Progress report for CDFA contract 10-278

I. PROJECT TITLE.

TOOLS TO IDENTIFY PGIPS TRANSMITTED ACROSS GRAPEVINE GRAFTS

PRINCIPAL INVESTIGATORS.

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II. Reporting Period:

1 March 2011 to 22 July 2011.

III. OBJECTIVES.

1. Using existing 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.

IV. SUMMARY OF MAJOR RESEARCH ACCOMPLISHMENTS AND RESULTS FOR EACH OBJECTIVE.

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). The purification steps are:

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^{o} 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^{o} 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.

In one preparation, 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 $MnCl_2$) and applied to Concanavalin A-sepharose to bind the glycosylated proteins, including pPGIP. Elution with 250 mM and 1 M methyl-α-D-mannopyranoside did not result in recovery of glycosylated pPGIP, probably due to improper binding to the Con A matrix. That preparation was abandoned and a second preparation was initiated. The preparation (ca. 100 ml) was similar and currently the ammonium sulfate precipitated proteins have been dialyzed and assayed for pPGIP activity

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 (*Bc*PG) 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). The concentration of proteins in the sample is low and the preparations still contain considerable polysaccharides which may impair binding to ConA sepharose. The excess polysaccharide may be because the pears used for this preparation were not completely green.

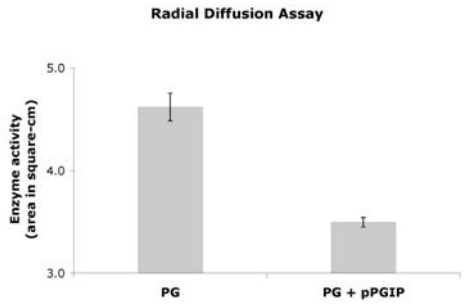

Figure 1. Results of a radial diffusion assay to determine the amount of pPGIP in a protein preparation from pear fruit.

Additional green pear fruit are now available and the purification will begin on these pears next week. The preparation of the protein is expected to take about 2 months and the generation of the antibody will take the remainder of the year.

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.

V. INTELLECTUAL PROPERTY ISSUES.

None are known.

VI. REFERENCES.

Abu-Goukh AA, Greve LC Labavitch JM 1983. Purification and partial characterization of "Bartlett" pear fruit polygalacturonase inhibitors. Plysiological Plant Pathology 23:111-122.

Taylor RJ and Secor GA. 1988. An Improved Diffusion Assay for Quantifying the Polygalacturonase Content of *Erwinia* Culture Filtrates. Phytopathology. 78: 1101-1103.

VII. HOW THIS WORK WILL CONTRIBUTE TO SOLVING THE PD PROBLEM IN CALIFORNIA.

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 PD control candidates, including polygalacturonase-inhibiting proteins (PGIPs), from grafted rootstocks. Four currently funded projects (two research projects and two field trials) use expression of PGIPs as a control

strategy to limit the spread of *X. fastidiosa* in the xylem network and thereby limit PD symptom progression in infected vines. A monoclonal antibody recognizing the pear fruit PGIP (pPGIP), the protein expressed in the grape lines in the four projects, is needed to detect, quantify, and observe the localization of the protein in transformed grapevines and in grafted vines with transformed rootstocks. Authentic pear PGIP protein from pear fruit is needed is needed to prepare this monoclonal antibody which can be maintained in perpetuity as a cell culture. 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 assessments of the potency of pPGIP for limiting PD symptoms.