"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, own-rooted or grafted with nontransgenic TS, were planted in 2010 and 2011 in Riverside and Solano Counties 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 in two field locations as ungrafted plants and as transgenic rootstocks grafted with wild-type scion. One

field location has PD pressure and plants were naturally infected with *Xf*. In another location with no PD pressure, grapevines 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 whole plants or used 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 both field locations 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 (40-41, 40-89, 40-92, and 41-151) and four expressing different PGIP constructs (31-25, 45-77, 52-08, and TS50) were planted at two locations. Initial planting of 210 transgenic or untransformed vines, own-rooted or grafted with untransformed TS scions, was completed in Riverside County on May 18, 2010. Ten more were planted on March 6, 2011, completing the plantings at this location **(Table 1)**. We also planted 110 transgenic and untransformed vines on their own roots on August 2, 2010 and 110 vines grafted with untransformed TS scions on June 27, 2011 in Solano County, completing the planting at this second location. Genotyping of NE-CB- and PGIP-expressing transgenic grapevine lines in Solano County has confirmed the presence of the inserted transgene in all lines.

Table 1. Transgenic and control grapevines planted in Riverside and Solano fields

Ungrafted		Grafted	
Event ID (Vector)	**# Planted**	**Event ID (Vector)**	**# Planted**
NE-CB lines			
40-41 (pDU04.6105)	12	40-41G (pDU04.6105)	12
40-89 (pDU04.6105)	12	40-89G (pDU04.6105)	12
40-92 (pDU04.6105)	12	40-92G (pDU04.6105)	12
41-151 (pDU04.6105)	12	41-151G (pDU04.6105)	12
PGIP Lines			
31-25 (pDU05.1002)	12	31-25G (pDU05.1002)	12
45-77 (pDU06-0201)	12	45-77G (pDU06-0201)	12
52-08 (pDU05.1910)	12	52-08G (pDU05.1910)	12
TS50 (pDU94.0928)	12	TS50G (pDU94.0928)	12
Control line			
TS	16	TS-G	12

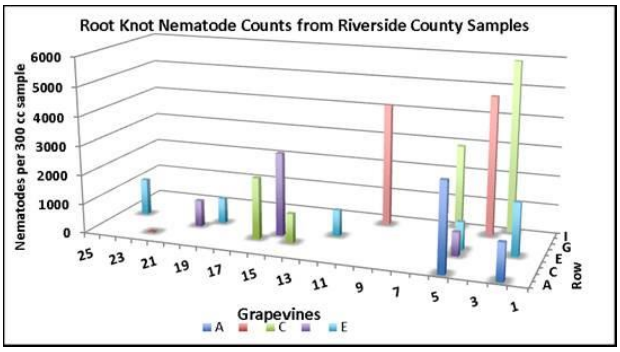
Activity 2. Evaluate preservation of varietal characteristics in transgenic grapevines grown as whole plants or used as rootstocks.

To verify that horticultural and varietal characteristics of the parental genotype were unchanged, NE-CB- and PGIP-expressing transgenic lines were evaluated phenotypically in Solano County in September 2011 and in Riverside County in November 2011. This examination was accomplished using the first 12 descriptors from the "Primary descriptor priority list" proposed by the International Organization of Vine and Wine (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. NE-CB and PGIP-expressing transgenic lines at the Riverside and Solano sites were also phenotypically evaluated in fall 2012 and 2013. No differences between 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 Riverside County site, grafted and ungrafted transgenic grapevine lines naturally infected in the field were scored for PD symptoms from 2011 to 2013. Severity or absence of PD symptoms and grapevine vigor was assessed: PD symptoms were rarely observed. *Xf* was detected in xylem sap, petiole, and stem extracts by ELISA and plating. Unexpectedly, during spring 2014 all 220 Thompson Seedless vines planted at Riverside were in decline; most had no new growth. Growth that did occur did not correlate with genotype: transgenic and control plants were equally affected. We dug up the soil about 18 inches from the trunk and observed that the root zone was limited to the first six inches of soil and was heavily infected with root-knot and citrus nematodes. Consulting with Andrew Walker, Howard Ferris, and Michael McKenry led us to a preliminary root-knot diagnosis.

