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UNIVERSITY OF CALIFORNIA
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The Use of Symbionts Expressing Pear PGIP to Delay or Possibly Prevent Development
of Pierce's Disease

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy
in

Cell, Molecular, and Developmental Biology
by

Surachet Charoenkajonchai

December 2011

Dissertation Committee:

Dr. Thomas A. Miller, Chairperson
Dr. Donald A. Cooksey
Dr. Anandasankar Ray
Dr. Caroline Roper

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2011

The Dissertation of Surachet Charoenkajonchai is approved:

Committee Chairperson

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This dissertation is dedicated with love to my parents,

Mr. Chai and Mrs. Jenjira Charoenkajonchai

ABSTRACT OF THE DISSERTATION

The Use of Symbionts Expressing Pear PGIP to Delay or Possibly Prevent Development
of Pierce's Disease

by

Surachet Charoenkajonchai

Doctor of Philosophy, Graduate Program in Cell, Molecular, and Developmental Biology

University of California, Riverside, December 2011

Dr. Thomas A. Miller, Chairperson

Polygalacturonase (PG) is produced by endophytic bacteria to degrade the pit membranes of xylem vessels to facilitate movement between xylem channels. Pear polygalacturonase-inhibiting protein (pPGIP) is produced by the pear plant to block the movement of pathogenic bacteria in the plant. pPGIP is an agent being examined to protect against the pathogen *X. fastidiosa* from causing PD in grapevine. In the present study, candidate symbiotic bacteria were genetically altered to express pPGIP to inhibit the movement of *X. fastidiosa* and prevent development of PD symptoms in grapevines. Here I report successful transformation of *P. agglomerans* and *X. fastidiosa* with pPGIP plasmids, pMCS5-PGIP and pXF20-PGIP respectively. Stability of plasmid pMCS5-

PGIP and pXF20-PGIP was verified by PCR analysis and gel electrophoresis. Expression of pPGIP by both of these bacteria was verified by Western blot analysis. However *P. agglomerans* pMCS5-PGIP did not secrete pPGIP, but *Xf* did secrete a very small amount of the protein. Moreover, pPGIP extracted from cell lysate and obtained from supernatant of *P. agglomerans* pMCS5-PGIP did not show inhibition of PG in a bioassay. Whereas, pPGIP extracted from cell lysate and obtained from supernatant of *X. fastidiosa* pXF20-PGIP did show inhibition of PG in a bioassay. This shows that in principle the delivery bacteria can synthesize and secrete an agent that could be use to prevent PD.

The growth of *X. fastidiosa* pXF20-PGIP *in planta* is significantly lower than *X. fastidiosa*. The presence of PGIP was detected in total plant extracts and in the xylem fluid of the plants inoculated with *X. fastidiosa* pXF20-PGIP using Western blot analysis. *X. fastidiosa* and *X. fastidiosa* pXF20-PGIP were inoculated in to grapevines. Ten weeks after inoculation, *X. fastidiosa* pXF20-PGIP at 45 cm above inoculation point (IP) (8.96 ± 1.04253 pg/ μ l) were found significantly less than *X. fastidiosa* (54.60 ± 12.01012 pg/ μ l) at the same distance. Bacteria in double inoculation plants, which were inoculated with *X. fastidiosa* pXF20-PGIP at day 0 and again with *X. fastidiosa* at week 4 at 45 cm above the first IP, were found significantly lower than bacteria in plants with only *X. fastidiosa* inoculation. The reduction of bacteria in plants with double inoculation may be due to the present of PGIP. Over time, the migration of *X. fastidiosa* was found in further distance in the double inoculation plants than the plant with only *X. fastidiosa* inoculation. In conclusion this *X. fastidiosa* pXF20-PGIP may contribute to inhibition of the growth and movement of *X. fastidiosa*, and possible delay development of Pierce's Disease.

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General Introduction

Pierce's Disease

Pierce's Disease (PD) of grapevines is an economically important disease that currently threatens the wine, table, and raisin grape industries in California because of the new arrival of the glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*) (Takiya et al., 2006). This insect is an important vector of *Xylella fastidiosa* (Wells et al., 1987), which is a bacterium that causes PD (Hopkins and Mollenhauer, 1973). Pierce's Disease was named after Newton B. Pierce, Special Agent to the Secretary of Agriculture and plant pathologist, who was the first to characterize the disease (Pierce, 1892). However, he was not able to find the actual cause of PD. Before the 1990s, outbreaks of PD were limited to the edges of vineyards near riparian habitats, and the pathogen was spread by native sharpshooters, including the blue-green sharpshooter (*Graphocephala atropunctata*). A new vector of *X. fastidiosa*, the glassy-winged sharpshooter (GWSS), arrived in California sometime in the mid-1980s and became a more serious threat to California vineyards because it flies faster, longer, and has a much wider host range than the native sharpshooters. Even though GWSS has lower transmission efficiency than the blue-green sharpshooter, GWSS increases the rate of disease transmission from vine to vine and has now become the dominant factor in PD in California. Currently GWSS is found in all ten counties of southern California, and a very vigorous and expensive quarantine program is in place to prevent spread from southern California.

Disease Symptoms

The Pierce's Disease caused by *X. fastidiosa* causes typically a drying or "scorching" of leaves. Infected leaves eventually fall from the plant, but the leaf petiole remains attached to the stem, a characteristic called "matchstick". Symptoms usually develop on one cane and spread to other canes as the season progresses. This phenomenon is associated with the upstream movement (toward the roots) of *X. fastidiosa* in the xylem of plants (Meng et al., 2005). *X. fastidiosa* moves upstream toward the roots of the grapevine to cause chronic infections (Almeida et al., 2005). In the early spring, the new leaves of *Xylella*-infected vines are stunted and deformed in shape. The leaves are more yellow in color than normal leaves. The infected vines usually die within two years in susceptible varieties. Most European grapevines are susceptible to the disease but they vary in the degree of susceptibility. Highly susceptible varieties include Barbera, Chardonnay, Emperor, Fiesta, Pinot noir, and Red Globe. Intermediate susceptible varieties are Cabernet sauvignon, Merlot, and Sauvignon blanc. Less susceptible varieties are Chenin blanc, Flame Seedless, Riesling, Sylvaner, Ruby cabernet, and Thompson seedless (Rashed et al., 2011).

Xylella fastidiosa

X. fastidiosa is a complex of xylem-limited, gram-negative, rod-shaped bacterial strains each of which can cause specific plant diseases, including, among others, Pierce's Disease (PD) of grapevines (Davis et al., 1980), phony disease of peach (PDP), plum leaf scald (PLS) (Davis et al., 1981), oleander leaf scorch disease (OLS) (Purcell et al., 1999),

citrus variegated chlorosis disease (CVC) (Beretta et al., 1997), and almond leaf scorch disease (ALSD) (Almeida and Purcell, 2003; Chen et al., 2005). There are differences in the host ranges of the different strains of *X. fastidiosa*. Strains that cause PD of grapevine do not cause disease in peach, and peach strains do not cause disease in grapevines (Hopkins, 1989). Oleander strains do not infect grape, nor do grape strains infect oleander (Purcell et al., 1999). However, PD, ALS and alfalfa dwarf are caused by the same strains (Redak et al., 2004).

In the host plants, *X. fastidiosa* colonizes, multiplies and spreads inside the xylem vessels (Mollenhauer and Hopkins, 1974). The xylem vessels are blocked by bacterial cells, tyloses, and gums. (Hopkins, 1989). If there is sufficient blockage of the petiole and leaf veins it can produce water stress resulting in leaf marginal necrosis (Chatelet et al., 2006; Goodwin et al., 1988).

For *X. fastidiosa* populations to become systemic, the bacteria have to cross the pit membrane that separates one xylem vessel from its neighbors. *X. fastidiosa* cells are able to pass-through smaller pit membrane pores (Chatelet et al., 2006; Chatterjee and Newman, 2008) but they are too big to move passively from one vessel to another. The movement of cells of *X. fastidiosa* appears to be dependent on its ability to degrade cell wall of pit membrane (Newman et al., 2003; Perez-Donoso et al., 2010). The *X. fastidiosa* genome contains genes encoding cell-wall degrading polygalacturonase (PG) (Simpson et al., 2000). Recent study by Roper et al., reported that polygalacturonase is required for

colonization and pathogenicity of *X. fastidiosa* in *Vitis vinifera* grapevines (Roper et al., 2007). Indeed, *X. fastidiosa* *pglA* mutant unable to move systemically in grapevine. Inhibition of PG by PG-inhibiting protein (PGIP) reduces PD symptom in grapevines (Agüero et al., 2005). Transgenic grapevines expressing pear-PGIP suppresses PD development (Agüero et al., 2005).

Vectors

In the temperate regions of the United States, the *X. fastidiosa* strain that causes PD in grapevines is transmitted to plants by several species of sap-feeding sharpshooters, including the glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis* (Purcell and Saunders, 1999; Takiya et al., 2006), the blue-green sharpshooter (*Graphocephala atropunctata*), the green sharpshooter (*Draeculacephala minerva*), the black winged sharpshooter (*Oncometopia nigricans*) (Adlerz, 1980), and the red sharpshooter (*Xyphon fulgida*). Sharpshooters have an efficient sap-sucking mechanism for feeding on xylem fluid. First, sharpshooters feed on xylem fluid and acquire *X. fastidiosa* from the infected plants. Second, *X. fastidiosa* uses fimbriae to attach itself inside the foregut of sharpshooters (Newman et al., 2004). Third, the pathogen multiplies in the foregut (Hill and Purcell, 1995; Purcell and Finlay, 1979). Lastly, infected adult sharpshooters inoculate the pathogen into other host plants. However colonization of bacteria is not required for transmission, since sharpshooter can acquire *X. fastidiosa* from an infected plant and immediately transmit it to a new host plant (Purcell and Finlay, 1979).

Disease Control Strategies

There are no cures for the scorch disease such as PD, ALS, CVC, PDP, and PLS. Over the past decades, there were many epidemics related to diseases caused by *X. fastidiosa* strains. In Northern California during 1995-98 \$33 million in losses were reported (Guilhabert and Kirkpatrick, 2003). In Southern California's Temecula Valley in 1995, an outbreak caused over \$6 million in losses. First, PD is partially managed in the United States by use of systemic insecticides introduced via drip irrigation (Purcell, 1979), but long-term use of insecticides can select for resistance of vector insects. Moreover, chronic infection of the disease (Baccari and Lindow, 2011; Meng et al., 2005) cannot be treated with insecticidal control of vectors. Second, pruning is a common practice when PD symptoms appear usually after first year of infection. The healthy grapevines are regularly monitoring for infection by an enzyme linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR). Third, genetically modified plants are a promising approach for incorporating resistance genes into host plants. Several genes that have antimicrobial activity have been inserted into grapevines; however PD resistance has not yet been perfected, which would require several years of research. Fourth, endophytic microorganisms and biological control have the potential to control pathogens. Several endophytic bacteria were isolated from citrus, including *Methylobacterium* spp., *Curtobacterium flaccumfaciens* (Andreote et al., 2006; Araújo et al., 2001; Lacava et al., 2007) and from grapevine, *Pantoea*, *Paenibacillus* spp. (Parker, 2008). We are proposing the use of symbiotic control as a new way of controlling PD.

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Chapter 1: Transformation of *P. agglomerans* and *X. fastidiosa* with pear PGIP

Abstract

Polygalacturonase (PG) is produced by endophytic bacteria to degrade the pit membranes of xylem vessels to facilitate movement between xylem channels. Pear polygalacturonase-inhibiting protein (pPGIP) is produced by the pear plant to block the movement of pathogenic bacteria in the plant. pPGIP is an agent being examined to protect against the pathogen *X. fastidiosa* from causing PD in grapevine. In the present study, candidate symbiotic bacteria were genetically altered to express pPGIP to inhibit the movement of *X. fastidiosa* and prevent development of PD symptoms in grapevines. Here I report successful transformation of *P. agglomerans* and *X. fastidiosa* with pPGIP plasmids, pMCS5-PGIP and pXF20-PGIP respectively. Both bacteria maintained the plasmids for 6 passages in vitro. Stability of plasmid pMCS5-PGIP was verified by PCR analysis of plasmid extracted from transformed *P. agglomerans* with primers pelB-PGIP-1F and pelB-PGIP-1R. Stability of plasmid pXF20-PGIP was verified by PCR analysis of plasmid extracted from transformed *X. fastidiosa* with primers ssPG-PGIP-1F and ssPG-PGIP-1R. This approach is designed to produce a symbiotic agent to control PD.

Introduction

Pierce's Disease (PD) of grapevines (Pierce, 1892) is an economically important disease that currently threatens the wine, table, and raisin grape industries in California

because of the new arrival of the glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*) (Takiya et al., 2006). This insect is an important vector of *Xylella fastidiosa* (Wells et al., 1987), which is a bacterium that causes PD (Hopkins and Mollenhauer, 1973). *X. fastidiosa* is a gram-negative bacterium that inhabits in the xylem of host plants. The water conducting system of infected plants is blocked by the pathogen (Hopkins, 1989), resulting in scorching and dieback symptoms of the plants (Chatelet et al., 2006; Goodwin et al., 1988).

There are no cures for the scorch diseases such as PD, ALSD, CVC, PDP, and PLS. Over the past decades, there were many epidemics related to diseases caused by *X. fastidiosa* strains. In Northern California during 1995-98 \$33 million in losses were reported (Guilhabert and Kirkpatrick, 2003). In Southern California's Temecula Valley in 1995, an outbreak caused over \$6 million in losses. In Brazil, CVC is responsible for losses of US \$100 million per year (Lacava et al., 2009; Lambais et al., 2000). PD is partially managed in the United States by use of systemic insecticides introduced via drip irrigation (Purcell, 1979), but long-term use of insecticides can select for resistance of vector insects. Moreover, chronic infection of the disease (Baccari and Lindow, 2011; Meng et al., 2005) cannot be treated with insecticidal control of vectors. Second, pruning is a common practice when PD symptoms appear which do not occur in the first year of infection. Thus the grapevines have to be regularly monitored for infection and identification by an enzyme linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR). Third, genetically modified plants are a promising

approach for incorporating resistance genes into host plants. Several genes that have antimicrobial activity have been inserted into grapevines; however PD resistance has not yet been perfected, which would require several years of research. Forth, endophytic microorganisms and biological control have the potential to control pathogens. Several endophytic bacteria were isolated from citrus, including *Methylobacterium* spp., *Curtobacterium flaccumfaciens* (Andreote et al., 2006; Araújo et al., 2001; Lacava et al., 2007) and from grapevine, *Pantoea*, *Paenibacillus* spp. (Parker, 2008).

