Renewal Progress Report for CDFA Agreement number 16-0559-SA

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 September 30, 2016 to April 1, 2017

INTRODUCTION

Collectively, 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. 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. The continuation of the basic research and the field trials to be described herein 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. The present field trial consisting of single gene constructs will be 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. 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. 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 2017.

Briefly, we describe information on the history and impact of the genes deployed as single transgenes currently in APHIS approved field trials where test plants are mechanically inoculated with Xf. 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 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.

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). 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 due to the ability of the 3'UTR of PR1 to bind to a region in the PR1 coding sequence to prevent translation. 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

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 8 copies of each for field planting at the APHIS approved site in Solano County.

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.

DESCRIPTION OF ACTIVITIES AND RESULTS

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. The binary backbone is based on pCAMBIA1300 (Hajdukiewicz et al.; 1994). Binaries were constructed to express two genes from two 35S promoters (Figure 1). 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 (duel expressers) were constructed by Dr. Lincoln and are of the general form shown in Figure 1.

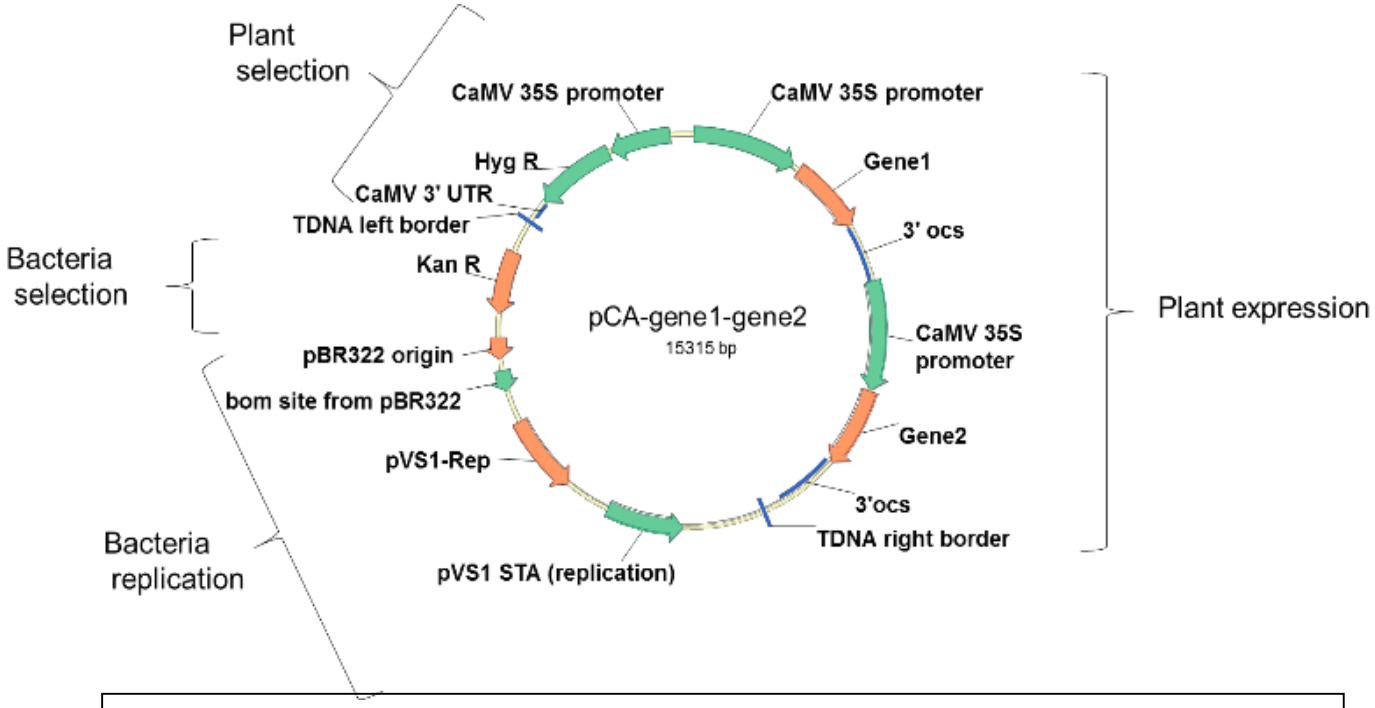

Figure 1. Dual expression binary expresses two genes within the same TDNA insert. This allows a single transformation event to generate plants that express two gene products.

