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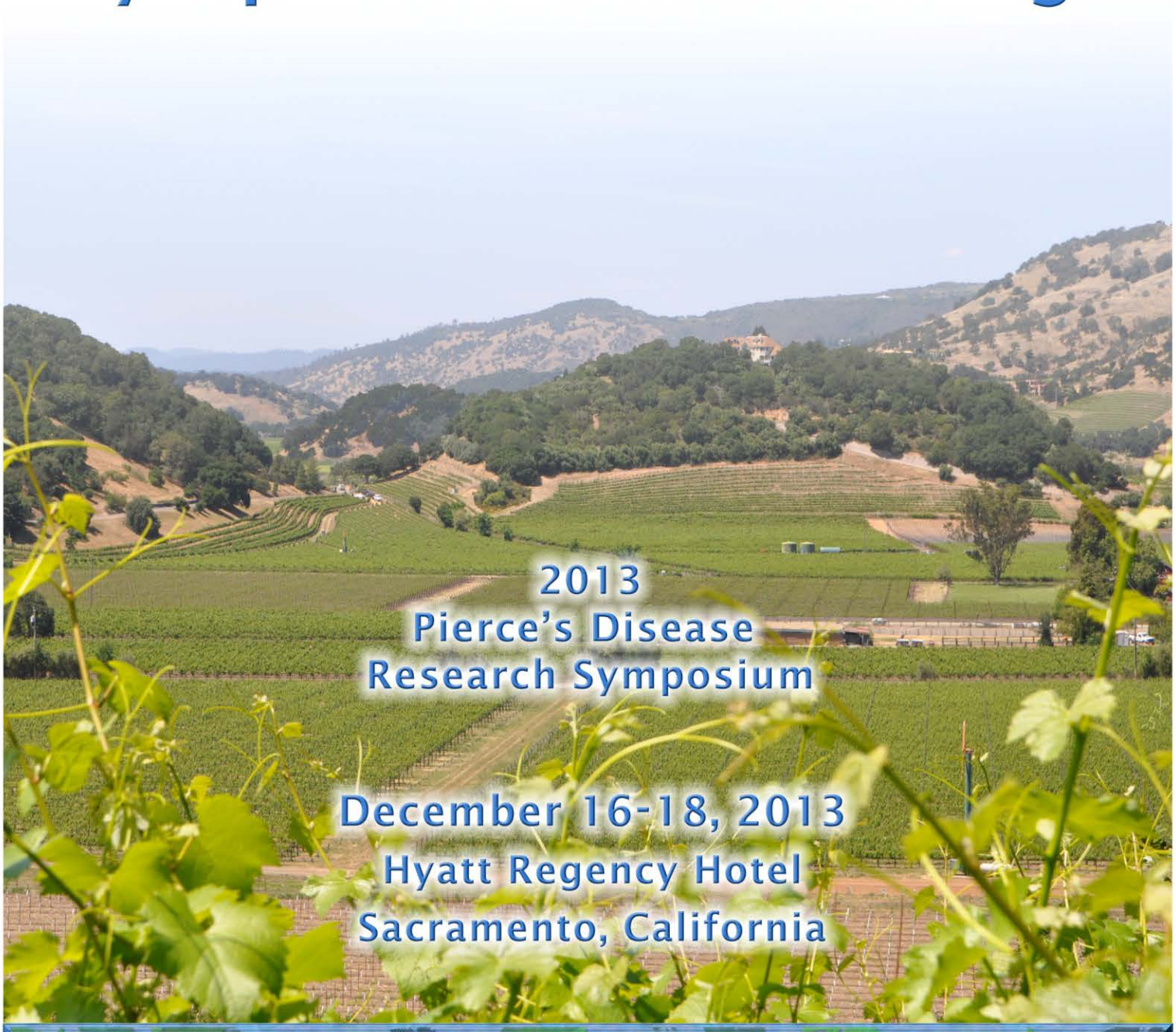

2013 Pierce's Disease Research Symposium

December 16-18, 2013
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Sacramento, California

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TABLE OF CONTENTS

Section 1:
Vector Biology and Ecology

Section 2:
Vector Management

Section 3:
Pathogen Biology and Ecology

REPORTS

ABSTRACTS

Section 4:
Pathogen and Disease Management

REPORTS

ABSTRACTS

Section 5:
Crop Biology and Disease Epidemiology

REPORTS

ABSTRACTS

Section 1:

Vector Biology and Ecology

OPTIMIZING ELECTRICAL PENETRATION GRAPH SETTINGS TO RECORD BLUE-GREEN SHARPSHOOTER X WAVES FOR FUTURE STUDIES OF GRAPE HOST PLANT RESISTANCE TO *XYLELLA FASTIDIOSA* INOCULATION

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ABSTRACT
The long-term goal of the Backus research program is to work with grape breeders to aid in development of a grape rootstock variety that is resistant to a sharpshooter's ability to inoculate *Xylella fastidiosa* (*Xf*) bacteria into healthy grapevines, thereby preventing later *Xf* infection. Such a trait would be quite different from the more common mechanism of resistance to *Xf*, i.e., resistance to systemic bacterial spread and multiplication, after vector inoculation has already occurred. Identification and selection of an anti-inoculation trait depends upon two research objectives: 1) understanding the behavioral mechanisms of *Xf* inoculation by sharpshooters, and 2) devising a rapid method for testing and identifying grapevines that possess a means of deterring those inoculation behaviors, so that such plants can be added to the resistance-selection process. Ten years of research has revealed the behavioral mechanism of *Xf* inoculation, using electrical penetration graph (EPG) monitoring of insect feeding. The inoculation behavior is represented by the sharpshooter X wave. The present research showed that the same X wave components are visible for the blue-green sharpshooter as for the glassy-winged sharpshooter, with very similar, recognizable appearances. Thus, this work supports that EPG will be a crucial technology for detection of *Xf* inoculation behaviors represented by the sharpshooter X wave. It also showed that EPG monitor settings can be optimized for future recordings of the blue-green sharpshooter X wave, and that those settings may be different from the best settings for glassy-winged sharpshooter recordings. The deliverable for grape growers from this research would be pyramiding of multiple resistance traits into one resistant rootstock variety, for greater permanence of resistance in the field against all types of sharpshooter vectors.

LAYPERSON SUMMARY
Pierce's disease of grape, a lethal plant disease caused by the bacterium *Xylella fastidiosa* (*Xf*), has the potential to devastate grape production in California, worth $4.1 billion/year. Due to the expense, environmental damage, and potential impact on honey bees caused by insecticides to control sharpshooter leafhoppers (vectors of *Xf*), there is a pressing need to develop grape varieties resistant to the bacterium and/or sharpshooters. The long-term goal of the Backus research program is to work with grape breeders to aid in development of a grape rootstock variety that is resistant to a sharpshooter's ability to inject *Xf* bacteria into healthy grapevines, thereby preventing later *Xf* infection. The present research provides the first step in achieving this goal, by preparing to use electrical penetration graph (EPG) monitoring as a means of testing grape varieties for resistance of *Xf* inoculation. The deliverable for grape growers from this research would be combining multiple resistance traits into one resistant rootstock variety, for longer-lasting resistance in the field.

INTRODUCTION
Pierce's disease of grape, a lethal plant disease caused by the bacterium *Xylella fastidiosa* (*Xf*), has the potential to devastate grape production in California, worth $4.1 billion/year in direct product and much more in tourist dollars. Over 337,000 hectares of vineyards are distributed throughout the state. The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is the most important vector of *Xf* in southern and central California vineyards, while the blue-green sharpshooter (BGSS; *Graphocephala atropunctata*) is the most important vector in northern California vineyards. Due to the expense, environmental damage, and potential impact on honey bees caused by insecticides to control *Xf* vectors, there is a pressing need to develop crop varieties resistant to the bacterium and/or sharpshooters.

The long-term goal of the Backus research program is to work with grape breeders to aid in development of a grape rootstock variety that is resistant to a sharpshooter's ability to inoculate *Xf* bacteria into healthy grapevines,

thereby preventing later *Xf* infection. Such a trait would be quite different from the more common mechanism of resistance to *Xf*, i.e., resistance to systemic bacterial spread and multiplication in grape, after vector inoculation has already occurred. Identification and selection of an anti-inoculation trait depends upon two research objectives: 1) understanding the behavioral mechanisms of *Xf* inoculation by sharpshooters, and 2) devising a rapid method for testing and identifying grapevines that possess a means of deterring those inoculation behaviors, so that such plants can be added to the resistance-selection process. The deliverable for grape growers from this research would be pyramiding of multiple resistance traits into one resistant rootstock variety, for longer-lasting resistance in the field.

Background on the Science.

For the last ten years, Backus has successfully sought and discovered the behavioral/ physiological mechanism of vector inoculation of *Xf* bacterial cells (Backus et al., 2009, Backus and Morgan 2011), which occurs during a specific portion of the sharpshooter piercing-sucking feeding process (also termed stylet penetration). The key to the success of this objective was use of electrical penetration graph (EPG) monitoring of insect feeding (Walker 2000). EPG makes an insect part of an electrical circuit that includes the plant and an EPG monitor. It depicts otherwise-invisible behaviors (activities of the stylets inside opaque plant tissues) as waveforms displayed on a computer. The waveform can later be measured by a researcher to provide exacting quantification of the time spent in the various stylet activities. EPG also is likely to be the primary means by which the second objective above (devising a testing method) will be achieved.

Through a lengthy process of research, Backus was able to characterize the EPG waveforms of GWSS (Backus et al., 2005, Joost et al., 2006), and also determine the biological meaning of each waveform. Complete details of this research, and the evidence for Backus's "salivation-egestion hypothesis" of *Xf* inoculation, can be read in a new review book chapter (Backus, *in press*), due out in a few months. Results of numerous experiments support that the most likely mechanism of *Xf* inoculation is a sequential process of: 1) salivation into a xylem cell, 2) uptake of minute amounts of xylem sap mixed with saliva, 3) swishing this fluid around inside the insect's "mouth" (or oral cavity [precibarium and cibarium], where the *Xf* bacteria live inside the insect), which causes some bacterial cells to be dislodged and suspended in the sap-saliva mixture, then 4) egestion ("spitting out") of the fluid back into the xylem cell. The EPG waveform that represents this inoculation behavior is the sharpshooter X wave, which is only performed when the vector's stylet tips are inside xylem cells. These are the plant cells in which the *Xf* bacteria reside.

A preliminary EPG study comparing GWSS feeding on resistant wild grape, *Vitis candicans*, and susceptible cultivated grape, *V. vinifera* cv. Chardonnay, found that both counts and durations of X waves were significantly lower on resistant than on susceptible grape (Backus and A. Walker, *unpub. data, ms. in prep*.). This finding supports the theory that some grape plants can manipulate the feeding of sharpshooters in such a way as to decrease the likelihood of inoculation. Thus, EPG detection of X waves will be the key technology in the search for grape plants resistant to vector inoculation of *Xf*.

Near-future tests are planned in which BGSS "loaded" with *Xf* in their oral cavities will be EPG-recorded on resistant and susceptible accessions of cultivated grapevines, to determine the degree to which differences in X waves correspond to reduced *Xf* inoculation. BGSS will be used because previous studies have shown that it is easier to "maximally load" BGSS with *Xf* for laboratory tests than to do so with GWSS. To prepare for such future tests with BGSS, the present study was performed to characterize BGSS waveforms and compare them with the better-known appearances of GWSS waveforms, especially X waves. Various settings of an AC-DC EPG monitor (e.g., amplifier sensitivities, applied signal type and voltages) were tested, to determine the optimal EPG settings for BGSS recordings, for use in these future tests.

In order to best understand the results described in this report, it is important to know some background on EPG science and sharpshooter X waves. The most important aspect of EPG science is the concept of electrical origins of waveforms. R (for electrical resistance) and emf (for electromotive force, a synonym of voltage) are two reciprocal electrical mechanisms (called 'origins') of all EPG waveforms that can explain how electrical signals are generated by an insect's stylet activities. R components of a waveform occur when highly conductive saliva is secreted, or valves and pumps inside the insect's head close or open during feeding, causing physical resistance to electrical signal flow through the insect. In contrast, emf components occur when minute voltages (also called biopotentials) spontaneously arise in the circuit, caused by aspects of feeding. For example, the sharpshooter's

stylets may break a plant cell membrane that would ordinarily cause an electrical charge separation that generates a tiny biopotential within the plant. Or a minute voltage (called a streaming potential) can become established as fluid rushes through the insect's capillary tube-like food canal in the stylets, e.g., during ingestion (uptake and swallowing) of xylem sap. Because the biological meanings of most R vs. emf components are now well understood, identifying the electrical origin of a waveform can actually explain what the waveform means, in biological (in addition to electrical) terms. Data about electrical origins has provided strong evidence for understanding the X wave, and how it represents *Xf* inoculation behavior.

The best way to identify which electrical origins are dominant in a certain waveform is to compare the appearance of the waveform at different amplifier sensitivities, also known as input impedance (or less accurately, input resistance, or Ri) levels. This is because different Ri levels emphasize R vs. emf components. The AC-DC EPG monitor in use in the Backus laboratory has six Ri levels, measured as 10^6, 10^7, 10^8, 10^9, 10^{10}, and 10^{13} Ohms (Ω). Waveforms recorded at 10^6 Ω (the "pure R" level) reveal exclusively R components whereas those recorded at 10^{13} Ω (the "pure emf" level) reveal exclusively emf components. Waveforms recorded at intermediate Ri levels reveal intermediate mixtures of R and emf components whose proportion varies with insect size. The smaller the body size of the insect, the higher the Ri level needs to be to record emf. Previous EPG recordings of GWSS feeding showed that the components of X waves (see below) were emf-dominated waveforms, and were best visualized for large-bodied sharpshooters like GWSS (~13 mm body length) at Ri 10^8 Ω. BGSS is a smaller sharpshooter (~8 mm body length), so the best Ri level to record X waves is likely to be different.

The sharpshooter X wave represents a stereotypical sequence of behaviors used by the insect to taste, test, and accept a xylem cell for sustained ingestion (pumping and swallowing of xylem sap). Several X waves are repeatedly performed when a xylem cell is tested, before sustained ingestion begins. The terms used are explained in **Figure 1**. An X wave is divided into two waveform sub-phases, XN and XC. XN is the more behaviorally complicated sub-phase. It is comprised of two waveform types, B1 and C1 (**Figure 1**). B1, in turn, is comprised of two sub-types, B1s and B1w (see subsequent figures). A special sub-sub-type of B1w, fB1w, is utterly diagnostic of an X wave, occurring at no other time in stylet penetration. XC is composed of only one waveform type, C2 (**Figure 1**). Each of these "X wave components" represents a tiny part of the behavioral/ physiological processes involved in testing and accepted a xylem cell. During XN, tasting of the cell contents occurs via minute fluid uptake (fB1w), swishing of fluid (B1s) and then egestion of fluid outward (C1), plus salivation (B1w) to seal the stylets tips firmly into the xylem cell. Salivary sealing prevents leakage of air into the cell (called cavitation) that would prevent subsequent uptake of fluid. During XC, a few pumps of fluid (C2) are performed for a very short-duration bout of "trial ingestion" to test the mechanical strength of the salivary seal into the cell. If the seal is not strong enough, the insect returns to tasting/salivary sealing (XN), then another bout of trial ingestion (XC). If the seal is strong enough this time, it continues pumping and swallowing (C2) that can last for several hours ("sustained ingestion"), to derive the insect's primary nutrition. Sub-phase XN, especially sub-type C1, is considered to be the most important waveform for *Xf* inoculation.

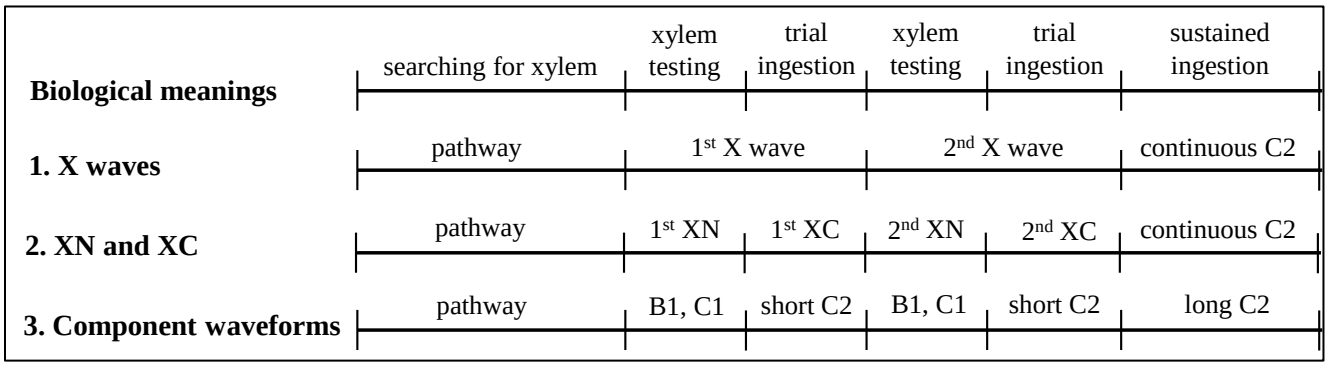

Figure 1. Biological meanings (first line) of EPG recording of a single stylet penetration, including X wave components. Nested, hierarchical waveform terminologies are explained in numbered lines below first line. Waveform type B1 is divided into sub-types described further in the text.

OBJECTIVES

1. Characterize the appearance and fine-structure of EPG waveforms, especially X waves, for BGSS; compare them with previously-recorded waveforms for GWSS.
2. Identify the input impedance (Ri) and applied voltage settings for best visualization of X waves for BGSS, for future comparisons of resistant vs. susceptible cultivated grapevine accessions.

RESULTS AND DISCUSSION

BGSS adults were originally field-collected in riparian areas near Malibu, CA, in March 2011 by T. Perring and colleagues (UC Riverside). A year later, some of their adult offspring were shipped under permit to the Backus laboratory in Parlier, CA, to start a colony. BGSS were lab-reared under spring photoperiods (16:8 L:D) and temperatures (24^{o} C.) in an artificially-lit rearing room, on cowpea, basil, and faba bean plants (in the same cage) previously raised in a USDA greenhouse under supplemental lighting with fertilization. The day before EPG recording, six to eight adult BGSS were caged on a healthy grapevine, cv. Chardonnay for 24 hrs of pre-test conditioning. The morning of recording, four conditioned insects were wired for EPG (Backus et al., 2009). Wired insects were recorded for 18 – 20 h using an AC-DC 4-channel EPG monitor; each of the four channels was set to a different input impedance (Ri) level, i.e., 10^6, 10^7, 10^8 or 10^9 Ω. The 10^6 and 10^7 Ω channels were set for 10 mV DC+ (direct current, positive) and the 10^8 and 10^9 Ω channels for 30 mV AC (alternating current) applied voltage. After 8 – 10 h at those voltages, each channel was switched to the opposite voltage type, so that low-Ri channels were then recorded with AC and vice versa. After 24 BGSS were recorded in this manner, the conditioning period was eliminated, and an additional 28 BGSS were recorded directly from colony plants without prior exposure to grape. This was done to increase the types and durations of pathway, ingestion, and X waveforms.

To date, waveform excerpts have been analyzed for eight of the 52 insects recorded, two insects per Ri level, emphasizing the fine structure of X waves. In total for the eight insects, 141 XN events were excerpted, copied into Excel, and analyzed. This report describes preliminary interpretations of the electrical origins and X wave components at each of the four recorded Ri levels.

X waves were barely distinguishable at Ri 10^6 Ω. As usual, the first XN event (at the end of pathway) was separable from earlier pathway waveforms due to C1 peaks at the start of XN (**Figure 2**); however, those peaks were very short in amplitude. In addition, B1w waves were very flat, fB1w was not seen, and B1s spikes were very short in amplitude and erratic. Subsequent XN events (interruptions of the XC events; **Figure 3**) were elevated toward positive voltage level, and often had even less detail than the first XN. The first XC event showed very tiny C2 plateaus, whose shape was distorted from the typical rectangular plateaus seen at higher Ri levels. The above characteristics demonstrate that C1 is a mixture of R and emf components because it is visible and undistorted (but short) at Ri 106 Ω. However, the flatness of B1w without a frequency component, i.e., fB1w, and the near-absence of regular B1s show that B1 is a highly emf-dominated waveform. C2 also is a highly emf-dominated waveform, barely visible as mostly a small spike on a tiny plateau at this low Ri level.

Clarity of X wave details improved greatly at Ri 10^7 Ω, with all components taller, less distorted and more distinguishable from pathway B1. The first XN was easily separated from pathway (**Figure 4**) by the size and clarity of B1 subtypes; B1s was more distinctly spikey and B1w was more wavy, with a very low-frequency undulation (**Figure 4** inset box). C1peaks were distinctly triangular, gradually evolving into the more rectangular plateaus of C2 in XC, the latter being no longer distorted. C2 plateaus also showed a small but prominent spike at the leading edge of each distinct plateau, an R component on the otherwise exclusively emf component of the rounded plateau. Second and subsequent XN (in interruptions of C2) were still elevated positive-ward. The above characteristics show that Ri 10^7 Ω, more sensitive to emf than Ri 10^6 Ω, added details attributable to emf.

Fine structures of the X wave components were slightly improved at Ri 10^8 Ω over Ri 10^7 Ω. In the XN portion (**Figure 6**), B1s spikes were straighter, with each more separate and distinct from the still-wavy B1w. Some B1w had a higher frequency, was less wavy, than at Ri 10^7 Ω (**Figure 7**). Yet, no fB1w was visible. C1 spikes were tall and triangular. XN voltage level was flat; even with level of C2. In XC, the C2 plateaus were much taller and more separable from C1, and still showed the R-component short spike at the leading edge of each plateau (**Figures 6 and 7**). The above electrical characteristics show that details of X wave components were easier to identify and separate from other components at Ri 10^8 Ω, with no loss of information compared with Ri 10^7 Ω.

The clarity of waveform appearances was because the waveforms are mostly due to emf, but have a small component of R as well. Both electrical origins were visible at this Ri level, but emf was emphasized.

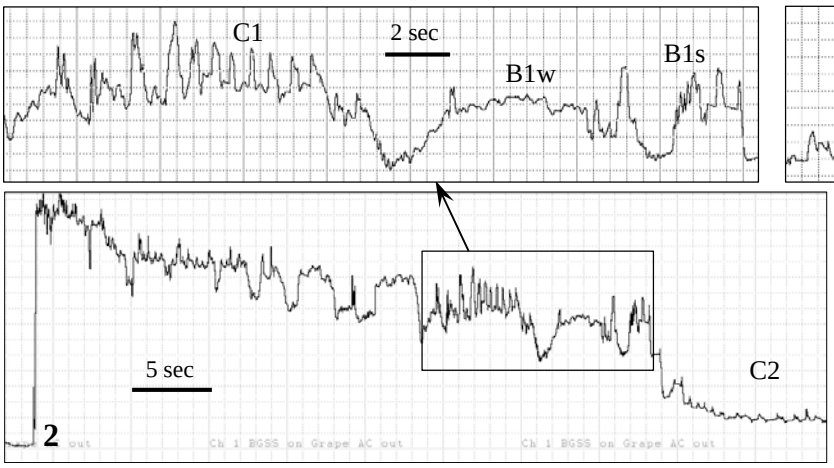

Figure 2. The beginning of a probe recorded using Ri 10^6 Ω, 30 mV AC applied signal, showing the first XN before the first XC (boxed). Inset box shows triangular C1 peaks, flat B1w and rare, short B1s. Other waveforms are indistinct. The start of the first XC event is also shown, with very small, distorted C2 plateaus.

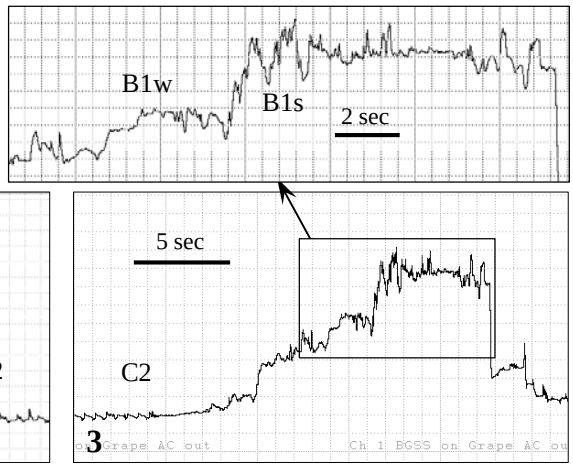

Figure 3. The second XN after the first XC from the same probe as **Figure 2**, showing upward polarity, indistinct B1 components.

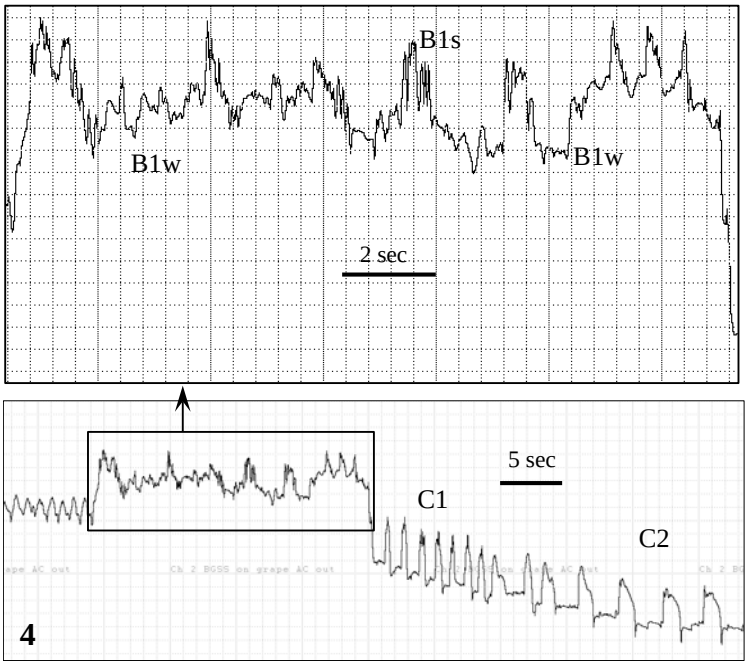

Figure 4. The middle of a probe recorded using Ri 10^7 Ω, 30 mV AC applied signal, showing the first XN (partly boxed) before the first XC. Inset box shows triangular wavy B1w and distinctly spikey B1s.

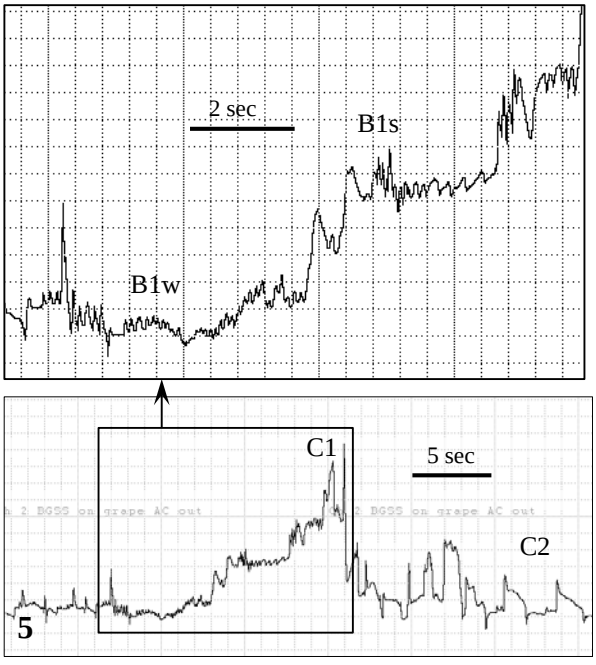

Figure 5. The second XN after the first XC from the same probe as **Figure 4**, showing upward polarity, more distinct B1 components (B1s and B1w).

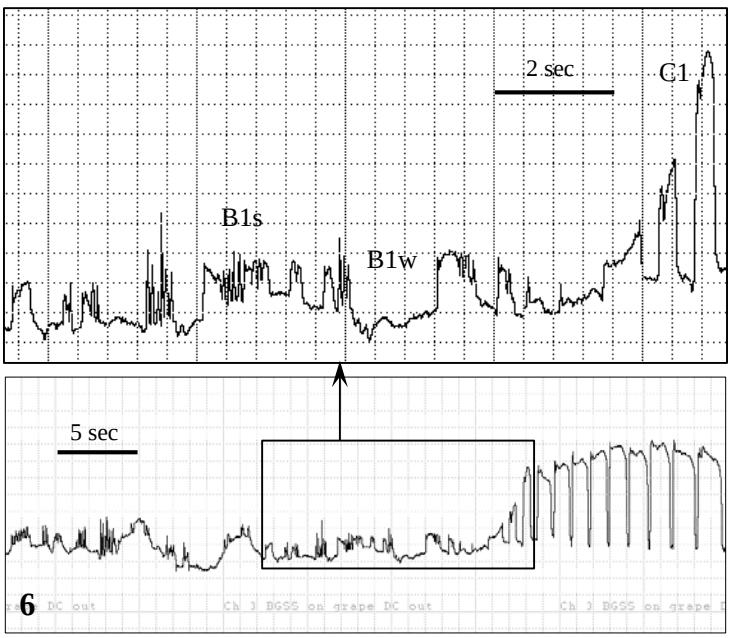

Figure 6. The middle of a probe recorded using Ri 10^8 Ω, 30 mV AC applied signal, showing the first XN before the first XC, showing triangular C1 before rectangular C2.

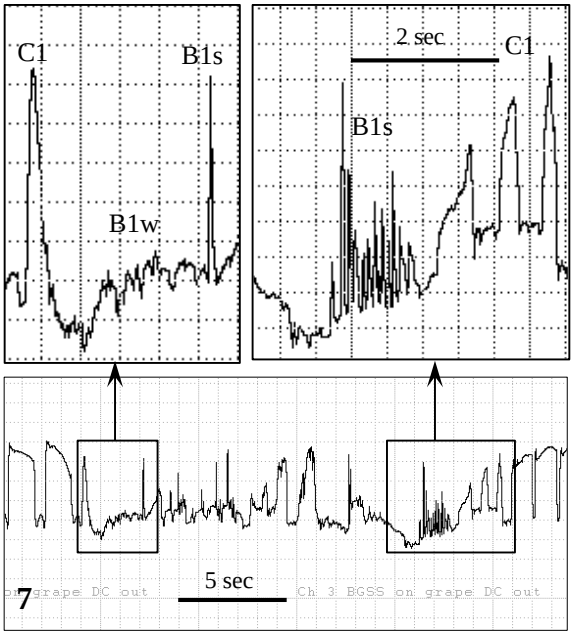

Figure 7. The second XN after the first XC in **Figure 6**, showing upward polarity, triangular C1 and rectangular C2.

Several drastic changes occurred at Ri 10^9 Ω, especially compared with Ri 10^6 and 10^7 Ω. In the last waveforms before C2 (normally considered the first XN event), B1 waveforms before C2 were amorphous and not detailed enough to distinguish the B1 sub-types B1w and B1s (**Figure 8**). Therefore, pathway waveforms before C2 were not considered the first XN. C2 plateaus were exceptionally tall compared with the height of B1. Also, C2 plateaus were rounded rectangles, completely lacking the R-component spike (**Figure 8**). When XN (interruption) events occurred, they were depressed to a more negative voltage level compared with C2. Most noticeably, the B1 sub-sub-type, fB1w, was visible for the first time, and represented a large proportion of the B1w in the first and subsequent XN events (**Figure 9**). There were two variants of fB1w, one with upward-pointed spikelets and another with downward-pointing spikelets (first and second inset boxes of **Figure 9**, respectively). The above findings support that Ri 10^9 Ω reveals almost exclusively emf components in BGSS, indicated by the extreme height of C2 plateaus, absence of the R-component spike on C2 plateaus, negative voltage levels, and presence of fB1w. This is similar to findings from earlier GWSS recordings for Ri 10^8 Ω. Thus, the smaller body size of BGSS (~8 mm) compared with GWSS (~13 mm) shifted the R-emf responsiveness curve toward lower Ri levels, as predicted by theory in EPG science (Backus and Bennett 2009).

Electrical origins of BGSS waveforms support similar biological interpretations for BGSS X waves as those of GWSS. B1 is the primary waveform of tasting and salivary sealing. Part of B1w (the wave) is R-dominated, supporting previous visual correlation with saliva secretion; the other part of B1w (the frequency component known as fB1w) is highly emf-dominated and therefore represents streaming potentials caused by cibarial diaphragm quivering, for minute fluid uptake into the oral cavity. B1s also originates as both R and emf; the R portion is thought to represent bursts of precibarial valve movements that very briefly block the precibarial part of the oral cavity, while the emf portion reflects streaming potentials kicked up by the value movements. These actions cause fluid to be "swished" around in the oral cavity, for tasting by the precibarial chemosensilla. Both C1 and C2 are emf-dominated, although C2 more so than C1. Therefore, both C waveforms represent large streaming potentials caused by the pumping motion of the large cibarial diaphragm (the muscular "lid" of the piston-like pump, the cibarium). C1 is triangular because the diaphragm is thought to be rapidly slammed downward to push fluid out of the oral cavity during egestion. Complete closure probably leads to the R-component portion of C1. C2 is rectangular because the diaphragm is held up to provide a vacuum to suck fluid into the cibarium during filling. Yet the diaphragm does not completely close during subsequent swallowing. The R-component spike on the C2 plateau marks the brief closure of the precibarial valve to facilitate swallowing.

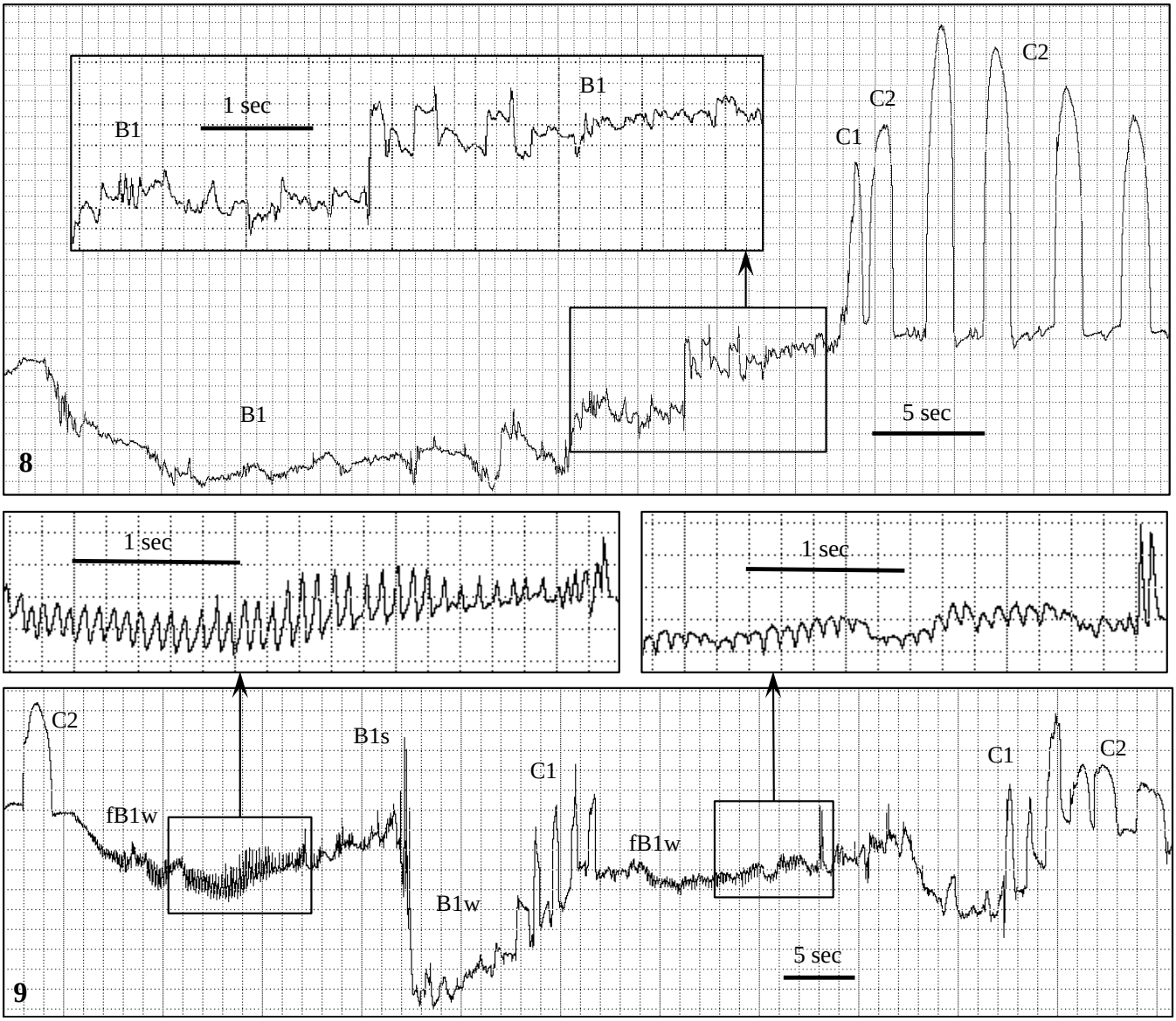

Figure 8. The middle of a probe recorded using Ri 10^9 Ω, 30 mV AC applied signal, showing the large portion of pathway before C2. There is no obvious XN at the end of pathway.

Figure 9. The first clear-cut XN after C2, showing downward polarity, triangular C1 and rectangular C2.

The present experiment confirmed that the same X wave components existed for BGSS as GWSS, and were similar in appearance and electrical origin. This demonstrates the fundamental similarity of feeding behaviors of two somewhat distantly related sharpshooter species from two different tribes of Cicadellinae. The present findings, although preliminary, also support predictions based on the R-emf responsiveness curve, i.e., that smaller body size of BGSS compared with GWSS would shift the optimal Ri level for X wave visualization, from Ri 10^8 Ω of GWSS to Ri 10^9 Ω for BGSS. Ri 10^9 Ω may be best Ri level for BGSS because it reveals fB1w, an important and diagnostic X wave component. However, Ri 10^9 Ω is not best for visualizing certain other components, especially B1s and the R-component spike on C2. Consequently, further analysis of more recordings will be required to confirm that Ri 10^9 Ω is the best setting to use for future EPG recordings of BGSS.

CONCLUSIONS

The present research showed that the same X wave components are visible for BGSS as for GWSS, with very similar, recognizable appearances. It also supported the same electrical origins and interpretations of stylet activities for BGSS X wave components as those of GWSS. Thus, the present work supported that EPG will be a crucial technology for detection of *Xf* inoculation behaviors represented by the sharpshooter X wave. It also

showed that EPG monitor settings can be optimized for best appearances of the BGSS X wave, and that those settings may be different from the best settings for GWSS recordings. Results from the present research will make possible future studies to identify experimental accessions of cultivated grapevine that resist the ability of a sharpshooter to perform the *Xf*-inoculation behaviors, thereby adding a new resistance trait to the grape rootstock varieties currently under development. Pyramiding multiple resistance traits will provide the greatest likelihood of success in sustaining host plant resistance traits in the field.

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SUITABILITY OF FERTILIZED AND UNFERTILIZED EGGS OF THE GLASSY-WINGED SHARPSHOOTER FOR THE EGG PARASITOID *GONATOCERUS MORRILLI*

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Reporting Period: The results reported here are from work conducted March 1, 2012 to September 30, 2012.

ABSTRACT
The glassy-winged sharpshooter [GWSS; *Homalodisca vitripennis* (Germar); Hemiptera: Cicadellidae] is a key vector of *Xylella fastidiosa*, the bacterium that causes Pierce's disease of grapevines and other diseases of high-valued crops in the Americas. GWSS invaded California in the late 1980s and continues to threaten the $4 billion grape industry. Control measures for GWSS in California include an area-wide insecticide (i.e., imidacloprid) application program and release of natural enemies, but, despite such efforts, the geographic distribution of GWSS continues to expand. *Gonatocerus morrilli* (Howard) (Hymenoptera: Mymaridae) is a natural enemy used in California, USA to control GWSS. Virgin GWSS females deposit unfertilized eggs and mated females can exhaust sperm reserves for egg fertilization. However, nothing is known about *Gonatocerus* spp. performance when using unfertilized GWSS eggs. Host age preference for oviposition and suitability of unfertilized GWSS eggs for *G. morrilli* reproduction were investigated. *G. morrilli* parasitized all ages of GWSS eggs (one to eight days old) regardless if the host egg was fertilized or not. However, parasitism rates and parasitoid emergence were reduced in older (> five-day-old) unfertilized host eggs compared to fertilized eggs. In choice tests (fertilized versus unfertilized eggs), parasitoids failed to emerge as adults from unfertilized eggs more often than from fertilized eggs. The results indicate that unfertilized eggs were accepted by *G. morrilli* as suitable hosts for oviposition, but were relatively unsuitable for immature development compared to fertilized eggs.

LAYPERSON SUMMARY
The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is a key vector of *Xylella fastidiosa*, the bacterium that causes Pierce's disease of grapevines and other diseases of high-valued crops in the Americas. GWSS invaded California in the late 1980s and continues to threaten the $4 billion grape industry. Control measures for GWSS in California include an area-wide insecticide (i.e., imidacloprid) application program and release of natural enemies, but, despite such efforts, the geographic distribution of GWSS continues to expand. Virgin GWSS females deposit unfertilized eggs and mated females need to re-mate to continue to produce progeny. Therefore, it is possible that egg parasitoids of GWSS, such as *Gonatocerus morrilli* that is used in California to control GWSS, are utilizing unfertilized eggs for reproduction in mass rearing facilities and/or field conditions, which may affect efficacy of the parasitoid for control of GWSS. Parasitoids failed to emerge as adults from unfertilized eggs more often than from fertilized eggs, indicating that unfertilized eggs were accepted by *G. morrilli* females as suitable hosts for oviposition, but were relatively unsuitable for immature development compared to fertilized eggs. Results suggest that reducing rates of GWSS egg fertilization through a mating disruption or sterilization program will have a negative impact on control of GWSS by natural enemies.

A METHOD TO QUANTIFY GLASSY-WINGED SHARPSHOOTER EGG MATURATION

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Reporting Period: The results reported here are from work conducted June 2012 to October 2013.

ABSTRACT
To identify factors affecting glassy-winged sharpshooter egg production, a method to accurately estimate the number of mature eggs produced during a short-term assay is needed. Egg production is typically quantified by determining the number of eggs deposited during the assay plus the number of mature eggs carried by the female at end of the assay (determined by dissection). However, this approach ignores confounding effects of variation in number of mature eggs carried by females entering an assay. Dissection of field collected females indicates that egg loads of reproductively active females are variable (range 0 to 37 eggs; Sisterson 2008). If the number of eggs carried by females entering an assay is variable, subsequent estimates of egg production during the assay will be inaccurate. Poor estimates of egg production during assays increase risk of making a Type II error (failure to reject a false null hypothesis). To address this issue, a pre-treatment designed to reduce variance in the number of eggs carried by females entering a feeding assay was evaluated. The pre-treatment consisted of providing females with a four-day oviposition period on sorghum. An oviposition period on sorghum was expected to reduce mean and variance in the number of eggs carried by females as previous tests documented that females readily deposit eggs on sorghum, but do not mature eggs when feeding on sorghum (Sisterson 2012). To determine if an oviposition period on sorghum reduced mean and variance in the number of eggs carried by a group of females, field collected females were split into two groups. The control group was dissected immediately to determine the number of eggs carried by each female. The pre-treatment group was provided with a four-day oviposition period on sorghum prior to dissection. The mean number of eggs carried by females in the pre-treatment group was 63% lower than for females in the control group. Similarly, the standard deviation in the number of eggs carried by females in the pre-treatment group was reduced by 44% compared to females in the control group. To demonstrate the importance of reducing variance in the number of eggs carried by females entering a feeding assay, results from feeding assays using females directly from the field were compared to results of feeding assays using females exposed to the pre-treatment. The feeding assay consisted of confining females to cowpea stems in parafilm enclosures for six days. Feeding was quantified during the assay by measuring excreta production. As females typically do not deposit eggs on stems, females were dissected at the end of six days to quantify egg production. A significant relationship between feeding (as measured by excreta production) and egg production was observed for females exposed to the pre-treatment before the feeding assay ($P = 0.04$), but was not observed for females placed directly into the feeding assay ($P = 0.35$). Thus, reducing mean and variance in the number of eggs carried by females entering the feeding assay resulted in detection of a significant positive relationship between feeding and egg production that otherwise would not have been observed. Use of the methodology proposed here will aid in completion of more in-depth studies designed to identify factors affecting glassy-winged sharpshooter egg production.

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Sisterson, M.S. 2012. Host selection by *Homalodisca vitripennis*: the interplay between feeding, egg maturation, egg load, and oviposition. *Arthropod-Plant Interactions* 6:351-360.

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ACKNOWLEDGEMENTS
I thank Sean Uchima and Donal Dwyer for assisting with experiments.

Section 2:

Vector Management

THE RIVERSIDE COUNTY GLASSY-WINGED SHARPSHOOTER PROGRAM IN THE TEMECULA VALLEY

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Reporting Period: The results reported here are from work conducted November 2012 to October 2013.

ABSTRACT
For more than 10 years the Temecula Valley has been part of an area-wide control program for an invasive vector, the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*). The goal of this program is to limit Pierce's disease spread by suppressing vector populations in commercial citrus, an important reproductive host for this insect, before they move out into vineyards. To achieve effective GWSS control, late spring applications of the systemic insecticide imidacloprid to citrus have made in years past. As part of this treatment program there is ongoing monitoring of GWSS populations to ensure that the treatments are effective. Notably, this year, reimbursements to citrus growers were not made. As a result, unlike previous years, apparently no Temecula Valley citrus acreage was treated specifically for GWSS – the consequences of which are not well understood. Approximately 140 yellow sticky traps were inspected on a biweekly basis throughout 2013 to monitor GWSS in citrus. The results show a typical phenology for this pest in the region, with a total of approximately 450 GWSS caught during the summer peak (July through September). Overall GWSS catch was modest compared to the highest years (2008, 2009), but higher than the lowest years (e.g., 2010, 2011).

LAYPERSON SUMMARY
The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) constitutes one of the primary threats to the wine, table grape, and raisin industries in California owing to its ability to spread a pathogen that causes Pierce's disease. In the Temecula Valley, an area-wide control program has been in place for more than 10 years, which relies on insecticides application in citrus groves to control GWSS before they move into vineyards. This program is viewed as critical for reducing the disease spread in vineyards. As part of the control program, citrus groves are monitored regularly for GWSS. This year, despite no insecticide applications being made to target GWSS, GWSS catch in Temecula was relatively modest; intermediate between very high years such as 2008 and very low years such as 2010 and 2011.

INTRODUCTION
The winegrape industry and its connecting tourist industry in the Temecula Valley generate $100 million in revenue for the economy of the area. Following the invasion of the glassy-winged sharpshooter (GWSS) into Southern California, from the Southeastern USA, a Pierce's disease (PD) outbreak occurred. This outbreak resulted in a 30% loss in overall vineyard production over a few years, with some vineyards losing 100% of their vines during the initial years of the outbreak. An area-wide GWSS management program initiated in the spring of 2000 saved the industry from even more dramatic losses. Since the initiation of the Temecula GWSS area-wide management program 300 new acres of grapes have been planted and six new wineries have been built. Only a continuation of an area-wide GWSS/PD management program will keep the vineyards viable in Temecula. GWSS has the potential to develop high population densities in citrus. Fortunately, GWSS is also highly susceptible to systemic insecticides such as imidacloprid. 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 disease.

As part of the area-wide treatment program monitoring of GWSS populations in citrus has been conducted since program inception. This monitoring data is needed to guide treatment decisions for citrus, to evaluate the efficacy of the treatments, and to guide vineyard owners, pest control advisers, and vineyard managers on the need for supplementary vector control measures within vineyards.

In the spring of 2008, 120 acres of citrus were identified and were treated for GWSS control in Temecula. In July 2008, Temecula GWSS trap catches reached over 2,000. This was the highest number of GWSS trapped since the area-wide program was initiated. Because of the phenology of GWSS, the summer citrus culture and the peculiarities of the uptake of the systemic imidacloprid it was decided that treatments in the citrus in July would not adequately reduce GWSS populations. Therefore, insecticide applications to control GWSS for the last two years were initiated in May 2011 and May-June 2012. Monitoring data suggest fairly robust control of GWSS using that treatment timing.

In 2013, the decision was made by state and federal regulators not to reimburse citrus growers for insecticide applications intended to target GWSS in the Temecula Valley. The effect this policy change might have on risk of disease spread is not known. Therefore, monitoring of sharpshooter populations is especially critical, to determine whether GWSS populations, which already show substantial interannual variability, appear to be rebounding.

OBJECTIVES

1. Monitor regularly GWSS populations in citrus groves throughout the Temecula Valley to evaluate the effectiveness of prior insecticide applications and to provide a metric of Pierce's disease risk for grape growers.
2. Disseminate a newsletter for stakeholders on sharpshooter seasonal abundance in citrus throughout the region.

Double-sided yellow sticky cards (7 inches x 9 inches) are being used to monitor for adult sharpshooters in citrus. 140 such sticky traps have been placed in citrus groves throughout the Temecula Valley. All traps are labeled, numbered, and bar coded to identify the site within the management program. Each trap is then georeferenced with a handheld GPS monitor. Most yellow sticky cards are placed at the edge of the groves at the rate of approximately one per 10 acres. Traps are attached with large binder clips to wooden stakes around the perimeter of the grove; in large groves traps are also placed in the interior. The total number of traps depends on the size of the orchard block. Sharpshooters found on the traps will be counted and then removed from the trap.

The yellow cards are inspected and replaced every two weeks. At each inspection the number of adult GWSS and smoke-tree sharpshooters (*Homalodisca liturata*) will be recorded, along with the abundance of common generalist natural enemy taxa.

After collecting all data for a given sharpshooter census date, these data are collated into a newsletter that shows the number of sharpshooters caught, where they were caught, and the seasonal phenology of sharpshooter populations to date. This newsletter is disseminated to stakeholders via e-mail and on a blog hosted by UC Riverside's Center for Invasive Species Research (http://cisr.ucr.edu/temeculagwss/).

RESULTS AND DISCUSSION

The results for 2013 are shown in **Figure 1**. This includes monthly censuses of GWSS in citrus through April, then biweekly censuses from May through October. Census results show seasonal patterns of GWSS abundance and activity that are typical for this region. GWSS catch is low for much of the year, it increases dramatically at the beginning of the summer, and then drops off through August and September. This year, nearly 25% of all GWSS caught were trapped on a single trap, suggesting a high degree of spatial heterogeneity in GWSS activity. As of mid-October, GWSS populations appear to have declined substantially.

Figure 2 shows GWSS catch in 2013 relative to other years. 2013 shows qualitatively the same seasonal phenology as in other years, with a moderate overall catch compared to others (i.e. 2008).

CONCLUSIONS

The results for 2013 suggest that, so far, there is no clear evidence of a GWSS resurgence in the Temecula Valley region. This result could be explained by carryover of systemic insecticides in citrus from applications made in prior years, which is known to occur. Alternatively, at least some of the explanation may be because of the potential for treatments made for another invasive insect, the Asian citrus psyllid (*Diaphorina citri*; ACP), which is controlled primarily via the same classes of insecticides as are used for GWSS. Although the recommended treatment timings are slightly different for ACP versus GWSS, applications made for its control may aid somewhat with GWSS control.

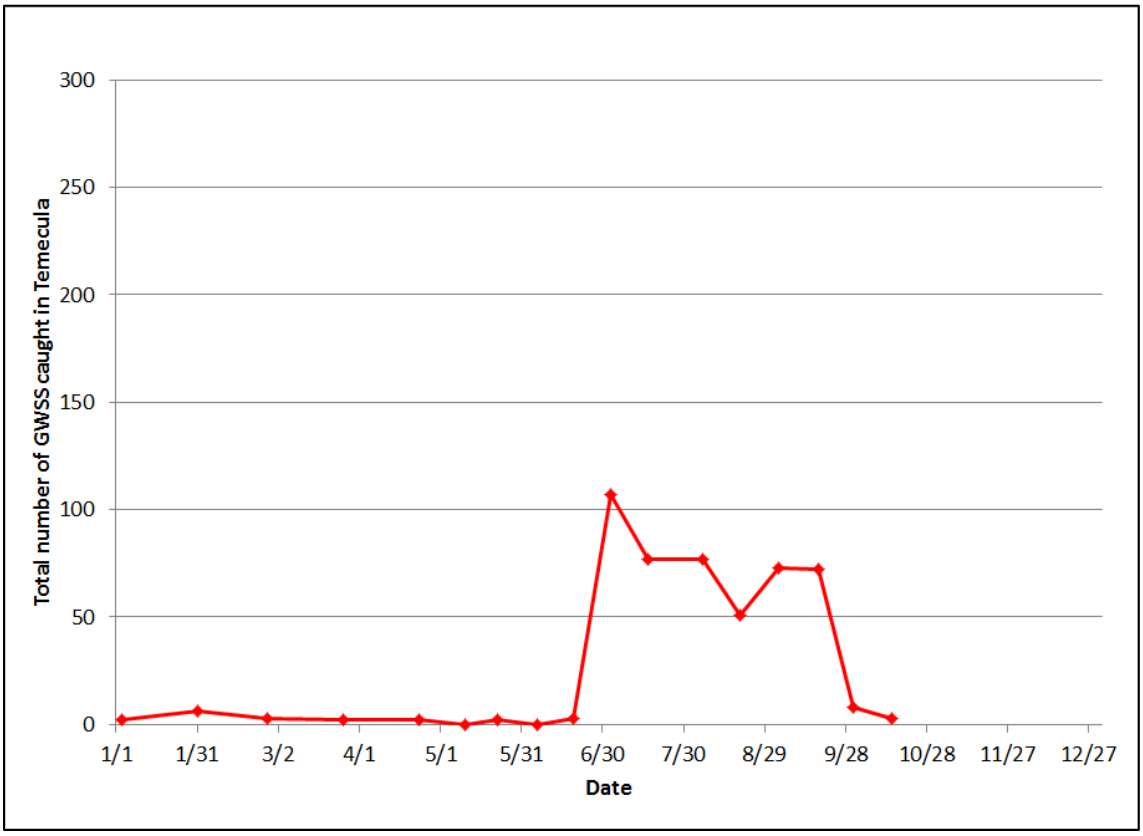

Figure 1. Seasonal total GWSS catch in 2013 for 140 traps throughout the Temecula Valley.

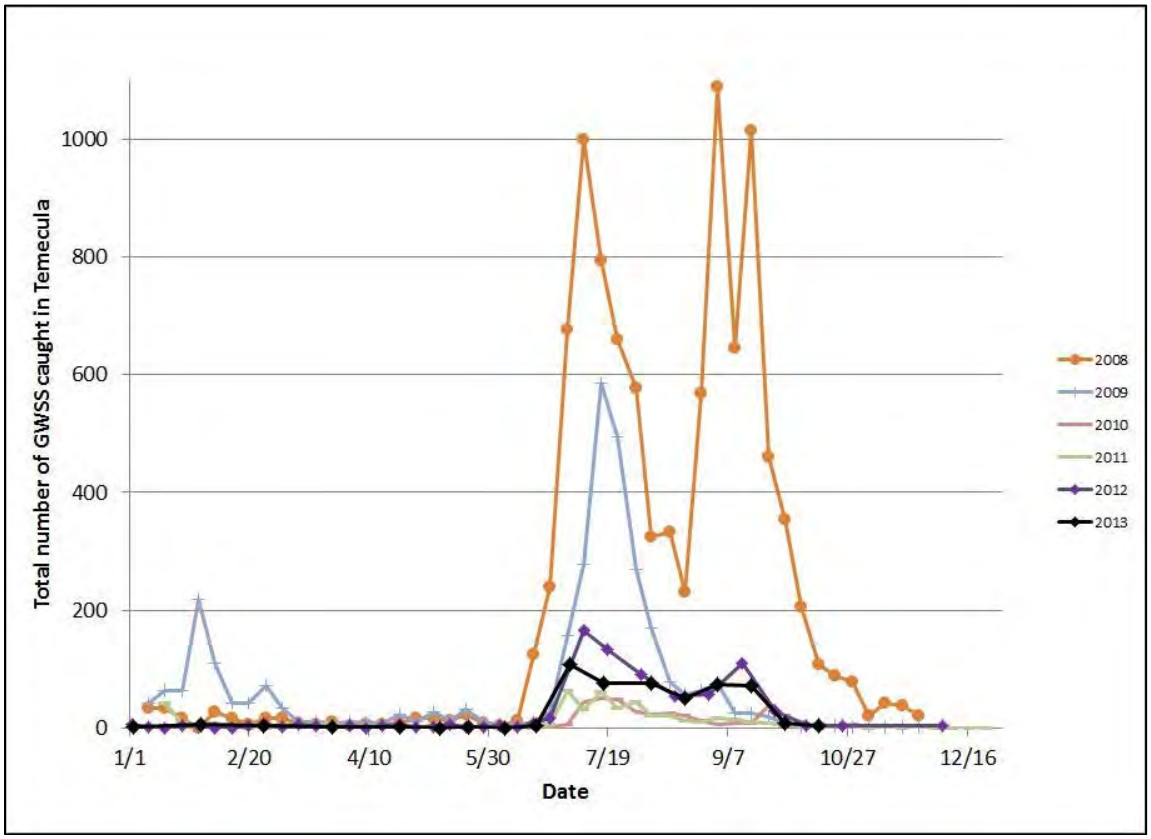

Figure 2. Seasonal total GWSS catch in the Temecula Valley from 2008-2012.

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

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ABSTRACT
RNAi is a natural biological activity for controlling gene expression and anti-viral defense in a majority of eukaryotic organisms, including insects [1]. The application of RNAi directed toward the control of different types of insect plant pests is becoming more feasible and promising [2-4]. RNAi has already been developed for use in various pest insect systems, both for reverse genetics and insect control [5-9]. In our efforts, we were able to induce RNAi in *Homalodisca vitripennis* and evaluate initial transgenic plants as a means to initiate RNAi to help control the glassy-winged sharpshooter and other leafhopper vectors of *Xylella fastidiosa*. RNAi is already used in commercial agriculture for plant virus control, and the many new publications demonstrating experimental successes with various plant-feeding insects suggest that RNAi could have a role in helping to manage Pierce's disease of grapevines.

LAYPERSON SUMMARY
This work presents fundamental efforts towards understanding the feasibility of applying RNA interference (RNAi), to help combat Pierce's disease of grapevines. Pierce's disease is a significant threat to grape production in California and other parts of the USA, and the causal agent, *Xylella fastidiosa* (*Xf*), a xylem-limited bacterium, also causes several other extremely important plant diseases worldwide [10-13]. 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 other sharpshooter vectors of *Xf*. We focused our efforts this year on evaluating transgenic potato plants for their potential to cause RNAi effects in GWSS, and for developing and identifying optimal RNAi inducer delivery systems. Potatoes are easier and faster to transform and regenerate than are grapes, and the GWSS feeds readily on these plants. We also have made good progress toward developing an efficient, rapid non-plant-based delivery system. We were also able to show the expression of the reporter genes in specific plant tissues (xylem, water conducting tissue) compared to our previous expression in the entire plant tissue. Apart from the above mentioned accomplishments, we have generated large scale genomic data that was further analyzed for the identification of GWSS targets which will help us gear towards GWSS control.

INTRODUCTION
Our primary objective is to evaluate and demonstrate RNA interference (RNAi) activity against the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*). We envision that RNAi approaches can be part of long term strategies to help control GWSS and other sharpshooter vectors of *Xylella fastidiosa* (*Xf*), the causal agent of Pierce's disease of grapevines [12]. We have made significant progress during the past year and are in excellent position to complete most of our objectives during the upcoming year. We have published three new refereed journal articles (Nandety et al., 2013a; Nandety et al., 2013b; and Kamita et al., 2013) and are working on a manuscript on RNAi on GWSS (Pitman et al., 2013). We have presented one new meeting abstract (Nandety et al., 2013) and are making excellent progress on our effort.

Here we present our efforts towards the development and application of an RNA interference (RNAi) based system aimed to target genes of GWSS, the vector of *Xf*. We have made stable potato transgenic plants using the constitutive, non tissue specific 35S promoter and a *Eucalyptus gunii* minimal xylem-specific promoter to control the spatial expression of candidate interfering RNAs. We showed expression of the GUS gene *in vivo* in the transgenic potato plants and were able to further test the localized xylem expression of the GUS marker gene. We have demonstrated the ability of stable transgenic plants to display gene expression through the use of RT-PCR and small RNA northern blots. We were thus far able to generate and evaluate potato plants transgenic to constitutive expression of GWSS-Actin, GWSS-cuticle, and GWSS-chitin deacetilase to produce dsRNAs (siRNAs) and the corresponding down regulation of their host specific mRNA targets in GWSS adult insects. Encouraged by the results of GUS transgene expression in the xylem tissues of potato transgenic plants (spatial restriction of the transgene), we have also developed the transgene constructs to generate small RNAs specific for GWSS mRNA that are driven by xylem expressing, ECAD promoter. These GWSS mRNA target gene specific hairpin constructs were further used to generate the stable transgenic lines in potatoes in a manner similar to the generation of constitutive promoter transgenic potato lines. Since our update last year, we found effective targets largely from the GWSS transcriptome sequencing project that we adopted (Nandety et al., 2013b, in press). Further, we are going to test the efficacy of these promoters to deliver the small RNAs (dsRNAs) into specific plant tissues that might streamline the delivery process.

OBJECTIVES

The specific objectives of our effort are:

1. To evaluate transgenic potato plants for their ability to generate small RNAs capable of inducing RNAi effects in GWSS.
2. To identify GWSS-interfering RNAs for use in transgenic plants.

RESULTS AND DISCUSSION

Generation of transgenic lines.

Transformation / regeneration was performed via recharge at the UC Davis Ralph M. Parsons plant transformation facility (http://ucdptf.ucdavis.edu/). To generate the potato transgenic lines we used the potato cultivars Desiree and Kennebec. In order to generate dsRNAs that can target the insect, GWSS target sequences previously described (**Table 1**) were cloned into a gateway-compatible binary vector pCB2004B [14, 15]. The target sequences were cloned in head to tail direction in the gateway vector with a non-homologous sequence between them forming the hairpin structure. Upon transcription in transgenic plants, these constructs yield double-stranded, hairpin RNAs of the desired sequence. We have performed screening of these transgenic potato plants for insert composition and have established the presence of the transgenes. The presence of GWSS-Chitin Deacetylase and GWSS- Actin transgenes in the potatoes resulted in the production of small RNAs in those transgenic plants. We are utilizing vegetative propagation to maintain the T_0 plants and for RNAi experiments with GWSS. Potatoes are an excellent host plant for GWSS, though they seem to be unable to complete their lifecycle feeding exclusively on potatoes. We have characterized these plants to ensure that they contain the desired transgene(s) and for some, that they generate the desired siRNAs.

Table 1. List of stable transgenic lines containing GWSS gene hairpin constructs or the reporter gene GUS.

Name of Construct	# constructs	Selection Method	Small RNA Northern
35S Actin	10	BASTA	Yes
35S Chitin Deacetylase	10	BASTA	Yes
35S GUS	2	BAR	Yes
ECAD Actin	6	BAR	In progress
ECAD Chitin Deacetylase	In production	BAR	In progress
ECAD GFP	2	BAR	Yes

Feeding experiments.

We performed feeding assays and assessed for RNAi effects on GWSS using our transgenic potatoes, and by basil stem infusion of dsRNA assays. Transgenic potato feeding assays were done using 3rd- 4th instar nymphs (**Figure 1**). Original cultivars of potatoes (non-transgenic) used to generate the transgenic lines were used as

control plants. We placed cuttings of the potato plants in individual cages in a growth chamber, released five nymphs per cutting, and observed mortality for two weeks (**Figure 2**). Higher mortality was observed in the nymphs that fed on one construct of chitin deacetylase and actin transgenic potato cuttings as compared to the controls. To determine if those observations were due to the effects of the transgene, we designed four-day feeding assays. Using the same potato cutting experiment design as for the mortality study, we allowed five 3rd-4th instar nymphs to feed on the cuttings for four days and then removed them from plants and dissected out the intestinal tracts of the nymphs. RNA was extracted from each sample, and cDNA generated from 500ng total RNA. Quantitative Real-Time PCR (qPCR) was then used to quantify relative expression of the genes targeted for down regulation, and was normalized with ubiquitin [16-18]. Gene expression, expression SEM, and corrected expression SEM were generated by the Bio-Rad CFX Manager 3.0 software (Carlsbad, CA).

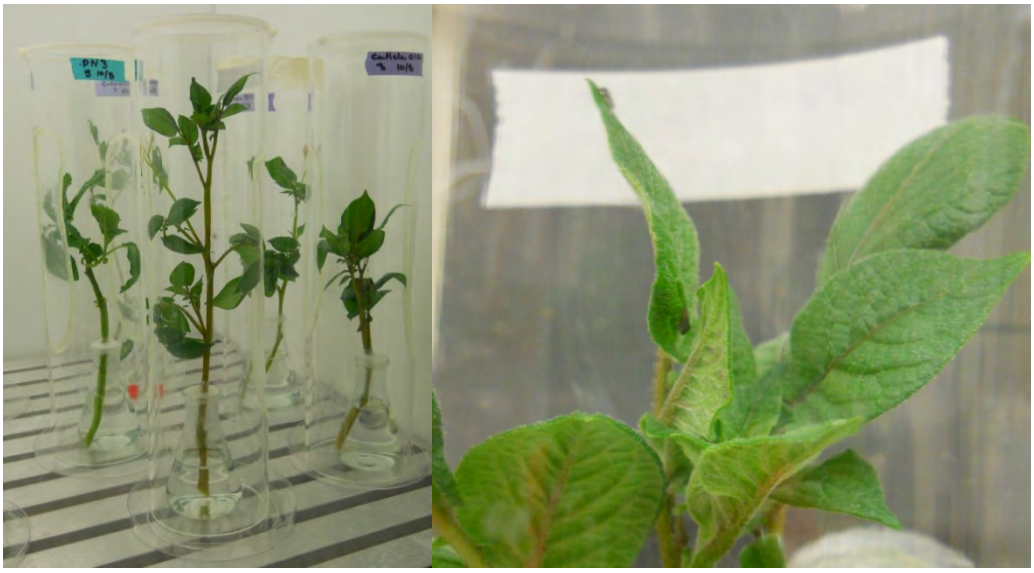

Figure 1. GWSS RNAi feeding assays on transgenic potato cuttings. **Left:** Stems in cylindrical cages, each containing five nymphs with three replications per treatment. **Right**: Close-up photo of a GWSS nymph feeding on upper potato foliage.

Initial results indicated down-regulation of target mRNAs after four days of GWSS nymphs feeding on transgenic potatoes as compared to wild-type potatoes (**Table 2**). We have completed feeding experiments with five of the transgenic potatoes; four events of chitin deacetylase, and one of actin. We are currently growing more potato plants from tubers, including a transgenic plant control with green fluorescent protein (GFP) gene insert. Once plants reach appropriate size they will be used in GWSS feeding assays. Initial results are correlating with the mortality study; there is decreased expression of target mRNAs as compared to controls for some chitin deacetylase and actin, but no significant difference for other events of chitin deacetylase (**Table 2**). Statistical analyses were performed using SAS 4.0 (Cary, NC) with the general linear model and Bonferroni correction to determine significance.

The promoter we used for our initial transgenic plants was the *Cauliflower mosaic virus* 35S promoter, which gives general constitutive gene expression across many tissue types. The second promoter, which we have been working with this year, is the EgCad2 xylem-specific promoter from *Eucalyptus gunii*. With the generation of EgCad2 promoter driven hairpin RNAi transgenic plants against GWSS mRNA targets, we hope to significantly alter the composition of xylem tissue RNAi effectors thus rendering them to provide significant amounts of small RNAs that are specific to GWSS adult and juvenile insects. Initially we examined mortality of 3rd and 4th instar nymphs in no-choice feeding assays on the 35S transgenic potatoes expressing: actin, chitin deacetylase, or cuticle dsRNAs; or the wild-type variety Kennebec. Based on these results we looked for differences in mRNA levels between 4th instar nymphs feeding on Kennebec as compared to chitin deacetylase or actin transgenic potatoes. We allowed the insects to feed for 5 days then collected samples from the remaining live insects, as the bodies degrade very quickly after death. The majority of evidence so far suggests that RNAi effects in insects are not systemic, that changes only occur in the cells in contact with the RNAi effector RNAs. Thus, we examined whether there were knockdown effects by dissecting out the digestive tracts of the experimental insects, extracting

RNA from the tissue, and performing quantitative RT-PCR. Real time assays were developed using Primer Express® software by Invitrogen (Grand Island, NY) with GWSS-ubiquitin as the endogenous control. Our preliminary efforts with transgenic potatoes expressing GWSS-chitin deacetylase when screened against GWSS insects showed a significant ten-fold reduction in the GWSS-chitin deacetylase mRNA target expression in the guts.

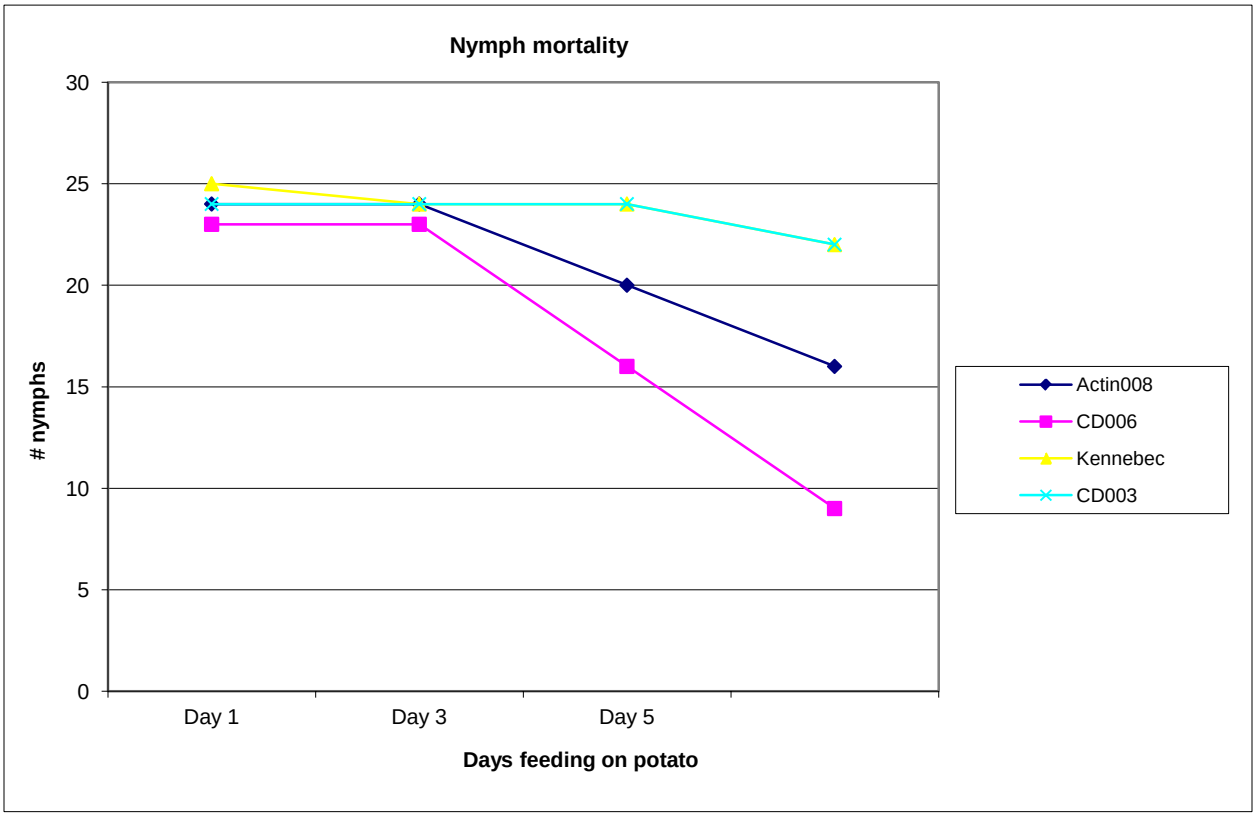

Figure 2. GWSS nymph mortality for seven days feeding on transgenic potato cuttings (Actin008, Chitin Deacetylase003, Chitin Deacetylase006,) or wild-type control potato (Kennebec).

Table 2. Real-time PCR results of gene expression in GWSS that fed on transgenic or wild-type potato plants. Statistical analyses were performed using Bonferroni test, α =0.05[19].

Target	Potato	Expression	Corrected Exp. SEM	Mean Cq	Bon Group
Experiment 1					
Chitin Deacetylase	Chitin Deacetylase002	0.41041	0.06843		A
Chitin Deacetylase	Chitin Deacetylase007	0.26919	0.05058		A
Chitin Deacetylase	Desiree	1	0.11864		B
Experiment 2					
Chitin Deacetylase	Chitin Deacetylase004	2.63164	0.19496	26.6	B
Chitin Deacetylase	Chitin Deacetylase008	1	.21406	28.74	AB
Chitin Deacetylase	Desiree	1.50313	0.06913	28.56	A
Experiment 3					
Actin	Actin008	0.2811	0.10858	29.79	A
Actin	Kennebec	1.1775	0.16807	23.47	B

In addition to the transgenic plant approaches, based on recent reports in the literature and personal communications from other scientists, we have evaluated *in vitro* feeding approaches for GWSS. We are utilizing basil stem cuttings in a feeding solution, as previously described, to introduce dsRNA to GWSS nymphs (**Figure 3**). We are currently evaluating the effects of dsRNA from the previously described actin and chitin deacetylase sequences have on GWSS nymphs, and whether the results are comparable to the transgenic potato feeding assays. If this method has similar treatment effects, we can utilize this method to more rapidly screen candidate sequences for RNAi approaches. In our initial experiment, bulk GFP PCR product 720bp in length was synthesized and purified. The PCR product was added to the water of one basil cutting for a final concentration of 10ng/uL, and a second cutting was used as a water control. Three nymphs were placed on each cutting. After four days of feeding, nymphs were ice anesthetized and their digestive tracts dissected out. Total DNA was extracted from the tissue, and then GFP specific PCR performed. We recovered the GFP fragment from treated nymphs, and are now using the feeding system to assess whether dsRNA fed to GWSS has similar RNAi effects to the transgenic potatoes we currently have.

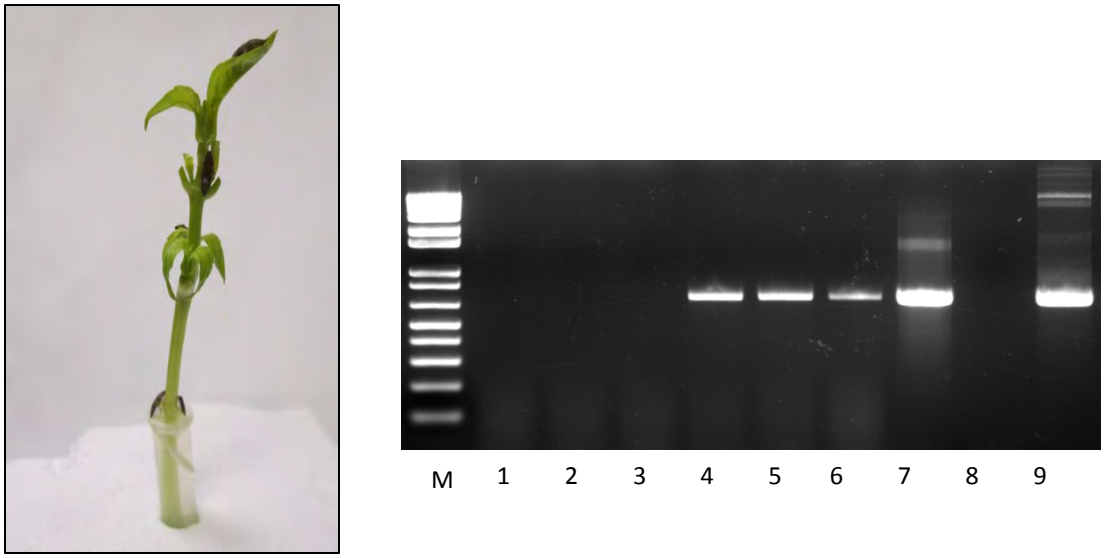

Figure 3. Left: Basil stem infusion setup with GWSS nymphs. A short terminal basil stem was cut and immersed in water in a 2mL micro centrifuge tube. The top of the tube was sealed with Parafilm. Tubes were secured in Styrofoam, then 50mL Eppendorf tubes were inverted over the cutting to act as a cage. Nymphs were allowed to feed for four days, and then ice anesthetized and digestive tracts dissected out for further analysis. **Right**: PCR results from GWSS nymphs fed on basil stem cuttings immersed in water with GFP PCR product at 10 ng/uL or water alone. Lanes 1-3 are individual nymphs from water treatment; lanes 4-6 are individual nymphs that fed on the basil immersed in a solution of GFP PCR product. Lane 7 is GFP PCR product; lane 8 is no template control; lane 9 is pGemTeasy plasmid with GFP insert.

Identify optimal interfering RNA forms for use in transgenic plants.

We have taken *in vitro* and *in vivo* approaches to identify optimal interfering RNAs. Since our update in 2012, we have attempted to identify additional effective RNA targets from the large scale GWSS transcriptome sequencing project that we adopted. We have a well-built transcriptome data set for GWSS insects that covers 35 Mb of the genome (Nandety et al., 2013b, in press). In addition we have generated a profile map of transcriptome with the available small RNA data and micro RNA data. The latter will help identify optimal interfering RNA forms for future targeting of GWSS insects.

Sequencing of adult GWSS small RNA libraries yielded 22,151,482 reads (Nandety et al., 2013a). Small RNA sequencing reads (43% of the total, ~9.5 million) were mapped to an artificial build of the GWSS transcriptome (Nandety et al., 2013b accepted, in press). 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 were analyzed for the virus discovery that resulted in the identification of *Homalodisca coagulata virus-1* (HoCV-1) and *Homalodisca reovirus* (HoVRV) that infect 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.

The small RNA reads were further investigated for the presence of conserved micro RNAs (~22nt) that can be identified through bioinformatic analysis based on their size and folding patterns. In our analysis thus far, we have identified the conserved and putatively novel micro RNAs. We were able to validate few of the micro RNAs through stem loop Real time-PCR. The following data were analyzed using GWSS-miR1692 as standard. The expression among the tested candidate microRNAs was found highest for GWSS-miR171 followed by GWSS-miR71.

In our analysis thus far, we have identified a diversified expression pattern of micro RNAs from each other in adult GWSS insects. Further, we were also able to identify the differential expression in relevance to the tissues chosen (**Figure 4**). The expression of microRNAs was found to be relatively higher for nymphs in comparison to adult insects (**Figure 4**). We were in the process of identifying novel microRNAs from the libraries we have generated.

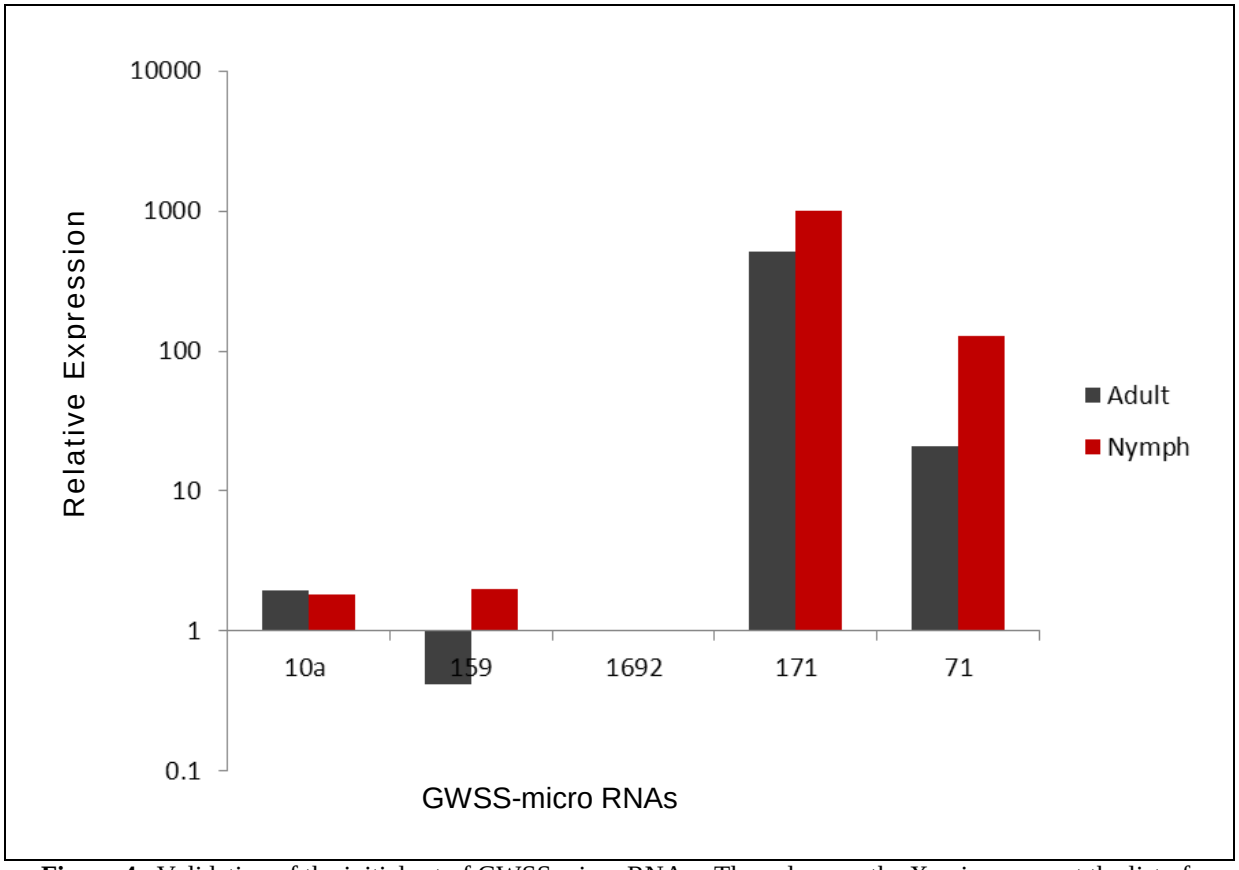

Figure 4. Validation of the initial set of GWSS micro RNAs. The values on the X axis represent the list of microRNAs chosen for validation. Values on the Y axis represent the relative quantification of the microRNAs in a logarithmic scale.

CONCLUSIONS

We have made stable potato transgenic plants using the constitutive, non tissue specific 35S promoter and a *Eucalyptus gunii* minimal xylem-specific promoter to control the spatial expression of candidate interfering RNAs. We have demonstrated the ability of stable transgenic plants to display gene expression through the use of RT-PCR and small RNA northern blots. We showed down-regulation of GWSS genes GWSS-Actin and GWSS-chitin deacetylase by dsRNAs (siRNAs) produced in transgenic potato plants. We also demonstrated expression of the GUS gene *in vivo* in the transgenic potato plants and were able to further test the localized xylem expression of GUS marker gene. Encouraged by the results of GUS transgene expression in the xylem tissues of potato transgenic plants (spatial restriction of the transgene), we have also developed the transgene constructs to

generate small RNAs specific for GWSS mRNA that are driven by xylem expressing, ECAD promoter. These GWSS mRNA target gene specific hairpin constructs were further used to generate the stable transgenic lines in potatoes in a manner similar to the generation of constitutive promoter transgenic potato lines. Since our update last year, we found effective targets largely from the GWSS transcriptome sequencing project that we adopted (Nandety et al., 2013b, in press). Further, we are going to test the efficacy of these promoters to deliver the small RNAs (dsRNAs) into specific plant tissues that might streamline the delivery process. Taken together, we have a research program that will allow for the rapid identification and screening of GWSS gene targets for RNAi.

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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*) transmits the bacterium *Xylella fastidiosa*, which causes Pierce's disease of grapevines. We are attempting to use natural, GWSS-infecting viruses as part of a strategy to control the GWSS population and thereby help to control Pierce's disease. We hope to engineer the 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 *Xylella fastidiosa*. In our studies, we are attempting to use recombinant *Homalodisca coagulata virus-1* (HoCV-1), a naturally-occurring virus that specifically infects GWSS. We have developed an infectious cloned cDNA for HoCV-1 which shows infection in cultured GWSS cells, but so far not in whole insects. Our efforts will continue to improve HoCV-1 in hopes to use it as part of a GWSS control strategy.

