

TRANSGENIC ROOTSTOCK-MEDIATED PROTECTION OF GRAPEVINE SCION BY SINGLE AND STACKED DNA CONSTRUCTS

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Important information on continuation of this project: The results reported here cover the period 07/01/2014 to 12/31/2016. It is important to note that, even though funding for this project ended 12/31/2016, the objectives and ongoing data collection are being continued with minor modifications from the original 07/01/2014 without interruption under a new grant, CDFA-16-0559-SA.

ABSTRACT

This document represents the final report on CDFA 14-0149-SA for which the grant period ended 12/31/2016 but the research described herein is continuing unabated under CDFA16-0559-SA. There was no lapse in support or in the research being conducted. Historically, a team of researchers (Lindow, Dandekar, 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 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. The continuation of the basic research and the field trials results from the field data indicating that several of the five DNA constructs, when incorporated into transgenic rootstock, have shown potential for protection of a non-transformed scion across a graft union (Figure 1). The present 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 rootstocks bearing paired combinations of the five constructs.

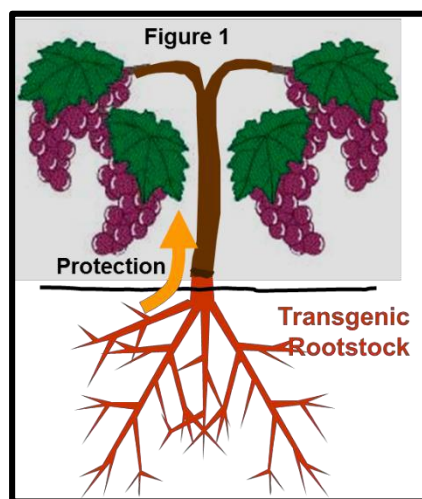


Figure 1, example scenario whereby a transgenic rootstock is being tested for ability to protect an untransformed scion from Pierce's Disease.

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. The approach taken under this grant involved “stacking,” a combination of distinct protective transgenes into single rootstock lines to which a non-transgenic PD susceptible scion (Chardonnay) will be grafted. The ongoing research under grant CDFA-16-0559-SA will test whether the combined DNA constructs first developed under grant CDFA 14-0149-SA will foster not only durability but also more robust protection of the non-transformed scion against PD. Stacked transgene rootstock lines are now being received for greenhouse whole plant PD evaluation, then grafting and PD evaluation, first under controlled greenhouse conditions and then to produce ramets of the most suppressive transgenic rootstock lines for field evaluation beginning in late 2017 or early 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) identified, constructed and advanced the evaluation of five (Table 1) novel genes (DNA constructs) that, when engineered into grapevines, suppressed 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 with mechanical inoculation of the test plants with pathogenic strains of *Xf*. Data collected during the course this grant period indicates that each of the five transgenes, introduced as single constructs, reduced the disease levels under field conditions. In addition, 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 in that any unmodified varietal scions can be grafted to and be protected by transformed rootstock lines. Hence, the objectives in this project also addressed the issue of durability of genetic resistance a critical factor for a long-lived perennial crop such as grapevine. The objective of this research and the current continuation of these objectives involved “stacking,” a combination of distinct protective transgenes in a single rootstock lines to assess durability and the level of protection of the non-transformed scion against PD. The stacking of genes is the next logical step toward achieving commercialization of transgenic resistance. Stacked transgene rootstock lines will be ready for evaluation in 2017 under controlled greenhouse conditions and for field testing of the rootstock-scion combinations in 2018 as projected under the continuing project funded in 2017..

INTRODUCTION

Briefly, we review here information on the history and impact of the genes deployed as single transgenes currently in APHIS approved field trials as funded by this concluding project. The subjects of this project are five specific DNA constructs (Table 1) that have shown to be effective in PD suppression under field conditions as single gene

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

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

constructs and also appear to have potential in cross-graft-union protection described by the Lindow, Dandekar and Gilchrist in previous reports and noted in the references.

