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TABLE OF CONTENTS

Section 1: Vector Biology and Ecology

REPORTS

- **Substrate-Borne Vibrational Signals in Intraspecific Communication of the Glassy-winged Sharpshooter**
  Rodrigo Krugner and Valerio Mazzoni 2

ABSTRACTS

- **Multiple, Stochastic Factors Can Determine Acquisition Success of the Foregut-Borne Bacterium, *Xylella fastidiosa*, By a Sharpshooter Vector**
  Elaine A. Backus and Elizabeth E. Rogers 6

- **A New Paradigm for Vector Inoculation of *Xylella fastidiosa*: Direct Evidence of Egestion and Salivation Supports that Sharpshooters Can Be "Flying Syringes"**
  Elaine A. Backus, Holly J. Shugart, Elizabeth E. Rogers, J. Kent Morgan, and Robert Shatters 7

- **Exploring Glassy-winged Sharpshooter Microbiota Using Deep 16S rRNA Sequencing from Individual Insects**
  Elizabeth E. Rogers and Elaine A. Backus 8

- **Effects of Feeding on Glassy-winged Sharpshooter Lipid Content and Egg Production**
  Mark Sisterson, Christopher Wallis, and Drake Stenger 9

Section 2: Vector Management

REPORTS

- **The Riverside County Glassy-winged Sharpshooter Program in the Temecula Valley**
  Matt Daugherty and Diane Soto 12

- **RNA-Interference and Control of the Glassy-winged Sharpshooter and Other Leafhopper Vectors of *Xylella fastidiosa***
  Bryce W. Falk 16

- **Development and Use of Recombinant *Homalodisca coagulata Virus-1* for Controlling the Glassy-winged Sharpshooter**
  Bryce W. Falk, Bryony Bonning, W. Allen Miller, and Drake Stenger 23

- **Selective Disruption of Glassy-winged Sharpshooter Maturation and Reproduction by RNAi**
  Shizuo George Kamita 29

- **Monitoring for Insecticide Resistance in the Glassy-winged Sharpshooter in California**
  Thomas M. Perring and Nilima Prabhaker 31

- **Management of Insecticide Resistance in Glassy-winged Sharpshooter Populations Using Toxicological, Biochemical, and Genomic Tools**
  Rick Redak, Frank Byrne, and Bradley White 36

ABSTRACTS

- **RNA-Interference Strategies Against the Glassy-winged Sharpshooter and Other Leafhopper Vectors of *Xylella fastidiosa***
  Raja Sekhar Nandety, Tera L. Pitman, and Bryce W. Falk 39

Section 3:
Pathogen Biology and Ecology

REPORTS

- **Identification of a New Virulence Factor Required for Pierce's Disease and Its Utility in Development of a Biological Control**
  Thomas J. Burr, Patricia Mowery, Luciana Cursino, and Lingyun Hao 42

- **Defining the Role of Secreted Virulence Proteins LesA and PrtA in the Pathobiology of *Xylella* and in the Development of Pierce's Disease**
  Abhaya M. Dandekar 50

- **Characterization of the *Xylella fastidiosa* PhoP/Q Two-Component Regulatory System**
  Bruce Kirkpatrick 57

- **Elucidating the Process of Cell-Cell Communication in *Xylella fastidiosa* to Achieve Pierce's Disease Control by Pathogen Confusion**
  Steven Lindow 62

- **Characterization and Inhibition of *Xylella fastidiosa* Proteins Secreted by the Type II Secretion System and Their Secretion Machinery**
  Caroline Roper 74

- **Characterization of the Lipopolysaccharide-Mediated Response to *Xylella fastidiosa* Infection in Grapevine**
  Caroline Roper and Dario Cantu 82

ABSTRACTS

- **Role of Cold Shock Proteins in *Xylella fastidiosa* Virulence**
  Lindsey P. Burbank and Drake C. Stenger 91

- **Analyses of *Xylella* Whole Genome Sequences and Proposal of *Xylella taiwanensis* sp. nov.**
  J. Chen 92

- **Population Genetic Structure of *Xylella fastidiosa* Infecting Coffee and Citrus Trees in Sympatric Regions of São Paulo State, Brazil**
  Carolina S. Francisco, Paula C. Ceresini, and Helvecio D. Coletta-Filho 93

- **Host Selection and Adaptation Are Major Driving Forces Shaping Almond Leaf Scorch *Xylella fastidiosa* Populations in the San Joaquin Valley of California**
  Hong Lin 94

- **Overexpression of the MQSR-XF Toxin from the *Xylella fastidiosa* Toxin/Antitoxin System Induces Sessile Growth and Persister Cell Formation**
  M.V. Merfa, B. Niza, and A.A. de Souza 95

Section 4:
Pathogen and Disease Management

REPORTS

- **Exploiting a Chitinase to Suppress *Xylella fastidiosa* Colonization of Plants and Insects**
  Rodrigo Almeida 98

- **Building a Next Generation Chimeric Antimicrobial Protein to Provide Rootstock-Mediated Resistance to Pierce's Disease in Grapevines**
  Abhaya M. Dandekar 99

- **Chimeric Antimicrobial Protein and Polygalacturonase-Inhibiting Protein Transgenic Grapevine Field Trial**
  Abhaya M. Dandekar 106

- **Development of a Standardized Methodology to Assess the Efficacy of Genetic Constructs for Suppression of *Xylella fastidiosa* in Grape**
  David Gilchrist 118

- **Field Evaluation of Grape Plants Expressing Potential Protective DNA Sequences Effective Against Pierce's Disease**
  David Gilchrist, Abhaya Dandekar, Ann Powell, and Steven Lindow 122

- **Field Evaluation of Grape Plants Expressing PR1 and UT456 Transgenic DNA Sequences for Protection Against Pierce's Disease**
  David Gilchrist and James Lincoln 126

- **Transgenic Rootstock-Mediated Protection of Grapevine Scion By Dual Stacked DNA Constructs**
  David Gilchrist, James Lincoln, Abhaya Dandekar, and Steven Lindow.....133

- **Pierce's Disease Symptom Evaluation at the Solano County Research Block**
  Deborah Golino.....139

- **Evaluation of Pierce's Disease Resistance in Transgenic *Vitis vinifera* Grapevines Expressing *Xylella fastidiosa* Hemagglutinin Protein**
  Bruce Kirkpatrick 141

- **Inhibition of *Xylella fastidiosa* Polygalacturonase to Produce Pierce's Disease Resistant Grapevines**
  Bruce Kirkpatrick 149

- **Comparison and Optimization of Different Methods to Alter Diffusible Signal Factor Mediated Signaling in *Xylella fastidiosa* in Plants to Achieve Pierce's Disease Control**
  Steven Lindow 155

- **Continued Field Evaluation of Diffusible Signal Factor Producing Grape for Control of Pierce's Disease**
  Steven Lindow 162

- **Field Evaluation of Grafted Grape Lines Expressing Polygalacturonase-Inhibiting Proteins**
  Ann L.T. Powell and John M. Labavitch.....172

- **Tools for Identifying Polygalacturonase-Inhibiting Protein Transmission from Grapevine Rootstock to Scion**
  Ann L.T. Powell, John M. Labavitch, and Abhaya Dandekar 180

- **Continuation of the Field Evaluation of New Strategies for the Management of Pierce's Disease of Grapevine**
  Philippe Rolshausen, Matt Daugherty, and Peggy Mauk 185

- **Evaluation of Natural Products Isolated from Grapevine Fungal Endophytes for Control of Pierce's Disease**
  Philippe Rolshausen, Caroline Roper, and Katherine Maloney.....193

ABSTRACTS

- **Strategy for Bacterial Disease Control Using Genes from Pathogens**
  R. Caserta, R.R. Souza-Neto, M.A. Takita, and A.A. de Souza 199

Section 5:
Crop Biology and Disease Epidemiology

REPORTS

- **Blocking *Xylella fastidiosa* Transmission**
  Rodrigo Almeida and Fabien Labroussaa 202

- **Development of a Grape Tissue Culture and Transformation Platform for the California Grape Research Community**
  David Tricoli 211

- **Breeding Pierce's Disease Resistant Winegrapes**
  Andrew Walker 220

- **Map-Based Identification and Positional Cloning of *Xylella fastidiosa* Resistance Genes from Known Sources of Pierce's Disease Resistance in Grape**
  Andrew Walker 227

- **Molecular and Functional Characterization of the Putative *Xylella fastidiosa* Resistance Gene(s) from b43-17 (*Vitis arizonica*)**
  Andrew Walker 240

- **Molecular Breeding Support for the Development of Pierce's Disease Resistant Winegrapes**
  Andrew Walker and Dario Cantu 249

ABSTRACTS

- **Glassy-winged Sharpshooter Oviposition Effects on Photinia Volatile Chemistry with Implications on Egg Parasitoid Effectiveness**
  Christopher Wallis 256

AUTHOR INDEX 257

Section 1:

Vector Biology and Ecology

SUBSTRATE-BORNE VIBRATIONAL SIGNALS IN INTRASPECIFIC COMMUNICATION OF THE GLASSY-WINGED SHARPSHOOTER

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Reporting Period: The results reported here are from work conducted July 1, 2014 to October 1, 2014.

ABSTRACT
Exploitation of vibrational signals for suppressing glassy-winged sharpshooter (GWSS) populations could prove to be a useful tool. However, existing knowledge on GWSS vibrational communication is insufficient to implement a management program for this pest in California. Therefore, the objective of this study is to identify and describe substrate-borne signals associated with intraspecific communication of GWSS. Sound and video recordings of male and female GWSS on plants revealed a complex series of behaviors linked to vibrational signals that lead to mating. Data are currently being analyzed to characterize the spectral and temporal features of signals such as frequency span, dominant frequency, intensity, and pulse repetition time.

LAYPERSON SUMMARY
The goal of this research project is to describe and characterize vibrational signals used in glassy-winged sharpshooter (GWSS) intraspecific communication. A substantial amount of research on other leafhopper species has shown that individuals communicate solely by substrate-borne vibrational signals. To our knowledge, vibrational communication in GWSS has not been investigated in detail. Fundamental understanding of these factors is important for achieving our deliverable: a new management strategy to suppress GWSS populations and Pierce's Disease incidence.

INTRODUCTION
Epidemiological models suggest that vector transmission efficiency, vector population density, and the number of plants visited per vector per unit time are key factors affecting rates of pathogen spread (Jeger et al. 1998). Measures to reduce glassy-winged sharpshooter (GWSS) population density in California include an area-wide insecticide application program and release of natural enemies. Despite such efforts, geographic distribution of GWSS continues to expand. Chemical control of GWSS in urban areas, organic farms, and crops under integrated pest management programs is problematic because insecticides are ineffective, not used, or incompatible with existing practices, respectively. Thus, long-term suppression of GWSS populations will rely heavily on novel methods.

In leafhoppers, mate recognition and localization are mediated exclusively via substrate-borne vibrational signals transmitted through the plant. Exploitation of attractive vibrational signals for trapping leafhoppers or disrupting mating, as well as excluding pests via emission of repellent signals have been considered, but not yet implemented in commercial agricultural landscapes (Polajnar et al. 2014). In Florida, an experimental prototype of a microcontroller-buzzer system attracted the Asian citrus psyllid, *Diaphorina citri*, to branches of citrus trees by playback of insect vibrational signals (Mankin et al. 2013). Recently, small-scale field studies on mating disruption of leafhoppers via playback of vibrational signals through grapevines have demonstrated promising results. Specifically, electromagnetic shakers attached to wires used in vineyard trellis successfully disrupted mating of *Scaphoideus titanus*, vector of the grapevine disease Flavescence dorée in Europe (Eriksson et al. 2012). Exploitation of attractive and/or repellent signals for suppressing GWSS populations could prove to be a useful tool. However, existing knowledge on GWSS vibrational communication is insufficient to implement a management program for this pest in California.

OBJECTIVES
1. To identify and describe substrate-borne signals associated with intraspecific communication of GWSS in the context of mating behavior.

RESULTS AND DISCUSSION

This research project is being conducted at the USDA-ARS San Joaquin Valley Agricultural Sciences Center (SJVASC) in Parlier, California. Discovery in the GWSS behavioral analyses has been bioassay-driven and focused on the identification of signals that initiate natural responses to conspecifics. Insects being used in the experiments are obtained from colonies maintained year-round at the SJVASC. Briefly, late-instar GWSS obtained from colonies are separated by gender in cages to generate virgin adult individuals. Only reproductively active females are being used in the recordings.

A series of laboratory studies are being conducted to describe vibrational signals used in GWSS communication. First, virgin males and females are placed on host plants individually to identify common and unique signals produced by each gender. Second, males and females are paired on host plants to identify signals used in advertisement and species recognition, male-female duetting that result [or not] in oriented movement of one individual to another, and courtship. Third, groups of individuals (males and females together and males and females separately) are placed on plants to identify potential rivalry or distress signals. Insects are monitored via video surveillance. Vibrational signals produced by individuals are recorded and measured using a laser vibrometer (NLV-2500, Polytec) and associated softwares (e.g. Raven, Adobe Audition). Recorded signals have been digitized with 44.1 kHz sample rate and 16 bits resolution. Data from vibrometry are currently being analyzed to characterize the spectral and temporal features of signals such as frequency span, dominant frequency, intensity, and pulse repetition time. Data are analyzed using a window size of 512 samples (124 Hz). Recorded signals are used to perform playback experiments conducted with an electrodynamic mini-shaker (Type 4810, Brüel & Kjær**)** driven by a computer, where individuals are stimulated with selected signals transmitted to host plants. Together with video analysis, the role of specific signals in GWSS intra- and inter-gender communication are being assessed to identify signals capable of influencing GWSS behavior for applicative purposes (e.g., disruption of mating communication, attraction).

Two different types of GWSS male calls are described in this report: MC1 and MC2 (**Table 1**). MC1 has two distinct parts, whereas MC2 has three distinct parts. In MC2, the first part consists of two or more pulses of comparatively high amplitude. The second part is a modulated signal with dominant frequency within 300 Hz and the third part is a train of pulses with variable length and constant amplitude (**Figure 1a**). The amplitude ratio among the three parts is variable, but the first part has always the highest intensity. MC1 is similar to MC2, except for the absence of the first part of the signal (**Table 2**). A female alone on the plant emitted only one type of signal (FC) with high variability in length. FC is a modulated signal characterized by low frequency. Amplitude peaked in the middle of the call (**Figure 1b**). Recordings of a virgin male and female placed together on the plant revealed a complex series of behaviors linked to vibrational signals that lead to mating, or not. Prior to mating, male and female communication ranges between 6 min to hours. In the example described here, a duet was established after the male replied to the female signal (**Figure 2**). After the duet was established, the male began searching for the female on the plant by alternating a walking behavior and short stops to emit additional signals; likely to maintain communication with the female. The female remained on the same the position on the plant during the mate finding process. Mating began a few minutes after the male found the female. The couple remained in copula for more than six hours. After mating, the female was kept individually on a new plant until fertility was confirmed by deposition of fertilized eggs.

CONCLUSIONS

The project is providing a detailed description of GWSS vibrational communication signals that are relevant for management of Pierce's disease. In the context of GWSS reproduction, descriptions are aiding identification of signals that influence mate recognition, finding, choice, and/or acceptance behaviors. These include signals produced by individuals in duets, trios, and quartets. Outside the context of reproduction, competitive or cooperative interactions may arise to facilitate access to feeding sites. These potential interactions may be mediated by signals used to repel or attract (e.g., aggregational signals) conspecifics to feeding sites. Finally, the research project will provide practical recommendations on the exploitation of GWSS vibrational communication as a novel method to suppress GWSS populations under field conditions.

Table 1. Acoustic parameters of GWSS signals. Pulse repetition time (PRT) is the ratio between the length of the phase call and the number of pulses composing the phase call. MC1 is a male call without part 1, MC2 is a male call with part 1, and FC is a female call. Data are expressed as (mean ± st. dev.) when more than one signal was present, as in the duet, or more pulses of the same part of male's call were analyzed.

Signal		Part	Length (s)	Dominant frequency (Hz)	Number of pulses	PRT
Individual male call		1	0.55	86.10 ± 0.00	6	0.09
		2	0.80	172.30		
		3	1.70	119.62 ± 43.24	18	0.09
		total	3.05			
Individual female			2.67	172.30		
Male and female paired on plant (duet)	MC1	2	0.92 ± 0.07	86.10 ± 0.00		
		3	0.74 ± 0.73	91.49 ± 21.55	7.50 ± 6.36	0.09
	MC2	1	0.40	86.10 ± 0.00	2	0.20
		2	0.82	86.1		
		3	1.14	100.46 ± 49.74	12	0.09
	FC		1.73 ± 0.41	86.1 ± 0.0		

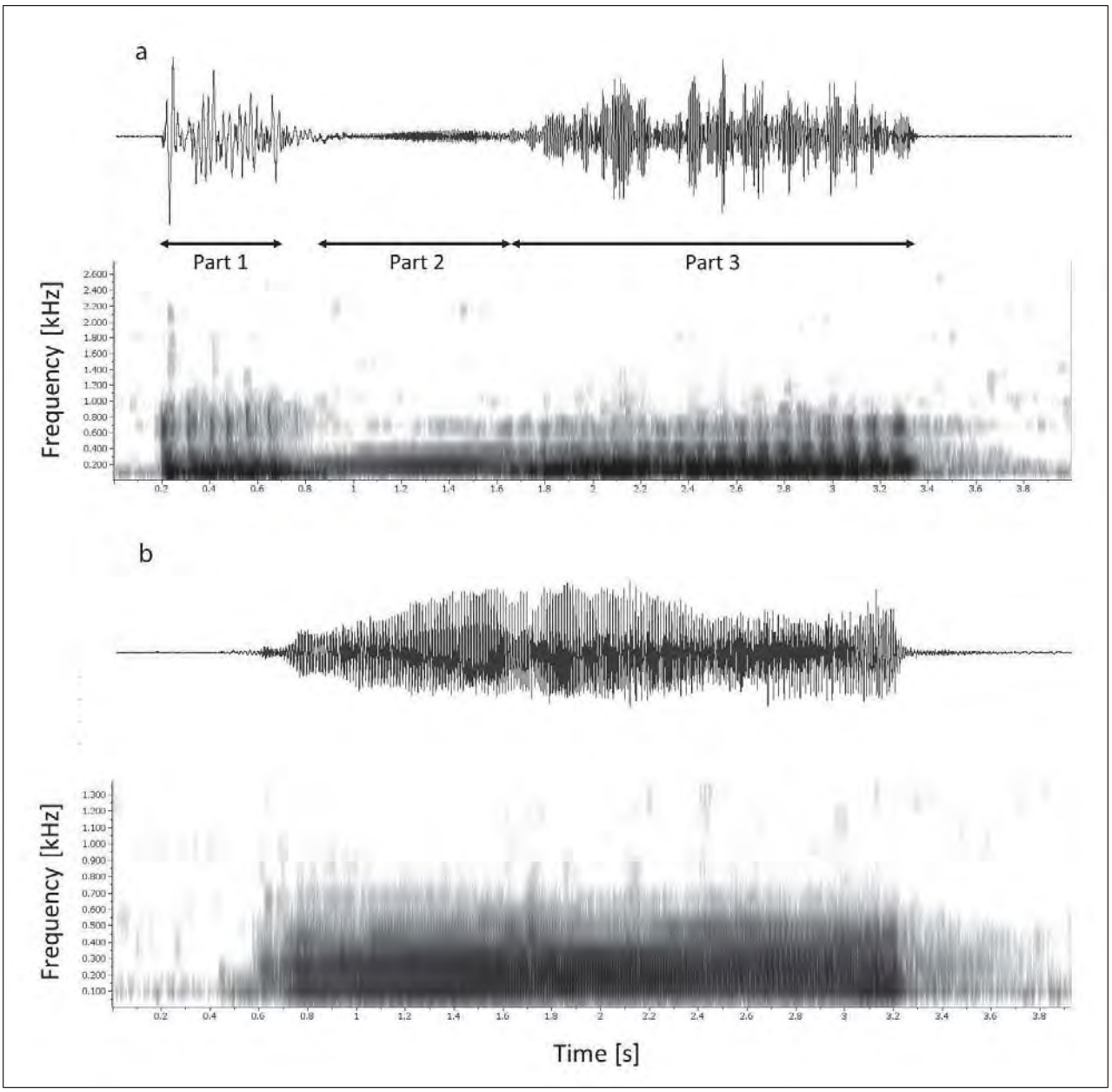

Figure 1. Oscillogram (above) and spectrogram (below) of a male (a) and a female (b) GWSS alone on the plant.

Table 2. Relative amplitude of male and female vibrational signals. Signals were recorded when male and female were together (duet) and alone on the plant. Amplitude is expressed as a relative measure, where the value 1 was assigned to the beginning of the signal. MC1 is a male call without part 1 and MC2 is a male call with part 1.

	Female signal					Male signal			
Position	Alone	With male (duet)			Part	Alone	With female (duet)		
							MC1		MC2
Start	1.00	1.00	1.00	1.00	1	1.00	-	-	1.00
Middle	4.19	4.74	1.27	3.36	2	0.12	1.00	1.00	0.37
End	3.25	4.18	1.41	3.30	3	0.77	8.57	0.88	0.23

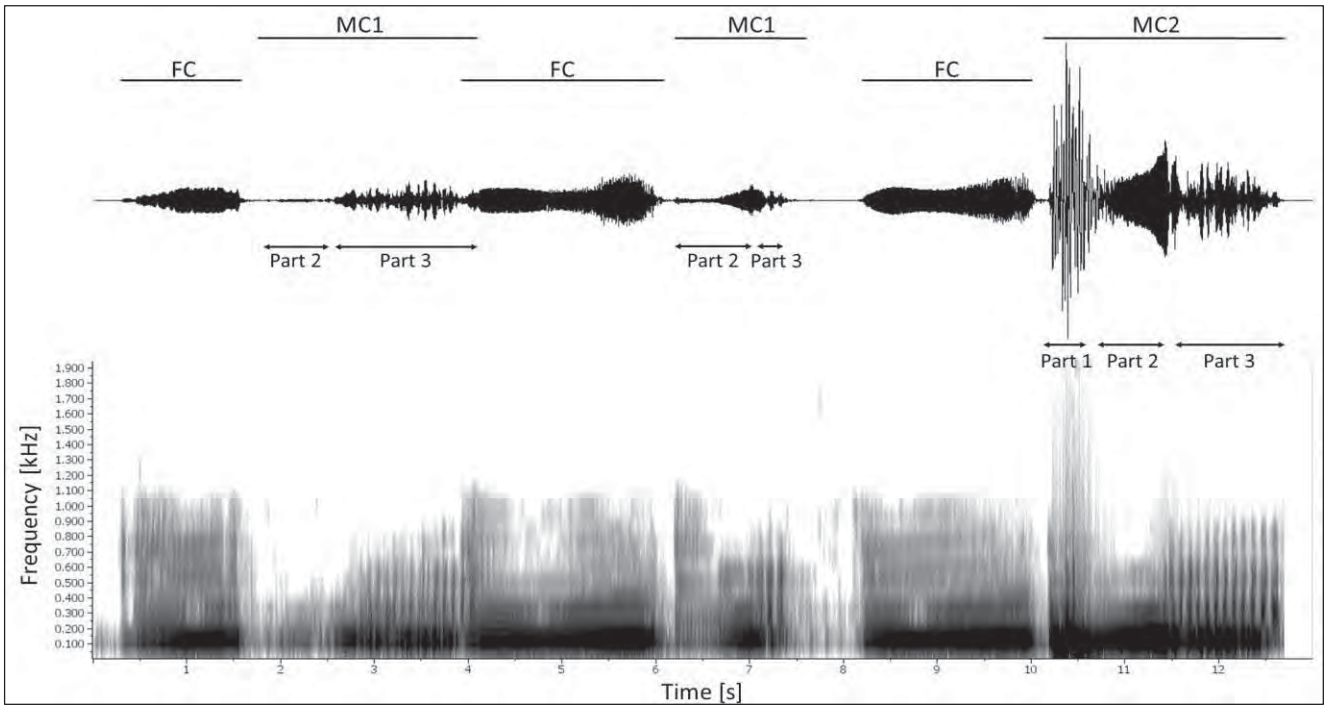

Figure 2. Duet of a male and female GWSS. Female call (FC) followed by a two-part male call (MC1). The third female call at about 8 seconds into the duet was followed by a three-part male call (MC2). Male and female mated shortly thereafter.

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MULTIPLE, STOCHASTIC FACTORS CAN DETERMINE ACQUISITION SUCCESS OF THE FOREGUT-BORNE BACTERIUM, *XYLELLA FASTIDIOSA*, BY A SHARPSHOOTER VECTOR

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Reporting Period: The results reported here are from work conducted October 2013 to September 2014.

ABSTRACT

Xylella fastidiosa (*Xf*) is a phytopathogenic foregut-borne bacterium whose vectors are sharpshooter leafhoppers. Despite several decades of study, the mechanisms of transmission (acquisition and inoculation) of *Xf* still are not fully understood. Studies of the inoculation mechanism depend upon reliable and consistent acquisition of the bacteria by test sharpshooters. Reliability of acquisition was recently improved by development of an *in vitro* method, using artificial diets in parafilm feeding sachets to deliver bacteria to sharpshooters. However, while *in vitro* acquisition is more controlled than acquisition from plants, the number of bacteria acquired from diets by individual sharpshooters is still highly variable. The effects of several underlying factors were assessed in an attempt to improve the consistency of *in vitro* acquisition methods. Blue-green sharpshooters, *Graphocephala atropunctata*, were allowed a 3 – 4 h acquisition access period on diets housing *Xf* 'Temecula' or control, plain diets. Insects then were removed from the diets and caged for a 10-day multiplication period, in groups of 20 on small (8 – 10 cm tall) Chardonnay grapevines. Sharpshooter heads were dissected and tested using qPCR to determine the number of bacterial genomes in each head. Experimental factors such as: 1) duration of bacterial culture, 2) age and 3) gender of sharpshooters were correlated with the number of bacterial cells detected in the sharpshooter heads. Results suggest that acquisition of *Xf* from diets can be made more reliable and consistent by standardizing the three factors listed above. Improving the reliability and consistency of *Xf* acquisition will facilitate future experiments using electropenetrography (EPG) to determine whether insects with and without acquired bacteria feed differently on resistant and susceptible grapevines. Ultimately, this research aims to improve host plant resistance to *Xf* by selecting grapevines resistant to inoculation of the bacterium by the vector.

FUNDING AGENCIES

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A NEW PARADIGM FOR VECTOR INOCULATION OF *XYLELLA FASTIDIOSA*: DIRECT EVIDENCE OF EGESTION AND SALIVATION SUPPORTS THAT SHARPSHOOTERS CAN BE "FLYING SYRINGES"

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Reporting Period: The results reported here are from work conducted April 2011 to September 2014.

ABSTRACT

Despite nearly 70 years of research, the inoculation mechanism of *Xylella fastidiosa* (*Xf*) by its sharpshooter vectors remains unproven. *Xf* is unique among insect-transmitted plant pathogens because it is propagative but non-circulative, adhering to and multiplying on the cuticular lining of the anterior foregut. Thus *Xf* is termed "foregut-borne." A non-circulative mechanism for inoculation of *Xf* must explain how bacterial cells exit the vector's stylets via the food canal and directly enter the plant. A combined egestion-salivation mechanism has been proposed to explain these unique features. Egestion is the putative outward flow of fluid from the foregut via hypothesized bidirectional pumping of the cibarium (part of the foregut). The present study traced green fluorescent protein-expressing *Xf* or fluorescent nanoparticles acquired from artificial diets by glassy-winged sharpshooters, *Homalodisca vitripennis*, as they were egested into simultaneously secreted saliva. *Xf* or nanoparticles were shown to mix with gelling saliva to form fluorescent deposits and salivary sheaths on artificial diets, providing the first direct, conclusive evidence of egestion by any hemipteran insect. Therefore, the present results strongly support an egestion-salivation mechanism of *Xf* inoculation. Evidence also suggests an additional model for inoculation: a column of fluid, potentially containing suspended bacteria, may be held in the foregut during the vector's transit from plant to plant. Thus, sharpshooters could be true "flying syringes," a new paradigm for *Xf* inoculation.

FUNDING AGENCIES

Funding for this project was provided by the USDA Agricultural Research Service, appropriated project 5302-22000-010D.

EXPLORING GLASSY-WINGED SHARPSHOOTER MICROBIOTA USING DEEP 16S rRNA SEQUENCING FROM INDIVIDUAL INSECTS

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Reporting Period: The results reported here are from work conducted September 2012 to September 2014.

ABSTRACT

The glassy-winged sharpshooter (GWSS) is an invasive insect species that transmits *Xylella fastidiosa (Xf),* the bacterium causing Pierce's disease of grapevine and other leaf scorch diseases. *Xf* has been shown to colonize the anterior foregut (cibarium and precibarium) of sharpshooters, where it may interact with other naturally-occurring bacterial species. To evaluate such interactions, a comprehensive list of bacterial species associated with the sharpshooter cibarium and precibarium is needed. Here, a survey of microbiota associated with the GWSS anterior foregut was conducted. Ninety-six individual GWSS, 24 from each of four locations (Bakersfield, CA; Ojai, CA; Quincy, FL; and a laboratory colony), were characterized for bacteria in dissected sharpshooter cibaria and precibaria by amplification and sequencing of a portion of the 16S rRNA gene using Illumina MiSeq technology. An average of approximately 150,000 sequence reads were obtained per insect. The most common genus detected was *Wolbachia*; sequencing of the *Wolbachia ftsZ* gene placed this strain in supergroup B, one of two *Wolbachia* supergroups most commonly associated with arthropods. *Xf* was detected in all 96 individuals examined. By multilocus sequence typing, both *Xf* subspecies *fastidiosa* and subspecies *sandyi* were present in GWSS from California and the colony; only subspecies *fastidiosa* was detected in GWSS from Florida. In addition to *Wolbachia* and *Xf*, 23 other bacterial genera were detected at or above an average incidence of 0.1%; these included plant-associated microbes (*Methylobacterium*, *Sphingomonas*, *Agrobacterium*, and *Ralstonia*) and soil- or water-associated microbes (*Anoxybacillus*, *Novosphingobium*, *Caulobacter*, and *Luteimonas*). Sequences belonging to species of the family Enterobacteriaceae also were detected but it was not possible to assign these to individual genera. Many of these species likely interact with *Xf* in the cibarium and precibarium.

FUNDING AGENCIES

Funding for this project was provided by the USDA Agricultural Research Service, appropriated project 5302-22000-010-00D.

EFFECTS OF FEEDING ON GLASSY-WINGED SHARPSHOOTER LIPID CONTENT AND EGG PRODUCTION

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Reporting Period: The results reported here are from work conducted June 2013 to the present.

ABSTRACT
Glassy-winged sharpshooter (GWSS) females emerge without mature eggs and females must feed to produce mature eggs. As a result, allocation of incoming resources must be balanced between egg production and maintenance of other critical biological functions. Central to this process is allocation of lipids stored in the fat body. Insect eggs are comprised of 16-40% lipid, which typically originate from the fat body. Lipids from the fat body also serve as an energy source during periods of starvation and may be mobilized during periods of sustained flight. To improve understanding of basic biological factors affecting GWSS egg production, effects of feeding on GWSS lipid content and egg production were assessed. Females were field collected and given a four-day oviposition period on sorghum to reduce variance in egg load among females. After the oviposition period on sorghum, females were divided into four groups. Females from the first group were frozen to provide an estimate of female egg load and lipid content at start of feeding assays. Females in the remaining three groups were allowed to feed on cowpea until approximately 12, 25, or 50 ml of excreta was produced. After producing the designated quantity of excreta, females were dissected to determine egg load. Mature eggs were separated from the body and dry weights of eggs and bodies (head, thorax, and abdomen) obtained. Lipid content of eggs and bodies were determined using a quantitative colorimetric assay. Dry weight and lipid content of GWSS bodies increased rapidly with low levels of feeding, but decelerated with additional feeding. In contrast, dry weight and quantity of lipid allocated to eggs increased slowly with low levels of feeding, but accelerated with additional feeding. Accordingly, egg production was preceded by an increase in body dry weight and body lipid content. Likewise, allocation of resources to the body decreased as resources were shifted to egg production. Collectively, the results suggest that variation in the rate of egg production among GWSS females partially may be explained by availability of lipid reserves at start of a feeding bout.

FUNDING AGENCIES
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Section 2:

Vector Management

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

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ABSTRACT
For approximately 15 years 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 have been made in years past. As part of this treatment program there is ongoing monitoring of GWSS populations to ensure that the treatments are effective. Notably, starting last year, reimbursements to citrus growers were not made. As a result, over the past two seasons, apparently no Temecula Valley citrus acreage was treated specifically for GWSS – the consequences of which are not well understood. Approximately 140 yellow sticky traps were inspected on a biweekly basis throughout 2014 to monitor GWSS in citrus. The results show a typical phenology for this pest in the region, with a total of approximately 480 GWSS caught during the summer peak (July through September). Overall GWSS catch was intermediate this year - modest compared to the highest years (2008, 2009) but slightly higher than the lowest years (e.g., 2010, 2011).

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

INTRODUCTION
The winegrape industry and its connecting tourist industry in 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 U.S., 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 several hundred new acres of grapes have been planted and multiple new wineries have been built. GWSS has the potential to develop high population densities in citrus. Fortunately, GWSS is also highly susceptible to systemic insecticides such as imidacloprid. Insecticide treatments in citrus groves, preceded and followed by trapping and visual inspections to determine the effectiveness of these treatments, are needed to manage this devastating insect vector and disease.

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

In the spring of 2008, 120 acres of citrus were identified and were treated for GWSS control in Temecula. In July, 2008, Temecula GWSS trap catches reached over 2,000. This was the highest number of GWSS trapped since the

area-wide program was initiated. Because of the phenology of GWSS, the summer citrus culture, and the peculiarities of the uptake of the systemic insecticide imidacloprid it was decided that treatments in citrus in July would not adequately reduce GWSS populations. Therefore, insecticide applications to control GWSS for the last two years were initiated in May 2011 and May-June 2012. Monitoring data suggest fairly robust control of GWSS using that treatment timing.

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

OBJECTIVES

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

Double-sided yellow-sticky cards (14x22 cm; Seabright Laboratories, Emeryville, CA**)** are being used to monitor for adult sharpshooters in citrus. 137 such sticky traps have been placed in citrus groves throughout the Temecula Valley. All traps are labeled, numbered, and barcoded to identify the site within the management program. Each trap is then georeferenced with a handheld GPS monitor. Most traps 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, and 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 are counted and then removed from the trap.

The yellow cards are inspected and replaced every two weeks during the summer and fall (May through October) and monthly the rest of the year. At each inspection the number of adult GWSS and smoke-tree sharpshooters (*Homalodisca liturata*) are recorded, and the abundance of common generalist natural enemy taxa.

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

RESULTS AND DISCUSSION

The results for 2014 are shown in **Figure 1**. This includes monthly censuses of GWSS in citrus through April, then biweekly censuses from May through October. Census results show seasonal patterns of GWSS abundance and activity that are typical for this region. GWSS catch is low for much of the year; it increases dramatically at the beginning of the summer and then drops off through August and September. As of early-October, GWSS populations appear to have declined substantially.

Figure 2 shows the GWSS catch in 2014 relative to other years. 2014 shows qualitatively the same seasonal phenology as in other years, with a moderate overall catch compared to others (i.e. 2008).

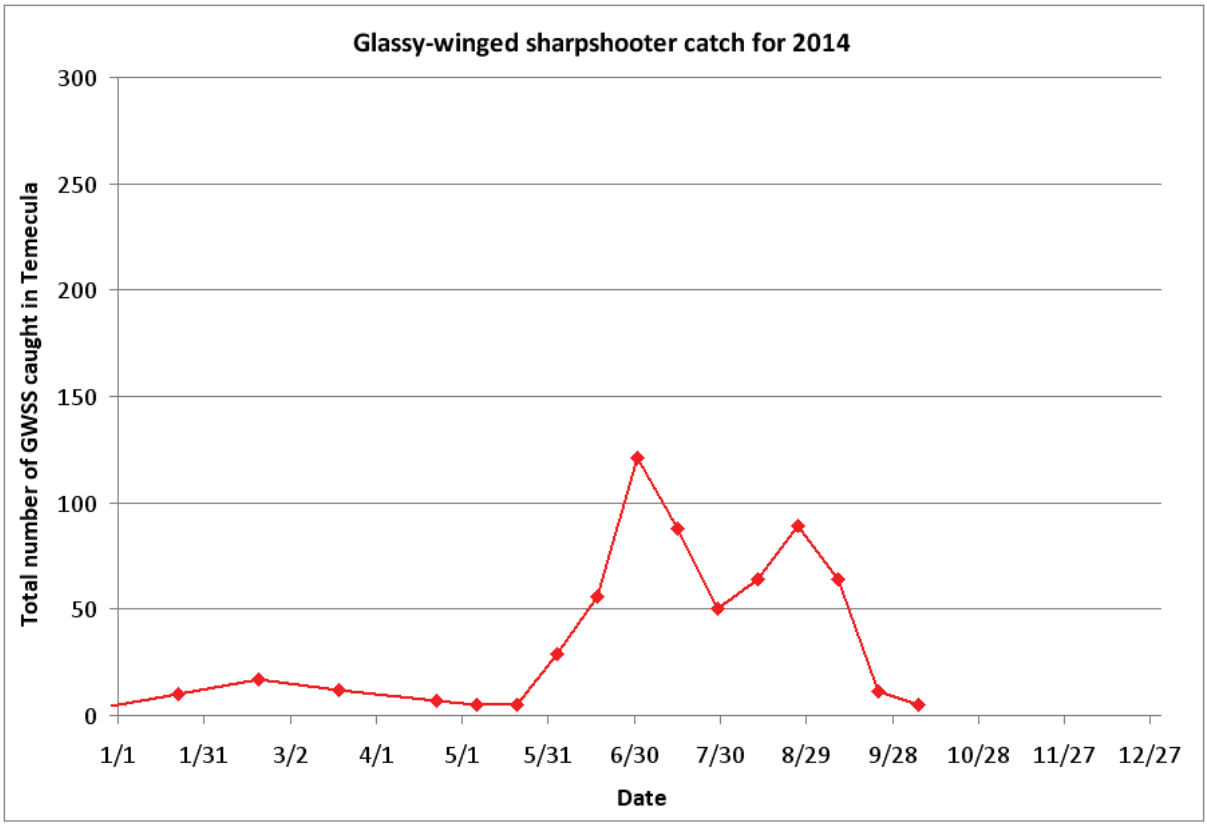

Figure 1. Seasonal total GWSS catch in 2014 for 137 traps throughout the Temecula Valley.

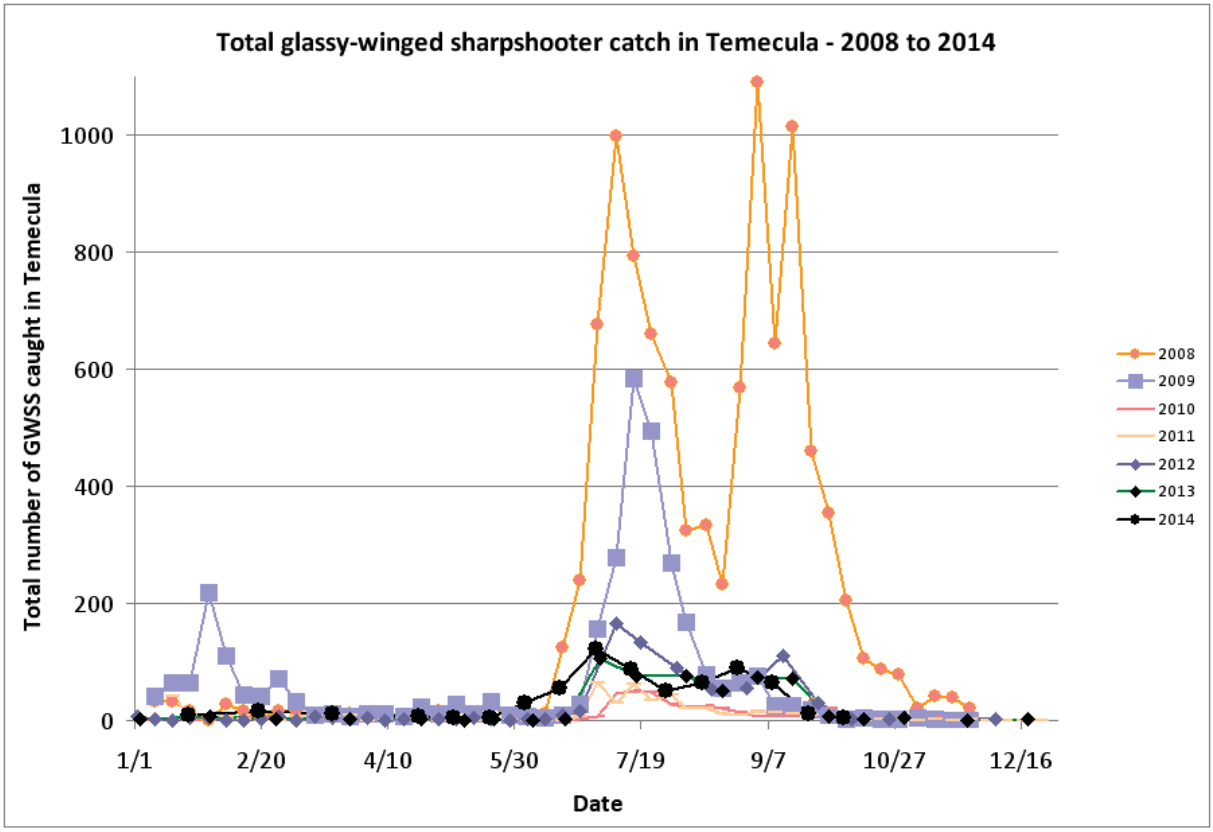

Figure 2. Seasonal total GWSS catch in the Temecula Valley from 2008-2014.

CONCLUSIONS

The results for 2014 continue to suggest that there is no clear evidence of a GWSS resurgence in the Temecula Valley region. At least some of the explanation may be because of the potential for treatments made for another invasive insect, the Asian citrus psyllid (*Diaphorina citri*), which is controlled primarily via the same classes of insecticides as are used for GWSS. Although the recommended treatment timings are slightly different for ACP versus GWSS, applications made for its control may aid somewhat with GWSS control.

FUNDING AGENCIES

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

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ABSTRACT
RNA interference (RNAi) in insects is a gene regulatory process that also plays a vital role in the maintenance and regulation of host defenses against invading viruses (1, 2). The application of RNAi directed toward the control of different types of insect plant pests is becoming more feasible and promising (3, 4). RNAi has already been used in various pest insect systems, both for reverse genetics and insect control. In our efforts, we were able to induce RNAi effects in the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) *in vitro* and evaluated different *in vivo* plant-based approaches to test RNAi in GWSS and other leafhopper vectors of *Xylella fastidiosa* (5, 6). RNAi is already used in commercial agriculture for plant virus control, and the recent publications demonstrate the experimental success with different plant-feeding insects (7, 8). Here we report our ongoing efforts at using RNAi for GWSS.

LAYPERSON SUMMARY
This work presents fundamental efforts towards understanding the feasibility of applying RNA interference (RNAi) to help combat Pierce's disease of grapevines. Pierce's disease is a significant threat to grape production in California and other parts of the U.S., and the causal agent, *Xylella fastidiosa (Xf)*, a xylem-limited bacterium, also causes several other extremely important plant diseases worldwide. Our effort here does not directly target *Xf*, but instead targets one of its most significant insect vectors, the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS).

We focused our recent efforts on evaluating transgenic potato plants (constitutive and spatial expression of transgenes) to evaluate their potential for inducing RNAi effects in GWSS, and for identifying optimal RNAi inducer delivery systems. Potatoes are easier and faster to transform and regenerate than grapes, and the GWSS feeds readily on these plants, thus, they are a good model herbaceous plant for our RNAi studies. More importantly, we were able to compare different transgenic events resulting from the use of different promoters for GWSS survival and gene expression reduction.

INTRODUCTION
Our primary objectives are to evaluate and demonstrate effective RNA interference (RNAi) activity against the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS). We envision that RNAi approaches can be part of long term strategies to help control GWSS and other sharpshooter vectors of *Xylella fastidiosa*, the causal agent of Pierce's disease of grapevines. We used *in vitro* and *in vivo* approaches towards the goal of achieving RNAi in GWSS. In particular, our *in vivo* approaches involve the generation of stable transgenic potato plants using the constitutive, non-tissue-specific CaMV 35S promoter, and a *Eucalyptus gunii* minimal xylem-specific promoter (Ecad) to control the spatial expression of candidate interfering RNAs in plants. We have demonstrated through RT-PCR analysis the ability of stable transgenic plants to express the transgene, and tested their ability to generate small RNAs through small RNA northern blots (previous reports). In our previous report, we have shown GWSS mortality and target mRNA level reduction in constitutively expressing GWSS-*actin* and GWSS-*chitin deacetylase* potato transgenic plants. Spatial gene expression of the Ecad promoter was verified through the GUS gene expression *in vivo* in the transgenic potato plants. Encouraged by the results of GUS transgene

expression in the xylem tissues of potato transgenic plants (spatial restriction of the transgene), we also developed the transgene constructs for GWSS-*chitin deacetylase* driven by xylem expressing, Ecad promoter to generate small RNAs specific for GWSS mRNAs in hopes of expressing these mostly in the xylem, and generated stable transgenic lines in potatoes. We were successful in generation of transgenic plants that produce small RNAs for GWSS *chitin deacetylase*. In the present reporting period, we tested and compared the ability of different transgenic potato plants (expressing GWSS *chitin deacetylase* RNAs) to induce the RNAi in GWSS insects (third instars). Further we were also able to understand the forms of RNA that were present in the stable transgenic potato plants.

We have made considerable progress during the past funding periods and have completed our objectives that we proposed for this grant period. We have published three new refereed journal articles (Nandety et al., 2013; Kamita et al., 2013 and Nandety et al., 2014) and are working on one manuscript. We have also presented an oral talk at International conference Plant and Animal Genome in San Diego, (Nandety et al., 2014).

OBJECTIVES

Our primary and sub-objectives are:

To assess the effectiveness of GWSS hairpin RNA transgenic plants against GWSS mRNA accumulation and insect fecundity, survival and development.

A. Temporal and spatial analysis of GWSS mRNA targeting.
B. Assessing RNAi effects on GWSS fecundity, development and survival.

RESULTS AND DISCUSSION

Objective A. Temporal and spatial analysis of GWSS mRNA targeting.

In order to generate dsRNAs (and siRNAs) against GWSS *chitin deacetylase* and GWSS *actin*, corresponding cDNAs were cloned into a Gateway-compatible binary vector pCB2004B under the 35S and Ecad promoters respectively (**Table 1**). Stable plant transformation with binary vectors for 35S-GWSS *chitin deacetylase*, 35S-GWSS *actin*, Ecad-GUS, Ecad-GWSS *chitin deacetylase* and 35S-GFP was done via recharge at the UC Davis Ralph M. Parsons plant transformation facility (http://ucdptf.ucdavis.edu/). Wild-type potato (Desiree) were used as the control plants for all experimental purposes.

We screened the transgenic potato plants for insert composition and showed the presence of the transgenes respectively. We previously reported the expression of GWSS *chitin deacetylase* and GWSS *actin* transgene-associated small RNAs in potato plants, expressing these anti-GWSS transgenes constitutively. The number of potato lines that we now have for the GWSS *chitin deacetylase* and GWSS *actin* expressed from 35S and Ecad promoters were presented in the table (**Table 1**).

To study the effects of RNAi activity on growth and development of GWSS insects, we used *in vitro* and *in vivo* approaches to identify optimal interfering RNAs for use in RNAi experiments. We performed gene expression studies on the Ecad-GWSS *chitin deacetylase* plants generated as described above (**Table 1**). Small RNA expression studies were performed by using northern blots to assess for the expression of the desired GWSS *chitin deacetylase* small RNAs in these plants (**Figure 1**). The small RNAs extracted from the above transgenic plants were analyzed using poly acrylamide- urea gels (PAGE-urea) and were probed with a GWSS *chitin deacetylase* small RNA probe synthesized *in vitro*. The results from the small RNA northern blot show the expression of 21 nucleotide (nt) small RNAs as represented in **Figure 1**. Based on the small RNA hybridization blot experiments, we were able to see the expression of small RNAs specific for GWSS *chitin deacetylase* in transgenic plants (**Figure 1**). To test this further, we checked whether the same transgenic plants were able to express transgene mRNAs for GWSS *chitin deacetylase*.

We performed northern blot to confirm different sizes of RNAs (**Figure 2**). These are the same plants that were shown to express anti GWSS *chitin deacetylase* small RNAs. The result in the northern blot shows that the transgenic plants express 0.9 kb long mRNAs, suggesting that these plants also express full length mRNAs of the transgene (**Figure 2**) along with the small RNA expression (**Figure 1**).

Table 1. List of 35S and Ecad promoter fusion GWSS transgenic potato plants along with their pedigree number. The list shows transgenic potato plants expressing GWSS *chitin deacetylase* and GUS under Ecad promoter and GWSS *actin*, GWSS *chitin deacetylase* and GFP under 35S promoter.

S.No:	Transgenic line/potato	Pedigree number
1	Ecad-GWSS *chitin deacetylase*	132052-002
2	Ecad-GWSS *chitin deacetylase*	132052-003
3	Ecad-GWSS *chitin deacetylase*	132052-004
4	Ecad-GWSS *chitin deacetylase*	132052-005
5	Ecad-GWSS *chitin deacetylase*	132052-006
6	Ecad-GWSS *chitin deacetylase*	132052-007
7	Ecad-GUS	122099-001
8	Ecad-GUS	122099-002
9	Ecad-GUS	122099-003
10	Ecad-GUS	122099-004
11	Ecad-GUS	122099-005
12	Ecad-GUS	122099-006
13	35S-GWSS *actin*	112064-004
14	35S-GWSS *actin*	112064-008
15	35S- GWSS *chitin deacetylase*	102203-001
16	35S- GWSS *chitin deacetylase*	102203-002
17	35S- GWSS *chitin deacetylase*	102203-004
18	35S-GFP	122009-004
19	35S-GFP	122009-005

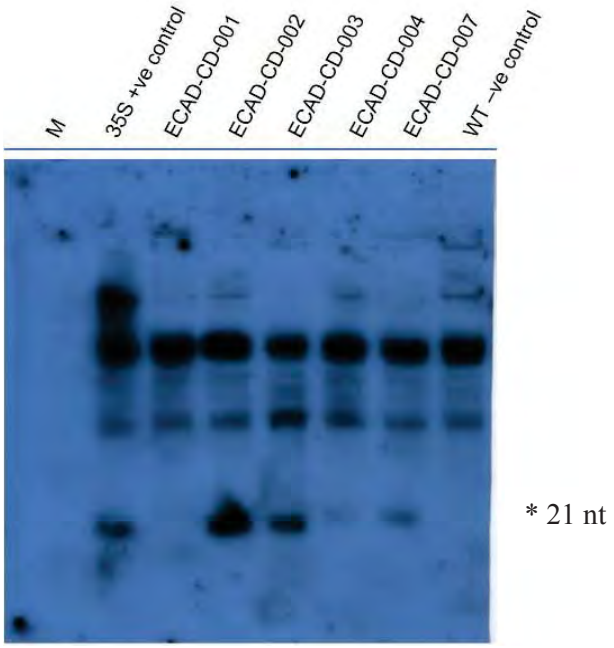

Figure 1. Transgenic potato plants expressing GWSS *chitin deacetylase* under Ecad promoter were tested for small RNA expression using small RNA northern blots. Probe: T7-CD-P^{32}. WT- Desiree potato, 35S-*chitin deacetylase*-potato transgenic plants with 35S promoter, ECAD-CD-potato transgenic plants with Ecad promoter, Marker- microRNA marker. Asterisk represents the 21 nt small RNAs expressed in the transgenic potato plants.

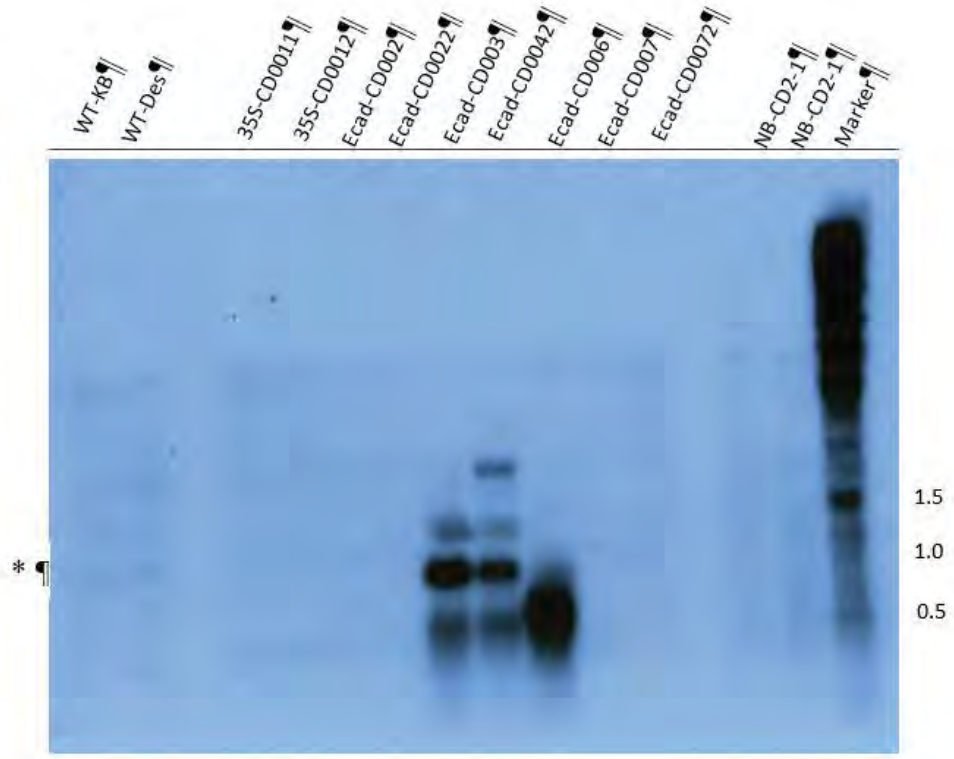

Figure 2. Transgenic potato plants expressing GWSS chitin deacetylase under Ecad promoter were tested for messenger RNA expression using northern blots. The asterisk represents the target mRNA hybridized with the probe. Probe: T7-CD-P^{32}. WT-KB – Kennebec potato, WT-Des- Desiree potato, 35S-CD: potato transgenic plants expressing anti GWSS *chitin deacetylase* with 35S promoter, Ecad-CD: potato transgenic plants expressing anti GWSS *chitin deacetylase* with Ecad promoter, Marker- 0.5 to 10 Kb RNA ladder.

Based on our small RNA expression data and messenger RNA northern blot, we identified plants that can generate small RNAs as well as express the GWSS *chitin deacetylase* messenger RNAs. Hence we screened the transgenic plants, Ecad *chitin deacetylase* 002, 003 and 35S- *chitin deacetylase* 004 for their ability to induce RNAi effects in GWSS.

Objective B. Assessing RNAi effects on GWSS fecundity, development, and survival.
We next evaluated the ability of transgenic potato plants (expressing GWSS transgenes either constitutively or spatial expression) to induce RNAi effects in GWSS insects. All the feeding assays on GWSS insects (3rd instar nymphs) were performed at the CRF. We previously reported the ability of transgenic plants constitutively expressing GWSS transgenes (*chitin deacetylase* and *actin*) to induce RNAi effects and mortality. In our initial assessment that we reported (previous year), we were able to observe a 70% reduction in the target gene expression in GWSS insects, when they were fed on 35S *actin* and *chitin deacetylase* transgene expressing potato plants.

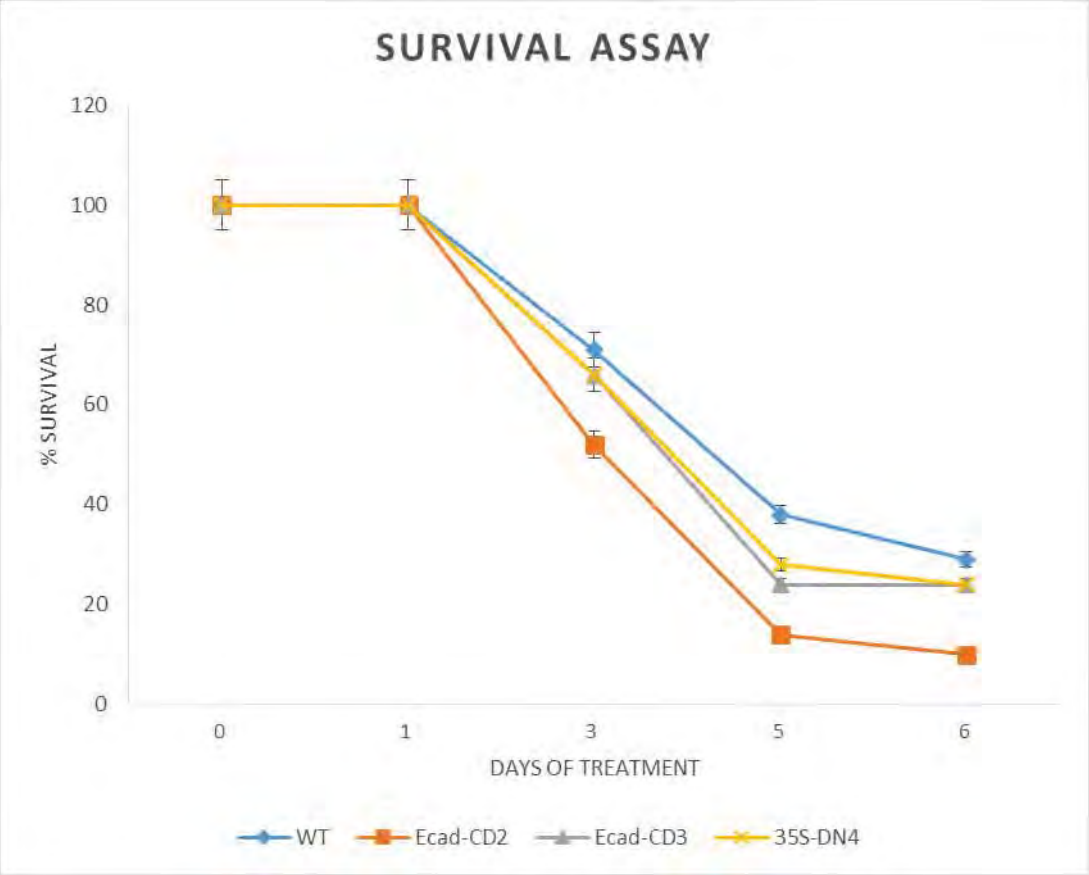

Figure 3. Survival assay measured as percent survival of GWSS 3rd instars against wild-type, Ecad-CD transgenic potato plants and 35S-CD transgenic potato plants. Annotation: Ecad-CD2: Ecad *chitin deacetylase* 002; Ecad-CD3: Ecad-*chitin deacetylase* 003; 35S-DN4: 35S *chitin deacetylase* 004.

Here we were able to test for the efficiency of the transgenic potato plants (spatial expression) against GWSS. Further, we were also able to compare the ability of 35S transgenic plants and Ecad promoter driven plants on GWSS insect development, survival and fecundity.

We performed the survival assay with GWSS on Ecad *chitin deacetylase* transgenic plants with six different biological replications. In our first set of mortality experiments, the total sample size was 50 insects (3rd instar nymphs) per event tested. As reported in one of our earlier reports, the survival assay did not suggest big changes across treatments except for enhanced mortality in the Ecad promoter driven plants. From our experience with our earlier experiments in the lab, this is not uncommon as we observe relatively higher change in the gene expression in the guts of the insects. Based on our first experimental assay, we have expanded the trial experiment to accommodate transgenic potato plants that are expressing under constitutive promoter as well as Ecad promoter. The experiment was designed to sufficiently include three replications per each event. Further, the mortality (% survival) assay was performed in the exact similar conditions at the CRF (**Figure 3**). The percent of survival is lowest in insects fed on Ecad *chitin deacetylase* 2 plants (10%) compared to the wild-type plants (35%) at six days after treatment.

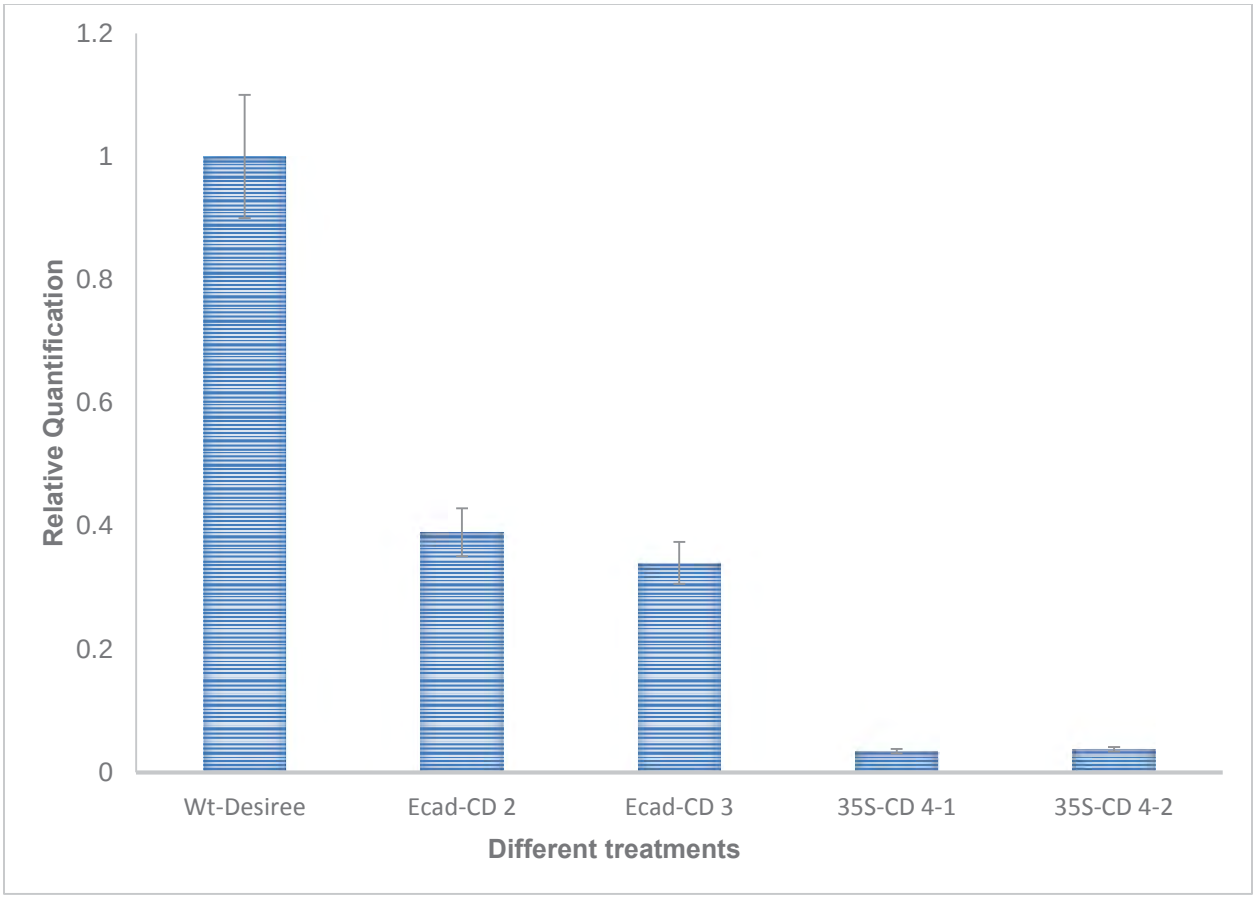

Figure 4. Gene expression study of chitin deacetylase from GWSS insects that fed on potato transgenic plants. Annotation: *Wt-Desiree* –Wild-type potato Desiree plants; *Ecad-CD* represents the Ecad *chitin deacetylase* transgenic potato plants; *35S-CD* represents the 35S *chitin deacetylase* transgenic potato plants. Bars show relative expression data, compared to wild-type Desiree (Wt-Desiree).

In addition to the above experiment, in order to fully understand the effective role of small RNAs generated in the transgenic potato plants and their effect on the GWSS mRNAs, RT quantitative Real-Time PCR (RT-qPCR) was used to quantify relative expression of the *chitin deacetylase* mRNAs targeted for down regulation (**Figure 4**). In this experiment, we were able to analyze the relative changes in the gene expression. The insects were dissected and the gut tissues were extracted into 1X PBS buffer and processed for total RNA. Quantitative realtime PCR was performed using a SYBR green assay. The data are normalized to the wild-type control (Desiree potato plants). Greater reduction was observed in insects fed on transgenic plants expressing GWSS *chitin deacetylase* gene either under Ecad promoter (60%) or 35S promoter (90%). This is somewhat similar to recent reports for *Nilavarpata lugens* when they were fed on transgenic rice plants expressing the *N. lugens* transgenes hexose transporter gene *NlHT1*, the carboxypeptidase gene *Nlcar*, and the trypsin-like serine protease gene *Nltry* (9). Insects fed on the transgenic rice plants did not show any mortality symptoms while their target genes were expressed at a very lower percentage compared to the wild-type controls.

CONCLUSIONS

We have made stable potato transgenic plants using the constitutive, non-tissue specific 35S promoter and a *Eucalyptus gunii* minimal xylem-specific promoter to control the spatial expression of candidate interfering RNAs. We have demonstrated the ability of stable transgenic plants to display gene expression through the use of RT-PCR, small RNA northern blots and northern blots. We showed down-regulation of GWSS genes GWSS *chitin deacetylase* by dsRNAs (siRNAs) produced in transgenic potato plants. We were able to test the ability and efficiency of our transgenic potato plants in inducing the RNAi effects on GWSS insects. Though we did not observe considerable mortality/survivability in the treatments, we did observe the higher levels of gene reduction in the third stage nymphs under the conditions tested by us.

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

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Reporting Period: The results reported here are from work conducted July1, 2011 to June 30, 2014.

INTRODUCTION

We received two, one-year grants, plus a one-year no-cost extension for this effort. We focused our collaborative efforts on attempting to develop glassy-winged sharpshooter (GWSS) infecting viruses for use as agents to help manage GWSS, and then indirectly help manage Pierce's disease of grapevines. Viruses are the most abundant microbes on earth, with estimates as high as 10^{31} [1], and although viruses are often identified as pathogens, their roles in nature are not always associated with disease. Viruses often exist without causing disease in specific hosts, and in many instances viruses have proven to be useful for a variety of beneficial applications including use as biological control agents for insect pests [2]. If we could identify viruses that caused disease, or those that did not, both would be useful for our goals. Initially in year one we attempted to use the naturally-occurring *Homalodisca coagulata virus – 1* (HoCV-1), and *Flock House virus* (FHV), a model system virus for our work. We envisioned that both could assist our efforts and allow for more rapid progress. We used GWSS cells (GWSS Z-15) and whole insects for our virus transmission assays. In year two we focused our efforts only on HoCV-1. This report presents our data from the past two years.

OBJECTIVES

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

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

RESULTS

Objective 1.

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

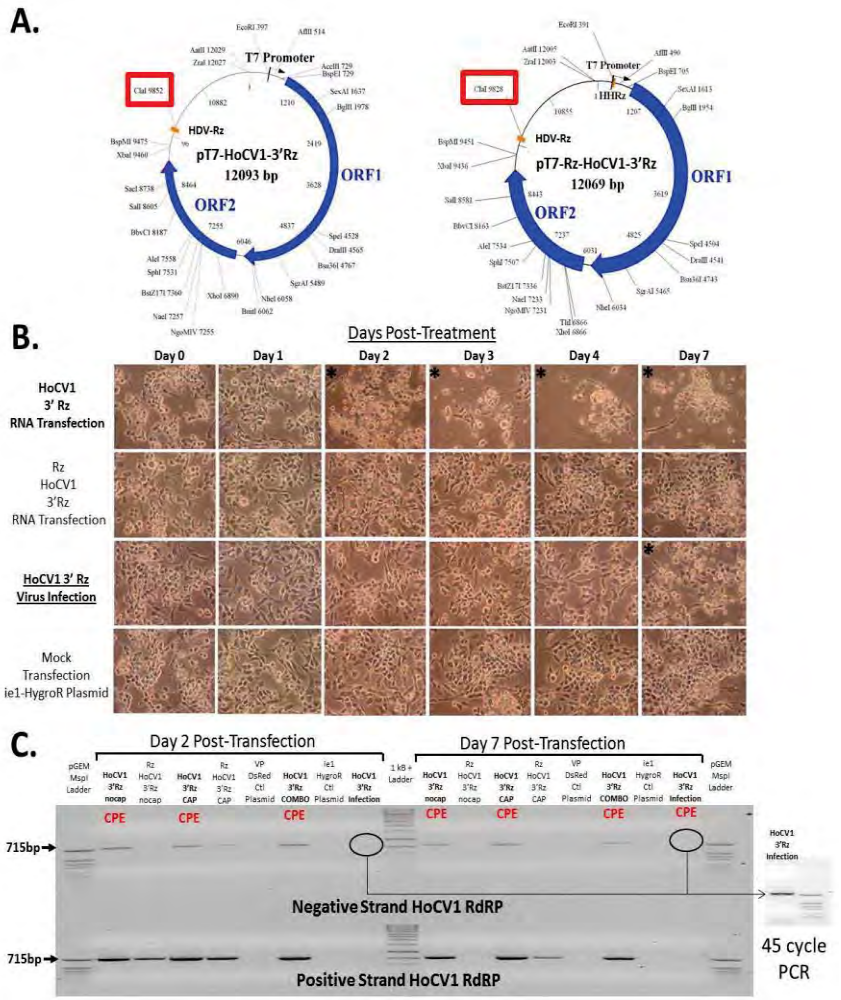

Figure 1. A. Vector diagram of pT7-HoCV1-3'Rz and pT7-Rz-HoCV1-3'Rz. The T7 promoter is indicated by the black bar and arrow. Hammerhead (HHRz) and Hepatitis Delta Virus (HDFV-Rz) ribozymes are indicated as orange boxes. pT7-HoCV1-3'Rz lacks the HHRz. HoCV-1 open reading frames (ORF) 1 and 2 are indicated as blue arrows. The *ClaI* restriction site (red box) is used to linearize plasmid for *in vitro* transcription. **B.** Cytopathic effects (black asterisks) were induced in GWSS Z-15 cells after transfection using HoCV-1 RNA transcripts and with HoCV-1 virus generated from the pT7-HoCV1-3'Rz plasmid. **C.** Negative and positive RNA strands for the HoCV-1 RNA-dependent RNA-polymerase (RdRP) were detected by 30 cycle RT-PCR for HoCV-1 RNA transfected from both plasmids, although only transfections with pT7-HoCV1-3'Rz RNA caused strong cytopathic effects (indicated by red CPE) in GWSS-Z15 cells. Weak signals for positive (30 cycles) and negative (45 cycles-indicated) strand RdRP RNA were also detected in virus overlayed from the HoCV-3'Rz transfection onto new GWSS-Z15 cells.

We successfully cloned full length HoCV-1 cDNAs (**Figure 1A**). We generated a series of different constructs to help increase the probability of success. *In vitro* transcription was performed using these constructs to generate HoCV-1 transcripts which were delivered to GWSS Z-15 cells (**Figures 1B and C**). After transfection with HoCV-1 transcripts with extended or unextended 5'-ends, Z-15 cells showed severe cytopathic effects (CPE; **Figure 1B**). Control cells did not, thus these results suggested that we most likely had generated infectious HoCV-1 cloned cDNAs. In order to support the cytopathology data, we also performed reverse-transcription polymerase chain reaction (RT-PCR) assays to identify specific RNAs resulting from HoCV-1 replication. Both

the HoCV-1 genomic-sense strand (positive-strand) and its complementary strand (negative-strand) RNAs were amplified by RT-PCR analysis following the transfection indicating that the virus was replicating (Fig. 1C). pT7-Rz-HoCV1-3'Rz generated transcripts were less efficient possibly due to enhanced RNA degradation following ribozyme cleavage at the 5' end. Thus, both the cell cytopathology and the RT-PCR analyses suggested that our cloned HoCV-1 cDNAs were infectious to GWSS Z-15 cells and offered an opportunity for us to move forward.

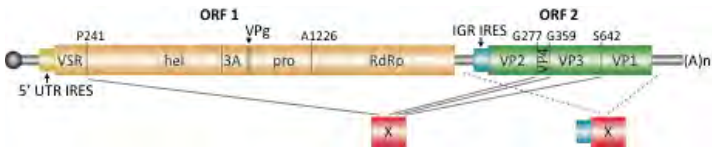

Figure 2. Potential insertions sites for expression of foreign proteins and RNAs from the RhPV and HoCV-1 genomes. Genome organization shows ORF 1 (orange) and ORF 2 (green) which encode proteins separated by cleavage sites indicated at estimated positions by vertical lines. Precise cleavage sites, estimated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

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

Objective 2.

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

Transfection assays in whole insects.
In year two we focused efforts on HoCV-1 and attempted to efficiently infect GWSS Z-15 cells and whole insects using both wild-type virus (from naturally-infected GWSS) and our HoCV-1 clones. We established HoCV-1 GWSS colonies at the UC Davis CRF. We attempted to infect healthy GWSS with the GWSS-Z15 transfected cell extracts both by injection and oral acquisition. RNA from five infectious clones of HoCV1 and two controls were used to transfect GWSS-Z15 cells: HoCV-3'Rz, HoCV-3'Rz old, Rz-HoCV-3'Rz, Rz-HoCV-3'Rz old, mutant Rz-HoCV-3'Rz, elongation factor RNA, and transfection buffer. An additional negative control for the GWSS infection experiments was untreated GWSS-Z15 cell suspension. For the injections, 1µL of needle homogenized cell suspension in injection buffer (10 mM Tris-HCl, pH 7.0,1 mM EDTA) was injected into adult GWSS between tergites three and four of the ventral aspect using a 33 gauge needle (**Figure 5**). Three insects were injected per HoCV-3'Rz construct. After injection the insects were put in cages with a basil plant for one week, and then RNA collected as a treatment group. For feeding assays we used basil cuttings approximately 5cm in length and submerged the cut end in a suspension of cell pellet and supernatant of approximately 1.5mL volume. Three insects per treatment were given an acquisition period of three days on the basil cuttings, then moved to basil plants for four days, after which RNA was extracted from each group. One-tube RT-PCR was used to detect infection with primer pairs specific to inter-genomic region 1 and coat protein of HoCV-1. The positive control was GWSS RNA from a naturally infected insect. All treatment groups tested negative for HoCV-3'Rz (**Figure 6**). Unfortunately, these data showed no evidence for replication of our HoCV-1 in adult GWSS.

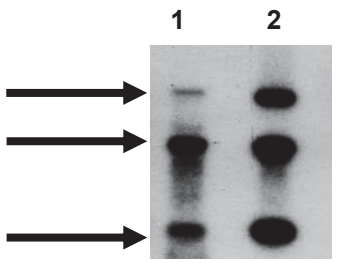

Figure 3. Lanes contain 1; FHV-inoculated S2 cells, 2; FHV-inoculated GWSS Z-15 cells. Arrows at left indicate FHV RNAs 1, 2 and 3, from top to bottom, respectively. RNA 3 is a subgenomic RNA from replicating RNA 1.

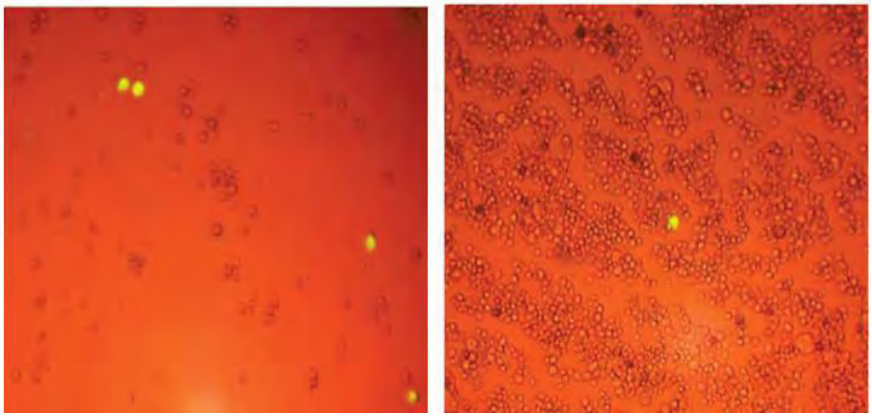

Figure 4. FHV infected S2 cells examined by fluorescence microscopy. S2 cells on the left panel were transfected with the plasmids that express FHV RNA1, FHV RNA2, and FHV DI RNA that contains GFP sequence. The transfected cells were collected, frozen and thawed three times, and filtered through a filter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

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

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

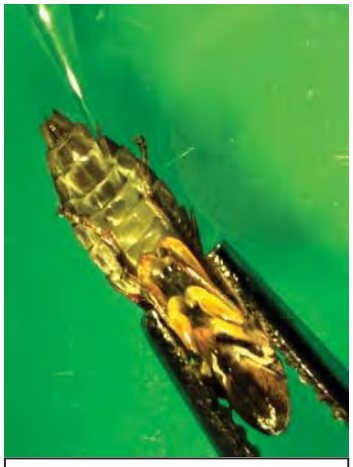

Figure 5. Injection of adult GWSS.

In the current reporting period we have been working on optimizing transfection assays in whole insects and the GWSS cell line Z15. We received cell pellets and cell culture supernatant from Iowa State to use in insect experiments. We attempted to inoculate healthy GWSS with the GWSS-Z15 transfected cells both by injection and oral acquisition. RNA from five infectious clones of HoCV1 and two controls were used to transfect GWSS-Z15 cells: HoCV-3'Rz, HoCV-3'Rz old, Rz-HoCV-3'Rz, Rz-HoCV-3'Rz old, mutant Rz-HoCV-3'Rz, elongation factor RNA, and transfection buffer. An additional negative control for the GWSS infection experiments was untreated GWSS-Z15 cell suspension. For the injections, 1µL of needle homogenized cell suspension in injection buffer (10 mM Tris-HCl, pH 7.0,1 mM EDTA) was injected into adult GWSS between tergites three and four of the ventral aspect using a 33 gauge needle. Three insects were injected per HoCV-3'Rz construct. After injection the insects were put in cages with a basil plant for one week, and then RNA collected as a treatment group. For feeding assays we used basil cuttings approximately 5cm in length and submerged the cut end in a suspension of cell pellet and supernatant of approximately 1.5mL volume. Three insects per treatment were given an acquisition period of three days on the basil cuttings, then moved to basil plants for four days, after which RNA was extracted from each group. One-tube RT-PCR was used to detect infection with primer pairs specific to inter-genomic region 1 and coat protein of HoCV-1. The positive control was GWSS RNA from a naturally infected insect. All treatment groups tested negative for HoCV-3'Rz.

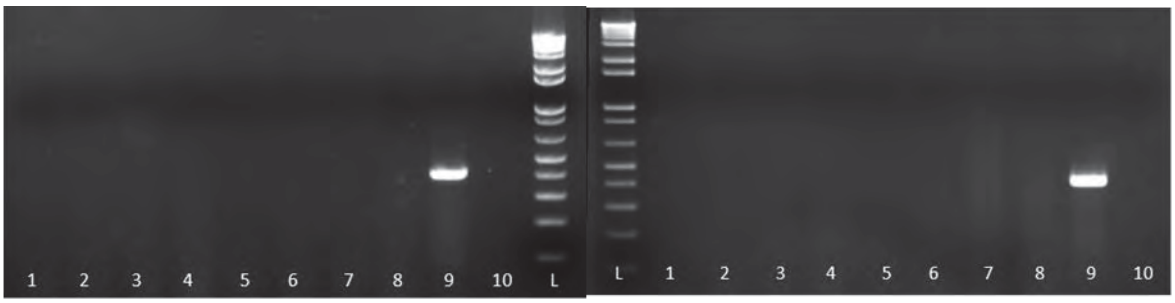

Figure 6. One-tube RT-PCR amplifying a segment of HoCV1 IGR (left) or coat protein (right) from insects that were fed transfected Z15 cell suspensions. Results from injection were identical. Treatments were Z15 cell transfected with constructs of HoCV1 infectious clones. **1.** HoCV-3'Rz **2.** HoCV-3'Rz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

We worked to optimize cell line transfection to generate infectious virus, however we did not have positive results. We used the initial protocol and plasmids developed at Iowa State University, in which single stranded RNA is generated from plasmid template for transfection experiments. We used *in vitro* transcription kits that generated both capped and uncapped messenger RNA (**Figure 8**). This was followed by cell transfection and analysis at three days post transfection. Using two-step RT-PCR we were unable to find both positive and negative sense HoCV1 RNA from these experiments, indicating that virus replication was not occurring. We tried using different transfection reagents, but were unable to generate positive results. To see if the problem was RNA degradation during transfection, we then tried to generate a plasmid with an insect derived promoter in front of the HoCV1 sequence, which would then be expressed in the Z15cells.

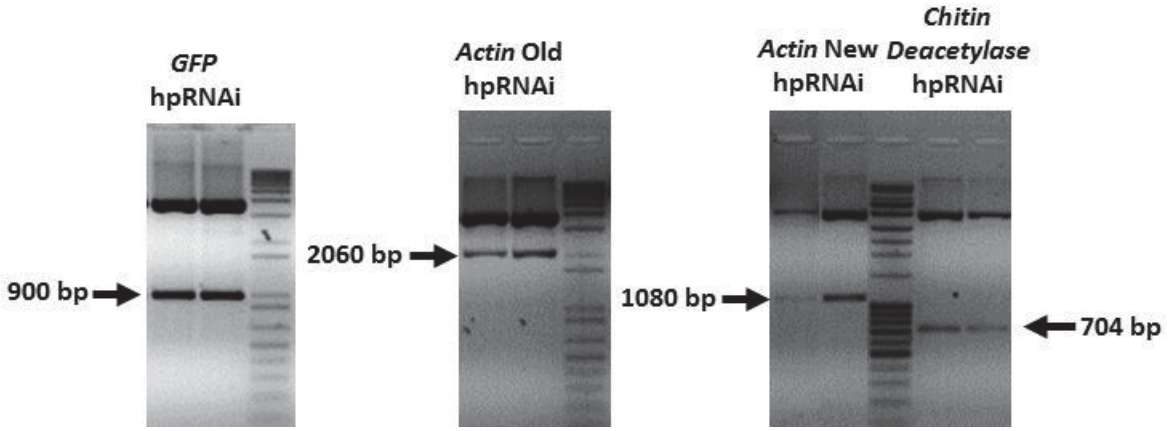

Figure 7. Positive pGEM-13 Zf+ clones obtained carrying hpRNAi cassettes against *GFP*, GWSS *actin*, and GWSS *chitin deacetylase*. Approximate sizes of hpRNAi when released from the vector are: *GFP*-900 bp; *Actin Old*-2060 bp; *Actin New*-1080 bp; *Chitin Deacetylase*-704 bp.

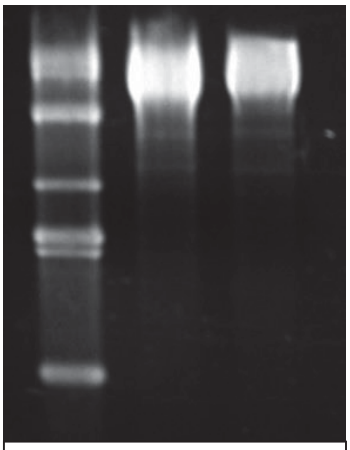

Figure 8. RNA gel of capped (left) and uncapped (right) HoCV1 from in vitro transcription that was used for transfection of Z15 cells.

We used a Baculovirus promoter, *ie1*, and the promoter to heat shock 70 gene in *Drosophila melanogaster*, *hsp70*, to use plasmid template for the transfection experiments instead of single stranded RNA. DNA is a more robust template to work with, and we already have a vector system that works with SF9 cells using this promoter. Previous research has shown the *ie1* system to work in *Bombyx moorei* and *Drosophila melanogaster*, so we designed experiments to see if they would be effective for the Z15 cell line (1). Initially, we used the plasmid pAcp+IE1lacZ, a plasmid with the promoter *ie1* and *lac-z* gene to determine if it can drive production of messenger RNA and protein in Z15 cells. We also used the plasmid pAcDZ1, which contains the heat shock 70 promoter, *hsp70*, with the *lacz* gene. We were able to transfect the Z15 cell line to express the *lac-z* gene with both constructs. Unfortunately, we found we would not be able to use *lac-z* as a reporter gene in whole insects because reporter substrates, such as x-gal, were hydrolyzed by naturally occurring enzymes in whole insects, causing no differentiation between treatments and controls. We then redesigned the constructs to have the *ie1* or *hsp70* promoters drive GFP synthesis, creating plasmids pAcP+IE1GFP and pAcDGFP. At the same time we used restriction digestion to take both promoters and insert them upstream of the HoCV1 sequence flanked by ribozymes, creating the plasmids pIE1-RzHoCV1Rz and pHSP70-RzHoCV1Rz. Unfortunately, this was as far as we got before June.

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FUNDING AGENCIES

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SELECTIVE DISRUPTION OF GLASSY-WINGED SHARPSHOOTER MATURATION AND REPRODUCTION BY RNAi

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Reporting Period: The results reported here are from work conducted July 2014 to October 2014.

ABSTRACT
The overall goal of this project is to develop an RNAi-mediated system to inhibit maturation and reproduction of the glassy-winged sharpshooter (GWSS). The initial target for RNAi will be GWSS *jheh* (also known as *hovi-meh1* (Kamita et al., 2013)), the gene that encodes juvenile hormone epoxide hydrolase (JHEH). GWSS *jheh* will be used as a model gene target to establish an efficient expression and screening system for characterizing RNAi effectors. This system will then be used to evaluate other JH metabolic genes including those that encode JH esterase, JH acid methyl transferase, and other identified genes as targets for RNAi. These gene sequences will be mined from the recently determined transcriptome sequence of GWSS (Nandety et al., 2013). Finally, plant virus- or insect virus-based systems for expression and delivery of the RNAi effectors in insects will be developed.

LAYPERSON SUMMARY
A natural process called RNAi is used by a wide range of organisms to regulate normal gene function and defend against viruses. RNAi can be artificially manipulated to knock down the activity of a targeted gene. In this project, an RNAi-based system will be developed to knock down the activity of key genes in the glassy-winged sharpshooter (GWSS) endocrine system. We predict that minor disruption of this highly sensitive regulatory system will lead to aberrant GWSS development and reduce its ability to spread Pierce's disease. Strategies for a field-applicable delivery system for inducing RNAi will be tested.

INTRODUCTION
Juvenile hormone (JH) and molting hormones are the key regulators of insect maturation. JH also regulates other important biological actions such as reproduction, mating behavior, feeding induction, and diapause (reviewed in Riddiford, 2008). The level of JH within an insect is determined by a combination of its biosynthesis and degradation. JH acid methyl transferase (JHAMT) is the enzyme that catalyzes the final step of JH biosynthesis. On the other hand, JH degradation occurs through the action two hydrolytic enzymes called JH epoxide hydrolase (JHEH) and JH esterase (JHE). JHEH and JHE metabolize the epoxide and ester moieties that are found on all JH molecules. Minor changes in normal JH levels can result in dramatic alterations in insect development or death. The sensitivity of the insect endocrine system to minor changes is a critical factor in the success of JH analog insecticides such as pyriproxyfen and methoprene. As a class, these insecticides mimic the chemical structure and/or biological action of JH. If insects are exposed to these compounds at a time during development when JH titer is normally undetectable, these compounds cause abnormal nymphal-pupal development and/or death. RNAi is a natural process that is found in a wide range of organisms that regulates gene function and protects against viruses (reviewed in Huvenne and Smagghe, 2010).

The natural RNAi process can be artificially induced in insects and other organisms by the introduction of RNAi effectors. These RNAi effectors are double-stranded RNA (dsRNA) or small interfering RNA (siRNA) that targets a specific messenger RNA. For example, RNAi is induced in insects that feed on artificial diet infused with dsRNA or on transgenic plants that express dsRNAs. RNAi-based technology will likely be developed into an effective and highly selective method of plant protection (reviewed in Burand and Hunter, 2013; Gu and Knipple, 2013). A key requirement of any RNAi approach for plant protection is the identification of a suitable gene target. JH metabolic genes such as those encoding JHEH, JHE, and JHAMT are ideal targets for RNAi-based insect control strategies because they are components of a critical, highly sensitive, insect-specific, developmental pathway. RNAi has already been used in laboratory experiments to knock down some of these gene targets in beetles (Minakuchi et al., 2008) and caterpillars (Asokan et al., 2013).

OBJECTIVES

1. Develop *jheh* as a model target for RNAi-based control of GWSS maturation.
2. Mine the GWSS transcriptome for other RNAi targets.
3. Develop virus-based dsRNA production and delivery systems for controlling GWSS.

RESULTS AND DISCUSSION

The primary focus of the initial four months of this project has been to mine full-length JH esterase and JH methyl transferase encoding sequences from GWSS. In order to do this, double-stranded cloned DNA (ds cDNA) libraries were generated from a developmentally mixed population of 5th instar GWSS (30 individuals) as well as individual GWSS at day 7, 8, and 9 of the 5th instar. The ds cDNAs are being used as template sequences for 3'- and 5'-random amplification of cDNA ends (RACE) procedures to generate full-length gene coding sequences. Degenerate as well as gene-specific primers (based on transcriptome sequences from 5th instar nymphs, Nandety et al., 2013) were designed for the 3'- and 5'-RACE. These primers have been used to generate partial sequences of several putative JHE-encoding cDNAs. Unfortunately, the full-length sequences remain elusive possibly because the 5' ends of the target cDNAs were not completely synthesized during the reverse transcription steps. Attempts to general full-length cDNAs are ongoing. A cDNA encoding JH epoxide hydrolase of GWSS, GWSS *jheh*, has been previously identified (Kamita et al. 2013). Primers to amplify various lengths of this cDNA for the generation of dsRNAs (using the MEGAscript T7 expression system) have been designed. The dsRNA generated will be tested in individual 5th instar GWSS for *jheh* knockdown by real time PCR.

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FUNDING AGENCIES

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MONITORING FOR INSECTICIDE RESISTANCE IN THE GLASSY-WINGED SHARPSHOOTER IN CALIFORNIA

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Reporting Period: The results reported here are from work conducted July to October 2014.

ABSTRACT
Insecticide bioassays were conducted on glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) populations collected in Kern and Riverside counties from July through September 2014 to evaluate relative susceptibilities to various insecticides. Of particular concern was the possibility of resistance to imidacloprid occurring after more than a decade of area-wide imidacloprid use. Mortality responses were high to imidacloprid and to a second neonicotinoid compound, dinotefuran. Other compounds tested included two pyrethroids, bifenthrin and fenpropathrin, and two organophoshates, chlorpyrifos, and dimethoate. Of these six insecticides, only dimethoate failed to elicit a strong mortality response. Comparison of 2014 bioassay results to test results obtained from 2001-2003 suggest that no significant change in responsiveness to insecticide treatments has occurred in GWSS populations from either region.

LAYPERSON SUMMARY
Management of the glassy-winged sharpshooter (*Homalodisca. vitripennis*; GWSS) in California for more than a decade has relied heavily on insecticide treatments to suppress populations and ultimately reduce vector pressure and incidence of Pierce's disease. The neonicotinoid insecticide imidacloprid has played a pivotal role in area-wide control programs in Kern and Riverside counties where a mix of citrus and grapes acreages contributed in the past to high populations of GWSS and epidemics of Pierce's disease. Reports in recent years about increased numbers of GWSS occurring in Kern County despite area-wide applications of imidacloprid have raised concerns about the possibility of resistance to imidacloprid being present in GWSS populations. The present study was undertaken to investigate responses of GWSS populations to imidacloprid and other insecticides in bioassays conducted in the laboratory using field-collected samples. Comparison of bioassay results from 2014 collections to similar results obtained from 2001-2003 indicate non-significant differences in susceptibilities. Further testing from additional sites in Kern and Riverside counties will be necessary before conclusively determining whether GWSS populations have developed resistance to one or more insecticides.

INTRODUCTION
Area-wide programs administered by the USDA Animal and Plant Health Inspection Service for glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) control first were implemented in 2000 and have been an integral part of its management ever since. Well-timed applications of imidacloprid in citrus orchards have proven to be highly effective at reducing the first generation of GWSS each year and limiting the dispersal out of citrus to grapes and other crops vulnerable to transmission of *Xylella fastidiosa* (*Xf*) (Castle et al. 2005). In addition, abatement programs conducted at the state and county levels have caused wide exposure of GWSS populations to a number of foliar insecticides representing different classes and modes of action. These programs have been driven by intense concerns about the capacity of GWSS to increase to conspicuous numbers and disperse across the landscape spreading *Xf*. The outfall from such concerns has been a prolonged exposure of GWSS populations to multiple classes of insecticides over a wide area of California for more than a decade. The legacy of insecticide resistance occurrence in over 500 species of arthropods (http://www.pesticideresistance.com/) has been that continuous exposure of insect populations to insecticide treatments eventually results in some level of resistance to one or more insecticides. Whether this phenomenon is responsible for control problems that have been reported in Kern County is unknown and will remain so without appropriate toxicological investigations. Successful management of insecticide resistance requires monitoring of populations to test their relative susceptibility to various insecticides and making appropriate adjustments in the insecticide regimen to reduce insecticide resistance selection pressure. Information gained by a resistance monitoring program on specific insecticides that are no longer effective is essential to a sustainable management program for GWSS.

At the time that concerted action first was mobilized to address the threat represented by the spread of GWSS in California, virtually no field efficacy data or toxicological information was available regarding relative susceptibilities to various insecticides. What soon became clear from research investigations as well as area-wide and local community control actions was that GWSS populations responded readily to insecticide treatments. Dramatic declines were observed in the area-wide control programs, and locally heavy infestations could be knocked down by a single treatment compared to other agricultural pests that continue to persist at economically high densities no matter what treatment is applied. For example, systemic uptake bioassays with imidacloprid produced LC_{50}s of 16 and 22 ng ml^{-1} (= parts per billion) in two different tests using GWSS adults collected from citrus at UC Riverside's Ag Ops. In comparison, the same type of bioassay performed on field populations of the silverleaf whitefly, *Bemisia argentifolii*, typically yielded LC_{50}s in the range of 3-115 µg ml^{-1} (e.g. Prabhaker et al. 2005), values approximately 1,000 times greater than those for GWSS. In the case of another compound widely used for GWSS control, the LC_{50} for chlorpyrifos was determined at 3.4 ng ml^{-1} (Prabhaker et al. 2006), whereas LC_{50}s against *B. argentifolii* for chlorpyrifos often requires 1,000-2,000 µg ml^{-1} (Castle et al. 2009), a concentration nearly one million times greater than that needed to produce the same level of mortality in a bioassay for GWSS. Remarkably, commercial rates for chlorpyrifos (Lorsban® 4E) have been in the neighborhood of 12 pts $acre^{-1}$ (formulated product) (Grafton-Cardwell 2003), the top label rate for this insecticide in citrus. At rates this high, the likelihood of potential overkill of both GWSS and beneficial insects is high. Unfortunately, toxicological data that puts into perspective the relative susceptibility of GWSS have not been available when determining what rates to use for combating GWSS infestations. Awareness of such information along with other field-based efficacy data is crucial to the development and deployment of an evidence-based insecticide use strategy.

Previous studies by the members of this research team have demonstrated the high susceptibility of GWSS to insecticides and the contributions of natural enemies to the control of GWSS populations (Castle et al. 2005 a,b; Prabhaker et al. 2006 a,b, 2007). However, toxicological tests for these studies were carried out relatively early within the time period that area-wide and local control efforts were carried out against GWSS. To our knowledge, there have been no subsequent studies on susceptibility of GWSS populations to insecticides even though insecticides have served as the primary defense against GWSS populations. Because pesticides are an integral part of the high-yielding production agriculture in citrus and grapes, pest resistance to pesticides must be evaluated. This potential is magnified when overreliance on a few select products occurs, such as has been the case with the use of imidacloprid in the area-wide control programs and by growers and pest control advisors protecting their orchards and vineyards. The repeated use of the same product(s) for control of a pest population results in continual selection pressure, which ultimately may result in resistance development. The continued successful implementation of insecticides in management programs require that their efficacy be carefully evaluated and monitored to ensure maximum benefit. Insecticide resistance poses the most serious threat to the long-term use of insecticides for controlling GWSS. There are both financial and environmental costs associated with resistant populations that can be mitigated by a resistance monitoring and management program.

OBJECTIVES

1. Conduct laboratory bioassays on field-collected GWSS from Kern and Tulare counties to determine susceptibility to neonicotinoid, pyrethroid, and organophosphate insecticides.
2. Investigate the geographic variation in susceptibility of GWSS to determine if a pattern of resistance emerges associated with insecticide use patterns.
3. Identify potential resistance evolution of the field populations of GWSS to insecticides by comparing the LC_{50} values to previously established LC_{50}s using the same methodology.
4. Evaluate relative toxicity of new insecticides such as spirotetramat, cyantraniliprole, and flupyradifurone as candidates for alternative treatments for GWSS.

RESULTS AND DISCUSSION

Collections of GWSS were made from the Bena Road area east of Bakersfield in July, August, and September. A mixed-age navel orange orchard yielded high numbers of adults the first two months, but then was treated by insecticides in September that virtually eliminated the once heavy infestation. An organic navel orange orchard was sampled for the September collection that also returned a high number of adults for bioassay purposes. In the Temecula region, GWSS adults were collected from organic citrus orchards but they were in much lower densities compared to the Bakersfield locations. Thus they required considerably more time before sufficient numbers were obtained for bioassay tests.

Differences in toxicities among insecticides were observed in the August and September collections from Kern County. A comparison between the two neonicotinoids, dinotefuran (Venom®) and imidacloprid (Admire® Pro), indicated the higher toxicity of dinotefuran (**Figure 1**). However, a progressive dose response at relatively low concentrations was still observed with the imidacloprid treated insects that suggests susceptibility to imidacloprid is still present in this population. A similar dose response was observed in GWSS collected from Temecula (**Figure 2**). High control mortality was observed with Temecula insects as well as in other bioassays that will have to be corrected by improving handling of insects in future tests.

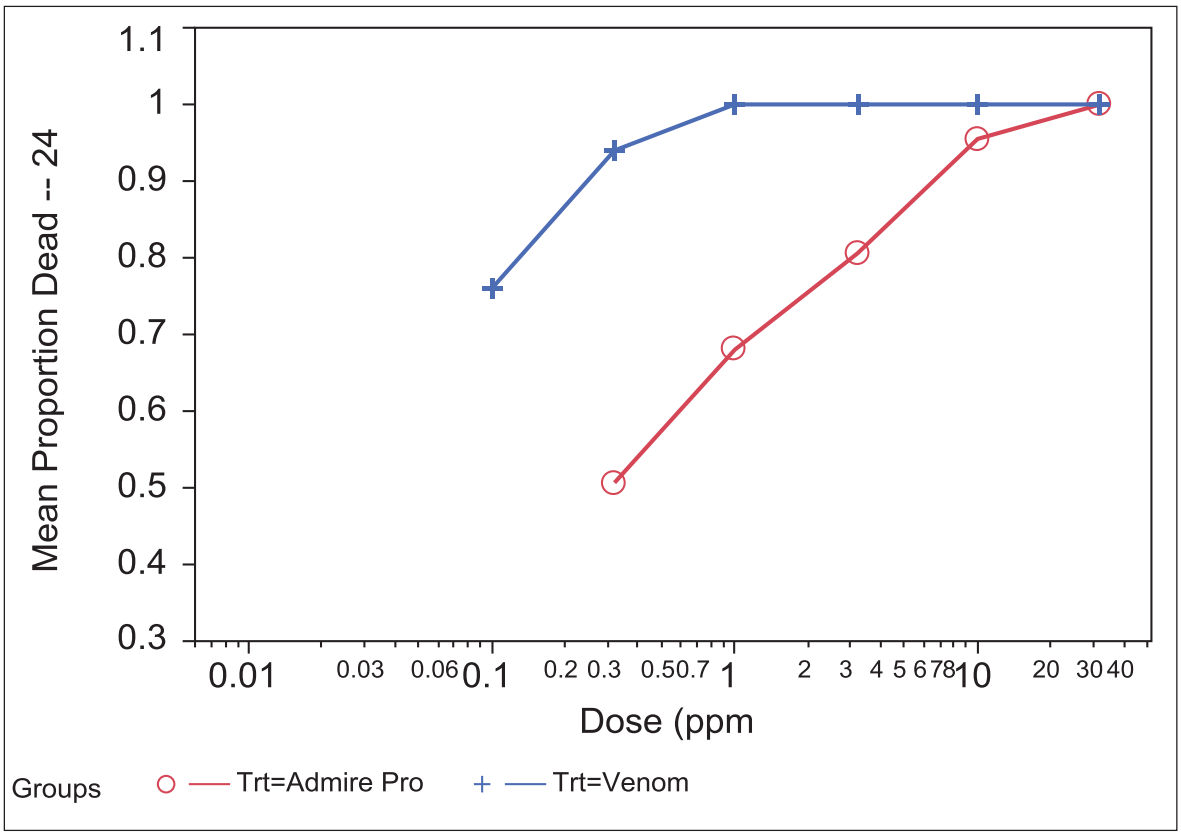

Figure 1. A comparison of mortality responses in GWSS from Kern County to the neonicotinoid insecticides dinotefuran and imidacloprid.

In addition to the neonicotinoids, two pyrethroid insecticides also showed extreme toxicity against GWSS (**Figure 3**). The concentration range was clearly set too high and will have to be adjusted lower for future testing. Of the two insecticides, bifenthrin appeared more toxic than fenpropathrin. Based on the toxicity observed in the bioassay, it seems possible that either insecticide could serve as an effective knockdown agent in situations where an infestation needed to be brought quickly under control.

CONCLUSIONS

Preliminary bioassays conducted in 2014 indicate that GWSS populations at two sites in Kern County and in Temecula remain susceptible to imidacloprid and even more so to dinotefuran, also a neonicotinoid insecticide. In addition, two pyrethroid treatments were highly toxic to GWSS.

