

RESULTS AND DISCUSSION

Objective 1. A path to commercialization of transgenic rootstocks

a. 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 test PGIP-based PD control strategies in vineyards in Solano and Riverside Counties. '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).

b. Grape transformation vector assembly is undergoing re-evaluation.

Objective 2. Identify plant PGIPs that maximally inhibit Xf PG

a. Propagation, grafting and susceptibility testing 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 UC Davis Core Greenhouse Complex. More individual plants of each cultivar expressing pPGIP and control plants not expressing pPGIP have been rooted with the help of an aeroponic cloner (EZ-Clone, Inc., Sacramento, CA). Details of the grafting procedure are described in the report, "Field evaluation of grafted grape lines expressing PGIPs" (PI Powell).

Collaborator, Victor Haroldsen, has shown that pPGIP protein is found across graft junctions of grapes and tomato plants. That is, it moves from transgenic rootstocks into wild-type tomato scion leaf tissue (**Figure 1**). For these experiments, he used existing stocks of polyclonal pPGIP antibodies after concentrating leaf extract samples 30-fold. Once the monoclonal antibody is available (see report, "Tools to identify PGIPs transmitted across grapevine grafts, PI Powell), its increased specificity will allow for quantification of the amount of pPGIP protein crossing the graft junction into wild-type tissues.

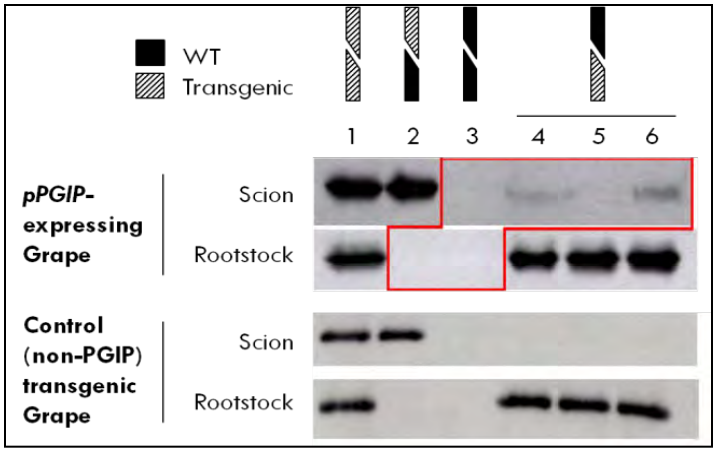

Figure 1. Western blot of leaf extracts taken from rootstock and scion portions of grafted 'Thompson Seedless' grapevines. Transgenic vines are expressing either pPGIP or NPTII (control). pPGIP is visualized crossing from transgenic rootstocks into wild-type (WT) scion tissue (lanes 4-6). This movement is not seen in the reciprocal graft (lane 2).

Testing of the susceptibility of the scion portions of plants to PD has begun using the plants in the field (details in the report "Field evaluation of grafted grape lines expressing PGIPs," PI Powell). Insufficient numbers of scions grafted with pPGIP expressing rootstocks are currently in the field for testing susceptibility, but additional grafted plants are in progress.

b. Selection of PGIPs as PD defense candidates and PGIP-*Xf*PG modeling

Fourteen candidate PGIPs were initially 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 (PGA) substrate for PG, and each of the candidate PGIPs provided predictive tools to interpret the inhibition mechanisms and physical interactions between *Xf*PG and the PGIPs (Labavitch, 2009). Dynamic *in silico* reaction simulations predicted that two clusters of amino acids, #63-74 and #223-226, must be unblocked for *Xf*PG to cleave PGA. The long columns of electronegative residues on the concave faces of the PGIP's leucine rich repeat structure bind to these critical regions (**Figure 2**). This information coupled with surface chemistry mapping predicts that pPGIP, CsiPGIP (citrus), and OsPGIP1 (rice) will be the best inhibitors of *Xf*PG.

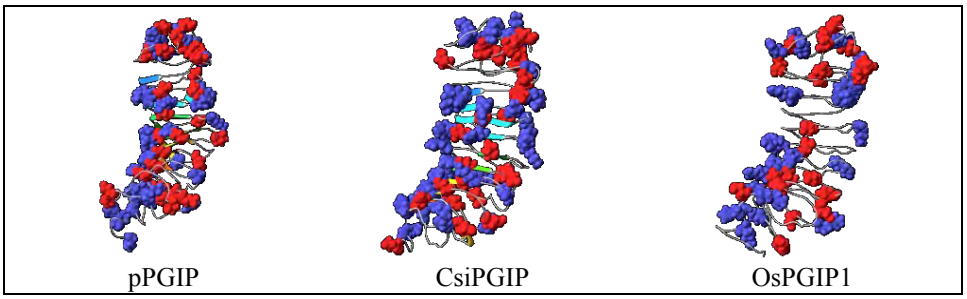

Figure 2. Homology models of three PGIPs predicted to be good candidates to inhibit *Xf*PG. The column of electronegative residues (red) on the concave faces of each protein may align with critical residues on *Xf*PG important for inhibition.

A closer look at the dynamic reaction simulations highlighted other 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 3**). Electrostatic repulsions between VvPGIP (grape PGIP) 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 evaluate the predicted interactions and infer other potentially useful interactions between the candidate PGIPs and other PGs.

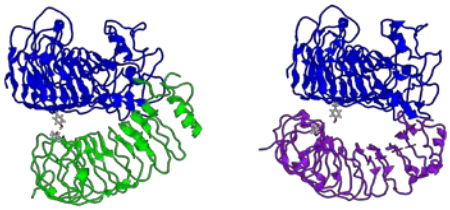

Figure 3. *Xf*PG-PGIP complexes. Tyr303 of *Xf*PG (blue) binds strongly with a region of pPGIP (green) which is not possible with VvPGIP (purple). Interactions such as this might influence PG-PGIP interaction and inhibition.

We are hoping to add unpublished PGIP sequences from non-vinifera *Vitis* varieties to model 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 determine how the models of these non-vinifera PGIPs compare to the modeled structure of VvPGIP from *Vitis vinifera* cv. 'Pinotage.'

Based on these modeling studies the two PGIPs (from rice and citrus) have been selected for further study of their inhibition of the PG produced by *Xf*. PCR primers for the amplification and cloning of the PGIP sequences from citrus and rice have been designed (**Table 1**) and are being tested. Genomic DNA has been prepared from rice and citrus and PCR reactions using these and other primers has begun.

Table 1.

Primer sequence*	Primer name	Gene amplified; Modifications
TCACagatcttccatggATGAGtAACACGTCA	CsiPGIP_F3	CsiPGIP; BglII and NcoI sites, alternate frame nonsense mutation
TTCAAAccATGgGCAACACGTCACTG	CsiPGIP_Falt2	CsiPGIP; NcoI site, S2G missense mutation
CCAGgctagcgcgaccctcaatTCTTTC	CsiPGIP_R3	CsiPGIP; NheI site, Xa site, removal of TGA
ccatggtATGCGCGCCATGGTaGTC	OsPGIP1_F	OsPGIP1; NcoI site, alternate frame nonsense mutation
ccATGgGCGCCATGGTCGT	OsPGIP1_Falt	OsPGIP1; NcoI site, R2G missense mutation
gctagcgcgaccctcaatATTGCAG	OsPGIP1_R	OsPGIP1; NheI site, Xa site, removal of TAA
cgagatctccATGGATGTGAAGCTCCTG	OsPGIP2_F2	OsPGIP2; BglII and NcoI sites
gctagcgcgaccctcaatTCGACGAC	OsPGIP2_R3	OsPGIP2; NheI site, Xa site, removal of TAA

*Uppercase bases are homologous to the reference sequence; lowercase bases are introduced changes.

c. *Xf*PG expression and purification

Two strategies have been developed to express active *Xf*PG to use to evaluate the PGIPs. First, an *Xf*PG expression system utilizing Drosophila S2 cells was developed to provide active, stable *Xf*PG protein for *in vitro* inhibition assays. The cloning strategy fused the coding sequence of *Xf*PG to a C-terminal polyhistidine tag for purification and an N-terminal targeting sequence for extracellular secretion of the protein (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 4**; Taylor and Secor, 1988). *Xf*PG was partially purified from the medium and pelleted Drosophila cell lysate and analyzed by Western blotting and Coomassie staining SDS-PAGE. Putative *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 5**). The protein bands in the cell medium preparation eluant were visualized at 68 kDa by Coomassie staining (**Figure 6**). Each of these preparations showed very slight PG activity, as measured by reducing sugar analysis (Gross, 1982). These activities, however, diminished over time.

The second strategy was to express *Xf*PG transiently in leaves. We have successfully cloned the *Xf*PG into pCAMBIA1301 and introduced this construct into *A. tumefaciens* for transient expression in tobacco leaves. The design of the vector is shown in **Figure 7**. To insure the extracellular localization of the *Xf*PG protein, the protein coding sequence was modified so that the pPGIP extracellular targeting sequence was linked to the 5' end of the *Xf*PG coding sequence. PGIPs are naturally targeted to the apoplast probably as a result of this targeting sequence. We anticipated that the fusion construct pPGIP::*Xf*PG would yield more obvious infiltration results than the unmodified native *Xf*PG construct because the pPGIP signal sequence has been shown to target proteins to the extracellular space. Thus, by targeting the pPGIP::*Xf*PG protein to the cell apoplastic space, it can degrade the pectin-rich middle lamellae and cell walls to simulate the situation in infections and also be inhibited by any co-infiltrated PGIP in or PGIP efficacy tests. It

has been reported that the infiltration assay will work on grape leaves, so the constructs will also be tested in grape leaves. However, the final pPGIP::*Xf*PG construct contained a single base change due to a PCR error that resulted in no active protein.

The advantage of the leaf infiltration assay is that it should be quicker than testing Arabidopsis lines expressing *Xf*PG and PGIPs so we have focused more on generating the material for testing in tobacco.

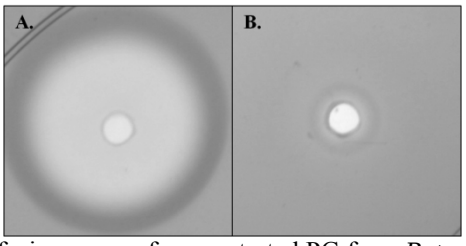

Figure 4. Radial diffusion assay of concentrated PG from *Botrytis cinerea* (A) or culture media from induced *Xf*PG-expressing Drosophila cells (B). The clearing zone diameter is related to amount of PG activity.

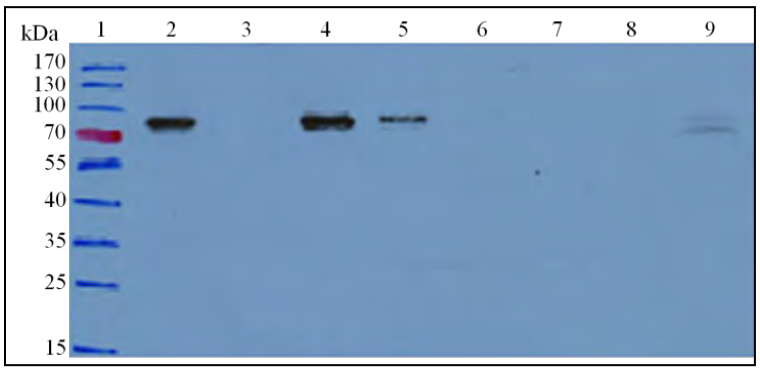

Figure 5. Western blot analysis of partially purified cell lysate after *Xf*PG protein expression. 15 mL crude *Xf*PG lysate was purified by column chromatography and selected fractions were analyzed by Western blotting. Lane 1 = pre-stained ladder, lane 2 = flow-through #4, lane 3 = wash #10, lanes 4-7 = elution fractions #1-4, lane 8 and 9 = cellular medium. Recombinant *Xf*PG protein was eluted with 250 mM imidazole and probed with the anti-V5 primary antibody and anti-mouse HRP secondary antibody.

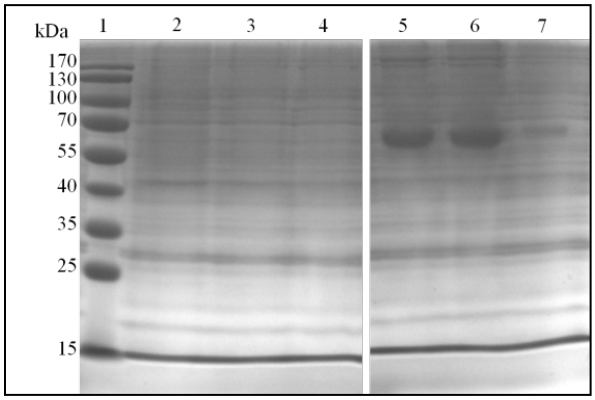

Figure 6. Partially purified *Xf*PG protein eluted with 250 mM imidazole. Coomassie stained polyacrylamide gel electrophoresis. Lane 1 = pre-stained ladder, lanes 2-4 = cell lysate fractions #1-3, lanes 5-7 = cellular medium fractions #1-3.

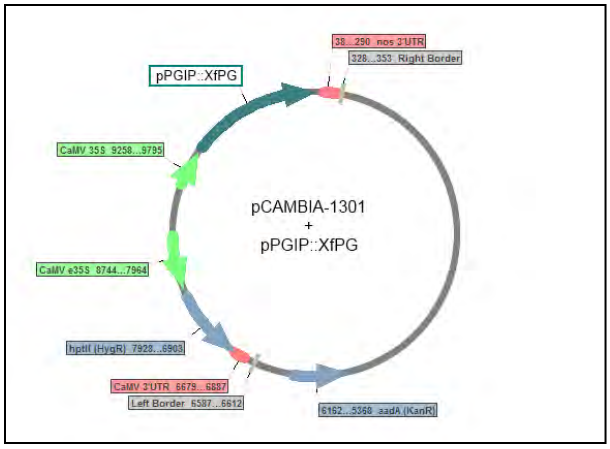

Figure 7. Transient *Xf*PG expression vector for agroinfiltration in tobacco leaves.

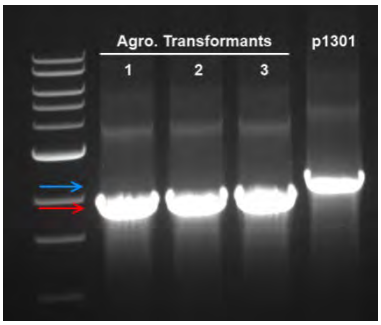

Figure 8. The DNA gel image shows the PCR products from *Agrobacterium* plasmid DNA. Three colonies containing the *Xf*PG:pPGIP sequence (1-3) were screened with p1301 primers flanking the insert site. The expected size fragment is 1924 bp (red arrow). The last lane used "empty" p1301 (gusA intact) as a control template and the same p1301 primers resulted in the gusA product with the expected size of 2204 bp (blue arrow).

d. Expression of PGIPs in *Arabidopsis* and tobacco for *Xf*PG inhibition 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 2**; Labavitch, 2009).

The *Xf*PG expression construct (**Figure 7**) provides a potential diagnostic tool to test the efficacy of each PGIP *in planta* using a tobacco leaf infiltration system. It has been reported that the infiltration assay will work on grape and tomato leaves and as this approach provides advantages in terms of time and cost, we will continue to develop and use this technique for testing the inhibition of PGs by different test PGIPs. 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 9**). Infiltration with cultures expressing *Xf*PG resulted in marked wilting, localized water soaking, and chlorotic lesions developing in the infiltration zone. Leaves co-infiltrated with *Xf*PG and PGIP expressing cultures displayed attenuated symptoms while leaves infiltrated with just PGIP or empty vector cultures showed no symptoms. LePGIP (tomato PGIP) 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 due to the targeted delivery of the *Xf*PG to the apoplast.

Table 2. 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	-	-	-	-
*Xf*PG (*Xylella*)	✓	✓	✓	✓	✓
pPGIP::*Xf*PG	✓	✓	✓	O	-

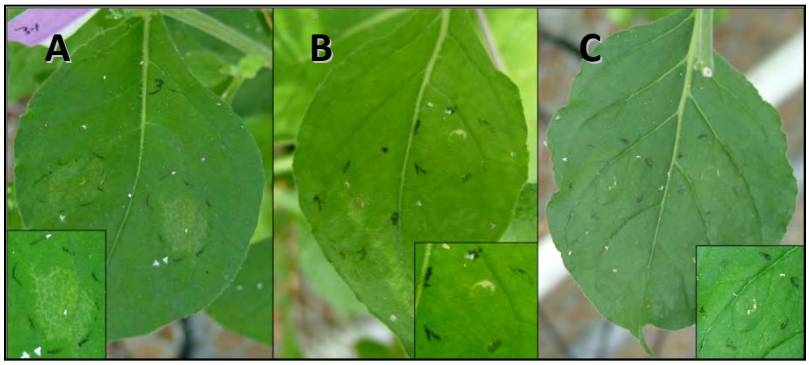

Figure 9. Transient expression of *Xf*PG, pPGIP, and LePGIP in *N. benthamiana* leaves by infiltration with *Agrobacterium* cultures. Chlorotic lesions and water soaking mark the site of agro-infiltrations with *Xf*PG (A). Symptoms are reduced when *Xf*PG is co-infiltrated with pPGIP (B) or LePGIP expressing *Agrobacterium* (C). Inserts show details of infiltration sites. Black marks indicate the borders of the initial zone infiltrated.

e. Modeling of PGIP:*Xf*PG interactions is covered under B above.

Objective 3. Maximize PGIP expression in and transport from roots

The transformation vector to be used in grape transformation has been reevaluated for its effectiveness. 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 as the optimal PGIP has not been evaluated *in planta.*

CONCLUSIONS

The comparisons of multiple PGIPs are key steps 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. A more efficient assay, a co-infilitration assay on tobacco leaves, has been developed to assess PGIP inhibition of *Xf*PG. Grape leaves will be tested for their suitability for this assay. *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*. The ability of one of the candidate PGIPs discussed here, pPGIP, to provide PD resistance to wild-type scions is currently being determined by the field trials.

The overall goal of the project is to develop transgenic grape rootstock lines that express PGIPs that effectively reduce the virulence of *Xf*, an approach that should help to solve the PD/GWSS problem. 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. 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.

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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 diffusitble 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 hence 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 apparently substantially increases 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 and is being investigated. We are currently determining the relative activity of these forms of DSF and if such molecules cooperate in regulating gene expression in *Xf*. The various forms of DSF may preferentially affect different behaviors of *Xf*. Since some reduction in disease severity was observed in grape scions grafted to DSF-producing rootstocks suggests that DSF produced by rootstocks can somewhat move to scions and confer disease control the control of disease, grafted plants are being made that have a relatively large rootstock to test the hypothesis that increased supply of DSF to the scion will be associated with a larger rootstock. 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 adhesin XadA and EPS have been developed. Gene expression in *Xf* exposed to various levels of DSF can also be directly assessed using *phoA* reporter gene fusions. Xanthomons campestris-based biosensors in which Rpf components have been replaced by those form *Xf* also selectively detect the DSF produced by *Xf*. The adherence of mutants of *Xf* to grape vessels is predictive of their virulence, indicating that adhesiveness is a major facor affecting the ability of *Xf* to cause disease. Such adhesive assays should enable us to more rapidly screen transgenic plants for their resistance to PD as well the efficacy of chemical analogs of DSF to induce resistance. The adherence of WT strains of *Xf* to transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *Xf* was much higher than non-transformed plants, indicating that DSF production in the plants has increased the adhesiveness of the pathogen, and thereby reduced it ability to move within the plant after inoculation.

LAYPERSON SUMMARY:
Xylella fastidiosa (Xf) produces an unsaturated fatty acid signal molecule called diffusible 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 the pathogen, but the overall effect is to suppress its virulence 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 DFS-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 erduces is movement 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 the pathogen 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 have developed 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 the pathogen makes at least one DSF molecule that is recognized by *Xanthomonas campestris pv. campestris (Xcc*) but slightly different than the DSF of *Xcc* (**Figure 1**).

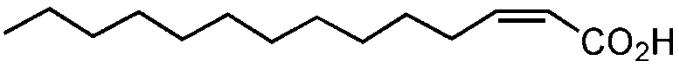


Figure 1 Structure of DSF: C14-cis.

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 pathogen 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 the pathogen 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 to 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 the pathogen 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 characterized the behavior of the pathogen 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 the pathogen itself.

OBJECTIVES

1. 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 Pierce's disease after inoculation into plants in various ways.
2. 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.
3. Optimize the ability of DSF-producing in rootstocks to confer resistance to Pierce's disease in the scion.
4. 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

Characterization of DSF made by *Xf*.

While the molecule C14-cis is one component of DSF made by *Xf*, 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 2**). 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 hyper-virulent in grape, *rpfB* mutants were nearly as virulent as WT strain. In contrast, while rpfF mutants are non-transmissible by sharpshooters, the *rpfB* mutants exhibit only a slight decrease in their transmission 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 plants 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.

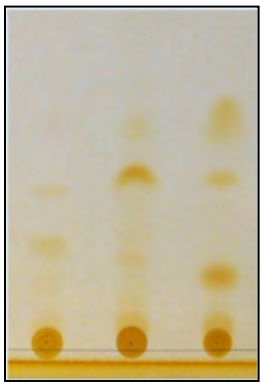


Figure 2. Fatty acids resolved by TLC from a RpfF mutant of *Xf* (left lane) a RpfB mutant (center lane) and a wild type strain of *Xf* (right lane). Fatty acids were visualized after exposure to iodine vapor.

Studies of adhesion of *Xf* to grape

Our studies have suggested strongly that adhesion of *Xf* to plant tissues inhibits movement of the pathogen through the plant, and hence tends to reduce the virulence of the pathogen. RpfF- mutants of the pathogen that do not produce DSF adhere to glass surfaces and to each other much less effectively than WT strain that produce DSF. This is consistent 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. 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 3**). There was a very strong inverse relationship between the adhesiveness of the cells to grape and their ability to cause disease (**Figure 3**). 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 quicker 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.

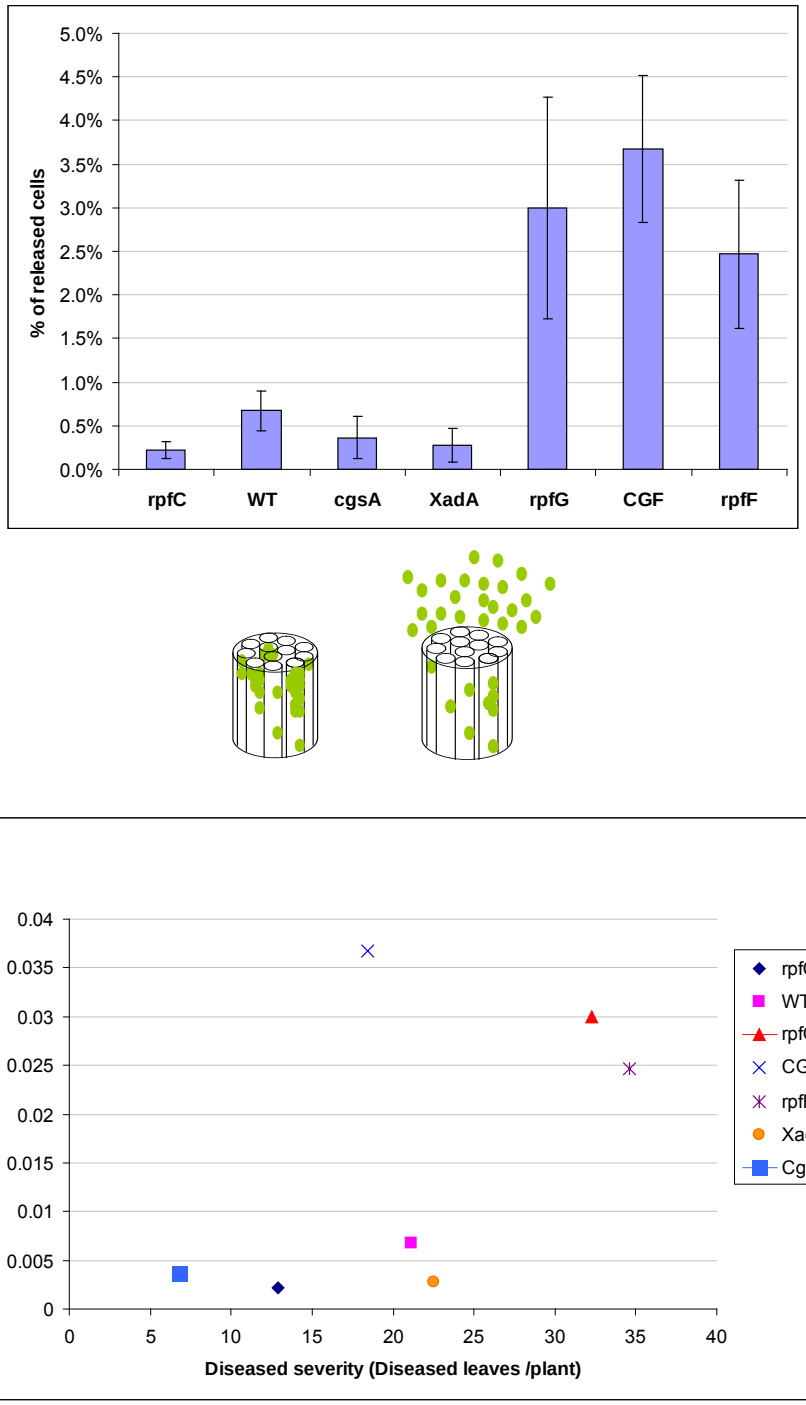


Figure 3. (top) Proportion of total cells of various mutants of *Xf* that were released during gentle washing of grape stem segments in buffer as depicted (middle). The vertical bars represent the standard error of estimates of the proportion of released cells for a given treatment. (bottom) Relationship between proportions of various *Xf* mutants released from tissues and the virulence of those strain in Thompson seedless grape.

To further address the usefulness of the cell release assay to assess treatments designed to limit the movement of *Xf* in plants to achieve disease control, we tested the adherence of WT strains of *Xf* to transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *Xf* compared with that to non-transformed plants. Plants were inoculated with a gfp-marked wild type strain of *Xf* and petioles were removed from plants at a distance of about 20 cm from the point of inoculation, and the percent of the cells removed during a brief washing step measured as above. The adherence of WT strains of *Xf* to transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *Xf* was much greater than that of cells in the non-transformed plants, indicating that DSF production in the plants has increased the adhesiveness of the pathogen, and thereby reduced it ability to move within the plant after inoculation. That is, the percentage of cells that was released from *rpfF*-expressing plants was from two to three-fold less than that of control plants (**Figure 4**). As seen before, cells of an *rpfF* mutant that does not produce DSF exhibited about three-fold higher percentage of cells released from a normal Thompson grape compared to the WT strain in the non-transgenic grape (**Figure 4**). These results suggest strongly that DSF production presumably has occurred in the *rpfF*-expressing plants, and that the enhanced adhesiveness of these cells is associated with their reduced ability to spread through the plant and cause disease. These results suggest that the release efficiency assay should be a useful tool to rapidly assess treatments designed to control PD.