LAYPERSON SUMMARY
We are attempting to engineer viruses to deliver toxic peptides and/or deliver glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*) RNAs that will activate the RNA interference (RNAi)-based immune system. During this project year, we successfully developed an infectious cDNA clone of *Homalodisca coagulata virus-1* (HoCV-1) and verified its biological activity in the GWSS Z-15 cell-line. We are currently modifying our virus constructs for delivering RNAs that can express toxic peptides or induce RNAi in GWSS insects and Z-15 cell-line. The knowledge gained from this study will be used further to develop a virus system to help control the GWSS population and contribute to Pierce's disease control.

INTRODUCTION
We received two, one-year grants for this effort. We focused our collaborative efforts on attempting to develop glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) infecting viruses for use as agents to help manage GWSS, and then indirectly help manage Pierce's disease of grapevines. Viruses are the most abundant microbes on earth, with estimates as high as 10^{31} [1], and although viruses are often identified as pathogens, their roles in nature are not always associated with disease. Viruses often exist without causing disease in specific hosts, and in many instances viruses have proven to be useful for a variety of beneficial applications including use as biological control agents for insect pests [2]. If we could identify viruses that caused disease, or those that did not, both would be useful for our goals. Initially in year one we attempted to use the naturally-occurring *Homalodisca coagulata virus – 1* (HoCV-1), and *Flock House virus* (FHV), a model system virus for our work. We envisioned

that both could assist our efforts and allow for more rapid progress. We used GWSS cells (GWSS Z-15) and whole insects for our virus transmission assays. In year two we focused our efforts only on HoCV-1. This report presents our data from the past year.

OBJECTIVES

Our long term objectives were to develop and utilize the naturally occurring virus, HoCV-1, and engineer it to be useful for GWSS control either by modifying HoCV-1 to express toxic peptides or to induce systemic RNA interference (RNAi) in recipient, recombinant HoCV-1-infected GWSS. Our specific objectives are:

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

Objective 1.

HoCV-1 does not cause obvious disease in GWSS, and although it is most commonly found in GWSS its natural host range includes other sharpshooters [3]. Thus, our idea was to engineer this virus to be an effective and specific pathogen. We had previous success with a virus similar to HoCV-1 (the aphid-infecting *Dicistrovirus*, *Rhopalosiphum padi virus* (RhPV) [4], where we developed an infectious clone of RhPV. Here we initially took the same approach with HoCV-1.

We successfully cloned full length HoCV-1 cDNAs (**Figure 1A**). We generated a series of different constructs to help increase the probability of success. *In vitro* transcription was performed using these constructs to generate HoCV-1 transcripts which were delivered to GWSS Z-15 cells (**Figures 1B** and **1C**). After transfection with HoCV-1 transcripts with extended or un-extended 5'-ends, Z-15 cells showed severe cytopathic effects (CPE; **Figure 1B**). Control cells did not, thus these results suggested that we most likely had generated infectious HoCV-1 cloned cDNAs. In order to support the cytopathology data, we also performed reverse-transcription polymerase chain reaction (RT-PCR) assays to identify specific RNAs resulting from HoCV-1 replication. Both the HoCV-1 genomic-sense strand (positive-strand) and its complementary strand (negative-strand) RNAs were amplified by RT-PCR analysis following the transfection indicating that the virus was replicating (**Figure 1C**). pT7-Rz-HoCV1-3'Rz generated transcripts were less efficient possibly due to enhanced RNA degradation following ribozyme cleavage at the 5' end. Thus, both the cell cytopathology and the RT-PCR analyses suggested that our cloned HoCV-1 cDNAs were infectious to GWSS Z-15 cells and offered an opportunity for us to move forward.

We next attempted to engineer the HoCV-1 infectious clones to express YFP (yellow fluorescent protein) and mCherry (modified red fluorescent protein) reporters as part of the transcribed viral sequence (refer to **Figure 2**). This would allow for simple, efficient testing of our constructs in both Z-15 cells and whole GWSS insects. We used sites that were predicted to tolerate insertion of foreign sequences. If this was successful, these sites could also be used for future efforts to insert foreign sequences coding for toxic peptides or interfering RNAs. Unfortunately, we failed in this approach.

Objective 2.

In year one, in addition to utilizing HoCV-1, we explored the possibility of using a second virus, *Flock house virus* (FHV). FHV belongs to the family *Nodaviridae*, and is a non-enveloped, positive-sense RNA virus that has a bipartite genome. This virus been shown to multiply in insects from four different orders (Hemiptera, Coleoptera, Lepidoptera, and Diptera) and even plants. We felt that if FHV infected GWSS or GWSS Z-15 cells, we could use it to more rapidly evaluate candidate peptides and/or RNA sequences. We obtained infectious, recombinant constructs producing FHV genomic RNAs 1 and 2 (pMT FHV RNA1 and pMT FHV RNA2; gift from Dr. Shou-wei Ding, UC Riverside). The plasmid backbone (pMT) of the constructs contains a copper-inducible *Drosophila* metallothionein promoter that drives an efficient transcription of FHV genomic RNA. We showed that FHV could infect *Drosophila* (S2) and Z-15 cells (**Figure 3**) in our initial experiments. We also engineered FHV to express GFP as a marker to use in our RNAi studies. This recombinant was useful in S2 cells (**Figure 4**), but not in Z-15 cells, thus FHV proved to not be useful for our longer term strategies and thus we terminated efforts with FHV and focused exclusively onHoCV-1.

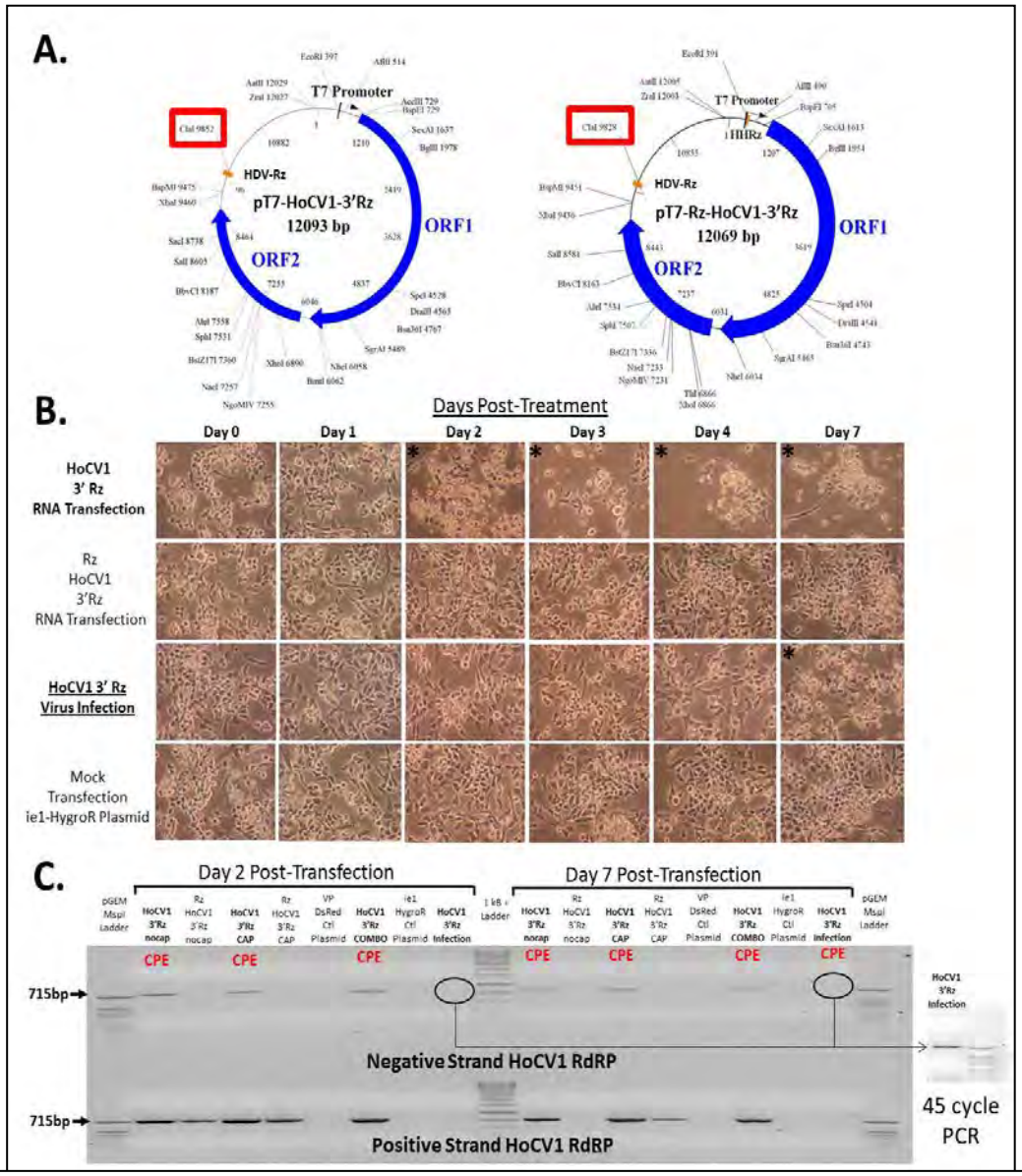

Figure 1. A. Vector diagram of pT7-HoCV1-3'Rz and pT7-Rz-HoCV1-3'Rz. The T7 promoter is indicated by the black bar and arrow. Hammerhead (HHRz) and Hepatitis Delta Virus (HDFV-Rz) ribozymes are indicated as orange boxes. pT7-HoCV1-3'Rz lacks the HHRz. HoCV-1 open reading frames (ORF) 1 and 2 are indicated as blue arrows. The *ClaI* restriction site (red box) is used to linearize plasmid for *in vitro* transcription. **B.** Cytopathic effects (black asterisks) were induced in GWSS Z-15 cells after transfection using HoCV-1 RNA transcripts and with HoCV-1 virus generated from the pT7-HoCV1-3'Rz plasmid. **C.** Negative and positive RNA strands for the HoCV-1 RNA-dependent RNA-polymerase (RdRP) were detected by 30 cycle RT-PCR for HoCV-1 RNA transfected from both plasmids, although only transfections with pT7-HoCV1-3'Rz RNA caused strong cytopathic effects (indicated by red CPE) in GWSS-Z15 cells. Weak signals for positive (30 cycles) and negative (45 cycles-indicated) strand RdRP RNA were also detected in virus overlayed from the HoCV-3'Rz transfection onto new GWSS-Z15 cells. Similar results were also obtained with RT-PCR against the HoCV-1 intergenic region and capsid sequences (not shown).

Transfection assays in whole insects.

In year two we focused efforts on HoCV-1 and attempting to efficiently infect GWSS Z-15 cells and whole insects using both wildtype virus (from naturally-infected GWSS) and our HoCV-1 clones. We established HoCV-1 GWSS colonies at the UC Davis CRF. We attempted to infect healthy GWSS with the GWSS-Z15 transfected cell extracts both by injection and oral acquisition. RNA from five infectious clones of HoCV1 and two controls were used to transfect GWSS-Z15 cells: HoCV-3'Rz, HoCV-3'Rz old, Rz-HoCV-3'Rz, Rz-HoCV-3'Rz old, mutant Rz-HoCV-3'Rz, elongation factor RNA, and transfection buffer. An additional negative control for the GWSS infection experiments was untreated GWSS-Z15 cell suspension. For the injections, one µL of needle homogenized cell suspension in injection buffer (10 mM Tris-HCl, pH 7.0,1 mM EDTA) was injected into

adult GWSS between tergites three and four of the ventral aspect using a 33 gauge needle (**Figure 5**). Three insects were injected per HoCV-3'Rz construct. After injection the insects were put in cages with a basil plant for one week, and then RNA collected as a treatment group. For feeding assays we used basil cuttings approximately five cm in length and submerged the cut end in a suspension of cell pellet and supernatant of approximately 1.5mL volume. Three insects per treatment were given an acquisition period of three days on the basil cuttings, then moved to basil plants for four days, after which RNA was extracted from each group. One-tube RT-PCR was used to detect infection with primer pairs specific to inter-genomic region 1 and coat protein of HoCV-1. The positive control was GWSS RNA from a naturally infected insect. All treatment groups tested negative for HoCV-3'Rz (**Figure 6**). Unfortunately, these data showed no evidence for replication of our HoCV-1 in adult GWSS.

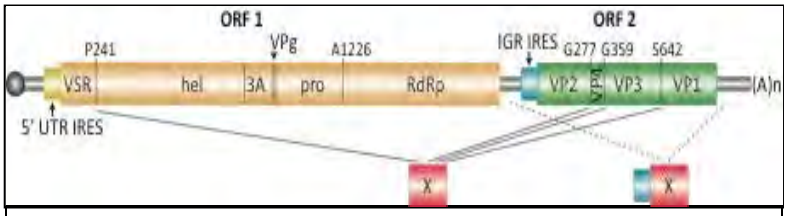

Figure 2. Potential insertions sites for expression of foreign proteins and RNAs from the RhPV and HoCV-1 genomes. Genome organization shows ORF 1 (orange) and ORF 2 (green) which encode proteins separated by cleavage sites indicated at estimated positions by vertical lines. Precise cleavage sites, estimated by alignment are labeled by the first amino acid downstream of the cleavage (e.g. P241 = proline at amino acid 241 in ORF 1). A1226 and S642 cleavages are from Nakashima and Nakamura (2008). We predict Q219 using alignment in Nayak et al. (2010), and G277, G359 using VP structures in Tate et al. (1999). Sequence encoding the protein of interest (e.g. toxin or fluorescent protein) indicated by red box (protein X) may be inserted at as a separate ORF, preceded by the IGR IRES (blue) at noncoding regions (gray bars) at sites indicated by dashed lines. Host RNA sequences can also be inserted at these sites for the RNAi strategy (objective 3). Alternatively, protein X coding region can be inserted within ORFs, at cleavage sites indicated by solid lines. Predicted functions of polyprotein cleavage products are viral suppressor of RNAi (VSR), helicase (hel), picornavirus-like protease 3A (3A), genome-linked protein (VPg), 3C-like protease (pro), RNA-dependent RNA polymerase (RdRp), virion proteins (VP1-4). Non-coding features include the VPg protein (sphere), 5' untranslated region IRES (5' UTR IRES), intergenic region IRES (IGR IRES), and poly(A) tail [(A)n)].

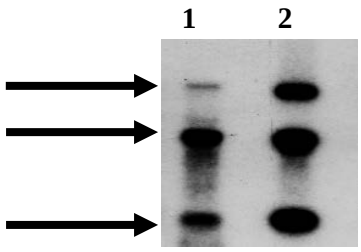

Figure 3. Lanes contain 1; FHV-inoculated S2 cells, 2; FHV-inoculated GWSS Z-15 cells. Arrows at left indicate FHV RNAs 1, 2 and 3, from top to bottom, respectively. RNA 3 is a subgenomic RNA from replicating RNA 1.

Our failure to transmit HoCV-1 from initially infected Z-15 cells suggested that something was wrong with our virus construct. We used transmission electron microscopy to assess HoCV-1 transfected Z-15 cells and failed to find virus particles in cells, even in those cells that were RT-PCR positive. Thus, although our data suggested HoCV-1 replication in Z-15 cells, HoCV-1 virus particles were not formed. This could explain our inability to transfer HoCV-1 from Z-15 cells to whole insects.

We attempted to engineer HoCV-1 cDNAs to contain and express GWSS cDNA sequences that could be used at least for RNA silencing studies in GWSS Z-15 cells. Hairpin RNAi cassettes against GFP (control), GWSS actin, and GWSS chitin deacetylase have been completed in the pGEM-13Zf+ vehicle (**Figure 7**). Sequences were verified by linearizing with an enzyme in the hairpin region (Xho I, Sac I, Sac II, Nru I, or Nar I) and sequencing linear templates. Hairpin RNAi cassettes can be transferred directly from the pGEM-13Zf+ vehicle to the HoCV1-3' Rz infective clone utilizing the enzyme XbaI to complete infective HoCV1-3'Rz clones carrying the RNAi cassettes. Due to time and funding constraints, we failed to complete this part of the project.

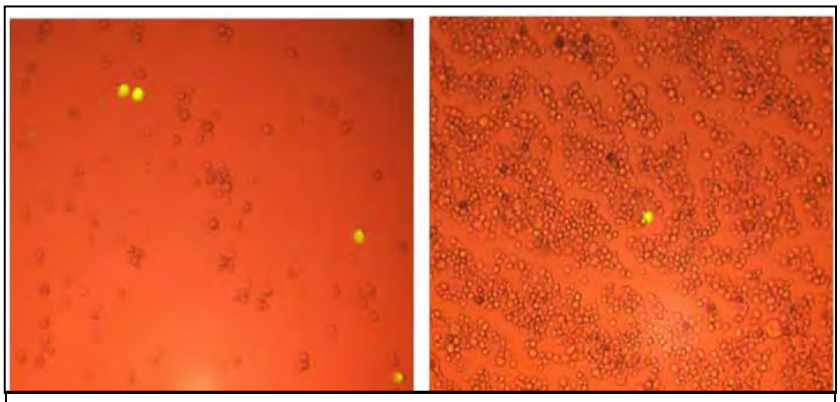

Figure 4. FHV infected S2 cells examined by fluorescence microscopy. S2 cells on the left panel were transfected with the plasmids that express FHV RNA1, FHV RNA2 and FHV DI RNA that contains GFP sequence. The transfected cells were collected, frozen and thawed 3 times, and filtered through a filter with 0.22um pores. The S2 cells on the right panel were inoculated with the cell lysate. The arrows indicate the cells expressing GFP from FHV DIeGFP.

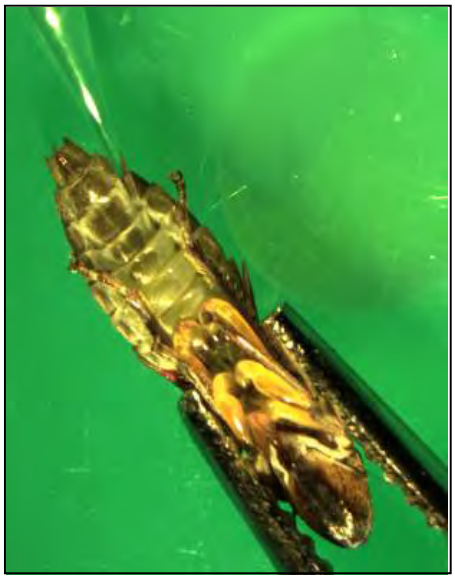

Figure 5. Injection of adult GWSS.

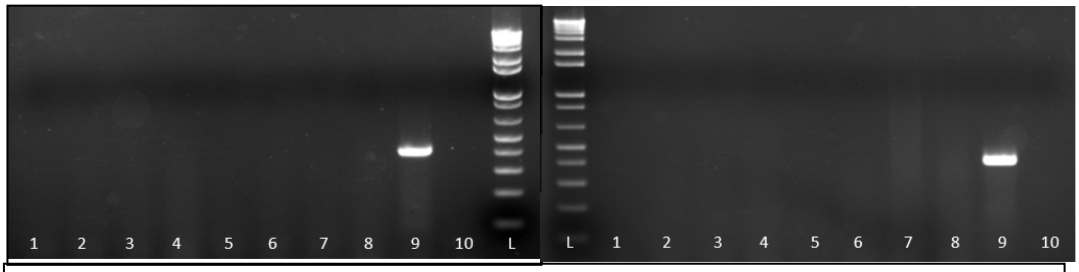

Figure 6. One-tube RT-PCR amplifying a segment of HoCV1 IGR (left) or coat protein (right) from insects that were fed transfected Z15 cell suspensions. Results from injection were identical. Treatments were Z15 cell transfected with constructs of HoCV1 infectious clones. **1.** HoCV-3'Rz **2.** HoCV-3'Rz old **3.** Rz-HoCV-3'Rz **4.** Rz-HoCV-3'Rz old **5.** Mutant Rz-HoCV-3'Rz **6.** Elongation factor RNA **7.** Transfection buffer Z15 cells **8.** No treatment Z15 cells **9.** Naturally infected HoCV1 GWSS **10.** No template control.

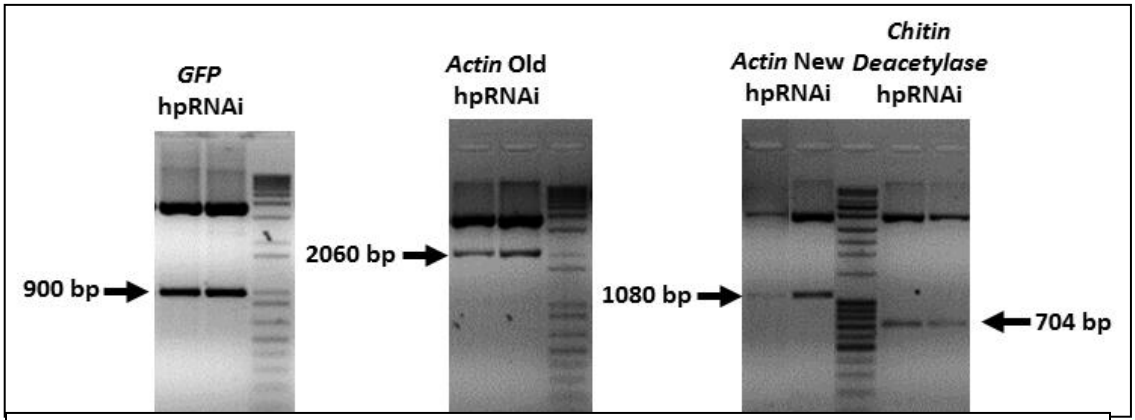

Figure 7. Positive pGEM-13 Zf+ clones obtained carrying hpRNAi cassettes against *GFP*, GWSS *actin*, and GWSS *deacetylase*. Approximate sizes of hpRNAi when released from the vector are: *GFP*-900 bp; *Actin Old*-2060 bp; *Actin New*-1080 bp; *Chitin Deacetylase*-704 bp.

CONCLUSIONS

We have developed a complete cDNA copy of the HoCV-1 genomic RNA. We can deliver *in vitro*-generated transcripts to cultured GWSS Z-15 cells and induce cytopathology and show evidence for limited replication. We have not yet been able to achieve similar results in whole GWSS insects. We also have not been able to detect HoCV-1 virus particles in inoculated Z-15 cells. Taken together these data suggest that we need to improve and re-engineer the HoCV-1 cDNA, and/or delivery methods. We will attempt to accomplish this during the upcoming year.

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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 June 2011 to September 2013.

ABSTRACT
The overall goal of this project is to identify and study targets within the endocrine system of the glassy-winged sharpshooter (GWSS) that can be used to control GWSS or reduce its vector competence. Juvenile hormone (JH) and molting hormones are key regulators of the insect endocrine system. The presence of JH in the insect hemolymph at low nanomolar levels maintains the *status quo* so that a juvenile insect will remain in the juvenile stage (e.g., nymph) during a molt. Minor changes in the *status quo* level of JH, however, can result in dramatic changes in development or cause death. Here we investigated two complementary approaches to alter the *status quo* level of JH. In our first approach, we cloned and characterized a JH-selective epoxide hydrolase-encoding gene, *hovimeh1*. The purpose is to develop *hovimeh1* as a selective and potent target for gene silencing. In our second approach, we determined the efficacy and effects of JH analog (JHA) insecticides against GWSS. JHA insecticides are green compounds that mimic the action of JH and selectively disrupt the insect endocrine system. In terms of mode of action, the effects of JHA application and knockdown of the *hovimeh1* gene are similar in that both approaches enhance "JH action" during periods of developmental when endogenous JH levels are exceptionally low.

LAYPERSON SUMMARY
Insects possess a simple endocrine system but one that is highly sensitive to minor changes. The overall goal of our project is to study and exploit targets within the endocrine system of the glassy-winged sharpshooter (GWSS) that can be used to control GWSS or reduce its ability to spread Pierce's disease. Juvenile hormone (JH) is a key component of the insect endocrine system. Minor changes in JH levels can result in dramatic changes in development, reproduction, behavior, and other insect biology. In this project we are taking two complementary approaches to target the JH regulatory system of GWSS. Our direct approach is to test the effects of various, commercially available, juvenile hormone analog insecticides against nymphal GWSS. Our indirect approach involves the identification and characterization of an enzyme called epoxide hydrolase that selectively metabolizes JH. The objective of this approach is to eventually use the gene encoding this epoxide hydrolase as a target for gene knockdown. If we can knock down this gene, the metabolism of JH should be significantly reduced so that JH levels do not fall below the threshold level that is required for normal insect development. The predicted result is abnormal development and death.

INTRODUCTION
Insect development is precisely regulated by the relative titers of juvenile hormone (JH) and molting hormones. JHs form a family of sesquiterpenoids (**Figure 1A**) that regulate key biological events in insects including reproduction, behavior, polyphenisms, and development (reviewed in Riddiford, 2008). Minor disruption of an insect's hemolymph JH levels can result in death or dramatic alterations in development. Juvenile hormone analog (JHA) insecticides such as methoprene, fenoxycarb, and pyriproxyfen (**Figure 1B**) are green compounds that selectively target the insect endocrine system by mimicking the biological action and/or structure of JH (reviewed in Dhadialla et al., 2005). When pest insects are exposed to JHA insecticides at a time during development when JH titer is normally undetectable, abnormal nymphal-pupal development and/or death is induced. JHA insecticides can selectively target insects within an order or even at the family level. This level of selectively is not obtained with more classical insecticides. High target selectivity is one reason that JHA insecticides are considered to be exceptionally safe.

The timely metabolism of JH by two hydrolytic enzymes, JH esterase (JHE) and JH epoxide hydrolase (JHEH), is critical for maintaining appropriate JH titers during development. When the enzymatic activity of JHE is

inhibited by a chemical inhibitor, developmental abnormalities are induced (Abdel-Aal and Hammock, 1985). These abnormalities are similar to those caused by JHA insecticides. Chemical inhibition of JHE putatively results in JH titers that are not below the threshold required for normal development. Similarly, we hypothesize that inhibition of JHEH will also cause abnormal nymphal-pupal development and/or death of GWSS. In this project we are focusing our efforts on the cloning and characterizing the JHEH of GWSS, an enzyme that metabolizes the epoxide moiety that is found on all known JHs.

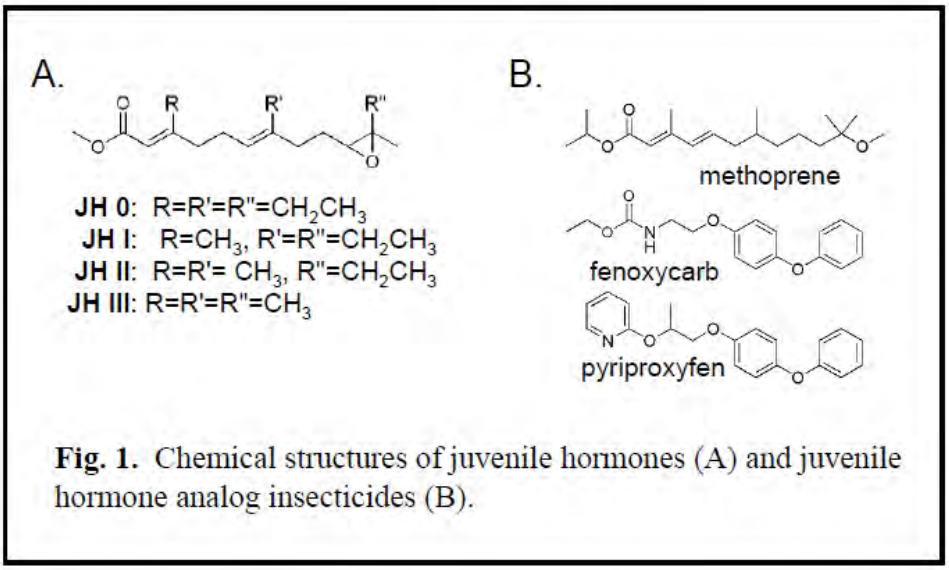

Fig. 1. Chemical structures of juvenile hormones (A) and juvenile hormone analog insecticides (B).

Gene silencing mediated by RNA interference (RNAi) is an effective method to knock down the activity of a targeted gene (reviewed in Huvenne and Smagghe, 2010). Recent studies show that RNAi effects can be induced in insects that feed on artificial diets containing double-stranded RNA (dsRNA) or even on transgenic plants that express dsRNAs (reviewed in Price and Gatehouse, 2008). These findings suggest a new and highly selective method of plant protection. One manifestation of this approach might be to generate a transgenic grapevine that expresses an appropriate effector RNA (and secretes this RNA into the xylem for systemic spread). Scion from this transgenic grape could then be grafted onto plants in the field as a form of 'inoculation' against the Pierce's disease causing bacterium. Our collaborator, Bryce Falk, has recently shown that RNAi is effective in cultured GWSS cells (Rosa et al., 2010) and in whole insects (Rosa et al., 2012). A key requirement of any RNAi approach is the identification of a suitable target. We believe that genes that encode proteins such as JHEH and JHE will be ideal targets for RNAi-based control strategies because they are insect selective and essential for normal insect development. In this study we have cloned and characterized a JHEH encoding cDNA (*hovimeh1*) from GWSS. We believe that the *hovimeh1* gene is an excellent target for developing an RNAi-based control strategy for GWSS.

OBJECTIVES

I. Investigate the delayed effects of low dose JHA insecticide exposure
   A. Determine sublethal dose in eggs and 1st instar nymphs
   B. Evaluate delayed effects of sublethal exposure on egg development
   C. Evaluate delayed effects of sublethal exposure on nymph development
II. Characterize recombinant JHEH from GWSS
   A. Clone full-length *jheh* gene of GWSS
   B. Biochemically characterize recombinant JHEH
   C. Screen JHA insecticides for JHEH inhibitory activity

RESULTS AND DISCUSSION

We have established a simple yet robust bioassay protocol to test the efficacy of JHA insecticides and assess nymphal development times. The assay is based on a single basil plant and developmentally synchronized insects (**Figure 2A**). Using this assay, we have determined precise nymphal developmental times (see **Figure 2B**) under

standard assay conditions. These data are a critical for life history assessments following low dose exposure experiments. We found that low dose exposure (i.e., 5.0 ppm for one hour) to methoprene during the 1st nymphal instar increased the duration of the 5th nymphal instar by up to two-fold. This finding is exciting because it indicates that short, low level exposure to a JHA insecticide can have long term (longer than four weeks) effects through multiple instars in GWSS. The implication is that although low-level exposure to JHA insecticides will not result in quick kill or even knockdown, it will alter normal GWSS development so that its competence as a disease vector is reduced. We also found that the duration of the 1st instar of GWSS is increased by up to one day (17% longer than untreated controls) following exposure to methoprene concentrations as low as 0.5 ppm. When 5th instar nymphs were exposed to 5.0 ppm of methoprene, the duration of the 5th instar was extended by about two days (21% longer in comparison to untreated controls). Interestingly, exposure of 5th instars at 5.0 ppm resulted in tremendous variation in the length of the 5th instar with some nymphs remaining as 5th instars for more than 20 days. These results also suggest that some insects retain the highly non-polar methoprene within their tissues resulting in the induction of long-term biological effects.

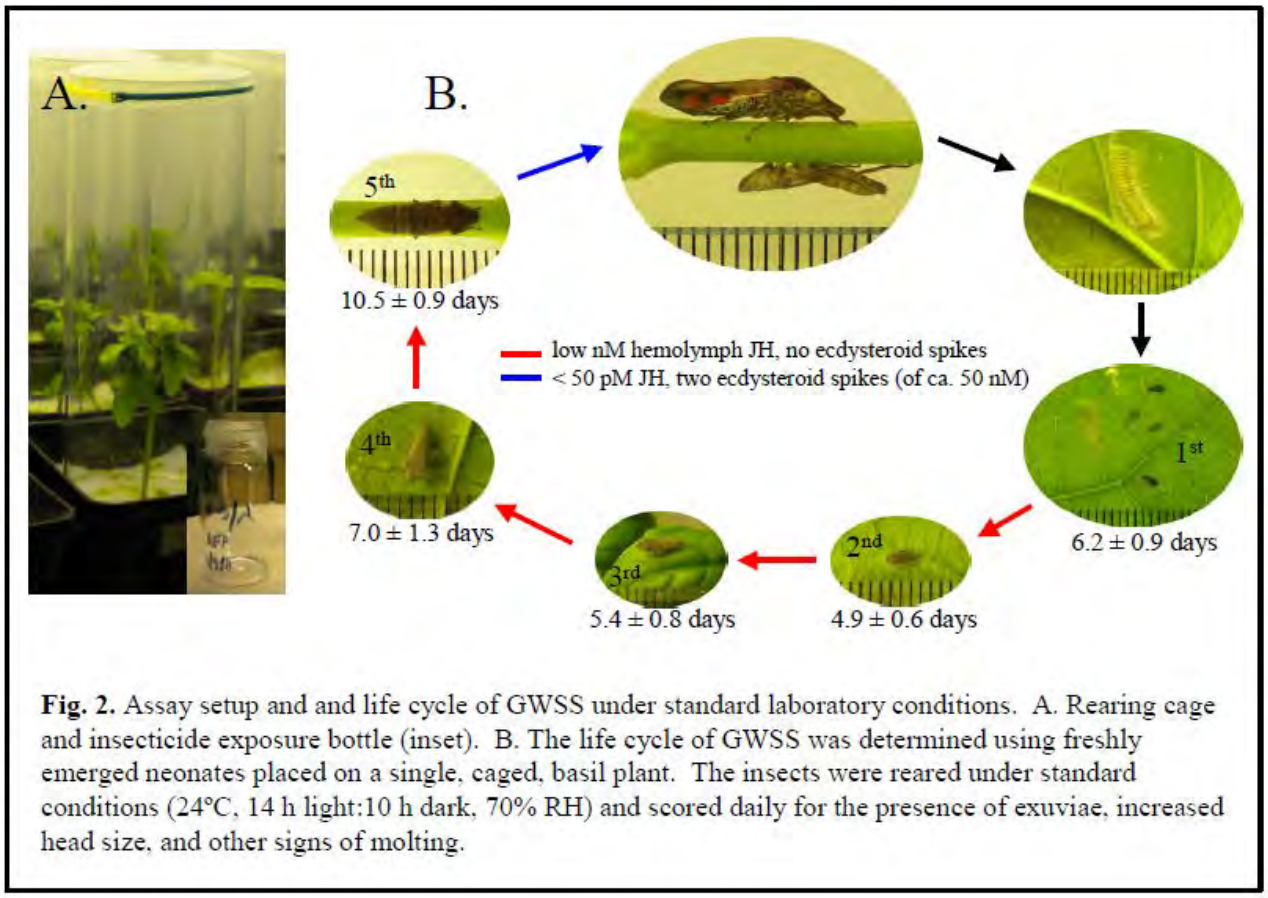

Fig. 2. Assay setup and and life cycle of GWSS under standard laboratory conditions. A. Rearing cage and insecticide exposure bottle (inset). B. The life cycle of GWSS was determined using freshly emerged neonates placed on a single, caged, basil plant. The insects were reared under standard conditions (24°C, 14 h light:10 h dark, 70% RH) and scored daily for the presence of exuviae, increased head size, and other signs of molting.

In order to confirm and quantify the activity of JH metabolic enzymes in GWSS, both JHEH and JHE enzyme activity levels in the hemolymph of 5th instar GWSS nymphs were measured. A partition assay (Hammock and Sparks, 1977) was used to determine the specific activity of HoviJHEH for JH III (PerkinElmer and Sigma-Aldrich). The assay time and/or enzyme concentration were adjusted so that no more than 10% of the substrate was metabolized during the incubation period. The assays were performed in triplicate and repeated at least three times for each concentration of substrate. 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, respectively. In contrast, JHE activity was dramatically lower outside of these days. This low JHE activity may help to explain the exceptional sensitivity that nymphal GWSS showed towards methoprene. 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.

In order to identify the coding sequence of JHEH, total RNAs were isolated from nymphs (at days six, eight, or ten of the 5th instar), and these RNAs were used to generate first strand cDNAs for 3'-RACE to identify the 3'-end and 3'-UTR of potentially JHEH-encoding cDNAs. On the basis of the 3'-end sequence, several gene-specific, nested primers were designed for 5'-RACE to identify the 5'-UTR and 5'-end of potentially JHEH-encoding cDNAs. On the basis of the 3'- and 5'-RACE results two gene-specific primers were designed and used to amplify a full-length cDNA that we named *hovimeh1* (**Figure 3A**).

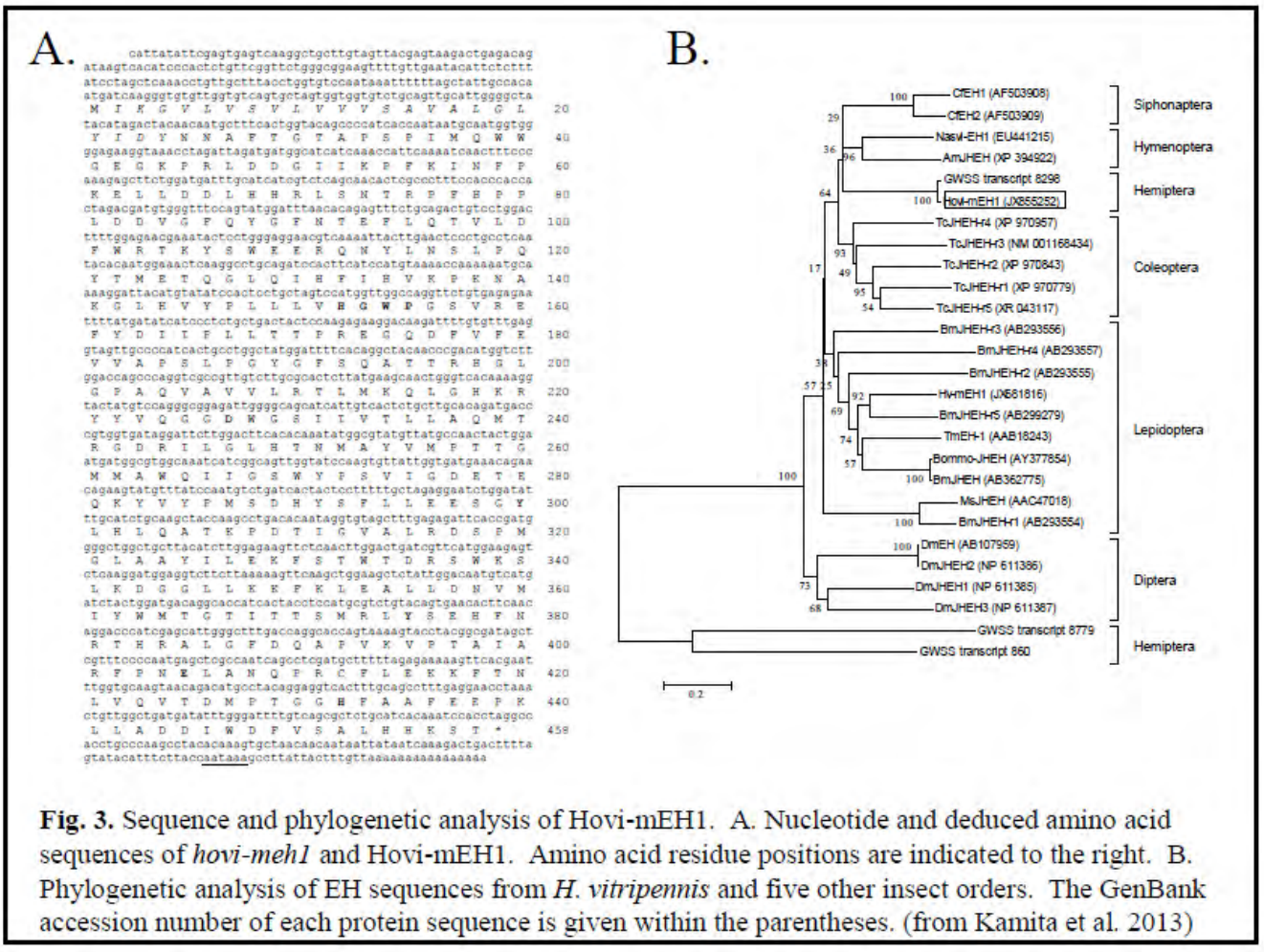

Fig. 3. Sequence and phylogenetic analysis of Hovi-mEH1. A. Nucleotide and deduced amino acid sequences of *hovi-meh1* and Hovi-mEH1. Amino acid residue positions are indicated to the right. B. Phylogenetic analysis of EH sequences from *H. vitripennis* and five other insect orders. The GenBank accession number of each protein sequence is given within the parentheses. (from Kamita et al. 2013)

Hovimeh1 is 1,668 nts-long and contains a 1,374 nts-long open reading frame flanked by 5'- and 3'-UTR sequences that are 174 and 101 nts-long, respectively. The deduced protein, Hovi-mEH1 (**Figure 3A**), of *hovimeh1* is 459 amino acid residues long and has a predicted mass of 52,108 Daltons and pI of 7.00. A putative membrane anchor domain (IKGVLVSVLVVVSAVALGLYIDY) of 23 amino acid residues is predicted by SOSUI v. 1.11 (Hirokawa et al., 1998) at the amino terminal of HoviJHEH. The catalytic residues of Hovi-mEH1 are predicted to be D-277, H-432, and E-405. In addition, core and lid domains, and an oxyanion hole motif that are found in known EHs are conserved in Hovi-mEH1. Phylogenetic analysis shows Hovi-mEH1 in a clade with two hymenopteran and siphonapteran EHs (**Figure 3B**). The deduced amino acid sequence of Hovi-mEH1 shows approximately 50% identity with Nasvi-EH1 and AmJHEH (but also 44-54% identity with other EHs that were investigated).

In order to characterize the protein encoded by *hovimeh1*, the coding sequence of *hovimeh1* was PCR-amplified and the resulting amplicon was subcloned into the baculovirus transfer vector plasmid pAcUW21 generating pAcUW21-hovimeh1. pAcUW21-hovimeh1 was subsequently transfected with *Bsu*36I-digested BacPAK6 baculovirus DNA (Clontech) into Sf-9 cells using Cellfectin Transfection Reagent (Invitrogen). This transfection generated AcHovimEH1, a recombinant baculovirus expression vector expressing recombinant Hovi-EH1.

AcHovimEH1 was isolated from the supernatant of the transfected Sf-9 cells by three rounds of plaque purification on Sf-9 cells. The recombinant Hovi-mEH1 protein was produced in insect High Five cells (1×10^6 cells ml^{-1}) that were inoculated with AcHovimEH1 at a multiplicity of infection of 0.8 and collected at 72 hours post inoculation. Microsomes were prepared from the infected High Five cells as described previously (Kamita et al., 2011) and stored at -80ºC until use. The protein concentration of the microsomal preparation was determined by the Bradford method using bovine serum albumin as a standard. The relative amount of Hovi-mEH1 in each preparation was analyzed by SDS-PAGE as described previously (Kamita et al., 2011).

The partition assay described above was used to determine the specific activity of Hovi-mEH1 for JH III. The kinetic constants (Michaelis constant (K_M) and V_{max}) of Hovi-mEH1 for JH III were determined using eight different concentrations of substrate (642 to 20,014 nM) that flanked the estimated K_M value. The reaction was repeated at least three times for each substrate concentration. The K_M and V_{max} were calculated using the Enzyme Kinetics module 1.1 of Sigma Plot (Systat Software). An estimated molecular weight of 52,108 Daltons was used to calculate the turnover value (k_{cat}) of Hovi-mEH1 for JH III.

Confirmation of the enzymatic activity of Hovi-mEH1 is critical because the GWSS transcriptome (Nandety and Falk, unpublished) and insect genomes in general (Tsubota et al., 2010; Crone et al., 2007) commonly carry multiple genes that appear to encode biologically authentic JHEH (or JHE). Although multiple genes are commonly found, only one of these genes encodes a biologically functional JHEH or JHE whereas the other genes may encode a general epoxide hydrolase or esterase with non-specific substrate preference, or a pseudo gene. By confirming that the *hovimeh1* encodes authentic GWSS JHEH, we can selectively target *hovimeh1* and not other EH-encoding genes in order to minimize off-target effects within not only GWSS and but other organisms.

The V_{max} of Hovi-mEH1 for JH III was 18.7±1.1 nmol of JH III diol formed min^{-1} mg^{-1} when determined in 100 mM sodium phosphate, pH 8.0, at 30ºC. This rate was about three-fold lower than that found with recombinant JHEHs from the lepidopterans *Manduca sexta* (Wojtasek and Prestwich, 1996) and *Bombyx mori* (Zhang et al., 2005), and five-fold lower than that of the coleopteran *Tribolium castaneum* (Tsubota et al., 2010). The K_M and k_{cat} values of Hovi-mEH1 for JH III were 5,400±830 nM and 0.02 s^{-1}, respectively. In comparison, the K_M of Hovi-mEH1 for JH III was 23- and 10-fold higher than that of *M. sexta* and *B. mori*, respectively. The turnover (i.e., k_{cat}) of JH III by Hovi-mEH1 was similar (about 2-fold lower) to these JHEHs. Biochemical characterization of Hovi-mEH1 with other substrates is described in detail in our recent publication (Kamita et al., 2013).

CONCLUSIONS

In this study, we found that low dose exposure to JHA insecticides such as methoprene results in long-term effects that project through multiple instars. Additionally, we identified a gene (*hovimeh1*) that encodes a biologically authentic JHEH. We propose *hovimeh1* as a target for future gene knockdown studies for control of GWSS.

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Section 3:

Pathogen Biology and Ecology

IDENTIFICATION OF A NEW VIRULENCE FACTOR REQUIRED FOR PIERCE'S DISEASE AND ITS UTILITY IN DEVELOPMENT OF A BIOLOGICAL CONTROL

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ABSTRACT
Xylella fastidiosa (*Xf*) is an important phytopathogen that infects a number of important crops including citrus, almonds, and coffee. The *Xf* Temecula strain infects grapevines and induces Pierce's disease. We recently deleted the *Xf* PD1311 gene and found that the strain was no longer pathogenic. Based on sequence analysis, PD1311 appears to encode an acyl CoA synthetase, which is a class of enzymes involved in many different processes including secondary metabolite production. Given the critical role of PD1311 in Pierce's disease development, we are exploring how it induces its phenotype. In addition, we are testing the ΔPD1311 strain as a potential biocontrol for preventing Pierce's disease.

LAYPERSON SUMMARY
We discovered that deleting the *Xylella fastidiosa* Temecula gene, PD1311, results in a strain that does not induce Pierce's disease. This project will examine how PD1311 plays such a central role in disease. Given the importance of Pierce's disease, it is critical to understand how PD1311 exerts its effects. In addition, we will determine if the strain deleted for PD1311 can function as a biocontrol. Options for managing Pierce's disease are limited, which makes possible new biocontrols critically important. Together the results from these aims will expand our understanding of Pierce's disease and provide information in relation to preventing disease.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a Gram-negative, xylem-limited bacterium that causes Pierce's disease (PD) of grapevines (Chatterjee et al., 2008a). *Xf* is transmitted to plants by insect vectors and once in the xylem, *Xf* is postulated to migrate, aggregate, and form biofilm that clogs the vessels leading to Pierce's disease. We, and others, have studied *Xf* proteins and regulators involved in these steps (Guilhabert and Kirkpatrick 2005, Meng et al., 2005, Feil et al., 2007, Li et al., 2007, Shi et al., 2007, da Silva Neto et al., 2008, Cursino et al., 2009, Cursino et al., 2011) with the goal of better understanding PD in order to develop prevention strategies.

While the steps leading to blocked vessels appear to be key to disease, we wanted to explore if additional virulence factors facilitate symptoms. Of particular interest were genes with potential roles in secondary metabolite production. We explored a *Xf* gene, PD1311, that is annotated as a putative peptide synthase (Altschul et al., 1990) or AMP-binding enzyme (Punta et al., 2012). PD1311 has the three motifs found in adenylate-forming enzymes (**Figure 1**), also known as the ANL superfamily, which is composed of ACSs (acyl- and aryl-CoA synthetases), NRPS (nonribosomal peptide synthetase) adenylation domains, and Luciferases (Chang et al., 1997, Gulick 2009). *Xf* does not have luciferase activity, nor the domains and size of NRPS megaenzymes (Strieker et al., 2010), suggesting that PD1311 is potentially an ACS. The most studied bacterial ACS is the *Escherichia coli* FadD, which catalyzes exogenous long-chain fatty acyl-CoA from fatty acid, coenzyme A, and ATP (Black et al., 1992). ACS metabolite intermediates are involved in ß-oxidation and phospholipid biosynthesis, and ACS proteins are also implicated in cell signaling, protein transportation, and protein acylation (Korchak et al., 1994, Glick et al., 1987, Gordon et al., 1991). Importantly, ACSs are known to be involved in

virulence factors, such as the *Xanthomonas campestris* ACS FadD homolog, RpfB, which appears to be involved in production of quorum-sensing molecule, DSF (diffusible signal factor) (Barber et al., 1997). We discovered that deleting PD1311 results in a nonpathogenic strain when inoculated in grapevines, indicating that PD1311 is fundamental for Pierce's disease development.

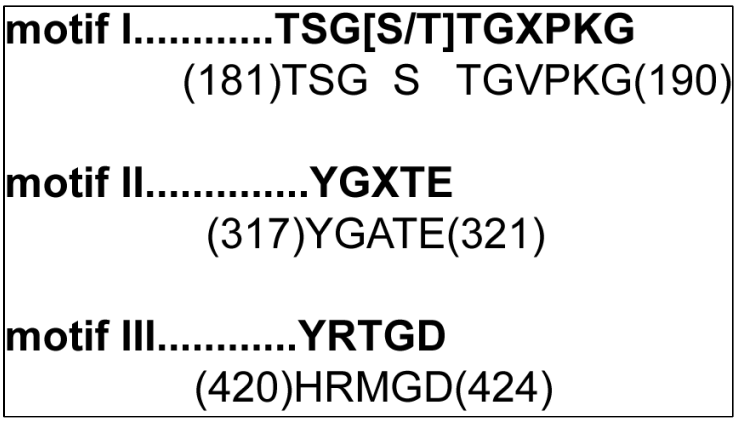

Figure 1. Sequence comparison of PD1311 to acyl-adenylate-forming enzyme superfamily signature motifs. For each motif, consensus sequence (bold) is shown above PD1311 (unbold) sequence with amino acid numbers in parenthesis. Motifs I/II are for ATP binding. Motif III is for substrate binding and catalysis at the invariant aspartate (Chang et al., 1997, Ingram-Smith et al., 2012).

Given our findings with the ΔPD1311 strain, we propose it has potential as a biocontrol for Pierce's disease. The weakly virulent *Xf* elderberry strain EB92-1 has been studied as a potential Pierce's disease biocontrol (Hopkins 2005, Hopkins 2012). Other approaches include naturally resistant rootstocks (Cousins and Goolsby 2011) or transgenic varieties (Dandekar 2012, Gilchrist and Lincoln 2012, Kirkpatrick 2012, Labavitch et al., 2012, Lindow 2012, Powell and Labavitch 2012). However, continued research for Pierce's disease controls is warranted. Given the avirulent phenotype of the ΔPD1311 strain, understanding how PD1311 orchestrates the disease response may also provide key insights into Pierce's disease development.

OBJECTIVES

The overall goal of this project is to understand how the PD1311 protein influences virulence, and test if the PD1311 mutant strain functions as a biocontrol for Pierce's disease. To examine these questions, we propose the following:

1. Characterize the *Xf* ΔPD1311 mutant.
   a. Complete *in vitro* behavioral assays critical for disease.
   b. Determine the role(s) of PD1311 in producing virulence factor(s).
2. Determine the effectiveness of ΔPD1311 Temecula strain as a biological control of Pierce's disease.
   a. Determine conditions for biological control.
   b. Examine spread of ΔPD1311 and wild-type strains simultaneously.

RESULTS AND DISCUSSION

1. Characterize the *Xf* ΔPD1311 mutant.

1a. Complete *in vitro* behavioral assays critical for disease.

We deleted 1308bp of the 1695bp PD1311 gene in *Xf* Temecula1 by double cross-over recombination of upstream and downstream flanking sequences of the target gene and replacement with a kanamycin cassette (Shi et al., 2009). The mutant was successfully complemented as previously described (Cursino et al., 2011). In a growth curve, the ΔPD1311 and complemented strains grew equal to wild-type *Xf* (data not shown), suggesting that PD1311 is not critical for basic metabolism. Key processes leading to Pierce's disease are motility, aggregation, and biofilm formation (Chatterjee et al., 2008a), and therefore we are examining these behaviors in the ΔPD1311 strain. Such information will help us determine if PD1311 exerts its effects by common virulence methods or has more specialized function.

i) Motility.
Weakly virulent strains have been found that are less motile than wild-type *Xf* strains (Cursino et al., 2009). Our preliminary results show that ΔPD1311 motility may be modestly affected, as examined by colony fringe assay (**Figure 2**); fringe around the bacterial colony directly correlates with type IV pilus twitching motility (Meng et al., 2005, Li et al., 2007). Given the potential reduced motility and that in grapevines *Xf* migrates against the transpiration stream (Meng et al., 2005), we will assess the speed of cells in microfluidic chambers and plants (Meng et al., 2005, de la Fuente et al., 2007a, de la Fuente et al., 2007b).

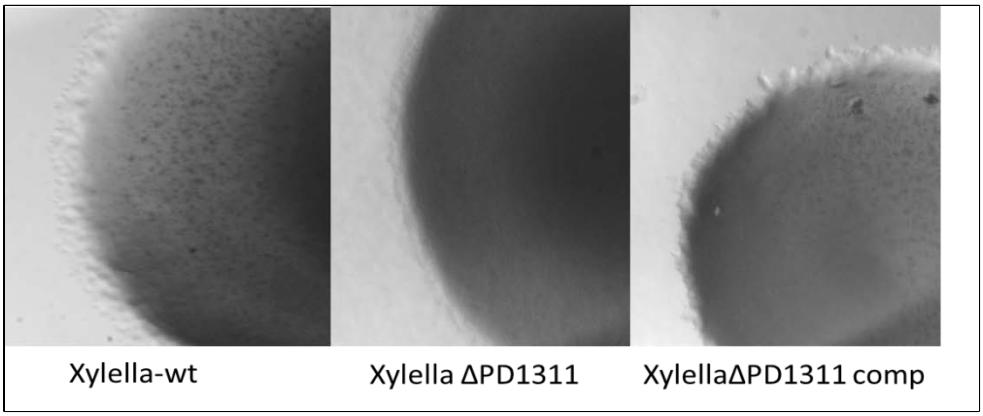

Figure 2. Motility of ΔPD1311 mutant strain. Colony fringes of wild-type (*Xylella*-wt), mutant (*Xylella*ΔPD1311), and complemented mutant (*Xylella*ΔPD1311comp) strains were assayed on PW agar overlaid with cellophane. Colonies were assessed after five days of growth (Meng et al., 2005, Li et al., 2007). Colonies photographed at 90X magnification. Experiment was repeated three times.

ii) Aggregation and biofilm formation.
Cell aggregation is a critical step in biofilm formation, which is proposed to clog xylem vessels and prevent transport of nutrients and water resulting in Pierce's disease (Chatterjee et al., 2008a). To study aggregation the bacteria was grown in test tubes for five days in three ml PD2 (Davis et al., 1980). After five days the OD_{540} was recorded (OD_T) and the bacteria resuspended before recording the OD (OD_S) again. The percent aggregation was calculated as $[(OD_T - OD_S)/ OD_T] \times 100$ (Burdman et al., 2000, Shi et al., 2007). Our studies showed that the ΔPD1311 strain has decreased aggregation in comparison to wild-type and complement strains ($P<0.03$) (**Figure 3**). We then examined biofilm production using a 96-well crystal violet assay (Zaini et al., 2009). In line with the aggregation findings, the ΔPD1311 strain appears to produce less biofilm than wild-type *Xf* ($P<0.0001$) (**Figure 4**). Decreased biofilm production generally correlates with decreased pathogenicity (Cursino et al., 2009, Shi et al., 2009, Cursino et al., 2011). However previously examined mutants that have reduced biofilm do not eliminate disease, unlike ΔPD1311. This result suggests that other factors besides altered biofilm production are involved in ΔPD1311 avirulence.

1b. Determine the role(s) of PD1311 in producing virulence factor(s).
Our preliminary results suggest that PD1311 affects virulence by methods beyond motility and biofilm formation, which are fundamental processes in Pierce's disease (Chatterjee et al., 2008a). Many bacteria produce secondary products that are critical to their pathogenic responses (Raaijmakers and Mazzola, 2012). In terms of *Xf*, these molecules may be involved in pathogen regulation or secondary metabolite production. The *Xf* DSF is a quorum-sensing product that coordinates motility, biofilm formation, and virulence (Chatterjee et al., 2008a). *Xf* is postulated to have multiple DSF products of which the known ANL protein, RpfB, appears only to be involved in the production of a subset (Almeida et al., 2012). Given that PD1311 appears to be an ANL member, it may also play a role in DSF production in conjunction with RpfB or in another DSF product. Determining if PD1311 is involved in DSF production will either place PD1311 within the growing class of *Xf* quorum-sensing associated molecules (Almeida et al., 2012) or direct our studies to other areas of ACS involvement.

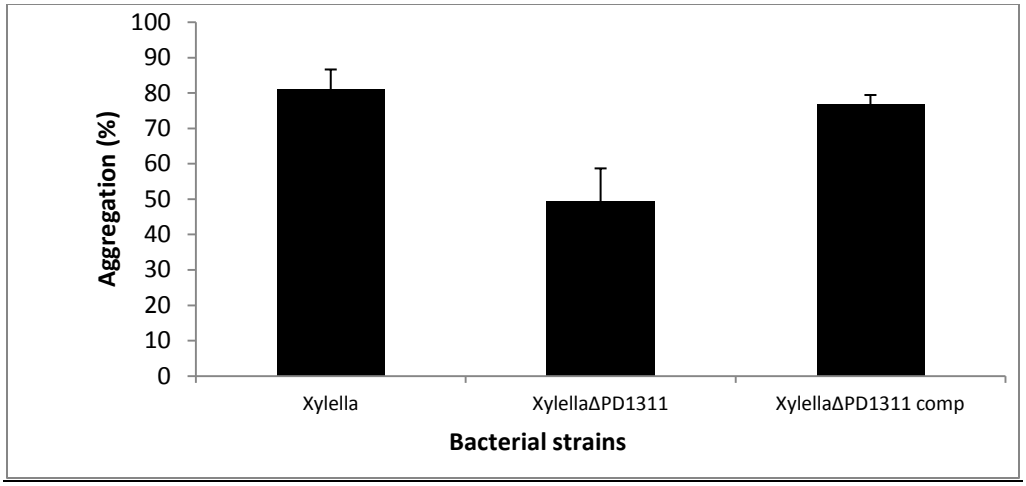

Figure 3. Aggregation of ΔPD1311 mutant strain. Aggregation of wild-type (*Xylella*), mutant (*Xylella*ΔPD1311) and complemented mutant (*Xylella*ΔPD1311comp) strains were assayed in test tubes (Burdman et al., 2000, Shi et al., 2007). The experiment was repeated three times.

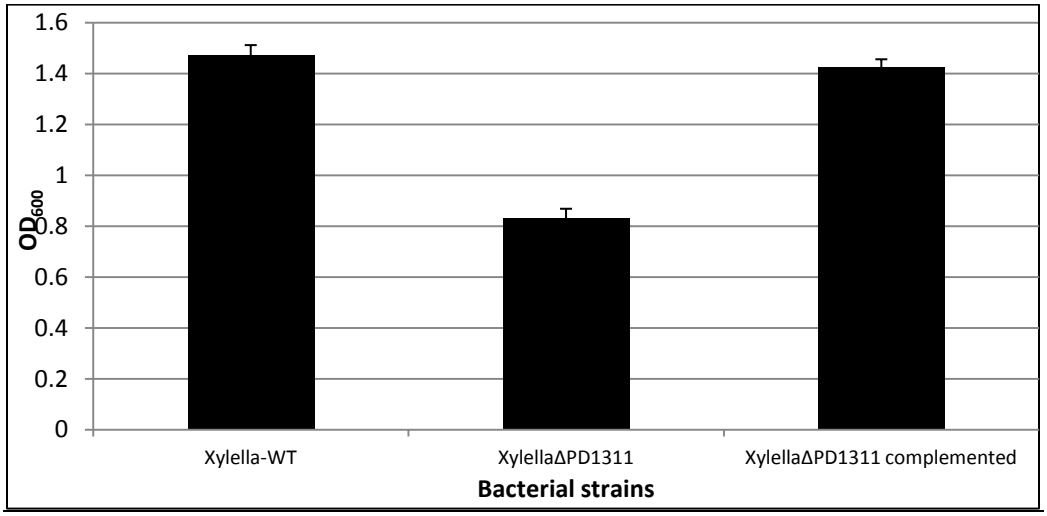

Figure 4. Biofilm formation by ΔPD1311 mutant strain. Quantification of biofilm formation in 96 well plates for wild-type (*Xylella*-WT), mutant (*Xylella*ΔPD1311), and complemented mutant (*Xylella*ΔPD1311complemented) strains (Zaini et al., 2009). The experiment was repeated three times with 24 replicates each.

To determine if PD1311 alters DSF production wild-type *Xf* and the ΔPD1311strain were streaked onto PW agar plates (Davis et al., 1981) for eight days to allow production of DSF. The *Xanthamonas campestris campestris* (*Xcc*) indicator strain 8523 (kindly provided by Prof. Steven Lindow, UC Berkeley) was streaked perpendicular to either the wild-type or ΔPD1311 for 24 hours (Newman et al., 2004). A suspension was made of the *Xcc* strain 8523 cells and fluorescence was visualized using a confocal microscope. Our findings showed no alterations in DSF production by the ΔPD1311 strain suggesting that PD1311 is not involved in quorum sensing molecule production (data not shown).

Objective 2. Determine the effectiveness of the ΔPD1311 Temecula strain as a biological control of Pierce's disease.

When we tested the PD1311 strain *in planta* we found that deleting it eliminated disease (**Figure 5**). As plants were started late in the season and the buffer control began to show signs of senescence around week 14, we do not believe the post-week 14 disease rating of ΔPD1311 was true Pierce's disease. These results indicate that PD1311 product is critical for Pierce's disease symptoms

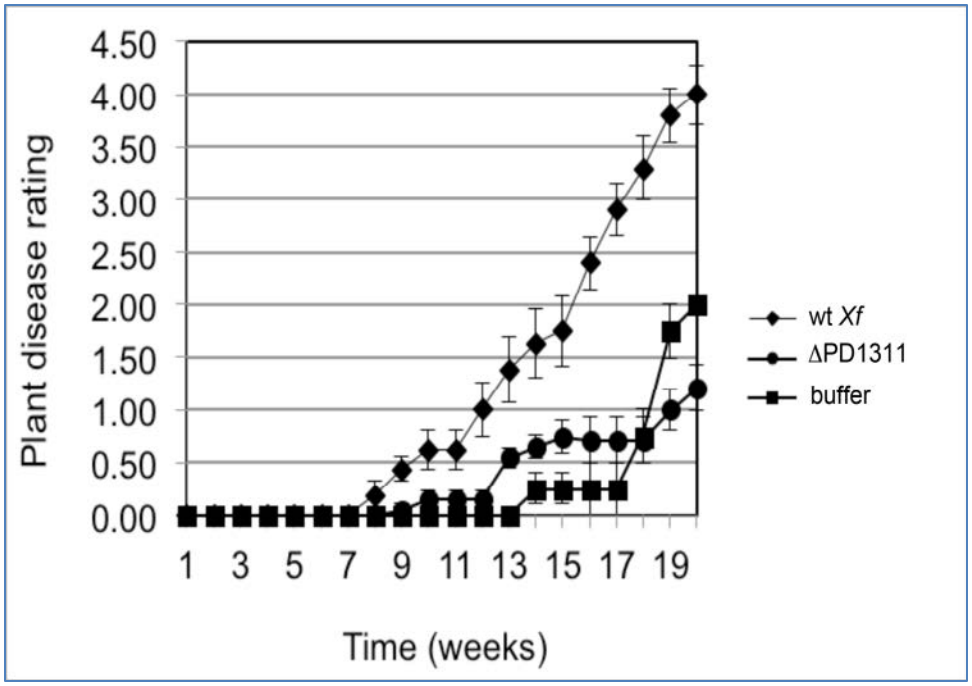

Figure 5. Development of Pierce's disease. Grapevines were inoculated with wild-type *Xf* (diamond), ΔPD1311 strain (circle), and buffer as a negative control (square). Symptoms were monitored on 10 plants for each treatment over a period of 20 weeks and rated on a scale of 0-5 (Guilhabert and Kirkpatrick 2005, Cursino et al., 2009). Plants were started late in the season and the buffer control showed symptoms at week 14, suggesting that ΔPD1311 symptoms may be due to senescence and not Pierce's disease.

We believe that the ΔPD1311 strain may act as a biocontrol as it appears to have an impact on wild-type *Xf* cells in relationship to an important Pierce's disease trait, biofilm formation. We have wild-type *Xf* cells constitutively expressing green fluorescent protein (wt-GFP) (kindly provided by Prof. Steven Lindow, UC Berkeley). We have used this strain before in a number of behavioral assays and found it to function like wild-type *Xf* (data not shown). To determine if ΔPD1311 affected biofilm formation by wild-type strain Temecula *Xf*, half the cells were wt-GFP and the other half were either non-fluorescent wild-type or the ΔPD1311 strain. As stated above, wild-type cells produce more biofilm than the ΔPD1311 strain (**Figure 4**) so mixtures of wt-GFP/ΔPD1311 should have equal or greater fluorescence than mixtures of wt-GFP/wt, if the strains did not impact each other. We observed that the wt-GFP/ΔPD1311 mixture had less fluorescence than the wt-GFP/wt mixture (**Figure 6**), suggesting that the ΔPD1311 strain has an ability to reduce the virulence-associated biofilm produced by wt *Xf*.

2a. Determine conditions for biological control.

To determine if the ΔPD1311 strain can act as a biocontrol we have begun *in planta* studies. First, we have grapevines infected with the ΔPD1311 strain from 2012 that were allowed to grow for 20 weeks post-inoculation and were placed in dormancy. We then inoculated these vines with the wild-type strain. Second, we inoculated plants with wild-type *Xf* after a short pre-treatment with the ΔPD1311 strain, following procedures used in *Xf* elderberry EB92.1 strain biocontrol studies (Hopkins 2005); the ΔPD1311 strain treatment was given approximately two weeks before inoculation with wild-type *Xf*. Third, the wild-type and the ΔPD1311 strains were inoculated simultaneously. Fourth, we inoculated vines with the wild-type strain for approximately two weeks followed by ΔPD1311. These various treatments with their appropriate controls will allow us to assess the effectiveness of the ΔPD1311 strain as a biocontrol and provide appropriate treatment protocol information. However, due to uncontrollable circumstances in the greenhouse that affected plant growth, we doubt that conclusive results on the effectiveness of biological control will be obtained this year. Inoculations will be performed again next spring.

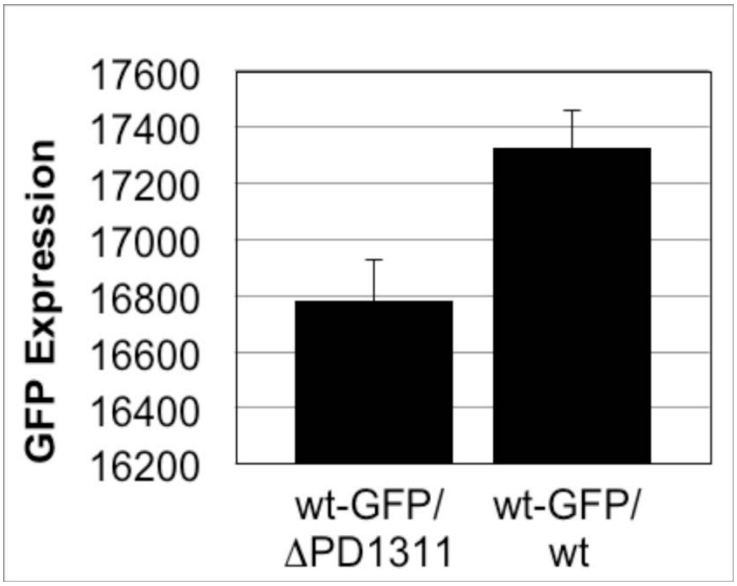

Figure 6. Mutant strain impact on wild-type strain biofilm formation. Quantification of biofilm in 96 well plates with agitation with equal amounts of wild-type *Xf* constitutively expressing green fluorescent protein (wt-GFP) and either wild-type *Xf* or the ΔPD1311 strain ΔPD1311). The experiment was performed once with 24 replicates. Fluorescence is in artificial units.

2b. Examine spread of ΔPD1311 and wild-type strains simultaneously.
We will soon begin organizing the experiments in order to address objective 2b.

CONCLUSIONS
Xf motility, aggregation, and biofilm formation are key steps in Pierce's disease development (Chatterjee et al., 2008a). PD1311 appears to play a role in biofilm formation, aggregation, and maybe motility. Other functions presumably explain its nonpathogenic response, as unlike ΔPD1311, *Xf* mutants with modified motility or biofilm still induce some disease (Cursino et al., 2009, Shi et al., 2009, Cursino et al., 2011). Because the ΔPD1311 strain appears to impact biofilm production by wild-type *Xf*, it may alter wild-type-induced disease *in vivo*. We will complete greenhouse studies within the next year to determine if the ΔPD1311 strain can block Pierce's disease development by wild-type *Xf*. We are currently performing studies to better understand the role of PD1311. Overall, this work will help further understanding of disease development and prevention.

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CHARACTERIZATION OF THE *XYLELLA FASTIDIOSA GCVR* GENE REQUIRED FOR PATHOGENICITY

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Reporting Period: The results reported here are from work conducted October 2012 to September 2013.

ABSTRACT
The transcriptional regulator *gcvR* of *Xylella fastidiosa* (*Xf*) is a putative pathogenicity gene. To elucidate the role of *gcvR* in Pierce's disease, mutant *XfΔgcvR* and complementary *XfΔgcvR*-C strains were constructed. The genetically-modified strains and wild-type *Xf* were inoculated into Cabernet Sauvignon grapevines. Three months after inoculation, grapevines inoculated with wild-type or *XfΔgcvR*-C showed typical Pierce's disease symptoms while plants inoculated with *XfΔgcvR* showed very mild symptoms. Quantitative PCR assays demonstrated that *Xf* titers in grapevines inoculated with wild-type or *XfΔgcvR*-C strains were significantly higher than that of grapevines inoculated with *XfΔgcvR*. *In vitro* studies showed that while all *Xf* strains had similar growth curves, *XfΔgcvR* exhibited significant reduction in biofilm formation. The results suggest that the knockout of *gcvR* in *Xf* reduces colonization of grapevines, resulting in reduced pathogenicity.

LAYPERSON SUMMARY
Xylella fastidiosa (Xf) is a plant pathogenic bacterium that causes Pierce's disease in grapevine. The virulence regulator, *gcvR*, is known to coordinate expression of virulence-related factors responsible for cellular aggregation and biofilm formation of *Xf* in host plants. The characterization of the virulence regulator *gcvR* of *Xf* improves the understanding of how *Xf* causes Pierce's disease.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a xylem-inhabited bacterium that has been identified in 28 plant families including grapevine (Raju et al, 1983; Costa et al., 2004). *Xf* growth and multiplication require nutritional support from its host. Studies showed that xylem sap from Pierce's disease susceptible grapevines provides better support for bacterial growth and biofilm formation than that from Pierce's disease resistant grapevines (Perring et al., 2001 and Chenge et al., 2009). Although xylem sap is relatively diluted, it contains various amino acids, organic acids, inorganic ions, and proteins (Buhtz et al., 2004). It has been reported that there were certain amino acids (e.g. glycine) and organic acids (e.g. tartaric acid) that appeared to be important for *Xf* growth (Leite et al., 2004). In a recent study, exposure of *Xf* to xylem fluids collected from different *Vitis* genotypes resulted in significant differences in both planktonic growth, biofilm formation and virulence gene expression (Andersen et al., 2007, Bi et al., 2007a; Bi et al., 2007b, Chenge et al., 2009, Shi et al., 2013). These data suggested that the survival and biofilm formation of *Xf* were affected by the differential chemical components of xylem fluid.

The analyses of the differentially expressed genes of *Xf* responding to xylem fluid showed that some virulence genes including transcriptional regulator, *gcvR* were greatly expressed in Pierce's disease susceptible xylem fluid as compared in Pierce's disease resistant xylem sap (Cooksey et al,. 2008; Shi et al., 2013). GcvR is a transcriptional regulator of the glycine cleavage operon in *Escherichia coli* (Wilson et al., 1993). It is the Gcv enzyme system that catalyzes the oxidative cleavage of intracellular glycine to NH_3, CO_2, and a one-carbon methylene group that is transferred to the acceptor, tetrahydrofolate, a major source of the biosynthesis of purines, thymine, and methionine (Kikuchi, 1973; Meedel and Pizer, 1974; Mudd and Cantoni, 1964). The expression activated by GcvR/GcvA complex depends on the presence of glycine (Wilson et al., 1993). The presence of glycine could disrupt the GcvA/GcvR complex, allowing GcvA to activate the synthesis of the glycine cleavage system. The *gcvR* has been shown to have involvement enhancing of the ability of the bacterium to survive in low pH by up-regulating the levels of the alternate sigma factor RpoS (Ye et al, 2009). However, little is known about the contribution of GcvR-mediated regulatory pathways to *Xf* pathogenesis in the resistance/susceptibility host. Since xylem-limited *Xf* is likely exposed to a various stresses such as changes in osmolarity and nutrient

starvation (Alves et al., 2004), it is expected that *Xf* may respond to these stresses through regulatory mechanisms involving specific regulatory genes. The goal of this study is to elucidate the mechanisms of GcvR regulatory pathways that are involved in the pathogenicity of *Xf*. Here we report the creations of *XfΔgcvR* mutant and complementary *XfΔgcvR*-C strains, and the role of GcvR in biofilm formation and pathogenicity in grapevine.

OBJECTIVES

1. Constructions of the *XfΔgcvR* mutant and complementary *XfΔgcvR*-C strains.
2. Assays of the role of *gcvR* in biofilm formation and pathogenicity of *Xf* in grapevine.

RESULTS

The generations of *XfΔgcvR* and *XfΔgcvR*-C *in vitro.*

A site-directed deletion method (Shi et al., 2007) was used to construct an *XfΔgcvR* mutant. The physical replacement of the *gcvR* ORF from start condon ATG to terminal condon TAA with a gentamicin (GM) cassette in the genome of *Xf* was reconfirmed by PCR and sequencing analysis (data not shown). The chromosome-based genetic complementation strategy (Matsumoto et al, 2009) was employed with a chloramphenicol (CM) cassette to create the complementary *XfΔgcvR*-C strain (data not shown). Stable *XfΔgcvR* and *XfΔgcvR*-C strains were obtained from selection medium supplemented with 10 µg/ml GM or 10 µg/ml CM, respectively. The bacterial genotypes were confirmed by *XfΔgcvR* and *XfΔgcvR*-C strain specific primers.

Pathogenicity of *XfΔgcvR* and *XfΔgcvR*-C

Grapevines inoculated with *Xf* wild-type and *XfΔgcvR*-C showed the typical symptoms of Pierce's disease (**Figure 1**). In contrast, grapevines inoculated with *XfΔgcvR* developed very mild symptoms 12 weeks after inoculation (**Figures 1 and 2**). All diseased grapevines were detected *Xf* positive using an *Xf* specific PCR. Quantitative PCR assay showed that grape plants infected with *Xf* wild-type and *XfΔgcvR*-C contained similar level of bacterial titers but were significantly higher than that infected by *XfΔgcvR*. These data suggest the mutated *gcvR* may affect the survival of *Xf* inside the xylem, resulting in reduced pathogenicity.

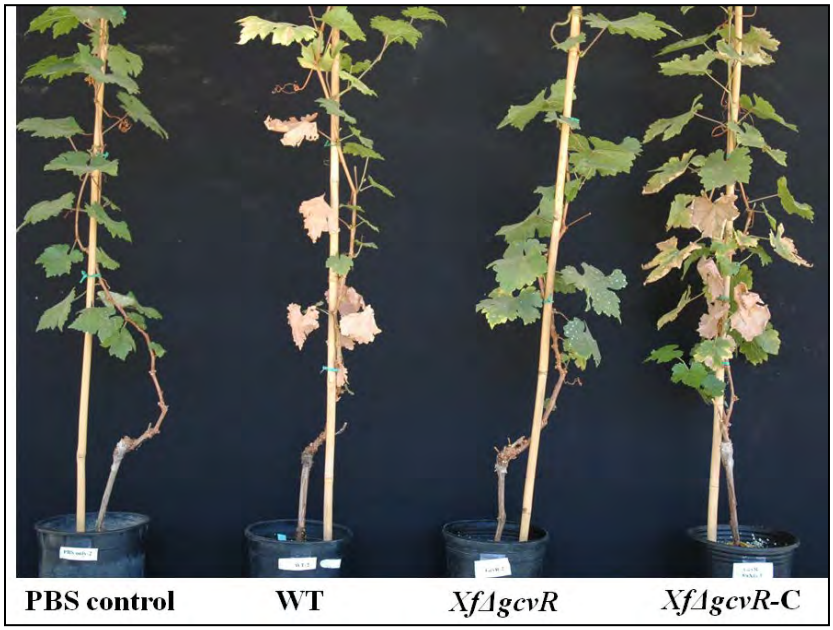

Figure 1. Pierce's disease symptom of Cabernet Sauvignon grapevines inoculated with *Xf* wild-type, *Xf*ΔgcvR, and *Xf*ΔgcvR-C. Grape plants infected by wild-type and *Xf*ΔgcvR-C showed severe Pierce's disease symptoms while plants infected by *Xf*ΔgcvR had very mild symptoms. All infected and control plants were confirmed by PCR.

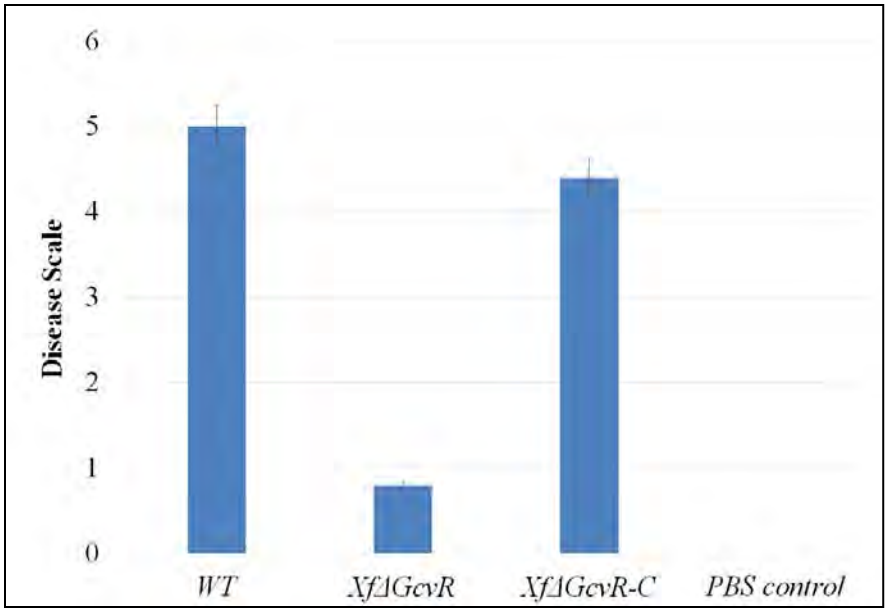

Figure 2. Disease severity of grapevines inoculated with *Xf* wild-type, *Xf*ΔgcvR, and *Xf*ΔgcvR-C was evaluated three months post infection. Disease severity was scaled based on a visual symptom ranged from 0 to 5. The data is represented in average of five repeats.

Physiological properties of *XfΔgcvR* and *XfΔgcvR*-C *in vitro*.

In vitro growth curves showed that *XfΔgcvR* and *XfΔgcvR*-C reached the exponential and stationary phase in a manner similar to the wild-type (data not shown). There was no obvious difference in colony morphology among all tested strains. The cells of *Xf* wild-type and *XfΔgcvR*-C attached to the wall of glass tubes and formed wide rings whereas *XfΔgcvR* cells showed much less ring formation (**Figure 3**). Furthermore, biofilm formation by *XfΔgcvR* was five-fold less than that of *Xf* wild-type after ten days of static incubation measured by a crystal violet assay, while *XfΔgcvR*-C had significantly improved biofilm formation (**Figure 4**). This suggests that *gcvR* mutant reduces the ability of the surface attachment, resulting in the reduction in biofilm formation.

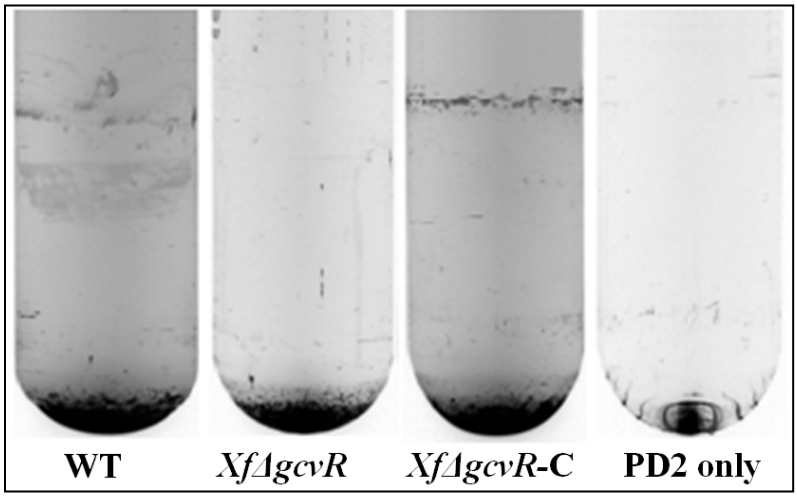

Figure 3. Biofilm formation analysis of *Xf* wild-type, *XfΔgcvR,* and *XfΔgcvR*-C cells in PD2 broth. *Xf* cells attached to the inside wall of the glass tubes forming a ring.

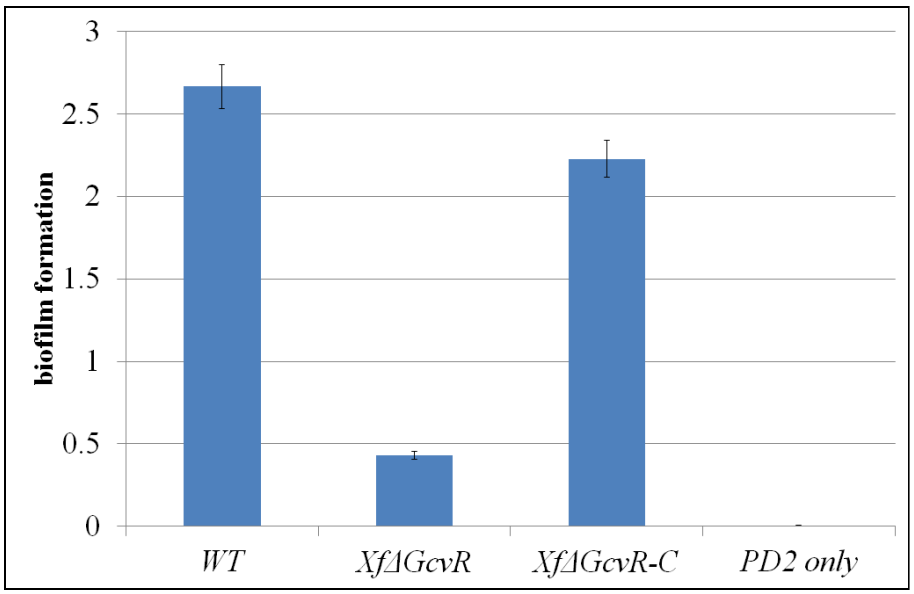

Figure 4. Biofilm formation was evaluated at OD_{600nm} in PD2 broth by a crystal violet assay method. All treatment groups were independently repeated three times. The significant differences were indicated at $P < 0.05$.

CONCLUSIONS

While the exact mechanism in which GcvR regulates pathways in *Xf* is not yet clear, we demonstrated that GcvR played an important role associated with the biofilm formation *in vitro* and pathogenicity in grapevines. Since *gcvR* was expressed high in xylem fluid of Pierce's disease susceptible grapevine but low in Pierce's disease resistant grapevines, it is needed to further investigate the regulatory mechanisms of *gcvR* influencing by xylem sap derived from Pierce's disease resistant and susceptible grapevines. Identifications of expression gene profiles regulated by GcvR and the target components in Pierce's disease resistant xylem fluid will reveal the mechanisms through which *Xf* -host plant interactions take place resulting in resistance or susceptibility. Xylem sap composition analysis also may be useful for screening grapevines for Pierce's disease resistance.

