Interim Progress Report for CDFA Agreement Number 12-0443-SA

Project Title Field evaluation of grape plants expressing PR1 and UT456 transgenic DNA sequences for protection against Pierce's Disease.

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Time period covered by the report October 2013 to March 2014

Project History Initiated in 2010 with proposed continuation through 2016.

Introduction.

The objective of the field experiments in Riverside and Solano counties is to evaluate transgenic grape plants and grape rootstocks expressing two DNA constructs designated PR1 and UT456 under field conditions for resistance to *Xylella fastidiosa* (Pierce's Disease strain) (Xf). Infection at the Solano site is by mechanical inoculation whereas infection at the Riverside site is by endemic sharpshooters that carry *Xf*. The basis for this experiment derives from four previous inoculation experiments in a controlled greenhouse over a two year period, involving more than 300 transgenic plants of several primary transformants of PR1 and UT456 in either susceptible Thompson Seedless or the rootstock Freedom These experiments indicated suppression of PD symptoms and reduction in bacterial titer occurred in the transgenics compared with untransformed control plants. Data collected in 2012-13 from both field sites indicate that the bacteria are present in all plants at the Riverside site and in the mechanically inoculated plants at the Solano site. Control plants containing *Xf* at both locations show symptoms of Pierce's Disease and cane or plant death. Both the PR1 and UT456 expressing plants show suppression of symptoms and reduced bacterial counts at both sites. Quantitative data collection is continuing at both sites.

Objectives of research ongoing

The objective is to continue to evaluate transgenic grape plants and grape rootstocks expressing two DNA constructs designated PR1 and UT456 at the Riverside and Solano county sites for resistance to *Xylella fastidiosa* (Pierce's Disease strain). A positive correlation between the PR1 and UT456 message level, suppression of bacterial titer and absence of PD symptoms was established first in controlled greenhouse experiments using qPCR to measure both the transgenic message level and the bacteria titer as indicated in the introduction.

The field experiment in Solano county is being conducted in two phases. The first phase of the experiment that began in 2010 was designed to evaluate clonal copies of the fully

transformed ungrafted plants that exhibited suppressed PD symptoms and low bacterial titers in the greenhouse. The second phase begun in 2011 consisted of untransformed commercial scions grafted onto the most resistant of the PR1 and UT456 lines as rootstocks. All plantings are in an APHIS approved field area with strict control on all movement of plant material. There is no history of any movement of the bacteria from plant to plant in this experimental location even though *Xf* has been present in an adjacent disease nursery for at least 3 decades. The test plants in both phases have been mechanically inoculated with two different pathogenic strains used in the greenhouse experiments (one from Temecula, one from Napa).

Specific objective with time lines.

A. The overall objective is to evaluate fully transformed grape plants and grape rootstocks expressing two DNA constructs designated PR1 and UT456 genes in a field site in Solano County for resistance to the Pierce's Disease strain of Xylella *fastidiosa* (*Xf*) following mechanical inoculation.

B. The field experiments in Solano County will be conducted in two phases. The first phase of the field experiment started in 2010 will evaluate clonal copies of the fully transformed ungrafted PR1 and UT 456 plants that exhibited suppressed PD symptoms and low bacterial titers. These experiments will consist of sets of inoculated and uninoculated control plants. All plants to be inoculated will be infected by stem puncture with ~20,000 *Xf* bacterial cells per inoculation site. Inoculations were done July 2011 and repeated in June of 2012.

C. The second phase of the Solano County field planting began in 2011 with planting the untransformed commercial scions grafted onto the most resistant of the PR1 and UT456 plants as rootstocks followed by inoculation in 2012 and 2014.

D. The second field experiment in Riverside County was planted in the Spring of 2011. The planting consisted of clonal copies of the fully transformed ungrafted plants expressing PR1 or UT 456 that were planted in 2010 in Solano County. These Riverside plants will not be inoculated with *X. fastidiosa* but have been exposed to infection via natural populations of the Glassywinged sharpshooter Xf vector.

Description of activities to accomplish objectives

There were no distinguishable morphological differences in the control plants compared with any of the transgenic lines using criteria of descriptors described by the International Organization of Vine and Wine. All plants have a normal phenotype, true to the untransformed control plants of each parental genotype (Figures 1, 2, 4). As of October 2013, the inoculated plants have been confirmed to harbor the introduced *Xf*. Uninoculated individuals are healthy, growing normally and tested free of *Xf*. The plants were confirmed to have been successfully infected in the 2011, 2012 and 2013 inoculations by sampling individual inoculated canes followed by qPCR analysis for relative bacterial populations (references 1 and 2). Bacterial numbers varied from 500-1500 cells per 1 cm of inoculated stem tissue. Uninoculated control plants appear healthy while the inoculated controls and some of the experimental lines are showing symptoms of PD, including bud and cane death (Figures 5 & 6). At this stage it is clear that there is a rich source of additional data to be

collected that will prove important as we move forward in experiments to combine the best of the transgenes into commercially accepted rootstocks to assess trans-graft protection.

