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

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Reporting Period: The results reported here are from work conducted October 2009 to October 2010.

ABSTRACT
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 candidates, including polygalacturonase-inhibiting proteins (PGIPs). Optimal PGIPs for inhibition of *Xylella fastidiosa* (*Xf*) polygalacturonase (PG) are being selected from several plant sources. Fourteen candidate PGIPs have been chosen and homology models were generated to predict interactions with and potential inhibition of *Xf*PG. 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 XfPG symptom development in tobacco leaf infiltration assays. Expression of additional PGIPs to test is underway and other non-vinifera *Vitis* PGIPs are being pursued.

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 Pierce's disease (PD) symptom development. Plant proteins called PG-inhibiting proteins (PGIPs) are produced by many plants and selectively inhibit PGs from bacteria, fungi, and insects. Pear fruit PGIP is known to inhibit *Xf*PG and to limit PD development in inoculated grapevines which have been transformed to express the pear protein. PGIPs are graft transmissible so we are interested to determine which PGIP best inhibits *Xf*PG and how well, when expressed in transgenic rootstocks, this PGIP prevents PD development in *Xf* inoculated wild-type scions. We have modeled 14 candidate PGIPs to predict how they physically interact with *Xf*PG and to combine this knowledge with *in vitro* and *in planta* assay results measuring the ability of each candidate PGIP to inhibit *Xf*PG. For these inhibition assays we are developing separate systems to generate high levels of active *Xf*PG and PGIPs. The best inhibiting PGIPs will be expressed in test grape rootstock germplasm and, after grafting, their ability to limit PD development in non-transgenic scions will be determined.

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 (Labavitch and Sun, 2009). 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 (*B. 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 (*Xf)* 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 wild-type scions. pPGIP also has been shown, this year, to be transported from rootstocks across grafts into the aerial portions of tomato plants.
The overall goal of the project is to develop transgenic grape rootstock lines that express PGIPs that effectively reduce the virulence of *Xf*. 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 is limited in infected scion tissues.

OBJECTIVES

1. Define a path to commercialization of a PD control strategy using PGIPs, focusing on IP and regulatory issues associated with the use of PGIPs in grape rootstocks.
2. Identify plant PGIPs that maximally inhibit *Xf*PG.
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.

RESULTS AND DISCUSSION

Objective 1: A path to commercialization of transgenic rootstocks

PIPRA IP analyst, Gabriel Paulino, has served as the main liaison for issues associated with the potential commercialization of transgenic grapevine rootstocks for several CDFA PD/GWSS Board funded projects. He has obtained the necessary APHIS-USDA authorizations to begin testing these PD control strategies in field locations. 'Thompson Seedless' and 'Chardonnay' grapevines expressing the pear fruit PGIP (pPGIP) gene were planted in a jointly operated field trial in Solano County during July, 2010. More details can be found in the report "Field evaluation of grafted grape lines expressing PGIPs" (PI Powell).

Objective 2A: Propagation and grafting 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 greenhouse. Previous attempts at vegetative propagation for grafting studies proved inconsistent. Total plant numbers for both cultivars and control plants not expressing pPGIP were increased during this reporting period with the help of an aeroponic cloner (EZ-Clone, Inc., Sacramento, CA). Non-lignified stem segments, three nodes in length, were transferred to individual sites within the cloner. Roots began forming on dark-grown, constantly misted basal regions in 1-2 weeks. The application of 1000 ppm IBA to basal regions immediately after cutting did not result in increased rooting time or yield.

Grafted plants are being generated to verify the transport of pPGIP protein from transgenic rootstocks, across the graft junction, into scion tissue not expressing any foreign PGIP. Grafting has been attempted with both green and semi-lignified stem segments for all graft combinations. Grafts of six 'Thompson Seedless'plants and one 'Chardonnay' plant have been formed by a modified wedge grafting technique whereby scion sections of 1 to 2 nodes were stripped of foliage and cut with perpendicular apical ends and wedge basal ends. These sections were fitted into notched rootstock stems of equal maturity. The grafts were secured with Parafilm M, a clothespin, and a translucent bag to prevent desiccation. Other green grafting techniques, such as chip budding, have been attempted with limited success.

Work in the project "Tools to identify PGIPs transmitted across grapevine grafts" (PI Powell) is developing a monoclonal antibody to recognize pPGIP, but not the native grape PGIP, in these own-rooted and grafted grapevines. UC Davis Biochemistry and Molecular Biology Ph.D. candidate, Victor Haroldsen, has shown pPGIP crossing graft junctions from transgenic tomato rootstocks into wild-type tomato scion leaf tissue. For these experiments, he used existing stocks of polyclonal pPGIP antibodies after concentrating leaf extract samples 30-fold. Similar experiments using the aforementioned grafted grapevines will probe isolated xylem sap from scion tissues. Combined with the monoclonal antibody, the increased reactivity will allow for quantification of pPGIP crossing the graft junction into wild-type tissues.

