Progress report for CDFA contract number 08-0171

UC Pierce's Disease Research Grants Program
CDFA PD/GWSS Board

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

II. Principal investigators and cooperators: ***Project Leader*****:** John Labavitch; ***Cooperators*****:** Ann LT Powell, Alan Bennett, Daniel King, Rachell Booth

III. List of objectives and description of activities conducted to accomplish each objective

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.

- **A.** Evaluate IP and licensing status of the plant expression construct components for the PGIP-based rootstock strategy (Year 1)
- **B.** Assemble grape transformation vectors utilizing PIPRA vectors with defined IP characteristics (Year 2)

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 (Year 1)
- **C.** Express PGIPs in *Arabidopsis thaliana* and test for optimal inhibition of *X. fastidiosa* PG (Years 1 and 2)
- **D.** Optimally express *X. fastidiosa* PG, using recombinant protein expression systems (Year 1)
- **E.** Model PGIP and *X. fastidiosa* PG interactions to identify optimal PGIPs for PD defense (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.** Make 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* (Year 3)

IV. Summary of major research accomplishments and results for each objective

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

PIPRA is evaluating the Intellectual Property (IP) around each of fourteen candidate genes (see Objective 2B). PIPRA is utilizing protein based queries to search the patent and patent application databases using the program GenomeQuest. The IP information will be used to determine if there is IP related to these particular genes. Additional information regarding other activities that relate to this objective are available in report #06-0224, "Enabling technologies for grape transformation", PI: Alan Bennett.

Objective 2: Identify plant PGIPs that maximally inhibit *Xf*PG

A. Propagation and generation of existing lines expressing pear PGIP

Transformed grape cultivars Thompson Seedless and Chardonnay expressing pear fruit PGIP (Agüero et al., 2005) have been maintained in our greenhouse facilities. PCR analysis verified the presence of the pear PGIP in all the transformed lines. Vegetative cuttings are being propagated to increase the total plant number for future experiments. The current propagation efforts have yielded 66% efficiency and we expect this to increase as Winter progresses into Spring. Grafting experiments will begin to test the effect of the pear fruit PGIP transgene exported from the rootstock into a non-transformed scion on Pierce's Disease in the coming months. Our grafting experiments rely on successful propagations and are being prepared now with the first round of newly cut transgenic rootstocks and existing non-transformed scions of the same cultivar.

B. Selection of PGIPs

An extensive search of public databases returned 68 proteins with sequence similarity to known and characterized PGIPs. These PGIPs represent diverse plant families, expression patterns, and known PG inhibition activities. To further analyze the relationships among them, 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 (Figure 1) is more robust than its predecessor (Labavitch, 2008) due to the inclusion of both monocot and dicot sequences. This tree is the largest comparison of PGIP sequences from several plant families and stems from a continuing interest in plant-pathogen interactions at the protein level which has provided a large array of sequence information.

Fourteen candidate PGIPs (Figure 1, Table 1) have been selected for transformation into *Arabidopsis* and subsequent characterization of their ability to inhibit *Xf*PG, *in vitro*. The candidate PGIPs represent the major clades of the phylogenetic tree, implying a wide variation in amino acid sequence. Plant PGIPs are typically characterized by 10 leucine rich repeats (LRR) in the region thought to influence binding to PGs. The majority of the candidates have known fungal PG inhibition specificities but lack bacterial PG inhibition data. The candidates OsPGIP1 and OsPGIP2 share 60% sequence homology but OsPGIP1 is lacking the seventh LRR (Janni et al., 2006) so it will be interesting to analyze its activity against *Xf*PG compared to the complete OsPGIP2. Other large PGIP families are well represented by the candidate selections. *Brassica napus* has a 16 member PGIP family, only four are included in this analysis, but the genes all cluster to either *BnPgip1* or *BnPgip2* with the BnPGIP2 family being close enough to AtPGIP1

to give only slight sequence variation (Hegedus et al., 2008). The common bean PvPGIP2 is the only PGIP with a solved crystal structure and serves as a good representative of the soybean PGIPs with 82% similarity to GmPGIP3, the protein responsible for all fungal PG inhibitions observed in soybean (D'Ovidio et al., 2006).

