TITLE OF REPORT: Interim Report for CDFA Agreement number 12-0442-SA

PROJECT TITLE: Field Evaluations of Grafted Grape Lines Expressing Polygalacturonase Inhibiting Proteins (PGIPs)

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TIME PERIOD COVERED BY THE REPORT: The results reported here are from work conducted from 1 February 2013 to 1 July 2013.

INTRODUCTION:

The project was designed to establish two field sites that would allow grape lines to be evaluated to assess whether polygalacturonase inhibiting proteins (PGIPs) restrict *Xylella fastidiosa* (*Xf*) spread and Pierce's Disease (PD).

The PI and co-PI had shown that the expansion of *X. fastidiosa* from the infection site throughout the vine, creates a systemic infection that causes PD and vine death (Krivanek and Walker, 2005; Labavitch 2006, 2007; Lin, 2005; Lindow, 2006, 2007a,b; Rost and Matthews, 2007). The grapevine water-conducting xylem elements are separated by pit membranes, cell wall "filters" whose meshwork is too small to permit movement of *Xf* (Labavitch et al., 2004, 2006, 2009a,b). *Xf* uses cell wall-degrading enzymes to digest the pit membrane polysaccharides (Labavitch et al., 2009b), opening the xylem elements and permitting systemic spread of the bacteria.

The *Xf* genome encodes a polygalacturonase (*XfPG*) and several β-1,4-endo-glucanase genes, whose predicted enzyme products could participate in the digestion pectin and xyloglucan polymers in pit membranes, thereby facilitating *Xf* systemic movement and PD development. Labavitch et al. (2006, 2007, 2009a; Perez-Donoso et al., 2010) reported that introduction of PG and EGase into uninfected grapevines caused pit membrane breakage. Roper et al. (2006, 2007) developed an *XfPG*-deficient *X. fastidiosa* strain and showed it was unable to cause PD symptoms, so *XfPG* is a PD virulence factor.

The over-all research aim is to use plant proteins, PGIPs, to limit *Xf* spread in grapevines. PGIPs are selective inhibitors of pathogen and pest PGs (Powell et al., 2000; Shackel et al., 2005; Stotz et al., 1993, 1994). Transformed grapevines expressing pear fruit PGIP (pPGIP) have reduced susceptibility to *Xf* and transgenic pPGIP is transported across the graft junction from a genetically engineered pPGIP expressing rootstock into wild-type scions (Agüero et al., 2005, Haroldsen et al., 2012).

This project has been designed to scale up the grafted and own-rooted pPGIP expressing grapevines, plant them in field settings, and evaluate their agronomic performance and their resistance to PD in settings comparable to commercial fields.

OBJECTIVES:

1. Scale up the number of grafted and own-rooted pPGIP expressing lines.
2. Plant and maintain grafted and own-rooted lines in two locations with different PD pressure.
3. Evaluate relevant agronomic traits of vines in two locations.
4. Determine PD incidence in pPGIP expressing grafted and own-rooted lines. Test for *X. fastidiosa* presence and, if present, determine the extent of infection.

DESCRIPTION OF ACTIVITIES AND ACCOMPLISHMENTS:

Objective 1: Generate enough grafted and own-rooted grapevines for the field trial

Progress: The pPGIP expressing 'Chardonnay' and 'Thompson Seedless' grapevines generated by Agüero et al. (2005) were maintained at the UC Davis Core Greenhouses. Vegetative cuttings of non-lignified stem sections from transgenic and control plants of both cultivars were rooted in an aeroponic cloning manifold (EZ-Clone Inc., Sacramento, CA). These plants are referred to as "own-rooted plants." Rooted cuttings were transferred to soil and maintained in the greenhouse before being transferred to the field sites. A sufficient number of grafted and "transgrafted" plants were generated for the field trials and were made by green grafting rootstock stem sections with budding scion tissue. Transgrafted plants had rootstocks from the pPGIP expressing lines and scions that do not express pPGIP. The number of plants of each genotype and grafting protocol for two field sites in Solano and Riverside Counties is shown in Table 1.

