- **Title of report:** Final Report for CDFA Agreement Number 08-0171
- **Title of Project:** OPTIMIZING GRAPE ROOTSTOCK PRODUCTION AND EXPORT OF INHIBITORS OF *XYLELLA FASTIDIOSA* POLYGALACTURONASE ACTIVITY
- **Principal Investigators:**

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- **Time Period Covered by Report:** The results reported are from work conducted from July 1, 2008 to June 31, 2013.
- **Objectives:**

Objective 1 **-** Define a path for commercialization of a PD control strategy using PGIPs, focusing on IP and regulatory issues associated with the use of PGIPs in grape rootstocks.
Objective 2 **-** Identify plant PGIPs that maximally inhibit *Xf*PG.
Propagate and graft grape lines expressing and exporting pPGIP for use in PD resistance assays
Identify and clone plant PGIPs that are efficient inhibitors of *Xf*PG
Develop a recombinant expression system for *Xf*PG
Express PGIPs, using plant recombinant systems, to assay *Xf*PG inhibition
Objective 3 **-** Assemble transcription regulatory elements, *Xf*-inducible promoters, and signal sequences that maximize PGIP expression in and transport from roots.
Objective 4 **-** Create PGIP-expressing rootstocks and evaluate their PD resistance.

- **Description of Activities:**

Objective 1. A path to commercialization of transgenic rootstocks

PIPRA evaluated the Intellectual Property (IP) around each of fourteen candidate PGIP genes selected for possible evaluation in this project (see Objective 2B). PIPRA utilized protein-based queries to search the patent and patent application databases using the program GenomeQuest. The IP information was used to determine if there were IP issues related to these particular genes and none were identified. The use of the PGIP sequences from non-*vinifera* grape varieties was not possible due to restrictions on their release by the wine and grape industry board associated with the Institute for Wine Biotechnology at Stellenbosch University, South Africa.

PIPRA acted as a liaison for the Board for issues associated with the potential commercialization of various approaches using transgenic grapevine rootstocks for several CDFA PD/GWSS Board-funded projects, including the strategy outlined in this project. Since 2010, PIPRA analysts managed the initial permitting process for the field trial testing of Thompson Seedless and Chardonnay varieties of grapevines expressing pPGIP and established the BQMS protocols. Some of their work was funded through a separate contract.

Objective 2. Identifying plant PGIPs that maximally inhibit XfPG

A. Propagation and grafting of grape lines expressing and exporting pPGIP

The pPGIP-expressing Chardonnay and Thompson Seedless grapevines described in Agüero *et al.* (2005) were maintained throughout the project in the UC Davis Core Greenhouse Complex. The propagation and grafting techniques used for this objective are described in detail in the progress reports for the project "Field Evaluation of Grafted Grape Lines Expressing pPGIP" (PI: Powell). These efforts provided more than sufficient material for the plots of grafted and own-rooted plants for the test sites in Solano and Riverside counties. Beginning in February 2013, using funding from the field evaluation project, David Dolan was engaged to complete the grafting work. All the plots at both locations were completely planted by June 2013.

Using grafted plants to capture active exogenous pPGIP translocated from transgenic rootstocks to the scion portion of the plants, we modified a pressure flow apparatus to flush long stem segments with water or high salt buffers. We were able to obtain xylem exudate containing a small amount of total proteins (26 μg/ml) from own-rooted, transgenic pear fruit PGIP (pPGIP)-expressing Thompson Seedless stems. We used the polyclonal pPGIP antibodies produced in rabbits several years ago and identified pPGIP in macerated grape leaves and stem segments, but did not identify cross-reactive pPGIP in xylem exudate (Figure 1).

There was insufficient protein to measure PG inhibiting activity in the proteins collected from the grapevine xylem exudate or macerate.

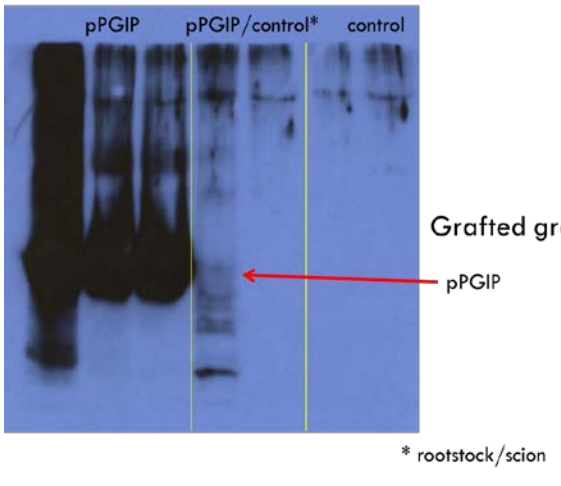

Figure 1. Western blot showing pPGIP protein collected from grafted and own-rooted grapevines. The rootstock in the graft, but not the scion, expressed pPGIP. The grafted samples were taken at least 30 cm beyond the graft junction, indicating translocation of the pPGIP protein.

