Interim- Progress Report for CDFA Agreement Number 18-0307-000-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 January 31, 2019 to July 15, 2019, which is an update of the renewal report submitted covering the period from July 1, 2018 to January 31, 2019

Introduction

This interim report will update activities from January 31, 2019 to July 15, 2019 and describe briefly the history, likely function, and impact of each of the genes deployed in the APHIS approved field trials wherein test plants were mechanically inoculated with *Xylella fastidiosa (Xf)* to induce Pierce's Disease. 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 *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 begun in 2010 and evaluated until discontinued at the end of the 2016 growing season. The current project is focused on untransformed scion protection by rootstocks bearing paired combinations of the five constructs. 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 experimental materials of this project are five specific DNA constructs (Table 1) that were shown to be effective in PD suppression under field conditions as single gene constructs are now being evaluated for

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

potential cross-graft-union protection as described by the Lindow, Dandekar and Gilchrist labs in previous Pierce's Disease Symposium reports (references 1-5). 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. 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.

Objectives of the Research

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" (Reference 6), describes the stacking of several genes for virus resistance in squash. Note, David Tricoli, the lead author in this paper, has done 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 (Reference 7). Experiments proposed here will evaluate potential synergism in suppression of PD symptoms and in reducing *Xf* titer for inoculations distant from the graft union under field conditions.

Specific Objectives

1. Complete introduction of 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 in the lab and selections moved later to the field.
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 inoculation 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. Establish a new planting area within the current APHIS approved site to contain a new set of lines bearing paired, PD suppressive, DNA constructs, referred to as stacked genes. The stacked genes will be transferred to two adapted rootstocks (1103 and 101-14). These rootstocks will be grafted to an untransformed PD susceptible Chardonnay scion prior to field planting. The goal is to assess the potential of cross graft protection against PD of a non-transgenic scion and to determine if the transgenic rootstocks are protected against bacterial movement from the scion to the rootstock thereby providing protection of the rootstock against *Xf* triggered death compared to native untransformed rootstock combinations. Planting was begun in 2018 (Figure 4) and will be completed by 2019 with field inoculations to begin in 2020.

Description of Activities Conducted to Accomplish each Objective

1. Construction and analysis of dual gene expression binaries:
The transgenic 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 et al., 2016). The binary backbone is based on pCAMBIA1300 (Hajdukiewicz et al.; 1994). Binaries were constructed to

express two genes from two 35S promoters. 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. Extensive analysis of each plasmid before delivery to the transformation facility confirmed the integrity of the dual binary plasmid, used to transform the grape embryos by David Tricoli. Each plasmid containing the dual protective DNA sequences were introduced into embryogenic grapevine culture in a single transformation event. At the time this protocol was developed, this was a novel attempt to expedite the simultaneous insertions of the two constructs rather than the traditional, much slower method of using two separate binary transformations. In grape this was a veritable nightmare since a flowering plant had to be produce from the first transformation. **This novel protocol was successful and has been adopted for future transformation of grape and other plants as a labor and time saving strategy.** The new transgenic dual gene expressing grape plant lines exhibit a phenotype indistinguishable from the untransformed wild type rootstock used as control. Analysis of the transgenic rootstocks to confirm production in the rootstock and potential movement across a graft union to the untransformed scion

2. As indicated in Objective 2 one goal is to conduct analysis of the integrity of the insertions in the rootstocks and to subsequently begin to develop methods to assess the expression and possible movement of the transgene products.

Analysis of dual insertions: This analysis is performed by isolating the RNA from transgenic grape leaves and purified using a modification of a CTAB protocol that 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 6 independently transformed lines bearing the dual sets of the 5 transgenes 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 1). 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. David Tricoli, transformation facility manager, confirmed that the aforementioned steps provided the highest success rate in transformation he has experienced with grape.

Table 1. 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
none	12	4

The grafted plants listed in tables 2 and 3, were completed successfully in the past 6 months and included the non-transgenic controls designed to measure any direct effect of the wild type 1103 and 101-14 rootstock compared with the transgenic rootstocks. These separate columns in the tables indicated the plants introduced in the field in 2018 and those to be planted in 2019.

