Interim Report for CDFA Agreement Number 12-0443-SA

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

Principal Investigator	David Gilchrist	Department of Plant Pathology, UC Davis	dggilchrist@ucdavis.edu
Co-Principal Investigator	James Lincoln	Department of Plant Pathology, UC Davis	jelincoln@ucdavis.edu

Reporting Period: The results reported here are from work conducted February1, 2013 to July 15, 2013

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). In eukaryotic systems, PCD is regulated by activators or inhibitors, which may be either endogenous or from exogenous sources including bacteria, fungi and viruses. In the case of *Xf*, the bacteria live predominantly as endophytes or epiphytes but occasionally as pathogens. We demonstrated previously that the death symptoms associated with PD exhibit characteristic molecular markers of PCD. Presumably, sensitivity to the presence of the *Xf* bacteria, expressed as cell death-dependent symptoms, is the result of signals expressed by the bacteria that lead to activation of PCD. Our research has focused on the effect of altering the expression of two different plant DNA sequences (PR1/P14a and UT456) that were obtained from a functional anti-PCD screen of grape and tomato cDNA libraries. The UT456 is a noncoding sequence that contains small RNA hairpin structure indicative of a potential regulatory microRNA. The nomenclature of PR1 gene can be confusing since PR1 refers to the coding sequence of the gene while the product of the PR1 gene is a 14 kilodalton protein referred to in the literature as p14a. Hence, there is a duplicative description of the construct inserted into the grape plants, but for simplicity and continuity we have chosen to refer to this construct of the gene in the transgenic plants as the protein product P14-x, with x referring to independent transgenic plants. Both PR1 and UT456, expressed transgenically, protected against PD symptoms and limited bacterial titer four to six orders of magnitude below that reached in untransformed plants of PD susceptible Thompson Seedless and the commercial rootstock Freedom in repeated inoculation experiments under greenhouse conditions. Current field experiments are in progress to evaluate transgenic grape plants and grape rootstocks expressing P14a and UT456 constructs in field sites in Solano and Riverside Counties for suppression of PD. We have own-rooted transformed Freedom and Thompson Seedless grape plants, and we have transformed rootstocks (Freedom and Thompson Seedless) expressing PR1 or UT456 grafted to untransformed Thompson Seedless scions to assess possible protection across a graft union. Initial greenhouse inoculation experiments indicated that the protection by PR1 and UT456 does move across the graft union

Infection at the Solano site is by mechanical inoculation, in contrast to the Riverside site, which depends on natural inoculation by endemic sharpshooters carrying *Xf*. The Solano field experiment is conducted in two phases. The first phase started in 2010 to evaluate clonal copies of the fully transformed own-rooted plants that exhibited suppressed PD symptoms and low bacterial titers. The second phase began in 2011 with planting the untransformed Thompson Seedless scions grafted onto the most resistant of the PR1 and UT456 plants as rootstocks. Over the course of the 3 year field evaluation at both sites, test plants in the first planting (2010) include own-rooted conventional Thompson Seedless and Freedom plants as controls to be compared with the transformed plants. Controls in the second planting phase (2011) include, untransformed rootstocks grafted to

the untransformed scions, which will be compared to equivalent combinations expressing the test genes grafted to untransformed PD susceptible scions. Inoculations were done in 2011, 2012 and 2013. Bacterial assays of conducted in 2012 from both sites indicate that bacteria are present in all plants at the Riverside site and in the mechanically inoculated canes at the Solano site. Some plants in Riverside are showing symptoms of leaf and cane death while the plants in Solano remained healthy in appearance until late September, early October of 2012 when a number of canes on the inoculated control plants appeared to be dying. Clearly, at least one more year of evaluation is needed to begin to develop an assessment of the possible field efficacy of the transgenes. Quantitative data collection is in progress at both sites.

OBJECTIVES 2010-2013

A. The first objective is to evaluate transgenic 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.

The field experiments in Solano County are being conducted in two phases. The first phase of the field experiment that started in 2010 will evaluate clonal copies of the fully transformed own-rooted PR1 and UT456 plants that exhibited suppressed PD symptoms and low bacterial titers under greenhouse conditions. These experimental materials consist of sets of inoculated and uninoculated control plants. Treated plants were inoculated with ~20,000 *Xf* bacterial cells per inoculation site by stem puncture in July 2011, then repeated on different canes with ~200,000 *Xf* bacterial cells per inoculation site in June of 2012 and a third inoculation with ~200,000 *Xf* bacterial cells per inoculation site was done in June, 2013.

B. 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. Inoculations per schedule in A above were carried out in 2012 and 2013.

