

Pierce's Disease Control Program



Pierce's Disease Research Progress Reports

*Pierce's Disease
Research
Progress Reports*

- December 2012 -

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Note to Readers:

The reports contained in this document have not been subject to scientific peer review.

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Section 1:

Vector Biology and Ecology



EFFECTS OF PLANT WATER STRESS ON GLASSY-WINGED SHARPSHOOTER FEEDING BEHAVIORS THAT CONTROL ACQUISITION AND INOCULATION OF *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted October 1, 2011 to September 30, 2012.

ABSTRACT

Feeding behaviors by vectors of *Xylella fastidiosa* (*Xf*) such as the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, directly control *Xf* transmission (especially acquisition and inoculation). The present study tested whether plant water stress affects vector performance of acquisition and/or inoculation behaviors. Feeding behaviors on well-watered vs. water-stressed plants were recorded using electrical penetration graph (EPG); plants studied were almond, *Prunus dulcis* cv 'Sonora,' and citrus, *Citrus sinensis* cv 'Navel.' EPG waveforms representing pathway phase (searching for xylem), X waves (xylem contact, likely to control *Xf* inoculation), and waveform C (ingestion of xylem fluid, *Xf* acquisition) were analyzed. Results showed that duration of xylem-sap ingestion per insect was longer on well-watered than on water-stressed plants. Numbers of X waves per insect also were higher when plants were well-watered. Thus, both acquisition and inoculation behaviors were decreased on water-stressed almond and citrus plants. These findings support other studies suggesting that diminished irrigation may impact Pierce's disease epidemiology by reducing bacterial acquisition and/or inoculation by the vector.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) is an economically important pathogen of grapevine (Pierce's disease), stone fruits, nursery trees, and ornamental plants (various scorch diseases) in California. The bacterium is transmitted by sharpshooter leafhopper vectors, such as the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS), an exotic, invasive species that is now established in many grape-growing areas in southern and central California. GWSS feeding behaviors directly control *Xf* transmission (acquisition and inoculation). The present study tested whether plant water stress affects vector performance of acquisition and/or inoculation behaviors on two agricultural host plants, citrus (*Citrus sinensis* cv 'Navel') and almond (*Prunus dulcis* cv 'Sonora'). Feeding behaviors on well-watered vs. water-stressed plants were recorded using electrical penetration graph (EPG) technology. EPG waveforms representing searching for xylem, xylem contact (likely to control *Xf* inoculation), and ingestion of xylem fluid (*Xf* acquisition) were significantly different among treatments. Results showed that duration of xylem-sap ingestion per insect was shorter on water-stressed than on well-watered plants. Also, numbers of X waves per insect were lower when plants were water-stressed. Thus, both acquisition and inoculation behaviors were decreased on water-stressed almond and citrus plants. These findings support other studies suggesting that diminished irrigation may impact Pierce's disease epidemiology by reducing bacterial acquisition and/or inoculation by the vector.

INTRODUCTION

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, is a xylem fluid-feeding, invasive leafhopper that transmits the bacterium *Xylella fastidiosa* (*Xf*) to grapevine, almond, citrus, and other crops, where it causes Pierce's disease, almond leaf scorch, citrus variegated chlorosis (CVC), and other related diseases, respectively. Grapes are considered one of the most economically important crops in California (\$4.1 billion/year), with almonds closely following (\$2 billion/year) (CDFA 2006). Over 337,000 ha of vineyards and 405,000 ha of almond orchards are distributed throughout the state, which are now threatened by the association of GWSS and *Xf*. Although CVC is not known to be present in North America, citrus is the most common overwintering and reproductive host of GWSS in southern California. Citrus plays an important

epidemiological role in Pierce's disease incidence and severity in adjacent vineyards because it influences the spatial distribution of GWSS populations (Park et al. 2006, Perring et al. 2001).

Recent laboratory research has shown that GWSS will settle and feed (as measured by excretion) significantly more on surplus-irrigated citrus trees than on citrus under continuous deficit irrigation (Groves et al. 2006). Under field conditions, deficit irrigation in citrus trees affected the population densities and spatiotemporal distribution of GWSS (Krugner et al. 2009, Krugner et al. *in press*). Short periods of deficit irrigation (termed Regulated Deficit Irrigation, or RDI) in citrus causes little to no impact on yield and, in some instances, increases gross yield, fruit load, and fruit quality (Peng and Rabe 1998, González-Altozano and Castel 1999). Therefore, the present project is part of a coordinated series of experiments designed to provide necessary information to develop management plans to deploy RDI in citrus orchards, to minimize the risk of Pierce's disease outbreaks in California. A sustainable use of RDI will not only save precious water, but substantially reduce the costs of current insecticide control measures by limiting applications to better-watered sections of the orchard that are highly attractive to GWSS.

The most rigorous means of studying GWSS feeding behavior is electrical penetration graph (EPG) monitoring (Walker 2000). Waveforms for all feeding behaviors for GWSS have been characterized (Backus et al. 2005, Joost et al. 2006), and those likely controlling acquisition and inoculation of *Xf* have recently been identified (Backus et al. 2009).

OBJECTIVE

1. Determine whether the GWSS feeding behaviors associated with acquisition and inoculation of *Xf* differ on young citrus vs. almond trees that were well-watered vs. water-stressed.

RESULTS AND DISCUSSION

GWSS were age-specific and lab-reared on cowpea, sunflower, basil, and sorghum plants raised in a USDA greenhouse under supplemental lighting with fertilization. All test plants were well-watered until the week before experimental recordings were begun. During the last week, the water-stressed plants were watered only once and the well-watered plants were watered three times. On the day of an experiment, the well-watered plants were watered an hour before recording began, while the water-stressed plants were not. Each afternoon, one plant for each plant-treatment combination was positioned under high-pressure sodium vapor lights. Four insects were wired for EPG (Backus et al. 2009) and placed on plants for wire and plant acclimation overnight, without lights on. Experimental design was a factorial, 2x2 comparison of host plant vs. amount of water, in a randomized complete block. Beginning 1 – 2 h after setup, insects were recorded for 20 – 22 h using an AC-DC 4-channel EPG monitor set at either 10^7 or 10^8 Ohms input impedance (depending on noise) and 25 – 30 mV AC substrate voltage. Sodium vapor lights were positioned above plants, and turned on or off hourly during recording. Once an hour, a leaf not being fed upon was removed and its xylem tension was recorded using a pressure chamber. EPG waveforms were manually measured using Windaq Pro+, databased in Excel, then durations and frequencies of all waveforms, across all hours, were analyzed using mixed-model ANOVA (Proc GLIMMIX, SAS). Data were log or square-root transformed, to improve heterogeneity. Pairwise comparisons were performed using Least Significant Difference tests, with $\alpha = 0.05$. Future analysis will regress EPG waveform and xylem tension data, by hour.

Overview of stylet penetration

Mean durations (\pm standard errors) of each period of stylet penetration period (termed a probe) were significantly different for both main effects (plant or water level) and also their interaction effects. Therefore, all four plant \times water treatments could be graphed (**Figure 1**; green color scheme). Probes were numerically longer on well-watered almond, though not significantly different from well-watered citrus. Probes were significantly shorter on water-stressed plants, both almond and citrus; water-stressed almond was not significantly different from well-watered citrus. Mean numbers of probes, and all other cohort-level variables (numbers and durations), were not significantly different among treatments.

Behaviors performed during those probes

All remaining significances were for main effects only, not interactions. Therefore, the remaining charts (orange-blue color scheme) portray plant and treatment results separately. Three main behaviors were significantly different: sheath branching, xylem contact (testing) and ingesting.

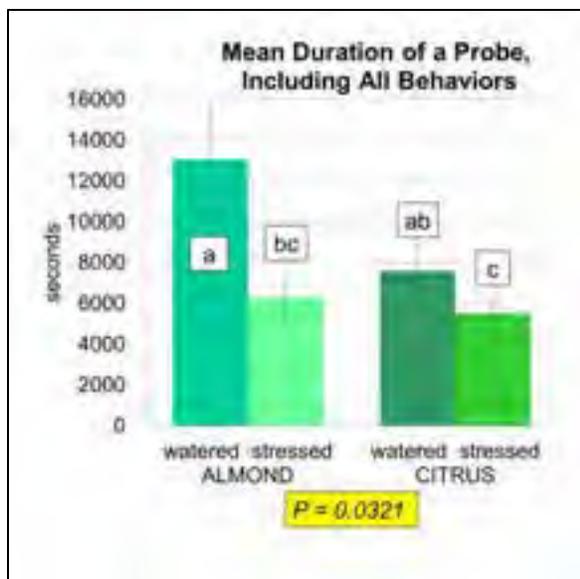


Figure 1. Results from interaction effects tests of probe duration data from insects in all four treatments, using ANOVA and LSD.

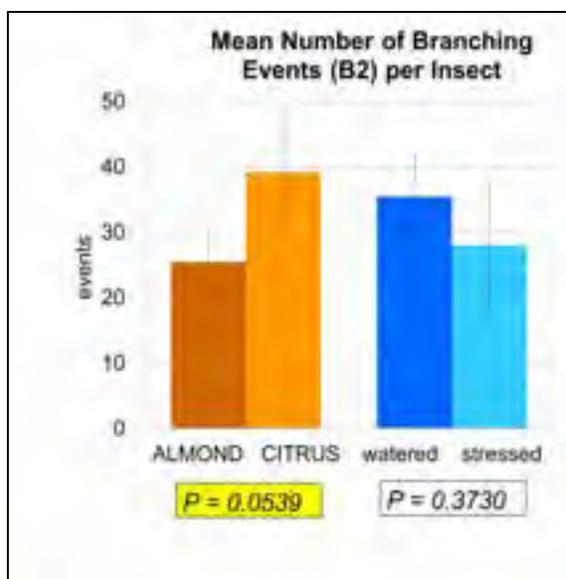


Figure 2. Results from main effects tests of B2 frequencies using ANOVA and LSD. Interaction effects were not significantly different.

Number of salivary sheath branches (Figure 2)

Sheath branching, represented by EPG waveform B2, occurs when the insect is searching for a xylem cell from which to ingest (imbibe) sap. A larger number of branches indicate that the insect is having difficulty locating a xylem cell. Significantly more branches were made on citrus than on almond. However, the same number of branches was made on well-watered as on water-stressed plants.

Number of X waves (xylem contacts; putative *Xf* inoculation) (Figure 3)

When its stylets first contact a xylem cell, the insect performs a complex series of behaviors to test the acceptability of the cell, including salivating, tasting, and egesting (rinsing and discharging fluid out). Each of these behaviors is represented by an EPG waveform; collectively, these waveforms comprise the XN portion of the sharpshooter X wave, a stereotypical pattern of repeating waveforms common in sheath-feeding hemipterans. Behaviors represented by XN are thought to control inoculation of *Xf*, after bacteria have been acquired onto the cuticle of the anterior foregut. The second portion of the X wave, XC, represents brief (trial) ingestion events that serve to test the mechanical seal of the stylets into a xylem cell. The same number of XN as XC events occur, therefore, XN frequencies are the same as overall X wave frequencies. Mean durations of X waves were not significantly different among treatments. However, numbers of X waves (frequencies) were significantly different. Insects performed significantly more xylem contacts on almond than on citrus, and on well-watered plants than on water-stressed plants (**Figure 3**).

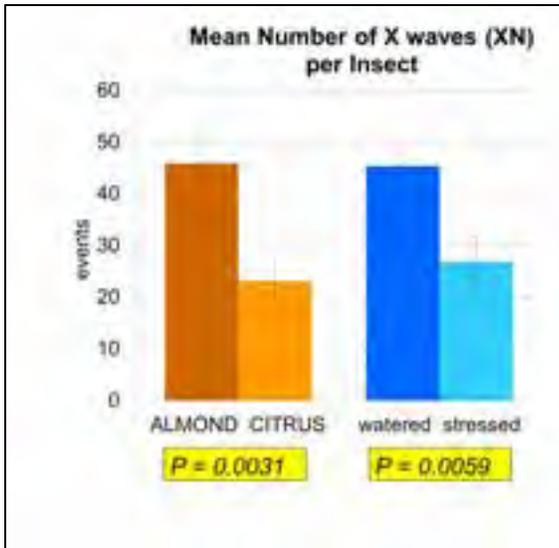


Figure 3. Results from main effects tests of XN frequencies using ANOVA and LSD. Interaction effects were not significantly different.

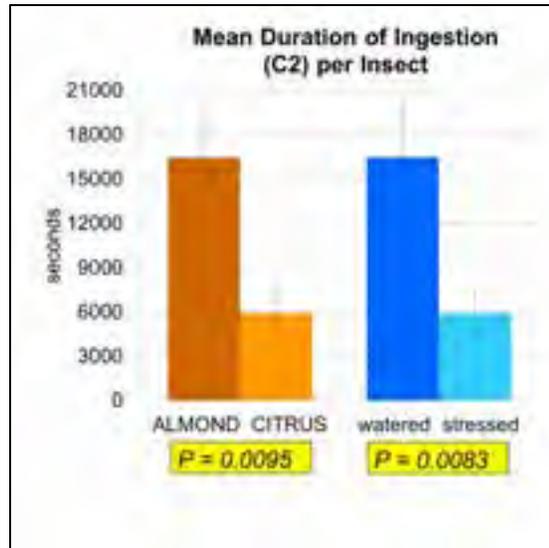


Figure 4. Results from main effects tests of C2 durations using ANOVA and LSD. Interaction effects were not significantly different.

Combined duration of sustained ingestion events (*Xf* acquisition) (Figure 4)

After xylem contact and testing during the X wave is complete and the cell has been found acceptable, the insect begins sustained ingestion of xylem sap, lasting for many minutes to hours. During sustained ingestion phase, the vector acquires bacteria from the xylem cell. Durations of all sustained ingestion events were summed, then averaged per insect; these mean durations were significantly greater on almond (compared with citrus) and on well-watered plants (compared with stressed plants) (Figure 4). That was because both numbers and durations of individual ingestion events were also significantly longer on both almond and well-watered plants.

In summary, GWSS feeding behaviors differed by both host plant treatment and watering status. The two treatments were not dependent upon one another except at the broadest level of analysis, mean duration of a probe per insect. All of the specific analyses of behaviors showed independence of the two treatment factors. Perhaps a larger sample size might have influenced independence. Alternatively, the two factors could be independent because many differences between host plants could be fixed in spite of watering status, such as anatomical differences between the host plants. This was probably the case for number of branching events, a behavior that indicates degree of difficulty in searching for a xylem cell. Physical impediments such as lignification of cell walls, location and depth of xylem cells, etc. could influence the amount of time needed for searching, which proved to be easier for the insects on citrus than almond plants. Amount of water did not influence the difficulty of searching. In contrast, both host plant and watering level had significant effects on xylem-related behaviors, especially number of xylem contacts and duration of sustained ingestion. The pattern of effects was similar for each of these behaviors, i.e. both occurred more on almond than citrus and on well-watered than water-stressed plants. Thus, plentiful xylem flux and almond were more favorable for GWSS feeding.

CONCLUSIONS

Both host plant species and irrigation treatment significantly affected feeding behaviors of GWSS in ways that could change an insect's transmission efficiency. For host plant species treatments, insects on almond reached xylem more quickly than on citrus, but performed more frequent X wave behaviors (potentially inoculating *Xf* in those cells). The insect then ingested xylem fluid for longer periods (potentially acquiring more bacteria) on almond than citrus. Thus, there is a greater likelihood of *Xf* acquisition and inoculation on almond than citrus. For irrigation treatments, insects took the same time to locate xylem on both well-watered and water-stressed plants. However, they performed more X wave behaviors and ingested for longer periods on well-watered

plants. Therefore, there is a greater likelihood of *Xf* acquisition and inoculation on well-watered than on water-stressed plants. Results support that application of RDI has the potential to reduce the incidence of Pierce's disease by suppressing GWSS populations and by decreasing pathogen transmission efficiency.

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

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ABSTRACT

This project is now almost complete; its main objectives were to label (using archival paper and unique plastic database numbers with barcodes), identify, database (including georeferencing), preserve, partially dry from ethanol, and point- and slide-mount specimens among at least 17,000 voucher specimens of mymarid and trichogrammatid egg parasitoids of the glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) (GWSS). These were either collected (reared) by the CDFA Pierce's Disease Control Program personnel in California since 2001 in the course of pre- and post-release surveys, are irreplaceable vouchers of the colonies of the biological control agents that were released in California (both exotic and native), or were collected by staff of Texas A&M in Texas since 2005. Taxonomic identifications were checked and, when necessary, specimens were identified by the PI for the California material (in more than 7,500 vials); particularly, all Trichogrammatidae were identified to genera and species. Specimens from Texas (in 930 vials) were all identified to genera and species and were transferred into leak-proof vials with good caps to prevent alcohol leakage, labeled properly, and databased using University of California, Riverside Entomology Research Museum (UCRC) numbering system which then can be made available online if desired. Valuable representatives of each species were dried from ethanol using a critical point dryer and point-mounted (and representatives of both sexes slide-mounted) as museum quality voucher specimens, and also were labeled using acid-free archival paper and databased. Most voucher specimens from Texas will be submitted at the end of 2012 to the CDFA Mt. Rubidoux Field Station in Riverside to be eventually transferred for storage at the California State Collection of Arthropods, CDFA in Sacramento, its permanent depository; some duplicate representatives and a few taxonomically important specimens will be also deposited in UCRC.

LAYPERSON SUMMARY

Important, irreplaceable, and numerous voucher specimens of the glassy-winged sharpshooter (GWSS) egg parasitoids from California and Texas were curated in the course of this two-year project. Museum-quality specimens were prepared and preserved on points, slides, and in ethanol, all properly labeled and databased.

INTRODUCTION

Recently (since 1997), major efforts have been undertaken by the CDFA Pierce's Disease Control Program to survey for egg parasitoids of the glassy-winged sharpshooter (GWSS) in California and to release egg parasitoid species (*Anagrus epos* Girault and several *Gonatocerus* spp.) from other states in the USA and also northeastern Mexico as part of classical biological control effort (CDFA 2012).

It is well known that the taxonomic impediment in identification of natural enemies may adversely affect the biological control efforts against agricultural pests. In the case of GWSS, early misidentifications (due to

objective reasons, such as partially inaccurate existing keys) of one of the species of the California native egg parasitoids of GWSS, as *Gonatocerus morrilli* (Howard), resulted in the inability of biological control practitioners to distinguish them from the introduced “real” *G. morrilli* from Texas and northwestern Mexico. Therefore, contamination of the colonies in the mass-rearing program was noticed only after the molecular methods distinguished them as two genetically different entities. The “California *G. morrilli*” was later described taxonomically as a new species, *G. walkerjonesi* Triapitsyn, based on the combination of molecular evidence and some morphological differences that are difficult to observe without special preparation of the specimens (Triapitsyn 2006). Another new species, *G. morgani* Triapitsyn, was also described from Orange Co. (Triapitsyn 2006); it is now being mass-produced and released in other parts of California infested with GWSS (Son et al. 2012). As proper part of the ongoing biological control program against GWSS, the CDFA Pierce’s Disease Control Program has conducted extensive pre- and post-surveys of the egg parasitoids of GWSS in California from 2001. These surveys, which also included egg parasitoids of the native leafhopper in California, the smoke-tree sharpshooter *Homalodisca liturata* Ball, resulted in collection of more than 10,000 specimens of egg parasitoids (Mymaridae and Trichogrammatidae) which are stored, along with voucher specimens of the numerous colonies of GWSS egg parasitoids maintained by the CDFA, in several thousand vials at the CDFA Mt. Rubidoux Field Station in Riverside. Also, Dr. Forrest L. Mitchell kindly donated to the CDFA Pierce’s Disease Control Program 930 vials of GWSS egg parasitoids collected in or near Fredericksburg, Gillespie Co., Texas, by Texas A&M staff. These insects were collected during 2005-2007, each vial containing parasitoids that emerged from a single egg mass (usually at least eight individuals per vial, but often many more); they were in need to be properly curated as museum quality voucher specimens.

OBJECTIVES

1. Check the taxonomic identities of all the specimens of GWSS egg parasitoids from California; pull out specimens of taxonomic and voucher interest.
2. Transfer the bulk of the voucher specimens from Texas into leak-proof vials for long-term storage; label properly and database all the vials using barcodes with unique numbers. Fully identify and catalog more than 7,000 GWSS parasitoids collected by Texas A&M in the native range of GWSS.
3. Prepare representatives of each species from both states by drying specimens from ethanol using a critical point dryer, point- and slide-mount as museum quality vouchers, label (using archival, acid-free paper), and database them.

RESULTS AND DISCUSSION

This project, which is almost complete, dealt with important, numerous voucher specimens of the GWSS egg parasitoids from California and Texas (Triapitsyn 2011). Taxonomic identifications were checked and, when necessary, specimens were identified by the PI for the California material (in more than 7,500 vials); particularly, all Trichogrammatidae were identified to genera and species. All specimens in 930 vials with GWSS egg parasitoids received from Texas AgriLife Research and Extension Center at Stephenville were identified to genera and species by the PI and then completely curated by the two technicians employed by the project. These were transferred from the original unsuited vials into leak-proof vials good for long-term storage; labeled properly (with the data label inside and the identification label and the database number and a barcode outside of each vial, **Figure 2**), databased using barcodes with unique UCRC ENT numbers, and arranged into boxes for cold storage (**Figure 1**).

Representatives of each species (at least 50 specimens of the common species from Texas, as many as possible of the less common species, all specimens of the rare species, and also 10 specimens of each species pulled out by Jessica Nichols from the collections in California) were critically point dried from ethanol, and point-mounted. Then representatives of both sexes of each species (both sexes) were selected and slide-mounted in Canada balsam (**Figure 3**). All the mounted specimens were properly labeled using acid-free archival paper and databased; this database can be made available online if desired. Most voucher specimens from Texas will be submitted at the end of 2012 to the CDFA Mt. Rubidoux Field Station in Riverside to be eventually transferred for storage at the California State Collection of Arthropods in Sacramento, its permanent depository; some duplicate representatives and a few taxonomically important specimens will be also deposited in UCRC.

The following species of GWSS egg parasitoids were identified from California: *Gonatocerus* (*Cosmocomoidea*) *ashmeadi* Girault, *G. (Cosmocomoidea) fasciatus* Girault (intentionally introduced, from a few release sites only), *G. (Cosmocomoidea) incomptus* Huber, *G. (Cosmocomoidea) morgani* Triapitsyn, *G. (Cosmocomoidea) morrilli* (Howard) (intentionally introduced), *G. (Cosmocomoidea) novifasciatus* Girault, *G. (Cosmocomoidea) triguttatus* Girault (intentionally introduced), and *G. (Cosmocomoidea) walkerjonesi* Triapitsyn (Mymaridae), as well as *Ufens ceratus* Owen and *U. principalis* Owen (Trichogrammatidae). Newly discovered and reported in California (four females from Riverside) is an apparently undescribed species of the genus *Pseudoligosita* Girault (Trichogrammatidae), which is morphologically different from *P. plebeia* (Perkins) from Sonora, Mexico, reported as *Pseudoligosita* sp. by Triapitsyn & Bernal (2009) and later identified as such by the PI. Also of taxonomic interest is an undescribed species of *Polynema* Haliday (subgenus *Doriclytus* Foerster) which was quite rare among the reared egg parasitoids of GWSS in California (only one female and two males from Pomona and two females from Riverside). The latter species was quite commonly collected in southern California by other (non-reared) methods. Most likely GWSS is not a primary host for both aforementioned species, and they attack its eggs only occasionally.

The following species of GWSS egg parasitoids were identified from Texas: *Gonatocerus* (*Cosmocomoidea*) *ashmeadi* Girault (very common), *G. (Cosmocomoidea) incomptus* Huber (quite rare), *G. (Cosmocomoidea) morrilli* (Howard) (less common), *G. (Cosmocomoidea) novifasciatus* Girault (quite rare), *G. (Cosmocomoidea) triguttatus* Girault (common) (Mymaridae), as well as *Burksiella spirita* (Girault) and *Ufens ceratus* Owen (Trichogrammatidae, both quite common).

CONCLUSIONS

These now fully-curated collections of GWSS egg parasitoids from California and Texas preserve the invaluable voucher specimens for further analyses (including molecular, distributional, taxonomic, biological, etc.), thus making them available. They also provide information on the species composition and their relative abundance. The specimens and information on them will be useful for the CDFA Pierce's Disease Control Program and biological control research practitioners in this state and beyond.

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Figure 1. Boxes with curated, labeled, and databased vials containing identified GWSS egg parasitoids from Texas.



Figure 2. Typical curated vials containing GWSS egg parasitoids from Texas.

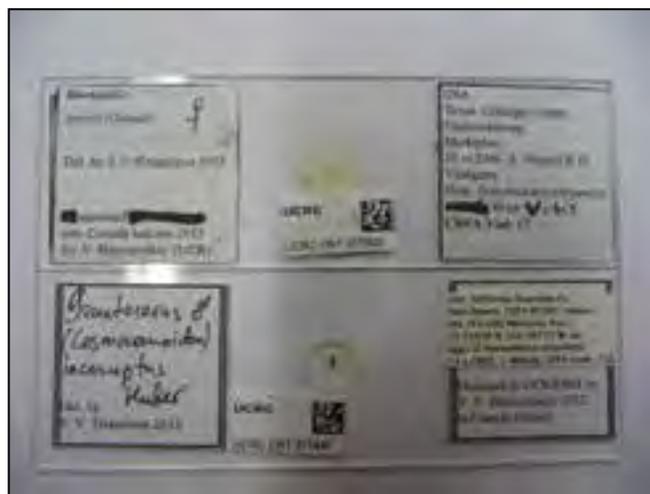


Figure 3. Typical slides of the identified GWSS egg parasitoids from California and Texas.

EVALUATION OF GRAPEVINE AS A HOST FOR THE GLASSY-WINGED SHARPSHOOTER

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Reporting Period: The results reported here are from work conducted from June 2010 to October 2012.

ABSTRACT

Grapevine was evaluated as a feeding and oviposition host for the glassy-winged sharpshooter (GWSS). Two sets of experiments were conducted. The first set compared performance and preference of GWSS females for grapevine (cv. Chardonnay) versus cowpea (*Vigna unguiculata* cultivar black eye). Cowpea was used as a reference host plant species as previous studies have documented that GWSS readily use cowpea as a feeding and oviposition host. The second set of experiments compared performance and preference of GWSS females for *Xylella fastidiosa* (*Xf*)-inoculated grapevine versus mock-inoculated grapevine. In choice tests comparing preference of females for grapevine versus cowpea, females were observed more frequently on grapevine than on cowpea with 78% of eggs deposited on grapevine. In no-choice tests with females confined to plant stems, females confined to cowpea stems produced 1.5 times more excreta than females confined to grapevine stems. Further, females confined to cowpea stems produced 1.6 times more mature eggs than females confined to grapevine stems. In no choice tests with females provided full access to plants, females produced similar numbers of eggs on grapevine and cowpea. As feeding affects egg maturation, differences in the results of no-choice tests with females confined to plant stems versus no-choice tests with females given full access to plants suggests that feeding site selection may be more important on grapevine than on cowpea. Collectively the results suggest grapevine is of similar quality and acceptability for adult feeding and oviposition as cowpea. Choice tests comparing preference of females for *Xf*-inoculated grapevine versus mock-inoculated grapevine produced variable results. In choice tests conducted in 2011, females were observed more frequently on mock-inoculated grapevines than on *Xf*-inoculated grapevines. In choice tests conducted in 2012, females were observed more frequently on mock-inoculated grapevines compared to *Xf*-inoculated grapevines displaying severe Pierce's disease symptoms, but females did not display any preference for mock-inoculated grapevines versus *Xf*-inoculated grapevines that were asymptomatic or displayed minor symptoms of Pierce's disease. In no-choice tests with females confined to grapevine stems, females on mock-inoculated grapevines produced numerically more excreta than females on *Xf*-inoculated grapevines, although the difference was not significant. Collectively, results comparing preference and performance of GWSS females for *Xf*-inoculated versus mock-inoculated grapevines suggest females prefer mock-inoculated grapevines but degree of preference may be related to disease severity. Disease severity may impact GWSS preference because plants with greater symptom severity may be of reduced host quality. Additional tests evaluating effects of disease severity on GWSS preference and performance are needed.

FUNDING AGENCIES

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We thank Sean Uchima and Donal Dwyer for assisting with experiments.

Section 2:

Vector

Management



DEVELOPMENT OF EFFECTIVE MONITORING TECHNIQUES FOR SHARPSHOOTERS AND THEIR PARASITOIDS

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Reporting Period: The results reported here are from work conducted October 2011 to August 2012.

ABSTRACT

Post-release monitoring of glassy-winged sharpshooter (GWSS) parasitoids currently involves the collection and incubation of field-collected GWSS egg masses. Since egg masses are removed from the field and cannot be further parasitized, this reduces the estimated rates of parasitism. In addition, optimal incubation conditions vary for each parasitoid species. Therefore, significant developmental mortality can occur during the two-week or longer incubation period needed for wasps and GWSS to eclose. Some species are being significantly underreported because of these factors. Further confounding the issue is the difficulty in differentiating GWSS eggs from those of the native smoke-tree sharpshooter. The development of a single-step multiplex high resolution melting curve real-time PCR assay for sharpshooters and their parasitoids will resolve these obstacles. This will lead to accurate reporting of GWSS parasitism.

LAYPERSON SUMMARY

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

INTRODUCTION

Gonatocerus morgani, *G. morrilli*, and *G. triguttatus* have been reared and released by the Pierce's Disease Control Program at sites throughout Southern California and the southern Central Valley since 2000. However, data concerning the extent of released species populations, the effects of parasitism by native competitors, and the host preferences of the parasitoids involved is still needed, even though data presented in the most recent CDFA report (2010) demonstrates the effectiveness of the release program. The post-release collection and incubation of field-collected glassy winged sharpshooter (GWSS) eggs is currently the only methodology available for monitoring the GWSS biocontrol program. Since the eggs are removed from the field before development has been completed, the possibility of further parasitism is eliminated and, therefore, parasitism rates are underestimated. In addition, because optimal incubation conditions vary for each parasitoid species, significant developmental mortality can occur during the two-week or longer incubation period needed for wasps and GWSS to eclose. This results in some species being significantly underreported. If there is no sharpshooter emergence, there currently is no economical method for identifying whether eggs are from GWSS or the native smoke-tree sharpshooter (STSS). Therefore, it is essential that a more efficient method for monitoring biological control activity be developed if we are to have more accurate, timely, and economic reporting of GWSS parasitism.

In conjunction with his research in comparative and functional genomics of *Xylella fastidiosa* (*Xf*), D. Cooksey has developed a multiplex PCR system for the simultaneous identification of *Xf* strains (Hernandez-Martinez et

al., 2006). D. Morgan, an expert in the biology, ecology, systematics, and identification of the host (Son et al., 2009) as well as the parasitoid species targeted in this study, is the supervisor of the release program. C. LeVesque directs a high throughput testing program for citrus Huanglongbing disease that employs high resolution melting curve analysis as developed by Lin et al., 2011. The development of a high resolution melting (HRM) real-time PCR system will greatly enhance the data acquisition of the CDFA parasitoid release biocontrol program which will assist in assessing the efficacy of the ongoing sharpshooter egg parasitoid strategy.

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

OBJECTIVES

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

RESULTS AND DISCUSSION

To develop HRM primers targeting the cytochrome c oxidase subunit I (COI) gene of the various *Gonatocerus* wasp species using published sequences, alignments were made using the DNASTAR (Madison, WI) Lasergene MegAlign program, followed by primer design using the Lasergene PrimerSelect program. A small region of 144 bp and 102 bp was targeted for cloning from each wasp and sharpshooter species, respectively, using these primers (**Figures 1 and 2**). Clones have been obtained for each wasp species and this region was the amplification target used for our preliminary HRM analyses. GWSS and STSS clones have yet to be obtained. Conventional PCR reactions for cloning were performed on a Bio-Rad (Hercules, CA) S1000 thermal cycler using Platinum Taq polymerase (Invitrogen, Grand Island, NY) and the following amplification program: 94°C 2 min, 35 cycles of 94°C 30 sec., 45°C 30 sec., 72°C 20 sec., 72°C 5 min. Products were analyzed on and isolated from Invitrogen E-Gel SizeSelect 2% agarose gels and both the pGem T-Easy (Promega Corporation, Madison, WI) and the TOPO TA (Invitrogen) kits were used to clone PCR products. Sequencing of the cloned PCR products was performed by the Genomics Core sequencing service of the Institute for Integrative Genome Biology on the U.C. Riverside campus. All plasmid concentrations were determined using the DTX 880 Multimode Detector (Beckman Coulter, Brea, CA) and adjusted to 60 ng/μl. A serial dilution was prepared for each plasmid. HRM analyses were performed using 2 μl of 10⁻⁵, 10⁻⁷, 10⁻⁹ and 10⁻¹¹ plasmid dilutions in duplicate with one sample at each dilution designated as standard. Genomic samples consisted of 2.0 or 0.2 ng of genomic DNA in 2 μl. MeltDoctor HRM 2X master mix (Applied Biosystems Life Technologies, Foster City, CA) was used for all reactions, which were 20 μl in volume. An Applied Biosystems 7500 Fast Real-Time thermal cycler was used. The amplification-melt program was: 95°C 10 min, 40 cycles of 95°C 15 sec., 45°C 30 sec., 60°C 20 sec., followed by 95°C 15 sec., 60°C 1 min., 95°C 15 sec., 60°C 15 sec. Analyses were performed using the ABI HRM software. To determine the level of detection within sharpshooter embryos, fresh egg masses of GWSS were subjected to parasitism by each wasp species. Eggs were dissected at 1 hr., 6 hr., 12 hr., 24 hr., 4 days, and 10 days after parasitism and after parasite eclosion. These samples are being extracted for genomic DNA and will be subjected to HRM analysis.

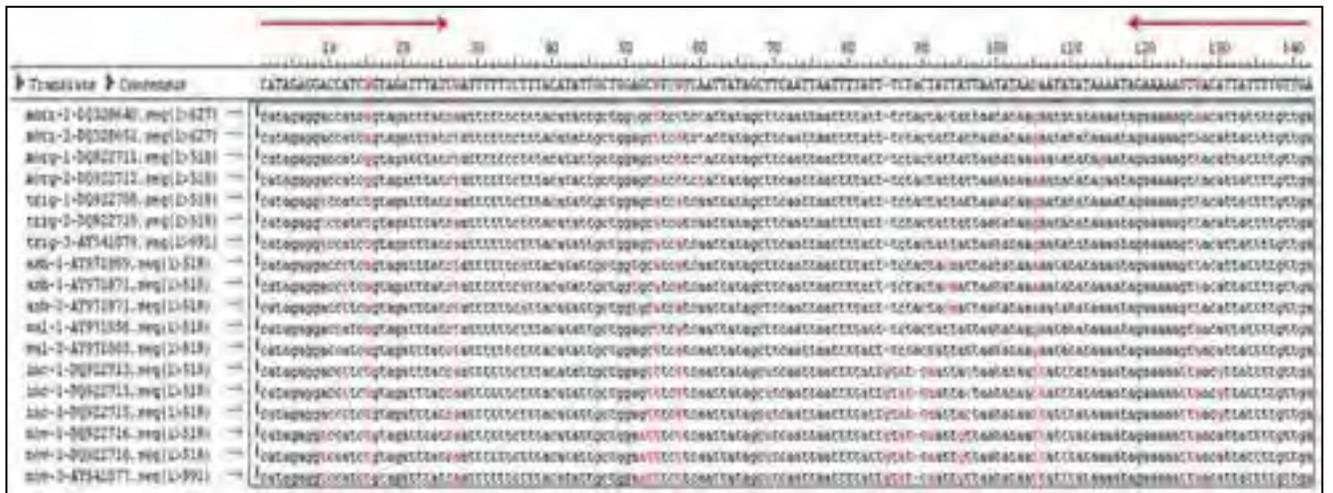


Figure 1. *Gonatocerus* wasp COI sequence alignments with HRM primers indicated by red arrows.

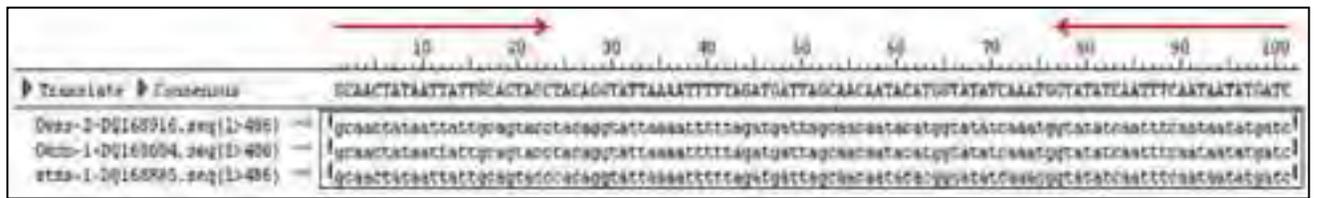


Figure 2. Sharpshooter COI sequence alignments with HRM primers indicated by red arrows.

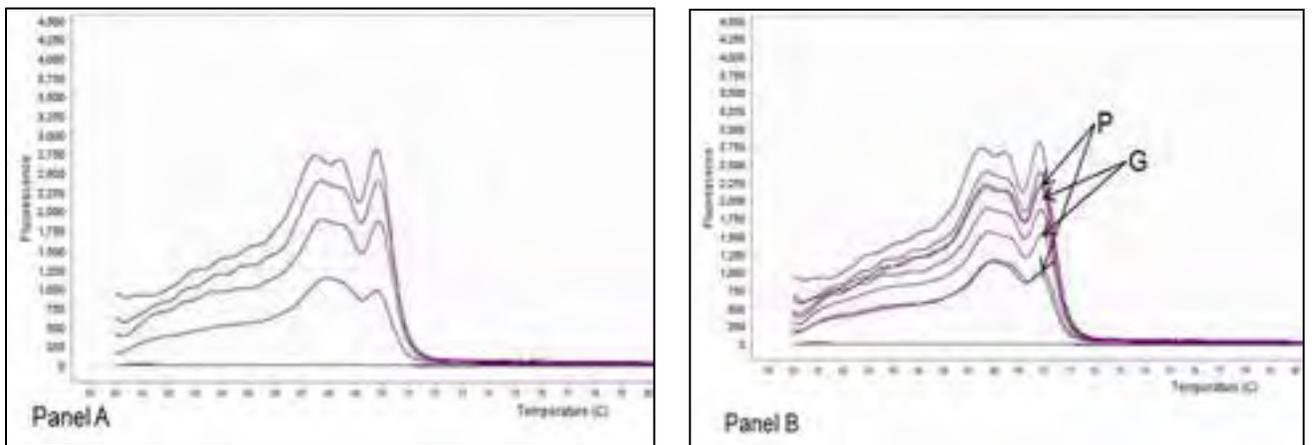


Figure 3. Panel A, melt curves for the four *G. ashmeadi* cloned plasmid standards. Panel B, melt curves for the standards and correctly identified plasmid (P) and genomic (G) samples.

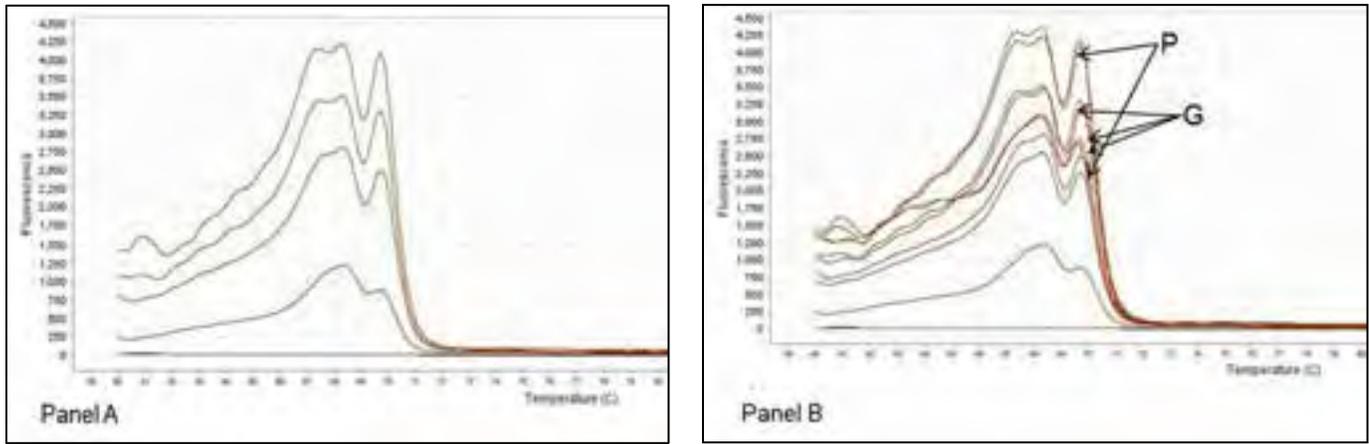


Figure 4. Panel A, melt curves for the four *G. morrilli* cloned plasmid standards. Panel B, melt curves for the standards and correctly identified plasmid (P) and genomic (G) samples.

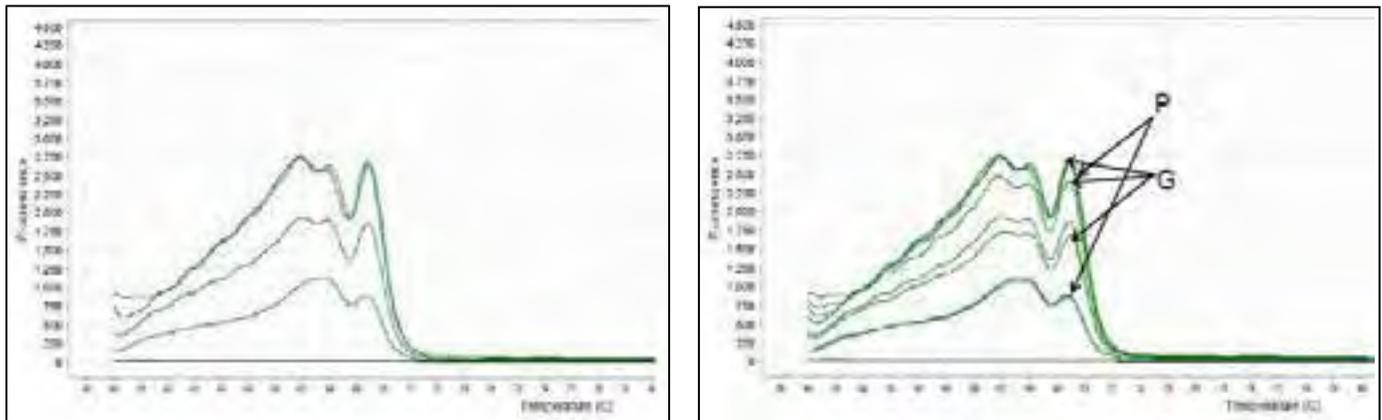


Figure 5. Panel A, melt curves for the four *G. morgani* cloned plasmid standards. Panel B, melt curves for the standards and correctly identified plasmid (P) and genomic (G) samples.

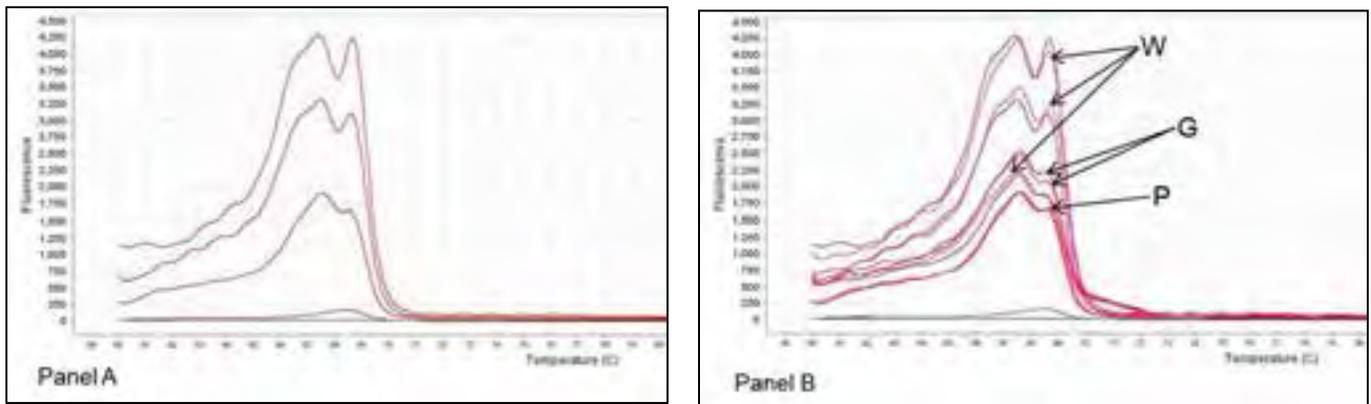


Figure 6. Panel A, melt curves for the four *G. novifasciatus* cloned plasmid standards. Panel B, melt curves for the standards and plasmid (P) and genomic (G) samples correctly identified and incorrectly identified as *G. walkeri* (W).

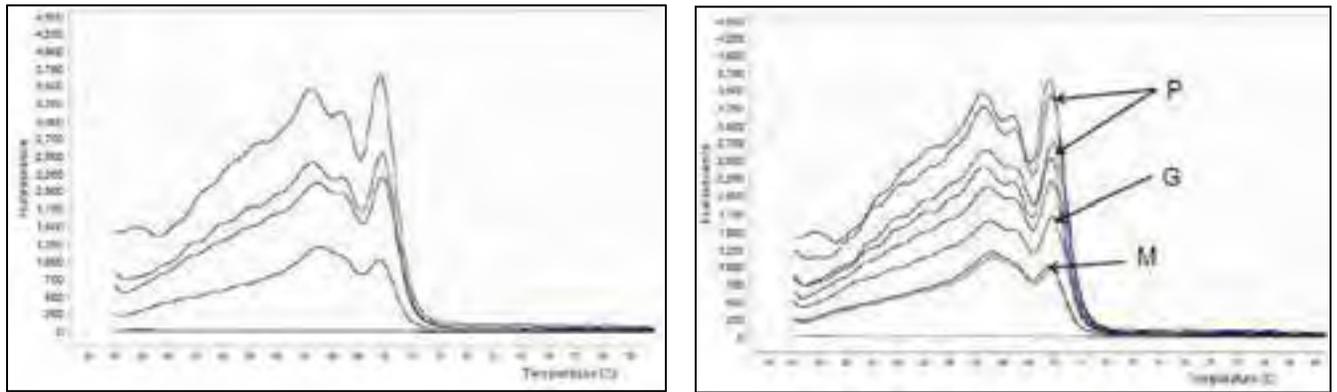


Figure 7. Panel A, melt curves for the four *G. triguttatus* cloned plasmid standards. Panel B, melt curves for the standards and correctly identified plasmid (P) and genomic (G) samples. One lowest concentration plasmid was incorrectly identified as *G. morrilli* (M).

CONCLUSIONS

Although more work is required to fully optimize the assay, we have been successful, with the exception of *G. novifasciatus*, in correctly identifying parasitoids in HRM analyses. We are in the process of developing additional primer pairs for the COI region as well as other targets which we are cloning using degenerate primers. We are hopeful that this assay will be able to identify the species of host, GWSS or STSS, as well. The use of the perfected assay will allow for the identification of the host and its parasitoids simultaneously within half a day of collection, rather than two weeks. In addition, since the pupal and sharpshooter egg casing can be analyzed, old egg masses should be able to be used after wasp eclosion. We are confident that better suppression of GWSS will be achieved by the refinement of control strategies. Determining the effectiveness of the different parasitoid species in the various environments encompassed in the current range of GWSS will facilitate this process.

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THE BENEFITS AND COSTS OF ALTERNATIVE POLICIES FOR THE MANAGEMENT OF PIERCE'S DISEASE

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ABSTRACT

We address economic questions related to Pierce's disease (PD) by developing and applying a quantitative model of the supply and demand for California wine and wine grapes. The economic component of this model combines existing wine market models with some entirely new elements. The biophysical component draws on a range of technical information and, where appropriate, evidence from crop and disease simulation modeling. We have consulted with scientists who study the disease and its vectors. Drawing on their advice, combined with information gleaned from interviews with vineyard managers, we have modeled the problem of the blue green sharpshooter in Northern California. By doing so, we have gained a better understanding of how to model the statewide PD problem. During the past year, we have shifted our emphasis to the role of the glassy-winged sharpshooter and the problems it poses in the southern regions of the state, and incorporated both vectors into a model that is designed specifically to evaluate the likely expected benefits from investments in alternative R&D projects related to the management of PD.

LAYPERSON SUMMARY

Our initial learning and progress led us to revise some aspects of the research strategy, but the work has gone generally according to plan, albeit after a delayed start. In the first two years of the project we concentrated on gathering data and other information, learning about Pierce's disease and the sharpshooters that spread it, and building vineyard-level models. We began by creating a vineyard-level model of the economic impacts of the disease in the Napa Valley. More recently we have worked to extend that model so that it can reflect the varying situation across the state and to incorporate the role of the glassy-winged sharpshooter. In the past year, in particular, we have worked to combine elements from the vineyard-level model with other elements to develop a three-region, statewide simulation model that can be used to evaluate the benefits from investments in alternative R&D projects.

INTRODUCTION

It is widely accepted that Pierce's disease (PD), with its vectors, including the glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*), has large current and very large potential economic consequences. However, we are not aware of other studies that have modeled and measured the economic consequences. Siebert (2001) discussed the economics and he estimated that the value of lost wine grape production—in Temecula, Riverside County, alone, in 1988 and 1989—was worth \$37.9 million to California. Echoing that sense of economic importance, the National Research Council of the National Academies (2004) undertook an extensive study and published a book on California research priorities focused on PD. That book does not contain estimates of the economic consequences of PD nor alternative management or control methods, but it does provide a comprehensive documentation of knowledge about the problem, as well as a useful classification of types of research, and priorities among them, including economic research. Further work is needed to develop a quantitative economic understanding of PD and alternative policies to address it.

OBJECTIVES

The overall objective of this project is to develop a detailed, practical, quantitative understanding of the economic consequences of PD and alternative management strategies. More specific objectives are to quantify the current and potential economic impact of the disease, to estimate the potential economic payoff to investments in PD R&D, to evaluate alternative management strategies including alternative research investments, and to guide policy decisions, including research priorities. Additionally, we aim to study the economic aspects of regional differences across California in PD and its associated vectors, allowing for a better economic understanding of the problem and a more precise evaluation of alternative management regimes. To pursue these objectives, we propose to develop an economic model of the California wine and wine-grape sector. The model will be structured to allow us to simulate market outcomes under alternative scenarios for the prevalence of PD, and alternative technologies and policies for its management, and to assess the economic consequences of these alternatives for various stakeholder groups. The model will be designed specifically with a view to evaluating the likely expected benefits from investments in alternative R&D projects related to the management of PD.

RESULTS AND DISCUSSION

Our project commenced formally on September 1, 2008. The project was originally due to end on June 30, 2011. In May 2011 we received approval for a 12-month no-cost extension to the project to allow us to complete the work and make best use of the expertise of the team to draw useful insights and publish (and publicize) the results. We are presently about six months behind the original schedule, reflecting the combination of a delayed start (while we waited for funding to be approved), a slow beginning (as we learned that we had to revise our modeling strategy), and some further delays as we sought to identify suitable assistance in view of the revised strategy. More recently, we have been making excellent progress having established a revised strategy and put together a very good team appropriate to that strategy, and being able to draw upon the necessary investment in learning about the issues. Kate Fuller has been employed as a Graduate Student Researcher to work on the project since the outset, and Kabir Tumber, Jonathan Kaplan, and Jim Sanchirico have been actively involved, especially during the current year. We have benefited on a continuing basis from advice from our co-operators and other experts, both in informal settings and in a half-day modelling workshop we held at Davis, in July 2011, including the project team members and other economists.

In the initial phases of work we emphasized investment in developing our own knowledge and information resources and creating preliminary models. One important element of this was to develop a detailed database on the economics of wine and wine grape production in California. We have completed the data-gathering phase, and are compiling the information into a report documenting by county and crush district for each important grape variety the area planted, yield, quantity produced (crush volume), and price over the past 30 years. We also made some investment in learning how to structure and use models of spatial-dynamic processes such as the spread of disease, through consulting with scientists and others and reviewing literature. As a result we have made significant progress in developing an understanding of the pest and disease problem, as well as an overview of the issues.

Having learned that the PD/GWSS problem would be difficult to model, we opted to focus initially on studying the issues as they arise in the north coastal valleys, where PD is spread by native sharpshooters (in particular, the blue green sharpshooter). To guide our efforts to understand the issue in that area, we conducted interviews with vineyard managers, utilizing a process known as “participatory mapping,” in which managers were asked to sketch out PD incidence, controls, and associated costs onto aerial images of their vineyards. This approach enabled us to develop some economic data and insight into the problem, management strategies, and costs of prevention, control, and eradication strategies, which has helped us in designing approaches to study the more general problem, including the role of the GWSS. In December 2010, Kate Fuller traveled to Temecula to conduct a series of interviews similar to those conducted in Napa. These interviews with growers, buttressed with discussions with scientists and others, were helpful in understanding the problem and have been useful in designing and parameterizing models. We have recently completed the development of a dynamic simulation model of the production and pricing of winegrapes in California. This integrated three-region supply and demand model can be used to simulate 50-year forward projections of the production and prices of California winegrapes, by region, under various scenarios of pest and disease prevalence and policy, and the associated pattern of benefits and costs to growers and others. We expect to have a range of initial simulation results in hand for presentation at the annual workshop. We plan to spend the first quarter of 2012 conducting further simulations and analysis, in light of things learned from the workshop among other things, and to spend the second quarter of 2012 writing up the results and wrapping up the project.

Based on the work to date, Kate Fuller has written two research essays (as required by the Ph.D. program in Agricultural and Resource Economics at UC Davis). These essays entailed a review of relevant literature and the development of the framework for a general economic model of vector-borne disease as applicable to PD. As well as providing a useful reference document for our project, they formed the basis for Kate’s oral qualifying examination and dissertation prospectus. Kate’s dissertation, which will be completed within the next six months, entails elements related to the main objectives of the project. We have drafted papers on the modeling of PD/GWSS in the Napa Valley and the costs of PD in California, which we expect to have submitted for publication before the end of 2011.

CONCLUSIONS

None to date.

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RIVERSIDE COUNTY GLASSY-WINGED SHARPSHOOTER PROGRAM IN THE TEMECULA VALLEY

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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 (*Homalodisca vitripennis*; GWSS). 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 are being made. As part of this treatment program there is ongoing monitoring of GWSS populations to ensure that the treatments are effective. In 2012 approximately 140 yellow sticky traps were inspected on a biweekly basis to monitor GWSS in citrus. The results for this year show a typical phenology for this pest in the region, with a total of approximately 600 GWSS caught in the 7 censuses since July. This overall GWSS catch was intermediate compared to previous years, far less than some (e.g. 2008, 2009), but more than the previous two seasons.

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 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/Pierce's disease management program will keep the vineyards viable in Temecula. At present there are no apparent biological or climatological factors that will limit the spread of GWSS or Pierce's disease. 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 bacterium.

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 in 1999-2000. Because of the phenology of GWSS, the summer citrus culture, and the peculiarities of the uptake of the systemic imidacloprid (AdmirePro) 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. The effectiveness of these applications will be evaluated throughout 2012. Previous work indicates that applications of Admire on citrus are not 100% efficacious; improper application of Admire will limit uptake by citrus trees; sick or weak trees will not take up Admire properly, therefore the systemic insecticide will not reach the target site; GWSS populations tend to be clumped, high numbers were found on weak trees; GWSS hotspots in citrus can be identified and only troublesome spots need to be treated; and GWSS populations have been dramatically reduced in Temecula valley since 2000, but still are a threat to vineyards. PyGanic treatments require multiple applications during the season, and provide little control of GWSS.

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, PCAs, and vineyard managers on the need for supplementary vector control measures within vineyards.

OBJECTIVES

1. Regularly monitor GWSS populations in citrus groves throughout the Temecula Valley to determine the need for and effectiveness of area-wide treatments.
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 geo-referenced with a handheld GPS monitor. Most yellow sticky cards are placed at the edge of the groves at the rate of approximately 1 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 will be disseminated to stakeholders via e-mail and on a blog hosted by UC Riverside's Center for Invasive Species Research (<http://civr.ucr.edu/temeculagwss/>).

RESULTS AND DISCUSSION

The results for 2012 are shown in **Figure 1**. This includes weekly censuses of GWSS in citrus up through June, then biweekly censuses from July through September. The 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. In 2012 there was a notable peak that occurred in September, and occasionally occurs in other years, presumably because of the unusually warm weather during that period this year.

Figure 2 shows the catch from this year put in perspective relative to other years. 2012 shows qualitatively the same seasonal phenology as in other years, with a moderate overall catch compared to others (i.e. 2008).

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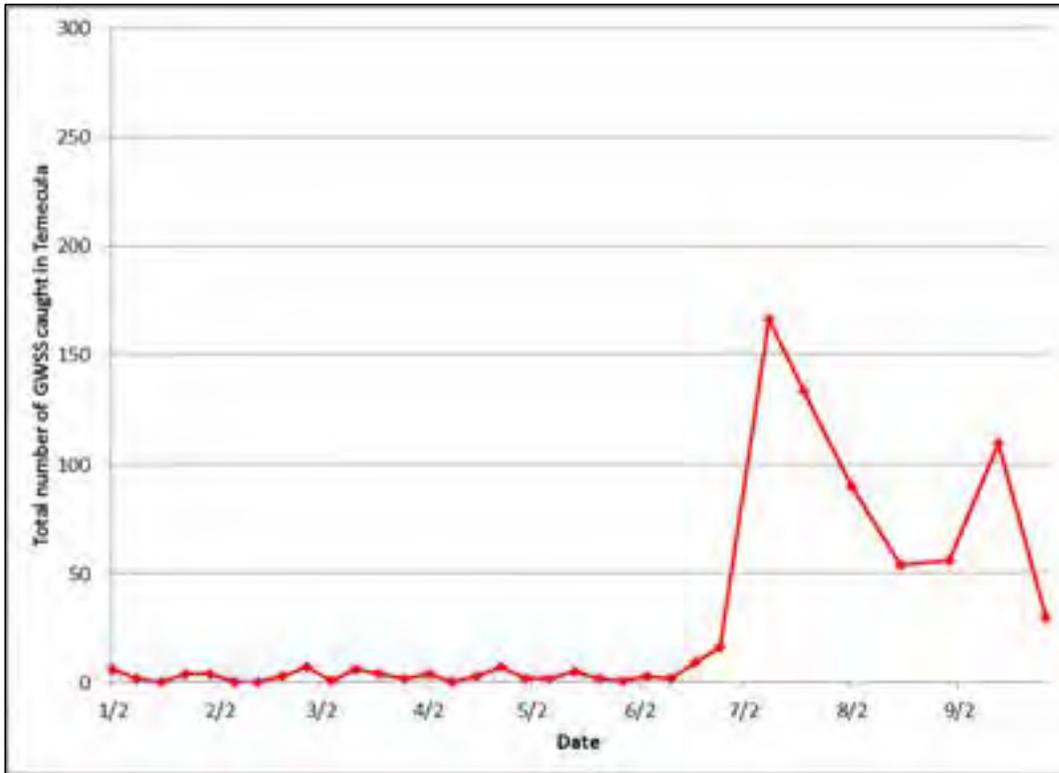


Figure 1. Seasonal total GWSS catch for 140 traps throughout Temecula Valley.

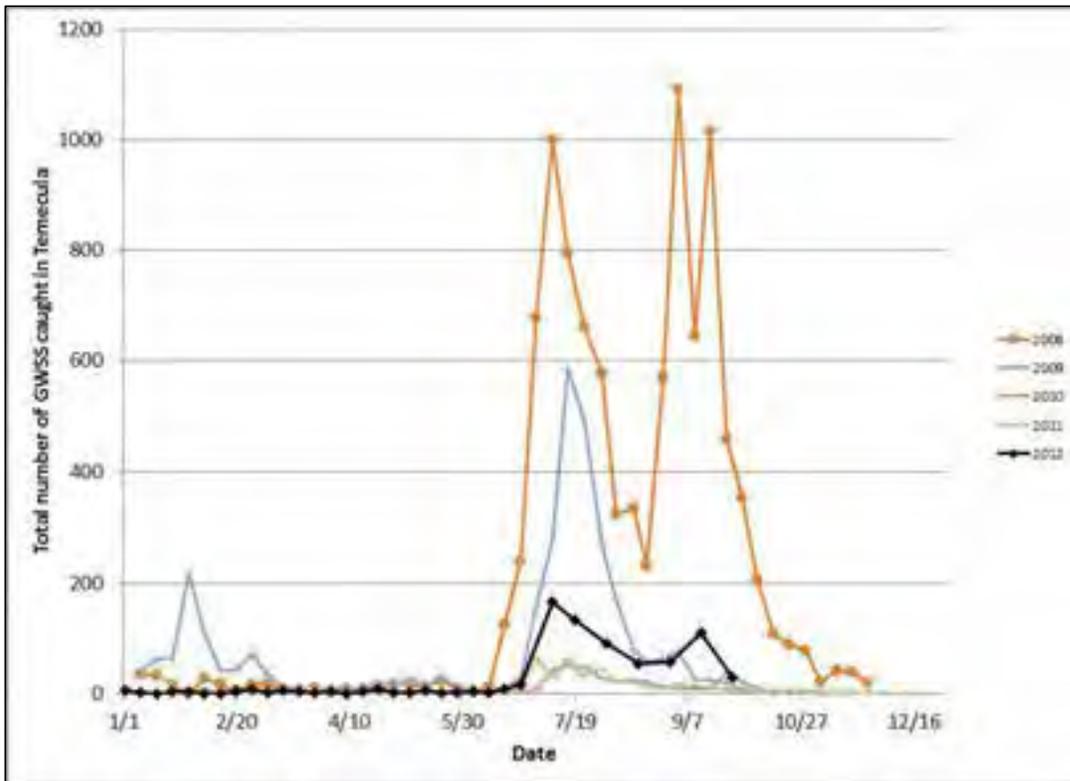


Figure 2. Seasonal total GWSS catch in Temecula Valley from 2008-2012.

LINKING WITHIN-VINEYARD SHARPSHOOTER CONTROL TO PIERCE'S DISEASE SPREAD

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ABSTRACT

Pierce's disease management in southern California vineyards hinges on chemical control of populations of the vector, the invasive glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS), residing in citrus. Growers also frequently apply systemic insecticides in vineyards, but the efficacy of these treatments for disease management is not known. We are conducting a series of surveys in treated and untreated vineyards in Temecula Valley to determine the relative economic value of within-vineyard chemical control for Pierce's disease management. In each of the past three seasons we have surveyed 34 vineyards in the Temecula Valley that differ in their use of systemic insecticides. As in previous years, in 2012 overall Pierce's disease prevalence was low; average 1.3% based on visual symptoms. Prevalence differed slightly among fields of different treatment categories with the lowest infection rates in those vineyards that were either consistently or irregularly treated with imidacloprid. Based on sticky trap monitoring, consistently or intermittently treated vineyards also had lower catches of sharpshooters than untreated fields, but natural enemy catch did not differ among the three treatment categories. Finally, tap sampling results showed slightly lower natural enemy abundance in treated sites, but the abundance of non-predatory arthropods was also substantially lower in the treated sites. Collectively, these results suggest that imidacloprid treatments may reduce slightly disease spread, at least in part due to reductions in vector pressure, but without any clear non-target effects on natural enemies that may lead to secondary pest outbreaks.

LAYPERSON SUMMARY

One of the main tools for dealing with the glassy-winged sharpshooter (GWSS) in southern California and the southern San Joaquin vineyards is the application of insecticides. Systemic insecticides (imidacloprid) are regularly applied to citrus, which is a preferred plant type for sharpshooter, to reduce insect abundance before they move into vineyards. These treatment programs have been successful, reducing sharpshooter populations to a fraction of what they once were. Grape growers frequently use systemic insecticides in vineyards as well to reduce further the threat of sharpshooters spreading Pierce's disease among vines. However, no measurements have been made about whether these costly insecticide treatments are effective at curbing disease spread. We have been conducting a series of disease and arthropod surveys in Temecula Valley to understand whether chemical control of GWSS in vineyards is justified. Results indicate that on average vineyards that employ

systemic insecticides tend to have slightly lower Pierce's disease prevalence. Yet, given that some of the untreated or intermittently treated fields had extremely low prevalence, it is plausible that systemic insecticides may not need to be applied every year – at least for sites or years with low vector pressure. We also see no evidence that these systemic insecticide applications are disrupting biological control of other grapevine pests.

INTRODUCTION

Chemical control of insect vectors plays a crucial role in many disease mitigation programs. This is true not only for the management of mosquito-borne diseases of humans, such as malaria and dengue fever, but also for limiting disease epidemics in a wide range of agricultural crops. In southern California vineyards chemical control at both the area-wide and local scales may affect the severity of Pierce's disease, by reducing the density or activity of the primary vector, the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) (Castle et al. 2005).

The bacterial pathogen *Xylella fastidiosa* (*Xf*) is endemic to the Americas, and is widespread throughout the western and southeastern USA. This xylem-limited bacterium is pathogenic to a wide variety of plants, including several important crop, native, ornamental, and weedy species (Purcell 1997). In the western USA the most economically significant host is grapevine, in which *Xf* causes Pierce's disease. Multiplication of the bacterium in vines plugs xylem vessels, which precipitates leaf scorch symptoms and typically kills susceptible vines within a few years (Purcell 1997).

Xf can be spread by several species of xylem sap-feeding insects, the most important being the sharpshooter leafhoppers (Severin 1949). Historically Pierce's disease prevalence has been moderate, with a pattern that is consistent with primary spread into vineyards from adjacent riparian habitats by the native blue-green sharpshooter (*Graphocephala atropunctata*). However, beginning in the late 1990s severe outbreaks occurred in southern California and the southern San Joaquin Valley that are attributable to the recent establishment of GWSS. This invasive sharpshooter is not inherently more efficient at transmitting the pathogen than are native sharpshooters (Almeida and Purcell 2003). Instead its threat as a vector appears to stem from a combination of ability to achieve extremely high densities (Blua et al. 1999) and promote vine-to-vine (i.e. secondary) disease spread (Almeida et al. 2005).

Citrus trees themselves are not susceptible to the strains of *Xf* found in the USA (though strains found in Brazil have caused significant economic losses to their citrus industry – Purcell 1997). Nonetheless, citrus plantings figure prominently in the epidemiology of *Xylella* diseases in California. Many portions of southern California and the southern San Joaquin Valley have vineyards in close proximity to citrus groves (Sisterson et al. 2008). This is important because citrus is a preferred habitat for GWSS at key times of the year, allowing this vector to achieve very high densities (Blua et al. 2001). High vector populations then disperse seasonally out of citrus into nearby vineyards, resulting in clear gradients of Pierce's disease prevalence (i.e. proportion of infected plants) as a function of proximity to citrus (Perring et al. 2001).

Given the importance of citrus in Pierce's disease epidemiology, citrus groves have been the focus of area-wide chemical control programs, initiated in the Temecula and Coachella Valley's in the early 2000s and shortly afterward in Kern and Tulare counties (Sisterson et al. 2008). The southern California programs use targeted application of systemic insecticides, such as imidacloprid, to limit GWSS populations residing within citrus. Census data in citrus show substantial year-to-year variation in sharpshooter abundance that may stem from incomplete application, the use of less effective organically-derived insecticides, or inadequate irrigation to facilitate uptake, which makes the consistent management of sharpshooter populations a challenge (Toscano and Gispert 2009). Nonetheless, trap counts have been much reduced compared to pre-area-wide counts. The effect of chemical control is clear in early insect surveys which found significantly fewer sharpshooters in treated relative to untreated citrus and in vineyards bordering treated versus untreated groves (R. Redak and N. Toscano, unpublished data). Thus, these area-wide control programs have been considered successful in southern California (Toscano and Gispert 2009), and the swift implementation of an area-wide management program in Kern County has been credited with limiting Pierce's disease outbreaks (Sisterson et al. 2008).

Research into imidacloprid uptake by grape also has been initiated, and target concentrations high enough to suppress GWSS activity (approximately 10 µg/L of xylem sap) can be achieved and will endure for several weeks in mature vines (Byrne and Toscano 2006). This information coupled with the success of area-wide programs in citrus appears to have led to relatively widespread adoption by grape growers of imidacloprid application in vineyards to reduce further exposure to *Xf*. In the Temecula Valley, for example, it is estimated that 70% of vineyards use imidacloprid, at an approximate cost of \$150-200 per acre (N. Toscano, personal communication). Yet consistent treatment of vineyards with systemic insecticides is neither universal, nor have there been any measures of how effective these costly treatments are at reducing Pierce's disease.

We are studying the epidemiological significance of chemical control in vineyards, via a multi-year series of field surveys in the Temecula Valley. This work will address gaps in empirically-derived observations regarding the cascading effects of vineyard imidacloprid applications on GWSS abundance and, ultimately, Pierce's disease severity.

OBJECTIVES

The overall goal of this project is to understand **does within-vineyard sharpshooter chemical control reduce vector pressure and Pierce's disease spread?** As part of this overall objective we have been evaluating the following set of research questions:

1. Do vineyards from different treatment categories (untreated, intermittent treatments, or consistently treated) differ in insecticide concentration?
2. Do imidacloprid applications reduce vector abundance or activity in vineyards?
3. Do treatments reduce disease spread in vineyards?
4. Are treatments disrupting biological control and contributing to secondary pest outbreaks?

We are currently in the middle of the final season of three fall disease surveys. Thus, the results presented below should be viewed as preliminary for the time being.

To verify insecticide use, in late summer 2012 we collected leaf samples from 10 vines at each site. For each site two vines were sampled in five different rows spread throughout the vineyard block. For each vine sampled we collected two healthy, fully expanded leaves from mid-cane. These samples were then subjected to an ELISA analysis to calculate imidacloprid concentration, using a slight modification to established methods (Castle et al. 2005, Byrne and Toscano 2006). Briefly, from each leaf we used a #6 cork borer to punch a disc of leaf tissue, weighed that disc, and then ground it in 1% methanol. After incubation dilutions were made and this material was added to Envirologix Imidacloprid Quantiplate Kit.

In 2012, we continued to survey Pierce's disease prevalence, imidacloprid concentrations *in planta*, and arthropod densities in the 34 sites we had identified and surveyed in previous years. To survey disease prevalence, we conducted visual surveys of vine symptoms in the fall for all vines in each site. As in previous years these visual surveys will be adjusted for false positives using plate culturing of bacteria and for false negatives using ELISA. For false positive testing, we collected petioles from 50 randomly selected vines that were visually categorized as symptomatic for Pierce's disease. We then plate cultured isolates from these petioles for detection of *Xf*. For false negative testing, we collected petioles from 100 randomly selected asymptomatic vines. We then conducted ELISA tests for the presence of *Xf*. We estimated a false positive and a false negative rate and used these to adjust the disease prevalence based on the visual surveys.

We surveyed population densities of GWSS, the native smoke-tree sharpshooter (*Homalodisca liturata*; STSS), and generalist natural enemies. We placed 4-8 yellow sticky traps at 0.5-1 m above the vines at each site. The number of traps depended on the area of vines planted. Traps were replaced monthly. For each trap, we counted the number of GWSS, STSS, and four groups of generalist predators: minute pirate bugs (*Orius* spp.), assassin bugs (Reduviidae), big-eyed bugs (*Geocoris* spp.), and spiders (Aranea). These four groups appear to be the most important generalist predators of GWSS (Fournier et al. 2008). The total seasonal density of *Homalodisca* spp. and predators was calculated for each site between February and September.

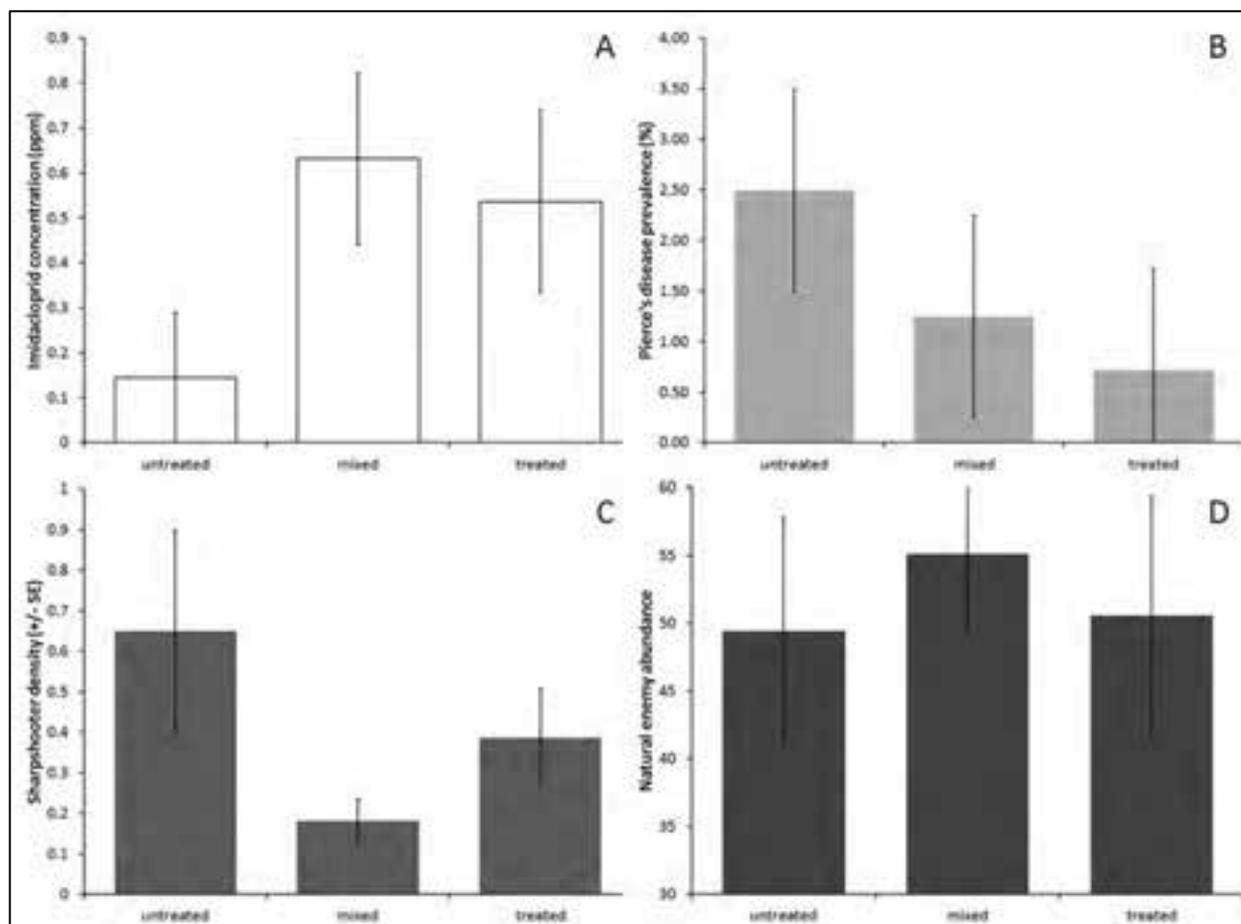


Figure 1. A) Mean imidacloprid concentration, B) mean estimated Pierce's disease prevalence, C) mean sharpshooter catch on sticky traps, and D) mean natural enemy catch on sticky traps among 34 Temecula vineyards based on treatment category: untreated, intermittently treated (i.e. "mixed") or consistently treated with imidacloprid.

In addition to sticky traps, over the summer of 2012, between July 10th and September 6th we conducted tap sampling from each of the sites to estimate the abundance of generalist predators and pest insects. At each site, six rows approximately evenly spaced throughout the vineyard block were chosen for sampling, avoiding edge rows. Within each row, five vines approximately spaced throughout the row were "tap-sampled," avoiding vines at the end of rows, with no neighbors, or which were obviously diseased or dying. Tap sampling was conducted by tapping the vine 40 times to dislodge arthropods from the upper canes, foliage, and branches into a shallow beat net. Arthropods collected within a single row were pooled and aspirated into plastic vials and placed on ice during transport. Upon return to the lab, vials were partially filled with 70% ethanol and placed in the freezer until identification.

RESULTS AND DISCUSSION

Our estimates of imidacloprid concentrations *in planta* match the classes for management practices. All but one of the untreated sites had no detectable imidacloprid in leaf tissue samples. Conversely, both the regularly treated and those treated intermittently (i.e. "mixed" treatment history) had markedly higher insecticide concentrations (**Figure 1A**). These results support the classification of most sites into the three treatment categories.

Estimates of disease prevalence were generally quite low, with a mean prevalence based on visual symptoms alone of 1.3%. Prevalence varied substantially, with patterns that correspond roughly with imidacloprid treatment class (**Figure 1B**). Disease prevalence tended to be higher in untreated sites, lowest in the

consistently treated sites, and intermediate in mixed sites. These results suggest at least a trend towards lower disease prevalence in sites that are treated with imidacloprid. Thus, within-vineyard imidacloprid applications may lead to modest reductions in *Xylella* spread among vines.

We collected more than 75 sharpshooters, both GWSS and STSS, among all sites between November 2011 and May 2012. Untreated sites had the highest sharpshooter catches, consistently treated sites were intermediate, and mixed sites had the lowest catches over this period (**Figure 1C**). These results support the idea that insecticide application reduces vector pressure, through mortality or by having anti-feedant effects, which likely explains the patterns seen in disease prevalence among the treatment categories.

Based on sticky trap catches there were little differences among the treatment categories with respect to natural enemy abundance in vineyards. We found similarly high catches of several natural enemy taxa, especially spiders, at all three types of sites (**Figure 1D**). Tap sampling results showed a slightly different pattern. Generalist natural enemies were more common in untreated sites than mixed or consistently treated sites (**Figure 2A**). However, this result does not appear to stem from disruption of natural enemy activity at treated sites. Rather, the abundance of all non-predatory arthropods (including several pest species such as grape leafhopper) were up to seven-fold higher on average at untreated sites compared to treated sites (**Figure 2B**). Collectively these two sources of data indicate that imidacloprid applications do not dramatically upset natural enemy activity in vineyards. Rather, if anything, natural enemy populations may track pest populations, which are strongly affected by whether fields were treated with imidacloprid.

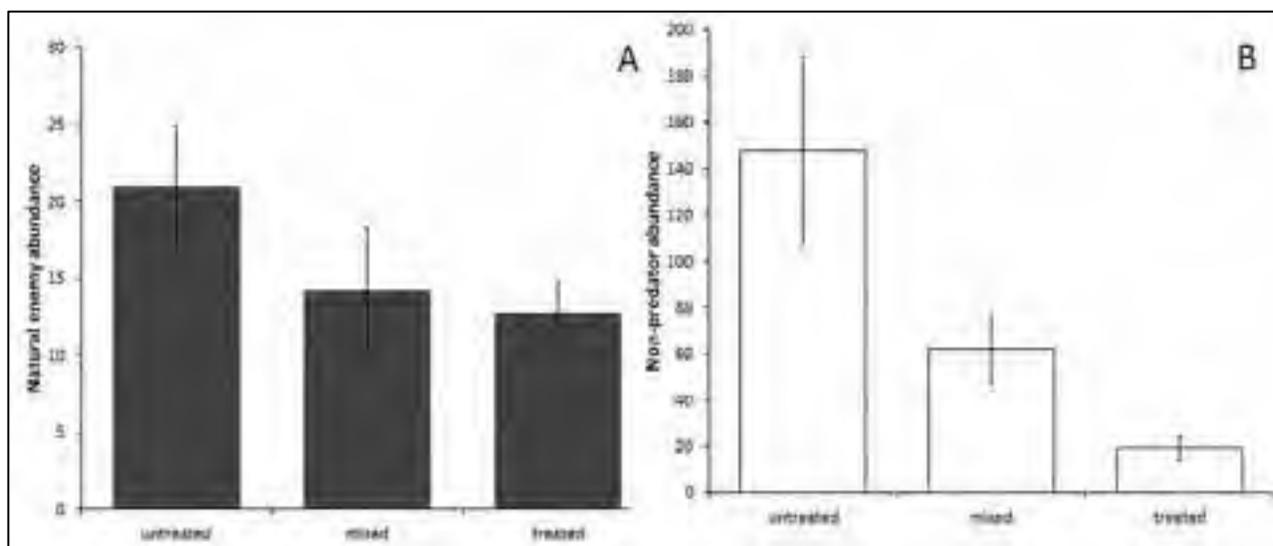


Figure 2. A) Mean natural enemy abundance, and B) mean non-predatory arthropod abundance (+/- SE) based on tap sampling in Temecula Valley vineyards in 2012.

CONCLUSIONS

Results so far suggest that current Pierce's disease prevalence throughout the Temecula Valley region is low. There is a trend for differences in prevalence based on chemical control strategies, with untreated vineyards having the highest average prevalence, but whether those differences are due to recent management, historical artifacts, or differences in vector pressure remains unclear. Ultimately estimates of year-to-year changes in prevalence are needed (i.e. incidence), which are ongoing, to determine the precise impact of within-vineyard systemic insecticides on disease spread. It is worth noting that such modest levels of disease are likely attributable to very low vector populations that exist currently relative to conditions during the peak of the GWSS outbreak in the region. Thus the apparently slight differences in disease incidence among treatment categories might be expected to be substantially greater should the effective area-wide control of sharpshooters be discontinued.

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FUNDING AGENCIES

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

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

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Reporting Period: The results reported here are from work conducted July 2011 to October 2012.

ABSTRACT

We have made significant progress during this past one year and are in excellent position to complete most of our objectives during the upcoming year. We have published two refereed journal articles (1) and (2), and have presented four Pierce's Disease Research Symposium reports (Falk et al., 2010), (Rosa et al., 2010), (Falk et al., 2011) and (Nandety et al., 2011) during the entire scope of this project. In addition we presented our work at various national and international meetings during this past year and half. RNA interference applications are at the forefront for new strategies aimed at controlling insect pests and vectors, and our work here is very timely. We show good progress towards the development and application of an RNA interference (RNAi) based system aimed to target genes of the vector of *Xylella fastidiosa* (*Xf*), the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS). After demonstrating that RNAi induction in GWSS cells and insects is achievable, we began screening a large pool of candidate genes by homology search to find the best targets to control the survival of GWSS. These targets were used to develop transgenic *Arabidopsis* and potato plants that express dsRNAs for the insect targets. We also made stable *Arabidopsis* transgenic plants that express glucuronidase (GUS) marker genes using the constitutive 35S and a *Eucalyptus gunii* minimal xylem-specific promoter. While we were able to show the expression of GUS gene *in vivo* in the T2 transgenic plants, other transgenic plants are being evaluated for their ability to produce dsRNAs and will be tested against GWSS adult insects. Recently, we were able to generate transgenic potato plants expressing the GUS gene under the control of a xylem specific promoter, E-cadherin (ECAD) and are in the process of testing them. Encouraged by our efforts to find effective targets, we have adopted large scale sequencing of the GWSS transcriptome as well as the small RNA complement from GWSS adult insects. We were able to generate 65 million reads and 22 million reads of the short read sequence data for transcriptomic and small RNA sequences in our initial run. We discovered new information in our sequence data which we are planning to publish shortly.

LAYPERSON SUMMARY

Pierce's disease of grapevines is one of the plant diseases caused by the Gram-negative bacterium *Xylella fastidiosa* (*Xf*). This bacterium, upon inoculation in the plant host, travels within and attaches to the plant xylem vessels where, after multiplication, produces biofilms which interfere with the water-flow in the infected plants. Resulting infected plants can die between one and five years after inoculation. *Xf* is vectored by many leafhoppers, but one of the most important vectors in many areas is the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS). The importance of GWSS can be attributed to its ability to colonize more than 100 species of plants, its propensity for long distance dispersal, and its capacity for ingesting large volumes of fluids from colonized plants. We are taking a contemporary molecular targeting approach to disrupt normal gene expression in GWSS and other sharpshooter vectors of *Xf* as a strategy to help control these important insect vectors of *Xf*. We are attempting to develop RNA-interference (RNAi) as a tool to target and kill GWSS

and other sharpshooter vectors of *Xf*. We have generated transgenic plants expressing anti-GWSS dsRNAs corresponding to the identified GWSS mRNAs. We have evaluated these plants for molecular markers and are in the process now of conducting experiments to assess their effects on GWSS. Our hope is that ingestion of these dsRNA molecules by GWSS will trigger RNAi activity in the recipient insects, resulting in the subsequent degradation of the targeted mRNAs and corresponding debilitating effects on sharpshooters, thereby contributing to strategies for Pierce's disease control. We have also taken a new strategy, next generation sequence analysis, as a strategy to help us identify new targets for RNAi approaches. Together, we feel that these approaches are contemporary and will contribute to long term approaches for GWSS and Pierce's disease control.

