Comprehensive Final Report for CDFA Agreement Number SA-16-0559

Project Title: Transgenic rootstock-mediated protection of grapevine scion by single and stacked DNA constructs

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Research Area Pathogen and Disease Management

Time Period of Project: This final report covers the period July 1, 2016 to December 31, 2018 to complete development of and analysis of the stacked gene transgenics and prepare selected individuals for moving to the field in the summer of 2018. The research described herein is now continuing under CDFA Agreement Number SA-18-0307.

Project History: This proposal continued the research under CDFA Agreement Number 14-0149-SA and that has now been extended to 2020 under CDFA Agreement Number SA-18-0307 with an end date of June 23, 2020.

Introduction: This continuing research project focused the goal of identifying plant genes, which when transformed into cultivated wine grape (*Vitus vinifera)* would depress symptoms of Pierce's Disease (PD) caused by the bacteria Xylella fastidiosa (Xf). Previously, a team of researchers (Lindow, Dandekar, and Gilchrist) (1,2,3,4,5,7,8,10) identified five novel DNA constructs (Table 1), constructed transgenic plants, and advanced the plants to field evaluation that, when engineered individually into grapevines, suppressed symptoms of Pierce's Disease (PD) (references 2-8, 10-11). These constructs appeared to function by several different mechanism: a) reducing the titer of *Xylella fastidiosa (Xf)* in the plant, b) reducing its systemic spread in the plant, or c) by blocking *Xf*'s ability to trigger tissue death symptoms of PD. Current data indicate that each of the five transgenes dramatically reduces the disease levels under field conditions. Having satisfied the initial objectives, this field trial was discontinued in July 2016.
The current project was designed prepare materials for and to begin a second field trial evaluating

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, presumed function

Gene	Function
CAP	Xf clearing/antimicrobial
PR1	grape cell anti-death, suppresses symptoms
rpfF	changing quorum sensing of Xf (DSF)
UT456	non-coding microRNA activates PR1 translation
PGIP	inhibits polygalacturonase/ suppressing Xf movement

whether pairs of these genes introduced into adapted rootstocks would suppress symptoms of PD across a graft union in an untransformed susceptible scion (Chardonnay 04) (6).

The objectives encompass the continuation of the basic research leading to development of the dual gene transfer vectors, transformation and confirmation of the two gene pair insertions in the rootstocks and develop the grafted plants for field evaluation. If successful, the obvious benefit would be that any unmodified (non-transgenic) varietal winegrape scion could be grafted to and be protected by transformed rootstock lines. This report confirms that the objectives were addressed successfully in the timelines proposed initially and initial planting of the grafted plants was begun in the summer of 2018 and that the continuation of this research is proceeding under CDFA Agreement Number SA-18-0307 with an end date of June 23, 2020.

Objectives of Proposed Research

The overall objective of this continuation project addresses the practical issue of durable resistance to PD; specifically, the capability of genetic resistance to avoid being overcome by evolving virulent versions of the pathogen, a critical factor for a long-lived perennial crop such as grapevine. Several steps were designed to be completed in 2018 leading to development of the grafted transgenic rootstock-based plants and advance them to the field in preparation for inoculation with *Xf*. The dual gene transfer binary vector was successfully constructed, the paired genes inserted into the vector and delivered to the UCD transformation facility (14). Transgenic plants were released from the facility beginning in 2016 and molecular analysis begun. From experience, we know that primary transgenic plants show varying levels of gene expression, hence the critical importance of preemptive molecular assays to confirm successful insertion of both genes and their expression. Selection will be based on precise confirmation of the molecular fidelity of the insert sequences. From these latter analyses, five (5) lines of each rootstock transgene combination were to be selected and expanded to ramets of six replications of each for field testing.

