Project Title: Inhibition of *Xylella fastidiosa* polygalacturonase to produce Pierce's disease resistant grapevines.

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Research conducted: 4/1/07 to 8/1/08

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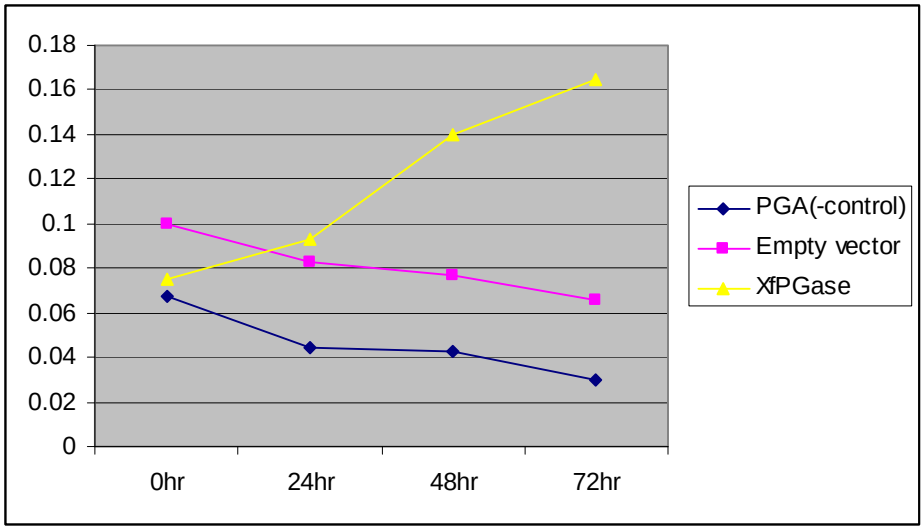
Objective 1: Isolate a sufficient amount of biologically active Xylella fastidiosa (Xf) polygalacturonase (PG) enzyme to conduct phage panning and PG-inhibition assays.

Although we now have in hand a functional Xf PG enzymatic activity assay we would still like to obtain greater amounts of active Xf PG. The first attempt at using a recently developed agroinfection-compatible tobacco mosaic virus protein expression system (Lindbow, 2007) did not provide us with active Xf PG. However, we produced two new Xf PG plant expression constructs that might improve our Xf PG yields using the plant expression system. The first involves changing the structure of the gene near the start codon to make it more conducive to use in plant expression systems. The second employs the use of a Rice Alpha Amylase signal peptide that will export Xf PG to plastids and extracellular compartments (Chen, et al., 2004). Targeting the Xf PG to these organelles could be important if the reason we are not getting active Xf PG is because the plant is recognizing it and degrading it in the cytoplasm. In addition to the plant expression system we are currently using to create a greater amount of active Xf PG, we are also generating constructs for *E. coli* expression systems that fuse Xf PG to Maltose Binding Protein (MBP) in the hopes the MBP will help overcome some of the insolubility issues we have encountered with previous bacterial fusion protein expression systems. The method we described in the previous reporting period for generating active Xf PG remains the method that delivers the most protein in active form, however we hope that one of these new strategies will provide us with an even greater amount of active protein. As was reported in the previous period we feel confident that the reducing sugar assays that we are using to detect Xf PG activity, dinitrosalicylic acid (Fig. 1) (Wang et al., 1997) and 3-Methyl-2-benzothiazolinonehydrazone methods (Anthon and Barrett 2002) will be suitable for the PG-inhibition assays.

Figure 1



Objective 2: Isolate M13 phage that possess high binding affinities to Xf PG from a M13 random peptide library.

We did extensive *in silico* analyses of the enzymatically active sites of several phytopathogenic bacterial and fungal PGs such as *Pectobacterium carotovora ssp. carotovora* and *Aspergillus aculeatus* (Pickersgill, et al., 1998, Cho, et al. 2001). The PGs from these other microbes have been well studied and structural studies have shown that the active site, consisting of roughly 8 amino acids, and the tertiary structure of the PGs are highly conserved across all fungal and bacterial PGs (Pickersgill, et al., 1998, Cho, et al. 2001, Shimizu, et al., 2002, Abbott and Boraston 2007). Furthermore, previous research using phage display technologies showed that many of the phage that bound to a variety of enzymes also bound to and inactivated the enzyme (Hyde-deRuyscher et al, 1999).

