PROGRESS REPORT FOR CDFA CONTRACT 08-0171

I. Project title

OPTIMIZING GRAPE ROOTSTOCK PRODUCTION AND EXPORT OF INHIBITORS OF *XYLELLA FASTIDIOSA* POLYGALACTURONASE ACTIVITY

Principal investigators and cooperators

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II. Time period: March 2011- July 2011

III. 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)

Summary of major research accomplishments and results for each objective

Objective 1: A path to commercialization of transgenic rootstocks

A. No additional work was done on this objective in this period.

B. No additional work was done on this portion of the proposal.

Objective 2: Identify plant PGIPs that maximally inhibit X. fastidiosa PG

A. Propagation, grafting and susceptibility testing of grape lines expressing and exporting pPGIP

The transgenic 'Thompson Seedless' and 'Chardonnay' grapevines expressing the pPGIP described in Aguero *et al.* (2005) have been maintained in the UC Davis Core Greenhouse Complex. Additional plants of each cultivar expressing pPGIP and control plants not expressing pPGIP have been rooted during this reporting period with the help of an aeroponic cloner (EZ-Clone, Inc., Sacramento, CA) as described in previous reports.

Grafted plants continue to be generated to verify the transport of pPGIP protein from transgenic rootstocks, across the graft junction, into scion tissue not expressing any foreign PGIP.

As we have reported previously, we have shown that pPGIP protein produced in transgenic rootstocks crosses the graft junction and can be identified in scions of grafted grape and tomato plants. The pPGIP protein has been identified in grafted wild-type grape and tomato scion leaf tissue (Figure 1). For these experiments, existing stocks of polyclonal pPGIP antibodies were used on a Western gel and blot after leaf protein extracts were concentrated 30-fold. Once the monoclonal antibody we are preparing (PI Powell) is available, its increased specificity will be used for quantification of pPGIP crossing the graft junction into wild-type tissues.

Once sufficient grafted plants have been generated, they will be transferred to the Solano vineyard to allow testing of the PD susceptibility of the scion portions of plants with pPGIP expressing rootstocks. Initial inoculations of ungrafted plants began in early July, 2011 and are being monitored.

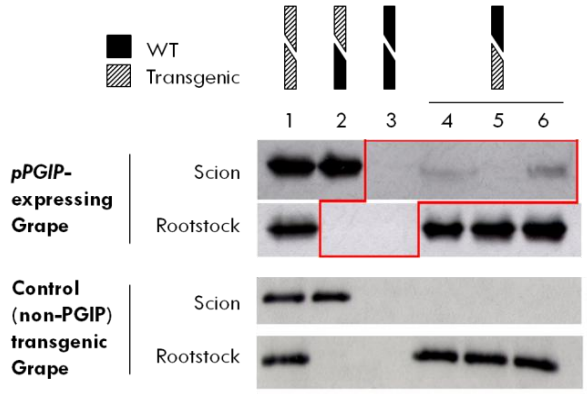

Figure 1. Western blot of leaf extracts taken from rootstock and scion portions of grafted 'Thompson Seedless' grapevines. Transgenic vines are expressing either pPGIP or NPTII (control). pPGIP is visualized crossing from transgenic rootstocks into wild-type (WT) scion tissue (lanes 4-6). This movement is not seen in the reciprocal graft (lane 2).

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

Fourteen candidate PGIPs were initially selected for *in vitro* and *in vivo* *Xf*PG inhibition assays based on predicted protein charge and phylogenetic analyses. The homology models created for *Xf*PG, the polygalacturonic acid (PGA) substrate for PG, and each of the candidate PGIPs provided predictive tools to interpret the inhibition mechanisms and physical interactions between *Xf*PG and the PGIPs (Labavitch, 2009). Dynamic *in silico* reaction simulations predicted that two clusters of amino acids, #63-74 and #223-226, must be unblocked for *Xf*PG to cleave PGA. The long columns of electronegative residues on the concave faces of the PGIP's leucine rich repeat structure bind to these critical regions (Fig. 2). This information coupled with surface chemistry mapping predicts that pPGIP, CsiPGIP (citrus), and OsPGIP1 (rice) will be the best inhibitors of *Xf*PG.

