- **TITLE OF REPORT: Interim Report for CDFA Agreement Number 10-0278**
- **TITLE OF PROJECT: TOOLS FOR IDENTIFYING PGIP TRANSMISSION FROM GRAPEVINE ROOTSTOCK TO SCION**
- **PRINCIPAL INVESTIGATOR:**

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- **TIME PERIOD COVERED BY REPORT:** The results reported here are from work conducted by March 2014.
- **INTRODUCTION:**

Pierce's Disease (PD) incidence has been associated with the spread of the causal agent, *Xylella fastidiosa* (*Xf*), throughout the xylem vasculature of infected grapevines. The spread from one vessel to the next utilizes cell wall modifying enzymes produced by the bacteria to degrade pit membranes separating adjacent vessels (Pérez-Donoso et al., 2010). One enzyme that degrades the polysaccharide portion of pit membranes is a polygalacturonase (XfPG), a well characterized PD virulence factor of *Xf* (Roper et al., 2007). Previous projects have analyzed the PG-inhibiting proteins (PGIPs) for their effectiviness in minimizing the damage caused by pathogens and pests on plants (Powell et al., 2000, Aguero et al., 2005), including damage caused by *Xf* in PD. Two field projects currently funded by the CDFA use pear fruit PGIP (pPGIP) to restrict *Xf* movement.

This project was designed to generate a new polyclonal antibody preparation that recognizes PGIPs in general and monoclonal antibodies that specifically recognize the pPGIP protein. The previous polyclonal antibody preparation was over 25 years old and little of the stock remained (Stotz et a., 1993). The monoclonal antibody is a necessary tool for the related field evaluation projects, including "Field evaluation of grafted grape lines expressing PGIPs" (PI Powell). The monoclonal antibodies will allow 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 from grafted rootstocks and comparisons of anti-PD strategies can be made knowing the amount of the active anti-PD protein in the tissues.

- **OBJECTIVES:**

Objective 1 – Purify pear fruit PGIP protein to use to generate new polyclonal and monoclonal antibodies.

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

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

- **ACTIVITIES, ACCOMPLISHMENTS AND RESULTS:**

Objective 1: Purify pear fruit PGIP protein to use to generate new polyclonal and monoclonal antibodies.

Because of budget limitations, we abandoned purification of the pear PGIP from transgenic Arabidopsis leaves engineered to express a tagged version of the protein.

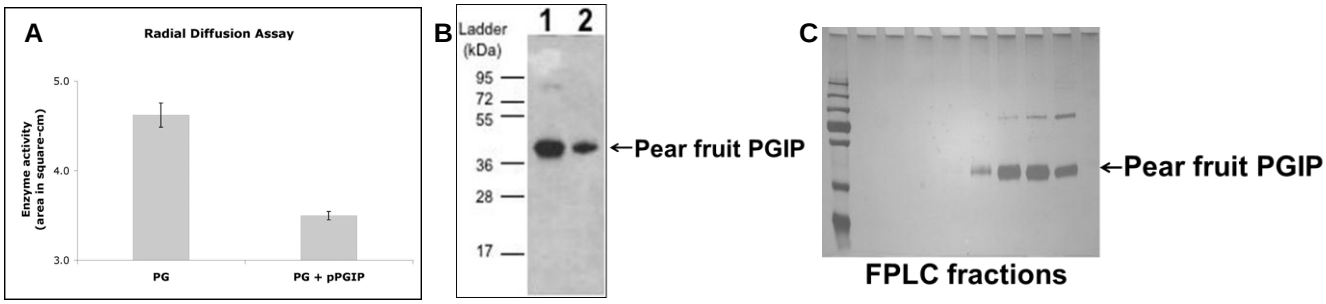

Figure 1. A. Partially purified pPGIP protein caused a 75% inhibition of *Botrytis cinerea* polygalacturonase activity. **B.** Immunoblot detection of pPGIP separated by SDS-PAGE (10%) and probed using a 25 year old polyclonal antiserum to pPGIP. Lane 1: 80 ng protein after ammonium sulfate precipitation. Lane 2: 10 ng protein after the ConA purification. **C.** Silver stained SDS-PAGE 10% gel. Lanes 2-10 are FPLC fractions. The PGIP protein band of 45 kDa molecular weight is seen. A band at 90 kDa is likely a PGIP dimer.

