

## Renewal Progress Report for USDA-UC PD Grant SA7627.

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

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**Reporting Period:** The results reported here are from work conducted July 1, 2011 to March 3, 2012

#### ABSTRACT

Two DNA sequences (VvPR1 and UT456) were selected by a functional screen to test for ability to suppress the Programmed Cell Death-dependent symptoms of PD. Greenhouse experiments confirmed that these two different anti-PCD DNA sequences prevented PD symptoms in the PD-susceptible cultivar Thompson Seedless and the commercial rootstock Freedom. Furthermore, the bacterial titer in the transgenic plants was reduced four to six orders of magnitude below that reached in untransformed control vines. In contrast, all untransformed control plants died within 3-4 months after inoculation while the transgenic plants were asymptomatic for 12 months. The net effect of these transgenes is to limit bacterial titer but not distribution of bacteria in the asymptomatic plants. From the perspective of the grape-bacterial interaction, it appears that the anti-PCD genes suppress PD symptoms and functionally confine (*Xf*) to an endophytic ecology in the xylem equivalent to that seen in the related asymptomatic host *Vitis californica*. Clonal copies of the transgenic and control plants were moved to two field locations under an APHIS permit secured by PIPRA and were inoculated July 21, 2011. Greenhouse data obtained from grafting experiments indicate the protective effect of these genes may be transferred across a graft union to protect a susceptible untransformed scion. Grafted plants expressing VvPR1 and UT456 in the rootstock, but not the scion, have been moved to the field site for inoculation in the spring of 2012.

#### INTRODUCTION

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

## OBJECTIVES 2011-2012

- A. 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. (2011-2012)
- B. Determine presence and movement of the mRNA and/or protein of VvPR1 and UT456 across the graft union into the untransformed Thompson Seedless O2A scion. (2011-2012)
- C. Perform inoculations of eight winegrape varieties, initially on their own rootstocks. Then chose three varieties that cover the range of susceptibility to test for cross protection by VvPR1 and UT456. (2012)
- D. Investigate the mechanism underlying the protection against PD by VvPR1 and UT456. (2011-2012)
- E. Collaborate with PIPRA to obtain permits to enable field evaluation of transgenic VvPR1 and UT456 in a location providing for controlled inoculation. **Done**
- F. Secure patent protection as intellectual property for those genes that prove to be capable of blocking PD in grape. (2011-2012)

## RESULTS AND DISCUSSION

### **Complete the evaluation of the additional four candidate anti-apoptotic genes transformed into PD susceptible Thompson Seedless plants.**

The protective genes or DNA sequences, isolated by a functional anti-PCD screen (1), have been described in (2,3,4). Greenhouse inoculations are complete and the results summarized in Table 1. 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  $\mu$ l of the Temecula strain of Xf at a concentration of  $10^5$  cfu/ml (2,000 cells). 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 (Figure 1).

**Evaluate the relative susceptibility of eight commercial winegrape varieties to Pierce's Disease and titer of Xf in the inoculated cane under controlled greenhouse inoculation conditions (2010-2012).** Experiments were concluded on a suite of commercial winegrape varieties to obtain quantitative data on bacterial population dynamics and relative PD susceptibility. The varieties tested include Chardonnay, Pinot Gris, Sauvignon Blanc, Cabernet Sauvignon, Pinot Noir, Zinfandel, Syrah and Merlot with untransformed Thompson Seedless, with VvPR1 and UT456 transgenic lines as reference controls. Data collected included bacterial titer, and "disease symptoms". Disease symptoms are herein defined as leaf defoliation, not marginal death of leaves that is generally considered but not proven to be true symptoms of PD under field conditions. NOTE: It has been our consistent observation over the past seven years that marginal leaf death, often associated with PD under field conditions is meaningless and misleading under our greenhouse conditions. Uninoculated control plants frequently exhibit marginal and interveinal death reminiscent of the field PD symptoms, while leaf drop occurs only in susceptible inoculated plants. The point is that after inoculating more than 500 plants in the greenhouse, the susceptible control plants always defoliate, show high bacterial titre, and die. In our experiments, only the transgenic protected plants retain their leaves and show low levels of bacterial titre. Selected clones of each variety were inoculated by the needle prick method with Temecula strain of Xf delivering 10-20  $\mu$ l at bacterial concentration of  $10^5$  cfu/ml (2,000 cells or less). All varieties were susceptible to PD in terms of leaf defoliation symptom expression and exhibited 1-3 orders of magnitude higher bacterial titers four months after inoculation than the asymptomatic *Vitis californica* or transgenic Vv PR1 or UT456 comparison plants (Table 1). Pinot Gris had the highest bacterial titer and exhibited the most severe defoliation while Syrah was the most tolerant with symptoms and bacterial titer nearly as low as *V. californica*. The Cabernet Sauvignon and Merlot winegrape varieties have now been grafted to transgenic rootstocks expressing VvPR1 and UT456 to determine if any cross-graft protection occurs. Inoculation of these grafted plants under greenhouse conditions, comparable to the previous Thompson Seedless transgenic rootstock: untransformed scion combinations will begin in the spring of 2012.

