A. Title of report: Renewal Progress Report for CDFA Agreement Number 08-0171

B. Title of project: OPTIMIZING GRAPE ROOTSTOCK PRODUCTION AND EXPORT OF INHIBITORS OF *XYLELLA FASTIDIOSA* POLYGALACTURONASE ACTIVITY

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D. Time period covered by the report: November 2011 to March 2012.

E. Introduction:

Xylella fastidiosa (*Xf*), the causative agent of Pierce's Disease (PD) in grapevines, has been detected in infected portions of vines. Several lines of evidence support the hypothesis that *Xf* uses cell wall-degrading enzymes to digest the polysaccharides of plant pit membranes separating the elements of the water-conducting vessel system, the xylem, of the vines. *Xf*'s cell wall degrading enzymes break down these primary cell wall barriers between cells in the xylem, facilitating the systemic spread of the pathogen. Recombinantly expressed *Xf* polygalacturonase (PG) and β-1,4-endo-glucanase (EGase), cell wall degrading enzymes that are known to digest cell wall pectin and xyloglucan polymers respectively, have been shown to degrade grapevine xylem pit membranes and increase pit membrane porosity enough to allow passage of the bacteria from one vessel to the next (Pérez-Donoso *et al.*, 2010). *Xf* cells have been observed passing through similarly degraded pit membranes without the addition of exogenous cell wall degrading enzymes, supporting the conclusion that the enzymes are expressed by *Xf* and allow its movement within the xylem (Sun *et al.,* 2011). Roper *et al.* (2007) developed a PG-deficient strain of *Xf* and showed that the mutant bacterial strain was unable to cause PD symptoms; thus, the *Xf*PG is a virulence factor of the bacteria that contributes to the development and spread of PD.

PG-inhibiting proteins (PGIPs) produced by plants are selective inhibitors of PGs and limit damage caused by fungal pathogens (*Botrytis cinerea*; Powell *et al.*, 2000) as well as by insects (*Lygus hesperus*; Shackel *et al.*, 2005). Agüero *et al.* (2005) demonstrated that by introducing a pear fruit PGIP (*pPGIP*) gene (Stotz *et al.*, 1993) into transformed grapevines, the susceptibility to both fungal (*B. cinerea)* and bacterial (*X. fastidiosa)* pathogens decreased. This result implied that the pPGIP provided protection against PD by inhibiting the *Xf*PG, reducing its efficiency as a virulence factor. In fact, recombinant *Xf*PG is inhibited *in vitro* by pPGIP-containing extracts from pear fruit (Pérez-Donoso *et al.*, 2010). In a key preliminary observation for the PD control approach investigated in this project, Agüero *et al.* (2005) demonstrated that transgenic pPGIP protein could be transported from transformed grapevine rootstocks, across a graft junction and into the grafted wild-type scions. pPGIP also has been shown to be transported from rootstocks across grafts into the aerial portions of tomato plants (Haroldsen *et al.,* 2012).

The overall goal of the project is to develop transgenic grape rootstock lines that express PGIPs that effectively reduce the virulence of *X. fastidiosa*. The project is designed to compare potential *Xf*PG inhibiting properties of PGIPs from a wide variety of plants in order to identify specific PGIPs that optimally inhibit the virulence factor, *Xf*PG. The goal is to express these PGIPs in grape rootstocks to provide PD protection in grafted scions. The expression of PGIPs in grape rootstocks will utilize transformation components with defined intellectual property (IP) and regulatory characteristics, as well

as expression regulating sequences that result in the maximal production of PGIPs in rootstocks and efficient transport of the proteins through the graft junctions to the aerial portions of vines so that *Xf* movement is limited in infected scion tissues.