We purified sufficient active pear fruit PGIP (pPGIP) from immature green pears for evaluation of the antibodies prepared by Antibodies Inc. Approximately 195 µg of protein was obtained and the preparation is actively inhibits PGs produced in culture by the Del 11 strain of *B. cinerea*, as expected. Figure 1 shows results documenting the activity and purity of the protein. As described in Objective 2, we decided not to use this protein itself to develop monoclonal antibodies because of its extensive glycosylation, typical of plant proteins. Instead we have used the protein to determine the specificity of the antibodies generated to the peptides.

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

Based on the concern noted above that authentic pPGIP protein may not result in the generation of sufficiently specific anti-pPGIP monoclonal antibodies, we worked with Richard Krogsrud, CEO of Antibodies Inc., to identify hydrophilic peptide sequences in the pPGIP protein sequence that could be used as antigens. We selected 3 peptides (Figure 2) that would be specific to pPGIP and would be likely to assure that the monoclonal antibodies would not recognize other PGIPs. The three peptides were combined when they were used to raise the antibodies in order to optimize the chances of getting a specific and robust antibody. We also identified a peptide from the conserved amino end of the PGIPs.

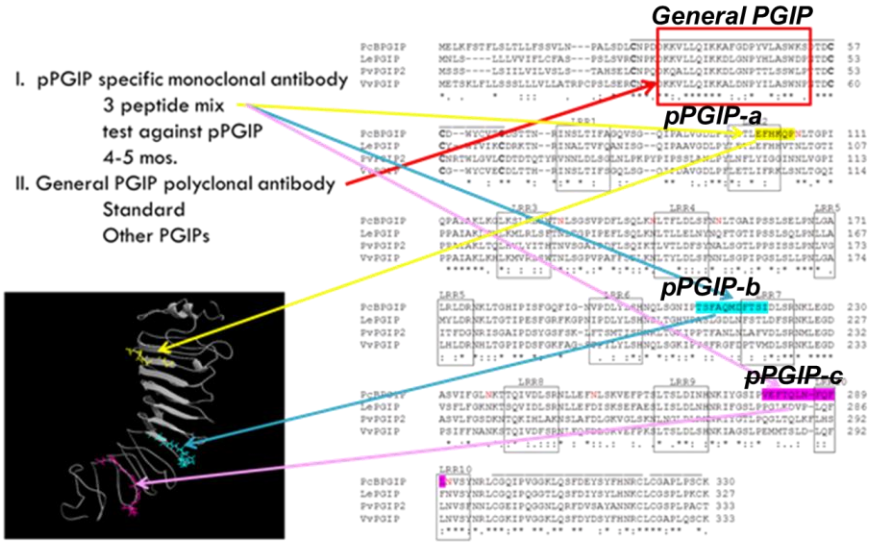

Figure 2. Amino acid sequence of pear (pPGIP), tomato (LePGIP), common bean (pvPGIP) and grape (vvPGIP) showing the location of the leucine-rich repeats (LRR) and the three pPGIP specific peptides (in yellow, blue and pink pPGIP-a, pPGIP-b, pPGIP-c) and the peptide common to all PGIPs (in red, General PGIP). Locations on the predicted 3-D structure of PGIP are shown.

We selected this peptide to generate a new polyclonal antibody to detect other PGIPs in addition to the pPGIP. The peptides were synthesized through subcontractors used by Antibodies Inc. although one of the pPGIP-specific peptides (pPGIPc) proved to be recalcitrant to conjugation. Antibodies Inc. developed hybridomas using the other two pPGIP-specific peptides (pPGIPa and pPGIPb) and delivered them to us in late September, 2013.

In May 2013, we received the first test bleed and pre-immune sera from the polyclonal antibody preparations generated against the general PGIP peptide. This antibody preparation is considered a general PGIP antibody because it is was generated in response to a conserved region at the amino end of the PGIP proteins. We have used the pre-immune serum on a western blot with protein extracts from tomato plants expressing pPGIP and the purified pear fruit pPGIP protein described above. The antibody specifically recognizes the purified pPGIP protein from pear fruit as well as the pPGIP protein expressed in the tomato variety "Cuatomate" lines 3-15 (Figure 3a). The antibody preparation does not detect tomato PGIPs in the "Cuatomate" material 3-8, which is not transformed and, therefore, does not express pPGIP. With the antibodies from the first test bleed, we were able to detect strongly just the pPGIP band in the same protein preparations used to check the pre-immune serum (Figure 3b). We detected no cross-reactivity with the pre-immune serum (Figure 3b). On 18 July, 2013, the final bleed sera were received from Antibodies Inc., and brought to UC Davis. They have been aliquoted, stored and distributed.