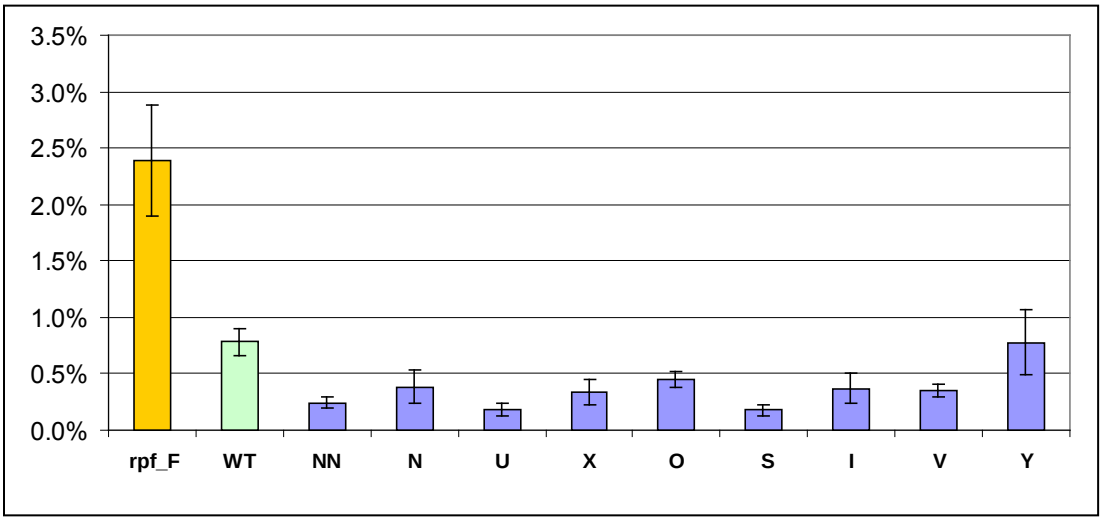


Figure 4. Percentage of total cells of a gfp-marked WT strain of *Xf* (blue and green) and a gfp-marked rpfF mutant of *Xf* (orange) in petioles of non-transformed Thompson (Orange and Green) or of transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *Xf* (blue) that were released 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. *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. We now have identified differently marked *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. 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 5**). 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 form 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 plant, 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. Plants have been inoculated and assessment of the ratios of cells of the two strains in the mixture are currently being made. 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*.

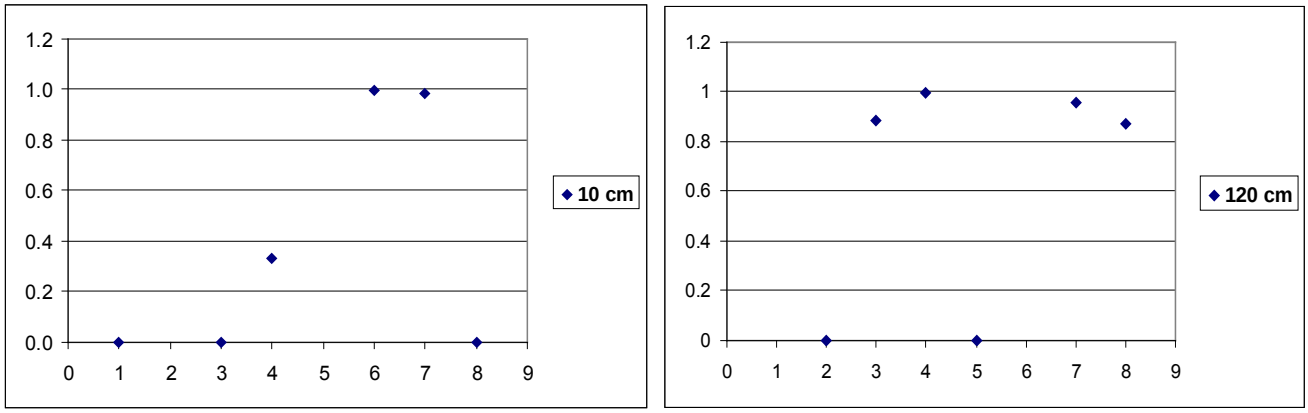


Figure 5. Proportion of cells recovered at 10 cm (left panel) or 120 cm (right panel) from the point of inoculation from individual plants that were inoculated with an equal mixture of a gentamycin-resistant PhoA mutant strain of *Xf* and a kanamycin–resistant PhoA mutant (abscissa) that were the kanamycin-resistant 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* gene 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* colonization as a rootstock than as a scion. Initial estimates of disease severity indicate that there were about 30% less symptomatic leaves of the normal Cabernet scion when grafted onto an *rpfF*-expressing rootstock compared with plants on a normal Freedom rootstock. 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 grafting experiment both with the non-targeted *rpfF* Freedom as a rootstock as well as the chloroplast-targeted *rpfF* Thomson seedless as a rootstock. We are testing the hypothesis that increasing the size of the rootstock will increase its potential to distribute DSF to the scion. We thus are producing wild-types scions grafted to rootstocks of differing sizes. This is proving difficult because the normal process is root the root stock at the same time that the scion is grafted onto the top. Establishing a large rootstock before grafting has made establishment of the grafted scion more difficult. Some success has now been achieved in these plants will soon be inoculated and their disease susceptibility will be related to the size of the rootstock.

Disease control with endophytic bacteria

The severity of PD can be 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. Hoowever, these bacteria do not spread well within the plant after 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. For that reason we have initiated a study of naturally-occurring 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. Our new biosensor, however has allowed us to screen large numbers of bacteria recovered from blue-green sharpshooter insect heads and grape plants for *f* DSF production. We are executing this part of the project using two approaches; the first approach is building our own endophyte 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. 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 than 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 as a relatively large library of DSF-producing bacteria has been assembled. Those strains found to produce the same DSF as *Xf* are then being 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 *Xcc*-based biosensors efficient in detecting *Xylella* DSF

For many of the objectives of this project, an improved bioindicator for DSF would be very valuable. Until recently we have used 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. 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. 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. We named the three different sensors based on their DSF sensing element, *Xcc*-rpfC, *Xf*-RpfC and *Xf-Xcc* chimeric RpfC. The *eng'::gfp* transcriptional fusion in *Xcc* 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*. The three *Xcc*-based DSF biosensors (*Xcc*RpfC (also called pKLN55), *Xf*RpfC (also called GCF), and *Xcc*chimericRpfC (also called Chimeric)) exhibited similar sensitivity to the DSF containing extract from *Xcc* but in sharp contrast, *Xcc*RpfC exhibited much lower sensitivity to the DSF containing extract from *Xf* than the two *Xf*-DSF specific biosensors(**Figure 6**). These results suggest that *Xf*-RpfC can interact with a wider range of molecules then *Xcc*-RpfC. In addition, these two sensors were found to become activated even in the presence of dilutions of the *Xf* extract that contain less than the fraction contributed by a single PWG plate from which the DSF was originally extracted.

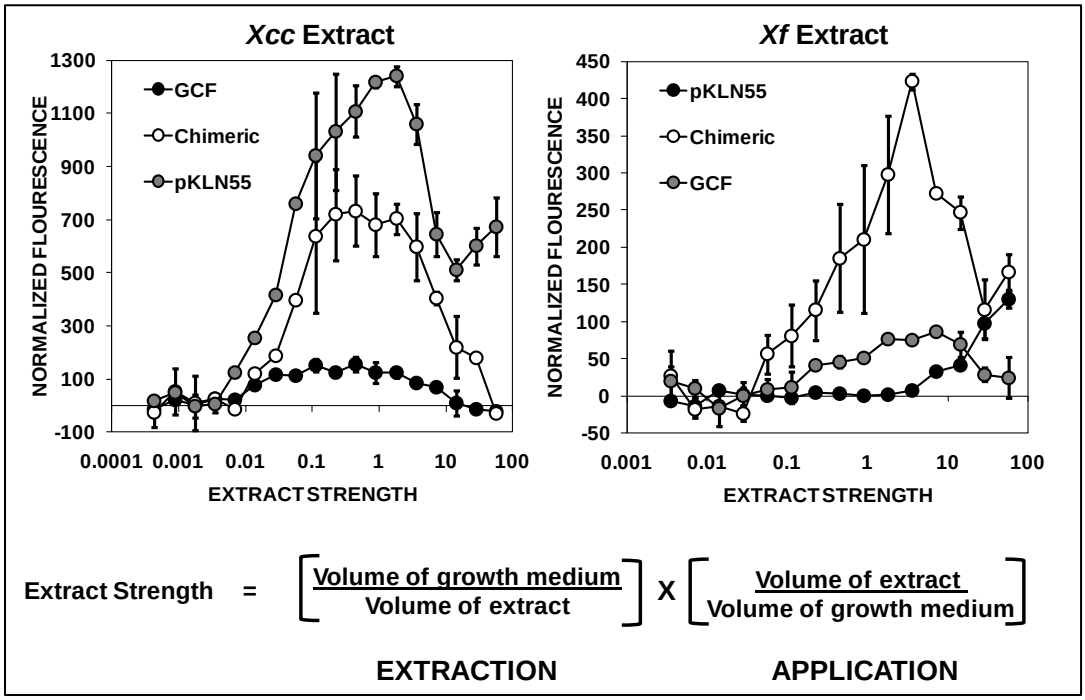


Figure 6. GFP fluorescence of different DSF biosensors to increasing concentrations of DSF from culture extracts of *Xcc* (left panel) or from *Xf* (right panel).

To test the specificity of the DSF biosensors we compared the activity of those sensors to a panel of pure DSF analogues. Quantification of the response of the three biosensors to various fatty acid molecules revealed a substantial difference in their selectivity to compounds of different chain length. The *Xf*RpfC and *Xcc*ChimericRpfC biosensors, based on the use of an *Xf* RpfC receptor were clearly more responsive than the XccRpfC biosensor for fatty acid molecules greater than 12 Carbon atoms long (**Figure 7**). Given that the DSF produced by *Xcc* is 12 Carbons in length, while at least one of the molecules made by *Xf* is apparently 14 Carbons in length, it appears that the RpfC receptor has evolved to bind fatty acid signal molecules of a particular length. This also suggests that while *Xcc* may be relatively unaffected by exposure to DSF made by *Xf*. The converse is probably not the case. 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.

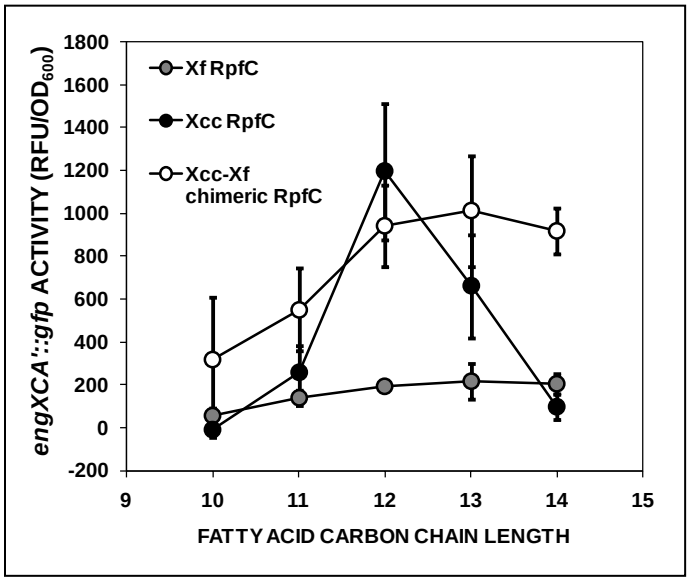


Figure 7. Relative responsiveness of three different DSF biosensors to unsaturated fatty acids of different chain lengths.

Since the various DSF sensors appeared to have high specificity and sensitivity for DSF, they are suitable for large-scale testing of DSF molecules and transgenic plants as well as for DSF produced by antagonistic bacteria. Methods were therefore tested to ascertain the most expedient way in which these biosensors could be deployed for the detection of DSF. A broth culture assay in which the biosensors were suspended in xylem sap of transgenic plants expressing RpfF from *Xf* successfully detected DSF. We were pleased to find that GFP fluorescence and hence DSF responsiveness of the biosensor could be easily monitored in such a high throughput manner.

Development of a *Xf*-based bioreporter for DSF

We have developed 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. Previous attempts to establish *gfp* or *inaZ*-based transcriptional fusions in *Xf* failed, presumably due to its incapability to express foreign genes properly we have successfully use the endogenous *phoA* gene (encoding alkaline phosphatase) as a bioreporter of gene expression in *Xf*. The PhoA-based biosensor in which *phoA* is driven by the *hxfA* promoter is quite responsive to exogenous DSF (**Figure 8**). We are currently conducting extensive tests of this *Xf*-based biosensor to screen various compounds produced by *Xf* to determine those that are active as DFS signaling molecules.

We also 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. The DSF-induced behaviors of *Xf* were found to be strongly dependent on the culture medium that the cells were grown in. All strains of *Xf* are much more responsive when cells are grown in a minimal medium such as PIM6 or PDA than in media such as PWG containing BSA. For example, cells of the WT strain which are not adherent in culture, 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 9** left). The attachment of *Xf* cells to tubes or wells is readily measured by estimating the number of attached cells by their ability to bind crystal violet. The amount of cells bound to the surface of tubes increased with increasing concentration of C14-cis above about 1 uM. The concentrations of C14-cis at which cell binding was increased as estimated by this assay was similar to that as measured in an *Xcc*-based bioassay (**Figure 9** right). Cells of *Xf* exposed to DSF in PIM6 minimal medium also appear to adhere to each other more than those in the medium with added DSF. The DSF-medated increase in adhesiveness is readily apparent as cells that clump together when visualized microscopically (**Figure 10**). Clearly, exogenous DSF stimulates an adhesive state in *Xf***.** The *Xf*-based cell binding assay therefore appears to be a very valuable and rapid method by which DSF response in *Xf* can be assessed.

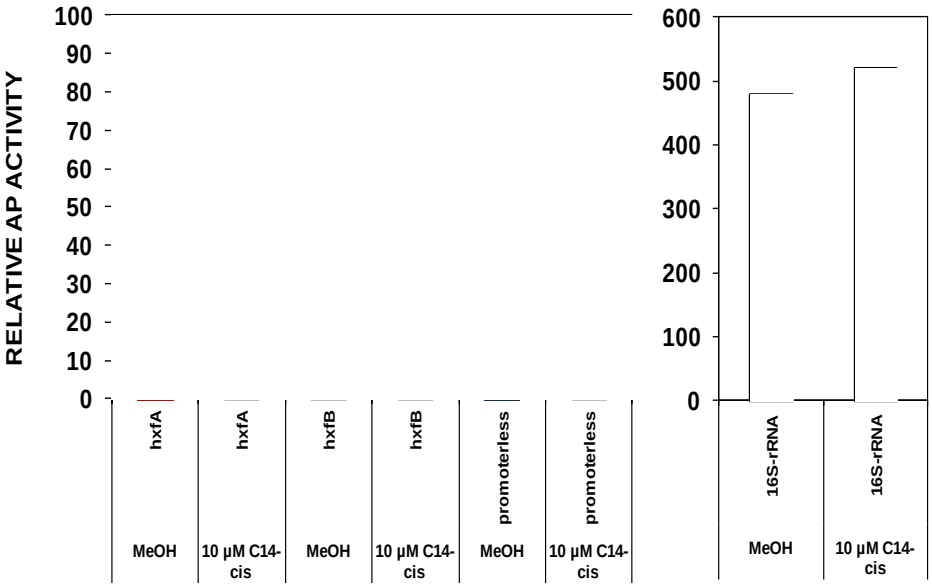
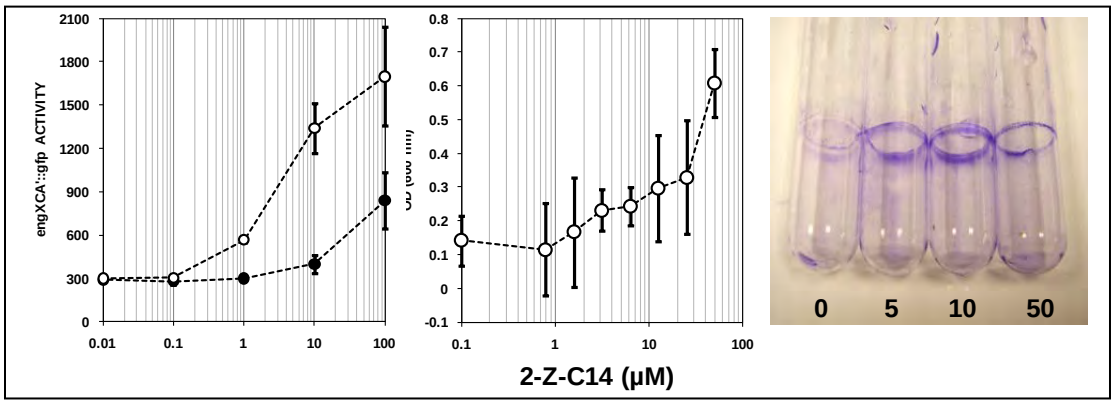


Figure 8. Increased alkaline phosphatase activity of cells of *Xf* harboring a fusion of *phoA* and *hxfA* and exposed to C14-cis (left box) and constitutive expression of *phoA* when fused to *rrnB* (right box).



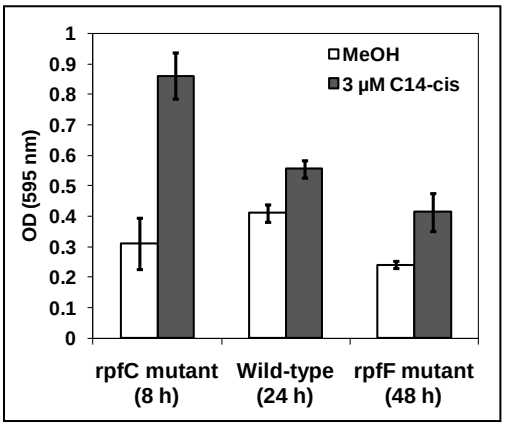


Figure 9. (top 3 panels). Binding of cells of *Xf* to the walls of glass tubes at the air-medium interface in cells grown in PIM6 medium containing up to 50 micromolar C14-cis as visualized by crystal violet staining (right panel). The quantification of cell binding as measured by crystal violet absorption is shown in the center panel for the cells. The responsiveness (gfp fluorescence) of an *Xcc*-based biosensor grown in PIM6 medium containing up to 50 micromolar C14-cis is shown in the left panel. (bottom panel). Quantification of attachment of cells of the RpfC mutant, wild type strain, and RpfF mutant of *Xf* to the walls of glass tubes to which 3 uM C14-cis had been added when assayed with a crystal violet binding procedure.

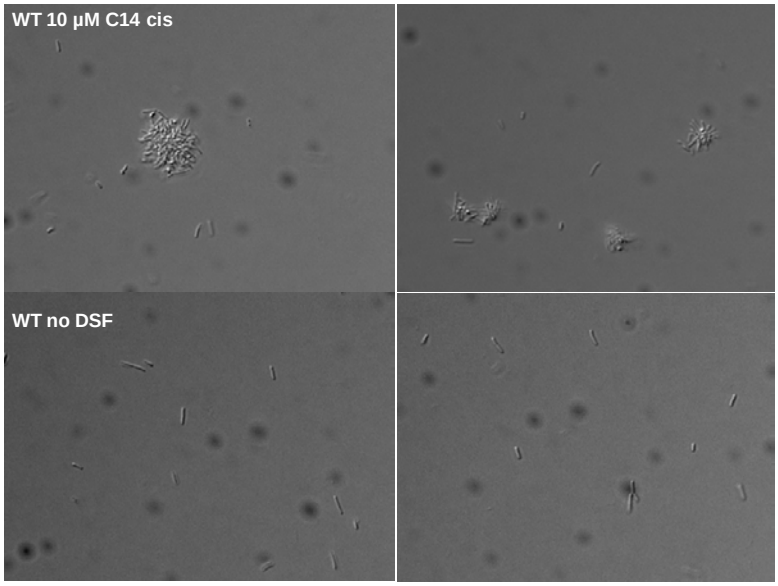


Figure 10. Microscopic depiction of cells of wild type *Xf* grown in PIM6 medium for three hours to which 10 uM C14-cis had been added (top panels) and cells grown in PIM6 medium without added DSF (bottom). Note the cellular aggregates found in the top panels in the presence of DSF.

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 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. 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. 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. We are still optimistic that chemically synthesized DSF molecules might also ultimately be the most useful strategy for controlling disease. The presence of more than one DSF base signal molecule suggests that perhaps more than one molecule might be needed to achieve changes in pathogen behavior. Our major advances in the development of biosensors to detect the responsiveness of *Xf* to signal molecules is a major breakthrough that hopefully will allow us to make rapid progress in ascertaining those transgenic plants most capable of altering pathogen behavior as well as in formulating synthetic molecules suitable for use in disease control. The biological sensors also have proven useful in screening naturally occurring bacteria associated with grape that might also be exploited to produce signal molecules

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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 2011.

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 pit membranes 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 in Solano County on August 2, 2010. Plants were planted at a Riverside County site on April 26, 2011. The plants established in the Solano County site have grown well and were in general in excess of 2 to 3 meters in length by July, 2011. All plants at the Solano County experimental site were needle-inoculated with a suspension of *Xf* on July 22, 2011. From one to four vines per plant were inoculated, each at a given site with a 20 ul droplet of *Xf* containing about 20,000 cells of *Xf*. As of October, 2011 no visible signs of disease are apparent in any of the plants. Population sizes of the pathogen are also being assessed in the plants as a means of determining their susceptibility to PD. Plants at the Riverside County plot are subject to natural infection, and any incidence of infection of the plants will be assessed in late October, 2011.

LAYPERSON SUMMARY:
Xylella fastdiosa coordinates its behavior in plants in a cell density-dependent fashion using a diffusible signal molecule (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 PD.
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 PD.

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

Figure 1. Overview of research plot in Solano County in soon after DSF-producing plants were established (left). Close-up of transgenic Freedom vines in mid-September 2010 (right).

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 one meter 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 tooted 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 all survived transplanting and are growing well (**Figure 1**). The plants were too small to inoculate in the 2010 growing season and hence were inoculated on July 26, 2011 (no natural inoculum of *Xf* occurs in this plot area and so manual inoculation of the vines with the pathogen will be performed. The plants established in the Solano County site have grown

well and were, in general, in excess of two to three meters in length by July, 2011 (**Figure 2**). All plants at the Solano County experimental site were needle-inoculated with a suspension of *Xf*. From one to four vines per plant were inoculated, depending on the size and number of vines per plant. Each inoculation site received a 20 ul droplet of *Xf* containing about 20,000 cells of *Xf* (**Figure 3**). 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 are being shared between research groups. All plants at UC-Davis were inoculated by needle puncture through drops of a common inoculum source of *Xf* of about 10^6 cells/ml as in previous studies. As of early October, no symptoms were apparent, although the plants continued to grow very well (**Figure 4**).

Figure 2. Images of Thompson Seedless grape (left) and Freedom grape (right) at the Solano County field trial in July 2011.

Figure 3. Process of inoculation of grape at the Solano County field trial in July, 2011. A needle was inserted through a vine and a droplet of inoculum applied to the needle tip. After withdrawal of the needle, the bacterial inoculum is drawn into the vine due to the tension of the water in the xylem vessels.

Some of the plants needed to establish the trial at Riverside county were damaged in the greenhouse at UC Berkeley in 2010 due to pesticide applications, and since plants from other researchers at UC Davis were also not ready for transplanting to the field in 2010, a decision was made to establish all of the plants from the UCB and UCD research groups together in early 2011. The plants for the Riverside County were generated and were transferred to a lath house at UC Davis on March 23, 2011 to harden off for about three weeks. The plants were then transported to Riverside County for establishment in the field experiment together with plants from researchers at UC Davis and were planted on April 26, 2011 (**Figure 5**). The plants at the Riverside County trial 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. Leaves exhibiting scorching symptoms characteristic of PD will be counted periodically at each location, 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 4. Appearance of transgenic Freedom grape in early October 2011.

Figure 5. Establishment of grape trial in Riverside County.

CONCLUSIONS

The transgenic plants have been successfully established at two field sites in California. The first disease assessments will be made in late-summer, 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

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 from grafted rootstocks of Pierce's disease (PD) control candidates, including polygalacturonase-inhibiting proteins (PGIPs). 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* in the xylem network and, thereby, limit PD symptom progression in infected vines. The aim of this project is to develop a monoclonal antibody to the pear fruit PGIP, the protein expressed by the aforementioned grape lines, 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*), the bacteria that causes Pierce's disease (PD) in grapevines, utilizes a key enzyme, polygalacturonase (*Xf*PG), to spread from one grapevine xylem vessel to the next, eventually leading to the development of PD symptoms in infected vines because the bacteria multiply and interrupt the flow of nutrients and water through the vessels in the plant. Plant proteins called PG-inhibiting proteins (PGIPs) selectively inhibit PGs from bacteria, fungi, and insets. Our work has identified a PGIP from pear fruits that at least partially inhibits the *Xf*PG and we demonstrated reduced 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 the PGIP protein delivered to the scion portion of grafted plants from rootstocks expressing the pear fruit PGIP. The monoclonal antibody allows the researchers to compare the amounts of the PGIP protein at different times and places and thereby determine the protein's role in *Xf*PG inhibition in grapevines. We are purifying active pear PGIP green pear fruit for commercial antibody production to meet the needs of the collaborating groups.

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 (*Xf*PG), 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 *Xf* 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 fresh green 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 plant cell wall proteins that require plant specified glycosylation for activity (Powell et al., 2000). The project "Optimizing grape rootstock production of and export of inhibitors of *Xylella fastidiosa* (*Xf*) polygalacturonase (PG) activity" (Labavitch, 2008) generated transgenic *Arabidopsis thaliana* plants expressing the pPGIP protein fused to a C-terminal histidine tag for purification. Leaves from these transgenic plants yielded a small amount of total protein, as determined by Bradford assays. Work to refine the transgenic protein purification process is ongoing.

pPGIP extraction has begun from fresh pear fruit flesh using the protocols in Stotz et al. (1993) with modifications. In 2010 we worked with two batches of green pear fruit to purify sufficient protein for the antibody preparation. The fruit (2 kg each) were homogenized in 2 L of extraction buffer (1 M PGIP extract preparation (Abu-Goukh *et al.,* 1983).

pPGIP activity was measured throughout purification by radial diffusion assays (Taylor and Secor, 1988). Samples of the initial pear homogenate were able to fully inhibit a PG (*Bc*PG) mixture from *Botrytis cinerea* culture filtrates. The pPGIP purification preparations after ammonium sulfate precipitation and subsequent dialysis resulted in a 75% reduction in BcPG activity in the assay (**Figure 1**). Although in the first attempt to purify the protein the concentration of proteins in the sample was low and the preparations still contained considerable polysaccharides which may impair binding to ConA sepharose. The excess polysaccharide could have been because the pears used for this preparation were not completely green. This procedure was repeated with a second preparation of pear fruit and the following purifications steps were followed:

1. Pears were peeled, cored, sliced, and homogenized for 1 minute in equal volume of 0.1 M sodium acetate pH 6, containing 0.2% sodium bisulfite and 1% PVPP.
2. Homogenate was filtered through cheese cloth, and pulp was collected. The filtrate was centrifuged at 15,000 g for 25 min and the pellet was collected and pooled with the pulp. The supernatant was discarded.
3. The pulp and pellet were suspended in 1 M sodium acetate pH 6 containing 1 M NaCl. The mixture was stirred overnight at 4^{o} C, and the pH was maintained at 8.0 with addition of 2N NaOH.
4. Step 2 above was repeated, and the filtrate and supernatant were collected. The pulp and pellet were resuspended in 1 M sodium acetate pH 6 containing 1 M NaCl and centrifuged to collect the wash. The salt supernatant and wash was pooled, and the pellet/pulp was discarded.
5. The pooled salt extract was filtered through Whatman #1 filter paper, and dialyzed using a 6-8000 kda cut off membrane at 4^{o} C for 72 h.
6. The dialyzed material was first precipitated with ammonium sulfate at 50% saturation. The solution was centrifuged. The pellet was resuspended and dialyzed using a 6-8000 kda cut off membrane.
7. The 50% saturated ammonium sulfate supernatant was saturated with ammonium sulfate to 100% saturation. The solution was centrifuged. The pellet was resuspended and dialyzed using a 6-8000 kda cut off membrane. The supernatant was discarded.
8. 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 $MnCl_2$) and applied to Concanavalin A-sepharose to bind the glycosylated proteins, including pPGIP. The proteins retained by the ConA seqpharose were eluted with 250 mM alpha-methyl mannoside. Fractions containing PGIP activity were pooled and dialyzed.
9. A western blot showing the pear PGIP (pPGIP) band using polyclonal anti-pPGIP antibody is shown in **Figure 2**. The pPGIP band corresponds to a molecular weight of approximately 45 kDa, which is the expected size for pPGIP.
10. The protein preparation was concentrated to 1 mg/ml protein concentration using ultra filtration device fitted with a 10 kDa cutoff membrane.
11. PGIP was further purified using fast protein liquid chromatography (FPLC) using a Resource S cation exchange column. The concentrated protein preparation was loaded and the column was run at a rate of 4 mL/min. The column was equilibrated with 50 mM sodium acetate buffer pH 4.5 (buffer A) and eluted with a linear gradient of sodium chloride to a final concentration of 500 mM sodium chloride in buffer A.
12. A protein trace from the FPLC is shown in **Figure 3**.
13. The fractions collected from FPLC are being analyzed for PGIP activity and further processing (dialysis, concentration, etc).

