Interim Progress Report for CDFA 16-0559-SA. Start date 07/01/2016; End date 12/31/2018

Transgenic Rootstock-Mediated Protection of Grapevine Scion by Introduced Single and Dual Stacked DNA Constructs

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Reporting Period: The results reported here are from January 1, 2018 to August 1, 2018

INTRODUCTION

Collectively, a team of researchers (Lindow, Dandekar, Labavitch/Powell, and Gilchrist) identified, constructed and advanced to field evaluation five novel DNA constructs (Table 1) that, when engineered into grapevines, suppress symptoms of Pierce's Disease (PD) by either a) reducing the titer of *Xylella fastidiosa (Xf)* in the plant, b) reducing systemic spread of the bacteria or c) blocking *Xf*'s ability to trigger PD symptoms. Each of the five transgenes, when expressed as single genes, reduced the disease levels under field conditions both as full plant transgenics and in transgenic rootstocks grafted to a non-transformed PD susceptible scion. This initial field trial consisting of single gene constructs was begun in 2010 and evaluated until discontinued at the end of the 2016 growing season. The field experiment is to be replaced with a second field trial designed to evaluate untransformed scion protection by rootstocks bearing paired combinations of the five constructs. If successful, the obvious benefit would be that any unmodified (non-transgenic) varietal wine grape scion could be grafted to and be protected by transformed rootstock lines. This approach involves "stacking," a combination of distinct protective transgenes in a single rootstock line, which is intended to foster not only durability but also more robust protection of the non-transformed scion against PD.

Briefly, this report will describe information on the history, likely function, and impact of each of the genes deployed as single transgenes in fully transformed plants in the initial field study in APHIS approved field trials wherein test plants were mechanically inoculated with *Xf* to induce Pierce's Disease. The experimental materials of this project are five specific DNA constructs (Table 1) that were shown to be effective in PD suppression under field conditions as single gene constructs to now be evaluated for potential cross-graft-union protection as described by the Lindow, Dandekar and Gilchrist in previous reports (see references).

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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

CAP and PGIP: (Abhaya Dandekar)

The Dandekar lab has genetic strategies to control the movement and to improve clearance of *Xylella fastidiosa* (*Xf*), the xylem-limited bacteria. The first strategy tests the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) from pear to inhibit the *Xf* polygalacturonase activity necessary for long distance movement (Aguero et al., 2006). The second strategy enhances clearance of bacteria from *Xf*-infected xylem tissues by expressing a chimeric antimicrobial protein (CAP), that consist of a surface binding domain that is linked to a lytic domain the composition and activity of these two protein components have been described earlier (Dandekar et al., 2012).

rpfF, DSF (Steven Lindow)

The Lindow lab has shown that *Xylella fastidiosa (Xf)* uses diffusible signal factor (DSF) perception as a key trigger to change its behavior within plants (Lindow, 2013. Accumulation of DSF in *Xf* cells, causes a change in many genes in the pathogen with the overall effect to suppress its virulence in plants by increasing its adhesiveness to plant surfaces and also suppressing the production of enzymes and genes needed for active movement through the plant.

PR1 and microRNA UT456 (David Gilchrist)

The Gilchrist lab is focused on the host response to *Xf* through identifying plant genes that block the inappropriate activation of a genetically conserved process of programmed cell death (PCD) common to many, if not all, plant diseases. Blocking PCD, either genetically or chemically, suppresses disease symptoms and bacterial pathogen growth in several plant-bacterial diseases Two of these grape sequences (PR1 and UT456), when expressed as transgenes in grape, suppressed Pierce's Disease (PD) symptoms and dramatically reduced bacterial titer in inoculated plants under greenhouse and field conditions. (Lincoln, Sanchez, and Gilchrist, 2018).

OBJECTIVES

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.

