SAVE THE WINE: EXPRESSION AND PARTIAL PURIFICATION OF XYLELLA FASTIDIOSA POLYGALACTURONASE

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ABSTRACT

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July 2010

SUPERVISING PROFESSOR: RACHELL E. BOOTH

Pierce's disease (PD) in grapevines was first identified as problematic in California around 1884, destroying 35,000 acres of grapevines and completely eliminating grape production southeast of Los Angeles. Pierce's Disease, caused by the gram-negative bacterium *Xylella fastidiosa* (*Xf*), is transmitted by xylem feeding insects called sharpshooters. *X. fastidiosa* has genes that encode an array of cell-wall-digesting enzymes including polygalacturonases, which catalyze the hydrolysis of the α1-4 glycosidic linkages of pectin (polygalacturonic acid).

Polygalacturonase inhibitor proteins (PGIPs) are glycoproteins located in plant cell walls that selectively bind and inhibit pathogen PGs. In order to screen PGIPs for

optimum inhibition, we have cloned and expressed functional polygalacturonase in a *Drosophila* expression system. The polygalacturonase (PG) gene, *Xf*PG, produced by, *X. fastidiosa,* was ligated into the expression vector pMT/BiP/V5-His A. Positive clones were transfected into *Drosophila S2* cells and expression was induced at 24 hours with copper sulfate. PG protein was detected with western blotting and partially purified utilizing a Ni^{2+} affinity chromatography column. Activity was then detected with a standard reducing sugar assay and radial diffusion assay.

There are few reports on the purification, characterization, and stability of *Xf*PG, however, these properties have been well documented on other species producing polygalacturonases. The results produced from this study will contribute to efforts of characterizing *Xf* polygalacturonase leading to the creation of transgenic grapevines and suppression of Pierce's Disease. Funding support provided by the Welch Foundation and Texas State University-San Marcos.

CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

California produces approximately 90% of U.S. wine supply, accounting for two-thirds of all wine sales in the U.S. and making it America's top wine producer. Behind France, Italy, and Spain, California is one of the leading wine producers in the world and has annually increased sales since 1993. California's wine industry not only benefits the state, but also the nation by producing $51.8 billion in revenue for California's economy and $123.3 billion in economic value for the U.S. This industry alone provides ~309,000 jobs in California and ~875,000 jobs nationwide (Baker 2005). In addition, the average annual consumption of wine in the U.S. has increased from 449 billion gallons in 1993 to 745 billion gallons in 2007 (Baker 2005). Thus, preventing disease and/or death of grapevines is a top priority.

Pierce's disease (PD), a grapevine disease, was first identified as problematic and characterized by Newton Pierce in 1892. Between 1884 and 1900 PD destroyed 35,000 acres of grapevines and completely eliminated grape production in southeast Los Angeles (Schaad 2002). During the twentieth century there have been three major epidemics in California pertaining to PD (CCANR 2004). One of the more recent PD outbreaks occurred between 1994 and 2000; northern California suffered a loss of over 1,000 acres of grapevines resulting in $30 million in damage (Kawamura 2008). Pierce's Disease, caused by the gram-negative bacterium *Xylella fastidiosa* (*Xf*), is transmitted by xylem

feeding insects. Once transmitted, the xylem, which are the water-conducting vessels in plants' vascular systems, become infected (Figure 1) and block the xylem by creating a biofilm reducing water and nutrient transport (Figure 2) (Ramirez *et al.* 2008).

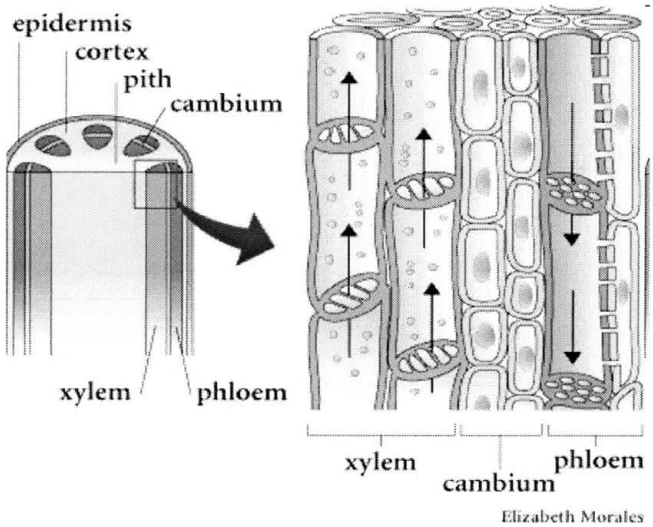

Figure 1. Vertical cross section of a plants vascular system (Lainew 2008).

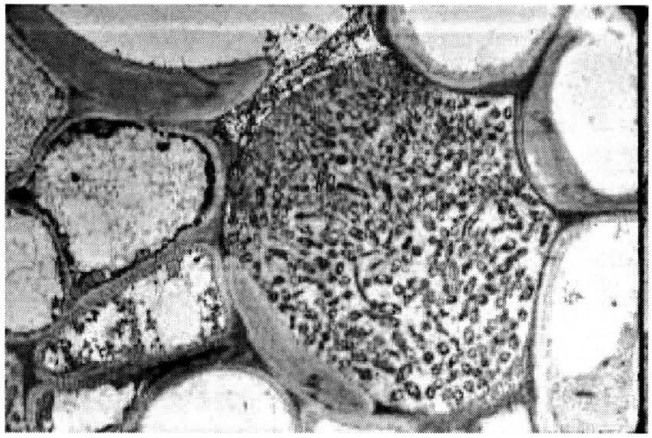

Figure 2. *Xylella fastidiosa* bacterium colonization in the xylem tissue of a grapevine (Purcell 2008).

Since the emergence of PD in 1892, an array of leafhopper species have become disease vectors in the spread of *X. fastidiosa* and, thus PD. During the feeding process the insects acquire sap contents from the xylem tissues and adopt *X. fastidiosa* (Meng 2005), which attaches to the esophagus and is transferred to healthy plants through saliva excretion (Purcell 1979). The first known insect to spread PD, the blue-green sharpshooter, has been considered a major threat only in the coastal regions of California due to its inability to fly long distances and preference of stream /river bank (riparian) vegetation which provides a natural containment. However, this species of sharpshooter is responsible for most of the damage accomplished by PD in coastal grape-growing regions located near riparian vegetation. In addition, California's Central Valley inhabitance of grapes by red-headed and green sharpshooters is usually accidental, due to their preference for bermuda grass and hayfields as breeding grounds; therefore, they do not pose a large threat to the economically important grapevines (Purcell 2001). However, the recent emergence of the glassy-winged sharpshooter, a highly mobile insect that also possesses the ability to transport *Xf*, is expected to amplify the spread of PD. These insects often travel in large numbers, fly up to a quarter of a mile, and survive temperatures as low as 20 °F (Meng *et al.* 2005).

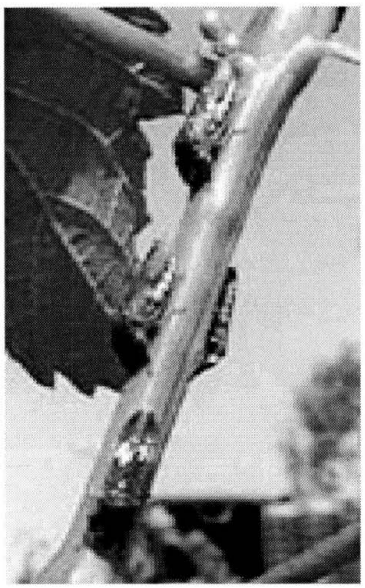

Figure 3. GWSS inhabiting and feeding on a grapevine (Williams 2008).

Figure 4. Symptoms of PD throughout the stages of infection. (1) leaf scorching (2) fruit clusters raisin (3) leafless stems (4) wood on new canes mature irregularly, producing patches, surrounded by mature brown bark (PDO 2008, Strand *et al.* 2008).

Sharpshooters feed on grapevines, and transmit the bacterium, which then colonizes the vascular tissues and causes water deprivation throughout the plant (Figure 3). *X. fastidiosa* is difficult to detect, and symptoms such as leaf scorching do not surface until late in the growing season, making PD challenging to diagnose early. The most common symptoms in grapevines acquired from PD are scorching along leaf margins, shriveled or raisined fruit clusters, leafless stems attached to the cane, and irregular maturation of wood on new canes, producing patches, surrounded by mature brown bark (Figure 4) (Schaad 2002). At any of these points of pathogenesis, it is too late to treat the grapevines for the disease.

During pathogenesis, the plant cell walls are the first line of defense against the bacterium. Cell walls provide plants with structural support, resistance to internal turgor pressure, carbohydrate storage, and protection against pathogens and other environmental factors. Primary composition of the cell walls consists of an array of complex polysaccharides. The major polysaccharides present in the primary and secondary walls are cellulose, hemicellulose, and pectin (Figure 5). Pectin consists of complex polysaccharides containing 1,4-linked α-D-galacturonic acids which can be randomly acetylated and methylated, and has been characterized into three different classes: homogalacturonans, rhamnogalacturonans, and substituted galacturonans (Figures 6, 7, and 8) (Zablackis *et al.*1995).

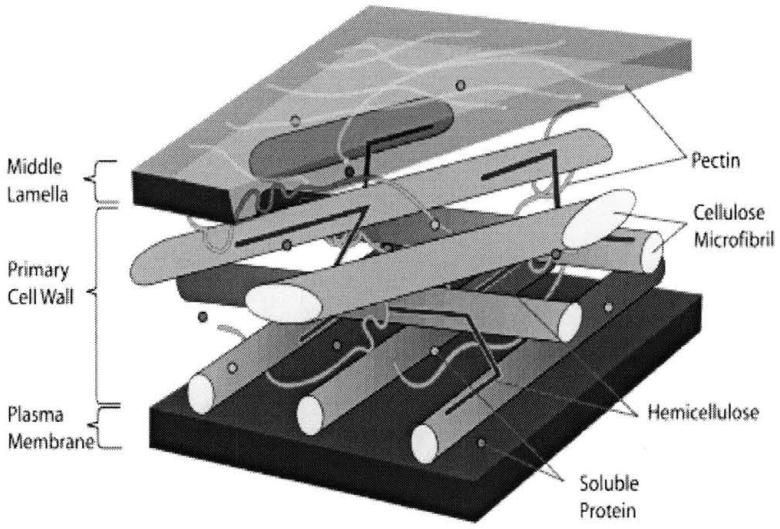

Figure 5. Plant cell wall and component assembly at the cellular level. The orange strings running throughout the cell are representative of pectin (Villarreal 2008).

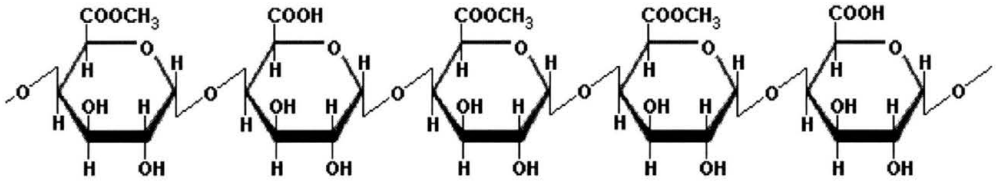

Figure 6. Homogalacturonan component of pectin (Zamora 2005).

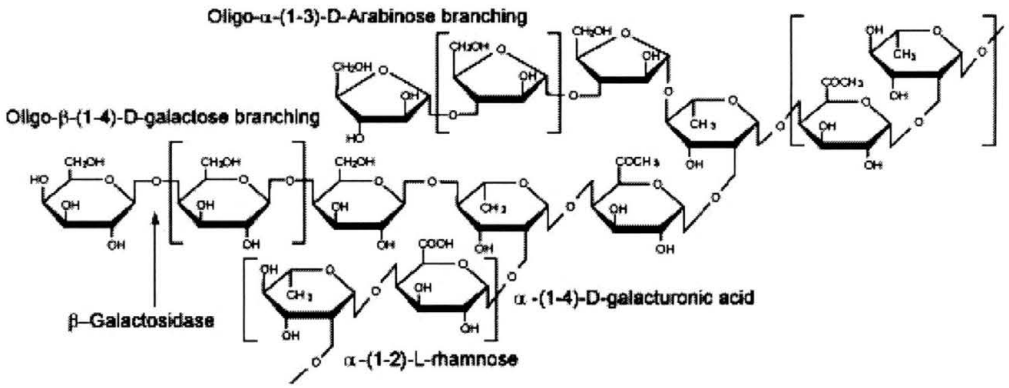

Figure 7. Rhamnogalacturonan I component of pectin (Sigma-Aldrich 2010).

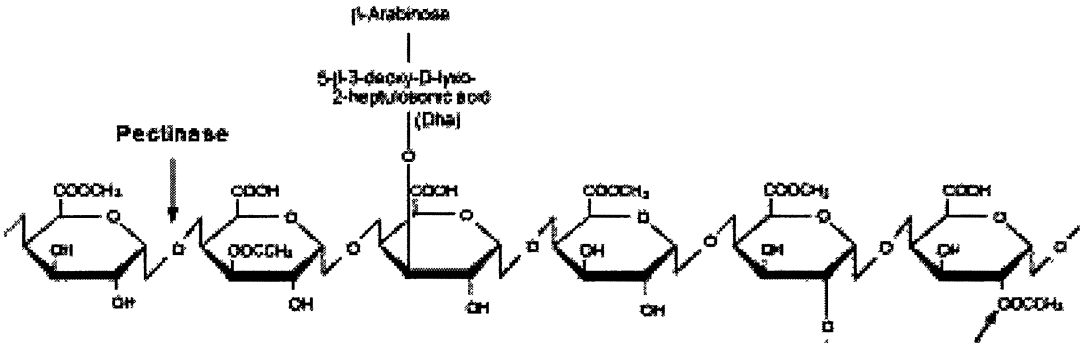

Figure 8. The Rhamnogalacturonan II component of pectin (Sigma-Aldrich 2010).

