Comprehensive Final 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: This final report covers the period from 7/1/2016 to 6/30/2019

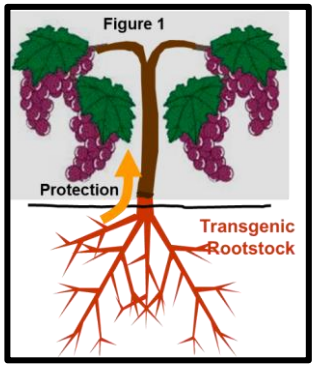
Introduction

The the field evaluation of DNA constructs integrated into grape plants to suppress the symptoms of Pierce's Disease was begun in 2010 and has been supported with consecutive renewals to the present time. Although the funding period of this project ended June 30, 2019, a renewal grant for the work described herein has been awarded from July 1, 2019 through June 30, 2022. Hence, the scope of this project and this report concludes the first generation of field studies begun in 2010 and now extends into the beginning of a second generation of field studies to evaluate genetically modified plants bearing Pierce's Disease suppressive constructs under disease conditions in APHIS approved field environments. The individual laboratories of the PI and Co-PI s established transgenic plants and field tested the genes shown in Table 1 as transgenes in a PD susceptible commercial grapevine variety (Thompson Seedless) and the Freedom grape rootstock. Each of the genes were selected based on laboratory, and greenhouse assays that revealed their capability to disrupt either the virulence of the bacterial pathogen or plant factors triggering the susceptible response in the grape host. There was strong evidence obtained that each of these genes can

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



protect, but to differing levels as transgenes and each appears to be able to exert suppressive action on the symptoms of PD in cultivated grapes.

By way of brief review, the initial planting, begun in 2010, consisted of own rootstock whole plant transgenics and a group consisting of transgenic rootstocks expressing single DNA constructs grafted to non-transformed PD susceptible scions. Promising results from both types of transgenic strategies provided the necessary impetus to move this program forward to the next logical step, begun in 2017 in which combinations of the transgenes have been introduced into two different rootstocks adapted to California grape growing regions (References 1-9). Prior to the initiation of the second phase, final observations were made of the first planting, followed by the destructive removal of the first planting according to requirements of the APHIS agreement (Figure 3) and re-tilling the entire field area, including a new contiguous area that had not been planted previously and into which, the first of set of new plants are now established (Figures 4 and 7).

The new rootstock combination with paired transgenes each were evaluated first in the laboratory and then the greenhouse before moving to the field. The highest expressing rootstocks were grafted to susceptible non-transgenic Chardonnay scions to assess potential cross graft protection of the scion (figures 5 and 6) .

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 late 2017 and completed by 2018. (Planting actually began in 2018 and will be completed in 2019).

Description of activities to accomplish the objectives

1. Completed the current field evaluation of transgenic grape and grape rootstocks (figures 1 and 2)
2. 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 1-9).

3. 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 3). This will be followed by establishment of second phase approved by the Product Development Committee to develop transgenic rootstocks incorporating 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) Maintenance of the experimental planting including irrigation, pruning, weed removal, and pest management was followed through 2016 season (Reference 3) 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 and data collected was reported annually in the

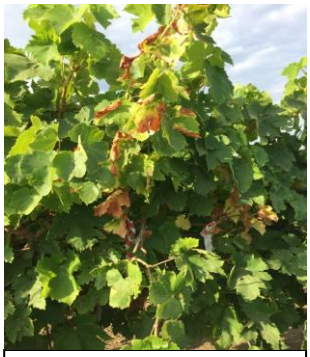


Figure 1 Symptoms of Pierce's Disease in 2015 on inoculated field

Figure 2. Inoculated non-transgenic Thompson Seedless foreground; essentially dead. Inoculated transgenic Thompson Seedless in the rear: asymptomatic. Image taken prior to destruction in 2017.

