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I. Project Title: Control of Pierce's Disease by altered expression of anti-apoptotic genes or their RNA-based regulatory elements

II. **Principle Investigator:** David Gilchrist (dggilchrist@ucdavis.edu) (530) 304-6007
Co-Principal Investigator: James Lincoln (jelincoln@ucdavis.edu) (530)752-6587

III. OBJECTIVES

A. Complete the evaluation of the additional four candidate anti-apoptotic genes transformed into PD susceptible Thompson Seedless plants. (2010-2011)
B. Evaluate the relative susceptibility of eight commercial winegrape varieties to Pierce's Disease and titer of Xf in the inoculated canes. (2010-2012)
C. Initiate experiments to assess the potential for protection against PD across a graft union by VvPR1 and UT456, first with Thompson Seedless as the untransformed scion. (2010-2012)
D. Determine presence and movement of the mRNA and/or protein of VvPR1 and UT456 across the graft union into the untransformed Thompson Seedless O2A scion. (2010-2012)
E. Perform inoculations the eight winegrape varieties, initially on their own rootstocks and subsequently on Freedom and Thompson Seedless rootstocks expressing VvPR1 and UT456. (2012)
F. Investigate the mechanism underlying the protection against PD by VvPR1 and UT456. (2010-2012)
G. Collaborate with PIPRA to obtain permits to enable field evaluation of transgenic VvPR1 and UT456 in a location providing for controlled inoculation. (2010)
H. Secure patent protection as intellectual property for those genes that prove to be capable of blocking PD in grape. (2011-2012)

IV. Summary of major research accomplishments for each objective

Each of the objectives has moved at the rate projected and interim goals for this fiscal year will be met. The overall goal of this research is evaluation the *in planta* efficacy of several DNA sequences isolated from a functional cDNA screen for DNA sequences that were capable of suppressing *Xylella fastidiosa*-induced symptoms resulting from induction of programmed cell death (PCD) in Pierce's Disease. A total of six putative anti-PCD cDNAs were isolated and two (PR1 and UT456) were chosen as first-pass candidates to be transformed into in the rootstock Freedom and Thompson Seedless and evaluated under controlled greenhouse inoculation for disease suppression. The transgenic expression of these sequences not only protected the transgenic plants against PD symptoms and plant death but maintained the population of *Xf* at four or more orders of magnitude below the level observed in untransformed plants that died within 3 months under the controlled inoculation conditions (1). Progress on each of the objectives of this two year funded project is detailed below.

Progress by Objective:

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

The protective genes or DNA sequences, isolated by a functional anti-PCD screen (2), have been described in (1). Table 1 and Figure 1 summarize 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 each of these genes indicates the presence of orthologs in other plants including potato and tomato (Table 1). Inoculation of individual canes by the needle prick method delivered 10-20 µl at of the Temecula strain of *Xf* at a concentration of 10^5 cfu/ml (2,000 cells) (1). Presence of bacteria in the inoculated tissue is determined by qPCR and reported as the number of cells per 0.1 gm of stem tissue (Table 1). All four candidate genes suppressed PD symptoms and reduced bacterial titer in the inoculated canes below that of the control but were not superior to VvPR1 or UT456 in either case. These genes will be maintained in clonally propagated plants and patent protection sought but will not be tested further. Ongoing greenhouse and field experiments are focused on VvPR1 and UT456.

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

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 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, VvPR1 and UT456 as reference lines. These experiments also provide baseline disease information for 2011-2012 experiments to test potential protection of these varieties when grafted to rootstocks expressingVvPR1 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 to 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 PR1 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 (Figure 2).

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

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 VvPR1 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 for Spring 2011 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 to be started late in 2011(see Objective E).