Xylem, located in the secondary cell wall, is a network of interconnected vessels or channels, called bordered pits. These pit membranes contain pores approximately 5 to 20 nm in size. Intact pit membranes block the passage of objects larger than 5 nm, such as *X. fastidiosa* (0.3 to 0.5 μm) (Roper *et al.* 2007). Newman et al. used a strain of *X. fastidiosa* expressing green fluorescent protein to analyze and better understand the bacterium's colonization patterns. The study revealed vessel-to-vessel movement through pit membranes which presented evidence of a cell-wall degrading enzyme (Sakellaris *et al.* 1989). *X. fastidiosa*, the causal agent of Pierce's disease, has genes that encode an array of cell-wall-digesting enzymes including polygalacturonases (PGs), pectate lyases, and glucanases (Zablackis *et al.* 1995, Newman *et al.* 2003). These enzymes hydrolyze plant cell walls to gain admittance to the plant cells during the early stages of pathogenesis (Van Sluys *et al.* 2003). The first of these enzymes secreted by pathogens when they encounter plant cell walls is the PGs (Meng *et al.* 2005). PGs specifically hydrolyze the α1-4 glycosidic bonds in pectin (also known as polygalacturonic acid) (Figure 9) (Di *et al.* 2006).

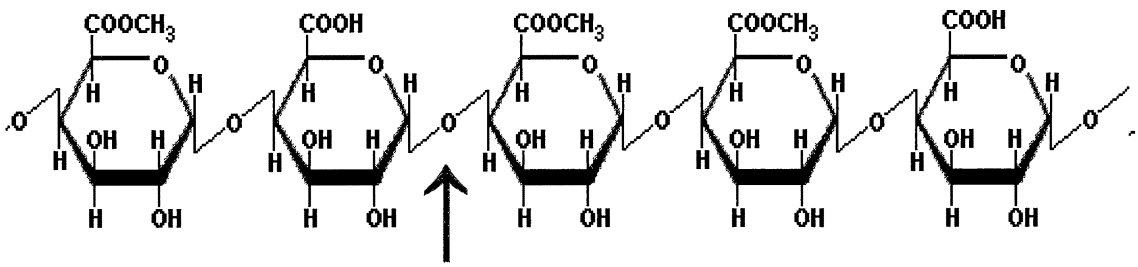

Figure 9. Structure and cleavage of polygalacturonic acid. The arrow indicates where polygalacturonase will hydrolyze the α1-4 glycosidic bonds in pectin, resulting in monogalacturonic acid molecules (Sicilia *et al.* 2005).

Roper *et al.* performed a pathogenecity assay to confirm that PG was a virulence factor of *X. fastidiosa*. Grapevines were individually inoculated with wild-type *X. fastidiosa (pglA)*, mutant *X. fastidiosa* (*pglA*$^-$, Tn*903*<kan-2> cassette marker created by a double crossover event in the *pglA* open reading frame), or water, and then analyzed for disease severity over several weeks. The mutant *X. fastidiosa* inoculated plants and water inoculated plants displayed identical growth patterns of healthy, disease free grapevines and never developed symptoms of PD. However, plants inoculated with wild-type *X. fastidiosa* displayed typical disease symptoms of PD, such as leaf scorching. This experiment demonstrated that PG is essential for PD development in grapevines (Roper *et al.* 2007).

Characterization studies previously performed by Sakellaris and associates have provided vital information pertaining to two proposed PG "charge isomers" (PG I and PG II) produced by the bacterium *Lactobacillus plantarum* (1989). These forms of polygalacturonase were found to possess enzymatic activity over a wide pH range (3.5-6), but were optimally active at pH 4.5. Similarly, enzymatic activity was functional up to

50 °C with optimum activity at 35 °C. The *L. plantarum* polygalacturonase assays using sodium polygalacturonate as the substrate resulted in K_m values of 1.63 and 1.78 mg/ml and specific activity of 206.52 and 169.53 U/mg, for PG I and PG II respectively. Differentiations in charge, due to amino acid or carbohydrate composition, may have accounted for the differences in K_m values and specific activities (Sakellaris *et al.* 1989). Similar studies were performed on PG produced by the filamentous fungus species *Tetracoccosporium*, which had comparable results with optimum activity at pH 4.3 and temperature at 40 °C. However, the K_m and specific activity varied slightly at 3.23 mg/mL and 6.17 U/mg, respectively. In addition, the effects of metal ions, metal chelators, and chemical compounds on *Tetracoccosporium* PG activity was explored. They discovered that Al^{3+}, Ba^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, and SDS inhibited polygalacturonase activity, whereas Ag^{+}, Co^{2+}, EDTA, Tween-20, Tween-80, and Triton X-100 stimulated activity. It was speculated that calcium inhibited PG activity by interfering with the substrate forming insoluble complexes. In addition, iodoacetamide and iodoacetic acid did not inhibit (nor did it stimulate) the enzymatic activity indicating that the catalytic site of the enzyme does not possess cysteine residues. However, all of the surface-active detergents tested most likely stimulated PG activity by lowering the surface tension of the aqueous medium, thereby increasing the contact frequency between the substrate and PG active site. Characteristics of the *Tetracoccosporium* PG were similar to those reported for other fungal PGs, such as *S. sclerotiorum* (Aminzadeh *et al.* 2006, 2007).

For over 30 years, polygalacturonase inhibiting proteins (PGIPs) extracted from the cell walls of red kidney beans, tomato stems, and suspension-cultured sycamore cells

have caused complete inhibition of PGs secreted by several pathogens (Van Sluys 2003). PGIPs are glycoproteins located in plant cell walls that selectively bind and inhibit the pathogen PGs' hydrolytic activity and prevent bacterial infestation (Figure 10). During systemic stress (i.e., wounding), salicylic acid is produced and induces PGIP activity (Zamora 2005). PGIPs are leucine-rich repeat (LRR) proteins which initiate signal transduction for plant defense gene activation (Bergmann 1994). PG inhibition by PGIPs imply that PGIPs may be an effective tool for preventing plant susceptibility to pathogenic species, as seen in preliminary experiments expressing PGIPs in plants (Powell *et al.* 2000).

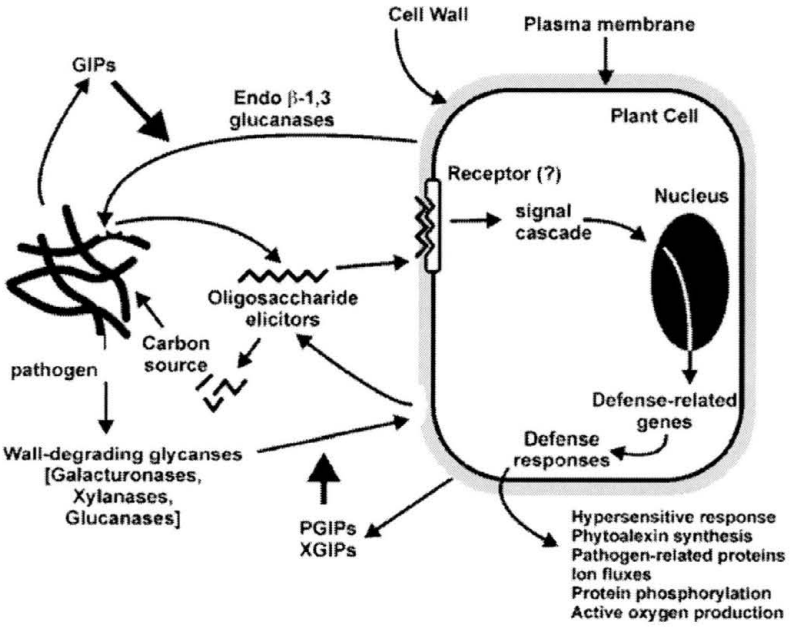

Figure 10. Pathogenesis and plant defense response. Pathogens fragment the plant cell wall with polygalacturonase (glycanase). In addition to the fragments produced during cell wall degradation, oligosaccharides are produced that induce plant defense responses. PGIPs interfere with the hydrolysis of the plant cell wall by polygalacturonase (Agüero 2005).

One strategy commonly used in agriculture to prevent the spread of diseases includes incorporating foreign genes into plants (i.e., transgenic plants). Thus, the overall goal of this thesis project was to determine the most effective polygalacturonase inhibiting proteins (PGIPs), which were used in future transgenic studies with grapevines. In order to screen and characterize PGIP inhibition of PG, (1) the polygalacturonase gene was cloned and expressed in a system designed to produce large quantities (μg amounts) of functional PG protein, (2) PG was purified from other proteins, and (3) potential PG inhibitory proteins will need to be screened to determine the inhibitory effects on PG *in vitro*. The current thesis aimed to complete items (1) and (2).

There are few reports on the purification, characterization, and stability of *Xf*PG, however, these properties have been well documented on other species producing polygalacturonases. In the present investigation, we report the production of an ample amount of protein utilizing the *Drosophila* expression system (Invitrogen 2003). To our knowledge, this is the first time the *Xf*PG enzyme has been expressed using this system. The results produced from this study will contribute to the production, purification, stability, future creation of transgenic grapevines, and suppression of Pierce's Disease.

CHAPTER II

MATERIALS AND METHODS

Transformation of Plasmid DNA into Top 10 *E. coli* Cells

Transformations were carried out according to a modified method of Chung and Miller (1998). Top 10 competent *Escherichia coli* cells from the -80 °C freezer were thawed on ice for seven min, and 1 μg of plasmid DNA was added. The cell/DNA mixture was incubated on ice for 20 min, then exposed to heat shock at 42 °C for 45 seconds, and succeeded by 2 min. ice incubation. One ml of Luria-Bertani (LB) broth was added to each transformation reaction and incubated in a 37 °C Max Q 4000 E-class shaker (Bearnstead Lab-Line) at approximately 225 rpm for 1 hrs. At the conclusion of the incubation period, a 500 μL aliquot of each transformation reaction was plated onto an LB plate containing 100 μg/mL ampicillin (AMP) or 50 μg/mL kanamycin (KAN). The plates were inverted and incubated at 37 °C for 24 hrs.

Plasmid DNA Isolation

Individual *E. coli* transformant colonies were selected from the LB AMP plates and placed in 5 ml of LB broth with 100 μg/mL AMP. The cultures were incubated at 37 °C for 16-20 hrs while shaking at 225 rpm (Max Q 4000 E-class shaker, Bearnstead Lab-

Line). Plasmid DNA purification was completed utilizing the QIAprep Spin Miniprep Kit per the manufacturer's protocol with the preference of eluting with water (Qiagen).

DNA Quantification

The isolated plasmid DNA concentrations were determined using the NanoDrop ND-1000 spectrophotometer and ND-1000 software. The nanodrop was blanked with the water solution the isolated plasmids had been eluted in. DNA concentrations were calculated according to the amount of light absorbed at 260 nm.

Horizontal Gel Electrophoresis

DNA samples were analyzed using a 1 % (w/v) agarose gel prepared in 1X Tris-acetate (TAE) buffer (20 mM acetic acid, 40 mM Tris base, and 2 mM EDTA, pH 7.4) with ethidium bromide (0.5 µg/mL final concentration). Purified DNA samples were electrophoresed at 100 V for one hrs in an IBI QS-710 Quick Screen (Shelton Scientific) Horizontal Gel Electrophoresis Unit containing 1X TAE buffer. Kodak Digital Sciences Image Station 440 and Kodak 1D imaging software were used to visualize and document the gel.

Custom *Xf*PG Primer Design

Custom primers were designed based on the following criteria: (1) primer length 18-22 nt, (2) consist of at least 50% GC content, (3) melting temperature (T_m) in the range of 52-58 °C with the forward and reverse primers no more than 2 °C different, and

(4) a GC clamp (two G or C bases grouped together) within the last five bases from the 3' end.

XfPG custom primers had the following sequences:

XfPG forward 5'- CAGATATCATGGACCTTGACCGTTTCC -3'

XfPG reverse 5'-GACTCGAGGATAGGATCAGGAAATACGC -3'

Polymerase Chain Reaction (PCR)

Each PCR reaction consisted of approximately 100 ng of DNA template, 1 μM each of the two (forward and reverse) complimentary custom primers (IDT Integrated DNA Technologies, Inc), 2.5 mM dNTPs (Stratagene), standard 1X Taq buffer (New England Biolabs, NEB), and 0.1 units Taq polymerase (NEB). The Applied Biosystems 2720 Thermo Cycler was utilized under the following conditions to perform the PCR temperature cycles: denaturation at 94 °C for 2 min; twenty-five cycles of replication [94 °C for 30 seconds for denaturation, 45 °C for 30 seconds for annealing, and 72 °C for 2 min for elongation]; 72 °C for 10 min for completion; 4 °C for infinity for storage.

Confirmed PCR products were purified with Wizard SV Gel and PCR Clean-Up System protocol (Promega). Alternatively, the target gene was also amplified using 1X ThermoPol Buffer (NEB), the high-fidelity Vent Polymerase, and additional 10 mM $MgSO_4$.

Digestion

Restriction digestions were performed on the purified PCR products and expression vectors to prepare for ligation and subcloning. Digestion reactions contained

the following components: ~100-200 ng of DNA, 1 U of restriction enzymes (20,000 units/ml) XhoI, EcoRV, EcoRI, or Hind III (selectively chosen per reaction), and 2.0 µL of the corresponding 1X NEB buffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9). The digestion was carried out for three hrss at 37 ºC and stopped using 1X EndoR Stop, (10 mM EDTA, 5% v/v glycerol, 0.1% w/v SDS, 0.01% w/v bromophenol blue, final concentrations, pH 8.0) or when appropriate were stopped by incubating the reaction at 65 ºC for 15 min.