Having a very good idea of where the Xf PG active site may be located on the protein, and which amino acids are involved in catalysis and substrate binding, we had commercially synthesized two 14mer peptides derived from the Xf PG sequence, one which will target the active site directly and a second that will target an area providing entry into the active site (Figure 2). Additionally these peptides were injected into rabbits to create polyclonal antibodies. These antibodies were used in a western blot that confirmed that the antibodies created against each 14-mer peptide could also recognize full length Xf PG (Figure 3).

Figure 2. Sequences of synthesized peptides

Peptide	Sequence
Peptide 1	DSPNSNGLQMKSDAC
Peptide 2	STGDDHVAIKARGKC

Figure 3. Western blot analysis of BSA-conjugated Xf PG peptide antibodies

1 2 ladder 1 2 ladder

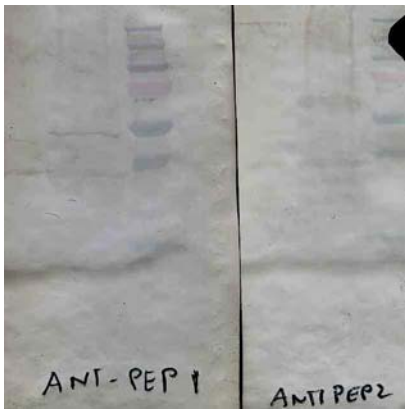


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Fig 3. Lane 1 is E.coli lysate containing no XF PG. Lane 2 is E. coli lysate containing recombinant Xf PG. Arrow represents the location of Xf PG band.

We have completed the phage panning procedure for peptide 2 using the Tomlinson I and J single chain fragment variable antibody (scFv) libraries provided by Paul Feldstein of the Bruening laboratory at UC Davis. These libraries are a collection of over 100 million different random scFv sequences attached to the P-3 binding protein of bacteriophage M13 with the J library being the more diverse. At the end of the third round of selection with BSA conjugated peptide 2 as the target, polyclonal ELISA analysis showed that each library (I and J) of scFvs showed a higher binding affinity to the BSA conjugated peptide 2 than to BSA alone, or other negative controls. Using the ELISA results, 90 individual colonies from each library were picked from the third round phage pool and used in a monoclonal ELISA to determine which monoclonal scFvs had the highest binding efficiencies to the BSA-conjugated PG peptide (Table 1). Eight clones that had the highest ELISA absorbance readings were chosen for sequencing. We have currently only sequenced the heavy chain variable portion of the scFv and although none of the eight clones from each of libraries shared the same exact sequence they did have similarities to each other. We are in the process of sequencing the light chain portion of these clones. Our next step is to use these monoclonal phages as the primary antibodies in western blot analyses to confirm that they can bind to Xf PG in ELISA and westrn blots analyses. Once all variable regions have been sequenced and Xf PG binding clones confirmed by western blot, positive PG binding clones will be tested for their ability to inhibit Xf PG activity *in vitro.*

Table 1. ELISA values of selected Xf PG binding phage.

clone	Pep2-BSA	BSA	Recombinant Xf PG E.coli lysate
I-1	0.594	0.204	0.437
I-2	0.632	0.168	0.502
I-3	0.44	0.228	0.616
I-4	0.542	0.302	0.669
J-1	0.44	0.28	0.402
J-2	0.434	0.185	0.405
J-3	0.476	0.166	0.572
J-4	0.327	0.186	0.431

Objective 3: Determine if selected M13 phage and the gp38 M13 protein which mediates phage binding to Xf PG can inactivate PG activity in vitro.

