**Interim Progress Report for** CDFA Agreement number 16-0558-SA

**Field Evaluation of Cross-Graft Protection Effective Against Pierce's Disease by Dual and Single DNA Constructs**


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**\Reporting Period:** The results reported here are from July 1, 2017 to March 19, 2019.

**Project History** CDFA 16-0558-SA, which concludes June 30, 2019 is a continuation of CDFA 12-0444-SA and is proposed to continue with a new grant submitted for 2019-2021 to extend the field evaluation of the transgenic rootstocks described herein

## Introduction

Genetic strategies for disease suppression and information characterizing the biology of the bacterial-plant interaction are high priority areas in the Pierce's Disease/GWSS Research Program. Research from Dandekar, Powell, Lindow, and Gilchrist identified several grape genes that, when expressed transgenically suppressed, Pierce's Disease (PD) in laboratory and greenhouse testing. The five genes illustrated in Table 1 now have been tested in transgenic grape plants under greenhouse and field conditions in APHIS permitted field environments. The transgenic plants and untransformed control plants were inoculated with *Xylella fastidiosa (Xf).* Symptoms of PD were observed within one year of infection on the control plants to the point of plant death within 2-3 years. Promising results from studies involving whole plant transgenics, begun in 2010 and terminated in 2017, provided the necessary impetus to move this program to the next logical step in which paired combinations of the transgenes have now be introduced into two rootstocks adapted to California grape growing regions (References 1-12). The dual gene expressing rootstocks have now been grafted to susceptible non-transgenic Chardonnay scions to assess potential cross graft protection of the scion under field conditions in the presence of *Xf.*

**Table 1. Genes selected to evaluate as dual genes in the 2nd generation field evaluation for suppression of Pierce's disease in grape**

The table lists gene names, abbreviation used, and presumed function

| Gene | Code | Function |
|---|---|---|
| CAP | C | *Xf* clearing/antimicrobial |
| PR1 | A | grape cell anti-death |
| rpfF | F | changing quorum sensing of *Xf* (DSF) |
| UT456 | B | non-coding microRNA activates PR1 translation |
| PGIP | D | inhibits polygalacturonase/ suppressing *Xf* movement |

**Objectives**

1. Complete the current field evaluation of transgenic grape and grape rootstocks expressing Pierce's disease suppressive DNA constructs in the APHIS-regulated field site in Solano County through the spring of 2016.
2. Remove the current planting per the APHIS agreement by dismantling trellising, uprooting the plants, and burning all grape plant material on site following the final July 2016 data collection, followed by cultivation and fumigation to ensure no living grape vegetative material remains.
3. Establish a new planting area within the current APHIS approved site to contain a new set of lines bearing paired, PD suppressive, DNA constructs, referred to as stacked genes, in two adapted rootstocks (1103 and 101-14). These rootstocks will be grafted to a PD-susceptible Chardonnay scion prior to field planting. The goal is to assess the potential of cross graft protection against PD of a non-transgenic scion. Planting to begin in 2017 and completed by 2018.

**Description of activities to accomplish the objectives**

1. **Complete the current field evaluation of transgenic grape and grape rootstocks**

Maintenance of the experimental planting including irrigation, pruning, weed removal, and pest management was followed through 2016 season (Reference 6) to enable evaluation of the plants for plant morphology, symptoms of Pierce's disease infection, and the presence of the bacteria. Visual rating of symptoms and detection of the amount and movement of the bacteria in plant tissues (mainly leaves and stems) by quantitative PCR (qPCR) assays in the Gilchrist lab was done by the same methods and laboratory personnel as previously. The assessment format and data collected has been reported annually in the Pierce's Disease Research Symposium Proceedings (References 1-12).

2. **Destruction of existing planting:**

In conjunction with the investigators, the Product Development Committee of the Pierce's Disease Control Board in October 2015, approved the decision to terminate the field evaluation of current transgenics as originally planned (Objective 2) and move to the second phase of transgenic PD resistance evaluation. Field data over the course of this experiment has been collected by all investigators and can be found in their individual reports from in the 2012- 2016 Pierce's Disease Symposium reports (Reference 1-12).