Eighteen root and soil samples were taken and all were positive for root-knot and citrus nematodes (**Table 1, Fig. 2**); root and soil knot nematode population data confirmed infection. Roots sampled had four knots per inch, in contrast to the normal one knot or gall per four inches (**Fig. 2**). The Riverside site was previously planted with Chardonnay and the vineyard was removed and replanted without fumigation. The field location was most likely selected for an aggressive population of root-knot nematode during the previous grapevine planting. Our vines subsequently suffered lethal infection soon after planting; physiology was disrupted, root growth stopped, and gall development likely happened one or two days after root knot nematodes penetrated the young root. Heavy nematode feeding likely resulted in root leakage in the first year after planting. Root-knot infection symptoms include suppressed shoot growth, decreased shoot-root ratio, nutritional deficiencies showing chlorosis in the foliage, and poor plant yield (Kassen and Moens 2006). Evaluation of PD resistance or suceptibility of trangenic grapevines under Riverside County field conditions will not be possible, given the combination of symptoms for root-knot and *Xf* infections.



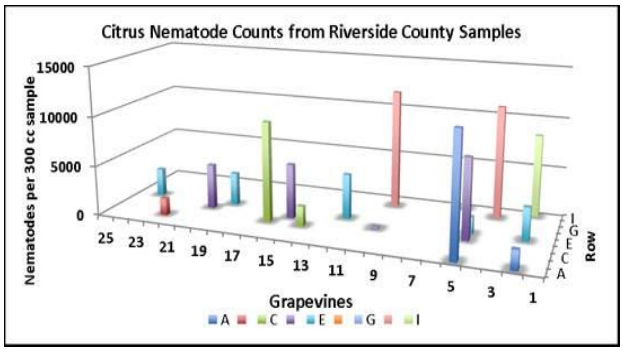


Figure 1. Root-knot (left) and citrus (right) nematode counts from Riverside County field soil samples (summer 2014).

Table 2. Root-knot and citrus nematode counts found in Riverside County site soil samples (per 300 cc soil sample)

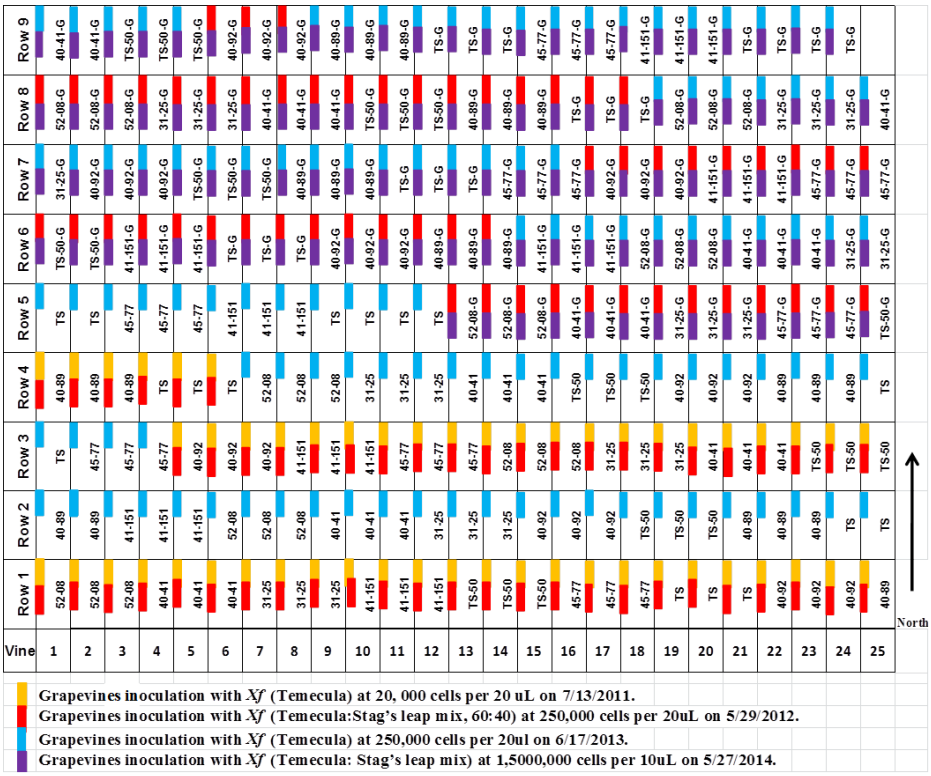
Location row-vine	Line ID	Genotype	Root-knot count	Citrus nematode count
1-2	40-41	NE-CB	1,260	1,920
1-5	31-25	PGIP	3,000	12,000
2-22	31-25	PGIP	30	1,700
3-14	45-77G	PGIP	1,000	2,000
3-16	TS50	PGIP	2,100	10,000
4-5	40-41G	NE-CB	800	8,000
4-10	52-08	PGIP	30	200
4-15	40-89G	NE-CB	2,840	5,460
4-20	41-151G	NE-CB	900	4,500
5-2	TS	WT control	1,800	3,300
5-5	45-77G	PGIP	954	1,700
5-12	TS-G	WT control	870	4,850
5-19	40-41	NE-CB	870	3,300
5-24	45-77	PGIP	1,250	2,830
8-4	45-77G	PGIP	4,750	11,200
8-10	40-41G	NE-CB	4,200	11,880
9-3	TS50	PGIP	5,850	8,250
9-6	40-89	NE-CB	1,400	2,700

Figure 2. Healthy grapevine root (upper left), root-knot infected roots with knots (upper right), microscopic view of grape root knots (lower left) and root-knot and citrus nematodes (lower right).