Pierce's Disease Research Symposium Proceedings (References 1-9). Figure 1 shows classical leaf symptoms of PD and Figure 2 illustrates differences between transgenic and non-transgenic control plants at the termination of the first generation field study.

4. Destruction of existing planting:

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 (Figure 3). The entire field was then cross disked multiple times and leveled in preparation for grafted transgenic rootstock planting begun in August 2018. Fumigation step, as indicated in objective3, was not done since APHIS approved destruction was deemed sufficient by the regulating agency ..

Figure 3. 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 in July 2017.

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

Figure 4 shows the physical location of the new planting (green border) in relation to the 2010 planting. The establishment of a new planting area within the current APHIS approved site was begun in August 2018 (Figure 7). 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 2018a) These rootstocks were grafted to PD-susceptible Chardonnay 04 scions prior to field planting (Figure 6). The goal is to assess the potential of cross graft protection against PD of a non-transgenic scion.

Figure 4. Solano planting area relationships. The green boxed is the site of the 2018/2019 planting. The red boxed area is the site of the first generation planting. Both areas are now part of the APHIS permitted area for planting transgenic grapes.

The first phase of field planting of the stacked gene rootstock combinations was completed on August 1, 2018 (figure 7) with the current funding, which expired 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 3). The development of the stacked gene rootstock transgenics was completed, including molecular confirmation by the Gilchrist lab 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 be conducted or coordinated by field superintendent Bryan Pellissier and Foundation Plant Services project manager Josh Puckett, Director Deborah Golino and David Gilchrist Project PI.

The selection, grafting, planting, and training of the vines has been guided by Josh Puckett (FPMS) (Figure 5) working with PI Gilchrist to produce clones for grafting non-transgenic scions to the transgenic rootstocks (Figure 6). The field planting, trellising and plant management will reflect commercial production standards.

Figure 5. Josh Puckett harvesting transgenic rootstock canes for bud grafting to untransformed Chardonnay. Packet tag indicates rootstock and paired gene combinations expressed in this rootstock

Figure 6 Example of Chardonnay scions grafted to transgenic rootstocks in preparation for field planting.

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 should the need arise. Row spacing will be 11feet between rows with 7 feet between plants. Each row contains 30 plants 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. All plants will be maintained under a drip irrigation system.

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. Inoculation of *Xylella fastidiosa* with be done in the spring of the second year after planting and will follow previous protocol, which successfully introduced the bacteria and elicited classic symptoms of PD (Figure 1), including death of susceptible control plants (Figure 2). 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 works 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.

Field planting of dual DNA insert transgenic rootstocks grafted to Chardonnay scions and untransformed control plants in the APHIS regulated field site

Figure 7. Planting configuration for the dual constructs. This image illustrates the new planting of the dual construct transformed rootstocks grafted with an untransformed scion of Chardonnay. This first phase of the planting was completed August 1, 2018.

The initial planting occurred in 2018 (Figure 7) and will be followed by final plantings in 2019 as experimental plants become available.

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 was proposed in the 2019 funding cycle and was approved for continuation of support for these field studies through June of 2022. 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 of the bacterial to move systemically within the plant.