SOLANO		Chardonnay					Thompson Seedless				
	Grafting Strategy (Scion/root) Hatch – pPGIP expressing										
Own-Rooted (#)	Inoculated	17	-	9	-	-	8	-	9	-	-
	Non-Inoculated	8	-	4	-	-	4	-	5	-	-
Grafted (#)	Inoculated	9	9	9	-	-	9	9	9	-	-
	Non-Inoculated	4	4	4	-	-	4	4	4	-	-
RIVERSIDE											
Own-Rooted (#)	Natural Infections	13	-	11	6	-	9	-	12	6	-
Grafted (#)	Natural Infections	15	6	8	6	3	7	14	7	3	3

Table 1. Total number of grapevines planted in, and prepared for Solano and Riverside Counties. Dashed shapes represent pPGIP expressing grapevine rootstocks and/or scions; solid black shapes are null-transformant controls (no pPGIP); solid white shapes are wild-type controls (not transformed). Own-rooted vines were mechanically inoculated in Solano County on 7/21/2011 and 5/29/2012; grafted vines will be mechanically inoculated in July, 2013. Vines planted in Riverside County will be assessed in response to "natural" infections.

DNA was prepared from the vines used as source tissue for grafting and the genotypes were confirmed by PCR (Figure 1).

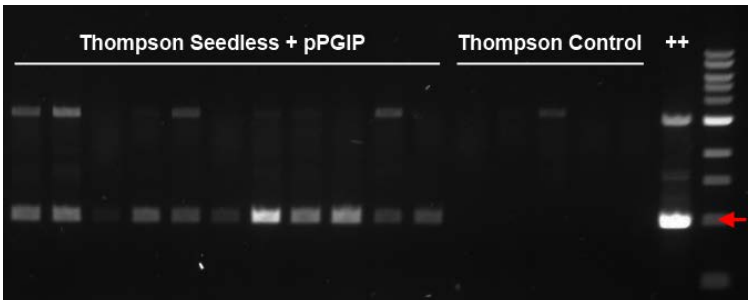

Figure 1. Sample of gel used to genotype by PCR with genomic DNA from grape leaf tissue from Thompson seedless vines expressing pPGIP and null-transformed (no pPGIP) controls used to generate the transgrafted vines. A 1 kb band (arrow) of pPGIP is expected only in samples used as rootstocks for transgrafts and pPGIP self-grafted controls. Each sample's quality was verified by amplifying a control fragment (not shown).

Results: Sufficient plants of both the Chardonnay and Thompson Seedless varieties have been self-grafted, transgrafted or propagated by own rooting to complete the Solano and Riverside County plots designed for the trial. The genotypes of the plants have been verified. All of the vines have been transplanted to the sites.

Objective 2: Establish field trial sites

Progress: Two field trial sites in Solano and Riverside Counties have been established to assess the PD resistance and general agronomic viability of own-rooted and grafted pPGIP expressing grapevines. The field plans of the Powell trial plots for Contract 12-0442-SA in Solano and Riverside Counties are shown in Figure 2. The field sites are shared by other projects testing PD resistance of other transgenic grapevines from PIs, D. Gilchrist, A. Dandekar, and S. Lindow. The vines satisfying our initial PCR analysis were hand-planted in a randomized block design with blocks consisting of two or three individuals in the same treatment (Table 1). The young plants were surrounded by protective grow tubes and hand-watered every two weeks in Solano County or as needed. The grapevines have been planted approximately 8 ft. apart and tied to wooden stakes with trellising wires at 40 in. and 52 in. A time-line showing when grafting, plantings, inoculations and assessments have been done is shown in Figure 3.

