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 in Vitis vinifera grapevines. 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 phage panning experiments 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* PG and *Aspergilus aculeatus* PG 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 which showed that the constructs are producing Xf PG, as 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 this processing is likely 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.

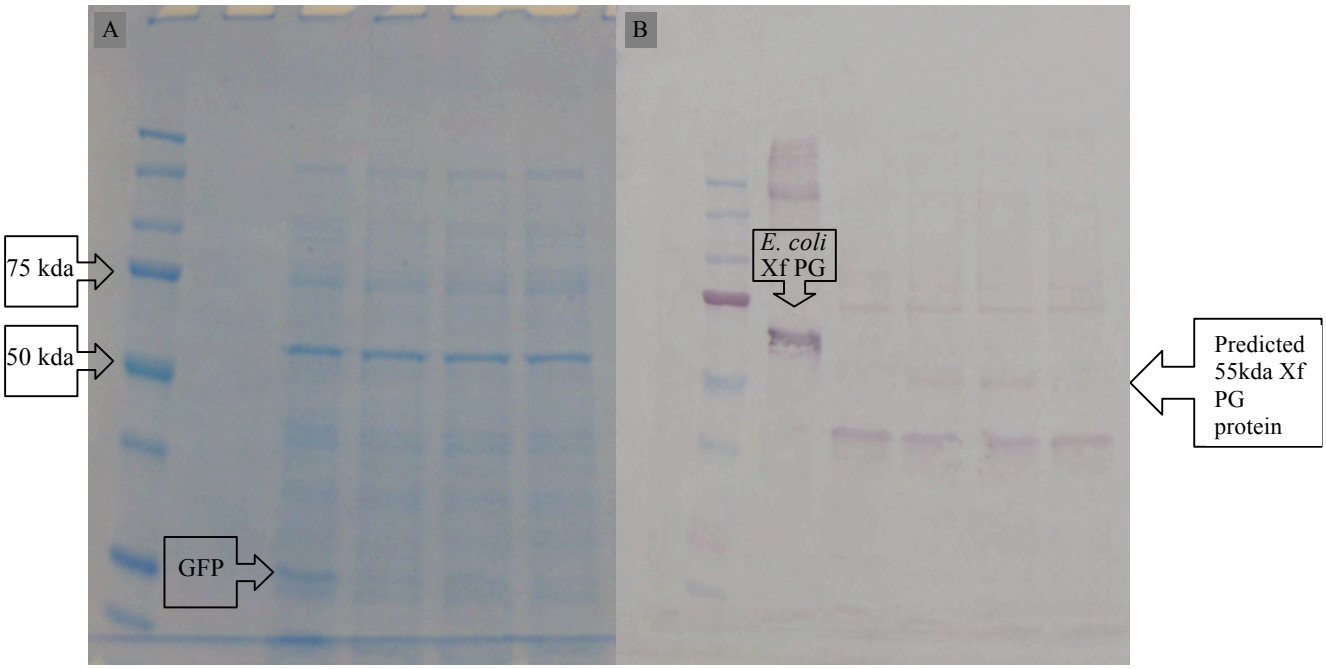

Figure 1. **A:** Coomassie stained polyacrylamide gel electrophoresis, **Lane 1**: Bio-rad dual color protein ladder, **lane 2**: Recombinant Xf PG produced in *E. coli*, **lane 3**: Xf over-expressing GFP, **lanes 4-6** Xf over-expressing XfPG **B:** Western blot analysis using polyclonal anti Xf PG 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 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 for crown gall disease of grapevines. For this reason we cloned the Av PG gene into an *E. coli* overexpression system to produce recombinant, enzymatically active Av PG fpr use in inhibition assays (**Figure 2**).