***Objective 2* - Calibrate the antibodies produced by the hybridoma clones to determine effective dilutions for use in detecting the pPGIP protein.**

Will commence once the antibody has been generated.

***Objective 3* - Use the antibody to detect transgenic pear PGIP in xylem sap of own-rooted and grafted grapevines.**

Will commence once the antibody has been generated.

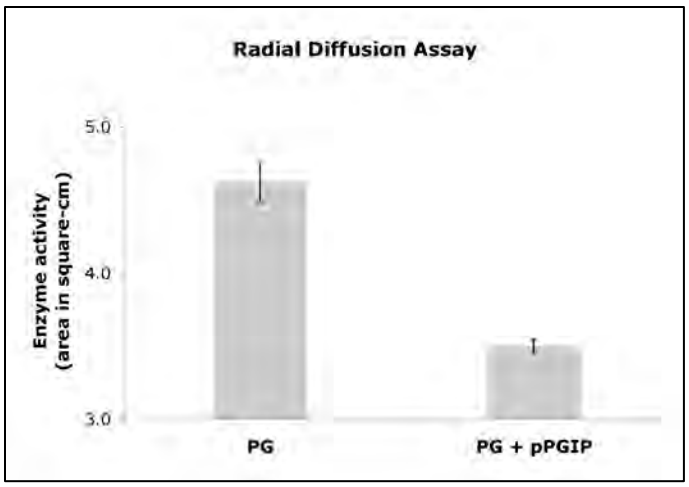

Figure 1. Results of a radial diffusion assay to determine the amount of pPGIP in a protein preparation from pear fruit.

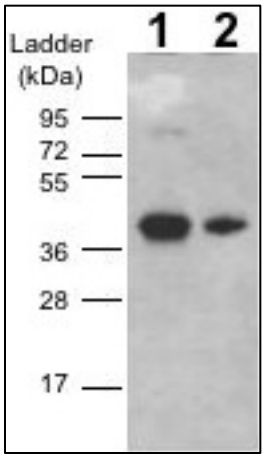

Figure 2. Immunoblot analysis of pPGIP. Lane 1: 80 ng protein after ammonium sulfate precipitation (50-100% fraction). Lane 2: 10 ng protein after the ConA purification step. Molecular weight ladder is indicated on the left. Proteins were separated on a SDS-PAGE (10%) gel and probed using pPGIP antiserum.

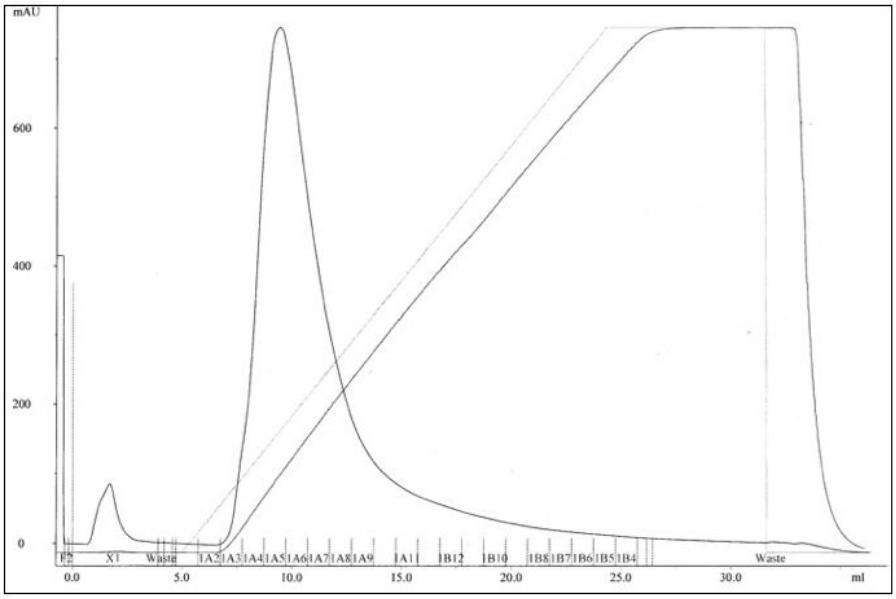

Figure 3. FPLC analysis of pPGIP preparation.

CONCLUSIONS

We are on track to complete the purification of the pear PGIP from green pear fruit tissue in the next month. We have consulted with Antibodies, Inc., about the amount of the protein needed to generate the monoclonal antibodies. The partially purified protein has been analyzed for its activity and been visualized by Western blots using the original polyclonal antibody we have used for more than 20 years. The protein migrates as a single band, and is largely separated from other proteins. This work should benefit a solution to the PD problem because it will allow for standard assays to evaluate the quantity, location and effectiveness of the PGIP delivered by the strategies of the collaborating groups. Since top priority has been given for the delivery from grafted rootstocks of PGIP as a PD control candidate, the four currently funded projects will be able to use the antibody to evaluate their strategies for delivering PGIPs to control the spread of *Xf* in the xylem network and thereby limit PD symptom progression in infected vines. Production of the monoclonal antibody by Antibodies, Inc. will begin once sufficient quantities of properly glycosylated, active PGIP have been purified. The monoclonal antibody will allow comparison of the approaches from different research groups and will allow accurate and PGIP-specific assessments of the potency of pear PGIP for limiting PD symptoms.

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FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

FIELD EVALUATION OF GRAFTED GRAPEVINE LINES EXPRESSING POLYGALACTURONASE-INHIBITING PROTEINS

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ABSTRACT

The aim of this project is to evaluate in two California field vineyards, the performance of grafted grapevine lines that produce in the rootstock, a protein that is a candidate for control of Pierce's disease (PD). The PD and Glassy-winged Sharpshooter Board's (GWSS-PD) Research Scientific Advisory Panel gave priority to the delivery of polygalacturonase-inhibiting proteins (PGIPs), from grafted rootstocks to control PD. Previously transformed 'Thompson Seedless' and 'Chardonnay' grapevines expressing a PGIP from pear fruit (pPGIP) show reduced PD incidence when inoculated with *Xylella fastidiosa (*Agüero *et al*., 2005*).* In this field evaluation, these grapevines were propagated vegetatively for PD assessment in field trial locations in Solano and Riverside Counties. Fifty-one transgenic and control, own-rooted, grapevines were planted in Solano County on 07/06/2010. Additional grafted plants, utilizing the pPGIP-expressing vines as rootstocks, have been prepared, rooted and they will be planted later in 2011 when field conditions are appropriate. The field plantings in the plot in Solano County were winter pruned in 2011 and currently are in the midst of their main growing season. PD resistance and plant growth characteristics are being assessed on the plants in the northern California location. The test field also contains plots from the three collaborating groups of D. Gilchrist, A. Dandekar and S. Lindow who were funded jointly. Inoculations and disease assessments of the vines developed in the four projects are being evaluated simultaneously. The plants from these trials have been planted at the same locations and the APHIS-USDA authorizations for all projects have been handled through PIPRA.

LAYPERSON SUMMARY

The goal of this project is to verify that the Pierce's disease (PD) resistance provided by expression of polygalacturonase inhibiting protein (PGIP) in grapevines is evident when the plants are grown in field vineyard settings in California. The overall health and robustness of the plants expressing the pear fruit (p) PGIP will be compared to the plants from other jointly funded groups evaluating other strategies to limit PD development. The resistance of plants expressing PGIPs in grafted rootstocks will be compared following manual infections with *Xylella fastidiosa* through stem inoculations in the Northern California location and as a result of natural infections in the Southern California site. Funding for this project was needed to develop sufficient plants for both locations, manage the vineyard plantings, confirm the genetic identity of rootstocks as well as the scion and do the resistance testing. The performance and resistance of the grapevines in the field are being evaluated as the vines become established in the vineyards.

INTRODUCTION

Grapevines transformed to express the pear fruit polygalacturonase inhibiting protein (pPGIP) were grrown in greenhouses prior to the work in this proposal. These vines displayed fewer symptoms of Pierce's disease (PD) infections when inoculated with *Xylella fastidiosa (Xf) (*Agüero *et al*., 2005*).* The additional PGIP in the grape plants inhibits the enzyme, polygalacturonase (PG), that *Xf* employs to spread infections throughout the vine (Roper *et al.,* 2007). In a separate glassy-winged sharpshooter (GWSS)-PD funded project aiming to optimize the activity, expression, and export of PGIP proteins expressed in transgenic grape rootstocks to provide optimal PD protection in the scion portions of the vines ("Optimizing grape rootstock production and export of inhibitors of *Xf* PG activity" (PI Labavitch)), PGIPs from various plants are being evaluated for their efficacy. While these evaluations strongly suggested that expression of additional PGIPs could be an appropriate strategy for improving grapevine resistance to PD, the vines had only been grown and evaluated in greenhouses and to be acceptable to the California grape industry, their growth performance and susceptibility to PD in vineyard settings comparable to commercial production locations was necessary. The goal of this project is to verify that the transgenic grapevines expressing pPGIP in grafted rootstocks (1) have increased resistance to PD and (2) maintain the appropriate performance traits necessary for commercial release when grown in field vineyard settings in California. The project was funded jointly with other groups evaluating anti-*Xf* strategies so that uniform field conditions could be achieved for all of the trials. Comparisons of protection and performance outcomes from the groups should be achieved.

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 performance 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) continue to be maintained at the UC Davis Core Greenhouses. Grafting has been done with green and semi-lignified stem segments for the possible graft combinations. A modified wedge grafting technique is used whereby scion sections of one to two nodes were stripped of foliage and cut into a wedge. These sections were fit into notched rootstock line stems of equal maturity, alternating the bud position. 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 similarly grafted vegetative cuttings - one node scions grafted onto three node, disbudded rootstock cuttings - were basally dipped in 5.7 µM IAA and 2.7 µM NAA for five min. before transferring to a loose perlite:vermiculite medium (1:1). We have utilized mist beds to increase the success of callusing these green grafted cuttings and have modified the EZ-Clone aeroponic system discussed in previous reports so that the grafted plants develop more robust roots before they are transferred to soil prior to transplantation to the field (**Figure 1**). We have made significant progress toward generating the grafted plants needed to complete the project design in the field trial. Our grafting techniques are continuously evolving to yield higher success rates. For this season, we have generated 21 potted, grafted plants with another 142 grafted cuttings currently callusing and hardening off.

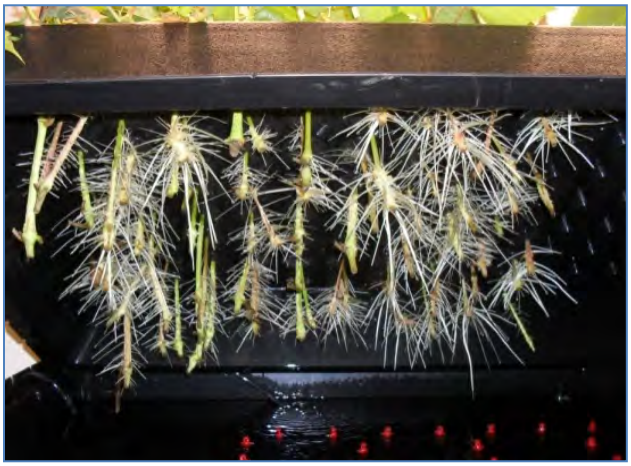

Figure 1. Grapevine cuttings rooting in the EZ-Clone aeroponic manifold.

Objective 2: Establish field trial sites.

Two field trial sites are being established to assess the PD resistance and general agronomic viability ("performance") of own-rooted and grafted pPGIP expressing grapevines. We have focused our efforts on generating sufficient high quality vines for the primary site in Solano County, CA. The Solano site has no natural PD pressure and the secondary site in Riverside County, CA has high natural PD pressure. The field sites are shared by projects testing other transgenic PD control grapevines from PIs, D. Gilchrist, A. Dandekar, and S. Lindow. All vines, both grafted and ungrafted, that by PCR analysis have the correct genotypes, from our portion of the field trial were hand-planted in a randomized block design with blocks consisting of two or three individuals in the same treatment beginning in July 2010. In **Table 1** the number of each combination of each genotype of grafted and ungrafted plants is shown. Not all of the plants that are needed for the experimental design have been transplanted to the field vineyards as of October, 2011 but most of the remaining plants have been grafted and are developing roots and hardening off in lathe houses. The remaining grafted lines expressing pPGIP in the rootstocks will be planted in the early fall 2011.

The grapevines are planted approximately eight ft. apart and tied to wooden stakes with trellising wires at 40 in. and 52 in. All plants in the field vineyard were winter pruned in February 2011 and have grown vigorously so far in 2011 (**Figure 2**). The vines in the Solano site have been weeded and monitored weekly throughout the 2011growing season. Grow tubes were initially placed around the vines to minimize damage by rabbits, mechanical weeding, and herbicides. We have lost none of the vines that we placed in the field in 2010, a result we attribute to planting robust vines that were sufficiently hardened off, combined with tending the vines in the field with appropriate care. The propagated vines were trained to one major shoot and pruning biweekly to encourage growth. The vines were topped and major cordons were extended bidirectionally, keeping additional positions to account for the vigorous nature of the vines at the site.

Table 1. Total number of grapevines planted in, and prepared for Solano County. Dashed shapes represent pPGIP expressing grapevine rootstocks and/or scions; solid shapes are null-transformant controls (no pPGIP). Vines were mechanically inoculated with *Xf* on 7/21/2011. Grafting progress numbers include all grafted cuttings at each checkpoint.

		Own-Rooted Plants (#)		Grafting Progress (#)			
Cultivar	Grafting Strategy	Inoculated	Non-Inoculated	Mist Beds	EZ-Clone	Potted in Greenhouse	Originally Planned
Chardonnay		8	4	0	2	2	13
		-	-	70	2	5	13
		9	4	0	1	1	13
Thompson Seedless		8	4	0	3	4	13
		-	-	58	4	8	13
		9	5	0	2	1	13
Subtotals		34	17	128	14	21	
Aggregate Totals		51		163			78

Figure 2. Examples of vines in the field in July, 2011. Vines have been trained to the central post and trellising wires. *Xf* mechanical inoculation sites are marked with orange tags (C).

The rooted cuttings of 'Chardonnay' and 'Thompson Seedless' grapevines engineered to express pPGIP were genotyped by PCR analysis prior to transplantation to the field vineyard to confirm the presence of the pPGIP sequences in the appropriate rootstock or scion portions of the plants. To confirm that the pPGIP sequences produced the expected pPGIP protein, total extracted proteins were cross-reacted with a pPGIP-specific polyclonal antibody on Western blots. The pPGIP protein was observed only in samples from scions grafted to pPGIP expressing rootstocks or where otherwise expected in scions expressing the pPGIP sequences (**Figure 3**). Graft translocation is not seen in control transgenic grapes expressing a cellular-localized protein.

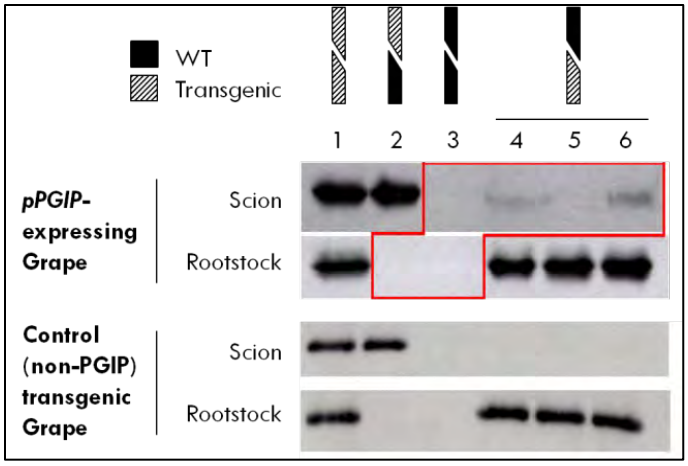

Figure 3. Western blot of leaf extracts taken from rootstock and scion portions of grafted 'Thompson Seedless' grapevines. Transgenic vines are expressing either pPGIP or NPTII (control). pPGIP is visualized crossing from transgenic rootstocks into wild-type (WT) scion tissue (lanes 4-6). This movement is not seen in the reciprocal graft (lane 2).

Objective 3. Evaluate relevant agronomic traits of vines in two locations.
The grapevines planted in Solano County have been monitored for general health and maintained on a weekly basis. Our regulatory permits require that all flowers be removed to prevent the potential for pollen escape. Because of this restriction, we have not been able to perform the agronomic fruit production measurements necessary for the commercial viability assessment. We are working with PIPRA to explore possible exceptions to this policy for future seasons. We however, monitor health and vigor of individual plants.

Objective 4. Determine PD incidence in pPGIP expressing grafted and own-rooted lines.
Two-thirds of the own-rooted vines at the Solano site have been mechanically inoculated with *Xf* to monitor PD incidence throughout the summer. Mechanical stem inoculations were utilized to ensure an even introduction of the bacteria in this site with no natural PD pressure. Each of the 34 own-rooted vines were inoculated three-four times per plant using a pin-prick technique by which a 20 µl (20,000 cells) drop was placed on a 21 gauge needle piercing the cane, and the needle was then withdrawn. The bacterial suspension was taken up into the xylem by the natural negative turgor pressure associated with evapotranspiration. The inoculations were performed in conjunction with the other field site collaborators. The bacterial suspension was provided by D. Gilchrist. A subset of the inoculated plants will be screened for *Xf* presence and movement by culturing xylem sap extracts on PD3 media.

CONCLUSIONS
Field vineyards are being established that contain grapevines that express pPGIP protein in rootstocks and these rootstock export the pPGIP protein to the scion portion of the plant. The efficacy of the exported pPGIP to reduce the PD damage caused by *Xf* is being evaluated in the field plantings to verify that the resistance observed in greenhouse settings can be replicated by plants grown in typical field settings in California. We are completing the generation of the remaining plants needed for the sites and confirming the genotype of the lines transplanted to the field. The general health and performance of the plants in the field vineyard continue to be monitored.
The results of the field evaluation will confirm that delivery of the pPGIP from rootstocks provides a means of controlling PD and *Xf* infection in a typical vineyard setting in California, an outcome that is important for the acceptance of this strategy to control PD in California. The evaluations of the performance and productivity of the plants will confirm that that expression and presence of pPGIP does not unintentionally adversely affect other characteristics of the vines. By using varieties grown for fresh fruit and for wine production in California, we are testing varieties important to California growers.

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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 (PD). We hypothesized that some of the fungal endophytes present in PD-escaped grapevines possess anti-*Xf* properties, likely due to the production of secondary metabolites. We sampled from vineyards located in Napa and Riverside Counties that are under high disease pressure and isolated fungal endophytes living in the xylem sap and in wood spurs, shoots, petioles of these PD-escaped vines. Fungi were identified by ribosomal DNA sequencing. We have selected thus far a total of nine fungal strains that have inhibitory effects on *Xf* growth *in vitro*. We introduced several of these fungi into grapevines and then challenged them with *Xf*. Two of these fungi reduced PD progression in grapevines inoculated with *Xf* (Temecula1). In addition, we have isolated and identified the chemical structure of two fungal natural compounds produced by our candidate biocontrol fungi that inhibited *Xf* growth *in vitro*. In future experiments we will repeat the biocontrol experiment in greenhouse to confirm the prophylactic effect provided by these fungi. We will also evaluate the curative treatment potential of the fungal natural products that we have isolated in PD-infected grapevines. These molecules and fungi are currently under review for patentability by the Executive Licensing Officer in the UC-Riverside Office of Research and, hence, their names cannot be disclosed in this report.

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 was 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, 2010 and 2011 we have sampled from vineyards in Napa and Riverside Counties that are under high disease pressure and identified fungi living in the xylem sap, shoots, petioles and wood spurs of diseased and PD-escaped grapevines. We have identified nine fungi that inhibit *Xf* growth in culture. Four fungi were re-introduced in grape cuttings that were inoculated with *Xf*, and two of them show a reduction in PD-disease progression. In addition, we also extracted natural compounds secreted by these fungi and identified two purified molecules inhibitory to the bacterium. In the future our goals are to; 1) screen more fungi with potential for *Xf* growth inhibition; 2) repeat the experiment with introduction of fungi in grape cuttings with additional potential biocontrols; 3) elucidate the chemical structure of additional fungal natural products antagonistic to *Xf* and test them as a curative treatment on PD-infected grapevines. These molecules and fungi are currently under review for patentability by the Executive Licensing Officer in the UC-Riverside Office of Research and, hence, their names cannot be disclosed in this report.

INTRODUCTION
Current Pierce's disease (PD) management strategies largely involve vector management through the use of insecticides (3). This has contained the spread of the disease (9). However, for sustained control of PD, strategies that either target *Xylella fastidiosa* (*Xf*) 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 (1, 5, 10, 11). Other approaches include traditional breeding focused on introducing PD resistance into *V. vinifera* (14). Integrated control strategies are also being investigated in natural vineyard settings. These include the use of natural parasitoids to the GWSS (4) and inoculation of grapevine with mild *Xf* strains that may provide cross protection prior to infection with virulent *Xf* strains (8). 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. Fungal endophytes are receiving increasing attention by natural product chemists due to their diverse and structurally unprecedented compounds which make them interesting candidates for discovery of novel molecules and for their potential in disease control (2, 12, 15). This project focuses on identifying endophytic fungi in grapevine and evaluating their potential as biocontrol agents against *Xf*. Our objectives are to characterize the microbial 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. We are assessing the ability of these endophytes and their natural products (i.e. secondary metabolites) for inhibitory activity against *Xf in vitro*. Finally, we are determining in greenhouse tests if 1) fungi have potential use as prophylactic biocontrol agents for control by inoculating grapevine cuttings with endophytic, *Xf*-antagonistic fungi and 2) if treatments PD-infected grapevine with fungal natural products have curative properties. If successful, we envision that these control strategies can be implemented at the nursery level (for biocontrols) 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

Objective 1. Identify fungal endophytes that are present in xylem sap and xylem tissues of PD-escaped grapevines but not in PD- symptomatic grapevines.

The goal is to identify the fungal endophytic populations inhabiting grapevines infected with PD and apparently healthy grapevines adjacent to PD-infected vines (PD-escaped) (**Figure 1**) with classical isolation techniques and DNA-based methods. Plant tissues/fluids (xylem sap, green shoots, petioles, and wood spurs) were collected at bud-break and before harvest from vineyards grown in Riverside and Napa Counties (**Table 1**) and brought back to the laboratory. Culturable fungi were isolated on fungal medium (Potato Dextrose Agar, PDA), and were identified after comparing the PCR-amplified rDNA sequence to homologous sequences posted in the GenBank database.

Results in **Table 2** show showed that based on our samplings to date, *Cladosporium* sp. and *Aureobasidium* sp. are the most widespread culturable fungi inhabiting grapevine xylem. Both of these species have a high incidence in both diseased and PD-escaped grapevines (xylem sap, green shoots and wood spurs). Furthermore, these are the only culturable fungi repeatedly identified in the xylem sap of grapevine. We also found other fungal species occurring in both diseased and PD-escaped grapevines, albeit, at a lower frequency. These include *Alternaria* sp., *Cryptococcus* sp., *Penicillium* sp., and a *Geomyces* sp. Some fungi were only present in PD-escaped or diseased grapevines. The fungal species found only in diseased vines include *Epicoccum* sp., *Phomopsis* sp., *Fusarium* sp., *Biscogniauxia* sp., *Cryptosporiopsis* sp., *Ulocladium* sp., *Pezizomycete* sp, and *Didymella* sp. Most interestingly, we found several species only inhabiting PD-escaped grapevines. These include *Peyronellae* sp., *Drechslera* sp., *Discostroma* sp., *Cochliobolus* sp., *Chaetomium* sp., *Phaeosphaeria* sp., *Oidodendron* sp., and *Diplodia* sp. Identification of fungi from sampling in September/October of 2012 from vineyards 2, 3, and 4 is currently underway. Additional sampling will occur in March/April of 2012 at the same vineyards to complement these results.

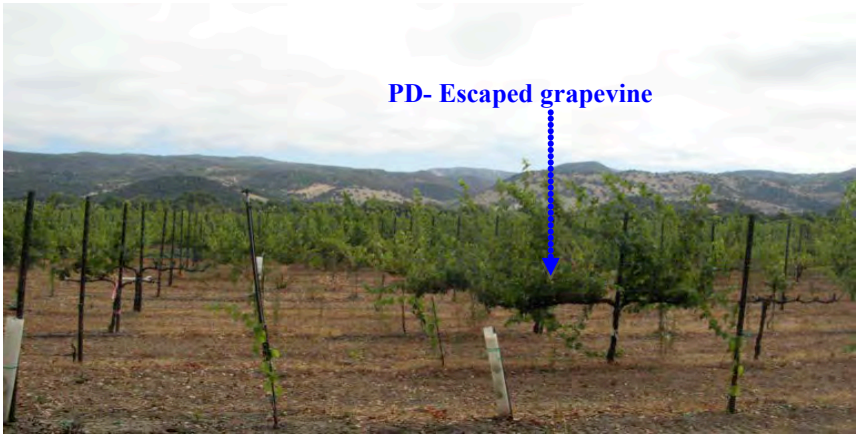

Figure 1. PD-escaped- grapevines in a Riesling block infected with Pierce's disease in Napa County.

Table 1. Location, variety and timing of the sampling of vineyards.

Location	Vineyard #	Variety	Timing of Sampling
Riverside	Vineyard 1	Cabernet Sauvignon Chardonnay	August 2009
County	Vineyard 2	Syrah	September 2010 March 2011 October 2011[a] March/April 2012[b]
Napa County	Vineyard 3	Riesling Chardonnay Merlot	August 2010 April 2011 October 2011[a] March/April 2012[b]
	Vineyard 4	Chardonnay	August 2010 April 2011 October 2011[a] March/April 2012[b]

[a:] sampling was completed and fungi are currently being identified
[b;] future sampling dates

Table 2. Identification and percent recovery of fungal taxa from PD-escaped and PD-infected grapevines. Results are based on sampling from 5 grapevine varieties (Merlot, Cabernet Sauvignon, Chardonnay, Riesling, Syrah), in 4 vineyards located in Napa and Riverside County for sampling times in August 2009, August/September 2010 and April/March 2011. Fungi were isolated from xylem sap, green shoots and wood spur.

Fungal Taxa	Percent Recovery	
	Escaped Grapevines (n=26)	Diseased Grapevines (n=19)
Cladosporium sp.	77	53
Aureobasidium sp.	81	74
Alternaria sp.	12	16
Cryptococcus sp.	12	11
Penicillium sp.	4	5
Geomyces sp.	4	5
Peyronellae sp.	8	
Drechslera sp.	4	
Discostroma sp.	4	
Cochliobolus sp.	4	
Chaetomium sp.	8	
Phaeosphaeria sp.	4	
Oidiodendron sp.	4	
Diplodia sp.	4	
Epicoccum sp.		5
Phomopsis sp.		5
Fusarium sp.		11
Biscogniauxia sp.		5
Cryptosporiopsis sp.		5
Ulocladium sp.		16
Pezizomycete sp.		11
Didymella sp.		5

Characterization of the fungal population using oligonucleotide-based fingerprinting of rRNA genes (13) is underway to characterize the total (culturable and non-culturable) fungal population inhabiting grapevines. We have extracted the total DNA from diseased and PD-escaped grapevines using Qiagen Plant DNA extraction kit, and were able to PCR amplify the total ribosomal DNA. We are currently conducting the DNA based population analysis.

