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

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ABSTRACT
Previous research in our lab first established a determinative role for a genetically regulated process of programmed cell death (PCD), in the leaf scorch and cane death symptoms in Pierce's disease (PD) and then developed a functional screen for PCD-suppressing plant genes from a cDNA library of grape genes. The functional screen identified six candidate DNA sequences potentially able to suppressing *Xylella fastidiosa* (*Xf*)- induced PCD when expressed as transgenes. Two of the sequences (VvPR1 and UT456) were selected to test for ability to suppress the PCD-dependent symptoms of PD. Greenhouse experiments over several years 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. All untransformed control plants died within 2-3 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.

LAYPERSON SUMMARY
Xylella fastidiosa 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, 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 were begun in July 2010 with additional plantings to be made in Solano and Riverside Counties in 2011.

INTRODUCTION
Susceptibility in most plant-microbe interactions depends on the ability of the pathogen to directly or indirectly regulate genetically determined pathways leading to apoptosis or programmed cell death (PCD). The role of altered cell stability in disease through an evolutionarily conserved program involving programmed cell death occurs in both animals and plants. Functionally, the induction of PCD results in an orderly dismantling of cells while maintaining integrity of the plasma membrane until internal organelles and potentially harmful contents including phenolics, reactive oxygen and hydrolytic enzymes have been rendered harmless to contiguous cells. Processed in this manner, the cell contents can serve as nutrients for microbial cells when they are present in the immediate environment of the pathogen (2). In the case of *Xylella fastidiosa* (*Xf)* and many other plant pathogenic bacteria, the bacteria live predominantly as endophytes or epiphytes but occasionally as pathogens. The relative susceptibility of the individual plant species is determined by unknown genetic factors. Presumably, sensitivity to the presence of the bacteria, expressed as cell death-dependent symptoms, is the result of signals expressed by the bacteria that lead to activation of PCD, as appears to be the case with Pierce's disease (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, While protection against PD appears to be feasible in plants where the transgenes are expressed constitutively throughout the plant, it remains critical to determine whether transfer of this protection can occur across a graft union to untransformed scions. Hence, we have constructed transformed rootstocks (Freedom and Thompson Seedless) expressing PR1 or UT456 grafted to untransformed Thompson Seedless and winegrape scions to be tested first by greenhouse inoculation and later in field plantings if the greenhouse tests are positive for protection. To identify possible winegrape varieties to use as differential scions we completed testing the relative susceptibility of 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 before any field evaluation is undertaken. The greenhouse assays for these eight varieties have been completed and are summarized herein. Initial greenhouse inoculation experiments indicated that the protection by PR1 and UT456 does move across the graft union. Final data on these experiments will conclude in 2011. 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

1. Complete the evaluation of the additional four candidate anti-apoptotic genes transformed into PD susceptible Thompson Seedless plants. (2010-2011)
2. Evaluate the relative susceptibility of eight commercial winegrape varieties to PD and titer of *Xf* in the inoculated canes. (2010-2011)
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. (2010-2012)
4. 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)
5. Perform inoculations the eight winegrape varieties, initially on their own rootstocks and subsequently on Freedom and Thompson Seedless rootstocks expressing VvPR1 and UT456. (2012)
6. Investigate the mechanism underlying the protection against PD by VvPR1 and UT456. (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. (2010-2011)
8. 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. (2010-2011)

The protective genes or DNA sequences, isolated by a functional anti-PCD screen (1), have been described in (3,4,5). Greenhouse inoculations were completed in 2010-2011 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 µl 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.

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. This experiment was conducted under controlled greenhouse inoculation conditions to avoid any vagaries associated with natural infection and GWSS preferences. 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 expressing VvPR1 and UT456. 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 to be 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 µl 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 2**). 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 symptom level and bacterial titers appeared to be well correlated as seen in the photos of representative plants of each variety (**Figure 1**). 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: scion combinations will begin in the Fall of 2011.

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. (2010-2012)

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 3** and **Figure 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 2011 (see Objective D).

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

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, know 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 be understood. 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. Other research in the lab has developed a highly sensitive Taqman-based assay capable of detecting specific and low abundant microRNA sequences in plant extracts. In addition, PR1 antibodies will be used to test directly for the presence of mobile PR1 protein from the rootstock into the grafted scions. In addition PR1 antibodies are being used in immunoprecipitation assays to detect potential PR1 interacting factors. To date we have been successful in identifying 3 PR1-interacting proteins, HP70, HP90 and RACK1 from plant extracts. Interestingly, these three proteins have previously been reported to interact directly with each other and occur in a membrane associated complex involved in innate immunity to rice blast in rice plants transgenic for RACK1 (7).

Collaboration with 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. 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 3**). 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 3**). 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 3C**) 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 $1x10^{2}$ to $7.5x10^{3}$ per 0.1 g of stem tissue in the inoculated plants. Untransformed control plants were negative for the transgenes. As of October 2, 2011, no typical field symptoms or defoliation of leaves was observed on any of the inoculated plants.

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. 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.

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Table 1. List of potential plant anti-apoptotic genes derived from functional cDNA screen. Each has now been 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.

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, found in all plant species examined	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 and in the animal kingdom	R=1; 10^3
Vv PR1	Pathogenesis related protein found in all plant species	R=1; 10^3

Figure 1. 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. Photos taken and *Xf* titers (red inset numbers) in 0.1g of stem tissue were measured by qPCR at 4 months after inoculation compare three of the varieties with the control plants. The quantitative comparisons of all the varieties are shown in **Table 3**.