INTRODUCTION

During the work supported by this research program, we developed tools to induce RNA interference (RNAi) in the insect vectors of Pierce's disease, and in particular in the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS). We were able to induce RNAi for specific genes *in vitro* in a GWSS cell line developed at the University of California, Davis (3, 4) and in whole insects (2). We also optimized protein, small and large RNA hybridization and real time PCR techniques to detect the extent of RNAi induced in the two systems. We finally were able to generate phenotypes in cells and GWSS 5th instars affected by RNAi. While our previous efforts were limited by the scarce amount of nucleotide sequences available in GenBank, our present research is based on the sequence analyses of extensive GWSS expressed sequence tag (EST) data released via GenBank. We cloned a series of genes expressed during insect digestion and during molting, and we inserted these genes in an easy to manipulate binary vector (**Figure 1**) set up for hairpin RNA transcription. We also modified this vector substituting the 35S promoter with a minimal xylem specific promoter cloned by us from *Eucalyptus gunii*. The potential of these series of vectors is great, in fact we are using these plasmids to generate transgenic plants of different species (*Arabidopsis*, grape, citrus, and potatoes) via the Ralph M. Parsons Foundation Plant Transformation facility of UC Davis and at the same time to generate in a fast and convenient way *Arabidopsis thaliana* plants via flower dipping.

OBJECTIVES

1. To assess the effectiveness of anti-GWSS transgenic plants against GWSS.
2. To identify optimal interfering RNA forms for use in transgenic plants.

RESULTS AND DISCUSSION

Objective 1: To assess the effectiveness of anti-GWSS transgenic plants against GWSS

We have generated a series of transgenic plants (potatoes and *A. thaliana*) for our RNAi studies so far (see **Table 1** for potatoes; we have 11 lines with more GWSS sequences for *A. thaliana*). We have characterized these plants to ensure that they contain the desired transgene(s) and for some, that they generate the desired siRNAs (**Figure 1**). They are engineered to generate dsRNAs (**Figure 2**), but as the dsRNAs are generated in plants, they are processed by the plant's RNAi machinery thereby yielding siRNAs. This is acceptable for our work here as siRNAs also induce RNAi activity in GWSS (1) and other insects (5).

Generation of transgenic lines

For the purpose of generating the *Arabidopsis* transgenic lines we used a different ecotype, Cape Verdi (Cvi). Compared to Columbia (Col-0) it has larger leaves and presents more robust growth, and will be more appropriate in supporting insects of large size such as GWSS. In order to generate dsRNAs that can target the insect, GWSS target sequences were cloned into a gateway-compatible binary vector pCB2004B (**Figure 1**). The target sequences were cloned in head to tail direction in the gateway vector with a non-homologous sequence between them. Upon transcription in transgenic plants, these constructs will yield double-stranded, hairpin RNAs of the desired sequence. The expression vectors carrying the insect target sequences of interest were first cloned into *E.coli* and *Agrobacterium tumefaciens* and they have been sequence verified. *A. tumefaciens* cultures carrying the sequences of interest were used to transform *A. thaliana* Cvi plant ecotypes through the floral dip process. *Arabidopsis* T₀ plants were screened for resistance against the selectable marker *BAR* gene, and we were able to confirm T₁ transgenics. Further sets of transformation of *Arabidopsis* plants are underway to generate more independent transgenic lines for the GWSS target genes that had less than three

independent transgenic lines. Also, efforts are underway to generate more transgenic lines for other target genes of GWSS that were not previously described. We are in the process of obtaining the homozygous transgenic *Arabidopsis* lines that will be used for screening against GWSS.

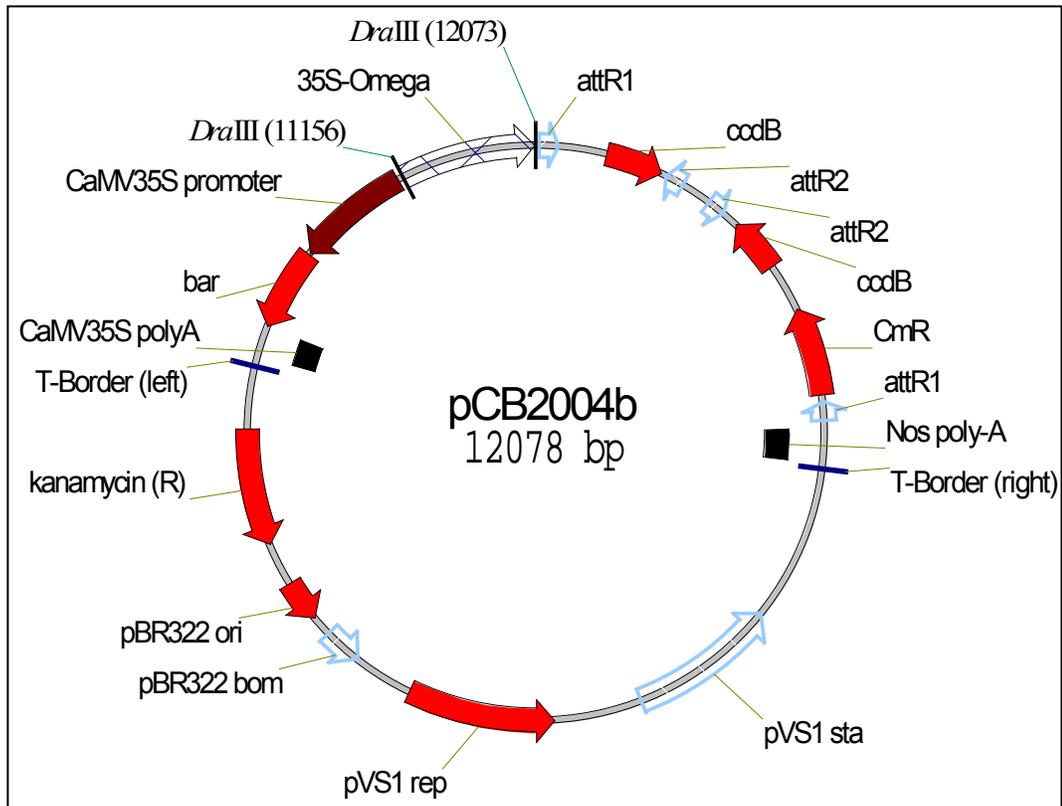


Figure 1. Diagrammatic representation of the vector pCB2004B used for generation of GWSS transgene constructs. The binary construct is designed to produce short hairpin between the sense and antisense target genes that will result in the production of small RNAs in the transgenic plants (*Arabidopsis*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

Table 1. Transgenic potato plants for GWSS-directed RNAi

GWSS Targets in Potato Plants	Pedigree	Potato Cultivar	Selectable Marker	Production of ds- and siRNAs
Chitin deacetylase	102203	Kennebec	BAR	Yes
Chitin deacetylase	102203	Desiree	BAR	In process
GWSS actin	112064	Desiree	BAR	Yes
GWSS cuticle	112073	Desiree	BAR	Yes

We have used three of the constructs (**Table 1**) to transform potato plants. Transformation/regeneration was performed via recharge at the UC Davis Ralph M. Parsons plant transformation facility (<http://ucdptf.ucdavis.edu/>) and approximately ten independent transgenic lines were obtained for each of the constructs. We have performed screening of these transgenic potato plants for insert composition and have established the presence of a transgene similar to the procedure as described for *Arabidopsis* transgenic lines. The presence of chitin deacetylase transgene in the potatoes resulted in the production of small RNAs in those transgenic plants. In contrast to the approach with *A. thaliana*, we are vegetatively propagating the T₀ potato plants and are in the process of testing the same for RNAi experiments with GWSS. Potatoes are an excellent host plant for GWSS so we expect them to be very useful for our efforts here. We have characterized these plants to ensure that they

contain the desired transgene(s) and for some, that they generate the desired siRNAs (e.g. see **Figure 2**). Finally, we have started generating transgenic potatoes where the transgene will be driven via the specific xylem promoter EgCAD2 which we previously cloned from *Eucalyptus gunii*.

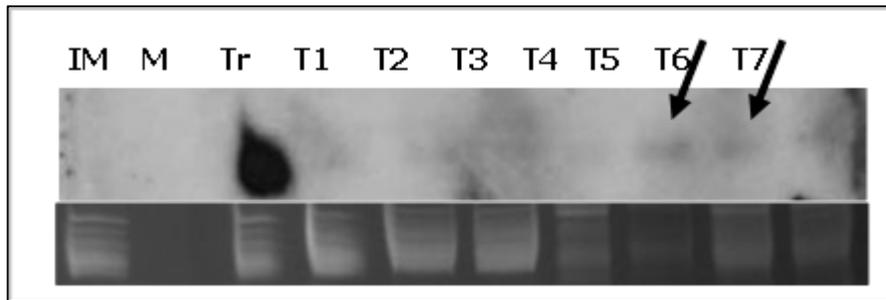


Figure 2. Small RNA northern hybridization analysis of transgenic potato plants. Arrows indicate positions of GWSS-specific actinsi RNAs.

Feeding assays for RNAi effects in GWSS

Transgenic potato feeding assays, using the plants listed in Table 1, have been carried out using 3rd-4th instar nymphs. Non-transgenic potatoes of the cultivars used for the transformation events were used as control plants. We set up cuttings of the potato plants in individual cages in a growth chamber, released five nymphs per cutting, and observed mortality for two weeks (**Figure 3**). We collected dead nymphs as they were observed and collected the remaining live nymphs at the end of the two weeks. RNA was extracted from all nymph samples and will be tested for differences in mRNA levels of target and non-target sequences using qRT-PCR. We are currently working to validate those assays using TaqMan® (Applied Biosystems, Carlsbad, CA) chemistry of quantitative real-time PCR.



Figure 3. GWSS RNAi feeding assays on transgenic potato cuttings. At left shows stems in cylindrical cages, each containing 5 nymphs times 5 replications per treatment. Right shows a close up photo of a GWSS nymph feeding on upper potato foliage.

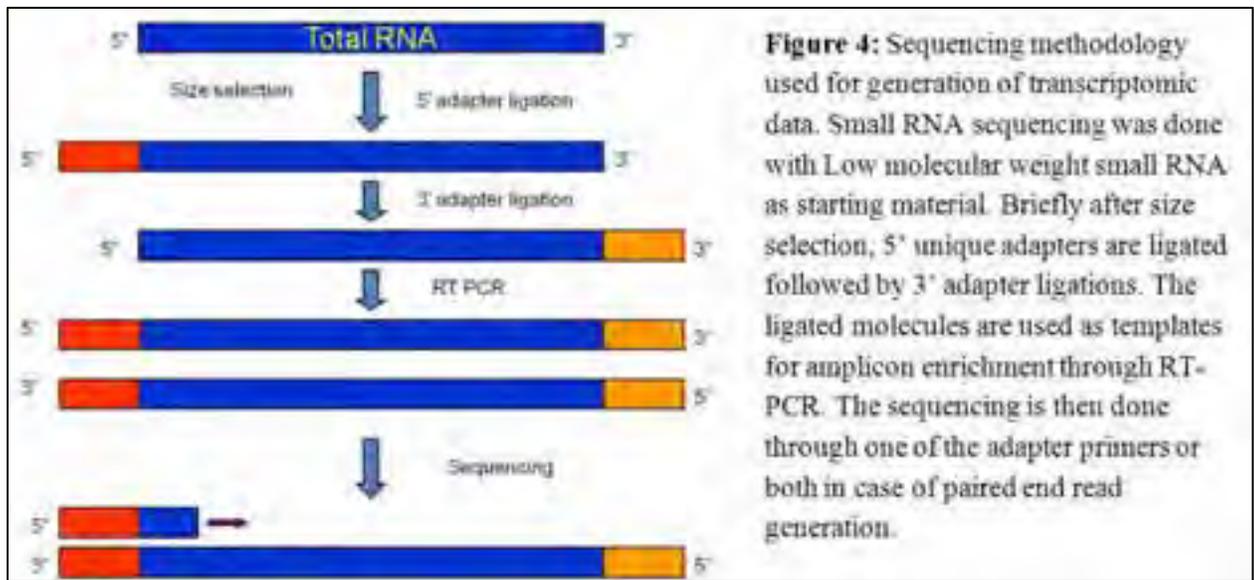
In addition to the transgenic plant approaches, based on recent reports in the literature and personal communications from other scientists, we have evaluated *in vitro* feeding approaches for GWSS. We have confirmed that basil stem infusion is a very efficient means to deliver specific effector RNAs to GWSS. We have used the basil infusion in the past and it offers some advantages as well as disadvantages. We have tested the GFP PCR product through this new method in comparison to the established basil feeding method and were able to detect the GFP PCR product in equal proportions inside the GWSS insects. We hope to rapidly screen target sequences without having to develop transgenic plants, thereby saving time and effort towards our ultimate goal.

Objective 2: To identify optimal interfering RNA forms for use in transgenic plants

We will engineer and express short, specific RNAi effectors in transgenic plants in order to compare their efficacy with more traditional, longer hairpin dsRNAs. The latter are more likely to give undesirable off-target effects and thus greater specificity is highly desirable. One way to gain some understanding of potential RNAi targets is to utilize next generation sequencing to identify the quality and quantity of specific small RNAs in GWSS. Therefore, we took this approach while simultaneously engineering new generation RNAi effectors.

Next generation sequencing of GWSS adult insects

The developmental regulation of insects through the use of small RNAs has been well studied. In our efforts to study the regulation of GWSS insect genes and identify RNAi targets, we took an alternate approach using high throughput parallel sequencing to identify the small RNAs from the GWSS insects. For our work, we noticed GWSS transcriptome data is lacking information for the identification of small RNA reads. To address this and identify the loci of the small RNAs that were originated from the short read sequencing, we sequenced the transcriptome of GWSS through the use of mRNA sequence methods as described in **Figure 4**. The sequencing of GWSS mRNA transcriptome was done through paired end sequencing on Illumina GA-II Platform. Both the mRNAseq library data and the small RNAseq library data were generated from the GWSS adult insects.



The sequencing reads from the transcriptomic data were assembled into scaffolds with a minimum size of 200 bases using Oases transcriptome assembler. We were able to assemble approximately 32.9Mb of the transcriptome across 47,265 loci and 52,708 transcripts. The average transcript length assembled was 624 nucleotides. Roughly 15 million of the total reads were found to be unique for the genome and 51% of the reads were incorporated into the assembly. The sequencing reads were then mapped back to the assembled transcripts with up to one mismatch. The reads that could not be mapped back to the reference assembly are being

analyzed for the possible discovery of new viruses that may be infecting the GWSS insects. With the help of these sequencing reads, we aim to study the GWSS insect target genes and we hope to identify the small RNAs that target the GWSS target genes in a highly specific manner.

CONCLUSIONS

We have generated transgenic experimental effects to now assess RNAi effects on GWSS by plant feeding assays. Based on our previous successes with feeding assays, we believe that we are well on our way to complete this part of our work. We also performed next generation small RNA and transcriptome sequence analysis on GWSS. The abundant information obtained from this strategy should lead to new opportunities for identification of GWSS RNAi targets.

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FUNDING AGENCIES

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

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ABSTRACT

During this period we were able to demonstrate HoCV-1 infection in the glassy winged sharpshooter GWSS Z-15 cells after transfection using in vitro-derived RNAs. HoCV-1 infection resulted in characteristic cytopathology in infected but not healthy cells. Furthermore, quantitative RT-PCR analyses showed accumulation of HoCV-1 positive and negative-sense RNAs, the latter being characteristic indicators of HoCV-1 replication. Because one of our long-term goals is to induce debilitating RNA interference responses in HoCV-1-infected GWSS, we used next generation small RNA sequence analyses to qualitatively and quantitatively assess small RNA responses in HoCV-1-infected GWSS. These analyses showed abundant response by GWSS to HoCV-1 infection, providing validity for our approaches here.

LAYPERSON SUMMMARY

During the past year, we successfully engineered infectious clones of HoCV-1 and verified the biological activity in the glassy winged sharpshooter (GWSS) Z-15 cell-line. We also developed another virus-system using FHV to be used in conjunction with the HoCV-1 system to expedite our efforts to control GWSS population in California to prevent the spread of *Xylella fastidiosa* (*Xf*). We are currently modifying HoCV-1 for delivering RNAs that can express toxic peptides or induce RNAi in GWSS insects and the Z-15 cell-line. We also demonstrated that GWSS whole insects respond to HoCV-1 infection with robust RNA interference activity. The knowledge gained from this study will be used further to develop a virus system to help with GWSS management.

INTRODUCTION

The glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS), transmits the bacterium *Xylella fastidiosa* (*Xf*), which causes Pierce's disease of grapevines. We are attempting to use a naturally-occurring GWSS-infecting virus, *Homalodisca coagulata virus-1* (HoCV-1, Hunnicutt et al., 2008), as part of a strategy to help control the GWSS population. We are attempting to develop infectious cloned HoCV-1 which we can then engineer to deliver toxic peptides to kill GWSS, and/or deliver GWSS RNAs that will activate the systemic RNA interference (RNAi)-based immune system in GWSS. We hope that one or both approaches will result in

GWSS mortality thereby preventing the spread of *Xf*. If successful, our studies may lead to new and effective methods to help control the GWSS population.

OBJECTIVES

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

RESULTS AND DISCUSSION

We made good progress on both objectives during this past year. We are on track to make more progress by the end of the funding period.

Objective 1: Development of *Homalodisca coagulata virus-1* (HoCV-1) infectious cloned cDNAs

We have successfully cloned full length HoCV-1 cDNA. *In vitro* transcription was performed using the constructs HoCV1-3'RZ (produces HoCV-1 RNA) and mutRZ-HoCV1-3'RZ (produces HoCV-1 RNA that can not produce functional proteins) and delivered to GWSS-Z15 cells. After transfection with HoCV-1 transcripts produced with HoCV1-3'RZ, GWSS-Z15 cells showed severe cytopathic effects that were not observed in the GWSS-Z15 control or in cells transfected with mutRZ-HoCV1-3'RZ (**Figure 1**). Both the genomic-sense strand (positive-strand) and the complementary strand (negative-strand) of HoCV-1 RNAs were amplified by RT-PCR analysis following the transfection indicating that the virus was replicating (data not shown).

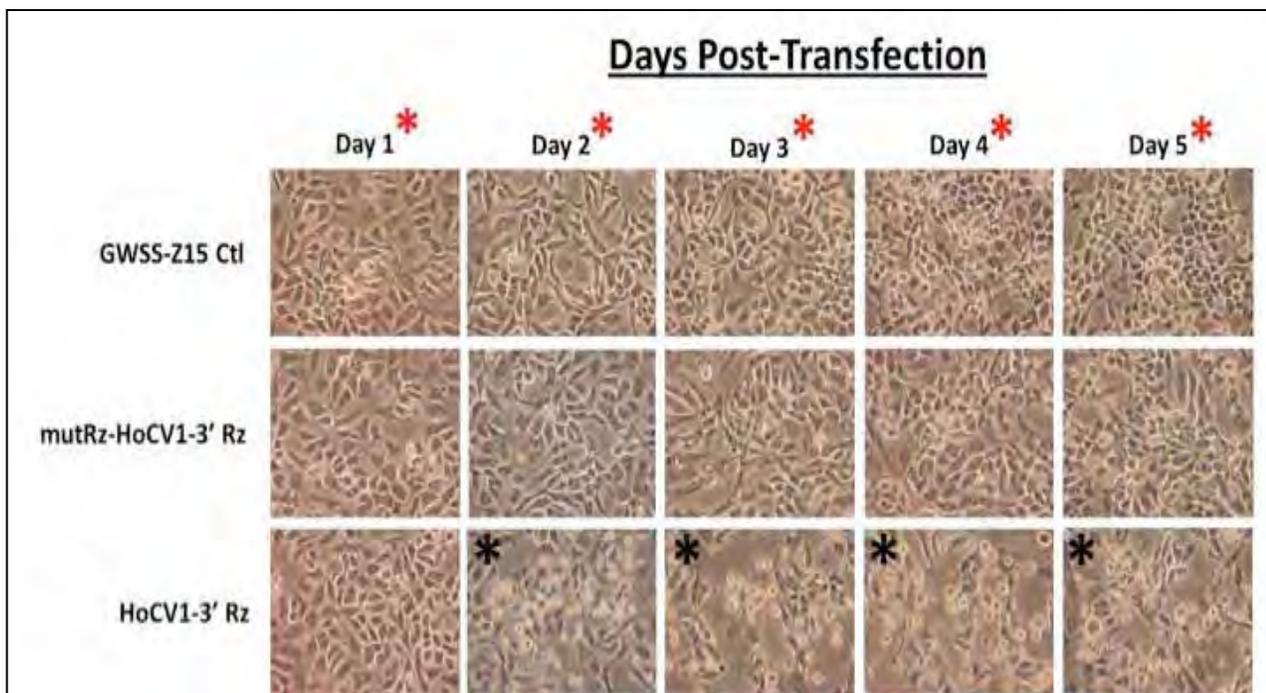
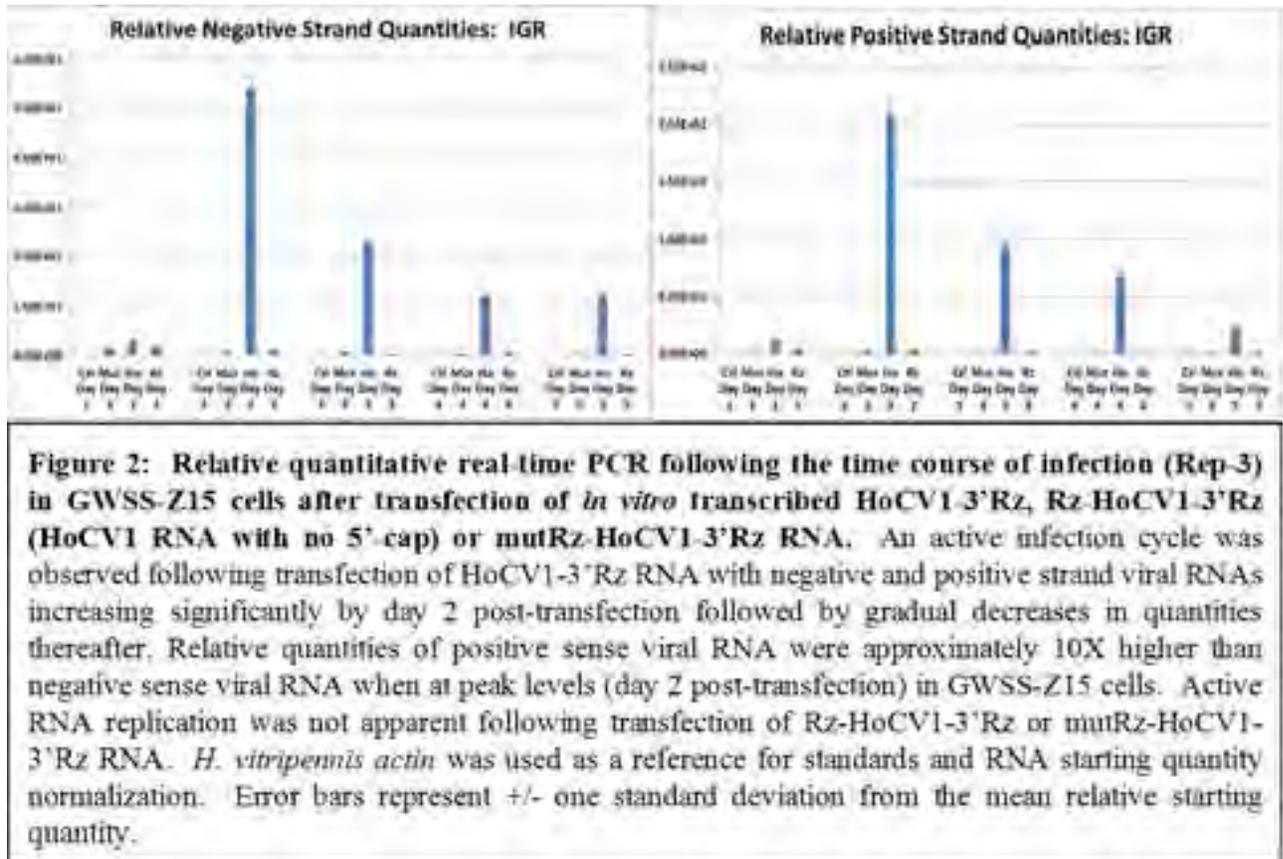


Figure 1. Time course of infection in GWSS-Z15 cells following transfection of in vitro transcribed HoCV1-3'Rz, or mutRz-HoCV1-3'Rz RNA. Cells (5 wells/construct) were transfected with 8 μ g of HoCV1-3'Rz RNA, or 8 μ g of mutRzHoCV1-3'Rz in vitro transcribed HoCV-1 viral RNA. Images were taken at day 1, day 2, day 3, day 4, and day 5 post-transfection. CPE were observed in GWSS cells transfected with HoCV1-3'Rz RNA on days 2, 3, 4, and 5 (black asterisks) that were not observed in the other wells. Samples of cells (1 mL) were removed from the wells on each day for quantitative real-time PCR analyses of negative and positive sense viral RNA (red asterisks). Images were taken at 40X objective magnification.

Relative quantitative real time PCR reactions were performed to detect infectivity and relative quantities of negative- and positive-sense HoCV-1 viral RNA (**Figure 2**). A significant increase in the amount of HoCV-1 negative and positive sense RNA was detected between days 1 and 2 post-transfection in GWSS-Z15 cells

transfected with HoCV1-3'Rz with a gradual decrease documented thereafter through day 5 post transfection. Positive-sense RNA appeared at levels up to 10X higher than negative sense viral transcript in duplicate qRT-PCR studies targeting the HoCV-1 IGR region. Taken together, these data strongly suggest that we have developed a full length HoCV-1 genomic RNA cDNAs, and that the transcripts generated *in vitro* are infectious at least to GWSS cells. More work must be done to optimize these cloned cDNAs and to evaluate them in whole GWSS insects.



Objective 2: Expression of GFP or other stable sequences in GWSS-Z15 cells or whole GWSS insects by using HoCV-1

We are in the process of engineering infectious clones to express YFP and mCherry reporters as part of the transcribed viral sequence (refer to **Figure 3**). We next will convert HoCV-1 into a highly specific, lethal virus by engineering it to express a peptide that is toxic only when expressed intracellularly within target sharpshooters. This includes primarily GWSS, but as HoCV-1 has a host range that is reported to include other sharpshooters (Hunnicuttt, 2008), our success here has broader applicability for *Xf* vector control. The toxic peptides to be employed are lethal only to insects, and only when expressed intracellularly. Thus, only sharpshooters infected by the modified virus will be negatively affected by the peptides.

In our previous work with *Flockhouse virus* (FHV) we were able to express GFP in insect (*Drosophila* S2) cells and we originally planned to use FHV as a model for our efforts with HoCV-1. 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, Diptera). We used 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 attempted to use this same strategy, as well as using direct RNA transfection for HoCV-1 into GWSS Z-15 cells.

Because our HoCV-1 so far has not been easy to manipulate, we used small RNA (sRNA) next generation sequencing to confirm our original sequence. This approach allowed for rapid, accurate sequencing and assembly of the HoCV-1 genome (**Figure 4**), and allowed for validation of the original sequence. Furthermore, these data clearly demonstrate that GWSS actively responds via RNA interference to HoCV-1 infection. This strengthens our rationale for taking the approach to induce systemic RNAi effects in GWSS by using recombinant HoCV-1.

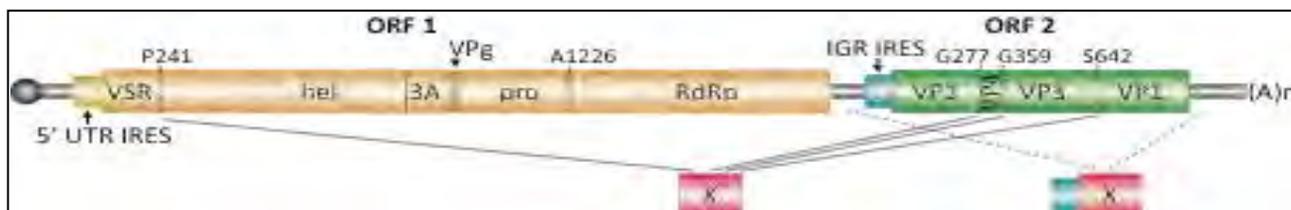


Figure 3. Potential insertions sites for expression of foreign proteins and RNAs from the RhPV and HoCV-1 genomes. Genome organization shows ORF 1 (orange) and ORF 2 (green) which encode proteins separated by cleavage sites indicated at estimated positions by vertical lines. Precise cleavage sites, estimated 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 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].

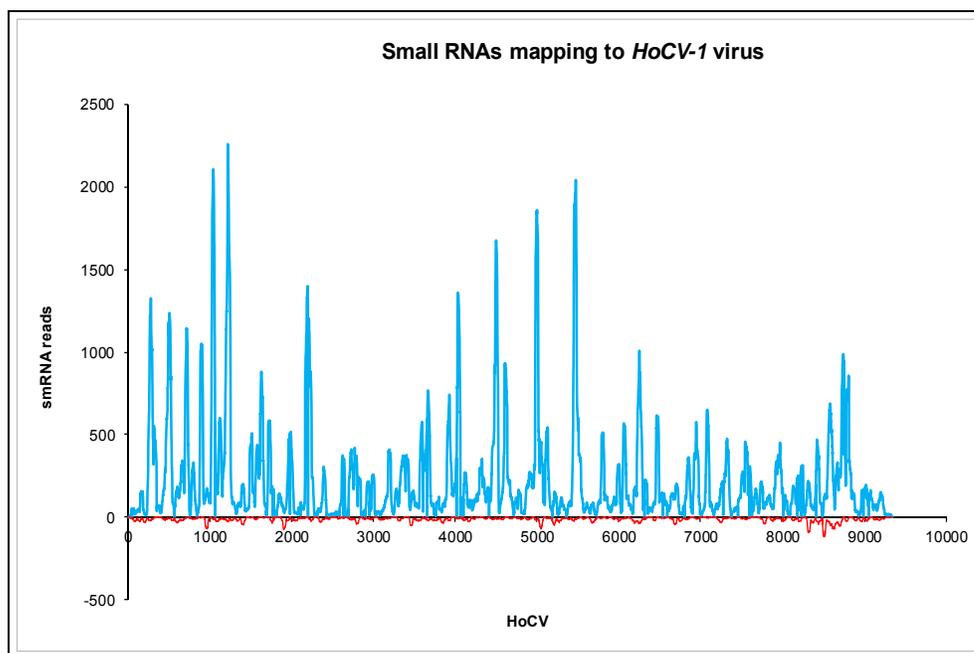


Figure 4. Small RNA mapping on the HoCV-1 positive (blue) and negative (red) sense RNAs. The number of small RNAs for a specific region are shown on the Y-axis and the HoCV-1 genome is represented across the X-axis.

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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 October 2011 to September 2012.

ABSTRACT

Death or dramatic changes in normal insect development can result from minor disruption of the insect endocrine system. Juvenile hormone (JH) is a key insect developmental hormone that induces biological effects when present at low nanomolar levels in the hemolymph. JH analog (JHA) insecticides are green compounds that can mimic the action of JH and selectively disrupt the insect endocrine system. In this project we are testing the efficacy of JHAs against the glassy winged sharpshooter (GWSS). We are also evaluating the potential of a JH metabolizing epoxide hydrolase (*jheh*) gene as a target for gene silencing-based control of GWSS. We hypothesize that knocking down the *jheh* gene and consequently juvenile hormone epoxide hydrolase (JHEH) activity will prevent JH titer from dropping below the threshold required for normal development. In terms of mode of action, the effects of JHA application and knockdown of the *jheh* 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 yet highly sensitive endocrine system. 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 (JHA) insecticides against GWSS eggs and nymphs. The results of this direct approach will have near-term applicability since the JHA insecticides that we are testing are US-EPA registered and commercially available. Our indirect approach involves the identification and characterization of an enzyme called epoxide hydrolase that selectively metabolizes JH. The objective of this approach is to characterize the epoxide hydrolase gene as a potential target for gene knockdown. If we can knock down the epoxide hydrolase gene, we will be able to reduce the normal metabolism of JH and thus prevent JH levels from falling below the normal threshold that is required for normal insect development.

INTRODUCTION

Insect development is precisely regulated by the relative titers of juvenile hormone (JH) and molting hormones. JHs are a family of sesquiterpenoids that regulate reproduction, behavior, polyphenisms, development, and other key biological events in insects (reviewed in Riddiford, 2008). Minor disruption of an insect’s hemolymph JH levels can result in insect death or dramatic alterations in insect development. Juvenile hormone analog (JHA) insecticides are green compounds that selectively target the insect endocrine system by mimicking the biological action of JH (reviewed in Dhadialla et al., 2005). When pest insects are exposed to JHAs at a time during development when JH titer is normally undetectable, abnormal nymphal-pupal development and/or death is induced. Abnormal developmental morphologies, similar to those induced by JHAs are also induced by inhibiting an esterase that selectively metabolizes JH (Abdel-Aal and Hammock, 1985). Inhibition of the JH-selective esterase putatively results in JH titers that are not below the threshold required for normal development. Similarly, we hypothesize that inhibition of another JH-metabolizing enzyme called JH epoxide

hydrolase (JHEH) will also result in the induction of abnormal nymphal-pupal development and/or death of GWSS.

In this project we are testing the efficacy of commercially available JHAs against GWSS eggs and nymphs. We are also attempting to characterize the JHEH of GWSS, an enzyme that metabolizes the epoxide moiety that is found on all known JHs. The gene that encodes this enzyme, *jheh*, could have potential as a target for gene silencing-based control of GWSS. In terms of mode of action, the effects of JHA application and JHEH knockdown by gene silencing are similar in that both approaches can enhance “JH action.” During the current reporting period our work has primarily focused on Objective 2, Characterization of recombinant JHEH from GWSS.

OBJECTIVES

1. Investigate the delayed effects of low dose JHA insecticide exposure.
 - a. Determine sublethal dose in eggs and first instar nymphs.
 - b. Evaluate delayed effects of sublethal exposure on egg development.
 - c. Evaluate delayed effects of sublethal exposure on nymph development.
2. 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

During the previous reporting period, we characterized JHEH enzyme activity levels in the hemolymph of fifth instar GWSS nymphs. Peak JHEH activity (9.3 ± 1.7 pmol of JH diol formed per min per ml of hemolymph) was found at day six of the fifth instar. During the current reporting period, total RNAs were initially isolated from nymphs at days three, four, and five of the fifth instar. These RNAs were used to generate first strand cDNAs. The first strand cDNAs were then used as template for random amplified cDNA end (RACE)-based attempts to identify the 3'-end and 3'-UTR of a potentially JHEH-encoding cDNA. These attempts were unsuccessful. Subsequently, total RNA was isolated from older fifth instars (days 6, 8, and 10), and these RNAs were used to generate first strand cDNAs for 3'-RACE. The 3'-RACE approach using these cDNAs was successful. On the basis of the 3'-end sequence of a potential JHEH, several gene-specific, nested primers were designed for 5'-RACE. The 5'-RACE identified the 5'-UTR and 5'-end of a potentially JHEH-encoding cDNA. On the basis of the 5'- and 3'-RACE results two gene-specific primers were designed and used to amplify a full-length cDNA, *hovijheh*, that potentially encoded a JHEH (**Figure 1**).

Hovijheh was 1,668 nts-long and contained a 1,374 nts-long open reading frame flanked by 5'- and 3'-UTR sequences that were 174 and 101 nts-long, respectively. The deduced protein, HoviJHEH, of *hovijheh* was 459 amino acid residues long and had a predicted mass of 52,108 Daltons and pI of 7.00. A putative membrane anchor domain (IKGVLVSVLVVVSVALGLYIDY) of 23 amino acid residues was predicted by SOSUI v. 1.11 (Hirokawa et al., 1998) at the amino terminal of HoviJHEH. The catalytic residues of HoviJHEH were 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 were also conserved in HoviJHEH (**Figure 1**). Phylogenetic analysis showed HoviJHEH in a clade with two hymenopteran EHs, Nasvi-EH1 and AmJHEH (**Figure 2**). The deduced amino acid sequence of HoviJHEH showed approximately 50% identity with Nasvi-EH1 and AmJHEH (but also 44-54% identity with other EHs investigated).

In order to characterize the protein encoded by *hovijheh*, the coding sequence of *hovijheh* was PCR-amplified and the resulting amplicon was subcloned into the baculovirus transfer vector plasmid pAcUW21 (Pharmingen) generating pAcUW21-hovijheh. pAcUW21-hovijheh was subsequently transfected with *Bsu*36I-digested BacPAK6 baculovirus DNA (Clontech) into Sf-9 cells using Cellfectin Transfection Reagent (Invitrogen). This transfection generated AcHoviJHEH, a recombinant baculovirus expression vector expressing HoviJHEH. AcHoviJHEH was isolated from the supernatant of the transfected Sf-9 cells by three rounds of plaque purification on Sf-9 cells following standard procedures (Merrington et al., 1999). The recombinant HoviJHEH

protein was produced in insect High Five cells (1×10^6 cells ml^{-1}) that were inoculated with AcHoviJHEH at a multiplicity of infection of 0.8 and collected at 72 h post inoculation. Microsomes were prepared from the infected High Five cells as described previously (Morisseau et al., 2011) and stored at -80°C . The protein concentration of the microsomal preparation was determined by the Bradford method using bovine serum albumin as a standard. The relative amount of HoviJHEH in the preparation was analyzed by SDS-PAGE as described previously (Kamita et al., 2011).

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. The kinetic constants (Michaelis constant (K_M) and V_{\max}) of HoviJHEH for JH III were determined using at least 6 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 HoviJHEH for JH III.

The V_{\max} of HoviJHEH for JH III was 18.7 ± 1.1 nmol of JH III diol formed $\text{min}^{-1} \text{mg}^{-1}$ when determined in 100 mM sodium phosphate, pH 8.0, at 30°C . This rate was about 3-and 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 5-fold lower than that of the coleopteran *Tribolium castaneum* (Tsubota et al., 2010). The K_M and k_{cat} values of HoviJHEH for JH III were $5,400 \pm 830$ nM and 0.02 s^{-1} , respectively. In comparison, the K_M of HoviJHEH for JH III was 23-fold and 10-fold higher than that of *M. sexta* and *B. mori*, respectively. The turnover (i.e., k_{cat}) of JH III by HoviJHEH was similar (about two-fold lower) to these JHEHs.

CONCLUSIONS

During the reporting period, we identified a full-length epoxide hydrolase encoding cDNA (i.e., *hovijheh*) from fifth instar, day 10 GWSS nymphs. We expressed and semi-purified the protein encoded by *hovijheh* using a recombinant baculovirus expression vector and characterized the ability of the recombinant protein, HoviJHEH, to metabolize juvenile hormone. HoviJHEH metabolized JH III at a rate that was three-to-five-fold slower than that shown by known recombinant lepidopteran and coleopteran JHEHs. We are currently in the process of developing a high throughput fluorescent substrate based assay for use in screening JH analogue insecticides and other compounds for their ability to inhibit HoviJHEH. The availability of chemical inhibitors will allow for experiments to investigate the *in vivo* function of HoviJHEH.

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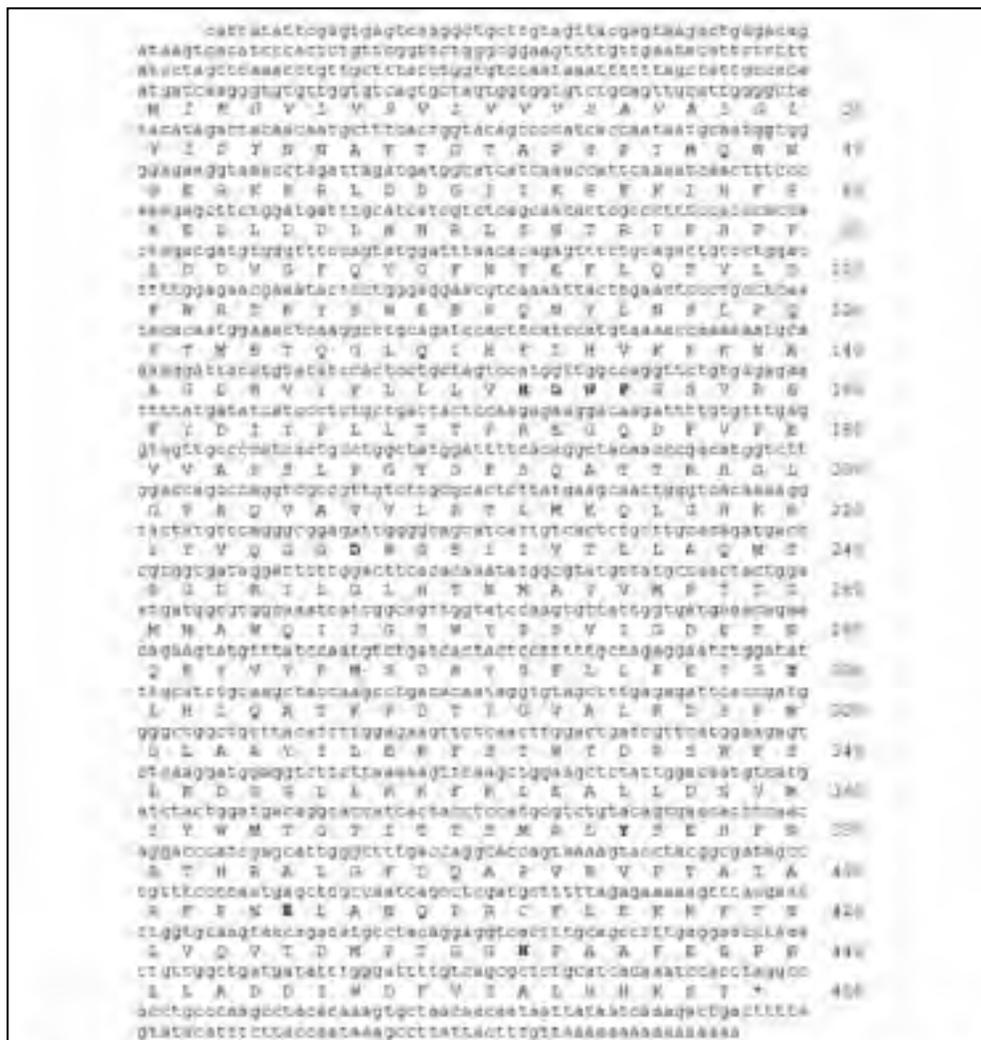


Figure 1. Nucleotide (lower case text) and deduced amino acid (upper case text) sequences of *hovp/wh* and HovJHEH, respectively. The 5'- and 3'-UTR sequences, and coding sequence of *hovp/wh* were 174, 101, and 1374 nts-long, respectively. Amino acid residues that form the putative catalytic triad (D-217, H-432, and E-405), lid domain (Y-300 and Y-375), and oxyanion hole (HGWP, residues 152-155) are shown in bold text. The asterisk indicates a stop codon (TAG). A putative membrane anchor domain (residues 2-24) that was predicted by SOSUI version 1.11 (Hirokawa et al., 1998) is shown in italic text. Amino acid residue positions are indicated to the right.

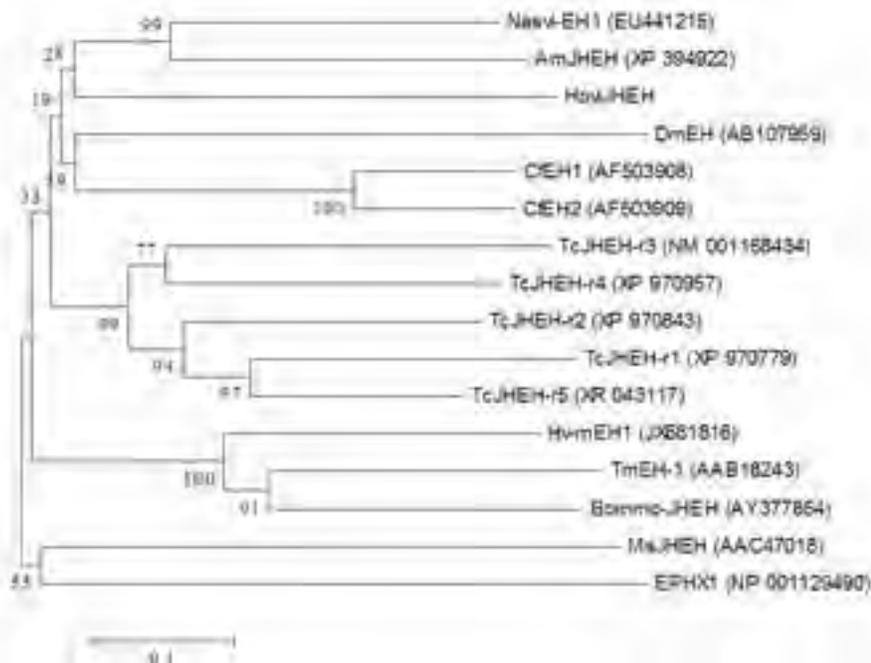


Figure 2. Phylogenetic analysis of HovJHEH and EH sequences from hymenopteran (Nasvi-EH1 and AmJHEH), dipteran (DmEH), siphonateran (CfeH1 and CfeH2), coleopteran (TcJHEH-r3, TcJHEH-r4, TcJHEH-r2, TcJHEH-r1, and TcJHEH-r5), and lepidopteran (HvmEH1, TmEH-1, Bommo-JHEH, and MsJHEH) insects and Primates (EPHX1). The GenBank accession number of each sequence is given within the parentheses. The phylogenetic analysis was performed using MEGA version 5.05 (Tamura et al., 2011). The tree was generated by the Neighbor-Joining method using a ClustalW generated alignment of 16 EH sequences. The percentage of replicate trees in which the sequences clustered together in the bootstrap analysis (1000 replicates) is shown at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances (computed using the Poisson correction method) used to infer the phylogenetic tree.

Section 3:

Pathogen Biology and Ecology



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

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ABSTRACT

Xylella fastidiosa (*Xf*), a xylem-limited plant pathogen, causes leaf scorch diseases in many plant hosts, but individual strains exhibit considerable host specificity. In this project that terminated in August 2012, we further defined the host range of a large number of strains of *Xf* and began to explore the hypothesis that host range of *Xf* may be influenced by differential expression of virulence genes in response to different host xylem chemistry. In multi-year host range tests, we found that some hosts (oleander, olive, and sweetgum) are infected by single genotypes of *Xf*, but strains from oleander, olive, and sweetgum may infect some other hosts. Some hosts (almond, plum, and blackberry) can be infected by many different genotypes of *Xf*, including strains with otherwise narrow host ranges, such as oleander and red oak strains. Recombinant genotypes of *Xf* (mulberry and blackberry strains) are associated with unique host specificities. *Xf* can grow in the xylem fluid of both host and non-host plants, but a number of key virulence genes, particularly those contributing to biofilm formation and twitching motility, are more highly expressed in xylem fluid of susceptible host species (Shi et al., 2010; Cooksey et al., 2011). A few genes are expressed at lower levels in host vs. non-host xylem fluid. Together with related collaborative work on differential expression of virulence genes in xylem fluid from different grapevine cultivars (Hoch et al., 2011), our data support the hypothesis that cultivar specificity and broader plant host range in *Xf* is determined by differential regulation of virulence genes in response to xylem fluid chemistry. While strains can grow in xylem fluid from host and non-host plant species and resistant and susceptible plant cultivars, differential expression of key virulence genes enables systemic movement of the pathogen only in susceptible hosts.

LAYPERSON SUMMARY

Host specificity varies greatly between subspecies and between strains within subspecies of *Xylella fastidiosa* (*Xf*). In some hosts, such as oleander, olive, and sweetgum, disease is caused only by specific strains of the pathogen, while certain other hosts, such as almond, plum, and blackberry, can be infected by many different strain types. Naturally-occurring recombinant strains of *Xf* (genetic combinations between two subspecies) have unusual host ranges. Strains of *Xf* can grow in extracted xylem fluid of susceptible and resistant plant species or cultivars within species, but key virulence genes are expressed differently in xylem fluid from susceptible plants, enabling the pathogen to move and cause systemic infections. Applications of these findings in the future might include the modification of host xylem chemistry through cultivation techniques, breeding, or genetic engineering to reduce disease caused by *Xf* in susceptible plants.

INTRODUCTION

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

Xf not only causes diseases in a variety of host plants, but it can grow in symptomless hosts that can serve as sources of inoculum (Costa et al., 2004). We showed that a Pierce's disease strain of *Xf* can grow when inoculated into pure xylem fluid from grapevine (a symptomatic host for Pierce's disease), citrus (symptomless with Pierce's disease), or mulberry (symptomless with Pierce's disease) (Shi et al., 2010; Cooksey et al., 2011), but had limited aggregation and biofilm formation. A number of virulence-related genes, such as those required for twitching motility, were expressed at a greater level in grapevine xylem fluid compared with citrus or mulberry xylem fluid. However, some genes had greater expression in both non-host xylem fluids (Shi et al. 2010; Cooksey et al., 2011). In collaborative work, a Pierce's disease strain was also shown to have lower aggregation, biofilm formation, and motility, when grown in xylem fluid from grapevine varieties tolerant of Pierce's disease vs. susceptible varieties (Hoch et al., 2011). Understanding which specific chemical components of plant xylem fluid influence virulence gene expression could lead to strategies for practical disease control.

This final report on the subject from the Cooksey laboratory will focus on unpublished data from a multi-year host range study by H. Azad that was just concluded in August 2012. The host range information should be useful in future projects that may further explore the specific chemistry of xylem fluid that *Xf* strains sense differentially.

OBJECTIVES

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

RESULTS AND DISCUSSION

Host range tests were conducted over a five-year period from 2007-2011, to support the goals of this project, as well as a collaborative project with L. Nunney on genetic typing and detection of recombination in different strains of *Xf*. Replicated greenhouse-grown plants were needle-inoculated, observed for symptoms, and tested by ELISA and by culturing for systemic movement of *Xf*. Strain/host combinations listed as positive (+) in **Table 1** were positive for leaf scorch symptoms and were both ELISA positive and positive for *Xf* by culturing at multiple points distal to the inoculation site. Those combinations listed as negative (-) did not have disease symptoms, and the bacterium was not detected away from the inoculation site by ELISA or by culturing. A few combinations (indicated with a # symbol) produced symptoms and positive ELISA detection, but we were not able to isolate *Xf* from those plants away from the inoculation site. Combinations listed as +/- had no disease symptoms, but systemic movement of *Xf* was detected by ELISA.

Several conclusions can be drawn from the strain and host combinations that were tested (**Table 1**). Leaf scorch disease and systemic infections of some hosts, such as olive, oleander, and sweetgum, were only caused by strains that were isolated from those specific hosts. The strains from those three hosts had relatively narrow host ranges but were not strictly limited to their host of origin. Oleander strains could also infect (cause disease and spread systemically) mulberry, for example, and the olive strain we tested could infect grape as well as olive. A few hosts were susceptible to a large number of *Xf* genotypes. These included almond, plum, and blackberry. Strains that infected blackberry included some with otherwise narrow host ranges, such as the Ann1 oleander strain and the strain from red oak. Recombinant strains, such as those from mulberry (Nunney, 2011) and blackberry (Nunney, personal communication), had unique host specificities. About one half of the mulberry strain genome is thought to be derived from *Xf* subsp. *fastidiosa* and the other half from *Xf* subsp. *multiplex*, but the host range of mulberry strains is unlike any of the individual strains from those two subspecies that we have tested.

Xf gene expression studies in xylem fluid were completed with only a few of these strain/host combinations and have been reported on previously (Shi et al., 2010; Cooksey et al., 2011). Together with the additional studies on gene expression by *Xf* in susceptible and resistant grapevine cultivars by Hoch et al. (2011), our work supports the hypothesis that both cultivar specificity and broader plant host range in *Xf* is determined by differential expression of key virulence traits in response to differences in xylem chemistry between hosts.

Unfortunately, our analysis of amino acid composition, organic acids, and sugars in different xylem fluids did not yield any definitive conclusions about what environmental signals are responsible for this differential gene expression in susceptible and resistant hosts.

Table 1.

<i>Xf</i> Strain	Host of Origin	Plant Hosts Inoculated																
		Pinot Noir	Coffee	Olive	Oleander	Almond	Plum	Peach	Mulberry	Sweetgum	Sycamore	Crape myrtle	Red Oak	Black Oak	Encelia	Elm	Blackberry	Alder
Temecula	Grape	+	-	-	-	-	-	-	-	-	-	-	#	-	-	-	+	-
Hopland	Grape	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
PD0308	Grape	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
PD0004	Grape	+	-	-	-	-	+	-	-	-	-	-	#	-	-	-	-	-
Dixon	Almond	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
276	Almond	-	-	-	-	+	+	-	-	-	-	#	-	-	-	-	+	-
Tulare ALS	Almond	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
M12	Almond	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-
MLS024	Mulberry	#	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-
267	Mulberry	#	-	-	-	+	+	-	+	-	#	-	-	-	-	-	-	-
MLS059	Mulberry	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	-
Ann1	Oleander	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-
Riverside	Oleander	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-
PC045	Plum	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
PC053	Plum	-	-	-	-	+	+	+	-	-	-	-	-	+/-	-	-	+	+/-
PC052	Plum	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
LA043	Sweetgum	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
LA20	Sweetgum	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-
Olive	Olive	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ATCC35874	Red Oak	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-
Bkb95-1	Blackberry	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
EDB92-1	Elderberry	-	-	-	-	+	+	-	+/-	-	-	-	-	-	-	-	+	-

CONCLUSIONS

All strains of *Xf* that we tested have reproducible host range patterns that differed between subspecies and between strains within subspecies. Some plant hosts were susceptible to many different strains of *Xf*, while other hosts were infected only by strains derived from those same hosts. Recombinant strains of the pathogen that contain genes from different subspecies of *Xf* had unique host ranges, and we are concerned that continued recombination between strains of *Xf* could lead to new compatible strain/host combinations and potential economic loss to new crops. Our work on *Xf* gene expression in xylem fluid from different host plants supports the hypothesis that both cultivar specificity and broader plant host range in *Xf* is determined by differential expression of key virulence traits in response to differences in xylem chemistry between hosts. The data set provided by our host range studies should be useful in future projects that may further explore the specific chemistry of xylem fluid that *Xf* strains sense differentially. It may be possible in the future to alter xylem chemistry in susceptible hosts to inhibit expression of key virulence traits by the pathogen and reduce disease expression.

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

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ABSTRACT

The rapid production and accumulation of reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂) and superoxide anions, is a key component of the initial plant response to bacterial infection. Because the elevated levels of ROS are highly toxic to the bacteria, most pathogens have evolved a variety of enzymes capable of detoxifying ROS. The goal of this project is to understand how *Xylella fastidiosa* (*Xf*) responds to different types of ROS and to characterize the enzymes and regulatory proteins induced in this response. We have generated *Xf* strains carrying mutations in the key scavenging enzymes and have examined the impact of hydrogen peroxide on their transcription. We have also examined the importance of the transcription factor OxyR in the *Xf* oxidative stress response. Strains carrying a null mutation in *oxyR* are more sensitive to killing by hydrogen peroxide and are impaired in their ability to attach to solid surfaces. Moreover, *oxyR* mutants do not colonize grapevines as well as wild-type strains and the defect appears to occur early in the infection process. Experiments are underway to determine the impact of the *oxyR* null mutation on transmission and acquisition of *Xf* by the insect vector.

LAYPERSON SUMMARY

Plants have developed a broad range of responses for dealing with invading microorganisms. One of the earliest observable responses is an oxidative burst, which involves the rapid release of reactive oxygen species (ROS). ROS are thought to serve as antimicrobial agents and as signals to activate further plant defense reactions. This project is designed to uncover the vulnerabilities of *Xylella fastidiosa* (*Xf*) to reactive oxygen species (ROS) during the initial stages of infection and during the later stages when the bacteria are protected by a biofilm. Our strategy has been to generate mutations in the genes involved in the *Xf* oxidative stress response and to examine how the absence of these gene products impacts *Xf* survival in grapevines and in the insect vector. Uncovering where and when in the infection process these gene products are required is essential if we wish to fully appreciate the importance of the oxidative stress response in *Xf* virulence, as well as the value of the individual genes as potential therapeutic targets.

INTRODUCTION

Xylella fastidiosa (*Xf*) is exposed to reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂) and superoxide anions, at potentially two points in the infection cycle. First, ROS are a normal part of both the plant defense response and certain developmental processes (Bolwell and Daudi, 2009). In xylem tissue, there are two primary sources of ROS (Barcelo, 2005). One is developmentally related and comes from the process of lignifying xylem. The second source is the living xylem parenchyma cells, which can diffuse H₂O₂ to the adjacent xylem vessels. *Xf* may also be exposed to the ROS produced by the immune systems of the insect vector (Vallet-Gely et al., 2008). Since elevated levels of ROS are highly toxic and can disrupt many cellular processes through their oxidation of lipids, modification of proteins and damage to DNA, most pathogenic bacteria have developed strategies to overcome this toxicity.

The immediate detoxification of ROS is accomplished, in part, by scavenging enzymes designed to cope with specific oxidative stresses (Imlay, 2008). Comparative genomics suggests that many of these enzymes are present in *Xf* (Table 1).

Table 1. Predicted *Xf* enzymes and regulators.

Enzyme	<i>Xf</i> gene(s)	Oxidative signal	Regulator
Alkyl hydroperoxide reductase	<i>ahpC, ahpF</i>	H ₂ O ₂ , organic peroxides	OxyR
Catalase	<i>cpeB</i>	H ₂ O ₂	OxyR
Superoxide dismutase	<i>sodA, sodM</i>	superoxides	unknown
Flavodoxin NADP ⁺ reductase	<i>fpr</i>	superoxides	unknown
Thiol-dependent peroxidase	<i>ohr</i>	organic peroxides	unknown

OBJECTIVES

The goal of this project is to understand how *Xf* responds to the different ROS it encounters in the xylem and to characterize the enzymes and regulatory proteins induced in this response. Understanding the vulnerability of *Xf* to oxidative stress could lead to targeted strategies for mitigating the devastating symptoms of Pierce's disease in grapevines.

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

RESULTS AND DISCUSSION

Many bacteria have evolved distinct sensing mechanisms to detect different forms of oxidative stress and to induce the synthesis of a particular set of scavenging enzymes. In Gram-negative bacteria, much of this regulation occurs at the transcriptional level through regulatory proteins such as OxyR, SoxRS, and Ohr (Imlay, 2008). Examination of the *Xf* genome revealed a potential homolog to OxyR. However, unlike other Xanthomonads, *Xf* is missing both the homolog to Ohr, which regulates the response to organic peroxides and the SoxRS system, which regulates the response to superoxide stress. In this project, we have been examining how *Xf* responds to different types of ROS, whether or not these responses are regulated, and the importance of OxyR in these responses.

Objective 1: Determine the key components in the response of *Xf* to ROS and the contribution of OxyR to this regulation

Xf is predicted to contain multiple scavenging enzymes that respond to different ROS (**Table 1**). During the past year, we have focused primarily on the response of *Xf* to H₂O₂ and the role of OxyR in the regulation of this response. We first examined how different exposure times and concentrations of H₂O₂ impacted *Xf* transcription, growth, and viability. For the transcriptional analysis, we chose sublethal H₂O₂ conditions that did not impact RNA quality. Liquid cultures of *Xf* Temecula1 (wild-type; WT) and the *Xf oxyR* mutant (*oxyR*) were grown in PD3 for three days and the cultures were split. One sample was exposed to H₂O₂ (0.5 mM final concentration) for 10 minutes; the other sample served as the untreated control. Total RNA was then extracted from all four cultures with TRIZOL reagent (Invitrogen) and cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). The resulting cDNA samples were subjected to qRT-PCR analysis using primer pairs exhibiting homology to *oxyR* and the seven genes listed in **Table 1** and the reagents in the SsoFast EvaGreen Supermix Kit (BioRad). The results from this analysis are shown in **Figure 1**.

As expected based on studies of other Gram-negative bacteria, transcription of four chosen target genes (*aphC*, *aphF*, *oxyR*, *cpeB*) was induced in WT cells following the addition of H₂O₂. The *aphC* and *aphF* genes encode alkyl hydroperoxide reductase, whereas the *cpeB* gene encodes catalase. These enzymes are known to play a critical role in the detoxification of H₂O₂. Given its location in the same operon as *aphC* and *aphF*, the induction of *oxyR* was also expected. Moreover, induction of these genes does not occur in the *oxyR* mutant,

confirming the role of OxyR in their regulation. In contrast, transcription of the three genes encoding scavenging enzymes for superoxides and organic peroxides (*sodM*, *fpr* and *ohr*) were not induced to a significant level by H₂O₂ or by the absence of the OxyR transcription factor.

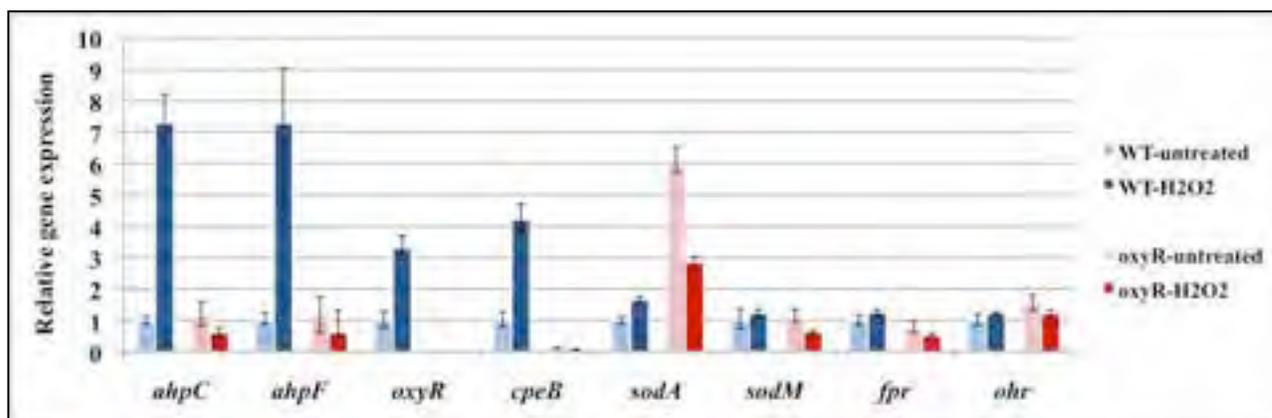


Figure 1. Impact of H₂O₂ and OxyR on transcription of oxidative stress genes.

The most intriguing result came from our analysis of *sodA*, which encodes the manganese-containing superoxide dismutase. In *E. coli*, *sodA* transcription is regulated by the superoxide-sensing transcription factor SoxR (Imlay, 2008). However, a number of bacteria that respond to superoxide stress do not contain a SoxR-homolog and have developed alternative strategies for regulating their superoxide-detoxifying enzymes. For example, the obligate anaerobe *Porphyromonas gingivalis* uses OxyR to positively regulate *sodA* transcription in response to superoxide stress (Ohara et al., 2006). It has been proposed that *P. gingivalis* OxyR triggers *sodA* transcription by functioning as an intracellular redox sensor rather than as a peroxide sensor. Like *P. gingivalis*, the *Xf* genome does not contain a homolog to SoxR and appears to use OxyR to sense superoxide stress. As shown in **Figure 1**, transcription of *Xf sodA* appears to be negatively regulated by OxyR. Whether this regulation occurs through a direct interaction between OxyR and the *Xf sodA* regulatory region or some indirect effect remains to be determined. However, this unusual regulatory pattern for *sodA* suggests that *Xf* may have developed novel methods for coping with certain types of oxidative stress.

Objective 2: Determine the role of the scavenging enzymes designed to cope with specific oxidative stresses in *Xf* cell physiology and virulence

Scavenging enzymes play a critical role in the response of bacteria to oxidative stress. In an earlier study, we established that deletion of the catalase-encoding *cpeB* gene ($\Delta cpeB$) resulted in increased sensitivity to H₂O₂ on solid media (Matsumoto et al., 2009). Moreover, the $\Delta cpeB$ mutant exhibits a lower survival rate compared to WT following treatment of a culture with 0.5 mM H₂O₂. As shown in **Figure 2A**, a dramatic decrease in *Xf* survival is observed following even a 1 minute exposure to 0.5 mM H₂O₂. Similar results were obtained when the strains were compared using the LIVE/DEAD cells staining method following treatment with 0.5 mM H₂O₂ or the superoxide generator, paraquat (PQ) for one hr (**Figure 2B**). Experiments are currently underway to determine how mutations in the other scavenging enzyme genes impact *Xf* survival following exposure to either peroxide or superoxide stress.

Objective 3: Determine the role of the transcription factor OxyR in oxidative stress sensing, biofilm formation and virulence

A bacterial biofilm is an aggregation of bacterial cells, which adheres to living or non-living surfaces (Hall-Stoodley et al., 2004). These adhered bacterial communities are usually embedded in a protective self-produced matrix exopolysaccharide (EPS). The stages of biofilm development generally include: (1) initial attachment, (2) microcolony formation, (3) maturation, and (4) dispersion. Recent studies have shown that OxyR is involved in biofilm formation by regulating fimbrial gene expression, a key aspect of the early attachment stage of biofilm formation (Shanks et al., 2007). The Roper laboratory has observed that OxyR also controls EPS

production in the xylem-dwelling phytopathogen, *Pantoea stewartii* (*unpublished data*). We hypothesize that OxyR may also play a similar role in regulating genes involved in biofilm formation in *Xf*. To test this hypothesis, Peng Wang (Roper laboratory) created a null mutation in the *oxyR* gene by site-directed mutagenesis and established that the resulting *oxyR* mutant exhibits a greater sensitivity to H₂O₂ on solid media than WT. Subsequently, Dr. Yunho Lee (Igo laboratory) compared the relative transcription levels of eight oxidative stress genes in the *oxyR* mutant to WT (**Figure 1**). This analysis indicated that OxyR is required for the transcriptional regulation of *ahpC*, *ahpF*, *oxyR*, *cpeB*, and possibly *sodA* in response to peroxide stress.

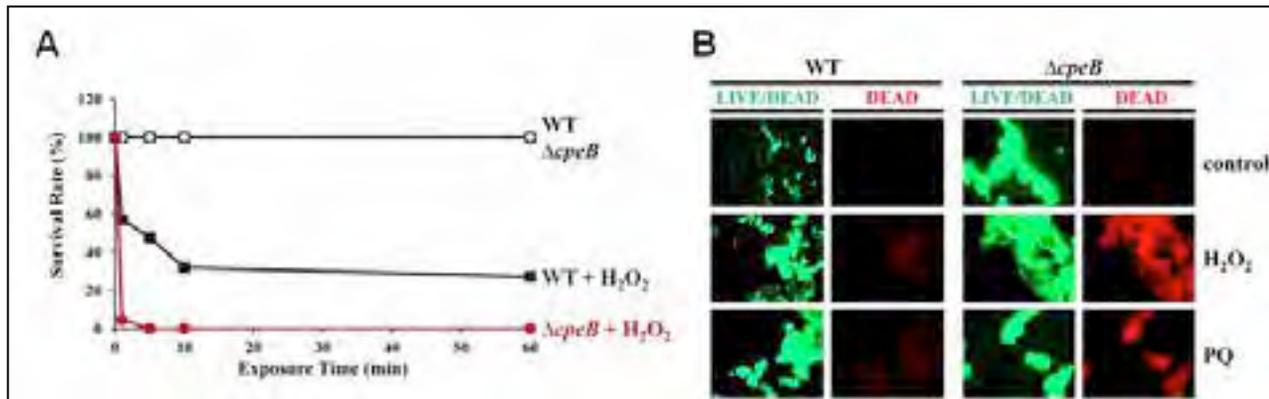


Figure 2. Importance of catalase for *Xf* survival following exposure to oxidative stress.

We also examined how the absence of OxyR impacts two biofilm related behaviors: surface attachment and cell-cell aggregation. To examine surface attachment, cultures of WT or the *oxyR* mutant were grown in polystyrene, glass, and polypropylene tubes without agitation. The presence of attached cells on these surfaces was assessed using crystal violet (Espinosa-Urgel et al., 2000). As shown in **Figure 3A**, the tubes containing WT had clear purple rings at the air-medium interface indicating a large number of attached cells. Notably, there was less of a purple ring observed for the *oxyR* mutant, indicating that the *oxyR* mutant did not attach as well as the WT parent to any surface tested.

The second phase of biofilm formation involves cell-cell aggregation, an important aspect of microcolony formation. We first observed the marked decrease in the aggregative ability of the *oxyR* mutant simply by growing them in liquid cultures and visually comparing them to WT liquid cultures. The decrease in the ability of the *oxyR* mutant to aggregate was visible to the naked eye. We then quantified cell-cell aggregation using an established protocol (Guilhbert and Kirkpatrick, 2005). Briefly, WT and *oxyR* mutant cells were grown in PD3 without agitation. At day 10, the cultures were gently agitated and allowed to settle for 20 minutes. The OD₅₄₀ of upper culture (OD_u) was measured and returned to the original tube. The aggregated cells were dispersed by briefly vortexing and the OD₅₄₀ of total cell culture (OD_t) was measured. The percentage of cell aggregation was then calculated (% aggregated cells = 100(OD_u-OD_s)/OD_t). The results indicate that a mutation in *oxyR* greatly affects cell-cell aggregation (**Figure 3B**).

Another important component of the maturing biofilm is exopolysaccharide (EPS) matrix production. In other bacterial systems, *oxyR* mutants have a marked decrease in EPS production (Roper, *unpublished data*). To determine if the *Xf oxyR* mutant is similarly affected, we are quantifying EPS production by the *oxyR* mutant using a Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS ELISA) method (Roper et al., 2007). This assay is very sensitive and even small amounts of EPS can be detected (detection limit is 1μg). We speculate that EPS production will be decreased based on findings in other bacterial systems in the Roper laboratory. We anticipate completion of this study by December 2012. Finally, we are visualizing *Xf* biofilm architecture of both the *oxyR* mutant and WT *Xf* using the confocal microscope available in the UCR core microscope facility. Based on the decreased ability of the *oxyR* mutant to attach and self-aggregate, we suspect

the *oxyR* mutant will form only a monolayer of cells and will be unable to achieve the 3-dimensional architecture of a WT biofilm.

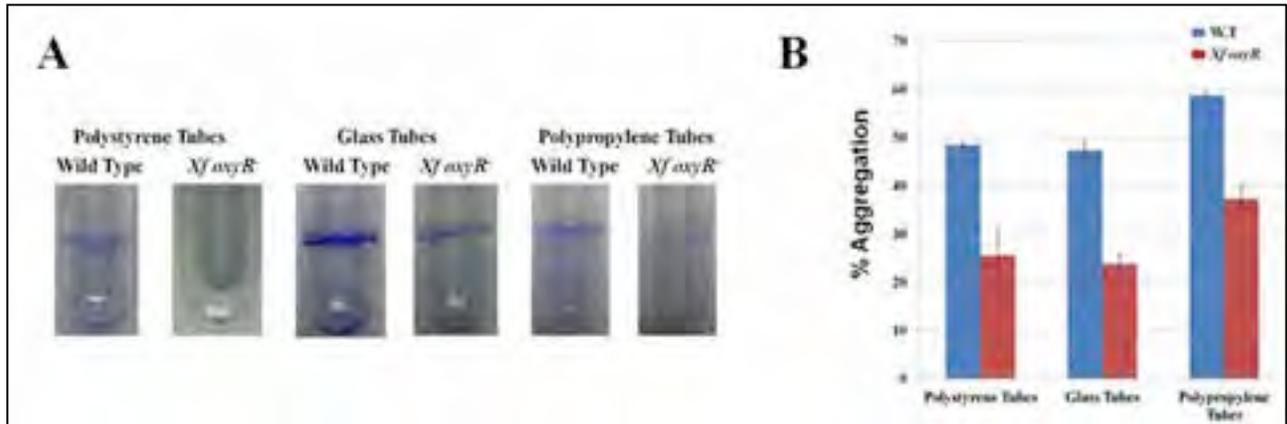


Figure 3. The *oxyR* mutation impacts attachment to solid surfaces and autoaggregation.

Objective 4: Test mutants generated in Objectives 2 and 3 for virulence in grapevines and for sharpshooter transmission

The *cpeB* mutant (**Objective 2**) and the *oxyR* mutant (**Objective 3**) are important for *Xf* survival under laboratory conditions. Furthermore, the *oxyR* mutant is defective in biofilm formation, a property known to be important for *Xf* virulence. The next step in our analysis was carried out at UC Riverside and examined how the *oxyR* mutation affects *Xf* colonization in host plants and its transmission by the insect vector. For this experiment, *Vitis vinifera* cv. Thompson Seedless grapevines were pin-prick inoculated as described by Hill and Purcell (1995). The populations of *Xf* WT and the *oxyR* mutant were quantified from petioles harvested 11 weeks and 14 weeks post-inoculation as previously described (Roper et al., 2007). As shown in **Table 2**, the *oxyR* mutant does not colonize grapevines as efficiently as WT.

Table 2. The *oxyR* mutant does not colonize grapevines as efficiently as *Xf* wild type.

Time post-inoculation*	<i>Xf</i> WT (CFU)	<i>Xf oxyR</i> (CFU)
11 weeks	$(28.65 \pm 7.92) \times 10^6$	$(11.39 \pm 4.3) \times 10^5$
14 weeks	$(1.20 \pm 0.19) \times 10^7$	$(6.10 \pm 3.60) \times 10^6$

* The populations of *Xf* wild type and *oxyR* mutant were determined in the leaf petioles at (11 weeks post-inoculation) or near (14 weeks post-inoculation) the point of inoculation (POI) in grapevines (Average CFU/g \pm SE) and the results analyzed using a Wilcoxon rank sums test.

However, we had anticipated a larger impact on colonization based on the apparent role of OxyR in the early steps of biofilm formation. Therefore, we decided to reexamine the properties of the *oxyR* mutant and included earlier time points in our analysis. Thus far, we have collected data two weeks post-inoculation (**Table 3**) and are currently assessing bacterial populations at four and six weeks post-inoculation. Although preliminary, the two weeks post-inoculation data suggest that the impairment in host colonization observed for the *oxyR* mutant occurs very early in the infection process.

Table 3. *In planta* colonization study at earlier time points of infection.

Time post-inoculation*	<i>Xf</i> WT (CFU)	<i>Xf oxyR</i> (CFU)
2 weeks	$(1.73 \pm 1.48) \times 10^5$	4.80×10^4

* The populations of *Xf* wild type and *oxyR* mutant were quantified from the leaf petioles near the point of inoculation (POI) in grapevines two weeks post-inoculation (Average CFU/g \pm SE).

The greenhouse experiments on the *cpeB* mutant were initiated at UC Davis last year using our standard protocol (Matsumoto et al., 2012). Unfortunately, we ran into two problems. First, the grapevines obtained for the experiment were already infected with a virus. Second, there was a major problem with insects in the greenhouse. As a result, no data was obtained from last year's grapevine experiments. Other UC Davis researchers using the same grapevine supplier and greenhouse ran into similar problems. To minimize these issues in our future experiments, we have rooted our own vines with the assistance of members of the Kirkpatrick laboratory (UC Davis Department of Plant Pathology). This should increase the probability that the starting vines will be healthy. We are also using a different greenhouse that is closer to the lab. This has allowed more frequent monitoring of the vines by the scientists conducting the experiments. With these changes, we anticipate that we will be able to complete the experiments concerning the importance of catalase (*CpeB*) for *Xf* survival in grapevines by December 2012.

During the past year, we also initiated the insect transmission studies for the *oxyR* mutant at UC Riverside. In cooperation with Dr. Matt Daugherty (UC Riverside Department of Entomology), we conducted insect acquisition and transmission studies using the glassy-winged sharpshooters (*Homalodisca vitripennis*; GWSS) that were collected and reared at the UC Riverside Agricultural Operations facility. For this analysis, we are using the artificial feeding sachet technique developed by Killiny and Almeida (2009), 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. Briefly, we confined individual sharpshooter adults in a feeding apparatus for 8 hours, which contained the artificial diet solution alone or a solution inoculated with either WT or *oxyR*. The sharpshooters were then transferred to healthy grape seedlings for four days. Insect acquisition rates were assessed by monitoring the presence or absence of *Xf* in sharpshooter heads using conventional PCR and the *Xf*-specific detection primers HL-5 and HL-6 (Francis et al., 2006). As shown in **Figure 4A**, *Xf* was detected in GWSS sharpshooters fed with either the WT or the *oxyR* mutant.

We also initiated studies to look at the impact of the *oxyR* mutation on *Xf* transmission by the GWSS. At eight and nine weeks, we were not able to detect WT or *oxyR* in the grapevine seedlings that the GWSS fed upon. This may simply be a consequence of the low rate of *Xf* transmission by the GWSS. We are currently incubating the plants longer in the growth chamber, which may allow *Xf* titer to reach a detectable level.

Due to the inherently low rate of *Xf* transmission by the GWSS, we are opting to move to a vector with a higher *Xf* transmission rate, the blue-green sharpshooter (*Graphocephala atropunctata*; BGSS). In cooperation with Dr. Tom Perring (UC Riverside Department of Entomology), we have initiated a BGSS colony at UC Riverside and will use these for future insect acquisition and transmission studies. This will allow us to more accurately quantify differences in acquisition and transmission rates between the WT and the *oxyR* mutant. In our pilot study, we confirmed that the artificial feeding system developed for GWSS (Killiny and Almeida, 2009) worked for BGSS with minor modifications. Specifically, we used an acquisition access period of six hours. The presence of *Xf* in the heads of the BGSS sharpshooters was then detected using the *Xf*-specific detection primers HL-5 and HL-6 (**Figure 4B**). Based on the success of our pilot study, we have initiated a full insect acquisition/transmission experiment. In this experiment, we have also included a multiplication period where fed BGSS are kept on healthy basil plants for 48 hours prior to moving them to healthy grapevine seedlings.

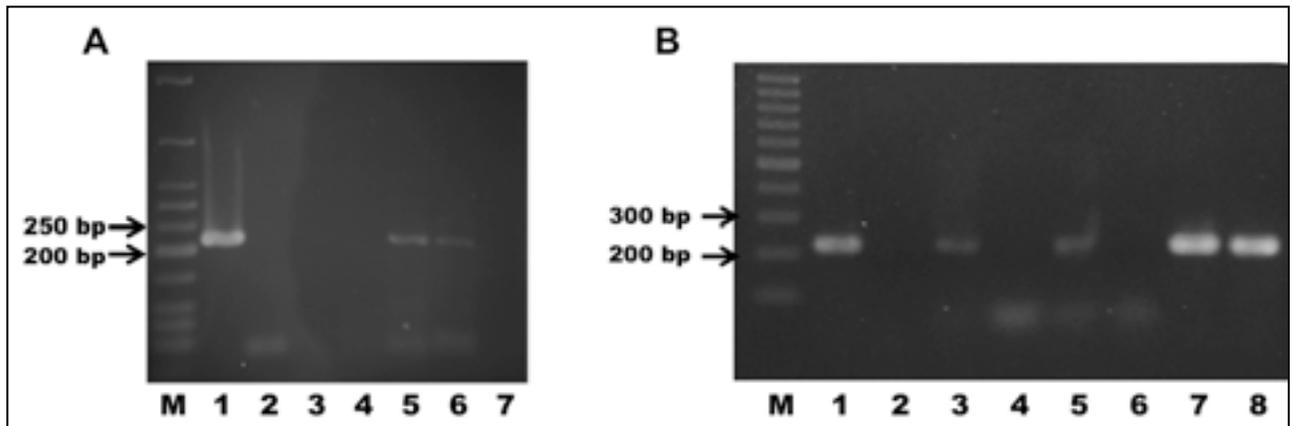


Figure 4. Using PCR to detect the presence of *Xf* in insect heads. PCR was used to detect the presence of *Xf* in the heads of two different types of sharpshooters: GWSS (A) and BGSS (B). The PCR products were separated by electrophoresis on 1.7% agarose gels. The lanes in **Figure 4A** contain the following: NEB Low Molecular Weight DNA ladder (Lane M); Positive control-WT *Xf* genomic DNA (Lane 1); Negative control-water (Lane 2); GWSS fed with diet solution only (Lane 3); GWSS fed with WT *Xf* (Lanes 4 & 5); GWSS fed with *Xf oxyR* mutant (Lanes 6 & 7). The lanes in **Figure 4B** contain the following: Fermentas 100 bp DNA ladder (Lane M); Positive control-WT *Xf* genomic DNA (Lane 1); Negative control-water (Lane 2); BGSS fed with WT *Xf* (Lanes 3, 4 & 5); BGSS fed with diet solution only (Lane 6); Positive Control, sharpshooter head manually mixed with *Xf* cells (Lanes 7 & 8).

Objective 5: Develop a bioluminescent (Lux) reporter system for *Xf*

Fusions to luciferase are excellent tools for tagging bacteria for *in vivo* studies and for monitoring dynamic changes in transcript or protein abundance both *in vitro* and *in vivo* (Gheysens and Mottaghy, 2009). The advantage of using the Lux system is that organisms produce light without the need of an exogenous substrate. We have constructed plasmids that carry various *Xf* promoters upstream of the Lux operon. However, when introduced into *Xf*, none of these constructs result in bioluminescence. The next step will be to determine if the *lux* operon is transcribed from the selected *Xf* promoters by conducting qRT-PCR analysis.

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

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

ABSTRACT

Previously we demonstrated that twitching motility in *Xylella fastidiosa* (*Xf*) is dependent on an operon, named Pil-Chp, encoding signal transduction pathway proteins (*pilG*, *pilI*, *pilJ*, *pilL*, *chpB* and *chpC*), which is related to the system that controls flagella movement in *Escherichia coli*. We report five advances in examining this system. First, we finished studying the Pil-Chp operon mutants *in planta*. We have shown that the first four genes are critical for twitching and that all genes play a role in biofilm formation and Pierce's disease symptoms. Second, we have found that the Pil-Chp operon and other type IV pili genes are down-regulated in sap from Pierce's disease resistant *Vitis* genotypes. Third, we are producing clean type I and type IV pili knockout *Xf* strains as controls for understanding how the Pil-Chp operon regulates pili protein expression. Fourth, we have explored the *chpY* gene, a putative degenerative GGDEF-EAL protein, which lies near the Pil-Chp operon and plays a role in Pierce's disease development. Fifth, we have created knockout *Xf* mutants in predicted virulence genes and have preliminary evidence on their role in Pierce's disease development.

LAYPERSON SUMMARY

This project involves studying the chemical sensing pathway by which the plant pathogen *Xylella fastidiosa* is able to control its movement within the plant environment. We examined a gene cluster essential for cell movement (twitching motility) and we explored expression of movement genes in sap from grapevines resistant or susceptible to Pierce's disease. In addition we are building strains to explore how motility genes regulate pili expression, exploring a gene that may be a global regulator of disease, and examining predicted virulence genes for their importance in symptom development. These results give insight into targets for preventing Pierce's disease.

INTRODUCTION

Bacteria sense and respond to changes in their environment, integrating the signals to produce a directed response. *Xylella fastidiosa* (*Xf*) is a non-flagellated, xylem-restricted Gram-negative bacterium that moves within grapevines via twitching motility that employs type I and type IV pili (Meng et al. 2005). Movement appears to be controlled by a system with similarities to that first reported in *Escherichia coli*, in which a group of *che* genes regulates the rotational movement of flagella. Transmembrane chemoreceptors bind chemical stimuli in the periplasmic domain and activate a signaling cascade in their cytoplasmic portion to ultimately control the direction of flagella rotation (see review Hazelbauer et al. 2008). We previously found that the

homologous gene cluster is an operon (named Pil-Chp) that regulates type IV pili, and that disruption of the operon leads to a decrease in Pierce's disease symptoms (**Figure 1**). Herein, we further characterize the genes in the Pil-Chp operon and describe our advances in understanding of genes involved in Pierce's disease development.