The field experiment that begun in 2010 was terminated under objective 2 of this proposal according to the regulations specified in the APHIS permit (Figure 1). This will be followed by establishment of second phase approved by the Product Development Committee to develop transgenic rootstocks incorporating

**Figure 1.** Final destruction of the plants at Solano field site by burning on June 7, 2017 following removal of poles and wires, undercutting and piling of plants, the material was burned and the ashes incorporated by disking.

stacked genes (dual constructs) to be grafted to non-transformed PD-susceptible Chardonnay scions to test for potential cross-graft protection against PD (Objective 3)

Destruction of existing planting was begun in the fall of 2016. All posts and wires were removed in November but early rains prevented the removal of the plants. The plant removal, burning of the plants and incorporation of the ashes was completed as soon as the field dried in the spring. The entire field was then cross disked multiple times and leveled in preparation for grafted transgenic rootstock planting begun in August 2018.

3. **Establishment and management of new planting with stacked gene transgenic rootstocks**

Figure 2 shows the physical location of the new planting (green border) in relation to the 2010 planting (red border).

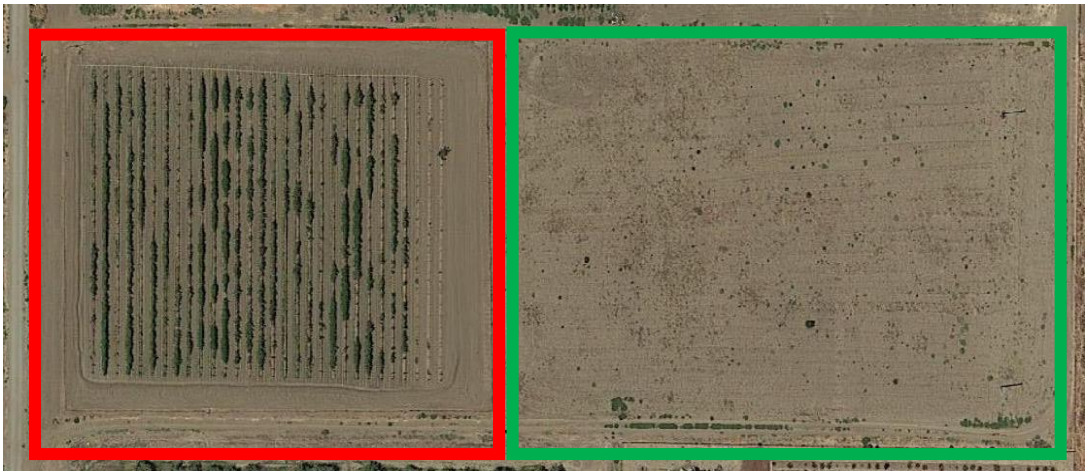

**Figure 2. Solano planting area.** Future area (green box) available to plant the next generation of transgenic plants expressing the dual constructs or new single genes: This area is 300 X 470 ft for planting, which equals 1.8 acres accommodating up to 32 new rows (excluding the 50 ft buffer areas surrounding the plots. The new area will accommodate ~900 new plants in 2018-19. Current area (rows) now planted to grapes: 300 X 370 ft equaling 1.6 acres including the 50 ft buffer areas surrounding the plots.

The establishment of a new planting area within the current APHIS approved site was begun in August 2018 (Figures 2&4). The experimental material is comprised of a new set of lines bearing paired PD suppressive, DNA constructs, referred to as stacked genes, in two adapted rootstocks (1103 and 101-14) (Gilchrist and Lincoln 2018 ) These rootstocks have been grafted to a PD-susceptible Chardonnay 04 scion prior to field planting. The goal is to assess the potential of cross graft protection against PD of a non-transgenic scion. The first phase of field planting of the stacked gene rootstock combinations was completed on August 1, 2018 (figure 1) with the current funding, which will expire on June 30, 2019. This current field experiment was approved by the Product Development Committee specifically to develop transgenic rootstocks consisting stacked genes (expressing dual constructs) that have been grafted with non-transformed PD-susceptible Chardonnay scions to test for potential cross-graft protection against PD (Objective 1). The development of the stacked gene rootstock transgenics was completed, including molecular analysis of the transgenic rootstock lines produced by the UC Davis Transformation Facility. All field activities described in the section on Methodology to Accomplish Objectives will conducted or coordinated by field superintendent Bryan Pellissier and Foundation Plant Services project manager Josh Puckett and David Gilchrist Project PI.