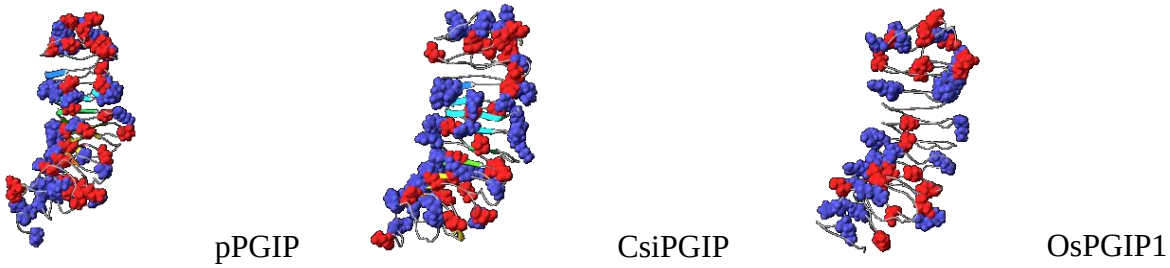

Figure 2. Homology models of three PGIPs predicted to be good candidates to inhibit *Xf*PG. The column of electronegative residues (red) on the concave face of each protein may align with critical residues on *Xf*PG that are important for inhibition.

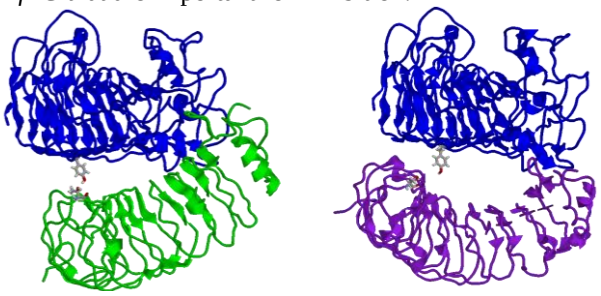

Figure 3. PG-PGIP complexes. Tyr303 of *Xf*PG (blue) binds strongly with a region of pPGIP (green); this is not possible with VvPGIP (purple). Interactions such as this might influence PG-PGIP interaction and inhibition.

A closer look at the dynamic reaction simulations highlighted other residues that may also influence PG-PGIP binding. Strong hydrogen bonding occurs between residues on pPGIP and Tyr303 of *Xf*PG, bringing them together in a potentially inhibitory manner (Fig. 3). Electrostatic repulsions between VvPGIP (grape PGIP) residues and *Xf*PG Tyr303 prevent a similar alignment and may predict a failure to inhibit *Xf*PG. Combining modeling predictions and future inhibition data will allow us to evaluate the predicted interactions and infer other potentially useful interactions between the candidate PGIPs and other PGs.

Based on these modeling studies two PGIPs have been selected for further study of their inhibition of the PG produced by *X. fastidiosa*. PCR primers for the amplification and cloning of the PGIP sequences from citrus and rice have been designed (Table 1) in order to evaluate the potential utility of these PGIPs.

Primer sequence*	**Primer name**	**Gene amplified; Modifications**
TCACagatcttccatggATGAGtAACACGTCA	CsiPGIP_F3	CsiPGIP; BglII and NcoI sites, alternate frame nonsense mutation
TTCAAAccATGgGCAACACGTCACTG	CsiPGIP_Falt2	CsiPGIP; NcoI site, S2G missense mutation
CCAGgctagcgcgaccctcaatTCTTTC	CsiPGIP_R3	CsiPGIP; NheI site, Xa site, removal of TGA

ccatggtATGCGCGCCATGGTaGTC	OsPGIP1_F	OsPGIP1; NcoI site, alternate frame nonsense mutation
ccATGgGCGCCATGGTCGT	OsPGIP1_Falt	OsPGIP1; NcoI site, R2G missense mutation
gctagcgcgaccctcaatATTGCAG	OsPGIP1_R	OsPGIP1; NheI site, Xa site, removal of TAA
cgagatctccATGGATGTGAAGCTCCTG	OsPGIP2_F2	OsPGIP2; BglII and NcoI sites
gctagcgcgaccctcaatTCGACGAC	OsPGIP2_R3	OsPGIP2; NheI site, Xa site, removal of TAA

*Uppercase bases are homologous to the reference sequence; lowercase bases are introduced changes.

C. *Xf*PG expression and purification

The *Xf*PG expression system utilizing Drosophila S2 cells was developed to yield sufficient amounts of active, stable *Xf*PG protein for *in vitro* inhibition assays. The cloning strategy fused the coding sequence of *Xf*PG to a C-terminal histidine tag for purification and an N-terminal targeting sequence for protein secretion (Labavitch, 2009). The medium from transiently transfected cells induced to express *Xf*PG had a small amount of PG activity, as shown by radial diffusion assay (Taylor and Secor, 1988); this activity decreased over time. Additional work on expressing *Xf*PG in cell lines has not occurred during this reporting period.

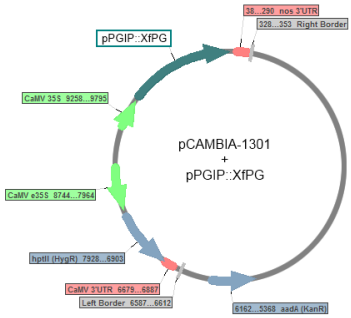

Figure 4. Transient pPGIP::*Xf*PG expression vector for agroinfiltration.