D. *Determine presence and movement of the mRNA and/or protein of VvPR1 and UT456 across the graft union into the untransformed Thompson Seedless O2A scion. (2010-2012.*

MicroRNAs are small endogenous RNA molecules (~21-25 nt) that are processed from longer transcripts into pre-microRNA hairpin structures with final steps completed by an enzyme called Dicer. MicroRNAs regulate gene expression by targeting by sequence homology one or more messenger RNAs (mRNAs) for translational regulation or degradation. Although the first microRNA was identified over ten years ago, it is only recently that the scope and diversity of these regulatory molecules have begun to be understood. One current method of analysis is "deep sequencing" which isolates all the small RNA in a tissue and converts it to cDNA for sequencing by standard molecular techniques. This type of sequencing generates databases of microRNA sequences that are the putative regulatory microRNAs active in that tissue (4). We have run computer comparisons of 456 and microRNA sequences and have found that a grape microRNA called 3637 has significant homology to a region in 456 (figure 4). This homology is found in a part of 456 that folds into a hairpin structure which is a known substrate for microRNA production by Dicer. We have successfully developed a protocol to amplify putative microRNAs from 456 transgenic grape tissue with primers designed to amplify 456 microRNAs from the region homologous to grape microRNA 3637. We are in the process of cloning and sequencing these 456 specific microRNAs.

E. *Perform inoculations the eight winegrape varieties, initially on their own rootstocks and subsequently on Freedom and Thompson Seedless rootstocks expressing VvPR1 and UT456. (2012).* The results of the first inoculation are presented in B) above. Grafting of the eight winegrape varieties is in progress with inoculations scheduled for late 2011.

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

These studies are just beginning but to date we have found two novel and possibly linked mechanisms for VvPR1 and UT456 action. First, the transgenic PR1 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 PR1 3'UTR. Specifically, searching with an RNA matching program (RNAhybrid) detected homology to known microRNAs embedded in these hairpins are shown with red arrows; the green arrow shows a region protected from RNAse digestion which suggests a strong RNA-RNA duplex stem-like structure in that region. Within the 456-2 hairpin is the mir3637 region, which, by sequence analysis, can anneal to the 3' UTR of PR1 (Figure 4). Initial *in vitro* protein translation studies indicate that the UT456 contains a signal sequence (22-23 base microRNA that is released by DICER) that activates translation. There is precedent for translational blockage by the 3'UTR in plant systems and for RNA movement from roots to tubers (3). Expression of the UT456 activated the translation of the PR1 protein in transgenic

tobacco leaves. In addition PR1 antibodies, used in immunoprecipitation assays to detect potential PR1 interacting factors, were successful in identifying 3 PR1-interacting proteins, HP70, HP90 and RACK1 from plant extracts. Interestingly, these three proteins have previously been reported to interact directly with each other and occur in a membrane associated complex involved in innate immunity in rice plants. Work has also begun to assess a role for the potential small RNA hairpin loops within UT456 to activate PR1translation using RNA protection assays. We have developed protocols for amplifying, cloning and sequencing microRNAs generated from transgenic expression of PR1 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. PR1 antibodies will also be used to test directly for the presence of transgenic PR1 protein in the grafted scions.

G. *Collaborate with PIPRA to obtain permits to enable field evaluation of transgenic VvPR1 and UT456 in a location providing for controlled inoculation. (2010)*
We and others collaborated with PIPRA in the permit process, which was successful. The first phase of the field planting in Solano County was completed in the summer of 2010. The remainder of the plants for this location, which include untransformed scions grafted to transgenic rootstocks expressing PR1 or 456 are ready to be moved to the field when the danger of frost is passed. In addition, the plants for our portion of the planting in Riverside Country are also ready to be moved to that location when plants from the other investigators are also ready for transport and planting.

H. *Secure patent protection as intellectual property for those genes that prove to be capable of blocking PD in grape. (2011-2012)*
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 Xylella fastidiosa in the transformed and untransformed grape plants.

V. Publications: Current results were published in the 2010 Pierce's Disease Symposium Proceedings. Two other publications for peer reviewed journals have been drafted and final submission is awaiting the completion of the 2011 experiments in the greenhouse.

VI. Presentations. The results described herein were presented to the 2010 Pierce's Disease Symposium and two additional extension meetings on the UC Davis Campus attended by members of the wine and vineyard industry.

