Progress report for CDFA contract number 08-0171

Project title: Optimizing grape rootstock production and export of inhibitors of *X. fastidiosa* PG activity

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Time period covered by the report: October, 2009 through February, 2010

List of objectives and description of activities conducted to accomplish each objective (Abbreviations used in this report are: **PD**, Pierce's disease; **PG**, polygalacturonase; **PGIP**, PG-inhibiting protein; ***Xf***, *Xylella fastidiosa*)

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 *X. fastidiosa* PG.

A. Use existing pear PGIP-expressing grapes, test PD susceptibility of normal scions grafted to PGIP-expressing and –exporting roots (Years 1 and 2)
B. Identify plant PGIPs that are efficient inhibitors of *Xf*PG and model the PGIP-PG interactions for optimal PGIP prediction (Years 1 and 2)
C. Optimally express *Xf*PG, using recombinant protein expression systems (Year 1)
D. Express PGIPs in *Arabidopsis thaliana* and *Nicotiana* sp. to test for optimal inhibition of *Xf*PG (Years 1 and 2)

Objective 3: Assemble transcription regulatory elements, *Xf*-inducible promoters and signal sequences that maximize PGIP expression in and transport from roots.

A. Generate transformed grape lines using the best PGIP candidates, promoters, etc. (Years 2 and 3)

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

A. Molecular analysis of putative marker free transgenic grape plants (Year 3)
B. Evaluate transgenic grape lines for optimal expression and export to scions of selected PGIPs (Year 3)
C. Evaluate transgenic lines for susceptibility to *X. fastidiosa*

Summary of major research accomplishments and results for each objective

Objective 1: Define a path for commercialization of a PD control strategy

Current work towards the objective of commercialization of transgenic rootstocks for PD control has been focused on gathering information and preparing for upcoming field trials. These trials will test the PD resistance of 'Thompson Seedless' and 'Chardonnay' transgenic grapevines expressing the 'Bartlett' pear fruit PGIP (PcBPGIP) gene (Agüero et al., 2005) as own-rooted and grafted plants in field settings under natural and manufactured PD pressure. A proposal for the field trial was submitted in January, 2010 to the PD/GWSS Board under the title "Field evaluations of grafted grape lines expressing polygalacturonase inhibiting proteins (PGIPs)," PI: Ann Powell. Since submission, propagation efforts (see below) have begun to increase the total plant number and establish the proper graft combinations for planting in the field sites.

Objective 2: Identify plant PGIPs that maximally inhibit *X. fastidiosa* PG

A. Propagation and grafting of existing grape lines expressing and exporting pear PGIP

The transgenic grapevine cultivars 'Thompson Seedless' and 'Chardonnay' expressing the PcBPGIP are being maintained in the UC Davis Core Greenhouse Complex. After dealing with the Panicle Rice Mite quarantine procedures described in prior reports, the experimental grapevines were moved out of isolation in November, 2009 and into facilities suitable for allowing work to commence. The plant numbers have been increased for grafting experiments by rooting vegetative cuttings of both cultivars in an aeroponic cloner (EZ-Clone, Inc., Sacramento, CA). Cuttings of green tissue, three nodes in length, were basally dipped in 1000 ppm IBA solution for five minutes and transferred to individual sites in the cloning box. Apical regions of the cuttings received continuous light while basal nodes received constant water misting in darkness. Roots began to form after 8 days and cuttings were transferred to soil after 17 days (Figure 1). Grafting experiments have begun in order to determine the extent of PD resistance imparted on wild-type, *Xf*-inoculated scions by PcBPGIP-producing rootstocks. Eight trial grafts have been attempted. The remaining work related to this objective will be completed after an appropriate number of grafted vines have been established.

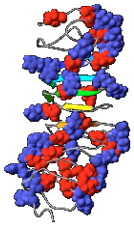
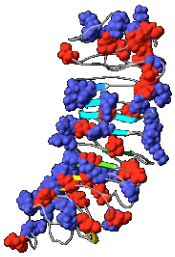
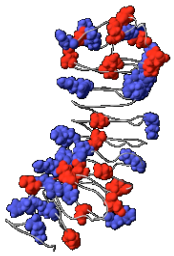
Figure 1. Grapevine cuttings rooting in the EZ-Clone aeroponic cloner.