Phosphatase Reaction

Calf Intestinal Alkaline Phosphatase (NEB) was added to the linearized expression vector to dephosphorylate the 5' phosphate termini in order to prevent recircularization. Reactions were incubated at 37 ºC for 1 hrs and halted by the addition of 1X EndoR Stop.

DNA Gel Extraction

DNA samples were extracted and purified from preparative agarose gels after separation utilizing the Wizard SV Gel and PCR Clean-Up System (Promega). Membrane Binding Solution was added to each agarose gel slice containing DNA samples at a ratio of 10 µl of solution per 10 mg of agarose gel slice and incubated at 65 ºC for 10 min to completely melt the gel slice. The melted gel solution was then placed into an SV Minicolumn with a collection tube provided in the kit. Two washes were then performed with Membrane Wash Solution followed by DNA elution with sterile water.

Ligation

The digested PCR products were ligated into the expression vector, pMT/BiP/V5-His A (Invitrogen). The ligation to create the recombinant plasmid was achieved by mixing 150 ng of PCR product with 50 ng of expression vector, a 3:1 insert to vector ratio, with 1 U (400,000 cohesive end units/ml)T4 DNA ligase and 1X T4 DNA ligase buffer. The ligation reactions were incubated at 4 °C for 24 hrs.

DNA Sequencing

Constructs were sent to Davis Sequencing for automated sequencing. Samples were prepared at a concentration of 400-500 ng/mL in water. Primers (3 μM) were designed against the flanking regions of the polygalacturonase gene (including engineered restriction enzyme sites EcoRV (forward primer) and XhoI (reverse primer) on the ends) and utilized in the sequence reactions (see sequences below).

Sequencing Primers for pMT/BiP/V5-His A/ *Xf*PG:

pMT Forward primer- 5'- CATCTCAGTGCAACTAAA -3'

BGH Reverse primer- 5'- TAGAAGGCACAGTCGAGG -3'

Transfection of pMT/BiP/V5-His A/ *Xf*PG into *Drosophila* S2 cells

The plasmids were transiently transfected into a cell line derived from *Drosophila melanogaster,* Schneider 2 (S2) cells for protein expression. Cultured S2 cells were prepared for transfection by seeding them into a 6-well 35mm plate at a concentration of 1 x 10^6 cells/ml in 3 ml complete Schneider's *Drosophila* Medium (Invitrogen,

containing 10% v/v heat-inactivated fetal bovine serum (FBS) and 5 ml/L of Penicillin-Streptomycin at 5000:5000 units/ml). The cells were then grown for approximately 16 hrs at 28 °C until they reached a density of 2-4 x 10^6 cells/ml. Transfection solutions A and B were prepared in the following manner. For solution A, the subsequent solutions were mixed together in a microcentrifuge tube: 2 M $CaCl_2$ (36 µl), recombinant DNA (19 µg), and sterile water bringing the solution to a final volume of 300 µl. In a second microcentrifuge tube, solution B consisted of 300 µl 2X HEPES-Buffered Saline (HBS; 50 mM HEPES, 1.5 mM Na_2HPO_4, 280 mM NaCl, pH 7.1). Solution A was slowly added dropwise to solution B with continuous mixing until solution A was depleted. The resulting solution was incubated at room temperature for approximately 30-40 min at which time a fine precipitate was formed. The solution was added dropwise to the cultured cells with continuous mixing and incubated for 24 hrs at 28 °C. The calcium phosphate solution was removed from the cells via centrifugation and decantation. The cells were then washed twice by resuspending with complete medium and centrifuging at 100 x g for approximately 5 min and decanting the medium. Fresh medium was used to resuspend the cells and replated into the same vessel to continue incubation at 28 °C. Expression of the transfected cells was induced by adding copper sulfate (500 µM). Induction should extend for a minimum of 24 hrs. Cells were harvested 2, 3, 4, and 5 days post-transfection, centrifuged and separated into supernatant and cell pellet, and assayed for expression of the target gene.

SDS-PAGE Vertical Gel Electrophoresis

Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was used to analyze the protein of interest. The pelleted cell samples were prepared by adding cell lysis buffer (50 mM Tris base, pH 7.8, 150 mM NaCl, and 1% Nonidet P-40 final concentrations) solution to the pelleted cells and incubating at 37 °C for 10 min, then the addition of 5X SDS-PAGE sample buffer (125 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue) to all of the protein samples, supernatant and pelleted cells. Samples were incubated for 5 min at 100 °C and centrifuged at 14,000 rpm for 5 min. The prepared samples were loaded into a polyacrylamide gel consisting of a 4% stacking gel (3.96% (v/v) bis-acrylamide, 126 mM Tris-HCl, pH 6.8, 0.1% (v/v) SDS, 0.01% (v/v) TEMED, and 0.05% (w/v) amonium persulfate) and a 10% resolving gel (12% (v/v) bis-acrylamide, 375 mM Tris-HCl, pH 8.8, 0.1% (v/v) SDS, 0.005% (v/v) TEMED, and 0.05% (w/v) ammonium persulfate), then electrophoresis was conducted at 150 V for 1.0 hrs in 1X Running buffer (25 mM Tris-Base, 40 mM glycine, and 0.1% v/v SDS, pH 8.0).

Western Blot

Once the proteins were separated via SDS-PAGE vertical gel electrophoresis, the gel was rinsed in dH_2O for 5 min and proteins were then transferred from the gel to a nitrocellulose membrane (Bio-Rad Trans-Blot Transfer Medium) by constructing a nitrocellulose/gel sandwich between two pieces of filter paper and pads in a blotting cassette. The blotting cassette was placed inside a vertical gel rig filled with 1X Transfer buffer (25 mM Tris Base, 40 mM glycine, and 20% v/v methanol) chilled to 4 ºC and was

transferred for 45 min at 100 V. After the proteins were transferred, the nitrocellulose was rinsed for 5 min in fresh 1X TBST (1.5 mM Tris-HCl, 50 mM NaCl, 0.1% (v/v) Tween 20, pH 7.4) followed by incubation in blocking solution (1% (w/v) dry milk in 1X TBST) for 30 min with agitation. The nitrocellulose was removed from the blocking solution and incubated at 4 °C overnight in a 1/5000 dilution of primary antibody (anti-V5) in blocking solution (1.0 g milk powder dissolved in 20 mL TBST). The following morning the primary antibody solution was removed and the blot was washed three times with TBST for 5 min each with agitation. Secondary antibody anti-mouse conjugated with horseradish peroxidase (HRP) was then added to the blot at a ratio of 1/20,000 dilution in blocking solution for 1 hr at room temperature while agitating. The blot was then washed two times with 1X TBST and one time with 1X TBS (1.5 mM Tris-HCl, 50 mM NaCl, pH 7.4) for 5 min each with agitation. To visualize the blot, 750 µl each of the two developer reagents contained in the Western Lighting Kit (Perkin Elmer LAS, Inc.) were mixed together and applied to the blot for 1 minute. The excess solution was removed and the blot wrapped in Saran wrap and placed in a film cassette. X-ray film (Thermo Scientific) was placed in the film cassette on top of the blot and exposed from 1 to 10 min, then developed in the Mini-Medical Series film developer (AFP Imagıng).

Stable Cell Lines

Drosophıla S2 stable cell lines were prepared and transfected as previously described, with the addition of pCoHygro (1 µg) to Solution A. After incubating the cells for 24 hrss the calcium phosphate solution was removed, and the cells were replated, without selection agent. Following a 2 day incubation period at 28 °C, the cells were

centrifuged and resuspended in complete growth medium containing the selective antibiotic (hygromycin-B) at the appropriate concentration (300 μg/μl). Selective medium is being replaced every 4 to 5 days until resistant colonies appear (approximately 3 to 4 weeks). Once resistant clones have been established, they will be replated into new culture flasks containing selective medium and passed once they reach a density of 6 to 20 x 10^6 cells/ml. After passing, the cells will grow to a density of ≥1 x 10^7 viable cells/ml at which time they will be harvested for frozen stocks. Frozen stocks will consist of a50% fresh, complete growth medium, 50% conditioned (includes fetal bovine serum) medium and 10% dimethyl sulfoxide (DMSO) and stored at -80°C.

Polyhistidine-tag Protein Purification

Purification was accomplished by means of affinity purification of the polyhistidine-tagged proteins. Crude protein containing media and pelleted cell extracts were prepared separately for purification. Crude protein containing media was dialyzed over night in 50 mM sodium phosphate buffer, pH 7.0 prior to purification. Pelleted cells were prepared by lysing the cells with cell lysis buffer as previously described. An empty gravity-flow column (Pierce) was loaded with 3.0 mL Ni-NTA resin and 5.0 mL Equilibration/Wash Buffer (50 mM sodium phosphate, 0.3 M sodium chloride, pH 7.0). Once the resin settled, Equilibration/Wash Buffer was released until ~0.5 mL remained in the column. Approximately 9.0 mL of the prepared enzyme extract was loaded and allowed to fractionate off the column with gravity. The flow-through was recovered in eppendorf tubes and saved for later analysis. A new set of eppendorf collection tubes was used to collect unbound protein washed from the column using 15.0 mL of

Elution/Wash Buffer (50 mM sodium phosphate, 0.3 M sodium chloride, pH 7.0); fractions were also saved for later analysis. The target protein was eluted with 6.0 mL Elution Buffer (150 mM Tris-HCl Buffer, pH 8.8) into new collection tubes and saved for later analysis.

Alternatively, the following buffers were also used for protein purification: Equilibration Buffer comprised of 50 mM sodium phosphate, 0.3 M sodium chloride, pH 8.0; Wash Buffer comprised of 50 mM sodium phosphate, 0.3 M sodium chloride, 5 mM imidazole, pH 8.0; Elution Buffer (250 mM imidazole) comprised of 50 mM sodium phosphate, 0.3 M sodium chloride, 250 mM imidazole, pH 8.0, and Elution Buffer (500 mM imidazole) comprised of 50 mM sodium phosphate, 0.3 M sodium chloride, 500 mM imidazole, pH 8.0. The purified protein samples were then analyzed using SDS-Page, Western blot protocols, radial diffusion assay, and/or spectrophotometer reducing sugar assay, as previously mentioned.

Spectrophotometer Reducing Sugar Assay

Analysis of enzymatic activity was based on the hydrolytic release of reducing sugars from polygalacturonic acid. Polygalacturonic acid substrate was dissolved in 50 mM sodium acetate buffer (0.2% w/v), pH 5.0. The substrate (50% of the 0.2 ml total reaction volume) was incubated with purified enzyme extracts (50% of reaction volume) at 35 °C for multiple time points (T=0 min, 1 min, 5 min, 15 min, 30 min, and 1 hrs). At the conclusion of the timed aliquots, 1 ml of 0.1 M borate buffer, pH 9.0 was added to each reaction mixture to terminate the reactions. The assays were developed with the addition of 0.2 ml of 2-cyanoacetamide (1% (w/v) in water) into each tube containing the

reaction mix plus borate buffer. Samples were heated in boiling water for 10 min and allowed to cool to room temperature before taking spectrophotometer readings at A_{276} with a BioRad SmartSpec 3000 spectrophotometer. A standard curve was prepared using a range of Gal A stock solution (0.2 mg in water) and sodium borate ratios in 200 µl volumes. Stock solution Gal A was prepared and aliquoted at 0, 50, 100, 150, and 200 µl of Gal A stock with 200, 150, 100, 50, 0 µl of sodium borate buffer. These ratios represented a range of 0, 10, 20, 30, and 40 µg of Gal A for the standard curve.

Radial Diffusion Assay (Performed at U.C. Davis, Davis CA)

The radial diffusion assay was performed in petri dishes containing 20 ml of assay medium (1% agarose (Type II), 0.5% ammonium oxalate, and 0.2% in sodium azide in 0.2 M phosphate buffer, pH 5.3 with 0.01% polygalacturonic acid as the substrate). The medium was heated to dissolve all of the components and transferred into the petri dishes. After agar solidification, 4.1 mm diameter wells (6 wells total) were punched into the agar utilizing a cork borer. Approximately 30 µl of enzyme extract (polygalacturonase), standard, or control was added to two of the wells and incubated at 37 °C for 17 hrs. The assay was then developed by adding 10 ml of 0.05% ruthenium red and incubated at room temperature for 30 min. At the conclusion of the incubation period, excessive dye was rinsed from the plate with several washes of deionized water and diameter of the clear areas of activity was measured. (Taylor 1988).

CHAPTER III

RESEARCH RESULTS AND DISCUSSION

Among the many pathogens encountered by grapevines, *Xylella fastidiosa* may potentially be the most threatening and difficult to manage. *X. fastidiosa* produces a polygalacturonase enzyme allowing the pathogen to degrade the pectin in the plant cell wall. In order to suppress Pierce's Disease, which is caused by *X. fastidiosa*, polygalacturonase inhibiting proteins (PGIPs) need to be discovered and screened *in vitro*. The current research aimed to clone, express, and partially characterize the *X. fastidiosa* polygalacturonase gene in a system capable of producing large quantities (µg amounts) of functional PG protein and to purify it from other proteins. Functional PG protein will then be used to screen efficacy of potential PGIPs.

The recombinant plasmids pCR-BluntII-TOPO/*Xf*PG4 and pET29b/*Xf*PG mutant (received from U.C. Davis) were transformed into Top 10 *E. coli* cells and plated onto LB/kanamycin for selection of positive transformants which possessed kanamycin resistance. Individual positive transformants were selected for plasmid DNA isolation. Overnight cultures were used in order to allow bacterial cell growth and amplification of the plasmid DNA. Plasmid isolation and purification was accomplished with the QIAprep Spin Miniprep Kit following the manufacturer's protocol (Qiagen) to complete the miniprep process. A minimum of ~5 µg of plasmid DNA was isolated from each overnight culture.