The grafting, planting, and training of the vines has been guided by Josh Puckett and Deborah Golino (FPMS) (figure 3) working with PI Gilchrist to produce clones for grafting non-transgenic scions, grafting the scions, field planting, trellising and plant management to reflect commercial production standards.

**Figure 3**. Josh Puckett harvesting transgenic rootstock canes for bud grafting to untransformed Chardonnay. Packet tag indicates rootstock and paired gene combinations expressed in this rootstock

The field plot design will enable experimental *X. fastidiosa* inoculations, pathogen and disease assessments, as well as grape yield. Land preparation and planting of the experimental area will be sufficient to accommodate and manage 900 new plants. Row spacing will be 9 feet between rows with 6 feet between plants. This spacing permits 32 rows of 28 plants each (up to 896 plants total) and includes a 50 foot open space around the planted area as required by the APHIS permit. The planting pattern will permit a 2 bud pruned bilateral cordon system of sufficient lengths for inoculation, real time sampling of inoculated tissue and determination of the fruit yield by the untransformed Chardonnay scions. Total fenced area occupied by plants and buffer zones as required by the APHIS permit will be ~3.4 acres (Figure 2). All plants will be maintained under a newly installed drip irrigation system. The initial planting began in August 2018 is shown in Figure 4.

**Figure 4. Planting configuration for dual constructs.** This image illustrates the new planting of the dual construct transformed rootstocks grafted with and untransformed scion of Chardonnay. Planting on August 1, 2018

**The following protocols have been followed as the planting proceeds:**

a. Experimental design will be a complete randomized block with six (6) plants per each of five (5) entries (replications), including all controls. Each plant will be trained as a single trunk up the wood stake as with the existing planting. When the shoot tip reaches about 12" past the cordon wire it will be topped to just above a node that is about 2-3 inches below the wire. Then, the laterals that push will be used to establish the bi lateral cordons. The plants will be allowed to grow vertically, or close to vertical, rather than tying them while green, which reduces their elongation and tends to force more lateral growth. Metal 9' highway stakes, inserted 3' into the ground every 18' will support the wires, including catch wires. A single 11 gauge wire will be used for the cordons and 13 gauge for the catch wires. Two pairs of moveable catch wires will be installed to tuck and position the shoots vertically for optimizing bacterial inoculation, bacterial analysis, and fruit production. The catch wires will be installed initially or after the first year of growth using 13 gauge wire to support the drip irrigation wire, about 18" off the ground.
b. After the first year, the canes will be tied down during the dormant season and trimmed to the appropriate length or shorter if the cane girth is not over 3/8" in diameter. The shoots that push will be suckered to remove double shoots and to achieve a shoot (and hence spur position) spacing of about 4-5 inches between them.
c. Grape fruit yield will be measured after second or third year depending on the fruit set.
d. Evaluation of the experimental plants for plant morphology, symptoms of Pierce's Disease infection, and the presence of the bacteria will follow past protocol. Each parameter will be determined overtime by visual monitoring of symptom development and detection of the amount and movement of the bacteria in plant tissues (mainly leaves and stems) by quantitative PCR (qPCR) assays. The analysis will be done in the Gilchrist lab by the same methods and laboratory personnel as has been done with the current planting. A comparative quantitative determination by qPCR of the presence of Xylella in non-transgenic scions and grape rootstocks will be compared with conventional grape and grape rootstocks.
e. Both symptom expression and behavior of the inoculated bacteria will provide an indication on the level of resistance to Pierce's Disease infection and the effect of the transgenes on the amount and movement of the bacteria in the non-transgenic scion area.
f. An important consideration with respect to experimental control over movement of the disease from transgenic to nontransgenic control plants is that grapes grown in an area is adjacent to this experimental grape planting have been infected with Pierce's Disease for the past two decades with no evidence of spread of the bacteria to uninfected susceptible grape plantings within the same experiment. Hence, there is a documented historical precedent for the lack of spread of the bacteria from inoculated to non-inoculated plants, an important consideration for the experiments carried out for this project and for the granting of the APHIS permit. In addition, the field area itself has never had grapes planted therein, which should avoid any potential confounding by soil borne diseases, including nematodes.
g. Irrigation and pest management, primarily powdery mildew, weeds and insects, will be coordinated by PI Gilchrist and conducted by Bryan Pellissier the Field Superintendent employed by the Department of Plant Pathology. The field crew work closely with PI Gilchrist to determine timing and need of each of the management practices, including pruning and thinning of vegetative overgrowth as necessary.
h. Regular tilling and hand weeding will maintain a weed-free planting area. As the experiment proceeds, plants will be pruned carefully in March of each year leaving all inoculated/tagged branches and numerous additional branches for inoculation and sampling purposes in the coming year. All pruning material will be left between the rows to dry, then flail chopped and later rototilled to incorporate the residue per requirements of the APHIS permit.
i. Application of the fungicides Luna Experience and Inspire will be alternated at periodic intervals to maintain the plants free of powdery mildew. Leafhoppers and mites will be treated with insecticides when needed. Neither powdery mildew nor insect pressure was has been observed with these ongoing practices throughout the past five growing seasons.