We have evaluated proteins collected from the xylem of non-transgenic tomato scions grafted onto transgenic tomato rootstocks expressing pPGIP in order to establish whether pPGIP protein can move into the grafted portions of plants. We used this system because we were able to gather more protein from the xylem and thus could detect the pPGIP protein in xylem sap. We confirmed that pPGIP protein is expressed in the rootstocks and were able to use the pressure device to force xylem sap out of the cut stems of own-rooted and grafted plants. The xylem sap

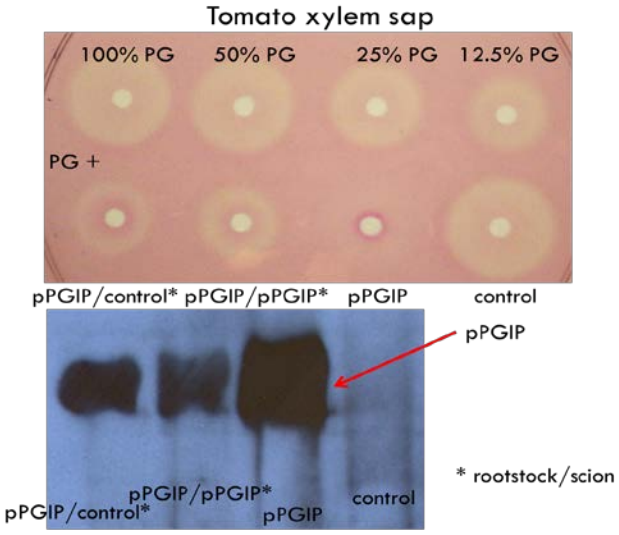

Figure 2. PG inhibiting activity (top panel) of xylem sap from grafted and control tomato plants expressing pPGIP. pPGIP protein is detected (bottom panel) with existing polyclonal antibodies in a western blot. Inhibiting activity and pPGIP protein is detected when the root portions of the plants express pPGIP.

fluid from non-transgenic scions grafted onto pPGIP-producing rootstocks contained detectable pPGIP protein and the collected protein was able, as expected for pPGIP, to inhibit the PGs collected from cultured *B. cinerea* Del 11 (Figure 2).

B. Selection of PGIPs as PD defense candidates

Based on phylogenetic (Figure 3), biochemical (Table 1), and structural analyses of PGIP sequences from 68 diverse plant varieties, the PGIPs from 'Roma' rice, 'Hamlin' orange, and 'Bartlett' pear were predicted to be the best candidates to evaluate for their inhibition of *Xf*PG. Since *Xf*PG is unusually and highly positively charged (+22.24 at pH 4.5, Table 2), we elected to focus our attention on PGIPs with the lowest overall positive charge at pH 4.5, the pH of most apoplastic plant fluids. This choice reflected our bias towards PGIPs that would be most likely to interact with *Xf*PG because of charge differences.

We modeled, with Dan King of Taylor University, the 3D structures of selected candidate PGIPs (Figure 4) and *Xf*PG proteins to try to understand the locations of relevant interactions (Figure 5). The homology models created for *Xf*PG, the polygalacturonic acid (PGA) substrate for PG, and each of the candidate PGIPs provided predictive tools to interpret the inhibition mechanisms and physical interactions between *Xf*PG and the PGIPs. Dynamic *in silico* reaction simulations predicted that two clusters of *Xf*PG amino acids, #63-74 and #223-226, must be unblocked for *Xf*PG to cleave PGA. The long columns of electronegative residues on the concave faces of the selected PGIPs' leucine rich repeat structures bind to these critical regions (Fig. 4). This information coupled with surface chemistry mapping predicts that pPGIP, CsiPGIP (citrus), and OsPGIP1 (rice) will be the best inhibitors of *Xf*PG. A closer look at the dynamic reaction