Only the peptide labelled pPGIPa in Figure 2 succeeded in raising antibodies that recognize that a peptide rather than the BSA-conjugate in an Elisa assay. Hybridoma clones 3H12, 6G2 and 7G5 recognize the pPGIPa peptide, LETLEFHKQPC. Material from clones 3H12 and 6G2 recognize pPGIP protein in ELISA analyses and will be assayed further once more material is available to find effective concentrations for Western blot analyses.

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

The western blot with the new polyclonal antibodies in Figure 3 contains proteins from the leaves of a tomato line expressing pPGIP; similar results have been obtained with xylem sap collected from the cut stem of the same plants. Efforts to collect xylem sap from pPGIP-expressing grapevines has yielded only a very small amount of protein and the expected greater sensitivity of the monoclonal antibodies is necessary to detect this pPGIP from grapevine xylem exudate, but we have not yet determined an appropriate dilutions of the cell line supernatants to do this. Howeve,r it is clear that the polyclonal antibody (Figure 4A) and at least one of the monoclonal antibody lines (6G2) recognize pPGIP protein in

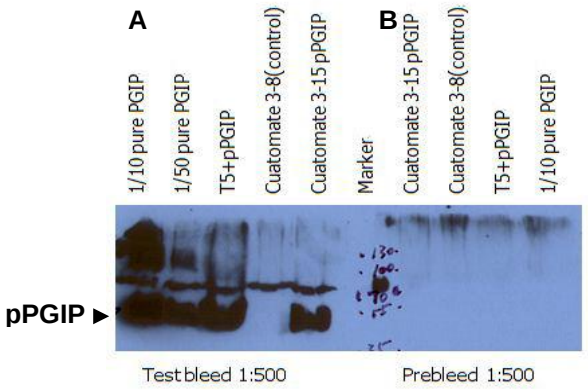

Figure 3. Image of western blot of proteins cross-reacted with (**A**) polyclonal antibodies from the first test bleed serum in response to the general PGIP peptide and (**B**) pre-bleed serum from the rabbits (B).

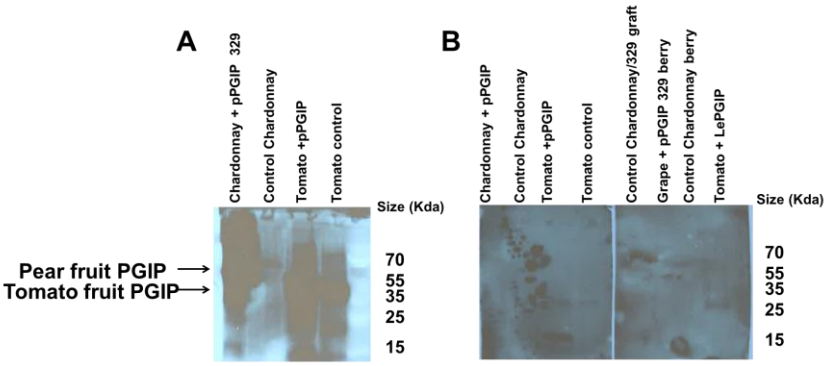

Figure 4. Western blots using (**A**) new polyclonal antibody to the General pPGIP peptide or (**B** left) the monoclonal antibody 6G2 or (**B** right) combined supernatants from the hybridoma lines. All proteins were from leaves except for two samples from grape berries. 10 mg protein was loaded per lane.

grape leaves and berries. Optimization of the dilutions of the monoclonal antibodies for use on Western blots continues. Activities for this objective will be concluded once more supernatants from the cell lines expressing the monoclonal antibodies are received at UC Davis.

- **CONCLUSIONS:**

In response to the strategy recommended by the Advisory Board to enhance the resistance of grapevines to PD, several field trial projects have used alternative approaches to optimally express plant genes for particularly effective PGIPs targeting the *X. fastidiosa* PG (*Xf*PG) in transgenic grape rootstocks. This project was designed to generate monoclonal antibodies that specifically recognize the pPGIP protein so that the amount of protein can be compared among different strategies, different plants and at different times. Using monoclonal antibodies is necessary for the multiple field trial projects evaluating the efficacy of pPGIP as an anti-Xf strategy. The antibodies allow for detection and quantification of pPGIP without cross-reactive interference from the native PGIP and will allow comparisons between groups. 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 from grafted rootstocks.

