- **TITLE OF REPORT**: Interim Report for CDFA Contract Number 12-0442

- **TITLE OF PROJECT: FIELD EVALUATIONS OF GRAFTED GRAPE LINES EXPRESSING POLYGALACTURONASE INHIBITING PROTEINS (PGIPS)**

- **PRINCIPAL INVESTIGATOR:**
  Ann L.T. Powell
  Department of Plant Sciences
  University of California
  Davis, CA 95616
  alpowell@ucdavis.edu

CO-PRINCIPAL INVESTIGATOR:
John M Labavitch
Department of Plant Sciences
University of California
Davis, CA 95616
jmlabavitch@ucdavis.edu

FIELD COOPERATORS:
David Gilchrist
University of California
Davis, CA 95616
dggilchrist@ucdavis.edu

Thomas Miller
Department of Entomology
University of California
Riverside, CA 92521
thomas.miller@ucr.edu

- **TIME PERIOD COVERED BY THE REPORT:** The results reported here are from work conducted from October 2013 to March 2014.

- **INTRODUCTION:**

The project was designed to establish two field sites with typical vineyard practices that would allow grape lines to be evaluated in order to assess whether polygalacturonase inhibiting proteins (PGIPs) restrict *Xylella fastidiosa* (*Xf*) spread and Pierce's Disease (PD) and whether expression of pPGIP impacted the performance and attributes of the vines.

The PI, co-PI and others 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, pectin-rich cell wall "filters" whose meshwork is too small to permit movement of *Xf* (Labavitch et al., 2004, 2006, 2009a,). *Xf* produces cell wall-degrading enzymes to digest the pit membrane polysaccharides (Labavitch et al., 2009b), opening xylem connections and permitting spread of the bacteria.

The *Xf* genome contains a polygalacturonase (*XfPG*) and several β-1,4-endo-glucanase (EGase) genes, whose predicted enzyme products could participate in the digestion of pectin and xyloglucan polymers in pit membranes and, thereby, facilitate PD development by the movement of *Xf* within vines. 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 PGIPs to limit *Xf* spread in grapevines. PGIPs are produced by plants, including in flowers and edible fruits, and are selective inhibitors of pathogen and pest PGs (Powell et al., 2000; Shackel et al., 2005; Stotz et al., 1993, 1994). Grapevines transformed by A. Dandekar's group to express the pPGIP-encoding gene from pears have reduced susceptibility to *Xf* and pPGIP is transported across the graft junction from pPGIP expressing grape and tomato rootstocks into wild-type scions (Agüero et al., 2005, Haroldsen et al., 2012).

Grafting pPGIP-producing rootstocks to scions, which do not contain an introduced pPGIP gene, is an opportunity to provide a beneficial plant fruit protein (i.e., pPGIP) without introducing a pPGIP gene into the scion itself. 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 ACTIVITES:**

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

Progress: The pPGIP expressing Chardonnay and Thompson Seedless (TS) grapevines (Agüero et al., 2005) have been maintained at the UC Davis 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." 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. Rooted plantlets maintained in the greenhouse before being transferred to the field sites. The number of plants of each genotype and grafting protocol for the Solano and Riverside sites are shown in Table 1.

Table 1.

SOLANO		Chardonnay					Thompson Seedless				
	Grafting Strategy (Scion/root) Hatch – pPGIP expressing										
Own-Rooted (#)	Inoculated	9	-	9	-	-	16	-	9	-	-
	Non-Inoculated	5	-	4	-	-	7	-	5	-	-
Grafted (#)	Inoculated	3	8	9	-	-	15	10	9	-	-
	Non-Inoculated	1	3	4	-	-	7	5	4	-	-
RIVERSIDE											
Own-Rooted (#)	Natural Infections	12	-	11	6	-	10	-	12	6	-
Grafted (#)	Natural Infections	8	5	8	6	3	15	15	7	3	3

Table 1. Numbers of grapevines planted in Solano and Riverside Counties. Dashed fill represents pPGIP expressing rootstocks and/or scions; black fill is no pPGIP transformed controls; white fill is non-transformed controls. In Solano County, own-rooted vines were inoculated in 2011-2013; grafted vines were inoculated in July, 2013. Vines planted in Riverside County are exposed to "natural" infections.

