

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

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Research conducted: 8/15/08 to 3/1/09

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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.

The method we described previously for generating active Xf PG remains the method that delivers the most protein in active form, however we would still like to obtain greater amounts of active Xf PG. The biggest problem we have had with expressing Xf PG in *E. coli* is that much of the recombinant Xf PG aggregates in inactive, insoluble, inclusion bodies. We have been able to overcome the solubility problem of expressing Xf PG in *E. coli* and can now produce large quantities of purified soluble Xf PG. We were able to first solubilize recombinant protein in a denaturing buffer. The Xf PG was then loaded onto a FPLC and bound to a metal affinity column (Ni^{2+}). While the Xf PG was bound to the column we slowly renatured it using buffer exchange to remove the denaturant. Once all of the denaturant was removed we eluted purified soluble Xf PG (Fig 1). Initial assays of the FPLC solubilize and purified Xf PG indicated that the imidazole present in the elution buffer is interfering with our reducing sugar Xf PG activity assays. We are currently in the process of removing the imidazole from the purified Xf PG so we can correctly access the activity of these products.

Despite considerable effort, our attempts at using the agroinfection-compatible tobacco mosaic virus protein expression system (Lindbow, 2007), did not provide us with active Xf PG. We were able to produce and purify Xf PG from our constructs, however we only obtained a small amount and it did not show adequate activity in our colorimetric assays.

As was reported previously we feel confident that the reducing sugar assays that we are using to detect XfPG activity dinitrosalicylic acid (Wang et al. 1997) and 3-Methyl-2-benzothiazolinonehydrazide methods (Anthon and Barrett 2002) will be suitable PG-inhibition assays.

Figure 1

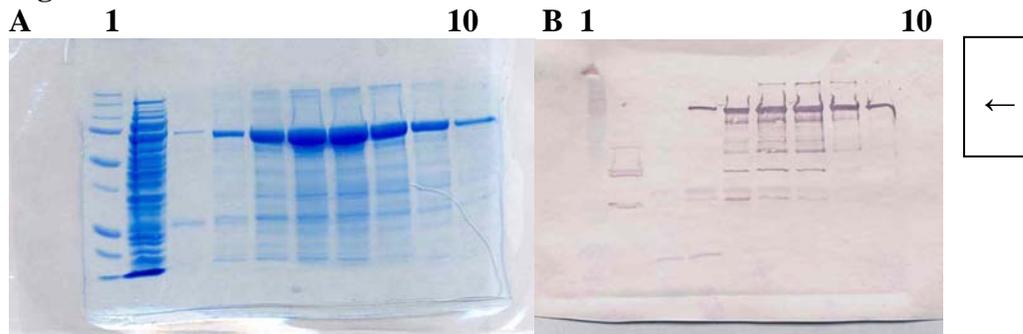


Figure 1. A: Coomassie stained Polyacrylamide gel electrophoresis of purified, soluble, recombinant Xf PG fractions from FPLC refolding/ purification experiments, arrow denotes the correct size of recombinant Xf PG. Lane 1 Bio-Rad dual color protein ladder, lane 2 column flow through, lanes 3-10 soluble Xf PG fractions **B:** Western blot analysis of purified, soluble Xf PG fractions from FPLC refolding/purification experiment using Xf PG polyclonal antibodies. Lane 1 Bio-Rad dual color protein ladder, lane 2 column flow through, lanes 3-10 soluble Xf PG fractions

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

We have done 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 amino acids, 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 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 substrate entry into the active site (Fig 2).

Peptide	Sequence
Peptide 1	DSPNSNGLQMKSDAC
Peptide 2	STGDDHVAIKARGKC

Figure 2. Sequences of synthesized peptides.

Additionally, these peptides were injected into rabbits to create polyclonal antibodies. These polyclonal 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 (Fig 3).

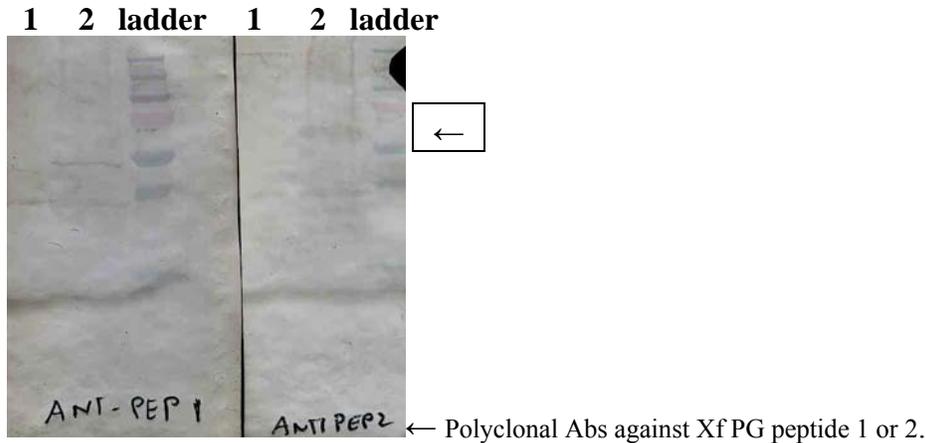


Figure 3. Western blot analysis of recombinant Xf PG developed with polyclonal Abs against 2 Xf PG peptides. Lane 1 is *E. coli* lysate containing no Xf PG. Lane 2 is *E. coli* lysate containing recombinant Xf PG. Arrow represents location of Xf PG band.

