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Transgenic rootstock-mediated protection of grapevine scion by stacked DNA constructs

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INTRODUCTION

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 or constructed and advanced the evaluation of five (Table 1) novel genes (DNA constructs) that, 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 dramatically reduces the disease levels under field conditions. These existing field trials will continue through 2016. Importantly, 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. Under the objectives, we will introduce pairs of protective constructs into an adapted grapevine rootstock 1103 and test for efficacy by inoculation with *Xf* in a preliminary greenhouse experiment to identify the most protective lines from each combination of genes. Secondly, the ability of transgenic rootstock to protect all or most of the scion, even at a distance from the graft union, will be tested.
These experiments are the next logical step toward achieving commercialization of transgenic resistance. Stacked transgene rootstock lines will be ready for evaluation in late 2015, with results from greenhouse testing by 2016.

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 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 with (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.

Briefly, we describe information on the history and impact of the genes deployed as single transgenes currently in APHIS approved field trials. The subjects of this proposal are five specific DNA constructs (Table 1) that show cross-graft-union protection described by the Lindow, Dandekar and Gilchrist laboratories as follows:

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

Table 1. Genes selected to suppress Pierce's disease in grape

The table lists gene names, presumed function

Gene	Function
CAP	Xf clearing/antimicrobial
PR1	grape cell anti-death
rpfF	changing quorumsensing of Xf (DSF)
UT456	non-coding microRNA activates PR1 translation
PGIP	inhibits poygalacturonase/ suppressing Xf movement

movement through the plant (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 these cells become numerous within xylem vessels, causes a change in many genes in the pathogen, 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.

CAP and PGIP: (Abhaya Dandekar)

The Dandekar lab has successfully participated in the two field plantings to investigate two greenhouse-tested 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 (Dandekar et al., 2012).

PR1 and microRNA UT 456 (David Gilchrist)

The Gilchrist lab is focused on the host response to *Xf* by 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 in which cell death is the visible symptom of disease. We have demonstrated previously that blocking PCD, either genetically or chemically, can block 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, we developed a functional screen and 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 conditions. Assays with various chemical and bacterial inducers of PCD confirmed that the PR1 was capable of blocking PCD in transgenic plant cells (Sanchez et al., 2015a). The library screen also identified a noncoding micro RNA that suppressed PD in planta. Additional experiments confirmed a functional link of the noncoding UT456 sequence to PR1 resides in the ability of the UT456 sequence, in the form of a microRNA, that binds to the PR1 3'UTR and release the translational block of PR1 translation, in this case the native grape PR1. Hence, in both transgenic plants 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 (Sanchez et al., 2015b).

OBJECTIVES

1. Introduce pairs of protective constructs into an adapted grapevine rootstock 1103.
2. The resulting lines will be tested for efficacy by inoculation with *Xf* in a preliminary greenhouse experiment to identify the most protective lines from each combination of genes.

Activities to accomplish objectives: progress to date

Construction of dual gene expression binaries: In the past six months, binaries were constructed to express two genes simultaneously each driven by 35S promoters on a single plasmid. The DNA fragments containing transcription units for expression of the transgenes are flanked by rare cutting restriction sites enabling direct ligation into the backbone.

Binary plasmids capable of expressing two genes from the same TDNA (duel expressers) were made by Jim Lincoln in the Gilchrist lab and are of the general form shown in figure 1. All dual gene plasmids are transformed into Agrobacterium strain EHA105 for transformation into grape. As a check on stability of the duel expresser binary plasmid, the plasmid was re-isolated from two Agrobacterium colonies for each construct and the plasmid transformed into E. coli. Six E. coli colonies from each Agrobacterium isolated plasmid (for a total of 12) are then analyzed by restriction digest to confirm that the plasmid in Agrobacterium is not rearranged. Table 2 shows the status of the constructs with regard to the UCD transformation facility of which six dual constructs are now being introduced into the 1103 root stock.

Table 2. Duel expressers to go into grape rootstock 1103

Table shows construct name and timing of submission to the Parsons Transformation Facility.