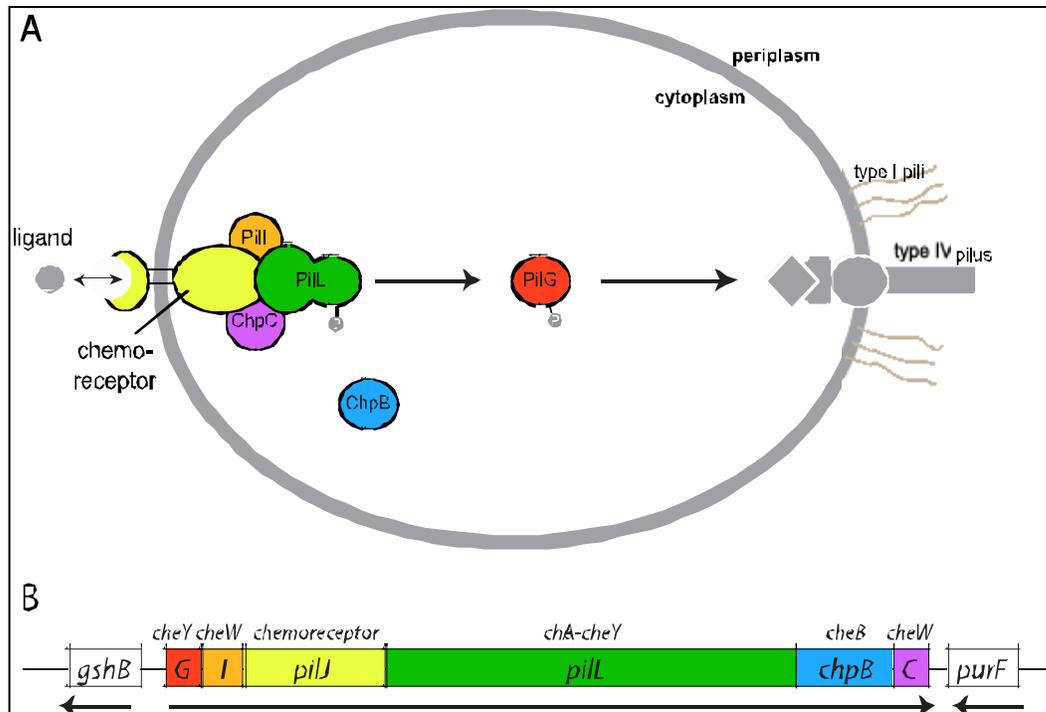


Figure 1. The *Xf* Pil-Chp operon. A) Model for operon protein products regulating twitching motility in *Xf*. The chemoreceptor PilJ senses environmental signal(s). ChpC/PilI couples PilL to PilJ. PilL phosphorylates its hybrid CheY-like receiver domain and PilG. ChpB is homologous to ligand adaptation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

OBJECTIVES

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

RESULTS AND DISCUSSION

In planta results with Pil-Chp operon null mutant strains

We previously discovered that the Pil-Chp transposon polar mutant cells induced less Pierce's disease symptoms *in planta* compared to inoculation with wild-type cells (Cursino et al. 2011). We completed our analysis of the

mutants in plants. Twitching minus non-polar Pil-Chp operon mutants *pilG*, *pilI*, and *pilJ* induced less Pierce's disease *in planta* and symptoms plateaued after 20 weeks. Conversely, the Pil-Chp operon twitching plus *chpB* and *chpC* mutants induced full Pierce's disease symptoms, however the disease was delayed compared to wild-type cells. We tested the non-polar *pilL* mutant *in planta* (Figure 2) and found that it gave similar results as non-motile mutants *pilG*, *pilI*, and *pilJ*.

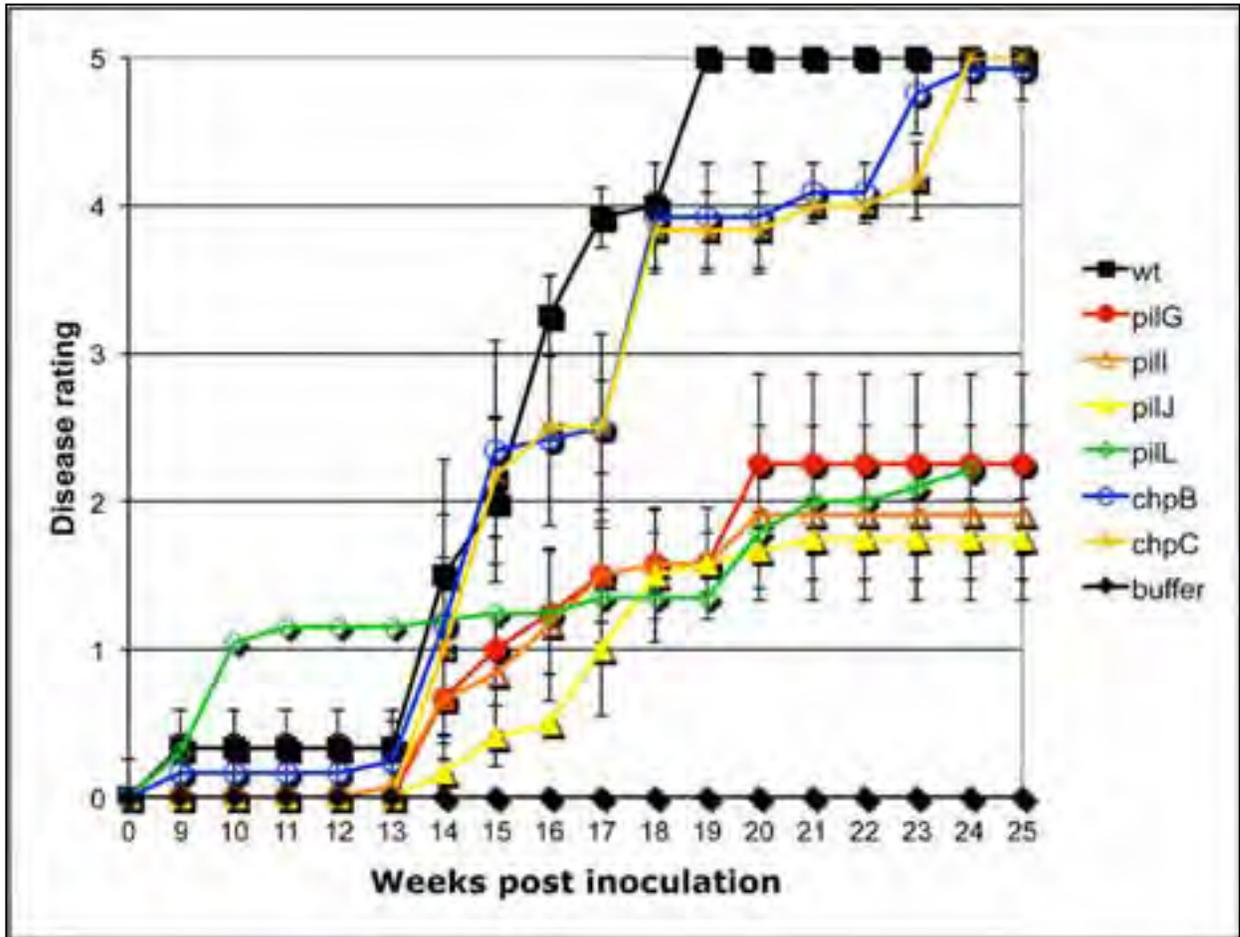


Figure 2. Pierce's disease development in Pil-Chp operon gene mutants. *Vitis vinifera* L. cv. Cabernet Sauvignon vines were inoculated with wild-type or Pil-Chp non-polar gene mutants and Pierce's disease was assessed over 25 weeks. Plant rating on a scale of 0-5 (Guilhabert and Kirkpatrick 2005).

Pil-Chp mutants and pili results

Disruption of the Pil-Chp operon could result in non-motility due to a number of factors. We examined the pili levels of mutants by TEM. Non-motile mutants either lacked pili or appeared hyperpiliated (data not shown), whereas motile mutants appeared like wild-type cells. Surprisingly, the cells lacking pili lacked both type I and type IV pili, suggesting that the Pil-Chp operon regulated both pili types. To further explore if the Pil-Chp operon mutant regulates pili expression, we have begun constructing clean type I pili (*fimA*) and type IV pili (*pilA*) knockout mutants as controls (Meng et al., 2005; Da Silva Neto et al., 2008). Once completed, we will test the mRNA expression of *fimA* and *pilA* in the various Pil-Chp mutants.

Pil-Chp genes and Pierce's disease resistant sap

Certain *Vitis* species exhibit either resistance or tolerance to Pierce's disease, however it is not known which mechanism(s) influence susceptibility. We examined the influence of xylem sap from susceptible and resistant grapevines on *Xf* behavior. *Xf* had reduced motility, aggregation, and biofilm formation in saps from resistant

genotypes. We also found that resistant saps lead to downregulation of numerous type IV pili genes (**Figure 3**): Pil-Chp chemotaxis homologous genes (*pilG*, *pilI*, and *pilJ*), type IV pili structural genes (*pilQ* and *pilA*), and type IV pili regulatory genes (*pilR*) (Meng et al., 2005; Li et al., 2007; da Silva Neto et al., 2008; Cursino et al., 2011).

Pil-Chp operon and *chpY* gene

The *chpY* gene lies downstream of the Pil-Chp operon and appears to be a degenerate GGDEF-EAL hybrid protein. GGDEF domains are found in diguanylate cyclases that catalyze the formation of c-di-GMP while EAL domains are found in phosphodiesterases involved in the degradation of c-di-GMP. C-di-GMP is a second messenger that is known to regulate bacteria motility and biofilm formation. Deletion of *chpY* results in loss of motility *in vitro* without altered pili biogenesis, increased biofilm formation, and increased Pierce's disease progression *in planta* (data not shown). The mutation in *chpY* also leads to increased expression of biofilm associated *gum* genes (data not shown) and extracellular polymeric substance (EPS) production but decreased pectin methylesterase production (**Table 1**). The numerous responses related to the *chpY* mutant suggest that ChpY has a global regulatory role, and degenerative GGDEF-EAL hybrid proteins have been implicated in regulation processes (Sondermann et al., 2012; Suzuki et al. 2006).

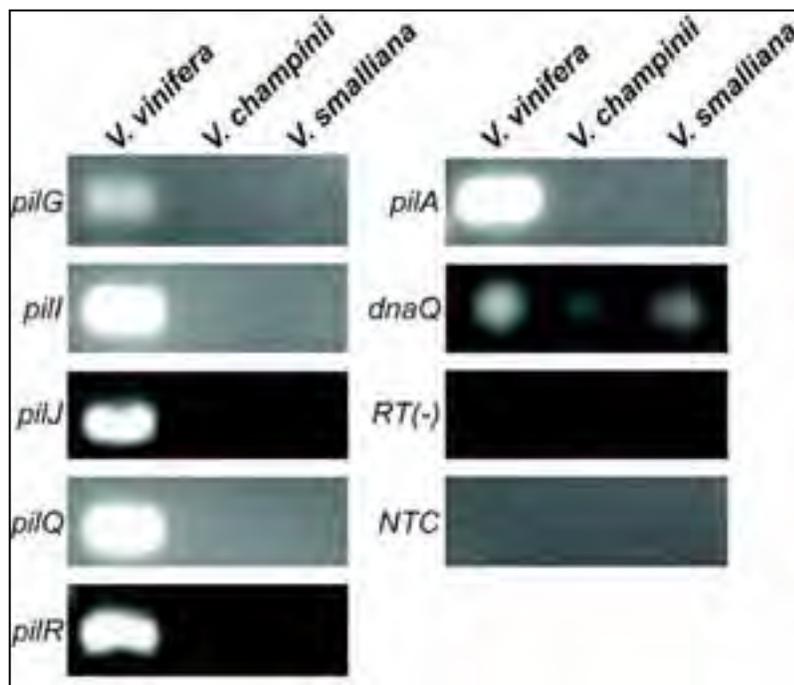


Figure 3. Expression of type IV pili-related genes in Pierce's disease susceptible sap (*V. vinifera*) and Pierce's disease resistant saps (*V. champinii* and *V. smailiana*) by reverse transcriptase polymerase chain reaction (RT-PCR). Positive control was *dnaQ* expression and negative controls were RT(-) and NTC, where RT(-) was a reaction with all components except reverse transcriptase, and NTC was a reaction with all components except RNA template.

Virulence gene studies

PD1199 and PD1311 encode a putative protein disulfide isomerase (PDI) and a putative non-ribosomal peptide synthetase (NRPS) respectively. Both proteins are predicted to play a role in the production of secondary metabolites that may have a role in virulence. Non-ribosomal peptide synthetases have been reported to affect secondary metabolite production in organisms such as *Pseudomonas syringae* pv. *syringae* where deletion of NRPS resulted in reduced virulence (Arrebola et al. 2007). PDI plays a role in maturation of proteins and mutations in PDI encoded by *Pseudomonas aeruginosa* resulted in attenuation of virulence on plants and

animals (He et al. 2004). We have created deletion mutants in both *Xf* PDI and NRPS genes and are currently determining the role of these proteins in virulence on grapevines.

Table 1. Release of virulence-related extracellular products by *Xf*.

Strains	Extracellular products ^a					
	CMCase ^b	EPS ^c	Mannanase	PG ^d	PME	Pr
Wild-type	1.01 ± 0.20	3.85 ± 0.78	0.0 ± 0.0	0.0 ± 0.0	3.2 ± 0.70	2.12 ± 0.2
<i>chpY</i>	1.1 ± 0.30	12.8 ± 1.08 ^e	0.0 ± 0.0	0.0 ± 0.0	2.3 ± 0.51 ^f	2.2 ± 0.3
<i>chpY-C</i> ^g	1.2 ± 0.25	3.98 ± 0.23	0.0 ± 0.0	0.0 ± 0.0	3.25 ± 0.83	2.10 ± 0.25

^a CMCase (carboxymethylcellulase), EPS (exopolysaccharide), endo- β -mannanase (mannanase), PG (polygalacturonase), PME (pectin methylesterase), and Pr (protease).

^b Extracellular enzyme activity for CMC, Mannanase, PME, and PTA were estimated from the diameter (mm) of zones surrounding each well. Enzyme experiments were done three times, with five replicate plates each. The standard deviations of the means for the enzyme in each assay are shown.

^c Average of dry weight of EPS (mg/ml). The experiments were done three times, with three replicates each. The standard deviations of the means are shown.

^d PG activity values (U/ml) were measured according to Taylor and Secor (1988).

^e Statistically significant compared to wild type ($X^2=6.56$, $P=0.01$).

^f Statistically significant compared to wild type ($X^2=5.05$, $P=0.03$).

^g Complemented *chpY* mutant.

CONCLUSIONS

Our results with the Pil-Chp mutants show that the operon is required for twitching motility in *Xf*. Interestingly, some of the genes in the operon may not play a role in twitching motility but all play a role in biofilm formation and Pierce's disease development. We are building control strains to explore the relationship between Pil-Chp and regulation of pili expression. We discovered that a component(s) in sap from Pierce's disease resistant grapevines turns off pili expression or from Pierce's disease susceptible grapevines turns on pili expression. Additionally, we report that *chpY* contributes to Pierce's disease development by regulating the gum proteins, extracellular polymeric substance, and pectin methylesterase, which leads to an increase in Pierce's disease symptoms. Finally, our studies have uncovered two proteins potentially involved in virulence factor production that are involved in Pierce's disease.

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CHARACTERIZATION OF *XYLELLA FASTIDIOSA* GENES REQUIRED FOR PATHOGENICTY

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ABSTRACT

Xylella fastidiosa (*Xf*) is a gram-negative, xylem-limited plant pathogenic bacterium and the causal agent of Pierce's disease of grapevine (Wells et al., 1981). *Xf* is closely related to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Recent findings indicate that the sulfated Type 1 secreted protein Ax21 is required for density-dependent gene expression and consequentially pathogenicity of *Xoo*. Two two-component regulatory systems (TCSs) are required for Ax21 mediated immunity: PhoP/Q and RaxR/H. Like many other bacteria, *Xf* possesses homologs to the two component regulatory system phoP/Q which differentially regulates genes in responses to divalent periplasmic cation concentration and other environmental stimuli. *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. Dispersed growth and lack of cell clumping are phenotypes of *Xf* mutants deficient in hemagglutinins; however western blot analysis showed both *Xf* hemagglutinins are expressed in the *Xf* phoP/Q mutants. Grapevine pathogenicity assays showed phoP/Q mutants are non-pathogenic and are unable to successfully colonize or move within the xylem vessels. These results may be due to the inability of *Xf* to successfully sense, respond, and adapt to the nutrient-poor environment of the xylem. A second TCS, ColR/S, plays a role in *Xf* density-dependent processes although further research is required. Based on research conducted to date, we have not yet identified the functional role of *Xf* Ax21.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) is a plant pathogenic bacterium and the causal agent of disease in a variety of economically important crops, including Pierce's disease of grapevine. *Xf* causes disease by colonizing the xylem vessels, blocking the flow of water in the grapevine. In many plant pathogenic bacteria communication among organisms plays an important role in successful colonization of the host environment. This communication may be mediated by small molecules released by neighboring bacteria in the environment. We are currently investigating the role of one communication compound, Ax21, and the means by which *Xf* recognizes Ax21. Bacteria must also be able to sense changes in the environment in order to adapt to stress such as nutrient starvation. One system used by bacteria to sense the environment is PhoP/Q, which senses specific nutrients and regulates responses in the bacteria. Loss of this sensing system renders *Xf* unable to induce Pierce's disease symptoms or survive in grapevines. Furthermore, loss of PhoP/Q alters the normal growth of *Xf*.

INTRODUCTION

Xylella fastidiosa (*Xf*) is a gram-negative, xylem-limited plant pathogenic bacterium and the causal agent of Pierce's disease of grapevine (Wells et al., 1981). *Xf* is found embedded in the plant matrix in clumps, which leads to the xylem vessel blockage. The formation of biofilms allows for bacteria to inhabit an area different

from the surrounding environment, potentially protecting itself from a hostile environment. Furthermore, biofilm formation is an important factor in the virulence of bacterial pathogens. Biofilm formation is a result of density-dependent gene expression (Morris and Monier, 2003). Density-dependent biofilm formation is triggered by the process of quorum sensing (QS). In QS, bacteria are able to communicate with each other via small signal compounds, generically called “auto-inducers” and the specific case of *Xanthomonas* and *Xylella* the molecules are referred to as diffusible signal factors (DSF). The auto-inducer is a means by which bacteria recognize population size, and mediate the expression of specific genes when bacterial populations reach a threshold concentration (Fuqua and Winans, 1994; Fuqua et al., 1996).

In *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), Ax21 is a sulfated, Type 1 secreted protein that is a quorum sensing compound. Ax21 was recently shown to be a requirement for induction of density-dependent gene expression, including biofilm formation (Lee et al., 2006; Lee et al., 2009). In *Xoo*, two two-component regulatory systems (TCSs) required for Ax21-mediated activity have been found and orthologs of the TCSs and Ax21 were identified in the *Xf* genome (Simpson et al., 2000). In order for an active Ax21 gene product to be produced, two TCSs are required: RaxR/H and PhoP/Q (Burdman et al., 2004; Lee et al., 2008).

The 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+} , among 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 (Groisman 2001). 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 of high Mg^{2+} inhibit the PhoP/Q system through dephosphorylation of PhoP (Groisman 2001). The goal of this research is to investigate the role of Ax21 and the associated two-component regulatory systems in *Xf*.

OBJECTIVES

1. Determine the functional role of the Ax21 homolog in *Xf*.
2. Determine the functional role of the RaxR/RaxH homologs 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 assays, there are no differences between *Xf*Δ*ax21* and wild type *Xf* (**Figure 2**). There was no significant difference (95% CI) in biofilm formation or cell aggregation between *Xf* fetzer (wt) and *Xf* Δ*ax21* (**Figure 1, 2**). Pathogenicity assays on Thompson seedless grapevines were conducted in the greenhouses in 2010 and 2011. We found no significant differences in colonies isolated from the point of inoculation or 25cm above the point of inoculation (**Figure 7**). Furthermore, we found similar levels of disease severity in *Xf* Δ*ax21* and wt *Xf* 18 weeks post-inoculation (**Figure 8**). We are repeating pathogenicity assays with low-density inoculum to look at the potential role of Ax21 as a low-density quorum sensing compound.

To test if *Xf* has Ax21 activity recognized by the XA21 protein in rice, we carried out an Ax21 activity assay (Lee, et al., 2006). Rice leaves from TP309, susceptible to *Xoo* PXO99, and TP309-XA21, resistant to PXO99, were cut at the tip and pretreated with supernatants from wild type *Xf* and *Xf*Δ*ax21* (knockout mutant). Supernatants from *Xoo* PXO99 and PXO99Δ*ax21* were used as positive and negative controls, respectively. Five hours later the pretreated leaves were inoculated with the *raxST* knockout strain (PXO99Δ*raxST*), which lacks Ax21 activity. Ax21 activity was evaluated by measuring lesion lengths three weeks after inoculation. If *Xf* had Ax21 activity, leaves of TP309-XA21 pretreated by supernatant from wild-type *Xf* would show resistance to subsequent inoculation by PXO99Δ*raxST* strain. However, both leaves pretreated by supernatants from *Xf* and *Xf*Δ*ax21* were susceptible to PXO99Δ*raxST*. This result suggests that *Xf* Ax21 is unable to trigger XA21-mediated immunity in the rice plant bioassay. A lack of secretion and/or sulfation system in *Xf* may be the

cause of the lack of Ax21 activity. We are currently in the process of determining whether or not Ax21 is secreted from *Xf*.

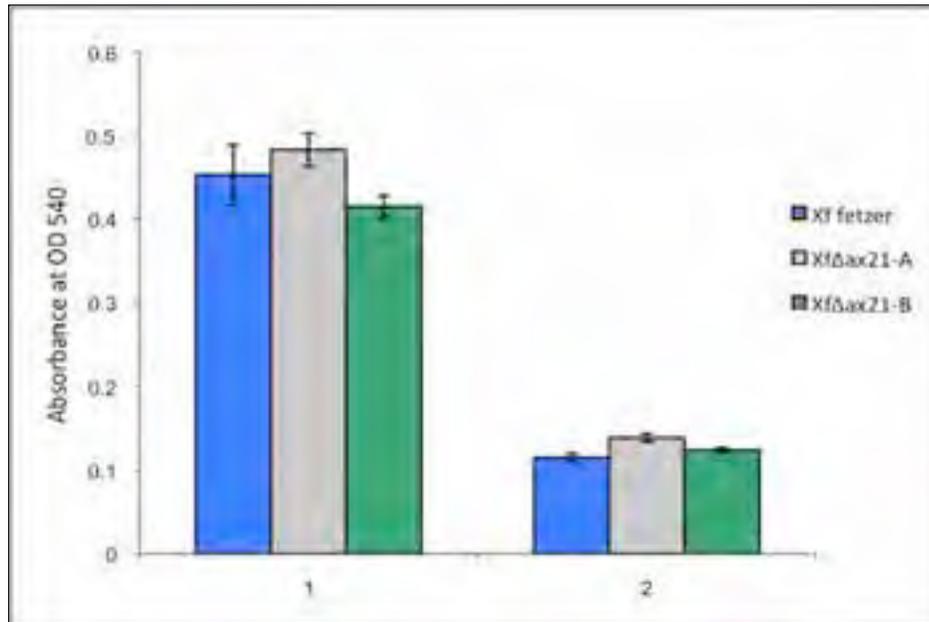


Figure 1. Comparison of biofilm formation in wild-type *Xf fetzer*, *XfΔax21-A*, and *XfΔax21-B* in stationary cultures as determined by the crystal violet staining method. Values shown are the means of 10 samples +/- error.

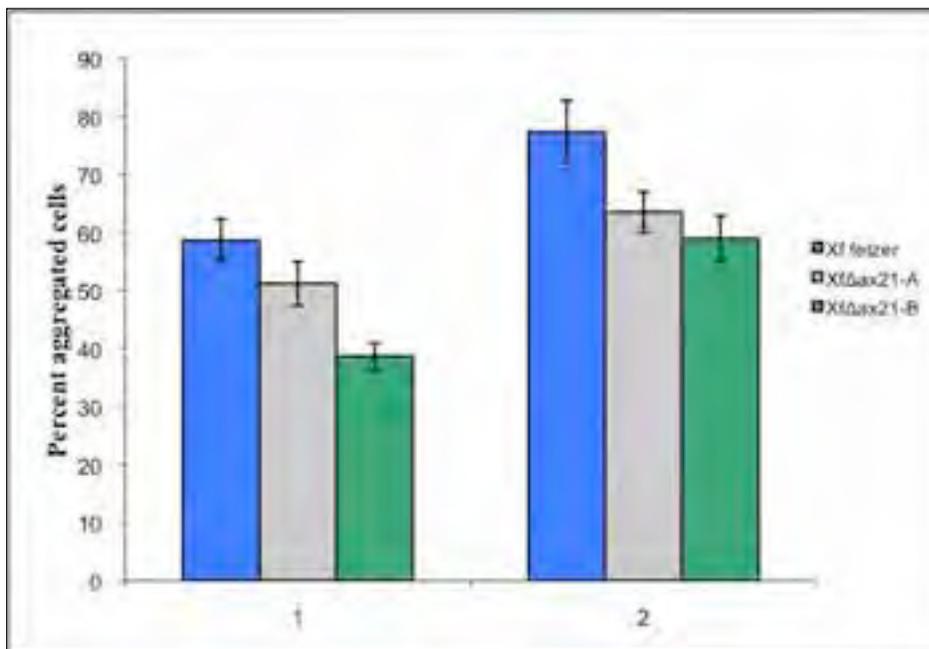


Figure 2. Comparison of percent aggregated cells in wild-type *Xf fetzer*, *XfΔax21-A*, and *XfΔax21-B*. Percentage of aggregated cells was determined as described by Guilhabert and Kirkpatrick, 2005. Values shown are the means of 10 samples +/- error.

Objective 2

We have begun characterizing the RaxR/RaxH homologs, *XfΔcolR* and *XfΔcolS* respectively. ColR is the response regulator and ColS is the histidine kinase. Mutation of *XfΔcolR* was lethal, although the *XfΔcolS* mutant was viable. TCS's can often be regulated by another TCS, thus based on the 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 biofilm formation or cell growth but did exhibit a significant reduction in biofilm formation. Pathogenicity assays with *XfΔcolS* are currently underway and will be completed this fall.

Objective 3

Both *XfΔphoP* and *XfΔphoQ* were found to have significantly less (95% CI) biofilm formation than wt *Xf* after ten days static incubation using the crystal violet assay (**Figure 4**). Furthermore, we also found that *XfΔphoP* and *XfΔphoQ* had significantly (95% CI) less cell-aggregation (**Figure 6**). We also found that there was no significant difference in biofilm formation or cell-cell aggregation between *XfΔphoP* and *XfΔphoQ*. This result would be expected, since PhoP and PhoQ collectively make up a two-component regulatory system (TCS). A mutant deficient in one gene should exhibit the same phenotype as a mutant deficient in the second gene of the TCS. When grown in culture, *XfΔphoP* and *XfΔphoQ* 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 3**). 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 (Guilhabert and Kirkpatrick 2005; Voegel et. al. 2010), secreted proteins were purified from *Xf* wild-type Fetzer, *XfΔax21*, *XfΔphoP*, and *XfΔphoQ* cells. Western blot analysis confirmed the presence of *Xf* hemagglutinin A and B in wild-type and all three mutants, thus the observed decrease in clumping is not due to lack of hemagglutinin proteins (**Figure 5**).

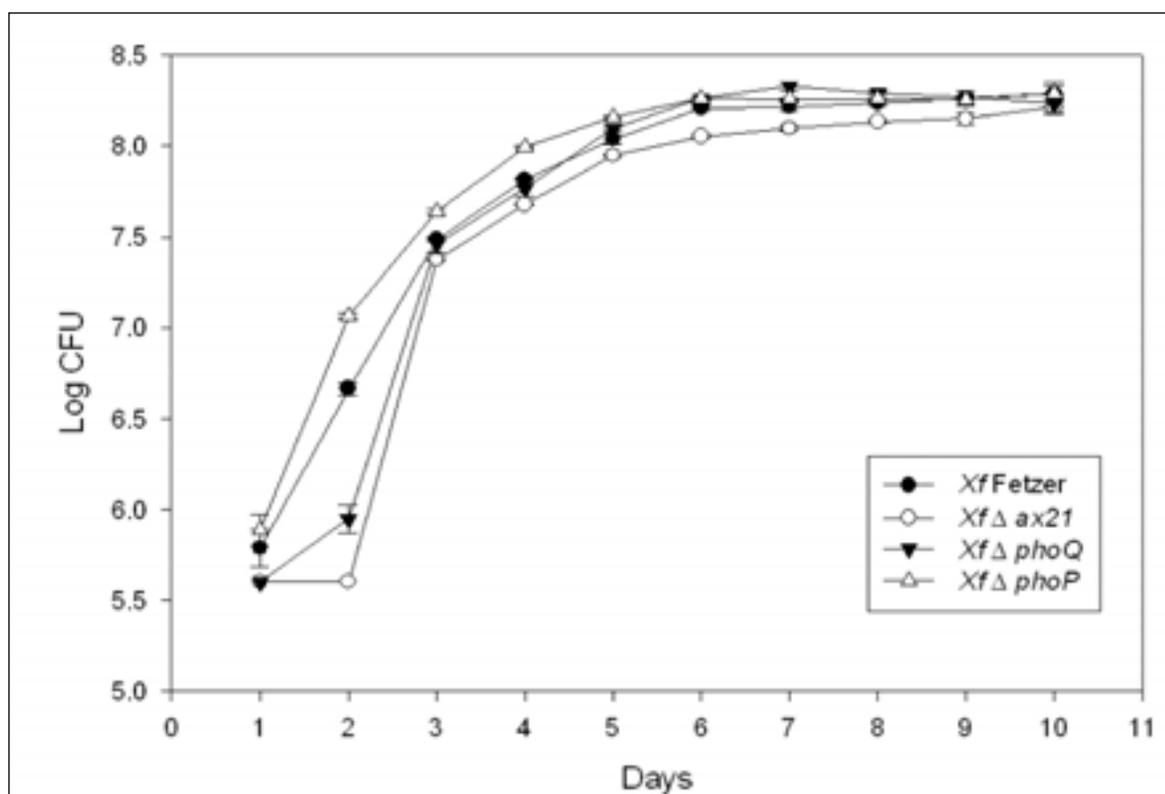


Figure 3. Bacterial growth of wild type *Xf* fetzer, *Xf* Δ*ax21*, *Xf* Δ*phoQ* and *Xf* Δ*phoP*. Values shown are mean of five samples +/- standard error. The assay was conducted twice.

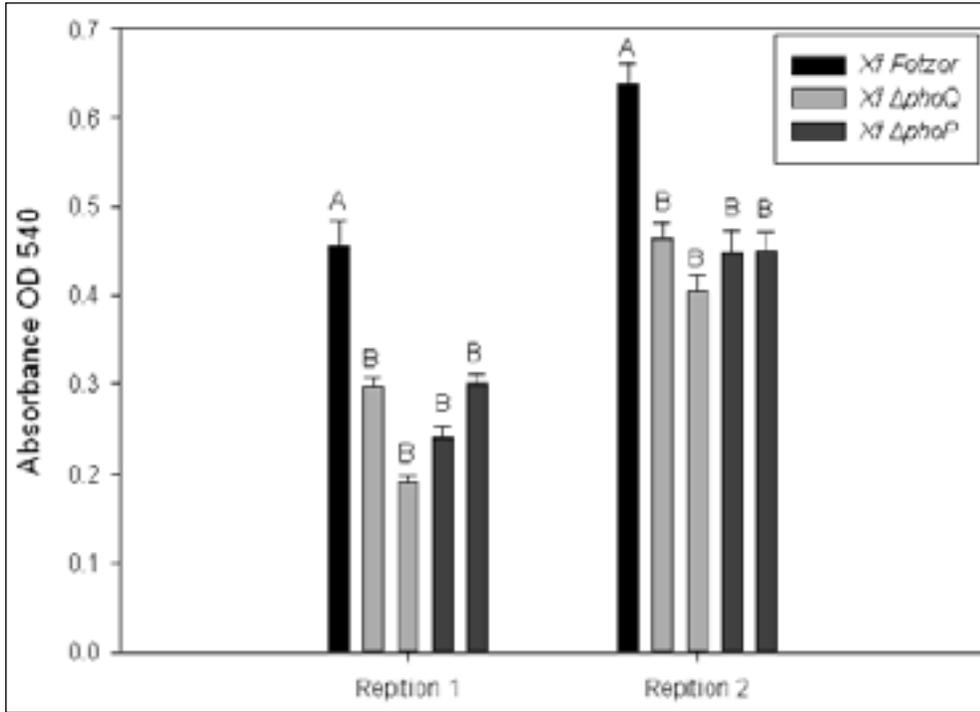


Figure 4. Comparison of biofilm formation by wild type *Xf fetzer*, *Xf ΔphoP* and *Xf ΔphoQ* mutants after 10 days growth in static liquid culture. Values shown are mean of 10 samples +/- standard error.

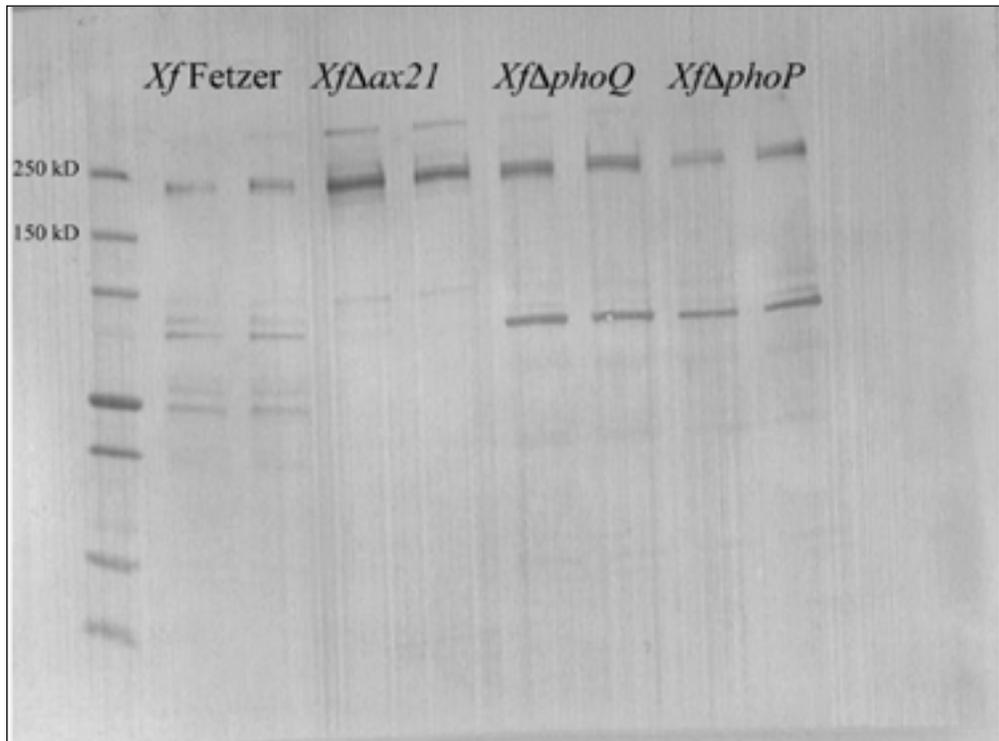


Figure 5. Western blot on type *Xf fetzer*, *Xf Δax21*, *Xf ΔphoQ*, *Xf ΔphoP* with *Xf* hemagglutinin antibodies showing hemagglutinin bands at 225 kD.

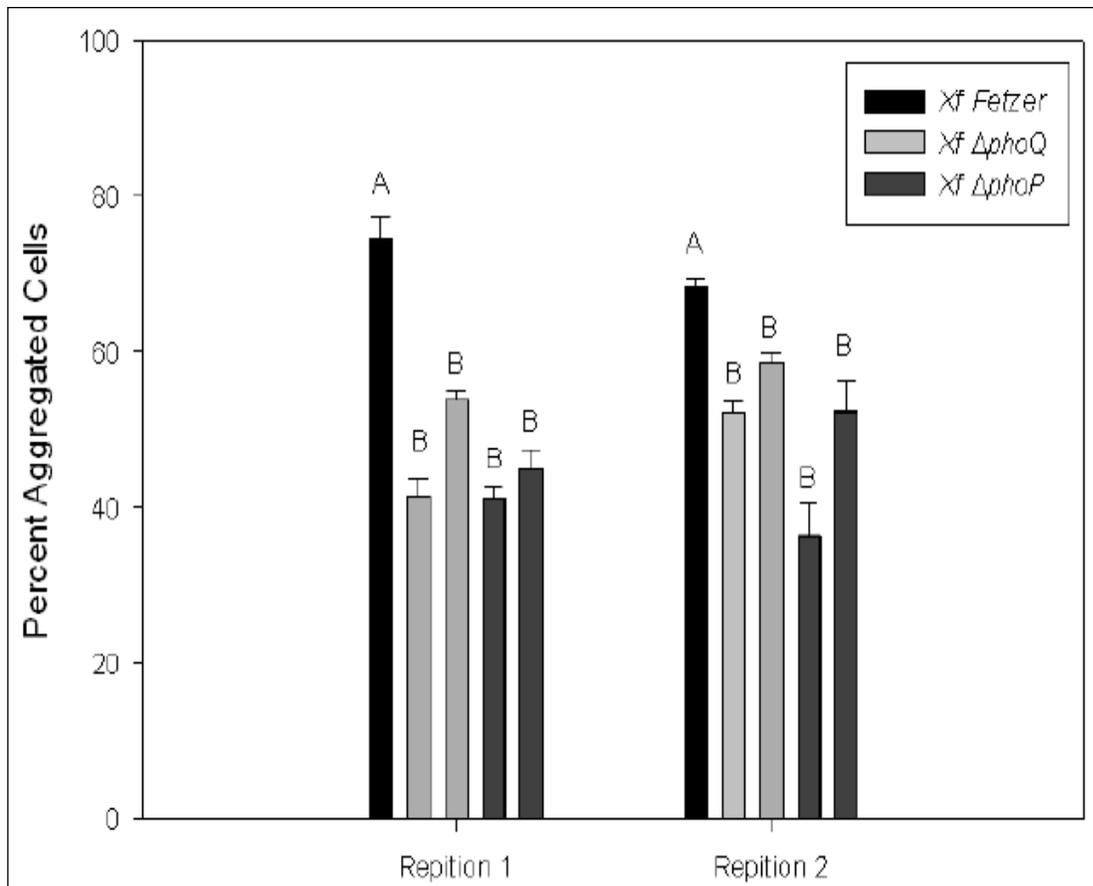


Figure 6. Comparison of percent aggregated cells by wild type *Xf fetzer*, *Xf ΔphoP* and *Xf ΔphoQ* mutants after 10 days growth in static liquid culture. Values shown are mean of 10 samples +/- standard error.

Pathogenicity assays on Thompson seedless grapevines were conducted in the greenhouse in 2011 and 2012 (not yet completed). We found no colonies isolated from the point of inoculation or 25cm above the point of inoculation (**Figure 7**). Furthermore, we found significantly reduced levels of disease severity compared to grapevines inoculated with wt *Xf* 18 weeks post-inoculation (**Figure 8**).

Although we isolated no live colonies from grapevines inoculated with *Xf ΔphoP*, and *Xf ΔphoQ*, it appeared that the vines exhibited mild Pierce's disease-like related symptoms. These mild symptoms are most likely due to a variety of non-Pierce's disease issues that were stressing the grapevines including nutrient deficiency, insect damage and scorching from the greenhouse lamps. Immunocapture PCR confirmed the inability of *Xf ΔphoP* and *Xf ΔphoQ* to move within the grapevine (**Figure 9**). In 2012, we repeated pathogenicity assays in the greenhouse with *Xf ΔphoP* and *Xf ΔphoQ* and our results through 18 weeks post-inoculation are in agreement with the absence of disease symptoms observed in 2011. The 2012 pathogenicity assays will be completed this fall.

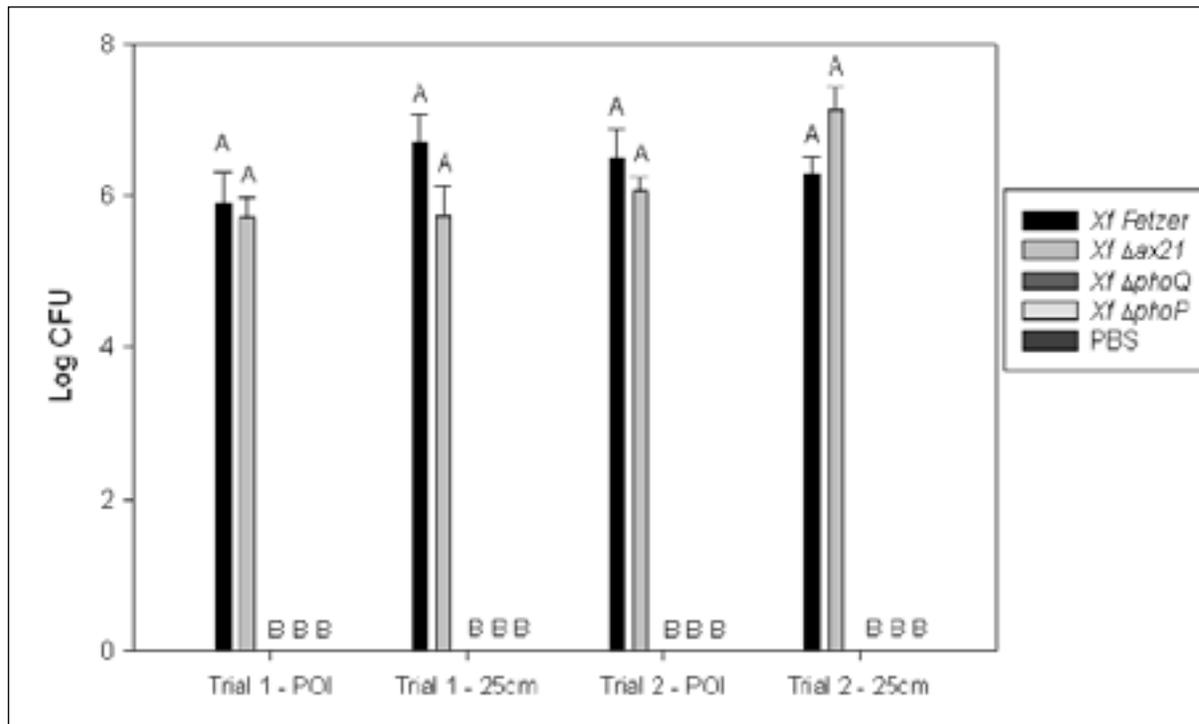


Figure 7. Log CFU isolated from Thompson Seedless grapevines inoculated with wild type *Xf fetzer*, *Xf Δax21*, *Xf ΔphoQ*, *Xf ΔphoP* and PBS (negative control) 18 weeks post-inoculation. Values shown are mean +/- standard error.

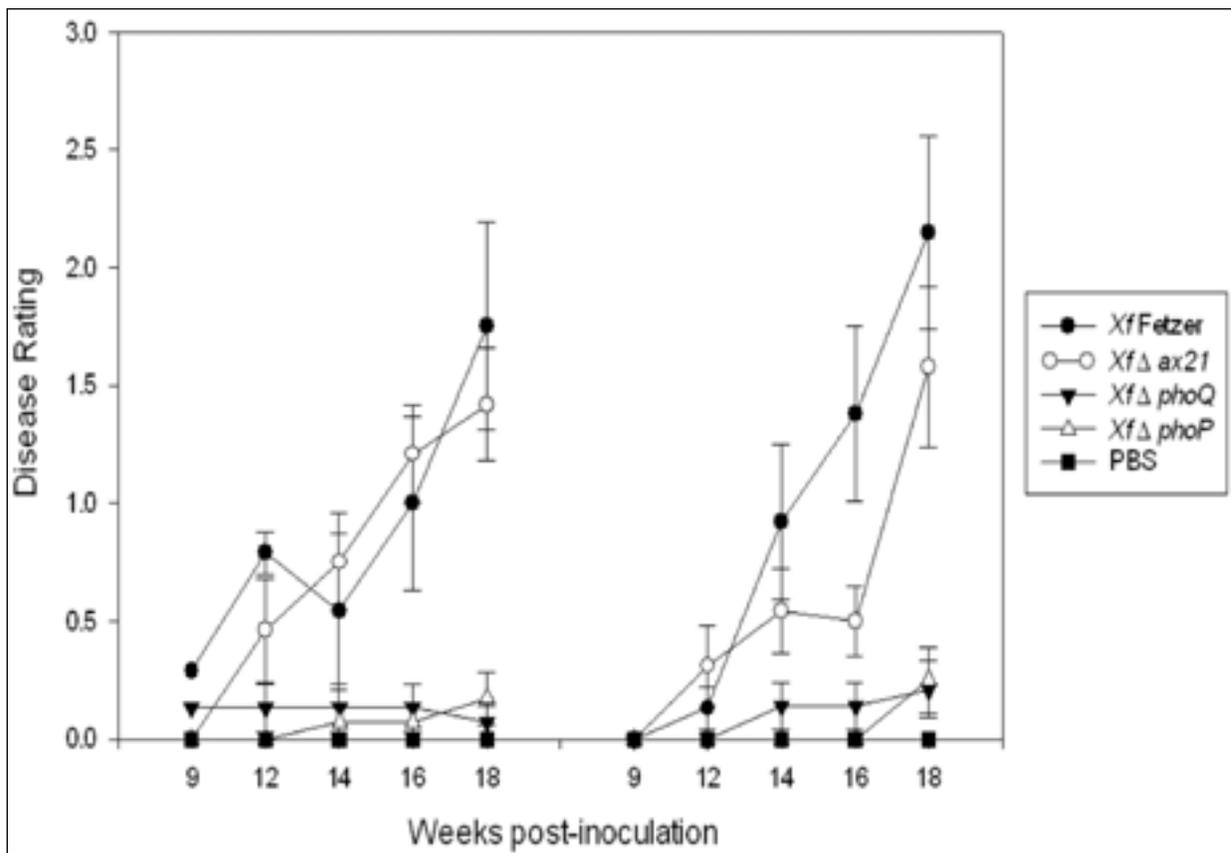


Figure 8. Disease ratings of Thompson Seedless grapevines inoculated with *Xf* wt, mutants or PBS (negative control) 18 weeks post-inoculation. Values shown are mean +/- standard error.

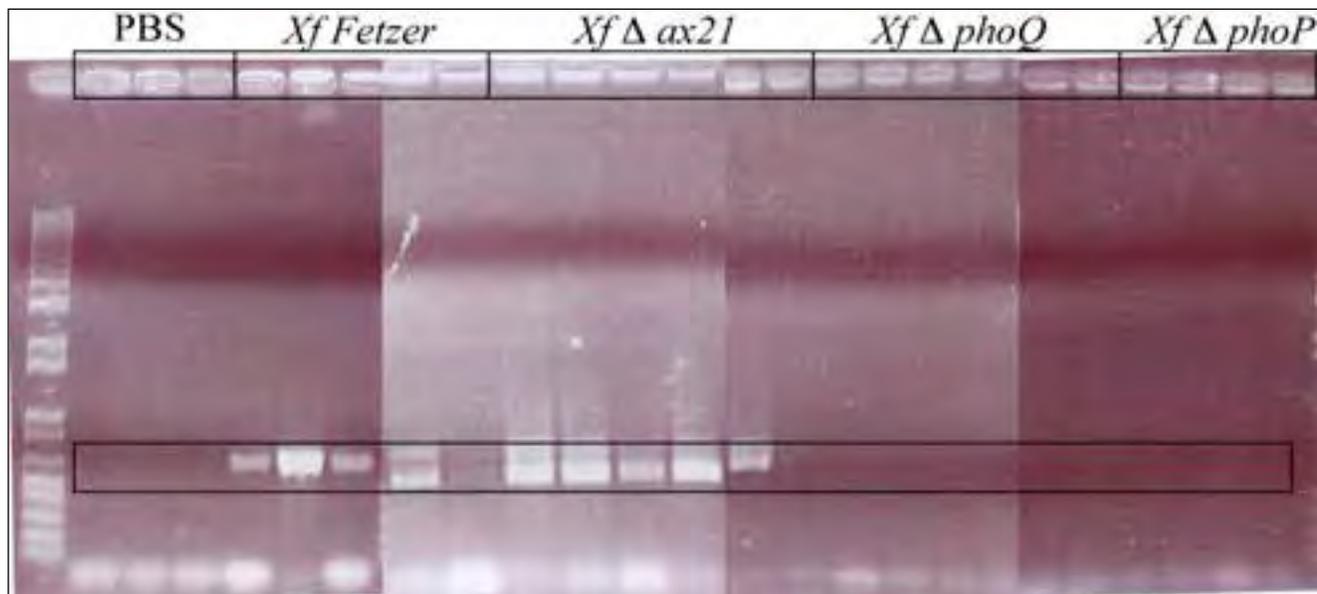


Figure 9. Gel electrophoresis of IC-PCR reaction with primers RST31 & RST33 on Thompson seedles grapevines inoculated with wild type *Xf fetzer*, *Xf Δax21*, *Xf ΔphoQ*, *Xf ΔphoP* and PBS (negative control weeks post-inoculation in the greenhouse).

CONCLUSIONS

We have made good progress on investigating the functional role of a potential peptide-based QS system in *Xf*. Thus far, we have found no role of Ax21 in *Xf*, but based on the role of Ax21 at low-cell densities in *Xoo* it is likely that the functional role of *Xf* Ax21 is apparent at low-cell density. In *Xf*, loss of PhoP/Q results in significant reductions in biofilm formation and cell-cell aggregation both of which are significant factors in successful colonization of the grape xylem. The grape xylem is a nutritionally limited environment that requires *Xf* to efficiently uptake nutrients. *Xf* mutants deficient in PhoP/Q are nonpathogenic and unable to colonize the xylem. Research conducted thus far is in agreement with the role of PhoP/Q as a regulator of virulence, survival and physiological response genes in other well-studied systems such as *Salmonella spp.*, *E. coli*, and *Xoo*. We are looking into the use of PhoP/Q as a potential control strategy. We are also further investigating the regulatory network of PhoP/Q in *Xf* to better understand the genes under the control of PhoP. So far, the ColR/S TCS has an interesting phenotype with the response regulator (ColR) being a lethal mutation in *Xf*. The results of pathogenicity assays with *Xf ΔcolS* will determine the future direction we take in investigating the ColR/S TCS.

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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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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, we are investigating the O-antigen portion because it 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 host specificity observed for this pathogen.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) is a bacterium capable of colonizing many different plant hosts. This bacterium is the causal agent of Pierce's disease of grapevine and also causes disease in other crops such as almond, citrus, and oleander. While all *Xf* isolates belong to the same species, some isolates can cause disease in one host, but not another. One goal of this project is to understand the bacterial traits that dictate this host specificity. This research is focused on elucidating the role of the cell surface component, lipopolysaccharide (LPS), in the pathogenic interaction between the bacterium and grapevine, almond, or oleander. LPS also imparts traits that may contribute to pathogenesis, such as the ability to attach to host cell walls. Thus far, we have shown that a region of the LPS molecule is 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 LPS biosynthesis exist and can 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

We are currently exploring the role of lipopolysaccharide (LPS) as both a virulence factor in grapevine and as a host specificity determinant for this pathogen. LPS is an integral part of the Gram-negative bacterial outer membrane and is displayed on the outer surface of the cell, thereby mediating interactions between the bacterial cell and its environment. LPS is composed of three parts: 1) Lipid A, 2) oligosaccharide core, and 3) O-antigen polysaccharide (**Figure 1**) (23). 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* (7, 10, 15). Because of its location in the outer membrane, LPS can also contribute to the initial adhesion of the bacterial cell to a surface or host cell (9, 17). Additionally, host perception of LPS occurs in both plants and animals (18). Host immune receptors can

recognize the LPS structure and mount a defense response 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 are focusing on the O-antigen portion of LPS in three *Xf* isolates: Temecula1, causal agent of Pierce's disease (PD) of grape; M12, causal agent of almond leaf scorch (ALS); and Ann-1, causal agent of oleander leaf scorch (OLS). Based on investigations of LPS in host-pathogen interactions in other bacterial systems (11, 21), we identified two genes, *waaL* (PD0077) and *wzy* (PD0814) in the genomes of the three *Xf* isolates. *Wzy* is a putative O-antigen polymerase that plays a role in chain length determination of the O-antigen. *WaaL* is a putative O-antigen ligase that ligates the preformed O-antigen to the oligosaccharide core. We predicted that mutants in *wzy* would produce a truncated O-antigen and that mutants in *waaL* would completely lack the O-antigen. Based on these alterations, we hypothesized that we would see profound differences in virulence, surface attachment, aggregation, and insect transmission. Thus far, we have demonstrated that mutations in *wzy* and *waaL* do affect O-antigen biosynthesis and cause a visible change in the O-antigen profiles as seen in **Figure 1**. LPS was extracted from Temecula1 wild-type (WT), *wzy*, and *waaL* using a hot-phenol extraction method (22) and then subjected to Tris-Tricine polyacrylamide gel electrophoresis (PAGE). Both strains had a significant reduction in O-antigen (**Figure 1**). We had expected the *waaL* mutant would be devoid of O-antigen, but this is not the case suggesting that there may be 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 (14) (**Figure 1**).

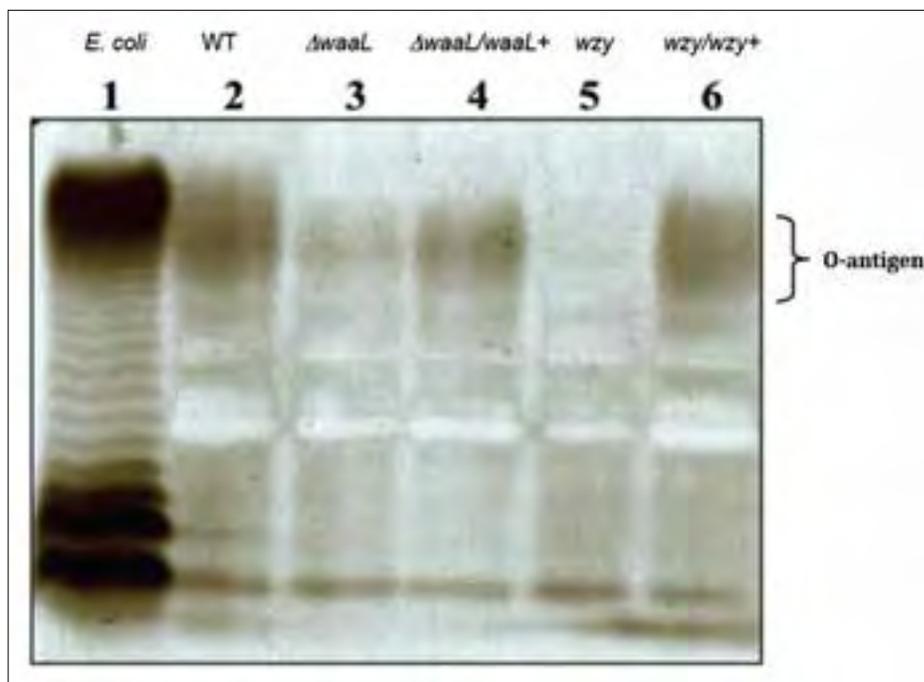


Figure 1. Mutations in key enzymes of the *Xf* LPS biosynthetic pathway result in reduction or abolishment of O-antigen. Both *waaL* (lane 3) and *wzy* (lane 5) mutant strains had less O-antigen than the wild-type (lane 2). Complementation of these mutants restored O-antigen quantity to near that of the wild-type parent (lanes 4 and 6). LPS was extracted from cells normalized to cell density using a modified hot phenol method and analyzed on a 4, 12% discontinuous Tricine-PAGE gel and silver stained. (1) *E. coli* standard, (2) Temecula1 wild-type, (3) *waaL*, (4) *waaL/waaL+*, (5) *wzy*, (6) *wzy/wzy+*.

Following the confirmation of the change in the O-antigen profiles for the *waaL* and *wzy* mutants, we tested these strains for differences in virulence. Temecula1 O-antigen mutant strains were needle-inoculated into grapevine, cv. 'Thompson Seedless' and symptoms were detected 4 weeks after inoculation. The *wzy* mutant

was significantly delayed in causing Pierce's disease symptoms on grapevine, 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 2**). **This indicates that alterations in the chemistry of the O-antigen correlates with a profound defect in *Xf* virulence.** We observed no difference in disease progress between the *waaL* mutant and the wild-type strain. Plants inoculated with 1X PBS buffer control did not develop any Pierce's disease symptoms.

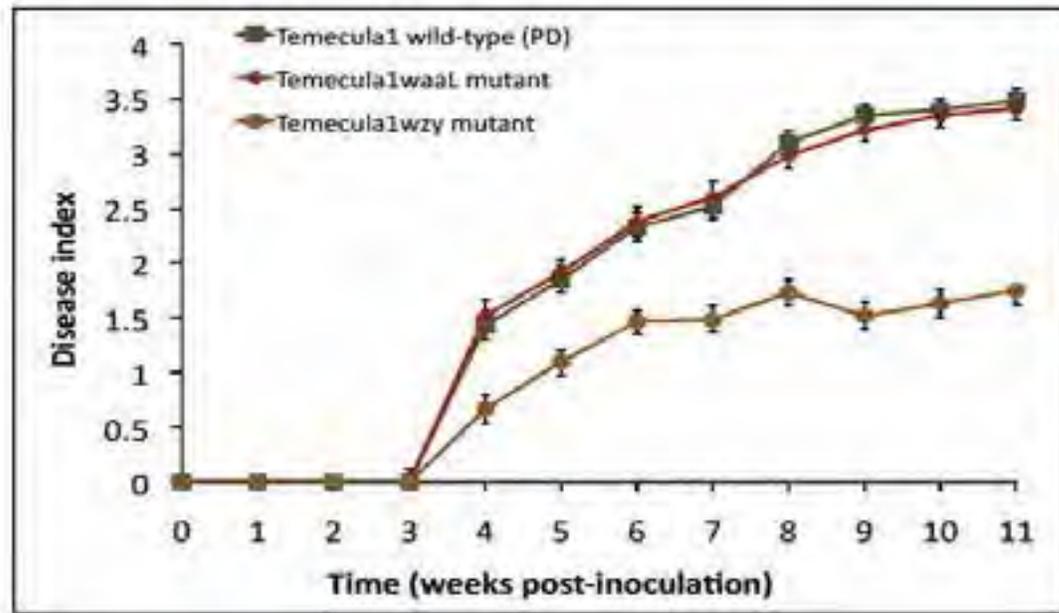


Figure 2. Disease progress of *Xf* Temecula1 wild-type and O-antigen mutant strains on grapevine cv. ‘Thompson Seedless.’ The *wzy* mutant strain lags behind the WT in causing leaf-scorching symptoms and does not cause WT levels of Pierce's disease throughout the disease progress curve. The *waaL* mutant is as virulent as WT. 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 WT 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 fewer bacteria than plants inoculated with WT (**Figure 3**). Populations were quantified by plate counts and the data were analyzed using a Wilcoxon rank test. The *waaL* mutants colonized the plants to similar levels as wild type *Xf*.

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*

We compared the LPS profiles of three *Xf* strains in this study. We hypothesized that there are strain specific differences among the O-antigen portion of the LPS molecules that contribute to the host specificity of these

three isolates. We isolated LPS from all three *Xf* isolates using a hot-phenol extraction method (22). The extracted LPS preparations were then subjected to Tris-Tricine PAGE. These analyses have confirmed that all three strains possess smooth LPS (ie. O-antigen), which was previously unknown (**Figure 4**). 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.

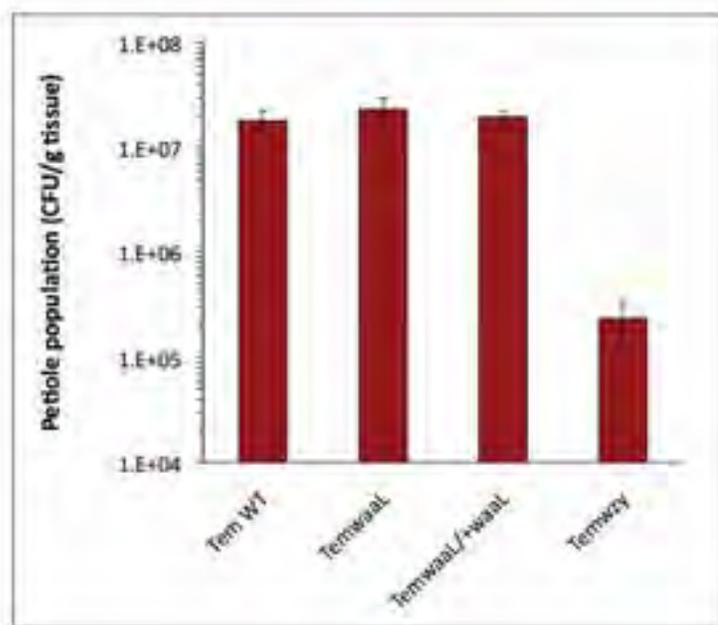


Figure 3. *In planta* populations of the *wzy* mutant after inoculation of grapevine petioles are significantly lower than those of the wild-type parent. Populations were quantified by plate counts. Five petioles from three independent inoculations. Bars represent the standard error of the mean.

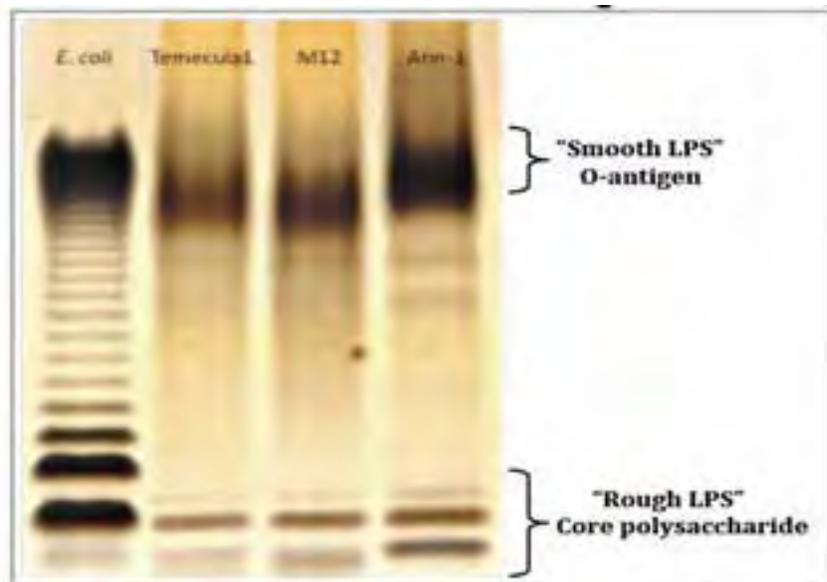


Figure 4. Tris-Tricine PAGE gel indicating the presence of both smooth (O-antigen) and rough (core polysaccharide) LPS in 1) Temecula1 (PD), 2) M12 (ALS), and 3) Ann-1 (OLS) strains of *Xf*.

We subsequently conducted a more detailed carbohydrate composition and linkage analysis of *Xf* LPS using Gas chromatography/Mass Spectrometry analysis in collaboration with the Complex Carbohydrate Research Center (CCRC) at the University of Georgia, Athens. Our studies included the Temecula1 strain and its respective *wzy* mutant. The CCRC performed O-antigen purification using a mild acid hydrolysis technique and conducted carbohydrate composition and glycosyl linkage analysis. Results of the glycosyl composition analysis revealed that *Xf* O-antigen is a heteropolymer consisting mostly of rhamnose and glucose, with smaller amounts of ribose, xylose and mannose (**Table 1**). The Temecula *wzy* mutant O-antigen was similar in carbohydrate composition to wild-type with regard to the residues present in the polysaccharide. However, there was a striking depletion in the percentage of rhamnose in the *wzy* mutant as compared to the wild-type, which decreased from 68.2% in the wild-type to 9.4% in the *wzy* mutant. In addition, the percentage of glucose increased from 19% in the WT to 84.6% in the *wzy* mutant (**Table 1**). Deletion of *wzy* in *Xf* resulted in an O-antigen composed primarily of glucose with much of the rhamnose absent. This suggests that the initial O-unit linked to the core LPS is composed primarily of glucose and the remainder of the polymer that extends out into the environment is a rhamnose-rich repeat. We are currently coordinating a series of experiments with the CCRC based on NMR spectroscopy to elucidate the absolute structure of the *Xf* O-antigen.

Table 1. Glycosyl composition analysis of O-antigen isolated from wild type or the *wzy* mutant. Values are expressed as mole percent of total carbohydrate.

O-antigen sugars (Mol %)					
	Rib	Rha	Xyl	Man	Glc
<u>Strain</u>					
Wild-type	6.5	68.2	5.5	0.8	19.0
<i>wzy</i>	4.9	9.4	0.5	0.6	84.6

Host specificity plant assays

While there are likely several factors that contribute to host specificity of *Xf*, we are investigating if O-antigen is involved. *Xf* Temecula1 can colonize and cause disease in grape and, to some extent, in almond. *Xf* M12, an ALS strain, causes disease in almond and elicits some symptoms in grape (1). Moreover, *Xf* Ann-1 cannot cause disease in grape or almond and neither Temecula 1 nor can M12 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 WT and the O-antigen mutant strains into host (grape) and non-host (almond and oleander) plants. We observed that the grape isolate can colonize and cause disease in almond, albeit, to a lesser extent than the WT almond leaf scorch (ALS) isolate (M12), which is consistent with what other research groups have observed (1). Interestingly, the Temecula1 *walL* mutant strain is **more virulent** in almond than its Temecula1 WT parent and is similarly virulent to the WT 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 5**). Symptoms of oleander leaf scorch develop much later than those in grape and almond (ten months post-inoculation) so we are just now collecting data from these plants.

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 quantified the Temecula1 *walL* 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. Interestingly, when grown in glass tubes, the

Temecula1 *wzy* mutant aggregated less, but attached more to a glass surface (**Figure 6**). The *wzy* mutant was significantly less virulent *in planta* which may be a result of its hyperattachment phenotype causing it to adhere more strongly to the xylem cell wall, which does not allow it to move as efficiently throughout the plant.

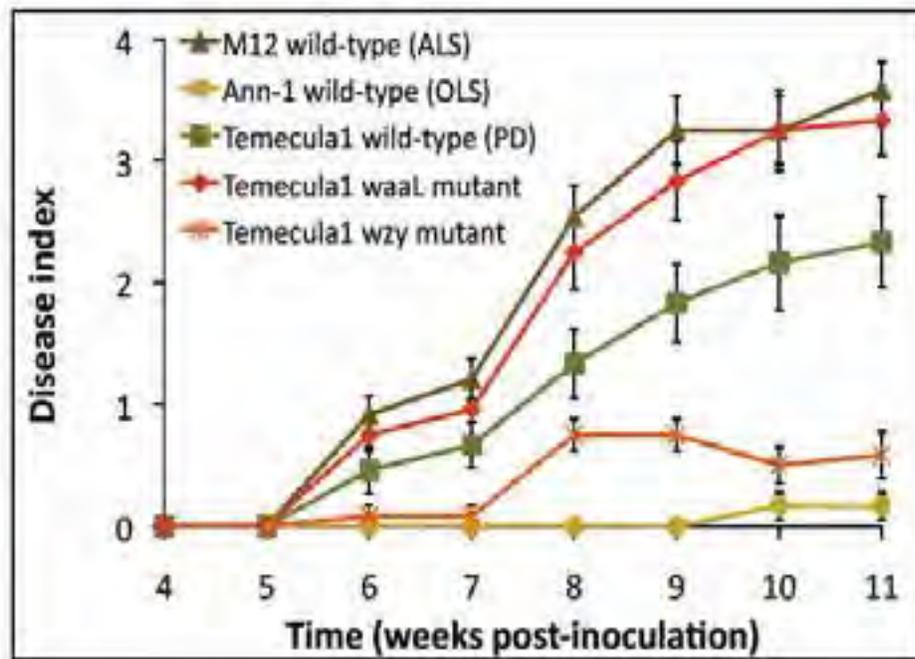


Figure 5. Disease progress of M12 (ALS), Ann-1 (OLS) Ann-1, Temecula1 (PD) isolates, and Temecula1 O-antigen mutants in almond cv. ‘Sonora.’ Loss of Waal increases virulence of a Pierce’s disease isolate of *Xf* in almond. Data are means of two independent assays with 6 replications each. Bars represent standard error.

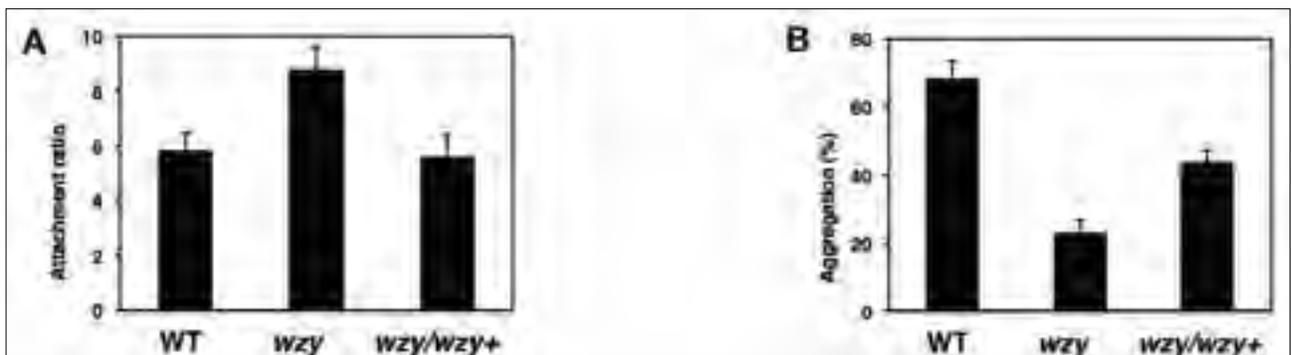


Figure 6. Surface attachment and cell-cell aggregation of the O-antigen mutant, *wzy*. A) The Temecula1 *wzy* mutant cells attach to a solid surface to a greater extent than the wild-type parent. Attachment assays involved crystal violet staining of cells attached to a glass surface at the medium/air interface. B) The Temecula1 *wzy* mutant cells are reduced in the ability to aggregate to each other compared to the WT. Aggregation assays reflect the proportion of the total cell population that remains in culture after 10 days of static incubation. At least three independent assays were performed in triplicate. Bars represent standard error of the mean.

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. These measurements indicated that 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 WT at -10.5 mV (**Table 2**). 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 approximately -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 WT strain. Particles with lower zeta potentials tend to flocculate or aggregate, which explains the high capacity for *Xf* to aggregate in culture (6). 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 WT, which explains the inability of this strain to aggregate *in vitro*. We are currently testing the *waaL* mutant for attachment to glass and cell-cell aggregation and will also measure its zeta potential.

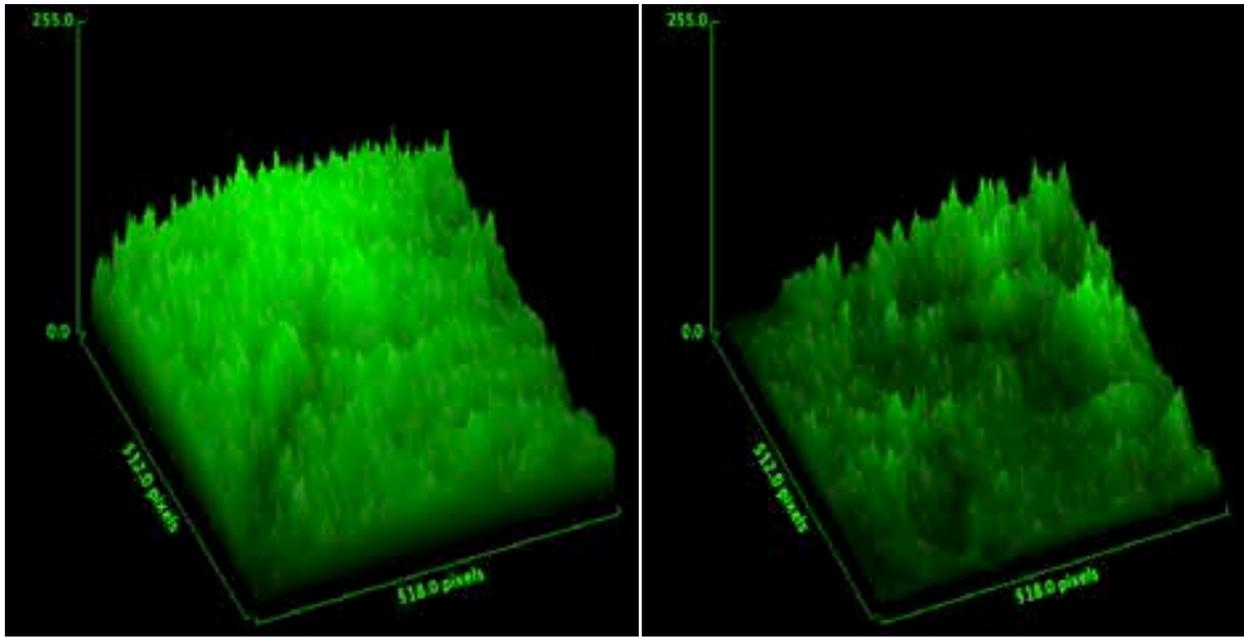
Table 2. Zeta potential measurements of *Xf* Temecula wild type, *wzy* mutant, and *wzy/wzy+* complemented strain. The *wzy* mutant bacterial cells exhibit a significantly more negative zeta potential measurement than the WT cells, which likely account for its hyperattaching and non-aggregative phenotype. Data are means of three independent assays with 5 replications each.

Strain	zeta potential (mV)
Wild type	-10.5±2.2
<i>wzy</i>	-27.12±1.91
<i>wzy/wzy+</i>	-10.9±0.97

In addition, we tested the ability of the *wzy* mutant to form 3-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. We observed mature *Xf* biofilms by confocal laser scanning microscopy (CLSM) and reconstructed the z-stack series using BitPlane Software. The *wzy* mutant biofilms were impaired in building the typical three-dimensional biofilm architecture (**Figure 7**). The *wzy* mutant was capable of attaching to the glass surface but was unable to build the towers characteristic of a WT biofilm. Moreover, the *wzy* biofilms were thinner on average than the WT biofilms. We are expanding the attachment studies to the more biologically relevant substrates, chitin and cellulose.

Objective 3: Test O-antigen mutants for insect transmissibility

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 (12) because differences in strain multiplication *in planta* may confound estimates of sharpshooter acquisition efficiency, which depends on plant infection level. In collaboration with Dr. Thomas Perring (UC Riverside Department of Entomology), we have initiated a colony of *Graphocephala atropunctata*, blue green sharpshooters (BGSS). Insects were fed 35 μ l aliquots of bacterial suspension or diet solution only (negative control) and given an acquisition access period of 6 hours and then placed on basil plants for a multiplication period of 48 hours. We are currently quantifying the bacterial cells within the sharpshooter heads using quantitative PCR. Concomitant inoculation studies will be performed by caging diet-fed sharpshooters onto healthy grapevine seedlings for an inoculation access period of 4 days. Seedlings will be observed for symptom development and bacterial populations within infected plants will be quantified. In collaboration with Dr. Elaine Backus (USDA ARS, Parlier, CA) we are imaging the colonization patterns of the *wzy*, *waaL* mutants as compared to WT in the BGSS foregut. These studies are underway and we hypothesize that we will see a difference in the manner in which these mutants colonize the insect based on our *in vitro* attachment and aggregation data.



Temecula1 wild- type

wzy mutant

Figure 7. *In vitro* biofilm assay of the wild-type and the *wzy* mutant. Images are 3-dimensional renderings of a z-series of images captured with the CLSM and representations of three independent assays with three replications each.

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

Variation in the O-antigen portion of the LPS molecule can aid in the adaptation or tolerance to different environmental stresses (2, 3, 16, 20). We are investigating whether the absence or truncation of the O-antigen affects tolerance to: 1) oxidative stress, 2) cold temperature, or 3) treatment with antimicrobial peptides. 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 (18). 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 performed a simple disc inhibition assay protocol as previously described (14) to test sensitivity to peroxide. We observed that the *wzy* mutant was more sensitive to peroxide treatment as compared to the WT (**Figure 8**). We are currently testing the *waaL* mutant for sensitivity to peroxide. In addition, we are testing the *waaL* and *wzy* mutants for increased susceptibility to cold temperature and treatment with antimicrobial peptides. We chose these two treatments based on current strategies being evaluated for control of Pierce's disease (4, 5, 13). We have chosen to test cecropin B because it has previously been identified to have antimicrobial activity against *Xf* (5) and polymyxin B because its mode of action is to bind directly to LPS (20). Antibacterial activity will be expressed as Minimum Inhibitory Concentrations (MIC), the concentration at which no growth is observed. We are initially conducting these experiments in liquid cultures, but the results will be confirmed by testing on solid PD3 medium overlaid with a Whatman paper disk containing the different concentrations of the AMP being tested. The MICs will be calculated by measuring the diameter of the inhibition zones around the disk containing the AMP. Cold temperatures have been associated with the geographic distribution of Pierce's disease (19). A test of the effects of cold temperature-mediated therapy on *Xf*-infected grapevines is an ongoing area of research, and certain aspects of this phenomenon are currently being exploited as possible control strategies for Pierce's disease (13, 19). Bacteria can modify their LPS in response to temperature (2, 16). These temperature-induced modifications are generally regarded as an adaptive response to the cells surrounding environment. We are basing our assay on previously established protocols (8). We anticipate that both the antimicrobial peptide and cold temperature studies will be completed by December of 2012.

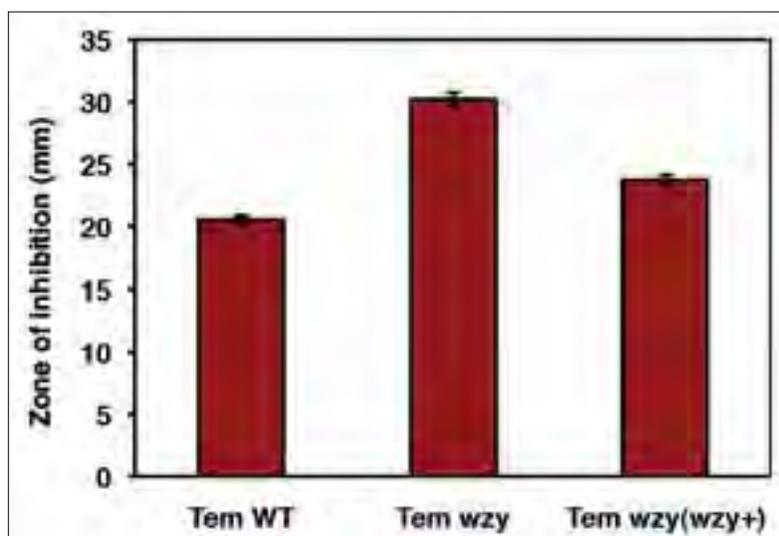


Figure 8. The role of O-antigen in the protection against oxidative stress. The *Xf wzy* mutant strain was less tolerant of hydrogen peroxide stress than the wild-type parent or the complemented mutant strain. Three independent assays were performed in triplicate.

CONCLUSIONS

By targeting key O-antigen biosynthetic genes, we demonstrated the contribution of the rhamnose-rich O-antigen to surface attachment, cell-cell aggregation, and biofilm maturation: critical steps for successful infection of the host xylem tissue. Moreover, we have demonstrated that a fully formed O-antigen moiety is an important virulence factor for Pierce's disease development in grape and that depletion of the O-antigen compromises its ability to colonize the host. It has long been speculated that cell surface polysaccharides play a role in *Xf* virulence and this study confirms that LPS is a major virulence factor for this important agricultural pathogen.

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SMALL RNAS IN *XYLELLA FASTIDIOSA* AND THEIR EPIDEMIOLOGICAL APPLICATIONS

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ABSTRACT

Xylella fastidiosa (*Xf*) causes many economically important crop diseases including almond leaf scorch disease and Pierce's disease of grapevine. Although non-coding small RNAs (sRNAs) are regarded as ubiquitous regulatory elements in bacteria, research attention to sRNAs in *Xf* has been limited. In *Xf* strain M23, 49 sRNA genes were predicted based on the availability of its whole genome sequence and computational analyses. *Xf* is, however, nutritionally fastidious and grows very slowly even in complex culture media. Compounded by the in general low expression levels of sRNAs, implementation of the commonly used techniques such as Northern-blotting to detect sRNAs has been a challenging task. In this study, an alternative method was developed to experimentally verify the presence of sRNAs in *Xf*. The technique took the advantage of the high sensitivity of PCR technology. Primers were designed within (internal) and outside (external) the putative sRNA genes. sRNAs in bacterial cultures were verified by real-time quantitative reverse- transcriptase PCR (qRT-PCR) when internal primer sets yielded positive amplifications and external primer sets yielded weak or no products. Detection of 16S rRNA was used as internal control. A total of nine sRNAs have been detected in *Xf* strain M23. Different sRNA types were observed depending on culture media. Results from this study provide the first experimental proof of sRNAs in *Xf*. The developed technique also has a potential to be used in sRNA detection in other bacteria.

FUNDING AGENCIES

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CHARACTERIZATION OF THE VIRULENCE-RELATED GENES OF *XYLELLA FASTIDIOSA*

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ABSTRACT

To elucidate the role of virulence genes of *Xylella fastidiosa* (*Xf*) responsible for Pierce's disease in grapes, a site-directed deletion method was employed to knock out virulence-related genes. Six virulence-related genes were selected, of which three genes were annotated to be transcriptional regulators; *hfQ*, *xrvA* and *gcvR*, and three genes were associated with two-component regulators; *algR*, *colR* (PD1919), and *colR* (PD1367). The mutant strains were obtained from selective media and the deleted loci were confirmed by PCR. To evaluate the pathogenicity of six mutant genotypes, greenhouse-grown Cabernet Sauvignon grapevines were mechanically inoculated with mutant bacteria, wild-type *Xf* Temecula strain served as a control. Three months after inoculation, grapevines inoculated with *algR*, *xrvA*, and *colR* (PD1919) showed only mild symptoms when compared with grapes inoculated with wild type *Xf*. However, no symptoms were observed in grapes inoculated with mutant strains of *gcvR*, *hfQ*, and *colR* (PD1367). Quantitative PCR analysis was conducted to estimate *Xf* titers in infected grapes. *In vitro* studies showed that while both mutants and wild-types of *Xf* had similar growth curves all mutants appeared to have significantly reduced abilities to adhere to the well of culture tubes. Biofilm production of *gcvR*, *hfQ*, *algR*, and *colR* (PD1367) was five-fold less than that of wild type whereas *xrvA* and *colR* (PD1919) mutants produced 2.5-fold less biofilm than that of wild type. To further investigate metabolic pathways that involve regulation of virulence genes, chemical profiles of mutant and wild type of *Xf* were analyzed by gas chromatography-mass spectrometry. All six virulence mutants have been complemented via chromosome based recombination. These complemented strains are currently being subjected to inoculation experiments along with wild type *Xf* Temecula strain to examine restoration of gene function. Characterization of virulence-related genes in *Xf* will provide insight into mechanisms of pathogenicity of *Xf*.

FUNDING AGENCIES

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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 investigating replacing the surface binding domain of our NE-CB chimeric antimicrobial protein with a protein (plant elastase, PE) that performs the identical function from a plant source. We will identify an appropriate plant protein using a recently described computational tool (CLASP, Chakraborty et al., 2011, 2012). The search for this protein will be based on the conformational similarity of its active site's 3D signature to that of human neutrophil elastase (HNE). We will verify the enzymatic activities of the top candidates, i.e. elastase activity and the ability to cleave *Xf mopB*, to confirm which are most suitable to serve as a surface-active domain in our chimera. We will then construct a synthetic gene encoding the chimeric antimicrobial protein, substituting PE for the NE domain and attaching it to the lytic domain CB, using the same flexible linker from the previous construct. The new PE-CB gene 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 the methods reported previously for NE-CB constructs (Dandekar et al., 2012). In addition, we will investigate how efficiently the chimeric antimicrobial protein disrupts *Xylella fastidiosa* - glassy-winged sharpshooter (*Xf*-GWSS) interaction. To accomplish this, we will isolate NE-CB and PE-CB proteins from plants and feed GWSS with *Xf* in the presence and absence of the individual CAP proteins. This study will measure the utility of this novel strategy to provide durable resistance to Pierce's disease in grapevines and aid in potential stacking with other disease resistance traits.