At the Solano County site (**Fig. 3**), half of the ungrafted transgenic lines were manually inoculated as described (Almeida et al. 2003) on July 13, 2011 and half on May 29, 2012. Half of the grafted transgenic lines were manually inoculated on the latter date. Ungrafted and grafted grapevines at the Solano site that were not previously inoculated were manually inoculated on June 17, 2013, completing the inoculations of all grapevines at this location. Cane survival for inoculated runners of ungrafted and grafted transgenic grapevines was scored using a 0−1 scale, where 0 = alive and 1 = dead. Vigor for Solano inoculated transgenic and control grapevines was scored using a 0−4 scale, where 0 = healthy, no PD symptoms; 1 = a few leaves on a few shoots on cane(s) with symptoms, 2 = many symptomatic leaves on multiple canes (in a mature bilateral cordon trained vine); 3 = dieback/death of canes/cordons; and 4 = death of whole vine. On May 28, 2014, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, at least four current-year canes from all grafted transgenic and control plants at this site were mechanically inoculated with *Xf* (**Table 3**).

Figure 3. Solano County transgenic grapevines inoculated in spring 2014 and winter 2015.

Table 3. Solano grape field trial map, color-coded by *Xf* inoculation date, 2011 to 2014.



Severity or absence of PD symptoms for all Solano County ungrafted and grafted grapevines inoculated in 2011, 2012, or 2013 (**Fig. 4**), grafted transgenic grapevines inoculated in 2012 or 2013 (**Fig. 5**), and grafted individual transgenic canes inoculated in 2014 (**Fig. 6**) was assessed on July 22, 2014, using a 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. On July 22, 2014, the absence or severity of PD was rated for all four canes. PD symptoms are present in ungrafted and grafted grapevines inoculated in 2011, 2012, and 2013 and in 2014-inoculated grafted individual canes, but the severity of the symptoms is lower in some ungrafted and grafted transgenic lines from each strategy than in untransformed controls.

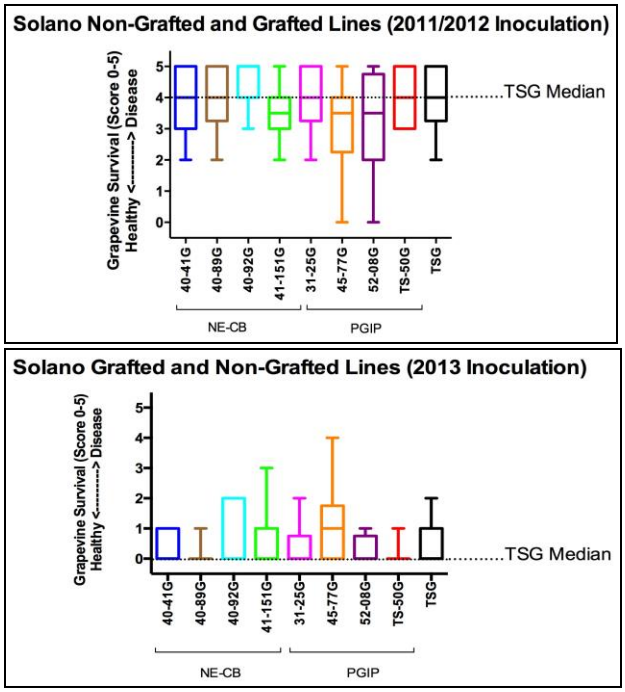


Figure 4. 2014 Pierce's disease symptom scoring for Solano ungrafted and grafted transgenic grapevines inoculated in 2011/12 (left) and 2012 (right) and scored in summer 2014.

