- **Title of report:** Interim Progress Report for CDFA Agreement Number 08-0171.
- **Title of Project:** OPTIMIZING GRAPE ROOTSTOCK PRODUCTION AND EXPORT OF INHIBITORS OF *XYLELLA FASTIDIOSA* POLYGALACTURONASE ACTIVITY
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- **Time Period Covered by Report:** The results reported are from work conducted October 2012 to March, 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

Work on this objective has been described in previous reports.

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) continue to be maintained in the UC Davis Core Greenhouse Complex. The propagation and grafting techniques used for this objective are described in the progress report for the project "Field Evaluation of Grafted Grape Lines Expressing pPGIP" (PI: Powell). These efforts have continued to provide source material

for grafted plants and assays. In February, 2013, because of funding from the field evaluation project, David Dolan has been engaged to complete the grafting work. He has grafted twice as many plants as needed to complete the Solano and Riverside County plots for our project. The plants are currently in the greenhouse.

We modified a pressure flow apparatus to flush long stem segments with water or high salt buffers, to capture exogenous or translocated pPGIP. We were able to obtain xylem exudate from own-rooted, transgenic pPGIP expressing Thompson Seedless stems containing a small amount of total proteins (26 μg/ml). We were able to identify a small amount of pPGIP protein from macerated grape tissue, but not the xylem exudate, that was recognized by our current stock of polyclonal anti-pPGIP antibodies (Figure 1).

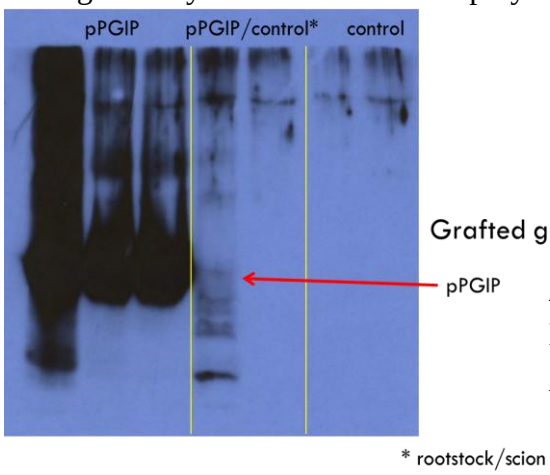

Figure 1. Western blot showing pPGIP protein from collected from grafted grapevines. The rootstock in the graft, but not the scion, expressed pPGIP.

There was insufficient protein to measure PG inhibiting activity of the grapevine xylem exudate or macerate. We evaluated inhibition of PGs produced by *B. cinerea* strains B05.10 or Del 11 in culture in the collected xylem exudate and using proteins from macerated Thompson seedless tissues expressing pPGIP.

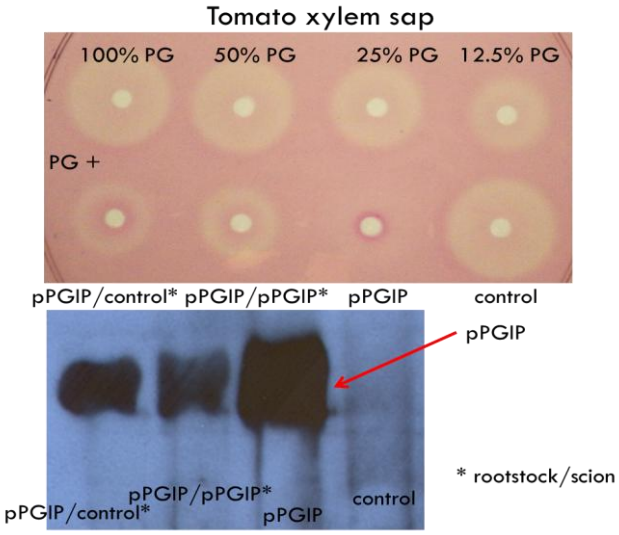

Figure 2. PG inhibiting activity (top panel) of xylem sap from grafted and control tomato plants expressing pPGIP. pPGIP protein is detected (bottom panel) with existing polyclonal antibodies in a western blot. Inhibiting activity and pPGIP protein is detected when the root portions of the plants express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

We have evaluated rootstocks of transgenic tomato plants expressing pPGIP in order to gather more material for detecting the pPGIP protein in xylem sap. We have confirmed that pPGIP protein is expressed in the rootstocks and were able to use a pressure device to force xylem sap out of the cut stems of own-rooted and grafted plants.

