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: March, 2010 through July, 2010

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

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
B. Identify plant PGIPs that are efficient inhibitors of XfPG and model the PGIP-PG interactions for optimal PGIP prediction
C. Optimally express XfPG, using recombinant protein expression systems
D. Express PGIPs in *Arabidopsis thaliana* and *Nicotiana* sp. to test for optimal inhibition of XfPG

Objective 2: Identify plant PGIPs that maximally inhibit XfPG

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

The transgenic grapevine cultivars 'Thompson Seedless' and 'Chardonnay' expressing the 'Bartlett' pear fruit PGIP (PcBPGIP) gene are being maintained in the UC Davis Core Greenhouse Complex. Grafting efforts are underway to generate plants that are to be used to verify the transport of PcBPGIP protein from the transgenic rootstock, across the graft junction, into scion tissue not expressing any foreign PGIP. Six 'Thompson Seedless' grafts and one 'Chardonnay' graft have been formed by a modified wedge grafting technique where a semi-lignified non-PcBPGIP expressing scion piece of 1 to 2 nodes is stripped of foliage, cut into a wedge, and fitted into a notched stem of the same diameter on one of two main branches of the established rootstock expressing PcBPGIP. The graft union is covered with Parafilm M, secured by a clothespin, and the entire scion piece is covered loosely by a translucent bag to prevent desiccation. This has been a major area of experimentation as the grafts are sensitive to several external factors and are prone to failure. We are testing alternative grafting techniques, such as chip budding and saddle grafts, with some success. The grafted vines generated from the PcBPGIP-expressing rootstock lines are also being used in the related field trial, funded by the CDFA PD/GWSS Board under the title "Field Evaluations of Grafted Grape Lines Expressing PGIPs," PI: Powell.

The grafted plants will be used to verify the movement of PcBPGIP across the graft junction and the protein's activity in inhibiting XfPG, both *in vitro* and *in planta*. As stated before, PcBPGIP will be detected in xylem sap collected from the scion tissue by Western blotting and ELISA. XfPG inhibition *in vitro* will be tested using recombinant PG (see Objective 2C) mixed with scion xylem sap, using our routine radial diffusion assay. *In planta* Xf inhibition will be scored by PD symptom development in inoculated, grafted vines. The proposal titled "Tools to Identify PGIPs Transmitted across Grapevine Grafts," PI: Powell, was accepted by the CDFA PD/GWSS Board. This grant will support the generation of a monoclonal antibody necessary for recognizing the PcBPGIP protein in the ELISA assays both for this project and for the aforementioned field trial as current polyclonal antibody stocks are limited.

Transgenic tomato plants expressing PcBPGIP were previously generated in the lab for another research project. To further the proof-of-concept stage and verify that transgenic PcBPGIP crosses a graft junction, these tomato plants have been grafted in the same manner as the experimental grapevines. Total protein was extracted from macerated scion and rootstock leaves and separated by SDS-PAGE. The resulting blot was cross-reacted with the polyclonal PcBPGIP antibody and a secondary antibody conjugated with horseradish peroxidase. The blot was developed to show that PcBPGIP is present in the scion tissue of own-rooted transgenic plants and wild-type scions that have been grafted to transgenic rootstocks (Fig. 1). The PcBPGIP protein is presumably present in the xylem of the macerated leaf tissue and would be more concentrated if the xylem sap were isolated and probed as planned for the grafted grapevines. The faint banding seen in the wild-type control scion (Fig. 1, Lane 3) is due to spillover from loading scion lane 2. This experiment confirms that, at least in tomato, PcBPGIP crosses the graft junction in a unidirectional manner and can be found in wild-type scion tissue that has been grafted onto a transgenic rootstock.