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains used in this study include *Escherichia coli* strain JM109 (Promega), *Pantoea agglomerans* strain E325 (rifampicin-resistant) and *Xylella fastidiosa* strain Temecula. *E. coli* was used as the host strain for cloning and preparing the template plasmids. *P. agglomerans* and *X. fastidiosa* were mainly used as symbionts, which were the ultimate goals of this research. *E. coli* was grown in Luria Bertani (LB) media (Fisher Scientific company, catalog number 50-751-6886) at 200 rpm and 37°C. Transformed *E. coli* strains were grown in LB media supplemented with suitable antibiotic to the plasmid containing antibiotic resistance genes. Final concentration of antibiotics was 100 µg/ml ampicillin, 50 µg/ml kanamycin. *P. agglomerans* strain E325 was grown in LB media supplemented with 30 µg/ml rifampicin at 200 rpm and 28°C. Transformed *P. agglomerans* were grown in LB media supplemented with suitable antibiotic. The *X. fastidiosa* strain Temecula was grown in PD 3 media (Davis et al.,

1981) which was modified from PD2 by substituting BSA with soluble potato starch. The bacteria were grown at 200 rpm and 30°C. Transformed *X. fastidiosa* were grown in PD3 media supplemented with 50 µg/ml kanamycin.

Design of optimized sequences

The PGIP sequence of pear (*Pyrus communis*) was obtained from Pubmed (GenBank: AAO29329.1). Its signal sequence was predicted with SignalP 3.0 software (Technical University of Denmark) and indicated the first 24 amino acids (melkfstflstlflfssvlnpals) as signal sequence. This signal sequence was substituted with a signal sequence of pelB gene from *E. coli* (mkylptaaagllllaaqpama) or PG gene from *X. fastidiosa* (mnldrflplvfglhcfaamg). At the c-terminus of PGIP sequence, the six amino acids of histidine were added for tracking purposes. The complete amino acid sequences (Figure 1.1 and 1.2) were reverse DNA sequenced using software Optimizer from website (<http://genomes.urv.es/OPTIMIZER/>). The resulting DNA sequence was fused with a kanamycin promoter, PnptII. The completed DNA sequences (Table 1.2 and 1.3) were sent to Genscript to synthesize the DNA. They were contained in the commercial plasmid pUC57.

Construction of plasmid for *P. agglomerans* E325

pUC-pelB-PGIP (or pUC57-PnptII-pelB-PGIP-6xHis) obtained from Genscript was maintained in *E. coli* strain JM109. The plasmid was cut by restriction enzymes

EcoRI and PstI (New England BioLabs) following manufacture protocol. The gene fragment was fused with pBB-MCS5, which was digested with EcoRI and PstI enzymes. The complete ligated plasmid was designated pMCS5-PGIP (or pBBR1-MCS5-PnptII-pelB-PGIP-6xHis) (Figure 1.3). The plasmid was transformed to competent cells of *E. coli*. It was grown on an LB agar plate with 10 µg/ml gentamycin. Colonies were selected and recultured in 5 ml LB media, containing 10 µg/ml gentamycin, overnight at 37°C. Bacterial plasmids were extracted and verified with EcoRI and PstI restriction enzyme digestion and gel electrophoresis.

The completed and verified plasmid, pMCS5-PGIP was inserted into *P. agglomerans* using electroporation in a GenePulser Xcell electroporator (Bio-Rad), 2000 voltage, 10 µsec. 100 µl of bacteria was transferred into 900 µl LB media, and grown for 3 hours. Then 100 µl of bacteria were spread on LB agar plate supplemented with 30 µg/ml rifampicin. Once grown, at least 10 colonies were selected and grown in individual vials. Each colony was verified for plasmid size and restriction enzyme digestion pattern.

Construction of plasmid for *X. fastidiosa*

pUC-ssPG-PGIP (or pUC57-PnptII-ssPG-PGIP-6xHis) obtained from Genscript was maintained in *E. coli* strain JM109. The plasmid was cut by restriction enzymes HindIII and BamHI (New England BioLabs) following the manufacturer's protocol. The gene fragment was fused with pXF20-PEMIK (6.0 kbp), which was digested with HindIII and BamHI enzymes. The complete ligated plasmid was designated pXF20-PGIP (or

pXF20-PEMIK-PnptII-ssPG-PGIP-6xHis) (Figure 1.4). The plasmid was transformed to competent cells of *E. coli*. It was grown on an LB agar plate with 100 µg/ml ampicillin. Colonies were selected and recultured in 5 ml LB media, containing 100 µg/ml ampicillin, overnight at 37°C. Bacterial plasmids were extracted and verified with HindIII and BamHI restriction enzyme digestion and gel electrophoresis.

The completed and verified plasmid, pXF20-PGIG was inserted into *X. fastidiosa* using electroporation in GenePulser Xcell electroporator (Bio-Rad), 2500 voltage, 10 µsec. 100 µl of bacteria was transferred to 900 µl PD3 media and grown for 12 hours. Then 20 µl of bacteria was plated on PD3 agar plate supplemented with 100 µg/ml ampicillin. Once grown, colonies were selected and grown in individual vials. Each colony was verified for plasmid size and restriction enzyme digestion pattern.

Plasmid stability analysis

Transformed *P. agglomerans* and *X. fastidiosa* were grown in LB liquid medium overnight and PD3 liquid medium for 7-10 days until turbid, respectively. Strains were subcultured (1:10 dilution) into media without antibiotics. A total of five passages in nonselective media were performed. Their plasmids were verified with restriction enzyme digestion and gel electrophoresis, and traditional PCR as described below.

Primer design and PCR

Primer3 software was used to design primers for optimized PGIP genes. Primers corresponding to each plasmid are shown in Table 1.1. Polymerase Chain Reaction (PCR) amplification was carried out in a 20 µl reaction mixture containing 10 µl of iQ Supermix (Bio-Rad, Catalogue number 170-8860), 1 uM of each primer, in a Mastercycler Gradient (Eppendorf, Westbury, NY). All PCR amplification protocols consisted of an initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 5 min. Products were analyzed by 1% agarose gel electrophoresis.

Results

P. agglomerans

Construction of the pMCS5-PGIP for *P. agglomerans*

pMCS5-PGIP (Figure 1.3) was constructed by cloning the 1108-bp of PnptII-pelB-PGIP-6xHis fragment from pUC57 into EcoRI-BamHI-digested pBBR1-MCS5 into *E. coli JM109*. Gel electrophoresis of final the construct verified insertion of the gene fragment into pBBR1-MCS5 as the correct size (1108 bp) (data not show). Gel electrophoresis of the PCR product of the final construct also verified the gene insertion (data not show). pMCS5-PGIP was maintained in *E. coli* grown in LB media supplemented with 10 µg/ml of gentamycin.

Transformation of *P. agglomerans*

pMCS5-PGIP was extracted from transformed *E. coli* and transferred into *P. agglomerans* E325 using electroporation. The successful transformed colonies were grown in 10 and 30 µg/ml of gentamycin and rifampicin, respectively. Plasmid insertion was verified by gel electrophoresis after enzyme digestion (data not show). 10 colonies were selected and individually grown in LB with antibiotic as described above.

Analysis of plasmid

The pMCS5-PGIP plasmids were extracted from transformed *P. agglomerans* E325 strains. A restriction enzyme digestion assay was performed and gel electrophoresis showed correct fragment size (1108-bp band; Figure 1.5). PCR analysis was also performed using primers pelB-PGIP-1F and pelB-PGIP-1R (Table 1.1). Gel electrophoresis of PCR products showed a 200-bp DNA fragment (Figure 1.6).

Plasmid stability

Transformed *P. agglomerans* was grown in non-selective LB medium. Bacteria were subculture for 6 passages. After passage, plasmid DNA was purified using the QIAprep Spin Miniprep Kit. PCR analysis was performed using primers pelB-PGIP-1F and pelB-PGIP-1R (Table 1.1). Gel electrophoresis of PCR products showed a 200-bp DNA fragment (Figure 1.7).

X. fastidiosa

Construction of the pXF20-PGIP for *X. fastidiosa*

pXF20-PGIP (Figure 1.4) was constructed by cloning the 1114-bp PnptII-ssPG-PGIP-6xHis fragment from pUC57 into HindIII-BamHI-digested pXF20-PEMIK. Gel electrophoresis of the final construct (data not show) verified insertion of the gene fragment into pXF20-PEMIK as the correct size (1114 bp). Gel electrophoresis of PCR products of the final construct (data not show) also verified gene insertion. pXF20-PGIP was maintained in *E. coli* grown in LB media supplemented with 100 and 30 µg/ml of ampicillin and kanamycin, respectively.

Transformation of *X. fastidiosa*

pXF20-PGIP was extracted from transformed *E. coli* and transferred into *X. fastidiosa* using electroporation. The successful transformed colonies were grown in PD3 agar plate supplemented with 100 and 30 µg/ml of ampicillin and kanamycin, respectively. Plasmid insertion was verified by gel electrophoresis of enzyme digestion (data not show). Ten colonies were selected and individually grown in LB with antibiotic as described above.

Analysis of plasmid

The pXF20-PGIP was extracted from transformed *X. fastidiosa*. A restriction enzyme digestion assay was performed. Gel electrophoresis showed the correct fragment

size (1114-bp band) (Figure 1.8). PCR analysis was also performed using primers ssPG-PGIP-1F and ssPG-PGIP-1R. Gel electrophoresis of the PCR product showed a 230-bp DNA fragment (Figure 1.9).

Plasmid stability

Transformed *X. fastidiosa* was grown in non-selective PD3 medium. Bacteria were subculture for 6 passages. After each passage, plasmid DNA was purified using the QIAprep Spin Miniprep Kit. PCR analysis was confirmed using primers ssPG-PGIP-1F and ssPG-PGIP-1R. Gel electrophoresis of the PCR product showed a 230-bp DNA fragment (Figure 1.10).

Discussion

Confirmation of the transformation of bacteria

Our results show successful transformation of *P. agglomerans* E325 and *X. fastidiosa* with pPGIP gene constructs. Moreover the transformation was stable in each strain. The pPGIP genes were synthesized by Genscript Company and inserted into plasmids, pBBR-MCS5 and pXF20-MEPIK. The results of the restriction enzyme digestion assay indicate that the transformed *P. agglomerans* strains contain plasmid pMCS5-PGIP. The inserted gene shows as a 1-kbp band in gel electrophoresis. Moreover the results of PCR analysis indicate that the pMCS5-PGIP plasmids extracted from transformed *P. agglomerans* strains contain the PGIP gene, which is 206-bp band produced by PCR with the pelB-PGIP-1F/1R primer set. PCR analysis of bacterial

subculture (4-6 passages) confirmed stability of the inserted plasmid in transformed *P. agglomerans* E325. In vitro, the clones grow at normal rate since there was no significant difference of growth curve (data not show).

The results of the restriction enzyme digestion assay indicate that the transformed *X. fastidiosa* strains contain plasmid pXF20-PGIP. The inserted gene shows as a 1.1-kbp band in gel electrophoresis. Moreover the results of PCR analysis indicate that the pXF20-PGIP plasmids extracted from transformed *X. fastidiosa* strains contain PGIP gene, which is 230-bp band produced by PCR with the ssPG-PGIP-1F/1R primer set. PCR analysis of bacterial subculture (4-6 passages) confirmed stability of the inserted plasmid in transformed *X. fastidiosa*. In vitro, the clones grow at normal rate since there was no significant difference of growth curve (data not show).

Figures and Tables

Table 1.1: Plasmids, primers and strains used in this study

Plasmids, primers, strains	Relevant characteristics or primer sequences	Source
Plasmids		
pUC57	Cloning vector in <i>E. coli</i> . 2,710 bp	GenScript
pUC57-pelB-PGIP-6xHis	pUC57 with synthesized 1108-bp PntpII-pelB-PGIP-6xHis gene	GenScript
pUC57-ssPG-PGIP-6xHis	pUC57 with synthesized 1114-bp PntpII-ssPG-PGIP-6xHis gene	GenScript
pBBR1-MCS5	Broad-host-range cloning vector, Gm ^r	(Kovach et al., 1995)
pMCS5-PGIP	pBBR1-MCS5 with 1108-bp PntpII-pelB-PGIP-6xHis gene	This study
pXF20-PEMIK	Suitable shuttle vector of <i>X. fastidiosa</i> , OriV, Kan ^r , Amp ^r	(Lee et al., 2010)
pXF20-PGIP	pXF20-PEMIK with 1114-bp PntpII-ssPG-PGIP-6xHis gene	This study
Primers and probes		
pUC57-F	GTAAAACGACGGCCAGTG	GenScript
pUC57-R	GGAAACAGCTATGACCATG	GenScript
nptII-F	ACAAGATGGATTGCACGCAGG	This study
nptII-R	AACTCGTCAAGAAGGCGATAG	This study
pelB-PGIP-1F	GTGCTGCTGCAAATCAAAAA	This study
pelB-PGIP-1R	TGAAATTCCAGGGTTTCCAG	This study
ssPG-PGIP-1F	GACTGCTGCGACTGGTACTG	This study
ssPG-PGIP-1R	GAGAGGTTTCGTCCACGAGAG	This study
XFF2-16S	CTCGCCACCCATGGTATTACTAC	(Schaad et al., 2002)
XFR2-16S	CTGGCGGCAGGCCTAAC	(Schaad et al., 2002)
Xfp2	5'-Quasar 670-ATGTGCTGCCGTCCGACTTGCATG-BHQ-2-3'	(Schaad et al., 2002)
Strains		
<i>Escherichia coli</i> DH5	Cloning bacteria	Invitrogen
<i>Pantoea agglomerans</i> E325	Rif ^r	Dr. Dave Lampe
<i>Xylella fastidiosa</i> Tem		Dr. Steven Lindow

Primer sequences are presented 5' to 3'; Amp^r, Kan^r, Rif^r and Gm^r indicated ampicillin, kanamycin, rifampicin, and gentamicin resistant, respectively.

Table 1.2: Optimized DNA sequence of PnptII-pelB-PGIP-6xHis for *P. agglomerans*.

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1  TTGACAATTA ATCATCGGCT CGTATAATTG TGAATTGTG AGCGGATAAC AATTCACAC
61 AGGAAACAGG ATCGATCAAG AGACAGGATG AGGATCGTTT CGCATGAAAT ATCTGCTGCC
121 GACGGCTGCT GCTGGTCTGC TGCTGCTGGC TGCCCAACCG GCTATGGCGG ATCTGTGCAA
181 CCCGGACGAT AAAAAAGTGC TGCTGCAGAT TAAAAAAGCA TTTGGCGATC CGTATGTTCT
241 GGCTTCATGG AAATCGGATA CCGACTGCTG TGATTGGTAC TGCCTGACGT GTGACTCAAC
301 CACGAACCGT ATTAATTCGC TGACCATCTT TGCGGGCCAA GTGAGCGGTC AAATTCGGC
361 CCTGGTTGGT GATCTGCCGT ATCTGGAAAC CCTGGAATTT CATAAACAGC CGAACCTGAC
421 GGGCCCGATT CAACCGGCGA TCGCCAAACT GAAAGGTCTG AAAAGTCTGC GCCTGTCTTG
481 GACCAACCTG AGTGGCTCCG TTCCGGATTT TCTGAGTCAG CTGAAAAATC TGACCTTCT
541 GGACCTGTCC TTCAACAATC TGACGGGTGC AATTCCGAGC TCTCTGAGTG AACTGCCGAA
601 CCTGGGCGCT CTGCGTCTGG ATCGCAATAA ACTGACCGGT CATATTCCGA TCAGCTTTGG
661 CCAGTTCATC GGTAATGTCC CGGACCTGTA CCTGAGCCAC AACCAGCTGT CTGGCAATAT
721 TCCGACCTCA TTTGCACAAA TGGATTTTAC GTCAATCGAC CTGTCGCGTA ACAAACTGGA
781 AGGCGATGCC TCTGTCATTT TTGGTCTGAA TAAAACCACG CAGATCGTGG ACCTGAGCCG
841 CAACCTGCTG GAATTTAATC TGTCTAAAGT CGAATTTCCG ACCAGCCTGA CGTCTCTGGA
901 TATTAACCAT AACAAAATCT ACGGCTCAAT CCCGGTGGA TTTACCCAGC TGAACCTTCA
961 ATTCCTGAAC GTTTCGTACA ATCGTCTGTG CGGTCAGATC CCCGTCGGCG GTAAACTGCA
1021 AAGTTTGTAT GAATACTCCT ACTTCCACAA CCGTTGTCTG TGTGGCGCTC CGCTGCCGTC
1081 CTGTAAACAT CACCATCACC ATCACTGA

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Figure 1.1: Diagram of PnptII-pelB-PGIP-6xHis



Table 1.3: Optimized DNA sequence of PnptII-ssPG-PGIP-6xHis for *X. fastidiosa*.