Table 2. Relative susceptibility of winegrape 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	5×10^6
Chardonnay	3	5×10^5
Merlot	2	7×10^6
Pinot Gris	5	1×10^7
Pinot Noir	4	1×10^5
Sauvignon Blanc	3	1×10^5
Syrah	1	7×10^4
Zinfandel	3	5×10^5

Figure 2. 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. Summer 2010 results of greenhouse PD 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.

Table 3. 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 *Vitis californica* vs those plants dead	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	8/8 healthy, none dead Rating 1-2	10^3 - 10^4
TS02A FDPR1-13	CaMV 35S-driven PR1 Freedom rootstock	5/5 healthy, none dead Rating 1-2	10^3 - 10^4
TS02A FD3 (wild type) Control	Untransformed Thompson Seedless scion	10/13 dead (R5), 3/13 barely) alive (R4	10^6 - 10^7
Vitis californica	Asymptomatic wild type untransformed host.	no death (R5) after 12 months post inoculation	10^4

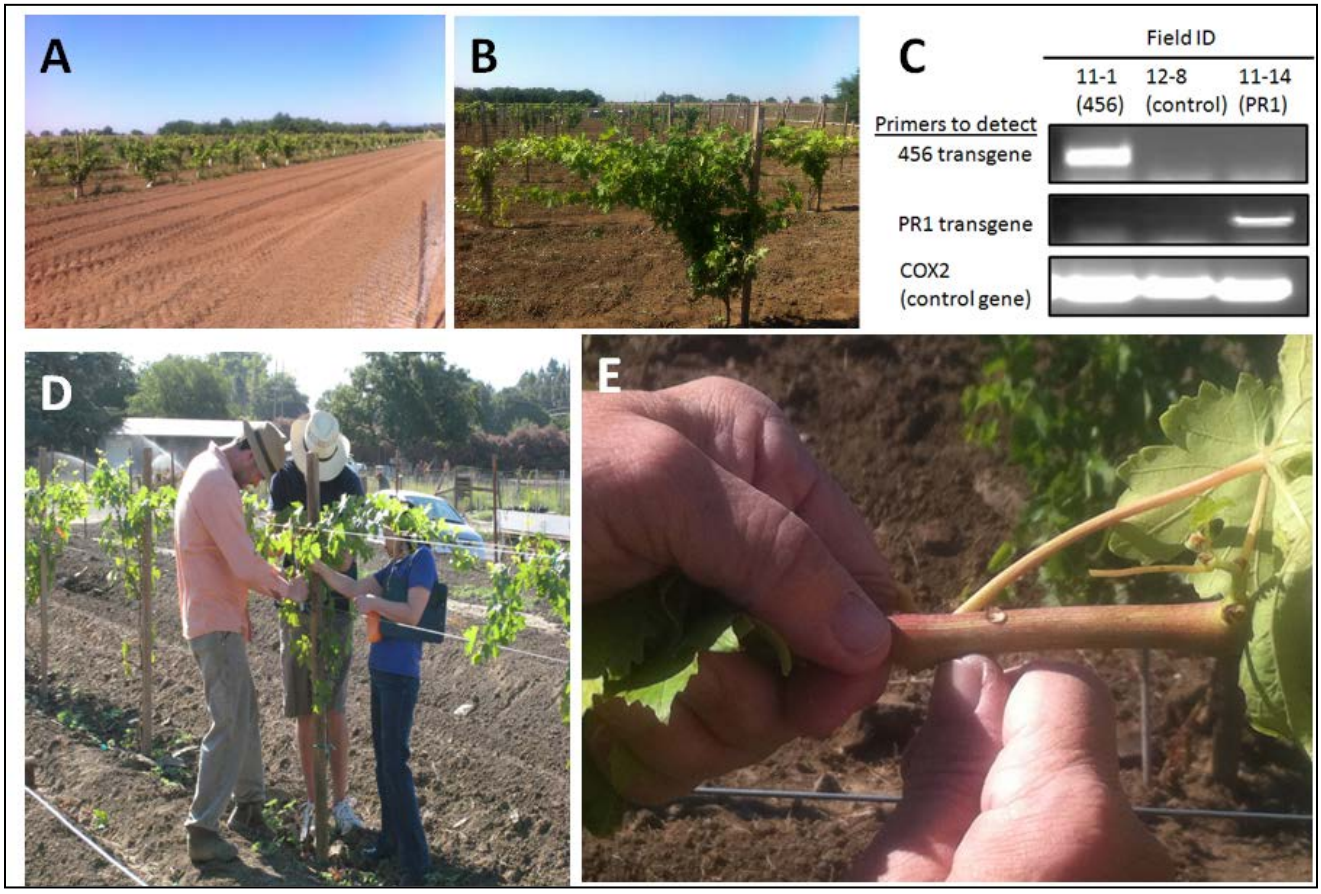

Figure 3. The Gilchrist section of the Solano Field has been completely planted, as have those of all the other investigators. Currently our planting consists of 75 own-rooted and 75 grafted plants including controls (A and B). Checks of genotype identity of several plants were performed by sampling genomic DNA and using specific primers in PCR. DNA was extracted from canes of field plants 11-1, 12-8 and 11-14 and analyzed by PCR using pairs of primers for the 456 transgene, the PR1 transgene and an endogenous grape gene cytochrome c oxidase (COX2) (C). Plants of all investigators were mechanically inoculated with 20,000 cells of *Xf* "Temecula" in a 20ul drop on July 21, 2011 (D and E). Preliminary sampling and analysis by qPCR of several inoculation sites confirmed the presence of *Xf* in each of the inoculation sites tested at 1 month after inoculation.