Objective 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).

The goal of this objective is to identify fungal species and fungal natural products produced by these species that can be used as treatments for control of PD. Fungal cultures recovered from xylem sap, shoot, petioles and spur isolations (Obj. 1) were evaluated in an *in vitro* inhibition assay for antagonism against *Xf*. In brief, *Xf* liquid cultures were adjusted to OD_{600nm}=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 sterile circle of agar was drawn from the margin of an actively growing pure fungal culture and was placed onto the plates previously inoculated with *Xf.* Plates were incubated at 28ºC for seven days and then observed for an inhibition zone around the fungal colony (**Figure 2**). Fungal species with a halo of inhibition were considered antagonistic to *Xf*. All the fungal specimens showing inhibition are being identified to the species level using multi-gene sequencing and morphological identification.

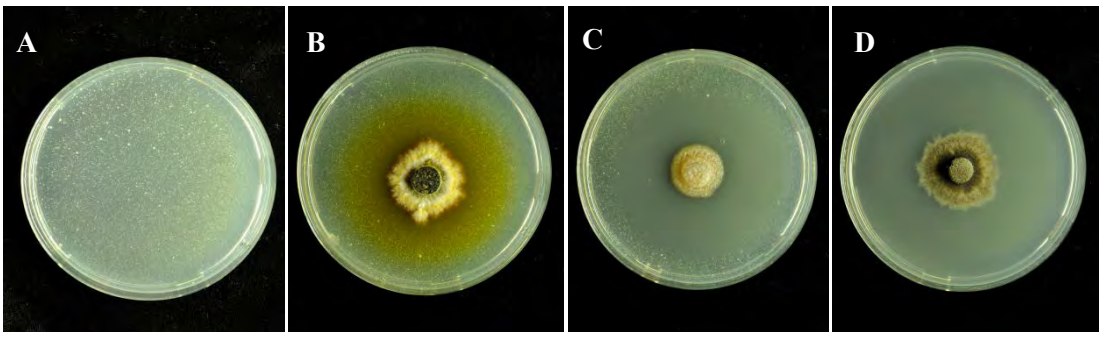

Figure 2. *In vitro* inhibition assay for fungi. Results show; (A) control; (B) no inhibition of *Xf* around fungal growth; (C) partial inhibition of *Xf* as shown with the halo around the fungal growth (D) total inhibition of *Xf.*

In addition, crude extracts collected from the fungal cultures showing inhibition towards *Xf* was collected for evaluation using the growth inhibition assay as described above. Fungal crude extracts were extracted as follows; agar plugs of 0.5 cm diameter of each fungus were used to inoculate 250 mL liquid media, and the fungi were cultivated at room temperature on a shaker. After seven days, each culture was filtered and further extracted with ethyl acetate, re-suspended in sterile methanol to an extract mass of 1mg, 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 and compared to control *Xf*-only plates and plates with paper discs treated with methanol only. The efficacy of fungal crude extracts was initially pre-screened in a high throughput method using three paper discs per plate (data not shown) and when inhibition of *Xf* growth was observed, the experiment was repeated using one disc per plate (**Figure 3**).

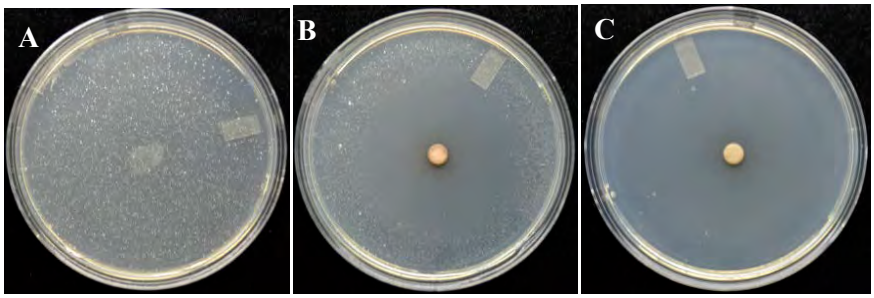

Figure 3. *In vitro* inhibition assay for fungal crude extracts. Results show; **A** Control *Xf* only; **B** intermediate inhibition of *Xf* as shown by the halo around the disc; **C** good inhibition of *Xf*, as shown by the absence of *Xf* growth around the disc. Note: no clearings were formed around the methanol-treated disc only control.

From the field sampling we have identified nine fungal taxa that inhibited *Xf* growth *in vitro* with various degrees of inhibition. We are currently testing the potential of four of these fungi as biocontrol agents *in planta* (see Objective 3). We have extracted crude extracts from six of the fungi that showed inhibition of *Xf* growth *in vitro.* We are currently fractionating the crude extracts from these six fungi in order to purify and identify the inhibitory molecule. Thus far, we have purified two individual molecules that are active against *Xf* growth *in vitro* and identified them to the chemical structural

level. These molecules and fungi are currently under review for patentability by the Executive Licensing Officer in the UC-Riverside Office of Research and, hence, their names cannot be disclosed in this report.

Objective 3. Evaluate biological control activity of the fungal candidates *in planta*.
The goal of this objective is to provide increased tolerance to PD by inoculating grapes with natural fungal endophytes that possessed anti-*Xf* properties. We have selected four fungal candidates that displayed two features; 1) they showed inhibitory effect of *Xf* in *in vitro* assays (Obj. 2); 2) they were heavily sporulating in culture. Spore formation is an important criteria. Because of the small size and shape of the spores, they are more likely to successfully infiltrate and colonize the plant xylem vessels as opposed to the larger vegetative structures, such as fungal hyphae. Spores of fungi 1 to 4 that were grown on PDA medium were harvested in sterile water and the concentration was adjusted to 10^5 to 10^6 to spores/µl. Grapes cuttings var. 'Merlot' (with 2 buds) were vacuum infiltrated (**Figure 4**) with the fungal spore suspension, planted and placed in the greenhouse. Control plants were infiltrated with sterile water only. In June of 2011, green shoots arising from these cuttings were inoculated with *Xf* (Temecula strain) by mechanical needle inoculation (7). A sub-sample of plants was left un-inoculated with *Xf* to determine if the concentration of fungal spore treatment used is detrimental itself to the grape cuttings. Plant symptoms were rated from 0 to 5 weekly (0= no symptoms; 5=Plant dead or dying) according to Guilhabert and Kirkpatrick (6). (**Figure 5**).

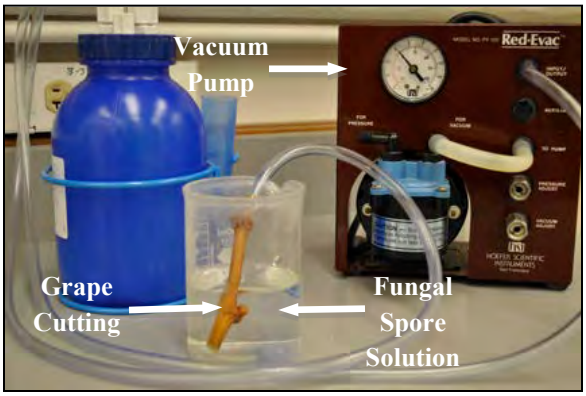

Figure 4. Technique used to vacuum infiltrate grape cuttings with spores of fungal endophytes that showed inhibitory effects in the *Xf in vitro* inhibition assay.

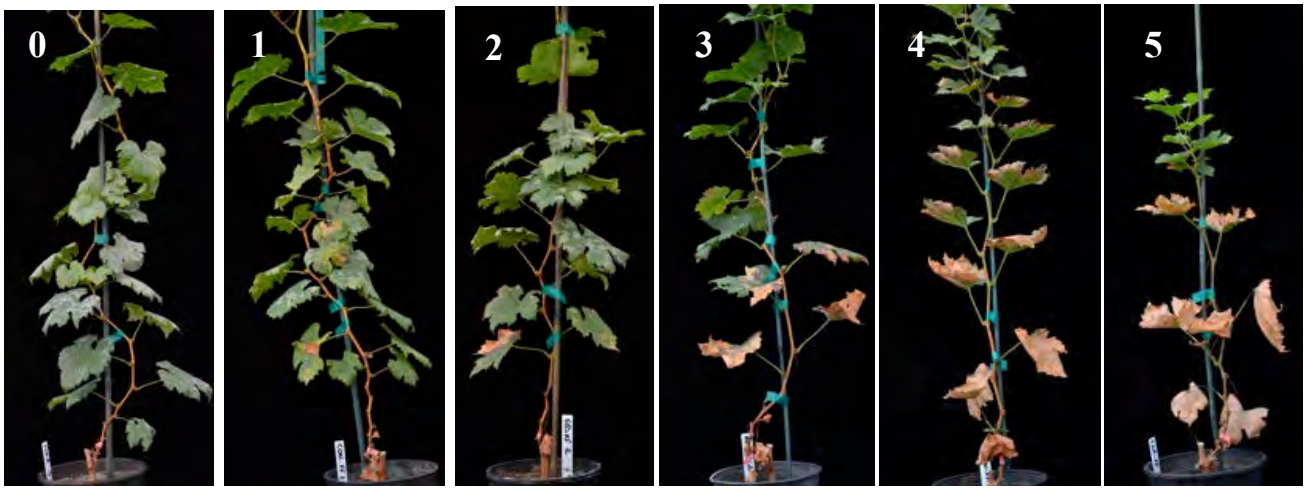

Figure 5. PD symptoms severity rating in grapevine cv. 'Merlot'; 0 = no symptoms (Mock inoculation); 1 through 5= grapes infected with the wild type strain Temecula showing an increase in the disease severity.

Our results (**Figure 6**) showed that the progression of the disease symptoms over a 14 week-period were less when grape cuttings were previously vacuum infiltrated with two fungal endophytes (fungus 1 and 3) that showed inhibition to *Xf* in the previous *in vitro* inhibition assay (Obj. 2). The two other fungal endophytes (fungus 2 and 4) show little to no reduction in disease progression. No fungi were detrimental to the plant. Plant will be rated for a total of 18 weeks post-*Xf* inoculation after which petioles will be sampled, and brought back to the laboratory to quantify *Xf* titer in the plants. Petioles will be

surface sterilized and plated on PD3 medium in order to quantify *Xf* populations per plant. We will also confirm that the inhibitory fungi were able to systemically colonize the plant by recovering them from cut petiole tissues on PDA medium. Statistical analyses will be performed at the end of the experiment to determine if the treatments are statistically different from the control. This experiment will be repeated in 2012 with additional potential biocontrols.

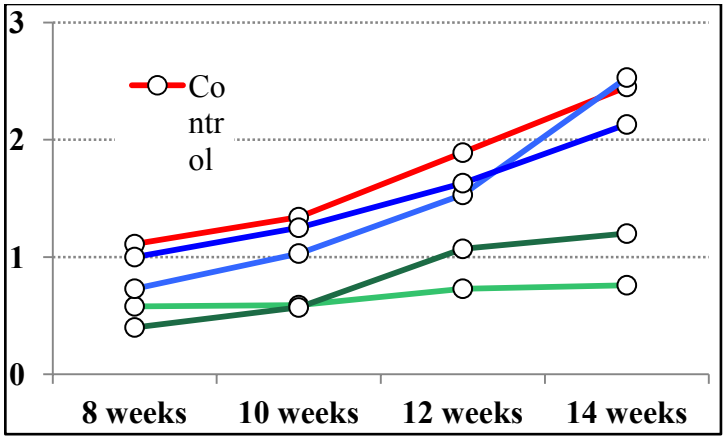

Figure 6: PD severity progression between eight and fourteen weeks post-inoculation with *Xf* 'Temecula' strain on grape cuttings cv. 'Merlot' previously vacuum infiltrated with four different fungal endophytes of grapevine showing inhibition to *Xf* in *in vitro* assays.

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 nine promising fungal candidates that inhibit *Xf in vitro*. Four fungi were evaluated as potential biocontrols and were vacuum-infiltrated into grape cuttings cv. 'Merlot' that were later inoculated with *Xf* Temecula strain. Our results showed that two fungi were able to reduce disease progress and severity after 14 weeks post-inoculation. The goal is to inoculate plant materials with these fungal biocontrols at the nursery level so that they can provide a prophylactic control against PD in natural vineyard settings. In addition, we are currently evaluating the antagonistic efficiency of the fungal natural products against *Xf* in *in vitro* inhibition assay and characterizing their structure and chemical properties. Thus far we have identified two molecules that inhibited *Xf in vitro*. The goal is to develop these natural products to commercial products that can be used as curative treatments for grapevines already infected with PD.

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FUNDING AGENCIES
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CAN PIERCE'S DISEASE *PDR1* RESISTANCE INTROGRESSED INTO *VITIS VINIFERA* BE TRANSLOCATED FROM A RESISTANT ROOTSTOCK TO A SUSCEPTIBLE SCION?

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ABSTRACT
The goal of this research is to evaluate the potential of a non-transgenic, Pierce's disease (PD) resistant *Vitis vinifera* selection used as an experimental rootstock to confer systemic resistance to PD susceptible *V. vinifera* scions. Source of PD susceptible plant material will be the wine grape variety 'Chardonnay', known to support high populations of *Xylella fastidiosa (Xf)* and exhibit severe PD symptoms. Source of PD resistant material will be a modified backcross generation 2 (mBC2) raisin selection with PD resistance locus *PdR1* introgressed from 89-F0908 (*V. rupestris* X *V. arizonica*). Scions will be mechanically inoculated with *Xf* strain Stags Leap. PD severity will be visually assessed using a nominal 0-5 rating scale where 0 corresponds to no visual symptoms and 5 corresponds to death of the plant. Following development of PD symptoms on the positive control ('Chardonnay' as both scion and rootstock), anticipated to be ~12-16 weeks post inoculation, tissue samples (petioles) will be assayed for *Xf* titer by real-time PCR.

LAYPERSON SUMMARY
Pierce's disease (PD) resistance from a wild grapevine species has been transferred into *Vitis vinifera* via classical (non-transgenic) breeding. However, given the extensive number of wine, raisin, and table grape varieties susceptible to PD, introgression into each will be time consuming and costly. In this research, proof of concept experiments will be conducted in greenhouse trials to determine if PD resistance in a *V. vinifera* selection used as a rootstock may be translocated to susceptible *V. vinifera* scions.

INTRODUCTION
This new project describes pilot experiments designed to test the hypothesis that a Pierce's disease (PD) resistant rootstock can affect PD development in susceptible scions. It is known that rootstock selection can affect symptom expression resulting from *Xylella. Fastidiosa (Xf)* infection of peach and citrus (Gould et al., 1991; He et al., 2000), and these observations were used as a rationale by Cousins and Goolsby (2010) to initiate examination of five grape rootstocks for potential to reduce PD symptoms in susceptible scions. The *V. vinifera* selection to be used as an experimental rootstock bears the *PdR1* resistance locus introgressed from 89-F0908 (*V. rupestris* X *V. arizonica*) that is known to confer high levels of PD resistance via reduction of *Xf* population levels (Buzkan et al., 2005; Krivanek et al., 2006; Riaz et al., 2009) and xylem sap from *PdR1* plants reduce growth of *Xf* in culture (Cheng et al., 2009). As the *PdR1* resistant rootstock to be used in these experiments is a second generation backcross with a genetic composition of ~87.5% *Vitis vinifera*, difficulties encountered by Lin and Walker (2004) in establishing sound graft unions between *V. vinifera* scions and rootstocks derived from wild *Vitis* species should be eliminated. The simple experimental design to be used will determine whether or not the *PdR1* resistance factor(s) is (are) capable of systemic protection of tissues beyond the graft union to affect pathogenesis of *Xf* in susceptible scions.

OBJECTIVE
Determine effect of rootstock genetic background (+/- *PdR1*) on disease severity and *Xf* population levels in PD susceptible scions following challenge inoculation of scions with *Xf*.

RESULTS AND DISCUSSION
Data are not yet available for this newly funded project.

CONCLUSIONS
No conclusions may be drawn at this time.

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FUNDING AGENCIES

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N-ACETYL-L-CYSTEINE, A NEW PERSPECTIVE FOR *XYLELLA FASTIDIOSA* CONTROL

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ABSTRACT

Xylella fastidiosa (Xf) multiply and attach to the vessel walls, forming biofilm that can, when sufficiently large, occlude xylem vessels, blocking water and nutrient transport in many different plant species such as citrus, grapevine, plum, almond, peach, and coffee. Fimbrial and afimbrial proteins seem to be essential for colonization and biofilm formation in *Xf*. Disulfide bonds play an important role in the folding and stability of such proteins. N-acetyl-L-cysteine (NAC), a thiol-containing antioxidant, is an analogue of cysteine that disrupts disulfide bonds in mucus, and has been used in medical treatment of humans with chronic bronchitis for instance. NAC also decreases biofilm formation by a variety of bacteria, and reduces the production of an extracellular polysaccharide matrix, while promoting the disruption of mature biofilms. This antibacterial property, together with the ability to avoid biofilm formation and to induce its detachment makes NAC an excellent candidate to be tested against *Xf*. The effect of this molecule in a phytopathogen has never been studied, thus, the aim of this work was, in general, to evaluate the activity of NAC against *Xf* biofilm cells. More specifically, we investigated the effects of NAC on (i) biofilm cellular mass and cell viability, (ii) production of extracellular polysaccharides (EPS), (iii) CVC symptoms in sweet orange (*C. sinensis*) infected plants, (iv) uptake of NAC by those plants, (v) its environmental degradation rate, and (vi) the variation of the number of viable *Xf* cells in plants treated or not with NAC. Results of biomass quantification, number of viable cells, total exopolysaccharide content, and microscope fluorescence images of *in vitro* cultured biofilms revealed that all the tested doses of NAC (1.0, 2.0, and 6.0 mg / mL) led to a decrease in biofilm formation, and inhibited growth of *Xf*, which indicates that this substance could also be toxic for this bacterium. *In vivo* experiments showed a strong reduction in CVC symptoms in *C. sinensis* treated with different doses of NAC three months after treatment. The amount of NAC added to the plant was monitored by HPLC and it seems that the plant absorbed the analogue. The population of the bacteria was lower in plants with NAC but was still possible to detect living cells. These results indicate that NAC may have an effect on *Xf* biofilm and the symptoms remission could be a possible consequence of restoration of the xylem flow. Approximately three months after stopping the treatment with NAC, the initial symptoms returned. These results open a new perspective for the use of this molecule on *Xf* control, where the improvement of NAC absorption by the plant (low absorption would increase the availability time of NAC), the NAC association with other molecules like Cu or Zn, and the time of application, could keep the plant in the field without the disease symptoms caused by *Xf*.

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Section 5:

Crop Biology and Disease Epidemiology

BLOCKING *XYLELLA FASTIDIOSA* TRANSMISSION

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ABSTRACT
This report describes work performed since October 2010. First, we summarize results on the transmission efficiency of 20 *Xylella fastidiosa* (*Xf)* mutants. Many mutants were deficient in vector transmission, but none resulted in complete abolishment of transmission. These results support previous results and indicate that *Xf* colonization of vectors is a complex process. Second, we present data on the interference of *Xf* vector transmission by disrupting cell-surface molecular interactions. Three groups of molecules were used to block transmission, i) lectins that would bind to receptors on the vector's foregut, ii) carbohydrates that would bind to adhesins on *Xf*'s surface, and iii) antibodies that bound to various *Xf* cell surface components. All these approaches resulted in significant blockage of vector transmission. These results not only provide confirmation of the nature of *Xf*-vector interactions, but also show that targeting specific interactions at the molecular level is a feasible approach to control disease spread.

LAYPERSON SUMMARY
Pierce's disease (PD) of grapevines and other *Xylella fastidiosa (Xf)* diseases are the outcome of complex interactions among plants, sharpshooters and bacterial pathogen. To move between hosts *Xf* must be transmitted by sharpshooter vectors, and although this represents an essential component of PD (i.e. no transmission no disease), it is also its most poorly studied aspect. Research on *Xf*-vector interactions will not only lead to novel approaches to limit pathogen transmission, but will also lead to new biological insights that may assist in the development of other control strategies. Here we report on the transmission efficiency of several *Xf* mutants and on efforts to block transmission. Results indicate that vector colonization is a complex process, as several mutants were impacted in vector transmission, but none resulted in zero transmission. One of the most important findings is that *Xf* transmission can be disrupted by blocking adhesins on the cell surface that interact with vectors, and receptors on the cuticle of vectors that cells use to initiate sharpshooter colonization. In other words, disrupting vector transmission of *Xf* is feasible and would lead to control strategies that are not aimed at killing vector or pathogen, but suppressing disease spread in a sustainable and environmentally sound manner.

INTRODUCTION
This report is divided into two sections: i) testing of several mutants for their transmissibility by vectors, and ii) work to show that transmission can be disrupted by the use of specific molecules that affect *Xylella fastidiosa (Xf)*-vector interactions. Although both sections address molecular aspects of *Xf* transmission, we provide a brief introduction to each, as the specific goals of these studies are different. Final results are pending for aspects of the study using mutants, while those are final for the transmission blocking work reported here; the latter is also followed by a detailed discussion. Sections of this report are redundant with previously submitted reports (unpublished) as there is an overlap in the reporting period (specially the work with mutants).

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

The long-term goal of this work is to develop tools to block *Xf* transmission by sharpshooter vectors, which we show here to be a feasible disease control strategy.

RESULTS AND DISCUSSION
In a previous report (2010 Proceedings) we report on initial findings part of this project. Essentially, we found that *Xf* has a functional chitin utilization machinery and is capable of utilization chitin as a carbon source. That work has been published and will not be discussed here (Killiny, N., Prado, S.S. and Almeida, R.P.P. 2010. Chitin utilization by the insect-transmitted

bacterium *Xylella fastidiosa*. Applied and Environmental Microbiology 76: 6134-6140). Here we focus on unpublished data. We have also submitted several libraries for RNA-Seq using the Illumina platform, those are currently pending in the UC Berkeley sequencing facility. The goal of those is to compare media that induce *Xf* transmission with those that do not, hopefully allowing us to identify novel transmission blocking candidate genes.

Transmission of *Xf* mutants

So far the role of very few genes on vector transmission of *Xf* is understood. To address this important knowledge gap we tested the transmission efficiency of 20 *Xf* mutants. We used a protocol developed by our group based on an artificial diet system that eliminates the need of using infected plants as a pathogen source to insects (Killiny and Almeida 2009b), which prohibits adequate analyses of vector-pathogen interactions because many mutants are deficient in plant colonization. We note that these mutants were generated by other research groups and kindly provided to us for this study.

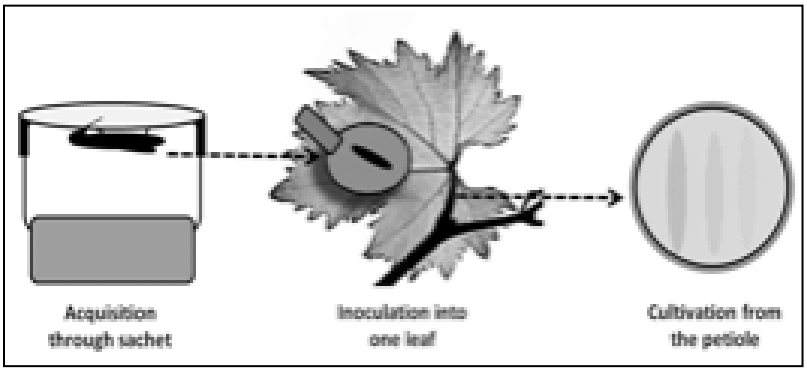

Figure 1. Experimental design to test *Xf* mutants for vector transmission.

Briefly, our experimental design was aimed at determining if strains were transmitted by vectors without considering plant-pathogen interactions (or minimizing its importance). Insects acquired cells from an artificial diet system for a few hours, then were transferred to a leaf on a healthy plant for inoculation (**Figure 1**). This leaf was tested two weeks after inoculation for *Xf*. We used plants for inoculation, but those were tested two weeks after insect access to reduce potential problems with mutants that cannot colonize plants. This approach allowed us to show that mutants that are deficient in plant colonization are transmissible by vectors. We were successful in obtaining positive inoculation events into artificial diets as well (Rashed et al. 2011), but we believe that vector probing behavior in plants and diets are reasonably different and could affect inoculation efficiency. Thus, the approach used looks at initial cell adhesion and colonization of vectors, followed by inoculation into plants, requiring short term survival/multiplication of cells, which is expected even from mutants that do not colonize grapevines. We have also quantified bacterial populations within sharpshooters with qPCR (Killiny and Almeida 2009a) after acquisition and one week later to gain insights on retention of the various strains. Data have been collected but are still being analyzed.

Because of the large number of mutants tested, different experiments (n=8) were performed and each one had its own wild type control (**Figure 2**). Transmission rates for the wild type ranged from 80 to 92%. The figure below summarizes our results. For comparative purposes, transmission rates of the wild type control in independent experiments were all normalized (to 100%), allowing a comparison among the various mutants. Statistical analyses need to be performed to compare the treatments tested, but for the purpose of discussion we may consider mutants with less than ~40% transmission as being deficient in this essential trait to *Xf*'s biology.

Mutants tested so far can be arbitrarily divided into four categories: adhesins, gene regulation, AT-1 transporters, and gum- and pectinase-deficient mutants. We will briefly discuss our results; a more complete discussion depends on the retention data we are yet to analyze. Among adhesins, all those tested affected transmission, except for *pilB*, which is part of type IV pilus involved in *Xf* twitching motility. Gum-deficient mutants were expected to be unable of biofilm formation within vectors, thus their lower transmission rates is not unexpected. We have previously shown that pectin degradation by the polygalacturonase (PglA) is required for vector transmission of *Xf* cells grown on the XFM-pectin medium used to induce vector transmissibility (Killiny and Almeida 2009). Thus, we did not expect much if any transmission of the *pglA* mutant. Regulatory genes involved in cell-cell signaling and within-cell signaling have been demonstrated to be deficient in transmission, results obtained here were similar to those with whole plants, for example, *rpfC* is affected in transmission, but less than *rpfF* (Newman et al. 2004, Chatterjee et al. 2008, 2010). Our results were within expectations, as much of *Xf*'s biology is affected by cell-cell signaling and within cell gene regulation. The AT-1 autotransporters tested were not affected as much, although the double mutant *xatA/B* was transmitted only 40% of the time. However, results with the complemented mutant *xatA* show that this protein has some role in transmission, as complementation revert transmission back to wild type level.

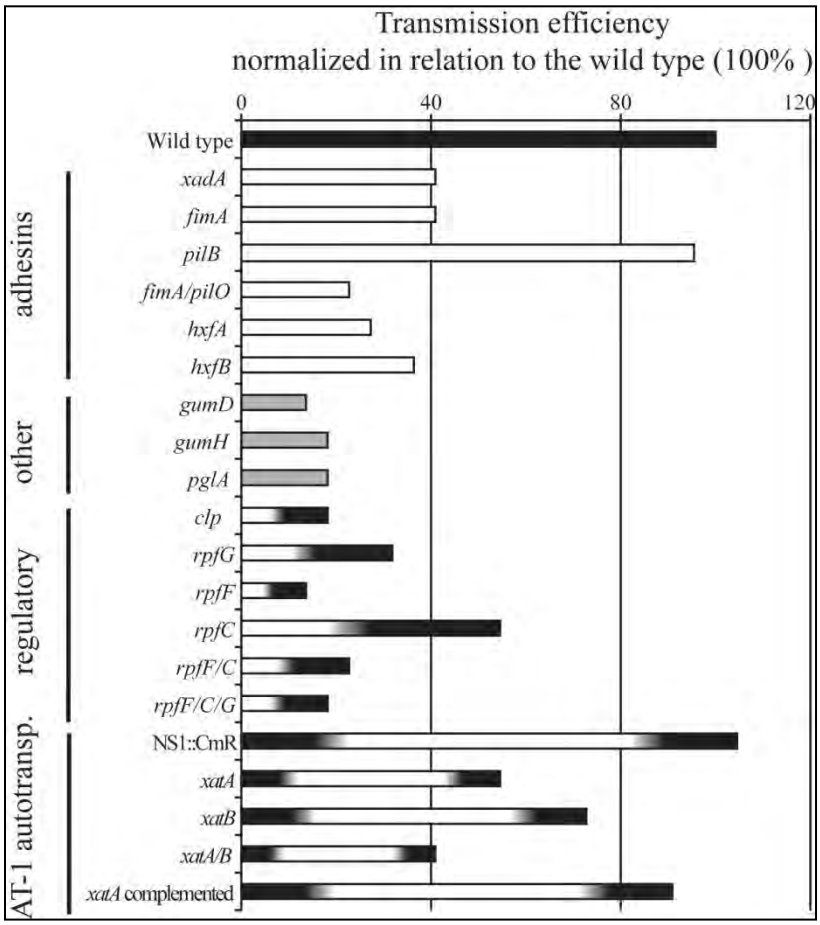

Figure 2. Transmission efficiency of *Xf* mutants in relation to the wild type.