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1  TTGACAATTA ATCATCGGCT CGTATAATTG TGAATTGTG AGCGGATAAC AATTTCACAC
61  AGGAAACAGG ATCGATCAAG AGACAGGATG AGGATCGTTT CGCATGAACC TCGACCGGTT
121 CCTCCCGCTC GTGTTCGGGC TCCACTGCTT CGCGGCGATG GGGGACCTCT GCAACCCGGA
181 CGACAAGAAG GTGCTCCTCC AGATCAAGAA GCGGTCGGG GACCCGTACG TGCTCGCGTC
241 GTGGAAGTCG GACACGGACT GCTGCGACTG GTACTGCGTG ACGTGCGACT CGACGACGAA
301 CCGGATCAAC TCGCTCACGA TCTTCGCGGG GCAGGTGTCT GGGCAGATCC CGGCGCTCGT
361 GGGGGACCTC CCGTACCTCG AGACGCTCGA GTTCCACAAG CAGCCGAACC TCACGGGGCC
421 GATCCAGCCG GCGATCGCGA AGCTCAAGGG GCTCAAGTCG CTCCGGCTCT CGTGGACGAA
481 CCTCTCGGGG TCGGTGCCGG ACTTCCTCTC GCAGCTCAAG AACCTCACGT TCCTCGACCT
541 CTCGTTCAAC AACCTCACGG GGGCGATCCC GTCGTCTGCTC TCGGAGCTCC CGAACCTCGG
601 GGCGTCCGG CTCGACCGGA ACAAGCTCAC GGGGCACATC CCGATCTCGT TCGGGCAGTT
661 CATCGGGAAC GTGCCGGACC TCTACCTCTC GCACAACCAG CTCTCGGGGA ACATCCCGAC
721 GTCGTTCTCG CAGATGGACT TCACGTCGAT CGACCTCTCG CGGAACAAGC TCGAGGGGGA
781 CGCGTCGGTG ATCTTCGGGC TCAACAAGAC GACGCAGATC GTGGACCTCT CGCGGAACCT
841 CCTCGAGTTC AACCTCTCGA AGGTGGAGTT CCCGACGTCG CTCACGTCGC TCGACATCAA
901 CCACAACAAG ATCTACGGGT CGATCCCAGT GGAGTTCACG CAGCTCAACT TCCAGTTCCT
961 CAACGTGTCG TACAACCGGC TCTGCGGGCA GATCCCAGTG GGGGGGAAGC TCCAGTCGTT
1021 CGACGAGTAC TCGTACTTCC ACAACCGGTG CCTCTGCGGG GCGCCGCTCC CGTCGTGCAA
1081 GCACCACCAC CACCACCAC

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Figure 1.2: Diagram of PnptII-pelB-PGIP-6xHis gene



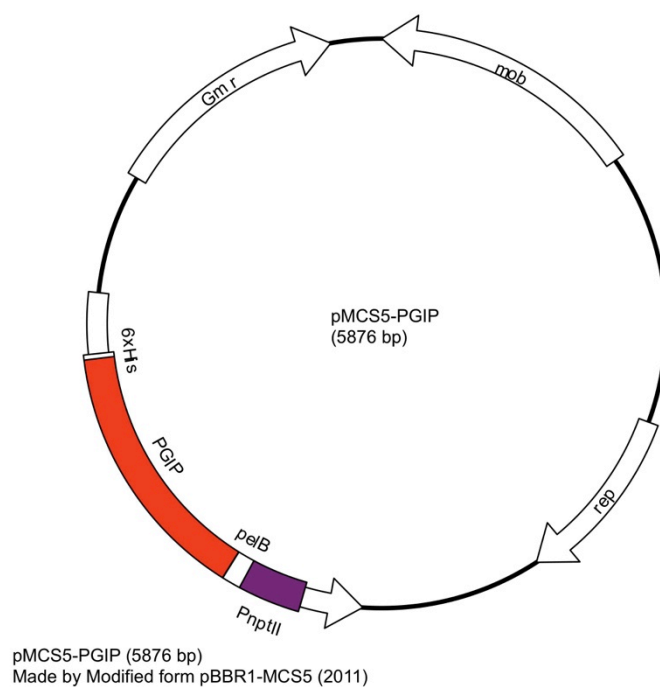


Figure 1.3: Map of pMCS5-PGIP (5876 bp) derived from pBBR1-MCS5 (4768 bp) (Kovach et al., 1995). The PnptII-pelB-PGIP-6xHis gene (1108 bp) was inserted in between EcoRI and PstI.

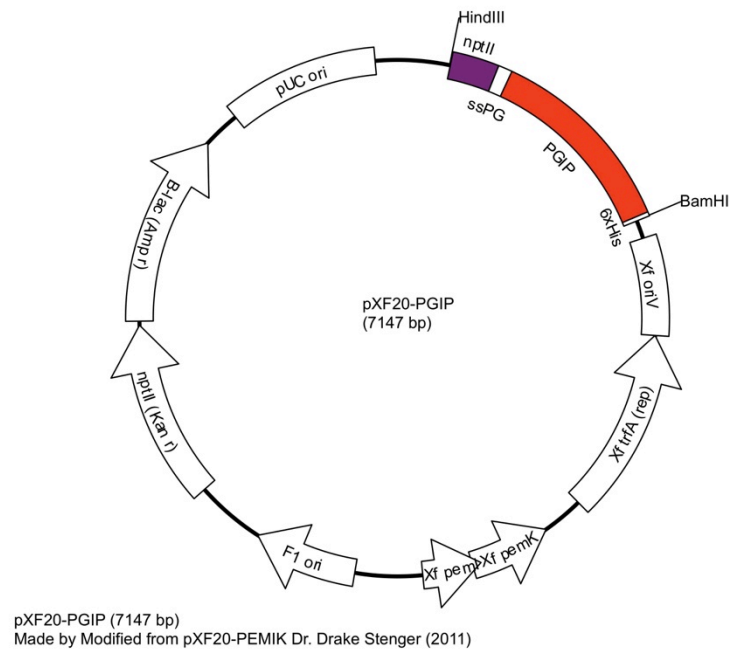


Figure 1.4: Map of pXF20-PGIP (7147 bp) derived from pXF20-PEMIK (6000 bp) (Lee et al., 2010). The PnptII-ssPG-PGIP-6xHis gene (1114 bp) was inserted in between HindII and BamHI.

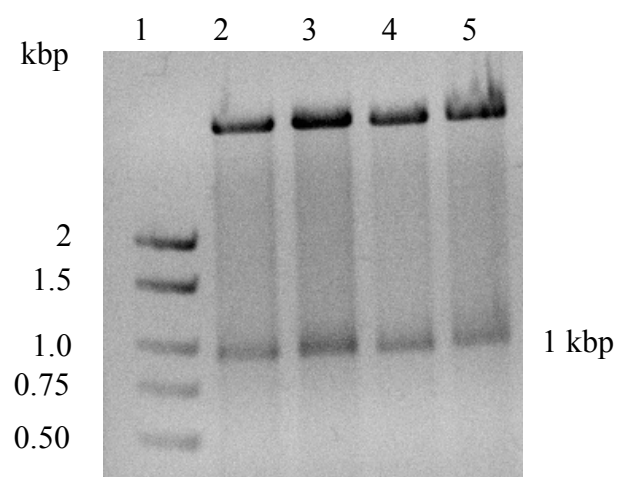


Figure 1.5: Restriction enzyme digestion of pMCS5-PGIP extracted from transformed *P. agglomerans* E325. Lane 1, DNA markers; lane 2-5, cloned *P. agglomerans* E325 clone 1-4. Gel electrophoresis shows the EcoRI and PstI restriction enzyme digestion. The fragment size was estimated 1 kbp.

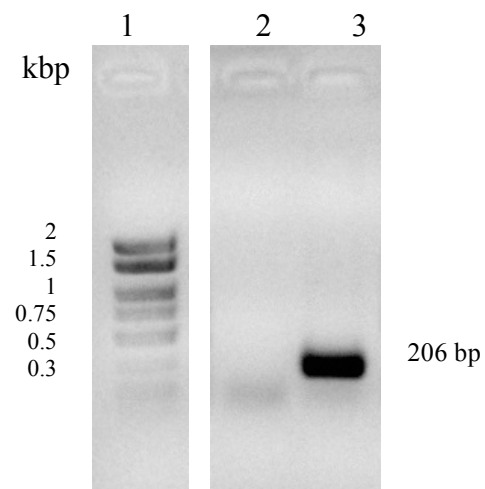


Figure 1.6: PCR analysis of pMCS5-PGIP extracted from transformed *P. agglomerans* E325. The primers are pelB-PGIP-1F and pelB-PGIP-1R. Lane 1, DNA marker; lane 2, pBBR1-MCS5 (no PGIP); lane 3, pMCS5-PGIP extracted from transformed *P. agglomerans* E325. The PCR product was 206 bp.

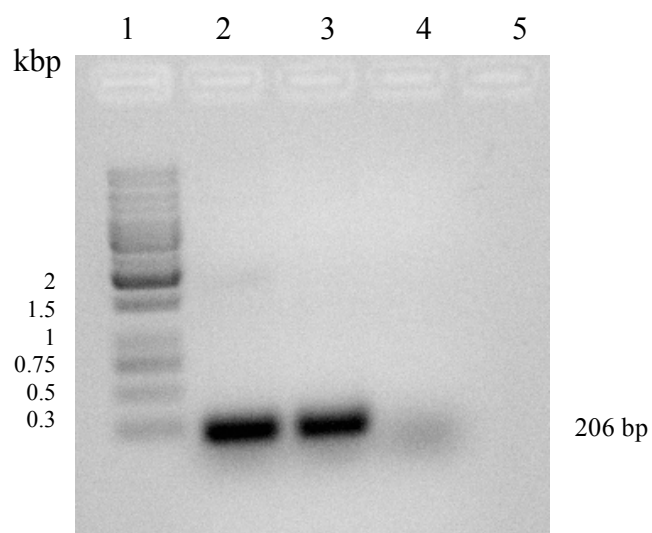


Figure 1. 7: Stability of pMCS5-PGIP of transformed *P. agglomerans* E325. It was verified by PCR analysis of plasmid extracted from transformed *P. agglomerans* E325 with primers pelB-PGIP-1F and pelB-PGIP-1R. Lane 1: DNA marker; lane 2, pMCS5-PGIP extracted from transformed *P. agglomerans* E325 passage 4; lane 3, passage 5; lane 4, passage 6; lane 5, pBBR1-MCS5 (no PGIP).

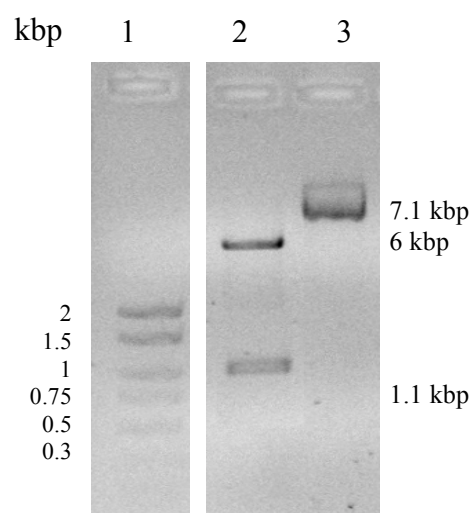


Figure 1.8: Restriction enzyme digestion of pXF20-PGIP extracted from transformed *X. fastidiosa*. Lane 1, DNA markers; lane 2, transformed *X. fastidiosa* pXF20-PGIP digested with HindIII and BamHI restriction enzymes; lane 3: transformed *X. fastidiosa* pXF20-PGIP without digestion.

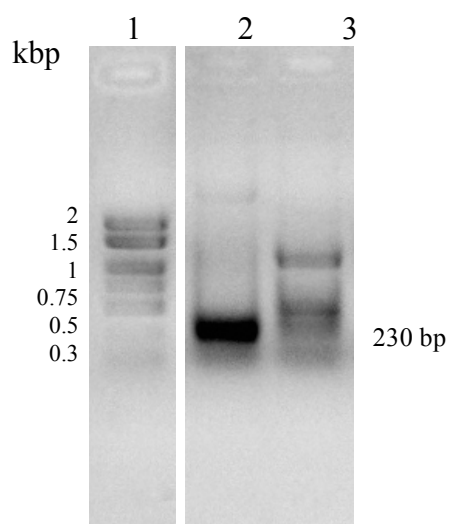


Figure 1.9: PCR analysis of pXF20-PGIP extracted from transformed *X. fastidiosa*. Primers are ssPG-PGIP-1F and ssPG-PGIP-1R. Lane 1, DNA marker; lane 2, pXF20-PGIP extracted from transformed *X. fastidiosa*. The PCR product was 230 bp; lane 3, pXF20-PEMIK (no PGIP).

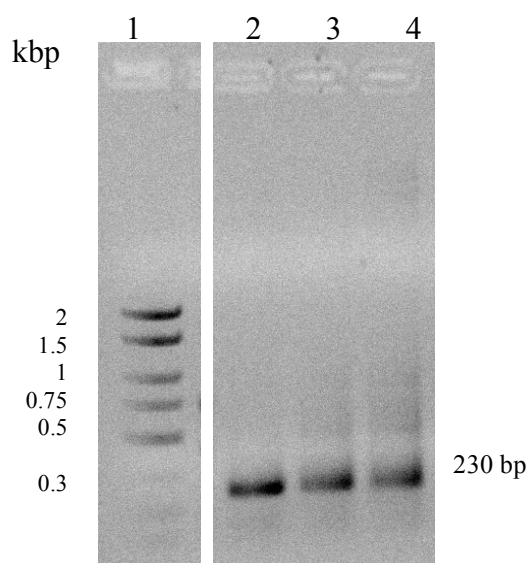


Figure 1.10: Stability of pXF20-PGIP of transformed *X. fastidiosa*. It was verified by PCR analysis of plasmid extracted from transformed *X. fastidiosa* with primers ssPG-PGIP-1F and ssPG-PGIP-1R. Lane 1, DNA marker; lane 2, pXF20-PGIP extracted from transformed *X. fastidiosa* passage 4; lane 3, passage 5; lane 4, passage 6; negative control was not shown.