VII. Research relevance.

Genetic strategies for disease suppression and information characterizing the bacterial-plant interaction are high priority areas in the Pierce's Disease/GWSS Research Program and the NAS report. The goal of this project is to identify novel genes from cDNA libraries of either grape or heterologous plants that, when expressed in grape, will prevent infection, spread or symptom

development due to the presence of *Xf* in the xylem. Currently, several laboratories including our own have begun to carry out systematic studies of the molecular basis of susceptibility of plants to a range of pathogens including bacteria and fungi. The objective of these studies is to identify genetic or chemical approaches that have the potential to block susceptibility, thereby effectively creating cells that are refractory or insensitive to the signals expressed by pathogens that lead to susceptibility. Recent information from our laboratory established that susceptibility of several plants to a range of pathogens depends on the ability of the pathogen to directly or indirectly trigger the activation of preformed, genetically determined pathways leading to apoptosis or programmed cell death (PCD).

VIII. LAYPERSON SUMMARY

Xylella fastidiosa induces Pierce's Disease symptoms that are the result of the activation of a genetically regulated process of programmed cell death. We identified six novel anti-PCD genes from a grape cDNA library functional screen for ability to suppress PCD. Two of these grape sequences, VvPR1 and UT456, when expressed as transgenes in the PD susceptible Thompson Seedless plants, suppressed PD symptoms and dramatically reduced bacterial levels in inoculated plants. The remaining four genes were tested this year, along with VvPR1 and UT456; each of the four provided substantial suppression of both PD symptoms and bacterial titer. However, none were as effective as VvPR1 and UT456. Currently in progress are a series of experiments designed to evaluate whether the protective effect of these two sequences can protect untransformed susceptible winegrape scions across a graft union. Preliminary data suggest that 50% or more of the susceptible scions grafted to either VvPR1 or UT456 showed less PD symptoms and had lower bacterial titers than the unprotected control plants. While these results are encouraging, they are not complete or definitive and the experiment is continuing. The relative susceptibility of the suite of eight commercial winegrape varieties is being tested under controlled greenhouse conditions prior to field testing these varieties as scions on the transgenic rootstocks. Mechanism of action experiments initiated recently suggests a genetically conserved basis for suppression of PCD and the protection against PD. This project is now moving from the proof-of-concept to potential application and characterization of these plants under field conditions with appropriate APHIS permits: initial field plantings were begun in July 2010 with additional plantings to be made in Solano and Riverside Counties in 2011.

IX. Status of funds. We anticipate that all funds allocated for fiscal year 2010-2011 will be expended by June 30, 2011.

X. Intellectual property.

As indicated in the results for Objective H above, 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 Xylella fastidiosa in the transformed and untransformed grape plants.

References:

1. Gilchrist, D.G., and J.E. Lincoln 2010. Resistance to Pierce's Disease by transgenic expression of plant-derived anti-apoptotic genes. Pierce's Disease Symposium Proceedings, San Diego, CA December 15-17.
2. Harvey, J. JW, J. E. Lincoln, K. Zumstein and D. G. Gilchrist 2007 Programmed cell death suppression in transformed plant tissue by cDNAs identified from an Agrobacterium rhizogenes-based functional screen. Molecular Genetics and Genomics 279:509-521.
3. Cornelissen, B. J. et al. 1987. Structure of tobacco genes coding pathogenesis related proteins from the PR1 group. Nucleic Acids Research. 15:6799-6811.
4. Pantaleo V, Szittya G, Moxon S, Laura Miozzi L, Moulton V, Dalmay T, Burgyan J. (2010); "Identification of grapevine microRNAs and their targets using high-throughput sequencing and degradome analysis" Plant J. 62:960-976.

Figure 1. Plant anti-apoptotic genes, derived from functional screens of cDNA libraries, have been transformed into grape plants and assayed for resistance to Pierce's disease. Titers of *Xf* in stem section and subjective ratings were performed four months after mechanical inoculations. *Xf* bacterial titers were determined by qPCR and are expressed as bacterial cells per 0.1gm of stem tissue. See Figure for representative pictures. Disease rating is a 1-5 scale with 1 = asymptomatic and 5 = defoliated

Figure 4. Map of UT456. This schematic shows the full 270bp of 456 RNA. When folded the RNA has 2 stem-loop-stem regions or hairpins that are marked with blue lines; Searching with an RNA matching program (RNAhybrid) detected homology to known microRNAs embedded in these hairpins are shown with red arrows; the green arrow shows a region protected from RNAse digestion which suggests a strong RNA-RNA duplex stem-like structure in that region. Within the 456-2 hairpin is the mir3637 region, which, by sequence analysis, can anneal to the 3' UTR of PR1.