The 14 candidate PGIPs also were chosen based on their total protein charge as determined by the amino acid sequence at a given pH. The charge was calculated for all 68 PGIPs but the candidates' lower charges, we believe, will be important in avoiding disruption of any potential interactions between the PGIP and the highly charged *Xf*PG. The large positive charge on AtPGIP2 and the minimal charge on OsPGIP2 will bracket the candidate PGIPs and provide correlative measurements between *Xf*PG inhibition and total PGIP charge.

The multiple sequence alignment suggests that strong relationships exist among the specific signal sequences of PGIPs belonging to different clades. Current work involves analyzing the 68 PGIPs for signal sequence identity and comparing the phylogeny of PGIP signal sequences to the current tree. An efficient apoplastic targeting sequence is critical to the export of the optimum PGIP from a grape rootstock. A new multiple sequence alignment algorithm is also being developed to accurately identify and show preference for the characteristic LRR structure of PGIPs.

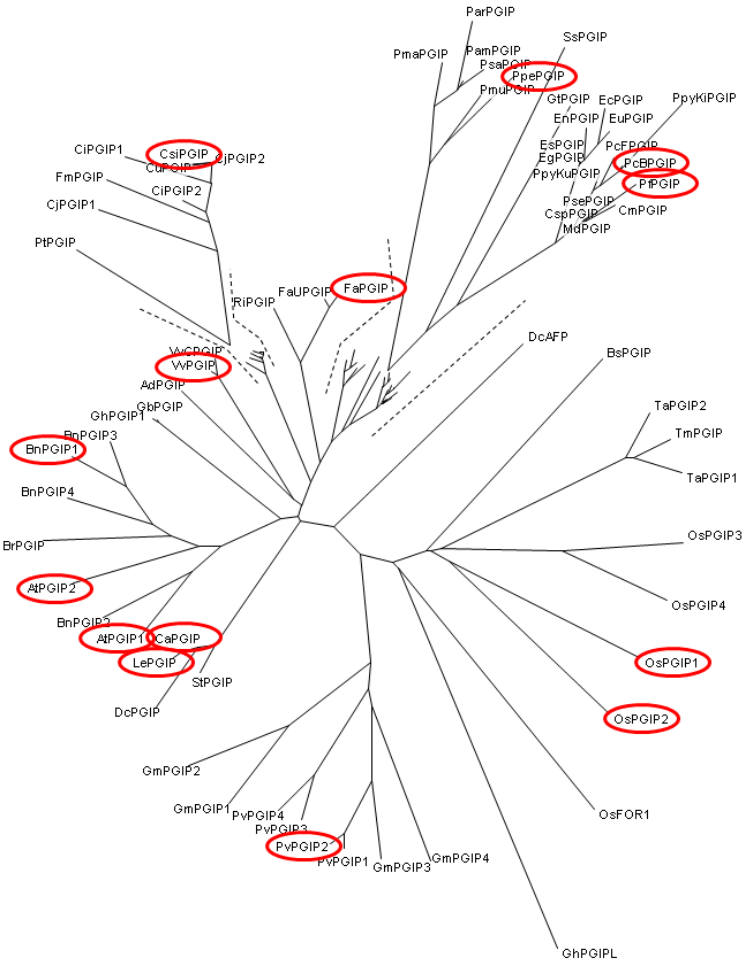

Figure 1. Unrooted phylogenetic tree of PGIPs. The 14 candidate PGIPs for *Arabidopsis* transformation are circled in red. The protein names and organisms are given in Labavitch, 2008.

Common name	Organism	Protein	Charge of Protein (at certain pH)					
			3.5	*4.0*	***4.5***	*5.0*	*5.5*	*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	PcBPGIP	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 1. Total protein charge analysis for the 14 candidate PGIPs in different pH environments.

C. Express PGIPs in *Arabidopsis thaliana*

Five PGIPs (4 tomato fruit PGIPs and the pear fruit PGIP) have been cloned and assembled into plant transformation vectors. All five were transformed into *Arabidopsis*. Three of the tomato PGIPs and the pear fruit PGIP transformations were successful and the plants have been self-crossed to obtain lines that are homozygous for the transgenes. Genomic DNA of the transgenic lines has been prepared and analyzed by PCR for the presence of the selectable marker, hygromycin resistance. The lines are being characterized further to confirm the full-length identity of the PGIP in each line and to confirm that the lines are homozygous and stable. New transgenic lines will be engineered with the fourth tomato fruit PGIP and two *Medicago truncatula* PGIP-like sequences in the next month. The additional selected PGIPs will be cloned from the appropriate plants, inserted into the plant transformation vector and new *Arabidopsis* lines generated.