PcBPGIP, exported from transgenic rootstocks, will be detected in otherwise wild-type scion tissue by ELISA. These assays are necessary for confirming the passage of the PGIP protein in the xylem sap across the graft union in this project and the aforementioned field trials.

A proposal to generate monoclonal antibodies recognizing PcBPGIP and antibodies recognizing the optimal PGIP for *Xf*PG inhibition – as determined by this project – was submitted in January, 2010 to the PD/GWSS Board under the title "Tools to identify PGIPs transmission from grapevine rootstock to scion," PI: Ann Powell. The current stocks of polyclonal antibodies recognizing PcBPGIP are limited in quantity and will be inadequate for the eventual testing of hundreds of grapevines, expressing either PcBPGIP or an optimized PGIP selected from several candidates (work in progress, below).

B. Selection of PGIPs as PD defense candidates and PGIP-*Xf*PG modeling

The 14 candidate PGIPs were previously selected for *in vitro* and *in vivo* *Xf*PG inhibition assays based on predicted protein charge and phylogenetic analyses (Labavitch, 2009). Homology models of all 14 PGIPs and *Xf*PG were created to visualize the potential interactions between each PGIP and *Xf*PG and to attempt to predict residues or regions crucial to *Xf*PG inhibition (Labavitch, 2009). The predicted models are shown in Table 1. Dynamic reaction simulations determined that two clusters of amino acids, 63-74 and 223-226, must be unblocked for *Xf*PG to cleave a polygalacturonic acid substrate in its active cleft. Long columns of electronegative residues on the concave faces of PGIPs may align with these *Xf*PG residues and prevent cleavage. Pear PGIP, orange PGIP, and rice PGIP1 have the most favorable alignments to these *Xf*PG residues. Other specific residues may also influence PG-PGIP binding. Strong hydrogen bonding occurs between the pear PGIP and Tyr303 of *Xf*PG, bringing them together in a potentially inhibitory manner (Figure 2), whereas electrostatic repulsions between grape PGIP and *Xf*PG (Table 1) prevent a similar alignment and subsequent interaction. Combining the modeling predictions and future inhibition data will allow us to score the result of predicted interactions and infer other potentially useful interactions between the PGIPs modeled and other PGs.