A few general points need to be addressed. First, it is now evident that the *in vitro* system developed to study transmission independently of plants mimics experiments with plants adequately. For example, general trends obtained with transmission of the following mutants with plants were reproduced with the artificial diet system: *hxfA*, *hxfB*, *rpfF*, *rpfC*. This is important as a majority of mutants, such as *gumD* and *pilB*, cannot be tested using plants, but this protocol allows them to be studied in relation to vector transmission. Second, all mutants were transmissible to some degree in this study. This indicates that transmission is complex and not dependent on a single factor, such as only one adhesin. The fact that multiple factors are important for vector colonization is not surprising, but the results identified novel targets to block transmission. It should be mentioned that we believe that cells provided to insects are 'super sticky' and offered at higher densities than if acquisition occurred from plants, potentially inflating the proportion of transmitting individuals. In other words, transmission rates could be lower if plant-to-plant assays were performed.

Blocking of *Xf* transmission

The surface colonized by *Xf* in insects is not well characterized, but the nature of cell-vector interactions has been demonstrated to depend on carbohydrate-protein interactions (Killiny and Almeida 2009a). Cell surface proteins mediated *Xf* attachment to various substrates, including leafhopper foregut extracts and hindwings. In addition, adhesion decreased when certain carbohydrates were added to suspensions in adhesion assays, indicating that carbohydrate-binding proteins on the cell surface are substrate specific and that saturation of these proteins affects adhesion. Much like a biofilm, however, *Xf* colonization of vectors is likely a complex multi-step process, in which different factors are important for each step of biofilm formation, from initial cell adhesion to colony maturation (Almeida and Purcell 2006, Killiny and Almeida 2009a). Work on *Xf*-vector interactions has focused primarily on the early stages of biofilm formation, i.e. initial cell adhesion. Because of the protein-carbohydrate nature of the *Xf*-vector interface, it should be possible to disrupt transmission by saturating carbohydrate-binding proteins on cell surfaces; alternatively, lectins (carbohydrate-binding proteins) could be used to mask carbohydrate coated surfaces on the foregut of leafhoppers. In addition, *Xf*-derived antibodies could also reduce transmission if they were to bind to proteins on the cell surface that are involved in vector adhesion. We performed a series of experiments testing different approaches to block leafhopper transmission of *Xf* to plants. The experimental design used here was similar to tests with mutants, except that molecules (lectins, carbohydrates or antibodies) were all mixed together with cells in the diet. We will focus on results instead of methods.

Lectins affect vector survivorship. We detected a significant among-treatment variation in insect survivorship (log rank X_6^2 = 165.63, $P < 0.001$) with lentil lectin (LL) and peanut lectin (PL) being the two treatments inflicting the highest mortality rate across all three concentrations over time. Neither concentration nor treatment caused insect mortality within the first 48 hours of exposure. Following this verification of survivorship within experimental time-frame for transmission experiments (4 hours of exposure followed by at 12-hour IAP on healthy grapevines) we proceeded to the competition assay with lectins using the 0.1% concentration. This intermediate concentration was selected to reduce potential effects of higher concentrations on insect feeding, meanwhile assuring the presence of a minimum number of competitor molecules in suspensions for the competition experiments.

Lectins reduce transmission efficiency. The probability of a successful transmission event was significantly affected by lectin treatments (Wald X_6^2 = 44.84, $P < 0.001$, **Figure 3**). Compared to the sharpshooters that fed in the control diet, insects belonging to all lectin treatments showed a significant reduction in transmission rate; PL (Wald X_1^2 = 4.93, P == 0.026), LL (Wald X_1^2 = 5.15, P = 0.023), wheat germ agglutinin (WGA) (Wald X_1^2 = 23.67, $P < 0.001$), and concanavalin A (CoA) (Wald X_1^2 = 11.71, $P < 0.001$). *Xf* transmission rate by leafhoppers fed on diets with either BSA or OV was not different from that of the control (BSA: Wald X_1^2 = 0.01, P = 0.975; OV: Wald X_1^2 = 1.49, P = 0.221). Transmission rate of the insects treated with WGA was significantly lower than all other treatments except for CoA. Insects treated with CoA also had lower transmission rates than both OV and BSA treatments, but not compared to peanut and lentil lectins.

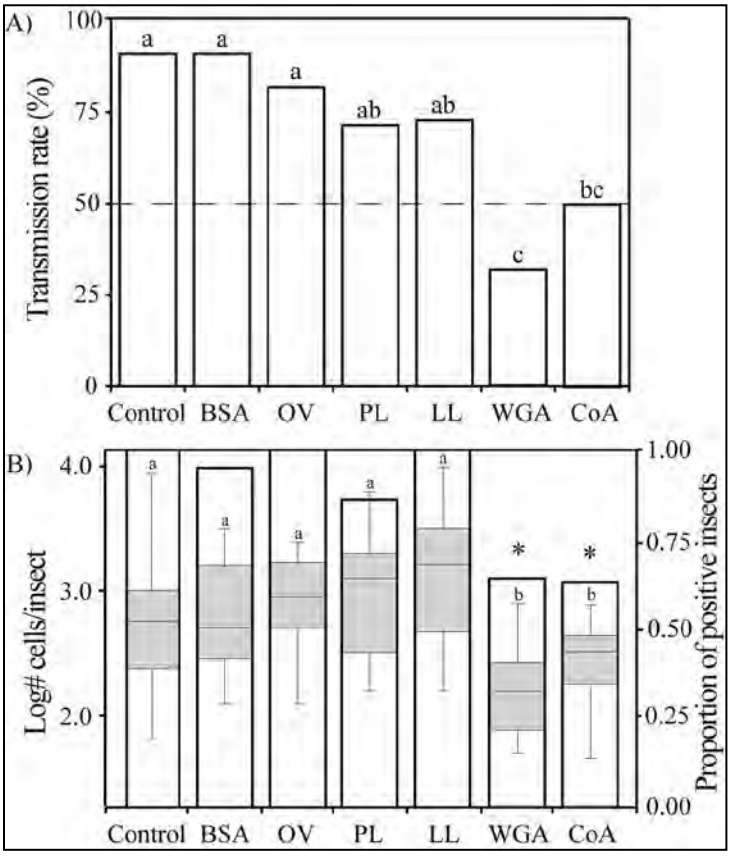

Figure 3. Lectins affect vector transmission of *Xylella fastidiosa* to plants. A) Lectins, BSA and OV were provided to insects through diet solutions containing *Xf* cells; control contained cells only. Treatments with different letters on bars indicate statistically different treatments. B) *Xf* retention and populations within vectors after the inoculation access period no plants. Y-axis on the right represents the proportion of insects that were positive for *Xf* (empty bars); WGA and CoA were the only treatments statistically different from the control (marked with asterisks). Y-axis on the left shows bacterial populations within insects as measure by quantitative PCR; boxes show the interquartile range including 50% of results, mid-horizontal line represents the median; different letters on top of error bars represent statistically significant different treatments. BSA – bovine serun albumine, OV – ovalbumine, PL – peanut lectin, LL – lentil lectin, WGA – wheat germ agglutinin and CoA – concanavalin A.

There was a significant variation in acquisition rates among the insects treated with different lectins (X_6^2 = 27.70, $P < 0.001$), which was measured by quantitative PCR after the 12-hour IAP on plants. The proportion of leafhoppers that acquired the

pathogen in WGA and CoA treatments was significantly lower than that of the control (Ps < 0.003). Acquisition rate for the other treatments was not statistically different from the control (Ps > 0.232). These differences are likely not associated with feeding deterrence due to the presence of lectins in the diet, as LL and PL were the lectins that were toxic to leafhoppers, not WGA or CoA. Similarly to the acquisition rates, the number of bacterial cells acquired and retained by the experimental insects varied significantly among-treatments (ANOVA: $F_{6, 126} = 7.71$, $P < 0.001$). Further pairwise comparisons indicated the number of cells recovered from the insect heads in WGA treatment was significantly lower than all other treatments (Tukey, Ps < 0.043), with the exception from CoA (Tukey, $P = 0.54$).

Carbohydrates with GlcNAc units affect transmission rate. Overall, carbohydrate groups with an acetyl amine group significantly reduced *Xf* transmission rate by vectors (**Figure 4**). For the insects treated with glucose, galactose or mannose, all lacking GlcNAc, the rate of successful transmission events was not statistically different from the control (binary logistic regression: Ps > 0.60). However, transmission rates varied significantly between vectors treated with the carbohydrates possessing GlcNAc and the control (Ps < 0.001). Transmission rates were not different among vectors treated with glucose, galactose, and mannose ($X_2^2 = 2.61$, $P = 0.12$). Likewise, no difference in transmission rates was detected among insects treated with GlcNAc, $GlcNAc_2$and $GlcNAc_3$ ($X_2^2 = 1.73$, $P = 0.42$).

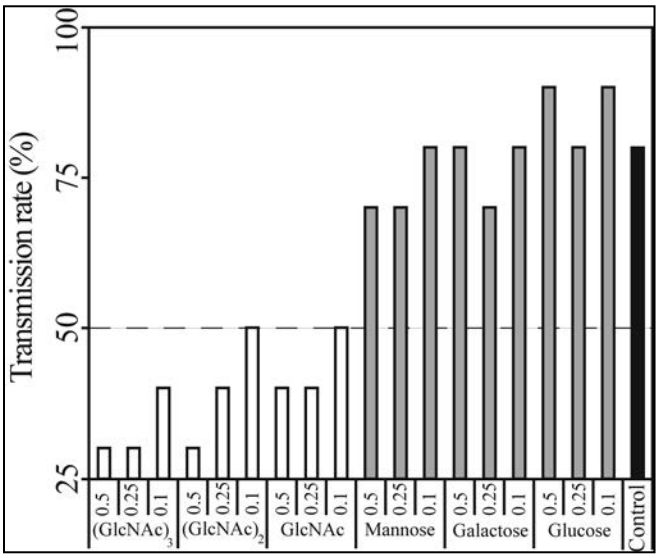

Figure 4. Carbohydrates with N-linked residues decrease *Xylella fastidiosa* vector transmission efficiency to plants. Three concentrations (0.1, 0.25 and 0.5M) were used for each saccharide tested in this competition assay, no differences among concentrations for any treatment were statistically significant; the control only included *Xf* cells. Carbohydrates with an *N*-acetylglucosamine (GlcNAc) residue were statistically equivalent to each other (white bars), but all were different from glucose, mannose and galactose, which also did not differ from each other (grey bars).

Antibody-mediated blocking of cell surface reduces transmission rate. There was a significant variation in transmission efficiencies among vectors treated with different antibodies (Wald $X_7^2 = 31.22$, $P < 0.001$; **Figure 5**). With the exception of PilA2 ($P = 0.068$) and PilC ($P = 0.111$), the two type IV pilus antibodies, all of the remaining antibody treatments significantly reduced transmission rate (Ps < 0.007). A separate Chi-square analysis revealed no variation in transmission rates of insects treated with antibodies against gum, whole cell, and the three different afimbrial adhesins ($X_4^2 = 4.53$, $P = 0.34$) .

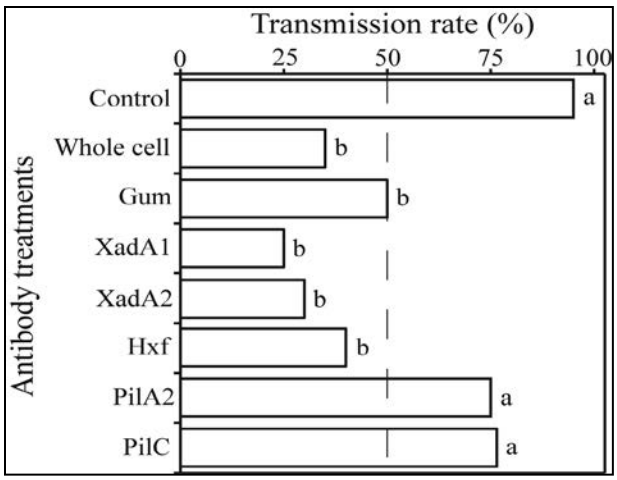

Figure 5. Cell surface antibodies reduce *Xylella fastidiosa* transmission to plants. Various antibodies were added to the diet solution containing *Xf*; different letters on bars indicate statistically different treatments. Antibodies prepared against whole cell, afimbrial adhesins (XadA1, XadA2, Hxf) and gum (EPS) all reduced transmission, while those against type IV pilus proteins did not.

Altogether, we tested three distinct approaches to determine if *Xf* transmission efficiency could be reduced by competitor molecules, based on the fact that carbohydrate-protein interactions appear to be essential in the pathogen-vector interface. Competition for receptors on the vector's foregut was performed using lectins with affinity for GlcNAc, which resulted in reduced transmission rates. Likewise, saturation of carbohydrate-binding proteins on the cell surface with GlcNAc-containing sugars reduced the rate of successful transmission events. Lastly, the use of various antibodies had a similar effect. Thus, various approaches significantly reduced vector transmission of *Xf*; however, no treatment completely abolished transmission. Similar results were observed with circulative viruses. Due to the biological complexity of biofilm establishment and development, it was expected that no individual treatment would result in total blockage of transmission.

Although two of the tested lectins, PL and LL, reduced insect survivorship down the road, this affect did not appear in the first 48 hours of the assay. However, it is possible that insect behavior was affected after acquisition of these molecules, resulting in the small but significant decrease in *Xf* transmission for those two treatments (PL and LL) in relation to the controls, as they were not expected to have much affinity for carbohydrates on the foregut surface. This possibility is highlighted by the fact that the number of cells in the foregut of vectors after the transmission experiment was not different from that of the control nor was the proportion of infective insects. In other words, vector colonization did not appear to be affected by PL or LL, and we interpret the small differences in transmission as being a consequence of changes in vector behavior. CoA and WGA, on the other hand, were lectins that resulted in significant decreases in bacterial populations in insects, a smaller proportion of positive individuals, and a decrease in transmission efficiency; neither was detrimental to insect survivorship. Although the number of vector colonization events is unknown when the artificial diet system employed here is used for pathogen acquisition, we expect that multiple colonization events occurred as insects were exposed to high densities of adhesive cells. Moreover, acquisition through artificial system occurs in a less turbulent environment compared to acquisition from plants. We interpret the results as a significant reduction in *Xf* initial adhesion to vectors, leading to fewer colonization events, as observed by a smaller proportion of positive insects. However, the smaller population in insects would be due to the fact that fewer cells were able to colonize the foregut, not an effect on those that successfully colonized that surface.

Pathogen adhesion to carbohydrates functioning as host receptors for attachment are common. For example, a streptococcal adhesin that recognizes and binds to a galactosyl-α 1-4-galactose-containing glycoconjugates host receptor has been described recently. Likewise, *Vibrio cholerae* surface proteins mediate cell attachment to chitin and the surface of copepods. In addition, the presence of chitin may induce the expression of proteins with strong affinity for chitin and chito-oligomers. Thus, lectin-carbohydrate interactions mediate the binding of several pathogens to their hosts; interfering with these interactions may lead to development of novel disease control strategies. *Xf* colonizes carbohydrate-rich surfaces in both plants and insects; therefore, it was not surprising that it adheres to such molecules. We have previously shown that cell adhesion to insect hindwings is reduced with the addition of GlcNAc, indicating that *Xf* carbohydrate-binding proteins are saturated by the presence of this monosaccharide. These *in vitro* findings were confirmed and successfully applied in multiple biological assays in the present study. GlcNAc-containing sugars significantly reduced transmission efficiency to plants, whereas mannose, galactose and glucose had no effect. The fact that the carbohydrates containing an acetyl amino group appeared to saturate adhesins on the cell surface indicates that the cuticular surface of leafhopper vectors is rich in

these molecules. The recent finding that *Xf* has a functional chitinase is further indication that GlcNAc is important for vector colonization.

Although assays based on competitor molecules as those with lectins and carbohydrates provide general information about the nature of *Xf*-vector interactions, they do not address the role of specific components of the cell's surface that may be required for vector colonization. Targeting specific genes via knockout mutants is one approach to identify factors required for transmission, such as work performed with the afimbrial adhesins HxfA and HxfB. An alternative approach is to use antibodies against the same targets, where masking of Hxfs, for example, would reduce transmission if they were involved in that process. Indeed, we found that antibodies generated against whole cells, gum, and afimbrial adhesins (XadA1, XadA2 and Hxf) all reduced vector transmission of *Xf* to plants. HxfA and HxfB have been shown to be involved in transmission, but no data are available for the other adhesins or gum. We propose that XadA1 and XadA2 are also involved in vector transmission. It is possible that gum is involved in initial attachment to vectors, it is also plausible that gum is evenly distributed on the cell surface and that these antibodies may have resulted in inadvertent masking of adhesins actually involved in this vector early colonization. This interpretation does not mean that gum is not required for successful vector colonization, but that it may not be as important for initial adhesion. Lastly, PilC is involved in type IV pilus assembly and appears to be bound to the cell membrane, therefore it does not appear to be located on the cell surface and should not affect cell attachment. PilA2 is a rod-forming unit and was observed both in cell membranes and outside cells, presumably on pili. The fact that the PilA2 antibody did not reduce transmission efficiency is suggestive that type IV pili are not involved in the initial colonization of vectors. Twitching motility is mediated by type IV pili in *Xf*, and is involved in cell movement within plants. As mentioned previously, because the experimental design may result in higher transmission rates than if plant-to-plant experiments were used, we would also expect that the efficiency of blocking molecules would be higher (i.e. lower transmission) if conditions for vector colonization had not been artificially optimized.

CONCLUSIONS

We report on the role of 20 *Xf* genes in vector transmission using an artificial diet system. Many mutants were impacted on transmission, although none was not transmissible. This may be a consequence of the fact that cells used were highly adhesive and provided in large number to insect vectors. Overall, results suggest that transmission is a complex process mediated by various factors, and that the experimental approach allows for meaningful comparisons in relation to the wild type. Proof-of-concept experiments were performed to determine if it was possible to disrupt *Xf* transmission by blocking pathogen-vector interactions. Results showed that carbohydrates can saturate adhesins on the cell surface so attachment to vectors is reduced, and that lectins can coat receptors on the vector's foregut so that cell can not bind to and colonize sharpshooters. These data show that blocking transmission is a feasible concept and represents a novel mechanism to suppress PD spread in vineyards. Current efforts are aimed at testing *Xf*-derived targets (i.e. adhesins that bind to vectors) because those would be ideal for field trials, as they are specific to this system and evolved to bind to sharpshooters.

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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 BC5 *Vitis arizonica* crosses (98.5% *V. vinifera*) were made in 2011 to continue creating germplasm with Pierce's disease (PD) resistance and high fruit quality. A total of 29 seedless x seedless crosses to develop BC3, BC4, and BC5 *V. arizonica* x *V. vinifera* families were made. The crosses consisted of 61,611 emasculations and produced 5,430 ovules and 877 (16.2%) embryos for PD resistance. An example of increased fruit quality is the selection for propagation in first stage production trials of six BC4 table, 8 BC4 and 3 BC3 raisin grape selections. One of the table grape selections is good enough to put in a 25 vine production trial. A total of 18 crosses to combine PM and PD resistance were made and consisted of 40,179 emasculations, 3,571 ovules and 651 embryos. The use of molecular markers to select for PD resistance and greenhouse screening to select for powdery mildew (PM) resistance allows selection of these two types of resistance before planting to the field. Two table and five raisin grapes were propagated into first stage production trials from 31 PD plus PM resistant seedlings. PD resistance of over 38 parents and selections from *V. arizonica* populations have been tested in greenhouse tests to insure resistance continues to co-segregate with markers. Two hundred and forty-three seedlings of the BD5-117 mapping family, with PD resistance different than *V. arizonica*, have been greenhouse tested. A preliminary rough molecular map based on 70 SSR primers and 144 seedlings indicates a major QTL for PD resistance on linkage group 2.

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 cultivars existing in markets today. Sixth generation (BC5) crosses to produce quality table and raisin grapes with *Vitis arizonica* source of PD resistance were made for the first time this year. These families will have high fruit quality as they consist of 98.5% *V. vinifera*. An example of increased fruit quality is the selection for propagation in first stage production trials of six BC4 table, 8 BC4 and 3 BC3 raisin grape selections. These selections show some commercial potential with one table selections showing enough promise for inclusion in 25 vine cultural production trial. Crosses to combine powdery mildew(PM) and PD resistance were also made. The use of molecular markers to select for PD resistance and greenhouse screening to select for powdery mildew PM resistance allows selection before planting seedlings to the field. Two table and five raisin grapes with PD plus PM resistance were selected and propagated in first stage production trials. PD resistance of over 38 parents and selections from *V. arizonica* populations have been tested in greenhouse tests to insure the molecular markers for PD resistance continue to function properly. Two hundred and forty-three seedlings of the BD5-117 mapping family, which has PD resistance different than *V. arizonica*, have been greenhouse tested. A preliminary rough molecular map based on 70 SSR primers and 144 seedlings indicates a major QTL for PD resistance on linkage group 2. This means that selected molecular markers from linkage group 2 might be useful as markers to select resistance from BD5-117 source of resistance. 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 table grape cultivars in the southeastern United States, but fruit quality is inferior to *V. vinifera* table grape cultivars grown in California. No PD resistant raisin grape cultivars exist. 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 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-nine of 33 crosses using *V. arizonica* source of resistance made in 2011 were successful, consisting of 61,611 emasculations, and produced 5,866 berries, 5,430 ovules, and 877 embryos (16.2% embryos/ovules) (**Table 1**). The majority of these were BC4 and the first BC5 crosses. The seedlings obtained from these crosses should have high fruit quality as they now have 97 to 98.5% *V. vinifera* in their background. An additional 18 crosses to pyramid PD (*V. arizonica)* resistance with powdery mildew (PM) (*V. romanetii*) resistance consisting of 40,179 emasculations produced 3,772 berries, 3,571 ovules and 651 (18.2%) embryos (**Table 1**). Ten and five seeded crosses made in 2011 produced 646 seed for PD resistance and 1,190 seed for PD plus PM resistance respectively (**Table 2**). Leaves from all 2010 *V. arizonica* PdR1 plants were taken when seedlings were still in test tubes starting in November, 2010. They were tested for resistance with molecular markers for the PdR1 locus on chromosome 14. Results for 11 BC3 and 13 BC4 seedless x seedless families is shown in **Table 3**. A total of 213 individuals were tested with SSR markers and 159 showed markers on both sides of the PdR1 region. A total of 73 individuals (46% of those showing markers) were resistant and 86 plants had susceptible markers. 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. From the crosses made in 2010 which combined PD resistance from *V. arizonica* with PM resistance from *V. romanetii*, 79 seedlings were screened with molecular markers for PD resistance and in the greenhouse for PM resistance. The segregation ratios are shown in **Table 4** and they are similar to the expected ratios for segregation of PD and PM as single dominant genes. 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 38 selected individuals from *V. arizonica* (**Table 5**) of which 14 are resistant to date. These seedlings represent the best table and raisin selections that have been used as parents or planted in production trials. 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 its use will continue to be emphasized.

In 2011 five BC3 table grape selections were treated with 20 ppm giberellic acid (GA) at berry set to determine berry response. In all cases the berries increased in weight, diameter and length (**Table 6**). GA treated berries from all selections were similar in size or larger than Thompson Seedless produced by standard commercial practices at the ARS Parlier research station. The treatments used for Thompson Seedless were 15ppm GA x 2 bloom sprays; 20ppm GA bump spray; 60ppm GA x 2 size sprays; girdle and tip. The selection 08-5001-34 (Fig. 1A) had larger berries than both samples of Thompson Seedless and had only one application of GA. 08-5001-34 has very acceptable fruit quality. The majority of 800 PD resistant BC3 and BC4 *V. arizonica* seedlings planted in 2009 and 2010 fruited and were evaluated. From these seedlings, the number of selections good enough for propagation into the first stage production trials is: 6 BC4 table selections, 8 BC4 and 3 BC3 raisin selections. Fruiting characteristics of these selections are shown in **Table 7**. One of the table grape selections, 09-5013-118 (**Figure 1B**), is good enough for inclusion in the 25 vine advanced cultural production trial. One table grape selection with PD plus PM resistance was also selected for propagation. In addition the following selections were made for use as parents: 3 BC4, 9 BC3 and 1 BC1 table grape; and 6 BC4 raisin grapes. In 2011, 6 table and 8 raisin PD resistant selections and 2 table and 5 raisin PD plus PM resistant selections were planted in production trials. Half of the planting (four of 8 replications) of 12 selections at the USDA ARS research station, Weslaco, Texas were inoculated with *Xf* on July 15 with the help of David Appel, Texas A&M University. Each plant was inoculated in twice. Leaf samples will be collected this fall to determine *Xf* levels.

Objective 2

The PD resistant grape selection BD5-117 from Florida was hybridized with the seedless table grape selection C33-30 to create the mapping population of over 500 individuals. Fruit samples are being taken from all seedlings to have three years data for berry weight and seed/seed trace weight as an indication of fruit quality. Flower type is also being recorded. Greenhouse testing for PD resistance has been accomplished on 243 individuals, with 112 rated clearly resistant or clearly susceptible. One hundred twenty-two individuals are being evaluated this year (**Table 5**). The 70 polymorphic markers tested on 144 individuals and greenhouse PD resistance evaluations were run in JoinMap which indicated a major QTL on linkage group 2. Forty additional polymorphic primers have been tested on the 144 seedlings for further refinement of the rough framework map. In 2011, nine seedless x seedless and three seeded by seedless BC1 crosses were made for table grape improvement using resistant F1 selections from BD5-117 (**Table 1 and 2**). A total of 22 and 8 clusters were pollinated for these crosses and resulted in 304 embryos and 526 seed from seedless x seedless and seeded x seedless crosses respectively.