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

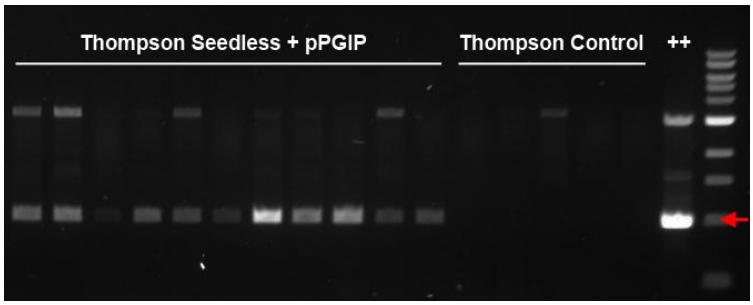

Figure 1. A gel used to genotype by PCR genomic DNA from grape leaf tissue from Thompson Seedless vines expressing pPGIP and null-transformed (no pPGIP) controls used to generate transgrafted vines. A 1 kb band (arrow) indicating the *pPGIP* sequence was detected only in material used as rootstocks for transgrafted and self-grafted controls. Each sample's quality was verified by amplifying a control fragment (not shown).

Results: Sufficient plants of both the Chardonnay and TS varieties have been own-rooted, grafted or transgrafted to complete the Solano and Riverside County plots. The genotypes of the plants have been verified. All of the vines for the trail were transplanted to the two sites by mid-summer 2013.

Objective 2: Establish field trial sites

Progress: Field trial sites in Solano and Riverside Counties have been established to assess the PD resistance and general agronomic viability of own-rooted, grafted and transgrafted pPGIP expressing grapevines. The field plans of the Powell trial plots in Solano and Riverside Counties are shown in Figure 2. The location of the plots for this trial is within fields shared by other projects testing PD resistance of other transgenic grapevines. 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 was observed weekly during the 2013 growing season. The vines in Riverside County were planted in early June, 2013 and allowed to grow during the 2013 season. Observations of the vines in Riverside were made by members of the Powell team midway through the 2013 growing season.