All plasmids were transformed into Agrobacterium strain EHA105, the transformation strain for grape plants. As a check on stability of the duel expresser 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 prepared. 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 being introduced into embryogenic grapevine culture in a single transformation event rather than sequentially as would normally be the conventional strategy at the Parsons Transformation Facility. The new transgenic dual gene expressing grape plant lines are normal looking (Figure 2) and transformation progress for each line is shown in Table 2.

Table 2. Progress in generation of the dual construct transformed transgenic rootstocks
The current status of grape transformations into the rootstocks 1103 and 101-14.

Genotype	Construct code	Construct	# Plants Delivered	Comment
1103	AB	pCA-5oP14HT-5oUT456	10	Complete
101-14	AB	pCK-5oP14HT-5oUT456	0	
1103	AC	pCA-5fCAP-5oP14HT	15	Complete
101-14	AC	pCK-5fCAP-5oP14LD	0	
1103	AD	pCA-5PGIP-5oP14HT	13	Complete
101-14	AD	pCK-5PGIP-5oP14LD	8	
1103	AF	pCA-5oP14HT-5orpfF	0	
101-14	AF	pCK-5oP14LD-5orpfF	0	
1103	BC	pCA-5fCAP-5oUT456	12	Complete
101-14	BC	pCA-5fCAP-5oUT456	0	
1103	BD	pCA-5PGIP-5oUT456	0	
101-14	BD	pCK-5PGIP-5oUT456	15	Complete
1103	BF	pCA-5oUT456-5orpfF	1	
101-14	BF	pCK-5oUT456-5orpfF	0	
1103	CD	pCA-5PGIP-5FCAP	7	
101-14	CD	pCK-5PGIP-5FCAP	0	
1103	CF	pCA-5fCAP-5orpfF	12	Complete
101-14	CF	pCK-5ofCAP-5orpfF	0	
1103	DF	pCA-5PGIP-5orpfF	15	Complete
101-14	DF	pCK-5PGIP-5orpfF	15	Complete

Analysis of the transgenic rootstocks to confirm dual insertions transcripts

RNA from transgenic grape leaves is 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 of each genotype to be placed in the field with 8 replications of each line. To meet this objective, we will determine the movement of the transgene products in the transformed rootstock plants prior to grafting to the non-transformed PD susceptible Chardonnay (Table 3, Figure 4). These assays, while time consuming and tedious, will ensure that each plant will have a full phenotypic and genotypic analysis enabling a severe selection of the most promising individual rootstock prior addition of the Chardonnay scion and moving them to the field site beginning in last 2017 and or early in 2108.

Following verification of the genotypic integrity of the transgenic rootstock plants, clonal ramets of each plant line will be made to enable two cane growth development of the rootstocks and grafting of the Chardonnay scions (Figures 2 and 3). Once the grafts have healed, one cane will be inoculated with Xf to assess relative resistance to greenhouse symptoms along with further qPCR to monitor the movement and relative amount of the bacterial in the inoculated susceptible scion as well as the movement of transgene products from transgenic rootstock to untransformed scion tissue and analysis of impact on bacteria presence and symptoms of PD.

These tests will be conducted both during and after the scions are inoculated in the greenhouse. 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 of moving only the most promising lines to the field. Dr. Lincoln and Aaron Jacobson with coordinate all activities described above. The time frame from receipt of plants, analysis and selection of the individuals for field planting will be 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. Progress in analysis of the dual construct transformed transgenic rootstocks
The current status of verification of transgenic RNA from transgenic rootstocks 1103 and 101-14.

Grape Dual Construct Code	Genotype	Number of Plants	Replicates Inoculated
AB: P14-456	101-14	0	
AB: P14-456	1103	8	
AC: P14-CAP	101-14	0	
AC: P14-CAP	1103	11	
AD: P14-PGIP	101-14	7	
AD: P14-PGIP	1103	10	
AF: P14-rpfF	101-14	0	
AF: P14-rpfF	1103	0	
BC: 456-CAP	101-14	0	
BC: 456-CAP	1103	10	8
BD: 456-PGIP	101-14	10	
BD: 456-PGIP	1103	0	
BF: 456-rpfF	101-14	0	
BF: 456-rpfF	1103	1	
CD: CAP-PGIP	101-14	0	
CD: CAP-PGIP	1103	5	
CF: PGIP-rpfF	101-14	0	
CF: PGIP-rpfF	1103	10	
DF: PGIP-rpfF	101-14	12	
DF: PGIP-rpfF	1103	12	7

The following images illustrate the status of the dual construct transgenic plants as they are managed in the greenhouse. 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. We are beginning to inoculate the first transgenic lines of 1103 in the greenhouse with *Xf* (figure 4)

Figure 2. Transgenic grape plants growing in greenhouse. Left side of image shows plants

Figure 3. Transgenic grape plants growing in greenhouse.