Objective 2B: Selection of PGIPs as PD defense candidates and PGIP-XfPG modeling

The 14 candidate PGIPs were previously 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 substrate for PG, and each of the candidate PGIPs have provided unique predictive tools to interpret the inhibition mechanisms and physical interactions between XfPG and the PGIPs (Labavitch, 2009). Dynamic reaction simulations predicted that two clusters of amino acids, #63-74 and #223-226, must be unblocked for *Xf*PG to cleave its substrate *in silico*. The long columns of electronegative residues on the concave faces of the PGIP's leucine rich repeat structure bind to these critical regions (**Figure 1**). This information coupled with surface chemistry mapping predicts pPGIP, CsiPGIP, and OsPGIP1 to be the best inhibitors of *Xf*PG.

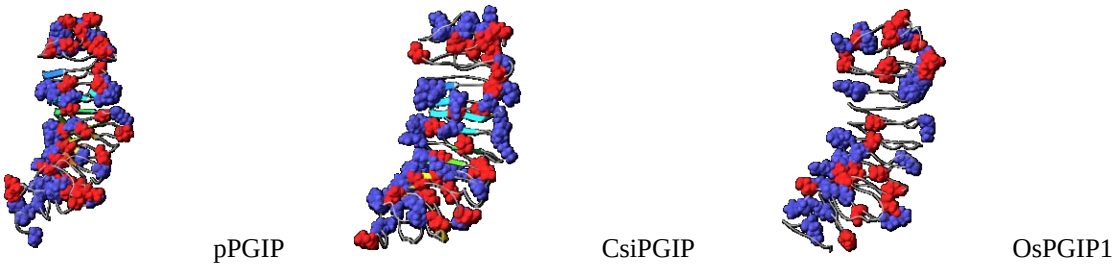

Figure 1. Homology models of 3 prime candidate PGIPs (CsiPGIP-orange;,OsPGIP-rice). The column of electronegative residues (red) on the concave faces of each protein may align with critical residues on XfPG important for inhibition.

A closer look at the dynamic reaction simulations highlighted other specific 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 (**Figure 2**). Electrostatic repulsions between VvPGIP 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 score the results of predicted interactions and infer other potentially useful interactions between the candidate PGIPs and other PGs.

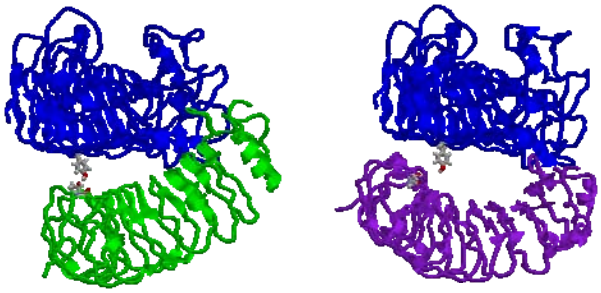

Figure 2. PG-PGIP complexes. Tyr303 of *Xf*PG (blue) binds strongly with a region of pPGIP (green) which is not possible with VvPGIP (purple). Interactions such as this might influence PG-PGIP interaction and inhibition.

Adding to the information gained from the 14 candidate PGIP homology models, other unpublished PGIP sequences from non-vinifera *Vitis* varieties will be modeled in the future. These sequences will be obtained as part of a collaboration, currently in negotiation, with a research group at Stellenbosch University, South Africa. The sequences are the property of an industry board associated with the Institute for Wine Biotechnology at Stellenbosch University. It will be of interest to note how the models of these non-vinifera PGIPs compare to the modeled structure of VvPGIP from *Vitis vinifera* cv. 'Pinotage.'

Objective 2C: XfPG expression and purification

The *Xf*PG expression system utilizing Drosophila S2 cells is being developed to yield large 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). Media from transiently transfected cells induced to express *Xf*PG has a small amount of PG activity, as shown by radial diffusion assay (**Figure 3**; Taylor and Secor, 1988). *Xf*PG was purified from the medium and pelleted Drosophila cell lysate by immobilized nickel column chromatography. The medium and resuspended cell lysate were separately loaded onto the gravity flow column and each was eluted with several volumes of EB (50 mM sodium phosphate, 0.3 M NaCl, 250 mM imidazole). The eluate was analyzed by Western blotting and Coomassie staining SDS-PAGE. Proposed *Xf*PG bands, cross-reacting with a tagged antibody recognition site on the recombinant protein, were visualized at 78 kDa in Western blots for cell lysate preparations (**Figure 4**). The protein bands in the cell medium preparation eluant were visualized at 68 kDa by Coomassie staining (**Figure 5**). Each of these preparations showed slight PG activity, as measured by reducing sugar analysis (Gross, 1982). These activities, however, diminished over time. Current work is focused on generating stably transfected recombinant cell lines to provide more consistent stocks of *Xf*PG. The methods for purifying and storing the protein are also being analyzed to reduce possible causes of the loss of PG activity.

