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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Introduction

Genetic strategies for disease suppression and information characterizing the bacterial-plant interaction are high priority areas in the Pierce's Disease/GWSS Research Program. Projects from laboratories of Dandekar, Powell, Lindow, and Gilchrist have been tested extensively under greenhouse and field conditions in APHIS approved field environments in Riverside and Solano Counties. Two types of genetically modified plants bearing single constructs of test genes have been evaluated under disease conditions; whole plant transgenics and graft transmissible transgenes in which transgenic rootstocks were grafted to non-transformed PD susceptible scions. Positive and promising results from both types of transgenic strategies provided the necessary impetus to move this program forward to the next logical step in which combinations of the transgenes will be introduced into individual rootstocks adapted to California grape growing regions (References 1-10).

The individual laboratories of the PI and Co-PI s have established transgenic plants and field tested the following genes as transgenes in a commercial grape rootstock and a commercial grapevine variety (Table 1). Each of the genes were selected based on laboratory, greenhouse and field data to address and disrupt known functions related to virulence of the bacteria or key factors triggering the susceptible response in the grape host. There is strong evidence that each of these genes can 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. The new rootstock combination with paired transgenes each will be evaluated first in the laboratory

and then the greenhouse before moving to the field. The highest expressing rootstocks will be grafted to susceptible non-transgenic Chardonnay scions to assess potential cross graft protection of the scion.

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 2016 and completed by 2018.

Description of activities to accomplish the objectives

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

Final field evaluations of this planting were completed in June of 2016.

2. Destruction of existing planting:

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 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 future planting.

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.

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

Figure 2 shows the physical location of the new planting in relation to the 2010 planting. All plants will be located in a secured, USDA-APHIS-approved, area in Solano County. The disease will be introduced into the cordon trained plants by mechanical injection of *Xylella fastidiosa* into stems after the first year of growth beginning in 2018. The plants are to be monitored regularly for quantity and movement of the bacteria along with symptoms of PD. Test plants included transgenic plants expressing genes from Dandekar, Powell, Lindow, and Gilchrist projects compared with non-transgenic PD-susceptible Thompson Seedless and Freedom rootstock plants as controls. The results through 2016 indicated that the mechanical inoculations introduced the bacteria into the plants with subsequent appearance of classic foliar symptoms and cane death within 24 months in susceptible controls. There is no evidence of spread of the bacteria to uninoculated and uninfected susceptible grape plants adjacent to infected plants, confirming tight

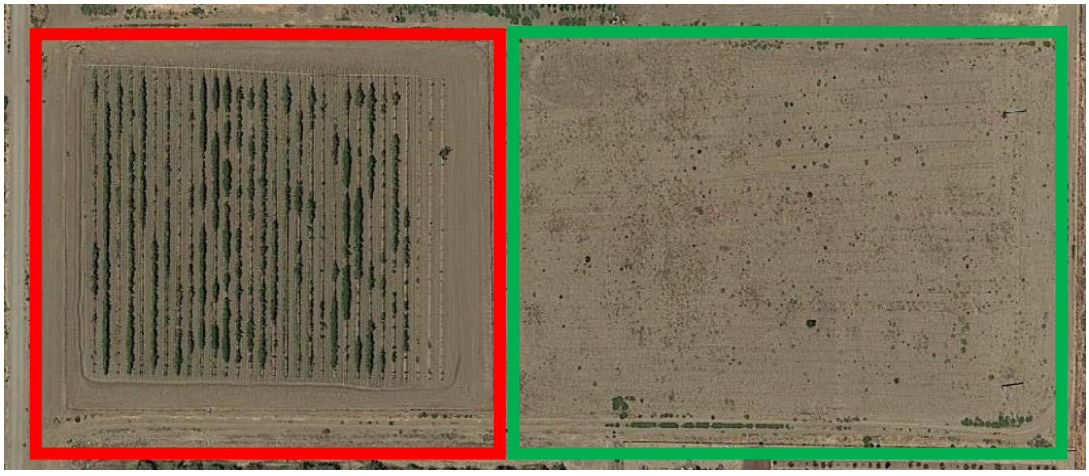

Figure 2. Solano planting area. Future area (green box) available to plant the next generation of transgenic plants expressing the dual constructs or new single genes: This area is 300 X 470 ft for planting, which equals 1.8 acres accommodating up to 32 new rows (excluding the 50 ft buffer areas surrounding the plots. The new area will accommodate ~900 new plants in 2018-19. Current area (red box) equaling 1.6 acres including the 50 ft buffer areas surrounding the plots is the area that is now cleared of plants and all plant material burned as shown in Figure 1.

experimental control on the pathogen and symptoms. Each of the transgenes tested suppress the symptoms of PD inoculated vines to varying degrees, including protection of untransformed scions on the grafted plants. This first phase of field research has been terminated and is now moving forward with the second generation of two new transgenic rootstocks (1103 and 101-14) expressing pairs of the disease suppressive genes in a gene stacking approach with the genes paired together by differential molecular function.

The grafting, planting, and training of the vines will be guided by Josh Puckett and Deborah Golino (FPMS) working with PI Gilchrist for grafting non-transgenic scions, grafting the scions and field planting. They also will provide guidance for trellising and plant management to reflect commercial production standards.