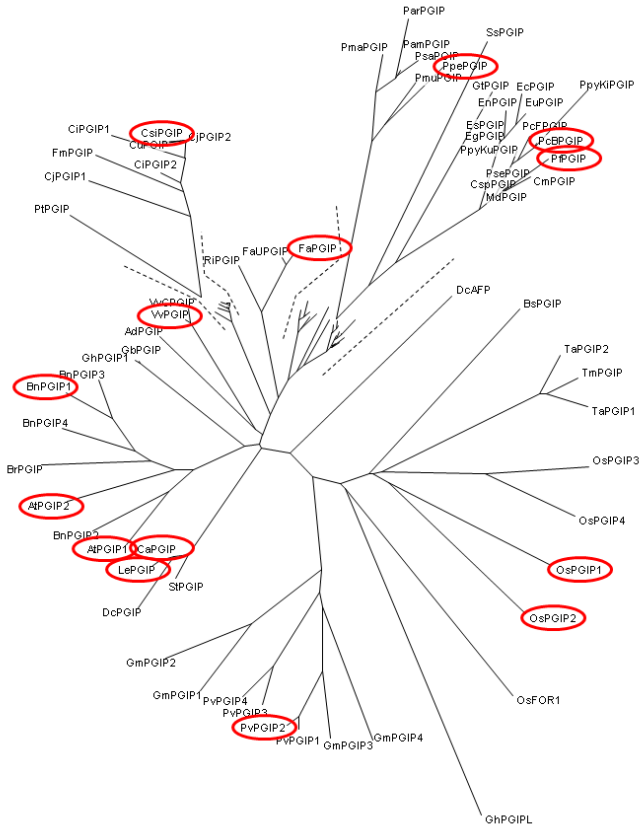

Figure 3. Unrooted phylogenetic tree of PGIPs. The full-length amino acid sequences were aligned using ClustalX 2.0.9. An unrooted, neighbor joining tree was constructed in ClustalX and visualized with TreeView 1.6. The resulting radial phylogeny is more robust than its predecessor due to the inclusion of both monocot and dicot sequences. The 14 sequences that were analyzed further in Table 1 are circled in red.

simulations highlighted other residues that may also influence PG-PGIP binding. Strong hydrogen bonding occurs between residues on pPGIP and Tyr303 of *Xf*PG, bringing them together in a potentially inhibitory manner (Figure 5). Electrostatic repulsions between VvPGIP (grape) residues and *Xf*PG Tyr303 prevent a similar alignment and may explain VvPGIP's failure to inhibit *Xf*PG. A traditional cloning strategy was applied to generate plant transformation vectors with each of these PGIPs inserted into pCAMBIA-1301. Based on the transformation construct, transcription of the PGIPs is constitutive because it would be driven by the CaMV- 35S promoter.

Genomic DNA was prepared from 'Kitaake' rice, 'Valencia' and 'Washington Navel' orange leaves and each PGIP was successfully PCR amplified. However, because of the sequence differences between the PGIPs we obtained from 'Valencia' and 'Washington Navel' and the published sequence, we amplified and cloned PGIP from 'Hamlin' orange. It has the same sequence we obtained for PGIP from 'Valencia' and so it appears that the sequence for 'Hamlin' orange in the database is incorrect; this recognotion modifies our conclusions about the apparent net charge of the protein. In amplifying and cloning the rice PGIPs, we again discovered discrepancies among the cloned PGIP sequences and those previously published. After requesting 'Roma' rice germplasm and cloning the PGIPs, we found only a single silent mutation in the coding sequence of *OsPGIP*. We made some progress towards preparing the transformation vector although additional mutations were found in the inserted pPGIP sequences and therefore we were not able to pursue this further. The coding sequence of *OsPGIP2* that we obtained does not appear to encode a *bona fide* PGIP and will not be pursued further.

Table 1. Total protein charge analysis for the 14 candidate PGIPs in different pH