Cumulative time line from 2015 through the current funding period to 2022

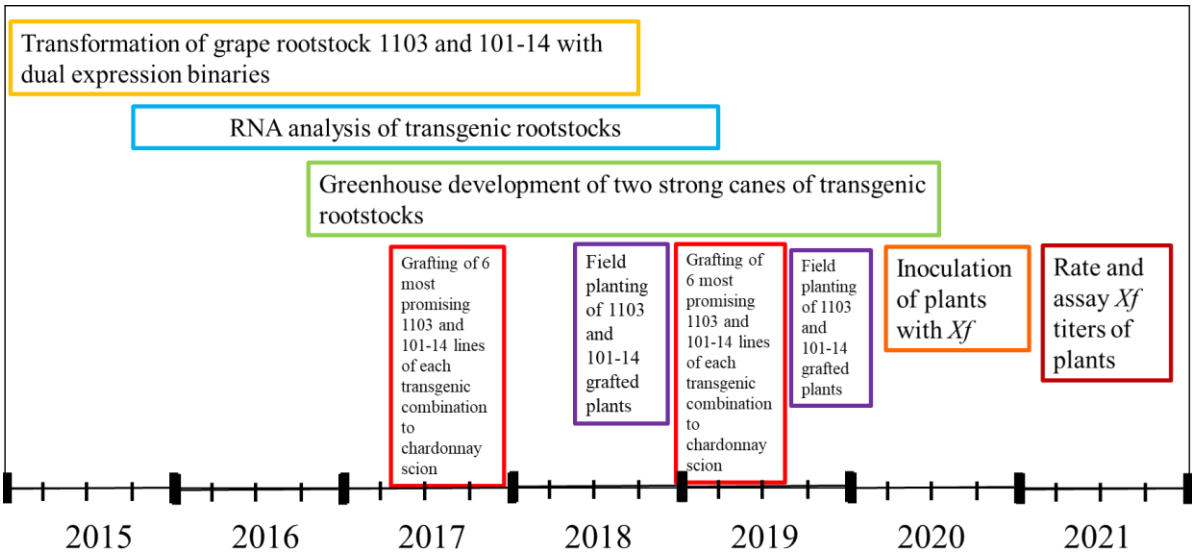


Figure 8. Anticipated Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock controls and untransformed susceptible control plants.

From the development of the dual construct vectors by Dr. Lincoln, introduction the dual constructs into the two rootstocks by David Tricoli (1103 and 110-14), followed by grafting of the untransformed Chardonnay scion by Josh Puckett and initial phase of planting, the projected time lines have 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 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 to 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 has now been initiated with the first planting in 2018. The current field 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 revealed positive protection against PD by five (5) different DNA constructs. A new planting has been established that consists 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 Chardonnay 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 were expended at the anticipated rate. Funding for the field management of this project has been approved through 2022.

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. Gilchrist, David G., James E. Lincoln, 2016a Transgenic Rootstock-Mediated Protection of Grapevine Scions by Single and Stacked DNA Constructs Proceedings of the Pierce's Disease Research Symposium. California Department of Food and Agriculture, San Diego, CA.

3. Gilchrist, David G., James E. Lincoln, 2016b Field Evaluation of Cross-Graft Protection Effective Against Pierce's Disease by Dual and Single DNA Constructs Proceedings of the Pierce's Disease Research Symposium. California Department of Food and Agriculture, San Diego, CA.

4. Gilchrist, David G., James E. Lincoln, 2017a Transgenic Rootstock-Mediated Protection of Grapevine Scions by Single and Stacked DNA Constructs Proceedings of the Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA.

5. Gilchrist, David G., James E. Lincoln, 2017b Field Evaluation of Cross-Graft Protection Effective Against Pierce's Disease by Dual and Single DNA Constructs Proceedings of the Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA.

6. Gilchrist, David G., James E. Lincoln, 2018a Transgenic Rootstock-Mediated Protection of Grapevine Scions by Single and Stacked DNA Constructs Proceedings of the Pierce's Disease Research Symposium. California Department of Food and Agriculture, San Diego, CA.

7. Gilchrist, David G., James E. Lincoln, 2018b Field Evaluation of Cross-Graft Protection Effective Against Pierce's Disease by Dual and Single DNA Constructs Proceedings of the Pierce's Disease Research Symposium. California Department of Food and Agriculture, San Diego, CA.

8. Lincoln, J. E., Sanchez, J. P., Zumstein, K. and Gilchrist, D. G. (2018), Plant and animal PR1 family members inhibit programmed cell death and suppress bacterial pathogens in plant tissues. *Molecular Plant Pathology*, 19: 2111-2123. doi:10.1111/mpp.12685

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.