Additionally, there could be favorable synergistic protection when two or more resistance-mediating DNA constructs are employed. There are data indicating synergism in other crops. For example, the paper, "Field Evaluation of Transgenic Squash Containing Single or Multiple Virus Coat Protein Gene Constructs for Resistance to Cucumber Mosaic Virus, Watermelon Mosaic Virus 2, and Zucchini Yellow Mosaic Virus" (Tricoli et al 1995), describes the stacking of several genes for virus resistance in squash. Note, David Tricoli, the lead author in this paper, will be doing the stacking transformations in this proposal. Additionally, the Dandekar laboratory has successfully stacked two genes blocking two different pathways synergistically to suppress crown gall in walnut (Escobar et al., 2001). Experiments proposed here will evaluate potential synergism in suppression of PD symptoms and in reducing *Xf* titer for inoculations distant from the graft union.

1. Complete introduction pairs of protective paired constructs via the dual insert binary vector into adapted grapevine rootstocks 1103 and 101-14 for a total of 20 independent transgenic lines to be evaluated.
2. Conduct extensive analysis, both by Northern analysis and PCR and RTqPCR experiments of each transgenic plant to verify the presence of the two stacked genes in the genome, the full RNA sequence and the expression level of each of the mRNAs expected to be produced by the inserted genes before they are subjected to grafting and greenhouse inoculation assays for transgene movement and resistance to PD.
3. The second major step in the process after verification of the genotypic integrity of the transgenic plants is production of the clonal ramets of each plant line to enable two cane growth development of the rootstocks and grafting of the Chardonnay scions
4. Evaluate the resulting lines for efficacy by inoculation with Xf in a preliminary greenhouse experiment to identify the most protective lines from each combination of genes. A total of three independent transgenic lines of each dual construct in each rootstock with be selected to be bulked up to five copies of each for field planting at the APHIS approved site in Solano County. **Note: the greenhouse inoculation step was eliminated once it was clear that greenhouse based foliar symptoms did not provide a reliable indicator of disease response. There were discernable differences among the individual plants in based on bacterial counts within each of the10 dual combinations but spurious leaf burn symptoms were confounding and not characteristic of Pierce's Disease. However, the PCR confirmation of dual transformation was successful and was carried forward as the selection criteria**

DESCRIPTION OF ACTIVITIES CONDUCTED TO ACCOMPLISH OBJECTIVES

Construction of dual gene expression binaries:
The strategy is to prepare dual plasmid constructs bearing a combination of two of the protective genes on a single plasmid with single selectable marker as described previously (Gilchrist and Lincoln, 2016). The binary backbone is based on pCAMBIA1300 (Hajdukiewicz et al.; 1994). Binaries were constructed to express two genes from two 35S promoters. The DNA fragments containing transcription units for expression of the transgenes are flanked by rare cutting restriction sites for ligation into the backbone. The nt-PGIP used in these constructs is a modified version of the Labavitch PGIP that was constructed in the Dandekar laboratory to include a signal peptide obtained from a grapevine xylem secreted protein (Aguero et al., 2006). Binary plasmids capable of expressing two genes from the same TDNA were constructed by Dr. James Lincoln (Gilchrist et al., 2016).

All plasmids were transformed into Agrobacterium strain EHA105, the preferred transformation strain for grape plants. As a check on integrity of the duel binary plasmid, the plasmid was isolated from two Agrobacterium colonies for each construct and the plasmid was used to transform E. coli. Six E. coli colonies from each Agrobacterium isolated plasmid (for a total of 12 for each construct) were analyzed by restriction digest to confirm that the plasmid in Agrobacterium is not rearranged. Table 2 shows successful transformations by the UCD transformation facility. To ensure optimum recovery of

the transgenic embryos, two versions of the plasmid with different antibiotic selectable markers were delivered to the transformation facility. Hence, the dual inserts can now be subjected to two different selections that enables transformation to move forward in the fastest manner depending on which marker works best for each dual or each rootstock. Each plasmid containing the dual protective DNA sequences are introduced into embryogenic grapevine culture in a single transformation event rather than sequentially as would normally be the conventional strategy at the transformation facility. The new transgenic dual gene expressing grape plant lines exhibit a phenotype indistinguishable from the untransformed wild type rootstock (Figure 1). The transformation progress, following verification of insert integrity, for each line is shown in Table 3.