This project represents the culmination of a decade of basic research and discovery regarding the molecular and genetic factors affecting the bacterial behavior in the grape plant and the basis for the disease symptoms. Additionally, there could be favorable synergistic protection when two or more resistance-mediating DNA constructs are employed. There are data indicating multigene 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" (12), describes the stacking of several genes for virus resistance in squash. Note, David Tricoli, the lead author in this paper, is doing the paired gene transformations. Additionally, the Dandekar laboratory has successfully stacked two genes blocking two different pathways synergistically to suppress crown gall in walnut (13). Experiments proposed here evaluated potential synergism in suppression of PD symptoms and in reducing *Xf* titer for inoculations distant from the graft union.

Specific steps to complete the development and pre-field analysis of the transgenic rootstocks

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 with at least 10 paired combinations from each rootstock line delivered by the transformation facility.
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 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 5 independent transgenic lines of each dual construct in each rootstock will be selected to be bulked up to 6 copies of each for field planting at the APHIS approved site in Solano County (11).

5. **Note: the greenhouse inoculation step was eliminated after several attempts 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 to accomplish objectives and vector development

1. **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 (9). The binary backbone is based on pCAMBIA1300 (14). 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 (1). Binary plasmids capable of expressing two genes from the same TDNA were constructed and analyzed by Dr. James Lincoln.

This was not a trivial exercise. All plasmids were transformed into Agrobacterium strain EHA105, the preferred transformation strain for grape plants. As a check on integrity of the duel genes in the 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. 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. (9).

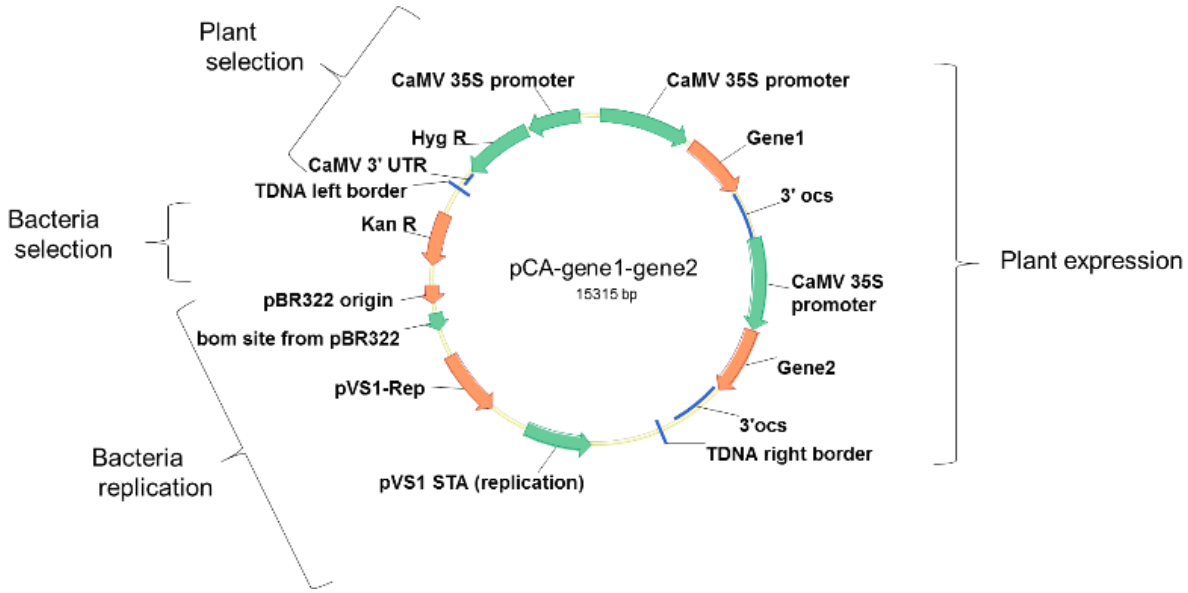

Figure 1. Dual expression binary expresses two genes within the same TDNA insert. This allows a single transformation event to generate plants that express two gene products.

The new transgenic dual gene expressing grape plant lines exhibit a phenotype indistinguishable from the untransformed wild type rootstock (Figure 2).