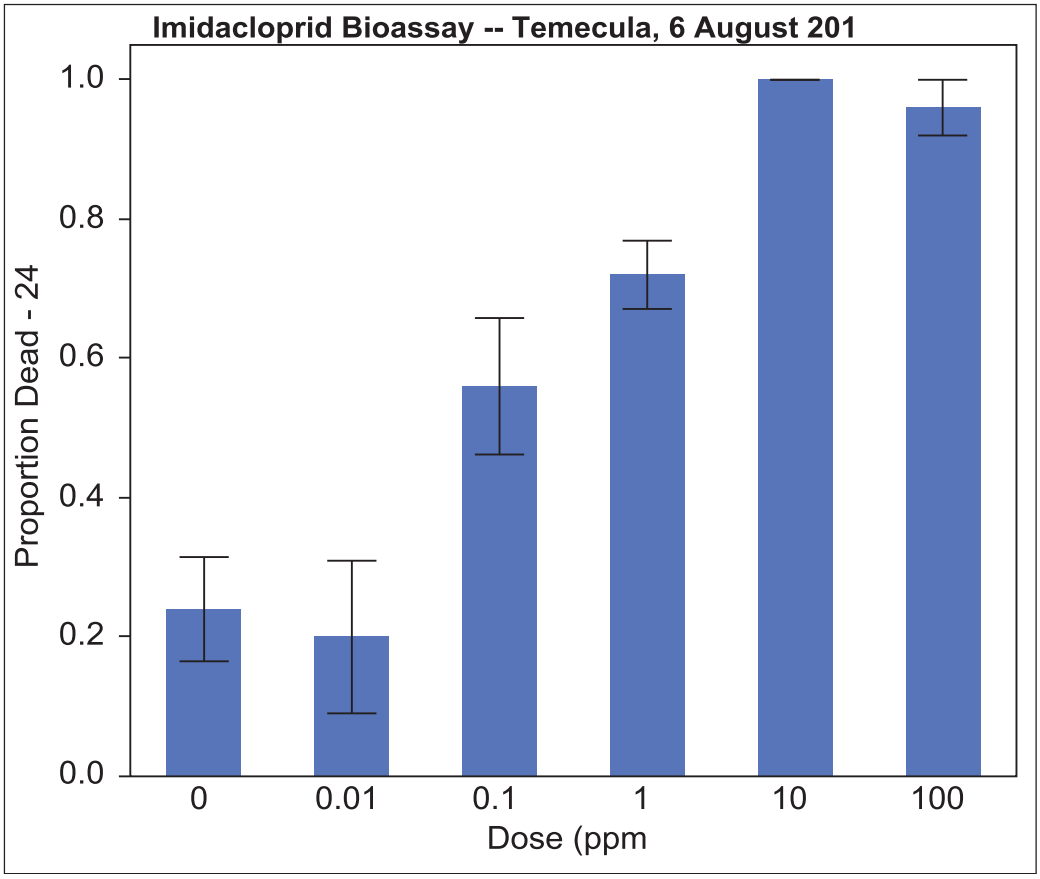

Figure 2. Mortality response to imidacloprid in GWSS collected from Temecula. The approximate 70% mortality at 1.0 ppm is similar to the mortality seen in Kern County insects at the same dose (see **Figure 1**).

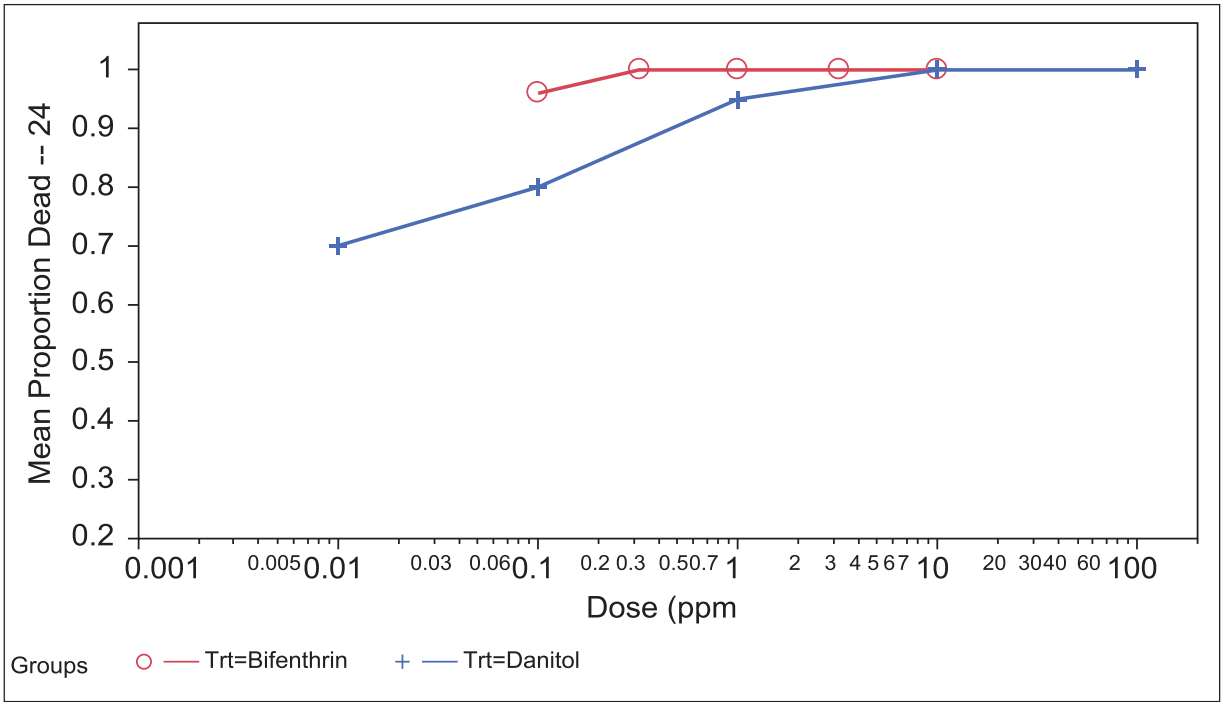

Figure 3. Comparison of mortality responses in GWSS adults to bifenthrin (Capture®) and fenpropathrin (Danitol®).

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MANAGEMENT OF INSECTICIDE RESISTANCE IN GLASSY-WINGED SHARPSHOOTER POPULATIONS USING TOXICOLOGICAL, BIOCHEMICAL, AND GENOMIC TOOLS

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LAYPERSON SUMMARY

Insecticide resistance is one of the major causes of pest control failures for growers. It is most likely to occur where there is reliance on one insecticide. In many cases, the selection for resistance to the principal insecticide used for pest management within a system may also confer cross-resistance to other insecticides. Our project will address the recent upsurge in glassy-winged sharpshooter numbers in Kern County where reliance on a small number of insecticides may have selected for resistance. Associated with this work, we will also investigate whether heavy insecticide use has selected for resistance in the Western Riverside County (Temecula area) and in Orange County (commercial nursery industry). We will use diagnostic tools that detect resistance, and the information generated will enable pest managers to refine existing control strategies and minimize the impact that resistance has on future management efforts.

INTRODUCTION

This is a new project initiated in October 2014. In previous studies funded by the Pierce's Disease Control Program, toxicology work was conducted to determine baseline susceptibility levels of the glassy-winged sharpshooter (GWSS) to different insecticide classes. The results of these studies have been published in peer-reviewed journals (Byrne & Toscano, 2007; Prabhaker *et al*., 2006, 2007). The bioassay techniques that were developed and the baseline toxicological data that were generated in those studies can be used in this proposed new study as a reference point in our efforts to detect any shifts in susceptibility that have occurred as a consequence of the continued use of insecticides for the statewide control of GWSS. In addition to the new assays we will develop in this study, previously developed GWSS-specific biochemical assays that measure qualitative and quantitative changes in putative insecticide resistance-causing enzymes will be used to evaluate the incidence of insecticide resistance in agricultural, nursery, and urban populations of GWSS.

Systemic imidacloprid treatments have been the mainstay of GWSS management in citrus, grapes, and commercial nursery operations. The treatments in citrus groves are generally applied post-bloom to suppress the newly-emerging spring populations. The use of winter or early spring foliar treatments of pyrethroid or carbamate treatments were introduced to the management program to suppress over-wintering adults and reduce the first early season cohort of egg-laying adults. The combination of early season foliar treatments combined with the more persistent systemic treatments has effectively managed GWSS populations in the Bakersfield area for many years.

In Kern County, GWSS populations have been monitored since the area-wide treatment program was instigated by the California Department of Food and Agriculture following an upsurge in GWSS numbers and an increase in the incidence of Pierce's disease. The data shows an interesting pattern of sustained suppression of GWSS populations, following the implementation of the area-wide treatment program, until 2009 when numbers began to increase again, culminating in a dramatic flare-up in numbers in 2012. In 2012, a single foliar treatment with either Lannate® (methomyl: carbamate insecticide class), Assail® (acetamiprid: neonicotinoid insecticide class) or Baythroid® (cyfluthrin: pyrethroid insecticide class) was applied in groves in late March while systemic treatments with imidacloprid (neonicotinoid insecticide class) were applied mid-March to early April. The application of systemic imidacloprid during 2012 mirrored the strategy used in 2001 when the imidacloprid treatments were highly effective in suppressing the GWSS populations. Despite the additional foliar treatments in 2012, the insecticide treatments failed to suppress the insect population at a level that had occurred previously. It is a worrying trend that in the two years prior to 2012, there was a steady increase in total GWSS numbers, an early indication that the predominant control strategy might be failing. The consequence of the increase in GWSS populations has been an increase in the incidence of Pierce's disease. In the Temecula area, this worrisome increase in GWSS has not occurred (yet); however the selection pressure in this area remains high as similar management approaches are in use here as in Kern County.

There is also significant concern for the development of insecticide resistance arising from the management of GWSS in commercial nursery production. The majority of commercial nurseries maintain an insect-sanitary environment primarily through the use of regular application of soil-applied imidacloprid or other related systemic neonicotinoids. For nursery materials to be shipped outside of the southern California GWSS quarantine area, additional insecticidal applications are required. Applications of fenpropathrin (class pyrethroid) or carbaryl (a carbamate) must be applied for all nursery stock shipped out of the quarantine area. As with citrus and vineyard production, here too, the potential for the development of insecticidal resistance to these three classes of materials (neonicotinoids, pyrethroids, and carbamates) is high.

OBJECTIVES

The focus of this proposal is to investigate the role of insecticide resistance as a contributing factor to the increased numbers of GWSS that have been recorded since 2009 in commercial citrus and grapes in Kern County. Although the primary focus of our research will be in Kern County, we propose broadening the scope of the project to include populations from agricultural, nursery, and urban settings. This broader approach will enable us to provide a more comprehensive report on the overall resistance status of GWSS within southern California and develop more effective resistance management plans. Our specific objectives are:

1. For commonly used pyrethroid, carbamate, and neonicotinoid insecticides, determine LC_{50} data for current GWSS populations and compare the response to baseline susceptibility levels generated in our previous studies.
2. Define diagnostic concentrations of insecticides that can be used to identify increased tolerance to insecticides in insects sampled from other locations (where numbers are relatively low).
3. Monitor populations for known molecular markers of resistance to pyrethroids
4. Monitor populations for target-site insecticide resistance, by testing enzymatic activity against carbamates using the AChE biochemical assay.
5. Monitor populations for broad-spectrum metabolic resistance, by comparing esterase levels in current populations of GWSS to baseline susceptibility levels we previously recorded.
6. Develop assays for additional resistance mechanisms not previously characterized in GWSS.

The detection and characterization of resistance to an insecticide will allow us to refine chemical-based management programs in order to minimize selection for that specific resistance mechanism. It may be possible to restore susceptibility to an insecticide through the implementation of a rotational strategy, or if cross-resistance is an issue, it may be necessary to replace the affected insecticide with a chemical having a different mode of action. The methods developed in this project will allow us to make that distinction, and will therefore have a direct impact on GWSS control efforts.

RESULTS, DISCUSSION, AND CONCLUSIONS

As this is an entirely new project (October 2014), no useful data have been collected to date. Our efforts over the last five weeks have focused upon hiring the necessary research personnel refining our insecticide resistance

bioassays, and developing tools for genetic analysis including an improved GWSS genome assembly, which will enable rapid identification of both canonical and novel insecticide resistance genes. Initial genetic analyses and genome assembly are ongoing and being performed on GWSS collected in the urban areas of Riverside in the late 1990s and early 2000s.

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

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

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ABSTRACT

RNA interference (RNAi) in insects is a gene regulatory process that also plays a vital role in the maintenance and regulation of host defenses against invading viruses. The key players in this multi-step disruptive process involve double-stranded RNAs (dsRNAs) that are known to be triggers for siRNA biogenesis. A robust systemic RNAi, though elegantly present in *Caenorhabditis elegans,* is absent in the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS), largely due to the absence of RNA-dependent RNA polymerase (RdRP). We developed plant-based *in vivo* small RNA delivery methods to deliver RNAi inducer small interfering RNAs (siRNAs) towards GWSS. We developed stable transgenic potato plants that either express GWSS genes *actin* or *chitin deacetylase* under constitutive promoter and that express GWSS *chitin deacetylase* with xylem specific Ecad promoter. We tested the ability of these stable transgenic plants for spatial and temporal expression of RNAi molecules and for their effectiveness on GWSS 3rd instar nymph survival, development and the gene target reduction.

Section 3:

Pathogen Biology and Ecology

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

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ABSTRACT
Xylella fastidiosa (*Xf*) is a serious phytopathogen that infects a number of important crops including citrus, almonds, and coffee. The *Xf* Temecula strain infects grapevines and induces Pierce's disease. We deleted the *Xf* PD1311 gene and found that the strain had significantly reduced pathogenicity. Based on sequence analysis, PD1311 appears to encode an acyl CoA synthetase, which is a class of enzymes involved in many different processes including secondary metabolite production. Given the critical role of PD1311 in Pierce's disease development, we are exploring how it induces its phenotype. We are also testing the ΔPD1311 strain as a potential biocontrol for management of Pierce's disease as it appears to dramatically reduce the symptoms when inoculated with wild-type *Xf*.

LAYPERSON SUMMARY
We discovered that deleting the *Xylella fastidiosa* (*Xf*) Temecula gene, PD1311, results in a strain that induces significantly less Pierce's disease. We are performing research to determine how PD1311 plays such a central role in disease development. Given the importance of Pierce's disease, it is critical to understand how PD1311 exerts its effects. We also have evidence that the strain deleted for PD1311 may function as a biocontrol. When inoculated with wild-type *Xf*, disease development becomes significantly reduced. Options for managing Pierce's disease are limited, which makes possible new biocontrols critically important. Together the results from these aims will expand our understanding of Pierce's disease and provide information in relation to preventing disease.

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

We have been examining an *Xf* gene, PD1311, that is annotated as a putative peptide synthase (Altschul et al. 1990) or AMP-binding enzyme (Punta et al. 2012). The putative PD1311 protein contains motifs found in ACSs (acyl- and aryl-CoA synthetases) (Chang et al. 1997, Gulick 2009). While ACS metabolite intermediates are involved in βeta-oxidation, phospholipid biosynthesis, cell signaling, protein transportation, and protein acylation (Korchak et al. 1994, Glick et al. 1987, Gordon et al. 1991), interestingly the *Xanthomonas campestris* ACS, RpfB, appears to be involved in production of quorum-sensing molecule, DSF (diffusible signal factor) (Barber et al. 1997). DSF is known to play an important role in Pierce's disease development (Chatterjee et al. 2008). We deleted the PD1311 gene and found that the resulting strain, ΔPD1311, is significantly attenuated in pathogenicity when inoculated in grapevines. These results indicate that the PD1311 protein is fundamental for Pierce's disease development and therefore warrants further study.

Preliminary studies with the ΔPD1311strain suggest that beyond its role in disease, it greatly reduces pathogenicity by the wild-type Temecula strain. Therefore we propose that it has potential as a biocontrol for Pierce's disease. The weakly virulent *Xf* elderberry strain, EB92-1, has been studied as a Pierce's disease biocontrol (Hopkins 2005, Hopkins 2012). Additional Pierce's disease control strategies that are, or have been, studied involve naturally resistant rootstocks (Cousins and Goolsby 2011) and transgenic varieties (Dandekar 2012, Gilchrist and Lincoln 2012, Kirkpatrick 2012, Labavitch et al. 2012, Lindow 2012, Powell and Labavitch 2012). However, continued research for Pierce's disease controls is warranted. Given the low virulent phenotype of the Δ*PD1311* strain, understanding how PD1311 is associated with the disease response may also provide key insights into Pierce's disease development.

OBJECTIVES

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

Objective 1. Characterize the *Xf* ΔPD1311mutant.

a. Complete *in vitro* behavioral assays critical for disease.
b. Determine the role(s) of PD1311 in producing virulence factor(s).

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

a. Determine conditions for biological control.
b. Examine spread of ΔPD1311and wild-type strains simultaneously.

RESULTS AND DISCUSSION

Objective 1. Characterize the *Xf* ΔPD1311mutant.

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

We deleted the PD1311 gene and complemented the ΔPD1311strain as previously described (Matsumoto et al. 2009, Shi et al. 2009). The ΔPD1311strain grows in PD2 (Davis et al. 1980) and xylem sap (**Figure 1**). We examined the ability of the mutant to move, aggregate, and form biofilm, which are key steps in Pierce's disease development (Chatterjee et al. 2008). Such information will help us determine if PD1311 affects these virulence related behaviors or if it has a more specialized function.

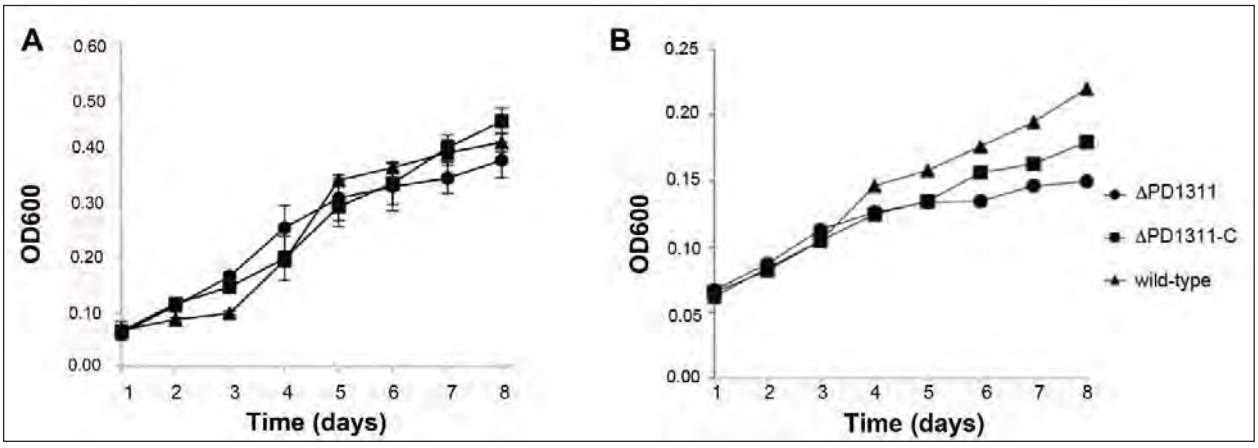

Figure 1. **ΔPD1311 strain growth.** Growth of wild-type *Xf* (triangle), mutant ΔPD1311 (circle), and complemented mutant (ΔPD1311-C; square) strains were examined for eight days in PD2 (A) or 100% *Vitis vinifera* cv. Chardonnay xylem sap (B) and growth was determined by OD_{600} readings.

We examined ΔPD1311movement by the *in vitro* fringe assay where fringe around the bacterial colony directly correlates with type IV pilus twitching motility (Meng et al. 2005, Li et al. 2007). The ΔPD1311strain is motile on PW plates, but it appears to be modestly affected (**Figure 2**). When tested on sap plates, however, it does not produce fringe, suggesting that the mutant requires a component in rich media not provided in nutrient poor sap. We will examine translocation in plants (Meng et al. 2005, De La Fuente et al. 2007a, De La Fuente et al. 2007b)

to determine if it has movement *in vivo*. *Xf* mutants with decreased motility have been shown to induce less Pierce's disease (Cursino et al. 2009, Cursino et al. 2011).

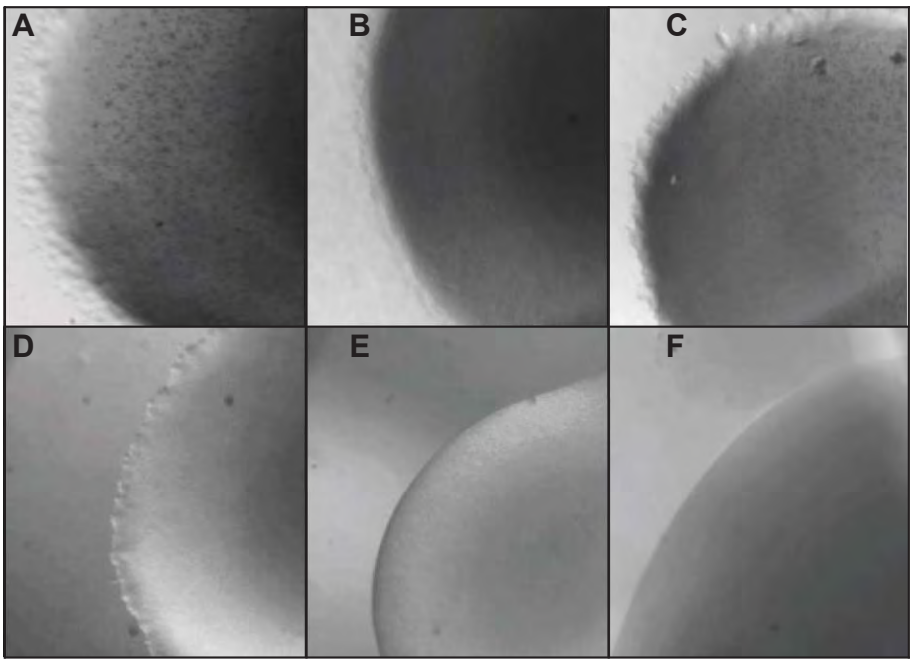

Figure 2. **ΔPD1311 strain fringe.** Colony fringes of wild-type (A, D), ΔPD1311 mutant strain (B, E), and complemented mutant strain (C, F) were assayed on PW agar (A-C) or sap (D-F). Colonies were assessed after five days of growth (Meng et al. 2005, Li et al. 2007). Colonies photographed at 90X magnification. Sap plates were composed of 80% Chardonnay sap, 20% water, and 12g/L agar. PW fringe tested three independent times, sap experiment tested two times.

Cell aggregation is a critical step in biofilm formation, which is proposed to clog xylem vessels and prevent transport of nutrients and water resulting in Pierce's disease (Chatterjee et al. 2008). We found that the ΔPD1311strain has decreased aggregation compared to the wild-type and complemented strains ($P<0.03$) (**Figure 3A**). In addition, the ΔPD1311strain produces less biofilm than wild-type *Xf* ($P<0.0001$) (**Figure 3B**); decreased biofilm production generally correlates with decreased pathogenicity (Cursino et al. 2009, Shi et al. 2009, Cursino et al. 2011).

Preliminary real-time RT-PCR data suggested the ΔPD1311strain has down-regulated expression of i) type I pili gene, *fimA*, which is important for cell adhesion (Li et al. 2007), ii) type IV pili gene, PD1926, which we have found to regulate motility (data not shown), and iii) afimbrial adhesion gene, *hxfB* (hemagglutinin *Xf* B) (Feil et al 2007) (**Table 1**). These results are consistent with the reduced aggregation, motility and biofilm formation phenotype seen in this mutant. Surprisingly, adhesion gene, *xadA* (*Xanthomonas* adhesin-like protein A) (Feil et al 2007), was upregulated in the mutant strain. This result requires further exploration. Overall our findings suggest a role of PD1311 in regulation of multiple virulence factors at the transcriptional level.

<u>1b. Determine the role(s) of PD1311 in producing virulence factor(s).</u>

Given that PD1311 may be an ACS protein and ACS proteins are implicated in the production of quorum sensing molecules (Barber et al. 1997; Chatterjee et al. 2008) we asked if it was involved in DSF production. We streaked wild-type *Xf* and the ΔPD1311 strain onto PW agar plates (Davis et al. 1981) for eight days to allow production of DSF. The *Xanthamonas campestris campestris* (*Xcc*) indicator strain 8523 (kindly provided by Prof. Steven Lindow, U. Cal., Berkeley) was streaked perpendicular to either the wild-type or the ΔPD1311 strain for 24 hours (Newman et al. 2004). A suspension was made of the *Xcc* strain 8523 cells and fluorescence was visualized using a confocal microscope, and we found no changes in fluorescence by the ΔPD1311 strain (data not shown). We are now performing additional DSF studies to confirm the relationship between PD1311 and production of DSF.

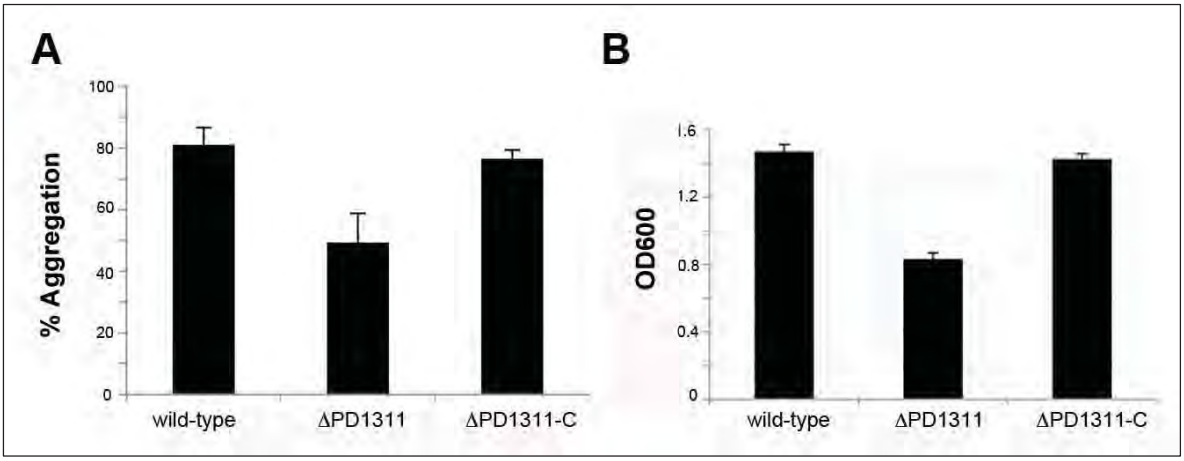

Figure 3. **ΔPD1311 strain aggregation and biofilm formation.** A) Aggregation of wild-type, mutant (ΔPD1311) and complemented mutant (ΔPD1311-C) strains grown in test tubes for five days in three ml PD2 (Davis et al. 1980). After five days the OD_{540} was recorded (OD_T) and the bacteria resuspended before recording the OD (OD_S) again. The percent aggregation was calculated as $[(OD_T - OD_S)/ OD_T] \times 100$ (Burdman et al. 2000, Shi et al. 2007). The experiment was repeated three times. B) Quantification of biofilm formation in 96 well plates for wild-type, mutant (ΔPD1311) and complemented mutant (ΔPD1311-C) strains (Zaini et al. 2009). Experiment was repeated three times with 24 replicates each.

Table 1. Expression of key genes in ΔPD1311 strain compared to wild-type strain. Wild-type or ΔPD1311 mutant cells were grown in PD2 broth for three days and collected for RNA extraction. The *petC* and *nuoA* genes were used as reference genes. Data represents expression in mutant compared to expression in wild-type cells. Three biological samples were included for each strain and the experiment was conducted once.

fimA	PD1926	*hxfB*	*xadA*
0.23± 0.11	0.03± 0.03	0.08± 0.02	15.15± 3.77

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

To determine the impact of the ΔPD1311 strain on Pierce's disease development *in planta*, we inoculated *V. vinifera* cv. Cabernet franc vines per standard procedures (Cursino et al. 2011) and recorded disease development of Pierce's disease using the five-scale assessment (Guilhabert & Kirkpatrick 2005). Our first trial gave promising results and lead to repeat experiments this year (**Figure 4**). We are near the end of two additional trials with this experiment. Trial two once again shows that PD1311 produces little disease. Trial three also suggests little disease but the wild-type strain is not showing the expected Pierce's disease phenotype at week 16, making conclusions difficult. To determine if the ΔPD1311 strain moves through the plant, we will examine translocation of wild-type and mutant strains following standard procedures (Meng et al. 2005).

We believe that the Δ*PD1311* strain may act as a biocontrol because we found that it reduced biofilm production by the wild-type *Xf* strain. For this study, we grew wild-type *Xf* cells constitutively expressing green fluorescent protein (wt-GFP) with either the ΔPD1311strain or wild-type cells. We previously used this strain (kindly provided by Prof. Steven Lindow, UC Berkeley) and found it to produce biofilm similar to wild-type *Xf* (data not shown). As stated above, wild-type cells produce more biofilm than the ΔPD1311strain so mixtures of wt-GFP/ΔPD1311should have equal or greater fluorescence than mixtures of wt-GFP/wt, if the strains did not impact each other. We found that the wt-GFP/ΔPD1311mixture had <u>less</u> fluorescence than the wt-GFP/wt mixture (**Figure 5**), indicating that the ΔPD1311 strain impacts biofilm produced by wt *Xf*.

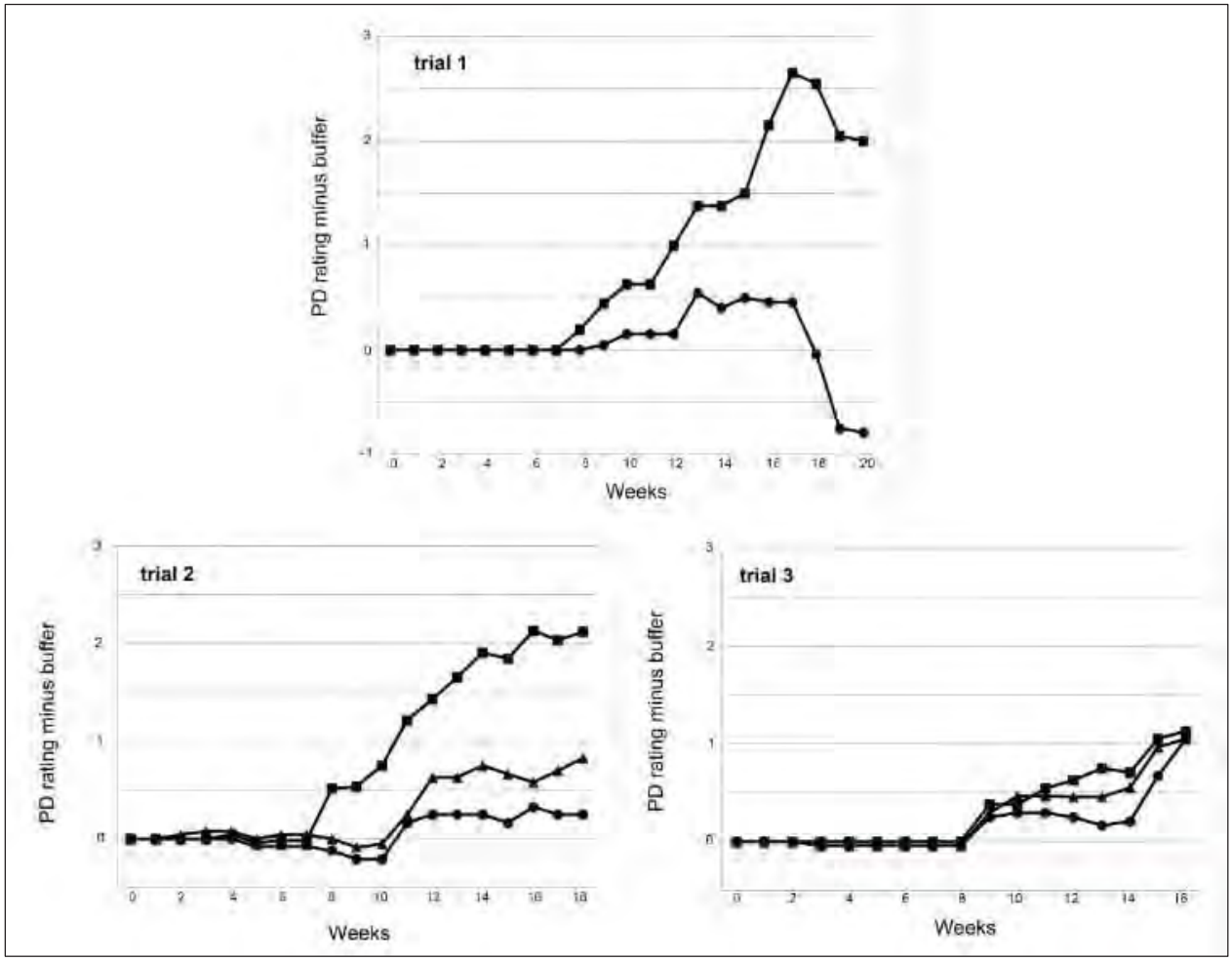

Figure 4. Development of Pierce's disease. Grapevines were inoculated with wild-type *Xf* (square), *Δ*PD1311 strain (circle), ΔPD1311 complement (triangle), and buffer. Symptoms were monitored and rated on a scale of 0-5 (Guilhabert and Kirkpatrick 2005, Cursino et al. 2009). Trial one was followed for 20 weeks, while trial two and three are still in process. Trial one did not include the complement strain.

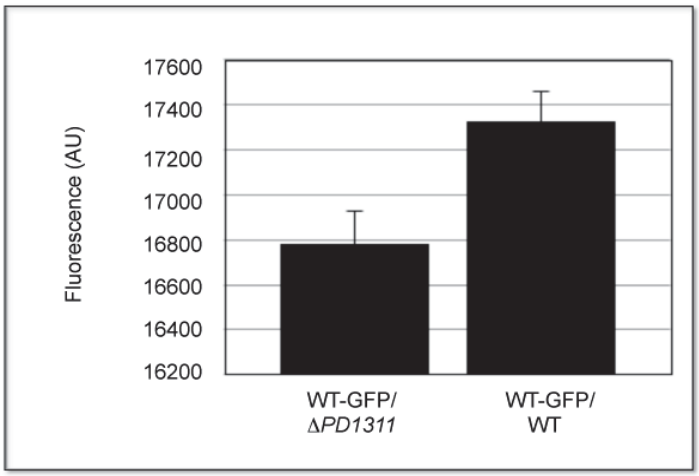

Figure 5. The ΔPD1311 strain impacts biofilm formation by wild-type cells. Quantification of biofilm in 96 well plates with agitation with equal amounts of wild-type *Xf* constitutively expressing green fluorescent protein (WT-GFP) and either wild-type *Xf* (WT) or the ΔPD1311 strain (Δ*PD1311*). Experiment was performed with 24 replicates. Fluorescence in arbitrary units (AU).

2a. Determine conditions for biological control.

Given our findings that the ΔPD1311strain induces low virulence and impacts biofilm production by wild-type cells, we began greenhouse studies to determine if the ΔPD1311 strain can be a viable biocontrol for Pierce's disease. We have three different inoculation conditions: i) co-inoculated wild-type and ΔPD1311strains, ii) inoculation with the ΔPD1311strain followed two weeks later by wild-type *Xf* [following procedures used in *Xf* elderberry EB92.1 strain biocontrol studies (Hopkins 2005)], and iii) inoculation of wild-type *Xf* followed two weeks later by the ΔPD1311 strain (**Figure 6**). The ΔPD1311 strain significantly reduces the disease impact of wild-type *Xf* whether co-inoculated or inoculated prior or after the wild-type strain. The mechanism by which this occurs is currently being investigated.

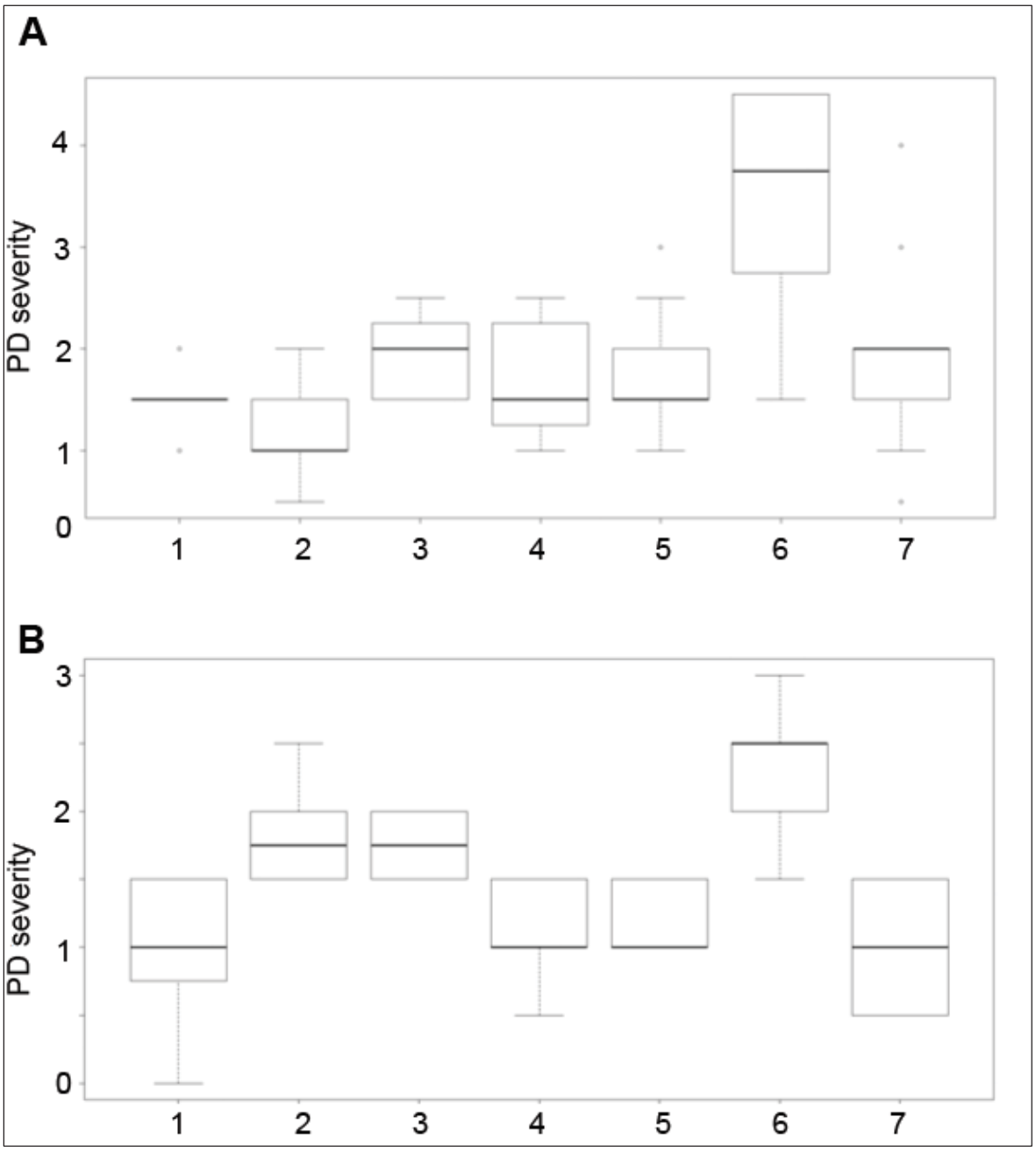

Figure 6. ΔPD1311 strain reduces Pierce's disease. Grapevines were inoculated with buffer (1), ΔPD1311strain (2), ΔPD1311 complement strain (3), wild-type two weeks after ΔPD1311 inoculation (4), a mixture of ΔPD1311 strain and wild-type cells (5), wild-type (6), and ΔPD1311 two weeks after wild-type inoculation (7). Bold lines represent the median values and circles representing outliers of each data group. Symptoms have been monitored on 12 plants for each treatment for 21 weeks (A) or 19 weeks (B) and rated on a scale of 0-5 (Guilhabert and Kirkpatrick 2005).

2b. Examine spread of ΔPD1311and wild-type strains simultaneously.
Our initial trial *in planta* indicates that the ΔPD1311strain can be detected in plants (**Table 2**), suggesting that limited Pierce's disease symptoms from ΔPD1311inoculation is not due to an inability of the ΔPD1311to survive in the grapevines. Given our findings of the impact of the ΔPD1311 strain on wild-type cells *in planta*, and the ability of the ΔPD1311 strain to impact wild-type biofilm production, further studies are needed to determine what specific disease inducing processes by wild-type *Xf* are curtailed by the ΔPD1311 strain.

Table 2. Δ*PD1311* strain detected *in planta*. Five microliters of 10^9 CFU/mL of wild-type (WT) or mutant (ΔPD1311) *Xf* were inoculated into young grapevines in the 6-7th node counting from the top. The petioles directly above (up) and below (down) the inoculation point were sampled 10 days post-inoculation for PCR detection using *Xf* specific primers. + or – represents the presence or absence of the characteristic band.

	Plant 1		Plant 2		Plant 3		Plant 4	
	up	down	up	down	up	down	up	down
WT	-	-	-	-	-	+	-	-
ΔPD1311	-	+	-	-	+	+	-	-

CONCLUSIONS
Xf motility, aggregation, and biofilm formation are key steps in Pierce's disease development (Chatterjee et al. 2008). Concerning objective 1a, we have shown that PD1311 plays a role in aggregation, biofilm formation, and motility. For objective 1b, our first study suggests that PD1311 may not be involved in DSF production, however, additional studies are needed to confirm this finding. For objective 2, we are nearing completion of additional greenhouse studies showing that the ΔPD1311 strain has low virulence. Turning to objective 2a, we have found that the ΔPD1311strain impacts biofilm production by wild-type *Xf*. Additionally, the mutant strain appears to inhibit wild-type virulence *in planta*. Objective 2b will be performed once Pierce's disease is more developed in the grapevines. Overall, this work will help further understanding of disease development and prevention.

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DEFINING THE ROLE OF SECRETED VIRULENCE PROTEINS LesA AND PrtA IN THE PATHOBIOLOGY OF *XYLELLA* AND IN THE DEVELOPMENT OF PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted July 2014 to September 2014.

ABSTRACT

The goal of this project is to understand the virulence mechanisms of *Xylella fastidiosa* (*Xf*) that lead to leaf scorching symptoms observed in Pierce's disease and to exploit this information to develop new strategies to control Pierce's disease in grapevines. The analysis of *Xf* Temecula 1 secreted proteins has enabled us to focus on two previously uncharacterized proteins, LesA and PrtA, that appear to be causal to the leaf scorching phenotype observed in Pierce's disease. We generated mutant *Xf* that are defective for each of these two genes and they show alterations in disease phenotype, lesA1 is less virulent while prtA1 is more virulent. LesA displays lipase/esterase activities and is the most abundant but is very similar to two additional less abundant proteins LesB and LesC also secreted by *Xf*. Expression of LesA, B, and C individually in *Escherichia coli* indicate that these proteins can induce scorching symptoms in grapevine and walnut leaves. These symptoms appear to be related to the lipase/esterase activity present in these proteins. The PrtA protein has protease activity and *Xf*-prtA1 mutants are highly virulent. We are building vectors to test this protein for anti-virulence activity in transgenic SR1 tobacco plants. An understanding of how these two proteins work will provide new insights into this disease and provide new avenues of therapy.

LAYPERSON SUMMARY

Pierce's disease of grapevines is caused by the bacterium *Xylella fastidiosa* (*Xf*), a xylem-limited bacterium. A characteristic symptom of Pierce's disease is leaf scorching, with marginal regions of leaves developing chlorosis progressing to necrosis. The blockage of xylem elements and interference with water transport by *Xf* has been posited to be the main cause of Pierce's disease symptom development. The analysis of *Xf* Temecula 1 secreted proteins has enabled us to focus on two previously uncharacterized proteins: LesA and PrtA. We generated mutant *Xf* that are defective for each of these two genes. Mutant *Xf* that do not make LesA are less virulent and the encoded proteins are enzymes that degrade specific lipids and fatty acids. Injecting the proteins in leaves can cause the scorching type of symptoms that appear to be related to the observed enzymatic activity. Mutant *Xf* that lack the ability to make PrtA protein are more virulent suggesting that this protein may somehow block disease. We are engineering tobacco plants to express this protein to test this hypothesis. An understanding of how these two proteins work will provide new insights into this disease and provide new avenues of therapy.

INTRODUCTION

Xylella fastidiosa (Xf) is a fastidious, xylem-limited gamma-proteobacterium that causes several economically important diseases in many plants including grapevine, citrus, periwinkle, almond, oleander, and coffee (Davis et al., 1978, Chatterjee et al., 2008). In the field, *Xf* is vector-transmitted by various xylem sap-feeding sharpshooter insects (Purcell and Hopkins, 1996; Redak et al., 2004). The *Xf* subspecies *fastidiosa* (*Xff*), as exemplified by the California strain Temecula 1, causes Pierce's disease in grapevine. The *Xf* life cycle and virulence mechanism are not entirely understood (Chatterjee et al., 2008). This research seeks to understand the pathobiology of *Xf* that leads to disease, specifically the underlying mechanism that leads to leaf scorching symptoms. Understanding the underlying mechanism could lead to the development of new strategies to control Pierce's disease in grapevines in California. The secretion of virulence factors by pathogens has been shown to be an important mechanism by which many plant diseases are triggered. Unlike its closely related pathogens from genus *Xanthomonas*, *Xff* does

not possess the type III secretion system (T3SS) (Van Sluys et al., 2002). However, *Xanthomonas* and *Xf* have in common a similar type II secretion system (T2SS) for a battery of important extracellular enzymes that are known to be responsible for virulence (Ray et al., 2000). In *Xff*, genes have been identified that code for plant cell wall degrading enzymes (CWDEs) such as polygalacturonase, cellulase, lipase/esterase, and several proteases (Simpson et al., 2000). These enzymes may play a role in *Xff* migration inside the xylem vessels through the degradation of the pit membrane and also in the release of carbohydrates necessary for the bacterial survival. The degradation of the cell wall by CWDEs releases oligosaccharides as products, which can induce potent innate immune responses of plants. The plant defense responses include the production of phytoalexins, fortification of cell walls through the deposition of callose, oxidative burst, and induction of programmed cell death (Darvill and Albersheim 1984; Ryan and Farmer, 1991; Braun and Rodrigues 1993). One of the T2SS secreted proteins, a polygalacturonase, is a virulence factor encoded by pglA, lost pathogenicity when it was mutated, and resulted in a *Xf* that was unable to colonize grapevine (Roper et al., 2007). This confirmed an earlier finding of gaining resistance to Pierce's disease through the expression of a polygalacturonase inhibitory protein that would block the action of pglA (Aguero et al., 2005).

OBJECTIVES

The goal of this project is to define the role that *Xylella* secreted proteins LesA and PrtA play in the Pierce's disease phenotype of grapevine.

Objective 1. Define the mechanism of action of LesA and PrtA gene products.

- Activity 1. Express LesA, B, C, and PrtA individually and examine their role in the virulence response of *Xylella* cultures.
- Activity 2. Metagenome analysis of xylem tissues infected by strains mutated for Les A, B, C, and PrtA.
- Activity 3. Develop transgenic SR1 tobacco expressing PrtA and evaluate protection against *Xylella* virulence.

RESULTS AND DISCUSSION

Objective 1. Define the mechanism of action of LesA and PrtA gene products.

Since lesA1 are more in a biofilm state and prtA1 are mostly planktonic cells our guiding hypothesis is that lesA promotes planktonic growth and this objective will clarify the mechanism by which lesA mediates planktonic growth and examine its relationship to virulence. Conversely prtA promotes biofilm growth as the prtA1 mutants that are disrupted for this gene display a mostly planktonic growth and are more virulent than wild-type *Xf*. The objective is to identify if the protein is able to mediate this behavior.

Activity 1. Express LesA, B, C, and PrtA individually and examine their role in the virulence response of *Xylella* cultures.

The most abundant secreted protein was annotated as an uncharacterized Pierce's disease protein that we have designated LesA. The protein is a lipase/esterase enzyme that appears to be highly conserved in both *Xanthomonas* and *Xylella*. Shown in **Figure 1** are the conserved active site residues in the LesA proteins from *Xanthomonas* or *Xylella*. Mutating the Ser residue at position 200 (**Figure 1**) to an Ala inactivates the enzyme activity as well as the pathogenicity of *Xanthomonas oryzae* pv *oryzae* in rice (Aparna et al., 2009).

Additionally, there are three secreted lipase/esterase proteins that are encoded by three genes in *Xf* that display a strong sequence similarity that we have designated lesA, lesB, and lesC. The lesA and lesB genes are located right next to each other while lesC is another location. We have successfully built vectors to express LesA, LesB, LesC, and PrtA in *Escherichia coli*. Each of the genes was chemically synthesized so the protein coding and secretory sequences are identical to that found in the *Xf* genome. A Flag tag was included at the C-terminal of these proteins to facilitate purification of these proteins. The *E.coli* strain carrying each of these genes was grown on plates containing tributryn to test for the secretory lipase activity. A zone of clearance was observed when the vector contained the coding sequence of LesA (**Figure 2**), the empty vector showed no zone of clearance. In addition we expressed a mutated version of LesA designated LesA2 where the serine residue at position 200 was mutated to Alanine and these strains showed no zone of clearance (**Figure 2**). We also measured for esterase activity using 4 methylumbelliferyl buterate and strong activity was observed with LesA but not with LesA2 or the empty vector (**Figure 2**). Antibodies were made against the LesA protein and *E.coli* that expressed LesA or LesA2 made the protein that could be detected on western blots even though no enzyme activity was observed with LesA2 (**Figure 2**).

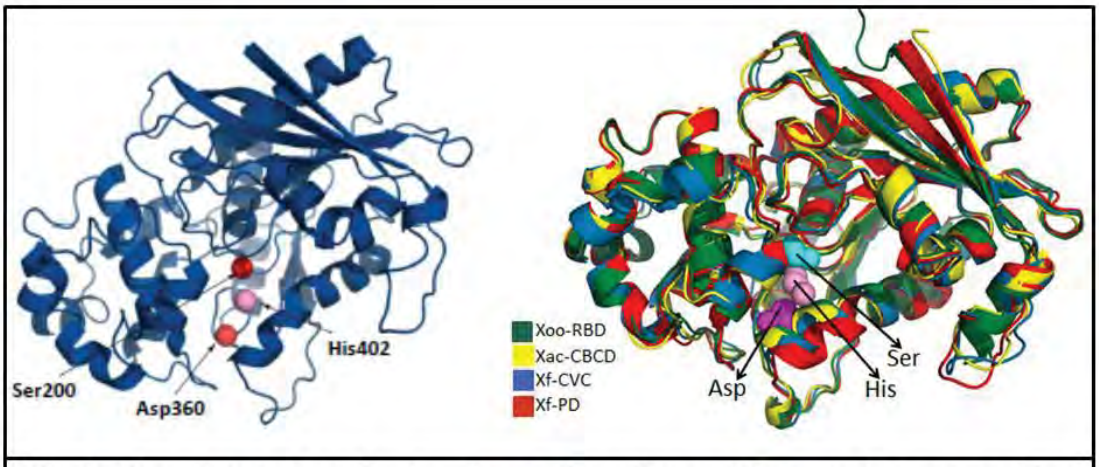

Fig 1: Structure of LesA showing active site residues and conservation of the structure in Xanthomonas and Xylella.

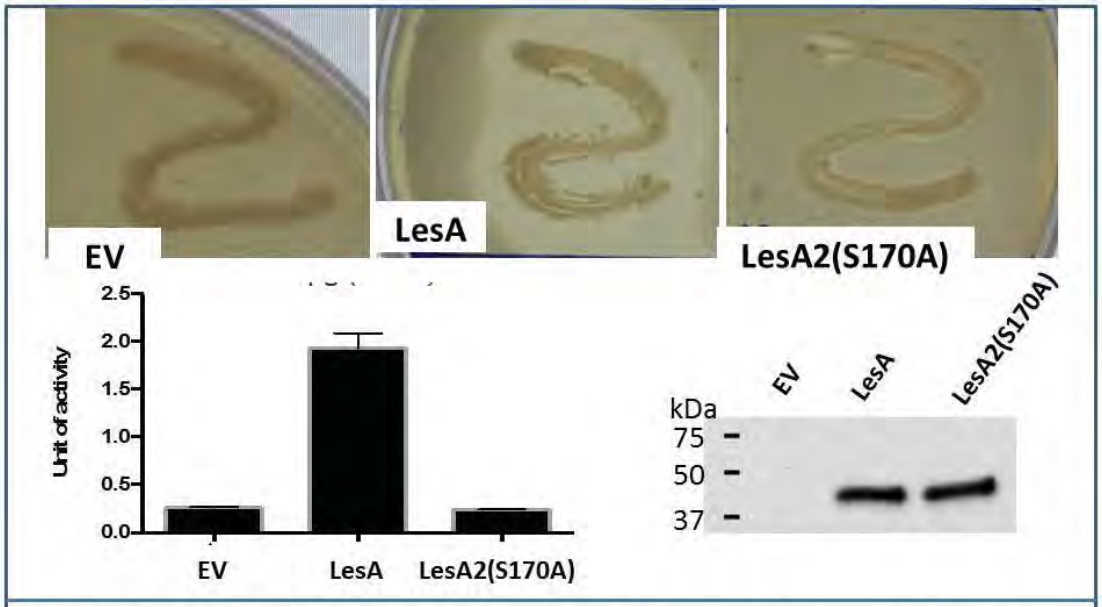

Fig 2: Expression of LesA in E.coli on medium containing tributryn to evaluate lipase activity, EV is the empty vector, wild type LesA and LesA2. Esterase activity measured with 4-methylumbelliferyl butyrate (4-MUB assay). Western blot using LesA antibody.

To evaluate the ability of these proteins to cause leaf scorching symptoms LesA, LesB, and Les C proteins were purified from the *E.coli* expression vectors. The proteins were first tested *in planta* in grapevine leaves using syringe infiltration were the presence of these proteins was sufficient to cause leaf scorch like symptoms. However, it was quite difficult to reproducibility get protein into grapevine leaves, so we used walnut leaves. After normalization of the protein based on weight (5 ug/uL), we infiltrated these proteins into walnut leaves. In Walnut leaves, LesA, LesB, and LesC were capable of causing lesion unlike PBS and proteins extracted from LesA2 (functional mutant: S200A) and empty vector (**Figure 3**). Although the infiltrated proteins are from *Xf* origin, the virulence effect is so severe that is capable of causing lesions in walnut leaves.

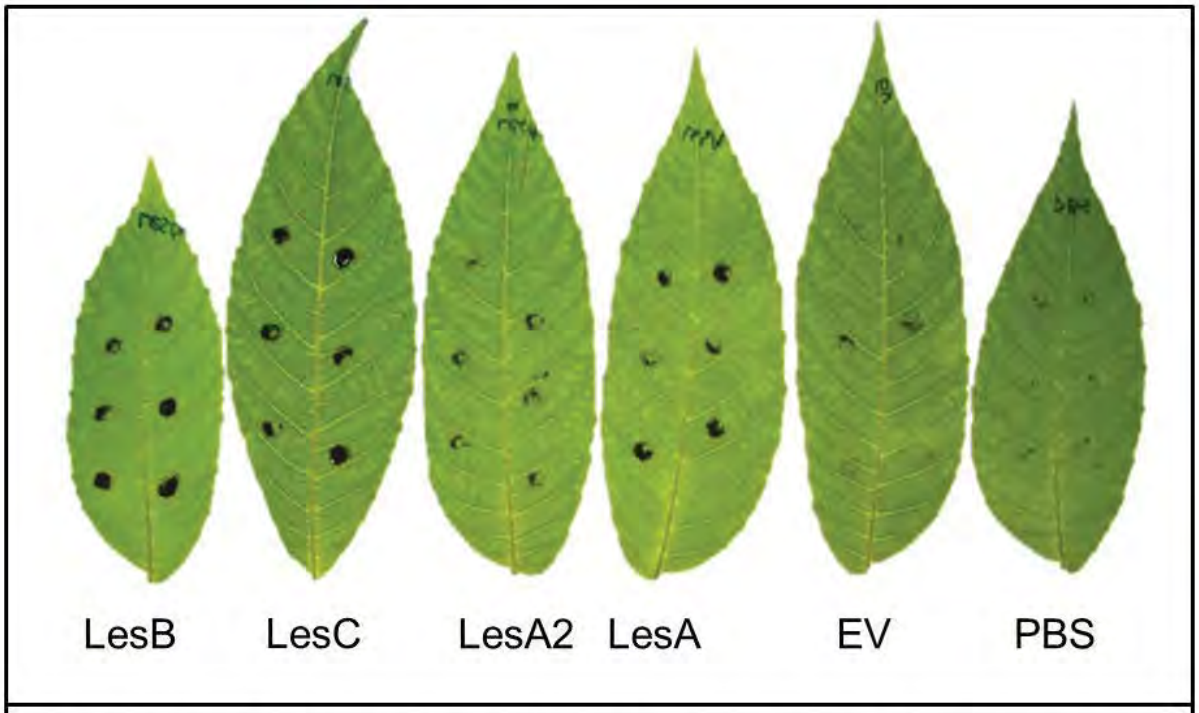

Fig 3: Walnut leaves infiltrated with LesA,B,C LesA2 (functional mutant of LesA) and empty vector (EV) proteins expressed in *E. coli*.

Activity 2. Metagenome analysis of xylem tissues infected by strains mutated for Les A, B, C, and PrtA. The secreted proteins could influence the grapevine microbiota and that interaction could influence the disease outcome. To investigate this possibility we have used mutants unable to make LesA (lesA1), LesAB (lesA2B3) and PrtA (prtA1). In order to investigate this approach we conducted a preliminary survey of the alpha diversity of the resident microbiome of Thompson Seedless (TS) grapevines infected with wild-type (Temecula 1), prtA1, and an uninfected control. TS grapevines were allowed to grow for 12 weeks and then five plants from each treatment were harvested. Briefly, we collected from the 1st offshoot above the 10th node of each plant (the 2nd and 3rd petiole and leaf from this offshoot). Samples were placed on ice and immediately brought to the lab where they were frozen in liquid nitrogen. Samples were kept frozen and ground into powder using Qiagen's grinding jar set and associated TissueLyser. DNA was extracted using the MoBio PowerPlant ®Pro DNA isolation kit. PCR and sequencing of the V4 region of the 16S rRNA gene using region-specific primers and PCR and sequencing were performed using standard protocols as agreed upon in the Earth Microbiome Project (http://www.earthmicrobiome.org/emp-standard-protocols/) and using Illumina MiSeq (Caporaso et al., 2012).

The immediate problem we observed when we examined the sequencing data obtained from samples was a high proportion of host chloroplast sequences that came from the extraction of the leaf samples. After removing chloroplast sequences from analysis the sequencing depth was not sufficient for analysis. As such, the PCoa plot (**Figure 4**) does not show large differences between samples. Additionally, due to sampling constraints our preliminary samples were obtained from new growth far from the initial point of infection. For our later infection study we will obtain non-leaf samples and will obtain tissue much closer to the inoculation point closer to where symptoms are located and where probable changes in the microbiota will be first evident. Additionally, for both the alpha diversity and infection study the depth of sequencing will be increased by dealing with the chloroplast sequences in one of three ways: 1) Chloroplast depletion; 2) Chloroplast-excluding primers (Redford et al., 2010); 3) PCR Blockers.

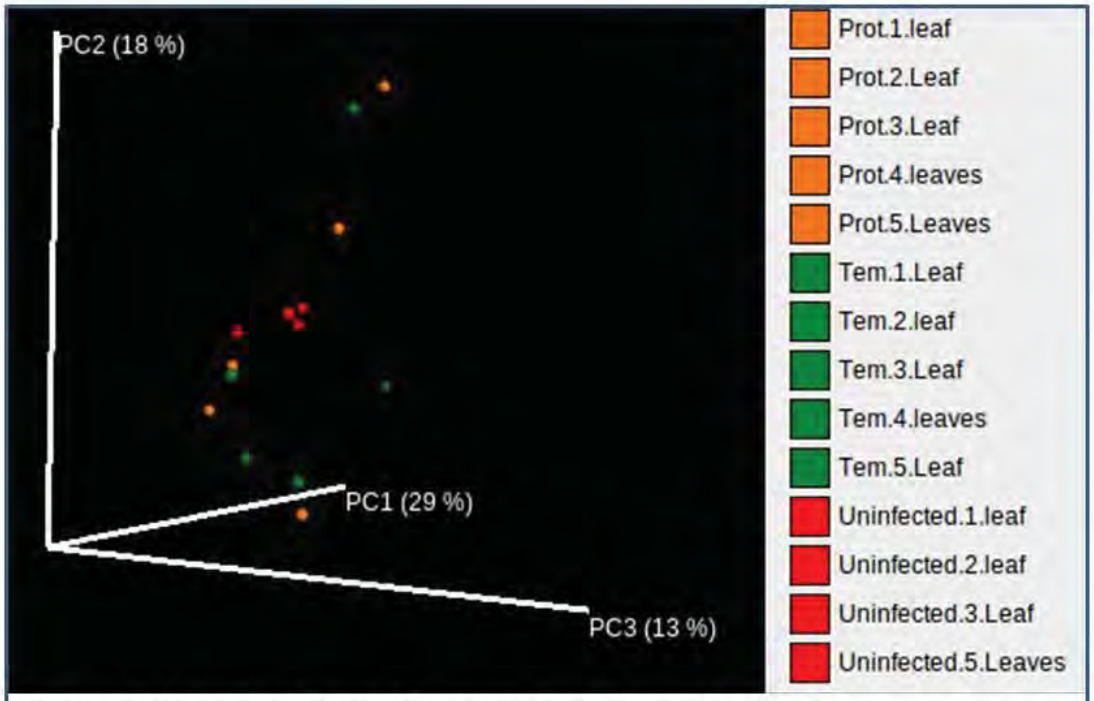

Figure 4: PCoA plot of Weighted Unifrac Values. Results show a possible correlation of *X.f. mt prtA* (Prot) and *X.f. Tem1* (WT) with possible clustering of uninfected samples.

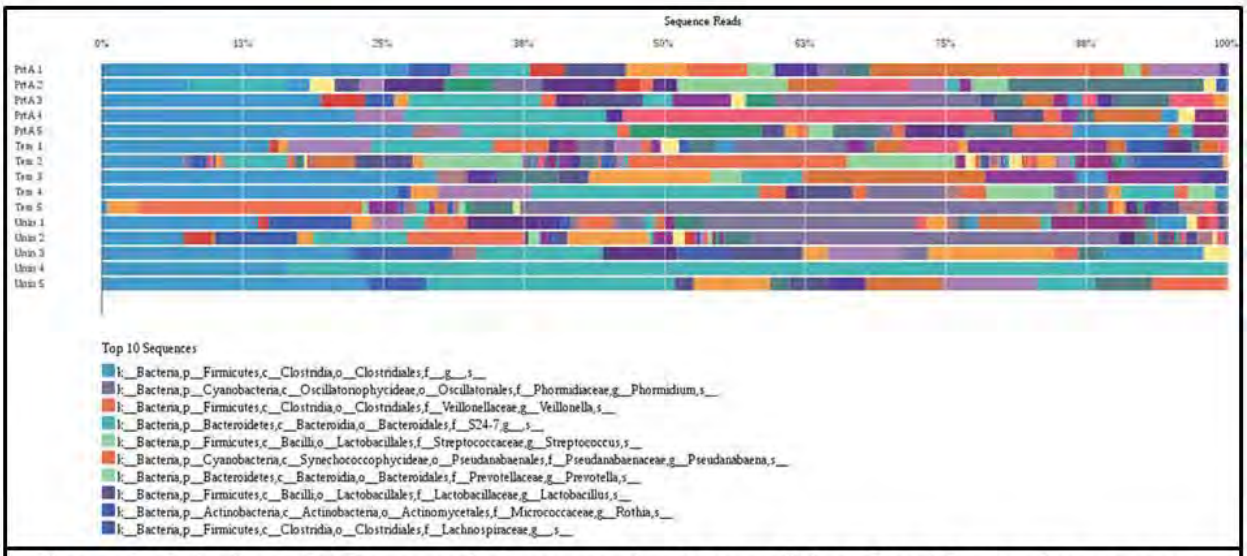

Figure 5: Relative 16S Taxonomic Community Composition. Top 10 constituents are identified to the Genus level where possible.

Results from preliminary samples are shown in **Figure 5** demonstrating the low number of sequences after chloroplast removal and showing the percentage of total sequences attributed to different microorganisms. We have begun sampling and DNA extraction for both the grapevine alpha diversity study across the inside of the grapevine and an infection study to determine differences in the microbiome structure upon infection of *Xf.*

Activity 3. Develop transgenic SR1 tobacco expressing PrtA and evaluate protection against *Xylella* virulence.

The PrtA protein is a secreted protein and a knockout of the gene encoding this protein in *Xf* designated prtA1 mutant is more virulent. This mutant displays an anti-virulence phenotype much like that which was observed for the haemagglutinin adhesins (HxfA and HxfB), where knockouts of these proteins also displayed a more virulent phenotype (Guilhabert and Kirkpatrick, 2005). The *Xf*-prtA1 strain displays less protease activity as compared to wild-type and the other mutants that we are using in the present study, lesA1 and lesA3B1(**Figure 6**). Preliminary structural analysis shows similarity to bacterial γ-glutamyl proteases especially for the active site residues (**Figure 6**). The prtA gene appears to be conserved only among *Xylella* and not in *Xanthomonas*.

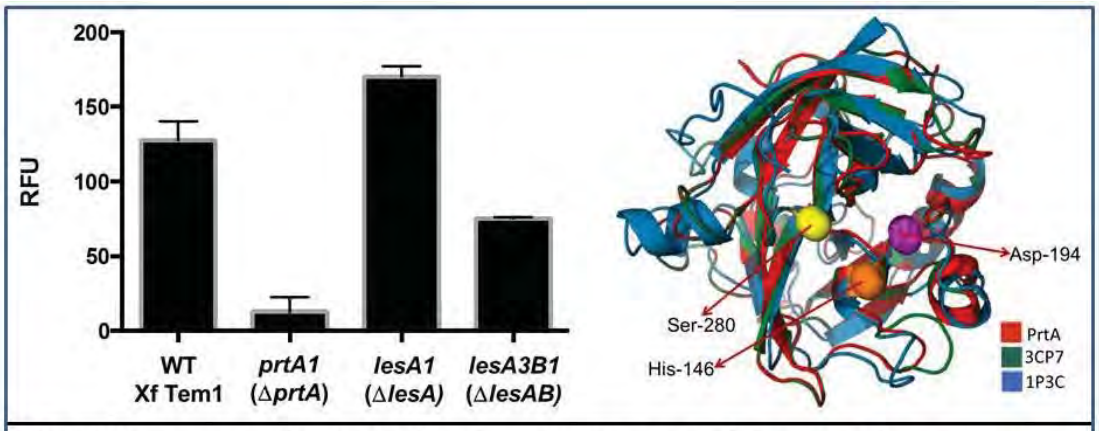

Fig 6: Mutant strain prtA1 has less protease activity. PrtA has some structural similarity to bacterial γ-glutamyl protease.

The objective of this activity is to test prtA for anti-virulence activity *in planta*. The prtA gene was chemically synthesized and codon optimized for expression in tobacco. The coding region has been linked to a CaMV35S regulatory region in a binary vector. The construct was electoroporated into a disarmed strain of *Agrobacterium* (EHA105). The resulting *agrobacterium* strain then was provided to the UC Davis Parson Transformation Facility to generate transgenic SR1 tobacco plants and the transgenic SR1 tobacco expressing prtA will be challenged with *Xf* as described earlier (Dandekar et al., 2012; Francis et al., 2008) to see if prtA expression can block the virulence and thus the scorching phenotype.

CONCLUSIONS

The goal is to understand the virulence mechanisms of *Xf* that lead to leaf scorching symptoms observed in Pierce's disease and to exploit this information to develop new strategies to control Pierce's disease in grapevines. The blockage of xylem elements and the interference with water transport by *Xf* is regarded to be the main cause of Pierce's disease symptom development. The analysis of *Xf* Temecula 1 secreted proteins has enabled us to focus on two previously uncharacterized proteins: LesA and PrtA. We generated mutant *Xf* that are defective for each of these two genes and they show alterations in disease phenotype, lesA1 is less virulent while prtA1 is more virulent. LesA displays lipase/esterase activities and is the most abundant but is very similar to two additional less abundant proteins LesB and LesC also secreted by *Xf*. Expression of LesA, B, and C individually in *E.coli* indicate that these proteins can induce scorching symptoms in grapevine and walnut leaves. These symptoms appear to be related to the lipase/esterase activity present in these proteins. The PrtA protein has protease activity and *Xf*-prtA1 mutants are highly virulent suggesting that this protein may somehow block disease. We are building vectors to test this protein for anti-virulence activity in transgenic SR1 tobacco plants. An understanding of how these two proteins work will provide new insights into this disease and provide new avenues of therapy.

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CHARACTERIZATION OF THE *XYLELLA FASTIDIOSA* PHOP/Q TWO-COMPONENT REGULATORY SYSTEM

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ABSTRACT
Xylella fastidiosa (Xf) is a gram-negative, xylem-limited plant pathogenic bacterium that causes disease in a variety of economically important agricultural crops including Pierce's disease of grapevine. *Xf* biofilms formed in the xylem vessels of plants play a key role in early colonization and pathogenicity by providing a protected niche and enhanced cell survival. Biofilm formation is induced by the process of quorum sensing and may be mediated by two-component regulatory systems. Like many other bacteria, *Xf* possesses homologs to the two component regulatory system PhoP/Q. PhoP/Q differentially regulates genes in responses to divalent periplasmic cation concentration and other environmental stimuli. Grapevine pathogenicity assays showed *phoP/Q* mutants and are non-pathogenic and significantly hindered in colonization or movement within the xylem vessels. The purpose of this research is to further our understanding of the PhoP/Q regulon in order to understand essential processes responsible for survival of *Xf* in *Vitis vinifera* grapevines.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*), the causal agent of Pierce's disease of grapevine, possesses many highly conserved bacterial regulatory systems, including the PhoP/Q system. This system has been shown in other bacteria to play an important role in survival and pathogenicity. In the case of *Xf*, we have previously shown that the PhoP/Q system is required for *Xf* to survive in the plant, rendering *Xf* unable to move or cause disease if PhoP or PhoQ are knocked out. We propose to further characterize this system using next generation molecular tools, such as RNAseq. This will allow us to identify *Xf* genes that are regulated by PhoP/Q and give us further insight into the processes essential for the pathogen to survive in grapevines and identify potentially novel disease control targets.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a gram-negative, xylem-limited plant pathogenic bacterium and the causal agent of Pierce's disease (PD) of grapevine (Wells et al., 1981). *Xf* forms aggregates in xylem vessels, which leads to the blockage of xylem sap movement. The formation of biofilms allows for bacteria to inhabit an area different from the surrounding environment. Furthermore, biofilm formation is an important factor in the virulence of bacterial pathogens. Biofilm formation is a result of density-dependent gene expression (Morris and Monier, 2003). Density-dependent biofilm formation is triggered by the process of quorum sensing (QS). Biofilm formation induced by QS is essential for survival and pathogenicity and may be regulated through a two-component regulatory system (TCS). TCS's are signal transduction systems through which bacteria are able to respond to environmental stimuli (Hoch, 2000). The TCS is comprised of a histidine kinase, responsible for sensing stimuli, and the response regulator, responsible for mediating gene expression (Charles et al, 1992).

The PhoP/Q TCS is a well-studied and highly conserved TCS responsible for regulation of genes involved in virulence, adaptation to environments with limiting Mg^{2+} and Ca^{2+}, and regulation of other genes. PhoQ is a transmembrane histidine kinase protein with a long C-terminal tail residing in the cytoplasm. The periplasmic domain of PhoQ is involved in sensing of Mg^{2+}, Ca^{2+}, and antimicrobial peptides. The cytoplasmic domain contains a histidine residue that is phosphorylated when physiological signals are detected in the periplasm. The PhoP/Q TCS is a phosphotransfer signal transduction system and upon activation by environmental stimuli, PhoQ phosphorylates the corresponding response regulator PhoP. In most bacteria, environments high in Mg^{2+} inhibit the PhoP/Q system through dephosphorylation of PhoP (Groisman, 2001). *Xf* contains homologs of the PhoP/Q system (Simpson et al. 2000). We have previously shown that the PhoP/Q system is essential for *Xf* survival *in planta* and plays a role in regulation of biofilm formation and cell-cell aggregation. The current aim of our research is to understand what genes are being controlled by PhoP/Q in *Xf*, especially genes involved in the early

adaptation processes essential for survival in the xylem. We are also investigating factors involved in induction or repression of the PhoP/Q system such as pH, cation concentration and peptides.

OBJECTIVES

1. Characterization of factors involved in induction and/or repression of the PhoP/Q system.
2. Identification and characterization of genes regulated by PhoP.
3. Determine if peptides in a library provided by Prof. Carlos Gonzalez are able to bind to *Xf* PhoQ and inhibit activation of PhoP.

RESULTS AND DISCUSSION

Objective 1.

We are currently working to further our understanding of factors that influence the PhoP/Q system in *Xf*. So far we have found that *XfΔphoP* and *XfΔphoQ* mutants show inhibited growth in Pim6 media containing 50 µM Mg^{2+} when compared to wild-type *Xf*. We also see a reduction in growth among the mutants compared to wild-type at a lowered pH of 5.0 (instead of pH 7.0) when the media contains 500 µM Mg^{2+}.

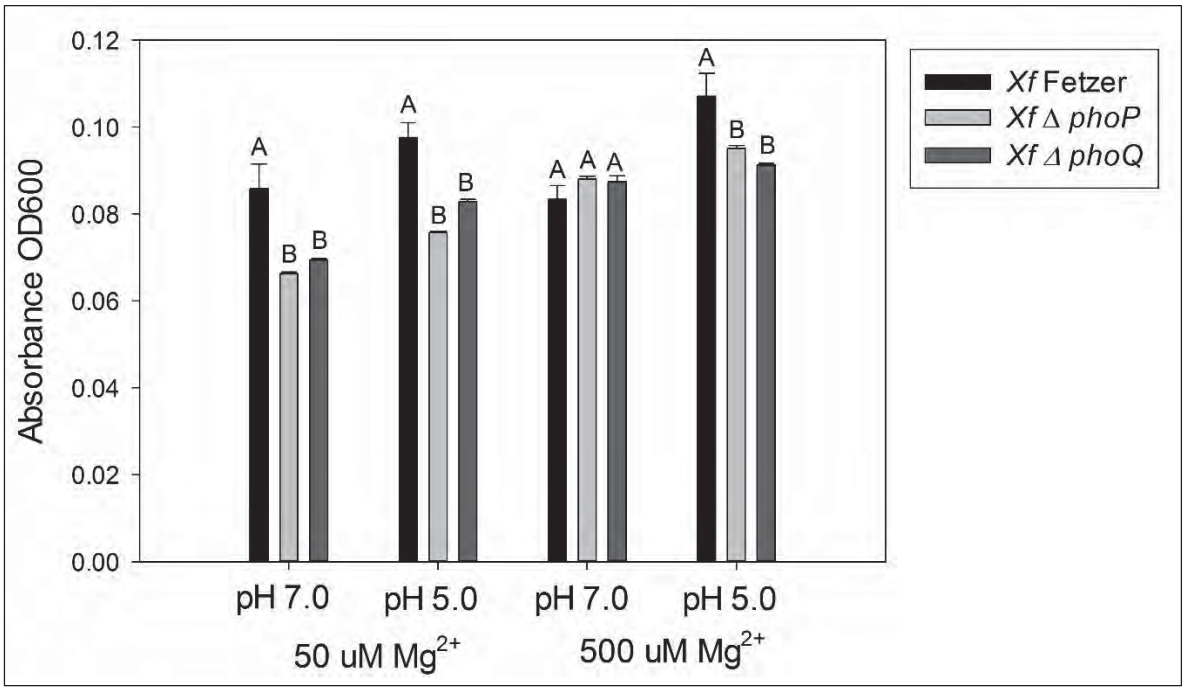

Figure 1. Absorbance OD600 of *Xf* fetzer, *XfΔphoP*, and *XfΔphoQ* in Pim6 media containing either 50 µM or 500 µM Mg^{2+} at a pH of 5.0 or 7.0. Absorbance was measured after 5 days growth at 28° C.

We are currently looking at other types of media, xylem sap, and different ranges of various ions.

Objective 2.

We will begin work on objective 2 in November 2014. We are in the final stages of selecting the optimal media for RNA isolation.

Objective 3.

We have begun work on objective 3 investigating whether two potential peptides, kindly provided by Professor Carlos Gonzalez, have an inhibitory effect on *Xf*. The two peptides tested are 66-10D: FRLKFH and 77-12D: FRLKFHI (Reed *et al.*, 1997) We found the peptides have an inhibitory effect on *Xf* Fetzer when grown for five days in Pim6 media (Michele Igo, personal communication) modified to contain 10 uM Mg^{2+}. *Xf* was grown in the presence of the peptides at varying concentrations, with an inhibitory effect observed at peptide concentrations as low as 10 ug/ml (**Figure 2**).

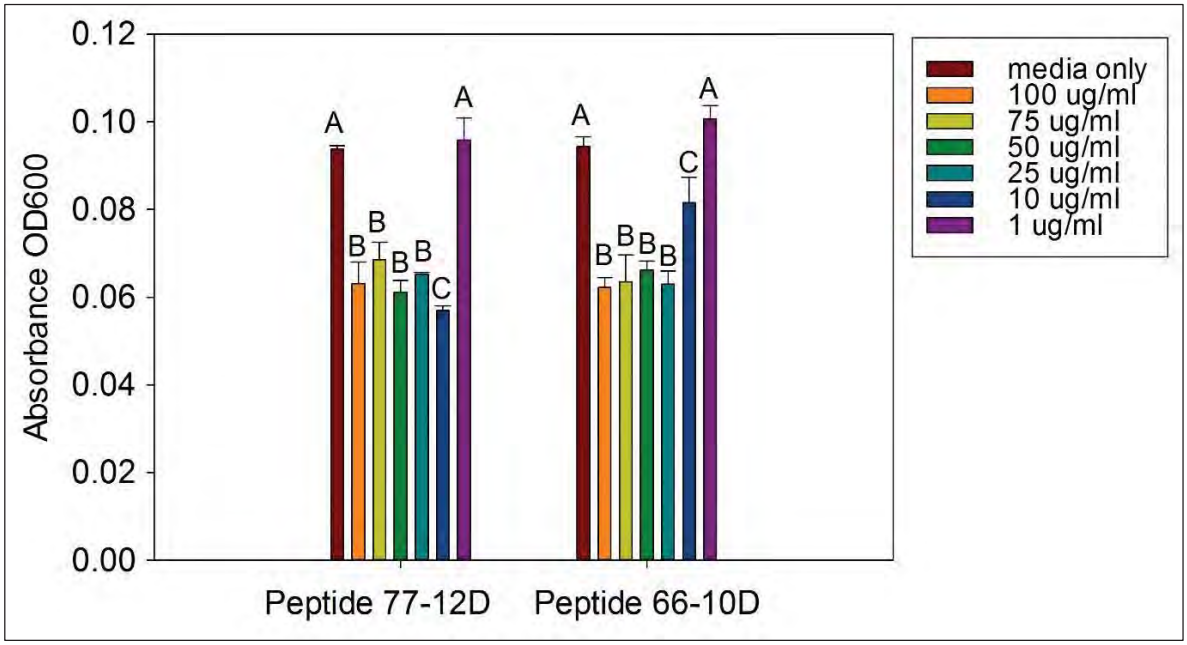

Figure 2. Absorbance OD600 of *Xf* Fetzer cells grown for five days at 28° C with shaking at 100 rpm in Pim6 media containing 10 µM Mg^{2+} and varying concentrations of 77-12D and 66-10D peptides. Different letters indicate significance ($P < 0.05$) as determined by the Tukey test.

We have observed a further reduction in growth (greater inhibitory effect) of these peptides on our *XfΔphoP* and *XfΔphoQ* mutants when grown under the same conditions as above (**Figure 3**).

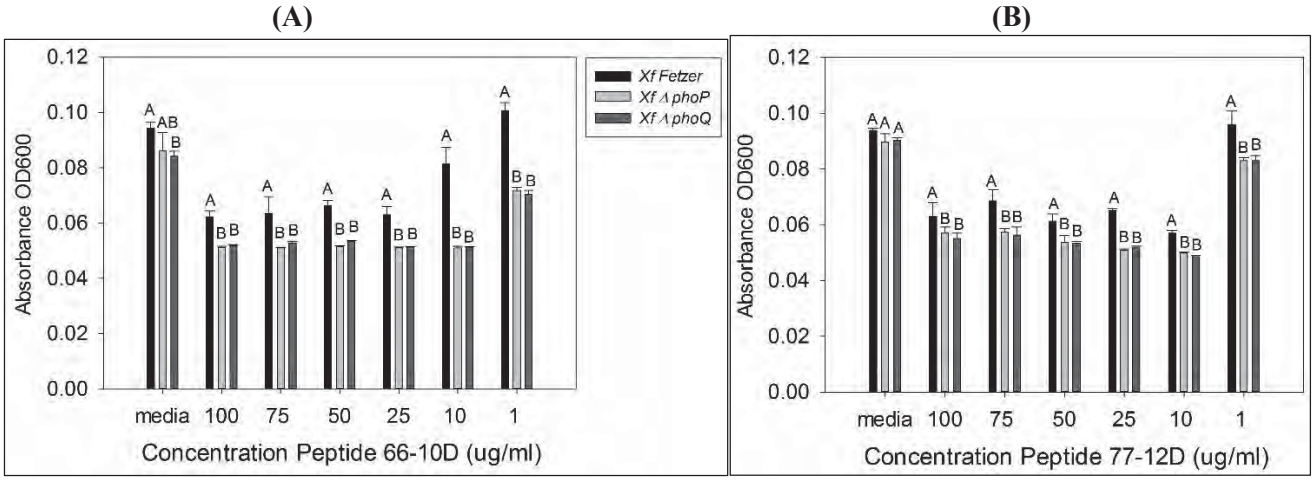

Figure 3. Absorbance OD600 of *Xf* Fetzer, *XfΔphoP* and *XfΔphoQ* cells grown for five days at 28° C with shaking at 100 rpm in Pim6 media containing 10 µM Mg^{2+} and varying concentrations of (**A**) Peptide 66-10D or (**B**) Peptide 77-12D. Different letters indicate significance ($P < 0.05$) as determined by the Tukey test.

We also tested the effect of these peptides on *Xf* Fetzer when *Xf* was incubated in the presence of the peptide for one hour in Pim6 media containing 10 µM Mg^{2+}. After the incubation period, 20 µl aliquots were plated onto solid PD3 media and growth was evaluated after seven days incubation at 28° C. We found that only peptide 77-12D was able to inhibit *Xf* growth after the one hour incubation period (**Table 1**).

Table 1. Evaluation of *Xf* Fetzer growth after 1 hour incubation with peptide 77-12D or 66-10D in Pim6 media containing 10 µM Mg^{2+}.

Peptide concentration	77-12D	66-10D
100 µg/ml	No growth	Growth
75 µg/ml	No growth	Growth
50 µg/ml	No growth	Growth
25 µg/ml	No growth	Growth
10 µg/ml	No growth	Growth
1 µg/ml	Growth	Growth

Future experiments will evaluate gene expression after *Xf* is incubated or grown in the presence of these peptides in order to determine whether these peptides are influencing phoP-mediated gene regulation. We will accomplish this using qRT-PCR with primers for gene targets identified in objective 2.

CONCLUSIONS

We have begun characterizing the PhoP/Q regulatory networks as well as roles this two-component system plays in adaptation of *Xf* to varying environmental and biological parameters. The two peptides, 77-12D and 66-10D, provided interesting results that we hope to explore further through qRT-PCR. The fact that peptide 77-12D can induce an inhibitory effect even after a one-hour incubation with *Xf* indicates it is likely binding to essential *Xf* gene products. These results are preliminary and further research is required for an understanding of what this peptide is doing to *Xf*.

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We would like to thank Professor Carlos Gonzalez for donating the peptides used in objective 3.

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

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ABSTRACT

Xylella fastidiosa (*Xf*) produces an unsaturated fatty acid signal molecule called diffusible signal factor (DSF) that modulates gene expression in cells as they reach high numbers in plants. By increasing the expression of a variety of afimbrial adhesins while decreasing the expression of pili involved in twitching motility as well as extracellular enzymes involved in degrading pit membranes and hence movement between vessels, DSF accumulation suppresses virulence of *Xf* in grape. We thus are exploring different ways to elevate DSF levels in plants to achieve disease control via "pathogen confusion." As exogenous sources of DSF applied in various ways to grape suppressed pathogen mobility and hence virulence we have further studied the chemical identity of DSF. *Xf* can respond to a variety of related unsaturated fatty acids, while it naturally produces at least three different DSF species. While the initial DSF species produced by *Xf* was identified as, 2-Z-tetradecenoic acid (hereafter called C14-cis), it can also produce a second compound termed C12-cis, as well as 2-Z-hexadecenoic acid (C16-cis). This latter molecule is the most active molecule. Gene expression in *Xf* exposed to various levels of DSF is a sensitive means of assessing DSF levels and *Xf* harboring *phoA* reporter gene fusions to hxfA has proven to be an excellent bioreporter. *Xf* can respond to *cis* unsaturated fatty acids with the site of unsaturation at the number 2 carbon molecule with chain lengths from 12 to 18 carbon atoms. The corresponding *trans* unsaturated fatty acids not only are not able to induce gene expression, but antagonize gene expression conferred by the corresponding *cis* fatty acid. The commercially available unsaturated fatty acid palmitoleic acid is also active as a signaling molecule and is being evaluated for its ability to reduce the susceptibility of plants to Pierce's disease when applied topically or introduced into the plant in different ways. The release of extracellular membranous vesicles by *Xf* is responsible for the suppression of its adherence to surfaces. These vesicles attach to surfaces such as that of the walls of the xylem vessels. By so attaching, these vesicles prevent the attachment of *Xf* cells themselves to such surfaces. Only upon reaching relatively high cell concentrations in a particular vessel would DSF concentrations increase to a level that would suppressed the release of the membranous vesicles, thereby retaining adhesive molecules on the surface of *Xf* cells themselves, thus allowing the bacterial cells themselves to attached to surfaces, such as that of insect vectors.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) produces an unsaturated fatty acid signal molecule called diffusible signal factor (DSF). Accumulation of DSF in *Xf* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in the pathogen, but the overall effect is to suppress its virulence in plants by increasing its adhesiveness to plant surfaces and also suppressing the production of enzymes and genes needed for active movement through the plant. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption (pathogen confusion) as a means of controlling Pierce's disease. Elevating DSF levels in plants artificially reduces its movement in the plant. In this study we have investigated the variety of different fatty acid molecules that can serve as cell-cell signaling agents in *Xf*. Several new DSF species have been found including a 16-carbon unsaturated fatty acid that appears to be far more active than the 14-carbon unsaturated fatty acid that we have previously investigated as well as a commercially available fatty acid (palmitoleic acid). The release of extracellular membranous vesicles by *Xf* is responsible for the suppression of its adherence to surfaces, thus facilitating its movement through the plant, and is therefore a virulence factor.

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 in most xylem vessels, and hence they do not express adhesins that would hinder their movement through the plant (but which are required for vector acquisition) but actively express extracellular enzymes and retractile pili needed for movement through the plant. Disease control can be conferred by elevating DSF levels in grape to "trick" the pathogen into transitioning into the non-mobile form that is normally found only in highly colonized vessels. While we have demonstrated the principles of disease control by so-called "pathogen confusion," our continuing work aims to understand how best to alter DSF levels in plants to achieve even higher levels of disease control. Until now we have suffered from a lack of sensitive methods to detect DSF levels in plants (the *Xanthomonas*-based bioassay we have used previously is relatively insensitive to the chemically distinct forms of DSF produced by *Xf*). That is, while we showed that DSF-producing endophytes, direct application of DSF, and transgenic plants producing DSF all conferred some resistance to disease, we had no way to know why they were not more resistant, nor what would be needed to improve control measures since we could not measure the direct effect of our efforts to increase DSF levels in plants. However, we have now developed several new sensitive biosensors that enable us to measure *Xf* DSF both in culture and within plants. We have recently found that *Xf* apparently produces more than one molecule that can act as a DSF signal molecule and that the molecules made by *Xf* are dependent on its growth environment. We thus need to ascertain which form is most active, whether the various forms all have the same effect on regulating traits in *Xf,* and what are their fates when applied to plants in various ways. Thus the overall goal of our work is to use these new biosensors to examine how DSF levels can best be altered by the various methods we have previously identified. As disease control should be directly proportional to both the concentration of and dispersal of DSF within plants we will quantitatively explore the effectiveness of different strategies to elevate DSF levels throughout plants.

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

OBJECTIVES

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

RESULTS AND DISCUSSION

Objective 1. Finding new DSF species.

We have optimized methods to use *Xf* itself to detect DSF**.** Among the several genes that we know to be most strongly regulated by DSF are genes such as *hxfA* and *HxfB* which are involved in cell-surface adhesion. We now have successfully used the endogenous *phoA* gene (encoding alkaline phosphatase) as a bioreporter of gene expression in *Xf*. The PhoA-based biosensor in which *phoA* is driven by the *hxfA* promoter is quite responsive to

exogenous DSF from extracts of *Xf* cultures as well as C14-cis (hereafter called *Xf*DSF) itself. Assay of *Xf* extracts by *Xf* DSF-specific biosensors provide evidence of more than one *Xf* DSF molecule. Our analysis of the material collected by HPLC from these cultures using electro-spray MS revealed it to be an unsaturated C16 fatty acid. We therefore chemically synthesized this presumptive derivative which we will call C16-cis or *Xf* DSF2 (**Figure 1**).

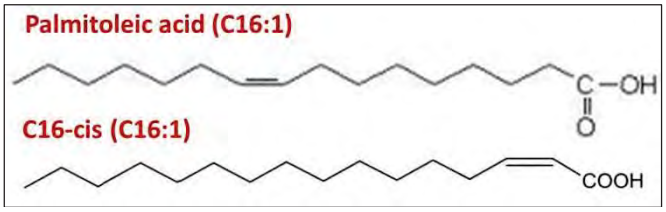

Figure 1. Structure of C16-cis (*Xf* DSF2) and palmitoleic acid

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

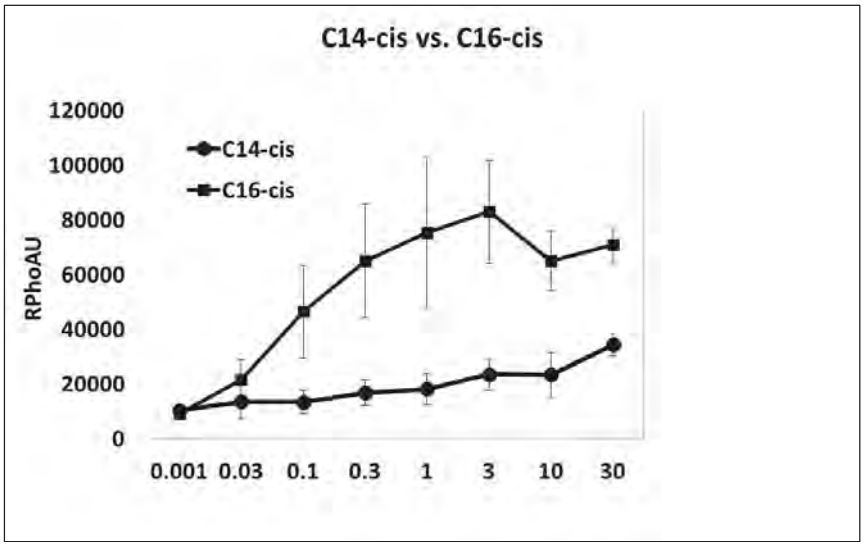

Figure 2. *Xf*DSF and *Xf*DSF2 Dose-dependent induction of the *Xf*-based DSF-biosensor (*rpfF**-*Xf*HA-biosensor).

To better understand how promiscuous the DSF synthase RpfF from *Xf* was, we synthesized a variety of different enoic fatty acids and assayed them with the *Xf:phoA* biosensor. A variety of related fatty acids having the site of unsaturation at the number 2 position but with different carbon chain lengths were assessed. In most cases, the double bond was constructed to be in a *cis* orientation, but a few corresponding *trans* unsaturated fatty acids were synthesized.

Several different patterns of response of the *Xf:phoA* biosensor to these various fatty acids was observed. Some relatively short chain-link fatty acids such as C10-cis were toxic, interfering with gene expression, but not bacterial growth, such that the alkaline phosphatase activity exhibited by the *Xf:phoA* biosensor *Xf:phoA* biosensor decreased with increasing concentration of the fatty acid (Figure 3). In contrast, the alkaline phosphatase activity exhibited by the *Xf:phoA* biosensor increased with increasing concentrations of fatty acid

such as for *Xf*DSF itself, thereby indicating a positive response, while there was no response to other fatty acids such as C19-cis (**Figure 3**).

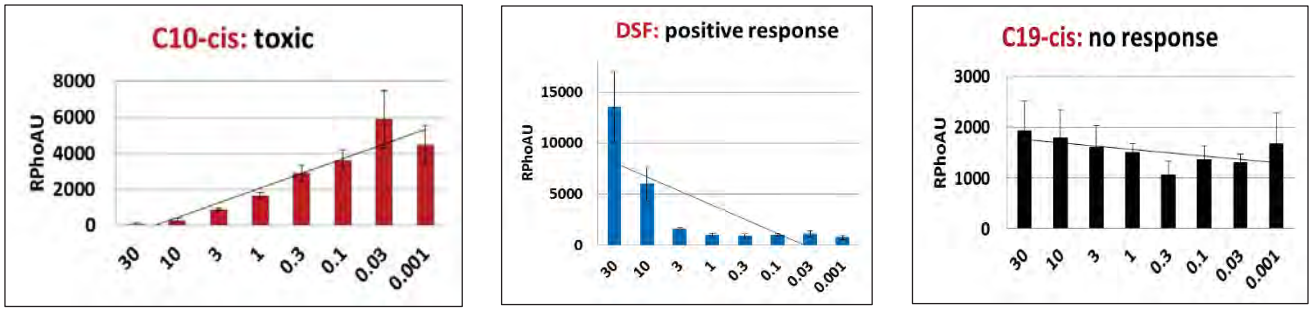

Figure 3. Various responses of the *Xf:phoA* biosensor has indicated by alkaline phosphatase activity (ordinate) as a function of the concentration of various synthetic fatty acids noted on the abscissa (uM).

Unsaturated fatty acids differed greatly in their ability to induce quorum sensing in *Xf*. While *Xf* responded positively to unsaturated fatty acids with chain lengths from 12 to 18 carbons, as long as the site of unsaturation was at the number 2 carbon position, those fatty acids less than 12 carbons in length tended to be toxic while there was no response to those greater than 18 carbons in length (**Table 1**). While *Xf* could respond positively to a wide range of different fatty acids, the lowest concentrations at which some response could be detected was highest for those of carbon lengths of 14-18.

Table 1. Activity of various unsaturated fatty acids as signal molecules in *Xf*.

Chain length	Orienta-tion	Location of unsatura-tion	Molecule name	*Xf* biosensor		*Xcc* biosensor	
				Minimum detection concentration (uM)	Fold induction	Minimum detection concentration (uM)	Fold induction
8	*cis*	2	2-z-octanoic acid	Toxic	-	No response	-
9	*cis*	2	2-z-nonanoic acid	Toxic	-	No response	-
10	*cis*	2	2-z-decanoic acid	Toxic	-	10	4.1
11	*cis*	2	2-z-undecanoic acid	Toxic	-	1	12.4
12	*cis*		2-z-dodecenoic acid (BDSF)	3	3.2	0.1	12.4
12	*trans*	2	2-E-dodecanoic acid	Toxic	-	3	7.9
13	*cis*	2	2-z-tridecanoic acid	Toxic	-	0.001	17.9
13	*cis*	2	2-z-11-methyldodecenoic acid (DSF)	3	17.5	0.01	17.9
14	*cis*	2	2-z-tetradecanoic acid (*Xf*DSF)	1	3.3	7	4.8
14	*cis*	5	5-z-tetradecanoic acid	No response	-	No response	-
14	*cis*	6	6-z-tetradecanoic acid	No response	-	No response	-
15	*cis*	2	2-z-pantadecanoic acid	10	4.2	No response	-
15		0	12-methyltetradecanoic acid (CVC-DSF)	1.5	0.32	No response	-
16	*cis*	2	2-z-haxadecanoic acid (*Xf*DSF2)	0.15	8.9	No response	-
17	*cis*	2	2-z-heptadecanoic acid	0.3	8.6	No response	-
18	*cis*	2	2-z-octadecanoic acid	1	6.5	No response	-
19	*cis*	2	2-z-nonadecanoic acid	No response	-	No response	-
20	*cis*	2	2-z-eicosanoic acid	No response	-	No response	-

Given that *Xf* appeared to be relatively promiscuous in its perception of a variety of unsaturated fatty acids, a number of different commercially available saturated and unsaturated fatty acids were evaluated for their ability to induce quorum sensing (**Table 2**). While no saturated fatty acid exhibited the ability to induce DSF-mediated quorum sensing in *Xf*, palmitoleic acid (**Figure 1**) was quite active as a signaling molecule (**Figure 4**). While approximately five times higher concentrations of palmitoleic acid were required to induce the *Xf:phoA* biosensor compared to *Xf*DSF2 (C16-cis), it conferred high levels of induction of the biosensor. This is a very exciting finding as it will allow us to proceed with tests to apply such as exogenous sources of DSF as a signal molecule to plants since large amounts of this material can be obtained relatively inexpensively.

Table 2. Commercially available fatty acids evaluated for signing activity in *Xf*.

Chain length	Orientation	Location of unsaturation	Chemical name	Common name
14	-	0	tetradecanoic acid	myristic acid
14	*cis*	9	9-z-tetradecenoic acid	myristoleic acid
14	*cis*	5	5-z-tetradecenoic acid	physeteric acid
16	-	0	hexadecenoic acid	palmitic acid
16	*cis*	9	9-z-hexadecenoic acid	palmitoleic acid
16	*cis*	6	6-z-hexadecenoic acid	sapenic acid
16	*trans*	9	9-E-hexadecenoic acid	palmitelaidic acid

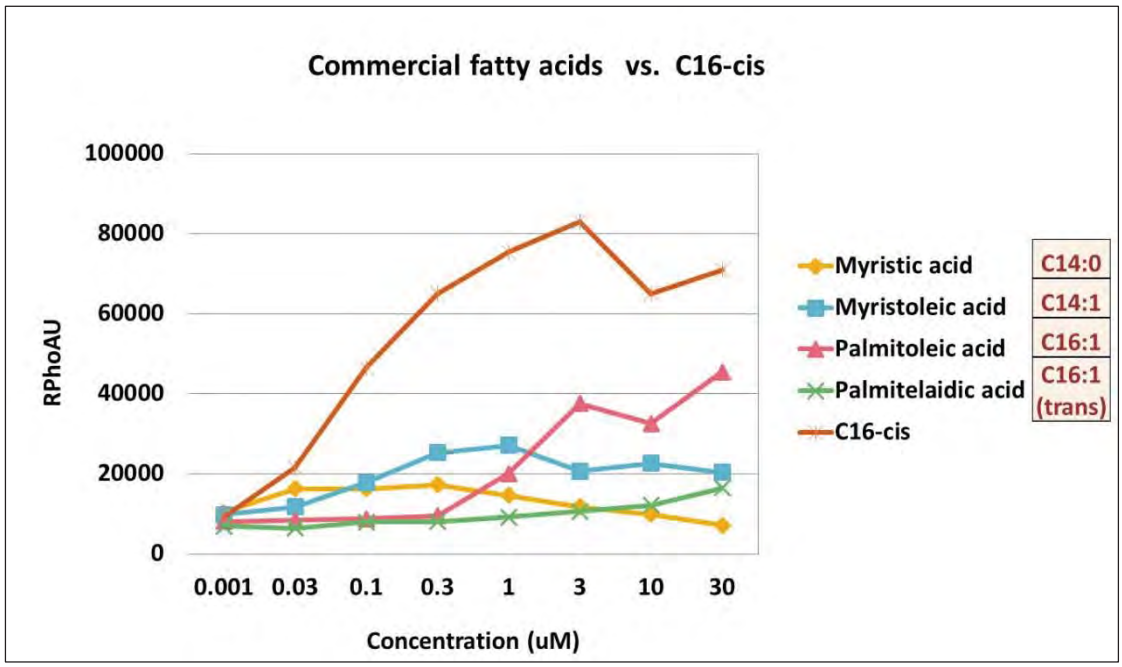

Figure 4. Alkaline phosphatase activity exhibited by different commercially available saturated and unsaturated fatty acids tested at various concentrations in the *Xf:phoA* biosensor.

Given that a commercially available unsaturated fatty acid is a potent inducer of quorum sensing in *Xf*, we further investigated the extent to which DSF signaling could be interfered with by the presence of other dissimilar saturated and unsaturated fatty acids. Not only does the grape strain of *Xf* not respond to the DSF from citrus variegated chlorosis (CVC) strains of *Xf*, this molecule is a powerful inhibitor of signaling in grape strains of *Xf* in the presence of its own DSF, C16-cis (**Figure 5**). In the presence of one micromolar C16-cis the induction of the *Xf:phoA* biosensor decreased steadily with increasing concentrations of CVC DSF in the range from 0.1 to 30 micromolar (**Figure 5**).

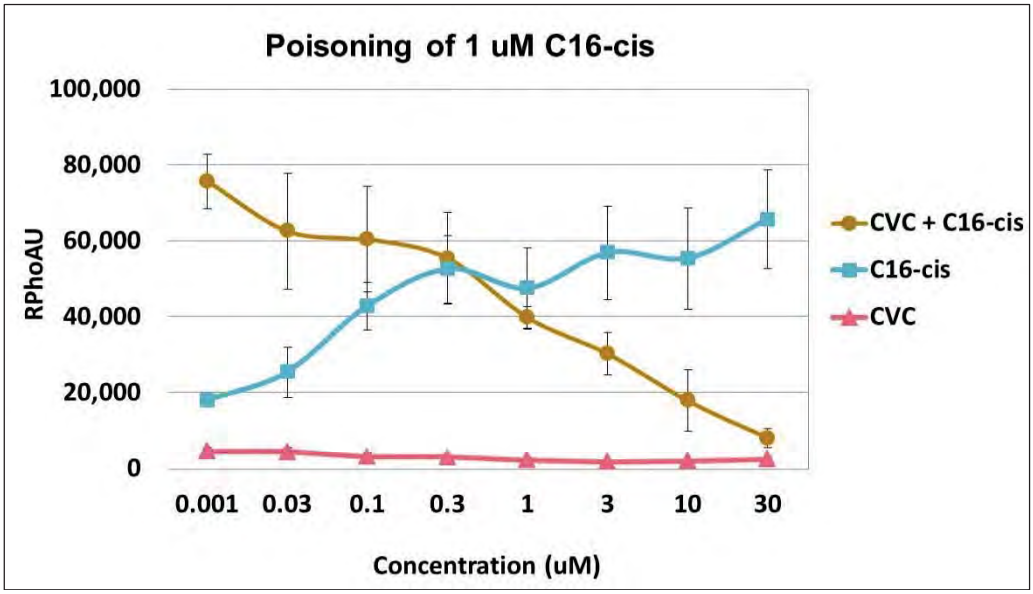

Figure 5. Alkaline phosphatase activity exhibited by cells of the *Xf:phoA* biosensor exposed to different concentrations of CVC DSF (red line), C16-cis (blue line), or to a combination of 1 uM C16-cis and different concentrations of CVC DSF as shown on the abscissa (brown line).