Figure 4. Xf inoculation of greenhouse grown grapes containing inserts of dual DNA constructs capable of expressing suppression of Pierce's Disease symptoms.

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

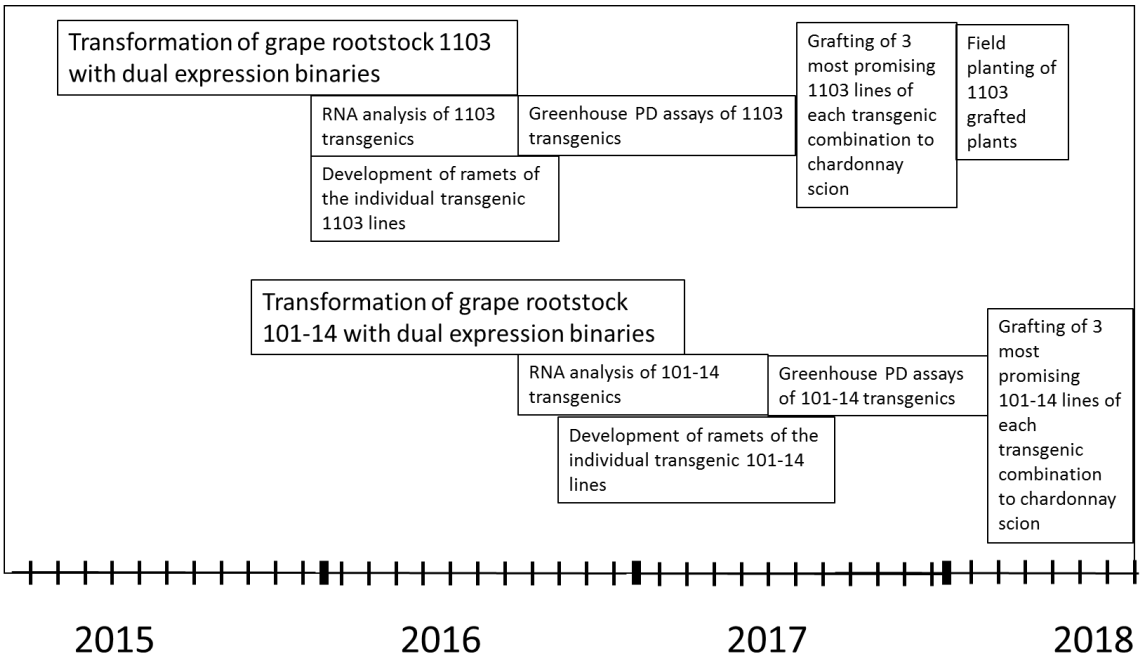

Figure 5. Anticipated Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.

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 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 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.

PUBLICATIONS AND PRESENTATIONS

Gilchrist, David G. and James E. Lincoln. 2016. Field Evaluation of Grape Plants Expressing Potential Protective DNA Sequences Effective against Pierce's Disease. Proceedings of the Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA.

Gilchrist, David G. and James E. Lincoln. 2016. Field Evaluation of Grape Plants Expressing PR1 and UT456 Transgenic DNA Sequences for Protection against Pierce's Disease Proceedings of the Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA.

Gilchrist, David G., James E. Lincoln, Abhaya M. Dandekar, and Steven Lindow. 2016. Transgenic Rootstock-Mediated Protection of Grapevine Scions by Single and Stacked DNA Constructs Proceedings of the Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA.

Lindow, Steven. 2016 Continued Field Evaluation of Diffusible Signal Factor Producing Grape for Control of Pierce's Disease Proceedings of the Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA.

Gilchrist, David. July 21, 2016. Pierce's Disease Research Update. Oral presentation to the Sonoma County Vineyard Technical Group. Santa Rosa, CA

RESEARCH RELEVANCE

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 2016-2017 under controlled greenhouse conditions while ramets of the most suppressive transgenic lines are being produced for field testing; initiated by 2017. The proposed changes are the next logical step toward achieving commercialization of transgenic resistance.

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 (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, 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. 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 2016 under controlled greenhouse conditions while ramets of the most suppressive transgenic lines are being produced for field testing; initiated by 2017. The proposed changes are the next logical step toward achieving

commercialization of transgenic resistance.

STATUS OF FUNDS

Funds are being expended consistent with timeline.

SUMMARY AND STATUS OF INTELLECUAL PROPERTY

No intellectual property or patent applications have been filed at this point but will be as results dictate

LITERATURE CITED

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FUNDING AGENCIES AND 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.