The vines have been pruned both to maximize potential cane number for inoculations and to establish vigorous positions for future growth. With the permit amendment granted by the BRS-USDA in March 2012, flowers and fruiting clusters have been allowed to persist. All own-rooted Chardonnay vines were cordon trained and spur pruned and the majority of the Thompson Seedless vines were cane pruned in an attempt to maintain proper vine balance and ensure fruit development. The Solano field site has been under weekly observation for the duration of the growing season and Tom Miller has reported that, in early July approximately 1 month after planting, the vines in Riverside County are growing robustly. Zac Chestnut and Constanza Jackson will visit Riverside on 29 July, 2013.

Results: As of 3 June, 2013, both the Riverside and Solano County sites have been established with all the planned plantings for this project.

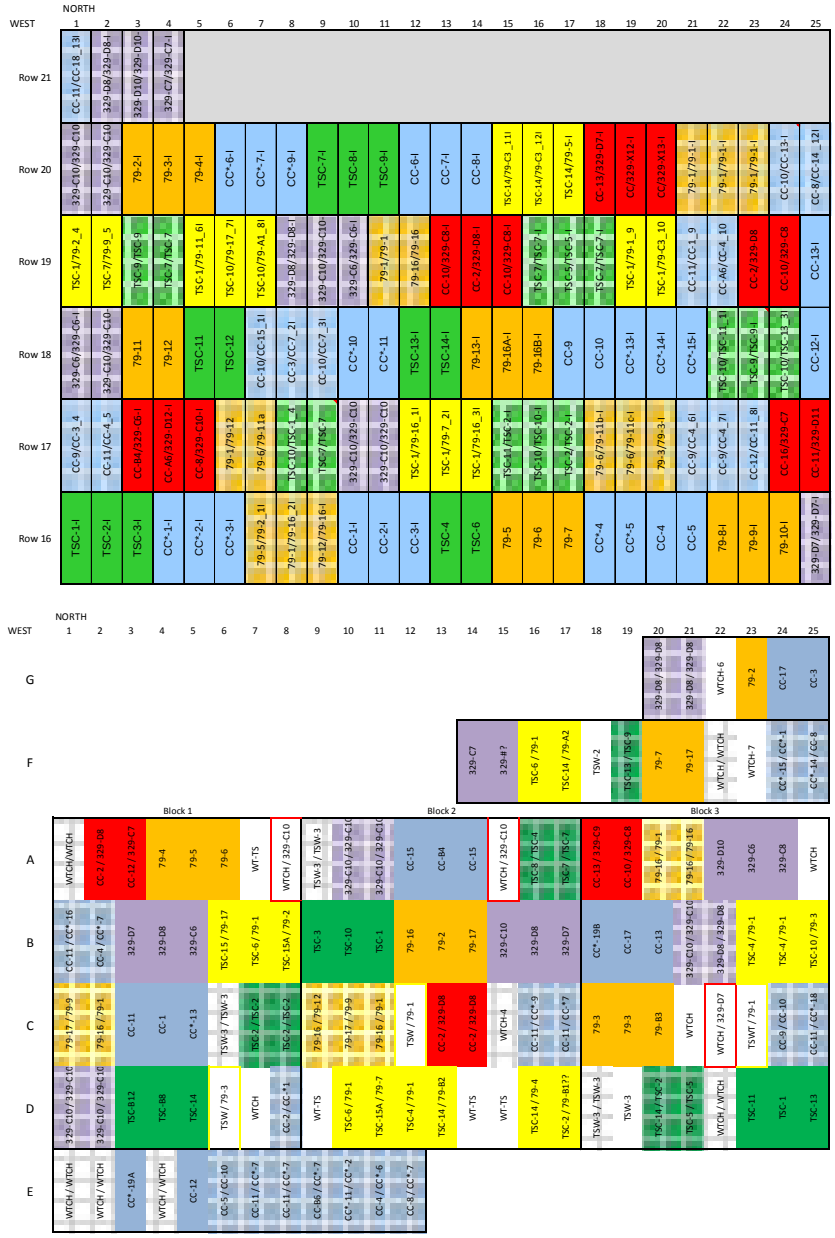

Figure 2. Field plans for Solano (A) and Riverside (B) County sites for Powell et al. trials. The color codes are as follows; O.R. = own-rooted, Gr. = grafted.