**Conduct experiments to assess the potential for protection against PD across a graft union by VvPR1 and UT456 with Thompson Seedless as the untransformed scion (2011-2012).** The purpose is to determine if the protective effect of these genes as observed in the primary transgenics is transferrable across a graft union to protect a susceptible scion. PD susceptible untransformed Thompson Seedless scions were grafted onto Freedom rootstocks transgenic for VvPR1 and UT456. A total of 13 untransformed control grafts were compared

with 13 transformed rootstock:untransformed scions. The preliminary data suggest that all 13 of the susceptible scions showed none or far less PD symptoms and had reduced bacterial titer than the untransformed control grafted plants, all of which were dead or nearly dead by four months after inoculation with approximately 20,000 Xf cells per branch (Table 2). While these results are encouraging, the experiment is continuing with two of the winegrape varieties, (Cabernet Sauvignon and Merlot) grafted to a transgenic rootstock. Currently, comparable grafted plants are being prepared in the greenhouse for field planting in the Spring 2012 (see Objective D).

#### **Investigate the mechanism underlying the protection against PD by VvPR1 and UT456 (2011-2012).**

We have found two novel and likely linked mechanisms for VvPR1 and UT456 action. First, the transgenic PR1 protein product will suppress PCD in several plants systems we have tested. However, the PR1 coding sequence is translationally blocked in healthy cells and an *in vitro* translation system, even when the message level is high 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 hairpins that show sequence conservation with the 3'UTR of PR1 and are projected to interact with each other by RNA modeling programs. *In vitro* protein translation studies indicated that the block in translation of PR1 RNA can be relieved by the addition of UT456 RNA to wheat germ extracts. The same result was obtained by agro- infiltration assays, whereby the expression of UT456 activated the translation of the PR1 protein in tobacco leaves expressing high levels of the PR1 message that was blocked until the UT456 RNA was present and processed into a microRNA. By functional definition, microRNAs are small endogenous RNA molecules (~22-24 bases) that are processed from longer transcripts into pre-microRNA hairpin structures with final steps completed by an enzyme called Dicer. The *in vitro* activation of PR1 translation results from the release of a 22-24 bases from the native 270 base UT456 hairpin by the endogenous nuclease DICER, known to be present in the wheat germ extracts. MicroRNAs regulate gene expression by targeting by sequence homology one or more messenger RNAs (mRNAs) for translational regulation or degradation. Although the first microRNA was identified over ten years ago, it is only recently that the scope and diversity of these regulatory molecules have begun to emerge. There is precedent for translational blockage by the 3'UTR in plant systems and for RNA movement from roots to tubers (6). The presence of the sequence in UT456 with annealing ability to the 3'UTR of PR1 is the basis for the current model of PR1 and UT456 function in the Xf-grape system. Currently, we are searching for mobile UT456 microRNA in extracts in the transgenic grapes and as the mobile element in the untransformed scions which appear to be protected against PD symptoms when grafted to transgenic rootstocks expressing UT456. We have developed a "hook assay" for PCR detection of the putative microRNA (figure 2). In addition, the lab has developed a highly sensitive Taqman-based assay capable of detecting this specific and low abundant microRNA sequence in plant extracts (figure 3). PR1 antibodies will be used to test directly for the presence of mobile PR1 protein from the rootstock into the grafted scions. In addition PR1 antibodies are being used in immunoprecipitation assays to detect potential PR1 interacting factors. To date we have been successful in identifying 3 PR1-interacting proteins, HP70, HP90 and RACK1 from plant extracts. Interestingly, these three proteins have previously been reported to interact directly with each other and occur in a membrane associated complex involved in innate immunity to rice blast in rice plants transgenic for RACK1 (5).