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Chapter 2: Enzyme activity of PGIP expressed by transformed *P. agglomerans* and

X. fastidiosa

Abstract

Polygalacturonase (PG) is produced by endophytic bacteria to degrade the pit membranes of xylem vessels to facilitate movement between xylem channels. The pear polygalacturonase-inhibiting protein (pPGIP) is produced by the pear plant to block the movement of pathogenic bacteria in the plant. The pPGIP is an agent being examined to protect against the pathogen *X. fastidiosa* from causing PD in grapevine. Previously I transformed *P. agglomerans* and *Xf* with pPGIP plasmids, pMCS5-PGIP and pXF20-PGIP, respectively. Expression of pPGIP by both of these bacteria was verified by Western blot analysis. However *Pa* did not secrete pPGIP, but *Xf* did secrete a very small amount of the protein. Moreover, pPGIP extracted from cell lysate and obtained from supernatant of *PA* did not show inhibition of PG in a bioassay. Whereas, pPGIP extracted from cell lysate and obtained from supernatant of *Xf* did show inhibition of PG in a bioassay. This shows that in principle the delivery bacteria can synthesize and secrete an agent that could be use to prevent PD.

Introduction

Pierce's Disease (PD) of grapevines is caused by *Xylella fastidiosa*, a gram-negative, xylem-limited bacterium (Davis et al., 1980; Hopkins and Mollenhauer, 1973; Pierce, 1892; Wells et al., 1987). In the host plants, *X. fastidiosa* colonizes, multiplies and spreads inside the xylem vessels (Mollenhauer and Hopkins, 1974). The xylem vessels are blocked by bacterial cells, tyloses, and gums. (Hopkins, 1989). Moreover *X. fastidiosa* moves upstream toward the roots of the grapevine to cause chronic infections (Almeida et al., 2005; Meng et al., 2005). The bacteria have to cross the pit membrane that separates one xylem vessel from its neighbors. *X. fastidiosa* cells are able to pass-through smaller pit membrane pores (Chatelet et al., 2006; Chatterjee and Newman, 2008) but they are too big to move passively from one vessel to another. The movement of cells of *X. fastidiosa* appears to be dependent on its ability to degrade cell wall of pit membrane (Newman et al., 2003; Perez-Donoso et al., 2010). The *X. fastidiosa* genome contains genes encoding cell-wall degrading polygalacturonase (PG) (Simpson et al., 2000). Inhibition of PG by PG-inhibiting protein (PGIP) reduced PD symptom in grapevines (Agüero et al., 2005). Transgenic grapevines expressing pear-PGIP suppresses PD development (Agüero et al., 2005).

Materials and Methods

Bacterial culture

P. agglomerans, which had been previously transformed, was grown in LB media. The cultures were incubated at 30°C, shaken at 200 rpm. *X. fastidiosa*, which had been previously transformed, was grown in PD 3 media. The cultures were incubated at 28°C, shaken at 200 rpm. When required, antibiotics were added as follows: ampicillin, 100 ug/ml; kanamycin, 50 ug/ml; gentamycin, 10 ug/ml; rifampicin, 30 ug/ml.

SDS-PAGE and Western blot analysis

Transformed *P. agglomerans* was grown in LB liquid medium overnight and *X. fastidiosa* was grown in PD3 liquid medium for 7-10 days until reaching an optical density at 600 nm (OD₆₀₀) of 0.6. The bacteria cells were harvested by centrifugation and lysed using a sonicator. Protein electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) and western blot assays were performed using standard methods (Sambrook, 2001). Proteins were transferred to a nitrocellulose membrane (Amersham) at 100 V for 1.5 h. Membranes were kept for 2 h at room temperature in blocking buffer [5% non-fat milk, 0.1% Tween 20, 1% PBS] and incubated for 12 h at 4°C with an anti-6xHis probe (Thermo Scientific Pierce) (1:2,000 dilution) in a wash buffer containing 0.5% non-fat milk. After washing 8 times, a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Thermo Scientific Pierce) (1:15,000 dilution) was added to the membrane in wash buffer containing 0.5% non-fat milk, and

incubated for 2 h at room temperature. Peroxidase activity was detected by enhanced chemiluminescence (Fast ECL kit, Amersham).

PGIP activity assay

PGIP activity of transformed bacteria was analyzed by the diffusion plate assay (Buescher 1992, Taylor and Secor 1988, Tamura, Gao, Tao, Labavitch 2004) with some modification. A 0.7% (wt/v) agarose solution was heated in a microwave oven until dissolved. A 0.1% solution of polygalacturonic acid (PGA) (Sigma-Aldrich, USA), 3.65 g/l EDTA and 0.2 g/l Na-azide in 0.1 M sodium acetate buffer (pH 5.0) were added to the agarose solution. Then 25 ml of agarose solution was poured into 100x15-mm disposable petri dish and allow to solidify. The 5.6 mm diameter wells were cut in the agar plate using a cork bored No. 3. Sample mixtures of 30 μ l total volume (15 μ l of cell lysate or supernatant) mixed with 15 μ l of 0.1% of PG from *Aspergillus niger* (Sigma-Aldrich P-4625) were loaded into wells. The plates were incubated for 17 h at 37°C. After incubation, the plates were stained with 10 mL of 0.05% ruthenium red for 30 min, followed by destaining in water several times over 1 h. The destained plates were allowed to stand for 3 h at room temperature prior to assessment of PGIP activity by measuring the diameter of the clear zone around the wells.

Results

Protein expression of transformed *P. agglomerans*

Protein production of the pelB-PGIP-6xHis in transformed *P. agglomerans* was verified by SDS-PAGE and Western blot. In the SDS-PAGE (Figure 2.1), the PGIP protein bands were not distinguishable from total proteins, but the Western blotting analysis of cell lysate confirmed expression of 36-kDa PGIP (Figure 2.2), the expected size of pear PIGP. Moreover the Western blotting analysis of the supernatant did not find detect a PGIP band (data not show).

PGIP activity of transformed *P. agglomerans*

The crude lysates and supernatant from *P. agglomerans* transformed with pMCS5-PGIP did not inhibit polygalacturonic (PG), as indicated by diffusion plate assay. We observed a small reduction of the clear zone produced by *P. agglomerans* pMCS5-PGIP (Figure 2.3). However, measurement of the clear zone in the diffusion plate assay did not indicate a significant difference in cell lysate and supernatant samples (Figure 2.4) (p-value = 0.03281 and 0.01550 respectively).

Protein expression of transformed *X. fastidiosa*

Protein production of the ssPG-PGIP-6xHis in transformed *X. fastidiosa* was verified by SDS-PAGE and Western blot. In the SDS-PAGE (Figure 2.5), the PGIP protein bands were not distinguishable from total proteins, but the Western blotting

analysis of cell lysate confirmed expression of 36-kDa PGIP (Figure 2.6), the expected size of pear PGIP. Concentrated supernatant of media culture from bacteria were performed western blot analysis. It confirmed expression of 36-kDa PGIP (Figure 2.7).

PGIP activity of transformed *X. fastidiosa*

The crude lysates and supernatant from *X. fastidiosa* transformed with pXF20-PGIP were able to inhibit polygalacturonic (PG), as indicated by the diffusion plate assay. We observed reduction of the clear zone produced by *X. fastidiosa* pXF20-PGIP (Figure 2.8). Additionally measurement of clear zone (Figure 2.9) of diffusion plate assay indicate significant different in cell lysate and supernatant samples (p-value = 2.3756E-07 and 0.00022 respectively).

Discussion

The purpose of this study was to determine if transformed endophytic bacteria expressed PGIP and if the protein inhibited PG activity in a diffusion plate assay. The results suggested that *X. fastidiosa* pXF20-PGIP expressed PGIP, which inhibited PG activity. On the other hand, *P. agglomerans* pMCS5-PGIP expressed PGIP but did not inhibit PGA activity.

Protein expression of *P. agglomerans* pMCS5-PGIP was found in the crude cell lysate, but not in the supernatant of culture media. This indicated that PGIP protein was

not secreted or not produced at high enough levels to detect with Western blot analysis. As shown in the agar diffusion assay, the PGIP from *P. agglomerans* pMCS5-PGIP clones were not able to inhibit activity of PGA. We did not use them in experiments in grapevines.

Protein expression of *X. fastidiosa* pXF20-PGIP was found in both the crude cell lysate and supernatant of culture media. This indicated that PGIP protein was expressed and secreted out of the *X. fastidiosa* cell. Furthermore, bioassay analysis of PGIP activity suggested that expressed PGIP by bacteria inhibited PGA activity. Thus it is expected that PGIP expressed by *X. fastidiosa* pXF20-PGIP can inhibit activity of PG of *X. fastidiosa* in grapevines.

Figures

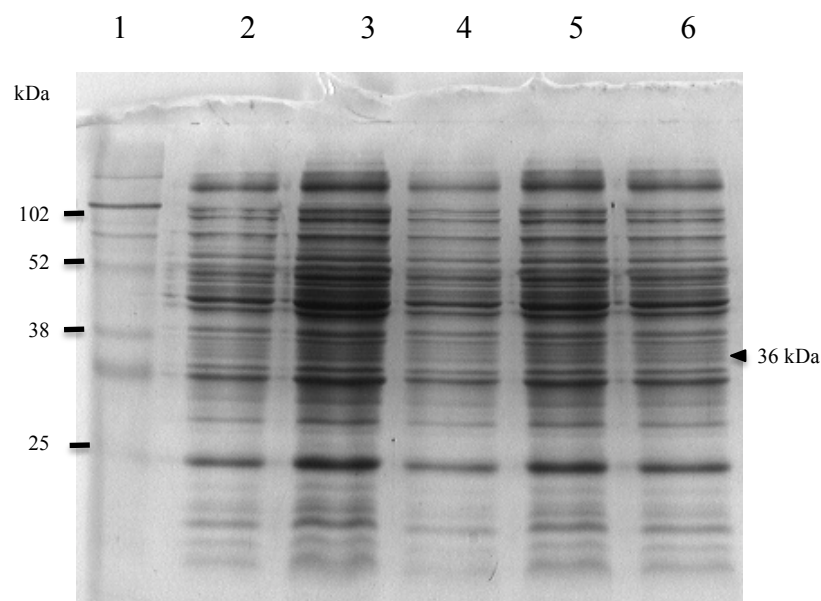


Figure 2.1: SDS-PAGE of total protein profile transformed *P. agglomerans* E325 clone 1-5. The expected 36-kDa PGIP bands are indicated. Lane 1, molecular marker; lane 2-6, total proteins of transformed *P. agglomerans* E325 clone 1-5 containing pMCS5-PGIP. Coomassie blue-stained 10% was used to visualize the protein bands.

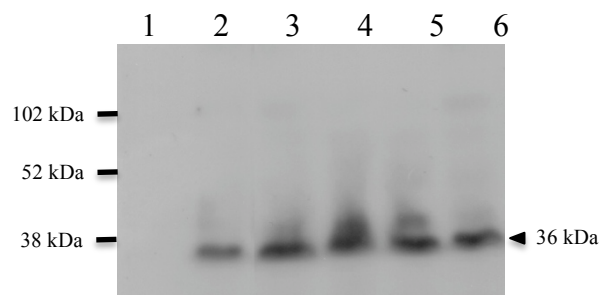


Figure 2.2: Western blotting analysis of cell lysate of transformed *P. agglomerans* E325 clone 1-5. Primary antibody was anti-6xHis. The expected 36-kDa PGIP bands are indicated. Lane 1, molecular marker; lane 2-6, cell lysate of transformed *P. agglomerans* E325 clone 1-5 containing pMCS5-PGIP.

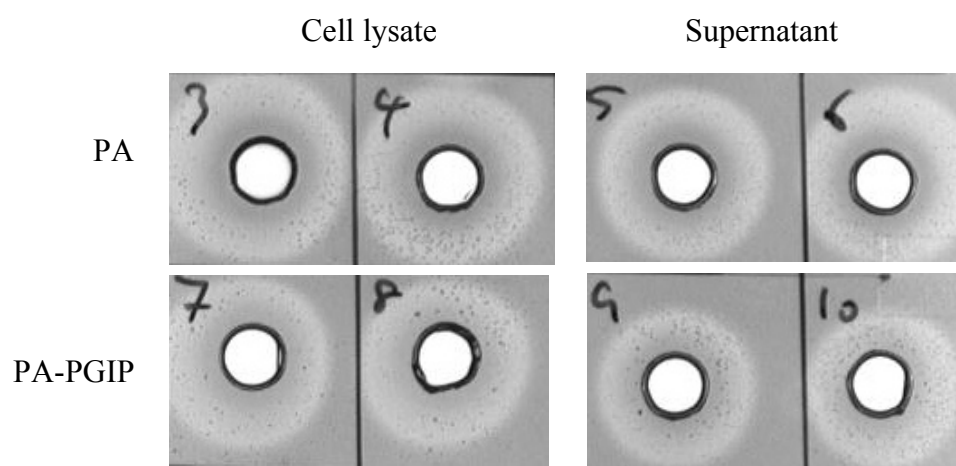


Figure 2.3: Polygalacturonic (PG) diffusion plate assay by PGIP from crude cell lysate and supernatant of culture of *P. agglomerans* E325 (PA) and *P. agglomerans* pMCS5-PGIP (PA-PGIP). As described in the text, the clear ring around the wells indicates PG activity. Assays for each combination of PG and cell lysate or supernatant were performed in duplicated. There was no significant difference of inhibition activity of PG in all samples.