The xylem sap fluid from grafted tomato plants produced detectable pPGIP protein that was able to inhibit the PGs collected from cultured *B. cinerea* Del 11 (Figure 2).

B. Selection of PGIPs as PD defense candidates

Based on phylogenetic, biochemical, and structural analyses of PGIP sequences from 68 plant species, PGIPs from 'Roma' rice, 'Hamlin' orange, and 'Bartlett' pear have been selected for further study of their inhibition of *Xf*PG. The same cloning strategy previously reported is being applied to generate plant transformation vectors with each of these PGIPs. Transcription will be constitutive, as driven by the CaMV-35S promoter, and the resulting proteins will have a C-terminal 6x-histidine tag for subsequent purification. As previously reported, 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' with 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. 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 *OsPGIP1* and will proceed with preparing the transformation vector. The coding sequence of *OsPGIP2* does not appear to encode a *bona fide* PGIP and will not be pursued further.

C. *Xf*PG expression and purification

The previously reported *Xf*PG expression system utilizing Drosophila S2 cells produced quantifiable amounts of PG protein with very slight activity that diminished over time. The second strategy was to express *Xf*PG transiently in leaves. A fusion construct of the apoplastic signal sequence from pPGIP and the coding sequence of *Xf*PG was generated for transient expression by *Agrobacterium tumefaciens*. Preliminary agroinfiltration assays with intact tobacco leaves indicated that the targeted 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 in the field, we have obtained a helper strain of *A. tumefaciens* that has been used to improve the expression of introduced genes.

Because our initial assays of PGIP have used *B. cinerea* PG as a standard, we have developed a new method for evaluating the activity of the PGIPs we are testing. In our assays, the PGs produced by the B05.10 strain 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 in our *in vitro* assay by pPGIP. One explanation for this difference could be that key amino acids recognized as part of the inhibition by pPGIP are different in the B05.10 and Del 11 versions of the primary 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 Assist. Prof. Dario Cantu (Dept. of Viticulture and Enology, UC Davis) and sequenced the genome of the Del 11 *B. cinerea* strain. 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 3 and it is clear that there are more amino acid sequence differences between the BcPG1s of these lines. We plan to do predictive protein modeling to determine whether these changes occur at sites likely to be involved in the interaction with pPGIP. This work will help us refine our analysis of key amino acids in the *Xf*PG sequence, which are crucial for inhibition by PGIPs.

Figure 3. 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) continues. The potential recombinant expression system for *Xf*PG (Obj. 2C) will be used to transiently express and

purify active candidate PGIPs. Observations of PGIP activity *in planta* will be made using transgenic model plants for agroinfiltration experiments. The genotypes of tomato and Arabidopsis plants constitutively expressing *pPGIP* or *LePGIP* have been confirmed by PCR. These pPGIP-expressing plants will be used to test the efficacy of pPGIP expressed *in planta*. Agroinfiltration with *Xf*PG-expressing bacterial strains will be done on the leaves of own-rooted and transgrafted plants.

Objective 3. Maximize PGIP expression in and transport from roots

Once we have identified a PGIP to "optimally" inhibit *Xf*PGs, improvements to the expression and delivery of this protein will 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 will be assayed for *Xf*PG inhibition *in planta* utilizing agroinfiltration and transgrafted tobacco and tomato plants. Grape rootstock transformation will commence once an optimal PGIP has been determined.

- **Publications:**

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 "Plant cell wall in ripening related susceptibility of fruits to necrotrophs" 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. 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 3 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:** From the original $520,478 award, as of 28 February 2013, $479,465.67 has been spent.
- **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 optimal 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.

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