"Interim Progress Report for CDFA Agreement Number 12-0445-SA"
Project Title: CAP and PGIP transgenic grapevines field trial.

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Introduction

Thompson Seedless (TS, *Vitis vinifera*) grapevines were transformed with a gene that encodes a chimeric anti-microbial therapeutic protein with a recognition domain from a neutrophil elastase (NE) and the lytic domain Cecropin B (CB). The NE domain specifically binds to the outer-membrane protein MopB of *Xylella fastidiosa* (*Xf*), the causative agent for Pierce's Disease (PD), while the CB domain clears *Xf* (Dandekar et al. 2012). We also transformed TS grapevines with a gene encoding polygalacturonase-inhibiting protein (PGIP). PGIP expression in transformed plants inhibits the action of polygalacturonase (PG), a virulence factor expressed by *Xf*. Inhibiting PG interferes with long-distance movement of *Xf*, providing resistance to PD (Agüero et al. 2005). Transgenic grapevines expressing NE-CB and different PGIP constructs were first tested under greenhouse conditions. Several lines that showed resistance to PD were identified by mechanically inoculating plants with *Xf* (Dandekar et al. 2012). Selected transgenic grapevines expressing either NE-CB or PGIP, grafted with non-transgenic TS, were planted in 2011 in Solano County to validate their PD resistance and horticultural characteristics under field conditions.

List of objectives

The goals of this project are to finish field-testing four NE-CB and four PGIP transgenic grapevine clones by evaluating their horticultural characteristics and resistance to PD. Transgenic grapevines were tested under field conditions as transgenic rootstocks grafted with wild-type scion. The field location has no PD pressure and plants were mechanically inoculated with *Xf*.

Objective 1. Validate the efficacy of *in planta*-expressed chimeric NE-CB and PGIP with different signal peptides to inhibit and clear *Xf* infection in xylem tissue and to pass through the graft union under field conditions.

Activity 1. Propagate, field plant, and graft NE-CB and PGIP transgenic grapevines.
Activity 2. Evaluate preservation of varietal characteristics in transgenic grapevines grown as rootstocks.
Activity 3. Evaluate PD resistance of NE-CB and PGIP transgenic grapevines after inoculation with *Xf*.

Objective 2. Assume permit-holder status for existing USDA-APHIS field permit 12-340-102r and maintain regulatory oversight and compliance with permit reporting requirements.

Activity 4. Participate with PIPRA during transition and assume permit-holder status.
Activity 5: Maintain regulatory oversight of the field location and compliance with reporting requirements.
Activity 6. Maintain active regulatory compliance inspections.

Description of activities conducted to accomplish each objective, and summary of accomplishments and results for each objective

Activity 1. Propagate, field plant, and graft NE-CB and PGIP transgenic grapevines. Four independent transgenic events expressing NE-CB (40-41G, 40-89G, 40-92G, and 41-151G), four expressing different PGIP constructs (31-25G, 45-77G, 52-08G, and TS50G) and a non-transgenic control (TS-G) grafted with non-transgenic TS scions **(Table 1)** were planted at Solano site on June 27, 2011. Genotyping of NE-CB- and PGIP-expressing grafted transgenic grapevine lines in Solano County has confirmed the presence of the inserted transgene in all lines.

Table 1. Grafted transgenic and control grapevines planted in Solano County		
Event ID	**Event ID (Vector)**	**# Planted**
NE-CB lines		
40-41G	pDU04.6105	12
40-89G	pDU04.6105	12
40-98G	pDU04.6105	12
41-151G	pDU04.6105	12
PGIP lines		
31-25G	pDU05.1002	12
45-77G	pDU06-0201	12
52-08G	pDU05.1910	12
TS50G	pDU94.0928	12
Control line		
TS-G	N/A	12

Activity 2. Evaluate preservation of varietal characteristics in transgenic grapevines grown as whole plants or used as rootstocks.
To verify that horticultural and varietal characteristics of the parental genotype were unchanged, NE-CB- and PGIP-expressing grafted transgenic lines were evaluated phenotypically in Solano County in 2012 and 2013. This examination was accomplished using the first 12 descriptors from the "Primary descriptor priority list" proposed by the Organisation Internationale de la Vigne et du Vin (OIV 1983). The descriptors used were 1) aperture of young shoot tip/opening of young shoot tip, 2) density of prostrate hairs between main veins on 4th leaf lower side of blade, 3) number of consecutive shoot tendrils, 4) color of upper side of blade on 4th young leaf, 5) shape of mature leaf blades, 6) number of lobes on mature leaf, 7) area of anthocyanin coloration on main veins on upper side of mature leaf blades, 8) shape of teeth on mature leaves, 9) degree of opening of mature leaves/overlapping of petiole sinuses, 10) mature leaf petiole sinus bases limited by veins, 11) density of prostrate hairs between main veins on lower side of mature leaf blades, and 12) density of erect hairs on main veins on lower sides of mature leaf blades. No differences between grafted transgenic and parental TS grapevines were observed.