Given that palmitoleic acid is a promising commercially available fatty acid that can serve as a signaling molecule in *Xf*, we tested to what extent its ability to act as a signaling molecule could be blocked in the presence of other fatty acids (**Figure 6**). Not only did the saturated fatty acids palmitic acid (C16) and myristic acid (C14) interfere with signaling induced by C16-cis or C14-cis, but it also interfered with signaling induced by palmitoleic acid as measured by the *Xf:phoA* biosensor (**Figure 6**). It thus appears that it might not be possible to use complex mixtures of fatty acids as signaling molecules, although we are continuing to investigate the ubiquity with which various saturated or *trans* fatty acids interfere with signaling in *Xf*.

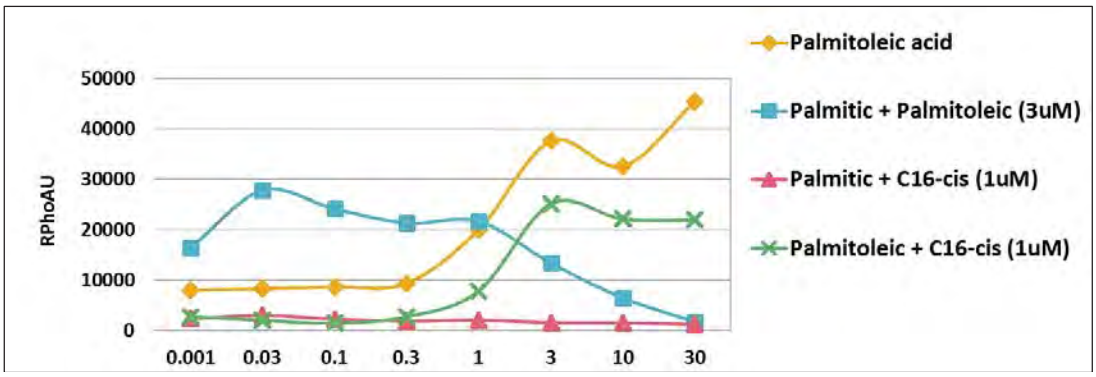

Figure 6. Alkaline phosphatase activity exhibited by cells of the *Xf:phoA* biosensor exposed to different concentrations of palmitoleic acid alone (gold line), various concentrations of palmitic acid and 3 µm palmitoleic acid (blue line), various concentrations of palmitoleic acid and 1 um C16-cis (blue line), or to a combination of 1 µM C16-cis and different concentrations of palmitic acid (red line) as shown on the abscissa.

Given that palmitoleic acid is a promising commercially available fatty acid that can serve as a signaling molecule in *Xf* we have investigated different ways in which it can be introduced into plants. We therefore have initiated large experiments in which we are assessing both the concentration of palmitoleic acid within the xylem tissue as well as any phytotoxicity of palmitoleic acid applied either by itself or in conjunction with various surfactants or solubilizing agents. We thus have inoculated grape with solutions of palmitoleic acid with different concentrations of the surfactants Breakthru and Triton X-100 as well is he solubilizing agents DMSO and Solutol. palmitoleic acid was applied at a concentration of 10 mM and to plants both as a foliar spray, as a soil drench, and as a stem injection. While high concentrations of several of these detergents or solubilizing agents caused phytotoxicity no, or limited cytotoxicity was observed at a concentration of less than 0.2% Breakthru, 0.2% Triton X-100, 1%

DMSO, or 1% Solutol. The effectiveness of these agents in introducing palmitoleic acid into grape tissue was assessed by assessing the ability of sap extracted from individual leaves using a pressure bomb to induce the expression of alkaline phosphatase activity in the *Xf Xf:phoA* biosensor. The initial results of these studies reveal that substantial amounts of palmitoleic acid could be introduced into grape leaves one applied as a foliar spray with 0.2% Breakthru (**Figure 7**). Lesser amounts could be introduced with foliar sprays including Solutol and DMSO. As a registered surfactant for use in agriculture, Breakthru has the potential to be a practical delivery agent. The efficacy of this material is probably associated with its extraordinarily low surface tension that enables spontaneous stomatal infiltration of leaves with aqueous solutions containing 0.2% of this detergent. Thus, solutions of fatty acid supplied with this concentration of surfactant appear to bypass the cuticular surface as a means to enter the intercellular spaces and presumably also the vascular tissue.

These most promising treatments were also applied to grape plants to evaluate their efficacy in reducing the symptoms of Pierce's disease. Initial application of palmitoleic acid was followed two weeks later by inoculation with *Xf*. The palmitoleic acid treatments were re-applied every three weeks until nine weeks. Just as disease symptoms were appearing, a malfunction of the deregulation system in the greenhouse caused the plants to severely damage due to desiccation. These experiments will therefore be repeated.

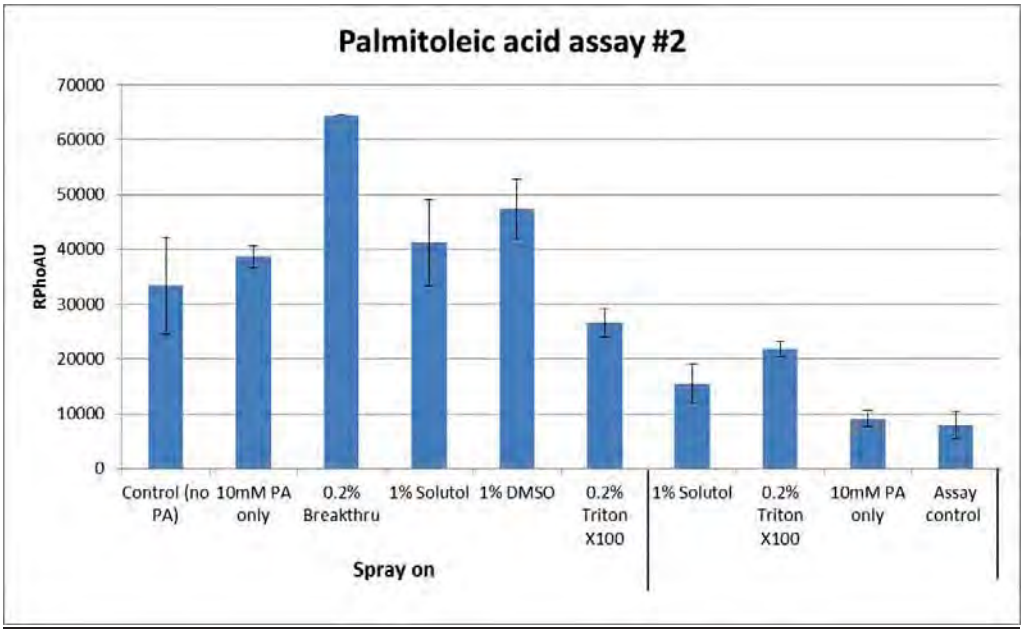

Figure 7. Alkaline phosphatase activity exhibited by 10 µl aliquots of xylem sap extracted under pressure from individual leaves of grape plants treated with 10 mM palmitoleic acid with the various surfactants noted when applied as a foliar spray or a stem injection.

Objective 2. Role of extracellular vesicles.

Our continuing work reveals that *Xf* is a very prolific producer of extracellular vesicles. A large number of vesicles (>400/cell) can be associated both with the surface of the bacterial cell, as well as a high portion that are shed by the cells to the extracellular environment (**Figure 8**). The vesicles are generally quite small, ranging in size from about 0.01 to 0.1 µm in diameter. Quantification of vesicles by both deconvolution fluorescence microscopy, flow cytometry, and a Nanovision particle counter reveals that the higher number of vesicles (normalized for the number of *Xf* cells) are present in RpfF mutants, suggesting strongly that DSF accumulation suppresses the release of such vesicles. The *rpfF* mutant produces as much as three times more vesicles (**Figure 9**). While some vesicles were as large as approximately 1000 nm, the average diameter of vesicles was only approximately 150 nm (**Figure 9**). We can estimate that each *Xf* cell has shed approximately 100 to 1,000 vesicles of different sizes. It is thus clear that vesicles constitute a major extracellular factor produced by *Xf*.

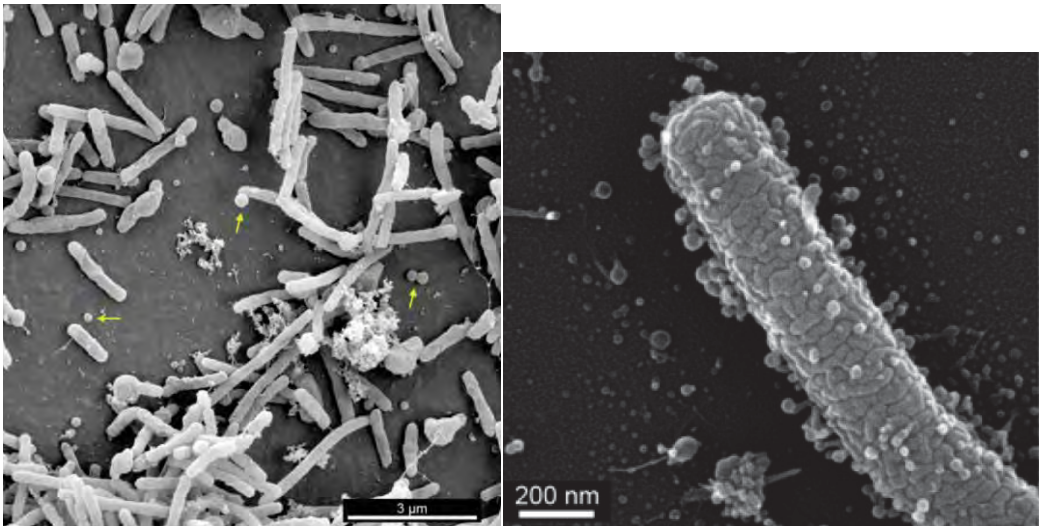

Figure 8. Membranous vesicles forming on the surface of cells of a wild-type strain of *Xf*.

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

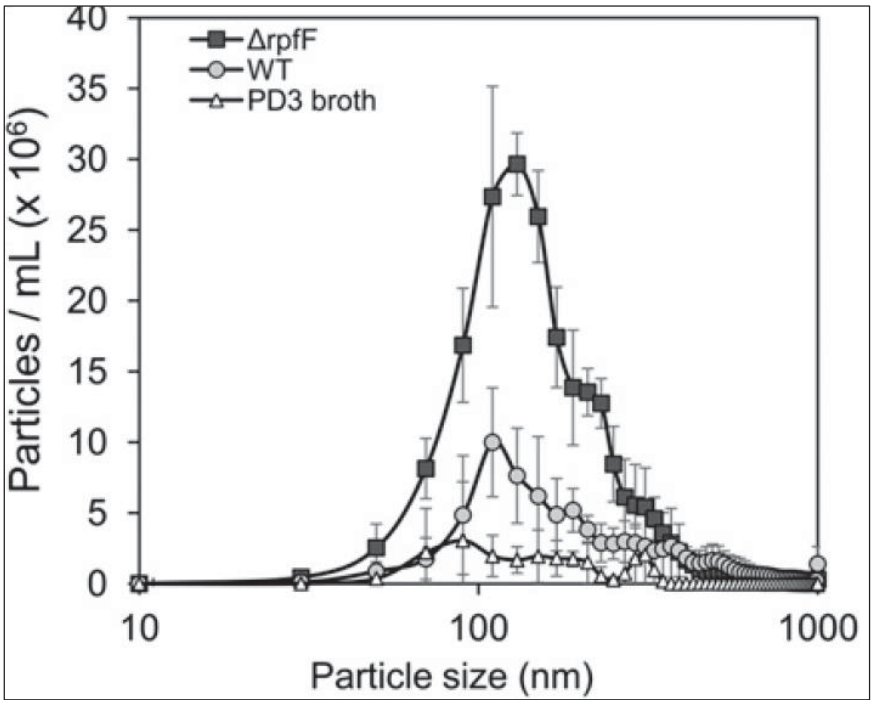

Figure 9. Distribution of sizes and abundance of vesicles of different sizes produced by a wild-type and an *rpfF* mutant of *Xf* when grown for two days in PD3 broth when assessed with a Nanovision device. The vertical bars represent the standard error of the estimate of the number of vesicles produced by a given strain.

Xf produced abundant vesicles while colonizing plants. Outer membrane vesicles could be readily detected in the xylem fluid of plants infected with the wild-type strain, and much higher numbers in plants infected with an RpfF

mutant strain. It is thus clear, that the production of outer membrane vesicles by *Xf* is not an artifact of their culture in laboratory media, but that it is an intrinsic trait of the pathogen while growing in host plants.

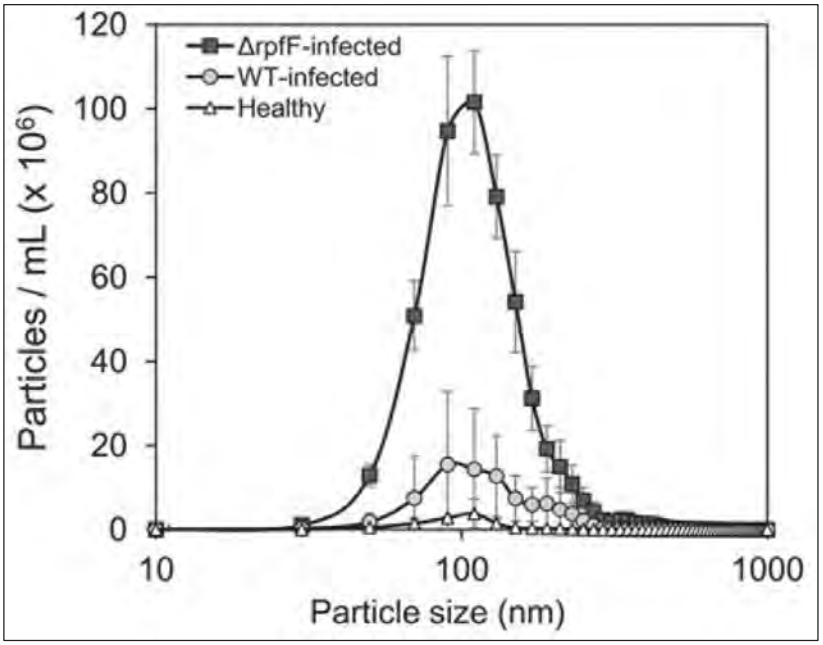

Figure 10. Numbers of particles of various sizes in xylem sap recovered from Cabernet Sauvignon grape infected with the wild-type (circles) or an RpfF mutant of *Xf* (squares), or from healthy plants. Particles of various sizes were enumerated with a Nanovision device. The vertical bars represent the standard error of the determination of mean particles of a given size.

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

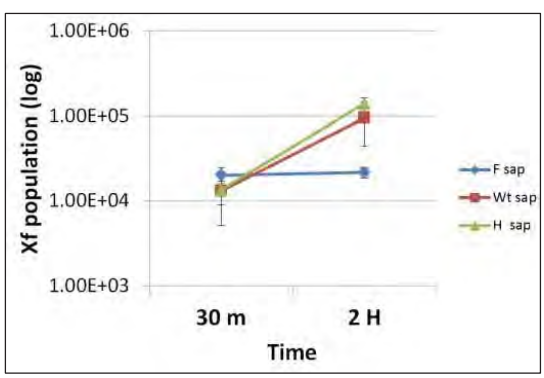

Figure 11. The number of cells of a wild-type strain of *Xf* that had adhered to wings of the GWSS suspended in xylem sap from plants infected with an RpfF mutant (blue), or a wild-type strain of *Xf* (red), or from healthy plants (green) after incubation for either 30 minutes or 2 hours.

The ability of vesicles to interfere with binding of *Xf* to surfaces such as insect wings also suggested that it would interfere with binding to plant surfaces, such as xylem vessels, thereby better enabling the movement of the pathogen through the plant. This was investigated by introducing cells of *Xf* to grape stem segments in the presence or absence of purified membrane vesicles. Vesicles were collected by ultracentrifugation of cell free supernatants. Vesicles were than either resuspended in buffer or in culture media and cells of *Xf* were then introduced into surface sterilized, two cm stem segments in buffer or culture media alone, or in such solutions containing membrane vesicles. After introduction into the stem segments and incubation for one hour, stem segments were flushed with sterile buffer to remove any unattached cells of *Xf*. Population size of the attached *Xf* cells were then determined by dilution plating. When cells were co-inoculated into stem segments with membrane vesicles suspended in buffer, there was a dramatic reduction (>20-fold) and the fraction of those cells which attached to the xylem vessels compared to that of cells introduced in buffer alone (**Figure 12**).

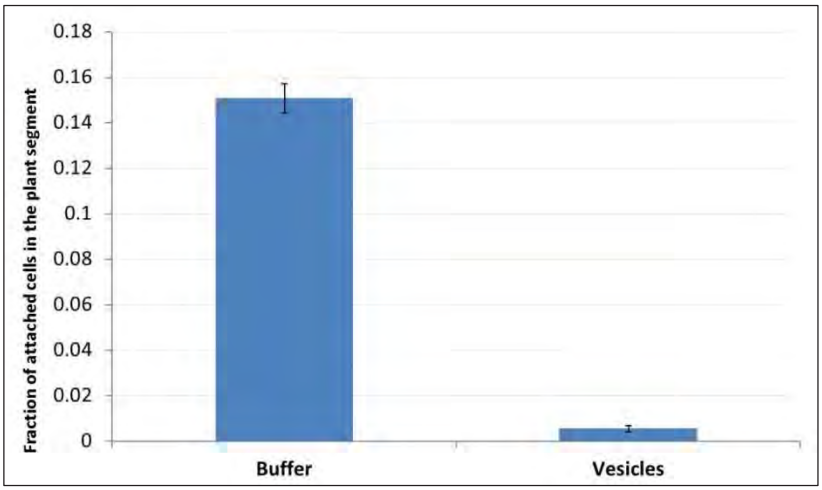

Figure 12. The fraction of total cells introduced into xylem vessels that were retained after one hour incubation when introduced in buffer alone or in phosphate buffer containing membranous vesicles of *Xf*. The vertical bars represent the standard error of the mean of the fraction of attached cells.

To better assess the process by which the vesicles were interfering with the attachment of *Xf* to plant tissues, we conducted similar experiments as above, but introduced the membrane vesicles in buffer alone, incubated the vesicles with the tissue for one hour before then flushing the vesicles out with buffer, before then introducing bacterial cells in buffer to the same stem segments. In this way, we enabled membrane vesicles to interact with plant tissue before, or instead of, bacterial cells themselves. This design enabled us to determine whether the process of finding of vesicles to plant tissues led to the blockage of finding of *Xf* to the plants, or whether binding of the vesicles to the bacterial cells then prevented their binding to the plant tissue. It was clear however that prior treatment of the plant tissue with the vesicles conferred the same dramatic reduction in the ability of *Xf* to bind to plants as well as the case when the cells and vesicles were co-inoculated into the plant (**Figure 13**). It thus seems clear that vesicles prevent binding of *Xf* to plant by preferential binding to the surfaces to which the bacteria themselves might otherwise have bound.

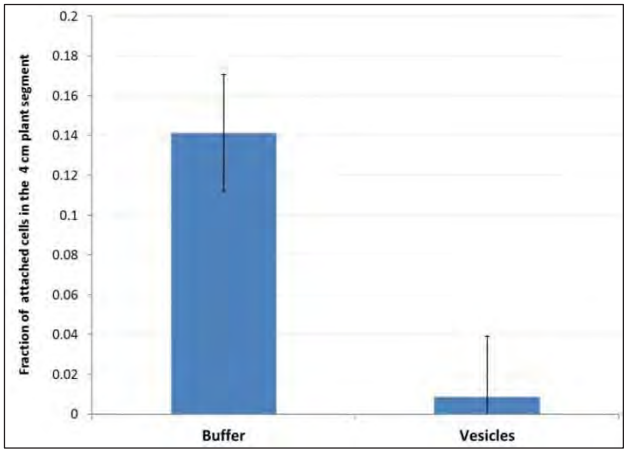

Figure 13. The fraction of total cells introduced into xylem vessels that were retained after one hour incubation when introduced into plants treated one hour earlier with either buffer alone or phosphate buffer containing membranous vesicles of *Xf*. The vertical bars represent the standard error of the mean of the fraction of attached cells.

To better understand the relative ability of outer membrane vesicles to block adhesion of *Xf* to various surfaces we performed experiments similar to that above in which hindwings of GWSS were immersed in cell suspensions of *Xf* either in PIM6 medium alone or in PIM6 medium containing membranous vesicles. While the number of *Xf* cells that attach to the insect hindwings was lower when suspended in membranous vesicles compared to medium alone (**Figure 14**), this effect of vesicles preventing attachment of *Xylella* to surfaces which much smaller than observed in blockage to xylem vessels. That is, while membranous vesicles reduced the proportion of *Xf* cells that would attach to xylem vessels by over 20-fold (see **Figure 13**), these vesicles reduced attachment to insect hindwings by only about three-fold. These results suggest that the membranous vesicles attach more strongly to plant surfaces, and thereby reduce the attachment of *Xf* to the same plant surfaces. It is sensible to speculate that this selective blockage of attachment would have been evolutionarily selected in *Xf*. Specifically, successful transmission of *Xf* from one infected plant to another is dependent on acquisition of the cells by the insect vector. While the membranous vesicles appear to facilitate movement of *Xf* throughout the plant by blocking its attachment to the plant , which would be expected to enter its movement, the vesicles are apparently do not strongly affect its acquisition by insect vectors, thereby enabling it to be acquired and thus transmitted.

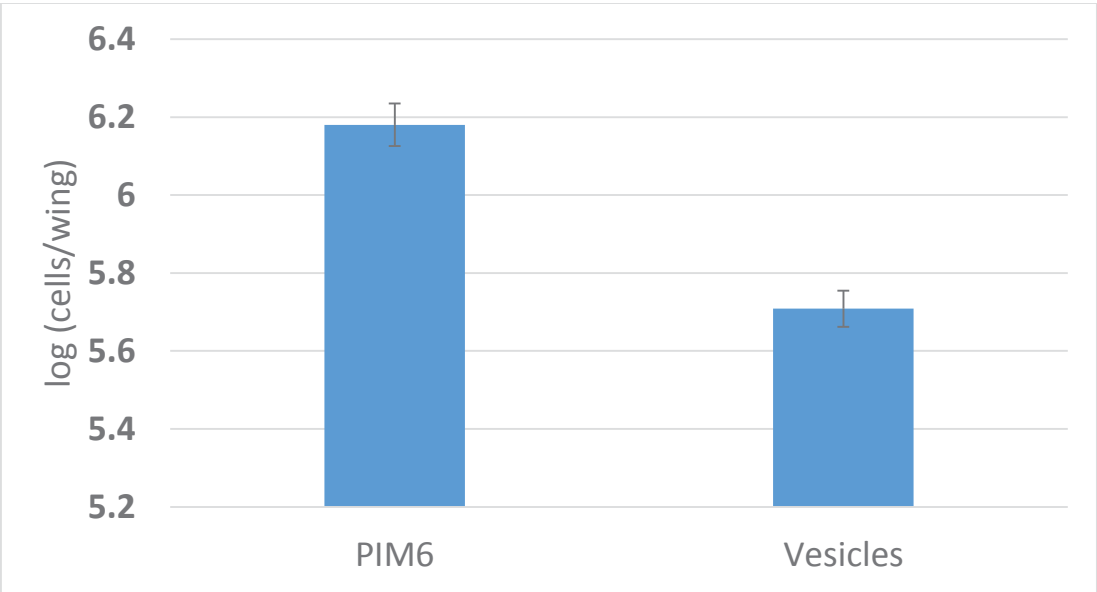

Figure 14. Population size of *Xf* attached to hindwings of GWSS when suspended for two hours in PIM6 culture medium alone or in membranous vesicles of *Xf* suspended in PIM6 medium. The vertical bars represent the standard error of the determination of mean numbers of cells attached as determined by dilution plating.

CONCLUSIONS

We are very excited about results to date that show that several means of elevating DSF levels in plants have provided disease control via a strategy of "pathogen confusion." Given the limitations in standard methods of disease control, we are optimistic that DSF interference represents a promising strategy for Pierce's disease control. Control of Pierce's disease by direct application of DSF is a very attractive disease control strategy since it could be quickly implemented and would utilize commonly used agricultural equipment and methods and would not require the use of transgenic technologies. Our earlier work had shown that C14-cis, a component of *Xf* DSF, conferred some reduction of disease after topical application, but less than might have been expected compared to application of crude DSF-containing extracts of *Xf*. Our recent studies using improved *Xf*-based DSF biosensors more responsive to the DSF molecules made by *Xf* reveal that at least two additional molecules related to C14-cis are biologically active in *Xf,* and DSF2 is much more active than C14-cis. We are very excited to find that a cheap, commercially available molecule palmitoleic acid also is quite active as a DSF signal molecule. We will determine which of these molecules are most biologically active, whether they all have similar effects on gene expression in *Xf*, and which are most abundant within plants infected with *Xf*. Our new sensitive biosensors will be used to document the absorption and translocation of these molecules by grape after application in various ways. This should enable us to greatly increase disease control by direct application of the most appropriate molecule.

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

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CHARACTERIZATION AND INHIBITION OF *XYLELLA FASTIDIOSA* PROTEINS SECRETED BY THE TYPE II SECRETION SYSTEM AND THEIR SECRETION MACHINERY

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ABSTRACT
The purpose of this study is to elucidate the contributions of host cell wall-degrading enzymes (CWDEs) produced by *Xylella fastidiosa* (*Xf*) to systemic colonization of grapevine, as well as the role of the Type II Secretion System (T2SS) in delivering these CWDEs into the xylem. Of the CWDEs predicted to be secreted by the T2SS, this project will focus on the endoglucanases (EGases) produced by *Xf*. We hypothesize that the T2SS secretes these EGases along with a polygalacturonase (PG), and that these enzymes collaborate to degrade the pit membranes that separate xylem vessels to facilitate the bacterium's systemic colonization of the grapevine via its xylem system. It has been previously reported that a purified PG and one of the *Xf* EGases are required to increase pore sizes of pit membranes in grapevine. Moreover, mutation of PG results in the loss of pathogenicity and movement for *Xf*. We also show that a loss of function in the T2SS results in a similar dramatic loss of pathogenicity. In addition, we are investigating the role of an EGase/expansin hybrid protein in pit membrane degradation. Ultimately, characterization of these EGases and the T2SS will help us to determine if they are suitable targets for Pierce's disease management.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) relies on degradation of the plant cell wall to move within the grapevine. This is accomplished by the cooperation of at least two classes of enzymes that target different components of the complex scaffold of the plant cell wall. A major goal of this research is to further elucidate the factors that lead to disassembly of the plant cell wall, thereby, allowing the bacteria to systemically colonize the plant. Systemic colonization is highly correlated with Pierce's disease development and preventing movement of the bacteria is critical to devising successful control strategies. We propose that characterizing and inhibiting *Xf* enzymes that facilitate movement throughout the plant and/or the secretion machinery responsible for delivering those *Xf* enzymes into the grapevines water pipes will provide a comprehensive approach to restriction of disease development.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a xylem-limited bacterial pathogen that is the causal agent of Pierce's disease of grapevine (Hopkins and Purcell, 2002, Chatterjee et al., 2008, Purcell and Hopkins, 1996). In order to systemically colonize the xylem, *Xf* must be able to move efficiently from one xylem vessel element to adjacent vessels. These xylem vessels are connected by pit membranes, which are porous primary cell wall interfaces that are composed of cellulose microfibrils embedded in a meshwork of pectin and hemicellulose (Buchanan, 2000, Sun et al., 2011). The pore sizes of these pit membranes range from 5 to 20 nM, and serve to prevent the movement of air embolisms and pathogens within the xylem (Mollenhauer & Hopkins, 1974, Buchanan, 2000). Indeed, these small pore sizes do prevent the passive movement of *Xf* between xylem vessels given that the size of the bacterium is 250-500 x 1,000-4,000 nM (Perez-Donoso et al., 2010, Mollenhauer & Hopkins, 1974). In order to move from one vessel to another, it has been shown through genomic and experimental evidence that *Xf* utilizes Cell Wall-Degrading Enzymes (CWDEs), including a polygalacturonase (PG) and at least one β-1,4 Endoglucanase (EGase), to break down the pit membranes network (Roper et al., 2007, Perez-Donoso et al., 2010). Furthermore, PG is necessary for pathogenicity in grape and has become a primary target for *Xf* inhibition

studies (Roper et al, 2007). However, PG alone is not sufficient for pathogenicity in grape and *Xf* requires both PG and an EGase for pit membrane degradation (Perez-Donoso et al., 2010). Therefore, elucidating the role of EGases in pit membrane degradation is critical for understanding systemic movement within the xylem. The *Xf* genome contains three genes that encode for canonical EGases: *egl* (PD2061) *rlpA* (PD1236) and *engXCA2* (PD1851). A fourth annotated EGase, *engXCA1* (PD 1856), putatively encodes a modular hybrid protein that contains both an EGase domain and an expansin domain (Simpson et al., 2000). Expansins are primarily plant proteins that function to non-enzymatically loosen the cell wall during development (e.g., cell elongation, fruit ripening). Recently, expansins have been found in several plant-associated bacteria, most of which have a significant xylem-dwelling phase in their lifestyle (Nikolaidis et al., 2014). It is predicted that these EGases and PG are delivered into the xylem by the Type II Secretion System (T2SS). Preliminary data demonstrate that *Xf* with a deficient T2SS display a non-pathogenic phenotype similar to that of the *Xf pglA* mutant that is deficient in production of PG, suggesting that the T2SS is essential for *Xf* pathogenicity. Therefore, our central hypothesis is that *Xf* utilizes other CWDEs and an endoglucanase/expansin hybrid protein in concert with PG to breach the pit membranes and that the majority of these are secreted by the Type II Secretion System. We will determine the role that each of these components plays in pit membrane degradation and systemic movement, and subsequently if they are good candidates for potential inhibition to limit Pierce's disease development.

OBJECTIVES

1. Characterization of *Xf* host cell wall degrading enzymes and an endoglucanase/expansin
2. Inhibition of *Xf* endoglucanases and the endoglucanase/expansin using endoglucanase-inhibiting proteins.
3. Characterization of the *Xf* Type II secretion system
4. Inhibition of the *Xf* Type II secretion system

RESULTS AND DISCUSSION

Objective 1. Characterization of *Xf* host cell wall degrading enzymes and an endoglucanase/expansin.

It was previously determined that *Xf* EngXCA2, which is one of the three *Xf* EGase-encoding genes, is a major contributor to the pit membrane dissolution, and the synergistic effect of both the PG and the *Xf* EGase was sufficient to increase pit membrane pore size (Perez-Donoso et al., 2010). Indeed, recombinant EngXCA2 was capable of digesting carboxymethyl cellulose (CMC) and xyloglucan (XyG) polymers, which both contain β1,4-linked glucan backbones and are representative of substrates *Xf* would likely encounter in grapevine primary cell walls (Roper, 2006; Perez-Donoso et al., 2010). Given the role EngXCA2 plays in pit membrane degradation, we hypothesize that other predicted EGases produced by *Xf* may impact pit membrane integrity as well. The *egl* gene is predicted to encode a β1,4 EGase belonging to the glycoside hydrolase family 5 as indicated in the CAZy (Carbohydrate Active Enzyme) database. Glycoside hydrolase family five proteins hydrolyze glycosidic bonds between two carbohydrates or a carbohydrate and non-carbohydrate moiety and have activities ranging from EGases to mannanases. *rlpA* putatively encodes a lipoprotein containing a Barwin-related EGase domain belonging to the glycoside hydrolase family 45. This family contains proteins with only EGase activity. The last gene annotated as an EGase is *engXCA1*, which encodes an EGase/expansin hybrid putatively involved in plant cell wall disassembly. This is of particular interest because expansins are primarily found in the plant kingdom and are non-enzymatic proteins that function to loosen the cell wall during plant growth without enzymatic digestion of the wall (Cosgrove, 2000). Expansins facilitate cell wall loosening by binding to their target polysaccharide and disrupting the weak bonds between the glycans and the cellulose microfibrils, allowing turgor pressure from within the cell to expand the cell wall (Cosgrove, 2000). Expansin-like proteins with similar structure and function were later found in a few bacterial species that associate with plants likely as a result of cross-kingdom horizontal gene transfer (Nikolaidis et al., 2014). These bacterial expansins are thought to enhance the activity of bacterial CWDEs by aiding in the loosening of the cell wall, thereby promoting wall breakdown, colonization and virulence. Interestingly, orthologs of at least one plant expansin (EXLX1) are found in several plant pathogens, including *Xylella, Xanthomonas*, *Ralstonia,* and *Erwinia* species (Kerff et al., 2008, Georgelis et al., 2014). While these are all phylogenetically diverse bacteria, they all share the commonality that they spend the majority of their lives in the xylem tissue of plants. It is hypothesized that they are involved in host colonization (Kerff et al., 2008). In the *Xf* pathosystem, they could potentially weaken the wall and more readily expose carbohydrate targets for digestion by the suite of other *Xf* CWDEs.

Characterization of the *Xf* EGase/Expansin hybrid protein.
In order to test the plant cell wall loosening properties and potential EGase function of the *Xf* EGase/expansin hybrid, we required the synthesis of recombinant protein. We cloned *engXCA1* into the Champion pET200 Directional TOPO expression vector (Life Technologies) and transformed that into *Escherichia coli* (strain BL21 Star). We induced the expression of EngXCA1 with IPTG, and confirmed expression by Western Blot (**Figure 1**) using an antibody against the N-terminal His-Tag. We confirmed the sequence of the protein by Mass Spectrometry and are now working on purifying the protein by Ni-NTA column chromatography. The purified recombinant protein will then be used to test for endoglucanase activity in radial diffusion assays and reducing sugar assays (Johnsen and Krause, 2014, Gross, 1982). We will also assess expansin activity of the recombinant protein (i.e., its ability to promote the extension of plant tissues that are subjected to stress) in close collaboration with the Cosgrove Laboratory (Penn State University). The cell wall elongation assay will then be performed using an extensometer apparatus as described by Cosgrove, D. J. (1989), and expansin activity will be determined by measuring the extension of wall specimens over a 2-h period.

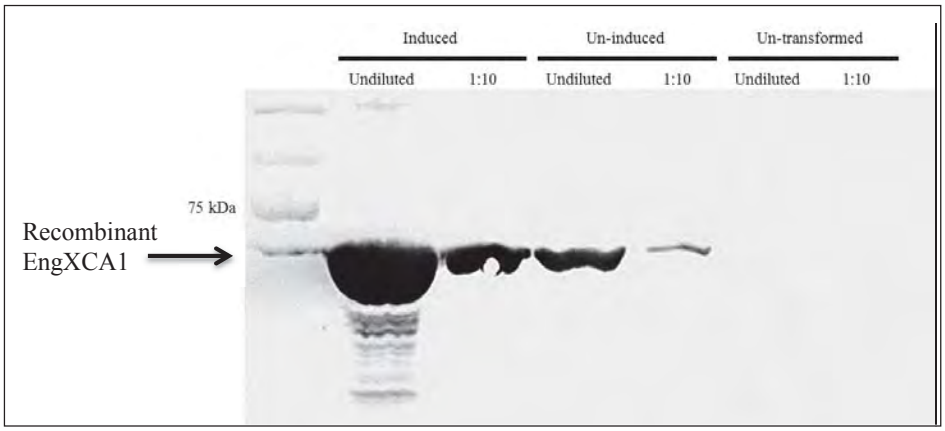

Figure 1. Western Blot of recombinant EngXCA1 expressed in *E. coli* strain BL21 Star. Lanes 1 and 2: *E. coli* was transformed with pET200::*engXCA1* and induced with 1 mM IPTG for six hours before cell lysis. Lanes 3 and 4: *E. coli* was transformed with pET200::*engXCA1* and incubated without IPTG. The presence of recombinant protein in these lanes is the result of leaky expression. Lanes 5 and 6: *E. coli* was neither transformed nor induced and served as the negative control. All lanes were probed with a primary 6x-His Tag mAb (ThermoFisher) at a dilution of 1:1000, followed by a goat anti-mouse secondary pAb conjugated to Alkaline phosphatase (ThermoFisher) at a dilution of 1:1000. The blot was developed using an Alkaline phosphatase development kit (Bio Rad). The molecular weight of the recombinant EngXCA1 protein is approximately 65 kDa.

Assessment of the biological contribution of the *Xf* EGase/Expansin to pathogenicity and host colonization.
To test the role of the *Xf* EGase/expansin *in planta*, we constructed a deletion mutant (Δ*engXCA1*) in the *Xf* Temecula 1 background using established mutagenesis techniques and confirmed the mutant via PCR (Matsumoto et al., 2009). We mechanically inoculated the Temecula 1 wild-type and Temecula Δ*engXCA1* mutant into grapevine (Cabernet Sauvignon variety) using the pin-prick method (Hill and Purcell, 1995). Grapevines inoculated with 1X phosphate buffered saline (PBS) were used as negative controls. Both the Temecula 1 wild-type and the Temecula Δ*engXCA1* mutant were inoculated into 10 plants each and the experiment was repeated three times (30 plants/treatment). This entire experiment was replicated in two grape varieties, Chardonnay and Cabernet Sauvignon. We quantified *Xf* populations in the plants by isolating *Xf* from the petioles at the point of inoculation (POI) (11 weeks post-inoculation) and ≥ 37 cm above the POI (12 weeks post-inoculation) to determine the ability of the EGase mutants to systemically colonize the host (**Figure 2**). The statistical differences between wild-type and Δ*engXCA1* mutant populations at both POI and ≥ 37 cm above the POI were determined by ANOVA. While there was a significant difference in colonization at the POI ($P = 0.027$), the difference in colonization at ≥ 37 cm above the POI was statistically insignificant ($P = 0.155$), indicating that the Δ*engXCA1*mutant is not impaired in systemic movement as we originally hypothesized. A similar trend was also observed in experiments conducted with the Chardonnay variety (*data not shown*). In these experiments, we inoculated at the base of the plants. However, in a natural scenario, sharpshooters feed on new green growth and the bacteria migrate in a basipetal direction against the flow of sap. We speculate that EngXCA1 may play a role in this basipetal movement and in future experiments we plan to inoculate plants closer to the shoot apex rather than at the base to assess for basipetal movement.

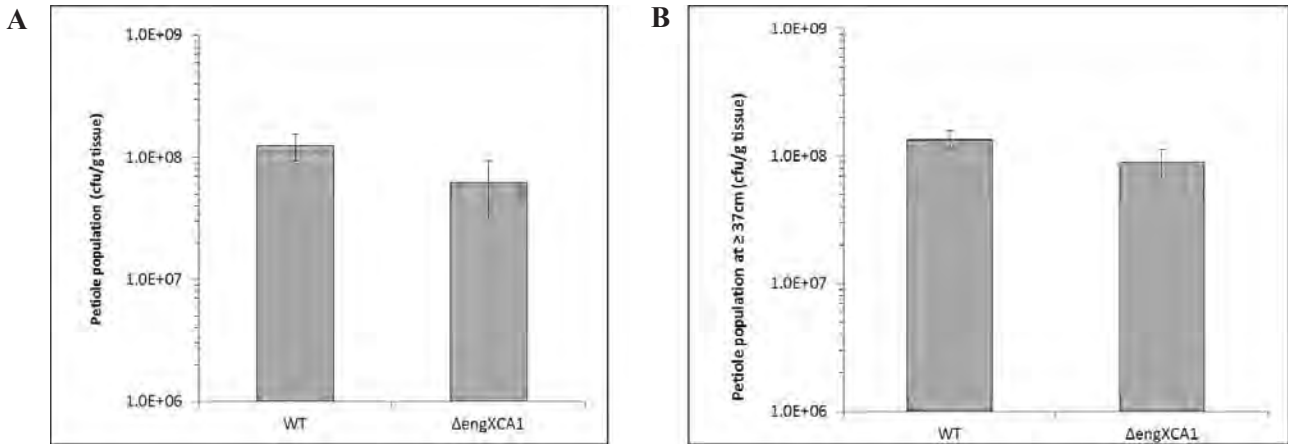

Figure 2. *In planta* populations of the Temecula Δ*engXCA1* mutant relative to the Temecula 1 wild-type strain. **A)** *In planta* populations of the Temecula Δ*engXCA1* mutant are significantly lower than those of the Temecula 1 wild-type strain at the point of inoculation (POI). **B)** *In planta* populations of the Temecula Δ*engXCA1* mutant are not significantly different from those of the Temecula 1 wild-type strain at ≥ 37 cm above the point of inoculation. Data are the means of three independent assays with ten replications each. Bars represent the standard error of the mean.

Disease ratings for all plants were taken, using a scale of 0-5 with 0=healthy and 5=dead as described by Guilhabert and Kirkpatrick (2005). Interestingly, the Δ*engXCA1* mutant strain is less virulent than the wild-type parent strain (**Figure 3**). Furthermore, the percentage of plants inoculated with the Δ*engXCA1* mutant strain rating two or higher on the disease index was consistently less than the percentage of plants inoculated with wild-type *Xf* rating two or higher over a 14-week period (**Figure 4**). This suggests that despite the ability of the Δ*engXCA1* mutant to systemically colonize the grapevine host similar to wild-type *Xf* as shown in **Figure 2**, the onset of disease in plants inoculated with the Δ*engXCA1* mutant is significantly delayed relative to plants inoculated with wild-type *Xf*. This experiment was also repeated in Chardonnay and a similar disease development trend was also observed (*data not shown*). It is very interesting that despite colonizing the plants to the same levels as wild-type *Xf*, the onset and severity of Pierce's disease symptoms is delayed in plants inoculated with the Δ*engXCA1* mutant.

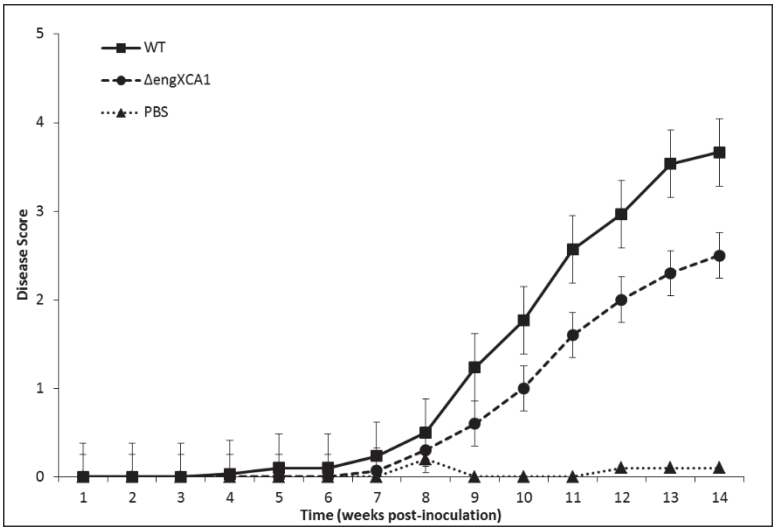

Figure 3. Disease progress of the Temecula Δ*engXCA1* mutant and the Temecula 1 wild-type strains over 14 weeks. The Temecula Δ*engXCA1* mutant strain lags behind the Temecula 1 wild-type strain in Pierce's disease symptom development. 1X PBS served as the negative control. Data are the means of three independent assays with ten replicates each. Bars represent the standard error of the mean.

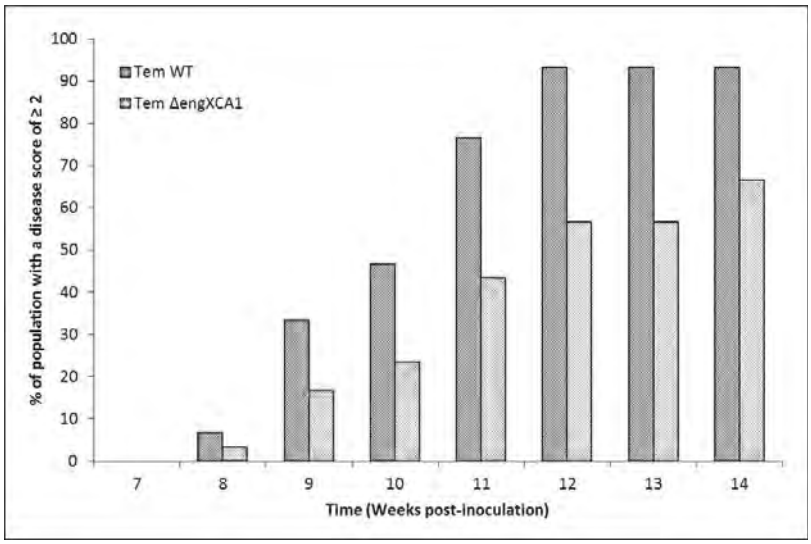

Figure 4. Percentage of plants rating a two or higher on the Pierce's disease scale. The percentage of plants inoculated with the Temecula Δ*engXCA1* mutant strain that rated two or higher was consistently less than the percentage of plants inoculated with the Temecula 1 wild-type strain over a period of 14 weeks.

Objective 2. Inhibition of *Xf* endoglucanases and the endoglucanase/expansin using endoglucanase inhibiting proteins.

As the combined action of a PG and an *Xf* EGase was required to digest pit membranes, both could be targets for inhibition. PG is a major pathogenicity factor for *Xf* and grapevines expressing a pear PGIP were more tolerant to *Xf* infection (Aguero et al., 2005). Several plant proteins have also been identified and characterized as xyloglucan-specific EGase inhibiting proteins (XEGIPs) that could potentially inhibit *Xf* EGases. These include XEGIPs from tomato and tobacco (Naqvi et al., 2005, Qin et al., 2003). We propose to assess the ability of the tobacco and tomato XEGIPs to inhibit the degradative ability of the *Xf* EGases and the EGase/expansin.

Currently, we are working on expressing and purifying these *Xf* EGases and assessing their activity as stated in Objective 1. Once these studies have been completed, we will test for inhibition using a radial diffusion assay performed in agarose containing either CMC or XyG as a substrate with increasing concentrations of each XEGIP. In addition, we will quantify the generation of reducing groups produced by the *Xf* EGases or EGase/expansin alone or in combination with each of the XEGIPs (Naqvi et al., 2005)

Objective 3. Characterization of the *Xf* Type II secretion system.

The T2SS is composed of 12-15 different proteins depending on the species that are involved either structurally or mechanistically involved in the function of the T2SS. These proteins are encoded in a single operon and the *Xf* genome contains a similar operon similar strongly suggesting a functional T2SS (Jha et al., 2005). The T2SS can be divided into four different subassemblies that are 1) the pseudopilus; 2) the Out membrane complex; 3) the inner membrane platform and 4) the secretion ATPase. The pseudopilus is composed primarily of the major pseudopilin protein, G (XpsG), and also contains the minor pseudopilins, S, H, I, J, and K (XpsH, I, J, and K). The XpsE ATPase harnesses the energy that drives secretion through the T2SS via hydrolysis of ATP. Proteins destined for secretion by the T2SS are first delivered to the periplasm via the Sec or Tat-dependent secretion pathway where they are folded (Slonczewski, 2014). The T2SS then uses a pilus-like piston to push proteins through the T2 channel. This piston action is a function of the cyclic assembly and disassembly of pilin subunits (primarily XpsG).

We have created a mutation in the *xpsE* gene, encoding the putative ATPase that powers the T2SS. Grapevines inoculated with the *xpsE* mutant never developed Pierce's disease symptoms and remained healthy, a phenotype similar to the *Xf pglA* mutant (**Figure 5**). Thus, we have compelling preliminary data indicating that *Xf* has a functional T2SS system and the proteins secreted by T2SS are critical for the infection process.

Figure 5. The *Xf* T2SS is necessary for Pierce's disease development in grapevine. **A)** the Δ*xpsE* mutant does not incite Pierce's disease symptoms in *Vitis vinifera* cv. Chardonnay grapevines. Disease severity was based on a visual disease scale from 0 (no disease) to 5 (dead). **B)** Representative images of plants from the virulence assay are shown here, 1 = Wild-type-inoculated, 2 = Δ*xpsE*-inoculated, 3 = 1X PBS buffer-inoculated. Plants shown are 11 weeks post-inoculation.

We hypothesize that the non-pathogenic phenotype of the *ΔxpsE* mutant is due largely to the inability to secrete host CWDEs. Indeed, we have indirect experimental evidence that *Xf* utilizes the T2SS to secrete PG. This is based on an assay performed on the defined growth medium, XFM. When XFM is supplemented with pectin as the sole carbon source, this induces production of copious amounts of the carbohydrate-based exopolysaccharide (EPS) (Killiny & Almeida, 2009). Pectin is a complex carbohydrate comprised in its simplest form of repeating galacturonic acid residues. Therefore, when grown on XFM with pectin as the sole carbon source, we hypothesize that *Xf* must first digest the pectin source utilizing its endo-polygalacturonase (Roper et al., 2007) and likely other pectin-digesting enzymes that eventually disassemble the pectin polymer into individual galacturonic acid residues that can then feed into various metabolic processes within the bacterium, such as EPS production.

In support of our hypothesis that PG (and other CWDEs) are secreted through the T2SS, we demonstrate that the Δ*xpsE* mutant produces visibly less EPS on XFM+pectin medium resulting in a much less mucoid phenotype. Furthermore, when wild-type *Xf* and Δ*xpsE* are grown on XFM+galacturonic acid (i.e., the monomeric sugar that makes up the pectin polymer), both strains produce similar amounts of EPS (*data not shown*). We infer from this that, indeed, breakdown of the pectin substrate is necessary to produce EPS and when the T2SS is disrupted this prevents secretion of PG and the subsequent breakdown of pectin.

Objective 4. Inhibition of the *Xf* Type II secretion system.
Proteins destined for secretion by the T2SS are first exported to the periplasm by the Sec or Tat pathways. *Xf* appears to only possess the Sec-dependent secretion pathway. Disruption of the T2SS by small molecule inhibitors was demonstrated in *Pseudomonas aeruginosa* and *Burkholderia pseudomallei*, and could be used to inhibit the *Xf* Sec-dependent pathway (Moir et al., 2011). A chemical compound library will be screened for Sec-inhibitory molecules, including those compounds used by Moir et al. (2011). Inhibition of the Sec-dependent pathway will be confirmed by monitoring the secretion of a CWDE using a polyclonal antibody raised against EngXCA2 and analyzed via Western Blot and ELISA.

CONCLUSIONS
Our goal is to first understand the roles each of the EGases produced by *Xf* has in pit membrane degradation, as well as the role of the T2SS in secreting these CWDEs. Ultimately, we speculate that inhibition of the EGases and/or the T2SS will significantly reduce the ability of *Xf* to systemically colonize its grapevine host. Preliminary results indicate that the EGase/expansin hybrid protein plays a role in virulence, and could possibly be an elicitor of the host defense response. These studies will be repeated with the addition of the *engXCA1/engXCA1+* complement strain to confirm these results. In addition, an *Xf* strain with a deficient T2SS (Δ*xpsE*) displayed reduced virulence than unmodified *Xf*, lending credence to the hypothesis that the T2SS secretes CWDEs such as PG and EngXCA2 that are necessary for systemic colonization. We speculate that further characterization of these EGases and the T2SS will elucidate significant targets for controlling Pierce's disease.

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CHARACTERIZATION OF THE LIPOPOLYSACCHARIDE-MEDIATED RESPONSE TO *XYLELLA FASTIDIOSA* INFECTION IN GRAPEVINE

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ABSTRACT
Xylella fastidiosa (Xf) is a gram-negative, xylem-limited bacterium that causes serious diseases in economically important crops, such as Pierce's disease of grapevine. Lipopolysaccharide (LPS) is the dominant macromolecule displayed on the bacterial cell surface. LPS acts as a selective barrier, preventing entry of toxic substances into the cell, and as an anchor for superficial structures. Finally, LPS is a well-described pathogen-associated molecular pattern (PAMP) and is known to elicit host basal defense responses in model plant systems. LPS is composed of a conserved lipid A-core oligosaccharide component and a variable O-antigen. Through mutations made in *wzy* (XP0836), which encodes an O-antigen polymerase, we have demonstrated that the *Xf* O-antigen contributes to plant and insect colonization, and depletion of the O-antigen causes a severe reduction in overall virulence *in planta*. This project aims to determine the role of the *Xf* O-antigen in modulation of the basal defense response in grapevine. Our goal is to determine if specific alterations to the LPS structure cause a change in the elicitation of the grapevine response to *Xf*, therefore affecting critical, early stages of *Xf* establishment *in planta*. We are also evaluating *Xf* O-antigen structural variants as a preventative treatment for the control of Pierce's disease.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*), a bacterial pathogen, is the causal agent of Pierce's disease of grapevine and poses a serious threat to the viticulture industry. We have demonstrated that truncation of the O-antigen portion of the lipopolysaccharide (LPS) entity alters the adhesive properties of the cell, leading to a defect in mature biofilm formation. Furthermore, depletion of the O-antigen results in a significantly less virulent pathogen that is severely compromised in host colonization**.** Additionally, LPS is a Pathogen-Associated Molecular Pattern (PAMP) that potentially triggers the grapevine basal immune response. We hypothesize that the long chain O-antigen allows *Xf* to circumvent the innate immune system by masking the conserved core and lipid A portions of the LPS chain from the host immune system. The goal of the proposed work is to further explore the role of LPS, specifically focusing on the O-antigen moiety, in the interaction between *Xf* and the grapevine and to use this information to develop and evaluate an environmentally sound preventative application for Pierce's disease.

INTRODUCTION
Xylella fastidiosa (Xf), a gram-negative, fastidious bacterium, is the causal agent of Pierce's disease of grapevine (*Vitis vinifera*) and several other economically important diseases (Chatterjee *et al.*, 2008; Varela, 2001). Pierce's disease has devastated some viticulture areas in California and there are currently no effective control measures available to growers targeted towards the bacterium itself. Lipopolysaccharide (LPS) is a tripartite glycolipid molecule that is an integral part of the Gram-negative bacterial outer membrane. It is primarily displayed on the outer surface of the cell, thereby mediating interactions between the bacterial cell wall and its environment. LPS plays diverse roles for the bacterial cell. It provides structural integrity to the cells and can act as a permeability barrier to toxic antimicrobial substances (Kotra *et al.*, 1999; Raetz & Whitfield, 2002). Because of its location in the outer membrane, it is also a key contributor to the initial adhesion to a surface or host cell (Goldberg & Pler, 1996; Walker *et al.*, 2004). We have been exploring the roles of LPS in the Pierce's disease cycle and in the Plant-Microbe-Insect (PMI) interactions of *Xf*. We targeted our studies towards the outermost exposed region of the LPS molecule, the O-antigen. By mutating a key O-antigen polymerase, *wzy* (XP0836), in the Temecula1 (Pierce's disease) isolate, we have demonstrated that severe truncation of the O-antigen, and subsequent termination in the synthesis of rhamnose-rich subunits, alters the adhesive and aggregative properties of the cell considerably, thus causing a marked defect in biofilm formation. Furthermore, the resulting mutation of the O-antigen caused increased sensitivity of the bacterium to hydrogen peroxide stress *in vitro* and resulted in a significantly less virulent pathogen that is severely impaired in host colonization (Clifford et al, 2013). It has long

been speculated that *Xf* surface polysaccharides play a role in the host-pathogen interaction with grapevine and our ongoing studies confirm that LPS is a major virulence factor for this important agricultural pathogen.

Contrary to the role of LPS in promoting bacterial survival *in planta*, the immune systems of plants have also evolved to recognize the LPS structure and mount a basal defense response to counteract bacterial invasion (Dow *et al.*, 2000; Newman *et al.*, 2000). LPS is considered a Pathogen Associated Molecular Pattern (PAMP). PAMPs, also known as Microbial Associated Molecular Patterns (MAMPs), are conserved molecular signatures that are often structural components of the pathogen (ie. LPS, flagellin, fungal chitin, etc.). These PAMPs are recognized by the host as "non-self" and can be potent elicitors of the basal defense response. This line of defense against invading pathogens is referred to as PAMP triggered immunity (PTI) and represents the initial layer of defense against pathogen ingress (Nicaise *et al.*, 2009). PTI is well studied in both mammalian and plant hosts. However, little is known about the mechanisms involved in perception of the LPS PAMP in grapevine, particularly the *Xf* LPS PAMP. *Xf* is introduced by its insect vector directly into the xylem, a non-living tissue, which cannot mount a defense response on its own. However, in other systems, profound changes do occur in the adjacent living parenchyma cells upon infection suggesting that these cells communicate with the xylem and are capable of recognizing the presence of a pathogen (Hilaire *et al.*, 2001). Bacteria can also circumvent the host's immune system by altering the structure of their LPS molecule. Specifically, bacteria can display different O-antigen profiles by varying the extent of polymerization or by completely abolishing synthesis of the O-antigen depending on the environment and developmental phase of the cell (Bergman *et al.*, 2006; Guerry *et al.*, 2002; Lerouge & Vanderleyden, 2002). We speculate that during the interaction between *Xf* and a susceptible grapevine host, the bacterium's long chain, rhamnose-rich O-antigen shields the conserved lipid A and core-oligosaccharide regions of the LPS molecule from being recognized by the grapevine immune system, providing an opportunity for it to subvert the basal defense response and establish itself in the host. A similar scenario occurs in *Escherichia coli*, where truncation of the O-antigen caused an increased sensitivity to serum suggesting the full length O-antigen provides a masking effect towards the host immune system (Duerr *et al.*, 2009; Guo *et al.*, 2005). *Salmonella enterica* subsp. *enterica* sv. (*S.*) Typhimurium also possesses an O-antigen that aids in evasion of the murine immune system (Duerr *et al.*, 2009).

Our main aim is to further explore the role of LPS, specifically focusing on the O-antigen moiety, in the interaction between *Xf* and the grapevine host and to use this information to develop and evaluate an environmentally sound preventative application for Pierce's disease. We hypothesize that the LPS molecule contributes not only to biofilm formation but also modulates the host's perception of *Xf* infection. The *Xf* O-antigen mutant we currently have, and the additional ones we propose to construct in this study, provide a unique platform designed to test this hypothesis. The fundamental goal is to elucidate the mechanism(s) that *Xf* uses to infect the grapevine host and exploit this knowledge to evaluate the use of LPS structural variants as a preventative treatment for control of Pierce's disease.

OBJECTIVES

1. Characterization of *Xf* LPS mutants *in vitro* and *in planta*.
2. Examination of the LPS-mediated response to *Xf* infection.
3. Evaluation of structural variants of LPS as a preventative treatment for Pierce's disease.

RESULTS AND DISCUSSION

Objective 1. Characterization of *Xf* LPS mutants *in vitro* and *in planta*.

We have determined that the wild-type *Xf* O-antigen is composed primarily of 2-linked rhamnose with smaller amounts of glucose, ribose, xylose, and mannose (Clifford et al, 2013). Most importantly, we demonstrated that mutation of the O-antigen polymerase, Wzy, results in a severely truncated O-antigen resulting from a depletion of the majority of the 2-linked rhamnose. This change was confirmed both electrophoretically and biochemically utilizing gas chromatography and mass spectrometry (GC/MS) techniques in collaboration with the Complex Carbohydrate Research Center (CCRC) at the University of Georgia. Notably, the depletion of rhamnose led to a marked reduction in virulence and host colonization (Clifford et al, 2013). This indicates that the process of rhamnose biosynthesis and its incorporation into the O-antigen is a vulnerable step in the *Xf* LPS biosynthetic pathway that could be exploited for disease control. Therefore, in this objective we are building on our current knowledge and continuing our studies by focusing on the process of rhamnose biosynthesis in *Xf*. We are presently creating mutants that we hypothesize will be unable to synthesize rhamnose, and we will structurally characterize the O-antigen from these mutants in collaboration with the CCRC. Following this, we will define the

biological impact of these mutations by conducting virulence and colonization studies in grapevine. We will also determine the effect of these mutations *in vitro* using substrate attachment, cell-cell aggregation, and visualized biofilm studies that reflect host colonization behaviors.

Using comparative genomics, we have identified five genes with high homology to those involved in rhamnose biosynthesis in other bacterial systems. The genes are designated $rmlB_1$ (XP0208), *rmlA* (XP0209), *rmlC* (XP0210), and *rmlD* (XP0211) (in map order) that encode proteins involved in the conversion of glucose-1-phosphate into dTDP-rhamnose (Jiang *et al.*, 1991; Koplin *et al.*, 1993; Rahim *et al.*, 2001). The *rml* genes are usually clustered within a single locus and our *in silico* analysis demonstrates the presence of a *rml* locus in *Xf*. We also identified an additional, unlinked copy of *rmlB*, designated $rmlB_2$ (XP1617). Mutations in the *rml* locus in *Xanthomonas campestris* pv. campestris, a close relative of *Xf*, resulted in a loss of rhamnose only in the O-antigen with no change in the sugars comprising the core oligosaccharide (Koplin *et al.*, 1993). We hypothesize that *rml* mutants in *Xf* will be similarly affected and be significantly deplete of O-antigen.

Mutant Construction.
We are currently constructing the *Xf* Δ*rml* mutants using site-directed mutagenesis, and we have a completed construct for the $\Delta rmlAB_1CD$ mutant. Our next step is to make an $rmlAB_1CD/rmlB_2$ double mutant. We predict that the O-antigen in the *rml* mutants will be completely devoid of rhamnose. We will confirm this by conducting glycosyl composition and linkage analyses in collaboration with the CCRC.

O-antigen purification and structural analysis.
We have isolated LPS from the *Xf* wild-type and our previously constructed O-antigen mutant strains (*wzy* and *waaL*). LPS from the *rml* mutant strain (from at least three biological replicates for each strain) will be isolated once complemented strains are made, and purified LPS will be sent to the CCRC for structural analysis. LPS was purified from whole cells using a modification of the hot phenol extraction method (Marolda, 2006). O-antigen will be isolated from the total LPS fraction by mild acid hydrolysis in 1% acetic acid for four hours at 100º C, followed by centrifugation at 8,000 rpm for 30 minutes. The supernatant will be removed and reserved for glycosyl composition and linkage analysis. Glycosyl composition analysis will be performed by combined gas chromatography/mass spectrometry (GC/MS) of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis as previously described (Merkle & Poppe, 1994). GC/MS analysis of the TMS methyl glycosides will be performed on an Agilent 7890N GC interfaced to a 5975C MSD, using a Supelco EC-1 fused silica capillary column (30m × 0.25 mm ID). For glycosyl linkage analysis, the sample will be permethylated, depolymerized, reduced, and acetylated; and the resultant partially methylated alditol acetates will be analyzed by GC-MS (York, 1985). These techniques will allow us to deduce a preliminary structure and determine any differences between the wild-type and mutant strains. Samples of the WT and *wzy* LPS are currently under analysis at the CCRC.

Surface attachment, aggregation and biofilm studies.
Once we have the LPS structural data, we will begin to link *Xf* LPS structure to function using *in vitro* assays. Attachment to a surface is the first step in successful biofilm formation and because of the location and abundance of LPS in the outer membrane we hypothesized that LPS plays a key role in mediating initial attachment to the cellulose and chitin substrates *Xf* encounters in the plant and insect, respectively. We previously demonstrated that a mutant in the Wzy polymerase was deficient in cell-cell aggregation and hyperattached to surfaces, which led to a defect in biofilm formation (Clifford et al., 2013). We will similarly test the $rmlAB_1CD$ *and* $rmlAB_1CD/rmlB_2$ (and single *rml* mutants if necessary) mutants for these behaviors to determine if the inability to synthesize rhamnose results in defective biofilm formation.

Virulence and host colonization assays.
Once we have obtained both the *rml* mutants and their complemented strains, we will mechanically inoculate *Vitis vinifera* Cabernet Sauvignon vines using the pin-prick method (Hill & Purcell, 1995). Each plant will be inoculated twice with a 20µL drop of a 10^8 CFU/mL suspension of either wild-type *Xf* or the *rml* mutants constructed in this objective. We will inoculate 10 plants/mutant and repeat each experiment three times. Plants inoculated with 1x PBS will be used as negative controls. All plants will be rated on a disease scale of 0-5 with 0 being healthy and 5 being dead (Guilhabert & Kirkpatrick, 2005). We will also assess the *Xf* populations in the plants by isolating cells from the petioles at the point of inoculation and 25cm above the point of inoculation to assess the ability of the *rml* mutants to systemically colonize the host. Isolations will be performed at five and

fourteen weeks post-inoculation. Petioles will be surface sterilized and ground in 2mL of sterile 1x PBS. The resulting suspension will be diluted and plated on solid PD3 medium and colonies will be counted and normalized to tissue weight.

Objective 2. Examination of the LPS-mediated response to *Xf* infection.
In grapevine, recognition of PAMPs other than LPS, such as the *Botrytis cinerea* endopolygacturonase BcPG1and β-glucans, trigger a cascade of signaling events including calcium ion influxes, reactive oxygen radical accumulations, and activation of protein kinases, that coordinate the transcriptional activation of defense genes (Aziz *et al.*, 2003; Aziz *et al.*, 2007; Poinssot *et al.*, 2003). The LPS PAMP can induce similar responses in other plant species, but these studies have been performed primarily in model systems, such as *Arabidopsis thaliana* or tobacco (Desaki *et al.*, 2006; Zeidler *et al.*, 2004). There is limited knowledge about the grapevine response to the LPS PAMP, particularly on the transcriptional level. However, one study demonstrated that a rhamnolipid MAMP from *Pseudomonas aeruginosa* could induce defense-related responses in grapevine cell suspensions (Varnier *et al.*, 2009).

The defense reactions activated upon PAMP recognition involve intricate networks of transcriptional regulators and phytohormone signaling. Genome-wide transcriptional profiling is a logical starting point to begin understanding this complex process in the *Xf*-grape pathosystem (Jones & Dangl, 2006). We speculate that mutated *Xf* LPS (deplete of O-antigen) recognition elicits a transcriptional response that results in the deployment of specific defense reactions in grape that results in less disease and host colonization. We hypothesize that the grapevine is recognizing the conserved core/lipid A portions of the *Xf* LPS molecule and that the long chain O-antigen serves to camouflage the rest of the LPS PAMP (the core-lipid A complex) from being recognized by the host innate immune system. Thus, we expect an increase in expression of defense-related genes in plants inoculated with the O-antigen mutants (*wzy::kan*, $rmlAB_1CD$ *and* $rmlAB_1CD/rmlB_2$) that are depleted of O-antigen as compared to wild-type *Xf.* The studies detailed below are designed to test our hypothesis that loss of the rhamnose-rich O-antigen allows the grapevine to more readily perceive the *Xf* LPS molecule and that this recognition leads to elicitation of a specific transcriptional response associated with defense.

LPS-induced oxidative burst in grapevine.
To explore the role of LPS as an elicitor of basal defense responses in grapevine, we first investigated reactive oxygen species (ROS) production *ex vivo* using a luminol assay. ROS production was measured through the chemiluminescence of luminol and monitored over 60 minutes. Due to the increased exposure of the Lipid A-Core oligosaccharide region of the *wzy* mutant, we hypothesized that we would see a quicker, stronger oxidative burst, compared with wild-type. As shown in **Figure 2**, both wild-type and *wzy* mutant LPS induced an oxidative burst in grapevine leaf disks. However, the burst induced by *wzy* mutant LPS was stronger and more prolonged than wild-type, peaking around five minutes and returning to near-basal levels around 32 minutes post-elicitation.

ROS production in response to live *Xf* cells.
Once we established that *Xf* LPS induced an oxidative burst in *V. vinifera* Cabernet Sauvignon leaf disks, we then turned our attention to ROS produced in response to living *Xf* wild-type and *wzy* mutant live cultures. LPS O-antigen moieties can adopt numerous conformations, sometimes bending to shield the cell from recognition. This is the case with species of *Salmonella*, where O-antigen-mediated evasion of innate immune activation significantly enhances bacterial survival *in vitro* and *in vivo* (Duerr *et al*., 2009). Our working hypothesis is that wild-type O-antigen helps mask *Xf* from recognition by the grapevine host, thereby facilitating *Xf* establishment *in planta*. Luminol assays were conducted in the same manner as described previously, except that suspensions of wild-type or *wzy* mutant culture (at $1x10^8$ CFU/mL) were added to each well. As shown in **Figure 2**, whole *wzy* mutant cells induced a strong response from grapevine leaf disks. ROS production peaked at around 12 minutes and lasted nearly 100 minutes. Whole Wild-type cells failed to produce a sharp peak, as compared with the *wzy* mutant, and ROS production plateaued much sooner (around 60 minutes).

Transcriptome profiling
High-throughput sequencing technologies provide a relatively inexpensive means to profile the expression of nearly all genes in a tissue simultaneously. The application of transcriptome profiling approaches using next generation RNA sequencing (RNA-seq) will allow us to monitor the activation or suppression of specific defense pathways at the genome scale. In early July of 2014, individual vines of *V. vinifera* Cabernet Sauvignon were inoculated with *Xf* wild-type or *wzy*::*kan* live culture. We inoculated nine vines for each treatment. Vines

inoculated with 1xPBS buffer alone served as the negative controls for the experiment. Using the pin-prick method described previously, each vine was inoculated 2x with a 20µL drop of a 10^8 CFU/mL suspension of either wild-type *Xf* or the *wzy* mutant. PTI usually causes major transcriptional reprogramming of the plant cells within hours after perception (Dow *et al.*, 2000; Tao *et al.*, 2003). Thus, petioles were harvested at the following four time points: 0,1,8, and 24 hours post-inoculation. To stabilize transcripts, petioles were submerged into liquid Nitrogen immediately after harvesting and stored at -80°C until RNA extraction. Currently, RNA extraction protocols are being optimized to ensure that yields are sufficient prior to the downstream applications. Before construction of the RnaSeq libraries is conducted, we plan to first use qPCR to fine-tune the timing of the transcriptional profiling. This will be done through monitoring the induction of known LPS-induced genes in *A. thaliana* (*PR-1,2,3,4,5*) at varying time points following inoculation. Using this information, we will finalize the time points we will use for the genome-wide transcriptional profiling.

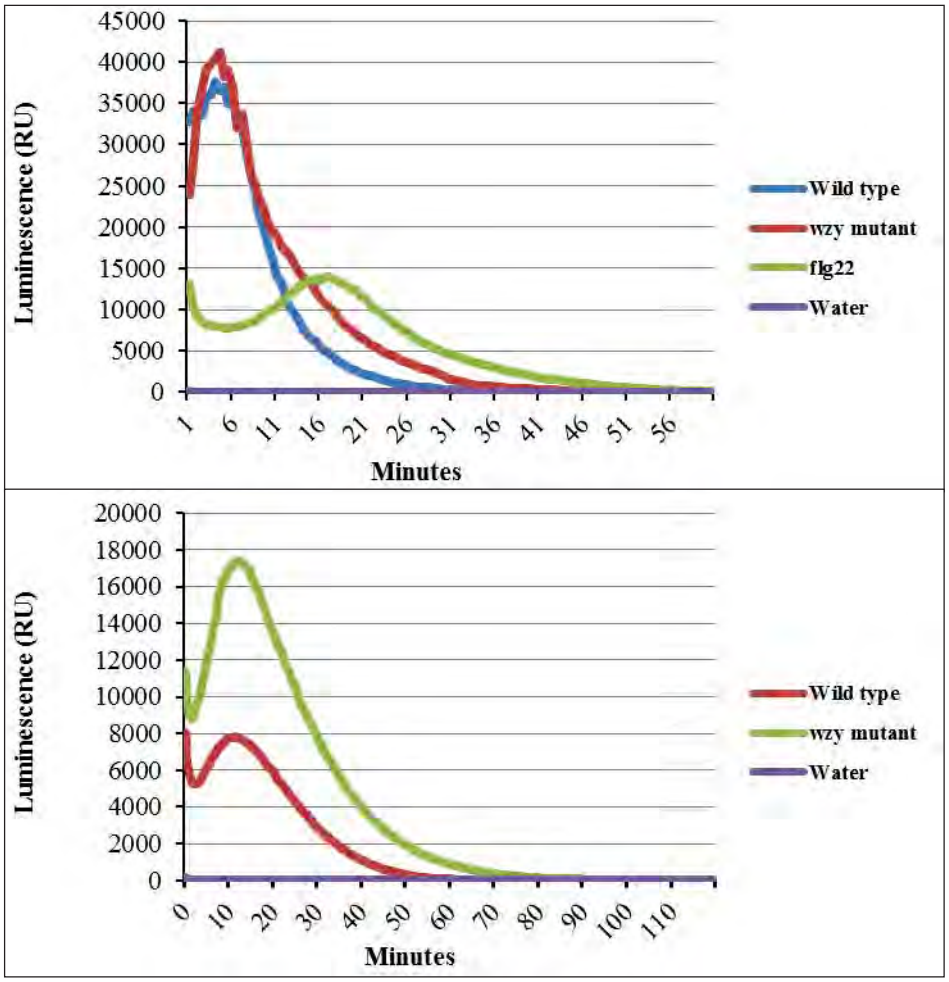

Figure 2. Purified LPS-induced ROS production in grapevine (top). Presence of an oxidative burst was determined through the chemiluminescence of luminol. The *wzy* mutant LPS elicits a stronger, more prolonged response in grapevine leaf disks, compared with *Xf* wild-type LPS. ROS production in response to live *Xf* cells (bottom). Suspensions of wild-type or *wzy* mutant culture were added to grapevine leaf disks, and ROS production was monitored through the chemiluminescence of luminol. The O-antigen mutant culture induced a strong response from grapevine leaf disks, peaking at around 12 minutes and plateauing around 100 minutes. Wild-type culture failed to produce a sharp peak, compared with *wzy* mutant cells, and ROS production plateaued at around 60 minutes. Data are means of three independent assays with eight replications per treatment.

Objective 3. Evaluation of structural variants of LPS as a preventative treatment for Pierce's disease.
In some systems, treatment with LPS alone does not induce a measurable difference in gene expression. However, it does potentiate a more robust and measurable defense response following challenge with a pathogen. Pre-treatment of plants with LPS can prime the defense system resulting in an enhanced response to subsequent

pathogen attack. This defense-related memory is called "priming" and stimulates the plant to initiate a faster and/or stronger response against future invading pathogens (Conrath, 2011). Priming often results in rapid and robust activation of defense responses such as the oxidative burst, nitric oxide synthesis, and expression of defense-related genes (Erbs & Newman, 2003; Newman *et al.*, 2000). The LPS PAMP has been specifically implicated in priming in the *X. campestris* pv. *vesicatoria* pathosystem. Pepper leaves pre-treated with LPS isolated from incompatible (non-virulent) xanthomonads had enhanced expression of several PR proteins after being challenged with virulent *X. campestris* pv. vesicatoria (Newman *et al.*, 2000). In this objective, we hypothesize that pre-treatment with LPS isolated from *Xf* O-antigen mutants results in a difference in the grapevine's tolerance to *Xf* by stimulating the host basal defense response.

Priming assays.
Grapevines were pre-treated with 40µL of either wild-type or *wzy* mutant LPS (50µg/mL). 1xPBS served as the negative control. After we mechanically inoculated the vines with LPS, we challenged with an inoculation of live wild-type *Xf* cells (40µL of a $1x10^8$ CFU/mL suspension). These inoculations were performed at 4 and 24 hours after the original inoculation with the LPS. These time points were established based on previously described assays (Newman *et al.*, 2002). We inoculated 24 vines/treatment/LPS concentration/time point. Once we have established that we can induce the primed state in grapevine, we will then begin assessing how long the temporal window of the primed state lasts by increasing the amount of time between the inoculation with the LPS and the challenge with live *Xf* cells.

Disease ratings.
To determine if the primed state affects the development of Pierce's disease symptoms, we documented disease progress in plants that were pre-treated with either wild-type or *wzy* LPS and then challenged with *Xf* either 4 or 24 hours later. Plants were rated on a disease index scale of 0-5, with 0 being healthy and 5 being dead or dying (Guilhabert & Kirkpatrick, 2005). Disease ratings were taken at 10 weeks post-inoculation. As shown in **Figure 3**, plants pre-treated with either wild-type or *wzy* LPS were delayed in Pierce's disease symptom development when challenged with *Xf* four hours later, compared to those plants inoculated solely with *Xf* (*Xf* only; no LPS pre-treatment). This indicates that treatment with either form of LPS (WT or *wzy*) does impart some form of defense against *Xf* within a four-hour time window. Interestingly, this effect seems to be transient in plants pre-treated with WT LPS because when plants were challenged with *Xf* 24 hours after pre-treatment with WT LPS PD symptom severity was the same as those that did not reeive any pre-treatment. However, a decrease in Pierce's disease severity was still observed in plants pre-treated with *wzy* LPS and challenged with *Xf* 24 hours later. These data are in agreement with the leaf disk assays that show that wzy LPS induces a longer and more prolonged ROS burst than WT LPS.

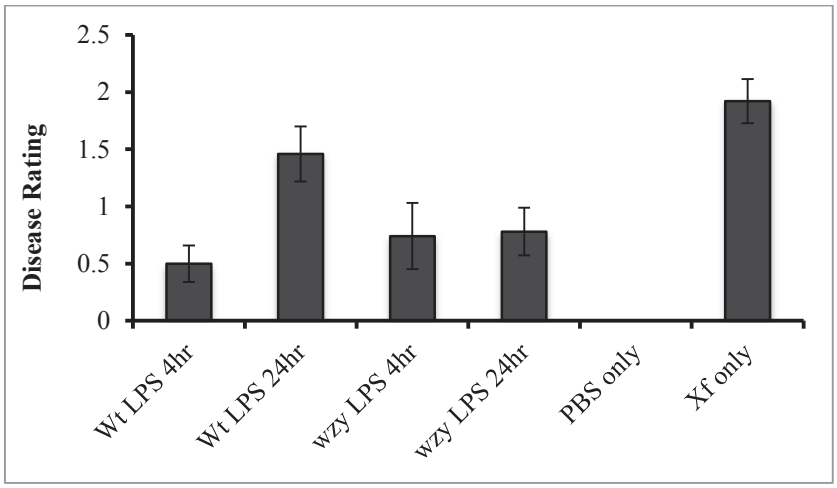

Figure 3. Pierce's disease ratings of LPS pre-treated plants. Mean disease rating at 10 weeks post-inoculation of Cabernet Sauvignon grapevines pre-treated with wild-type or *wzy* mutant LPS (50µg/mL), followed by inoculation with *Xf* Temecula1 wild-type culture at 4 or 24hr post-inoculation with LPS. *Xf* only plants had no pre-treatment. The LPS pre-treated plants are significantly delayed in symptom development, compared with *Xf* only plants. Plants inoculated with *Xf* at 4hr post-inoculation with LPS showed fewer symptoms than 24hr-inoculated plants. PBS only plans represent negative controls. Bars represent standard error of the mean.

Bacterial colonization *in planta.*

Pre-treatment of LPS can also restrict *in planta* growth (Erbs & Newman, 2003; Newman *et al.*, 2002). We reason that purified *Xf* LPS deplete of the O-antigen could potentiate the host defense response resulting in a decrease in bacterial proliferation in the plant. To determine if pre-treatment of plants with mutated *Xf* LPS does, in fact, lead to a decrease in bacterial growth, we isolated *Xf* populations at the point of inoculation at six weeks post-inoculation. *Xf* is a slow growing organism, which is why we chose this long time point. Interestingly, we observed no significant difference in bacterial titer amongst the treatments at six weeks post-inoculation (**Figure 4**). We speculate this may be because we performed isolations solely at the point of inoculation. In the future, we also plan to isolate from 25cm above the point of inoculation to assess differences in movement as well. Perhaps *Xf* was restricted in movement, which lead to the observed decrease in Pierce's disease symptoms (**Figure 3**). We will repeat these experiments in spring 2015.

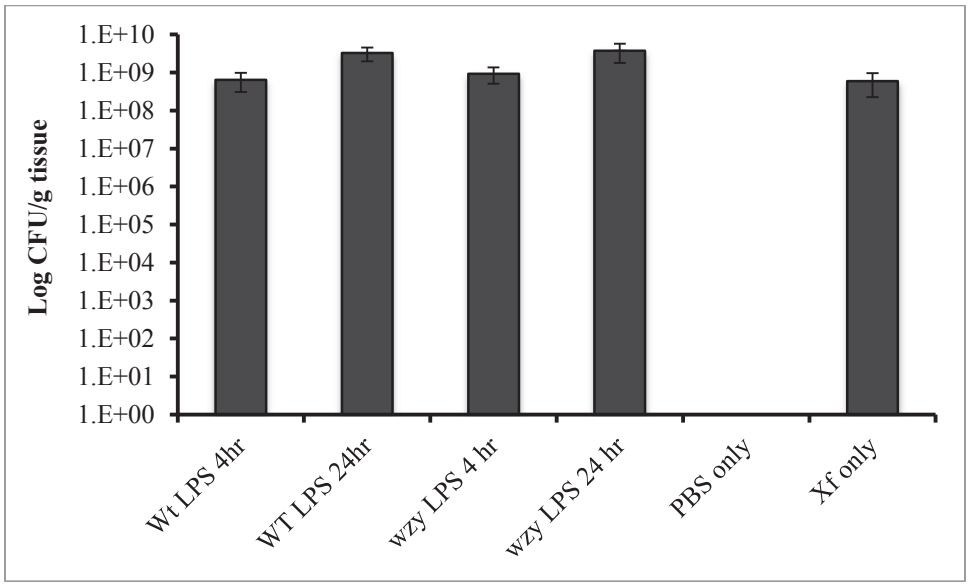

Figure 4. Bacterial colonization in LPS pre-treated plants. Log CFU isolated from the point of inoculation on Cabernet Sauvignon grapevines pre-treated with wild-type or *wzy* mutant LPS (50µg/mL), followed by inoculation with *Xf* Temecula1 wild-type culture at 4 or 24hr post-inoculation with LPS. *Xf* only plants had no pre-treatment. PBS only plants represent negative controls. Data are means of three independent assays with eight replications per treatment. Bars represent standard error of the mean.

CONCLUSIONS

The proposed project will address a key aspect of the interaction of *Xf* with its grapevine host. In addition, it will provide knowledge about basal resistance to disease in grapevines and plant hosts in general. Notably, we will also test a potential preventative measure for Pierce's disease. Information gleaned from this project could also help guide traditional breeding programs aimed at disease resistance by identifying potential resistance markers. The overall outcome will result in a foundation of fundamental knowledge about Pierce's disease at the molecular level that we will use to develop an innovative and environmentally sound approach to controlling this disease.

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ROLE OF COLD SHOCK PROTEINS IN *XYLELLA FASTIDIOSA* VIRULENCE

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ABSTRACT

Xylella fastidiosa (*Xf*), causal agent of Pierce's disease of grapevine, is mainly prevalent in warmer climates. Subjecting *Xf* infected grapevines to cold temperatures can, in many cases, effectively eliminate the bacterial population, a phenomenon known as cold curing. However, very little is known regarding the physiological response of *Xf* to cold temperatures. Cold shock proteins (CSPs), a family of nucleic acid binding proteins, are known to be an important component in the response of bacteria to temperature downshift. Genes encoding CSPs are often present in multiple copies; expression is strongly induced by cold temperature or in stationary phase as part of a general stress response. In some cases, bacterial CSPs are recognized by the plant host as an elicitor of the basal defense response. Two putative CSP homologs (Csp1 and Csp2) with conserved cold shock and nucleic acid binding domains were identified. A deletion mutant of Csp1 (Δ*csp1*) in *Xf* strain Stag's Leap had a decreased rate of survival at 4°C, as compared with wild type. Wild type survival rates at 4°C were partially restored in Δ*csp1* by complementation with the predominant CSP of *Escherichia coli* (CspA), or the CspA homolog of *Xanthomonas campestris*. Most notably, Δ*csp1* was significantly less virulent in grapevine, as compared with wild-type. Further study of the role of CSPs in *Xf* survival and virulence *in planta* will lead to a better understanding of the cold curing phenomenon observed in Pierce's disease affected grapevines and interactions of *Xf* with the plant host.

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ANALYSES OF *XYLELLA* WHOLE GENOME SEQUENCES AND PROPOSAL OF *XYLELLA TAIWANENSIS* SP. NOV.

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ABSTRACT

Xylella fastidiosa (*Xf*) is a group of Gram negative, xylem limited and nutritionally fastidious plant pathogenic bacteria that cause diseases in many economically important plants. A single species, *fastidiosa*, with three subspecies, *fastidiosa*, *multiplex*, and *pauca,* have been described. Most *Xylella* strains were reported from North or South America. However, reports from other continents are emerging. For example, a *Xylella* strain was found in Taiwan causing pear leaf scorch (PLS) disease in the area where the low-chilling pear cultivar Hengshan (*Pyrus pyrifolia*) was grown. The current taxonomy of *Xf* is anchored around whole genome DNA-DNA hybridization (DDH) relativeness. DDH is a gold standard for bacterial species delineation. Yet, DDH is labor intensive and its use for analyses of multiple *Xf* strains is limited or even prohibitive. Thanks to the advancement of next generation sequencing (NGS), whole genome sequencing of *Xf* strains is feasible and sequences are accumulating. Average Nucleotide Index (ANI) calculated from whole genome sequence comparisons has been introduced for the replacement of DDH in bacterial taxonomy. The current version of GenBank sequence database has 17 whole genome sequences of *Xf* including a PLS strain, PLS229. In this study, ANI values were calculated from available whole genome sequences of *Xylella* strains and a phylogenic relative strain, *Xanthomonas campestris* pv. *campestris*. Substantiated by the analyses of 16S rRNA gene and 16S-23S intergenic transcribed spacer (ITS) sequences, ANI analyses have supported previous taxonomic establishment of grouping all American strains of *Xf* into a single species (ANI>95). ANI analyses also have further identified thresholds to define the three previously established subspecies. Lastly, ANI has delineated a proposed new species, *Xylella taiwanesis*, for the PLS strain from Taiwan.

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POPULATION GENETIC STRUCTURE OF *XYLELLA FASTIDIOSA* INFECTING COFFEE AND CITRUS TREES IN SYMPATRIC REGIONS OF SÃO PAULO STATE, BRAZIL

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ABSTRACT

Population-structure-based studies focusing on the systemic-colonizer, vector-dependent, multi-host plant pathogenic bacterium *Xylella fastidiosa* (*Xf*) have provided remarkable information about their ecology and biology. Here we studied the population structure of *Xf* infecting coffee trees in São Paulo State, Brazil, sampling from geographic regions sympatric to citrus plantations. Using 14 genomic microsatellite markers, we found that populations of *Xf* from coffee were similarly geographically structured as the sympatric populations of *Xf* from citrus, with the exception of the populations from central and northwestern (Ce - Tabatinga and Nw – Jales), which were in gene flow. Overall, the populations had a strong admixture component (38% of admixture individuals). Populations from Ce - Tabatinga also has contributed with the higher number of migrants while the population Nw from Jales has received most of the migrant genotypes. The coffee-associated *Xf* populations had also higher gene diversity and allelic richness than citrus-, typical of an evolutionarily older population. A predominant non-recombining reproductive system could not be ruled out for the four populations of coffee-associated *Xf.* Compared to sympatric populations of *Xf* from citrus no one admixture event was observed between citrus and coffee isolates, which reinforce that are two different strains. Cross inoculations assays shown no infection between both hosts. How particular ecological traits of the coffee-infecting *Xf* and the related agricultural practices could play a role in structuring the bacteria populations are discussed

HOST SELECTION AND ADAPTATION ARE MAJOR DRIVING FORCES SHAPING ALMOND LEAF SCORCH *XYLELLA FASTIDIOSA* POPULATIONS IN THE SAN JOAQUIN VALLEY OF CALIFORNIA

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ABSTRACT

Xylella fastidiosa (*Xf*) causes disease in many commercial crops including almond leaf scorch (ALS) disease in susceptible almond (*Prunus dulcis*). In this study, genetic diversity and population structure of *Xf* associated with ALS disease were evaluated. Strains isolated from two almond production sites in the San Joaquin Valley of California were analyzed with multiple locus DNA markers. The distribution of genotypes, combined with UPGMA and PCA analyses, identified two major genetic clusters that were associated with the cultivars 'Sonora' and 'Nonpareil,' regardless of the year of study or location. These relationships suggest that host selection and adaptation are major driving forces that are forming ALS *Xf* population structure in the San Joaquin Valley. This finding may provide insight into understanding pathogen adaptation and host selection in the context of ALS disease dynamics.

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OVEREXPRESSION OF THE *MQSR-XF* TOXIN FROM *XYLELLA FASTIDIOSA* TOXIN/ANTITOXIN SYSTEM INDUCES SESSILE GROWTH AND PERSISTER CELL FORMATION

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ABSTRACT

Bacterial toxin/antitoxin (TA) systems encode two genes located in the same operon - a stable protein toxin and an unstable molecule antitoxin that inhibits the toxin action. It has been recently observed by our team that, in *Xylella fastidiosa* (*Xf*), the causal agent of citrus variegated chlorosis, the formation of persister cells involves the expression of TA systems, being the *MqsR-Xf*/*MqsA-Xf* system the most induced under these conditions (Muranaka et al., 2012). The *MqsR*/*MqsA* TA system from *Escherichia coli*, which is homologous to *MqsR-Xf*/*MqsA-Xf*, is also the most induced loci in persister cells, and also has regulatory function. The aim of this work is to analyze the functional role of the *MqsR-Xf* toxin from the *Xf* TA system, since there is few information about the role of any of these systems in a plant pathogen bacterium. The toxin overexpression was made by cloning the *MqsR-Xf* gene under control of its native promoter in the pXF20 vector (*MqsR-Xf-XF* cells), which has been described as efficient to express and maintain the plasmid in *Xf* (Lee et al., 2010), and subsequent transformation by electroporation in *Xf* competent cells. Real time quantitative PCR (qPCR) was used to confirm the overexpression of the *MqsR-Xf* gene. The ability to form biofilm of the *MqsR-Xf-XF* and wild-type (WT) cells was evaluated by growing both in liquid PW medium in polysterene plates for 7, 10, and 15 days and subsequent staining of the biofilm cells with a solution of 1% crystal violet to measure the optical density (OD). To analyze the capability of cell-cell aggregation, both strains had their OD standardized and, every hour, for six hours, the OD of the supernatant was measured to verify their aggregation level. The cell movement (twitching motility) of *MqsR-Xf-XF* and WT cells was assessed by serial dilution of both and plating them on PWG medium containing three different BSA concentrations. After one month of growth, the isolated colonies were visualized in an OPT Medical 2 T magnifier in search of the characteristic fringe that forms in *Xf* cells that present twitching motility. To assess the gene regulation capability of *MqsR-Xf*, RNA from *MqsR-Xf-XF* and WT were extracted and submitted to qPCR using *gumB* (associated with biofilm formation), *fimA* (related to adhesion induction), *pilP*, *pilS* and *pilA* (type IV pili, which are associated with cell motility) and *eal* (involved in c-di-GMP degradation) genes. To check the relation of *MqsR-Xf* with persister cell formation, cells from *MqsR-Xf-XF* and WT were grown in liquid PW medium for 15 days, when copper sulfate was added to the culture in different concentrations. After an additional 24 hours of growth, cells were collected and plated on PWG medium to check the CFU growth on *MqsR-Xf-XF* compared with WT. RNA from these same conditions were extracted from *MqsR-Xf-XF* and WT and submitted to qPCR to evaluate the *MqsR-Xf* expression under different copper stress conditions. The phenotypic evaluation revealed that *MqsR-Xf-XF* cells showed significant more biofilm formation, as well as cell-cell aggregation, and less movement in relation to WT; and the qPCR results showed an upregulation of *gumB*, *fimA,* and *eal* and a downregulation of *pilP*, *pilS,* and *pilA*. The copper sulfate stress assays showed that *MqsR-Xf-XF* forms more persister cells than WT under stress conditions and that, within a certain limit, the *MqsR-Xf* expression is higher as the copper concentration (stress) increases. Taken together, the results suggest that *MqsR-Xf* is involved in persister cell formation under stress conditions and also has regulatory functions that induce biofilm formation.

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Section 4:

Pathogen and Disease Management

EXPLOITING A CHITINASE TO SUPPRESS *XYLELLA FASTIDIOSA* COLONIZATION OF PLANTS AND INSECTS

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ABSTRACT
We previously identified a chitinase (ChiA) while studying *Xylella fastidiosa* (*Xf*) sharpshooter interactions (Killiny et al., 2010). We have shown that *Xf* is able to use chitin as its sole carbon source *in vitro*, and that ChiA is required for both plant and insect colonization. Lastly, we have demonstrated that ChiA itself does not bind to its substrate and that other proteins are necessary for its enzymatic activity. In other words, all data available indicate that ChiA plays an essential role in *Xf* biology, but also that it is not biologically active by itself. We initially assumed that ChiA would only be associated with insect colonization; it was surprising to find out that a *chiA* mutant is completely unable to colonize plants. It is now clear that ChiA represents a unique target for control of both *Xf* colonization of the host and Pierce's disease spread. Disruption of ChiA activity should not only reduce virulence to plants, but also affect vector colonization. Furthermore, it is feasible that the same mechanism leading to within-plant blockage of ChiA activity may also block it in insects, effectively reducing Pierce's disease incidence in vineyards. Here, we will identify proteins associated with ChiA, identify substrates catalyzed by this enzyme, and demonstrate their role in plant and insect colonization. We propose that ChiA activity is a target that will lead to a control strategy affecting both plant and insect colonization by *Xf*, effectively reducing within- and between-plant spread of this pathogen.

LAYPERSON SUMMARY
We have shown that a chitinase (ChiA) plays a central role in the *Xylella fastidiosa* (*Xf*) transmission cycle. It is also essential for the successful colonization of both plant and insect hosts of *Xf*. However, there are several important questions related to the activity of this enzyme. First, it is not clear what substrates it cleaves, especially in plants. Second, ChiA is not active by itself, apparently requiring a partnership with substrate-binding proteins. We propose to characterize ChiA so that we can suppress its activity in both plants and insects.

OBJECTIVES
This project has three objectives:
1. Identify *X. fastidiosa* proteins or protein complexes that bind to ChiA and are required for its activity.
2. Screen potential substrates cleaved by ChiA.
3. Functionally demonstrate the role of ChiA partners during insect and plant colonization.

RESULTS AND DISCUSSION
The project is being initiated, there are no new results to report at this stage.

CONCLUSIONS
The project was just started, there are no conclusions available. The project will explore ChiA activity as a novel target that will lead to a control strategy affecting both plant and insect colonization by *Xf*, effectively reducing within- and between-plant spread of this pathogen.

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

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ABSTRACT
We are identifying grapevine-derived replacement components for both the surface recognition (SRD) and lytic (LD) domains of the NE-CB chimeric antimicrobial proteins that perform identical functions as these individual protein components. Using a novel computational tool CLASP, we found a good match for NE with the pathogenesis-related protein P14a from tomato and its conserved *Vitis* homolog. We focused on the version of this gene in *Vitis shuttleworthii* that we have designated VsP14a, as a good replacement for NE. Two plant expression vectors have been successfully constructed to express VsP14a by itself and a second vector that expresses as a CAP with CB (VsP14a-CB). The plant-expressed VsP14a protein clears *Xylella fastidiosa* (*Xf*) and inhibits growth of *Escherichia coli* (*Ec*). We have developed two new computational tools, PAGAL and SCALPEL that have been used successfully to identify portions of three *Vitis* proteins that could be used to replace the lytic peptide component CB. These have been designated; VvHAT52, VvISS15 and VvPPC20. Peptides corresponding to these proteins were synthesized and evaluated for their antimicrobial activity against *Xf* and *Ec*. Two of these, VvHAT22 and VvPPC20, displayed antimicrobial activity against *Xf* but not VvISS15. A binary vector to express VvHAT52 as a new CAP has been completed and has been designated VsP14a-VvHAT52. Once constructed the new grapevine CAP expressing vectors will be used for *Agrobacterium*-mediated transformation of grapevine rootstock and tobacco to confirm resistance to Pierce's disease using methods reported previously for NE-CB constructs (Dandekar et al., 2012).

LAYPERSON SUMMARY
We have engineered transgenic grapevines that can protect themselves from Pierce's disease by making a chimeric antimicrobial protein, NE-CB, that kills the causative agent, *Xylella fastidiosa* (*Xf*). We build on that success in this project by seeking to identify grapevine components that are similar in structure to the human neutrophil elastase (NE) or insect Cecropin B (CB) protein components. Since the 3D structural details of both NE and CB are known, we used recently developed computational tools (CLASP, PAGAL and SCALPEL) to identify structurally/functionally similar proteins from grapevine based upon specific structural features present in NE and CB. We have identified a grapevine P14a protein as a replacement for NE and two candidates that can replace CB (VvHAT52 and VvPPC20). We have tested these proteins and they can inhibit and/or kill *Xylella* cells. We are making the vectors to generate grapevine plants expressing these to grapevine proteins to confirm that they can provide 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 foster its ability to cause disease in grapevines (Chatterjee et al., 2008). Resistance to this pathogen must be multifaceted to block the pathogen at different stages of its complex lifestyle. A key issue is the reservoir of bacterial inoculum already present in California that poses an immediate threat in the presence of a significant insect vector like the glassy-winged sharpshooter (GWSS). Chemical pesticides are still used to suppress the GWSS population, which is effective but does not reduce this inoculum reservoir. Resistance mechanisms capable

of degrading the reservoir could prevent further spread of the disease. It is critical to know whether any resistance mechanism under consideration can clear *Xf* and if so, by what mechanism. The resistance mechanism must limit spread and movement of the bacterium *in planta* and block transmission of the disease by insect vectors. We have previously shown that *Xf* exposed to xylem fluid from resistant lines expressing 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. A surface-binding domain recognizes outer membrane proteins; we previously showed that it recognizes and cleaves mopB, a major *Xf* outer membrane protein (Dandekar et al., 2012). This surface-recognition domain is encoded by a synthetic gene derived from 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 (CB), 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 surface of *Xf* via its mopB outer membrane protein, bringing the cecropin B 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 and CB proteins derived from plants, ideally from grapevine, would be less controversial.

OBJECTIVES

The goal of this project is to redesign our existing therapeutic NE-CB CAP, replacing the human NE and insect CB domains with plant/grapevine orthologs, and to validate the efficacy of the new CAP components in providing resistance to Pierce's disease in grapevine. We are now following the goals, objectives, and activities as proposed in the revised proposal submitted last year (2013) and approved for two years.

Goal: Redesign the NE-CB chimeric antimicrobial with a plant elastase and plant-derived lytic domain and test its efficacy to combat Pierce's disease in transgenic tobacco and grapevines.

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

- Activity 1. Identify a suitable plant elastase candidate that is comparable to human neutrophil elastase in active site structure using the CLASP computational tool.
- Activity 2. Construct vectors and test the *in planta*-produced protein for efficacy in killing *Xf* in culture.
- Activity 3. *In planta* efficacy testing: construct binary vectors (PE-CB), transform grapevine and tobacco, and test transgenic tobacco and grapevine for clearance of *Xf* and resistance to Pierce's disease symptoms.

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

- Activity 4. Identify a suitable PLD candidate that is comparable to insect-derived Cecropin B in primary and secondary structure using CLASP and other computational tools.
- Activity 5. Test the synthetic PLD protein for efficacy in killing *Xf* in culture.
- Activity 6. *In planta* testing of the efficacy of grape-derived CAP components using transient expression.

Objective 3. Construct and test a fully plant-derived CAP (PE-PLD) and test its ability to confer resistance to Pierce's disease in grapevine rootstocks.

- Activity 7. Construct a PE-PLD binary vector, transform grapevine and tobacco, and evaluate *Xf* resistance and Pierce's disease development.

RESULTS AND DISCUSSION

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

Since the CAP components work synergistically we will replace them one at a time, maintaining the other original component. In this time period, we focused our efforts in replacing the human neutrophil elastase (NE) with a plant/grapevine version of NE (PE). To do this we sought an appropriate protein in plants and possibly more appropriately in grapevine that had the same activity as NE.

Activity 1. Identify a suitable plant elastase candidate that is comparable to human neutrophil elastase in active site structure using the CLASP computational tool.
This activity has been successfully accomplished. A plant PE candidate protein was identified using the CLASP package (Chakraborty et al., 2011, http://www.sanchak.com/clasp/). Details of the protocol and workflow used to make this selection have been described (Chakraborty et al., 2013). The P14a from *Solanum lycopersicum* (tomato) was the protein of choice since we found similar, highly conserved proteins from other plant species, including grapevine. Additionally, the PR superfamily is widely distributed in animals, plants, and fungi. From several matching criteria, we chose the P14a from *Vitis shuttleworthii* (*Vs*), as this species is resistant to Pierce's disease (Walker, personal communication). More recently, 21 different PR-1 genes from grapevine, including those from *Vs*, were characterized and shown to confer resistance to bacterial disease (Li et al., 2011).

Activity 2. Construct vectors and test *in planta*-produced protein for efficacy in killing *Xf* in culture.
To test the efficacy of *Vs*P14a in clearing *Xf,* we codon-optimized and chemically synthesized *Vs*P14a after adding a 3xFlag purification tag (Sigma Aldrich) and cloned it into the expression vector pEAQ-HT and into a binary vector for transient protein expression in tobacco (*N. benthamiana;* Sainsbury et al., 2009). Total protein was extracted using an apoplastic wash method and tested for the ability inhibit growth of *Escherichia coli* and *Xf*. After four hours, the *E. coli* with the extract from the plant expressing the empty vector showed growth, while growth of those exposed to *Vs*P14 was completely inhibited. *Vs*P14a also inhibited the growth of *Xf* while protein obtained from the empty vector did not. By 50 minutes, 50% of the population was killed, but mortality reached a plateau at ~ 60%. These results are encouraging and indicate that we have found the desired replacement for NE.

Activity 3. *In planta* efficacy testing: construct binary vectors (PE-CB), transform grapevine and tobacco, and test transgenic tobacco and grapevine for clearance of *Xf* and resistance to Pierce's disease symptoms.
We have completed the construction of two binary vectors, one for expressing *Vs*P14a by itself (**Figure 1**) and another which links the *Vs*P14a sequence to CB. This recreates the original CAP protein, but with the SRD domain replaced by the P14a protein (**Figure 1**). In the first construct, the P14a coding sequence was fused to a signal peptide from the Ramy3D protein which is cleaved upon expression in the plant. Next to the signal peptide cleavage site and at the N-terminal of P14a is a 3XFLAG sequence to improve the immune detection of the P14a protein. In the second construct, the coding sequence is fused to the CB sequence via the four-amino acid flexible linker sequence used in the original CAP design (Dandekar et al., 2012). Both coding regions have a TMV omega sequence in the 5' non-coding region to improve translation efficiency. The regulatory sequence in both constructs is a double CaMV35S promoter sequence. The binary vectors were introduced into a disarmed *Agrobacterium* strain to reconstitute a functional plant transformation system. Both vectors are being used to transform grapevine rootstock and SR1 tobacco to evaluate the efficacy of these two constructs.