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

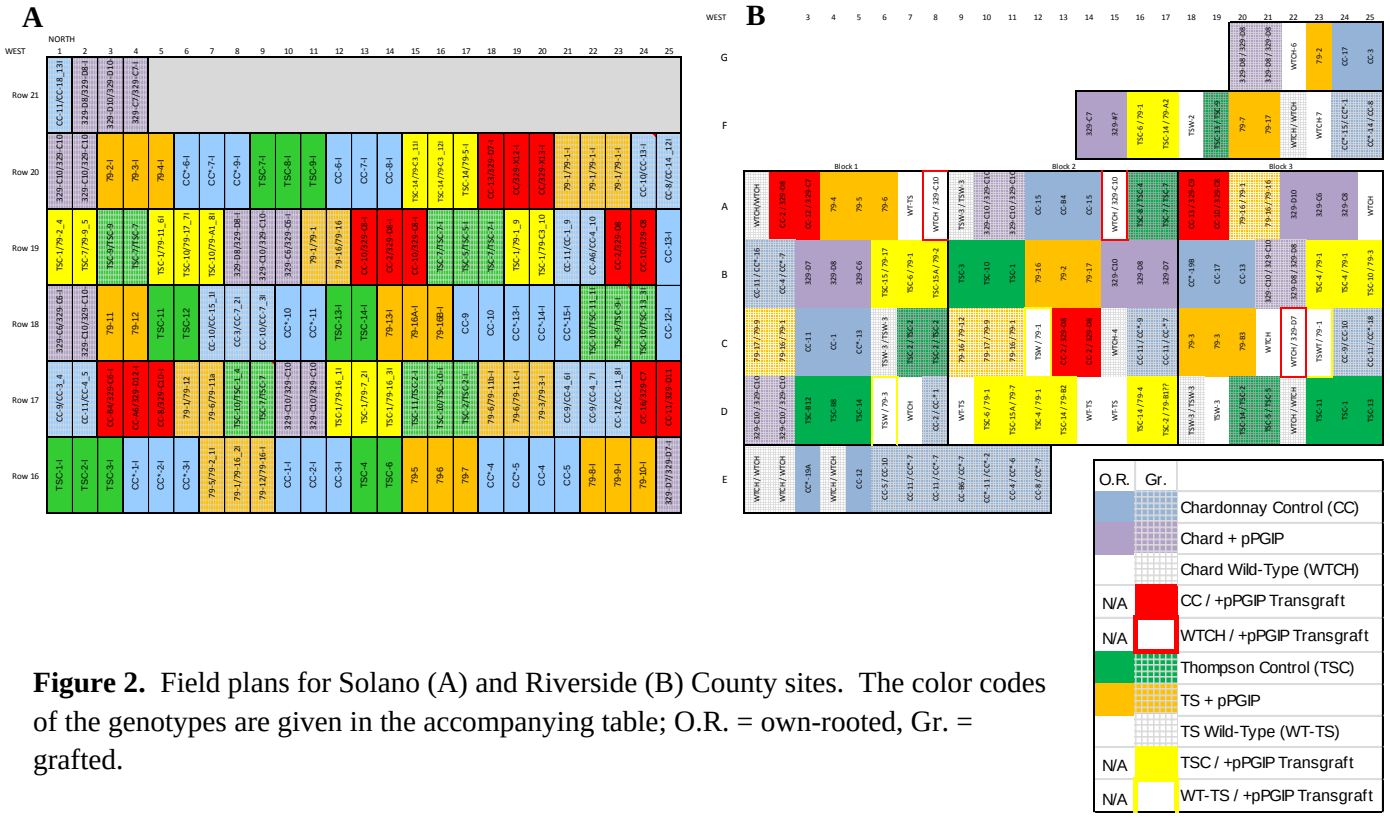

Figure 2. Field plans for Solano (A) and Riverside (B) County sites. The color codes of the genotypes are given in the accompanying table; O.R. = own-rooted, Gr. = grafted.

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

Progress: The Solano and Riverside vineyards continue to be maintained by appropriately pruning and training the vines. Eleven uninoculated grafted plants did not survive the exceptionally dry 2013 summer in Solano – these were replaced in Fall, 2013. Otherwise, growth of the vines at both locations has been vigorous (Figure 4). We reclassified in the Fall of 2013 11 own-rooted vines originally labeled Chardonnay control to TS control based on cluster morphologies. The previously observed leaf morphologies were supportive of this reclassification, but fruiting habit and form permitted definitive identification. Data for the agronomic and phenotypic observations in 2013 has been included in previous reports. Prior to annual pruning on 20 March, 2014, visual assessments of bud growth throughout the plants and on inoculated canes were made twice on 14 and 19 March on all vines in the Solano County site. Photos of representative vines on 20 March 2014 are shown in Figure 5.