Activity 3. Evaluate PD resistance of NE-CB and PGIP transgenic grapevines after inoculation with *Xf*.
At the Solano County site (**Fig. 1**), half of the grafted transgenic lines were mechanically inoculated as described by Almeida et al. (2003) on May 29, 2012, and half on June17, 2013. On May 27, 2014 and on May 27, 2015, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, at least four new canes per year from all grafted transgenic and control

plants were mechanically inoculated with *Xf*. Inoculation dates from 2012 to 2015 are shown in a color-coded map (**Table 2**).

Figure 1. Solano County grafted transgenic grapevines inoculated annually from 2012 to 2015. Winter 2016.

Table 2. Solano County grape field map, color-coded by *Xf* inoculation date, from 2012 to 2015.

	Vine 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Row 9	40-41-G	40-41-G	TS-50-G	TS-50-G	TS-50-G	40-92-G	40-92-G	40-92-G	40-89-G	40-89-G	40-89-G	TS-G	TS-G	TS-G	45-77-G	45-77-G	45-77-G	41-151-G	41-151-G	41-151-G	TS-G	TS-G	TS-G	TS-G	
Row 8	52-08-G	52-08-G	52-08-G	31-25-G	31-25-G	31-25-G	40-41-G	40-41-G	40-41-G	TS-50-G	TS-50-G	TS-50-G	40-89-G	40-89-G	40-89-G	TS-G	TS-G	TS-G	52-08-G	52-08-G	52-08-G	31-25-G	31-25-G	31-25-G	40-41-G
Row 7	31-25-G	40-92-G	40-92-G	40-92-G	TS-50-G	TS-50-G	TS-50-G	40-89-G	40-89-G	40-89-G	TS-G	TS-G	TS-G	45-77-G	45-77-G	45-77-G	40-92-G	40-92-G	40-92-G	41-151-G	41-151-G	41-151-G	45-77-G	45-77-G	45-77-G
Row 6	TS-50-G	TS-50-G	41-151-G	41-151-G	41-151-G	TS-G	TS-G	TS-G	40-92-G	40-92-G	40-92-G	40-89-G	40-89-G	40-89-G	41-151-G	41-151-G	41-151-G	52-08-G	52-08-G	52-08-G	40-41-G	40-41-G	40-41-G	31-25-G	31-25-G
Row 5													52-08-G	52-08-G	52-08-G	40-41-G	40-41-G	40-41-G	31-25-G	31-25-G	31-25-G	45-77-G	45-77-G	45-77-G	TS-50-G

North

Grapevine inoculatation with Xf (Temecula:Stag's leap mix, 60:40) at 250,000 per 20ul on 5/29/2012.
Grapevine inoculatation with Xf (Temecula) 250,000 per 20ul on 6/17/2013.
Grapevine inoculatation with Xf (Temecula:Stag's leap mix, 60:40) at 500,000 per 20ul on 5/27/2014.
Grapevine inoculatation with Xf (Temecula) at 500,000 per 20ul on 5/27/2015.

On July 22, 2014, one 2014-inoculated cane per grafted transgenic plant was harvested for quantification of *Xf* by qPCR using an Applied Biosystems SYBR Green fluorescence detection system. *Xf* DNA was extracted using a modified CTAB (hexadecyltrimethyl-ammonium-bromide) method that allowed us to obtain DNA with quantity and quality suitable for qPCR. The *Xf* 16s primer pair (Forward 5'-AATAAATCATAAAAAATCGCCAACATAAACCCA-3' and (Reverse 5'-AATAAATCATAACCAGGCGTCCTCACAAGTTAC-3') was used for *Xf* quantification. qPCR standard curves were obtained using concentrations of *Xf* ranging from 10^2 to 10^6 cells per 0.1 gm of tissue. *Xf* was detected in grafted transgenic vines, but with *Xf* counts lower than in grafted control grapevines (**Fig. 2)**. Another set of canes from the 2014-inoculated grafted transgenic and control individual canes was harvested in fall 2015, *Xf* DNA extraction was finished and *Xf* quantification is undergoing.