Currently, we are requesting extension of support for the field experiment an additional two years to continue collecting data from this site through June 30, 2016. It is critical that more extended data collection occur for several reasons. First, the need to determine the sustainability of PD disease symptom suppression observed to date over at least a 4 year period since the plants were last exposed the *Xf*, which will be covered by this extension. Secondly, we will be collecting new additional information on the behavior of the bacteria in the control and transgenic plants. Third, it is critical that we collect data on the movement of the transgenes (signals) across the graft union from the transgenic rootstock to the untransformed scion in the plants that have been exposed to the bacteria for two years. We also need to continue monitoring bacterial concentration and movement in the untransformed scion tissue of the grafted plants with repeated samplings from spring to fall of 2014 and 2015. These are all important data sets to determine when or if there is movement as key data required in pursuing Federal deregulation of these genes.

Test plants were planted in a complete randomized block design. Evaluation of the experimental plants for plant morphology, symptoms of Pierce's Disease infection, including dead or dying canes , and the presence of the bacteria will be over a time course by visual monitoring of symptom development (Figures 3 & 7) and sampling inoculated tissue for bacteria plant tissues (mainly leaves and stems) by quantitative PCR (qPCR) assays. A comparative quantitative determination by qPCR of the presence of Xylella in transgenic grape and grape rootstocks compared with conventional grape and grape rootstocks will provide an indication of the level of resistance to Pierce's Disease infection and the impact on the bacterial load in the respective transgenic and control plants.

- Assessment of symptom expression in Xf-inoculated grape plants and plant phenotype Inoculated grape plants are monitored for symptom development (leaf scorch and leaf senescence) and rated according to a subjective 5 point scale illustrated in previous PD Symposium reports. Growth characteristics of the inoculated canes both on transformed and untransformed rootstocks will include; cane elongation rate (cm/day over 90 days), internode length (cm) and leaf shape (photos)
- qPCR analysis of *X. fastidiosa* presence, movement, and population dynamics in the inoculated plants and compared with inoculated untransformed plants of each of the winegrape varieties and cuttings of the original transformed PR1 and UT456 plants *X. fastidiosa*-GFP movement and relative concentration will be determined by quantitative PCR (qPCR) and of stem segments and petioles of inoculated canes. The inoculated material is assayed over a time course following *Xf* inoculation. The qPCR assays have been and will continue to be used to accurately assess the level and amount of *X. fastidiosa* multiplication and movement in field plants. In our application the qPCR assay consists of quantifying the *Xf* 16S ribosomal amplification product from grape stem DNA extracted from inoculated stems compared to standards generated by diluting known amounts of *Xf* cells into healthy grape stem extractions. The method is tightly controlled and highly reproducible in our hands.

Conclusions:

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. In addition, we have proposed to stack these genes along with those found to be effective under field conditions by other researchers.

Publications in preparation:

- Juan Sanchez, James Lincoln, and David Gilchrist, 2014. Pathogenesis-related protein PR-1 interferes with programmed cell death and is synthesized under translational control
- Juan Sanchez, James Lincoln, and David Gilchrist, 2014. The translation of pathogenesis-related-PR-1 is triggered by by a miRNA excised from grape coding sequences and the coding sequence of grape fanleaf virus.
- James Lincoln and David Gilchrist. 2014. Pierece's Disease suppression in grape by transgenic expression of DNA sequences capable of blocking programed cell death.

Layperson summary of project accomplishments

Previously, we identified novel anti-PCD genes by a functional screen from cDNA libraries of grape. Two of these grape sequences (PR1 and UT456) expressed as transgenes in grape, suppressed Pierce's Disease (PD) symptoms and dramatically reduced bacterial titer in inoculated plants under greenhouse conditions. Field experiments underway in Solano and Riverside counties, conducted with an APHIS permit, will evaluate clonal copies of the most resistant transgenic plants under field conditions for resistance to PD. The field evaluation will be conducted at the respective sites will involve mechanical inoculation with *X. fastidiosa* in Solano County and Glassy Winged Sharpshooter inoculation in Riverside County. Data sets will include visual monitoring of plant morphology, PD symptoms and bacteria titer by quantitative PCR (qPCR) assays. To date, PCR data and plating assays confirm the presence of Xf in the plants at both locations. Differential protection against defoliation was observed at the Riverside site and PCR assays confirmed bacterial populations in the plants. Bacteria are present in inoculated plants at the Solano site and there is definitive evidence of extensive symptom differences between several of the transgenic plants and the non-transgenic control. Clearly both sites will need to be monitored and assays taken over a longer period of time for conclusive results to be obtained.