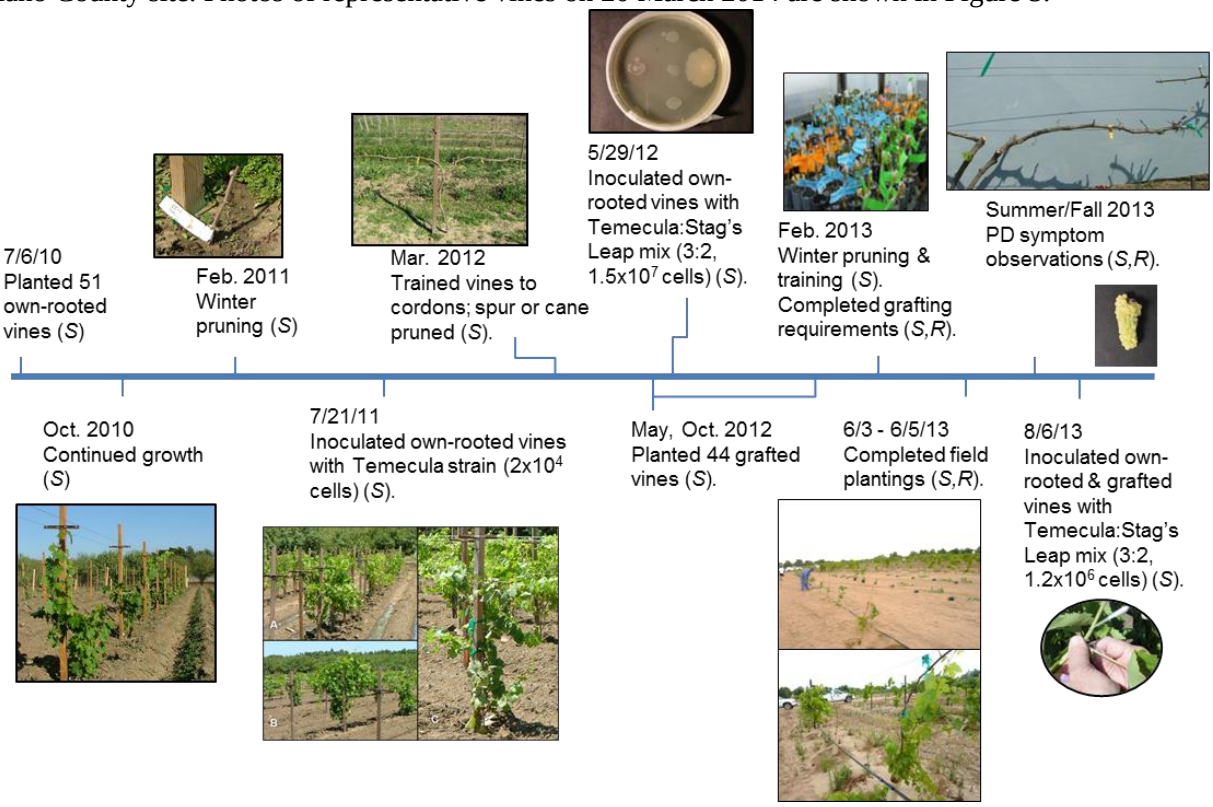

Figure 3. Timeline depicting the grafting, planting, inoculation and assessments of vines at the Solano (*S*) and Riverside (*R*) sites.

Figure 4. Representative inoculated Thompson Seedless plants in Solano County: top row, pPGIP expressing vine; bottom row, control vine. Pictures taken (from left to right) May 3, June 26, Sept. 24, and Sept. 11, 2013.