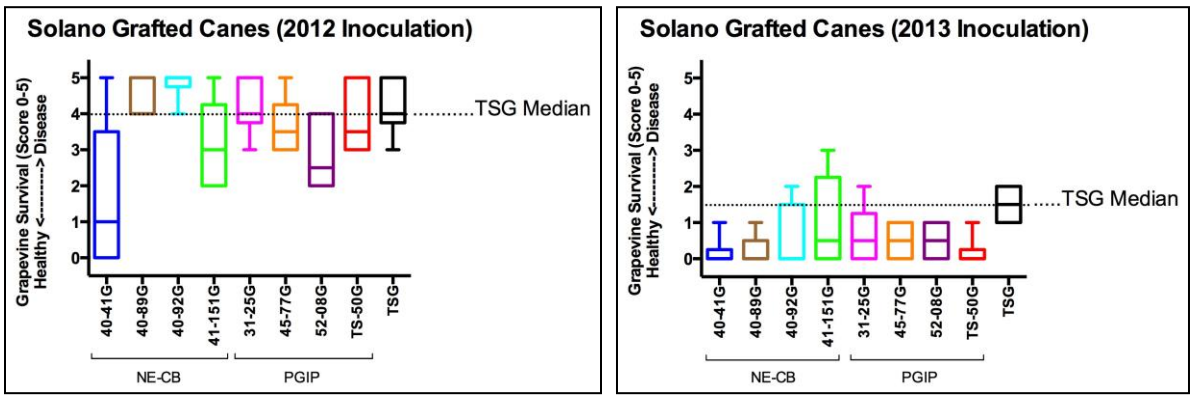


Figure 5. 2014 Pierce's disease symptom scoring for Solano grafted transgenic grapevines inoculated in 2012(left) and 2013 (right) scored in summer 2014.

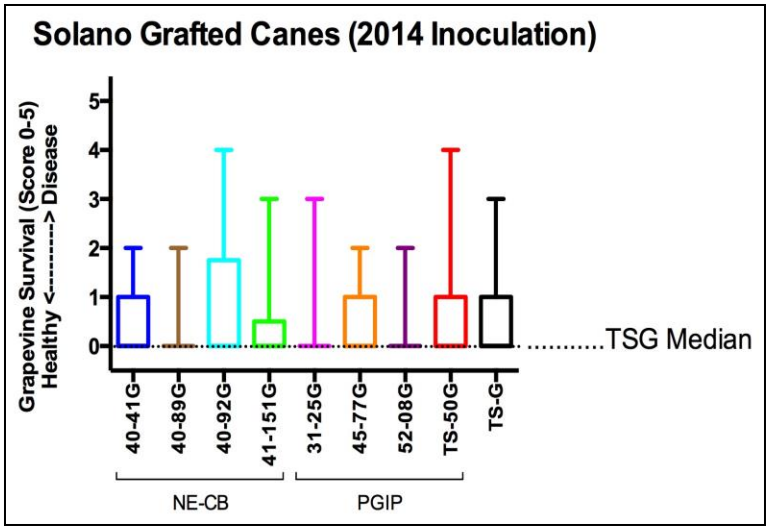


Figure 6. 2014 Pierce's disease symptom scoring for Solano grafted transgenic canes inoculated in spring 2014 and scored in summer 2014.

Xf cell counts for petioles and stem samples from runners of ungrafted or grafted vines in the Solano plot inoculated in 2011, 2012, and 2013 and harvested in spring and fall 2013 were determined using ELISA; the standard curve was created using *Xf* cells obtained from liquid culture. *Xf* infection was confirmed.

On July 22, 2014, one 2014-inoculated cane per grafted plant was harvested for quantification of *Xf* by qPCR using an Applied Biosystems SYBR Green fluorescence detection system. Grape stem and *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'-AATAAATCATAAAAAAATCGCCAACATAAACCCA-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. *Xf* was detected in grafted transgenic vines, but with *Xf* counts lower than in control grapevines (**Fig. 7)**. Another set of canes will be harvested for *Xf* quantification in spring 2015.

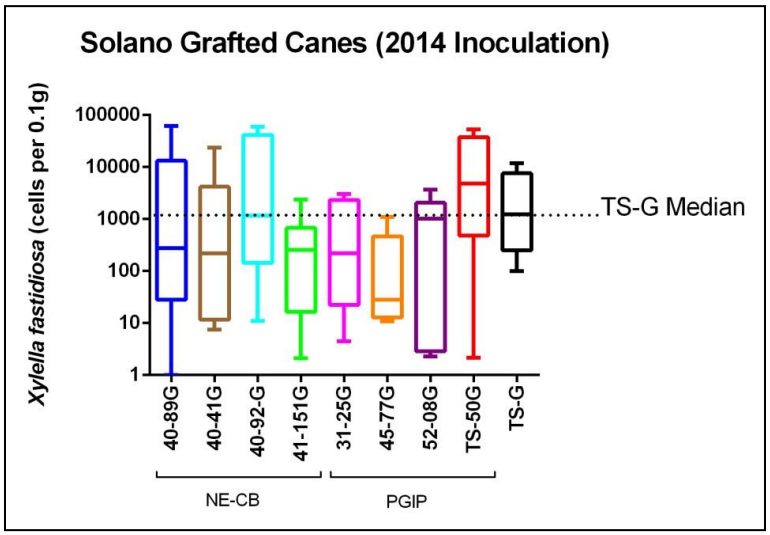


Figure 7. *Xf* quantification by qPCR for Solano grafted individual transgenic canes inoculated in spring 2014 and harvested in summer 2014.