The goal of the project is to provide the resources needed for the field trial projects that are designed to help the California grape industry develop a strategy that uses plant genes to limit the damage caused by *Xf* and to mobilize this technology with non-transgenic vines grafted on the disease limiting rootstocks. The project's outcomes should provide growers with plants that resist PD and produce high quality grapes.

- **PUBLICATIONS:** None specifically on this but the material will be used in other publications.

- **RESEARCH RELEVANCE:**

The CDFA Pierce's Disease (PD) and Glassy-winged Sharpshooter Board's Research Scientific Advisory Panel review and RFPs gave priority to delivery proteins, including polygalacturonase-inhibiting proteins (PGIPs), from grafted rootstocks to control PD. Two currently funded projects use expression of PGIPs as control strategies to limit the spread of *X. fastidiosa* in the xylem network and, thereby, reduce PD symptom progression in infected vines. Monoclonal antibodies recognizing pear fruit PGIP (pPGIP), the protein expressed in the grape lines that are currently under evaluation in field trial studies, was needed to detect, quantify, and observe the localization of the protein in the transformed grapevines and in grafted vines with transformed rootstocks. In order to make comparisons between the different strategies to control *X. fastidiosa* spread, the amounts and efficacy of the pPGIP in the infected parts of the plant must be determined and a pPGIP recognizing monoclonal antibody allows measurements of the amounts of the protein. Authentic pPGIP protein from pear fruit could have been used prepare this monoclonal antibody which will be maintained in perpetuity as a cell culture, but we modified the approach and synthesized synthetic peptides from specific regions of the pPGIP protein to use as antigens. This approach assures that that antibodies recognize only pPGIP and not the endogenous grape PGIPs. Production of the monoclonal antibodies has been accomplished and has been partly tested for the specificity and strength of their recognition of properly glycosylated, active pPGIP protein from pear fruit and pPGIP expressed grape plants which we have been purified.

- **LAYPERSON SUMMARY:**

X. fastidiosa (*Xf*), the bacteria that causes Pierce's Disease (PD) in grapevines, utilizes a key enzyme, polygalacturonase (XfPG), to spread from one grapevine xylem vessel to the next, eventually leading to the development of PD symptoms because the bacteria multiply and interrupt the flow of nutrients and water through the vessels of the plant. PG-inhibiting proteins (PGIPs) produced by plants selectively inhibit PGs from bacteria, fungi, and insets. Our work (Abu-Goukh et al., 1983) identified a PGIP

(pPGIP) from pear fruit that at least partially inhibits the XfPG and we demonstrated that expression of pPGIP reduced PD symptom development in grapevines (Aguero et al., 2005). Current projects, including field trial evaluations, require a monoclonal antibody specifically recognizing the pPGIP protein in order to detect, quantify, and characterize the pPGIP protein delivered to the scion portion of grafted plants from rootstocks expressing the p PGIP (Aguero et al., 2005). The monoclonal antibody will allow the researchers to compare the amounts of the pPGIP protein at different times and places and thereby determine the protein's role in XfPG inhibition in grapevines. We have received a new polyclonal antibody that recognizes pPGIP and we have received monoclonal antibody preparations made to recognize specific and unique parts of the pPGIP protein. We purified active pPGIP from green pear fruit to test the antibody preparations.

- **STATUS OF FUNDS:**

Budget for Contract Extension Time Period – July 1, 2013 to June 30, 2014

	Amount in original budget ($)	Amount spent to date (1 July 2010-28 Feb 2014) ($)	Amount to be spent through current ending date 30 June 2014
Personnel			
Professional	950	771	0
SRA/Tech			
Lab Assistant			
Other			
Employee Benefits	29	204	0
SUBTOTAL (Personnel + Benefits)	979	975	0
Supplies and Expenses (Incl. Antibodies Inc. charges)	13,091	11,798	1,297
Equipment			
Travel			
Computer Time			
Other			
Indirect Costs*			
SUBTOTAL (Supplies, Expenses, Equipment, etc.)	13,091	11,798	1,297
TOTAL	14,070	12,773	1,297

- **INTELLECTUAL PROPERTY:** None appropriate for this material.

- **LITERATURE CITED:**

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