Table 2. Production of wild type control rootstocks grafted to untransformed scions

rootstock type	scion type	# lines grafted 2018	# plants to field 2018	# lines grafted 2019	expected # plants in field
wildtype (WT) 1103	WT chardonnay	1	10	1	20
WT 101-14	WT chardonnay	1	10	1	20
WT 101-14	WT Cabernet Sauvignon	0	0	1	6
WT 101-14	WT Pinot noir	0	0	1	6
WT 101-14	WT Merlot	0	0	1	6
WT 101-14	WT Sauvignon blanc	0	0	1	6

Table 3. July 1, 2019 updated list of transgenic paired gene and rootstock combinations including controls both in the field and those expected to be planted in 2019

Genotype	Construct code	Construct	# lines grafted 2018	# plants to field 2018	# lines grafted 2019	expected # lines in field
1103	AB	pCA-5oP14HT-5oUT456	6	36	0	6
101-14	AB	pCK-5oP14HT-5oUT456	4	24	2	6
1103	AC	pCK-5fCAP-5oP14LD	6	36	0	6
101-14	AC	pCK-5fCAP-5oP14LD	no transformants	no transformants	no transformants	0
1103	AD	pCA-5PGIP-5oP14HT	6	36	0	6
101-14	AD	pCK-5PGIP-5oP14LD	6	36	0	6
1103	AF	pCA-5oP14HT-5orpfF	see 2019	see 2019	3	3
101-14	AF	pCK-5oP14LD-5orpfF	1	6	2	3
1103	BC	pCA-5fCAP-5oUT456	6	36	0	6
101-14	BC	pCA-5fCAP-5oUT456	see 2019	see 2019	6	6
1103	BD	pCA-5PGIP-5oUT456	see 2019	see 2019	3	3
101-14	BD	pCK-5PGIP-5oUT456	6	36	0	6
1103	BF	pCA-5oUT456-5orpfF	4	24	0	4
101-14	BF	pCK-5oUT456-5orpfF	see 2019	see 2019	4	4
1103	CD	pCA-5PGIP-5FCAP	see 2019	see 2019	4	4
101-14	CD	pCK-5PGIP-5FCAP	see 2019	see 2019	4	4
1103	CF	pCA-5fCAP-5orpfF	6	36	0	6
101-14	CF	pCK-5ofCAP-5orpfF	see 2019	see 2019	2	2
1103	DF	pCA-5PGIP-5orpfF	6	36	0	6
101-14	DF	pCK-5PGIP-5orpfF	6	36	0	6
101-14	DF	pCK-5PGIP-5orpfF	6	36	0	6
totals			69	414	30	99

Detection of transgene products: Preliminary experiments were conducted to develop protocols for detecting the protein products expressed in both the rootstock tissues and the grafted scion. Figure 4 shows soluble proteins of the p14 gene isolated from the rootstock and grafted scions The analysis consists of using p14 antibodies we developed to visualize the presence of the protein by immunological detection by classic Western analysis. A positive detection is revealed by a dark spot indicating the presence and relative amount of the product isolated from the respective tissues. Lane A shows the p14 protein expressed by a E. *coli* bacterial expression vector using the same coding sequence as was transformed into the grape plants (Lane A). Lanes B-D show high levels of the p14 protein expressed by each of the three rootstocks. Lane E is a sample taken from the Chardonnay scion grafted to the rootstock D indicates presence of the p14 product across the graft union. Lane F is a control of wild type rootstock grafted to the Chardonnay scion, confirming that there is no evidence of the p14 product in either the untransformed rootstock or scion.

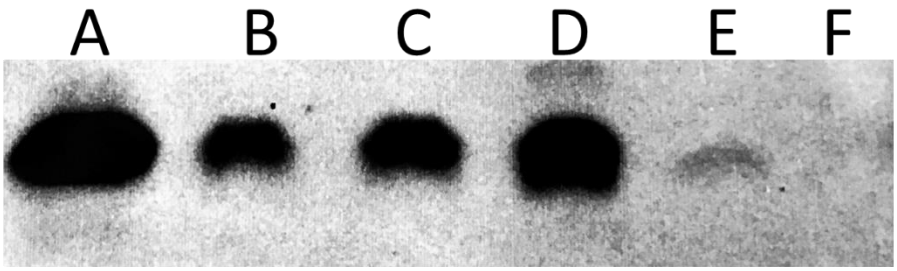

Figure 1. Detection of protein products expressed in the 3 independent transgenic rootstocks and in the untransformed Chardonnay scion. A) E. coli produced p14 (positive control); B-D) 3 independent 101-14 rootstocks expressing p14 in a stem sample from each rootstock; E) Chardonnay scion grafted to plant D; E) chardonnay scion grafted to wildtype 101-14 (negative control)

3. After verification of dual inserts by RTqPCR, the selected lines were moved to a lath house (Figure 2) for final stem development prior to rooting of the transformed rootstock and grafting of the untransformed Chardonnay scions. (Figures 3 and 4).