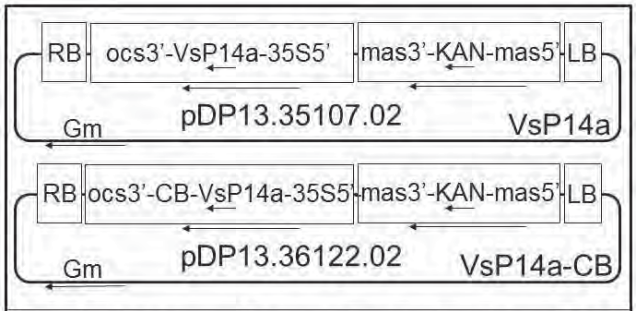

Fig. 1: Binary vector constructs developed for *in planta* efficacy testing of VsP14a based CAP proteins in tobacco and grapevine plants

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

The goal of this objective is to identify a CB-like protein in plants to replace the lytic domain of CAP described earlier (Dandekar et al., 2012). Unlike the search for the HNE, where the focus was the congruence of the active site (spatial and electrostatic), a similar approach cannot be used for CB as it has no enzymatic function and thus no active site. The approach for finding a CB-like protein in plants focused instead on the highly structured nature of CB.

Activity 4. Identify a suitable PLD candidate that is comparable to insect-derived Cecropin B in primary and secondary structure using CLASP and other computational tools.

Initially, we used an approach similar to that described above in Activity 1 to identify a replacement component for CB. However, instead of comparing the reactive atoms as was done for the NE matching algorithm, we sought matches for the Cα atoms of the four Lys residues, Lys10, Lys11, Lys16, and Lys29, as the input motif from CB (PDBid:2IGR), allowing Lys to matched by Lys, Arg, or His. As indicated in our last report, this strategy revealed several good candidate proteins. We are now focusing on VvHAT52, a 52-amino acid segment of the HAT protein from *Vitis vinifera*, whose structure matches very well with the CB. However, we have refined our approach and focused instead on the alpha-helical structure itself to generate a greater diversity of candidates for our previously described CAP (Dandekar et al., 2012). To better understand the functionality of the alpha-helical domains of CB, we have developed two new computational tools, PAGAL (Chakraborty et al., 2014) and SCALPEL, to better predict antimicrobial activities in portions of existing proteins. PAGAL (**P**roperties **a**nd corresponding **g**raphics of **al**pha helical structures in proteins) is open source software that implements previously known and established methods of evaluating the properties of alpha-helical structures, providing very useful information of the amphipathicity, hydrophobicity and charge moments within these structures. A key feature of lytic peptides is the distribution of hydrophobic and charged residues on the surface of the protein. To find proteins like cecropin that have an alpha-helical structure, we used PAGAL to evaluate CB. CB contains two alpha helixes (AHs) joined by a short stretch of random coil. **Figures 2A and B** show the Edmundson wheel and hydrophobic moment of the two AHs. The N-terminal AH has a large hydrophobic moment and a specific positive charge distribution. This can also be seen in a Pymol rendering of the peptide surface (**Figure 3**). The Pymol script for this rendering is automatically generated by PAGAL. On the other hand, the C-terminal AH of CB has neither of the above two properties.

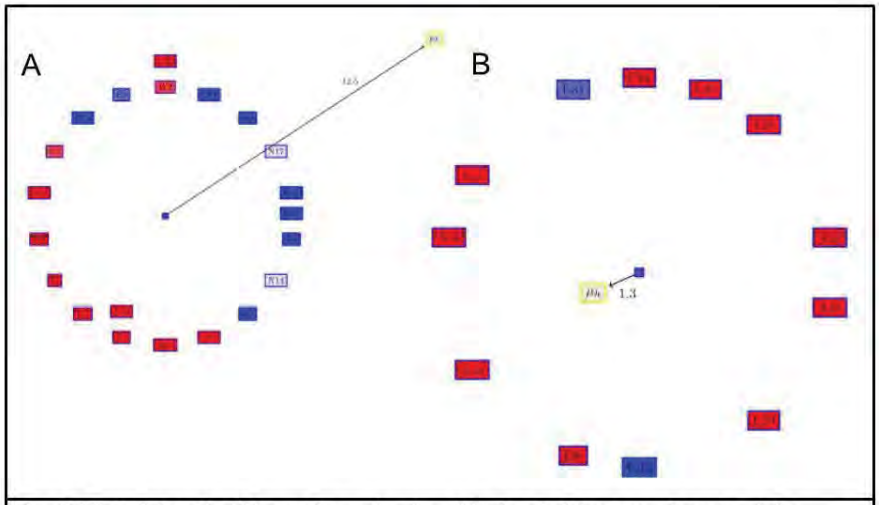

Fig. 2: Visualizing the Edmundson wheel and hydrophobic moment some alpha helices: The color coding is as follows: all hydrophobic residues are colored red, while hydrophilic residues are colored blue - dark blue for positively charged residues, medium blue for negatively charged residues and light blue for amides. (A) N-Terminal helix of CB. (B) C-Terminal helix of CB.

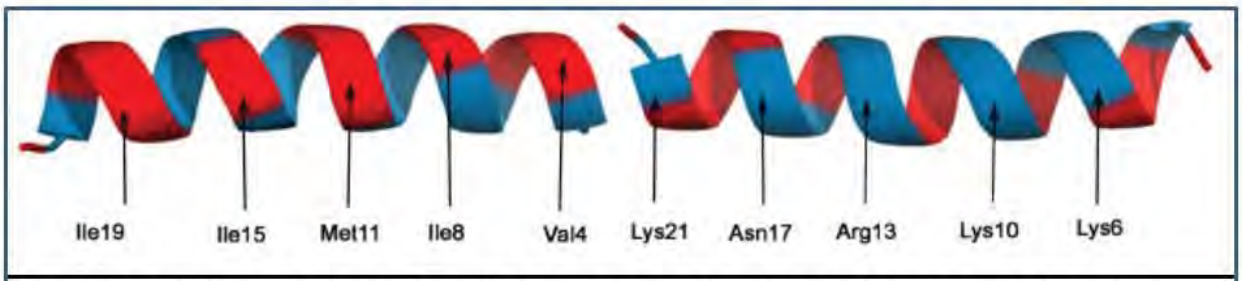

Fig. 3: Pymol rendering of peptides showing the hydrophobic and charged surfaces for the N-terminal helix of CB. All hydrophobic residues are colored red, while hydrophilic residues are colored blue.

We then developed the second program, SCALPEL to search for alpha-helical structures of a particular type. We searched for the smallest peptide with a large hydrophobic moment and a high proportion of positively charged residues on the hydrophilic side. Here, we identified a 20-aa region of the protein PPC from *Vitis vinif*era, a key enzyme in the C4-photosynthetic carbon cycle from grapevine that we call PPC20 (**Figure 4A**). We also searched for the smallest peptide with a large hydrophobic moment and a high proportion of negatively charged residues on the hydrophilic side and identified a 15-aa region of isoprene synthase from grapevine that we call ISS15 (**Figure 4B**). Both of these proteins have a very large hydrophobic moment. **Figure 5A** is a PYMOL rendering that shows the highly hydrophobic surface of PPC20 and **Figure 5B** clearly shows the positively charged surface, with the exception of a single Asp that is the only negative residue in a positive surface. **Figure 5C** shows a PYMOL rendering of the highly hydrophobic protein surface of ISS15 and in **Figure 5D** one can clearly see the negatively charged surface.

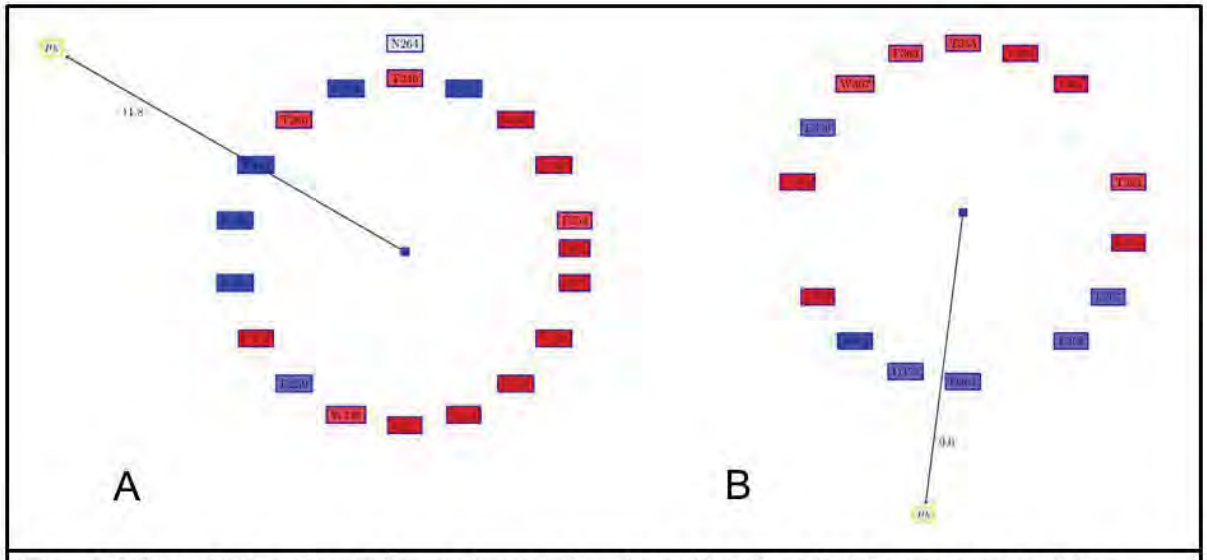

Fig. 4: Visualizing the Edmundson wheel and hydrophobic moment of two Vitis alpha helices: The color coding is as follows: all hydrophobic residues are colored red, while hydrophilic residues are colored blue - dark blue for positively charged residues, medium blue for negatively charged residues and light blue for amides. (A) The 20 aa residue PPC20 with positively charged residues. (B) 15 aa residue ISS15 with negatively charged residues.

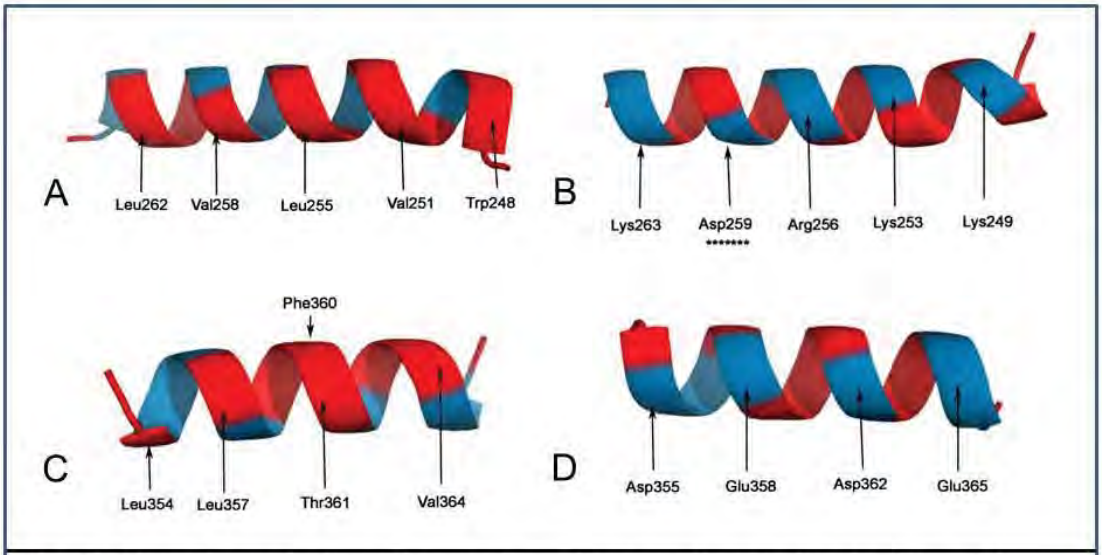

Fig. 5: Pymol rendering of peptides showing the hydrophobic and charged surfaces for the two potential lytic peptides extracted from grapevine genes. All hydrophobic residues are colored red, while hydrophilic residues are colored blue. (A) hydrophobic surface for PPC20 (B) charged surface for PPC20 (C) hydrophobic surface for ISS15 (D) charged surface for ISS15.

Activity 5. Test synthetic PLD protein for efficacy in killing *Xf* in culture.
Using our recently developed bioinformatics tools PAGAL and SCALPEL, we have conducted our phase 1 search and identified three potential grapevine candidate proteins that could replace the CecB lytic peptide domain of our previously described chimeric antimicrobial protein (CAP; Dandekar et al., 2012 PNAS 109(10): 3721-3725). Using the same tools, we have further refined our search within these particular proteins to identify a smaller segment that was then tested for antimicrobial activity after chemical synthesis of each of the protein candidates. The following grapevine proteins were chemically synthesized: a 22-aa version of HAT (VvHAT22; a 52-aa segment of this protein was previously identified), a 15-aa segment of ISS (VvISS15), and 20-aa segment of PPC (VvPPC20). These proteins were successfully tested for antimicrobial activity using to the Temecula strain of *Xf*. Using the same search criteria, the search flagged a 22-aa N-terminal segment of the 34-aa Cecropin B (CBNT22) protein and a 12-aa segment of cathaylecitin (CATH15); these proteins with known antimicrobial activity served as a positive control for our bioassays. VvHAT22 and VvPPC22 inhibited *Xf* growth at levels comparable to CBNT22 and CATH15; however, VvISS15 displayed no detectable antimicrobial activity.

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Activity 6. Conduct *in planta* efficacy testing of the grape-derived CAP components using transient expression.
We have successfully constructed a binary vector to express *Vs*P14a-PLD and test the efficacy of the 52-aa H+ ATPase sequence described above (**Figure 6**). This must be incorporated into an *Agrobacterium* host to create a function transformation system for transient expression in tobacco and for the transformation of grapevine (**Activity 7**).

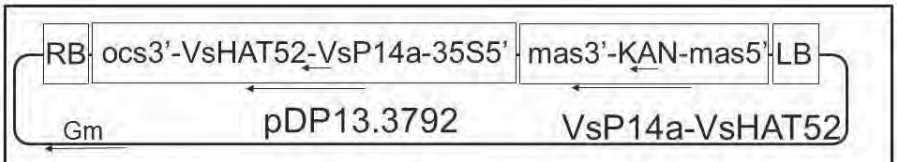

Fig. 6: Binary vector constructs developed for *in planta* efficacy testing of VsP14a-VsHAT52 CAP protein in tobacco and grapevine plants

CONCLUSIONS

Using novel computational tools CLASP, PAGAL, and SCALPEL, we successfully identified grapevine proteins that can be used to replace the NE and CB components in a CAP design to provide resistance to Pierce's disease. The *Vs*P14a protein replacement for NE was expressed in plants. This protein was isolated and shown to cause lysis of *Xf*. We have identified two potential grapevine protein candidates, VvHAT22 and VvPPC20, which can lyse *Xf* at levels comparable to that observed for CB. We have begun designing vectors to test combinations of these two proteins to confer resistance in transgenic grapevine and tobacco.

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

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Reporting Period: The results reported here are from work conducted March 2010 to September 2014.

ABSTRACT

We successfully established two field trials to validate two greenhouse-tested strategies to control the movement and clearance of *Xylella fastidiosa* (*Xf*). *Xf* is a xylem-limited, Gram-negative bacterium and is the causative agent of Pierce's disease. Key to the virulence of *Xf* is its ability to digest pectin-rich pit pore membranes that interconnect the host plant's xylem elements. This action enhances long-distance movement and vector transmission. Our first strategy evaluated the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) derived from pear to counteract *Xf* virulence associated with PG activity. Our second strategy enhanced clearance of bacteria from *Xf*-infected xylem tissues using a chimeric antimicrobial protein, NE-CB. Expressing these proteins is expected to 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 NE-CB, on their own roots or as rootstocks grafted with untransformed Thompson Seedless (TS) scions, were planted together with untransformed controls in two locations in Riverside and Solano counties. Planting was completed in 2011, with 220 vines at each location. The transgenic vines were evaluated phenotypically using the first 12 descriptors from the "Primary descriptor priority list" proposed by the International Organization of Vine and Wine (OIV). No phenotypic or horticultural differences were observed between transgenic and untransformed TS vines. NE-CB- and PGIP-expressing transgenic grapevine lines in Solano County were genotyped, confirming the presence of the inserted transgene in all lines.

In Riverside County, the plants were naturally infected by wild populations of the glassy-winged sharpshooter (GWSS). Presence of *Xf* in petiole extracts was confirmed by ELISA and plate cell count in 2011, in xylem sap, petiole and stem extracts in 2012, and in stem extracts in 2013. In these years, Pierce's disease symptoms were assessed using a standardized score based on percentage of leaf area scorching. In 2013, we used a 0-4 scale to evaluate grapevine vigor. During spring 2014, we observed that all 220 transgenic and control vines were in decline. Symptoms of root knot nematode infection were present, like suppressed shoot growth, limited root zone, and gall formation in roots. Eighteen soil samples were collected and all were positive for root knot and citrus nematodes. Roots taken had four knots per inch, in contrast to the normal one knot or gall per four inches. Nematode population data in soil and root samples confirmed a heavy root knot nematode infection. The Riverside site was previously planted with Chardonnay and the vineyard was removed and replanted without fumigation. From this, we surmised that the field location had selected for an aggressive population of root knot nematode during the previous planting. Our vines subsequently suffered lethal root knot infection soon after they

were planted. Root knot infection at the Riverside County site will not allow us to evaluate our transgenic grapevines for Pierce's disease resistance or susceptibility.

At the Solano County site, half of the ungrafted vines were mechanically inoculated with *Xf* in 2011 and again in 2012 to validate resistance to Pierce's disease under field conditions. *Xf* presence was confirmed by ELISA in 2011. However, neither *Xf* growth in plates nor Pierce's disease symptoms were detected. Half of the Solano County grafted plants were inoculated with *Xf* for the first time in 2012. Leaf scorching, a characteristic symptom of Pierce's disease, was observed in Solano Country for the first time in fall 2012. *Xf* presence was in petiole extracts and confirmed by ELISA that season; its presence was also detected in stem extracts from own-rooted and grafted lines in 2013. Ungrafted and grafted grapevines not previously inoculated at the Solano site were manually inoculated in 2013, completing the inoculations at this site. In previous years, Pierce's disease symptoms were assessed using a standardized score based on percentage leaf area scorching, cane survival, and grapevine vigor. On May 28, 2014, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, four current-year canes from all grafted transgenic and control plants at the Solano site were mechanically inoculated with *Xf*. Severity or absence of Pierce's disease symptoms in ungrafted and grafted transgenic grapevines inoculated in 2011, 2012, and 2013 and the individual transgenic grafted canes inoculated in 2014 were rated for the absence or severity of Pierce's disease using the Pierce's disease symptom severity rating system 0-5 in summer 2014. In the same season, one cane per grafted plant was harvested for *Xf* quantification; the results are pending.

In January 2014, the USDA APHIS permit holder for the Solano and Riverside fields was changed from Professor Alan Bennett to Professor Abhaya Dandekar; the permit end date is April 1, 2016. Personnel from the Dandekar laboratory are maintaining regulatory oversight of the field trials. Timely reporting and inspections are conducted to maintain compliance with USDA APHIS.

LAYPERSON SUMMARY

Four hundred and forty (440) transgenic grapevines expressing either polygalacturonase-inhibiting protein (PGIP; 192 plants) or a chimeric antimicrobial protein (NE-CB; 192), and 56 untransformed control vines were planted in two locations: Riverside County (220 plants) and Solano County (220 plants). Half of the transgenic grapevines are being evaluated as plants on their own roots and half as rootstocks grafted with untransformed Thompson Seedless (TS) scions to demonstrate the field efficacy of two strategies to control Pierce's disease in California grapevines. The first uses transgenic rootstocks, through expression of PGIP, to control the movement of the bacterium *Xylella fastidiosa (Xf)* in water-conducting xylem. The second strategy tests whether transgenic rootstocks can clear *Xf* infections in xylem tissue by expressing NE-CB.

At the Riverside County site, natural *Xf* infection was confirmed in petioles, stems, and xylem sap by ELISA, and infections appeared uniform through 2013. During spring 2014, all 220 transgenic and control vines at the Riverside site were in decline; most had no new growth. Growth that did occur did not correlate with genotype; transgenic and control plants were equally affected. The root zone was limited to the first six inches of soil and was heavily infected with root knot nematodes. The Riverside site was previously planted with Chardonnay and the vineyard was removed and replanted without fumigation. The field location had selected for an aggressive population of root knot nematode during the previous planting. Our vines subsequently suffered lethal infection soon after they were planted. Nematode population data and number of knots per inch of root obtained from 18 soil and root samples confirm a heavy root knot nematode infection. Evaluating the resistance or susceptibility of our transgenic grapevines to Pierce's disease under field conditions will not be possible due to the combined root knot and Pierce's disease infections.

At the Solano County site, about 25% of the plants were mechanically inoculated in 2011 and again in 2012. Another 25% were inoculated in 2012 and the remaining 50% in 2013. The presence of *Xf* was confirmed in petiole and stem extracts using the ELISA assay. In addition, we evaluated Pierce's disease symptoms, cane survival, and grapevine vigor and found that some transgenic lines from each strategy consistently scored better than the control and others did not. Lines that show resistance can transmit their resistance from the rootstock to the wild scion. However, resistance transmitted from transformed rootstock is weaker than that achieved in a transformed plant. On May 28, 2014, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, four current-year canes from all grafted transgenic and control plants at the Solano site were mechanically inoculated with *Xf*. Severity or absence of Pierce's disease symptoms

was evaluated in summer 2014 for all inoculated canes using a Pierce's disease symptom severity rating system 0-5, where 0 = healthy vine, all leaves green with no scorching; 1= first symptoms of disease, light leaf scorching on one or two leaves; 2 = about half the leaves on the cane show scorching; 3 = the majority of the of the cane shows scorching; 4 = the whole cane is sick and declining and 5 = the cane is dead. In the same season, one cane per grafted plant was harvested for *Xf* quantification; results are pending.

The current Solano and Riverside field permit was changed from Professor Alan Bennett to Professor Abhaya Dandekar in January 2014. The USDA APHIS permit end date is April 1, 2016. Personnel from the Dandekar laboratory are maintaining regulatory oversight of the field trials. Timely reporting and inspections are conducted to maintain compliance with USDA APHIS.

INTRODUCTION

Thompson Seedless (TS, *Vitis vinifera*) grapevines were transformed with a gene that encodes a chimeric anti-microbial therapeutic protein with a recognition domain from a neutrophil elastase (NE) and the lytic domain Cecropin B (CB). The NE domain specifically binds to the *Xylella fastidiosa* (*Xf*) outer-membrane protein MopB, while the CB domain clears *Xf,* the causative agent for Pierce's disease (Dandekar et al., 2012). We also transformed TS grapevines with a gene encoding polygalacturonase-inhibiting protein (PGIP). PGIP expression in transformed plants inhibits the action of polygalacturonase (PG), a virulence factor expressed by *Xf*. Inhibiting PG interferes with long distance movement of *Xf,* providing resistance to Pierce's disease (Agüero et al., 2005). Transgenic grapevines expressing NE-CB and different PGIP constructs were first tested under greenhouse conditions. Several lines that showed resistance to Pierce's disease were identified by mechanically inoculating plants with *Xf* (Dandekar et al., 2012). Selected transgenic grapevines expressing either NE-CB or PGIP, own-rooted or grafted with nontrangenic TS, were planted in 2010-11 in Riverside and Solano counties.

At the site in Riverside County, which has natural Pierce's disease pressure, plants were naturally infected. From 2011 to 2013, severity or absence of Pierce's disease symptoms and grapevine vigor was assessed. *Xf* was detected in xylem sap, petiole, and stem extracts by ELISA and plating. During spring 2014, all 220 Thompson Seedless transgenic and control vines were in decline and presented symptoms of root knot nematode infection like suppressed shoot growth, limited root zone, and gall formation in roots. The Riverside site was previously planted with Chardonnay and the vineyard was removed and replanted without fumigation. The field location had selected for an aggressive population of root knot nematode during the previous planting. Our vines subsequently suffered lethal root knot infection soon after planting. Eighteen soil samples were collected and all were positive for root knot and citrus nematodes. Roots had four knots per inch, instead of the normal one knot or gall per four inches. Nematode population data in soil and root samples confirmed root knot nematode infection. Root knot infection at the Riverside County site will not allow us to evaluate our transgenic grapevines for Pierce's disease resistance or susceptibility.

At our second site in Solano County, half of the ungrafted transgenic grapevine lines were manually inoculated as described (Almeida et al., 2003) in July 2011 and May 2012, when half of the grafted transgenic grapevine lines were inoculated. Ungrafted and grafted grapevines not inoculated during 2011 and 2012 were inoculated on June 2013, completing the inoculation of all grapevines at the Solano site. *Xf* was detected in petiole and stem extracts using ELISA assays. Pierce's disease symptoms were scored based on leaf area scorching, grapevine vigor, and cane survival. On May 28, 2014, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, at least four current-year canes from all grafted transgenic and control plants at this site were mechanically inoculated with *Xf*. Severity or absence of Pierce's disease symptoms was recorded in summer 2014 for all inoculated canes using the Pierce's disease symptom severity rating system 0-5, where 0 = healthy vine, all leaves green with no scorching; 1= first symptoms of disease, light leaf scorching on one or two leaves; 2 = about half the leaves on the cane show scorching; 3 = the majority of the of the cane shows scorching; 4 = the whole cane is sick and is declining and 5 = the cane is dead.. One cane per grafted plant was harvested in summer 2014 for *Xf* quantification by qPCR; results are pending.

OBJECTIVES

The goals of this project are to finish field-testing four NE-CB and four PGIP transgenic grapevine clones by evaluating their horticultural characteristics and resistance to Pierce's disease. Transgenic grapevines were tested in two field locations as ungrafted plants and as transgenic rootstocks grafted with wild-type scion. One field

location has Pierce's disease pressure and plants were naturally infected with *Xf*. In another location with no Pierce's disease pressure, grapevines were mechanically inoculated with *Xf*.

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

Activity 1. Propagation, field planting, and grafting of NE-CB and PGIP transgenic grapevines.

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

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

Objective 2. Assume permit holder status for existing USDA APHIS field permit 12-340-102r and maintain regulatory oversight and compliance with permit reporting requirements.

Activity 4. Participate with PIPRA during transition and assume permit holder status.

Activity 5: Maintain regulatory oversight of both field locations and compliance with reporting requirements.

Activity 6. Maintain active regulatory compliance inspections.

RESULTS AND DISCUSSION

Activity 1. Propagation, field planting, and grafting of NE-CB and PGIP transgenic grapevines.

Four independent transgenic events expressing NE-CB (40-41, 40-89, 40-92, and 41-151) and four expressing different PGIP constructs (31-25, 45-77, 52-08, and TS50) were planted at two locations. Initial planting of 210 transgenic or untransformed vines, own-rooted or grafted with untransformed TS scions, was completed in Riverside County on May 18, 2010. Ten more were planted on March 6, 2011, completing the plantings at this location **(Table 1)**. We also planted 110 transgenic and untransformed vines on their own roots on August 2, 2010 and 110 vines grafted with untransformed TS scions on June 27, 2011 in Solano County, completing the planting at this second location. Genotyping of NE-CB- and PGIP-expressing transgenic grapevine lines in Solano County has confirmed the presence of the inserted transgene in all lines.

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

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

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

To verify that horticultural and varietal characteristics of the parental genotype were unchanged, NE-CB- and PGIP-expressing transgenic lines were evaluated phenotypically in Solano County in September 2011 and in Riverside County in November 2011. This examination was accomplished using the first 12 descriptors from the "Primary descriptor priority list" proposed by the International Organization of Vine and Wine (OIV, 1983). The descriptors used were 1) aperture of young shoot tip/opening of young shoot tip, 2) density of prostrate hairs between main veins on 4th leaf lower side of blade, 3) number of consecutive shoot tendrils, 4) color of upper side of blade on 4th young leaf, 5) shape of mature leaf blades, 6) number of lobes on mature leaf, 7) area of anthocyanin coloration on main veins on upper side of mature leaf blades, 8) shape of teeth on mature leaves, 9) degree of opening of mature leaves/overlapping of petiole sinuses, 10) mature leaf petiole sinus bases limited by

veins, 11) density of prostrate hairs between main veins on lower side of mature leaf blades, and 12) density of erect hairs on main veins on lower sides of mature leaf blades. NE-CB and PGIP-expressing transgenic lines at the Riverside and Solano sites were also phenotypically evaluated in fall 2012 and 2013. No differences between transgenic and parental TS grapevines were observed.

Activity 3. Evaluate PD resistance of NE-CB and PGIP transgenic grapevines after inoculation with *Xf*. At the Riverside County site, grafted and ungrafted transgenic grapevine lines naturally infected in the field were scored for Pierce's disease symptoms for the last time in May 2013. Stem samples harvested from grapevines at this location and date were assayed for *Xf* cell counts by ELISA, with a standard curve created using *Xf* from liquid culture. Pierce's disease symptoms were rarely observed, but ELISA cell counts confirmed *Xf* infection. Unexpectedly, during the 2014 spring season all 220 Thompson Seedless vines planted at Riverside were in decline; most had no new growth (**Figure 1**). Growth that did occur did not correlate with genotype: transgenic and control plants were equally affected. We dug up the soil about 18 inches from the trunk and observed that the root zone was limited to the first six inches of soil and was heavily infected with root knot and citrus nematodes. Consulting with Andrew Walker, Howard Ferris, and Michael McKenry led us to a preliminary root-knot diagnosis.

Figure 1. Riverside County transgenic grapevine field trial, fall 2014.

Eighteen root and soil samples were taken and all were positive for root knot and citrus nematodes (**Table 2, Figures 2 and 3**); root knot nematode population data confirmed infection. Roots sampled had four knots per inch, in contrast to the normal one knot or gall per four inches (**Figure 4**). The Riverside site was previously planted with Chardonnay and the vineyard was removed and replanted without fumigation. The field location was most likely selected for an aggressive population of root knot nematode during the previous grapevine planting. Our vines subsequently suffered lethal infection soon after planting; physiology was disrupted, root growth stopped, and gall development likely happened one or two days after root knot nematodes penetrated the young root. Heavy nematode feeding likely resulted in root leakage in the first year after planting. Root knot infection symptoms include suppressed shoot growth, decreased shoot-root ratio, nutritional deficiencies showing chlorosis in the foliage, and poor plant yield (Kassen and Moens 2006). Evaluation of Pierce's disease resistance or suceptibility of trangenic grapevines under Riverside County field conditions will not be possible, given the combination of symptoms for root knot and *Xf* infections.

Table 2. Root knot and citrus nematode counts found in Riverside County site soil samples (per 300 cc soil sample).

Location row-vine	Line ID	Genotype	Root knot count	Citrus nematode count
1-2	40-41	NE-CB	1,260	1,920
1-5	31-25	PGIP	3,000	12,000
2-22	31-25	PGIP	30	1,700
3-14	45-77G	PGIP	1,000	2,000
3-16	TS50	PGIP	2,100	10,000
4-5	40-41G	NE-CB	800	8,000
4-10	52-08	PGIP	30	200
4-15	40-89G	NE-CB	2,840	5,460
4-20	41-151G	NE-CB	900	4,500
5-2	TS	WT control	1,800	3,300
5-5	45-77G	PGIP	954	1,700
5-12	TS-G	WT control	870	4,850
5-19	40-41	NE-CB	870	3,300
5-24	45-77	PGIP	1,250	2,830
8-4	45-77G	PGIP	4,750	11,200
8-10	40-41G	NE-CB	4,200	11,880
9-3	TS50	PGIP	5,850	8,250
9-6	40-89	NE-CB	1,400	2,700

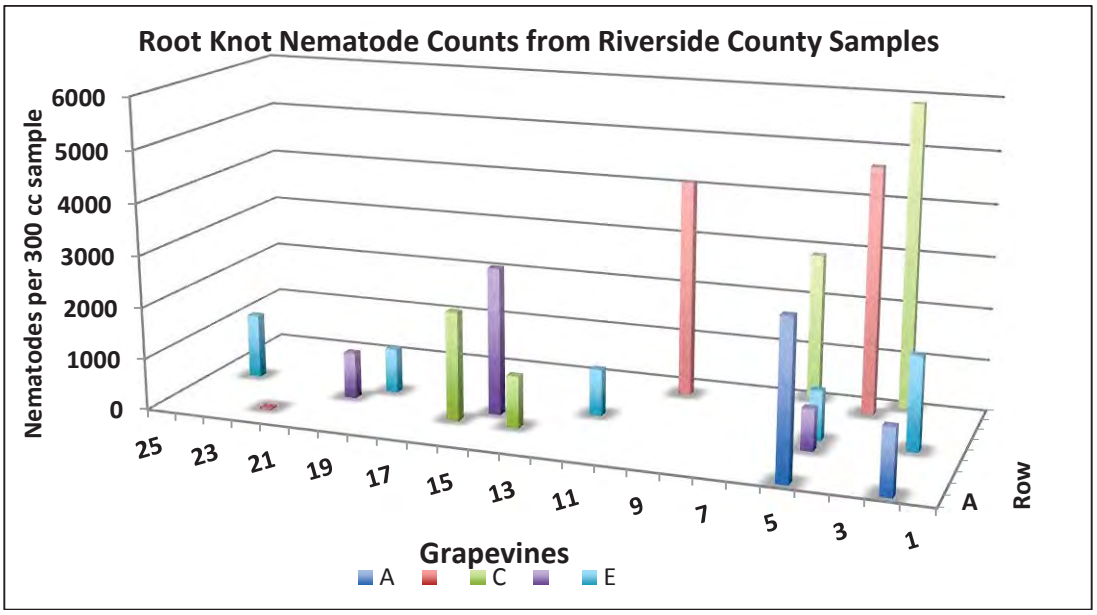

Figure 2. Root knot nematode counts from Riverside County field soil samples (summer 2014).

At the Solano County site (**Figure 5**), half of the ungrafted transgenic lines were manually inoculated as described (Almeida et al., 2003) on July 13, 2011 and half on May 29, 2012. Half of the grafted transgenic lines were manually inoculated on the latter date. Ungrafted and grafted grapevines at the Solano site that were not previously inoculated were manually inoculated on June 17, 2013, completing the inoculations of all grapevines at this location. Cane survival for inoculated runners of ungrafted and grafted transgenic grapevines was scored using a 0−1 scale, where 0 = alive and 1 = dead. Vigor for Solano inoculated transgenic and controls grapevines was scored using a 0−4 scale, where 0 = healthy, no Pierce's disease symptoms; 1 = a few leaves on a few shoots on cane(s) with symptoms, 2 = many symptomatic leaves on multiple canes (in a mature bilateral cordon trained vine); 3 = dieback/death of canes/codons; and 4 = death of whole vine. On May 28, 2014, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, at least

four current-year canes from all grafted transgenic and control plants at this site were mechanically inoculated with *Xf* (**Table 3**).

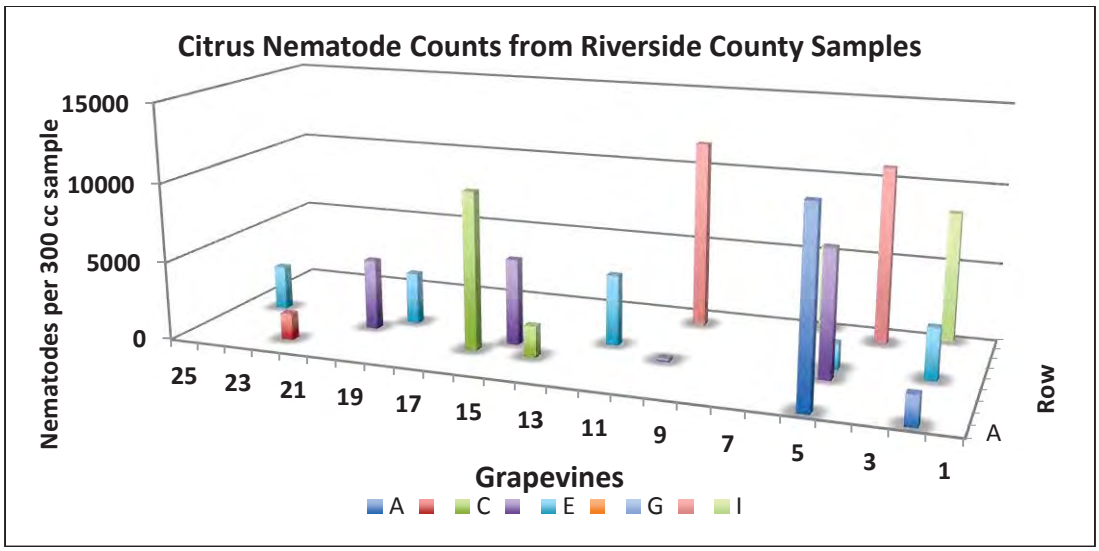

Figure 3. Citrus nematode counts from Riverside County field soil samples (summer 2014).

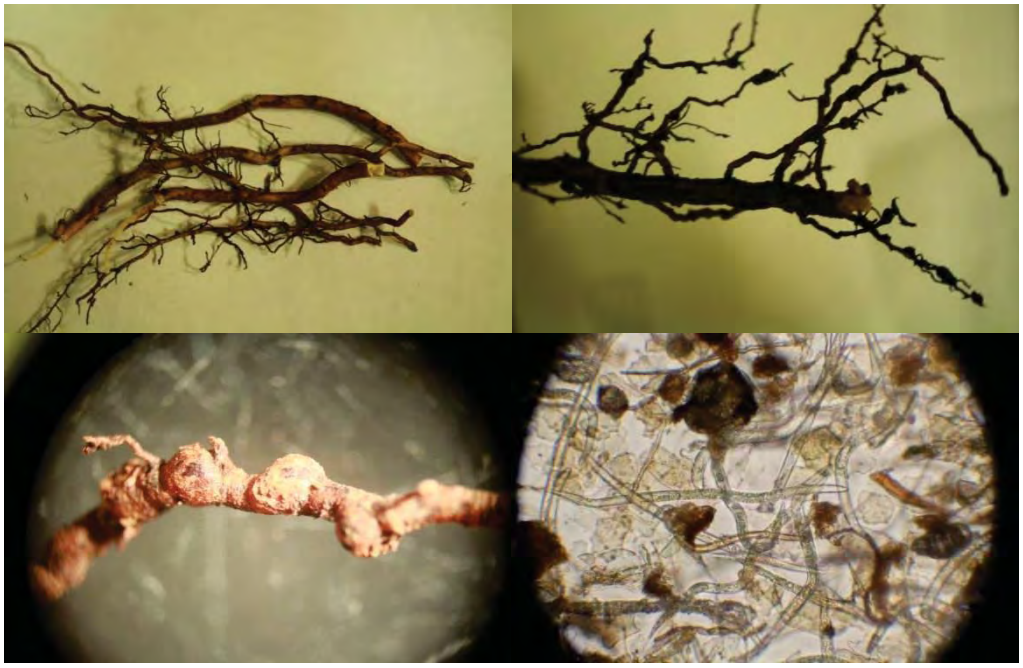

Figure 4. Healthy grapevine root (upper left), root knot infected roots with knots (upper right), microscopic view of grape root knots (lower left) and root knot and citrus nematodes (lower right).

Severity or absence of Pierce's disease symptoms for all Solano County ungrafted (**Figures 6 and 7**) and grafted (**Figures 8 and 9**) grapevines inoculated in 2011, 2012, 2013, and 2014 inoculated grafted canes (**Figure 10**) was assessed on July 22, 2014, using a Pierce's disease symptom severity rating system 0-5, where 0 = healthy vine, all leaves green with no scorching; 1= first symptoms of disease, light leaf scorching on one or two leaves; 2 = about half the leaves on the cane show scorching; 3 = the majority of the of the cane shows scorching; 4 = the whole cane is sick and is declining and 5 = the cane is dead. On July 22, 2014, the absence or severity of Pierce's disease was rated for all four canes. Pierce's disease symptoms are present in ungrafted and grafted grapevines inoculated in 2011, 2012, 2013, and in 2014 inoculated grafted canes, but the severity of the symptoms is lower in some ungrafted and grafted transgenic lines from each strategy than in untransformed controls.

Figure 6. Solano County transgenic grapevines inoculated in Spring 2014, fall 2014.

Table 3. Dandekar's Solano grape field map, color coded by *Xf* inoculation date, 2011 to 2014.

Vine	Row 1	Row 2	Row 3	Row 4	Row 5	Row 6	Row 7	Row 8	Row 9
1	52-08	40-89	TS	40-89	TS	TS-50-G	31-25-G	52-08-G	40-41-G
2	52-08	40-89	45-77	40-89	TS	TS-50-G	40-92-G	52-08-G	40-41-G
3	52-08	41-151	45-77	40-89	45-77	41-151-G	40-92-G	52-08-G	TS-50-G
4	40-41	41-151	45-77	TS	45-77	41-151-G	40-92-G	31-25-G	TS-50-G
5	40-41	41-151	40-92	TS	45-77	41-151-G	TS-50-G	31-25-G	TS-50-G
6	40-41	52-08	40-92	TS	41-151	TS-G	TS-50-G	31-25-G	40-92-G
7	31-25	52-08	40-92	52-08	41-151	TS-G	TS-50-G	40-41-G	40-92-G
8	31-25	52-08	41-151	52-08	41-151	TS-G	40-89-G	40-41-G	40-92-G
9	31-25	40-41	41-151	52-08	TS	40-92-G	40-89-G	40-41-G	40-89-G
10	41-151	40-41	41-151	31-25	TS	40-92-G	40-89-G	TS-50-G	40-89-G
11	41-151	40-41	45-77	31-25	TS	40-92-G	TS-G	TS-50-G	40-89-G
12	41-151	31-25	45-77	31-25	TS	40-89-G	TS-G	TS-50-G	TS-G
13	TS-50	31-25	45-77	40-41	52-08-G	40-89-G	TS-G	40-89-G	TS-G
14	TS-50	31-25	52-08	40-41	52-08-G	40-89-G	45-77-G	40-89-G	TS-G
15	TS-50	40-92	52-08	40-41	52-08-G	41-151-G	45-77-G	40-89-G	45-77-G
16	45-77	40-92	52-08	TS-50	40-41-G	41-151-G	45-77-G	TS-G	45-77-G
17	45-77	40-92	31-25	TS-50	40-41-G	41-151-G	40-92-G	TS-G	45-77-G
18	45-77	TS-50	31-25	TS-50	40-41-G	52-08-G	40-92-G	TS-G	41-151-G
19	TS	TS-50	31-25	40-92	31-25-G	52-08-G	40-92-G	52-08-G	41-151-G
20	TS	TS-50	40-41	40-92	31-25-G	52-08-G	41-151-G	52-08-G	41-151-G
21	TS	40-89	40-41	40-92	31-25-G	40-41-G	41-151-G	52-08-G	TS-G
22	40-92	40-89	40-41	40-89	45-77-G	40-41-G	41-151-G	31-25-G	TS-G
23	40-92	40-89	TS-50	40-89	45-77-G	40-41-G	45-77-G	31-25-G	TS-G
24	40-92	TS	TS-50	40-89	45-77-G	31-25-G	45-77-G	31-25-G	TS-G
25	40-89	TS	TS-50	TS	TS-50-G	31-25-G	45-77-G	40-41-G	

North

Grapevines inoculation with *Xf* (Temecula) at 20, 000 cells per 20 uL on 7/13/2011.
Grapevines inoculation with *Xf* (Temecula:Stag's leap mix, 60:40) at 250,000 cells per 20uL on 5/29/2012.
Grapevines inoculation with *Xf* (Temecula) at 250,000 cells per 20ul on 6/17/2013.
Grapevines inoculation with *Xf* (Temecula: Stag's leap mix) at 1,5000,000 cells per 10uL on 5/27/2014.

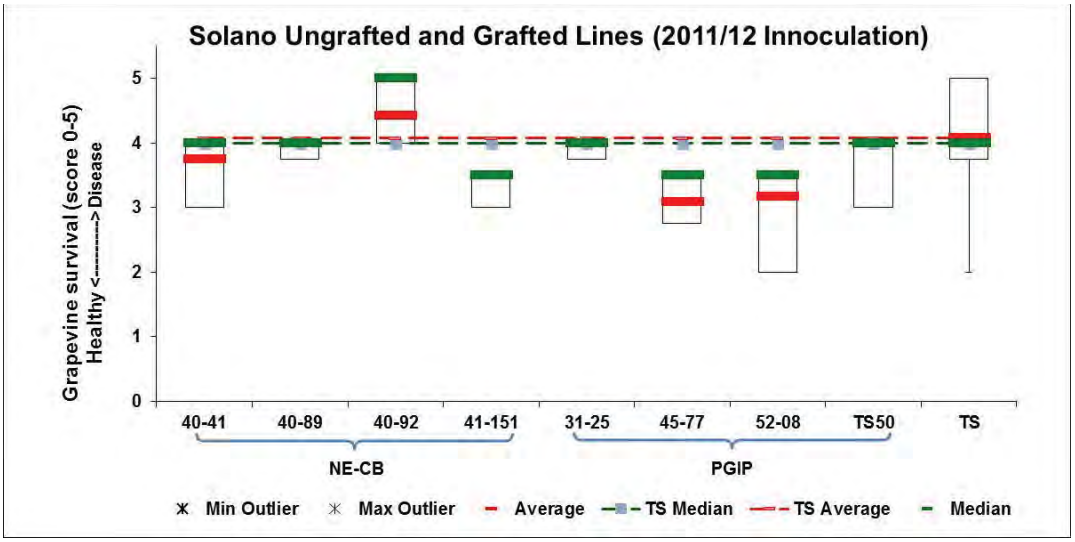

Figure 6. 2014 Pierce's disease symptoms scoring for Solano ungrafted and grafted transgenic grapevines inoculated in 2011 and 2012 and scored in summer 2014.

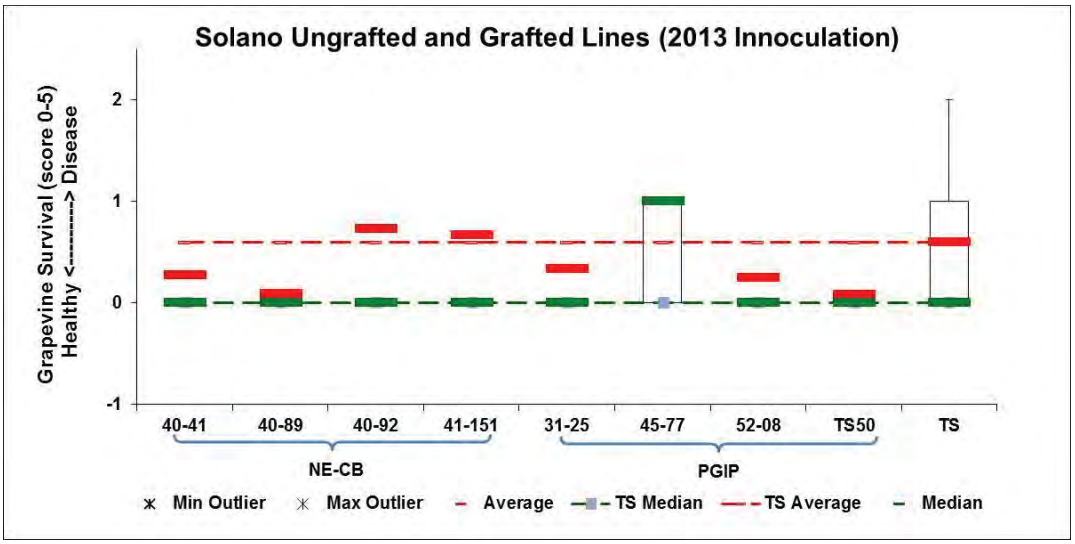

Figure 7. 2014 Pierce's disease symptom scoring for Solano ungrafted and grafted transgenic grapevines inoculated in 2013 and scored in summer 2014.

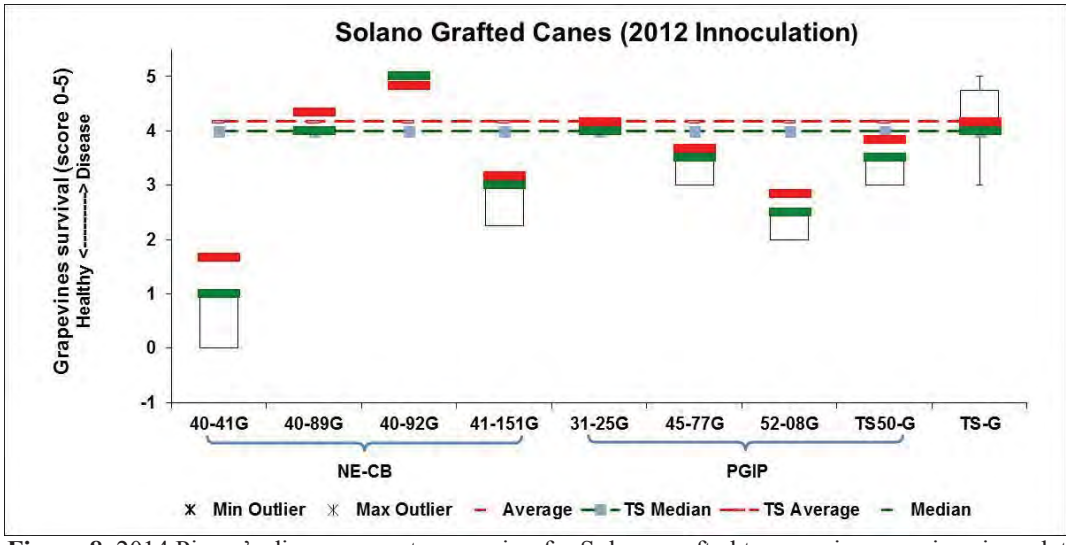

Figure 8. 2014 Pierce's disease symptom scoring for Solano grafted transgenic grapevines inoculated in 2012 and scored in summer 2014.

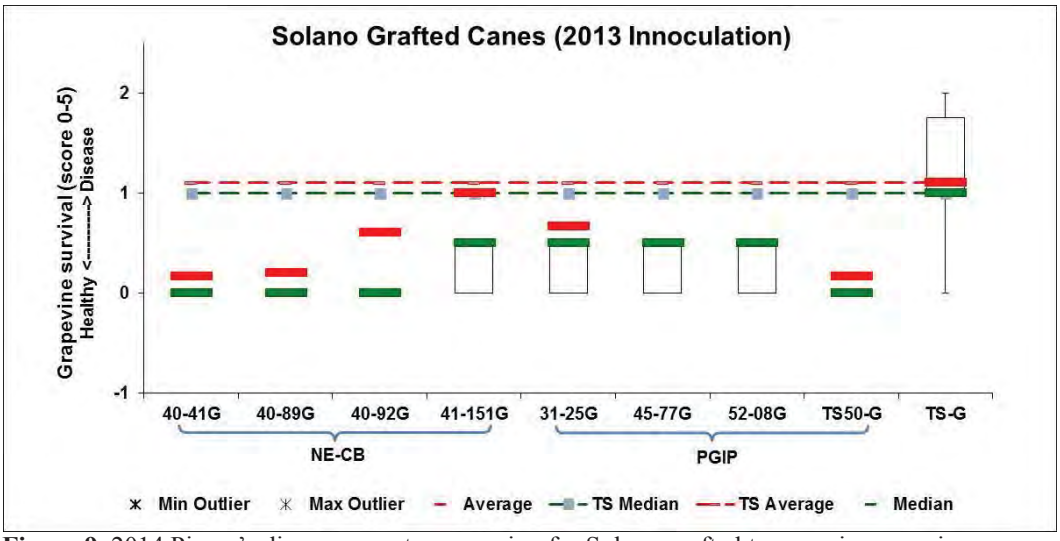

Figure 9. 2014 Pierce's disease symptoms scoring for Solano grafted transgenic grapevines inoculated in summer 2013 and scored in summer 2014.

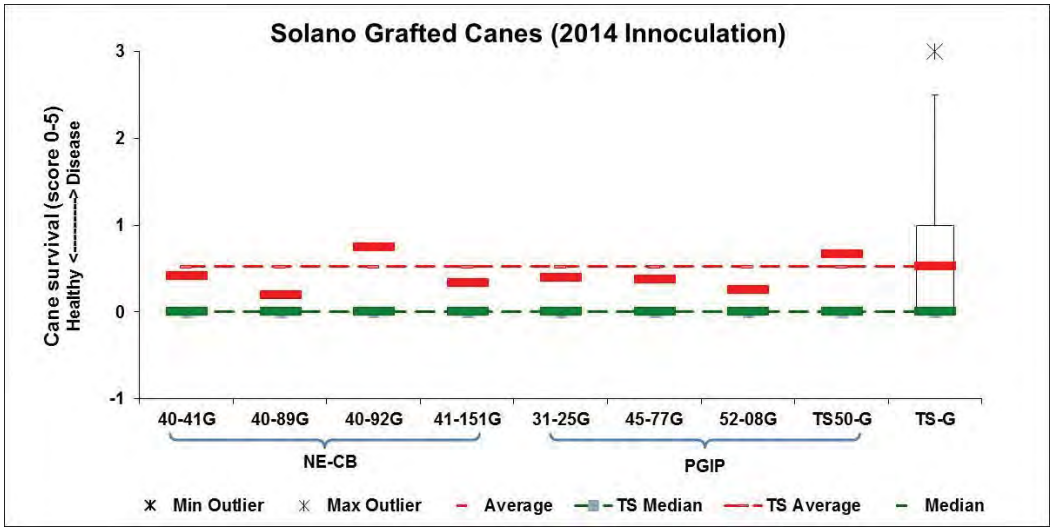

Figure 10. 2014 Pierce's disease symptoms scoring for Solano grafted transgenic canes inoculated in spring 2014 and scored in summer 2014.

Stem samples from runners of ungrafted vines in the Solano plot inoculated in 2011, 2012, and 2013 were harvested in spring and fall 2013 and *Xf* cell counts were determined using ELISA; the standard curve was created using *Xf* cells obtained from liquid culture. *Xf* infection was confirmed. On July 22, 2014, one cane per grafted plant was harvested for quantification of *Xf* by qPCR; results are pending. Another set of canes will be harvested for *Xf* quantification in spring 2015.

Objective 2. Assume permit holder status for existing USDA APHIS field permit 12-340-102r and maintain regulatory oversight and compliance with permit reporting requirements.

Activity 4. Participate with PIPRA during transition and assume permit holder status.
The current Solano and Riverside counties field APHIS permit #12-340-102r was transferred from Professor Alan Bennett to Professor Abhaya Dandekar in January 2014. The permit was extended by APHIS, with a new end date of April 1, 2016.

Activity 5. Maintain regulatory oversight of both field locations and compliance with reporting requirements.
During the transition period beginning Oct. 1, 2013, personnel from the Dandekar laboratory worked with PIPRA personnel to obtain all documentation and records necessary to maintain regulatory oversight of the field trial. This process was completed in January 2014 with the transfer of full responsibility to the new permit holder. We have worked closely with UC Davis Environmental Health and Safety to modify our existing Biological Use Authorization (BUA) to include this permit, a process that integrated the institutional biosafety committee into the chain of custody for regulatory oversight compliance management. Although the responsibility for regulatory compliance rests with the new permit holder, UC Davis was included during the transition to maintain their oversight of campus BUAs. Personnel from the Dandekar laboratory are maintaining regulatory oversight of the field trials. The issues requiring regulatory oversight are listed in the permit.

Activity 6. Maintain active regulatory compliance inspections.
Timely reporting and inspections are conducted to maintain compliance with USDA APHIS. Regulatory compliance is enforced by working closely with the two field coordinators and their crew, obtaining monitoring and activities information from Pierce's disease field trial participant investigators. Two individuals from the Dandekar lab are entrusted with the tasks of documentation, training, and inspection to ensure regulatory compliance.

CONCLUSIONS
We successfully established two field trials to validate two greenhouse-tested strategies to control 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 polygalacturonase-inhibiting protein (PGIP) from pear to counter virulence associated with *Xf* PG activity. Our second strategy enhances clearance of bacteria from *Xf*-infected xylem tissues using a chimeric antimicrobial protein, NE-CB. The expectation is that expressing these proteins will prevent *Xf* movement and reduce inoculum size, curbing the spread of Pierce's disease in California vineyards.

Transgenic grapevine plants expressing either PGIP or NE-CB along with untransformed controls were successfully planted in two locations. In Riverside and Solano counties, planting was completed in 2011 with 220 vines in the ground at each location. These transgenic grapevines were evaluated as plants on their own roots and as rootstocks grafted with untransformed scions. NE-CB- and PGIP-expressing transgenic lines in Riverside and Solano counties were evaluated phenotypically using the first 12 descriptors from the "Primary descriptor priority list" proposed by the International Organization of Vine and Wine (OIV). No phenotypic/horticultural differences were observed between transgenic and untransformed TS vines. NE-CB- and PGIP-expressing transgenic grapevine lines in Solano County were genotyped, confirming the presence of the inserted transgene in all lines. At the Riverside County site, the plants were naturally infected by wild populations of GWSS and *Xf* presence in petiole extracts was confirmed by ELISA, PCR, and plate cell count in fall 2011. *Xf* presence was also confirmed in Riverside xylem sap samples collected in spring 2012, in petiole extracts collected in fall 2012, and in stem extracts collected in spring 2013. Pierce's disease symptoms were also assessed using standardized scores based on percentage of leaf area scorching and grapevine vigor to validate resistance to Pierce's disease under field conditions. At the Riverside County site, the confirmed root knot infection present concurrently with *Xf* infection will not allow us to evaluate Pierce's disease resistance or suceptibility of trangenic grapevines under field conditions with natural Pierce's disease pressure.

At the Solano County site, ungrafted vines were mechanically inoculated with *Xf* in 2011 to validate resistance to Pierce's disease under field conditions. *Xf* presence was confirmed by ELISA in 2011, but no *Xf* growth in plate or Pierce's disease symptoms were detected. Solano County ungrafted plants were re-inoculated and grafted plants were for the first time mechanically inoculated with *Xf* on spring 2012. Leaf scorching, the characteristic symptom of Pierce's disease, was observed in Solano Country for the first time 2012 and *Xf* presence was confirmed by ELISA in petiole extracts collected in the same season and in stem samples collected in 2013. Solano ungrafted and grafted grapevines that were not inoculated previously were manually inoculated in 2013, completing the manual inoculation of all grapevines. In previous years, Pierce's disease symptoms were assessed using standardized scores based on percentage leaf area scorching, cane survival, and grapevine vigor. On May 28, 2014, following the recommendation of the Product Development Committee (PDC) of the Pierce's

Disease Control Program, four current-year canes from all grafted transgenic and control plants at this site were mechanically inoculated with *Xf*. Severity or absence of Pierce's disease symptoms was recorded in summer 2014 for all inoculated canes using the Pierce's disease symptom severity rating system 0-5, where 0 = healthy vine, all leaves green with no scorching; 1 = first symptoms of disease, light leaf scorching on one or two leaves; 2 = about half the leaves on the cane show scorching; 3 = the majority of the of the cane shows scorching; 4 = the whole cane is sick and is declining and 5 = the cane is dead. One inoculated cane per transgenic grafted vine was harvested for *Xf* quantification by qPCR; results are pending. Severity or absence of Pierce's disease symptoms on all ungrafted and grafted transgenic grapevines inoculated in 2011, 2012, and 2013 was also rated in summer 2014 using the Pierce's disease symptom severity rating system 0-5. At least two NE-CB and two PGIP transgenic lines are scored better under field conditions than untransformed controls.

In January 2014, the USDA APHIS permit holder for the Solano and Riverside county fields was changed from Professor Alan Bennett to Professor Abhaya Dandekar; the permit end date is April 1, 2016. Personnel from the Dandekar laboratory are maintaining regulatory oversight of the field trials. Timely reporting and inspections are conducted to maintain compliance with USDA APHIS.

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FUNDING AGENCIES

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DEVELOPMENT OF A STANDARDIZED METHODOLOGY TO ASSESS THE EFFICACY OF GENETIC CONSTRUCTS FOR SUPPRESSION OF *XYLELLA FASTIDIOSA* IN GRAPE

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ABSTRACT
The current field trial of transgenic grapevine (*Vitis vinifera*) tests several genetic constructs, at least some of which appear to operate under distinct mechanisms to provide protection against *Xylella fastidiosa (Xf)*, the causative agent of Pierce's disease. A stacked gene strategy is in progress in which two or more constructs are being incorporated into a commercial grapevine rootstock to evaluate their potential for protection against an untransformed scion. Stacked genes against other host-pathogen interactions have been shown to provide not only more effective protection against disease than could be achieved with one gene alone but also more durable protection. It is the goal of this project to provide data, and to provide a protocol for obtaining additional data that will assist the researchers and the Product Development Committee in decision-making. Experimentally, the aim is to determine the titers of live and dead *Xf* cells in *Xf*-infected untransformed scion tissue and transformed rootstock tissue, to accurately reflect the total number of live (infectious) bacterial cells at various points in the grapevine.

LAYPERSON SUMMARY
This project is intended to support the current transformed grapevine field trials. These field trials are testing the degree of protection provided by various genetic constructions against *Xylella fastidiosa*, the bacterial causative agent of Pierce's disease. Methods are being developed to provide additional information on the behavior of living infectious bacteria in the inoculated vines, particularly the extent of accumulation of live bacterial cells at various locations.

INTRODUCTION
Xylella fastidiosa (*Xf*), the bacterium that is the causative agent of Pierce's disease of grapevine, is unevenly distributed in the infected vine, and its populations in the plant include both live and dead cells. Greenhouse experiments, and to a limited extent field experiments, have demonstrated that specific genetic constructs inserted into commercial grapevine, *Vitis vinifera*, can strongly protect the vines against infection by *Xf* and/or reduce the symptoms of Pierce's disease. At least some of these constructs have shown, as transgenic rootstock of a grafted plant, the ability to protect the untransformed scion (see reports from the laboratories of A.M. Dandekar, D.G. Gilchrist, B.C. Kirkpatrick, S.E. Lindow, and A.L.T. Powell in the Pierce's Disease Control Program 2013 Symposium Proceedings, http://www.cdfa.ca.gov/pdcp/research.html). If adequate protection of untransformed scion by transgenic rootstock can be achieved under vineyard conditions, this has the obvious advantage of flexibility by allowing any of many varietals to be the scion grafted onto a given transformed rootstock. A goal of this project is to assess the titers of *Xf* cells, particularly live *Xf* cells, in vines in field test plots, especially vines with non-transgenic scions on transgenic rootstock.

This project is intended to provide data for evaluating the relative merits of various genetic constructs in altering the titer of bacteria associated with a *Xf* infection and the extent of symptom expression. Our selected source of tissue for analysis of titer of *Xf* cells is the petiole. Petioles can be harvested without much collateral damage to the plant. The tissue is relatively easily disrupted for DNA purification. *Xf* tagged with green fluorescent protein was found by confocal microscopy to occur more frequently and to be in higher concentrations in petioles compared to leaf lamina (Gilchrist and Lincoln, 2008). However, not all petioles on a vine will be representative of the *Xf* cell content of the entire vine or even of a cane, particularly in the early stages of infection. The xylem architecture of the stem is such that not all xylem bundles transport to all petioles. Therefore, some petioles of an infected vine will show a high *Xf* titer, and others will have only a low or no detected titer. Thus, several petioles should be sampled from a given cane.

The most common tool for measuring *Xf* titers in infected grapevine tissue is quantitative polymerase chain reaction (qPCR). Given the unquantified persistence of DNA in plant tissue, qPCR results may reflect the DNA content of *Xf* cells, both living and dead. Hence, accuracy in measuring the amount of live bacteria will address issues of biological behavior associated with the differences in the mode of action of respective transgenes.

Approaches to assessing live *Xf* cell titers include:

1. CFU: plating of tissue extracts for detection of colony forming units (cfu),
2. RT-qPCR: reverse transcription of RNA followed by qPCR (RT-qPCR) to detect *Xf* cell RNA (since unlike DNA, RNA is not expected to persist in dead *Xf* cells), and
3. EMA-qPCR: following treatment of grapevine tissue or extracts with ethidium monazide bromide (EMA) or similar reagent reactive toward DNA in dead bacterial cells.

The disadvantages of plating are low and variable efficiency of plating and the lack of a selective medium for *Xf*. The disadvantage of RT-qPCR is difficulty in relating observed RNA amounts to *Xf* cell counts. However, RT-qPCR or simple RT-PCR would reveal, for example, the situation in which all or almost all of the *Xf* cells in the tissue are dead.

Conversely, EMA-qPCR has the potential to be a relatively simple and straightforward method for evaluating the live *Xf* cell population in grapevine tissues. The important features of EMA are its exclusion from live bacterial cells and its ability to react with DNA in dead cells, thereby making the DNA from dead cells unsuitable as a template for amplification by PCR. A disadvantage of EMA-qPCR is the dearth of published information on the use of EMA to assess living bacteria from inside actual plant tissue, although bacteria on the plant surface have been analyzed (Liang, Dong, et al., 2011). Given that most reports use cultured bacterial cells, we are working to remedy this situation by developing EMA-qPCR as a method for assessing dead and live titers of *Xf* in grapevine tissue. We also are using RT-qPCR and RT-PCR, as a reference point for EMA-qPCR and as an alternative if EMA-qPCR proves to be unworkable for bacteria embedded in plant tissue

OBJECTIVES

1. Develop a protocol for assessing live *Xf* accumulation in transgenic grapevines and suited to the evaluation of the relative efficacy of various transgenic constructs
2. Use the developed protocol in early 2015 on bud emergence samples from the current field trials of transgenic grapevine
3. Advance and standardize the protocol to the point that the protocol could be applied by a designated laboratory to evaluate grapevine lines bearing stacked transgenes

RESULTS AND DISCUSSION

Initial attempts at quantitating dead *Xf* cells and total *Xf* cells in grapevine petiole tissue.

In the three months that this project has been active, a variety of conditions have been explored, including a demonstration of inactivation of naked *Xf* DNA and cultured *Xf* cells by a EMA plus light treatment pursuant to distinguishing living from dead *Xf* cells in plant tissue. The results from an experiment in which several petioles were cut into 1-2 mm slices is shown in **Figure 1**. The petioles were pooled and divided into six samples for the three treatments, each in duplicate. As expected from published reports from cultured cells, the genome equivalents of recovered DNA by qPCR from plant tissues were much greater for slices exposed to just light than for slices exposed to EMA and light. However, unexpectedly, the slices exposed to EMA but not irradiated with white light also yielded few genome equivalents of *Xf* DNA from this control treatment. We do not have an explanation for this result on this singular treatment but note that the EMA-no-illumination control that we performed here does not appear in publications on the use of EMA to distinguish live from dead bacterial populations.

Actinometry for lamp calibration.

If the photo-inactivation of DNA bound to EMA or other photoinactivating agent is to be accomplished under reproducible conditions, the flux of photons at the tissue sample must be reproducible from experiment to experiment. Additionally, there should be a method for comparing the flux for chambers of different geometry. A Lithonia Lighting #OFL 300/500Q 120 LP lamp was operated with a 500 W bulb in a glass housing. The housing glass surface was located 85 mm from a Petri plate containing a solution of 12 mM potassium ferrioxalate [synthesized from ferric chloride and potassium oxalate and recrystallized from water (Hatchard and Parker,

1956)] in 50 mM sulfuric acid. In two min of irradiation, 0.6 µmole of ferrous ions were produced from the ferrioxalate per square cm of liquid surface, as determined colorimetrically (A_{510}) by complex formation with ortho-phenanthroline. This result shows that we have a chemical actinometer of the desired capability.

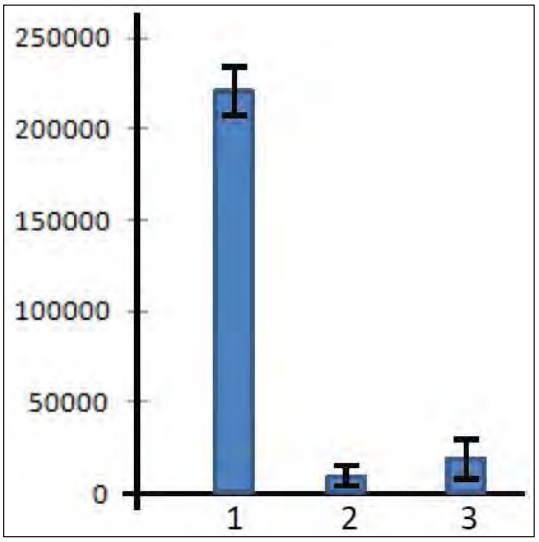

Figure 1. Genome equivalents (ordinate scale) of *Xf* detected after irradiation of petiole slices imbibed or not imbibed with EMA. DNA was purified from the petiole slices by a hot cetyltrimethylammonium bromide (CTAB) method. qPCR primers were HL5 and HL6 (Francis, Lin, et al., 2006), and qPCR was performed in triplicate. The standard curve for determination of genome equivalents used template prepared by dilution of *Xf* DNA in a constant concentration of DNA from uninfected grapevine (Francis, Civerolo, et al., 2005). The average and range of genome equivalents detected are shown for duplicate samples. Sample 1 was derived from petiole slices not exposed to EMA but irradiated with visible light. Petiole slices for samples 2 and 3 were exposed to 100 µg/mL EMA for 30 min in the dark and then were either continued in the dark (sample 2) or were irradiated with white light (sample 3).

CONCLUSIONS

Our initial attempts at quantitating dead *Xf* cells and total *Xf* cells in grapevine petiole tissues have yielded promising results for experiments carried out under similar conditions. We have successfully recovered PCR amplifiable DNA from infected tissue. Conditions are being modified in an effort to improve the EAM-based analyses of *Xf* DNA and are exploring an RNA-based method as an alternative live-dead assay. These include gently grinding tissue to release minimally damaged *Xf* cells for EMA treatment, rather than imbibing or vacuum infiltrating EAM. We also are applying a different inactivation agent, propidium azide (Liang, Dong, et al., 2011) along with continuing the developing the RT-qPCR methods..

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

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ABSTRACT
The objective of this field experiment is to evaluate transgenic grape and grape rootstocks expressing various transgenes for protection against *Xylella fastidiosa* (*Xf*; Pierce's disease strain) in a field site in Solano County. The pathogen is introduced into individual vines by mechanical injections of Xf into the grape stems of transgenic and non-transgenic control plants. The experiment is now in the fourth year after inoculations were initiated. Test plants include own-rooted transgenic and non-transgenic plants and grafted plants with non-transgenic scions of a Pierce's disease susceptible variety grafted to root stocks bearing transgenes. The plants have been maintained under optimum field conditions with respect to water management and powdery mildew and insect control. Following the third (2013) and fourth (2014) years after inoculations began, control plants are showing clear symptoms of Pierce's disease (**Figure 6**) and many inoculated canes are dying or dead. Several lines bearing transgenes of each of the investigators show promise in terms of Pierce's disease symptom suppression compared with the susceptible non-transgenic controls.

LAYPERSON SUMMARY
The purpose of the field planting is to evaluate grape and grape rootstocks expressing transgenes with different modes of action directed towards suppression of Pierce's disease. The site in Solano County was selected and approved by USDA APHIS to enable controlled inoculation and close monitoring of the host response in terms of symptoms, bacterial behavior, and plant morphology. Over the course of the multi-year field evaluation, test plants included ungrafted conventional Thompson Seedless and Freedom rootstock plants as controls, transgenic plants from investigators Dandekar, Powell, Lindow, and Gilchrist and later transgenic rootstocks expressing some of the test genes were grafted to untransformed Pierce's disease susceptible scions to assess potential for disease suppression in an untransformed scion from signals originating in the transformed rootstocks. The results to date of this field experiment indicate that the mechanical inoculations successfully introduced the bacteria into the plants with subsequent appearance of foliar symptoms and cane death. There are transgenes from each of the investigators that appear to be suppressing the symptoms of Pierce's disease inoculated vines. Dr. Dandekar has now assumed responsibility for the USDA APHIS permit and Dr. Gilchrist will continue to manage the field operations at this site.

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 multi-year field evaluation, test plants will include ungrafted conventional Thompson Seedless and Freedom plants as controls, transgenic plants from Dandekar, Powell, Lindow, and Gilchrist projects and, as plant material availability permits, transgenic rootstocks expressing some of the test genes grafted to untransformed Pierce's disease susceptible scions were introduced in 2011 and 2012. All plants are located in a USDA APHIS approved field area with no risk of pollen or seed dispersal. The area is adjacent to experimental grape plantings that have been infected with Pierce's disease for the past two decades with no evidence of spread of the bacteria to uninfected susceptible grape plantings within the same experiment. Hence, there is a documented historical precedent for the lack of spread of the bacteria from

inoculated to uninoculated plants, an important consideration for the experiments carried out for this project and for the granting of the USDA APHIS permit. The field area chosen has never had grapes planted therein, which is to avoid any potential confounding by soil-borne diseases, including nematodes.

OBJECTIVES

A. Land preparation, planting, and management of the experimental resources to accommodate 500 plants. Plants occur with a row spacing of 15 feet between rows and 4 feet between plants in a row. There is a 50-foot open space buffer area surrounding the field, which is fenced to protect against rabbits. Each row is staked with 7-foot grape stakes supporting 13-gauge wire in a two-wire trellis system with a stake at each plant site. Wires are stretched and anchored by 7-foot pressure-treated posts at the end of each row. The plants are irrigated by surface furrow in accordance with standard practices for maintaining grapes for experimental purposes at this site. Furrow irrigation will be continued on the existing plots, although a drip irrigation system was installed in 2014 and will be used in all future plantings. Irrigation and pest management, primarily for powdery mildew, weeds and insects, is coordinated by PI Gilchrist and conducted by Tom Kominek, the Field Superintendent employed by the Department of Plant Pathology. Mr. Kominek recently retired after 30 years of service and has been replaced by Mike Eldridge, who has 20 years of experience working with grapes and other perennial crops. The field crew work closely with PI Gilchrist to determine timing and need of each of the management practices.

B. Principal Investigators, with assistance from contract field crews, are responsible for pruning in the spring of each year and within the season as needed to maintain a reasonable canopy permitting sun exposure to leaves on inoculated canes. Periodic trimming is necessary, given that the transgenic plants are derived from Freedom (a common rootstock) and Thompson Seedless, both of which exhibit tremendous vegetative growth during the season. In addition, annual pruning deviates from conventional practice in that multiple cordons have been established with a separate new cordon retained from each successive inoculation. This enables differential experimental materials for evaluation and sampling in the form of seasonal canes associated each succeeding annual inoculation. The objective is to provide sufficient inoculated and control material for destructive sampling over years to assess both timing of symptom development after successive inoculations and to assess bacterial presence and movement over time.

C. Plants have been mechanically inoculated with *Xylella fastidiosa* by the Investigators beginning in 2011 and subsequent years. Uniform inoculum has been produced by PI Gilchrist and provided to all investigators. All inoculations occurred simultaneously on the same date by all investigators. Hence, inoculum type, concentration, and timing has been and will continue to be uniform across all grape plants.

RESULTS AND DISCUSSION

All of the above objectives set out for the establishment and management of this field planting were completed in the timelines proposed in 2010. 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**) with all plants surviving the winter as shown in **Figure 2**. The second phase of the planting, including grafted transgenics was completed May 2011 and June 2012.

Extensive polish trimming during the season was quickly recognized as necessary to manage the Freedom and Thompson Seedless plants in a fashion to allow ease of mechanical inoculation and recovery of experimental samples (**Figure 3**).

As of July 21, 2014, all transgenic individuals exhibited a normal phenotype, true to the untransformed control plants of each parental genotype (**Figure 4**). Symptoms of Pierce's disease did not appear until two years after inoculation. Evaluations in the summer of 2014 indicated inoculated controls and some transgenic plants showed symptoms of Pierce's disease. It is clear that this field planting will provide important data on the effectiveness of any of the transgenic strategies employed by the respective researchers.

As of March 2014, many inoculated canes on control plants and some transgenics did not survive the winter but the uninoculated canes on these plants still appear healthy. Visual observation and destructive sampling of inoculated canes indicates that mechanical inoculation was successful in infecting inoculated canes (**Figure 5**). As of July 2014, several uninoculated canes adjacent to inoculated canes showed foliar symptoms indicating that the

bacteria had moved systemically through the plants and, in the case of some non-transformed control plants, the entire plant is now dead.

There are two points to be made regarding the appearance of symptoms. First, plant turgor has been maintained throughout the growing season with timely irrigation and there has been no evidence of wilt or epinasty symptoms prior to appearance of classic foliar symptoms (**Figure 6**) or even death of inoculated control susceptible canes. Symptomatic leaves occur on inoculated canes without the appearance of water stress (**Figure 6**). This belies the long-held anecdotal effect of vascular plugging leading to the classic foliar symptoms of sectored death within green areas of leaves. Second, excellent symptoms associated with the presence of the pathogenic bacteria are readily seen in the spring of each year from buds emerging on inoculated canes. Buds break, push tiny leaves, and then die in tissues confirmed in the laboratory to harbor bacteria from inoculations that occurred one to two years prior.

As of September 2014, it is clear that there is a rich source of additional data to be collected from this field experiment. There are now substantial differences between inoculated control plants compared with plants expressing some of the transgenes. There is no evidence of any spread of the bacteria from inoculated to uninoculated control plants, but there is now evidence of systemic spread within some of the plants representing different genetic composition (different transgenes). The positive result of effective mechanical inoculation over time suggests that plants consisting of transgenic rootstocks grafted to non-transgenic scions will enable experimental assessment of cross-graft protection. Field data over the course of this experiment has been collected by all investigators and can be found in their individual reports.

We are now approved and funded to continue maintenance and data collection from this site for the coming two years through June 30, 2016. This time period matches the time extension proposed by Dr. Dandekar, who has now assumed responsibility for the USDA APHIS permit. Dr. Gilchrist will continue to manage the field operations at this site.

Solano County Pierce's Disease Field Work 2014.
All field activities are conducted or coordinated by field superintendent Tom Kominek. Regular tilling and hand weeding maintained a weed-free planting area. Plants were pruned carefully in March leaving all inoculated/tagged branches and numerous additional branches for inoculation and sampling purposes in the coming year. All pruning material was left between the rows to dry, then flail chopped and later rototilled to incorporate the residue per requirements of the USDA APHIS permit. Frequent trimming of the plants was done to ensure that leaves on inoculated canes were exposed to sunlight and shading of the associated leaves was avoided. Surface irrigation was applied as needed to maintain the soil at field capacity and turgor in the plants. Application of the fungicides Luna Experience and Inspire were alternated at periodic intervals to maintain the plants free of powdery mildew. Leafhoppers and mites were treated with insecticides when needed. Neither powdery mildew nor insect pressure was noted throughout the growing season.

CONCLUSIONS

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

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

FUNDING AGENCIES

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

Figure 1. Field view July 2010 Solano planting.

Figure 2. Field view March 2011 Solano planting.

Figure 3. Field view April 2012 Solano planting.

Figure 4. Field view July 2014 Solano planting.

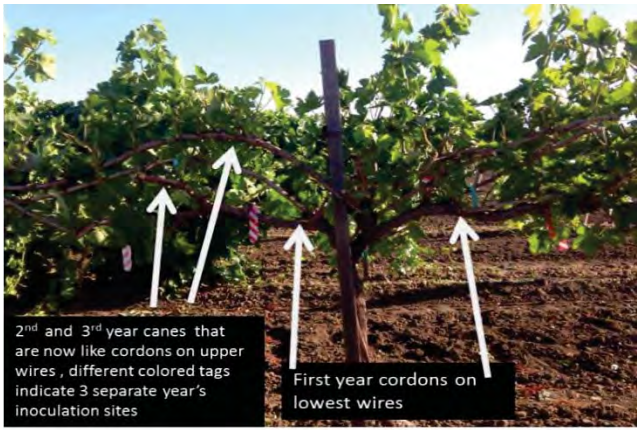

Figure 5. Illustration of successive cordons retained from successive inoculations done in 2011-2014.

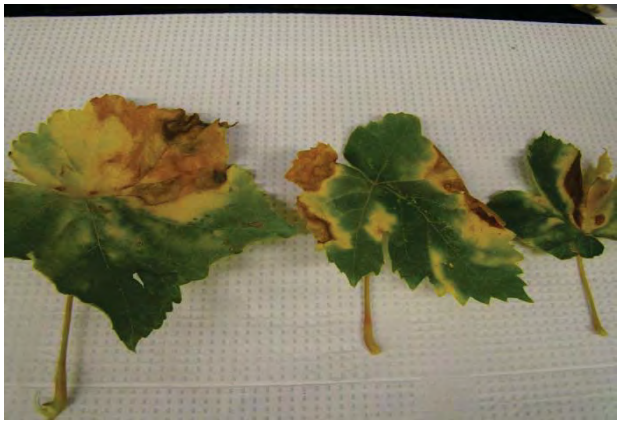

Figure 6. Illustration of classical Pierce's disease foliar symptoms observed July 2014 resulting from mechanical inoculation of *Xf* at the Solano County site.

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 2013 to October 2014.

ABSTRACT
Field experiments were initiated in Riverside and Solano counties to evaluate transgenic grape plants and grape rootstocks expressing two DNA constructs, PR1 and UT456, in several different transgenic lines of each construct for resistance to *Xylella fastidiosa* (*Xf*; Pierce's disease strain). Mechanical inoculation was employed at the Solano site whereas natural infection occurred at the Riverside site by endemic sharpshooters that carry *Xf*. The Solano field experiment was conducted in two phases. The first phase of the field studies started in 2010 to evaluate clonal copies of the fully transformed own-rooted plants that exhibited suppressed Pierce's disease symptoms and low bacterial titers in greenhouse assays (1). The second phase began in 2011 with planting the untransformed Thompson Seedless scions grafted onto PR1 and UT456 primary transformants as rootstocks. Data collected in 2012-14 from both sites indicate that the bacteria are present in plants at the Riverside site, and in the mechanically inoculated canes on plants at the Solano site (2). Results indicate that both PR1 and UT456 transgenes provide protection against Pierce's disease, while the level of protection varies with location and individual transgenic line.

LAYPERSON SUMMARY
Previously, we identified novel genes that suppress Pierce's disease symptoms by blocking programmed cell death (PCD), elicited by *Xylella fastidiosa* (*Xf*) through use of a functional screen from cDNA libraries of grape and tomato. Two of these sequences (PR1 and UT456) expressed as transgenes in grape, suppressed Pierce's disease symptoms and dramatically reduced bacterial titer in inoculated plants under greenhouse conditions. Field experiments underway in Solano and Riverside counties, conducted with a USDA APHIS permit, are designed to evaluate clonal copies of several of these transgenic lines under field conditions for resistance to Pierce's disease. The field evaluation includes mechanical inoculation with *Xylella fastidiosa* in Solano County and glassy-winged sharpshooter (GWSS) inoculation in Riverside County. Data sets 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 initially at the Riverside County site and PCR assays confirmed bacterial populations in the plants, although the plants were generally unthrifty from the outset. However, by 2013-14 all plants at the Riverside County site are dead or nearly dead but have not shown extensive foliar symptoms of Pierce's disease. Recently, it has been discovered in adjacent plots that the root systems of the plants are infected with the root-knot nematode, presenting a serious confounding with any bacterial infection. Conversely, plants at the Solano County site remain vigorous with normal phenotypes. Inoculated plants are now showing typical symptoms of Pierce's disease. Bacteria are present in inoculated plants at the Solano County site and there is definitive evidence of symptom differences between several of the transgenic lines compared with the non-transgenic control. Evaluations at the Solano County site are ongoing.

OBJECTIVES
The overall objective is to continue to evaluate several lines of transgenic grape plants and grape rootstocks expressing two DNA constructs designated PR1 and UT456 for resistance to *Xylella fastidiosa* (*Xf*) at sites in Riverside and Solano counties (1,2). Controlled mechanical inoculation is used at the Solano County site and natural infection via the glassy-winged sharpshooter (GWSS) is the infection source at the Riverside site. The background research on selected transgenic lines leading to these field trials is from four controlled inoculation experiments in a greenhouse over a two-year period, involving more than 300 transgenic plants of five lines derived from independent transformation events bearing PR1and UT456. Each of these transgenes in several lines suppressed Pierce's disease symptoms and reduced bacterial titer compared with untransformed controls of the same genotype. A positive correlation between the P14 and UT456 message level, suppression of bacterial titer,

and absence of Pierce's disease symptoms was established using qPCR to measure both the message and the bacteria titer.

A. The Solano experiment will evaluate transgenic grape plants and grape rootstocks/scion combinations expressing two DNA constructs designated PR1 and UT456 in a field site in Solano County for resistance to the Pierce's disease strain of *Xf* following mechanical inoculation (**Figure 1**). A first planting of fully-transformed plants was established in 2010 and a second set of plants consisting of rootstocks transformed with PR1 and UT456 genes grafted to untransformed Pierce's disease susceptible Thompson Seedless scions. The grafted plants are designed to assess the potential for trans-graft protection against Pierce's disease.

B. The Riverside County field experiment 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 were exposed to *Xf* infection via natural vector populations of GWSS.

RESULTS AND DISCUSSION

Plant Phenotypes

There were no distinguishable morphological differences in the control plants compared with any of the transgenic lines using criteria of descriptors described by the International Organization of Vine and Wine. All plants have a normal phenotype, true to the untransformed control plants of each parental genotype and all produced fruit. The Thompson Seedless transgenic plants are fully fruited with no visually distinguishable differences in fruit set, fruit size, or maturity from the untransformed control plants. The field map in **Figure 1** shows the genotypes and colored bars indicating the various inoculation dates and bacterial populations introduced at each inoculation date.

Solano County Planting

As of August 2014, all inoculated plants at the Solano site are confirmed to harbor the introduced *Xf*. Uninoculated individuals are healthy, growing normally and tested free of *Xf* (**Figure 2**). Inoculated plants were confirmed to have been successfully infected in the 2011, 2012, and 2013 inoculations (**Figure 4**) by sampling individual inoculated canes followed by qPCR analysis for relative bacterial populations. Bacterial numbers varied from 500-1500 cells per 1 cm of inoculated stem tissue. On May 28, 2014, 3-4 young shoots were inoculated with *Xf* in each of the plants by all investigators, including the grafted and non-grafted plants expressing PR1 and UT456 in our specific set of plants (**Figure 3**). Evaluation of this set of inoculations occurred in late summer (**Figures 9 and 10**).

Earlier we observed in March of 2014 that excellent definitive differences were present at bud break in inoculated canes (now essentially cordons) in the form of dying buds and very young shoots that died quickly after emergence in 2011, 2012, or 2013. **Figures 6, 7 and 8** show examples of bud and shoot death only on previously inoculated canes, which was confirmed by confirming the presence of the bacteria by quantitative PCR (qPCR) assays in the inoculated canes. From this data, we conclude that evaluation of bud health in the spring is an important criteria to reveal the presence of sufficient bacteria in perennial tissues to cause serious disease and death in emerging new annual vegetative growth (**Figure 8**).

By late June of 2014 all the inoculated untransformed control plants showed foliar symptoms of Pierce's disease (**Figure 9**), along with some of the experimental plants. Uninoculated control plants appear healthy in all cases. There is no evidence of plant to plant spread and only limited movement of bacteria from an inoculated cordon to uninoculated adjacent cordons or canes. The young canes of untransformed scions grafted to transgenic rootstocks, inoculated in May 2014 (**Figure 3**), began to show Pierce's disease symptoms within 90 days (**Figure 9**). Eight leaves from the point of inoculation were rated for foliar symptoms at 120 days revealed significant differences Pierce's disease symptoms between control and transgenic rootstocks (**Figure 10**). These evaluations will be continued when the plants emerge from dormancy. However, at this stage it is clear that there is a rich source of additional data to be collected that will prove important as we move forward in experiments now ongoing to combine (stack) the best of the transgenes into commercially accepted rootstocks. For future reference, the transgenic rootstocks expressing multiple constructs will be grafted to untransformed scions to assess trans-graft protection. The paired genes exhibit different mechanisms of action either acting as a bactericide, suppressing bacterial movement, blocking plant cell death, or by a quorum sensing modification of the bacterial behavior.

Riverside County Planting

Test plants were introduced to the field site in Riverside County in the spring of 2011 in a location reputed to have a history of severe presence of GWSS carrying *Xf*. Sampling for presence of the bacteria in these plants in the fall of 2011 confirmed infection but there was no evidence of classical foliar Pierce's disease symptoms at that time. Over the three years since planting, the plants remained unthrifty and many began to die in the summer of 2012. Classical foliar symptoms of Pierce's disease were rarely observed, especially in comparison to the mechanically inoculated plants in the Solano County experiment. Initially, the plants expressing PR1 and UT456 transgenes were rated as more healthy that the non-transgenic controls. By the summer of 2013, it was clear that all the living plants, transgenic and non-transgenic, were not growing normally, the trunks were spindly and many had dead or dying cordons. Again, there was an absence of typical foliar Pierce's disease symptoms on the canes that were clearly dying. In the spring of 2014, most all of the aerial portions of the plants, transgenic and non-transgenic, were dead, although, suckers were emerging from the base of many of these plants (**Figure 10, panels A and B**).

Coincidentally, the plants in the Dandekar planting directly adjacent to the Gilchrist plants also were all dead by the spring of 2014, with only random plants showing emergence of suckers. The Dandekar laboratory conducted evaluation of the roots of the dead plants and confirmed extensive colonization by root-knot nematodes. The conclusion is that infestation by the root-knot nematode both confounded any data interpretation relative to Pierce's disease and was the cause of the premature death of many, if not all, of the plants. In addition, the foliar portions of all plants with living tissue from the suckers were aggressively pruned by the Riverside field crews in the summer of 2014 without informing any of the PIs with plants in the field. So, even if there were foliar symptoms of Pierce's disease on the regrowth sucker branches, the pruning removed them and no data was collected. In summary, the Riverside County planting was not useful in evaluating the transgenic plants of any of the investigators for response to Pierce's disease over the period that plants were in this field. The lack of information was due primarily to the confounding impact of the root-knot nematode, nutrient deficiency (Riverside analysis), improper management, and general unthriftiness of the plants from the outset (**Figure 10, panels A & B**).

CONCLUSIONS

Xf induces Pierce's disease symptoms that result from activation of a genetically regulated process of programmed cell death. We have identified two DNA sequences from a cDNA library screen, 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 and tomato. 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 in controlled greenhouse studies. Similar results are being seen under field conditions. Current data from the Solano County site suggests that protective sequences may function across a graft union to protect an untransformed and susceptible wild-type scion, although this data is preliminary. Both the PR1 and UT456 expressing plants show suppression of symptoms and reduced bacterial counts. Individual plants within UT456 and PR1 lines have remained asymptomatic. While some lines are less suppressive, all lines are rated more suppressive of Pierce's disease than the controls.

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. In addition, we have initiated a project to stack these genes in pairs, along with those found to be effective under field conditions by other researchers with functionally different transgenes, into commercially desired rootstocks. The stacked gene constructs will be tested for efficacy as whole plant transgenics and for ability to protect untransformed scions across a graft union.

FUNDING AGENCIES

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

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1. David Gilchrist and James Lincoln. 2011. Disease control and bacterial population dynamics in winegrape varieties grafted to rootstocks expressing anti-apoptotic sequences. Proceedings of the Pierce's Disease research symposium. Sacramento, CA December 13-15.

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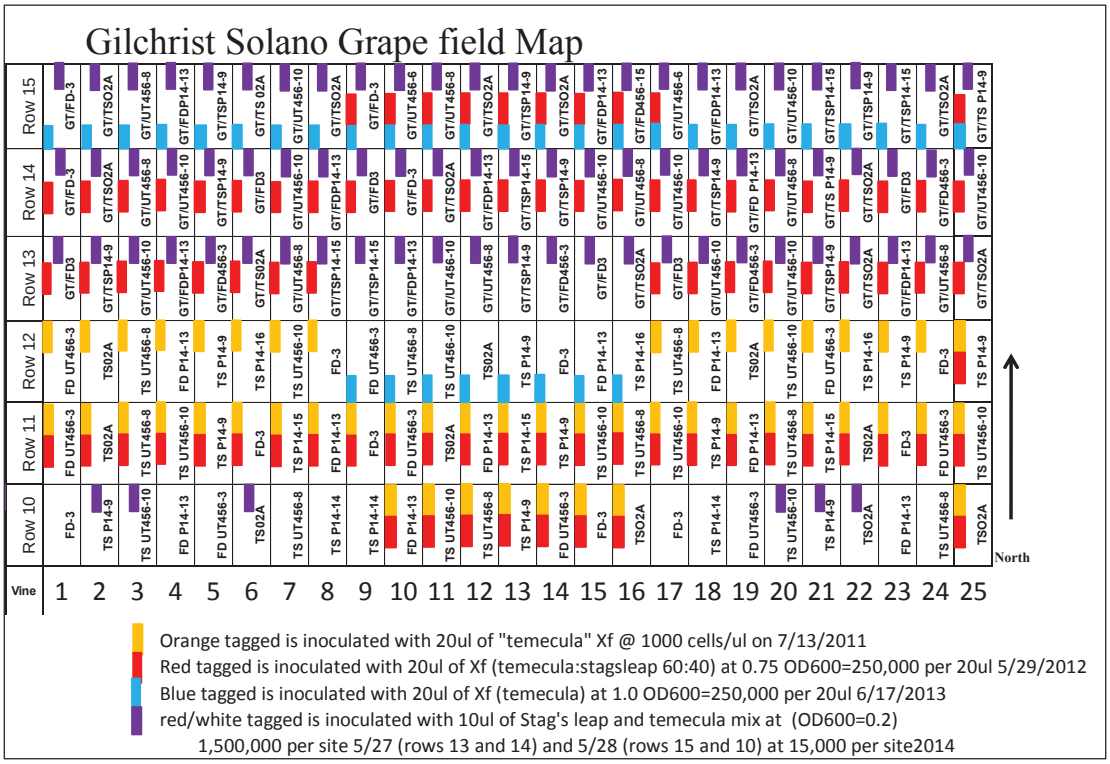

Figure 1.

Figure 2. Solano County grafted grapes, June 2014, trimmed to expose the tagged inoculation sites to permit sun exposure and avoid shading of inoculated branches.

Figure 3. Young shoots of grafted plants mechanically inoculated by skilled worker in May 2014 to assess potential protection across a graft union.

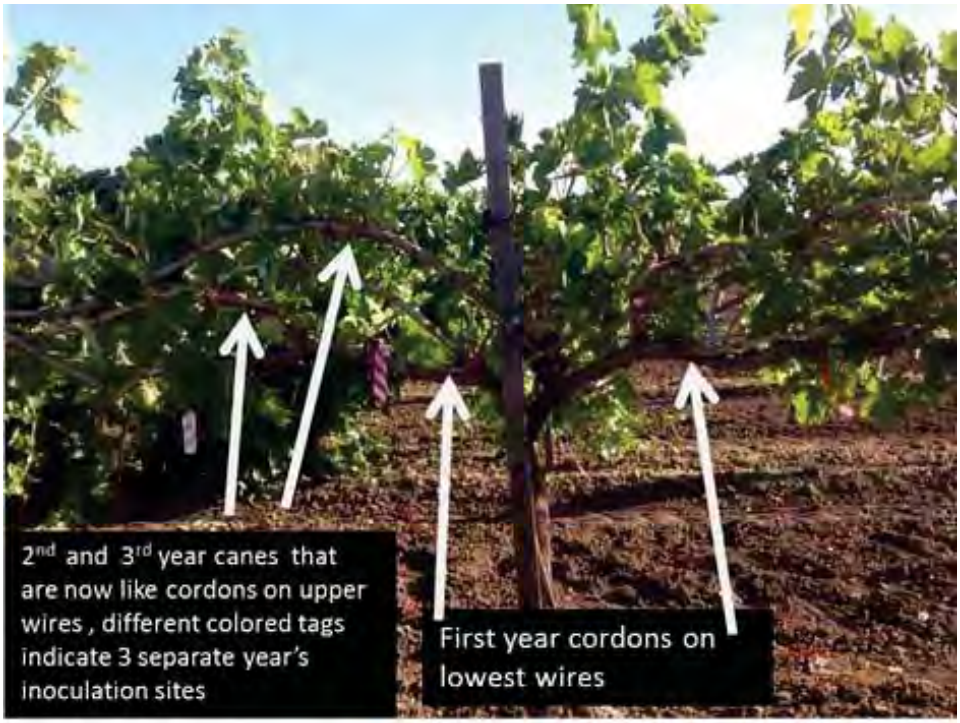

Figure 4. Illustration of multiple cordons retained from successive inoculations done in 2011-2014. Photo taken late June 2014. Tags visible on branches reflect the date of inoculation as see also in **Figure 1.**

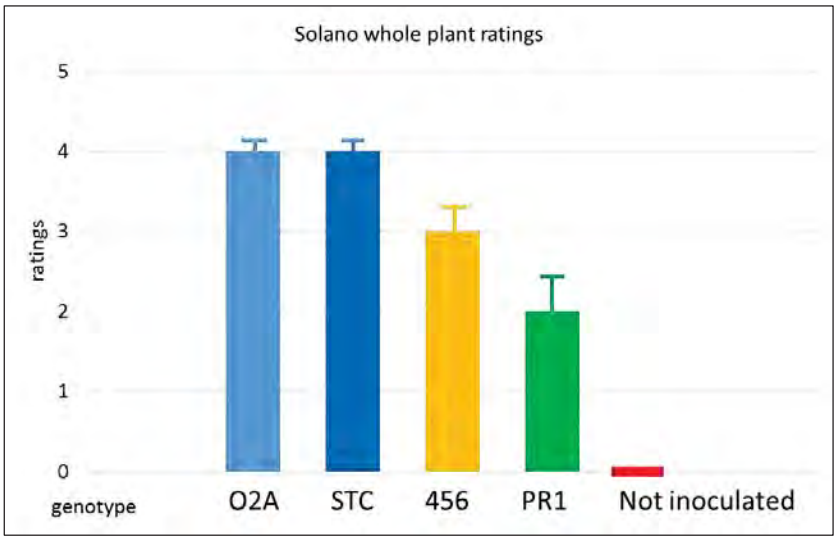

Figure 5. Evaluation of whole plant transgenics at the Solano County site in the summer of 2014. The susceptible controls are untransformed Thompson Seedless (O2A) and a susceptible transgenic line (STC). Many plants within the controls were dead or dying at the end of the 2014 growing season. Individual plants within UT456 and PR1 lines have remained asymptomatic. While some lines are less suppressive, all the transgenic lines are rated more suppressive of Pierce's disease than the controls. There are no Pierce's disease symptoms nor bacteria in the uninoculated susceptible controls. Rating scale is 0-5 with 0 being asymptomatic and 5 is a dead plant.

Figure 6. Illustration of bud and shoot death in the spring of 2014 due to Pierce's disease on cordons inoculated in 2011 and 2012. Untransformed control Thompson Seedless (O2A) and a susceptible transgenic control (STC) plant showing shoot death shortly after emergence, compared with transgenic Pierce's disease plant lines expressing different anti-programmed cell death genes.

Figure 7. Close-up of spring Pierce's disease symptoms where buds or very young shoots die shortly after emergence.

Figure 8. March 17, 2014 evaluation of inoculated canes on untransformed Thompson Seedless (TS02A), a transgenic susceptible control (STC) and transgenic lines expressing disease suppressive genes. 15 shoots were assessed in 3 plants per genotype as the plants emerged from dormancy. Figures 4 and 5 illustrate the stage of growth and appearance of the shoots

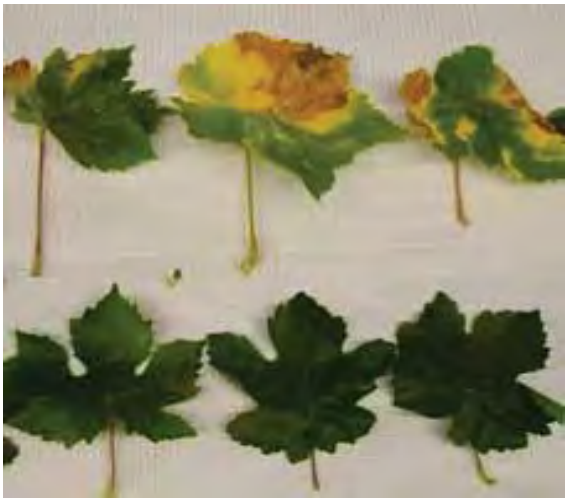

Figure 9. Illustration of foliar symptoms on young shoots of a grafted susceptible control plant (**A**) compared with susceptible scion on a transgenic PR1 rootstock (**B**). Shoots were inoculated in March 2014 (see **Figure 3**).

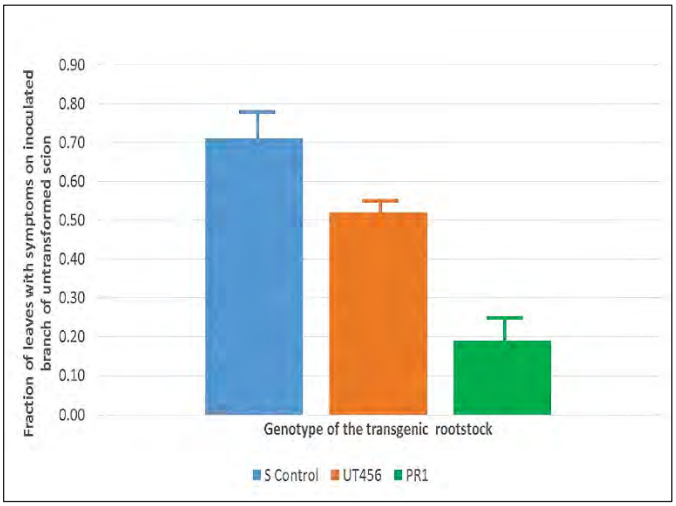

Figure 10. Evaluation of leaves on young shoots of a susceptible control plant grafted to untransformed and transformed rootstocks expressing either UT456 or PR1 transgenes; shoots were inoculated in spring of 2014 (see **Figure 3**).

