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 lose pathogenicity and are compromised in their ability to systemically infect grapevines. We have cloned the *Xf 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 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 protect the plant against PD.

LAYPERSON SUMMARY
This period we have made significant progress on Objectives 1-3. Most importantly we seem to have made progress on what has been the biggest obstacle thus far in this project, which is creating enough enzymatically active *Xf* PG to pan and test our putative inhibitory phage against. Initial *Xf* PG over-expression experiments in *Xylella fastidiosa* (*Xf)* have been encouraging. Once we confirm the results by repeating our activity assay we should have the ability to produce a large quantity of enzymatically active *Xf* PG. We can then test the efficacy of the inhibitory phage we have obtained from panning against the peptides representing the active site of *Xf* PG.

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* polygalacturonase (PG) enzyme to conduct phage panning and PG-inhibition assays.
2. Isolate M13 phage that possess high binding affinities to *Xf* and/or 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
4. which mediates phage binding to *Xf* PG can inactivate PG activity in vitro.
5. Clone anti-*Xf* PG gp38 protein into an Agrobacterium binary vector and provide this construct to the
6. UCD Plant Transformation facility to produce transgenic SR1 tobacco and Thompson Seedless
7. grapevines.
8. Determine if anti-*Xf* PG gp38 protein is present in xylem sap of transgenic plants.

9. Mechanically inoculate transgenic plants with *Xf* and compare PD development with inoculated, non-transgenic control plants.

RESULTS

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

Our previous work in trying to express *Xf* PG using commercially available *E. coli* and *P. pastoris* expression systems, as well as, an agroinfection-compatible Tobacco mosaic virus protein expression system (Lindbo 2007) did not provide us with a sufficient amount of active enzyme. An inducible expression system has not yet been developed for *Xf*; however previous work has shown that it is possible to over-express proteins in *Xf* (Newman et al 2003). Newman et al. were able to stably over-express the green fluorescent protein (GFP) in *Xf*; because of this we decided it may be a viable option to over-express *Xf* PG in a similar manner.

Modifying the same vectors used to construct GFP over-expressing *Xf* with the *Xf* PG gene and using the transformation protocols of Matsumoto et al. (2009) we have generated 22 putative *Xf* PG over expressing transformants. Transformants were tested by colony PCR to confirm that they contained the correct size insert. Fourteen of the 22 transformants were grown in PD3 Kan for 10 days at 28C. The cultures were centifuged at 10,000g for 10 minutes and the media fraction was collected. One ml of media fraction was concentrated using TCA precipitation resulting in a 20X concentration of the media fraction, these samples were then analyzed by polyacrylamide gel electrophoresis and Western blot analysis. Media fractions from two of the transformants were concentrated 20X using centrifugal concentrators with a 9 Kd molecular weight cut off. These concentrated samples were used in a PG cup plate assay and one of the transformants *XF*-6 appears to be enzymatically active (**Figure 1**) (Taylor and Secor 1988). We have repeated this experiment with similar results; however, the amount of PG produced is still rather small. In order to create a larger amount of active enzyme we are currently moving the constructs onto plasmids known to replicate in *Xylella*, e.g., pBBR1MCS-5 and p*XF*20-PemIK (Kovach et al. 1995, Reddy et al. 2007, Stenger in press). If we can show these results to be repeatable we are confident that using these transformants we can easily produce a large amount of enzymatically active *Xf* PG for use in this experiment as well as to others who need this enzyme for use in their work.

Additionally, we are using the pProbe vector series to characterize the *Xf* PG promoter region (Miller et al., 2000). When a promoter on the plasmid is active or activated *gfp* will be produced, giving a visual indication of gene expression from the promoter. The promoter region of *Xf* PG was PCR amplified and inserted into pProbe *gfp* tagless and we are in the process of testing them. These constructs should allow us to determine if there is a compound that can induce the production of *Xf* PG from the native *Xf* PG promoter.

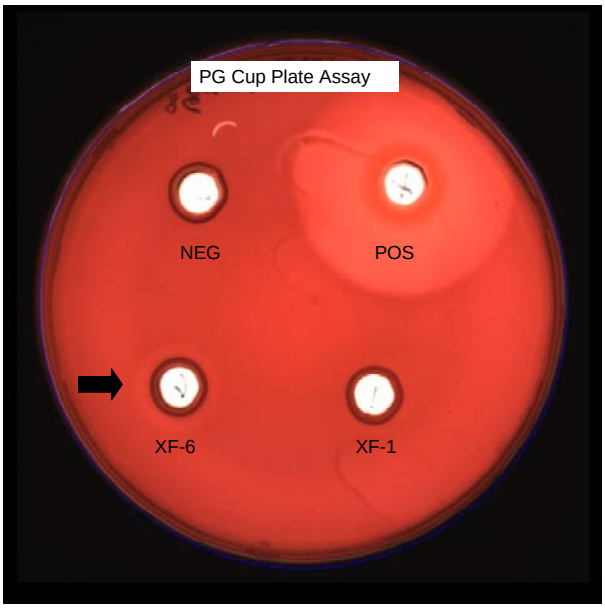

Figure 1. PG cup plate assay, a clear halo representing enzyme activity is evident in the positive control (POS) from *A. acleatus* and PG over-expressing transformant *XF*-6.

Objective 2: Isolate M13 phages 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 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 (**Figure 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 (data not shown).

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. 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 identify full length recombinant PG (**Figure 3**) (Tanaka et al 2002).

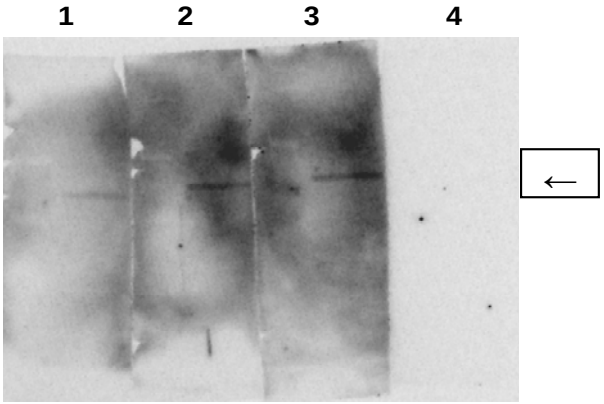

Figure 3. Western blot analysis of 3 representative monoclonal scFv phages (J-library).Lanes 1-3 are *E. coli* lysate containing recombinant *Xf* PG each membrane strip was reacted with a single monoclonal phage from the third round of panning . Arrow represents location of *Xf* PG band. Molecular weight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

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. Once we obtain enough active *Xf* PG 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.

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 PD development with inoculated, non-transgenic control plants.
All previous objectives must be completed before we can start objective 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 PD.

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