Common name	Organism	Protein	Charge of Protein					
			pH 3.5	*pH 4.0*	*pH **4.5***	*pH 5.0*	*pH 5.5*	*pH 6.0*
Thale cress	*Arabidopsis thanliana* (Col.)	AtPGIP1	27.5	20.9	**14.2**	10.0	7.4	5.2
Thale cress	*Arabidopsis thanliana* (Col.)	AtPGIP2	35.4	28.5	**21.6**	17.0	14.2	11.8
Rape	*Brassica napus* cv. DH12075	BnPGIP1	30.5	22.2	**14.2**	9.4	6.8	4.8
Pepper	*Capsicum annum* cv. arka abhir	CaPGIP	20.7	15.2	**9.5**	5.9	3.8	2.2
Sweet orange	*Citrus sinensis* cv. Hamlin	CsiPGIP	28.0	21.7	**15.2**	11.1	8.7	6.7
Strawberry	*Fragaria x ananassa*	FaPGIP	25.4	18.7	**12.1**	8.0	5.6	3.7
Rice	*Oryza sativa* cv. Roma	OsPGIP1	18.4	12.9	**7.6**	4.3	2.2	0.2
Rice	*Oryza sativa* cv. Roma	OsPGIP2	17.5	9.3	**1.6**	-3.1	-6.1	-8.8
Common bean	*Phaseolus vulgaris* cv. Pinto	PvPGIP2	22.7	17.6	**12.9**	10.2	8.5	7.1
Peach	*Prunus persica*	PpePGIP	28.7	21.9	**14.9**	10.3	7.5	5.3
Chinese Firethorn	*Pyracantha fortuneana*	PfPGIP	16.9	11.7	**6.6**	3.4	1.4	-0.3
Bartlett pear	*Pyrus communis* cv. Bartlett	pPGIP	23.1	16.1	**9.3**	5.0	2.6	0.7
Tomato	*Solanum lycopersicum* cv. VFNT Cherry	LePGIP	29.8	23.4	**17.0**	12.8	10.1	7.7
Grape	*Vitis vinifera* cv. Pinotage	VvPGIP	30.5	24.0	**17.7**	13.6	11.1	8.7

Table 2. Total protein charge analysis for the fungal, bacterial and plant PGs in different

	PG								
pH	*X. fastidiosa* PG	*F. moniforme* PG	*A. niger* PGC	*A. niger* PGB	*A. niger* PGA	*A. niger* PG2	*A. niger* PG1	Tomato PG	Grape PG
3.50	40.99	17.90	4.79	27.25	3.88	21.94	10.46	30.88	32.19
4.00	31.30	11.25	-9.53	18.45	-11.40	12.86	-2.13	25.51	27.26
4.50	22.24	5.44	-23.56	9.92	-26.43	4.08	-14.38	20.03	22.4
5.00	16.39	2.07	-32.08	4.67	-35.56	-1.35	-21.80	16.26	19.23
5.50	11.90	-0.13	-36.17	1.86	-39.77	-4.36	-25.41	13.54	17.15
6.00	6.79	-2.36	-38.46	-0.20	-41.90	-6.69	-27.53	10.68	15.14

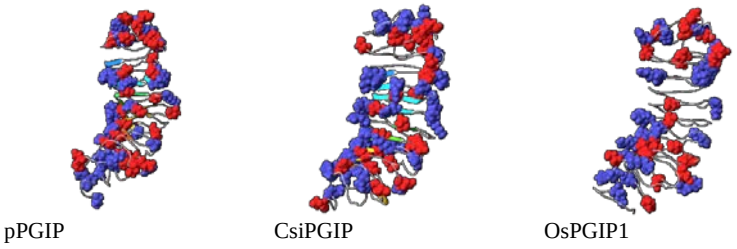

Figure 4. Homology models of three PGIPs predicted to be good candidates to inhibit *Xf*PG. The column of electronegative residues (red) on the concave faces of each protein may align with critical residues on *Xf*PG important for inhibition.

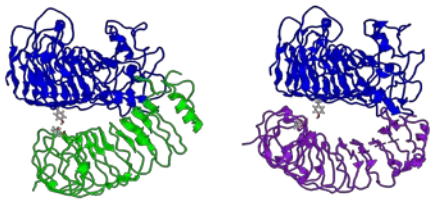

Figure 5. *Xf*PG-PGIP complexes. Tyr303 of *Xf*PG (blue) binds strongly with a region of pPGIP (green) which is not possible with VvPGIP (purple). Interactions such as this might influence PG-PGIP interaction and inhibition.

C. *Xf*PG expression and purification

Two strategies were used to obtain active *Xf*PG for assays to compare the inhibition efficiencies of the PGIPs. In one approach, Rachell Booth and her group at Texas State University, San Marcos, tried to express active *Xf*PG protein using expression in heterologous cells. Drosophila S2 cells produced quantifiable amounts of PG protein (Figure 6) but it had only very slight activity and this activity diminished over time (Figure 7). These efforts did not result in sufficient *Xf*PG for further experiments.