***Xylella fastidiosa* Polygalacturonase Gene Amplification Utilizing PCR:** The purified *Xf*PG DNA plasmids were used as the template for polymerase chain reaction (PCR) to amplify the target polygalacturonase gene while excluding the stop codon. Primers were designed against the flanking regions of the polygalacturonase gene and include engineered restriction enzyme sites EcoRV (forward primer) and XhoI (reverse primer) on the ends. Theses custom designed primers were used in the cloning process to ensure correct orientation of the gene into the cloning vector. VENT polymerase was chosen for the PCR reactions due to its proofreading capabilities, and PCR products lacking adenine overhangs were satisfactory for the expression vector used. The expected PCR products were 1695 bp based on theoretical sequence analysis. The PCR products were separated in a 1% (w/v) agarose gel stained with ethidium bromide and confirmed at approximately1570 bp (calculated with a standard curve of the ladder) utilizing horizontal gel electrophoresis techniques (Figure 11, Figure 12). The migration patterns for the PCR products (1570 bp) were comparable to results from work previously performed with the *X. fastidiosa* wild-type polygalacturonase gene, *pgl*A, which had determined the gene to be 1695 bp in length (29).

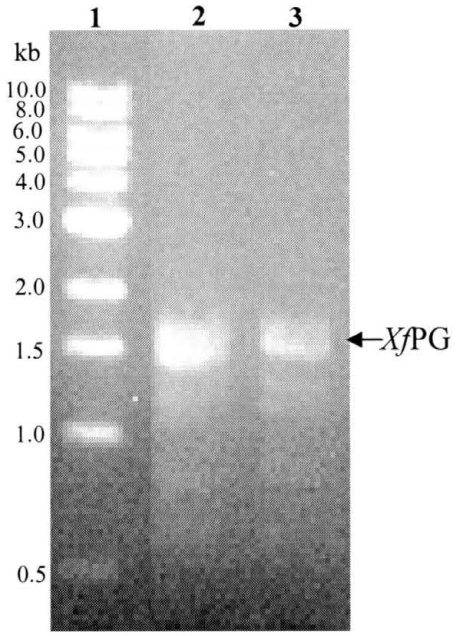

Figure 11: PCR amplification of *Xf*PG4 gene. Lane 1- 1 kb ladder, lane 2- *Xf*PG4 PCR product utilizing VENT polymerase, and lane 3- *Xf*PG4 PCR product utilizing VENT polymerase with 5 mM Mg^{2+}. Agarose gel (1%) stained with ethidium bromide.

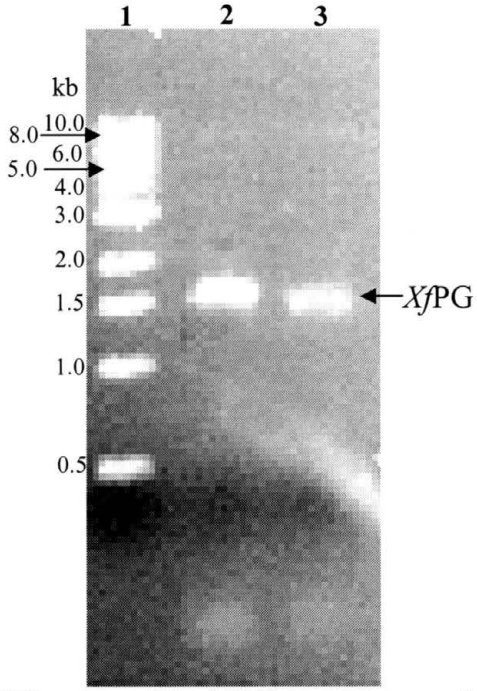

Figure 12: PCR amplification of the *Xf*PG mutant gene. Lane 1- 1 kb ladder, lane 2- *Xf*PG mutant PCR product utilizing standard Taq polymerase, and lane 3- *Xf*PG mutant PCR product utilizing VENT polymerase. Agarose gel (1%) stained with ethidium bromide.

After confirmation that the correctly sized PCR products were produced, they were purified with the Wizard SV Gel and PCR Clean-Up System (Promega) followed by digestion with XhoI and EcoRV restriction enzymes (Table 1) for 3 hours to create ends complementary to the digested expression vector. Digested *Xf*PG gene fragments were subjected to horizontal gel electrophoresis in a preparative agarose gel for the purpose of gel extraction. The DNA-containing gel extracts were then purified using Promega Wizard SV Gel and PCR Clean-Up System protocol (Figure 13).

Digestion of the Expression Vector pMT/BiP/V5-His A: Isolated *Xf*PG gene was incorporated into an insect expression vector, pMT/BiP/V5-His, creating a recombinant plasmid. The expression vector offered an inducible metallothionein promoter which allowed controlled expression of the inserted gene. The *Xf*PG gene followed a secretion signal sequence, BiP, naturally found in *Drosophila,* resulting in improved protein yield and quality. Proceeding behind the gene was an antibody tag, V5, and a poly-histidine tail.

The cloning and expression vector pMT/BiP/V5-HisA was chosen for *Xf*PG expression, and was transformed into Top 10 *E. coli* cells and selected for on LB/AMP media. Positive transformants were amplified for 20 hours in LB broth containing ampicillin with continuous shaking at 37 °C and purified using QIAprep Spin Miniprep kit. The isolated plasmids were digested utilizing the restriction enzymes, XhoI and EcoRV, which were used to digest the PCR products. Digestion products were treated with alkaline phosphatase (calf intestinal) to dephosphorylate of the 5' ends in order to prevent vector recyclization during the ligation process. The digested, dephosphorylated

vector products were analyzed by horizontal gel electrophoresis in 1% (w/v) agarose gel containing ethidium bromide. According to the vector map provided by Invitrogen's Vector NTI program, migration of the digested expression vector was expected to be at 3624 bp and was observed at approximately 3.8 kb. The digested fragments were gel extracted, purified with Promega Wizard SV Gel and PCR Clean-Up System following the manufacturer's protocol, and quantitated (Figure 13). Quantitation revealed DNA concentrations for the extracted and purified products to be 39 ng/µl of pMT expression vector, 11 ng/µl of *Xf*PG mutant, and 23 ng/µl of *Xf*PG4.

Table 1: Restriction Enzyme used to Digest Samples.

Plasmid Name	**Enzyme 1**	**Enzyme 2**	**NEB Buffer**	**Expected (bp)**	**Actual Size (bp)**
pMT/BiP/V5-His A	*Xho* I	*EcoRV*	3	3624	~3776
*Xf*PG	*Xho* I	*EcoRV*	3	1695	~1730

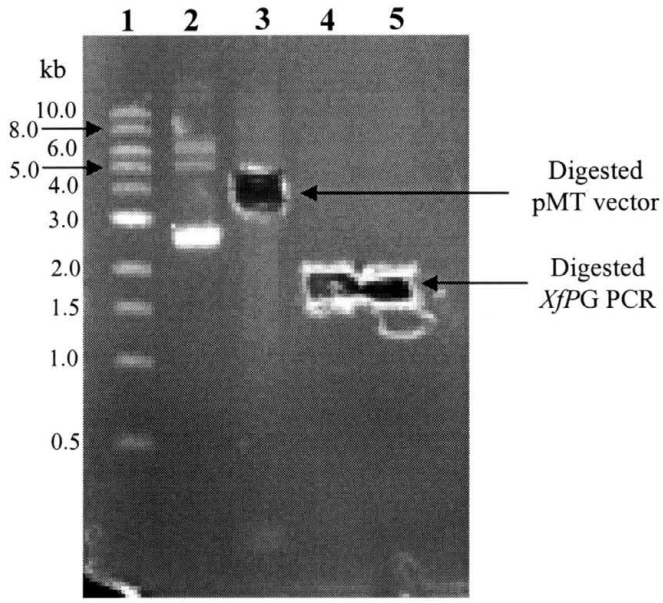

Figure 13: Digested and gel extracted PCR amplified *Xf*PG mutant gene products and pMT expression vector. Lane 1- 1 kb ladder, lane 2- uncut pMT vector, lane 3- digested pMT vector, lane 4- *Xf*PG PCR amplification with Taq polymerase, and lane 5- *Xf*PG PCR amplification with VENT polymerase. 1% agarose gel stained with ethidium bromide

***X. fastidiosa* Polygalacturonase Sub-cloning:** The digested *Xf*PG gene and pMT/BiP/V5-His A vector DNAs were ligated at a 3:1 concentration ratio using T4 DNA ligase at 4 °C overnight. The ligation reactions were then transformed into competent Top 10 *E. coli* cells and spread on LB/Amp plates for selection and incubated for 24 hours at 37 °C. Positive transformants were used to innoculate LB/Amp cultures, which were grown overnight at 37 °C with constant shaking to encourage cell proliferation for gene amplification. The Qiagen Plasmid Purification kit was used to isolate and purify the plasmids from the overnight culture.

Two different confirmation digestions utilizing (1) XhoI and EcoRV or (2) HindIII were performed on six recombinant plasmids, pMT/BiP/V5-His A/*Xf*PG4, and one confirmation digest utilizing EcoRI was performed on pMT/BiP/V5-His A/*Xf*PG

mutant, to ensure correct orientation of the PG gene in the pMT expression vector. The expected DNA fragments for digested pMT/BiP/V5-His A/*Xf*PG4 were (1) 3624 bp and 1695 bp when digested with XhoI and EcoRV and (2) 1101 bp, 1269 bp, and 2887 bp when digested with HindIII. The new constructs were also compared to fragment sizes of undigested recombinant plasmid, pMT/BiP/V5-His A/*Xf*PG4 (Figure 14, lanes 2, 4, 6, 9, 11, and 13). Analysis of XhoI and EcoRV digested products with horizontal gel electrophoresis revealed DNA fragments of approximately ~1620 bp and ~3440 bp (Figure 14, lanes 7, 10, and 12), which was expected in the ligation of *Xf*PG4 into pMT/BiP/V5-His A. Further digestion analysis were performed on the previously confirmed pMT/BiP/V5-His A/*Xf*PG4 ligations by digesting with HindIII and the results revealed products at ~2880 bp, ~1276 bp, and 1106 bp (Figure 15, lanes 5 and 7). The results, in part, confirm that the PG gene had been correctly ligated into the pMT expression vector. The DNA fragments that migrated to approximately 2000 bp represent supercoiled undigested expression vector lacking the *Xf*PG gene (Figure 14, lanes 2 and 4) 3600 bp was expression vector lacking *Xf*PG gene that had been digested with XhoI and EcoRV (Figure 14, lanes 3 and 5). In addition, the DNA products at approximately 4000 bp were supercoiled undigested recombinant plasmid (expression vector with the *Xf*PG gene) (Figure 14, lanes 13 and 14).

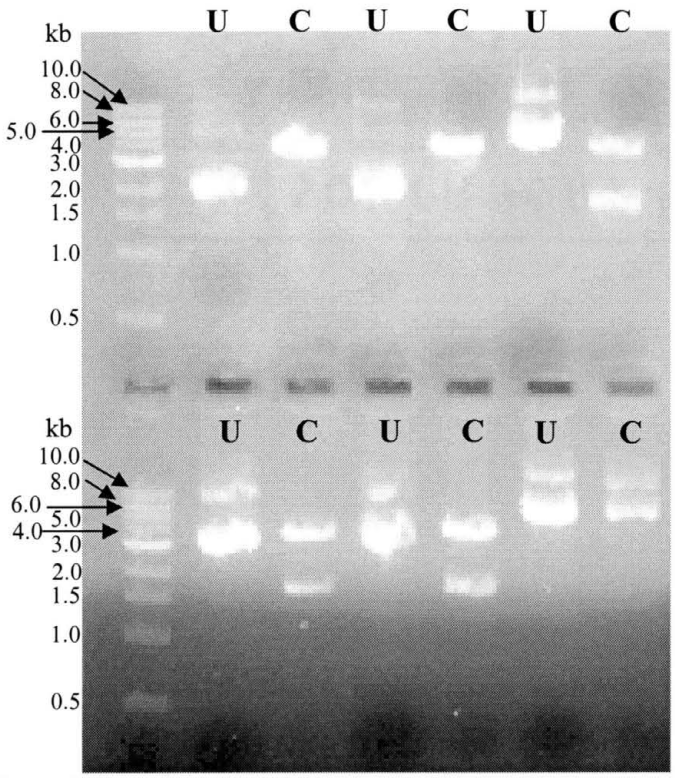

Figure 14: Confirmed digestion of pMT/BiP/V5-His A/*Xf*PG4. Verified ligation of *Xf*PG into the pMT expression vector. C=Cut/digested, U=Uncut/undigested. Lane 1 & 8- 1 kb ladder; lanes 2, 4, 6, 9, 11, and 13 were undigested positive transformants, and lanes 3, 5, 7, 10, 12, 14 were positive transformants digested with EcoRV and XhoI. 1% agarose gel stained with ethidium bromide.