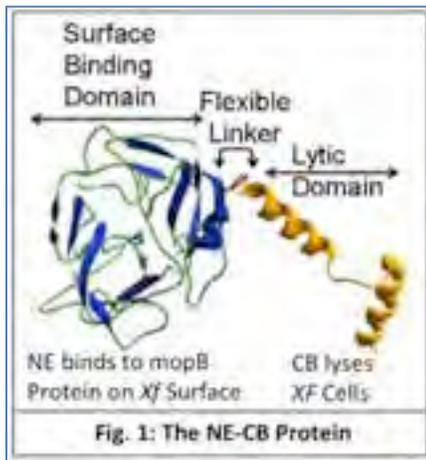
LAYPERSON SUMMARY

We have successfully expressed a chimeric antimicrobial protein NE-CB in grapevines to protect against Pierce's disease. In this project, we are swapping the NE component based on the design of the human neutrophil elastase (HNE) with a protein with the same activity from a plant. The structure of HNE is known in detail, and we will use a recently developed computational tool (CLASP) developed by our collaborator that can identify a plant protein with the same active site configuration as found in HNE. We can test for enzymatic activity like that of HNE in our candidate proteins, which we designate PE. We will then construct a synthetic gene encoding the new chimeric antimicrobial protein. The new chimeric protein will have PE in place of the NE domain, attached as before to the lytic domain CB with the flexible linker used previously. The new PE-CB gene will be inserted in to a vector and used for *Agrobacterium*-mediated transformation of grapevine rootstock and tobacco. The goal will be to confirm resistance to Pierce's disease using the methods reported previously for NE-CB 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). Thus, any control or resistance measure must by necessity be multifaceted to block this pathogen at different stages in 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 reservoir of bacterial inoculum. Resistance mechanisms must be directed to degrade this inoculum and prevent further disease spread. 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 NE-CB shows significant mortality. Our group has successfully designed and tested a NE-CB 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 (**Figure 1**). 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 domain is a synthetic gene that encodes the human innate defense protein neutrophil elastase (NE)



(Dandekar et al., 2012; Kunkel et al., 2007). The second, CB domain is a clearance domain, connected with a flexible linker to the C-terminal of NE. 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 NE binds to the 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 NE (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 GMOs. Therefore, substituting NE with PE found in a familiar edible plant may be less controversial.

OBJECTIVES

The goal of this project is to redesign the NE-CB chimeric antimicrobial protein by swapping out the NE domain with plant elastase (PE) domain and testing for the efficacy with respect to the NE-CB therapeutic protein for disease development and transmission.

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

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

Activity 2: Construct vectors and test *in planta*-produced protein for efficacy in killing *Xf* in culture.

Objective 2: Compare the efficacy of PE-CB with NE-CB in plants with *Xf* challenge.

Activity 3: Construct binary vectors and transform grapevine and tobacco.

Activity 4: Test transgenic tobacco and grapevine for clearance of *Xf* and resistance to Pierce's disease symptoms.

RESULTS AND DISCUSSION

We initiated our project three months ago and have focused on the first objective outlined above.

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

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

We have initiated the process of finding a plant elastase (PE) candidate protein by identifying a similar active site configuration to that found in NE, a serine protease with elastase activity. We are working with Sandeep Chakraborty and B.J. Rao in the Biosciences Department at the Tata Institute of Fundamental Research in Mumbai (India), who have developed the novel computational tool CLASP (Catalytic Active Site Prediction) used in a manner similar to BLAST to search all known protein structures for a desired active site conformation (Chakraborty et al., 2011). A protein active site motif from a known function and 3D structure provide the initial template for searching potential matches by this approach. The theoretical foundation of CLASP lies in the conserved electrostatic potential difference and spatial distances in cognate pairs of active site residues in proteins with the same functionality. Similarity can be observed in structures solved independently over many years, and also holds true for convergently evolved proteins (for example, in the two major families of serine proteases, chymotrypsin and subtilisin). CLASP enabled us to identify a plant protein which has elastase function after no such protein was found in the literature. We created an active site motif using the catalytic residues from HNE (PDB: 1BOF), and searched for this motif among ~300 *plant* proteins extracted from the Protein Data Bank.

This gave us a list of significant matches (**Table 1**). We chose the pathogenesis-related protein (P14A, PDB: 1CFE). Its protease function has also been associated with the pathogenesis-related proteins (Milne et al., 2003). Furthermore, there was a striking structural homology shared between P14A and a snake venom protein, which was previously demonstrated to be an elastase (Bernick and Simpson 1976).

Table 1.

PDB	function	sequence length	score
1QMG	ACETOHYDROXY-ACID ISOMEROREDUCTASE	524	0.017
1CFE	PATHOGENESIS-RELATED PROTEIN P14A	135	0.038
2JEM	ENDO-BETA-1,4-GLUCANASE	261	0.047
3HGO	12-oxophytodienoate reductase 3	402	0.050
2O7R	CXE carboxylesterase	338	0.051
1DX6	ACETYLCHOLINESTERASE	543	0.053
1IYN	Chloroplastic ascorbate peroxidase	295	0.053
1YHZ	Acetolactate synthase	590	0.066
1USW	FERULOYL ESTERASE A	260	0.070
2HOR	Alliin lyase 1	427	0.089
1SQD	4-hydroxyphenylpyruvate dioxygenase	424	0.094
1GP6	LEUCOANTHOCYANIDIN DIOXYGENASE	356	0.098
1T3K	Dual-specificity tyrosine phosphatase	152	0.101
3I6S	Subtilisin-like protease	649	0.101
1XKL	salicylic acid-binding protein 2	273	0.113
2EFC	Similarity to vacuolar protein sorting-associ	267	0.113
3H2G	esterase	397	0.114
1NAR	NARBONIN	290	0.116
1IEQ	BETA-D-GLUCAN GLUCOHYDROLASE ISOENZYME EXO1	605	0.122
1F2N	CAPSID PROTEIN	238	0.129
2CC0	ACETYL-XYLAN ESTERASE	195	0.131
3E5N	D-alanine-D-alanine ligase A	386	0.131
2J5C	1,8-CINEOLE SYNTHASE	569	0.138
2C71	GLYCOSIDE HYDROLASE, FAMILY 11:CLOSTRIDIUM CE	216	0.141
1N10	Pollen allergen Pnl p 1	241	0.142
1QRR	sulfolipid biosynthesis (SQD1) PROTEIN 394	0.153	

Table 1. Best matches of plant proteins queried with a 5 residue motif from a HNE (PDB id: 1BOF)

Since P14 is a protein from tomato, we decided to look for a similar pathogenesis-related protein in grape. Extensive BLAST searches resulted in a list of grape proteins with sequence similarity to P14, including a pathogenesis-related protein (PR-1) from a wild variety of grape which is also resistant to Pierce's disease (Fritschi et al., 2007). We then analyzed the active sites of PR-1 using computational approaches. We realized that this protein has very similar structure and active site configuration as NE. Recently Li et al. (2011) showed that a PR-1 duplicated from a *Vitis* interspecific hybrid confers a high level of resistance to a bacterial pathogen.

Activity 2: Construct vectors and test *in planta*-produced protein for efficacy in killing *Xf* in culture.

To study and to demonstrate the efficacy of our PR-1 protein candidate from grapevine, we are developing a synthetic gene construct. We have fused this protein to a purification tag (3xFlag, Sigma) to facilitate detection and purification. The PR-1 gene is currently being synthesized and will then be cloned into a binary vector (Figure 2).

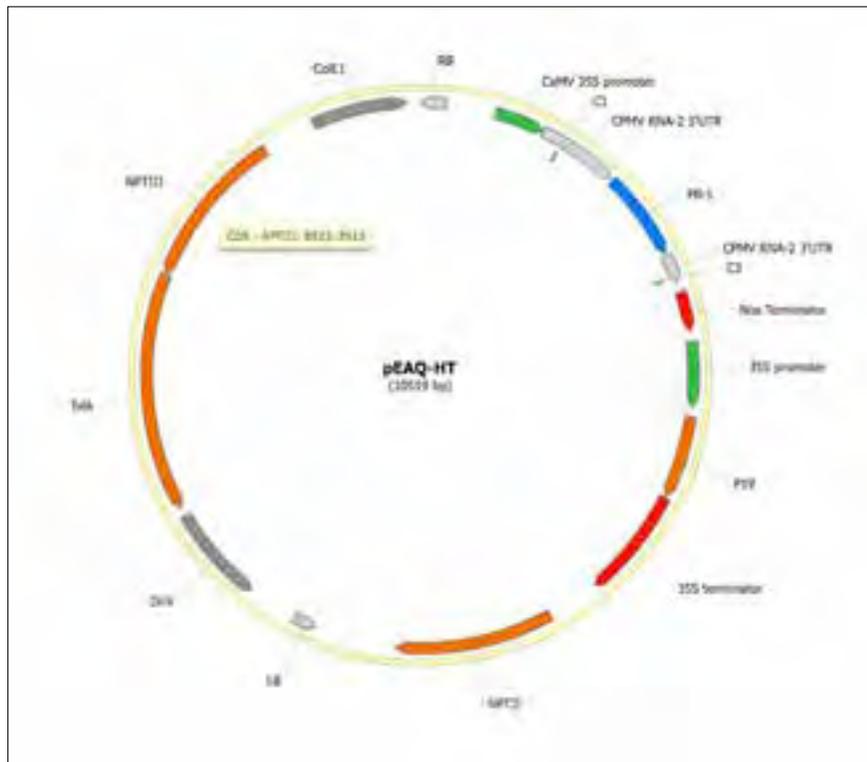


Figure 2. Map of potential binary vector containing PR-1 protein. This construct will be used for transient expression of the gene in tobacco.

CONCLUSIONS

Using a novel computational program called CLASP (Catalytic Active Site Prediction), we have identified a protein from a wild variety of grape that has similar catalytic active sites as NE. This protein is a pathogenesis-related protein (PR1) present in some varieties of wild grape which are resistant to *Xf* infection. Potentially, this protein could function as a capture domain owing to its NE-like structure and its ability to confer resistance to bacterial diseases in transgenic tobacco. This protein will be characterized *in vitro* for its efficacy in clearing *Xf*.

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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 2012.

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, 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 5/18/2010, with the remaining 10 planted on 03/06/2011. In Solano County, 110 own-rooted transgenic and untransformed control vines were planted on 08/02/2010 and 110 untransformed TS scions grafted onto transgenic or untransformed rootstocks were planted on 06/27/2011. These transgenic grapevines are being evaluated both as plants on their own roots and as rootstocks grafted with untransformed TS 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 and xylem sap by ELISA. Pierce's disease symptoms were assessed using a standardized score based on percentage of leaf area scorching to validate resistance to Pierce's disease under field conditions. At the Solano County site, plants on their own roots were mechanically inoculated with the *Xf* type strain (Temecula 1) on 6/27/2011 and re-inoculated on 05/29/2012, to validate resistance to Pierce's disease under field conditions. At the Solano site, field-grafted transgenic plants were also mechanically inoculated for the first time on 05/29/2012. The presence of *Xf* was confirmed in petiole extracts, but not in xylem sap from mechanically inoculated grapevines using the ELISA assay. *Xf* growth was not observed when petiole extracts were plated and no Pierce's disease symptoms have been detected to date. HNE-CecB- and PGIP-expressing transgenic grapevine lines in Solano County have also been tested to confirm the presence of the transgene.

LAYPERSON SUMMARY

Transgenic grapevine plants expressing either polygalacturonase-inhibiting protein (PGIP) or a chimeric antimicrobial protein (HNE-CecB) have been planted in two locations, one in Riverside County and the other in Solano County. These transgenic grapevines are being evaluated both as plants on their own roots and as

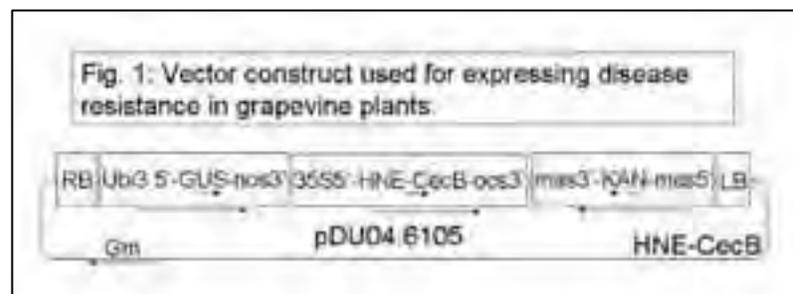
rootstocks grafted with untransformed Thompson Seedless (TS) scions to demonstrate the field efficacy of two strategies to control Pierce's disease in California grapevines. The first strategy uses transgenic rootstocks to control the movement of the bacterium *Xylella fastidiosa* (*Xf*) in the water-conducting 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. PGIP- and HNE-CecB-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, natural *Xf* infection has been confirmed in petioles and xylem sap by ELISA. Pierce's disease symptoms were scored using a standardized score based on percentage of leaf area scorching to validate resistance to Pierce's disease under field conditions. At the Solano County site, non-grafted plants were mechanically inoculated with *Xf* type strain Temecula 1 in 2011. The presence of *Xf* was confirmed in petiole extracts but not in xylem sap from mechanically inoculated grapevines using the ELISA assay. *Xf* growth was not observed when petiole extracts were plated and no Pierce's disease symptoms have been detected to date. HNE-CecB- and PGIP-expressing transgenic grapevine lines in Solano County have also been tested to confirm the presence of the transgene. At the Solano County site, grafted plants were mechanically inoculated with *Xf* and non-grafted plants were re-inoculated on May 29, 2012.

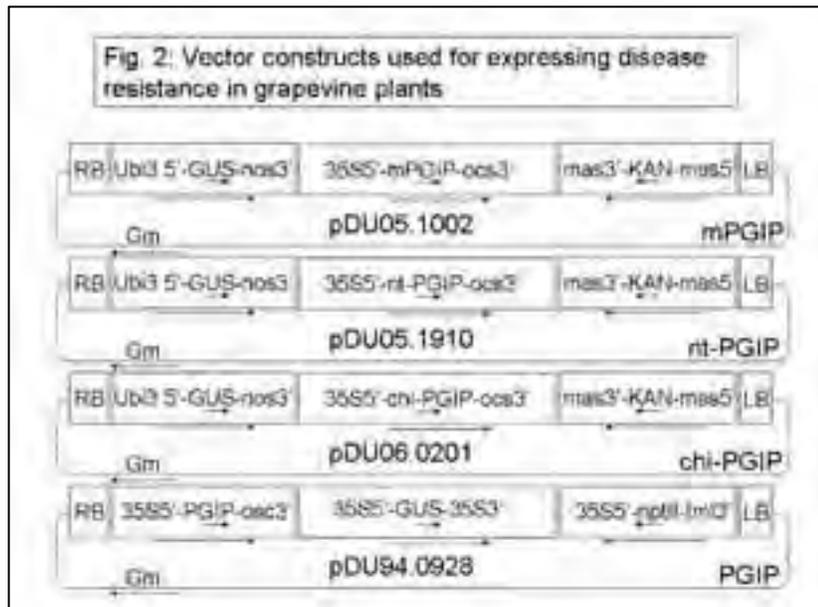
INTRODUCTION

Xylella fastidiosa (*Xf*), a xylem-limited, Gram-negative bacterium, is the causative agent of Pierce's disease. A key feature of *Xf* virulence is its ability to digest pectin-rich pit pore membranes that connect individual xylem elements (Roper et al., 2007), enhancing long distance movement and vector transmission. In this project, we are examining the ability of xylem-targeted polygalacturonase inhibiting protein (PGIP, Aguero et al., 2005, 2006) and a chimeric antimicrobial protein (HNE-CecB, Kunkel et al., 2007) to restrict bacterial movement and clear *Xf* under field conditions (Dandekar et al., 2009, 2012). The expectation is that expression of these proteins will prevent *Xf* movement and reduce its inoculum, decreasing spread of Pierce's disease.

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

We have also planted vines carrying four different constructs of PGIP (Figure 2). The four different modifications allow us to better understand how to control/restrict *Xf* spread and thus disease virulence. Two versions have different signal peptide sequences to identify which most efficiently localizes PGIP to xylem tissues and which provides the best distribution through the graft union into untransformed scion tissues. In vector pDU05.1910 (event 52-08), the pear PGIP signal peptide was replaced with a signal peptide from a grapevine xylem-secreted protein that is similar to the PRp27-like protein from *Nicotiana tabacum*. In vector pDU06.0201 (event 45-77), the pear PGIP protein was linked to a signal peptide from the Ch1b chitinase protein found in the xylem of grapevine (*Vitis vinifera*). The remaining two vectors, with and without the endogenous signal peptide, will serve as controls. The construct pDU94.0928 (event TS50), which uses the pear PGIP's own endogenous peptide, serves as a control to evaluate the efficiency of exogenous signal peptides in targeting PGIP to the xylem tissue. Vector pDU05.1002 (event 31-25) eliminates the endogenous signal peptide; the expressed PGIP cannot be secreted and should not limit *Xf* spread.





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

OBJECTIVES

The goals of this project are to field-test four HNE-CecB- and four PGIP-expressing transgenic TS grapevine lines to evaluate their horticultural characteristics and resistance to Pierce’s disease. Transgenic grapevines are being evaluated at two field locations as own-rooted plants and as transgenic rootstocks grafted with untransformed TS scions. One field location has endemic Pierce’s disease pressure and plants have been naturally infected with *Xf*. In the location with no Pierce’s disease pressure, grapevines have been mechanically inoculated with *Xf*.

Objective 1: Validate the efficacy of *in planta*-expressed chimeric HNE-CecB 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.

RESULTS AND DISCUSSION

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

Four selected transgenic grapevine lines expressing HNE-CecB and four expressing different PGIP constructs were propagated from cuttings in the greenhouse to obtain 48 clones of each line. After the root system developed, cuttings were transferred to 5.5-inch pots to develop into plants. Twenty-four clones were grafted with untransformed TS scions. Well-established plants were transferred to the lath house to acclimatize and then planted in two experimental fields. Two hundred and ten transgenic or untransformed vines, own-rooted or grafted with untransformed TS scions, were planted in Riverside County on 05/18/2010 and the remaining 10 were planted on 03/06/2011, completing the planting at this location (**Figure 3, Table 1**). We also planted 110 transgenic and untransformed vines on their own roots on 08/02/2010 and 110 vines grafted with untransformed TS scions on 06/27/2011 in Solano County, completing the planting at this location (**Figure 3, Table 1**).



Figure 3. Riverside (left) and Solano County (right) transgenic grapevine plantings (Summer 2012).

Table 1. Transgenic and control grapevines planted at Riverside and Solano fields.

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

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

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 TS 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 (**Figure 4**), 5) shape of mature leaf blades, 6) number of lobes on mature leaf (**Figure 4**), 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. In Riverside and Solano Counties, HNE-CecB- and PGIP-expressing transgenic grapevines lines will also be phenotypically evaluated in 2012.

Table 2. PCR genotyping of Solano County transgenic grapevine lines.

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

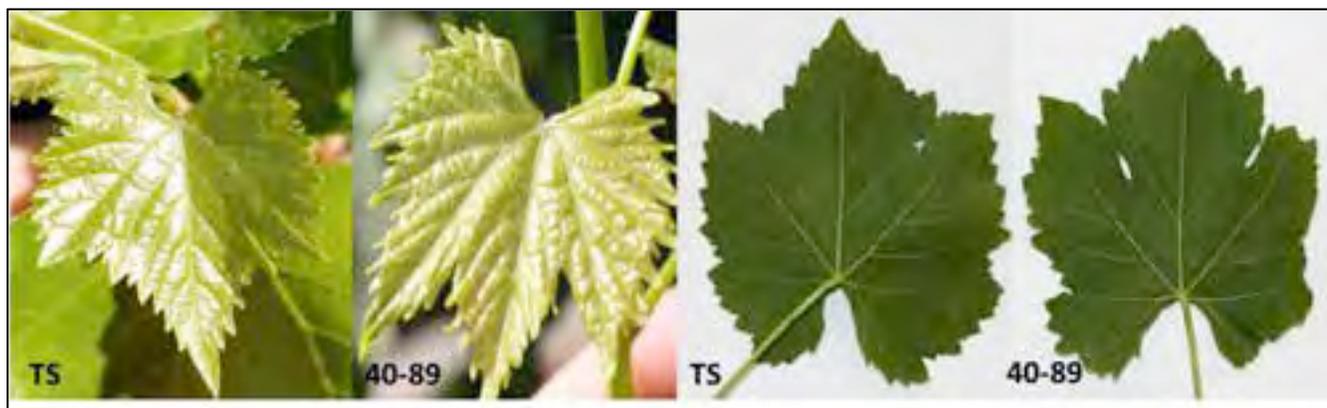


Figure 4. Color of upper side of blade on 4th young leaf (left) and number of lobes of mature leaf of TS and 40-89 transgenic line (right).

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

Two hundred twenty-four petiole samples from grafted and ungrafted transgenic and control grapevines planted in Riverside County were evaluated for *Xf* using a commercial ELISA kit (Agdia, Elkhart, IN) in fall 2011. The ELISA assay is based on a mixture of *Xf* antibodies against eight grape *Xf* isolates. Sample extracts were also plated on PD3 medium and *Xf* growth was verified by PCR using EFTu and 16s primers. The ELISA cell count (**Figure 5**), plate cell count (**Figure 6**) and PCR assay (**Figure 7**) results confirmed *Xf* infection in Riverside County.

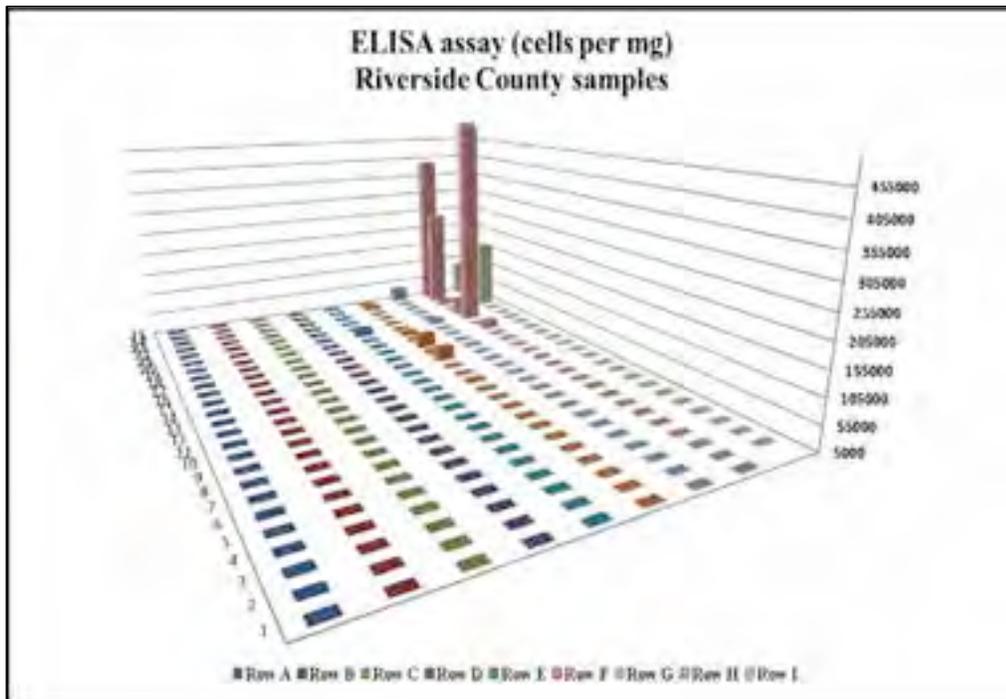


Figure 5. ELISA *Xf* detection in 2011 Riverside County's petiole samples.

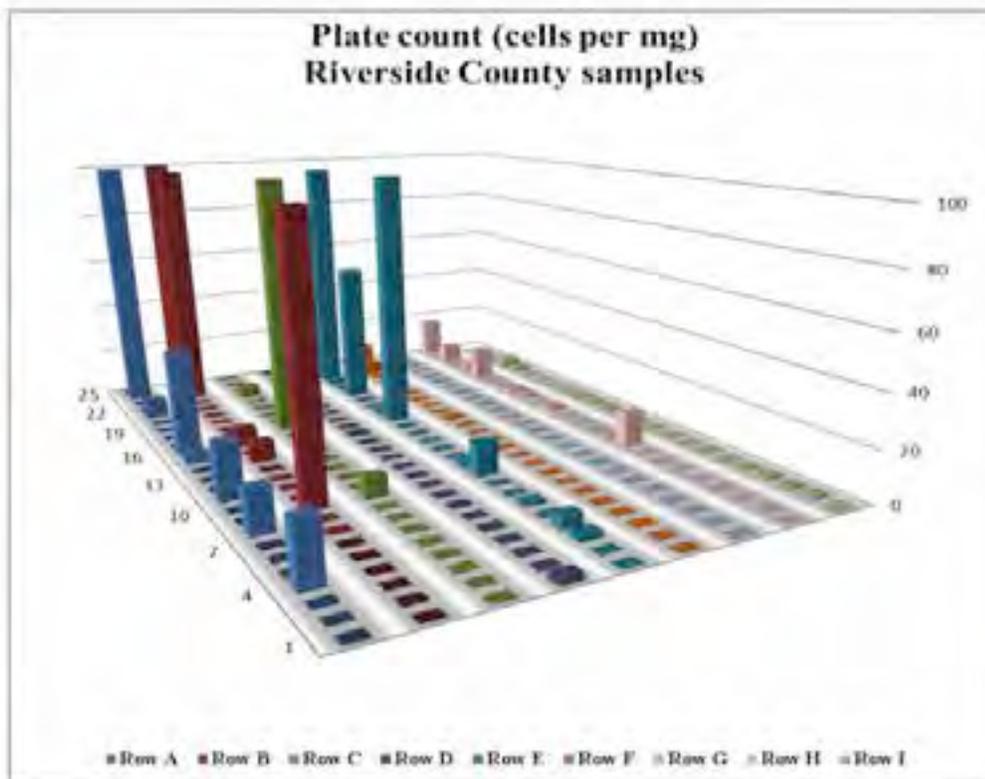


Figure 6. Plating *Xf* cell counts from 2011 Riverside County's petiole samples.

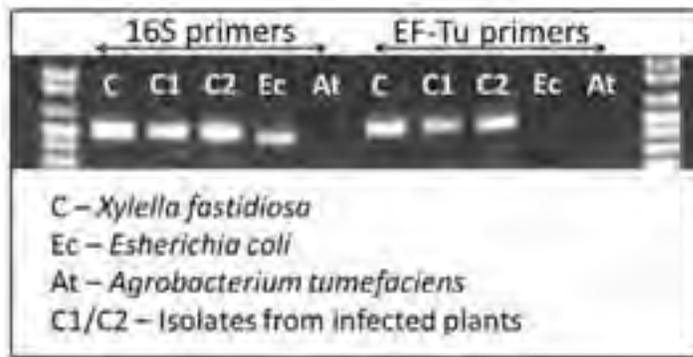


Figure 7. *Xf* detection in Riverside County samples using PCR.

Pierce's disease symptoms in each single Riverside County HNE-CecB- and PGIP-expressing grapevine were scored using a standardized score based on percentage of leaf area scorching (Figure 8), a characteristic of Pierce's disease (Krivanek et al., 2005a, 2005b). The following scoring system was used: 0 = no infection, 1 = potential infection, 2 = definitive infection (1-5 leaves infected), 3 = 5-10 leaves infected, 4 = more than 10 leaves infected, 5 = systematic infection on 1 runner, 6 = systematic infection in more than one runner, 7 = systematic infection in all runners, 8 = completely systematic with less than 50% leaf loss and 10 = dead plant. Riverside field average score was 3.25, the highest scoring line was 40-92 at 4.8 and the lowest was 40-41 grafted at 1.7.

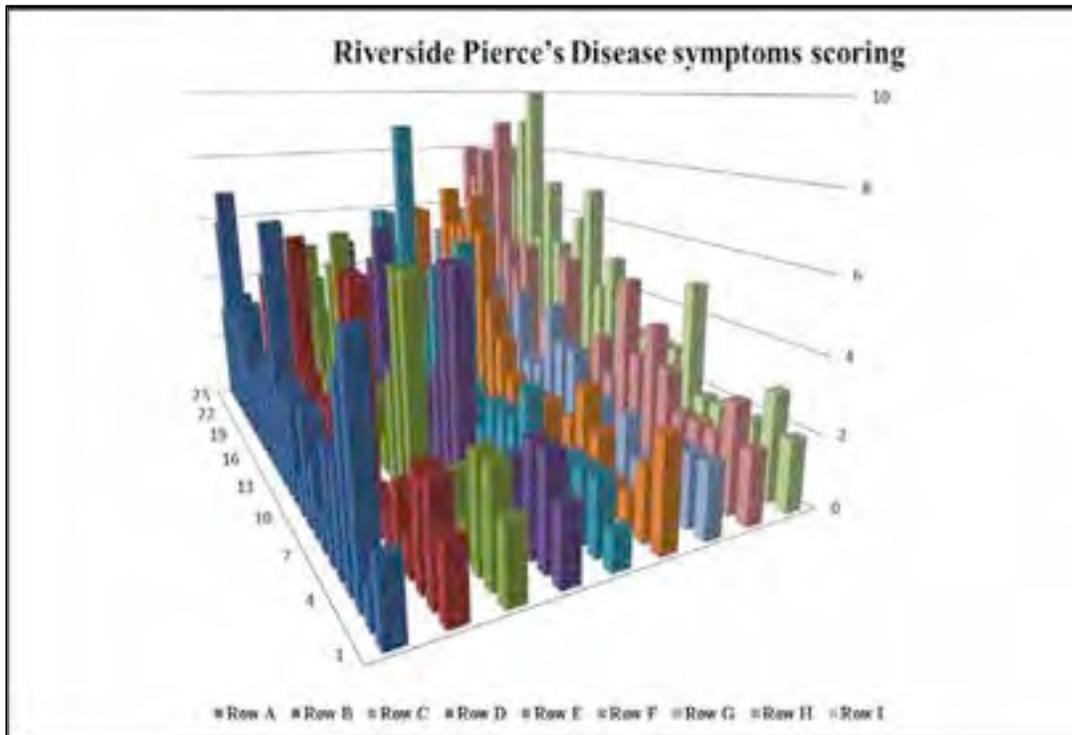


Figure 8. Pierce's disease symptoms scoring in 2011 Riverside County's plants.

One hundred and nineteen xylem sap samples from grafted and non-grafted transgenic and control grapevines planted in Riverside County were evaluated using ELISA for *Xf* detection in spring 2012. *Xf* was found in every single xylem sap sample collected: the average *Xf* cell number was 32,092 cells/50 μ L. The results confirmed once again the presence of Pierce's disease at the Riverside County site (Figure 9).

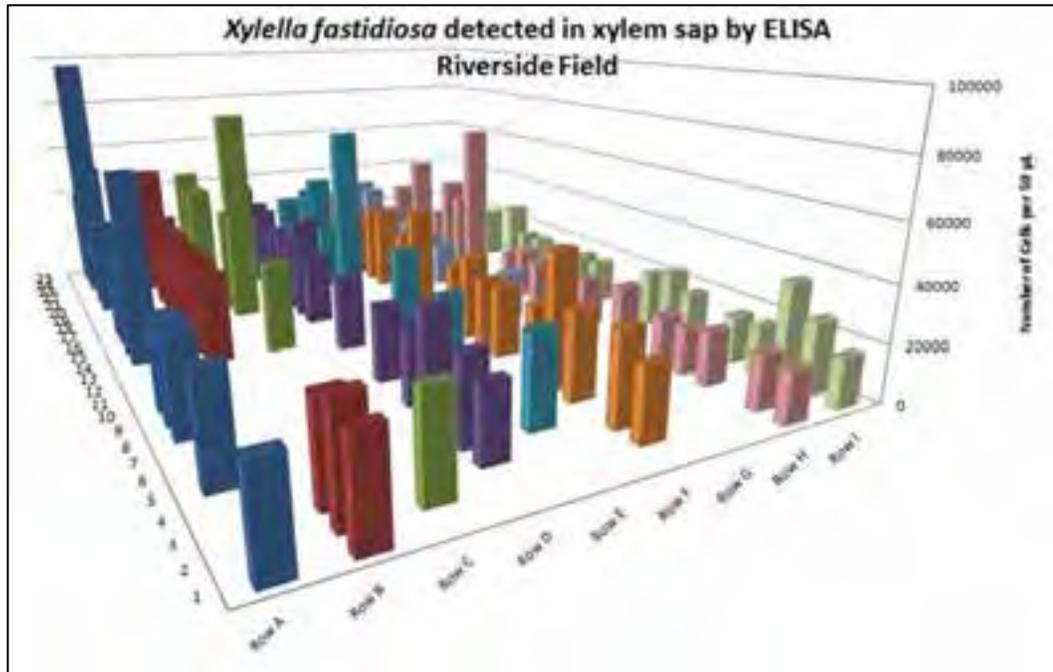


Figure 9. ELISA *Xylella fastidiosa* detection in Riverside County’s 2012 xylem sap samples.

At the Solano County site, petioles from transgenic and non-transgenic plants that were mechanically inoculated with *Xf* (Almeida and Purcell, 2003) in July 2011 and from TS and TS50 non-inoculated plants were evaluated for *Xf* infection using the commercial ELISA kit. Solano County sample extracts were also plated on PD3 medium. *Xf* was detected in petiole extracts by ELISA (**Figure 10, Table 3**), but no growth was observed when petiole extracts were plated. Solano County grafted plants were mechanically inoculated for the first time with *Xf* and non-grafted plants were re-inoculated on May 29, 2012. Two runners per plant were inoculated with an inoculum size of 2.5×10^5 cells/20 μ L.

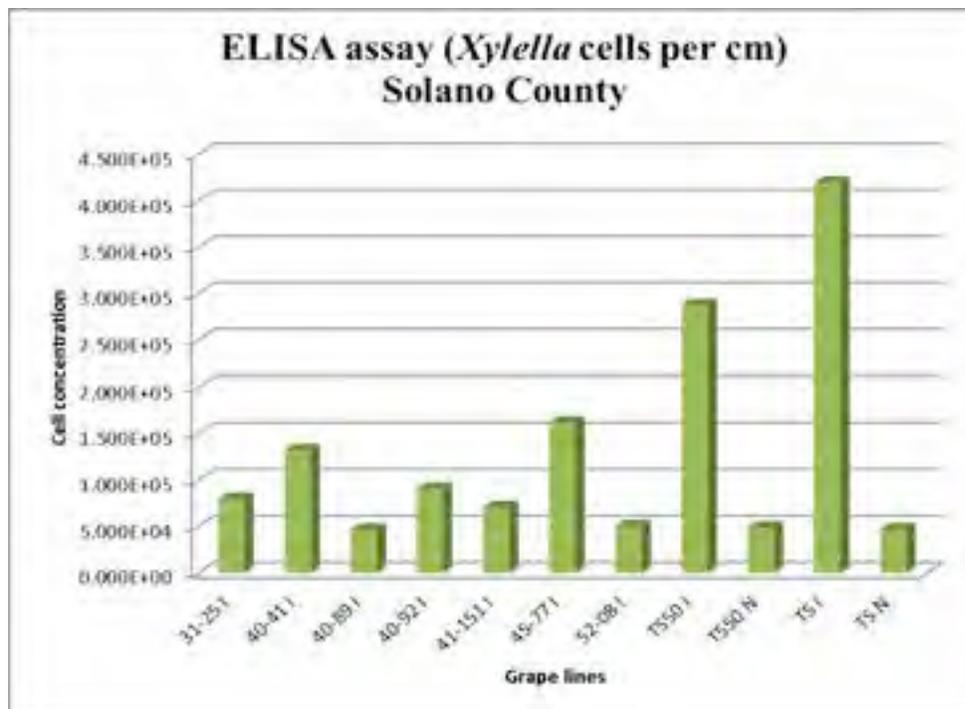


Figure 10. ELISA *Xf* detection in Solano County's petiole samples.

Table 3. ELISA cell count on Solano County petiole extracts.

Line	Gene	Cell/cm
31-25 inoculated	mPGIP	8.026E+04
40-41 inoculated	HNE	1.329E+05
40-89 inoculated	HNE	4.728E+04
40-92 inoculated	HNE	9.104E+04
41-155 inoculated	HNE	7.136E+04
45-77 inoculated	chiPGIP	1.625E+05
52-08 inoculated	ntPGIP	5.099E+04
TS50 inoculated	Control	2.877E+05
TS50 non-inoculated	Control	4.931E+04
TS inoculated	Wild type	4.199E+05
TS non-inoculated	Wild type	4.768E+04
TS non-inoculated + <i>Xf</i>	Positive control	3.675E+12

CONCLUSIONS

We have successfully established two field trials to validate two greenhouse-tested strategies to control the movement and clearance of *Xf*, a xylem-limited, Gram-negative bacterium that is the causative agent of 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 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.

Transgenic grapevine plants expressing either PGIP or HNE-CecB along with untransformed controls have been successfully planted in two locations. In Riverside County, planting is now complete at 220 vines in the ground: 210 planted on 05/18/2010 with the remaining 10 planted on 03/06/2011. In Solano County, where planting is also completed with all 220 vines in the ground, 110 were planted on 08/02/2010 and the remaining 110 on 06/27/2011. These transgenic grapevines have been evaluated as plants on their own roots and as rootstocks grafted with untransformed Thompson Seedless (TS) scions. HNE-CecB- and PGIP-expressing transgenic grapevine lines in Riverside and Solano County have been evaluated phenotypically using the first 12 descriptors from the "Primary descriptor priority list" proposed by the International Organization of Vine and Wine (OIV). No phenotypical/horticultural differences were observed between transgenic and untransformed TS vines. 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. At the Riverside County site, the plants have been naturally infected by wild populations of GWSS and *Xf* presence in petioles extracts was confirmed by ELISA, PCR, and plate cell count in fall 2011. *Xf* presence was also confirmed in Riverside xylem sap samples collected on April 2012. Pierce's disease symptoms were assessed using a standardized score based on percentage of leaf area scorching to validate resistance to Pierce's disease under field conditions. At the Solano County site, non-grafted vines were mechanically inoculated with the *Xf* type strain (Temecula 1) in 2011 to validate resistance to Pierce's disease under field conditions, *Xf* presence was confirmed by ELISA, but no *Xf* growth in plate or Pierce's disease symptoms have been detected to date. Solano County grafted plants were mechanically inoculated with *Xf* and non-grafted plants were re-inoculated on 05/29/2012.

PUBLICATIONS

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FUNDING AGENCIES

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

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ABSTRACT

We propose to develop transgenic grapevine rootstocks resistant to Pierce's disease using new transformation protocols for the commercially important grapevine rootstocks '101-14' and '1103-P.' Our genetic approach involves developing transgenic rootstocks that deliver therapeutic proteins, like the chimeric antimicrobial protein HNE-CecB, that can protect an untransformed scion from Pierce's disease. Our approach is to improve grapevine transformation by exploring the possibility of *in planta* transformation of shoot apical meristems (SAM). We have successfully isolated SAM tissues from grapevine rootstocks '101-14' and '1103-P.' SAM explants are being tested for regeneration potential using different media and hormone concentrations that have worked in other grapevine cultivars. We have successfully obtained '101-14' and '1103-P' callus, embryos, and plants from shoot apical meristems, using solid medium supplemented with the cytokinin benzyladenine (BA) and the auxin 2,4-dichlorophenoxyacetic acid (2,4-D). We are currently confirming the reproducibility of a SAM-based grapevine regeneration system for more efficient production of transgenic plants. The outcome of this research would be the successful development of a more efficient transformation system for commercially relevant grapevine rootstock and scion varieties using SAM.

LAYPERSON SUMMARY

Current strategies for Pierce's disease control emphasize deploying transgenic rootstocks that deliver Pierce's disease control to the untransformed scion. We propose to develop transgenic grapevine rootstocks resistant to Pierce's disease using new transformation protocols for the commercially relevant grapevine rootstocks '101-14' and '1103-P.' Our genetic approach involves developing transgenic rootstocks that deliver therapeutic proteins like HNE-CecB that can protect an untransformed scion from Pierce's disease. Our approach includes improving grapevine transformation by exploring the possibility of *in planta* transformation of shoot apical meristems (SAM). We have successfully isolated SAM tissues from grapevine rootstocks '101-14' and '1103-P.' These SAM tissues are being tested for regeneration potential using various media and hormone additions that have worked in other grapevine cultivars. We have successfully obtained '101-14' and '1103-P' callus, embryos, and plants. We are currently confirming the reproducibility of a SAM-based grapevine regeneration system for more efficient production of transgenic plants.

INTRODUCTION

Several presentations at the 2010 Pierce's Disease Symposium highlighted transgenic strategies using various promising transgenes with potential for conferring resistance to *Xylella fastidiosa* (*Xf*), the causal agent of Pierce's disease. However, most such projects have not yet demonstrated such control in commercially significant rootstocks (Dandekar, 2010; Gilchrist, 2009; Labavitch, 2010; and Lindow, 2009). This is partly because the current grapevine transformation and regeneration system was developed at UC Davis a decade ago in rootstocks and scion varieties like *Vitis vinifera* 'Thompson Seedless' (Aguero et al., 2005, 2006). This system is cumbersome and slow because it uses embryogenic callus developed from young anthers, a tissue available for one brief period during each growing season. It takes six to eight months to generate transgenic somatic embryos from callus lines derived from anther tissue. Additionally, somatic embryogenic callus lines are not available for some widely used commercial rootstocks such as '101-14' and '1103-P.' To overcome this hurdle, we are developing a transformation system using meristematic stem cells present in the shoot apical meristem (SAM). In plants like grape, all aboveground plant parts are generated from a cluster of stem cells present in the central dome of the SAM (Sablowski, 2007, Gordon et al., 2009). Genetic factors regulated through cytokinin signaling determine and control the number of stem cells (Gordon et al., 2009). Several research- and commercial-scale transformation systems use meristem tissue from different crops. Use of SAM for transformation has occurred with a limited number of grapevine varieties. Mullins et al. (1990) co-cultivated adventitious buds of *Vitis rupestris* 'St. George' rootstock with *Agrobacterium* and produced transgenic plants. However, the methodology was never repeated. Mezzetti et al. (2002) transformed *V. vinifera* 'Silcora' and 'Thompson Seedless', cultivars with a strong capacity to differentiate adventitious shoots, using a meristematic tissue culture system. The culture type was unique and the overall application to other cultivars is unclear. Levenko and Rubtsova (2000) used *in vitro* internode explants to transform three *V. vinifera* scions and a rootstock, but did not provide sufficient details for the technique to be repeated. Dutt et al. (2007) described a simple transformation system for 'Thompson Seedless' using explants from readily obtainable micropropagation cultures. Tissues from etiolated cultures and meristem wounding using fragmented meristems gave the best results. This latter system has not been tested in many rootstocks. Taken together, these studies indicate that SAM is an interesting tissue to investigate, particularly since it is available all year. Additionally, much is now known about various developmentally regulated genes in plants like *Arabidopsis* that suggests how hormone input can be used to manipulate the developmental patterns of SAM (Galinha et al., 2009).

OBJECTIVES

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

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

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

RESULTS AND DISCUSSION

Activity 1: Develop a SAM regeneration system for grapevines that provides faster, more efficient production of transgenic plants

Our first step involved developing expertise and proficiency at dissecting and excising the meristematic dome from field-grown '101-14' and '1103-P' rootstocks. A similar technique is used routinely for pathogen elimination at UC Davis Foundation Plant Services. We have worked closely with Adib Rowhani and his colleagues to learn the best technique for excising a SAM. A pictorial outline of the process using '101-14' is shown (**Figure 1**). The sterile meristematic explants are then cultured to examine their potential for organogenesis or somatic embryogenesis using different hormone concentrations and combinations and different medium composition. We are currently investigating various hormone and medium compositions to identify those that lead to proliferation of the SAM; some are described below.

For experiment one we followed published protocols that use different hormone concentrations and combinations to proliferate SAMs to induce "meristematic bulk" (MB) (Mezzetti et al. 2002, Dhekney et al 2011). This was done using benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), and a specific medium composition. For experiments 2 and 3, we investigated the role of hormone concentrations and combinations in one medium to induce embryogenesis from '101-14' and '1103-P' SAMs.

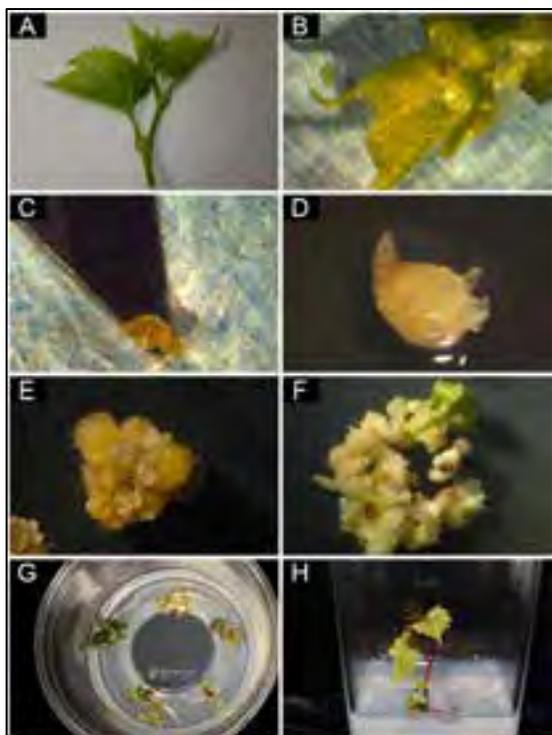


Figure 1. Regeneration system for '101-14' grape rootstock shoot apical meristem.

Experiment 1. '101-14' shoot tips were harvested and immediately dissected to produce explants composed of the apical meristem plus microscopic leaf primordia. Explants were immediately placed in Petri dishes containing induction media composed of Murashige and Skoog (MS) salts and vitamins, 0.1 g/L *myo*-inositol, 30 g/L sucrose and the hormones 2,4-D and BA at four and five different concentrations, respectively (**Table 1**). There were three explants per treatment, replicated three times (nine explants total per hormone combination). The explants were transferred to fresh medium every three weeks and incubated in the dark at 25°C. We obtained callus, embryos, and plants from '101-14' meristems in treatments B8 and B9.

Experiment 2. Observations and data from experiment 1 allowed us to design the second experiment using meristematic explants from '101-14' and '1103-P' rootstocks. Grapevine shoot tips were harvested from field-grown material and immediately dissected to produce explants composed of the apical meristem plus microscopic leaf primordia. Explants were immediately placed in Petri dishes containing five different induction media, N1 to N5, composed of Nitsch and Nitsch (NN) salts and vitamins, 0.1 g/L *myo*-inositol, 20 g/L sucrose, 5

μM BA, and 0 μM (N1), 1 μM (N2), 5 μM (N3), 10 μM (N4), or 50 μM (N5) 2,4-D (**Table 2**). Here, we were testing the ability of 2,4-D to induce somatic embryogenesis. There were 15 explants per treatment, replicated three times, for 45 explants per treatment. The explants were transferred to fresh medium twice every three weeks and incubated in the dark at 25°C

Table 1. BA and 2,4-D concentrations used for specific '101-14' SAM treatments.

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

Table 2. Concentrations of BA and 2,4-D used to investigate somatic embryogenesis of TS, '101-14' and '1103-P' grape rootstocks.

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

The resulting callus was transferred every three weeks to fresh MS salts and vitamins medium supplemented with 0.1 g/L *myo*-inositol, 20 g/L sucrose, 1 μM BA, and 11 μM naphthaleneacetic acid (NAA) and maintained at 26°C in light (65 μmol/s · m²) for development of somatic embryos. Proliferating embryogenic material was then transferred every three weeks to X6 medium supplemented with 3.033 g/L KNO₃, 0.364 g/L NH₄Cl, 60.0 g/L sucrose, 1.0 g/L *myo*-inositol, 7.0 g/L TC agar, and 0.5 g/L activated charcoal and maintained at 26°C in

light ($65 \mu\text{mol s}^{-1} \text{m}^{-2}$) for development and proliferation of somatic embryos. In this second experiment, we successfully obtained ‘101-14’ and ‘1103-P’ callus, embryos (**Figure 2**), and plants

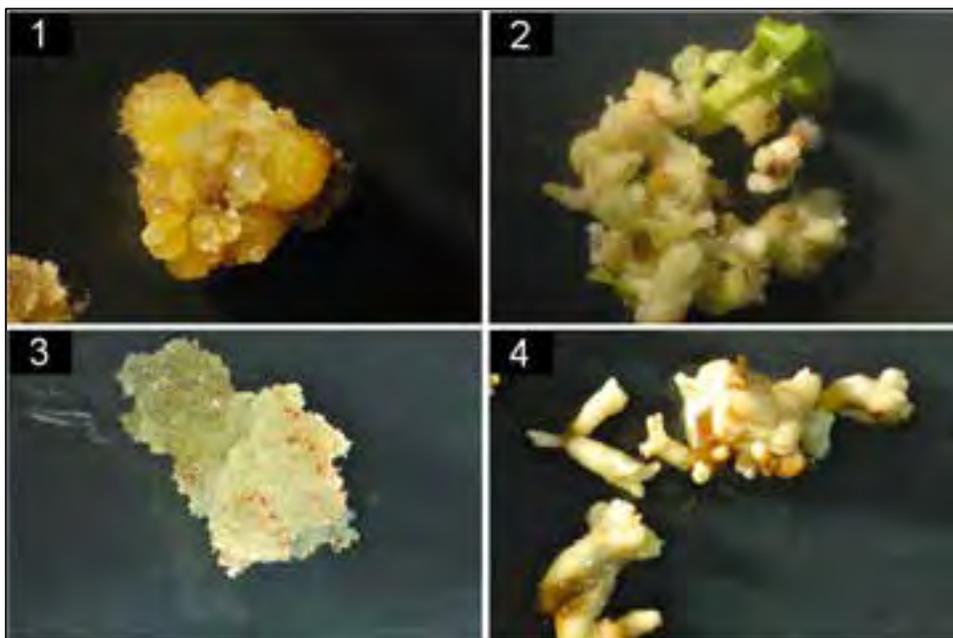


Figure 2. Grape callus and embryos from ‘101-14’ (1-2) and ‘1103-P’ (3-5) rootstocks regenerated from shoot apical meristems.

Experiment 3. ‘101-14’ and ‘1103-P’ shoot tips were harvested in 2012 from field-grown material and immediately dissected to produce explants composed of the apical meristem plus microscopic leaf primordia. Explants were immediately placed in Petri dishes containing induction media under the same conditions as in experiment 2. We are also confirming the reproducibility of the regeneration system and optimizing the time required for the process. There were 50 meristem explants per treatment, replicated three times, for each medium and each cultivar.

Activity 2: Transform, select, regenerate, and confirm transgenic grapevine plants using this new system. ‘101-14’ and 1103-P embryogenic callus (obtained in activity 1) will be infected with *Agrobacterium* containing the existing HNE-Cecropin B vector (pDU04.6105) to produce transgenic grape rootstocks.

CONCLUSIONS

Transgenic rootstocks have been proposed as the best strategy to develop Pierce’s disease resistant grapevines. However, current transformation protocols use embryogenic callus lines developed from anther culture for transformation and available cultures do not include rootstock genotypes currently used by growers in California. Also, current callus lines have been in culture for a long time and fresh new cultures must be selected, a task limited to a single season each year. This research seeks to overcome this seasonal limitation by developing a transformation system using a SAM, an initial explant material that is available year round. We have successfully obtained ‘101-14’ and ‘1103-P’ callus, embryos, and plants from SAMs using solid medium, the cytokinin benzyladenine (BA), and the auxin 2,4-dichlorophenoxyacetic acid (2,4-D). We are currently confirming the reproducibility of a SAM-based grapevine regeneration system for more efficient production of transgenic plants. We have also initiated transformation of the ‘101-14’ and ‘1103-P’ embryogenic callus with an existing HNE-Cecropin B vector (pDU04.6105).

The results of this research will benefit other research groups working on transgenic strategies to control Pierce’s disease and has the potential to benefit research in other crops where transgenic approaches are sought

to create pathogen resistance in rootstocks. The objectives described in this progress report directly address research priorities outlined in ‘Attachment A’ of the 2010 PD/GWSS proposal RFA. They also address the top RSAP priority in the “Enabling tools- Development of grape regeneration and transformation systems for commercially important rootstocks” handout released in December 2009. This document outlines the “Top 5 to 10 Project Objectives to Accelerate Research to Practice” and updates the priority research recommendations provided in the report “PD/GWSS Research Scientific Review: Final Report” released in August 2007 by the CDFA’s Pierce’s Disease Research Scientific Advisory Panel.

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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 1, 2011 to October 10, 2012.

ABSTRACT

The objective is to evaluate transgenic grape and grape rootstocks expressing various genes from different constructs in a field site in Solano County for resistance to *Xylella fastidiosa* (*Xf*), Pierce's disease strain following mechanical injections of *Xf* into the plant stems. Over the course of the three-year field evaluation, test plants in the first planting (2010) included ungrafted conventional Thompson Seedless and Freedom plants as controls, transgenic plants of the same varieties from Dandekar, Labavitch, Lindow and Gilchrist projects as experimental units. Transgenic rootstocks expressing some of the test genes grafted to untransformed Pierce's disease susceptible scions were planted in 2011. The 2010 planting was inoculated in July 2011 and re-inoculated in June 2012, along with the 2011 plantings of the untransformed scions grafted to transgenic rootstocks. All field operations and the handling of plant material proceeds under an APHIS permit for these materials and over a specified time period.

LAYPERSON SUMMARY

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

INTRODUCTION

The objective is to evaluate transgenic grape and grape rootstocks expressing various genes from different constructs in a field site in Solano County for resistance to *Xylella fastidiosa* (*Xf*), Pierce's disease strain following mechanical injections of *Xf* into the plant stems. Over the course of the 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, transgenic rootstocks expressing some of the test genes grafted to untransformed Pierce's disease susceptible scions. 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 6 years. The same *Xf* strain will be used to inoculate the plants in this experiment.

OBJECTIVES

1. Prepare land area and manage the experimental planting under APHIS permit to provide for up to 500 plants from Lindow, Gilchrist, Labavitch, and Dandekar.
2. Layout will have rows 15 feet apart with 4 feet between plants. This spacing required 18 rows of 28 plants each and includes a 50 foot open space around the planted area. Total area occupied by plants and buffer zones will be a minimum of 1.8 acres.
3. Area is fenced to protect against rabbit invasion. The plants are irrigated by surface furrow. Field crews will be provided from these funds to assist in planting, weeding. And irrigation
4. Irrigation and pest management will be provided by Cooperator Tom Kominek.
5. First planting was completed in late July 2010 with the second planting completed May 17, 2011.
6. Plants were mechanically inoculated with *Xf* in 2011 and subsequent years.

RESULTS AND DISCUSSION

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

The 2010 and 2011 plantings of all four investigators survived each winter without loss (**Figure 4**). The attachment of new shoots to the trellis system, cultivation, and irrigation management progressed in a normal and effective manner. Extensive pruning was done to manage the plants in a fashion compatible with mechanical inoculation. All prunings were collected, bagged, and autoclaved before disposal.

As of October 2012, the 2010 planting and the second 2011 planting individuals are healthy, growing normally and all plants have a normal phenotype, true to the untransformed control plants of each parental genotype (**Figure 3**). Inoculations of the 2010 plants occurred on July 2011 and June 2012 (**Figures 4 and 5**). Field data has been collected by all investigators and can be found in their individual reports.

FUNDING AGENCIES

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



Figure 1. This image shows the field preparation, trellis and staking arrangement and a portion of the initial planting at the Solano County site in July 2010.



Figure 2. This shows the Solano Co. site as of July 2011 including the newly-planted grafted plants seen as smaller plants in rows adjacent to larger ungrafted more mature transformed plants, which were planted one year earlier.



Figure 3. Image taken July 10, 2012 of the Solano County site including the 2011 planted grafted plants in the front rows and the Fall 2010 planting in the back rows. Plants are healthy, growing normally and all plants have a normal phenotype.



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



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



Figure 6. July 2011 inoculated grapevine canes, marked with orange tags, photographed as the plants leafed out in May 2012 at the Solano County site.

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 1, 2011 to October 10, 2012.

ABSTRACT

The objective 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 PR1 and UT456 indicated that suppression of Pierce's disease symptoms and reduction in bacterial titer was consistent in the transgenic compared with untransformed control plants. The Solano field experiment is conducted in two phases. The first phase started in 2010 to evaluate clonal copies of the fully transformed ungrafted plants that exhibited suppressed Pierce's disease symptoms and low bacterial titers. The second phase began in 2011 with planting the untransformed Thompson Seedless scions grafted onto the most 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 will include ungrafted conventional Thompson Seedless and Freedom plants as controls to be compared with the transformed plants. Controls in the second phase will include, untransformed rootstocks grafted to the untransformed scions, which will be compared to equivalent combinations expressing the test genes grafted to untransformed Pierce's disease susceptible scions. Data collected in 2012 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. Some plants in Riverside are showing symptoms of leaf death while the plants in Solano remain healthy in appearance. Clearly, at least one more year of evaluation is needed to begin to develop an assessment of the possible field efficacy of the transgenes. Quantitative data collection is in progress at both sites.

LAYPERSON SUMMARY

Previously, we identified novel anti-programmed cell death (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. This project will evaluate clonal copies of these same plants under field conditions for resistance to Pierce's disease. The field evaluation will be conducted in Solano and Riverside Counties and will include 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 site and PCR assays confirmed a correlation between protection, transgene presence, and bacterial populations in the plants. While bacteria are present in inoculated plants at the Solano site, there is no definitive evidence of extensive symptoms or bacterial population differences. Clearly both sites will need to be monitored and assays taken over a longer period of time for meaningful results to be obtained.

INTRODUCTION

Susceptibility in most plant-microbe interactions depends on the ability of the pathogen to directly or indirectly regulate genetically determined pathways leading to apoptosis or programmed cell death (PCD). The role of altered cell stability in disease through an evolutionarily conserved program involving programmed cell death

occurs in both animals and plants. Functionally, the induction of PCD results in an orderly dismantling of cells while maintaining integrity of the plasma membrane until internal organelles and potentially harmful contents including phenolics, reactive oxygen and hydrolytic enzymes have been rendered harmless to contiguous cells. Processed in this manner, the cell contents can serve as nutrients for microbial cells when they are present in the immediate environment of the pathogen (2). In the case of *Xylella fastidiosa* (*Xf*) and many other plant pathogenic bacteria, the bacteria live predominantly as endophytes or epiphytes but occasionally as pathogens. The relative susceptibility of the individual plant species is determined by unknown genetic factors. Presumably, sensitivity to the presence of the bacteria, expressed as cell death-dependent symptoms, is the result of signals expressed by the bacteria that lead to activation of PCD, as appears to be the case with Pierce's disease. Our research has focused on the effect of altering the expression of two different plant DNA sequences (PR1 and UT456). Both of these putative anti-PCD sequences protected both against Pierce's disease symptoms and limited bacterial titer four to six orders of magnitude below that reached in untransformed control vines of the susceptible cultivar Thompson Seedless and the commercial rootstock Freedom. In the past year we constructed transformed rootstocks (Freedom and Thompson Seedless) expressing PR1 or UT456 grafted to untransformed Thompson Seedless and winegrape scions to be tested for efficacy of protection across a graft union. Initial greenhouse inoculation experiments indicated that the protection by PR1 and UT456 does move across the graft union. In summary, experimental results to date confirm progress in identifying DNA transcripts of grape which, if regulation of the natural transcripts is altered in transgenic plants, result in the suppression of symptoms of Pierce's disease with an associated limitation in bacterial titer to levels generally associated with a benign endophytic association. Initial data on potential for transmission of protection by these anti-PCD sequences across a graft union to protect an untransformed wild type scion is positive.

OBJECTIVES for 2011-2012

1. The overall objective is to evaluate 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.
2. The field experiments in Solano County will be conducted in two phases. The first phase of the field experiment started in 2010 will evaluate clonal copies of the fully transformed ungrafted PR1 and UT 456 plants that exhibited suppressed Pierce's disease symptoms and low bacterial titers. These experiments will consist of sets of inoculated and uninoculated control plants. All plants to be inoculated will be infected by stem puncture with ~20,000 *Xf* bacterial cells per inoculation site. Inoculations were done July 2011 and repeated in June of 2012.
3. 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 plants as rootstocks.
4. The field experiment in Riverside County was planted in the spring of 2011. The planting consisted of clonal copies of the fully transformed ungrafted plants expressing PR1 or UT 456 that were planted in 2010 in Solano County. These Riverside plants will not be inoculated with *Xf* but will be exposed to infection via natural populations of the glassy-winged sharpshooter (GWSS) *Xf* vector.

RESULTS AND DISCUSSION

The first phase of the field experiment started in 2010 will evaluate clonal copies of the fully transformed ungrafted PR1 and UT 456 plants that exhibited suppressed Pierce's disease symptoms and low bacterial titers (2010-2013)

This phase took place as planned with the planting occurring on July 12, 2010. Plants were placed in plastic sleeves to protect against sunburn and wind damage. The young plants had all emerged from the sleeves within two months and appeared to be growing normally. Selections of canes to form cordons were made in spring 2011. Test plants were planted in a complete randomized block design. Field maps were prepared prior to planting and each plant is labeled with a permanent metal tag. Evaluation of the experimental plants for plant morphology, symptoms of Pierce's disease infection, and the presence of the bacteria will be a time course evaluation by visual monitoring of symptom development and sampling inoculated tissue (mainly leaves and stems) for *Xf* by quantitative PCR (qPCR) assays. A comparative quantitative determination by qPCR of the presence of *Xylella* in transgenic grape and grape rootstocks compared with conventional grape and grape rootstocks will provide an indication of the level of resistance to Pierce's disease infection and the impact on the

bacterial load in the respective transgenic and control plants. All these procedures have been used successfully in the ongoing greenhouse experiments for the past five years.

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 plants as rootstocks (2010-2013)

Transgenic rootstocks for grafting were made by removing green shoots from greenhouse-grown plants of Thompson Seedless and Freedom expressing either PR1 or UT456, surface sterilized for 30 seconds in 70% ethyl alcohol, followed by 1% sodium hypochlorite solution containing 0.2% Tween 80 for 20 min with shaking, on a rotary shaker (50 rpm). The surface sterilized shoots are cut into single node pieces and placed into solid growth media to stimulate root formation. All the grafting is conducted in sterile Magenta GA-7 Plant Culture Boxes (3 x 3 x 4") containing 50 ml media under a 16 h light, 8 h dark photoperiod at 25°C. Rootstock plantlets obtained *in vitro* are allowed to grow until several leaves are produced (4-6 weeks) and divided into 3-4 explants, each containing a single node. A scion with a single node and a leaf was selected to match the size of the rootstock; cut into a wedge to match a cleft made in the rootstock and was carefully fitted on to the cleft of the rootstock on the medium. After four weeks incubation healing in a magenta box, the rooted plantlet is transferred to sterile soil, allowed to heal and then transferred to the greenhouse for assays. Success rate is greater than 95% using this procedure, 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.

Establish a field planting in Riverside, County consisting of clonal copies of the fully transformed ungrafted PR1 and UT 456 plants that were planted in Solano County in 2010 (2011-2013)

Field space was prepared in Riverside County and grape plants grown in our greenhouse were transported to Riverside for planting. We coordinated the movement of plants to Riverside County with Professor Steven Lindow from UC Berkeley, who also planted his materials for the first time in Riverside County. The planting occurred April 2011.

Secure patent protection as intellectual property for those genes that prove to be capable of blocking Pierce's disease in grape

The grape plants containing the anti-PCD genes and the grafted rootstocks will require the use of several patented enabling technologies. Record of invention disclosures have been submitted to the UC Office of Technology Transfer. The research proposed reported herein will provide data on the activity and mechanism of action of the protective transgenes in grape relative to the presence, amount and movement of *Xf* in the transformed and untransformed grape plants.

CONCLUSIONS

Xf induces Pierce's disease symptoms that result from activation of a genetically regulated process of programmed cell death. We have identified grape DNA sequences, which when constitutively expressed in transgenic grapes suppress the death-dependent symptoms of Pierce's disease and reduce the bacterial titre to a level found in Pierce's disease resistant wild grapes. We identified six novel anti-PCD genes from cDNA libraries of grape. Two of these grape sequences expressed as transgenes in grape, suppressed Pierce's disease symptoms and dramatically reduced bacterial titer in inoculated plants in full plant transgenics. Initial data suggest that protective sequences may function across a graft union to protect an untransformed and susceptible wild type scion. This project has identified a basis for Pierce's disease symptoms and a genetic mechanism to suppress symptoms and bacterial growth with an infected plant. If needed in the future, a transgenic strategy exists to address 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, also under field conditions.

INTELLECTUAL PROPERTY

Record of invention disclosures have been submitted to the UC Office of Technology Transfer.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Row DGG5	TS UT 456-6	TS02A	TS UT 456 #8	FD P14 #13	TS P14 #9	TS P14 #16	TS UT 456 #10	FD#3	TS UT 456-5	TS UT 456 #8	TS UT 456 #10	TS02A	TS P14-9	FD#3	TS UT 456-6	TS P14 #16	TS UT 456 #8	TS UT 456-5	TS02A	TS UT 456 #10	TS P14-9	TS P14 #16	TS P14 #9	FD#3	TS P14 #9
Row DGG6	TS UT 456-6	TS P14 #9	TS UT 456 #10	FD P14 #13	FD UT 456 #3	TS02A	TS UT 456-8	TS UT 456-5	FD P14 #13	FD#3	TS P14-9	TS UT 456 #10	TS02A	TS UT 456 #8	TS UT 456 #10	TS UT 456 #8	TS UT 456 #10	TS P14 #9	TS P14-9	TS UT 456 #10	TS P14-16	TS02A	FD#3	TS UT 456 #8	TS P14-9
Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek	Creek

Figure 2. Gilchrist Riverside Field Grape Map. Plants are planted on a sloping river bank with a creek at the bottom. *Xylella*-spreading sharpshooters are common to this site.



Figure 3. Inoculated grapevine canes at the Solano County site.



Figure 4. Example grape plants growing in Riverside county August 2012, subject to feeding by GWSS confirmed to carry *Xf*. Plant on the left is transgenic TS UT 456-6 compared with a control plant on the right expressing substantial symptoms of leaf death. Complete data set collection is in progress.

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

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Reporting Period: The results reported here are from work conducted October 1, 2011 to October 10, 2012.

ABSTRACT

Two DNA sequences (VvPR1 and UT456) were selected by a functional screen to test for ability to suppress the programmed cell death (PCD)-dependent symptoms of Pierce's disease. Greenhouse experiments confirmed that these two different anti-PCD DNA sequences prevented Pierce's disease symptoms in the Pierce's disease susceptible cultivar Thompson Seedless and the commercial rootstock Freedom. Furthermore, the bacterial titer in the transgenic plants was reduced four to six orders of magnitude below that reached in untransformed control vines. In contrast, all untransformed control plants died within 3-4 months after inoculation while the transgenic plants were asymptomatic for 12 months. The net effect of these transgenes is to limit bacterial titer but not distribution of bacteria in the asymptomatic plants. From the perspective of the grape-bacterial interaction, it appears that the anti-PCD genes suppress Pierce's disease symptoms and functionally confine *Xylella fastidiosa* to an endophytic ecology in the xylem, equivalent to that seen in the related asymptomatic host *Vitis californica*. Thompson Seedless, Merlot, and Cabernet Sauvignon were grafted to the respective transgenic rootstocks and subjected to greenhouse inoculation. In each case, the untransformed scions grafted to the transformed rootstocks exhibited less disease than the untransformed control plants. Clonal copies of the transgenic and control plants were moved to two field locations under a USDA-APHIS permit secured by the Public Intellectual Property Resource for Agriculture (PIPRA) in Solano and Riverside counties. The plants at Solano County were inoculated on July 21, 2011 and June 26, 2012. The plants at the Riverside site were subject to infection via glassy-winged sharpshooter feeding. PCR and plating assays confirmed bacteria at both site. Greenhouse data obtained from grafting experiments indicate the protective effect of these genes may be transferred across a graft union to protect a susceptible untransformed scion. Grafted plants expressing VvPR1 and UT456 in the rootstock, but not the Thompson Seedless scion, were moved to the Solano County field site for inoculation on June 26, 2012.

LAYPERSON SUMMARY

Xylella fastidiosa induces Pierce's disease symptoms that are the result of the activation of a genetically regulated process of programmed cell death. We identified six novel anti-programmed cell death (PCD) genes from a grape cDNA library functional screen for ability to suppress PCD. Two of these grape sequences, VvPR1 and UT456, when expressed as transgenes in the Pierce's disease susceptible Thompson Seedless plants, suppressed Pierce's disease symptoms and dramatically reduced bacteria levels in inoculated plants. The remaining four genes were tested this year, along with VvPR1 and UT456; each of the four provided substantial suppression of both Pierce's disease symptoms and bacterial titer. However, none were as effective as VvPR1 and UT456. Currently in progress are a series of experiments designed to evaluate whether the protective effect of these two sequences can protect untransformed susceptible winegrape scions across a graft union. Preliminary data suggest that 50% or more of the susceptible scions grafted to either VvPR1 or UT456 showed less Pierce's disease symptoms and had lower bacterial titers than the unprotected control plants. While these results are encouraging, they are not complete or definitive and the experiment is continuing. The relative susceptibility of the suite of eight commercial winegrape varieties was tested under controlled greenhouse conditions prior to field testing these varieties as scions on the transgenic rootstocks. Mechanism of action experiments initiated recently suggests a genetically conserved basis for suppression of PCD and the protection against Pierce's disease. This project is now moving from the proof-of-concept to potential application and

characterization of these plants under field conditions with appropriate APHIS permits: initial field plantings in Solano County began in July 2010 with additional plantings made in Solano and Riverside Counties in 2011.

INTRODUCTION

Susceptibility in most plant-microbe interactions depends on the ability of the pathogen to directly or indirectly regulate genetically determined pathways leading to apoptosis or programmed cell death (PCD). The role of altered cell stability in disease through an evolutionarily conserved program involving PCD occurs in both animals and plants. Functionally, the induction of PCD results in an orderly dismantling of cells while maintaining integrity of the plasma membrane until internal organelles and potentially harmful contents including phenolics, reactive oxygen, and hydrolytic enzymes have been rendered harmless to contiguous cells. Processed in this manner, the cell contents can serve as nutrients for microbial cells when they are present in the immediate environment of the pathogen (2). In the case of *Xf* and many other plant pathogenic bacteria, the bacteria live predominantly as endophytes or epiphytes but occasionally as pathogens. The relative susceptibility of the individual plant species is determined by unknown genetic factors. Presumably, sensitivity to the presence of the bacteria, expressed as cell death-dependent symptoms, is the result of signals expressed by the bacteria that lead to activation of PCD, as appears to be the case with Pierce's disease. Our research has focused on the effect of altering the expression of two different plant DNA sequences (PR1 and UT456). These putative anti-PCD sequences protected both against Pierce's disease symptoms and limited bacterial titer four to six orders of magnitude below that reached in untransformed control vines of the susceptible cultivar Thompson Seedless and the commercial rootstock Freedom. In the past year we constructed transformed rootstocks (Freedom and Thompson Seedless) expressing PR1 or UT456 grafted to untransformed Thompson Seedless and winegrape scions to be tested for efficacy of protection across a graft union. Initial greenhouse inoculation experiments indicated that the protection by PR1 and UT456 does move across the graft union. In summary, experimental results to date confirm progress in identifying DNA transcripts of grape which, if regulation of the natural transcripts is altered in transgenic plants, result in the suppression of symptoms of Pierce's disease with an associated limitation in bacterial titer to levels generally associated with a benign endophytic association. Initial data on potential for transmission of protection by these anti-PCD sequences across a graft union to protect an untransformed wild type scion is positive.

OBJECTIVES (2011-2013)

1. Conduct experiments to assess the potential for protection against Pierce's disease across a graft union by VvPR1 and UT456, first with Thompson Seedless as the untransformed scion. (2011-2013)
2. Determine presence and movement of the mRNA and/or protein of VvPR1 and UT456 across the graft union into the untransformed Thompson Seedless O2A scion. (2011-2013)
3. Perform inoculations of eight winegrape varieties, initially on their own rootstocks. Then chose three varieties that cover the range of susceptibility to test for cross protection by VvPR1 and UT456. (2012). Chosen varieties are Thompson Seedless, Merlot, and Cabernet Sauvignon. Inoculations were initiated in May 2012 and first evaluations were done on October 10, 2012. (2012-2013)
4. Investigate the mechanism underlying the protection against Pierce's disease by VvPR1 and UT456. (2011-2013)
5. Secure patent protection as intellectual property for those genes that prove to be capable of blocking Pierce's disease in grape. (2013)

RESULTS AND DISCUSSION

Conduct experiments to assess the potential for protection against Pierce's disease across a graft union by VvPR1 and UT456 with Thompson Seedless as the untransformed scion (2011-2012)

The purpose is to determine if the protective effect of these genes as observed in the primary transgenics is transferrable across a graft union to protect a susceptible scion. Pierce's disease susceptible untransformed Thompson Seedless scions were grafted onto Freedom rootstocks transgenic for VvPR1 and UT456. A total of 13 untransformed control grafts were compared with 13 transformed rootstock:untransformed scions. The preliminary data suggest that all 13 of the susceptible scions showed none or far less Pierce's disease symptoms and had reduced bacterial titer than the untransformed control grafted plants, all of which were dead or nearly dead by four months after inoculation with approximately 20,000 *Xf* cells per branch. While these results are

encouraging, the experiment is continuing with two of the winegrape varieties grafted to a transgenic rootstock. Cabernet Sauvignon (susceptible) and Merlot (tolerant) winegrape varieties, along with the highly susceptible Thompson Seedless were grafted to transgenic rootstocks expressing VvPR1 and UT456 and inoculated to determine if any cross-graft protection occurs. Inoculation of these grafted plants under greenhouse conditions, comparable to the previous Thompson Seedless transgenic rootstock: untransformed scion combinations were begun in May 2012 and rated on October 10, 2012 (**Table 1**). Taqman assays are in progress to attempt to detect and quantify the presence of the UT456 microRNA in the rootstocks and scions of grafted plants that appear to be protected against Pierce's disease.

Determine presence and movement of the mRNA and/or protein of VvPR1 and UT456 across the graft union into the untransformed Thompson Seedless O2A scion (2011-2013)

By functional definition, microRNAs are small endogenous RNA molecules (~22-24 bases) that are processed from longer transcripts into pre-microRNA hairpin structures with final steps completed by an enzyme called Dicer. The *in vitro* activation of PR1 translation results from the release of a 22-24 bases from the native 270 base UT456 hairpin by the endogenous nuclease DICER, known to be present in the wheat germ extracts. MicroRNAs regulate gene expression by targeting by sequence homology one or more messenger RNAs (mRNAs) for translational regulation or degradation. Although the first microRNA was identified over ten years ago, it is only recently that the scope and diversity of these regulatory molecules have begun to emerge. There is precedent for translational blockage by the 3'UTR in plant systems and for RNA movement from roots to tubers (6). The presence of the sequence in UT456 with annealing ability to the 3'UTR of PR1 is the basis for the current model of PR1 and UT456 function in the *Xf*-grape system. In ongoing experiments we developed a PCR-based assay, termed a "hook assay", wherein a synthetic oligonucleotide that is designed to anneal on itself leaving a 6 nucleotide un-annealed (single stranded) that has sequence homology to the 3' end of the UT456 RNA (**Figure 1 A**). This restricts amplification by PCR to RNAs containing a unique set of bases such as those found only in the UT456 as shown in **Figure 1 B** where the arrow points to the band confirmed by isolating and sequencing to be the UT456 microRNA.

Once we confirmed that the hook assay was capable of detecting the UT456 microRNA in an RNA pool from grape extracts this sequence was used to develop a so-called "Taqman" assay obtained from Invitrogen (Life Technologies Inc) for detection of microRNAs. We contracted with Invitrogen to construct a Taqman probe for the mobile UT456 microRNA to detect it in extracts of the transgenic grapes and as a mobile element in untransformed scions. Confirmation of the ability to detect the UT456 microRNA in grape extracts is seen in **Figure 1B**. This assay is now being applied to the UT456 transgenic rootstocks and the untransformed scions, which appear to be protected against Pierce's disease symptoms when grafted to transgenic rootstocks as performed expressing UT456 (**Table 1**).

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

Experiments were concluded on a suite of commercial winegrape varieties to obtain quantitative data on bacterial population dynamics and relative Pierce's disease susceptibility. The varieties tested include Chardonnay, Pinot Gris, Sauvignon Blanc, Cabernet Sauvignon, Pinot Noir, Zinfandel, Syrah, and Merlot with untransformed Thompson Seedless, with VvPR1 and UT456 transgenic lines as reference controls (5). Data collected included bacterial titer, and "disease symptoms." Disease symptoms are herein defined as leaf defoliation, not marginal death of leaves that is generally considered but not proven to be true symptoms of Pierce's disease under field conditions. NOTE: It has been our consistent observation over the past seven years that marginal leaf death, often associated with Pierce's disease under field conditions is meaningless and misleading under our greenhouse conditions. Uninoculated control plants frequently exhibit marginal and interveinal death reminiscent of the field Pierce's disease symptoms, while leaf drop occurs only in susceptible inoculated plants. The point is that after inoculating more than 500 plants in the greenhouse, the susceptible control plants always defoliate, show high bacterial titer, and die. In our experiments, only the transgenic protected plants retain their leaves and show low levels of bacterial titer. Selected clones of each variety were inoculated by the needle prick method with Temecula strain of *Xf* delivering 10-20 μ l at bacterial concentration of 10^5 cfu/ml (2,000 cells or less). All varieties were susceptible to Pierce's disease in terms of leaf defoliation

symptom expression and exhibited 1-3 orders of magnitude higher bacterial titers four months after inoculation than the asymptomatic *Vitis californica* or transgenic Vv PR1 or UT456 comparison plants (5). Pinot Gris had the highest bacterial titer and exhibited the most severe defoliation while Syrah was the most tolerant with symptoms and bacterial titer nearly as low as *V. californica*. Cabernet Sauvignon (susceptible) and Merlot (tolerant) winegrape varieties, along with the highly susceptible Thompson Seedless were grafted to transgenic rootstocks expressing VvPR1 and UT456 and inoculated to determine if any cross-graft protection occurs. Inoculation of these grafted plants under greenhouse conditions, comparable to the previous Thompson Seedless transgenic rootstock: untransformed scion combinations were begun in May 2012 and rated on October 10, 2012 (Table 1). Taqman assays are in progress to attempt to detect and quantify the presence of the UT456 microRNA in the rootstocks and scions of grafted plants that appear to be protected against Pierce's disease.

Investigate the mechanism underlying the protection against Pierce's disease by VvPR1 and UT456 (2011-2012)

As indicated above, we have found two novel and likely linked mechanisms for VvPR1 and UT456 action. First, the transgenic PR1 protein product will suppress PCD in several plants systems we have tested. However, the PR1 coding sequence is translationally blocked in healthy cells and in an *in vitro* translation system, even when the message level is high. The key element in these experiments is that the message is readily translated into the functional anti-PCD protein when the tobacco, tomato, or grape cells are under chemical or pathogenic (death) stress. The biological impact in disease is to block death in the cells where pathogen -induced lesion spread is restricted and disease is limited. Secondly, the noncoding UT456 sequence contains small RNA hairpins that show sequence conservation with the 3'UTR of PR1 and are projected to interact with each other by RNA modeling programs. *In vitro* protein translation studies indicated that the block in translation of PR1 mRNA can be relieved by the addition of UT456 RNA to wheat germ extracts. The same result was obtained by agro- infiltration assays, whereby the expression of UT456 activated the translation of the PR1 protein in tobacco leaves expressing high levels of the PR1 message that was blocked until the UT456 RNA was present and processed into microRNA. PR1 antibodies will be used to test directly for the presence of mobile PR1 protein from the rootstock into the grafted scions. In addition PR1 antibodies are being used in immunoprecipitation assays to detect potential PR1 interacting factors. To date we have been successful in identifying 3 PR1-interacting proteins, HP70, HP90 and RACK1 from plant extracts. Interestingly, these three proteins have previously been reported to interact directly with each other and occur in a membrane associated complex involved in innate immunity to rice blast in rice plants transgenic for RACK1 (7).

Collaboration with the Public Intellectual Property Resource for Agriculture (PIPRA) to obtain a USDA APHIS permit for field planting

A USDA APHIS permit to enable field planting of transgenic plants from our laboratory as well as those of PIs Dandekar, Lindow, and Labavitch is in place and the experiments are underway. Planting of the primary transgenic plants from the respective programs was completed in July 2010. The 2010 plantings of all four investigators survived the winter without loss. The attachment of new shoots to the trellis system, cultivation, and irrigation management progressed in a normal and effective manner. Pruning was done to manage the plants in a fashion compatible with mechanical inoculation. As of September 21, 2012, the initial planting and the second 2011 planting individuals are healthy, growing normally, and all plants have a normal phenotype, true to the untransformed control plants of each parental genotype. Plants were maintained under clean field conditions, with furrow irrigation on a regular schedule. The plants were well established in the spring of 2012, which reduced in-season irrigation to twice during the season. Regular monitoring was conducted for weeds, insects, and non-Pierce's disease disease. Weeds were managed by cultivation and minimal hand weeding. No significant insect or disease pressure was noted, although the plants were sprayed once for powdery mildew once in the spring. Plants from all four laboratories were inoculated on July 21, 2011 and again on June 26, 2012. The inoculation was by needle-prick method with a delivery of ~20,000 *Xf* cells per inoculation site. Sampling of a limited number of inoculated canes near the inoculation site on control and VvPR1 and UT456 transgenic plants assayed by PCR confirmed the presence of the respective transgenes and then determined, by qPCR, to harbor a low level of *Xf* in the sampled inoculated plants. Bacterial titer ranged from undetectable in the uninoculated control plants to 1×10^2 to 7.5×10^3 per 0.1 g of stem tissue in the inoculated plants. Untransformed control plants were negative for the transgenes. During the growing season of 2011 and 2012,

no typical field symptoms or defoliation of leaves was observed on any of the inoculated plants until late in the fall of 2012.

Secure patent protection as intellectual property for those genes that prove to be capable of blocking Pierce's disease in grape

The grape plants containing the anti-PCD genes and the grafted rootstocks will require the use of several patented enabling technologies. Record of invention disclosures have been submitted to the UC Office of Technology Transfer. The research proposed reported herein will provide data on the activity and mechanism of action of the protective transgenes in grape relative to the presence, amount, and movement of *Xf* in the transformed and untransformed grape plants.

CONCLUSIONS

Xf induces Pierce's disease symptoms that result from activation of a genetically regulated process of PCD. We have 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. We identified six novel anti-PCD genes from cDNA libraries of grape. Two of these grape sequences expressed as transgenes in grape, suppressed Pierce's disease symptoms and dramatically reduced bacterial titer in inoculated plants in full plant transgenics. Initial data suggest that protective sequences may function across a graft union to protect an untransformed and susceptible wild type scion. This project has identified a basis for Pierce's disease symptoms and a genetic mechanism to suppress symptoms and bacterial growth within an infected plant. If needed in the future, a transgenic strategy exists 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, also under field conditions.

INTELLECTUAL PROPERTY

Record of invention disclosures have been submitted to the UC Office of Technology Transfer.