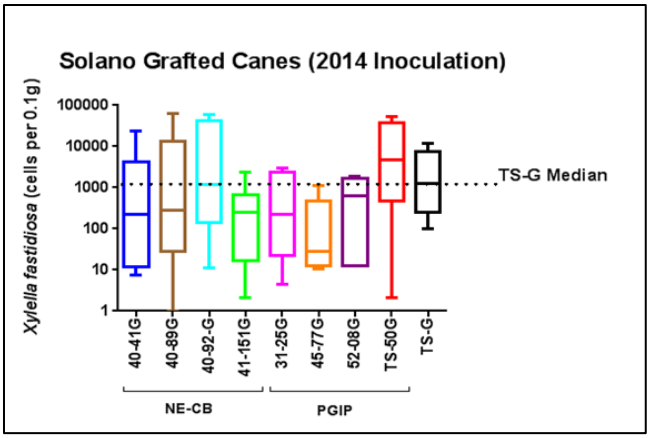

Figure 2. *Xf* quantification by qPCR for Solano grafted individual transgenic canes inoculated in spring 2014 and harvested in summer 2014.

Bud break success of grafted individual transgenic canes inoculated in 2014 was assessed on March 26th 2015 (**Fig. 3**). Bud break success was higher in most of the grafted inoculated transgenic lines from each strategy than in grafted non-transgenic control. Bud break success of grafted individual transgenic canes inoculated in 2014 and 2015 will be assessed on March 2016.

Grapevine survival of grafted transgenic grapevines, inoculated in 2012/2014, and in 2013/2014 was assessed on April 28th 2015 using a 1 to 5 score, where 1 = very healthy and vigorous grapevine; 2 = healthy grapevine and slightly reduced vigor; 3 = slightly reduced spring growth; 4 = much reduced spring growth and 5 = dead grapevine (**Fig. 4**). Grapevine survival was higher in some of the grafted inoculated transgenic lines from each strategy than in grafted non-transgenic control. Grapevine survival of grafted individual transgenic canes inoculated in 2014 and 2015 will be assessed on April 2016.

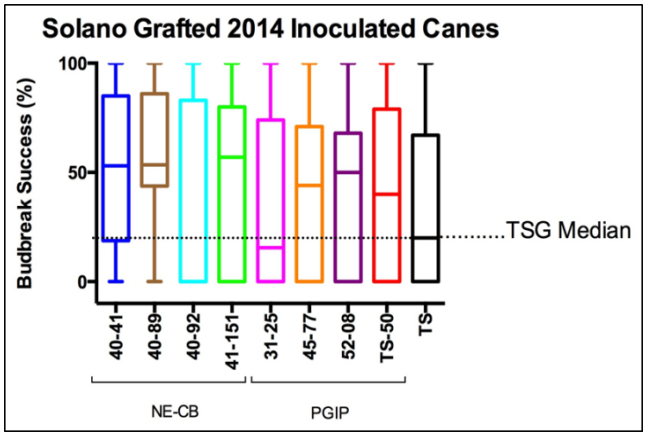

Figure 3. Budbreak success for Solano grafted inoculated grapevines scored on March 26th, 2015.

Severity or absence of PD symptoms for all Solano County grafted transgenic grapevines inoculated from 2012 to 2015 was assessed on 2015 fall season using the PD disease symptom severity rating system 0-5, where 0 = healthy vine, all leaves green with no scorching; 1= first symptoms of disease, light leaf scorching on one or two leaves; 2 = about half the leaves on the cane show scorching; 3 = the majority of the of the cane shows scorching; 4 = the whole cane is sick and is declining and 5 = the cane is dead (**Fig. 5**). PD disease symptoms severity score was lower in most of the grafted inoculated transgenic lines from

each strategy than in grafted non-transgenic control. Severity or absence of PD symptoms for all inoculated Solano County grafted transgenic grapevines will be assessed on 2016 fall season.