**Collaboration with PIPRA to obtain an APHIS permit for field planting.** An APHIS permit to enable field plant of transgenic plants from the our laboratory as well as those of PIs Dandekar Lindow and Labavitch is in place and the experiments have been started. Planting of the primary transgenic plants from the respective programs was completed in July, 2010. The 2010 plantings of all four investigators survived the winter without loss. The attachment of new shoots to the trellis system, cultivation, and irrigation management progressed in a normal and effective manner. All flowers were removed before opening and extensive pruning was done to manage the plants in a fashion compatible with mechanical inoculation. All flowers and prunings were collected, bagged, and autoclaved before disposal. As of July 21, 2011, the initial planting and the second 2011 planting individuals are healthy, growing normally and all plants have a normal phenotype, true to the untransformed control plants of each parental genotype (Figure 4). Plants were maintained under clean field conditions, with furrow irrigation on a regular schedule. Regular monitoring was conducted for weeds, insects, and non-PD disease. Weeds were managed by cultivation and minimal hand weeding. No significant insect or disease pressure was noted. Plants from all four laboratories were inoculated on July 21 (Figure 4). The inoculation was by needle prick method with a delivery of ~ 20,000 Xf cells per inoculation site. Sampling of a limited number of inoculated canes near the inoculation site on control and VvPR1 and UT456 transgenic plants assayed by PCR confirmed the presence of the respective transgenes (Figure 4C) and then determined, by qPCR, to harbor a low level of Xf in the sampled inoculated plants. Bacterial titre ranged from undetectable in the uninoculated control plants to  $1 \times 10^2$  to  $7.5 \times 10^3$

per 0.1 g of stem tissue in the inoculated plants. Untransformed control plants were negative for the transgenes. During the growing season of 2011, no typical field symptoms or defoliation of leaves was observed on any of the inoculated plants. The plants should begin to leaf out mid-March 2012 and data collection will begin. Inoculation of new vegetative tissue will be repeated when the first growth leaves are fully expanded.

**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 *Xylella fastidiosa* in the transformed and untransformed grape plants.

**Publications:** Gilchrist D.G. and J.E. Lincoln 2011 Pierce's Disease control and bacterial population dynamics in winegrape varieties grafted to rootstocks expressing anti-apoptotic sequences. Sacramento, CA December 2011

**Research relevance:** *Xylella fastidiosa* induces PD symptoms that result from activation of a genetically regulated process of programmed cell death. We have identified grape DNA sequences, which when constitutively expressed in transgenic grapes suppress the death-dependent symptoms of PD and reduce the bacterial titre to a level found in PD resistant wild grapes. We identified six novel anti-PCD genes from cDNA libraries of grape. Two of these grape sequences expressed as transgenes in grape, suppressed PD symptoms and dramatically reduced bacterial titer in inoculated plants in full plant transgenics. Initial data suggest that protective sequences may function across a graft union to protect an untransformed and susceptible wild type scion. This project has identified a basis for 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 plan for the coming year is to continue the field evaluation of transgenic grapes expressing PR1 and UT456 and to test for cross graft protection by these two sequences, also under field conditions.

#### REFERENCES:

1. 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. *Mol.Gen.Gen.*, 279, 509-521.
2. Gilchrist, D.G., and J.E. Lincoln 2008 Systemic Resistance to Pierce's Disease by transgenic expression of plant-derived anti-apoptotic genes. San Diego, CA December 15-17.
3. Gilchrist, D.G., and J.E. Lincoln 2009 Systemic Resistance to Pierce's Disease by transgenic expression of plant-derived anti-apoptotic genes. Sacramento, CA December 9-11.
4. Gilchrist D.G. and J.E. Lincoln 2010 Pierce's Disease control and bacterial population dynamics in winegrape varieties grafted to rootstocks expressing anti-apoptotic sequences. San Diego, CA December 2010
5. Ayako Nakashima, Letian Chen, Nguyen Phuong Thao, Masayuki Fujiwara, Hann Ling Wong, Masayoshi Kuwano, Kenji Umemura, Ken Shirasu, Tsutomu Kawasaki and Ko Shimamoto (2008). RACK1 Functions in Rice Innate Immunity by Interacting with the Rac1 Immune Complex. *The Plant Cell* 20:2265-2279.