F. List of objectives:

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
    b. Assemble grape transformation vectors utilizing PIPRA vectors with defined IP characteristics
2. Identify plant PGIPs that maximally inhibit *X. fastidiosa* PG.
    a. Using existing pear PGIP-expressing grapevines, test PD susceptibility of normal scions grafted to PGIP-expressing and -exporting rootstocks
    b. Identify plant PGIPs that are efficient inhibitors of *Xf*PG
    c. Express PGIPs in *Arabidopsis thaliana* and test for optimal inhibition of *X. fastidiosa* PG
    d. Optimally express *X. fastidiosa* PG, using recombinant protein expression systems
3. Assemble transcription regulatory elements, *Xf*-inducible promoters and signal sequences that maximize PGIP expression in and transport from roots.
4. Create PGIP-expressing rootstocks and evaluate their PD resistance.
    a. Molecular analysis of putative marker free transgenic grape plants
    b. Evaluate transgenic grape lines for optimal expression and export to scions of selected PGIPs
    c. Evaluate transgenic lines for susceptibility to *X. fastidiosa*

G. Description of activities and summary of accomplishments:

Objective 1. A path to commercialization of transgenic rootstocks

PIPRA has acted as a liaison for issues associated with the potential commercialization of transgenic grapevine rootstocks for several CDFA PD/GWSS Board funded projects. Mark Szczerba, and Gabriel Paulino before him, have managed the permitting process for the field trial testing of Thompson Seedless and Chardonnay grapevines expressing pPGIP, in progress since 2010. The details of the field trial are discussed in the renewal progress report for CDFA contract 09-0746 (PI: Powell). Further work with grape transformation vector assembly is being re-evaluated for its usefulness to future transgenic line development and commercialization.

Objective 2. Identify plant PGIPs that maximally inhibit XfPG

A. Propagation, grafting, and susceptibility testing 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 renewal progress report for CDFA contract 09-0746 (PI: Powell). These efforts have maintained 137 own-rooted vines for assays and grafting source material: 29 pPGIP-expressing Thompson Seedless vines, 35 Thompson Seedless control vines, 37 pPGIP-expressing Chardonnay vines, and 36 Chardonnay control vines (Figure 1).

Figure 1. Own-rooted grapevines expressing pPGIP These are to be used in PD susceptibility and grafting experiments.

As previously reported, Victor Haroldsen has shown that pPGIP protein is found in wild-type scion tissues grafted to transgenic tomato and grape rootstocks expressing the pPGIP gene. These results are limited by the relatively low sensitivity of the polyclonal antibody that we have used for detection of pPGIP presence. (**Note**: This led us to propose development of a monoclonal antibody that recognizes the pPGIP protein. That project, PI: Powell, is currently underway.) We are currently extracting samples from different scion tissue types and stem xylem sap to determine the extent of the pPGIP translocation. Trans-grafted grapevines will soon be inoculated with *X. fastidiosa* in the greenhouse to examine their degree of resistance to PD. These tests have been delayed due to our experience that inoculation tests are less predictable when carried out in the fall and winter months.

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

Based on phylogenetic, biochemical, and structural analyses of PGIP sequences from 68 plant species, PGIPs from rice, orange, and pear have been selected for tests of their ability to inhibit *Xf*PG. The 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 PGIP purification. Genomic DNA has been prepared from rice and orange leaves and each PGIP-encoding sequence was successfully PCR amplified. The resulting *pgip* genes have been cloned into plasmids for sequencing and transformed into *E. coli* for DNA modifications. More colonies must be sequenced to find *pgip* clones without mutations from the modeled sequence.

C. *Xf*PG expression and purification

Constructs Infiltrated	Bacterial Media	Agrobacterium Growth Phase*	Infiltration Media	Infiltration Density **	Species and leaf stage	Symptoms	Time to symptoms
XfPG	YEP	1.2 (late log)	Sterile H_2O	0.6	N.t. & N.b.; mature leaves	Water soaking in N.t. & N.b., chlorosis in N.b. only	48 hpi
XfPG, pPGIP::XfPG	YEP	1.7-2.6 (stationary)	MgCl + MES (MM), pH 5.6	1.0	N.t. & N.b.; mature leaves	Mild necrosis in N.b. only + pPGIP::XfPG	4 dpi
XfPG, pPGIP::XfPG	YEP	3.0 (decline)	MM + Acetosyringone (MMA), pH 5.6	1.0, incubated 30 min.	N.b.; 4- or 6-leaf stage	None, plants too small	-
XfPG, pPGIP::XfPG	LB	2.2 (stationary)	MMA, pH unknown	1.0, incubated 2 hrs.	N.t.; mature	Mild chlorosis + pPGIP::XfPG	3 dpi

Table 1. Infiltration variables and results. *$O.D._{600}$ at harvest; **$O.D._{600}$ after resuspension; N.t. = *Nicotiana tabacum*; N.b. = *N. benthamiana*.