Figure 3. Grafted PD protection. Images of untransformed Thompson seedless (Ts) scions grafted to PR1 and 456 transgenic Freedom rootstocks, then inoculated with 20,000 Xf cells. Control grafts of Ts02A grafted back to itself is the plant on the left. Images were taken 9 months post-inoculation.

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

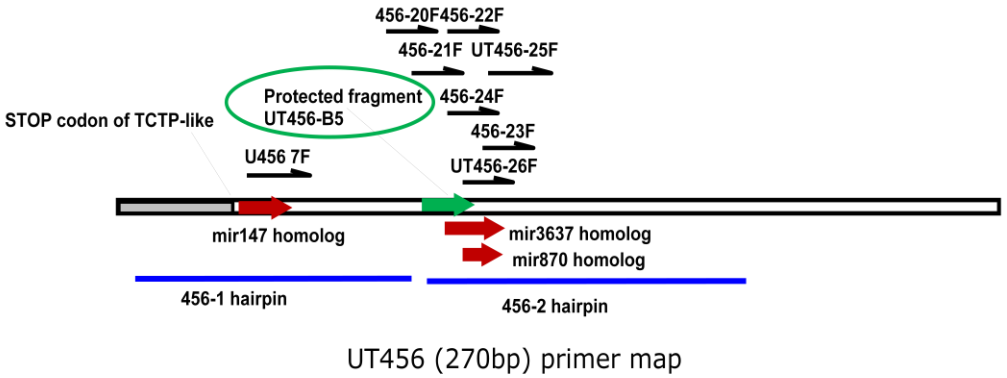
Construct	Gene	PD resistance rating average	Xf / 0.1g cane by qPCR (average)
CB390	metallothionein	3	10^4
UT456	270bp sequence from 3'UTR of "nematode-inducible" p23 gene	5	10^2
WG23	cupin-like	4	10^3
WG71	cytokine-like gene (MIF)	4	10^4
O2A	Untransformed Thompson seedless	1	10^6
PR1A	VvPR1	5	10^2
I35	Intron p35 (anti-PCD control gene)	3	10^4
PR1	PR1 (homolog of PR1)	5	10^2
PR1B	Secretory leader deletion of PR1	1	10^6
CB376	Mycorrhizal induced gene	3	10^4
9353	GFP (transformed control)	1	10^6

Table 2. Titers of Xf in grape scions (Ts 02A) grafted to Freedom or Thompson seedless transgenic for PR1 or UT456. Four months after mechanical inoculations, Xf bacterial titers were determined by qPCR and are expressed as bacterial cells per 0.1g of stem tissue. See Figure X for representative pictures.

Transgenic notation for the grafted plants: Scion / Rootstock	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
S02A / FDPR1-13	CaMV 35S-driven PR1 Freedom rootstock	50%	10^3 - 10^4
TS02A / TS456-8	CaMV 35S-driven 456 Thompson seedless rootstock	66%	10^3 - 10^4
TS02A / TSPR1-9	CaMV 35S-driven PR1 Thompson seedless rootstock	100%	10^4 - 10^5
TS02A Control	Untransformed and ungrafted TS . Dead at 4 months.	none	10^6 - 10^7
TS02A / TS02A	Untransformed Grafted Control (dead at 7 mo.)	none	10^6 - 10^7
Vitis californica	Asymptomatic wild type untransformed host.	no death after 12 months post inoculation	10^4

Table 3. Winegrape varietal Xf titers evaluated by qPCR following mechanical inoculations. *Xf* 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.

Varietal	Titer of Xf
Cabernet Sauvignon	5×10^6
Chardonnay	5×10^5
Merlot	7×10^6
Pinot Gris	1×10^7
Pinot Noir	4×10^6
Sauvignon Blanc	1×10^5
Syrah	7×10^4
Zinfandel	5×10^5
V. californica	4×10^4
Thompson seedless	5×10^6



UT456 (270bp) primer map

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, erlot, 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.