Title of Report: Interim Progress Report for CDFA Agreement Number 12-0129

Title of Project: INHIBITION *OF XYLELLA FASTIDIOSA* POLYGALACTURONASE TO PRODUCE PIERCE'S DISEASE RESISTANT GRAPEVINES

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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 PD.

To accomplish this we will use phage display of a random heptapeptide library and a scFv antibody library attached to the coat protein gp38 of M13 phage in a phage panning experiment using enzymatically active recombinant Xf PG as the target. After 3 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 to see if they can also provide resistance to grafted scions.

OBJECTIVES

Objective 1: Localization and isolation of sufficient amounts of biologically active *Xylella fastidiosa* (Xf) polygalacturonase (PG) enzyme to conduct phage panning and PG-inhibition assays.

Objective 2: : Isolate M13 phages that possess high binding affinities to Xf PG, as well as *Agrobacterium vitis* and *Aspergilus aculeatus*, from a M13 random peptide or scFv antibody libraries

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

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 Thompson Seedless grapevines.

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

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

Summary of Accomplishments and Results:

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

Xf does not produce a detectable amount of PG when grown in biological media. Furthermore, attempts at expressing Xf PG in *E. coli*, yeast, and plant based viral expression systems have not produced active Xf PG (see previous PD/GWSS Proceeding reports). Because of this, Xf strains have been engineered that will constitutively express the PG gene. The pBBR1MCS and pPROBE broad host range cloning vectors provide the Xf expression plasmid backbone and the constitutive nptII promoter was utilized to drive protein expression (Miller et al. 2000, Kovach et al. 1995). GFP reporter constructs made using this plasmid system stably expressed GFP in Xf under antibiotic selection. Additionally, the amount of GFP produced using this system is readily detectable on a Coomassie stained polyacrylamide gel (Figure 1). Xf PG-expressing constructs have been tested for the production of Xf PG and western blot analysis using polyclonal anti-Xf PG antibodies indicates that the constructs are producing Xf PG, indicated by the presence of a 55kd band in the Xf PG over-expression strains that is not present in the Xf strain over-expressing GFP (Figure 1). Tandem mass spec analysis of Xf produced PG indicates that Xf PG is being processed in Xf and likely is a major factor in the apparent size difference between *E. coli* produced recombinant PG and Xf produced PG. We have begun testing these strains for PG activity and there seems to be some activity associated with the Xf PG containing fractions. However, the plasmids generated seem to be somewhat unstable in Xf as GFP and PG expression drops off after repeated transfers on selective media. This fact is hampering our efforts to produce the large amounts of active Xf PG we need for the subsequent objectives in this project.

Surprisingly, this plasmid does not suffer the same instability issues in *E. coli,* as both plasmids are stable even after many subsequent transfers on selective medium. Interestingly, Xf remains resistant to the antibiotic resistance provided on the plasmid suggesting that the DNA on the plasmid is being rearranged as opposed to losing the plasmid entirely. The strain of *E. coli* we are using has a mutated *recA* gene, which is involved in homologous recombination of DNA and plasmid stability. Xf has a single copy of the *recA* gene and there is a strong possibility that mutating the Xf *recA* gene may eliminate the DNA rearrangement in the plasmid that is leading to its instability. We have created a *recA* mutant integration vector which will be transformed into Xf to test this hypothesis. Additionally, we have moved the expression machinery into pBBR-MCS5 which should not suffer from the same homologous recombination problems as the original plasmid.

Activity of Xf produced PG will be assayed using two methods. The first is the 2-cyanoacetamide reducing sugar assay, a spectrophotometric method which quantitatively measures the increase in reducing end accumulation due to PG enzymatic degradation of polygalacturonic acid (Gross 1982). The second is a cup plate diffusion assay in which activity is represented by a colorimetric clearing around where the enzyme is introduced into a plate of agarose containing polygalacturonic acid (Taylor and Secor 1988). As Xf PG has not previously been detected in *in vitro* culture supernatants or xylem sap from Xf-infected grapevines, we feel it is important to determine where PG is present in the newly developed Xf PG-producing strains.