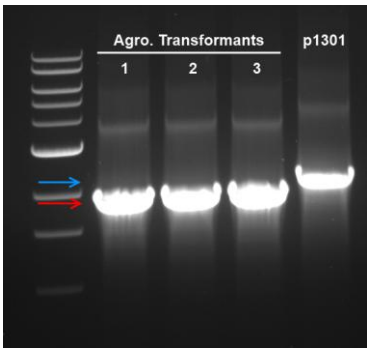

Figure 5. The DNA gel image shows the PCR products from from Agrobacterium miniprep plasmid DNA. Three colonies containing the *Xf*PG:pPGIP sequence (1-3) were screened with p1301 primers flanking the insert site. The expected size fragment is 1924 bp (red arrow). The last lane used "empty" p1301 (gusA intact) as a control template and the same p1301 primers resulted in the gusA product with the expected size of 2204 bp (blue arrow).

In previous reporting periods we have reported our successful cloning and delivery of *Xf*PG into plants via *A. tumefaciens*. In this reporting period we have successfully cloned the *Xf*PG linked to the pPGIP apoplastic targeting signal into pCAMBIA1301 (Figure 5) and introduced this construct into A. tumefaciens for plant expression. The design of the vector is shown in Figure 4. The pPGIP extracellular targeting sequence was linked to the 5' end of the *Xf*PG coding sequence in order to optimize delivery of the protein to the extracellular region (apoplast) of the plant cell, including the cell wall. We anticipate that the fusion construct pPGIP::*Xf*PG will yield more obvious infiltration results than the native *Xf*PG construct because the pPGIP signal sequence has been shown to target the translated *Xf*PG protein to the cell apoplastic space where it can either degrade the pectin-rich middle lamellae and cell walls or be inhibited by any co-infiltrated PGIP. PGIPs are naturally targeted to the apoplast.

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

Protein (Organism)	**Cloning Progress Checkpoints**				
	Source tissue acquired	PGIP cDNA isolated	Transformed into *E. coli*	Transformed into *Agrobacterium*	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	-	-	-
pPGIP (Pear)	✓	✓	✓	✓	✓
LePGIP (Tomato)	✓	✓	✓	✓	O
VvPGIP (Grape)	O	-	-	-	-
*Xf*PG (Xylella)	✓	✓	✓	✓	✓
pPGIP::*Xf*PG	✓	✓	✓	O	-

Table 2. Cloning progress chart. Checkmarks indicate completed checkpoints while circles indicate work in progress.

The strategy for cloning selected PGIPs for transformation into *A. tumefaciens* (EHA105 pCH32) will continue once the PCR products have been obtained (Table 1; Labavitch, 2009). The full-length *Xf*PG construct was successfully cloned into the transformation vector which was then transformed into *Agrobacterium*. This pPGIP::*Xf*PG fusion construct (Figure 4) provides a potential diagnostic tool to test the efficacy of each PGIP *in planta* using a tobacco leaf infiltration system that maximizes the possibility that the *Xf*PG is exported to the extracellular space. The advantage of this assay is that it should be quicker than testing the lines in Arabidopsis or grape plant lines stably transformed to express PGIPs. It has been reported that the infiltration assay will work on grape and tomato leaves and as this approach provides advantages in terms of time and cost, we will continue to develop and use this technique for testing the inhibition of PGs by different test PGIPs.

Co-infiltration of *Agrobacterium* cultures harboring *Xf*PG and a PGIP in pCAMBIA-1301 was carried out as described by Joubert *et al.* (2007). Fully formed leaves of *Nicotiana benthamiana* and *N. tabacum* were infiltrated with constant manual pressure using a needle-less syringe, thus forcing bacterial cells into the abaxial leaf tissue. In most cases, initial infiltration zones were marked on the adaxial surface and had measured areas of approximately 35 mm^2. Visual symptom development was observed at 24 and 72 hours post infiltration (hpi, Figure 6). Infiltration with cultures harboring the *Xf*PG construct resulted in marked wilting, localized water soaking, and chlorotic lesions developing in the infiltration zone. Leaves co-infiltrated

with *Xf*PG and PGIP cultures displayed attenuated symptoms while leaves infiltrated with just PGIP or empty vector cultures showed no symptom development. LePGIP (tomato PGIP) was less effective than pPGIP at inhibiting wilting and lesion development when co-infiltrated with *Xf*PG. Currently we are evaluating the wilting symptoms that occur as a result of infiltration with the *Xf*PG:pPGIP fusion protein. We plan to use the infiltration assay with our existing lines that express the pPGIP (grape and tomato lines) or the LePGIP (tomato lines). We also anticipate doing this assay with leaves from the scions of the grafted lines which translocate the pPGIP from the rootstock into the scion (described above).