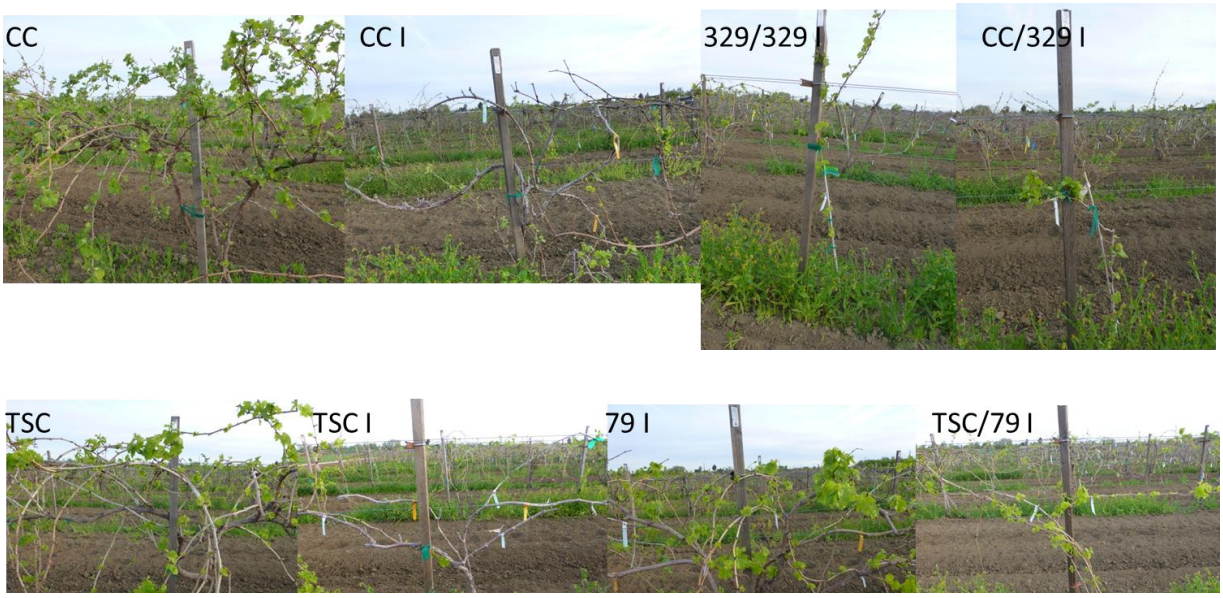

Figure 5. Representative uninoculated (left panels) and inoculated (I, right three panels) Chardonnay (CC, top row) and Thompson Seedless (TSC, bottom row) plants in Solano County on 20 March 2014, just before pruning. 329 is Chardonnay expressing pPGIP , 79 is Thompson Seedless expressing pPGIP, genotypes of grafted vines are represented as scion/rootstock.

In 2013, detailed analysis of plant performance and phenotypes was done on berries collected from 3 uninoculated and inoculated plants of each own-rooted genotype at the Solano site. The grafted plants in the plot were too juvenile to bear fruit in 2013 and had been inoculated three weeks earlier; they were not sampled. Total cluster numbers per plant were counted and one cluster per plant was harvested per plant. Twenty-five berries were removed for further analysis after counting the total number of healthy and raisined berries per cluster (Figure 6). 5 berries from 1-2 clusters inside the fruiting zone per plant were combined within plots to reduce plant-to-plant variation. The pH and °Brix of samples of crushed and free-run filtered juice were measured and reported previously. Soluble solids ranged from 21.7-24.4 °BRIX and pH values were 3.56-4.00 and showed no significant differences due to the pPGIP. In addition, cluster weight, length, and peduncle length were measured. Assessments of the subsamples included the weight of 25 berries, retention of pedicels, number and class of seeds (trace, rudimentary, or mature), dimensions of 5 berries, soluble solids, titratable acidity, and pH of juice. Each cluster and 5 individual berries were photographed for assessment of cluster density and berry color and shape. A smaller subsample was repeated on 4 September 2013 with similar results. The data collected in 2013 (Figure 6) will be critically analyzed in 2014 to select appropriate measurements for meaningful comparisons between genotypes, treatments and plots.

Results: Based on observations in 2013, the own-rooted TS plants expressing pPGIP had a slightly larger average yield than control vines. Expression of pPGIP in the Thompson Seedless variety did not significantly affect the berry cluster morphology or the juice characteristics. In 2014, initial bud growth data has just been collected and is currently being analyzed. In general, inoculated plants of all genotypes produced fewer buds and leaves than uninoculated control plants.