C. The second field experiment, located in Riverside County, was planted in the spring of 2011. The planting consisted of clonal copies of the fully transformed own-rooted plants expressing PR1 or UT456 that were planted in 2010 in Solano County. The Riverside plants were infected with *Xf* via natural populations of GWSS (*Xf* vector).

RESULTS AND DISCUSSION

A. *The first phase of the field experiment started in 2010 to evaluate clonal copies of the fully transformed own-rooted (ungrafted) PR1 and UT456 plants that exhibited suppressed PD symptoms and low bacterial titer under extensive greenhouse testing (2010-2013).*

This phase took place as planned with the planting occurring on July 12, 2010. Evaluation of the experimental plants for plant morphology, symptoms of Pierce's Disease infection, and the presence of the bacteria involve a time course by visual monitoring of symptom development and sampling inoculated tissue (mainly stems) for *Xf with* assessment 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. All procedures have been used successfully in the ongoing greenhouse experiments for the past 5 years. The plants were confirmed to have been successfully infected in both the 2011 and 2012 inoculations by sampling individual inoculated canes followed by qPCR analysis for relative bacterial populations. Bacterial numbers varied from 500-1500 cells per 1 cm of inoculated stem tissue in the fall of 2011 sampling and no difference between the control plants and transgenic plants. Also,

there were no distinguishable morphological differences in the control plants compared with any of the transgenic lines using criteria of descriptors produce by the International Organization of Vine and Wine. There were no detectable symptoms of PD in leaf or stem tissue at the end of the 2011 season nor when buds and leaves emerged in the spring of 2012. Vines were pruned to retain inoculated canes and to provide for 2-4 additional canes for inoculation in the early summer of 2012. The second sets of inoculations were done in June of 2012. Some inoculated canes began to express leaf and stem symptoms of cell death compared with uninoculated canes at end of the 2012 growing season. Individual canes were showing symptoms consistent with PD as the plants emerged from dormancy in the spring of 2013. Individual inoculated cane were rated as dead or alive in April 2013 (figure 1). Both the P14-9 and UT456-10 showed substantial reduced death compared with the susceptible control canes. Bacterial sampling again showed Xf to be present in the surviving canes but no qPCR positive data was recovered from the dead canes (tissue too dried out). There was no evidence of cane death in any plants and canes that had not been inoculated. All cane death was restricted to inoculated canes, with a few inoculated plants showing death of the entire plant but, again, no plant or cane death in uninoculated plants. Representative plant images are shown in figures 2-6. Captions on individual figures describe the typical symptoms or plant appearance in relation to disease-associated tissue death. Conclusion is that both P14-9 and UT456-10 exhibited greatly reduced cane death with the P14-9 less impacted than the UT456. The plants were re-inoculated in June of 2013. Samples for bacterial analysis and scoring of individual inoculated canes will be done in August of 2013.

B. ***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 (2010-2013).*** Transgenic rootstocks for grafting were made by removing green shoots from greenhouse-grown plants of Thompson Seedless and Freedom expressing either PR1 or UT456, surface sterilized for 30 seconds in 70% ethyl alcohol, followed by 1% sodium hypochlorite solution containing 0.2% Tween 80 for 20 min with shaking, on a rotary shaker (50 rpm). The surface sterilized shoots are cut into single node pieces and placed into solid growth media to stimulate root formation. All the grafting is conducted in sterile Magenta GA-7 Plant Culture Boxes (3 x 3 x 4") containing 50 ml media under a 16 h light, 8 h dark photoperiod at 25^{o}C. Rootstock plantlets obtained *in vitro* are allowed to grow until several leaves are produced (4-6 weeks) and divided into 3–4 explants, each containing a single node. A scion with a single node and a leaf was selected to match the size of the rootstock; cut into a wedge to match a cleft made in the rootstock and was carefully fitted on to the cleft of the rootstock on the medium. After 4 weeks incubation healing in a magenta box, the rooted plantlet is transferred to sterile soil, allowed to heal and then transferred to the greenhouse for assays. Success rate is greater than 95% using this procedure, is more space efficient relative to greenhouse grafting, can be done anytime of the year, and is as rapid as green grafting. The plants for the Solano County phase two were planted in the field May 17, 2011. These plants were first inoculated in June of 2012. The plants were confirmed to have been successfully infected in 2012 by sampling individual inoculated canes followed by qPCR analysis for relative bacterial populations. Bacterial numbers varied from 200-1000 cells per 1 cm of inoculated stem tissue in the fall of 2012 sampling and there were no differences between the control plants and transgenic grafted plants. There were no detectable symptoms of PD in leaf or stem tissue at the end of the 2012 season nor when buds and leaves emerged in the spring of 2013. However, as the plants began to leaf out, there were noticeable areas of bud failure on the inoculated control plants (figure 6) compared with the transgenic grafted P14-9 shown in figure 5. Plants were re-inoculated in June of 2013 and evaluations will continue during the summer and fall for symptoms and bacterial population levels.