D. *Xf*PG Cloning and Expression Progress (Rachell Booth, Texas State Univ.)

*Xf*PG in the pET 29b vector was received from our collaborators at UC Davis. The *Xf*PG gene was amplified using PCR that included custom primers, *Xf*PG forward and *Xf*PG reverse (Table 2). The *Xf*PG reverse primer had an *XhoI* restriction site engineered into it. Once the PCR reaction was complete, the products were analyzed using horizontal gel electrophoresis (Figure 2). The amplified product was purified and digested with *NcoI* and *XhoI*. Additionally, the Invitrogen pMT/BiP/V5-His A expression vector was digested with the same enzymes. The

digestion products were then gel purified (Figure 3) and concentrations were determined. The *Xf*PG gene was ligated into an Invitrogen pMT/BiP/V5-His A expression vector and the resulting plasmids (pMT/*Xf*PG) were transformed into Top 10 *E. coli* cells. Single colonies were selected for amplification and plasmid isolation. Isolated plasmids were screened for proper ligation in a confirmation digest using *BglII* and *NheI* (Figure 4). The recombinant plasmid, pMT/*Xf*PG, is currently in the early stages of transfection for production in the *Drosophila* S2 cell expression system.

Table 2. Engineered primer sequences for *Xf*PG forward and *Xf*PG reverse. The XhoI restriction site is indicated by brackets in the *Xf*PG reverse primer sequence.

Primer Name	Sequence
*Xf*PG forward	5'- CCATGGACCTTGACCGTTTC -3'
*Xf*PG reverse	5'- [CCACTCGAG] GCGAAT CAGGAAATACGCTGG -3'

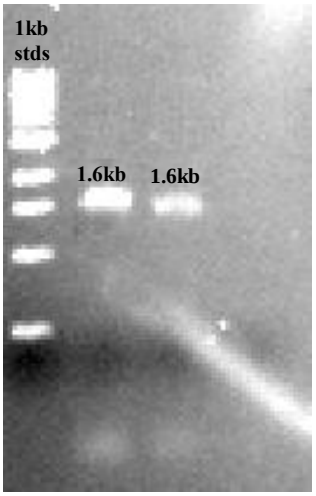

Figure 2. Analysis of amplified *Xf*PG gene.

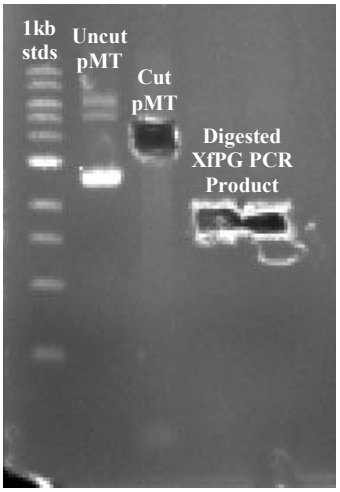

Figure 3. Gel purification of the digestion products.

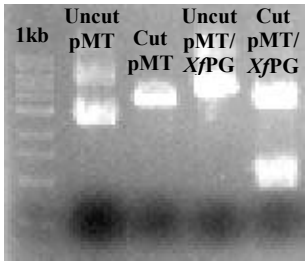

Figure 4. Confirmation digest of recombinant plasmid.

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

No activity planned in Year 1 of project.

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

No activity planned in Year 1 of project.

V. Publications or reports resulting from the project

Optimizing grape rootstock production and export of inhibitors of *X. fastidiosa* PG activity. Proceedings, 2008 Pierce's Disease Research Symposium. California Department of Food and Agriculture, San Diego, CA.

VI. Presentations on research

Poster, Optimizing grape rootstock production and export of inhibitors of *X. fastidiosa* PG activity. Proceedings, 2008 Pierce's Disease Research Symposium. California Department of Food and Agriculture, San Diego, CA.