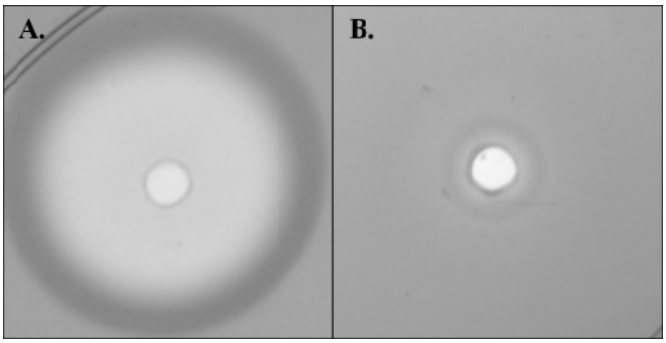

Figure 3. Radial diffusion assay of concentrated PG from *Botrytis cinerea* (A) or culture media from induced *Xf*PG-expressing Drosophila cells (B). The clearing zone diameter is related to amount of PG activity.

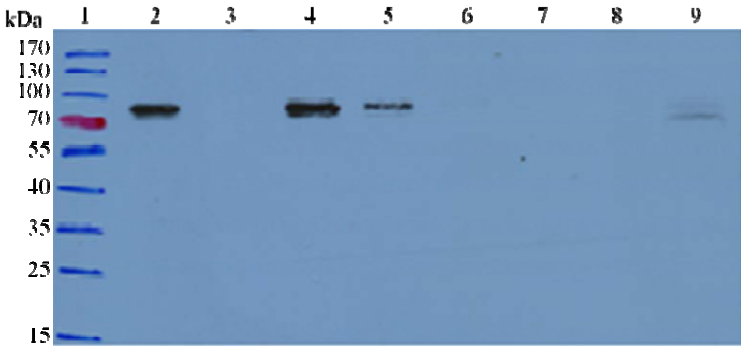

Figure 4. Western blot analysis of partially purified cell lysate after XfPG protein expression. 15 mL crude XfPG lysate was purified by column chromatography and selected fractions were analyzed by Western blotting. Lane 1 = pre-stained ladder, lane 2 = flow-through #4, lane 3 = wash #10, lanes 4-7 = elution fractions #1-4, lane 8 and 9 = cellular medium. Recombinant XfPG protein was eluted with 250 mM imidazole and probed with the anti-V5 primary antibody and anti-mouse HRP secondary antibody.

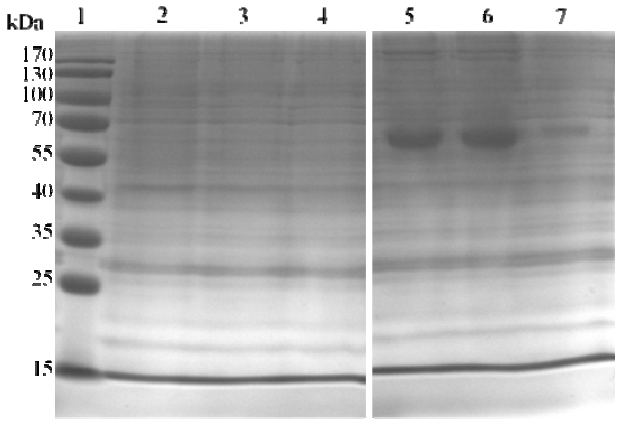

Figure 5. Partially purified XfPG protein eluted with 250 mM imidazole. Coomassie stained polyacrylamide gel electrophoresis. Lane 1 = pre-stained ladder, lanes 2-4 = cell lysate fractions #1-3, lanes 5-7 = cellular medium fractions #1-3.

Objective 2D: Expression of PGIPs in Arabidopsis and tobacco for XfPG assays
The previously reported strategies for cloning each of the 14 candidate PGIPs into pCAMBIA-1301 and transformation into *Agrobacterium tumefaciens* (EHA105 pCH32) continues (**Table 1**; Labavitch, 2009). The full-length *Xf*PG construct was successfully cloned into the transformation vector and transformed into *Agrobacterium*. This construct, and soon the pPGIP::*Xf*PG fusion construct (still in progress) provide a potential diagnostic tool to test the efficacy of each PGIP *in planta*.

