TOOLS TO IDENTIFY POLYGALACTURONASE-INHIBITING PROTEINS TRANSMITTED ACROSS GRAPEVINE GRAFTS

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Reporting Period: The results reported here are from work conducted August 18, 2010 to October 1, 2010.

ABSTRACT
The CDFA Pierce's Disease and Glassy-winged Sharpshooter Board's Research Scientific Advisory Panel review in 2007 and subsequent RFPs have given top priority to delivery of Pierce's disease (PD) control candidates, including polygalacturonase-inhibiting proteins (PGIPs), from grafted rootstocks. Four currently funded projects – two scientific research projects and two field trials of transgenic PD control lines – use PGIPs as a control strategy to limit the spread of *Xylella fastidiosa (Xf)* in the xylem network and thereby limit PD symptom progression in infected vines. A monoclonal antibody to the pear fruit PGIP, the protein expressed by the aforementioned grape lines, is being developed to detect, quantify, and observe the localization of the pear PGIP in transformed grapevines and grafted vines with transformed rootstocks. Pear PGIP is being isolated from previously transformed *Arabidopsis thaliana* plants and from mature green 'Bartlett' pear fruit tissue. Monoclonal antibody production by Antibodies, Inc. will begin once sufficient quantities of properly glycosylated, active PGIP have been purified.

LAYPERSON SUMMARY
Xylella fastidiosa (Xf) utilizes a key enzyme, polygalacturonase (*Xf*PG), to spread from one xylem vessel to the next, eventually leading to the development of Pierce's disease (PD) symptoms in infected vines. Plant proteins called PG-inhibiting proteins (PGIPs) selectively inhibit PGs from bacteria, fungi, and insets. Our collective work has identified a PGIP from pear fruits as partially inhibiting PD symptom development in grapevines expressing the pear fruit PGIP. Current projects, including field trial evaluations, require a monoclonal antibody specifically recognizing the pear fruit PGIP to detect, quantify, and characterize the protein's role in *Xf*PG inhibition in transformed grapevines. We are currently purifying active pear PGIP from two plant sources for commercial antibody production.

INTRODUCTION
Pierce's disease (PD) incidence has been associated in several studies with the spread of the causal agent, *Xylella fastidiosa* (*Xf*), throughout the xylem vasculature of infected grapevines. The spread of bacteria from one vessel to the next utilizes bacterial cell wall modifying enzymes to degrade the pit membranes separating adjacent vessels (Pérez-Donoso et al., 2010). One such enzyme, a polygalacturonase (XfPG), has been well characterized and is a PD virulence factor (Roper et al., 2007). Several previous projects have analyzed the effectiveness of PG-inhibiting proteins (PGIPs) in minimizing the detrimental effects of pathogen and pest attack on various plants. Two currently funded projects both use pear fruit PGIP (pPGIP) to restrict Xf movement: "Optimizing grape rootstock production and export of inhibitors of *X. fastidiosa* PG activity" (PI Labavitch) and "*In planta* testing of signal peptides and antimicrobial proteins for rapid clearance of *Xylella*" (PI Dandekar).

This project was developed to generate a monoclonal antibody that selectively recognizes the pear fruit pPGIP protein. The monoclonal antibody is a necessary tool for both aforementioned research projects and the related project "Field evaluation of grafted grape lines expressing PGIPs" (PI Powell) and will allow for detection and quantification of pPGIP without cross-reactive interference from the native PGIP. Plants can therefore be more efficiently screened for the presence of the pPGIP protein, whether directly produced in, or transported to the plant tissue of interest.

OBJECTIVES
1. Using existing plants expressing histidine-tagged pPGIP and fresh pear flesh, prepare pPGIP protein and provide it to Antibodies, Inc. to develop mouse hybridoma lines expressing monoclonal antibodies against the pear PGIP.
2. Calibrate the antibodies produced by the hybridoma clones to determine effective dilutions for use in detecting the pPGIP protein.
3. Use the antibody to detect transgenic pear PGIP in xylem sap of own-rooted and grafted grapevines.