Research Timetable Inclusive of the two years from 7/1/14 to 6/30/16, the evaluations will continue through the June of 2016, at which point the planting will be destroyed following the protocol in the APHIS permit.

Research Capacity and Likelihood of Accomplishing Objectives The field area has been designated as an APHIS approved site under 7CFRE340. Commercialization of the currently effective anti-PCD containing vines and/or All experimental protocols have been used successfully to evaluate the field plants over the past 2 years and will be expected to continue to 2016.

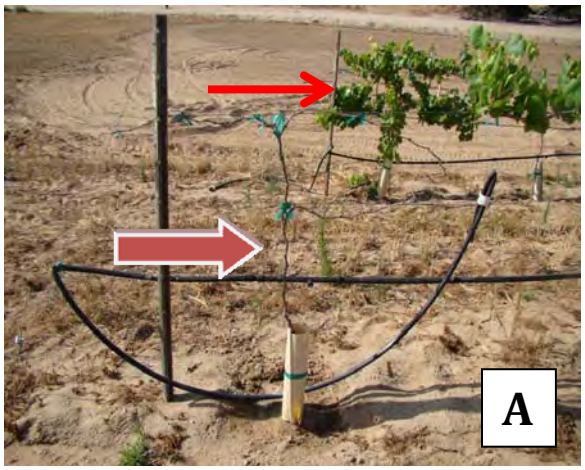
Status of funds. All funds budget for these projects will be expended at the end of the current funding cycle as proposed.

Summary and status of intellectual property 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. David. Gilchrist, and James.Lincoln 2011. Disease control and bacterial population dynamics in winegrape varieties grafted to rootstocks expressing anti-apoptotic sequences. Proceedings of the Pierce's Disease research symposium. Sacramento, CA December 13-15.
2. David. Gilchrist, and James.Lincoln 2013. Field evaluation of grape plants expressing PR1 and UT456 transgenic DNA sequences for protection against Pierce's Disease. Proceedings of the 2013 Pierce' Disease Research Symposium

Figure 1. Untransformed control Thompson Seedless plant showing leaf death and coloration considered to be associated with Pierce's Disease in the Riverside plots in 2012.



August 2013
Riverside County

Figure 2. Untransformed control Thompson Seedless plant in the foreground (maroon arrow) above in panel A showing complete plant collapse and death compared with the plant in the background (red arrow) expressing the PR1 transgene that appears free of PD symptoms, which is also seen in a close-up image in panel B.