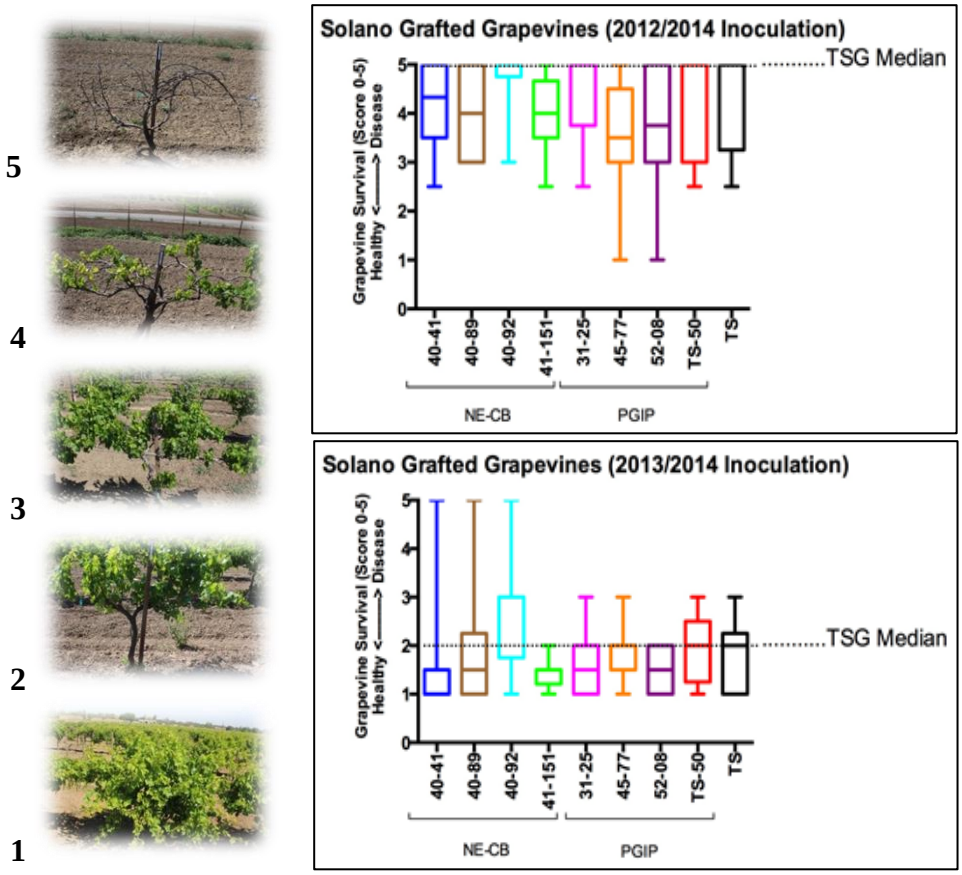

Figure 4. Grapevine survival of Solano grafted transgenic grapevine inoculated in 2012/2014 (upper right) and 2013/2014 (lower right), scored on April 28th 2015, using a scale of 1 to 5 (left).

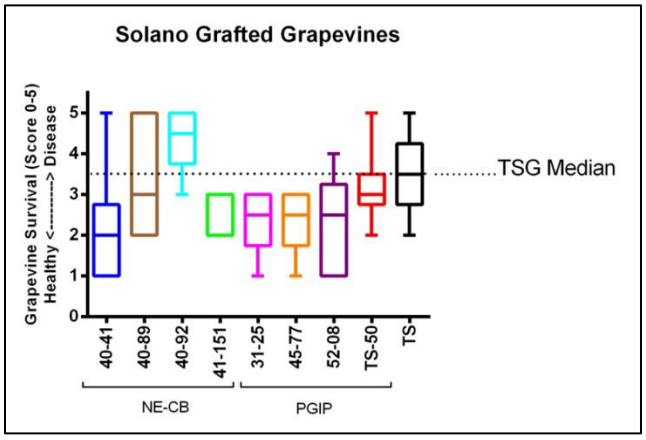

Figure 5. Severity or absence of PD symptoms for all Solano grafted inoculated grapevines scored on September 29th, 2015.

Objective 2. Assume permit-holder status for existing USDA-APHIS field permit 12-340-102r and maintain regulatory oversight and compliance with permit reporting requirements.

Activity 4. Participate with PIPRA during transition and assume permit-holder status.
The current USDA-APHIS field permit was transferred from Professor Alan Bennett to Professor Abhaya Dandekar in January 2014, the permit was extended by APHIS until April 1, 2016. In October 2015 a renewal permit's application was submitted to APHIS, the permit will be extended until April 1, 2018.

Activity 5. Maintain regulatory oversight of the field locations and compliance with reporting requirements.
During the transition period beginning Oct. 1, 2013, personnel from the Dandekar laboratory worked with PIPRA personnel to obtain all documentation and records necessary to maintain regulatory oversight of the field trial. This process was completed in January 2014 with the transfer of full responsibility to the new permit holder. We have worked closely with UC Davis EH&S to modify our existing BUA to include this permit, a process that integrated the institutional biosafety committee into the chain of custody for regulatory oversight compliance management. Although the responsibility for regulatory compliance rests with the new permit holder, UC Davis was included during the transition to maintain their oversight of campus BUAs. Personnel from the Dandekar laboratory are maintaining regulatory oversight of the field trial. The issues requiring regulatory oversight are listed in the permit.