**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 was tested under controlled greenhouse conditions prior to field testing these varieties as scions on the transgenic rootstocks. Mechanism of action experiments initiated recently suggests a genetically conserved basis for

suppression of PCD and the protection against 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 began in July 2010 with additional plantings made in Solano and Riverside Counties in 2011.

**Status of funds.** We anticipate that funds allocated to this project will be expended by the end of the grant period, June 30, 2013.

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

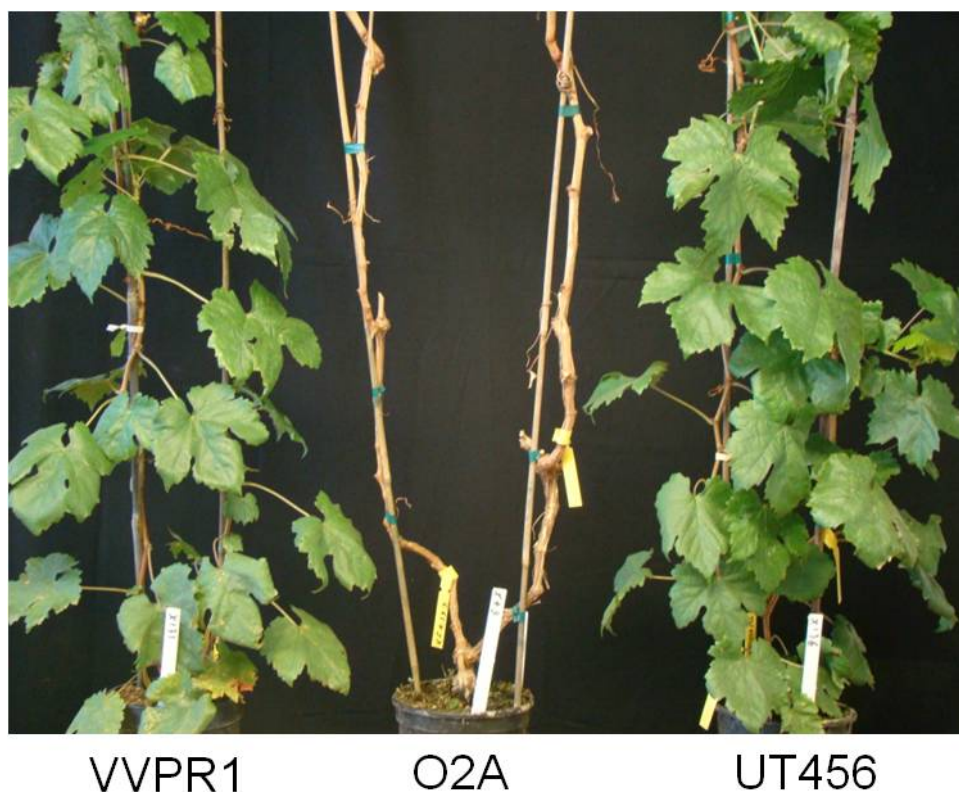


Figure 1. Protection of transgenic Thompson seedless plants by expression of VVPR1 (left) or UT456 (right) compared to control (center). Plants were mechanically inoculated with 200,000 *Xf* cells 4 months prior to photographing.

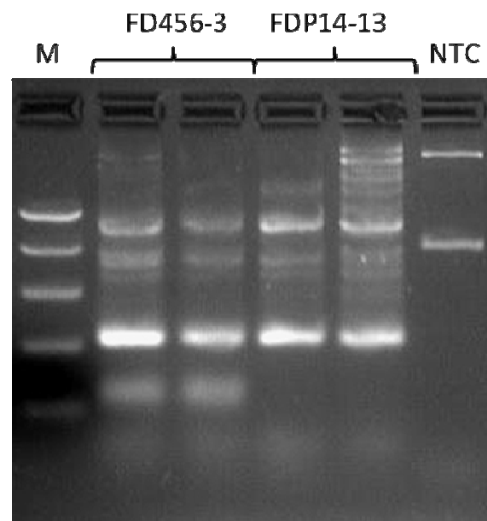
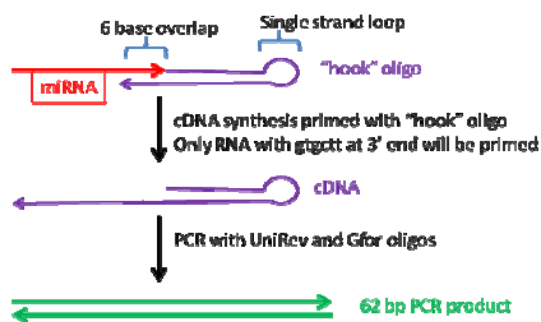