O.R.	Gr.	
		Chardonnay Control (CC)
		Chard + pPGIP
		Chard Wild-Type (WTCH)
N/A		CC / +pPGIP Transgraft
N/A		WTCH / +pPGIP Transgraft
		Thompson Control (TSC)
		TS + pPGIP
		TS Wild-Type (WT-TS)
N/A		TSC / +pPGIP Transgraft
N/A		WT-TS / +pPGIP Transgraft

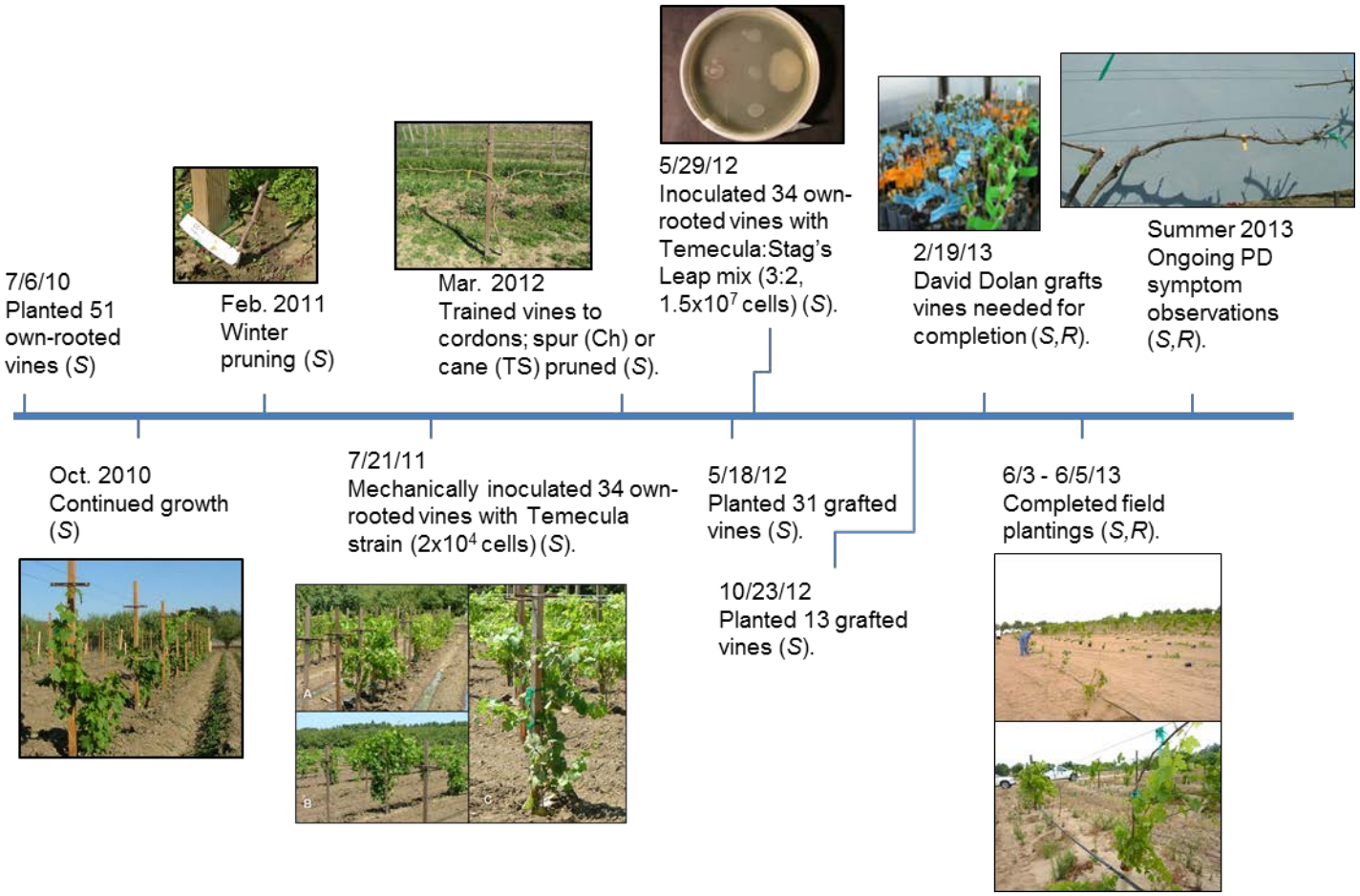

Figure 3. Timeline depicting the grafting, planting, inoculation and assessments of vines in the Solano (*S*) and Riverside (*R*) sites.

Objective 3: Evaluate relevant agronomic traits of vines in two locations.

Progress: Growth of the vines at both locations during the 2012 and 2013 growing seasons has been vigorous (Figure 3). The grapevines planted in Solano County have been monitored for general health and maintained on a weekly basis. PD symptoms were first observed on the inoculated vines in Solano County on April 24, 2013. The most frequent symptoms were a lack of bud break along formerly inoculated shoots (Figure 4) and excessive growth from the base of plants, potentially indicating a disruption in the vasculature or more severe die-back of cordons and mature canes. Outside viticulturists and pathologists confirmed that these vines were afflicted with PD. Their opinions were sought