VII. Research relevance statement

In response to the strategy recommended by the Advisory Board to enhance the resistance of grapevines to Pierce's Disease (PD), the project uses integrated approaches to optimally express plant genes for particularly effective polygalacturonase inhibiting proteins (PGIPs) targeting *X. fastidiosa* (*Xf*) polygalacturonase (PG) in transgenic grape rootstocks. To ease the path to commercialization, PIPRA investigators are examining relevant intellectual property and regulatory issues associated with the use of this strategy. A majority of the annotated PGIPs in dicot and monocot plants have been identified and are being screened *in silico* for their charge and predicted molecular structures to identify a short list of 14 PGIPs to be expressed in plants and tested for their ability to inhibit *Xf*PG. Grafts of existing grape lines expressing a PGIP are being propagated to test 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 PGIP expression in the rootstock and its 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.

VIII. Lay summary of current year's results

By expressing in grape rootstocks plant proteins that are effective inhibitors of the *X. fastidiosa* polygalacturonase (PG) enzyme, we aim to reduce the symptoms of Pierce's Disease. In the first six months of this project we have assembled for the first time sequences for a majority of the PG inhibiting proteins (PGIPs) expressed in plants. Based on their amino acid sequences, we have grouped similar PGIPs. We have analyzed the total charge of all the PGIPs because it is unlikely that PGIPs which have the same charge as the *Xf*PG will be effective inhibitors. This is the first time that all the known PGIPs have been analyzed using these criteria and the information will help us select a smaller group of 14 PGIPs that are likely to inhibit the *Xf*PG. The intellectual property issues of this subset of PGIPs is to be determined next. In order to establish that PGIPs expressed in grape rootstocks are effective in reducing Pierce's Disease, we are grafting non-transgenic scions onto pear fruit PGIP-expressing transgenic rootstocks. We know that when pear fruit PGIP is expressed in the scion, the symptoms of Pierce's Disease are reduced and we know from earlier grafted plants that pear fruit PGIP protein is transported across the graft junction (Agüero et al., 2005). Now we will be able to test whether enough of the transgenic pear fruit PGIP is translocated across the graft junction to improve the Pierce's Disease resistance of the wild-type scion portion of the plant.

IX. Status of funds

Our 3-year budget is $520,478, with $179,901 allocated for year 1 expenses. As of March 9, 2009 our actual expenditures have been $41,136 and funds encumbered amount to $54,650. Included in the encumbrances is the year 1 $8,595 sub-contract to Co-PI R. Booth at Texas State Univ., San Marcos. Our current year 1 budget balance is $84,115 or 47% of our year 1 funds. This appears to be on target, particularly given that expenditures did not begin until UCD accounts were established in August, 2008.

X. Summary and status of intellectual property produced during this research project

No new IP has thus far been generated in this project.

Relevant Publications

Agüero, C. B., S. L. Uratsu, C. Greve, A. L. T. Powell, J. M. Labavitch, C. P. Meredith, and A. M. Dandekar. 2005. Evaluation of tolerance to Pierce's Disease and *Botrytis* in transgenic plant of *Vitis vinifera* L. expressing pear PGIP gene. Mol Plant Pathology 6: 43-51.

D'Ovidio, R., S. Roberti, M. Di Giovanni, C. Capodicasa, M. Melaragni, L. Sella, P. Tosi, and F. Favaron. 2006. The characterization of the soybean polygalacturonase-inhibiting proteins (*Pgip*) gene family reveals that a single member is responsible for the activity detected in soybean tissues. Planta 224: 633-645.

Hegedus, D. D., R. Li, L. Buchwaldt, I. Parkin, S. Whitwill, C. Coutu, D. Bekkaoui, and S. R. Rimmer. 2008. *Brassic napus* possesses an expanded set of polygalacturonase inhibitor protein genes that are differentially regulated in response to *Sclerotinia sclerotiorum* infection, wounding and defense hormone treatment. Planta 228: 241-253.

Janni, M., M. Di Giovanni, S. Roberti, C. Capodicasa, and R. D'Ovidio. 2006. Characterization of expressed *Pgip* genes in rice and wheat reveals similar extent of sequence variation to dicot PGIPs and identifies an active PGIP lacking an entire LRR repeat. Theor Appl Genet 113: 1233-1245.

Labavitch, J.M. 2008. Optimizing grape rootstock production and export of inhibitors of *X. fastidiosa* PG activity. Proceedings, 2008 Pierce's Disease Research Symposium. California Department of Food and Agriculture, San Diego, CA.