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ELUCIDATING THE PROCESS OF CELL-CELL COMMUNICATION IN *XYLELLA FASTIDIOSA* TO ACHIEVE PIERCE'S DISEASE CONTROL BY PATHOGEN CONFUSION

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ABSTRACT

Xylella fastidiosa (*Xf*) produces an unsaturated fatty acid signal molecule called diffusible signal factor (DSF) that modulates gene expression in cells as they reach high numbers in plants. By increasing the expression of a variety of afimbrial adhesins while decreasing the expression of pili involved in twitching motility as well as extracellular enzymes involved in degrading pit membranes and 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." 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 2-Z-hexadecenoic acid (C16-cis) which seems to be the most active molecule. A *Burkholderia* strain capable of abundant growth and movement in grape has been identified and is being evaluated as a surrogate host for the production of DSF in grape to achieve disease control. Gene expression in *Xf* exposed to various levels of DSF is a sensitive means of assessing DSF levels and *Xf* harboring *phoA* reporter gene fusions to hxfA has proven to be an excellent bioreporter. The adhesiveness of wild-type strains of *Xf* grown in a minimal medium rapidly increases upon addition of DSF. The extent of increase in the adhesiveness of the strain, as measured by binding to the walls of glass tubes, increases with concentration of DSF added. Adherence of mutants of *Xf* to grape vessels is predictive of their virulence, indicating that adhesiveness is a major factor affecting the ability of *Xf* to cause disease. Such adhesive assays should enable us to more rapidly screen transgenic plants for their resistance to Pierce's disease as well the efficacy of chemical analogs of DSF to induce resistance. The release of extracellular membranous vesicles by *Xf* is responsible for the suppression of its adherence to surfaces. These vesicles attach to surfaces such as that of the walls of the xylem vessels. By so attaching, these vesicles prevent the attachment of *Xf* cells themselves to such surfaces. Only upon reaching relatively high cell concentrations in a particular vessel would DSF concentrations increase to a level that would suppressed the release of the membranous vesicles, thereby retaining adhesive molecules on the surface of *Xf* cells themselves, thus allowing the bacterial cells themselves to attached to surfaces, such as that of insect vectors

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 by increasing its adhesiveness to plant surfaces and also suppressing the production of enzymes and genes needed for active movement through the plant. 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. Elevating DSF levels in plants artificially reduces its movement in the plant. In this study we have investigated the variety of different fatty acid molecules that can serve as cell-cell signaling agents in *Xf*. Several new DSF

species have been found including a 16-carbon unsaturated fatty acid that appears to be far more active than the 14-carbon unsaturated fatty acid that we have previously investigated. The release of extracellular membranous vesicles by *Xf* is responsible for the suppression of its adherence to surfaces.

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 they 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," more work is needed to understand how best to alter DSF levels in plants to achieve even higher levels of disease control. Until now we have suffered from a lack of sensitive methods to detect DSF levels in plants (the *Xanthomonas*-based bioassay we have used previously is relatively insensitive to the chemically distinct forms of DSF produced by *Xf*). That is, while we showed that DSF-producing endophytes, direct application of DSF, and transgenic plants producing DSF all conferred some resistance to disease, we had no way to know why they were not more resistant, nor what would be needed to improve control measures since we could not measure the direct effect of our efforts to increase DSF levels in plants. However, we have now developed several new sensitive biosensors that enable us to measure *Xf*DSF both in culture and within plants. We have recently found that *Xf* apparently produces more than one molecule that can act as a DSF signal molecule and that the molecules made by *Xf* are dependent on its growth environment. We thus need to ascertain which form is most active, whether the various forms all have the same effect on regulating traits in *Xf*, and what are their fates when applied to plants in various ways. Thus the overall goal of our work is to use these new biosensors to examine how DSF levels can best be altered by the various methods we have previously identified. As disease control should be directly proportional to both the concentration of and dispersal of DSF within plants we will quantitatively explore the effectiveness of different strategies to elevate DSF levels throughout plants.

We also have made the discovery that *Xf* produces abundant extracellular membranous vesicles which are shed from the cell. Importantly, the content of outer membrane proteins including the adhesion XadA are controlled by DSF accumulation in cultures of *Xf*, and even more importantly, the shedding of these vesicles from the cell are apparently suppressed by the accumulation of DSF. We therefore are testing the model that DSF signaling in *Xf* involves two very different processes both of which lead to a rapid, cell density dependent change in its adhesiveness. When cells of *Xf* are found in relatively low numbers within the xylem vessel they have accumulated little DSF, and because of this they do not produce large amounts of the cell surface adhesins including XadA, HxfA, and others, yet such cells shed large numbers of vesicles. However, when cell density, and thus DSF concentrations increase membrane vesicles are not shed by the cell, and the higher concentration of afimbrial adhesins would be retained on the surface of the *Xf* cells rather than being fed into the environment. Such a process would tend to maximize the adhesiveness of *Xf* when DSF levels increased. This increased adhesiveness is apparently needed for their acquisition by insect vectors but would be expected to suppress their ability to move in the plant. In contrast, the shedding of vesicles would tend to prevent access from adhering to surfaces because most cell surface adhesins would no longer be attached to the cell. In this project, we are testing the role of the membranous vesicles, since preliminary data revealed that they may interfere with the adherence of *Xf* to surfaces. That is, by shedding adhesive vesicles which themselves adhere to the surface of plants, access may prevent its own adherence to such surfaces because they are now coded would such vesicles.

OBJECTIVES

1. Identify additional DSF molecules made by *Xf* that contribute to cell-cell signaling and determine their movement and stability when applied to plants in various ways to improve disease control.
2. Determine the contribution of membrane vesicles shed by *Xf* in the absence of DSF to its virulence and the utility of measurement of vesicular presence within plants as a sensitive means to assess the success of strategies of disease control by pathogen confusion.

RESULTS AND DISCUSSION

Objective 1. Finding new DSF species.

We have optimized methods to use *Xf* itself to detect DSF**.** Among the several genes that we know to be most strongly regulated by DSF are genes such as *hxfA* and *HxfB* which are involved in cell-surface adhesion. We now have successfully used 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 from extracts of *Xf* cultures as well as C14-cis (hereafter called *Xf*DSF) itself. Assay of *Xf* extracts by *Xf*DSF-specific biosensors provide evidence of more than one *Xf*DSF molecule. Our analysis of the material collected by HPLC from these cultures using electro-spray MS revealed it to be an unsaturated C16 fatty acid. We therefore chemically synthesized this presumptive derivative which we will call C16-cis or *Xf*DSF2 (**Figure 1**).

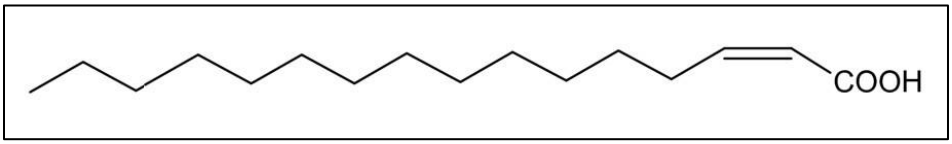

Figure 1. Structure of C16-cis (*Xf*DSF2).

The biological activity of the synthetic *Xf*DSF2 was tested by the addition of this material at various concentrations to an rpfF* mutant strain of *Xf* harboring the hxfA:*phoA* reporter gene fusion and grown in PD3 medium. The rpfF* mutant is unable to synthesize DSF due to two mutations introduced into the catalytic site of the DSF synthase, yet this mutant is still able to respond to exogenous DSF. Importantly, this *Xf:phoA* biosensor exhibited very high alkaline phosphatase activity upon the addition of as little as 100 nM C16-cis (**Figure 2**). Importantly *Xf*DSF2 also conferred much higher induction of hxfA, as indicated by a higher alkaline phosphatase activity at a given concentration than *Xf*DSF, and also induced other adhesins more highly. Both *Xf*DSF and *Xf*DSF2 strongly induced adhesion of wild-type cells of *Xf* to glass tubes (**Figure 2**). Thus *Xf*DSF2 seems to be a particularly powerful signal molecule in *Xf*. We have applied *Xf*DSF to plants in various ways and are in the process of evaluating changes in behavior of *Xf* and disease severity in these plants.

To better understand the chemical species of DSF produced by *Xf* in various conditions in culture and when in infected grape plants, we examined ethyl acetate extracts of chemical species obtained from both culture and *in planta* conditions using electrospray ionization mass spectrometry (ESI-MS). It was possible to identify all of the potential DSF species which we observed in *Xf* as well as those observed by others in other bacterial species such as *Xanthomonas* species based on their highly precise and distinctive ratio of mass to charge. We could detect both *Xf*DSF1 and *Xf*DSF2 in culture supernatants of *Xf* grown in PD3 media (**Figure 3**). Given that *Xf*DSF2 had never been detected in PWG medium, these results confirm that the production of DSF by *Xf* is rather plastic and somewhat dependent on the growth environment of cells.

Surprisingly, we also detected small amounts of a molecule which had previously been described as the DSF from CVC strains of *Xf* (which we term CVCDSF). While our identification of this molecule is tentative, it is noteworthy that it was found only in wild-type and a RpfC mutant of *Xf* but not in an RpfF mutant. Assays of this material in *Xf* revealed that it does not induce expression of genes such as *hxfA*, and instead acts as a potent inhibitor of the expression of such genes (**Figure 4**).

To address which DSF species that *Xf* would produce under natural conditions such as within the xylem of grape plants, we extracted DSF-like molecules from plants infected with a wild-type strain as well as from an RpfF mutant as well as from healthy plants and subjected these chemicals to identification of separation by ESI-MS. We could easily detect *Xf*DSF1 in plants infected with the wild-type strain of *Xf* but this DSF species was not present in either plants infected with the RpfF mutant of *Xf* or in healthy plants. We also could detect elevated concentrations of a molecule presume to be *Xf*DSF2 in plants infected with the wild-type *Xf* strain. Further characterization of the DSF species found in plants infected with the wild-type strain of *Xf* are proceeding to identify other DSF species that are produced under these natural conditions. We find it exciting however that *Xf*DSF2 can be detected in infected plants, suggesting that this highly active DSF species is biologically relevant

and may contribute to much of the cell density dependent behavior of *Xf*. For this reason, we are continuing studies to alter the abundance of *Xf*DSF2 in plants by topical applications, injections, and soil drenches.

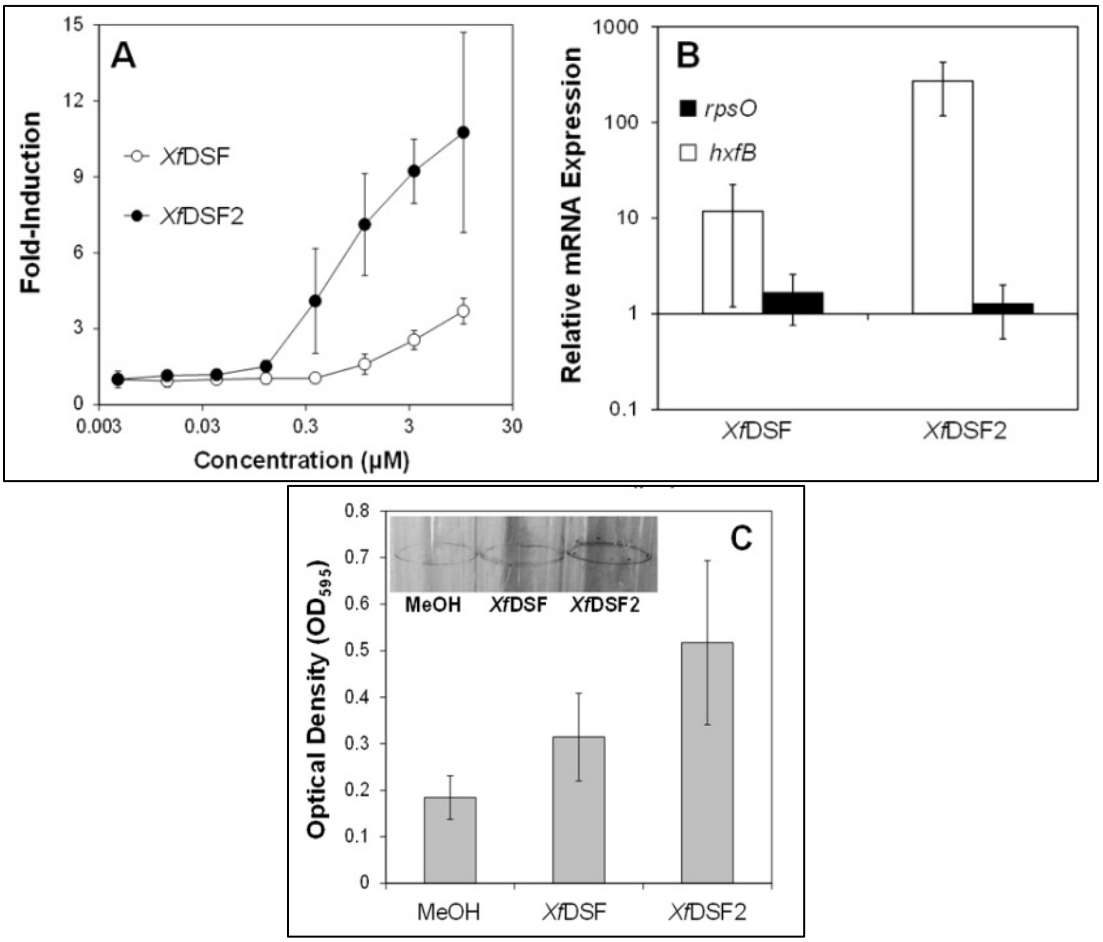

Figure 2. **(A)** *Xf*DSF and *Xf*DSF2 **d**ose-dependent induction of the *Xf*-based DSF-biosensor (*rpfF**-*Xf*HA-biosensor). **(B)** qRT-PCR analysis of *hxfB* expression in *Xf rpfF** strain after 72 hr of growth in PD3 broth supplemented with 10 μM *Xf*DSF, 10 μM *Xf*DSF2, or MeOH. *rpoD* and *rpsO* were used endogenous control genes. **(C)** Biofilm formation at the liquid-air interface of shaken glass tubes by *Xf rpfF** strain after 24 hr of growth in PD3 broth supplemented with 10 μM *Xf*DSF, 10 μM *Xf*DSF2, or MeOH.

To better understand the variety of different DSF species that might be produced by *Xf*, we chemically fractionated cultures of *Erwinia herbicola* harboring RpfF, the DSF synthase from *Xf*. *E. herbicola* was chosen as a surrogate host for the DSF synthase because much larger numbers of cells of the species can be produced than that of *Xf* itself. Ethyl acetate extracts of culture supernatants were separated using HPLC. Several chemical entities were identified in extracts of *E. herbicola* containing RpfF or containing both RpfF and RpfB from *Xf* but not in control cultures of *E. herbicola* lacking these genes from *Xf*. To obtain putative chemical structures for these chemical species that were present only in the presence of the DSF synthase we physically collected fractions of the separated material and subjected to ESI-MS to obtain high-resolution M/Z ratios for these materials. A precise molecular formula for these molecules could be obtained this process and their putative structure can be obtained given the constraints that the DSF species are very likely to be 2-enoic acids (**Figure 5**).

Objective 2. Role of extracellular vesicles.

Our continuing work reveals that *Xf* is a very prolific producer of extracellular vesicles. For a large numbers of vesicles (>400/cell) can be associated both with the surface of the bacterial cell, as well as a high portion that are shed by the cells to the extracellular environment (**Figure 16**). The vesicles are generally quite small, ranging in size from about 0.01 to 0.1 μm in diameter.

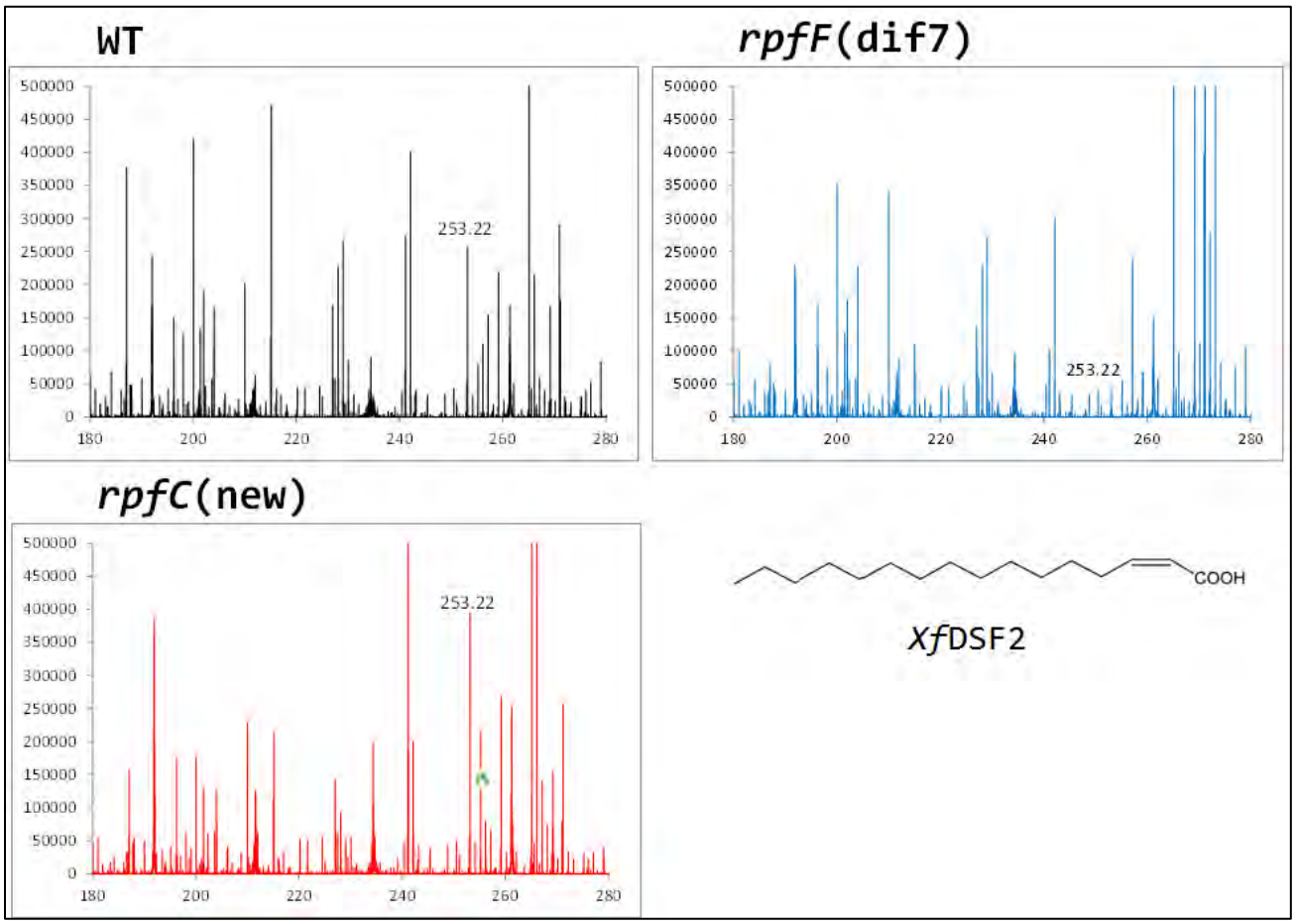

Figure 3. Abundance of ions with the M/Z ratios shown on the abscissa or culture extracts of a wild-type strain of *Xf* (upper left panel), of an RpfF mutant unable to produce DSF (upper right panel), of an RpfC mutant which overproduces DSF (lower left panel). Note that *Xf*DSF2 has a M/Z ratio of 253.22.

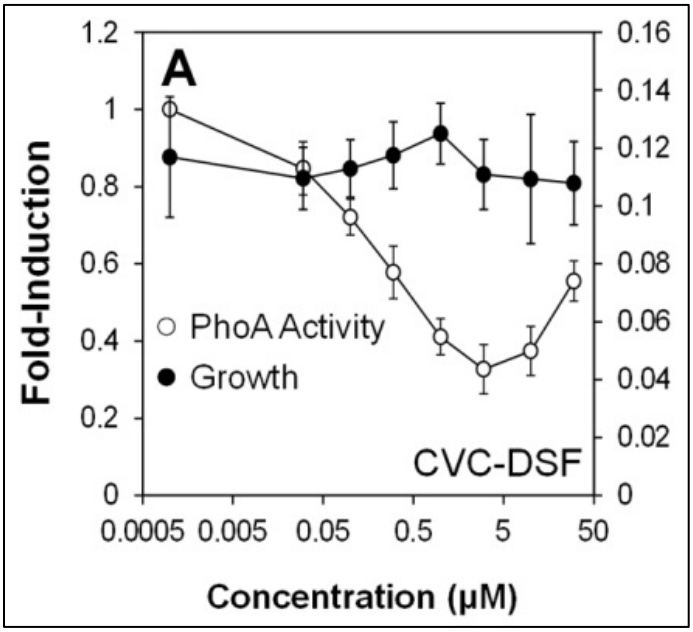

Figure 4. Silencing of the activity of the *Xf*-based DSF-biosensor (*rpfF**-XfHA) by CVC-DSF. PhoA Activity units were measured at T = 96 h and is shown on the left Y-axis. Optical density (OD_{600}) of culture at the time of PhoA Activity is shown on the right Y-axis.

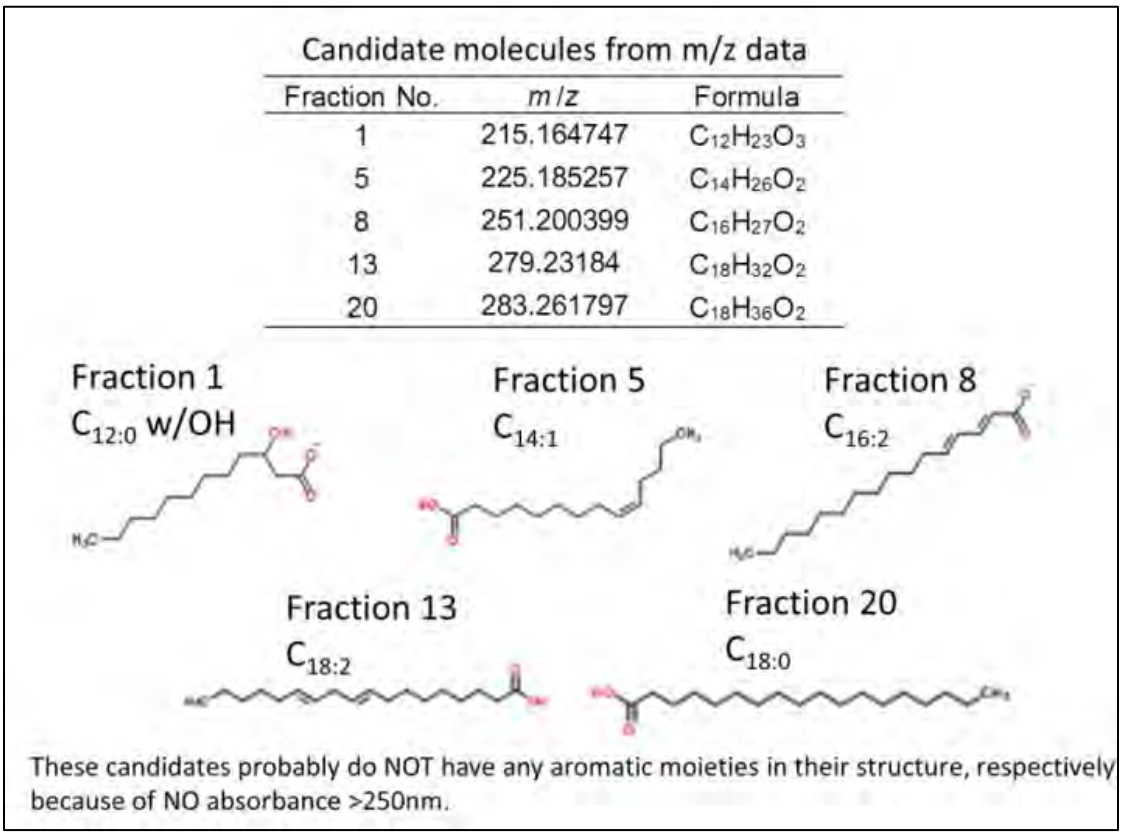

Figure 5. Tentative structures for novel DSF species produced in *E. herbicola* harboring RpfF from *Xf.*

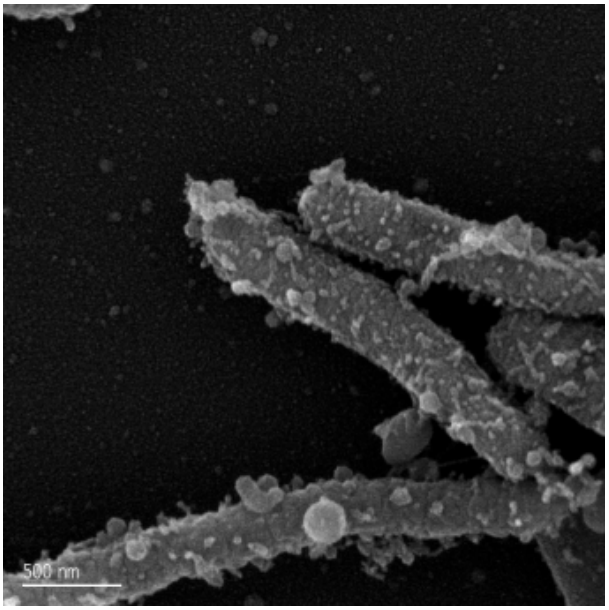

Figure 6. Membranous vesicles forming on the surface of cells of a wild-type strain of *Xf.* Not shown due to the method of preparation are those additional vesicles that have been shed from cells.

Our ability to quantify membrane vesicles and to determine those factors which control their production and release from cells has been facilitated by our finding that a major outer membrane adhesins XadA is a significant component in these membranous vesicles. Since we have obtained antibodies specific to XadA, it is possible to visualize membrane vesicles using light microscopy (**Figure 7**). Using anti-XadA antibodies with a red

fluorescent tag it is clear that the surface of *Xf* cells harbors a large constellation of membranous vesicles which surround the cell has somewhat of a "cloud." In addition to those vesicles which are relatively closely associated with the cell (although apparently not physically linked), are vesicles that can be found at further distances away from cells (**Figure 7**). These distantly located vesicles are clearly not simply "mini-cells" of *Xf* since they do not contain DNA as determined with a DNA-binding dyes such as DAPI (**Figure 7**).

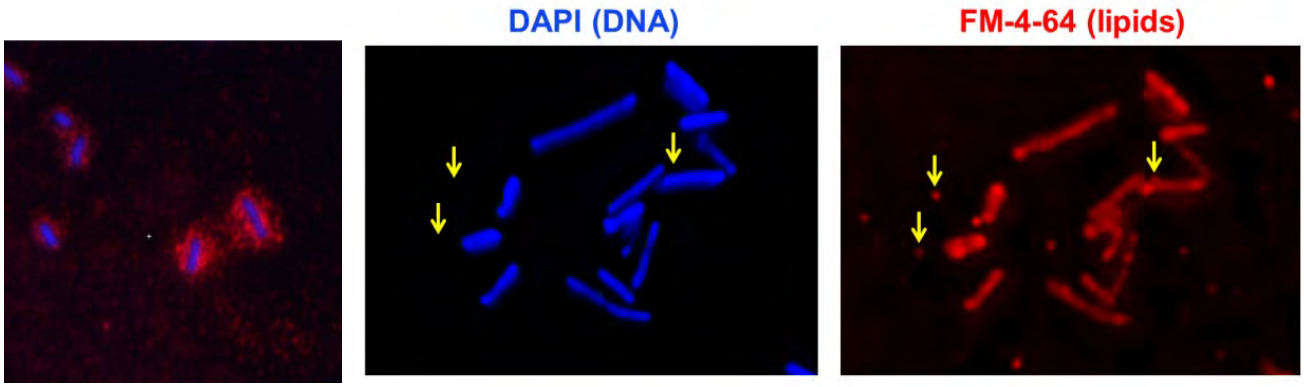

Figure 7. (Left panel) Visualization of membranous vesicles produced by cells of *Xf* whose DNA is stained with DAPI and appearing blue in this image. Vesicles are red in this image due to their binding to fluorescently-labeled anti-XadA antibodies. (Center panel) Visualization of cells of *Xf* stained with DAPI as well as with the lipid binding stain FM-4-64 (Right panel). Note the location of small circular red objects indicating presence of membranous material distal to the location of adjacent *Xf* cells.

To utilize the outer membrane protein XadA as a molecular marker for the presence of extracellular vesicles it was necessary to demonstrate that it is not secreted as a free protein into the extracellular environment, and is always found associated with membranous vesicles. We isolated total proteins from vigorously washed cells of an RpfF mutant of *Xf*, from a pallet of extracellular material could be recovered after high-speed centrifugation (150,000 x g), as well as free protein that was not pelleted after high-speed centrifugation. While small amounts of XadA were attached to the *Xf* cells, large amounts of XadA were found in membranous vesicles that could be obtained after high-speed centrifugation, and importantly, no XadA was free and solution of cultures of *Xf* (**Figure 8**). These results clearly show that XadA is abundantly released in the form of membranous vesicles from cells of *Xf*, and since it is always associated with membranes, it makes an excellent marker for membranous vesicles.

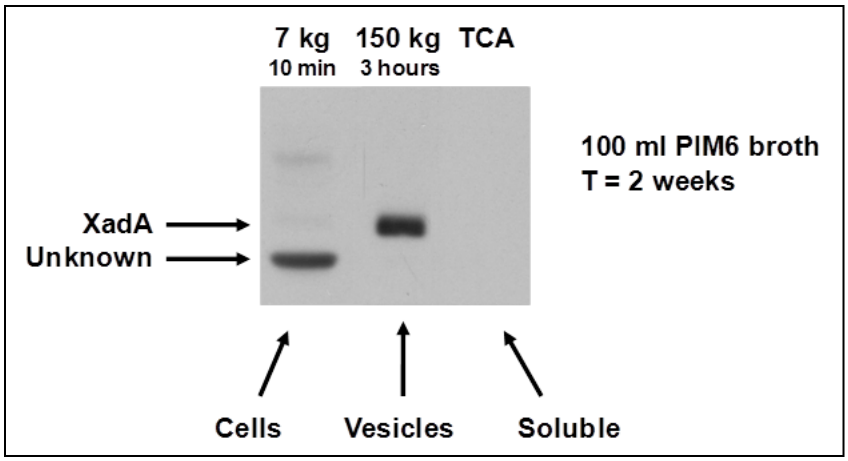

Figure 8. Quantification of the outer membrane protein XadA by Western blot analysis using anti-XadA antibodies from washed cells of *Xf* (left lane) as well as from material recovered from high-speed centrifugation of culture supernatants (center lane), or in proteins precipitated from culture supernatants after high-speed centrifugation by TCA (right lane).

Quantification of vesicles by both deconvolution fluorescence microscopy, flow cytometry, and a Nanovision particle counter reveals that the higher number of vesicles (normalized for the number of *Xf* cells) are present in RpfF mutants, suggesting strongly that DSF accumulation suppresses the release of such vesicles. The *rpfF* mutant produces as much as three times more vesicles (**Figure 9**). While some vesicles were as large as approximately 1,000 nm, the average diameter of vesicles was only approximately 150 nm (**Figure 9**). We can estimate that each *Xf* cell has shed approximately 100 to 1,000 vesicles of different sizes. It is thus clear that vesicles constitute a major extracellular factor produced by *Xf*.

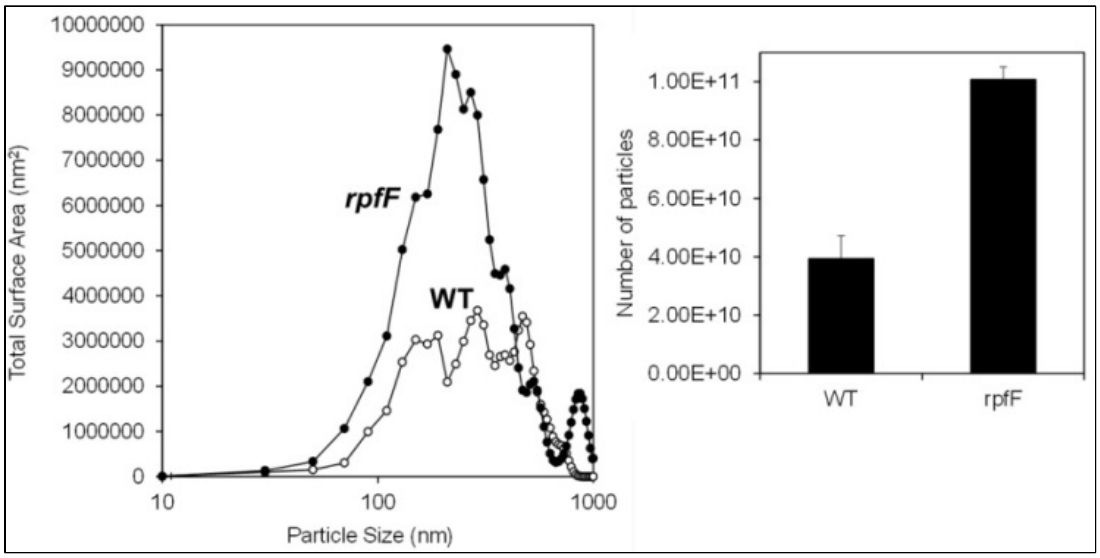

Figure 9. Distribution of sizes and abundance of vesicles of different sizes produced by a wild-type and an *rpfF* mutant of *Xf* when grown for two days in PIM6 minimal media when assessed with a Nanovision device. Vesicles were determined directly in culture supernatants after removal of whole cells by low-speed centrifugation. The absolute number of vesicles of all sizes are shown in the right-hand panel. The vertical bars represent the standard error of the estimate of the number of vesicles produced by a given strain.

Evidence was obtained that XadA, as an outer membrane protein of *Xf* also can act as an adhesin. To test this hypothesis the gene encoding XadA was cloned into an *E. coli* strain lacking strong surface adhesins. The comparative ability of this wild-type *E. coli* and *E. coli* overexpressing *Xf* XadA to adhere to glass surfaces was then assessed by quantifying the number of bounds cells by their ability to bind crystal violet. A much higher number of *E. coli* harboring XadA adhered to glass services, forming a biofilm than of *E. coli* itself.

Strong evidence has been obtained that *Xf* releases factors into the xylem fluids of plants that it is colonizing that prevents their adherence to surfaces. XadA that was not associated with intact cells of *Xf* (and hence associated with membranous vesicles), was readily detected in the xylem sap of plants infected with both the wild-type strain as well as an RpfF mutant of *Xf* (**Figure 10**). Substantially more vesicles were apparently present in the sap of plants infected with the RpfF mutant, consistent with our finding that such a mutant produces more membranous vesicles in culture.

Xylem sap containing membranous vesicles was shown to reduce the adherence of various bacteria to surfaces. Xylem fluid was collected by pressure bomb from healthy Thompson Seedless grape, as well as from plants infected with a wild-type strain of *Xf* or with an RpfF mutant of *Xf*. To test the differential adherence of wild-type cells of *Xf* to surfaces such as insects in the presence of these different sample collections, we immersed small sections of hindwings of the glassy-winged sharpshooter in each of these xylem sap samples to which we also added a wild-type strain of *Xf*. Many more cells that had been suspended in samples from healthy plants attached them from cells suspended in sap from plants infected with the wild-type *Xf* strain, particularly from sap infected with an RpfF mutant of *Xf* (**Figure 11**). Over 20-fold more bacterial cells were attached when suspended in sap from the healthy plant compared to that of sap from the plant infected with the RpfF mutant of *Xf* (**Figure 11**). It is thus clear that the xylem sap environment of plants infected with an RpfF mutant of *Xf* is much less conducive

to the adherence of *Xf* to surfaces than that of healthy plants. In fact, the sap environment of plants infected with the wild-type strain of *Xf* is also somewhat less conducive to adherence.

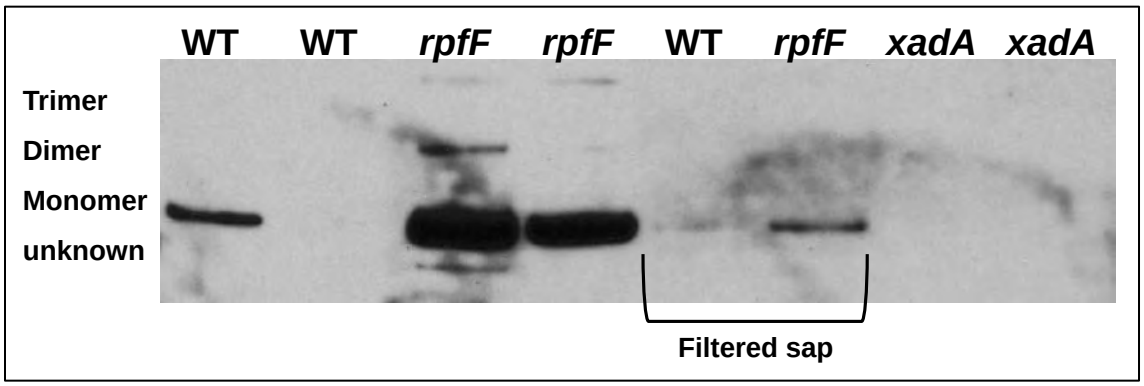

Figure 10. Abundance of XadA, indicative of the presence of membranous vesicles, as determined by western blot analysis in xylem sap of Thompson Seedless grapes infected with either a wild-type or an RpfF mutant of *Xf*. Total XadA in sap which had not been filtered to remove intact cells are shown in the leftmost four lanes, while that in sap that had been filtered to remove intact cells (retaining only membranous vesicles) are shown in lanes 5 and 6. Note the high abundance of XadA in filtered xylem sap, especially from plants infected with the RpfF mutant.

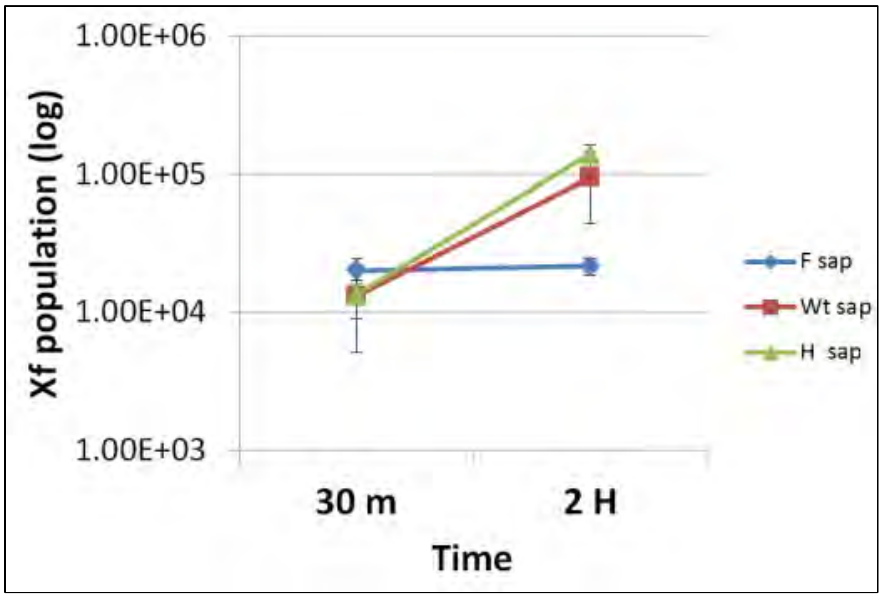

Figure 11. The number of cells of a wild-type strain of *Xf* that had adhered to wings of the glassy-winged sharpshooter suspended in xylem sap from plants infected with an RpfF mutant (blue), or a wild-type strain of *Xf* (red), or from healthy plants (green) after incubation for either 30 minutes or two hours.

CONCLUSIONS

We are very excited about results to date that show that several means of elevating DSF levels in plants have provided disease control via a strategy of "pathogen confusion." Given the limitations in standard methods of disease control, we are optimistic that DSF interference represents a promising strategy for Pierce's disease control. Control of Pierce's disease by direct application of DSF is a very attractive disease control strategy since it could be quickly implemented and would utilize commonly used agricultural equipment and methods and would not require the use of transgenic technologies. Our earlier work had shown that C14-cis, a component of *Xf*DSF, conferred some reduction of disease after topical application, but less than might have been expected compared to application of crude DSF-containing extracts of *Xf*. Our recent studies using improved *Xf*-based DSF biosensors more responsive to the DSF molecules made by *Xf* reveal that at least two additional molecules related

to C14-cis are biologically active in *Xf*, and DSF2 is much more active than C14-cis. Our new sensitive biosensors will be used to document the absorption and translocation of these molecules by grape after application in various ways. This should enable us to greatly increase disease control by direct application of the most appropriate molecule.

Strong circumstantial evidence suggests that the release of extracellular membranous vesicles by the RpfF mutant is responsible for the suppression of adherence of *Xf* to surfaces. Since the RpfF mutant of *Xf* does not accumulate DSF, which in turn suppresses the release of extracellular vesicles, a higher concentration of extracellular vesicles would be expected in plants infected with the RpfF mutant. At least some extracellular vesicles would also be expected in the sap of plants infected with the wild-type strain as well. A higher concentration of extracellular vesicles, as estimated by the abundance of XadA (which we can use as a marker protein for these membranous vesicles), is found in plants infected with the RpfF mutant of *Xf* compared to that of the wild-type strain. These results further support our model of a "Teflon mechanism" of virulence of *Xf* whereby it releases adhesive vesicles, especially one found at relatively low cell densities where DSF would not have accumulated. These vesicles would be expected to attach to surfaces such as that of the walls of the xylem vessels. By so attaching, these vesicles would prevent the attachment of *Xf* cells themselves to such surfaces. Only upon reaching relatively high cell concentrations in a particular vessel would DSF concentrations increase to a level that would suppressed the release of the membranous vesicles, thereby retaining adhesive molecules on the surface of *Xf* cells themselves, thus allowing the bacterial cells themselves to attach to surfaces, such as that of insect vectors. The presumptive anti-adhesive factor apparently plays a major role in facilitating the movement of *Xf* throughout the plant, and further work to elucidate its nature and contributions to this process are warranted.

FUNDING AGENCIES
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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 that causes disease in a variety of economically important agricultural crops including Pierce's disease of grapevine. *Xf* biofilms formed in the xylem vessels of plants play a key role in early colonization and pathogenicity by providing a protected niche and enhanced cell survival. Biofilm formation is induced by the process of quorum sensing and may be mediated by two-component regulatory systems. Like many other bacteria, *Xf* possesses homologs to the two component regulatory system PhoP/Q and ColR/S. PhoP/Q differentially regulates genes in responses to divalent periplasmic cation concentration and other environmental stimuli while ColR/S has been shown to play a role in membrane regulation. *Xf* knockout mutants deficient in the production of PhoP and PhoQ exhibit phenotypic differences in cell dispersal and clumping when grown in liquid culture. *Xf phoP/Q* mutants had a 42% and 47% reduction in biofilm formation, and a 42% and 36% reduction in cell-cell aggregation, respectively. Grapevine pathogenicity assays showed *phoP/Q* mutants and *colS* are non-pathogenic and significantly hindered in colonization or movement within the xylem vessels. These results may be due to the inability of *Xf* to successfully sense, respond and adapt to the nutrient-limited environment of the xylem.

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, forming a protective niche in which the bacteria can reproduce and subsequently block the flow of water in the grapevine. In many plant pathogenic bacteria, biofilm formation plays a key role in virulence. The production of biofilm is regulated by quorum sensing system, in which bacteria communicate with one another via small molecular weight compounds. Quorum sensing is important for the ability of an organism to transition between various lifestyles. Furthermore, complex regulatory networks, such as two-component systems, play an essential role in microbial response to changes in the environment. This research has explored the role of two different two-component systems in basic processes such as biofilm formation and cell aggregation. The role of these systems in disease development has also been explored. We found that the systems investigated play an important role in the colonization of grapevines and subsequent disease development.

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* forms aggregates in xylem vessels, which leads to the blockage of xylem sap movement. 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). Biofilm formation induced by QS is essential for survival and pathogenicity and may be regulated through a two-component regulatory system (TCS). TCS's are signal transduction systems through which bacteria are able to respond to environmental stimuli (Hoch, 2000). The TCS is comprised of a

histidine kinase, responsible for sensing stimuli, and the response regulator, responsible for mediating gene expression (Charles et al, 1992).

The PhoP/Q TCS is a well-studied and highly conserved TCS responsible for regulation of genes involved in virulence, adaptation to environments with limiting Mg^{2+} and Ca^{2+}, and regulation of other genes. PhoQ is a transmembrane histidine kinase protein with a long C-terminal tail residing in the cytoplasm. The periplasmic domain of PhoQ is involved in sensing of Mg^{2+}, Ca^{2+}, and antimicrobial peptides. The cytoplasmic domain contains a histidine residue that is autophosphorylated when physiological signals are detected in the periplasm. The PhoP/Q TCS is a phosphotransfer signal transduction system and upon activation by environmental stimuli, PhoQ phosphorylates the corresponding response regulator PhoP. In most bacteria, environments high in Mg^{2+} inhibit the PhoP/Q system through dephosphorylation of PhoP (Groisman, 2001). The ColR/S TCS has not been as extensively characterized as the PhoP/Q TCS but, as a regulatory network, is similar to PhoP/Q in that it is also a phosphotransfer TCS. It is involved, in general, in regulation of membrane processes including membrane permeability and tolerance to various compounds (Kivistik et al., 2006; Kivistik et al., 2009).

OBJECTIVES

1. Determine the functional role of the *Xf* putatively secreted protein, PD1063.
2. Determine the functional role of the ColR/S two-component regulatory system in *Xf*.
3. Determine the functional role of the PhoP/PhoQ two-component regulatory system in *Xf*.

RESULTS AND DISCUSSION

Objective 1.

Based on cell growth, cell-cell aggregation, and biofilm production assays, we found some differences between the wild-type (WT) *Xf* and *XfΔ1063*. Based on cell growth assays, *XfΔ1063* grows to a lower population density than wild-type *Xf* (**Figure 12**). There was no significant difference in biofilm formation or cell aggregation between wild-type *Xf* fetzer and *Xf Δ1063* (**Figures 1 and 2**). Pathogenicity assays on Thompson Seedless grapevines were conducted in the greenhouse. We found similar levels of disease severity in *Xf Δ1063* and wild-type *Xf* 18 weeks post-inoculation (**Figure 3**) and no significant differences in colony titers isolated from the point of inoculation (POI) or 25cm above the POI (**Figure 4**).

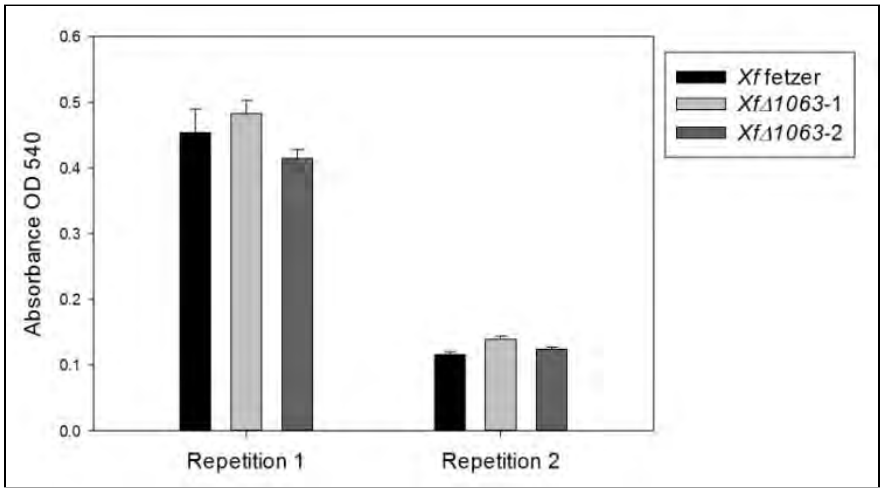

Figure 1. Comparison of biofilm formation in wild-type *Xf* fetzer, *XfΔ1063*-A, and *XfΔ1063*-B in stationary cultures as determined by the crystal violet staining method. Values shown are the means of 10 samples +/- standard error.

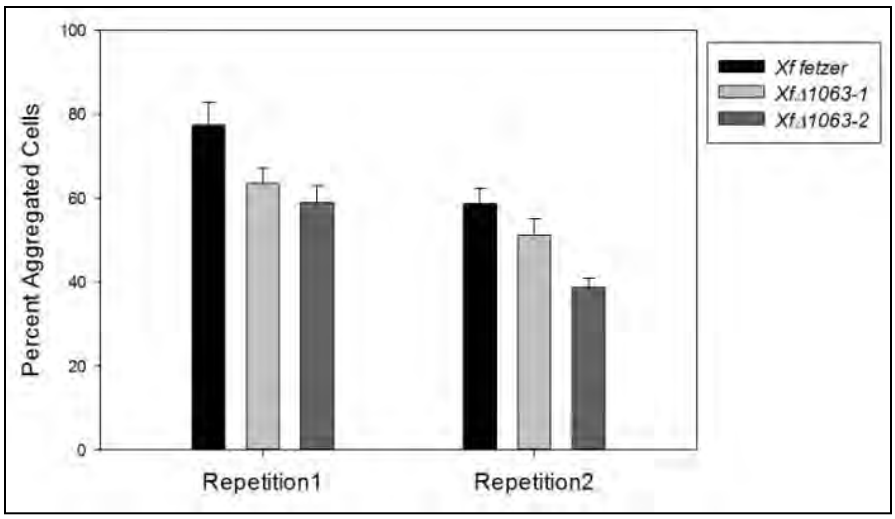

Figure 2. Comparison of percent aggregated cells in wild-type *Xf* fetzer, *XfΔ1063*-A, and *XfΔ1063*-B. Percentage of aggregated cells was determined as described by Guilhabert and Kirkpatrick, 2005. Values shown are the means of 10 samples +/- standard error.

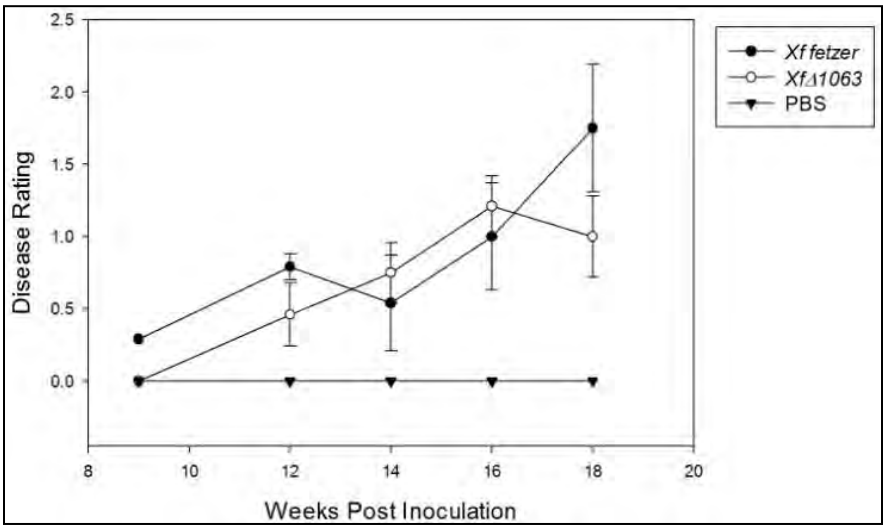

Figure 3. Disease ratings of Thompson Seedless grapevines inoculated with wild-type *Xf* fetzer, *XfD1063*, and PBS (negative control) 18 weeks post-inoculation. Values shown are the mean +/- the standard error.

To further our understanding of PD1063, we did experiments to localize the protein. Anti-PD1063 monoclonal antibodies were made and used for these studies. We have found, thus far, that PD1063 is secreted in wild-type *Xf* and not secreted in the *XfΔ1063* mutant (**Figure 5**). We are in the process of looking at *XfΔtolC* mutants to determine whether the type one secretion system mediates secretion of PD1063 (Reddy et al., 2007). We have recently discovered that the *Xoo* orthology of *XfΔ1063* is not secreted through the type I system as previously hypothesized (Lee et al., 2013; Han et al., 2013). We have now shown that the leader sequence of this protein is processed by the general secretory system in *Xoo* and that it is secreted outside the cell as a component of outer membrane vesicles (in prep).

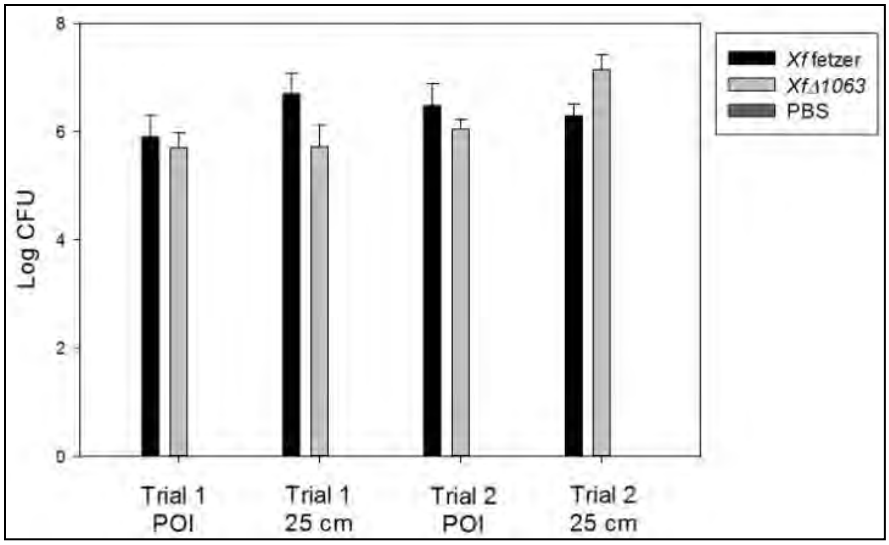

Figure 4. Log CFU isolated from Thompson Seedless grapevines inoculated with *Xf* fetzer, *XfΔ1063*, and PBS (negative control). Isolations from petioles at the POI or 25 cm above POI 18 weeks post-inoculation. Values shown are the mean +/- the standard error.

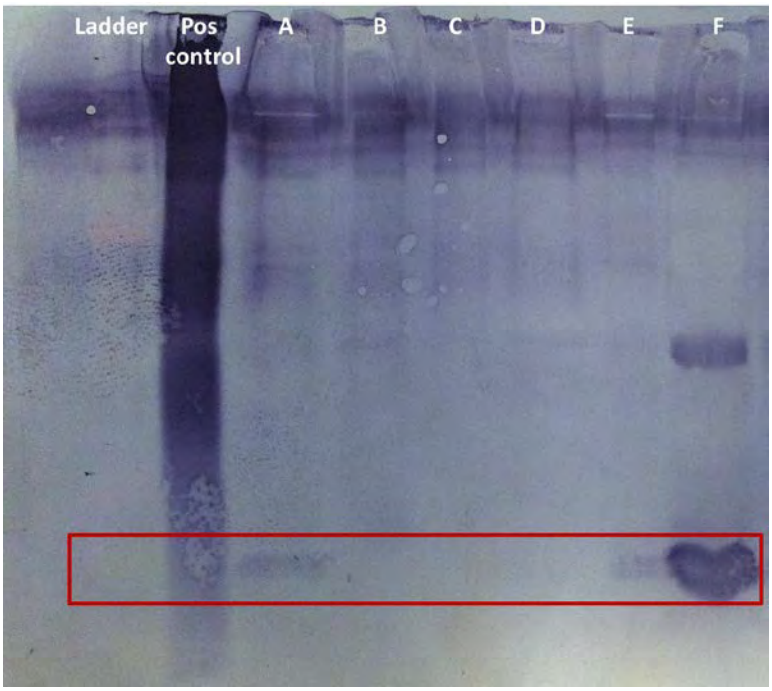

Figure 5. Western blot with anti-PD1063 antibodies on *Xf* secreted proteins from A) *Xf* fetzer, B) *XfΔ1063*, C & D) uninoculated media controls. Samples A-D were concentrated 25x. E & F are *Xf* fetzer concentrated 10x and 50x respectively. Positive bands can be seen at approximately 21 kD in lanes A, E, and F.

Objective 2.

We have begun characterization of the ColR/ColS TCS. ColR is the response regulator and ColS is the histidine kinase. Mutation of *colR* was lethal although the *XfΔcolS* was viable. TCS's can often be regulated by another TCS, thus based on ColR mutation being lethal for *Xf* while ColS mutation is not, the ColR response regulator is likely being regulated by another TCS. *XfΔcolS* exhibited no significant differences in biofilm formation (**Figure 6**) or cell growth but did exhibit a significant reduction in cell-cell aggregation (**Figure 7**). Pathogenicity assays found *XfΔcolS* caused no Pierce's disease symptoms when mechanically inoculated into Thompson

Seedless grapevines (**Figure 8**). Furthermore, *XfΔcolS* was only isolated from the POI in approximately 10% of plants (**Figure 9**). When *XfΔcolS* was recovered, populations were significantly lower than wild-type *Xf*.

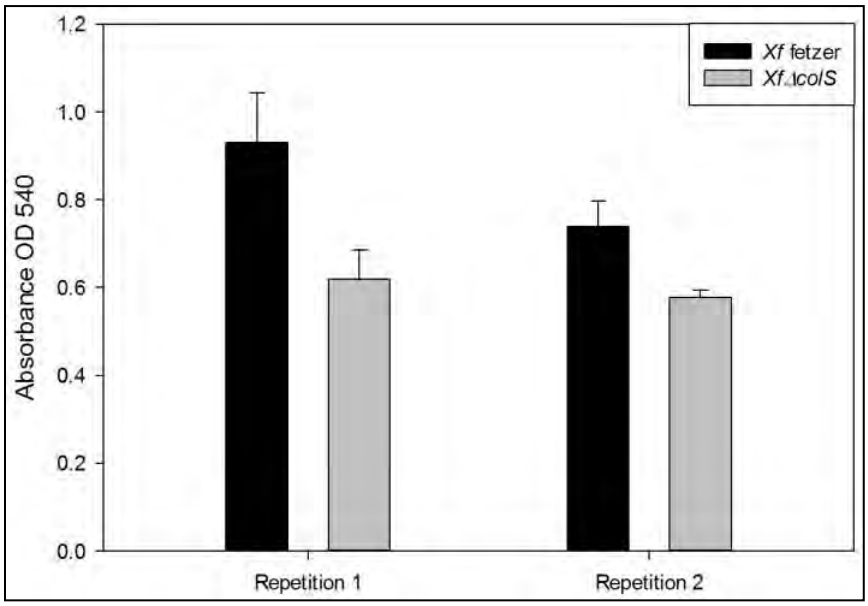

Figure 6. Comparison of biofilm formation by wild-type *Xf* and *XfΔcolS* after 10 days growth in static liquid culture. Values shown are the mean of five samples +/- the standard error.

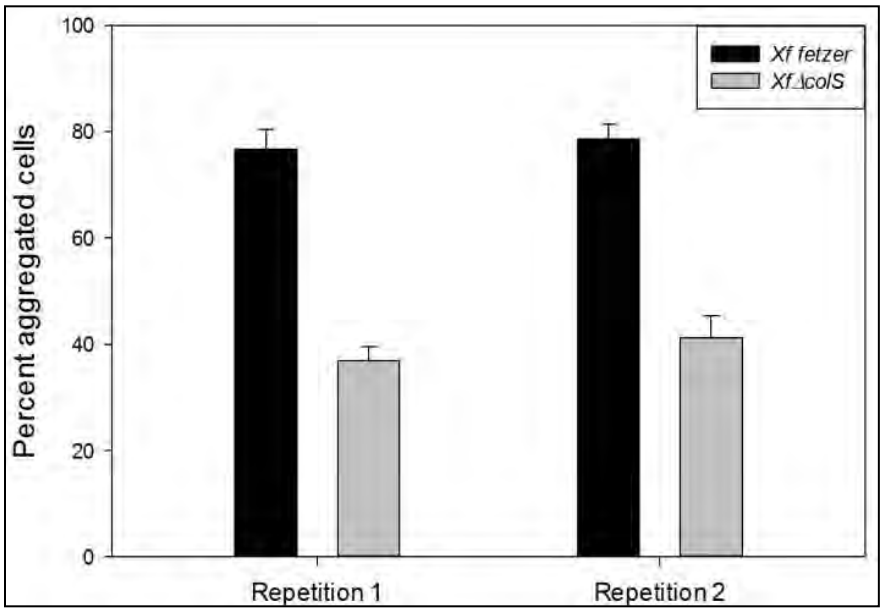

Figure 7. Comparison of percent aggregated cells by wild-type *Xf* and *XfΔcolS* after 10 days growth in static liquid culture. Values shown are the mean of five samples +/- the standard error.

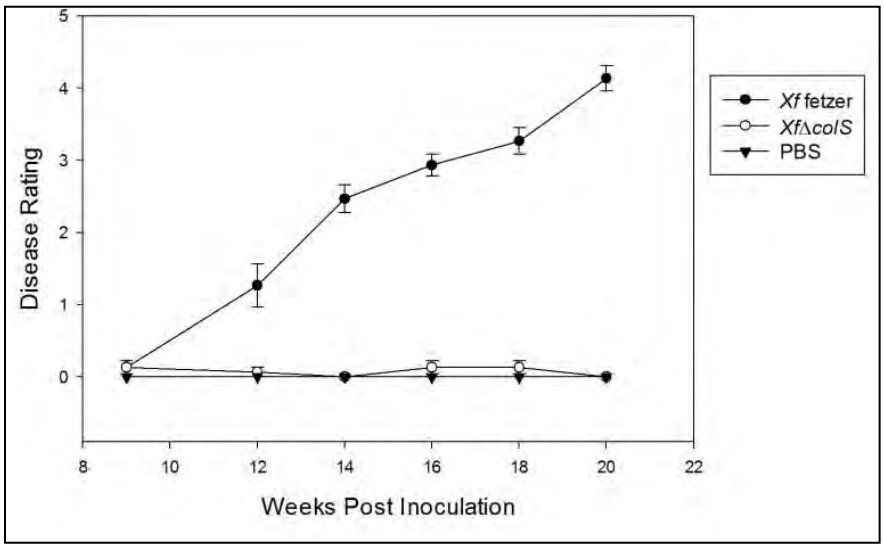

Figure 8. Disease ratings of Thompson Seedless grapevines inoculated with wild-type *Xf* fetzer, *XfΔcolS*, and PBS (negative control) 20 weeks post-inoculation. Values shown are the mean +/- the standard error.

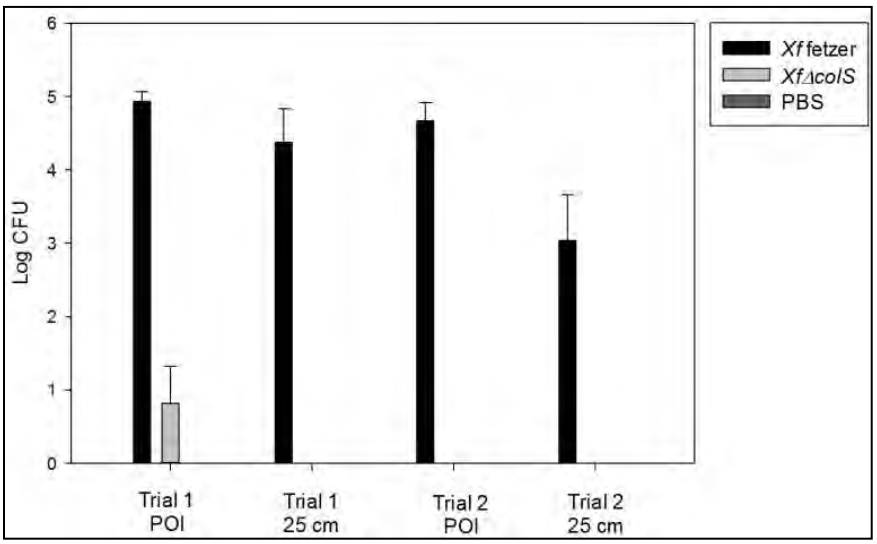

Figure 9. Log CFU isolated from Thompson Seedless grapevines inoculated with wild-type *Xf* fetzer, *XfΔcolS*, and PBS (negative control) 20 weeks post-inoculation. Isolations from petioles at the POI or 25 cm above the POI. Values shown are the mean +/- the standard error.

Objective 3.

Both *Xf*Δ*phoP* and *Xf*Δ*phoQ* were found to have significantly less biofilm formation than wild-type *Xf* after ten days static incubation using the crystal violet assay (**Figure 10**). In addition *Xf*Δ*phoP* and *Xf*Δ*phoQ* had significantly less cell-aggregation (**Figure 11**). We also found that there was no significant difference in biofilm formation or cell-cell aggregation between *Xf*Δ*phoP* and *Xf*Δ*phoQ*. Results of biofilm formation and cell aggregation found with mutated PhoP and PhoQ would be expected, since PhoP and PhoQ collectively make up a TCS. A mutant deficient in one gene should exhibit the same phenotype as a mutant deficient in the second gene of the TCS. When grown in culture, *Xf*Δ*phoP* and *Xf*Δ*phoQ* exhibited a dispersed phenotype, as indicated by the cell-cell aggregation assay, although there was no significant difference from wild-type in total cell growth (**Figure 12**). In order to determine if the dispersed growth phenotype was caused by a lack of hemagglutinin proteins, which are known to mediate cell-cell clumping, secreted proteins were purified from *Xf* wild-type Fetzer, *XfΔ1063*, *XfΔphoP,* and *XfΔphoQ* cells (Guilhabert and Kirkpatrick 2005; Voegel et. al. 2010). Western blot

analysis confirmed the presence of *Xf* hemagglutinin A and B in wild-type *Xf*, *XfΔphoP*, *XfΔphoQ*, and *XfΔ1063*, thus the observed decrease in clumping is not due to lack of hemagglutinin (**Figure 13**).

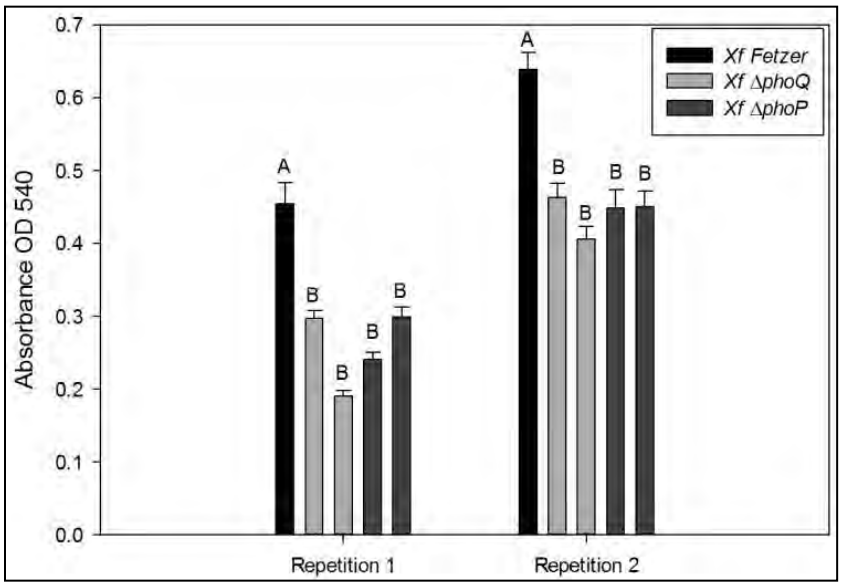

Figure 10. Comparison of biofilm formation by wild-type *Xf* fetzer, *XfΔphoP*, and *XfΔphoQ* mutants after 10 days growth in static liquid culture. Values shown are the mean of ten samples +/- the standard error.

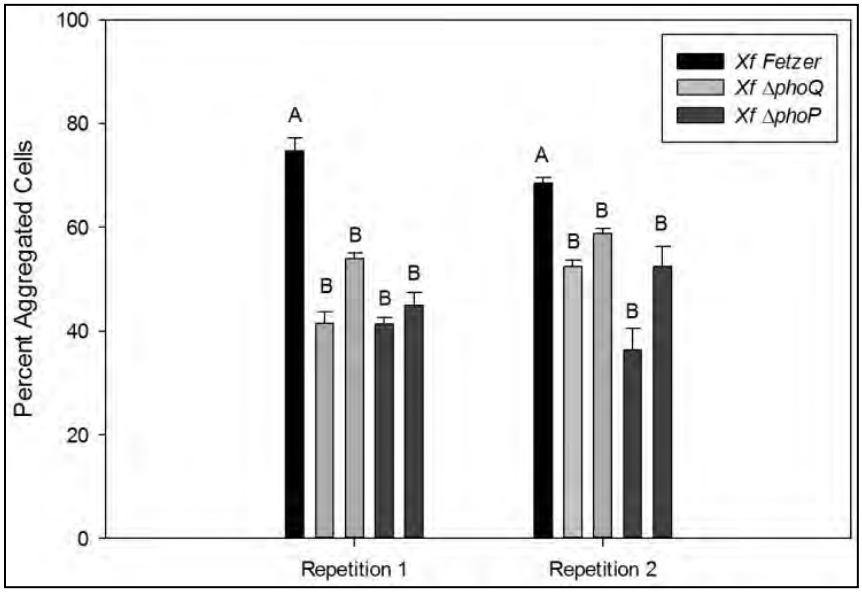

Figure 11. Comparison of cell aggregation by wild-type *Xf* fetzer, *XfΔphoP*, and *XfΔphoQ* mutants after 10 days growth in static liquid culture. Values shown are the mean of ten samples +/- the standard error.

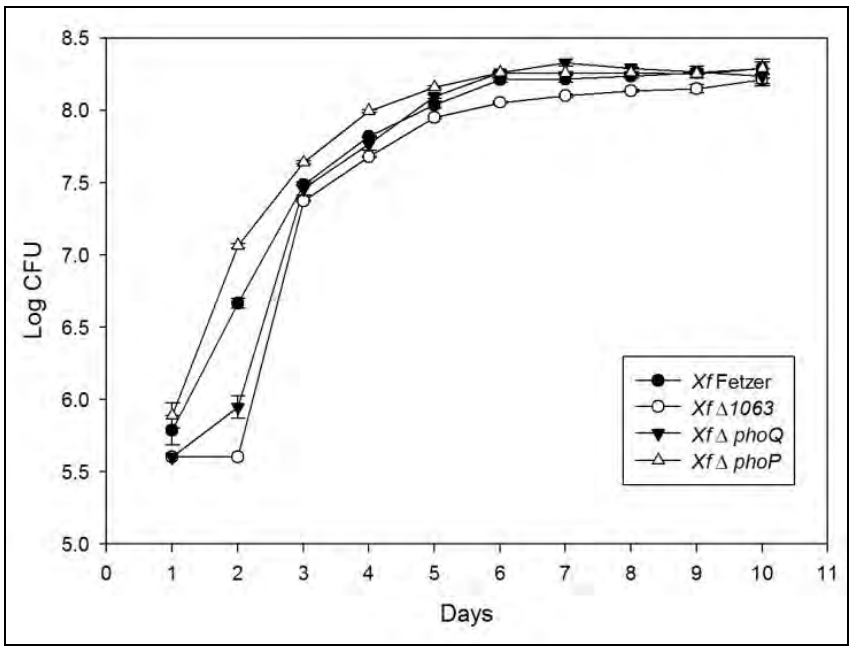

Figure 12. Bacterial growth of wild-type *Xf* fetzer, *XfΔ1063*, *XfΔphoQ*, and *XfΔphoP*. Values shown are the mean of five samples +/- the standard error.

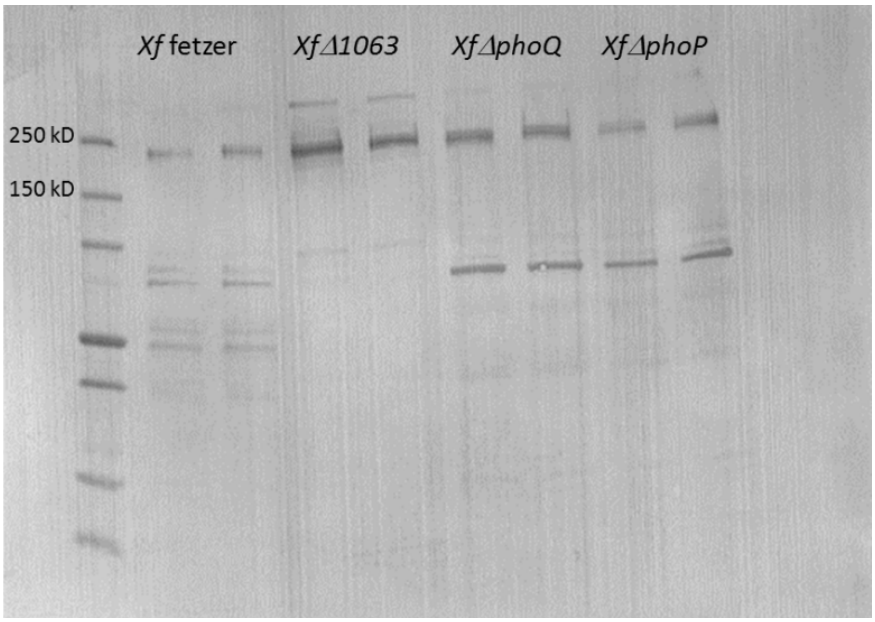

Figure 13. Western blot on type *Xf* fetzer, *XfΔ1063*, *XfΔphoQ*, and *XfΔphoP* with *Xf* hemagglutinin antibodies showing hemagglutinin bands at 225 kD.

Pathogenicity assays on Thompson Seedless grapevines were conducted in the greenhouse in 2011 and 2012. We found that no colonies could be isolated from the point of inoculation or 25cm above the point of inoculation (**Figure 14**). Furthermore, PhoP and PhoQ mutants produced no disease symptoms 20 weeks post-inoculation (**Figures 15 and 16**).

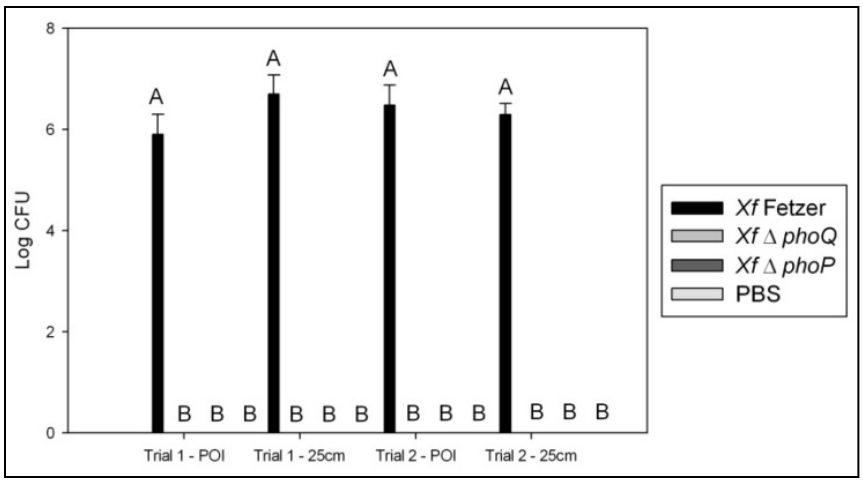

Figure 14. Log CFU isolated fromThompson Seedless grapevines inoculated with wild-type *Xf* fetzer, *Xf*Δ*phoQ*, *Xf*Δ*phoP*, and PBS (negative control) 18 weeks post-inoculation. Isolations from petioles at the POI or 25cm above the POI. Values shown are the mean +/- the standard error.

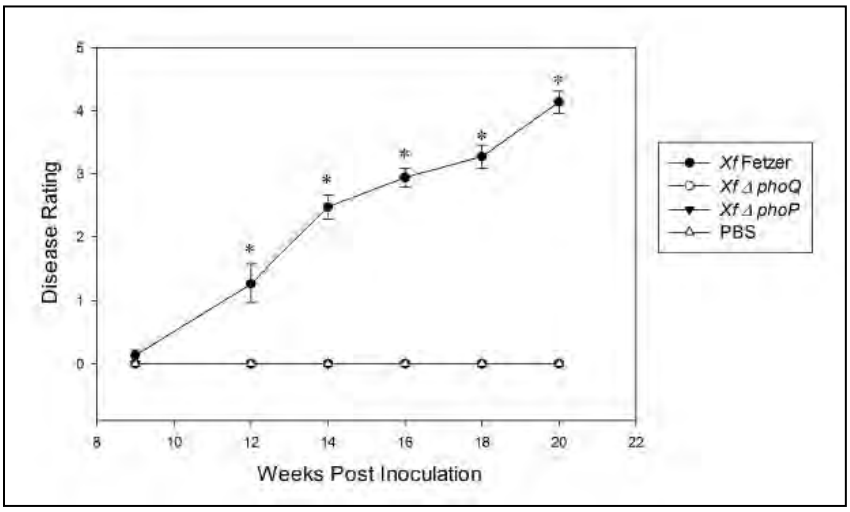

Figure 15. Disease ratings of Thompson Seedless grapevines inoculated with wild-type *Xf* fetzer, *Xf*Δ*phoQ*, *Xf*Δ*phoP*, and PBS (negative control) 10 weeks post-inoculation. Values shown are the mean +/- the standard error.

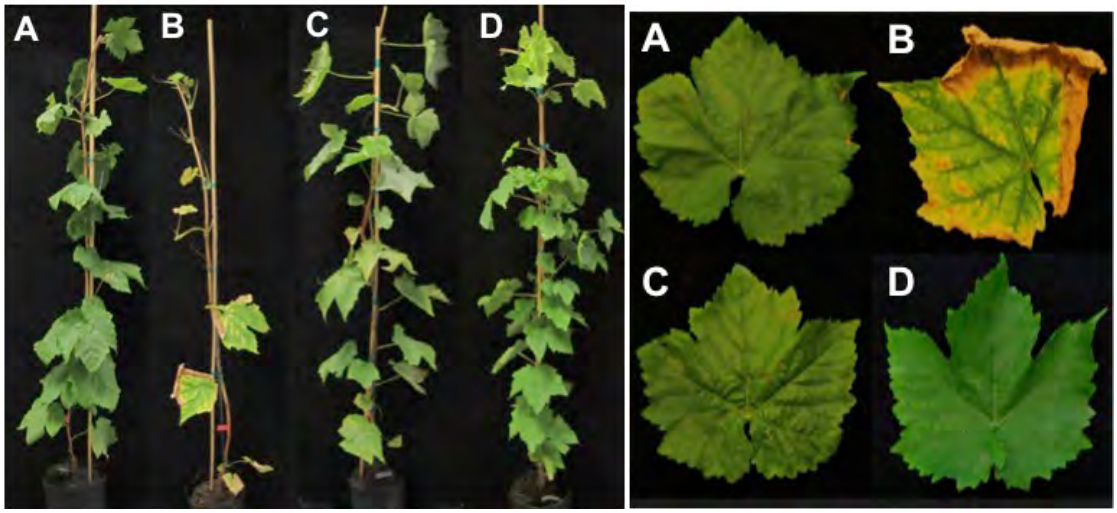

Figure 16. Representative grapevines from 2012 pathogenicity assays on Thompson Seedless grapevines inoculated with A) PBS (negative control), B) wild-type *Xf* fetzer, C) *Xf*Δ*phoQ*, D) *Xf*Δ*phoP* 20 weeks post-inoculation in the greenhouse.

CONCLUSIONS

We have made good progress on investigating the functional role of the hypothetical protein PD1063, ColR/S TCS, and the PhoP/Q TCS. We have found that the *Xf* PhoP/Q TCS plays an important role in normal physiological processes of *Xf* such as cell-cell aggregation and biofilm formation. Furthermore, PhoP/Q plays an essential role in adaptation and survival in the grapevine xylem. We are looking into strategies to inactivate PhoP/Q as a potential control for Pierce's disease. We are also further investigating the regulatory network of PhoP/Q in *Xf*. Similar to the case with the PhoP/Q mutants, mutation of the ColS protein results in significantly decreased survival in grapevines. The lethal mutation of ColR further indicates that the ColR/S TCS is involved in regulation of essential processes for *Xf* survival. In the future, we will exploit these two-component regulatory systems to develop novel Pierce's disease control strategies.

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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 October 2012 to September 2013.

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, which consists of lipid A, core oligosaccharides, and a variable O-antigen moiety. Specifically, the O-antigen portion has been implicated as a virulence factor in several other 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 insect acquisition and transmission.

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 (PD), which has caused major losses for the California grape industry. 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. Thus far, we have shown that certain components of the LPS molecule are essential for the *Xf* interaction with its host, which together with 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 alone or in combination with other anti-*Xf* compounds to control disease.

INTRODUCTION
Xylella fastidiosa (Xf) is a Gram-negative bacterium comprised of subspecies that cause disease on several different economically important crops, such as grapevine, almond, and oleander. We are currently exploring the role of lipopolysaccharide (LPS) as both a virulence factor in grapevine and its involvement in insect acquisition/ transmission. LPS is a structural component of the Gram-negative bacterial outer membrane and is primarily displayed on the outer surface of the cell, thereby mediating interactions between the bacterial cell wall and its environment. LPS is a tripartite glycolipid composed of Lipid A, an oligosaccharide core and O-antigen polysaccharide (32). Both Lipid A and the oligosaccharide core are highly conserved among all Gram-negative bacteria, whereas, the O-antigen can be varied even among subspecies. LPS has been implicated as a major virulence factor in both plant and animal pathogens, such as *Escherichia coli, Xanthomonas campestris* pv. *campestris*, and *Ralstonia solanacearum* (8, 12, 22). LPS can also contribute to the initial adhesion of the bacterial cell to a surface or host cell (10, 24). Additionally, host perception of LPS is well documented and occurs in both plants and animals (25). Host immune receptors can recognize several regions of the LPS structure and mount a defense response following bacterial invasion based on this recognition. Bacteria can also circumvent the host's immune system by altering the structure of the O-antigen moiety or by masking it with

capsular or exopolysaccharides.

We identified two genes, *waaL* (PD0077) and *wzy* (PD0814), both predicted to encode proteins required for production of a full O-antigen moiety in *Xf* (15, 30). We have, thus far, demonstrated that Wzy is an O-antigen polymerase that plays a role in chain length determination of the O-antigen. A mutation in *wzy* results in a truncated O-antigen polymer. Furthermore, glyclosyl composition and linkage analysis determined that deletion of Wzy results in a dramatic depletion of the 2-linked rhamnose-rich portion of the O-antigen (33). WaaL is a putative O-antigen ligase that ligates the preformed O-antigen onto the oligosaccharide core. Therefore, we predicted that mutants in *wzy* would produce a truncated O-antigen and that mutants in *waaL* would be completely lacking the O-antigen. However, despite a marked reduction in O-antigen accumulation, there is still some O-antigen present in the *waaL* mutant. This suggests to us that there may be a protein(s) that shares a redundant function with WaaL. Both mutant phenotypes were restored by introducing *waaL* or *wzy* into their respective mutant genomic backgrounds using the chromosomal complementation vector, pAX1Cm (19) (33).

Following the confirmation of the change in the O-antigen profiles for the *waaL* and *wzy* mutants, we then tested these strains for differences in virulence *in planta* (33). Temecula1 O-antigen mutant strains were inoculated into grapevine, cv. Thompson Seedless according to the method of Hill and Purcell (1995) (13). Plants were rated weekly on a disease index scale of 0-5, with 0 being healthy and 5 being dead or dying (11). Pierce's disease symptoms were detected four weeks after inoculation, similar to other greenhouse Pierce's disease virulence studies conducted in Riverside County. The Temecula1 *wzy* mutant was significantly delayed in causing Pierce's disease symptoms, and after 11 weeks of incubation, it did not cause the extensive Pierce's disease symptoms observed in plants inoculated with wild-type (as determined by an ANOVA analysis with Tukey's pairwise comparisons) (**Figure 1**) (33). **This indicates that depletion of the O-antigen correlates with a profound defect in *Xf* virulence.** We observed no difference in disease progress between the Temecula1 *waaL* mutant and the wild-type strain. Plants inoculated with 1X PBS buffer control did not develop any Pierce's disease symptoms.