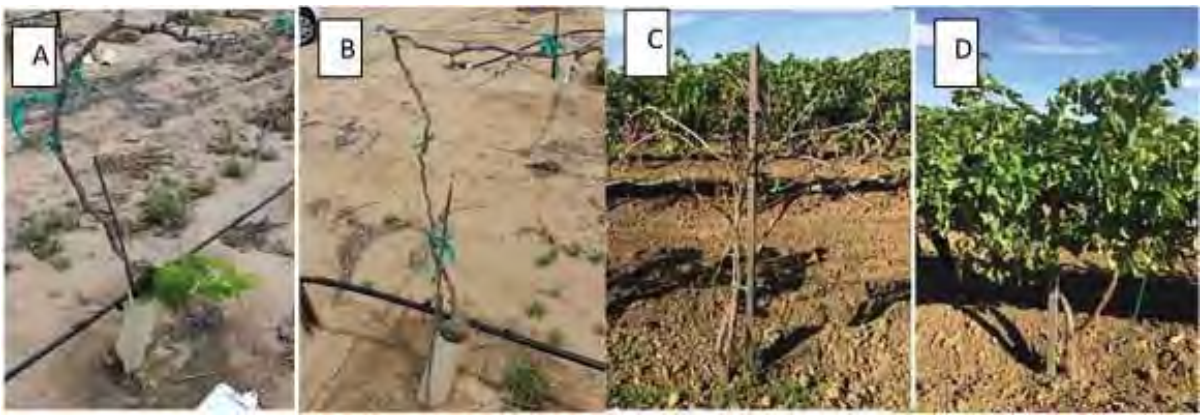

Figure 11. Comparison of growth characteristics of plants at the Riverside and Solano county sites three years after planting. Panels A and B are examples of Riverside plants with images taken in summer of 2014 indicating the spindly nature and general unthriftiness of the plants at this site from the time they were planted. Currently, all aerial portions are dead with limited suckers emerging from the base of the plant. Panels C and D illustrate plants at the Solano site of a similar age and genotype which are vigorous and reveal classical Pierce's disease foliar symptoms and presence of *Xf* in the inoculated canes (C) but not in uninoculated plants and many of the transgenic genotypes (D).

TRANSGENIC ROOTSTOCK-MEDIATED PROTECTION OF GRAPEVINE SCION BY DUAL STACKED DNA CONSTRUCTS

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ABSTRACT
Genetic strategies for disease suppression and information characterizing the bacterial-plant interaction are high priority areas in the Pierce's disease and glassy-winged sharpshooter research program. Five potential Pierce's disease suppressive DNA constructs from the laboratories of the investigators listed above have been tested extensively as transgenes in grape plants under greenhouse conditions and in USDA APHIS approved field environments in Riverside and Solano counties. Two types of genetically modified plants bearing single DNA constructs of test genes have been evaluated under disease conditions: (a) whole plant transgenics and (b) transgenic rootstocks grafted to non-transformed Pierce's disease susceptible scions to assess cross-graft protection. Positive and promising results from both environments and both types of transgenic strategies provide the necessary impetus to advance this program both in the field and with augmented transgenic strategies using combinations of the individual transgenes as stacked constructs in both whole plant transgenics and in an adapted rootstock to evaluate cross-graft protection from transformed rootstocks to untransformed winegrape scions. Dual gene plasmids have been constructed over the past two months and are in the process of being introduced into the adapted 1103 rootstock.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) is the causative agent of Pierce's disease of grapevines. Collectively, a team of researchers (Lindow, Dandekar, Labavitch/Powell, and Gilchrist) has identified or constructed and advanced the evaluation of five (**Table 1**) novel genes (DNA constructs) that, when engineered into grapevines, suppress symptoms of Pierce's disease by reducing the titer of *Xf* in the plant, reducing its systemic spread in the plant, or blocking *Xf*'s ability to trigger Pierce's disease symptoms. These projects have moved from the proof-of-concept stage in the greenhouse to characterization of Pierce's disease resistance under field conditions where current data indicate that each of the five transgenes, introduced as single constructs, reduces the disease levels under field conditions. These existing field trials will continue through 2016. Importantly, preliminary data indicates that each of the five DNA constructs, when incorporated into transgenic rootstock, has shown the ability to protect non-transformed scions, with obvious benefit: any of many unmodified varietal scions can be grafted to and be protected by any of a small number of transformed rootstock lines. The ability of transgenic rootstock to protect all or most of the scion, even at a distance from the graft union, is currently being tested. The objective described herein addresses the issue of durability, the capability of genetic resistance to avoid being overcome by evolving virulent versions of the *Xf* pathogen, a critical factor for a long-lived perennial crop such as grapevines. This approach involves "stacking," a combination of distinct protective transgenes in a single rootstock line, which is intended to foster not only durability but also more robust protection of the non-transformed scion against Pierce's disease. The proposed changes are the next logical step toward achieving commercialization of transgenic resistance. Stacked transgene rootstock lines will be ready for evaluation in 2015 under controlled greenhouse conditions while ramets of the most suppressive transgenic lines are being produced for field testing to be initiated by 2016.

INTRODUCTION
The primary motive for combining genes in combination is to create durable resistance, resistance to *Xylella fastidiosa* (*Xf*) that will last the life of the vine. Since the five DNA constructs (**Table 1**) have biochemically

distinct mechanisms of action, having two or more such distinctly acting DNA constructs "stacked" in the rootstock should reduce the probability of *Xf* overcoming the resistance. With multiple, distinct transgenes, *Xf* would be required to evolve simultaneously multiple genetic changes in order to overcome the two distinct resistance mechanisms.

Additionally, there could be favorable synergistic protection when two or more resistance-mediating DNA constructs are employed. There are data indicating synergism in other crops. For example, the paper, "Field Evaluation of Transgenic Squash Containing Single or Multiple Virus Coat Protein Gene Constructs for Resistance to Cucumber Mosaic Virus, Watermelon Mosaic Virus 2, and Zucchini Yellow Mosaic Virus" (Tricoli et al., 1995), describes the stacking of several genes for virus resistance in squash. Note that David Tricoli was the lead author in that paper, and he will be doing the stacking transformations in this proposal. Additionally, the Dandekar laboratory has successfully stacked two genes blocking two different pathways synergistically to suppress crown gall (Escobar et al., 2001). Experiments to be conducted here will evaluate potential synergism in suppression of Pierce's disease symptoms and in reducing *Xf* titer for inoculations distant from the graft union.

Table 1. Genes selected to suppress Pierce's Disease in grape.
The table lists genes names, presumed function and the selectable marker of the current binary in which the genes reside.

Gene	function
CAP	clearing/antimicrobial
PR1	host anti-death
rpfF	changing quorum sensing (DSF)
456	non-coding microRNA activates PR1 translation
PGIP	inhibits polygalacturonase; suppressing movement

Briefly, we describe below information on the history and impact of the genes deployed as single transgenes currently in USDA APHIS approved field trials.

rpfF and DSF -- Steven Lindow
The Lindow lab has shown that *Xf* uses diffusible signal factor (DSF) perception as a key trigger to change its behavior within plants (Lindow, 2013). Under most conditions DSF levels in plants are low since cells are found in relatively small clusters, and hence they do not express adhesins that hinder their movement through the plant and are needed for vector acquisition, but instead actively express extracellular enzymes and retractile pili needed for movement through the plant (Chatterjee et al., 2008). Accumulation of DSF in *Xf* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in the pathogen, but the overall effect is to suppress its virulence in plants by increasing its adhesiveness to plant surfaces and also suppressing the production of enzymes and genes needed for active movement through the plant (Beaulieu, 2012)

CAP and PGIP -- Abhaya Dandekar
The Dandekar lab has successfully participated in the two field plantings to investigate two greenhouse-tested strategies to control the movement and to improve clearance of *Xf*, the xylem-limited, Gram-negative bacterium that is the causative agent of Pierce's disease in grapevine (Dandekar, 2013). 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* PG activity necessary for long distance movement (Aguero et al., 2005). The second strategy enhances clearance of bacteria from *Xf*-infected xylem tissues by expressing a chimeric antimicrobial protein (CAP), that consist of a surface binding domain that is linked to a lytic domain. The composition and activity of these two protein components have been described earlier (Dandekar et al., 2012).

PR1 and microRNA UT 456 -- David Gilchrist
The Gilchrist lab is focused on the host response to *Xf* by identifying plant genes that block a critical aspect of grape susceptibility to *Xf*, namely the inappropriate activation of a genetically conserved process of programmed

cell death (PCD) that is common to many, if not all, plant diseases in which cell death is the visible symptom of disease. We have demonstrated previously that blocking PCD, either genetically or chemically, can block disease symptoms and bacterial pathogen growth in several plant-bacterial diseases (Richael et al., 2001, Lincoln et al., 2002, Harvey et al., 2007). In the current project with Pierce's disease, we developed a functional screen and identified novel anti-PCD genes from cDNA libraries of grape and tomato. Two of these grape sequences (PR1 and UT456), when expressed as transgenes in grape, suppressed Pierce's disease symptoms and dramatically reduced bacterial titer in inoculated plants under greenhouse conditions. Assays with various chemical and bacterial inducers of PCD confirmed that the PR1 was capable of blocking PCD in transgenic plant cells (Sanchez et al., 2014a). The results with PR1 led to a second discovery of a novel mechanism linking PR1 and UT456 in mode of action. Initially, we discovered that the mechanism blocking PR1 translation is due to the ability of the PR1's 3'UTR to bind to a region in the PR1 coding sequence to prevent translation. Sequence analysis of UT456 revealed a strong sequence complementarity to a region in the PR1 3'UTR. Additional experiments confirmed a functional link of the noncoding UT456 sequence to PR1 resides in the ability of the UT456 sequence, in the form of a microRNA, to bind to the PR13'UTR and release the translational block of PR1 translation. Hence, in both transgenic plants the mechanism of suppression of Pierce's disease symptoms depends on translation of either the transgenic or the endogenous PR1 message in the face of *Xf* -trigger cell stress (Sanchez et al., 2014b; Gilchrist et al., 2011, 2013).

OBJECTIVES

The objective is to introduce pairs of protective constructs into an adapted grapevine rootstock. The resulting lines will be tested for efficacy by inoculation with *Xf* in a preliminary greenhouse experiment to identify the most protective lines from each combination of genes. As new starting materials for this effort, current constructs (**Table 1**) will be transferred in combinations of two constructs per new dual binary with a single selectable marker, e.g., hygromycin. The nt-PGIP to be used in these constructs is a version of the Labavitch PGIP that was modified in the Dandekar laboratory to include a signal peptide obtained from a grapevine xylem secreted protein (Aguero et al., 2008).

Dual gene expression binaries

The strategy is to prepare dual plasmid constructs bearing a combination of two of the protective genes on a single plasmid with single selectable marker. The binary backbone is based on pCAMBIA1300 (Hajdukiewicz,P, et.al., 1994). Binaries will be constructed to express two genes from two 35S promoters as has been done for apple and walnut (Dandekar et al., 2004; Walawage et at., 2013). The DNA fragments containing transcription units for expression of the transgenes will be flanked by rare cutting restriction sites to be ligated into the backbone. These dual expression vectors will be analyzed by sequence and PCR to verify integrity and orientation of the transcription units to ensure all transcription going in one direction. Each dual protective gene plasmid will be introduced into embryogenic grapevine culture in a single transformation, i.e., conventional grapevine transformation in the Parsons Plant Transformation Facility.

Timeline for individual steps for the stacked transformations (Figure 1)

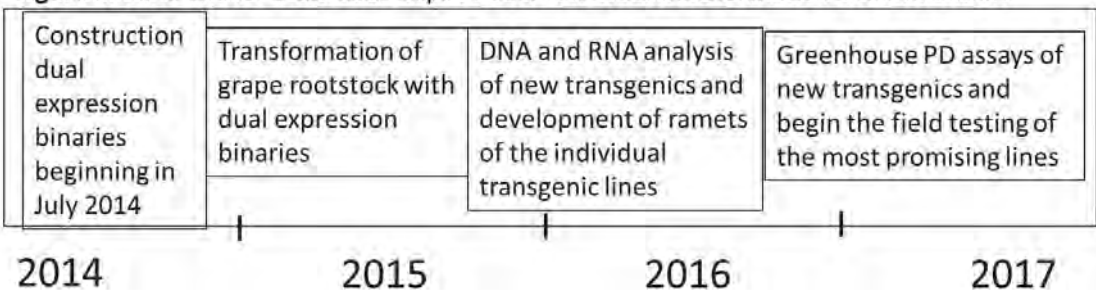
The transformation should take six months to confirm that the selection of callous is effective. The regeneration of plants will take another 10 months. Following regeneration, individual transgenic plants (12-15) will be expanded to ramets of 20 to 30 rootstock plants to be used as for testing of the level of expression before grafting to susceptible scions for inoculation first under greenhouse conditions (20 months), and then moved to the USDA APHIS approved field in Solano County for field evaluation, with mechanical inoculation where infection has been successful with definitive differentiation of resistance due to the respective transgenes compared with non-transgenic controls. The current field experiment has confirmed that the mechanical inoculation method will lead to complete death of unprotected (non-transgenic) control plants compared with the transgenic lines.

Assay of transgenic plants containing combinations of Pierce's disease suppressive transgenes

The first three dual transgenic events now in progress are expected to yield 12-15 individual transgenic plants (36-45 plants) that will be assayed for the relative level of RNA expression of both transgene pairs with only the three highest expressers of each construct expanded to the clonal propagation stages (ramets). Each of the three highest expressing plants will be clonally copied into 10 ramet sets of each selected individuals (3 highest expressers x 10 plants x 3 events = 90 initial plants) and moved to the greenhouse for growth to 50 cm before mechanical inoculation. The "mother" plant of each of three highest expressers will be retained and available for expanding to an additional set of ramet plants for grafting, if the infection data indicates Pierce's disease suppression. This

latter population of plants will undergo grafting in the second year of this grant by the micro propagation procedure developed and used successfully in the Gilchrist lab to produce the grafted plants now in the Solano County field experiment. The original transgenics (mother plants) will be retained in a greenhouse archive after production of the clonal copies.

Figure 1. Time line for individual steps of dual construct transformation and evaluation



Preliminary rapid evaluation of the transformed plants under controlled conditions by RNA analysis of the new transgenics, ramet production, and greenhouse inoculation.
Ten plants of each of the three highest expressers will be inoculated along with untransformed control plants of each transgenic line under controlled greenhouse conditions using inoculation and evaluation techniques established in previous experiments. The greenhouse inoculation step will use the GFP-tagged *Xf* used previously to measure bacterial population levels and movement in the xylem, along with monitoring the plants for Pierce's disease symptoms. We will use established methods, including quantitative PCR (qPCR) and confocal laser scanning fluorescence microscopy, to monitor GFP-tagged *Xf* methods to measure bacterial population levels and movement in the xylem.

This technique has been employed successfully under the same conditions to measure the effect of the single transgene-based grafted plants currently in the field experiments. The greenhouse inoculation experiments will provide useful data on transcription and translation of the paired transgenes, potential protection against Pierce's disease, and detailed biological data to support pursuit of intellectual property protection prior to the longer-term field experiment. Based on past experience, these experiments can be concluded in one year following delivery of the transgenic plants.

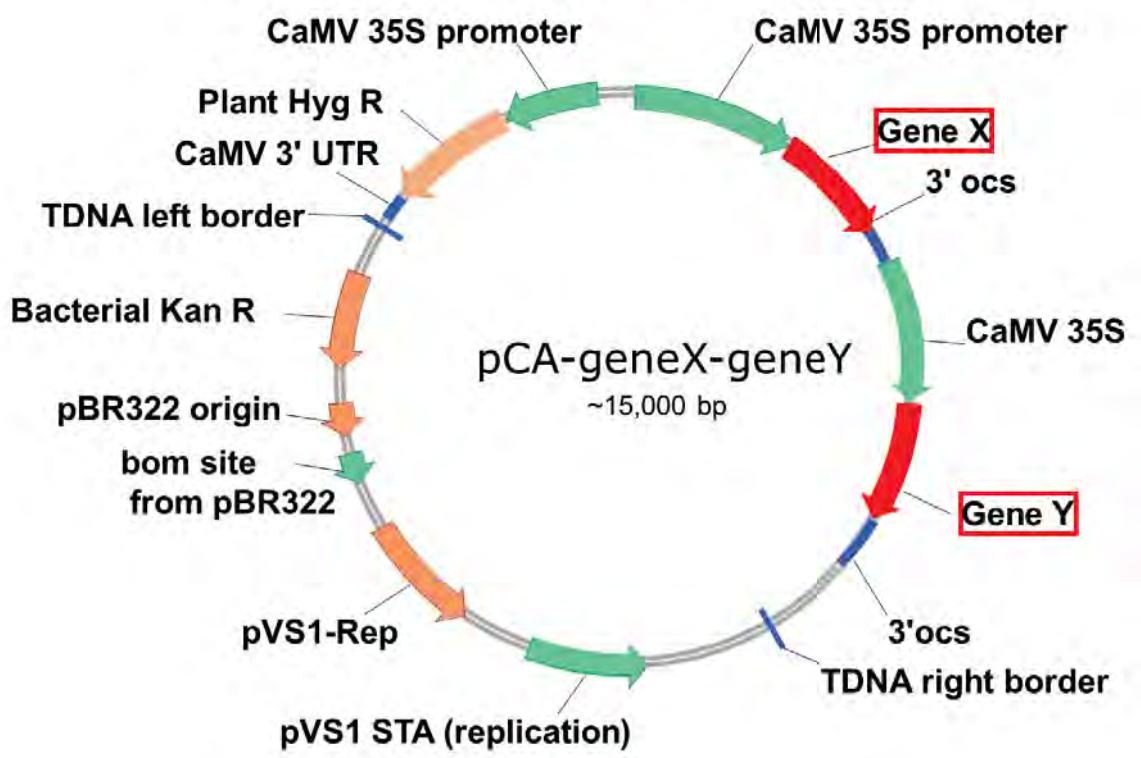
RESULTS AND DISCUSSION
This project began in July 2014. To date, we have prepared dual binary plasmids bearing a combination of two of the following protective genes on a single plasmid with a single selectable marker.

1. CAP:PR1	6. PR1:456
2. PGIP:456	7. rpfF: PR1
3. CAP:456	8. CAP: rpfF
4. PGIP:PR1	9. PGIP:rpfF
5. PGIP:CAP	10. RpfF: 456

The binary backbone is based on pCAMBIA1300 and was constructed to express two genes from two 35S promoters by Dr. Lincoln (**Figure 2**). The DNA fragments containing transcription units for expression of the transgenes are flanked by rare cutting restriction sites to be ligated into the backbone. These dual expression vectors were analyzed by sequence analysis and PCR to verify integrity and orientation of the transcription units to ensure all transcription going in one direction. All those listed were confirmed to have the proper sequence and orientation. Each of these dual protective gene plasmids 1-3 above have been provided to David Tricoli in the transformation facility and are now being introduced into embryogenic rootstock 1103 grapevine cultures in a single transformation via the conventional grapevine transformation procedure in the Parsons Transformation Facility. Combinations 1-3 have been constructed by Dr. Lincoln and delivered to the transformation facility.

Combinations4-6 have been constructed and are awaiting in the queue for transformation. Combinations 7-10 are in the process of being constructed and analyzed for sequence and orientation.

Figure 2. Dual expressing binary plasmid with genes X and Y indicated



CONCLUSIONS

Our capacity to achieve all the objectives is essentially assured based on prior accomplishments. All techniques and resources are available in the lab and proven reliable, informative, and reproducible. This project will bring together a full time research commitment for this team of experienced scientists to Pierce's disease. Each of the senior personnel, including Dr. Lincoln, have been with this project since 2007 and have different skills and training that complement changing needs of this project in the areas of molecular biology, plant transformation, and analysis of transgenic plants. This includes both greenhouse and field evaluation of protection against Pierce's disease. Commercialization of the currently effective anti-Pierce's disease containing vines and/or rootstocks could involve partnerships between the UC Foundation Plant Services, nurseries, and, potentially, with a private biotechnology company.

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FUNDING AGENCIES

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CONDUCTING PIERCE'S DISEASE SYMPTOM FIELD EVALUATIONS AT THE SOLANO COUNTY RESEARCH BLOCK

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OBJECTIVES

The Product Development Committee (PDC) of the California Department of Food and Agriculture (CDFA) Pierce's Disease and Glassy-winged Sharpshooter Board requested research into uniform evaluation of Pierce's Disease symptoms exhibited by grapevines developed by four Principal Investigators (PIs) as part of the Board's research portfolio. These vines are planted in a single research block in Solano County. The PI and a team of grape pathologists monitored these blocks and took data on disease severity in September 2014, and will again in May 2015. An analysis of the variation in the data overall and between individuals will be calculated. This research will help the PDC make future decisions about evaluating products of the research funded by the Board.

INTRODUCTION AND DISCUSSION

Evaluation Team

PI Golino and 5 Foundation Plant Services (FPS) plant pathologists with many years of grape disease experience made up the core evaluation team. Two plant pathology PhD graduate students with grape pathology thesis research were also invited to participate. A Viticulture Consultant scored the vines as well. Each individual participated in training in evaluating Pierce's disease symptoms according to the scoring system described below. That training included 'calibration' by examining a subset of vines including healthy and *Xylella fastidiosa* inoculated controls to ensure that ratings were as uniform as possible. All vines were evaluated in mid-September, and will be again in spring 2015.

Scoring Technique

A visual rating system on a scale of 1-5 was used by each member of the team to rate every vine individually. All vines were labelled by row and vine number. Data was collected by row and vine number without any information about the particular treatment that vine had received. This is a slightly modified version of the rating system used by the Kirkpatrick lab.

Golino / Gilchrist Simplified Rating System

0 – Healthy vine. All leaves green with no scorching, good cane growth, no cordon dieback or failure to push canes at bud positions. Dry or yellowing leaves may be present but do not show characteristic Pierce's disease symptoms.

1 – Leaves on one or two canes showing characteristic Pierce's disease scorched leaf symptoms. No evidence of physical damage to leaf petiole(s) or cane(s). On cane in question, at least TWO leaves are symptomatic, 1 single leaf is NOT enough to warrant a rating of #1.

2 – More than 2 canes possess multiple scorched leaves. HOWEVER, canes with symptomatic leaves are still confined to just one area of the vine.

3 – Canes with clearly scorched leaves are found on several canes including canes which have not been inoculated.

4 – Ends of cane(s) begin dying back; some canes failed to push in the spring. Vine is clearly symptomatic on all or nearly all surviving canes. Main point is that the vine is NOT yet dead but is clearly facing a terminal fate.

5 –Dead vine or a vine that had a few canes weakly push in the spring but those canes later died with onset of hot temps in July or August. There are NO visible signs of other potential problems such as gophers, crown gall, P*hytophthora*, or *Eutypa*/*Bot* dieback of cordons.

If a vine appears to have died for reasons other than Pierce's disease, that will be entered in the comments field for that vine and no score will be entered in the rating field.

Analysis of Data

The disease scores will be analyzed using a chi-squared test and contingency table analyses, since it is the frequency of scores that is being evaluated rather than quantitative data (Eskridge, 1995). An overall chi-square statistic (χ^2) will be calculated to determine if the null hypothesis (no difference between treatments) can be rejected or not. If the null hypothesis is rejected, the contingency table will be subdivided into smaller tables and chi-square analyses will be used to identify how the treatments differ.

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EVALUATION OF PIERCE'S DISEASE RESISTANCE IN TRANSGENIC *VITIS VINIFERA* GRAPEVINES EXPRESSING *XYLELLA FASTIDIOSA* HEMAGGLUTININ PROTEIN

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Reporting Period: The results reported here are from work conducted October 1, 2013 to October 1, 2014.

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 HfxA 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 four years we produced transgenic HA-expressing tobacco and grapevine lines; these transgenic lines, grown in the greenhouse, exhibited less severe symptoms of Pierce's disease following mechanical inoculation of *Xf* cells. With the assistance of the Public Intellectual Property Resource for Agriculture (PIPRA) we secured all the necessary permits to plant these lines in the field in spring 2013. These vines grew well and were trained up to the wire and established as a conventional bilateral cordon vines. We cut back the shoots to two buds and then mechanically inoculated four shoots/vine with a mixture of Temecula and Stag's Leap *Xf* strains in April 2014, the same timeframe that other Pierce's disease workers inoculated their transgenic vines a couple of years ago. Pierce's disease symptoms were rated in September 2014 on the inoculated shoots and we noted whether adjacent uninoculated shoots developed Pierce's disease symptoms. Over 90% of the inoculated canes showed scorch symptoms typical of Pierce's disease in September 2014, indicating that our inoculations were successful. In only one instance did we find Pierce's disease symptoms on an adjacent, uninoculated shoot.

In January 2015 the shoots will be trimmed to two buds and the emerging shoots will be rated for Pierce's disease symptoms in August 2015. This second year of rating Pierce's disease symptoms will be the greatest test of this potential Pierce's disease resistance approach to determine whether the expression of the *Xf* HA genes would slow or prevent the movement of *Xf* back down into the permanent cordon to cause systemic Pierce's disease.

LAYPERSON SUMMARY

Our 7+ 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 that vines inoculated with wild-type (wt) *Xylella fastidiosa* (*Xf*) cells (Guilhabert and Kirkpatrick, 2005). HA mutants no longer clumped together in liquid cultures like wt cells, nor did HA mutants attach to inert substrates like glass or polystyrene 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 wt cells and they were compromised in binding to chitin and sharpshooter tissues compared to wt cells (Killany and Almeida, 2009). 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 wt cells, thus providing another line of evidence regarding the importance of these proteins in *Xf* pathogenesis and insect transmission.

We are now evaluating our hypothesis that HAs expressed in transgenic grapevine xylem sap may act as a "molecular glue" that would aggregate and thus slow the movement of wt *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. We finished a greenhouse Pierce's disease disease severity screening of the

eight HA transgenic lines that were produced. The results were encouraging in that all of the HA-transgenic lines had lower disease ratings than non-transgenic controls.

With the assistance of the Public Intellectual Property Resource for Agriculture (PIPRA) we secured all the necessary permits to plant these lines in the field in spring 2013. These vines grew well and were trained up to the wire and established as conventional bilateral cordon vines. We cut back the shoots to two buds and then inoculated four shoots/vine with the Fetzer strain of *Xf* in April 2014, the same timeframe that other Pierce's disease workers inoculated their transgenic vines a couple of years ago. Pierce's disease symptoms were rated in September 2014 on the inoculated shoots and we noted whether adjacent uninoculated shoots developed Pierce's disease symptoms. Over 90% of the inoculated canes showed scorch symptoms typical of Pierce's disease in September 2014, indicating that our inoculations were successful. In only one instance did we find Pierce's disease symptoms on an adjacent, uninoculated shoot. Pierce's disease symptom severity was lower in the inoculated HA-transgenic grapevines than in the *Xf*-inoculated non-transgenic controls.

In January 2015 the shoots will be trimmed to two buds and the emerging shoots will be rated for Pierce's disease symptoms in August 2015. This second year of rating Pierce's disease symptoms will be the greatest test of this potential Pierce's disease resistance approach for determining whether the expression of the *Xf* HA genes would slow or prevent the movement of *Xf* back down into the permanent cordon to cause systemic Pierce's disease.

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 have shown that Hxfs were regulated by an *Xf*-produced compound know as diffusible signal factor (DSF) (Newman et al., 2004), and that they were important factors in insect transmission (Killiny and Almeida, 2009). The *Xf* HAs essentially act as a "molecular glue" that are essential for cell-cell attachment and likely play a role in *Xf* attachment to xylem cell walls and contribute 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 (Voegel and Kirkpatrick, 2010). In the third year we identified a two-component transport system that mediates the secretion of *Xf* HAs. In the final years of the initial project we expended considerable time and effort in constructing transgenic tobacco and grapevines that expressed HA. We conducted pathogenicity evaluations of our nine HA-transgenic lines. Disease severity ratings in greenhouse grown vines were considerably less in the transgenic lines than the non-transgenic controls. Permits to establish a field planting of the HA vines were obtained with the assistance of the Public Intellectual Property Resource for Agriculture (PIPRA) and a field trial was established in April 2013. The vines were inoculated with *Xf* in spring 2014 and Pierce's disease symptoms of HA-transgenics were compared to non-transgenic, *Xf*-inoculated controls in September 2014. Vines will then be pruned back to two buds and allowed to go through the winter; symptoms on the vines will then again be rated in September 2015.

OBJECTIVES

Revised as per instructions of the 2013 Panel Review Committee

1. Complete the characterization of grape transgenic plants over-expressing *Xf* hemagglutinin (Hxf) protein.
2. Mechanically inoculate HA-transgenic grapevines growing in the greenhouse with wild-type (wt) *Xf* and evaluate the effect on Pierce's disease symptom expression and movement in the xylem by culture and quantitative PCR (qPCR).

3. Secure permits to plant HA transgenic lines in the field in Solano County. Plant transgenic vines in the field and train them into a traditional bilateral cordon.
4. Inoculate four canes on each HA-transgenic field vine with wt *Xf* in spring 2014. Rate Pierce's disease symptoms in September 2014 on inoculated canes. Take samples for qPCR.
5. Cut back all canes to two buds and rate cane growth in spring 2015 and Pierce's disease symptoms in September 2015 to determine if the expression of *Xf* HA in the transgenic vines retarded or prevented movement of the inoculated *Xf* into the cordons, which typically results in systemic Pierce's disease.

RESULTS AND DISCUSSION

PLEASE NOTE: Results described below for Objectives 1, 2, and 3 were reported in the 2013 Proceedings. I have included this for the reader's information only. Objective 4 Results represents new data obtained in 2014.

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

Twenty-one transgenic Thompson Seedless grape plants that potentially over-expressed the Hxf protein in the xylem using a binary plasmid with a polygalacturonase secretory leader sequence were obtained from the UCD Plant Transformation Facility in September 2010. These were initially obtained as small green 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 5 of 9 transgenic lines containing *Xf* HA adhesion domains (AD 1-3) labeled as SPAD1 and 3 of 12 transgenic lines of the full-length HA, labeled PGIP220 in **Table 1** below, had the HA gene inserted into the grapevine chromosome.

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

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

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

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

We were very interested in determining whether any of these lines possessed Pierce's disease resistance by testing the lines in the greenhouse as soon as we had sufficient plants, rather than waiting for the results of extensive ELISA and Western blot analysis of transgenics to determine if HA protein could be detected in grapevine xylem sap. On December 8th and 9th of 2011 we inoculated 10 reps of each of the 9 PCR-positive transgenic lines with 40 ul of a 108 suspension of *Xf* Fetzer in PBS, typically done as two separate 20 ul inoculations on each vine, an amount of inoculum that would be far greater than what a sharpshooter injects into a vine.

We also inoculated untransformed Thompson Seedless and two transgenic lines that did not contain HA inserts by PCR analysis, shown as Transformed Non-transgenic TS 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 TS control, inoculated at the same time as the transgenic vines, had a mean disease rating of 3.65 while two of the lines, one containing the truncated HA fragment AD1-3 and one line containing the full-length native HA protein, had the lowest disease ratings of 1.5. Most of the other lines had mean disease severity ratings below 2.0 and the average disease ratings for all of the lines representing the two HA constructs had disease ratings below 2.0.

Considering the large amount of inoculum that was used, we are pleased with this promising preliminary result. We will soon be quantifying by culture and qPCR the amount of *Xf* in each of these lines. While clearly some disease symptoms were evident, the severity was much less than the control and this could very well reflect lower *Xf* populations in the transgenic lines. If this does indeed turn out to be true then we might have produced a moderately resistant grapevine that could very well end up being like a Muscadine grapevine, i.e. they can be infected with *Xf* but populations are not high enough to compromise fruit quality. The original hypothesis was that transgenic vines producing HA in the xylem sap might facilitate clumping of *Xf* cells and slow their ability to colonize a mature vine during a growing season such that the incipient infection might very well be pruned off in the dormant season. It will take a couple of years to plant and train to a cordon system that would be then mechanically inoculated, or hopefully with the assistance of the Almeida lab insect inoculated with *Xf*. These initial greenhouse results with young vines certainly warrant further evaluations.

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

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

Transgenic lines highlighted in tan color are the 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 3a. Secure permits to plant HA transgenic lines in the field site in Solano County.
This objective was completed with the assistance of PIPRA.

Objective 3b. Plant transgenic vines in the field.
Approximately 50 HA-transgenic vines representing all the transgenic lines that were produced were planted in the field in April 2013 and trained as bilateral cordons (**Figure 2**).

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

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

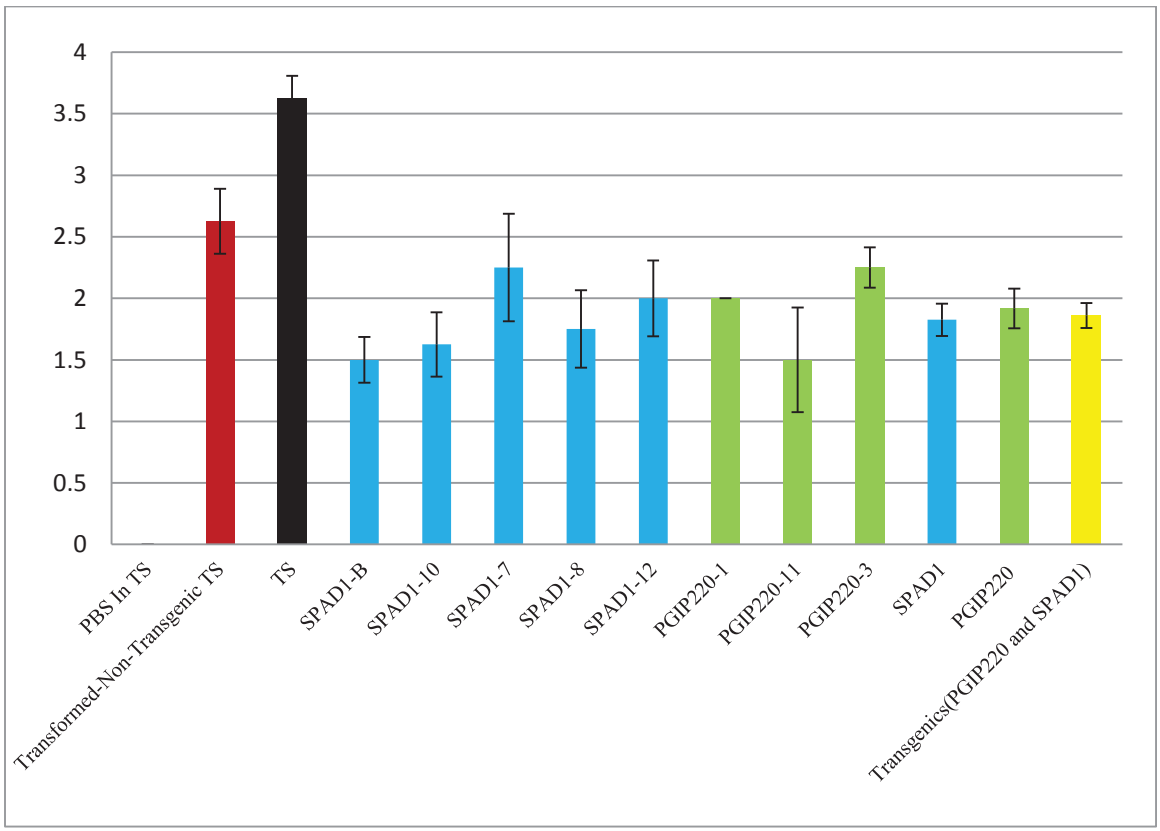

Figure 1. Graph showing the mean disease ratings from 0 to 5 (0 is healthy; 5 is dead) of Pierce's disease symptoms in Thompson Seedless (TS) and transgenic (SPAD1 and PGIP220) vines inoculated with *Xf* Fetzer at 16 weeks post inoculation, except for the Transformed-Non-Transgenic TS, which was inoculated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

Objective 4. Inoculate four canes on each HA-transgenic field vine with wt *Xf* in spring 2014.
Rate Pierce's disease symptoms in September 2014 on each inoculated cane. Inoculate non-transgenic Thompson Seedless canes as positive controls, leave two vines of each transgenic line as uninoculated controls.

A combination of *Xf* Temecula and Stags Leap strains were grown on solid PD3 medium and the cells were harvested and suspended in 1XPBS to a concentration of 10 X 8th. Four canes on replicates of each transgenic line were labelled and then mechanically inoculated 1X with a 20 ul drop of *Xf* cell suspension. Inoculations were done in mid-May 2014 and inoculum droplets were quickly taken up by the transpiring canes.

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

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

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

Overall success in inoculating canes in transgenic and non-transgenic vines was very high. In some cases the tags marking inoculated canes in HA-transgenic vines were missing so no rating was made. 0 ratings of canes on HA-transgenic canes occurred on vines where at least two of the other canes on that vine expressed some Pierce's disease symptoms; thus we believe the inoculum that was used to inoculate 0 scoring canes was viable. However, it is certainly possible that the inoculum was not taken into actively transpiring xylem vessels which could result in an unsuccessful inoculation.

Overall Pierce's disease symptom severity was higher in the non-transgenic positive controls than in the HA-transgenic vines. These results were similar to what we observed in the greenhouse inoculations. It was also clear from the field inoculations that none of the transgenic lines completely prevented the onset of Pierce's disease symptoms, again results that were observed in greenhouse trials.

Cane samples were collected from all *Xf*-inoculated lines for testing by qPCR. This should give us some information concerning the relative *Xf* titers in transgenic vs. non-transgenic inoculated vines.

Xylem sap was also extracted from all uninoculated transgenic controls and the sap will be tested for expressed HA protein by ELISA and western blot analysis to determine if detectable amounts of HA are present in transgenic xylem sap.

Objective 5. Cut back all canes to two buds and rate cane growth in spring 2015 and Pierce's disease symptoms in September 2015 to determine if expression of *Xf* HA in the transgenic vines retarded or prevented movement of the inoculated *Xf* into the cordons, thus preventing systemic Pierce's disease. Canes will be cut back to two buds once vines are completely dormant in January/February 2015.

Table 3. Pierce's disease symptom ratings of HA-transgenic grapevines.

Transgenic Lines	# Inoculated Vines	# of PD Rated Canes	Mean Disease Rating
			Ratings individual canes
Adhesion Domain Lines			
AD 6	3	10	0=5; 1=4; 2=2 **0.7**
AD 7	4	15	0=7; 1=2; 2=6 **0.9**
AD 8	5	20	0=2; 1=5; 2=12; 3=1 **1.6**
AD 10	3	10	0=1; 1=2; 2=6; 3=1 **1.7**
AD 12	5	19	0=5; 1=5; 2=9 **1.2**
Complete HA Gene			
220-1	4	10	0=4; 1=1; 2=6; 3=1 **1.6**
220-3	3	12	0=4; 1=1; 2=6; 3= 1 **1.3**
220-11	3	10	0=8; 1=1; 2=1 **0.3**

Note: Pierce's disease symptoms of inoculated transgenic canes were made by Kirkpatrick on Sep. 14, 2014. Symptoms ratings of individual canes were as follows:
0 = no symptoms of Pierce's disease, i.e., no scorched leaves on cane
1 = 2 to <10% scorched leaves on cane
2 = >10% to <75% scorched leaves on cane
3 = all leaves showing Pierce's disease scorch symptoms, no cane dieback observed
4 = cane dieback, cane still alive
5 = dead cane

Rating of inoculated NON-transgenic Thompson canes. Ratings of 18 inoculated canes on NON-transgenic Thompson vines were made by Lincoln and Gilchrist on Sep. 12, 2014. Symptoms of canes were as follows.

0 = no symptoms of Pierce's disease, i.e. no scorched leaves on cane	NO canes were rated 0
1 = 2 to < 25% of leaves with scorched leaves	NO canes were rated 1
2 = 25% to 50% of leaves with scorched leaves	NO canes were rated 2
3 = all leaves on cane were showing PD scorch symptoms	2 canes were rated 3
4 = all leaves scorched and some terminal cane dieback	3 canes were rated 4
5 = cane near death or dead	13 canes were rated 5

CONCLUSIONS

Eight HA-trangenic lines were shown by qRT-PCR to express HA mRNA. Greenhouse inoculations of the eight HA-transgenic Thompson Seedless grapes with cultured *Xf* cell showed all lines expressed less severe symptoms of Pierce's disease than inoculated, non-transgenic controls. All transgenic lines as well as non-transgenic Thompson Seedless vines that were used as positive and negative controls were planted in the field in spring 2013. The vines grew well and were trained as bilateral cordons. Four shoots on each vine were mechanically inoculated with wt *Xf* in May 2014. Pierce's disease symptoms on inoculated and uninoculated shoots were evaluated in September 2014. A high percentage of the inoculated shoots developed scorched leaves typical of Pierce's disease symptoms. However, disease severity ratings were lower among HA-transgenic lines than

inoculated non-transgenic grapevine controls. Canes from transgenic and non-transgenic vines were collected to determine *Xf* titers by qPCR. Xylem sap was extracted from uninoculated transgenic controls and the sap will be analyzed for *Xf* HA by ELISA and western blot analyses. All shoots will be pruned back to two buds in January/February 2015 and allowed to push during the 2015 growing season. Spring shoot growth will be rated and Pierce's disease symptoms will be recorded in September 2015 to determine if the *Xf* infections overwintered and systemically infected the vines. 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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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
We have identified a peptide that is able to inhibit the activity of *Agrobacterium vitis* (*Av*) polygalacturonase (PG), a PG closely related to *Xylella fastidiosa* (*Xf*) PG. It's possible that this peptide could also inhibit *Xf* PG activity. Additionally, we have shown that *Xf*/*Av* polygalacturonase chimeras can be produced as active and soluble proteins in *Escherichia coli* expression systems and they might be used as targets for phage panning to identify *Xf* PG inhibitory peptides.

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 ultimate goal 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 identify a PG inhibitory peptide 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 panning experiments using active recombinant *Xf* PG as the target. After three rounds of panning, phage that show a high binding affinity for *Xf* PG will be screened for their ability to inactivate PG using *in vitro* in reducing sugar assays. Once a suitable inhibitory peptide is discovered it will be cloned into an *Agrobacterium* binary vector and used to transform tobacco and grapevines by the UCD Plant Transformation Facility. These transgenic plants will then be inoculated with *Xf* and Pierce's disease symptoms, if any, in transgenic grapevines will be compared to non-transgenic plants. If significant disease inhibition is shown we will use these transgenic grapevines as rootstock to determine if they can also provide resistance to non-transgenic grafted scions.

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

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

RESULTS

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

Xf does not produce a detectable amount of PG when grown in biological media. Furthermore, attempts at expressing *Xf* PG in *Escherichia coli*, yeast, plant-based viral expression systems, and *Xf* PG based protein expression systems have not produced active *Xf* PG. An additional issue further complicating this situation is the fact the *Xf* PG enzyme seems to be unique among all other described active PG enzymes in that it has a different substrate binding amino acid motif. While *Xf* PG contains all the catalytic amino acids for the hydrolysis of 1,4-alpha-D-galactosiduronic linkages it has very different substrate binding amino acids. It has been shown in previous research with *Aspergillus niger* PG that mutation of this motif results in only 14% residual PG activity. This information suggests that it could be likely that *Xf* PG will have a substantially lower activity than other PGs, as well as a different manner of substrate binding, or perhaps preference for a different pectic substrate other than polygalacturonic acid.

The experiments we conducted focused on two questions. First, can we produce soluble *Xf* PG *in vitro* and second, do the altered amino acids in *Xf* PG result in a reduced enzyme activity, different degradation product sizes, or different substrate specificity? We decided to address both of these questions through the creation of a protein chimera using the catalytic and substrate binding domains of *Xf* PG to replace the catalytic and substrate binding domains of an active PG enzyme from a different prokaryotic plant pathogen. *Agrobacterium vitis* (*Av*) PG is the same size as *Xf* PG and likewise it is important in the virulence of *Av* to grapevines. Furthermore, a soluble, and more importantly and active form of *Av* PG can be easily produced in recombinant *E. coli* expression systems.

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

The N terminal and C terminal regions of both *Av* and *Xf* PG genes possess the most sequence variation. It follows that these highly variable regions are likely preventing recombinant *Xf* PG from being produced in a soluble form. If this is true it should follow that a chimera protein containing the variable N terminal and C terminal regions from the *Av* PG combined with the catalytic and substrate binding regions from the *Xf* PG could have an increased likelihood of being expressed in *E. coli* as a soluble protein. At the same time, such a chimera would allow us to assess the active site amino acids of *Xf* PG and determine if they can catalyze the degradation of PG. Furthermore, we hypothesized that using two chimeras, one constituting the major amino acids involved in catalysis (AX1APG) and the other containing the catalytic amino acids as well as the substrate binding amino acids (AX2APG) would allow us to determine if indeed these specific amino acids are biologically relevant for substrate binding (**Figure 1**). Moreover, if the *Xf* PG motif was mutated to the standard PG motif used by all other known active PGs we would expect to see a more biologically active enzyme. If the chimeric *Xf*/*Av* PGs were active, these could function as surrogates for *Xf* PG in inhibition assays. Initial results show that both chimeras are showing activity; however, the second chimera which contains more *Xf* PG sequence is less active (**Figure 2**).

AX1APG:

AX2APG:

Figure 1. Gene diagrams for each of the chimera constructs.

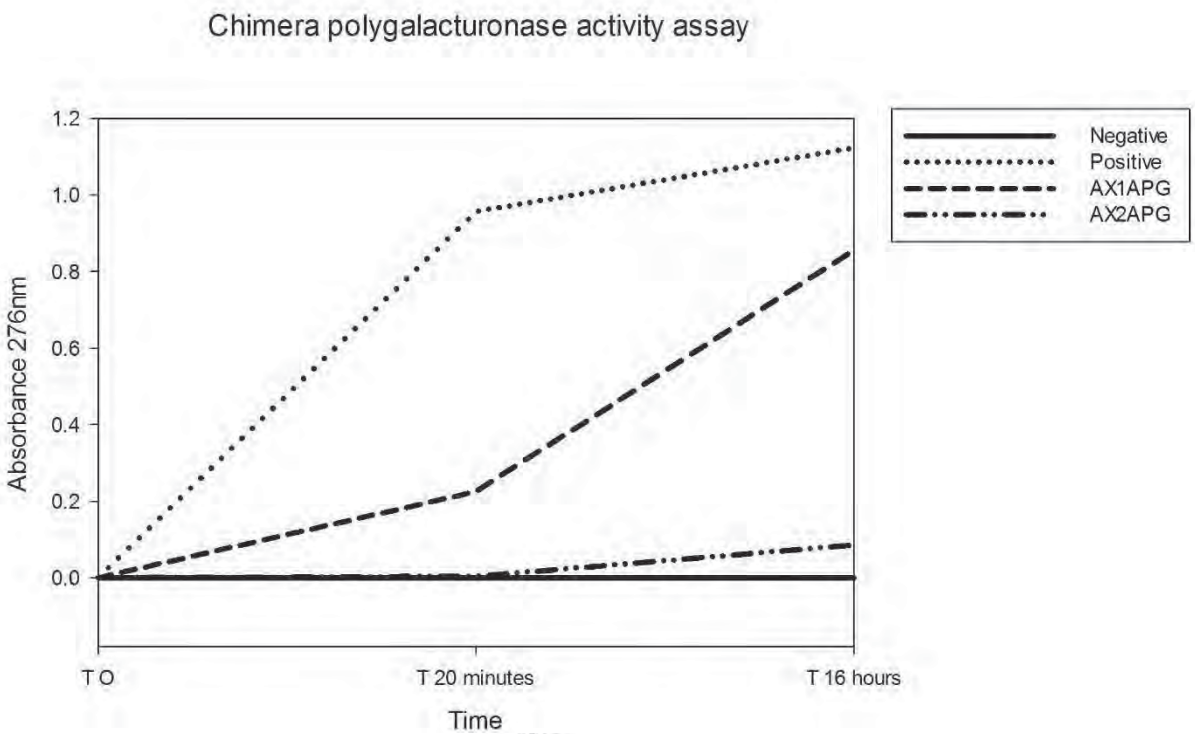

Figure 2. PG activity assay showing that both PG chimeras, AX1APG and AX2APG, are enzymatically active.

Objective 2. Isolate M13 phages that possess high binding affinities to *Av* PG from M13 random peptide antibody libraries.

Previous phage panning experiments conducted using smaller peptides constituting sections of the active site of *Xf* PG, FPLC purified recombinant *Xf* PG, and *Aspergillus aculeatus* PG as surrogates did not provide us with PG inhibitory peptides. For this reason we cloned the *Av* PG gene into an *E. coli* overexpression system to produce recombinant *Av* PG to use in inhibition assays. Experiments showed recombinant *Av* PG is produced in large amounts and is enzymatically active in cup plate assays. Phage panning was carried out according to a standard protocol using *Av* PG as the target but instead of eluting with pH or trypsin, the phage were eluted with the PG substrate, polygalacturonic acid. This should provide us with phage that are interacting with the substrate binding cleft.

Twenty individual phages from each library (PhD 7 linear and PhD 7 circular (New England Biolabs)) were isolated from blue plaques after the final round of panning and single stranded phage DNA was extracted and sequenced to identify the peptide sequences. No clear consensus sequence was determined for all of the phages in either the linear or circular libraries, however 10 of the phages were found to contain portions of a common motif, and one of the phage sequences represented 25% of the linear peptide phage pool. Six peptides were chosen as candidates for use in the inhibition assays, peptides A-F and a phage ELISA was performed which confirmed specific binding to *Av* PG (**Figure 3**).

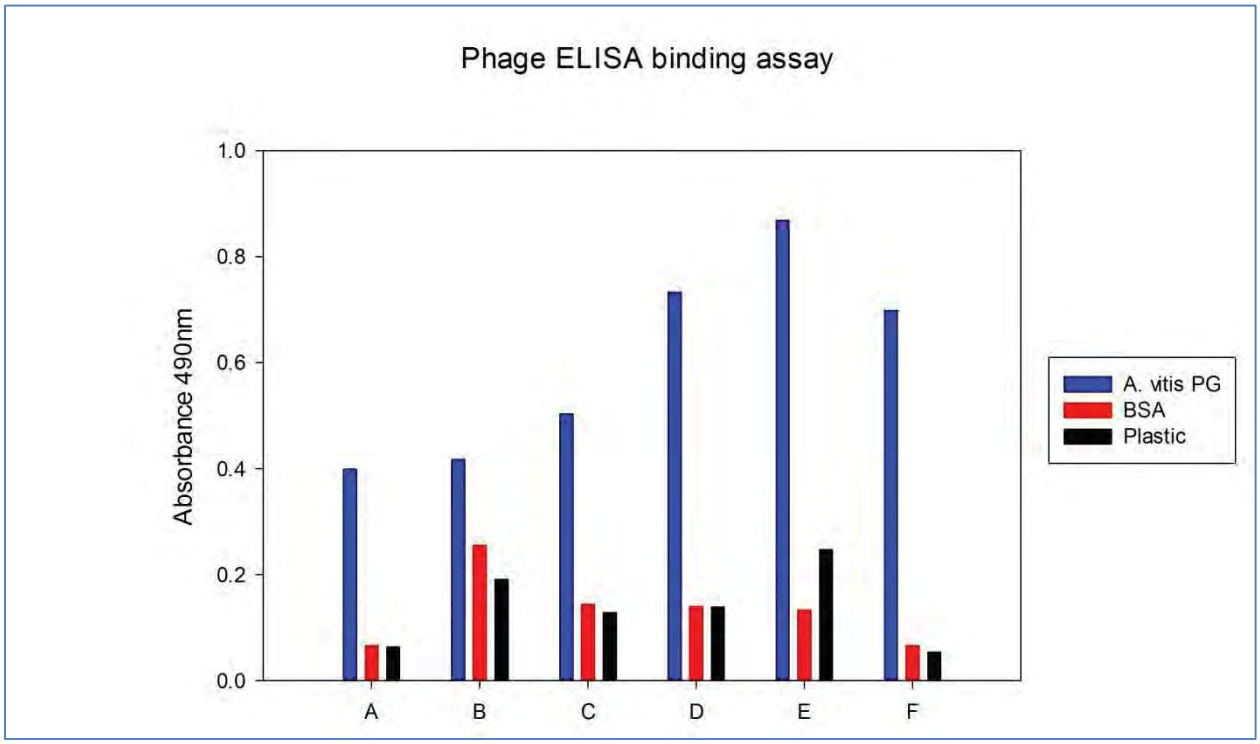

Figure 3. Monoclonal phage ELISA using *Av* PG, bovine serum albumin (BSA), and plastic as the targets. Each of the phage screened has a higher binding affinity for *Av* PG than BSA or plastic.

Objective 3. Determine if selected M13 phage and the gp38 M13 protein that mediates phage binding to *Xf* PG and surrogate PGs can inactivate PG activity *in vitro*.
All peptides (A-F) were synthesized with the C-terminal GGGS linker sequence included, as well as amidation of the C-terminus to negate the negative charge that a free C-terminus would generate. This negative charge would not have been present when the peptide linker was fused to the PIII phage protein. *Av* PG activity in the presence of each peptide was monitored and peptide A was the only peptide that showed an inhibitory effect on *Av* PG activity (**Figure 4**); peptide A was able to reduce enzyme activity approximately 20% compared to the positive control. This result does provide a proof of concept that phage panning can identify polygalacturonase inhibitory peptides.

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

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

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

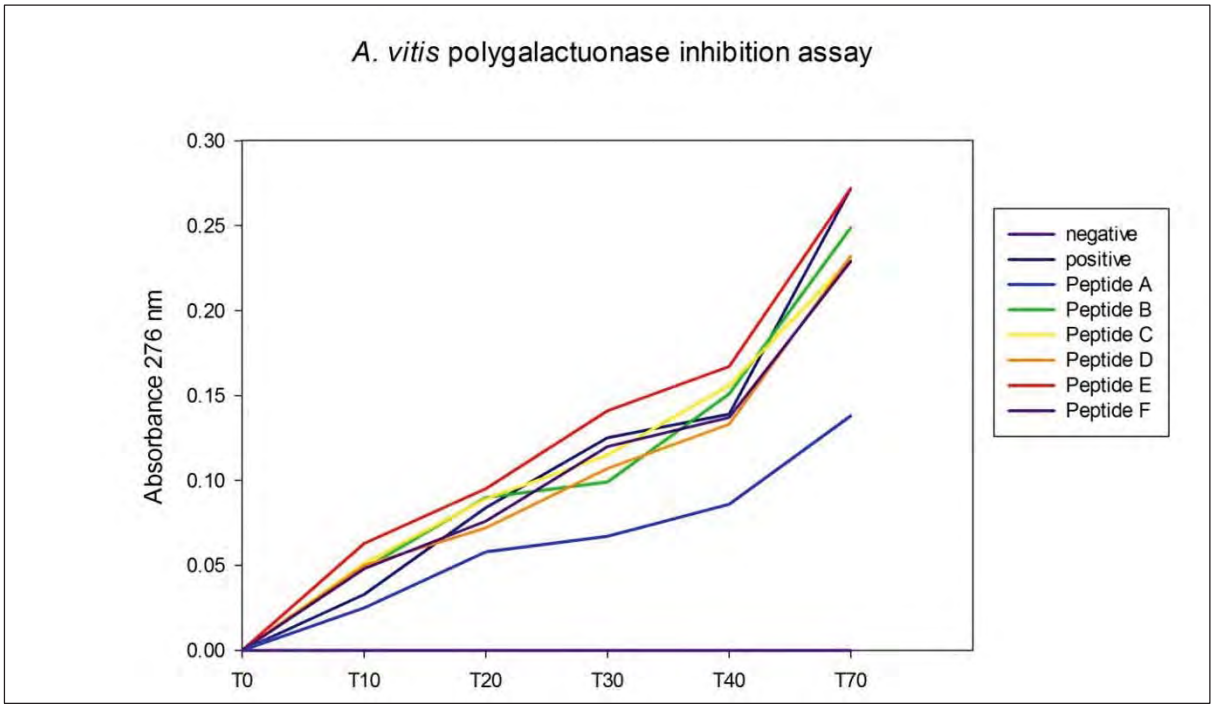

Figure 4. 2-cyanoacetamide reducing sugar activity assay showing Peptide A is able to inhibit *Av* PG activity. None of the other peptides were able to inhibit the activity of *Av* PG at the concentrations tested.

CONCLUSIONS

We have shown that phage panning is a reliable method to identify peptides which can inhibit PG activity. Additionally, we have shown that *Av*/*Xf* PG chimeras can be produced as active and soluble proteins in *E. coli* expression systems. Additionally, the active site amino acids of *Xf* PG are able to hydrolyze 1,4-alpha-D-galactosiduronic linkages of polygalacturonic acid and *Xf* PG possesses unique substrate binding amino acids. These chimeras should also be able to be used in phage panning experiments to select for inhibitors that target the active site of the enzymes.

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COMPARISON AND OPTIMIZATION OF DIFFERENT METHODS TO ALTER DIFFUSIBLE SIGNAL FACTOR MEDIATED SIGNALING IN *XYLELLA FASTIDIOSA* IN PLANTS TO ACHIEVE PIERCE'S DISEASE CONTROL

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ABSTRACT
Xylella fastidiosa (*Xf*) coordinates its behavior in plants in a cell density-dependent fashion using a diffusible signal factor (DSF) molecule which acts to suppress its virulence in plants. Artificially increasing DSF levels in transgenic grape greatly reduced disease severity in both greenhouse and field trials. We are investigating DSF production in additional transgenic grape varieties to determine the robustness of this strategy of disease control. *Xf* is relatively promiscuous in its production and perception of various unsaturated fatty acids as DSF signal molecules and we will explore ways to introduce the common, inexpensive fatty acid palmitoleic acid and other DSF homologs into plants following direct application. Improved DSF biosensors that we have developed will enable us to monitor the uptake and redistribution of such molecules in plants. Initial results suggest that the use of penetrating surfactants introduces sufficient amounts of this DSF-like molecule to alter behavior of *Xf* in plants. A naturally occurring *Burkholderia* strain capable of DSF production that is also capable of growth and movement within grape has been found that can confer increased resistance to Pierce's disease. We are exploring the biological control of disease using this strain. Initial results indicate that the movement of *Xf* within plants and disease symptoms are greatly reduced in plants in which this *Burkholderia* strain was inoculated either simultaneously with or prior to that of *Xf*.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) produces an unsaturated fatty acid signal molecule called diffusible signal factor (DSF). Accumulation of DSF in *Xf* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in the pathogen, but the overall effect is to suppress its virulence in plants by increasing its adhesiveness to plant surfaces and also suppressing the production of enzymes and genes needed for active movement through the plant. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption (pathogen confusion) as a means of controlling Pierce's disease. Elevating DSF levels in plants artificially reduces its movement in the plant. We will be introducing the gene conferring DSF production and to a variety of different grape cultivars to determine if they also will exhibit high levels of disease resistant as did the Freedom cultivar previously constructed. Topical application of commercially available unsaturated fatty acids capable of altering gene expression in *Xf* with penetrating surfactants can introduce sufficient amounts of these materials to reduce the virulence of the pathogen. A naturally-occurring *Burkholderia* strain reduces the movement of *Xf* and thereby its virulence in plants when inoculated prior to or simultaneously with *Xf*. By comparing disease control by these three methods the most efficacious and practical means of control can be identified.

INTRODUCTION
Our work has shown that *Xylella fastidiosa* (*Xf*) uses diffusible signal factor (DSF) perception as a key trigger to change its behavior within plants. Under most conditions DSF levels in plants are low since cells are found in relatively small clusters, and hence they do not express adhesins that would hinder their movement through the plant (but which are required for vector acquisition) but actively express extracellular enzymes and retractile pili needed for movement through the plant. Disease control can be conferred by elevating DSF levels in grape in various ways to "trick" the pathogen into transitioning into the non-mobile form that is normally found only in highly colonized vessels – "pathogen confusion." Transgenic 'Freedom' grape expressing the DSF synthase RpfF from *Xf* are much more resistant to disease than the wild-type plants in both greenhouse and field trials. Our work has shown, however, that RpfF is rather promiscuous and that *Xf* can both produce and respond to a variety of unsaturated fatty acids, and that the DSF species produced is influenced apparently by the particular substrates available within cells. It is possible that grape varieties might differ in their ability to produce DSF molecules

perceived by *Xf*. It will be important therefore to determine whether commercial grape cultivars can all produce DSF species capable of altering pathogen behavior in high amounts if transformed with the DSF synthase. Non-transgenic strategies of achieving pathogen confusion might be preferred by the industry. While endophytic bacteria capable of producing DSF species is an attractive strategy, until recently, strains capable of growth and movement within grape could not be found. However, we have now found a *Burkholderia* strain capable of DSF production that both colonizes grape and has conferred substantial disease control in preliminary studies. We will investigate the interactions of this endophyte with grape to optimize disease control and determine practical methods of its explication. We have found that *Xf* produces additional DSF species including 2-Z-hexadecenoic acid (C16-cis) that are much more active than C14-cis previously found, and that the common, inexpensive, unsaturated fatty acid palmitoleic acid is also reasonably active as a signal molecule in *Xf*. Using a new *Xf* biosensor for DSF in conjunction with such an abundant, inexpensive molecule means we can now thoroughly investigate methods by which such a molecule can be directly applied to plants to achieve concentrations sufficiently high in the xylem to alter pathogen behavior and thus achieve disease control.

OBJECTIVES

1. Compare DSF production and level of disease control conferred by transformation of *Xf* RpfF into several different grape cultivars.
2. Evaluate efficacy of direct applications of palmitoleic acid, C16-cis, and related DSF homologs to grape in various ways to achieve disease control.
3. Evaluate the potential for *Burkholderia phytofirmans* to multiply, move, and produce DSF in grape plants to achieve Pierce's disease control.

RESULTS AND DISCUSSION:

Objective 1. Production of DSF in a variety of grape cultivars.

While Freedom grape transformed with the *Xf rpfF* gene encoding the DSF synthase produced DSF species to which *Xf* was responsive, considerable evidence has been accumulated that RpfF is a rather promiscuous enzyme capable of producing a variety of DSF-like molecules. For example, we detected the production of C14-cis (*Xf*DSF1), C16-cis (*Xf*DSF2), and surprisingly, even DSF (normally produced only by *Xanthomonas* species) in transgenic RpfF-expressing freedom grape. Likewise, introduction of *Xf* RpfF into *Erwinia herbicola* yielded the production not only of *Xf*DSF1 and *Xf*DSF2, but other apparently related enoic acids not seen in *Xf* itself (data not shown). The enzymatic activity of Bcam0581, a protein highly homologous to *Xf* RpfF, that mediates biosynthesis of DSF in *Burkholderia cenocepacia* was recently shown to both catalyze the dehydration of 3-hydroxydodeca-noyl-ACP to cis-2-dodecenoyl-ACP as well as to cleave the thioester bond to yield the corresponding free acid. We presume that *Xf* RpfF also possesses these same features, although it probably shows a preference for longer chain 3-hydroxyacyl-ACPs since the DSF species produced by of *Xf* include 2-*Z*-tetradecenoic acid. The process by which such a compound could be produced by the expression of RpfF in plants remains somewhat unclear. Plant fatty acid synthesis is not prominent within the cytosol, and occurs primarily in the plastid, although some synthesis can also occur in the mitochondria. However, plant tissues are capable of incorporating exogenously provided fatty acids into their endogenous lipids indicating that fatty acids are mobile in the plant. In *B. cenocepacia* it appears that DSF synthesis results from a branch of the more classical fatty acid biosynthesis pathway by diverting 3-hydroxydodecanoyl-ACP. In plants, the majority of such corresponding acyl-ACP substrates for RpfF would be expected to be found within plastids, as a thioesterase is normally involved in converting such compounds to the free acid for release from the plastid. Thus there is either sufficient 3-hydroxyacyl-ACP of either plastid or mitochondrial origin in the cytoplasm of plants to enable RpfF resident in the cytoplasm to produce the DSF observed in the transgenic plants, or the expression of *Xf rpfF* in Freedom grape may have allowed some transport of RpfF into the chloroplast. Given that both *Xf*DSF and *Xf*DSF2 were produced in grape harboring RpfF we presume that the corresponding 3-hydroxyacyl-ACP substrates were available in Freedom grape. The production of various DSF species in grape might therefore be somewhat contextual, and different grape cultivars may differ in their ability to provide suitable substrates for RpfF. The various enoic acids that can be produced by RpfF differed substantially in their ability to induce gene expression in *Xf*, with those of longer chain lengths such as C16-cis being much more active than those of shorter chain lengths. We have also observed that DSF-mediated signaling in *Xf* by active DSF species such as C16-cis can be blocked in the presence of certain other *trans* unsaturated fatty acids. It is therefore possible that in some plants other fatty acid species indigenous to the plant or induced upon transformation of RpfF might interfere with signaling that would otherwise be conferred by the production of C16-cis and other “active” DSF species. To verify that the strategy of production of DSF in RpfF-containing transgenic grape is a robust one, widely

applicable in a variety of grape cultivars we propose to compare and contrast the production of DSF species in such a variety of grape cultivars. In addition, it seems likely that targeting RpfF to cellular compartments where the substrates for DSF synthesis may be more abundant could lead to enhanced production of this signal molecule. We thus also will compare the amount and types of DSF produced, and disease susceptibility, in transgenic plants in which RpfF is targeted to plastids and in plants in which it is not targeted.

RpfF was initially introduced only into Freedom grape, because it was the only variety for which transformation was feasible at that time. Continuing work by Dr. Davis Tricoli at the Plant Transformation Facility at UC Davis has now made it possible to transform Thompson Seedless as well as the winegrapes Chardonnay and Cabernet Sauvignon and the advanced rootstock varieties 1103 and 101-14. In addition to untargeted expression of RpfF, we will produce constructs which target RpfF to the chloroplast of grape by fusing the small subunit 78 amino acid leader peptide and mature N-terminal sequences for the *Arabidopsis* ribulose bisphosphate carboxylase (which is sufficient to target the protein to the chloroplast) to RpfF. For these studies we will use the recently created plant transformation vector pPIPRA561 which contains the FMV34S promoter driving the selectable marker NPTII that has been developed by Cecelia Chi-Ham of PIPRA to be free of Intellectual Property restrictions. Thus, the resultant transgenic grape will be directly usable for eventual commercial application. Most of the genetic constructs have now been made. Transformation of the various grape varieties will be conducted at the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis. The following lines will thus be produced and tested:

Variety	Untargeted RpfF	Gene Introduced Chloroplast-targeted RpfF	Vector Only
Thompson	+	+	+
Chardonnay	+	+	+
Cabernet Sauvignon	+	+	+
1103	+	+	+
101-14	+	+	+
Freedom	done		done

It is expected that the process of transformation of the various varieties will take at least eight months. Between 5 and 10 individual transformants will be produced for each variety/construct combination. Because the expression of *rpfF* in a given transformant of a given plant line will vary due to the chromosomal location of the randomly inserted DNA, it will be necessary to identify those lines with the highest levels of expression. It is not practical to directly test disease susceptibility in each of the many transformed lines; each line would have to be grown to a sufficiently large size that vegetative clones could be produced (three months) and then each plant would need to be propagated and assessed for disease susceptibility (five additional months). Instead, to most rapidly identify those transformants with high levels expression of *rpfF* and production of DSF, three assays that can be rapidly employed on seedling plants will be conducted to identify the most promising transformants. 1) The expression of *rpfF* will be assessed by quantitative RT-PCR of RNA isolated from individual leaves of the transformed plants after they are grown to a height of approximately 40 cm. 2) The distal 20 cm of each 40 cm high plant will be excised, placed in a pressure bomb, and xylem sap extruded under pressure. The approximately 30 µL of xylem sap collected from each plant by this method will be assessed for the presence of DSF species capable of inducing gene expression in *Xf* by adding it to micro-cultures (200 µL) of a *phoA* mutant of *Xf* harboring a *hxfA:phoA* reporter gene fusion. The alkaline phosphatase activity of the cells of this *Xf* DSF biosensor, measured as in our other studies, will be proportional to the concentration of various DSF species. This assay will not only identify those transformants within a given variety that maximally express the introduced *rpfF* gene, but will provide early evidence of those species capable of producing DSF species to which *Xf* is maximally responsive. 3) A functional "cell release" assay to determine those transformed lines in which *Xf* exhibits the highest adhesiveness, (expected of DSF-producing lines) will also be performed on the decapitated plant after extraction of xylem sap. Each excised plantlet will be rooted by placing the excised stem in moist vermiculite in a humid chamber for two weeks. Cells of *Xf* (ca. 10^7) will be injected into the petioles of three leaves for each plantlet. The leaves will be excised three days after inoculation, the petiole surface sterilized, and the cut end of the petiole introduced into sterile water and gently agitated for 20 min. to release cells from within the xylem vessels. The proportion of cells released from a petiole will be calculated as the ratio of those released from the total number of cells within that

petiole (determined by macerating petiole after cell release). The proportion of cells released from plants in such an assay is inversely proportional to the concentration of DSF in those plants (DSF producing plants induce stickiness of *Xf* and they are thus not released).

The disease susceptibility of the two transformed lines from each treatment combination having highest *rpfF* expression or apparent DSF production will then be assessed. At least 15 vegetative clones each of the lines will be produced from green cuttings of plants developing from the remaining transgenic plant remaining after the assays above. Plants (ca. 30 cm high) will be inoculated with *Xf* by needle puncture as in earlier studies. Disease severity will be assessed visually each week. After 14 weeks, when substantial disease will have appeared in untransformed lines, population sizes of *Xf* in petioles of leaves collected at 30 cm intervals from the point of inoculation will be assessed as before. We also will assess the efficacy of the best of the two RpfF-expressing rootstock varieties to confer disease control to normal Cabernet Sauvignon scions grafted onto them as in other studies.

The composition of DSF species present in xylem sap and their aggregate signaling activity will be assessed by extracting xylem sap from mature (two m tall) plants of each of the two best transformed lines of a given variety/construct forwarded for further analysis. Vines will be cut into 40 cm segments and placed in a pressure chamber and subjected to about 20 bar pressure. The xylem sap obtained will be collected into glass containers containing ethyl acetate, mixed vigorously for five min, and the ethyl acetate (now containing DSF) will be separated from the aqueous phase. The ethyl acetate will be concentrated by evaporation and the dried residue dissolved in methanol. Mass spectrometry analysis of the plant xylem sap-extracts will be performed using an LTQ Orbitrap XL mass spectrometer equipped with an electrospray ionization (ESI) source. Mass spectra will be recorded in the negative ion mode over the range m/z = 100-500 using the Orbitrap mass analyzer and spectra processed using Xcalibur software. DSF species will be identified by their m/Z ratio, with *Xf*DSF, XfDSF2, and DSF (having m/Z ratios of 225.18, 253.22, and 211.17, respectively) being readily distinguished in xylem sap of RpfF-expressing Freedom. We will also resolve other chemical species found in RpfF-expressing lines that are not found in control plants by a similar procedure. We expect that more than one enoic acid will be produced in a given line expressing RpfF. Because of this, aggregate DSF signaling activity will be determined in samples of xylem sap collected as above using the *Xf phoA*-based DSF biosensor as described above. Control sap samples in which different concentrations of *Xf*DSF2 (C16-cis) are spiked will enable the DSF signaling activity of xylem sap from a given line to be expressed as that of *Xf*DSF2 equivalents. It is possible that some transgenic lines will exhibit little aggregate DSF signaling activity because of their production of antagonistic fatty acids. Such lines will be identified in two ways: 1) Such a line might contain relatively high concentrations of *Xf*DSF2 and other known inducers of signaling in *Xf* (determined by ESI-MS analysis of sap) yet not induce expression of the *Xf* DSF biosensor strain. 2) Direct evidence for such antagonism will be obtained by spiking xylem sap samples from such transgenic lines with *Xf*DSF2 and comparing the alkaline phosphatase activity of the *Xf* DSF biosensor in such samples with those of spiked samples of xylem sap from control plants; reduced biosensor activity in the test samples would provide direct evidence for such antagonism.

Objective 2. Direct application of DSF to plants.

Several recent findings in our laboratory of the process of DSF-mediated signaling in *Xf* suggest that Pierce's disease control by direct application of DSF to plant surfaces is both feasible and practical. Studies of the context-dependent production of DSF reveals that DSF species such as *Xf*DSF2 are far more active than *Xf*DSF1 which was originally described (**Figure 1**). While topical applications of *Xf*DSF1 to grape provided modest reductions in disease severity, applications of *Xf*DSF2 should be far more efficacious. Studies of applications of *Xf*DSF2 were hindered by a limitation of the amount of this material that we could chemically synthesize. Fortunately, our studies of the promiscuity of DSF signaling in *Xf* reveal that it is quite responsive to the cheap, commercially available enoic acid palmitoleic acid (**Figure 1**).

While about eight-fold more palmitoleic acid is required to induce gene expression in *Xf* than *Xf*DSF2, it is much more active than *Xf*DSF1 itself. We therefore will conduct a variety of studies to address how such molecules could be introduced into plants in different ways to achieve pathogen confusion. While most studies will use palmitoleic acid, we also will conduct comparative studies using synthetic *Xf*DSF2 and *Xf*DSF1.

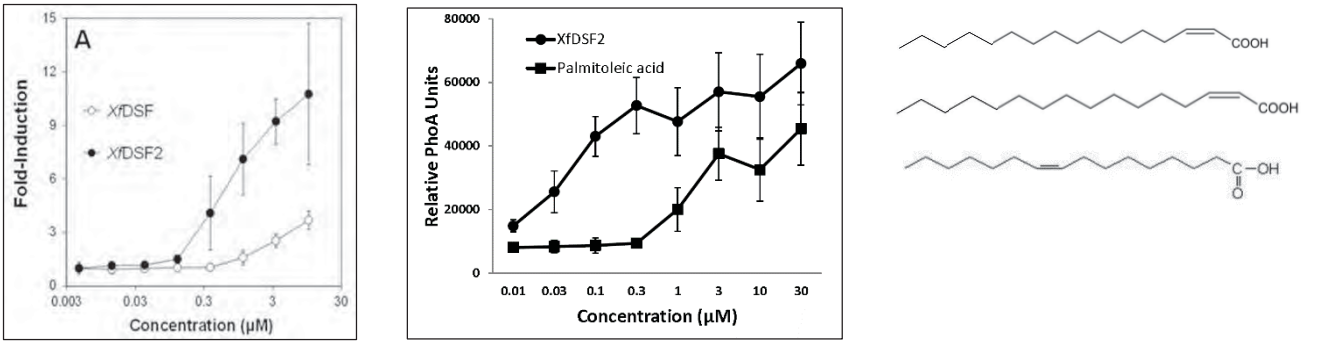

Figure 1. Responsiveness of a PhoA-based *Xf* DSF biosensor to different concentrations of *Xf*DSF1 (top molecule), *Xf*DSF2 (middle molecule), and palmitoleic acid (bottom molecule).

We are investigating several strategies by which direct application of DSF molecules can reduce Pierce's disease. While we will determine the effects of application of DSF homologs on disease severity of plants inoculated with *Xf* in some studies, direct monitoring of DSF levels in treated plants will be a MUCH more rapid and interpretable strategy of assessing this strategy of disease control. As DSF must enter the xylem fluid in order to interact with the xylem-limited *Xf* in plants we will assess DSF levels in xylem sap of plants treated in different ways using the PhoA-based *Xf* biosensor as described above. We will address four main issues that we hypothesize to limit the direct introduction of DSF into plants: 1) The penetration of DSF through leaves and other plant tissues may be slow or inefficient, 2) DSF may readily enter plant tissues but only slowly enter the xylem sap, 3) DSF may be degraded after introduction into plants, and 4) DSF may enter plants more readily via certain tissues than others (e.g., it may readily be taken up via the roots but more slowly from leaves). We thus will measure DSF species levels in 1) xylem sap as well as in 2) leaf, stem, and root tissue after removal of xylem sap after applying synthetic DSF to (A) foliage, (B) direct injection into stems, and (C) application to roots as a drench. DSF will be measured directly in xylem sap expressed from plants under pressure at various times after topical treatments as above. DSF content of treated leaves however will require the extraction of DSF from treated plants with ethyl acetate, followed by assay of the extract using the *Xf* DSF biosensor. Enoic acids will be used in two forms: 1) the free acid which is relatively hydrophobic and which might thus more readily penetrate waxy leaves, and 2) the sodium salt which is freely water soluble and which has mild surfactant activity which might allow them to more readily be taken up and dispersed from stem injections or root application. In addition to the use of purified fatty acids we will also evaluate mixtures of fatty acids. For example, macadamia oil contains a very high concentration of palmitoleic acid (23%) which will be saponified by treatment with sodium hydroxide to yield the sodium salts of the constituent fatty acids. We expect that such a fatty acid mixture will have high DSF signaling activity as we have no evidence that other saturated fatty acids that would be found in the lipids of macadamia oil would interfere with DSF signaling in *Xf*. Saponified plant oils such as this are very attractive as inexpensive sources of DSF homologs that could be directly applied to grape.

As DSF species are somewhat hydrophobic, a variety of adjuvants will be tested for their effects on enhancing their introduction into plants. For example, detergents and solubilizing materials such as Solutol HS15 may greatly increase the penetration and dispersal of DSF and its analogs. We thus will suspend the hydrophobic materials in such carriers prior to foliar sprays or soil drenches. Considerable preliminary results of already been obtained on the ability of such a topically applied palmitoleic acid solutions to enter into the plants. Apparent DSF signaling activity was measured using the biosensors noted above. These results were presented in the progress report for project 12–0224–SA ("Elucidating the process of cell-cell communication in cell *Xf* to achieve Pierce's disease control by pathogen confusion"), and hence will not be reproduced here. The silicon-based surfactant Breakthru having very low surface tension, thereby enabling spontaneous to model infiltration, was a most effective agent in introducing palmitoleic acid into leaves and petioles of grape.

In addition to directly assessing DSF levels within plants as described above, the adhesiveness of *Xf* cells inoculated into treated plants will also be determined using the cell release assay described above. Since the virulence of various *Xf* mutants is inversely related to their release efficiency, and cells are released at a much lower rate from transgenic RpfF-expressing grape that produce DSF that are resistant to disease, we expect that treatments with exogenous DSF that reduce the release efficiency of *Xf* cells when measured two weeks or more after inoculation will also be the most resistant to disease. This assay is far quicker than assays in which disease

symptoms must be scored after several months of incubation, and will be employed during those times of the year such as the fall and winter when disease symptoms are difficult to produce in the greenhouse. Disease assessment assays in which *Xf* will be inoculated via a droplet puncture method into treated vines will also be employed to test the efficacy of those topical treatments that best introduce exogenous DSF into plants. Disease severity and *Xf* populations will be measured at various times after inoculation as described above and in our other studies.

Objective 3. Biological control with *Burkholderia phytofirmans* PsJN.
While the biological control of Pierce's disease with endophytic bacteria that would grow within grape and produce DSF has been an attractive strategy, until recently we have been unable to find bacteria capable of exploiting the interior of grape. All of hundreds of strains isolated from within grape by our group as well as that of Dr. Kirkpatrick exhibited no ability to grow and move beyond the point of inoculation when re-inoculated. We have recently, however, found that *B. phytofirmans* strain PsJN, which had been suggested to be an endophyte of grape seedlings, multiplied and moved extensively in mature grape plants (**Figure 2**). It's population size and spatial distribution in grape within six weeks of inoculation was similar to that of *Xf* itself, suggesting that it is an excellent grape colonist. Furthermore, DSF production has been demonstrated in certain other *Burkholderia* species and the genome sequence of *B. phytofirmans* revealed that it has a homologue of *Xf rpfF*. While little evidence for its production of a DSF species to which *Xf* could respond was obtained in preliminary studies in culture, the promiscuous nature of RpfF in *Xf* and other species suggested that it might make DSF species to which *Xf* would respond under some circumstances. Preliminary results suggest that co-inoculation of *Xf* and *B. phytofirmans* resulted in greatly reduced disease symptoms compared to plants inoculated with *Xf* alone (**Figure 3**), suggesting that *B. phytofirmans* can produce a suitable DSF *in planta*. We propose to follow up these exciting results by conducting studies that 1) Further elucidate the potential for *B. phytofirmans* to multiply within grape varieties and produce DSF and thus to confer biological control of Pierce's disease, 2) Account for the role, if any, of its endogenous production of DSF on the biological control of *Xf*, and 3) Address whether it's potential to confer biological control of *Xf* via DSF production can be enhanced.

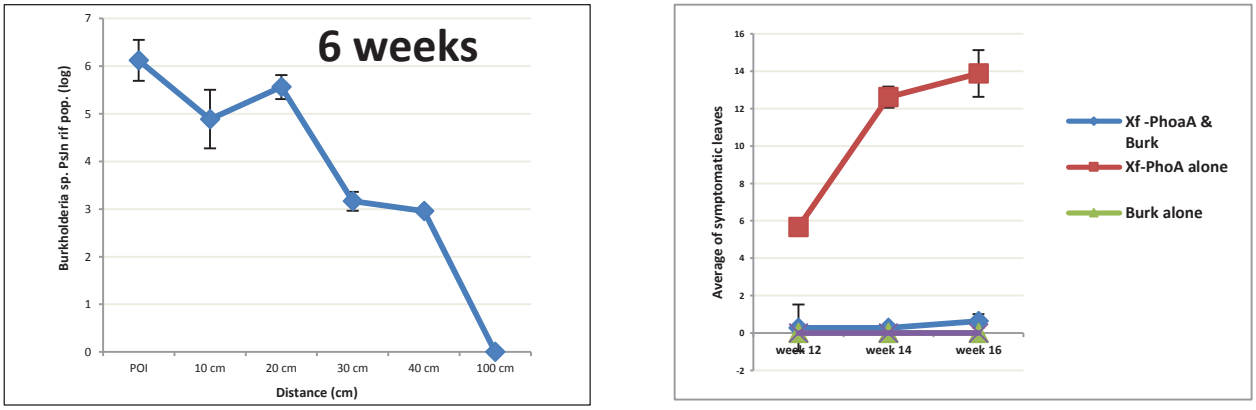

Figure 2. (Left). Population size of *B. phytofirmans* in Cabernet Sauvignon grape at various distances from the point of inoculation after six weeks incubation. (Right). Severity of Pierce's disease on Cabernet Sauvignon at various times after inoculation with *Xf* alone (red) or when co-inoculated with *B. phytofirmans* (blue).

While the droplet puncture method used in **Figure 2** is an effective way to introduce bacteria into the xylem we have investigated the potential to introduce *B. phytofirmans* into the vascular tissue by topical application to leaves using 0.05% Silwet L77, an organo-silicon surfactant with sufficiently low surface tension that spontaneous invasion of plant tissues can be achieved. The population size of *B. phytofirmans* in the petioles of leaves distal from the leaf on which cell suspensions in L77 (10^8 cells/) have been applied were used as a measure of growth and movement potential from such an inoculation site. Substantial numbers of cells of *Burkholderia* could be recovered from petioles within one or two weeks after topical application to leaves in the presence of Silwet L77 or Breakthru (**Figure 3**). Very few cells were present with and petioles when the bacterium was applied without a penetrating surfactants. Topical application of such an endophyte thus appears to be a very practical means of inoculating plants in the field.

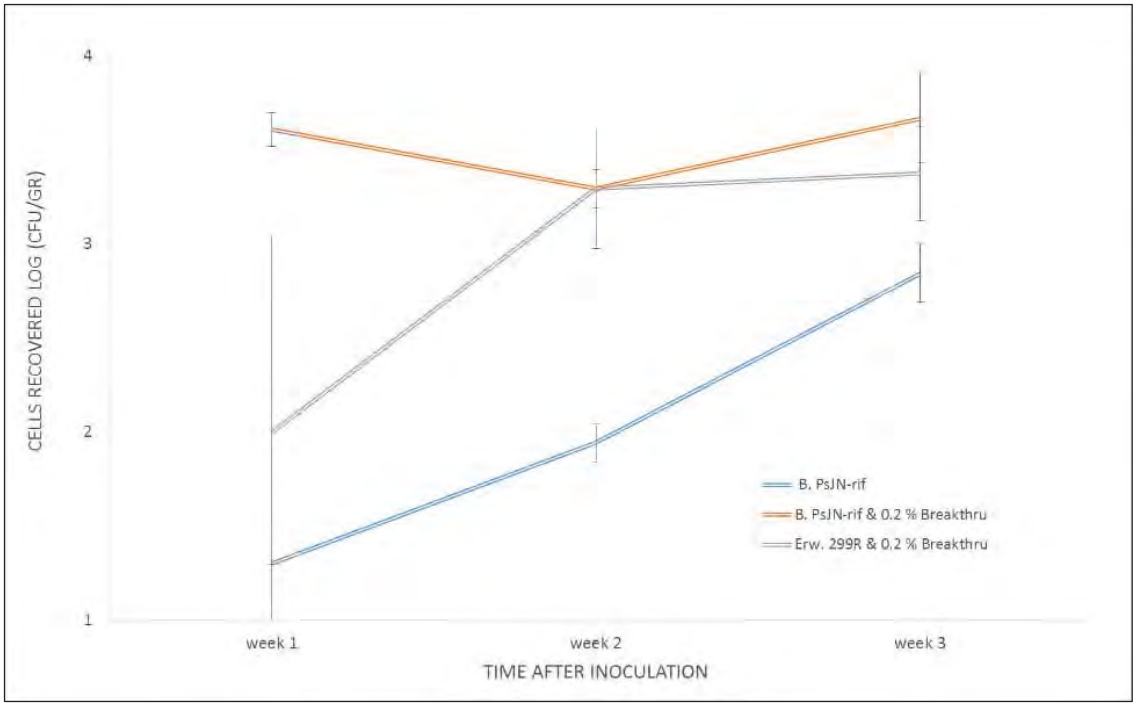

Figure 3. Population size of *B. phytofirmans* in petioles of Cabernet Sauvignon of plants sprayed with this strain alone (blue line) or this strain applied with 0.2% Breakthru (gray line), or of *Erwinia herbicola* strain 299R applied with 0.2% Breakthru (orange line). Vertical bars represent the mean of log population size at a given sampling time.

The ability of *B. phytofirmans* to confer biological control of Pierce's disease when co-inoculated with *Xf* and when applied at various times prior to that of *Xf* will be assessed by measuring both *Xf* population sizes in petioles distal to the point of *Xf* inoculation as well as of disease symptoms at weekly intervals as above. Evidence for any DSF production by *B. phytofirmans* in grape plants will be obtained by assaying xylem fluid collected from plants colonized by the strain using the PhoA-based *Xf* DSF biosensor as above. Ideally, as a biological control agent *B. phytofirmans* should persist in inoculated plants for several years. Different grape cultivars inoculated with *B. phytofirmans* in the greenhouse will be established in field trials at the Gill Tract at UC Berkeley and the population size of *B. phytofirmans* measured monthly for the two-year period of the study. The population size of *B. phytofirmans* can be readily determined by plating of appropriate dilutions of plant tissues onto rifampicin-containing KB medium.

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

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ABSTRACT

A cell density-dependent gene expression system in *Xylella fastidiosa* (*Xf*) mediated by a small signal molecule called diffusible signal factor (DSF) which we have now characterized as 2-Z-tetradecenoic acid (hereafter called C14-cis) and 2-Z-hexadecenoic acid (C16-cis) controls the behavior of *Xf*. The accumulation of DSF attenuates the virulence of *Xf* by stimulating the expression of cell surface adhesins such as HxfA, HxfB, XadA, and FimA (that make cells sticky and hence suppress its movement in the plant) while down-regulating the production of secreted enzymes such as polygalacturonase and endogluconase which are required for digestion of pit membranes and thus for movement through the plant. Artificially increasing DSF levels in plants in various ways increases the resistance of these plants to Pierce's disease. Disease control in the greenhouse can be conferred by production of DSF in transgenic plants expressing the gene for the DSF synthase from *Xf*; such plants exhibit high levels of disease resistance when used as scions and confer at least partial control of disease when used as rootstocks. This project is designed to test the robustness of disease control by pathogen confusion under field conditions where plants will be exposed to realistic conditions in the field and especially under conditions of natural inoculation with insect vectors. We are testing two different lineages of DSF-producing plants both as own-rooted plants as well as rootstocks for susceptible grape varieties in two field sites. Plants were established in one field site in Solano County on August 2, 2010. Plants were planted at a Riverside County site on April 26, 2011. All plants at the Solano County experimental site were needle-inoculated with a suspension of *Xf* in May 2012; at least four vines per plant were inoculated, each at a given site with a 20 ul droplet of *Xf* containing about 10^6 cells of *Xf*. The incidence of infection of the inoculated vines was reduced about three-fold in assessments made in August and September. Disease was observed only near the point of inoculation in transgenic Freedom, but had spread extensively in wild-type Freedom grape. Only a modest reduction in incidence or severity of Pierce's disease was seen in Thompson Seedless grafted onto DSF-producing Freedom rootstocks compared to those grafted on wild-type Freedom. The incidence of infection of transgenic Thompson Seedless plants was similar to that of wild-type Thompson, 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 Seedless, while the incidence and severity of Pierce's disease on Thompson Seedless grafted onto DSF-producing Thompson Seedless rootstocks was less than that of plant grafted onto wild-type Thompson Seedless rootstocks. Similar levels of resistance of the rpfF-expressing Freedom grape relative to wild-type Freedom have been seen in continuing evaluations in 2013 and 2014.

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 grapes which were highly resistant to Pierce's disease in greenhouse trials are also much less susceptible to disease in field trials, especially in plants naturally infected by sharpshooter vectors. No mortality of the transgenic Freedom plants has been seen, and they remain more highly resistant to Pierce disease than the untransformed plants.

INTRODUCTION

Our work has shown that *Xylella fastidiosa* (*Xf*) uses diffusible signal factor (DSF) perception as a key trigger to change its behavior within plants. Under most conditions DSF levels in plants are low since cells are found in relatively small clusters, and hence cells do not express adhesins which are required for vector acquisition but would hinder their movement through the plant. Instead, the cells 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		
1	FT	Non-targeted RpfF Freedom
2	TT	Chloroplast-targeted RpfF Thompson
3	FW	Non-targeted RpfF Freedom as rootstock with normal Thompson scion
4	TTG	Chloroplast-targeted RpfF Thompson as rootstock with normal Thompson scion
5	FWG	Normal Freedom rootstock with normal Thompson scion
6	TWG	Normal Thompson rootstock with normal Thompson scion
7	FW	Normal Freedom
8	TW	Normal Thompson

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

One field trial was established in Solano County on August 2, 2010. Twelve plants of each treatment were established in a randomized complete block design. Self-rooted plants were produced by rooting of cuttings (about three cm long) from mature vines of plants grown in the greenhouse at UC Berkeley. The plants were inoculated in May 2012 (no natural inoculum of *Xf* occurs in this plot area and so manual inoculation of the vines with the pathogen was performed by needle-inoculated with a suspension of *Xf)*. At least four vines per plant were inoculated. Each inoculation site received a 20 ul droplet of *Xf* containing about 10^6 cells of *Xf*.

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

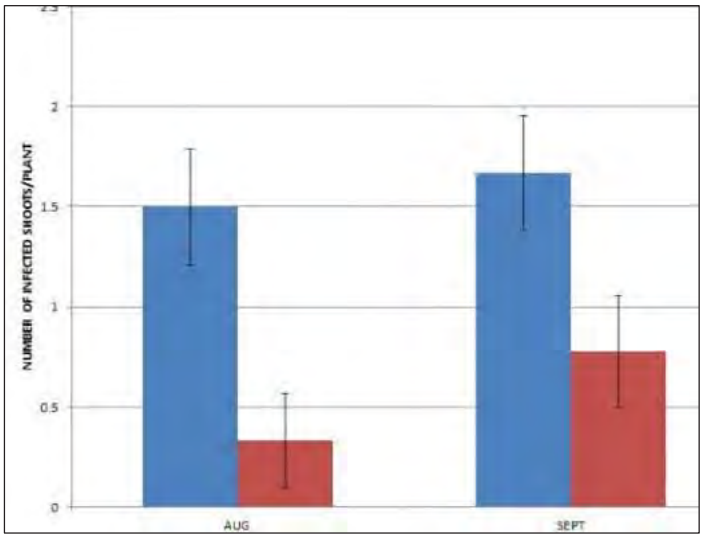

Figure 1. Incidence of vines of DSF-producing transgenic Freedom grape (red) or wild-type Freedom having any symptoms of Pierce's disease when rated in August or September, 2012. A total of 3 vines per plant were assessed. The vertical bars represent the standard error of the mean.

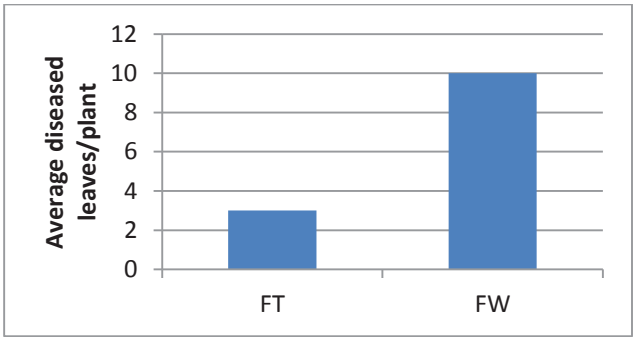

Figure 2. Severity of Pierce's disease on transgenic Freedom grape (FT) and on wild-type Freedom grape assessed in August 2012 in the Solano country trial.

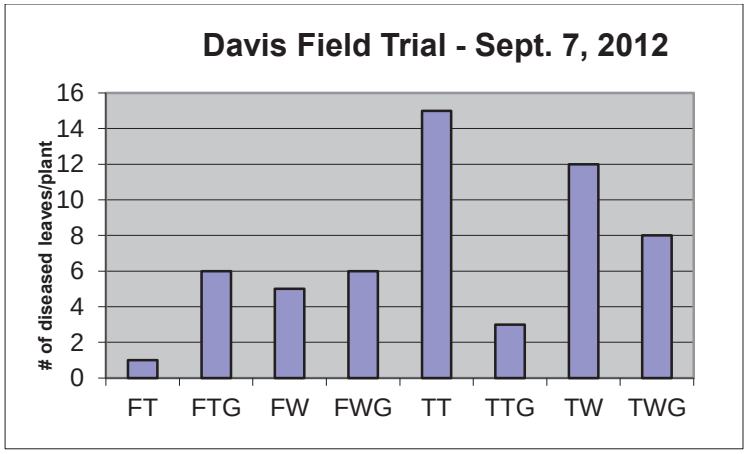

Figure 3. Severity of Pierce's disease on grape assessed in September 2012 in the Solano country trial. See treatment codes above for treatment comparisons.

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 4**). The plants at the Riverside County trial were subjected to natural infection from infected sharpshooter vectors having access to *Xf* from surrounding infected grapevines. Very high levels of Pierce's disease were seen in the summer of 2012, although much less symptoms were seen on the transgenic DSF-producing Freedom grape compared to other plants (**Figure 5**).

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

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

The incidence of infection of transgenic DSF-producing Freedom was about three-fold less than that of wild-type Freedom grape (**Figure 6**), while the number of infected leaves per vine was about five-fold less (**Figure 9**), suggesting that the pathogen had spread less in the DSF-producing plants after insect inoculation. Only a modest reduction in incidence or severity of Pierce's disease was seen in Thompson Seedless grafted onto DSF-producing

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

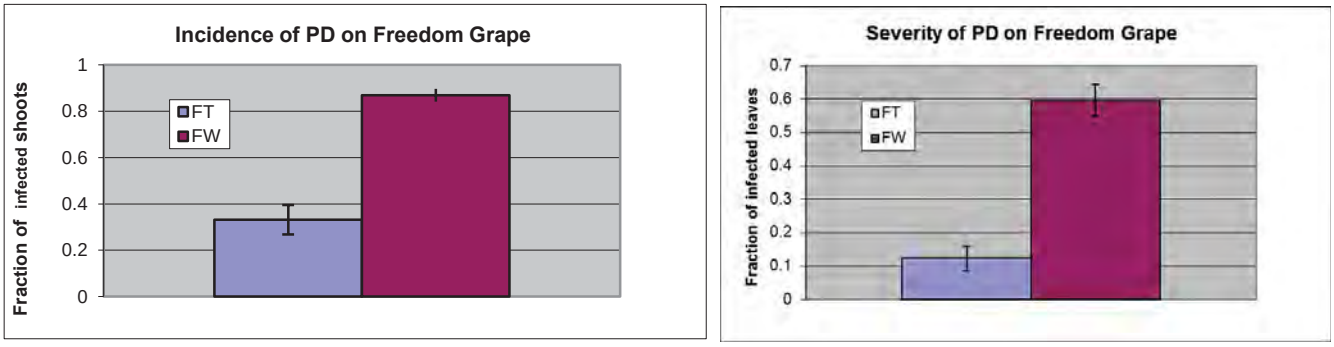

Figure 6. Incidence of Pierce's disease of transgenic DSF-producing Freedom grape (blue bars) or wild type Freedom (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited symptoms (right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

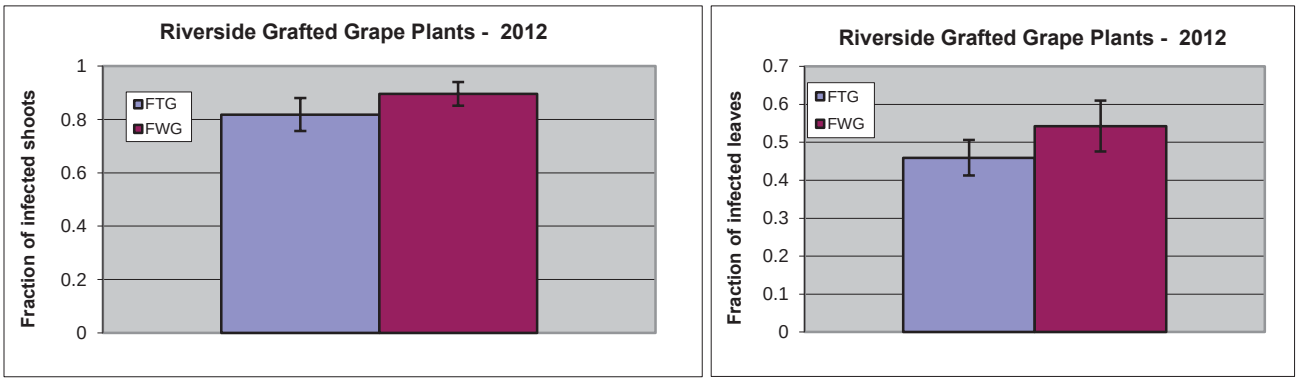

Figure 7. Incidence of Pierce's disease of normal Thompson Seedless grape grafted onto transgenic DSF-producing Freedom grape rootstocks (blue bars) or wild type Freedom rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured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

On May 15, 2013 plants at the Solano County field trial were evaluated for both the incidence of survival over winter, as well as any symptoms of Pierce's disease that were apparent at that early date. Vines that had been inoculated in 2012 had been marked with a plastic tie. The vines were pruned during the winter of 2012/2013 in a way that retained the inoculation site and the plastic marker for each of the vines inoculated in 2012. Thus, in May 2013 the return growth on those inoculated but pruned vines was assessed. One or more new shoots had emerged from such vines, and the incidence as to whether at least one new shoot had emerged was assessed (**Figure 10**). Nearly all of the inoculated vines from both Freedom and transgenic DSF-producing Freedom gave rise to new shoots as of May 2013 (**Figure 10**). In contrast, many vines of Thompson Seedless inoculated in 2012 were dead, and no shoots emerged in 2013. While most new shoots emerging in 2013 appeared asymptomatic at the time of assessment in May, a few exhibited discoloration, possibly indicating early stages of Pierce's disease. A separate assessment of such possibly symptomatic shoots from that of completely asymptomatic shoots was

made (**Figure 11**). It is noteworthy that no symptomatic new shoots were observed on transgenic Freedom, while about 10% of the new shoots emerging from vines of wild-type Freedom exhibited some symptoms (**Figure 11**). It was also noteworthy that a much higher proportion of the vines from Thompson Seedless scions grafted onto a transgenic Freedom rootstock gave rise to new shoots in 2013 compared to that on Freedom rootstocks (**Figures 10 and 11**). Likewise, a higher proportion of vines from Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks gave rise to new shoots in 2013 compared to that of scions grafted onto normal Thompson Seedless rootstocks (**Figures 10 and 11**). Thus, infection of Thompson Seedless vines by inoculation in 2012 had led to some morbidity of those vines (and even of the cordon on which they were attached, in some cases), but Thompson Seedless when grafted onto either transgenic DSF-producing Freedom or transgenic DSF-producing Thompson Seedless rootstocks had a higher likelihood of surviving inoculation in 2012. Continued assessments of disease severity of those new shoots emerging on vines inoculated in 2012 were made in early October 2013, but the data was not fully analyzed at the time of preparation of this report.

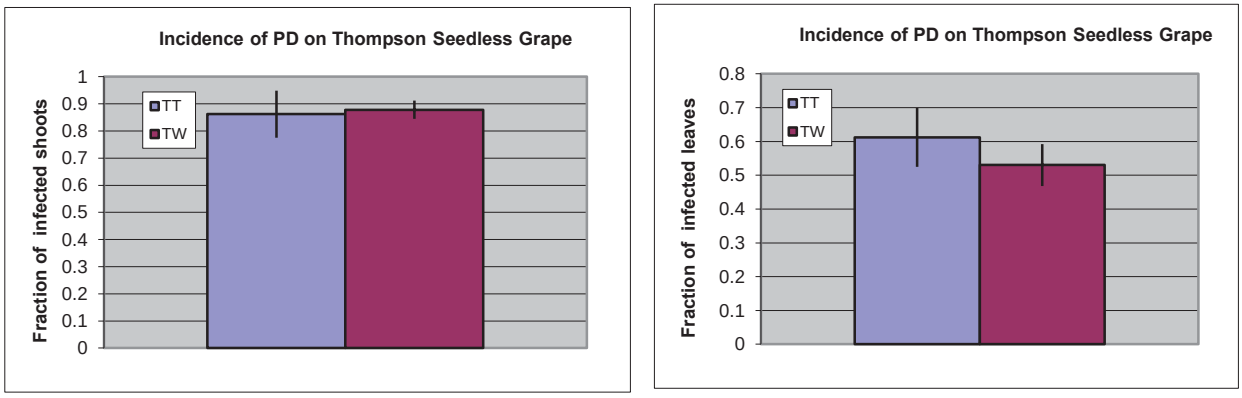

Figure 8. Incidence of Pierce's disease of transgenic DSF-producing Thomson Seedless grape (blue bars) or wild-type Thompson Seedless (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity of disease as measured as the fraction of leaves per shoot that exhibited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

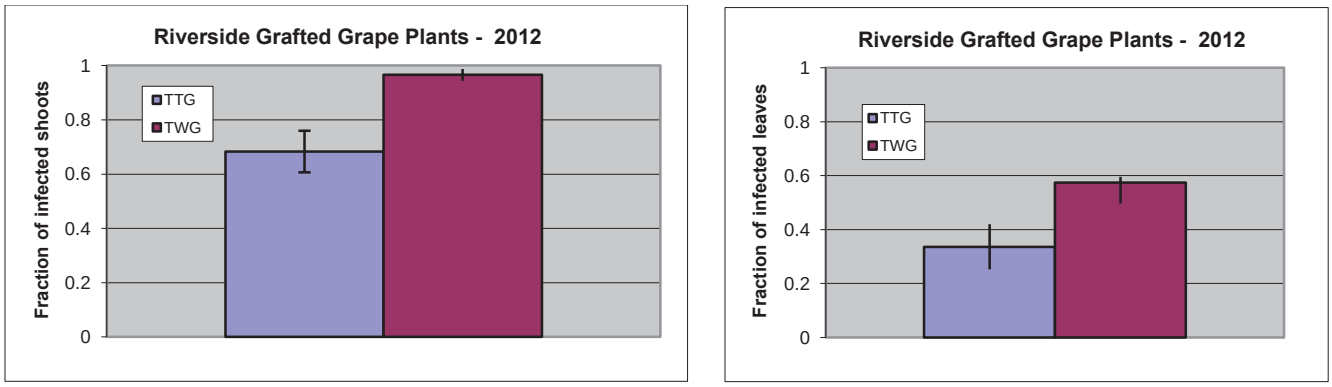

Figure 9. Incidence of Pierce's disease of normal Thompson Seedless grape grafted onto transgenic DSF-producing Thompson Seedless grape rootstocks (blue bars) or wild-type Thompson Seedless rootstocks (red bars) as measured as the fraction of vines with any disease symptoms (left box) or the severity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

Vines of transgenic and wild-type Freedom, as well as wild-type and transgenic Thompson Seedless, and Thompson Seedless scions grafted onto the various transgenic or wild-type rootstocks that were apparently healthy and derived from cordons not showing disease in 2013 were again inoculated with *Xf* at the Solano County trial on May 28, 2014. The goal of these continuing experiments is to verify the enhanced disease resistance exhibited by transgenic Freedom, and to further quantify the differential susceptibility of Thompson Seedless scions grafted onto various transgenic rootstocks. Disease severity was assessed on August 8 and Sept. 15. In addition, disease incidence and severity that developed in 2014 from vines inoculated in previous years, was measured. A uniform rating scale for rating of all vines in both the Solano and Riverside county trials

was developed by Lindow and Kirpatrick. This rating scale will allow the severity of disease on inoculated vines in the year of inoculation to be assessed as the fraction of leaves on a given inoculated vine that are symptomatic. Furthermore, on vines that have been infected for more than one year, this new 0-5 rating scale accounts for return growth and vigor of growth of vines in years subsequent to that year in which it was originally inoculated.