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

Figure 3 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 4. 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.

4. **As put forth in the original proposal, the first phase of the field plant was completed in August of 2018 with the final planting proceeding in 2019 under Objective 4:**

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 with the final planting of the remaining constructs listed in table 3 to occur in summer of 2019.

The planting and management protocols remain the same as originally proposed

a. **Experimental design** is a complete randomized block with six (6) plants per each of six (6) entries (replications), including all controls. Individual plants will be spaced 9 feet apart in rows on 11 feet centers (11 x 9). Each plant will be trained as a single trunk with bilateral cordons. When the shoot tip reaches about 12" past the cordon wire it will be topped to just above a node that is about 2-3 inches below the wire. Then, the laterals that push will be used to establish the bilateral cordons. The plants will be allowed to grow vertically, or close to vertical, rather than tying them while green, which reduces their elongation and tends to force more lateral growth. Metal 9' highway stakes, inserted 3' into the ground every 18' will support the wires, including catch wires. A single 11 gauge wire will be used for the cordons and 13 gauge for the catch wires. Two pairs of moveable catch wires will be installed to tuck and position the shoots vertically for optimizing bacterial inoculation, bacterial analysis, and fruit production. The catch wires will be installed initially or after the first year of growth and using 13 gauge wire to support the drip irrigation wire, about 18" off the ground (Figure 4).

b. After the first year, the canes will be tied down during the dormant season and trimmed to the appropriate length or shorter if the cane girth is not over 3/8" in diameter. The shoots that push will be suckered to remove double shoots and to achieve a shoot (and hence spur position) spacing of about 4-5 inches between them.

c. **Mechanical inoculation of *X. fastidiosa*** into vegetative shoots will follow the same protocol used to effectively establish the pathogen in the plant tissue and elicit PD symptoms as done successfully in the previous planting on this field site (Gilchrist and Lincoln, 2016).

d. Grape fruit yield will be measured after second or third year depending on the fruit set.

e. Evaluation of the experimental plants for plant morphology, symptoms of Pierce's Disease infection, and the presence of the bacteria will follow past procedures.

f. **Molecular analysis of bacterial dynamics**: Each parameter will be determined overtime by visual monitoring of symptom development and detection of the amount and movement of the bacteria in plant tissues (mainly leaves and stems) by quantitative PCR (qPCR) assays. The analysis will be done in the Gilchrist lab by the same methods and laboratory personnel as has been done successfully with the previous field planting (Gilchrist and Lincoln, 2018). Stem tissue containing the xylem-based bacteria will be pulverized in liquid nitrogen to preserve the native state of the bacteria in the grape tissue. DNA is isolated by a reproducible CTAB-based extraction method. Quantitative detection of Xylella genome uses specific 16S ribosomal primers. A quantitative qPCR detection method of *Xf* cells in non-transgenic scions and grape rootstocks will be compared with the untransformed grape scions and grape rootstocks.

g. Both symptom expression and behavior of the inoculated bacteria will provide an indication on the level of resistance to Pierce's Disease infection and the effect of the transgenes on the amount and movement of the bacteria in the non-transgenic scion area and the movement into the rootstocks.

h. In relation to natural spread of the pathogen from infected plants to adjacent plants, there was no evidence of movement between nearby mechanically infected plants in the previous experiment over a 6 year period. Hence, this lack of spread of the bacteria from inoculated to non-inoculated plants, is an important consideration for the experiments carried out for this project and for the granting of the APHIS permit. The field area chosen has never had grapes planted therein, which is to avoid any potential confounding by soil borne diseases, including nematodes.

i. **Plant and Pest management**: Irrigation and pest management, primarily powdery mildew, weeds and insects, will be coordinated by PI Gilchrist and conducted by Bryan Pellissier the Field Superintendent employed by the Department of Plant Pathology. The field crew work closely with PI Gilchrist to determine timing and need of each of the management practices, including pruning and thinning of vegetative overgrowth as necessary.

j. Regular tilling and hand weeding will maintain a weed-free planting area. Plants will be pruned carefully in March of each year leaving all inoculated/tagged branches and numerous additional branches for inoculation and sampling purposes in the coming year. All pruning material will be left between the rows to dry, then flail chopped and later rototilled to incorporate the residue per requirements of the APHIS permit.

k. Application of the fungicides Luna Experience and Inspire will be alternated at periodic intervals to maintain the plants free of powdery mildew. Leafhoppers and mites will be treated with insecticides when needed. Neither powdery mildew nor insect pressure was has been observed with these ongoing practices throughout the past five growing seasons.