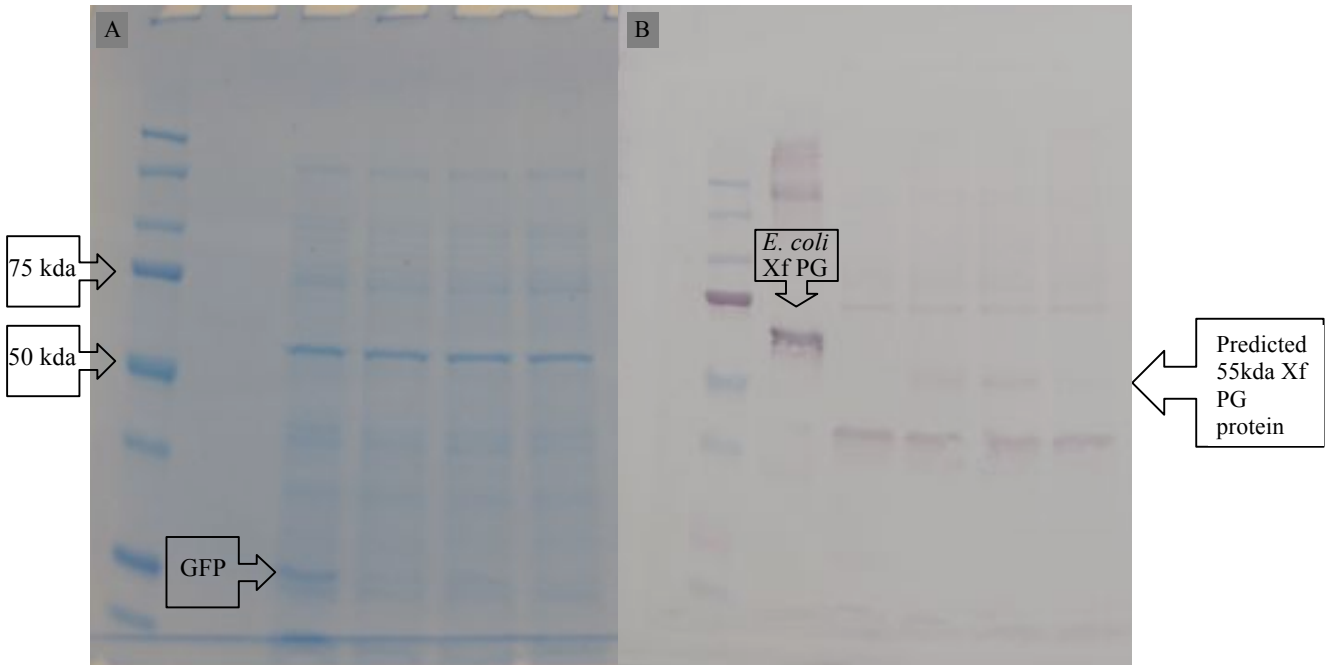

Figure 1. **A:** Coomassie stained polyacrylamide gel electrophoresis, **Lane 1**: Bio-rad dual color protein ladder, **lane 2**: Recombinant Xf PG produced in *E. coli*, **lane 3**: Xf over-expressing GFP, **lanes 4-6** Xf over-expressing XfPG **B:** Western blot analysis using polyclonal anti Xf PG antibodies, **Lane 1**: Bio-rad dual color protein ladder, **lane 2**: Recombinant Xf PG produced in *E. coli*, **lane 3**: Xf over expressing GFP, **lanes 4-6**: Xf over-expressing Xf PG. Right arrow denotes bands in Xf PG over-expressing Xf strains corresponding to predicted 55kda protein that are not present in GFP producing Xf strain.

Objective 2: Isolate M13 phages that possess high binding affinities to Xf PG, as well as *Agrobacterium vitis* and *Aspergilus aculeatus*, from a M13 random peptide or scFv antibody libraries

Do to the difficulties encountered in producing enzymatically active Xf PG enzyme we have decided to use two other PGs, as well as smaller peptides constituting sections of the active site of Xf PG, as surrogates to confirm that an enzymatic inhibitor can be isolated using phage panning techniques.

Agrobacterium vitis (Av) is a plant pathogenic bacterium that causes crown gall disease in grapevines. Like Xf Av also requires a PG in order to move from xylem element to xylem element. The Av PG gene has been previously cloned and shown to be active in *in vitro* activity assays (Herlache et al 1997). In addition, because the active sites of PGs are so highly conserved and need to degrade the same substrates in the same host plant (*V. vinifera*); a peptide which inhibits Av PG may also inhibit Xf PG. Furthermore, an inhibitor of Av PG activity would also prove useful for California grape growers as a possible control method far crown gall disease of grapevines.
For this reason we have cloned the Av PG gene into an *E. coli* overexpression system to produce recombinant Av PG to use in inhibition assays (**Figure 2**).

Figure 2

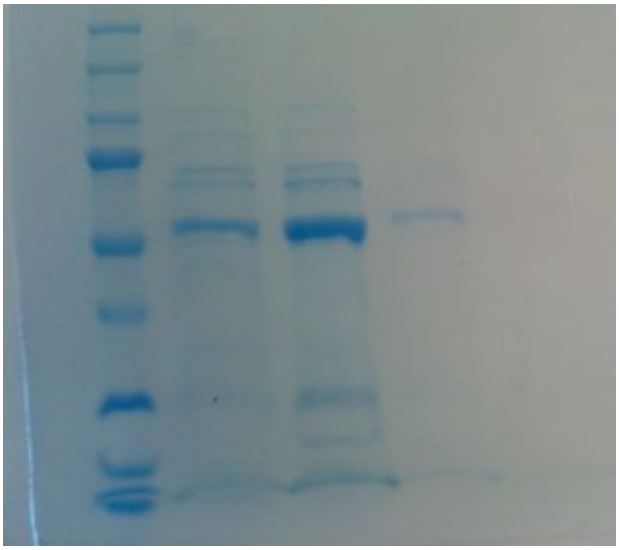

Figure 2. Coomassie stained 10% SDS polyacrylamide gel containing his column purified recombinant Av PG elutions. Note distinct Av PG bands at the 55 kda predicted size. Lane 1: Protein Mass ladder, Lane 2: Elution 1, Lane3: Elution 2, Lane4: Elution 3, Lane 5: Elution 4.