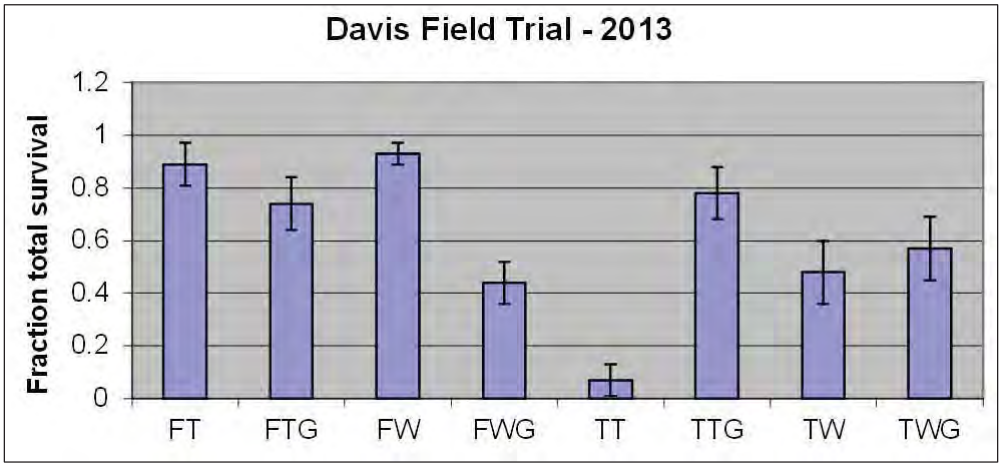

Figure 10. The fraction of vines in the Solano County field trial inoculated in 2012 with *Xf* that gave rise to at least one new shoot by May 2013. Treatments include: transgenic DSF-producing Freedom as an own-rooted plant (FT); wild-type freedom as an own-rooted plant (FW); Thompson Seedless scions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

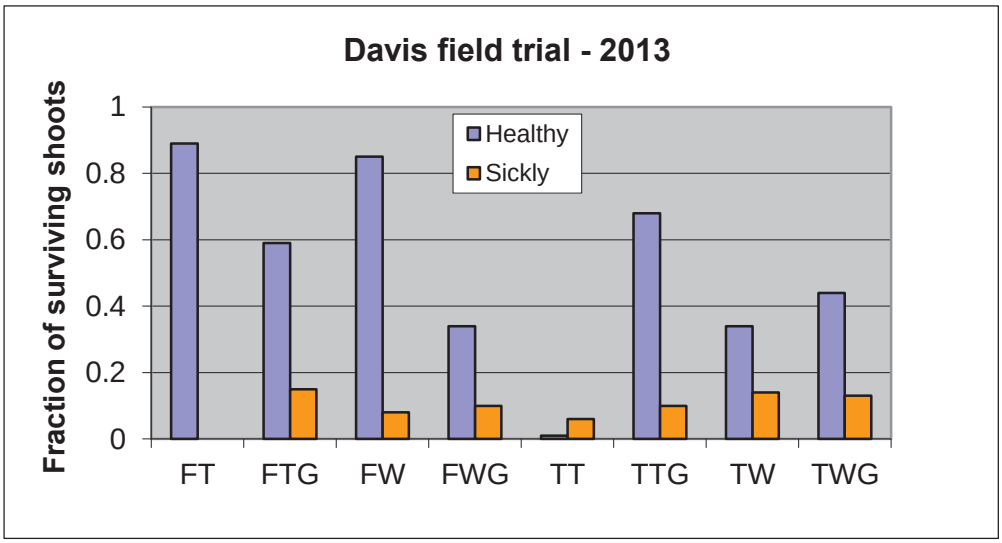

Figure 11. The fraction of vines in the Solano County field trial inoculated in 2012 with *Xf* that gave rise to at least one new shoot by May 2013 that exhibited some abnormalities possibly indicative of early stages of Pierce's disease infection (orange bars). Treatments include: transgenic DSF-producing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

Disease incidence and severity on plants was rated on both August 8 and September 15, 2014. No symptoms were apparent on inoculated vines of either wild-type or transgenic Freedom plants. However, symptoms were apparent on Thompson Seedless vines that had been inoculated earlier in the season. A lower incidence of symptomatic leaves were found on Thompson Seedless vines grafted onto transgenic Freedom rootstocks compared to those on wild-type Freedom rootstocks (**Figure 12**). The incidence of symptomatic leaves on Thompson Seedless vines grafted onto wild-type Thompson Seedless rootstocks did not differ from that on transgenic Thompson Seedless rootstocks. Similarly, the incidence of symptomatic leaves was similar on own rooted Thompson Seedless plants compared to that on transgenic Thompson Seedless plants (**Figure 12**). The overall vigor of Thompson Seedless scions grafted onto transgenic Freedom rootstocks was similar to that of those grafted onto wild-type Thompson Seedless rootstocks (**Figure 13**). The overall disease severity exhibited by wild-type and transgenic Thompson Seedless plants was also similar, and disease severity on Thompson Seedless scions grafted onto either wild-type or transgenic Thompson Seedless rootstocks also did not differ (**Figure 13**). Thus, some evidence for protection of scions grafted onto RpfF-expressing freedom rootstocks was again seen in 2014 as in earlier years.

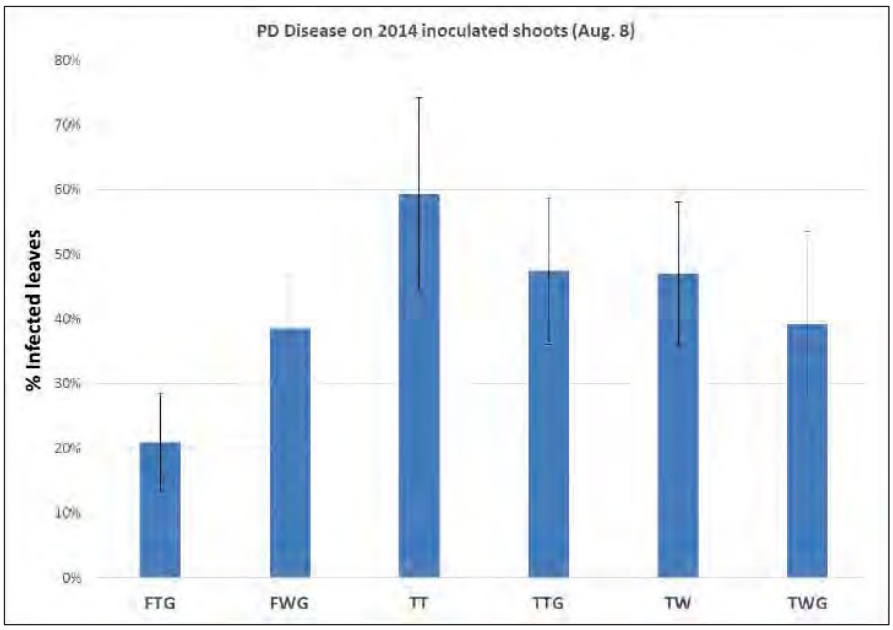

Figure 12. The percentage of leaves on vines in the Solano County field trial inoculated in 2014 with *Xf* that exhibited symptoms of Pierce's disease on August 8, 2014. Treatments include: Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG); transgenic DSF-producing Thompson Seedless as own-rooted plants (TT); normal Thompson Seedless as own-rooted plants (TW); Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG); and Thompson Seedless scions grafted onto normal Thompson Seedless rootstocks (TWG). The vertical bars represent the standard error of the mean.

The incidence of symptomatic leaves had increased by September 15 from the low levels seen in August. A dramatic difference in the incidence of symptomatic leaves was observed between wild-type and RpfF-expressing Freedom grape. While no symptomatic leaves were observed on the transgenic freedom plants, over 15% of the leaves on the vines of wild-type Freedom plants that had been inoculated in May were showing symptoms of Pierce's disease (**Figure 14**). As observed in the August evaluation, the incidence of leaves on Thompson Seedless vines grafted to a transgenic Freedom rootstock was lower than that on Thompson Seedless vines grafted onto a wild-type Freedom rootstock (**Figure 14**). An assessment was also made in September of the overall appearance of plants. The disease rating for transgenic freedom plants was significantly lower than that for wild-type freedom plants (**Figure 15**). In contrast, while numerically lower, the severity of Thompson Seedless scions grafted onto transgenic Freedom rootstocks did not differ from that of Thompson Seedless scions grafted onto wild-type Freedom rootstocks (**Figure 15**). Thus, the transgenic RpfF-expressing Freedom plants continue to show relatively high resistance to Pierce's disease both in the same season that they are inoculated as well as over several years compared to the wild-type Freedom plants.

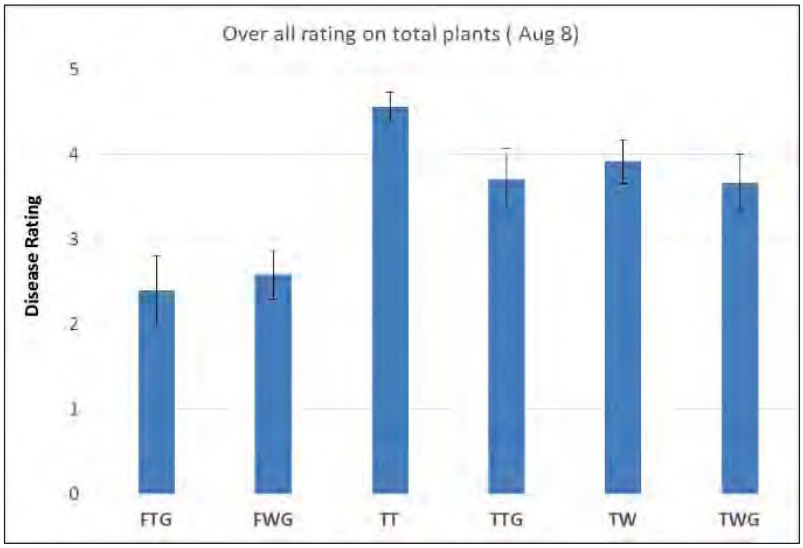

Figure 13. The overall disease rating of vines in the Solano County field trial when assessed on August 8, 2014. Treatments include: Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG); transgenic DSF-producing Thompson Seedless as own-rooted plants (TT); normal Thompson Seedless as own-rooted plants (TW); Thompson Seedless scions grafted onto transgenic DSF-producing Thompson Seedless rootstocks (TTG); and Thompson Seedless scions grafted onto normal Thompson Seedless rootstocks (TWG). The vertical bars represent the standard error of the mean.

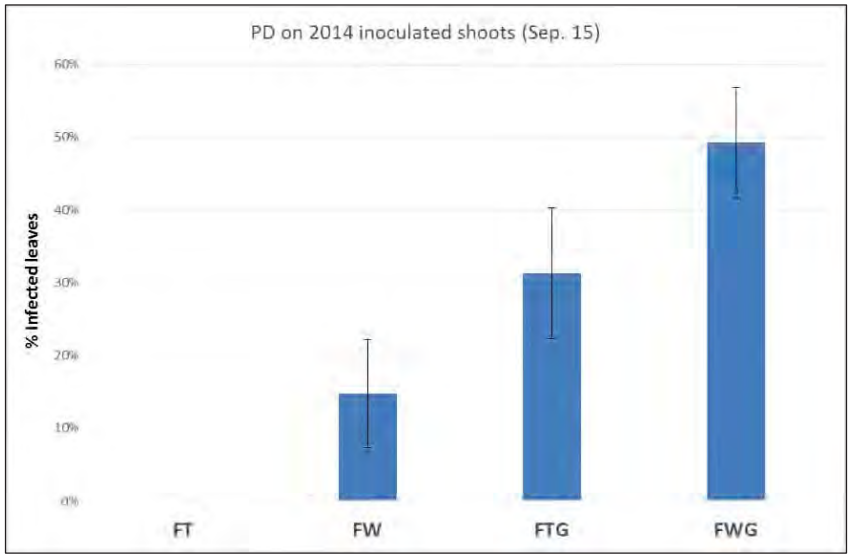

Figure 14. The percentage of leaves of vines in the Solano County field trial inoculated in 2014 with *Xf* that exhibited symptoms of Pierce's disease on September 15, 2014. Treatments include: transgenic DSF-producing Freedom as an own-rooted plant (FT); wild-type freedom as an own-rooted plant (FW); Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); and Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG). The vertical bars represent the standard error of the mean.

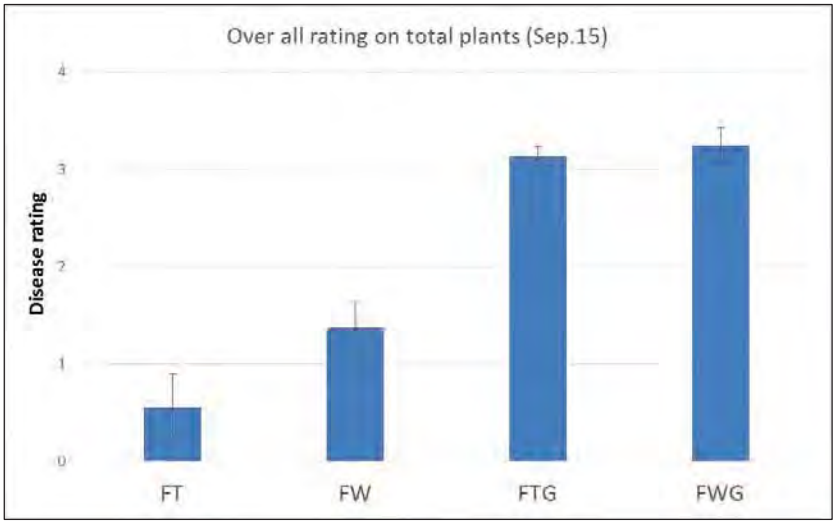

Figure 15. The overall disease rating of vines in the Solano County field trial that exhibited symptoms of Pierce's disease on September 15, 2014. Treatments include: transgenic DSF-producing Freedom as an own-rooted plant (FT); wild-type freedom as an own-rooted plant (FW); Thompson Seedless scions grafted onto transgenic DSF-producing Freedom rootstocks (FTG); and Thompson Seedless scions grafted onto normal Freedom rootstocks (FWG). The vertical bars represent the standard error of the mean.

FUNDING AGENCIES
Funding for this project was provided by the CDFA Pierces Disease and Glassy-winged Sharpshooter Board.

FIELD EVALUATIONS OF GRAFTED GRAPE LINES EXPRESSING POLYGALACTURONASE- INHIBITING PROTEINS (PGIPS)

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Reporting Period: The results reported here are from work conducted January 1, 2014 to October 31, 2014.

ABSTRACT

The project was designed to establish and evaluate grapevines in typical commercial vineyard settings in order to assess whether a protein that is naturally produced in edible fruit can restrict *Xylella fastidiosa* (*Xf*) spread and Pierce's disease symptoms without altering plant performance. Work in this project evaluates the performance and susceptibility to Pierce's disease of two varieties of grapevines that produce an introduced protein which had been selected by the Pierce's Disease Research Scientific Advisory Panel as a promising candidate to consider for advancement towards commercialization. The aim of the project is to determine whether a polygalacturonase (PG)-inhibiting protein (PGIP) naturally expressed in pear fruit (pPGIP), when delivered from grafted rootstocks, can control Pierce's disease in the scion, fruit bearing parts of the grapevines. Prior to this project, transformed Thompson Seedless and Chardonnay grapevines expressing pPGIP throughout the vine showed reduced Pierce's disease incidence and symptoms after inoculation with *Xf,* which produces a PG that is inhibited by pPGIP (Agüero et al., 2005). Cuttings from the two varieties of grapevines that had been transformed to express pPGIP were grafted as rootstocks with non-pPGIP producing Chardonnay or Thompson Seedless scions so that comparisons between vines producing pPGIP in their grafted rootstocks (transgrafted), those producing pPGIP throughout the vine, and vines with no pPGIP could be made. Active pPGIP protein that had been produced in transgrafted rootstocks had been detected in the xylem exudates that were collected from scions, which had not been modified to produce pPGIP (Agüero et al., 2005; Haroldsen et al., 2012). Once the vineyards were established, an objective of the project was to determine whether sufficient pPGIP that reduces Pierce's disease symptoms is delivered from rootstocks expressing pPGIP to scions, which have not been modified to produce pPGIP. Vineyards approximating commercial settings were established with own-rooted and transgrafted vines in regions of Solano and Riverside counties with low and high Pierce's disease pressure, respectively. Evaluations of performance and susceptibility continue to be made that enable comparisons of scion susceptibility to Pierce's disease based on the mode of infection (introduced vs. natural), varietal background (Thompson Seedless and Chardonnay), and origin of pPGIP (rootstock only vs. entire vine).

LAYPERSON SUMMARY

In order to determine whether polygalacturonase (PG)-inhibiting proteins (PGIPs) have potential for the commercial development and deployment to reduce Pierce's disease, vineyards were established in two locations in California. The model PGIP evaluated in this project is produced naturally in pear fruit and inhibits the PG that *Xylella fastidiosa* (*Xf*) produces as it spreads and causes damage in infected grapevines. Each vineyard contained Chardonnay and Thompson Seedless grapevines that were growing on their own roots (own-rooted) and others that were "transgrafted" (with rootstocks expressing pPGIP grafted to fruit producing non-modified scions that, thus, do not themselves produce pPGIP); plantings were completed by June 2013. The genetic and varietal identities of the vines were confirmed by the end of summer 2013. The vineyards were designed to enable comparisons of plant performance and susceptibility to Pierce's disease based on mode of infection (deliberate vs. natural introductions of *Xf*), varietal background (Thompson Seedless vs. Chardonnay), and origin of the pPGIP (transgrafted rootstock delivery to grafted non-PGIP producing scions vs. entire plant producing PGIP). Mechanical inoculations with *Xf* bacteria were done yearly from 2011-2014 in Solano County and, beginning with the establishment of the vineyard in June 2013, natural infections occurred in Riverside County. Data describing

the total vine and disease characteristics of the own-rooted or transgrafted vines of both varieties were collected in 2013 and 2014 in both locations.

INTRODUCTION

Pierce's disease, caused by *Xylella fastidiosa* (*Xf*), can result in the death of grapevine tissues, including scorching along leaf margins and premature abscission of infected leaves. The *Xf* bacteria move from infection sites throughout the vine in the xylem and this spread creates systemic infections and may contribute to xylem occlusions (Krivanek and Walker, 2005; Labavitch 2007; Lin, 2005; Lindow, 2007a,b; Rost and Matthews, 2007). The grapevine water-conducting xylem elements are separated by pit membranes, "filters" composed of cell wall polysaccharides whose meshwork is too small to permit movement of *Xf* (Labavitch et al., 2004, 2007, 2009a,). However, *Xf* produces enzymes that can digest the polysaccharides of pit membranes (Labavitch et al., 2009b), thereby opening xylem connections and permitting spread of the bacteria from the site of introduction. Xylem occluding tylose protrusions from adjacent xylem parenchyma cells may be another consequence of compromising pit membranes by polysaccharide-digesting enzymes produced by *Xf*.

The *Xf* genome encodes a polygalacturonase (*XfPG*) and several β-1,4-endo-glucanase (EGase) genes, whose predicted enzyme products could digest pectin and xyloglucan polymers in pit membranes. Labavitch et al. (2006, 2007, 2009a; Perez-Donoso et al., 2010) reported that introduction of PG and EGase into uninfected grapevines caused sufficient pit membrane breakage to allow movement of *Xf*. Roper et al. (2007) developed an *XfPG*-deficient *Xf* strain that did not cause Pierce's disease symptoms; *XfPG* is a Pierce's disease virulence factor.

Plant proteins that selectively inhibit pest and pathogen polygalacturonases (PGs) have been identified. PG-inhibiting proteins (PGIPs) have been found naturally in the flowers and edible fruits of many plants prior to contact with pathogens and PGIPs are induced in most plant tissues upon infection with microbial pathogens (Powell et al., 2000). PGIPs are extracellular proteins that, therefore, are available to move in the apoplastic stream as it is transported through the xylem. Grapevines, which have been modified to produce in all tissues a PGIP normally expressed in pear fruit (pPGIP), have reduced susceptibility to *Xf*, probably because pPGIP inhibits the *Xf*PG enzyme. Previous work has shown that the active pPGIP protein is transported across graft junctions from pPGIP-expressing grape and tomato rootstocks into wild-type (i.e. not expressing pPGIP) scion stem sections (Agüero et al., 2005, Haroldsen et al., 2012). Therefore, because infectious *Xf* populates the xylem, the presence of pPGIP in the xylem but coming originally from the roots, may reduce *Xf*PG activity and thereby, limit spread of and damage by*Xf*.

This project was originally designed to generate sufficient grafted and own-rooted pPGIP expressing Thompson Seedless and Chardonnay grapevines to plant commercial-type vineyards, and to evaluate their performance and resistance to Pierce's disease. The goals of establishing and identifying the fields have been met and the plantings are now being evaluated for their responses to natural or introduced infections with *Xf*.

OBJECTIVES

1. Scale up the number of grafted and own-rooted pPGIP expressing grapevine plants.
2. Plant and maintain grafted and own-rooted vines in two locations with different Pierce's disease pressures.
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 determine the extent of infection.

RESULTS AND DISCUSSION

Objective 1. Generate enough grafted and own-rooted grapevines for the field trials.

The pPGIP expressing Chardonnay and Thompson Seedless (TS) grapevines originally generated by Agüero et al. (2005) were maintained at the UC Davis Core Greenhouses. Vegetative cuttings of non-lignified stem sections from transgenic and control plants of both cultivars ("own-rooted") were rooted in aeroponic cloning manifolds (EZ-Clone Inc., Sacramento, CA), acclimated, and transferred to field sites. Grafted and "transgrafted" plants were generated for the field trials and were made by green grafting rootstock stem sections with budding scion tissue. Transgrafted plants had rootstocks from the pPGIP expressing lines and scions that do not express pPGIP. Grafted plants had the rootstocks and scions with the same genotypes. The number of plants of each genotype and grafting protocol for the field sites in Solano and Riverside counties are shown in **Table 1**. DNA was prepared from the vines used as source tissue for grafting and the genotypes were confirmed by PCR.

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

Table 1. Numbers of grapevines planted in Solano and Riverside counties. Upper portion of the graphic is scion genotype, lower portion is rootstock phenotype; nongrafted plants have no break. Hatched fill represents pPGIP expressing rootstocks and/or scions; black fill is null-transformants (no pPGIP) controls; white fill is non-transformed controls. In Solano County, own-rooted vines were mechanically inoculated in the summers of 2011-2013; transgrafted vines were inoculated in 2013 and 2014. Vines planted in Riverside County had "natural" infections.

SOLANO		'Chardonnay'								'Thompson Seedless'							
	Strategy (Scion/root)																
Own-Rooted	Inoculated (2011-2013)		17								10			9			
	Non-Inoculated		8								2			5			
Grafted	Inoculated (2013, 2014)	9		8	9					9		9	9				
	Non-Inoculated	4		4	4					4		4	4				
RIVERSIDE																	
Own-Rooted	Natural Infections		13			11		6			9			12		6	
Grafted	Natural Infections	16		6	8		6		3	7		14	7		3		3

Objective 2. Establish field trial sites.

Field trial sites in Solano and Riverside counties have been established to assess the Pierce's disease resistance and general agronomic viability of own-rooted and grafted pPGIP expressing grapevines. The field plans of the Powell trial plots in Solano and Riverside counties are shown in **Figure 1**.The vines satisfying our initial PCR analysis were hand-planted in a randomized block design with blocks consisting of two or three individuals in the same treatment (**Table 1**). The young plants were placed in protective grow tubes and hand-watered every two weeks in Solano County or as needed. In Riverside County, the plants were watered by drip irrigation. In Riverside County, the plot is at the bottom of a small hill and the soil is very sandy and porous; irrigation water accumulates in the lowest row (Row E). At both sites, grapevines were planted approximately eight ft. apart and tied to wooden stakes with trellising wires at 40 and 52 inches.

In Solano County, the vines were pruned by the PI and the field crews to maximize potential cane numbers for inoculations and to establish vigorous positions for future growth. In 2014, the first pruning was done in March, and the vines were re-pruned in April, July, and late September/early October. This pruning schedule is unconventional but was done to try to standardize vine growth in our plots with the practices of the other PIs with plots in the same field. With the permit amendment granted by the BRS-USDA in 2012, flowers and fruiting clusters were allowed to persist. Initially, all of the own-rooted Chardonnay vines were cordon trained and spur pruned and the majority of the Thompson Seedless vines were cane pruned in an attempt to maintain proper vine balance and ensure fruit development in our field in the Solano County site. Subsequent prunings have not taken into account varietal differences. The vines at the Riverside County site were pruned according to the schedule established at UC Riverside and varietal differences were not addressed in the prunings. The Solano County site has been observed approximately monthly for the duration of the growing season and the vines in Riverside County established themselves well and were continuing to grow robustly as of late October 2014. Vines at the Riverside County site were evaluated by this group in April and October. The activities in 2014 at both field sites are shown in **Table 2**.

Since June 3, 2013, both the Riverside and Solano county sites have been established with all the planned plantings for this project. A consistent pruning regime remains a goal for this plot so comparisons can be made with other evaluators.

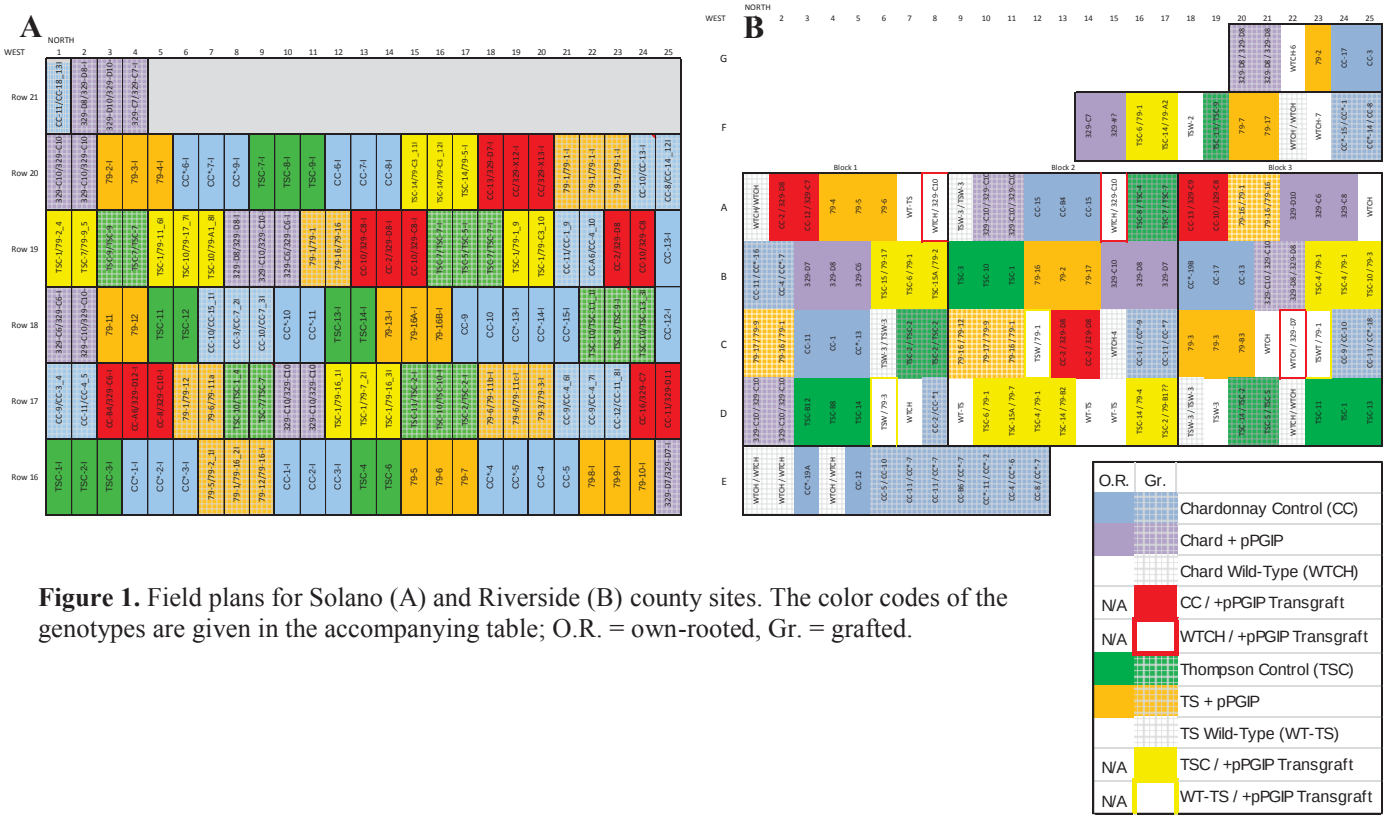

Figure 1. Field plans for Solano (A) and Riverside (B) county sites. The color codes of the genotypes are given in the accompanying table; O.R. = own-rooted, Gr. = grafted.

Table 2. Activities at the Solano and Riverside county sites for this project in 2014.

Date	Location	Activity
14 March 2014	Solano	Visual scoring of symptoms from 2011-2013 infections at each year's inoculation site on each grafted plant
19 March 2014	Solano	Visual re-scoring of symptoms from 2011-2013 infections (see above)
20 March 2014	Solano	Photos, light pruning since vines have buds that have broken; first pruning since 2013
4 April 2014	Riverside	Disease scoring of symptoms on each plant; photos taken (CJ UCD)
28 May 2014	Solano	Inoculate ca. 4 fresh canes/grafted vine for 2014; no pruning
9 July 2014	Solano	Visit field to assess disease on each plant
27 July 2014	Solano	Take cane samples of ca. 1 cane/ genotype/plot for qPCR of canes infected in 2014; prune vines again
29 July 2014	Solano	Count scorched leaves on infected canes; photos taken
3 September 2014	Solano	Disease assessment by D. Golino (UCD)
ca. 1 October 2014	Solano	Vines pruned again
6 October 2014	Riverside	Disease scoring of all plants by P. Rolshausen (PR, UCR)
9 October 2014	Solano	Count infected leaves
24 October 2014	Riverside	Disease re-scoring of all plants, photos taken by A. Powell (AP, UCD)

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

Because of inconsistent and atypical pruning this year, detailed analyses of overall vine growth was not pursued in detail in Solano County. Other than differences due to the variety (Chardonnay or Thompson Seedless), in general, however, no difference in overall growth, time to flower, fruit set, or yield was noticed in the vines. All produced buds in mid-March and flower buds broke by the end of March. Non-grafted vines had been inoculated for three years by March 2014, and clear examples of death in non-PGIP producing vines were apparent. Numbers of bud producing, no-bud producing, and scorched leaves along canes inoculated in 2011, 2012, and 2013 were recorded in March 2014 and will be analyzed for further details. The data has not yet been analyzed for statistical significance or for effects due to grafting. Photos of each vine were taken in March and will be analyzed in time for the Annual meeting.

At the Riverside County site, vine vigor was analyzed on April 4, 2014. Not much growth was visible on the vines and so the scores were very low; assessments will be made in the future later in the season. Assessments of the number of visible canes were made in late October 2014 and are shown in **Figure 2**. In general, vines that had been transformed, either with the empty vector (Control) or with the pPGIP construct (pPGIP and transgrafted) produced slightly fewer canes than the wild-type vines that had never been transformed. The two varieties Chardonnay and Thompson Seedless did not differ in the number of visible canes, but this could be a result of similar pruning regimes applied to the vines. Variation due to grafting vs. own rooting has not yet been analyzed but is available in the data collected.

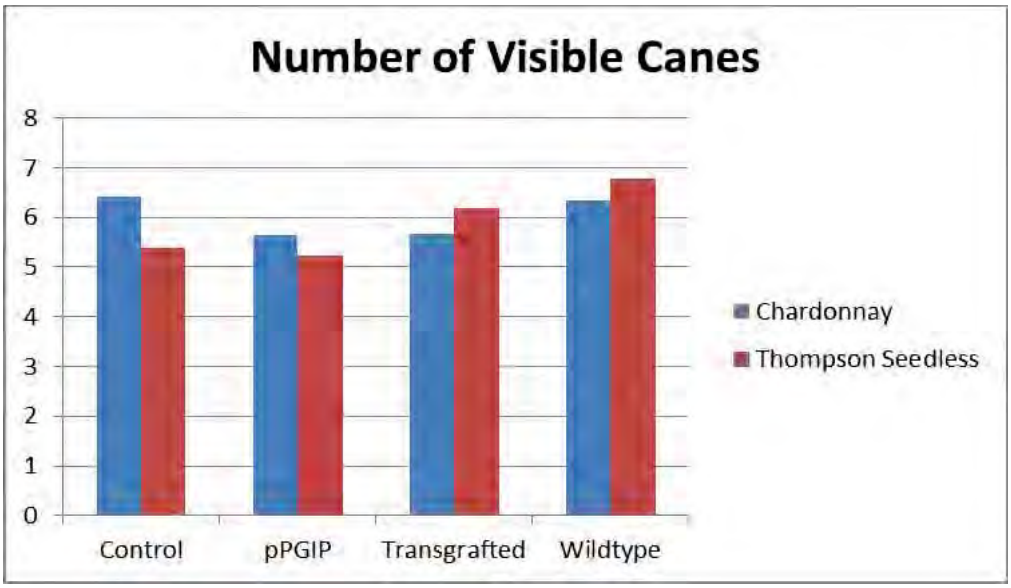

Figure 2. The average number of visible canes (without regard to the viability or extent of Pierce's disease) of vines in the Riverside County plot. Measurements were taken in late October 2014.

In general, the expression of pPGIP either in the scion or the rootstock or both does not impact the phenotype of the plant. However, based on the number of canes in the vines at the Riverside County site, transgenic manipulation, either introducing an empty vector (Control) or engineering the expression of pPGIP with the pPGIP construct could have negative impacts on the number of canes produced by the vines, particularly in the Thompson Seedless variety. However, the vines were not pruned in the format generally accepted for table grapes, such as Thompson Seedless, so the effect may be less apparent when the vines are appropriately pruned. In the Chardonnay variety, the negative effect on cane number was only seen in material engineered to express pPGIP.

Objective 4. Determine Pierce's disease incidence in pPGIP expressing grafted and own-rooted lines. Test for *Xf* presence and determine the extent of infection.

At the Riverside County plot, assessments of disease throughout the vines were made twice in October 2014 and initially in April 2014. The April assessment probably was too early in the season. Evaluators, PR and AP, used the same general assessment scale going from 0 (no disease) to 5 (dead) to evaluate the vines. Additionally, AP counted the total number of canes per vine and the number of canes with scorched leaves or no growth (diseased canes). The initial analyses of the results are given in **Figure 3**. In general, expression of pPGIP throughout the vines or via grafting to pPGIP expressing rootstocks reduced slightly the disease score and reduced the number of infected canes. The data has not yet been analyzed for statistical significance or for effects due to grafting.

At the Solano County plot, the leaves/petioles with evidence of Pierce's disease were counted in March 2014 along canes which had been infected in 2011, 2012, and 2013. The data has not been analyzed yet. The vines were reinoculated along with the vines in the plots of the other PIs on May 28, 2014. Up to four canes per vine were inoculated as previously with inoculum provided by D. Gilchrist. In our plot only vines that were grafted or transgrafted were inoculated in 2014. Previous inoculations in 2011-2013 had included vines that were own-rooted. The extent of disease along the canes inoculated in 2014 was measured three times during the 2014 season

on July 9, July 29, and October 9, 2014. As is shown in **Figure 4**, the number of leaves or petioles along canes infected in 2014 was greater in the October 9 assessment than in the July 29 observations. These results indicated that Pierce's disease was developing in these canes. However, vines with pPGIP in the scion portion had a slower increase in disease symptoms, especially in the Chardonnay variety. Notably, vines with pPGIP in the rootstocks also showed fewer numbers of diseased leaves or petioles along the infected canes, although the increase during 2014 was about what was observed in the controls (vines that had been grafted using material that had been transformed with the empty vector construct). The data has not yet been analyzed for statistical significance.

Infected cane material was collected on July 27, 2014 and ground to a fine powder for storage at -80°C prior to extraction of DNA to quantitate the amount of *Xf* in the samples. This analysis is not yet complete.

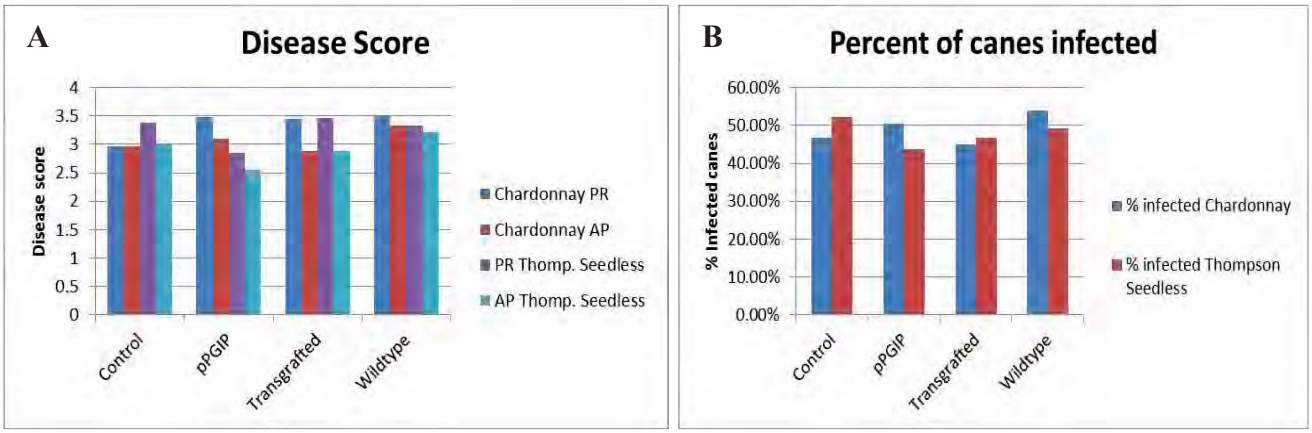

Figure 3. Disease incidence in the Riverside County plot of Chardonnay and Thompson Seedless vines measured in October 2014. **A.** Disease score based on 0-5 scale. **B.** Percent of vine canes with symptoms or evidence of Pierce's disease.

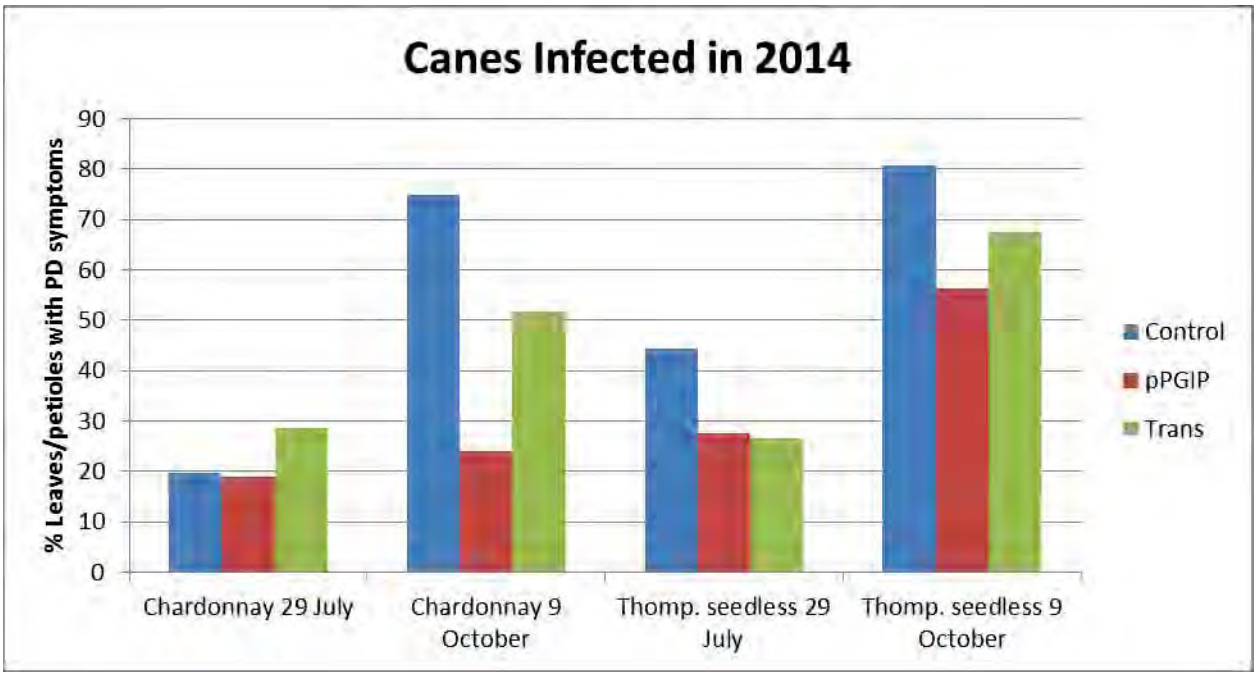

Figure 4. The average percentages of the number of leaves or aborted petioles distal to the point of infection in 2014 of vines in the Solano County site. Pierce's disease symptoms were defined as leaf edge scorching or matchstick petioles.

The disease scoring analyses done by PR and AP at the Riverside County site produced approximately equivalent scores. Analysis of the actual number of infected canes generally supported the overall disease score analyses. The results suggest that some beneficial effects of pPGIP express ion can be seen in rootstocks as well as in the

scion portions of the vines, although the Thompson Seedless variety showed a more pronounced positive effect than the Chardonnay variety. In Solano County, analysis of the vines infected in 2014 demonstrated disease progression over the summer and suggested that pPGIP expression in the scion portions of the vines provided greater reduction in disease development than expression of pPGIP just in the rootstock portions of the vines. In this field, the beneficial effects of PGIP expression were more pronounced in the Chardonnay variety than in the Thompson Seedless variety. Examples of infected vines are shown in **Figure 5**.

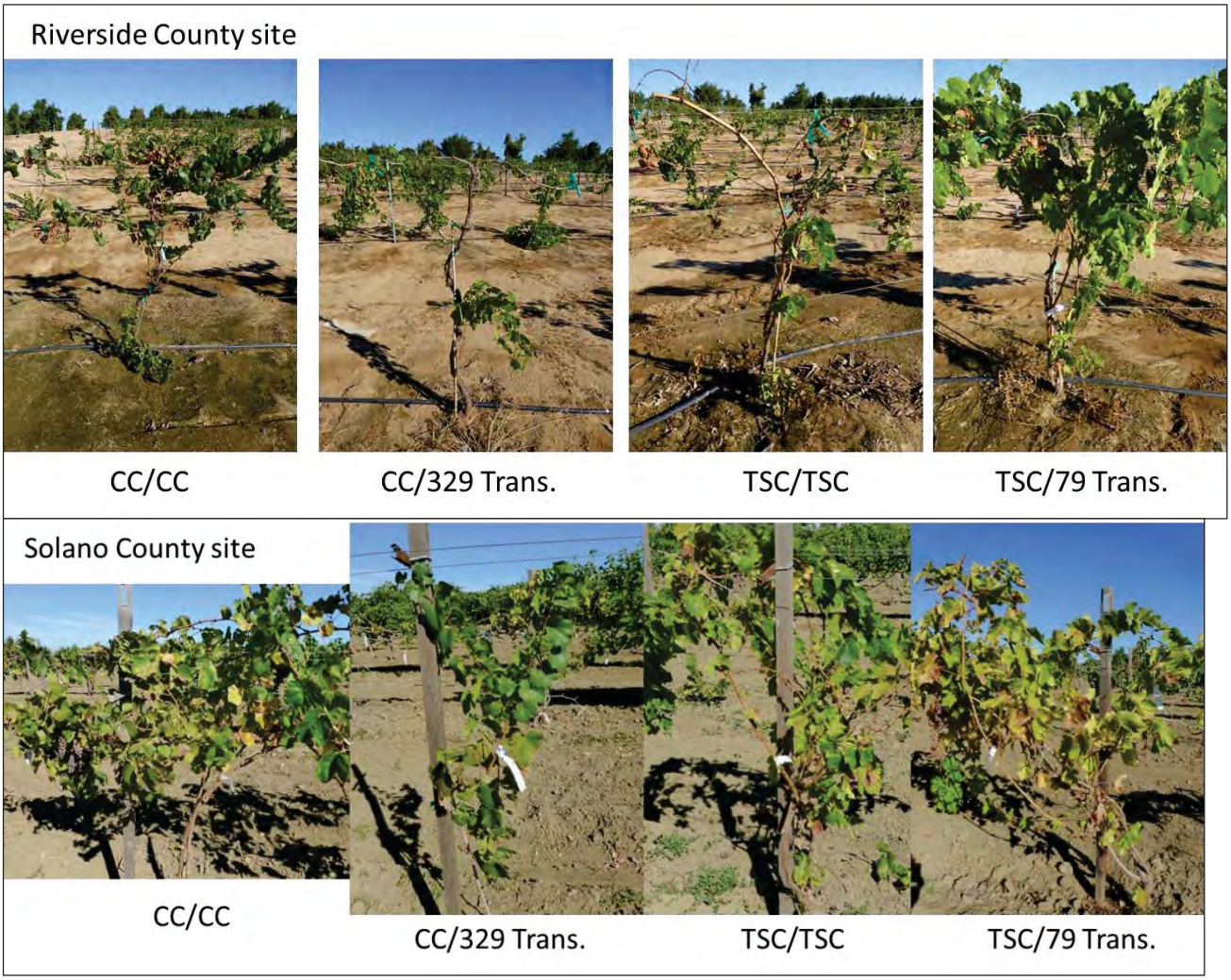

Figure 5. Selected examples of vines from the Riverside and Solano county sites. Photos taken in October 2014. CC/CC and TSC/TSC are vines that were grafted Chardonnay and Thompson Seedless controls in which the rootstocks and scions had been transformed with the control empty vector. CC/329 are transgrafted Chardonnay with pPGIP expressing rootstocks and TSC/79 are transgrafted Thompson Seedless with pPGIP rootstocks

CONCLUSIONS

All of the grafted plants necessary for the studies in Solano and Riverside counties were generated, planted, and inoculated according to the plans of the project. The genotypes of the grafted plants were confirmed. Initial infections in 2011 of the vines in Solano County produced no visible symptoms over a year. The second set of inoculations in Year 2 resulted in detectable *Xf* DNA in infected vines in November 2012 and visual symptoms of Pierce's disease in April 2013. Mechanical inoculations with *Xf* bacteria in 2011 and 2012 in Solano County resulted in the accumulation *Xf* DNA sequences only in the inoculated, but not in the uninoculated, cane material. Symptoms of Pierce's disease infection were visible on the inoculated vines beginning generally in the spring of the year following the introduction of *Xf*. Inconsistent or atypical pruning schedules have made determinations of similarities of vine phenotype and vigor to commercially propagated fields difficult, However, the overall

performance of the own-rooted Chardonnay and Thompson Seedless vines in the field seems to be unaffected by the expression of pPGIP either in the scion or the rootstocks. The evaluations of the leaf and cane phenotypes of the plants suggest that pPGIP expression improves resistance of vines to Pierce's disease, probably more in the Thompson Seedless than in the Chardonnay variety during natural infections in Riverside County, but the Chardonnay vines with pPGIP had fewer Pierce's disease symptoms than the Thompson Seedless variety when mechanically inoculated in Solano County. By using varieties grown for fresh fruit and for wine production in California, we are comparing the impacts of these changes using varieties which grow with different habits and which are important to different segments of the community of California grape growers.

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TOOLS FOR IDENTIFYING POLYGALACTURONASE-INHIBITING PROTEIN TRANSMISSION FROM GRAPEVINE ROOTSTOCK TO SCION

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Reporting Period: The results reported here are from work conducted July1, 2010 to June 30, 2014.

ABSTRACT

Pierce's disease incidence has been associated with the spread of the causal agent, *Xylella fastidiosa* (*Xf*), throughout the xylem vasculature of infected grapevines. The spread from one vessel to the next utilizes cell wall modifying enzymes produced by the bacteria to degrade 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 of *Xf* (Roper et al., 2007). Previous projects have analyzed the PG-inhibiting proteins (PGIPs) for their potential to minimize the damage caused by pathogens and pests on plants (Powell et al., 2000, Aguero et al., 2005), including damage caused by *Xf* in Pierce's disease. Two field projects currently funded by the CDFA use pear fruit PGIP (pPGIP) to restrict *Xf* movement.

This project was designed to generate a new polyclonal antibody preparation that recognizes PGIPs in general and new preparations of monoclonal antibodies that specifically recognize the pPGIP protein. The previous polyclonal antibody preparation used in initial analyses of plants was over 25 years old and little of the stock remained (Stotz et al., 1993). The monoclonal antibody is necessary for the related field evaluation projects, including "Field Evaluation of Grafted Grape Lines Expressing PGIPs" (PI Powell). The monoclonal antibodies allow 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 and comparisons of anti-Pierce's disease strategies can be made knowing the amount of the active anti-Pierce's disease protein in the tissues. The goal of the work was to design, generate and evaluate the new antibody preparations.

LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*), the bacterium that causes Pierce's disease of 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 of the plant. PG-inhibiting proteins (PGIPs) produced by plants selectively inhibit PGs from bacteria, fungi, and insets. Our work (Abu-Goukh et al., 1983) identified a PGIP (pPGIP) from pear fruit that at least partially inhibits the *Xf*PG and we demonstrated that expression of pPGIP reduced Pierce's disease symptom development in grapevines (Aguero et al., 2005). Current projects, including field trial evaluations, require a monoclonal antibody specifically recognizing the pPGIP protein in order to detect, quantify, and characterize the pPGIP protein delivered to the scion portion of grafted plants from rootstocks expressing the pPGIP (Aguero et al., 2005). The new monoclonal antibodies allow the researchers to compare the amounts of the pPGIP protein at different times and places and, thereby, determine the protein's role in *Xf*PG inhibition in grapevines. We have received a new polyclonal antibody that recognizes pPGIP and we have received monoclonal antibody preparations made to recognize specific and unique parts of the pPGIP protein. We purified active pPGIP from green pear fruit to develop and test the antibody preparations.

OBJECTIVES

1. Purify pear fruit PGIP protein to use to generate new polyclonal and monoclonal antibodies.
2. Calibrate the antibodies produced 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. Purify pear fruit PGIP protein to use to generate new polyclonal and monoclonal antibodies. Because of budget limitations, we abandoned purification of the pear PGIP from transgenic *Arabidopsis* leaves engineered to express a tagged version of the protein.

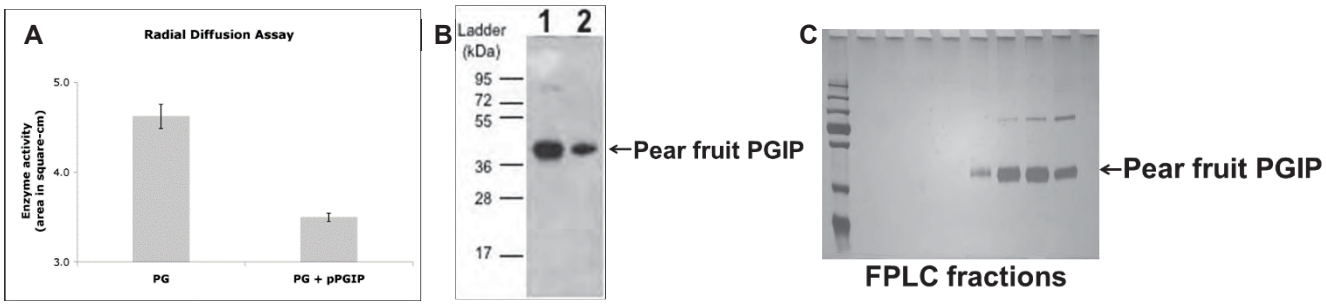

Figure 1. A. Partially purified pPGIP protein caused a 75% inhibition of *B. cinerea* PG activity. **B.** Immunoblot detection of pPGIP separated by SDS-PAGE (10%) and probed using a 25-year-old polyclonal antiserum to pPGIP. Lane 1: 80 ng protein after ammonium sulfate precipitation. Lane 2: 10 ng protein after the ConA purification. **C.** Silver stained SDS-PAGE 10% gel. Lanes 2-10 are FPLC fractions. The PGIP protein band of 45 kDa molecular weight is seen. A band at 90 kDa is likely a PGIP dimer.

We purified sufficient active pear fruit PGIP (pPGIP) from immature green pears for evaluation of the antibodies prepared by Antibodies Inc. Approximately 195 µg of protein was obtained and the preparation actively inhibits PGs produced in culture by the Del 11 strain of *B. cinerea*, as expected. **Figure 1** shows results documenting the activity and purity of the protein. As described in Objective 2, we decided not to use this protein itself to develop monoclonal antibodies because of its extensive glycosylation, typical of plant proteins. Instead we have used the protein to determine the specificity of the antibodies generated to the peptides.

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

Based on the concern noted above that authentic pPGIP protein may not result in the generation of sufficiently specific anti-pPGIP monoclonal antibodies, we worked with Richard Krogsrud, CEO of Antibodies Inc., to identify hydrophilic peptide sequences in the pPGIP protein sequence that could be used as antigens. We selected three peptides (**Figure 2**) that would be specific to pPGIP and would be likely to assure that the monoclonal antibodies would not recognize other PGIPs. The three peptides were combined when they were used to raise the antibodies in order to optimize the chances of getting a specific and robust antibody. We also identified a peptide from the conserved amino end of the PGIPs. We selected this peptide to generate a new polyclonal antibody to detect other PGIPs in addition to the pPGIP. The peptides were synthesized through subcontractors used by Antibodies Inc. although one of the pPGIP-specific peptides (pPGIPc) proved to be recalcitrant to conjugation. Antibodies Inc. developed hybridomas using the other two pPGIP-specific peptides (pPGIPa and pPGIPb) and delivered them to us in late September 2013.

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

Only the peptide labelled pPGIPa in **Figure 2** succeeded in raising antibodies that recognize a peptide rather than the bovine serum albumin (BSA)-conjugate in an Elisa assay. Hybridoma clones 3H12, 6G2, and 7G5 recognize the pPGIPa peptide LETLEFHKQPC. Material from clones 3H12 and 6G2 recognize pPGIP protein in ELISA analyses and will be assayed further once more material is available to find effective concentrations for Western blot analyses.

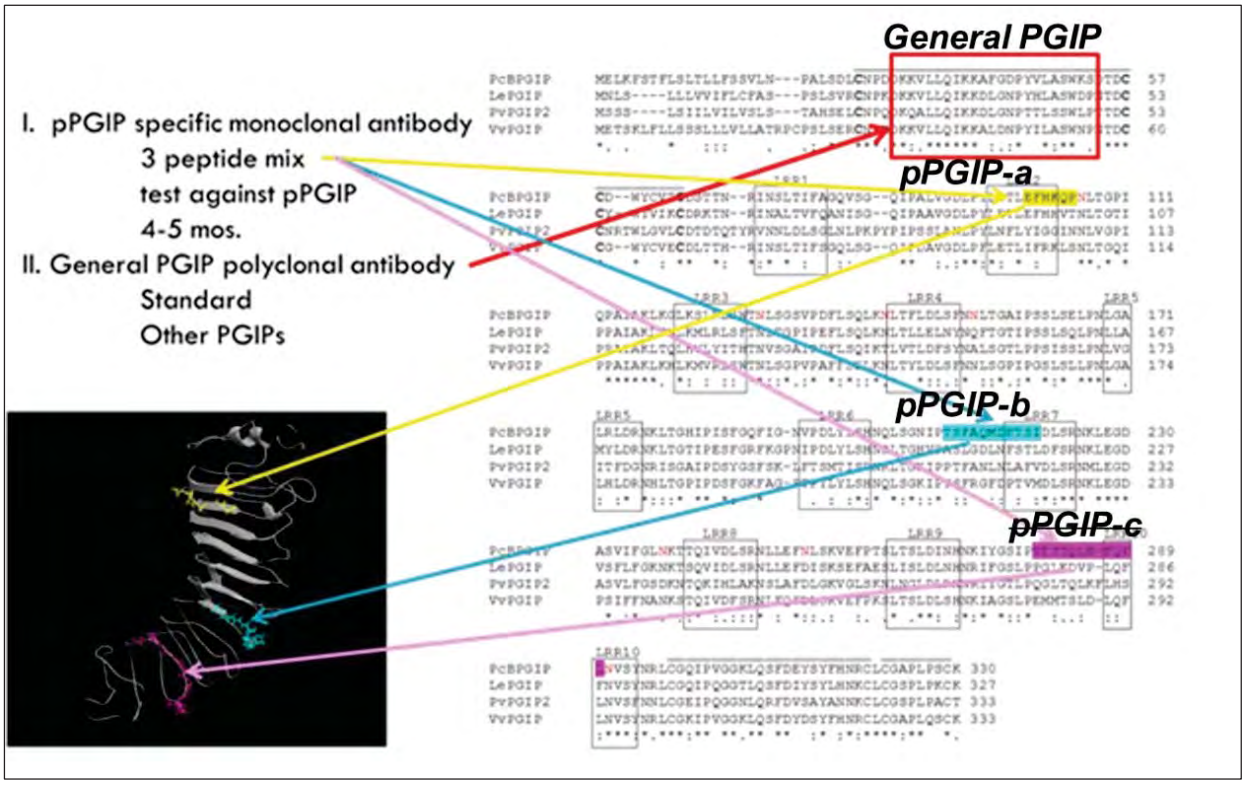

Figure 2. Amino acid sequences of pear (pPGIP), tomato (LePGIP), common bean (pvPGIP), and grape (vvPGIP) showing the location of the leucine-rich repeats (LRR) and the three pPGIP specific peptides (in yellow, blue, and pink pPGIP-a, pPGIP-b, pPGIP-c) and the peptide common to all PGIPs (in red, General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

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

The western blot with the new polyclonal antibodies in **Figure 3** contains proteins from the leaves of a tomato line expressing pPGIP; similar results have been obtained with xylem sap collected from the cut stem of the same plants. Efforts to collect xylem sap from pPGIP-expressing grapevines has yielded only a very small amount of protein and the expected greater sensitivity of the monoclonal antibodies is necessary to detect this pPGIP from grapevine xylem exudate, but we have not yet determined an appropriate dilutions of the cell line supernatants to do this. However, it is clear that the polyclonal antibody (**Figure 4A**) and at least one of the monoclonal antibody lines (6G2) recognize pPGIP protein in grape leaves and berries. Optimization of the dilutions of the monoclonal antibodies for use on Western blots continues. Activities for this objective will be concluded once more supernatants from the cell lines expressing the monoclonal antibodies are received at UC Davis.

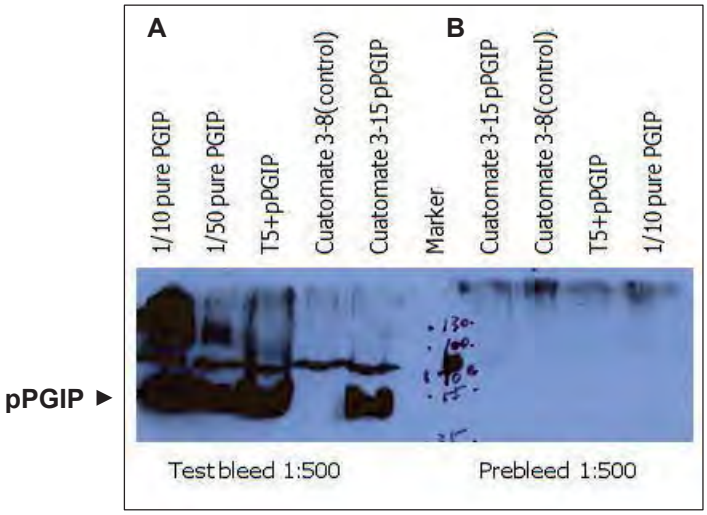

Figure 3. Image of western blot of proteins cross-reacted with (**A**) polyclonal antibodies from the first test bleed serum in response to the general PGIP peptide and (**B**) pre-bleed serum from the rabbits (B).

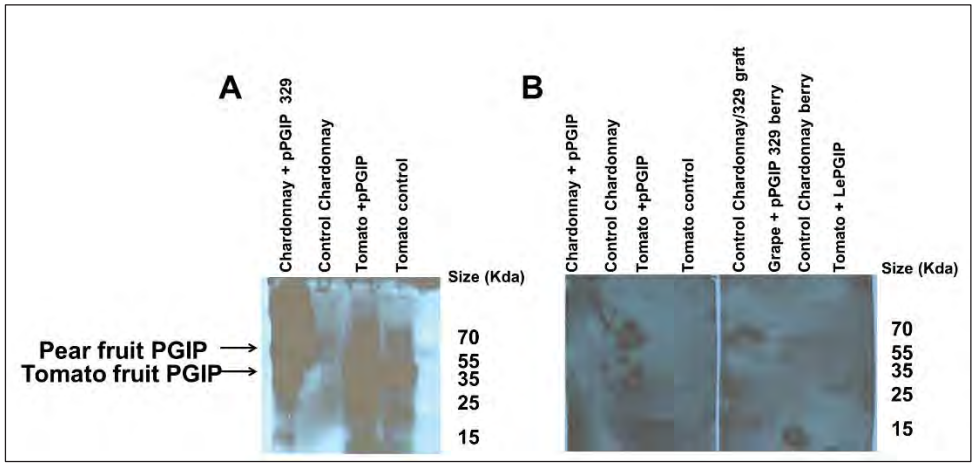

Figure 4. Western blots using (**A**) new polyclonal antibody to the General pPGIP peptide or (**B** left) monoclonal antibody 6G2 or (**B** right) combined supernatants from the hybridoma lines. All proteins were from leaves except for two samples from grape berries. 10 mg protein was loaded per lane.

CONCLUSIONS

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

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

The relevance of the research was based on the RSAP review and RFPs which gave priority to delivery proteins, including PGIPs, from grafted rootstocks to control Pierce's disease. Two currently funded projects use expression of PGIPs as control strategies to limit the spread of *Xf* in the xylem network and thereby reduce Pierce's disease symptom progression in infected vines. Monoclonal antibodies recognizing pear fruit PGIP (pPGIP), the protein expressed in the grape lines that are currently under evaluation in field trial studies, was needed to detect, quantify, and observe the localization of the protein in the transformed grapevines and in grafted vines with transformed rootstocks. In order to make comparisons between the different strategies to control *Xf* spread, the amounts and efficacy of the pPGIP in the infected parts of the plant must be determined and a pPGIP recognizing monoclonal antibody developed to allow measurements of the amounts of the protein. Authentic pPGIP protein from pear fruit could have been used to prepare this monoclonal antibody which would be maintained in perpetuity as a cell culture, but we modified the approach and synthesized synthetic peptides from specific regions of the pPGIP protein to use as antigens. This approach assures that antibodies recognize only pPGIP and not the endogenous grape PGIPs. Production of the monoclonal antibodies has been accomplished and has been partly tested for the specificity and strength of their recognition of properly glycosylated, active pPGIP protein from pear fruit and pPGIP expressed grape plants which have been purified.

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FUNDING AGENCIES

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

CONTINUATION OF THE FIELD EVALUATION OF NEW STRATEGIES FOR THE MANAGEMENT OF PIERCE'S DISEASE OF GRAPEVINE

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Reporting Period: The results reported here are from work conducted July 2014 to October 2014.

ABSTRACT

Our goal is to maintain the experimental vines currently being evaluated for management strategies of Pierce's disease. We have been implementing viticulture production standards in order to reduce the impact of abiotic and biotic stresses on vine health, thereby providing an objective assessment of the efficacy of each strategy. Those strategies were previously developed by project investigators (see list of collaborators) and funded by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and include different transgenic approaches and the use of a biocontrol agent. Here we present all the field activities that were conducted since July 2014, including irrigation water, soil, and plant tissue analyses. Based on these analyses, we identified several problems that may have limited the establishment and growth of those vines after planting. Abiotic stresses that were identified included slightly alkaline soil and water, nutrient deficiencies in the soil (i.e., nitrogen, magnesium, boron, and phosphorous), and in the plant (i.e., nitrogen and phosphorous), as well as boron toxicities in plant tissues. Crop load was also an issue in the Hopkins/Kirkpatrick plot. Biotic stresses other than Pierce's disease included the nematode *Tylenchulus semipenetrans* in the Dandekar plot and powdery mildew (*Erysiphe necator*) in the Hopkins/Kirkpatrick plot. As expected, disease pressure increased over the summer, as indicated by an increased number of glassy-winged sharpshooters (*Homalodisca vitripennis*) caught on yellow sticky traps. Pierce's disease severity was recorded in all research plots and the results are presented in this report, but will be discussed in reports by individual PIs.

LAYPERSON SUMMARY

Alternative strategies for control of Pierce's disease are currently being evaluated in a field trial in Riverside County. Vines are subjected to natural disease pressure because of the presence of populations of the glassy-winged sharpshooter (GWSS), an insect vector of the bacterium *Xylella fastidiosa* which causes Pierce's disease of grapevines. Here we present all the field activities that were done since July 2014, including irrigation water, soil, and plant tissue analyses. Based on these analyses, we identified several problems that may have limited the establishment and growth of those vines after planting. Abiotic stresses that were identified include slightly alkaline soil and water, nutrient deficiencies in the soil (i.e., nitrogen, magnesium, boron, and phosphorous), and in the plant (i.e., nitrogen and phosphorous), as well as boron toxicities in plant tissues. Crop load was sometimes an issue. Biotic stresses other than Pierce's disease included nematodes and powdery mildew. As expected, disease pressure increased over the summer, as indicated by an increased number of GWSS caught on yellow sticky traps. Pierce's disease severity was recorded in all research plots and results are presented in this report, but will be discussed in reports by individual PIs.

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). Current Pierce's disease management strategies primarily involve vector management through the use of insecticides. Several alternative strategies are currently being evaluated in field trials. One of the field trials is located in Riverside County. The experimental grapevines grown there are subjected to natural populations of the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*), a serious vector of the *Xf* bacterium. The strategies developed by principal investigators Dandekar, Lindow, Gilchrist, Powell, and Kirkpatrick/Hopkins that are currently being evaluated include various transgenic grapes and grape rootstocks expressing genes from different constructs, as well as the use of a non-virulent *Xf* strain as a biocontrol agent (see individual PIs reports for more information). Our goal is to maintain the vines growing at the site and record data on insect vector and disease pressure and Pierce's disease incidence and severity in order to identify the most effective control strategies moving forward.

OBJECTIVES

1. Maintain grapevines and research plots.
2. Monitor sharpshooter populations and disease pressure.
3. Record Pierce's disease severity.

RESULTS AND DISCUSSION

Objective 1. Maintain grapevines and research plots.

Field activities since July of 2014 are reported in **Table 1**. Water, soil, and tissue samples from each experimental plot were sent to Fruit Growers Lab, Inc. for analyses (**Tables 2, 3, and 4**). The Ever-Green Nematode Testing Lab, Inc. also performed nematode analyses from soil samples. For the irrigation water, no obvious problem was noticed besides a slightly alkaline pH (**Table 2**). The soil from shallow (0-25 cm) and deep (25-50 cm) samples around grapevine roots as well as background soil from middle rows also showed that soils were slightly alkaline. This condition is likely affecting cation exchange capacity (CEC) as higher pH decreases cation availability (**Table 3**). Overall, the deep soil seems to be more deficient in magnesium and zinc. In addition, boron, nitrate-nitrogen, and phosphorous availability were limited in deep and shallow soils. Some of these carried over to tissue analyses whereby nitrogen and sometimes phosphorous and zinc were lower than the optimum range (**Table 4**). However, toxic levels of boron were recorded in vines from all experimental plots. Mineral nutrient imbalance was also previously reported in grapevines and host plants infected with Pierce's disease (Lu et al., 2003; De La Fuente et al., 2013), but never for boron. Those deficiency or toxicity levels may have confounded Pierce's disease symptoms, as older leaves with boron toxicity can appear scorched. Thus improper disease severity rating may have resulted from it. Vine tissue analyses will be repeated in the spring of next year to confirm some of these preliminary data and vines will be fertilized accordingly to mitigate the deficiencies and toxicities observed. Nematode analysis showed that *Tylenchulus semipenetrans* was present in Dandekar's plot (2,254 nematodes per kg of soil) and they may have stressed the vines and caused them to decline (Verdejo-Lucas and Mckenry, 2004). Interestingly, these nematodes were only found in Dandekar's block. Abiotic stresses such as heavy crop load that was only observed on some vines in the Hopkins/Kirkpatrick plot may also have stressed the vines and caused them to decline (**Figure 1B**).

Objective 2. Monitor sharpshooter populations and disease pressure.

Sharpshooters were monitored at the experimental site in all three blocks (Dandekar, Gilchrist/Lindow/Powell, and Kirkpatrick/Hopkins). For each block, six 6" x 9" double-sided yellow sticky traps were placed randomly throughout the plots. Traps were mounted on wooden stakes slightly above the vine canopy. These traps were collected every month and returned to the laboratory to identify under the stereomicroscope the number of GWSS. Results (**Figure 2**) showed that a low insect vector population was recorded early in the season (March to May 2014) but that that population drastically increased over the summer of 2014. In addition to monitoring sharpshooter populations, we will monitor their natural infectivity. The GWSS collected from the sticky traps will be subjected to qPCR (Yang et al., in preparation) to determine the fraction testing positive for *Xf*. This information, together with sharpshooter seasonal counts, will allow for estimates of disease pressure at each experimental plot.

Table 1. Field activities for all grapevine experimental plots located in Riverside County.

Date	**Activity**
July 2	Traps collected, sharpshooters censused, new traps deployed
July 11	Rodent control
July 12	Grape tissue sampling for analysis by Fruit Growers Lab
July 17	Fungicide application (Rally + stylet oil) for powdery mildew control; weed control
August 7	Traps collected, sharpshooters censused, new traps deployed
August 8	Pruning and burying grape cuttings; fungicide application (stylet oil) for powdery mildew control
August 21	Pruning and burying grape cuttings
August 22	Soil and root sampling for nematode count; Ever-Green Nematodes Testing Lab Soil sampling for analysis by Fruit Growers Lab
August 12-26	Weeding and vine training
August 26	Weed control
September 4	Traps collected, sharpshooters censused, new traps deployed
September 17	Water sampling from drip irrigation for analysis by Fruit Growers Lab
September 22	Pierce's disease severity rating
September 23	Weed control
September 29	Pierce's disease severity rating
October 6	Pierce's disease severity rating; sampling petioles for *Xf* detection by qPCR

Figure 1. (A) Grapevine cv. Pinot improperly trained, also showing powdery mildew symptoms. (B) Over-cropped grapevine cv. Pinot showing signs of stress.

Table 2. Grape irrigation suitability analysis, Fruit Growers Laboratory, Inc.
Values highlighted in red represent higher than optimal levels.

Test Description	Results mg/L	Meq/L
Cations		
Calcium	50	2.5
Magnesium	10	0.82
Potassium	3	0.077
Sodium	40	1.7
Anions		
Carbonate	< 10	0
Bicarbonate	170	2.8
Sulfate	57	1.2
Chloride	29	0.82
Nitrate	16.1	0.26
Nitrate Nitrogen	3.6	
Fluoride	0.5	0.026
Minor Elements		
Boron	0.1	
Copper	0.01	
Iron	0.04	
Manganese	0.04	
Zinc	< 0.02	
TDS by Summation	376	
Other		
pH	7.6	
E.C.	0.513 dS/m	
SAR	1.4	
Crop Suitability		
No amendments	Fairly good	
With amendments	Good	
Amendments		
Gypsum requirement	0.2 Tons/AF	
Sulfuric acid (98%)	9.8 oz/1000Gal	
Leaching requirement	3.3 %	

Table 3. Soil analysis, Fruit Growers Laboratory, Inc. Soil samples representing shallow soil (SS-R) and deep soil (DS-R) of grapevine roots as well as background soil from middle row (BS), were collected from each experimental block. Values highlighted in yellow and red represent lower and higher levels than the optimal requirements, respectively.

Test Description	Block								
	Dandekar			Kirkpatrick / Hopkins			Lindow/Gilchrist/Powell		
	BS	SS-R	DS-R	BS	SS-R	DS-R	BS	SS-R	DS-R
Primary Nutrients									
Nitrate-Nitrogen (ppm)	11.8	1.5	2.5	7.4	1.9	1.5	111	1.4	1.4
Phosphorus-P_2O_5 (ppm)	48.1	25.2	45.8	20.6	25.2	18.3	38.9	20.6	29.8
Potassium-K_2O *Exch* (ppm)	157	84	133	72	84	60	145	72	169
Potassium-K_2O *Sol* (meq/L)	1.06	0.304	0.496	0.112	0.103	0.041	2.29	0.394	0.618
Secondary Nutrients									
Calcium *Exch* (ppm)	560	620	640	1120	1480	1140	560	580	660
Calcium *Sol* (meq/L)	3.06	2.33	1.88	3.42	5.78	3.34	26.5	4.28	1.95
Magnesium *Exch* (ppm)	78	92	84	132	174	139	79	85	96
Magnesium *Sol* (meq/L)	0.933	0.892	0.611	0.927	1.7	0.98	7.71	1.61	0.625
Sodium *Exch* (ppm)	20	40	30	40	80	50	30	30	50
Sodium *Sol* (meq/L)	0.678	4.48	2.57	2.4	7.72	4.09	5.37	4.66	3.89
Sulfate (meq/L)	0.627	3.01	1.86	1.25	7.69	3.65	6.17	2.31	2.95
Micronutrients									
Zinc (ppm)	0.8	1	0.8	0.5	0.8	0.3	0.6	0.5	0.3
Manganese (ppm)	6.4	4.1	3.2	4.2	5.4	4.1	5.3	5.3	4.8
Iron (ppm)	17.5	15.3	14.1	11.2	11.3	15	15.5	9.5	10.2
Copper (ppm)	0.6	0.7	0.6	0.7	0.9	0.5	0.6	0.6	0.5
Boron (ppm)	0.26	0.17	0.18	0.3	0.22	0.15	0.243	0.13	0.13
Chloride (meq/L)	0.45	1.65	0.69	0.68	4.59	2.6	4.04	1.54	1.72
CEC (meq/100g)	3.77	4.24	4.3	7.02	9.38	7.19	3.87	3.9	4.64
% Base Saturation									
CEC – Calcium (%)	74.3	73.1	74.4	79.8	78.8	79.3	72.4	74.4	71.1
CEC – Magnesium (%)	17	17.9	16	15.5	15.2	15.9	16.8	17.9	17
CEC – Potassium (%)	8.78	4.43	6.28	2.35	1.96	1.61	7.78	4.1	7.59
CEC – Sodium (%)	0.1	4.41	3.28	2.32	3.93	3.31	2.97	3.54	4.22
CEC – Hydrogen (%)	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Others									
pH	6.67	7.46	7.35	7.52	7.57	7.69	6.74	6.85	7.34
Soil Salinity (dS/m)	0.62	0.76	0.54	0.69	1.48	0.84	4.18	0.98	0.73
SAR	0.5	3.5	2.3	1.6	4	2.8	1.3	2.7	3.4
Limestone (%)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Moisture (%)	2.7	8.1	6.5	2.8	11.4	6.5	3.5	6.2	3.7
Saturation (%)	27.2	21.6	23.4	24.5	30.7	28.6	25.2	25	26.2

Table 4. Grapevine leaf blades and petioles nutrient analyses from the three experimental blocks at the Riverside County field trial site. Samples were collected in July 2014 and sent to the Fruit Growers Lab, CA. Values highlighted in yellow and red represent lower and higher levels than the optimal requirements, respectively.

Sample	Test Description	Block		
		Dandekar	Kirkpatrick Hopkins	Lindow Gilchrist Powell
Leaf blades	**Macronutrients**			
	Total Nitrogen (%)	3.3	2.62	2.88
	Phosphorus (%)	0.46	0.17	0.38
	Potassium (%)	1.37	0.47	1.78
	Calcium (%)	2.19	2.88	2.38
	Magnesium (%)	0.35	0.36	0.38
	Micronutrients			
	Zinc (ppm)	38.4	23.2	32.1
	Manganese (ppm)	111	100	121
	Iron (ppm)	251	290	187
	Copper (ppm)	15	8	14
	Boron (ppm)	91.3	69	102
	Sodium (%)	0.024	0.014	0.022
Petioles	**Macronutrients**			
	Total Nitrogen (%)	--	0.83	0.77
	Nitrate-Nitrogen (ppm)	--	840	710
	Phosphorus (%)	--	0.12	0.68
	Potassium (%)	--	0.98	4.30
	Calcium (%)	--	2.46	1.54
	Magnesium (%)	--	0.76	0.62
	Micronutrients			
	Zinc (ppm)	--	58.8	42.7
	Manganese (ppm)	--	223	218
	Iron (ppm)	--	47	72
	Copper (ppm)	--	7	7
	Boron (ppm)	--	34.2	42.9
	Sodium (%)	--	0.075	0.166

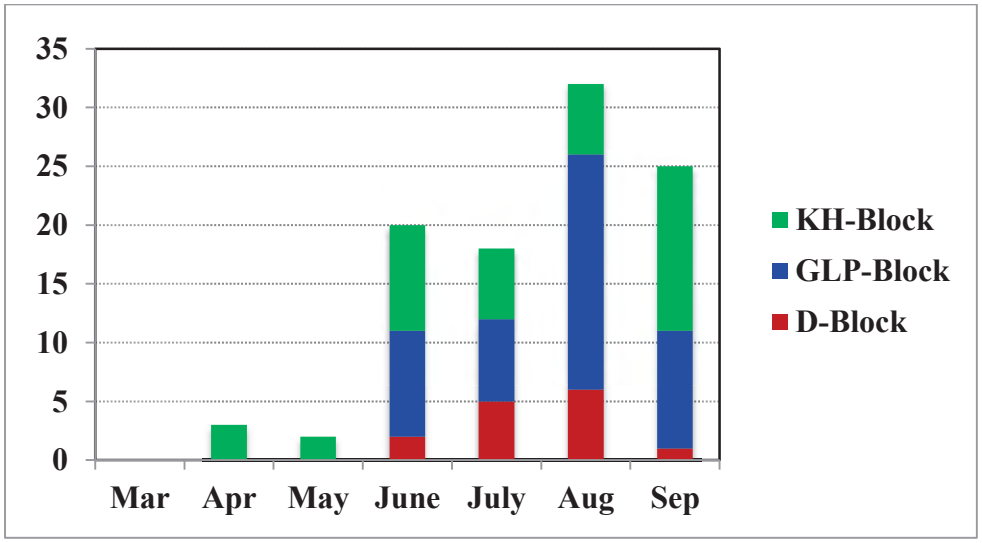


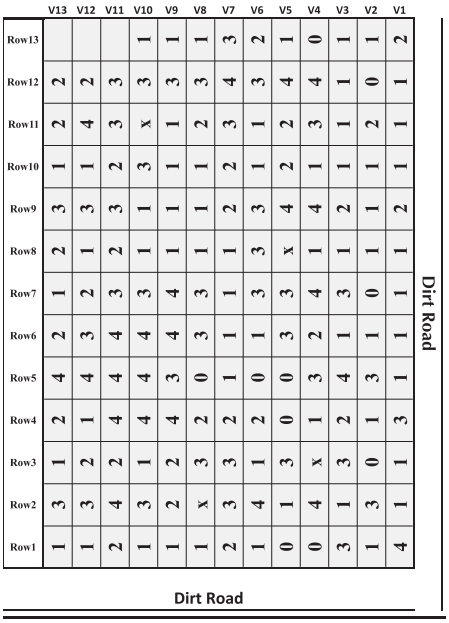
Figure 2. Total number of GWSS captured on yellow sticky traps from all three experimental blocks (D: Dandekar; KH: Kirkpatrick/Hopkins; GLP: Gilchrist/Lindow/Powell). Results are based on a total of 18 traps (6 traps per block).

Objective 3. Record Pierce's disease severity.

Pierce's disease rating was assessed based on a rating scale developed by Dr. Bruce Kirkpatrick. This Pierce's disease rating scale requires that vines are cordon trained. However, because the grapevines were not always trained with cordons and were sometimes pruned improperly (**Figure 1A**), it was difficult to use that disease rating scale, so it was modified (**Table 5**). Besides improper training, powdery mildew leaf symptoms (**Figure 1B**) and boron toxicity may have confounded Pierce's disease symptoms, especially in the Hopkins/Kirkpatrick plot.

Table 5. Rating scale used to score Pierce's disease severity on grapevines from all experimental plots at the Riverside County field trial site.

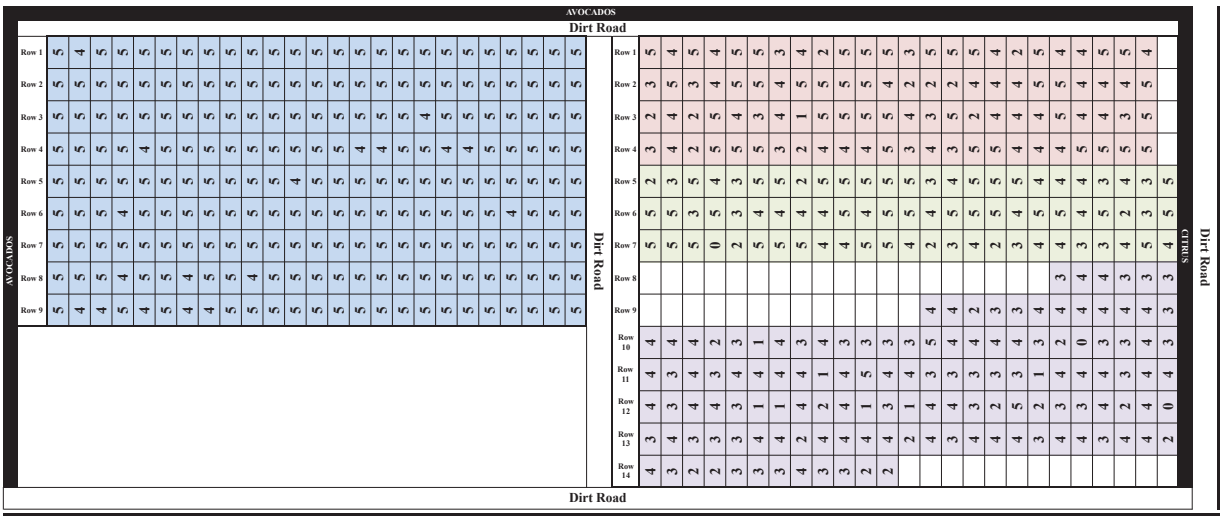
Pierce's Disease Rating	Symptoms
0	None
1	One shoot/cane expresses Pierce's disease symptoms
2	Two shoots/canes express Pierce's disease symptoms
3	Less than 50% of the grapevine is symptomatic
4	More than 50% of the grapevine is symptomatic
5	Grapevine is dead



	V13	V12	V11	V10	V9	V8	V7	V6	V5	V4	V3	V2	V1
Row13				1	1	1	3	2	1	0	1	1	2
Row12	2	2	3	3	3	3	4	3	4	4	1	0	1
Row11	2	4	3	x	1	2	3	1	2	3	1	2	1
Row10	1	1	2	3	1	1	2	1	2	1	1	1	1
Row9	3	3	3	1	1	1	2	3	4	4	2	1	2
Row8	2	1	2	1	1	1	1	3	x	1	1	1	1
Row7	1	2	3	3	4	3	1	3	3	4	3	0	1
Row6	2	3	4	4	4	3	1	1	3	2	1	1	1
Row5	4	4	4	4	3	0	1	0	0	3	4	3	1
Row4	2	1	4	4	4	2	2	2	0	1	2	1	3
Row3	1	2	2	1	2	3	3	1	3	x	3	0	1
Row2	3	3	4	3	2	x	3	4	1	4	1	3	1
Row1	1	1	2	1	1	1	2	1	0	0	3	1	4

Dirt Road

Dirt Road



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CONCLUSIONS

The field trial experimental site in Riverside County is the perfect site to evaluate strategies for control of Pierce's disease, because of the natural presence of GWSS, the disease vector. Our observations and results indicated that the management practices at the experimental site need to be modified so one could fully assess the efficacy of each strategy. However, the symptoms and decline of the grapevines that we recorded are mostly caused by the presence of *Xf*, although additional stressors may have caused those vines to decline faster.

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EVALUATION OF NATURAL PRODUCTS ISOLATED FROM GRAPEVINE FUNGAL ENDOPHYTES FOR CONTROL OF PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted July 2012 to October 2014.

ABSTRACT
The goal of this research is to identify fungal natural products antagonistic to *Xylella fastidiosa* (*Xf*) that could be developed into curative treatments for Pierce's disease. We previously showed in *in vitro* bioassays that eight fungal endophytes inhabiting grapevines possess anti-*Xf* properties, likely due to the production of natural products. We have purified and characterized two of those natural products (radicinin and molecule 'C') produced by *Cochliobolus* sp. and *Dreschlera* sp., and showed that they were effective inhibitors of *Xf*. However, these molecules have poor solubility in water and thus were not systemic when injected *in planta,* and therefore did not reduced Pierce's disease symptoms development. The next step is to develop water-soluble derivatives of those molecules so they can be applied as foliar sprays or trunk injections in vines and become active in the xylem, where the bacteria reside. First we developed a procedure for purifying radicinin by recrystallization instead of chromatography to increase yield and purity of the product. Second, we have produced several semi-synthetic molecule derivatives of radicinin, with one molecule showing increased water solubility. These molecules 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 developed, but as of today successful management largely involves vector control through the use of insecticides. Here we propose to test an alternative control strategy to complement those currently in place or being developed. We have identified eight fungi naturally inhabiting grapevines that are antagonistic to *Xylella fastidiosa* (*Xf*) *in vitro*. The strategy is to use the natural compounds produced by these fungi as potential curative treatments for Pierce's disease. We have been extracting, purifying, and characterizing these fungal compounds and have identified two molecules that are inhibitory to the bacterium in an *in vitro* bioassay. However, those molecules have poor water solubility and could not be used successfully as treatment for Pierce's disease infected grapevines. We are working towards increasing water solubility of these molecules so they can be applied as foliar sprays or trunk injections in vines and become active in the xylem where the pathogen resides. These natural products 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 that is the causal agent of Pierce's disease of grapevine (Hopkins and Purcell, 2002). The recent introduction of a more effective vector, the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*), to southern California shifted the epidemiology of Pierce's disease from a monocylic to a polycyclic disease. This led to a Pierce's disease epidemic with severe economic consequences for the southern California grape industry. The potential for the GWSS to move north and become established throughout the state remains a severe threat to the other major grape-growing regions (central and northern California). Current Pierce's disease management strategies largely involve vector management through the use of insecticides.

Control of Pierce's disease with fungi or fungal metabolites is a largely unexplored research area. Fungi are receiving increasing attention from natural product chemists due to the diversity of structurally distinctive compounds they produce, together with the fact that many fungal species remain chemically unexplored. Fungi are excellent sources of interesting novel molecules that may be candidates with potential for control of bacterial diseases. Indeed, using fungi as biocontrol agents against plant disease is an active area of research (Amna 2010; Proksch et al., 2010; Xu et al., 2008). We first characterized the microbial diversity in grapevines that escaped Pierce's disease in natural vineyard settings, and compared this population to Pierce's disease-infected grapevines with the goal of identifying fungi that are unique to Pierce's disease escaped vines. We identified eight fungal endophytes that possess anti-*Xf* properties, likely due to the production of natural products. Our objective is to identify anti-*Xf* fungal endophyte natural products and natural product derivatives that we could use as curative treatments for Pierce's disease.

OBJECTIVES

1. Identify fungal natural products and semisynthetic derivatives active against *Xf*.
2. Evaluate fungal natural products and semisynthetic derivatives for their potential as curative treatments for vines already infected with Pierce's disease.

RESULTS AND DISCUSSION

Objective 1. Identify fungal natural products and semisynthetic derivatives active against *Xf*.

The goal of this objective is to identify fungal species and fungal natural products produced by endophytes that can be used as curative treatments for control of Pierce's disease. We previously identified eight fungal specimens inhabiting grapevine tissues (xylem sap, shoot, petioles, and spur) that were able to inhibit *Xf* in a bioassay (Rolshausen and Roper, 2011). In brief, *Xf* liquid cultures are adjusted to OD_{600nm}=0.1 (approx. 10^7 CFU/ml); 300 µl of the *Xf* cell suspension are added to three ml of PD3 medium containing 0.8% agar and briefly vortexed. This mixture is then overlayed onto a petri plate containing PD3 medium. A sterile circle of agar is drawn from the margin of an actively growing pure fungal culture and is placed onto the plates previously inoculated with *Xf*. Plates are incubated at 28ºC for seven days and then observed for an inhibition zone around the fungal colony (**Figure 1**).

In addition, crude extracts collected from the fungal cultures showing inhibition towards *Xf* were collected for evaluation using a similar growth inhibition assay as described above. In brief, agar plugs of 0.5 cm diameter of each fungus were used to inoculate 250 mL liquid media, and the fungi were cultivated at room temperature on a shaker. After 10 days, each culture was filtered and further extracted with ethyl acetate, re-suspended in sterile methanol to an extract mass of 1mg, pipetted onto sterile paper discs, and allowed to dry in a laminar flow hood. Once dry, the paper discs containing the crude extracts were placed onto the *Xf* cultures and incubated at 28ºC for seven days. Following this, plates were observed for a halo of inhibition around the paper disc and compared to control *Xf*-only plates and plates with paper discs treated with methanol only. Crude extracts showing inhibition were further processed to purify and identify the inhibitory molecules. Thus far, we have purified two molecules (radicinin and molecule 'C') that are active against *Xf* growth *in vitro* and have characterized their chemical structure. Radicinin is produced by *Cochliobolus* sp. and molecule 'C' is produced by *Dreschlera* sp. These molecules 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.

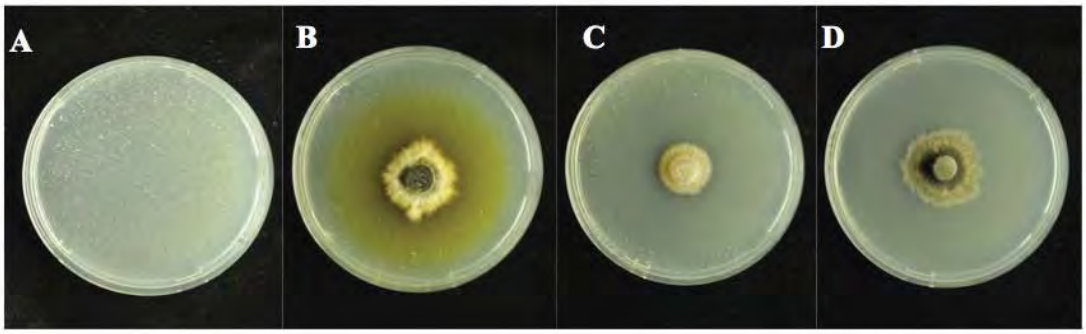


Figure 1. *In vitro* inhibition assay used to evaluate fungal activity towards *Xf*. *Xf* cells were plated in top agar and agar plugs containing fungi were placed on top. Inhibition was evaluated after 8 days of incubation at 28˚C. A) *Xf*-only control; B) No *Xf* inhibition; C) Mild *Xf* inhibition; D) Total *Xf* inhibition.

Radicinin showed great potential *in vitro* (Aldrich et al., in preparation). Hence, in an *in vitro* dose-response assay, where *Xf* cells are submitted to an increasing concentration of a fungal molecule, radicinin was able to inhibit *Xf* growth (**Figure 2**). We have developed a more efficient procedure for isolating radicinin from *Cochliobolus* sp. This is a critical step, as it allows us to produce substantial amounts of water-soluble derivatives and further test them *in planta*. Radicinin is not commercially available, and we had been employing a multistep isolation procedure involving liquid-liquid extraction of *Cochliobolus* cultures followed by an expensive and time-consuming chromatography step to obtain pure radicinin for all our studies to date. Recently, we developed a procedure for purifying radicinin by recrystallization instead of chromatography. In this way, we were able to increase our yield of radicinin from 60.5 mg/liter of culture to 150 mg/liter of culture. This procedure also makes scaling up of the isolation for commercial use much more practical. In addition, the radicinin obtained by this new procedure is significantly more pure, as observed by NMR spectroscopy.

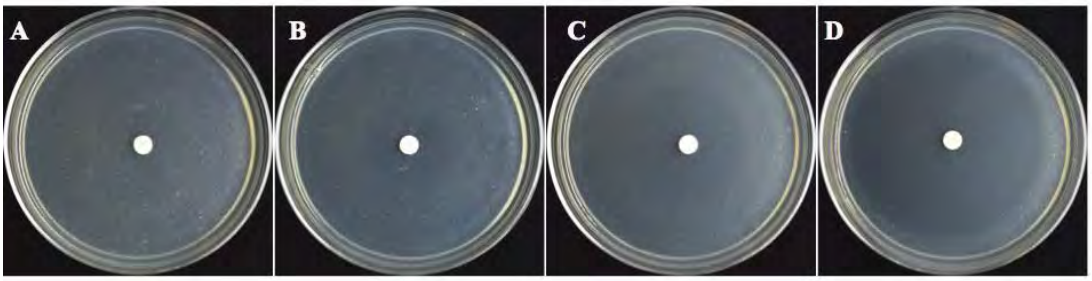
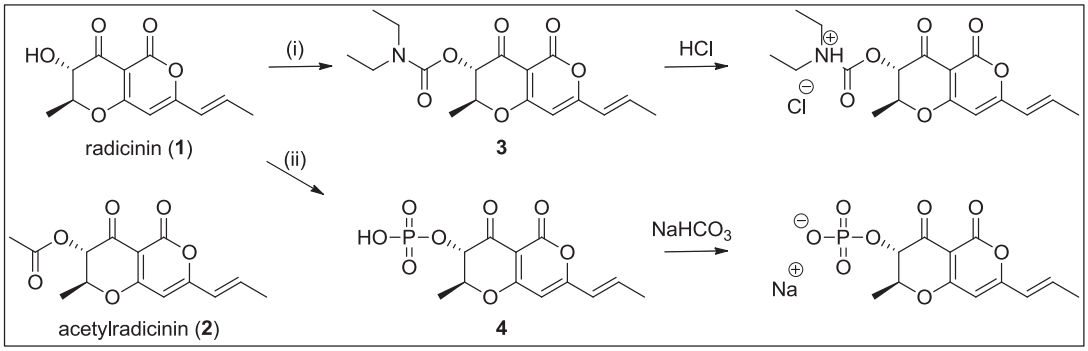


Figure 2. Dose response assay to evaluate *in vitro Xf* inhibition at increasing concentration of a fungal molecule. A) 0 µg molecule R1 (control); B) 50 µg molecule R1; C) 100 µg molecule R1; D) 250 µg molecule R1.

After figuring out how to scale up radicinin production and purification, the next step was to prepare water-soluble semisynthetic derivatives of radicinin to facilitate testing *in planta*. We determined the solubility of radicinin in water to be 0.15 mg/mL, which is considered very slightly soluble. We have shown that acetylradicinin ('2', **Scheme 1**), which was modified at the hydroxyl group of radicinin, retains its anti-*Xf* activity (**Figure 3**). This result suggests that modification of this position may provide a viable strategy for increasing the water solubility of radicinin without loss of activity. Adding ionizable groups is a commonly employed strategy for improving the water solubility of bioactive molecules (Kumar and Singh, 2013), so we had proposed to add two such groups at the hydroxyl position of radicinin (**Scheme 1**). The carbamate (3) is weakly basic and should form a water-soluble salt in low pH solutions, while the phosphate (4) is acidic and should form a water-soluble salt at high pH. Both carbamates and organophosphates are commonly found in pesticides, so we had good reason to believe that one or both of these compounds would be able to move into the xylem of grapevines. However, attempts to prepare the weakly basic carbamate (3) and the acidic phosphate (4) described in our proposal were unsuccessful. Specifically, the reaction with diethylcarbamoyl chloride (i) did not go to completion, while the phosphate reaction (ii) gave a mixture of products that we were unable to separate.



Scheme 1: *Xf*-inhibitory natural product radicinin (**1**), and semisynthetic derivatives (**2-4**). Reagents: (i) *N,N*-diethylcarbamoyl chloride, triethylamine (Vougogiannopoulou et al., 2008). (ii) 1. Cl_3CCN, 2. $(n\text{-Bu})_4NH_2PO_4$, CH_3CN, 3. DOWEX 50WX8, NH_4HCO_3 (Lira et al., 2013).

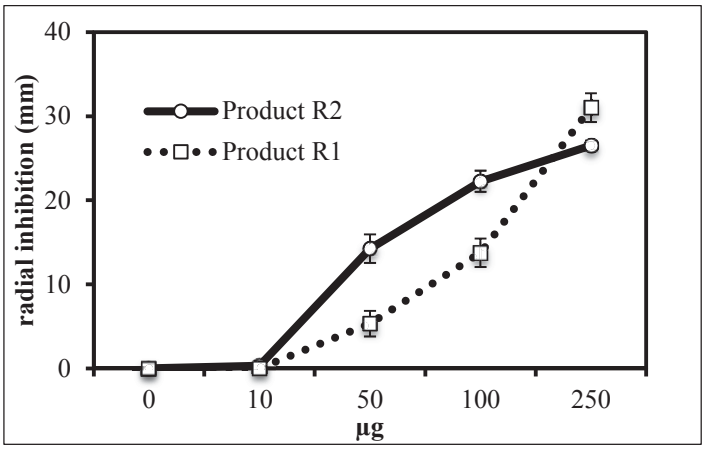
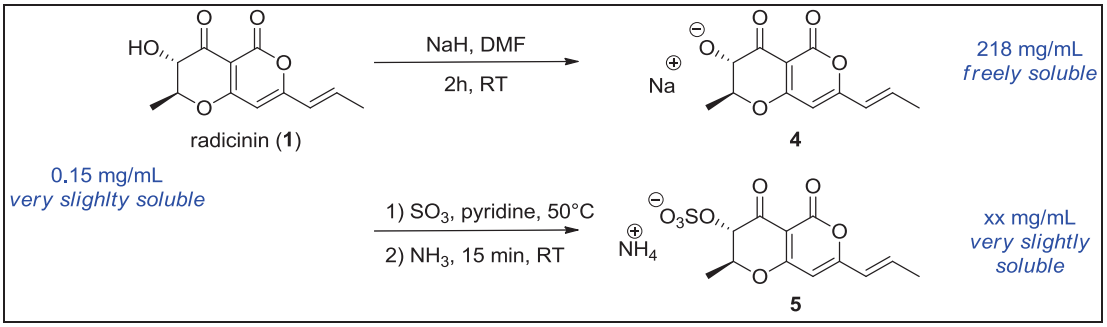


Figure 3: *In vitro* dose response assay. This lab assay quantifies inhibition of *Xf* growth as a measure of a halo around a disc (mm) containing increasing concentration of 'R1' (radicinin) and molecule derivative 'R2' (acetylradicinin).

In an effort to force the formation of carbamate 2, we prepared the alkoxide 4 in quantitative yield (**Scheme 2**). We also tested the water solubility of this derivative and found it to be more than a thousand-fold more water-soluble than radicinin, at 218 mg/mL, which is considered freely soluble. However, the high pH of the alkoxide solution leads us to be concerned about possible nonspecific toxicity. We also doubt that this high water solubility would be maintained in a cellular environment, which is buffered at neutral pH. We then prepared the sulfate of radicinin, '5' (**Scheme 2**). This reaction proceeded to completion and the product proved easy to isolate; we obtained pure '5.' We tested the water solubility and found it to be about twice that of radicinin. The next steps are to find additional derivatives of radicinin that have increased water solubility, while retaining anti-*Xf* activity, and test those molecules *in planta*.



Scheme 2. Alternative radicinin derivatives prepared, and their solubility in water.

In addition to radicinin, we have identified molecule 'C' (**Figure 4**) as another fungal natural product that inhibits *Xf* in our laboratory bioassay. However, low yields of 'C' from our strain of *Drechslera* in potato dextrose broth (PDB) prevented further studies. We are trying to enhance its production by growing the fungus in alternative liquid media including a grape wood extract-based medium and a rice-based medium. In the event that we are unable to increase production of 'C,' it is also commercially available from Sigma-Aldrich. With sufficient quantities of 'C' in hand, we will perform *in planta* studies as described for radicinin. Like radicinin, 'C' has an accessible hydroxyl group, and a similar strategy for improving its water solubility may be employed if necessary.

Figure 4. *In vitro* bioassay showing inhibition of *Xf* with 250 µg of molecule 'C' as indicated by the halo around the disc.

Objective 2. Evaluate fungal natural products and semi-synthetic derivatives for their potential as curative treatments for vines already infected with Pierce's disease.

The goal of this objective is to evaluate the anti-*Xf* efficacy of fungal natural products derivatives *in planta*. Once this proof of concept is established in the greenhouse, the experiment will be carried over to the field. The deliverable for this objective is the development of a commercial product for the cure of PD. We have currently identified two fungal natural products as an active molecule inhibitory to *Xf* (see objective 1). We had previsouly developed greenhouse assays to test radicinin on PD-infected vines. However, we observe no reduction of PD symptoms development because we established that radicinin was not water-soluble. When we will have sufficient quantities of the water-soluble radicinin derivatives and/or other natural product derivatives and confirmed that they maintained the anti-*Xf* activity, we will evaluate those products in the greenhouse assays using vascular injection techniques and spray on leaves.