 AtPGIP1	 AtPGIP2	 BnPGIP1	 CaPGIP
 CsiPGIP	 FaPGIP	 OsPGIP1	 OsPGIP2

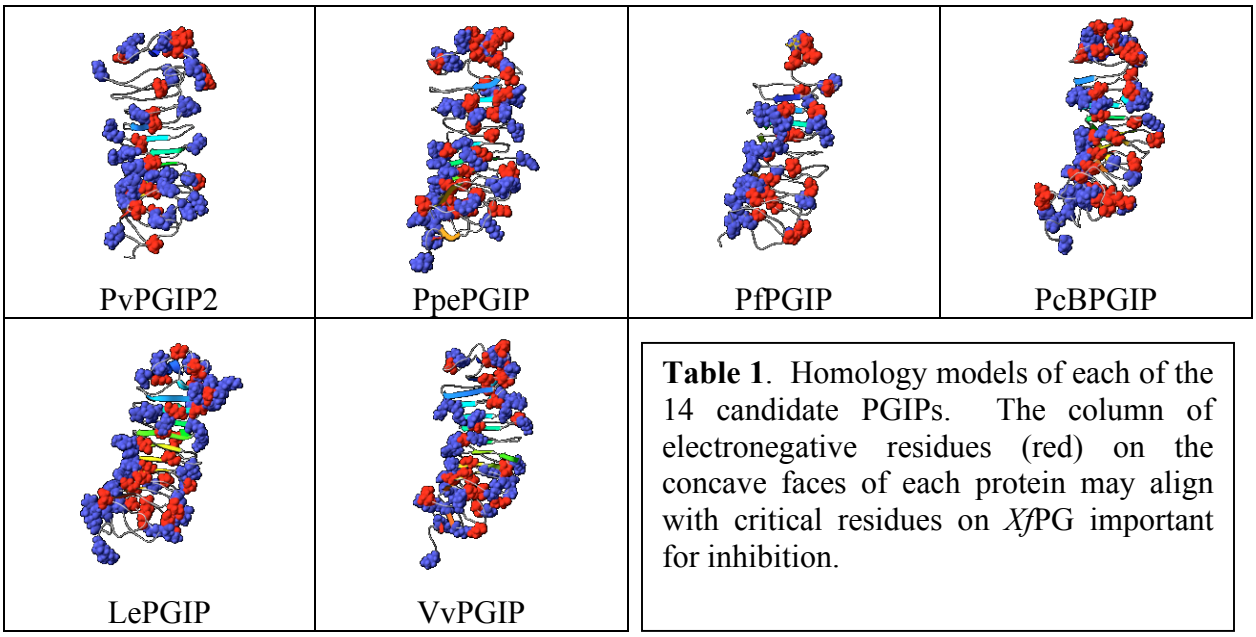


Table 1. Homology models of each of the 14 candidate PGIPs. The column of electronegative residues (red) on the concave faces of each protein may align with critical residues on *Xf*PG important for inhibition.

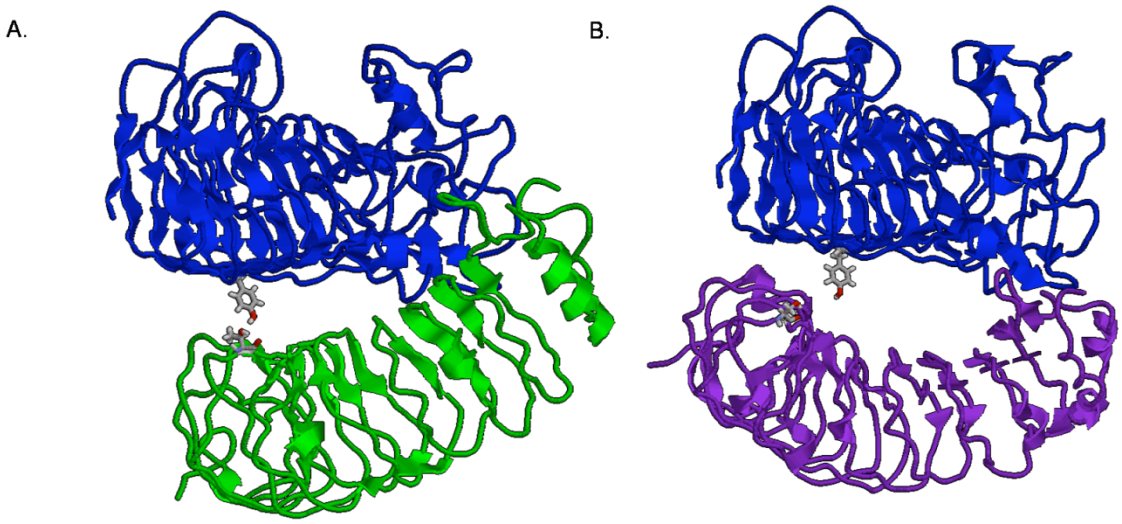


Figure 2. PG-PGIP complexes. Tyr303 of *Xf*PG (blue) binds strongly with a region of PcBPGIP (A, green) which is not possible with VvPGIP (B, purple). Interactions such as this one might influence PG-PGIP interaction and inhibition.

C. *Xf*PG cloning and expression

The *Xf*PG protein expression system is being further optimized to generate large quantities of stable protein for *in vitro* inhibition assays. Previously, the *Xf*PG gene was subcloned into pMT/BiP/V5-HisA for expression in Drosophila S2 cells and transient transfections resulted in a 70 kDa protein secreted into the media (Labavitch, 2009). Fractions of the cellular media were tested for PG activity using a radial diffusion assay (Taylor and Secor, 1988). The media from transiently transfected cells induced to express *Xf*PG shows a small clearing zone around the site of inoculation, indicating some PG activity (Figure 3). The *Xf*PG

produced by the Drosophila cells is tagged with the V5 epitope for Western blotting and a histidine tail for nickel affinity purification. Expression of PG was induced in transiently transfected cells and media collected 3-5 days after induction was purified using nickel affinity agarose resin columns. *Xf*PG containing media was washed with 50mM phosphate buffer (pH 8) and eluted from the column by adding 250mM imidazole to the wash buffer. A protein of the expected size was collected and verified by Western blot (Figure 4). Current work is focused on generating stably transfected Drosophila cell lines that will express and secrete a larger quantity of *Xf*PG.

