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

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ABSTRACT

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 discontinued at the end of the 2016 growing season 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. The propagative materials are currently in production, following the timeline in figure 5 with field planting of the rootstock/scion combinations (figures 3 and 4) to begin spring of 2018.

LAYPERSON 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. The obvious benefit: is that any unmodified varietal scions could be grafted to and protected by any of a small number of transformed rootstock lines. The ability of transgenic rootstock to protect 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," sets of dual transgenes (table 1) in a single rootstock lines, to test the protection of the non-transformed scion against PD

INTRODUCTION

This project began in 2010 to study the impact of selected single transgenes in the initial APHIS approved field trials where test plants are mechanically inoculated with *Xf*. The experimental materials of this project are five specific DNA constructs (Table 1) that were shown to be effective in PD suppression first under greenhouse and then field conditions as single gene constructs. Several of these genes also appear to have potential in cross-graft-union protection of untransformed scions as described by the Lindow, Dandekar and Gilchrist in previous reports and noted in the references.

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, Gram-negative bacterium that is the causative agent of Pierce's disease in grapevine Dandekar, 2013). 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), as 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, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in the pathogen, but the overall effect is 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 a critical aspect of grape susceptibility to *Xf*, namely the inappropriate activation of a genetically conserved process of programmed cell death (PCD) of many, if not all, plant diseases. Blocking PCD, either genetically or chemically, suppresses disease symptoms and bacterial pathogen growth in several plant-bacterial diseases (Richael et al., 2001, Lincoln et al. 2002, Harvey et al. 2008). A functional genetic screen identified novel anti-PCD genes from cDNA libraries of grape and tomato (Gilchrist and Lincoln 2011). 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 and field tests. The mechanism of suppression of PD symptoms depends on translation of either the transgenic or the endogenous PR1 message in the face of *Xf* –triggered cell stress.

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 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 three independent transgenic lines of each dual construct in each rootstock will be selected to be bulked up to five copies of each for field planting at the APHIS approved site in Solano County.

RESULTS AND DISCUSSION

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 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 2). The transformation progress, following verification of insert integrity, for each line is shown in Table 2.

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 of two genes into a given transgenic plant was 60 percent of the total plants provided by the transformation facility (Table 3). The fact that 40 percent of received transgenic rootstock plants lacked one or both of the transgenes underscores the need for molecular analysis of transcript integrity prior to moving plants forward to grafting and subsequent analysis for movement of the transgenic 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. Transcript profiling of the dual construct transformed transgenic rootstocks

Genotype	Construct code	Construct	# Plants both transcripts	Dual Transcript Analysis
1103	AB	pCA-5oP14HT-5oUT456	8	Complete
101-14	AB	pCK-5oP14HT-5oUT456	8	Complete
1103	AC	pCA-5fCAP-5oP14HT	10	Complete
101-14	AC	pCK-5fCAP-5oP14LD	0	
1103	AD	pCA-5PGIP-5oP14HT	10	Complete
101-14	AD	pCK-5PGIP-5oP14LD	9	Complete
1103	AF	pCA-5oP14HT-5orpfF	2	
101-14	AF	pCK-5oP14LD-5orpfF	5	
1103	BC	pCA-5fCAP-5oUT456	10	Complete
101-14	BC	pCA-5fCAP-5oUT456	7	complete
1103	BD	pCA-5PGIP-5oUT456	2	
101-14	BD	pCK-5PGIP-5oUT456	12	Complete
1103	BF	pCA-5oUT456-5orpfF	4	
101-14	BF	pCK-5oUT456-5orpfF	6	complete
1103	CD	pCA-5PGIP-5FCAP	4	
101-14	CD	pCK-5PGIP-5FCAP	6	complete
1103	CF	pCA-5fCAP-5orpfF	10	Complete
101-14	CF	pCK-5ofCAP-5orpfF	1	
1103	DF	pCA-5PGIP-5orpfF	12	Complete
101-14	DF	pCK-5PGIP-5orpfF	12	Complete

Table 3. 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
two	138
one	79
none	11

Table 4. Dual construct transformed 1103 and 101-14 rootstocks now in a lathe house for making rooted cuttings prior to grafting

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

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

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, 3 and 4).

Preliminary inoculations were initiated in the greenhouse and selections made based on qPCR analysis of *Xf* titre in the tissue above the inoculation site. These tests will be repeated after the scions are inoculated in the field. 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.

The following images illustrate the status of the dual construct transgenic plants as they are managed in the greenhouse (Figures 1+2). Each plant is staked to support vegetative growth for inoculation, symptom expression and sampling. Each pot is individually irrigated with a nutrient solution and plants are trimmed as necessary to avoid excessive branching under these growth conditions. A preliminary greenhouse inoculation experiment was initiated wherein samples were taken to determine the population of bacteria at the inoculations site, 10 cm and 30 cm from the inoculation site. Unfortunately the foliar symptoms are not reliably diagnostic of relative bacterial titer in the inoculated canes. Hence, we have found the more reliable indicator was the insert-depended suppression of bacterial titre. Table 4 and Figure 4 show the selected lines now in the lathe house for final stem development prior to rooting of the transformed rootstock prior grafting.