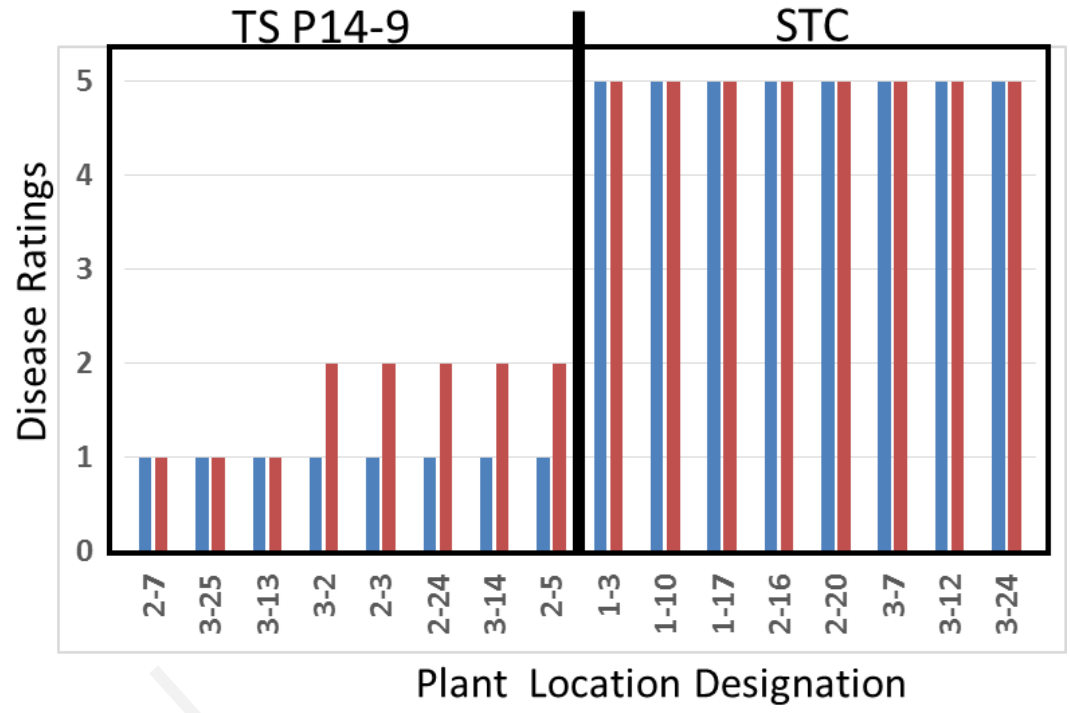


Figure 3. Plants established in Riverside County in 2010 were rated for symptoms of Pierce's Disease in June and August of 2013 on a 1 to 5 scale with 1 being healthy and 5 dead. June 2013 ratings are blue and August 2013 ratings are red. STC designates a susceptible transgenic control into which the 456 transcript was inserted but is not expressed and is used in this planting as a susceptible transgenic control. P14-9=PR1-9

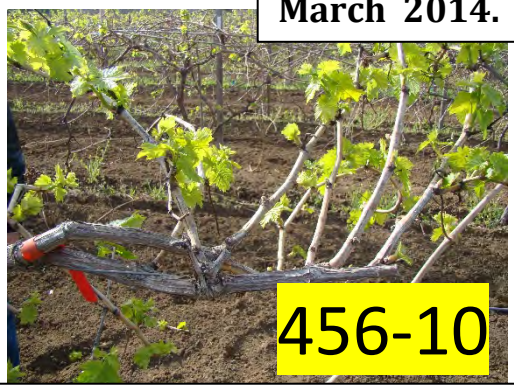
Non transgenic control

April 2013
Solano County

PR1-9 transgenic

Figure 4. Inoculated cane of an untransformed control Thompson Seedless plant showing death of shoots shortly after emergence in April of 2013 (A). Inset shows close-up view of the shoot on an inoculated cane that died shortly after emergence. Plant on the right (B) is an inoculated transgenic PR-1that is free of any dead shoots. See figures 5, 6, and 7 for 2014 data on current stat us of these

March 2014. Solano County



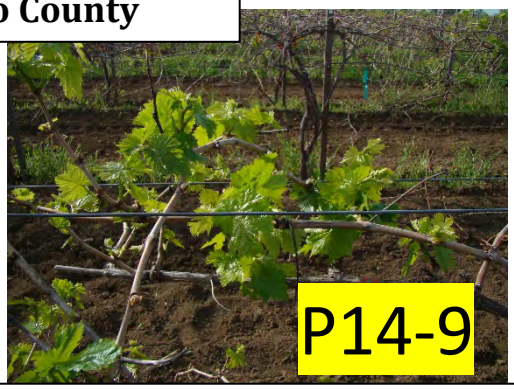


Figure 5 Untransformed control Thompson Seedless (O2A), a susceptible transgenic control (STC) plant showing shoot death shortly after emergence compared with transgenic PD suppressive expressing difference anti-programmed cell death genes. See figures 6 and 7 for additional images and data