Experiments have shown recombinant Av PG is being produced in large amounts and is enzymatically active in cup plate assays (Figure 3).

Figure 3

Figure 3. Ruthenium red stained cup plate assay showing Av PG activity. Note cleared region around Av PG well that is not present in negative control well indicating PG enzymatic activity.

We have completed the phage panning procedure for Av PG using the Tomlinson I and J scFv libraries as well as the PHD7 phage library. At the end of the third round of selection a monoclonal ELISA with Av PG as the target was run which showed a majority of the monoclonal phages from each library (I ,J, and PHD7) showed a higher binding affinity to Av PG than to casein (blocking protein), or to the wells of the plate. With this knowledge 16 clones from each library (I ,J, and PHD7) providing the highest ELISA absorbance readings were chosen for sequencing. The results of the PHD7 sequencing revealed that 5 unique phage sequences account for over 50% of the phage pool and on phage sequence represent 25% of the phage. Additionally these phages share a 4 amino acid consensus sequence. The peptide sequences encoded by these 5 phages are currently being synthesized and will be used in inhibition assays upon receipt. We are currently awaiting the sequence data for the I and J phage libraries and we will express the respective scFvs for inhibition assays after analyzing the sequence data.

Additionally we have completed panning with the commercially available *Aspergilus aclueatus* (Aa) PG using the Tomlinson I and J scFv phage libraries. At the end of the third round of selection a monoclonal ELISA with Aa PG as the target was run which showed a majority of the monoclonal phages from each library (I ,J) showed a higher binding affinity to Aa PG than to casein (blocking protein), or to the wells of the plate. With this knowledge 16 clones from each library (I ,J) providing the highest ELISA absorbance readings were chosen for sequencing. As with the Av panning experiment we are currently awaiting sequence data before expressing the scfvs and preforming inhibition assays.

Furthermore, 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 consists 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 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 (Figure 2).

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 to 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 (Tanaka et al 2002).

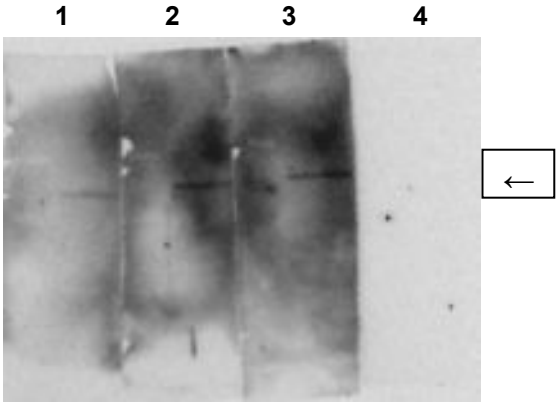

Figure 2. Western blot analysis of 3 representative monoclonal scFv phages(J-library) .Lanes 1-3 are *E. coli* lysate containing recombinant Xf PG each membrane strip was reacted with a single monoclonal phage from the third round of panning . Arrow represents location of Xf PG band. Molecular weight 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 that mediates phage binding to Xf PG and surrogate PGs can inactivate PG activity *in vitro*

Once a candidate phage is found that can inhibit any of the PGs *in vitro* we will then test it's activity against each of the other PGs. It may be possible to isolate a single sequence that could inhibit all of the PGs due to the similarities of the active site amino acids. We will then clone the anti-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:

Kirkpatrick, B. an J. Warren. 2012. Inhibition odf Xylella fastidiosa polygalacturonase to produce Piere's disease resistant grapevine. . Pierce's disease Research Progress Reports, California Department of Food and Agriculture. pp. 141 – 146.

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.

LAY SUMMARY OF CURRENT YEAR'S RESULTS

We have made progress on what has been the biggest obstacle thus far in this project, which is creating enzymatically active Xf PG to pan and test our putative inhibitory phage against. Xf PG over-expression experiments using Xf as the cloning host have shown that we can produce recombinant PG in Xf and initial tests identified some fractions that may contain active Xf PG. Once we overcome some of the instability issues of this plasmid system, perhaps by expressing these plasmids in a Xf *rec*A mutant or different plasmid, 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. Additionally we have completed our panning experiments against Av PG and Aa PG and will begin inhibition assays as soon as we receive all of the sequencing data.

STATUS OF FUNDS

As per 7/24/2013 there remained $48,064 in this grant which was granted a no cost extension until 6/30/2014.

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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