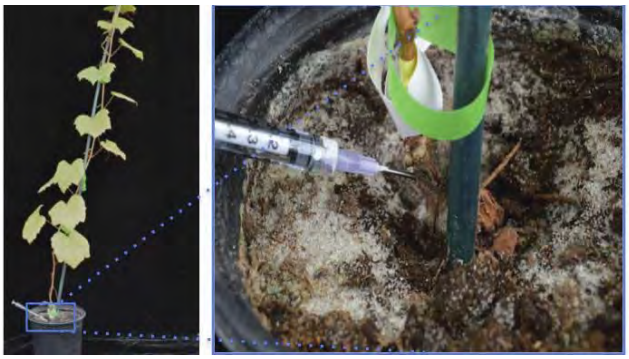


Figure 5. Needle-injection of an anti-*Xf* molecule in the xylem of Pierce's disease infected grapevine cuttings.

CONCLUSIONS

We aim to investigate curative measures for management of Pierce's disease as part of a sustainable Pierce's disease management program. Our strategy is to evaluate the use of anti-*Xf* fungal natural products produced by grapevine endophytic fungi. The commercialization of such of product will provide a solution to growers that have vineyards already infected with Pierce's disease. We have already discovered two active anti-*Xf* fungal natural products, radicinin and molecule 'C.' However, radicinin did not show efficacy in our greenhouse trials on Pierce's disease infected vines, likely because it is not water-soluble. We are now synthesizing semi-synthetic derivative molecules to increase water solubility of these products, which should increase their movement in the plant xylem where *Xf* resides. In addition to these two products, we are also searching for additional active water-soluble natural anti-*Xf* compounds. 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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STRATEGY FOR BACTERIAL DISEASE CONTROL USING GENES FROM PATHOGENS

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ABSTRACT

Pathogen-derived resistance (PDR) is a term that refers to the use of genes from the pathogen in plant transformation strategies to increase resistance against the disease caused by the pathogen. Initially used against viruses, this approach was recently demonstrated to be successful against *Xylella fastidiosa* (*Xf*), the bacterium that causes Pierce's disease in grapevines (Lindow et al., 2014). Transgenic grapevines overexpressing the *rpfF* gene reduced the incidence of disease and the colonization of bacteria in the plant, suggesting that excess diffusible signal factor (DSF) caused confusion in the pathogen, altering its ability to colonize transgenic plants. Since the *rpfF* gene from *Xf* that causes citrus variegated chlorosis (CVC) shares 92% identity with the *Xf* that causes Pierce's disease, we investigated the possibility of citrus transgenic plants overexpressing *rpfF* (*Xf*CVC-RpfF) to be CVC resistant. Besides *rpfF*, other genes from the pathogen, such as those encoding the toxin from the toxin/antitoxin (TA) system could also change the behavior of *Xf*. TA systems are organized in an operon encoding a stable toxin that is lethal to cells, and the corresponding antitoxin, which prevents the toxin lethality. Under stress conditions, the instability of the antitoxin facilitates its degradation and the lethal toxin exerts its function, killing the cell or inducing the formation of persistent cells (Melderen & Bast, 2009). Thus the other hypothesis of PDR is that citrus transgenic plants overexpressing this toxin could induce *Xf* cell death or impair the bacterial multiplication in plants. To test the potential use of this toxin (*mqsR-Xf*) in altering *Xf* pathogenicity we used *Nicotiana tabacum* as an alternative host, since it is susceptible to *Xf* and is easy to genetically transform. Therefore, the goal of this study was to use genes from two different *Xf* mechanisms in the PDR approach to develop tolerance to *Xf*. Both *rpfF* and *mqsR-Xf* were isolated from the 9a5c genome. The expression cassettes were cloned into the vector pCambia2301, generating pCambia2301_*rpfF* and pCambia2301_*mqsR-Xf*. *Agrobacterium tumefaciens* carrying vectors were used for transformation of *N. tabaccum* with pCambia2301_*mqsR-Xf* and sweet orange citrus varieties (Hamlin and Pineapple) with pCambia2301_*rpfF*. Seedlings from positive tobacco transgenic plants and buds from positive citrus transgenic plants were challenged with *Xf*, and non-transgenic plants (wild-type; WT) were used as controls. The assessment of symptoms caused by *Xf* in tobacco and the evaluation of CVC in citrus were determined by comparing the incidence and severity of symptoms relative to the WT. The incidence of symptoms was calculated as the ratio of the number of symptomatic leaves to the total number of leaves per plant, and the severity was assessed using a score of 1 to 6, according to diagrammatic scales developed for tobacco and citrus. In citrus, bacterial movement was also evaluated in infected plants through qPCR. Population sizes in each collection point were estimated by comparison to a standard curve. The results showed that the incidence and severity in four tobacco transgenic lines were significantly lower than in the WT, suggesting that the toxin may be affecting bacterial multiplication, reducing the severity of symptoms. In relation to *rpfF* citrus transgenic plants, the mean of disease severity score was also lower in the six transgenic citrus lines when compared to the WT. Movement was also reduced in transgenic citrus lines since 60 cm above the inoculation point, bacterial populations in one transgenic line of Hamlin and Pineapple sweet oranges was ten times lower than the population assessed in WT plants. For the evaluated Pineapple sweet orange transgenic line, this difference was kept until 120 cm above the inoculation point. However, for the evaluated Hamlin transgenic line this difference was even greater, since around 100 times more bacteria were detected in the WT plants. The reduction in movement and population sizes observed in transgenic citrus plants can explain the reduction in CVC incidence and severity in citrus plants overexpressing *rpfF,* suggesting that as observed for grapevines transformed with *rpfF,* the virulence of *Xf* in citrus transgenic plants may have been compromised due to the production of DSF. This work shows that PDR is a promising strategy for developing tolerance to *Xf*, since two different genes showed great potential in increasing the

tolerance of the disease caused by *Xf*. Curiously, these transgenic plants also showed increased tolerance to *Xanthomonas citri* (Caserta *et al*., 2014), another citrus bacterium pathogen, demonstrating that this approach may have broad spectrum bacterial control.

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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 July 2012 to June 2014.

ABSTRACT
The main goal of this project was to identify and test if *Xylella fastidiosa* (*Xf*) proteins with chitin-binding activity could be used to block the vector transmission of this bacterium from plant to plant. This final report summarizes the research done during the last two years, and is divided into three section: i) biological role of a *Xf* chitinase, ii) *in vitro* characterization of a transmission-blocking protein (PD1764) and other candidates (e.g., hemagglutinin-like protein), and iii) biological testing of specific constructs as transmission blocking agents. Due to space limitations, details were not included but can be found in previous reports. Briefly, the work showed that a chitinase mutant is deficient in both *Xf* plant and vector colonization, identifying a new target for future research. It also identified PD1764 as a protein with a LysM domain, associated with disruption of *Xf* transmission by vectors under greenhouse conditions. Finally, several candidate transmission blocking molecules were tested *in vitro*, and those with chitin-binding activity were later tested in relation to their transmission blocking activity. The work done demonstrates experimentally the potential of this concept to control the spread of *Xf* by vectors.

LAYPERSON SUMMARY
The goal of this project was to identify targets (i.e., proteins) to block the vector transmission of *Xylella fastidiosa* (*Xf*). The research led to the identification of one new target (i.e., a chitinase) that is required for *Xf* colonization of both plant and insect hosts. In addition, it identified a protein (PD1764) that, through the effect of a chitin-binding domain named LysM, significantly blocks the vector transmission of *Xf* under experimental greenhouse conditions. Lastly, a series of transmission-blocking candidate proteins were tested *in vitro* and in several greenhouse trials, and results showed that HAD (i.e., hemagglutination activity domain), a domain of a hemagglutinin-like protein, alone or associated with LysM domain in a fused peptide, led to reduced transmission of *Xf* to grapevines in the greenhouse when delivered to insects through an artificial diet system. Altogether the research not only identified putative candidates but also demonstrated, under experimental greenhouse conditions, that the concept of blocking the vector transmission of *Xf* to plants is feasible.

INTRODUCTION
Xylella fastidiosa (*Xf*), the etiological agent of Pierce's disease of grapevines, is a bacterium that colonizes plant and insect hosts (Chatterjee et al., 2008). Although the biology of *Xf* within plants is reasonably well understood, knowledge of how it colonizes insects is limited. Improved understanding of how *Xf* colonizes insects is vital because xylem-sap sucking insects, primarily sharpshooter leafhoppers, act as vectors of *Xf* and are the only means of natural dispersal for this pathogen (Almeida et al., 2005). Furthermore, because *Xf* colonization of insect vectors is expected to be as complex as its colonization of plants (Chatterjee et al., 2008), there should be multiple mechanisms through which transmission could be disrupted, reducing disease spread in the field. Most current strategies targeting Pierce's disease control are based on two principles: control of vector populations or reduction/elimination of *Xf* infections after grapevine infection. This research project focuses on a third approach – the disruption of *Xf*-vector interactions, so that the transmission of *Xf* from one plant to another is affected. Our work has demonstrated that this is feasible and that we can disrupt sharpshooter transmission of *Xf* to grapevines.

Insect vectors of *Xf* include sharpshooter leafhoppers (Hemiptera, Cicadellidae) and spittlebugs (Cercopidae), although sharpshooters are considered of economic importance for most disease systems (Severin 1949, 1950). Although *Xf* is genotypically and phenotypically diverse, as are sharpshooter vector species, there is no evidence of vector-pathogen specificity in relation to transmission (Almeida et al., 2005). In other words, all sharpshooter

species are expected to be capable of transmitting all *Xf* strains. This is relevant to this project, as it is expected that the technology developed here should be applicable to all vector species associated with Pierce's disease.

Although *Xf* is persistent in vectors, it is not transovarially nor transstadially transmitted, meaning that nymphs that carry the pathogen have to reacquire it after molting for transmission to occur (Purcell and Finlay, 1979, Almeida and Purcell, 2003). In adults, *Xf* is persistent for life (Severin 1949, Almeida and Purcell 2003). This biological observation, together with the fact that a latent period is not required for transmission, indicates that *Xf* does not circulate within vectors (reviewed in Almeida et al., 2005). Microscopy studies showed that *Xf* colonizes the foregut of sharpshooters, specifically the precibarial canal and the cibarium (Purcell et al., 1979, Brlansky et al., 1983). More recently, a correlation between colonization of the precibarium and transmission to plants was observed (Almeida and Purcell, 2006). Altogether this body of work indicates that *Xf* colonizes the cuticular lining of the foregut of sharpshooters, and that is the retention site of this bacterium within vectors.

Our group has studied *Xf*-vector interactions and identified various factors associated with colonization patterns and those involved in transmission. A summary of those findings has been recently summarized in a review article. More recently, we identified a chitinase (ChiA) in *Xf* (Killiny et al., 2010). This is interesting, as it had been thought that *Xf* did not use its insect vectors as a source of nutrients. The work described here addressed that question, and it also led to the surprising discovery that a mutant of ChiA is deficient in plant colonization.

Previously, we showed that biochemical competition assays with *N*-acetylglucosamine (GlcNAc), the main subunit of chitin polymers, reduced *Xf* binding to vector surfaces (Killiny and Almeida, 2009a). Adhesion was reduced with saturation of *Xf*'s surface adhesins by free molecules in solution. In addition, we showed that surface proteins were involved in cell adhesion to chitin and other polysaccharides. Those observations were tested *in vivo* by delivering *Xf* and lectins (carbohydrate-binding proteins) or carbohydrates to vectors using artificial diets, and then transferring insects to healthy grape plants to estimate the impact of various treatments on transmission efficiency by individual leafhoppers. All treatments expected to disrupt vector transmission reduced transmission efficiency when compared to controls (Killiny et al., 2012). Adding GlcNAc to *Xf* suspensions results in adhesins binding to free molecules before acquisition by vectors. Adhesins cannot recognize receptors on the foregut as they are already bound to a carbohydrate, and cells fail to colonize vectors. Another proposed scenario is to block the receptors on the foregut of vectors to which *Xf* surface proteins bind; that was successfully accomplished by adding lectins to the suspension. This later model, based on masking vector foregut receptors used by *Xf* to colonize sharpshooters, has much potential for field applications and is the focus of this proposal. Importantly, cells delivered to sharpshooters *in vitro* appear to be, as a population, much 'stickier' than their counterparts colonizing plants (Killiny and Almeida, 2009b). Furthermore, the artificial diet system used probably results in a much less turbulent environment in the foregut compared to ingestion of sap from xylem vessels, facilitating attachment and colonization. In fact, in these assays transmission was blocked to frequencies equivalent to *Xf* mutants that are not transmissible from plant to plant, indicating that blockage is efficient. The work described below is a continuation of these findings, and led to the identification of a protein that significantly reduced the vector transmission of *Xf* from plant to plant.

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.

To facilitate the presentation of our results, given that most of these data are already available in previous reports, this final report is divided into two sections. The first focuses on the characterization of ChiA, while the second on the identification and testing of transmission-blocking proteins/peptides. We note that a substantial amount of data are not included in this report due to space limitations, and can be obtained in previous reports.

RESULTS AND DISCUSSION

Biological characterization of ChiA.

The identification of ChiA in *Xf* (Killiny et al., 2010) led to the generation of a *chiA* mutant strain, as well as a complemented strain. We first studied the role of chitin on the *in vitro* population growth of this bacterium. Results demonstrate that the addition of chitin to a basal medium increases *Xf* bacterial populations (**Figure 1A**), that the *chiA* mutant is unable to grown on chitin alone, while the wild-type control reaches high populations as in a medium with other carbon sources (**Figure 1B**), and that the complemented strain is capable of growing on

chitin alone, as expected (**Figure 1C**). Lastly, we demonstrated that growth on a medium with chitin prior to exposure to another growth cycle in a chitin medium decreased the lag period of *Xf* on this substrate (**Figure 1D**).

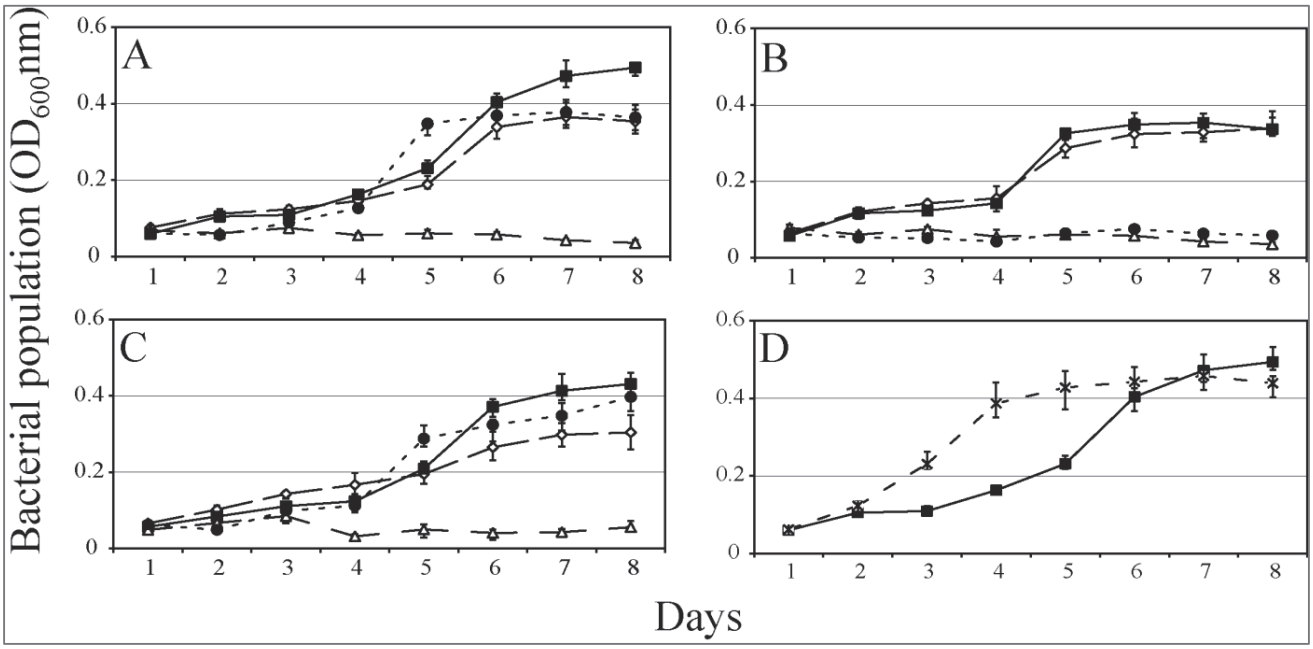


Figure 1. *In vitro* growth of *Xf* wild-type, *chiA* mutant, and its complemented strains, in the presence and absence of chitin. A) Wild-type growth in basal media, triangle represents medium without a carbon source, squares medium supplemented with chitin. B) *chiA* mutant strain, note no growth in medium with chitin as the sole carbon source (circles). C) comple-mented *chiA* mutant strain, the population grows in the medium with chitin as the sole carbon source (circles). D) lag phase is shorter in medium within chitin if cells were previously grown in the presence of that substrate (top curve).

Once the role of *chiA* on *Xf* chitin utilization was demonstrated *in vitro*, in addition to the evidence demonstrating that *Xf* can grow on chitin as a sole carbon source, we proceeded to study the role of ChiA on *Xf* colonization of insects and plants, as well as on plant-to-plant vector transmission. Vector transmission efficiency of the *chiA* mutant was less than half of that of the wild-type (~40% in comparison), while the complemented strain recovered its phenotype and was ~80% of the rate observed for the control (**Figure 2A**). Vector colonization followed a similar trend, with the complemented strain having bacterial populations within vectors similar to the wild-type at three and ten days after acquisition, while the *chiA* mutant had similar populaitons at three days post acquisition, but that population did not increase over time (**Figure 2B**). These data strongly indicate that ChiA is essential for bacterial multiplication within vectors, suggesting that *Xf* consumes chitin while colonizing the cuticular surface of sharpshooters.

Because we performed the characterization of most *Xf* mutant strains in both of its hosts, namely plants and insects, we also tested if the *chiA* mutant strain could colonize and cause disease in plants. To our surprise, we found that the *chiA* mutant did not cause disease and, more interestingly, did not colonize grapevines (**Figure 3A**). These results indicate that ChiA is not chitin specific and that it may cleave other polysaccharides. One of the interesting features of ChiA is that it only has a catalytic domain, and not a chitin-binding domain. We attempted to identify a chitin-binding domain using different approaches unsuccessfully. Thus, we hypothesized that ChiA uses other proteins as bridges between itself and its substrate(s). **Figure 3B** shows that *Xf* protein extracts are capable of cleaving a chitin substrate (lane 1), while the *chiA* mutant is not (lane 2). Interestingly, recombinant ChiA did not cleave the substrate (lane 3), but ChiA mixed with the protein extract of the *chiA* mutant results in cleavage. These results support the hypothesis that ChiA must interact with chitin-binding proteins to be biologically active.

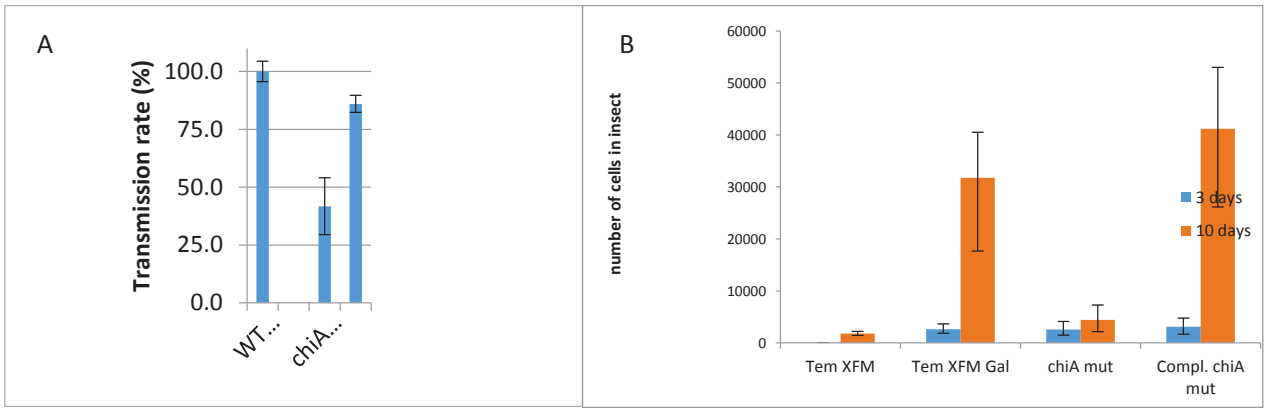
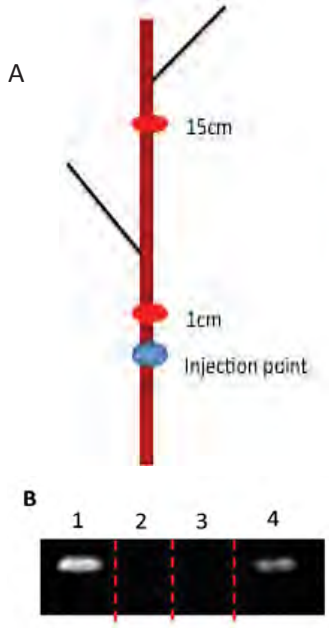


Figure 2. Role of ChiA on *Xf* vector transmission and colonization. A) Transmission of *Xf* wild-type strain grown on XFM (negative control), XFM supplemented with galacturonic acid (inducer of transmission), *chiA* mutant, and the complemented *chiA* mutant. Insects acquired *Xf* feeding on sachet membranes before being placed on basil for 10 days. Then insects were allowed to inoculate to healthy test plants for 24 hours. B) Number of cells of *Xf* that were detected by qPCR after three days (red bars) and ten days (blue bars) post-acquisition.



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

Figure 3. A) Partial results (others yet to be analyzed) showing that the *chiA* mutant of *Xf* does not cause Pierce's disease of grapevines, nor does it move within plants. B) Detection of the chitinolytic activity; incubation of the substrate (4-MU(GlcNAc)3 ; Sigma-Aldrich) with an active chitinase results in the cleavage of the substrate into a fluorescent product (4-MU) which can be visualized under UV lights. Visualization of the fluorescent bands on a native gel corresponding to the degradation of the substrate, by the chitinase. 1: Total proteins of *Xf* wild-type Temecula cells; 2: total proteins of *chiA* mutant; 3: recombinant ChiA and 4: recombinant ChiA incubated with total proteins of *chiA* mutant.

Identification and testing of transmission-blocking proteins.

The aim of this study was to identify *Xf* proteins able to recognize and interact with chitin on the cuticle of insect vectors. During acquisition of the bacteria by its vectors, such chitin-binding proteins will outcompete with *Xf* cells for accessibility to those receptors, thus leading to the blocking of the bacteria's transmission. In order to trap *Xf* proteins having affinities for insect-related polysaccharides, we performed a chromatography using chitin columns. Using *Xf* cells grown on medium inducing vector transmissibility, we were able to detect 10 bands after elution on the silver-stained SDS gel. In parallel, no bands were detected for conditions that do not induce *Xf* transmission. This is in accordance with previous results that imply that pectin, or its main subunit GlcNAc, induces the transmission of *Xf* by up-regulating genes expressing proteins involved in *Xf* transmission (Killiny et al., 2009b).

One band/protein was selected for further work. This protein shares some similarities with peptidoglycan-binding LysM proteins in other gamma-proteobacteria. In the sequenced genome of *Xf* Temecula strain, this protein is named PD1764 and annotated as a conserved hypothetical protein. 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, see 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 bacterium *Lactococcus lactis* was able to bind to fungal chitin cell wall material. Thus, PD1764 could be a highly interesting candidate for interactions of *Xf* with chitin.

In order to test specific *Xf* proteins as transmission-blocking molecules to disrupt its transmission by vectors, we expressed different proteins or domains as recombinant proteins. Several *Xf* adhesins (FimA, ChiA, and two constructs of HxfB) were targeted based on previous works showing their involvement in vector colonization and transmission (Killiny and Almeida, 2014). In addition, two additional molecules targeting PD1764 protein previously identified were also constructed carrying or not the LysM domain (**Figure 4**).

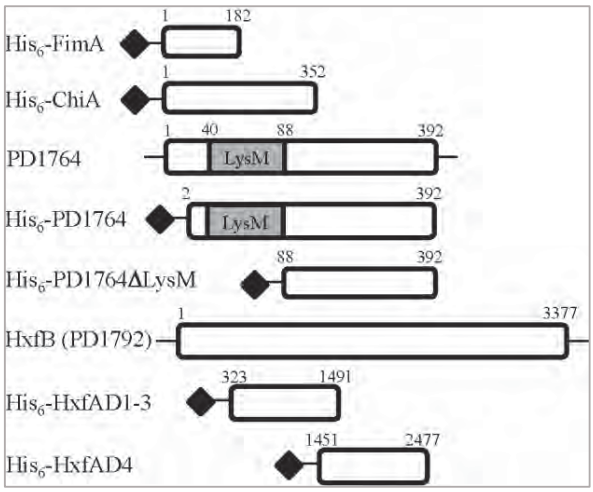


Figure 4. Diagram illustrating some of the constructs tested. Others include LysM alone, and a LysM-HAD fusion.

Before testing the effect of those peptides on *Xf* vector transmission, we tested their capacities to bind to different polysaccharides, insect-related or not (i.e., Avicel as a control). Based on our binding assays, ChiA, FimA, HxfAD4, and PD1764ΔLysM were not able to interact with any of the four polysaccharides tested. This is in accordance with previous analyses that showed no already-described chitin-binding domain in any of those peptides. On the other side, PD1764 full-length and HxfAD1-3 strongly interacted with chitin (**Figure 5**). In addition to the potential role of LysM domain, we also detected an interesting domain on HxfAD1-3. This construct expresses the 1,168 amino acids (aa) N-terminal part of the hemagglutinin-like protein HxfB (Voegel et al., 2010). Interestingly, according to SMART (http://smart.embl-heidelberg.de/), this region contains a 120 aa domain called haemagglutination activity domain (HAD), which has been suggested to be a carbohydrate-dependent haemagglutination activity site. It has been found in a number of adhesins or filamentous haemagglutinins such as the FHA of *Bordetella pertussis* and plays a role in adhesion to host cells (Kajava et al., 2001).

This is in accordance with previous results showing that PD1764 could be specifically trapped on a chitin column whereas Hxfs proteins act as adhesins important for binding on insect cells (Killiny et al., 2009). Interestingly, HxfAD4 but not PD1764 full-length interacts with chitosan. The main difference between chitin (or colloidal chitin) and chitosan polysaccharides is the presence of an acetyl group (COCH3) on the main chitin subunit. Based on this result, the acetyl residue seems to play an important role in the binding of PD1764 on chitin-related polysaccharides, whereas it doesn't seem to be a requirement for HxfAD1-3 interaction with such molecules. This is of interest because it could mean that these two peptides recognize different domains or different receptors. Interestingly, most of the interactions between chitin and PD1764 and HxfAD1-3 respectively occurred in the first

minute of the binding assay and remain stable in our conditions for at least 16 h. This result suggests that both proteins have affinity for chitin, which seems to be a requirement *in vivo* where interactions between *Xf* proteins and insect receptors occur in a highly turbulent environment due to the simultaneous ingestion of xylem sap by the insect when feeding.

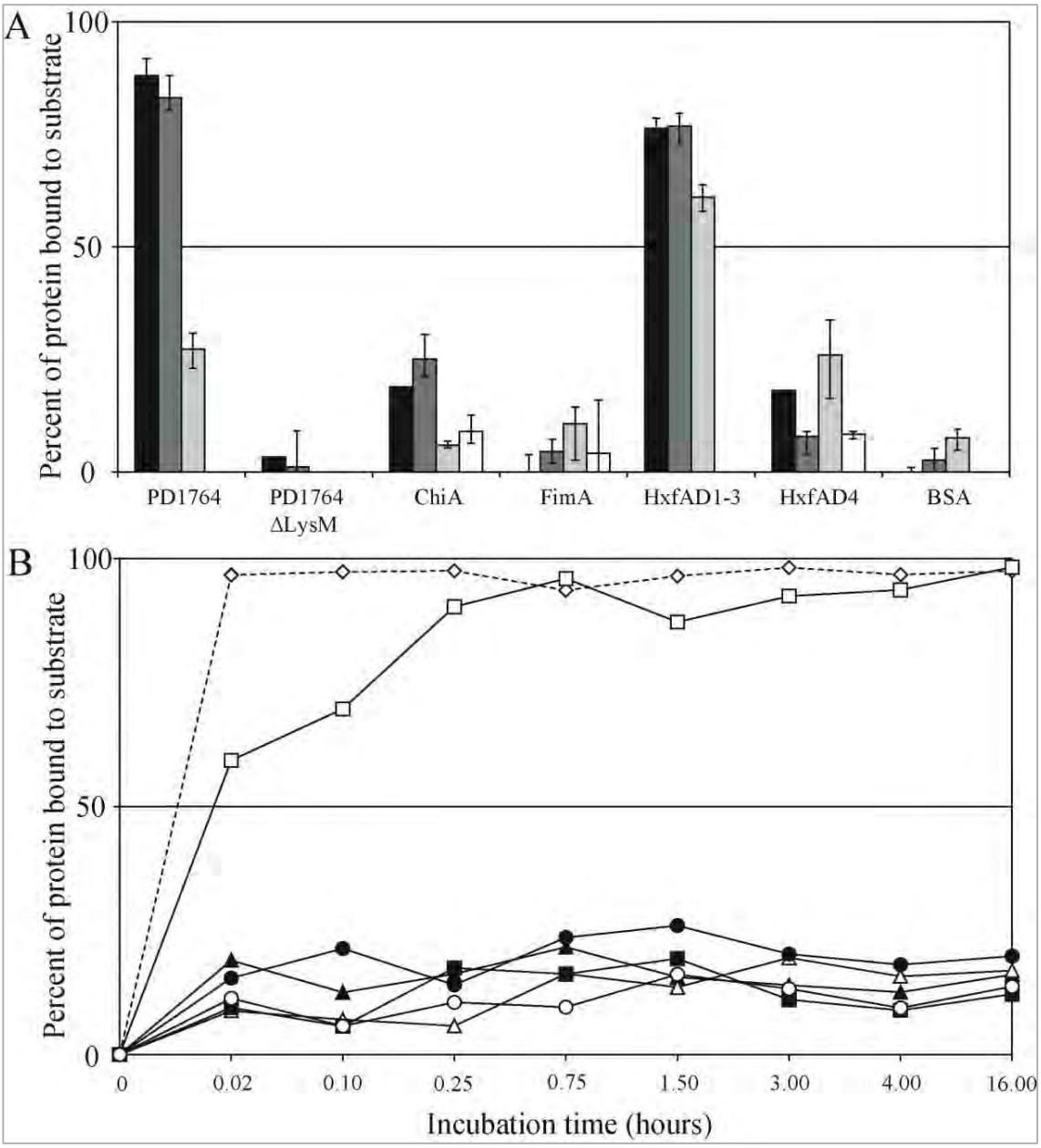


Figure 5. Comparative binding of various recombinant proteins tested to four polysaccharides of interest.

To test the effect of those peptides as transmission-blocking peptides, we checked that none of the molecules could have a detrimental effect on the physiology of the insects that fed on those diets, and we tested their effect on *Xf* transmission (**Figure 6A**). Interestingly, the two candidates (PD1764 and HxfAD1-3) that had an effect on the disruption of *Xf* were the same ones that we found could interact with insect-related polysaccharides. Interestingly, PD1764 had the highest affinity for chitin, completely blocking *Xf* transmission using a 100µM concentration. This result confirms the feasibility of our approach to control the spread of *Xf*, and validates the proteomics pipeline to identify new transmission-blocking proteins. This result is highly encouraging because this is the first time that one treatment focusing on disrupting *Xf*-vector interactions completely blocked the transmission of *Xf*. **Figure 6B** shows that proteins also resulted in smaller bacterial populations within vectors, which may or may not have had an impact on overall transmission; these results are still being interpreted due to

potential problems associated with the interpretation of transmission data when artificial diets are used as sources of *Xf* for vectors (see Killiny and Almeida, 2014 for more details).

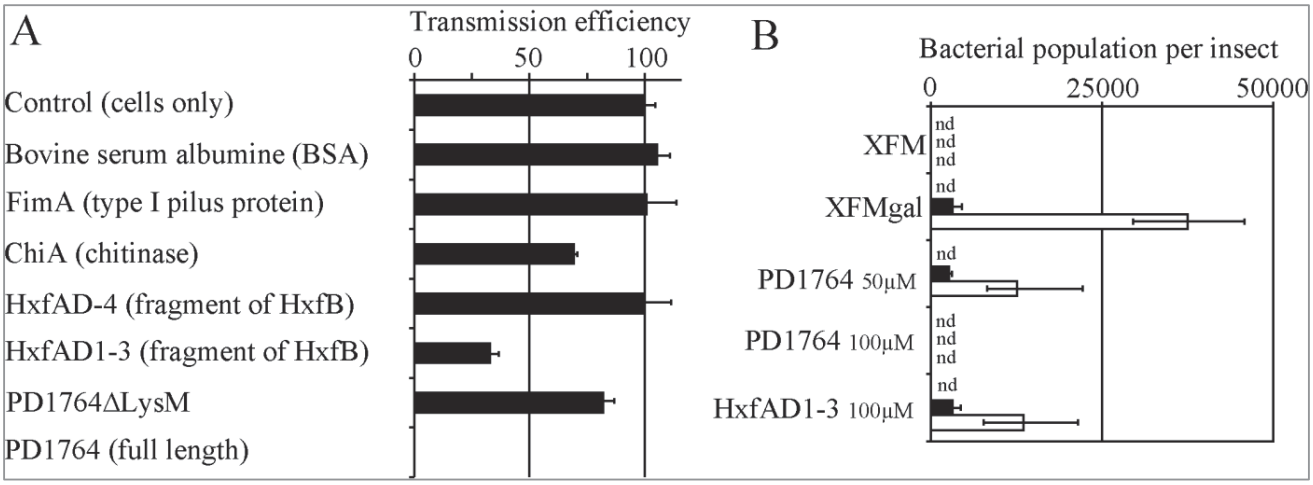


Figure 6. A) Vector transmission of *Xf* to grapevines after cells were provided to insects through an artificial diet system supplemented with various proteins. B) Bacterial populations within insects immediately after acquisition, then three and ten days after acquisition. Note that transmission-blocking peptides did not abolish bacterial multiplication within vectors, indicating that blocking activity occurs only at the initial stages of vector colonization. Nd = not detected.

To confirm or not the possible role of PD1764 in *Xf* transmission, we disrupted *pd1764* in the *Xf* genome by double-crossing-over following standard protocols used in the laboratory (Kung and Almeida, 2011). Such a *Xf* mutant was still transmissible in our system, meaning that PD1764 protein is not essential in *Xf* transmission by vectors. Even if there is no doubt concerning the specificity of the interaction between PD1764 and chitin, which results in the blocking of *Xf* transmission, those interactions do not appear to be biologically important for *Xf* to spread.

Identification of domains on HxfAD1-3 and PD1764 (respectively named HAD and LysM) involved in blocking *Xf* transmission by insects would lead to the construction of shorter transmission-blocking peptides. This is of great importance because utilization of small peptides could greatly enhance the efficiency of our transgenic system in the field, as the medium-term goal of this system is to develop transgenic grapevines expressing these transmission-blocking molecules constitutively. In addition, pathogen-host interactions could be highly multivalent and our attempts required high dose of transmission-blocking molecules. Combining the effects of multiple domains which can have different and higher affinities for insect-related polysaccharides could also allow us to overcome this challenge by reducing the concentration of molecules needed. We pursued this by studying the role of HAD, LysM, and a fusion LysM-HAD on the binding activity (**Figure 7**) and vector transmission of *Xf* to plants (**Figure 8**).

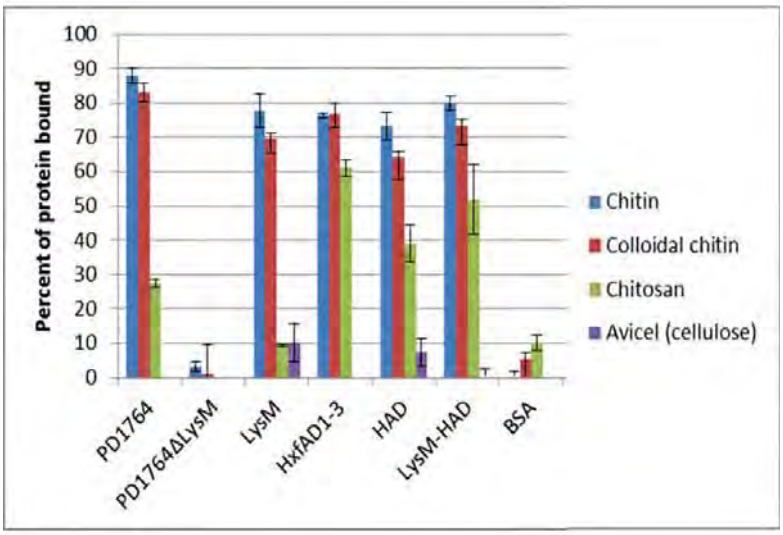


Figure 7. LysM and HAD have similar yet distinct binding activity in the presence of different substrates. However, the fusion had high activity *in vitro* to all chitin-based substrates.

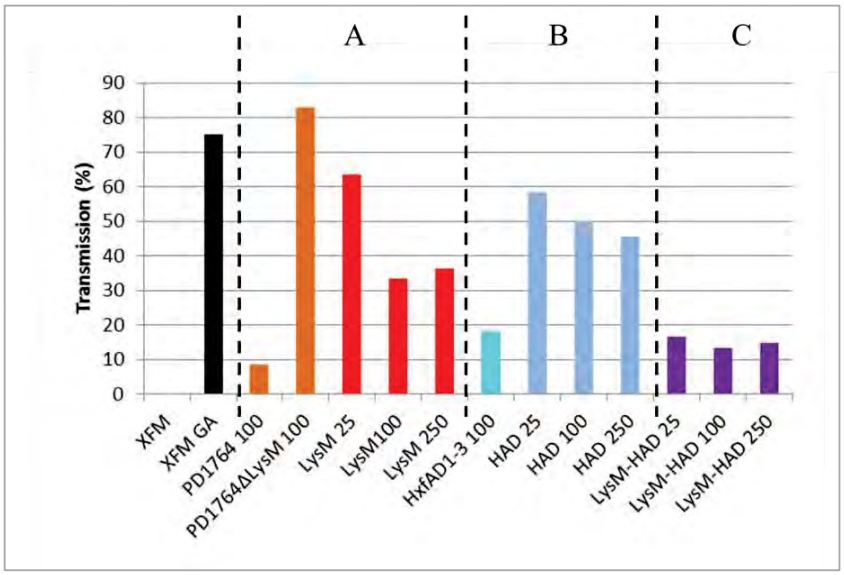


Figure 8. *Xf* vector transmission efficiency when different transmission-blocking peptides were provided to insects (number indicates µM concentration of peptides). Best transmission-blocking results were obtained by the LysM-HAD fusion, where all concentrations used generated less ≤15% transmission of *Xf* to grapevines. Because blocking activity was equivalent within one order of magnitude difference in peptide concentration, we believe that lower concentrations, which were not tested, would also yield significant blocking activity.

CONCLUSIONS

This project had two major results. First, it identified one target (ChiA) that apparently is essential for the colonization of both plant and insect hosts of *Xf*. Determining why ChiA is associated with the colonization of plants and insects may lead to new strategies to control disease spread within and between plants. Second, the work used greenhouse experiments with an efficient vector and grapevines to show that blocking the transmission of *Xf* to plants is a feasible approach to reduce disease spread. Furthermore, we have identified specific proteins involved in this process, and demonstrated that one specific fusion of two peptides we demonstrated to be active in chitin-binding led to efficient disruption of vector transmission of *Xf*.

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

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Reporting Period: The results reported here are from work conducted October 2013 to October 2014.

ABSTRACT

Tissue culture of grape plants remains an inefficient process for many genotypes. The procedure is labor intensive, limited to specific genotypes, and requires a significant amount of time to establish and maintain embryogenic cell cultures and convert cell cultures into whole plants. We are leveraging the expertise of the National Research Laboratory of Chile, (INIA), and the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis (UCDPTF) to significantly increase the efficiency of tissue culture and transformation technology in grape genotypes important to their respective countries. The two labs are sharing their latest protocol improvements for generating and increasing high quality embryogenic callus using germplasm important to their particular country. This combined effort, has allowed us to make significant advances in our ability to grow and maintain embryogenic callus cultures for use in tissue culture and transformation experiments for rootstock genotypes 1103, 101-14, and the winegrapes Chardonnay and Cabernet Sauvignon. We have successfully established high-quality, rapidly-multiplying grape suspension stock cultures for 1103, 101-14, Cabernet Sauvignon, and Chardonnay for each of the past three years 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 developed a method for maintaining somatic embryos for extended periods of time in a quiescent state by plating cell suspension on medium containing high concentrations of sorbitol. This system allows us to maintain a germplasm bank of embryos for numerous grape genotypes which provides a constant supply of embryos for use in tissue culture and transformation experiments. These embryos provide an excellent source of material for transformation and has allowed us to routinely transform rootstocks 1103 and 101-14. Using embryogenic suspension cultures, we are developing a high frequency transformation protocol based on direct transformation of 1103 and 101-14 suspension cultures which when optimized should allow us to produce transgenic rootstocks with less labor than is currently required for embryo transformation. We have also developed a sequential transformation protocol which allows stacking of multiple transgenes into grape. Although regeneration of whole plants from non-transgenic embryos of 1103 and 101-14 is routine, regeneration of transgenic embryos of 1103 and 101-14 remains inefficient and is the most significant bottleneck in grape transformation. We have made some improvements in efficiency of plant regeneration from transgenic embryos, but further improvements would greatly streamline the protocol.

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 cultures using germplasm important to their particular country. Using both cell suspension cultures and bioreactors, we have made significant advances in our ability to establish and increase embryogenic cultures for 1103, 101-14, Cabernet Sauvignon, and Chardonnay for use in tissue culture and transformation experiments. We have developed a long-term storage medium which allows grape somatic embryos to be stored for over six months which will allow for easy maintenance of numerous genotypes with minimal labor. Using these embryos, we have achieved high transformation frequencies for 1103 and 101-14. We have also demonstrated that we can directly transform grape suspension cultures, which bypasses the need to generate embryos prior to transformation. Although our results with direct transformation of grape suspensions are still inconsistent from run to run, experiments that are successful using this technique allow us to produce transgenic material with minimal input of labor. We have also developed a sequential transformation protocol which will allow us to re-transform transgenic grape embryos with a second disease gene. This will allow researchers to stack resistance genes for additional disease protection. Although regeneration from non-transgenic 1103 and 101-14 embryos is routine, regeneration of transgenic 1103

and 101-14 into whole plants remains inefficient. We continue to make progress developing new media formulations which allow for more rapid regeneration of plantlets from non-transgenic embryos of 1103 and 101-14, which if applicable to transgenic embryos, should reduce the time required to generate transgenic grape plants for the Pierce's disease research community.

INTRODUCTION

The development and transformation of embryogenic cultures in grape has historically been labor intensive with the establishment of embryogenic cell cultures requiring many months and limited to only a few genotypes, most notably the table grape Thompson Seedless. Once established, maintaining healthy embryogenic callus is difficult, with the quality of the cultures deteriorating over time. The efficiency of establishing embryogenic cultures and regenerating plants for important wine and rootstock genotypes remains low and are not at the level required to allow for cost-effective recovery of tissue culture or transgenic plants. Historically, because it is one of the only genotypes that could 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 in other grape genotypes. For grape rootstock mediated resistance strategies, efficient transformation of rootstock genotypes is required. Challenges involved in expanding the range of genotypes that can be successfully manipulated in culture include the reliable establishment of embryogenic cultures, the labor intensive methods required to increase and maintain high quality embryogenic cultures, prevention of tissue necrosis caused by oxidation, conversion of embryos into true-to-type plants, transformation of embryogenic callus, and rapid regeneration of non-chimeric transgenic plants from embryogenic cells. There is also a need to reduce the timeframe required to generate transgenic grape plants in order to test new strategies in a timely manner. 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 important to the Pierce's disease research community. Results of this collaboration will allow the Pierce's disease research community to test transgenic strategies in rootstock genotypes that are relevant to the industry through the establishment of a self-sustaining service facility.

OBJECTIVES

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

In the spring of 2011, 2012, 2013, and 2014, we harvested anthers from grape genotypes 11-03, 101-14, Chardonnay, and Cabernet Sauvignon. In 2014, we also collected anthers from Freedom and Richter 110R. Anthers were plated onto two different callus induction media; Nitsch and Nitsch minimal organics medium (1969) 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). A summary of the responses of the various genotypes on the two different media over the years is shown in **Table 1**.

Table 1. The number and percentage of anther cultures plated in 2011, 2012, 2013, and 2014 that developed embryogenic cultures on PIV or NB medium.

Genotype	2014	2013		2012		2011
	PIV	PIV	NB	PIV	NB	PIV
Cabernet	2/539 (0.4)	1/287 (0.3)	0/217 (0)	0/200 (0)	0/280 (0)	3/400 (0.8)
Chardonnay	11/539 (2.0)	22/344 (6.4)	18/344 (5.2)	9/184 (4.9)	2/156 (3.6)	4/400 (1.0)
1103	17/539 (3.0)	3/294 (1.0)	0/287 (0)	0/75 (0)	1/196 (0.5)	2/150 (1.3)
101-14	2/539 (0.4)	3/322 (0.9)	0/409 (0)	0/140 (0)	0/275 (0)	NT
Freedom	1/539 (0.2)	NT	NT	NT	NT	NT
Richter 110	4/438 (1.8)	NT	NT	NT	NT	NT

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

INIA has developed a method of rapidly increasing embryogenic cultures by cycling the cells between agar-solidified medium and liquid media in shake flasks. This technique allows for rapid increases in callus fresh weight while minimizing oxidation and the development of detrimental phenolic compounds in the cultures. Using a modification of INIA protocol, we have significantly improved the production of embryogenic grape cultures across a range of genotypes including 1103, 101-14, Cabernet Sauvignon, and Chardonnay. In order to avoid somaclonal variation, we re-initiated new suspensions each year from embryos generated from anther filaments harvested the previous year. Currently our suspensions are from anther filament callus generated in 2013. We are now routinely maintaining suspension cultures on liquid WPM medium (Lloyd and McCown, 1981) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 10.0 mg/l Picloram, 2.0 mg/l metatopolin, 2g/l activated charcoal, 100 mg/l ascorbic acid, and 120 mg/l reduced glutathione (Pic/MTag) and grown on a gyratory shaker at 100 rpms in the dark. Once established, 10 ml of the suspension is withdrawn each week from the flask and replaced with 10 ml of fresh medium. One ml of suspension that is removed from the flask is plated onto agar solidified Woody Plant Media (WPM) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 0.5 mg/liter BAP, 0.1 mg/liter NAA, 5% sorbitol, and 14 g/l phytoagar (BN-sorb). Embryogenic cells plated on BN-sorb medium produces high quality embryogenic cultures at the appropriate stage for use in transformation in approximately 4-8 weeks and embryos can be maintained on this medium for up to six months without further manipulation. This system has allowed us to efficiently generate large numbers of somatic embryos which can be used in transformation experiments. Although we have also successfully established temporary immersion technology for increasing embryogenic callus, we are now concentrating our efforts on suspension technology. These suspensions reliably provide a consistent supply of high quality somatic embryos for use in transformation experiments, and allow us to maintain a wide range of genotypes with minimal handling.

Objective 1c. Enhance the efficiency of whole plant regeneration from embryogenic cultures of grape.

Although non-transgenic embryos of 1103 and 101-14 consistently germinate within a few weeks and quickly regenerate into plants after plating on germination medium, regeneration of whole plants from transgenic embryos of 1103 and 101-14 has proven to be more difficult and is now the rate-limiting step in the production of transgenic grape plants. One of the main differences between the medium used for regeneration of transgenic vs. non-transgenic grape embryos is the presence of the selective agent (kanamycin or hygromycin) and the counter selective agents (carbenicillin, timentin, and plant preservative mixture) used to suppress *Agrobacterium* in the culture. We have evaluated each counter selective agent used for suppression of *Agrobacterium* (carbenicillin, timentin, and plant preservative mixture) individually and in combination for their effect on regeneration of non-transgenic embryos and have not found them to be detrimental.

In other species, the basal salt formulation of the tissue culture medium can have a dramatic effect on the regeneration efficiency. Therefore, we evaluated eight different salt formulations in an attempt to improve the efficiency of whole plant regeneration from embryos of 1103 and 101-14. Salt formulation tests included Andersons, Chee and Pool, DKW, Gamborg's B5, MS, WPM, SH, and X6. All media were supplemented with 1.0 g/l casein, 500 mg/l activated charcoal, and 0.1 mg/l BAP. Significant differences were seen between the various salt mixtures with the best regeneration occurring on DKW, SH, and WPM (**Figure 1**). We are testing transgenic embryos on these salt mixtures to determine if similar enhancements in regeneration can be achieved.

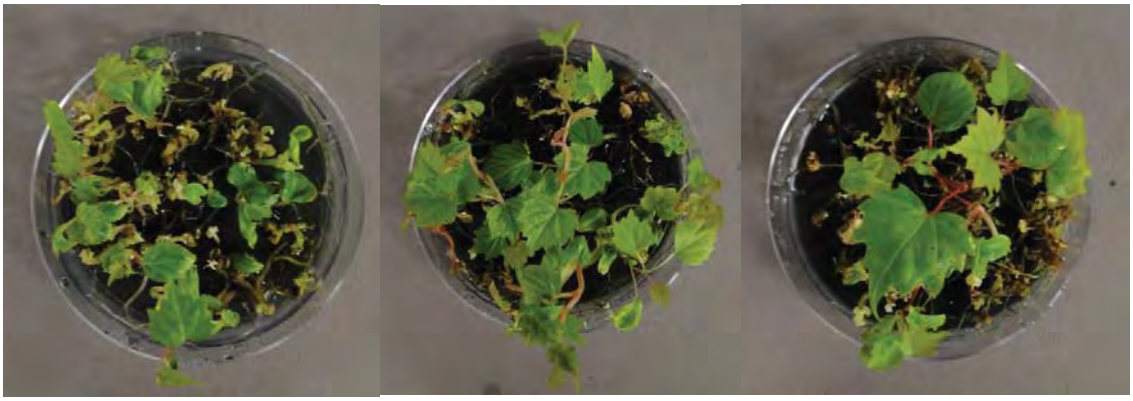

Figure1. Regeneration of non-transgenic embryos of 1103 generated on WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA 50 g/l sorbitol, and 14 g/l agar medium and transferred to DKW medium (left), SH medium (middle), and WPM (right), supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, and 0.1 mg/l BAP.

Objective 2. **Develop a cost-effective grape tissue culture and transformation platform for at least one priority California winegrape and one California grape rootstock which will provide the Pierce's disease research community with a predictable supply of experimental plant material while reducing labor and maximizing tissue culture and transformation efficiency.**

Objective 2a. Improve grape rootstock transformation efficiency for 1103 and 101-14 using embryos harvested from robust-growing suspension cultures.

We have developed a robust suspension system for 1103 and 101-14 which provides a continuous source of somatic embryos for transformation. Embryogenic cell suspensions are harvested from cell suspension cultures on a weekly basis as part of the process required for feeding the suspension cultures. As described above, one milliliter of the suspension can be plated on sorbitol containing medium for regeneration of somatic embryos which enter a quiescent state and can be stored for later use for over six months without any additional manipulation. Large quantities of embryos can then be collected from the plates and transformed with *Agrobacterium* when transformations are requested. Secondary transgenic embryos arise from the epidermis of the inoculated embryos while the remainder of the inoculated embryo turns necrotic due to the selective agent, kanamycin or hygromycin (**Figure 2**). The surviving secondary embryo can be harvested and transferred to regeneration medium for plant production. Using this system, we have been able to generate transgenic embryos for both 1103 and 101-14 (**Table 2**). We are currently utilizing this technique for transformation requests for Pierce's disease researchers.

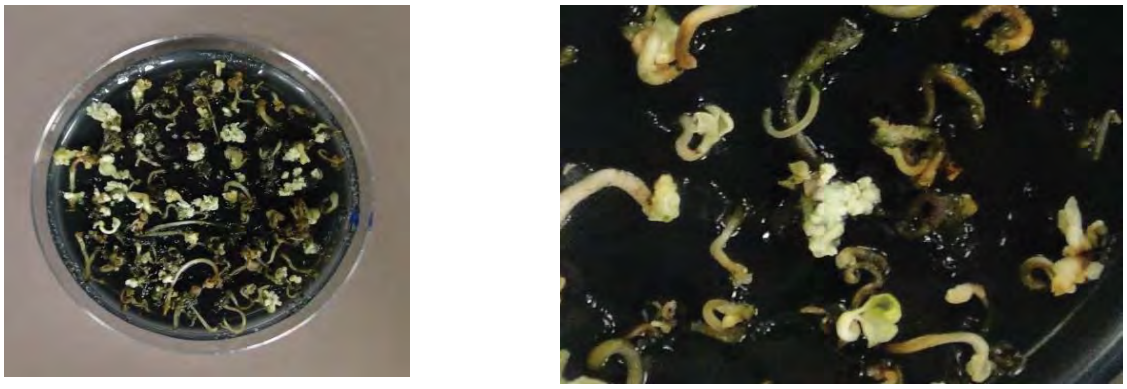

Figure 2. Clusters of transgenic secondary embryos developing from *Agrobacterium*-inoculated 110 somatic embryos plated on 200 mg/liter kanamycin sulfate (left) and close up of one secondary embryo cluster (right).

Table 2. Inventory of transgenic 1103 and 101-14 plants generated with various genes of interest.

Genotype	Transgene	Number of Plants Produced
1103	35s HNE-CecB	15
101-14	35s HNE-CecB	25
101-14	pDU10.1818	10
101-14	HNE-CecR	6

Objective 2b. Leverage the progress we have made in developing high quality suspension cultures that have the ability to rapidly regenerate whole plants when plated onto agar-solidified medium by testing direct transformation of cell suspension cultures.

We are leveraging the progress we have made in developing high quality grape suspension cultures that have the ability to rapidly regenerate whole plants when plated onto agar-solidified medium by exploring direct transformation of our grape suspension cultures. One to two ml of a grape cell suspension grown in liquid Pic/MTag medium and containing pre-embryogenic masses or small globular embryos are collected in a 15 ml conical centrifuge tube and pelleted by centrifugation at 1,000 x G for three minutes. The supernatant is removed and the cells are washed by re-suspending them in WPM medium without charcoal. Cells are pelleted by centrifugation at 1,000 x G for three minutes and are washed two additional times in WPM medium. After the last wash, the cells are subjected to heat shock by placing the 15 ml conical tube in a 45 degree water bath for five minutes. After heat shock the supernatant is removed and the cells are re-suspended in five ml liquid BN medium containing 200 uM acetosyringone and the *Agrobacterium* strain EHA105 carrying the desired vector at an OD 600 of 01.-0.2. The suspension is centrifuged at 1,000 x G for five minutes and allowed to incubate for 25 minutes at room temperature. After 25 minutes, all but 0.5-1.0 ml of the supernatant is removed. The grape and *Agrobacterium* cells are then re-suspended and transferred onto sterile Whatman filter paper in an empty 100 x 20 mm petri dish. Any excess fluid was carefully blotted up with a second sterile filter paper. The plates are co-cultured for 2-3 days at 23 degrees and then transferred to selection medium consisting of WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA, 400 mg/l carbenicillin, 150 mg/l timentin, 4 ml PPM, 50 g/ sorbitol, 14 g/l agar, and 200 mg/l kanamycin, or 25 mg/l hygromycin. Sub-culturing of the plated cells is achieved by simply transferring the filter paper with the cells onto fresh medium on a biweekly basis. Within 8 to 12 weeks transgenic embryos develop (**Figure 3**). Developing embryos are transferred to WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP, 400 mg/l carbenicillin, 150 mg/l timentin, 0 g/l sorbitol, 8 g/l agar, and 200 mg/l kanamycin or 25 mg/l hygromycin for germination. The time from inoculation to the recovery of transgenic embryos can be as short as 10 weeks. We have successfully used this technique to produce transgenic embryos of 1103 and 101-14 (**Figure 4**). The system has been employed successfully using both kanamycin and hygromycin selection. Currently we are seeing a significant amount of experiment to experiment variability in the number of transgenic embryos developing, with numerous experiments yielding no transgenic colonies and other experiments generating variable numbers of colonies (**Table 3**). However, if this protocol can be made more consistent, it represents a significant advance in our transformation system since it greatly increases transformation efficiencies while minimizing labor inputs. We believe some of the variability may be related to the growth stage of the suspension culture at the time the aliquots are harvested for transformation. We are now exploring the transformation efficiency between aliquots collected at 0, 2, 3, 4, and 5 days after sub-culturing the suspension to determine if the growth phase of the suspension influences transformation frequency. It also appears that the plating density of the cells (too high or too low) may impact transformation efficiency. Therefore, we are evaluating if the plating densities, as measured by settled cell volume, can impact transformation efficiencies. As with our embryo-based transformation system, the limiting step in this protocol is also the regeneration of whole plants from transgenic embryos, and we continue to explore media modification to enhance regeneration potential.

Figure 3. Transformation of suspension cultures of 1103 plated on BN sorbitol medium supplemented with 400 mg/l carbenicillin, 150 mg/l timentin 4 ml PPM and 25 mg/l hygromycin. Note the formation of small white transgenic cell colonies (A). Grape embryo expressing the DsRed gene, confirming the transgenic status of the developing embryos (B). Germination of transgenic embryos after transformation to WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP, and 8 g/l agar; bright field (C) and fluorescence (D). Regeneration of whole plants (E) expressing DsRed (F).

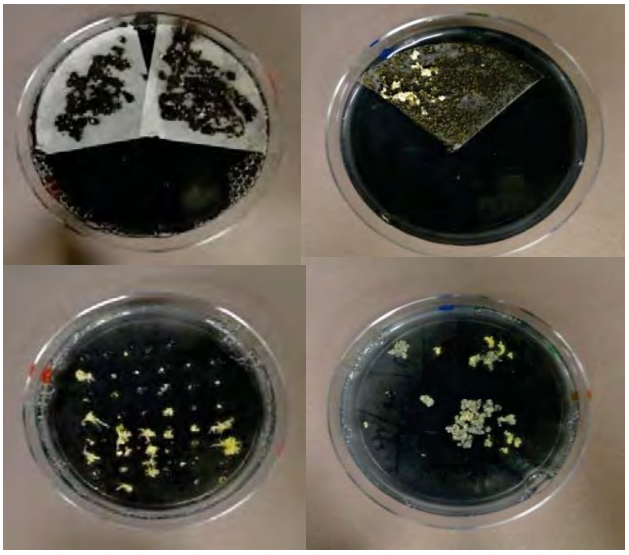

Figure 4. Transgenic colonies of 1103 forming on hygromycin selection (upper left) and transgenic colonies of 101-14 forming on kanamycin selection (upper right) after *Agrobacterium*-mediated transformation of aliquots of grape suspension cultures were plated on BN sorbitol medium. Germinating embryos of 1103 (lower left) and 101-14 (lower right) from colonies after transfer to regeneration medium consisting of Preece medium supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 0.1 mg/liter BAP, and 8 g/l phytoagar.

Table 3. The number of transgenic embryogenic colonies forming after inoculating grape suspension cultures with *Agrobacterium* and plating onto selection medium

Experiment	Germplasm	Flask #	Selection	Transgenic colonies/ml of plated suspension
139275	101-14	1	Kanamycin	3
149018	101-14	1	Kanamycin	24
149040	101-14	2	Kanamycin	3
141139	101-14	1	Hygromycin	6
141023	101-14	2	Kanamycin	17
149011	1103	5	Kanamycin	1
149041	1103	4	Kanamycin	7
141063	1103	5	Kanamycin	7
141080	1103	1	Hygromycin	>50
141087	1103	1	Hygromycin	14
141096	1103	mixed	Hygromycin	16

We currently are comparing the efficiencies of our two transformation methodologies; direct suspension transformation verse transformation of stored embryos produced from plated aliquots of suspensions (**Figure 5**).

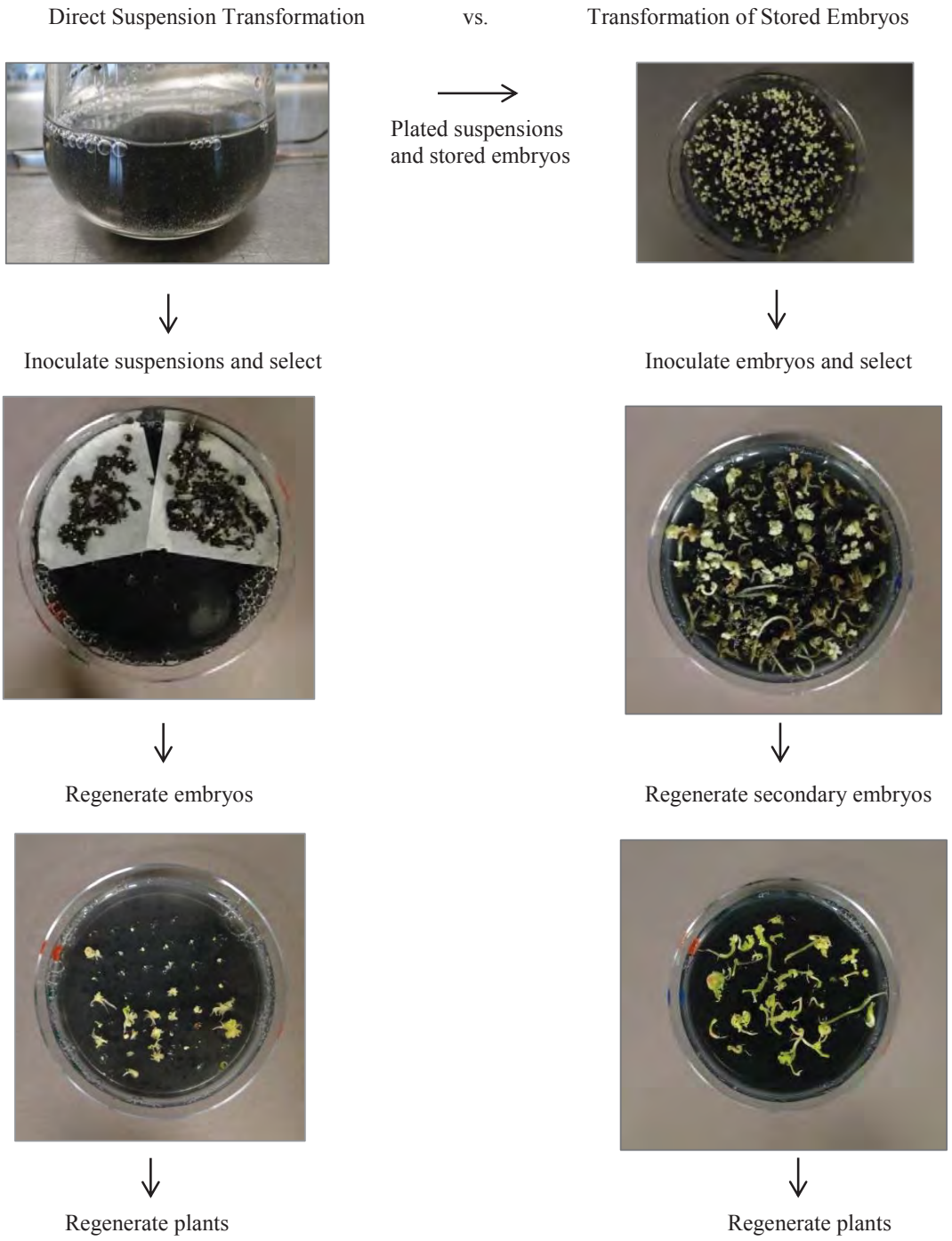

Figure 5. Two alternate paths for transformation of grape; direct suspension transformation (left) and transformation of stored embryos produced from plated aliquots of suspensions (right).

Objective 2c. Develop methods for transforming multiple trait genes into grape through sequential transformation using two different plant selectable marker genes.

Researchers have expressed an interest in stacking multiple resistant strategies in a single transgenic grape line. Although this can be accomplished by stacking traits in a single T-DNA, sometimes issues related to cloning can limit the researcher's ability to stack genes in a single T-DNA. There are also challenges associated with generating transgenic plants with stacked genes since it is difficult to recover transgenic plants where all of the stacked transgenes express at a high level. We first attempted to co-transform grape embryos by inoculating embryos with two *Agrobacterium* cultures each containing a different construct. One construct contained the nptii

plant selectable marker gene and the other construct contained the hpt plant selectable marker gene. After co-cultivation of the grape embryos with the two *Agrobacterium* cultures, we selected for transgenic embryos on 200 mg/liter kanamycin and 25 mg/liter hygromycin. We were unable to recover resistant embryos using co-transformation and double selection in grape. Therefore we performed sequential transformations in which we transformed grape embryos with the first construct containing a gene of interest and the hpt plant selectable marker gene and select for hygromycin resistant transgenic secondary embryos on medium containing hygromycin. Once hygromycin secondary embryos developed, they were increased on sorbitol containing medium which allows them to multiply without germinating. Once sufficient numbers of embryos were produced, they were re-inoculated with an *Agrobacterium* culture containing a second construct with a gene of interest and the kanamycin plant selectable marker gene (nptii) and cultured on medium containing both kanamycin and hygromycin. Developing embryos should contain both selectable marker genes and both genes of interest. We have produced putatively sequentially transformed embryos for 101-14 and we are currently testing the technology on 1103 (**Figure 6**). Once plants are recovered they can be tested for the presence of both genes.

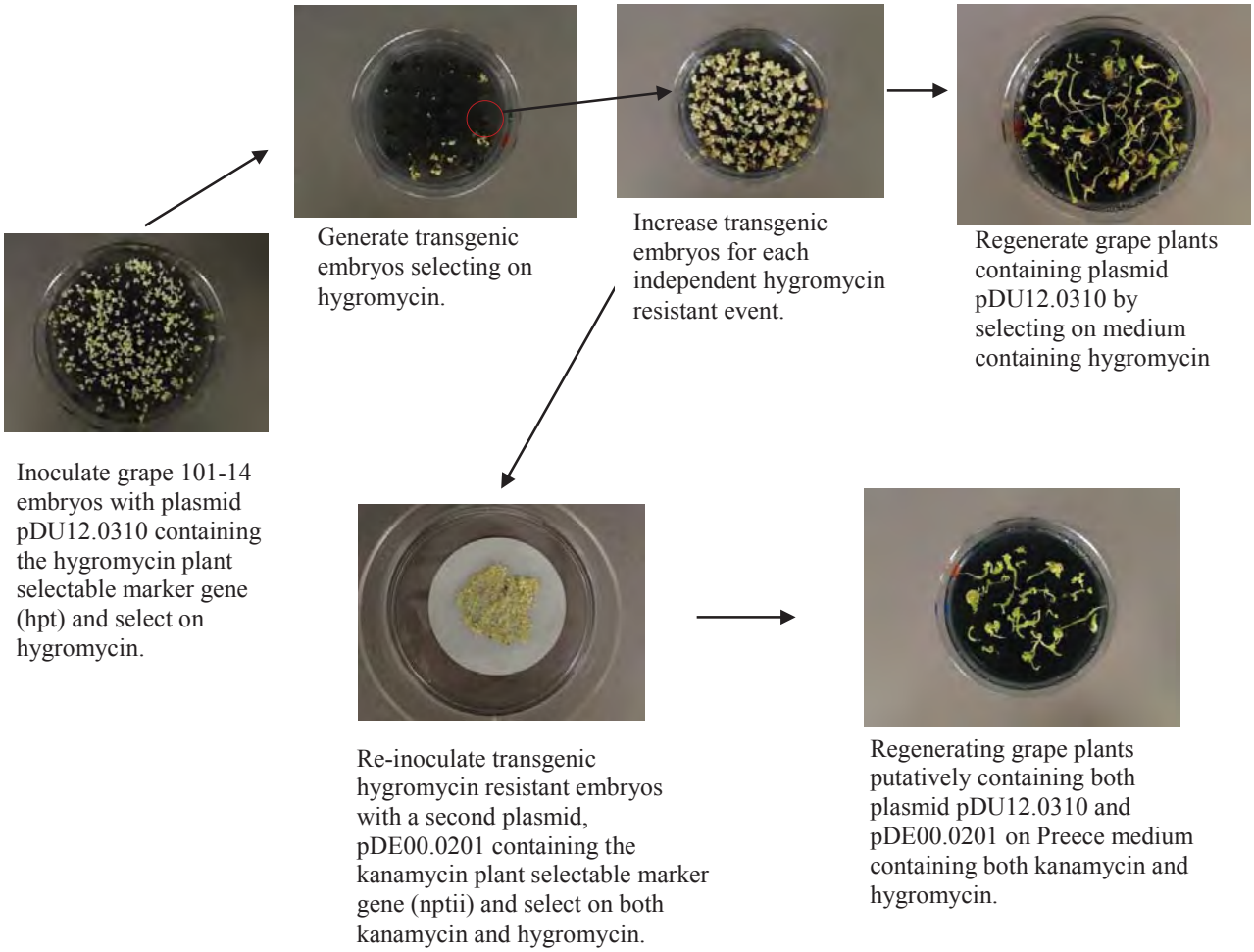

Figure 6. Sequential transformation of 101-14 somatic embryos inoculated initially with plasmid pDU12.0310 and selected on hygromycin. Some hygromycin resistant embryos were induced to regenerate plants while a subset of embryos were re-inoculated with plasmid pDE00.0201 and double selected on hygromycin and kanamycin.

CONCLUSIONS

We have made substantial progress producing high quality embryogenic cultures of 1103, 101-14, Cabernet Sauvignon, and Chardonnay by maintaining embryogenic suspension cultures and plating them onto high osmotic agar-solidified medium on a weekly basis. The system allows for continuous production of highly embryogenic, non-oxidized stock cultures that can serve as a constant supply of starting tissue for use in tissue culture and transformation experiments. This system also provided an efficient means of germplasm storage since embryos can be on sorbitol containing medium for extended periods of time, allowing us to store cultures of different grape

genotypes without using cryopreservation. Using these embryos, high transformation frequencies have been obtained for 1103 and 101-14. Although regeneration of non-transgenic embryos of 1103 and 101-14 is routine, regeneration of transgenic 1103 and 101-14 embryos into whole plants remains inefficient and is currently the rate-limiting step in establishing a more rapid production system for transgenic grape plants. We are developing a suspension-based transformation system and although results are still variable with this transformation method, if perfected, it could significantly enhance the transformation efficiency of grape rootstocks. Lastly, we have developed a sequential transformation methodology which allows transgenes to be stacked by retransforming transgenic embryogenic callus.

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BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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Reporting Period: The results reported here are from work conducted October 2013 to October 2014.

ABSTRACT
We continue to make rapid progress breeding Pierce's disease resistant winegrapes. Aggressive vine training and selection for precocious flowering have allowed us to reduce the seed-to-seed cycle to two years. We are also using marker-assisted selection (MAS) for the Pierce's disease resistance gene, *PdR1* (see companion report) to select resistant progeny as soon as seeds germinate. These two practices have greatly accelerated the breeding program and allowed us to produce four backcross generations with elite *Vitis vinifera* winegrape cultivars in 10 years. We have screened through about 2,000 progeny from the 2009, 2010, and 2011 crosses that are 97% *V. vinifera* with the *PdR1b* resistance gene from *V. arizonica* b43-17. Seedlings from these crosses continue to crop and others are advanced to greenhouse testing. We select for fruit and vine quality and then move the best to greenhouse testing, where only those with the highest resistance to *Xylella fastidiosa*, after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and two Napa sites. The best of these will be advanced to small-scale commercial wine testing, the first of which was planted in Napa in June 2013. We advanced three additional selections to Foundation Plant Services (FPS) this winter to begin the certification and release process. Three Pierce's disease resistant rootstocks were also advanced to FPS for certification. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 are being pursued but progress is limited by their multigenic resistance and the absence of tightly-linked genetic markers. Other forms of *V. arizonica* are being studied and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable resistance. Very small scale batches of wines from 94% and 97% *V. vinifera PdR1b* selections have been very good and have been received well at public tastings in Sacramento (California Association of Winegrape Growers) and Santa Rosa (Sonoma Winegrape Commission), Napa Valley (Napa Valley Grape Growers and Winemakers Associations), Temecula (Temecula Valley Winegrape Growers and Vintners), and Healdsburg (Dry Creek Valley and Sonoma Grape Growers and Winemakers).

LAYPERSON SUMMARY
One of the most reliable and sustainable solutions to plant pathogen problems is to create host plants naturally resistant to the disease. We use a traditional plant breeding technique called backcrossing to bring resistance to Pierce's disease from wild grape species into a diverse selection of elite winegrape backgrounds. In the case of our most advanced vines we have identified the genomic location of a very strong source of Pierce's disease resistance from a grape species native to Mexico. Using marker-assisted selection (MAS) for this Pierce's disease resistance region called *PdR1* (Krivanek et al., 2006), we are able to select resistant progeny shortly after seeds germinate. MAS and aggressive growing of the selected seedling vines have allowed us to produce new varieties that are more than 97% *V. vinifera* winegrape cultivars in only 10 years. We have evaluated thousands of resistant seedlings for horticultural traits and fruit quality. The best of these are advanced to greenhouse testing, where only those with the highest resistance *to Xylella fastidiosa*, after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and at Pierce's disease hot spots around California. The best of these are advanced to 25- to 50-vine plots for commercial wine testing. We have sent 13 advanced selections to Foundation Plant Services (FPS) over the past two winters to begin the certification and release process. Three Pierce's disease resistant rootstocks were also sent to FPS for certification. Other wild grape species are being studied and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable Pierce's disease resistance. Very small scale batches of wines made from our advanced *PdR1* selections have been very good, and have been received well at professional tastings throughout California.

INTRODUCTION
The Walker lab is uniquely poised to undertake this important breeding effort, having developed rapid screening techniques for *Xylella fastidiosa* (*Xf*) resistance (Buzkan et al., 2003, Buzkan et al., 2005, Krivanek et al., 2005a, 2005b, Krivanek and Walker, 2005, Baumgartel, 2009), and having unique and highly resistant *Vitis rupestris* x *V. arizonica* selections, as well as an extensive collection of southwestern grape species, which allows the introduction of extremely high levels of *Xf* resistance into commercial grapes. We have genetically mapped and

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

OBJECTIVES

1. Breed Pierce's disease resistant winegrapes through backcross techniques using high quality *V. vinifera* winegrape cultivars and *Xf* resistant selections and sources characterized from our previous efforts.
2. Continue the characterization of *Xf* resistance and winegrape quality traits (color, tannin, ripening dates, flavor, productivity, etc.) in novel germplasm sources, in our breeding populations, and in our genetic mapping populations.

RESULTS AND DISCUSSION

As in 2012, in 2013 we made F1 crosses to five new Pierce's disease resistant *Vitis* species accessions from the southwestern USA and Mexico to develop mapping populations so that genetic markers could be generated to expedite breeding. The resistant genotypes were chosen based on their low ELISA values, minimal expression of Pierce's disease symptoms in the greenhouse screen, and their diverse geographic origins. We germinated a subset of these seeds in late fall 2013, made copies of seedlings growing in 4" pots in early 2014, and greenhouse tested them to characterize the inheritance of Pierce's disease resistance. All five populations were tested with an average of about 50 progeny each. Statistically, the progeny families separated into three groups: Those from b41-13 being the least resistant; b47-32, SC36, and T03-16 intermediate; and the population from b43-57 having the highest level of resistance, based on ELISA values. Due to the haplotype and LG14 similarity between b43-17 and b43-57 we believe that the Pierce's disease resistance mechanism is similar so won't pursue it further. In 2014 we expanded the most promising population made in 2012, and made crosses to other promising *Vitis* species from this same region as detailed in **Table 1** below.

We have now evaluated more than 2,000 *PdR1b* 97% *V. vinifera* winegrape progeny from which we are selecting the best and most resistant for release. Our breeding efforts in 2014 continued with the stacking of the *PdR1b* and b42-26 sources that were advanced one generation to the 92% *V. vinifera* level. Selfing of selections at this level will create individuals homozygous at both resistance loci for a final crossing to pure *V. vinifera* to produce cultivars ≥ 96% *V. vinifera* (**Table 2a**).

Based on preliminary DNA sequence data and Pierce's disease phenotypic symptom differences with *PdR1b*-containing genotypes, we are renewing efforts in the b40-14 line, which is the source of *PdR1c* on LG14. **Table 2b** reflects crosses made in this line. Crosses to 09-367 go back to our most resistant 75% *V. vinifera* individuals to ensure we bring along minor resistance loci as we advance to higher backcross levels. Crosses to 12-326 and 12-327 will produce progeny ≥ 94% *V. vinifera*.

Advancing promising new southwest USA resistance sources for both breeding and mapping is summarized in **Table 2c**. Of particular interest is the b46-43 line, where all F1 individuals were resistant by both lack of symptoms and ELISA values in our greenhouse test. This population will also be used for mapping this promising new resistance source.

Table 1. Crosses made in 2014 to develop genetic maps in new accessions from southwestern USA and Mexico germplasm. 08326-61 is a self of Cabernet Franc, and F2-35 is a cross of Carignane and Cabernet Sauvignon – both are 100% *V. vinifera* and female flowered to expedite breeding.

Resistance Source	Geographic Origin - Species, Appearance Phenotype	Pure *V. vinifera* Types Used in 2014 Crosses	# of Seeds Produced
A14	Nogales, AZ *V. arizonica*	French Colombard Nero d' Avola Pinot blanc	220 311 407
A28	Willcox, AZ *V. arizonica*	F2-35 Rosa Minna	429 97
ANU67	Mohave, AZ *V. arizonica*, glabrous	F2-35	735
ANU71	Mohave, AZ *V. arizonica-riparia*	French Colombard Grenache blanc	43 146
C23-94	Sedona, AZ *V. arizonica*, glabrous	Nero d' Avola Pinot blanc	221 151
DVIT 2236.2	Veracruz, Mexico *V. cinerea*, long cordate leaves	F2-35 Malaga Rosada	1056 285
SAZ 7	San Rafael Valley, AZ *V. arizonica*	F2-35	1007

Table 2d and 2e show crosses made to stack both Pierce's disease and powdery mildew (PM) resistance from multiple sources into single cultivars. *Ren1* is a reliable, strain-specific PM resistance source found in a number of pure *V. vinifera* cultivars from Central Asia. The breeding efforts with alternative resistance sources and the complexing of these resistances is being done to broaden *Xf* resistance and address X*f*'s ability to overcome resistance.

Table 2. Pierce's disease breeding crosses made in 2014. A) Crosses made to stack *PdR1b* (F8909-08) Monterrey *V. arizonica/candicans* and b42-26 *V. arizonica/girdiana* resistance lines to produce 92.2% *V. vinifera* progeny. B) Chihuahua *V. arizonica* resistance source (*PdR1c*) b40-14 to produce progeny with 87.5% (09-367 crosses) or 94.75% *V. vinifera* parentage. C) Cross made to three new promising southwest USA resistance lines to produce BC1 75% *V. vinifera* progeny. 08319-07 and 08326-61 are self's of Zinfandel and Cabernet Franc respectively, and are 100% *V. vinifera* and female flowered. D) Monterrey *V. arizonica / candicans* resistance source *PdR1b* (F8909-08) to produce progeny with approximately 91% *V. vinifera* parentage with PM resistance from advanced *Ren1* and *Ren4* lines. Some crosses will produce homozygous resistant progeny at one or more loci. E) Monterrey *V. arizonica/candicans* resistance source *PdR1b* (F8909-08) stacked with b42-26 *V. arizonica/girdiana* resistance lines to produce progeny with about 90% *V. vinifera* parentage with PM resistance from *Ren4* or both *Ren1* and *Ren4* lines. Some crosses will produce homozygous resistant progeny at one or more loci.

	Resistant Type	***Vinifera* Parent \ Grandparent of Resistant Type**	***Vinifera* Types Used in 2014 Crosses**	**# of Seeds Produced**
A	09-331	Zinfandel, Grenache\Petite Sirah, F2-35	Zinfandel\Petite Sirah; Chardonnay\Grenache	3,603
B	09-367	Cabernet Sauvignon\Airen	Carignane, Nero d'Avola, Zinfandel	777
	12-326, 12-327	Carignane, Grenache\Cabernet Sauvignon	Carignane, F2-35, Nero d'Avola	1,350
C	ANU5, b40-29, b46-43	F2-35, Rosa Minna	08319-07, 08326-61, Alicante Bouschet	3,748
D	07-365, 08-335, 11-326, 11-715	F2-35\Karadzhandal	Zinfandel\Petite Sirah	1,316
E	11-394	F2-35\Karadzhandal	Zinfandel\Petite Syrah	2,317

Table 3 provides a list of the Pierce's disease resistance greenhouse screens analyzed, initiated, and/or completed over the last six months. We are making every effort in new lines to bring minor genes along with those for which we have markers. In group A we tested 28 BC1 genotypes from the b43-17 line that were selected with markers to be missing *PdR1* to elucidate the role of minor resistance genes that may have been lost during the early breeding generations. Data analysis revealed about 50% of the genotypes to be intermediate in resistance between our standard *PdR1b* resistant genotype and the susceptible genotype Chardonnay. The other half were as susceptible

as Chardonnay. Interestingly one genotype without *PdR1* tested as resistant as b43-17, the source of *PdR1*. Retesting of this anomalous genotype in the greenhouse and with markers is in progress. In groups A and B we continued testing of *PdR1b* selections at the 97-98% *V. vinifera* level. The special focus of these trials was on white-fruited selections and those that descend from Nero d'Avola. Results from these and earlier screens have helped us to decide on the selection of the most resistant genotypes to advance to field trials (for example, **Table 4**) and to Foundation Plant Services for certification. In part of group B we retested 20 of the most promising F1 genotypes from the 2012 crosses from the new southwest USA species selections ANU5, b40-29, and b46-43, first tested last August, and results were consistent between screens. BC1 crosses were made with these in spring 2014 to expedite their incorporation into our breeding efforts (**Table 2c**). In group C we tested individuals from the *PdR1b* x b42-26 stacked line as well as 25 Pierce's disease rootstock genotypes from four crosses made in 2011. Advanced *PdR1b* rootstocks were crossed to Ramsey, 420A, or Schwarzmann. Based on phenotype scores, five of the crosses to Ramsey look particularly promising. ELISA results are partially complete and are tracking closely with the phenotypic scores. In group D we tested 23 BC2 individuals in the b42-26 multigenic resistance source and identified two particularly promising genotypes for further advancement. New southwestern USA species were tested in groups A, C, D, F, G, and I to facilitate Pierce's disease resistance gene discovery work being done in our companion Pierce's disease mapping project. Upon completion of these trials we will have tested a total of 284 different accessions, the most resistant among them multiple times. BC2 progeny in the b40-14 line from crosses made in 2012 were tested in group F and based on encouraging results additional progeny are being tested in group G. We initiated test group H to confirm previous results in the b40-14 line and are testing multiple backcross levels in the same trial.

Table 3. Greenhouse Pierce's disease screens analyzed, completed, and/or initiated during the reporting period.

Group	Genotypes	No. of Genotypes	Inoculation Date	ELISA Sample Date	Resistance Source(s)
A	SWUS Species, *PdR1-,* 09-10 PdR1b advanced	132	11/19/2013	2/18/2014	*V.* species, b43-17
B	2010 Cross *PdR1b*, 2009 *PdR1b* cross final, 2012 SWUS Cross F1 most PDR	99	3/13/2014	6/12/14	b43-17, b40-29, b46-43, ANU05
C	*PdR1b* x b42-26 pyramided, new PD rootstocks, *PdR1a* advanced, SWUS species	165	3/20/2014	6/19/14	F8909-08, *PdR1a*, b42-26, Ramsey, *V.* species
D	b42-26 BC2 & SWUS Species	47	4/8/2014	7/8/14	b42-26, *V.* species
E	2013 SWUS F1 Cross Seedlings	190	4/24/2014	7/29/14	b41-13, b43-57, SC36, T03-16
F	88% b40-14, Additional 2013 SWUS Cross Seedlings	146	5/15/2015	8/14/14	b40-14, b43-57, b47-32
G	SEUS Xs, SWUS Species	107	9/11/14	12/11/14	B40-14, Haines City BD5-117
H	b40-14 F1, BC1, BC2; 2014 Cross Parents	163	9/24/14	12/24/14	F8909-08, b40-14, b42-26
I	SWUS Species & Promising Genotypes	223	10/2/14	1/1/15	*V.* species, F88909-08, Ramsey

In our program we test selections with the potential for release multiple times in the greenhouse screen to ensure that only selections with the greatest levels of resistance are considered for release. These selections have much better resistance than two selections with long histories of field survival in the southern USA – Blanc du Bois and Lenoir (Jacquez). We want to avoid releasing selections that are tolerant to *Xf* and therefore act as hosts for disease spread within a vineyard. This process involves passing our severe greenhouse screen multiple times. 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 vine field trials in Davis and in Pierce's disease hot spots around California complete the evaluation process. Pierce's disease resistant scions need Pierce's disease resistant rootstocks in case low levels of the bacteria work their way into a susceptible rootstock. Three such rootstock selections were sent to FPS in the spring of 2013 and another 25 genotypes were tested in 2C as discussed above.

Table 4 presents 10 promising Pierce's disease resistant genotypes that were advanced to field trials and/or sent to FPS at UC Davis in 2014 for certification and possible release. The later three will join the 10 *PdR1b* -based Pierce's disease resistant scion selections sent to FPS in the spring of 2013, the details of which were given in a previous interim progress report. The two new field trials were established with cooperators in Pierce's disease hot spots in both Temecula and Napa earlier this year. Twenty-five vine reps of each of eight advanced *PdR1b* accessions were planted (**Table 4**). No pictures are provided since they are newly planted. Extensive details on the encouraging results from our Beringer field trial in Napa were provided in our March 2014 progress report. In short, our crosses with Pierce's disease resistance from *PdR1b* continue to thrive and produce at our Beringer field trial in Napa Valley while the pure *V. vinifera* control vines and the surrounding commercial Chardonnay and Riesling vines continue to decline.

Table 4. *PdR1b* selections being advanced to field trials (FT) or FPS. All are at the 97% *V. vinifera* level.

Group	Genotype	Parentage	Color	Berry Wt (g)	Cluster Wt (g)	Season	Flavor
FT	09314-102	07370-028 x Cabernet Sauvignon	W	1.1	250	Late	Like Sauv blanc but more fruity
FT	09330-07	07370-039 x Zinfandel	B	1.4	300	Mid-Late	Berry
FT & FPS	09331-047	07355-020 x Zinfandel	B	1.1	150	Early	Berry, spice
FT	09331-133	07355-020 x Zinfandel	B	1.4	200	Early	Fruity, spice
FT	09333-178	07355-020 x Chardonnay	B	1.2	175	Mid	Like Cab Sauv but more fruity
FT	09333-253	07355-020 x Chardonnay	B	1.3	240	Early-Mid	Like Cab Sauv but more fruity
FT	09333-331	07355-020 x Chardonnay	B	1.2	225	Early	Fruity
FPS	09333-358	07355-020 x Chardonnay	B	1.1	150	Mid	Fruity
FT	09333-370	07355-020 x Chardonnay	B	1.5	310	Mid-Late	Fruity
FPS	09356-235	07371-19 x Sylvaner	B	1.2	175	Late	Fruity

Tables 5a through 5c detail the vine, fruit, and juice characteristics for the ten 97% *V. vinifera PdR1b* selections used to make wine lots in 2014. Two additional lots pair wines made from 94% *V. vinifera PdR1b* selections grown in Davis with those from our field trial at the Treasury Wine Estates (Beringer) vineyard in Yountville, Napa Valley (data not show). In addition, we made a number of *V. vinifera* controls and Blanc du Bois from both Davis and Napa. Lenoir was made from Davis fruit.

In 2012 we conducted the first industry tastings of our advanced selections. In August, we presented the best of our 87 and 94% *V. vinifera PdR1b* wines to about 200 people as part of a Sonoma County Winegrape Growers Commission meeting. We presented wines made in 2012 with our favorite 94% *V. vinifera PdR1b* selections in a blended format to show how these wines would be used as blending grapes to fill in chronic Pierce's disease hotspots and still stay within the 75% / 25% varietal wine labeling requirement. We did this with one of our favorite whites (07713-51), blended it with Chardonnay from Yountville, and compared these wines with those made at the same scale with Yountville Chardonnay and Blanc du Bois (top southern USA Pierce's disease resistant winegrape). One of our favorite reds at the 94% level is 07355-075 and it was blended with Oakville Merlot. We also presented a wine made from three of our advanced 97% *V. vinifera PdR1b* selections (there was not enough fruit from any of them individually, but all have now been replicated for future wine-making): 09331-047, 09332-165, and 09333-178. These wines were well received and many liked the 97% blend the best. The first tasting in 2013 was with select growers and winemakers in the Napa Valley at the UCD Oakville Station, a second was on July 17th in Temecula to the Winegrowers Association, and a third in early August at Healdsburg

(Dry Creek Valley and Sonoma Grape Growers and Winemakers). These wines were also presented at the Napa Grape Expo on November 14th. The next step is to make larger scale wines in multi-ton lots. These vines are being planted as noted above. Once selections clear FPS testing, the best selections will be ready for release.

Table 5a. 97% *V. vinifera* Pierce's disease resistant selections used in small scale winemaking in 2014: background and fruit characteristics.

Genotype	Parentage	2014 Bloom Date	2014 Harvest Date	Berry Color	Berry Size (g)	Ave Cluster Wt. (g)	Prod 1=v low, 9=v high
09314-102	07370-028 x Cab Sauvignon	5/13/2014	8/21/2014	W	1.6	400	9
09330-07	07370-039 x Zinfandel	5/18/2014	9/2/2014	B	1.3	275	8
09331-047	07355-020 x Zinfandel	5/8/2014	8/12/2014	B	1.4	400	5
09331-103	07355-020 x Zinfandel	5/13/2014	8/19/2014	B	1.2	333	7
09333-039	07355-020 x Chardonnay	5/13/2014	8/21/2014	B	1.3	400	7
09333-178	07355-020 x Chardonnay	5/13/2014	9/2/2014	B	1.9	350	6
09333-253	07355-020 x Chardonnay	5/15/2014	8/21/2014	B	1.2	225	6
09333-313	07355-020 x Chardonnay	5/15/2014	8/21/2014	B	1.3	350	8
09333-370	07355-020 x Chardonnay	5/18/2014	9/2/2014	B	1.4	282	6
09356-235	07371-19 x Sylvaner	5/13/2014	8/28/2014	B	1.4	325	7

Table 5b. Juice analysis of 97% *V. vinifera* Pierce's disease resistant selections used in small scale winemaking in 2014.

Genotype	°Brix	TA (g/L)	pH	L-malic acid (g/L)	potassium (mg/L)	YAN (mg/L, as N)	catechin (mg/L)	tannin (mg/L)	Total antho-cyanins (mg/L)
09314-102	24.0	6.5	3.67	3.92	2340	150	-	-	-
09330-07	23.0	6.0	3.55	2.61	1930	185	88	588	1667
09331-047	28.5	4.6	3.87	2.12	2670	284	4	572	1658
09331-103	23.9	7.3	3.30	1.6	1450	210	11	634	1253
09333-039	23.9	7.2	3.40	2.52	2020	162	36	470	648
09333-178	27.2	4.9	3.69	1.62	2090	57	51	429	520
09333-253	25.8	5.9	3.51	2.43	1840	147	40	425	566
09333-313	26.4	7.0	3.43	2.22	2160	76	36	298	811
09333-370	23.2	4.9	3.63	1.65	1630	205	17	469	628
09356-235	24.8	5.4	3.58	1.97	1890	143	56	787	1534

CONCLUSIONS

We continue to make rapid progress breeding Pierce's disease resistant winegrapes through aggressive vine training, marker-assisted selection, and our rapid greenhouse screening procedures. These practices have allowed us to produce four backcross generations with elite *V. vinifera* winegrape cultivars in 10 years. We have screened through thousands of seedlings that are 97% *V. vinifera* with the *PdR1* resistance gene from *V. arizonica* b43-17. Seedlings from these crosses continue to crop and others are advanced to greenhouse testing. We select for fruit and vine quality and then move the best to greenhouse testing, where only those with the highest resistance to *Xf*, after multiple greenhouse tests, are advanced to multi-vine wine testing at Davis and in Pierce's disease hot spots around California. The best of these are advanced to 50- to 100-vine commercial scale testing with the first selection planted this year. We have sent 13 advanced scion selections to FPS over the past two winters to begin the certification and release process. Three Pierce's disease resistant rootstocks were also sent to FPS for certification. Pierce's disease resistance from *V. shuttleworthii* and BD5-117 is also being pursued, but progress and effort is limited because their resistance is controlled by multiple genes without effective resistance markers. Other forms of *V. arizonica* are being studied and the resistance of some will be genetically mapped for future efforts to combine multiple resistance sources and ensure durable resistance. Very small-scale lots of wines from 94% and 97% *V. vinifera PdR1b* selections have been very good, and have been received well at tastings in the campus winery and at public tastings throughout California.

Table 5c. 97% *V. vinifera* Pierce's disease resistant selections used in small scale winemaking in 2014: berry sensory analysis.

Genotype	Juice Hue	Juice Intensity	Juice Flavor	Skin Flavor	Skin Tannin Intensity (1=low, 4= high)	Seed Color (1=gr, 4= br)	Seed Flavor	Seed Tannin Intensity (1=high, 4= low)
09314-102	Gr-gold tch brown	Med	Pear, yellow apple, sl spice	Sl melon, pear	1	3	Earthy, ashy	4
09330-07	Clear Bright pink-red	Lt+	Raspberry, tart cherry	Berry, fruity	2	3	Astringent neutral	1
09331-047	Red, tch orange	Med	Plum, candy, vs fresh jam	Berry, spice, chalky	2	4	Woody, ashy	1
09331-103	Red, typical clear	Med	Strawberry, cranberry, spice	Berry, fruity	3	4	Hot, woody, bitter	1
09333-039	Red	Lt	Strawberry, spice	Berry, plum, sl grass	2	4	Spicy, bitter	2
09333-178	Brown	Med	Spice, pear	Plum, berry	1	4	Woody, nutty, bitter	2
09333-253	Pink-orange	Lt+	Berry, apple	Berry, warm,	2	2	Woody, spicy, hot	3
09333-313	Brown orange	Med-	Spice, plum	Sl blk cherry,	1	4	Chalky, sl bitter	2
09333-370	Pink-orange	Lt+	Mellon, plum	Neutral, spicy	4	3	Woody, spicy	2
09356-235	Red, tch orange	Med	Fruity, berry	Mildly fruity, sl hay	4	3	Woody, vs bitter	2

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MAP-BASED IDENTIFICATION AND POSITIONAL CLONING OF *XYLELLA FASTIDIOSA* RESISTANCE GENES FROM KNOWN SOURCES OF PIERCE'S DISEASE RESISTANCE IN GRAPE

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INTRODUCTION

Genetic maps are powerful tools that enable the tagging of regions of interest, identification of markers that are tightly linked to that region for breeding, and map-based positional cloning of disease resistance genes. Tightly linked markers to resistance are extremely valuable for plant breeders. Firstly, they can avoid environmental affects by using indirect selection for a resistance trait through selection for a marker associated with the resistance allele. Secondly, marker-assisted selection accelerates the breeding process for relatively long generation time perennials such as grape, saving time, labor, and money. Thirdly, stacking of resistance genes from multiple backgrounds into a single line is only possible with the help of markers. Stacking of resistance genes will allow durable long-lasting resistance in the field.

The Walker lab possesses a wide range of Pierce's disease resistant germplasm that was collected from Mexico and the southeastern and western USA over a period of 50 years. This germplasm is a very valuable gene pool for not only Pierce's disease resistance, but also for resistance to other insects and pests and salt and drought tolerance. This unique germplasm is present only in our collection and not represented anywhere else in the world. To exploit the resistant accessions for breeding, it is important to understand the inheritance and genetics of resistance, develop phenotyping assays for the disease screening, and develop mapping populations that can be used to tag the resistant regions and identify linked markers.

The history of the genetic mapping in our lab goes back to the project that was funded by the USDA-funded UC Pierce's Disease Research Grant Program. It expanded on a genetic mapping effort originally funded by the California Grape Rootstock Improvement Commission, the Fruit Tree, Nut Tree, and Grapevine Improvement Advisory Board, the California Table Grape Commission, and the American Vineyard Foundation. The initial project examined the genetics of resistance within several *Vitis rupestris* x *Muscadinia rotundifolia* F1 populations that were later identified to be crosses of *V*. rupestris and *V*. arizonica hybrids. From 2003 to 2014, we have explored a wide range of resistant material, fully understood the mode of inheritance, and genetically mapped Pierce's disease resistance from the homozygous resistant selection *V. arizonica/candicans* b43-17 and identified very tightly-linked markers for use in marker-assisted selection (MAS), which has greatly expedited our Pierce's disease resistance breeding program (Krivanek et al., 2006, Riaz et al., 2006, Riaz et al., 2007, Riaz et al., 2008, Riaz et al., 2009). We have used these markers to rapidly select at each generation of the backcross breeding program as we increase the percentage of *V. vinifera* in the hybrid progeny (F1 = 50% *V. vinifera*; backcross (BC) 1 = 75%; BC2 = 87%; BC3 = 94%; BC4 = 97% *V. vinifera*). This project supported the grape breeding companion project by screening 1,000's of seedlings with markers each year from 2005 onward. Marker-assisted screening (MAS) allowed the breeding program to reduce the seed-to-seed cycle to two years and greatly accelerated the development of Pierce's disease resistant grapes.

We developed four different populations with b43-17 (F1 and BC1), separated resistance haplotypes F8909-08 and F8909-17 (each a different allele from the homozygous b43-17), developed framework maps, and verified resistance in both. In 2005, we initiated crosses with the second resistant accession, b42-26, which is a complex mix of *V. arizonica, V. girdiana, and V. candicans*. Inheritance studies in later years indicated that resistance in b42-26 is multigenic and complex and required a large mapping population. Crosses with the third resistance accession, a pure form of *V. arizonica* (b40-14), were initiated in 2007. All F1 progeny of b40-14 were resistant to Pierce's disease in the greenhouse screen and multiple BC1 populations were created with different susceptible *V. vinifera* parents. Framework maps with Simple Sequence Repeat (SSR) based markers were initiated in both resistant sources, b42-26 and b40-14, and a single major resistant locus was found in b40-14. Apart from tagging the resistance loci via genetic mapping, we also initiated BAC library development in order to pursue physical mapping and cloning of resistance genes from b43-17 and b40-14. The physical map was completed for b43-17 haplotype F8909-08 and candidate resistance genes were identified (manuscript in progress). The BAC library of

b40-14 was also developed; screening and physical map development is part of the next funding cycle. In 2011, following the recommendations of the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, we screened over 200 accessions in our collection to identify new Pierce's disease resistance sources to broaden the genetic base of the breeding program under the project title "Genetic mapping of *Xylella fastidiosa* resistance gene(s) in grape germplasm from the southern United States." Multiple new resistant accessions were identified and further work on them is the scope of the next funding cycle.

Over the past 11 years, this mapping project has exploited multiple resistance sources, developed framework and high resolution fine-scale maps, tagged resistance loci, and generated markers that were used to carry out MAS on 1,000's of seedlings each year to support the companion breeding program. BAC libraries were developed to pursue map-based positional cloning of the resistance genes, a physical map was completed, and candidate genes were identified from the F8909-08 resistant selection. The results from this project also fueled the second companion project "Molecular characterization of the putative *Xylella fastidiosa* resistance gene(s) from b43-17 (*V. arizonica*)." The necessary symbiotic bond of this project to two other projects made it possible to move forward to begin field and wine testing leading to the release of new varieties and to strengthen resistance by complexing multiple forms into one line.

OBJECTIVES

These objectives summarize those from the 11-year duration of this project.

1. Characterize resistance from *V. arizonica/candicans* b43-17.
2. Genetically map resistance from b43-17 and use tightly-linked markers in MAS for the Pierce's disease resistant wine, table, and raisin grape breeding programs.
3. Physically map resistance from b43-17 and b40-14 and provide resistance genes to our companion project testing the function of *PdR1* by transforming *PdR1* into susceptible grapes.
4. Evaluate southern USA and Mexican *Vitis* germplasm for alternative sources of Pierce's disease resistance.
5. Characterize and map alternative forms of resistance and use them in MAS with *PdR1* to complex or stack resistance genes in an effort to produce more durably resistant cultivars.
6. Physically map new forms of resistance to better characterize Pierce's disease resistance and confirm their function by transforming susceptible *V. vinifera* with them.

RESULTS

Research highlights.

2003 – 2004

The refinement of the 9621 population map was pursued with publicly available SSR, EST-SSR, and resistance gene ortholog markers. Resistance was mapped to chromosome 14 in the F8909-17 parent and was flanked by both AFLP and SSR markers. Crosses were made with resistant selections F8909-08 and F8909-17 with susceptible *V. vinifera* parents to create populations for mapping in order to study the resistance in the absence of *V. ruspestris* or b42-26 in the background. Markers were identified that were linked to the resistance and efficacy of their use for MAS was tested. **Table 1** shows the species information for populations and genotypes used for mapping. **Table 2** shows the 9621 consensus map details.

2004 – 2005

DNA extractions of four different mapping populations were completed. Framework mapping was initiated for the 04190 (F2-35 x F8909-08) population. Below is the detail of different populations that were investigated in 2004-2005.

Expected or known segregation patterns.

1. 9621 Population: *PdR1* single locus for F8909-17 and multiple quantitative trait loci (QTL) for D8909-15.
2. 0023 Population: multiple QTLs.
3. 03-300 population: *PdR1* resistance segregates 1:1 (single gene model) *Xf* greenhouse screening for entire population was initiated.
4. 04-190 population: results based on resistant alleles from six markers, *PdR1* segregates as 1:1 (single gene model), *Xf* greenhouse screening for the entire population underway.
5. 04-191 population: *PdR1* resistance should segregate 1:1; plant DNA extraction and addition of Pierce's disease group markers underway.
6. 04-373 population: *PdR1* resistance should segregate 1:1; plant DNA extraction and addition of Pierce's disease group markers underway.

We also determined and verified the true parents of multiple populations of the 89-series with the help of SSR markers. This was the pivotal milestone in our understanding of Pierce's disease resistance in germplasm collected from Mexico in 1961 by Harold Olmo (**Table 3**). The correct identification of these accessions was also verified in the National Clonal Germplasm Repository. The 9621 population was expanded with 300 individuals to allow the identification of recombinants, DNA isolation was carried out, and plants were tested with markers.