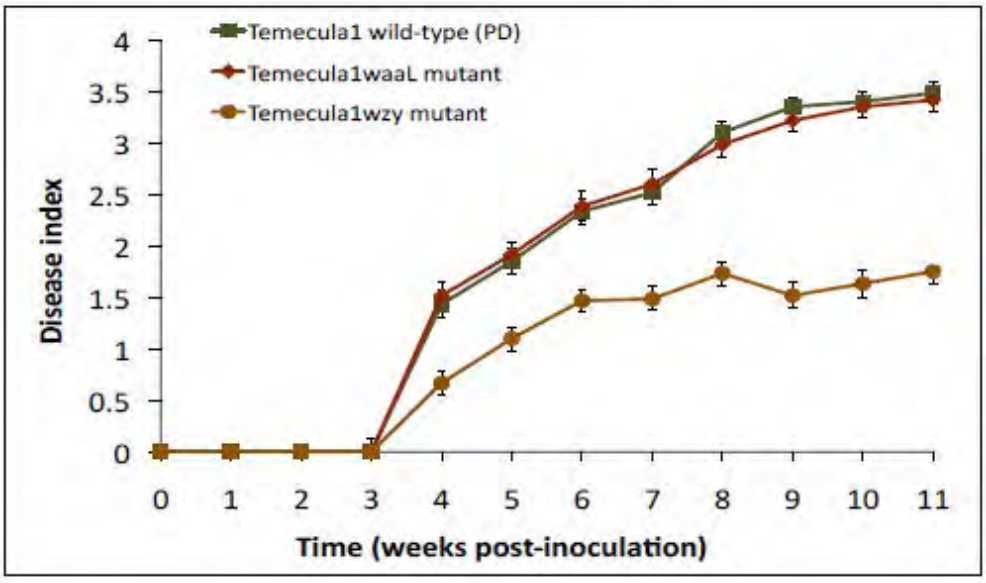


Figure 1. Disease progress of *Xf* Temecula1 wild-type and O-antigen mutant strains on grapevine cv. Thompson Seedless. The wzy mutant strain lags behind the wild-type in causing leaf scorching symptoms and does not cause wild-type levels of Pierce's disease throughout the disease progress curve. The waaL mutant is as virulent as the wild-type. Data are means of three independent assays with 10 replications each. Bars represent standard error.

In addition, we quantified bacterial population levels to assess any colonization differences between Temecula1 wild-type and the O-antigen mutant strains that may be occurring *in planta*. We isolated bacteria from surface-sterilized petioles at 13 weeks post-inoculation and found that plants inoculated with the *wzy* mutant harbored

significantly less bacteria than plants inoculated with wild-type Temecula1 (33). The *waaL* mutants colonized the plants to similar levels as wild-type *Xf*.

LPS is considered to be a Pathogen Associated Molecular Pattern (PAMP) that is recognized by the immune system of both plants and mammals causing the host to mount a defense response. One strategy that pathogens use to evade the host defense response is to mask the conserved portions of the LPS molecule (core and Lipid A) with a varying O-antigen. We speculate that the high molecular weight O-antigen chain in the *Xf* LPS molecule may serve to mask the rest of the LPS molecule from the host to avoid triggering a defense response, and the removal of this protective O-antigen may trigger the host defense response, resulting in reduced colonization and disease symptoms as observed for the *wzy* mutant. In a continuation of this project, we are gearing up to investigate the grapevine response to purified *Xf* LPS and LPS variants. This work will allow us to determine if LPS elicits an innate immune response by acting as a PAMP as it does in other systems. We will also determine if variations in the structure of the O-antigen affect the elicitation of the defense response. We are determining the effective LPS concentration and timing to study the immune responses and the Cantu lab has optimized a high throughput protocol for extraction of high quality RNA from woody material.

OBJECTIVES

1. Expand the characterization of the LPS profiles from the grape, almond, and oleander strains of *Xf*.
2. Test attachment and biofilm formation phenotypes of *Xf* O-antigen mutants to the biologically relevant substrates, chitin and cellulose.
3. Test O-antigen mutants for insect transmissibility.
4. Test O-antigen mutants for increased vulnerability to environmental stress and antimicrobial compounds.

RESULTS AND DISCUSSION

Objective 1. Expand the characterization of the LPS profiles from the grape, almond, and oleander strains of *Xf*.

A bacterium with only lipid A and core oligosaccharide is said to have "rough" LPS and one with lipid A, core oligosaccharide, and O-antigen is said to have "smooth" LPS. There can be both smooth and rough variants of the same bacterial species. We compared the LPS profiles of three *Xf* strains (Temecula1 (grape), M12 (almond), and Ann-1 (oleander) by Tris-Tricine polyacrylamide gel electrophoresis (PAGE). We hypothesized that there are strain specific differences among the variable O-antigen portion of the LPS molecules that contribute to the host specificity. We isolated LPS from all three *Xf* isolates using a hot-phenol extraction method (31). The extracted LPS preparations were then subjected to Tris-Tricine PAGE. These analyses confirmed that all three strains possess smooth LPS (ie. O-antigen). The gels also revealed small shifts in the molecular weights of the smooth LPS for each strain, indicating a fundamental difference among the O-antigen chain length or composition.

Host specificity plant assays.

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 (2). Moreover, *Xf* Ann-1 cannot cause disease in grape or almond and neither Temecula1 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. Furthermore, perhaps changes in O-antigen may enable Ann-1 to become a pathogen of grape or almond while M12 and Temecula1 strains become pathogens of oleander.

We inoculated Temecula1 wild-type and the Temecula O-antigen mutant strains into host (grape) and non-host (almond and oleander) plants. We observed that the grape isolate (Temecula1 wild-type) can colonize and cause disease in almond, albeit, to a lesser extent than the wild-type almond leaf scorch (ALS) isolate (M12), which is consistent with what other research groups have observed (1). Interestingly, the Temecula1 *waaL* mutant strain is **more virulent** in almond than its Temecula1 wild-type parent and is similarly virulent to the wild-type ALS isolate (M12). This suggests that an alteration in the O-antigen moiety of the LPS molecule does affect host specificity. The Temecula1 *wzy* mutant is similarly less virulent in almond as in grape. As expected, *Xf* Ann-1, the OLS isolate, does not cause scorch symptoms on almond (**Figure 2**).

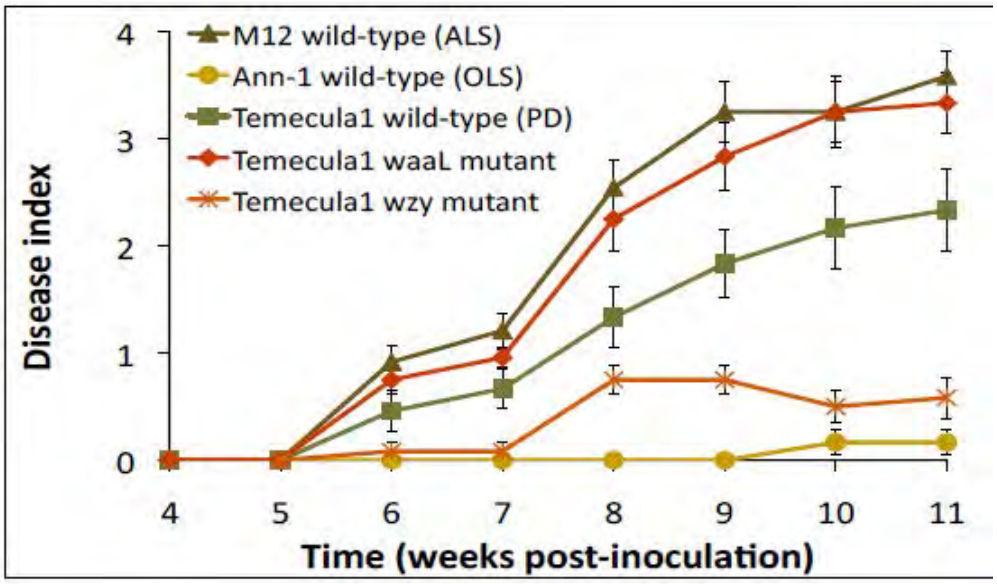


Figure 2. Disease progress of M12 (ALS), Ann-1 (OLS) Ann-1, Temecula1 (PD) isolates, and Temecula1 O-antigen mutants in almond cv. Sonora. Loss of WaaL increases virulence of a Pierce's disease isolate of *Xf* in almond. Data are means of two independent assays with six replications each. Bars represent standard error.

Objective 2. Test attachment and biofilm formation phenotypes of *Xf* O-antigen mutants to the biologically relevant substrates, chitin and cellulose.

Biofilm formation is an important component of the plant-microbe and plant-insect interaction. To test the role of LPS in *Xf* biofilm formation, we initially quantified the Temecula1 *waaL* and *wzy* mutant strains in two biofilm related behaviors: 1) surface attachment and 2) cell-cell aggregation. Both of these phenotypes are critical early steps in the formation of a mature biofilm. We hypothesized that LPS may contribute to these behaviors because of its location and abundance in the outer membrane. Glass is a hydrophilic surface and the polysaccharide components (ie. pectin, cellulose, hemicellulose) that make up the xylem primary cell wall are also highly hydrophilic. This similarity allows us to extrapolate our *in vitro* results to what might be occurring in portions of the plant xylem tissue that have an abundance of exposed primary cell wall polysaccharides, such as xylem pit membranes. Interestingly, when grown in glass tubes, the Temecula1 *wzy* mutant aggregated less, but attached more to a glass surface (**Figure 3**) (33). The *wzy* mutant was also significantly less virulent *in planta* which may be a result of its hyperattaching phenotype causing it to adhere more strongly to the xylem primary cell wall, which does not allow it to move as efficiently throughout the plant.

We performed a series of zeta potential measurements to determine if depletion of O-antigen correlated with a change in the net charge on the surface of the bacterial cell, which in turn could account for the differences in attachment and aggregation that we observed. Zeta potential is calculated based on the electrophoretic mobility of particles in a given solution. These values provide information about the net charge on the surface of a particle, in this case, a bacterial cell. The zeta potential was quantified for wild-type, *wzy* and *wzy/wzy+* mutant strains grown on solid PD3 medium. The surface of the *wzy* mutant was more negatively charged as indicated by an average zeta potential measurement of -27.1 mV compared to the zeta potential of wild-type at -10.5 mV (33). The ionic strength of PD3 medium was estimated to be 85 mM and the zeta potential of a glass microscope slide submerged in PD3 medium was estimated to be -12mV. Therefore, under the growth conditions tested here, it is logical that the more negatively-charged *wzy* mutant would adhere more strongly to the glass surface than the more positively-charged wild-type strain. Particles with lower zeta potentials tend to flocculate or aggregate, which explains the high capacity for *Xf* to aggregate in culture (7). However, if particles have a large negative or positive zeta potential this causes high repulsion among the particles, and will cause them to resist flocculation or aggregation. The *wzy* mutant has a significantly large negative zeta potential as compared to the wild-type, which explains the inability of this strain to aggregate *in vitro*.

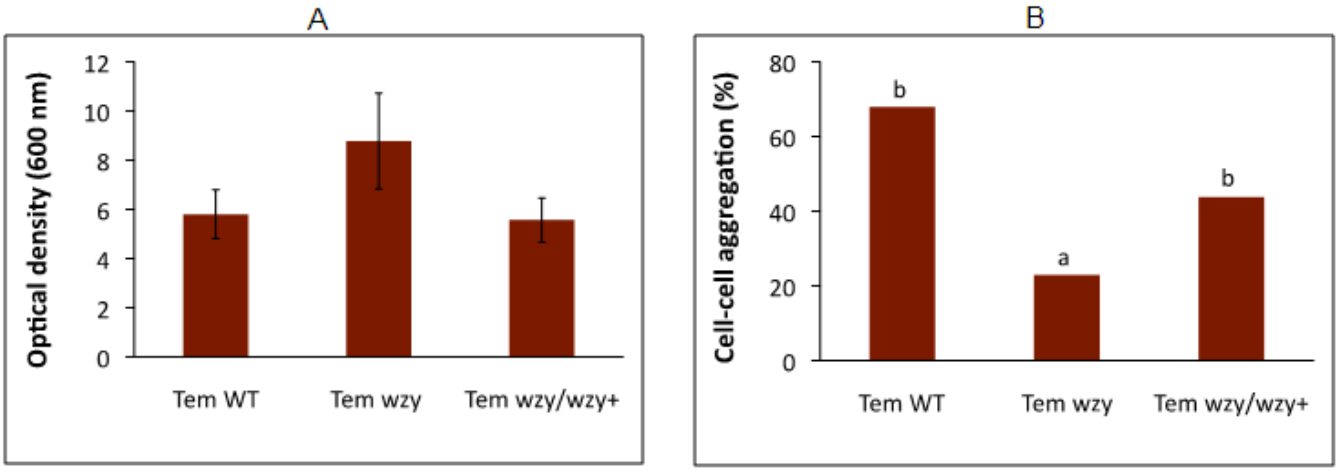
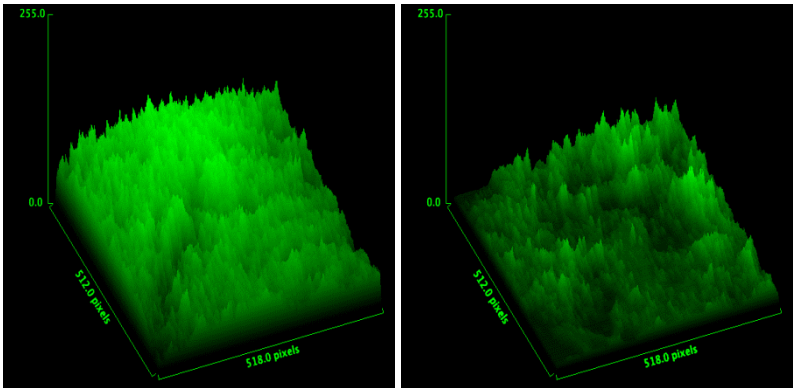


Figure 3. Surface attachment and cell-cell aggregation of the O-antigen mutant, wzy. **A**) The Temecula1wzy mutant cells attach to a solid surface to a greater extent than the wild-type parent. Attachment assays involved crystal violet staining of cells attached to a glass surface at the medium/air interface (2) after seven days of incubation at 28°C, 100 rpm. **B**) The Temecula1wzy mutant cells are reduced in the ability to aggregate to each other compared to the wild-type. Aggregation assays reflect the proportion of the total cell population that remains in culture after 10 days of static incubation (3). At least three independent assays were performed in triplicate. Bars represent standard error of the mean.

In addition, we tested the ability of the *wzy* mutant to form three-dimensional biofilms *in vitro*. Because the *wzy* mutant is impaired in cell-cell aggregation and hyperattaches to surfaces, we hypothesized that it would be impaired in biofilm formation. Both the wild-type and the *wzy* mutant were grown in liquid culture at 28°C for 2, 4, 6, or 8 days with constant shaking. A glass microscope slide was placed vertically into each tube, allowing biofilms to form at the air-liquid interface as previously described (27). Specimens were mounted in Slow Fade mounting fluid and observed using a Zeiss 510 Confocal Laser Scanning Microscope. As shown in **Figure 4**, *wzy* mutant biofilms were impaired in building the typical three-dimensional biofilm architecture (33). The *wzy* mutant was capable of attaching to the glass surface but was unable to build the towers characteristic of a wild-type biofilm. Moreover, the *wzy* biofilms were, on average, significantly thinner (120µm) than the wild-type biofilm (145µm), as determined by a student's t-test ($P<0.05$). Biofilm heterogeneity of the *wzy* mutant was also further examined using roughness coefficients. Biofilm surface roughness characterizes the variation in mean thickness. Higher roughness coefficients indicate a rough (or irregular) biofilm, while smaller coefficients indicate a smoother (or uniform) biofilm. Roughness coefficients were calculated according to the methods of Murga et al. (34) and revealed that the wild-type biofilms have a significantly smaller roughness coefficient (0.019) than the *wzy* mutant biofilms (0.133), further supporting that the *wzy* mutant is impaired in biofilm formation.

We also visualized biofilm formation of the Temecula1 wild-type and *wzy* mutant *in planta* using scanning electron microscopy. As shown in **Figure 5**, xylem vessels in *wzy*-inoculated plants were less occluded as compared with wild-type.

Lastly, we have initiated chitin attachment assays. We are currently using a technique developed by Killiny et al. (35) with slight modifications, that uses blue-green sharpshooter hindwings as a proxy for the chitinous surface of the insect foregut. Using this technique will allow us to better understand what role *Xf* O-antigen plays in surface attachment and biofilm formation within its insect hosts. Briefly, sharpshooter hindwings were removed and placed onto agar plates. 2µL droplets of *Xf* Temecula1 wild-type or *wzy* mutant were pipetted onto each hindwing. Following a six-hr incubation period, culture droplets were removed and wings were gently rinsed with distilled water. Bacterial populations attached to the hindwings were quantified using qPCR. Our preliminary data show that both the *wzy* and *waaL* mutants attach less to the chitinous surface of the hindwings.



Temecula1 wild-type *wzy* mutant

Figure 4. *In vitro* biofilm assay of the wild-type and the wzy mutant. Images are representations of three independent assays with three replications each.

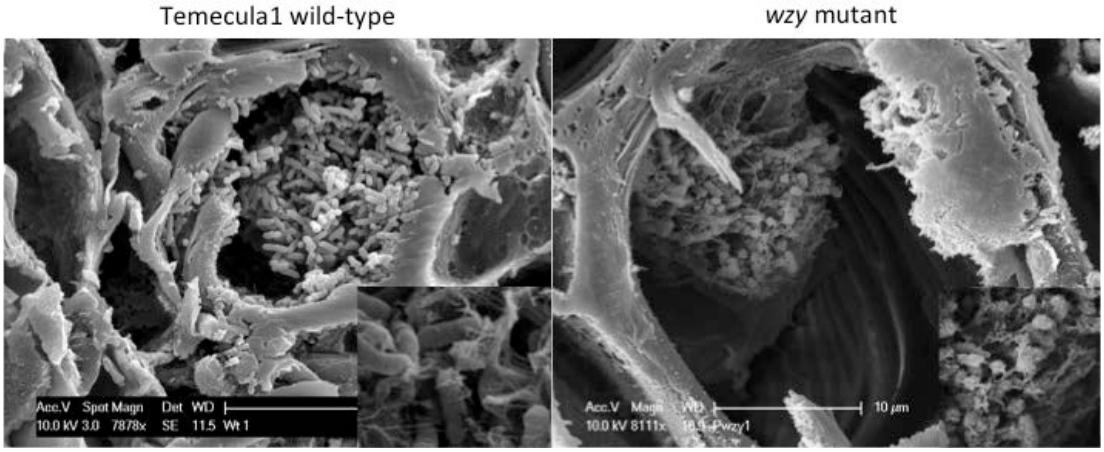


Figure 5. *In planta* biofilm formation of the wild-type and wzy mutant. Images are representations of three independent assays with three replications each.

Objective 3. Test O-antigen mutants for insect transmissibility.
In this objective, we are comparing sharpshooter transmission rates for wild-type versus either the *waaL* or *wzy* mutant. We are using the artificial feeding sachet technique developed by Killiny and Almeida (16). The artificial sachet technique is extremely useful because it allows us to normalize all strains to the same starting cell density in the individual feeding sachets, thereby avoiding any *in planta* multiplication differences.

In collaboration with Dr. Thomas Perring (UC Riverside, Dept. of Entomology), we have initiated a blue-green sharpshooter colony. We conducted insect acquisition studies using the artificial sachet technique, according to the methods of Killiny and Almeida (17). Insects were fed 35µl aliquots of bacterial suspension or diet solution only (negative control) and given an acquisition access period of six hours. Following the acquisition access period, insects were removed from the feeding sachets and placed onto clean basil plants for a clearing and multiplication period of 48 hours. Following this period, half of the insects were stored at -20°C for quantitative PCR (qPCR), and the other half were placed into glutaraldehyde fixative (4%) for examination of *Xf* colonization within the insect foreguts using scanning electron microscopy (SEM).

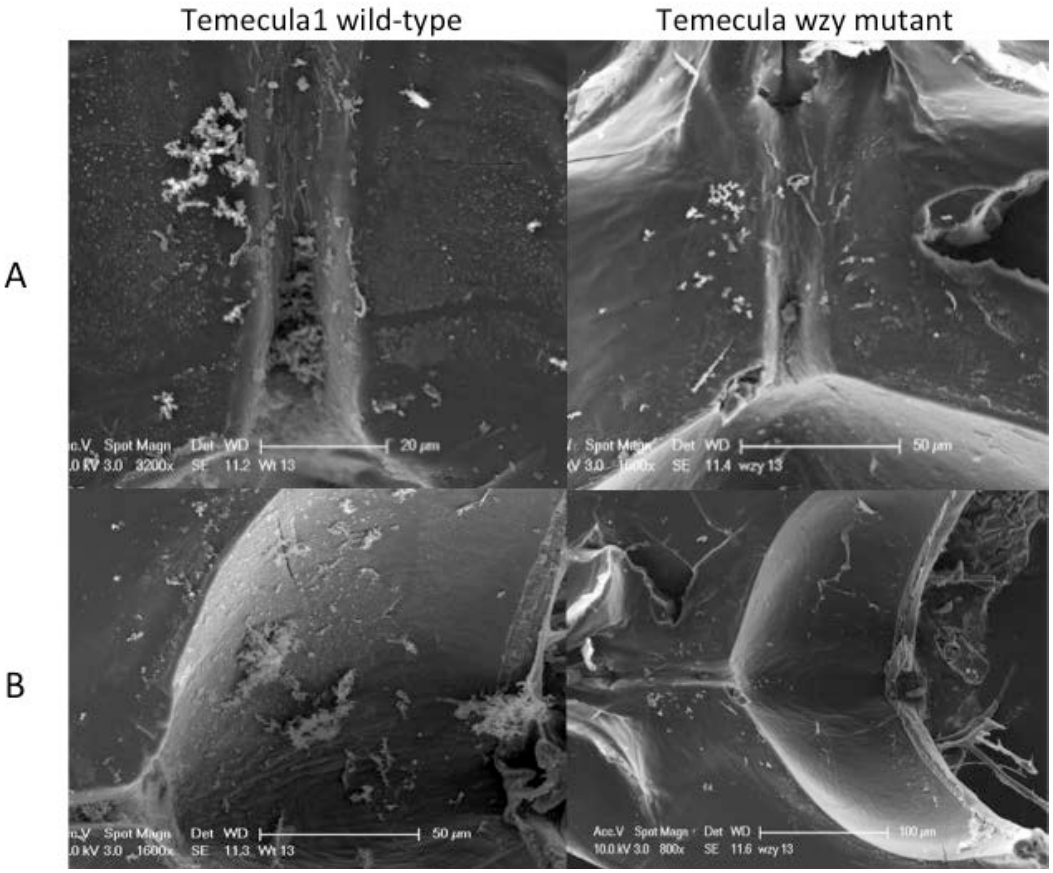


Figure 6. Scanning electron microscopy of sharpshooter foreguts. The *wzy* mutant was significantly impaired in colonization of sharpshooter foreguts. **A**) Close-up of colonization within the precibarial trough. **B**) Overview of colonization within the cibarium and parts of the precibarium. Images are representations of three independent assays with 10 replications each.

We have collected preliminary images of the insect foreguts using scanning electron microscopy. In brief, in collaboration with Dr. Elaine Backus (USDA ARS, Parlier), insects were prepped for SEM examination using a series of dehydrations, followed by critical point drying. Insect foreguts were dissected out from the heads and mounted onto copper tape attached to aluminum stubs. Stubs were sputter-coated in gold/palladium prior to analysis, and insects were imaged using the Philips XL30-FEG scanning electron microscope. As shown in **Figure 6**, the *wzy* mutant was significantly impaired in colonization within sharpshooter foreguts, while the Temecula1 wild-type readily attached and formed microcolonies. We are currently in the process of quantifying the bacterial cells within the sharpshooter foreguts using qPCR. Concomitant transmission studies were performed this summer by limiting fed sharpshooters onto healthy grapevine seedlings for an inoculation access period of four days. Seedlings are currently in the greenhouse and will be observed for symptom development over the coming weeks. Bacterial populations within infected plants and sharpshooter foreguts will also be quantified using qPCR.

Objective 4. Test O-antigen mutants for increased vulnerability to environmental stress and antimicrobial compounds.

The outer membrane of a bacterial cell is strongly influenced by its environment, and variation in the O-antigen portion of the LPS molecule can aid in the adaptation or tolerance to different environmental stresses such as oxidative stress, temperature, and resistance to antimicrobial peptides (3, 4, 23, 28). We are investigating if the absence or truncation of the O-antigen affects tolerance to environmental stress and antimicrobial compounds by testing the wild-type Temecula1, *waaL*, and *wzy* mutants for increased susceptibility to three environmental stresses: 1) oxidative stress, 2) cold temperature, and 3) treatment with antimicrobial peptides.

We rationalized that LPS is likely involved in resistance to oxidative stress because of its physical location in the outer leaflet of the bacterial cell membrane. During the plant infection process, bacteria encounter oxidative stress in the form of reactive oxygen species (ROS). ROS can be a product of the elicitation of the host defense

response or a by-product of normal plant metabolism and development (25). In any case, oxidative stress is detrimental to the bacterial cell, and the cells must have a mechanism to cope with this environmental insult. We hypothesize that LPS, in particular the high molecular weight O-antigen chain, provides some protective effect to the cells and that the *waaL* and *wzy* mutants will be more vulnerable to ROS than the wild-type Temecula1 strain. We performed a simple disc inhibition assay protocol as previously described (20) to test sensitivity to peroxide. In brief, the wild-type Temecula1, *wzy*, and complemented strains were plated in PD3 top agar. A Whatman paper disk impregnated with 100 µM H_2O_2 was overlaid onto the top agar. Plates were incubated for seven days at 28ºC and observed for zones of inhibition around the disk containing the H_2O_2. The diameter of the zone of inhibition correlates with sensitivity to H_2O_2. We observed that the *wzy* mutant was more sensitive to peroxide treatment as compared to the Temecula1 wild-type (**Figure 7**). We are currently testing the *waaL* mutant for sensitivity to peroxide.

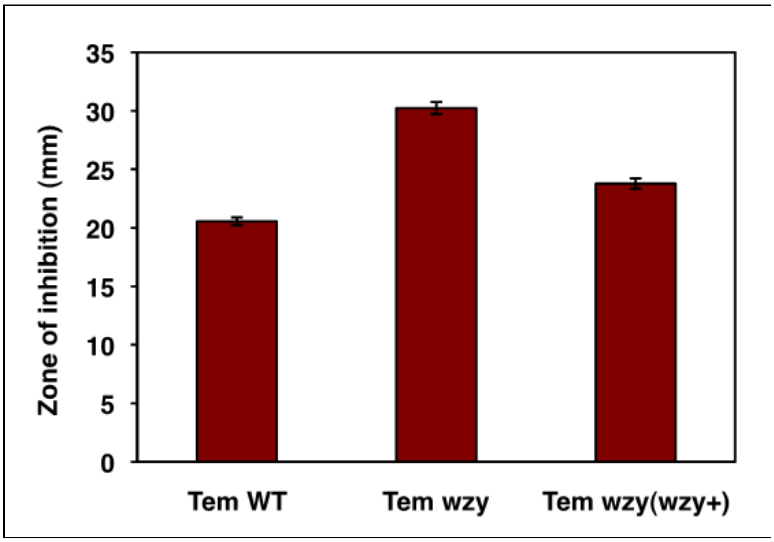


Figure 7. The role of O-antigen in the protection against oxidative stress. In the disk inhibition assay, the *Xf* Temecula1 *wzy* mutant strain was less tolerant of hydrogen peroxide stress than the wild-type parent or the complemented mutant strain. Three independent assays were performed in triplicate. Error bars represent the standard error of the mean.

CONCLUSIONS

We are working toward understanding the role of the O-antigen component of LPS in contributing to virulence and those behaviors associated with xylem colonization, such as host cell wall attachment and cell-cell aggregation required for biofilm formation. In addition, 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. Most importantly, we have demonstrated that O-antigen in an important factor in Pierce's disease development and host colonization and because of its abundance in the outer membrane, LPS may provide a target for disease control.

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INITIATION OF A PAN-GENOMIC RESEARCH PROJECT FOR *XYLELLA FASTIDIOSA*

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ABSTRACT
The variations in DNA material, together with differences in genomic structure and nucleotide polymorphisms among strains, form the genetic basis for phenomenal adaptability of a bacterial species. This can be described by a bacterial pan-genome, which is defined as the full complement of genes in all strains of a species. The pan-genome is typically composed by a "core genome," shared by all strains and characteristic of the species, and a "dispensable genome" that accounts for many of genotypic and phenotypic differences between strains. Sequencing a large number of strains from a single species is a major effort and an easiest way to study the bacterial pan-genome. The more genome sequences available, the more comprehensive a bacterial pan-genome is described. For *Xylella fastidiosa* (*Xf*), there are nine whole genome sequences available in public databases (Strains 9a5c, Temecula1, M12, M23, GB514, ATCC 35871, Dixon, EB92.1, and Ann-1). To enrich the pan-genome information, we have initiated a project to sequence at least ten more *Xf* strains from different geographical and host origins. In the past research period, whole genome sequencing was performed on two *Xf* strains: Griffin-1, an oak leaf scorch strain isolated from a symptomatic red oak tree, and Mus-1, a strain isolate from muscadine grape showing Pierce's disease symptoms. Both *Xf* strains were isolated from Georgia, USA. The genomes of the two *Xf* strains were sequenced using pure culture DNA. Efforts were made for gap closure. The two whole genome sequences were annotated and deposited in GenBank. The taxonomy status of the two *Xf* strains was established through multi-locus comparison. It is expected that the pan-genome research efforts will have important consequences for the way we understand bacterial evolution, adaptation, and population structure, as well as for more applied issues such as pathogen detection and identification.

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IDENTIFICATION AND CHARACTERIZATION OF *XYLELLA FASTIDIOSA* ISOLATED FROM COFFEE PLANTS IN FRANCE

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ABSTRACT

Xylella fastidiosa (*Xf*) has been occurring in the Americas where more than 200 plant species have been reported to be infected. Some of these plant species have a major economical impact as fruit crops (*Vitis vinifera, Prunus persica, Citrus sinensis, Coffea* spp., etc.), ornamentals (*Nerium oleander*, *Platanus occidentalis*, etc.) and forest trees (*Quercus* spp., *Ulmus* spp., etc.). *Xf* is a quarantine bacterium for the European Union (EU) listed on the directive 2000/29/EC. *Xf* has been absent from the EU territory. Nevertheless, a risk of introduction is still pending due to high number of imported plant hosts from contaminated areas and the occurrence of asymptomatic plants. In 2012, in France, the Plant Health Laboratory (Anses-LSV) detected by immunofluorescence four infected plants and isolated three strains from coffee plants (*Coffea arabica* et *C. canephora*) growing in a confined glasshouse. These plants were used as mother plants in breeding programs. This outbreak was eradicated (OEPP RS N° 8 2012/165).

Detection methods were set up based on PCR (Minsavage et al., 1994) and real time PCR (Harper et al., 2010) on various plant matrices such as coffee (*Coffea* spp.), grapevine (*Vitis vinifera*), and peach (*Prunus persica*) in order to provide efficient tools for imported plants test.

Strains isolated from coffee plants in the eradicated outbreak were characterized by multiplex PCR (Hernandez-Martinez R et al., 2007) and by multilocus sequence typing / multilocus sequence analysis (MLST / MLSA) on seven housekeeping genes. The three strains isolated from the coffee plants were placed in a phylogenetic tree constructed with a collection of strains representative of the diversity of *Xf*. Two strains isolated from *C. arabica* imported from South America were allocated to a new genetic lineage different from *Xf* subsp. *pauca* and one strain isolated from *C. canephora* imported from Central America was allocated to a phylogenetic group that includes *Xf* subsp. *fastidiosa* strains.

Section 4:

Pathogen and Disease Management

BUILDING A NEXT GENERATION CHIMERIC ANTIMICROBIAL PROTEIN TO PROVIDE ROOTSTOCK-MEDIATED RESISTANCE TO PIERCE'S DISEASE IN GRAPEVINES

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ABSTRACT

We are identifying grapevine-derived replacement components for both the surface recognition domain (SRD) and lytic domains (LD) of our neutrophil elastase - cecropin B (NE-CB) chimeric antimicrobial protein (CAP) that perform identical functions as the individual protein components of our current NE-CB CAP. Our ability to identify appropriate grapevine proteins is based upon comparing protein shapes (3D structures) using a recently described computational tool [Catalytic Active Site Prediction (CLASP), Chakraborty et al., 2011]. We used this tool to identify an appropriate grapevine protein based on the conformational similarity of its key structural features. We also compare 3D conformation of candidate proteins to that of human neutrophil elastase or the insect-derived cecropin B protein. Using the active site configuration of neutrophil elastase, we found a good match with the pathogenesis-related protein P14a from tomato. Based on the tomato sequence, we found the conserved protein in grapevines. We focused on the version of this gene in *Vitis shuttleworthii* (*Vs*), designated *Vs*P14a, as a good replacement for neutrophil elastase. The plant-expressed *Vs*P14a protein can lyse *Xylella* and inhibits growth of *Escherichia coli*. Using the same approach, we identified a potential protein in grapevine that has the same structure as cecropin B. Once we confirm comparable activity of both components, we will construct a synthetic gene encoding the chimeric antimicrobial protein, substituting both the neutrophil elastase and cecropin B domains. The new chimeric antimicrobial protein with plant/grapevine components will be incorporated into a binary vector and used for *Agrobacterium*-mediated transformation of grapevine rootstock and tobacco to confirm resistance to Pierce's disease using methods reported previously for neutrophil elastase - cecropin B constructs (Dandekar et al., 2012).

LAYPERSON SUMMARY

We have engineered transgenic grapevines that can protect themselves from Pierce's disease via the expression of the chimeric antimicrobial protein neutrophil elastase - cecropin B that kills the causative agent, *Xylella fastidiosa*. We build on that success in this project by seeking to identify grapevine components that are similar in structure to the human neutrophil elastase (NE) or insect cecropin B (CB) protein components. Since the 3D structural details of both NE and CB are known, we used a recently developed computational tool (Catalytic Active Site Prediction; CLASP) that identifies structurally similar proteins based upon specific structural features present on the surface of neutrophil elastase or cecropin B. We have identified a grapevine P14a protein as a replacement for neutrophil elastase. Like neutrophil elastase, it can inhibit or kill bacterial cells. We have identified a grapevine replacement candidate for cecropin B and are in the process of testing its efficacy. The goal is to replace the neutrophil elastase and cecropin B components with proteins from grapevine that perform the same function and to confirm resistance to Pierce's disease using the methods reported previously for neutrophil elastase - cecropin B constructs (Dandekar et al., 2012).

INTRODUCTION

Xylella fastidiosa (Xf), the causative agent of Pierce's disease, has a complex lifestyle requiring colonization of plant and insect. Its growth and developmental stages include virulence responses that stimulate its movement *in planta* and its ability to cause disease in grapevines (Chatterjee et al., 2008). Resistance to this pathogen must be multifaceted to block the pathogen at different stages of its complex lifestyle. A key issue is the reservoir of bacterial inoculum already present in California that poses an immediate threat in the presence of a significant insect vector like the glassy-winged sharpshooter (GWSS). Chemical pesticides are now used to suppress the GWSS population, which is effective but does not reduce this inoculum reservoir. Resistance mechanisms capable of degrading the reservoir could prevent further spread of the disease. It is critical to know whether any resistance mechanism under consideration can clear *Xf* and if so, by what mechanism. The resistance mechanism must limit spread and movement of the bacterium *in planta* and block transmission of the disease by insect vectors. We have previously shown that *Xf* exposed to xylem fluid from resistant lines expressing neutrophil elastase – cecropin B (NE-CB) shows significant mortality. Our group has successfully designed and tested a neutrophil elastase – cecropin B chimeric protein that specifically targets *Xf* in plant xylem (the site of infection), rapidly clears the pathogen, and blocks infection (Dandekar et al., 2009, 2012; Kunkel et al., 2007). The protein contains two separate domains. A surface-binding domain recognizes outer membrane proteins; we have previously shown that it recognizes and cleaves mopB, a major *Xf* outer membrane protein (Dandekar et al., 2012). This surface-binding domain is encoded by a synthetic gene derived from the human innate defense protein neutrophil elastase (Dandekar et al., 2012; Kunkel et al., 2007). The second, cecropin B domain is a clearance domain, connected with a flexible linker to the C-terminal of neutrophil elastase. This domain is a synthetic gene that encodes an antimicrobial peptide, cecropin B, that specifically lyses Gram-negative bacteria like *Xf* (Andrès and Dimarcq, 2007). The two domains work in tandem to recognize and lyse *Xf*. Our current hypothesis for the mode of action is that neutrophil elastase binds to the surface of *Xf* via its mopB outer membrane protein, bringing the cecropin peptide close to the bacterial surface where it can lyse and kill the pathogen. Transgenic expression of this protein in tobacco and grape has provided phenotypic evidence of bacterial clearance and biochemical evidence of mopB degradation by neutrophil elastase (Dandekar et al., 2012). A major concern is that the presence of a protein of human origin in grapevines is potentially controversial to groups opposed to genetically-modified organisms (GMOs). Therefore, substituting neutrophil elastase and cecropin B proteins derived from plants, ideally from grapevine, would be less controversial.

OBJECTIVES

The goal of this project is to redesign the neutrophil elastase – cecropin B chimeric antimicrobial protein (CAP) with a plant elastase and plant-derived lytic domain and test its efficacy to combat Pierce's disease in transgenic tobacco and grapevines.

1. Redesign the chimeric antimicrobial protein by substituting a plant counterpart [plant elastase (PE)] for the human neutrophil elastase component and demonstrate its efficacy for bacterial clearance.
    a. Activity 1. Identify a suitable plant elastase candidate that is comparable to human neutrophil elastase in active site structure using the Catalytic Active Site Prediction (CLASP) computational tool.
    b. Activity 2. Construct vectors and test *in planta*-produced protein for efficacy in killing *Xf* in culture.
    c. Activity 3. Conduct *in planta* efficacy testing: construct binary vectors (plant elastase – cecropin B), transform grapevine and tobacco, and test transgenic plants for clearance of *Xf* and resistance to Pierce's disease symptoms.
2. Redesign the chimeric antimicrobial protein by substituting a plant/grapevine counterpart [plant lytic domain (PLD)] for the insect-derived cecropin B component in the lytic domain and demonstrate its efficacy for bacterial clearance.
    a. Activity 4. Identify a suitable plant lytic domain candidate that is comparable to insect-derived cecropin B in primary and secondary structure using CLASP and other computational tools.
    b. Activity 5. Test the synthetic plant lytic domain protein for efficacy in killing *Xf* in culture.
    c. Activity 6. Conduct *in planta* efficacy testing of the grape-derived chimeric antimicrobial protein components using transient expression of *Vs*P14-LPD and *Vs*P14m-LPD.
3. Construct and test a fully plant-derived chimeric antimicrobial protein (plant elastase – plant lytic domain) and test its ability to confer resistance to Pierce's disease in grapevine rootstocks.
    a. Activity 7. Construct a plant elastase – plant lytic domain binary vector, transform grapevine and tobacco, and evaluate *Xf* resistance and Pierce's disease development.

RESULTS AND DISCUSSION

Objective 1. Redesign the chimeric antimicrobial protein by substituting a plant counterpart [plant elastase (PE)] for the human neutrophil elastase component and demonstrate its efficacy for bacterial clearance.

The strategy here was to identify a plant version of the surface-binding domain (PE) corresponding to the neutrophil elastase domain of our chimeric antimicrobial protein and to test the efficacy of this component outlined in the three activities associated with this objective.

Activity 1. Identify a suitable plant elastase candidate that is comparable to human neutrophil elastase in active site structure using the CLASP computational tool.

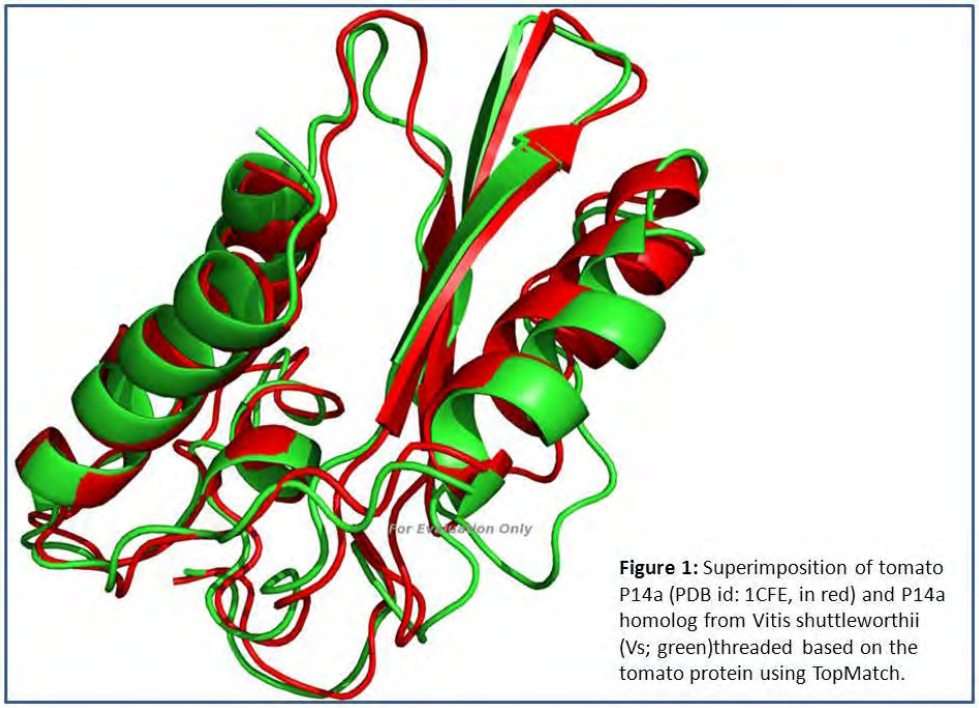

Figure 1: Superimposition of tomato P14a (PDB id: 1CFE, in red) and P14a homolog from Vitis shuttleworthii (Vs; green)threaded based on the tomato protein using TopMatch.

This activity has been successfully accomplished. A plant PE candidate protein was identified using the CLASP (Chakraborty et al., 2011) and PROMISE packages (http://www.sanchak.com/clasp/). The potential differences between reactive atoms in the active site of the target proteins were calculated using the Adaptive Posson-Boltzmann Solver (APBS) and PDB2PQR packages (Baker et al., 2001 and Dolinsky et al., 2004). The neutrophil elastase structure (1B0F) was chosen from Protein Data Bank (PDB). The elastase scaffold residues Ser195, His57, Asp102, Ser214, and Gly193 were chosen based on their known interaction with the elastase enzymatic function. The best plant candidate based on this analysis was a pathogenesis-related (PR) protein P14a from tomato (1CFE). Details of the protocol and workflow used to make this selection have been recently described (Chakraborty et al., 2013). The P14a from *Solanum lycopersicum* (tomato) is a promising choice since we found similar, highly conserved proteins from other plant species, including grapevine. Additionally, the PR superfamily is widely distributed in animals, plants, and fungi. Some of these related to cysteine-rich secretory proteins (CRISP) have demonstrated, substrate-specific protease activity (Milne et al., 2003). Furthermore, a striking structural homology was observed between P14a and a protein found in snake venom that has been demonstrated to be an elastase (Bernick and Simpson 1976). The structure of the 135 amino acid mature P14a protein from tomato was also determined using nuclear magnetic resonance (NMR) (Fernandez et al., 1997). Based on these considerations, we used the tomato P14a to find an appropriate P14a in *Vitis*. We chose, from several matching these criteria, the P14a from *Vitis shuttleworthii* (*Vs*), as this species is resistant to Pierce's disease (Walker, personal communication). More recently, 21 different PR-1 genes from grapevine, including those from *Vs*, were characterized and shown to confer resistance to bacterial disease (Li et al., 2011). Further analysis of the predicted active site of *Vs*P14a using CLASP indicated that removal of amino acids 74 and 77 had

the potential to improve elastase/protease activity. The sequence of *Vs*P14a without the two amino acids is designated *Vs*P14m.

Activity 2. Construct vectors and test *in planta*-produced protein for efficacy in killing *Xf* in culture.
To test the efficacy of *Vs*P14a and *Vs*P14m proteins in the clearing of *Xf*, we codon-optimized and chemically synthesized the two versions of *Vs*P14a after adding a 3xFlag purification tag (Sigma Aldrich). *Vs*P14a-3xFlag and *Vs*P14m-3xFlag genes were cloned into pEAQ-HT, a binary vector specific for transient protein expression system in the tobacco species *Nicotiana benthamiana* (Sainsbury et al., 2009) (**Figure 2A**). The constructed binary vectors were transformed into a super-virulent *Agrobacterium* strain (EHA105). As a negative control, an empty vector (pEAQ-HT) was also transformed into the same strain of *Agrobacterium*. Tobacco leaves were harvested from greenhouse-grown plants and vacuum-infiltrated with *Agrobacterium* suspensions containing *Vs*P14a and the empty vector. Agro-infiltration conditions were optimized as described earlier (Huang et al., 2009). The infiltrated leaves were harvested six days post-infiltration. Total protein was extracted using an apoplastic wash method to extract secreted proteins present in the leaf apoplast. Using Anti-Flag M2 antibody (Sigma), we detected *Vs*P14a on the first run. We tested the ability of the crude leaf protein extract from plants transformed with both constructs to inhibit growth of *Escherichia coli* (**Figure 2C**) and *Xylella* (**Figure 2D**). After four hours, the *E. coli* with the extract from the plant expressing the empty vector showed growth, while growth of those exposed to *Vs*P14A was completely inhibited (**Figure 2C**). Proteins *Vs*P14a and *Vs*P14m also inhibited the growth of *Xylella* while protein obtained from the empty vector did not. By 50 minutes, 50% of the population was killed, but mortality reached a plateau at ~ 60% mortality (**Figure 2D**). These results are encouraging and indicate that we may have found the desired replacement for neutrophil elastase. Now it must be tested *in planta* for protection against *Xf* infection. We also developed antibodies against *Vs*P14a and used these to detect the protein in the plants using Western blots. Proteins isolated from Thompson Seedless (TS) and *V. shuttleworthii* infected and not infected with *Xylella* showed that the P14a in *V. shuttleworthii* was expressed in both infected and uninfected tissues. In Thompson Seedless, it was only expressed in infected tissues and un-infected tissues expressed no protein corresponding to P14a (**Figure 2B**).

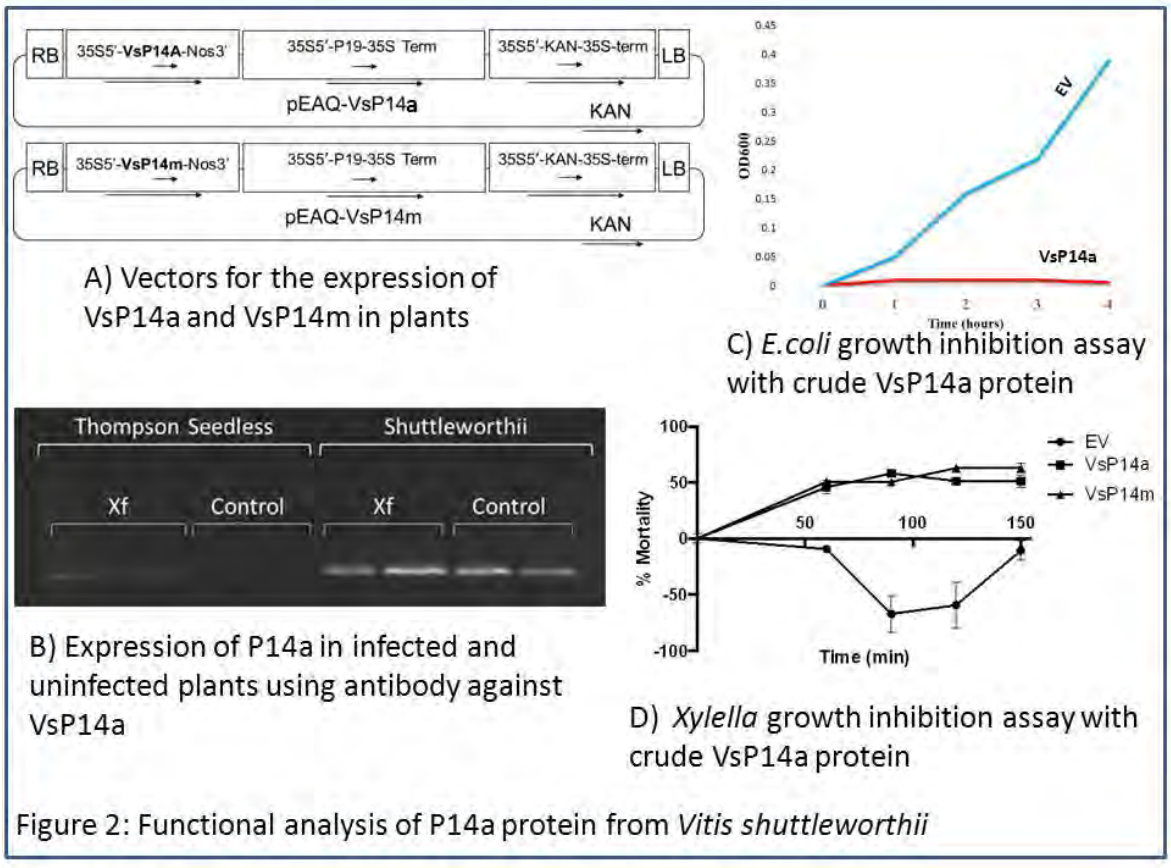

Figure 2: Functional analysis of P14a protein from *Vitis shuttleworthii*

Activity 3. Conduct *in planta* efficacy testing: construct binary vectors (plant elastase – cecropin B), transform grapevine and tobacco, and test transgenic plants for clearance of *Xf* and resistance to Pierce's disease symptoms.
We have initiated the construction of a binary vector for the expression of *Vs*P14a.

Objective 2. Redesign the chimeric antimicrobial protein by substituting a plant/grapevine counterpart [plant lytic domain (PLD)] for the insect-derived cecropin B component in the lytic domain and demonstrate its efficacy for bacterial clearance.

Activity 4. Identify a suitable plant lytic domain candidate that is comparable to insect-derived cecropin B in primary and secondary structure using CLASP and other computational tools.
We have used an approach similar to that described above in Activity 1 to identify a replacement component plant lytic domain for cecropin B. Our focus here is to identify a plant/grapevine version of the cecropin B lytic domain. We chose the structural motif Lys10, Lys11, Lys16, and Lys29 from cecropin B (PDBid:2IGR) (**Figure 3**). Our analysis has identified good candidates. It is noteworthy that several defense-like proteins feature in the list, allowing us to speculate that another peptide might be a good replacement for cecropin B in the chimera. We have evaluated eight protein candidates. We are now focusing on a 52 amino acid segment of the plasma membrane H^+ ATPase, whose structure matches very well with the cecropin B structure shown below (**Figure 3**).

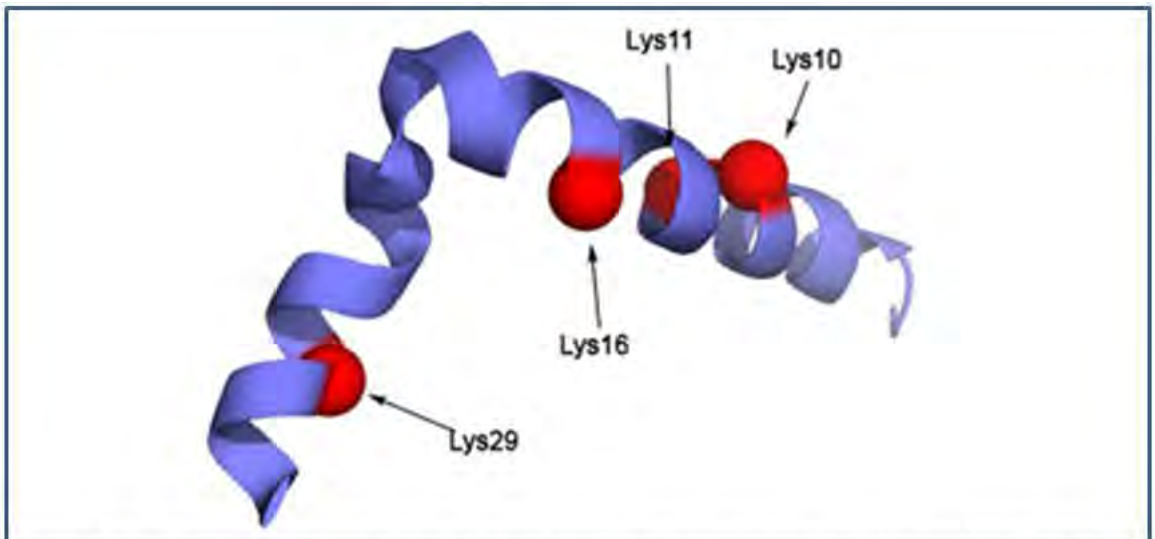

Figure 3: CecropinB structure (CB; PDBid: 2IGR) showing chosen motif (Lys10, Lys11, Lys16 and Lys29). A short list of 8 candidate proteins were identified as the best matches in a set of approximately 200 proteins identified when queried using the motif (Lys10, Lys11, Lys16 and Lys29) from CecropinB (PDBid:2IGR).

CONCLUSIONS
Using a novel computational tool called CLASP, we have identified two proteins in grapevine that can be used to replace the neutrophil elastase and cecropin B components in a chimeric antimicrobial protein designed to provide resistance to Pierce's disease. The *Vs*P14a protein replacement for neutrophil elastase was expressed in plants. This protein was isolated and shown to inhibit growth of *E. coli* and to cause lysis of *Xylella*. We have identified a potential candidate to replace the cecropin B domain, a 52 amino acid segment of a grapevine plasma membrane H^+ ATPase. We have begun designing vectors to test combinations of these two proteins to confer resistance in transgenic tobacco.

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

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Reporting Period: The results reported here are from work conducted March 2010 to September 2013.

ABSTRACT

We have successfully established two field plantings to investigate two greenhouse-tested strategies to control the movement of and to clear *Xylella fastidiosa* (*Xf*), a xylem-limited, Gram-negative bacterium that is the causative agent of Pierce's disease 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 the *Xf* polygalacturonase activity necessary for long distance movement. Our second strategy enhances clearance of bacteria from *Xf*-infected xylem tissues by expressing a chimeric antimicrobial protein, human neutrophil elastase – cecropin B (HNE-CecB). The expectation is that expressing these two proteins will prevent *Xf* movement and reduce *Xf* inoculum, curbing the spread of Pierce's disease in California vineyards. Transgenic grapevine plants expressing either PGIP or HNE-CecB have been planted in two locations, one in Riverside County and the other in Solano County. Two hundred and ten transgenic or untransformed control vines, own-rooted or grafted with untransformed Thompson Seedless (TS), were planted in Riverside County on May 18, 2010, with the remaining 10 planted on March 6, 2011. In Solano County, 110 own-rooted transgenic and untransformed control vines were planted on August 2, 2010 and 110 untransformed Thompson Seedless scions grafted onto transgenic or untransformed rootstocks were planted on June 27, 2011. These transgenic grapevines are being evaluated both as plants on their own roots and as rootstocks grafted with untransformed Thompson Seedless scions. HNE-CecB- and PGIP-expressing transgenic grapevine lines in Riverside and Solano counties have been evaluated phenotypically; no visible differences were seen between transgenic and untransformed vines. At the Riverside County site, the plants have been naturally infected. *Xf* has been detected in petiole extracts, xylem sap, and stems by ELISA. Pierce's disease symptoms were previously assessed using a standardized score based on percentage of leaf area scorching; now we are using a 0-1 and a 0-4 scale to validate resistance to Pierce's disease under field conditions. Pierce's disease symptoms and ELISA cell count results confirmed *Xf* infection in the Riverside County field. At the Solano County site, plants on their own roots were mechanically inoculated with the *Xf* type strain (Temecula 1) on June 27, 2011 and re-inoculated on May 29, 2012, to validate resistance to Pierce's disease under field conditions. At the Solano County site, field-grafted transgenic plants were also mechanically inoculated for the first time on May 29, 2012. The presence of *Xf* was confirmed by ELISA in petiole extracts in fall 2011, but no *Xf* growth on plates or Pierce's disease symptoms were detected. Leaf scorching, the characteristic symptom of Pierce's disease, was observed in Solano County for the first time in fall 2012 and *Xf* presence was confirmed by ELISA in petiole extracts collected in the same season and in stem samples collected in spring 2013. Non-grafted and grafted grapevines at the Solano County site that were not inoculated previously were manually inoculated on June 2013, completing the manual inoculation of all grapevines at this location. HNE-CecB- and PGIP-expressing transgenic grapevine lines in Solano County have also been tested to confirm the presence of the transgene.

LAYPERSON SUMMARY

Four hundred and forty (440) transgenic grapevine plants expressing either polygalacturonase-inhibiting protein (PGIP; 192 plants) or a chimeric antimicrobial protein, human neutrophil elastase – cecropin B (HNE-CecB; 192 plants) and 56 untransformed control vines have been planted in two locations, one in Riverside County (220 plants) and the other in Solano County (220 plants). One half of these transgenic grapevines are being evaluated as plants on their own roots and the other half as rootstocks grafted with untransformed Thompson Seedless (TS) scions to demonstrate the field efficacy of two strategies to control Pierce's disease in California grapevines. The first strategy uses transgenic rootstocks to control the movement of the bacterium *Xylella fastidiosa* (*Xf*) in the water-conducting xylem of the vine through expression of PGIP. The second strategy tests whether transgenic rootstocks can clear *Xf* infections in xylem tissue by expressing HNE-CecB. At the Riverside County site, natural *Xf* infection has been confirmed in petioles, xylem sap, and stems by ELISA and appears to be uniform. At the Solano County site, about 25% of the plants were mechanically inoculated in 2011; another 25%, in 2012; and the remaining 50%, in 2013. The presence of *Xf* was confirmed in petiole and stem extracts of grapevines using the ELISA assay. We have used various phenotype scoring techniques and while these methods are not perfect, we see lines that consistently score better than the control for both strategies and others that do not. We also observe that those lines that show resistance are also able to transmit their resistance from the rootstock. However, the resistance transmitted from the rootstock is weaker than that obtained with a transformed plant. Further observations in the next two years will help quantify the ability of the elite lines that we have identified to provide resistance to Pierce's disease.

INTRODUCTION

Thompson Seedless (TS, *Vitis vinifera*) grapevines were transformed with a gene that encodes a chimeric anti-microbial therapeutic protein with a recognition domain from a neutrophil elastase (NE) that specifically binds to the *Xylella fastidiosa* (*Xf*) outer-membrane protein MopB and a lytic domain, cecropin B (CB), that clears *Xf,* the causative agent of Pierce's disease in grapevines (Dandekar et al., 2012). We have similarly transformed Thompson Seedless grapevines with a gene encoding polygalacturonase-inhibiting protein (PGIP) that results in the expression of a PGIP that inhibits the action of polygalacturonase (PG), a virulence factor expressed by *Xf,* which interferes with long distance movement of *Xf* and provides resistance to Pierce's disease in grapevine (Aguero et al., 2005, 2006). Transgenic grapevines expressing NE-CB and different PGIP constructs were first tested under greenhouse conditions and several lines that showed increased resistance to Pierce's disease over controls were identified by mechanical inoculation with *Xf* (Dandekar et al., 2012).

Selected transgenic grapevine plants expressing either NE-CB or PGIP, own-rooted or grafted with untransformed Thompson Seedless, were planted in 2010-11 and are being tested for Pierce's disease resistance under field conditions in two locations. At the Riverside County site, with Pierce's disease pressure, plants have been naturally infected, Pierce's disease symptoms have been detected, and *Xf* has been confirmed present in petiole extracts and xylem sap by ELISA and plating. At the Solano County site where plants were mechanically inoculated, Pierce's disease symptoms have also been detected and *Xf* has been detected in petiole extracts using an ELISA assay.

OBJECTIVES

The goals of this project are to finish the field test of four NE-CB and four PGIP transgenic grapevine clones by evaluating their horticultural characteristics and their resistance to Pierce's disease. Transgenic grapevines have been tested in two field locations as non-grafted plants and as transgenic rootstocks grafted with wild type scions. One field location has Pierce's disease pressure and plants were naturally infected with *Xf;* in the other location with no Pierce's disease pressure, grapevines were mechanically inoculated with *Xf*.

1. Validate the efficacy of *in planta*-expressed chimeric NE-CB and PGIP with different signal peptides to inhibit and clear *Xf* infection in xylem tissue and to pass through the graft union under field conditions.
    a. Activity 1. Propagation, field planting, and grafting of HNE-CecB and PGIP transgenic grapevines.
    b. Activity 2. Evaluate preservation of varietal characteristics in transgenic grapevines grown as whole plants or used as rootstocks.
    c. Activity 3. Evaluate Pierce's disease resistance of HNE-CecB and PGIP transgenic grapevines after inoculation with *Xf*.

RESULTS AND DISCUSSION

Activity 1. Propagation, field planting, and grafting of HNE-CecB and PGIP transgenic grapevines.

Four independent transgenic events expressing HNE-CecB (40-41, 40-89, 40-92, and 41-151) and four expressing different PGIP constructs (31-25, 45-77, 52-08, and TS50) were planted in two experimental fields. Two hundred and ten transgenic or untransformed vines, own-rooted or grafted with untransformed Thompson Seedless scions, were planted in Riverside County on May 18, 2010 and the remaining 10 were planted on March 6, 2011, completing the planting at this location **(Table 1)**. We also planted 110 transgenic and untransformed vines on their own roots on August 2, 2010 and 110 vines grafted with untransformed Thompson Seedless scions on June 27, 2011 in Solano County, completing the planting at this location (**Table 1**). HNE-CecB- and PGIP-expressing transgenic grapevine lines in Solano County have also been genotyped, confirming the presence of the inserted transgene in all lines.

Table 1. Transgenic and control grapevines planted at Riverside and Solano county field sites.

Non-grafted		Grafted	
Event ID (Vector)	**# Planted**	**Event ID (Vector)**	**# Planted**
HNE-CecB lines			
40-41 (pDU04.6105)	12	40-41G (pDU04.6105)	12
40-89 (pDU04.6105)	12	40-89G (pDU04.6105)	12
40-92 (pDU04.6105)	12	40-92G (pDU04.6105)	12
41-151 (pDU04.6105)	12	41-151G (pDU04.6105)	12
PGIP Lines			
31-25 (pDU05.1002)	12	31-25G (pDU05.1002)	12
45-77 (pDU06-0201)	12	45-77G (pDU06-0201)	12
52-08 (pDU05.1910)	12	52-08G (pDU05.1910)	12
TS50 (pDU94.0928)	12	TS50G (pDU94.0928)	12
Control line			
TS	16	TS-G	12

Activity 2. Evaluate preservation of varietal characteristics in transgenic grapevines grown as whole plants or used as rootstocks.

To verify that horticultural and varietal characteristics of the parental genotype Thompson Seedless were unchanged, HNE-CecB- and PGIP-expressing transgenic grapevine lines in Solano and Riverside counties were evaluated phenotypically in September 2011 and November 2011, respectively. 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 1) aperture of young shoot tip/opening of young shoot tip, 2) density of prostrate hairs between main veins on 4th leaf lower side of blade, 3) number of consecutive shoot tendrils, 4) color of upper side of blade on 4th young leaf, 5) shape of mature leaf blades, 6) number of lobes on mature leaf, 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. HNE-CecB and PGIP-expressing transgenic grapevine lines at both sites were also phenotypically evaluated in the fall of 2012 and will be evaluated in the fall of 2013; this evaluation will include fruit shape, color, and size. To date, no difference between transgenic and untransformed Thompson Seedless was observed.

Activity 3. Evaluate Pierce's disease resistance of HNE-CecB and PGIP transgenic grapevines after inoculation with *Xf*.

Grafted and non-grafted transgenic grapevine lines naturally infected in the field in Riverside County were scored for Pierce's disease symptoms using a 0-4 scale, where 0 = healthy no Pierce's disease symptoms, 1 = a few leaves on a few shoots that are symptomatic on cane(s), 2 = Many symptomatic leaves on multiple canes (in a mature bilateral cordon trained vine), 3 = dieback/death of canes/cordons, and 4 = death of whole vine (**Figure 1**). Stem samples from the Riverside County site grapevines were harvested and the number of *Xf* cells was

determined using an ELISA kit from Agdia; the standard curve was created using *Xf* from liquid culture (**Figure 2**). Pierce's disease symptoms and ELISA cell count results confirmed *Xf* infection in the Riverside County field.

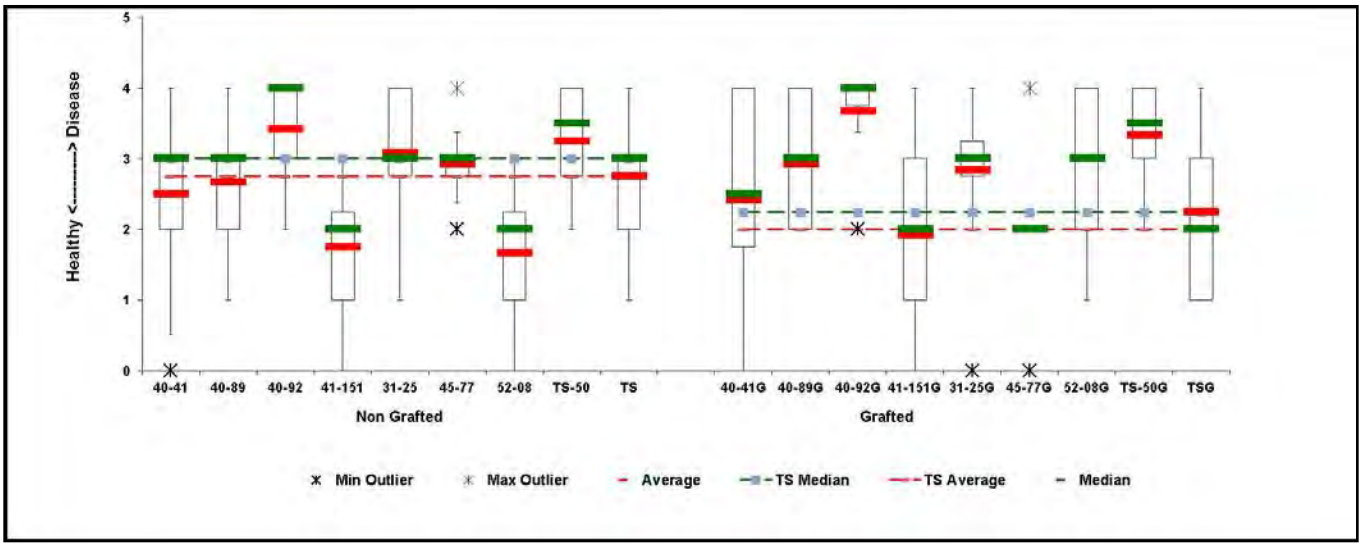

Figure 1. Riverside Pierce's disease symptom scoring. Spring of 2013

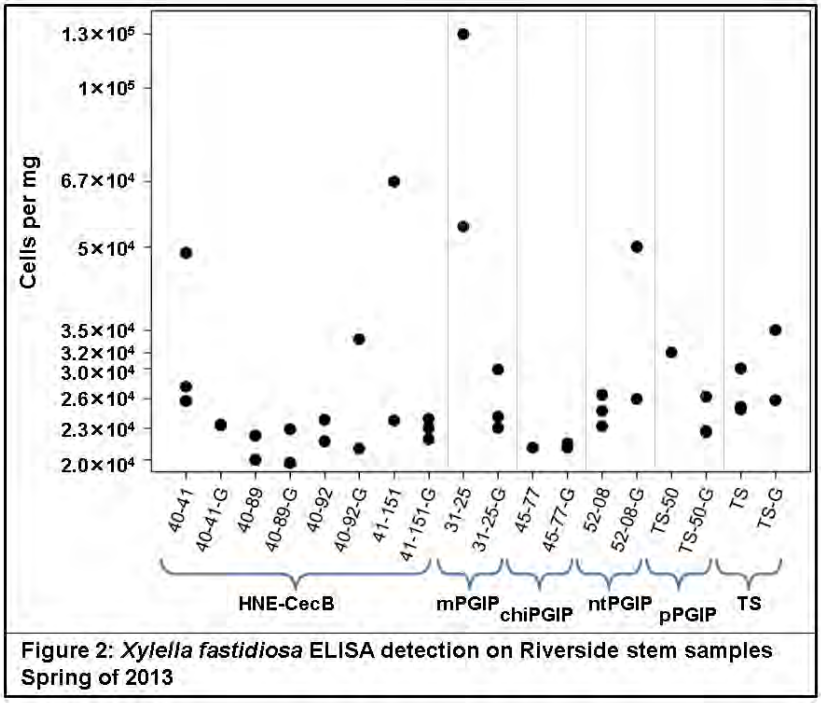

Figure 2: *Xylella fastidiosa* ELISA detection on Riverside stem samples Spring of 2013

Non-grafted transgenic grapevine lines in the Solano County field site were manually inoculated as described by Almeida et al. (2003) for the first time in July 2011, for a second time in May 2012, and for a third time in June 2013. The manually inoculated runners of grafted and non-grafted transgenic grapevines were scored for Pierce's disease symptoms using a 0-1 scale, where 0 = live runner and 1 = dead runner (**Figure 3**). Stem samples from runners in the Solano County site inoculated in 2011 or 2012 were harvested in spring 2013 and the number of *Xf* cells was determined using an ELISA kit from Agdia; the standard curve was created using *Xf* cells obtained from liquid culture (**Figure 4**). Pierce's disease symptoms and ELISA cell count results confirmed *Xf* infection in the

Solano County field site. Solano non-grafted and grafted grapevines that were not inoculated in 2011 or 2012 were manually inoculated on June 17, 2013, completing the manual inoculation of all grapevines at this location.

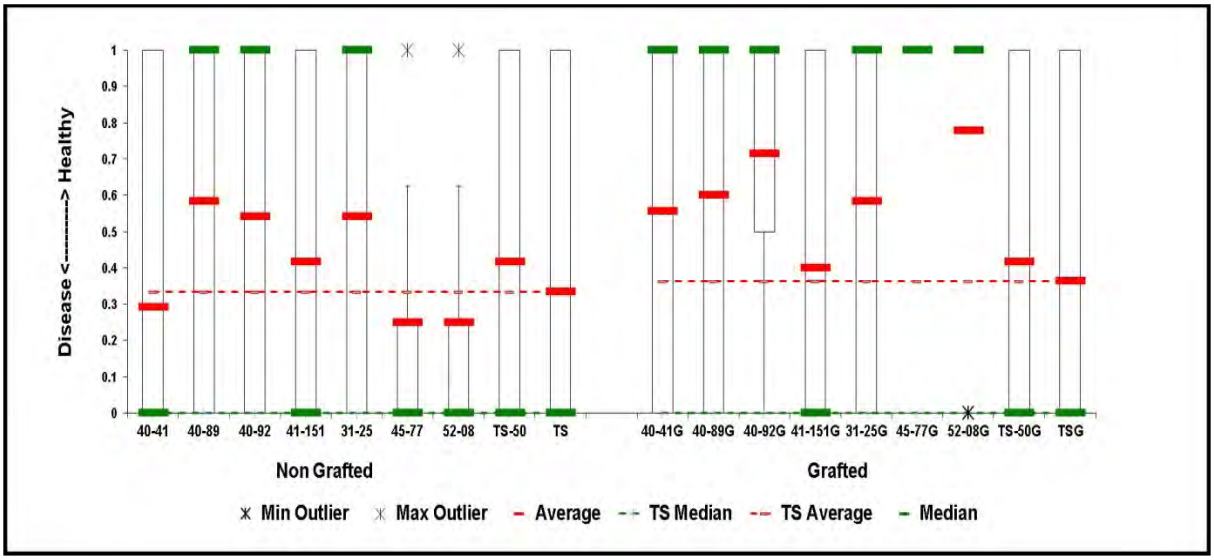

Figure 3. Solano Pierce's disease symptoms scoring. Spring of 2013

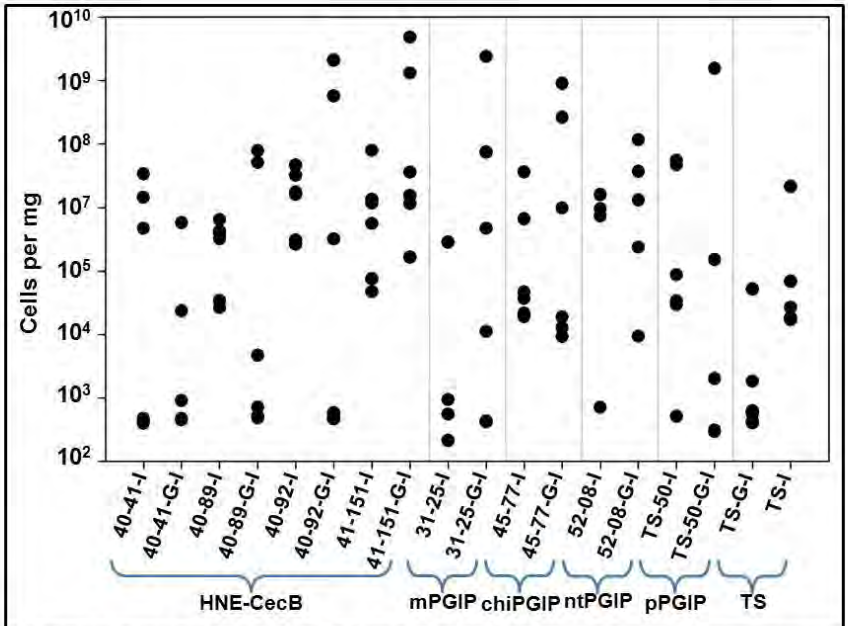

Figure 4: *Xylella fastidiosa* ELISA detection on Solano stem samples. Spring of 2013

CONCLUSIONS

We have successfully established two field trials to validate two greenhouse-tested strategies to control the movement and clearance of *Xylella fastidiosa* (*Xf*), a xylem-limited, Gram-negative bacterium that is the causative agent of Pierce's disease. 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 evaluated 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, HNE-CecB. The expectation is that expressing these proteins will prevent *Xf* movement and reduce its inoculum size, curbing the spread of Pierce's disease in California vineyards.

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FUNDING AGENCIES

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

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Reporting Period: The results reported here are from work conducted October 2012 to October 2013.

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 protection against *Xylella fastidiosa* [*Xf*; Pierce's disease strain] following mechanical injections of *Xf* into the grape stems of transgenic and non-transgenic control plants. The experiment is now in the third year after inoculations were initiated. Test plants include own-rooted transgenic and non-transgenic plants and grafted plants with non-transgenic scions of a Pierce's disease-susceptible variety grafted to rootstocks bearing transgenes from the respective investigators. The plants have been maintained under optimum field conditions with respect to water management and powdery mildew and insect control. Following the third year (2013) after inoculations began, control plants are showing clear symptoms of Pierce's disease and many inoculated canes are dying or dead.

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. 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. 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 Pierce's disease-susceptible scions to assess potential for disease suppression in an untransformed scion from signals in the transformed rootstocks. The results of this field experiment to date indicate that the mechanical inoculations successfully introduced the bacteria into the plants with subsequent appearance of foliar symptoms and cane death. There are several of the transgenes from several investigators that appear to be suppressing the symptoms of Pierce's disease-inoculated vines.

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 three-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 susceptible scions will be introduced. All plants were moved as vegetative material in 2010 and 2011 to the USDA 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 Pierce's disease 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 Pierce's disease six 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 six years. Hence, there is a documented historical precedent for the lack of spread of the bacteria from inoculated to non-inoculated plants, an important consideration for the experiments planned for this project.

OBJECTIVES

A. Land preparation, planting, and management of the experimental resources. Land to accommodate 500 plants from the investigators was designated for this project. Plants were introduced with a row spacing of 15 feet between rows and four feet between plants in a row. There is a 50-foot open space buffer area surrounding the field, which is fenced to protect against rabbits. Each row is staked with seven-foot grape stakes supporting 13 gauge wire in two wire trellis system with a stake at each plant site. Wires are stretched and anchored by seven-foot pressure-treated posts at the end of each row. The plants are irrigated by surface furrow in accordance with standard practices for maintaining grapes for experimental purposes at this site, which is used for other experimental planting not related to this project. Irrigation and pest management, primarily powdery mildew and insects, is conducted by the Collaborator Tom Kominek, Field Superintendent for the Department of Plant Pathology, who has 30 years of experience working with grapes under experimental conditions for USDA and UC Davis scientists.

B. Principal and Co-Principal Investigators, with assistance from contract field crews, are responsible for pruning in the spring of each year to provide for multiple canes for inoculation and destructive sampling. Note the pruning strategies will deviate from conventional production strategies in that many additional cordons will be maintained over seasons to enable multiple inoculations of canes for destructive sampling of the bacterial presence and movement in the plant throughout the seasons.

C. Irrigation and pest management is to be provided by Collaborator Tom Kominek as needed.

D. Plants were mechanically inoculated with *Xf* by the Investigators, beginning in 2011 and again in years 2012 and 2013.

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 (F**igures 1 and 2**). The second phase of the planting, including grafted transgenics, was completed May 17, 2011.

The 2010 and 2011 plantings of all four investigators survived the winter of 2011 without loss. The attachment of new shoots to the trellis system, cultivation, and irrigation management progressed in a normal and effective manner. Extensive pruning during the season is now recognized as necessary to manage the plants in a fashion to allow ease of mechanical inoculation.

As of July 21, 2011, the initial 2010 planting and the second 2011 planting individuals are healthy, growing normally, and all plants have a normal phenotype that is indistinguishable from untransformed control plants of each parental genotype. Inoculation of individual canes was done on July 12, 2011 (F**igures 5 and 6**), on July 12 and July 21 in 2012 and again in May of 2013. The field planting is now providing important data on the effectiveness of any of the transgenic strategies employed by the respective researchers.

As of June 2012 (F**igure 3**), the inoculated controls and transgenic plants appeared healthy. Additional canes were inoculated in July of 2012. By October 2012, some inoculated canes of the control plants were showing symptoms consistent with Pierce's disease and appeared to be dying at this point and several were dead as the plants leafed out in 2013 (**Figure 4**).

As of October 2013, many inoculated canes on control plants and some transgenics were symptomatic or were dead in contrast to the non-inoculated canes on these plants, which appeared healthy and asymptomatic. This observation indicates that the mechanical inoculations were successful and further suggests that, in most cases, the

bacteria have not yet moved systemically through the plants. Plant turgor has been maintained throughout the growing season with regular watering. Images below show the progression of the plants in the field from planting in 2010 to the summer of 2013. Included are representative plants illustrating the appearance of inoculated vs. uninoculated control plants. Within each of those two categories are plants that were either carried a putatively protective transgenes or were non-transgenic plants of the same parental background. The selected images in F**igures 7 and 8** illustrate that some, but not all, transgenes in the respective plants appear to afford protection against Pierce's disease.

Solano County Pierce's disease Field Work 2013.
All field activities are conducted or coordinated by field superintendent Tom Kominek. Regular tilling and hand weeding maintained a weed-free planting area. Plants were pruned carefully in March leaving all inoculated/ tagged branches and numerous additional branches for inoculation and sampling purposes in the coming year. All pruning material was left between the rows to dry, then flail chopped and later rototilled to incorporate the residue per requirements of the USDA APHIS permit. Surface irrigation was applied as needed to maintain the soil at field capacity and turgor in the plants. Application of the fungicides Luna Experience and Inspire were alternated at periodic intervals to maintain the plants free of powdery mildew. Leafhoppers and mites were treated with insecticides when needed. Neither powdery mildew nor insect pressure was noted throughout the growing season.

CONCLUSIONS

The results to date of this field experiment indicate that the mechanical inoculations successfully introduced the bacteria into the plants with subsequent appearance of foliar symptoms and cane death. There are several of the transgenes from several investigators that appear to be suppressing the symptoms of Pierce's disease inoculated vines.

Images below illustrate the status the field experiment from planting in 2010 to the summer of 2013. The caption to each figure indicates the date the image was obtained and together they represent the both asymptomatic inoculated transgenic and symptomatic inoculated non-transgenic control plants at the Solano County site.

FUNDING AGENCIES

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

Figure 1. July 2010 Solano planting.

Figure 2. July 2010 Solano planting.

Figure 3. April 2012 Solano planting.

Figure 4. July 2013 Solano planting.

Figure 6. Close-up field inoculation June 2011.

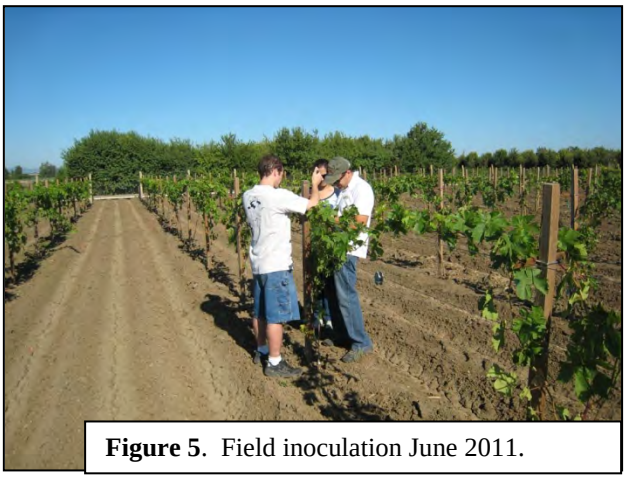

Figure 5. Field inoculation June 2011.

Figure 7. Inoculated untransformed control; May 2013.

Figure 8. Inoculated transformed P14-9; May 2013.

FIELD EVALUATION OF GRAPE PLANTS EXPRESSING PR1 AND UT456 TRANSGENIC DNA SEQUENCES FOR PROTECTION AGAINST PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted October 2012 to October 2013.

ABSTRACT
The objective of the field experiments is to evaluate transgenic grape plants and grape rootstocks expressing two DNA constructs designated PR1 and UT456 genes in field sites in Solano and Riverside counties for resistance to *Xylella fastidiosa* (*Xf*; Pierce's disease strain). Infection at the Solano site will use mechanical inoculation and will depend on natural inoculation at the Riverside site where endemic sharpshooters carry *Xf*. The basis for this experiment derives from four previous inoculation experiments in a controlled greenhouse over a two-year period, involving more than 300 transgenic plants of several primary transformants of PR1 and UT456 in either susceptible Thompson Seedless or the rootstock Freedom. Backgrounds indicated that suppression of Pierce's disease symptoms and reduction in bacterial titer occurred in the transgenics compared with untransformed control plants. The Solano field experiment has been conducted in two phases. The first phase started in 2010 to evaluate clonal copies of the fully transformed own-rooted plants that exhibited suppressed Pierce's disease symptoms and low bacterial titers in greenhouse assays. The second phase began in 2011 with planting the untransformed Thompson Seedless scions grafted onto the most resistant of the PR1 and UT456 primary transformants as rootstocks. Over the course of the multiyear field evaluation at both sites, test plants in the first planting included own-rooted conventional Thompson Seedless and Freedom plants as controls to be compared with the transformed plants. Controls in the second phase included untransformed rootstocks grafted to the untransformed scions. Data collected in 2012-13 from both sites indicate that the bacteria are present in all plants at the Riverside site and in the mechanically-inoculated plants at the Solano site. Control plants at both locations show symptoms of Pierce's disease and cane or plant death. Both the PR1 and UT456 expressing plants show suppression of symptoms and reduced bacterial counts. Quantitative data collection is in progress from samples collected at both sites.

LAYPERSON SUMMARY
Previously, we identified novel anti-PCD genes by a functional screen from cDNA libraries of grape. Two of these grape sequences (PR1 and UT456) expressed as transgenes in grape, suppressed Pierce's disease symptoms and dramatically reduced bacterial titer in inoculated plants under greenhouse conditions. Field experiments underway in Solano and Riverside counties, conducted with a USDA APHIS permit, will evaluate clonal copies of the most resistant transgenic plants under field conditions for resistance to Pierce's disease. The field evaluation will be conducted at the respective sites will involve mechanical inoculation with *Xylella fastidiosa* (*Xf*) in Solano County and glassy-winged sharpshooter inoculation in Riverside County. Data sets will include visual monitoring of plant morphology, Pierce's disease symptoms, and bacteria titer by quantitative PCR (qPCR) assays. To date, PCR data and plating assays confirm the presence of *Xf* in the plants at both locations. Differential protection against defoliation was observed at the Riverside County site and PCR assays confirmed bacterial populations in the plants. Bacteria are present in inoculated plants at the Solano County site and there is definitive evidence of extensive symptom differences between several of the transgenic plants and the non-transgenic control. Clearly both sites will need to be monitored and assays taken over a longer period of time for conclusive results to be obtained.