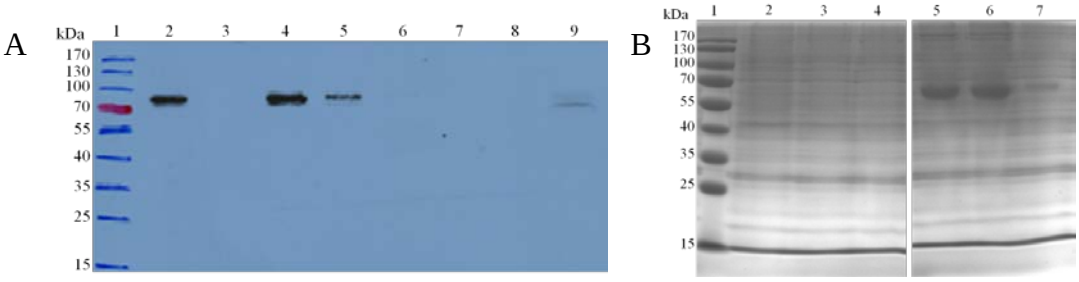

Figure 6. **A.** Western blot analysis of partially purified insect cell lysate after *Xf*PG protein expression. 15 mL crude *Xf*PG lysate was purified by column chromatography and selected fractions were analyzed by Western blotting. Lane 1 = pre-stained ladder, lane 2 = flow-through #4, lane 3 = wash #10, lanes 4-7 = elution fractions #1-4, lane 8 and 9 = cellular medium. Recombinant *Xf*PG protein was eluted with 250 mM imidazole and probed with the anti-V5 primary antibody and anti-mouse HRP secondary antibody. **B.** Partially purified *Xf*PG protein eluted with 250 mM imidazole analyzed by polyacrylamide gel electrophoresis and Coomassie staining. Lane 1 = pre-stained ladder, lanes 2-4 = cell lysate fractions #1-3, lanes 5-7 = cellular medium fractions #1-3.

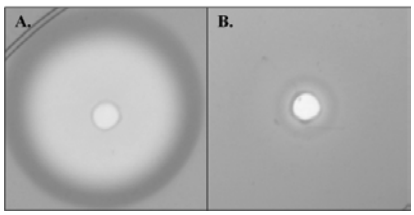

Figure 7. Radial diffusion assays of concentrated PG from *Botrytis cinerea* (A) or culture medium from induced *Xf*PG-expressing Drosophila cells (B). The clearing zone diameter is related to amount of PG activity.

The second strategy was to express *Xf*PG transiently in leaves. A fusion construct with the apoplastic signal sequence from pPGIP was linked to the coding sequence of *Xf*PG for transient expression by *Agrobacterium tumefaciens* of *Xf*PG targeted to the extracellular space. Preliminary agroinfiltration assays (Figure 8) with intact tobacco leaves indicated that the targeted *Xf*PG had a similar activity to the non-targeted protein, both resulting in necrotic lesions in the infiltrated tissue, although the necrotic response did not appear for several days. The strain of *A. tumefaciens* used in agroinfiltration experiments has been shown to influence the appearance and severity of necrosis in different plant species and tissues (Wroblewski *et al.*, 2005). Therefore, after conferring with Jan Van Kan (Dept. of Phytopathology, Wageningen University) and other researchers, we obtained and tested other strains of *A. tumefaciens.* All strains yielded similarly confounding background necrosis when infiltrated as empty vector controls.

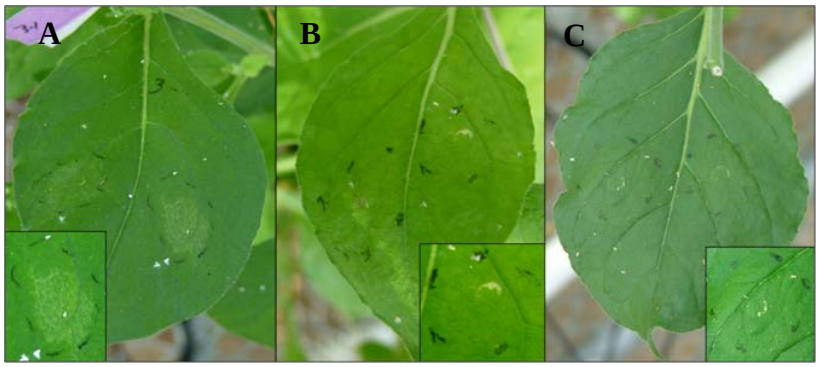

Figure 8. Transient expression of *Xf*PG, pPGIP, and LePGIP in *N. benthamiana* leaves by infiltration with *Agrobacterium* cultures. Chlorotic lesions and water soaking mark the site of agro-infiltrations with *Xf*PG (A). Symptoms are reduced when *Xf*PG is co-infiltrated with pPGIP (B) or with LePGIP *Agrobacterium* (C). Inserts show details of infiltration sites. Black marks indicate the borders of the initial zone infiltrated.

