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

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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 result in lost pathogenicity and 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 enzymatically 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 antibody (scFv) libraries 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 root stock to determine if the peptides can provide protection to the plant from Pierce's disease.

LAYPERSON SUMMARY
This period we have again made progress on Objectives 1-3. We have been able to overcome the solubility issues with expression of *Xylella fastidiosa (Xf)* PG in E. coli using FPLC techniques. We now are able to produce large quantities of soluble purified *Xf* PG. We also hope that the *Pichia pastoris* yeast expression system we are currently using will provide us with active *Xf* PG. Additionally; we have completed the panning procedure for peptide 2 and the commercially available *Aspergillus aculeatus* PG. We have shown that the antibodies raised against peptide 2 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

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 lost 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 Pierce's disease (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 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 compared to non transgenic plants in PD symptom progression. If significant disease inhibition is shown we will use these transgenic grapevines as rootstock and see if they can also provide resistance to grafted scions.

OBJECTIVES:
1. Isolate a sufficient amount of biologically active *Xf* 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 peptidesspecific 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 PD development with inoculated, non-transgenic control plants.

RESULTS AND DISCUSSION

Objective 1.

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 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 bound to a FPLC 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 the all of the denaturant was removed we eluted purified soluble *Xf* PG (**Figure 1**). We have not been able to detect polygalacturonase activity in assays of the FPLC solubilized and purified *Xf* PG thus far, however because the protein is soluble we are continuing our efforts to detect activity using this preparation. We are also currently using this solubilized *Xf* PG in panning experiments with the Tomlinson I and J phage libraries.

Additionally, we are using a *Pichia pastoris* yeast expression system to produce active PG. The *Pichia pastoris* expression system has a few advantages; it can form disulfide bonds in the cytoplasm unlike *E. coli* and it is able produce glycosolated proteins which an E. coli expression system cannot. Additionally, *Pichia pastoris* is able to secrete recombinant proteins into the medium making the following protein purification and activity assays steps easier. We are currently screening our *P. pastoris* expression constructs for *Xf* PG production.

As was reported previously 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.

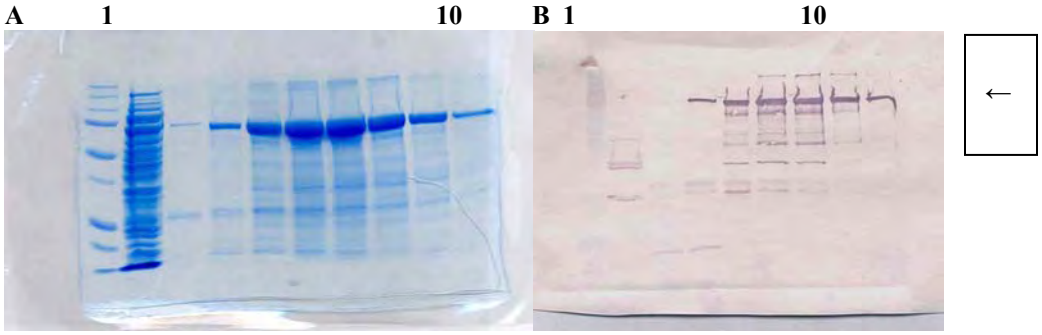

Figure 1. **A:** Coomassie stained Polyacrylamide gel electrophoresis of purified, soluble *Xf* PG fractions from FPLC refolding/ purification experiment, arrow denotes correct *Xf* PG band size. Lane 1 Bio-rad dual color protein ladder, lane 2 column flow through, lanes 3-10 soluble *Xf* PG fractions **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.

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 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 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 entry into the active site. 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 (**Figure 2**).

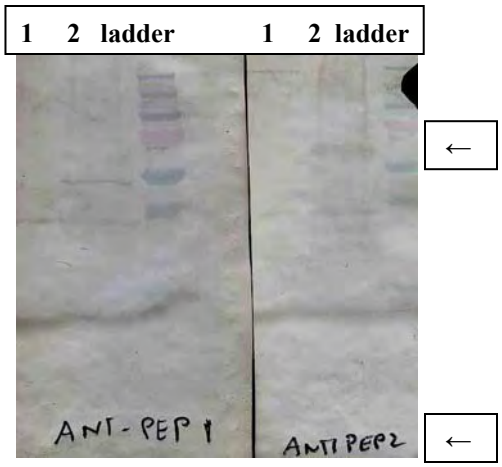

Figure 2. Lane 1 is *E.coli* lysate containing no *Xf* PG. Lane 2 is *E. coli* lysate containing recombinant *Xf* PG. Arrow represents the location of the *Xf* PG band.

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 monoclonal scFvs had the highest binding efficiencies. 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 raised against the 14-mer peptide 2 conjugated to BSA would also be able to indentify full length recombinant PG (**Figure 3**) (Tanaka et al 2002).

Additionally, we have finished phage panning against the commercially available *Aspergillus aculeatus* PG and are nearly finished panning against the solublized *Xf* PG. We are currently screening the monoclonal phages for specificity to *Aspergillus aculeatus* PG and *Xf* PG. Once we confirm specificity for either target we will use these phages in PG inhibition assays.

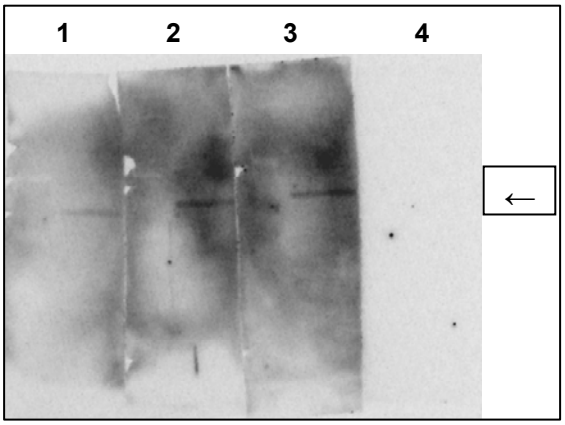

Figure 3. Western blot analysis of three representative monoclonal scFv phages (J-library). Lanes 1-3 are *E. coli* lysate containing recombinant *Xf* PG each membrane strip was reacted with a single monoclonal phage from the third round of panning. Arrow represents location of *Xf* PG band. Molecular 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 finding suitable PG activity assays to use in the PG-inhibition assays. We are currently exploring different expression systems to generate more active PG to use in phage panning and activity assays. We have acquired 16 candidate scFv phage, by panning against peptide 2 conjugated to BSA that are capable of indentifying full length *Xf* PG that we will be using in *Xf* PG inhibition assays, as we have described previously. If one of the candidate phage can inhibit *Xf* PG activity *in vitro* then we can transform grapevines with the peptide and determine if they provide plants with resistance to Pierces disease.

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