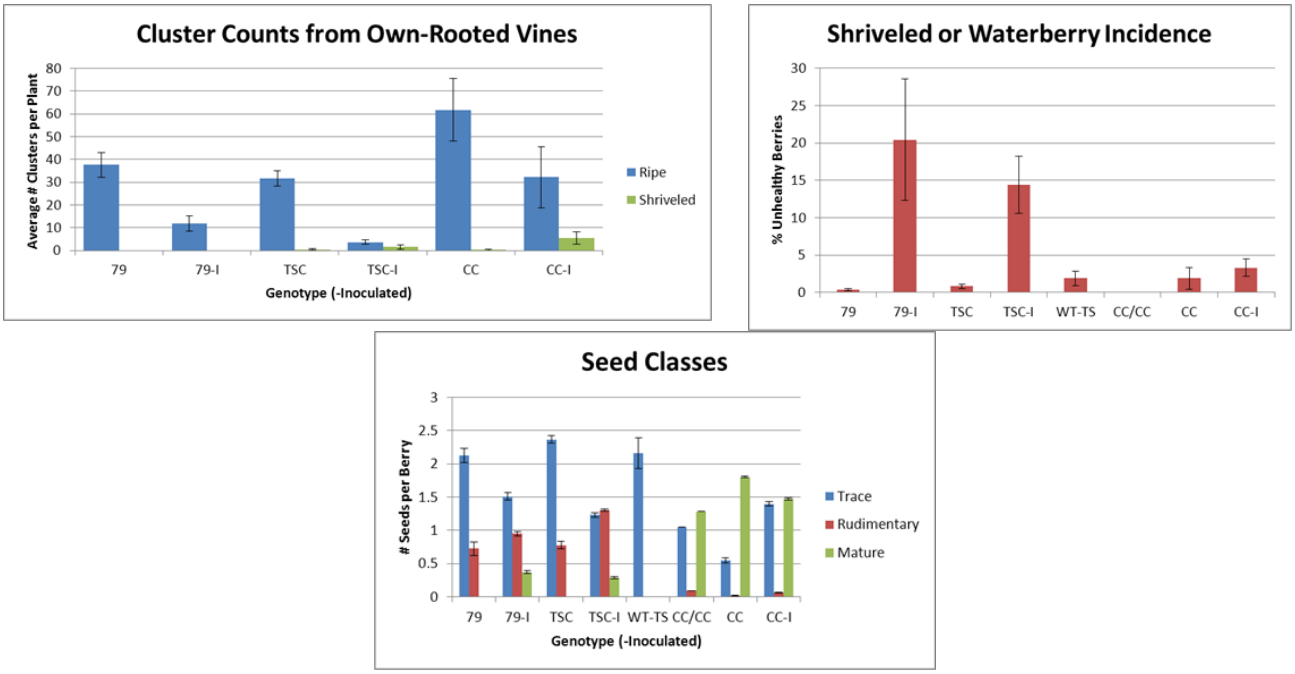

Figure 6. Number of clusters per plant (A), Percentage of shriveled unhealthy berries per cluster (B) and seed morphologies within clusters (C) of own-rooted TS expressing pPGIP ("79"), TS control (TSC) and Chardonnay (CC) from uninfected and infected (-I) plants.

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 first mechanically inoculated with approximately 2 x 10^4 *X. fastidiosa* Temecula cells on 21 July 2011. On the inoculated vines, no visual evidence of PD infection was observed in the 2011 growing season or early in 2012 after bud break. The same 34 own-rooted vines were re-inoculated on 29 May 2012 with a mixture of *X. fastidiosa* Temecula and Stag's Leap strains (3:2, v:v). 3-4 canes of young, green tissue per vine were mechanically inoculated with approximately 1.5 x 10^7 cells. The inoculations were performed simultaneously with the other field site collaborators in 2011 and 2012. The bacterial suspensions were provided by D. Gilchrist.

Once the remaining grafted and transgrafted vines at the Solano County site were transplanted in the summer of 2013, the complete trial of pPGIP -expressing vines was mechanically inoculated on 8 August 2013. The own-rooted vines previously infected and the selected newly planted grafted vines were inoculated with a mixture of the *X. fastidiosa* Temecula and Stag's Leap strains (3:2, v:v) prepared in our laboratory from glycerol stocks provided by the Kirkpatrick and Gilchrist labs. Inoculations were performed as in previous years, except only one site was inoculated per grafted vine because the vines were quite small (they had only been transplanted in June, 2013); larger, own-rooted vines were inoculated at 2 to 3 sites per plant. The inoculum cell density was estimated to be 1.2 x 10^6 cells per inoculation site by optical density and confirmed by serial plating.