The previously reported *Xf*PG expression system utilizing Drosophila S2 cells produced quantifiable amounts of PG protein that, unfortunately, had very slight activity that diminished over time. The second strategy was to express *Xf*PG transiently in leaves. The *Xf*PG coding sequence was successfully cloned into the plant transformation vector pCAMBIA-1301 and transformed into *Agrobacterium tumefaciens* (EHA105 pCH32) for transient expression in tobacco leaves. To ensure the extracellular localization of the *Xf*PG protein, the 5' region of the coding sequence was modified to append the pPGIP apoplastic signal sequence. This modified pPGIP::XfPG fusion construct has also been cloned into pCAMBIA-1301 and transformed into *A. tumefaciens*. Preliminary agroinfiltration assays indicate that the targeted PG has a similar activity to the non-targeted protein, both resulting in necrotic lesions in the infiltrated tissue. The infiltration assay conditions are currently being modified to yield maximal transformation events which will result in increased expression of PG activity. These modifications include altering *A. tumefaciens* growth media, growth phase, infiltration conditions and

tobacco species and leaf stage. Past infiltration procedures, not including replicate attempts, are listed in Table 1.

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

Cloning and expression of the rice and orange PGIPs selected in Objective 2B continues as previously reported. The *Xf*PG and pPGIP::XfPG constructs discussed in Objective 2C provide a potential diagnostic tool to test the efficacy of XfG inhibition of each test PGIP *in planta* using a tobacco leaf co-infiltration strategy. Co-infiltration of *A. tumefaciens* cultures harboring *Xf*PG and either pPGIP or LePGIP (from tomato) in pCAMBIA-1301 was carried out as described by Joubert *et al.* (2007). Infiltration with cultures expressing *Xf*PG resulted in marked wilting, localized water soaking, and chlorotic lesions developing in the infiltration zone. Leaves co-infiltrated with XfPG- and PGIP-expressing cultures displayed attenuated symptoms while leaves infiltrated with just PGIP or empty vector cultures showed no symptoms. LePGIP (tomato PGIP) was less effective than pPGIP at inhibiting wilting and lesion development when co-infiltrated with XfPG, a result that is not surprising because the tomato PGIP is not predicted to be a particularly effective inhibitor of XfPG. Further work to quantify these infiltration test results will provide a measure of the inhibition of XfPG by each cloned PGIP. We anticipate that the fusion construct pPGIP::XfPG will yield more easily scored results due to the targeted delivery of the XfPG to the apoplast.

Agroinfiltration assays have also been reported using grape, tomato, and *Arabidopsis* leaves. We are continuing to develop this technique for testing the inhibition of PGs by different test PGIPs *in planta* by utilizing transgenic grape, tomato, and *Arabidopsis* plants over-expressing either pPGIP or LePGIP. Both own-rooted transgenic and trans-grafted plants are being propagated for infiltration with *Xf*PG-expressing *A. tumefaciens* cultures. Once the candidate PGIP constructs have been prepared, stable transgenic tobacco plants will be generated for agroinfiltration with *Xf*PG cultures and for inoculation with *X. fastidiosa*. In our request for a no-cost extension on project 08-0171 we are proposing to generate transgenic tobacco lines that express the three *pgip* genes that are predicted to encode PGIPs effective against XfPG (i.e., PGIPs from pear, citrus and rice). These lines are expected to be useful in infiltration assays because tests would only require that the XfPG gene be expressed in the tobacco test system.

Protein (Organism)	Cloning Progress Checkpoints				
	Source Tissue Acquired	PGIP Sequence Isolated	Transformed into E. coli	Transformed into Agrobacterium	Ready for Plant Transformation
pPGIP (Pear)	✓	✓	✓	✓	✓
OsPGIP1 (Rice)	✓	✓	✓	**O**	**-**
CsiPGIP (Orange)	✓	✓	✓	**O**	**-**
AtPGIP1 (Arabidopsis)	✓	✓	✓	✓	✓
AtPGIP2 (Arabidopsis)	✓	✓	✓	✓	✓
BnPGIP1 (Rapeseed)	✓	✓	-	-	-
CaPGIP (Pepper)	✓	✓	-	-	-
FaPGIP (Strawberry)	✓	✓	-	-	-
LePGIP (Tomato)	✓	✓	✓	✓	✓
OsPGIP2 (Rice)	✓	✓	✓	O	-
PvPGIP2 (Bean)	✓	✓	O	-	-
PpePGIP (Peach)	O	-	-	-	-
PfPGIP (Firethorn)	✓	-	-	-	-
VvPGIP (Grape)	O	-	-	-	-
XfPG (Xylella)	✓	✓	✓	✓	✓
pPGIP::XfPG	✓	✓	✓	✓	✓

Table 2. Cloning progress to date. ✓ = completed tasks; O = in progress.