Submitted January 2015	To be submitted April 2015
pCA-CAP-PGIP	pCA-PGIP-rpfF
pCA-PGIP-PR1	pCA-CAP-rpfF
pCA-UT456-PR1	pCA-PR1-rpfF
pCA-CAP-PR1	pCA-UT456-rpfF
pCA-CAP-UT456	
pCA-UT456-PGIP	

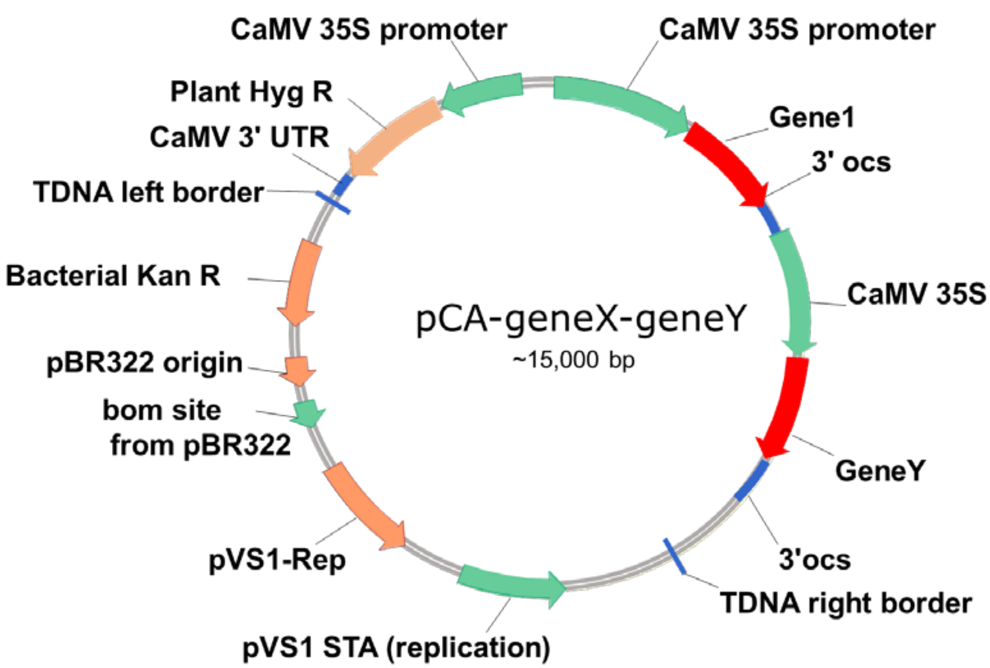

Figure 1. Dual expression binary expresses two genes within the same TDNA insert. This allows a single transformation event to generate plants that express two gene products.

CONCLUSIONS

Progress on creating a dual construct (gene) plasmid for transformation of grape rootstock 1103 has moved at a rapid pace. The binary plasmid required for shuttling the dual gene constructs has been developed and tested successfully for stability and integrity. All techniques and resources in the lab have proven reliable, informative, and reproducible. At this point, six of the paired constructs have been made and confirmed to contain the genes of interest. These six listed in table 1 are now being introduced into embryonic grape cells as the first step leading to the production of the transgenic plants. The remaining four dual constructs will be completed 30-60 days. This project has combined 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. 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.

Publications:

- 2014 Pierce's disease Symposium
- Sanchez, Juan, James Lincoln, and David Gilchrist, 2015a. Pathogenesis-related protein PR-1 interferes with programmed cell death and is synthesized under translational control (pending)
- Juan Sanchez, James Lincoln, and David Gilchrist, 2015b. The translation of pathogenesis related-PR-1 is triggered by a miRNA excised from grape coding sequences and the coding sequence of grape fanleaf virus. (pending)
- Lincoln, James and David Gilchrist. 2015. Pierce's Disease suppression in grape by transgenic expression of DNA sequences capable of blocking programed cell death. (pending)

RESEARCH RELEVANCE

The primary goal 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. In addition, each of the five DNA constructs used in these experiments haves shown the ability to protect non-transformed scion based on preliminary data from field infected plants. The benefit: of cross-graft protection of an unmodified varietal scions is of interest to the industry as well as the scientists.

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 or constructed and advanced the evaluation of five novel genes (DNA constructs) that, when engineered singularly 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 (Table 1). 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. However, 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 the current transgenic rootstock genotypes to protect all or most of the scion, even at a distance from the graft union will continue through 2016.. The objective of the current "stacking" experiment 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 transgenes with different modes of action in a single rootstock line The objective is determine if the combined genes can lead to protection and durability and also more robust protection of the non-transformed scion against PD. The proposed changes are the next logical step toward achieving commercialization of transgenic resistance. Stacked transgene rootstock lines will be ready for evaluation in late 2015 under controlled greenhouse conditions while ramets of the most suppressive transgenic lines are being produced for field testing; initiated by 2016-2017.

Status of funds. All funds budget for these projects will be expended at the end of the current funding cycle as proposed.

Summary and status of intellectual property. The grape plants containing the anti-PCD genes and the grafted rootstocks will require the use of several patented enabling technologies. Record of invention disclosures have been submitted to the UC Office of Technology Transfer. The research proposed reported herein will provide data on the activity and mechanism of action of the protective transgenes in grape relative to the presence, amount and movement of Xylella fastidiosa in the transformed and untransformed grape plants.

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