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

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

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

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

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

OBJECTIVES 2010-2012

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

RESULTS AND DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

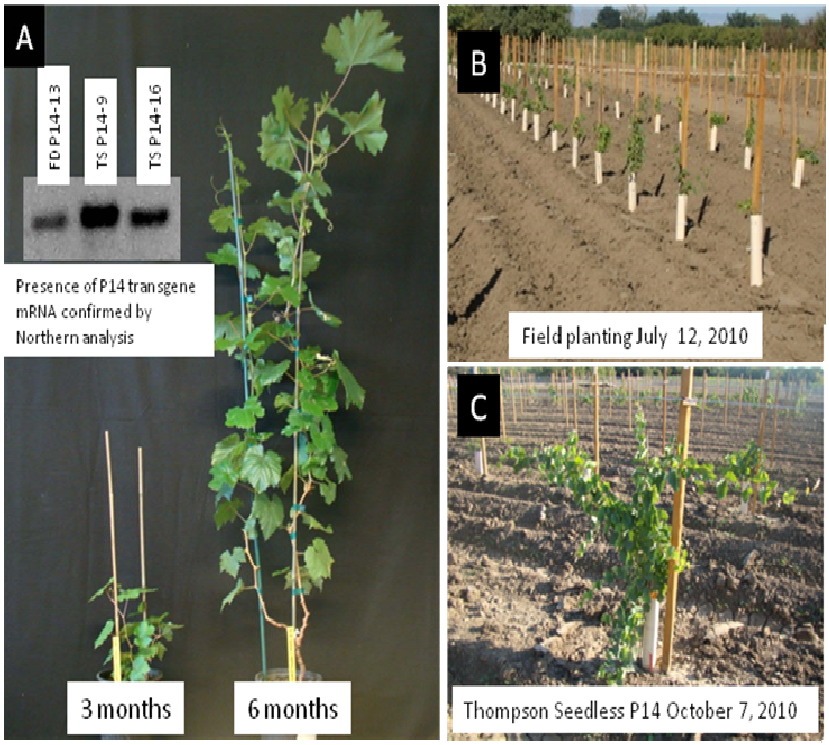

Figure 4. Field Trial. Panel A illustrates the time –course development of transgene expressing rootstocks grafted to untransformed PD susceptible Thompson Seedless scions. The inset shows northern blot analysis of the P14 transgenic lines currently planted in the field trial confirming the presence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

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

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

CONCLUSIONS

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

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