

TOOLS TO IDENTIFY POLYGALACTURONASE-INHIBITING PROTEINS TRANSMITTED ACROSS GRAPEVINE GRAFTS

Principal Investigator:

Ann L.T. Powell
Department of Plant Sciences
University of California
Davis, CA 95616
alpowell@ucdavis.edu

Principal Investigator:

Abhaya Dandekar
Department of Plant Sciences
University of California
Davis, CA 95616
amdandekar@ucdavis.edu

Principal Investigator:

John M. Labavitch
Department of Plant Sciences
University of California
Davis, CA 95616
jmlabavitch@ucdavis.edu

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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 from grafted rootstocks of Pierce's disease (PD) control candidates, including polygalacturonase-inhibiting proteins (PGIPs). 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* in the xylem network and, thereby, limit PD symptom progression in infected vines. The aim of this project is to develop a monoclonal antibody to the pear fruit PGIP, the protein expressed by the aforementioned grape lines, 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*), the bacteria that causes Pierce's disease (PD) in grapevines, utilizes a key enzyme, polygalacturonase (*Xf*/PG), to spread from one grapevine xylem vessel to the next, eventually leading to the development of PD symptoms in infected vines because the bacteria multiply and interrupt the flow of nutrients and water through the vessels in the plant. Plant proteins called PG-inhibiting proteins (PGIPs) selectively inhibit PGs from bacteria, fungi, and insects. Our work has identified a PGIP from pear fruits that at least partially inhibits the *Xf*/PG and we demonstrated reduced 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 PGIP protein delivered to the scion portion of grafted plants from rootstocks expressing the pear fruit PGIP. The monoclonal antibody allows the researchers to compare the amounts of the PGIP protein at different times and places and thereby determine the protein's role in *Xf*/PG inhibition in grapevines. We are purifying active pear PGIP green pear fruit for commercial antibody production to meet the needs of the collaborating groups.

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 (*Xf*/PG), 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 *Xf* 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 fresh green 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 plant cell wall proteins that require plant specified glycosylation for activity (Powell et al., 2000). The project “Optimizing grape rootstock production of and export of inhibitors of *Xylella fastidiosa* (Xf) polygalacturonase (PG) activity” (Labavitch, 2008) generated transgenic *Arabidopsis thaliana* plants expressing the pPGIP protein fused to a C-terminal histidine tag for purification. Leaves from these transgenic plants yielded a small amount of total protein, as determined by Bradford assays. Work to refine the transgenic protein purification process is ongoing.

pPGIP extraction has begun from fresh pear fruit flesh using the protocols in Stotz et al. (1993) with modifications. In 2010 we worked with two batches of green pear fruit to purify sufficient protein for the antibody preparation. The fruit (2 kg each) were homogenized in 2 L of extraction buffer (1 M PGIP extract preparation (Abu-Goukh et al., 1983).

pPGIP activity was measured throughout purification by radial diffusion assays (Taylor and Secor, 1988). Samples of the initial pear homogenate were able to fully inhibit a PG (BcPG) mixture from *Botrytis cinerea* culture filtrates. The pPGIP purification preparations after ammonium sulfate precipitation and subsequent dialysis resulted in a 75% reduction in BcPG activity in the assay (**Figure 1**). Although in the first attempt to purify the protein the concentration of proteins in the sample was low and the preparations still contained considerable polysaccharides which may impair binding to ConA sepharose. The excess polysaccharide could have been because the pears used for this preparation were not completely green. This procedure was repeated with a second preparation of pear fruit and the following purifications steps were followed:

1. Pears were peeled, cored, sliced, and homogenized for 1 minute in equal volume of 0.1 M sodium acetate pH 6, containing 0.2% sodium bisulfite and 1% PVPP.
2. Homogenate was filtered through cheese cloth, and pulp was collected. The filtrate was centrifuged at 15,000 g for 25 min and the pellet was collected and pooled with the pulp. The supernatant was discarded.
3. The pulp and pellet were suspended in 1 M sodium acetate pH 6 containing 1 M NaCl. The mixture was stirred overnight at 4° C, and the pH was maintained at 8.0 with addition of 2N NaOH.
4. Step 2 above was repeated, and the filtrate and supernatant were collected. The pulp and pellet were resuspended in 1 M sodium acetate pH 6 containing 1 M NaCl and centrifuged to collect the wash. The salt supernatant and wash was pooled, and the pellet/pulp was discarded.
5. The pooled salt extract was filtered through Whatman #1 filter paper, and dialyzed using a 6-8000 kda cut off membrane at 4° C for 72 h.
6. The dialyzed material was first precipitated with ammonium sulfate at 50% saturation. The solution was centrifuged. The pellet was resuspended and dialyzed using a 6-8000 kda cut off membrane.
7. The 50% saturated ammonium sulfate supernatant was saturated with ammonium sulfate to 100% saturation. The solution was centrifuged. The pellet was resuspended and dialyzed using a 6-8000 kda cut off membrane. The supernatant was discarded.
8. 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 CaCl₂, 2 mM MgCl₂, 2 mM MnCl₂) and applied to Concanavalin A-sepharose to bind the glycosylated proteins, including pPGIP. The proteins retained by the ConA sepharose were eluted with 250 mM alpha-methyl mannoside. Fractions containing PGIP activity were pooled and dialyzed.
9. A western blot showing the pear PGIP (pPGIP) band using polyclonal anti-pPGIP antibody is shown in **Figure 2**. The pPGIP band corresponds to a molecular weight of approximately 45 kDa, which is the expected size for pPGIP.
10. The protein preparation was concentrated to 1 mg/ml protein concentration using ultra filtration device fitted with a 10 kDa cutoff membrane.
11. PGIP was further purified using fast protein liquid chromatography (FPLC) using a Resource S cation exchange column. The concentrated protein preparation was loaded and the column was run at a rate of 4 mL/min. The column was equilibrated with 50 mM sodium acetate buffer pH 4.5 (buffer A) and eluted with a linear gradient of sodium chloride to a final concentration of 500 mM sodium chloride in buffer A.
12. A protein trace from the FPLC is shown in **Figure 3**.
13. The fractions collected from FPLC are being analyzed for PGIP activity and further processing (dialysis, concentration, etc).

Objective 2 - Calibrate the antibodies produced by the hybridoma clones to determine effective dilutions for use in detecting the pPGIP protein.

Will commence once the antibody has been generated.

Objective 3 - Use the antibody to detect transgenic pear PGIP in xylem sap of own-rooted and grafted grapevines.

Will commence once the antibody has been generated.

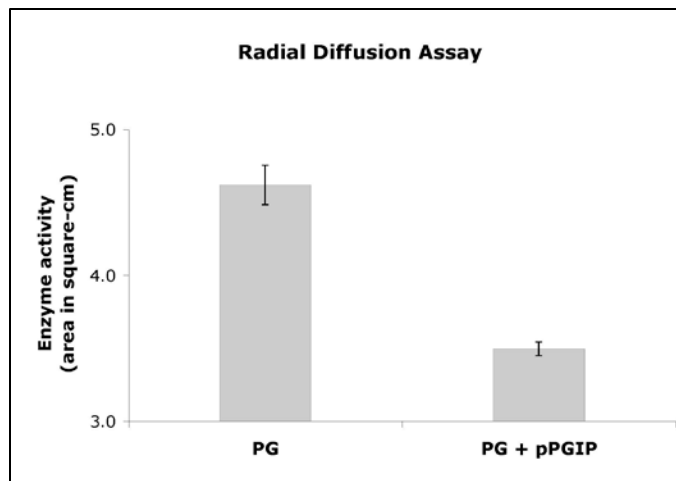


Figure 1. Results of a radial diffusion assay to determine the amount of pPGIP in a protein preparation from pear fruit.