**Research timetable for the new planting of dual constructs and untested single constructs.** Four years beginning with initial planting in 2018 (Figure 5) to be followed by addition plantings as experimental plants become available in 2019. Inoculation and evaluation will begin when the plants have been in the ground for

one year and will continue annually until PD is established in the untransformed control plants. Funding for completion of the fourth and any following years has been proposed in the 2019 funding cycle. The field area has been designated legally available for planting the specified transgenic grapes by USDA-APHIS under permit number 7CFRE340 that is held by Professor Abhaya Dandekar. The protocols for managing the existing and the new plantings with the dual constructs have used successfully over the past 5 years (References 1-9). These protocols include the plant management, inoculation with *Xylella fastidiosa*, development of classical symptoms of Pierce's Disease exhibiting the range from foliar symptoms to plant death and the assessment of protection by a set of transgenes selected by molecular techniques to suppress the symptoms of Pierce's Disease and/or reduce the ability. So far, this timeline has been met.

**Publications produced and pending:**


1. James Lincoln, Sanchez, Juan, and David Gilchrist, 2018. Pathogenesis-related protein PR-1 interferes with programmed cell death and is synthesized under translational control. Molecular Plant Pathology. Vol 19, Issue 9, page 2111-2123.
2. Gilchrist, David et al. 2018. Transgenic rootstock-mediated protection of grapevine scions by introduced single and dual stacked DNA constructs. Proceedings of the Pierce's Disease Research Symposium. San Diego, CA, December 17-19
3. Gilchrist, David et al. 2016. Transgenic rootstock-mediated protection of grapevine scions by introduced single and dual stacked DNA constructs. Proceedings of the Pierce's Disease Research Symposium. San Diego, CA, December 12-14.
4. Sanchez, Juan, James Lincoln, and David Gilchrist, 2019. The translation of pathogenesisrelated-PR-1 is triggered by4 a miRNA excised from grape coding sequences and the coding sequence of grape fan leaf virus. (pending)


**Research Relevance.** This translational research conducted herein will test for potential cross-graft protection of a PD susceptible Chardonnay 04 scion against the development of Pierce's Disease symptoms by expression of dual combinations of five PD suppressive transgenes in two adapted rootstocks. The protocol includes planting, training, inoculating to evaluate both disease and yield components specifically in the PD susceptible scions. It also will enable assessing both potential cross-graft protection of a non-transformed scion and the effect of the transgenes to protect the