INTRODUCTION
Susceptibility in most plant-microbe interactions depends on the ability of the pathogen to directly or indirectly alter genetically determined pathways leading to apoptosis or programmed cell death (PCD). In eukaryotic systems, PCD is regulated by activators or inhibitors, which may be either endogenous or from exogenous sources including bacteria, fungi and viruses. In the case of *Xylella fastidiosa* (*Xf*), the bacteria live in a wide range of

plant species predominantly as endophytes or epiphytes but occasionally as pathogens. We demonstrated previously that the death symptoms associated with Pierce's disease exhibit characteristic molecular markers of PCD. Presumably, sensitivity to the presence of the *Xf* bacteria, expressed as cell death-dependent symptoms, is the result of signals expressed by the bacteria that lead to activation of PCD. Our research has focused on the effect of altering the expression of two different plant DNA sequences (PR1 and UT456) that were obtained from a functional anti-PCD screen of grape and tomato cDNA libraries. The UT456 is a noncoding sequence that contains small RNA hairpin structure indicative of a potential regulatory microRNA. The nomenclature of PR1 gene can be confusing since PR1 refers to the coding sequence of the gene while the product of the PR1 gene is a 14 kilodalton protein referred to in the literature as p14a. Hence, there is a duplicative description of the construct inserted into the grape plants, but for simplicity and continuity we have chosen to refer to this construct of the gene in the transgenic plants as the protein product PR1-x, with x referring to independent transgenic plants. Both PR1 and UT456, expressed transgenically in either susceptible Thompson Seedless or the rootstock Freedom, protected against Pierce's disease symptoms and limited bacterial titer four to six orders of magnitude below that reached in untransformed control plants in repeated greenhouse experiments. Current field experiments are in progress to evaluate transgenic grape plants and grape rootstocks expressing PR1 and UT456 constructs in field sites in Solano and Riverside counties for suppression of Pierce's disease. The plants include own-rooted transformed Freedom and Thompson Seedless grape plants, and transformed rootstock of each expressing PR1 or UT456 grafted to untransformed Thompson Seedless scions. The grafted plants are designed to assess possible protection across a graft union of the respective transgenic sequence. Once again, preliminary greenhouse inoculation experiments indicated that the protection by PR1 and UT456 does move across the graft union, although the extent of protection will only be clear after more field data

Infection at the Solano County site is by mechanical inoculation versus the Riverside County site, which depends on natural inoculation by endemic glassy-winged sharpshooters (GWSS) carrying *Xf*. The Solano field experiment is conducted in two phases. The first phase started in 2010 to evaluate clonal copies of the fully transformed own-rooted Thompson Seedless or Freedom plants expressing PR1 or UT 456. The second phase began in 2011 with planting the untransformed Thompson Seedless scions grafted onto clonal copies of the resistant of the PR1 and UT456 plants as rootstocks. Over the course of the three-year field evaluation at both sites, test plants in the first planting (2010) include own-rooted conventional Thompson Seedless and Freedom plants as controls to be compared with the transformed plants. Controls in the second planting phase (2011) included untransformed rootstocks grafted to the untransformed scions. Mechanical inoculations were done in 2011, 2012, and 2013 on separate canes. Bacterial assays conducted in 2012 from both sites indicate that bacteria are present in the mechanically inoculated canes at the Solano County site and in all plants at the Riverside County site. Quantitative data collection is in progress at both sites.

OBJECTIVES 2010-2013

A. Evaluation of transgenic grape plants and grape rootstocks expressing two DNA constructs designated PR1 and UT456 genes in a field site in Solano County for resistance to the Pierce's disease strain of *Xf* following mechanical inoculation.

   The field experiments in Solano County are being conducted in two phases. The first phase of the field experiment started in 2010 to evaluate clonal copies of the fully transformed own-rooted PR1 and UT456 plants of several independent transformants that exhibited suppressed Pierce's disease symptoms and low bacterial titers under greenhouse conditions. These experimental materials consist of sets of inoculated and uninoculated control plants. Treated plants were inoculated with ~20,000 *Xf* bacterial cells per inoculation site by stem puncture in July 2011, then repeated on different canes with ~200,000 *Xf* bacterial cells per inoculation site in June of 2012, and a third inoculation with ~200,000 *Xf* bacterial cells per inoculation site was done in June 2013.

B. The second phase of the Solano County field planting began in 2011 with planting the untransformed commercial scions grafted onto the most resistant of the PR1 and UT456 independent transformants as rootstocks. Inoculations per schedule in A above were carried out in 2012 and 2013.

C. The second field experiment, located in Riverside County, was planted in the spring of 2011. The planting consisted of clonal copies of the fully transformed own-rooted plants expressing PR1 or UT456 that were

planted in 2010 in Solano County. The Riverside plants were infected with *Xf* via natural populations of GWSS (*Xf* vector).

RESULTS AND DISCUSSION

A. **The first phase of the field experiment started in 2010 to evaluate clonal copies of the fully transformed own-rooted (ungrafted) PR1 and UT456 plants that exhibited suppressed Pierce's disease symptoms and low bacterial titer under extensive greenhouse testing (2010-2013).**
This phase took place as planned with the planting occurring on July 12, 2010. Evaluation of the experimental plants for plant morphology, symptoms of Pierce's disease infection, and the presence of the bacteria involve a time course of visual monitoring of symptom development and assessment by quantitative PCR (qPCR) assays of bacteria in the inoculated tissue (mainly stems) for *Xf*. A comparative quantitative determination by qPCR of the presence of *Xylella* in transgenic grape and grape rootstocks compared to equivalent untransformed grape and grape rootstocks will provide an indication of the impact on the bacterial load in the respective transgenic and control plants. All procedures have been used successfully in the ongoing greenhouse experiments for the past five years. The plants were confirmed to have been successfully infected in the 2011, 2012, and 2013 inoculations by sampling individual inoculated canes followed by qPCR analysis for relative bacterial populations. Bacterial numbers varied from 500-1500 cells per one cm of inoculated stem tissue in the fall of 2011 sampling and no difference between the control plants and transgenic plants. Further, there were no distinguishable morphological differences in the control plants compared with any of the transgenic lines using criteria of descriptors described by the International Organization of Vine and Wine. There were no detectable symptoms of Pierce's disease in leaf or stem tissue at the end of the 2011 season or when buds formed and leaves emerged in the spring of 2012. Vines were pruned to retain inoculated canes and to provide for two to four additional canes for inoculation in the early summer of 2012. The second sets of inoculations were done in June of 2012. Some inoculated canes on the control plants began to express leaf and stem symptoms of cell death compared with uninoculated canes at end of the 2012 growing season. Individual inoculated canes showed symptoms consistent with Pierce's disease as the plants emerged from dormancy in the spring of 2013. Uninoculated canes on all plants were asymptomatic. Individual inoculated canes were rated as dead/dying or alive in April 2013 (**Figure 1**). Two of the independent transformants, PR1-9 and UT456-10, cloned plants showed substantial reduced death or no death compared with the susceptible control canes. Bacterial sampling again showed *Xf* to be present in the surviving canes but no qPCR positive data was recovered from the dead canes (tissue too dried out). There was no evidence of cane death in any plants and canes that had not been inoculated. All cane death was restricted to inoculated canes, with a few inoculated plants showing death of the entire plant but, again, no plant or cane death in uninoculated plants. Representative plant images are shown in **Figures 2-6**. Captions on individual figures describe the typical symptoms or plant appearance in relation to disease-associated tissue death. The results to date indicate that both PR1-9 and UT456-10 exhibited greatly reduced cane death with the PR1-9 less impacted than the UT456. The plants were re-inoculated in June of 2013. Samples for bacterial analysis and scoring of individual inoculated canes from all three inoculations are being analyzed at the present time.

B. **The second phase of the Solano County field planting began in 2011 with copies of the untransformed scions grafted onto the most resistant of the PR1 and UT456 plants as rootstocks (2010-2013).**
Transgenic rootstocks grafted to untransformed Pierce's disease susceptible Thompson Seedless scions were produced *in vitro* under sterile conditions in magenta boxes, then transferred to the greenhouse to grow in preparation for field planting. Using this procedure, success rate is greater than 95%, is more space efficient relative to greenhouse grafting, can be done anytime of the year, and is as rapid as green grafting. The plants for the Solano County phase two were planted in the field May 17, 2011 and first inoculated in June of 2012. The plants were confirmed to have been successfully infected in 2012 by sampling individual inoculated canes in the fall of 2012 followed by qPCR analysis. Bacterial numbers varied from 200-1000 cells per one cm of inoculated stem tissue sampling and there were no differences between the control plants and transgenic grafted plants at this time. There were no detectable symptoms of Pierce's disease in leaf or stem tissue at the end of the 2012 season nor when buds and leaves emerged in the spring of 2013. However, as the plants began to leaf out, there were noticeable areas of bud failure on the inoculated control plants (**Figure 6**) compared with the transgenic grafted P14-9 shown in **Figure 5**. Plants were re-inoculated in June of 2013 and evaluations will continue during the fall for symptoms and bacterial population levels.

C. Establish a field planting in Riverside County consisting of clonal copies of the fully transformed ungrafted PR1 and UT456 plants that were planted in Solano County in 2010. (2011-2013)

Field planting occurred April 2011. The GWSS populations were sufficiently high to initiate infections in all plants in this location based on both symptom and bacterial assays in June 2012 and the GWSS populations were recorded as high in 2011 and 2012 by field surveys conducted by Riverside personnel. Samples of cane tissue analyzed by qPCR confirmed infection by *Xf*. Symptoms of Pierce's disease associated death were evident in 2012 and early spring of 2013. All plants were rated for disease severity. **Figure 7** shows a comparison of PR1-9 with the untransformed Thompson Seedless control. Images were captured of all plants, examples of which are seen in **Figures 8-9** for time course comparisons. Bacterial assays are in progress for the 2013 samplings. Clearly, the level of disease impact is high and this site appears to have afforded infection levels that affect plant survival in the presence of Pierce's disease. As has been the case at the Solano County site, plants expressing the transgenes PR1 (especially PR1-9) and UT456 (UT 456-10) have been consistently rated as more resistant than the untransformed control plants under a circumstance of heavy disease pressure.

CONCLUSIONS

Xylella fastidiosa (Xf) induces Pierce's disease symptoms that result from activation of a genetically regulated process of programmed cell death (PCD). We identified grape DNA sequences, which when constitutively expressed in transgenic grapes suppress the death-dependent symptoms of Pierce's disease and reduce the bacterial titer to a level found in Pierce's disease resistant wild grapes. Two of these grape sequences, constitutively expressed as transgenes in grape, suppressed Pierce's disease symptoms and dramatically reduced the titer of *Xf* in inoculated plants under greenhouse conditions. Field experiments ongoing in Solano and Riverside counties under a USDA APHIS permit appear to confirm the greenhouse results. Greenhouse and current field data indicate that the transgenic sequences referred to as PR1 and UT 456 suppress the disease by preventing the PCD without eliminating the bacteria, only reducing the bacterial population to a level found in the naturally resistant wild grape, *Vitis californica*. Hence, the mode of action is not antibiotic. The field experiment includes an evaluation of the potential for these respective transgenes to function across a graft union. The first year assessment of cross-graft protection indicates that one or more of the transgenic sequences may function across a graft union to protect an untransformed and susceptible wild type scion. This project has identified a molecular and biochemical basis for Pierce's disease symptoms and a genetic mechanism to suppress symptoms and bacterial growth within an infected plant. Given these results and results from other transgenes being tested by other Principal Investigators, there very likely will be a gene-based transgenic strategy to address Pierce's disease. 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 on the plants at the Solano County site.

FUNDING AGENCIES

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

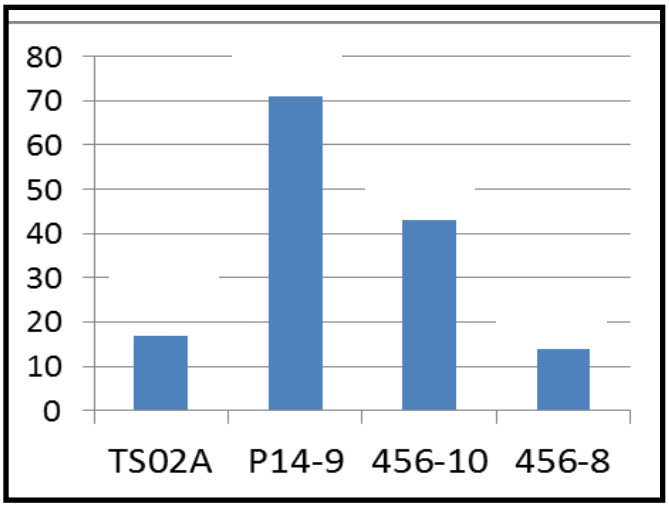

Figure 1. Percent inoculated canes alive two years post inoculation at the Solano County site. Ratings were done May 13, 2013. Plants are own-rooted Thompson seedless transgenic and non-transgenic. N= number of canes rated.

Figure 2. Solano County: Non-transgenic control plant (TS02A) Inoculated in 2011 and 2012. Image taken April 2013 showing shoots dying shortly after emergence. Colored tags indicate dates of inoculation. See **Figure 3** for view of the entire plant with additional death of inoculated canes.

Figure 3. Solano County: non-transgenic control plant (TS02A) inoculated July 2012 showing cane death visible after emergence of uninoculated canes on the same plant. Image illustrates the observation that the bacteria can kill inoculated canes but do not move rapidly to other canes on the same plant.

Figure 4. Solano County: own-rooted transgenic PR1-9 that was inoculated in 2011 and 2012. There is no evidence of death in inoculated canes or shoots emerging from the inoculated canes. Image recorded May 13, 2013.

Figure 5. Solano County: non-transgenic scion grafted to a transgenic PR1-9 rootstock. The scion was inoculated in 2012 currently showing no evidence of death in inoculated canes or shoots emerging from the inoculated canes. Image recorded in May 2013. See **Figure 6** for control comparison with substantial cane death.

Figure 6. Solano County: non-transgenic scion grafted to a non-transgenic control rootstock. The scion was inoculated in 2012. Evidence of death is visible in inoculated canes. Note red flags for reference to limited cane death. Image recorded in May 2013. See **Figure 5** for comparison to grafted scion on a PR1-9 rootstock.

Figure 7. Example of grape plants growing in Riverside County in June 2013, subject to feeding by GWSS confirmed to carry *Xf*. Plant in the foreground of the right picture (red arrow) is a non-transgenic Thompson Seedless control, which is essentially dead. The plant in background is a transgenic PR1 Thompson Seedless plant, which, although infected (5673 bacterial cells per cm of stem tissue) shows no symptoms of cane or leaf death, seen here also as the image on the left.

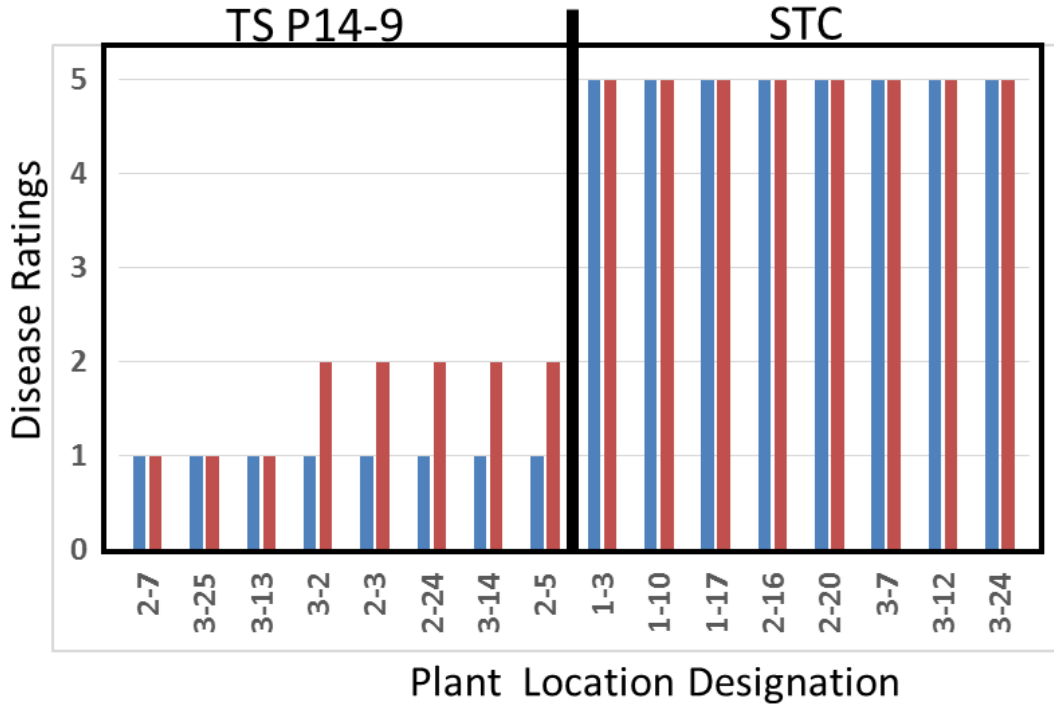

Figure 8. Plants established in Riverside County in 2010 were rated for symptoms of Pierce's disease in June and August of 2013 on a 1 to 5 scale, with 1 being healthy and 5 being dead. June 2013 ratings are blue and August 2013 ratings are red. STC designates a susceptible transgenic control into which the 456 transcript was inserted but is not expressed and is used in this planting as a susceptible transgenic control. P14-9=PR1-9.

Figure 9. Image of grape plants growing in Riverside County June 2013 showing both asymptomatic and severely diseased plants (cone in foreground) that were subject to infection by GWSS that were confirmed to carry *Xf* by UC Riverside personnel.

Figure 10. Example grape plants growing in Riverside County June 2013 showing both asymptomatic and severely diseased plants that were subject to infection by GWSS, which were confirmed to carry *Xf*. Arrows indicate each plant base. P14-9 =PR1-9.

EVALUATION OF PIERCE'S DISEASE RESISTANCE IN TRANSGENIC *VITIS VINIFERA* GRAPEVINES EXPRESSING EITHER GRAPE THAUMATIN-LIKE PROTEIN OR *XYLELLA FASTIDIOSA* HEMAGGLUTININ PROTEIN

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ABSTRACT

Previous research in our lab identified two hypervirulent mutants of *Xylella fastidiosa* (*Xf*). These mutations were in large hemagglutinin (HA) adhesion genes that we named *Hfx*A and *Hfx*B. *Hxf* mutants also showed a marked decrease in cell-cell clumping when grown in liquid culture. We hypothesize that if Hxf protein, or a portion of the Hxf protein that mediates adhesion, could be expressed in the xylem fluid of transgenic grapevines then perhaps insect-inoculated *Xf* cells would clump together and be less capable of colonizing grapevines. During the past three years we produced transgenic HA-expressing tobacco and grapevine lines; these transgenic lines exhibited less severe symptoms of Pierce's disease following mechanical inoculation of *Xf* cells. With the assistance of the Public Intellectual Property Resource for Agriculture (PIPRA) we secured all the necessary permits to plant these lines in the field in spring 2013. These vines grew well and were trained up to the wire and established as conventional bilateral cordon vines. We will cut back the shoots to two buds and then inoculate the shoots with *Xf* next spring at the same time that other Pierce's disease workers inoculated their transgenic vines a couple of years ago. Symptoms will be rated in September on the inoculated shoots and we will score whether adjacent non-inoculated shoots develop Pierce' disease symptoms. In January 2015 the shoots will be trimmed to two buds and the emerging shoots will be rated for Pierce's disease symptoms in August 2015.

In a five-year project that sought to better understand the cold curing phenomenon of *Xf* infected grapevines, we found that cold-treated vines had significantly elevated levels of phenolics compounds as well as a grapevine thaumatin-like protein (TLP). TLPs from other plant species have been shown to possess antimicrobial activity, and grapevine TLP cloned and expressed in *Escherichia coli* possessed some anti-*Xf* activity *in vitro*.

Even though additional funding was not allocated to evaluate the potential resistance of TLP-expressing transgenic grapevines, we had already submitted the appropriate TLP transformation constructs to the UC Davis Plant Transformation Facility. Thirteen distinct TLP lines were obtained from the transformation facility. RT-PCR confirmed that the vines were transcribing elevated levels of TLP mRNA and 10 reps of each TLP-transgenic line were inoculated with *Xf* in the greenhouse. Pierce's disease symptoms at 17 weeks post inoculation were the same in the TLP-transgenics as non-transgenic vines, suggesting that these transgenic vines did not possess resistance to Pierce's disease. The inoculated vines will be placed in a screen house this winter and Pierce's disease symptoms of inoculated vines and non-transgenic controls will be rated in September 2014 to determine if the TLP-transgenics might increase cold-curing rates higher than non-transgenic controls.

LAYPERSON SUMMARY

Our 7+ year research effort on the role hemagglutinins (HAs), large proteins that mediate the attachment of bacteria to themselves and to various substrates, play in Pierce's disease pathogenicity and insect transmission has been very fruitful. Our early work showed that HA mutants were hypervirulent, ie. they caused more severe symptoms and killed vines faster that vines inoculated with wild-type (WT) *Xylella fastidiosa* (*Xf*) cells. HA

mutants no longer clumped together in liquid cultures like wild-type cells, nor did HA mutants attach to inert substrates like glass or polyethylene when grown in liquid culture. ALL of these properties show that HAs are very important cell adhesion molecules. Research conducted in the Almeida lab also showed that HA mutants were transmitted at lower efficiencies than wild-type cells and they were compromised in binding to chitin and sharpshooter tissues compared to wild-type cells. Thus they have a very important role in insect transmission. Lindow's lab showed that diffusible signal factor (DSF) mutants, which are also hypervirulent, produced much less HA than wild-type cells, thus providing another line of evidence regarding the importance of these proteins in *Xf* pathogenesis and insect transmission.

We are now evaluating our hypothesis that HAs expressed in transgenic grapevine xylem sap may act as a "molecular glue" that would aggregate and thus slow the movement of wild-type *Xf* cells introduced into grapevines by an infectious insect vector. If this happens then it is possible that HA-aggregated *Xf* cells would remain close to the site of inoculation and if that site is in the terminal portion of a cane, which is where *Xf* is introduced by our native blue-green, green, and red-headed sharpshooters, then that cane would likely be pruned off in the winter and the infection removed from the vine. Our most optimistic hope is that HAs could be expressed in transgenic rootstocks and the HAs would be translocated into a non-genetically modified organism (GMO) fruiting scion and afford similar levels of functional Pierce's disease resistance. We finished a greenhouse Pierce's disease severity screening of the nine HA transgenic lines that were produced. The results were very encouraging, with all of the HA-transgenic lines having much lower disease ratings than non-transgenic controls. In spring 2013, twelve reps of each HA-transgenic line were planted in the field. These vines grew well and were established as bilateral cordons. Four shoots on each vine will be inoculated with *Xf* in April 2014 and Pierce's disease symptoms will be evaluated every two weeks after eight weeks post inoculation.

Greenhouse pathogenicity testing of TLP-transgenic vines showed no difference in Pierce's disease symptom severity in the TLP-transgenic vines vs. non-transgenic positive control vines.

INTRODUCTION

Xylella fastidiosa (*Xf*) cell-cell attachment is an important virulence determinant in Pierce's disease.. Our previous research has shown that if two secreted hemagglutinin (HA) genes which we have named HxfA and HxfB are mutated, *Xf* cells no longer clump in liquid medium and the mutants form dispersed "lawns" when plated on solid PD3 medium (Guilhabert and Kirkpatrick, 2005). Both of these mutants are hypervirulent when mechanically inoculated into grapevines, i.e. they colonize faster, cause more severe disease symptoms, and kill vines faster than wild-type (WT) *Xf*. If either HxfA OR HxfB is individually knocked out there is no cell-cell attachment, which suggests that BOTH HA genes are needed for cell-cell attachment. It is clear that these proteins are very important determinants of pathogenicity and attachment in *Xf*/plant interactions. Research by other Pierce's disease researchers has shown that *Hxf*s were regulated by an *Xf*-produced compound known as diffusible signal factor (DSF) (Newman et al., 2004) and that they were important factors in insect transmission (Killiny and Almeida, 2009). The *Xf* HAs essentially acts as a "molecular glue" that is essential for cell-cell attachment and likely plays a role in *Xf* attachment to xylem cell walls and contributes to the formation of *Xf* biofilms.

Our initial objectives proposed to further characterize these HAs using some of the techniques that were used to identify active HA binding domains in *Bordetella pertussis*, the bacterial pathogen that causes whooping cough in humans. *B. pertussis* HA was shown to be the most important protein that mediates cell attachment of this pathogen to epithelial host cells (Liu, et al., 1997; Keil, et al., 2000). In the first two years of research we identified the specific HA domain(s) that mediate *Xf* cell-cell attachment and determined the native size and cellular location of *Xf* HAs. In the third year we identified a two-component transport system that mediates the secretion of *Xf* HAs. In the final years of the initial project we expended considerable time and effort in constructing transgenic tobacco and grapevines that expressed HA. We conducted pathogenicity evaluations of our nine HA-transgenic lines. Disease severity ratings in greenhouse grown vines were considerably less in the transgenic lines than the non-transgenic controls. Permits to establish a field planting of the HA vines were obtained with the assistance of the Public Intellectual Property Resource for Agriculture (PIPRA) and a field trial was established in April 2013. The vines will be inoculated with *Xf* in spring 2014 and Pierce's disease symptoms of HA-transgenics will be compared to non-transgenic, *Xf*-inoculated controls in September 2014. Vines will then be pruned back to two buds and allowed to go through the winter. Symptoms on the vines will again be rated in September 2015.

OBJECTIVES

1. Complete the characterization of grape transgenic plants over-expressing *Xf* hemagglutinin (Hxf) protein.
2. Mechanically inoculate wild-type *Xf* and evaluate the effect on Pierce's disease symptom expression, and the effect of Hxf expression on *Xf* bacterial population levels and movement in the xylem by quantitative PCR (qPCR).
3. Secure permits to plant HA transgenic lines in the field at UCD. Plant transgenic vines in the field.
4. Following greenhouse testing, graft promising *Hxf* transgenic rootstocks to untransformed scions.

(Please Note: Objectives 5-7, described below, were not recommended for funding during the 2012 grant cycle. However, as previously noted, we had already submitted transformation constructs to the UC Davis Plant Transformation Facility, and they have since supplied us with 13 TLP-transgenic lines that were propagated and grown in the greenhouse. Reps of each transgenic line were inoculated with *Xf* and Pierce's disease symptoms were rated every two weeks from 6-17 weeks post inoculation.)

5. Generate grape transgenic plants over-expressing the grape thaumatin-like protein (TLP).
6. Screen putative TLP-transgenic lines for quantitative gene expression by RT-PCR, and protein expression by ELISA and western blot analysis, testing both leaves and expressed xylem fluid for the presence of TLP.
7. Mechanically inoculate greenhouse TLP-transgenics with wild-type and GFP-tagged *Xf* and evaluate the effect on Pierce's disease symptom expression, and the effect of TLP expression on *Xf* bacterial population levels and movement in the xylem by quantitative PCR (qPCR).

RESULTS AND DISCUSSION

Objective 1. Complete the characterization of grape transgenic plants over-expressing *Xf* hemagglutinin (Hxf) protein.

Twenty-one transgenic Thompson Seedless grape plants that potentially over-expressed the Hxf protein in the xylem using a binary plasmid with a polygalacturonase secretory leader sequence were obtained from the UCD Plant Transformation Facility in September 2010. These were initially obtained as small green three-inch plants that needed to be grown in growth chambers and later in the greenhouse to produce hardened woody shoots that could be vegetatively propagated. It took approximately four months for each of the propagated shoots to grow up sufficiently to allow them to be further propagated or inoculated with *Xf*. By July 2011 we had propagated sufficient numbers of transgenic grapevines that we could begin analyzing them for HA expression. Analysis by **standard and qPCR** for the presence of the HA transgene in genomic grapevine DNA from each of the 22 lines showed that six of nine transgenic lines containing *Xf* HA adhesion domains (AD 1-3) labeled as SPAD1 and 3 of 12 transgenic lines of the full-length HA, labeled PGIP220 in **Table 1** below, had the HA gene inserted into the grapevine chromosome.

The construct used to transform grapevines, which was recommended by the Plant Transformation Facility, contained two copies of the 35S promoter flanking the HA construct. We hypothesize that recombination occurred within the *Agrobacterium* plasmid that allowed the HA insert to be deleted but the kanamycin selection marker was still inserted into the grape genome. This would explain why a number of the kanamycin resistant transgenics did not actually have the truncated or full-length form of *Xf* HA inserted into the grape chromosome.

RT-qPCR analysis on mRNA isolated from these lines confirmed the presence of AD1-3 or full-length HA mRNA in the lines that tested positive by standard or qPCR PCR, thus the HAs inserted into the grape genome are being expressed (**Table 2**).

Objective 2. Mechanically inoculate transgenic grapevines with wild-type *Xf* cells. Compare disease progression and severity in transgenic grapevines with non-protected controls.

We have gone through five rounds of vegetatively propagating the lignified transgenic grapevine lines. We attempted to propagate green shoots but only 10-15% of the green shoots became established, thus we are now propagating only lignified wood.

Table 1. Results of PCR testing of transgenic grapevines for the presence of full-length (PGIP 220) of AD1-3 fragment of *Xylella fastidiosa* HA genes in grape chromosome.

DNA ID#	Genotype	Standard PCR	qPCR
1	PGIP 220-E	—	—
2	PGIP 220-5	—	—
3	PGIP 220-11	†	†
4	PGIP 220-1	—	†
5	PGIP 220-9	—	—
6	PGIP 220-14	—	—
7	PGIP 220-3	†	†
8	PGIP 220-13	—	—
9	PGIP 220-A	—	—
10	PGIP 220-D	—	—
11	SPAD1-4	NT	NT
12	SPAD1-10	†	†
13	SPAD1-6	—	†
14	SPAD1-7	†	†
15	PGIP 220-42A	†	—
16	SPAD1-I	†	†
17	SPAD1-B	†	†
18	SPAD1-8	†	—
19	SPAD1-12	†	†
20	SPAD1-1A	†	†
21	PGIP 220-15	—	—
22	SPAD1-2	—	—

Transgenic lines highlighted in tan color are the 3 full-length transgenic lines while lines highlighted in purple contain the AD1-3 HA fragment.
†= this line tested positive for a *Xf* HA insert by standard and/or qPCR
— = this transgenic line tested negatively for a *Xf* hemagglutinin insert by PCR
NT = not tested by PCR for presence of HA gene

Table 2. RNA RT-qPCR of Thompson Seedless HA transgenic lines*.

Line ID	Relative transgenic *Hxf* RNA level
SPAD1-B	28.9
SPAD1-10	28.1
PGIP 220-01	27.9
PGIP 220-11	26.6
SPAD1-07	25.8
PGIP 220-03	19.8
SPAD1-08	19
SPAD1-12	14.7
Untransformed Thompson Seedless	0

* Total RNA was isolated from leaves of transgenic grape plants, converted to cDNA by reverse transcriptase and quantified by qPCR with HA specific primers. SPAD1 lines express short constructs and PGIP220 lines express long constructs. The higher the number the higher the RNA level in the leaves.

We were very interested in determining whether any of these lines possessed Pierce's disease resistance by testing the lines in the greenhouse as soon as we had sufficient plants, rather than waiting for the results of extensive ELISA and western blot analysis of transgenics to determine if HA protein could be detected in grapevine xylem sap. On December 8 and 9 of 2011 we inoculated 10 reps of each of the nine PCR-positive transgenic lines with 40 ul of a 10^8 suspension of *Xf* Fetzer in PBS, typically done as two separate 20 ul inoculations on each vine, an amount of inoculum that would be far greater than what a sharpshooter injects into a vine.

We also inoculated untransformed Thompson Seedless and two transgenic lines that did not contain HA inserts by PCR analysis, shown as Transformed Non-transgenic Thompson Seedless in **Figure 1**, as positive controls. **Figure 1** shows the results of disease severity in transgenic and non-transgenic control 16 weeks post inoculation with *Xf*. The Thompson Seedless control, inoculated at the same time as the transgenic vines had a mean disease rating of 3.65 while two of the lines, one containing the truncated HA fragment AD1-3 and one line containing the full-length native HA protein, had the lowest disease ratings of 1.5. Most of the other lines had mean disease severity ratings below 2.0 and the average disease ratings for all of the lines representing the two HA constructs had disease ratings below 2.0. Considering the large amount of inoculum that was used, we are pleased with this promising preliminary result. We will soon be quantifying by culture and qPCR the amount of *Xf* in each of these lines. While clearly some disease symptoms were evident, the severity was much less than the control and this could very well reflect lower *Xf* populations in the transgenic lines. If this does indeed turn out to be true then we might have produced a moderately resistant grapevine that could very well end up being like a Muscadine grapevine, i.e. they can be infected with *Xf* but populations are not high enough to compromise fruit quality. The original hypothesis was that transgenic vines producing HA in the xylem sap might facilitate clumping of *Xf* cells and slow their ability to colonize a mature vine during a growing season such that the incipient infection might very well be pruned off in the dormant season. It will take a couple of years to plant and train to a cordon system that would be then mechanically inoculated, or hopefully with the assistance of the Almeida lab insect inoculated with *Xf*. These initial greenhouse results with young vines certainly warrant further evaluations.

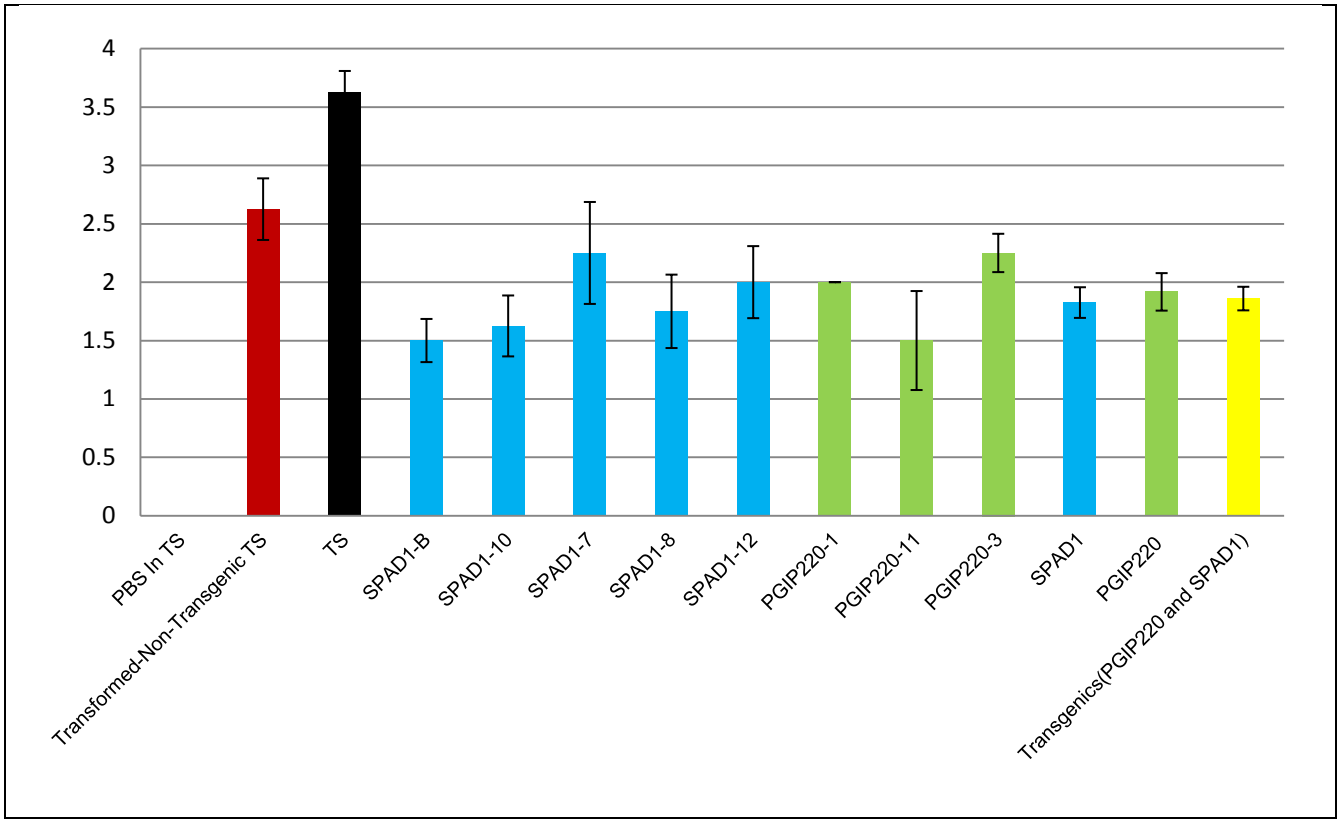

Figure 1. Graph showing the mean disease ratings from 0 to 5 (0 is healthy, 5 is dead) of Pierce's disease symptoms in Thompson Seedless (TS) and transgenic (SPAD1 and PGIP220) vines inoculated with *Xf* Fetzer at 16 weeks post inoculation, except for the Transformed-Non-Transgenic Thompson Seedless, which was inoculated four weeks later and its disease rating is for 12 weeks post inoculation, we anticipated these vines will have disease ratings similar to the Thompson Seedless control at 16 weeks post inoculation. The last three columns are the averages of all inoculated vines of the specified type of construct used, either transformed with AD1-3 (SPAD1) or the full length native HA (PGIP220). Error bars are the standard error of the 10 reps, all PGIP220-1 vines had the same disease rating.

Objective 3a. Secure permits to plant HA transgenic lines in the field at UCD.
This objective was completed with the assistance of PIPRA.

Objective 3b. Plant transgenic vines in the field.
Approximately 120 HA-transgenic vines representing all the transgenic lines that were produced were planted in the field in April 2013 and trained as bilateral cordons (**Figure 2**).

Figure 2. HA-transgenic and non-transgenic control vines planted in the field.

Objective 4. Following greenhouse testing, graft promising *Hxf* transgenic rootstocks to untransformed scions.
Additional vines from all nine transgenic lines are being propagated in the greenhouse and when they obtain sufficient size they will be top grafted with non-transgenic Thompson Seedless scions.

Objective 5. Generate grape transgenic plants over-expressing the grape thaumatin-like protein (TLP).
The wild-type TLP gene from Thompson Seedless grapevines was cloned into an *Agrobacterium* transformation vector that was developed in the Dandekar lab. This vector has signal peptide sequences that facilitate the expression and translocation of the TLP into grapevine xylem. We received 15 independently transformed TLP transgenic lines from the UC Davis Plant Transformation Facility. Two of those lines died before they could be vegetatively propagated. RNA was extracted from the remaining 13 lines as well as non-transformed Thompson Seedless grapevine controls using standard procedures. Equal amounts of total RNA were analyzed by a semi-quantitative RT PCR (qRT PCR) using primers specific for the grapevine TLP. TLP mRNA expression was elevated from 2X to 15X higher in the transgenic vines compared to TLP expression in the non-transformed controls. **Figure 3** below shows the results of this analysis.

Ten reps of the TLP transgenic vines (13 distinct lines) and wild-type Thompson Seedless grapevines were mechanically inoculated using two 20 µl drops of 10^7 (as measured by optical density) *Xf* Fetzer cells suspended in PBS. Control Thompson Seedless and TLP vines were inoculated with PBS. These vines were kept in the greenhouse and were observed for Pierce's disease symptom development over 17 weeks. When symptoms first became apparent, two of the TLP lines appeared to develop symptoms more rapidly than the Thompson Seedless plants. These differences proved to be not statistically significant. The Thompson Seedless and TLP progressed through symptom development with no significant differences (**Figures 4 and 5**). At 15 weeks bacterial cells were isolated from the point of inoculation (POI) as well as 25cm above POI. These results are still being processed at the time of writing.

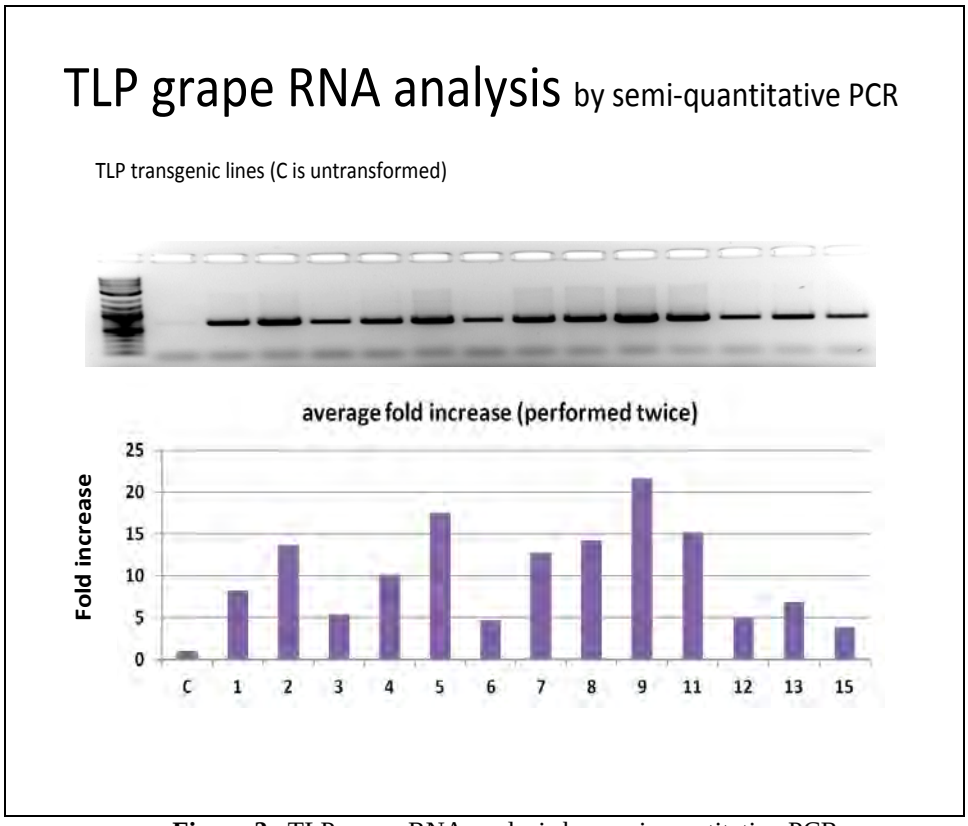

Figure 3. TLP grape RNA analysis by semi-quantitative PCR.

Due to our past observations with TLP in non-transgenic grapes we will be over-wintering both the Thompson Seedless and TLP transgenic vines in an outdoor screen house at UCD to determine if elevated TLP levels could possibly encourage cold curing at temperatures that are not sufficient to produce cold curing in non transgenic grapes.

Figure 4. Representative Pierce's disease symptoms in TLP-transgenic and non-transgenic Thompson Seedless vines mechanically inoculated with wild-type Fetzer *Xf* or PBS controls 15 weeks post-inoculation.

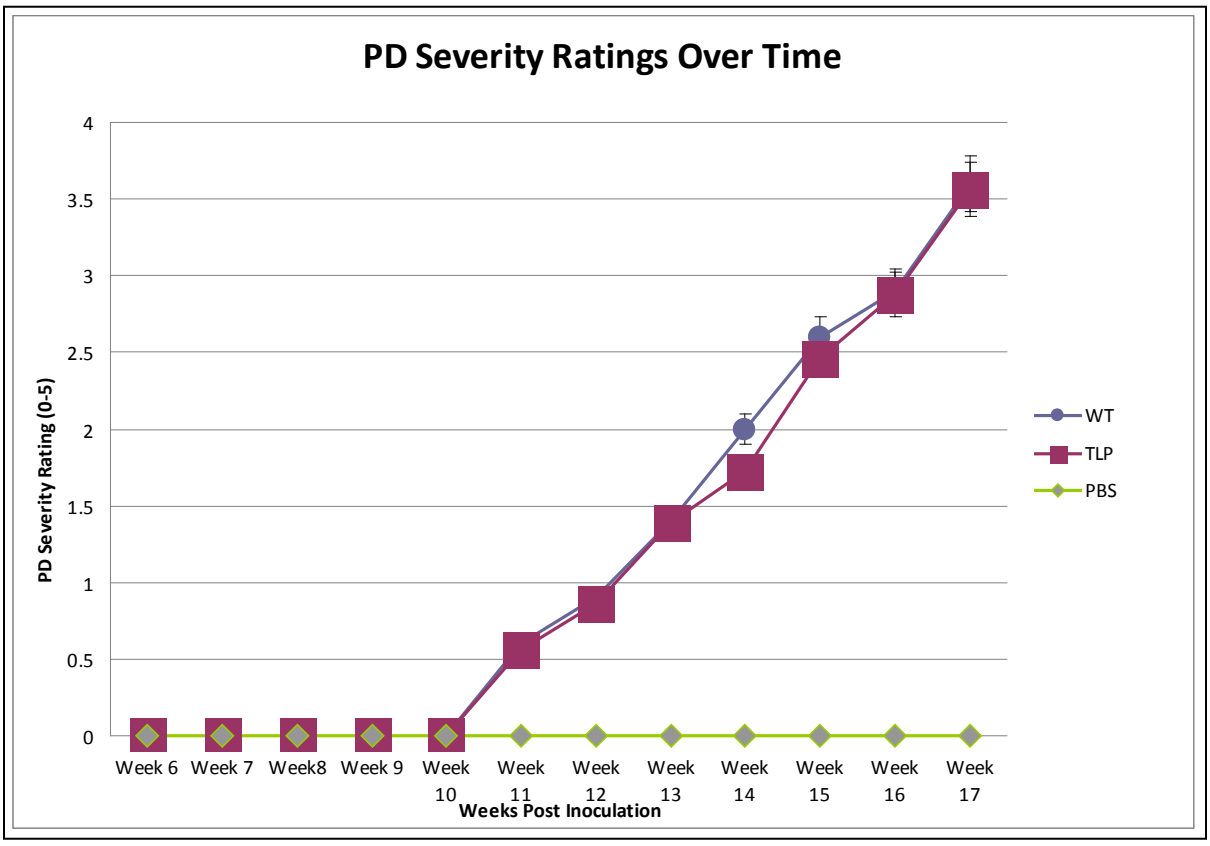

Figure 5. Pierce's disease symptom severity following mechanical inoculation with wild-type Fetzer *Xf*.

CONCLUSIONS

Ten HA-transgenic lines were shown by qRT-PCR to express HA mRNA. Greenhouse inoculations of the nine HA-transgenic Thompson Seedless grapes with cultured *Xf* cell showed all lines expressed less severe symptoms of Pierce's disease than inoculated, non-transgenic controls. All transgenic lines as well as non-transgenic Thompson Seedless vines that will be used as positive and negative controls were planted in the field in spring 2013. The vines grew well and were trained as bilateral cordons. Two to four shoots on each vine will be mechanically inoculated with wild-type *Xf* in April 2014. Pierce's disease symptoms on inoculated and non-inoculated shoots will be evaluated in September 2014. All shoots will be pruned back to two buds and allowed to push during the 2015 growing season. Final Pierce's disease symptoms will be recorded in September 2015. If *Xf* populations in HA-transgenic lines are low enough to prevent fruit symptoms and vine dieback we may have produced transgenic vines that are functionally tolerant of *Xf* infection. Their possible use as rootstocks grafted with non-transgenic scions will be evaluated in the coming years.

Thompson Seedless grapevines were transformed with a grapevine thaumatin-like protein (TLP) gene. Elevated TLP mRNA was found in all transgenic lines as compared to non-transformed controls. TLP vines and non-transformed vines were mechanically inoculated with *Xf* in the greenhouse. Unfortunately there was no difference in Pierce's disease symptom severity or disease onset in the transformed versus non-transformed controls. All TLP and control vines will be moved into a screen house and allowed to undergo dormancy this winter. Disease symptoms will again be recorded in September 2014 to determine if there are any differences in the reoccurrence of Pierce's disease symptoms in the transformed versus non-transformed vines.

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

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

LAYPERSON SUMMARY
We have obtained phages that putatively bind to each of the polygalacturonase (PG) targets. The peptides and scFvs encoded by these phages will be used in PG inhibition assays. If any of the candidate phages can inhibit PG activity *in vitro* then we will test their efficacy versus the other PGs used in this study. If we are able to obtain a peptide or scFv which can inhibit multiple PGs, it is likely binding to the active site amino acids. This would suggest activity against *Xf* PG and we would proceed to transform grapevines with the peptide and determine if they inactivate *Xf* PG *in planta* and possibly confer resistance to Pierces disease.

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

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

OBJECTIVES
1. Localization and isolation of sufficient amounts of biologically active *Xf* PG enzyme to conduct phage panning and PG-inhibition assays.
2. Isolate M13 phages that possess high binding affinities to *Xf* PG, as well as *Agrobacterium vitis* PG and *Aspergilus aculeatus* PG from a M13 random peptide or scFv antibody libraries.

3. Determine if selected M13 phages and the gp38 M13 protein that mediates phage binding to *Xf* PG and surrogate PGs can inactivate PG activity *in vitro*.
4. Clone anti-*Xf* PG gp38 protein into an *Agrobacterium* binary vector and provide this construct to the UCD Plant Transformation Facility to produce transgenic Thompson Seedless grapevines.
5. Determine if anti-*Xf* PG gp38 protein is present in xylem sap of transgenic plants.
6. Mechanically inoculate transgenic plants with *Xf* and compare Pierce's disease development with inoculated, non-transgenic control plants.

RESULTS

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

Xf does not produce a detectable amount of PG when grown in biological media. Furthermore, attempts at expressing *Xf* PG in *Escherichia coli*, yeast, and plant-based viral expression systems have not produced active *Xf* PG. Because of this, *Xf* strains have been engineered that will constitutively express the PG gene. The pBBR1MCS and pPROBE broad host range cloning vectors provided the *Xf* expression plasmid backbone and the constitutive nptII promoter was utilized to drive protein expression (Miller et al., 2000, Kovach et al., 1995). Green fluorescent protein (GFP) reporter constructs made using this plasmid system stably expressed GFP in *Xf* under antibiotic selection. Additionally, the amount of GFP produced using this system is readily detectable on a Coomassie stained polyacrylamide gel (**Figure 1**). *Xf* PG-expressing constructs have been tested for the production of *Xf* PG and western blot analysis using polyclonal anti-*Xf* PG antibodies indicates that the constructs are producing *Xf* PG, indicated by the presence of a 55kd band in the *Xf* PG over-expression strains that is not present in the *Xf* strain over-expressing GFP (**Figure 1**). Tandem mass spec analysis of *Xf* produced PG indicates that *Xf* PG is being processed in *Xf* and likely is a major factor in the apparent size difference between *E. coli* produced recombinant PG and *Xf* produced PG. We have begun testing these strains for PG activity and there seems to be some activity associated with the *Xf* PG containing fractions. However, the plasmids generated seem to be slightly unstable in *Xf* as GFP and PG expression drops off after repeated transfers on selective media. This fact is hampering our efforts to produce the large amounts of active *Xf* PG we need for the subsequent objectives in this project. Surprisingly, this plasmid does not suffer the same instability issues in *E. coli*, as both plasmids are stable even after many subsequent platings on selective medium. Interestingly, *Xf* remains resistant to the antibiotic resistance provided on the plasmid suggesting that the DNA on the plasmid is being rearranged as opposed to losing the plasmid entirely. The strain of *E. coli* we are using has a mutation of the *recA* gene, which is involved in homologous recombination of DNA and plasmid stability. *Xf* has a single copy of the *recA* gene and there is a strong possibility that mutation of this gene may eliminate the DNA rearrangement in the plasmid that is leading to the instability of this plasmid in *Xf*. We are currently creating *recA* mutants in *Xf* to use with our expression plasmids to test this hypothesis. Activity of *Xf* produced PG will be assayed using two methods. The first is the 2-cyanoacetamide reducing sugar assay, a spectrophotometric method which quantitatively measures the increase in reducing end accumulation due to PG enzymatic degradation of polygalacturonic acid (Gross 1982). The second is a cup plate diffusion assay in which activity is represented by a colorimetric clearing around where the enzyme is introduced into a plate of agarose containing polygalacturonic acid (Taylor and Secor 1988). As *Xf* PG has not previously been detected in *in vitro* culture supernatants or xylem sap from *Xf* infected grapevines, we feel it is important to determine where *Xf* is present in the newly developed *Xf* PG-producing strains.

Objective 2. Isolate M13 phages that possess high binding affinities to *Xf* PG, as well as *Agrobacterium vitis* PG and *Aspergilus aculeatus* PG from a M13 random peptide or scFv antibody libraries.

Due to the difficulties encountered in producing active *Xf* PG enzyme we have decided to use two other PGs, as well as smaller peptides constituting sections of the active site of *Xf* PG, as surrogates to confirm that an enzymatic inhibitor can be isolated using phage panning techniques.

Agrobacterium vitis (*Av*) is a plant pathogenic bacterium that causes crown gall disease in grapevines. Like *Xf*, *Av* also requires a PG in order to move from xylem element to xylem element. The *Av* PG gene has been previously cloned and shown to be active in *in vitro* activity assays (Herlache et al 1997). In addition, because the active sites of PGs are so highly conserved and need to degrade the same substrates in the same host plant (*Vitis vinifera*); a peptide which inhibits *Av* PG may also inhibit *Xf* PG. Furthermore, an inhibitor of *Av* PG activity would also prove useful for California grape growers for a possible control method of crown gall of grapevines.

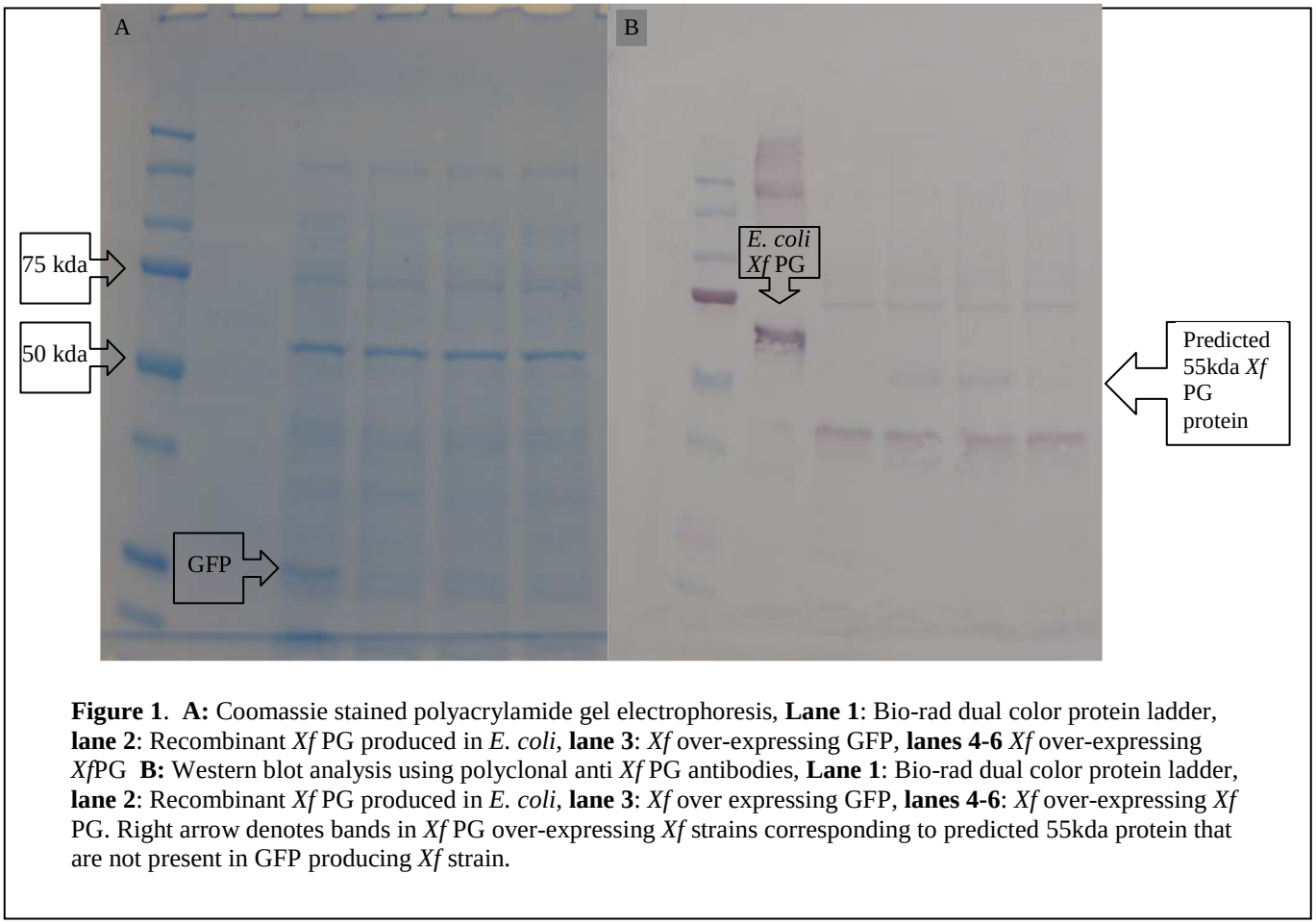

Figure 1. **A:** Coomassie stained polyacrylamide gel electrophoresis, **Lane 1**: Bio-rad dual color protein ladder, **lane 2**: Recombinant *Xf* PG produced in *E. coli*, **lane 3**: *Xf* over-expressing GFP, **lanes 4-6** *Xf* over-expressing *Xf*PG **B:** Western blot analysis using polyclonal anti *Xf* PG antibodies, **Lane 1**: Bio-rad dual color protein ladder, **lane 2**: Recombinant *Xf* PG produced in *E. coli*, **lane 3**: *Xf* over expressing GFP, **lanes 4-6**: *Xf* over-expressing *Xf* PG. Right arrow denotes bands in *Xf* PG over-expressing *Xf* strains corresponding to predicted 55kda protein that are not present in GFP producing *Xf* strain.

For this reason we have cloned the *Av* PG gene into an *E. coli* over-expression system to produce recombinant *Av* PG to use in inhibition assays. Experiments have shown recombinant *Av* PG is being produced in large amounts and is enzymatically active in cup plate assays. Panning was carried out according to a standard protocol and at the end of the third round of selection a monoclonal ELISA with each respective PG as the target was run. The results indicated a majority of the monoclonal phages from each library (I, J, and Ph.D7) showed a higher binding affinity to each respective PG than to casein (blocking protein), or to the wells of the plate. Additionally, the monoclonal phages from each selection were then used individually as the primary antibodies in western blots to confirm that monoclonal phage were binding to the correct target. With this knowledge 16 clones from each library (I, J, and Ph.D7:*Av* PG only) providing the highest ELISA absorbance readings were chosen for sequencing. The sequencing results for panning against *Av* PG with the Ph.D7 library revealed that five unique phage sequences account for over 50% of the phage pool and one phage sequence represents 25% of the phage. Additionally these phages share a four amino acid consensus sequence. *Av* PG panning for I&J libraries resulted in a consensus sequence but no single phages dominated the phage pool. Panning with FPLC purified *Xf* PG as the target resulted in a consensus phage sequence and there were five phage sequences that accounted for nearly 50% of the phage pool. Interestingly an alignment of *Av* PG and FPLC purified *Xf* PG monoclonal phage also resulted in a consensus sequence. Which was not the case when aligning to Aa PG. Aa PG panning with I&J libraries resulted in a consensus sequence and nearly 60% of the phages in the phage pool belonged to one of five different phage sequences. The peptides similarly to *Av* PG presented a consensus sequence but no phages dominated the phage pool. The peptide/scFv sequences encoded by these phage are currently being synthesized/expressed and will be used in inhibition assays.

We have also completed the phage panning procedure for peptide 2 using the Tomlinson I and J scFv libraries. At the end of the third round of selection a polyclonal ELISA with bovine serum albumin (BSA) conjugated peptide 2 as the target was run which showed that each library (I and J) of scFvs showed a higher binding affinity to BSA conjugated peptide 2 than to BSA alone, or to the wells of the plate. With this knowledge 90 individual colonies

from each library were picked from the third round phage pool and used in a monoclonal ELISA to determine which monoclonal scFvs had the highest binding efficiencies. The eight clones from each library (I and J) providing the highest ELISA absorbance readings were chosen for sequencing. We have currently only sequenced the heavy chain variable portion of the scFv and although none of the eight clones from each of libraries shared the exact same sequence they did have similarities to each other. The eight monoclonal phages from each library (I and J) were then used individually as the primary antibodies in a western blot to confirm that monoclonal phage raised against the 14-mer peptide 2 conjugated to BSA would also be able to indentify full length recombinant PG (Tanaka et al 2002) (Figure 2).

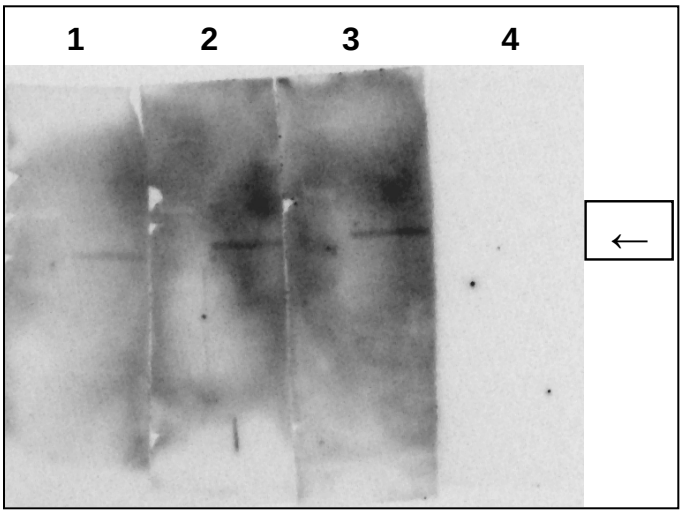

Figure 2. Western blot analysis of three representative monoclonal scFv phages (J-library). Lanes 1-3 are *E. coli* lysate containing recombinant *Xf*PG. Each membrane strip was reacted with a single monoclonal phage from the third round of panning. Arrow represents location of *Xf*PG band. Molecular weight markers are on the left side of each gel strip. Lane 4 is a conjugate control that was not reacted with any monoclonal phage.

Objective 3. Determine if selected M13 phage and the gp38 M13 protein that mediates phage binding to *Xf* PG and surrogate PGs can inactivate PG activity *in vitro*.

We have begun screening the peptides obtained in the phage panning in objective 2 against their corresponding PG targets to test for enzymatic inhibition. Of the limited number of phages we have screened so far none have shown any inhibitory properties toward any of the PGs we are using. However, one of the peptides obtained using the Ph.D. phage library appears to show an increase in PG activity in the cup plate assays (**Figure 3**). While this result was unexpected it does show that phage panning can provide us with peptides that can modulate the activity of PGs *in vitro.* We are continuing to screen the remaining peptides we have to determine if any of them will inhibit PG activity. Furthermore, since this peptide seems to increase PG activity we are in the process of testing to see if it will also boost the activity of the small amount of *Xf* PG we can obtain.

Once a candidate phage is found that can inhibit *Xf* PG *in vitro* we will then express the scFv protein alone and determine if the protein itself can also inhibit *Xf* PG activity *in vitro*. We will then be able to clone the anti-*Xf* PG protein into an *Agrobacterium* binary vector and provide this construct to the UCD Plant Transformation Facility to produce transgenic SR1 tobacco and Thompson Seedless grapevines. Once we have transgenic plants we will be able to complete objectives 5 and 6.

Objective 4. Clone anti-*Xf* PG gp38 protein into an *Agrobacterium* binary vector and provide this construct to the UCD Plant Transformation Facility to produce transgenic SR1 tobacco and Thompson Seedless grapevine.

Once suitable inhibitory phage peptides are discovered in objective 3 we can begin objective 4.

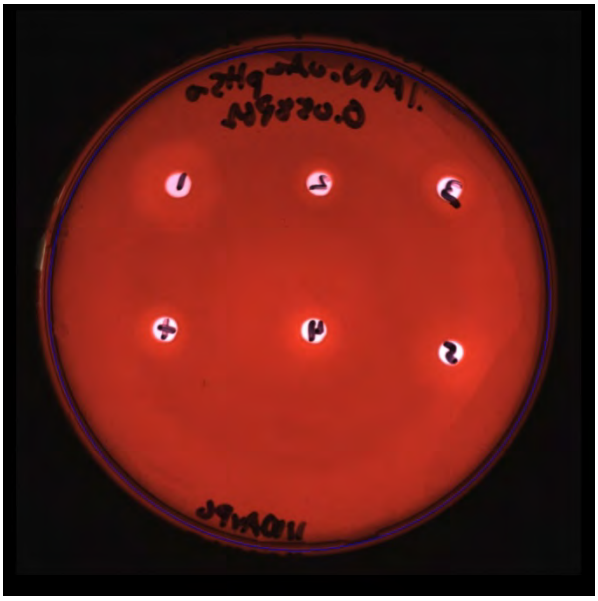

Figure 3. PG cup plate assay, a small clear halo representing enzyme activity is evident in the positive control (+) from *A. vitis*. Cups 1-5 contain *Av* PG, as well as, synthesized peptides based on sequencing results of Ph.D. phage panning experiments using *Av* PG as the target. Note the larger halo surrounding cup one suggesting increased activity.

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

Objective 6. Mechanically inoculate transgenic plants with *Xf* and compare Pierce's disease development with inoculated, non-transgenic control plants.
All previous objectives must be completed before we can start objective 6.

CONCLUSIONS

We have made progress on what has been the biggest obstacle thus far in this project, which is creating enzymatically active *Xf* PG to pan and test our putative inhibitory phage against. *Xf* PG over-expression experiments in *Xf* have shown that we can produce recombinant PG in *Xf* and initial tests point to some fractions that may contain active *Xf* PG. Once we overcome some of the instability issues of this plasmid system we can then test the efficacy of the inhibitory phage we have obtained from panning against the peptides representing the active site of *Xf* PG. Additionally, we have acquired a large pool of putative inhibitors from our panning experiments to screen our PGs against. If one of the candidate phage can inhibit *Xf*PG activity *in vitro* then we can transform grapevines with the peptide and determine if they provide plants with resistance to Pierces disease.

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

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ABSTRACT
The development of Pierce's disease control strategies utilizing transgenic rootstocks expressing and exporting candidate factors to wild-type scions has been a well-received and promising focus of research, from both scientific and public appeal standpoints. Plant-derived polygalacturonase-inhibiting proteins (PGIPs) target specific pathogen and pest polygalacturonases (PGs). Several PGIPs were selected to determine the best candidates for *Xylella fastidiosa* (*Xf*) PG inhibition. Three PGIP sequences, one each from pear, orange, and rice, were chosen based on predicted protein-protein interactions with *Xf*PG. Recombinant protein expression systems were developed to provide active *Xf*PG and each PGIP. Preliminary *in planta* activity assays have shown that when transiently expressed in tobacco leaves, the pear fruit PGIP is a more effective inhibitor of *Xf*PG than is tomato PGIP. The transient expression systems have been expanded by cloning the enzyme sequences into different *Agrobacterium tumefaciens* strains. Current work involves evaluating the inhibitory capacity of the PGIPs in grape plants with initial evaluations in tobacco and tomato because tissues from these plants are continuously available. We were able to detect *Xf*PG activity in plant tissues and we have been able to detect pPGIP protein in non-transgenic grape scion leaves at least 70 centimeters away from the graft site with pPGIP expressing rootstocks.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) uses a key enzyme, polygalacturonase (PG), to spread throughout the grapevine from the initial point of inoculation; this spread leads to Pierce's disease symptom development. Proteins called polygalacturonase-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 Pierce's disease development in inoculated grapevines which have been transformed to express the pear PGIP protein. PGIPs are secreted from cells and can passively travel across graft junctions. The goal of the project is to identify the PGIP that best inhibits *Xf*PG and ascertain how well, when this PGIP is expressed in transgenic rootstocks, it prevents Pierce's disease development in grafted wild-type scions inoculated with *Xf*. We modeled the protein structures of 14 candidate PGIPs to predict how the PGIPs physically interact with *Xf*PG and we selected three candidate PGIPs. We use *in vitro* and *in planta* assays to measure the ability of the candidate PGIPs to inhibit *Xf*PG. To do these assays we have had to develop systems to generate high levels of active *Xf*PG and PGIPs. The aim of the project is to identify the best candidate PGIP and evaluate grafted grape rootstocks, so that we can develop a strategy to limit Pierce's disease development in non-transgenic grape scions.

INTRODUCTION
Xylella fastidiosa (*Xf*), the causative agent of Pierce's disease (PD) in grapevines, has been detected in infected portions of grapevines after spreading systemically from the point of inoculation. Several lines of evidence support the hypothesis that *Xf* uses cell wall degrading enzymes to digest the polysaccharides of plant pit membranes separating the elements of the water-conducting vessel system, the xylem, of the vines. Recombinantly expressed *Xf* polygalacturonase (PG) and β-1,4-endo-glucanase (EGase) have been shown to

degrade grapevine xylem pit membranes and increase pit membrane porosity enough to allow passage of the bacteria from one vessel to the next (Pérez-Donoso et al., 2010). *Xf* cells have been observed passing through similarly degraded pit membranes without the addition of exogenous cell wall degrading enzymes, supporting the conclusion that the enzymes are expressed by *Xf* and allow its movement within the xylem (Sun et al., 2011). Roper et al. (2007) developed a PG-deficient strain of *Xf* and showed that the mutant bacterial strain was unable to cause Pierce's disease symptoms; thus, the *Xf*PG is a virulence factor that contributes to the development and spread of Pierce's disease.

Polygalacturonase-inhibiting proteins (PGIPs) are naturally produced by plants and are selective inhibitors of PGs; they limit damage caused by many types of pathogens. Agüero et al. (2005) demonstrated that a pear fruit PGIP (pPGIP) expressed in grapevines, reduced susceptibility to both fungal (*Botrytis cinerea*) and bacterial (*Xf*) pathogens. This result implied that the pPGIP provided protection against Pierce's disease 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). Agüero et al. (2005) also demonstrated that the pPGIP protein could be transported from rootstocks, across a graft junction and into the grafted wild-type grape scions. We have shown that pPGIP is transported from grafted tomato rootstocks (Haroldsen et al., 2012).

The overall goal of the project is to develop transgenic grape rootstock lines that express PGIPs that effectively reduce the virulence of *Xf*. To do this, we proposed to compare potential *Xf*PG inhibiting properties of PGIPs from a wide variety of plants in order to identify PGIPs that optimally inhibit *Xf*PG. The most effective PGIPs will be expressed in grape rootstocks to provide Pierce's disease 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.

OBJECTIVES

1. Define a path for commercialization of a Pierce's disease control strategy using PGIPs, focusing on IP and regulatory issues associated with the use of PGIPs in grape rootstocks.
2. Identify plant PGIPs that maximally inhibit *Xf*PG.
3. Assemble transcription regulatory elements, *Xf*-inducible promoters, and signal sequences that maximize PGIP expression in and transport from roots.
4. Create PGIP-expressing rootstocks and evaluate their Pierce's disease resistance.

RESULTS AND DISCUSSION

Objective 1. A path to commercialization of transgenic rootstocks.

The Public Intellectual Property Resource for Agriculture (PIPRA) evaluated the intellectual property (IP) around each of 14 candidate PGIP genes (see Objective 2B) using protein-based queries to search the patent and patent application databases with the program GenomeQuest. The use of the PGIP sequences from non-*vinifera* grape varieties was not possible due to restrictions on their release by the wine and grape industry board associated with the Institute for Wine Biotechnology at Stellenbosch University, South Africa.

PIPRA acted as a liaison for the CDFA Pierce's Disease and Glassy-winged Sharpshooter (PD/GWSS) Board for issues associated with the potential commercialization of various approaches using transgenic grapevine rootstocks for several CDFA PD/GWSS Board-funded projects, including the strategy outlined in this project. Beginning in 2010, PIPRA analysts managed the initial permitting process for the field trial testing of Thompson Seedless and Chardonnay varieties of grapevines expressing pPGIP and established the Biotechnology Quality Management System (BQMS) protocols. Some of their work was funded through a separate contract. The IP information obtained determined that there were no IP issues related to these particular genes and none were identified.

Objective 2. Identifying plant PGIPs that maximally inhibit *Xf*PG.

Objective 2A. Propagation and grafting of grape lines expressing and exporting pPGIP.

The pPGIP-expressing Chardonnay and Thompson Seedless grapevines described in Agüero et al. (2005) were maintained throughout the project in the UC Davis Core Greenhouses. The propagation and grafting techniques used for this objective are described in the progress reports for the project "Field Evaluation of Grafted Grape Lines Expressing pPGIP" (PI: Powell). These efforts provided more than sufficient grafted, transgrafted, and

own-rooted plants for the field test sites in Solano and Riverside counties. All the plots for this project at both locations were completely planted by June 2013.

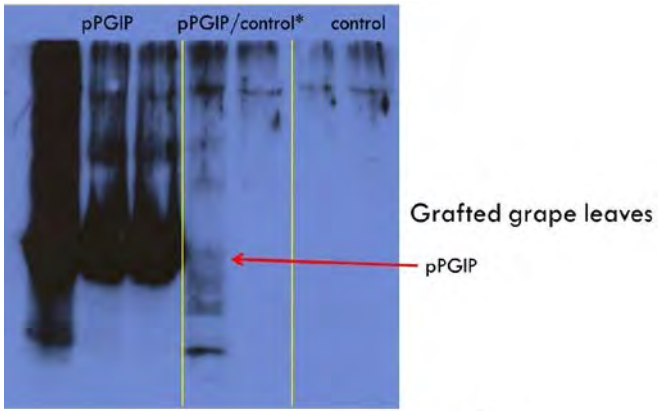

Figure 1. Western blot showing pPGIP protein collected from grafted and own-rooted grapevines. The rootstock in the graft, but not the scion, expressed pPGIP. The grafted samples were taken at least 30 cm beyond the graft junction, indicating translocation of the pPGIP protein.

Using grafted plants to detect active exogenous pPGIP translocated from transgenic rootstocks to the scion portion of the plants, we modified a pressure flow apparatus to flush long stem segments with water or high salt buffers. We were able to obtain xylem exudate containing a small amount of total proteins (26 μg/ml) from own-rooted, transgenic pear fruit PGIP (pPGIP)-expressing Thompson Seedless (TS) stems. We used the polyclonal pPGIP antibodies and identified pPGIP in macerated grape leaves and stem segments, but could not identify cross-reactive pPGIP in xylem exudate (**Figure 1**). There was insufficient protein to measure PG inhibiting activity in the proteins collected from the grapevine xylem exudate or macerate.

We also evaluated proteins collected from the xylem of non-transgenic tomato scions grafted onto transgenic tomato rootstocks expressing pPGIP. We used this system because we were able to gather more protein from the xylem and thus could detect the pPGIP protein in xylem sap. We confirmed that pPGIP protein is expressed in the rootstocks and were able to use the pressure device to force xylem sap out of the cut stems of own-rooted and grafted plants. The xylem sap fluid from non-transgenic scions grafted onto pPGIP-producing rootstocks contained detectable pPGIP protein and the collected protein was able, as expected for pPGIP, to inhibit the PGs collected from cultured *B. cinerea* Del 11 (**Figure 2**).

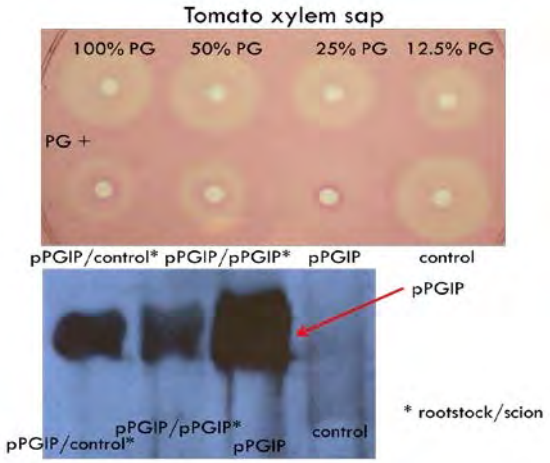

Figure 2. PG inhibiting activity assay (top) of xylem sap from grafted and control tomato plants expressing pPGIP. pPGIP protein is detected (bottom) with polyclonal antibodies in a western blot. Inhibiting activity and pPGIP protein is detected when the root portions of the plants express pPGIP.

All experimental plots at both field locations were planted. pPGIP protein was detected in the scions of grafted grape and tomato plants.

Objective 2B. Selection of PGIPs as Pierce's disease defense candidates.
Based on phylogenetic (**Figure 3**), biochemical (**Table 1**), and structural analyses of PGIP full-length protein sequences from 68 diverse plant varieties, the 'Roma' rice, 'Hamlin' orange, and 'Bartlett' pear PGIPs were selected. Since *Xf*PG is unusually and highly positively charged (+22.24 at pH 4.5, **Table 2**), we focused our attention on PGIPs with the lowest overall positive charge at pH 4.5, the pH of most apoplastic plant fluids. This reflected our hypothesis that the PGIPs that would be most likely to interact with *Xf*PG would have the largest charge differences with *Xf*PG.

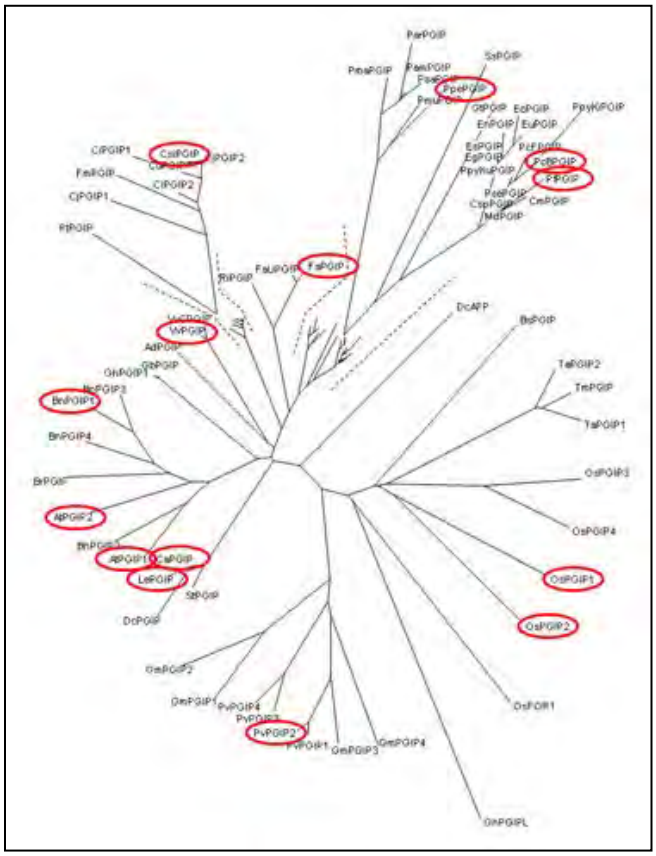

Figure 3. The PGIP amino acid sequences were aligned using ClustalX 2.0.9. An unrooted, neighbor joining phylogenetic tree was constructed in ClustalX and visualized with TreeView 1.6. The resulting radial phylogeny is more robust due to the inclusion of both monocot and dicot sequences. The 14 sequences that were analyzed further in **Table 1** are circled in red.

pPGIP CsiPGIP OsPGIP1

Figure 4. Homology models of three candidate PGIPs. The column of electronegative residues (red) on the concave faces of each protein may align with critical residues on *Xf*PG important for inhibition.

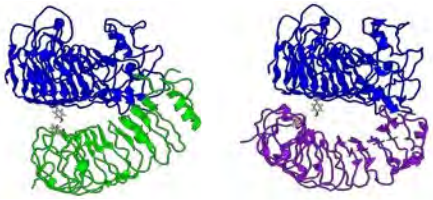

Figure 5. *Xf*PG-PGIP complexes. Tyr303 of *Xf*PG (blue) binds strongly with part of pPGIP (green) which is not possible with VvPGIP (purple). These interactions might influence PG-PGIP interaction and inhibition.

We modeled, with Dan King of Taylor University, the 3D structures of selected candidate PGIPs (**Figure 4**) and *Xf*PG proteins to try to understand the locations of relevant interactions (**Figure 5**). 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. Dynamic *in silico* reaction simulations predicted that two clusters of *Xf*PG 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 PGIPs' leucine-rich repeat structures bind to these critical regions (**Figure 4**). This information coupled with surface chemistry mapping predicts that pPGIP, CsiPGIP (citrus), and OsPGIP1 (rice) will be the best inhibitors of *Xf*PG. 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 5**). Electrostatic repulsions between VvPGIP (grape) residues and *Xf*PG Tyr303 prevent a similar alignment and may explain VvPGIP's failure to inhibit *Xf*PG.

Table 1. Total protein charge analysis for the 14 candidate PGIPs in different pH conditions.

Common name	**Organism**	**Protein**	**Charge of Protein**					
			pH 3.5	*pH 4.0*	*pH* ***4.5***	*pH 5.0*	*pH 5.5*	*pH 6.0*
Thale cress	*Arabidopsis thanliana* (Col.)	AtPGIP1	27.5	20.9	**14.2**	10.0	7.4	5.2
Thale cress	*Arabidopsis thanliana* (Col.)	AtPGIP2	35.4	28.5	**21.6**	17.0	14.2	11.8
Rape	*Brassica napus* cv. DH12075	BnPGIP1	30.5	22.2	**14.2**	9.4	6.8	4.8
Pepper	*Capsicum annum* cv. arka abhir	CaPGIP	20.7	15.2	**9.5**	5.9	3.8	2.2
Sweet orange	*Citrus sinensis* cv. Hamlin	CsiPGIP	28.0	21.7	**15.2**	11.1	8.7	6.7
Strawberry	*Fragaria x ananassa*	FaPGIP	25.4	18.7	**12.1**	8.0	5.6	3.7
Rice	*Oryza sativa* cv. Roma	OsPGIP1	18.4	12.9	**7.6**	4.3	2.2	0.2
Rice	*Oryza sativa* cv. Roma	OsPGIP2	17.5	9.3	**1.6**	-3.1	-6.1	-8.8
Common bean	*Phaseolus vulgaris* cv. Pinto	PvPGIP2	22.7	17.6	**12.9**	10.2	8.5	7.1
Peach	*Prunus persica*	PpePGIP	28.7	21.9	**14.9**	10.3	7.5	5.3
Chinese Firethorn	*Pyracantha fortuneana*	PfPGIP	16.9	11.7	**6.6**	3.4	1.4	-0.3
Bartlett pear	*Pyrus communis* cv. Bartlett	pPGIP	23.1	16.1	**9.3**	5.0	2.6	0.7
Tomato	*Solanum lycopersicum* cv. VFNT Cherry	LePGIP	29.8	23.4	**17.0**	12.8	10.1	7.7
Grape	*Vitis vinifera* cv. Pinotage	VvPGIP	30.5	24.0	**17.7**	13.6	11.1	8.7

Table 2. Total protein charge analysis for the fungal, bacterial and plant PGs in different pH environments.

	PG								
pH	***X. fastidiosa* PG**	***F. moniforme* PG**	***A. niger* PGC**	***A. niger* PGB**	***A. niger* PGA**	***A. niger* PG2**	***A. niger* PG1**	**Tomato PG**	**Grape PG**
3.50	40.99	17.90	4.79	27.25	3.88	21.94	10.46	30.88	32.19
4.00	31.30	11.25	-9.53	18.45	-11.40	12.86	-2.13	25.51	27.26
4.50	**22.24**	5.44	-23.56	9.92	-26.43	4.08	-14.38	**20.03**	**22.4**
5.00	16.39	2.07	-32.08	4.67	-35.56	-1.35	-21.80	16.26	19.23
5.50	11.90	-0.13	-36.17	1.86	-39.77	-4.36	-25.41	13.54	17.15
6.00	6.79	-2.36	-38.46	-0.20	-41.90	-6.69	-27.53	10.68	15.14

Genomic DNA was prepared from Kitaake rice and Valencia and Washington Navel orange leaves, and PGIPs were PCR amplified. Because of the nucleotide differences between Valencia and Washington Navel and the published PGIP sequence (cited as 'Hamlin' orange), we amplified and cloned PGIP from Hamlin leaves. The resulting PGIP sequence for Hamlin was identical to that of Valencia, indicating that the database is incorrect; this observation modifies our conclusions about the apparent net charge of the protein. In amplifying and cloning the rice PGIPs, we again discovered discrepancies among the cloned and published PGIP sequences. After requesting 'Roma' rice germplasm and cloning the PGIPs, we found a single silent mutation in the published coding sequence of *OsPGIP*. We made some progress towards preparing the transformation vector although additional mutations were found in the inserted pPGIP sequences and therefore we did not pursue this further. The coding sequence of *OsPGIP2* that we obtained does not appear to encode a *bona fide* PGIP and was pursued.

Objective 2C. *Xf*PG expression and purification.

Two strategies were used to obtain active *Xf*PG for assays to compare the inhibition efficiencies of the PGIPs. In one approach, Rachell Booth and her group at Texas State University, San Marcos, tried to express active *Xf*PG protein using expression in heterologous cells. *Drosophila* S2 cells produced quantifiable amounts of PG protein (**Figure 6a, b**) but it had only very slight activity and this activity diminished over time (**Figure 6c**). These efforts did not result in sufficient *Xf*PG for further experiments.