Figure 2

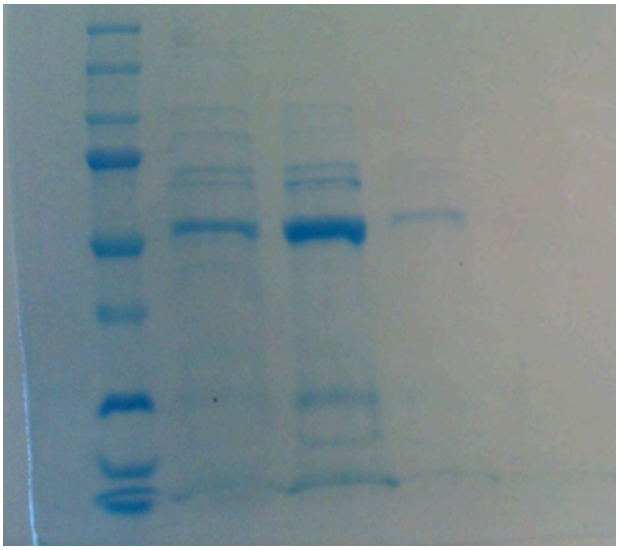

Figure 2. Coomassie stained 10% SDS polyacrylamide gel containing His column purified recombinant Av PG elutions. Note distinct Av PG bands at the 55 kda predicted size. Lane 1: Protein Mass ladder, Lane 2: Elution 1, Lane3: Elution 2, Lane4: Elution 3, Lane 5: Elution 4.

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 and recombinant Xf PG using the Tomlinson I and J scFv libraries as well as the PHD7 and PHD7circular phage libraries. 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, PHD7, PHD7C) showed a higher binding affinity to these PGs than to casein (blocking protein), or to the wells of the plate. With this knowledge 20 clones from each library (I ,J, PHD7, PHD7C) providing the highest ELISA absorbance readings were chosen for sequencing. The resulting sequences were analyzed and the 14 most promising sequences were synthesized for use in inhibition assays in objective 3. Interestingly, some of the peptides sequences show similarities to portions of the sequences of known polygalacturonase inhibiting proteins (PGIPs). Suggesting that these panning results could provide a way to determine which PGIP may inhibit these PGs most effectively.

Additionally, we have completed panning against 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.

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*

We have begun screening the 14 peptides synthesized from the phage panning in objective 2 against their corresponding PG targets to test for enzymatic inhibition. One of the synthesized peptides from the AVPG/PHD7 panning experiments has shown the ability to inhibit the activity of Av PG (**Figure 3)**.