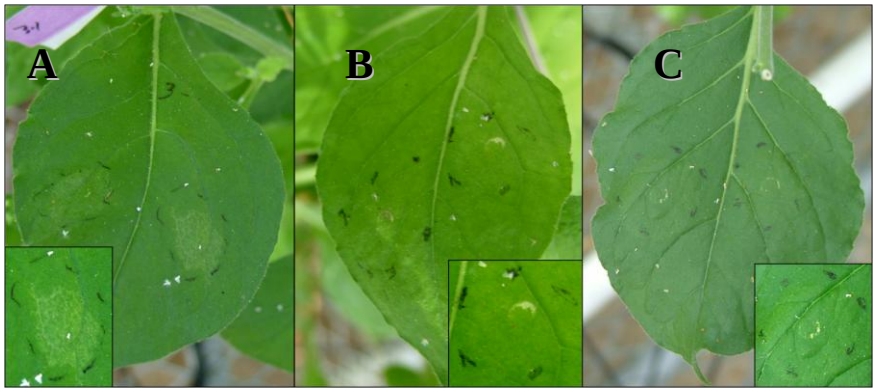

Figure 6. Transient expression of *Xf*PG, pPGIP, and LePGIP in tobacco *N. benthamiana* leaves by infiltration with *Agrobacterium* cultures. Chlorotic lesions and water soaking mark the site of agro-infiltrations with *Xf*PG (A). Symptoms are reduced when *Xf*PG is co-infiltrated with *Agrobacterium* expressing the pPGIP (B) or LePGIP (C). Inserts show details of infiltration sites. Black marks indicate the borders of the initial leaf zone infiltrated.

E. Modeling of PGIP:*Xf*PG interactions is covered under **B** above.

Objective 3: Maximize PGIP expression in and transport from roots

The transformation vector to be used in grape transformation has been reevaluated for its effectiveness. Information pertaining to potential signal sequences targeting PGIPs to xylem tissues for transport to and across graft junctions into wild-type scions has been reported by the project "*In planta* testing of signal peptides and anti-microbial proteins for rapid clearance of *Xylella*" (PI: A. Dandekar).

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

Multiple rootstock genotypes are being considered for transformation with the optimized vectors containing the candidate PGIP constructs. Different rootstock varieties are adapted to diverse climates, soil conditions, and disease pressures. St. George (Rupestris du Lot), 101-14 (Millardet et de Grasset), and Freedom rootstocks are transformable by the UC Davis Plant Transformation Facility, providing time for production of embryogenic calli. St. George and 101-14 are both adapted for moist, clay soils and have moderate to high phylloxera resistance; 101-14 is less resistant to high salinity and drought-prone, deep soils, but provides greater nematode resistance. Freedom provides a high level of nematode resistance, but is very susceptible to phylloxera and imparts higher than average scion vigor so it should be used in sandier soils. We continue to evaluate the predicted usefulness of each of these varieties for our field evaluations in Solano and Riverside counties.

Conclusions

The ability of selected PGIPs to provide PD resistance to wild-type scions will eventually be determined by the field trials. This evaluation, while a key step in advancing the use of transgenic rootstocks for PD control in commercial applications, requires careful assessment of PGIP effectiveness in model plant situations. We have developed many of the tools and resources needed for these evaluations. Homology models of all 14 candidate PGIPs have been constructed and critical residues for *Xf*PG-PGIP interaction were discovered. Recombinant *Xf*PG, produced from transiently transfected Drosophila cells, was purified and shown to have a low level of PG activity. We have developed an *in planta* assay for *Xf*PG inhibition by PGIPs that allows identification of optimal PGIPs.

IV. Intellectual property issues: Evaluations of intellectual property considerations for the selection of appropriate PGIPs have been described in previous reports. No patents have been filed for the PGIPs selected for further evaluations.

V. References cited:

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Publications or reports resulting from the project: None

VI. Research relevance statement for solving PD in California

The CDFA Pierce's Disease (PD) and Glassy-winged Sharpshooter Board's Research Scientific Advisory Panel review in 2007 and subsequent RFPs have given top priority to delivery from grafted rootstocks of PD control factor candidates, including polygalacturonase-inhibiting proteins (PGIPs). Optimal candidate PGIPs for inhibition of *Xylella fastidiosa* (*Xf*) polygalacturonase (PG) have been selected from several plant sources. From fourteen candidate PGIPs, PGIPs from pear, rice, and orange were determined to be the most likely inhibitory proteins for *Xf*PG. Recombinant protein expression systems have been developed for *Xf*PG and each candidate PGIP. Initial inhibition assays have shown that the pear fruit PGIP is a more effective inhibitor of *Xf*PG than the PGIP from tomato, however both PGIPs limit *Xf*PG symptom development in tobacco leaf infiltration assays. Expression of additional PGIPs to test is underway.

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 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