because traditional PD symptoms were mostly absent during the previous two growing seasons. Since the initial discovery, each vine has been photographed and scored for the presence of similar stunting or "blind" phenotypes (Table 2).

Table 2

Genotype		Plants (#)	% Plants with No Growth from Buds on Inoculated Canes		% Buds with No Growth on Inoculated Canes Only		% Plants with No Growth from Buds on Un-inoculated Canes		% Plants with Stunted Growth from Buds on Inoculated Canes		% Plants with Stunted Growth from Buds on Un-inoculated Canes	
			Spring	Summer	Spring	Summer	Spring	Summer	Spring	Summer	Spring	Summer
Inoculated Thompson+pPGIP	79-I	9	100 (9/9)	100 (9/9)	50-75	71.8	55.6 (5/9)	44.4 (4/9)	44.4 (4/9)	55.6 (5/9)	0 (0/9)	11.1 (1/9)
Thompson+pPGIP	79	5	-	-	-	-	40 (2/5)	20 (1/5)	-	-	0 (0/5)	20 (1/5)
Inoculated Thompson	TSC-I	8	100 (8/8)	100 (8/8)	75-100	84.5	75 (6/8)	87.5 (7/8)	25 (2/8)	25 (2/8)	50 (4/8)	50 (4/8)
Thompson Control	TSC	4	-	-	-	.	75 (3/4)	0 (0/4)	-	-	0 (0/4)	0 (0/4)
Inoculated Chardonnay	CC-I	17	94.1 (16/17)	100 (17/17)	75-100	79.0	47.1 (8/17)	52.9 (9/17)	47.1 (8/17)	52.9 (9/17)	23.5 (4/17)	17.7 (3/17)
Chardonnay Control	CC	8	-	-	-	-	75 (6/8)	0 (0/8)	-	-	12.5 (1/8)	0 (0/8)