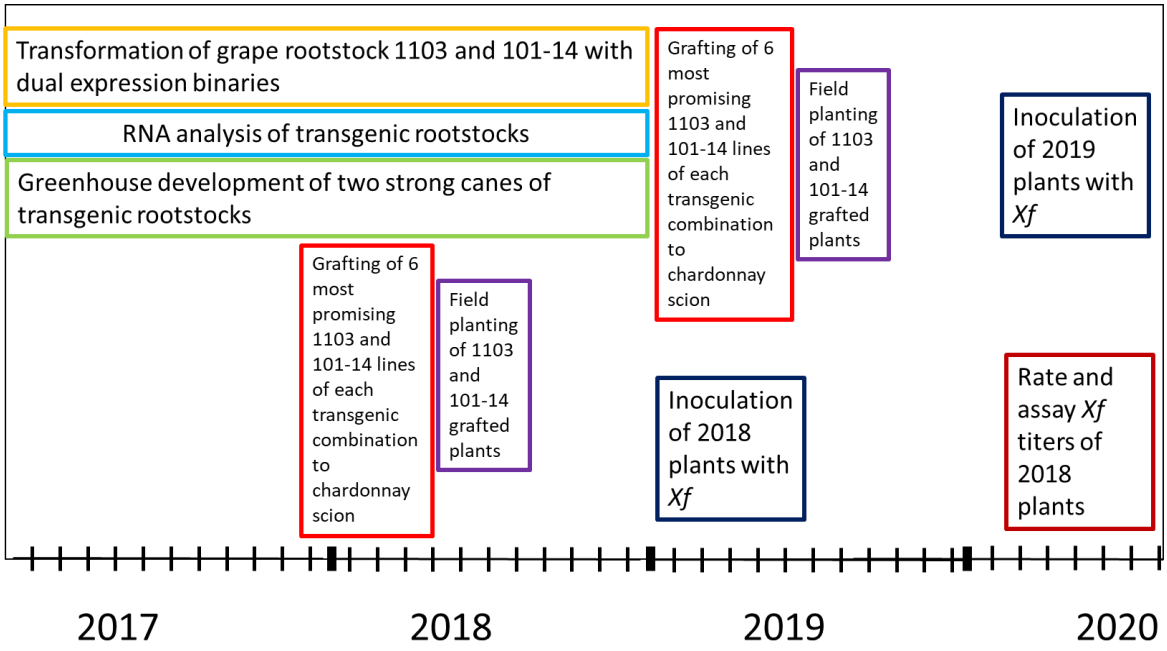


**Figure 5.** Anticipated Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.

rootstocks against bacterial movement and death compared to equivalent combinations of untransformed rootstock/scion control combinations.

**Layperson summary**

This first phase field project began in 2010 to evaluate grapevines expressing potential Pierce's Disease (PD) suppressive transgenes under field conditions was terminated in 2017. A second phase field experiment will continue evaluation of resistance to Pierce's Disease (PD) in transgenic grape and grape rootstocks by expressing dual combinations of five unique transgenes under field conditions. The evaluation continues in an USDA-APHIS-regulated Solano County site where the plants are mechanically injected with *X. fastidiosa*. Pierce's Disease symptoms including classical foliar symptoms and cane death occur within 24 months. The initial field tests have shown positive protection against PD by five (5) different DNA constructs. A new planting is in progress that will consist of untransformed PD susceptible scions grafted to transgenic rootstocks (1103 and 110-14) expressing the paired constructs of the five genes to assess cross-graft protection of a non-transformed scion that is otherwise highly susceptible to Pierce's Disease. This research also will address the ability of the pathogenic bacteria to colonize and move within the xylem of the grape plant downward from the inoculated scion to the transgenic rootstock. The latter analysis will determine if the transgenic rootstock is differentially protected against Xylella induced death of the rootstock.

**Status of funds:** Funding for this project is provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board and the Regents of the University of California. Funds are being expended at the anticipated rate. Remaining funds to be allocated will be used as described under Objective 3 for planting, maintaining and evaluating the field plants through at June 30, 2019.

**Summary and status of intellectual property:** No intellectual property is expected from the field maintenance aspect of these research studies. Pending the stacked genes protection results of the unmodified scion, appropriate disclosures will be filed with the Office of Research Innovation Access for the disease suppression

**Literature cited**

1. Gilchrist, D.G., and J.E. Lincoln. 2014. Field evaluation of grape plants expressing PR1 and UT456 transgenic DNA sequences for protection against Pierce's disease. *Proceedings of the 2014 Pierce's Disease Research Symposium*. California Department of Food and Agriculture, Sacramento, CA.
2. Dandekar, Abhaya M. 2015 Chimeric Antimicrobial Protein and Polygalacturonase-Inhibiting Protein Transgenic Grapevine Field Trial. Proceedings of the 2015 Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA.
3. Gilchrist, David G. and James E. Lincoln. 2015a Field Evaluation of Grape Plants Expressing Potential Protective DNA Sequences Effective against Pierce's Disease. Proceedings of the Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA.
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9. Lindow, Steven. 2015 Continued Field Evaluation of Diffusible Signal Factor Producing Grape for Control of Pierce's Disease Proceedings of the Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA.
10. James Lincoln, Sanchez, Juan, and David Gilchrist, 2018. Pathogenesis-related protein PR-1 interferes with programmed cell death and is synthesized under translational control. Molecular Plant Pathology. Vol 19, Issue 9, page 2111-2123.
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