The second strategy was to express *Xf*PG transiently in leaves. A fusion construct with the apoplastic signal sequence from pPGIP was linked to the coding sequence of *Xf*PG for transient expression by *Agrobacterium tumefaciens* of *Xf*PG targeted to the extracellular space. Preliminary agroinfiltration assays (**Figure 7**) with intact tobacco leaves indicated that the targeted *Xf*PG had a similar activity to the non-targeted protein, both resulting in necrotic lesions in the infiltrated tissue, although the necrotic response did not appear for several days. The strain of *A. tumefaciens* used in agroinfiltration experiments has been shown to influence the appearance and severity of necrosis in different plant species and tissues (Wroblewski et al., 2005). Therefore, after conferring with Jan Van Kan (Dept. of Phytopathology, Wageningen University) and other researchers, we obtained and tested other strains of *A. tumefaciens.* All strains yielded similarly confounding background necrosis when infiltrated as empty vector controls.

Because our initial assays of PGIP have used *Botrytis cinerea* PG as a standard, we altered our method for evaluating the activity of PGIPs. In our assays, the PGs produced by the B05.10 strain of *B. cinerea* in culture are not inhibited by pPGIP in our *in vitro* assays. Therefore, we have gone back to the Del 11 *B. cinerea* strain and collected the PGs it produces in culture. We have confirmed that they are inhibited by pPGIP in our *in vitro* assay. One explanation for this difference could be that key PG amino acids recognized by pPGIP as part of the inhibitory protein-protein interaction are different in the B05.10 and Del 11 versions of the key PGs, BcPG1and BcPG2, produced by *B. cinerea* in culture. Alternatively, the two strains could express different amounts of the BcPGs. To test the first hypothesis, we worked with Asst. Prof. Dario Cantu (Dept. of Viticulture and Enology, UC Davis) and sequenced the genome of the Del 11 *B. cinerea* strain. The genome of this strain had not been

sequenced before. Comparisons of the Del 11, B05.10 and SAS56 (another grapevine strain of *B. cinerea*) are shown in **Figure 8**. It is clear that there are several amino acid sequence differences between the PG1 enzymes of these *B. cinerea* lines. We plan to do predictive protein modeling to determine whether these changes occur at sites likely to be involved in the PG1 interaction with pPGIP. This work helped us refine our analysis of key amino acids in the *Xf*PG sequence and identify those that are crucial for inhibition by diverse PGIPs.

Objective 2D. Expression of PGIPs to test *Xf*PG inhibition.

The cloning and expression of candidate PGIPs (Obj. 2B) could be continued. The potential *A. tumefaciens* expression system for *Xf*PG (Obj. 2C) could be used to transiently express and purify active candidate PGIPs. Observations of PGIP activity *in planta* could be made using transgenic model plants for agroinfiltration experiments. The genotypes of tomato and *Arabidopsis* plants previously transformed to express *pPGIP* or *LePGIP* constitutively were confirmed by PCR and these could be used to assay *Xf*PG inhibition activity. These pPGIP-expressing plants will be used to test the efficacy of pPGIP constitutively or transiently expressed *in planta*. Agroinfiltration with *Xf*PG-expressing bacterial strains also could be done on the leaves of own-rooted and transgrafted plants.

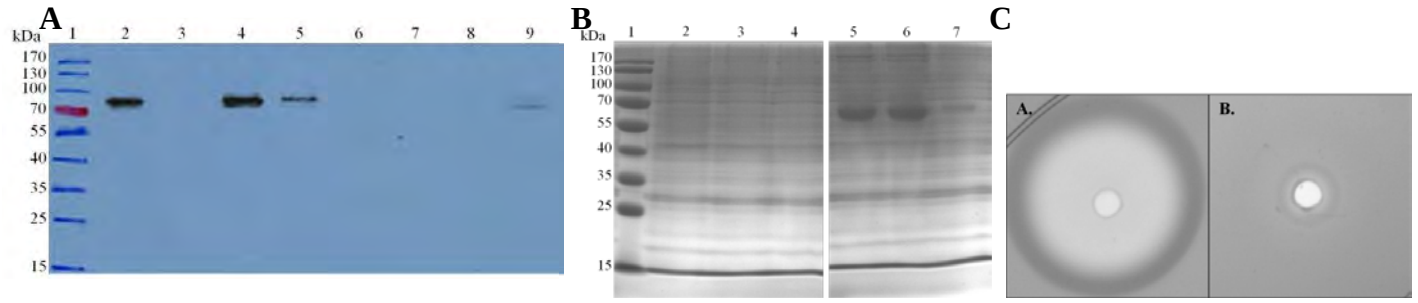

Figure 6. A. Western blot of selected fractions of 15 mL of crude insect cell lysate expressing *Xf*PG protein purified by column chromatography. Lane 1 = pre-stained ladder, lane 2 = flow-through #4, lane 3 = wash #10, lanes 4-7 = elution fractions #1-4, lane 8 and 9 = cellular medium. Recombinant *Xf*PG protein was eluted with 250 mM imidazole and probed with the anti-V5 primary antibody and anti-mouse HRP secondary antibody. **B.** Partially purified *Xf*PG protein eluted with 250 mM imidazole analyzed by polyacrylamide gel electrophoresis and Coomassie staining. Lane 1 = pre-stained ladder, lanes 2-4 = cell lysate fractions #1-3, lanes 5-7 = cellular medium fractions #1-3. **C.** Radial diffusion assays of PG from *Botrytis cinerea* (a) or culture medium from induced *Xf*PG-expressing *Drosophila* cells (b). The area of the cleared zone is proportional to amount of PG activity.

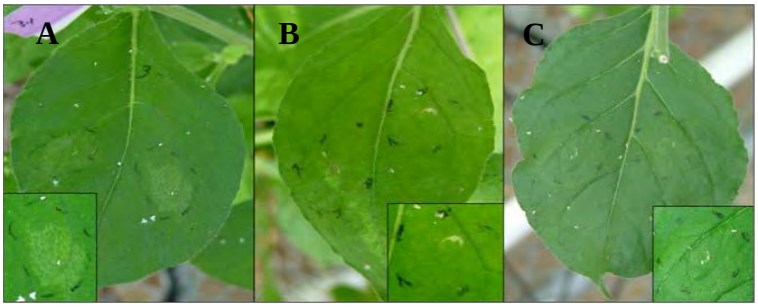

Figure 7. Transient expression of *Xf*PG, pPGIP, and LePGIP in *Nicotiana benthamiana* leaves by infiltration with *Agrobacterium* cultures. Chlorotic lesions and water soaking mark the site of agro-infiltrations with *Xf*PG (**A**). Symptoms are reduced when *Xf*PG is co-infiltrated with pPGIP (**B**) or with LePGIP *Agrobacterium* (**C**). Inserts show details of infiltration sites. Black marks indicate the borders of the initial zone infiltrated.

Figure 8. For BcPG1 (left panel) and BcPG2 (right panel) the closest match in Del11 was aligned to BcPGsfrom B05.10 and SAS56. The gene and coding sequence accessions from SAS56 (Wubben et al., 1999) were used to determine intron positions in SAS56. The coding sequences of B05.10 and Del11 were inferred by comparing the genomic sequences with SAS56 and assuming the same intron-exon junctions. Amino acid changes are highlighted in yellow.

Objective 3. Maximize PGIP expression in and transport from roots.
If we had identified a PGIP to "optimally" inhibit *Xf*PGs, improvements to the expression and delivery of this protein would utilize information being developed in this and other projects.

Objective 4. Create PGIP-expressing rootstocks and evaluate their Pierce's disease resistance.
As discussed previously, the candidate PGIPs would have been assayed *in vitro* for inhibition of *Xf*PG had enough *Xf*PG been available or *in planta* utilizing agroinfiltration and transgrafted tobacco and tomato plants. Grape rootstock transformation could commence once an optimal PGIP has been identified.

CONCLUSIONS
Towards the goal of enhancing Pierce's disease resistance, we have determined that PGIPs from 'Bartlett' pear, but not as we expected, orange, or 'Roma' rice are likely to be very good candidates for *Xf*PG inhibition. We narrowed considerably the possible PGIPs to pursue. Although we have been able to express *Xf*PG in tobacco and have shown that this source of *Xf*PG is active, sufficient, reliable sources of *Xf*PG continue to be a problem plaguing us and other groups. We detected pPGIP protein crossing the graft junctions from rootstocks to non-transgenic scion leaves in grafted grape and tomato plants. The information about the sequence differences in the *Bc*PGs from different strains of *B. cinerea* helps to identify portions of the *Xf*PG that are important targets of PGIPs. The ability of pPGIP, one of the candidates investigated in this proposal, to provide Pierce's disease resistance to transgrafted scions is being addressed by the corresponding field trial. A paper was published based on this work (Haroldsen et al., 2012)

We advanced towards our goal to develop transgenic grape rootstocks that express PGIPs that effectively reduce the virulence of *Xf*, an approach that will help manage the Pierce's disease problem without targeting the growing insect vector population. To achieve this goal, we have had to overcome some information and technical difficulties in this complex system. Because several other pathogens of grapes (both vines and fruit) utilize PG as a part of their tissue infection strategies, it is reasonable to presume that the strategy examined here for Pierce's disease management may have additional beneficial impacts in the vineyard.

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FUNDING AGENCIES

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CONTINUED 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 July, 2011 to October 2013.

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) and 2-Z-hexadecenoic acid (C16-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. 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. All plants at the Solano County experimental site were needle-inoculated with a suspension of *Xf* in May, 2012; at least four vines per plant were inoculated, each at a given site with a 20 ul droplet of *Xf* containing about 10^6 cells of *Xf*. The incidence of infection of the inoculated vines was reduced about three-fold in assessments made in August and September. Disease was observed only near the point of inoculation in transgenic Freedom, but had spread extensively in wild-type Freedom grape. Only a modest reduction in incidence or severity of Pierce's disease was seen in Thompson Seedless grafted onto DSF-producing Freedom rootstocks compared to those grafted on wild-type Freedom. The incidence of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson Seedless, while the incidence and severity of Pierce's disease on Thompson Seedless grafted onto DSF-producing Thompson Seedless rootstocks was less than that of plant grafted onto wild-type Thompson Seedless rootstocks. Plants at the Riverside County plot were subject to high levels of natural infection in 2012. The incidence of infection of transgenic DSF-producing Freedom was about three-fold less than that of wild-type Freedom grape, while the number of infected leaves per vine was about 5-fold less, suggesting that the pathogen had spread less in the DSF-producing plants after insect inoculation. Only a modest reduction in incidence or severity of Pierce's disease was seen in Thompson Seedless grafted onto DSF-producing Freedom rootstocks compared to those grafted on wild-type Freedom. The incidence of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson, while the incidence and severity of Pierce's disease on Thompson Seedless grafted onto DSF-producing Thompson Seedless rootstocks was less than that of plant grafted onto wild-type Thompson Seedless rootstocks.

LAYPERSON SUMMARY

Xylella fastidiosa 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. Plots in both Solano and Riverside counties reveal that DSF-producing Freedom grape, which was highly resistant to Pierce's disease in greenhouse trials is also much less susceptible to disease in field trials, especially in plants naturally infected by sharpshooter vectors. No mortality of the transgenic Freedom plants has been seen, and they remain more highly resistant to Pierce disease than the untransformed plants.

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 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.

OBJECTIVES

1. Determine the susceptibility of DSF-producing grape as own-rooted plants as well as rootstocks for susceptible grape varieties for Pierce's disease.
2. Determine population size of the pathogen in DSF-producing plants under field conditions.
3. Determine the levels of DSF in transgenic *rpfF*-expressing grape under field conditions as a means of determining their susceptibility to Pierce's disease.

RESULTS AND DISCUSSION

Disease susceptibility of transgenic DSF-producing grape in field trials.

Field tests are being performed with two different genetic constructs of the *rpfF* gene in grape and assessed in two different plant contexts. The *rpfF* has been introduced into Freedom (a rootstock variety) in a way that does not cause it to be directed to any subcellular location (non-targeted). The *rpfF* gene has also been modified to harbor a 5' sequence encoding the leader peptide introduced into grape (Thompson Seedless) as a translational fusion protein with a small peptide sequence from RUBISCO that presumably causes this RpfF fusion gene product to be directed to the chloroplast where it presumably has more access to the fatty acid substrates that are required for DSF synthesis (chloroplast-targeted). These two transgenic grape varieties are thus being tested as both own-rooted plants as well as rootstocks to which susceptible grape varieties will be grafted. The following treatments are thus being examined in field trials (**Table 1**).

Table 1. Field trial treatments.

Treat-ment	Code	Type
1	FT	Non-targeted RpfF Freedom
2	TT	Chloroplast-targeted RpfF Thompson Seedless
3	FW	Non-targeted RpfF Freedom as rootstock with normal Thompson Seedless scion
4	TTG	Chloroplast-targeted RpfF Thompson Seedless as rootstock with normal Thompson Seedless scion
5	FWG	Normal Freedom rootstock with normal Thompson Seedless scion
6	TWG	Normal Thompson Seedless rootstock with normal Thompson Seedless scion
7	FW	Normal Freedom
8	TW	Normal Thompson Seedless

Treatments 5-8 serve as appropriate controls 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 a randomized complete block design. Self-rooted plants were produced by rooting of cuttings (about three cm long) from mature vines of plants grown in the greenhouse at UC Berkeley. The plants were inoculated in May 2012 (no natural inoculum of *Xf* occurs in this plot area and so manual inoculation of the vines with the pathogen was performed by needle-inoculated with a suspension of *Xf*. At least four vines per plant were inoculated. Each inoculation site received a 20 ul droplet of *Xf* containing about 10^6 cells of *Xf*.

The incidence of infection of the inoculated vines at the Solano County trial was reduced about three-fold in assessments made in August and September (**Figure 1**). Disease was observed only near the point of inoculation in transgenic Freedom, but had spread extensively in wild-type Freedom grape. Because of the shading of the inoculated vines by subsequent growth of uninoculated vines of the same plant many of the older leaves had died or had fallen from the plant, especially by the September rating, making it difficult to quantify the number of infected leaves per vine. In August, however, we found that there were about three times as many symptomatic leaves on each inoculated vine of wild-type Freedom than on DSF-producing transgenic Freedom (**Figure 2**). Only a modest reduction in incidence or severity of Pierce's disease was seen in Thompson Seedless grafted onto DSF-producing Freedom rootstocks compared to those grafted on wild-type Freedom. The severity of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson Seedless, while the incidence and severity of Pierce's disease on Thompson Seedless grafted onto DSF-producing Thompson Seedless rootstocks was less than that of plant grafted onto wild-type Thompson Seedless rootstocks (**Figure 3**).

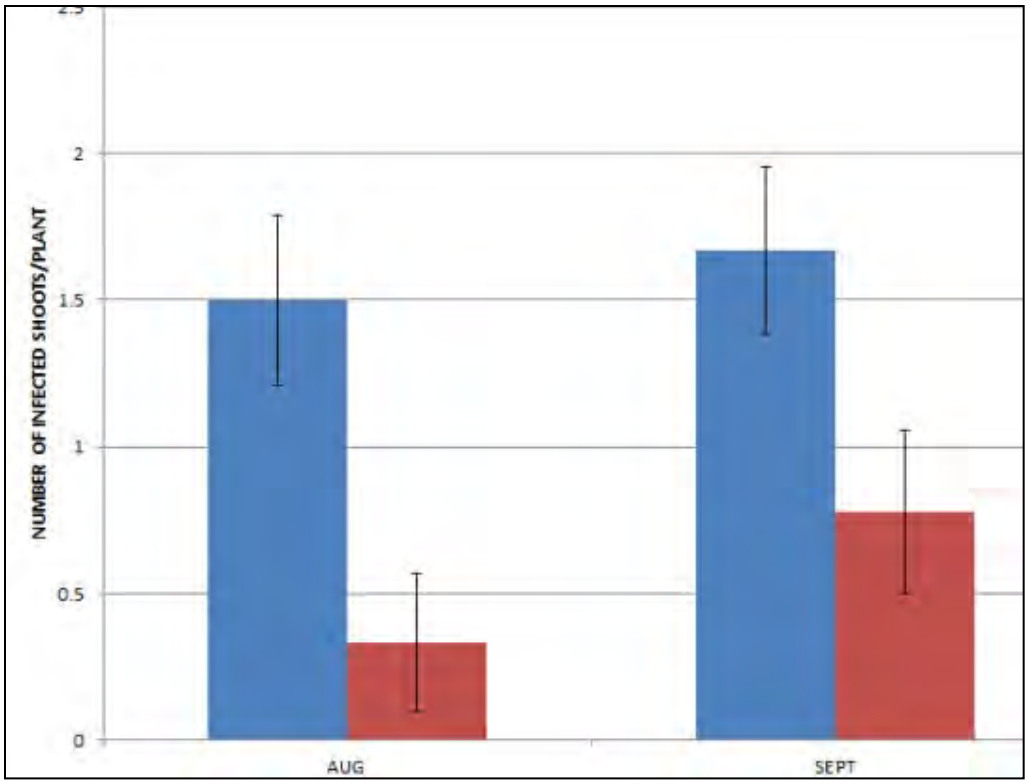

Figure 1. Incidence of vines of DSF-producing transgenic Freedom grape (red) or wild-type Freedom having any symptoms of Pierce's disease when rated in August or September, 2012. A total of three vines per plant were assessed. The vertical bars represent the standard error of the mean.

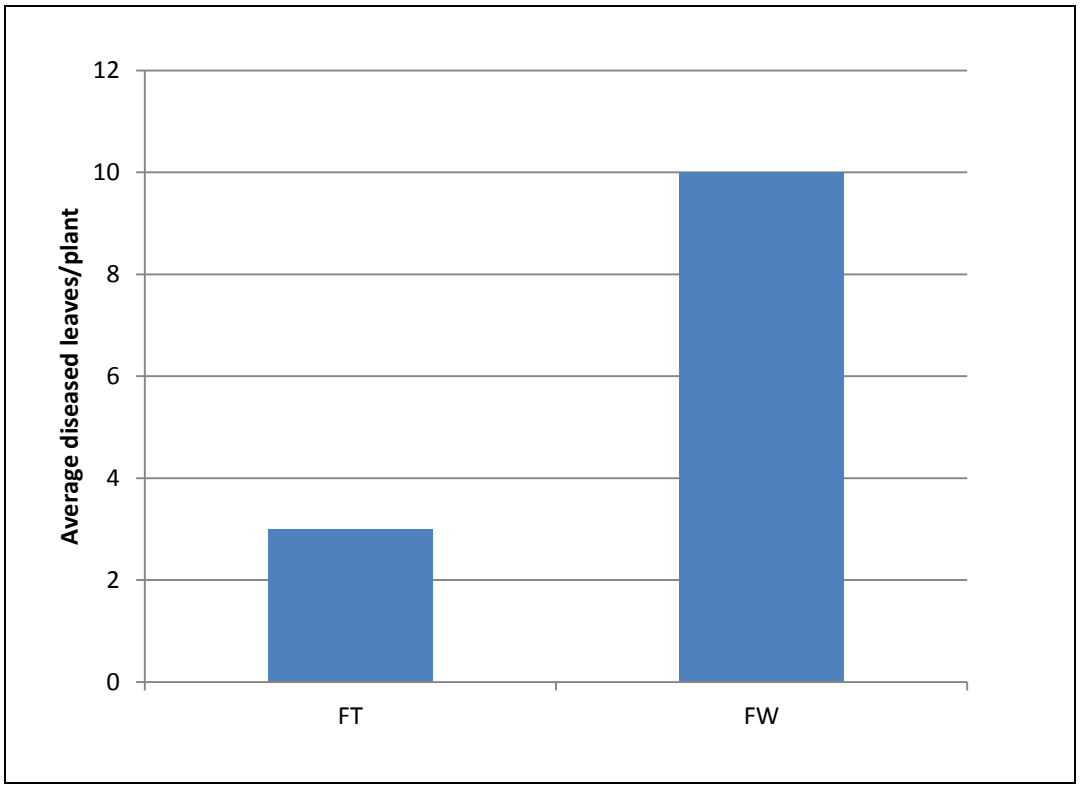

Figure 2. Severity of Pierce's disease on transgenic Freedom grape (FT) and on wild-type Freedom grape assessed in August 2012 in the Solano County trial.

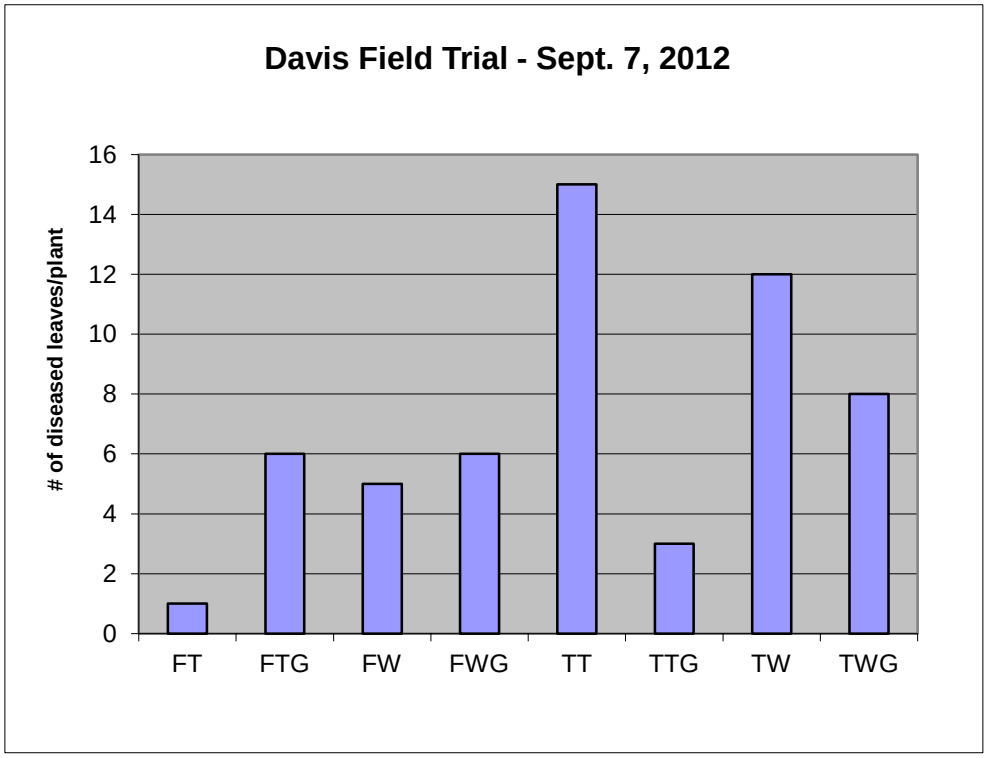

Figure 3. Severity of Pierce's disease on grape assessed in September 2012 in the Solano County trial. See treatment codes above for treatment comparisons.

The plants for the Riverside County trial were planted on April 26, 2011 (**Figure 5**) and have exhibited much less growth than those at the Solano County trial (**Figure 4**). The plants at the Riverside County site were subjected to natural infection from infested sharpshooter vectors having access to *Xf* from surrounding infected grapevines. Very high levels of Pierce's disease were seen in the summer of 2012, although much less symptoms were seen on the transgenic DSF-producing Freedom grape compared to other plants (**Figure 5**).

Figure 4. Establishment of grape trial in Riverside County in April 2010 (left) and image of plot in October 2012 (right).

Figure 5. Pierce's disease symptoms on transgenic DSF-producing Freedom grape (left) and wild-type Freedom grape (right) on October 4, 2012.

The incidence of infection of transgenic DSF-producing Freedom was about three-fold less than that of wild-type Freedom grape (**Figure 6**), while the number of infected leaves per vine was about five-fold less (**Figure 9**), suggesting that the pathogen had spread less in the DSF-producing plants after insect inoculation. Only a modest reduction in incidence or severity of Pierce's disease was seen in Thompson Seedless grafted onto DSF-producing Freedom rootstocks compared to those grafted on wild-type Freedom (**Figure 7**). The incidence of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson Seedless (**Figure 8**), while the incidence and severity of Pierce's disease on Thompson Seedless grafted onto DSF-producing Thompson Seedless rootstocks was less than that of plant grafted onto wild-type Thompson Seedless rootstocks (**Figure 9**). The effectiveness of transgenic Thompson Seedless rootstocks in reducing Pierce's disease was surprising, given that the transgenic Thompson Seedless scions were similar in susceptibility to that of the normal Thompson Seedless scions. We have seen evidence that in addition to DSF chemical species that serve as agonists of cell-cell signaling in *Xf* that transgenic Thompson Seedless may also produce chemical antagonists of cell-cell signaling. It is possible that the DSF agonist is more readily transported into the scion than any antagonists, and thus that DSF-mediated inhibition of pathogen mobility can be conferred by grafted DSF-producing rootstocks.

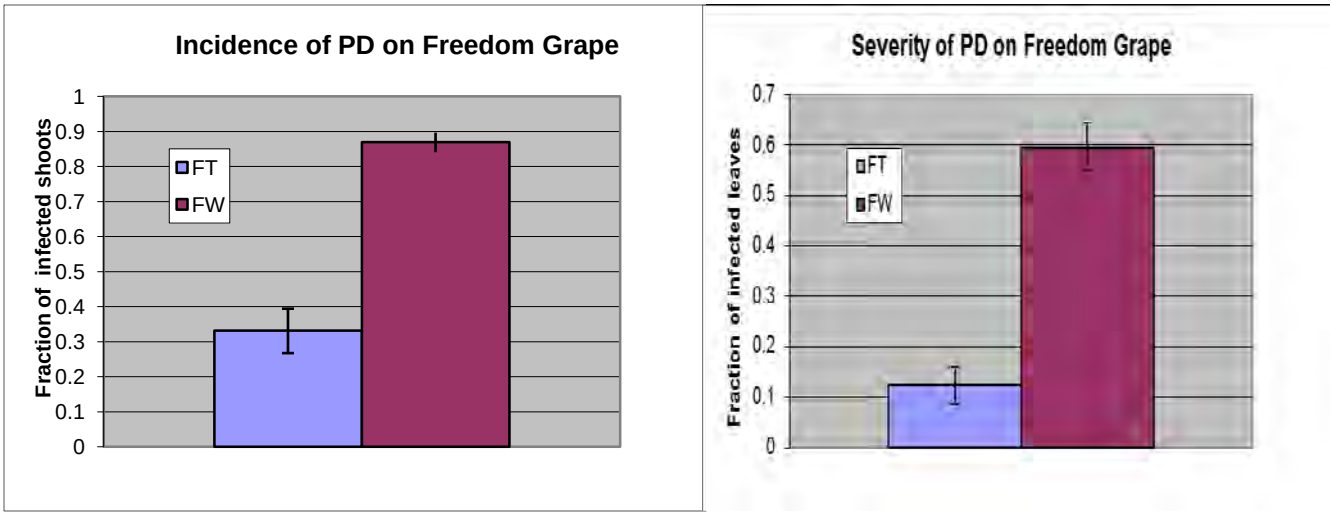

Figure 6. Incidence of Pierce's disease of transgenic DSF-producing Freedom grape (blue bars) or wild-type Freedom (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.

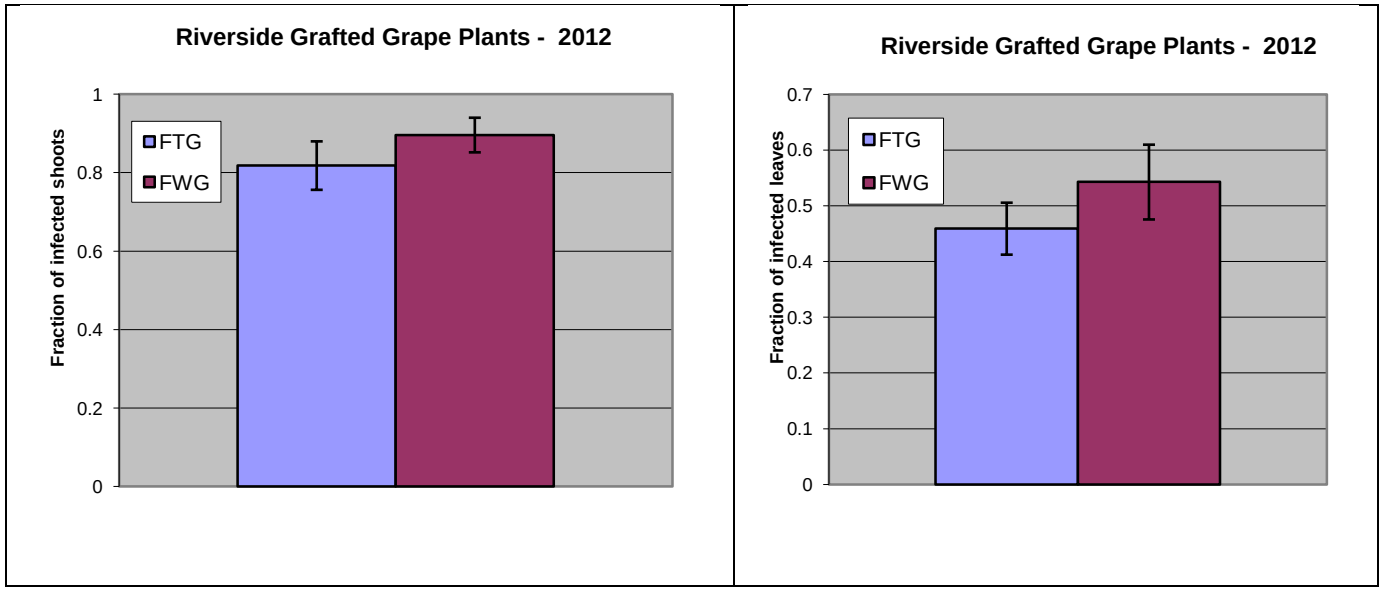

Figure 7. Incidence of Pierce's disease of normal Thompson Seedless grape grafted onto transgenic DSF-producing Freedom grape rootstocks (blue bars) or wild-type Freedom rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.

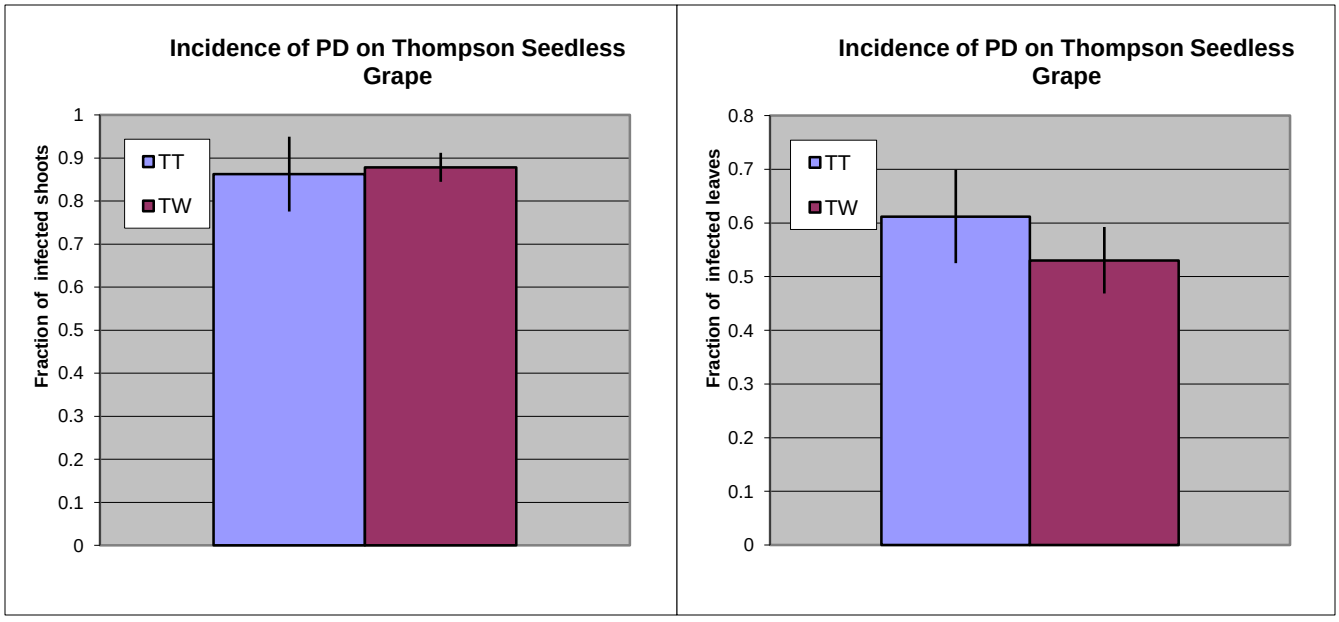

Figure 8. Incidence of Pierce's disease of transgenic DSF-producing Thomson Seedless grape (blue bars) or wild-type Thompson Seedless (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.

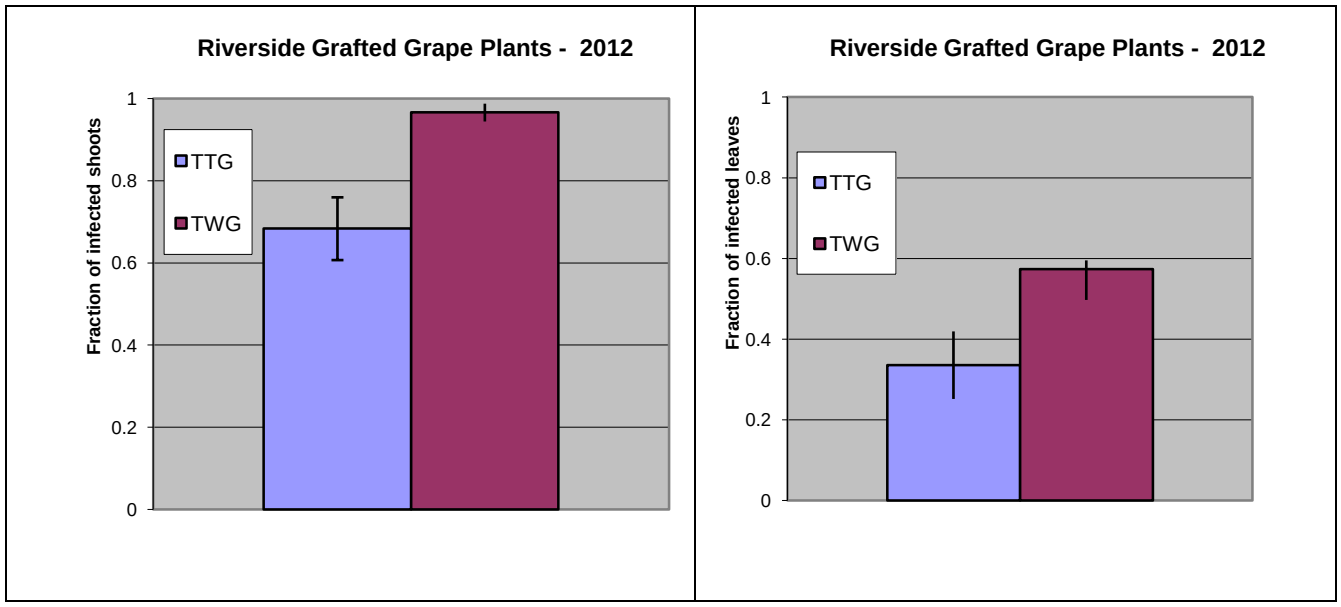

Figure 9. Incidence of Pierce's disease of normal Thompson Seedless grape grafted onto transgenic DSF-producing Thompson Seedless grape rootstocks (blue bars) or wild-type Thompson Seedless rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.

On May 15, 2013, plants in the Solano County field trial were evaluated for both the incidence of survival over winter, as well as any symptoms of Pierce's disease that were apparent at this early date. Vines that had been inoculated in 2012 had been marked with a plastic tie. The vines were pruned during the winter of 2012/2013 in a way that retained the inoculation site and the plastic marker for each of the vines inoculated in 2012. Thus, in May 2013 the return growth on those inoculated, but pruned, vines was assessed. One or more new shoots had emerged from such vines, and the incidence as to whether at least one new shoot had emerged was assessed

(**Figure 10**). Nearly all of the inoculated vines from both Freedom and transgenic DSF-producing Freedom gave rise to new shoots as of May 2013 (**Figure 10**). In contrast, many vines of Thompson Seedless inoculated in 2012 were dead, and no shoots emerged in 2013. While most new shoots emerging in 2013 appeared asymptomatic at the time of assessment in May, a few exhibited discoloration, possibly indicating early stages of Pierce's disease. A separate assessment of such possibly symptomatic shoots from that of completely asymptomatic shoots was made (**Figure 11**). It is noteworthy that no symptomatic new shoots were observed on transgenic Freedom, while about 10% of the new shoots emerging from vines of wild-type Freedom exhibited some symptoms (**Figure 11**). It was also noteworthy that a much higher proportion of the vines from Thompson Seedless scions grafted onto a transgenic Freedom rootstock gave rise to new shoots in 2013 compared to that on Freedom rootstocks (**Figures 10 and 11**). Likewise, a higher proportion of vines from Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks gave rise to new shoots in 2013 compared to that of scions grafted onto normal Thompson Seedless rootstocks (**Figures 10 and 11**). Thus, infection of Thompson Seedless vines by inoculation in 2012 had led to some morbidity of those vines (and even of the cordon on which they were attached in some cases), but Thompson Seedless when grafted onto either transgenic DSF-producing Freedom or transgenic DSF-producing Thompson Seedless rootstocks had a higher likelihood of surviving inoculation in 2012. Continued assessments of disease severity of those new shoots emerging on vines inoculated in 2012 were made in early October 2013, but the data was not fully analyzed at the time of preparation of this report.

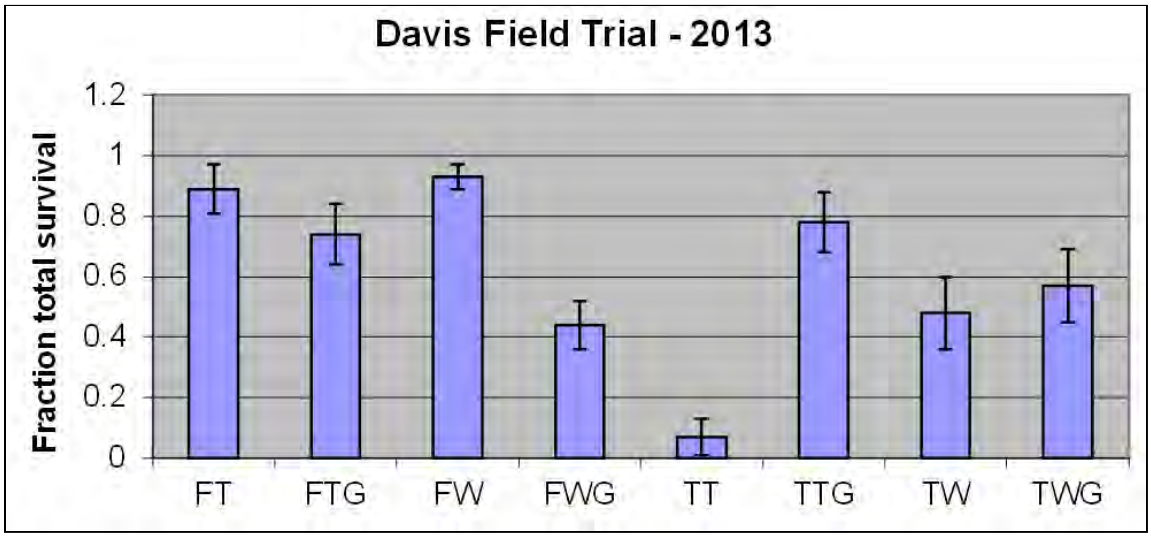

Figure 10. The fraction of vines in the Solano County field trial inoculated in 2012 with *Xf* that gave rise to at least one new shoot by May 2013. Treatments include: transgenic DSF-producing Freedom as an own-rooted plant (FT); wild-type freedom as an own-rooted plant (FW); Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG); transgenic DSF-producing Thompson Seedless as own-rooted plants (TT); normal Thompson Seedless as own-rooted plants (TW); Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG); and Thompson Seedless scions grafted onto normal Thompson Seedless rootstocks (TWG). The vertical bars represent the standard error of the mean fraction of inoculated vines that gave rise to new shoots in 2013.

All plants at the Riverside County trial were also rated on October 14, 2013. While the data from this final rating of the 2013 growing season was not fully analyzed at the time of preparation of this report, it was clear that both the severity and incidence of Pierce's disease symptoms on transgenic DSF-producing Freedom grape was much lower than that on wild-type Freedom plants, as had been seen in 2012. In addition, the severity of Pierce's disease symptoms on Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks was also lower than that of Thompson Seedless scions grafted onto wild-type Thompson Seedless rootstocks, again as was noted in 2012.

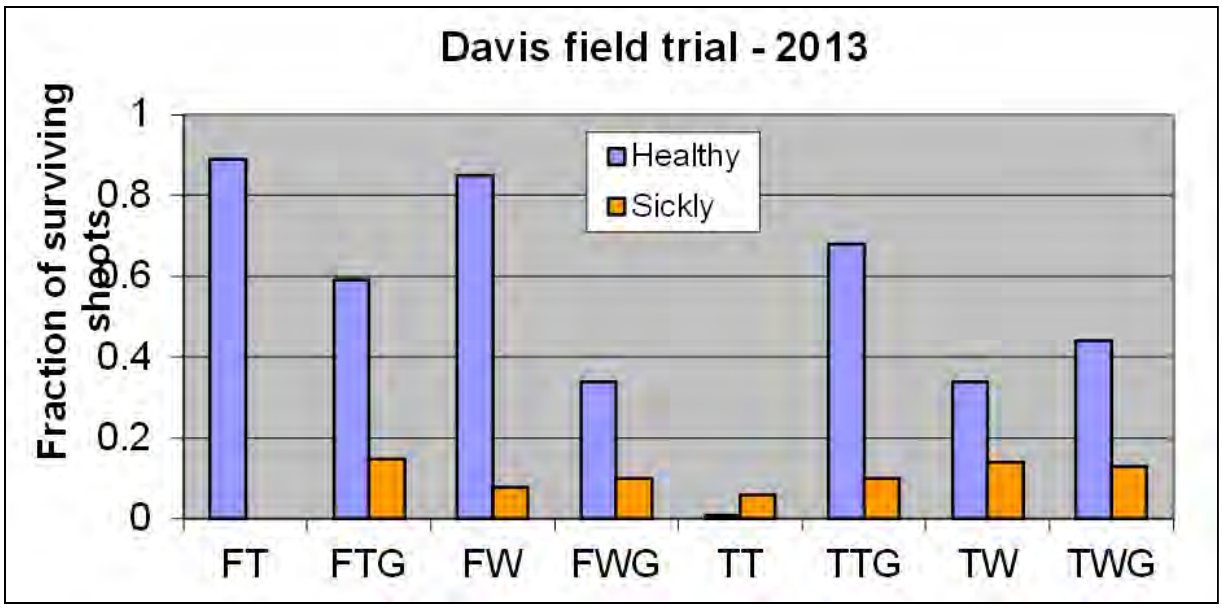

Figure 11. The fraction of vines in the Solano County field trial inoculated in 2012 with *Xf* that gave rise to at least one new shoot by May 2013 that exhibited some abnormalities possibly indicative of early stages of Pierce's disease infection (orange bars). Treatments include: transgenic DSF-producing Freedom as an own-rooted plant (FT); wild-type Freedom as an own-rooted plant (FW); Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG); transgenic DSF-producing Thompson Seedless as own-rooted plants (TT); normal Thompson Seedless as own-rooted plants (TW); Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG); and Thompson Seedless scions grafted onto normal Thompson Seedless rootstocks (TWG).

CONCLUSIONS

Substantial disease control was conferred by transgenic DSF-producing Freedom grape in both the Solano County and Riverside County field trails. In neither trial did the transgenic Freedom rootstock confer substantial disease control, similar to the observations seen in greenhouse trials. While the transgenic Thompson Seedless scion was similar in susceptibility to the wild-type Thompson Seedless grape, it conferred substantial disease control when used as a rootstock. This work is a direct demonstration of the utility of disease control by a process of pathogen confusion. The work demonstrates that in two different grape varieties to behavior of the pathogen can be altered by the production of the signal molecule in plants. The results are quite promising and provide substantial evidence that this strategy will be effective in reducing the severity of Pierce disease in a wide variety of grape varieties.

FUNDING AGENCIES

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

FIELD TRIAL FOR RESISTANCE TO PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted July 2012 through September 2013.

ABSTRACT
This project was constituted to establish experimental vineyards in Riverside County to test for the ability to withstand vector insects transmitting the pathogens causing Pierce's disease. A third vineyard was added this past season and is flourishing. All operations are meant to duplicate a commercial vineyard except the grapes themselves are not harvested. Originally flowers were removed, but in the past season, some of the flowers were left intact and will not be removed from now on. Because of the current infestation of Asian citrus psyllid in Southern California, the citrus near the field site is being treated with systemic insecticides that affect the population of the vector insect, the glassy-winged sharpshooter (GWSS). However, the biological control plot is left untreated and GWSS may still be found year-round.

LAYPERSON SUMMARY
The experimental grapevines have been in place since they were planted in spring of 2010. A second vineyard was added in 2011 and a third in 2012. Many control grapevines are dead by now in the original planting, confirming the high incidence of Pierce's disease. Most if not all of the experimental grapevines with resistance to Pierce's disease are doing well. The stems of the oldest surviving grapevines are well above an inch in diameter and appear to be thriving. The grapes themselves are not harvested. Flowers were at first removed, but this was discontinued. The coming season will be the first in which all of the flowers are left intact.

INTRODUCTION
There is no cure for plant diseases caused by pathogens transmitted by insects. That leaves controlling the vector insect or developing plant resistance to the pathogens as the only two viable options. Advances in genetic engineering of plants mean the second option is readily available in the modern era. Several such strategies are the result of investments in Pierce's disease research (Dandekar, et al., 2012). The current project was designed as a "pass through" to allow the Pierce's disease program to field test several varieties of transgenic plants from collaborators at UC Davis and UC Berkeley. Funds pass though the Principal Investigator (PI) and Co-PI to cover construction of trellises, installation of drip irrigation equipment, scheduled automatic irrigation, weed abatement, yearly pruning, and to pay for planting of experimental grape plants in the vineyard and all maintenance of the vineyard throughout the experimental period as well as destruction of materials stipulated by the protocols in place at the end of the project. Riverside County was desired as a venue because of the very high incidence of Pierce's disease. The extensive collection of citrus also provides a winter harborage for the vector insect, the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*).

OBJECTIVES
A major focus of Pierce's disease management includes attempts to develop grapevine varieties that are less susceptible to *Xylella fastidiosa*. We are providing support for field trials of newly developed grapevine varieties that show promising reductions in Pierce's disease severity. The field trial is intended to duplicate a commercial operation to determine how grapevines will fare in the presence of pressure from the sharpshooter leafhopper vectors that transmit the pathogen causing Pierce's disease. The specific objectives of the project are as follows:
1. Prepare the vineyard. Rogue out existing plants and prepare additional trellises as needed.
2. Transplant test grapevines to the experimental vineyard.
3. Maintain the grapevines exactly as handled by commercial vineyards.
4. Dispose of plants at the end of trials according to protocols provided.

RESULTS AND DISCUSSION

The field trials in Riverside County are considered a success. Control grapevines lacking resistance to Pierce's disease died after the first year. This confirms the presence of high pressure from Pierce's disease. After extensive surveys of insect presence during the first two years by an undergraduate volunteer, Candice Sanscartier, the survey yellow sticky cards have been used less frequently during the past year. We are convinced the vector insect population is still significant despite wide-spread insecticide treatment of the experimental citrus holdings for Asian citrus psyllid (ACP). This is being done in part to prevent huanglongbing disease (citrus greening) from becoming established in Southern California. The host range of ACP is restricted to citrus mainly and is not a problem on grapevines or other crops.

The PI is hosting a separate study testing a symbiotic control of Pierce's disease in greenhouses on campus and being conducted by graduate student Arinder Arora who just passed his qualifying exam in the Ph.D. graduate program at University of New Mexico, Albuquerque. This project requires hundreds of GWSS for testing. Arinder and colleagues have had little trouble securing the insects from the area near the field trial. Arinder has spent the last two summers in Riverside conducting these studies. He expects to finish his Ph.D and graduate by next June in Albuquerque.

The laboratory of Abhaya Dandekar has kept in excellent contact with the PI. He and his personnel have visited the experimental vineyards several times after the first planting in 2010. Occasionally digital images were taken of the vineyards and sent to him as a way of keeping him informed of the appearance of the plants.

Francisco Quintana, USDA APHIS, paid an official oversight visit to the experimental vineyards on September 11, 2013. Between this season and last, a separate experimental vineyard was installed near the experimental vineyard. The distance between the plots appears to be in excess of 50 feet. Mr. Quintana stopped by the office to find out the nature of this new vineyard. We updated Biological Use Authorization documentation through Patricia Steen in the UC Riverside Research Office to cover activities on this project.

CONCLUSIONS

Grapevines can be genetically engineered to carry resistance to Pierce's disease in areas where there is high pressure for the disease and susceptible grapevines do not survive more than one year. Since the product of the grapevines was not harvested, the quality of the fruit of the grape was not assessed.

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FUNDING AGENCIES

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FIELD EVALUATIONS OF GRAFTED GRAPE LINES EXPRESSING POLYGALACTURONASE-INHIBITING PROTEINS

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ABSTRACT
Work in this project evaluates the performance of grafted grapevine lines that produce a protein that is a candidate for control of Pierce's disease. The vines have been established in vineyards in a manner that approximates typical commercial settings in regions of Solano and Riverside counties with low and high Pierce's disease disease pressure, respectively. The CDFA Pierce's Disease and Glassy-winged Sharpshooter Board's Research Scientific Advisory Panel had established a priority to evaluate the potential commercial use of the strategy to deliver polygalacturonase-inhibiting proteins (PGIPs) from grafted rootstocks to control Pierce's disease in the scion, fruit-bearing portions of grapevines. Established transformed Thompson Seedless and Chardonnay grapevines expressing a PGIP from pear fruit (pPGIP) showed reduced Pierce's disease incidence when inoculated with *Xylella fastidiosa* (Agüero *et al*., 2005). The pPGIP that was produced in the transformed rootstock was identified in samples of xylem exudate that were collected from grafted, but not transformed scions (Agüero *et al*., 2005). Therefore, cuttings from these grapevines were grafted with non-pPGIP producing scions to make comparisons of the effectiveness and outcomes between vines producing pPGIP in grafted rootstocks, those producing pPGIP throughout the vine, and vines with no pPGIP.

LAYPERSON SUMMARY
Two vineyard plots containing own-rooted and transgrafted [rootstocks expressing pear polygalacturonase-inhibiting protein (pPGIP) grafted to fruit producing scions with no genetic modifications that, thus, do not themselves produce pPGIP] combinations of Chardonnay and Thompson Seedless grapevines were established and the identities of the genotypes were established by June 2013. Mechanical inoculations with *Xylella fastidiosa* bacteria were done in 2011, 2012, and 2013 in Solano County and natural infections were allowed to occur in Riverside County. Data describing the agronomic and disease traits of the vines have been collected.

INTRODUCTION
The project was designed to establish two field sites with typical vineyard practices that would allow grape lines to be evaluated in order to assess whether polygalacturonase inhibiting proteins (PGIPs) restrict *Xylella fastidiosa* (*Xf*) spread and Pierce's disease, and whether expression of pear polygalacturonase-inhibiting protein (pPGIP) impacted the performance and attributes of the vines.

The Principal Investigator (PI), Co-PI, and others had shown that the expansion of *Xf* from the infection site throughout the vine creates a systemic infection that causes Pierce's disease and vine death (Krivanek and Walker, 2005; Labavitch 2006, 2007; Lin, 2005; Lindow, 2006, 2007a,b; Rost and Matthews, 2007). The grapevine water-conducting xylem elements are separated by pit membranes, cell wall "filters" whose meshwork is too small to permit movement of *Xf* (Labavitch et al., 2004, 2006, 2009a,). *Xf* produces cell wall-degrading enzymes to digest the pit membrane polysaccharides (Labavitch et al., 2009b), opening xylem connections and permitting spread of the bacteria.

The *Xf* genome encodes a polygalacturonase (*XfPG*) and several ß-1,4-endo-glucanase (EGase) genes, whose predicted enzyme products could participate in the digestion of pectin and xyloglucan polymers in pit membranes, thereby facilitating *Xf* movement and Pierce's disease development. Labavitch et al. (2006, 2007, 2009a; Perez-Donoso et al., 2010) reported that introduction of polygalacturonase (PG) and EGase into uninfected grapevines caused pit membrane breakage. Roper et al. (2006, 2007) developed an *XfPG*-deficient *Xf* strain and showed it was unable to cause Pierce's disease symptoms, so *XfPG* is a Pierce's disease virulence factor.

The over-all research aim is to use plant PGIPs to limit *Xf* spread in grapevines. PGIPs are produced by plants, including in flowers and edible fruits, and are selective inhibitors of pathogen and pest PGs (Powell et al., 2000; Shackel et al., 2005; Stotz et al., 1993, 1994). Transformed grapevines expressing the pPGIP-encoding gene from pears have reduced susceptibility to *Xf* and pPGIP is transported across the graft junction from pPGIP expressing grape and tomato rootstocks into wild-type scions (Agüero et al., 2005, Haroldsen et al., 2012).

This project has been designed to scale up the grafted and own-rooted pPGIP expressing grapevines, plant them in field settings, and evaluate their agronomic performance and their resistance to Pierce's disease in settings comparable to commercial fields.

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 Pierce's disease pressure.
3. Evaluate relevant agronomic traits of vines in two locations.
4. Determine Pierce's disease 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 (TS) grapevines generated by Agüero et al. (2005) were maintained at the UC Davis Core Greenhouses. Vegetative cuttings of non-lignified stem sections from transgenic and control plants of both cultivars were rooted in an aeroponic cloning manifold (EZ-Clone Inc., Sacramento, CA). These plants are referred to as "own-rooted plants." Rooted cuttings were transferred to soil and maintained in the greenhouse before being transferred to the field sites. A sufficient number of grafted and "transgrafted" plants were generated for the field trials and were made by green grafting rootstock stem sections with budding scion tissue. Transgrafted plants had rootstocks from the pPGIP expressing lines and scions that do not express pPGIP. The number of plants of each genotype and grafting protocol for the field sites in Solano and Riverside counties are shown in **Table 1**.

Table 1. Numbers of grapevines planted in Solano and Riverside counties. Dashed fill represents pPGIP expressing rootstocks and/or scions; black fill is null-transformants, no pPGIP, controls; white fill is non-transformed controls. In Solano County, own-rooted vines were mechanically inoculated in the summers of 2011-2013; grafted vines were inoculated in July 2013. Vines planted in Riverside County were assessed in response to "natural" infections.

SOLANO		Chardonnay					Thompson Seedless				
	Grafting Strategy (Scion/root) Hatch – pPGIP expressing										
Own-Rooted (#)	Inoculated	9	-	9	-	-	16	-	9	-	-
	Non-Inoculated	5	-	4	-	-	7	-	5	-	-
Grafted (#)	Inoculated	3	8	9	-	-	15	10	9	-	-
	Non-Inoculated	1	3	4	-	-	7	5	4	-	-
RIVERSIDE											
Own-Rooted (#)	Natural Infections	12	-	11	6	-	10	-	12	6	-
Grafted (#)	Natural Infections	8	5	8	6	3	15	15	7	3	3

DNA was prepared from the vines used as source tissue for grafting and the genotypes were confirmed by PCR (**Figure 1**).

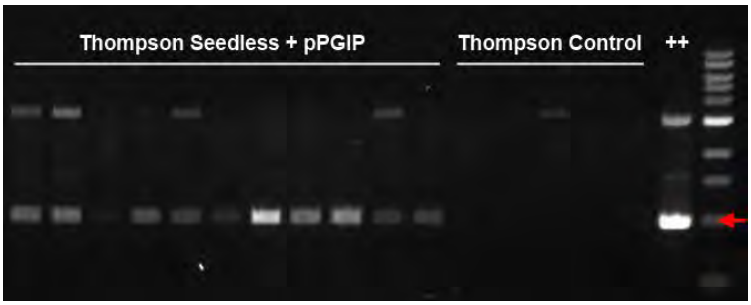

Figure 1. A gel used to genotype by PCR with genomic DNA from grape leaf tissue from Thompson Seedless vines expressing pPGIP and null-transformed (no pPGIP) controls used to generate transgrafted vines. A 1 kb band (arrow) indicating the pPGIP DNA sequence is expected only in samples used as rootstocks for transgrafts and pPGIP self-grafted controls. Each sample's quality was verified by amplifying a control fragment (not shown).

Sufficient plants of both the Chardonnay and Thompson Seedless varieties have been self-grafted, transgrafted or propagated by rooting to complete the Solano and Riverside county plots. The genotypes of the plants have been verified. All of the vines have been transplanted to the sites.

Objective 2. Establish field trial sites.
Field trial sites in Solano and Riverside counties have been established to assess the Pierce's disease resistance and general agronomic viability of own-rooted and grafted pPGIP expressing grapevines. The field plans of the Powell trial plots in Solano and Riverside counties are shown in **Figure 2**. The field sites are shared by other projects testing Pierce's disease resistance of other transgenic grapevines. The vines satisfying our initial PCR analysis were hand-planted in a randomized block design with blocks consisting of two or three individuals in the same treatment (**Table 1**). The young plants were surrounded by protective grow tubes and hand-watered every two weeks in Solano County or as needed. The grapevines were planted approximately eight ft. apart and tied to wooden stakes with trellising wires at 40 in. and 52 in. A time-line showing when grafting, plantings, inoculations and assessments have been done is shown in **Figure 3**.

The vines have been pruned both to maximize potential cane number for inoculations and to establish vigorous positions for future growth. With the permit amendment granted by the USDA's Biotechnology Regulatory Services in March 2012, flowers and fruiting clusters have been allowed to persist. All own-rooted Chardonnay vines were cordon trained and spur pruned and the majority of the Thompson Seedless vines were cane pruned in an attempt to maintain proper vine balance and ensure fruit development. The Solano County field site has been under weekly observation for the duration of the growing season and the vines in Riverside County established themselves well and were growing robustly as of late July 2013. As of June 3, 2013, both the Riverside and Solano county sites have been established with all the planned plantings for this project.

Objective 3. Evaluate relevant agronomic traits of vines in two locations.
Both the Solano and Riverside vineyards will continue to be maintained by properly pruning and training the vines this winter. The grapevines planted in Solano County have been monitored for general health and maintained (hand watered) on a weekly basis. Eleven uninoculated grafted plants did not survive the arid 2013 summer in Solano – these are being replaced in Fall 2013. Otherwise, growth of the vines at both locations has been vigorous (**Figure 3**). We reclassified 11 own-rooted vines from Chardonnay control to Thompson Seedless control based on cluster morphologies. The previously observed leaf morphologies were supportive of this reclassification, but fruiting habit and form permitted definitive identification. Data for the agronomic and phenotypic observations is included in Objective 4 (**Figure 5**), when comparisons were made between infected and uninfected vines. Own-rooted Thompson Seedless plants expressing pPGIP have a slightly larger average yield than control vines. Expression of pPGIP did not significantly affect the Thompson Seedless cluster morphology or the juice characteristics measured.

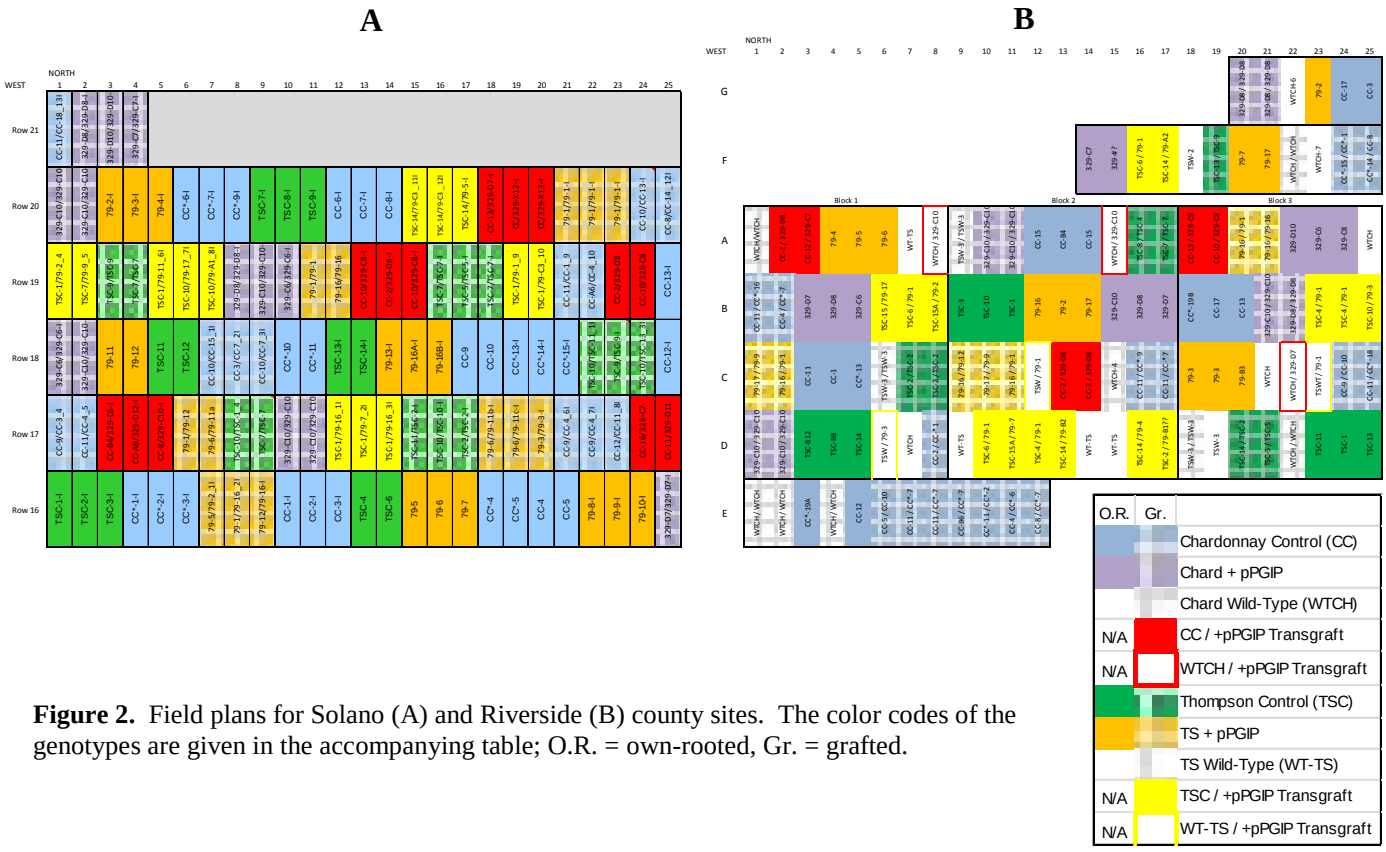

Figure 2. Field plans for Solano (A) and Riverside (B) county sites. The color codes of the genotypes are given in the accompanying table; O.R. = own-rooted, Gr. = grafted.

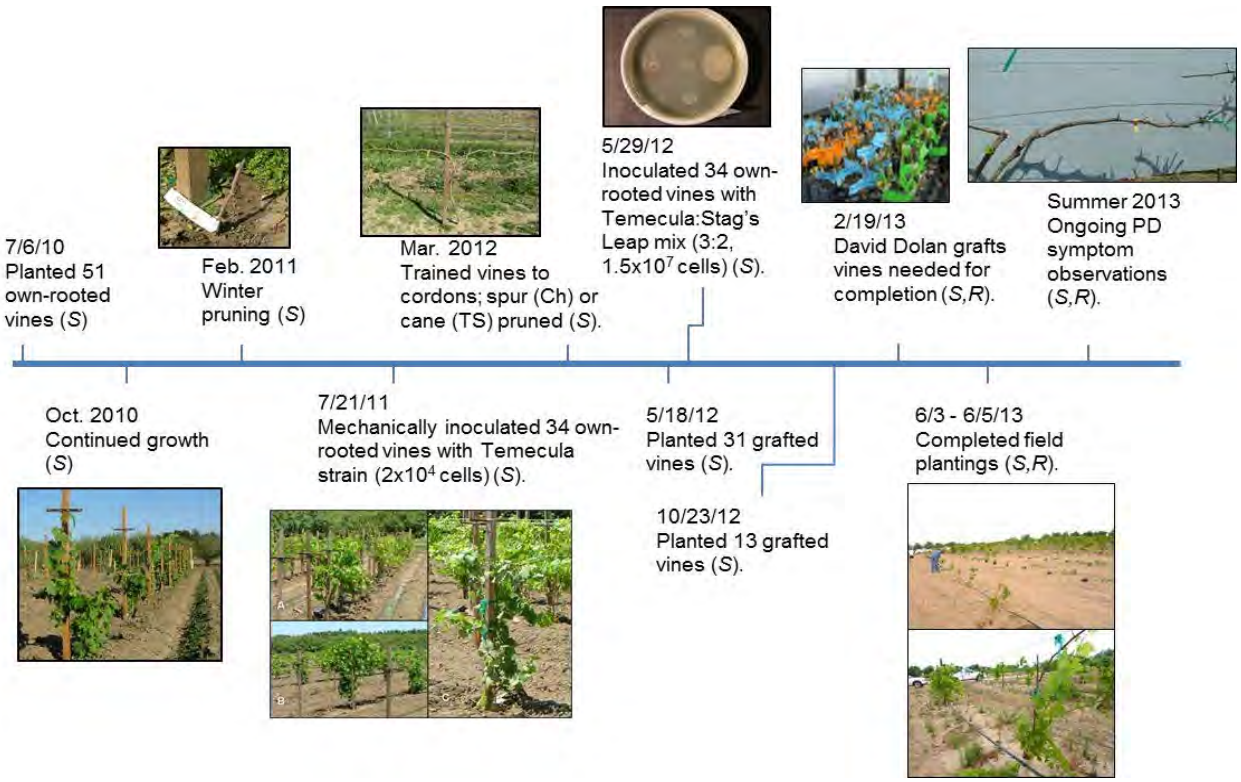

Figure 3. Timeline depicting the grafting, planting, inoculation, and assessments of vines at the Solano (*S*) and Riverside (*R*) sites.

Figure 4. Representative inoculated Thompson Seedless plants in Solano County: top row, pPGIP expressing vine; bottom row, control vine. Pictures taken (from left to right) May 3, June 26, Sept. 24, and Sept. 11, 2013.

Table 2. Observations of own-rooted vines taken in the spring and summer of 2013 at the Solano County site. Each characteristic was counted as "positive" for the disease trait if one instance was observed on the plant. "Buds" refer to bud positions selected for by winter pruning and does not include buried buds.

Genotype		Plants (#)	% Plants with No Growth from Buds on Inoculated Canes		% Buds with No Growth on Inoculated Canes Only		% Plants with No Growth from Buds on Uninoculated Canes		% Plants with Stunted Growth from Buds on Inoculated Canes		% Plants with Stunted Growth from Buds on Uninoculated Canes	
			Spring	Summer	Spring	Summer	Spring	Summer	Spring	Summer	Spring	Summer
Inoculated Thompson+pPGIP	79-I	9	100 (9/9)	100 (9/9)	50-75	71.8	55.6 (5/9)	44.4 (4/9)	44.4 (4/9)	55.6 (5/9)	0 (0/9)	11.1 (1/9)
Thompson+pPGIP	79	5	-	-	-	-	40 (2/5)	20 (1/5)	-	-	0 (0/5)	20 (1/5)
Inoculated Thompson	TSC-I	8	100 (8/8)	100 (8/8)	75-100	84.5	75 (6/8)	87.5 (7/8)	25 (2/8)	25 (2/8)	50 (4/8)	50 (4/8)
Thompson Control	TSC	4	-	-	-	.	75 (3/4)	0 (0/4)	-	-	0 (0/4)	0 (0/4)
Inoculated Chardonnay	CC-I	17	94.1 (16/17)	100 (17/17)	75-100	79.0	47.1 (8/17)	52.9 (9/17)	47.1 (8/17)	52.9 (9/17)	23.5 (4/17)	17.7 (3/17)
Chardonnay Control	CC	8	-	-	-	-	75 (6/8)	0 (0/8)	-	-	12.5 (1/8)	0 (0/8)

Genotype		Plants (#)	% Plants with Excessive Base Growth		% Plants with Marginal Leaf Necrosis on Inoculated Canes		% Plants with Marginal Leaf Necrosis on Un-inoculated Canes		% Plants with Atypical Berry Clusters (partial, aborted, or absent)	
			Spring	Summer	Spring	Summer	Spring	Summer	Spring	Summer
Inoculated Thompson+pPGIP	79-I	9	77.8 (7/9)	66.7 (6/9)	0 (0/9)	33.3 (3/9)	0 (0/9)	11.1 (1/9)	-	44.4 (4/9)
Thompson+pPGIP	79	5	0 (0/5)	0 (0/5)	-	-	0 (0/5)	0 (0/5)	-	20 (1/5)
Inoculated Thompson	TSC-I	8	25 (2/8)	100 (8/8)	0 (0/8)	12.5 (1/8)	0 (0/8)	0 (0/8)	-	75 (6/8)
Thompson Control	TSC	4	0 (0/4)	50 (2/4)	-	-	0 (0/4)	0 (0/4)	-	0 (0/4)
Inoculated Chardonnay	CC-I	17	17.7 (3/17)	82.4 (14/17)	0 (0/17)	11.8 (2/17)	0 (0/17)	0 (0/17)	-	58.8 (10/17)
Chardonnay Control	CC	8	0 (0/8)	37.5 (3/8)	-	-	0	0	-	25 (2/8)

Objective 4. Determine Pierce's disease incidence in pPGIP expressing grafted and own-rooted lines. Two-thirds of the own-rooted vines at the Solano County site were first mechanically inoculated with *Xf* Temecula on July 21, 2011. No visual evidence of Pierce's disease infection was observed throughout the 2011 growing season or early in 2012 after bud break. The same 34 own-rooted vines were resubmitted to mechanical inoculations on May 29, 2012 with a mixture of *Xf* Temecula and Stag's Leap strains (3:2, v:v). Young, green tissue was chosen for inoculation with three-four canes chosen per plant. Mechanical inoculations were performed as in 2011 except that approximately 1.5 x 10^7 cells were used per inoculation, an increase of 750-fold over the previous year. The inoculations were performed simultaneously with the other field site collaborators. The bacterial suspension was provided by D. Gilchrist.

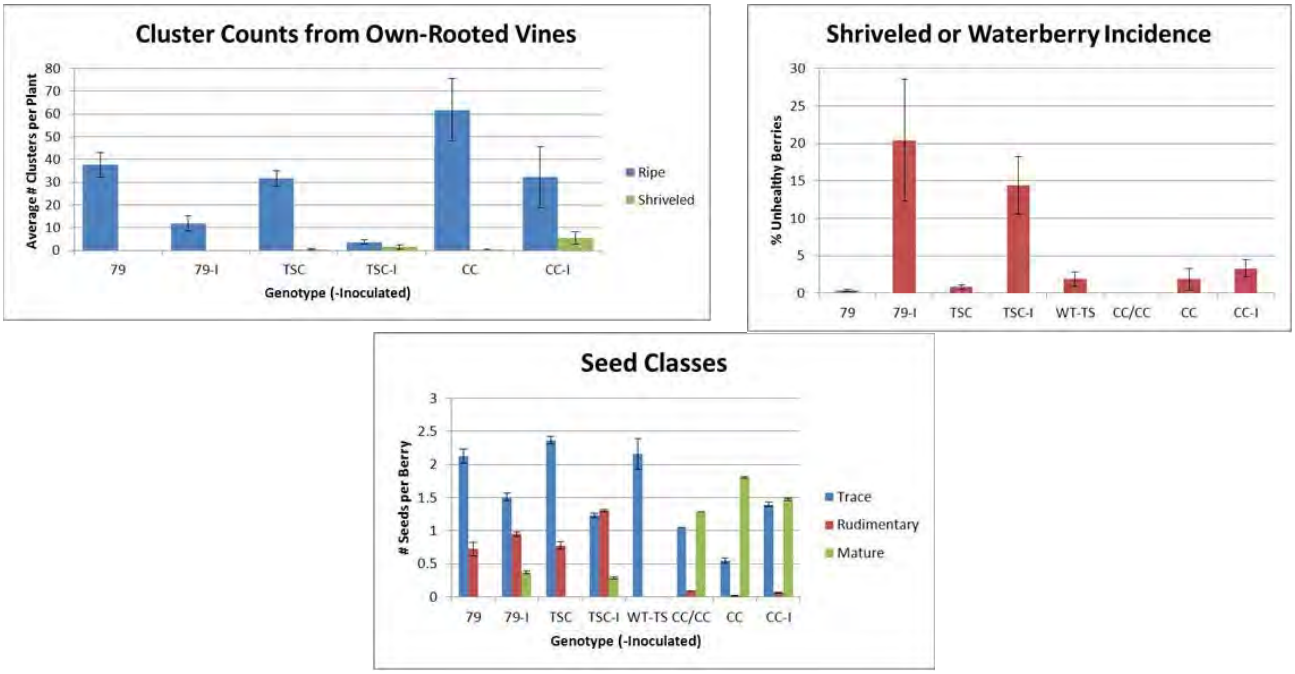

Figure 5. Number of clusters per plant (A), percentage of shriveled unhealthy berries per cluster (B), and seed morphologies within clusters (C) of own-rooted Thompson Seedless expressing pPGIP ("79"), Thompson Seedless control (TSC), and Chardonnay (CC) from uninfected and infected (-I) plants.

Pierce's disease symptoms were first observed on the inoculated vines in Solano County on April 24, 2013. The most frequent symptoms were inhibition of bud break along inoculated shoots (**Figure 4**) and excessive growth from the base of plants, potentially indicating a disruption in the vasculature or more severe die-back of cordons and mature canes. Outside viticulturists and pathologists confirmed that these vines were afflicted with Pierce's disease. Their opinions were sought because traditional Pierce's disease symptoms were mostly absent during the previous two growing seasons. Since the initial discovery, each vine has been photographed and initially scored for the presence of similar stunting or "blind" phenotypes (**Table 2**).

Vines in the Solano County field site were mechanically inoculated for the third consecutive year on August 6, 2013. The own-rooted vines previously infected and the newly-planted grafted vines tagged for treatment were inoculated with a mixture of Temecula and Stag's Leap strains of *Xf* (3:2, v:v). The inoculum was prepared in our laboratory from glycerol stocks provided by the Kirkpatrick and Gilchrist labs. Inoculations were performed as in previous years, except only one site was inoculated per grafted vine due to the smaller size of the vines; larger, own-rooted vines were inoculated at two to three sites per plant. The inoculum cell density was first estimated by optical density and later confirmed by serial plating to be 1.2 x 10^6 cells per inoculation site.

For more detailed analysis of plant performance and phenotypes, on August 29, 2013, twenty-five berries total were collected from three plants of each own-rooted genotype and inoculation state at the Solano County site; grafted plants were too juvenile to bear fruit in 2013 and were not sampled. Sample collection was randomized

by choosing five berries spread across one to two clusters per plant. Clusters were chosen from inside the fruiting zone on each plant. Berries were crushed by hand and the free-run juice was combined with juice pressed from the solids, strained through cheesecloth. Sediments were precipitated overnight at 4°C and clarified juice was sampled for pH and °Brix. Soluble solids ranged from 21.7-24.4 °BRIX and pH values were 3.56-4.00. A smaller subsample was repeated on September 4, 2013 with similar results. After one week, total cluster numbers were counted (**Figure 5a**) and one cluster was harvested per plant. Some inoculated own-rooted vines did not bear fruit; grafted plants, with one exception, were fruitless in 2013. Cluster weight, length, and peduncle length were measured upon returning to the lab. Twenty-five berries were removed from each cluster for further analysis after counting the total number of healthy and raisined berries per cluster (**Figure 5b**). Assessments of the subsamples include the weight of 25 berries, retention of pedicels, number and class of seeds (trace, rudimentary, or mature, **Figure 5c**), dimensions of five berries, soluble solids, titratable acidity, and pH of juice. Each cluster and five individual berries were photographed for assessment of cluster density and berry color and shape.

PCR was used to detect *Xf*DNA sequences in leaves and petioles from inoculated and un-inoculated vines (**Figure 6**). *Xf*DNA sequences were only detected in inoculated, and not in uninoculated, plant leaves. All DNA preparations were checked to see that PCR amplification of grape DNA sequences was possible. Efforts to quantify the amount of *Xf* in the inoculated material by qRT-PCR continue.

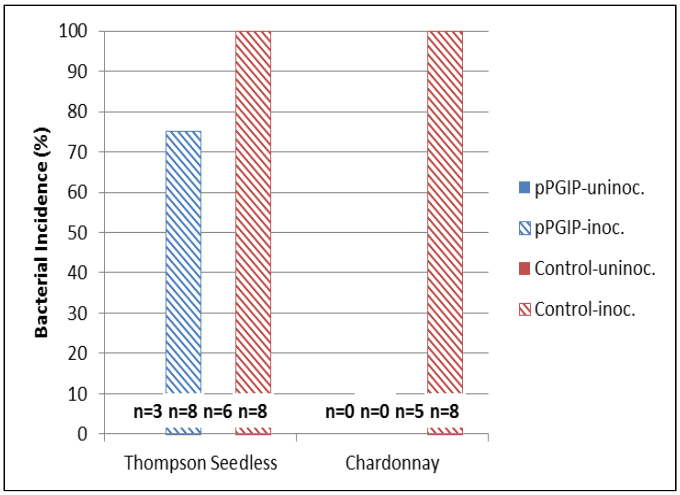

Figure 6. Results of PCR detection of *Xf* DNA sequences in inoculated vines from the Solano County site.

Initially, a higher percentage of bud positions were able to grow out on mechanically inoculated pPGIP expressing Thompson Seedless vines than on control Thompson Seedless vines. Inoculations with *Xf* promote shoot growth from the base of the vines. Inoculated pPGIP expressing Thompson Seedless vines had 40% fewer clusters with aborted or abnormal berries than infected controls. However, one of five uninoculated pPGIP expressing Thompson Seedless vines had abnormal berry clusters and the uninoculated controls had none. Three times as many mechanically inoculated pPGIP expressing Thompson Seedless vines had leaves with signs of marginal necrosis than infected control vines.

Later in the season, inoculations with *Xf* resulted in a noticeable decline in clusters for all genotypes. However, this decline was 20% less in Thompson Seedless plants expressing pPGIP. Shriveled clusters were only observed in control genotypes and did not always correspond to infection with *Xf*. An increased percentage of unhealthy berries per cluster was measured in inoculated plants, but there was not a significant difference with or without pPGIP. Berries from inoculated plants contained slightly more developed seeds than those from uninoculated plants; Thompson Seedless plants expressing pPGIP had fewer rudimentary seeds. Other observations reflect that healthy plants produce longer clusters with more berries.

Xf DNA sequences were detected by PCR in the inoculated samples. No *Xf* DNA sequences were detected in uninoculated controls. Visual assessments indicate that expression of pPGIP reduces Pierce's disease symptoms (bud outgrowth and abnormal berry clusters).

CONCLUSIONS

All of the grafted plants necessary for the studies in Solano and Riverside counties have been generated. The genotypes of the grafted plants were confirmed. An initial attempt to infect the vines in Solano County was made but no symptoms were observed. A second attempt in 2012 resulted in detectable *Xf* DNA in infected vines in November 2012 and visual symptoms of Pierce's disease in April 2013. The performance of the own-rooted Chardonnay and Thompson Seedless vines in the field thus far has been appropriate for commercial settings. Mechanical inoculations with *Xf* bacteria in 2011 and 2012 in Solano County resulted in the accumulation of *Xf* DNA sequences only in the inoculated, but not in the uninoculated, cane material. Symptoms of Pierce's disease infection were visible on the inoculated vines beginning in the spring of the year following the introduction of *Xf*. The evaluations of the performance and productivity of the plants suggest that pPGIP expression improves resistance of vines to Pierce's disease. The evaluations of the performance and productivity of the plants thus far confirm that expression and presence of pPGIP does not affect unintentionally 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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TOOLS FOR IDENTIFYING POLYGALACTURONASE-INHIBITING PROTEIN TRANSMISSION FROM GRAPEVINE ROOTSTOCK TO SCION

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Reporting Period: The results reported here are from work conducted January 2013 to October 2013.

ABSTRACT
The CDFA Pierce's Disease and Glassy-winged Sharpshooter Board's Research Scientific Advisory Panel review and requests for proposals (RFPs) gave priority to the delivery of proteins, including polygalacturonase-inhibiting proteins (PGIPs), from grafted rootstocks to scions in order to control Pierce's disease. Two currently-funded projects use expression of PGIPs as control strategies to limit the spread of *Xylella fastidiosa* (*Xf*) in the xylem network and, thereby, reduce Pierce's disease symptom progression in infected vines. Monoclonal antibodies recognizing pear fruit PGIP (pPGIP), the protein expressed in the grape lines that are currently under evaluation in field trial studies, are needed to detect, quantify, and observe the localization of the protein in the transformed grapevine rootstocks and the scions that are grafted to them. In order to make comparisons between the different strategies to control *Xf* spread, the amounts and efficacy of the pPGIP in the infected parts of the plant must be determined and a pPGIP recognizing monoclonal antibody allows measurements of the amounts of the protein. Authentic pPGIP protein from pear fruit could be used to prepare this monoclonal antibody, which then could be maintained in perpetuity as cultures of antibody-producing cells. However, we modified the approach and synthesized synthetic peptides from specific regions of the pPGIP protein to use as antigens. This approach assures that antibodies recognize only pPGIP and not the endogenous grape PGIPs. Production of the monoclonal antibodies has been accomplished; these soon will be tested for their recognition of properly glycosylated, active pPGIP protein from pear fruit that normally express the pPGIP-encoding gene and tissues of transformed pPGIP-expressing grape rootstocks and scions that have been grafted with the transformed rootstocks.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*), the bacterium that causes Pierce's disease 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 Pierce's disease symptoms because the bacteria multiply and cause the interruption of the flow of nutrients and water through the vessels of the plant. PGIPs produced by plants selectively inhibit PGs from bacteria, fungi, and insects. Our work (Abu-Goukh et al., 1983) identified a PGIP (pPGIP) from pear fruit that at least partially inhibits the *Xf*PG and we demonstrated that expression of pPGIP reduced Pierce's disease symptom development in grapevines (Aguero et al., 2005). Current projects, including field trial evaluations, require a monoclonal antibody specifically recognizing the pPGIP protein in order to detect, quantify, and characterize the presence in and delivery of the pPGIP protein that has been delivered to scions grafted to the available (Aguero et al., 2005) pPGIP-expressing rootstocks. The monoclonal antibody will allow us to compare the amounts of the pPGIP protein at different times and grapevine tissues and, thereby, determine the protein's role in *Xf*PG inhibition in grapevines. We now have received (1) a new polyclonal antibody that recognizes pPGIP and (2) monoclonal pPGIP antibodies that recognize different unique structural features of the pPGIP protein epitope recognizing antibody preparations and have purified active pPGIP from green pear fruit to test these antibody preparations.

INTRODUCTION
Pierce's disease 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 enzyme that degrades the polysaccharide portion of pit membranes is a polygalacturonase (*Xf*PG), a well-characterized Pierce's disease virulence factor (Roper et al., 2007). Several previous projects have analyzed the effectiveness of polygalacturonase-inhibiting proteins (PGIPs) in minimizing the damage caused by pathogens and pests on plants (Powell et al., 2000; Aguero et al., 2005), including damage

caused by *Xf* in Pierce's disease. Two currently-funded field projects use pear fruit PGIP (pPGIP) to restrict *Xf* movement in infected grapevines.

This project was designed to generate a new polyclonal antibody preparation that recognizes PGIPs in general and monoclonal antibodies that specifically recognize the pPGIP protein. The previous polyclonal antibody preparation was over 25 years old and little of the stock remains (Stotz et al., 1993). The monoclonal antibody is a necessary tool for the related field evaluation projects, including "Field evaluation of grafted grape lines expressing PGIPs" (Principal Investigator: Powell) and will enable the detection and quantification of pPGIP without cross-reactive interference from the native grape PGIP. Plants can, therefore, be more efficiently screened for the presence of the pPGIP protein, whether directly produced in rootstocks or transported grafted scion tissues. Consequently, the effectiveness of anti-Pierce's disease strategies can be made knowing the amount of the active anti-Pierce's disease protein in the tissues of *Xf*- and mock-inoculated test tissues.

OBJECTIVES

1. Using existing fresh pear flesh, prepare pPGIP protein. Design appropriate antibodies for 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.