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FUNDING AGENCIES

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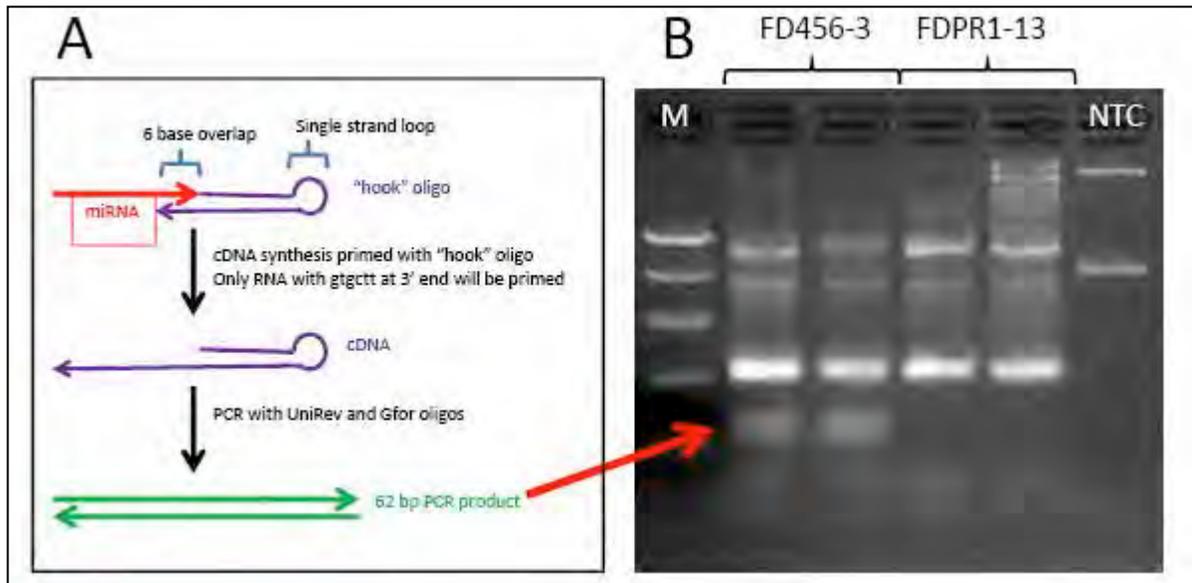


Figure 1. “Hook assay” for detection of UT456 microRNA. Schematic of method (left) shows “hook” oligo used to prime cDNA synthesis and PCR primers to amplify a 62bp fragment. Ethidium bromide stained gel (right) shows presence of UT456G microRNA only in UT456 expressing plants. M is a set of DNA markers, NTC is no template control. No endogenous signal of 456 is detected in the FDPR1-13 transgenic control, confirming the absence of the 456 microRNA in this genotype. The FD 456-3 extracts will be assayed by PR1 antibodies for translation of the endogenous PR1 mRNA.

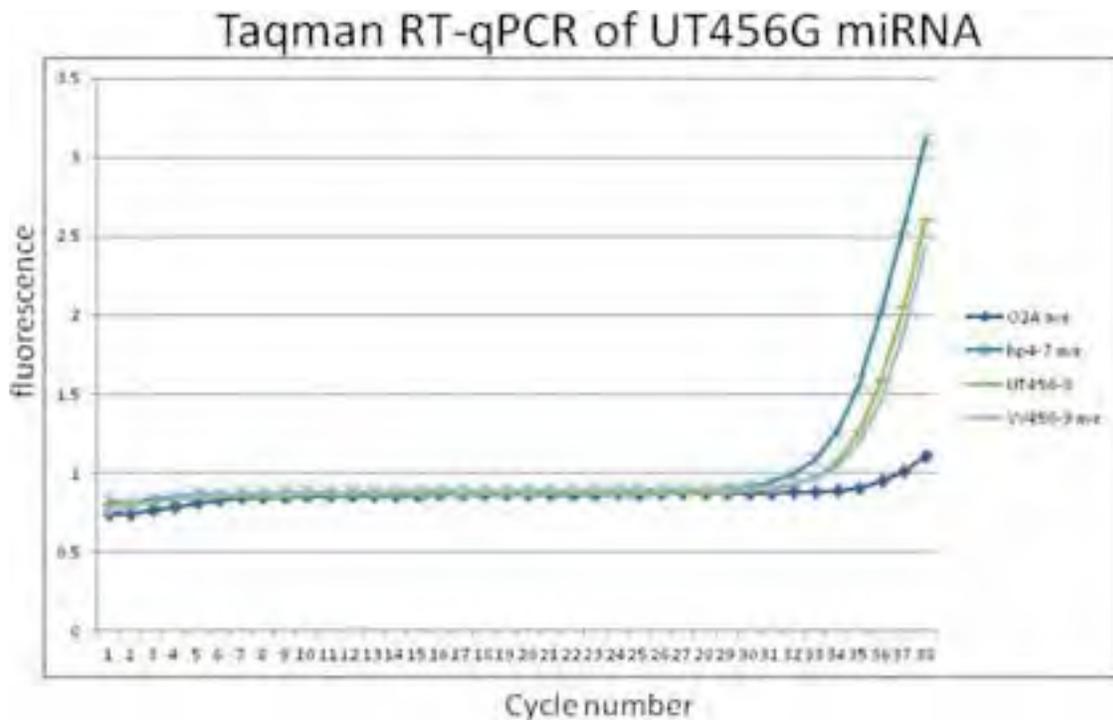


Figure 2. RNA was purified from leaves of each genotype and converted to cDNA with oligo (dT) primer and reverse transcriptase. The cDNA corresponding to the small RNA UT 456G was quantified in the cDNA by qPCR Taqman assay.



Figure 3. Potential protection across a graft union. Representative control and transgenic plants expressing the genes indicated in **Table 2**. All grafts have untransformed Thompson seedless “02A” scions. FD is untransformed Freedom rootstock control. All plants photographed and *Xf* titers taken 4 months after inoculation with *Xf*. Age of plants at the time of inoculation was approximately 22 months. Samples and photos were taken at four months after inoculation. Examples of greenhouse Pierce’s disease assay of transgenic grapes expressing PCD blocking genes. Photos taken and *Xf* titers were measured by qPCR at four months after inoculation. See **Table 1** for details.

Table 1. Freedom rootstock expressing transgenes grafted to untransformed Thompson Seedless scions and mechanically inoculated with 20,000 *Xf* “Temecula” in a 20 ul drop. Disease rating is a 1-5 scale with 1 = asymptomatic and 5 = defoliated. Bacterial titers are expressed as bacterial cells per 0.1gm of stem tissue. Evaluations were done at 12 months post inoculation. (See **Figure 3** for a representative image of control and transgenic rootstock/wild scion plants)

Transgenic notation	Relevant genotype (transgenic rootstocks grafted to untransformed Thompson Seedless scions)	Ratio transgenic graft-protected plants with leaf retention equal to <i>Vitis californica</i> vs. those plants dead	Range of bacterial load per 0.1 gm of stem in at 4 months post inoculation
<u>TS02A</u> FD456-15	CaMV 35S-driven 456 Freedom rootstock	8/8 healthy, none dead Rating 1-2	$10^3 - 10^4$
<u>TS02A</u> FDPR1-13	CaMV 35S-driven PR1 Freedom rootstock	5/5 healthy, none dead Rating 1-2	$10^3 - 10^4$
Merlot	Untransformed control	3/3 relatively healthy Rating 2	$10^5 - 10^6$
Cabernet Sauvignon	Untransformed control	3/3 relatively diseased Rating 4	$10^5 - 10^6$
<u>Merlot</u> FD PR1	Untransformed Merlot grafted to Freedom rootstock/PR1-9	4,4 healthy with an average rating of 1	In Progress
<u>Cabernet Sauvignon</u> FD PR1	Untransformed Cabernet sauvignon grafted to Freedom rootstock/PR1-9	Mixed response with one dead and one healthy out of 4 plants with an average rating of 3	In Progress
<u>Merlot</u> FD UT456	Untransformed Merlot grafted to Freedom rootstock/UT456-10	4/6 are healthy with an average rating of 1.4	In Progress
<u>Cabernet Sauvignon</u> FD UT456	Untransformed Cabernet sauvignon grafted to Freedom rootstock/PR1-9	3 out of 4 plants were healthy with an average rating of 2	In Progress
<u>TS02A</u> FD3 (wild type) Control	Untransformed Thompson Seedless scion	10/13 dead (R5), 3/13 barely) alive (R4)	$10^6 - 10^7$

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

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Reporting Period: The results reported here are from work conducted July 2007 through September 2012.

ABSTRACT

After three years of no increase in Pierce's disease in Preston Vineyards in Sonoma, there was a large increase in incidence in both the untreated and treated vines in 2012, with more than 50% of the Voignier vines developing symptoms typical of Pierce's disease. More important than the incidence of Pierce's disease may be the incidence of severe Pierce's disease symptoms, which we define in this study as incidence of vines with a disease rating of 2-4. These vines do not recover. Twelve untreated vines had severe Pierce's disease symptoms compared to eight vines treated with EB92-1. However, statistical analysis of disease severity ratings showed that there was no difference between the treated and untreated. In the fourth season in the Beringer Vineyard in Napa, there still was only one Chardonnay vine out of 82 that had any Pierce's disease symptoms. However in 2012, twenty-one Reisling vines out of 85 developed some possible Pierce's disease symptoms. In the Bella Vista Vineyard in Temecula, loss of plants from extreme water stress and nutritional problems has forced abandonment of the trials on Orange Muscat and Cabernet Sauvignon. To evaluate *Xylella fastidiosa* strain EB92-1 for the biocontrol of Pierce's disease in Southern California, 50 treated and 50 untreated Merlot and Pinot Noir vines were transplanted into the UC Riverside vineyard in mid-October 2011. In 2012, there was very little Pierce's disease development and no differences between treatments, with 4% incidence in the plots. In September 2010, forty mature Chardonnay vines in the Beringer Vineyard were inoculated with strain EB92-1 by boring a small hole into the trunk with an electric drill and injecting 2 ml of bacterial suspension into the hole with a nail-injector syringe. In 2012, one treated and 1 untreated vine had Pierce's disease symptoms. In a Florida trial, transfer of the biological control from the mother plant through scion wood was less effective than direct injection. This evaluation of scion from treated mother vines is especially significant, because scion wood from infected mother vines would be a less laborious treatment method than injection of every vine by pin pricking. After three years of no increase in Pierce's disease incidence, disease became more prominent in Voignier in Preston Vineyard and was beginning to develop in the Reisling in Beringer Vineyard in the 2012 season.

LAYPERSON SUMMARY

After three years of a very low level of Pierce's disease in the Voignier trial in Preston Vineyards in Sonoma, more than 50% of the vines had Pierce's disease symptoms in 2012. Many of these plants had minor symptoms and could be healthy in 2013; however, 12 untreated vines and eight vines treated with EB92-1 developed severe symptoms of Pierce's disease. Grapevines with severe Pierce's disease symptoms do not recover and eventually die. In this fourth year of the test in Beringer Vineyards in Napa, there still was only one vine with Pierce's disease symptoms out of 82 total Chardonnay vines; however, Pierce's disease did spread rapidly in the Reisling trial. Twenty-five percent of the Reisling vines had some symptom of Pierce's disease, most symptoms were mild. In the Bella Vista Vineyard in Temecula, loss of plants from extreme water stress and nutritional problems has forced abandonment of the trials on Orange Muscat and Cabernet Sauvignon. In 2011, the lost Temecula trials were replaced with trials on Merlot and Pinot Noir in the UC Riverside Vineyard. In 2012,

Pierce's disease began to develop, with no differences between treatments. A trial to evaluate the effectiveness of the biocontrol strain in protecting mature, producing grapevines against infection with Pierce's disease was established in Beringer Vineyard in 2010. Mature Chardonnay vines were inoculated with biocontrol strain EB92-1 by boring a small hole into the trunk with an electric drill and injecting 2 ml of bacterial suspension into the hole. In 2012, one treated and 1 untreated vine had developed Pierce's disease. In tests in Florida to determine the most efficient and effective way to apply the biocontrol strain, direct injection of EB92-1 into the vines still appeared to be the most effective method. In summary, after three years of no increase in Pierce's disease in the trials, Pierce's disease began to spread in the Sonoma and Napa trials in 2012. Data on the effectiveness of EB92-1 for the biocontrol of Pierce's disease in California should be obtained in the 2013 season.

INTRODUCTION

Pierce's disease of grapevine is an endemic, chronic problem in the southeastern USA where it is the primary factor limiting the development of a grape industry based on the high-quality European grapes (*Vitis vinifera* L.) (Hopkins and Purcell, 2002). Pierce's disease is also endemic in California and has become more of a threat to the California grape industry with the introduction of the glassy-winged sharpshooter (GWSS). While vector control has been effective for Pierce's disease control in some situations, the only long-term, feasible control for Pierce's disease has been resistance. Almost 20 years of research on the biological control of Pierce's disease of grapevine by cross protection with weakly virulent strains of *Xylella fastidiosa* (*Xf*) has demonstrated that this is a potential means of controlling this disease (Hopkins, 2005). One strain of *Xf* that was able to control Pierce's disease in *V. vinifera* for 14 years in Central Florida has been identified. We are testing this strain in commercial vineyards in several states and, if these tests are successful, the strain will be ready for commercial use. In most trials with the biocontrol strain, the bacteria were injected into the grapevines either in the greenhouse or in the vineyard after transplanting. This is a labor-intensive procedure. Treatment methods that would make the technology less labor-intensive, less costly, and more consistent are being evaluated. The overall goal of this project is to develop a biological control system for Pierce's disease of grapevine that would control the disease in California and other areas where Pierce's disease and GWSS are endemic.

OBJECTIVES

1. To evaluate strain EB92-1 of *Xf* for the biological control of Pierce's disease of grapevine in new plantings in the vineyard in California.
2. To evaluate strain EB92-1 of *Xf* for the protection of older established grapevines against Pierce's disease in California vineyards.
3. To evaluate rapid, efficient methods of treatment with strain EB92-1 of *Xf* for the biocontrol of Pierce's disease in *V. vinifera* in the vineyard.

RESULTS AND DISCUSSION

Field trials evaluating strain EB92-1 for biological control of Pierce's disease in vineyards in California

Sonoma tests

For Preston Vineyards in Sonoma, 50 Barbera/110R and Viognier/110R from were inoculated with EB92-1 and 50 vines of each were left as untreated controls. These plants were transported to Sonoma and transplanted as replants for missing vines in a mature vineyard the last week of July 2008. On August 26, 2009, these vines were mapped for symptoms. All of the Barbera vines appeared to be healthy with no Pierce's disease symptoms. The block of Barbera did not appear to have any Pierce's disease symptoms, even in the older vines and this test was abandoned because of the lack of disease.

In the Viognier test, there were a few vines that had minor yellow and/or necrotic leaf margins on the basal leaves in 2009, but there were no definitive symptoms. Minor Pierce's disease symptoms began to develop in a very few vines in the Viognier test in 2010. However, there were fewer vines with Pierce's disease symptoms in 2011 than in 2010. After three years, the Pierce's disease incidence in the test vines was very low, but there was a large increase in incidence in both the untreated and treated vines in 2012 (**Table 1**). There were no significant differences between treatments.

Table 1. Effect of EB92-1 on the incidence of Pierce’s disease in young “Voignier” grapevines in Preston Vineyards in Sonoma California.¹

Year	Untreated vines	EB92-1 treated vines
2011	6/48 (12%)	5/48 (10%)
2012	32/48 (67%)	29/48 (60%)

¹Pierce’s disease incidence is the number of Pierce’s disease symptomatic vines over total vines in treatment.

More important than the incidence of Pierce’s disease symptoms may be the incidence of severe Pierce’s disease symptoms, which we define in this study as incidence of vines with a disease rating of 2-4 (**Table 2**). These severely infected vines have 50%, or more, of the leaves with symptoms and will usually die. Vines with fewer symptomatic leaves may not show any symptoms the following year. For example, six Voignier vines had a disease rating of one in 2011 and four of these six vines had no Pierce’s disease symptoms in 2012. This could be the result of vine recovery or misdiagnosis of plants with only a few symptomatic leaves. The disease rating incorporates both the incidence and severity of the Pierce’s disease symptoms. Incidence of severe Pierce’s disease was much lower than total Pierce’s disease (compare **Table 1** with **Table 2**). Untreated vines had 25% incidence of severe symptoms; whereas, EB92-1 vines had 17% incidence of severe symptoms (**Table 2**). Statistical analysis of the disease ratings showed that there was no difference between the treated and untreated. Interestingly, this trial is next to a riparian area and there were 3 times as many vines with severe Pierce’s disease symptoms in the five rows next to the river as in the 5 rows further from the river.

Table 2. Effect of EB92-1 on the incidence of severe Pierce’s disease (disease rating of 2 - 4) in young “Voignier” grapevines in Preston Vineyards in Sonoma California.

Year	Untreated vines:		EB92-1 treated vines:	
	Incidence ¹	Disease rating ²	Incidence ¹	Disease rating ²
2011	3/48 (6%)	0.21	2/48 (4%)	0.15
2012	12/48 (25%)	0.98	8/48 (17%)	0.81

¹Incidence of severe Pierce’s disease is the number of vines with a rating of 2 or greater over total vines in a treatment.

²Disease rating was an average per vine on a scale of: 0 = no symptoms; 1 = any symptom of Pierce’s disease, such as marginal necrosis (MN) on a basal leaf; 2 = definite, moderate symptoms on <50% of vine; 3 = severe symptoms on >50% of vine; 4 = dead plant.

Napa tests

For transplanting into the Beringer Vineyard in Napa, 50 Reisling/3309 and 50 Chardonnay/3309 were treated with EB92-1 on June 25, 2008 and 50 vines of each were left untreated as controls. The vines were transplanted as replants for missing vines in Beringer Vineyard in early April 2009. In 2011, there still was essentially no disease in either the Chardonnay or Reisling. Only one Chardonnay vine and two Reisling vines were considered to have the beginning of Pierce’s disease symptoms, but these were still questionable. In 2012, there still is only one Chardonnay vine with minor Pierce’s disease symptoms (**Table 3**). However, there was a considerable increase in the number of Reisling vines with Pierce’s disease symptoms. These were mostly vines with a disease rating of one and could have no symptoms next year (see Sonoma test section above). There were no significant differences in disease ratings between the untreated and treated plots. Differences should be apparent in 2013.

Table 3. Effect of EB92-1 on the incidence of Pierce's disease in young grapevines in California in October 2012.

Location and Cultivar	Untreated vines:		EB92-1 treated vines:	
	Incidence ¹	Disease rating ²	Incidence ¹	Disease rating ²
<i>Beringer Vineyard, Napa</i>				
Chardonnay	0/40 (0%)	0	1/42 (2%)	0.02
Reisling	9/42 (21%)	0.24	12/43 (28%)	0.40
<i>UCR Vineyard, Riverside</i>				
Merlot	0/26 (0%)	0	3/45 (7%)	0.07
Pinot Noir	3/43 (7%)	0.09	1/49 (2%)	0.02

¹Pierce's disease incidence is the number of Pierce's disease symptomatic vines over total vines in treatment.

²Disease rating was an average per vine on a scale of: 0 = no symptoms; 1 = any symptom of Pierce's disease, such as marginal necrosis (MN) on a basal leaf; 2 = definite, moderate symptoms on <50% of vine; 3 = severe symptoms on >50% of vine; 4 = dead plant.

Southern California tests

For transplanting into the Bella Vista Vineyard in Temecula, 50 Orange Muscat were inoculated with the biocontrol strain (EB92-1) on June 26, and 50 were left untreated as controls. Fifty Cabernet Sauvignon/110R were treated and 50 were untreated controls. These plants were transported to Temecula and transplanted into plots in the Bella Vista Vineyard on July 21-22, 2008.

In September 2010, all the young plants in the Bella Vista vineyard appeared to have severe water and nutritional stress. Many plants died without ever having any visible Pierce's disease symptoms, probably due to the lack of water and poor nutrition. In the Orange Muscat test, 35-40% of the vines had died after two years from something other than Pierce's disease, probably lack of water. Twenty-two percent of the Cabernet Sauvignon also had died, probably from water stress. In both the Cabernet Sauvignon and Orange Muscat, many of the vines were severely stunted and barely reached the trellis wire after three seasons. Therefore, the trials were abandoned.

To replace the lost tests in southern California, a replacement test was established in 2011 at UC Riverside. For transplanting into the UC Riverside vineyard, 100 Merlot/1103 plants and 100 Pinot Noir/1103 plants were obtained from Sunridge Nursery in March 2011 and maintained in UC Davis greenhouse. Fifty Merlot and 50 Pinot Noir were inoculated with EB92-1 in July 2011 and fifty plants of each cultivar were kept as untreated controls. These plants were maintained in the greenhouse for six weeks and then moved outside to harden them off. These plants were transported to Riverside in mid-October and transplanted into the plots at UCR. In 2012, there was very little Pierce's disease development and no differences between treatments (**Table 3**).

Field trial evaluating EB92-1 for the protection of older established grapevines against Pierce's disease in California vineyards

Since Pierce's disease is rapidly developing in the mature Chardonnay block at Beringer Vineyard in Napa, it was chosen for an evaluation of EB92-1 for the prevention of Pierce's disease development in mature, producing grapevines. Randomly, forty vines were inoculated with EB92-1 and 40 vines were chosen as controls. On September 8, 2010, the vines were inoculated with strain EB92-1 in the main trunk, approximately equidistant from the graft and the trellis wire. Vines were injected by boring a small hole into the trunk with an electric drill. Two ml of the bacterial suspension will be injected into each hole using a nail-injector syringe. In the first year, none of the mature vines, treated or untreated, had developed any Pierce's disease symptoms. In October 2012, there was one severely diseased vine of 43 in the untreated and one vine of 49 with mild symptoms in the EB92-1 treated plot.

Comparison of treatment methods with strain EB92-1 for biocontrol of Pierce's disease

Plants of Chardonnay/Salt Creek were obtained by grafting green cuttings from Chardonnay plants from the vineyard onto rooted cuttings of Salt Creek. The grafting was done between May and July in 2007. Grafted plants were transplanted into the vineyard on August 14, 2007. Treatments included (1) Cuttings from Chardonnay not infected with EB92-1 on Salt Creek, (2) Cuttings from EB92-1 inoculated Chardonnay on Salt Creek, and (3) Cuttings from Chardonnay not infected with EB92-1 on Salt Creek, but injected in the vineyard with EB92-1 on August 29.

Table 4. Transmission of biocontrol in scion from infected Chardonnay mother plant grafted onto Salt Creek rootstock.

	% PD incidence:
Treatment	8/2/2012
Scion from clean Chardonnay	90
Scion from clean Chardonnay injected with EB92-1 in the field	27
Scion from EB92-1 Chardonnay mother plant	64

In 2012, the incidence of Pierce's disease symptoms in the scion from clean Chardonnay was high (**Table 4**). As expected, field injection of these clean scion plants with EB92-1 reduced the incidence of Pierce's disease from 90% to 27%. The scion wood from mother vines of Chardonnay infected with EB92-1 developed less Pierce's disease than did the uninfected scion but more than the field injected scion. Thus, transfer of the biological control from the mother plant through scion wood was less effective than direct injection. Further development of the symptoms will be observed.

CONCLUSIONS

Due to the loss of trials in Temecula and the lack of Pierce's disease development in trials in Sonoma and Napa, no definitive information on the biological control of Pierce's disease with EB92-1 in California was obtained through 2011. To replace the lost tests in southern California, a test was established in 2011 at UC Riverside. With the Pierce's disease pressure in southern California, this test should yield conclusive results over the next two-three years. In the 2012 season, Pierce's disease increased significantly in the Sonoma and Napa test. If this continues, data should be obtained on the effectiveness of the biocontrol in these vineyards in 2013. With the successful trials in other states, this project could yield results within the next two-three years that would provide a commercial biological control for Pierce's disease for vineyards in California.

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**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 *HxfA* and *HfxB*. *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 have secured all the necessary permits to plant these lines in the field in Solano County in spring 2013. After they have grown sufficiently, the transgenic as well as non-transgenic grapevines will be inoculated with *Xf* and Pierce's disease symptom severity will be compared between the HA-expressing transgenic lines and non-transgenic, *Xf*-inoculated control vines.

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 *E. coli* possessed some anti-*Xf* activity *in vitro*. Even though additional funding was not recently 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 and have recently obtained 12 putative TLP transgenic lines. Progress on the TLP plants is described in our other 2012 Progress Report entitled, "Identification and Utilization of Cold Temperature Induced Grapevine Metabolites to Manage Pierce's Disease."

LAYPERSON SUMMARY

Our seven+ year research effort on the role hemagglutinins (HA), 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 than vines inoculated with wild-type *Xylella fastidiosa* 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 HA 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 comprised 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 HAs 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 grapevines 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-GMO fruiting scion and afford similar levels of functional Pierce's disease resistance. The evaluation of HA-expressing transgenic grapevines acting as rootstocks is an objective of a recent proposal that was submitted in 2012. We have recently finished a first Pierce's disease severity screening of the nine HA transgenic lines we produced with funding for this project. The results were very encouraging with all of the HA-transgenic lines having much lower disease ratings than non-transgenic control. We are in the process of repeating the pathogenicity assays and we are now in the process of determining the sizes of *Xf* populations in the transgenic lines versus the non-transgenic control. If we find that *Xf* populations are suppressed below levels that induce fruit raisining or cordon dieback the HA lines might provide some level of functional resistance/tolerance against Pierce's disease. If this occurs, we will have obtained the goal we set five years ago.

INTRODUCTION

Xylella fastidiosa (*Xf*) cell-cell attachment is an important virulence determinate 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 *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 *Hxfs* were regulated by an *Xf*-produced compound diffusible signal factor (DSF) (Newman et al. 2004) and that they were important factors in insect transmission (Killing and Almeida, 2009). The *Xf* HAs essential 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 consider time and effort in constructing transgenic tobacco and grapevines that expressed HA. We recently completed our first pathogenicity evaluations of our 9 HA-transgenic lines. Disease severity ratings were considerably less in the transgenic lines than the non-transgenic controls. We are currently in the process of evaluating *Xf* population levels in all of the plants used in this trial. It is possible that *Xf* populations will be considerably less in the transgenic lines than in non-transgenic vines. If *Xf* levels are below the level where fruit raisining and cordon dieback don't occur, similar to what happens in muscadine grape vines infected with *Xf*, these vines might possess a tolerance to *Xf*. A second round of pathogenicity assays will be performed in the greenhouse this summer and arrangements are being made through the Public Intellectual Property Resource for Agriculture (PIPRA) to establish a field plot at UC Davis.

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 in Solano County. Plant transgenic vines in the field.
4. Following greenhouse testing, graft promising *Hxf* transgenic root stocks 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; they have since supplied us with 12 putative thaumatin-like protein (TLP)-transgenic lines that we are now propagating and characterizing as time and plant materials permit.

5. Generate grape transgenic plants over-expressing the grape TLP.
6. Screen putative TLP-transgenic lines for quantitative gene expression by RT-PCR, 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 UC Davis Plant transformation facility in September 2010. These were initially obtained as small green 3" 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 hemagglutinin transgene in genomic grapevine DNA from each of the 22 lines showed that six of nine transgenic lines of containing *Xf* HA AD 1-3, labeled as SPAD1 and three 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 HA inserted into the grape genome are being expressed (**Table 2**).

As per 4/12 we had produced sufficient numbers of HA-transgenic grapevines to destructively sample and test for the presence of HA protein in foliar tissue as well as extracted xylem sap by ELISA and western blot analysis. This work should be completed within the next two months.

Table 1. Results of PCR testing transgenic grapevines for presence of full-length (PGIP 220) of AD1-3 fragment of *Xf* hemagglutinin 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	—	—

Notes:

Transgenic lines highlighted in tan color are the three full-length transgenic lines while lines highlighted in purple contain the AD1-3 HA fragment.

† = this line tested positive for a *Xf* hemagglutinin 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 hemagglutinin gene.

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 three 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.

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 could be detected in grapevine xylem sap. On December 8th and 9th of 2011 we inoculated 10 reps of each of the nine PCR-positive transgenic lines with 40 ul of a 10⁸ suspension of *Xf* Fetzner 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 inject into a vine.

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

Line ID	Relative transgenic <i>Hxf</i> 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

RNA analysis of HA expressing grapevines. 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 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 the incipient infection might very well be prune 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 the Almeida lab insect inoculated with *Xf*. These initial greenhouse results with young vines certainly warrant further evaluations.

Objective 3a: Secure permits to plant HA transgenic lines in the field at UC Davis

This objective was completed with the assistance of PIPRA.

Objective 3b: Plant transgenic vines in the field

Andy Walker and other viticulture experts strongly suggested we not plant our trial until next spring because of the danger of frost killing the young, newly-transplanted vines. Considering the time and effort it took to produce the 140 vines that will be planted in the field we will keep the vines in a screen

house where they will undergo natural dormancy and then plant them in the field as early as conditions permit in the spring.

Objective 4: Following greenhouse testing, graft promising *Hxf* transgenic root stocks 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.

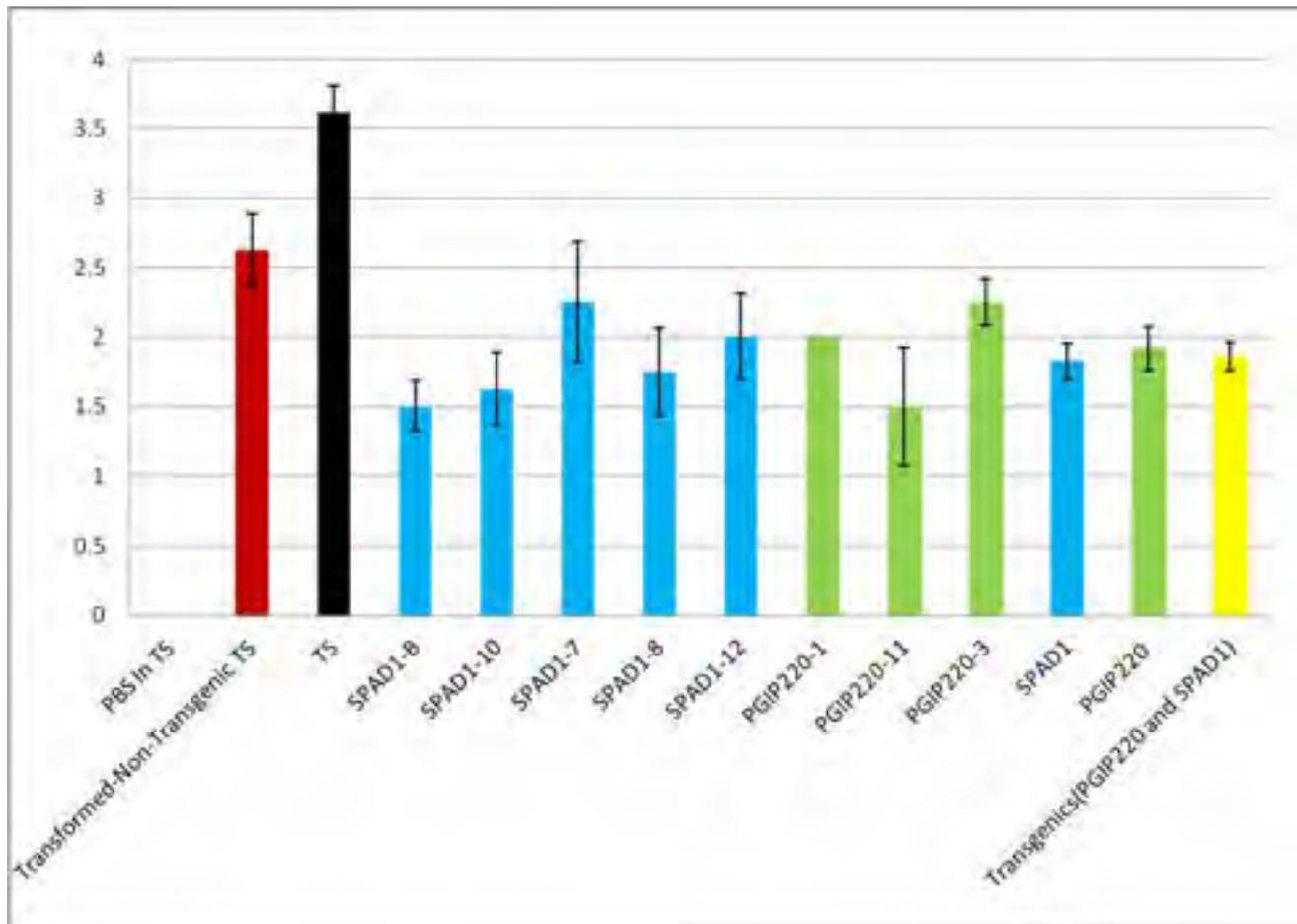


Figure 1. Graph showing the mean disease ratings from 0 to 5 (0 is healthy 5 is dead) of Pierce’s disease symptoms in Thompson Seedless (TS) and transgenic (SPAD1 and PGIP220) vines inoculated with *Xf*Fetzer at 16 weeks post inoculation, except for the Transformed-Non-Transgenic TS, which was inoculated four weeks later and its disease rating is for 12 weeks post inoculation, we anticipated these vines will have disease ratings similar to the TS 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.

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* cells showed all lines expressed less severe symptoms of Pierce’s disease than inoculated, non-transgenic controls. All transgenic lines are under constant propagation and sufficient materials are now in hand to establish field plots with these lines in spring 2013; PIPRA successfully obtained all of the necessary permits to allow these HA-transgenics to be planted in the field. 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.

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FUNDING AGENCIES

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

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

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Reporting Period: The results reported here are from work conducted November 2011 to October 2012.

ABSTRACT

This work builds on discoveries made in the past 8 years of research on better understanding the mechanism(s) responsible for the Pierce's disease cold curing phenomenon. A thaumatin-like grape protein (TLP) was found in elevated levels in the xylem sap from cold-exposed vines and we cloned and expressed TLP in *E.coli*. With the assistance of the UC Davis Plant Transformation Facility, we now have 12 lines of putatively TLP-over expressing transgenic Thompson Seedless grapevines. These are now being propagated in the greenhouse to produce sufficient material to characterize TLP gene expression. Greater amounts of total phenolics were measured in xylem sap extracted from cold-exposed vines. In collaboration with the Waterhouse lab we characterized these phenolic compounds and assessed their potential *anti-Xylella fastidiosa* activity *in vitro*. Results from the phenolic project were reported in previous Pierce's disease Research Symposium Proceedings. Previously, greenhouse grown Pinot Noir and Cabernet Sauvignon vines treated with commercial abscisic acid (ABA) were shown to have higher levels of winter recovery from Pierce's disease than non-treated vines, as well as producing higher levels of polyphenolic compounds. In fall 2010 and 2011 we treated Riesling vines growing in a vineyard in Napa that had light to moderate Pierce's disease symptoms vines with a foliar spray or a soil drench treatment of ABA. The severity of Pierce's disease symptoms in the treated vines was not significantly different than untreated controls in fall of 2011 and 2012.

LAYPERSON SUMMARY

Research conducted in this project showed that cold exposed grapevines produced significantly higher concentrations of a number of phenolic compounds in xylem sap when compared to the concentrations of phenolics in xylem sap extracted from vines exposed to less severe cold temperatures. Several of these compounds were shown in this study, as well as studies by other researchers, to be toxic to *Xylella fastidiosa* when tested *in vitro*. Pierce's disease affected grapevines growing in one gallon pots in a lath house at UC Davis experienced higher rates of winter recovery when treated with foliar and soil drenches of abscisic acid (ABA), a naturally occurring plant hormone. However, following two years of foliar sprays and soil applications to field grown Riesling grapevines with mild to moderate severity of Pierce's disease, no statistically significant differences were found in the severity of Pierce's disease symptoms in treated vs. non-treated grapevines. It is likely that the size of the grapevines, as well as the confinement of the roots in the 1 gallon potted vines, prevented efficacious levels of ABA being taken up by the field grown grapevines.

INTRODUCTION

Previous research conducted in the Purcell laboratory at UC Berkeley definitively demonstrated that *Vitis vinifera* grapevines that were infected with *Xylella fastidiosa* (*Xf*), the bacterial pathogen that causes Pierce's disease, could often be cured of the infection if exposed to freezing temperatures for some period of time. This "cold curing" phenomenon likely explains why Pierce's disease is restricted to areas that have mild winter temperatures. Research conducted in our laboratory by Dr. Melody Meyer confirmed and expanded the work performed by Purcell, et. al. She found that grapevines exposed to cold temperatures had elevated levels of a thaumatin-like protein (TLP) that has been shown to have antimicrobial properties in other plant host/pathogen interactions. We cloned and expressed the grapevine TLP in *E. coli* and showed that incubation of the cloned TLP with cultured *Xf* cells considerably decreased the viability of the *Xf* cells compared to incubating the *Xf* cells with other appropriate controls. We then cloned the TLP gene in an *Agrobacterium* binary vector with the intention of over-expressing the TLP in transgenic grapevines. We have very recently obtained 12 lines of putative TLP-transgenic grapevines and are in the process of propagating these transgenic grapevines in the greenhouse. When sufficient material is available we will assess the level of TLP mRNA in the transgenic vines and then mechanically inoculate the vines with *Xf*. The severity of Pierce's disease symptoms in the transgenic vines will then be compared with the severity of symptoms in non-transgenic controls.

Dr. Meyer's research also showed elevated levels of polyphenolic compounds in xylem sap extracted from cold exposed grapevines. In collaboration with the Waterhouse lab we characterized the phenolic compounds in the xylem fluid from cold and warm-exposed grapevines. Dr. Meyer's research also showed elevated levels of the plant hormone abscisic acid (ABA) in xylem sap of cold exposed grapevines. She showed that exogenous application of ABA greatly increased the Pierce's disease curing rates of potted grapevines exposed to the comparatively mild winter temperatures in Davis. In 2010 and 2011 we applied ABA in the fall to mildly Pierce's disease infected Riesling vines growing in a vineyard in Napa. The severity of Pierce's disease symptoms in the ABA-treated vines was compared to non-treated controls in October 2011 and 2012. No statistically significant differences in the severity of Pierce's disease symptoms were found between ABA-treated and non-treated vines.

OBJECTIVES

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

RESULTS AND DISCUSSION

Objectives 1 and 2

We successfully cloned and expressed grapevine TLP in *E. coli*, results of *in vitro* assays testing the toxicity of the cloned TLP protein against *Xf* cells grown were extensively described in the Proceedings of the 2010 and 2011 Pierce's Disease Research Symposia. We also prepared an *Agrobacterium* binary vector system for over-expressing TLP in transgenic grapevines. Our initial plan was to use the same vector we used to generate our hemagglutinin (HA) transgenic lines, however our results using these plasmids, which use two 35S promoters to express the HA gene and uses hygromycin resistance, produced a number of lines in which crossing over by the two promoters ended up deleting all or part of the HA gene construct while maintaining the antibiotic selection. To avoid these deletion events we have decided to change the binary system to a neomycin (kanamycin) resistant plasmid driven by the nptII promoter to eliminate the chance of promoter crossing over/deletion events. We used the pUNCB5omega plasmid for initial cloning of the grape TLP gene in *E. coli*. We then moved the

TLP construct into the low copy pCB4NN plasmid. This plasmid provides the neomycin resistance and is in low copy in case there are any toxicity issues resulting from expression of TLP in *Agrobacterium*. We submitted this TLP transformation construct to the UC Davis plant transformation facility at the end of October, 2011. Due to advancements in their production of transgenic grapevine we now have 12 independent lines that are being propagated in the greenhouse. Once sufficient material is available we will characterize the level of TLP expression in the transgenic lines using qRT-PCR as we did with our *Xf* hemagglutinin transgenic vines. When sufficient material is available we will mechanically inoculate the TLP transgenic vines and non-transformed controls with cultured *Xf* and compared the severity of symptoms in the TLP-transgenics to the controls.

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

Objective 3

Results of this Objective were previously reported in the Proceedings of the 2010 and 2011 Pierce's Disease Research Symposia.

Objective 4

Foliar and soil drench applications of ABA were made in October 2010 and 2011 to Riesling vines growing in a vineyard located in Napa. All vines had mild to moderate symptoms of Pierce's disease. A commercially available formulation of a naturally occurring ABA was kindly provided by Valent Chemical Company. Disease ratings of treated and non-treated vines were made in early October 2011 and 2012. No significant differences were found between ABA-treated vs. non-treated controls (**Figure 1**).

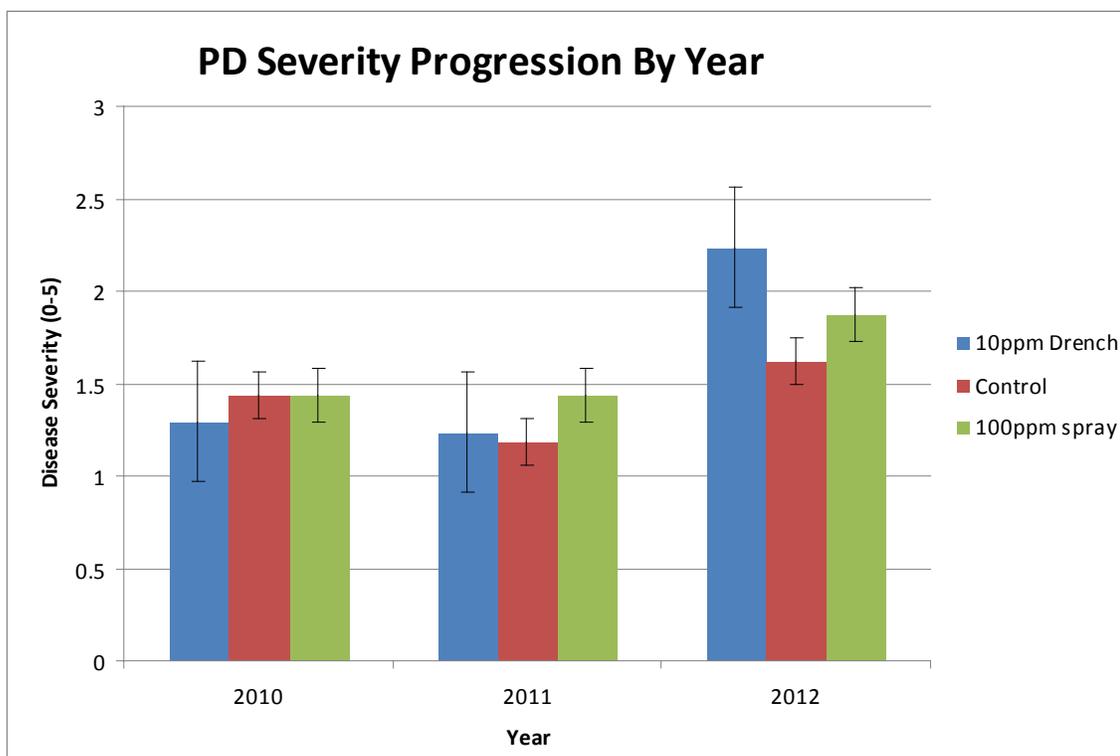


Figure 1.

CONCLUSIONS

Research conducted in this project demonstrated a correlation between increased levels of phenolic compounds in the xylem sap of cold-exposed grapevines compared to phenolic levels in sap extracted from vines exposed to moderate winter temperatures. Several of these phenolic compounds were shown to be toxic to *Xf* in *in vitro* studies. However it is difficult to envision a method of elevating phenolic compound levels to sufficient levels to be toxic to *Xf* without potentially changing the characteristic of juice extracted from berries to make wine. Similarly, higher levels of antimicrobial thaumatin-like protein (TLP) were found in the xylem sap of cold exposed vines as compared to xylem sap extracted from vines exposed to more moderate winter temperatures. Given the correlation between elevated levels of TLP in cold-exposed vines and higher rates of cold-curing we produced transgenic grapevines that should over express TLP as compared to non-transgenic vines. Once sufficient material is obtained from the transgenic lines we will evaluate TLP gene expression, mechanically inoculate transgenic and non-transgenic vines with *Xf* and compare the development of Pierce's disease symptoms in TLP-transgenic lines with non-transgenic controls. Field applications of ABA, which showed efficacy in curing Pierce's disease in potted grapevines, were not successful. This is most likely due to the much larger mass of roots and canopy in the field vines compared to potted vines. Pressure injections of ABA into field vines may be one option for increasing the efficacy of ABA application, but it is unlikely that a grower would be willing to go to the extra cost of vine trunk injections.

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FUNDING AGENCIES

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

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ABSTRACT

Polygalacturonases (PGs) (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

This period we have made significant progress on Objectives 1-3. Most importantly we seem to have made progress on what has been the biggest obstacle thus far in this project, which is creating enzymatically active *Xylella fastidiosa* (*Xf*) PG to pan and test our putative inhibitory phages 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 phages we have obtained from panning against the peptides representing the active site of *Xf* PG.

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 breakdown the pectin in these membranes. The premise of this research is to identify a peptide that can be expressed in the xylem of a grapevine that can suppress *Xf* PG activity thus limiting the ability of *Xf* to spread systemically through grapevines and cause Pierce's disease.

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 UC Davis 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 to see if they can also provide resistance to grafted scions.

OBJECTIVES

1. Localization and isolation of sufficient amounts of biologically active PG enzyme to conduct phage panning and PG-inhibition assays.
2. Isolate M13 phages that possess high binding affinities to *Xf* PG from a M13 random peptide or scFv antibody libraries.
3. Determine if selected M13 phage and the gp38 M13 protein that mediates phage binding to *Xf* PG can inactivate PG activity *in vitro*.
4. Clone anti-*Xf* PG gp38 protein into an *Agrobacterium* binary vector and provide this construct to the UC Davis 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 *E. coli*, yeast, and plant based viral expression systems have not produced active *Xf* PG (see previous Pierce's Disease Research Symposium Proceedings reports). Because of this, *Xf* strains have been engineered that will constitutively express the PG gene. The pBBR1MCS and pPROBE broad host range cloning vectors provide the *Xf* expression plasmid backbone and the constitutive nptII promoter was utilized to drive protein expression (Miller et al. 2000, Kovach et al. 1995). 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 somewhat 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 transfers 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 mutated *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 mutating the *Xf recA* gene the mutation may eliminate the DNA rearrangement in the plasmid that is leading to its instability. 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 PG is present in the newly developed *Xf* PG-producing strains.

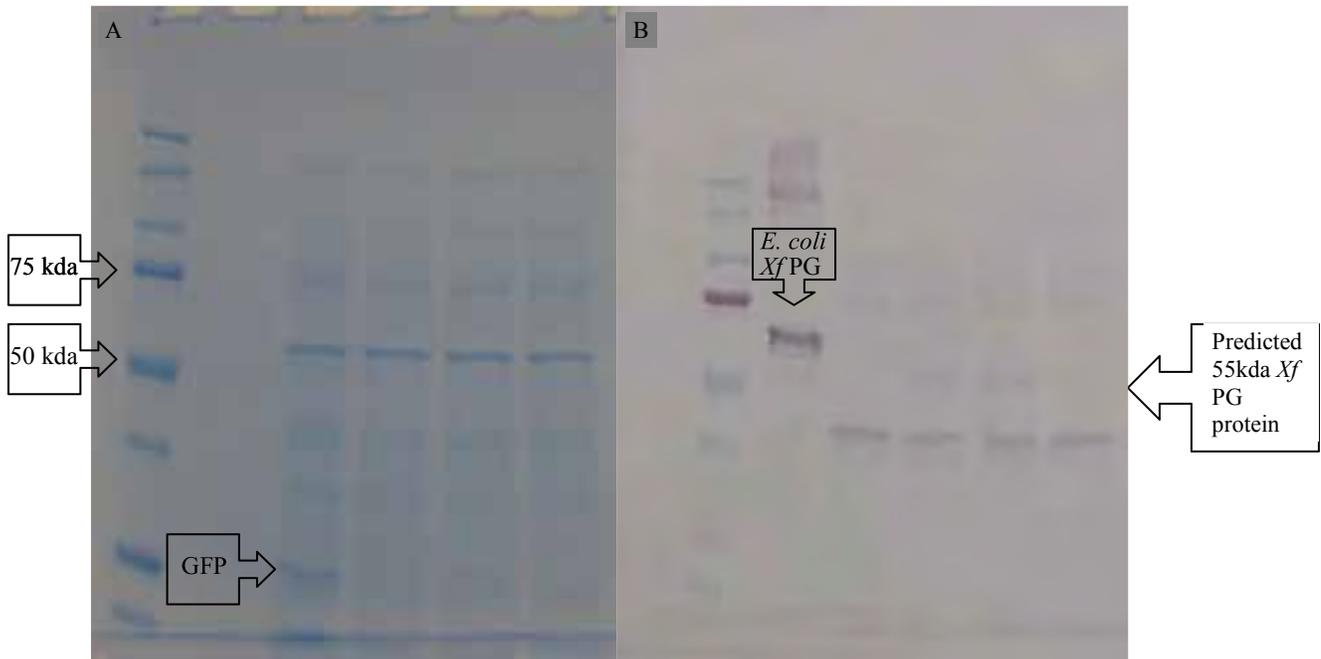


Figure 1. A: Coomassie stained polyacrylamide gel electrophoresis, **Lane 1:** Bio-rad dual color protein ladder, **lane 2:** Recombinant *Xf* PG produced in *E. coli*, **lane 3:** *Xf* over-expressing GFP, **lanes 4-6** *Xf* over-expressing *Xf*PG **B:** Western blot analysis using polyclonal anti *Xf*PG 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.

Objective 2: Isolate M13 phages that possess high binding affinities to *Xf* PG from a M13 random peptide library

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

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

Peptide	Sequence
Peptide 1	DSPNSNGLQMKSDAC
Peptide 2	STGDDHVAIKARGKC

Figure 2. Sequences of synthesized peptides.

Additionally, these peptides were injected into rabbits to create polyclonal antibodies. These polyclonal antibodies were used in a western blot that confirmed that the antibodies created against each 14-mer peptide could also recognize full length *Xf* PG (**Figure 2**).

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

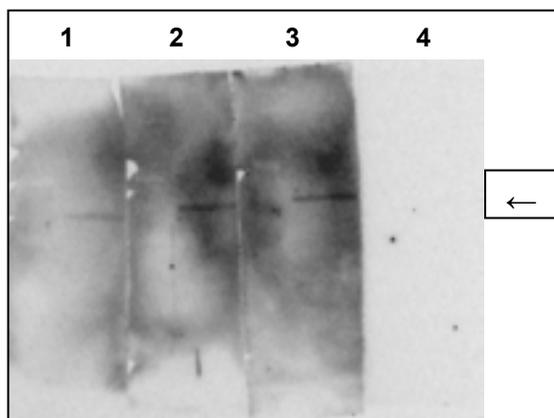


Figure 2. Western blot analysis of three representative monoclonal scFv phages(J-library). Lanes 1-3 are *E. coli* lysate containing recombinant *Xf*PG each membrane strip was reacted with a single monoclonal phage from the third round of panning. Arrow represents location of *Xf*PG band. Molecular weight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

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 (*V. 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. For this reason we have cloned the *Av* PG gene into an *E. coli* overexpression system to produce recombinant *Av* PG to use in inhibition assays. Initial results show that *Av* PG is being produced in large amounts and is enzymatically active in cup plate assays. We are currently producing a large amount of this recombinant *Av* PG that will be used in additional phage panning experiments.

Objective 3: Determine if selected M13 phage and the gp38 M13 protein which mediates phage binding to *Xf* PG can inactivate PG activity *in vitro*

We have sequenced the heavy chain variable regions of the 16 candidate monoclonal phage and although none of the eight clones from each of libraries shared the exact same sequence they did have similarities to each other. Once we obtain enough active *Xf* PG we will use the monoclonal phages in *Xf* PG inhibition assays. Once a

candidate phage is found that can inhibit *Xf*PG *in vitro* we will then express the scFv protein alone and determine if the protein itself can also inhibit *Xf*PG activity *in vitro*. We will then clone the anti-*Xf*PG protein into an *Agrobacterium* binary vector and provide this construct to the UC Davis 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 UC Davis Plant Transformation facility to produce transgenic SR1 tobacco and Thompson Seedless grapevine

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

Objective 5: Determine if anti-*Xf*PG gp38 protein is present in xylem sap of transgenic plants

Objective 4 needs to be completed before work on objective 5 can begin.

Objective 6: Mechanically inoculate transgenic plants with *Xf* and compare 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 using *Xf* as the cloning host have shown that we can produce recombinant PG in *Xf* and initial tests identified some fractions that may contain active *Xf*PG. Once we overcome some of the instability issues of this plasmid system, perhaps by expressing these plasmids in *Xf* *recA* mutants, we can then test the efficacy of the inhibitory phages we have obtained from panning against the peptides representing the active site of *Xf*PG. We have acquired 16 candidate scFv phages, by panning against peptide 2 conjugated to BSA that are capable of binding to full length *Xf*PG. We will be using these candidate phages in *Xf*PG inhibition assays as we have described previously. If one of the candidate phages can inhibit *Xf*PG activity *in vitro* then we can transform grapevines with the peptide and determine if they provide plants with resistance to Pierce's 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). Here, several PGIPs were analyzed 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 have been developed to provide active quantities of *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. In this reporting period we have been 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 PG-inhibiting proteins (PGIPs) are produced by many plants and these PGIPs selectively inhibit PGs from bacteria, fungi, and insects. The PGIP expressed in pear fruit is known to inhibit *Xf*PG and limit 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. We are interested in identifying the PGIP that best inhibits *Xf*PG and ascertaining 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 fourteen candidate PGIPs to predict how the PGIPs physically interact with *Xf*PG and we selected three candidate PGIPs. We expect to use *in vitro* and *in planta* assays to measure the ability of three 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 PGIPs that are most effective in inhibiting *Xf*PG by expressing and testing them first in tobacco and tomato and then evaluating grape rootstock germplasm after grafting, so that we can predict their ability to limit Pierce's disease development in non-transgenic grape scions.

INTRODUCTION

Xylella fastidiosa (*Xf*), the causative agent of Pierce's disease 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 of the bacteria that contributes to the development and spread of Pierce's disease.

The PGIPs that are naturally produced by plants are selective inhibitors of PGs; they limit damage caused by many types of pathogens. Agüero *et al.* (2005) demonstrated that by introducing a pear fruit PGIP (pPGIP) gene into transformed grapevines, vine susceptibility to both fungal (*Botrytis cinerea*) and bacterial (*Xf*) pathogens decreased. 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). In a key preliminary observation for the Pierce's disease control approach investigated in this project, Agüero *et al.* (2005) demonstrated that transgenic pPGIP protein could be transported from transformed grapevine rootstocks, across a graft junction and into the grafted wild-type scions. pPGIP also has been shown to be transported from rootstocks across grafts into the aerial portions of tomato plants (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 specific PGIPs that optimally inhibit the virulence factor, *Xf*PG. The PGIPs that after our evaluations seem likely to be the most effective 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 to the aerial portions of vines so that *Xf* movement in infected scion tissues is limited.

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.
 - a. Propagate and graft grape lines expressing and exporting pPGIP for use in Pierce's disease resistance assays
 - b. Identify and clone plant PGIPs that are efficient inhibitors of *Xf*PG
 - c. Develop a recombinant expression system for *Xf*PG
 - d. Express PGIPs, using plant recombinant systems, to assay *Xf*PG inhibition
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) has acted as a liaison for issues associated with the potential commercialization of transgenic grapevine rootstocks for several CDFA PD/GWSS Board-funded projects. PIPRA analysts have managed the permitting process for the field trial testing of Thompson

Seedless and Chardonnay grapevines expressing pPGIP, in progress since 2010. The details of the field trial are discussed in the progress report for the project “Field Evaluation of Grafted Grape Lines Expressing pPGIP” (PI: Powell).

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

a. Propagation and grafting of grape lines expressing and exporting pPGIP

The pPGIP-expressing Chardonnay and Thompson Seedless grapevines described in Agüero *et al.* (2005) continue to be maintained in the UC Davis Core Greenhouse Complex. The propagation and grafting techniques used for this objective are described in the progress report for the project “Field Evaluation of Grafted Grape Lines Expressing pPGIP” (PI: Powell). These efforts have continued to provide source material for grafted plants and assays and we have seen a 3% increase in grafting success over the past reporting period.

In grafted plants that we have generated, we now report that we have evidence of pPGIP protein in the leaves at least 70 centimeters from the site of the graft. While the amount of this protein is significantly less than that produced in tissue containing the pPGIP genes, this is an important accomplishment as a key goal of the project was to establish that the protein is translocated across the grafts. While we have previously published that pPGIP protein is found in wild-type scion tissues grafted to transgenic tomato and grape rootstocks that express the pPGIP gene (Haroldsen *et al.*, 2012), these results used tissue that was quite close to the grafting site. Furthermore, these results are limited by the sensitivity of the polyclonal antibody used to detect pPGIP presence. We have expanded our efforts to detect the translocated pPGIP protein by examining the proteins in xylem sap. Cut stems of greenhouse-grown vines do not exude xylem sap. Centrifugation of small, denuded stem segments, as in Wallis and Chen (2012), has yielded irregular protein concentrations of 14-179 µg/mL in volumes less than 5 µl. Future samples will be pooled to test for PGIP activity. We are modifying a pressure flow apparatus to flush long stem segments with high salt buffers, which we believe will provide a source of exogenous or translocated pPGIP, depending on the genotype assayed. We have also evaluated rootstocks of transgenic tomato plants expressing pPGIP in order to gather more material for detecting the pPGIP protein in xylem sap. We have confirmed that pPGIP protein is expressed in the rootstocks and are also testing the pressure device to force xylem sap out of the roots.

b. Selection of PGIPs as Pierce’s disease defense candidates

Based on phylogenetic, biochemical, and structural analyses of PGIP sequences from 68 plant species, PGIPs from ‘Roma’ rice, ‘Hamlin’ orange, and ‘Bartlett’ pear have been selected for further study of their inhibition of *Xf*PG. The same cloning strategy previously reported is being applied to generate plant transformation vectors with each of these PGIPs. Transcription will be constitutive, as driven by the CaMV-35S promoter, and the resulting proteins will have a C-terminal 6x-histidine tag for purification. Genomic DNA has been prepared from ‘Kitaake’ rice, ‘Valencia’ and ‘Washington Navel’ orange leaves and each PGIP was successfully PCR amplified. The resulting PGIP genes were cloned into plasmids for sequencing and transformed into *E. coli* for DNA modifications. The homology models for rice and orange PGIPs were built based on publicly available sequences for less common cultivars of each species. Our cloned PGIPs from the more studied reference varieties have identified multiple single nucleotide polymorphisms (SNPs) in the modeled sequences. Some of these are missense mutations, confirmed by sequencing multiple colonies from independent PCR and bacterial transformation events (**Figure 1**). New source material from the modeled plant cultivars has been obtained for cloning with a proofreading polymerase to minimize discrepancies between the modeled PGIP sequences and those used for inhibition assays.