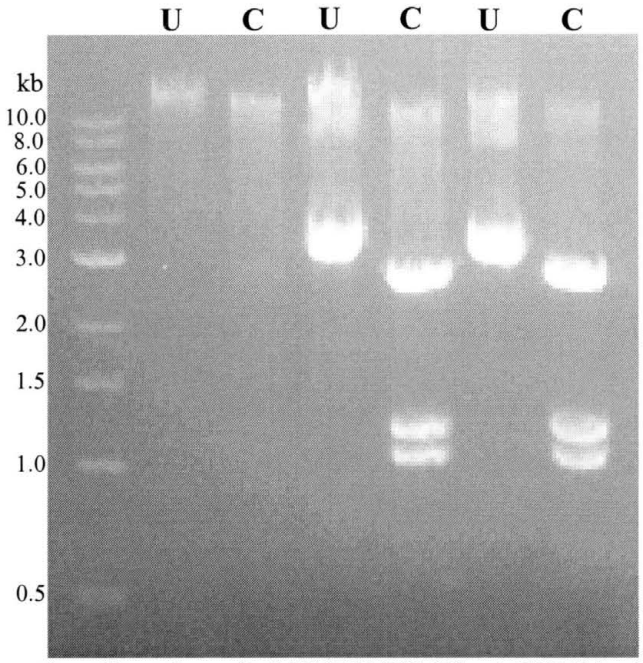

Figure 15: Confirmation digestion of pMT/BiP/V5-His A/*Xf*PG4. C=Cut/digested, U=Uncut/undigested. Lane 1- 1 kb ladder, lane 2, 4, and 6- uncut recombinant plasmid (pMT/BiP/V5-His A/*Xf*PG4), lane 3, 5, and 7- recombinant plasmid digested with HindIII. Gel stained with ethidium bromide.

The expected fragment sizes for pMT/BiP/V5-His A/*Xf*PG mutant DNA digested with EcoRI were 1280 bp and 3800 bp. The new constructs digestion fragments were also compared to the size of digested pMT/BiP/V5-His A (no PG), undigested pMT/BiP/V5-His A (no PG), and undigested recombinant plasmid (pMT/BiP/V5-His A/*Xf*PG mutant). Preliminary analysis of *EcoR* I digested products with horizontal gel electrophoresis revealed DNA fragments at approximately ~1250 bp and ~4000 bp (data not shown) indicating that the PG gene had been correctly ligated into the pMT expression vector. The pMT/BiP/V5-His A/*Xf*PG mutant undigested supercoiled plasmid migrates similar to a 5200 bp fragment. A plasmid midi-prep isolation was performed on each confirmed clone, followed by DNA quantitation (Table 2) and automated sequencing at Davis Sequencing (Davis, CA). Midi-preps resulted in ample isolated

DNA: 1.6 µg/µl (487 total µg) and 1.7 µg/µl (506 total µg) for the pMT/BiP/V5-His A/*Xf*PG mutant and 1.3 µg/ µl (400 total µg) for pMT/BiP/V5-His A/*Xf*PG4.

Table 2: Plasmid Quantitation with the Nano Drop.

Recombinant Plasmid	**DNA Concentration (µg/µl)**
pMT/BiP/V5-His A/*Xf*PG mutant	1.6, 1.7
pMT/BiP/V5-His A/*Xf*PG4	1.3

Sequence analysis using Vector NTI and BLAST software revealed that the PG gene in the recombinant pMT/BiP/V5-His A/*Xf*PG4 construct was 100% identical to the XfPG gene in the original clone (provided by J. Labavitch at U.C. Davis). The recombinant mutant construct, pMT/BiP/V5-HisA/*Xf*PG mutant, had a single point mutation in the gene at position 576 resulting in a threonine to alanine amino acid change; the effects of this mutation on physical properties and functionality have yet to be determined. In addition, there is an additional point mutation that results in an amino acid change directly following the start codon. This results in conversion of an asparagine (Asn) to an aspartate (Asp) residue in each of the *Xf*PG constructs. Prior studies demonstrated that this mutation (Asn to Asp) does not affect *Xf*PG activity (personal communication per Zac Chestnut at U.C. Davis). The pMT/BiP/V5-His A/*Xf*PG mutant and pMT/BiP/V5-His A/*Xf*PG4 constructs were used for protein expression in the *Drosophila* S2 cells expression system (Figure 16).

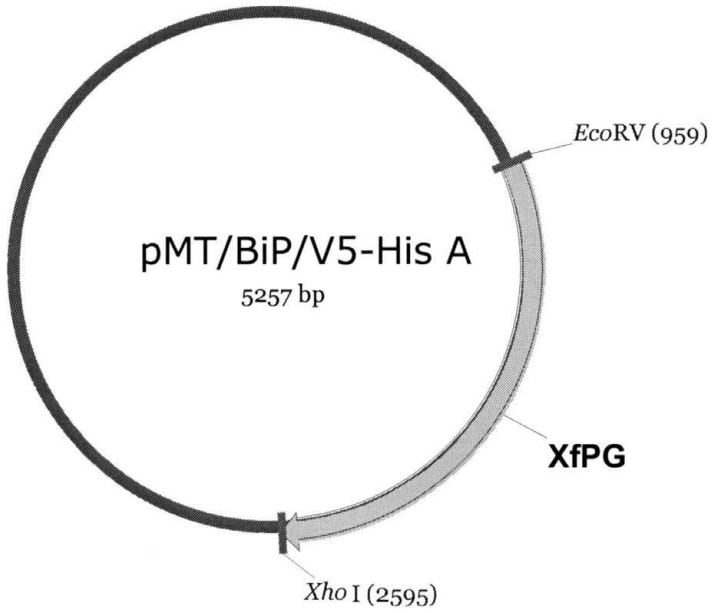

Figure 16: Recombinant plasmid vector map.

Expression of *Xf*PG in the *Drosophila* S2 Cells Expression System: The recombinant plasmids, pMT/BiP/V5-His A/*Xf*PG mutant and pMT/BiP/V5-His A/*Xf*PG4, and a positive control, pMT/BiP/V5-His A/GFP, were transfected into *Drosophila* S2 cells to produce measurable amounts of active protein. These cells were chosen because they are easy to maintain at room temperature, require minimal maintenance, are economically affordable, do not require CO_2, and yield higher protein than other systems, such as *E. coli*. The cells were cultured and seeded at a concentration of 1 x 10^6 cells/ml and grown for approximately 16 hours at 22-25 °C until they reached a density of 2-4 x 10^6 cells/ml. Transfection solutions and reagents were prepared including $CaCl_2$, recombinant DNA, and water for solution A and 2X HEPES Buffer for solution B. Combining the two solutions and incubating for 40 minutes created a fine, white precipitate which was then mixed with the cultured cells. After the transfected cells were incubated for 16-24 hours at 22-25 °C, the calcium phosphate solution was removed from

the cells. The cells were returned to the same culture dishes with fresh complete medium. Expression of the *Xf*PG genes was induced with the addition of copper sulfate to the medium (500 μM) in order to activate the metallothionein promoter, pMT, and initiate transcription. The signal sequence incorporated in the expression vector directed the expressed PG protein to be secreted outside of the cell as in native systems. Media containing induced cells were collected and harvested on post-transfection day 3.

The harvested cells were pelleted by centrifugation at 100 x g for 5 to 10 minutes and then separated from the supernatant (medium). Protein was extracted from the pelleted cells by resuspending them in Cell Lysis Buffer followed by an incubation period of 10 minutes at 37 °C. Once protein had been extracted from the cells, both samples, supernatant and cell extracted protein, were prepared for SDS-PAGE Analysis by incubating at 100 °C for 5 minutes in 1X SDS Sample Buffer, and centrifuging in order to remove cellular debris. Protein samples were then loaded onto an SDS-PAGE gel, electrophoresed, and transferred to nitrocellulose for Western blotting analysis. The V5 tag incorporated in the expression vector enabled detection of the protein with antibodies via Western blotting.

For visualization of *Xf*PG, the blot was probed with anti-V5 primary antibody, followed by an anti-mouse HRP (horse radish peroxidase) secondary antibody. *Xf*PG and GFP were expected at 62.2 kDa (calculated by converting the number of amino acids to kDa) and 27.0 kDa, respectively. A substantial amount of the *Xf*PG mutant protein was observed in the supernatant samples when compared to the pellet samples at approximately 79.0 kDa. Although the actual molecular weight was higher than that of the calculated molecular weight, it was in agreement with the range (34-140 kDa) of

pectinolytic enzyme molecular weights reported in the literature (Clausen 1996, Riou 1992). In addition, the *Xf*PG4 (non-mutant) protein in the pelleted cell lysate samples were also observed at approximately 78.0 kDa. GFP protein was visualized at 27.0 kDa as expected (Figure 17 and 18) (Amenzadeh *et al.* 2006, Thakur 2010, Osteryoung *et al.* 1990). The location and increased sizes of the two proteins could potentially be due to differences in protein folding, the BiP secretion signal (1.8 kDa), V5-His C-terminal tag (2.6 kDa), and possible post-translational modifications, such as glycosylation patterns (Spiro 2002, Stotz 1993). Similar molecular mass to *Xf*PG mutant of approximately 78.0 kDa were also observed by Bruce Kirkpatrick and associates when expressing *Xf*PG in *E.coli* (Kirkpatrick 2009). Furthermore, literature supports that native exopolygalacturonase proteins produced by *Sclerotinia sclerotiorum* yielded a molecular mass of 68.0 kDa similar to that determined here for the *Xf*PG4 protein (Riou 1992).

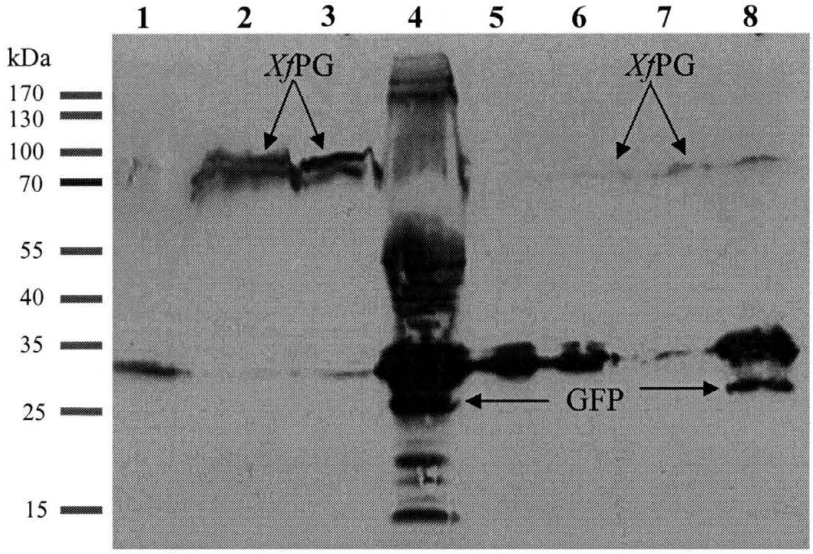

Figure 17: Western blot analysis of crude *Xf*PG mutant protein expression samples. This blot represents the media which the *Xf*PG protein was secreted in. Lane 1- media containing non-transfected *Drosophila* S2 cells, lane 2 and lane 3- media containing transfected *Drosophila*, lane 4- media containing GFP transfected *Drosophila* cells, lane 5- pelleted non-transfected *Drosophila* S2 cell lysate, lane 6 and lane 7- pelleted transfected *Drosophila* cell lysate, and lane 8- pelleted GFP transfected *Drosophila* cell lysate. Visualized with chemiluminescence using the Western Lighting Kit.

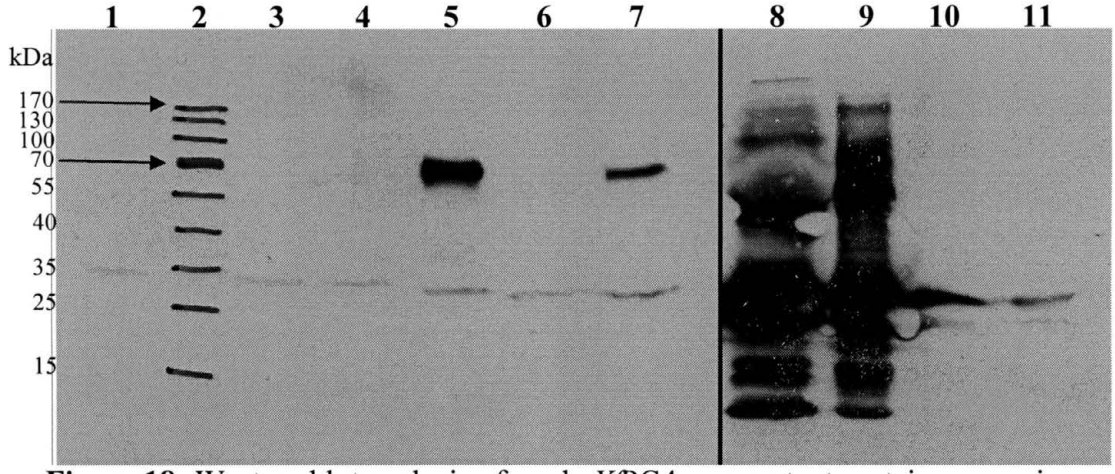

Figure 18: Western blot analysis of crude *Xf*PG4 non-mutant protein expression samples. This blot represents the media which the *Xf*PG protein was secreted in and protein produced in the cells, that was not secreted. Lane 1- media containing transfected *Drosophila* S2 cells, lane 2- Pre-stained Page Ruler, lane 3- pelleted transfected *Drosophila* cell lysate, lane 4- media containing transfected *Drosophila* S2 cells, lane 5- pelleted transfected *Drosophila* cell lysate, lane 6- media containing transfected *Drosophila* S2 cells, lane 7- pelleted transfected *Drosophila* cell lysate, lane 8- media containing GFP transfected *Drosophila* cells, lane 9- pelleted GFP transfected *Drosophila* cells, lane 10- media containing non-transfected *Drosophila* S2 cells, and lane 11-pelleted non-transfected *Drosophila* S2 cells lysate. Visualized with chemiluminescence using the Western Lighting Kit.

