**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, 2009 through July, 2009

**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

PIPRA is in the process of determining if there are any intellectual property rights related to any of the 14 candidate PGIP genes. Part of this work includes searching patent and patent application databases with protein based queries. Additional information regarding activities related to the objective are available in report #06-0224, PI: Alan Bennett.

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

A. Grafting of existing lines expressing and exporting pear PGIP

Work related to this objective has been delayed substantially due to quarantine measures implemented in response to a Panicle Rice Mite infestation in the greenhouse facilities. See the "note" at the end of this report for more details. Prior to these unforeseen and untimely circumstances, propagation efforts were underway to increase the numbers of pear PGIP-expressing transgenic 'Chardonnay' and 'Thompson Seedless' plants that were to have been used as rootstocks for grafting combinations and subsequent *X. fastidiosa* inoculation experiments. The CDFA and UC Davis issued directives for treating the affected grape vines including drastic pruning and isolation, resulting in rootstocks with only one or two viable buds remaining. Due to the considerable amount of time and effort to follow these directives and regenerate the plants, the grafting experiments to be conducted in years 1 and 2 have been delayed until the affected grape vines can be moved back to the appropriate greenhouse facilities.

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

The 14 candidate PGIPs selected for characterization of their ability to inhibit *Xf*PG were chosen as part of the work included in the last report. Efforts have begun to prepare these PGIPs for subsequent inhibition assays (Table 1). Of the 14 candidates, 4 have been successfully cloned into *E. coli* for maintenance. Appropriate plant material and DNA have been collected to clone nine of the remaining candidates and primers to amplify the PGIPs have been designed. Once sub-cloned into *Agrobacterium tumefaciens*, the constructs containing the candidate PGIPs will be used in both *in vitro* and *in planta* inhibition assays.

In order to identify PGIPs with optimal potential to inhibit *Xf*PG, additional work has been done to model the interactions between *Xf*PG and each of the candidate PGIPs. Prof. Daniel King (Taylor University) and his undergraduate student, Andrew Davisson, have created homology models for each of the 14 candidate PGIPs by threading the amino acid sequences onto the known crystal structure for PvPGIP2. Using a model of *Xf*PG with a polygalacturonan (its pectin substrate) polymer in its active site, King and Davisson have predicted one region of amino acids which may bind potential inhibitors, that is, PGIPs. Currently they are refining their analysis of this region through dynamic reaction simulations in an environment mimicking apoplastic pH conditions; by the end of the summer they expect to simulate the possible interactions between each of the candidate PGIPs and *Xf*PG (Figure 1). These models will enable them to predict regions of hydrophobicity, hydrogen donors, and hydrogen acceptors on the surfaces of *Xf*PG and the PGIPs that can have important implications for protein-protein

interactions and subsequent inhibition. Thus far, OsPGIP1, PcBPGIP, and CsiPGIP have the best predicted interactions of the 14 selected PGIPs with *Xf*PG. These *in silico* modeling studies will provide predictions of inhibition of *Xf*PG by PGIPs which can be compared with the experimental results (Objective 2D) and may, ultimately, identify mechanisms of *Xf*PG inhibition that can be predicted by simulated PG-PGIP interaction models.

| **Common name** | **Organism** | **Protein** | **Cloning Progress Checkpoints** | | | | |
|---|---|---|---|---|---|---|---|
| | | | Plant tissue acquired | PGIP cDNA isolated | Transformed into *E. coli* | Transformed into *A. tumefaciens* | PGIP ready for plant transformation |
| Thale cress | *Arabidopsis thaliana* | AtPGIP1 | X | X | X | O | - |
| Thale cress | *Arabidopsis thaliana* | AtPGIP2 | X | X | X | O | - |
| Rapeseed | *Brassica napus* | BnPGIP1 | O | - | - | - | - |
| Pepper | *Capsicum annum* | CaPGIP | O | - | - | - | - |
| Sweet orange | *Citrus sinensis* | CsiPGIP | X | O | - | - | - |
| Strawberry | *Fragaria x ananassa* | FaPGIP | X | X | - | - | - |
| Rice | *Oryza sativa* | OsPGIP1 | X | O | - | - | - |
| Rice | *Oryza sativa* | OsPGIP2 | X | O | - | - | - |
| Common bean | *Phaseolus vulgaris* | PvPGIP2 | X | O | - | - | - |
| Peach | *Prunus persica* | PpePGIP | O | - | - | - | - |
| Chinese Firethorn | *Pyracantha fortuneana* | PfPGIP | - | - | - | - | - |
| Bartlett pear | *Pyrus communis* | PcBPGIP | X | X | X | X | O |
| Tomato | *Solanum lycopersicum* | LePGIP | X | X | X | X | O |
| Grape | *Vitis vinifera* | VvPGIP | O | - | - | - | - |

**Table 1.** Research progress map for the 14 candidate PGIPs. "x" = completed checkpoints, "o" = work in progress, "-" = checkpoints to be completed.