CONCLUSIONS

Additional families for the development of PD resistant seedless table and raisin grape cultivars are being produced. Emphasis was placed on making the first BC5 and additional BC4 *V. arizonica* PD resistant families. These families will have high fruit quality as they consist of 97 – 98.5% *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 BC4 and BC3 *V. arizonica* and F1 BD5-117 families. For example, six new table and 11 new raisin PD resistant and one new table PD plus PM resistant selections were made from BC4 and BC3 *V. arizonica* families and will be propagated for production trials. Two hundred and forty-three 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. Initial mapping indicated a major QTL for resistance on linkage group 2 in the BD5-117 family. The development of PD resistant table and raisin grape cultivars will make it possible to keep the grape industry 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. 2011 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*							
04-5015-5	C 61-123	Table	BC2	1201	60	46	21
04-5514-144	Y125- 39--05	Table	BC2	1 bag	185	315	36
06-5503-107	Y129-161--05	Table	BC4	619	90	92	2
Y152-128--08	08-5001-20	Table	BC4	3797	27	47	25
06-5501-229	Y536- 53	Table	BC4	2702	408	270	28
05-5501-69	B 26-120	Table	BC4	5 bags	197	5	0
06-5503-126	Y537-131--06	Table	BC3	694	227	136	30
06-5501-423	05-5502-15	Table	BC3	1737	212	257	65
06-5501-423	05-5501-27	Table	BC3	3064	80	108	20
06-5503-126	C 61-123	Table	BC4	1814	458	650	114
06-5503-107	Y142- 54--09	Table	BC4	2029	152	166	2
06-5503-121	06-5503-121	Table	BC4	1755	200	63	2
08-5001-41	Y525- 60--07	Table	BC4	4243	356	500	86
09-5013-013	Y530- 5--07	Table	BC5	5 bags	450	500	122
09-5013-067	Y530- 5--07	Table	BC5	3015	21	0	0
A 51- 36	05-5551-116	Raisin	BC4	2744	422	341	53
07-5052-033	A 63- 85	Raisin	BC4	4202	550	483	57
A 51- 36	07-5061-072	Raisin	BC4	2437	340	182	68
09-5057-048	B 82- 43	Raisin	BC5	5 bags	455	500	26
09-5064-006	B 82- 43	Raisin	BC5	4 bags	3	0	0
A 63- 85	09-5056-012	Raisin	BC5	2113	43	81	7
A 63- 85	09-5064-038	Raisin	BC5	2502	218	268	46

Table 1 continued. 2011 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
A 63- 85	09-5072-24	Raisin	BC5	2552	102	33	0
B 82- 43	09-5056-012	Raisin	BC5	2550	135	0	0
B 82- 43	09-5063-023	Raisin	BC5	2561	27	22	1
Y143- 26	09-5063-023	Raisin	BC5	2489	53	14	2
Y143- 26	09-5064-016	Raisin	BC5	1301	70	11	3
Y143- 26	09-5072-024	Raisin	BC5	2315	25	23	4
Y144-132--04	09-5074-002	Raisin	BC5	2636	300	317	57
Total				**61,611**	**5,866**	**5,430**	**877**
PM resistance combined with 89-0908 *V. rupestris* x *V. arizonica*							
05-5501-69	Y312-187--06	Table	BC3	3 bags [z]	143	50	2
05-5501-69	Y313-157--08	Table	BC3	4 bags	47	36	1
06-5501-229	Y313-191--08	Table	BC3	86	14	7	1
06-5501-238	Y308-311--06	Table	BC3	1395	200	75	87
Y315-400--04	05-5501-27	Table	BC3	2098	73	122	41
Y520- 71--07	08-5001-20	Table	BC3	1957	190	198	20
08-6003-002	Y152- 84--08	Table	BC5	3453	191	200	38
07-5052-033	Y308-312--06	Raisin	BC4	2723	381	487	72
07-5052-033	Y308-329--06	Raisin	BC4	2100	195	205	19
A 49- 82	08-6053-020	Raisin	BC4	2500	21	32	2
A 51- 36	08-6053-012	Raisin	BC4	4374	189	159	23
A 63- 85	08-6053-020	Raisin	BC4	3646	214	116	10
08-6052-003	A 51- 60	Raisin	BC4	1998	550	500	71
08-6053-028	A 51- 43	Raisin	BC4	2627	304	386	73
08-6053-028	A 63- 85	Raisin	BC4	2866	520	500	87
B 82- 43	08-6053-012	Raisin	BC4	2571	300	265	62
B 82- 43	08-6053-020	Raisin	BC4	2481	200	195	36
09-5013-013	Y308-329--06	Raisin	BC5	5 bags	40	38	6
Total				**40,179**	**3,772**	**3,571**	**651**
PD resistance from BD5-117							
03-5003-103	Y540-193--05	Table	BC1	3 bags	241	400	75
03-5003-108	Y140- 54--08	Table	BC1	3 bags	225	400	17
03-5003-103	Y534-101--06	Table	BC1	2 bags	284	472	86
03-5003-108	Y537-168--06	Table	BC1	2 bags	115	156	5
03-5003-090	Y534- 91--06	Table	BC1	3 bags	126	90	15
03-5003-110	Y538-181--06	Table	BC1	2 bags	27	4	0
03-5003-090	Y537- 32--06	Table	BC1	2 bags	180	107	20
03-5003-090	03-5003-082	Table	BC1	2 bags	211	104	12
03-5003-103	03-5003-082	Table	BC1	3 bags	353	500	74
Total				**22 bags**	**1,762**	**2,233**	**304**

[z] Clusters bagged because flowers are female and do not need emasculation.

Table 2. 2011 table and raisin grape PD resistant seeded crosses and the number of seeds produced.

Female	Male	Type	No. Emas-culations	No. seed
89-0908 *V. rupestris* x *V. arizonica*				
04-5002-18	Y525- 60--07	Table BC1	388	96
04-5514-144	05-5501-27	Table BC2x3	3 bags [z]	13
05-5501-26	Y127-111--05	Table BC3	8 bags	21
05-5501-06	Y152-128--08	Table BC3	5 bags	235
07-5060-134	Y525- 60--07	Table BC3	2 bags	69
07-5060-134	Y540-193--05	Table BC3	3 bags	153
07-5060-134	05-5501-27	Table BC3x3	3 bags	1

Table 2 continued. 2011 table and raisin grape PD resistant seeded crosses and the number of seeds produced.

Female	Male	Type	No. Emas-culations	No. seed
07-5060-134	C 61-123	Table BC3	1 bags	30
07-5060-134	C 75- 4	Table BC3	1 bags	28
Total			**29 bags +388**	**646**
PM resistance combined with PD resistance				
07-5060-134	Y308-148--06	Table BC3	1 bags	315
05-5501-57	06-3551-226	Table BC3	1076	231
Y308- 14	Y520- 73--07	Table BC3	2354	269
Y308- 39	Y520- 73--07	Table BC3	1162	184
07-5052-61	Y308-311--06	Table BC4	2999	191
Total			**7,591**	**1,190**
PD resistance from BD5-117				
03-5003-052	Y537-168--06	Table BC1	3 bags	172
03-5003-052	Y139-139--04	Table BC1	3 bags	246
03-5003-052	Y131-181--05	Table BC1	2 bags	108
Total			**8 bags**	**526**

[z] Clusters bagged because flowers are female and do not need emasculation.

Table 3. Determination of seedling resistance based on PdR1 molecular markers for all 89-0908 families made in 2010.

Family	Type Cross	No. Resistant[a]	No. Susceptible[b]	No. Recombinant[c]	No data[d]	Off Types	Total
10-5004	Table BC4	3	1				4
10-5005	Table BC3	5	2	1			8
10-5006	Table BC3					1	1
10-5007	Table BC3	3	1	3		2	8
10-5008	Table BC3	1	9	1		21	32
10-5009	Table BC3	1	1				2
10-5013	Table BC4					3	3
10-5015	Table BC4	5	1			1	7
10-5052	Raisin BC4	1					1
10-5054	Raisin BC4	2	2				4
10-5063	Raisin BC4	2				1	3
10-5065	Raisin BC4	4	1		1		6
10-5073	Raisin BC4	2	3				5
10-5074	Raisin BC4	6	4	1			11
10-5076	Raisin BC4	2	14	1			20
10-6001	Table BC3	8	17	1			26
10-6002	Table BC3		3				3
10-6003	Table BC3	2	3				5
10-6005	Table BC3	1					1
10-6007	Table BC4					4	4
10-6051	Raisin BC3	1					1
10-6053	Raisin BC3	22	23	2		5	52
10-6056	Raisin BC4	1				1	2
10-6058	Raisin BC4	1	1			2	4
Total		**73**	**86**	**10**	**1**	**41**	**213**

[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. Segregation of PD and PM resistance in seedlings from 2010 crosses combining resistance from *V. arizonica* and *V. romanetti*, respectively.

		PD resistance from *V. arizonica*		
		PD Resistant Obs./Exp.	PD Susceptible Obs./Exp.	Total Obs./Exp.
PM resistance from *V. romanetii*	PM Resistant Obs./Exp.	18/20	28/20	46/40
	PM Susceptible Obs./Exp.	17/20	16/20	33/40
	Total Obs./Exp.	35/40	44/40	79/80

Table 5. Results of greenhouse test for determination of PD reaction in 2011.

Population	Resistance Source	Testing Compete		In greenhouse test
		No. tested	No. resistant	For evaluation by December
BD5-117 map	BD5-117	30	3	89
Arizonica	PdR1	22	14	16
Other PD	SEUS	2	0	6
TOTAL		54	17	111

Table 6. Berry size of PD resistant table grape selections treated with 20ppm gibberellic acid at berry set or no treatment (NT).

Name	Treatment	mean ber. Wt. (g)	mean ber. Dia. (mm)	mean ber. Len. (mm)
08-5001-34	NT	7.5	22.05	24.5
08-5001-34	GA	11.7	23.52	33.32
08-5001-21	NT	4.4	18.13	19.6
08-5001-21	GA	7.1	21.07	24.5
08-5001-38	NT	3.7	16.66	17.64
08-5001-38	GA	6.4	20.58	24.01
08-5001-47	NT	3.6	16.17	17.64
08-5001-47	GA	6.0	20.09	21.56
TS ARS Plot	GA	6.8	19.2	31.2
TS commercial	GA	10.2	22.0	36.0

Table 7. New PD resistant grape selections made and evaluated 2011.

Name	generation	color	trace	berry size	crop	cluster size	berry set	firmness	status	comment
Table PD *V. arizonica* resistance										
09-5013-070	BC4	W8	10	13	2	2	4	8	prop	P1 GH test
09-5013-075	BC4	R8	10	14	4	4	5	3	prop	P1 GH test
09-5013-118	BC4	R7	7	13	3	5	5	6	prop*	GH test
09-5013-122	BC4	W7	10	12	3	4	4	5	prop	GH test
09-5013-125	BC4	W8	5	12	2	4	6	7	prop	GH test
09-5064-022	BC4	W7	5	14	4	5	5	4	prop	P1 GH test
PD +PM resistance										
08-6002-089	BC3	W6	6	13	2	5	4	2	prop	P1 GH test

Table 7 continued. New PD resistant grape selections made and evaluated 2011.

Name	generation	trace	berry size	crop	cluster size	berry set	flavor	status	Type	comment
Raisin PD *V. arizonica resistance*										
09-5066-063	BC4	7	midget	1	1		neutral	prop	DOV	GH test
09-5066-097	BC4	8	TS					prop	DOV	P1
09-5068-023	BC4	9	TS	1	1		muscat	prop	DOV	GH test
09-5070-101/015	BC4	10	TS	6	5	5	neutral	prop	TD	GH test
09-5070-015/5071-007	BC4	10	TS	6	5	5	neutral	prop	TD	GH test
09-5063-018	BC4	7	TS	3	4	5	neutral	prop	DOV	GH test
09-5063-021	BC4	8	TS	2	4	4	neutral	prop	DOV	GH test
09-5066-039	BC4	7	midget	4	3	5	neutral	prop	DOV	GH test
09-5056-089	BC3	10	TS	6	5	5	neutral	prop	TD	GH test
08-5056-059	BC3	8	midget	6	4	5	neutral	prop	TD	GH test
08-5056-072	BC3	10	midget	5	4	5	neutral	prop*	TD	GH test
08-5054-047	BC1	10	TS	4	4	4	neutral	prop	TD	GH test

Trace: 10=none, 9=Thompson Seedless (TS) size, 7= Fiesta size, 6=Flame Seedless size
Berry size = 1/16" for table grapes and Thompson Seedless (TS) or midget size for raisins.
Crop: 9=v. heavy, 7=heavy, 5=average, 3=light, 1=v. light. Cluster size: 9=>2.5 lb, 7=2 lb, 5=1 lb, 3=0.5 lb, 1=widow size.
Berry set: 9=v. tight, 7=tight, 5=average, 3=loose, 1=v. loose. Firmness: 9=v. firm, 7=firm, 5=average, 3=soft, 1=v. soft.
Prop = propagate in first 2 vine plot; prop* = propagate table grape in 25 vine or raisin in 7 vine production trial.
DOV = natural dry on the vine type. TD = tray dried. GH test = test resistant reaction to *Xylella* in greenhouse.

Figure 1. A. Fruit of BC3 PD resistant table grape selection 08-5001-34 treated with 20ppm size GA. B. Natural fruit of BC4 PD resistant table grape selection 09-5013-118, first crop on seedling.

MOLECULAR CHARACTERIZATION OF THE PUTATIVE *XYLELLA FASTIDIOSA* RESISTANCE GENE(S) FROM B43-17 (*V. ARIZONICA/CANDICANS*).

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LAYPERSON SUMMARY

We maintain and characterize many populations while breeding Pierce's disease (PD) resistant wine grapes, some of which have been used to develop genetic maps. These maps have been used to identify genetic markers that are tightly linked with PD resistance, which have allowed classical breeding to be greatly expedited through marker-assisted selection. Genetic maps allow the construction of physical maps to identify resistance genes (Riaz et al. 2008; Riaz et al. 2009). The physical map of the *V. arizonica/candicans* b43-17 resistance region allowed us to identify six candidate genes responsible for PD resistance. Sequence comparisons with other plant genomes indicated that multiple tandem repeats of a disease resistance gene family of receptor-like proteins (Leucine Rich Repeats; LRR) were present in the resistance region. This category of genes is involved in the recognition of microbes and in the initiation of a defense response (Bent and Mackey 2007). We completed the cloning of four of the candidate genes: *PdR1b*-1, 2, 5 and 6. We also developed embryogenic callus cultures of the PD susceptible *V. vinifera* Chardonnay and Thompson Seedless and the PD susceptible rootstock *V. rupestris* St. George. These embryogenic callus cultures will be used for the transformation/complementation studies to verify the function of these candidate PD resistance genes. Transformation experiments with *PdR1b-1* and *PdR1b-6* have been initiated. 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 reduce the time required to generate transformed plants by four months. We also initiated total RNA extraction experiments to allow time course examinations of gene function from leaf and stem tissues. These were successfully completed and we are now ready to evaluate gene function over time in inoculated and un-inoculated plants of the *PdR1* containing resistant selections F8909-08 and F8909-17, their resistant parent b43-17, their susceptible parent *V. rupestris* A. de Serres, and the susceptible control Chardonnay. These plants have been established in the greenhouse and will soon be inoculated.

INTRODUCTION

New cultivars bred to resist *Xylella fastidiosa (Xf)* infection and subsequent expression of Pierce's disease (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 and 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 single genes from 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 cisgene approach in grapes is similar to the natural clonal variation that exists in many winegrape cultivars. This linkage-drag-free 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). The physical mapping 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 in the recognition of microbe-associated molecular patterns (MAMP) like compounds, which initiate 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 progress on the cloning and testing of four candidate resistance genes.

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. A refined genetic map of chromosome 14, which contains the PD resistance locus, was generated from three grape mapping populations derived from b43-17. The resistance locus segregates as a single dominant gene and mapped as *PdR1a* in the F1 selection 8909-17 and as *PdR1b* (allelic forms) in its sibling F8909-08. Clone H69J14 from a b43-17 BAC library, containing both markers flanking the *PdR1b* resistance locus, was sequenced using 454 sequencing. Further detailed analysis of the assembled, as well as unassembled sequences, revealed the presence of a high number of transposable elements (TE). Chromosome 14 is known to have the second largest number of TEs in the Pinot noir grape genome sequence (Moisy et al. 2008). TE's play a key role in the diversification of disease resistance genes through a process termed TE-induced gene alteration (Michelmore 1995). Given the complexity of this region due to the large number of TE tandem repeats, a fosmid library was generated with an insert size of 35-40kb from the H69J14 BAC clone. The second round of sequencing was performed using fosmid end-sequencing as well as shotgun reads.

The first assembly generated 10 contigs, with a portion of the sequence remaining unassembled. Analysis of assembled and unassembled sequences revealed the presence of four candidate genes, *PdR1b*.1–4, which appear to be receptor-like proteins, a class of resistance proteins. *PdR1b*.1-2 and 4 were cloned into a pGEM-T easy vector (Promega) and subcloned into vector pDE00.0113 containing the 35S promoter and ocs3' terminator (**Figure 1**). The second assembly allowed the identification of two new candidate genes and showed that *PdR1b*.1 was longer than previously found. As a consequence, we repeated the amplifications from BAC H69J14, using primers that hybridize to regions flanking the open reading frames (ORFs). So far, we have re-amplified and confirmed the sequences of *PdR1b*.1, *PdR1b*.2, *PdR1b*.5 and *PdR1b*.6. We have sublconed *PdR1b*.1 and *PdR1b*.6 into binary vectors pCAMBIA-1303 (www.cambia.org) and pDU99.2215 (**Figure 1**). PdR1b.1 is the largest gene, sharing a high degree of homology with *PdR1b*.2, 3, 4, and 5.. *PdR1b*.6 is significantly different from the rest. It has a kinase domain, which suggests it might be involved in PD resistance in combination with *PdR1b*.1 or one of the other candidates. pCAMBIA-1303 was included in the experiments because it carries a hygromicin resistance gene that improves the selection of transformants (D. Tricoli, pers. comm.). An additional advantage is that it allows the subcloning of the gene in one step, by replacing the gus gene with the gene of interest. The resulting plasmids were used for transformation via *Agrobacterium tumefaciens* of Chardonnay, Thompson Seedless, St. George and tobacco SR1. A similar procedure will be followed with the remaining four genes.

Sequence analysis and alignments to identify introns and exons on the *PdR1b*.1 gene were performed using the GeneQuest module of Lasergene v 8.1, which facilitates the prediction of coding regions using the Borodovsky's Markov method and predicts intron/exon boundaries using species-specific patterns by aligning to known genes. We also utilized the GeneMark (http://exon.biology.gatech.edu/) program using both *Arabidopsis thaliana* and *Medicago truncatula* settings. By using both systems, we identified two small introns from position 1-168 and position 3128-3191 that are 167bp and 361bp, respectively. With *A. thaliana* as the model system, the size of predicted protein was 976 amino acids, and with *M. truncatula* it was 964 amino acids. It is interesting to note that all four genes from the 12X assembly of PN40024 carry large introns. We plan to make sequence comparisons and identify protein domains for other putative candidate genes. Experiments are underway with specific primers to amplify the regions of the genes using total extracted RNA and making make sequence comparisons among Pinot noir and the five different genes[MSOffice1].

Objective 2. To conduct expression studies of the candidate genes, hardwood cuttings were collected in November to generate at least 15 plants of the susceptible *vinifera* control (Chardonnay), resistant and susceptible parents (b43-17 and *V. rupestris* A. de Serres) and two resistant F1 selections (F8909-08 and F8909-17). Stem and leaf tissues were used for the total RNA from both resistant and susceptible genotypes using a cetyltrimethylammonium bromide (CTAB)-based RNA extraction protocol as described previously (Iandolino et al., 2004). Two other methods were tested to get better yield and quality of total RNA with less genomic DNA. The best yield of total RNA was obtained by the procedure described by Reid et al. 2006.

We used a time course analysis to evaluate expression and to determine when the resistance gene(s) is activated. Total RNA was extracted from the leaves and stem tissue of un-inoculated plants at 1, 3 and 5 weeks after inoculations with the *Xf*. ELISA screening was carried out after 12 weeks to quantify the amount of *Xf* in tissues. **Figure 2** presents the results of RNA extracted from the young leaves of the four genotypes before the bacterial inoculations and stem tissue two weeks after inoculations. First-strand cDNA synthesis will be performed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Three different control housekeeping reference genes were tested as controls (Actin, Chitinase and B-actin). The actin gene (F-actgctgaactggaaattgt; R- acggaatctctcagctccaa) as described Vasanthaiah et al. 2008 worked very well in our system. We designed specific primers for quantitative PCR from two of our candidate genes (*PdR1*.1 and *PdR1*.6) and used comparative RT-PCR to check the effectiveness of the primers and the whole setup. We observed differences in the level of expression of the genes indicating that our RT-PCR system is working. Experiments that are more detailed are underway to monitor the gene expression of all six candidate PD resistant genes.

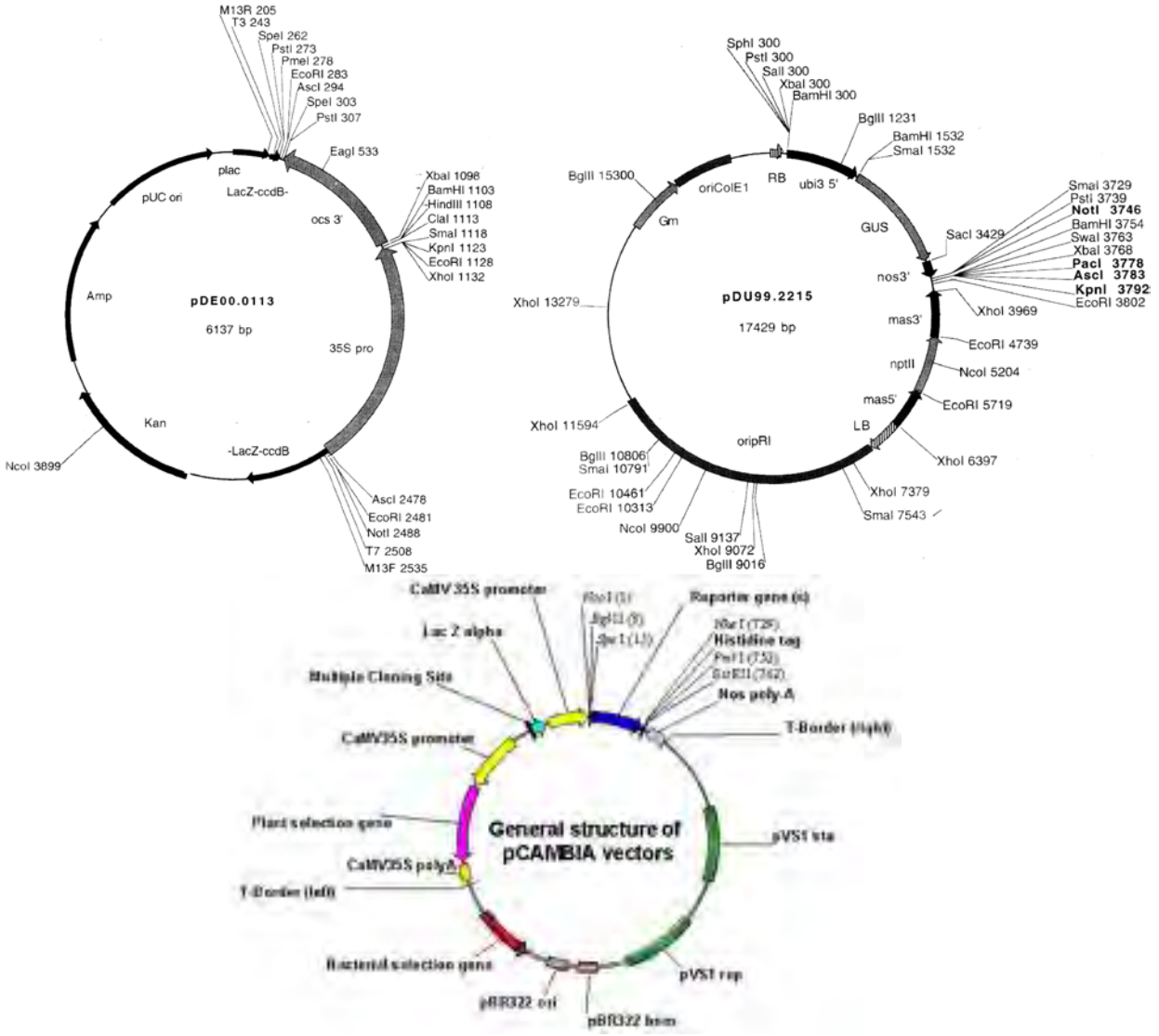

Figure 1. Vectors used in genetic transformation with *PdR1b* candidate genes. *PdR1b*.1 was subcloned into pDE00.0113 and then the expression cassette was moved into the binary plasmid pDU99.2215. *PdR1b*.1 and *PdR1b*.6 were also subcloned directly into the binary plasmid pCAMBIA 1303 in the place of the reporter gene. Because the two binary plasmids have different plant selection genes, it is possible to co-transform tobacco with both genes.

Objective 3. Development of alternative protocols for genetic transformation for the validation of gene constructs. Once the gene constructs are completed, they must be tested to see if they contain the resistance genes. This is done by inserting the gene(s) into a susceptible plant and testing to see if the inserted gene makes it resistant. Currently the most widely used method for the production of transgenic/cisgenic grapes is based on *Agrobacterium* transformation followed by regeneration of plants from embryogenic callus. We have established cultures of pre-embryogenic callus derived from anthers of *V. vinifera* Thompson Seedless and Chardonnay and the rootstock *V. rupestris* St. George. These cultures of embryogenic calli can be readily used for transformation (Agüero et al. 2006).

Two alternative transformation techniques via organogenesis have been tested to reduce the time needed to produce transgenic grapes. These methods were developed in Thompson Seedless and are based on the use of meristematic bulks or etiolated meristems as explants for inoculation with *Agrobacterium* (Mezzetti et al. 2002, 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 an effective system. The second method employs genetic transformation of *V. vinifera* via organogenesis (Mezzetti et al 2002). In this method, shoot apical meristem slices are prepared from meristematic bulks for *Agrobacterium*-mediated transformation of grape plants. Using this procedure, transgenic plants can be produced in a much shorter time interval. We have streamlined this procedure and have obtained transgenic shoots using Mezzetti's method in three months (**Figure 3**). With the successful modification and adoption of this technique, we expect that the time required for

transformation will be shortened to approximately six months instead of one year or longer via embryogenic callus. The green fluorescent protein (GFP) was used as a reporter gene for monitoring the occurrence of transformed and chimeric plants. **Figure 3** shows both procedures in comparison with transformation via somatic embryogenesis. No plants were regenerated from etiolated meristems and the procedure was laborious and time consuming. Transformation via meristematic bulks represented a better method to produce transgenic plants in a shorter period of time, although its efficiency was very low (1 plant in 50 sections). GFP expression in transgenic leaves was uniform; indicating a stable non-chimeric transformation of shoots regenerating in selection medium.

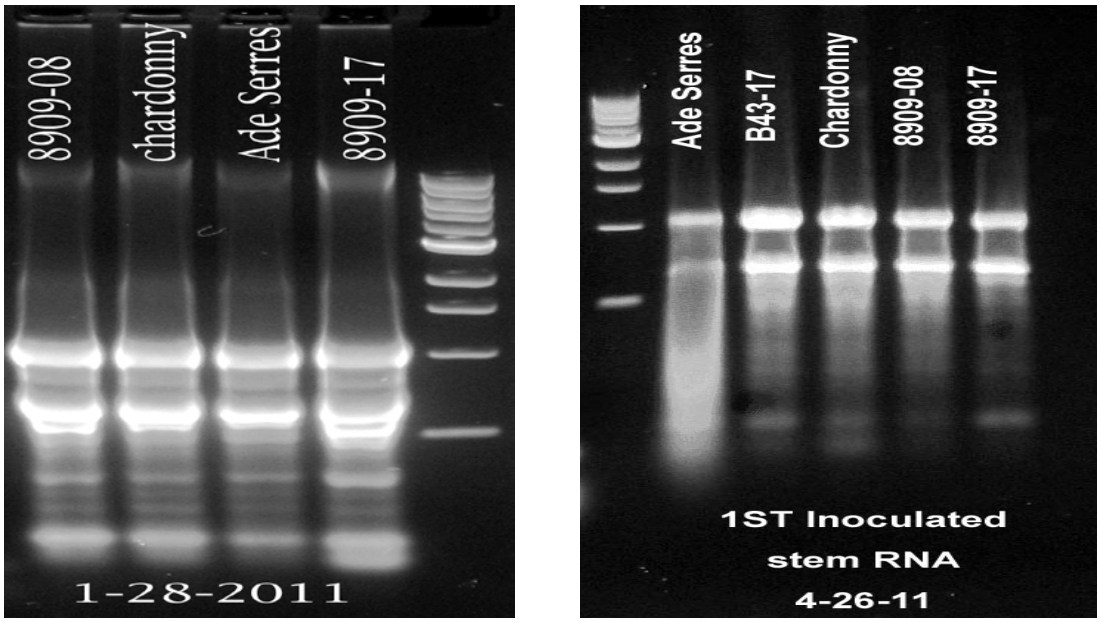

Figure 2. Total RNA extracted from the leaves and stem tissue of susceptible control and resistant plants.

