California Department of Food and Agriculture
Pierce's Disease Research
Final Report for CDFA Contract Number 07-0299
Funding Period June 2007 to December 2008

Project Title: Inhibition of Xylella fastidiosa polygalacturonase to produce Pierce's disease resistant grapevines.

Research conducted: is 7/1/07 to 6/31/08

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Project Summary:

The purpose of this project was to identify peptides that can bind and inactivate *Xylella fastidiosa* (Xf) polygalturonase (PG), an essential virulence factor that Xf needs to cause PD. With the year of funding we received for this project we have completed parts of objectives one and two. For objective one we were able to obtain a small amount of active recombinant Xf PG that we were able to detect using reducing sugar assays. Since most of the protein that we were able to produce was in the form of insoluble inactive inclusion bodies, we also tried a number of different expression constructs to increase the amount of active Xf PG we could generate. While we were working on increasing the amount of active Xf PG we could produce we went ahead with objective two. We synthesized two 14mer peptides derived from the Xf PG sequence, one which targeted the active site directly and a second that targeted an area providing entry into the active site. Polyclonal antibodies raised against each of these peptides were used in a western blot analysis that confirmed that the antibodies created against each 14-mer peptide could also recognize full length recombinant Xf PG. Peptide 2 was immobilized on a polystyrene surface and used as a "target" in a phage panning experiment using a scFv antibody library attached to the pIII protein of M13 phage. 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. We then sequenced the heavy chain variable portion of the scFvs giving the highest absorbance reading and although none of the eight clones selected from each of libraries shared the same sequence they did have similarities to each other.

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

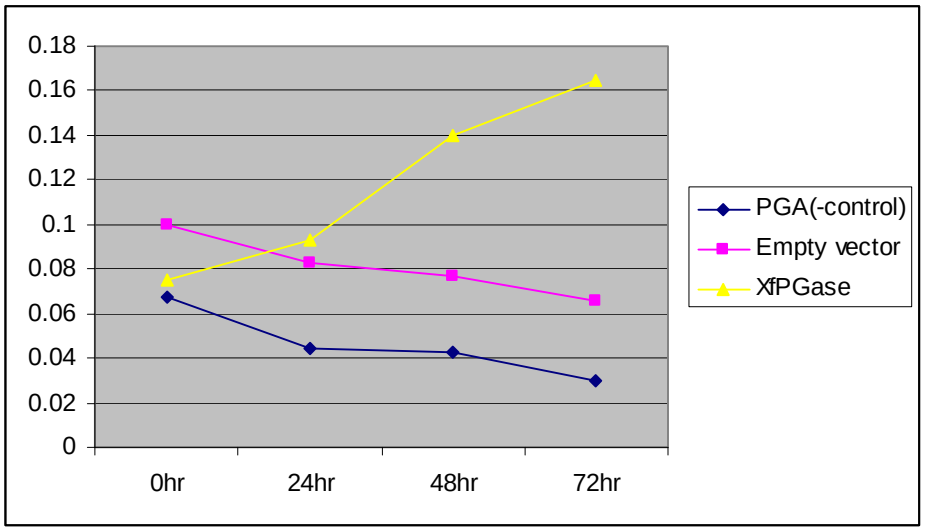
et al. 2007). In order for Xf to systemically infect a grapevine it must break down the pit membranes that separate individual xylem elements. Pectic polymers determine the porosity of the pit membrane (Baron-Epel, et al. 1988; Buchanan et al. 2000) and Xf PG allows the bacterium to breakdown the pectin 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 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 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 and see if they can also provide resistance to grafted scions.

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

We were able to produce a small amount of active recombinant Xf PG, however, most of the expressed protein was localized in insoluble and inactive inclusion bodies. Fortunately, we were able to measure the activity of the small amount of active PG that we have obtained by using visible spectrum reducing sugar assays such as the dinitrosalicylic acid (Figure 1) (Wang et al 1997, Sumner 1921) and 3-Methyl-2-benzothiazolinonehydrazone methods (Anthon and Barrett 2002, Honda et al 1981). This represents a significant improvement over the tedious High-performance liquid chromatography (HPLC) assays that were previously used to demonstrate Xf PG activity (Roper et al. 2007).

Additionally, we produced three XfPG plant expression constructs using a recently developed agroinfection-compatible tobacco mosaic virus protein expression system (Lindbow, 2007). The first was the Xf PG gene inserted into the expression vector in its native state. The second involved changing the structure of the gene near the start codon to make it more conducive to use in plant expression systems. The third employed the use of a Rice Alpha Amylase signal peptide to export Xf PG to plastids and extracellular compartments (Chen et al. 2004). At this time we have only assayed the first construct for Xf PG production and activity, unfortunately this construct did not produce any active Xf PG. However, we hope our other constructs will prove more effective.

Figure 1



Objective 2: Isolate M13 phage 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 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 (Fig2).

Figure 2

Peptide	Sequence
Peptide 1	DSPNSNGLQMKSDAC
Peptide 2	STGDDHVAIKARGKC

Fig 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 XfPG (fig 3).

Figure 3

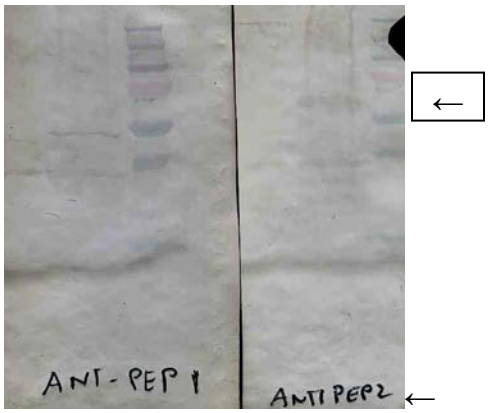


Fig 3. Lane 1 is E.coli lysate containing no XFPG. Lane 2 is E. coli lysate containing recombinant XfPG. Arrow represents Location of XfPG band.

We have completed the 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 providing the highest absorbance reading from the ELISA were chosen to use 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 same sequence they did have similarities to each other.

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 currently have candidate monoclonal phage that we will soon be using in inhibition assays using the methods described in objective #1

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:
2007 Pierces Disease Research Symposium Report
Presentations on Research:
2007 Pierces Disease Research Symposium

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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