Figure 1. Transgenic rootstock grape plants growing in greenhouse.

Figure 2. 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

Images from January 2018

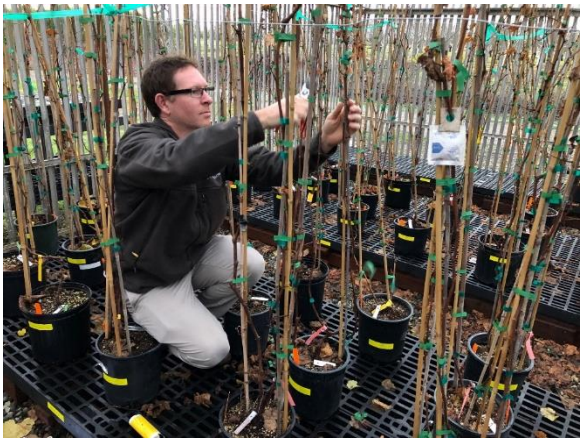

Figure 3. Josh Puckett harvesting transgenic rootstock canes for bud grafting to untransformed Chardonnay. Packet tag indicates rootstock and paired gene

Images from March 2018

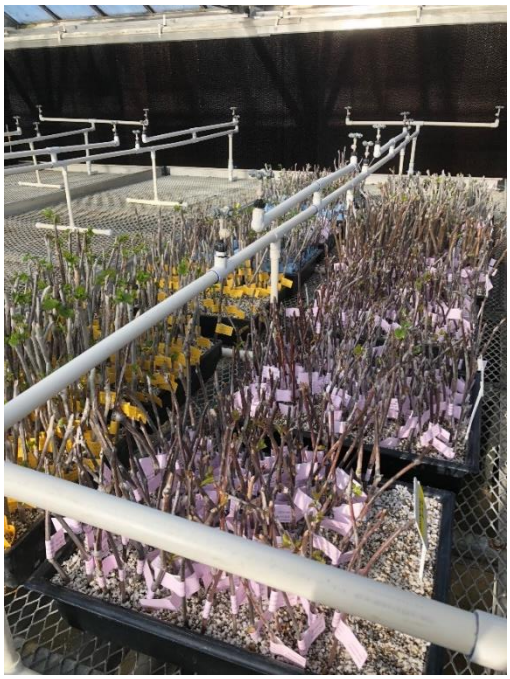

Figure 4. Transgenic rootstock canes rooting for bud grafting to untransformed Chardonnay. Tags indicate rootstock and paired gene combinations expressed in

Timeline for delivery of the transgenic rootstock plants, laboratory analysis, followed by grafting of untransformed scions and field planting for evaluation of the PD susceptibility of the non-transgenic Chardonnay scions

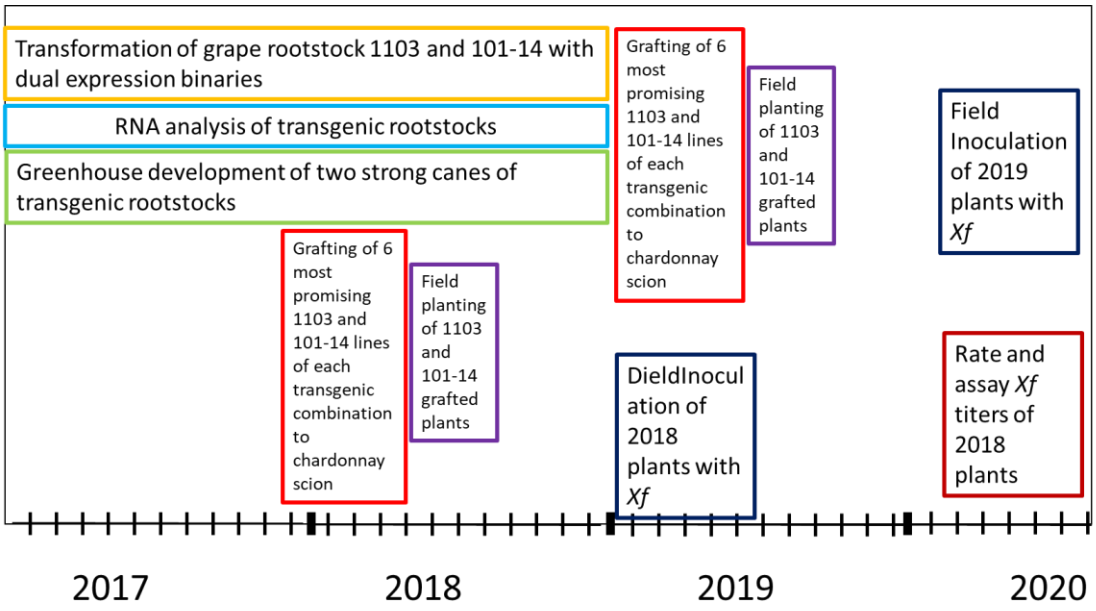

Figure 4. Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.
NOTE: This timeline remains accurate as of this March 2018 report.

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.

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