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). A key virulence feature of *Xf* resides in its ability to digest pectin-rich pit pore membranes that connect adjoining xylem elements, enhancing long-distance movement and vector transmission. 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). Under most conditions DSF levels in plants are low since cells are found in relatively small clusters, and hence they do not express adhesins that would hinder their movement through the plant (but which are required for vector acquisition) but actively express extracellular enzymes and retractile pili needed for movement through the plant (Chatterjee et al. 2008). 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) that is 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 (Richael et al., 2001, Lincoln et al. 2002, Harvey et al. 2007). In the current project with PD, 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. Assays with various chemical and bacterial inducers of PCD confirmed that the PR1 was capable of blocking PCD in transgenic plant cells when translated the presence of a bacterial secreted death signal Sequence analysis of UT456 revealed a strong sequence complementarity to a region in the PR1 3'UTR that released the translational block of PR1 translation. Hence, the mechanism of suppression of PD symptoms depends on translation of either the transgenic or the endogenous PR1 message in the face of *Xf*-trigger cell stress.

OBJECTIVES under CDFA 14-0149-SA

- 1: The first objective was to assess whether and to what degree each transgenic rootstock, bearing a DNA construct, can protect the entire non-transgenic scion from symptom development and reduce *Xf* accumulation.
- 2: The second objective was to generate grapevine rootstock lines bearing various combinations of 2, 3, or possibly 4, constructs from the Table 1 set (and possibly constructs from other investigators). This objective was later modified to include introduce the genes set out in Table 1 in paired combinations of two.

The primary motive 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 differentially acting DNA constructs “stacked” in the rootstock should theoretically 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, we pointed to historical examples of synergism between multiple transgenes in other crops when two or more resistance-mediating DNA constructs are employed. 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 with (Escobar et al., 2001). Experiments proposed here are designed to evaluate potential synergism in suppression of PD symptoms and reducing *Xf* titer distant from the graft union.

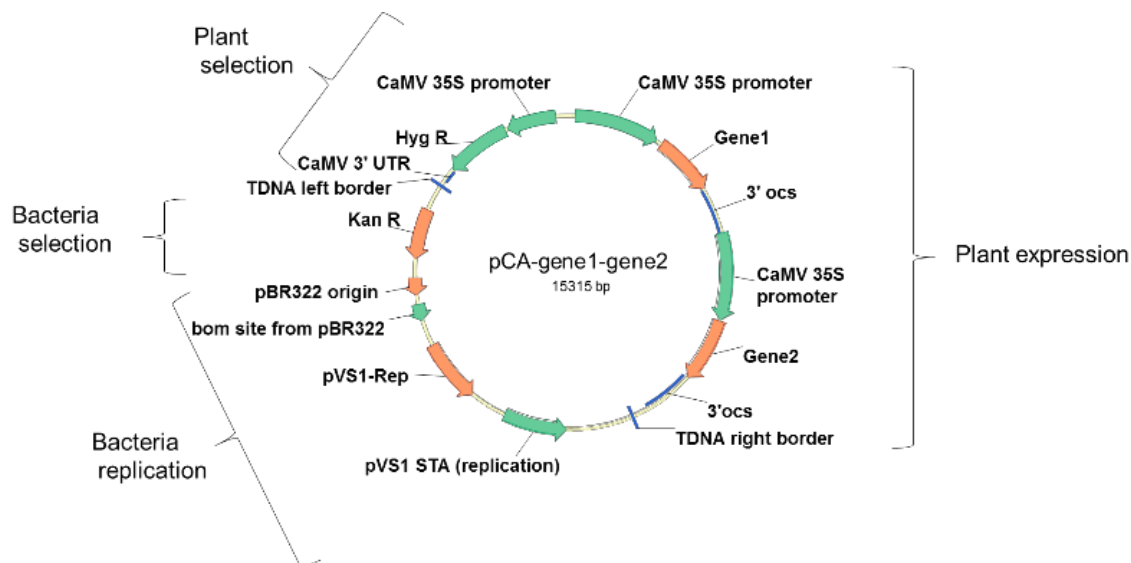


Figure 2. Dual expression binary expresses two genes within the same TDNA insert. This allows a single transformation event to generate plants that express two gene products.

Analysis of the transgenic rootstocks to confirm dual insertions conducted during this project.

RNA from transgenic grape leaves was purified by a modification of a CTAB protocol and included LiCl precipitation. In this approach, 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 resulting products are separated by agarose gel electrophoresis (Figure 4). In this figure the bands shown correspond to two amplification targets in each transgenic plant. Progress on the RNA verification is shown in Table 3.

Table 3. Progress in RNA analysis of the dual construct transformed transgenic rootstocks

The current status of verification of transgenic RNA from transgenic rootstocks 1103 and 101-14.