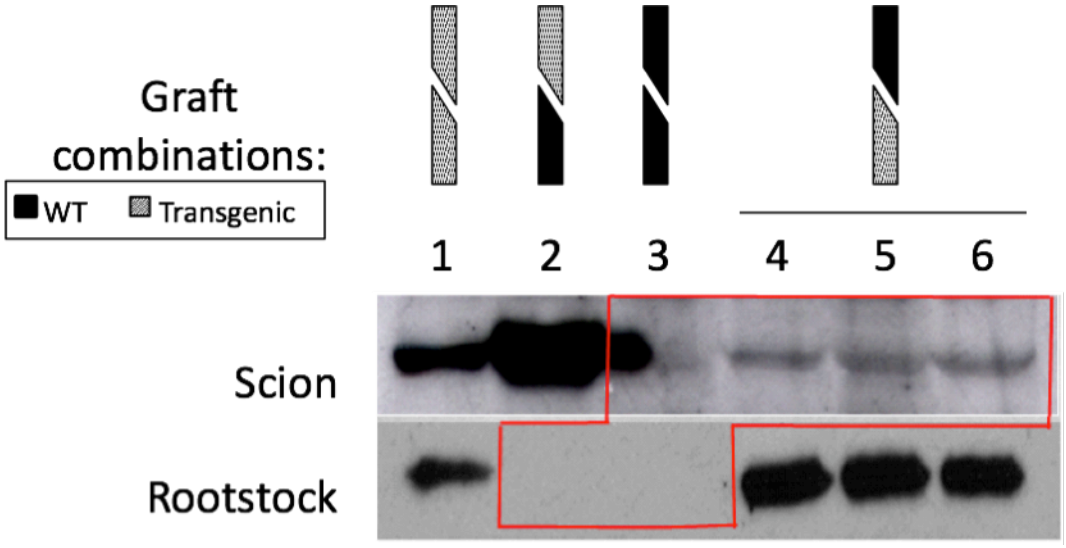

Figure 1. Western blot of PcBPGIP extracted from tomato leaves positioned on either scion or rootstock regions. PcBPGIP protein is seen in non-transgenic scion leaf xylem only when scions were grafted to transgenic rootstocks (lanes 4-6). Samples boxed in red are concentrated 30x to visualize cross-reactive bands.

B. Selection of PGIPs as PD defense candidates and PGIP-XfPG modeling

The homology models of the 14 candidate PGIPs reported in the last update provide unique predictive tools to interpret the inhibition mechanisms at work in the physical interactions of XfPG and several plant PGIPs. The selected PGIP candidates represent a broad array of sequence and species diversity. In addition to the 68 PGIP-like sequences included in the previous phylogenetic and protein charge analyses, we have reinitiated contact with the research group in Stellenbosch University, South Africa, for studies of the previously unpublished PGIP sequences from non-vinifera *Vitis* varieties. Discussions began in June to gain access to these sequences, as they are the property of an industry panel associated with the Institute for Wine Biotechnology at Stellenbosch University in South Africa and we are expecting a response in August or September. Many examples exist of PD-resistant non-vinifera species and while the PD susceptibility of these lines in question remains unknown for now, homology modeling will highlight any structural features which may influence PG binding by the non-vinifera PGIPs. How these differ from the already modeled VvPGIP from *Vitis vinifera* cv. 'Pinotage' is also of interest. We are currently waiting for access to the PGIP sequences before the modeling, inhibition profiling, and phylogenetic analysis can begin.

C. XfPG Expression Analysis

The XfPG expression system in Drosophila S2 cells is being optimized to yield large amounts of active protein for *in vitro* inhibition analyses with the candidate PGIPs. XfPG from transient transfections was purified from both pelletted Drosophila cell lysate and supernatant on an immobilized nickel affinity column using the C-terminal poly-His tag on the recombinant protein. The resuspended cell lysate was loaded onto the gravity flow column and eluted with elution buffer 1 (EB, 50 mM sodium phosphate, 0.3 M sodium chloride, 250 mM imidazole). The eluant was analyzed by Western blotting, utilizing the C-terminal V5 epitope tag (Fig. 2). The 78 kDa purification product is a slightly larger size than expected for XfPG, but the tagged,

expressed protein was shown primarily in the cell lysate fraction and faintly in the probed cellular medium. Seeing XfPG present in the cellular pellet, the supernatant fractions were purified and re-analyzed for any recombinant XfPG. After nickel affinity column purification using EB1, the supernatant eluant was analyzed by Coomassie staining SDS-PAGE (Fig. 3). Proposed XfPG bands were visualized at approximately 68 kDa in all three supernatant elution fractions.