Analysis of the transgenic rootstocks to confirm dual insertions transcripts

This analysis is performed by isolating the RNA from transgenic grape leaves and purified by a modification of a CTAB protocol and includes LiCl precipitation. The RNA is converted to cDNA by oligo dT priming and reverse transcriptase. PCR reactions are set up using the synthesized cDNA as template and specific pairs of primers designed against each of the 5 putative transgenes. The goal is to identify 5 independently transformed lines bearing the dual sets of the 5 transgene to confirm the genotype of each rootstock to be placed in the field with 6 replications of each line. The aforementioned analysis indicated that the successful insertion two genes into a given transgenic plant was 67 percent of the total plants provided by the transformation facility (Table 2). This underscores the need for dual transcript verification prior to moving plants forward to grafting and subsequent analysis for product movement across a graft union and symptom suppression of the untransformed Chardonnay. These assays, while time consuming and tedious, will ensure that each plant will have a full phenotypic and genotypic analysis prior to inoculating them in the field.

Table 2. Frequency of dual gene transcripts as confirmed in transgenic plants delivered by the Parsons Transformation Facility by reverse transcription and PCR analysis.)

Transgene Transcripts	Number of Plants	Percent of Plants
two	230	67
one	99	29
none	12	4

Following verification of the genotypic integrity of the transgenic rootstock plants, clonal copies of each plant line were made to enable two cane growth development for production of rootstocks to be grafted with Chardonnay scions (Figures 2 and 3).

Evaluation of the lines by inoculation with Xf in the greenhouse

Preliminary inoculations were initiated in the greenhouse and selections made based on qPCR analysis of *Xf* titre in the tissue above the inoculation site under original objective 4. The qPCR tests will be repeated after the scions are inoculated in the field, which our experience deems more reliable. In total, over the two years of transgenic rootstock delivery and greenhouse evaluations, there will be approximately 7,000 molecular analyses conducted to minimize time and maximize the likelihood correlating the field results on bacterial dynamics with PD symptom scoring. The time frame from receipt of plants, analysis and selection of the individuals for field planting has been 9-13 months. Total number of plants to screen if all plants are verified transgenics will be at least 1,070 including 70 untransformed control plants

Table 3. Transcript profiling of the dual construct transformed transgenic rootstocks. The totals do not include the both whole transformed and untransformed PD susceptible controls (200 plants).

Genotype	Construct code	Construct	# lines grafted 2018	# plants to field 2018
1103	AB	pCA-5oP14HT-5oUT456	6	36
101-14	AB	pCK-5oP14HT-5oUT456	4	24
1103	AC	pCA-5fCAP-5oP14HT	6	36
101-14	AC	pCK-5fCAP-5oP14LD	0	0
1103	AD	pCA-5PGIP-5oP14HT	6	36
101-14	AD	pCK-5PGIP-5oP14LD	6	36
1103	AF	pCA-5oP14HT-5orpfF	0	0
101-14	AF	pCK-5oP14LD-5orpfF	1	6
1103	BC	pCA-5fCAP-5oUT456	6	36
101-14	BC	pCA-5fCAP-5oUT456	0	0
1103	BD	pCA-5PGIP-5oUT456	0	0
101-14	BD	pCK-5PGIP-5oUT456	6	36
1103	BF	pCA-5oUT456-5orpfF	4	24
101-14	BF	pCK-5oUT456-5orpfF	0	0
1103	CD	pCA-5PGIP-5FCAP	0	0
101-14	CD	pCK-5PGIP-5FCAP	0	0
1103	CF	pCA-5fCAP-5orpfF	6	36
101-14	CF	pCK-5ofCAP-5orpfF	0	0
1103	DF	pCA-5PGIP-5orpfF	6	36
101-14	DF	pCK-5PGIP-5orpfF	6	36
			63	378

Table 4. Dual construct transformed 1103 and 101-14 rootstocks grafted to untransformed Chardonnay for planting in the APHIS regulated field on August 1, 2018.