Activity 6. Maintain active regulatory compliance inspections.
Timely reporting and inspections are conducted to maintain compliance with USDA-APHIS. Regulatory compliance is enforced by working closely with the participant investigators, the two field coordinators and their crews. PD Field trials activities information is updated quarterly using the PIs activity monitoring logs. Two individuals from the Dandekar lab are entrusted with the tasks of documentation, training, and inspection to ensure regulatory compliance.

Publications produced and pending, and presentations made that related to the funded project.

Dandekar, A.M., D. Gilchrist, P. Rolshausen, A.M. Ibanez, A. Jacobson D. Dolan, R. Just and H. Gouran. 2015. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines filed trial. Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes. December 2015. pp. 18-26.

Dandekar, A.M. D. Gilchrist, T. Miller, A.M. Ibanez, D. Dolan and H. Gouran. 2014. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines filed trial. Proceedings of Pierce's Disease Research Symposium held December 15-17, 2014 at the Sheraton Grand Sacramento Hotel, Sacramento, California. pp. 106-117.

Dandekar, A.M. D. Gilchrist, T. Miller, A.M. Ibanez, D. Dolan and H. Gouran. 2013. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines filed trial. Proceedings of Pierce's Disease Research Symposium held December 16-18, 2013 at the Hiatt Regency Hotel, Sacramento, California. pp. 95-100.

Dandekar, A.M., H. Gouran, A.M. Ibáñez, S.L. Uratsu, C.B. Aguero, S. McFarland, Y. Borhani, P.A. Feldstein, G. Bruening, R. Nascimento, L.R. Goulart, P.E. Pardington, A. Chaudhary, M. Norvell, E. Civerelo and G. Gupta. 2012. An engineered innate defense protects grapevines from Pierce's disease. Proc. Nat. Acad. Sci. USA 109: 3721-3725.

Dandekar, A.M., A.M. Ibáñez, D. Dolan, H. Gouran, D. Gilchrist and T. Miller. 2012. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines field trial. Proceedings of the Pierce's Disease Research Symposium, Dec 2012, pp. 94-103.

Dandekar, A.M., A.M. Ibáñez, H. Gouran, S. Uratsu, D. Gilchrist and T. Miller. 2011. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines field trial. Proceedings of the Pierce's Disease Research Symposium, Dec 2011, pp. 101-106.

Dandekar, A.M., A.M. Ibáñez, H. Gouran, S. Uratsu. D. Gilchrist and T. Miller. 2010. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines field trial. Proceedings of the Pierce's Disease Research Symposium, Dec 2010, pp. 161-164.

Research relevance statement, indicating how this research will contribute toward finding solutions to Pierce's disease in California.

This research aims to provide a transgenic solution to the Pierce's disease problem for the grower community of California. The objectives described in this proposal directly address the number 1 RSAP priority outlined under "Accelerate regulatory process: Establish and facilitate field trials of current PD control candidate vines / endophytes / compounds in multiple locations" handout released at the December 2009 Pierce's Disease Research symposium that outlined the "Top 5 to 10 Project Objectives to Accelerate Research to Practice". This document updates the priority research recommendations provided in the report "PD/GWSS Research Scientific Review: Final Report" released in August 2007 by the CDFA's Pierce's Disease Research Scientific Advisory Panel.

Layperson summary of project accomplishments.

We successfully established two field trials to validate two greenhouse-tested strategies to control movement and clearance of *Xylella fastidiosa* (*Xf*), a xylem-limited, Gram-negative bacterium that is the causative agent of Pierce's Disease (PD). A key virulence feature of *Xf* resides in its ability to digest pectin-rich pit pore membranes that interconnect the host plant's xylem elements, enhancing long distance movement and vector transmission. The first strategy evaluated the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) from pear to counter virulence associated with *Xf* PG activity. Our second strategy enhances clearance of bacteria from *Xf*-infected xylem tissues using a chimeric antimicrobial protein, NE-CB. The expectation is that expressing these proteins will prevent *Xf* movement and reduce inoculum size, curbing the spread of PD in California vineyards.