Because of budget limitations, we abandoned purification of the pPGIP from transgenic *Arabidopsis* leaves engineered to express a tagged version of the protein.

We purified sufficient active pear fruit PGIP (pPGIP) from immature green pears for evaluation of the antibodies being prepared by Antibodies Inc. Approximately 195 μg of protein was obtained and is active against (i.e., inhibits) PGs produced in culture by the Del 11 strain of *Botrytis cinerea*, as expected. **Figure 1** shows results from a previous report documenting the purity of the protein. As described in Objective 2, we decided not to use this protein itself to develop monoclonal antibodies because of its extensive glycosylation, typical of plant proteins.

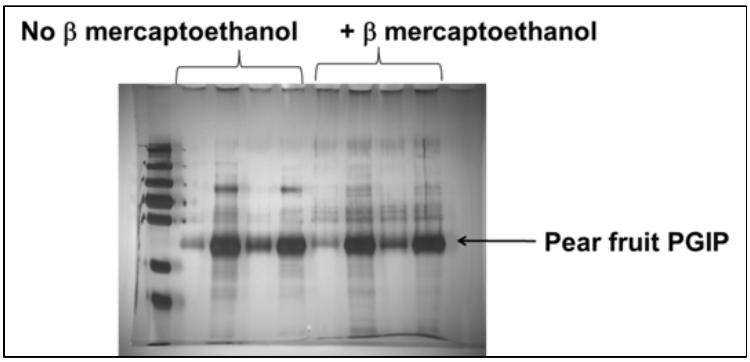

Figure 1. Silver stained SDS-PAGE gel showing pPGIP collected from cation exchange column fractions. Loading dye contains ß mercaptoethanol to reduce the multimeric 90 kDa PGIP proteins to the 45 kDa native pPGIP bands. Differences in glycosylation may account for PGIP sub-bands around 45 kDa.

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

Based on the concern noted above that authentic pPGIP protein may not result in the generation of sufficiently specific anti-pPGIP monoclonal antibodies, we worked with Richard Krogsrud, CEO of Antibodies Inc., to identify hydrophilic peptide sequences in the pPGIP protein sequence that could be used as antigens. We selected three peptides (**Figure 2**) that would be specific to pPGIP and, thus, likely assure that the antibodies would not recognize other PGIPs. We intended to mix the three peptides when they are administered to the mouse cells to optimize the chances of getting a specific and robust antibody. We also identified a peptide from the conserved

amino end of the PGIPs. We selected this peptide to generate a new polyclonal antibody that can be used to detect other PGIPs in addition to the pPGIP. The peptides have been synthesized through subcontractors used by Antibodies Inc. although one of the pPGIP-specific peptides proved to be recalcitrant to conjugation. Antibodies Inc. has gone ahead and developed hybridomas using the other two pPGIP-specific peptides and delivered them to us in late September 2013.

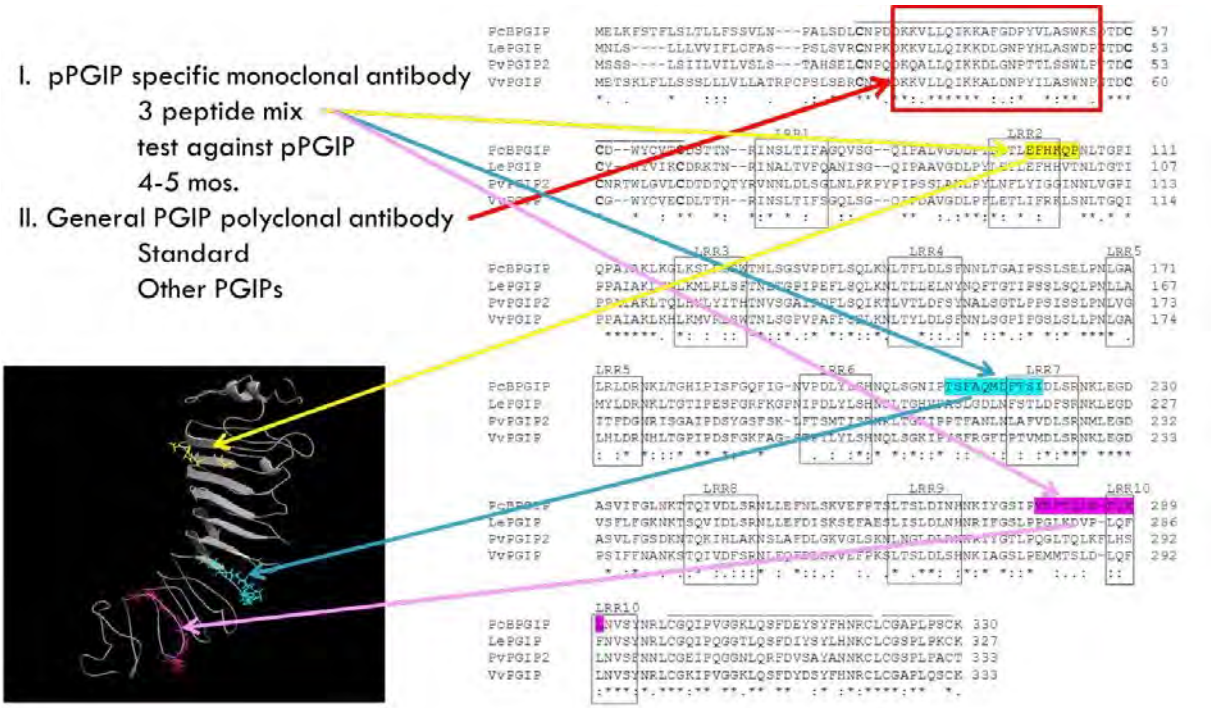

Figure 2. Amino acid sequence of pear (pPGIP), tomato (LePGIP), common bean (pvPGIP) and grape (vvPGIP) showing the location of the leucine-rich repeats (LRR) and the three pPGIP specific peptides (in yellow, blue, and pink) and the peptide common to all PGIPs (in red). Locations on the predicted 3-D structure of PGIP are shown.

In May 2013, we received the first test bleed and pre-immune sera from the polyclonal antibody preparations generated against the general PGIP peptide. This antibody preparation is considered a general PGIP antibody because it is was generated in response to a conserved region at the amino end of several PGIP proteins. We have used the pre-immune serum on a western blot with protein extracts from tomato plants expressing pPGIP and the purified pear fruit pPGIP protein described above. The antibody specifically recognizes the purified pPGIP protein from pear fruit as well as the pPGIP protein expressed in the tomato variety "Cuatomate" lines 3-15 (**Figure 3a**). The antibody preparation does not detect tomato PGIPs in the "Cuatomate" material 3-8, which is not transformed and, therefore, does not express pPGIP. With the antibodies from the first test bleed, we were able to detect clearly only the pPGIP band in the same protein preparations used to check the pre-immune serum (**Figure 3b**). We detected no cross-reactivity with the pre-immune serum (**Figure 3b**). On July 18, 2013 the final bleed sera were received from Antibodies Inc. and brought to UC Davis. They will be divided into several sub-samples, stored and distributed.

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

Activities for this objective will be concluded now that the monoclonal antibodies have been received at UC Davis. The western blot with the new polyclonal antibodies in **Figure 3** contains proteins from the leaves of a tomato line expressing pPGIP; similar results have been obtained with xylem sap collected from the cut stems of the same plants. Efforts to collect xylem sap from pPGIP-expressing grapevines has yielded only a very small amount of protein and the expected greater sensitivity of the monoclonal antibodies is necessary to detect pPGIP present in grapevine xylem exudate.

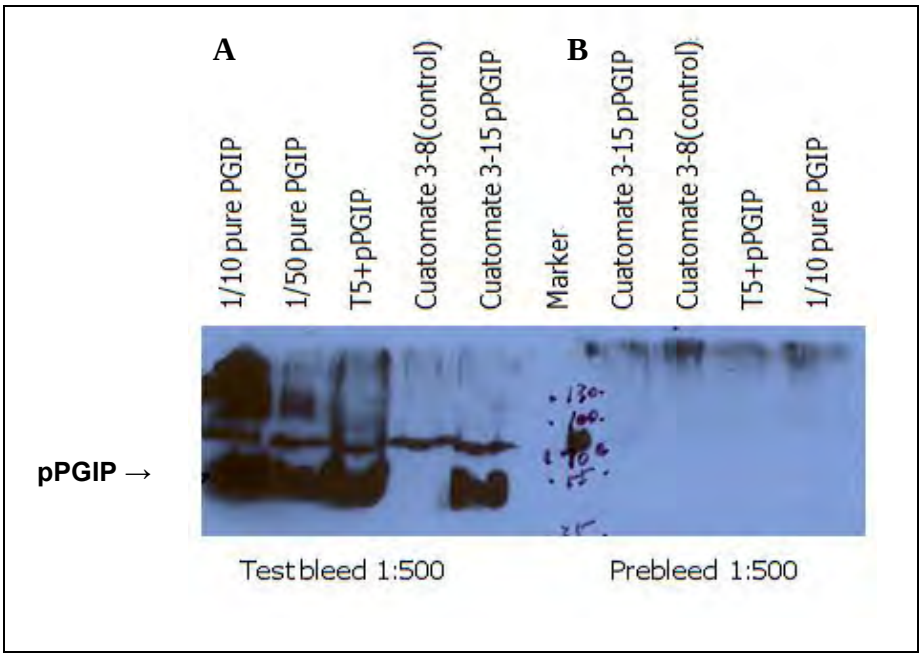

Figure 3. Image of western blot of proteins cross-reacted with antibodies from the first test bleed serum in response to the general PGIP peptide (A) and the pre-bleed serum from the rabbits (B).

CONCLUSIONS

In response to the strategy recommended by the Pierce's Disease Research Scientific Advisory Panel to enhance the resistance of grapevines to Pierce's disease, several field trial projects have used alternative approaches to optimally express plant genes encoding particularly effective PGIPs that target the *Xf* PG (*Xf*PG) in transgenic grape rootstocks. This project was designed to generate monoclonal antibodies that specifically recognize the pPGIP protein so that the amount of protein can be compared among different different plants used for testing different Pierce's disease protection strategies and at different times. The monoclonal antibody is a necessary tool for the multiple field trial projects evaluating the efficacy of pPGIP as an anti-*Xf* strategy. The antibodies will allow for detection and quantification of pPGIP without cross-reactive interference from the native PGIP (*Xf*PG) and will allow comparisons between groups. Plants can, therefore, be more efficiently screened for the presence of the pPGIP protein, whether directly produced in, or transported from rootstocks to the scion tissues of interest.

The goal of the project is to provide the resources needed for the field trial projects that are designed to help the California grape industry develop a strategy that uses plant genes to limit the damage caused by *Xf* and to mobilize this technology by using non-transgenic vines grafted on the disease-limiting rootstocks. The project's outcomes should provide growers with plants that resist Pierce's disease and produce high quality grapes.

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

GREENHOUSE EVALUATION OF GRAPEVINE FUNGAL ENDOPHYTES AND FUNGAL NATURAL PRODUCTS ANTAGONISTIC TO *XYLELLA FASTIDIOSA* FOR CONTROL OF PIERCE'S DISEASE

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ABSTRACT
The goal of this research is to identify fungal endophytes and fungal natural products that are antagonistic to *Xylella fastidiosa* (*Xf*) that could be implemented as a preventive or curative treatment for Pierce's disease, respectively. We previously showed that some fungal endophytes inhabiting grapevines possess anti-*Xf* properties, likely due to the production of secondary metabolites. To date, we have selected a total of eight fungal specimens and one bacterium that showed inhibitory effects on *Xf* growth *in vitro*. Our goal is to prove that these organisms can be used as biocontrols for Pierce's disease when introduced in grapevine cuttings before planting in vineyards. In a greenhouse experiment in 2012 and 2013, we introduced all nine putative biocontrols into grapevine cuttings cv. 'Merlot' and then challenged them with *Xf*. We are currently evaluating the ability of these biocontrol agents to mitigate Pierce's disease symptom development and decrease *Xf* titer *in planta*. In order to do so, we have developed and validated a new qPCR assay to quantify *Xf in planta*. The other objective is to use the natural compounds produced by the eight fungi as a curative treatment for Pierce's disease. We have identified two molecules (molecules 'R1' and 'C') and a semi-synthetic molecule derivative (molecule 'R2'), as an effective inhibitor of *Xf*. We are currently testing the ability of the molecule 'R1' to cure Pierce's disease infected grapevine cvs. 'Chardonnay' and 'Merlot' in a greenhouse bioassay following trunk injections. 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 are currently being deployed, but as of today successful management largely involves vector control through the use of insecticides. Here we propose to test two alternative control strategies to complement those currently in place or being developed. The first strategy is to use biocontrol agents that can be introduced in grapevine cuttings prior to field planting. We have identified eight fungi and one bacterium naturally inhabiting grapevines that are antagonistic to *Xylella fastidiosa* (*Xf*). We are currently evaluating in a greenhouse assay the ability of these biocontrols to mitigate Pierce's disease symptom progression as well as the *Xf* titer *in planta*. The second strategy is to use the natural compounds produced by the eight fungi as a curative treatment for Pierce's disease. We are currently extracting and characterizing these fungal compounds. Thus far, we have identified three compounds that are inhibitory to the bacterium in an *in vitro* assay. These natural compounds 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
Xylella fastidiosa (*Xf*) is a Gram negative, xylem-limited, insect-vectored bacterium and is the causal agent of Pierce's disease of grapevine (Hopkins and Purcell, 2002). The recent introduction of a more effective vector, the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*), to Southern California shifted the epidemiology of Pierce's disease from a monocylic to a polycyclic disease. This led to a Pierce's disease epidemic with severe

economic consequences for the Southern California grape industry. The potential for the GWSS to move north and become established throughout the state remains a severe threat to the other major grape-growing regions (Central and Northern California). Current Pierce's disease management strategies largely involve vector management through the use of insecticides.

Control of Pierce's disease with fungi or fungal metabolites is a largely unexplored research area. Fungi are receiving increasing attention from natural product chemists due to the diversity of structurally distinctive compounds they produce, together with the fact that many fungal species remain chemically unexplored. Fungi are excellent sources of interesting novel molecules that may be candidates with potential for control of bacterial diseases. Indeed, using fungi as biocontrol agents against plant disease is an active area of research (Amna 2010; Proksch et al., 2010; Xu et al., 2008).

Our objectives are to characterize the microbial diversity in grapevines that escaped Pierce's disease in natural vineyard settings, and compare this population to Pierce's disease infected grapevines with the goal of identifying fungi that are unique to Pierce's disease 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 of Pierce's disease by inoculating grapevine cuttings with endophytic, *Xf*-antagonistic fungi and 2) if fungal natural products have curative properties for vines already infected with Pierce's disease. 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. Evaluate grapevine fungal endophytes for *in planta* inhibition of *Xf* and Pierce's disease development using our established greenhouse bioassay.
2. Purify and characterize natural products produced by the inhibitory fungi.
3. Evaluate natural products for their potential as curative treatments for vines already infected with Pierce's disease.

RESULTS AND DISCUSSION

Objective 1. Evaluate grapevine fungal endophytes for *in planta* inhibition of *Xf* and Pierce's disease development using our established greenhouse bioassay.

The goal of this objective is to provide increased tolerance to Pierce's disease by inoculating grapes with natural fungal endophytes that possessed anti-*Xf* properties. From 2010 to 2012 we collected plant tissue samples (sap, petioles, canes, spurs) from Pierce's disease escaped and Pierce's disease infected grapevines and isolated fungi inhabiting these samples. We identified these specimens to the genus level by comparing the ribosomal DNA sequences to specimens deposited in the GenBank database. We then tested the ability of all the organisms recovered to inhibit *Xf* growth using an *in vitro* bioassay (Rolshausen and Roper, 2011), which allowed us to select a total of nine biocontrol candidates (eight fungi and one bacterium).

In 2012 and 2013, these organisms were subsequently re-introduced in grapevines prior to planting. Fungi were grown on PDA medium for two weeks and spores were harvested in sterile water and the concentration was adjusted to 50 spores/µl. Grapevine cuttings var. 'Merlot' were vacuum infiltrated (**Figure 1**) with the fungal spores, and planted in the greenhouse. Control plants were infiltrated with sterile water only. After a few weeks, the green shoots arising from these cuttings (20 to 30 cm long) were inoculated with *Xf* (Temecula strain) by mechanical needle inoculation (Hill and Purcell, 1995). A subsample of plants were left uninoculated 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 every two weeks (0= no symptoms; 5=Plant dead or dying) according to Guilhabert and Kirkpatrick (2005) (**Figure 2**).

Figure 1. Technique used to vacuum infiltrate grape cuttings with spores of the fungal endophytes. Grape cuttings are placed in the spore suspension on one end (blue arrow) and are attached to a tube on the other end that is connected to a vacuum pump (white arrow).

Figure 2. Pierce's disease 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.

In addition, our lab has developed a qPCR assay for *Xf* detection and quantification in order to quantify bacterial titer *in planta*. This molecular test is a key to evaluate the biocontrol agents' ability to suppress Pierce's disease in greenhouse bioassays. We first utilized an assay developed by Schaad et al. (2002). We optimized PCR conditions for the primer set *Xf*F1 (AAAAATCGCCAACATAAACCCA) and *Xf*R1 (CCAGGCGTCCTCACAA GTTAC), using SyBr green fluoresce detection. However, the qPCR data coupled with sequencing of the amplicons results revealed low detection specificity in environmental samples leading to false positives. Therefore, we developed a new primer sets targeting the ITS region of ribosomal genes and the RNA polymerase sigma-70 factor gene of *Xf* were developed using the PRISE software program (Fu et al., 2008; Huang et al., 2013). A Taqman quantitative PCR assay was developed specifically targeting a 90-bp long fragment of the ITS region of ribosomal genes of *Xf*. The selective primers and probe are *Xf*ITSF6 (5'- GAGTATGGTGAATATAA TTGTC-3'), *Xf*ITSR6 (5'-CAACATAAACCCAAACCTAT-3') and *Xf*ITS6-probe1 (5'- [6-FAM] CCAGGCGTC CTCACAAGTTA [BHQ1a-6FAM] -3'), where BHQ1a is Black Hole Quencher 1 (Eurofins MWG Operon, Huntsville, AL, USA). We were able to detect *Xf* in samples from infected grapevines in commercial vineyards and artificially inoculated grape cuttings in the greenhouse. Chromosome walking was performed to confirm the

specificity of the PCR assay. Universal bacterial primers hybridize to ribosomal gene 1507F (5'-GGTGAAGT CGTAACAAGGTA -3') (Yamamoto et al., 2013) and 23Sr (5'-GGGTTBCCCCATTCRG -3') (Fisher & Triplett, 1999), and were paired with *Xf*ITSR6 and *Xf*ITSF6 accordingly to perform PCR on environmental DNA extracted from grape shoot tissue. Sequence analysis was performed on Geneious Pro 6.0.5 (Geneious, Biomatters Ltd, Auckland, New Zealand). The sequence identities were determined by analyses using BLAST (NCBI) (Altschul et al., 1997) and confirmed the detection of *Xf.*

In 2012, we recorded a lot of background noise with plants expressing leaf-reddening symptoms that confounded the Pierce's disease symptoms. As a consequence it was difficult to rate Pierce's disease accurately and severity data are not presented. However, the experiment was repeated successfully in 2013. Our results showed that four fungi (fungus 2, 4, 6, and 8) and the bacteria were able to mitigate Pierce's disease severity (**Table 1**). Petioles were collected from plants in 2012 and 2013, and samples are currently being analyzed for bacterial titer.

Table 1. Pierce's disease incidence in grapevine cv. 'Merlot' infiltrated with biocontrols. Data in 2013 show the average disease severity rating (n=10 plants) and the percent of vines with a severity >3 at 12 weeks post-inoculation.

	Not *Xf* inoculated		***Xf* inoculated**	
Treatment	Avg. severity rating	Percent of vines with rating > 3	Avg. severity rating	Percent of vines with rating > 3
Control	1 ± 0.7	0	4.2 ± 0.8	90
Bacteria 1	0.4 ± 0.5	0	2.3 ± 1.8	50
Fungus 1	1.1 ± 0.7	0	4.4 ± 0.7	100
Fungus 2	0.9 ± 0.9	0	2 ± 1.8	30
Fungus 3	0.7 ± 0.5	0	3.6 ± 1.3	70
Fungus 4	0.7 ± 0.7	0	1.7 ± 1.3	40
Fungus 5	0.3 ± 0.5	0	3.6 ± 1.3	70
Fungus 6	0.4 ± 0.5	0	2 ± 0.9	20
Fungus 7	0.6 ± 1	0	4 ± 1.2	80
Fungus 8	1 ± 0.7	0	2.7 ± 1.2	50

Objective 2. Purify and characterize natural products produced by the inhibitory fungi.
The goal of this objective is to identify fungal species and fungal natural products produced by endophytes that can be used as curative treatments for control of Pierce's disease. We identified eight fungal specimens inhabiting grapevine tissues (xylem sap, shoot, petioles, and spur) that were able to inhibit *Xf* in a bioassay (Rolshausen and Roper, 2011). In brief, *Xf* liquid cultures are adjusted to OD_{600nm}=0.1 (approx. 10^7 CFU/ml); 300 µl of the *Xf* cell suspension are added to three ml of PD3 medium containing 0.8% agar and briefly vortexed. This mixture is then overlayed onto a petri plate containing PD3 medium. A sterile circle of agar is drawn from the margin of an actively growing pure fungal culture and is placed onto the plates previously inoculated with *Xf.* Plates are incubated at 28ºC for seven days and then observed for an inhibition zone around the fungal colony (**Figure 3**).

In addition, crude extracts collected from the fungal cultures showing inhibition towards *Xf* were collected for evaluation using a similar growth inhibition assay as described above. In brief, 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 10 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 are 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. Crude extracts showing inhibition were further processes to identify the composition in natural products. We are currently fractionating the crude extracts from some of these fungi in order to purify and identify the inhibitory molecules. Thus far, we have purified two individual molecules (molecules R and C) that are active against *Xf* growth *in vitro* and have characterized their chemical structure. Molecule 'R' is produced in large quantity by the fungus in Potato

Dextrose Broth medium, unlike molecule 'C.' We are currently testing different growth media (rice medium, grape cane extract medium) to increase the yield of molecule 'C' in culture in order to run our bioassays.

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.

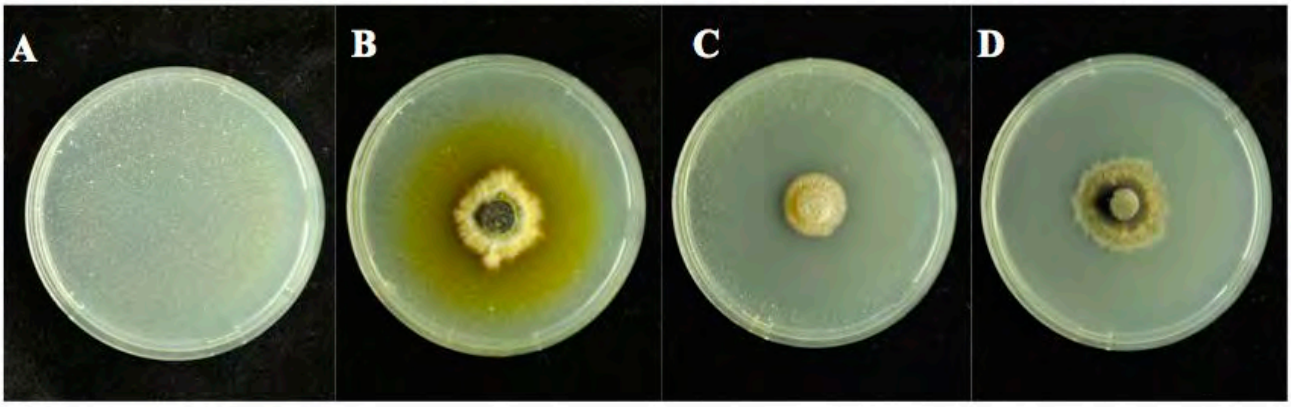

Figure 3. *In vitro* inhibition assay used to evaluate fungal activity towards *Xf*; *Xf* cells were plated in top agar and agar plugs containing fungi were placed on top. Inhibition was evaluated after eight days of incubation at 28˚C. **A**) *Xf*-only control; **B**) No *Xf* inhibition; **C**) Mild *Xf* inhibition; **D**) Total *Xf* inhibition.

We have been working with molecule 'R' specifically because it showed great potential *in vitro*. Hence, in an *in vitro* dose response assay, where *Xf* cells are submitted to an increasing concentration of a fungal molecule, molecule R1 was able to inhibit *Xf* growth (**Figure 4**). A semi-synthetic derivative of molecule 'R1' was also prepared (molecule 'R2') and was also able to reduce *Xf* cells growth (**Figure 5**). The dose response assay will also be repeated with molecule 'C' once we get enough material to work with. We are currently working on synthesizing derivative molecules that are water-soluble so they can be sprayed or injected on plants more readily. Currently these molecules are only soluble in dimethyl sulfoxide (DMSO), which may limit the ability of the molecules to become systemic in the plant following infection and act on site in the xylem, where the bacterium resides.

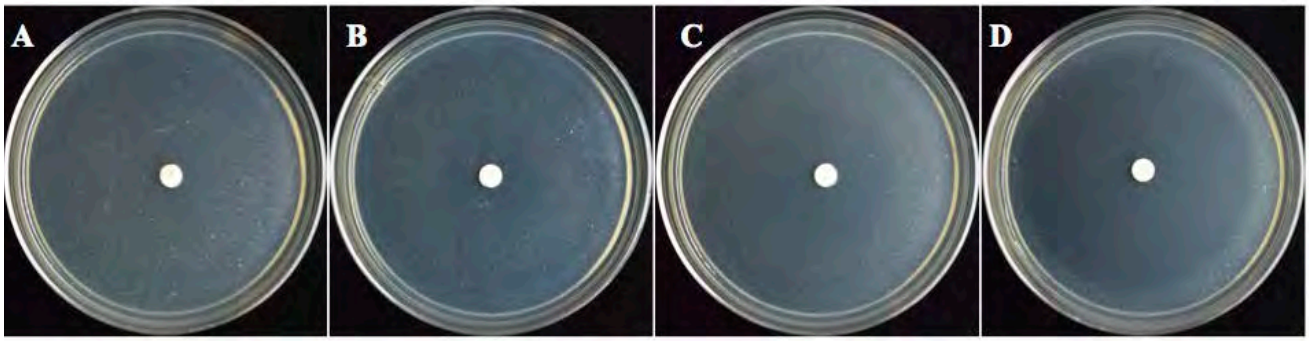

Figure 4. Dose response assay to evaluate *in vitro Xf* inhibition at increasing concentration of a fungal molecule. **A**) 0 µg molecule R1 (control); **B**) 50 µg molecule R1; **C**) 100 µg molecule R1; **D**) 250 µg molecule R1.

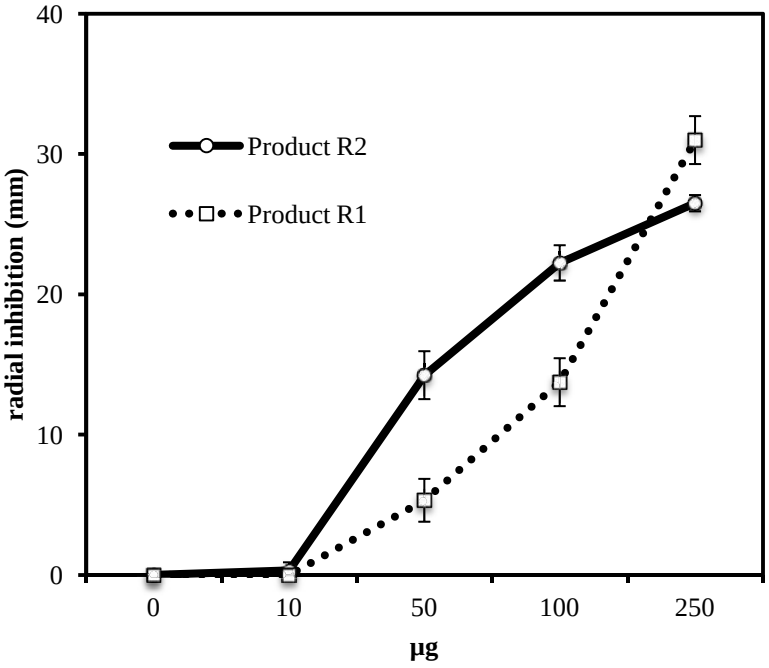

Figure 5. *In vitro* dose response assay. This lab assay quantifies inhibition of *Xf* growth as a measure of a halo around a disc (mm) containing increasing concentration of fungal molecule R1 and molecule derivative R2.

Objective 3. Evaluate natural products for their potential as curative treatments for vines already infected with Pierce's disease.

The goal of this objective is to develop a greenhouse bioassay to evaluate the anti-*Xf* efficacy of fungal natural compounds *in planta*. Once this proof of concept is established in the greenhouse, the experiment will be carried over to the field. The deliverable for this objective is the development of a commercial product for the cure of Pierce's disease.

We have currently identified one fungal natural compound (molecule 'R1') as an active molecule inhibitory to *Xf* (see Objective 2). In 2012 and 2013 our greenhouse trials were designed to test the molecule 'R1' on Pierce's disease-infected vines and determine if Pierce's disease symptoms and *Xf* titer *in planta* is reduced after injection of the molecule 'R1.' Grapevines cuttings cvs. 'Chardonnay' and 'Merlot' were infected with *Xf* (Temecula strain) by mechanical needle inoculation (Hill and Purcell, 1995). In 2012, the molecule 'R1' was injected in both cvs. after appearance of the first Pierce's disease symptoms (rating of 1 or 2 on Pierce's disease rating severity chart **Figure 2**) at eight weeks post-inoculation, while in 2013 the molecule 'R1' was injected in both cvs. at two weeks post-inoculation. Molecule 'R1' (80% pure) was suspended in pure DMSO and 10 mg was injected in healthy (control) and *Xf*-infected grapevines. Mock injections of healthy and Pierce's disease-infected plants consisted of pure DMSO only. Injection was made using a 1 ml 16-gauge needle. Injection was performed in the xylem tissue of the grape cutting right below the shoot (**Figure 6**). Our results indicated there was no reduction in the severity of the Pierce's disease symptoms following injection of molecule 'R1' (data not shown). We believe that because the molecule is not soluble in water, that it can't be metabolized and transported through the plant systemically. We are currently in the process of developing a water soluble 'R' molecule.

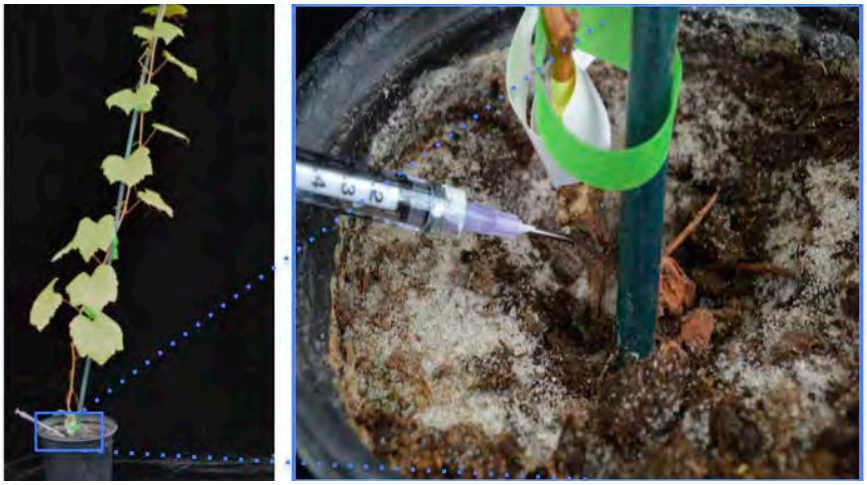

Figure 6. Needle injection of an anti-*Xf* molecule in the xylem of Pierce's disease-infected grapevine cuttings.

CONCLUSIONS

We aim to investigate both prophylactic and curative measures for Pierce's disease that will ultimately contribute to a sustainable Pierce's disease management strategy. We have developed a qPCR molecular tool to be able to detect and quantify *Xf* in plant samples to measure the efficacy of our strategies. Practically, we envision that the biocontrol organisms could be applied into grapevine cuttings at the nursery level through vacuum infiltration of fungal spores into the xylem tissue, thereby, providing enhanced protection against Pierce's disease. We are currently evaluating this strategy in the greenhouse. The remaining step is to confirm that the beneficial organisms provide Pierce's disease control in a field setting. As a curative strategy, we are evaluating the use of anti-*Xf* fungal natural products to provide a solution to growers that have vineyards already infected with Pierce's disease. We have developed a xylem injection prototype in which to deliver a sustain supply of the anti-*Xf* compounds effectively into the xylem of an infected vine. We have already discovered three active anti-*Xf* compounds. However, one compound did not show activity in our greenhouse trials likely because it is not water-soluble. The next step is to discover additional active natural anti-*Xf* compounds, and synthesize semi-synthetic derivative molecules to make them more active *in planta*. In the event that these compounds mitigate Pierce's disease in the greenhouse, we will test their efficacy in natural vineyard settings in the future.

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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 was 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. The source of the PD-susceptible plant material was the winegrape variety Chardonnay, known to support high populations of *Xylella fastidiosa* (*Xf*) and exhibit severe PD symptoms. The source of the PD-resistant material was a modified backcross generation 2 (mBC2) raisin selection with PD resistance locus *PdR1* introgressed from 89-F0908 (*V. rupestris* X *V. arizonica*). Scions were mechanically inoculated with *Xf* strain Stags Leap. PD severity was 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) at 14 weeks post inoculation, tissue samples (petioles and stems) were assayed for *Xf* titer by real-time PCR. Results indicated that PD symptom expression and the *Xf* titer in Chardonnay scions were not significantly different when grafted onto PD-susceptible or PD-resistant rootstocks. Thus, the answer to the question posed in the title is "no."

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 were 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. Results indicated that a PD-susceptible scion grafted onto a PD-resistant rootstock remains susceptible to PD.

INTRODUCTION

This 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 *Vitis vinifera* selection 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). Further, xylem sap from *PdR1* plants reduce growth of *Xf* in culture (Cheng et al., 2009). As the *PdR1* resistant rootstock used in these experiments is a second generation backcross with a genetic composition of ~87.5% *V. vinifera*, difficulties encountered by Lin and Walker (2004) in establishing sound graft unions between *V. vinifera* scions and rootstocks derived from wild *Vitis* species were avoided. This simple experimental design can 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 scions genetically predisposed to be susceptible.

OBJECTIVES

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

RESULTS AND DISCUSSION

The basic experimental design evaluated PD symptom development and *Xf* population levels in PD-susceptible scions grafted onto rootstocks that are either resistant or susceptible to PD (**Table 1**). The first year experiment was conducted in a greenhouse at the USDA ARS SJVASC facility in Parlier, CA. The source of PD-susceptible plant material was the winegrape variety Chardonnay, known to support high populations of *Xf* and exhibit severe PD symptoms (Buzkan et al., 2005). The source of PD-resistant material (Ramming and Walker, 2010) was a modified backcross generation 2 raisin selection (referred to here as PDR1) with PD resistance locus *PdR1* (Krivanek et al., 2006) introgressed from 89-F0908 (*V. rupestris* x *V. arizonica*). Each treatment consisted of 10 plants (replicates).

Table 1. Basic experimental design to evaluate effect of PD-resistant rootstocks on PD susceptible scions following challenge inoculation of scion with *Xf*.

Rootstock		Scion		
Variety	PD response	Variety	PD response	Expected scion response to PD
Chardonnay	Susceptible	Chardonnay	Susceptible	Susceptible
PDR1	Resistant	PDR1	Resistant	Resistant
PDR1	Resistant	Chardonnay	Susceptible	?
Chardonnay	Susceptible	PDR1	Resistant	?

The *Xf* strain Stag's Leap was used as challenge inoculum, as this strain was recovered from PD-symptomatic grape in California and is known to cause severe PD symptoms in inoculated plants (Hendson et al., 2001). Plants were mechanically inoculated above the graft union (e.g., scions) using a 20 µl droplet of bacterial culture suspension (10^8 colony forming units/ml) placed on a partially lignified stem just above the petiole junction. Bacteria were introduced into the xylem using a 25 gauge needle pushed through the droplet, penetrating about $1/3^{rd}$ of the width of the shoot. Plants were maintained in the greenhouse and evaluated visually for PD symptoms after 14 weeks. PD severity was visually assessed using a nominal 0-5 rating scale (Roper et al., 2007), where 0 corresponds to no visual symptoms and 5 corresponds to death of the plant (**Figure 1**). In all cases, the response of the scion to PD remained unaltered, regardless of rootstock genotype. Chardonnay scions expressed similar levels of PD severity on both PD-susceptible (mean severity rating 3.2) and PD-resistant rootstocks (mean severity rating 3.6), whereas PDR1 scions expressed only mild symptoms on PD-susceptible (mean severity rating 1.0) or PD-resistant (mean severity rating 1.4) rootstocks. PD symptom severity ratings for mock inoculated plants of all scion/rootstock combinations had means of less than 1.0, but greater than 0, presumably due to water stress at some point post-inoculation.

Real-time PCR was used to quantify bacterial titers in stems and petiole samples collected 25 cm above the point of inoculation. DNA samples were extracted from lyophilized tissue as previously described (Ledbetter and Rogers, 2009) and used as template. Real-time PCR reactions were run in triplicate to determine technical variability and standard curves were included in each plate to facilitate normalization. Real-time PCR data were converted to the equivalent number of *Xf* genomes; mean population levels were compared among scion/rootstock combinations for both stem and petiole samples (**Figure 2**). *Xf* population levels in Chardonnay scions were similar regardless of rootstock genotype ($\sim 10^7$) in both stem and petiole samples. In contrast, *Xf* population levels were substantially lower in PDR1 scions ($\sim 10^4 - 10^5$), but no significant differences were noted for PDR1 scions based on rootstock genotype. In the second year of the project, the experiment described above was repeated. Unfortunately, too few inoculated plants became infected; no useable data were obtained.

CONCLUSIONS

Based on one year of data, it is unlikely that *PdR1* germplasm will be suitable for development of commercial rootstocks that confer systemic resistance to existing PD-susceptible grape varieties used as scions. Nonetheless, *PdR1* scions remained resistant when grafted onto a PD-susceptible rootstock. This observation suggests that new table, raisin, and winegrape varieties bearing *PdR1*-mediated PD resistance may be deployed as scions onto existing PD-susceptible rootstocks. However, low bacterial population levels (but greater than zero) in PD-resistant material used as scions could eventually allow for systemic movement of *Xf* into PD-susceptible rootstocks. Thus, PD-resistant scions could eventually decline if susceptible rootstocks become systemically

infected and develop high population levels of *Xf* that interfere with xylem transport. Therefore, the industry may want to proceed with breeding programs aimed at developing PD-resistant rootstocks to complement efforts already in progress on development of PD-resistant scions.

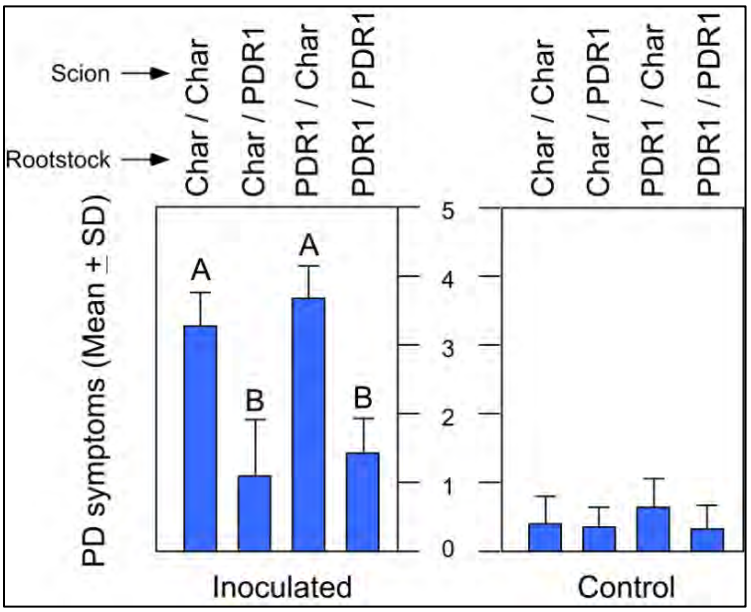

Figure 1. PD symptom ratings (0 = no disease, 5 = death) for scions at 14 weeks post inoculation. Means (+/- standard deviation) with different letters are significantly different ($P < 0.05$) based on a non-parametric rank sum test. Char [Chardonnay] (PD-susceptible); PDR1 (PD-resistant).

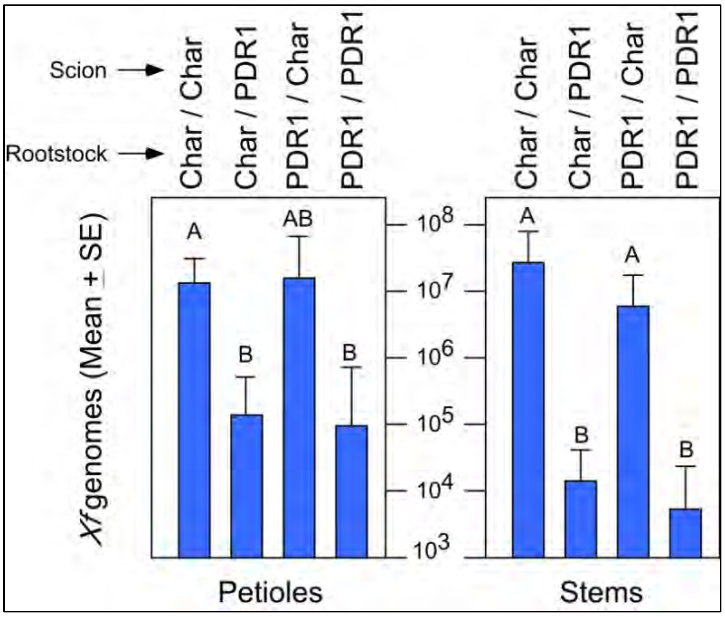

Figure 2. Estimation of *Xf* titer in scions 14 weeks post inoculation. Results of quantitative real time PCR are presented as the equivalent number of *Xf* genomes present in petiole or stem samples taken ~25 cm above the point of inoculation. Values refer to mean ± standard error for entire petioles or 2.5 cm sections of stem. Statistical analysis was by ANOVA followed by Tukey's HSD; means with different letters are significantly different ($P < 0.05$).

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VIRULENT PHAGES OF *XYLELLA FASTIDIOSA*: PROPHYLACTIC AND THERAPEUTIC EVALUATION

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ABSTRACT

Xylella fastidiosa (*Xf*) is the causal agent of Pierce's disease, a major threat to the wine industry in the USA. There are currently no effective control measures to prevent infection or manage the disease once it is established in a vineyard, short of aggressive culling of infected vines. Bacteriophages (phages) are viruses that attack bacteria. Phages specifically target the underlying pathogen, without posing any harmful effects to humans, animals, or plants. However, it is imperative that only virulent phages be implemented for preventative or therapeutic treatment to control disease, since the use of temperate phages can lead to lysogenic conversion and potential transfer of bacterial pathogenicity genes.

We have reported the isolation and propagation of the first *Xf* temperate phages. Here we describe the isolation and characterization of the first virulent phages for *Xf* and illustrate their utility as a prophylactic or therapeutic treatment for Pierce's disease. Four representative *Xf* phages, Sano, Salvo, Prado, and Paz, were chosen for this study. Siphophages Sano and Salvo have isometric heads with non-contractile tails, whereas podophages Prado and Paz have isometric heads with short-stubby tails. All four phages form plaques on lawns of *Xf* subsp. *fastidiosa*, *Xf* subsp. *multiplex*, and *Xf* subsp. *sandyi*. Phages Sano and Salvo are syntenic to *Burkholderia* phage Nazgul, and constitute a distinct phage type. Phages Prado and Paz are new members of the phiKMV-like phage type in the T7 superfamily. The four phages were determined to be Type IV pilus-dependent. In addition to genome analysis, we examined the potential for abortive lysogeny, using infection at a high multiplicity of infection (MOI), and measured survival. Our results indicated no evidence for lysogeny or repression, and supported the conclusion that the four phages are virulent and thus attractive candidates for the development of phage cocktails to control disease caused by *Xf*.

The therapeutic and preventative efficacy of a phage cocktail composed of phages Sano, Salvo, Prado, and Paz to protect grapevines (*Vitis vinifera* cv. Cabernet Sauvignon) against Pierce's disease caused by *Xf* was evaluated in greenhouse experiments. The results clearly demonstrated the efficacy of both applications. An ongoing trial will test efficacy under field conditions. Application of phages as a biocontrol agent for *Xf* offers a novel biological treatment for control of Pierce's disease.

FUNDING AGENCIES

Funding for this project was provided by a grant to the Center for Phage Technology and Texas A&M AgriLife Research from Otsuka Pharmaceutical Co., Ltd.

IDENTIFICATION OF NOVEL SECRETED VIRULENCE FACTORS FROM *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted September 2010 to September 2013.

ABSTRACT

Xylella fastidiosa (Xf) is a bacterium that causes Pierce's disease of grapevine and other leaf scorch diseases of agriculturally important crops. Little is known about virulence factors that are necessary for *Xf* to grow and cause disease in the xylem vessels of a plant host. Any protein secreted by the bacterium has the potential to interact with the plant host and affect pathogen virulence and/or recognition. Fifty-eight *Xf* proteins with putative secretion signals were identified, cloned into a tobacco rattle virus (TRV) expression vector, and tested for effects on the pathogenesis of TRV in *Nicotiana benthamiana*. Eight proteins conferred increased pathogenicity to TRV. These proteins are annotated as proteins of unknown function or having a metabolic role suggesting that these proteins may have multiple functions in *Xf* pathogenicity. An *Escherichia coli* expression system was used to determine protein localization and confirm secretion for the majority of identified proteins. Phenotypic analyses of the identified putative virulence factors are being performed. Additionally, mutations have been constructed in most of the eight genes and disease assays in grapevine are underway. Understanding the functions and mechanisms of virulence factors will aid in the development of control strategies for Pierce's disease.

FUNDING AGENCIES

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Section 5:

Crop Biology and Disease Epidemiology

BLOCKING *XYLELLA FASTIDIOSA* TRANSMISSION

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Reporting Period: The results reported here are from work conducted November 2012 to October 2013.

ABSTRACT
This report describes results achieved since November 2012. We focus on two specific topics in this report, as they have been better developed and most of the results are final. Other components of this project, which are ongoing but so far preliminary, will be ignored as final results may change with new data that are being gathered at the moment. First, the *Xylella fastidiosa* (*Xf*) chitinase A (ChiA) that we previously identified was characterized in more detail; one important finding is that we demonstrate that it is required for *Xf* colonization of both insects and plants. Second, we used greenhouse transmission experiments with an efficient vector to show that one of the chitin-binding proteins previous identified (PD1764) functions as a transmission-blocking peptide. Results also demonstrate that a domain of HxfB (HxfAD1-3) also blocks transmission, but less efficiently than PD1764. In summary, during this reporting period we finalized efforts to characterize the basic role of a chitinase in *Xf*, and performed greenhouse experiments that demonstrated the feasibility of using specific peptides to block *Xf* vector transmission to plants.

LAYPERSON SUMMARY
This project is based on the hypothesis that an understanding of how *Xylella fastidiosa* (*Xf*) interacts with its insect vectors at the molecular level will allow the development of strategies to disrupt such interactions, effectively leading to reduced disease spread in the field. This report focuses on two components of this project. First, we previously identified a chitinase in *Xf*; now we demonstrate it is required for effective colonization of both its insect and plant hosts. The finding that this chitinase is important for both of the life stages of *Xf* (in sharpshooters and grapevines) opens an important opportunity for the development of control strategies that could affect both disease development in plants and vector transmission between plants. Second, we propose that *Xf* proteins associated with vector colonization also block *Xf* transmission to plants. Here we show that two tested proteins reduce *Xf* transmission to plants under greenhouse conditions. This work has resulted in findings that impact our understanding of *Xf* colonization of plants and insect vectors. It also identified specific peptides that outcompete *Xf* for binding sites in its vector, effectively blocking transmission.

INTRODUCTION
This report is divided into two sections, which address the original Objectives of our project. First, we present data on the biological characterization of a chitinase (ChiA) previously identified in *Xylella fastidiosa* (*Xf*) (Killiny et al., 2010). Results show that both insect and plant colonization are severely impacted by the disruption of *chiA*. Second, we summarize trials aimed at identifying transmission-blocking peptides. Experiments performed during this year show that the LysM domain of a putative coding region (PD1764) in the genome of *Xf* Temecula, identified through a proteomics pipeline, blocks vectors transmission of *Xf* to plants. Sections of this report have been included in previously submitted yet unpublished reports.

OBJECTIVES

This project had two original objectives:

1. Continue efforts to identify additional targets implicated in *Xf* transmission by insects.
2. Test specific and efficient molecules to disrupt vector transmission.

RESULTS AND DISCUSSION

Objective 1. Continue efforts to identify additional targets implicated in *Xf* transmission by insects.

This Objective had two major objectives. First, the characterization of a chitinase (ChiA) in *Xf*, which was hypothesized to be essential for vector colonization and transmission, therefore being a good candidate as a transmission-blocking molecule. The second goal is to identify new targets to block *Xf* transmission; experiments, which are similar to those described below that resulted in the identification of PD1764 as a transmission-blocking molecule, are ongoing. Results of those efforts will be included in future reports.

***Xf* chitinase is important for plant and insect colonization.**

Previous work on *Xf*-insect interactions highlighted the importance of chitin in *Xf* colonization of vectors. First, results showed that *N*-acetylglucosamine (GlcNAc), the monomer of chitin, acted as a strong competitor when vector foregut extracts were used as a substrate, reducing cell adhesion (Killiny and Almeida, 2009). Second, competition between *Xf* adhesins and GlcNAc reduced the overall transmission of the bacteria by its vectors, effectively functioning as blocking molecules (Killiny et al., 2012). The observation that *Xf* possesses a functional chitinase (ChiA; PD1826) and all the machinery required to assimilate it (Killiny et al., 2010) led us to further test if *Xf* could use chitin as a carbon source for successful colonization of vectors.

***chiA* mutant is affected in its vector colonization and transmission.**

Early results with a *chiA* mutant suggested that it was impact in both plant and insect colonization. Complementation of the chitinase mutant was done following Kung et al. (2011). Briefly, the entire *chiA* sequence, along with its own promoter, was cloned into a pAX1-Cm plasmid from Matsumoto et al., and then *Xf* Temecula strain was transformed. The insertion of the *chiA* gene in the non-coding region NS1 (neutral site 1) of *Xf* chromosome was verified by PCR (data not shown).

To address the importance of *chiA* on *Xf* vector transmission we used the same protocol previously described to assess *chiA* mutant vector colonization. Ten days after acquisition, instead of testing vectors using qPCR (data show in **Figure 1B**, insects were individually transferred to healthy grapes to test their ability to transmit *Xf* to another plant (**Figure 1A**).

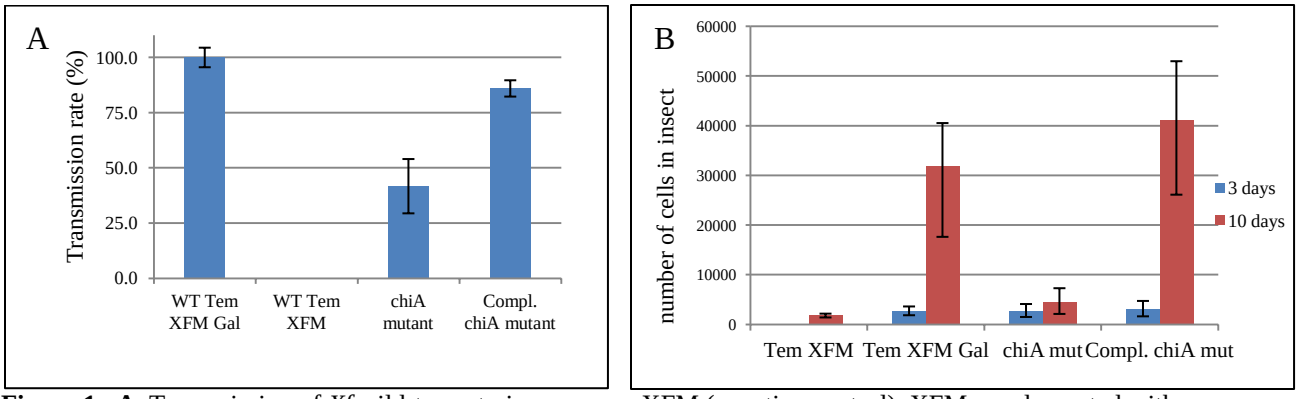


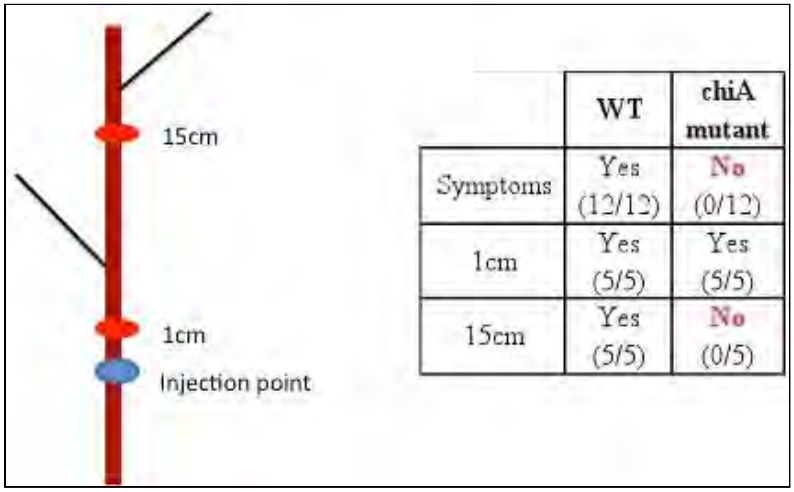
Figure 1. **A**. Transmission of *Xf* wild-type strain grown on XFM (negative control), XFM supplemented with galacturonic acid (inducer of transmission), *chiA* mutant and the complemented *chiA* mutant. Insects acquired *Xf* feeding on sachet membranes before being placed on basil for 10 days. Then insects were allowed to inoculate to healthy test plants for 24 hours. **B**. Number of cells of *Xf* that were detected by qPCR after three days (red bars) and 10 days (blue bars) post acquisition. Insects were placed on basil during this colonization period.

At 10 days post-acquisition, the transmission rate for the *chiA* mutant was significantly lower than the control. Transmission rate for *chiA* mutant was only 25.8% (6 out 22 plants tested positives, 41.7% once normalized) so a reduction of 58.3% in comparison to the wild type (14/23, 61%, normalized to 100%). As expected, the

transmission of the complemented *chiA* mutant is not different from the control (12 plants out of 23 tested positives, 86% once normalized). *Xf* cells grown on XFM medium (no induction of transmission) showed no transmission as were used as a negative control. This reduced transmission observed for the *chiA* mutant should be linked to the different abilities of the *chiA* mutant and the wild-type to colonize the insect. Ten days post-acquisition, the bacterial population colonizing insects was ~10 times greater for the wild type than for the *chiA* mutant. This difference could explain the reduction of the transmission for *chiA* mutant due to a lower number of inoculation events or a lower numbers of bacteria inoculated during each inoculation event. The explanation is less straightforward at three days post-inoculation (see previous report). Results for insect colonization, where no difference was observed between the *chiA* mutant and the wild type strain, cannot explain the difference in transmission obtained here. One possible explanation is that, due to the absence of ChiA, attachment of cells is still possible (as shown by the same number of cells colonizing the vector) but somehow less functional in relation to transmission. This result argues in favor of a probable implication of ChiA in *Xf* attachment process to the insect foregut even if ChiA should not be involved in the first steps of this adhesion.

***chiA* mutant is deficient in plant colonization.**

Thanks to our artificial diet system, no *Xf* acquisition through plants by insects was required to characterize the *chiA* mutant in relation to vector transmission. Therefore, its capacity to colonize plants still had to be determined. To address that question we mechanically inoculated grapevines (n=12) with wild-type cells, *chiA* mutant, or the complemented *chiA* mutant as previously described (Almeida et al., 2001). Two months after inoculation, we monitored symptom development and cultured plants at two sites to assess the colonization of *Xf* strains in plant. Results are presented in **Figure 2** below.



	WT	chiA mutant
Symptoms	Yes (12/12)	No (0/12)
1cm	Yes (5/5)	Yes (5/5)
15cm	Yes (5/5)	No (0/5)

Figure 2. Two sites of sampling were used to determine *Xf* colonization persistence within inoculated plants. Petioles at 1cm and 15 cm away from the injection point were cultured for assessing *Xf* persistence within plants.

Surprisingly, none of the plants infected with the *chiA* mutant developed symptoms. In comparison, plants infected with the wild type strain developed characteristic symptoms of *Xf* infection. In addition, cultures obtained from test plants 1cm above the inoculation site allowed us to recover both strains in their respective plants, meaning that the *chiA* mutant was able to survive *in planta* and the absence of symptoms previously described was not due to the death of the mutant. However, at 15cm above the inoculation site only the wild type strain was recovered. Altogether, these results show that the *chiA* mutant, even if capable of surviving in grapevines, is not able to move over long distances, and is therefore not capable of inducing Pierce's disease symptoms. This was unexpected because no role for ChiA in plants has been suggested; additional experiments are needed to define what its function is. This complemented strain will be used to confirm properties tested here, especially to restore its ability to successfully colonize plants.

ChiA requires a chitin-binding protein in order to degrade insect polysaccharides.

The ChiA in *Xf* lacks a chitin-binding domain. We initially made this observation through computational analyses, but also tested it experimentally. No binding of ChiA to chitin, colloidal chitin, chitosan or cellulose

was observed *in vitro*. In order to better understand the mechanism used by ChiA to degrade insect polysaccharides, we designed an experiment to detect the chitinolytic activity of ChiA alone or in *Xf* cells. Detection of chitinolytic activity (**Figure 3A**) was performed according to Killiny et al. (2010).

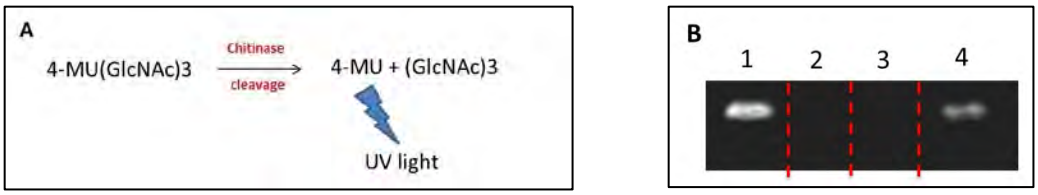


Figure 3. Detection of the chitinolytic activity. **A**. Incubation of the substrate (4-MU(GlcNAc)3; Sigma-Aldrich) with an active chitinase results in the cleavage of the substrate into a fluorescent product (4-MU) which can be visualized under UV lights. **B**. Visualization of the fluorescent bands on a native gel corresponding to the degradation of the substrate, by the chitinase. 1; Total proteins of *Xf* wild-type Temecula cells; 2: total proteins of *chiA* mutant; 3: recombinant ChiA and 4: recombinant ChiA incubated with total proteins of *chiA* mutant.

As already described by Killiny et al. (2010), ChiA present in *Xf* Temecula strain is able to degrade the chitinous substrate present in this test (**Figure 3B**, lane1). As expected because of the absence of ChiA, no fluorescence was visualized for the *chiA* mutant (lane2). However, the same result was also obtained with the recombinant protein alone (lane3). That result, in addition to the absence of a chitin-binding domain of *Xf* ChiA, reinforces our idea that ChiA needs a chitin-binding protein to degrade insect polysaccharides. One other possibility was that the recombinant protein was inactive and so, no chitin degradation was possible. To determine which of these two possibilities was correct, we tested the capacity of the recombinant ChiA to degrade the chitinous substrate in presence of total proteins of the *chiA* mutant. In lane 4 a degradation band was observed. This allowed us to conclude that the recombinant ChiA was active after the purification process. In addition, based on these results, it is very likely that *Xf* ChiA needs to interact with at least one *Xf* protein in order to degrade insect polysaccharides. Those proteins could play a role in chitin degradation linking ChiA to its substrate (chitin-binding proteins) or by "activating" ChiA in presence of chitinous substrates.

Objective 2. Test specific and efficient molecules to disrupt vector transmission.
Binding of transmission blocking molecules to different polysaccharides.

We used different candidate peptides, previously tested in our transmission-blocking experiment, in a binding assay to assess or confirm their ability to bind to chitin or other related insect polysaccharides. Binding of seven *Xf* candidates (PD1764, PD1764ΔLysM, FimA, ChiA, HxfAD1-3, HxfAD4, and BSA) has been tested on four different polysaccharides (chitin, colloidal chitin, chitosan, and Avicel). Briefly, one mg of each insoluble polysaccharide was incubated with 100µg of each of the seven candidates in a one ml final reaction volume. Incubation was conducted at room temperature for one hour under shaking conditions (60rpm; rotary shaker with vertical orbital motion). Reaction mixtures were centrifuged at low-speed and supernatants were used to determine concentration of proteins not bound to polysaccharides. In order to normalize our results, the same binding assay was conducted for each candidate in absence of polysaccharides as the protein concentration measurable when no interactions occur in the system. Results of those experiments are summarized in **Figure 4**.

PD1764 and HxdAD1-3 strongly interacted with chitin (88% and 76% respectively) and colloidal chitin (83% and 77% respectively). This is in accordance with previous results showing that PD1764 could be specifically trapped on a chitin column whereas Hxfs proteins act as adhesins important for binding on insect cells (Killiny et al., 2009). No interactions were found with PD1764ΔLysM nor HxfAD4 reinforcing the suggested role for LysM and HAD domains respectively in chitin interaction. Interestingly, these two candidates were the ones having an effect on the disruption of *Xf* leading to the conclusion that reduction of the transmission rate previously observed is a direct consequence of the interaction of transmission-blocking molecules on insect putative receptors. No or a slight interaction was observed for ChiA and FimA whatever the polysaccharide used.

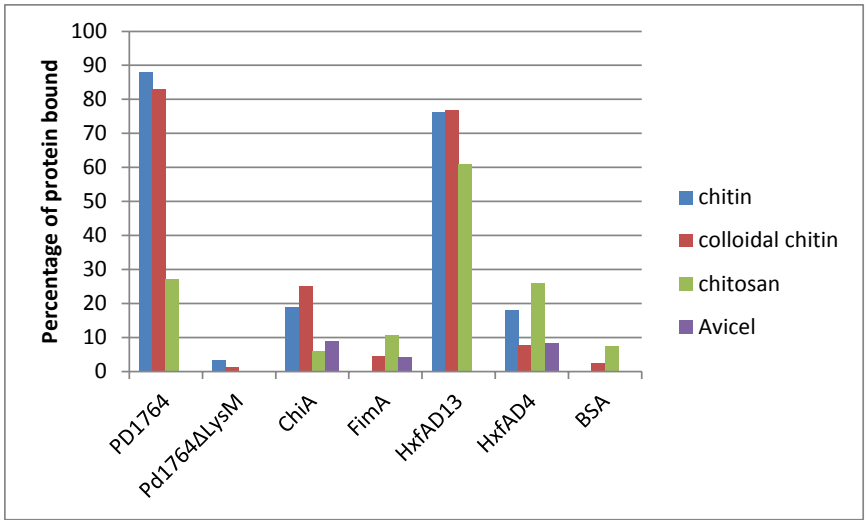
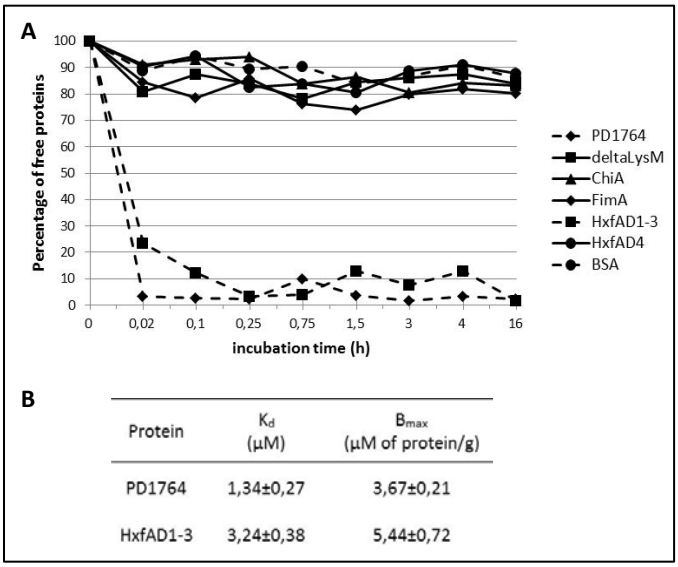


Figure 4. Binding of different *Xf* proteins to insect related polysaccharides.

In addition, HxfAD1-3 also interacts with chitosan (61% of proteins bound). The difference between chitin (or colloidal chitin) and chitosan polysaccharides is the presence of an acetyl group ($COCH_3$) on the main chitin subunit. Based on this result, the acetyl residue seems to play an important role in the binding of PD1764 on chitin-related polysaccharides whereas it doesn't seem to be a requirement for HxfAD1-3 interaction with such molecules. This is of interest because it could mean that these two peptides recognize different domains or different receptors.

In parallel, the same experiment as described above was conducted but interactions between different candidates and chitin was measured over the time (time course interaction). Results are presented in **Figure 5A**. Interestingly, more than 95% and 75% of interactions between chitin and PD1764 and HxfAD1-3 respectively occurred in the first minute of the binding assay. In addition, interactions were stable in our conditions for at least 16h. No significant interaction was detected for the other candidates whatever the time considered. This result suggests that both proteins have affinity for chitin which seems to be a requirement *in vivo* where interactions between *Xf* proteins and insect receptors occur in a highly turbulent environment due to the simultaneous ingestion of xylem sap by the insect when feeding. This suggestion was confirmed in a third experiment in which dissociation constant (Kd, ligand concentration that binds to half the receptor sites at equilibrium) and Bmax (ligand concentration to saturate maximum number of binding sites) were calculated.



Protein	K_d (µM)	B_{max} (µM of protein/g)
PD1764	1,34±0,27	3,67±0,21
HxfAD1-3	3,24±0,38	5,44±0,72

Figure 5. Determination of chitin interaction parameters for different *Xf* proteins. **A**. Graph showing the interaction between chitin and 6 *Xf* proteins previously tested as transmission-blocking peptides from 5 minutes to 16 hours. BSA was included as a control. **B**. Determination of the Kd and Bmax of the two candidates, PD1764 and HxfAD1-3, showing the best affinities among *Xf* proteins tested for chitin.

Blocking of the transmission using peptides able to bind to insect polysaccharides.
Following protocols previously published (Killiny et al., 2012), we performed transmission experiments in the greenhouse with an efficient vector to test the transmission-blocking properties of the various peptides tested *in vitro*. **Figure 6** summarizes those experiments.

According to these results most candidates did not reduce transmission efficiency. This is particularly true for ChiA, FimA, and HxfAD-4 domain. However, in addition to the blocking effect previously observed for PD1764, one candidate (HxfAD1-3) had a significant effect on transmission. This construct expresses the 1,168 amino acids (aa) N-terminal part of the hemagglutinin-like protein HxfB. Interestingly, according to SMART (http://smart.embl-heidelberg.de/), this region contains a 120 aa domain called haemagglutination activity domain (HAD; Voegel et al., 2010), which has been suggested to be a carbohydrate-dependant haemagglutination activity site. It has been found in a number of adhesins or filamentous haemagglutinins such as the FHA of *Bordetella pertussis* and plays a role in adhesion to host cells (Kajava et al., 2001). Concerning PD1764, the second construct tested here named PD1764ΔLysM, in which the LysM domain is absent, showed no significant effect in reducing *Xf* transmission. Thus, the region of PD1764 involved in transmission disruption could be restricted to the first 89aa on the N-terminal of the protein. Within this region, the LysM domain, which is known to have chitin-binding activity (Vieweswaran et al., 2012), was identified between aa 41 and 89. Identification of domains on HxfAD1-3 and PD1764 (respectively named HAD and LysM) involved in blocking *Xf* transmission by insects will lead to the construction of shorter transmission-blocking peptides. This is of great importance because utilization of small peptides could greatly enhance the efficiency of our transgenic system in the field, as the medium-term goal of this system is to develop transgenic grapevines expressing these transmission-blocking molecules constitutively.

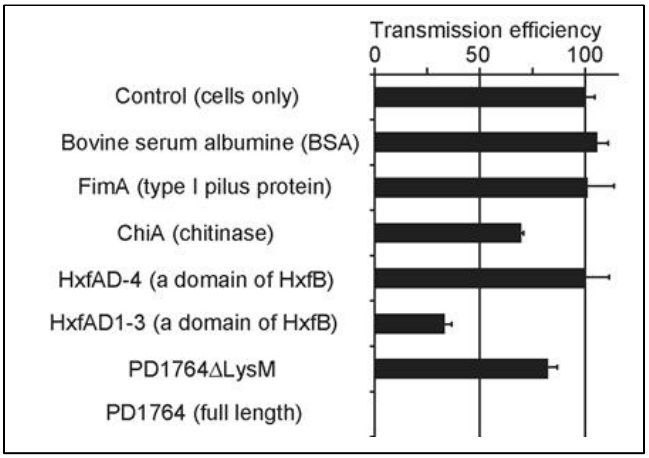


Figure 6. Effect of different transmission-blocking peptides on *Xf* transmission.

Construction of optimized peptides and transgenic plants.
We took advantage of the results obtained with PD1764 and the hemagglutinin-like protein HxfB (HxfAD1-3; PD1792) as transmission-blocking molecules to continue our research for smaller optimized peptides. This is of great importance because utilization of small peptides could greatly enhance the blocking efficiency of our molecules but also be more adequate for a transgenic system in the field. Based on *in silico* analyses, we designed three additional peptides corresponding to the sequences of domains potentially having the blocking activity (**Figure 7**). These peptides are being synthesized and constructs built for the generation of transgenic grapevines for future greenhouse experimentation.

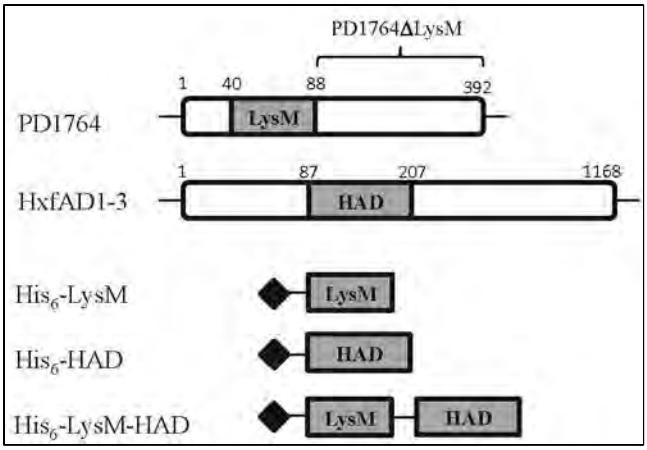


Figure 7. Representation of the location of the two candidate domains, LysM and HAD, on each protein but also the construction of a fused peptide including both domains.

CONCLUSIONS

This project is proceeding very well. First, we showed that the chitinase of *Xf* is affected in its transmission by vectors and in its ability to colonize plants. Based on these results, ChiA is unlikely involved in *Xf* adhesion to insect foregut but is probably involved is transmission and plant colonization, in addition to its role in insect colonization. More importantly, we also succeed to test several *Xf* proteins as transmission-blocking molecules. Very promising results were obtained with HxfAD1-3 but especially with PD1764, the candidate identified using our proteomic-based approach, which completely disrupted the transmission of *Xf*. *In silico* analyses also identified two domains (HAD and LysM) that could be highly promising peptides to continue our search for optimized transmission-blocking molecules.

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DEVELOPMENT OF A GRAPE TISSUE CULTURE AND TRANSFORMATION PLATFORM FOR THE CALIFORNIA GRAPE RESEARCH COMMUNITY

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ABSTRACT

Tissue culture of grape plants remains an inefficient process for many genotypes. The procedure is labor intensive, limited to specific genotypes, and requires a significant amount of time to establish and maintain embryogenic cell cultures and convert cell cultures into whole plants. We are leveraging the expertise of the National Research Laboratory of Chile, (INIA), and the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis (UCDPTF) to significantly increase the efficiency of tissue culture and transformation technology in grape genotypes important to their respective countries. The two labs are sharing their latest protocol improvements for generating and increasing high quality embryogenic callus using germplasm important to their particular country. This combined effort, has allowed us to make significant advances in our ability to grow and maintain embryogenic callus cultures for use in tissue culture and transformation experiments for rootstock genotypes 1103, 101-14, and the winegrapes Chardonnay and Cabernet Sauvignon. We have successfully established high quality, rapidly multiplying grape cell suspension stock cultures for 1103, 101-14, Cabernet Sauvignon, and Chardonnay by employing a modification of INIA's liquid/agar cell cycling system allowing us to maintain a constant supply of cells needed for tissue culture and transformation studies. We have also shown that we can grow stock embryogenic callus cultures of 1103, 101-14, Cabernet Sauvignon, and Chardonnay in a temporary immersion system (TIS) bioreactor, which requires minimal labor and should significantly increase the efficiency of maintaining stock callus for use in transformations. We have also shown that somatic embryos can be maintained for extended periods of time in a quiescent state by plating cell suspensions on medium containing high concentrations of sorbitol. Using high quality embryogenic cultures produced in cell suspension culture we have developed high frequency transformation of embryos of 1103 and 101-14 and we are using the scorable marker genes, DsRed to explore the impact of various protocol modifications such as pre-inoculation heat shock on *Agrobacterium*-mediated transformation frequency. Regeneration of whole plants from 1103 and 101-14 remains inefficient and we are testing the effect of various medium addenda on regeneration of transformed and non-transformed embryos of 1103 and 101-14 in order to increase regeneration efficiencies.

LAYPERSON SUMMARY

This project is aimed at establishing an international collaboration between leading laboratories in the USA and Chile to reduce the time and cost of tissue culture and transformation for grape varieties of importance to the viticulture industries in their respective countries. The collaboration leverages pre-existing expertise and technical know-how to expedite the development of efficient tissue culture and transformation protocols for grape varieties of importance to the Pierce's disease and glassy-winged sharpshooter (PD/GWSS) research community. The two labs are sharing their latest protocol improvements for generating and increasing high quality embryogenic cultures using germplasm important to their particular country. Using both cell suspension cultures and bioreactors, we have made significant advances in our ability to establish and increase embryogenic cultures for 1103, 101-14, Cabernet Sauvignon, and Chardonnay for use in tissue culture and transformation experiments. We have developed a long term storage medium which allows grape somatic embryos to be stored for over six months which will allow for easy maintenance of numerous genotypes with minimal labor. Using these embryos, we have achieved high transformation frequencies for 1103 and 101-14. Transformation frequencies for Cabernet Sauvignon and Chardonnay remain low. Regeneration of transgenic 1103 and 101-14 into whole plants remains inefficient, however we have made progress developing new media formulations which allow for more rapid regeneration of plantlets from non-transgenic embryos of 1103 and 101-14, which if applicable to transgenic embryos, should reduce the time required to generate transgenic grape plants for the PD/GWSS research community. These advances should significantly improve tissue culture and transformation of grape.

INTRODUCTION

The development and transformation of embryogenic cultures in grape has historically been labor intensive with the establishment of embryogenic cell cultures requiring many months and limited to only a few genotypes, most notably the table grape Thompson Seedless. Once established, maintaining healthy embryogenic callus is difficult, with the quality of the cultures deteriorating over time. The efficiency of establishing embryogenic cultures and regenerating plants for important wine and rootstock genotypes remains low and are not at the level required to allow for cost effective recovery of tissue culture or transgenic plants. Historically, because it is one of the only genotype that can be readily manipulated in tissue culture, Thompson Seedless has been used by most Pierce's disease and glassy-winged sharpshooter (PD/GWSS) researchers to test transgenic strategies for pathogen and disease management. However for many projects, it would be valuable to test strategies directly in rootstock or winegrape genotypes. Challenges involved in expanding the range of genotypes that can be successfully manipulated in culture include the reliable establishment of embryogenic cultures, the labor intensive methods required to increase and maintain high quality embryogenic cultures, prevention of tissue necrosis caused by oxidation, conversion of embryos into true-to-type plants, transformation of embryogenic callus, and rapid regeneration of non-chimeric transgenic plants from embryogenic cells. There is also a need to reduce the timeframe required to generate transgenic grape plants in order to test new strategies in a timely manner. Previously, even for Thompson Seedless, the production of transgenic plants normally requires a minimum of 12 months. Working with our collaborators at the National Research Laboratory of Chile (INIA), we are attempting to increase the efficiency of tissue culture and transformation technology in grape genotypes importance to the PD/GWSS research community. Results of this collaboration will allow the PD/GWSS research community to test transgenic strategies in genotypes that are relevant to the industry through the establishment of a self-sustaining service facility.

OBJECTIVES

1. To establish an international collaboration between leading laboratories in the USA and Chile that share a common goal of accelerating the development of efficient tissue culture and transformation protocols for grape varieties of importance to the viticulture industries in their respective countries.
    a. Adapt tissue culture and transformation methodologies developed by our Chilean partner, for grape genotypes of importance to California including; 11-03, 101-14, Cabernet Sauvignon, and Chardonnay.
    b. Increase the efficiency of maintaining embryogenic cultures and reduce the time required for *in vitro* regeneration of grape plants from embryogenic cultures by adapting INIA's cell suspension technology and UC Davis Plant Transformation Facility's temporary immersion system (TIS) for use in grape tissue culture and transformation.
    c. Enhance the efficiency of whole plant regeneration from embryogenic cultures of grape.
2. Develop a cost-effective grape tissue culture and transformation platform for at least one priority California winegrape, and one California grape rootstock which will provide PD/GWSS research community with a predictable supply of experimental plant material while reducing labor and maximizing tissue culture and transformation efficiency.

RESULTS AND DISCUSSION

Objective 1a. Adapt tissue culture and transformation methodologies developed by our Chilean partner, for grape genotypes of importance to California including; 11-03, 101-14, Cabernet Sauvignon, and Chardonnay

In the spring of 2013, we again harvested anthers from grape genotypes 11-03, 101-14, Chardonnay, and Cabernet Sauvignon and have plated them onto two different callus induction media; Murashige and Skoog minimal organics medium (Murashige and Skoog, 1962) supplemented with 60 g/l sucrose, 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and 2.0 mg/l benzylaminopurine (BAP) (PIV), or Murashige and Skoog minimal organics medium supplemented with 20 g/l sucrose, 1.0 mg/l naphthoxyacetic acid (NOA), and 0.2 mg/l BAP (NB medium). In addition to establishing new embryogenic callus for 11-03, Chardonnay, and Cabernet Sauvignon this year we were successful in obtaining embryos from anthers of genotype 101-14. A summary of the response of the various genotypes on the two different media is shown in **Table 1**. Overall, in our hands, grape anthers demonstrated greater development of embryogenic callus on PIV than on NB medium.

Table 1. The number and percentage of anther cultures plated in 2011, 2012, and 2013 that developed into embryogenic cultures on PIV or NB medium.

Genotype	2013		2012		2011	
	PIV	NB	PIV	NB	PIV	NB
Cabernet	1/287 (0.3)	0/217 (0)	0/200 (0)	0/280 (0)	3/400 (0.8)	NT
Chardonnay	22/344 (6.4)	18/344 (5.2)	9/184 (4.9)	2/156 (3.6)	4/400 (1.0)	NT
1103	3/294 (1.0)	0/287 (0)	0/75 (0)	1/196 (0.5)	2/150 (1.3)	NT
101-14	3/322 (0.9)	0/409 (0)	0/140 (0)	0/275 (0)	NT	NT

The National Research Laboratory of Chile (INIA) and the UC Davis Plant Transformation Facility (UCDPTF) are exploring an alternative method to generating embryogenic cultures utilizing leaf pieces from *in vitro* grown plants. Unlike generating callus from anthers which have a short window of availability in the spring of each year, leaf tissue from *in vitro* plantlets are available year round. In addition, unlike meristem explants which are time consuming and difficult to excise, leaf explants are easy to isolate and can be secured from known pathogen-free tissue culture plantlets. We received disease-free cultures of Chardonnay, 101-14, and Cabernet Sauvignon from Foundation Plant Services (FPS), and have established *in vitro* shoot cultures from field plantings of 11-03 that came from disease indexed cultures. These shoot cultures are being maintained in culture and used as a source of tissue for experiments designed to establish embryogenic callus culture from young leaf explants. Young non-expanded leaf explants have been harvested and transferred to INIA's recommended medium and are being incubated at 26 degrees centigrade until embryogenic callus is produced. To date, we have been successful with this protocol using Thompson Seedless, but not with any other genotype. We are currently evaluating the response of younger leaf primordial.

Objective 1b. Increase the efficiency of maintaining embryogenic cultures and reduce the time required for in vitro regeneration of grape plants from embryogenic cultures by adapting INIA's cell suspension technology and UC Davis Plant Transformation Facility's temporary immersion system (TIS) for use in grape tissue culture and transformation

INIA has developed a method of rapidly increasing embryogenic cultures by cycling the cells between agar-solidified medium and liquid media in shake flasks. This technique allows for rapid increases in callus fresh weight while minimizing oxidation and the development of detrimental phenolic compounds in the cultures. Using a modification of INIA protocol, we have significant improved the production of embryogenic grape cultures across a range of genotypes including 1103, 101-14, Cabernet Sauvignon, and Chardonnay. Using anther filament callus generated in 2011 and 2012, we are now routinely maintaining cell suspension cultures on liquid WPM medium (Lloyd and McCown, 1981) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 10.0 mg/l Picloram, 2.0 mg/l metatopolin, 2g/l activated charcoal, 100 mg/l ascorbic acid, and 120 mg/l reduced glutathione (Pic/MTag) and grown on a gyratory shaker at 110 rpms in the dark. Once established, 10 ml of the suspension is withdrawn each week from the flask and replaced with 10 ml of fresh medium. One ml of suspension that is removed from the flask is plated onto agar solidified Woody Plant Media (WPM) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 0.5 mg/liter BAP, 0.1 mg/liter NAA, 5% sorbitol, and 14 g/l phytoagar (BN-sorb). Embryogenic cells plated on BN-sorb medium produces high quality embryogenic cultures at the appropriate stage for use in transformation in approximately 4-8 weeks. One of the advantages of our new suspension protocol is that it does not require cycling material between agar and liquid medium. Instead, cells are maintained in suspension by continually sub-culturing them on a weekly basis which results in the continual production of non-phenolic embryogenic cells. These cultures have now been maintained for eighteen months without loss of regeneration potential or phenolic oxidation. New cell suspension cultures have been established from 2012 cultures demonstrating that the procedure is repeatable. We are also continuing to explore UCDPTF's TIS for use in rapidly increasing embryogenic callus. As reported previously, although this method of increasing embryogenic callus has proven very efficient, we were concerned because the callus produced was highly oxidized exhibiting excessive accumulation of phenolic compounds. However, we have found that by adding ascorbic acid to the culture medium and growing the cultures in the dark, we can reduce phenolic development in the callus. Growth rate studies of 1103, 101-14, and Chardonnay callus cultures were repeated this period and we again demonstrated that robust cell growth can be achieved for a minimum of 3 months without addition of fresh medium to the bioreactor (**Figure 1**). Based on these results, we are now using this temporary immersion system in parallel with our shake flask cultures to increase 1103, 101-14, Chardonnay, and Cabernet Sauvignon cultures. This system is advantageous from a labor management perspective, since it allows one to maintain stock cultures indefinitely in temporary immersion with medium exchanges occurring only

once every three months. When needed, sample of callus can be removed and transferred to agar-solidified medium a few weeks prior to initiating transformations.