Genotype		Plants (#)	% Plants with Excessive Base Growth		% Plants with Marginal Leaf Necrosis on Inoculated Canes		% Plants with Marginal Leaf Necrosis on Un-inoculated Canes		% Plants with Atypical Berry Clusters (partial, aborted, or absent)	
			Spring	Summer	Spring	Summer	Spring	Summer	Spring	Summer
Inoculated Thompson+pPGIP	79-I	9	77.8 (7/9)	66.7 (6/9)	0 (0/9)	33.3 (3/9)	0 (0/9)	11.1 (1/9)	-	44.4 (4/9)
Thompson+pPGIP	79	5	0 (0/5)	0 (0/5)	-	-	0 (0/5)	0 (0/5)	-	20 (1/5)
Inoculated Thompson	TSC-I	8	25 (2/8)	100 (8/8)	0 (0/8)	12.5 (1/8)	0 (0/8)	0 (0/8)	-	75 (6/8)
Thompson Control	TSC	4	0 (0/4)	50 (2/4)	-	-	0 (0/4)	0 (0/4)	-	0 (0/4)
Inoculated Chardonnay	CC-I	17	17.7 (3/17)	82.4 (14/17)	0 (0/17)	11.8 (2/17)	0 (0/17)	0 (0/17)	-	58.8 (10/17)
Chardonnay Control	CC	8	0 (0/8)	37.5 (3/8)	-	-	0	0	-	25 (2/8)

Table 2. Solano field observations of own-rooted vines taken on May 3, 2013 (Spring) and from June 19-July 1, 2013 (Summer). Each characteristic was counted as "positive" for the disease trait if one instance was observed on the plant. "Buds" refer to bud positions selected for by winter pruning and does not include buried buds. Inoculated vines were mechanically inoculated on marked canes in the summers of 2011 and 2012. Grafted vines were too young to score and did not show visual symptoms.

Figure 4. Inoculated Chardonnay control plant (no pPGIP) observed on April 24 (left), May 3 (center), and June 20, 2013 (right) at the Solano County site.

Results: None of the vines planted in our plots have been lost due to shock or accidental mishaps. All have been appropriately trained. Agronomic trait evaluation is on-going.

Objective 4: Determine PD incidence in pPGIP expressing grafted and own-rooted lines.

Progress: Two-thirds of the own-rooted vines at the Solano County site were mechanically inoculated with *X. fastidiosa* Temecula on 21 July 2011, to monitor PD incidence during the late summer 2011. No visual evidence of PD infection was observed throughout the 2011 growing season or early in 2012 after bud break. The same 34 own-rooted vines were resubmitted to mechanical inoculations on 29 May 2012 with a mixture of *X. fastidiosa* Temecula and Stags Leap strains (3:2, v:v). Young, green tissue was chosen for inoculation with 3-4 canes chosen per plant. Mechanical inoculations were performed as in 2011 except that approximately 1.5 x 10^7 cells were used per inoculation, an increase of 750-fold over the previous year. The inoculations were performed simultaneously with the other field site collaborators. The bacterial suspension was provided by D. Gilchrist.

Systematic evaluation of infected vines has been done weekly on the vines in the Solano site throughout July 2013. The results are shown in Table 2. At this point in the evaluation scheme, we have four preliminary observations of infected and un-infected vines from our trial in the Solano field:

1. A higher percentage of bud positions were able to grow out on mechanically inoculated pPGIP expressing Thompson seedless vines as on control Thompson Seedless vines. A similar comparison with the Chardonnay vines is not yet possible because of the selection of vines that were inoculated in 2012.
2. Inoculations with *Xf* promote shoot growth from the base of the vines.
3. Inoculation pPGIP expressing Thompson Seedless vines had 40% fewer berry clusters with aborted or abnormal berry clusters than infected controls. However 1 of 5 un-inoculated pPGIP expressing Thompson Seedless vines had abnormal berry clusters and the un-inoculated controls had none (0 of 4 vines).
4. However, three times as many mechanically inoculated pPGIP expressing Thompson Seedless vines had leaves with signs of marginal necrosis than infected control vines. A similar comparison with Chardonnay has not yet been made.