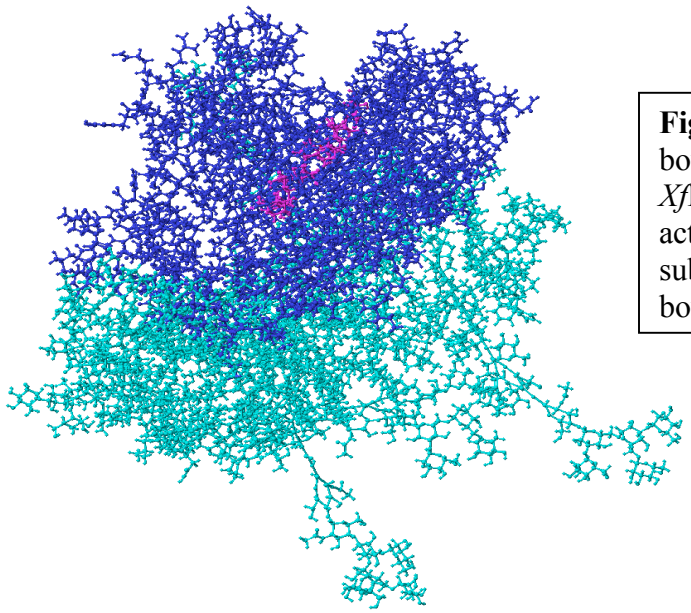

**Figure 1**. PcBPGIP (green) shown bound to a predicted binding site on *Xf*PG (blue) that is away from the active cleft, which is shown with a substrate (polygalacturonan, pink) bound in it,

C. *Xf*PG Cloning and Expression (Rachell Booth, Texas State University)

As of the last update report, the *Xf*PG gene has been successfully cloned into the Invitrogen pMT/BiP/V5-HisA expression vector. Problems with PCR-induced DNA sequence mutations and sequence verification have necessitated replicating some of the original cloning work presented in the previous report. The recombinant plasmid (pMT/*Xf*PG) has been transformed into *E. coli* and the sequence verified. The transfection process has begun for production of the plasmid in the *Drosophila* S2 cell expression system.

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

The progress made in assembling each of the PGIPs into plant transformation vectors for Arabidopsis transformation is outlined in Table 1. The *in vitro* radial diffusion assays to test *Xf*PG inhibition by each of the expressed PGIPs requires heterologous expression of active *Xf*PG described in Objective 2C. Assembly of the PGIP-containing constructs for plant transformation is in progress as is the expression of stable and abundant *Xf*PG. Once transformed Arabidopsis lines are generated, *in vitro* assays can be used to test for PGIP activity against known fungal PGs, in addition to the *XfPG* inhibition studies that will be done once sufficient *XfPG* is available, to confirm inhibition activity and specificity. These results will be used to characterize some of the PGIP proteins for PD defense as well as defenses against other known pathogens and pests that utilize PGs to cause diseases and plant destruction.

The aforementioned *in vitro* experiments with Arabidopsis plants expressing different PGIPs require the successful expression of *Xf*PG discussed in Objective 2C and generation of homozygous stably transformed Arabidopsis lines. We have developed an additional means of assessing PGIP inhibition of *Xf*PG; using a transient *in planta* approach we will examine quickly the ability of each of the candidate PGIPs to inhibit *Xf*PG in an environment similar to that of the potential Pierce's Disease interaction. Plant transformation vectors for PGIP and *Xf*PG expression will be used to coexpress *Xf*PG and PGIPs in tobacco. An analogous assay was used to test the effectiveness of a grape PGIP in inhibiting a fungal PG when both were transiently expressed in tobacco leaves (Joubert et al., 2007). Both the PG and PGIP were transiently expressed by using *A. tumefaciens* plant expression vectors that were co-infiltrated into tobacco leaves. When the PG was expressed alone, PG-dependent lesions on the treated leaf tissues were visible and could be measured; infiltration of the PG- and PGIP-encoding vectors together resulted in inhibition of PG-mediated lesion development.

We have modified this assay for use with the candidate PGIPs and *Xf*PG. *X. fastidiosa* is known to cause local lesions after infection of tobacco leaves so any reduction in lesion size caused by the co-expression of a PGIP with the PG can be measured. The candidate PGIPs cloned into plant transformation vectors for expression in Arabidopsis will be used in this transient assay for expression in *Nicotiana benthamiana* and *Nicotiana tobaccum* leaves. Two additional plant transformation constructs have been developed containing *Xf*PG for this assay: one with the intact, native *Xf*PG and one with a modified signal sequence targeting the PG protein to the apoplastic space. Inhibition data from this *in planta* assay will be used to complement the *in vitro* assays in order to select a PGIP which maximally inhibits *Xf*PG activity in varying conditions.