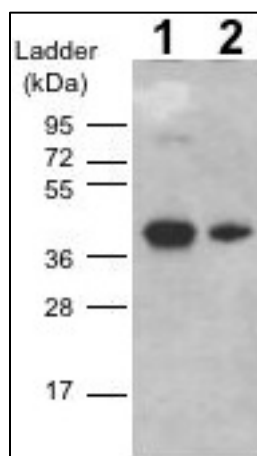


Figure 2. Immunoblot analysis of pPGIP. Lane 1: 80 ng protein after ammonium sulfate precipitation (50-100% fraction). Lane 2: 10 ng protein after the ConA purification step. Molecular weight ladder is indicated on the left. Proteins were separated on a SDS-PAGE (10%) gel and probed using pPGIP antiserum.

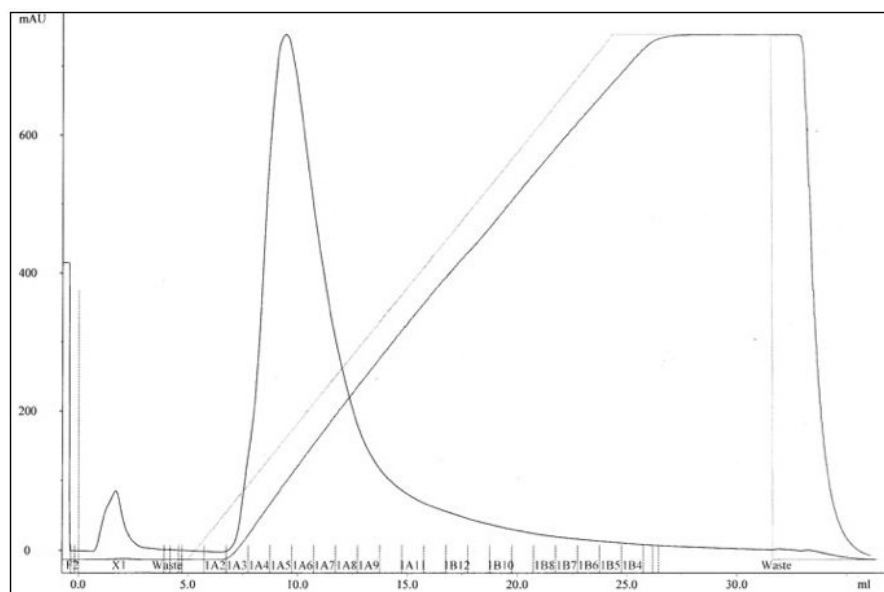


Figure 3. FPLC analysis of pPGIP preparation.

CONCLUSIONS

We are on track to complete the purification of the pear PGIP from green pear fruit tissue in the next month. We have consulted with Antibodies, Inc., about the amount of the protein needed to generate the monoclonal antibodies. The partially purified protein has been analyzed for its activity and been visualized by Western blots using the original polyclonal antibody we have used for more than 20 years. The protein migrates as a single band, and is largely separated from other proteins. This work should benefit a solution to the PD problem because it will allow for standard assays to evaluate the quantity, location and effectiveness of the PGIP delivered by the strategies of the collaborating groups. Since top priority has been given for the delivery from grafted rootstocks of PGIP as a PD control candidate, the four currently funded projects will be able to use the antibody to evaluate their strategies for delivering PGIPs to control the spread of *Xf* in the xylem network and thereby limit PD symptom progression in infected vines. Production of the monoclonal antibody by Antibodies, Inc. will begin once sufficient quantities of properly glycosylated, active PGIP have been purified. The monoclonal antibody will allow comparison of the approaches from different research groups and will allow accurate and PGIP-specific assessments of the potency of pear PGIP for limiting PD symptoms.

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FUNDING AGENCIES

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