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 Solano and Riverside County field APHIS permit #12-340-102r was transferred from Professor Alan Bennett to Professor Abhaya Dandekar in January 2014. The permit was extended by APHIS, with a new end date of April 1, 2016.

Activity 5. Maintain regulatory oversight of both 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 trials. 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 two field coordinators and their crews to obtain monitoring and activities information from PD field trial participant investigators. 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., A.M. Ibáñez, A. Jacobson, D. Dolan, R. Just, H. Gouran, D. Gilchrist and P. Rolshausen. 2014. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines field trial. Proceedings of the Pierce's Disease Research Symposium, Dec 2014, p. 95.

Dandekar, A.M., A.M. Ibáñez, D. Dolan, H. Gouran, D. Gilchrist and T. Miller. 2013. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines field trial. Proceedings of the Pierce's Disease Research Symposium, Dec 2013, pp. 101-106.

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.

Four hundred and forty (440) transgenic grapevines expressing either polygalacturonase-inhibiting protein (PGIP; 192 plants) or a chimeric antimicrobial protein (NE-CB; 192 plants) and 56 untransformed control vines were planted in two locations: Riverside County (220 plants) and Solano County (220 plants). Half of the transgenic grapevines are being evaluated as plants on their own roots and half as rootstocks grafted with untransformed Thompson Seedless (TS) scions to demonstrate the field efficacy of two strategies to control Pierce's disease (PD) in California grapevines. The first uses transgenic rootstocks, through expression of PGIP, to control the movement of the bacterium *Xylella fastidiosa (Xf)* in water-conducting xylem. The second strategy tests whether transgenic rootstocks can clear *Xf* infections in xylem tissue by expressing NE-CB.

At the Riverside County site, natural *Xf* infection was confirmed in petioles, stems, and xylem sap by ELISA and infections appeared uniform through 2013. During spring 2014, all 220 transgenic and control vines at the Riverside site were in decline; most had no new growth. Growth that did occur did not correlate with genotype: transgenic and control plants were equally affected. The root zone was limited to the first six inches of soil and was heavily infected with root-knot nematodes. The Riverside site was previously planted with Chardonnay and the vineyard was removed and replanted without fumigation. The field location had selected for an aggressive population of root-knot nematode during the previous planting. Our vines subsequently suffered lethal infection soon after they were planted. Nematode population data and number of knots per inch of root obtained from 18 soil and root samples confirm a heavy root-knot nematode infection. Evaluating the resistance or susceptibility of our transgenic grapevines to PD under field conditions will not be possible due to the combined root-knot and PD infections.

At the Solano County site, about 25% of the plants were mechanically inoculated in 2011 and again in 2012. Another 25% were inoculated in 2012 and the remaining 50% in 2013. The presence of *Xf* was confirmed in petiole and stem extracts using the ELISA assay. In addition, we evaluated PD symptoms, cane survival, and grapevine vigor and found that some transgenic lines from each strategy consistently scored better than the control and others did not. Lines that show resistance can transmit their resistance from the rootstock to the wild scion. However, resistance transmitted from transformed rootstock is weaker than that achieved in a transformed plant. On May 28, 2014, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, four current-year canes from all grafted transgenic and control plants at the Solano site were mechanically inoculated with *Xf*. Severity or absence of PD symptoms was evaluated in summer 2014 for all inoculated canes using a 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 declining and 5 = the cane is dead. In the same season, one cane per grafted plant was harvested for *Xf* quantification by qPCR. Grape stem and *Xf* DNA 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.

The current Solano and Riverside field permit was changed from Professor Alan Bennett to Professor Abhaya Dandekar in January 2014. The USDA-APHIS permit end date is April 1, 2016. Personnel from the Dandekar laboratory are maintaining regulatory oversight of the field trials. Timely reporting and inspections are conducted to maintain compliance with USDA-APHIS.

Status of funds.

We have exhausted all funds from July 2014 to February 2015 and have begun spending the March 2015 through June 2015 funding.

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 the UC Innovation Assessor, which could develop these further as a US patent variety.

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