Figure 2. “Hook assay” for detection of UT456 microRNA. Schematic of method (left) shows “hook” oligo used to prime cDNA synthesis and PCR primers to amplify a 62bp fragment. Ethidium bromide stained gel (right) shows presence of UT456G microRNA only in UT456 expressing plants.

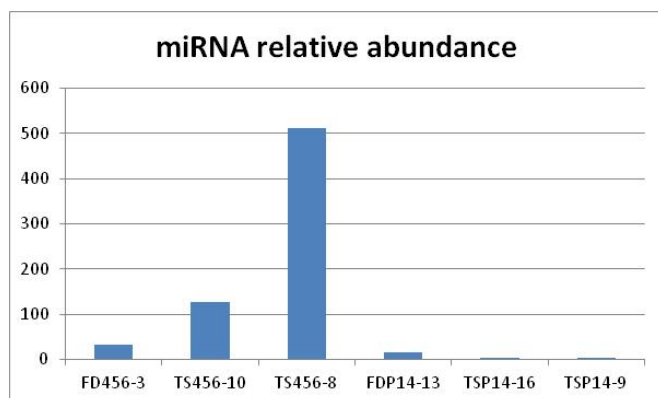


Figure 3. Taqman assay for detection of microRNA UT456-G. RNA was isolated from leaves of the respective genotypes and the abundance of UT456-G was measured.

**Table 1.** Relative susceptibility of Wine grape Varietals were evaluated by qPCR following mechanical inoculations of greenhouse grown plants. Disease rating is a 1-5 scale with 1 = asymptomatic and 5 = defoliated. *Xf* bacterial titers are expressed as bacterial cells per 0.1gm of stem tissue. Evaluations were done at 4 months post inoculation. See Figure 1 for representative pictures.

Varietal	Rating (5 is highest)	Xf titer
Cabernet Sauvignon	4	$5 \times 10^6$
Chardonnay	3	$5 \times 10^5$
Merlot	2	$7 \times 10^6$
Pinot Gris	5	$1 \times 10^7$
Pinot Noir	4	$1 \times 10^5$
Sauvignon Blanc	3	$1 \times 10^5$
Syrah	1	$7 \times 10^4$
Zinfandel	3	$5 \times 10^5$



**Table 2.** Freedom rootstock expressing transgenes grafted to untransformed Thompson Seedless scions and mechanically inoculated with 20,000 Xf “Temecula” in a 20ul drop. Disease rating is a 1-5 scale with 1 = asymptomatic and 5 = defoliated. Bacterial titers are expressed as bacterial cells per 0.1gm of stem tissue. Evaluations were done at 12 months post inoculation. (See Figure 3 for a representative image of control and transgenic rootstock/ wild scion plants)

Transgenic notation	Relevant genotype (transgenic rootstocks grafted to untransformed Thompson seedless scions)	Ratio transgenic graft-protected plants with leaf retention equal to <i>Vitis californica</i> vs those plants dead	Range of bacterial load per 0.1 gm of stem in at 4 months post inoculation
<u>TS02A</u> FD456-15	CaMV 35S-driven 456 Freedom rootstock	8/8 healthy, none dead Rating 1-2	$10^3 - 10^4$
<u>TS02A</u> FDPR1-13	CaMV 35S-driven PR1 Freedom rootstock	5/5 healthy, none dead Rating 1-2	$10^3 - 10^4$
<u>TS02A</u> FD3 (wild type) Control	Untransformed Thompson Seedless scion	10/13 dead (R5), 3/13 barely alive (R4)	$10^6 - 10^7$
<i>Vitis californica</i>	Asymptomatic wild type untransformed host.	no death (R5) after 12 months post inoculation	$10^4$