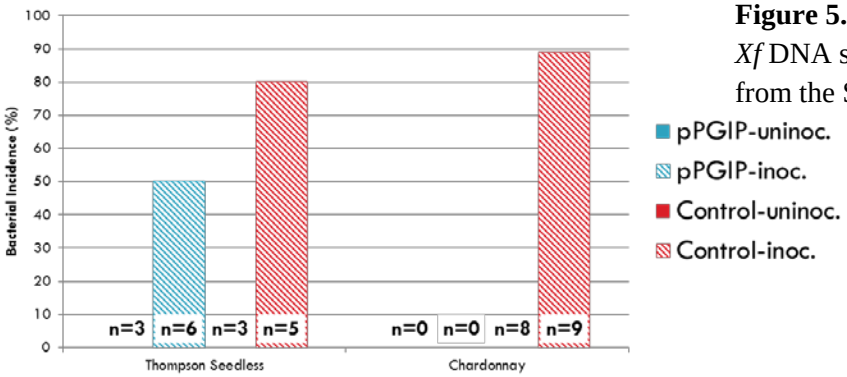

Figure 5. Results of PCR detection of *Xf* DNA sequences in inoculated vines from the Solano County site.

PCR has been used to detect *Xf*DNA sequences in leaves and petioles from inoculated and un-inoculated vines (Figure 5). XfDNA sequences were only detected in the inoculated plant leaves. All DNA preparations were checked to see that PCR amplification of grape DNA sequences was possible to insure that the material was appropriate for this evaluation.

Results: *Xf* DNA sequences were detected by PCR in the inoculated samples. No *Xf* DNA sequences were detected in un-inoculated controls (Figure 5). qRT-PCR efforts continue to quantify the amount of *Xf* in the inoculated material. Some of the visual assessments indicate that expression of pPGIP reduces PD symptoms (bud outgrowth and abnormal berry clusters) but another observation (necrosis of leaf margins) did not support this conclusion.

CONCLUSIONS:

All of the grafted plants necessary for the studies in Solano and Riverside Counties have been generated. The genotypes of the grafted plants were confirmed by PCR analysis of DNA from the plants. An initial attempt to infect the vines in Solano County was made but no symptoms were observed. A second attempt in 2012 resulted in detectable *Xf* DNA in infected vines in November, 2012 and visual symptoms of PD in April, 2012. The evaluations of the performance and productivity of the plants are on-going and will determine whether expression and presence of pPGIP affects unintentionally other characteristics of the vines.

PUBLICATIONS PRODUCED:

Haroldsen VM, Szczerba MW, Aktas H, Lopez-Baltazar J, Odias MJ, Chi-Ham CL, Labavitch JM, Bennett AB and Powell ALT (2012) Mobility of transgenic nucleic acids and proteins within grafted rootstocks for agricultural improvement. *Frontiers in Plant Science* **3**:39, Published 2 March 2012.

This publication describes the use of transgrafting for agricultural plants. Examples from the work with grapes are cited.

RESEARCH RELEVANCE STATEMENT:

The results of the field evaluations now that the field trials are established will provide the means of determining whether delivery of the pPGIP from rootstocks is effective for controlling PD and *X. fastidiosa* infection in a typical vineyard setting in California. The evaluations of the performance and productivity of the plants thus far confirm that expression and presence of pPGIP does not affect unintentionally other characteristics of the vines. By using varieties grown for fresh fruit and for wine production in California, we are testing varieties important to California growers.

LAYPERSON SUMMARY OF PROJECT ACCOMPLISHMENTS:

Own-rooted, self-grafted and transgrafted Chardonnay and Thompson Seedless grapevines, including those expressing pPGIP, were generated by vegetative propagation, genotyped by PCR, and planted as part of a field trial in Solano County. The performance of the plants in the field thus far has been appropriate for commercial settings. Mechanical inoculations with *X. fastidiosa* bacteria were done in 2011 and 2012 in Solano County and *Xf* DNA sequences have been detected only in the inoculated, but not in the un-inoculated, cane material. These results suggest that no false positives were encountered. Symptoms of PD infection were visible on the inoculated vines in the Spring of the year following the introduction of *Xf*.

INTELLECTUAL PROPERTY ASSOCIATED WITH PROJECT: This has been addressed in other projects associated with the PIs.

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