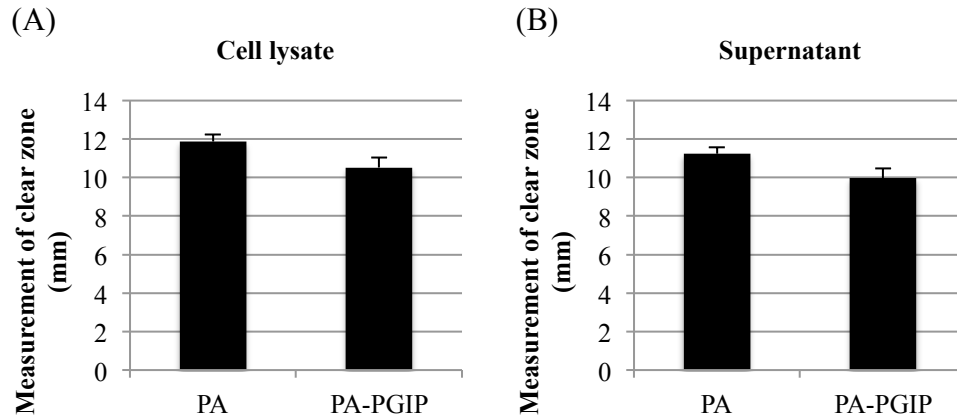


Figure 2.4: Inhibition of polygalacturonic (PG) by PGIP from crude cell lysate and supernatant of culture of *P. agglomerans* E325 (PA) and *P. agglomerans* pMCS5-PGIP (PA-PGIP). (A) Comparison of measurement of clear area from cell lysate samples between *P. agglomerans* E325 (PA) and *P. agglomerans* pMCS5-PGIP (PA-PGIP). There was no significant difference (p-value = 0.03281). (B) Comparison of measurement of clear area from supernatant samples between PA and PA-PGIP. There was no significant difference (p-value = 0.01550)

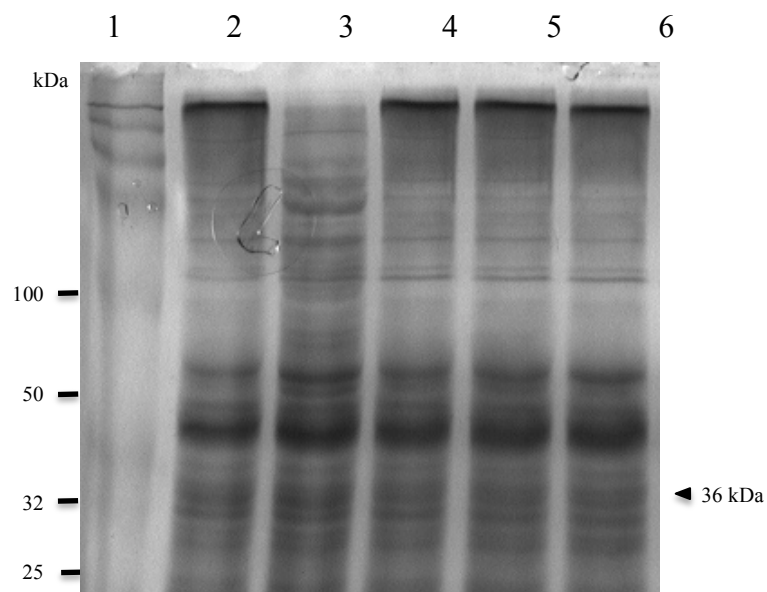


Figure 2.5: SDS-PAGE of total protein profile transformed *X. fastidiosa* clone 1-5.

The expected 36-kDa PGIP bands are indicated. Lane 1, molecular marker; lane 2-6, total proteins of transformed *X. fastidiosa* clone 1-5 containing pXF20-PGIP. Coomassie blue-stained 10% was used to visualize the protein bands.

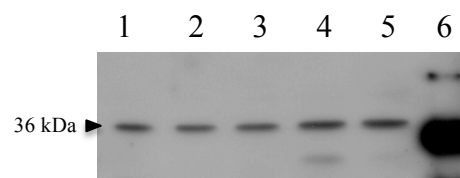


Figure 2.6: Western blotting analysis of crud extracts from transformed *X. fastidiosa* clone 1-5. Primary antibody was anti-6xHis. The expected 36-kDa PGIP bands are indicated. Molecular marker does not show. Lane 1-5, crude extracted proteins of transformed *X. fastidiosa* clone 1-5 containing pXF20-PGIP; lane 6, positive control as a purified PGIP-6xHis protein obtained from Genscript Company.

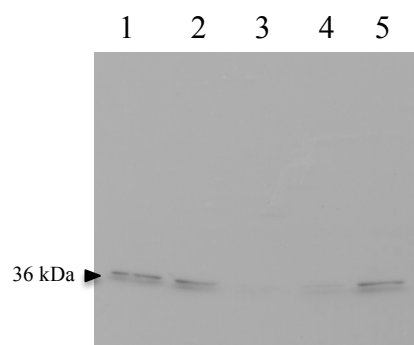


Figure 2.7: Western blotting analysis of supernatant culture of transformed *X. fastidiosa* clone 1-5. Primary antibody was anti-6xHis. The expected 36-kDa PGIP bands are indicated. Molecular marker does not show. Lane 1-5, supernatant culture of transformed *X. fastidiosa* clone 1-5 containing pXF20-PGIP.

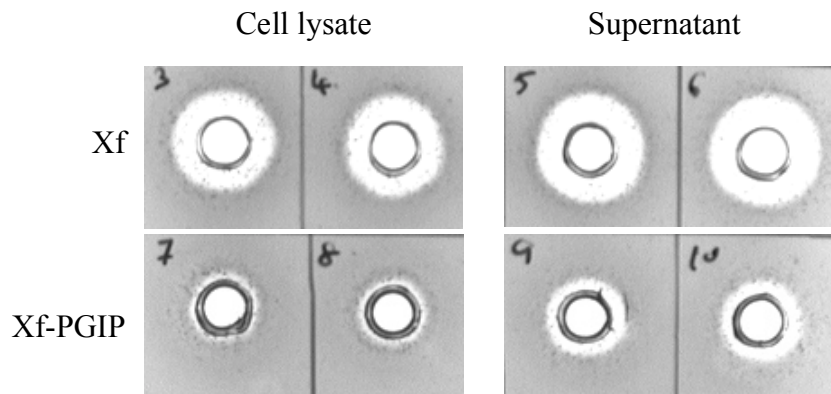


Figure 2.8: Polygalacturonic (PG) diffusion plate assay by PGIP from crude cell lysate and supernatant of culture of *X. fastidiosa* (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP). As described in the text, the clear ring around the wells indicates PG activity. Assays for each combination of PG and cell lysate or supernatant were performed in duplicated. There was significant difference of inhibition activity of PG between transformed and non-transformed bacteria.

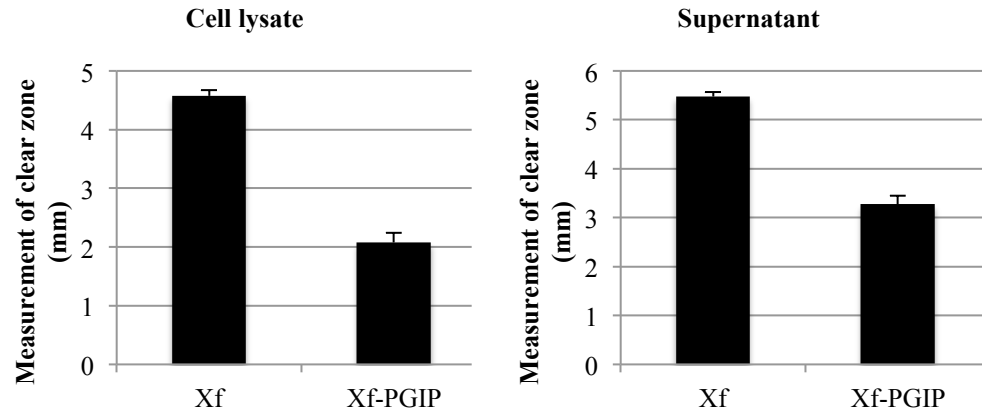


Figure 2.9: Inhibition of polygalacturonic (PG) by PGIP by PGIP from crude cell lysate and supernatant of culture of *X. fastidiosa* (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP). (A) Comparison of measurement of clear area from cell lysate samples between *X. fastidiosa* (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP). There was significant difference (p-value = 2.3756E-07). (B) Comparison of measurement of clear area from supernatant samples between Xf and Xf-PGIP. There was significant difference (p-value = 0.00022)

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Chapter 3: Colonization and PGIP expression in grapevines by *Xylella fastidiosa*

pXF20-PGIP

Abstract

pXF20-PGIP was inserted into *Xylella fastidiosa*. This less-virulent construct was intended to block the movement of pathogenic *X. fastidiosa* in grapevines. Four weeks after inoculation of *X. fastidiosa* pXF20-PGIP (Xf-PGIP) into grapevines, 29.83 ± 7.471280 and 8.73 ± 4.07573 pg/ μ l were found in total plant extracts and xylem, respectively. When *X. fastidiosa* (Xf) was inoculated under the same conditions, 77.60 ± 16.9365 and 26.38 ± 5.5815 pg/ μ l were found in total plant extracts and xylem, respectively. The bacteria growth of Xf-PGIP *in planta* was significantly lower than that of Xf. The presence of PGIP was detected in total plant extracts and in the xylem fluid of the plants inoculated with Xf-PGIP using Western blot analysis. A PG enzyme activity assay was inhibited significantly by the presence of xylem fluid from plants inoculated with Xf-PGIP compared to the xylem fluid from plants inoculated with Xf. The inhibition activity was attributed to PGIP secretion by Xf-PGIP into the xylem fluid. In principle this Xf-PGIP avirulent strain of bacteria can reduce the symptoms of Pierce's Disease in grapevines, but requires further testing.

Introduction

Pierce's Disease (PD) of grapevines is caused by *Xylella fastidiosa*, a gram-negative, xylem-limited bacterium (Davis et al., 1980; Hopkins and Mollenhauer, 1973; Pierce, 1892; Wells et al., 1987). For *X. fastidiosa* populations to become systemic, the bacteria have to cross the pit membranes that separate one xylem vessel from its neighbors. *X. fastidiosa* cells are able to pass-through smaller pit membrane pores (Chatelet et al., 2006; Chatterjee and Newman, 2008). The plant cell wall contains polysaccharides, which are sources of carbon and nitrogen for pathogens. Polygalacturonases (PGs) are produced by *X. fastidiosa* (Roper et al., 2007; Simpson et al., 2000) to facilitate utilization and colonization of host tissue. Thus PG is required for colonization and pathogenicity of *X. fastidiosa* in susceptible *Vitis vinifera* grapevines (Roper et al., 2007). Polygalacturonase inhibiting proteins (PGIPs) are produced by the plant cell-wall and can inhibit endo-PG (PGs) from pathogenic bacteria (Stotz et al., 1993). Pear PGIP in transgenic tomato plants increased their tolerance to *Botrytis cinerea* and decreased development of symptoms (Powell et al., 2000). We hypothesized that *X. fastidiosa* expressing pear PGIP can inhibit *X. fastidiosa* movement and delay PD symptom development.

Materials and Methods

Bacterial culture

Xylella fastidiosa pXF20-PGIP, which had been previously transformed, was grown in PD3 media. The bacterial cultures were incubated at 28°C, shaken at 200 rpm for 7-10 days. When required, antibiotics were added as follows: ampicillin, 100 ug/ml; kanamycin, 30 ug/ml. The bacterial cultures were collected and suspended to a final concentration of 10^8 - 10^9 CFU/ml.

Plant inoculation

Grapevines (*Vitis vinifera* cvs. Chardonnay) were propagated from seed or cuttings, which were obtained from the University of California, Davis. Plants were grown in greenhouse 56-H in Agricultural Operations at the University of California, Riverside. Inoculations with *X. fastidiosa* and *X. fastidiosa* pXF20-PGIP (Figure 1) were conducted via stem puncture with 21-gauge syringe needles with a 20µl droplet of bacteria culture at a concentration of 10^8 - 10^9 CFU/ml (Li et al., 2001). PBS inoculation served as a negative control. Plants were allowed to absorb the inoculum. Three replicated plant cuttings were inoculated with each treatment and placed in a randomized block design. The inoculated seedlings and cuttings were kept in a greenhouse for four weeks.

SDS-PAGE and Western blot analysis

Infected seedling stems were collected 15-cm from the inoculation point. Plant samples were macerated by grinding in sample mesh plastic bags (Agdia, Elkhart, IN). Protein electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) and western blot assays were performed as described (Sambrook, 2001). Proteins were transferred to nitrocellulose membranes (Amersham) and electrophoresis was performed at 100V for 1.5 h. Membranes were then kept for 2 h at room temperature in blocking buffer [5% non-fat milk, 0.1% Tween 20, 1% PBS] and incubated for 12 h at 4°C with an anti-6xHis probe (Thermo Scientific Pierce) (1:2,000 dilution) in a wash buffer containing 0.5% non-fat milk. After washing 8 times, a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Thermo Scientific Pierce) (1:15,000 dilution) was added to the membrane in wash buffer containing 0.5% non-fat milk, and incubated for 2 h at room temperature. Peroxidase activity was detected by enhanced chemiluminescence (Fast ECL kit, Amersham).

Quantitative real-time PCR

Plants samples were collected as described above. Surface sterilization was performed prior to DNA extraction to eliminate possible contamination. Surface sterilization steps included 2-min immersions in 75% ethanol, 10% household bleach, followed by 3 rinses of sterile distilled water. Plant samples were macerated by grinding in sample mesh plastic bags (Agdia, Elkhart, IN). Samples were extracted for DNA using GeneClean DNA extraction kit (MP Biomedicals, OH). Detection and quantification of *X*.

fastidiosa titers using quantitative real-time PCR (qPCR) assay was done in a Rotor Gene 3000 (Corbett Research 2005) with specific primers, forward primer XFF2-16S (5'-CTC GCCACCATGGTATTACTAC-3'), reverse primer XFR2-16S (5'-CTGGCGGCAGGCC TAA C-3') and a TaqMan probe Xfp2 (5'-Quasar 670-ATGTGCTGCCGTCCGACTTG CATG -BHQ-2-3') as described in Table 1.1. The qPCR 20- μ l reaction assay consisted of 10 μ l iQ Supermix (Bio-Rad, Hercules, CA), which included 100 mM KCL, 40 mM Tris-HCl, pH 8.4, 16 mM dNTPs, iTaq DNA polymerase, 50 U/ml, and 6 mM MgCl₂, a concentration of 100 nM forward primer, a concentration of 200 nM reverse primer, a concentration of 100 nM TaqMan probe, and water. Real time PCR conditions were 10 min at 95°C for enzyme activation, 15 sec at 95°C for denaturing, 1 min at 60°C for extension and annealing. Forty cycles were run. Each run was normalized and quantified with standard concentrations as described (Ramirez et al., 2008).

Xylem extraction by Pressure Chamber

Grapevine cuttings were collected and xylem fluid extracted using a pressure chamber. The whole plant was inserted in the chamber with one stem outside. Pressure inside the chamber was increased and maintained at 20 kPa for 5 min. Xylem fluid was collected from the protruding stem for further analysis, including Western blot analysis, qPCR analysis, and PG-inhibition assay in a diffusion agar plate.

PG-inhibition assay

Polygalacturonase-inhibiting protein (PGIP) activity of xylem samples was analyzed by the Polygalacturonase (PG)-inhibition assay or the diffusion agar plate assay (Buescher 1992, Taylor and Secor 1988, Tamura, Gao, Tao, Labavitch 2004) with some modification. A 0.7% (wt/v) agarose solution was heated in a microwave oven until dissolved. A 0.1% solution of polygalacturonic acid (PGA) (Sigma-Aldrich, USA), 3.65 g/l EDTA and 0.2 g/l Na-azide in 0.1 M sodium acetate buffer (pH 5.0) were added to the agarose solution. Then 25 ml of agarose solution was poured into 100x15-mm disposable petri dish and allow to solidify. The 5.6 mm diameter wells were cut in the agar sheet using a cork bored No. 3. Sample mixtures of 30 µl total volume (15 µl of cell lysate or supernatant) mixed with 15 µl of 0.1% of PG from *Aspergillus niger* (Sigma-Aldrich P-4625) were loaded into wells. The plates were incubated for 17 h at 37°C. After incubation, the plates were stained with 10 mL of 0.05% ruthenium red for 30 min, followed by destaining in water several times over 1 h. The destained plates were allowed to stand for 3 h at room temperature prior to assessment of PGIP activity by measuring the diameter of the clear zone around the wells.