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

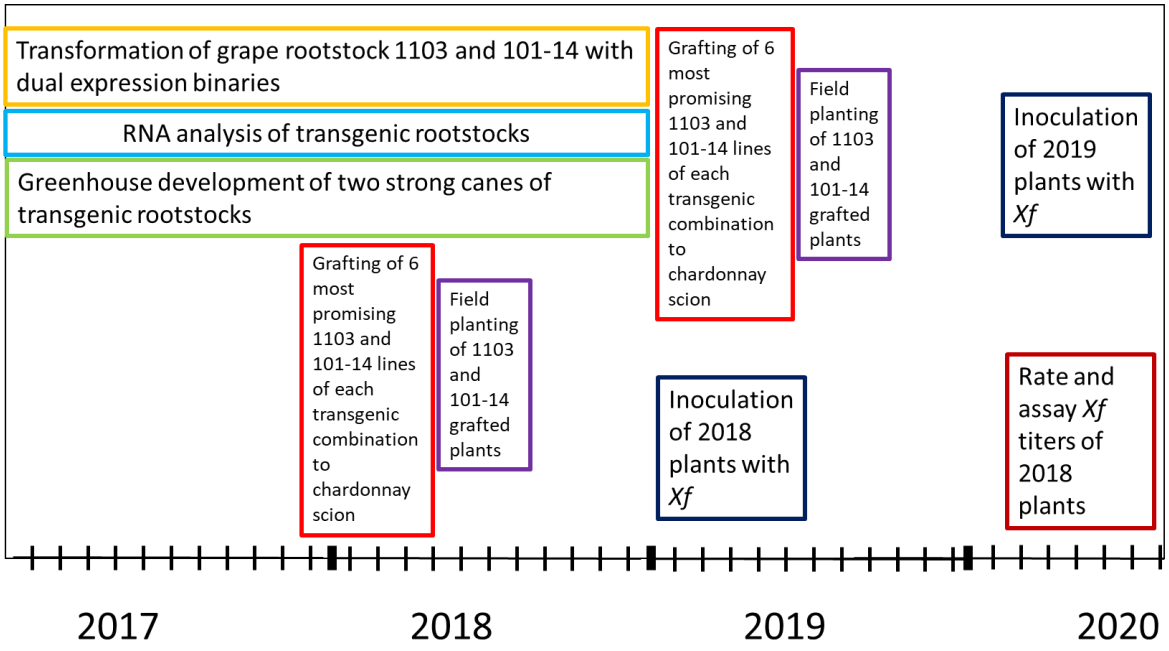

Figure 5. Anticipated Timeline for evaluation, propagation and planting of dual construct/susceptible scion combinations, fully transformed rootstock control, and untransformed susceptible control plants.

Research Timetable. This project began with an initial planting in 2018 (Figure 4) and will be followed by an additional plantings in 2019 as experimental plants become available in the second year. Inoculation and evaluation will begin when the plants have been in the ground for one year and will continue annually until the field planting is terminated. Funding for completion of the third and following years will depend on the results of the field evaluation up to that point to be proposed in the 2020 funding cycle. The field area has been designated legally available for planting the specified transgenic grapes by USDA-APHIS under permit number 7CFRE340 that is held by Professor Abhaya Dandekar. The protocols for managing the existing and the new plantings with the dual constructs have used successfully over the past 5 years (Gilchrist 2016). These protocols include the plant management, inoculation with *Xylella fastidiosa,* development of classical symptoms of Pierce's Disease exhibiting the range from foliar symptoms to plant death and the assessment of protection by a set of transgenes selected by molecular techniques to suppress the symptoms of Pierce's Disease and/or reduce the ability of the pathogenic bacteria to colonize and move within the xylem of the grape plant. Management of the vines by commercial standards will be directed by Debora Golino and PI Gilchrist. All timelines indicated above have been completed within the proposed periods.

Publications produced or pending related to this project and presentations made.

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

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.

Layperson summary of project accomplishments and perspective

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

Current funding is being expended at the anticipated rate and is expected to be sufficient to complete the objectives as presented.

Intellectual Property Associated with the Project

No intellectual property document has been filed at this point but will be done in a timely manner if results dictate there is a basis for any claims.

Literature cited

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4. 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.
5. 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

6. Tricoli, David M, Kim J. Carney, Paul F. Russell, J. Russell McMaster, David W. Groff, Keisha C. Hadden, Phyllis T. Himmel, Jon P. Hubbard, Maury L. Boeshore & Hector D. Quemada *NatureBiotechnology* 13, 1458 - 1465 (1995) . doi:10.1038/nbt1295-1458.
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