We have inoculated pre-embryogenic cultures of Thompson Seedless, Chardonnay and *V. rupestris* St George with *A. tumefaciens* EHA105 pCH32, carrying binary plasmids with *PdR1b*.1 and *PdR1b*.6 coding sequences. Overnight cultures of the bacteria in LB medium + antibiotics were diluted to 10^8 cells·ml^{-1} using liquid co-cultivation medium. Pre-embryogenic calli were placed on a sterile glassfiber filter (GFF) overlaid on co-cultivation medium. The *Agrobacterium* culture was poured over the callus and excess was blotted with sterile filter paper after five min. The callus on GFF was then transferred onto fresh co- cultivation medium. After 48 hr in the dark, the callus pieces were sub-divided into small clumps, about 2 mm in diameter, and cultured on selection medium. Currently, calli are being selected with 100 ug/ml kanamycin or 15 ug/ml hygromicin. We have also transformed meristematic bulks of Thompson Seedless following a similar procedure except that meristematic bulk slices were submerged in the *Agrobacterium* solution. We are producing meristematic bulks from Chardonnay and St. George (**Figure 4**) that will be tested as soon as they are ready for inoculation.

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 is transformed into a 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 six *PdR1* region gene candidates. We have also tested another technique to speed the development of transgenic tissue 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.

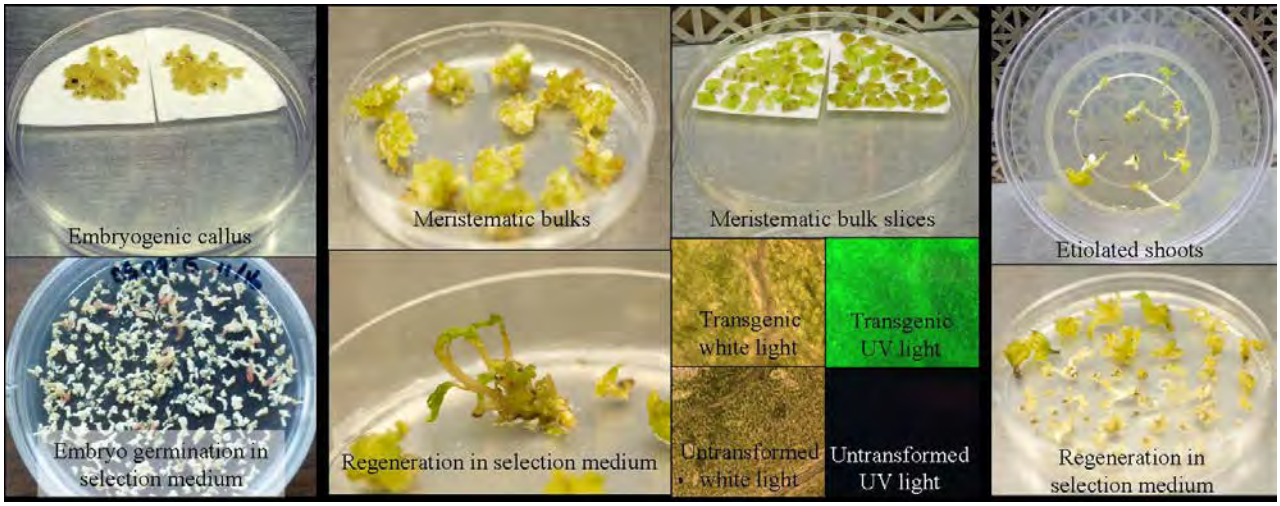

Figure 3. (A) Embryogenic callus developed from anthers are inoculated with *A. tumefaciens*. Transformed embryogenic callus is selected after about four months. An additional 4-6 months are needed for embryo germination and plant development. (B) Meristematic bulks, developed from shoot apical meristems, are sliced and inoculated with *A. tumefaciens*. Transformed bulks are selected after about 3 months. An additional four months are needed for shoot elongation and rooting. Pictures on the bottom right show green fluorescence protein (GFP) expression in transgenic leaflets. (C) Fragmented shoot apices from etiolated shoots are inoculated with A. tumefaciens. Transformed meristems are selected after 2-3 months. An additional 2-3 months are needed for plant regeneration and rooting.

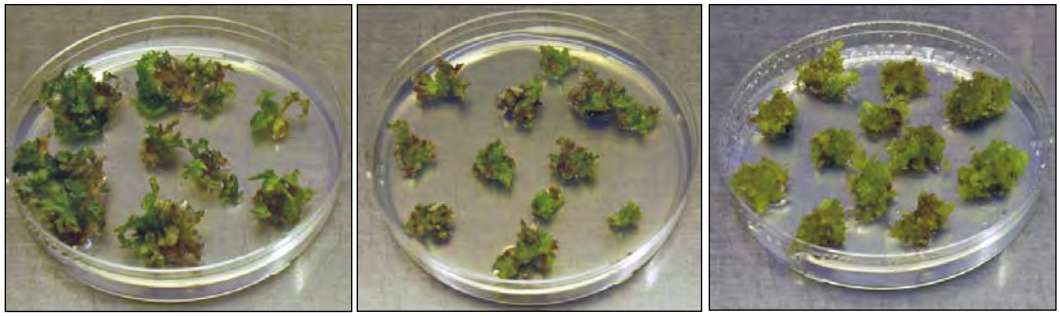

Figure 4. Meristematic bulks of Chardonnay (left), St. George (middle) and Thompson Seedless (right).

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GENETIC MAPPING OF *XYLELLA FASTIDIOSA* RESISTANCE GENE(S) IN GRAPE GERMPLASM FROM THE SOUTHERN UNITED STATES.

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Reporting Period: The results reported here are from work conducted September 2010 to October 2011

LAYPERSON SUMMARY

Genetic mapping of two different forms of *Vitis arizonica* has identified a region on chromosome 14 that is responsible for Pierce's disease (PD) resistance, we named it *PdR1*. We mapped two forms of *PdR1* from *V. arizonica/candicans* b43-17, identified a minor gene on chromosome 19 (*PdR2*), and have mapped a third form, *PdR1c*, which originated from *V. arizonica* b40-14. These forms are both single dominant genes for PD resistance. The resistance of *V. arizonica/girdiana* b42-26 is controlled by multiple genes. This resistance is being studied to determine if fine-scale mapping will allow markers to be placed closely enough to these multiple resistance genes to be useful for marker-assisted selection (MAS) in our breeding program. We plan to combine these varying resistance sources in our breeding program to ensure broad and durable resistance to PD. These mapping efforts are also essential to physically locating and characterizing PD resistance genes, so that we can study how they work and predict how well or how long they will function. We expanded our search for plant material that possesses resistance to PD by selecting and greenhouse screening 52 wild grape species accessions that were collected from different parts of the southern US and Mexico. Greenhouse screening identified 10 accessions with good resistance and that can be used to developing breeding and mapping populations. These populations will be used to determine the inheritance of PD resistance, develop framework genetic maps, and identify resistance regions for the development of markers to facilitate the breeding program.

This research project provides the genetic markers critical to the successful classical breeding of PD resistant wine, table and raisin grapes. Identification of markers for the PD resistance gene, *PdR1,* has allowed us to reduce the seed-to-seed cycle to two years and attain four backcross generations to produce resistant vines with 97% *vinifera* in 10 years. The development of these markers also led to the identification of six genetic sequences that house the resistance gene. These sequences are in the process of testing to verify their function. These efforts will help us better understand how, and which of, these genes function, and could lead to the identification of PD resistance genes from grape that would be available to genetically engineer PD resistance in *V. vinifera* cultivars

INTRODUCTION

We are mapping Pierce's disease (PD) resistance in different forms of *Vitis arizonica*. The breeding program produces and screens the seedling populations upon which the genetic mapping efforts depend. The tightly linked genetic markers generated in these mapping efforts are used to optimize and greatly accelerate the PD breeding program. These markers are essential to the successful introgression of resistance from multiple sources, because although the resistance genes may vary, the expression of resistance (the phenotype) is the same. Only the markers can verify that different resistance sources are being successfully combined. We are also identifying resistance in other southern grape species in an effort to discover new resistance genes. Once the species are identified we will genetically map the resistance, identify genetic markers that are tightly linked to the resistance and use them to pyramid resistance from different backgrounds into a 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 distinct from b43-17, they both posses strong resistance to PD and greatly suppress *Xylella fastidiosa (Xf)* levels in stem tissue after greenhouse screening. We have also widened the search for additional resistance sources by screening species collections from different parts of southern US and Mexico. Initial greenhouse screen results indicate that we have 10 other accessions that possess strong PD resistance.

OBJECTIVES

1. Complete the genetic mapping of additional QTLs in the 04191 (*V. vinifera* F2-7 x F8909-17 (*V. rupestris* x *V. arizonica/candicans* b43-17) population.
2. Greenhouse screen and genetically map PD resistance from other forms of *V. arizonica*: b42-26 (*V. arizonica/girdiana*) and b40-14 (*V. arizonica*).
3. Evaluate *Vitis* germplasm collected (250 accessions) from across the southwestern US to identify accessions with unique forms of PD resistance for grape breeding. Determine the inheritance of PD resistance from *Muscadinia rotundifolia*, develop new and exploit existing breeding populations to genetically map this resistance.
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. The genetic mapping of the 04191 (*V. vinifera* F2-7 x F8909-17 (*V. rupestris* x *V. arizonica/candicans* b43-17)) population was carried out to identify additional minor QTL(s) other than the major locus on chromosome 14 (*PdR1a*). A total of 139 SSR markers representing all 19 chromosomes were added to the set of 153 seedlings, of which 141 were greenhouse screened for resistance. A genetic map was constructed with a LOD score of 5.0 and a recombination frequency of 0.40. A total of 136 markers were grouped on 19 chromosomes. We confirmed the major locus, *PdR1a*, on chromosome 14 that explained 78% of the variation with a 95% confidence level (**Figure 1**). This work also identified a minor QTL (*PdR2*) with a LOD 2.3 that explains 7% of the phenotypic variation on chromosome 19, and which peaks at marker CB918037 (**Figure 2**). This QTL is within a 10 cM interval – a very large genetic distance for map-based positional cloning purposes. In order to narrow this region, we developed seven SSR markers based on the Pinot noir genome sequence (PN 40024). These markers should allow us to reduce the gap from 10 cM. In order to test whether the QTL on chromosome 19 has an additive effect in conjunction with the *PdR1a* locus, we analyzed the resistant and susceptible genotypes with a least square means test for chromosomes 14 and 19 (**Table 1**). It was clear that both loci work independently of each other and they do not have an additive impact. The mean ELISA values of resistant and susceptible plants with the *PdR1a* locus were very different, however, the mean values of resistant and susceptible plants for the *PdR2* locus were higher for the resistant plants.

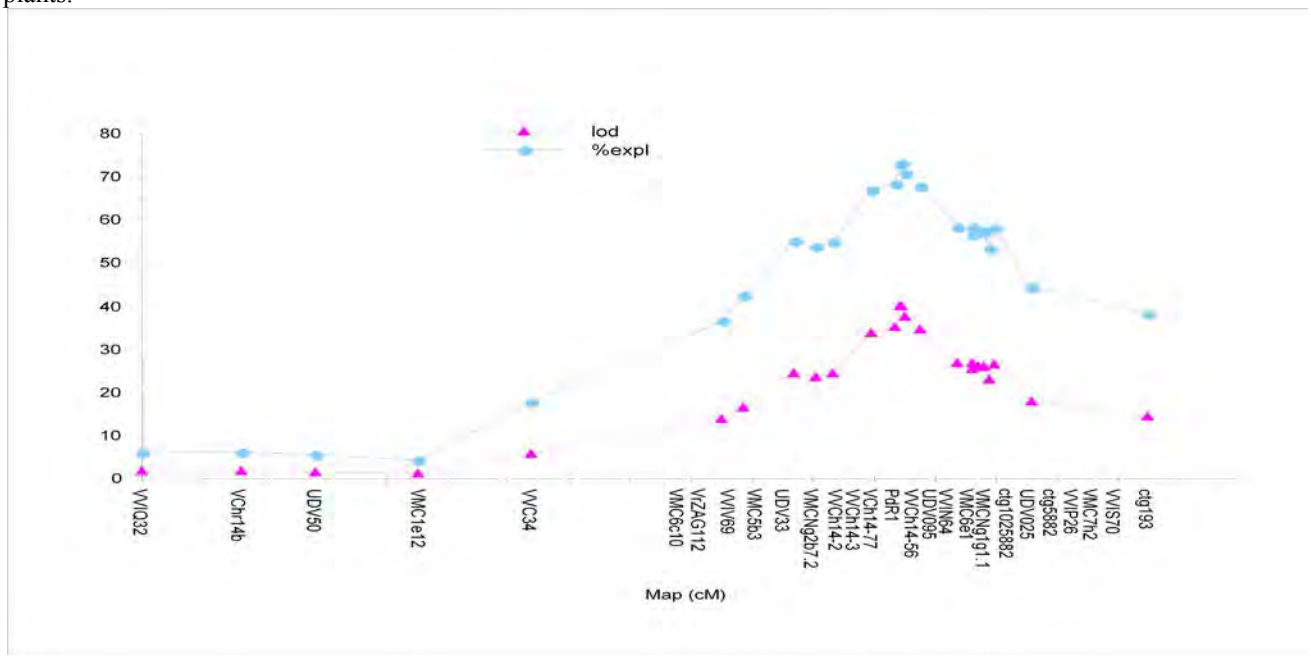


Figure 1. Updated interval mapping analysis of the *PdR1a* locus on LG 14.

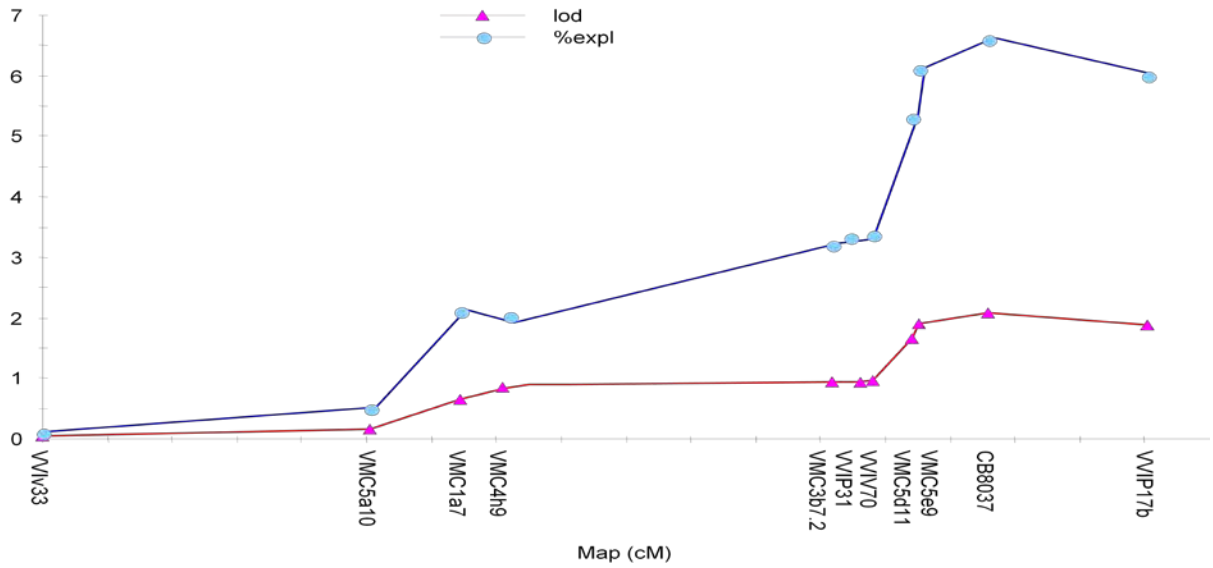
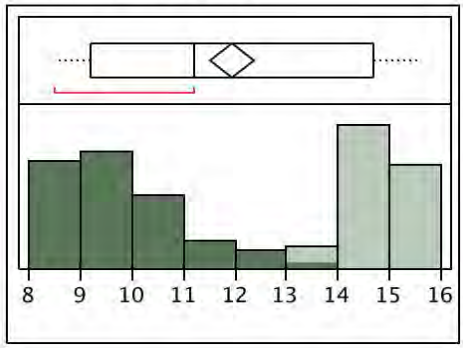


Figure 2. Interval mapping analysis of the *PdR2* locus on LG 19.

Table 1. The distribution of natural log transformed ELISA values from a greenhouse testing of the 04191 population.

Mean (ln cfu/ml)



Quantiles			Moments	
100.0%	maximum	15.6	Mean	11.941844
99.5%		15.6	Std Dev	2.6467273
97.5%		15.6	Std Err Mean	0.2228946
90.0%		15.28	Upper 95% Mean	12.382519
75.0%	quartile	14.7	Lower 95% Mean	11.501169
50.0%	median	11.2	N	141
25.0%	quartile	9.2		
10.0%		8.8		
2.5%		8.5		
0.5%		8.5		
0.0%	minimum	8.5		

Objective 2. Resistance to PD in *V. arizonica/girdiana* b42-26 is strong but is controlled by multiple genes. We completed preliminary QTL analysis with 64 greenhouse screened genotypes (**Figure 3**) from the 05347 population (*V. vinifera* F2-35 x b42-26) using data from 71 SSR markers. They were analyzed with the Kruskal-Wallis test, which allows association of each marker to the phenotypic trait. Because we know the chromosomes the markers reside on, we can get a rough map from this analysis. The results indicated that markers from chromosome 10 and 14 (and to a lesser extent 2 and 11) are associated with PD resistance. This allows us to focus mapping efforts on markers known to exist on these chromosomes, which will greatly accelerate the identification of genomic regions responsible for b42-26's resistance.

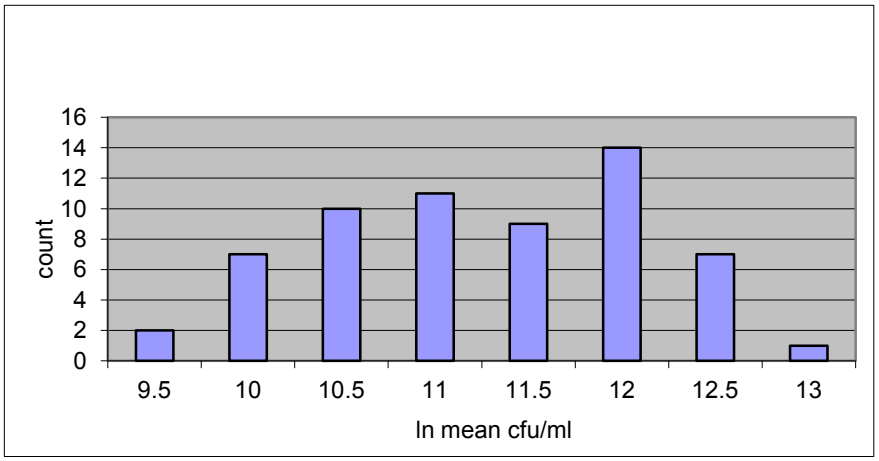


Figure 3. Distribution of ELISA values (ln means in cfu/ml) of greenhouse tested progeny from the 05347 population.

The genome of b42-26 is very homozygous, which is unusual for *Vitis* species and suggests that this collection is from a very localized population which has been inbred over time. This is likely given its collection location from a coastal valley along Baja California's east desert coast. This also means that many of the homozygous SSR markers are not useful for mapping. Thus, we developed 71 new SSR markers from clone sequences generated by the Vitis Microsatellite Consortium (the original source of SSR markers for grape). We acquired primer sequences of an additional 200 markers that have not been used to test b42-26. Marker testing on small set progeny and the parents is underway. We are also adding markers to develop a framework map for the entire population. We are now mapping with the complete set of 239 seedlings with 30 to 50 markers known to exist on chromosomes 2, 10, 11 and 14.

A single dominant gene controls PD resistance in *V. arizonica* b40-14. Two resistant siblings from the R8918population (*V. rupestris* x *V. arizonica* b40-14) were used to develop the 07388 (R8918-02 x *V. vinifera*) and 07744 (R8918-05 x *V. vinifera*) populations. Two hundred and twenty-seven 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*) 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 4**). In 2009, crosses were made with F1 resistant selections from 07744 population.

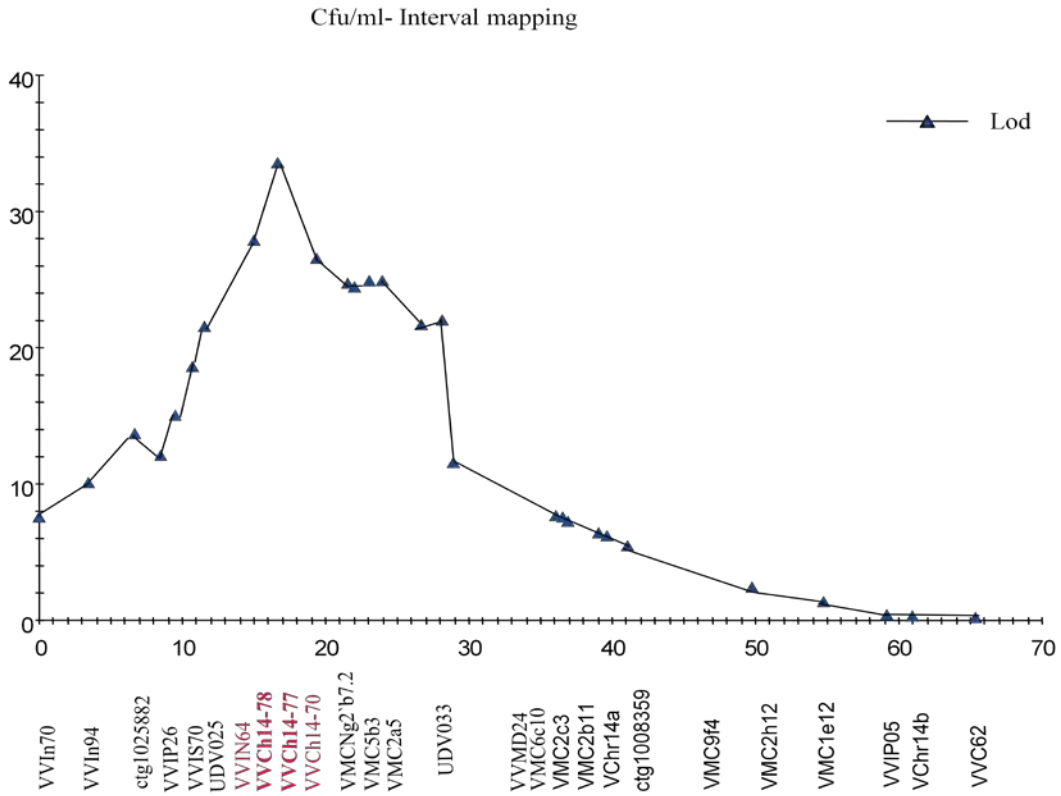


Figure 4. Interval mapping of *PdR1c* from the 07744 population indicating a peak at LOD 34.0 with a 95% confidence interval. The X-axis indicates the position of the markers; LOD values are plotted on the Y-axis.

Objective 3. *Vitis* species growing in the southern US have co-evolved with *Xf* and resist PD. To date we have focused on accessions of *Vitis* species that Olmo collected in northern Mexico in 1960. In addition to these accessions, we have more than 250 accessions collected from PD hot spots in Texas, New Mexico, Arizona, Nevada and California. Fifty-two of these from across this geographic range (including the 15 accessions from Mexico) are being evaluated with our greenhouse screen. Based on early evaluations of cane maturation and leaf scorch and leaf loss, we have identified 10 accessions that had very good scores for these two parameters. Three of these accessions were collected collected from Texas and Arizona (**Table 2**). The ANU5 accession was collected near Utah along the Virgin River and is a very easterly selection of *V. girdiana*. A recent collection trip to southwest Utah found this population is expanding and may prove very valuable. This accession also has excellent salt tolerance and is being utilized in our rootstock breeding program. ELISA results will be ready in November or early December 2011. **Figure 5** shows where the *Vitis* accessions current under test were collected. Many of the collection sites have multiple accessions associated with them, but they are not seen in this depiction. The green dots indicate accessions with promising resistance.

Working with this germplasm will expand the pool of resistance genes available for breeding, identify potentially unique sources of resistance, and identify geographic regions with high levels of resistance so that areas can be identified for future collections. Studying the inheritance of resistance in these accessions will be the next area for investigation followed by characterizing the nature of resistance so that multiple forms can be combined to broaden PD resistance.

Objective 4. We have employed three categories of sequencing (shotgun reads, fosmid reads and 454 sequencing) to localize the BAC clone H69J14 that carries the *PdR1* gene(s). Now that this sequence is assembled, we have been able to identify six genes ranging in size from 2Kb to 3.1Kb in the resistance region. Copies 1 through 4 are 97 to 99% similar and may be tandem repeats of one gene. They are also up to 78% similar to four copies of genes on the Pinot noir PN40024 sequence (**Figure 6**). We utilized CENSOR software to screen query sequences against a reference collection of repeats to generate a report classifying all of the detected repeats. All four PN40024 genes carry DNA transposons as well as LTR retrotransposons confirming that the region is very complex.

Table 2. List of promising accessions from Mexico and US that performed well in the greenhouse screen. CMSSI is the cane maturation index, LS-LL is an index of leaf scorch and leaf loss, both values are recorded before samples are run to detect *Xf* in stems with ELISA.

Genotype	Mean (CMSSI)	Mean (LS-LL Index)	Sex	Source
ANU5	0.0	1.5	F	Littlefield, AZ - near I15 bridge crossing Virgin River
B40-14	3.8	2.1	M	80km n Chihuahua
B40-29	0.0	0.8	M	80km n Chihuahua
B41-13	1.0	0.8	F	near Ciudad Mante - Ciudad de Maiz
B42-26	0.8	1.4	M	Loreto, B.C - 200 km N La Paz
B43-17	2.5	2.3	M	Guadalupe, near Monterrey
B46-43	0.0	1.0	M	Big Bend Park, 250km W San Antonio
B46-48	0.0	0.8	F	Big Bend Park, 250km W San Antonio
B47-28	0.0	1.9	F	Big Bend Park, 250km W San Antonio
B47-32	0.0	0.9	M	Big Bend Park, 250km W San Antonio
B47-5	0.0	1.7	F	Big Bend Park, 250km W San Antonio
T 03-16	0.0	1.4		Hwy 170 W of Lajitas, TX
TX9714	2.7	1.0	M	N. Hondo, Medina Co., TX

Figure 5. Source of *Vitis* species collections currently under greenhouse evaluation for resistance to PD. The green dots indicate accession with promising resistance based on foliar symptoms. ELISA results are due in mid to late November 2011.

We utilized different tools from www.expasy.org/tool/ to conduct pattern and profile searches of the PD resistance genes. There is very strong evidence of a leucine rich repeat (LRR) region in five of the candidate genes. **Figure 7** displays differences in the LRR regions in the *PdR1b-1* gene. There is no signal sequence in the protein sequence, which suggests that the resistance gene product is not secreted. There is also no indication of a coiled-coil, which suggests that the PD resistance gene is not a member of the CC-NB-LRR class of resistance proteins. The protein sequences do carry trans-membrane domains, however they lack the kinase domain. Interestingly, the PdR1b-6 gene candidate that was very different from the other candidates with protein kinase domains.

Currently we have cloned and verified the sequence of copy 1 and copy 6 candidate genes and are developing constructs for transformation experiments to determine which of these gene candidates confers resistance to PD. (See companion report "Molecular characterization of the putative *Xylella fastidiosa* resistance gene(s) from b43-17 (*V. arizonica*)).