C. ***Establish a field planting in Riverside, County consisting of clonal copies of the fully transformed ungrafted PR1 and UT456 plants that were planted in Solano County in 2010. (2011-2013)***
Field planting occurred April 2011. The GWSS populations were sufficiently high to initiate infections in all plants in this location based on both symptom and bacterial assays in June 2012 and the GWSS populations were high in 2011 and 2012. Samples of cane tissue are currently being analyzed. Symptoms of PD associated death were evident in 2012 and early spring of 2013. All plants were rated for disease severity (Figure 7 shows a comparison of P14-9 with the untransformed Thompson Seedless control. Images were captured of all plants, example of which are seen in Figures 8-9. Bacterial assays are in progress and further sampling is planned for late summer or early fall. Clearly, the level of disease impact is high and this site appears to have afforded infection levels that affect plant survival in the presence of PD.

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

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 titer 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. Current data suggest that some, but not all, of the transgenic sequences suppress the disease without eliminating the bacteria (not antibiotic in action). In addition, there this preliminary observations that one or more of the transgenic 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 on the plants at the Solano County site.

LAYPERSON SUMMARY

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. This project will evaluate clonal copies of these same plants under field conditions for resistance to (PD). The field evaluation will be conducted in Solano and Riverside Counties and will include 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 definitive results to be obtained.

INTELLECTUAL PROPERTY Record of invention disclosures will be submitted to the UC Office of Technology Transfer.

STATUS OF FUNDS: Funds are being expended at a rate consistent with the project proposal and budget.

Figure 1. % inoculated canes alive two years post inoculation at the Solano County site. Plants are own rooted Thompson Seedless transgenic (P14-9 and 456-10) and non-transgenic controls.

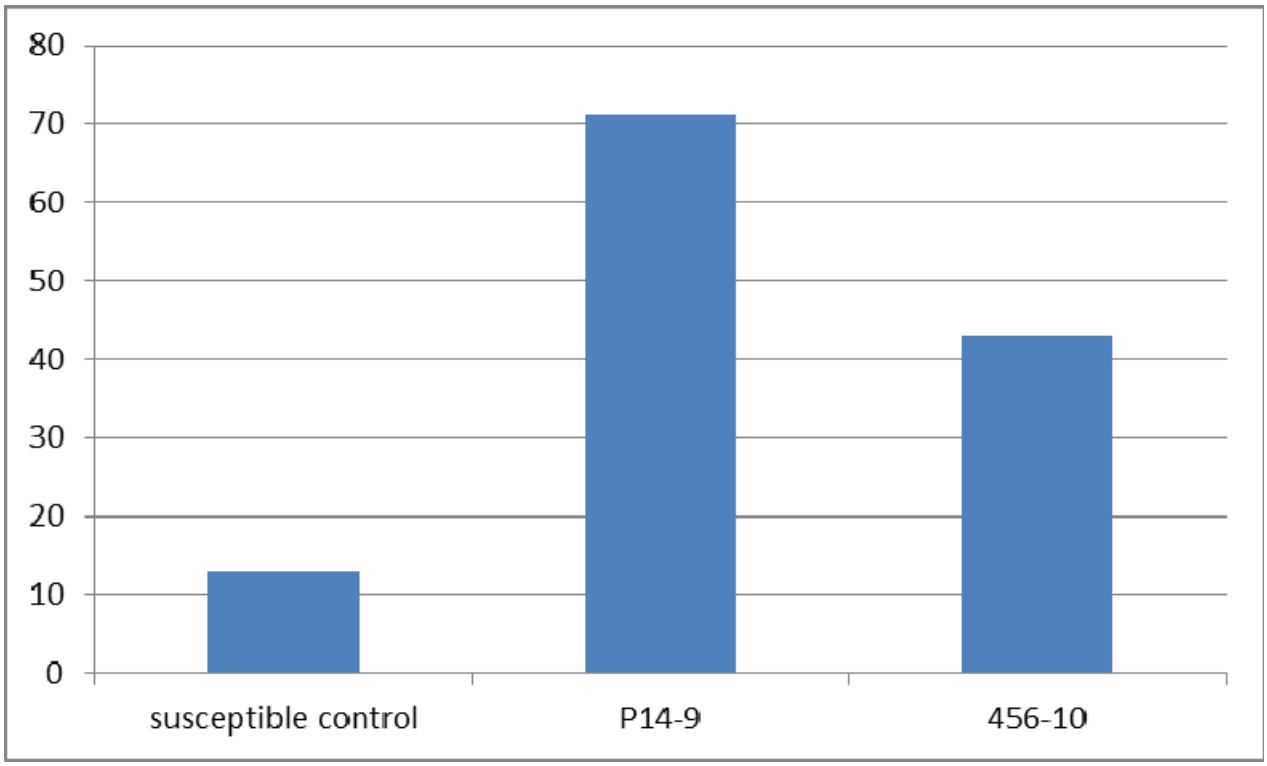

Figure 2. Solano County: Non-transgenic control plant (TS02A) Inoculated in 2011 and 2012. Image taken April 2013 showing shoots dying shortly after emergence.