c. *Xf*PG expression and purification

The previously reported *Xf*PG expression system utilizing *Drosophila* S2 cells produced quantifiable amounts of PG protein with very slight activity that diminished over time. The second strategy was to express *Xf*PG transiently in leaves. A fusion construct of the apoplastic signal sequence from pPGIP and the coding sequence of *Xf*PG was generated for transient expression by *Agrobacterium tumefaciens*. Preliminary agroinfiltration assays with intact tobacco leaves indicated that the targeted PG had a similar activity to the non-targeted protein, both resulting in necrotic lesions in the infiltrated tissue, although the necrotic response could not always be distinguished from the tissue appearance where control solutions had been infiltrated. 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). For this reason, we have cloned the pPGIP::*Xf*PG fusion construct and the empty vector pCAMBIA-1301 into 5 different *A. tumefaciens* strains (EHA105:pCH32, GV3101, C58:pMP90, C58C1:pCH32, and the wild strain 1D1249) to optimize transgene expression and PG-induced necrosis in infiltrated leaves.

```

Hamlin_PGIP      121  TLRISWTNISGPVPDFIRQLTNLTFLELSFNNLSTIPGSLSKLQKLGALHLDRNKLTGS 180
Valencia_PGIP   121  TLRISWTNISGPVPDFISQLTNLTFLELSFNNLSTIPGSLSKLQKLGALHLDRNKLTGS 180
Wash_PGIP       121  TLRISWTNISGLVPDFISQLTNLTFLELSFNNLSTIPSSLSKLQKLGALHLDRNKLTGS 180
Wash_UCR_PGIP   121  TLRISWTNISGPVPDFISQLTNLTFLELSFNNLSTIPSSLSKLQKLGALHLDRNKLTGS 180
CTG1108280      121  TLRISWTNISGPVPDFISQLTNLTFLELSFNNLSTIPSSLSKLQKLGALHLDRNKLTGS 180
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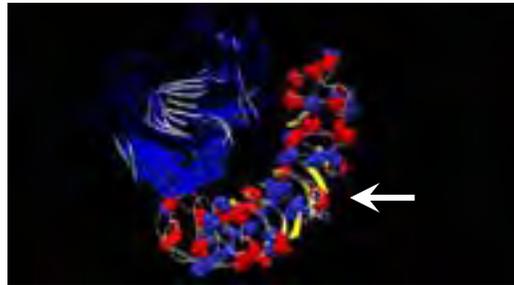


Figure 1. Location of the missense mutation in cloned citrus PGIPs. A SNP in the Hamlin orange coding sequence changes a serine residue to arginine (top, highlighted). This is the only non-synonymous substitution between the Valencia and Hamlin sequences. The arginine residue highlighted is located within an α -helix on the convex face of the PGIP (white arrow). Its significance for PG inhibition is not known.

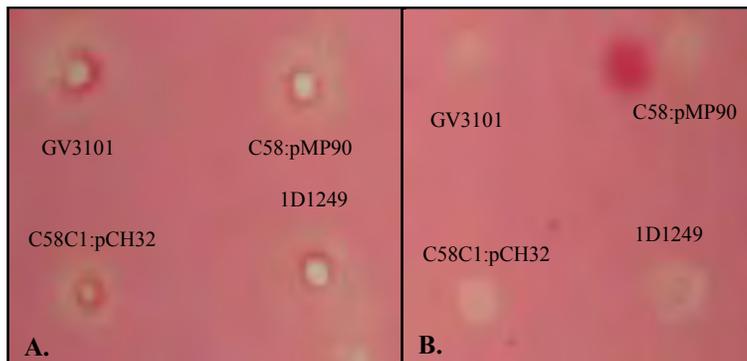


Figure 2. Radial diffusion assay indicating *Xf*PG activity. Concentrated leaf extracts (A) showed high activity, evidenced by the center clearing circle digested after 30 minutes and the more diffuse clearing zone apparent after 18 hours. Intercellular washing fluid (B) generated diffuse clearing zones only after overnight incubation.

Background necrosis was previously reported for our infiltrations using EHA105:pCH32 harboring the empty vector control. We conducted agroinfiltrations in tobacco (*Nicotiana benthamiana*) leaves with the four other strains, infiltrating the majority of the surface of three leaves per plant, one plant per *A. tumefaciens* genotype. Agroinfiltrations with GV3101, C58:pMP90, and C58C1:pCH32 did not produce marked necrotic zones when the strains contained either pPGIP::*Xf*PG or pCAMBIA-1301. The leaves infiltrated with both 1D1249 genotypes displayed dramatic necrosis and water soaking. Infiltrated regions were subjected to further analysis

to confirm transient expression. Frozen tobacco leaf tissue, excised from each infiltration zone, was ground to a powder and divided for analysis. Genomic DNA was prepared and the infiltrated construct PCR amplified in each sample to confirm the presence and genotype of the infiltrated *Agrobacterium* strain. Crude protein extracts and intercellular washing fluid were individually prepared from the excised infiltration zone of a tobacco leaf and assayed, one infiltration zone per assay, as described by Joubert *et al.* (2007) with minor modifications. Total protein recovered in the crude extracts ranged from 66-168 µg per sample. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of 2 µg of the crude leaf lysate confirmed that infiltrations of the four *A. tumefaciens* strains transformed with pPGIP::XfPG in different leaves produced a protein of approximately 61.9 kDa, as indicated by Coomassie staining; the predicted size of the fusion protein is 60.9 kDa. The overall banding seen by SDS-PAGE was faint and the crude lysate analyzed was too dilute to show activity in *in vitro* assays. Therefore, protein extracts were concentrated by passing the material once through Microcon centrifugal filters with Ultracel YM 10,000 MWCO membranes (Millipore, Billerica, MA). PG activity was detected in each of the concentrated extracts (**Figure 2A**) and to a lesser extent, the intercellular washing fluid (**Figure 2B**) from pPGIP::XfPG-expressing leaves, collected 7 days after infiltration. The ability to produce useful quantities of XfPG has been a major hurdle for multiple research groups attempting to study XfPG as a virulence factor. The system described here is the first report of measurable XfPG activity *in planta* and therefore is evidence of progress towards establishing an effective expression system to generate the PG needed for *in vitro* PGIP inhibition assays.

d. Expression of PGIPs to test XfPG inhibition

The cloning and expression of candidate PGIPs (**Objective 2b**) continues. The potential recombinant expression system for XfPG (**Objective 2c**) will be used to transiently express and purify active candidate PGIPs. Observations of PGIP activity *in planta* will be made using transgenic model plants for agroinfiltration experiments. Tomato and Arabidopsis plants constitutively expressing pPGIP or LePGIP have been germinated and confirmed by PCR. These lines will be used to test the efficacy of the PGIPs in leaves agroinfiltrated with XfPG-expressing strains in own-rooted and transgrafted plants. Once the candidate PGIP constructs have been prepared, stable transgenic tobacco plants will be generated for agroinfiltration with XfPG cultures and for inoculation with Xf.

Objective 3: Maximize PGIP expression in and transport from roots

Based on the assumption that the native signal sequence of pPGIP is sufficient for the targeted transport of active inhibitor protein to xylem tissues, we have tested the proteins extracted from leaves of transgrafted grapevines for the presence of pPGIP protein. First, the genotypes of all the grafts were confirmed by PCR analysis of genomic DNA. Then, own-rooted and transgrafted Thompson Seedless grapevines were evaluated for the presence of pPGIP protein in either the known transgenic tissue or in scion non-transgenic leaves from transgrafted vines. Two grafted vines and their respective donor rootstock and scion own-rooted plants (transgenic and wild-type, respectively) were subjected to protein extraction and probed by Western blotting (**Figure 3**). Proteins were extracted from newly expanded and young, expanding grape leaves by grinding 0.8 – 1.7 g FW in five volumes of 1 M NaCl, 0.1 M NaAcetate (pH 5), 1% polyvinylpolypyrrolidone [w/v], 0.1% NaHSO₃ [w/v] and shaking overnight at 4°C. The tissue homogenate was centrifuged and proteins in the collected supernatants were precipitated in five volumes of cold acetone. Total proteins were separated by 10% SDS-PAGE and electrotransferred to a PVDF membrane. Western detection used a primary polyclonal antibody to purified pPGIP and a secondary antibody conjugated to horseradish peroxidase.

Information pertaining to potential signal sequences targeting PGIPs to xylem tissues for transport across graft junctions has been reported by the project “*In planta* testing of signal peptides and anti-microbial proteins for rapid clearance of *Xylella*” (PI: A. Dandekar).

Objective 4: Create PGIP-expressing rootstocks and evaluate their Pierce’s disease resistance

As discussed previously (**Objective 2d**), the candidate PGIPs will be assayed for XfPG inhibition *in planta* utilizing transgrafted tobacco plants. Grape rootstock transformation will commence once an optimal PGIP has been determined.

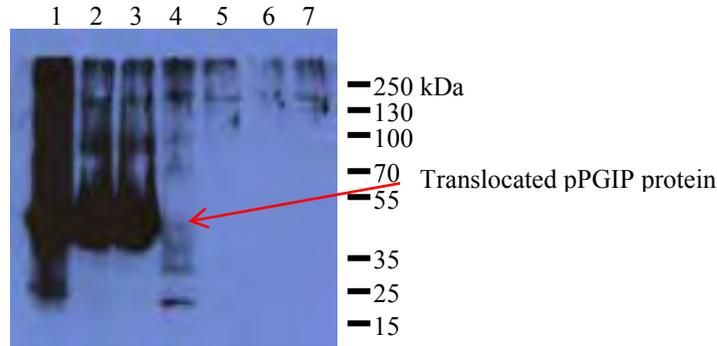


Figure 3. pPGIP protein in own-rooted transgenic, control, and transgrafted grapevine leaves. Total proteins from young leaves of own-rooted pPGIP-expressing (lanes 2, 3), control (lanes 6, 7) or transgrafted grapevines (lanes 4, 5). Protein bands were visualized by Western blotting with a polyclonal antibody to pPGIP. Intense cross-reactivity to a protein of approximately 43 kDa, the expected molecular weight of pPGIP, was observed from transgenic tomato leaves expressing pPGIP (lane 1), as well as from the own-rooted transgenic grapevines expressing pPGIP (lanes 2, 3). A signal of the same molecular weight was observed from scion leaf tissue (lane 4), implying the transport of cross-reactive pPGIP protein from the transgenic rootstock (lane 2) into the wild-type scion. Scion tissue for the graft represented in lane 4 was taken from the wild-type vine sampled in lane 6.

CONCLUSIONS

The ability to compare multiple PGIPs to determine an optimal inhibitor for specific PGs is a keystone for developing transgenic rootstocks as targeted strategies against pathogens that utilize PG(s) for virulence. In the scope of Pierce's disease resistance, we have determined that PGIPs from Bartlett pear, Hamlin orange, and Roma rice are optimal candidates for *Xf*/PG inhibition. These candidates were selected based on sequence diversity and homology modeling to predict which of the analyzed PGIPs most closely interacts with *Xf*/PG *in silico*. *Xf*/PG has been recombinantly expressed in tobacco leaves by agroinfiltration and proven to have activity. This source of protein will allow for immediate *in vitro* comparison of the PGIP candidates; their cloning and subsequent expression is in progress to correct sequence errors. We have detected pPGIP crossing the graft junctions from transgenic rootstocks to non-transgenic scion leaves in grafted grape plants we have developed for this project. The ability of pPGIP, one of the candidates discussed here, to provide Pierce's disease resistance to transgrafted scions is being addressed by the corresponding field trial.

The goal of the project is 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 needing to target the growing vector population. The project is designed to identify specific PGIPs that target the virulence factor, *Xf*/PG, and to express them in rootstocks to provide protection to the grafted wild-type scion tissues.

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ENHANCING CONTROL OF PIERCE'S DISEASE BY AUGMENTING PATHOGEN SIGNAL MOLECULES

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ABSTRACT

Xylella fastidiosa (*Xf*) produces an unsaturated fatty acid signal molecule called 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." Plants expressing *rpfF* from *Xf* produce low levels of DSF and are highly resistant to Pierce's disease. Chloroplast targeting of RpfF apparently substantially increases DSF production. *Xf* moved much less rapidly in *rpfF*-transformed grape, colonized many fewer xylem vessels, and achieved a much lower population size indicating that elevated DSF levels suppressed movement within the plant. As exogenous sources of DSF applied in various ways to grape suppressed pathogen mobility and hence virulence we have further studied the chemical identity of DSF. Preliminary evidence suggests that DSF is comprised of three closely related fatty acid molecules. One component is 2-Z-tetradecenoic acid (hereafter called C14-cis) while a second compound termed C12-cis is apparently also produced. The chemical identity of a third component is as yet undetermined and is being investigated. We are currently determining the relative activity of these forms of DSF and if such molecules cooperate in regulating gene expression in *Xf*. The various forms of DSF may preferentially affect different behaviors of *Xf*. Since some reduction in disease severity was observed in grape scions grafted to DSF-producing rootstocks suggests that DSF produced by rootstocks can somewhat move to scions and confer disease control the control of disease, grafted plants are being made that have a relatively large rootstock to test the hypothesis that increased supply of DSF to the scion will be associated with a larger rootstock. Naturally-occurring endophytic bacteria within grape are being assessed for DSF production; only about 1% of the endophytic bacteria in grape produce DSF and these are being tested for their ability to move within plants after inoculation. As studies of pathogen confusion will be greatly facilitated by having an improved bioassay for the DSF produced by *Xf*, we have been developing several immunological and biochemical means to assay for the presence of DSF using *Xf* itself as a bioindicator. Bioassays based on immunological detection of the cell surface adhesin XadA and EPS have been developed. Gene expression in *Xf* exposed to various levels of DSF is also being directly assessed using *phoA* reporter gene fusions. *Xanthomons campestris*-based biosensors in which Rpf components have been replaced by those from *Xf* also selectively detect the DSF produced by *Xf*. An improved *X. campestris*-based biosensor in which a mutant allele of *RpfF* from *Xf* that does not confer production of DSF but which apparently still interact with

RpfC has been produced; since this biosensor strain does not produce DSF, it has much lower background GFP reporter gene activity as well as a high responsiveness to exogenous DSF. 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. The cell adhesive assay therefore makes a valuable means of detection for DSF. DSF was readily detected and transgenic grape expressing the *Xf* rpfF gene when the *Xanthomonas campestris*-based biosensor was applied to agar in which intact leaves were detected. The use of intact leaves therefore appears attractive as a method to screen transgenic plants for DSF production. 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 adherence of wild-type strains of *Xf* to transgenic Thompson Seedless expressing a chloroplast-targeted *rpfF* gene from *Xf* was much higher than non-transformed plants, indicating that DSF production in the plants has increased the adhesiveness of the pathogen, and thereby reduced its ability to move within the plant after inoculation.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) produces an unsaturated fatty acid signal molecule called diffusible signal factor (DSF). Accumulation of DSF in *Xf* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in the pathogen, but the overall effect is to suppress its virulence in plants. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption (pathogen confusion) as a means of controlling Pierce's disease. We have investigated both the role of DSF-production by *Xf* on its behavior within plants, the manner in which other bacterial strains affect such cell signaling, the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, performed genetic transformation of grape to express DSF, and explored other means to alter DSF abundance in plants to achieve Pierce's disease control. Elevating DSF levels in plants reduces its movement in the plant. We have found naturally-occurring bacterial endophyte strains that can produce large amounts of DSF; we are testing them for their ability to move within plants and to alter the abundance of DSF sufficiently to reduce the virulence of *Xf*. Given that DSF overabundance appears to mediate an attenuation of virulence in *Xf* we have transformed grape with the *rpfF* gene of the pathogen to enable DSF production in plants; such grape plants produce at least some DSF and are much less susceptible to disease. Some reduction in disease severity was observed in grape scions grafted to DSF-producing rootstocks suggesting that DSF produced by rootstocks can somewhat move to scions and confer disease control. The chemical composition of DSF itself is being determined so that synthetic forms of this signal molecule can be made and applied to plants in various ways. We have found that the adherence of *Xf* to grape tissue is much more tenacious in the presence of DSF, and we thus have developed assays to more rapidly screen transgenic plants for their resistance to Pierce's disease as well as the efficacy of chemical analogs of DSF to induce resistance.

INTRODUCTION:

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

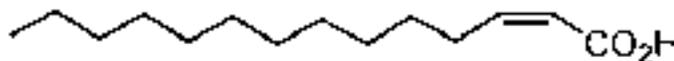


Figure 1. Structure of DSF: C14-cis.

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

OBJECTIVES

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

RESULTS AND DISCUSSION

Characterization of DSF made by *Xf*

While the molecule C14-cis is one component of DSF made by *Xf*, further support for the possibility that more than one fatty acid signal molecule is made by RpfF was obtained by the use of a Thin Layer Chromatography (TLC) method to assess the fatty acids produced by *Xf*. In this method, acidified ethyl acetate extracts of culture supernatants of a wild-type *Xf* strain and an *RpfF*- mutant and a *RpfB* mutant were subjected to TLC and fatty acids visualized by iodine vapors. Interestingly, three different fatty acids were visualized in the wild-type strain, while these were largely missing in an *RpfF*- mutant, with only very small amounts of two other putative fatty acids present (**Figure 2**). It also was of interest to see that the *RpfB* mutant produced an altered pattern of putative fatty acids, with the major chemical species produced by the wild-type strain missing, and much larger amounts of one of the other species produced. The top-most spot observed in extracts of a wild-type strain of *Xf* co-migrates with C14-cis, a chemical form of DSF that we have previously characterized. Interestingly, the

middle band found in the wild-type strain, which migrated similarly to the topmost, and most abundant band seen in the *rpfB* mutant, co-migrates with C12-cis. It is noteworthy, that a re-examination of the DSF species produced by *Xanthomonas campestris* pv. *campestris* using different methods have revealed that this species also produces C12-cis. The most prominent, lower, band seen in the wild-type strain is not observed in the *rpfB* mutant. This supports the model that RpfB, a putative long-chain fatty acyl CoA ligase, serves to produce suitable substrates for RpfF, the DFS synthase. Although RpfB is not required for DSF synthesis in *Xf*, it presumably aids in DSF synthesis by encoding long chain fatty acyl CoA ligase which might increase availability of the appropriate substrates for DSF synthesis by RpfF. It is interesting to note that *rpfB* mutants have an altered behavior compared to *rpfF* mutants and wild-type strains of *Xf*. While *rpfF* mutants are hyper-virulent in grape, *rpfB* mutants were nearly as virulent as wild-type strain. In contrast, while *rpfF* mutants are non-transmissible by sharpshooters, the *rpfB* mutants exhibit only a slight decrease in their transmission suggesting that they are retained by sharpshooters more efficiently than the *rpfF* mutant. Given that the *rpfB* mutant appears to make C12-cis but not C14-cis, this suggests that C12-cis is sufficient to enable signaling that leads to insect transmission but does not greatly affect virulence. We expect that co-expression of RpfB and RpfF in the chloroplast will further enhance the DSF levels in plants. We have produced transgenic *Arabidopsis* plants with such a construct and find evidence of high levels of DSF production. Pathogenicity assays with the *rpfF* mutant of *Xcc* indicated that the transgenic plants expressing both *rpfB* and *rpfF* transgenic plants can better complement the virulence of the non-pathogenic *rpfF* mutant of *Xcc*. Based on this and other data, we are expressing both RpfF and RpfB simultaneously in transgenic plants for optimum production of suitable DSF molecules. We thus are preparing genetic constructs to transform grape with these two genes to further enhance DSF production.

We have also used improved DSF biosensors (see below) to screen for active fractions in chemically separated culture supernatants of *Xf*. As noted above, C14-cis is produced by *Xf*, but is probably not the only DSF species produced by this bacterium. We therefore have further investigated the conditions under which DSF is

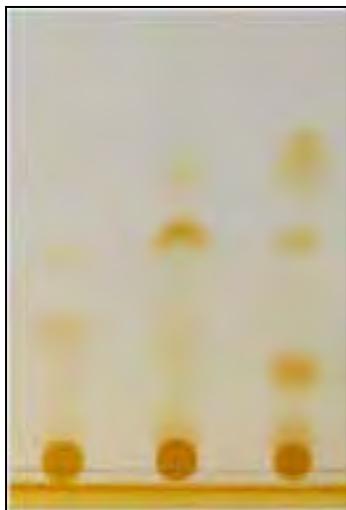


Figure 2. Fatty acids resolved by TLC from a RpfF mutant of *Xf* (left lane) a RpfB mutant (center lane) and a wild-type strain of *Xf* (right lane). Fatty acids were visualized after exposure to iodine vapor.

produced by *Xf*, as well as performed chemical fractionation of the molecules produced by *Xf* to determine the identity of additional chemical species having signaling activity in the pathogen. Nearly all of the work we have conducted to date has utilized the growth medium PWG to culture *Xf*. While *Xf* grows well on PWG medium, our recent results indicate that it either produces relatively little DSF on that medium or that the DSF is bound to medium ingredients, most likely bovine serum albumin which is a major medium ingredient. We therefore have studied the production of DSF in cells of *Xf* grown on PD3 medium. Not only does *Xf* exhibit abundant growth on this medium, but the yield of DSF seems to be much higher than on PWG medium. Chemical fractionation of PD3 medium in which *Xf* has been grown yield a variety of fractions, differing in polarity, that have biological activity as measured by the *Xcc*-based biosensor (**Figure 3**). NMR and Mass Spectral analysis of these fractions are currently underway to identify the chemical species associated with biological activity. Evidence to date suggests that longer chain enoic acids such as C16-cis might have strong biological activity. After identification, these chemical species will be synthesized and applied to plants. The biological sensors used for their initial detection will then be used to determine their stability and movement within the plant as part of objective 4. These plants will also be challenge inoculated with cultures of *Xf* to determine whether they are more or less effective than C14-cis in altering the behavior of *Xf* and thus the incitation of disease symptoms.

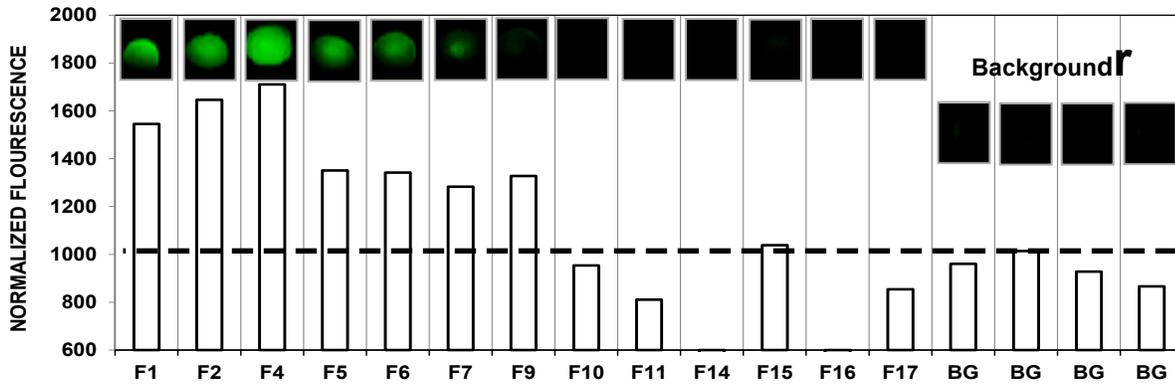


Figure 3. Biological activity of different fraction of a culture extract of *Xf* grown in PD3 media as detected by gfp fluorescence of a *Xccs*-based biosensor

Studies of adhesion of *Xf* to grape

Our studies have suggested strongly that adhesion of *Xf* to plant tissues inhibits movement of the pathogen through the plant, and hence tends to reduce the virulence of the pathogen. RpfF- mutants of the pathogen that do not produce DSF adhere to glass surfaces and to each other much less effectively than wild-type strain that produce DSF. This is consistent with the apparent rpfF-regulation of adhesins such as HxfA, HxfB and XadA etc. To better correlate levels of DSF in the plant and the stickiness of the *Xf* cells we have developed a practical assay to measure and compare stickiness of *Xf* cells in grapes infected with *Xf gfp-Wt* and *Xf* mutants. In this assay, the release of cells of *Xf* from stems and petioles tissue from grape infected with *Xf* wild-type strain and gfp-rpfF mutant were compared. Tissues from infected Thompson Seedless grapes were surface sterilized. From the sterile tissues, 5mm stem or petiole segments were cut and placed individually in sterile buffer and shaken gently for 20 minute. After 20 minutes the number of cells released from the cut end of the segment were estimated by dilution plating on PWG. To determine the total number of cells in a given sample (the number of cells that potentially could have been released by washing) the washed segment was macerated and *Xf* populations again evaluated by dilution plating. Total cell populations were calculated by summing the cells removed by washing and those retained in the segment. The ratio of easily released cells to the total cells recovered in the samples was termed the release efficiency. In both stems and petioles the release efficiency of the *rpfF* mutant was much higher than that of the wild-type strain (**Figure 4**). There was a very strong inverse relationship between the adhesiveness of the cells to grape and their ability to cause disease (**Figure 4**). This very striking difference in the adhesiveness of the *Xf* cells experiencing different levels of DSF in the plant suggest that this release efficiency assay will be valuable for rapidly assessing the susceptibility of grapes treated in various ways. For example, the adhesion of cells could be measured within a couple of weeks after inoculation of wild-type *Xf* cells into transgenic plants harboring various constructs designed to confer DSF production in plants, or in plants treated with DSF producing bacteria or topical application of chemicals with DSF-like activity. Such an assay would be far quicker than assays in which disease symptoms must be scored after several months of incubation, and could be employed during those times of the year such as the fall and winter when disease symptoms are difficult to produce in the greenhouse.

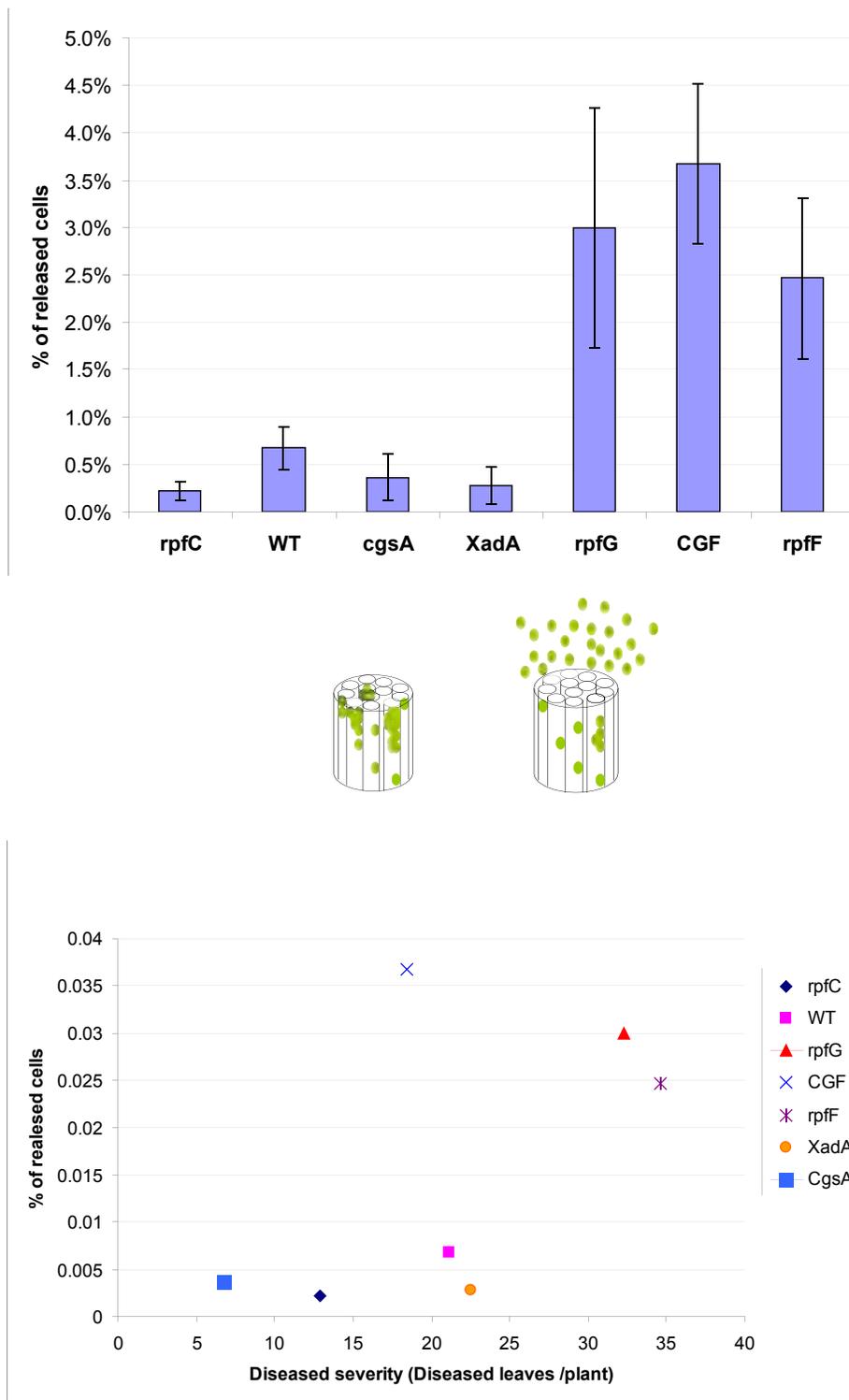


Figure 4. Top: Proportion of total cells of various mutants of *Xf* that were released during gentle washing of grape stem segments in buffer as depicted (Center). The vertical bars represent the standard error of estimates of the proportion of released cells for a given treatment. Bottom: Relationship between proportions of various *Xf* mutants released from tissues and the virulence of those strain in Thompson Seedless grape.

To further address the usefulness of the cell release assay to assess treatments designed to limit the movement of *Xf* in plants to achieve disease control, we tested the adherence of wild-type strains of *Xf* to transgenic Freedom expressing a non-targeted *rpfF* gene from *Xf* compared with that to non-transformed plants. Plants were inoculated with a gfp-marked wild-type strain of *Xf* and petioles were removed from plants at a distance of about 20 cm from the point of inoculation, and the percent of the cells removed during a brief washing step measured as above. The adherence of wild-type strains of *Xf* to transgenic Freedom was much greater than that of cells in the non-transformed plants, indicating that DSF production in the plants has increased the adhesiveness of the pathogen, and thereby reduced its ability to move within the plant after inoculation. That is, the percentage of cells that was released from *rpfF*-expressing plants was from 2 to 3-fold less than that of control plants (**Figure 5**). As seen before, cells of an *rpfF* mutant that does not produce DSF exhibited about 3-fold higher percentage of cells released from a normal Thompson grape compared to the wild-type strain in the non-transgenic grape (**Figure 4**). These results suggest strongly that DSF production has occurred in the *rpfF*-expressing plants, and that the enhanced adhesiveness of these cells is associated with their reduced ability to spread through the plant and cause disease. These results suggest that the release efficiency assay should be a useful tool to rapidly assess treatments designed to control Pierce's disease.

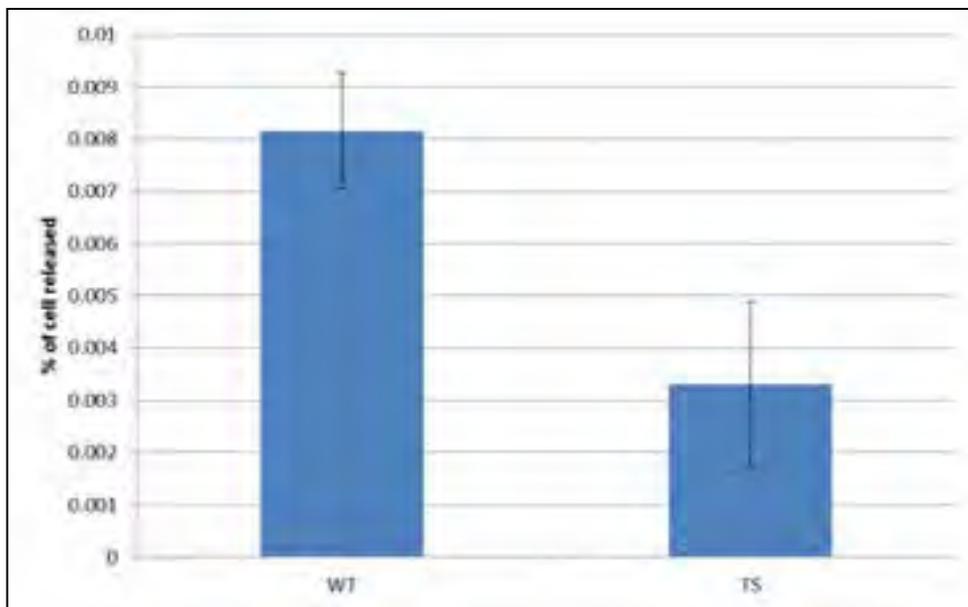


Figure 5. Percentage of total cells of a gfp-marked wild-type strain of *Xf* in petioles of non-transformed Freedom (wild-type) or of transgenic Freedom expressing the *rpfF* gene from *Xf* (Thompson Seedless) that were released during gentle washing of the segments in buffer. The vertical bars represent the standard error of estimates of the proportion of released cells for a given treatment.

Our studies of DSF-dependent traits in *Xf* indicate that elevation of DSF in transgenic plants should increase the adhesiveness of the pathogen, thereby reducing its potential to move throughout the plant. To better quantify these parameters, we have made detailed measurements of appearance of disease symptoms in transgenic Freedom grape transformed with the *rpfF* gene from *Xf*. Temporal measurements of disease severity reveal that initially both wild-type and DSF-producing grape had similar incidence of symptomatic leaves (about two leaves per plant) (**Figure 6**). After approximately seven weeks after inoculation however the incidence of symptomatic leaves increased rapidly in wild-type plants to over 12 leaves per plant by week 11, while the number of symptomatic leaves in the DSF producing plants remain low. These results strongly support the model that *Xf* could move passively short distances (15 cm or less) and neither plant however, active movement was restricted in the DSF producing plants, limiting infection of only those leaves found close to the point of inoculation.

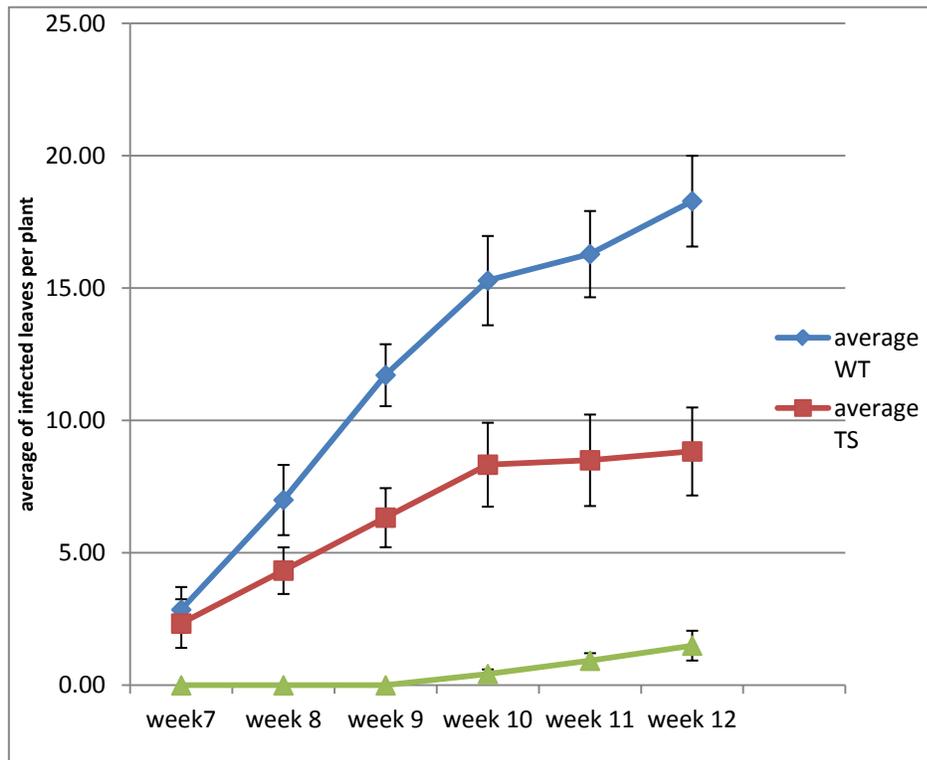
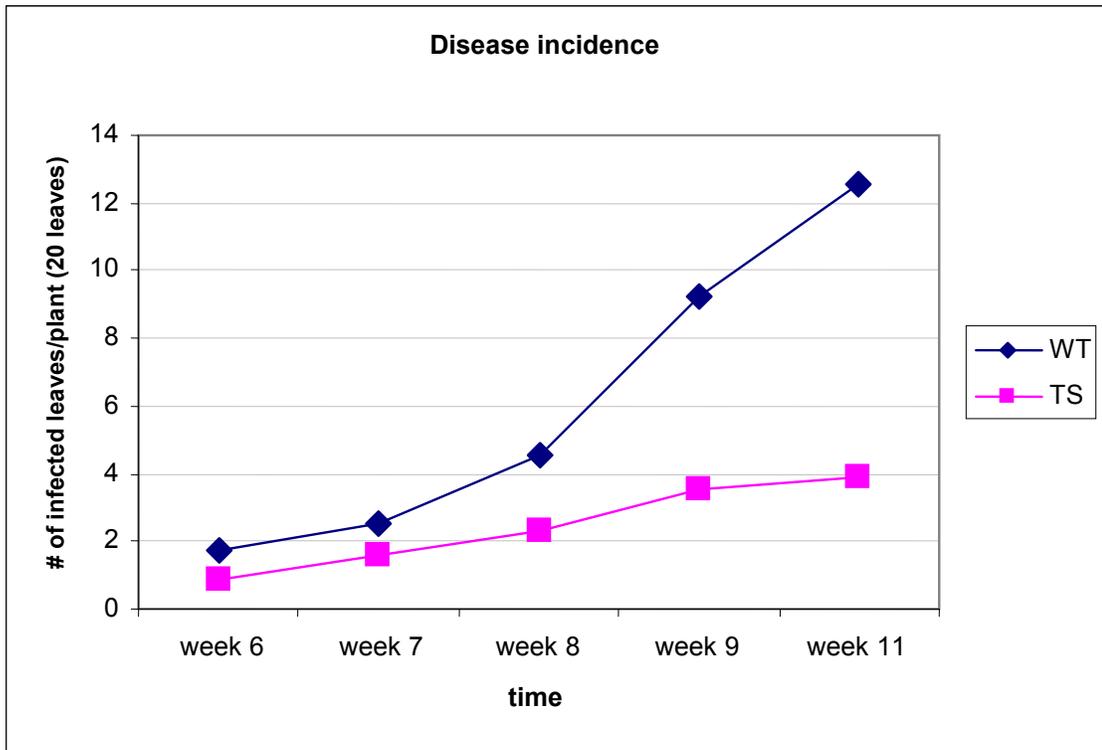


Figure 6. Incidence of leaves exhibiting symptoms of Pierce’s disease on wild-type Freedom grape (blue) or transgenic DSF-producing Freedom (red) assessed at various times after inoculation in two different experiments with *Xf*. Disease symptoms on uninoculated plants are shown in green (Bottom). The vertical bars represent the standard error of the mean.

In contrast to the effect of DSF on increasing the adhesiveness of cells of *Xf*, particularly as they would reach high population sizes in the plant, *Xf* also appears to produce a molecule or to induce the production of a molecule by the plant that reduces its ability to self-aggregate and to adhere to surfaces. Cells of a GFP-marked *Xf* wild-type strain were suspended in xylem sap recovered from both uninfected plants, as well as plants infected with a wild-type strain of *Xf* or a plant infected with an RpfF mutant. While many cells of the wild-type strain formed relatively large cellular aggregates after one day of suspension in samples from healthy plants, the cells remain dispersed and occurred primarily as single cells when suspended in sap from a plants infected with the RpfF mutant (**Figure 7**). Cells suspended in sap collected from a plant infected with a wild-type strain of *Xf* exhibited intermediate levels of cellular aggregation (data not shown). Since most of the sap recovered from infected plants would likely have been from vessels that had relatively low population sizes of *Xf* (more heavily infected vessels would have been plugged and therefore xylem fluid would have been difficult to collect), we presume that the factors present in the sap of the plants infected with the wild-type strain and particularly the RpfF mutant of *Xf* had produced one or more materials that interfered with the adhesiveness of the cells. Quantification of the process of cellular aggregation as a function of time after cells were suspended in sap from grape infected with different strains of *Xf*, revealed that sap from plants infected with the RpfF mutant was particularly effective in preventing cellular aggregation of *Xf* (**Figure 8**). Such anti-adhesive factors produced by the RpfF mutant might account for its enhanced ability to move within plants that had been previously observed. It thus seems likely that *Xf* alters the chemical environment within the xylem vessels to restrict its ability to adhere to plants, thereby maximizing its ability to move along vessels but also between vessels through pit membranes.

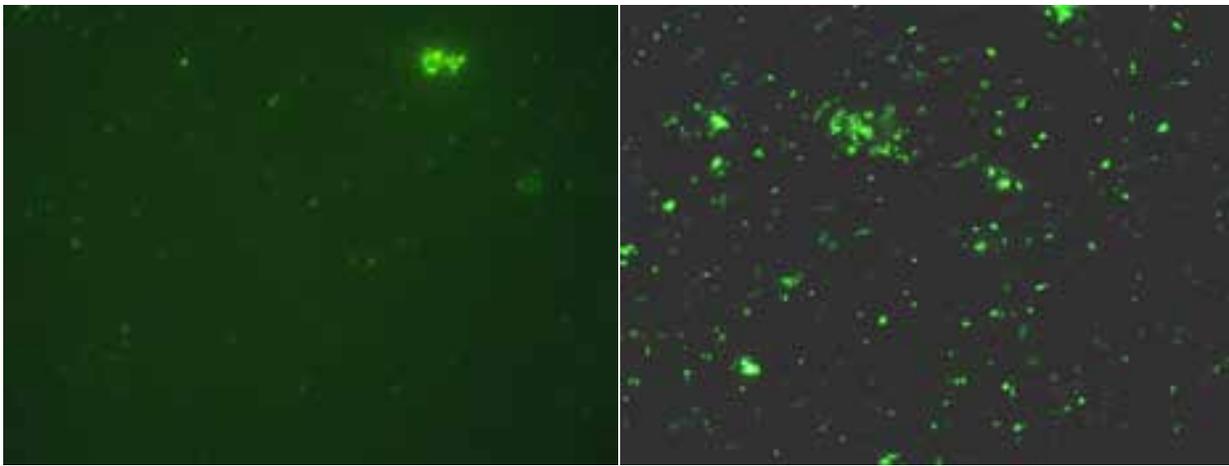


Figure 7. Cellular aggregates of a GFP marked strain of *Xf* suspended in sap from grape infected with an RpfF mutant of *Xf* (left panel) or from staff from an uninfected plant (right panel) when visualized at 20 x magnification. At this magnification only cellular aggregates are visible as relatively large green spots.

Graft transmissibility of DSF

To test whether DSF is mobile within the plant we are performing grafting experiments in which DSF-producing Freedom grape transformed with the *rpfF* gene of *Xf* are used as rootstocks to which normal Cabernet Sauvignon grape were green-grafted as a scion. As a control, normal Freedom was also used as a rootstock. These plants were inoculated with *Xf* to test whether normal scions on DSF-producing rootstocks have a lower susceptibility to *Xf* colonization as a rootstock than as a scion. Initial estimates of disease severity indicate that there were about 30% less symptomatic leaves of the normal Cabernet scion when grafted onto an *rpfF*-expressing rootstock compared with plants on a normal Freedom rootstock. Thus it appears that DSF production in the scion is more efficacious for disease control than is the expression of *rpfF* in the rootstock. We are repeating these grafting experiments and testing the hypothesis that increasing the size of the rootstock will increase its potential to distribute DSF to the scion. We thus are producing wild-types scions grafted to rootstocks of differing sizes. Green grafting is proving difficult because the normal process is root the rootstock

at the same time that the scion is grafted onto the top. Establishing a large rootstock before grafting has made establishment of the grafted scion more difficult. Some success has now been achieved in these plants will soon be inoculated and their disease susceptibility will be related to the size of the rootstock. In another strategy to address objective 3, we are using dormant transgenic grape from our field trial in Solano County to produce grafted plants with a very large rootstock. The plants that were established in 2010 in the Solano County trial are now quite large. The USDA –APHIS permit was amended to enable us to collect dormant vines from this field trial for terminal used in our greenhouses at UC Berkeley. A large amount of dormant vines from both transgenic Freedom as well as transgenic Thompson seedless plants that had not been inoculated with *Xf* during the summer of 2011 were collected. Non-transgenic Cabernet are being grafted onto the top of dormant twigs of different lengths, and the lower rootstock sections are being rooted in the greenhouse. We are optimistic that this strategy will be successful in producing large rootstocks up to 50 cm or more.

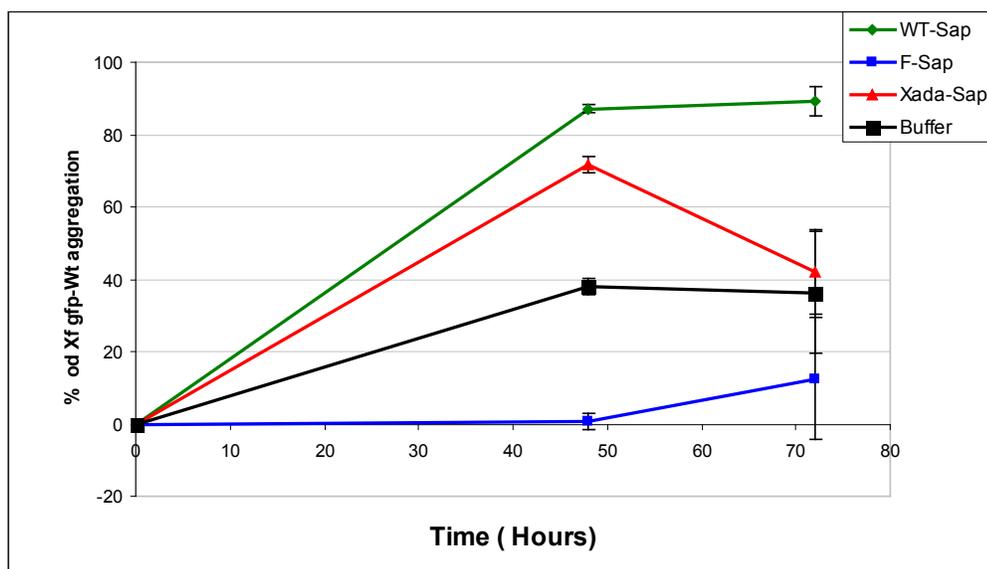


Figure 8. Increases in cellular aggregation of cells of a wild-type *Xf* strain suspended in xylem sap of grape plants inoculated with different strains of *Xf*, as measured by a decrease in turbidity of cells over time. Note the substantially lower level of aggregation of cells suspended in sap from plants infected with an RpfF mutant compared to that in buffer alone (blue versus black line).

Development of *Xcc*-based biosensors efficient in detecting *Xylella* DSF

We constructed three *Xcc*-based DSF biosensors specific to the DSF produced by *Xf* RpfF and which is sensed by *Xf* RpfC one of which we replaced the Rpf-DSF detection system of *Xcc* with that of homologous components from *Xf* and a second in which an *Xcc* *rpfF* and *rpfC* double mutant into which *Xf* *rpfF* and *rpfG* and a hybrid *rpfC* allele composed of the predicted trans-membrane domain of *Xf* RpfC and the cytoplasm domain of the *Xcc* RpfC was been added. A third is a *Xcc*-based GCF biosensor in which the *Xf* *rpfF* gene is replaced by with a mutant allele in which glutamate codons 141 and 161 which are essential for DSF production activity are replaced by Alanine codons. This mutant form of RpfF is no longer capable of DSF synthesis, but is still apparently capable of interaction with RpfC, and thus proper response to DSF; these have been termed *Xf*-RpfGCF, *Xf*-*Xcc* chimeric, and *Xf*-RpfGCF*. The *Xcc*-based *Xf*-*Xcc* chimeric, and *Xf*-RpfGCF biosensors exhibit very strong *gfp* fluorescence in response to DSF molecules and have been very useful in detecting DSF in transgenic plants (**Figure 9**).

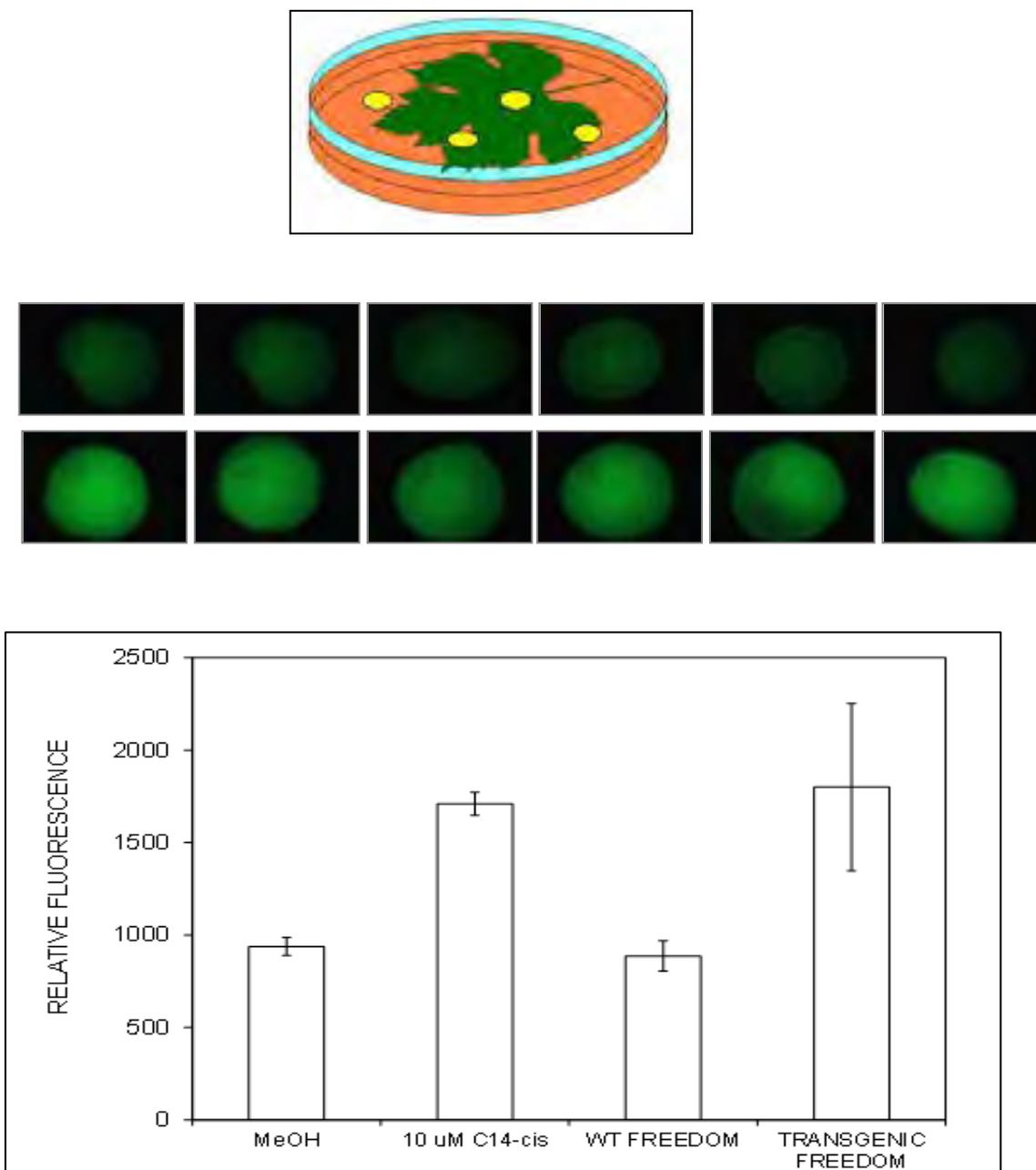


Figure 9. Assay for DSF content of intact plants performed using the *Xcc* rpfCGF DSF biosensor. Top: Contact grape leaves are embedded in agar and cells of the biosensor applied as spots above the leaf tissue. Middle: GFP fluorescence exhibited by this biosensor that had been placed above either wild-type plants (top row) or above these of transgenic freedom expressing the *Xf rpfF* gene (bottom row). Bottom: Quantification of alkaline phosphatase activity of sensor depicted in center panel and positive control of added C14-cis.

Development of a *Xf*-based bioreporter for DSF.

We have developed methods to use *Xf* itself to detect DSF by using its endogenous *phoA* gene (encoding alkaline phosphatase) as a bioreporter of gene expression in a *phoA* mutant. The PhoA-based biosensor in which *phoA* is driven by the *hxfA* promoter is quite responsive to exogenous DSF. Both *hxfA* and *hxfB* expression in *Xf* was strongly induced by C14-cis but not by the related molecule myristic acid as assessed using alkaline phosphatase as an assay (**Figure 10**).

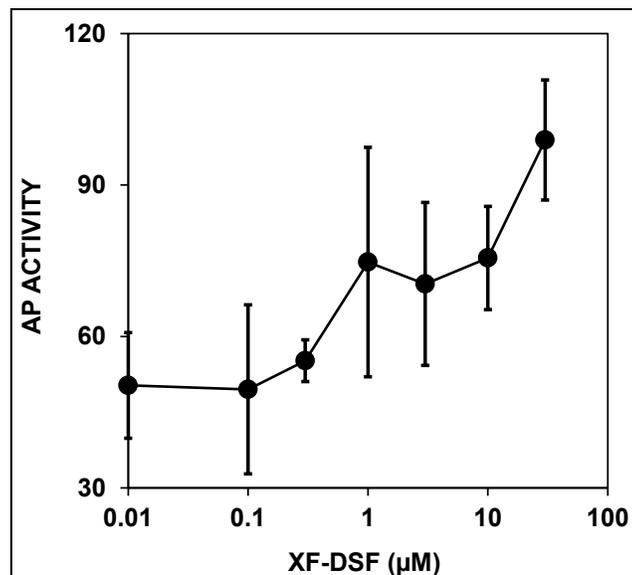
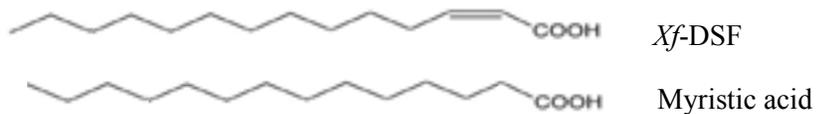
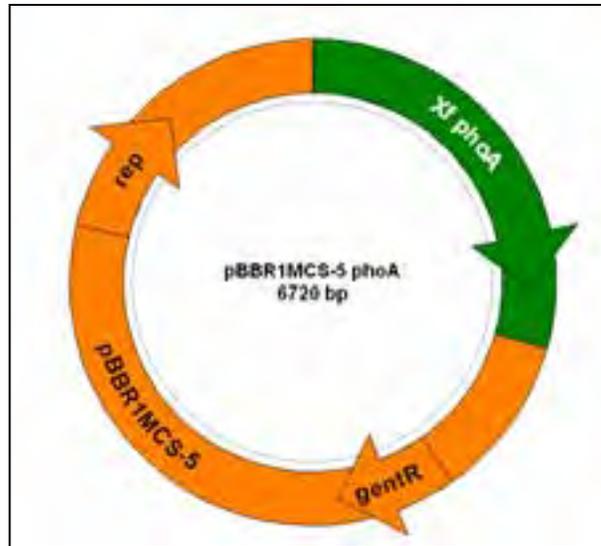


Figure 10. Top: Vector construct which enables the use of alkaline phosphatase as a reporter gene for monitoring gene expression of DSF responsive genes such as *hxfA* in *Xf* (Middle). Bottom: Alkaline phosphatase activity exhibited by cells of *Xf* harboring a *hxfA:phoA* fusion when grown on culture medium containing various concentrations of C14-cis when measured 24 hours after inoculation.

CONCLUSIONS

Since we have shown that DSF accumulation within plants is a major signal used by *Xf* to change its gene expression patterns and since DFS-mediated changes all lead to a reduction in virulence in this pathogen we have shown proof of principle that disease control can be achieved by a process of “pathogen confusion.”

Several methods of altering DSF levels in plants, including direct introduction of DSF producing bacteria into plants, and transgenic DSF-producing plants appear particularly promising and studies indicate that such plants provide at least partial protection when serving as a rootstock instead of a scion. While the principle of disease control by altering DSF levels has been demonstrated, this work addresses the feasibility of how achieve this goal, and what are the most practical means to achieve disease control by pathogen confusion. The tools we have developed to better detect the specific DSF molecules made by *Xf* will be very useful in our on-going research to test the most efficacious and practical means to alter DSF levels in plants to achieve disease control. We are still optimistic that chemically synthesized DSF molecules might also ultimately be the most useful strategy for controlling disease. The presence of more than one DSF base signal molecule suggests that perhaps more than one molecule might be needed to achieve changes in pathogen behavior. Our major advances in the development of biosensors to detect the responsiveness of *Xf* to signal molecules is a major breakthrough that hopefully will allow us to make rapid progress in ascertaining those transgenic plants most capable of altering pathogen behavior as well as in formulating synthetic molecules suitable for use in disease control. The biological sensors also have proven useful in screening naturally occurring bacteria associated with grape that might also be exploited to produce signal molecules

FUNDING AGENCIES

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

FIELD EVALUATION OF DIFFUSIBLE SIGNAL FACTOR PRODUCING GRAPE FOR CONTROL OF PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted March 2010 to October 2012.

ABSTRACT

A cell density-dependent gene expression system in *Xylella fastidiosa* (*Xf*) mediated by a small signal molecule called diffusible signal factor (DSF) which we have now characterized as 2-Z-tetradecenoic acid (hereafter called C14-cis) controls the behavior of *Xf*. The accumulation of DSF attenuates the virulence of *Xf* by stimulating the expression of cell surface adhesins such as HxfA, HxfB, XadA, and FimA (that make cells sticky and hence suppress its movement in the plant) while down-regulating the production of secreted enzymes such as polygalacturonase and endoglucanase 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, 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 five-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.

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:

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



Figure 1. Overview of research plot in Solano County.

Treatments 5-8 serve as appropriate control to allow direct assessment of the effect of DSF expression on disease in own rooted plants as well as to account for the effects of grafting per se on disease susceptibility of the scions grafted onto DSF-producing rootstocks.

One field trial was established in Solano County on August 2, 2010. Twelve plants of each treatment were established in randomized complete block design. Self-rooted plants were produced by rooting of cuttings (about three cm long) from mature vines of plants grown in the greenhouse at UC Berkeley. Cuttings were placed in a sand/perlite/peatmoss mixture and subjected to frequent misting for about four weeks, after which point roots of about 10 appeared. Plants were then be transferred to one-gallon pots and propagated to a height of about 1 m before transplanting into the field. Grafted plants were produced in a similar manner. Twenty cm stem segments from a susceptible grape variety were grafted onto 20 cm segments of an appropriate rootstock variety and the graft union wrapped with grafting tape. The distal end of the rootstock variety (harboring the grafted scion) was then be placed in rooting soil mix and tooted as described above. After emergence of roots, the grafted plant were then transplanted and grown to a size of about 1 m as above before transplanting into the field site. The plants all survived transplanting and are growing well (**Figure 1**). The plants were 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* (**Figure 2**). The plants at the Solano County trial have grown luxuriantly, and by the time of rating in August and September, vines had overgrown the older vines inoculated in May, making disease rating difficult (**Figure 3**).



Figure 2. Process of inoculation of grape at the Solano County field trial in July 2011. A needle was inserted through a vine and a droplet of inoculum applied to the needle tip. After withdrawal of the needle, the bacterial inoculum is drawn into the vine due to the tension of the water in the xylem vessels.



Figure 3. Appearance of transgenic Freedom grape in early October 2011.

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 4**). 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 5**). 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, 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 6**).

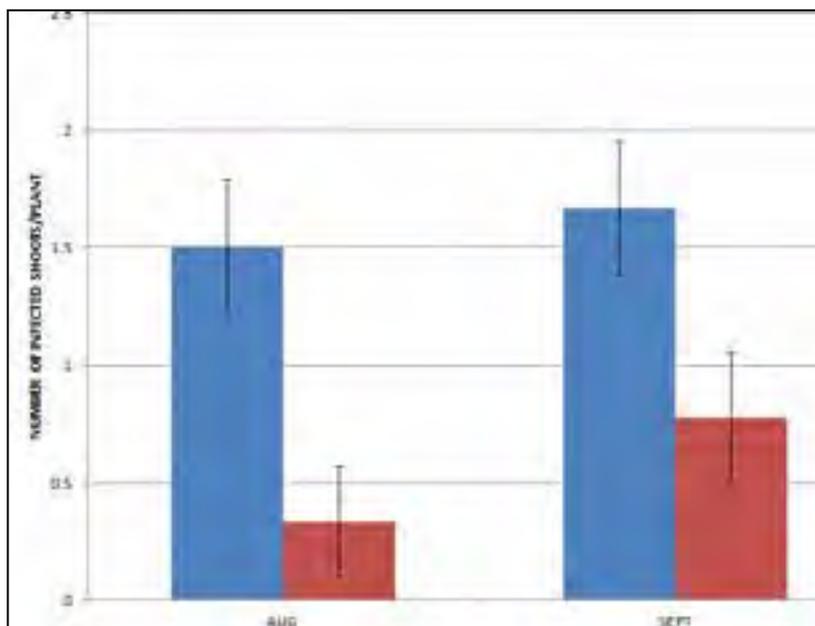


Figure 4. Incidence of vines of DSF-producing transgenic Freedom grape (red) or wild type Freedom having any symptoms of Pierce’s disease when rated in August or September 2012. A total of three vines per plant were assessed. The vertical bars represent the standard error of the mean.

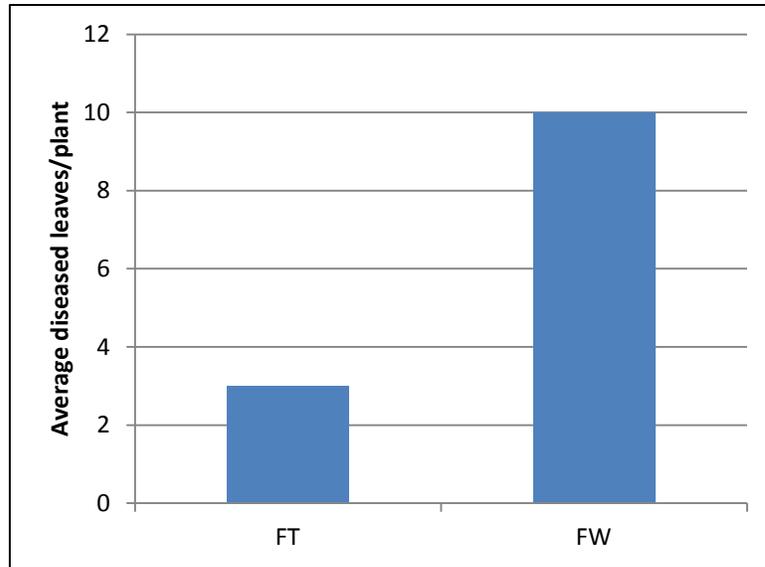


Figure 5. Severity of Pierce’s disease on transgenic Freedom grape (FT) and on wild type Freedom grape assessed in August 2012 in the Solano country trial.

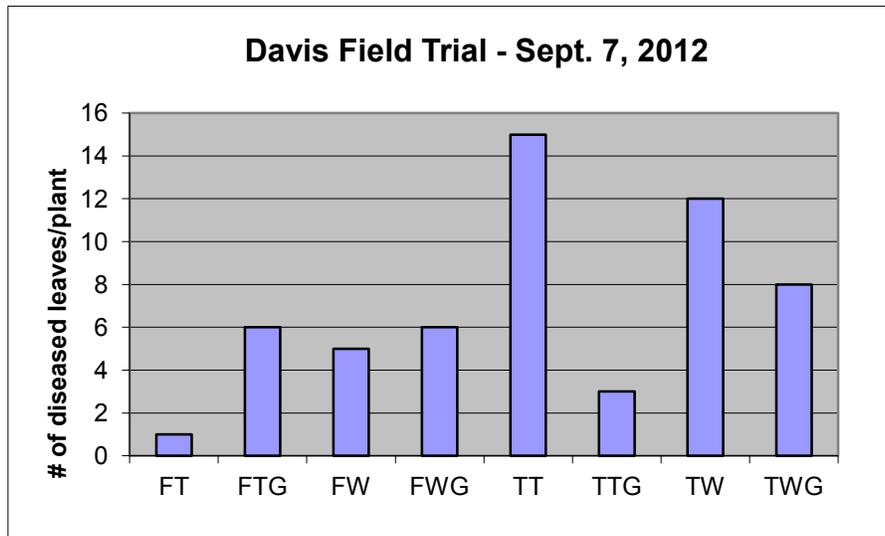


Figure 6. Severity of Pierce’s disease on grape assessed in September 2012 in the Solano country trial. See treatment codes above for treatment comparisons.

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 Country trial (**Figure 7**). The plants at the Riverside County trial 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 8**).



Figure 7. Establishment of grape trial in Riverside County in April 2010 (left) and image of plot in October 2012 (right).



Figure 8. 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 9**), 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 10**). The incidence of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson (**Figure 11**), 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 12**). The effectiveness of transgenic Thompson Seedless rootstocks in reducing Pierce's disease was surprising, given that the transgenic Thompson scions were similar in susceptibility to that of the normal Thompson 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.

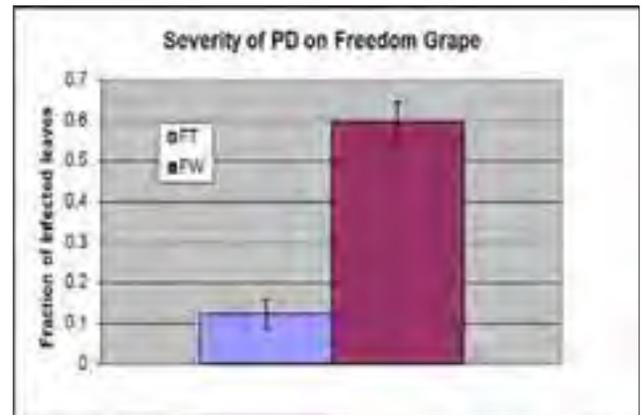
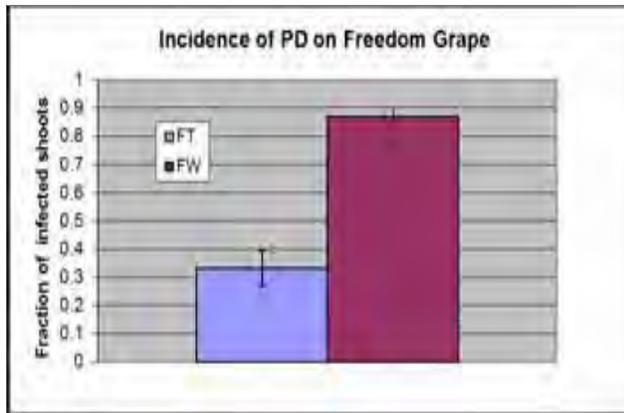


Figure 9. Incidence of Pierce’s disease of transgenic DSF-producing Freedom grape (blue bars) or wild type Freedom (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right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

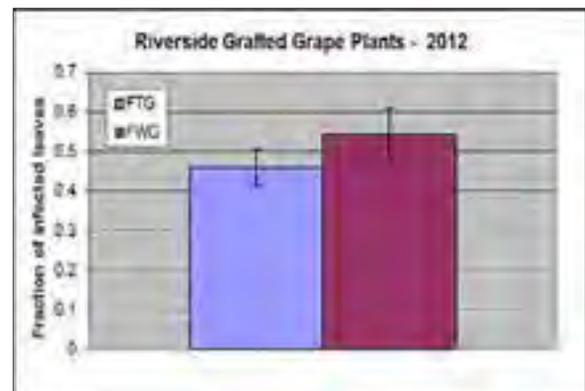
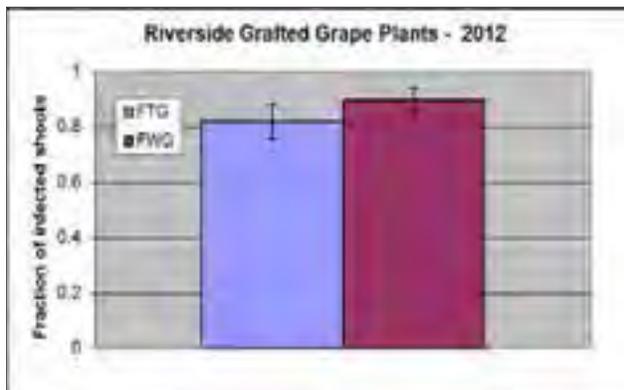


Figure 10. Incidence of Pierce’s disease of normal Thompson Seedless grape grafted onto transgenic DSF-producing Freedom grape rootstocks (blue bars) or wild type Freedom rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.

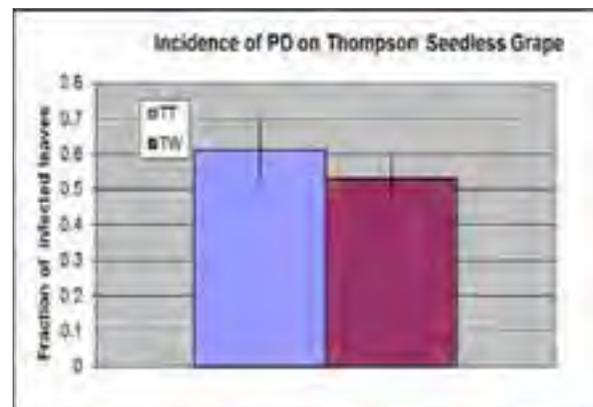
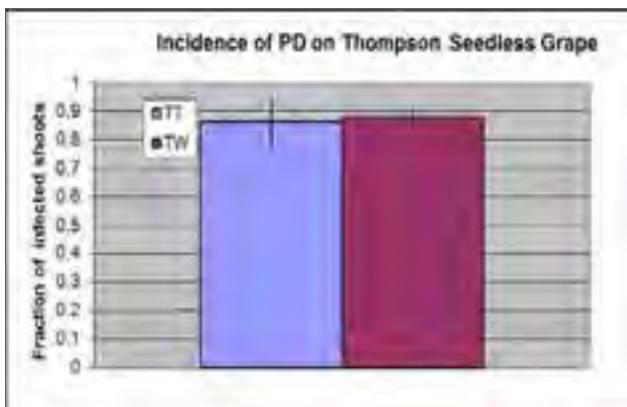


Figure 11. Incidence of Pierce’s disease of transgenic DSF-producing Thomson Seedless grape (blue bars) or wild type Thomson Seedless (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right box). The vertical bars represent the standard error of the mean.

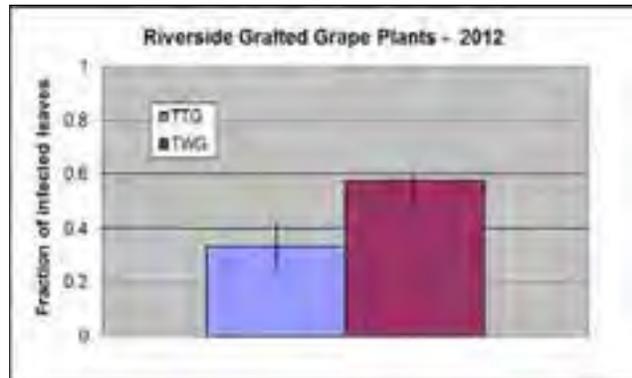
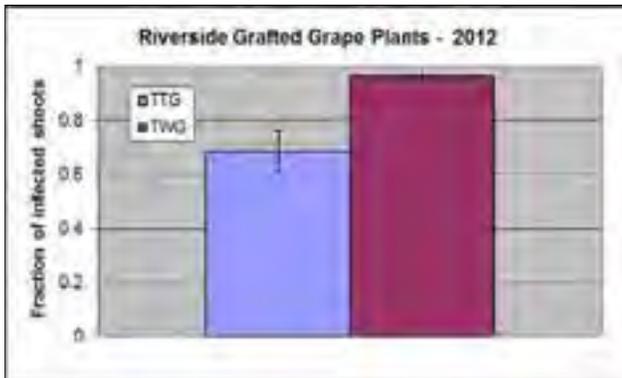


Figure 12. Incidence of Pierce’s disease of normal Thompson Seedless grape grafted onto transgenic DSF-producing Thompson Seedless grape rootstocks (blue bars) or wild type Thompson Seedless rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity 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.

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 grape, it conferred substantial disease control when used as a rootstock.

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 September 2011 through September 2012.

ABSTRACT

Experimental vineyards, some containing transgenic grapevines with Pierce's disease resistance elements inserted, were maintained for another year. Some of the test plants show Pierce's disease symptoms. The grapevines lasting through three seasons now have trunks of about one-inch in diameter.

LAYPERSON SUMMARY

This project is designed to monitor test plants in an experimental vineyard in Riverside County. The request was to treat the grapevines in a manner as nearly like a commercial vineyard as possible. The cooperating PIs will report their results separately.

INTRODUCTION

We have been monitoring grapevines in an experimental vineyard created in Riverside County. The biological materials come from laboratories at UC Davis and UC Berkeley. The oldest vineyard has been through three seasons and the younger one two seasons. We have been monitoring the presence of insects with yellow sticky traps replaced weekly and the information is shared with cooperating laboratories. We tested the mouth parts of a percentage of the insects trapped for the presence of *Xylella fastidiosa* (*Xf*) bacteria, the pathogen responsible for Pierce's disease. Insect numbers peak yearly in July and there has been an abundance of the pathogen. This continues to indicate heavy pressure for Pierce's disease.

OBJECTIVES

A major focus of Pierce's disease management includes attempts to develop grapevine varieties that are less susceptible to *Xf*. 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. Maintain the grapevines exactly as handled by commercial vineyards.
2. Monitor for pests and diseases.
3. Dispose of plants at the end of trials.

RESULTS AND DISCUSSION

The project was initiated in the spring of 2010, which included the first phase of planting. Since that time we have continued to maintain the research plot, which has included irrigation, pruning and training vines on the trellis, removal of flowers if necessary to meet compliance requirements, and occasional fertilization and fungicide application to control powdery mildew. Moreover, we have regularly inspected vines in the research plot to evaluate insect pest abundance and evidence of pathogen damage. **Figure 26** shows the percent of glassy-winged sharpshooters (GWSS) containing pathogen.

In April of 2011 the second phase of planting occurred at the site. After planting, drip irrigation and trellises were setup. Since that time we have continued to maintain and monitor the new planting in a manner that is consistent with vines from the first planting. Pierce's disease became evident in a number of the grapevines in

the vineyards during the 2012 season. Seasonal sharpshooter appearance began in June and peaked in July exactly as in previous years.



25

Figures 1-24b. Appearance of individual grapevines in one row of vineyard and sticky cards. 25 = blow up of 5.

Professor Dandekar visited his vineyard on 27 July 2012. He determined that Pierce's disease symptoms were "widespread" with only a few plants free of symptoms. He thought the thickness of the vine trunks was outstanding, some being over an inch in diameter. The vineyard to the east was planted a year later and all trunks are under an inch in diameter.

Figures 1-24 are individual images taken of the northern most row of the vineyard looking south and starting from the grapevine on the eastern side of the row. These were taken while Dr. Dandekar was present during his July visit. Three sticky cards are also shown. There are GWSS on two of them, **Figures 15b** and **24b**. The Pierce's disease symptoms Dandekar mentioned were not that obvious, so one of the images below was blown up and is shown in **Figure 25**. This is the same grapevine as shown in **Figure 5**.

Note the brown stubble present in most of the pictures between the trellises. This is the remains of barley planted to conserve moisture for the grapevines, prevent erosion, and displace weeds that might grow. On visual inspection alone it has to be considered a huge success. Without it, this time of year the vineyard is full of weeds requiring occasional plowing for removal.

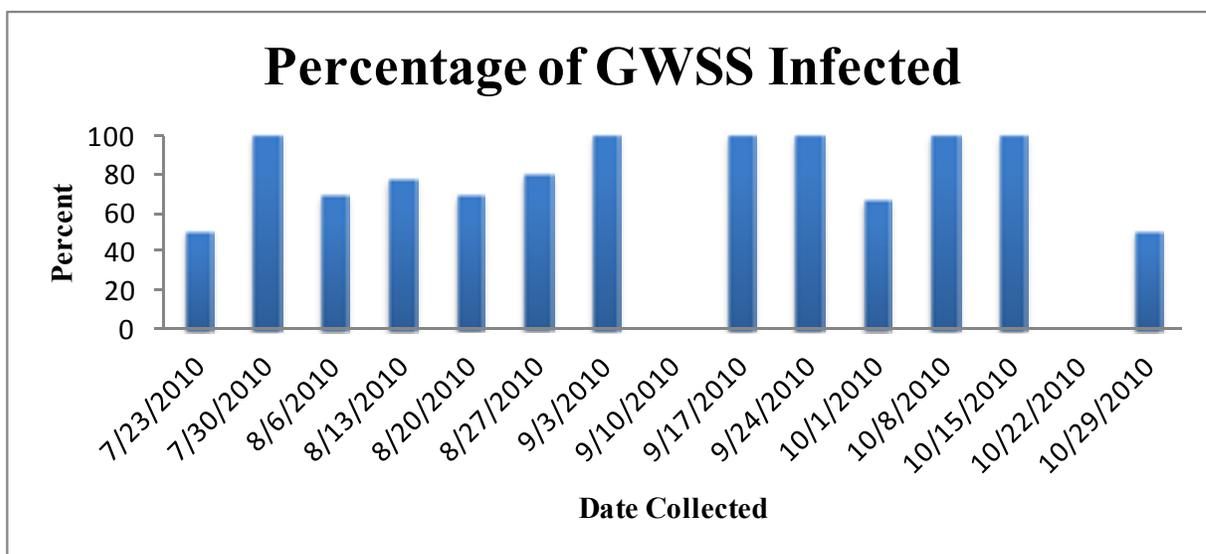


Figure 26. Percent of GWSS caught in sticky traps positive for *Xf*.

CONCLUSIONS

This project is providing support for a field trial of novel grapevine varieties that show promising reductions in their susceptibility to Pierce's disease. Over the past year we continued to maintain grapevines, and monitor for sharpshooters and disease symptoms within the initial planting in Riverside County.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and by grants from UC Riverside for undergraduate research.

TOOLS TO IDENTIFY POLYGALACTURONASE-INHIBITING PROTEINS TRANSMITTED ACROSS GRAPEVINE GRAFTS

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Reporting Period: The results reported here are from work conducted October 2011 to October 2012.

ABSTRACT

The CDFA Pierce's Disease and Glassy-winged Sharpshooter Board's Research Scientific Advisory Panel review and subsequent RFPs gave top priority to delivery of Pierce's disease control candidates, including polygalacturonase-inhibiting proteins (PGIPs), from grafted rootstocks. Four currently funded projects (two research projects and two field trials) use expression of PGIPs as a control strategy to limit the spread of *Xylella fastidiosa* in the xylem network and thereby limit Pierce's disease symptom progression in infected vines. A monoclonal antibody recognizing pear fruit PGIP (pPGIP), the protein expressed in the grape lines in the four projects, is needed to detect, quantify, and observe the localization of the protein in transformed grapevines and in grafted vines with transformed rootstocks. Authentic pPGIP protein from pear fruit is needed to prepare this monoclonal antibody which can be maintained in perpetuity as a cell culture. Production of the monoclonal antibody will begin now that sufficient quantities of properly glycosylated, active pPGIP have been purified although an alternative strategy using synthetic peptides generated from the pPGIP sequence is also being pursued to ensure that the antibodies have the greatest possible specificity and titer.

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 interrupt the flow of nutrients and water through the vessels in the plant. Plant proteins called PG-inhibiting proteins (PGIPs) selectively inhibit PGs from bacteria, fungi, and insects. Our work (Abu-Goukh et al., 1983) has identified a PGIP from pear fruits that at least partially inhibits the *Xf*PG and we demonstrated reduced Pierce's disease symptom development in grapevines expressing the pear fruit PGIP. Current projects, including field trial evaluations, require a monoclonal antibody specifically recognizing the pear fruit PGIP protein in order to detect, quantify, and characterize the PGIP protein delivered to the scion portion of grafted plants from rootstocks expressing the pear fruit PGIP (Aguero et al., 2005). The monoclonal antibody will allow the researchers to compare the amounts of the PGIP protein at different times and places and thereby determine the protein's role in *Xf*PG inhibition in grapevines. We have purified active pear PGIP from green pear fruit for commercial antibody production to meet the needs of the collaborating groups and we have set up an alternative strategy to generate more candidate monoclonal antibody candidates.

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 PG-inhibiting proteins (PGIPs) in minimizing the damage caused by pathogens and pests on plants (Powell et al., 2000), including damage caused by *Xf* in Pierce's disease. Two currently funded projects use pear fruit PGIP (pPGIP) to restrict *Xf* movement: "Optimizing grape rootstock production and export of inhibitors of *Xf*PG activity" (PI Labavitch) and "*In planta* testing of signal peptides and antimicrobial proteins for rapid clearance of *Xylella*" (PI Dandekar).

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

OBJECTIVES

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

RESULTS AND DISCUSSION

Objective 1: Purification of pear PGIP from transgenic *Arabidopsis* leaves and pear fruit

The generation of a monoclonal antibody requires purified protein to be used as the antigen. The project “Optimizing grape rootstock production of and export of inhibitors of *Xy*PG activity” (PI Labavitch) generated transgenic *Arabidopsis thaliana* plants expressing the pPGIP protein fused to a C-terminal histidine tag for purification. Leaves from these transgenic plants yielded a small amount of pPGIP protein, as determined by Bradford assays. Work to refine the purification of pPGIP protein from these plants is ongoing.

pPGIP protein extraction was done using fresh pear fruit and the protocols in Stotz et al. (1993) and Abu-Goukh et al., (1983) with modifications as described in the quarterly reports reports. **Figure 1** shows the fractions from the FPLC separation which were pooled. In **Figure 2**, the purity of the protein from the pooled fractions is shown. The yield of purified pPGIP protein is 1.7 mls (in 20 mM NaAcetate) of 115 µg/ml or a total of 195µgs protein, which should be sufficient for the generation of the monoclonal antibody by Antibodies Inc. We have contacted Antibodies Inc., and are discussing with the staff the best methods to use to generate the monoclonal antibodies.

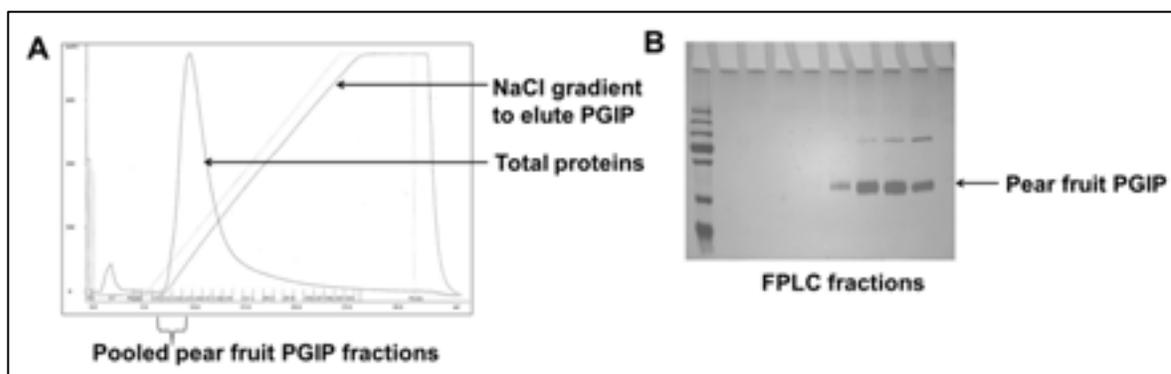


Figure 1. (A) FPLC analysis of pPGIP preparation. Proteins eluted from ConA column were applied to a cation exchange column and eluted by FPLC in 50 mM NaAcetate pH 4.5 and a 0-500 mM NaCl gradient. (B) Silver stained SDS-PAGE gel showing relative purification of PGIP. Lanes 2-10 contain various fractions from the FPLC separations. PGIP band corresponding to a molecular weight of 45 kDa is seen. A band corresponding to 90 kDa is also visible and corresponds to the molecular weight of a PGIP dimer.

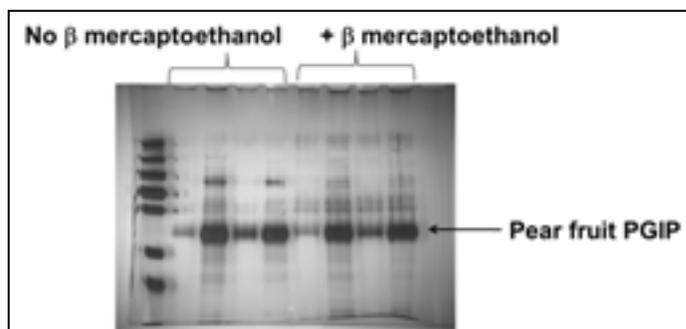


Figure 2. Silver stained SDS-PAGE gel showing pPGIP collected from cation exchange column fractions. Loading dye containing β mercaptoethanol causes a reduction of, presumably, multimeric PGIP proteins (90 kDa). The 90 kDa band in the absence of β mercaptoethanol, in the presence of β mercaptoethanol is resolved into the 45 kDa pPGIP bands. Differences in glycosylation may account for PGIP sub-bands around 45 kDa. Chemical deglycosylation of the pear PGIP was done but not evaluated on a gel.

A western blot showing the pear PGIP (pPGIP) band using polyclonal anti-pPGIP antibody is shown in **Figure 3**. The pPGIP band corresponds to a molecular weight of approximately 45 kDa, which is the expected size for pPGIP.

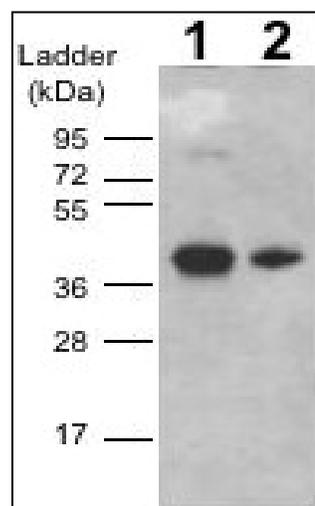


Figure 3. Immunoblot analysis of pPGIP. Lane 1: 80 ng protein after ammonium sulfate precipitation (50-100% fraction). Lane 2: 10 ng protein after the ConA purification step. Molecular weight ladder is indicated on the left. Proteins were separated on a SDS-PAGE (10%) gel and probed using pPGIP antiserum.

Inhibitor activity the pPGIP protein was measured throughout purification by radial diffusion assays (Taylor and Secor, 1988). Samples of the initial pear homogenate inhibited a PG (BcPG) mixture from *Botrytis cinerea* culture filtrates. The pPGIP purification preparations after ammonium sulfate precipitation and subsequent dialysis resulted in a 75% reduction in BcPG activity in the assay (**Figure 4**).

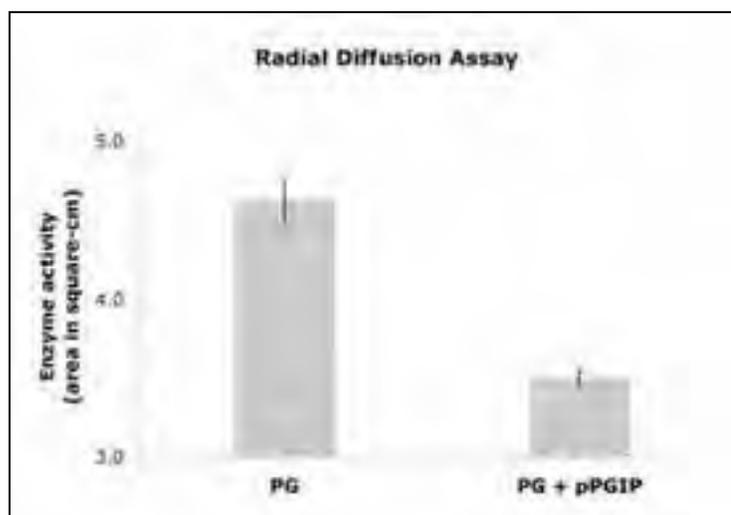


Figure 4. Results of a radial diffusion inhibition activity assay to determine the amount of pPGIP in a protein preparation from pear fruit.

During this period we have also contacted Ab-mart because they have proposed to generate multiple monoclonal antibodies for the pear fruit (p)PGIP protein. To do this they will divide the protein into smaller peptides and then develop monoclonal antibodies to each peptide. The advantage of this approach is that it is possible that antibodies recognizing unique epitopes of pPGIP as well as antibodies that recognize common features among PGIPs should be generated. The cost of doing this is about the same as developing a single monoclonal antibody through Antibodies Inc. We have provided the pPGIP amino acid sequence to Ab-mart and they are currently selecting regions of the protein to target. They will synthesize the peptides and generate the antibodies. If their progress is not acceptable we will revert to our previous plan. This opportunity is a good opportunity to leverage the CDFA-GWSS support to generate more resources for the community.

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

Will commence once the antibody has been generated.

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

Will commence once the antibody has been generated.

CONCLUSIONS

Authentic and active pPGIP protein from pear fruit has been prepared to generate this monoclonal antibody which can be maintained in perpetuity as a cell culture. An alternative strategy using synthetic peptides generated from the pPGIP sequence is also being pursued to ensure that the antibodies have the greatest possible specificity and titre. The monoclonal antibody will allow comparison of the approaches from different research groups and will allow accurate assessments of the relative amounts of the pPGIP protein achieved by the various approaches and thus will allow evaluation of the potency of pPGIP for limiting Pierce's disease symptoms.

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FUNDING AGENCIES

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

FIELD EVALUATION OF GRAFTED GRAPEVINE LINES EXPRESSING PEAR POLYGALACTURONASE-INHIBITING PROTEINS

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Reporting Period: The results reported here are from work conducted October 2011 to October 2012.

ABSTRACT

Work in this project evaluates the performance in fields in Solano and Riverside counties of grafted grapevine lines that produce in the rootstock a protein that is a candidate for control of Pierce's disease. The CDFA Pierce's Disease and Glassy-winged Sharpshooter Board's Research Scientific Advisory Panel gave priority to the delivery of polygalacturonase-inhibiting proteins (PGIPs) from grafted rootstocks to control Pierce's disease. Previously transformed 'Thompson Seedless' and 'Chardonnay' grapevines expressing a PGIP from pear fruit (pPGIP) show reduced Pierce's disease incidence when inoculated with *Xylella fastidiosa* (Agüero *et al.*, 2005). Therefore, cuttings from these grapevines have been grafted with non-pPGIP producing scions to make comparisons between the efficacy of pPGIP produced in grafted rootstocks vs. throughout the plant for Pierce's disease control.

LAYPERSON SUMMARY

Fifty-one own-rooted Chardonnay and Thompson Seedless grapevines, including those expressing pear polygalacturonase-inhibiting proteins (pPGIP), were generated by vegetative propagation, genotyped by PCR, and planted as part of a field trial in Solano County in 2010. Transgrafted (rootstocks expressing pPGIP grafted to fruit producing scions with no genetic modifications to produce pPGIP) combinations have been generated and planted as part of the Solano site in 2012. Grafted vines are being generated to add to the existing site in Riverside County. Mechanical inoculations with *Xylella fastidiosa* bacteria were done in 2011 and 2012 in Solano County but evidence of infections is being assessed.

INTRODUCTION

Grafted and non-grafted grapevines have been propagated vegetatively for Pierce's disease assessments in fields in Solano and Riverside counties. Fifty-one transgenic and control, own-rooted, grapevines were planted in Solano County on 6 July 2010. The field plantings in the plot in Solano County were severely pruned in the winter of 2011 and were pruned with standard protocols to establish primary canes and cordon positions in early spring 2012. Thirty-one grafted plants, utilizing the pear polygalacturonase-inhibiting proteins (pPGIP)-expressing vines as rootstocks or the appropriate control combinations, were planted in Solano County on 18 May 2012 and closely monitored to ensure survival throughout the dry summer season. The established, own-rooted vines were inoculated with *Xylella fastidiosa* (*Xf*) on 29 May 2012 and Pierce's disease resistance and plant growth characteristics are currently being assessed.

The grapevines transformed to express the pPGIP protein are also being analyzed in a separate project to optimize the activity, expression, and export of PGIP proteins from transgenic rootstocks to provide Pierce's disease protection in the scion portions of the vines. PGIP provides the potential for enhanced resistance to *Xf*

by inhibiting the enzyme, polygalacturonase (PG) that *Xf* uses to spread infections (Roper *et al.*, 2007). The proposal “Optimizing grape rootstock production and export of inhibitors of *Xf* PG activity” (PI Labavitch), addresses this approach. The plants with grafted rootstocks expressing pPGIP were previously only observed in greenhouse settings. The goal of this project is to verify that the transgenic grapevines expressing pPGIP as grafted rootstocks (1) have increased resistance to Pierce’s disease and (2) maintain the appropriate agronomic traits necessary for commercial release in field settings.

This field trial proposal was funded jointly with proposals from D. Gilchrist, A. Dandekar and S. Lindow. The plants from these trials have been planted at the same locations and the USDA APHIS authorizations have been handled through the Public Intellectual Property Resource for Agriculture (PIPRA).

OBJECTIVES

1. Scale up the number of grafted and own-rooted pPGIP expressing lines.
2. Plant and maintain grafted and own-rooted lines in two locations with different 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’ grapevines generated by Agüero *et al.* (2005) continue to be maintained at the UC Davis Core Greenhouses. Vegetative cuttings of non-lignified stem sections from transgenic and control plants of both cultivars have been rooted in an aeroponic cloning manifold (EZ-Clone Inc., Sacramento, CA (**Figure 1**)), as described in previous reports. These plants are referred to as “own-rooted plants.” Rooted cuttings were transferred to soil and maintained in the greenhouse.



Figure 1. Grafted grapevine cuttings rooting in the EZ-Clone aeroponic device manifold.

Since reusing the EZ-Clone tanks during the second season was problematic as the foam plugs were a source of contamination, a new system using different media combinations has been utilized beginning in mid-2011.

Grafted plants were made by green grafting rootstock stem sections with budded scion material as described in previous reports. As of 10 October 2012, 44 grafted plants and an additional 8 grafts (by grafting budded scions onto rooted plants) are currently in 1 gal. pots in greenhouses. The current inventory of potted grafted plants are: 14 Chardonnay trans-grafts (Chardonnay pPGIP expressing rootstock grafted with Chardonnay scion not expressing pPGIP), 3 Thompson Seedless transgrafted plants (Thompson seedless pPGIP expressing rootstock grafted with Thompson seedless scion not expressing pPGIP), 16 Chardonnay null-transformant control grafted plants (Chardonnay rootstock not expressing pPGIP grafted onto Chardonnay scion not expressing pPGIP), 4 Thompson seedless null control grafted plants (non-transformed Thompson seedless rootstock grafted onto Thompson seedless scion not expressing pPGIP), and 7 Thompson seedless pPGIP expressing control grafted plants (Thompson seedless expressing pPGIP rootstock grafted onto Thompson seedless expressing pPGIP scion). DNA was prepared from the vines used as source tissue for grafting and tested genotype confirmed by PCR (**Figure 2**). In addition to the grafts listed previously, 31 grafted plants were generated, confirmed, and transferred to the Solano field site in May 2012. Although about 50% of the plants needed for the population for the Riverside site have been generated but because of powdery mildew and mealy bug infestations in our greenhouses at UC Davis grafting efforts continue to complete the population grafting work continues.

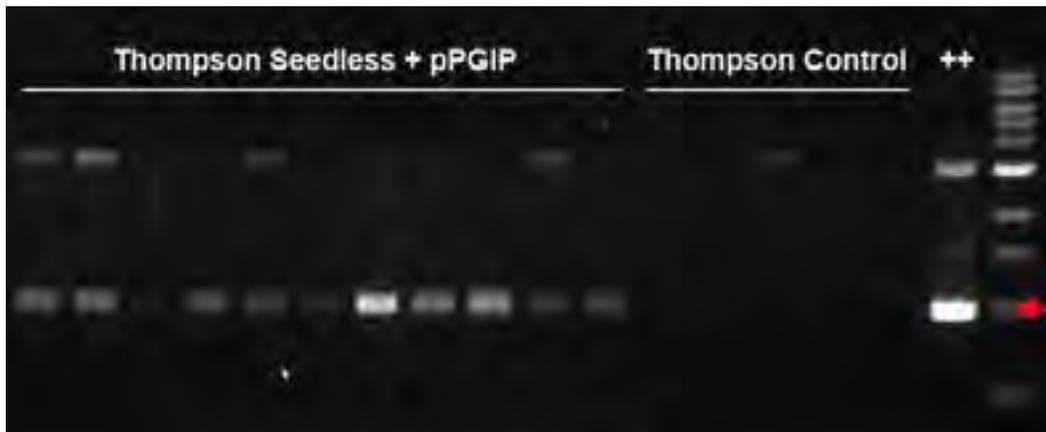


Figure 2. Sample genotyping PCR of grape leaf tissue from Thompson Seedless vines expressing pPGIP and null-transformed (no pPGIP) controls used to generate the transgrafted vines planted in May 2012. A 1 kb band (arrow) corresponding to pPGIP is expected only in samples used as rootstocks for transgrafts and pPGIP self-grafted controls. Each sample's quality was verified by amplifying a control fragment (not shown).

Objective 2: Establish field trial sites

Two field trial sites in Solano and Riverside counties are being used to assess the Pierce's disease resistance and general agronomic viability of own-rooted and grafted pPGIP expressing grapevines. The field sites are shared by projects testing Pierce's disease resistance of other transgenic grapevines from PIs D. Gilchrist, A. Dandekar, and S. Lindow. The vines satisfying our initial PCR analysis in 2010 for our portion of the field trial were hand-planted in a randomized block design with blocks consisting of two or three individuals in the same treatment in July 2010 (**Table 1**). Thirty-one grafted plants, either utilizing the pPGIP expressing material as rootstocks or the appropriate control graft combinations, were prepared as described above and hardened in a lath house for two months prior to planting in Solano County in May 2012. These younger, grafted plants were surrounded by protective grow tubes and hand-watered every two weeks or as needed. The grapevines are planted approximately 8 ft. apart and tied to wooden stakes with trellising wires at 40 in. and 52 in. Their growth during the 2012 growing season has been vigorous (**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 to M. Szczerba by the USDA Biotechnology Regulatory Services in March 2012, we have been able to allow flowers and fruiting clusters to persist. All own-rooted Chardonnay vines were cordon trained and spur pruned whereas the majority of the Thompson Seedless vines were cane pruned in an attempt to maintain proper vine balance and ensure fruit development. The Solano field site has been under weekly observation for the duration of the growing season.



Figure 3. Examples of vines in the field in March 2012 (left) and August 2012 (right). *Xf* mechanical inoculation sites are marked with tags.

Table 1. Total number of grapevines planted in, and prepared for, Solano County. Dashed shapes represent pPGIP expressing grapevine rootstocks and/or scions; solid shapes are null-transformant controls (no pPGIP). Own-rooted vines were inoculated on 7/21/2011 and 5/29/2012; grafted vines have not been mechanically inoculated. Grafting in progress numbers include all grafted cuttings at each checkpoint. Parentheses indicate vines that have not yet been planted in the field.

	Grafting Strategy	Chardonnay			Thompson Seedless		
							
Own-Rooted Plants (#)	Inoculated	17	-	(9)	8	-	9
	Non-Inoculated	8	-	(4)	4	-	5
Grafted Plants (#)	Inoculated	9	5 (4)	(9)	6 (3)	9	7 (2)
	Non-Inoculated	4	(4)	(4)	(4)	4	(4)
Grafting In Progress (#)	EZ-Clone	0	0	0	3	0	0
	Potted	16	14	0	4	3	7

Objective 3: Evaluate relevant agronomic traits of vines in two locations

The grapevines planted in Solano County have been monitored for general health and maintained on a weekly basis. With the permit amendment mentioned above, agronomic trait analyses are being discussed. Any measurements of fruit number or quality will be made later in the fall 2012.

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 mechanically inoculated with *Xf* Temecula on 21 July 2011, to monitor Pierce’s disease incidence during the late summer 2011. No visual evidence of Pierce’s disease infection was observed throughout the 2011 growing season or in the early 2012 months following bud break. The same 34 own-rooted vines were resubmitted to mechanical inoculations on 29 May 2012 with a mixture of *Xf* Temecula and Stags Leap strains (3:2, v:v). Young, green tissue was chosen for inoculation with 3-4 canes chosen per plant. Mechanical inoculations were performed as in 2011 except that approximately 1.5×10^7 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. An example of the match-stick response seen in Pierce’s disease infected vines is shown in **Figure 4**.



Figure 4. Typical match-stick symptoms of Pierce’s disease infected grapevines.

CONCLUSIONS

All of the grafted plants necessary for the study in Solano County have been generated and about 50% of the plants for the site designed for Riverside County are in greenhouse pots. The genotypes of the grafted plants were confirmed by PCR analysis of DNA from the plants. An initial attempt to infect the vines in Solano County was made but not symptoms were observed. A second attempt in 2012 is currently being evaluated. The results of the field evaluation will confirm that delivery of the pPGIP from rootstocks provides a means of controlling Pierce's disease and *Xf* infection in a typical vineyard setting in California. The evaluations of the performance and productivity of the plants will 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.

PUBLICATIONS

Haroldsen VM, Szczerba MW, Aktas H, Lopez-Baltazar J, Odias MJ, Chi-Ham CL, Labavitch JM, Bennett AB and Powell ALT (2012). Mobility of transgenic nucleic acids and proteins within grafted rootstocks for agricultural improvement. *Frontiers in Plant Science* **3**:39, Published 2 March 2012.

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FUNDING AGENCIES

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

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 *Xf* when introduced in grapevines cuttings before planting in vineyards. In a greenhouse experiment in 2012, we introduced all nine putative biocontrols into grapevines cuttings cv. 'Merlot' and then challenged them with *Xf*. We are currently evaluating the ability of these biocontrol agents to mitigate Pierce's disease symptoms development and decrease *Xf* titer *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 had previously identified one compound (compound 'R'), as an effective inhibitor of *Xf*. This year we tested the ability of the compound 'R' to cure Pierce's disease infected grapevine cv. 'Chardonnay' in a greenhouse bioassay. We are currently rating the plants for symptoms development and *Xf* titer. In addition, we are currently identifying the chemical structure and activity produced by the remaining seven fungal biocontrol candidates. 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 involve 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 8 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 one compound inhibitory to the bacterium in an *in vitro* assay. This year we have injected this compound in Pierce's disease infected grapevine and are currently monitoring the plants to see if we have a reduction in symptoms development and bacterial titer. 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 monocyclic 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 identify fungi inhabiting grapevine and fungal natural products antagonistic to *Xf* that could be used as a preventive or curative treatment for Pierce's disease, respectively. 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 for inhibitory activity against *Xf* in greenhouse assays on grapevine cuttings. If successful, we envision that these control strategies can be implemented at the nursery level (for biocontrols) or directly in the field (for the fungal 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 isolated fungi inhabiting these samples, and identify these specimens to the genus level by comparing the ribosomal DNA sequences to specimen posted in the GenBank database. We tested the ability of all the organisms recovered to inhibit *Xf* growth using an *in vitro* bioassay (Rolshausen and Roper, 2011), which did allow us to select a total of nine biocontrol candidates (eight fungi and one bacterium).

These organisms were subsequently re-introduced in grapevines cuttings 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/ μ l. Grape 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 were inoculated with *Xf* (Temecula strain) by mechanical needle inoculation (Hill and Purcell, 1995). A sub-sample of plants was left un-inoculated with *Xf* to determine if the concentration of fungal spore treatment used is detrimental itself to the grape cuttings. Plant symptoms were rated from 0 to 5 every two weeks (0 = no symptoms; 5 = Plant dead or dying) according to Guilhabert and Kirkpatrick (2005) (**Figure 2**).