RESULTS AND DISCUSSION
Objective 1: Purification of pear PGIP from transgenic Arabidopsis leaves and pear fruit.
The generation of a monoclonal antibody requires purified protein to be used as the antigen. PGIPs are heavily glycosylated plant cell wall proteins that require certain glycosylation levels for activity (Powell et al., 2000). The project "Optimizing grape rootstock production of and export of inhibitors of *X. fastidiosa* PG activity" (Labavitch, 2008) generated transgenic *Arabidopsis thaliana* plants expressing the pPGIP protein fused to a C-terminal histidine tag for purification. Rosette leaves

from these transgenic plants were frozen in liquid nitrogen and ground with a mortar and pestle. The resulting powder was mixed with a binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) and centrifuged at 500 x g to remove the leaf debris. The supernatant was mixed with an equal volume of immobilized nickel-sepharose matrix for separation and purification of the histidine tagged pPGIP from the crude extract. The matrix-pPGIP slurry was washed with several volumes of binding buffer and bound protein was eluted with several volumes of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). A minimal amount of total protein was found in the eluate after centrifugation and removal of the matrix, as determined by Bradford assays. Work to refine the transgenic protein purification process is ongoing.

In addition to obtaining pPGIP from the *Arabidopsis* protein expression system, pPGIP extraction from fresh pear fruit flesh is underway. pPGIP was purified from mature green 'Bartlett' pears according to Stotz et al. (1993) with modifications. Peeled, cored, and sliced pears (2 kg) were homogenized in 2 L of extraction buffer (1 M sodium acetate, pH 6, 1 M NaCl, 1 % [w/v] polyvinylpolypyrrolidone, 0.2% [w/v] sodium bisulfite). The homogenate was stirred at 4°C for 1 hour then filtered through three layers of Miracloth with manual pressure. The liquid fraction was centrifuged (10,000 rpm, 20 min, 4°C) and the supernatant collected. The pellet fraction and Miracloth rententate were combined and resuspended in 1 L extraction buffer, stirring 1 hour at 4°C. In some aliquots, the initial homogenate was degassed prior to centrifugation, eliminating the need for the Miracloth filtration. After centrifugation, the supernatants were combined. Total protein precipitating between 50% and 100% ammonium sulfate saturation was collected and resuspended in 100 mM sodium acetate, pH 6, and extensively dialyzed at 4°C against 10 mM sodium acetate, pH 6, using 6000-8000 molecular weight cut-off membranes.

The dialyzed ammonium sulfate fraction was mixed with an equal volume of 2x ConA buffer (200 mM sodium acetate, pH 6, 2 M NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM $MnCl_2$). A small volume was applied to a Concanavalin A-sepharose column which was then washed with several volumes of 1x ConA buffer. Bound protein was eluted with 250 mM methyl-α-D-mannopyranoside, followed by 1 M methyl-α-D-mannopyranoside. The initial column chromatography purification has not separated the glycosylated pPGIP protein from the dialyzed fraction, potentially due to improper binding conditions. Current efforts center on concentrating pPGIP protein and optimizing the column chromatography protocol.

pPGIP activity was measured throughout purification by radial diffusion assays (Taylor and Secor, 1988). Samples of the pear homogenate were able to fully inhibit a PG (BcPG) mixture from *Botrytis cinerea* (B05.10) culture filtrates. For BcPG preparation, fungal cultures were grown for 12 days in total darkness in 250 mL modified Pratt's medium, supplemented with 1 g/L Difco yeast extract and 3 g/L citrus pectin, inoculated with $1x10^5$ spores (Fergus, 1952). The fungus and media were filtered through 11 µm cellulose filters then 1.2 µm glass microfiber filters to remove any residual fungus and concentrated by dialysis against polyethylene glycol through 6000-8000 molecular weight cut-off membranes. The pPGIP purification preparations resulted in 66% and 59% reductions in BcPG activity after ammonium sulfate precipitation and subsequent dialysis, respectively.

CONCLUSIONS

Tagged pPGIP protein is being isolated from *Arabidopsis thaliana*. Concurrently, native pPGIP is being purified from 'Bartlett' pear fruits. pPGIP activity against *B. cinerea* PG has been maintained in all purification steps. Purified pPGIP protein will be delivered to Antibodies, Inc. for monoclonal antibody production.

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FUNDING AGENCIES

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