Once *Xf*PG mutant and non-mutant protein expression was confirmed via Western blot analysis, a time course was performed with the *Xf*PG mutant to determine the optimal expression time of *Xf*PG. Transient transfections were performed as previously described and induced cells were harvested on days 2, 3, 4, 5, 6, and 7, and analyzed utilizing Western blot analysis (Figure 19 A and B). *Xf*PG protein was visualized on each day samples were collected at approximately 78.0 kDa, however protein expression on days 2-4 appeared to be more abundant. Thus, the optimal expression time was determined to be 48 to 96 hrs post-induction. All future protein samples were collected during this optimal expression time.

Established stable cell lines for large-scale production were then generated by cotransfecting the expression vector with a selection vector (pCoHygro) containing the

antibiotic, hygromycin B (Figure 20). This system selects for only the stable transfectants and kill cells not containing the gene of interest (Howard *et al.* 2007).

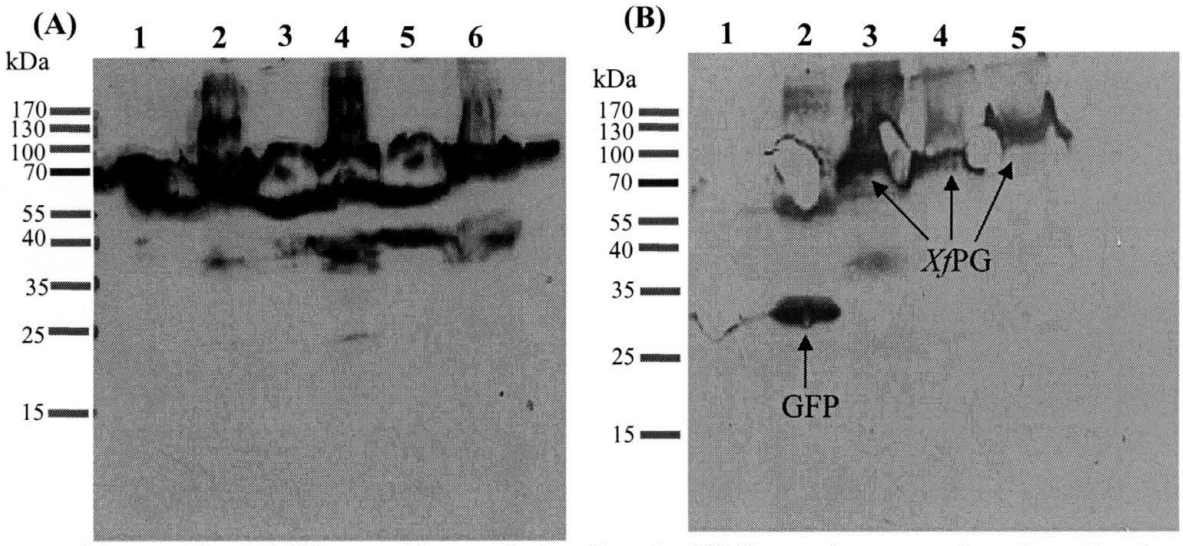

Figure 19: (A) Western blot analysis of crude *Xf*PG protein expression. Samples day 2-4 cell lysate and media from time course. Lane 1- media containing transfected *Drosophila* S2 cells harvested on day 2, lane 2- pelleted transfected *Drosophila* S2 cell lysate harvested on day 2, lane 3- media containing transfected *Drosophila* S2 cells harvested on day 3, lane 4- pelleted transfected *Drosophila* S2 cell lysate, lane 5- media containing transfected *Drosophila* S2 cells harvested on day 4, lane 6- pelleted transfected *Drosophila* S2 cell lysate. (B) Western blot analysis of crude *Xf*PG protein expression. Samples day 5-7 media containing transfected cells from time course. Lane 1- media containing non-transfected *Drosophila* S2 cells harvested on day 5, lane 2- media containing GFP transfected *Drosophila* S2 cells harvested on day 5, lane 3- media containing transfected *Drosophila* S2 cells harvested on day 5, lane 4- media containing transfected *Drosophila* S2 cells harvested on day 6, lane 5- media containing transfected *Drosophila* S2 cells harvested on day 7. Both blots were visualized with chemiluminescence.

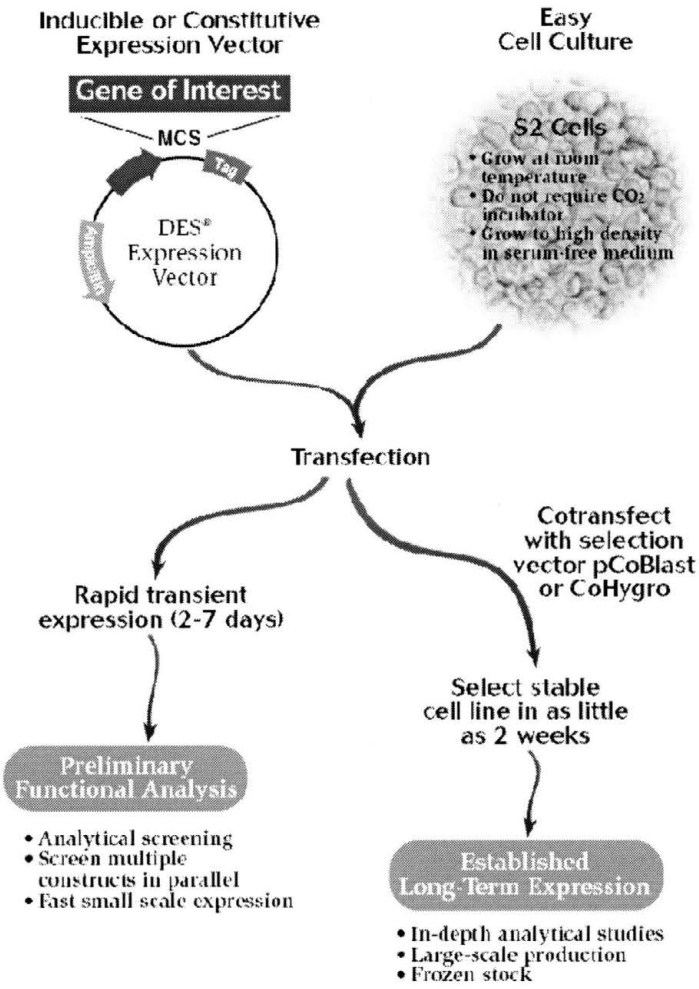

Figure 20. Overview of expression using the *Drosophila* S2 cell expression system (Howard 2007).

His_6-tag Purification: While stable cell lines are currently being established, transient transfections have provided protein. Confirmed *Xf*PG4 pelleted cell lysate (prepared by lysing cells pelleted from 15.0 mL of transfected media with 3.0 mL cell lysate buffer as previously described) and *Xf*PG mutant protein samples (15.0 mL of transfections) were partially purified utilizing the incorporated poly-His C-terminal tag in the expression vector by means of binding to an immobilized nickel, metal affinity column. Elution Buffer (50 mM sodium phosphate, 0.3 M sodium chloride) containing

imidazole (250 mM) was used to elute the protein off of the column. Western blot analysis with anti-V5 primary antibody and anti-mouse HRP secondary antibody was used to probe the *Xf*PG4 cell lysate and *Xf*PG mutant supernatant His-tag purification products which were observed at approximately 78.0 kDa (Figure 21, 22). This analysis revealed a large amount of *Xf*PG4 protein remaining unbound and being discarded in the flow-through. This observation could be due to incorrect binding conditions in the column, misfolding, or an inaccessible or degraded His_6-tag.

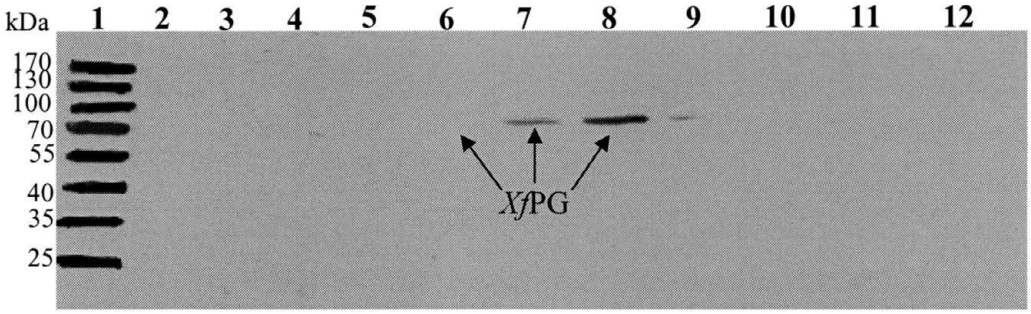

Figure 21: Western blot analysis of partially purified *XfP*G mutant protein expression. 3.0 mL crude XfPG sample was run in a Nickel affinity column. All samples were analyzed with Western Blot technique, including select flow-through and wash collections. Lane 1- Prestained PageRuler, lane 2- *Xf*PG flow-through 1, lane 3- *Xf*PG flow-through 2, lane 4- *Xf*PG flow-through 3, lane 5- *Xf*PG wash 10, lane 6- *Xf*PG wash 13, lane 7- *Xf*PG elution 1 with 250 mM imidazole, lane 8- *Xf*PG elution 2 with 250 mM imidazole, lane 9- *Xf*PG elution 3 with 250 mM imidazole, lane 10- *Xf*PG elution 1 with 500 mM imidazole, lane 11- *Xf*PG elution 2 with 500 mM imidazole, and lane 12- *Xf*PG elution 3 with 500 mM imidazole. The purified expressed protein was probed with anti-V5 primary antibody and anti-mouse (HRP) secondary antibody.

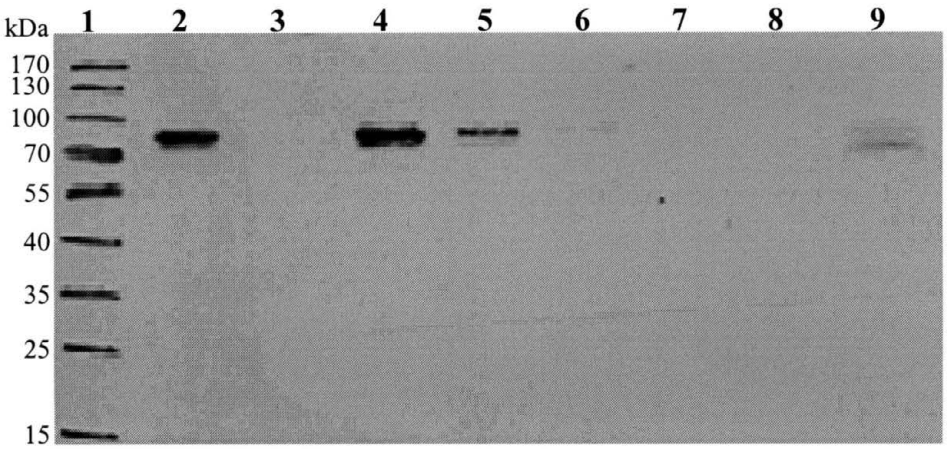

Figure 22: Western blot analysis of partially purified cell lysate *Xf*PG4 protein expression. 15.0 mL crude *Xf*PG sample was run in a Nickel affinity column. Selected samples were analyzed with Western Blot technique, including the fourth flow-through fraction and tenth wash collection fraction. Lane 1- Prestained PageRuler, lane 2- *Xf*PG flow-through #4, lane 3- *Xf*PG wash #10, lane 4- *Xf*PG elution I, lane 5- *Xf*PG elution II, lane 6- *Xf*PG elution III, and lane 7- *Xf*PG elution IV, lane 8 and 9- media containing expressed *Xf*PG protein. The purified expressed protein was probed with anti-V5 primary antibody and anti-mouse HRP secondary antibody.

Crude *Xf*PG4 supernatant samples, which had been analyzed and initially determined to not possess protein, were re-analyzed for confirmation. In the latter Western blot (Figure 22, lane 9), a faint protein appeared close to 78.0 kDa thus, these supernatants were purified and analyzed further. The *Xf*PG4 supernatant (SN) protein samples were purified following two different protocols. The first samples were purified identical to the lysate purifications and analyzed on the same SDS-PAGE as the cell lysate elution fractions. The SDS-PAGE analysis revealed a lack of protein in the pellet samples, but it was present in the supernatant elution fractions (Figure 23).

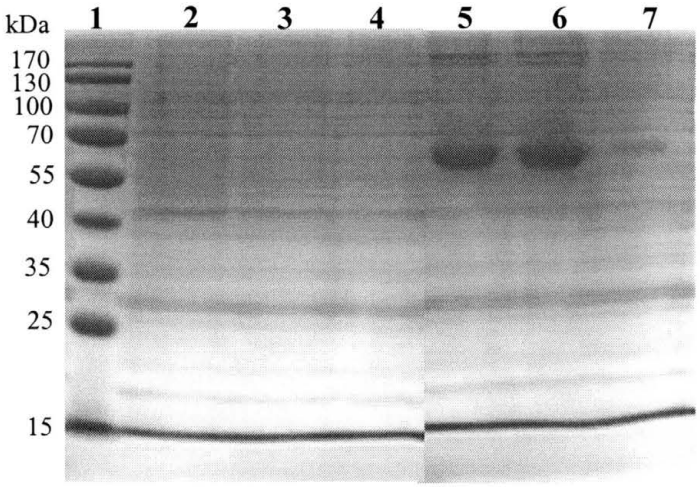

Figure 23: Partially purified *Xf*PG4 protein with 250 mM imidazole. Lane 1- pre-stained ladder, lane 2- purified pellet elution fraction I, lane 3- purified pellet elution fraction II, lane 4- purified pellet elution fraction III, lane 5- supernatant purification elution fraction I, lane 6- supernatant purification elution fraction II, and lane- 7 supernatant purification elution fraction III. Coomassie stained polyacrylamide gel electrophoresis.