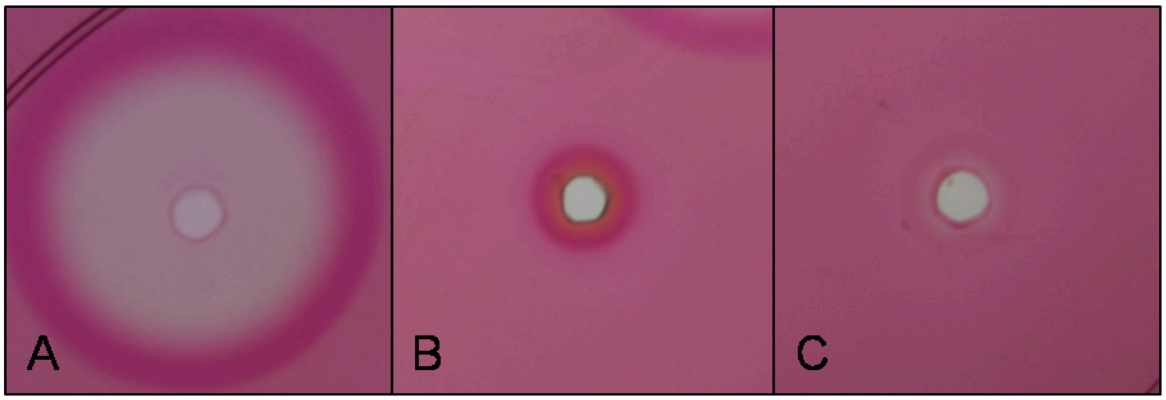


Figure 3. Radial diffusion assay of *Xf*PG containing culture media. The clearing zone diameter is related to polygalacturonase activity, degrading the polygalacturonic acid substrate. (A) Concentrated *Botrytis cinerea* PG; (B) culture media from GFP transfected cells; (C) culture media from *Xf*PG transfected cells. The red rings outlining the clearing zone are indicative of esterase activity, not polygalacturonase activity.

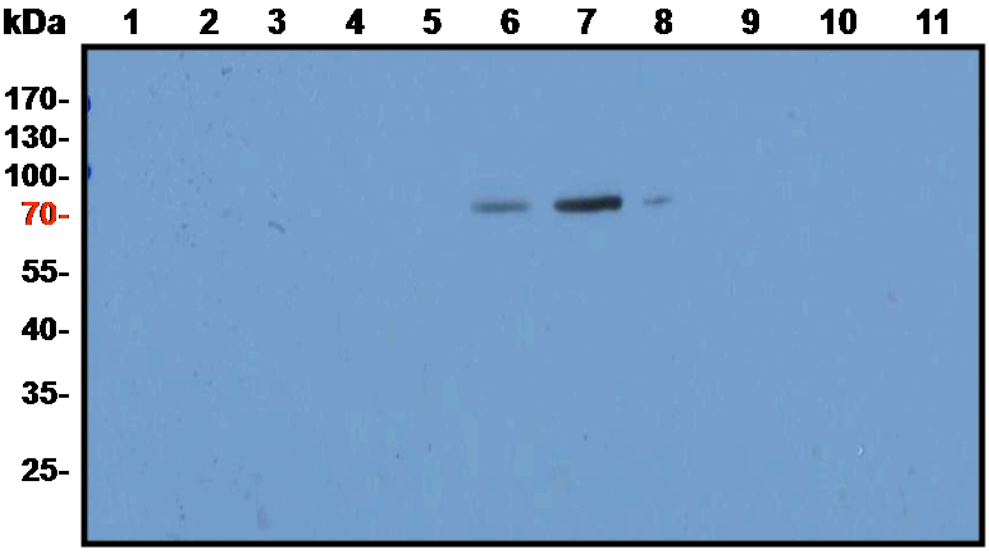


Figure 4. Western blot of nickel column purified *Xf*PG protein from transiently transfected Drosophila cells. Lanes 1-3 = initial flow through; lanes 4-5 = wash buffer; lanes 6-8 = elution buffer; lanes 9-11 = column washed with 500mM imidazole.