Results

***X. fastidiosa* pXF20-PGIP protein expression and colonization in grapevines**

X. fastidiosa (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP) from grapevine seedlings were detected via quantitative real-time PCR (qPCR) using the same primer set, including XFF2-16S, XFR2-16S primers, and XFP2 TaqMan probe (Figure 3.1). The

amount of Xf-PGIP (29.83 ± 7.471280) was significantly less than the amount of Xf (77.60 ± 16.9365 pg/ μ l) and P-value is 0.06128 (Figure 3.2). Moreover positive expression of PGIP was found in seedlings, which were inoculated with Xf-PGIP (Figure 3.3). The protein bands were estimated at 36 kDa, which is the expected size of pear PGIP. These corresponded to previous results obtained by in vitro assay (Chapter 2).

***X. fastidiosa* pXF20-PGIP protein expression and colonization in xylem**

X. fastidiosa (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP) were inoculated into grapevine cuttings. Four weeks after inoculation, xylem fluid was extracted from cuttings. Xf and Xf-PGIP were detected via qPCR using the same primer set, including XFF2-16S, XFR2-16S primers, and XFP2 TaqMan probe (Figure 3.4). The amount of Xf-PGIP (8.73 ± 4.07573 pg/ μ l) was significantly less than the amount of Xf (26.38 ± 5.5815 pg/ μ l) and P-value is 0.06305 (Figure 3.5). Moreover positive expression of PGIP was found in xylem of cuttings, which were inoculated with Xf-PGIP (Figure 3.6). The protein bands were estimated at 36 kDa, which is the expected size of pear PIGP. These results corresponded to previous results obtained by in vitro assay (Chapter 2).

PGIP activity of *X. fastidiosa* pXF20-PGIP in xylem

X. fastidiosa (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP) were inoculated into grapevine cuttings. Four weeks after inoculation, cuttings were extracted for xylem fluid. Polygalacturonase (PG)-inhibiting assay (diffusion agar plate assay) was performed.

Inhibition of PG activity was greater in the xylem from Xf-PGIP inoculated plants than in the xylem from Xf inoculated plants (Figure 3.7A). Clear circles around wells to which the positive standard, xylem from Xf-inoculated plants and xylem from Xf-PGIP-inoculated plants were added were 1.6025 ± 0.00479 , 1.405 ± 0.04193 , and 1.5825 ± 0.01315 cm in diameter, respectively (P-value = 0.00681) (Figure 3.7B). The clear zones on the agar plates provide quantitative measure of the inhibition of PG by PGIP thought to be produced by Xf-PGIP.

Discussion

Plasmid containing the gene encoding the pear (*Pyrus communis*) Polygalacturonase inhibiting protein (PGIP) (pXF20-PGIP) was inserted into *X. fastidiosa* (Xf). We conducted a series of experiments that allowed us to conclude that *X. fastidiosa* pXF20-PGIP (Xf-PGIP) can colonize the grapevines and express PGIP. Although the growth curves of Xf-PGIP and Xf are not significantly different *in vitro*, bacteria growth of Xf-PGIP *in planta* is significantly lower than Xf. We concluded that Xf-PGIP secreted PGIP inhibiting PG. Bacteria could not use nutrients from a degraded cell wall because *X. fastidiosa* requires PG for such degradation and thus for colonization and pathogenicity in grapevines (Roper et al., 2007).

Figures and Tables

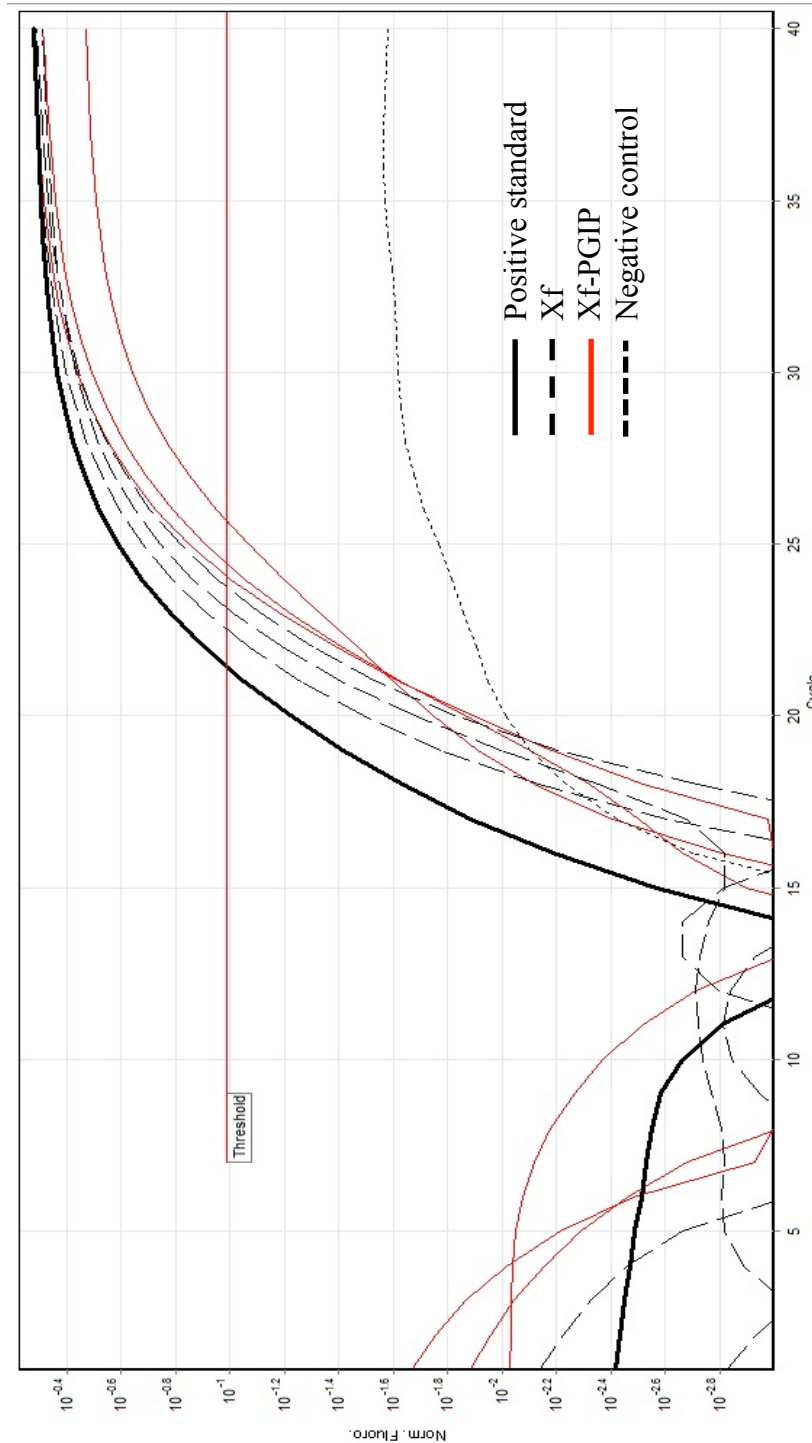


Figure 3.1: Detection of *X. fastidiosa* using quantitative real-time PCR (qPCR). DNA samples were extracted from seedlings inoculated with *X. fastidiosa* (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP) at the fourth week after inoculation. qPCR analysis was performed with the same primer set, including XFF2-16S, XFR2-16S primers, and XFP2 TaqMan probe. Threshold was calculated from known-concentration positive standards *X. fastidiosa* DNA. Negative control was distilled water.

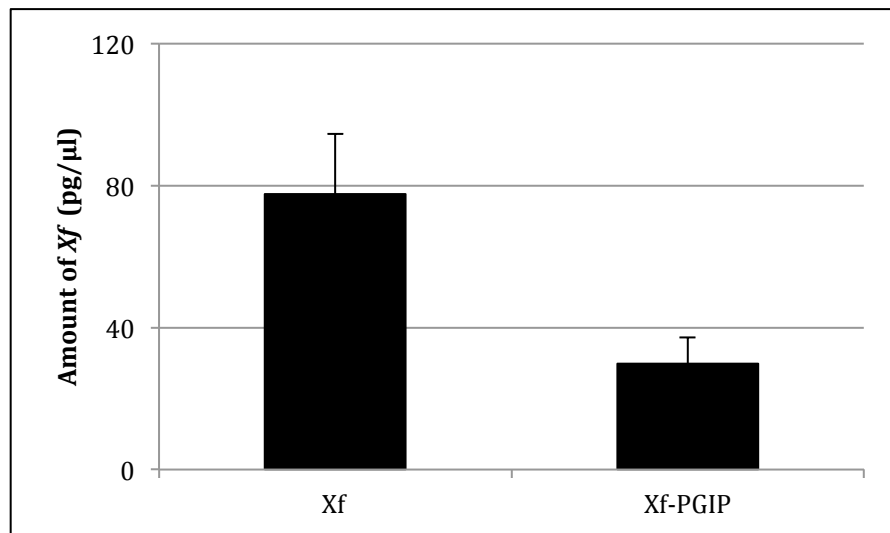


Figure 3.2: The amount of *X. fastidiosa* from quantitative real-time PCR (qPCR) analysis. DNA samples were extracted from seedlings inoculated with *X. fastidiosa* (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP) at the fourth week after inoculation. The amount of Xf-PGIP was significantly less than the amount of Xf (P-value = 0.06128).

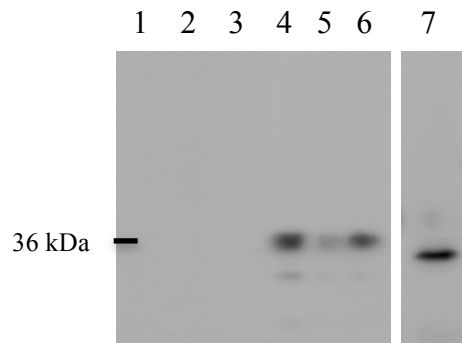


Figure 3.3: Western blot analysis of total protein extracted from seedling grapevines inoculated with *X. fastidiosa* (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP). Lane 0, Molecular marker (not show); lane 1-3, Plant inoculated with Xf; lane 4-6, Plant inoculated with Xf-PGIP; lane 7, Purified PGIP protein from Genscript Company.

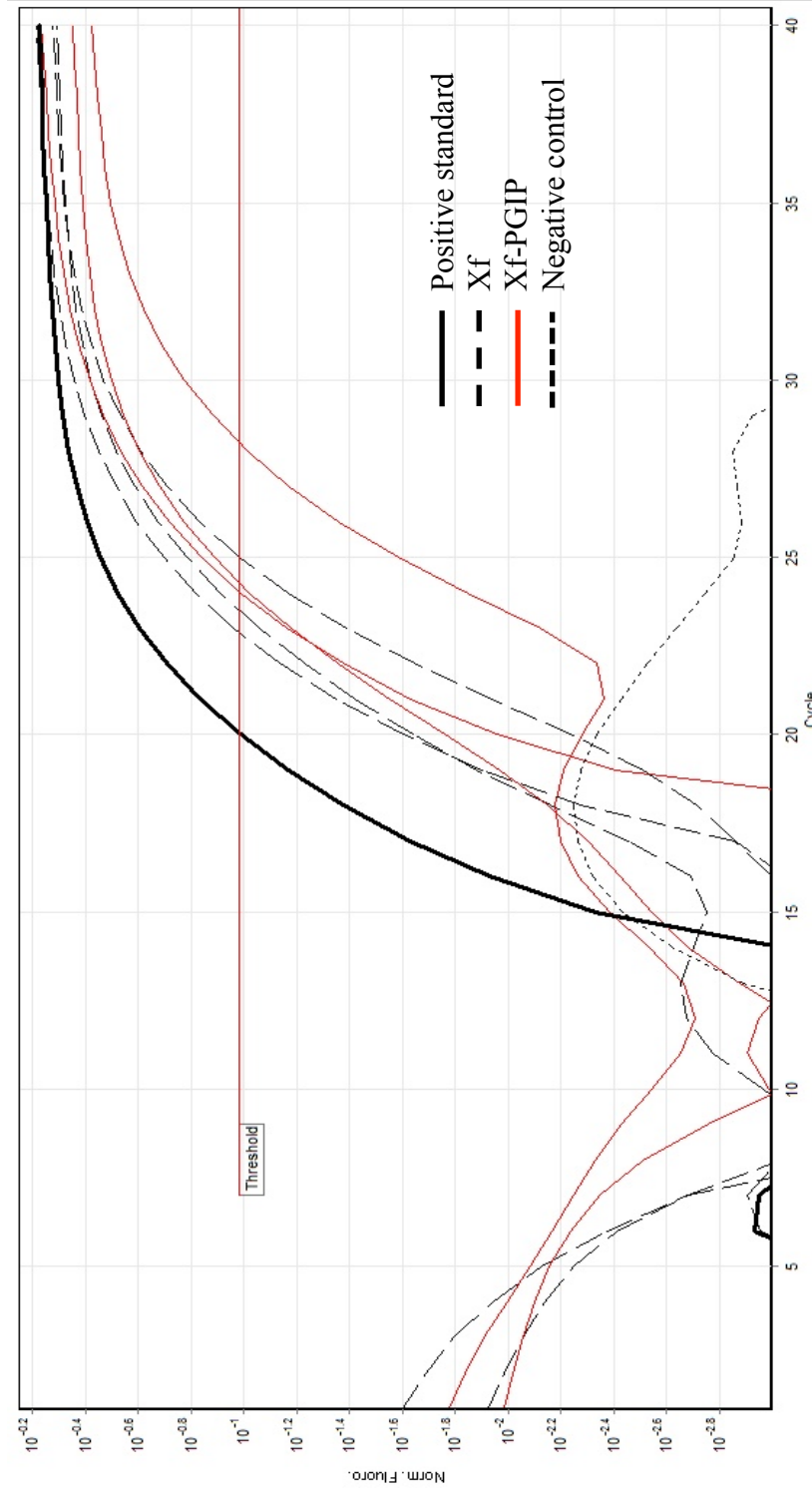


Figure 3.4: Detection of *X. fastidiosa* using quantitative real-time PCR (qPCR). DNA samples were extracted from xylem fluid of cuttings inoculated with *X. fastidiosa* (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP) at the fourth week after inoculation. qPCR analysis was performed with the same primer set, including XFF2-16S, XFR2-16S primers, and XFP2 TaqMan probe. Threshold was calculated from known-concentration positive standards *X. fastidiosa* DNA. Negative control was distilled water.