Table 1. Cloning progress chart. Checkmarks indicate completed checkpoints while circles indicate work in progress.

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	-	-	-	-
XfPG (Xylella)	✓	✓	✓	✓	✓
pPGIP::XfPG	✓	✓	✓	O	-

Co-infiltration of *Agrobacterium* cultures harboring *Xf*PG and either pPGIP or LePGIP 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, forcing bacterial cultures 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 was less effective than pPGIP at inhibiting wilting and lesion development when co-infiltrated with *Xf*PG. Further work to quantify the results will provide a measure of the inhibition of *Xf*PG by each cloned PGIP. We anticipate that the fusion construct pPGIP::*Xf*PG will yield more easily scored results than the native *Xf*PG construct due to the signal sequence from pPGIP predicted 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. PGIP is naturally targeted to the apoplast.

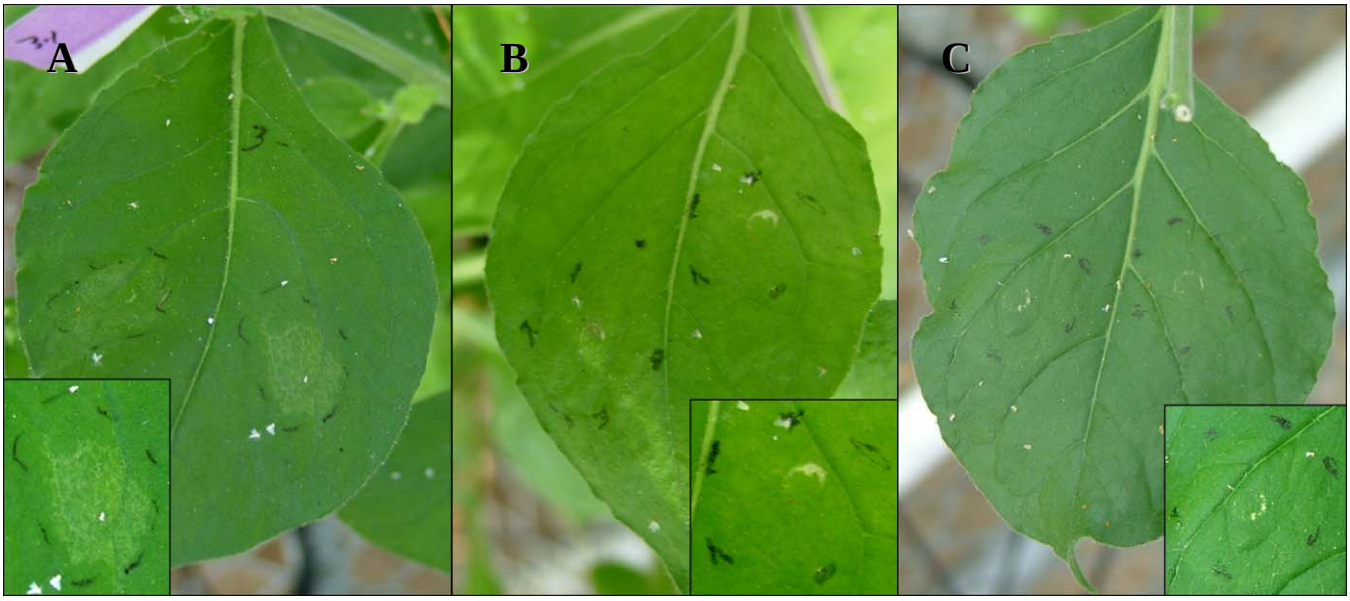

Figure 6. Transient expression of *Xf*PG, pPGIP, and LePGIP in *N. benthamiana* leaves by infiltration with *Agrobacterium* cultures. Chlorotic lesions and water soaking mark the site of agro-infiltrations with *Xf*PG (A). Symptoms are reduced when *Xf*PG is co-infiltrated with pPGIP (B) or LePGIP *Agrobacterium*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

Objective 3: Maximize PGIP expression in and transport from roots
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: No activity for this reporting period.

CONCLUSIONS
The ability of one of the candidate PGIPs discussed here, pPGIP, to provide PD resistance to wild-type scions will be determined by the recently initiated field trial. This will be a key step in advancing the use of transgenic rootstocks for PD control in commercial applications. 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. Further work to clone and express the candidate PGIPs continues. *In planta* co-infiltration assays have shown that both pPGIP and LePGIP are able to inhibit the chlorotic lesion development in tobacco leaves that is caused by *Xf*PG-harboring *Agrobacterium*.

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FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.