We have completed the phage panning procedure for peptide 2 using the Tomlinson I and J scFv libraries. At the end of the third round of selection a polyclonal ELISA with BSA conjugated peptide 2 as the target was run which showed that each library (I and J) of scFvs showed a higher binding affinity to BSA conjugated peptide 2 than to BSA alone, or to the wells of the plate. With this knowledge 90 individual colonies from each library were picked from the third round phage pool and used in a monoclonal ELISA to determine which phage monoclonal scFvs had the highest binding efficiencies to the Xf PG peptides. The eight clones from each library (I and J) providing 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 exact same sequence they did have similarities to each other. We are in the process of sequencing the light chain portion of these clones.

The eight monoclonal phages from each library (I and J) were then used individually as the primary antibodies in a western blot to confirm that monoclonal phage selected as binding to the 14-mer peptide 2 conjugated to BSA would also be able to bind to full length recombinant PG (Fig 4). Now that we have monoclonal phage that can bind to XfPG we will finish sequencing the variable regions and begin testing the efficacy of each monoclonal phage to inhibit Xf PG activity *in vitro*.

Because the active sites in many fungal and bacterial PGs are highly conserved, we decided to conduct parallel panning experiments using highly purified, biologically active, *Aspergillus aculeatus*. We have finished the first round of panning with the *A. aculeatus* PG. Once we have candidate inhibitory phage from this panning experiment we will test their ability to inhibit Xf PG *in vitro*.

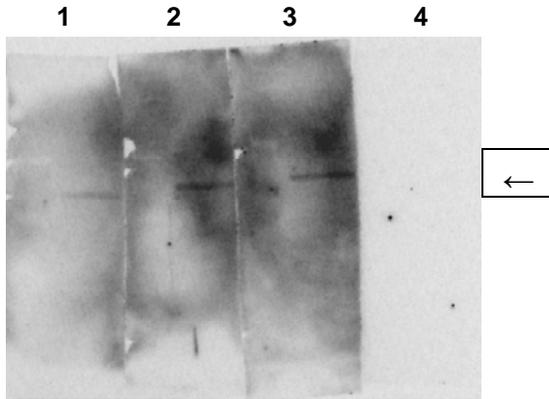


Figure 4. Western blot analysis of 3 representative monoclonal scFv phages(J-library).Panels 1-3 are *E. coli* lysate containing recombinant Xf PG each membrane strip was reacted with a single, unique monoclonal phage from the third round of panning . Arrow represents location of XfPG band. Molecular weight markers (not visualized) are on the left side of each gel strip. Panel 4 is a conjugate control that did not use any monoclonal phage as the primary antibody.

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 have sequenced the heavy chain variable regions of the 16 candidate monoclonal phage and although none of the eight clones from each of libraries shared the exact same sequence they did have similarities to each other. We are in the process of obtaining the sequences of the light chain variable portions. Once all variable region sequences have been determined we will use the monoclonal phages in Xf PG inhibition assays. Once a candidate phage is found that can inhibit Xf PG *in vitro* we will then express the scFv protein alone and determine if the protein itself can also inhibit XfPG activity *in vitro*. We will then be able to clone the anti-Xf PG protein into an *Agrobacterium* binary vector and provide this construct to the UCD Plant Transformation facility to produce transgenic SR1 tobacco and Thompson Seedless grapevines. Once we have transgenic plants we will be able to complete Objectives 5 and 6.

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 discovered 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:

2008 Pierces Disease Research Symposium Report

Presentations on Research:

2008 Pierces Disease Research Symposium

A poster describing results to date on this project will be presented at the 2009 American Phytopathological Society meetings in Portland, OR.

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 a strong rationale 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.

Lay Summary of Current Year's Results:

During this reporting period we have again made considerable progress on Objectives 1-3. We have been able to overcome the solubility issues with expression of Xf PG in *E. coli* using FPLC techniques. We now are able to produce large quantities of soluble purified Xf PG and hope our activity assays will prove that it is also in an active form. Additionally, we have completed the panning procedure for peptide 2, shown that the antibodies raised against it can bind to full length Xf PG, and thus have produced our first candidate monoclonal phage to use in the inhibition assays. We hope to soon have enough active Xf PG to test these phages in inhibition assays.

Status of Funds: Approximately ½ of the first year's funds have been spent to date.

Summary and status of intellectual property produced during this research project:

No intellectual property has been produced during this research period.

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