The second *Xf*PG4 supernatant protein samples were initially dialyzed in 50 mM sodium phosphate buffer, pH 7.0 for 24 hours at 4°C. Purified on an immobilized nickel, metal affinity column, and washed with 50 mM sodium phosphate buffer pH 7.0 containing 50 mM sodium chloride. Elutions were attempted with 50 mM sodium phosphate buffer containing 300 mM sodium chloride, pH 7.0, and 50 mM sodium phosphate buffer containing 500 mM sodium chloride, pH 7.0, but were found unsuccessful when the fractions were analyzed for protein content utilizing the BioRad Smart Spec 3000 at 280 nm (the wavelength at which protein absorbs light); therefore, the elution buffer was changed to 150 mM Tris-HCl Buffer, pH 8.8 which produced fractions containing protein.

The *Xf*PG4 supernatant fractions eluted with 150 mM Tris-HCl were analyzed utilizing SDS-PAGE analysis. Staining with Coomassie revealed molecular masses at

78.0 and 68.0 kDa (Figure 24, lanes 6, 7, 8, and 9). Protein visualized at 78.0 kDa represented the *Xf*PG protein while the band at 68.0 kDa was assumed to be bovine serum albumin (BSA) which has a molecular weight of 66.0 kDa. This SDS-PAGE analysis also revealed a large amount of *Xf*PG4 protein not binding the column and remaining in the flow-through (Figure 24, lane 2), as was previously seen with the purified *Xf*PG4 cell lysate analysis. This observation could again be due to incorrect binding conditions or an inaccessible or degraded His_6-tag (Figure 24). Once a sufficient amount of PG had been purified, activity studies were performed (Shackel 2005).

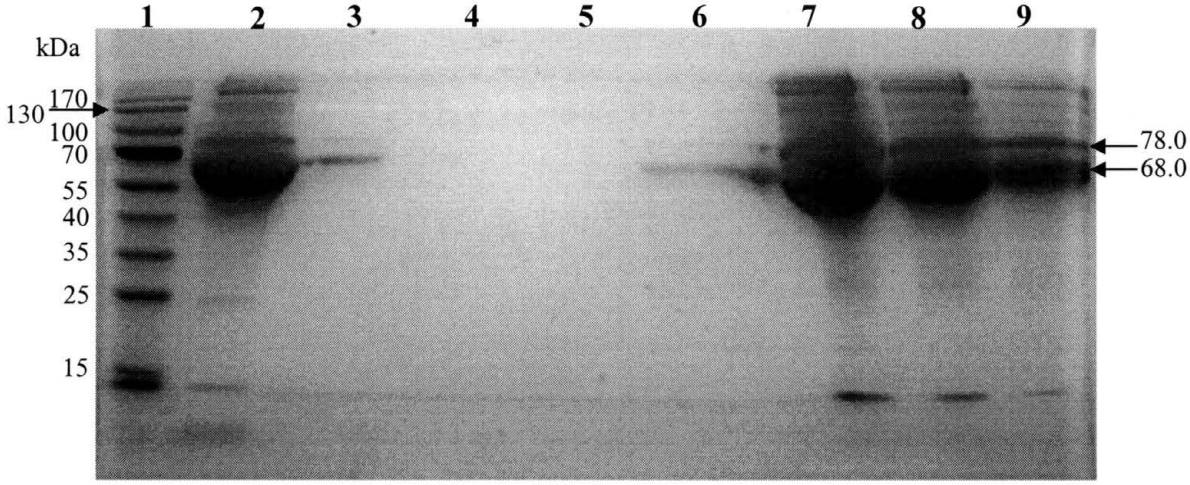

Figure 24: Partially purified *Xf*PG4 protein from supernatant. Lane 1- pre-stained ladder, lane 2- flow-through #3, lane 3- wash #6, lane 4- elution #2 with 300 mM NaCl, lane 5- elution #2 with 500 mM NaCl, lane 6- elution #1 with 150 mM Tris-HCl, lane 7- elution #2 with 150 mM Tris-HCl, lane 8- elution #3 with 150 mM Tris-HCl, and lane 9- elution #4 with 150 mM Tris-HCl. Coomassie stained polyacrylamide gel electrophoresis.

Radial Diffusion Assay: Confirmed purified supernatant PG protein samples, *Xf*PG4 and *Xf*PG mutant, were sent to collaborators at U. C. Davis to analyze PG activity by performing the radial diffusion assay. Their findings concluded that the *Xf*PG mutant

possessed minimal hydrolyzing activity when compared to the positive (pectinase) and negative controls (purified media collected from non-transfected cells). In a previous study, the radial diffusion assay was capable of detecting low PG activities (7 × 10-5 PG units/mL) (Buescher 1992) based on the amount of digested pectin due to the presence of PG when compared to a (+) control; larger "cleared" zones indicated greater PG activity and small zones indicated low levels of PG activity (Figure 25). Further analysis was performed with spectrophotometer reducing sugar assay to confirm PG activity.

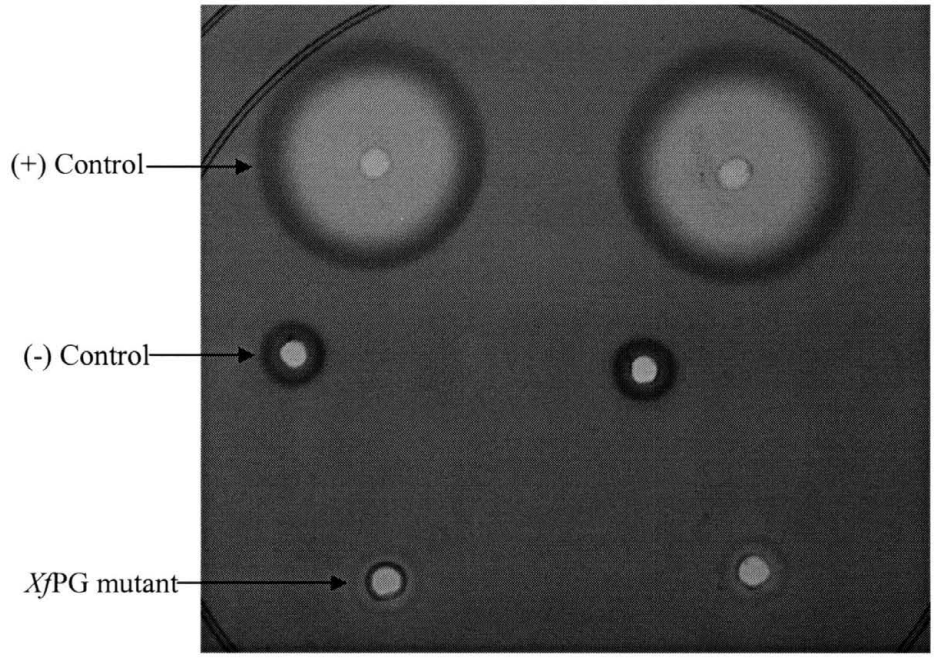

Figure 25: *Xf*PG mutant protein radial diffusion assay. 30 µl of *Xf*PG mutant protein was tested against a (-) control (media from non-transfected cells) and (+) control. Minimal hydrolyzing activity was observed.

Spectrophotometer Reducing Sugar Assay: Polygalacturonase catalyzes the hydrolysis of polygalacturonan polymer to yield new, free galacturonosyl C-1 groups

which can exist as an open chain "aldehydo" form or as the closed ring form. In the presence of 2-cyanoacetamide the uncyclized terminal GalA residue on a monosaccharide, oligosaccharide, or smaller polymeric pectin product hydrolyzed by PG forms an adduct that absorbs at 276 nm (Gross 1982). Honda *et al.* (1988) speculated that this adduct was one of two derivatives: 3-cyano-2-pyridone or 3-cyano-2-pyrrolidone. The latter compound also identified as a conjugated diene-ol. This reaction has been effective in quantifying 5 to 750 nmol amounts of reducing sugar (Carrillo-Lopez *et al.* 2002). Activity of PG was determined using a standard curve of galacturonic acid under the same conditions as previously described (Figure 26). Analysis of the standard curve indicated that as protein concentration increased the absorbance at 276 nm increased in a parallel fashion (Roper 2007).

Purified *Xf*PG4 protein samples (cell lysate and supernatant) utilizing elution buffers containing imidazole, which had been previously confirmed by Western blot and/or SDS-PAGE analysis, were assayed for 1 hour (aliquots were removed for analysis at T= 0, 5, 15, 30, and 60 min) utilizing the spectrophotometer-based reducing sugar assay to determine enzymatic activity (Figure 27). The standard curve obtained for Gal-A (free sugar) showed great linearity for sample concentrations between 0 μg and 40 μg (Figure 26). According to the fluorescent intensities produced by the assay, elution fractions #2 for both supernatant (E2 SN) and pelleted (E2 Pellet) samples in addition to SN fraction #3 (E3 SN) showed positive linearity indicating polygalacturonase hydrolyzing activity increased over time at 35 °C.

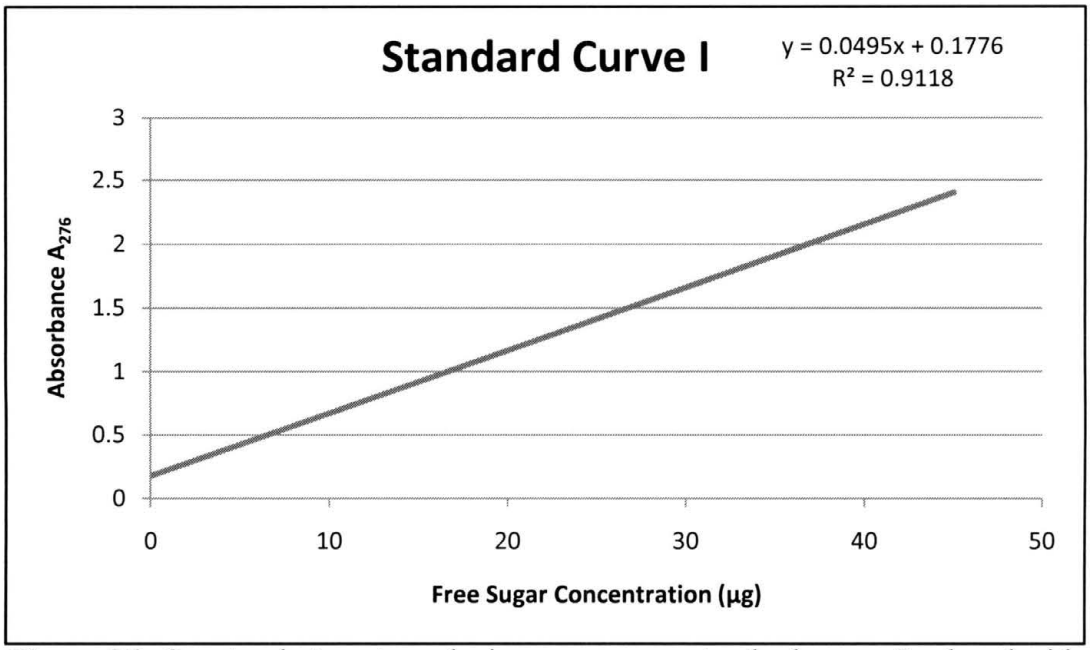

Figure 26: Spectrophotometer reducing sugar assay standard curve. Produced with different ratios of galacturonic acid to borate buffer and was used to determine efficiency of polygalacturonase activity. Higher concentrations of galacturonic acid produced higher absorbance at 276nm.

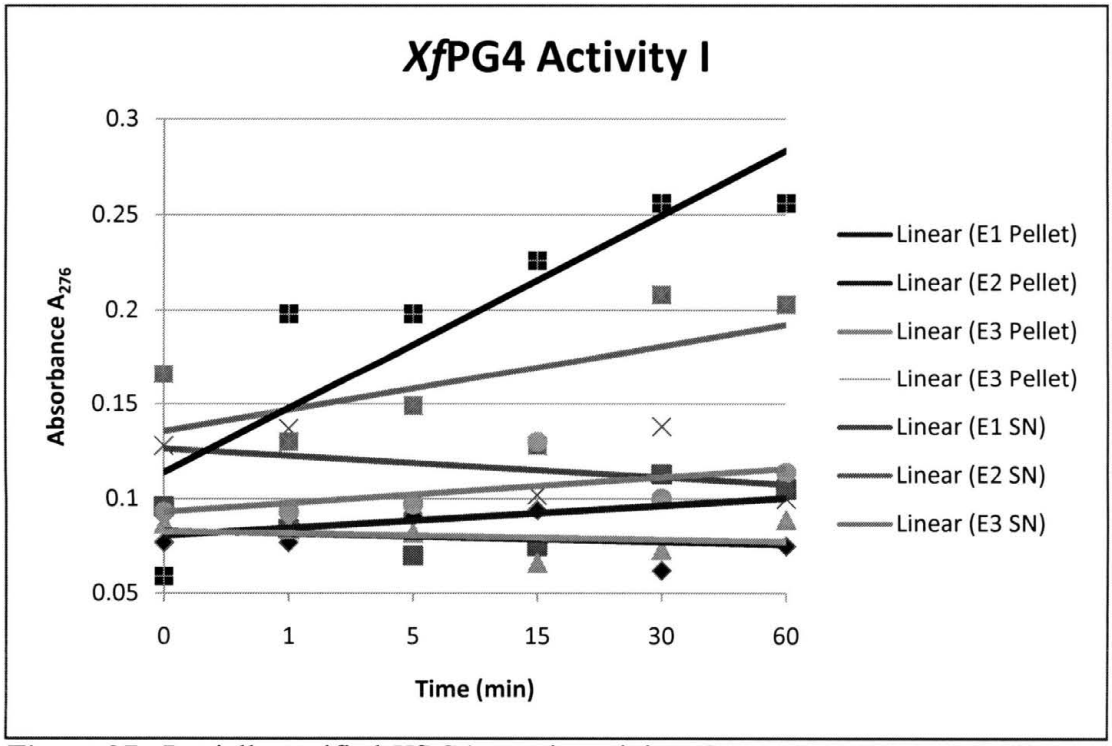

Figure 27: Partially purified *Xf*PG4 protein activity. Supernatant (SN) and cell lysate (pellet) elution fractions purified with imidazole containing buffers were analyzed at A_{280} for products produced by PG hydrolyzing activity. SN E2 & E3 both displayed potential PG activity in addition to Pellet E2 over a time of 60 min.