We currently have candidate monoclonal phage that we will soon be using in inhibition assays using the methods described in Objective #1

Objective 4: Clone anti-Xf PG gp38 protein into an Agrobacterium binary vector and provide this construct to the UCD Plant Transformation facility to produce transgenic SR1 tobacco and Thompson Seedless grapevine.

Once suitable inhibitory phage peptides are identified in Objective 3 we can begin Objective 4.

Objective 5: Determine if anti-Xf PG gp38 protein is present in xylem sap of transgenic plants.

Objective 4 needs to be completed before work on Objective 5 can begin.

Objective 6: Mechanically inoculate transgenic plants with Xf and compare Pierce's disease development with inoculated, non-transgenic control plants.

All previous objectives must be completed before we can start Objective 6.

Publications or reports resulting from the project:
2007 Pierces Disease Research Symposium Report
Presentations on Research:
2007 Pierces Disease Research Symposium

Research Relevance

Xylella fastidiosa (Xf) is a xylem-limited, gram-negative bacterium that causes Pierce's Disease (PD) in grapevines. In order to cause disease the bacterium must multiply and colonize xylem elements of a susceptible plant host such as *Vitis vinifera*. In order to move from one xylem element to another Xf must degrade xylem pit membranes that separate adjacent elements. Pit membranes (primary cell walls) are composed of cellulose and hemi-cellulose microfibrils and pectic polymers that mediate the porosity of the pit membrane (Baron-Epel, et al. 1988; Buchanan et al. 2000; Zwienecki et al. 2001). The primary enzyme that is needed to degrade pectic polymers in the xylem pit membrane is polygalacturonase (PG). Dr. Caroline Roper showed that if the gene encoding Xf PG was disrupted that the resulting PG-mutant was completely non-pathogenic in grapevines (Roper, et al, 2007). This result suggests that if grapevines expressed sufficient amounts of a "factor", such as a peptide or protein in xylem sap that could inactivate Xf PG, then Xf cells introduced by insect vectors could not move and subsequently cause PD. Additional evidence supporting this hypothesis was provided by Aguero, et al., (2005) who produced transgenic grapevines that expressed a pear fruit polygalacturonase-inhibiting protein (PGIP). Following mechanical inoculation with virulent strains of Xf, the transgenic vines had reduced leaf scorching, lower Xf titers and better re-growth after pruning than untransformed controls. They also found that PGIP was detected and functional in non-transformed grapevines that were grafted onto transgenic PGIP vines that served as rootstocks. This suggests that the constructs they used could deliver an anti-PG protein through the graft union and into a commercial grape scion variety; an observation that suggests transformed rootstocks could be developed while leaving the horticultural properties of wine varieties unchanged. While the PGIP transgenics were not completely immune to Xf, the significant reduction in disease severity, coupled with the PG knockout experiments performed by Roper, provides strong support for identifying additional molecules that can effectively knock out the enzymatic function of Xf PG. We propose to identify unique peptides or single chain fragment variable (scFv) antibodies (Nissim, et al, 1994) expressed on the surface of gp38 protein that mediate binding of M13 to *E. coli* using commercially available library kits. Recombinant gp38 proteins expressing anti-PG activity will then be expressed in transgenic grapevine rootstocks using vectors similar to those used by Aguero, et al, 2005. Transgenic plants will then be inoculated with Xf and disease progression compared with Xf-inoculated non-transgenic controls. The ultimate goal of this project is to provide a novel form of resistance against Pierce's disease that can be expressed in grapevine rootstocks and provide disease protection to grafted, fruit-bearing cultivars.

References cited

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Lay Summary of Current Year's Results:

This period we have again made progress on Objectives 1-3. We are continuing to explore different methods of expression to further increase the amount of soluble, active XF PG using a plant expression system and MBP fusion constructs. Additionally, we have completed the panning procedure for peptide 2 and have produced our first candidate monoclonal phage to use in the inhibition assays. We plan on starting the panning procedure on the rest of our proposed targets as soon as we get the results from our first set of inhibition assays.

Summary and status of intellectual property produced during this research project:
No intellectual property has been produced during this research period.