INHIBITION *OF XYLELLA FASTIDIOSA* POLYGALACTURONASE TO PRODUCE PIERCE'S DISEASE RESISTANT GRAPEVINES

Principal Investigator:
Bruce Kirkpatrick
Department of Plant Pathology
University of California
Davis, CA
bckirkpatrick@ucdavis.edu

Cooperators:
Jeremy Warren
Department of Plant Pathology
University of California
Davis, CA
jgwarren@ucdavis.edu

Paul Feldstein
Department of Plant Pathology
University of California
Davis, CA
pafeldstein@ucdavis.edu

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ABSTRACT
Polygalacturonases (PG) (EC 3.2.1.15), catalyze the random hydrolysis of 1, 4-alpha-D-galactosiduronic linkages in pectate and other galacturonans. *Xylella fastidiosa* (*Xf*) possesses a single PG gene, *pglA* (PD1485) and *Xf* mutants deficient in the production of PG lose pathogenicity and have a compromised ability to systemically infect grapevines. We have cloned the *pglA* gene into a number of protein expression vectors and a small amount of active recombinant PG has been recovered, unfortunately most of the protein expressed is found in inclusion bodies in an inactive form. The goal of this project is to use phage panning to identify peptides or single chain fragment variable (scFv) antibodies that can bind to and inhibit *Xf* PG. Once peptides or scFvs are discovered that can inhibit PG activity *in vitro* these peptides will be expressed in grapevine rootstock to determine if the peptides can provide protection against Pierce's disease (PD).

INTRODUCTION
Polygalacturonases (PG) have been shown to be virulence factors of a number of plant pathogenic bacteria including *Ralstonia solanacearum*, *Xanthomonas campestris*, and *Erwinia carotova* (Huang and Allen 2000; Dow et al. 1989; Lei et al. 1985). *Xylella fastidiosa* (*Xf*) possesses a single PG gene *pglA* (PD1485), and mutation of this gene results in a loss of pathogenicity and reduced ability to systemically infect grapevines (Roper et al. 2007). In order for *Xf* to systemically infect a grapevine, it must break down the pit membranes that separate individual xylem elements. Pectic polymers determine the porosity of the pit membrane (Baron-Epel, et al. 1988; Buchanan et al. 2000) and *Xf* PG allows the bacterium to breakdown the pectin in these membranes. The premise of this research is to identify a peptide that can be expressed in the xylem of a grapevine that can suppress *Xf* PG activity thus limiting the ability of *Xf* to spread systemically through grapevines and cause PD.

To accomplish this we will use phage display of a random dodecapeptide library and a scFv antibody library attached to the coat protein gp38 of M13 phage in a phage panning experiment using active recombinant *Xf* PG as the target. After three rounds of panning, phage that show a high binding affinity for *Xf* PG will be screened for their ability to inactivate PG activity *in vitro* in reducing sugar assays. Once a suitable inhibitory peptide, or the peptide embedded in a small protein carrier, is discovered it will be cloned into an Agrobacterium binary vector and used to transform tobacco and grapevines by the UCD Plant Transformation Facility. These transgenic plants will then be inoculated with *Xf* and the progression of PD symptoms will be compared to non-transgenic plants. If significant disease inhibition is shown, we will use these transgenic grapevines as rootstock to determine if they can also provide resistance to grafted non-transgenic *Vitis vinifera* scions.

OBJECTIVES
1. Isolate a sufficient amount of biologically active *Xf* polygalacturonase (PG) enzyme to conduct phage panning and PG-inhibition assays.
2. Isolate M13 phage that possess high binding affinities to *Xf* and/or *Aspergillus aculeatus* (AA) PG, or synthetic peptides specific for the active sites of several PGs, from a M13 random peptide and scFv library.
3. Sequence candidate binding phage and determine if selected M13 phage and the gp38 M13 protein which mediates phage binding to *Xf* PG can inactivate PG activity *in vitro*.
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 grapevines.
5. Determine if anti-*Xf* PG gp38 protein is present in xylem sap of transgenic plants.
6. Mechanically inoculate transgenic plants with *Xf* and compare Pierce's disease development with f-inoculated, non-transgenic control plants.

RESULTS
Objective 1.
Although we now have in hand a 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 (Lindbo, 2007) did not provide us with active *Xf* PG. However, we have produced a new *Xf* PG plant expression construct to help improve our yields using the plant expression system. This construct 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

areas 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 also generating constructs for an *E. coli* expression system that fuses *Xf* PG to Maltose Binding Protein (MBP) in the hopes the MBP will help overcome some of the insolubility issues we have encountered with other *E. coli* protein expression systems. The method we described previously 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 a greater amount of active protein.