Objective 3. Maximize PGIP expression in and transport from roots

The transformation vector to be used in grape transformation is being re-evaluated for its effectiveness and freedom to operate. Information pertaining to potential signal sequences targeting PGIPs to xylem tissues for transport across graft junctions has been reported by the project "*In planta* testing of signal peptides and anti-microbial proteins for rapid clearance of *Xylella*" (PI: A. Dandekar). Our project would make use of promising findings from Dandekar and his colleagues.

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

There is no activity for this reporting period as the identity of the "optimal" PGIP has not been determined or evaluated *in planta.*

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Haroldsen VM, Szczerba MW, Aktas H, Lopez-Baltazar J, Odias MJ, Chi-Ham CL, Labavitch JM, Bennett AB, Powell ALT. 2012. Mobility of transgenic nucleic acids and proteins within grafted rootstocks for agricultural improvement. Frontiers in Plant Science. 3: 39.

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Sun Q, Greve LC, Labavitch JM. 2011. Polysaccharide compositions of Intervessel Pit Membranes Contribute to Pierce's Disease Resistance of Grapevines. Plant Physiol. 155: 1976-1987.

H. PUBLICATIONS AND PRESENTATIONS

"Optimizing Grape Rootstock Production and Field Trial Evaluation of PGIPs." Poster presented at the 2011 Pierce's Disease Research Symposium.

"Preventing PD with Rootstock Derived Wall Proteins." Presentation to the UC Davis Plant Biology Graduate Group, 10 February 2012.

Haroldsen VM, Szczerba MW, Aktas H, Lopez-Baltazar J, Odias MJ, Chi-Ham CL, Labavitch JM, Bennett AB, Powell ALT. 2012. Mobility of transgenic nucleic acids and proteins within grafted rootstocks for agricultural improvement. Frontiers in Plant Science. 3: 39. Published 2 March 2012.

I. RESEARCH RELEVANCE STATEMENT

The overall goal of the project is to develop transgenic grape rootstock lines that express PGIPs that effectively reduce the virulence of *X. fastidiosa* (*Xf*). Rootstock delivery of PD control factors, including PGIPs, has been given top priority for the increased likelihood for commercial deployment and success of those strategies. The project is designed to identify specific PGIPs that optimally inhibit the virulence factor, *Xf*PG, and to express these PGIPs in grape rootstocks to provide PD protection in scions. The expression of PGIPs will utilize transformation components with defined intellectual property (IP) and regulatory characteristics, as well as expression regulating sequences that result in the maximal production of PGIPs in rootstocks and efficient transport of the proteins through the graft junctions to the aerial portions of vines so that *Xf* movement (i.e., PD symptom development) is delayed and limited in infected scion tissues.

J. LAYPERSON SUMMARY

Xylella fastidiosa (*Xf*) uses a key enzyme, polygalacturonase (PG), to spread from the initial point of inoculation throughout the grapevine; 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 they can 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 scions inoculated with *Xf*. We have modeled the protein structures of fourteen candidate PGIPs to predict how the PGIPs physically interact with *Xf*PG. We will combine this knowledge with *in planta* assay results measuring the ability of the candidate PGIPs to inhibit *Xf*PG. For these inhibition assays we are developing systems to generate high levels of active *Xf*PG and PGIPs. The best inhibiting PGIPs will be expressed in test tobacco and grape rootstock germplasm and, after grafting, their ability to limit PD development in non-transgenic scions will be determined.

K. Status of Funds: As of March 8 the budget for project 08-0171 had $160,969 remaining. A no-cost extension will be requested. What the fund balance as of June 30, 2012 will be is not certain at this time.

L. Summary and status of intellectual property associated with the project: No IP claims specific to the results of this research have been filed as of March 20, 2012.