Ninety six (96) grafted transgenic grapevine plants expressing either NE-CB or PGIP along with 12 grafted non-transgenic controls were successfully planted in Solano County in 2011. These grafted transgenic grapevines were evaluated as rootstocks grafted with non-transgenic TS scions. NE-CB- and PGIP-expressing grafted transgenic lines in Solano County were evaluated phenotypically using the first 12 descriptors from the "Primary descriptor priority list" proposed by the Organisation Internationale de la Vigne et du Vin (OIV). No phenotypic/horticultural differences were observed between grafted transgenic and non-transgenic TS vines. Grafted grapevines were also genotyped, confirming the presence of the inserted transgene in all lines. At the Solano County site, grafted vines were mechanically inoculated with *Xf* in 2012 and 2013 to validate resistance to PD under field conditions. Leaf scorching, the characteristic symptom of PD, was observed in Solano Country grafted transgenic and control lines in 2013 and *Xf* presence was confirmed by ELISA in stem extracts from samples collected in the same season. On May 27, 2014 and on May 27, 2015, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, at least four new canes per year from all grafted transgenic and control plants were mechanically inoculated with *Xf*.

Budbreak success of grafted individual canes inoculated in 2014 was assessed on March 26th 2015 using a 0-5 score. The data showed that budbreak success is higher in most of the grafted inoculated transgenic lines from each strategy than in grafted non-transgenic control. Grapevine survival of grafted transgenic grapevines, inoculated in 2012/2014, and in 2013/2014 was assessed on April 28th 2015 using a 1 to 5 score. Grapevine survival is higher in some of the grafted inoculated transgenic lines from each strategy

than in grafted non-transgenic control grapevines. Severity or absence of PD symptoms was recorded in summer 2014 and fall 2015 for all inoculated canes using the PD disease symptom severity rating system 0-5. PD disease symptoms severity score was lower in at least two NE-CB and two PGIP grafted inoculated transgenic lines than in grafted non-transgenic control. Bud break success, grapevine survival and severity of absence of PD symptoms for grafted individual transgenic canes inoculated in 2014 and 2015 will be assessed during the 2016 growing season.

In the 2014 summer season, one cane per grafted plant was harvested for *Xf* quantification by qPCR. *Xf* DNA from grape stem was extracted using a modified CTAB method that allows us to obtain DNA with quantity and quality suitable for qPCR. An *Xf* 16s primer pair was used for *Xf* quantification. qPCR standard curves were obtained using concentrations of *Xylella* ranging from 10^2 to 10^6 cells. *Xf* was detected in grafted transgenic vines, but with *Xf* counts lower than in grafted non-transgenic control grapevines. Another set of canes from the 2014-inoculated grafted individual canes was harvested in fall 2015 and *Xf* quantification is currently ongoing.

The current USDA-APHIS field permit was transferred from Professor Alan Bennett to Professor Abhaya Dandekar in January 2014, the permit was extended by APHIS until April 1, 2016. In October 2015 a renewal permit's application was submitted to APHIS., the permit will be extended until April 1, 2018. Timely reporting and inspections are conducted to maintain compliance with USDA-APHIS permit reporting requirements.

Status of funds.

We have expended all the funds available for the period July 2015 to Feb 2016; funds remain unspent for the period March 1, 2016 to June 30, 2016.

Summary and status of intellectual property associated with the project.

The intellectual property issues connected with the specific constructs and approach have not been the subject of any formal investigation. However, this needs to be done when elite lines are identified and must be patent-protected. Disclosures will be made at that point to UC Innovation Assess, which could develop these further as a US patent variety.

Literature cited.

Agüero, C.B., C.P. Meredith, and A.M. Dandekar. 2006. Genetic transformation of *Vitis vinifera* L. cvs. 'Thompson Seedless' and 'Chardonnay' with the pear PGIP and GFP encoding genes. Vitis 45:1-8.

Almeida, R.P.P., and A.H. Purcell. 2003. Biological traits of *Xylella fastidiosa* strains from grapes and almonds. App. Env. Microbiol. 68:7447-7452.

Dandekar, A.M., H. Gouran, A.M. Ibáñez, S.L. Uratsu, C.B. Aguero, S. McFarland, Y. Borhani, P.A. Feldstein, G. Bruening, R. Nascimento, L.R. Goulart, P.E. Pardington, A. Chaudhary, M. Norvell, E. Civerelo and G. Gupta. 2012. An engineered innate defense protects grapevines from Pierce's disease. Proc. Nat. Acad. Sci. USA 109:3721-3725.

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