As reported in the previous PD/GWSS Proceedings, we feel confident that the reducing sugar assays that we are using to detect *Xf* PG activity dinitrosalicylic acid (Wang et al. 1997) and 3-Methyl-2-benzothiazolinonehydrazone methods (Anthon and Barrett 2002), will be suitable for the PG-inhibition assays.

Objective 2.
We have done extensive *in silico* analyses of the enzymatic 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 consists of roughly eight 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 is likely located on the PG protein, and which amino acids are involved in catalysis and substrate binding, we had synthesized two 14-mer 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. Additionally, these peptides were injected into rabbits to create polyclonal antibodies. These polyclonal antibodies were used in Western blot analyses that confirmed that the antibodies created against each 14-mer peptide could also recognize full length *Xf* PG (**Figure 1**).

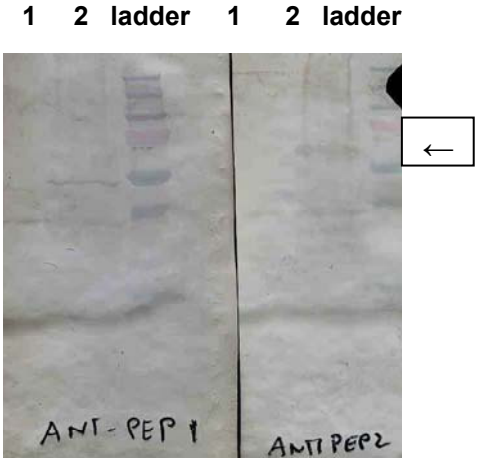

Figure 1. Western blot analysis of polyclonal antibodies to *Xf* PG peptides 1 and 2. Lane 1 is *E.coli* lysate containing no *Xf* PG. Lane 2 is *E. coli* lysate containing recombinant *Xf* PG. Arrow represents location of *Xf* PG band.

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 scFv's showed a higher binding affinity to BSA conjugated peptide 2 than to BSA alone, or to other negative control wells in the plate. With this knowledge, 90 individual colonies from each library were picked from the third round phage pool and used in a monoclonal based ELISA to determine which monoclonal scFvs had the highest binding efficiencies for the *Xf* PG peptides. Eight clones from each library (I and J) providing the highest ELISA absorbances 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 raised against the 14-mer peptide 2 conjugated to BSA would also be able to indentify full length recombinant PG (**Figure 2**) (Tanaka et al. 2002). Now that we have monoclonal phage that can bind to *Xf* PG, we will finish sequencing the variable regions and begin testing the efficacy of each monoclonal phage to inhibit *Xf* PG activity *in vitro*.

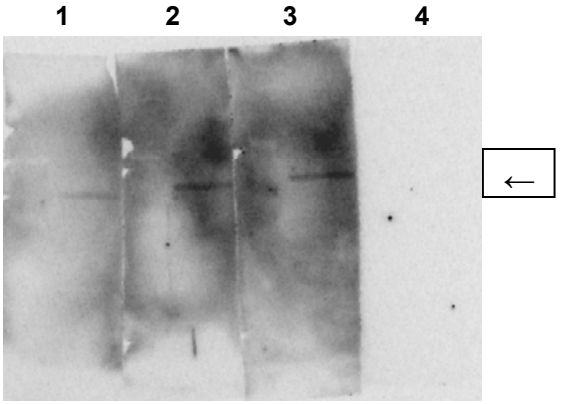

Figure 2. Western blot analysis of three representative monoclonal scFv phages from the J-library. Lanes 1-3 are *E. coli* lysate containing recombinant Xf PG, each membrane strip was reacted with a single monoclonal phage from the third round of panning. Arrow shows the location of the *Xf* PG protein. Molecular weight markers are on the left side of each gel strip. Lane 4 is a conjugate control that was not reacted with any monoclonal phage.

Objectives 3-6.

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 *Xf* PG 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.

CONCLUSIONS

We have made good progress thus far in identifying suitable PG activity assays to use in the PG-inhibition assays. We are currently exploring different plant and *E. coli* protein expression systems to generate more active PG to use in phage panning and activity assays. We have identified 16 candidate scFv phage, by panning against peptide 2 conjugated to BSA, that are capable of binding to full length *Xf* PG in Western blot analyses. These phage will now be used in *in vitro Xf* PG inhibition assays identified in Objective 1. If one of the candidate phage can inhibit *Xf* PG activity *in vitro,* then we will transform tobacco and grapevines with the peptide(s) and determine if the expressed anti-PG peptides are present in xylem sap and evaluate their potential for providing resistance to Pierce's disease.

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