1103 rootstocks						**101-14 rootstocks**	
AB15-01	AC35-01	AD13-04	BC36-03	CF07-02	DF108-03	BD23-05	DF85-01
AB15-02	AC62-01	AD13-06	BC36-05	CF07-03	DF108-07	BD58-01	DF85-02
AB15-04	AC62-02	AD13-07	BC36-06	CF07-04	DF108-08	BD58-02	DF85-04
AB15-05	AC62-04	AD33-01	BC36-09	CF07-05	DF108-09	BD58-08	DF85-06
AB15-06	AC62-06	AD33-02	BC36-11	CF07-06	DF108-10	BD80-05	DF85-08
AB 15-03	AC35-05	AD13-02	BC36-13	CF07-12	DF108-04	BD23-01	DF85-10

Initially each of first transgenic lines of 1103 were inoculated with *Xf* Inoculation in the greenhouse per the original objectives 4. Within the inoculation experiment, samples are taken to determine the population of bacteria at the inoculations site, 10 cm and 30 cm from the inoculation site. Unfortunately the foliar symptoms under these greenhouse conditions were not reliably diagnostic of the disease severity nor related to the relative bacterial titer in the inoculated canes. Hence, we have found the more reliable indicator of the integrity of the transformation was the insert-depended suppression of bacterial titre. **Hence the greenhouse inoculations were discontinued and molecular analysis used to select**

the transgenic rootstocks moved forward to grafting. After verification of dual inserts the selected lines were moved to a lath house for final stem development prior to rooting of the transformed rootstock prior to grafting (Figure 1).

Production two cane growth development of each plant line to enable of collection of rootstock cuttings for grafting of the Chardonnay scions (objective 3)

Figure 1. Plants selected as rootstock source material. Image shows selected dual construct containing plants in lath house as final site to produce material for rootstock development, for grafting of non-transgenic scions and field evaluation

Figure 2. 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 3. Bud grafting of wild type Pierce's Disease susceptible Chardonnay to the dual construct transformed rootstocks and planting of the grafted individuals in the APHIS regulated field.

Figure 4. Planting of 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.

Timeline for completion of delivery of the transgenic rootstock plants, the greenhouse and laboratory analysis, followed by the field planting the selected rootstocks grafted to the non-transgenic Chardonnay scions