Table 3: R^2values and slope equations of *Xf*PG4 spectrophotometer reducing sugar assay activity I.

Sample	Slope Equation	R^2 Values
(+) Control	y = 0.0339x + 0.0801	0.7502
E1 Pellet	y = -0.0015x + 0.0845	0.0567
E2 Pellet	y = 0.0039x + 0.0768	0.185
E3 Pellet	y = -0.0015x + 0.0845	0.0567
E1 Supernatant	y = -0.0038x + 0.1302	0.1327
E2 Supernatant	y = 0.0114x + 0.1242	0.3687
E3 Supernatant	y = 0.0045x + 0.0886	0.3339

The E2 elution fractions from the previous study were re-analyzed over a longer time period (0-240 min) to confirm PG activity. Another standard curve was generated in the same manner as before for an accurate evaluation (data not shown). The results were not replicated in this assay. Neither SN E2 nor Pellet E2 displayed PG hydrolysis activity when compared to the positive control (*Aspergillus niger* pectinase) which depicted increased hydrolyzing activity over the allotted time (Figure 28). From this analysis it was speculated that the protein may not be stable at the -20 °C storage conditions or a biochemical compound may be causing inhibitory effects on the protein.

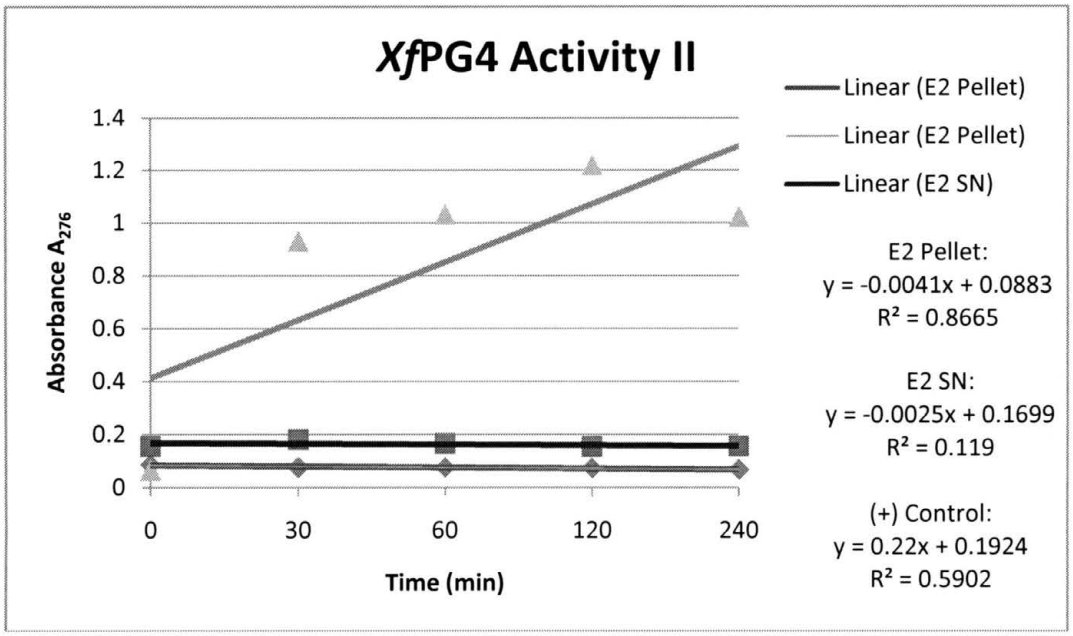

Figure 28. Partially purified *Xf*PG4 fractions SN E2 and Pellet E2 activity. These samples were re-analyzed using the spectrophotometer reducing sugar assay. (+) control displayed positive linearity with absorbance over time. SN E2 and Pellet E2 displayed no PG activity.

Due to the results of the previous spectrophotometer analysis, inhibitory effects during the protein purification process were analyzed first. Imidazole was the eluting compound utilized during the elution step of *Xf*PG protein purification, and therefore was studied first. As a preliminary study, imidazole was tested against the positive control by adding it to the substrate of (+) control sample (*A. niger*) in the assay at the same concentration as the elution buffer. The imidazole addition resulted in extensive inhibition of the positive control's hydrolyzing capabilities when compared to the positive control sample lacking imidazole. These results strongly suggested that imidazole could strongly inhibit the hydrolyzing capabilities of the *Xf*PG protein of interest (Figure 29).

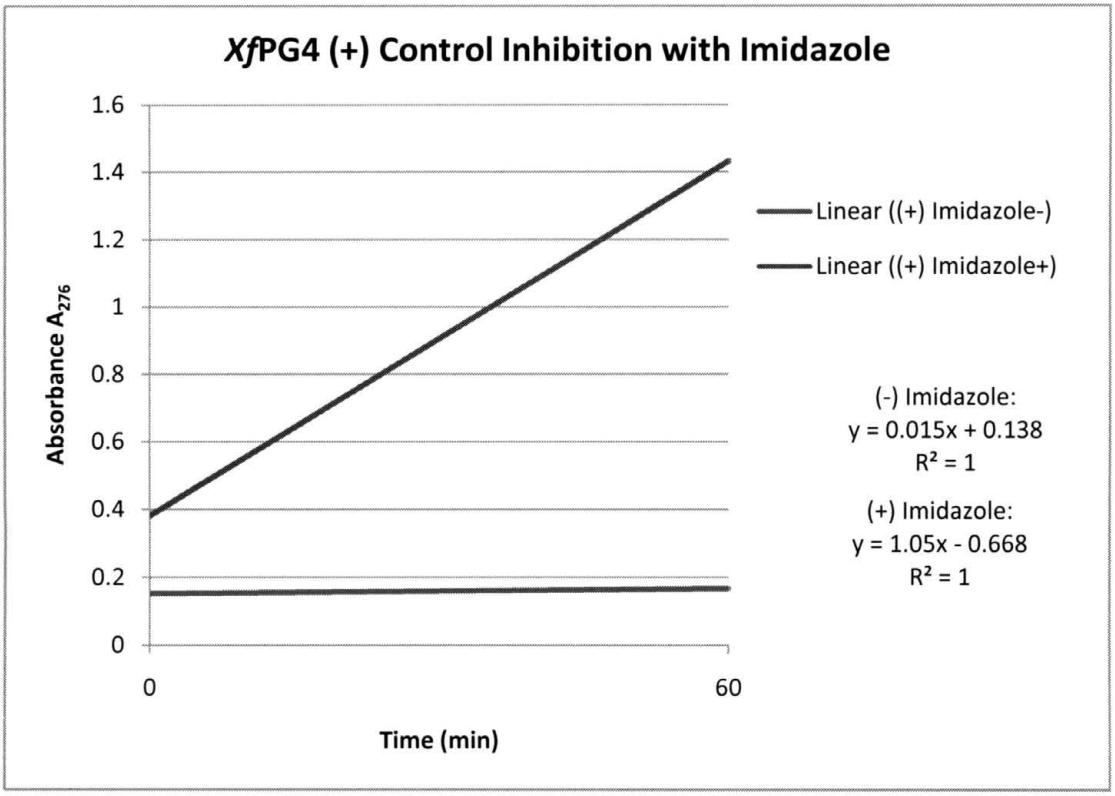

Figure 29. Inhibition of PG (+) control by imidazole. Imidazole containing substrate inhibited the (+) control's hydrolyzing capabilities when compared to imidazole lacking substrate sample.

To avoid imidazole inhibition and possible interference from other biochemical compounds contained in the transfection media, the collected *Xf*PG protein SN samples were dialyzed in sodium phosphate buffer, and then purified with buffers lacking imidazole (Tris-HCl buffer, pH 8.8). The elution fractions were initially analyzed with the BioRad SmartSpec at A_{280} (data not shown) and SDS-PAGE analysis to confirm the presence of *Xf*PG protein (Figure 24). Assay results revealed minimal to no PG activity for the purified protein fractions (Figure 30).

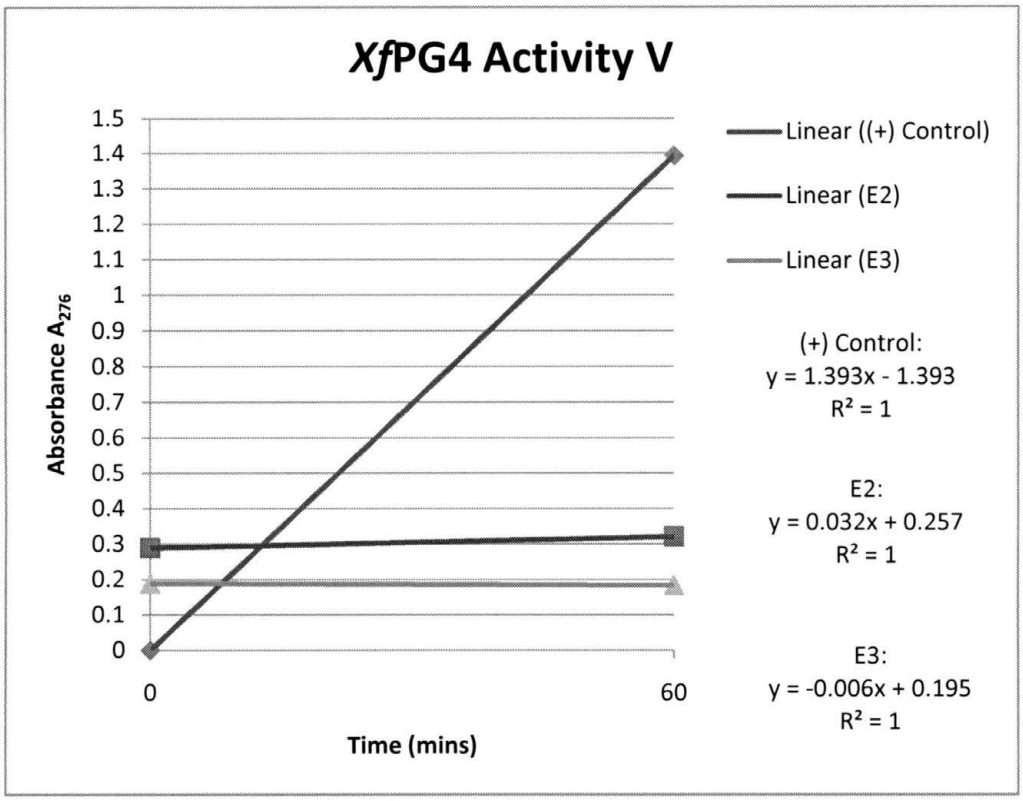

Figure 30. Partially purified dialyzed *Xf*PG4 SN samples activity. Dialyzed and purified *Xf*PG4 SN samples were subjected to the spectrophotometer reducing sugar assay. There was little to no activity demonstrated.

In previous studies, polygalacturonase naturally found in tomato (*Lycopersicon esculentum*) and prickly pear and PG produced by the mold, *Penicillium expansum*, have been successfully assayed using 2-cyanoacetamide and the spectrophotometer reducing sugar assay using the same technique described here (Roper 2007, Gross 1982, Conway 1988). However, two different studies have showed evidence that several biochemical compounds containing aldehydes (formaldehyde and acetaldehyde), amino acids (cysteine, glycine, histidine, threonine, alanine, glutamic acid, phenols, and high amounts of urea and sodium chloride (thousand-fold excess amounts) may interfere with the formation of the gal A/2-cyanoactemide complexes and thereby the results of the spectrophotometer assay (Honda 1980, 1982).

It may be of interest in future studies to examine other means of protein purification, such as cation or anion exchange chromatography in combination with gel filtration (Riou 1992). Also, the parameters of the spectrophotometer reducing sugar assay may be applied to fluorometry and enhanced with the addition of potassium dihydrogen phosphate (Honda 1980). The results from the radial diffusion assays and spectrophotometer reducing assay in this study revealed that *Xf*PG has potential hydrolyzing capabilities; however, for optimal activity analysis, the purification methods, optimal storage conditions, and biochemical compound interference with the spectrophotometer assay need to be further explored.

CHAPTER IV

CONCLUSION

X. fastidiosa, the causal agent of Pierce's disease of grapevines, is spread by an array of xylem feeding sharpshooter species. The bacterium utilizes pectinolases such as polygalacturonase to gain access to the xylem and systemically infect the grapevines. However, the mechanism by which polygalacturonase mediates infection is still unknown. This study aimed to clone, express, and partially characterize the *X. fastidiosa* polygalacturonase gene in a system capable of producing large quantities (μg amounts) of functional PG protein. *Xf*PG was successfully subcloned into the pMT/BiP/V5-His A expression vector, expressed in *Drosophila* S2 cells, and partially purified utilizing a Ni^{2+} affinity chromatography column.

Additional studies need to be performed on the optimum purification methods, optimal storage conditions, and biochemical compound interference with the spectrophotometer assay before activity and characterization studies can be successfully accomplished. The results produced from this study will contribute to efforts of characterizing *Xf* polygalacturonase leading to the creation of transgenic grapevines and suppression of Pierce's Disease.

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