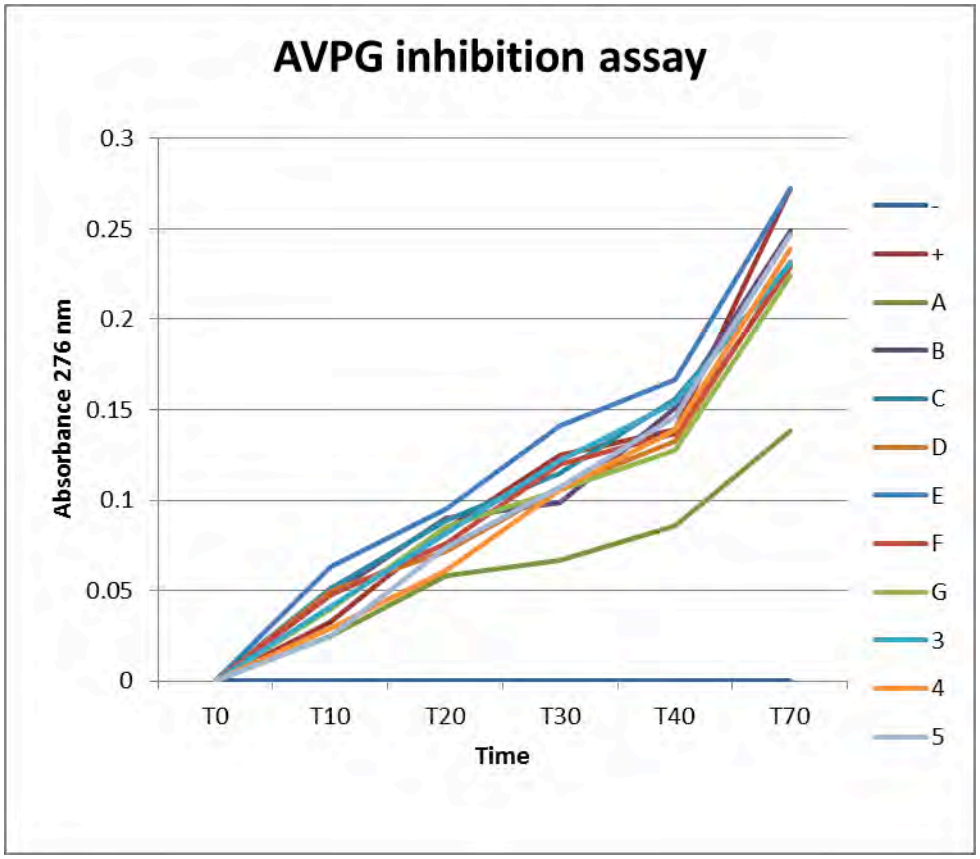

Figure 3. Av PG was incubated with each synthesized peptide for 30 minutes, this solution was then added to a solution of 0.05% polygalacturonic acid and the reaction progress was followed using the 2-cyanoacetamide reducing sugar assay. Peptide A shows the ability to inhibit the activity of AvPG when compared to the positive control and other peptides tested.

While not completely inhibitory under the conditions tested this does provide a proof of concept that phage panning can be used to select peptides that are inhibitory toward polygalacturonase enzymes. We are currently in the process of determining which amino acids are essential for this inhibitory effect. Understanding which amino acids are playing a role in the inhibition should allow us to increase the inhibitory effect of this peptide or peptide derivatives. We are also testing peptide A against other PGs to determine if this inhibitory action is specific to Av PG or if it is able to inhibit other bacterial or fungal PGs. Additionally, one of the peptides obtained using the Ph.D. phage library appears to show an increase in PG activity in the cup plate assays (**Figure 4**). While this result was unexpected it does show that phage panning can provide us with peptides that can modulate the activity of PGs *in vitro*. Furthermore, since this peptide seems to increase PG activity we are in the process of testing to see if it will also boost the activity of the small amount of Xf PG we can obtain.

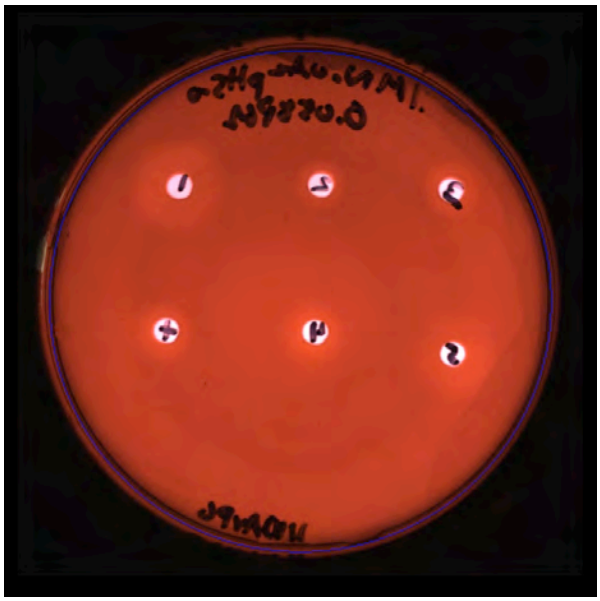

Figure 4. PG cup plate assay, a small clear halo representing enzyme activity is evident in the positive control (+) from *A. vitis*. Cups 1-5 contain Av PG, as well as, synthesized peptides based on sequencing results of Ph.D. phage panning experiments using Av PG as the target. Note the larger halo surrounding cup one suggesting increased activity.

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. and J. Warren. 2012. Inhibition of *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 completed panning experiments against Av PG, Xf PG and Aa PG using a number of different phage libraries. The results show that one of the peptides derived from these experiments is able to inhibit Av PG activity in *in vitro* inhibition assays. This provides a proof of concept that phage panning is a viable method to isolate inhibitors of PG activity. We are currently testing to determine if this peptide has any inhibitory effect on other PGs and which amino acids are necessary for the inhibitory effects.

STATUS OF FUNDS

As per March, 2013, 422,597 remained in this grant HOWEVER ALL of these funds have been encumbered and no funds will remain on 6/30/14.

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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