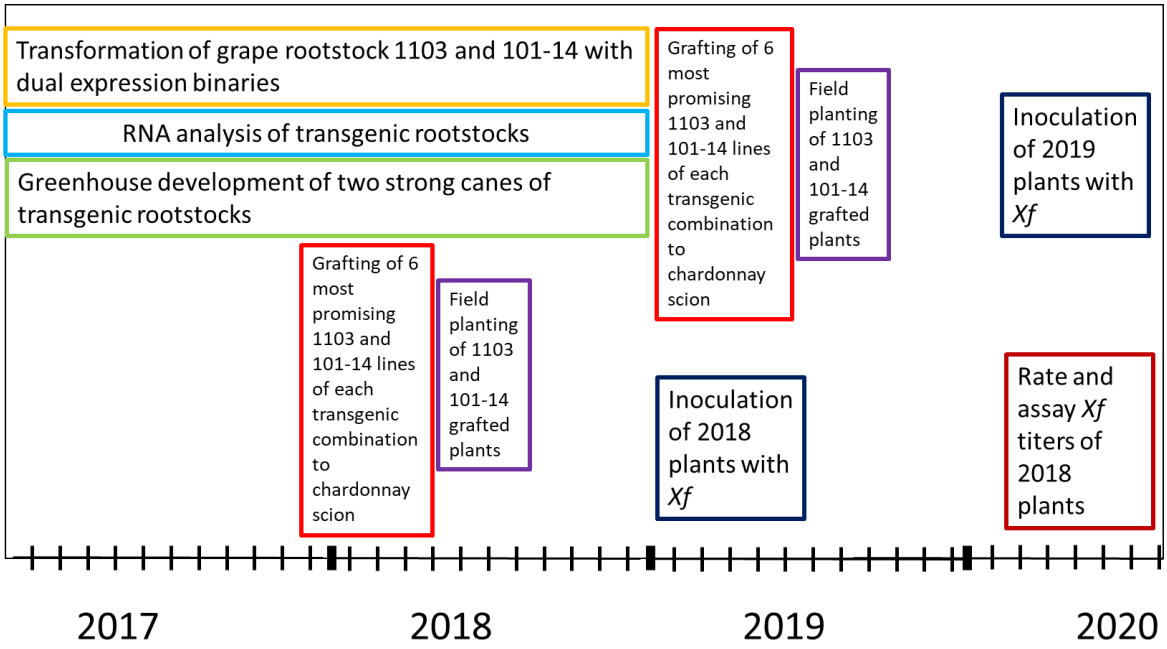

Figure 5. Anticipated Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.

CONCLUSIONS

Our capacity to achieve all the objectives is essentially assured based on prior accomplishments and the fact that we are exactly where we are projected to be within the timeline indicated in Figure 5. All techniques and resources are available in the lab and have proven reliable, informative, and reproducible. This project has consolidated a full time research commitment for this team of experienced scientists to Pierce's Disease. Each of the senior personnel, including Dr. Lincoln have been with this project since 2007. Collectively the team brings a full range of skills and training that complement changing needs of this project in the areas of molecular biology, plant transformation and analysis of transgenic plants.

The scope of research includes both greenhouse and field evaluation of the transgenic rootstocks for suppression of Pierce's Disease in the non-transgenic scions. Commercialization of the currently effective anti-PD containing vines and/or rootstocks could involve partnerships between the UC Foundation Plant Services, nurseries, and, potentially, with a private biotechnology company. As indicated above the dual constructs have been assembled and forwarded to David Tricoli at the Parsons' Plant Transformation facility. The transgenic plants are being delivered to Dr. Lincoln as indicated in table 2 and evaluations have begun as indicated in table 3 and figure 4. The first step in the analysis of the transcribed RNA to verify that each plant contains both of the intended constructs. The timeline shown in Figure 5 for both transformation and analysis is on track. . If successful, the stacking of genes is the next logical step toward achieving commercialization of transgenic resistance.

LAY PERSON SUMMARY

Xylella fastidiosa (*Xf*) is the causative agent of Pierce's Disease (PD). Collectively, a team of researchers (Lindow, Dandekar, Labavitch/Powell and Gilchrist) has identified five novel genes (DNA constructs) (Table 1) when engineered into grapevines, suppress symptoms of PD by reducing the titer of *Xf* in the plant, reducing its systemic spread in the plant, or blocking *Xf*'s ability to trigger PD symptoms. These projects have moved from the proof-of-concept stage in the greenhouse to characterization of PD resistance under field conditions where current data indicate that each of the five transgenes, introduced as single constructs, reduces the disease levels under field conditions. Importantly, preliminary data indicates that each of the five DNA constructs, when incorporated into transgenic rootstock, has shown the ability to protect non-transformed scion, with obvious benefit: any of many unmodified varietal scions can be grafted to and be protected by any of a small number of transformed rootstock lines. The ability of transgenic rootstock to protect all or most of the scion, even at a distance from the g r a f t union, is currently being tested. The objective described herein addresses the issue of durability, the capability of genetic resistance to avoid being overcome by evolving virulent versions of the *Xf* pathogen, a critical factor for a long-lived perennial crop such as grapevine. This approach involves "stacking," a combination of distinct protective transgenes in a single rootstock line, which is intended to foster not only durability but also more robust protection of the non-transformed scion against PD

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

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