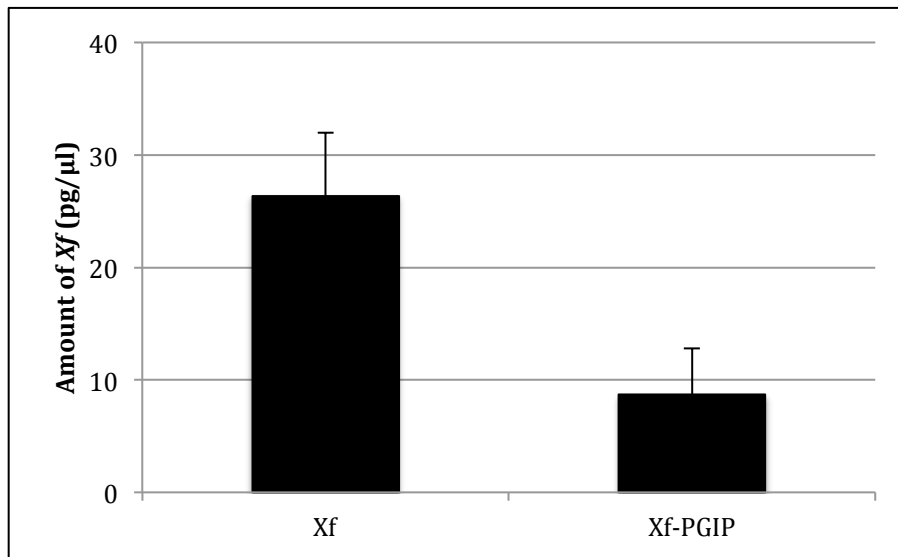


Figure 3.5: The amount of *X. fastidiosa* from quantitative real-time PCR (qPCR) analysis. DNA samples were extracted from xylem fluid from cuttings inoculated with *X. fastidiosa* (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP) at the fourth week after inoculation. The amount of Xf-PGIP was significantly less than the amount of Xf (P-value = 0. 06305).

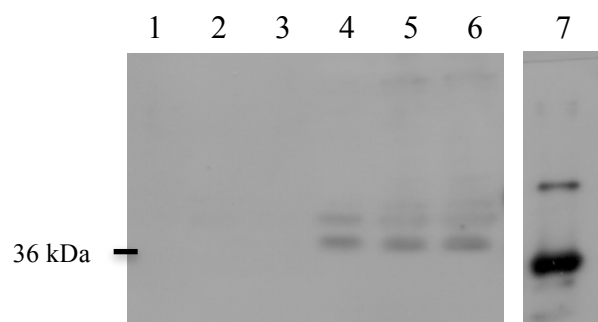


Figure 3.6: Western blot of protein extracted from xylem fluid of cuttings inoculated with *X. fastidiosa* (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP). Lane 1-3, Xylem extracted from plants inoculated with Xf; lane 4-6, Xylem extracted from plants inoculated with Xf-PGIP; lane 7, Purified PGIP protein from Genscript Company

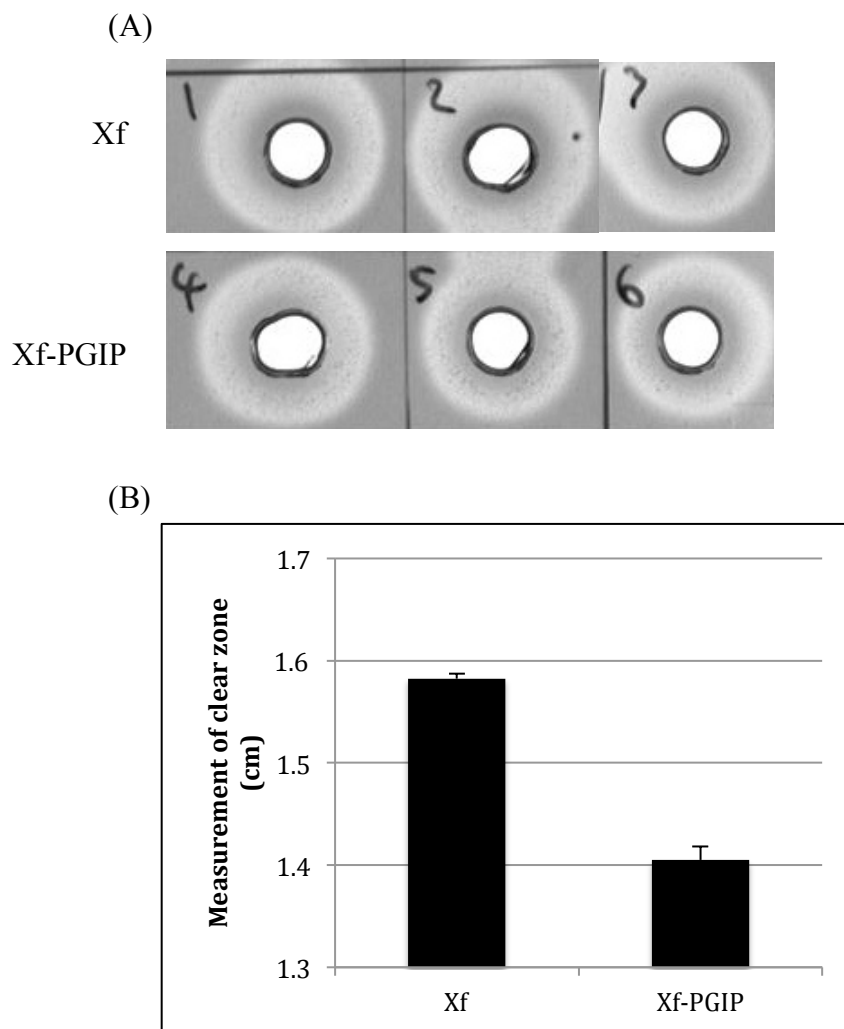


Figure 3.7: PG-inhibition assay of *X. fastidiosa* (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP) in xylem of inoculated grapevine cuttings. Panel (A): Diffusion agar plate. 1-3, Xylem extracted from cuttings that were inoculated with Xf; 4-6, Xylem extracted from cuttings that were inoculated with Xf-PGIP. Panel (B): Diameter of clear zones on the agar plates provide quantitative measure of the inhibition of PG by PGIP thought to be produced by Xf-PGIP. P-value is 0.00681.

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Chapter 4: Inhibition of *X. fastidiosa* colonization and movement by *X. fastidiosa* pXF20-PGIP in grapevines.

Abstract

Clean grapevines were inoculated with *X. fastidiosa* (Xf) alone or with *X. fastidiosa* pXF20-PGIP (Xf-PGIP) to test the hypothesis that the PGIP strain affects the growth or movement of Xf in the grapevines. If Xf-PGIP is avirulent, then demonstration of the control of growth and movement of the pathogen Xf would serve as proof of concept of symbiotic control of PD caused by Xf. Grapevines were inoculated with Xf and Xf-PGIP. (i) Ten weeks after inoculation, Xf-PGIP at 45 cm above inoculation point (IP) (8.96 ± 1.04253 pg/ μ l) was found present in significantly lower concentrations than Xf (54.60 ± 12.01012 pg/ μ l) at the same distance. (ii) The PGIP expression of Xf-PGIP was detected from IP to 75 cm above IP at week 6, from sections IP to 105 cm above IP at week 8 and 10. (iii) Bacteria in double inoculation plants, which were inoculated with Xf-PGIP at day 0 and again with Xf at week 4 at 45 cm above the first IP, were found present in significantly lower concentrations than bacteria in plants with only Xf inoculation at week 4. The overall reduction of bacteria in plants compared to Xf alone with double inoculation may be due to the presence of PGIP. (iv) Over time, the migration of Xf without Xf-PGIP inoculation was found in farther length than in the double inoculation plants at week 4. In conclusion this Xf-PGIP may contribute to inhibition of the growth and movement of Xf.

Introduction

Xylella fastidiosa, a gram-negative, xylem-limited bacterium causes Pierce's Disease (PD) in grapevines (Davis et al., 1980; Hopkins and Mollenhauer, 1973; Pierce, 1892; Wells et al., 1987). *X. fastidiosa* is transmitted from plant to plant by xylem-feeding sharpshooters. Once inside the xylem, *X. fastidiosa* multiply and spread throughout the plant's vascular (Chatterjee and Newman, 2008). For pathogen populations to become systemic, the bacteria have to cross the pit membrane that separates one xylem vessel from its neighbors. *X. fastidiosa* cells are able to pass-through smaller pit membrane pores (Chatelet et al., 2006). *X. fastidiosa* requires Polygalacturonases (PGs) for colonization and pathogenicity in *Vitis vinifera* grapevines (Roper et al., 2007; Simpson et al., 2000). Indeed, *X. fastidiosa pglA* mutant unable to move systemically in grapevine (Roper et al., 2007). Polygalacturonase inhibiting proteins (PGIPs) are plant cell-wall proteins that inhibit pathogen endo-PG (PGs) (Stotz et al., 1993). Pear PGIP in transgenic tomato plants increase their tolerance to *Botrytis cinerea* and decrease development of symptoms (Powell et al., 2000). We hypothesized that *X. fastidiosa* expressing pear PGIP can inhibit *X. fastidiosa* movement and delay PD symptom development.

Materials and Methods

Bacterial culture

Xylella fastidiosa pXF20-PGIP, which had been previously transformed, was grown in PD3 media. The bacterial cultures were incubated at 28°C, shaken at 200 rpm for 7-10 days. When required, antibiotics were added as follows: ampicillin, 100 ug/ml; kanamycin, 30 ug/ml. The bacterial cultures were collected and suspended to a final concentration of 10^8 - 10^9 CFU/ml.

Plant inoculation

Grapevines (*Vitis vinifera* cvs. Chardonnay) were propagated from cuttings, which obtained from the University of California, Davis. Plants were grown in greenhouse 56-H in Agricultural Operations at the University of California, Riverside. Inoculations with *X. fastidiosa* (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP) were conducted via stem puncture with 21-gauge syringe needle through 20µl droplet of bacteria culture at a concentration of 10^8 - 10^9 CFU/ml (Li et al., 2001). PBS inoculation served as a negative control. Plants were observed until the plants absorbed the inoculum. Plant inoculation conditions described in Table 4.1. In the first condition, the plants were inoculated with Xf-PGIP at 5 cm above soil. Then four weeks after the first inoculation, the plants were inoculated with Xf at 45 cm above the first inoculation point. Samples were collected at week 6, 8, and 10. In the second condition, the plants were inoculated with Xf-PGIP at 5 cm above soil. Samples were collected at week 6, 8, and 10. In the

third condition, the plants were inoculated with Xf at 5 cm above soil. Samples were collected at week 6, 8, and 10. In the fourth condition, the plants were inoculated with Xf at 45 cm above soil. Samples were collected at week 6, 8, and 10. Nine replicated plants were inoculated in each of these conditions (above) and placed in a randomized block design.

PGIP detection by ELISA

PGIP-6xHis (PGIP) from plant samples was detected by a modified microplate assay. Briefly, plant samples were macerated by grinding with 800 µl PBS buffer in sample mesh plastic bag (Agdia, Elkhart, IN). 100 µl of crude extract were added into 96-well plates. They were incubated overnight at 4°C and then in PBST for 1 h at room temperature. The plates were washed with 100 µl PBST three times. The PGIP protein was detected by incubation with Anti-6xHis-horseradish peroxidase (HRP) conjugate (1:1,500) for 1 h. After washing three times with PBST, HRP activity was revealed with a freshly prepared luminol substrate (Supersignal enzyme-linked immunosorbent assay [ELISA] pico; Thermo Scientific). An X-ray film was placed over the microplate in a darkroom for 30 min, and the data were quantified with NIH Image J software and analyzed using Origin (Origin Lab).

Quantitative real-time PCR

A 15-cm section of infected grapevines stems was collected from the inoculation point to 150 cm above. Surface sterilization was performed prior to DNA extraction in order to eliminate possible contamination. Surface sterilization steps included 2-min immersions in 75% ethanol, 10% household bleach, followed by 3 rinses of sterile distilled water. Plant samples were macerated by grinding in sample mesh plastic bags (Agdia, Elkhart, IN). Samples were extracted for DNA using GeneClean DNA extraction kit (MP Biomedicals, OH). Detection and quantification of *X. fastidiosa* titers using quantitative real-time PCR (qPCR) assay was done in a Rotor Gene 3000 (Corbett Research 2005) with specific primers, forward primer XFF2-16S (5'-CTCGCCACCATG GTATTACT AC-3'), reverse primer XFR2-16S (5'-CTGGCGGCAGGCCTAAC-3') and a TaqMan probe XfP2 (5'-Quasar 670-ATGTGCTGCCGTCCGACTTGCATG-BHQ-2-3') as described in Table 1.1. The qPCR 20- μ l reaction assay consisted of 10 μ l iQ Supermix (Bio-Rad, Hercules, CA), which included 100 mM KCL, 40 mM Tris-HCl, pH 8.4, 16 mM dNTPs, iTaq DNA polymerase, 50 U/ml, and 6 mM MgCl₂, a concentration of 100 nM forward primer, a concentration of 200 nM reverse primer, a concentration of 100 nM TaqMan probe, and water. Real time PCR conditions were 10 min at 95°C for enzyme activation, 15 sec at 95°C for denaturing, 1 min at 60°C for extension and annealing. Forty cycles were run. Each run was normalized and quantified with standard concentrations as described (Ramirez et al., 2008).

Results

Colonization of *X. fastidiosa* pXF20-PGIP in grapevines

Quantitative real-time PCR analysis was performed with DNA extracted from the plants inoculated with *X. fastidiosa* pXF20-PGIP (Xf-PGIP) and *X. fastidiosa* (Xf) after 10 weeks. The population of bacteria cells in *X. fastidiosa* pXF20-PGIP inoculated plants were 38.11 ± 9.46678 , 37.62 ± 9.68432 , 17.35 ± 4.27731 , 8.96 ± 1.04253 pg/ μ l at inoculation point (IP), 15, 30 and 45 cm above IP, respectively (Figure 4.1). The population of *X. fastidiosa* pXF20-PGIP declined in the further distance from IP. The population of bacteria cells in *X. fastidiosa* inoculated plants were 54.46 ± 11.41822 , 54.67 ± 17.12851 , 56.35 ± 11.49425 , 54.60 ± 12.01012 pg/ μ l at IP, 15, 30 and 45 cm above IP respectively. The population of *X. fastidiosa* pXF20-PGIP at 30 and 45 cm above IP was significantly lower than the population of *X. fastidiosa* at the same distance.

Detection of PGIP produced by *X. fastidiosa* pXF20-PGIP in grapevines

ELISA assay was performed to detect PGIP production of samples from plants with inoculation of *X. fastidiosa* pXF20-PGIP. We found PGIP positive at 0-75 cm above inoculation point (IP) after 6 weeks, and 0-105 cm above IP after 8 and 10 weeks (Figure 4.2). These results verified PGIP production of Xf-PGIP, which had been inoculated into grapevines. We also found PGIP positive by ELISA assay of xylem fluid, which was extracted from Xf-PGIP inoculated grapevines (data not show). These suggested that Xf-PGIP colonized the xylem and secreted PGIP.

Inhibition of the growth of *X. fastidiosa* by *X. fastidiosa* pXF20-PGIP

Inhibition of the growth of *X. fastidiosa* by *X. fastidiosa* pXF20-PGIP was determined by estimate of *X. fastidiosa* populations by quantitative real-time PCR (qPCR) (Figure 4.3). Conditions of plants and inoculation were described in Table 4.1. Eight weeks after inoculation, the total amount of bacteria in Xf-PGIP+Xf plants did not increase. This suggested that *X. fastidiosa* may be inhibited by PGIP. Ten weeks after inoculation, 18.09 ± 3.301310 pg/ μ l *X. fastidiosa* was found in Xf-PGIP+Xf plants and 33.08 ± 5.48518 pg/ μ l *X. fastidiosa* was found in Xf plants. These results suggested that the presence of Xf-PGIP inhibited the growth of *X. fastidiosa*.