Because our initial assays of PGIP have used *B. cinerea* PG as a standard, we altered our method for evaluating the activity of PGIPs. In our assays, the PGs produced by the B05.10 strain of *B. cinerea* in culture are not inhibited by pPGIP in our *in vitro* assays. Therefore, we have gone back to the Del 11 *B. cinerea* strain and collected the PGs it produces in culture. We have confirmed that they are inhibited by pPGIP in our *in vitro* assay. One explanation for this difference could be that key PG amino acids recognized by pPGIP as part of the inhibitory protein-protein interaction are different in the B05.10 and Del 11 versions of the key PGs, BcPG1and BcPG2, produced by *B. cinerea* in culture. Alternatively, the two strains could express different amounts of the BcPGs. To test the first hypothesis, we worked with Asst. Prof. Dario Cantu (Dept. of Viticulture and Enology, UC Davis) and sequenced the genome of the Del 11 *B. cinerea* strain. The genome of this strain had not been sequenced before. Comparisons of the Del 11, B05.10 and SAS56 (another grapevine strain of *B. cinerea*) are shown in Figure 9. It is clear that there are several amino acid sequence differences between the PG1 enzymes of these *B. cinerea* lines. We plan to do predictive protein modeling to determine whether these changes occur at sites likely to be involved in the PG1 interaction with pPGIP. This work helped us

refine our analysis of key amino acids in the *Xf*PG sequence, which are crucial for inhibition by diverse PGIPs.