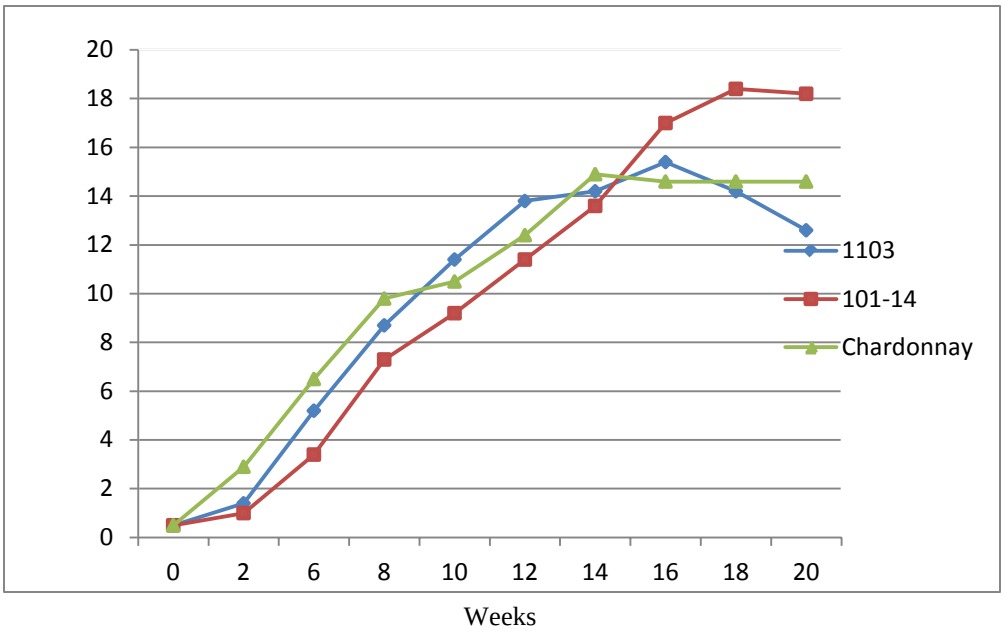

Figure 1. Embryogenic callus growth (grams fresh weight y axis) of 1103, 101-14, and Chardonnay in bioreactor demonstrating that callus can be maintained in bioreactors for sixteen to twenty weeks without medium additions or culture manipulation.

While we are exploring temporary immersion bioreactors for growing grape embryogenic cultures, our Chilean colleagues are investigation stir tank bioreactors as a cost effective improvement to their airlift bioreactor system. Embryos were generated from apical (leaf) buds in NB2 plates ('Red Globe' and 'Crimson') and transferred into X6 solid medium. Once they reach pro-embryo stage (usually 2 months), biomass is used as inoculum for the reactors. The bioreactors are filled with 400 mL of X6 medium. Significant biomass production is seen after 10-15 days of stirring (100 rpm) which can be filtered to re-inoculate the reactors or for return into regular solid media (**Figure 2**).

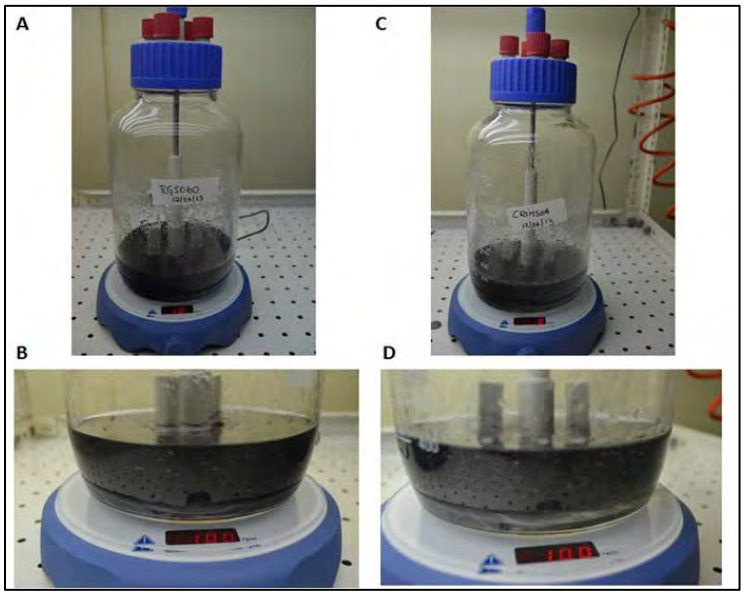

Figure 2. INIA is developing stirred tank reactors for growing grape somatic embryo genotypes the Red Globe (A and B) and Crimson (C and D) genotypes.

Objective 1c. Enhance the efficiency of whole plant regeneration from embryogenic cultures of grape
In addition to using cell suspension and temporary immersion techniques to reduce labor, we have been investigating methods for storing high quality embryogenic cultures over an extended period of time. Although we initially evaluated storing embryos at four degrees centigrade on agar solidified plates or at minus 80 degrees centigrade using cryopreservation, we have found that increasing the osmotic strength of the medium offers a simple solution for maintaining high quality somatic embryos over an extended period of time. One ml of embryo suspension can be plated onto WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA 50 g/l sorbitol, and 14 g/l agar and cultured in the dark at 26 degrees centigrade. Cells plated onto this medium will develop somatic embryos within approximately 4-8 weeks. Embryos do not germinate into plants, but remain as quiescent somatic embryos. They can be maintained in this state for up to six months without loss of viability and upon transferred to WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP lacking sorbitol, they germinate into whole plants (**Figure 3**). These embryos serve as an excellent source of embryos for use in transformation.

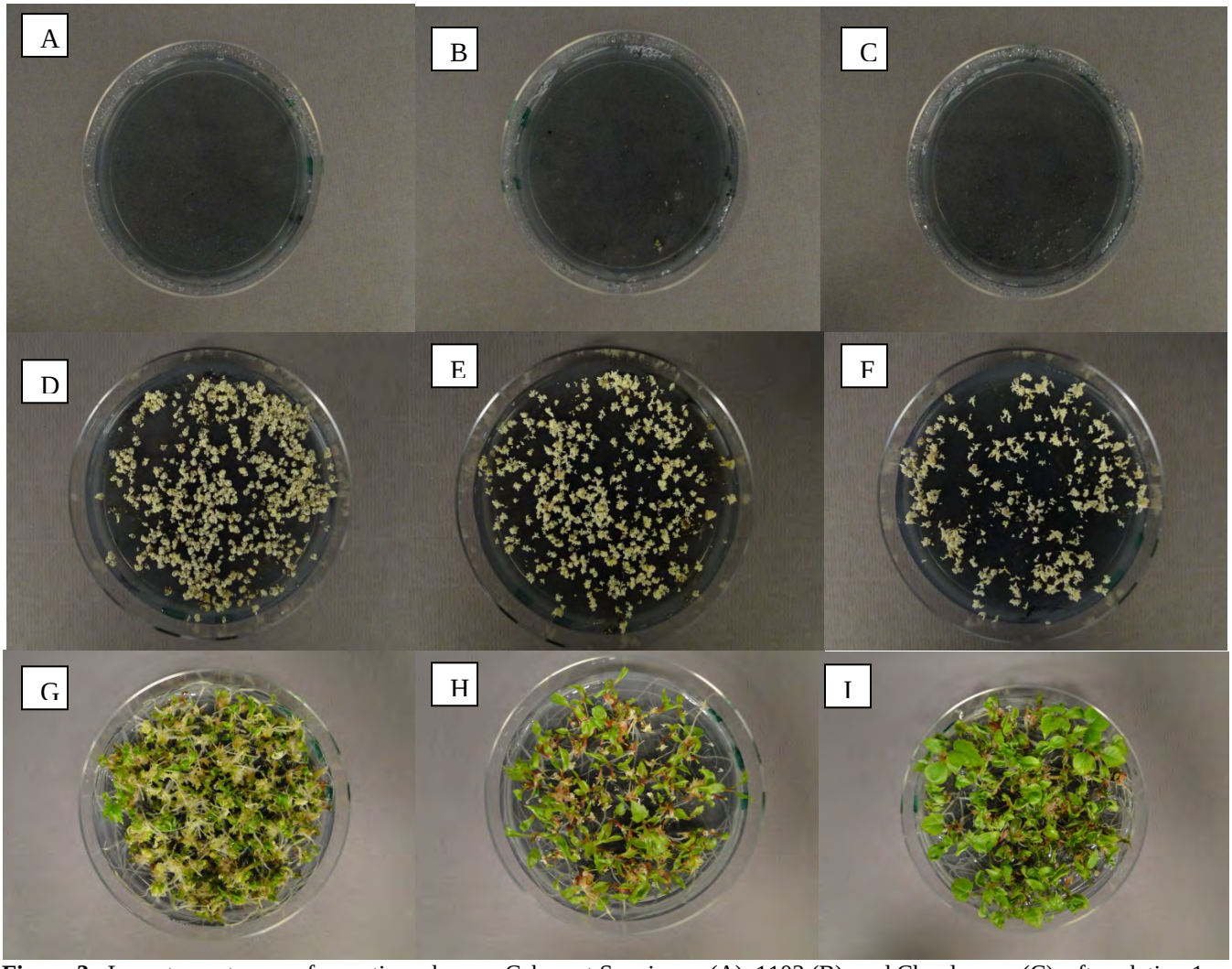

Figure 3. Long-term storage of somatic embryos. Cabernet Sauvignon (A), 1103 (B), and Chardonnay (C), after plating 1 ml of cell suspensions onto WPM supplemented with 20 g/l sucrose, 1g/l casein,1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA, 50 g/l sorbitol, and 14 g/l agar. Quiescent embryos of Cabernet Sauvignon (D), 1103 (E), and Chardonnay (F) five months after storage in the dark without sub-culturing. Cabernet Sauvignon (G), 1103 (H), and Chardonnay (I) fifteen days after transferring embryos stored for five months onto WPM supplemented with 20 g/l sucrose, 1g/l casein,1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP, and 8 g/l agar and cultured in the light.

We evaluated numerous media components in an attempt to improve the efficiency of whole plant regeneration from embryos of 1103 and 101-14. As reported last period, preliminary results indicate that our standard grape regeneration medium is not optimal for genotype 1103. We found that although genotype 1103 benefits

significantly from a reduction in the BAP concentrations further improvements were still required. Studies this period demonstrated that further reductions in the levels of the cytokinin, BAP, and the elimination of the auxin NAA from the regeneration medium significantly enhanced regeneration of non-transformed 1103 embryos and decrease the time required to regenerate whole plants. We also evaluated the addition of GA3 into the regeneration medium to determine if it would enhance embryo conversion. However we observed a strong negative effect of GA3 on embryo germination (**Figure 4**)

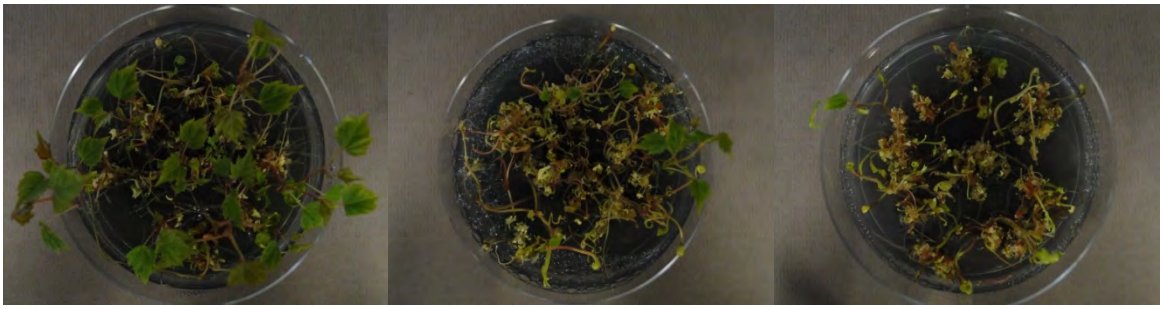

Figure 4. Regeneration of non-transformed rootstock 1103 embryos on WPM, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP, and 0.0 mg/l (left), 10 mg/l (middle), and 25 mg/l (right) GA3.

Objective 2. **Develop a cost effective grape tissue culture and transformation platform for at least one priority California winegrape, and one California grape rootstock which will provide PD/GWSS research community with a predictable supply of experimental plant material while reducing labor and maximizing tissue culture and transformation efficiency**

We have been evaluating the use of a heat shock treatment on somatic embryos prior to inoculating with *Agrobacterium tumefaciens.* We have preliminary results that indicate that a 10 minute heat shock treatment at 45 degrees centigrade increased the transformation frequency in Thompson Seedless (**Table 2**). We are now testing heat shock pretreatment in 1103 and 101-14 and preliminary results also show an increase in transformation frequency (**Table 3 and 4**). While transformation frequencies for 101-14 and 1103 are relatively high, transformation frequencies of Cabernet Sauvignon and Chardonnay remain low and further improvements are needed.

Table 2. Thompson Seedless transformation experiments comparing transformation efficiencies after subjecting embryos to 10 minutes of heat shock at 45 degrees centigrade verses no heat shock application prior inoculation with *Agrobacterium tumefaciens***.**

Experiment	Heat Shock	Plant Selection	# (%) transgenic colonies
121092	+	hygromycin	13/16 (81)
121090	-	hygromycin	1/16 (6)
121029	+	kanamycin	17/32 (53)
121028	-	kanamycin	3/30 (10)

Table 3. Transformation experiments with 1103 embryos comparing transformation efficiencies after exposure to 10 minutes of heat shock at 45 degrees centigrade verses no application of heat shock prior inoculation with *Agrobacterium tumefaciens.*

Experiment	Heat Shock	Genotype	# (%) transgenic colonies
121084	-	1103	3/20 (15)
129005	-	1103	9/28 (32)
129040	-	1103	5/25 (20)
129012	+	1103	46/50 (92)
129019	+	1103	12/25 (48)
129039	+	1103	3/25 (12)
131006	+	1103	11/24 (46)
139012	+	1103	0/16 (0)

Table 4. Transformation efficiencies with 101-14 embryos after exposure to 10 minutes of heat shock at 45 degrees centigrade heat shock prior inoculation with *Agrobacterium tumefaciens.*

Experiment	Heat Shock	Genotype	# (%) transgenic colonies
139023	+	101-14	29/32 (91)

When using heart and torpedo staged embryos as the target tissue for transformation, there is always the concern that chimeric plants will be generated. We have initiated transformation studies on of 101-14, 1103, Cabernet Sauvignon, and Chardonnay embryo cultures produced in cell suspension and plated onto sorbitol containing medium using a construct containing the scorable marker gene dsRed (Limpens et al., 2004). The scorable marker gene DsRed, allows us to non-destructively track transformation over the course of the transformation process and visualize the presence or absence of chimeric plants. Using DsRed we inoculated grape embryos after subjecting the embryos to a heat shock treatment for ten minutes at 45 degrees centigrade and observed the development of kanamycin resistant dsred transgenic embryos using a fluorescent stereo microscope. Preliminary results indicate that if the tissue in maintained on high levels for selection (200 mg/l kanamycin) the production of chimeric plants does not appear to be a significant issue (**Figures 5 and 6**).

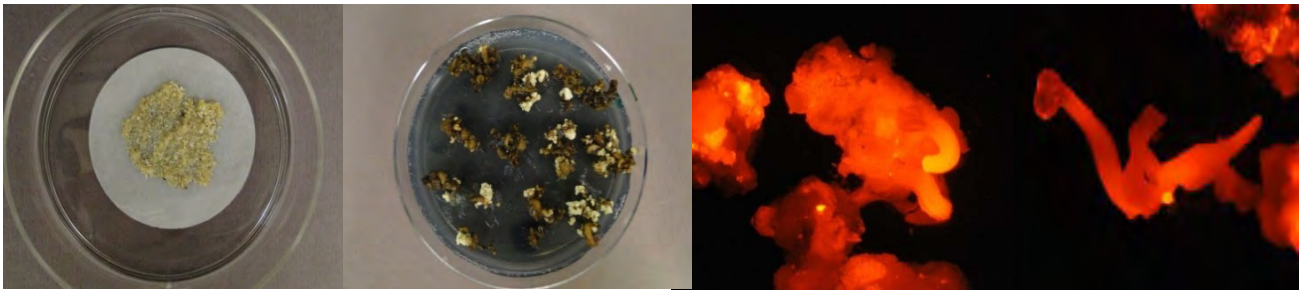

Figure 5. 1103 embryos plated onto filter paper soaked with liquid BN sorb medium and inoculated with an *Agrobacterium tumefacien* strain EHA105 containing the scorable dsred marker gene (A). Kanamycin resistant colonies developing on 200 mg/l kanamycin (B) Dsred expressing callus (C) non-chimeric embryos (D) are clearly visible.

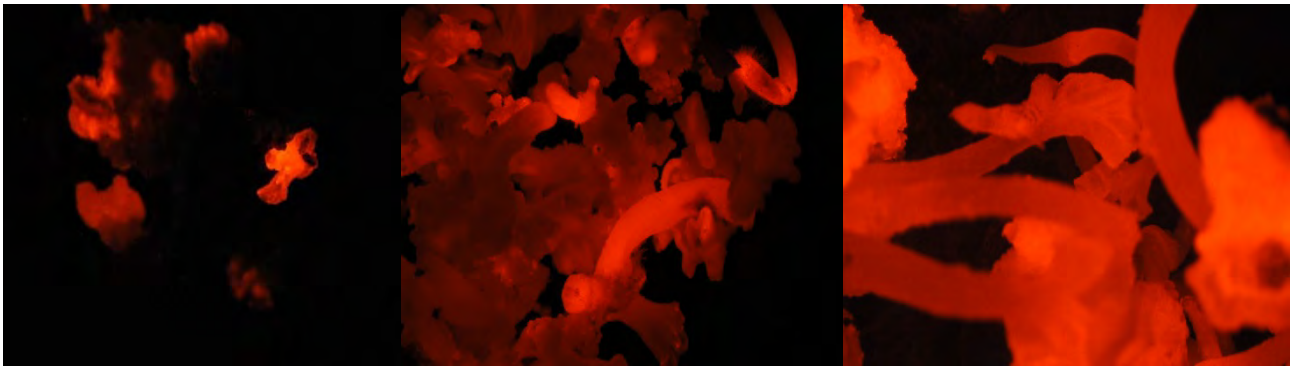

Figure 6. Transformation of Cabernet Sauvignon (left), 101-14 middle and Chardonnay (right) with the scorable marker gene deRed.

We have begun using the information we have learn to date regarding grape transformation of rootstock genotypes to generate transgenic 101-14 and 1103 embryos containing the transgenes provided by Dandekar's lab (**Figure 7**). Although high transformation frequencies can be achieved with 1103 and 101-14, regeneration of whole plants from transgenic embryos remains difficult and we continue to explore parameters to improve regeneration.

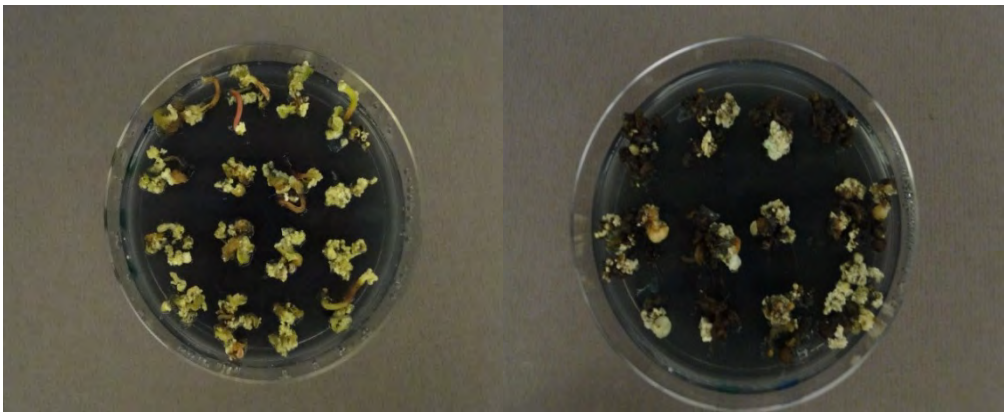

Figure 7. Sixteen putative independent transgenic events of 101-14 (left) and 12 putative independent transgenic events of 1103 (right) transformed with HNE.

CONCLUSION

With significant contributions from INIA, we have made substantial progress producing high quality embryogenic cultures of 1103, 101-14, Cabernet Sauvignon, and Chardonnay by maintaining embryogenic cell suspensions and plating them onto agar-solidified medium on a weekly basis. The system allows for continuous production of highly embryogenic, non-oxidized stock cultures that can serve as a constant supply of starting tissue for use in tissue culture and transformation experiments. Grape embryogenic callus can also be grown under temporary immersion with little to no physical manipulations of the cultures, resulting in significant reduction in labor. We have found that we can maintain high quality grape somatic embryos on sorbitol containing medium for extended periods of time, allowing us to store cultures of different grape genotypes without using cryopreservation. Relatively high transformation frequencies have been obtained for 1103 and 101-14, while transformation frequencies for Cabernet Sauvignon and Chardonnay are still low. Regeneration of transgenic 1103 and 101-14 into whole plants remains inefficient, however we have made progress developing new media formulations which allow for more rapid regeneration of plantlets from non-transgenic embryos or 1103 and 101-14, which if applicable to transgenic embryos, should reduce the time required to generate transgenic grape plants for the PD/GWSS research community.

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BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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Reporting Period: The results reported here are from work conducted October 2012 to October 2013.

ABSTRACT

We continue to make rapid progress breeding Pierce's disease (PD) resistant winegrapes. Aggressive vine training and selection for precocious flowering have allowed us to reduce the seed-to-seed cycle to two years. We are also using marker-assisted selection (MAS) for the Pierce's disease 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 *Vitis vinifera* winegrape cultivars in 10 years. We have screened through about 2,000 progeny from the 2009, 2010, and 2011 crosses that are 97% *V. vinifera* with the *PdR1b* resistance gene from *V. arizonica* b43-17. Seedlings from these crosses continue to crop and others are advanced to greenhouse testing. We select for fruit and vine quality and then move the best to greenhouse testing, where only those with the highest resistance to *Xylella fastidiosa*, after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and at a Pierce's disease hot spot in Napa. The best of these will be advanced to 100 vine commercial wine testing the first of which was planted in Napa this past June. We advanced 10 selections to Foundation Plant Services this winter to begin the certification and release process. Three Pierce's disease resistant rootstocks were also advanced to FPS for certification. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 are also being pursued but progress is limited by their multigenic resistance and the absence of corresponding genetic markers. Other forms of *V. arizonica* are being studied and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable resistance. Very small scale wines from 94% and 97% *V. vinifera PdR1b* selections have been very good and have been received well at industry tastings in Sacramento, Santa Rosa, the Napa Valley, Temecula, and Healdsburg.

LAYPERSON SUMMARY

One of the most reliable and sustainable solutions to plant pathogen problems is to create host plants naturally resistant to the disease. We use a traditional plant breeding and a technique called backcrossing to bring resistance to Pierce's disease (PD) from wild grape species into a diverse selection of elite winegrape backgrounds. In the case of our most advanced vines we have identified the genetic location of a very strong source of Pierce's disease resistance from a grape species native to Mexico. Using marker-assisted selection (MAS) for this Pierce's disease resistance region called *PdR1* (Krivanek et al., 2006), we are able to select resistant progeny as soon as seeds germinate. MAS and aggressive growing of the selected seedling vines have allowed us to produce new varieties that are more that 97% *Vitis vinifera* winegrape cultivars in only 10 years. We have evaluated thousands of resistant seedlings for horticultural traits and fruit quality. The best of these are advanced to greenhouse testing, where only those with the highest resistance *to Xylella fastidiosa*, after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and at a Pierce's disease hot spot in Napa. The best of these are advanced to 100 vine plots for commercial wine testing. We sent 10 advanced selections to Foundation Plant Services this past winter to begin the certification and release process. Three Pierce's disease resistant rootstocks were also sent to FPS for certification. Other wild grape species are being studied and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable Pierce's disease resistance. Very small-scale wines made from our advanced *PdR1* selections have been very good, and have been received well at industry tastings throughout California.

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, Baumgartel 2009), and having unique and highly resistant *Vitis rupestris* x *V. arizonica* selections, as well as an extensive collection of southwestern grape species, which allows the

introduction of extremely high levels of *Xf* resistance into commercial grapes. We have genetically mapped and identified what seems to be a single dominant gene for *Xf* resistance and named it *PdR1*, which was found in *V. arizonica*/*candicans* b43-17. This resistance has been backcrossed through four generations to elite *V. vinifera* cultivars (BC4) and we now have 97% *V. vinifera* Pierce's disease (PD) resistant material to select from. Individuals with the best fruit and vine characteristics are then tested for resistance to *Xf* under our greenhouse screen. Only those with the highest levels of resistance are advanced to small-scale winemaking trials by grafting them onto resistant rootstocks and planting six to eight vines set on commercial spacing and trellising. We have made wine from vines that are 94% *V. vinifera* level from the same resistance background for five years and from the 97% *V. vinifera* level for three years. They have been very good and don't have the hybrid flaws (blue purple color and herbaceous aromas and taste) that were prevalent in red wines from the 87% *V. vinifera* level. There are two forms of *PdR1* that descend from sibling progeny of b43-17 and they have different alleles of *PdR1* designated *PdR1a* and *PdR1b*. Screening results reported previously showed no significant difference in resistance level in genotype with either one or both alleles. We have narrowed our focus to *PdR1b* but retain a number of selections at various BC levels with *PdR1a* in the event that there is an as yet unknown *Xf* strain-related resistance associated with the *PdR1*alleles. 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

In 2012 we made F1 crosses to five new Pierce's disease resistant *V. arizonica* accessions from the southwestern USA and Mexico to develop mapping populations so that genetic markers could be generated to expedite breeding. The resistant genotypes were chosen based on their low ELISA values, minimal expression of Pierce's disease symptoms in the greenhouse screen and their diverse geographic origins. We germinated a subset of these seeds in late fall 2012, made copies of seedlings growing in four-inch pots in early 2013 and greenhouse tested them to characterize the inheritance of Pierce's disease resistance. Three of the five populations were tested with about 50 progeny each. There was clear statistical separation of the progeny families with those from ANU5 being the least resistant; b40-29 intermediate; and the population from b46-43 had unprecedented levels of resistance by both disease phenotype and ELISA. BC1 and full sibling crosses will be made in 2014 to better explore this very promising source of resistance. In 2013 we expanded the populations made in 2012, and made crosses to other promising *Vitis* species from this same region as detailed in **Table 1** below.

Table 1. Crosses made in 2013 to develop genetic maps in new accessions from southwestern USA and Mexico germplasm. 08326-61 is a self of Cabernet Franc, F2-35 is a cross of Carignane and Cabernet Sauvignon and both are 100% *V. vinifera.*

Resistance Source	Geographic Origin - Species, Appearance Phenotype	Pure *vinifera* Types Used in 2013 Crosses	# of Seed Produced
b41-13	Ciudad Mante - Ciudad de Maiz, MX *V. arizonica* (like b42-26)	F2-35	283
b43-57	Rinconada, Nuevo Leon, MX	Rosa Minna	312
	V. arizonica-champinii-acerifolia-girdiana	Malaga Rosada	544
b47-32	Big Bend, TX	08326-61	30
	glabrous *V. arizonica-monticola* (like b46-43)	F2-35	1586
SC36	San Diego, CA	Palomino	571
	V. girdiana	Grenache	945
T03-16	Lajitas, TX	Palomino	236
	V. arizonica	Grenache	17

We have now evaluated more than 2,000 *PdR1b* 97% *V. vinifera* winegrape progeny from which we are selecting the best and most resistant for release. Our breeding efforts in 2013 focused on increasing the *V. vinifera* content of our other Pierce's disease resistant lines with an emphasis on the b42-26 line: F1, BC1 intercross, BC2 self, and BC2 intercross (**Table 2**). Over the last several years, greenhouse screening has identified a number of highly Pierce's disease resistant genotypes in several of our BC1- and BC2-generation b42-26 lines. Crosses made this year aim to exploit these findings and take advantage of a developing b42-26 genetic map. Additional crosses were made to combine (pyramid) *PdR1b* x b42-26 both with (934 seeds) and without (390 seeds) powdery mildew resistance from *V. romanetii*. We also produced selfed *PdR1b* x *V. romanetii* pyramided lines (1,340 seeds) to create breeding sources homozygous resistant at both loci to backcross in the last generation to elite *V. vinifera* wine types. The breeding efforts with alternative resistance sources and the complexing of these resistances is being done to broaden *Xf* resistance and address *Xf*'s ability to overcome resistance.

Table 2. Crosses made in 2013 to advance and further refine the b42-26 Pierce's disease resistance line.

BC level	Cross type	Elite *V. vinifera* varieties in background	Number of Seeds
F1	F1	Cabernet Sauvignon, Carignane	944
BC1	Intercross	Those in the F1 plus Grenache	369
BC2	Self	Those in the BC1 plus Carignane	44
BC2	Intercross	Same as BC2	26

Table 3 presents the status of greenhouse screening for Pierce's disease resistance during this reporting period. Group B, part of Group E, and results from a previous greenhouse screen have been analyzed to provide statistically consistent results on 199 F1 progeny from the b42-26 resistance line. These results should allow a framework genetic map of Pierce's disease resistance loci in this multigenic background – work that is being done in our companion Pierce's disease mapping project. Genetic markers in this line are essential if we are to successfully combine resistances from different sources as is being attempted in the crosses listed in Groups C, D, and E. Groups C, G, H, J, and L test advanced selections from our *PdR1b* line. If they continue to have favorable confirmatory greenhouse testing they will be advance to release consideration with their counterparts in **Table 4**. In an initial greenhouse screen of 16 *PdR1b* x b42-26 genotypes (Group C) at approximately the 84% *V. vinifera* level, 75% of those with the *PdR1b* marker were rated as resistant with two genotypes having ELISA levels lower than our most resistant *PdR1b* biocontrol. In the same trial only 25% were rated resistant while missing *PdR1b* markers. From these early results, pyramiding these two Pierce's disease resistance sources looks promising. Unfortunately a series of greenhouse issues (poor temperature control and spray damage outside of the lab's control) made interpretation of the larger trial in Group D unreliable and the trial will need to be repeated. Also included in Group C were 10 BC2 genotypes in the b42-26 line to evaluate their resistance pattern and 45 *PdR1b* x *V. romanetii* PD x powdery mildew (PM) crosses at greater than the 90% *V. vinifera* level. In the former we found that only one genotype had significant Pierce's disease resistance, although at a lower level than our best *PdR1b* selections at even higher BC levels. This finding again underscores the necessity of markers associated with resistance in the b42-26 line. In the latter cross we found four promising Pierce's disease and PM resistant genotypes that fruited for the first time this year. This low (~10 percent) level of Pierce's disease resistant selections in the *PdR1b* x *V. romanetii* PD x PM line, although disappointingly low, is consistent with results from similar crosses we have tested in our collaboration with David Ramming for their PD/PM resistant table and raisin grape breeding efforts. In Group F, we are testing the impact of environmental conditions, spacing, and our standard cutback protocols in three different greenhouses with a dual purpose – to make our screening less costly and to shorten the test duration. Preliminary ANOVA results show 'genotype' and 'greenhouse' are both significant variables. Interpretation of multiple variable interactions is ongoing. Group G included 13 southeastern US (SEUS) cultivars from various historic Pierce's disease breeding programs in the southeastern USA as part of our effort to better understand the relationship between Pierce's disease resistance in the field and *Xf* titer in our GH screen. Results will be reported when the SEUS genotypes in Group L have completed testing at the end of this year. Also in Groups K and L we further explore and advance our b40-14 PD resistance line.

Table 3. 2012-13 Greenhouse testing.

Group	Genotypes	# Genotypes	Inoculation Date	ELISA Sample Date	Resistance Source(s)
B	05347 b42-26 F1 Mapping Population	84	07/03/2012	10/4/2012	b42-26
C	2012 Parents, 97% *V. vinifera* 2nd tests, PD pyramiding	75	10/02/2012	01/03/2013	F8909-08, b42-26
D	*PdR1b* x b42-26 pyramided	76	11/29/2012	02/28/2013	F8909-08, b42-26
E	05347, PD x PM *V. romanetii*, b42-26 BC2	222	12/13/2012	03/14/2013	F8909-08, b42-26
F	GH, Spacing, Cutback Trials	3	varies	varies	U0505 BC Group
G	94% & 97% *PdR1b* Vin advanced tests, SEUS	106	02/05/2013	05/09/2013	F8909-08, SEUS
H	97% *PdR1b vinifera* advanced tests	89	03/19/2013	06/20/2013	F8909-08
I	New *arizonica* mapping populations 2012 crosses and mist-propagated plants	159	05/21/2013	08/08/2013	Varies
J	97% *V. vinifera PdR1*	78	06/11/2013	09/12/2013	F8909-08, b42-26
K	SWUS Species, additional b42-26 mapping, 2012 b40-14 line recombinants	92	08/06/2013	11/05/2013	Species, b42-26, b40-14
L	b40-14 line F1 and BC1; SEUS named cultivars, *PdR1b* advanced selections	180	09/17/2013	12/17/2013	Species, b42-26, b40-14

In the last interim progress report we gave details on the 10 new *PdR1b* -based Pierce's disease resistant scion varieties and three Pierce's disease resistant rootstock genotypes that were advanced to UCD's Foundation Plant Services (FPS) in March for virus testing, certification, and possible release. At that time there were insufficient numbers of cuttings to provide to FPS of several of our most highly regarded 97% *V. vinifera* scion selections. In addition a number of other selections completed their testing in the greenhouse screen show in **Table 3J**. Although ELISA results are pending, based on the phenotype scores we anticipate sending the selections shown in **Table 4** and **Figure 1** to FPS this spring. Additional selections may be advanced based on results from the trial in **Table 3L** and others initiated but not yet inoculated. The focus of these later trials is to find promising white fruited varieties as we have yet to advance any white selections at the 97% *V. vinifera* level.

Table 4. Likely *PdR1b* releases for transfer to FPS.

Genotype	Parentage	% *V. vinifera*	Color	# Years small lot wine made	Multiple vine trials Davis	Multiple vine trials Napa
09311-160	07371-20 x Cabernet Sauvignon	97%	B	0	Yes	-
09330-07	07370-039 x Zinfandel	97%	B	1	Yes	-
09331-047	07355-020 x Zinfandel	97%	B	2	Yes	-
09331-133	07355-020 x Zinfandel	97%	B	0	Yes	-
09333-111	07355-020 x Chardonnay	97%	B	0	Yes	-
09333-370	07355-020 x Chardonnay	97%	B	0	Yes	-

Figure 1. Cluster shots of 97% *V. vinifera* PdR1b selections destined for Foundation Plant Services this winter prior to release and larger scale winemaking tests. Clockwise from upper left: 09331-160; 09330-07; 09331-047; 09333-370; 09333-111; 09331-133.

Table 5a through 5c detail the vine, fruit, and juice characteristics for the 7 *PdR1b* selections used to make wine lots in 2013. Three additional lots pair wines made from 94% *V. vinifera PdR1b* selections grown in Davis with those from our field trial at the Treasury Wine Estates (Beringer) vineyard in Yountville, Napa Valley. In addition, we made a number of *V. vinifera* controls and Blanc du Bois from both Davis and Napa. Lenoir was made from Davis fruit.

Table 5a. Pierce's disease resistant selections used in small scale winemaking in 2013: background and fruit characteristics

Genotype	Parentage	% *V. vinifera*	2012 Bloom Date	Berry Color	Berry Size (g)	Ave Cluster Wt. (g)	Ripening Season	Prod 1=v low, 9=v high
07338-37	U0505-01 x LCC	94%	05/03/2013	B	1.8	177	early-mid	6
07355-075	U0505-01 x Petite Sirah	94%	04/30/2013	B	1.4	129	early-mid	7
07355-075 Napa	U0505-01 x Petite Sirah	94%	05/17/2013	B	1.0	311	mid	7
07370-084	F2-35 x U0502-38	94%	05/03/2013	W	1.1	167	v. early	7
07370-084 Napa	F2-35 x U0502-38	94%	05/17/2013	W	1.3	179	early	7
07713-51	F2-35 x U0502-48	94%	05/03/2013	W	1.5	432	early	8
07713-51 Napa	F2-35 x U0502-48	94%	05/17/2013	W	1.3	160	early-mid	7
09330-07	07370-039 x Zinfandel	97%	05/03/2013	B	1.6	400	mid-late	8
09331-047	07355-020 x Zinfandel	97%	04/30/2013	B	1.0	127	early	7
09333-253	07355-020 x Chardonnay	97%	05/05/2013	B	1.5	304	mid	7

Table 5b. Juice analysis of Pierce's disease resistant selections use in small-scale winemaking in 2013. Cabernet Sauvignon and Pinot Noir were from previous vintages and added 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)
07338-37	23.7	5	3.56	1.15	1730	175	21	507	1621
07355-075	23.4	6.8	3.25	1.41	1550	160	25	496	1394
07355-075 Napa	23	7.4	3.33	3.07	1520	105	22	759	1863
07370-084	22.3	5.6	3.52	3.38	1950	170	-	-	-
07370-084 Napa	25.5	8.5	3.33	5.21	1840	112	-	-	-
07713-51	22.8	4.4	3.6	1.46	1400	114	-	-	-
07713-51 Napa	23.9	5.7	3.54	2.77	1840	108	-	-	-
09330-07	24	6.9	3.56	3.36	2240	234	72	527	1231
09331-047	27.5	4.8	3.78	2.32	2100	187	7	514	1520
09333-253	24.9	7.1	3.58	3.99	2360	239	80	326	708
Cab. Sauv.	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

Our crosses with Pierce's disease resistance from *PdR1b* continue to thrive and produce at our Beringer field trial in Napa Valley while the pure *V. vinifera* control vines and the surrounding commercial Chardonnay and Riesling vines continue to decline. This year Pierce's disease phenotype scores and cane samples for ELISA measurement of *Xf* levels were completed October 4th, 2013. Statistical analysis was performed of the cane maturity index scores used to evaluate Pierce's disease symptom severity on 33 *PdR1b* genotypes at the 88% and 94% *V. vinifera* level and 3 pure *V. vinifera* varieties (Chardonnay, Petite Sirah, and F2-35). Results indicated that only the *V. vinifera* varieties were statistically susceptible to Pierce's disease relative to b43-17, the source of *PdR1b* resistance. ELISA results for this year will be reported at the next reporting period. In June of this year at this same field trial, we planted 100 vines of our 94% *PdR1b* scion selection 07355-075 split equally between our 08314-15 and 08314-46 PD resistant rootstocks. Large-scale wine lots from this plot are two years away.

Finally, this past year saw the first industry tastings of our advanced selections. Last August in Santa Rosa we presented the best of our 87 and 94% *V. vinifera PdR1b* wines to about 200 people as part of a Sonoma County Winegrape Commission meeting. This year we presented wines made in 2012 with our favorite 94% *V. vinifera PdR1b* selections in a blended format to show how these wines would be used as blending grapes to fill in chronic Pierce's disease hotspots and still stay within the 75/25% varietal wine labeling. We did this with one of our favorite whites 07713-51 and blended it with Chardonnay from Yountville and compared these wines made at the same scale with Yountville Chardonnay and Blanc du Bois (top southern USA Pierce's disease resistant winegrape). One of our favorite reds at the 94% level is 07355-075 and it was blended with Oakville Merlot. Also from 2012 wines, we presented a wine made from three of our advanced 97% *V. vinifera PdR1b* selections (there was not enough fruit from any of them individually, but all have now been replicated for future winemaking) 09331-047, 09332-165, and 09333-178. These wines were well received and many liked the 97% blend the best. The first tasting in 2013 was with select growers and winemakers in the Napa Valley at the UCD Oakville Station, a second was July 17th in Temecula to the winegrowers association, and a third in early August at Healdsburg. These wines will also be presented at the Napa Grape Expo on November 14th. The next step is to make larger scale wines in multi-ton lots and these vines are being planted as noted above. Once the selections clear FPS testing the best selections will be ready for release.

Table 5c. Pierce's disease resistant selections used in small scale winemaking in 2013: berry sensory analysis.

Genotype	Juice Hue	Juice Intensity	Juice Flavor	Skin Flavor	Skin Tannin Intensity (1=low, 4= high)	Seed Color (1=gr, 4= br)	Seed Flavor	Seed Tannin Intensity (1=high, 4= low)
07338-37	Pink	Lt	Fruity, spicy	Fruity, berry	3	3	Woody, spicy, sl bitter	1
07355-075	Red, clear	Lt+	Apple, tea, cranberry	Herbal	2	3	Spicy, woody	4
07355-075 Napa	Bright red, clear	Med	Berry, plum	Neutral, fruity	3	4	Woody, bitter	1
07370-084	Gr-gold touch brown	Med	Melon, yellow apple	Neutral, straw	2	4	Spicy, hot, woody	2
07370-084 Napa	Pale green-yellow	Lt	Gr Apple, melon	Neutral, straw	1	3	Spicy, hot, woody	2
07713-51	Green-gold	Med	Apple juice	Neutral, fruity	2	4	Woody, warm	4
07713-51 Napa	Green-gold	Med+	Ripe yellow apple	Straw, sweet hay	2	4	Woody, hot	3
09330-07	Bright pink-red, clear	Lt+	Berry, apple	Neutral, fruity, sl hay	1	3	Bitter, ashy, sl spice	1
09331-047	Cloudy red brown	Med	Strawberry, raspberry, spice	Berry, spice, chalky	2	3	Spicy, woody	1
09333-253	Pink very slight brown	Lt	Berry, apple	CS veg, spice-hot	3	3	Woody, bitter	2

CONCLUSIONS

We continue to make rapid progress breeding Pierce's disease resistant winegrapes through aggressive vine training, MAS, and our rapid greenhouse screen procedures. These practices have allowed us to produce four backcross generations with elite *V. vinifera* winegrape cultivars in 10 years. We have screened through thousands of seedlings that are 97% *V. vinifera* with the *PdR1* resistance gene from *V. arizonica* b43-17. Seedlings from these crosses continue to crop and others are advanced to greenhouse testing. We select for fruit and vine quality and then move the best to greenhouse testing, where only those with the highest resistance to *Xf*, after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and at Beringer's Yountville vineyard. The

best of these will be advanced to 100 vine commercial scale testing with the first selection planted this year. We have sent 10 advanced selections to FPS this winter to begin the certification and release process. Three Pierce's disease resistant rootstocks were also sent to FPS for certification. Additional scion varieties are slated for FPS this winter. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 are also being pursued, but progress and effort is limited because their resistance is controlled by multiple genes without resistance markers. Other forms of *V. arizonica* are being studied and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable resistance. Very small-scale wines from 94% and 97% *V. vinifera PdR1b* selections have been very good, and have been received well at tastings in the campus winery and at industry tastings throughout California.

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

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ABSTRACT

The major objective of this project is to broaden the genetic base of Pierce's disease (PD) resistance by searching for and characterizing new forms of Pierce's disease resistance. Previously, we reported on the screening of 52 accessions of grape species that were collected from across the southern USA and northern Mexico. Greenhouse screening of these plants identified 20 new resistant accessions. We expanded this work to over 200 accessions that were acquired from states along the Gulf of Mexico, and utilized 22 simple sequence repeat (SSR) markers and 14 chloroplast markers to develop fingerprint profiles for them. Analysis with two different programs revealed three major groups. The *V. arizonica*-like group was composed of several species with distinct maternal and paternal inheritance. The species within this group are also very distinct from southeastern Pierce's disease resistant species, once thought to be the only source of Pierce's disease resistance. Greenhouse screening was completed on a subset of genotypes, and crosses with eight new resistant lines were made in 2012 and 2013; the remaining germplasm is in the process of being screened. This germplasm screening provides opportunities to explore and identify resistance loci that may provide different resistance mechanisms allowing us to expand the genetic base of the Pierce's disease resistance breeding program. To date, we have utilized three different genetic resources to identify Pierce's disease resistance. Progress was made with b43-17 and b40-14 both of which carry a major locus on chromosome 14, as well as minor quantitative trait loci (QTLs) on different chromosomes. For *V. arizonica/candicans* b43-17, a minor QTL has identified on chromosome 19 (*PdR2*) and for *V. arizonica* b40-14, a minor QTL was identified on chromosome 5. Mapping of a multigenic source of Pierce's disease resistance from *V. arizonica/girdiana* b42-26 continues. A total of 916 markers have been tested, and 185 polymorphic markers (60 more since the previous report) have been added to the entire population of 239 seedlings. A framework genetic map was developed for 198 seedlings, which had repeated greenhouse screening. Preliminary QTL analysis identified QTLs on chromosome 8, 12, and 14 that explained over 25% phenotypic variation. Currently, we are saturating maps of these three chromosomes and associating SSR markers that are in linkage with resistance for marker-aided breeding. We plan to combine these multiple resistance sources in our breeding program to ensure broad and durable Pierce's disease resistance. This project provides the genetic markers critical to the successful classical breeding of Pierce's disease resistant wine, table, and raisin grapes. Identification of markers for *PdR1* has allowed us to reduce the seed-to-seed cycle to two years and produce selections that are Pierce's disease resistant and 97% *V. vinifera*. These markers have also led to the identification of six genetic sequences that may house the Pierce's disease resistance gene, and which are being tested to verify their function. These efforts will help us better understand how these genes function and could also lead to Pierce's disease resistance genes from grape that would be available to genetically engineer Pierce's disease resistance in *V. vinifera* cultivars.

LAYPERSON SUMMARY

We have made rapid progress breeding Pierce's disease (PD) resistant winegrapes that are now approaching release. This progress could not have been made without the development and use of DNA markers for Pierce's disease resistance and the discovery of strong single gene resistance in forms of *Vitis arizonica*. The next phase of the breeding program is now underway – combining multiple Pierce's disease resistance sources into one background. Although single gene resistance is easy to breed with, it is often overcome by aggressive pathogens and pests. With this in mind, our Pierce's disease breeding is now characterizing resistance from other backgrounds and developing DNA markers so that we can combine these resistances into a single individual. Combining these genes together will require good markers since the resistant progeny resulting from efforts will appear the same – resistant. We will need the markers to the multiple sources to verify different genes have been

combined. We have discovered more sources of strong resistance and are now mapping and developing markers to determine if these new genes control different types or forms of resistance.

INTRODUCTION

This project provides the genetic support to molecular breeding efforts [see companion Pierce's disease (PD) breeding project]. Identification, understanding, and manipulation of novel sources of resistance are the foundation of a successful breeding program. We are exploring multiple genetic backgrounds for Pierce's disease resistant grape breeding, developing and testing breeding populations via a greenhouse screen, carrying out genetic mapping of segregating populations to identify genomic regions that carry disease resistance genes, and developing physical sequence maps of resistance regions to identify and characterize grape resistance genes. We have completed mapping a major Pierce's disease resistance locus originating from *V. arizonica/candicans* b43-17, which is the foundation of our Pierce's disease breeding efforts. 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, Sonora. These accessions are morphologically and genetically (both paternally and maternally) different than b43-17, and both possess strong resistance to Pierce's disease and greatly suppress *Xylella fastidiosa* levels in stem tissue after greenhouse screening. We made strong progress in identifying one major locus and one minor QTL in the b40-14 background. New populations were developed with eight newly identified Pierce's disease resistant plant material. The breeding part of the program produces and greenhouse screens the seedling populations, while the tightly-linked genetic markers generated in these mapping efforts are being used to optimize and greatly accelerate the Pierce's disease breeding program. These markers are essential to the successful introgression of resistance from multiple sources, and thus for the production of durably resistant grapevines. In response to recommendations from the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board and reviewer recommendations to broaden resistance, we have expanded the search for additional resistance sources by screening wide germplasm collected from different parts of the USA and Mexico. Initial greenhouse screen results indicate that we have twenty other accessions that possess strong Pierce's disease resistance. Analysis indicates that the southeastern resistant material is genetically distinct from the species in Mexico, and the extension of the Rocky Mountains (Sierra Madre) has acted as a physical barrier for grape species evolution over a time period of thousands of years.

OBJECTIVES

1. Fine-scale mapping of additional quantitative trait loci (QTLs) for Pierce's disease resistance in the 04191 ((F2-7 x F8909-17) population.
2. Greenhouse screen and genetically map Pierce's disease resistance from other forms of *V. arizonica*: b42-26 (*V. arizonica /girdiana*) and b40-14 (*V. arizonica*).
3. Evaluate *Vitis* germplasm collected from across the southwestern USA to identify accessions with unique forms of Pierce's disease resistance for grape breeding. Determine the inheritance of Pierce's disease 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 bacterial artificial chromosome (BAC) clones that carry *PdR1a* gene candidates (in cooperation with our companion resistance gene characterization project).

RESULTS AND DISCUSSION

Objective 1. **Fine scale mapping of additional QTL for Pierce's disease resistance in the 04191 ((F2-7 x F8909-17) population** (see details in March 2013 report).

In brief, a framework genetic map of the 04191 population (*V. vinifera* F2-7 x F8909-17) was used to identify a QTL with a minor impact on resistance (contributing 7% of the phenotypic variation to Pierce's disease resistance) on chromosome 19. This QTL is within a 10 cM interval – a relatively long genetic distance to be effective in marker assisted screening. A total of 1.783 Mbp of sequence of PN40024 was used to develop seven simple sequence repeat (SSR) primers in this region. Three of the seven tested primers gave clean amplifications with polymorphism for the F8909-17 Pierce's disease resistant parent. These markers were added to the population of 150 seedlings. We are in the process of completing this analysis. Two crosses with 04373-02 and 04373-22 and Pinot blanc were made to study the impact of this minor QTL from chromosome 19 without impact of the major locus from chromosome 14. A total of 100 plants were screened with SSR markers and 43 plants were planted in the field in Spring 2012. These plants are scheduled to be greenhouse screened during summer 2013.

Objective 2. Greenhouse screen and genetically map Pierce's disease resistance from other forms of *V. arizonica*: b42-26 (*V. arizonica /girdiana*) and b40-14 (*V. arizonica*).

The accession b40-14, a pure form of *V. arizonica*, is homozygous resistant to Pierce's disease. All seedlings from the F1 cross tested resistant to Pierce's disease. Two resistant siblings of this population were used to develop the 07388 (R8918-02 x *V. vinifera*) and 07744 (R8918-05 x *V. vinifera*) populations. In the previous report, we described the preliminary results with 07744 and genetic mapping with 152 markers. From March to July, we tested a total of 606 SSR markers and 224 polymorphic markers were added on the entire set of 122 plants (**Table 1**). A total of 216 markers were polymorphic for the female resistant parent – R8918-05.

Table 1. List of markers tested and completed for the 07744 population derived from the b40-14 background.

Marker series	Tested	Amplified	Polymorphic	Completed
VMC, VMCNg	271	161	133	106
VVI	93	84	56	50
UDV	55	54	35	26
VChr	3	3	3	3
VVMS, VVMD, VrZAG	35	34	25	22
Other unpublished	4	4	2	2
EST-SSR (SCU, VVC, CTG)	145	108	68	15
Total	606	448	322	224

Table 2. Characteristics of the framework map of R8918-05, a Pierce's disease resistant selection used as the maternal parent in the 07744 population.

Chromosome	Mapped Markers	Length (cM)
Chr1	15	72.7
Chr2	4	59.6
Chr3	6	37.9
Chr4	11	98.3
Chr5	13	60.6
Chr6	11	40.8
Chr7	12	88.0
Chr8	11	54.7
Chr9	10	87.7
Chr10	10	74.5
Chr11	9	79.7
Chr12	8	52.5
Chr13	11	71.9
Chr14	26	97.9
Chr15	8	35.9
Chr16	9	67.5
Chr17	12	56.2
Chr18	13	136.2
Chr19	13	55.8
Total	212	1328.4
Ave marker distance (cM)	6.3 cM	
Number of gaps > 20 cM	14	

A framework genetic map of R8918-05 was produced with JoinMap (4.0). A total of 212 markers mapped to 19 grape chromosomes with average distance of 6.3 cM between markers. The updated map did not have fragmented groups and provided adequate genome coverage when comparisons were made to the previously published integrated *Vitis* genetic maps.

QTL analysis was carried out with MapQTL. A major locus for Pierce's disease resistance was identified on chromosome 14. Pierce's disease resistance from b40-14 (which we have named *PdR1c*) maps in the same general region as *PdR1a* and *PdR1b* between flanking markers VVCh14-77 and VVIN64 and within 1.5 cM. The logarithm of the odds (LOD) threshold for the presence of this QTL was 39 and this major locus explained 80% of the phenotypic variation. Using the updated genetic map, we also identified a minor QTL with LOD 2.0 on chromosome five that explained 8.3% phenotypic variation for resistance (**Figure 2**). We did not find evidence for any other QTL on the remaining 17 chromosomes. Both QTLs explained total of 88% phenotypic variation for resistance within the b40-14 background.

We designed more primers to reduce the gap between markers on chromosome five by utilizing the Pinot noir genome sequence. A total of 275 seedlings from five different crosses were also tested with markers that are in linkage with the major locus on chromosome 14; seven of recombinant lines were saved and planted in the field in Spring of 2013. These recombinant lines are scheduled for greenhouse testing and the addition of other flanking markers. These recombinant lines will help to reduce the gap between the markers on chromosome 14. The updated data will be used for a manuscript describing the genetic map and QTL identification from the b40-14 background.

The F1 population 05347 (F2-35 x b42-26) represents b42-26 background, a third resistant accession that was collected from Loreto, Baja California in 1960. A total of 918 SSR primers were tested, 763 amplified b42-26 DNA successfully, and 185 markers were polymorphic. The level of polymorphic markers was relatively low. We have not observed such a low level of polymorphism in any other grape genotype so far, likely the result of this plant being from an isolated and now inbred population. A framework genetic map was developed for 198 seedlings with 185 markers. A large number of markers showed segregation distortion. There were only four chromosomes with relatively low marker coverage; all other chromosomes have evenly distributed markers. We have repeated and completed the greenhouse screen on 199 seedlings that rooted successfully. Thirty-five of the seedlings were tested three times, 77 tested twice, and 87 were tested once. An ANOVA on the 35 genotypes tested in all three trials indicated that only genotype matters and there we no significant interactions. The same was true for the 77 genotypes tested twice when compared pairwise. The updated results were used for the QTL analysis. One-way ANOVA and interval mapping revealed QTLs on chromosome 8, 12, and 14 that explained over 25% phenotypic variation. Currently, we are refining the maps of these three chromosomes with more markers and establishing the association of markers that are in linkage with the resistance for potential use in marker-aided screening.

Objective 3. Evaluate *Vitis* germplasm collected from across the southwestern USA to identify accessions with unique forms of Pierce's disease resistance for grape breeding.

We have made tremendous progress in assessing diversity and population structure of southwestern US accessions from March to July time period. It is thought that southeastern germplasm co-evolved with *Xylella fastidiosa* and developed resistance to this disease. Our focus has been on three accessions of *Vitis* that Olmo collected in northern Mexico in 1960 (as reported in Objectives 1 and 2). Two of these accessions are complexes of multiple *Vitis* species and it is not known which particular species is controlling Pierce's disease resistance. This point is confounded by the complete fertility among the *Vitis* species and the great number of hybrids that occur in the wild. It is extremely important for a breeding program to incorporate multiple unique resistance mechanisms, understand genetic diversity, and the mode of inheritance to facilitate decision making for resistance breeding.

In order to better understand the nature of Pierce's disease resistance and the genetic diversity and gene flow of grape species in the southern USA and Mexico, we examined a diverse collection of species from these areas. One of the objectives was to determine whether Pierce's disease resistance from the Gulf states and southeastern USA is similar to that of eastern coastal (and wet) Mexico. And whether this eastern resistance differs from Pierce's disease resistance found in the southwestern USA and central (and drier) Mexico. We examined a

collection of 219 (sixty more from previous report) accessions of these species, including Olmo's Mexico collections (which he collected across northern and central Mexico from the west to the east). DNA was collected from all those genotypes and six *V. vinifera* accessions were added as outliers. A total of 22 SSR markers were selected for their polymorphism and coverage of all 19 grape chromosomes (**Table 3**). All amplified products were run on the ABI 3500 genetic analyzer and analyzed with the Gene Mapper program to obtain fingerprint profiles. Hierarchical clustering (Ward method) and Principal Coordinate Analysis were carried out with DARWIN software (version 5.0.158) to determine the number of groups. STRUCTURE V2.3.1 was used to infer the number of pseudo-populations or clusters with 22 markers.

Table 3. List of SSR markers used for fingerprint analysis of 165 selections.

Marker name	Dye label	Chromosome	Amplified product size range
VVIp60	HEX	1	>300
VrZAG93	NED	2	180-240
APT3	6-FAM	2	266-466
VVIb23	6-FAM	2	250-320
VVMD28	6-FAM	3	216-270
VVMD32	NED	4	240-280
VVMD27	NED	5	170-220
VrZAG79	6-FAM	5	230-280
VVMD21	6-FAM	6	240-260
VVMD31	6-FAM	7	200
VVMD7	NED	7	220-270
VrZAG62	HEX	7	175-215
VMC1b11	HEX	8	150-190
VVIq52	6-FAM	9	70-90
VVIv37	6-FAM	10	140-180
VVS02	6-FAM	11	120-170
VMC4f3.1	HEX	12	160-290
UDV124	6-FAM	13	170-230
VVIP26	HEX	14	120-180
VVIv67	HEX	15	350-400
VVMD5	6-FAM	16	210-290
VVIn73	6-FAM	17	240-270
UDV108	HEX	18	200-276
VVIp31	6-FAM	19	150-220

After exclusion of those accessions that did not have enough representation in the study set, analysis was carried out on set of 180 accessions with three methods. All three methods [hierarchical clustering (Ward method), principle coordinate analysis (PCA), and a model-based clustering method implemented in the program STRUCTURE] revealed three main groups. **Figure 1** presents the results of PCA with three distinct groups and **Figure 2** presents the groupings revealed by STRUCTURE displayed on a map. Most of the accessions from the Mexican species collections appear to be introgressive hybrids among *V. arizonica*, *V. berlandieri*, *V. candicans* (*V. mustangensis*), *V. cinerea* var. *blancoii*, *V. girdiana*, and *V. monticola*. Strong resistance to Pierce's disease occurs in *V. arizonica/candicans, V. arizonica/girdiana, and V. arizonica/monticola* forms.

All accessions that are part of this study are being greenhouse-tested summer/fall 2013, and results will be available in Fall 2013. The goals of this study are to investigate the phylogeographic diversity of plant material collected from Gulf coast states and the southern USA, and determine the relationships between species, Pierce's disease resistance, and the genetic control of that resistance, so that we can better understand the evolution of

resistance and the range of resistance mechanisms and their control. With this information we can more effectively combine different resistance sources to achieve more durably resistant plant material.

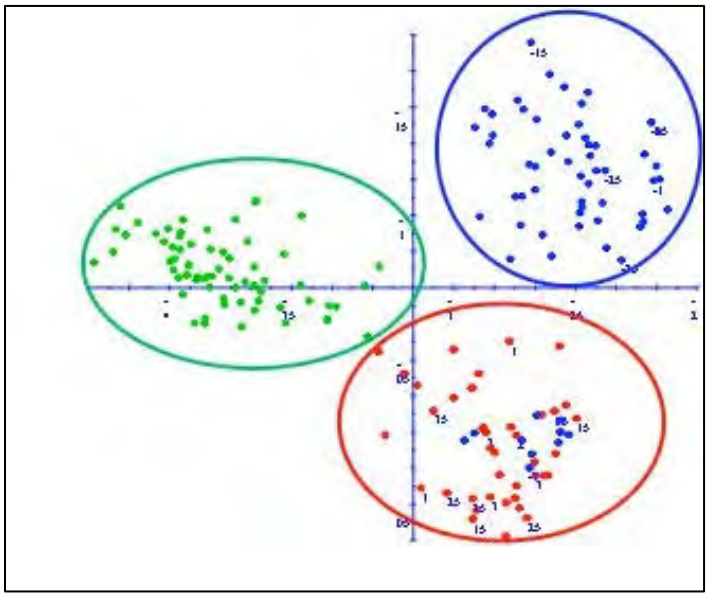

Figure 1. Principle Coordinate Analysis constructed with genotypic data from 22 SSR markers on 159 accessions using DARWIN software. Blue represents the *V. cinerea*-like accessions; red the *V. aestivalis*-like accessions; and green the *V. arizonica*-like accessions. The axis 1 and 2 presents 9.13 and 5.74 percent of the variation, respectively.

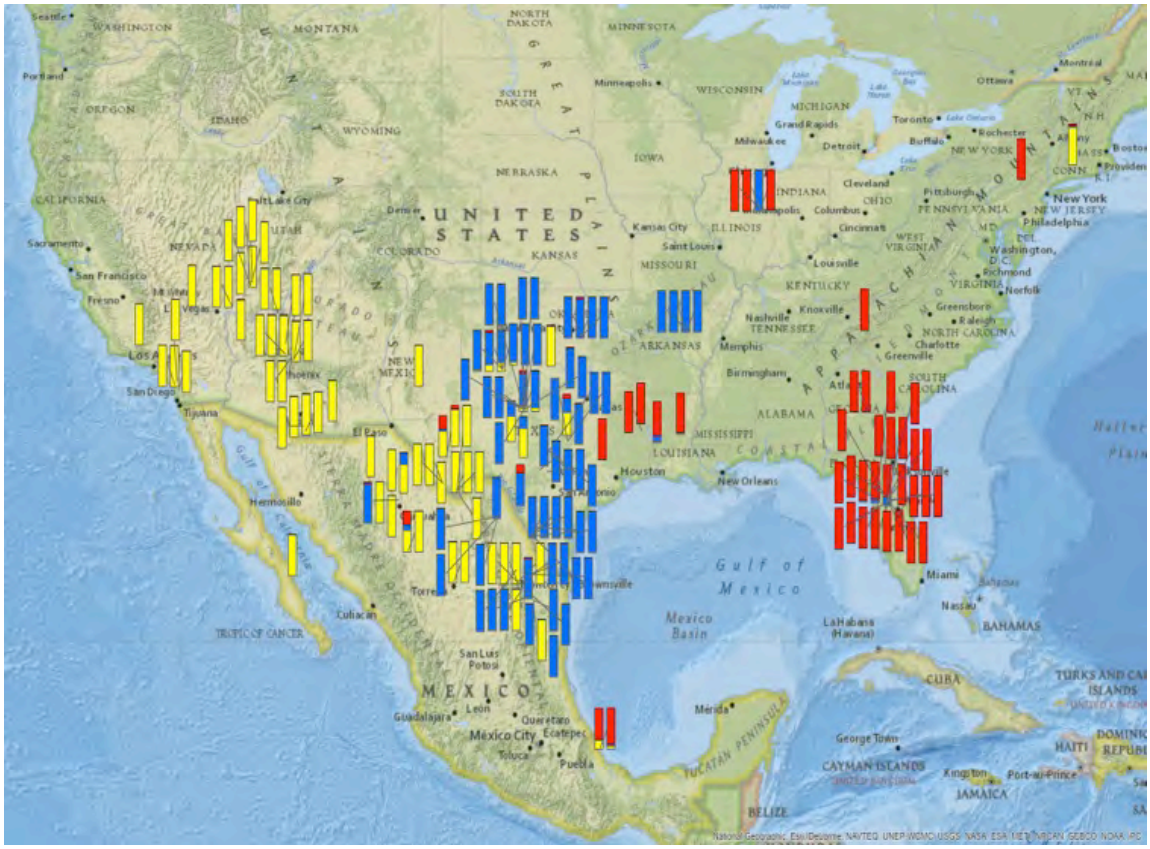

Figure 2. Grouping of accessions revealed by the clustering program STRUCTURE. The genetic composition of each accession is represented as a bar chart with different colors indicating the percentage of different species in these hybrid forms. Yellow represents *V. arizonica*-like accessions, blue = *V. cinerea*-like, and red = *V. aestivalis*-like accessions. It is noted that *V. arizonica* is complex mix of different species and further analysis only with that group separates these species into different clades.

To determine the inheritance and nature of resistance of the best forms, we made crosses in 2012 to develop breeding lines with four of the most resistant accessions. Small breeding populations were planted in Spring 2013. In 2013, we made additional crosses to expand the existing populations as well as used four new Pierce's disease resistant accessions to develop breeding populations (**Table 4**). Seedlings that were generated from the 2012 crosses were tested with markers and true-to-cross seedlings were transferred to the field. Currently these small populations are scheduled for greenhouse testing. All crosses made in 2013 will be evaluated in 2014.

Table 4. Crosses made in 2013 to develop genetic maps in new accessions from southern USA and Mexico germplasm. Crosses 08-319-29 and 08326-61 are female flowered selfed progeny of Zinfandel and Cabernet franc, respectively. F2-35 is also female and a cross of Cabernet Sauvignon x Carignane.

Resistant Source/ new or existing	Geographic Origin - Appearance Phenotype	Pure *Vinifera* Types used in 2013 crosses	Estimated # of Seed
ANU5	Littlefield, AZ	Alicante Bouschet	250
expands existing	*V. girdiana*		
b40-29	Chihuahua, MX	F2-35	1250
expands existing	*V. arizonica*	08319-29	2000
b41-13	Ciudad Mante, MX	F2-35	750
new	*V. arizonica-mustangensis-champinii*		
b43-57	Guadalupe, MX	Malaga Rosada	1000
new	*V. arizonica-mustangensis-champinii*	Rosa Minna	900
b46-43	Big Bend, TX	08326-61	850
expands existing	*V. arizonica glabra-monticola*		
b47-32	Big Bend, TX	F2-35	1950
expands existing	*V. arizonica glabra-monticola*	08326-61	70
SC36	San Diego, CA	Palomino	350
new	*V. girdiana*	Grenache	600
T03-16	Lahitas, TX	Palomino	175
new	*V. arizonica*	Grenache	20

Objective 4. Complete the physical mapping of PdR1a and PdR1b and initiate the sequencing of bacterial artificial chromosome (BAC) clones that carry PdR1a gene candidates (in cooperation with our companion resistance gene characterization project).

We have used three categories of sequences (shotgun reads, fosmid reads, and 454) to work on the BAC clone H69J14 that carries the Pierce's disease resistance gene(s). From the assembly of this sequence, we identified six copies ranging from 2Kb to 3.1Kb in the resistance region. Copies 1 – 4 are 97-99% similar and differ in size (potentially tandem repeats of one gene), they were up to 78% similar to the four copies of genes from the Pinot noir (PN40024) sequence. We utilized CENSOR software to screen query sequences against a reference collection of repeats to generate a report capable of classifying detected repeats. All four PN40024 genes carry DNA transposons as well as LTR retrotransposons indicating that the region is quite complex.

A detailed comprehensive comparison of the H69J14 clone sequence to the PN40024 sequence is not possible due to major re-arrangement of repetitive elements between the two genomes, and the presence of gaps in the contigs of the H69J14 BAC clone. We are in the process of using FGS technology, which helps close these gaps. For this purpose, we identified three overlapping BAC sequences (H15B20, H69J14 and H64M16) that span about 450Kb of the physical sequence. Complete assembly of this region will allow a more precise comparison to susceptible PN40024, which will help identify differences in the expressed and non-expressed regions and help us identify the susceptible allele of the PdR1b gene. We have received the partial results of the FGS technology, sequence assembly will be carried out when all data is available.

CONCLUSIONS

There are many strong sources of Pierce's disease resistance in the southwestern *Vitis* species particularly within forms of *V. arizonica* and *V. girdiana*. We are now combining (pyramiding) these forms of resistance to broaden the base of Pierce's disease resistance. However, we need to combine different forms of resistance not additional forms that have the same function. Thus, we are developing genetic maps for several of these resistant plants to confirm their resistances map to different locations on the genome. These efforts lay the groundwork for full characterization of these resistance genes and allow the combined resistant phenotype to be identified and confirmed with DNA markers. This project does all the marker-assisted selection for our Pierce's disease resistant winegrape breeding program and has enabled our rapid progress. It also provides the genetic material being utilized in our Pierce's disease resistance gene characterization project, which is now confirming the function of *PdR1* gene candidates by transforming them into the susceptible *V. vinifera* cultivar Chardonnay. The function of any new Pierce's disease resistance candidate genes derived from this mapping project will be evaluated through the same process.

FUNDING AGENCIES

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MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF THE PUTATIVE *XYLELLA FASTIDIOSA* RESISTANCE GENE(S) FROM B43-17 (*VITIS ARIZONICA*)

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ABSTRACT
The Walker lab has identified and cloned candidate Pierce's disease resistance genes from *Vitis arizonica* b43-17 by map-based positional cloning. In order to understand how these genes evolved and impart resistance, functional characterization and complementation by transforming susceptible plants is essential. We have generated five candidate gene constructs, which were used to transform tobacco leaf discs and grape embryogenic callus. These tissues were developed to produce transgenic tobacco and grape plants carrying the candidate *PdR1* genes. Transgenic tobacco plants have been tested against *Xylella fastidiosa* (*Xf*) in the greenhouse. Promising results have been obtained with two of the candidate genes in tobacco, which displayed significantly reduced symptom expression compared to the untransformed controls. Transgenic Chardonnay, Thompson Seedless, and St. George grapevines have been produced *in vitro*. Transgenic Chardonnay plants have been multiplied in the greenhouse and are ready to be inoculated with *Xf*.

LAYPERSON SUMMARY
We maintain and characterize many populations while breeding Pierce's disease (PD) resistant winegrapes, some of which have been used to develop genetic maps. These maps were used to identify genetic markers that are tightly linked with Pierce's disease resistance, and have allowed classical breeding to be greatly expedited through marker-assisted selection. Genetic maps allow the construction of physical maps, which can be used to identify resistance genes (Riaz et al., 2008; Riaz et al., 2009). The physical map of the b43-17 resistance region allowed us to identify candidate genes responsible for Pierce's disease resistance. Comparisons with plant genomes indicated that the gene candidates were in a repeated region and were R genes, which are known to recognize microbes and trigger a disease response limiting the pathogen (Bent and Mackey 2007). We completed the cloning of five candidate genes: *PdR1b.1, 2, 3, 4,* and *5* and confirmed their sequence. We also developed embryogenic callus cultures of Pierce's disease susceptible Chardonnay and Thompson Seedless and the rootstock St. George for genetic transformation to verify candidate Pierce's disease resistance gene function. *PdR1b.1, 2, 3, 4,* and *5* have been genetically engineered into tobacco and grape. Transgenic tobacco plants have been tested against *Xylella fastidiosa* (*Xf*) in the greenhouse and promising results have been obtained with two candidate genes. Transgenic grape plants have been acclimated to greenhouse conditions and are ready for *Xf* testing. Although the current transgenic grape plants were produced using the traditional procedure, we are also testing another technique to speed the development of transgenic tissue from meristems that will allow *PdR1* gene candidates to be tested faster.

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 Pierce's disease. Disease resistant cultivars can be obtained by conventional breeding through the introgression of resistance from Native American species into elite *Vitis 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 Pierce's disease resistant. This linkage-drag-free (just the specific resistance gene is introduced into a *V. vinifera* cultivar, not other non-desirable genes from the resistant wild species) 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 comparisons indicated that the *PdR1* region contains multiple tandem repeats of the Serine Threonine Protein Kinase with a leucine-rich repeats (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 five 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 bacterial artificial chromosome (BAC) clone H64J14.
2. Expression studies of candidate genes.
3. Complementation tests of candidate gene(s) to test their function using: a) *Agrobacterium*-mediated transformation of the susceptible *Vitis* cultivars (Chardonnay and Thompson Seedless, and the rootstock St. George); and b) Transformation of tobacco.

RESULTS AND DISCUSSION

Objective 1. Cloning, structural analysis, and gene annotation via comparison of the *PdR1b* locus to the susceptible Pinot noir genome sequence using the assembled sequence of the BAC clone H64J14.

A refined genetic map of chromosome 14, which contains the *PdR1* locus, was generated from three grape mapping populations derived from *V. arizonica/candicans* b43-17. The resistance locus segregates as a single dominant gene and mapped as *PdR1a* in the F1 selection 8909-17 and as *PdR1b* 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, and unassembled sequences, revealed the presence of a high number of transposable elements (TE). Considering 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. The second assembly allowed the identification of two new candidate genes and showed that *PdR1b.1* was longer than the sequence previously found. We have amplified and confirmed the sequences of five candidate genes *PdR1b.1 - 5*. *PdR1b.1* is the largest gene (3198 bp), sharing a high degree of homology with *PdR1b.2, 3, 4, and 6*. *PdR1b.5* is significantly different from the rest. It has a kinase domain that suggests it might be involved in Pierce's disease resistance in combination with *PdR1b.1* or one of the other candidates. *PdR1b.6* will be continued when more flanking sequence information is available.

Objective 2. Expression studies of candidate genes.

This objective was covered in last year's report and data was presented there that demonstrated the gene candidates were being expressed in the transgenic tissues and plants.

Objective 3. Complementation tests of candidate gene(s) to test their function.

Once the gene constructs are completed, they must be tested to see if they contain the resistance genes. This is done by inserting the genes into a susceptible plant and testing to see if the insertion results in resistant plants. 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 that have been used for transformation (Agüero et al., 2006).

PdR1b candidate genes were amplified using Phusion high-fidelity DNA polymerase (Finnzymes), cloned into pGEM-T easy vector (Promega) and sequenced at the UC Davis Sequencing Facility. After sequence verification, genes were subcloned into binary vector pCambia 1303 (www.cambia.org) containing the 35S cauliflower mosaic virus promoter, the nopaline synthase terminator, and an hptII-selectable marker gene. PdR1b.1 was also subcloned into binary vector pDU99.2215 containing an ntpII-selectable marker gene. The resulting plasmids were transformed into disarmed *A. tumefaciens* EHA105 pCH32 by electroporation and used for transformation of Chardonnay, Thompson Seedless, and St. George.

Pre-embryogenic calli of Thompson Seedless, Chardonnay, and St George transformed with the five candidate genes were selected in medium with antibiotics, then subcultured to germination medium for plant regeneration. The presence of the genes was checked in some callus through PCR and tested again in plants transferred to the greenhouse. For each gene, we expect to produce, at least 10 independent lines that will be subsequently propagated clonally to six plants per line and tested under greenhouse conditions. **Table 1** shows the number of independent lines that have been obtained at present. Because P2 binary plasmid was the last to be constructed, no P2 plants have been produced yet, but several P2 embryogenic callus cultures have developed in selection medium and are growing in germination medium. Chardonnay has the highest number of transgenic lines, some of which have been acclimated to greenhouse conditions and multiplied through green cuttings. Genomic DNA was isolated from these plants with DNeasy Plant Mini Kit (Qiagen). A primer that binds the CaMV 35S promoter and a primer that binds the coding region of each *PdR1b* candidate were used in combination for PCR amplification to verify the presence of the transgene. Candidate genes have amplified successfully in all the plants transferred to the greenhouse (**Figure 1**). These plants were cut back in September and will be inoculated with *Xf* in October 2013. Pierce's disease resistance analysis will be performed through symptom screening (leaf scorch and uneven cane maturation) and ELISA (Krivanek and Walker 2005).

Table 1. Number of independent lines produced until July 2013; lines in the greenhouse are shown in parentheses.

	Chardonnay	Thompson Seedless	St George
P1 pDU 99.2215	4 (3)	0	0
P1 pCambia1303	15 (5)	3	13
P2 pCambia1303	0	0	0
P3 pCambia1303	20 (5)	2	4
P4 pCambia1303	13 (5)	2	2
P5 pCambia1303	17 (5)	8	1

Tobacco transformation.

To speed the functional analysis, MS student Carolina Bistue transformed the tobacco variety SR1, which was recently demonstrated to be a susceptible host for *Xf* and is much easier and quicker to transform and test (Francis et al., 2008). Transgenic tobacco plants carrying each candidate gene (9-10 independent lines per gene) were produced at the UC Davis Transformation Facility and multiplied *in vitro* in our lab. Genomic DNA was isolated from plants of each line for PCR amplification to verify the presence of the transgene as described previously. All candidate genes amplified successfully with exception of PdR1b.1 sublcloned into pDU99.2215.

Preliminary experiments conducted to establish the best screening method for tobacco showed that pin-prick inoculation of the stem was best when compared to inoculations on the base of leaves, either through pin-prick or incision. The plants were pin-prick inoculated two times, one week apart. Each time, 20 µl of a water suspension of the Beringer strain (OD600=0.25), was inoculated on the second or third node on both sides of the stem. Symptoms were scored on a 5-point scale and stem tissue was collected and ELISA tested every four weeks (**Figure 2**).

Figure 1. Left to right from top left: Chardonnay embryos growing in germination medium, regenerated plantlets growing *in vitro*, *in vitro* plants transferred to substrate in greenhouse, transgene detection through PCR, green cuttings in mist bed, plants after cut back –will be ready for inoculation in two weeks.

Transgenic tobacco plants were acclimated to greenhouse conditions for testing against *Xf*. **Figures 3 and 4** show the results obtained from the testing of 4-5 lines of all five candidate genes, using five replicates per line. Two additional plants / line were inoculated with water. Untransformed plants were subjected to the same treatments. No significant differences were observed in stem *Xf* counts between untransformed controls and transformed plants 12 weeks post inoculation (**Figure 3**). However, candidate genes *PdR1b.1* and *PdR1b.5* displayed significantly lower symptoms compared to the untransformed controls (**Figures 4 and 5**). Other candidate genes did not show significant symptom differences with the untransformed control (**Figure 4**). Dried leaves at the base of water-inoculated plants were scored as Pierce's disease symptoms although this could be a consequence of the water restriction imposed on the plants to facilitate the development of Pierce's disease

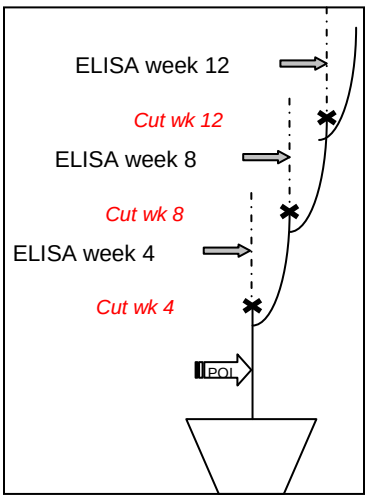

Figure 2. Schematic representation of tobacco sampling after inoculation.

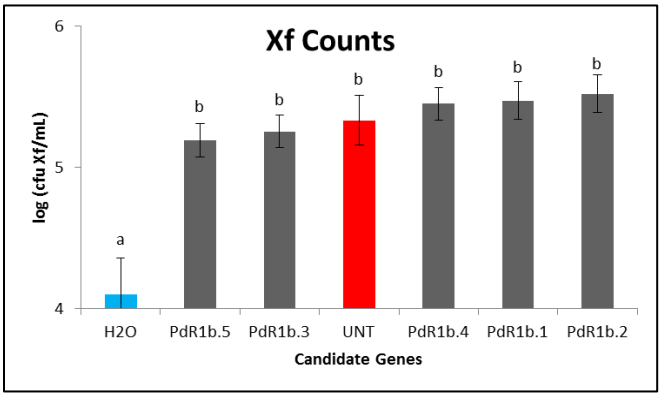

Figure 3. ELISA results for transformed candidate genes as well as negative controls (H2O) and positive controls (UNT). Samples were stem sections collected 50 cm above the POI.

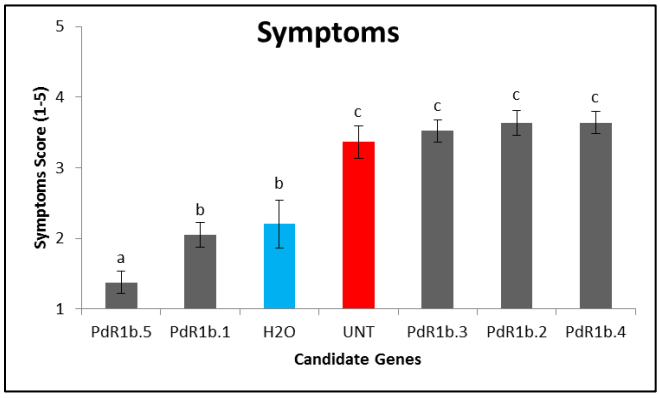

Figure 4. Symptom results for transformed candidate genes as well as negative controls (H2O) and positive controls (UNT).

Figure 5. Symptoms 12 weeks post -inoculation for untransformed controls (A) and for plants transformed with *PdR1b.5* (B).

PhD student Xiaoqing Xie is screening the transgenic lines by qPCR to ensure transgene expression. Total RNA is isolated from grape tissues using a small-scale method based on the RNeasy plant mini kit (Qiagen). Genomic DNA contamination is removed using the RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. DNAse-treated total RNA (3 µg) is reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Expression analysis is conducted by real-time PCR analysis using a SYBR Green method on a 7500 Real Time PCR System (Life Technologies). The expression of each target gene is calculated relative to the expression of the housekeeping gene (Nt-Act2; GenBank Accession number X69885) using StepOne Software v2.0. The first experiments focused on P1 and P5 expression and resulted in the amplification of single products of the expected size, which were confirmed by melt-curve analysis and agarose gel electrophoresis.

Genetic transformation via organogenesis.

Inoculation with *A. tumefaciens* of meristematic bulks (MB) is being tested as an alternative transformation technique via organogenesis to reduce the time needed to produce transgenic grapes (Mezzetti et al., 2002). In our lab, transgenic plants of Thompson Seedless expressing green fluorescent protein were produced in three months using MB and kanamycin as the selective agent. Based on these results, Thompson Seedless MB slices were inoculated with *A. tumefaciens* carrying *PdR1b.5* in pCAMBIA 1303 using three initial levels of hygromicin: 5, 10, and 15 µg/ml. Since no regeneration was produced at any of the concentrations tested, experiments assaying 0 µg / ml in the first subculture after inoculation, followed by 2.5 µg /ml hygromicin were initiated last January. This lower hygromicin concentration regenerated two MB out of 50 initial explants that are now forming adventitious buds. These buds will be tested for the presence of the transgene as soon as they produce shoots.

The partial success obtained with the use of hygromicin and the production of MB from Chardonnay and St. George have led Xiaoqing Xie to test different hormone ratios to adapt the protocol to these cultivars and study the use of different antibiotics. MS medium augmented with 17.6 µM BA and 0.5 µM NAA resulted in the

production of high quality MB of Chardonnay (**Figure 6**) that will be transformed using vectors provided by the Public Intellectual Property Resource for Agriculture (PIPRA) to compare the effect of kanamycin and hygromicin as selective agents. Media containing TDZ were unsuccessful with all genotypes. Higher BA and NAA concentrations are being assayed with rootstocks St. George and 101-14. Transformation of pre-embryogenic cultures or MB via *Agrobacterium* has been described in previous reports.

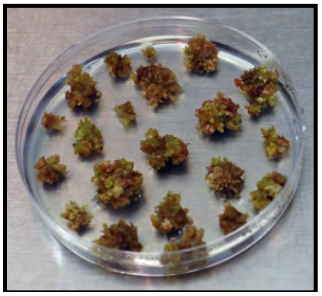

Figure 6. Embryogenic cultures from meristematic bulks of Chardonnay.

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. We have transformed grape and tobacco with five *PdR1* candidate genes. Results obtained with *Xf* inoculations of tobacco point to two potential sequences that might be involved in the resistance. We are currently testing the transgenic grapes. 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 backcrossing and simultaneous selection steps. Cisgene micro-translocation is a single-step gene transfer without linkage drag. Such engineering techniques also provide a possible means of stacking resistance genes in existing winegrape varieties

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ROOTSTOCK EFFECTS ON THE PROGRESSION OF PIERCE'S DISEASE SYMPTOM DEVELOPMENT, AND POSSIBLE MECHANISMS

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Reporting Period: The results reported here are from work conducted May 2012 to October 2013.

ABSTRACT

Rootstocks are utilized to reduce the incidence and severity of root-associated diseases. However, the ability of rootstocks to limit the progression of stem-afflicting diseases has not been studied extensively. Rootstocks potentially could slow progression of scion-afflicting diseases such as Pierce's disease, which is caused by infection by *Xylella fastidiosa* (*Xf*). In this experiment, greenhouse-grown Chardonnay or Cabernet Sauvignon grapevines were grafted to a variety of different rootstocks and infected with *Xf*. Pierce's disease symptoms and *Xf* titers were assessed six months later. Chardonnay grafted to Freedom or Salt Creek (Ramsey) rootstocks had reduced disease severity compared to Chardonnay grafted to RS-3 or Schwarzmann. Cabernet Sauvignon grafted to 1103 Paulsen, 101-14 Millardet et de Grasset, 3916, 420A, or Schwarzmann had reduced Pierce's disease severity compared to 110 Richter, 5BB Kober, or SO4. Chardonnay grafted to RS-3 had greater *Xf* titer than Chardonnay grafted to 101-14 Millardet et de Grasset, Freedom, or Salt Creek. No other significant differences in *Xf* titers were observed. Although reductions in disease severity were not always consistent between cultivars (Schwarzmann), certain rootstocks were considered promising for reduced Pierce's disease symptom progression (101-14 Millardet et de Grasset and Salt Creek). In order to observe the potential mechanism for reduced symptom development, phenolic compounds were analyzed within scion xylem sap. However, with the exception of caftaric acid (greater levels in rootstocks with fewer Pierce's disease symptoms) and quinic acid (positively associated with Pierce's disease symptoms), no consistent relationships between constitutive or induced phenolic levels and Pierce's symptoms were found. Field studies would be necessary to observe whether or not rootstocks can consistently slow the development of Pierce's disease.

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AUTHOR INDEX