We are currently rating the progression of the disease in the biocontrols-infiltrated ‘Merlot’ cuttings. At the end of the experiment, we will also quantify the bacterial titer *in planta*. Petioles will be harvested, surface-sterilized and ground in 1X PBS buffer. The grindate will be serially diluted and plated on PD3 medium and allowed to incubate at 28°C for 10 days. Following incubation, colonies will be enumerated and normalized to petiole weight to give a measure of CFU (colony forming units)/g tissue.



Figure 1. Technique used to vacuum infiltrate grape cuttings with spores of the fungal endophytes.



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.

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 fungal 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 μ l of the *Xf* cell suspension are added to 3 ml of PD3 medium containing 0.8% agar and briefly vortexed. This mixture is then overlaid 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 28°C for 7 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* was 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 28°C for 7 days. Following this, plates are 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 (**Figure 4**).

Currently, we have identified eight fungal taxa and one bacterium that provided good inhibition of *Xf* growth *in vitro*, either directly when co-cultured with *Xf* or indirectly when *Xf* was grown in the presence of the fungal natural products. We are currently testing the potential of these fungi and this bacterium as biocontrol agents *in planta* (see **Objective 1**). We are currently fractionating the crude extracts from these fungi in order to purify and identify the inhibitory molecules. Thus far, we have purified one individual molecule that is active against *Xf* growth *in vitro* and characterized its chemical structure. 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.

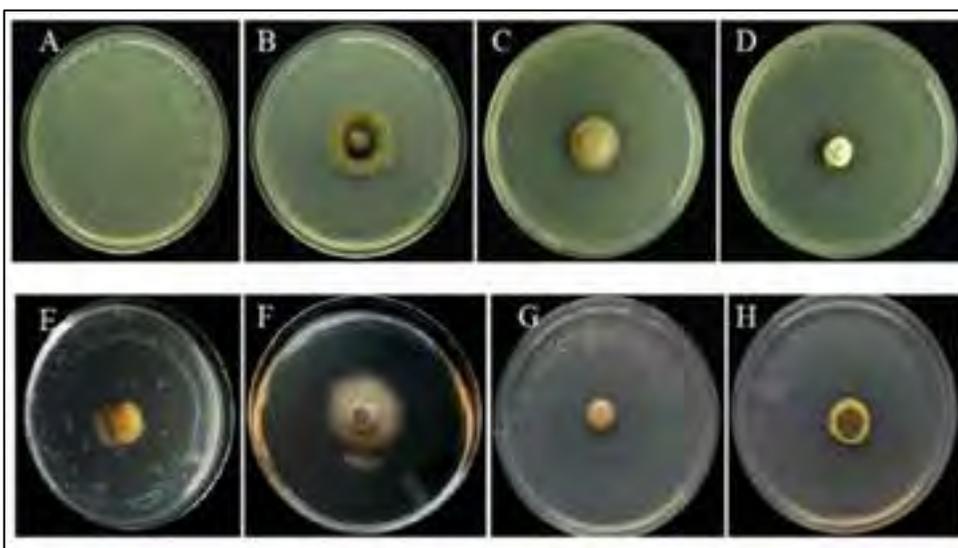


Figure 3. *In vitro* inhibition assay used to evaluate fungal activity towards *Xf*. A) *Xf*-only Control; *Xf* cells were plated in top agar and agar plugs containing fungi were placed on top. Following 8 days of incubation, several fungi strongly inhibited *Xf* growth as indicated by the large zone of inhibition around the fungal colony (Panels C, D, E, G, and H). Two fungi completely inhibited the growth of *Xf* (Panels B, F).

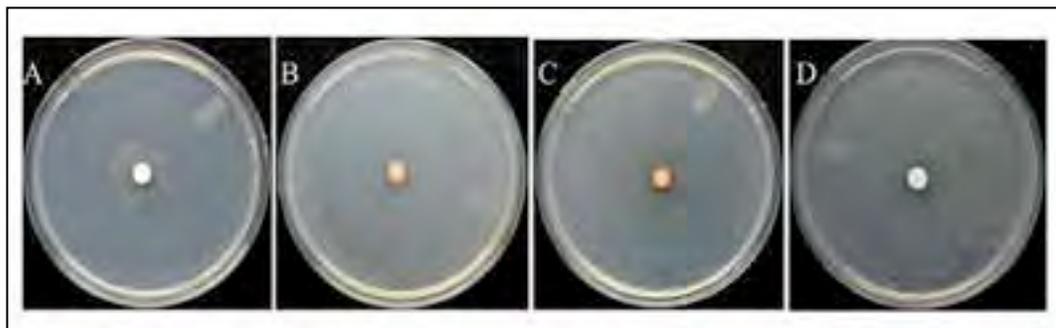


Figure 4. *In vitro* inhibition assay used to evaluate crude ethyl acetate extracts from culture supernatants of fungi. These extracts strongly inhibit *Xf* growth at concentrations of 1 mg/ml as indicated by either a complete lack of growth (Panel A) or a large inhibition zone around the paper

disc containing the crude extracts (Panel B, C). Panel D indicates the negative control disc that contained methanol only. No inhibition zone was observed for the negative control.

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 in order 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 control Pierce's disease.

We have currently identified one fungal natural compound (molecule 'R') as an active molecule inhibitory to *Xf* (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). In 2012 our greenhouse trial was designed to test the molecule 'R' 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 'R.' Grapevines cuttings cv. 'Chardonnay' were infected with *Xf* (Temecula strain) by mechanical needle inoculation (Hill and Purcell, 1995). The molecule 'R' was injected after appearance of the first Pierce's disease symptoms (rating of 1 or 2 on Pierce's disease rating severity chart **Figure 2**), which was 8 weeks post-inoculation. Molecule 'R' was extracted from a 3-week-old fungal culture grown in 250 ml of PD broth as previously described (**Objective 2**). Molecule 'R' (80% pure) was suspended in pure di-methyl sulfoxide (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 1ml 16 gauge needle. Injection was performed in the xylem tissue of the grape cutting right below the shoot (**Figure 5**). We are currently recording the progression of Pierce's disease severity so no data are available at this point.

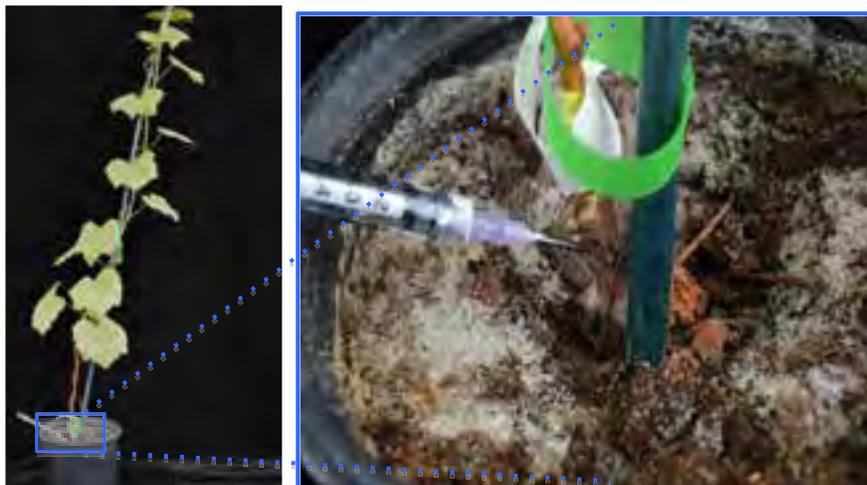


Figure 5. Needle-injection of an anti-*Xf* molecule in the xylem of Pierce's disease infected grapevine cuttings.

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. 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 assessing the efficacy of 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 are currently developing 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 one active anti-*Xf* compound that we are currently testing in the greenhouse. The next step is to discover additional active

natural anti-*Xf* compounds and evaluate their efficacy in greenhouse experiments with Pierce's disease infected grapevines. 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

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

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

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ABSTRACT

The goal of this research is to evaluate the potential of a non-transgenic, Pierce's disease resistant *Vitis vinifera* selection used as an experimental rootstock to confer systemic resistance to Pierce's disease susceptible *V. vinifera* scions. The source of Pierce's disease susceptible plant material was the winegrape variety 'Chardonnay,' known to support high populations of *Xylella fastidiosa* (*Xf*) and exhibit severe Pierce's disease symptoms. The source of Pierce's disease resistant material was a modified backcross generation 2 (mBC2) raisin selection with Pierce's disease resistance locus *PdRI* introgressed from 89-F0908 (*V. rupestris* x *V. arizonica*). Scions were mechanically inoculated with *Xf* strain Stags Leap. Pierce's disease 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 Pierce's disease 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 of the first year experiment indicated that Pierce's disease symptom expression and *Xf* titer in 'Chardonnay' scions were not significantly different when grafted onto Pierce's disease susceptible or Pierce's disease resistant rootstocks. Thus, the preliminary answer to the question posed in the title is "no." Replication of the experiment in the second year is in progress.

LAYPERSON SUMMARY

Pierce's disease 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 Pierce's disease, introgression into each will be time consuming and costly. In this research, proof of concept experiments were conducted in greenhouse trials to determine if Pierce's disease resistance in a *V. vinifera* selection used as a rootstock may be translocated to susceptible *V. vinifera* scions. Results of the first year experiment indicate that a Pierce's disease susceptible scion grafted onto a Pierce's disease resistant rootstock remains susceptible to Pierce's disease.

INTRODUCTION

This project describes pilot experiments designed to test the hypothesis that a Pierce's disease resistant rootstock can affect Pierce's disease 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 Pierce's disease symptoms in susceptible scions. The *V. vinifera* selection used as an experimental rootstock bears the *PdRI* resistance locus introgressed from 89-F0908 (*V. rupestris* X *V. arizonica*) that is known to confer high levels of Pierce's disease resistance via reduction of *Xf* population levels (Buzkan et al., 2005; Krivanek et al., 2006; Riaz et al., 2009). Further, xylem sap from *PdRI* plants reduces growth of *Xf* in culture (Cheng et al., 2009). As the *PdRI* resistant rootstock used in these experiments is a second generation backcross with a genetic composition of ~87.5% *Vitis vinifera*, difficulties encountered by Lin and Walker (2004) in establishing sound graft unions between *V. vinifera* scions and rootstocks derived from wild *Vitis* species were avoided. The simple experimental design can determine whether or not the *PdRI* resistance factor(s) is (are) capable of systemic protection of tissues beyond the graft union to affect pathogenesis of *Xf* in susceptible scions.

OBJECTIVE

1. Determine effect of rootstock genetic background (+/- *PdRI*) on Pierce's disease severity and *Xf* population levels in Pierce's disease susceptible scions following challenge inoculation of scions with *Xf*.

RESULTS AND DISCUSSION

The basic experimental design evaluated Pierce's disease symptom development and *Xf* population levels in Pierce's disease susceptible scions grafted onto rootstocks that are either resistant or susceptible to Pierce's disease. (**Table 1**). The first year experiment was conducted in a greenhouse at the USDA ARS SJVASC facility in Parlier, CA. The source of Pierce's disease susceptible plant material was the wine grape variety 'Chardonnay,' known to support high populations of *Xf* and exhibit severe Pierce's disease symptoms (Buzkan et al., 2005). The source of Pierce's disease resistant material (Ramming and Walker, 2010) was a modified backcross generation 2 raisin selection (referred to here as PDR1) with Pierce's disease resistance locus *PdRI* (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 Pierce's disease resistant rootstocks on Pierce's disease susceptible scions following challenge inoculation of scion with *Xf*.

Rootstock		Scion		Expected scion response to Pierce's disease
Variety	Pierce's disease response	Variety	Pierce's disease response	
'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 Pierce's disease symptomatic grape in California and is known to cause severe Pierce's disease symptoms in inoculated plants (Hendson et al., 2001). Plants were mechanically inoculated above the graft union (e.g., scions) using a 20 μ l 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 one-third of the width of the shoot. Plants were maintained in the greenhouse and evaluated visually for Pierce's disease symptoms after 14 weeks. Pierce's disease 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 Pierce's disease remained unaltered, regardless of rootstock genotype. 'Chardonnay' scions expressed similar levels of Pierce's disease severity on both Pierce's disease susceptible (mean severity rating 3.2) and Pierce's disease resistant rootstocks (mean severity rating 3.6), whereas PDR1 scions expressed only mild symptoms on Pierce's disease susceptible (mean severity rating 1.0) or Pierce's disease resistant (mean severity rating 1.4) rootstocks. Pierce's disease 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.

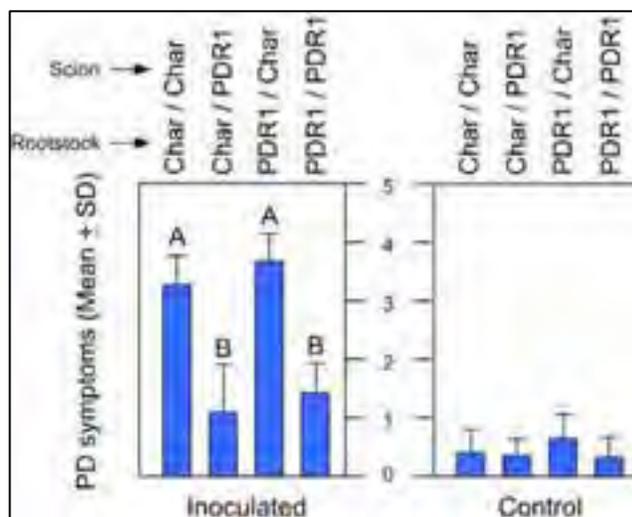


Figure 1. Pierce's disease symptom ratings (0 = no disease, 5 = death) for scions at 14 weeks post inoculation. Means (\pm standard deviation) with different letters are significantly different ($P < 0.05$) based on a non-parametric rank sum test. Char ['Chardonnay'] (Pierce's disease susceptible); PDR1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

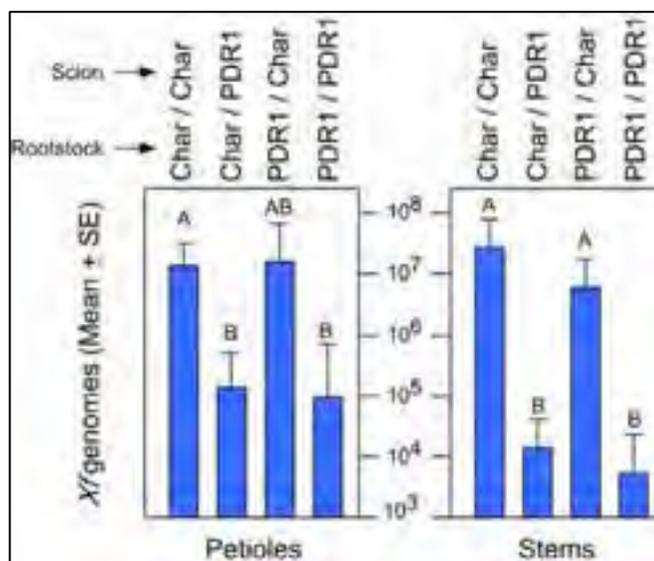


Figure 2. Estimation of *Xf* titer in scions 14 weeks post inoculation. Results of quantitative real time PCR are presented as the equivalent number of *Xf* genomes present in petiole or stem samples taken ~25 cm above the point of inoculation. Values refer to mean \pm standard error for entire petioles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

CONCLUSIONS

Based on one year of data (the experiment is being repeated in year 2), it is unlikely that *PdR1* germplasm will be suitable for development of commercial rootstocks that confer systemic resistance to existing Pierce's disease susceptible grape varieties used as scions. Nonetheless, *PdR1* scions remained resistant when grafted onto a Pierce's disease susceptible rootstock. Therefore, new table, raisin, and wine grape varieties bearing *PdR1*-mediated Pierce's disease resistance likely may be deployed as scions onto existing Pierce's disease susceptible

rootstocks. If verified, this observation may facilitate use of *PdR1* resistance, deployed as Pierce's disease resistant scions. However, low bacterial population levels (but greater than zero) in Pierce's disease resistant material used as scions could eventually allow for systemic movement of *Xf* into Pierce's disease susceptible rootstocks. Thus, Pierce's disease 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 Pierce's disease resistant rootstocks to complement efforts already in progress on development of Pierce's disease resistant scions.

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FUNDING AGENCIES

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

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 2012.

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. A number of novel *Xf* proteins with predicted signal sequences for secretion were investigated. An *E. coli* model system and/or *Xf* itself was used to confirm secretion for the majority of tested proteins. Next, a tobacco rattle virus (TRV) vector was used to express these proteins and examine effect on pathogenicity of TRV in *Nicotiana benthamiana*. Of 58 proteins assayed, eight were shown to reproducibly and significantly increase the virulence of TRV, implying they function as pathogenicity factors in *Xf*. Currently, mutations are being constructed in each of the eight candidate pathogenicity factors in *Xf*; once knock-out strains are available, disease assays in grapevine will be performed.

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 June 2012-September 2012.

ABSTRACT

This report describes results achieved since June 2012. First, we continued to characterize the role of a *Xylella fastidiosa* (*Xf*) chitinase (*chiA*), which allows the bacterium to use chitin as a carbon source. A *chiA* mutant was deficient in its ability to colonize *Xf* vectors. Second, we tested several serine protease mutants for their ability to be transmitted by insect vectors; some of these mutants were affected in vector transmission. Lastly, we summarized our efforts to identify chitin-binding proteins, and have prepared proteins for transmission experiments to test if they function as specific blocking molecules that disrupt *Xf* transmission. Those results are pending.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*), the etiological agent of Pierce's disease of grapevines and other diseases, is transmitted from plant to plant by xylem-sap feeding insects. Management of such diseases relies heavily on pesticide applications mainly due to a poor understanding of their biology. Improving our knowledge on mechanisms used by this bacterium during its transmission by vectors will lead to the development of new and environmentally sound strategies to control Pierce's disease. Because *Xf* colonization of vectors is a complex process, we decided to focus our attention on several steps required for efficient transmission. Blocking initial adhesion of *Xf* on insect cells and reducing the ability of the bacteria to successfully colonize insect mouthparts are two of the most promising approaches. Previous findings on the nature of *Xf*-insect interactions, using bacterial lectins recognizing insect receptors on its cuticle, allowed us to demonstrate that a blocking technology is a feasible approach to disrupt pathogen transmission. Identification of specific proteins of *Xf* involved in these interactions will improve the specificity of this technology allowing us to test it under field conditions in the near future. We are now waiting for results of ongoing greenhouse experiments that are testing our strongest candidates obtained through in vitro research.

INTRODUCTION

This report is divided into two sections: i) search for proteins implicated in the transmission of *Xylella fastidiosa* (*Xf*) including work conducted on the characterization of a chitinase mutant, the transmission of several serine proteases mutants and the identification of chitin-binding proteins, and ii) construction of recombinant proteins to use them as competitors to block *Xf* transmission by vectors under greenhouse conditions. A brief introduction will be provided for each section as they focus on different approaches. Results for some transmission experiments done under greenhouse conditions this summer are pending, especially those involving recombinant proteins.

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: Identification of targets implicated in *Xf* transmission by insects

Xf chitinase is important for 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) and all the machinery required to assimilate it (Killiny et al., 2010) leads us to further test if *Xf* could use chitin as a carbon source for successful colonization of vectors.

A chitinase mutant (*chiA*) is not able to grow with chitin as a sole carbon source

To test our hypothesis, we constructed a chitinase mutant (*chiA*) and tested its growth in media that differ in their composition in carbon sources. Four media were used: XFM, which is composed of two carbon sources (disodium succinate and sodium citrate); XFM Δ , where the two carbon sources of the XFM medium were omitted; XFM-chitin, where colloidal chitin was added as an additional (third) carbon source; and XFM Δ -chitin, where chitin was present as the sole carbon source. Optical density at 600nm corresponding to *Xf* growth was determined every day over an 8-day period and results were compared to the wild-type (WT) strain (Temecula) grown into same media.

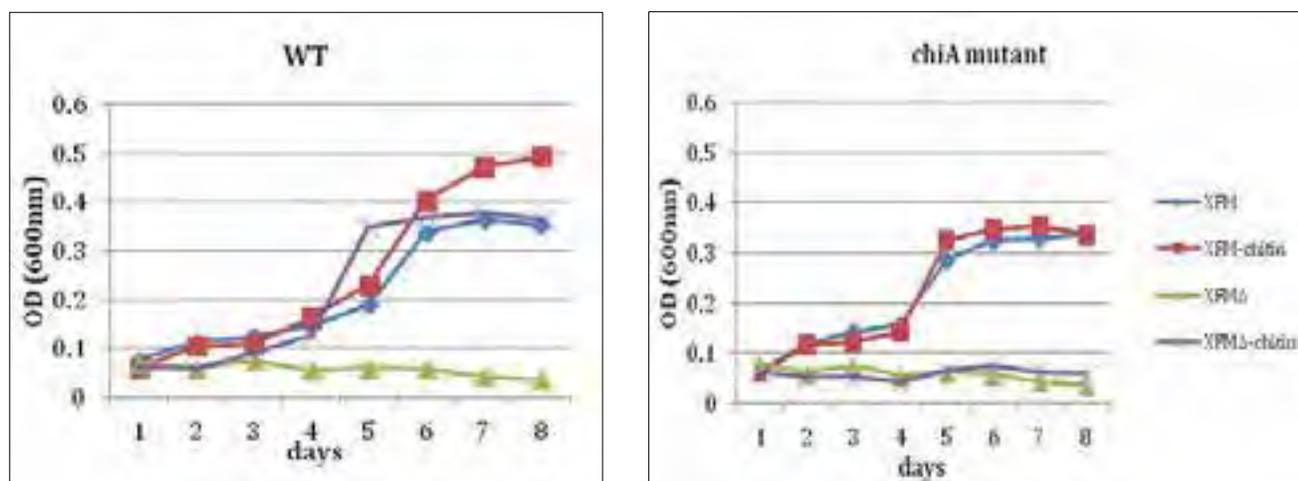


Figure 1. Growth comparison between WT and *chiA* mutant for their ability to use chitin as a carbon source.

First, no differences were found in the ability of both bacteria to grow in XFM (blue curves) meaning that the absence of ChiA doesn't have any effect on the capacity of *Xf* to grow when other carbon sources were available in the medium. As expected, no growth was observed into XFM Δ medium where no carbon sources were present. WT cells grew to larger populations in XFM-chitin than in XFM confirming results previously found (Killiny et al., 2010) but such increase in bacterial population was not observed for the *chiA* mutant. Interestingly, when colloidal chitin is present as the only carbon source in the medium (XFM Δ -chitin), only the WT is able to grow. These results confirmed our hypothesis that *Xf* has a functional chitinase allowing it to assimilate chitin as a carbon source.

The *chiA* mutant is affected in its vector colonization

The capacity of the *chiA* mutant to successfully colonize plants is yet to be determined (results currently pending). To address the role of chitin utilization during insect colonization, we used a protocol based on an artificial diet system that eliminates the need of using infected plants as infection source (Killiny et al., 2009). Briefly, insects acquired the *chiA* mutant or WT cells from this artificial diet system, with cell suspension between two Parafilm layers for 4 hours. Insects were removed from these sachets and transferred to basil plants for cells to colonize insects. Three and 10 days after acquisition, insects were recovered from basil plants, for each of these two time periods, insects were separated into two numerically identical populations. One of these populations (n=12) was tested with quantitative PCR (qPCR) to quantify bacterial population in the vectors' foregut. Remaining insects were individually transferred on healthy grapes to test their ability to transmit *Xf* to another plant. Results for this latter experiment are still pending but those concerning qPCR quantification are summarized in figure 2 below.

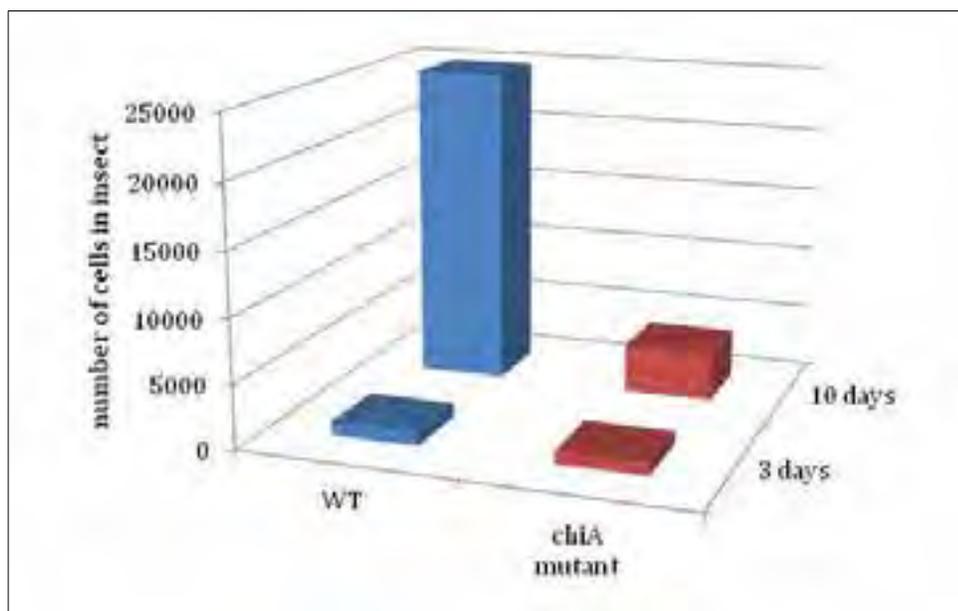


Figure 2. Comparison of insect colonization between WT and *chiA* mutant at 3 and 10 days post-acquisition.

At three days post-acquisition, bacterial populations of the *chiA* mutant were not different from that of the control ($P>0.1$). At 10 days post-acquisition a significant reduction in bacterial population were observed for the *chiA* mutant compared to the control ($P<0.001$). This difference indicates that the *chiA* mutant cannot successfully colonize insects. Indeed, from three days to 10 days after acquisition, a 25-fold increase in population size was observed for the control ($P<0.001$), whereas bacterial populations only slightly increased (3 to 5-fold, $P<0.05$) for the *chiA* mutant.

Altogether, we suggest that the *chiA* mutant is deficient in insect colonization because of it cannot use chitin as a carbon source. Results from transmission experiments conducted with these insects will determine if chitin utilization during insect colonization is relevant for the transmission of *Xf*. Additional experiments are still require to understand in detail the chitin degradation process, in particular if ChiA is able to bind and degrade chitin polymers by itself or if other(s) protein(s) help it in this task.

Effect of serine proteases on *Xf* transmission

As previously mentioned, chitin has a primordial role in *Xf* transmission by vectors. However, many important questions remain unclear. During early colonization of insect mouthparts, how does *Xf* reach chitin polymers to adhere to insects? Due to the composition of the cuticle of insects (**Figure 3**), *Xf* may not have direct access to chitin polymers when it first attaches to the insect's precibarium.

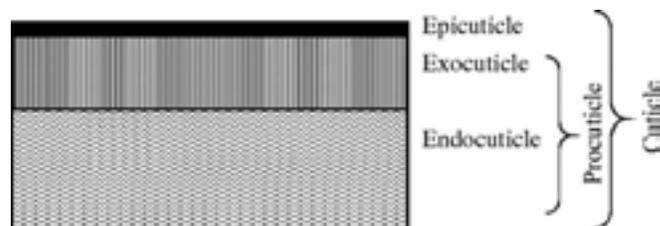


Figure 3. Composition of insect cuticle.

Cuticle is composed of different layers. The epicuticle is the outermost part of it, its function is to reduce water loss and block the invasion of foreign matter. It is composed of lipoproteins and chains of fatty acids embedded in a protein-polyphenol complex. This very thin layer is free of chitin polymers. Microfibers of chitin are contained in the procuticle, surrounded by a matrix of protein that varies in composition from insect to insect and even from place to place within the body of a single insect. We note that chitin has served well as a proxy for attachment experiments as a proxy for compounds in the epicuticle for many bacterial systems, we found that it also functions well for *Xf*. The difficulty is that we don't know exactly what the outermost layer of the cuticle looks like in sharpshooters, and that is what *Xf* initially binds to. Thus, chitin appears to work well as a proxy for this layer, but we are also trying to understand these early stages of colonization in more detail.

In order to colonize its insect vectors, *Xf* probably needs to degrade this first barrier composed by the epicuticle. *Xf* has proteases with similarities to known S8 subtilisin-like serine proteases named PD0218, PD0313, PD0950. Multiple functions have been associated with these proteases but among the most interesting ones is the possibility that *Xf* insect colonization is related to the ability of these enzymes to degrade the insect cuticle. The best understood model is based on a fungal endoprotease designated Pr1 (St.Leger et al., 1995), which degrades the cuticle of its insect hosts as a key step for fungal attachment on chitinous surfaces leading to the initiation of the infection process.

As a first step in the confirmation of a role for these enzymes in *Xf* insect colonization, we tested different mutants for these proteins kindly provided by Dr. Igo's group with our artificial transmission system (as previously described). Mutations carried by each of these mutants as well as transmission rates obtained for those tested are summarized in the **Figure 4** (Nt: non tested).

Due to an impossibility to reach adequate populations on plates for transmission experiments, some strains were not tested. According to the results presented in **Figure 4**, differences between the potential role of the three proteases are highlighted. First, no differences were observed between transmission rates for wild-type and TAM147 meaning that PD0218 has no effect in insect colonization. According to previous work conducted by Dr. Igo's group, PD0218 could potentially have a role in the maturation of a bacteriocin, molecules known to increase the competitiveness of a pathogen (2010 Proceedings). On the contrary, transmission rates were significantly lower for the two other strains tested. Indeed, transmission rate for TAM150 was only 50%, so a reduction of 30% approximately in comparison to the control. Based on that, we can argue that, contrary to PD0218, PD0313 has an effect in the transmission of *Xf* by vectors. The triple-mutant (TAM153), deleted in all three proteases, showed a highly reduced transmission rate close to 30% confirming the role of PD0313. In addition, due to this significant difference between these two last transmission rates (50% vs 30% for TAM150 and TAM153 respectively), we speculate that PD0950 also has a role in bacterial transmission, potentially in collaboration with PD0313.

Strain	Mutation(s)	Number of plants tested positive
Temecula	Wildtype	13/16
TAM147	PD0218::Cm ^R	12/14
TAM152	PD0313::Gm ^R	Nt
TAM146	PD0950::Em ^R	Nt
TAM148	PD0218::Cm ^R , PD0950::Em ^R	Nt
TAM150	PD0218::Cm ^R , PD0313::Gm ^R	8/15
TAM151	PD0313::Gm ^R , PD0950::Em ^R	Nt
TAM153	PD0218::Cm ^R , PD0313::Gm ^R , PD0950::Em ^R	4/14

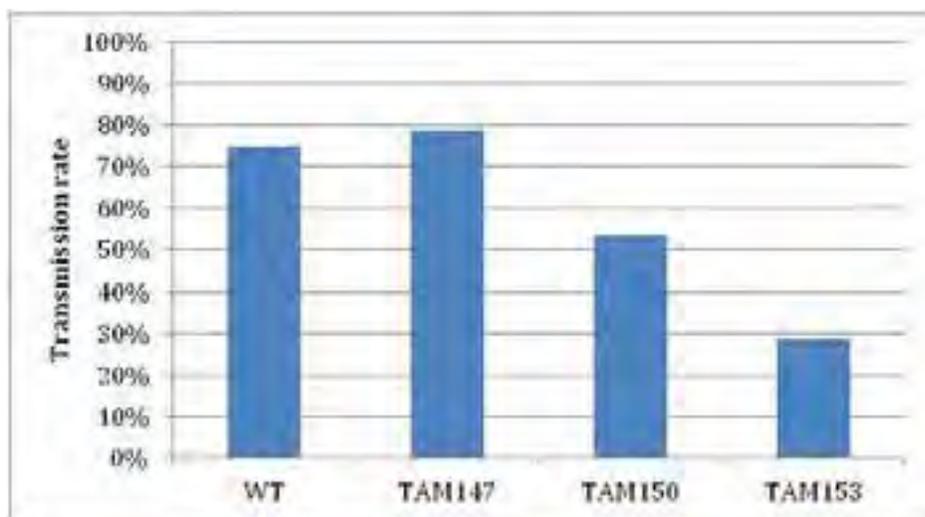


Figure 4. Transmission rates obtained for mutants tested in our experimental transmission system.

These encouraging results will allow us to move forward our analysis concerning these proteases, especially PD0313 and PD0950. Additional experiments will be necessary to test their activities *in vivo* and to confirm our hypothesis on the role of these enzymes on insect cuticle degradation.

Identification of chitin-binding proteins

As previously mentioned, chitin has a central role in *Xf* transmission. *Xf* binds to and degrades chitin *in vitro*, *Xf* binds to the chitinous cuticular surface of the foregut of sharpshooters *in vivo*, and chitin's subunit (GlcNAc) disrupts *Xf* transmission to plants. In that way, we used chitin as a proxy for sharpshooter vectors in order to identify *Xf* proteins able to bind to the chitin. We believe that these chitin-binding proteins could be good candidates for adhesins involved in an interaction with insect receptors.

Briefly, we grew cells under conditions that allow *Xf* to colonize vectors, extracted total proteins and purified those adhering to chitin. Utilization of whole cell extract proteins has the advantage of potentially identifying most of the chitin-binding proteins. However, additional washings steps were required to obtain a specific elution profile. We also repeated these assays for conditions that do not induce *Xf* transmission (Killiny et al. 2009b). Good correlation was found between proteins specifically bound to chitin and the medium inducing transmissibility. In other words, no proteins specifically bound to chitin were found for samples from a medium (XFM) poorly capable of inducing *Xf* transmission. The protocol described above allowed us to specifically elute ten bands of *Xf* proteins using N-acetylglucosamine (GlcNAc) as a specific competitor. Unfortunately, due to incompatibilities between proteins concentrations after trypsinization and the limit of detection of the mass spectrometer used for proteins identification (UC Davis facility), subsequent analyses enable us to only identify two new candidates out of the ten bands. Efforts are currently made to circumvent this limitation.

Nevertheless, mass spectrometry analyses allowed us to identify two proteins in proteins bands located at 30kDa and 42kDa named respectively MopB (PD1709) and PD1764 based on the genome of the *Xf* Temecula strain. First, MopB is the major outer membrane protein of *Xf*, distributed over the surface of the bacteria (Bruening et al., 2005). This property gives to this protein a particular interest in the interaction of *Xf* with insect cuticle, and chitin in particular. On the other hand, as a major outer membrane protein, MopB is also abundant in *Xf* proteome compare to others proteins. Identification of abundant proteins at this point requires additional verification in order to confirm the specificity of this binding. The other candidate, PD1764, is a hypothetical protein conserved in several *Xf* strains. BlastP analyses showed a highly conservation rate with a LysM containing-domain protein, present in several species of *Xanthomonas* (99% of identities). LysM (for lysin motif, Pfam PF01476) domains are especially known for their role in plant immune responses where they can serve as receptors for the recognition of common microbe associated molecular patterns (MAMPs) (for a review, Gust et al., 2012). Interestingly, those MAMPs are generally composed of N-acetylglucosamine (GlcNAc)-containing molecules and LysM motifs have been shown to recognize and bind to numerous of those compounds, such as chitin of pathogenic fungi (Ohnuma et al., 2008). More recently, Visweswaran et al. (2012) found that the LysM motif of the well-known bacteria *Lactococcus lactis* was able to bind to fungal chitin cell wall material. Thus, we think that PD1764 could be a highly interesting candidate for interactions of *Xf* with chitin. To our knowledge, this is the first example of an *Xf* protein containing an already described chitin binding domain.

Objective 2: Test specific and efficient blocking molecules to disrupt vector transmission

The central idea of this objective is that *Xf* colonization of insects can be disrupted by molecules that bind receptors on the sharpshooter foregut, the site of pathogen retention within vectors. These molecules will essentially compete with *Xf* for receptors on the vector; if those are masked the cells are not capable of attaching to and colonizing vectors, effectively disrupting transmission and Pierce's disease spread. As a result of intensive research conducted these past few years in our laboratory, we demonstrated that blocking *Xf* transmission following that way is feasible and could reasonably lead to control disease spread (Killiny et al., 2012). The current goal is to develop this strategy with specific and more efficient molecules identified during previous conducted research. Based on the assumption that *Xf* proteins we have identified as important for sharpshooter colonization could be used to block transmission to plants, several targets have been constructed and tested this summer. This is the case of previously implicated candidates for *Xf* transmission by vectors Hxfs, XadA, ChiA and FimA. In addition, because of its high interest, PD1764 was added to this study.

Production of His-tagged recombinant proteins

Several constructs were generated (see **Table 1** for a complete list) for proteins candidates to block *Xf* transmission. All were constructed following the same strategy. Full-length proteins or peptides were cloned into pET28b+ (Novagen) and expressed as N-terminal His-fusion peptides using *E. coli* Rosetta strain. An exception has been done for hemagglutinin constructs which were cloned into pET30b+ (provided by B. Kirkpatrick group). Proteins were induced for four hours at 28°C under shaking with 1mM IPTG. *E. coli* cells were lysed and loaded on top of a Ni-NTA column. After several washes, protein elution was made with increasing concentration of imidazole, a specific competitor of histidine residues for nickel. After a desalting step in order to eliminate imidazole, His6-tag was removed by the action of a protease (thrombin), which specifically cleaves between the histidine tag and the protein thanks to a specific cleavage sequence. Thrombin was added to the protein suspension (five units NIH per mg of protein) at RT for two hours. Action of the protease was stopped by addition of 1mM of PMSF.

The table below summarizes proteins targeted in this study and current status in order to test these molecules as competitors for disrupting *Xf* transmission by insect.

Proteins Steps	ChiA	XadA	FimA	HxfB			PD1764		
				AD1-3	AD4	AD5	PD1764-1 (full-length)	PD1764-2 (without LysM domain)	PD1764-3 (LysM domain only)
PCR amplification	✓	✓	✓	✓	✓	✓	✓	✓	✓
Cloning	✓	✓	✓	✓	✓	✓	✓	✓	✓
Sequence verification	✓	⚠	✓	✓	✓	✓	✓	✓	✓
Expression test	✓		✓	✓	✓	✓	✓	✓	✓
Purification (Ni-NTA column)	✓		✓	✓	✓	✓	✓	✓	✓
Tag excision	✓		✓	✓	✓	✓	✓	✓	✓
Blocking experiment	✓		✓	✓	✓	⚠	✓	✓	⚠
Recovering <i>Xf</i> from petioles	⚠		⚠	⚠	⚠		⚠	⚠	

Except for XadA, for which we encountered cloning difficulties, others constructions were successfully produced. Unfortunately, protein concentrations for HxfB-AD5 and PD1764-3 recovered after protein purification and tag excision did not allow us to test these two constructs in our *in vivo* artificial diet system. All six others constructs were tested as transmission-blocking molecules this summer, results are pending.

Transmission experiments with blocking recombinant proteins

Prior to test these blocking molecules in our artificial diet system, we tested the impact of different concentrations of cells on the transmission rate. Our goal was to select the optimal concentration of cells that result in a good transmission rate (>70%) without using too many cells, resulting in an increased risk of non-specific transmission. To do that, we allowed insects to acquire *Xf* on diet solutions containing different populations of *Xf* cells. Acquisitions with populations from 10^2 cells/ml to 10^9 cells/ml were performed. After this four-hour acquisition period, insects were individually transferred to a single leaf on a healthy plant and were allowed to inoculate the bacterium for 24 hours. One month later, the presence of *Xf* was checked in each plant by culturing the petiole from leaves on which insects were placed. **Figure 5** summarizes results of this experiment.

Bacteria concentration (cells/ml)	Number of plants tested positive
10^2	0/21
10^4	0/20
10^6	3/22
10^8	15/19
$5 \cdot 10^8$	17/22
10^9	16/19

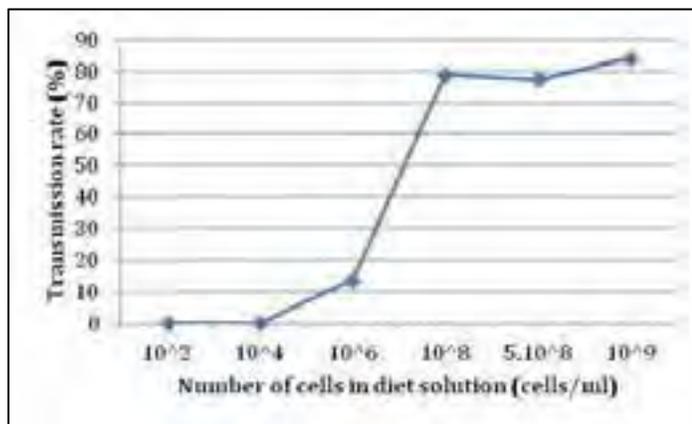


Figure 5. Effect of different *Xf* populations on the transmission rate.

Interestingly, the relationship between transmission rate and number of cells in the system was not linear; 10^6 cells/ml was the first concentration of cells with detectable transmission (3/22, 13,6%). On the contrary, high transmission rates (>75%) were detected for the 3 highest population levels. Based on criteria mentioned above, 10^8 cells/ml was the concentration selected for all our future experiments.

In parallel, survival of insect acquiring different concentrations of recombinants proteins was also tested under greenhouse conditions. Briefly, insects were allowed to feed on a diet solution containing 10^8 *Xf* cells/ml complemented with 250mM, 100mM, 50mM, 25mM or 10mM of recombinant protein for four hours. Such experiments have been conducted for PD1764 full-length, ChiA and FimA. Insects were immediately transferred to basil plants and their survival assessed everyday over a four-day period. This time is sufficient in our case because all our experiments have a limited time (four hours of acquisition followed by 24 hours transmission). During these four days, no death was observed for any of the three proteins or any of the five concentrations.

The same experiments were conducted to assess the effect of all the constructs listed in **Table 1** (with the exceptions of XadA, HxfB-AD5 and PD1764-3) as blocking molecules for *Xf* transmission. The same five proteins were tested and results for *Xf* transmission to grapes are currently pending. We will start soon to culture plants for pathogen presence and will soon better idea is this strategy is working using recombinant proteins and if one of the candidates used here could be an interesting target for the optimization of a field applicable approach.

CONCLUSIONS

This project is proceeding very well. Additional *Xf* mutants were tested for their vector transmissibility, adding to a large list that includes most of the available mutant strains produced by all researchers working with this pathogen. Additional mutants were tested but not included in this report. We also showed that chitin utilization is essential for successful vector colonization by *Xf*. We have now shown that several mutants are affected in vector colonization, similarly to observations made with plant infections, demonstrating that vector colonization is as complex as plant colonization. More importantly, we selected candidates from this list to test if specific recombinant proteins block *Xf* transmission to plants. Several of these candidate proteins have now been produced and experiments performed under greenhouse conditions are ongoing. Results from these experiments will be available for our next report.

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BREEDING PIERCE'S DISEASE RESISTANT TABLE AND RAISIN GRAPES AND THE DEVELOPMENT OF MARKERS FOR ADDITIONAL SOURCES OF RESISTANCE

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Reporting Period: The results reported here are from work conducted September 2011 to September 2012.

ABSTRACT

A total of 10 (6 table and 4 raisin) seedless x seedless crosses to develop mBC3, mBC4, and mBC5 *Vitis arizonica* x *V. vinifera* families were made. The crosses consisted of 44,187 emasculations and produced 4,974 ovules and 871 (17.5%) embryos for Pierce's disease resistance. An example of increased fruit quality is the propagation of three Pierce's disease resistant table grape selections in 27 vine and five Pierce's disease resistant raisin selections in 7 vine production trials. A total of 16 crosses to combine powdery mildew and Pierce's disease resistance were made and consisted of 47,347 emasculations, 4,712 ovules and 675 embryos. An additional 10 seeded x seedless crosses of 11,680 emasculations were made. From 2011 crosses, 1,232 leaf samples from plants still in test tubes were sent to University of California (Walker) laboratory for molecular marker screening. A total of 505 had resistant markers flanking the PdR1 locus and were planted in the field. The use of molecular markers to select for Pierce's disease resistance and greenhouse screening to select for powdery mildew resistance allows selection of these two types of resistance before planting to the field. Three raisin grape selections with dual resistance were selected and propagated in seven vine production trials. Cuttings of 102 parents and selections from *V. arizonica* populations were sent to University of California (Walker) laboratory for greenhouse tests to insure resistance continues to co-segregate with markers. Two hundred and forty-three seedlings of the BD5-117 mapping family, with Pierce's disease resistance different than *V. arizonica*, have been greenhouse tested. An additional 42 primers were tested on the 144 original seedlings to increase saturation of the 19 linkage groups. Last year's data indicated a major QTL on linkage group 2 (LG2). Of the 112 polymorphic primers screened to date, six are located on LG2.

LAYPERSON SUMMARY

Although Pierce's disease has existed in California since the late 1800s, the introduction of the glassy-winged sharpshooter to California in the late 1990's significantly increased the spread and damage caused by Pierce's disease. A collaborative breeding program was started in 2000 to develop Pierce's disease resistant table and raisin grapes with high fruit quality comparable to that existing in markets today. Sixth generation (mBC5) crosses to produce quality table and raisin grapes with *V. arizonica* source of Pierce's disease resistance were made again this year. These families will have high fruit quality as they consist of 98.5% *V. vinifera*. An example of increased fruit quality is the propagation of three Pierce's disease resistant table grape selections in 27 vine and five Pierce's disease resistant raisin selections in 7 vine production trials. These selections show some commercial potential. Crosses to combine powdery mildew and Pierce's disease resistance were also made. The use of molecular markers to select for Pierce's disease resistance and greenhouse screening to select for powdery mildew resistance allows selection of these two types of resistance before planting seedlings to the field. Three raisin grape selections with dual resistance were selected and propagated in seven vine production trials. Pierce's disease resistance of over 185 parents and selections from *V. arizonica* populations have been verified in greenhouse tests to insure the molecular markers used to select for Pierce's disease resistance continue to function properly. Cuttings of 102 parents and selections from *V. arizonica* populations were sent to University of California (Walker) laboratory for greenhouse tests to insure resistance continues to co-segregate with markers. Two hundred and forty-three seedlings of the BD5-117 mapping family, with Pierce's disease resistance different than *V. arizonica*, have been greenhouse tested. An additional 42 primers were tested on the 144 original seedlings to increase saturation of the 19 linkage groups. Last year's data indicated a major QTL on linkage group 2 (LG2). Of the 112 polymorphic primers screened to date, six are located on LG2. These

selected molecular markers from linkage LG2 might be useful as markers to select for resistance from BD5-117 source of resistance. This collaborative research between USDA Agricultural Research Service, Parlier and University of California, Davis has the unique opportunity to develop high quality Pierce's disease resistant table and raisin grape cultivars for the California grape industry where Pierce's disease could restrict the use of conventional table and raisin grape cultivars.

INTRODUCTION

Pierce's disease has existed in California since the late 1800s when it caused an epidemic in Anaheim. A number of vectors for Pierce's disease already exist in California, and they account for the spread and occurrence of the disease. The introduction of the glassy-winged sharpshooter to California in the 1990's significantly increased the spread and damage caused by Pierce's disease. Other vectors exist outside California and are always a threat. All of California's commercially grown table and raisin grape cultivars are susceptible to Pierce's disease. An effective way to combat Pierce's disease and its vectors is to develop Pierce's disease resistant cultivars so that Pierce's disease epidemics or new vectors can be easily dealt with. Pierce's disease resistance exists in a number of *Vitis* species and in *Muscadinia*. Pierce's disease resistance has been introgressed into table grape cultivars in the southeastern United States, but fruit quality is inferior to *V. vinifera* table grape cultivars grown in California. No Pierce's disease resistant raisin grape cultivars exist. Greenhouse screening techniques have been improved to expedite the selection of resistant individuals (Krivanek et al. 2005, Krivanek and Walker 2005). Molecular markers have also been identified that make selection of Pierce's disease resistant individuals from *V. arizonica* even quicker (Krivanek et al. 2006). The USDA Agricultural Research Service (ARS) grape breeding program at Parlier, CA has developed elite table and raisin grape cultivars and germplasm with high fruit quality. Embryo rescue procedures for culturing seedless grapes are being used to quickly introgress the seedless trait with Pierce's disease resistance (Emershad et al. 1989). This collaborative research gives the unique opportunity to develop high quality Pierce's disease resistant table and raisin grape cultivars for the California grape industry.

OBJECTIVES

1. Develop Pierce's disease resistant table and raisin grape germplasm/cultivars with fruit quality equivalent to present day cultivars.
2. Develop molecular markers for *Xylella fastidiosa* (*Xf*)/Pierce's disease resistance in a family (SEUS) other than *V. arizonica*.

RESULTS AND DISCUSSION

Objective 1

Ten (six table and four raisin) seedless x seedless crosses were made in 2012 to develop modified backcross 3 (mBC3), mBC4, and mBC5 *V. arizonica* (Pierce's disease resistance) x *V. vinifera* families. They consisted of 44,187 emasculations, and produced 4,425 berries, 4,974 ovules, and 871 embryos (17.5% embryos/ovules) (**Table 1**). This is a lower number of cross combinations than 2011 but a similar number of embryos were developed. The seedlings obtained from these crosses should have high fruit quality as they now have 93.8 to 98.5% *V. vinifera* in their background. An additional 16 crosses to pyramid Pierce's disease (*V. arizonica*) resistance with powdery mildew (*V. romanetii*) resistance consisting of 47,347 emasculations produced 4,358 berries, 4,712 ovules and 675 (14.3%) embryos (**Table 1**). Ten seeded table grape crosses were made in 2012 to combine Pierce's disease plus powdery mildew resistance (**Table 2**). Leaves from all *V. arizonica* PdR1 plants produced from 2011 crosses were taken when seedlings were still in test tubes starting in November 2011. They were tested for resistance with molecular markers for the PdR1 locus on chromosome 14. Results for Pierce's disease seedless x seedless families are shown in **Table 3**. A total of 494 individuals were tested with SSR markers and 457 showed markers on both sides of the PdR1 region with 230 (50%) being resistant. This is very similar to the ratio of resistant and susceptible plants obtained for over 1,600 F1, mBC1, mBC2 table and raisin seedlings reported by Riaz et al. 2009. The Pierce's disease + powdery mildew seedless x seedless families are also shown in **Table 3**. A total of 263 were tested and 67 individuals were resistant. The number of seedlings tested with molecular markers from seeded x seedless crosses made in 2011 is shown in **Table 4**. The ratio of resistant to susceptible is about 1:1 which is as expected. The susceptible and recombinant individuals were discarded, making more efficient use of greenhouse and field space. From the crosses made in 2011 which

combined Pierce's disease resistance from *V. arizonica* with powdery mildew resistance from *V. romanetii*, 45 seedlings with molecular markers for Pierce's disease resistance and powdery mildew resistance in the greenhouse were planted to the field. Inoculation of plants with *Xylella* in the Greenhouse (method of Krivanek et al. 2005, Krivanek and Walker 2005) was started to determine resistance of 102 selected individuals from *V. arizonica* (**Table 5**). Thirteen of 61 are resistant to date. These seedlings represent the best table and raisin selections that have been used as parents or planted in production trials. Greenhouse testing is absolutely necessary to make the final decision about resistance of individual selections. The highest level of resistance is being obtained from *V. arizonica* and its use will continue to be emphasized.

The spring of 2012, three table grape and five raisin selections with Pierce's disease resistance were propagated in 27 and 7 vine production trials respectively. After evaluation of all fruiting Pierce's disease seedlings in 2012, three raisin selections have been identified for propagation in seven vine production trials and eight selections in two vine plots in 2013. Fruiting characteristics of these selections are shown in **Table 6**. Three raisin selections with resistance to Pierce's disease and powdery mildew were propagated into seven vine production trials this spring. Another three raisin selections were identified this year for propagation into seven vine trials and one for two vine trials. Half of the planting (four of eight replications) of 12 Pierce's disease resistant selections at the USDA ARS research station, Weslaco, Texas were inoculated with *Xylella* on July 15, 2011 with the help of David Appel, Texas A&M University. Each plant was inoculated twice. Leaf samples were collected the fall of 2011 but no *Xylella* found. As of October 1, 2012, no Pierce's disease symptoms have been identified on the inoculated shoots.

Objective 2

The Pierce's disease resistant grape selection BD5-117 from Florida was hybridized with the seedless table grape selection C33-30 to create the mapping population of over 500 individuals. Fruit samples have been taken from all seedlings for three years data on berry weight and seed/seed trace weight as an indication of fruit quality. Flower sex as female or hermaphrodite was determined this spring. One hundred twenty-two individuals were evaluated in the greenhouse by inoculating with *Xylella* this year. Only seven were resistant (**Table 5**). Greenhouse testing for Pierce's disease resistance has been completed on 243 individuals, with 112 rated clearly resistant or susceptible. Fifty-one useful markers from the first 70 polymorphic markers tested on 144 seedlings with greenhouse Pierce's disease resistance evaluations were run in JoinMap and Map QTL which indicated a major QTL on linkage group 2. This is the same linkage group that flower sex is located on. Thirty-two useful markers from forty-two additional polymorphic primers tested on the 144 seedlings have been identified with JoinMap. They will help refine the framework map. All linkage groups are covered with useful primers with one to 13 per linkage group. Six primers have been located on linkage group 2. Thirteen primers were located on linkage group 14, which contains PdR1 resistance locus in *V. arizonica* but not in the BD5-117 germplasm.

CONCLUSIONS

Additional families for the development of Pierce's disease resistant seedless table and raisin grape cultivars have been produced in 2012. Emphasis was placed on making mBC3, mBC4, and mBC5 *V. arizonica* Pierce's disease resistant families. These families will have high fruit quality as they consist of 93.8 to 98.5% *V. vinifera*. The use of molecular markers has simplified and sped up the identification of Pierce's disease resistant individuals from *V. arizonica*. Seedless table and raisin grape selections with Pierce's disease resistance and improved fruit quality have been selected from both mBC4 and mBC3 *V. arizonica* and F1 and mBC1 BD5-117 families. For example, three table and five Pierce's disease resistant table and raisin selections were planted in 27 and 7 vine production trials respectively. Two hundred and forty-three seedlings from the BD5-117 family have been evaluated in greenhouse inoculations for Pierce's disease resistance. A rough framework map with 83 primers on 144 individuals has been developed. A major QTL for resistance is indicated on linkage group 2 which also contains flower sex. The development of Pierce's disease resistant table and raisin grape cultivars will make it possible to keep the grape industry viable in Pierce's disease infested areas. Molecular markers will greatly aid the selection of Pierce's disease resistant individuals from SEUS populations.

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Table 1. 2011 table and raisin grape Pierce's disease resistant seedless crosses that set fruit and the number of ovules and embryos produced.

Female	Male	Type	No. Emasculations	No. Berries Opened	No. Ovules	No. Embryos
89-0908 <i>V. rupestris</i> x <i>V. arizonica</i>						
Y128- 71--05	08-5001-21R	Table BC4	4979	470	475	84
Y537-168--06	08-5001-21R	Table BC4	5079	726	852	180
Y537-168--06	08-5001-34R-R	Table BC4	2231	312	445	116
09-5013-091	08-5001-21R	Table BC4	5613	517	817	160
09-5013-118	Y139- 31--08	Table BC5	6320	395	546	112
09-5064-022	Y129-161--05	Table BC5	5378	480	569	58
07-5059-194R-R	Y143- 98--04	Raisin BC3	3 Bags ^z	0	0	0
09-5056-072R	07-5051-09R-R	Raisin BC3	4410	772	255	22
A 50- 91	07-5052-109R-R	Raisin BC4	5141	347	505	99
09-5063-018R	08-5055-04R-R	Raisin BC4	5036	406	510	40
Total			44,187	4,425	4,974	871
Powdery mildew resistance combined with 89-0908 <i>V. rupestris</i> x <i>V. arizonica</i>						
05-5501-69R-R	Y308-311--06	Table BC3	4 Bags ^z	163	72	0
05-5501-69R-R	Y308-314--06	Table BC3	5030	542	472	10
05-5501-69R-R	Y315-55--04	Table BC3	2 Bags	34	12	0
05-5502-15R-R	Y308-311--06	Table BC3	5612	461	595	163
05-5502-15R-R	Y308-314--06	Table BC3	5957	280	336	34
Y310- 34--09	09-5013-075	Table BC4	2035	110	162	42
Y315-415--04	08-5001-21R	Table BC4	7608	172	88	9
Y315-415--04	08-5001-34R-R	Table BC4	1270	21	18	2
07-5051-028R-R	Y309-166--09	Raisin BC3	3773	373	472	28
07-5059-194R-R	Y308-344--06	Raisin BC3	1 Bag	0	0	0
09-5056-072R	Y315-264--08	Raisin BC3	2816	650	583	99
Y302-178--06	07-5051-09R-R	Raisin BC4	5572	786	1053	96
Y308-344--06	08-5505-04R-R	Raisin BC3	3100	15	10	0
09-5066-097R	09-6054-006	Raisin BC4	2551	337	269	29
09-5066-103R	09-6054-006	Raisin BC4	2023	414	570	163
Total			47,347	4,358	4,712	675

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

Table 2. 2012 seeded crosses of Pierce's disease resistant table grape combined with powdery mildew resistance.

Female	Male	Type	No. Emasculations	No. Seed
89-0908 <i>V. rupestris</i> x <i>V. arizonica</i>				
05-5502-32	Y308-314--06	Table BC3	440	- ^y
07-5060-134	Y308-344--06	Table BC3	4 bags ^z	-
07-5060-134	Y309-397--06	Table BC3	3 bags	-
07-5059-093	Y308-314--06	Table BC3	4 bags	-
05-5501-06	Y308-281--06	Table BC3	5 bags	-
05-5501-06	Y308-312--06	Table BC3	1 bags	-
05-5501-06	Y308-314--06	Table BC3	4 bags	-
05-5501-26	Y308-314--06	Table BC3	7 bags	-
05-5501-06	Y308-311--06	Table BC3	4172	-
05-5501-06	Y308-314--06	Table BC3	6412	-
Total			28 bags +11,024	-

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

^y Seed not extracted from fruit as of 10-9-12.

Table 3. Determination of seedling resistance based on PdR1 molecular markers for seedless x seedless 89-0908 families made in 2011.

Family	Type Cross	No. Resistant ^a	No. Susceptible ^b	No. Recombinant ^c	No Data ^d	Off Types	Total
Pierce's disease families							
11-5001	Table BC1	9	9		1	1	20
11-5002	Table BC3	1			1		2
11-5003	Table BC4	8	3	1			12
11-5004	Table BC3	8	6	3	1		18
11-5006	Table BC3	4	9				13
11-5007	Table BC3	31	7	4	3		45
11-5008	Table BC3	8	2		1		11
11-5009	Table BC3	8	3		1		12
11-5010	Table BC3	24	34	2	5	2	67
11-5013	Table BC1	20	21	1	4		46
11-5015	Table BC5	35	37	1	1		74
11-5051	Raisin BC3	5	20		1	7	33
11-5052	Raisin BC3	5	4	1	2		12
11-5053	Raisin BC4	20	12	2			34
11-5054	Raisin BC5	6	5				11
11-5057	Raisin BC5	1	1	1			3
11-5058	Raisin BC5	10	17	1	4		32
11-5063	Raisin BC5		1				1
11-5065	Raisin BC5	2	1				3
11-5066	Raisin BC5	25	17	1	2		45
Total		230	209	18	27	10	494

Table 3 (continued). Determination of seedling resistance based on *PdR1* molecular markers for seedless x seedless 89-0908 families made in 2011.

Family	Type Cross	No. Resistant ^a	No. Susceptible ^b	No. Recombinant ^c	No Data ^d	Off Types	Total
Pierce's disease + powdery mildew families							
11-6001	Table BC3		1				1
11-6002	Table BC3		1				1
11-6003	Table BC3		1				1
11-6004	Table BC3	19	27	3		2	51
11-6005	Table BC3		28				28
11-6007	Table BC3	3	2	1			6
11-6009	Table BC4	8	11	1	1		21
11-6051	Raisin BC4	6	5				11
11-6052	Raisin BC4	1	7				8
11-6053	Raisin BC3		1				1
11-6054	Raisin BC3		5	4	2		11
11-6055	Raisin BC3		2	1			3
11-6056	Raisin BC3	10	9	2			21
11-6057	Raisin BC3	5	5	3			13
11-6058	Raisin BC3	14	15	5	1		35
11-6059	Raisin BC3		20	13			33
11-6060	Raisin BC3	1	7			6	14
11-6061	Raisin BC5		3		1		4
Total		67	150	33	5	8	263

^aResistant = marker on both sides of *PdR1* region; ^bSusceptible = no *PdR1* markers.; ^cRecombinant = genotypes that amplified with one *PdR1* marker; ^dNo data = genotypes that failed to amplify properly.

Table 4. Determination of seedling resistance based on *PdR1* molecular markers for seeded x seedless 89-0908 families made in 2011.

Family	Type Cross	No. Resistant ^a	No. Susceptible ^b	No. Recombinant ^c	No Data ^d	Off Types	Total
Seeded families							
11-5501	Table BC1	32	25	1	5		63
11-5503	Table BC3	8	2				10
11-5504	Table BC3	61	59	6	2		128
11-5505	Table BC3	1	1				2
11-5506	Table BC3	16	10	2			28
11-5507	Table BC3	19	29	5	1		54
11-5509	Table BC3			2			2
11-5510	Table BC3			1			1
11-5517	Table BC2			17			17
11-6502	Table BC3	11	11	1			23
11-6504	Table BC3	6	21	2	1		30
Total		154	158	37	9	0	358

^aResistant = marker on both sides of *PdR1* region; ^bSusceptible = no *PdR1* markers; ^cRecombinant = genotypes that amplified with one *PdR1* marker; ^dNo data = genotypes that failed to amplify properly.

Table 5. Results of greenhouse test for determination of Pierce's disease reaction in 2012.

Population	Resistance Source	Testing Compete		In Greenhouse Test for Evaluation by December
		No. Tested	No. Resistance	
BD5-117 map	BD5-117	122	11	0
Arizonica	PdR1	61	13	41
Other PD	SEUS	15	1	20
TOTAL		198	25	61

Table 6. New Pierce's disease resistant grape selections made and evaluated 2012.

Name	Generation	Trace	Berry Size	Crop	Cluster Size	Berry Set	Flavor	Status	Type	Comment
Raisin Pierce's disease <i>V. arizonica</i> resistance										
09-5066-019/21	BC4	9	TS	7	6	6	neutral	prop*	TD	GH test
09-5066-079/95	BC4	10	midget	4	3	5	neutral	prop	TD	GH test
09-5066-121	BC4	10	Zante	5	4	5	neutral	prop	DOV	GH test
09-5066-144	BC4	6	midget	6	5	6	neutral	prop	TD	GH test
09-5068-007	BC4	10	midget	4	4	5	neutral	prop	TD	GH test
09-5068-011/16	BC4	9	Zante	6	5	4	neutral	prop	TD	GH test
09-5068-018	BC4	10	Zante	4	4	4	neutral	prop	DOV	GH test
09-5068-023	BC4	10	midget	5	5	5	neutral	prop*	TD	GH test
09-5068-042	BC4	10	TS	5	4	6	neutral	prop	TD	GH test
08-5074-003	BC4	10	TS	7	5	5	neutral	prop*	TD	GH test
08-5066-103	BC4	6	TS	5	5	5	neutral	prop	DOV	GH test
Raisin Pierce's disease + powdery mildew resistance										
08-6053-090	BC3	10	Zante	5	5	4	neutral	prop*	TD	GH test
08-6053-151	BC3	10	Zante	6	5-6	5-6	neutral	prop*	TD	GH test
09-6054-067	BC4	10	midget	3	3	5	muscat	prop*	DOV	GH test
09-6054-100	BC4	8	TS	6	5	5	neutral	prop	DOV	GH test

Trace: 10 = none, 9 = Thompson Seedless (TS) size, 7 = Fiesta size, 6 = Flame Seedless size

Berry size = 1/16" for table grapes and Thompson Seedless (TS) or midget size for raisins.

Crop: 9 = v. heavy, 7 = heavy, 5 = average, 3 = light, 1 = v. light. Cluster size: 9 = >2.5 lb, 7 = 2 lb, 5 = 1 lb, 3 = 0.5 lb, 1 = widow size.

Berry set: 9 = v. tight, 7 = tight, 5 = average, 3 = loose, 1 = v. loose. Firmness: 9 = v. firm, 7 = firm, 5 = average, 3 = soft, 1 = v. soft.

Prop = propagate in first 2 vine plot; prop* = propagate raisin selection in 7 vine production trial.

DOV = natural dry on the vine type. TD = tray dried. GH test = test resistant reaction to *Xylella* in greenhouse.

CLARIFICATION OF XYLEM FEATURES LIMITING THE ENTRY AND SYSTEMIC SPREAD OF *XYLELLA FASTIDIOSA* IN GRAPEVINES

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ABSTRACT

Pierce's disease of grapevines is a vascular disease caused by the xylem-limited bacterium *Xylella fastidiosa* (*Xf*). Understanding of possible interactions between xylem tissues and the pathogen is essential to clarify disease resistance/susceptibility mechanism of vines. In this report, two xylem factors, vascular occlusions and pit membrane (PM) polysaccharide compositions, were analyzed with grapevine genotypes displaying different Pierce's disease resistances in order to elucidate their roles in the disease symptom development. *Xf*-induced vascular occlusions occurred in both Pierce's disease susceptible and resistant grapevines, but they differed in their quantity and distribution among the genotypes with systemic, extensive occurrence in the susceptible grapevines but localized, minute presence in the resistant grapevines. The extensive vascular occlusions significantly reduce a host vine's hydraulic conductivity, thus highly likely exacerbating the disease symptoms of infected vines. We also established an immunogold-transmission electron microscopy technique to study cell wall polysaccharide compositions across the PM thickness with high resolution. With this technique, we have revealed differences in ultrastructure of three PMs (intervessel, vessel-parenchyma and interfiber PMs) and also successfully studied the composition, quantity and distribution of some pectic and hemicellulosic polysaccharides with test grapevines. This has further emphasized the need for analyzing the roles of PM polysaccharides in Pierce's disease resistance/susceptibility of host vines.

LAYPERSON SUMMARY

Efficient approaches in control of Pierce's disease based on the understanding of the natural Pierce's disease resistance mechanisms of grapevine are being sought as management strategies for Pierce's disease in vineyards. We recently focused on two important grapevine factors affecting the vine's Pierce's disease resistance: vessel (the water-conduits in a vine) occlusions and the ultrastructure and cell wall polysaccharide composition of pit membranes (PMs, porous thin cell wall regions found where two cells contact and communicate). By using grapevine genotypes differing in Pierce's disease resistances we investigated how vascular occlusions may affect water conduction in infected vines. Our results indicate that the abundant occluded vessels observed in Pierce's disease susceptible grapevines significantly suppressed the vines' water conduction, highly likely contributing to their Pierce's disease symptom progression. Also included in this report is our success in developing an immunogold-transmission electron microscopy technique for a detailed structural and compositional characterization of grapevine PMs. We have revealed the ultrastructures of three types of PMs: intervessel, vessel-parenchyma, and interfiber PMs. The first two are relevant to tylose development and *Xylella* spread in an infected vine, respectively. With the new technique, we were able to identify the quantity and distribution of certain cell wall polysaccharides that *Xylella's* cell wall-digesting enzymes may attack in the three types of PMs. This analysis has demonstrated the effectiveness of this technique, which is being used to analyze other cell wall polysaccharides with multiple grapevine genotypes displaying different Pierce's disease resistances. These data will help us understand how bacterial cells may

digest PMs to achieve the pathogen's systemic spread in an infected vine. They are also important in evaluating Pierce's disease resistance of new grape germplasm obtained from traditional breeding programs as well as genetic engineering. All of these are essential for the development of effective approaches for controlling this devastating disease.

INTRODUCTION

Pierce's disease of grapevines caused by the xylem-limited bacterium *Xylella fastidiosa* (*Xf*) has posed an unprecedented threat to the wine and table grape industries in the United States (Purcell and Hopkins, 1996). In nature, the pathogen is initially introduced by xylem sap-feeding insects (e.g., glassy-winged sharpshooter) to only very few vessels in the xylem tissue of a grapevine (Varela et al., 2001). Some studies have indicated that the initially introduced pathogen can spread through the vine's vessel system and eventually becomes systemic, resulting in Pierce's disease symptom development followed by the death of the infected grapevine (Purcell and Hopkins, 1996).

It is reasonable to believe that any factors affecting the *Xf*'s systemic expansion through a host vine's vessel system should be relevant to the resistance/susceptibility of the vine. One factor that has attracted a lot of attention is the role of the structures of the vessel system *per se* in Pierce's disease resistance/susceptibility of grapevine (e.g., Chatelet et al., 2006, 2011; Sun et al., 2006, 2007; Thorne et al., 2006). It is known that individual vessels forming the vessel system in a grapevine are relatively short with an average length of 3-4 cm (Thorne et al., 2006). This also suggests that the systemic spread of *Xf* in a vine requires consecutive intervessel movement of the pathogen. Adjacent vessels have specialized thin wall regions called pit pairs on their contacting lateral walls that facilitate intervessel movement of water and minerals and are believed to be the only passages of *Xf*'s intervessel movement. Each pit pair is separated by a middle lamella and the two vessels' primary cell wall regions, collectively called the pit membrane (PM). Intervessel PMs in grapevines are found to have pore sizes of less than 20 nm (Choat et al., 2003; Pérez-Donoso et al., 2010) and if intact, should be barriers to the intervessel movement of much larger *Xf* (0.25-0.5 μm x 1-4 μm , Mollenhauer and Hopkins, 1974).

It has been hypothesized that the pathogen cells should use their cell wall degrading enzymes (CWDEs) to remove some structural polysaccharides of the PM, enlarging the PM porosity for the pathogen's spread across adjacent vessels (Newman et al., 2003; Labavitch et al., 2006; Labavitch, 2007, Roper et al., 2006). This hypothesis has been supported by a few studies. Newman et al. (2003) and Ellis et al. (2010) observed the *Xf* cells traversing intervessel PM regions. By infusing certain CWDEs to an explant of healthy grapevine, Pérez-Donoso et al. (2010) demonstrated that PM porosity can be enlarged to sizes that allow passage of the pathogen cells. The degraded intervessel PMs and enlarged PM pores with the pathogen cells on site were also observed in susceptible Pierce's disease infected grapevines (Sun et al., 2011). These studies indicate that the pathogen-PM interactions permit *Xf* cells to achieve their movement between adjacent vessels. On the other hand, some studies have been carried out to further identify the PM polysaccharides that *Xf* attacks. Sun et al. (2011) established an immunohistochemical technique with confocal laser scanning microscopy and revealed that intervessel PMs of grapevine genotypes displaying differential Pierce's disease resistances differ in the quantity of weakly methyl-esterified homogalacturonans and fucosylated xyloglucan, potential substrates of *Xf*'s CWDEs. We also combined the immunohistochemical technique with scanning electron microscopy to study the distribution and quantity of those polysaccharides at much higher resolutions (Sun and Labavitch, 2010). Both studies have indicated that those polysaccharides were abundantly present in intervessel PMs of the susceptible grapevines and were absent or masked in those of resistant grapevines. Both techniques have helped us in effectively identifying the polysaccharides that are exposed on or close to the PM surfaces. In order to explore those polysaccharides across the thickness of the intervessel PMs, we recently developed an immunogold-transmission electron microscopy technique. We tested the effectiveness of this technique in identifying cell wall polysaccharides across a PM thickness and also investigated the quantity and distribution of fucosylated XyG with much higher resolution than on our previous reports; this result is included in the current report. Our goal on this is to reveal possible relationships between PM structures and polysaccharides and the Pierce's disease resistance/susceptibility of host grapevines.

Another major concern in grapevine Pierce's disease research is the possible role(s) of vascular occlusions in the disease resistance/susceptibility. Vascular occlusions, especially tyloses (outgrowths of parenchyma cells into a vessel lumen, Esau, 1977), have been reported in the vessel system of grapevines displaying external Pierce's disease symptoms (Esau, 1948; Stevenson et al., 2004; Lin, 2005), however, there is controversy regarding the vascular occlusions' roles in grapevine disease resistance/susceptibility. The three current viewpoints include: 1) vascular occlusions contribute to Pierce's disease resistance of a host vine by blocking *Xf* cells from further spreading in the vessel system (Mollenhauer and Hopkins, 1976), 2) vascular occlusions exacerbate Pierce's disease symptom development of a host vine by suppressing the vine's water transport (Stevenson et al., 2004; Krivanek et al., 2005) and 3) vascular occlusions are irrelevant to the Pierce's disease resistance/susceptibility of a host vine (Fry and Milholland 1990). However, it must be pointed out that more convincing evidence is still needed for any of the three viewpoints. Our previous data have indicated that vascular occlusions developed in both Pierce's disease susceptible and resistant grapevines in response to *Xf* infection and occurred extensively throughout infected vines of susceptible genotypes, but developed only in small quantity in the internodes close to the *Xf* inoculation site in resistant grapevines (Labavitch and Sun, 2009). We also found that the extensive formation of vascular occlusions in the susceptible genotypes did not stop the systemic spread of the pathogen (Sun and Labavitch, 2010; Sun et al., 2011), suggesting that the quantity of vascular occlusions have a limited role of in disease resistance of Pierce's disease susceptible grapevines. Here we report our investigation on how the vascular occlusions may affect water transport in infected Pierce's disease susceptible and resistant grapevines. We expect this will help to better understand the role of vascular occlusions in disease resistance/susceptibility of grapevines.

OBJECTIVES

1. Determine whether xylem structural features vary among grape genotypes with different Pierce's disease resistance and clarify what structural features are related to the Pierce's disease resistance of grapevines.
2. Determine whether PM polysaccharide composition and porosity and the extent of *Xf*'s spread from the inoculation site vary in grape genotypes with different Pierce's disease resistance, and clarify the extent to which PM polysaccharide structure and integrity are affected by *Xf* inoculation of these genotypes.

RESULTS AND DISCUSSION

Effects of vascular occlusions on water conduction in *Xf*-infected grapevines

We used two susceptible (Chardonnay and U0505-35) and two resistant (U0505-01 and 89-0917) grapevine genotypes to investigate possible differences in vascular occlusions among grapevines with different Pierce's disease resistance. Growth and inoculation of experimental vines were described in Sun and Labavitch (2010). Briefly, each vine was grown in the greenhouse from a grafted rootstock and trained to have only two shoots developed from a common trunk. When each vine was about 4 weeks old, one of the vine's two shoots was inoculated at the 5th or 6th internode from the shoot base. Inoculation for each of the two genotypes included two types of inocula: phosphate buffered saline (PBS-0.138 M NaCl, 0.0027M KCl, pH 7.4) as experimental control and a liquid containing *Xf* inoculum as treatment. Samples were collected from the inoculated vines of each genotype after 12 more weeks when the *Xf*-inoculated susceptible vines displayed severe external Pierce's disease symptoms. The samples were then used for the analyses of vascular occlusions (predominantly tyloses). Previously, we reported the qualitative and quantitative vascular occlusions of those genotypes in response to inoculation. We found that vascular occlusions occurred in the *Xf*-inoculated grapevines but not in the PBS-inoculated vines, indicating that the pathogen introduction caused the vascular occlusions in the xylem. Another important finding was that a significant amount of occluded vessels were present throughout *Xf*-inoculated susceptible vines; i.e., involving over 60% of the total vessels in any given transverse section of stem. The percentage of vessels occluded was mostly less than 20% in the *Xf*-inoculated, Pierce's disease resistant vines.

In order to find out if this difference in vascular occlusion formation has any impact on the hydraulic conductivity of the related vines, we measured the specific conductivity (Sperry et al., 1988) of the inoculated shoot for each of these genotypes, using both the control and treatment vines. Instrument setup for this method was fully described in Pérez-Donoso et al. (2010). In this experiment, the inoculated shoot was excised from an intact vine while the site where the cut was to be made was submerged in degassed, distilled water. While in the water the cut end was then connected to a degassed water-filling tube which was connected to a water reservoir.

The acropetal end of the shoot was cut off, leaving a 20~25 cm long explant connected to the tube. Reservoir water pressurized at 33kPa was then pushed into and through the explant, exiting from its distal end. After allowing the water flow through the explant for 3 min, the weight of water flowing through the explant over a 3 min period was measured. Measurements were used to calculate specific conductivity (Sperry et al., 1988), which is the water flow rate of the stem in water weight (gram) per unit sectional stem area at the lower end (mm^2) per minute under the pressure gradient (kPa). This parameter was compared between the control and treatment of each of these genotypes as well as between the four genotypes' vines inoculated with *Xf*. Our data indicated that the PBS-inoculated control vines, either Pierce's disease susceptible or resistant grapevines, had higher values in their explants' specific conductivity, while the values decreased in the *Xf*-inoculated vines (**Figure 1**). However, the extent of the hydraulic conductivity reduction was different between the genotypes of differing Pierce's disease resistance. The conductivity in the infected Pierce's disease susceptible vines was less than 10% of that in the appropriate control vines, while that in infected Pierce's disease resistant vines was about 70% of that in their control vines (**Figure 1**). This demonstrates that *Xf* infection can greatly reduce the hydraulic conductivity of xylem tissues in Pierce's disease susceptible grapevines, and has a much reduced impact on Pierce's disease resistant grapevines. This also indicated that the extensive vascular occlusions we have reported can significantly affect the water conduction in susceptible grapevines, further demonstrating that vascular occlusions may exacerbate Pierce's disease symptom development of susceptible vines by suppressing their water conduction.

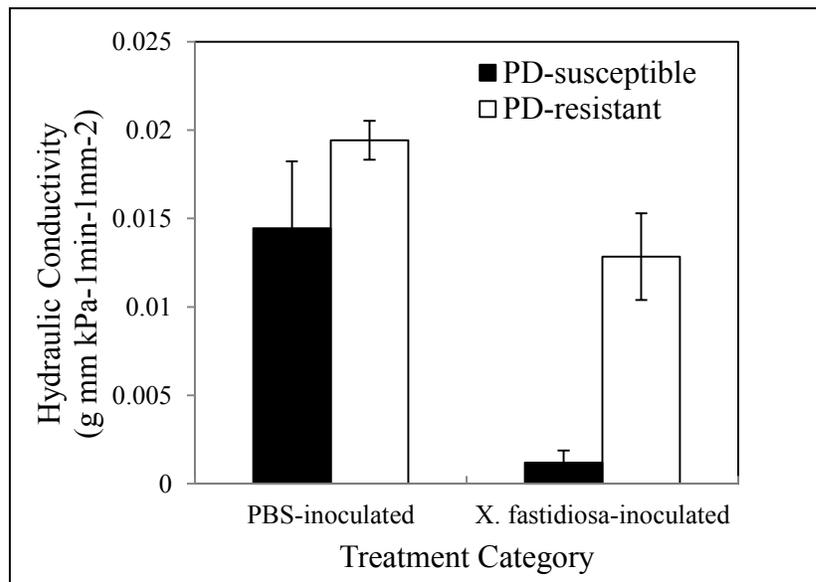


Figure 1. Comparison of specific hydraulic conductivity between PBS- and *Xf*-inoculated grapevines for Pierce's disease susceptible (Chardonnay and U0505-35) and -resistant (89-0917 and U0505-01) genotypes. Explants used for measurement were 18-25 cm and 4-5.5 mm in length and diameter, respectively and were obtained from the inoculated shoots containing the internode with the point of inoculation. Each datum is based on six or eight grapevines (three or four grapevines for each genotype) and presented as mean with standard deviation.