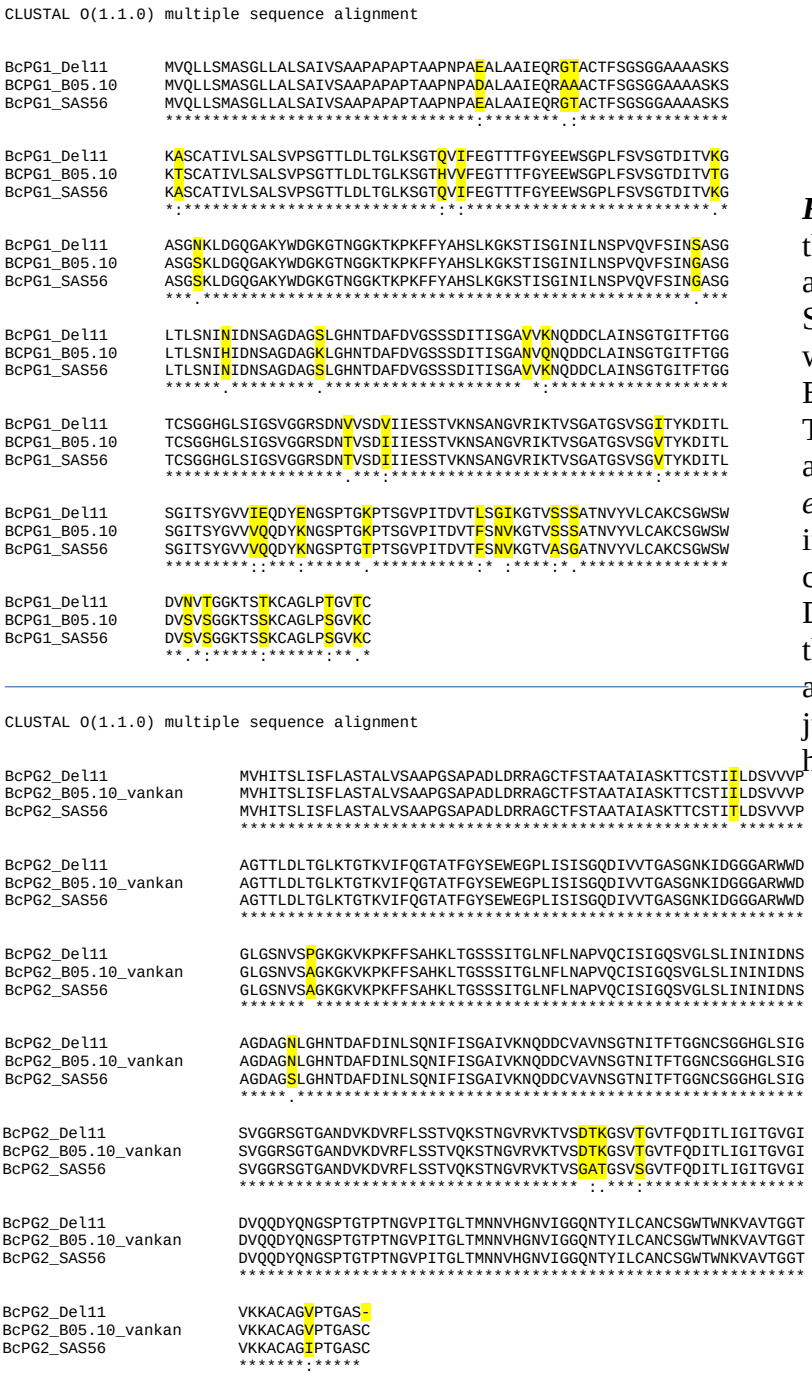

Figure 9. For BcPG1 (top panel), the closest match in Del11 was aligned to BcPG1 from B05.10 and SAS56. BcPG2 (bottom panel) was not correctly annotated in the Broad Institute's B05.10 release. The gene and coding sequence accessions from SAS56 (Wubben *et al.*, 1999) were used to determine intron positions in SAS56. The coding sequences of B05.10 and Del11 were inferred by comparing the genomic sequences with SAS56 and assuming the same intron-exon junctions. Amino acid changes are highlighted in yellow.

D. Expression of PGIPs to test *Xf*PG inhibition

The cloning and expression of candidate PGIPs (Obj. 2B) could be continued. The potential *A. tumefaciens* expression system for *Xf*PG (Obj. 2C) could be used to transiently express and purify active candidate PGIPs. Observations of PGIP activity *in planta* could be made using transgenic model plants for agroinfiltration experiments. The genotypes of tomato and Arabidopsis plants previously transformed to express *pPGIP* or *LePGIP* constitutively were

confirmed by PCR and these could be used to assay *Xf*PG inhibition activity. These pPGIP-expressing plants will be used to test the efficacy of pPGIP constitutively or transiently expressed *in planta*. Agroinfiltration with *Xf*PG-expressing bacterial strains also could be done on the leaves of own-rooted and transgrafted plants.

Objective 3. Maximize PGIP expression in and transport from roots

If we had identified a PGIP to "optimally" inhibit *Xf*PGs, improvements to the expression and delivery of this protein would utilize information being developed in this and other projects.

Objective 4. Create PGIP-expressing rootstocks and evaluate their PD resistance

As discussed previously, the candidate PGIPs would have been assayed *in vitro* for inhibition of *Xf*PG had enough *Xf*PG been available or *in planta* utilizing agroinfiltration and transgrafted tobacco and tomato plants. Grape rootstock transformation could commence once an optimal PGIP has been identified.

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

The PI, coPI and the graduate student working on this project have been asked by the editors of Frontiers in Plant Sciences to submit a manuscript for the special issue on *Plant cell wall in pathogenesis, parasitism and symbiosis*. The authors have agreed to produce a manuscript entitled "Polygalacturonase-Inhibiting Protein Sequence and Structural Analyses to Identify Regions Enabling Inhibition Specificity" by December, 2013. The work from CDFA and GWSS funding for this project will be the subject of this publication.

- **Research relevance statement, indicating how this research contributes towards finding solutions to Pierce's disease in California:**

In response to the strategy recommended by the Advisory Board to enhance the resistance of grapevines to PD, the project uses integrated approaches to optimally express plant genes for particularly effective PGIPs targeting the *X. fastidiosa* PG (*Xf*PG) in transgenic grape rootstocks. To ease the path to commercialization, PIPRA investigators examined relevant intellectual property and regulatory issues associated with the use of this strategy. A narrowed list of PGIPs was selected from national databases of annotated PGIPs in dicot and monocot plants and these PGIPs are being prepared to be expressed in plants and tested for their ability to inhibit *Xf*PG. Homology modeling revealed potential interaction sites that could be useful in predicting inhibition efficiency. Grafts of existing grape lines expressing 'Bartlett' pear PGIP will be tested to determine whether sufficient PGIP is transported from transgenic rootstocks into scions to affect the course of the disease. Eventually new grape rootstock lines will be transformed with the most effective PGIPs with signal and target sequences that maximize (1) PGIP expression in the rootstock and (2) PGIP export to the non-transgenic scions. The goal of the project is to help the California grape industries develop a strategy that uses plant genes to limit the damage caused by *Xf* and to mobilize this technology with non-transgenic vines grafted on the disease-

limiting rootstocks. The project's outcomes should provide growers with plants that resist PD and produce high quality grapes.