2005 – 2006

Marker-assisted selection was started to support the Pierce's disease resistance grape breeding program at Davis and also for David Ramming at the USDA ARS at Parlier. The framework map of the 9621 population was completed with over 200 SSR markers. The consensus map spanned 1154 cM across 19 linkage groups. Linkage group 14 was the largest group with 30 markers. Fifteen markers were tightly associated with the major locus – *PdR1*. An additional 276 genotypes were added to make a core population of 457 genotypes in the 9621 population. We continued screening to select EST-SSR markers with known function with the main goal of finding markers that were polymorphic for parents of two main mapping populations (9621 and 04190) and map to linkage group 14 only. We chose a subset of 20 RGA-STS primers to screen parental samples for polymorphism and to be used for mapping. We chose resistant genotype b43-17 to develop a BAC (Bacterial Artificial Chromosome) library that would enable the identification of the resistance gene. F8909-08 and F8909-17 are progeny of b43-17 and they inherited different alleles of *PdR1*. However, both alleles are associated with resistance locus. This information indicated that there might be cluster of genes associated to resistance and progeny F8909-08 and F8909-17 inherited different copies of resistant genes. Therefore, b43-17 was the right candidate to develop the BAC library. We collected 100 cuttings of b43-17 and grew them in the greenhouse to collect young leaves for isolation of high molecular weight DNA from which the BAC library was constructed.

2006 – 2007

Genetic marker work was completed on the newly-added genotypes of the 9621 population, which identified a subset of 60 progeny (primarily recombinants with a few resistant and susceptible genotypes as controls) capable of being used for fine-scale mapping. Recombinant plants within these populations are critical as they allow loci of interest (Pierce's disease resistance in this case) to be mapped fully enough to allow characterization and identification of the responsible gene(s). The greenhouse screening for Pierce's disease resistance of these plants was completed. This increased the number of individuals and helped us to refine the position of the *PdR1* locus and make the genetic window to less than 1 cM. We also screened an additional 400 9621seedlings for two markers flanking *PdR1* to find more recombinants. Fifty recombinants were detected and they were planted in the field. A subset of 20 RGA-STS primers were tested with a subset of five different restriction enzymes to create/find restriction site based polymorphism in the *PdR1* locus. Three markers were polymorphic with different restriction enzymes. They were added to the core 9621 population.

Genetic mapping of the 04190 population, a cross of *V. vinifera* F2-7 (Carignane x Cabernet Sauvignon) x F8909-08 was completed on a core set of 220 plants. Marker order for LG 14 was consistent between F8909-17 (9621 paternal map) and F8909-08 (04-190 paternal map). These genotypes inherit different alleles from the homozygous b43-17, and could represent different copies of the resistance gene(s). This work enabled us to choose easily-scored, highly polymorphic markers for use in marker-assisted selection (MAS) based breeding of Pierce's disease resistant winegrapes. The 04190 population was expanded from 220 to 366 individuals and all marker work was completed.

2007 – 2008

New markers were developed from the publicly available grape genome sequence of Pinot noir PN40024 (**Table 4**). These markers were tested and added to the key set of recombinants in different mapping populations. A major segregation distortion region was identified on chromosome 14 in progeny from the F8909-08 and b43-17 maps. Marker-assisted screening support was provided to the companion-breeding program by testing over 1,500 seedlings to identify resistant and susceptible plants. The greenhouse screen was repeated for the key recombinants in different mapping populations. Framework mapping was initiated in the 04191 population to identify any minor genes that might contribute to resistance. This population provides genotypes with a 50% *V. vinifera* background for breeding wine and table grapes as well as more recombinant plants for use in genetic mapping. This population also enables the study of resistance from F8909-17 to be examined without possible confounding effects from D8909-15. We also initiated genetic mapping in the F1 population from the b42-26 background (05347 – **Table 1**). A total of 337 markers were tested on a small parental data set. Results found a high level of homozygosity for b42-26 (only 113 markers were polymorphic); 184 markers were homozygous for

the male parent b42-26, and 40 markers did not amplify. Polymorphic markers were added to the 64 progeny. Crosses were made to increase the population size to over 200 progeny.

b40-14 is the third promising homozygous resistant genotype. We screened 45 genotypes from an F1 cross of *V. rupestris* x b40-14 and all were resistant except three genotypes with intermediate results. Crosses were made in the spring of 2007. We completed DNA extractions from 122 seedlings from 07744 and 105 seedlings for 07386 with b40-14 in their background. Marker testing was started to find a framework set of markers that span 19 chromosomes.

Two BAC libraries (each with a different restriction enzymes) from the homozygous resistant b43-17 were developed. Library screening was carried out twice with two markers (VVCh14-10 and VVCh14-56), which are tightly linked to *PdR1* as per the previous location of the locus. A total of 24 positive clones were identified – four of the positive clones were positive with both markers.

2008 – 2009

Marker-assisted selection support to the Pierce's disease breeding program continued. The genetic position of the *PdR1a* resistance locus was refined between marker VVCh14-56 and VVCh14-70. **Table 5** shows the key recombinants from two populations. VVCh14-70 is the new marker developed from the sequence obtained from the Pinot noir genome sequence. The new markers were added to the set of key recombinants in all populations. Testing of markers and adding polymorphic markers to the 04191, 07744, and 05347 populations continued. DNA extractions of expanded 05347 populations were completed. We screened 240 SSR markers on a small subset of eight genotypes from the 07744 population, including parents and a few progeny with VMC series, VVMD, and VVS markers. A total of 100 markers were polymorphic in the preliminary screen. Eighty polymorphic markers were completed on an entire set of 122 plants of the 07744 population.

Ten new primer sets were developed to screen the b43-17 BAC library. These primers spread across 60 to 80Kb of the 695Kb sequence from PN40024. Nine of these markers amplified the genomic DNA of resistant b43-17 successfully. This work localized the resistance locus between Ch14-56 and Ch14-70, at a physical distance of 340Kb.

2009 – 2010

Marker-assisted selection support to the Pierce's disease breeding program continued. Mapping work progressed in three populations (04191, 05347, and 07744). These populations were expanded and data were analyzed as the greenhouse screen results became available.

Genetic mapping and QTL analysis was completed in the 07744 population. 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. A major locus mapped on chromosome 14 – the same chromosome where *PdR1a* and *PdR1b* mapped. Pierce's disease resistance from b40-14 (which we have named *PdR1c*) also maps in the same general region between flanking markers VVCh14-77 and VVIN64 and within 1.5 cM. This locus explains 82% of the phenotypic variation. A minor QTL was identified on chromosome five. A shotgun library of BAC clone H64M16 was Sanger sequenced. Clone H69J14 was selected for 454 sequencing. However, the sequenced region was highly enriched with repetitive elements that complicated the assembly. Newbler software as well as the Lasergene program SEQMAN, which enable sequence analysis, do not work well with sequences containing many repeated regions. In order to generate longer sequence fragments, a shotgun library was constructed for clone H69J14. This generated 384 sequences in both directions to develop paired ends capable of filling the gaps between the contigs from the 454 sequence data. We then masked the repetitive region from all the sequences (both H69J14 and H64M16 clones) to carry on the assembly with the MIRA assembler program. This effort improved the assembly, but the contig number was still very high and not suitable for primer walking. Moreover, all the major contigs had masked repetitive regions on both ends indicating that the primer design effort would not generate sequence specific results capable of bridging the sequence gaps. A fosmid library with an insert size of 35-40Kb was initiated in order to allow us to better understand and assemble this highly repetitive region.

2010 – 2011

Genetic mapping was completed for the 04191 population. The genetic map was constructed with 5.0 LOD and a 0.40 recombination frequency. 136 markers were grouped on 19 chromosomes (2n=38). QTL analysis was carried out with the natural log of the ELISA values. We reconfirmed a major locus *PdR1*a on chromosome 14 and

identified a minor QTL explaining 7% phenotypic variation on chromosome 19 that peaks at marker CB918037 (**Figure 1**). The map for this minor resistance locus on chromosome 19 was refined by adding five markers that reduced the distance between the markers from 12 cM to 8 cM on one side and from 5 cM to 3 cM on the other side of the locus from the previous report. The locus explained 7% of the phenotypic information and may play an important role in Pierce's disease resistance. More markers were developed using the PN40024 sequence for the resistance region on chromosome 19.

We also worked on the 05347 population. 71 new SSR markers were developed from clone sequences generated from the *Vitis* Microsatellite Consortium (the original source of SSR markers for grape). These clones had been discarded as not useful for marker development because of the presence of microsatellite repeats at the beginning or end of the sequence, leaving no room for primer design. We also acquired primer sequences of an additional 200 markers that had not been tested with b42-26. Marker testing on a small set of parents and progeny proceeded. We also added markers to develop a framework map for the entire population.

We pursued the physical map of the BAC clone H69J14. To overcome the repetitive elements and to produce longer contigs, a Fosmid library was generated with an insert size of 35-40Kb. The assembled sequence (454 reads, shotgun reads, and fosmid library reads) produced more than 80 contigs. A search was carried out to identify the genes on all of them using the sequence builder module and the results were confirmed by blasting with the NCBI database. We identified six copies ranging from 2Kb to 3.1Kb in the resistance region. Copies 1 through 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. Detailed analyses based on the NCBI protein search identified four tandem repeats of serine threonine protein kinase with a leucine-rich repeat domain gene family in the resistance region. These proteins are involved in eliciting disease resistance responses. A direct comparison of the H69J14 clone sequence to the PN40024 sequence was not possible due to major re-arrangement of repetitive elements between the two genomes.

2011 – 2012

To develop the genetic map of the F1 population 05347 (F2-35 x b42-26), we tested a total of 916 markers, 763 SSR primers amplified b42-26 DNA, and 180 markers were polymorphic. The level of polymorphic markers was only 23% (very low compared to the other populations we have mapped and suggesting that b42-26 is inbred). A set of 173 of polymorphic markers was added to the entire population of 239 progeny. A framework map with 125 markers was developed. All 125 markers were grouped into 18 linkage groups; no marker was polymorphic for chromosome 6. For three linkage groups, markers did not map because of the large distance between them. Greenhouse screen was completed on 164 accessions, however, results are not conclusive due to large temperature variations during the greenhouse screen. This population was tested again in 2012. Reviewers of our CDFA Pierce's Disease and Glassy-winged Sharpshooter Board proposal suggested we expand the genetic base of resistance to enable breeding more durably resistant cultivars. To begin this process, we selected 52 *Vitis* accessions from Pierce's disease hot spots in Texas, New Mexico, Arizona, Nevada, and California (including fifteen accessions from Mexico). These were evaluated in the greenhouse screen, which identified 22 accessions with high Pierce's disease resistance. Three of these accessions were collected from the trips made over the past 10 years and were collected from Texas and Arizona. Crosses were made in 2012 to develop small breeding populations. Over the next two years over 250 accessions from across the southern USA will be tested.

2012 – 2013

The genetic mapping with accession b40-14, a pure form of *V. arizonica*, was completed in the 07744 population. We tested a total of 606 SSR markers and 224 polymorphic markers were added on the entire set of 122 plants (**Table 6**). A total of 216 markers were polymorphic for the female resistant parent R8918-05. A framework genetic map of R8918-05 was produced with Joinmap (4.0). A total of 212 markers mapped to 19 grape chromosomes with an average distance of 6.3 cM between markers (**Table 7**). The updated map did not have fragmented groups and provided adequate genome coverage when comparisons were made to the previously published integrated *Vitis* genetic maps. A major locus for Pierce's disease resistance was identified on chromosome 14 (**Figure 2**). Pierce's disease resistance from b40-14 (which we have named *PdR1*c) maps in the same general region as *PdR1*a and *PdR1*b between flanking markers VVCh14-77 and VVIN64 and within 1.5 cM. The LOD threshold for the presence of this QTL was 39 and this major locus explained 80% of the phenotypic variation. Using the updated genetic map, we also identified a minor QTL with LOD 2.0 on chromosome 5 that explained 8.3% phenotypic variation for resistance (**Figure 3**). We did not find evidence for any other QTL on the remaining 17 chromosomes. Both QTLs explained a total of 88% phenotypic variation for resistance within the b40-14 background.

A framework genetic map was also developed for 198 seedlings in the 05347 population with 185 markers. A large number of markers showed segregation distortion. We have repeated and completed the greenhouse screen on 199 seedlings that rooted successfully. Thirty-five of the seedlings were tested three times, 77 tested twice, and 87 were tested once. An ANOVA on the 35 genotypes tested in all three trials indicated that only the genotype matters and there were no significant interactions. The updated results were used for the QTL analysis. One-way ANOVA and interval mapping revealed QTLs on chromosome 8, 12, and 14 that explained over 25% phenotypic variation. We are currently refining the maps of these three chromosomes with more markers and establishing the association of markers that are in linkage with the resistance for potential use in marker-assisted screening.

Strong progress was made in accessing the diversity and population structure of the southwestern USA and Mexican *Vitis* accessions, examining 219 accessions of these species. DNA was collected from all those genotypes and six *V. vinifera* accessions were added as outliers. A total of 22 SSR markers were selected for coverage of all 19 grape chromosomes. Three methods: hierarchical clustering (Ward method); principle coordinate analysis (PCA); and a model-based clustering method implemented in the program STRUCTURE, revealed three main groups. **Figure 4** presents the results of PCA with three distinct groups and **Figure 5** presents the groupings revealed with the STRUCTURE program displayed on the geographic location map. Most of the accessions from the Mexican species collections appear to be introgressive hybrids among *V. arizonica, V. berlandieri, V. candicans (V. mustangensis), V. cinerea* var. *tomentosa, V. girdiana,* and *V. monticola*. Strong resistance to Pierce's disease occurs in *V. arizonica/candicans, V. arizonica/girdiana*, and *V. arizonica/monticola* forms.

To determine the inheritance and nature of resistance of the best forms, we made crosses in 2012 to develop breeding lines with four of the most resistant accessions. Small breeding populations were planted in spring 2013. In 2013, we made additional crosses to expand the existing populations as well as used four new Pierce's disease resistant accessions to develop breeding populations (**Table 8**). Seedlings that were generated from 2012 crosses were tested with markers and true-to-cross seedlings were transferred to the field.

2013 – 2014

The genetic map of 04191 was completed in 2012-2013. The 04191population segregates for both major and minor QTL. In order to study the impact of the minor QTL in isolation of *PdR1*, we made two crosses with 04373-02 and 04373-22 and Pinot blanc. The goal is to discard all those plants that carry the *PdR1* 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 were greenhouse screened. Cane lignification index and leaf scorch symptoms indicated 11of the seedlings were as resistant as U0505-01 (moderate to strong resistance) and 17 seedlings were susceptible.

Maternally inherited chloroplast SSR markers were added to the study set of southwestern USA accessions to identify unique resistant maternal haplotypes and enable study of evolutionary and taxonomic relationships. Greenhouse screening was completed for this large collection of *Vitis* species and a manuscript is in progress. Crosses were made with new resistant selections to generate new populations and to expand existing populations.

Greenhouse screening was repeated on the key recombinants from the 07744 population, five new markers developed from the sequence of b43-17 were added to the complete study set, and final analysis before the manuscript is written is underway. We also retested some of the marker data on a new Applied Biosystem setup for the 05347 population, and once all tested markers are added, the final analysis will be carried out.

In December 2013, we first used the PacBio RS II system sequencing to produce long reads with average lengths of 4,200 to 8,500 bp and the longest reads at over 30,000 base pairs. As no amplification is required, the read accuracy is very high and *de novo* assembly of the genome can be performed with up to 99% accuracy. We have isolated four BAC clones that overlap with each other and provide an approximately 500Kb long stretch of genomic region that compares to the PN40024 sequence (**Figure 6**). To date, we completed the sequencing and assembled the four BAC clones. The assembly generated a 604 Kb long sequence without any gaps, covering the entire resistance region, and is 126 Kb more than the corresponding sequence from PN40024, which is 491.2 Kb. The expansion of this resistance region is due to transposable elements (both type I and II). We identified multiple open reading frames of the leucine-rich repeat receptor kinase gene family, which regulate a wide variety of functions in plants including stem cell maintenance, hormone perception, wounding response, and both host and

non-host specific defense. The next step is to fully annotate the sequence, carry out comparative sequence analysis (manuscript in progress), and proceed to promoter isolation and characterization of the resistant genes. The results of this work will feed into the project "Molecular Breeding Support for the Development of Pierce's Disease Resistant Winegrapes" that is the continuation of this work and is funded by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board from 2014-2017.

LAYPERSON SUMMARY

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

We plan to combine these multiple resistance sources in our breeding program to ensure broad and durable Pierce's disease resistance. This project provides the genetic markers critical to the successful classical breeding of Pierce's disease resistant wine, table, and raisin grapes. Identification of markers for *PdR1* has allowed us to reduce the seed-to-seed cycle to two years and produce selections that are Pierce's disease resistant and 97% *V. vinifera*. These markers have also led to the identification of six genetic sequences that may house the Pierce's disease resistance gene, and which are being tested to verify their function. These efforts will help us better understand how these genes function and could also lead to Pierce's disease resistance

Table 1. Parentage and species information for populations and genotypes being used to map Pierce's disease resistance.

Population / Genotype	Species / Parentage
b42-26	*V. arizonica*
b43-17	*V. arizonica/candicans*
D8909-15	*V. rupestris* A. de Serres x *V. arizonica* b42-26
F8909-08 and F8909-17	*V. rupestris* A. de Serres x *V. arizonica/candicans* b43-17
F2-7 and F2-35 (both females)	*V. vinifera* (Carignane x Cabernet Sauvignon)
9621	D8909-15 x F8909-17
0023	F8909-15 x *V. vinifera* B90-116
03300	101-14Mgt (*V. riparia* x *V. rupestris*) x F8909-08
04190	F2-7 x F8909-08
04191	F2-7 x F8909-17
04373	F2-35 x b43-17
07744	R8915-05 x Airen
05347	F2-35 x b42-26

Table 2. 9621 consensus map details.

Chromo-some	Linked Markers	Mapped	Unmapped		Distance (cM)	New Markers
1	18	16	m-VMC8a7, fm-AF378125	2	91.2	8
2	11	10	VMC5g7	1	50.97	0
3	8	8		0	65.87	4
4	15	14	VMC2e10	1	79.95	4
5	17	11	f-VrZag89a, fm-VMC16d4, m-VrZag89b, f-VrZag79a, West-9, VMC4c6	6	46.77	4
6	16	10	f-VMC3f12, m-VMC3a8, fm-VVC7, fm-CF205720, f-VMC2h9	6	75.8	3
7	9	8	fm-VMC16f3	1	71.38	1
8	9	7	f-VMC1b11, f-VMC1e8	2	56.34	2
9	10	10		0	71.05	2
10	9	7	fm-ctg9946, f-vest235	2	30.87	3
11	8	8		0	48.86	4
12	13	12	fm-VMC5c6	1	33.16	4
13	9	9		0	57.29	3
14	30	28	m-VVIQ32, fm-ctg1008359	2	76.83	5
15	4	4		0	17.8	0
16	9	9		0	51.5	2
17	9	9		0	61.13	4
18	15	15		0	105.66	4
19	17	15	fm-VVIM03, m-VMC1a7	2	61.25	3
	236	210		26	1153.68	60

Table 3. Status of Olmo Mexico Collection genotypes (from the Armstrong block) at the USDA National Clonal Germplasm Repository.

Correctly transferred, labeled and verified	b40-14, b40-29, b40-50, b42-11. b42-33, b43-17, b43-42, b43-56, b43-57, b44-11, b44-16, b44-21, b44-44, b45-05, b45-15, b46-01, b46-21, b46-22, b46-48, b47-05
Mix-up corrected from Repository to Armstrong	b41-23 = **b41-47**
Olmo Collection plants not in the Repository	b-42-24, b42-26, b42-34
Misidentified or without a matching standard at Armstrong	b40-13, b40-34, b40-51, b40-59, b41-13, b41-23, b41-47, b42-11, b42-51, b42-55, b43-15, b43-36, b47-33, b47-06
Repository genotypes that no longer exist at Armstrong	b40-61, b44-22, b44-52, b44-53, b45-02, b45-26, b45-45, b45-63, b45-53, b45-28, b45-35, b46-07, b47-06, b47-27, b47-28, b47-32

Table 4. List of new markers that were developed from Pinot Noir genome sequence and were utilized on four different populations.

Name	PN contig id	new marker	amp size	04190	9621	04373	04191
A010	VV78X214158.8	VVCh14-02	170	Y	Y	N	Y
		VVCh14-56			Y		Y
UDV095	VV78X004565.11	VVCh14-09	170	Y		Y	
		VVCh14-10	210	Y	N	Y	N
VMCNg2b7.2	VV78X072246.8	VVCh14-27	193	Y	Y	Y	Y
VMCNg3h8	VV78X190796.4	VVCh14-28	167	Y	Y	Y	Y
		VVCh14-29	200	Y	Y	N	Y
		VVCh14-30	206	Y	Y	N	Y
		VVCh14-70	193	Y	Y	N	Y

Table 5. Key recombinants from the 9621 (*PdR1a*) and 04190 (*PdR1b*) populations. The genotypes in bold red font are key recombinants with a recombination event between the marker and the resistance locus. "0" indicates a susceptible allele and "1" indicates a resistant allele.

Genotypes with *PdR1a* background	A0101	VVCh14-56	*PdR1a*	VVCh14-70	VVCh14-29	VMCNg2b7.2
-416	0	0	0	0	1	1
-426	0	0	0	0	1	1
-470	0	0	0	0	1	1
-8	0	0	0	1	1	1
-194	0	0	0	1	1	1
-554	0	0	0	1	1	1
-629	0	0	0	1	1	1
-28	0	0	1	1	1	1
-38	0	0	1	1	1	1
-15	1	1	1	1	0	0
-23	1	1	1	1	0	0

Genotypes with *PdR1b* background	VVCh14-10	VVCh14-02	PdR1b	VVCh14-70	VVCh14-30	VVCh14-27
06314-24	0	0	0	0	1	1
06328-05	0	0	0	0	1	1
04190-026	0	0	0	0	1	1
06317-50	1	1	1	1	0	0
04190-383	1	1	1	1	0	0
06317-50	1	1	1	1	0	0
04190-381	1	1	1	0	0	0
04190-320	1	1	0	0	0	0
04190-065	1	1	0	0	0	0
06315-49	1	0	0	0	0	0
06326-23	1	0	0	0	0	-
06711A-60	0	0	?	1	1	1

Table 6. List of markers tested and completed for the 07744 population derived from the b40-14 background.

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

Table 7. Salient features of framework map of R8918-05, a Pierce's disease resistant selection used as the maternal parent in the 07744 population.

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

Table 8. Crosses made in 2013 to develop genetic maps in new accessions from southern USA and Mexico germplasm. Crosses 08-319-29 and 08326-61 are female flowered selfed progeny of Zinfandel and Cabernet franc, respectively. F2-35 is also female and a cross of Cabernet Sauvignon x Carignane.

Resistant source / new or existing	Geographic origin - appearance phenotype	Pure *V. vinifera* types used in 2013 crosses	Estimated # of seeds
ANU5	Littlefield, AZ	Alicante Bouschet	250
expands existing	*V. girdiana*		
b40-29	Chihuahua, MX	F2-35	1,250
expands existing	*V. arizonica*	08319-29	2,000
b41-13 new	Ciudad Mante, MX *V. arizonica-mustangensis-champinii*	F2-35	750
b43-57 new	Guadalupe, MX *V. arizonica-mustangensis-champinii*	Malaga Rosada Rosa Minna	1,000 900
b46-43 expands existing	Big Bend, TX *V. arizonica glabra-monticola*	08326-61	850
b47-32 expands existing	Big Bend, TX *V. arizonica glabra-monticola*	F2-35 08326-61	1,950 70

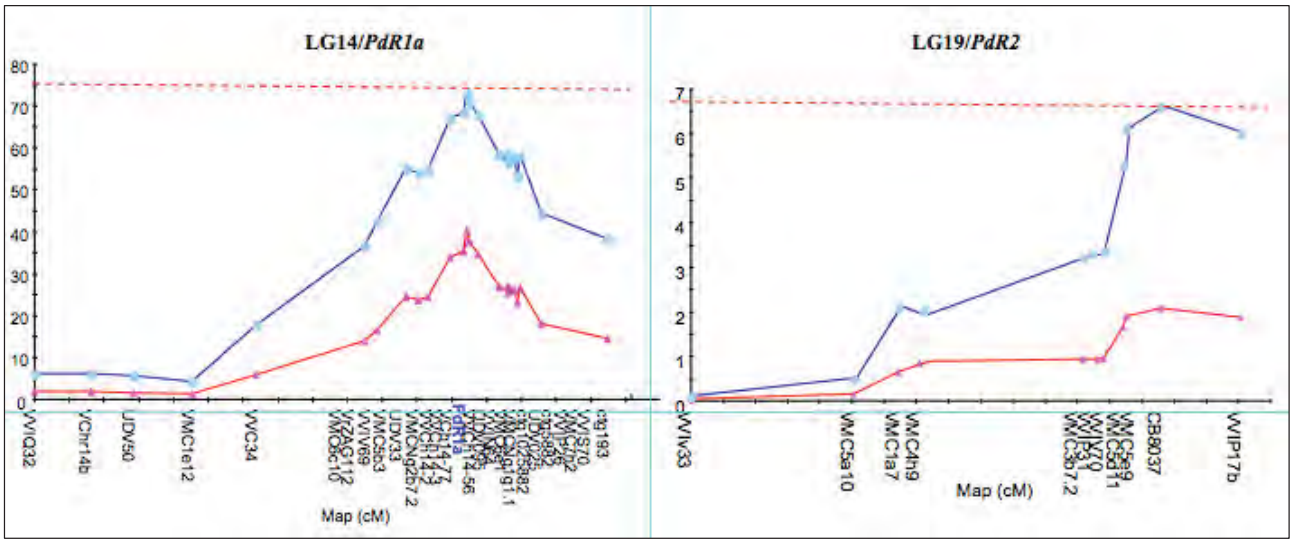

Figure 1. Interval mapping analysis of the 04191 population. We verified the *PdR1*a locus on LG 14 and identified a minor QTL (*PdR2*) on LG 19. Blue line display the phenotypic variation explained by the loci.

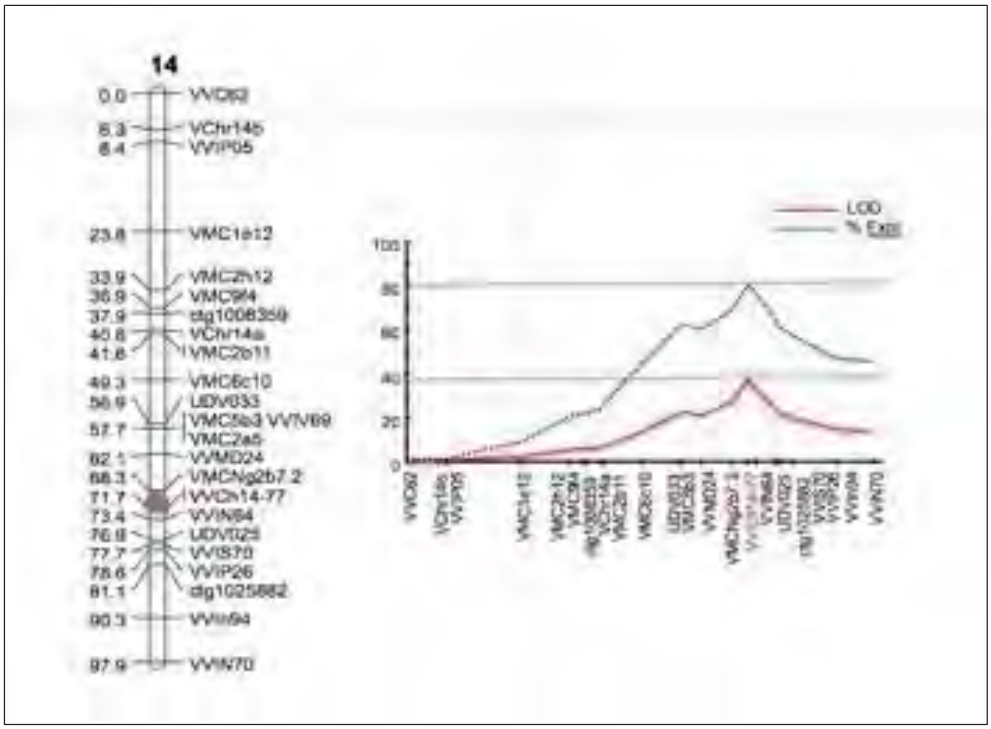

Figure 2. Interval mapping of *PdR1* indicating a peak at LOD 34.0 with a 95% confidence interval. The X-axis indicates the position of the markers; LOD values are plotted on the Y-axis.

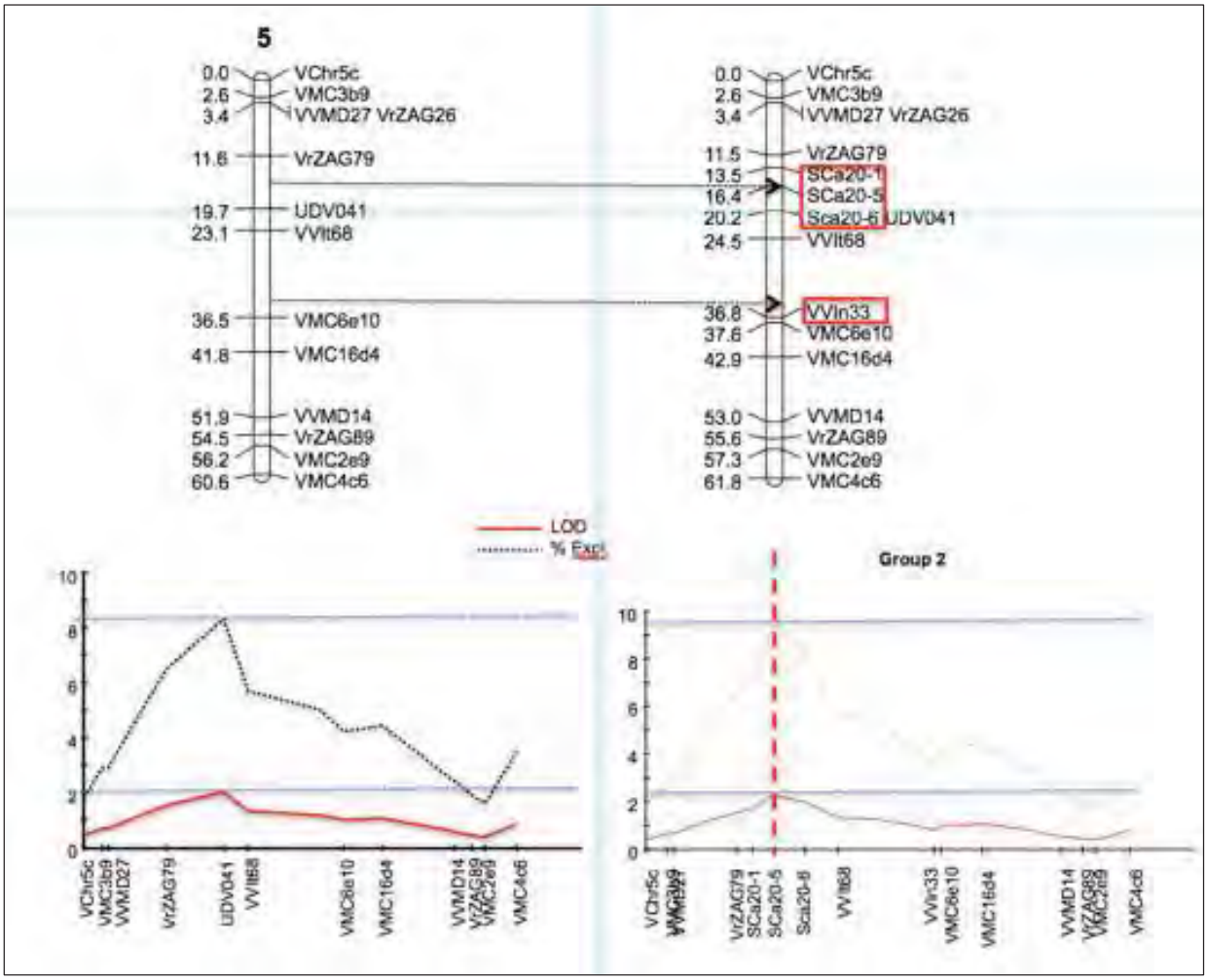

Figure 3. Interval mapping of QTL on chromosome 5 from the b40-14 background. More markers were developed using the PN40024 sequence. The X-axis indicates the position of the markers; LOD values are plotted on the Y-axis.

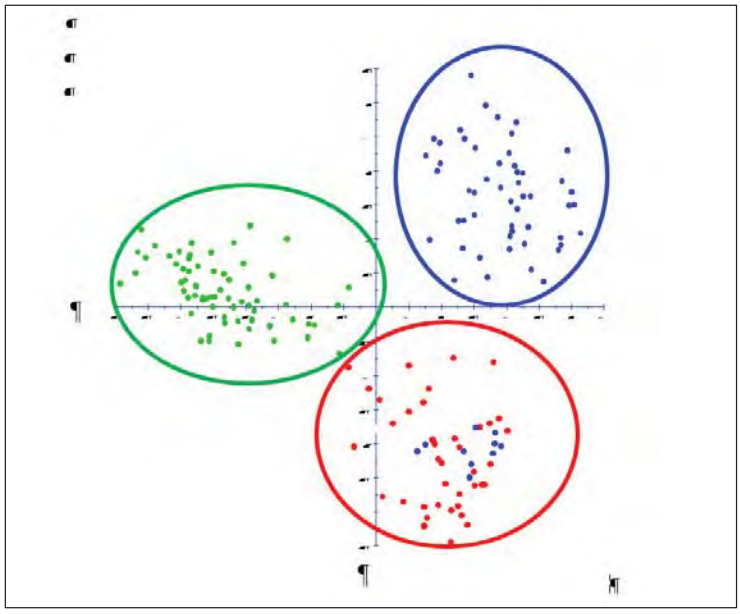

Figure 4. Principle Coordinate Analysis constructed with genotypic data from 22 SSR markers on 159 accessions using DARWIN software. Blue represents the *V. cinerea*-like accessions; red the *V. aestivalis*-like accessions; and green the *V. arizonica*-like accessions. The axis 1 and 2 presents 9.13 and 5.74 percent of the variation, respectively.

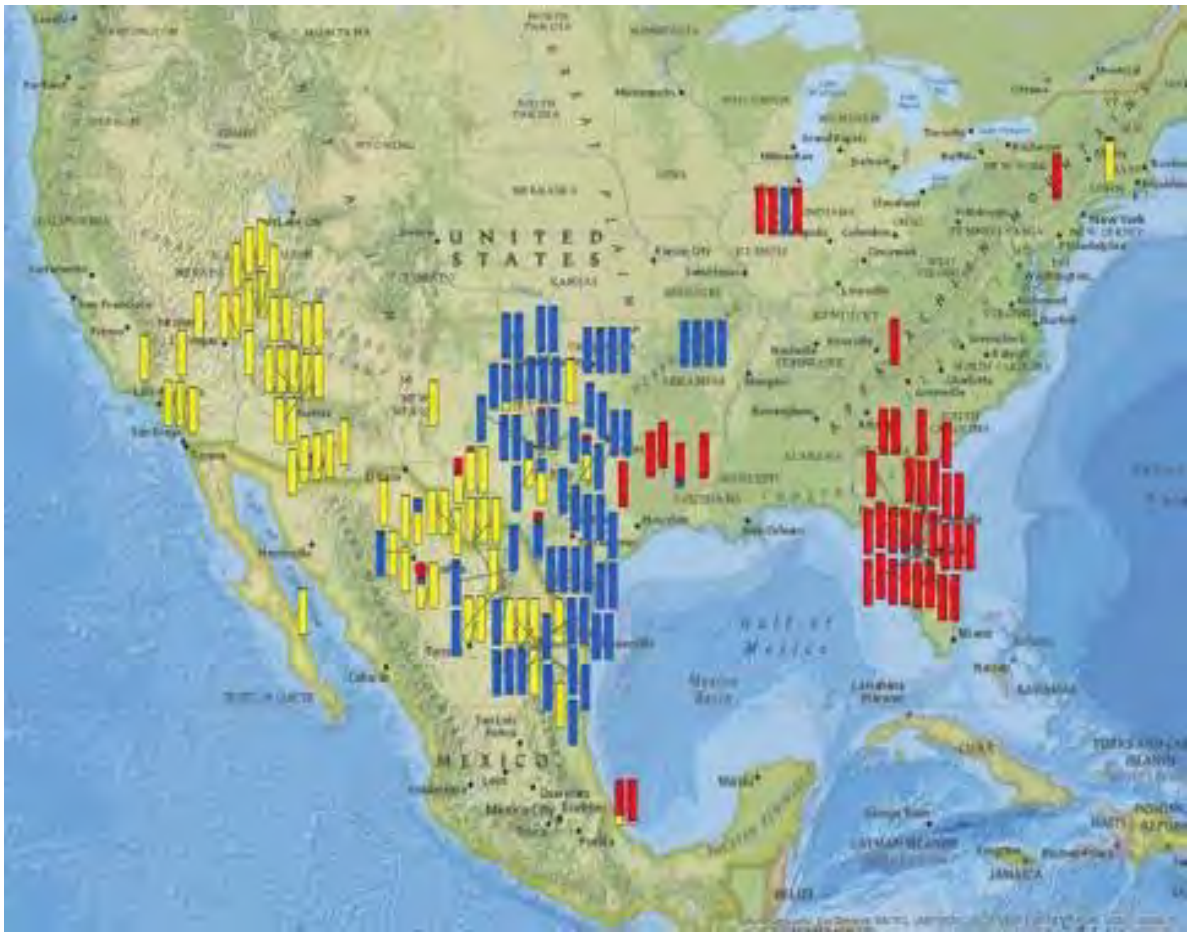

Figure 5. Grouping of accessions revealed by clustering program STRUCTURE. Representation of genetic composition of species for each accession is represented as a bar chart. Yellow color represents *V. arizonica*-like accessions, blue is for *V. cinerea*-like accessions, and red is for *V. aestivalis*-like accessions. It is noted that *V. arizonica* is a complex mix of different species and further analysis only with that group separates these species into different clades.

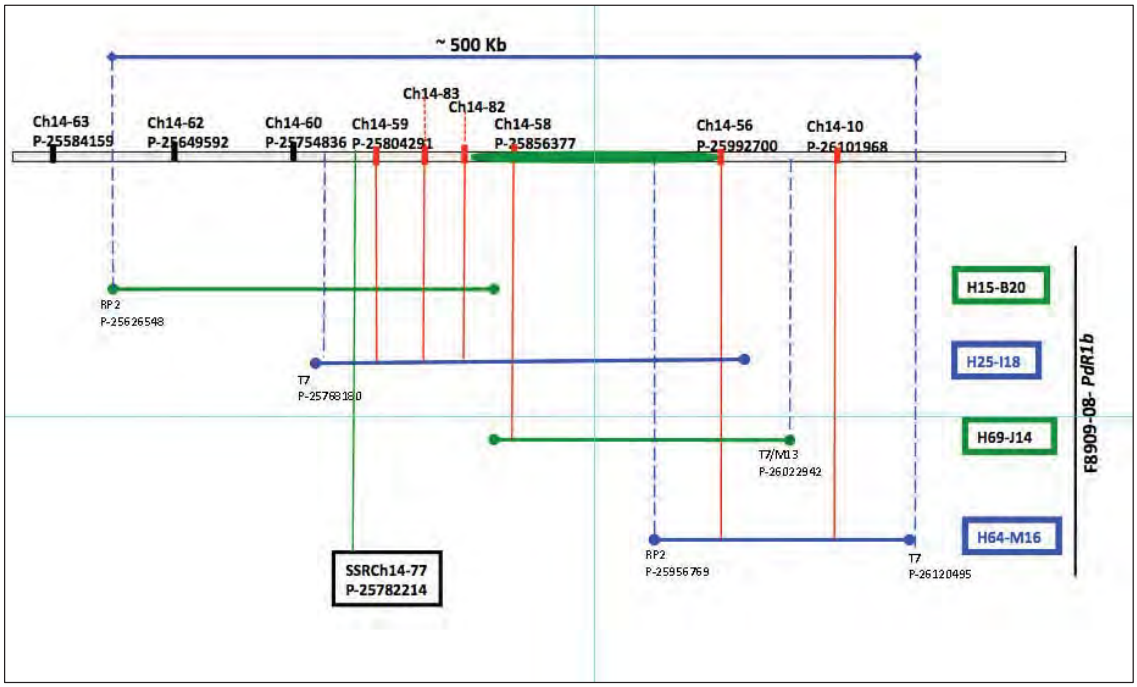

Figure 6. The position of four BAC clones relative to each other and to the PN40024 sequence. Orange lines are markers that were used to screen the BAC library. All four clones represent the *PdR1*b haplotype.

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

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Reporting Period: The results reported here are from work conducted July 2012 to June 2014.

ABSTRACT

Pierce's disease is a deadly disease of grapevines caused by the bacterial pathogen *Xylella fastidiosa* (*Xf*). Resistance to Pierce's disease is present in North American *Vitis* species. Resistance from *V. arizonica* accession b43-17 has been mapped as a single major locus (*PdR1*) onto linkage group 14. The physical mapping of the *PdR1b* allele allowed the identification of potential candidate resistance gene(s). We cloned candidate genes *PdR1b.1,2,4,5* and *6* and generated five constructs that were used to transform leaf discs of tobacco and embryogenic callus of *V. vinifera* Chardonnay and Thompson Seedless and *V. rupestris* St. George via *Agrobacterium tumefaciens*. Tobacco and transgenic plants of Chardonnay carrying the candidate genes under the control of 35S CaMV promoter were acclimated for testing against *Xf* in the greenhouse. Nine to ten independent lines of each gene were pinprick inoculated in two basal nodes with 10 ul of 108 cfu/ml of *Xf* Beringer strain. Symptoms based on leaf scorch and cane maturation index (CMI) were scored three months after inoculation. Transgenic tobacco plants exhibited promising results at symptom level with candidate genes *PdR1b.1* and *6*. Some transgenic Chardonnay also had phenotype scores comparable to those of resistant biocontrols, however *Xf* counts evaluated by ELISA showed high concentrations in most transgenic lines. Chardonnay PdR1b5-7a showed the lowest CMI and bacteria concentrations among the transgenics, although not as low as the resistant biocontrols. Transgenic plants of Chardonnay-PdR1b2, Thompson Seedless and St. George will be tested next. Some lines transformed with *PdR1b.6* displayed a dwarf phenotype indicating that the constitutive expression of this gene is affecting normal growth. Meristematic bulks of Thompson Seedless, Chardonnay, and St. George have been produced to accelerate genetic transformation via organogenesis.

LAYPERSON SUMMARY

We maintain and characterize many populations while breeding Pierce's disease resistant winegrapes, some of which have been used to develop genetic maps. These maps were used to identify genetic markers that are tightly linked with Pierce's disease resistance, and 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 candidate genes responsible for Pierce's disease resistance. Comparisons with plant genomes indicated that multiple tandem repeats of the disease resistance gene family Receptor-like proteins with leucine rich repeats (LRR) domains were present in the resistance region. This category of genes is involved in the recognition of microbes and in the initiation of defense responses (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 Chardonnay and Thompson Seedless and the rootstock St. George for genetic transformation to verify candidate Pierce's disease resistance gene function. *PdR1b.1,2,4,5* and *6* were used in transformation of tobacco and grape. Transgenic tobacco plants were tested against *Xylella fastidiosa* (*Xf*) in the greenhouse and promising results were obtained with *PdR1b.1* and *6* candidate genes. Transgenic grape plants have been acclimated to greenhouse conditions and *Xf* inoculations have been initiated. Screening of two sets of plants of Chardonnay, comprising a total of 9-10 lines for each gene, was completed in February and July of 2014. *Xf* counts showed high concentrations in most transgenic lines. PdR1b.5-7a showed the least severe disease symptoms and bacteria concentrations among the transgenics, although not as low as the resistant biocontrols. Testing of transformed Chardonnay-*PdR1b2*, Thompson Seedless and St. George is scheduled next. Although the current transgenic grape plants were produced using the traditional procedure, we are also testing another

technique to speed the development of transgenic tissue from meristematic bulks that will allow *PdR1* gene candidates to be tested faster.

This research is focused on demonstrating whether Pierce's disease resistance genes developed from genetic and physical mapping efforts function when transformed into susceptible host plants. These transformations are underway in tobacco (an easily used model system) and susceptible grape (Chardonnay, Thompson Seedless, and St. George). These studies will lay the foundation to understanding how these resistance genes work, and may provide a tool to genetically engineer grape resistance genes into susceptible grapevines.

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 North American *Vitis* species into elite *V. vinifera* wine and table grapes. Another approach is "cisgenesis" – the transformation of elite *V. vinifera* varieties with grape resistance genes and their native promoters, cloned from disease resistant American *Vitis* species. The cisgenic approach may have a more limited impact on the genome of the elite *V. vinifera* parent since single genes from the *Vitis* species genome would be added to the elite parent, thus limiting the impact on its fruit and wine quality while making it Pierce's disease resistant. The cisgene approach in grapes could be considered to be 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 comparisons 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 five candidate resistance genes.

OBJECTIVES

1. Cloning, structural analysis, and gene annotation via comparison of the *PdR1b* locus to the susceptible Pinot Noir genome sequence using the assembled sequence of the BAC clone H64J14.
2. Expression studies of candidate genes (previously reported on).
3. Complementation tests of candidate gene(s) to test their function using *Agrobacterium*-mediated transformation of the susceptible *Vitis* cultivars Chardonnay, Thompson Seedless, and the rootstock St. George, and transformation of tobacco.

RESULTS AND DISCUSSION

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

A refined genetic map of chromosome 14, which contains the Pierce's disease resistance locus, was generated from three grape mapping populations derived from *V. arizonica/candicans* b43-17. The resistance locus segregates as a single dominant gene and mapped as *PdR1a* in the F1 selection 8909-17 and as *PdR1b* in its sibling F8909-08. Clone H69J14 from a b43-17 BAC library, containing both markers flanking the *PdR1b* resistance locus, was sequenced using 454 and paired-end Sanger sequencing on two different libraries (fosmid and shotgun). The assembly of the sequence data generated 10 contigs, with a portion of the sequence remaining unassembled. Analysis of assembled and unassembled sequences revealed the presence of four candidate genes, *PdR1b.1 – 4*, which appear to code for receptor-like proteins, a class of resistance proteins. Earlier in 2014, we employed a PAC BIO RSII sequencing approach to sequence H69J14 and three other overlapping BAC clones. The assembled sequence data generated a 604Kb-long fragment without any gaps (**Figure 1**).

In comparison to the susceptible sequence, the resistant line has 126 Kb more sequence than susceptible PN40024 corresponding sequence that is 491.2 Kb. In the next step, we will fully annotate the sequence, carry out comparative sequence analysis (manuscript in progress), and proceed to promoter isolation and characterization of the resistant genes. The results of this work will feed into the project "Molecular-Functional Approach to Facilitate the Discovery of Novel *Xylella fastidiosa* Resistance Gene(s) and Markers in Native American Species" that is a continuation of this work and is funded by the CDFA Pierce's Disease and Glassy-winged Sharpshooter

Board from 2014 - 2017. We have amplified and confirmed the sequences of five candidate genes: *PdR1b.1-2-4-5-6. PdR1b.1* (P1) is the largest gene (3198 bp), and shares a high degree of homology with *PdR1b.2* (P2), *4* (P4) and *5* (P5). *PdR1b.6* (P6) is significantly different from the other four. It has a kinase domain, which suggests it might be involved in Pierce's disease resistance in combination with P1 or one of the other candidates.

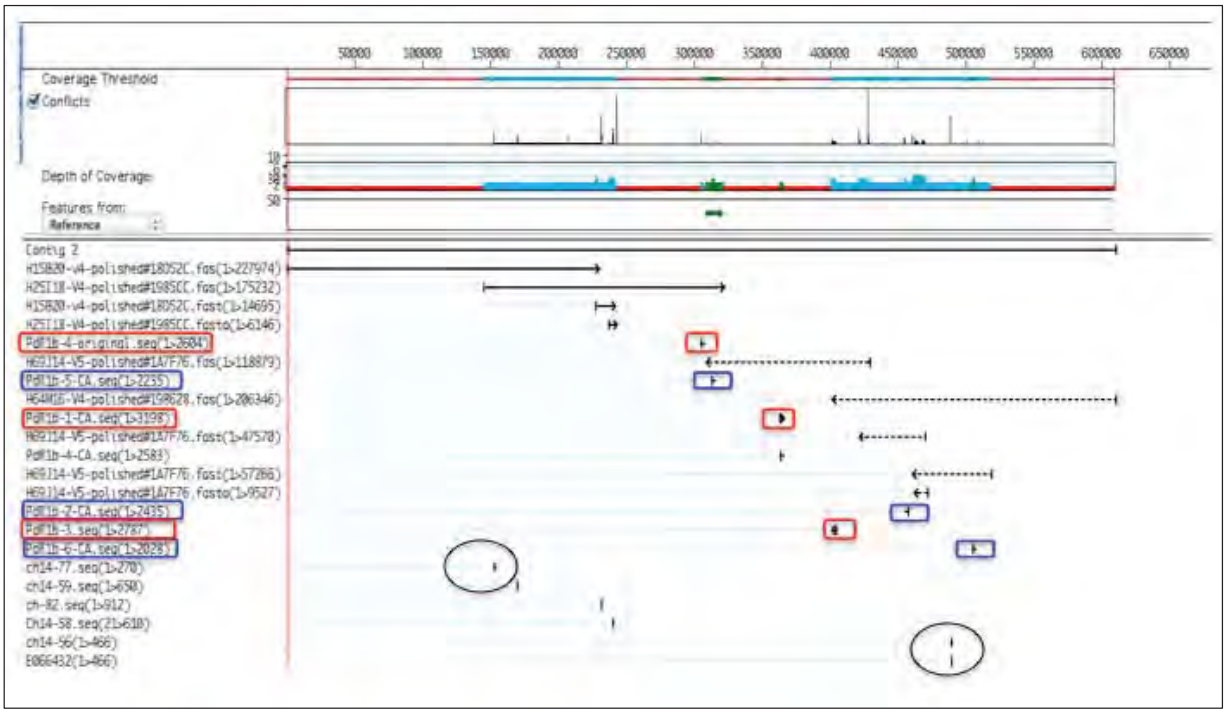

Figure 1. Assembled sequence of four BAC clones including H69J14 for 604Kb region of the resistant line. Blue and red boxes are the respective identified Open Reading Frames of candidate resistant genes. Circles show the placement of markers that flank the resistant locus.

Objective 3. Complementation tests of candidate gene(s) to test their function using *Agrobacterium*-mediated transformation of the susceptible *Vitis* cultivars Chardonnay, Thompson Seedless, and the rootstock St. George, and transformation of tobacco.

Once the gene constructs are completed, they must be tested to see if they maintain their function and provide resistance. This is done by inserting the genes into a susceptible plant and testing to see if the insertion results in resistant plants. Currently, the most widely used method for the production of transgenic/cisgenic grapes is based on *Agrobacterium* transformation followed by regeneration of plants from embryogenic callus. We have established cultures of pre-embryogenic callus derived from anthers of *V. vinifera* Thompson Seedless (TS) and Chardonnay (CH) and the rootstock *V. rupestris* St. George (SG) that have been used for transformation (Agüero et al., 2006).

PdR1b candidate genes were amplified using Phusion high-fidelity DNA polymerase (Finnzymes), cloned into pGEM-T easy vector (Promega) and sequenced at the UC Davis Sequencing Facility. After sequence verification, genes were sub-cloned into binary vector pCambia 1303 (www.cambia.org) containing the 35S cauliflower mosaic virus promoter, the nopaline synthase terminator, and an *hptii*-selectable marker gene. P1 was also sub-cloned into binary vector pDU99.2215 containing an *ntpii*-selectable marker gene. The resulting plasmids were transformed into disarmed *A. tumefaciens* EHA105 pCH32 by electroporation and used for transformation of CH, TS, and SG.

Pre-embryogenic calli of TS, CH, and SG transformed with the five candidate genes were selected in medium with antibiotics, then sub-cultured to germination medium for plant regeneration. The presence of the genes was checked in callus cultures through PCR and tested again in plants transferred to the greenhouse. For each gene, we attempted to produce, at least 10 independent lines that will be subsequently propagated clonally to six plants per line and tested under greenhouse conditions. **Table 1** shows the number of independent lines that have been obtained at present. Genomic DNA was isolated from these plants with DNeasy Plant Mini Kit (Qiagen). A primer that binds the caMV 35S promoter and a primer that binds the coding region of each *PdR1b* candidate

were used in combination for PCR amplification to verify the presence of the transgene in the plants transferred to the greenhouse (**Figure 2**).

Two sets of screenings were completed in February 2014 and July 2014. A third screening is underway that is scheduled to end in November 2014. Each line was multiplied through green cuttings to produce six replicates. They were cut back to two buds and re-grown. Inoculations via the pinprick procedure were performed eight weeks after the grapevines had been cut back, when all plants had reached a height of about 1m. Plants were inoculated below and above the node within 5 to 10 cm from the base of the main shoot, using 10 µl of the Beringer strain (OD600=0.25) each time. Pierce's disease resistance was analyzed through phenotype scoring and ELISA. Symptoms of Pierce's disease were evaluated using a 0-5 score for leaf scorch-leaf loss (LS-LL) and a 0-6 score for cane maturation index (CMI). For ELISA, plants were sampled 12 weeks post-inoculation by taking 0.5 g sections of stem tissue from 30 cm above the point of inoculation (Krivanek and Walker, 2005; Krivanek et al., 2005).

The first screening included lines of Chardonnay transformed with P1, P4, P5 and P6, with five independent lines per gene. All transgenic lines tested in this experiment displayed disease symptoms with different degrees of intensity. Line CH P5-7a had the lowest bacteria concentrations among the transgenics, although not as low as the resistant biocontrols. It also exhibited low cane maturation index (CMI) and leaf scorching-leaf loss scores (LS-LL; **Table 2**). The second round of screenings started in March 2014 and ended in July 2014. It included the remaining lines of CH P1, P4, P5 and P6, plus five lines of TS P6 and one line of SG P6. Symptom scoring is shown in **Table 3**. This was a severe screen, with high CMI and LS-LL scores, not only in transgenics but biocontrols as well. ELISA tests determined that bacteria concentrations were high in all transgenic lines. Both SG P6-20 and SG-untransformed grouped with the resistant genotypes. A third round of screenings started in July to test of CH- P2 and the rest of the TS and SG lines. Transgene expression was confirmed in randomly picked plants through qPCR analysis. Several lines of CH transformed with P6 exhibited an altered phenotype characterized by stunted growth (**Figure 3**). Gene expression analysis through qPCR showed that lines with higher expression levels had lower main shoot growth (**Figure 4**). Blast analysis of P6 showed a high degree of homology with lysine motif receptor-like kinases (LYK), which have been implicated in the recognition of bacterial peptidoglycans (Gust et al., 2012).

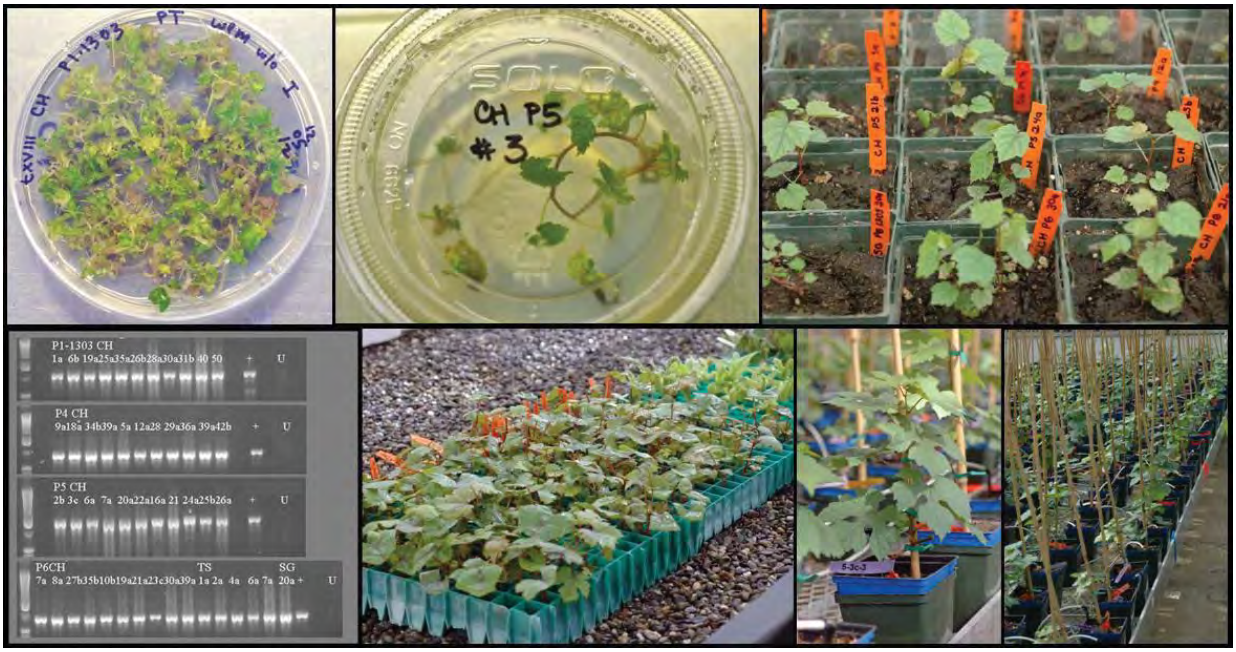

Figure 2. Clockwise from top left: Chardonnay (CH) embryos growing in germination medium, regenerated plantlets growing *in vitro, in vitro* plants transferred to substrate in greenhouse, transgene detection through PCR, green cuttings in mist bed, and plants after being cut back prior to inoculation.

Figure 3. Dwarf phenotype of P6 transgenic (left) vs. phenotype of untransformed Chardonnay (right).

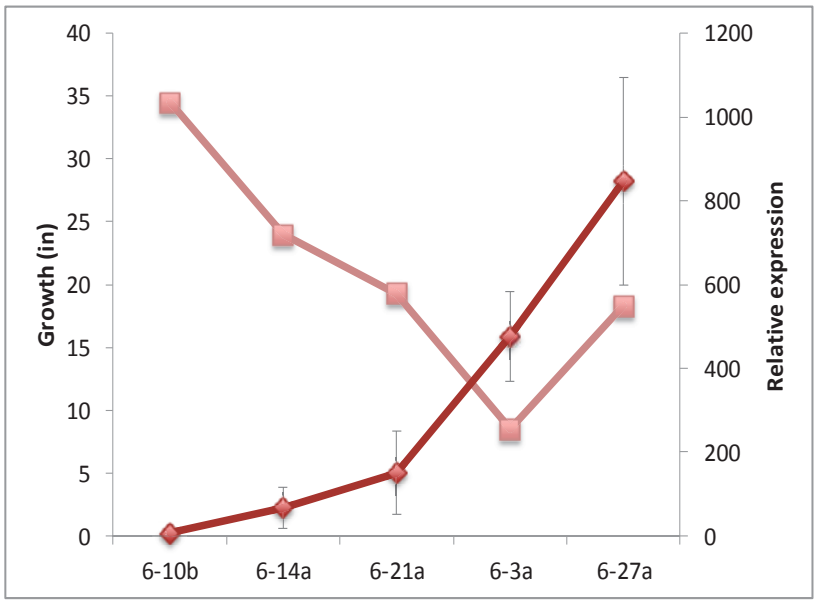

Figure 4. Expression levels of P6 (red) and growth of main shoot (pink) in five independent lines of Chardonnay. Expression levels were analyzed by qPCR, relative to un-transformed Chardonnay.

Table 1. Number of independent lines produced until June 2014. Lines in the greenhouse are shown in parentheses. CH = Chardonnay; TS = Thompson Seedless; SG = St. George.

	CH	TS	SG
P1 pDU 99.2215	4 (3)	0	0
P1 pCambia1303	15 (10)	5 (2)	13 (10)
P2 pCambia1303	6 (2)	0	0
P4 pCambia1303	20 (10)	7 (2)	4 (4)
P5 pCambia1303	13 (10)	5 (2)	2 (2)
P6 pCambia1303	18 (10)	8 (7)	2 (1)

Table 2. Greenhouse screen results for Chardonnay transformed with P1, P4, P5 and P6. U0505-01 is the resistant biocontrol. U0505-35, U0505-22, b43-17, Roucaneuf, and Blanc du Bois are additional biocontrols. CH uninoculated is the negative control

Genotype	Reps	Geometric mean (cfu/ml)	Mean (ln cfu/ml)	Std Error (ln cfu/ml)	CMI Mean	CMI Std Err	LS-LL Mean	LS-LL Std Err
CH uninoc	6	9,897	9.2	0.0	0.2	0.2	0.9	0.2
b43-17	6	10,232	9.2	0.0	1.8	0.2	2.7	0.4
Roucaneuf	6	16,592	9.7	0.5	0.0	0.0	0.7	0.2
U0505-01	6	27,356	10.2	0.5	0.8	0.7	1.7	0.3
Blanc du Bois	5	43,478	10.7	0.8	2.2	0.4	2.4	0.2
U0505-35	6	107,291	11.6	0.8	0.2	0.2	2.2	0.3
CH P5-7a	6	705,527	13.5	1.0	0.3	0.2	2.2	0.5
CH P1-19a	6	1,518,601	14.2	0.5	1.3	0.6	2.7	0.3
CH P4-9a	5	1,559,694	14.3	0.4	1.0	0.3	2.4	0.2
CH P6-14a	6	1,764,363	14.4	0.4	1.5	0.5	2.2	0.3
CH P1U-20a	6	1,794,075	14.4	0.4	1.1	0.5	2.7	0.3
CH P4-39a	4	2,032,953	14.5	0.4	1.6	0.9	2.8	0.3
CH P5-6a	6	2,303,638	14.7	0.4	2.0	0.4	2.7	0.2
CH P1U-10c	6	2,421,748	14.7	0.5	2.0	0.7	2.7	0.3
CH P5-2b	6	2,421,748	14.7	0.5	1.8	0.6	2.2	0.3

Table 3. Greenhouse screen results for Chardonnay transformed with P1, P4, P5, and P6, and Thompson Seedless and St. George transformed with P6. U0505-01 is the resistant biocontrol. U0505-35, U0505-22, b43-17, and Roucaneuf are additional biocontrols. CH uninoculated is the negative control.

Genotype	Reps	Geometric mean (cfu/ml)	Mean (ln cfu/ml)	Std Error (ln cfu/ml)	CMI Mean	CMI Std Err	LS-LL Mean	LS-LL Std Err
CH uninoc	6	10,757	9.3	0.1	0,2	0,2	3,8	0,5
b43-17	6	40,135	10.6	0.4	3,3	0,2	1,5	0,4
SG P6-20	6	393,682	12.9	0.6	4,8	0,2	4,2	0,4
U0505-01	6	638,387	13.4	0.7	1,3	0,3	3,0	0,0
SG untrans	6	984,609	13.8	0.3	3,8	0,5	3,5	0,4
U0505-35	6	2,303,638	14.7	0.4	2,7	0,6	3,2	0,2
Roucaneuf	6	5,300,438	15.5	0.1	4,3	0,3	5,0	0,0
CH P4-12a	6	5,666,034	15.6	0.2	3,8	0,2	4,0	0,4
CH P5-16a	6	5,761,452	15.6	0.1	3,7	0,3	4,2	0,4
U0505-22	6	5,761,452	15.6	0.1	4,2	0,2	3,8	0,3
CH P5-25b	6	5,956,538	15.6	0.1	3,3	0,3	4,0	0,3
CH P1-28a	6	6,158,230	15.6	0.0	3,7	0,2	4,3	0,3
CH P1-40	6	6,261,936	15.7	0.1	3,7	0,2	3,8	0,3
CH P4-36a	6	6,367,389	15.7	0.0	4,2	0,4	4,5	0,2
CH P6-19a	6	6,473,969	15.7	0.0	4,0	0,0	3,8	0,4
CH untrans-1	6	6,582,993	15.7	0.0	3,0	0,4	3,8	0,3
CH P1-26b	5	6,582,993	15.7	0.0	4,0	0,0	3,8	0,2
CH P1-30a	6	6,582,993	15.7	0.0	3,7	0,3	4,0	0,3
CH P4-28a	6	6,582,993	15.7	0.0	3,3	0,3	4,0	0,3
CH P4-42b	6	6,582,993	15.7	0.0	3,5	0,2	4,2	0,2
CH P4-5a	6	6,582,993	15.7	0.0	3,7	0,3	4,0	0,4
CH P5-21b	6	6,582,993	15.7	0.0	3,3	0,2	4,2	0,2
CH P5-24a	6	6,582,993	15.7	0.0	3,3	0,5	4,5	0,2
CH P6-30a	6	6,582,993	15.7	0.0	4,0	0,0	4,2	0,3
CH P6-39a	6	6,582,993	15.7	0.0	3,7	0,3	4,0	0,0
CH untrans-0	6	6,582,993	15.7	0.0	3,8	0,2	4,3	0,2
TS untrans	6	6,582,993	15.7	0.0	5,7	0,2	5,0	0,0
TS P6-1a	6	6,582,993	15.7	0.0	5,0	0,4	5,0	0,0
TS P6-2a	6	6,582,993	15.7	0.0	5,7	0,2	5,0	0,0
TS P6-4a	6	6,582,993	15.7	0.0	5,8	0,2	5,0	0,0
TS P6-6a	6	6,582,993	15.7	0.0	6,0	0,0	5,0	0,0
TS P6-7a	6	6,582,993	15.7	0.0	5,8	0,2	5,0	0,0

Tobacco transformation.
To speed the functional analysis, MSc student Carolina Bistue also transformed the tobacco variety SR1, which was recently demonstrated to be a susceptible host for *Xf* and is much easier and quicker to transform and test (Francis et al., 2008). Transgenic tobacco plants carrying each candidate gene (9-10 independent lines per gene) were produced at the UC Davis Transformation Facility and multiplied *in vitro* in our lab. No significant differences were observed in stem *Xf* counts between untransformed controls and transformed plants 12 weeks post inoculation (**Figure 5**). However, candidate genes P1 and P6 displayed significantly lesser symptoms compared to the untransformed controls (**Figure 6**). Expression analysis by real-time PCR confirmed expression of both genes.

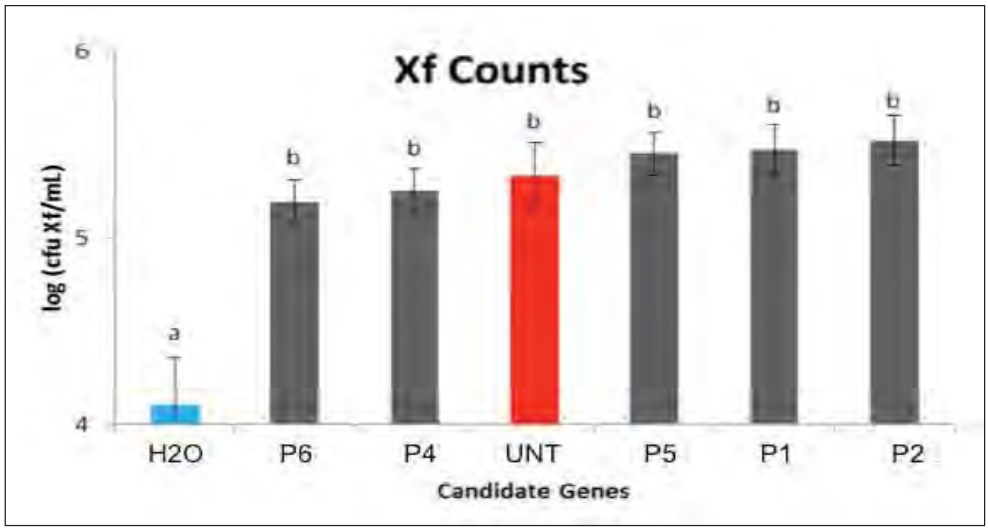

Figure 5. ELISA results for transformed candidate genes as well as negative controls (H2O) and positive controls (UNT). Samples were stem sections collected approx. 50 cm above the POI.

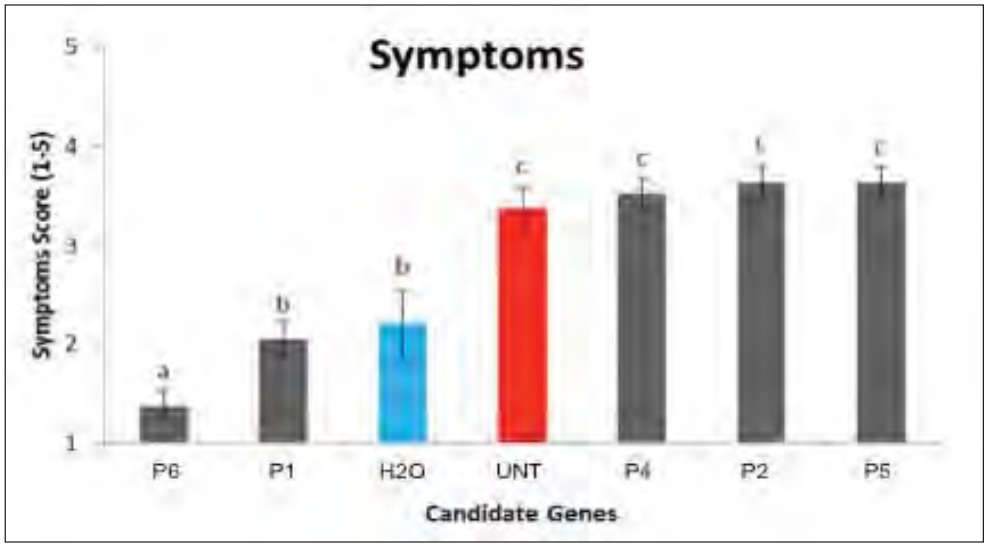

Figure 6. Symptom results for transformed candidate genes as well as negative controls (H2O) and positive controls (UNT).

Genetic transformation via organogenesis.
Inoculation with *A. tumefaciens* of meristematic bulks (MB) is being tested as an alternative transformation technique via organogenesis to reduce the time needed to produce transgenic grapes (Mezzetti et al., 2002). In our lab, transgenic plants of Thompson Seedless expressing GFP were produced in three months using MB and kanamycin as the selective agent. Based on these results, Thompson Seedless MB slices were inoculated with *A. tumefaciens* carrying P5 in pCAMBIA 1303 using three initial levels of hygromicin: 5, 10, and 15 ug/ml.

Since no regeneration was produced at any of the concentrations tested, experiments assaying 0 ug/ml in the first subculture after inoculation followed by 2.5 ug/ml hygromicin were initiated. Two out of 50 initial MB regenerated at this lower concentration, but efforts to establish regenerated plants were unsuccessful.

The partial success obtained with the use of hygromicin and the production of MB of CH and SG prompted PhD student Xiaoqing Xie to test different hormone ratios to adapt the process to these cultivars and study the use of different antibiotics. She has developed protocols to produce MB of TS, CH, SG, and 101-14 Mgt (**Figure 7**) and has transformed them with *A. tumefaciens* carrying plasmids pCambia 1303 and pCambia 2303 to compare the use of hygromicin and kanamycin as selectable markers (**Figure 8**). Regeneration efficiency has been greatly improved by delaying selection 1 or 2 weeks (**Table 4**), although this might increase the risk of producing chimeric plants, which will be checked through the *gus* reporter gene.

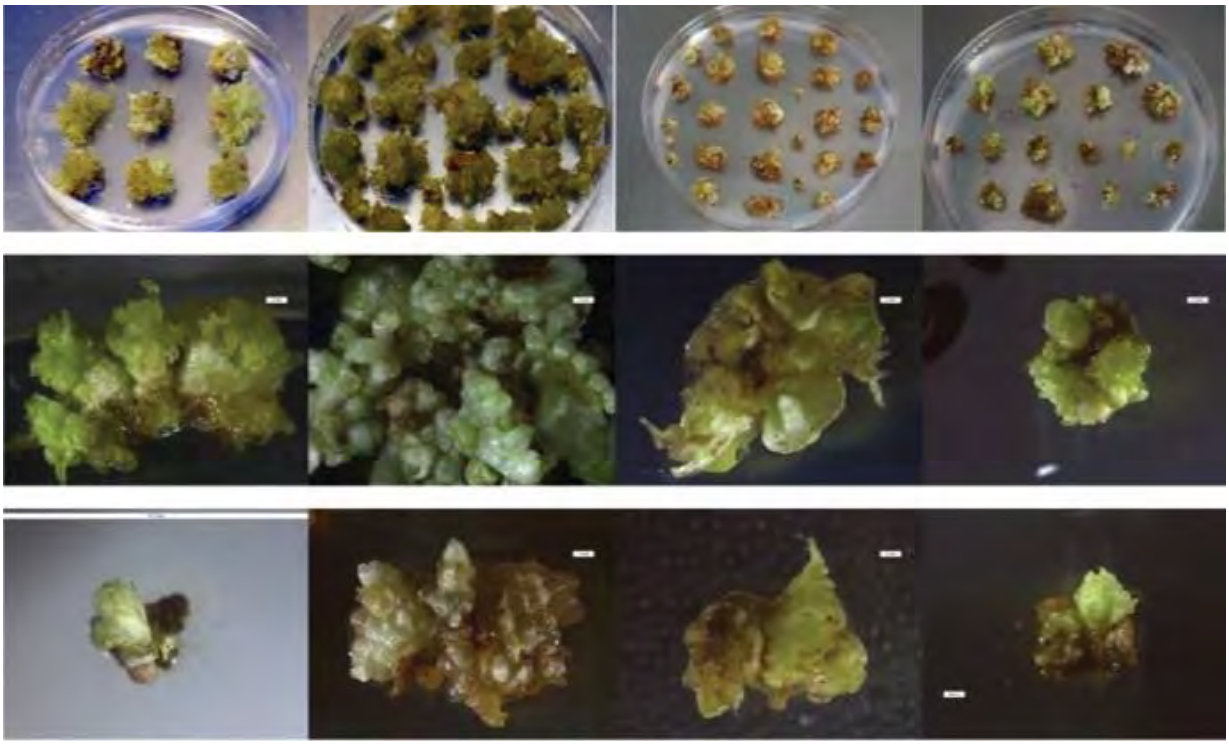

Figure 7. Genetic transformation of CH, TS, SG and 101-14 via organogenesis. Row 1, Meristematic Bulk (MB) induction; row 2, MB before transformation; row 3, shoot regeneration from transgenic meristematic slices; column 1, CH; column 2, TS; column 3, SG; column 4, 101-14 Mgt.

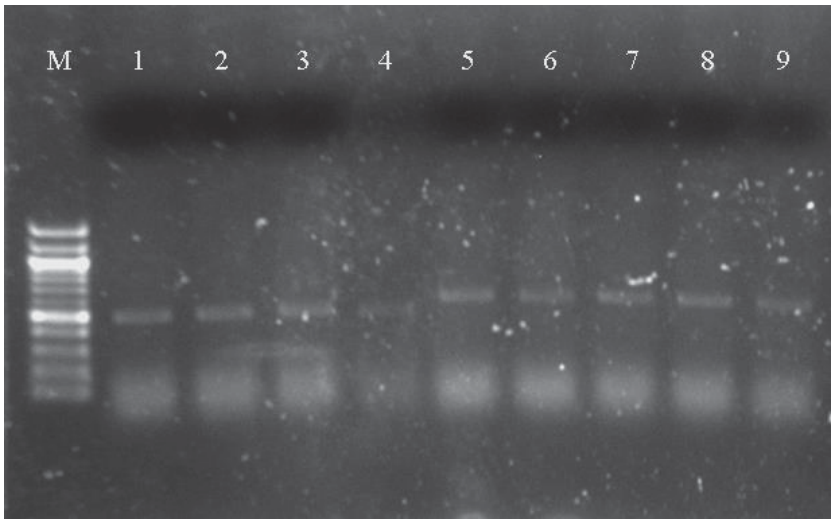

Figure 8. PCR analysis of tissue from regenerated shoots from Meristematic Bulks inoculated with *A. tumefaciens* carrying pCambia 2301-kan (lanes 1- 4) and pCambia 1303-hygr (lanes 5-9).

Table 4. Percentage survival rate after one month of selection in kanamycin (KAN) or hygromicin (HYG), calculated relative to the number of treated explants. Each value represents the mean ± SE of three different experiments

Weeks before selection	Antibiotics (µg/ml)	Genotype			
		TS	CH	SG	101-14 Mgt
0 W	KAN 100	42.34±2.2	51.67±18.37	41.56±0.56	29.9±6.68
0 W	HYG 2.0	15.15±0.61	16.26±1.25	14.95±0.74	14.44±1.36
1 W	KAN 100	90.67±1.89			
1 W	HYG 2	77.14±4.28			
2 W	KAN 100	97.33±1.15			
2 W	HYG 2	92.8±6.23			

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MOLECULAR BREEDING SUPPORT FOR THE DEVELOPMENT OF PIERCE'S DISEASE RESISTANT WINEGRAPES

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ABSTRACT
This project is a continuation of the project titled "Genetic Mapping of *Xylella fastidiosa* Resistance Gene(s) in Grape Germplasm from the Southern United States," which was funded by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board. To date, we have completed greenhouse-based Pierce's disease resistance screening, genotyping (SSR and chloroplast markers), and population analysis of over 200 accessions that were acquired from states along the Gulf of Mexico. Twenty new highly resistant accessions were identified and crosses with eight resistant lines were made in 2012, 2013, and 2014. DNA was isolated from the new breeding populations to test with markers to assure their correct identity. This germplasm screening provides opportunities to explore and identify resistance loci that may provide different resistance mechanisms and allow us to expand the genetic base of the Pierce's disease resistance-breeding program. To date, we have exploited three different genetic sources of Pierce's disease resistance (b43-17, b40-14, and b42-26). Resistance loci were identified on genetic maps, markers were developed for breeding, and physical mapping was completed for b43-17 to clone and characterize resistance genes. Constructs were made with the constitutive 35S promoter and transformations were carried out on leaf discs of tobacco and embryogenic callus of *Vitis vinifera* cvs. Chardonnay and Thompson Seedless and *V. rupestris* St. George via *Agrobacterium tumefaciens*. We now have the complete sequence available, allowing us to design and utilize the native promoter to determine if switching the promoter will improve the performance of the *PdR1* resistance gene. 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 into *V. vinifera* cultivars. 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* allowed us to reduce the seed-to-seed cycle to two years and produce selections that are Pierce's disease resistant and 97% *V. vinifera*.

LAYPERSON SUMMARY
This project provides molecular genetic support to our Pierce's disease resistant winegrape breeding program by developing DNA markers linked to resistance genes that accelerate the breeding process. We have identified and cloned Pierce's disease resistance genes from *Vitis arizonica* b43-17 by map-based positional cloning and transformed Chardonnay, Thompson Seedless, the rootstock St. George, and tobacco with five candidate genes. We continue to identify and genetically characterize novel resistance sources from southwestern USA and Mexican *Vitis* species collections, use genome sequence information to identify unique resistance genes, clone and characterize these resistance genes with native promoters, and develop resistance gene constructs and transform them into susceptible *V. vinifera* grapes to test their function. Creating genetic maps with DNA markers allows us to use marker-assisted selection and to incorporate (stack) multiple resistance genes into a single background to create more durably resistant varieties. Genetic mapping can also lead to the identification and characterization of grape Pierce's disease resistance genes under control of native promoters that could be used to genetically engineer resistance into elite *V. vinifera* cultivars.

INTRODUCTION
Identification, understanding, and manipulation of novel sources of resistance are the foundation of a successful breeding program. This project evolved from two previously funded projects: 1) Genetic Mapping – "Genetic Mapping of *Xylella fastidiosa* Resistance Gene(s) in Grape Germplasm from the Southern United States"; and

2) Functional Characterization – "Molecular and Functional Characterization of the *Xylella fastidiosa* Resistance Gene(s) from b43-17 (*V. arizonica*)." Both of these projects supported the Pierce's disease resistance grape breeding project titled "Breeding Pierce's Disease Resistant Winegrapes." Genetic markers linked to *Xylella fastidiosa* (*Xf*) resistance from the former two projects were used to perform marker-assisted selection (MAS) to accelerate our Pierce's disease resistant winegrape and the table and raisin grape breeding of David Ramming in the past. Outcomes from these projects include BAC libraries of the highly resistant *V. arizonica* accessions b43-17 and b40-14. The b43-17 BAC library was used to physically map the *PdR1* locus and several candidate genes were identified. Five genes were cloned and constructs were developed to transform tobacco, Chardonnay, Thompson Seedless, and St. George that are being tested for function.

The new merged project has four key objectives: to identify novel sources of Pierce's disease resistance for use in broadening the genetic base of resistance; to utilize improved sequencing technology to facilitate and accelerate marker discovery and the identification of new and unique resistance genes; to clone and characterize unique DNA sequences (promoters) that regulate the expression of candidate Pierce's disease resistant grape genes cloned from the *PdR1b* locus; and to evaluate and compare lines with native and 35S promoters. To broaden the genetic base of Pierce's disease resistance breeding, we surveyed over 250 accessions of *Vitis* species growing in the southern USA and Mexico to identify new Pierce's disease resistant accessions. Analysis using population genetics methods allowed us to better understand gene flow among resistant species and their taxonomic and evolutionary relationships. Pierce's disease resistance in southeastern *Vitis* spp. seems to be different than the resistance in *Vitis* from the southwest and Mexico. We have already identified new Pierce's disease resistant accessions that are genetically and phenotypically different, were collected from different geographic locations, and have different maternal inheritance. We are continuously developing and expanding breeding populations from new promising resistant lines. These populations will be tested to study the inheritance of resistance. Next generation sequencing will then be used on the recently identified resistant accessions to expedite marker discovery and confirm that they are unique. Genetic maps will then be developed to identify genomic regions associated with resistance, and genetic markers will be used for the stacking of multiple resistance genes to breed winegrapes with durable Pierce's disease resistance.

The identification and characterization of resistance genes and their regulatory sequences will help determine the basis of resistance/susceptibility in grape germplasm. In addition, these genes and their promoters can be employed in production of 'cisgenic' plants. Cisgenesis is the transformation of a host plant with its own genes and promoters (Holmes et al., 2013). Alternatively, other well characterized *V. vinifera*-based promoters, either constitutive (Li et al., 2012) or activated by *Xf* (Gilchrist et al., 2007) could be utilized. Development of *V. vinifera* plants transformed with grape genes and grape promoters might mitigate concerns about transgenic crops harboring genetic elements derived from different organisms that cannot be crossed by natural means. Proven resistance gene constructs could be transformed into a broad array of elite *V. vinifera* cultivars.

OBJECTIVES

The overall goal of this project is to provide molecular genetic support to the Pierce's disease resistant winegrape breeding program. These efforts include discovering new sources of Pierce's disease resistance, identifying functionally unique loci or genes with the help of population genetics and comparative sequence analysis, creating genetic maps with SSR and SNP markers to tag resistance regions, and providing genes and sequences to validate and characterize the function of candidate Pierce's disease resistance genes. These genes under the control of promoters derived from grape will then be transformed into elite *V. vinifera* cultivars.

The specific objectives of this project are:

1. Provide genetic marker testing for mapping and breeding populations produced and maintained by the Pierce's disease resistance breeding program, including characterization of novel forms of resistance.
2. Complete a physical map of the *PdR1c* region from the b40-14 background and carry out comparative sequence analysis with b43-17 (*PdR1a* and *b*).
3. Employ whole genome (WG) sequencing (50X) of recently identified Pierce's disease resistant accessions and a susceptible reference accession, use bioinformatics tools to identify resistance genes, perform comparative sequence analysis, and develop SNP markers to be used for mapping.
4. Clone *PdR1* genes with native promoters.
5. Compare the Pierce's disease resistance of plants transformed with native vs. heterologous promoters.

RESULTS AND DISCUSSION

Objective 1. Provide genetic marker testing for mapping and breeding populations produced and maintained by the Pierce's disease resistance breeding program, including characterization of novel forms of resistance.

New cultivars bred to resist *Xf* infection and the subsequent expression of Pierce's disease symptoms will provide long-term sustainable control of Pierce's disease. Funding for the mapping and breeding programs has made it possible to maintain and characterize breeding populations. These populations allowed the construction of genetic maps based on DNA markers, the identification of genomic regions associated with Pierce's disease resistance, the selection of markers that were tightly linked to Pierce's disease resistance, and the use of these markers to greatly expedite breeding through MAS. Tightly-linked markers have been used to accelerate our Pierce's disease breeding program, reducing the seed-to-seed generation time to two years and allowing us to attain BC4 97% *V. vinifera PdR1b* populations that are now being evaluated for release as resistant winegrapes (Walker and Tenscher, 2010, 2011, 2012, and 2013). These genetic maps also lay the foundation for the construction of physical maps that allow us to identify and then functionally characterize resistance genes. We have characterized the *V. arizonica/candicans* selection b43-17, which is homozygous resistant, and two of its heterozygous resistant progeny (F8909-17 and F8909-08). The resistance locus *PdR1* has been mapped to chromosome 14 (Riaz et al., 2006; Riaz et al., 2008). Selections from these populations have been key parents in the breeding program (Riaz et al., 2009). The *PdR1b* locus has been physically mapped through the use of a b43-17 BAC library, and five cloned candidate genes are currently under investigation. In addition, we mapped Pierce's disease resistance from *V. arizonica* b40-14, *PdR1c*, to the same region on chromosome 14. b40-14 was collected in Chihuahua, Mexico and appears to be typical of the *V. arizonica* found in parts of southern Arizona. We are also mapping another form of *V. arizonica* b42-26 that has multigenic resistance. Genetic mapping and tagging of this source found that its resistance is on two other chromosomes and the markers are being used to support the breeding program.

Pierce's disease is common across the southern US and Mexico. *Vitis* species growing in this region have co-evolved with *Xf* and developed natural resistance to the disease. Some of these resistant forms may have different mechanisms of resistance that could add to the repertoire of resistance genes and loci available for the breeding program. We completed a survey of over 250 southwestern USA and northern Mexico *Vitis* accessions using SSR and chloroplast markers to evaluate their genetic diversity and establish their relationships with known sources of resistance being used in the breeding program (Riaz and Walker, 2013). A subset of this germplasm was greenhouse screened for Pierce's disease resistance and preliminary results identified multiple new sources of resistance. In 2012, we made crosses with five new Pierce's disease resistant *V. arizonica* accessions from the southwestern USA and Mexico to develop small breeding populations. The resistant accessions were chosen based on low ELISA values, lack of Pierce's disease symptoms in the greenhouse screen, and diverse geographic origins. A subset of seeds from these crosses were germinated in late 2012, multiple copies of each seedling plant were grown from green cuttings in 4" pots in early 2013, and they were greenhouse screened to characterize the inheritance of their Pierce's disease resistance. Results indicated clear separation of progeny families into resistant, intermediate, and susceptible groups, and identified an unprecedented level of resistance in b46-43 based on disease phenotype and ELISA values. More crosses were made in 2013 with five additional resistant accessions: b41-13, b43-57, b47-32, SC36, and T03-16. Small seedling populations are currently being prepared for greenhouse screening.

We have developed F1 and BC1 breeding populations using two of the resistant accessions, b46-23 and T03-16, that are geographically unique, have different maternal origins, and are genetically diverse. Seeds have been extracted from the 2014 crosses and they are being treated for germination. We plan to complete the greenhouse testing of the F1 and BC populations over the next year. We will isolate the genomic DNA and employ a limited mapping strategy by focusing mapping on linked chromosomes identified from the sub-population screening and then saturate with SSR markers that reside on those chromosomes. We have used this approach to scan and identify powdery mildew resistance loci from different genetic backgrounds (Riaz et al., 2011). The identification of other genomic resistance regions is critically important, since it is not genetically possible to stack more than two LG14 resistance sources. **Figure 1** shows the flow and integration of information among three different aspects of the Pierce's disease resistant grape breeding program.

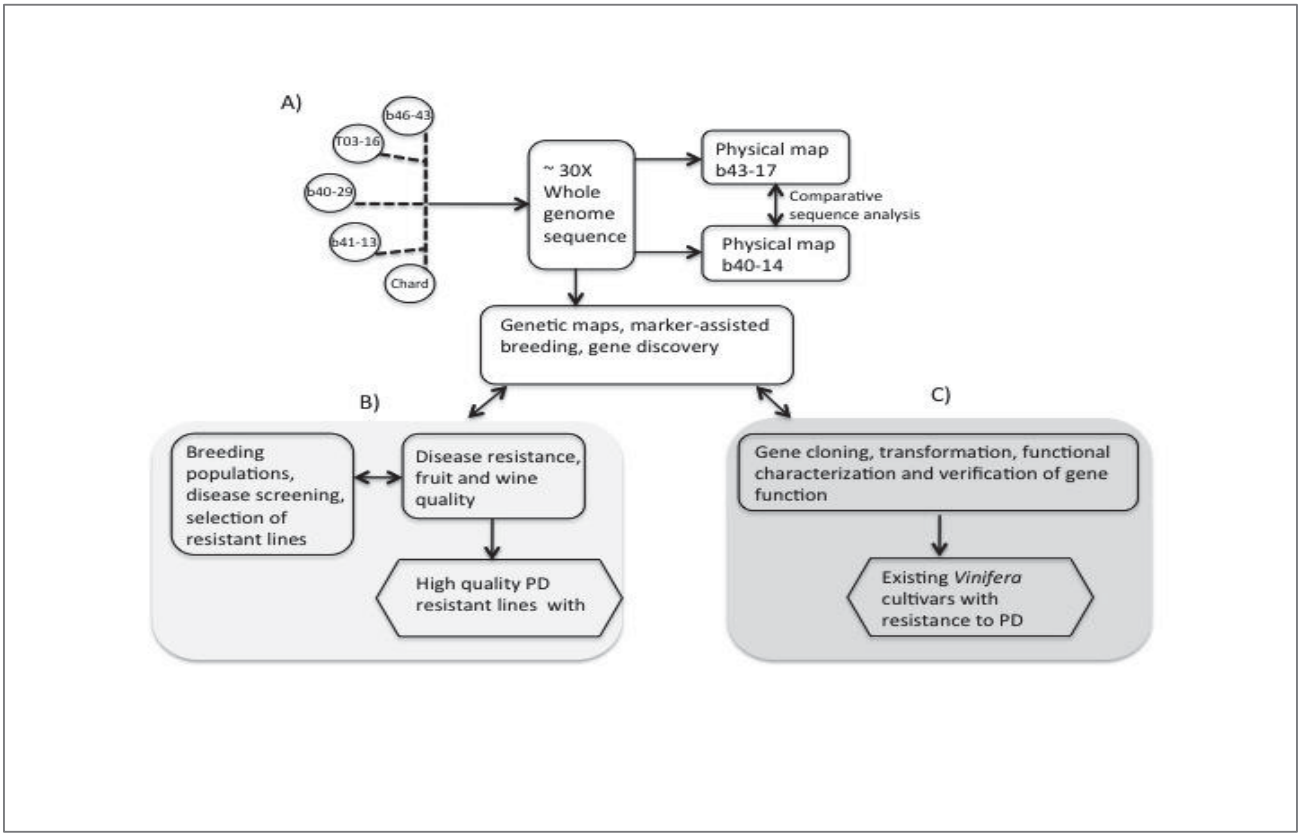

Figure 1. The flow of information within this project and a companion project on grape breeding. A) The identification of novel resistant material, trait inheritance, marker development using traditional and high throughput systems to assist the breeding program, development of genetic maps and resistance tagging, and ultimately gene identification. Results from this project feed into our companion winegrape breeding program (B) and the functional characterization, expression, and validation of resistant genes (C).

Objective 2. Complete a physical map of the *PdR1c* region from the b40-14 background and carry out comparative sequence analysis with b43-17 (*PdR1a* and *b*).
The accession b40-14, a pure form of *V. arizonica*, was tested and found to be homozygous resistant to Pierce's disease. Two resistant siblings of this population were used to develop the 07386 (R8917-02 x *V. vinifera*) and 07744 (R8918-05 x *V. vinifera*) populations. Genetic mapping and QTL analysis with the 07744 population identified a major locus for Pierce's disease resistance on chromosome 14. Pierce's disease resistance from b40-14 (which we have named *PdR1c*) maps in the same region as *PdR1a* and *PdR1b* between flanking markers VVCh14-77 and VVIN64 and within 1.5 cM (see previous reports). The allelic comparison of SSR markers within the 20cM region including the *PdR1c* locus revealed that the *PdR1c* locus is unique and its sequences and genomic features would be distinct from b43-17.

We developed a BAC library from b40-14 genomic DNA. To complete the physical map of the *PdR1c* locus, we have initiated the screening of the BAC library. By utilizing the b43-17 sequence, we have designed probes that amplify a single amplicon of 600-650 bp using b40-14 genomic DNA. The complete library of b40-14 is on nylon filters. We are in the process of screening the library by hybridization to identify positive BAC clones that carry the *PdR1c* locus to pursue the physical map of this region.

Objective 3. Employ whole genome sequencing (WGS) (50X) of recently identified Pierce's disease resistant accessions and a susceptible reference accession, use bioinformatics tools to identify resistance genes, perform comparative sequence analysis, and develop SNP markers to be used for mapping.
We identified multiple new Pierce's disease resistant accessions that were used to develop small breeding populations in 2012-2013. More crosses were made in 2013 and 2014 to expand existing and make new breeding populations (see final report). Our focus is on two new resistant accessions, b46-43 and T03-16. Both of them have shown very low bacterial levels in repeated greenhouse screens. Resistant accession b46-43 is homozygous resistant to Pierce's disease. Crosses to develop BC1 populations were made in 2014. We have extracted the DNA

of the F1 population to test it with markers to determine the true nature of the cross. Our approach of traditional bi-parental mapping populations has played an important role in gene discovery and understanding of Pierce's disease resistance in North American *Vitis* species, and both bi-parental and multi-parental breeding populations remain the foundation of our breeding program. In this project, we want to combine traditional SSR marker system and next generation sequencing using Illumina HiSeq and MiSeq platforms to carry out SNP discovery and potential SNP markers will be developed (**Figure 1**). We will pursue the WGS approach only on those resistant lines for which we have strong greenhouse screen information, heritability of the Pierce's disease resistance, and potential screening of the population using the limited mapping strategy. The BC1 populations with b46-43 and T03-16 background are under testing and will be ready for WGS approach in summer/fall 2015.

Objective 4. Cloning of *PdR1* genes with native promoters.
Earlier in 2014, we employed the PAC BIO RSII sequencing approach to sequence H69J14 and three other overlapping BAC clones. The assembled sequence data generated a 604Kb long fragment without any gaps (see previous reports). We identified multiple open reading frames of the Leucine-Rich Repeat Receptor Kinase gene family that regulates a wide variety of functions in plants including stem cell maintenance, hormone perception, and defense and wounding response for both host as well as non-host specific defense. With the help of molecular markers, we have limited the genetic region that carries the five open reading frames (ORF) to 82 Kb – these ORFs are associated with disease resistance and other plant functions described above. There are multiple ORF's that are outside this genetic region and have 99% sequence similarity to the candidate genes. Currently the major challenge is to isolate and clone the specific ORFs within the region, and verify it with the cDNA sequence. The next step is to fully annotate the sequence, and carry out comparative sequence analysis (manuscript in progress). Promoter isolation and characterization of the resistance genes will occur after verification of the most likely candidate gene (see previous reports for more detail).

Objective 5. Comparing the Pierce's disease resistance of plants transformed with native vs. heterologous promoters.
Once the gene constructs are completed, they must be tested to see if they confer resistance. This is done by inserting the genes into a susceptible plant and testing to see if the insertion results in resistant plants. Currently, the most widely used method for the production of transgenic/cisgenic grapes is based on *Agrobacterium* transformation followed by regeneration of plants from embryogenic callus. We have established cultures of pre-embryogenic callus derived from anthers of *V. vinifera* Thompson Seedless (TS) and Chardonnay (CH) and the rootstock *V. rupestris* St. George (SG) (Agüero et al., 2006). We have transformed these varieties with five candidate genes containing the 35S cauliflower mosaic virus promoter, the nopaline synthase terminator, and an *hptII*-selectable marker gene (see previous reports for details). To date, we have moved five lines forward by growing and multiplying the plants to create enough replicates for our greenhouse assay/ELISA screen. Two sets of screenings have been completed in February 2014 and July 2014. A third screening is underway that is scheduled to end in November 2014. Each line was multiplied through green cuttings to produce six replicates. Plants are needle inoculated near the basal nodes with 20 ul of 10^8 CFU/ml of the Beringer *Xf* strain. In 2014-2015, plants of the remaining five independent lines will be established and multiplied so that greenhouse screening and gene expression analysis of the transgenic lines can be completed.

Analysis of the expression of candidate genes in transgenic plants has also started. Total RNA was isolated using a cetyltrimethylammonium bromide (CTAB)-based RNA extraction protocol as described by Iandolino et al. (2004) with minor modifications. DNAse-treated total RNA was reverse transcribed using the SuperScript III First-Strand kit (Invitrogen). Expression analysis was conducted by real-time PCR analysis using a SYBR Green method on a 7500 Real Time PCR System (Life Technologies). The expression of each target gene was calculated relative to the expression of the housekeeping gene (18S rRNA – AF321771) using StepOne Software v2.0. Product size was confirmed by melt-curve analysis and agarose gel electrophoresis. Similar screening strategy for the transformed lines with complete Pierce's disease resistance gene (promoter-coding region-terminator) will be used once we have promoter region cloned and sequence is verified.

CONCLUSIONS
The development of breeding and mapping populations with the two new Pierce's disease resistance sources, b46-43 and T03-16, is proceeding. These two accessions support the lowest levels of bacteria of any we have tested. They are geographically isolated from b43-17 and genetically different based on a recent genetic diversity study of over 250 accessions from the southern USA and northern Mexico. We have started the screening of F1 and BC1 populations with these two backgrounds. Marker testing and a limited mapping strategy will proceed in the spring of 2014. The results from this work will allow us to use markers to facilitate stacking of these resistance

sources with *PdR1* from b43-17 – the multiple resistance should make resistance more durable. We have completed the genetic mapping of Pierce's disease resistance from b40-14 and named it *PdR1c*. This resistance source maps within the *PdR1b* locus, and may be an alternative gene within this complex replicated locus. We are physically mapping this gene to improve our understanding of the locus. We are using whole genome sequencing to generate single nucleotide polymorphism (SNP) markers to accelerate the genetic mapping in b46-43, which has shown to have exceptional resistance in multiple greenhouse trials. The use of SNP markers in combination with our SSR-based mapping will accelerate the identification of closely-linked markers for breeding and should also allow more rapid characterization of b46-43's resistance. Finally, we have been sequencing the *PdR1* locus to better define the five candidate genes and prepare them for complementation tests. This effort is also identifying their promoters so that we can avoid the use of constitutive non-grape promoters like CaMV 35S. We have tested versions of the *PdR1* candidate genes with 35S and they have not worked. We hope that the sequencing efforts we have employed recently to fine-tune these gene candidates and the addition of *PdR1's* native promoter will allow one of more of the five gene candidates to confer resistance in transformed Chardonnay.

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GLASSY-WINGED SHARPSHOOTER OVIPOSITION EFFECTS ON PHOTINIA VOLATILE CHEMISTRY WITH IMPLICATIONS ON EGG PARASITOID EFFECTIVENESS

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ABSTRACT
An effective way to limit the spread of Pierce's disease of grapevine is to reduce populations of the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis* (Germar); Hemiptera: Cicadellidae), which transmits the pathogenic bacterium *Xylella fastidiosa*. One strategy is to utilize egg parasitoids such as *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae) to consume GWSS eggs. However, greater knowledge is needed about how parasitoids find hosts to improve effectiveness of this strategy to control GWSS. Previous work showed that *G. ashmeadi* preferred plants with egg masses to those without, with greater preference for red-tip photinia (*Photinia* x *fraseri*) with egg masses than egg-mass colonized grapevine or citrus (Krugner et al., 2008). Likewise, *G. ashmeadi* was observed to be preferentially attracted to mixes of two grapevine volatile compounds, β-ocimene and α-farnesene, in choice assays (Krugner et al., 2014). Both of these compounds were found to increase in volatile profiles taken from egg-infested grapevines compared with non-infested controls (Krugner et al., 2014). Photinias are commonly planted throughout California as ornamental plants and are used by GWSS for oviposition. Therefore, the current study examined GWSS egg-infested photinia to observe if additional parasitoid-attracting compounds were present in that GWSS host compared to grapevines. To this end, three to six photinia plants each were either exposed to egg-laying GWSS females or left non-exposed for three days in cages in a containment greenhouse at USDA ARS in Parlier, CA. After the three-day oviposition period, leaves were collected from non-infested and egg-mass infested grapevines, the tissue was pulverized and extracted in methyl tert-butyl ether, and volatile terpenoids were analyzed using gas chromatography-mass spectrometry. The experiment was replicated in full three times. Of over 30 compounds analyzed, six were observed to occur at significantly greater levels in egg-infested leaves than leaves from control plants, including tentatively identified β-ocimene, α-pinene, Δ-3-carene, linalool, α-farnesene, and an additional farnesene enantiomer. Once all compounds are definitively identified, they will be tested for relative attractiveness to female parasitoids in wind tunnels and/or Y-tube olfactometry. These findings could be eventually utilized in the development of lures useful in monitoring egg parasitoid populations and effectiveness. The production of these compounds in response to oviposition also could be selected as a desirable plant trait in programs that aim to reduce GWSS populations.

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AUTHOR INDEX

Almeida, R. 98, 202
Backus, E 6. 7, 8
Bonning, B 23
Burbank, L 91
Burr, T. 42
Byrne, F 36
Cantu, D 82, 249
Caserta, R 199
Ceresini, P 93
Chen, J 92
Coletta-Filho, H 93
Cursino, L. 42
Dandekar, A. 50, 99, 106, 122, 133, 180
Daugherty, M 12, 185
de Souza, A 95, 199
Falk, B. 16, 23, 39
Francisco, C 93
Gilchrist, D. 118, 122, 126, 133
Golino, D 139
Hao, L 42
Kamita, S 29
Kirkpatrick, B. 57, 141, 149
Krugner, R. 2
Labavitch, J. 172, 180
Labroussaa, F. 202
Lin, H. 94
Lincoln, J. 126, 133
Lindow, S. 62, 122, 133, 155, 162
Mauk, P 185
Maloney, K. 193
Mazzoni, V. 2
Merfa, M 95
Miller, W. 23
Morgan, J 7
Mowery, P. 42
Nandety, R 39
Niza, B 95
Perring, T. 31
Pitman, T 39
Powell, A. 122, 172, 180
Prabhaker, N. 31
Redak, R 36
Rogers, E 6, 7, 8
Rolshausen, P 185, 193
Roper, C. 74, 82, 193
Shatters, R. 7
Shugart, H. 7
Sisterson, M 9
Soto, D 12
Souza-Neto, R 199
Stenger, D. 9, 23, 91
Takita, M 199
Tricoli, D 211
Walker, A. 220, 227, 240, 249
Wallis, C. 9, 256
White, B 36