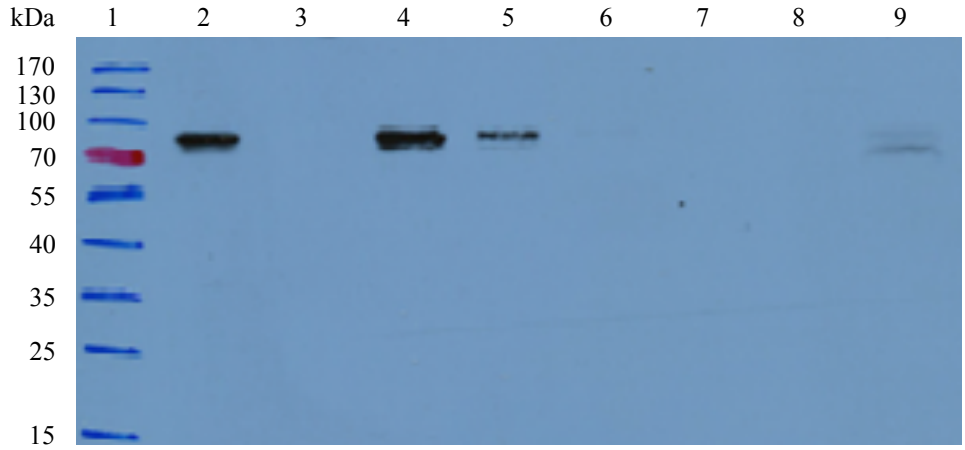

Figure 2. Western blot analysis of partially purified cell lysate after XfPG protein expression. 15.0 mL crude XfPG lysate was purified by column chromatography and selected samples were analyzed with Western blotting. Lane 1 = Prestained Page Ruler, lane 2 = XfPG flow-through #4, lane 3 = XfPG wash #10, lane 4 = XfPG elution fraction 1, lane 5 = XfPG elution fraction 2, lane 6 = XfPG elution fraction 3, lane 7 = XfPG elution fraction 4, lanes 8 and 9 = cellular medium. XfPG protein was probed with the anti-V5 primary antibody and anti-mouse HRP secondary antibody.

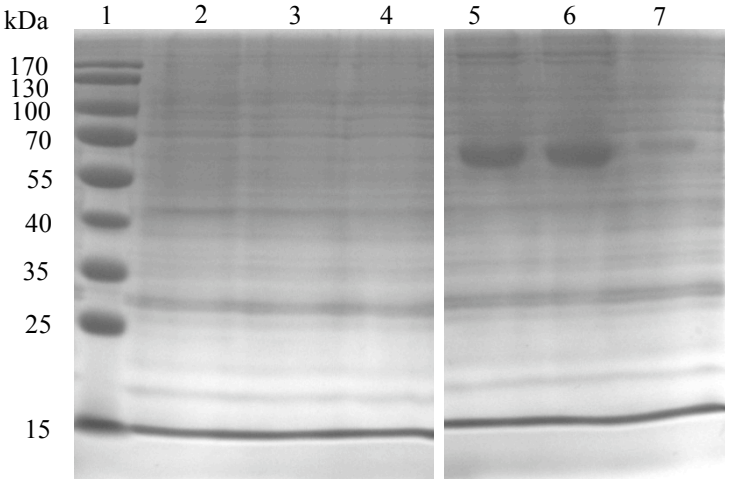

Figure 3. Partially purified XfPG protein eluted with 250 mM imidazole. Coomassie stained polyacrylamide gel electrophoresis. Lanes 1 = pre-stained ladder, lanes 2-4 = purified pellet elution fractions 1-3, lanes 5-7 = purified supernatant elution fractions 1-3.

PG activity was measured by reducing sugar analysis whereby the PG-catalyzed release of free galacturonic acid residues (both oligomeric products and monosaccharides) from a polygalacturonic acid substrate provide a reducing agent for 2-cyanoacetamide, yielding an increase in absorbance at A_{276} as PG activity increases (Gross, 1982). Cell lysate elution fraction 2 and supernatant fractions 2 and 3 showed minimal to moderate activity, at one-third the activity of the control PG from *Aspergillus niger*. These XfPG activities diminished over time of protein storage.

Recombinant XfPG activity and stability have been recurring issues since the original cloning and expression work in *Escherichia coli* (Roper *et al.*, 2007). Current work is focused on generating stably transfected recombinant cell lines that should provide larger, more consistent stocks of active XfPG. The methods for purifying and storing the protein are also being altered to minimize possible denaturing pH, buffer, and temperature conditions.

D. Expression of PGIPs in Arabidopsis and Tobacco for XfPG assays

The previous work of cloning the 14 candidate PGIPs from the different plant species into the appropriate sequencing and transformation vectors continues (Table 1). The full-length XfPG construct was successfully cloned into pCAMBIA-1301 and transformed into *Agrobacterium tumefaceans* (EHA105 pCH32). This construct, in addition to the PcBPGIP::XfPG fusion cloning still in progress, provides a potential diagnostic tool to test the efficacy of each PGIP *in planta*.

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

Table 1. Cloning progress chart. Checkmarks indicate completed checkpoints while circles indicate work in progress.