Figure 3. Solano County: non-transgenic control plant (TS02A) inoculated July 2012 showing cane death visible after emergence of uninoculated canes on the same plant. Illustrates the observation that the bacteria can kill inoculated canes but does not move rapidly to other canes on the same plant

Figure 4. Solano County: own-rooted transgenic P14-9 inoculated in 2011 and 2012 showing no evidence of death in inoculated canes or shoots emerging from the inoculated canes. Image recorded May 2013

Figure 5. Solano County: non-transgenic scion grafted to a transgenic P14-9 rootstock. The scion was inoculated in 2012 currently showing no evidence of death in inoculated canes or shoots emerging from the inoculated canes. Image recorded in May 2013.

Figure 6. Solano County: non-transgenic scion grafted to a non-transgenic control rootstock. The scion was inoculated in 2012. Evidence of death is visible in inoculated canes. Note red flags for reference to limited cane death. Image recorded in May 2013.

Figure 7. Disease ratings of individual plants in Riverside County exposed to natural infection via GWSS. Plants are own rooted Thompson Seedless transgenic (P14-9) and non-transgenic (TSO2A).

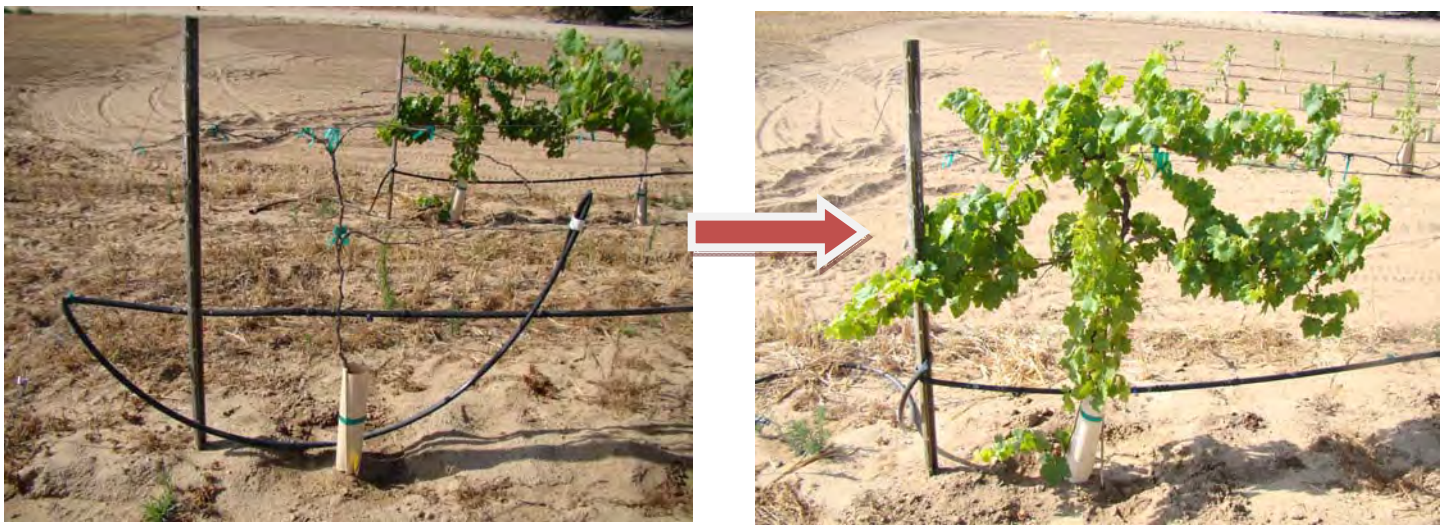

Figure 8 Example of grape plants growing in Riverside county in June 2013, subject to feeding by the Glassy-winged Sharpshooter confirmed to carry *Xylella fastidiosa*. Plant in the foreground of the left picture is a non-transgenic Thompson Seedless control, which is essentially dead. The plant in background is a transgenic P14 Thompson Seedless plant, which, although infected (5673 bacterial cells per cm of stem tissue) shows no symptoms of cane or leaf death, seen here also as the image on the right.

Figure 9. Example grape plants growing in Riverside County June 2013 showing both asymptomatic and severely diseased plants that were subject to infection by the Glassy-winged Sharpshooter, which were confirmed to carry *Xylella fastidiosa*.