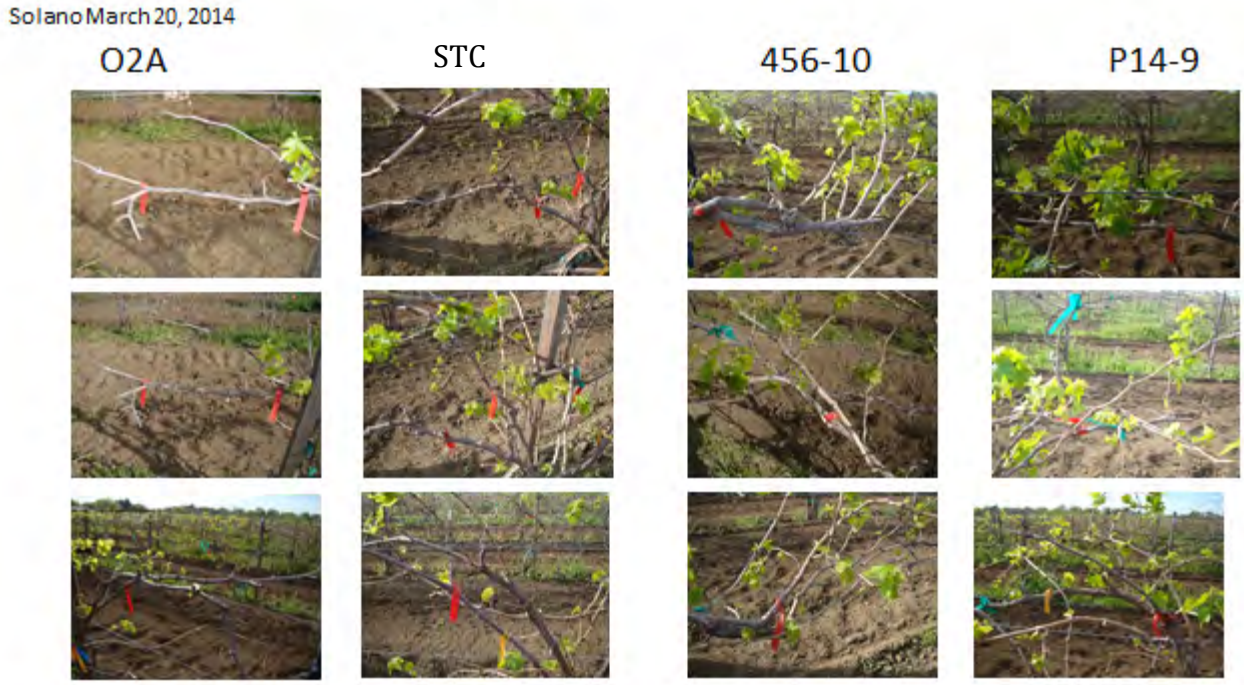


Figure 6. Additional images, including the ones in Figure 5, of untransformed control Thompson Seedless (O2A), a susceptible transgenic control (STC) plant showing shoot death shortly after emergence compared with transgenic PD suppressive expressing difference anti-programmed cell death genes. See figure 7 for summarized data

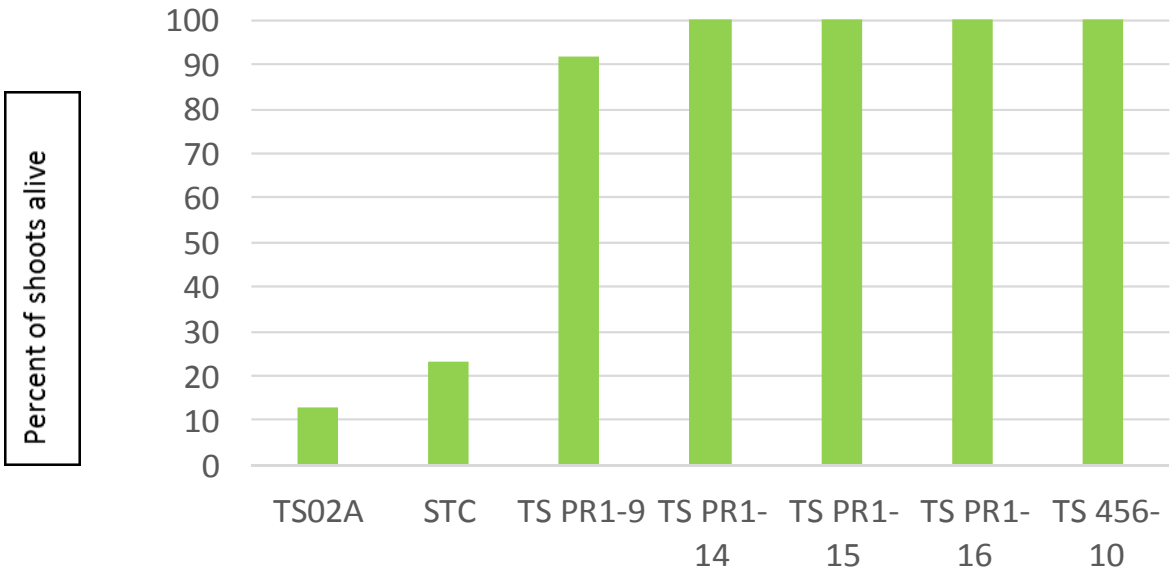


Figure 7. March 17, 2014 evaluation of inoculated canes on untransformed Thompson Seedless (TS02A) , a transgenic susceptible control (STC) and transgenic lines expressing disease suppressive genes . 15 shoots were assessed in 3 plants per genotype as the plants emerged from dormancy. Figures 5 and 6 illustrate the stage of growth and appearance of the shoots.