- **Layperson summary:**
  Xylella fastidiosa (*Xf*) uses a key enzyme, polygalacturonase (PG), to spread throughout the grapevine from the initial point of inoculation; this spread leads to PD symptom development. Proteins called PG-inhibiting proteins (PGIPs) are produced by many plants and these PGIPs selectively inhibit PGs from bacteria, fungi, and insects. The PGIP expressed in pear fruit is known to inhibit *Xf*PG and limit PD development in inoculated grapevines that have been transformed to express the pear PGIP protein. PGIPs are secreted from cells and can passively travel across graft junctions *via* the plant's water-conducting system. We are interested in identifying the PGIP that best inhibits *Xf*PG and ascertaining how well, when this PGIP is expressed in transgenic rootstocks, it prevents PD development in grafted wild-type *Xf*-inoculated scions. We modeled the protein structures of fourteen candidate PGIPs to predict how the PGIPs physically interact with *Xf*PG and we selected 3 candidate PGIPs. We are using *in vitro* and *in planta* assays to measure the ability of the candidate PGIPs to inhibit *Xf*PG. To do these assays we have had to develop systems to generate high levels of active *Xf*PG and PGIPs. The aim of the project is to identify PGIPs that are most effective in inhibiting *Xf*PG by expressing and testing them first in tobacco and tomato and then evaluating grape rootstock germplasm after grafting, so that we can predict their ability to limit PD development in non-transgenic grape scions.

- **Status of funds:**
  This project was initially funded by the CDFA for a 3-year period (July 1, 2008 through June, 30, 2011). Because of unanticipated difficulties obtaining sufficient *Xf*PG for activity assays and for identifying PGIP clones with sequences identical to those in public databases, initial progress was slow. The project twice was extended through no-cost extensions (July 1, 2011 through June 30, 2012 and then July 1, 2012 through June 30, 2013. As indicated above, work on a few specific aspects of the project will continue. Almost all of the initially budgeted funds have been spent in the budget categories indicated in the initial proposal and the revised budgets for the approved no-cost extensions. The only exception to this is ca. $2,200 from the allocation to Co-PI Dan King (Taylor University). These funds will be returned to CDFA.

- **Summary and status:**
  The ability to compare multiple PGIPs to determine an optimal inhibitor for specific PGs is a key for developing transgenic grape rootstocks as targeted strategies against pathogens that utilize PG(s) for virulence. Towards the goal of enhancing PD resistance, we have determined that PGIPs from 'Bartlett' pear, 'Hamlin' (or as we recently have established 'Valencia') orange, and 'Roma' rice are likely to be very good candidates for *Xf*PG inhibition. By selecting these candidates, we have narrowed considerably the possible PGIPs to pursue. Although we have been able to express (and extract from agro-infected leaves) *Xf*PG in tobacco and have shown that this source of *Xf*PG is active, sufficient and reliable sources of *Xf*PG continue to be a problem plaguing us and other groups. We have detected pPGIP protein crossing the graft junctions from transgenic rootstocks to non-transgenic scion leaves in grafted grape and tomato

plants in this project. In the course of doing this work, we have had to refine our inhibition assay protocol and have therefore identified sequence differences in two strains of *B. cinerea*. The information about the sequence differences in the *Bc*PGs from different strains of *B. cinerea* will help us to identify portions of the *Xf*PG that are important targets of PGIPs. The ability of pPGIP, one of the candidates investigated in this proposal, to provide PD resistance to transgrafted scions is being addressed by the corresponding field trial.

We are advancing towards our goal to develop transgenic grape rootstocks that express PGIPs that effectively reduce the virulence of *Xf*, an approach that will help manage the PD problem without targeting the growing insect vector population. The project is designed to identify specific PGIPs that target the virulence factor, *Xf*PG, and to express them in rootstocks to provide protection to the grafted wild-type scion tissues. To achieve this goal, we have had to overcome some information and technical difficulties in this complex system. Furthermore, because several other pathogens of grapes (both vines and fruit) utilize PG as a part of their tissue infection strategies, it is reasonable to presume that the strategy examined here for PD management may have additional beneficial impacts in the vineyard.

- **Literature cited**

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