Figure 2. Illustration of transgenic rootstock, 1103 containing the paired genes CAP and PR1 (P14LD)

1103 pCA-5fCAP-5oP14HT

2 <u>Analysis of the transgenic rootstocks to confirm dual insertion transcripts</u>

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 (Tables 3 and 4).

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

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).

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

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

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

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 transcript analysis. **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).

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

3. 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).
4. Grafted plants were then moved to the field in August 2018 (Figure 4).

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 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.

Research Timetable

Timeframe proposed for accomplishment of the objectives leading to field evaluation

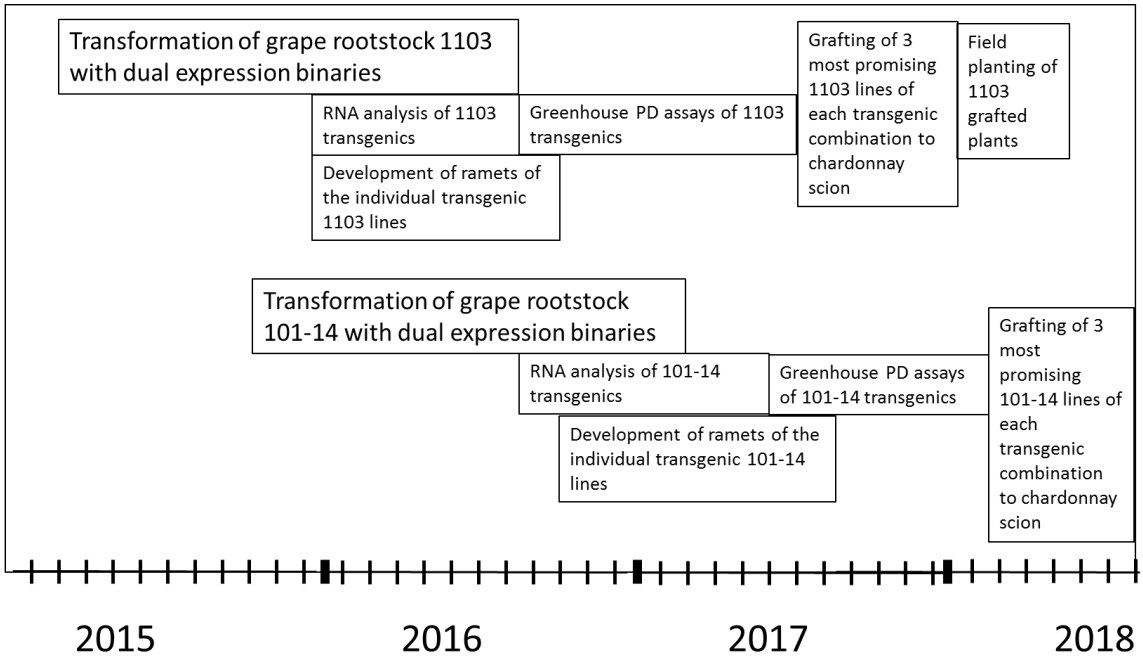

Figure 5. Anticipated Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.

Research Capacity and Accomplishment of Objectives within the Timelines

The anticipated timeline shown in Figure 5 was completed per expectations and is continuing under CDFA Agreement Number SA-18-0307 with an end date of June 23, 2020. The transgenic rootstocks were delivered as anticipated which confirmed that our capacity to achieve all the objectives was assured based on prior accomplishments. All techniques and resources are available in the lab were proven reliable, informative, and reproducible. This project brought together 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 and have different skills and training that complement changing needs of this project in the areas of molecular biology, plant transformation and analysis of transgenic plants. This includes both greenhouse and field evaluation of protection against Pierce's Disease.

Publications produced and pending, and presentations made that relate to this funded project

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, 2015b. 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)

Relevance Statement

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.

Laypersons 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. 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: all funds were expended following the 6 months no cost extension, which ended December 31, 2018.

Intellectual property. The grape plants containing the anti-PCD genes and the grafted rootstocks will require the use of several patented enabling technologies. The research proposed reported herein will provide data on the activity and mechanism of action of the protective transgenes in grape relative to untransformed control plants.

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