PD symptoms were first observed on the twice-inoculated vines in Solano County on April 24, 2013. The most frequently observed symptoms were inhibition of bud break along 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 had PD. Their opinions were sought because traditional PD symptoms were mostly absent during the previous two growing seasons. Since the initial observation, each vine was photographed and initially scored for the presence of similar stunting or "blind" phenotypes (see previous reports). In March, 2014, initial observations of the thrice-inoculated own-rooted and the once-

inoculated grafted and transgrafted vines, indicates that, in general, bud growth is retarded on inoculated vines; inhibition of bud growth is especially severe on non-pPGIP-expressing or non-transgrafted vines (Figure 5).

PCR was used to detect *Xf*DNA sequences in leaves and petioles from inoculated and un-inoculated vines in 2012 (Figure 7). *Xf*DNA sequences were only detected in inoculated, and not in uninoculated, plant leaves. All DNA preparations were checked to see that PCR amplification of grape DNA sequences was possible.

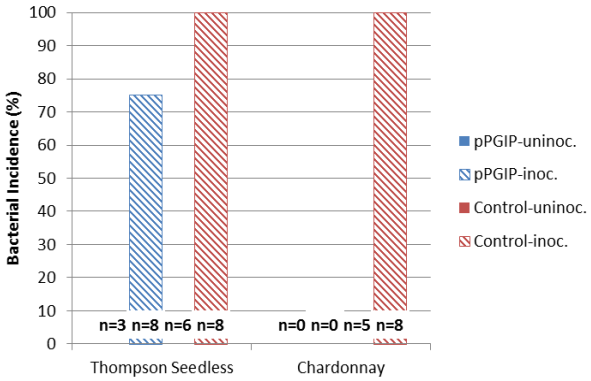

Figure 7. Results of PCR detection of *Xf* DNA sequences in inoculated vines in Solano County.

Results: In both 2013 and 2014, more bud positions were able to grow out on mechanically inoculated pPGIP expressing vines than on control vines. Preliminary analysis of 2014 observations suggests that both Thompson Seedless and Chardonnay vines had similar pPGIP-dependent differences. In the assessments in 2013, inoculated pPGIP expressing TS vines had 40% fewer clusters with aborted or abnormal berries than infected controls. However, 1 of 5 uninoculated pPGIP expressing TS vines had abnormal berry clusters and the un-inoculated controls had none. Three times as many mechanically inoculated pPGIP expressing TS vines had leaves with signs of marginal necrosis than infected control vines. In 2014, we expect to collect similar data with all genotypes and grafted and transgrafted plants. Preliminary visual assessments indicate that expression of pPGIP reduces PD symptoms (bud outgrowth and abnormal berry clusters).

Later in the 2013 season, inoculations with *Xf* resulted in a noticeable decline in clusters for all genotypes. However, this decline was 20% less in TS plants expressing pPGIP. Shriveled clusters were only observed in control genotypes and did not always correspond to infection with *Xf*. An increased percentage of unhealthy berries per cluster was measured in inoculated plants, but no significant difference in clusters from plants with or without pPGIP was observed. Berries from inoculated plants contained slightly more developed seeds than those from uninoculated plants; TS plants expressing pPGIP had fewer rudimentary seeds (Figure 6). Other observations reflect that uninoculated plants produce longer clusters with more berries.

Xf DNA sequences were detected by PCR in the inoculated samples. No *Xf* DNA sequences were detected in un-inoculated controls.

CONCLUSIONS:

All of the own-rooted, transgrafted and grafted plants necessary for the studies in Solano and Riverside Counties for this project have been generated and translplanted. The genotypes of the grafted plants were confirmed. An initial attempt to infect the vines in Solano County in 2011 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, 2013. Symptoms of the PD infections over 3 years were visible on the inoculated vines beginning in the Spring of 2014 The initial evaluations of the performance and productivity suggest that pPGIP expression in a table grape variety (Thompson seedless) or a wine grape (Chardonnay) improves resistance of vines to PD but does not otherwise affect vine growth or berry

characteristics. The performance of the own-rooted Chardonnay and Thompson Seedless vines in the field thus far has been appropriate for commercial settings.