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 is 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. An image of the completed phase 1 of the field planting is shown in Figure 3.

Figure 3. Planting configuration for the dual constructs.. This image illustrates the new planting of the dual construct transformed rootstocks grafted with an untransformed clone of Chardonnay. This first phase of the planting was completed August 1, 2018.

The following protocols will be 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. The area is adjacent to experimental grape plantings that 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. The field area chosen has never had grapes planted therein, which is to 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. Plants were 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 was 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 4) to be followed by addition plantings as experimental plants become available in the second and third years. Inoculation and evaluation will begin when the plants have been in the ground for one year and will continue annually until the field planting is terminated. Funding for completion of the fourth and any following years will be proposed in the 2018-2019 funding cycle and will depend on the results of the field evaluation up to that point. 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.

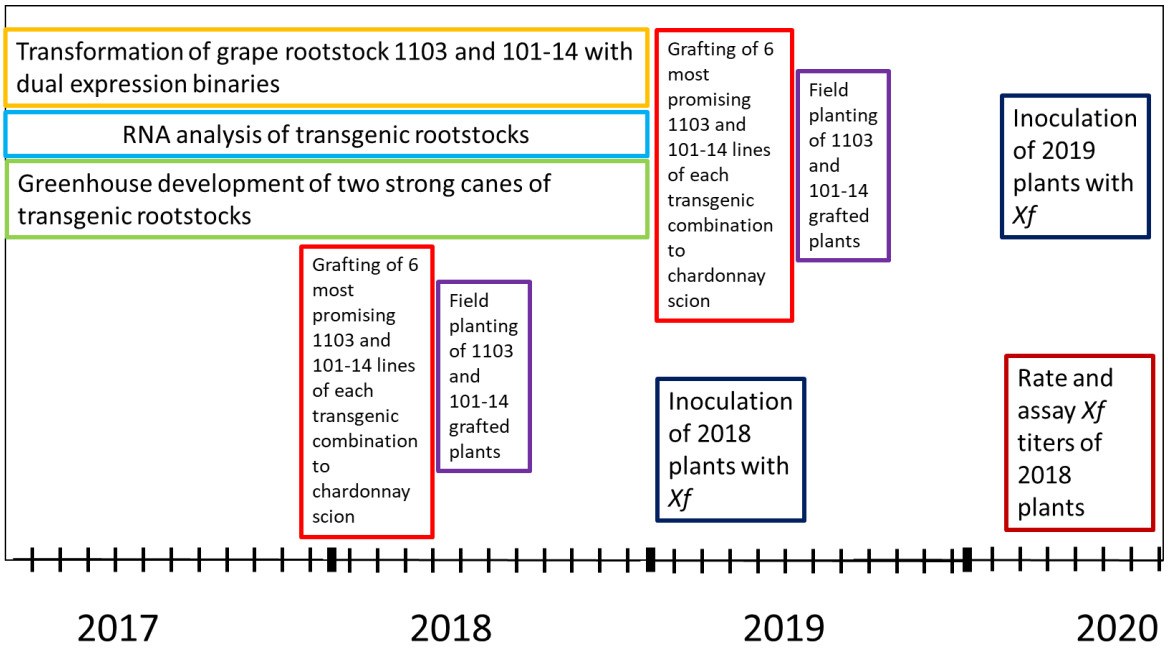

Figure 4 Anticipated Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.

Publications produced and pending: see literature cited

Research Relevance. The primary objective for expressing genes in combination is to create durable resistance, resistance to *Xf* that will last the life of the vine. Since at least several of the five DNA constructs (Table 1) have biochemically distinct mechanisms of action, having two or more such distinctly acting DNA constructs "stacked" in the rootstock should drastically reduce the probability of *Xf* overcoming the resistance. With multiple, distinct transgenes, *Xf* would be required to evolve simultaneously multiple genetic changes in order to overcome the two distinct resistance mechanisms.

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. The grafting, planting, and training of the vines will be guided by Josh Puckett and Deborah Golino (FPMS) for trellising and plant management to reflect commercial production standards.

Conclusions

The current planting of transgenic grapes was fully terminated in the spring of 2017 per the APHIS agreement by dismantling trellising, uprooting the plants and burning all grape plant material on site. The complete removal of the plants was followed by cultivation and the area will be fumigated when conditions permit to ensure no living grape vegetative material remains. The field research using PD suppressive transgenes is moving forward with the generation of new transgenic rootstocks expressing pairs of the disease suppressive genes in a gene stacking approach with the genes paired together by differential molecular function. The new rootstocks with two transgenes each were evaluated first in the laboratory and then the greenhouse before moving to the field. The dual gene expressing rootstocks were grafted to susceptible non-transgenic PD susceptible Chardonnay scions to assess potential cross graft protection against PD. The field area has been permitted by the USDA-APHIS for this experiment. The protocol for constructing the rootstocks and grafted scions planting and commercial style management of the vines is in place and will be coordinated by Josh Puckett and Debora Golino. Beginning with initial planting in 2018 and followed by addition plantings as experimental plants become available in the second year. Inoculation and evaluation will begin when the plants have been in the ground for one year and will continue annually until the field planting is terminated. Funding for completion of the fourth and any following years will be proposed in the 2018-2019 funding cycle and will depend on progress of the field evaluation up to that point.

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 least 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

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