**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.

**Publications or reports resulting from the project**

No new publications or reports.

**Presentations on research**

No new presentations.

**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. To ease the path to commercialization, PIPRA investigators are examining relevant intellectual property and regulatory issues associated with the use of this strategy. Most of the annotated PGIPs in dicot and monocot plants have been identified and were narrowed to a list of 14 PGIPs to be expressed in plants and tested for their ability to inhibit *Xf*PG. Homology modeling of these candidates has revealed potential interaction sites which 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. 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.

**Lay summary of current year's results**

By expressing in grape rootstocks plant proteins that are effective inhibitors of the *X. fastidiosa* polygalacturonase (*Xf*PG) enzyme, we aim to reduce the symptoms of Pierce's Disease. In the first six months of this project we have created a short list of 14 PG inhibiting proteins (PGIPs) which represent a wide diversity of amino acid sequences. Since this list was created we have begun cloning these candidate PGIPs from their host plants into vectors for expression and plant transformation with the intent of testing for *Xf*PG inhibition both *in vitro*

and *in planta*. Structural modeling of potential interactions between *Xf*PG and the candidate PGIPs has revealed certain regions and features which might be necessary for a positive interaction and successful inhibition. We will be able to compare this *in silico* prediction data with the actual inhibition data from the expressed PGIPs and *Xf*PG to verify our computer models and possibly predict a maximally inhibiting PGIP for Pierce's Disease defense.

**Summary and status of intellectual property produced during this research project**

No new intellectual property has thus far been generated by this work.

**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.

**Joubert, D.A., Kars, I., Wagemakers, L., Bergmann, C., Kemp, G., Vivier, M.A., and J.A.L. van Kan. 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 Interactions. 20: 392-402.

**Note:**

In late Winter/early Spring, 2009 we encountered a greenhouse management problem that directly affected progress in our Pierce's Disease research contract 08-0171 and, because it demanded time from personnel involved in my entire research program, delayed progress in our other two PD research contracts (06-0225 and 08-0174). In the greenhouse that we shared with another UC Davis research colleague, we were propagating a series of transgenic 'Thompson Seedless' and 'Chardonnay' grapevines that expressed the pear fruit PGIP gene. These PGIP-expressing vines had been used to demonstrate (Agüero et al., 2005) that PGIPs could provide partial protection of vines against PD. Our plan for project 08-0171 was to graft non-transgenic scions onto transgenic roots and test the scions for PD susceptibility. Work was progressing well until a serious problem arose.

The greenhouse where the transgenic grape plants were grown was also being used to grow Miscanthus (*Miscanthus giganteus*), a sub-tropical perennial grass that has the potential to be a useful "energy" crop, a producer of "lignocellulosic" residues from which biofuels could be produced. The Panicle Rice Mite (*Steneotarsonemus spinki*) was identified on rice grown in the adjacent greenhouse used by a rice research program. Subsequently, although no rice was in our shared greenhouse, the mite was found on the Miscanthus in our greenhouse. Panicle Rice Mite does not infect grapes and there was no evidence that the pest was growing on our grape plants. The Panicle Rice Mite had not been found in CA rice fields but it can cause a great deal of loss in rice-growing areas where it is prevalent. Therefore, the CDFA took action to eliminate the problem in the UC Davis greenhouse facility so that the pest would not be spread to commercial rice plantings in the Davis area. That action was coordinated through the UCD Panicle Rice Mite Task Force led by Prof. Richard Bostock (Plant Pathology Dept., UC Davis). The original

sanitation action decision was that the grapevines should be destroyed merely because they were in the same greenhouse where the Panicle Rice Mite had been found on the Miscanthus. This led to considerable follow-up discussion and eventually a decision was reached to (1) cut the transgenic vines back severely (to 6 inches with no residual leaves, leaving one or two viable buds), (2) treat the cut vine stubs with pesticides, (3) completely wrap the stubs and pots in plastic, and (4) transport the grape material to a clean isolation greenhouse away from the facility where the mite was originally identified. The vines now have regrown well in isolation and the previously infected and current isolation greenhouses contain clean "sentinel" rice plants to determine that both facilities have no persistent mite contamination. Regrowth of the grape lines has taken 4 months and a few replicates of the lines were lost during the transition. The isolation greenhouse where the grapevines expressing the pear PGIP are located is very small and propagation efforts to scale up the number of plants for grafting has not been possible.

This rescue operation has, for the moment, saved the considerable investment that went into the generation of the pear PGIP-expressing vines and their maintenance and propagation over several years. However it did prevent some of the work in project 08-0171 that was planned for this summer and took a great deal of scientist time (notably that of Dr. L.C. Greve and Ph.D. candidate Z. Chestnut) away from the work planned for other PD projects and other research in the lab.