- **PUBLICATIONS AND PRESENTATIONS PRODUCED:** Results as of mid-December 2013 were presented orally at the Annual Pierces Disease Symposium in Sacramento by Ann Powell.

- **RESEARCH RELEVANCE STATEMENT:**

Work in this project evaluates the performance of grafted grapevine lines that produce a protein that is a candidate for control of Pierce's Disease (PD). The vines have been established in vineyards in a manner that approximates typical commercial settings in Solano and Riverside Counties with low and high PD disease pressure, respectively. The CDFA PD and GWSS Board's Research Scientific Advisory Panel had established a priority to evaluate the potential commercial use of the strategy to deliver polygalacturonase-inhibiting proteins (PGIPs) from grafted rootstocks to control PD in the scion, fruit-bearing portions of grapevines. Established transformed 'Thompson Seedless' and 'Chardonnay' grapevines expressing a PGIP from pear fruit (pPGIP) showed reduced PD incidence when inoculated with *X. fastidiosa* (Agüero *et al*., 2005). The pPGIP that was produced in the transformed rootstock was identified in samples of xylem exudate that were collected from grafted, but not transformed scions (Agüero *et al*., 2005). Therefore, cuttings from these grapevines were grafted with non-pPGIP producing scions to make comparisons of the effectiveness and outcomes between vines producing pPGIP in grafted rootstocks, those producing pPGIP throughout the vine, and vines with no pPGIP.

- **LAY PERSON SUMMARY:**

Two vineyard plots containing own-rooted and transgrafted (rootstocks expressing pPGIP grafted to fruit producing scions with no genetic modifications that, thus, do not themselves produce pPGIP) combinations of Chardonnay and Thompson Seedless grapevines were established and the identities of the genotypes were confirmed by June 2013. Mechanical inoculations with *X. fastidiosa* bacteria were done in 2011, 2012 and 2013 in Solano County and natural infections were allowed to occur in Riverside County. Data describing the agronomic and disease traits of the vines have been collected. Since this trial evaluates grape varieties grown for fresh fruit and for wine production in California, we are testing varieties important to most California grape growers; these varieties have different growth habits and products so this trial examines the efficacy of pPGIP across wine and fresh sectors of the grape industry. The initial evaluations of the performance and productivity suggest that pPGIP expression in a table grape variety (Thompson seedless) or a wine grape (Chardonnay) improves resistance of vines to PD but does not otherwise affect vine growth or berry characteristics.

- **STATUS OF FUNDS:**

	Budget TOTAL	Feb 1, 2013 – Feb 28, 2014	March 1, 2014 – June 30, 2014
Personnel			
Professional, 8% Ann Powell, Feb 13 – Oct 13, 16% Nov 13 – June 14 (Monthly base $7,741.67)	**19,510**	12,589	$1,264
Lab Asst. I, 1 month (monthly base $2,368)	**2,368**	4,355	$0

Student Asst., 150 hrs at $10/hr	**1,500**	3,538	$960
Employee Benefits (30.3%, 33.3%)	**7,559**	6,571	$631
SUBTOTAL **(**Personnel + Benefits)	**30,937**	27,053	$2,855
Supplies and Expenses	**9,907**	5,710	$5,736
Equipment			
Travel	**2,000**	1,490	$0
Computer Time			
Other			
Indirect Costs*			
SUBTOTAL (Supplies, Expenses, Equipment, etc.)	**11,907**	7,200	$5,736
TOTAL	**42,844**	34,253	$8,591

- **SUMMARY AND STATUS OF INTELLECTUAL PROPERTY ASSOCIATED WITH THE PROJECT:** None is relevant.

- **LITERATURE CITED:**

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