Ultrastructures of three types of PM in grapevines

A pit is a thin cell wall region found where two cells contact and communicate with one another. Pits always occur in pairs with a pit on each of the contacting cells positioned "in opposition" to that on the neighboring cell. A PM is the wall region separating the two opposing pits in a pair. Due to the absence of a secondary wall covering where the opposing pits are located, a PM is structurally composed of two thin primary cell walls and one middle lamella and this cell wall "sandwich" is continuous with the primary cell wall regions that extend underneath the surrounding secondary walls. Secondary xylem of grapevine stem contains four major types of cells: vessel elements, axial parenchyma cells, ray parenchyma cells and fibers. Pits and their PMs occur in the

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 embryogenic cell cultures and convert cell cultures into whole plants. The efficiency of establishing and regenerating plants for many important grape genotypes remains very low and are not at the level required to allow for the predictable, cost effective and timely recovery of tissue culture plants needed to successfully offer grape tissue culture and transformation through a self-sustaining service-based facility. 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 for use in transformation experiments for rootstock genotypes 1103, 101-14, and the wine grape Chardonnay. We have successfully established high quality, rapidly multiplying grape cell suspension stock cultures for 1103, 101-14 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 and Chardonnay in a temporary immersion system (TIS). Maintaining callus in a TIS required minimal labor and could significantly increase the efficiency of maintaining stock callus for use in transformations. Using high quality embryogenic callus produced in both cell suspension culture and the TIS, we are exploring transformation parameters for 1103, 101-14 and Chardonnay.

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 research community. The two labs are sharing their latest protocol improvements for generating and increasing high quality embryogenic callus using germplasm important to their particular country. To date, we have made significant advances in our ability to establish, increase, and maintain embryogenic callus for 1103, 101-14, and Chardonnay for use in tissue culture and transformation experiments using cell suspensions and bioreactors. Using high quality embryogenic callus produced in both cell suspension culture and bioreactors, we will explore regeneration and transformation parameters for 1103, 101-14 and Chardonnay.

INTRODUCTION

The development and transformation of embryogenic cultures in grape is currently 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 callus and regenerating plants for important wine and rootstock genotypes remains very low and are not at the

level required to allow for cost-effective recovery of tissue culture or transgenic plants. Currently, 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 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 wine grape 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 high quality embryogenic callus, 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 this timeframe required to generate transgenic grape plants in order to test new strategies in a timely manner. Currently, even for Thompson Seedless, the production of transgenic plants normally requires a minimum of 8 to 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 Pierce's disease research community. Results of this collaboration will allow the Pierce's disease 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 and reduce the time required for *in vitro* regeneration of grape plants from embryogenic callus by adapting INIA's cell suspension technology and UC Davis Plant Transformation Facility's (UCDPTF) temporary immersion system (TIS) for use in grape tissue culture and transformation.
 - c. Enhance the efficiency of whole plant regeneration from embryogenic callus of grape cultures.
2. Develop a cost-effective grape tissue culture and transformation platform for at least one priority California wine grape, and one California grape rootstock which will provide the Pierce's disease 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

We 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 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). We have established robust embryogenic callus for 11-03, Chardonnay and Cabernet sauvignon from anther explants and we have obtained 101-14 embryos from meristem cultures.

The INIA and the UC Davis Plant Transformation Facility (UCDPTF) are exploring an alternative method to generating embryogenic callus 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 and 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 will be maintained in culture and used as a source of tissue for experiments designed to establish embryogenic callus culture from young leaf explants.



Figure 1. From left to right, somatic embryo cultures of 101-14 from cultured meristems, somatic embryo cultures 11-03, Chardonnay and Cabernet Sauvignon from cultured anther.

Objective 1b: Increase the efficiency and reduce the time required for *in vitro* regeneration of grape plants from embryogenic callus by adapting INIA’s cell suspension technology and UCDPTF’s TIS for use in grape tissue culture and transformation

INIA has developed a method of rapidly increasing embryogenic callus by cycling the callus between agar-solidified medium and liquid media in shake flasks. This technique allows for rapid increase in callus fresh weight while minimizing oxidation and the development of detrimental phenolic compounds in the callus. Eduardo Tapia Rodríguez from Dr. Prieto’s lab at INIA, visited the UCDPTF and instructed us on INIA’s method for increasing embryogenic callus by cycling tissue between solid and a liquid media. Eduardo compared growth rates of 101-14 and Thompson Seedless (TS) in INIA’s liquid RM medium consisting of 1/4 MS minimal organics medium supplemented with 60 g/L sucrose, 1 mg/L 2,4-D, 1 mg/L kinetin, 400 mg/L glutamine and 0.25 g/L activated charcoal to growth rates in UCDPTF’s callus induction medium consisting of Lloyd and McCown minimal organics medium, supplemented with 20 g/liter sucrose, 1g/liter casein, 1 mM MES, 10 mg/liter picloram, 2 mg/liter tridiazuron (TDZ) and 600 mg/liter activated charcoal (PT) during the liquid phase of INIA’s solid to liquid cycling methodology. Increase in fresh weights during the liquid phase was recorded over a 2 week period. The statistical kinetic growth comparison in Tukey HSD test, confirmed the advantage of using UCDPTF’s PT medium during the liquid phase of the process with significantly greater increases in callus fresh weigh in PT medium verses RM medium for TS and 101-14 (**Figure 2**).

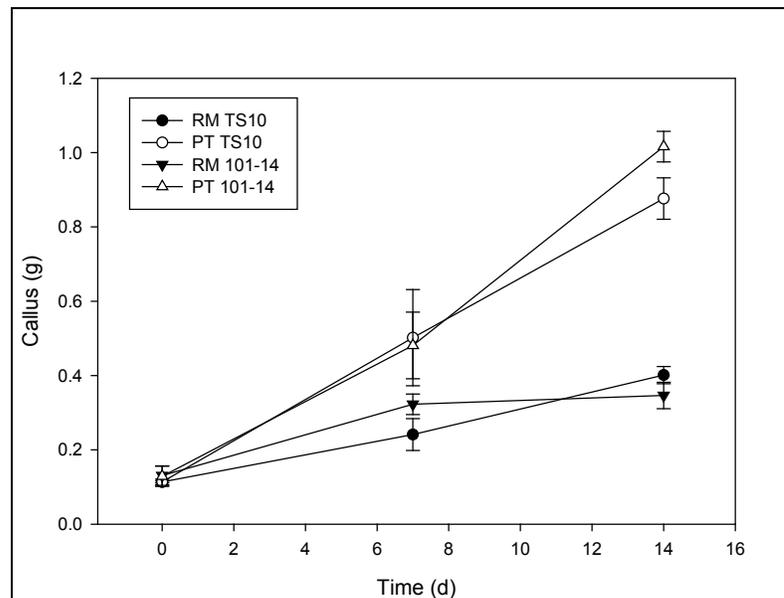


Figure 2. The statistical growth kinetics comparison in Tukey HSD test, showing the mean increase in fresh weight (g/liter) and confirming the improvement in biomass production using PT verses RM medium during the liquid phase of INIA’s methodology

Dr. Prieto's lab is now testing embryogenic callus growth rates of additional grape cultivars in suspension cultures on INIA's RM medium and UCDPTF's PT medium. Cultivars under investigation at INIA include Freedom, 110-R, Salt Creek and Moscatel de Alejandria. Images of the cell suspensions growing on the two media are shown in **Figure 3**.

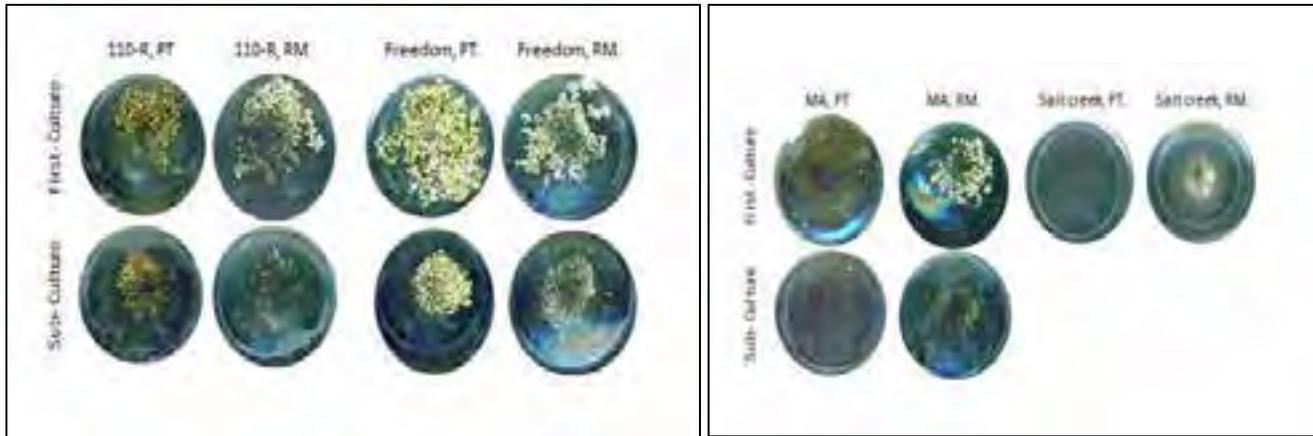


Figure 3. Results from Dr. Tapia at INIA comparing cell suspension growth of Freedom, 110-R, Moscatel de Alejandria and Salt Creek on INIA's RM medium compared to UCD's Pic/TDZ (PT) liquid medium. Suspensions on the top row are initial cultures whereas cultures on the bottom row have been sieved and subcultured.

We have made significant progress on improving the production of embryogenic grape callus across a range of genotypes including 1103, 101-14 and Chardonnay using a modification of INIA's method of cycling the callus between agar-solidified medium and liquid media in cell suspensions (**Figure 4**). We are now routinely maintaining stock suspension cultures as per Prieto's lab, but we are using a modification of UCDPTF's Pic/TDZ medium formulation, replacing TZD with the cytokinin meta-topolin; (MT) a highly active aromatic cytokinin from leaves of *populus x canadensis moench*, cv. Robusta (Aremu et al., 2011) and further supplemented with 100 mg/l ascorbic acid, 30 mg/l reduced glutathione and an additional 1500 mg/l activated charcoal (Pic/MTag). Embryogenic grape callus grown on agar-solidified medium is transferred to 25 ml of Pic/MTag in 125 ml shake flasks and grown on a gyratory shaker at 110 rpms in the dark. After the suspension is established, 10 ml of the suspension is withdrawn each week from the flask and replaced with 10 ml of fresh medium. The 10 ml of suspension that is removed from the flask is transferred to a 15 ml centrifuge tube or 100x20mm petri dish and allowed to settle under gravity. All but 0.5 ml of the supernatant is carefully removed and the final 0.5 ml of medium and cells are collected and transferred onto agar solidified Woody Plant Media (WPM) (Lloyd and McCown, 1981), 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 400 mg/l (BN). Embryogenic callus plated on BN medium produces extremely high quality embryogenic callus at the appropriate stage for use in transformation in approximately four weeks. If the callus is not required for transformation it can be discarded rather than continually subcultured, since new agar-solidified plates are established each week as part of the routine weekly subculture process required for the cell suspension cultures. Previously we maintained stock callus exclusively on agar-solidified plates which were subcultured every 3-4 weeks. Sub-culturing these plates was labor intensive since the callus often becomes oxidized when maintained on plates for long time intervals, requiring non-oxidized callus to be carefully separated from oxidized callus. By maintaining cells in suspension and plating 10 ml of the suspension during each weekly subculture, one can easily generate high quality callus while eliminating the need to maintain and subculture large numbers of stock callus plates. It appears that once high quality suspensions are created, this process can be repeated indefinitely, allowing for a constant and reliable supply of embryogenic callus for use in transformation experiments. The process does not require cycling material between agar and liquid medium but maintains cells in suspension by continually sub-culturing them on a weekly basis and allows for the continual production of non-phenolic producing embryogenic callus.

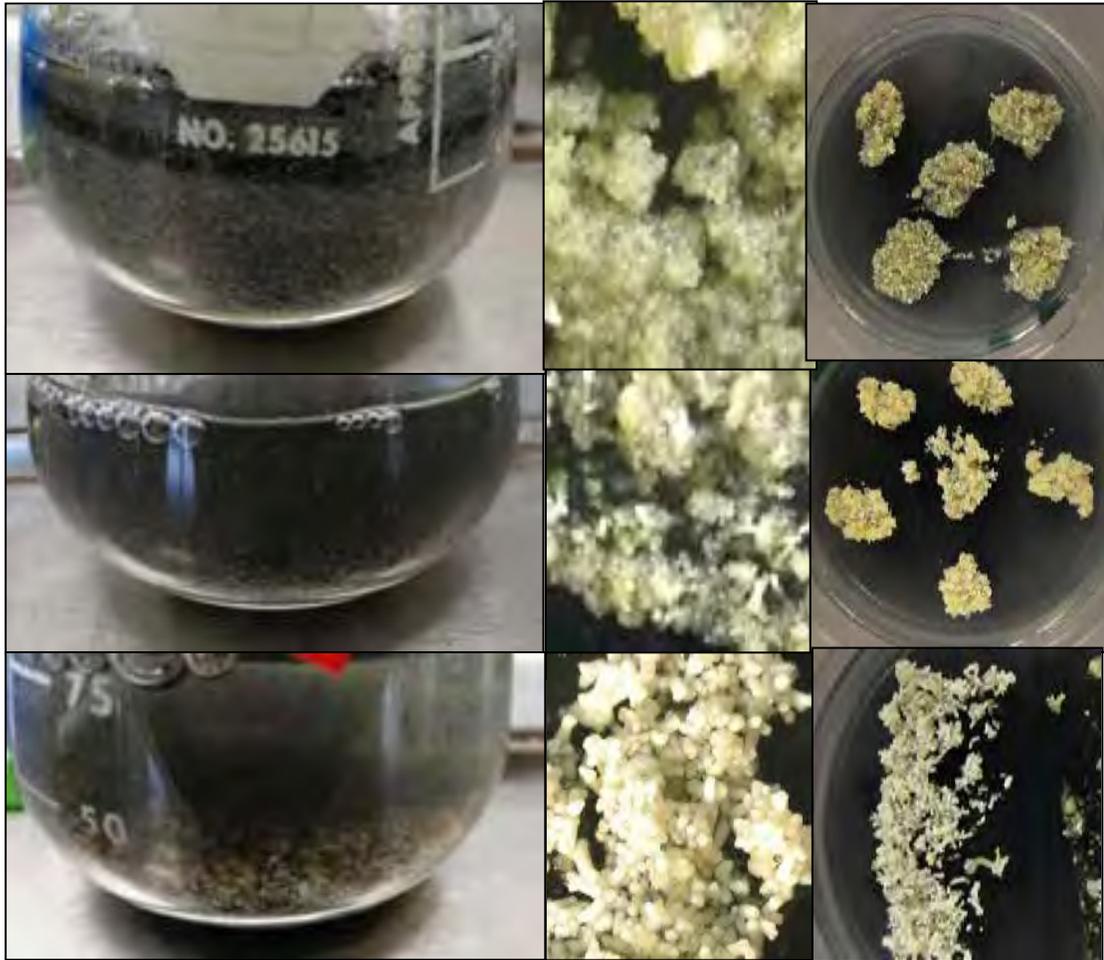


Figure 4. Chardonnay (top), 1103 (middle) and 101-14 (bottom) in cell suspension cultures on WPM medium supplemented with 1g/l casein, 1M MES, 1000 mg/l activated charcoal, 10 mg/l picloram, 2 mg/l meta-topolin (left), close up of cell suspension cultures (middle) and embryogenic callus developing 4 weeks after plating 1 ml of suspensions on WPM medium supplemented with 1g/l casein, 1M MES, mg/l activated charcoal, 0.5 mg/l BAP and 0.1 mg/l NAA.

In addition to evaluating INIA's liquid shake flasks methodology on grape genotypes 101-14, 1103, Chardonnay and Cabernet Sauvignon, we are exploring UCDPTF's TIS for use in rapidly increasing embryogenic callus. We have inoculated TIS bioreactors with 0.5 g fresh weight of Chardonnay, 101-14 or 1103 embryogenic callus. The TIS vessels contain Woody Plant Media (WPM) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 10 mg/l picloram, 2 mg/l thidiazuron, (PicTDZ medium) or WPM supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal 10.0 Picloram, 2mg/l meta-topolin (Pic/MT medium). Fresh weight measurements and observations were taken at 2 week intervals. Callus increased on both Pic/TDZ and Pic/MT media with more rapid growth seen on Pic/TDZ medium than on Pic/MT medium. However, the quality of the callus on both media formulations exhibited excessive accumulation of phenolic compounds, discoloring the callus. Although labor input to maintain callus in the TIS is significantly reduce, we were concerned because of the high accumulation of phenolic compounds in the callus. It was not known if this phenomenon would be detrimental to further callus growth and development. To test this, we transferred samples of discolored callus which had been growing in TIS vessels for three months to agar solidified BN medium and cultured them in the dark. Within 3-4 weeks new white non-phenolic embryogenic callus began to develop (**Figure 5**). Therefore, it appears that despite the phenolic accumulation in callus grown in the TIS vessels, the ability to produce non-phenolic cultures from this callus on

agar-solidified medium is not compromised. This system could prove advantageous from a labor management perspective, since it may allow one to maintain stock embryogenic 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 several weeks prior to initiating transformations.

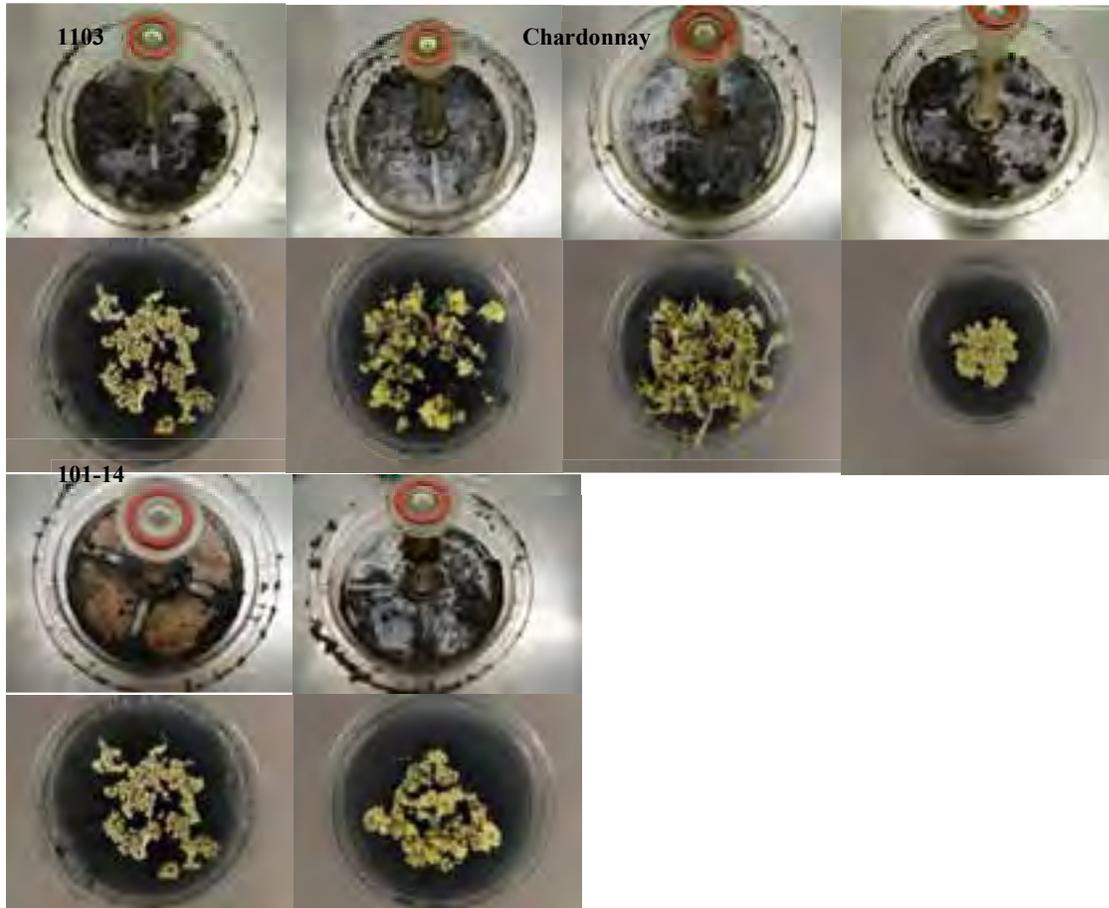


Figure 5. Callus growth of 1103, Chardonnay and 101-14 in TIS on Pic/TDZ (top left of each panel) or Pic/MT (top right of each panel) and subsequent callus development after transfer to agar-solidified BN medium (lower left and lower right of each panel).

Objective 1c: Enhance the efficiency of whole plant regeneration from embryogenic callus of grape cultures

In our hands, the generation of transgenic callus and regeneration of plants from that callus is a long term process often requiring 4-6 months for transgenic embryogenic callus to form and up to 6 to 8 additional months for transgenic embryogenic callus to convert into whole plants. We believe that these problems may be related to the extended length of time that callus remains in a non-differentiated state on high hormone containing medium such as our Pic/TDZ medium formulation. Therefore we are evaluating media formulations (particularly the cytokinin component) to determine if we can speed up the process of transgenic embryogenic callus production and whole plant regeneration. We transferred embryogenic Thompson Seedless callus to WPM medium containing 10 mg/l picloram and 2.0 mg/l TDZ, BAP or MT. After a 2-3 subculture, calli were inoculated with the agrobacterium strain EHA105 carrying the kanamycin plant selectable marker gene and the scorable marker gene, dsRed, co-cultivated for 2-3 days and then transferred to selection medium composed of WPM supplemented with 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin 10 mg/l picloram and 2.0 mg/l TDZ, BAP or MT. Callus was cultured in the dark and sub-cultured to fresh medium of the same

formulation every 3 to 4 weeks and monitored for the presence of transformed embryogenic cells. Preliminary data suggests that transgenic dsred expressing callus colonies develop faster on medium supplemented with Pic/MT than on experimental medium containing Pic/BAP or on our standard callus induction medium containing Pic/TDZ (**Figures 6 and 7**). If these results can be extrapolated to 1103 and Chardonnay, this new medium formulation could reduce the time required to generate transgenic grape callus colonies.

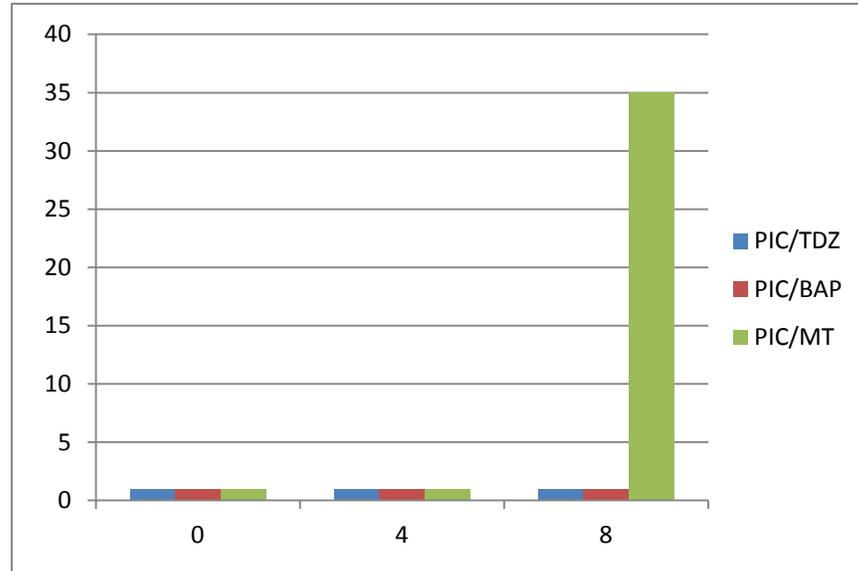


Figure 6. The percentage of transgenic Thompson Seedless callus colonies produced, eight weeks after co-cultivation and cultured on induction medium composed of WPM supplemented with 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin 10 mg/l picloram and 2.0 mg/l TDZ, BAP or MT.

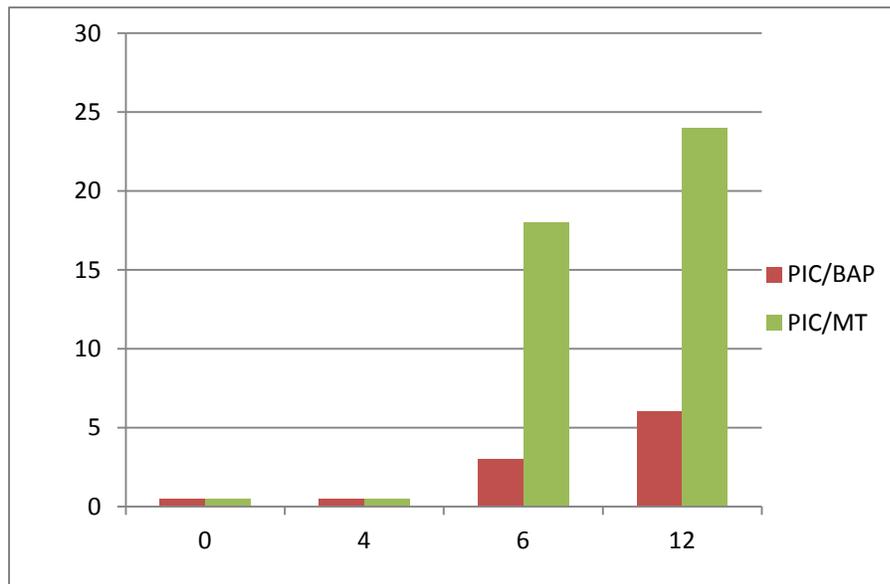


Figure 7. The percentage of transgenic Thompson Seedless callus colonies produced, twelve weeks after co-cultivation and cultured on induction medium composed of WPM supplemented with 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin 10 mg/l picloram and 2.0 mg/l BAP or MT.

Objective 2: Develop a cost effective grape tissue culture and transformation platform for at least one priority California wine grape, and one California grape rootstock which will provide the Pierce’s disease research community with a predictable supply of experimental plant material while reducing labor and maximizing tissue culture and transformation efficiency

We have initiated preliminary transformation studies on callus of 1103 and Chardonnay using a construct containing the scorable marker gene dsRed. This protein allows us to non-destructively track transformation over the course of the transformation process and access the presence or absence of chimeric plants. We have noticed excessive discoloration of Chardonnay but not with 1103 callus after inoculation with *agrobacterium* (**Figure 8**). Cultures will be sub-culture every three weeks to fresh medium and monitored for the production of dsred expressing transgenic callus.



Figure 8. Co-culture of embryogenic callus of 1103 (left) and Chardonnay (right) with the scorable marker gene dsRed. Note discoloring of Chardonnay callus after co-cultivation compared to 1103 due to excessive oxidation.

CONCLUSIONS

With significant contributions from INIA, we have made substantial progress producing high quality embryogenic callus of 1103, 101-14, and Chardonnay by maintaining embryogenic cell suspensions and plating them to agar-solidified medium on a weekly basis. The system allows for continuous production of highly embryogenic, non-oxidized stock callus that can serve as a constant supply of starting tissue for use in transformation experiments. Preliminary results suggest that grape embryogenic callus can also be grown under temporary immersion with little physical manipulations of the cultures resulting in significant labor savings. We have also begun developing new media formulation which allow for more rapid production of transgenic callus which should reduce the time required to generate transgenic grape plants for the Pierce’s disease research community. These advances should significantly improve tissue culture and transformation of grape.

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BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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ABSTRACT

We continue to make rapid progress breeding Pierce's disease resistant winegrapes. Aggressive vine training and selection for precocious flowering has allowed us to reduce the seed-to-seed cycle to two years. We are also using marker-assisted selection (MAS) for the 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 1,000 progeny from the 2009 and 2010 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, will be 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. We plan to move nine advanced selections to Foundation Plant Services (FPS) this winter to begin the certification and release process. Two Pierce's disease resistant rootstocks will also be advanced to FPS certification. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 are also being pursued but progress is limited by their multigenic resistance. 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 public tastings in Sacramento and Santa Rosa.

INTRODUCTION

The Walker lab is uniquely poised to undertake this important breeding effort, having developed rapid screening techniques for *Xylella fastidiosa* (*Xf*) resistance (Buzkan et al. 2003, Buzkan et al. 2005, Krivanek et al. 2005a 2005b, Krivanek and Walker 2005), and having unique and highly resistant *V. rupestris* x *V. arizonica* selections, as well as an extensive collection of southeastern grape hybrids, to allow the introduction of extremely high levels of *Xf* resistance into commercial grapes. We have selected progeny with the Pierce's disease resistance gene *PdR1* from the b43-17 *V. arizonica* / *candicans* resistance source for fruit quality at the backcross 4 (BC4), 97% *V. vinifera* level. They are also undergoing greenhouse testing to verify their resistance and those with the highest levels of resistance will be prepared for small-scale winemaking this winter by grafting them onto Pierce's disease resistant rootstocks and planting six to eight vines sets on commercial spacing and trellising. We have made wine from vines that are 94% *V. vinifera* level from the same resistance background for two years. They have been very good and do have the hybrid flaws (blue purple color and herbaceous aromas and taste) that were prevalent in wines from the 87% *V. vinifera* level. There are two forms of *PdR1*, 8909-08 and 8909-17 – sibling progeny of b43-17 and they have different alleles of *PdR1*. These selections have been introgressed into a wide range of winegrape backgrounds over multiple generations, and resistance from southeastern United States (SEUS) species is being advanced in other lines. However, the resistance in these later lines is complex and markers have not yet been developed to expedite breeding.

OBJECTIVES

1. Breed Pierce's disease resistant winegrapes through backcross techniques using high quality *V. vinifera* winegrape cultivars and *Xf* resistant selections and sources characterized from our previous efforts.

- 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

Table 1 shows the F1 crosses made in 2012 to 5 new Pierce's disease resistant *V. arizonica* accessions from the southern USA and Mexico to develop possible mapping populations so that genetic markers can be developed 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 plan to germinate a subset of these seeds in late fall, make copies of seedlings growing in gallon pots in early 2013 and greenhouse test them to characterize the inheritance of Pierce's disease resistance.

Table 1. Crosses made in 2012 to develop genetic maps in new accessions from southern USA and Mexico germplasm. Crosses 08-319 and 08-326 are selfs of Zinfandel and Cabernet Franc respectively and 100% *V. vinifera*.

Resistance source	Geographic origin - Appearance phenotype	Pure <i>V. vinifera</i> types used in 2012 crosses	# of seeds produced
ANU5	Littlefield, AZ Unique	Alicante Bouschet	211
		Grenache	129
b40-29	Chihuahua, MX like b43-17	08319-07	134
		08319-29	44
b31-13	Ciudad Mante, MX like b42-26	08319-07	163
		08319-29	65
b46-43	Big Bend, TX Similar to b47-32	08319-07	350
		08319-12	19
		08326-61	266
b47-32	Big Bend, TX	08319-07	243
		08326-61	189

We have now evaluated more than 2,000 *PdR1b* 97% *V. vinifera* winegrape progeny from which we will select the best and most resistant for release. Our breeding efforts are focusing on increasing the *V. vinifera* content of our other Pierce's disease resistant lines. **Table 2a** summarizes the number of seeds produced from nine different crosses that advance the *PdR1a* allele from b43-17 to the 97% *V. vinifera* level. Previously we identified a Pierce's disease resistance locus *PdR1c* from *V. arizonica* b40-14 that maps to the same region of LG14 as *PdR1* from b43-17. In the absence of an understanding of gene function and given the significant geographic difference from which the b43-17 and b40-14 resistance sources originated, we are continuing to advance the *PdR1c* line as a future breeding resource. **Table 2b** summarizes the six crosses in two groups in this background we continue to advance. The 08-331 set eliminates the *V. rupestris* heritage that was used with the *PdR1* sources before. They may help unravel difference in resistance from b40-14. **Table 2c** is a remake of the 86% *V. vinifera* *PdR1b* x b42-26 line cross made last year to combine resistance from b43-17 and b42-26. These seeds can increase the population size should the greenhouse screen results from the initial group prove promising. Although resistance from the complex BD5-117 selection is inherited as a multigenic trait, some progeny have excellent resistance. **Table 2d** shows the crosses made to create a group of selfed and intercrossed individuals with very good wine variety phenotypes as an option to explore incorporating BD5-117's resistance into other backgrounds. We have seen similar loss of Pierce's disease resistance in backcross lines with *V. shuttleworthii*. However, we have found a very rare individual that is at least as resistant as our most resistant *PdR1* genotypes. We have selfed this particularly resistant BC1 individual to find out whether there is any opportunity to exploit this resistance source (**Table 2e**). The Pierce's disease resistance in BD5-117 descends in part from *V. shuttleworthii*. In **Table 2f** we summarize 3 crosses involving highly resistant individuals with promising wine variety phenotype in these two resistance sources.

Table 2. Crosses made in 2012.

Resistant type	<i>V. vinifera</i> parent / grandparent of resistant type	<i>V. vinifera</i> types used in 2012 crosses	# of seeds produced
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2a. Monterrey *V. arizonica* / *candicans* resistance source *PdR1a* (F8909-17) to produce progeny with 97% *V. vinifera* parentage. 08-319 and 08-326 are selfs of Zinfandel and Cabernet Franc, respectively and 100% *V. vinifera*.

08-312, 08-318	Cabernet Sauvignon, Carignane	08-319, 08-326, Cabernet Sauvignon, Riesling, Zinfandel	2972
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2b. Chihuahua *V. arizonica* resistance source *PdR1c* b40-14 to produce 87% *V. vinifera* progeny. The 08331 line is free of *V. rupestris*.

08-331	Tannat	08319, 08326, Grenache, Zinfandel	297
09-367	Cabernet Sauvignon	Carignane, Grenache, Palomino	349

2c. Cross made to pyramid *PdR1b* (F8909-08) Monterrey *V. arizonica* / *candicans* and b42-26 *V. arizonica* resistance lines to produce 86% *V. vinifera* progeny.

09-331	Zinfandel/Petite Syrah	Grenache\F2-35	474
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2d. Cross made to self the BD5-117 SEUS resistance lines to produce 75% *V. vinifera* progeny. F2-7 is a cross of Cabernet Sauvignon x Carignane and 100% *V. vinifera*.

03-182	F2-7	Selfed & Intercrossed	2900
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2e. Cross made to self the Haines City *V. shuttleworthii* resistance lines to produce 75 % *V. vinifera* progeny

08-364	Tannat	Selfed	142
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2f. Cross made to combine the BD5-117 SEUS and Haines City *V. shuttleworthii* resistance lines to produce 75% *V. vinifera* progeny.

03-182	F2-7	08364 (Tannat), 08352 (Cabernet Sauvignon)	748
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Table 3 provides a list of Pierce's disease greenhouse screens initiated and/or completed over the last 18 months. Group A is testing genotypes in which *PdR1a* and *PdR1b* are being combined. The greenhouse screen is complete and MAS results are being processed. Group B tested BC1 and BC2 progeny and their parents in the Haines City *V. shuttleworthii* line. Results from this screen were used to select the resistant parents for crosses shown in **Tables 2e and 2f**. Of the 54 accessions tested in the *V. arizonica* Group C, we selected five highly Pierce's disease resistant individuals from four diverse geographic locations for new mapping crosses made in 2012 (**Table 1**). Results from Group D were used to select the three Pierce's disease resistant rootstocks advanced for possible release (**Table 4**). In Group E, we explored Pierce's disease resistance from the *V. rotundifolia* background. Given the apparent complex Pierce's disease resistance we observed and the difficult nature of working in a *V. rotundifolia* background, no crosses were made in this line this year. Group F tests the last of our elite 94% *PdR1b* genotypes and the phenotypically most promising selections of our wine types backcrossed to BD5-117. Results from this trial allowed the identification of parents that were used in crosses in **Tables 2d and 2f** and served as an additional resistant result for the five 94% *V. vinifera PdR1b* genotypes that may be advanced for release (**Table 4**). Our 97% *V. vinifera* level *PdR1b* resistance line fruited for the first time in 2011. The phenotypically most promising selections were tested in Group G. This was an extremely severe test with an average temperature of 84 °F and 40% RH. This allowed us to select 7 very resistant genotypes. If they have a favorable confirmatory greenhouse test (Group I) they will be moved to release consideration in **Table 4**. Group H is our third greenhouse screen in the F1 b42-26 line as we continue our mapping efforts of Pierce's disease resistance loci in this multigenic background. Genetic markers in this line are essential as we try to stack/combine resistance lines as in the crosses made in **Table 2c**. Group I screens our 2012 cross parents, our group of 15 advanced scion selections (**Table 4**) and several additional 97% *V. vinifera* scion selections from 2009 crosses remaining in the field (**Table 3**).

Table 3. 2012 Greenhouse testing.

Group	Genotypes	# Genotypes	Inoculation date	ELISA sample date	Resistance source(s)
A	<i>PdR1a</i> & <i>PdR1b</i> together	122	01/13/2011	04/14/2011	b43-17
B	Haines City BC1 & BC2 Progeny	173	03/31/2011	07/12/2011	<i>V. shuttleworthii</i>
C	New <i>V. arizonica</i> sources	54	05/12/2011	08/11/2011	<i>V. arizonica</i>
D	Pierce's Disease Rootstocks	15	06/14/2011	09/15/2011	F8909-08
E	<i>V. rotundifolia</i> and <i>V. rotundifolia</i> Hybrids	94	11/03/2011	02/02/2012	<i>M. rotundifolia</i>
F	94% <i>PdR1b</i> & BD5-117 Source Saves	109	12/15/2012	03/16/2012	F8909-08, BD5-117
G	97% <i>PdR1b</i> Elite Selections	77	03/10/2012	06/09/2012	F8909-08
H	05347 b42-26 F1 Mapping Population	84	07/03/2012	10/04/2012	b42-26
I	2012 Parents & 97% <i>V. vinifera</i> 2nd tests	75	10/02/12	01/03/2013	F8909-08, b42-26

The horticultural and wine phenotype evaluation efforts on our most advanced *PdR1b* genotypes are detailed in **Table 4**, where they are organized by *V. vinifera* parent. This table starts in the first row with the number of crosses made in 2009 and the resulting numbers of seedlings planted in 2010. Row 3 details the number remaining now following two years of fruit and vine quality evaluation. These are all slated for greenhouse testing in the coming year. The percent remaining gives some insight into the relative parental value of the different elite *V. vinifera* cultivars used in the crosses. The bottom section of **Table 4** details the evaluation progress on our 2010 crosses in the *PdR1b* line that fruited for the first time this year. It is important to remember that in all likelihood many additional promising genotypes will emerge from these crosses next year. Several hundred of these 2010 cross genotypes will fruit for the first time in 2013 and some that had low productivity this year may fruit better in their second year and warrant a closer look at their fruit quality parameters.

Table 4. 97% *V. vinifera PdR1b* evaluations: 2009 (2nd season of horticultural selection) and 2010 crosses evaluated for 1st season.

Evaluation level	<i>V. vinifera</i> parent									
	Barbera	Cab. Sauv.	Char-donnay	Chenin Blanc	Muscat Blanc	Pinot Noir	Riesling	Sylvaner	Zinfandel	Total
# 2009 Crosses	-	7	4	6	-	3	1	3	4	28
# Seedlings (sdls)	-	489	309	517	-	107	11	218	217	1868
# Sdls retained fall 2012	-	40	64	41	-	3	3	29	27	207
% Remaining	-	8.2%	20.7%	7.9%	-	2.8%	27.3%	13.3%	12.4%	11.1%
# 2010 Crosses	2	-	1	-	2	-	1	-	-	6
# Seedlings (sdls)	263	-	67	-	31	-	222	-	-	583
# Precocious sdls	151	-	44	-	21	-	165	-	-	381
# Sdls making first cut	24	-	24	-	0	-	23	-	-	71
# Sdls making 2nd cut	23	-	12	-	0	-	17	-	-	52
% Pass 2nd cut in 1st yr	9%	-	18%	-	0%	-	8%	-	-	9%

Table 5 presents promising Pierce’s disease resistant genotypes being considered for release. This process involves passing our severe greenhouse screen multiple times. The number following the “R” in the “# GH Screens” column tells how many times a selection has passed a severe greenhouse screen. To make this list selections must also possess desirable horticultural traits and have potential for high quality wine production. Producing small lot wines from multiple vines field trials in Davis and in Pierce’s disease hot spots in the North Coast complete the evaluation process. Pierce’s disease resistant scions need Pierce’s disease resistant rootstocks. On the list are three selections that have been tested for Pierce’s disease resistance, their ability to root and graft, and resistance to nematodes. The two 08314 selections have good nematode resistance in addition to their Pierce’s disease resistance.

Table 5. Possible *PdR1b* releases for transfer to Foundation Plant Services.

Screen rep	Genotype	Parentage	% <i>V. vinifera</i>	Color	# Years small lot wine made	Multiple vine trials Davis	Multiple vine trials Napa
R3	07713-51	F2-35 x U0502-48	94%	W	3	Yes	Yes
R2	07329-01	U0505-01 x Chardonnay	94%	B	0	Yes	Yes
R2	07329-31	U0505-01 x Chardonnay	94%	B	1	Yes	
R2	07338-37	U0505-01 x LCC	94%	B	0	Yes	Yes
R2	07355-75	U0505-01 x Petite Syrah	94%	B	3	Yes	Yes
R1	09330-07	07370-039 x Zinfandel	97%	B	0		
R1	09331-047	07355-020 x Zinfandel	97%	B	0	Yes	
R1	09331-133	07355-020 x Zinfandel	97%	B	0		
R1	09333-117	07355-020 x Chardonnay	97%	B	0		
R1	09333-178	07355-020 x Chardonnay	97%	B	1	Yes	
R1	09333-253	07355-020 x Chardonnay	97%	B	0		
R1	09333-370	07355-020 x Chardonnay	97%	B	0		
R1	06325-42	A81-138 x Alicante Bouschet	88%	B	0		Yes
R1	06325-43	A81-138 x Alicante Bouschet	88%	B	0		Yes
R1	U0502-20	A81-138 x Chardonnay	88%	W	4	Yes	
R3	03300-099	101-14 Mgt x F8909-08	0%	stock	NR		
R2	08314-15	03300-048 x 06301-93	0%	stock	NR		
R2	08314-46	03300-048 x 06301-93	0%	stock	NR		

Table 6a-c details the vine, fruit and juice characteristics for the 9 *PdR1* selections used to make wine lots in 2012: one lot at the 88% *V. vinifera* level; 5 lots at the 94% level; and one lot at the 97% level. For the first time we made wine from 94% *V. vinifera PdR1b* selections in our field trial at the Treasury Wine Estates (Beringer) vineyard in Yountville, Napa Valley: a pure lot of our red 07355-075 and a 25/75% blend of our white 07713-51 co-fermented with Chardonnay. We made a number of *V. vinifera* controls and Blanc du Bois from both Davis and Napa. Lenoir was made from Davis fruit.

Table 6a. 2012 Pierce's disease resistant selections used in small scale winemaking: background and fruit characteristics.

Genotype	Parentage	% <i>V. vinifera</i>	2012 bloom date	Berry color	Berry size (g)	Avg. cluster wt. (g)	Ripening season	Prod 1 = v low, 9 = v high
07329-01	U0505-01 x Chardonnay	94%	05/12/12	B	1.0	160	early-mid	5
07329-31	U0505-01 x Chardonnay	94%	05/12/12	B	0.9	160	early-mid	5
07338-37	U0505-01 x LCC	94%	05/12/12	B	1.6	210	early-mid	6
07355-75	U0505-01 x Petite Sirah	94%	05/16/12	B	1.3	185	mid	7
07355-75 Napa	U0505-01 x Petite Sirah	94%	06/01/12	B	1.2	300	early	7
07713-51	F2-35 x U0502-48	94%	05/08/12	W	1.3	325	early	8
09331-047	07355-020 x Zinfandel	97%	05/16/12	B	1.1	190	mid	5
09332-165	07355-020 x Chenin Blanc	97%	05/16/12	B	0.9	215	mid	6
09333-178	07355-020 x Chardonnay	97%	05/20/12	B	1.0	168	mid-late	5
U0502-20	A81-138 x Chardonnay	87%	05/12/12	W	1.1	215	early-mid	7
Lenoir	<i>V. aestivalis</i> Hybrid	<50%	05/29/12	B	1.3	145	late	6

Table 6b. 2012 Pierce's disease resistant selections used in small scale winemaking: juice chemical analysis.

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 anthocyanins (mg/L)
07329-01	26.3	9.3	3.44	4.80	2420	275	209	410	939
07329-31	27.9	8.0	3.46	2.99	2280	337	45	644	1091
07338-37	23.5	4.8	3.66	1.66	2010	183	20	392	1438
07355-75	27.8	6.8	3.44	1.89	1930	191	18	512	1382
07355-75 Napa	22.0	9.5	3.18	4.72	1650	86	27	496	1436
07713-51	20.3	5.1	3.49	1.31	1640	134	-	-	-
09331-047	24.4	4.6	3.75	1.11	2050	298	8	569	1556
09332-165	20.3	6.3	3.24	1.03	1410	135	9	378	625
09333-178	23.4	4.9	3.53	0.70	1670	137	51	338	862
U0502-20	22.9	7.7	3.30	2.85	1630	334	-	-	-
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
Lenoir	24.8	12.1	3.22	7.03	2240	183	186	268	2486

-- Cab. Sauvignon, Pinot Noir, and Lenoir from previous vintages for comparison.

Table 6c. 2012 Pierce's disease resistant selections used in small scale winemaking: berry sensory analysis.

Genotype	Juice hue	Juice intensity	Juice flavor	Skin flavor	Skin tannin (1=low, 4= high)	Seed color (1 = gr, 4= br)	Seed flavor	Seed tannin (1 = high, 4 = low)
07329-01	orange-red	low	fruit-jam, sl hay	fruity, sl grass, spice	1	4	nutty, woody	2
07329-31	bright pink	low	fruity, vinous	CS veg	2	3	spicy, toasted, sl bitter	1
07338-37	bright pink	low-med	fruity, berry	sl fruity	1	2	spicy, hot	1
07355-75	pink-red	med	fruity, straw	sweet hay	1	4	woody, spicy	3
07355-75 Napa	pink-red	med+	sl fruity	sl fruity	2	2.5	spicy	3
07713-51	green-gold	med	neutral, apple	straw, grass	1	4	dusty, grain	2
09331-047	red	med	fruity, spicy	ripe jam, plum	3	3	nutty, sl bitter	2
09332-165	pink-orange	low-med	strawberry, cranberry	chalky, hay	2	3	sawdust, spicy	3
09333-178	brown	med	spicy apple juice	sl CS veg, fruity	2	4	chalky, bitter	3
U0502-20	brown	med	floral, honey, apple	neutral, ast	1	3	spicy	2
Lenoir	red	med-dark	mildly fruity	fruity	1	4	hot	4

FUNDING AGENCIES

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Figure 1. Cluster shots of *PdR1b* selections destined for Foundation Plant Services this winter as larger scale wine making tests begin. Clockwise from upper left: U0502-20 (87% *V. vinifera*); 07338-37 (94% *V. vinifera*); 07355-75 (94% *V. vinifera*); 07713-51 (94% *V. vinifera*); 09331-47 (97% *V. vinifera*); 09333-178 (97% *V. vinifera*); 09333-117 (97% *V. vinifera*); and 09330-07 (97% *V. vinifera*).

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

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LAYPERSON SUMMARY

A major component of this project is to broaden the base of Pierce's disease resistance by searching for and characterizing new forms of resistance. Fifty-two accessions were selected from collections we have made from across the southern USA and northern Mexico. Greenhouse screening identified 20 new resistant accessions. Next we used 18 simple sequence repeat markers to enable an assessment of the diversity and commonalities among these accessions. We identified four major groups of resistant germplasm. The breeding program has used resistance from two of these groups, but resistance from the other two groups has not been used. This germplasm screening provides opportunities to explore and identify resistance loci that may provide different resistance mechanisms to expand the genetic base of the Pierce's disease resistance breeding program. Without any prior knowledge of the recent genetic grouping study, crosses were made with five of the most resistant accessions in spring 2012. This genetic diversity study will allow us to pick the populations with the greatest potential to have different resistance sources and guide next year's crosses with the two unrepresented resistance groupings. To date, we have utilized 3 different genetic resources to identify Pierce's disease resistance. Progress was made with b43-17 and b40-14 both of which carry a major, as well as a minor locus for Pierce's disease resistance. Genetic mapping of these two different forms of *Vitis arizonica* have identified a Pierce's disease resistance region on chromosome 14, which we termed *PdR1*. We have mapped two forms of *PdR1* from *V. arizonica* / *candicans* b43-17, identified a minor gene on chromosome 19 (*PdR2*) and have mapped a third form, *PdR1c*, which originated from *V. arizonica* b40-14. Mapping of a multigenic source of Pierce's disease resistance from *V. arizonica* / *girdiana* b42-26 continues – a total of 916 markers were tested, and 170 polymorphic markers (45 more since the previous report) were added to the entire population of 239 seedlings. An initial genetic map was developed to assess the level of coverage on all 19 chromosomes. All markers were grouped and 15 linkage groups were established. The whole population is currently being screened for Pierce's disease resistance due to the relatively high variation in ELISA values we encountered in previous tests. We have 4-5 reps of 86 plants with results expected in December 2012. Mapping analysis with previous screen results indicate that chromosome 12 and 14 are involved in Pierce's disease resistance. Our focus is to refine the genetic map to get better coverage of all chromosomes, and obtain consistent greenhouse screen data in b42-26 background. We plan to combine these multiple resistance sources in our breeding program to ensure broad and durable resistance to Pierce's disease. 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 2 years and produce selections that are Pierce's disease resistant and 97% *V. vinifera*. These markers have also led to the identification of 6 genetic sequences that may house the Pierce's disease resistance gene, which we will soon be 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

INTRODUCTION

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. This project provides

the genetic support to enable our successful molecular breeding efforts (see companion Pierce's disease breeding project). We have initiated and completed mapping of a major Pierce's disease resistance locus originating from *V. arizonica* / *candicans* b43-17 that is the basis of our Pierce's disease breeding efforts. b43-17 is highly resistant with very low bacterial titers in the stem and without any disease symptoms. 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. Although they are morphologically different than b43-17, they both possess strong resistance to Pierce's disease and greatly suppress *Xylella fastidiosa* levels in stem tissue after greenhouse screening. The breeding part of the program produced and greenhouse screened 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 US and Mexico. Initial greenhouse screen results indicate that we have twenty other accessions that possess strong Pierce's disease resistance.

OBJECTIVES

1. Fine-scale mapping of additional quantitative trait loci (QTL) for Pierce's disease resistance in the 04191 (F2-7 x F8909-17) population (*Completed*)
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 US 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*, and develop new and exploit existing breeding populations to genetically map this resistance.
4. Complete the physical mapping of PdR1a and PdR1b and initiate the sequencing of BAC clones that carry PdR1a gene candidates.

RESULTS AND DISCUSSION

Objective 1

We developed a framework genetic map of the 04191 (F2-7 x F8909-17) population that carries the major locus *PdR1a*. The main objective of this map was to identify any other minor QTL contributing to Pierce's disease resistance. A total of 139 simple sequence repeat (SSR) markers representing all 19 chromosomes were added to a set of 150 genotypes. QTL analysis confirmed a major locus *PdR1a* on chromosome 14, and identified a minor QTL (*PdR2*) on chromosome 19. *PdR2*'s LOD score was 2.3 and it explains 7% of the phenotypic variation, which peaks at marker CB918037 (Fig 1). This QTL is within a 10 cM interval – a relatively long genetic distance for effective marker assisted screening. To shorten the genetic distance between the markers, we utilized the Pinot noir 40024 genome sequence for that region to develop more markers. The positions of marker UDV023, CB918037, and VMC5e9 on the genome sequence are at 2,346,119, 2,974,668, and 4,182,806 bp, respectively. This provided us 1.783 Mbp to develop 7 SSR primers from this region. These primers are in the process of being tested for polymorphism. Useful markers will be added to the entire population of 150 seedlings. These markers would allow us to reduce the gap from 10 cM.

Statistical analysis of LG 14 and LG 19 indicated that both loci work independently of each other and do not have an additive impact. Identification of this minor QTL is important to understand genetic interactions and to spot epistatic interactions. Further work would allow us to narrow down the region and facilitate the screening of the PN40024 genome sequence to identify the nature of genes in that region. In order to study the impact of minor QTL, we made two crosses with 04373-02 and 04373-22 and Pinot Blanc. The goal is to discard all those plants that carry the *PdR1a* locus, greenhouse screen all other plants to test their level of resistance to Pierce's disease and use these populations to study and verify the *PdR2* region without interactions with the *PdR1* locus. A total of 100 plants were screened with SSR markers and 43 plants were planted in the field in spring 2012. These plants will be greenhouse screened in 2013.

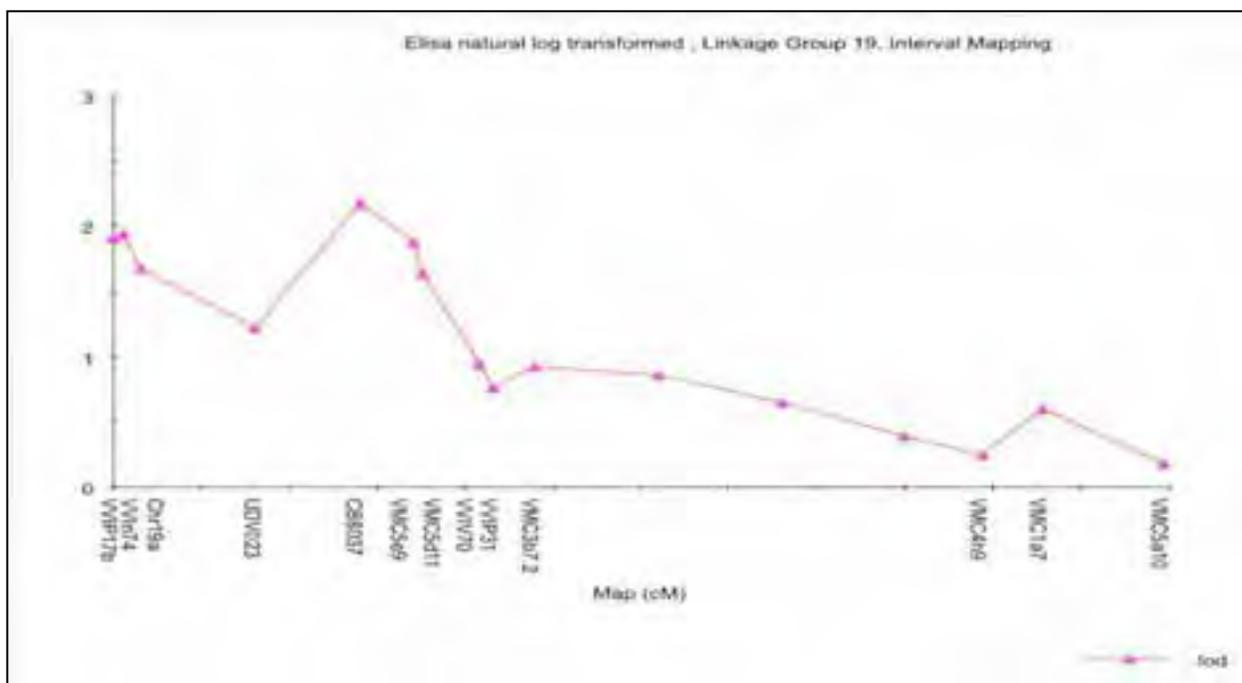


Figure 1. Updated interval mapping analysis of the *PdR2* locus on LG 19.

Objective 2

We have 918 SSR markers in our database to test the 05347 (F2-35 x b42-26) F1 population; 763 of these SSR primers amplified b42-26 DNA successfully, and 180 markers were polymorphic. The level of polymorphic markers is relatively low at 23%. We have not observed such a low level of polymorphism in any other genotype so far. Because the main focus of the work is to develop a genetic linkage map of the resistant parent, we are interested only in those markers that could be used for b42-26. We have completed 173 of polymorphic markers on the entire population of 239 progeny – an additional 48 markers since the previous report. A framework map with 125 markers was developed. Figure 2 represents the framework map of b42-26. The 125 markers grouped into 18 linkage groups; no marker was polymorphic for chromosome 6. For three linkage groups, markers were not mapped due to the large distance between them. A genetic map with 180 markers will be complete by the next reporting period. Our goal is to obtain average distance between markers below 5 cM for QTL analysis.

Greenhouse screening was completed on 164 accessions, however results were not conclusive due problems maintaining greenhouse temperatures. We have observed that the severity of the greenhouse screen is much more pronounced in the hot summer months. For traits that segregate quantitatively, it is extremely important to obtain cleaner phenotypic data. We are repeating the greenhouse screen for this population. For this purpose, we have made 4-5 reps of green cuttings from 82 genotypes and inoculations were carried out. ELISA sampling is scheduled for 10/4/2012. Greenhouse space is the limiting factor to screen whole population at one time period to reduce the environmental variance. As more greenhouse space becomes available, we plan to screen the rest of the population so results can be used for the QTL analysis.

A single dominant gene controls resistance to Pierce's disease in *V. arizonica* b40-14. 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. 227 markers were polymorphic for one of the parents; 152 were analyzed on the entire set of 122 plants; a framework map of R8918-05 was produced with MAP QTL (4.0) and the Kruskal-Wallis approach was used to complete the preliminary analysis. Pierce's disease resistance mapped only on chromosome 14 – the same chromosome where *PdR1a* and *PdR1b* mapped. 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

Table 1. Crosses made in 2012 to develop genetic maps in new accessions from southern USA and Mexico germplasm. Crosses 08-319 and 08-326 are selfings of Zinfandel and Cabernet Franc respectively and are 100% *V. vinifera*.

Resistant source	Geographic origin - Appearance phenotype	Pure <i>Vinifera</i> types used in 2012 crosses	Estimated # of seeds
ANU5	Littlefield, AZ	Alicante Bouschet	140
	Unique	Grenache	100
b40-29	Chihuahua, MX	08319-07	280
	like b43-17	08319-29	45
b41-13	Ciudad Mante, MX	08319-07	250
	like b42-26	08319-29	95
b46-43	Big Bend, TX	08319-07	320
	Unique	08319-12	150
		08326-61	200
b47-32	Big Bend, TX	08319-07	190
	Unique	08319-12	90
		08326-61	140



Figure 3. Map indicating collection locations of recently tested *Vitis* species. Green dots indicate promising resistant material.

In order to understand the genetic diversity and gene flow in the material collected from southwestern USA and Mexico, DNA was collected from all those genotypes that were screened with ELISA, and additional genotypes

including *V. vinifera* were added. Eighteen SSR markers selected for their polymorphism were used to group these accessions four clades (**Figure 4**). These preliminary results revealed that resistance exists in several groups, which will enable us to expand the genetic base of Pierce's disease resistance in our breeding program. So far resistant material from only two clades has been exploited.

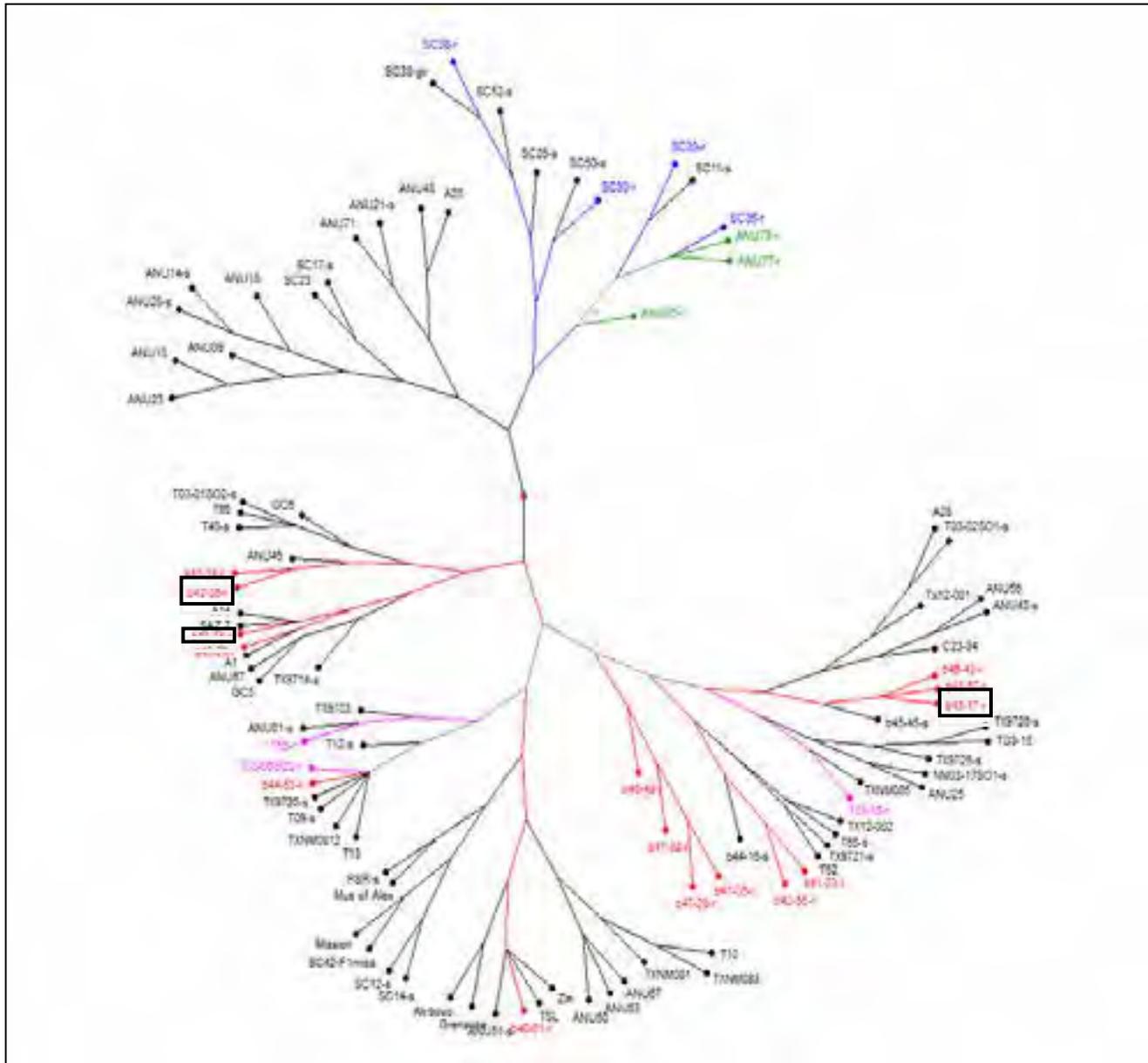


Figure 4. Hierarchical clustering (Ward method) based on the allele data with 18 SSR markers. Square boxes represent genotypes that are currently being used in the breeding program. All red accessions were collected from Mexico as seeds. Blue, green, and pink resistant accessions were collected from three recent collection trips.

Objective 4

We have used three categories of sequences (shotgun reads, fosmid reads and 454) for the BAC clone H69J14 that carries the Pierce's disease resistance gene(s). From the assembly of this sequence, we have identified 6 copies ranging from 2Kb to 3.1Kb in the resistance region. Copies 1 thru 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 on the 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 confirming the complexity of the region.

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 process of using FGS technology allows gap closure. For this purpose, we identified three overlapping BAC sequences (H15B20, H69J14 and H64M16) that span about 450Kb of physical sequence. Complete assembly of this region will allow a comparison to susceptible PN40024, identify differences in the expressed and non-expressed regions helping to identify the susceptible allele of the *PdR1b* gene. Results are expected within 2 months.

FUNDING AGENCIES

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

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

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LAYPERSON SUMMARY

We maintain and characterize many populations while breeding Pierce's disease resistant wine grapes, some of which have been used to develop genetic maps. These maps have been used to identify genetic markers that are tightly linked with Pierce's disease resistance, which have allowed classical breeding to be greatly expedited through marker-assisted selection. Genetic maps allow the construction of physical maps to identify resistance genes (Riaz et al. 2008; Riaz et al. 2009). The physical map of the b43-17 resistance region allowed us to identify six candidate genes, which may be responsible for conferring Pierce's disease resistance. Comparisons with plant genomes indicated that multiple tandem repeats of the disease resistance gene family Receptor-like proteins with LRRs domains were present in the resistance region. This category of genes is involved in the recognition of microbes and in the initiation of a defense response (Bent and Mackey 2007). We completed the cloning of five candidate genes: *PdR1b.1, 2, 4, 5 and 6* and confirmed their sequence. We also developed embryogenic callus cultures of Pierce's disease susceptible *Vitis vinifera* Chardonnay and Thompson Seedless and rootstock *Vitis rupestris* St. George for genetic transformation to verify candidate Pierce's disease resistance gene function. *PdR1b.1, 2, 4, 5, and 6* have been used in transformation of tobacco and grape. Tobacco plants transformed with all *PdR1b* candidates have been multiplied and are ready to be tested against *Xylella fastidiosa* in the greenhouse; preliminary testing has been conducted with untransformed plants. Transformed embryogenic callus of grape are producing plants. To reduce the time-span for generating healthy transgenic plants we also tested two different methods that employ organogenesis for *Agrobacterium*-mediated transformation. We were successful in streamlining one method that will allow us reduce the time required to generate transformed plants by four months. We also initiated total RNA extraction experiments to allow time course examinations of gene function from leaf and stem tissues. These were successfully completed and we are now ready to evaluate gene function over time in inoculated and uninoculated plants of the *PdR1* containing resistant selections F8909-08 and F8909-17, their resistant parent b43-17, their susceptible parent *V. rupestris* A. de Serres, and the susceptible control Chardonnay. These plants have been established in the greenhouse and have been inoculated later this summer.

INTRODUCTION

New cultivars bred to resist *Xylella fastidiosa* (*Xf*) infection and subsequent expression of Pierce's disease 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 *vinifera* wine and table grapes. Another approach is "cisgenesis" – the transformation of elite *Vitis 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. The cisgene approach in grapes is similar to the natural clonal variation that exists in many winegrape cultivars. This linkage-drag-free approach is attractive, and also allows the opportunity to stack additional resistance genes from other *Vitis* sources, even if these genes originate from the same chromosomal position in different species or accessions (Jacobsen and Hutten 2006). The physical mapping of the resistance region from *V. arizonica* / *candicans* b43-17, *PdR1*, allowed the identification of potential candidate resistance gene(s). Preliminary comparison indicated that the *PdR1* region contains multiple tandem repeats of Serine Threonine Protein Kinase with a LRR domain (STPK-

LRR) gene family. This category of genes belongs to a group involved in plant resistance. Their defense mechanism is based on compounds involved in the recognition of microbe-associated molecular patterns (MAMP) like compounds, which initiate a defense response (Bent and Mackey 2007). In order to gain insight and to verify the function of resistance gene(s), cloning and functional characterization is required. In this report, we present the progress on the cloning and testing of four candidate resistance genes.

OBJECTIVES

1. Cloning, structural analysis and gene annotation via comparison of the *PdR1b* locus to the susceptible Pinot noir genome sequence using the assembled sequence of the BAC clone H64J14.
2. Expression studies of candidate genes.
3. Development of alternative protocols for genetic transformation for the validation of gene constructs
 - a. *Agrobacterium*-mediated transformation of the susceptible *Vitis* cultivars (Chardonnay and Thompson Seedless, and the rootstock St. George).
 - b. Transformation of tobacco.

RESULTS AND DISCUSSION

Objectives 2 & 3: Genetic transformation for the validation of gene constructs / development of alternative protocols

Once 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 makes it resistant. Currently the most widely used method for the production of transgenic / cisgenic grapes is based on *Agrobacterium* transformation followed by regeneration of plants from embryogenic callus. We have established cultures of pre-embryogenic callus derived from anthers of *V. vinifera* Thompson Seedless and Chardonnay and the rootstock *V. rupestris* St. George. These cultures of embryogenic calli can be used readily for transformation (Agüero et al. 2006).

Candidate genes have been cloned successfully. We have subcloned *PdR1b.1* into binary vectors pCAMBIA-1303 (www.cambia.org) and pDU99.2215 and *PdR1b.2, 4, 5* and *6* into pCAMBIA-1303. *PdR1b.3* will not be pursued until flanking sequence information is available. pCAMBIA-1303 was included in the experiments because it carries a hygromycin resistance gene that improves the selection of transformants (D. Tricoli, pers. comm.). An additional advantage is that it allows subcloning the gene in one step, by replacing the *gus* gene with the gene of interest. The resulting plasmids have been used for transformation via *Agrobacterium tumefaciens* of Chardonnay, Thompson Seedless, St. George and tobacco SR1.

Transgenic tobacco plants carrying each candidate gene (9-10 independent lines per gene) have been produced at the UC Davis Transformation Facility and multiplied *in vitro* in our lab. Genomic DNA was isolated from plants of each line with DNeasy Plant Mini Kit (Qiagen). A primer that binds the CaMV 35S promoter and a primer that binds within the coding region of each *PdR1b* candidate were used in combination for PCR amplification to verify the presence of the transgene. All candidate genes amplified successfully (**Figure 1**) with the exception of *PdR1b.1* subcloned into pDU99.2215 (not shown).

Preliminary experiments have been conducted with untransformed plants based on the work by Francis et al., 2008. Plants were inoculated with 10 µl of a Beringer *Xf* strain suspension on water of (OD₆₀₀=0.25) on both sides of the stem using the pinprick technique as in grapevines. Because plants were symptomless 8 weeks after inoculation, they were cut to 10 cm and a second inoculation was performed on new growth leaves. This inoculation consisted of applying 20 µl of bacterium suspension to a 1 cm long incision made at the base of the main vein of the leaf. This type of inoculation produced symptoms on that particular leaf approx. 3-4 weeks after inoculation. *Xf* infection was later verified with ELISA.

Micro-propagated transgenic tobacco plants have been acclimated to greenhouse conditions for testing against *Xf*. They are being tested in groups that include 5 replicates of 3 independent lines of 3 candidate genes due to greenhouse space availability. Two additional plants / line are inoculated with water. Untransformed plants are subjected to the same treatments (**Figure 2**).

Embryogenic calli of Thompson Seedless, Chardonnay, and St George transformed with the 5 candidate genes have been selected in medium with antibiotics and subcultured to germination medium for plant regeneration (**Figure 3**). A summary of the progress achieved for each candidate gene is shown in **Table 2**.

Two alternative transformation techniques via organogenesis have been tested to reduce the time needed to produce transgenic grapes. These methods have been developed in Thompson Seedless and are based on the use of meristematic bulks (MB) or etiolated meristems (EM) as explants for inoculation with *Agrobacterium* (Mezzetti et al. 2002, Dutt et al. 2007). In the first method, *Agrobacterium* is inoculated on slices of MB. Using this procedure, transgenic plants of Thompson Seedless expressing GFP were produced in 3 months. No plants were regenerated from etiolated meristems and the procedure was laborious and time consuming. We have produced meristematic bulks of Chardonnay and St. George. They were inoculated with *Agrobacterium* carrying *PdR1b.4* in pCAMBIA 1303. We tested three initial levels of hygromycin, 5, 10, and 15 ug/ml. Hygromycin concentration was then increased gradually up to 25 ug/ml with each subculture. Since callus didn't grow with any of the concentrations tested we are now assaying 0 ug/ml at the first step after inoculation.

Transformation of pre-embryogenic cultures or MB have been performed with *A. tumefaciens* EHA105 pCH32, carrying binary plasmids with *PdR1b* coding sequences. Overnight cultures of the bacteria in LB medium + antibiotics are diluted to 10^8 cells·ml⁻¹ using a liquid co-cultivation medium. Pre-embryogenic calli are placed on a sterile glassfiber filter (GFF) overlaid on co-cultivation medium. The *Agrobacterium* culture is poured over the callus and excess is blotted with sterile filter paper after 5 min. Meristematic bulk slices are dipped in bacteria suspension for 10 minutes. Pre-embryogenic callus or MB are then transferred onto fresh co-cultivation medium. After 48 h in the dark, MB or callus pieces, sub-divided into clumps of about 2 mm in diameter, are cultured on selection medium containing 100 ug/ml kanamycin or 0-15-25 ug/ml hygromycin.

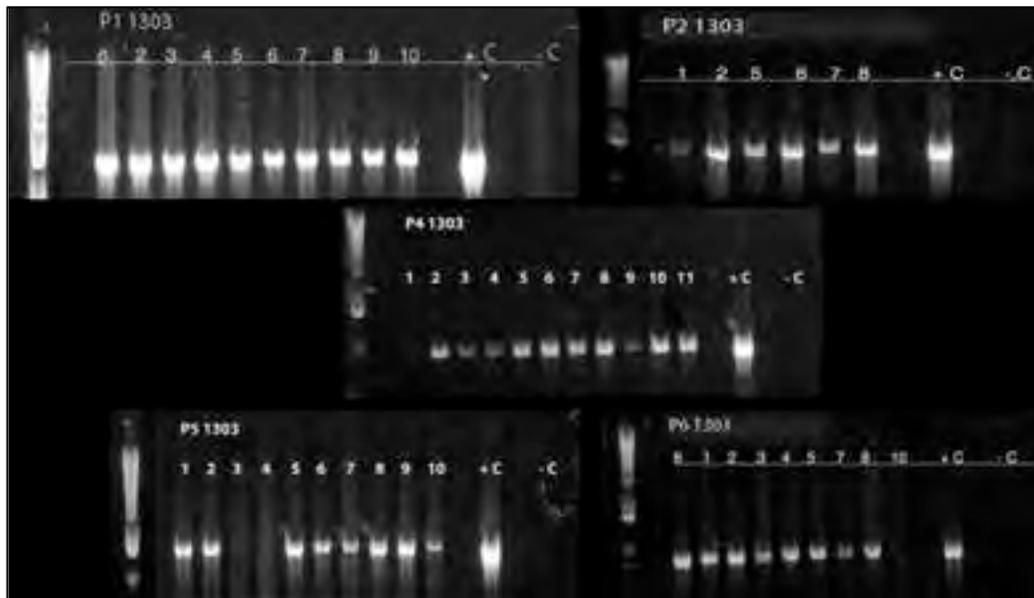


Figure 1. PCR amplification of *PbRI* transgenes in tobacco.



Figure 2. **a.** Tobacco plants after greenhouse acclimation; **b.** Tobacco growing in the greenhouse after inoculation with *Xf*; **c.** Untransformed tobacco leaf showing symptoms of Pierce's disease infection.

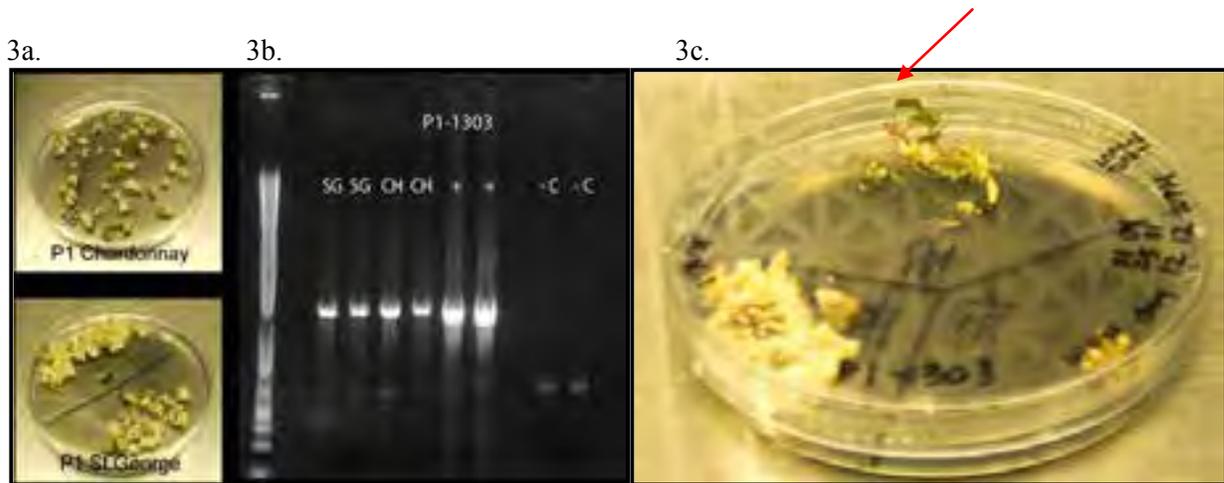


Figure 3. **a.** *PdR1b.1* embryogenic callus growing in germination medium, **b.** PCR amplification of *PdR1b.1* in transformed embryogenic callus of St George (SG) and Chardonnay (CH), **c.** Plant regeneration from a *PdR1b.1* embryogenic callus

Table 2. Progress status of transformation experiments

	Cloned into binary plasmid	Grape transformation / plants	Tobacco transformation / plants
PdR1b.1	x	x / plants <i>in vitro</i>	x / plants in GH, inoculations underway
PdR1b.2	x	x	x / plants in GH, inoculations underway
PdR1b.4	x	x	x / plants in GH, inoculations underway
PdR1b.5	x	x	x / plants in GH, inoculations underway
PdR1b.6	x	x / plants <i>in vitro</i>	x / plants in GH, inoculations underway

CONCLUSIONS

The last step in the characterization of a resistance gene is to verify that the isolated gene functions in a host plant. This process requires that the gene is transformed into a susceptible host and challenged by the disease agent. *Agrobacterium*-based transformation can be used with grape, but initiating transformable and regenerable tissue is often a problem with grape. We have cloned 5 *PdR1b* candidate genes and used them in genetic transformation of tobacco and preembryogenic callus of Chardonnay, Thompson Seedless, and St. George to produce transgenic plants for use in testing the *PdR1b* candidates. Plants of transformed tobacco with *PdR1b.1*, 2, 4, 5, and 6 have been obtained and are ready to be tested against *Xf* in the greenhouse. We are also

testing another technique to speed the development of transgenic tissue from meristematic bulks that will allow *PdR1* gene candidates to be tested in a much broader range of genotypes. Although we are currently producing transgenic grape plants using the traditional procedure.

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INDUCTION OF PHENOLIC COMPOUNDS IN RESPONSE TO *XYLELLA FASTIDIOSA* INFECTION IN FIVE DIFFERENT GRAPEVINE CULTIVARS

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Reporting Period: The results reported here are from work conducted April 2011 to September 2012.

ABSTRACT

Previously, Thompson Seedless grapevines infected with *Xylella fastidiosa* (*Xf*), the causal agent of Pierce's disease, were observed to possess greater phenolic levels in xylem sap and tissues than non-infected plants shortly after inoculation (Wallis and Chen 2012). Plants often produce greater levels of phenolic compounds in response to pathogen infections, and commercially-available phenolic compounds were observed to inhibit *Xf* growth *in vitro* (Maddox et al. 2010). Therefore, an improved understanding of induced phenolic production is needed in order to optimize efforts to breed Pierce's disease tolerant cultivars. Phenolic compounds in xylem sap were examined by high-performance liquid chromatography at two-, four-, and six-months after mock- or *Xf*-inoculation for five different cultivars (Chardonnay, Flame Seedless, Grenache Noir, Merlot, and Rubired). No differences overall were observed at two months post-inoculation between mock- and *Xf*-inoculated grapevines, but Flame Seedless and Grenache Noir grapevines possessed greater levels of sap phenolics than Cardonnay, Merlot, or Rubired grapevines. Four months after mock- or *Xf*-inoculation, levels of catechins (catechin and epicatechin), procyanidins (various catechin dimers and trimers), and hydroxycinnamoyltartaric acids (coumaric acid and ferulic acid) were greater in xylem sap from *Xf*-infected versus control grapevines. Merlot grapevines had greater sap phenolic levels than the other cultivars four months after inoculation. Six months post-inoculation, levels of coumaric acid, hydroxyphenylpropionic acid, patulein, and a procyanidin B isomer were greater in *Xf*-inoculated versus mock-inoculated grapevines. Also six months after inoculation, Grenache Noir grapevines had lower phenolic levels than the four other cultivars. Coumaric acid, ferulic acid, epicatechin, and four procyanidins were negatively associated with *Xf* titer suggesting antibiotic activity. Disease symptoms did not appear until six months after inoculation, at which time Merlot grapevines had greater symptom severity than Rubired. Disease severity did not otherwise differ between cultivars. Sap levels of procyanidins were positively correlated with disease severity measurements. It was possible that increased phenolic levels in xylem sap resulted in *Xf* aggregation and biofilm formation, which in turn resulted in greater Pierce's disease symptom progression. This could explain why Merlot had significantly greater symptom expression than Rubired even though Merlot possessed more putatively antibiotic phenolic compounds than the other cultivars at four months post-inoculation.

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contacting walls of almost any of these secondary xylem cell types. We recently investigated three types of pits and their PMs (**Figures 2-4**): intervessel, vessel-parenchyma, and interfiber pit. Intervessel pits and PMs are the barriers that *Xf* cells must pass through for their systemic spread (**Figure 2**). Intervessel pit pairs were bordered pits and occur very densely in the contact walls of adjacent vessels (**Figure 2A**). Along the contacting primary cell walls of two adjacent vessels were narrow regions covered by secondary walls and much wider regions without secondary walls—PMs (**Figure 2A**). Secondary wall borders of each pit arched over the majority of the PM of each pit pair (**Figure 2A-D**). Compared with the primary wall regions underneath the secondary walls (sw), intervessel PMs (pm) had the same thickness and density (**Figure 2B**), were swollen (**Figure 2C**), or appeared less dense (**Figure 2D & E**). The less dense intervessel PMs may have resulted from their partial disintegration (**Figure 2E**).

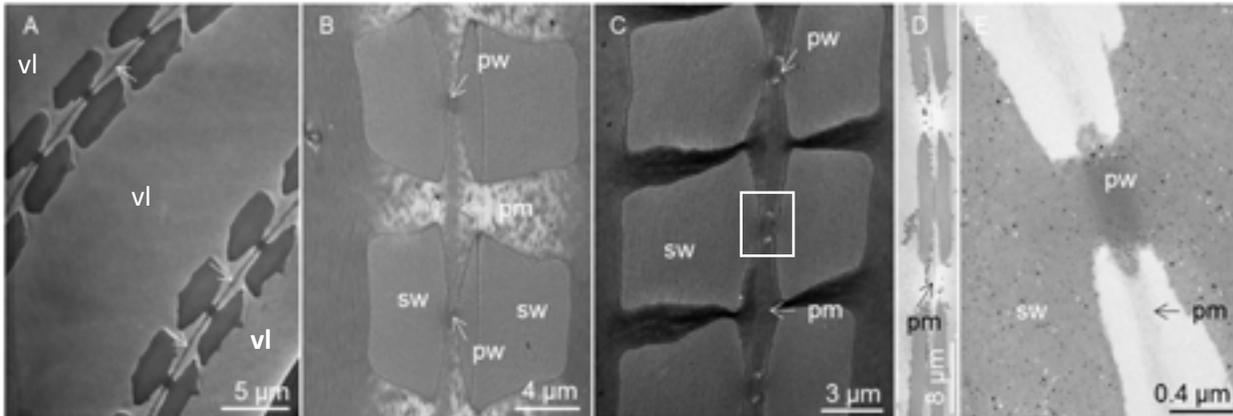


Figure 2. Intervessel pits and PMs in longitudinal section. A. Part of three adjacent vessels, showing densely arranged intervessel pit pairs and PMs (arrows) along the contact walls of adjacent vessels (vl: vessel lumen). B-D. Structural details of intervessel pits and PMs. Compared to the primary wall extension regions (pw) directly underneath the secondary walls (sw), intervessel PM (pm) regions are the same in thickness and density (B), swollen (C), or thinner and looser in appearance (D). E. The enlargement of the pit region indicated by the white frame in D, showing the PM (pm) is less dense and thinner than the primary wall extension (pw) covered by secondary walls (sw).

Vessel-parenchyma pits and PMs are related to the formation of vascular occlusions, including tyloses and gels. Vessel-parenchyma pit pairs were half-bordered with a bordered pit on the vessel side and a simple pit on the parenchyma cell side (**Figure 3A**). Vessel-parenchyma PMs could be distinguished into two more or less density-distinct regions across its thickness from the parenchyma cell wall side to its vessel wall side. The PM wall region facing the parenchyma lumen was less dense than the wall region facing the vessel lumen (**Figure 3B**). Pits were also sparsely present between adjacent fiber cells (**Figure 4**). Interfiber pits formed simple pit pairs with a simple pit on each of the two fiber cells (**Figure 4A**). Interfiber PMs were dense and also similar in density to the regions of the fibers' contacting primary walls underneath the secondary walls, but the former are usually thinner than the latter (**Figures 4B and C**).

Immunogold-transmission electron microscopy for detection of fucosylated xyloglucans (XyG) in the three types of PMs

In our previous projects, we successfully combined an immunohistochemical method with confocal laser scanning microscopy (CLSM) and scanning electron microscopy (immunogold-SEM), respectively, to analyze the PM polysaccharide compositions and distributions. With those techniques, we have revealed that some potential substrates of *Xf*'s cell wall degrading enzymes were abundantly present as components of the intervessel PMs of Pierce's disease susceptible grapevines, but were absent or occurred at low levels in PMs of Pierce's disease resistant grapevines. This indicates that the polysaccharide compositions of intervessel PMs may contribute to Pierce's disease resistance of the grapevines. These techniques involve the usage of both primary and secondary antibodies. Since the antibodies have relatively large molecular sizes, their entry to the interior of PMs might be restricted. Therefore, what the two methods have revealed reflects the status of the

polysaccharides closer to the PM surface. In order to reveal the compositions and distribution of the polysaccharides through the thickness of PMs at high resolution, we recently developed an immunogold-transmission electron microscopy (TEM) technique. Xylem samples used for this method were fixed in buffered paraformaldehyde. After being trimmed to small blocks (1mm x 1mm x 1mm), they were dehydrated via an ethanol series until 95% ethanol. Following this the blocks were infiltrated with LR White resin. Specimens were then embedded in the same resin and the resin containing samples was cured with UV light at -20 °C. After being trimmed, the resin blocks were cut with an ultratome to make sections of 80 -120 nm in thickness. Sections were picked up onto grids and treated with a specific monoclonal cell wall antibody followed by a matched colloidal gold-conjugated secondary antibody. After being air-dried, the sections were examined using TEM. The presence and distribution of the specific cell wall polysaccharide(s) is indicated by the defined gold particles appearing as dark dots in the TEM view.

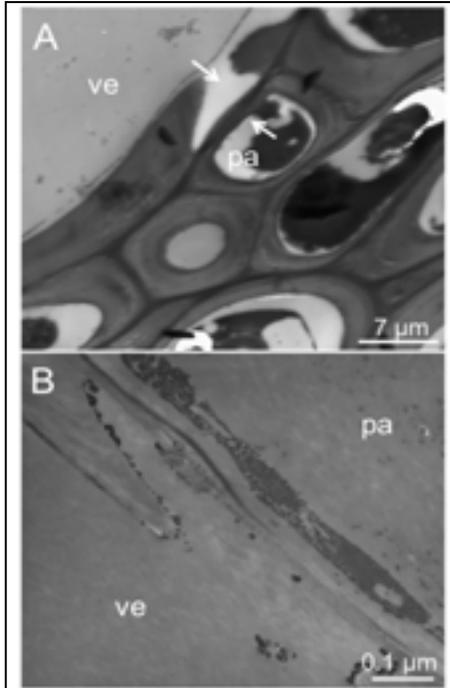


Figure 3. Vessel-parenchyma pits and PMs in transverse section. A. A half bordered pit pair (arrows) between a vessel (ve) and a parenchyma cell (pa). B. Enlargement of a vessel-parenchyma PM, showing the less dense region toward the vessel lumen (ve) and more compact region toward the parenchyma cell (pa).

The quantity and distribution of fucosylated XyG in interfiber PMs was also tested with the immunogold-TEM method (**Figure 7**). It was found that the polysaccharide was not evenly distributed across a PM's thickness, but mostly restricted to the two parallel strip regions of a PM, which were also parallel to the PM's surface. Each of the strips was approximately 0.15 µm from the PM's surface and 0.2 µm in thickness (**Figure 7**). The two strips were 3 µm separated from each other. Fucosylated XyG was abundant and more or less randomly distributed in the two regions (**Figure 7A**), but seldom occurred either outside the regions (i.e., nearer the PM's surface; **Figure 7B**) or between the two regions (**Figure 7C**). The quantity and distribution of fucosylated-XyG in the primary cell wall extension of the PM under the secondary cell walls had a pattern similar to that in the actual PM.

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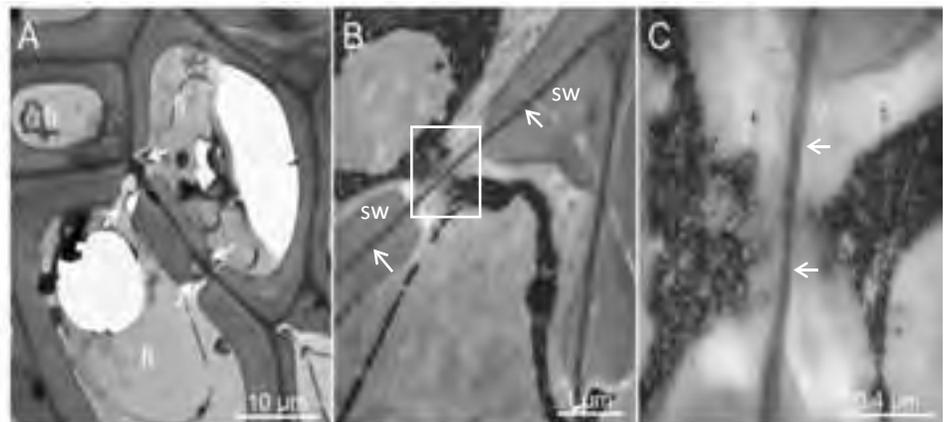


Figure 4. Interfiber pits and PMs in transverse section. A. Two simple pit pairs (arrows) between two fiber cells (fi). B. Enlargement of one interfiber pit pair and PM. The PM is thinner than its extended primary wall regions (arrows) underneath secondary walls (sw). C. Enlargement of the PM region framed in B, showing its dense appearance.

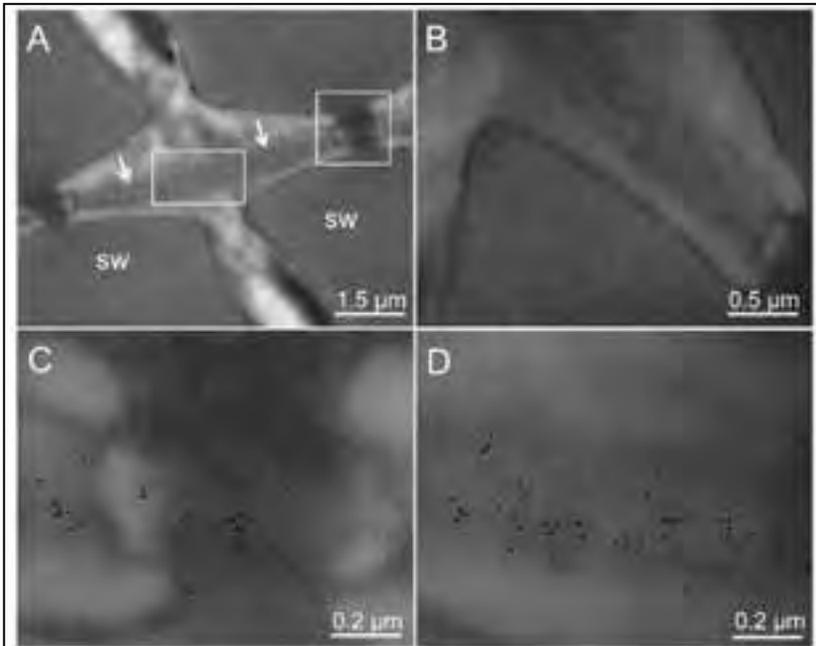


Figure 5. Distribution of fucosylated XyG in intervessel PMs of U0505-35 grapevines. A. An intervessel pit and PM (arrows). The square and rectangular frames indicate the PM region (uncovered) and its primary wall extension underneath secondary walls (sw), respectively. B. Enlargement of part of the intervessel pit and PM, showing gold particles (dark dots) randomly distributed throughout the PM's width and thickness. C-D. Enlargements of the square (C) and rectangular (D) regions in A, indicating the presence of XyG in the primary wall region covered by secondary wall (C) and the PM region (D).

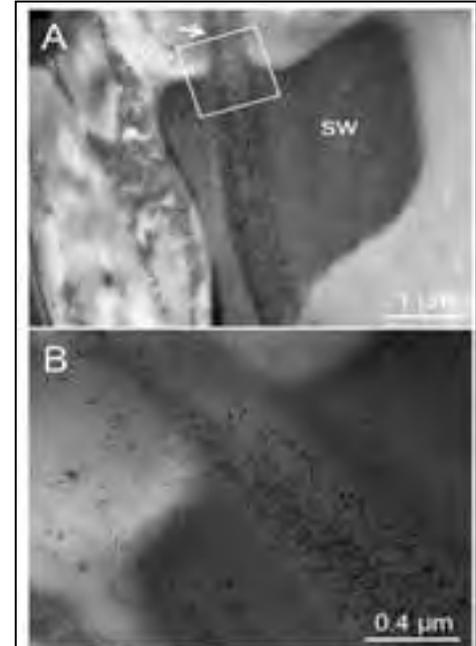


Figure 6. Distribution of XyG in vessel-parenchyma PMs. A. Part of a vessel-parenchyma PM and its extension underneath secondary walls, showing presence of XyG in both regions. B. Enlargement of the square region in A, showing that XyGs are more abundant in the primary wall region covered by secondary walls than in the PM region *per se*.

CONCLUSIONS

1. Vascular occlusions in *Xf*-infected Pierce's disease susceptible grapevines may significantly reduce the hydraulic conductivity of the vines and likely exacerbate the disease symptom development. This suggests that a solution to control/mitigate Pierce's disease symptom might be sought through the control of vascular occlusion formation in infected vines.
2. The immunogold-TEM method we established is proven to be effective in studying the quantity and distribution of pectic and hemicellulosic polysaccharides across the PM thickness with high resolution. This technique provides a way to clarify the Pierce's disease resistance mechanism of host vines from cell wall structure and composition.

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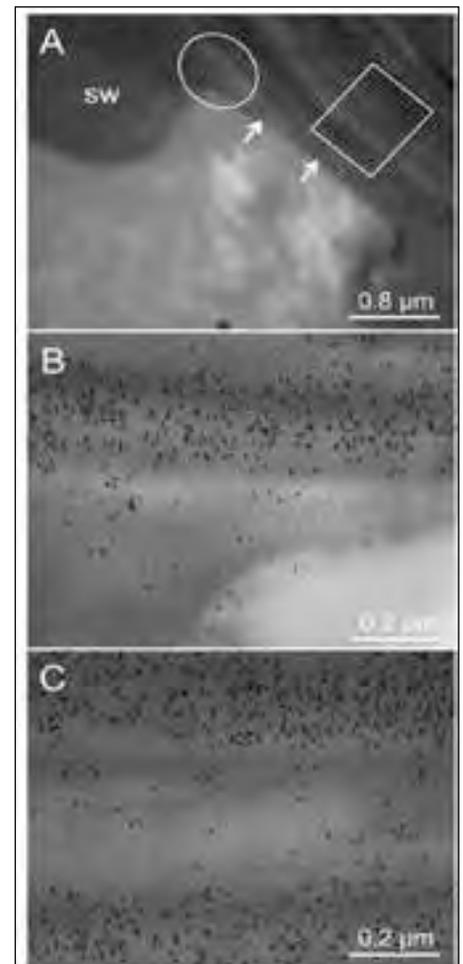


Figure 7. Distribution of fucosylated XyG in interfiber PMs. A. Part of an interfiber PM and its extension underneath the secondary walls, showing abundant fucosylated XyG presence in two strip regions across the PM's thickness that extend through the PM width to the primary wall region covered by secondary wall. B. Enlargement of the circled region indicated in A, showing only a small amount of fucosylated XyG in the superficial region of a PM. C. Enlargement of the square region indicated in A, showing the two strip regions with abundant fucosylated XyG and a wide region between the strips that lacks of fucosylated XyG.

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