Co-infiltration of Agrobacterium cultures harboring XfPG and full-length PcBPGIP in pCAMBIA-1301 was carried out as described in Joubert *et al.* (2007). Fully formed leaves of *Nicotiana benthamiana* and *Nicotiana tabacum* were infiltrated with constant manual pressure using a needle-less syringe, this forcing bacterial cultures into the abaxial leaf tissues. Initial infiltration zones measured approximately 35 mm^2. Plants were maintained in the UC Davis Core Greenhouse Complex under partial shade at 25°C, 60% relative humidity. Visual symptom development was scored 24 hours post infiltration (hpi, Fig. 4). Infiltration with Agrobacterium cultures containing the XfPG construct resulted in a phenotype that included marked wilting and localized water soaking. Leaves infiltrated with both PcBPGIP and XfPG in overlapping zones displayed less wilting and fewer soaked lesions. Leaves infiltrated with PcBPGIP alone did not show any wilting. *N. benthamiana* leaves showed a clearer response to infiltration with XfPG than did *N. tabacum*. Further work is being done to optimize the infiltration protocol and quantify the results in a way that usefully reflects the inhibition of XfPG by different PGIPs. We anticipate that the fusion construct PcBPGIP::XfPG will yield more easily scored results, both alone and in the presence of a PGIP, than the current construct with the native XfPG signal sequence. The PcBPGIP signal sequence will target the translated XfPG protein to the plant cell apoplastic space where it can either degrade the pectin-rich middle lamellae and cell walls (creating larger lesions than the native XfPG) or be inhibited by the co-infiltrated transgenic PGIP, naturally targeted to the apoplast.

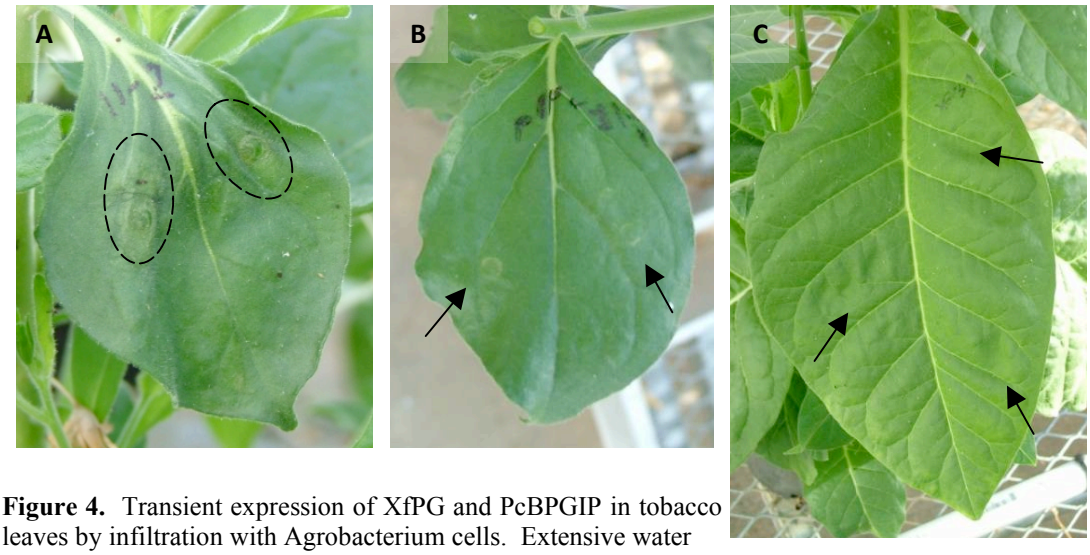

Figure 4. Transient expression of XfPG and PcBPGIP in tobacco leaves by infiltration with Agrobacterium cells. Extensive water soaking and necrotic lesions marked the site of agro-infiltrations with XfPG constructs in *N. benthamiana* (A) but similar symptoms were limited when co-infiltrated with cultures carrying PcBPGIP only (B). Water soaking in infiltrated *N. tabacum* leaves is restricted to areas surrounding the infiltration zone (C). Arrows, when present, mark sites of infiltration. All pictures taken 24 hpi.

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

Browning, JL. 2010. Save the wine: Expression and partial purification of *Xylella fastidiosa* polygalacturonase. Masters Thesis, Texas State University, San Marcos. 53p.

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 (XfPG) in transgenic grape rootstocks. Transgenic pear fruit PGIP has been shown to cross a graft junction into non-transformed scion tissue in one plant system. XfPG will be produced using an optimized protein expression system with Drosophila cells. Each candidate PGIP will be screened for effectiveness to inhibit XfPG *in vitro* and *in vivo*. Initial *in vivo* screening of the pear PGIP suggests it is able to transiently inhibit XfPG in infiltrated tobacco leaves. 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

Gross KC. 1982. A rapid and sensitive spectrophotometric method for assaying polygalacturonase using 2-cyanoacetamide. HortScience. 17(6):933-934.

Joubert DA, Kars I, Wagemakers L, Bergmann C, Kemp G, Vivier MA, van Kan JAL. 2007. A polygalacturonase-inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from *Botrytis cinerea* in *Nicotiana benthamiana* leaves without any evidence for in vitro interaction. Mol Plant Microbe In. 20(4):392-402.

Roper MC, Greve LC, Warren JG, Labavitch JM, Kirkpatrick BC. *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in *Vitis vinifera* grapevines. Mol Plant Microbe In. 20(4):411-419.