D. Expression of PGIPs in Arabidopsis and *Nicotiana* sp. for *Xf*PG inhibition assays

Current work cloning the candidate PGIPs from their host plants is underway (Table 2). The previously reported strategies for cloning and the inhibition assays (Labavitch, 2008; Labavitch 2009) are being used to generate *Agrobacterium tumefaciens* cultures harboring each of the PGIPs and the two *Xf*PG constructs for use in transforming *Arabidopsis thaliana* (Col.) and for transient expression in *Nicotiana tabacum* and *Nicotiana benthamiana*. These plants are being maintained in growth chambers and at the UC Davis Core Greenhouse Complex under standard conditions in anticipation of transformation.

Protein (Organism)	**Cloning Progress Checkpoints**						
	Source tissue acquired	cDNA isolated	Construct into TOPO expression vector	Transformed into *E. coli*	Construct into pCAMBIA-1301 transformation vector	Transformed into *A. tumefaciens*	Ready for plant transformation
AtPGIP1 (Arabidopsis)	X	X	X	X	X	O	-
AtPGIP2 (Arabidopsis)	X	X	X	X	X	O	-
BnPGIP1 (Rapeseed)	X	X	O	-	-	-	-
CaPGIP (Pepper)	X	O	-	-	-	-	-
CsiPGIP (Orange)	X	O	-	-	-	-	-
FaPGIP (Strawberry)	X	X	O	-	-	-	-
OsPGIP1 (Rice)	X	X	O	-	-	-	-
OsPGIP2 (Rice)	X	X	O	-	-	-	-
PvPGIP2 (Bean)	X	O	-	-	-	-	-
PpePGIP (Peach)	O	-	-	-	-	-	-
PfPGIP (Firethorn)	X	O	-	-	-	-	-
PcBPGIP (Pear)	X	X	X	X	X	X	O
LePGIP (Tomato)	X	X	X	X	X	X	O
VvPGIP (Grape)	O	-	-	-	-	-	-
*Xf*PG (Xylella)	X	X	X	X	O	-	-
PcBPGIP::*Xf*PG	X	X	X	X	O	-	-

Table 2. Cloning progress chart. "X" = completed checkpoint; "O" = work in progress; "-" = checkpoint to be completed.

Objective 3: Assemble transcription regulatory elements, *Xf*-inducible promoters and signal sequences that maximize PGIP expression in and transport from roots

No work has been completed this period.

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

No work has been completed this period.

Intellectual property issues associated with the project

No known intellectual property issues exist and no new intellectual property has been generated from this work.

Publications or reports resulting from the project

No new publications.

Research relevance statement

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. Homology models of each candidate PGIP were constructed to identify possible regions of interaction between the PGIP and *Xf*PG. Simulated interactions will be compared to inhibition assays to verify these integral regions. *Xf*PG will be produced using an optimized protein expression system with Drosophila cells. Each candidate PGIP will be screened for effectiveness to inhibit *Xf*PG *in vitro* and *in vivo*. 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. At the conclusion of the project, the capacity of the non-transgenic vines grafted on the transgenic rootstock to resist PD and produce high quality grapes will be tested.

References

Agüero CB, Uratsu SL, Greve LC, Powell ALT, Labavitch JM, Meredith CP, Dandekar AM. 2005. Evaluation of tolerance to Pierce's Disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing pear PGIP gene. Mol Plant Pathology 6: 43-51.

Labavitch JM. 2008. Optimizing grape rootstock production and export of inhibitors of *Xylella fastidiosa* polygalacturonase activity. Proceedings of the 2008 Pierce's Disease Symposium, p. 214-219.

Labavitch JM. 2009. Optimizing grape rootstock production and export of inhibitors of *Xylella fastidiosa* polygalacturonase activity. Proceedings of the 2009 Pierce's Disease Symposium, p. 167-173.

Taylor RJ, Secor GA. 1988. An improved diffusion assay for quantifying the polygalacturonase content of *Erwinia* culture filtrates. Phytopathology 78: 1101-1103.