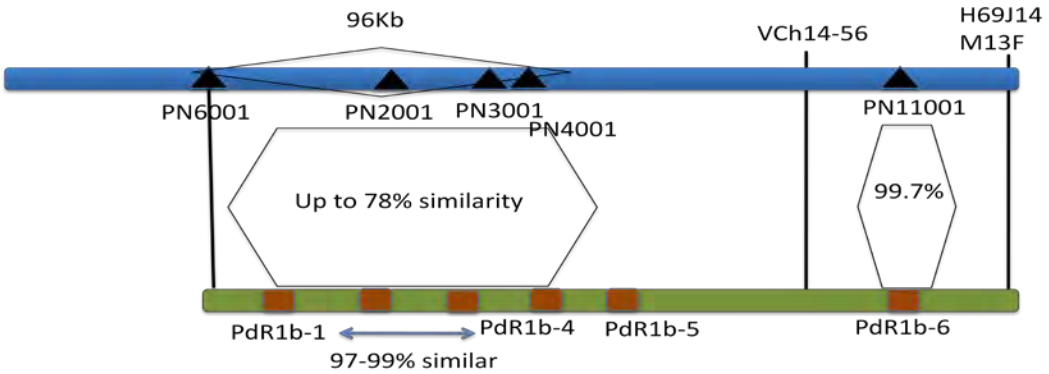


Figure 6. A direct comparison of the H69J14 clone sequence to the Pinot noir PN40024 sequence is not possible due to major re-arrangement of repetitive elements between the two genomes.

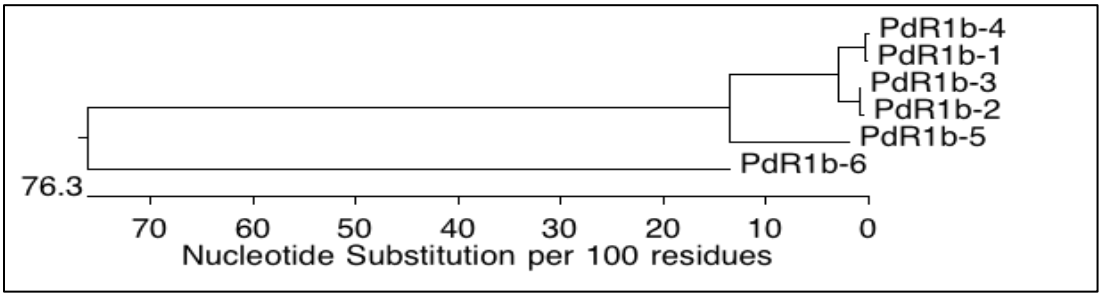


Figure 7. An alignment tree of the six candidate PD genes displaying their similarity.

Figure 7. Interpro scan results of the *PdR1b-1* gene.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board. Additional support from the Louis P. Martini Endowed Chair in Viticulture is also gratefully acknowledged.

BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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Reporting Period: The results reported here are from work conducted September 2010 to October 2011.

ABSTRACT
We continue to make rapid progress breeding Pierce's disease (PD) resistant winegrapes. Aggressive vine training and selection for precocious flowering has allowed us to reduce the seed-to-seed cycle to two years. We are also using marker-assisted selection (MAS) for the PD resistance gene, *PdR1* (see companion report) to select resistant progeny as soon as seeds germinate. These two practices have greatly accelerated the breeding program and allowed us to produce four backcross generations with elite *V. vinifera* wine grape cultivars in 10 years. In Spring 2010, we planted about 2,000 97% *vinifera* seedlings with *PdR1*. We finished the evaluating the fruit quality of over 1,200 of these in September 2011, and produced a small-scale wine of one, 09333-178. We are preparing to greenhouse test the best of these to verify which have the highest level of resistance to PD prior to multiplication and grafting for larger scale field trials. We plan to release commercially useful varieties from populations at this 97% *vinifera* level. The resistance above is based on 8909-08, which has one of the two alleles, *PdR1b*, from the *Vitis arizonica/candicans* b43-17 resistance source. The other resistance allele, *PdR1a*, is in 8909-17 and we have advanced this resistance to the 94% *vinifera* level and have combined it with the *PdR1b* allele to determine whether resistance with both alleles is stronger. There is also strong resistance in b42-26 a form of *V. arizonica/ girdiana* form Baja California. b42-26's resistance is controlled by multiple genes, as opposed to the single gene resistance found in b43-17. We made crosses this year to advance the b42-26 resistance to the 87% *vinifera* level and have been surprised no only by the strength of resistance but also by the relatively large number of resistant progeny each generation. We are now re-evaluating its resistance markers to verify that it is not another form of *PdR1*. Finally, we evaluated the first set of about 50 accessions collected across the southwestern US for PD resistance. There were many with very strong resistance and these will be tested to verify that their resistance is different from b43-17's. This year's wine making also included wines made as blends with elite vinifera winegrapes, a likely use of our eventual releases. These selections could be used in severe PD hot spots and the fruit could be blended into the rest of the vineyard in a 25/75% ratio. We used Napa Valley (Oakville Station) Sauvignon blanc and Merlot with 07713-051 and 07355-075, respectively. We also made wine at the same scale with the Napa Sauvignon blanc and Merlot, and Davis Sauvignon blanc and Merlot (as growing region controls).

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 *Vitis 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 selected progeny with *PdR1* from the b43-17 *V. arizonica*/*candicans* resistance source for fruit quality at the backcross 4 (BC4), 97% *vinifera* level. They are also undergoing greenhouse testing to verify their resistance and those with the highest levels of resistance will be prepared for small-scale winemaking this winter by grafting them onto Pierce's disease (PD) resistant rootstocks and planting six to eight vines sets on commercial spacing and trellising. We have made wine from vines that are 94% *vinifera* level from the same resistance background for two years. They have been very good and do have the hybrid flaws (blue purple color and herbaceous aromas and taste) that were prevalent in wines from the 87% *vinifera* level. There are two forms of *PdR1*, 8909-08 and 8909-17 – sibling progeny of b43-17 and they have different alleles of *PdR1*. 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 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

Table 1 presents the 2011 PD crosses made, although the list does not include crosses made to pyramid PD resistance with powdery mildew resistance. We used Nero d' Avola, a red wine grape from southern Italy and Sicily with very good color and tannin, and Pinot blanc, a Burgundian white wine grape to make crosses to increase our populations that contain PD resistance from 8909-08 (*PdR1b* from *V. arizonica/candicans* b43-17) at the 97% *vinifera* (backcross 4; BC4), and the 98.4% *vinifera* BC5 levels (**Table 1a**.). These crosses should generate over 500 seedlings of which half should have markers for, and resistance to, PD. These seedlings will add to our original planting of 97% *PdR1b* containing seedlings from our 2009 crosses (first fruit results are presented in **Table 3**).

There were 583 97% *vinifera PdR1b* containing seedlings planted in Spring 2011 from the 2010 crosses. These seedlings are 50% of their last *vinifera* parent and they largely express that last parent in their appearance and fruit quality. Those parents and the number of seedlings MAS for *PdR1b* that were planted include Barbera (263), Chardonnay (67), Muscat Blanc (31), and Riesling (222). If we combine the 2009-2011 *PdR1b* crosses, the last generation *vinifera* parents now number 11: Barbera, Cabernet Sauvignon, Chardonnay, Chenin blanc, Muscat blanc, Nero d'Avola, Pinot blanc, Pinot noir, Riesling, Sylvaner and Zinfandel.

In our greenhouse screening trials we always include b43-17 as a highly resistant standard to judge the effectiveness of the screen. In these screens we find that b43-17 is more resistant in terms of lower *Xf* levels (mean cfu/ml) in inoculated stems. These results prompted efforts to increase the durability or breadth of resistance by integrating resistance from other backgrounds and to combine both alleles of *PdR1* into one background. We have made the most progress with *PdR1b* from 8909-08, but we are now advancing populations with *PdR1a* from 8909-17 and exploring other facets of b43-17'S resistance. **Table 1b** presents crosses made with 75% *vinifera PdR1* selections back to *vinifera* to create mapping populations designed to examine the impact of minor genes associated with *PdR1*.

Resistance in *V. arizonica/girdiana* b42-26 tested as a quantitative multi-gene trait, which greatly suppresses *Xf*. Because resistance is quantitative backcross breeding is less effective. However, we continue to breed and map with this resistance (**Table 1c**) and have made our first crosses to combine b42-26 resistance to our advanced selections with *PdR1* (**Table 1d**). These efforts have been promoted by promising greenhouse screen results with b42-26 at the 75% *vinifera* level (**Table 2, group D**). Ultimately we want to combine these resistance sources to broaden and strengthen resistance. This Spring we broadened the wine quality background at the 87.5% *vinifera* level with crosses to F2-35 and Zinfandel.

Table 1. Pierce's disease resistant crosses made in 2011.

Resistant Type	*Vinifera* Parent\Grandparent of Resistant Type	*Vinifera* Types used in 2011 crosses	Estimated # of Seed
1a. Monterrey *V. arizonica/candicans* resistance source (F8909-08) to produce progeny between 97% and 98.4% *V. vinifera* parentage. F2-35 is 100% *vinifera* cross of Cabernet Sauvignon x Carignane.			
07355-020	Petite Sirah\Cabernet Sauvignon	Nero d'Avola	260
07370-039	F2-35\Chardonnay	Nero d'Avola	220
09-331	Zinfandel\Petite Sirah	Nero d'Avola, Pinot blanc	315
1b. Monterrey *V. arizonica/candicans* resistance source (b43-17) to produce progeny with 75% *V. vinifera* parentage for the discovery of minor genes for PD resistance.			
09373-01	F2-35	08319-12 (Zinfandel selfed), Rosa Minna	70
04373-02	F2-35	Pinot blanc, Zinfandel	565
04373-22	F2-35	Pinot blanc, Zinfandel	1,025
1c. Crosses to the b42-26 *V. arizonica* resistance source to produce progeny that are 87.5% *vinifera* and 12.5% the resistance source.			
07344A-10	Grenache	F2-35	175
07344A-11	Grenache	Zinfandel	175
07344A-24	Grenache	F2-35	220
07344A-35	Grenache	F2-35	700
1d. Cross made to pyramid *PdR1b* b43-17 Monterrey *V. arizonica/candicans* and b42-26 *V. arizonica* resistance lines to produce progeny between 84% and 86% *vinifera*.			
09-331	Zinfandel\Petite Syrah	Grenache\F2-35	725
07355-020	Petite Sirah\Cabernet Sauvignon	Grenache\F2-35	175
07370-039	F2-35\Chardonnay	Grenache\F2-35	60

Table 2 details the greenhouse screening we have done in 2010-11. Groups A and H were tests of populations being pursued as PD resistant rootstocks. Although we screen our selections for the highest level of resistance and the lack of PD symptoms, there is a chance that *Xf* could move downwards into the rootstock and damage a susceptible rootstock. Thus, we are breeding rootstocks with *PdR1* to ensure that they will be equally resistant. We are also incorporating resistance to a broad range of nematodes and screening for good rooting and vineyard performance.

Group B was a test of 97% *vinifera PdR1b* selections from our 2009 populations. These 23 selections were made based on the presence markers for *PdR1* and their vigor and appearance. They were grafted and moved to our larger Y trellis on commercial spacings to get more fruit for small-scale winemaking. The greenhouse testing found that seven of these selections had relatively high *Xf* levels even though they had *PdR1* markers. We have seen this level of resistance dilution in these later generations. However, the rest had high levels of resistance. This group included 09333-178, which was fruitful enough to allow very small wine making this year (**Table 4a-c**) and is very promising (**Figure 3**). The rest will be ready for small-scale winemaking in 2012 and we hope to choose selections for field testing from them. We have many more selections from these populations that will be evaluated when they fruit more reliably next year.

The Groups C, D, F and G provided screening results for our genetic mapping efforts with sources of PD resistance other than b43-17. Group G was the first of several tests examining our southwestern *Vitis* collections to identify additional sources of PD resistance. These efforts are important, as we want to broaden resistance by combining multiple resistance sources. The results of this screen are pending but they include 54 accessions and are elaborated in our companion report on the genetic mapping of *Xf* resistance. Screening results with b42-26 suggest that a major resistance locus may have a greater impact than previously thought and that resistance is not only strong, but more individuals are resistant than expected. Mapping studies are examining these progeny to determine whether this resistance is another form of *PdR1*. The 2010 crosses included several populations with the most resistant b42-26 progeny crossed to Cabernet Sauvignon, Carignane and Chardonnay, which resulted in 214 seedlings that were planted this Spring. The *V. shuttleworthii* 'Haines City' resistance source was tested at the 75% *vinifera* level in Group F. Only nine of the 97 tested progeny were resistant. However, they were strongly resistant so we will continue to advance this population to the next generation, although fewer resistant progeny are being detected in each successive generation.

Group E tested progeny from crosses to combine advanced lines of both alleles of *PdR1* (a and b from 8909-17 and 8909-08, respectively). We are waiting for MAS results to verify which allele is in which seedling. The data will be very interesting and help determine whether stronger resistance is achieved with both alleles of the homozygous resistant b43-17. This Spring we planted 180 progeny with *PdR1a* at the 94% *vinifera* level, which should begin fruiting in 2012. Advanced selections from this population will be crossed to 97% *vinifera PdR1b* selections, resulting in individuals with both alleles.

Table 2. Groups of plants greenhouse screened for *Xf* resistance.

Group	Genotypes	# Genotypes	Inoculation Date	ELISA Sample Date	Resistance Source(s)
A	08 PD Rootstocks & Recombinants	22	7/15/10	10/14/10	F8909-08
B	97% *vinifera*, Y-trellis	23	7/26/10	11/23/10	F8909-08
C	05347 Mapping	122	9/23/10	1/6/11	b42-26
D	07344A, 07744RT, 2010 Parents	79	11/9/10	2/10/11	b42-26, b40-14, b43-17
E	*PdR1a* & *PdR1b* together	122	1/13/11	5/3/11	b43-17
F	Haines City & Supplemental	97	3/24/11	7/12/11	*V. shuttleworthii*
G	*V. arizonica* and southwestern *Vitis*	54	5/12/11	8/11/11	*V. arizonica* accessions
H	PD Rootstocks Adv. Selections	15	6/14/11	9/15/11	F8909-08

Objective 2

Table 3 presents results of the 2011 evaluation of the 97% *vinifera PdR1b* progeny fruit. This was the first year that our most advanced material fruited. The table lists the progeny by the last *vinifera* parent in the crosses – the progeny are 50% of that parent. The first row of the table presents the number of crosses made with each parent, followed by the number of seedling progeny tested. The number of precocious progeny is next – these are the seedlings that flowered this year. We will evaluate the seedlings that did not fruit this year in 2012. We then selected based on flavor and appearance over two selection passes (cuts) and advanced as many as possible to juice and berry evaluations. Seventy-seven seedlings made the second cut and berry evaluations were made on 65 of these (the other 12 were very early and will have to be tested next year). Based on cluster and berry size, and juice flavor and color, 23 were selected as the best (**Figures 1 and 2**). The entire set of 77 will be greenhouse tested this Winter to identify those with the strongest resistance to *Xf* before we begin propagating the best for multiple vine testing and small-scale wine making. Hundreds of more progeny from the 2009 97% *vinifera PdR1b* crosses will begin fruiting next year. There should be many more advanced selections available from these evaluations. In addition, the first of the progeny from the 2010 97% *vinifera PdR1b* crosses will begin fruiting.

Table 3. 97% *vinifera PdR1* 2009 cross seedlings evaluated for fruit quality for the first time in 2011.

Evaluation Level	*Vinifera* Parent							
	Cab Sauv	Chard	Chenin blanc	Pinot noir	Riesling	Sylvaner	Zinfandel	Total
# Crosses	7	4	6	3	1	3	4	28
# Seedlings (sdlg)	489	309	517	107	11	218	217	1868
# Precocious Sdlg	313	216	366	74	10	125	174	1278
# Sdlg making first cut	62	76	77	22	6	40	61	344
# Sdlg making 2nd cut	9	25	13	3	2	4	21	77
# Sdlg juice/berry evaluation	9	21	11	4	2	3	15	65
# Sdlg elite rating post juice/berry evaluation	5	5	4	1	1	-	7	23

Figure 1. Juice samples from 97% *vinifera PdR1b* selections.

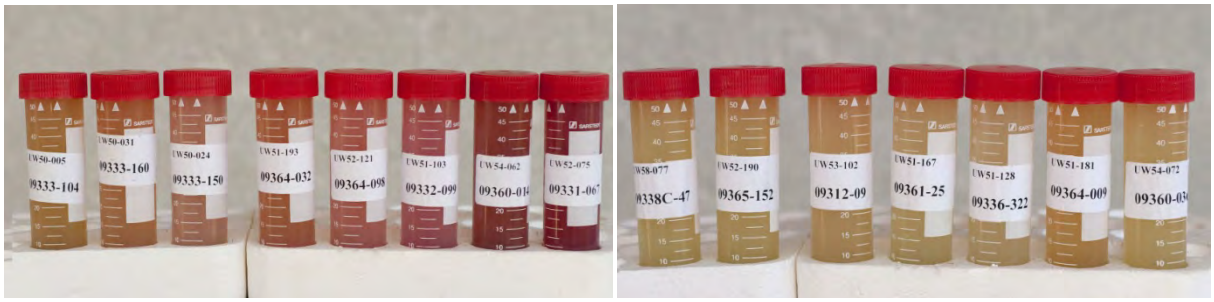

Figure 2. Range of juice color intensity from red and white 97% *vinifera PdR1b* selections.

Figure 3. 09333-178, the first 97% *vinifera PdR1b* selection used for small scale wine making in 2011.

We made 15 wine lots in 2011. Seven were from *PdR1* progeny (two at the 87% *vinifera* level, four at the 94% level and one at the 97% level) and included Blanc du Bois and Lenoir as our "state of the art" PD resistant southeastern US standards. We also made blended wines this year in anticipation of the likely use of many of our eventual releases. These selections could be used in severe PD hot spots and the fruit could be blended into the rest of the vineyard in a 25/75% ratio. We used Napa Valley (Oakville Station) Sauvignon blanc and Merlot with 07713-051 and 07355-075, respectively. We also made wine at the same scale with the Napa Sauvignon blanc and Merlot, and Davis Sauvignon blanc and Merlot (as growing region controls).

Table 4a-c provides the details of the vine, fruit and juice characteristics for the seven *PdR1* wine lots made in 2011. Two of the favorites from the 87% *vinifera* level were included; two 94% *vinifera* level selections that we made wine from last year, and two new 94% *vinifera* level selections; and our first effort at the 97% *vinifera* level, 09333-178.

Table 4a 2011 PD resistant wine lots background and fruit characteristics

Genotype	Parentage	% *vinifera*	2011 Bloom Date	Berry Color	Berry Size (g)	Avg Cluster Wt. (g)	Ripening Season	Prod 1=v low, 9=v high
07329-31	U0505-01 x Chardonnay	94%	5/24/11	B	1.0	136	mid	5
07355-48	U0505-01 x Petite Sirah	94%	5/24/11	B	1.1	262	mid	6
07355-75	U0505-01 x Petite Sirah	94%	5/27/11	B	1.1	245	late	7
07713-51	F2-35 x U0502-48	94%	5/19/11	W	1.2	229	early	8
09333-178	07355-020 x Chardonnay	97%	5/24/11	B	1.3	168	mid-late	5
U0502-20	A81-138 x Chardonnay	87.5%	5/21/11	W	1.1	183	early-mid	7
U0502-38	A81-138 x Chardonnay	87.5%	5/19/11	B	1.0	358	early	7
Lenoir	*V. aestivalis* hybrid	<50%	6/2/11	B	1.3	157	late	6

Table 4b 2011 PD wine lot juice chemical analysis. Data from Cab. Sauvignon, Pinot noir and Lenoir are form previous vintages for comparison.

Genotype	°Brix	TA (g/L)	pH	L-malic acid (g/L)	potassium (mg/L)	YAN (mg/L, as N)	catechin (mg/L)	tannin (mg/L)	Total antho-cyanins (mg/L)
07329-31	29.2	6.6	3.63	2.05	1810	548	2	395	1160
07355-48	26.2	5.9	3.54	1.89	1850	213	4	480	1624
07355-75	27.2	6.8	3.52	1.98	2050	324	<1	558	1397
07713-51	24.1	5.8	3.50	1.97	1540	210	-	-	-
09333-178	25.1	5.0	3.67	1.17	1800	265	61	328	595
U0502-20	23.9	6.9	3.52	2.92	1660	419	-	-	-
U0502-38	27.9	9.0	3.58	6.36	2570	449	18	737	1346
Cab. Sauvignon	24.9	6.2	3.65	2.19	2460	227	59	250	404
Pinot noir	26.5	4.9	3.83	2.43	2190	279	321	842	568
Lenoir	24.8	12.1	3.22	7.03	2240	183	186	268	2486

Table 4c 2011 PD wine lot berry sensory analysis. Skin tannin, seed color and seed tannin rated from 1 = low to 4 = high.

Genotype	Juice Hue	Juice Intensity	Juice Flavor	Skin Flavor	Skin Tannin	Seed Color	Seed Flavor	Seed Tannin
07329-31	pink-red	medium	fruity-stemmy	currant	1	4	woody	2
07355-48	red-pink	light	cherry jam	cassis	4	3	wood, astringent	2
07355-75	red	dark	jammy	prune	1	3	warm, spicy	1
07713-51	gold, sl brwn	dark	floral, melon	herbal	3	3	spicy, mildly hot	3
09333-178	orange	light	fruity, red apple	black plum	1	3	woody, sl bitter	2
U0502-20	green-brwn	medium	grn apple, melon	neutral	1	3	sl spicy, sl bitter	4
U0502-38	pink-red	med-dark	cherry, berry	plum	2	3	woody, ripe	1
Lenoir	red	med-dark	mildly fruity	fruity	1	4	hot	1

CONCLUSIONS

Strong progress continues to made breeding PD resistant winegrapes. We evaluated our first fruit at the 97% *vinifera PdR1* resistance level and were even able to get enough fruit from one selection to make a small-scale wine. We are advancing other forms of PD resistance to later generations with the goal of combining multiple resistance sources to broaden resistance. These include the other allele of *PdR1*, resistance from b42-26, and resistance from *V. shuttleworthii* 'Haines City'. We made small-scale wines again this year at the 87.5, 94 and 97% *vinifera* levels. We also made blended wines with a white 94% *vinifera* selection (07713-051) with Napa Valley Sauvignon blanc (25/75%) and a red 94% *vinifera* selection (07355-075) with Napa Valley Merlot to mimic how these selections might be used in North Coast PD hot spots.

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IDENTIFICATION AND CHARACTERIZATION OF PROTEOMIC EXPRESSION OF GRAPEVINES IN RESPONSE TO *XYLELLA FASTIDIOSA* INFECTION

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ABSTRACT
Xylella fastidiosa (*Xf*) is the bacterial causal agent of Pierce's disease (PD) of grapevines, as well as of other economically important diseases in a number of agronomic, horticultural and ornamental plants. In this study, comparative proteomic analyses were carried out to identify proteins differentially expressed in *Xf*- infected grape stems from a pair of siblings of 9621-67 (highly resistant) and 9621-94 (highly susceptible) grapevines from a cross of *Vitis rupestris* x *V. arizonica* (Yang et al., 2011). The identification of proteins and their expression pattern under given physiological conditions is of fundamental importance for functional analyses of cellular processes associated with PD development and resistance. The proteins were isolated from the stems of healthy and *Xf*-inoculated plants at one, six, and 12 weeks after inoculation. The total proteins were then separated by a 2D-PAGE system and the spots representing differentially expressed proteins were analyzed by an oMALDI-TOF-MS/MS Mass Spectrometry. Protein identification was performed using BLASTp and tBLASTn against NCBI non-redundant protein databases and EST databases. Ten proteins that were differentially expressed at different time points after inoculation were identified. For example, a thaumatin-like protein and the pathogenesis-related protein 10 from both genotypes, and the 40S ribosomal protein S25 from the susceptible genotype were up-regulated in response to *Xf*-infection. Furthermore, the expression of the thaumatin-like protein increased sharply 12 weeks post-inoculation in the PD-resistant genotype only. Three heat shock proteins, 17.9 kDa class II, protein 18 and 21 were highly expressed in healthy tissues compared with those in tissues infected with *Xf*. In addition, a down-regulated putative ripening related protein was found in the *Xf*-inoculated PD-susceptible genotype. Glycoprotein and formate dehydrogenase were identified in the PD-resistant genotype and their expression was constant during plant development. A putative GTP-binding protein was down-regulated in the PD-susceptible genotype. Our results revealed that differential expression of proteins in response to *Xf*-inoculation was genotype and tissue development stage dependent. The specific roles of these candidate proteins in alleviation or aggravation of this disease are under investigation. The information obtained in this study will aid in the understanding of the mechanisms related to the host-pathogen interactions involved in PD.

LITERATURE CITED
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TESTING *XYLELLA FASTIDIOSA* PATHOGENESIS MUTANTS IN *ARABIDOPSIS THALIANA*

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Reporting Period: The results reported here are from work conducted September 2010 to September 2011.

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 model 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. 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. The *Arabidopsis* ecotype Tsu-1 was inoculated with the *Xf* mutants *tolC*, *pglA*, and *rpfF* and their corresponding wild-type parents. The *tolC* mutant did not grow in *Arabidopsis* and no live cells were recovered, which is similar to results from *tolC* infection of grapevine. The *rpfF* mutant did grow to higher titer, again similar to growth in grape. However, growth of the *pglA* mutant was indistinguishable from that of the corresponding Fetzer wild type; this contrasts with the situation in grape where *pglA* is hypervirulent. It may be concluded that *Xf* growth in Arabidopsis is not enhanced by a functional polygalacturonase protein, perhaps because of differences in xylem and pit membrane structure between grape and *Arabidopsis*. Because two of the three mutants tested (*tolC* and *rpfF*) did behave similarly in *Arabidopsis* and in grape, it appears that *Arabidopsis* is an informative model host for the evaluation of at least some *Xf* mutants.

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XYLELLA FASTIDIOSA INFECTION OF GRAPEVINES AFFECTS XYLEM LEVELS OF PHENOLIC COMPOUNDS AND PATHOGENESIS-RELATED PROTEINS

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Reporting Period: The results reported here are from work conducted December 2010 to present.

ABSTRACT

Pierce's disease (PD), caused by the xylem-dwelling pathogen *Xylella fastidiosa* (*Xf*), is a serious threat to grape production. The effects of *Xf* infection six months post-inoculation on defense-associated proteins and phenolic compounds found in xylem sap and tissue were evaluated. Defense-associated protein (peroxidase, polyphenol oxidase, exo-chitanase, and beta-1,3-glucanase) levels from xylem sap and ground tissues were compared between non-inoculated and *Xf*-inoculated grapevine (cv. Thompson Seedless) using enzyme kinetic assays. Phenolic compound levels were compared using high performance liquid chromatography (HPLC). Peroxidase activity was greater in infected grapevines, whereas activities of polyphenol oxidase, exo-chitanase, and beta-1,3-glucanase levels were greater in non-infected grapevines. Grapevines infected with *Xf* had greater sap levels of three phenolics, including resveratol, than non-infected controls. Pooled levels of flavonoid glycoside and stilbenoid compounds also were greater in infected than in non-infected grapevine sap. In contrast, levels of phenolic acids and proanthocyanidins were lower in *Xf* infected than in non-infected grapevines. Methanol extracts of ground xylem tissue revealed that infected plants had lower levels of phenolic acids and cell wall macromolecules lignin and tannin. Previous work observed commercially available phenolic compounds found in grapevines significantly reduced *Xf* growth *in vitro* (Maddox et al. 2010). Therefore, reductions in sap and xylem tissue levels of phenolic acids/proathocyanidins observed in *Xf* infected plants may have compromised grapevine defense against *Xf*, allowing PD to proceed despite induction of anti-biotic flavonoids and stilbenoids in response to *Xf* infection. Infection of grapevine with *Xf* could have adversely impacted primary host physiology, resulting in less available host resources for phenolic acid production. Regardless, a better understanding of how *Xf* affects phenolic and defense-associated protein levels in the xylem could aid in the development of management strategies and novel chemistry-based PD detection methods.

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Section 6:

Economics