Grapevine Genotype	construct	verified RNA	ramets started
1103	CAP-PR1	10	X
	PGIP-456		
	CAP-456	10	X
	PGIP-PR1	10	X
	PGIP-CAP	5	
	PR1-456	10	X
	rpF-PR1		
	CAP-rpF	4	
	PGIP-rpF	10	X
	rpF-456	1	
101-14	CAP-PR1	2	
	PGIP-456		
	CAP-456		
	PGIP-PR1	2	
	PGIP-CAP	3	
	PR1-456		
	rpF-PR1		
	CAP-rpF		
	PGIP-rpF	5	
	rpF-456		

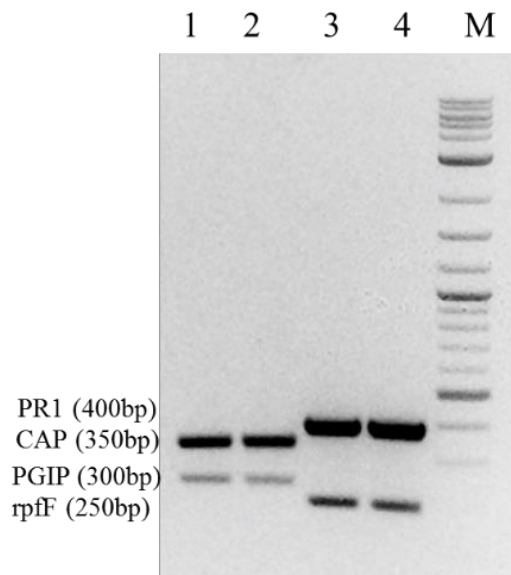


Figure 4. An example of leaf RNA analysis of 4 independent transgenic grape lines. Lanes 1 and 2 are putative CAP and PGIP dual expression lines. Lanes 3 and 4 are putative PR1 and rpF dual expression lines. Sizes of the expected products are shown.

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.

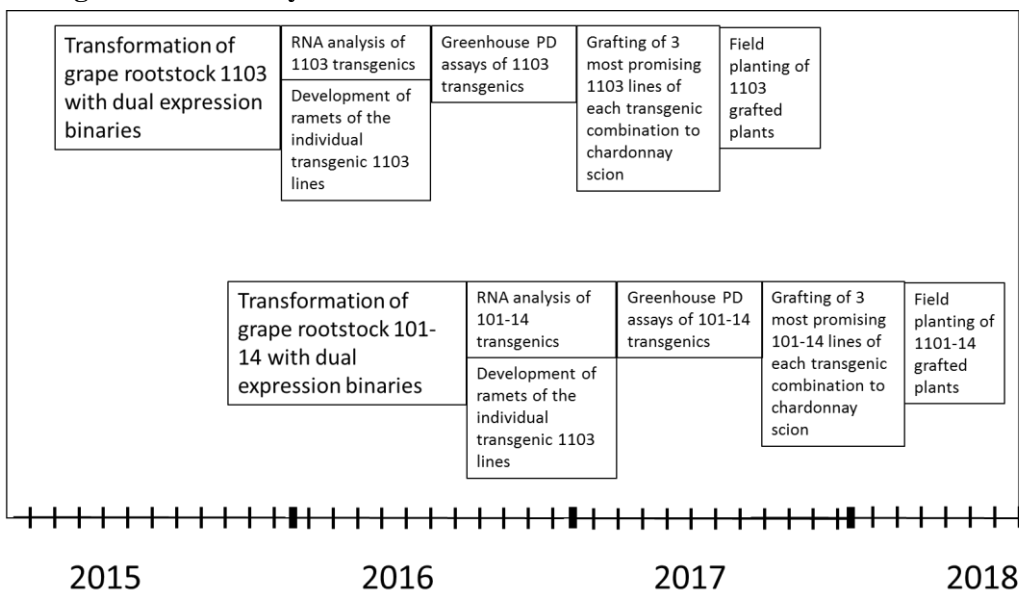


Figure 5. Anticipated Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.

CONCLUSIONS FOR CDFA 14-0149-SA

We were able to achieve all the objectives within the timeline indicated in Figure 5. All aspects of this research are being continued with the succeeding grant CDFA 16-0559-SA. All techniques and resources were available in the lab and have proven to be 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 concluded here and continuing under CDFA-16-0559-SA includes both greenhouse and field evaluation of the transgenic rootstocks for relative 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 are being introduced by 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 is 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.

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