Movement of *X. fastidiosa* in the plants inoculated with *X. fastidiosa* pXF20-PGIP

Movement of *X. fastidiosa* was determined by amount of *X. fastidiosa* detected by quantitative real-time PCR. Conditions of plants and inoculation were described in Table 4.1. Downstream migration of *X. fastidiosa* in Xf plants was found at 75 and 90 cm after week 8 and 10, respectively (Figure 4.4). On the other hand, downstream migration of *X. fastidiosa* in Xf-PGIP+Xf plants was found at 60 cm after week 8 and 10. Upstream migration of *X. fastidiosa* in Xf plants was found in 30 cm after week 8 and 10. Upstream migration of *X. fastidiosa* in Xf-PGIP+Xf plants was found in 30 cm after week 10 at much lower cell numbers than in Xf plants.

Discussion

A *pglA* *X. fastidiosa* mutant neither spread systemically in grapevines nor cause Pierce's Disease (PD) (Roper et al., 2007). Moreover the expression of PGIP in grapevine delayed development of PD (Agüero et al., 2005). These results suggest that PGIP inhibits the pathogen's PG. We concluded that PGIP expressed by Xf-PGIP inhibited growth and movement of *X. fastidiosa* as well.

Migration of *X. fastidiosa* was delayed by the presence of Xf-PGIP. *X. fastidiosa* did not migrate away from the point of inoculation as well as in the absence of Xf-PGIP. The partial inhibition of *X. fastidiosa* by Xf-PGIP suggested that inhibition of pathogen PG did not prevent the establishment and colonization of *X. fastidiosa* in grapevines, but it delayed the growth of *X. fastidiosa*. By delaying the growth of pathogen, it could possibly delay the development of Pierce's Disease (PD).

Even though systematic migration of bacteria is unlikely in Xf-PGIP infected plants, the bacteria colony significantly increased over time at inoculation point. However, its migration was limited to 60 cm above inoculation point after week 10.

Although we hypothesize that reduced colonization of Xf by Xf-PGIP is due to PGIP production, another possible explanation is that the inhibition of *X. fastidiosa* is possibly due to competition for nutrient by both bacteria. We actually set up an experiment to test for competitive using grapevines inoculated with *X. fastidiosa* GFP and *X. fastidiosa* compared to grapevines inoculated with Xf-PGIP and Xf. Unfortunately our plants died during the experiment.

We demonstrated that Xf-PGIP is less virulent than demonstrated reduction of growth and movement of the pathogen *X. fastidiosa*. This serves as proof of concept of symbiotic control of PD caused by *X. fastidiosa*.

Figures and Tables

No	Name	Inoculation ¹	Week after inoculation									
			1	2	3	4	5	6	7	8	9	10
1	Xf-PGIP + Xf	<i>Xf</i> pXF20-PGIP	-	-	-	<i>Xf</i> ²	-	x	-	x	-	x
2	Xf-PGIP	<i>Xf</i> pXF20-PGIP	-	-	-	-	-	x	-	x	-	x
3	Xf (wk0)	<i>Xf</i>	-	-	-	-	-	x	-	x	-	x
4	Xf (wk4)	-	-	-	-	<i>Xf</i> ²	-	x	-	x	-	x
5	PBS	PBS	-	-	-	-	-	-	-	-	-	x

x = Plant samples were collected.

¹ = Plants were inoculated with bacteria at 5 cm above soil. This called first inoculation point (IP).

² = Plants were inoculated with Xf at 45 cm above first IP

Table 4.1: Experimental condition of grapevines inoculated with bacteria. 1,

grapevines were inoculated with Xf-PGIP at the first inoculation point (1stIP) at 5-cm above soil on day 0. After inoculation for 4 weeks, the same plants were inoculated with *X. fastidiosa* at the second inoculation point (2ndIP) at 45-cm above 1stIP. **2,** grapevines were inoculated with Xf-PGIP at the first inoculation point (1stIP) at 5-cm above soil on day 0. **3,** grapevines were inoculated with Xf at the first inoculation point (1stIP) at 5-cm above soil on day 0. **4,** grapevines were inoculated with Xf at the second inoculation point (2ndIP) at week 4. **5,** grapevines were inoculated with PBS at the first inoculation point (1stIP) at 5-cm above soil on day 0. All the plants were placed in a greenhouse in randomized block design. Three plants of each treatment were taken for deconstructive sampling at every two weeks (week 6, 8 and 10), except the PBS inoculated plants were taken at week 10.

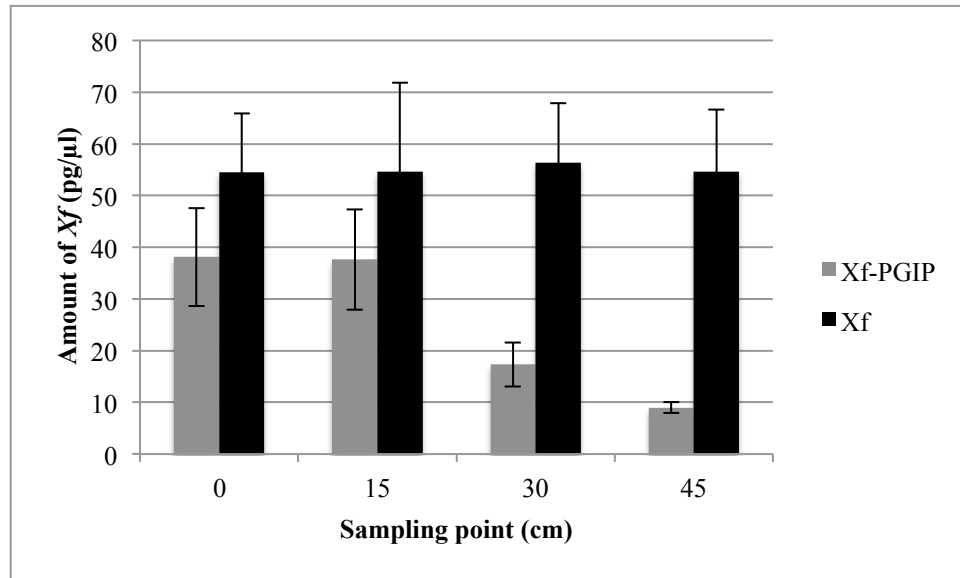


Figure 4.1: Detection of *X. fastidiosa* using quantitative real-time PCR (qPCR).

DNA samples were extracted from grapevine inoculated with *X. fastidiosa* (Xf) and *X. fastidiosa* pXF20-PGIP (Xf-PGIP) at the beginning of experiment (day 0). Sampling points: 0 is at inoculation point (IP), 15, 30, 45 cm above IP respectively. qPCR analysis was performed with the same primer set, including XFF2-16S, XFR2-16S primers, and XFP2 TaqMan probe. Threshold was calculated from known-concentration positive standards *X. fastidiosa* DNA. Negative control was distilled water.

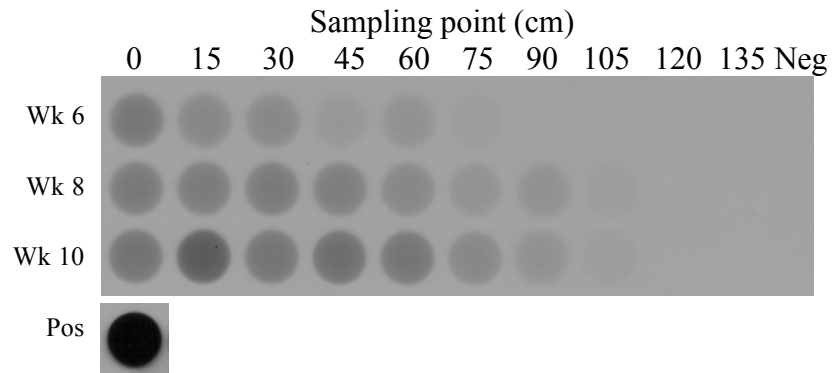


Figure 4.2: PGIP detection by ELISA of plants inoculated with *X. fastidiosa* pXF20-PGIP. Inoculation point (IP) was at 0 cm. Each point increased by 15 cm. After inoculation for 6 weeks, PGIP was detected up to 75 cm above IP. After inoculation for 8 weeks, PGIP was detected up to 105 cm above IP. After inoculation for 10 weeks, PGIP was detected up to 105 cm above IP. Detection in sampling point from week 6 to 8 increase, but stable in week 8 to 10. Negative controls (Neg) were plants inoculated with *X. fastidiosa*. Positive control (Pos) was purified PGIP from GenScript Company. Exposure time of ELISA was 30 min.

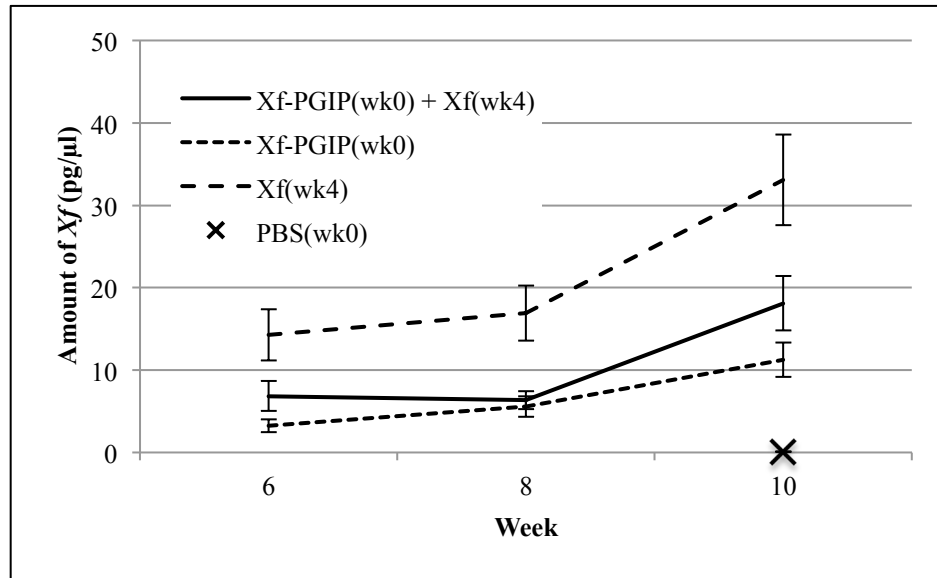


Figure 4.3: Average infection level (pg/ μ l) by week. Detection of *X. fastidiosa* in grapevines (Table 4.1) was determined by quantitative real-time PCR (qPCR). **Xf-PGIP + Xf**, grapevines were inoculated with *X. fastidiosa*-PGIP (*Xf*-PGIP) at the beginning of the experiment and inoculated with *X. fastidiosa* (*Xf*) 45-cm above first inoculation after week 4; **Xf-PGIP**, grapevines were inoculated with *Xf*-PGIP at the beginning of the experiment; **Xf**, grapevines were inoculated with *Xf* after week 4; **PBS**, grapevines were inoculated with PBS as negative control.

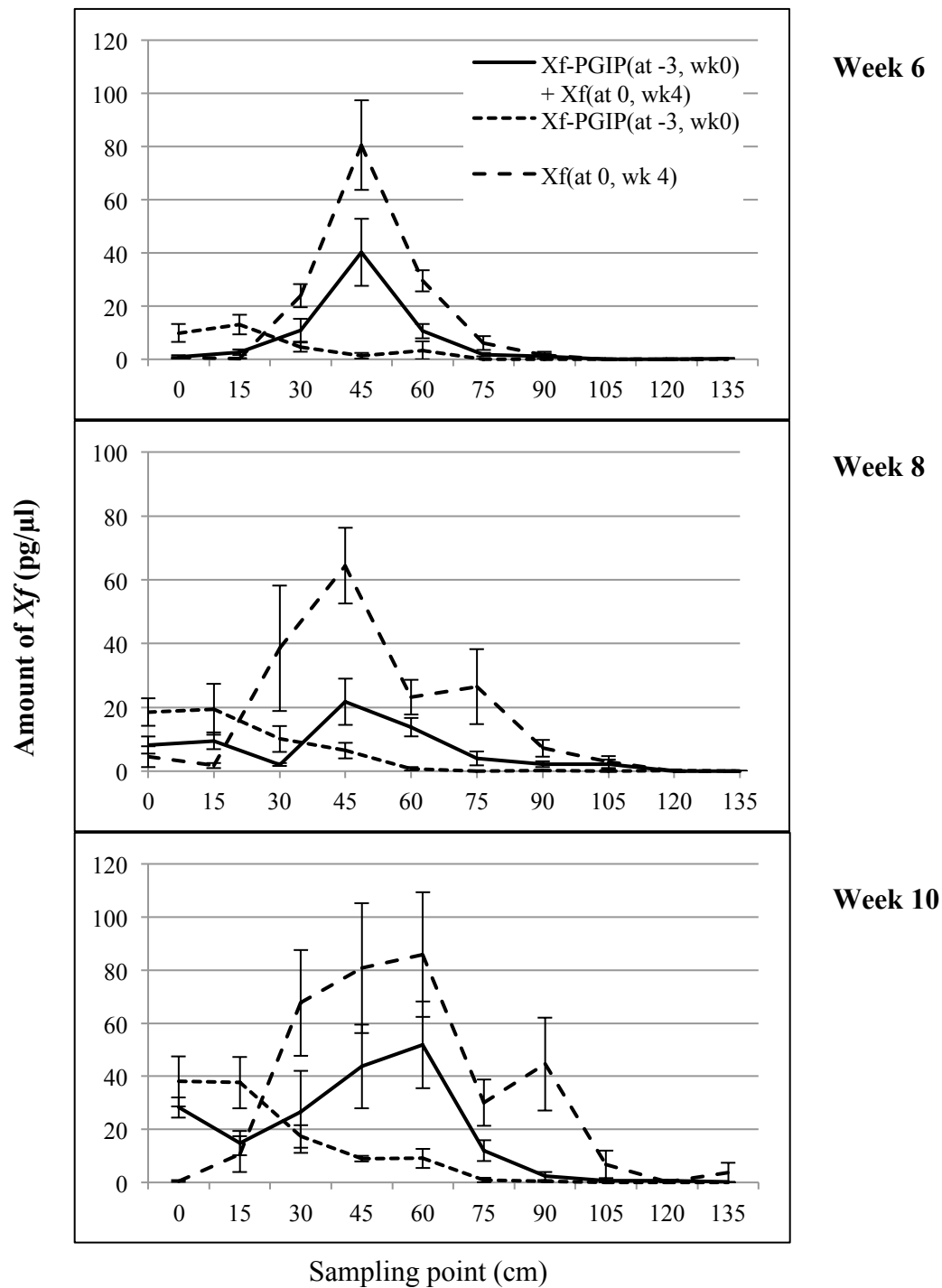


Figure 4.4: Average infection level (pg/μl) by sampling point and week of grapevines.

Detection of *X. fastidiosa* in grapevines (Table 4.1) was determined by quantitative real-time PCR (qPCR). **Xf-PGIP + Xf**, grapevines were inoculated with *X. fastidiosa*-PGIP (*Xf*-PGIP) at the beginning of the experiment and inoculated with *X. fastidiosa* (*Xf*) 45-cm above first inoculation after week 4; **Xf-PGIP**, grapevines were inoculated with *Xf*-PGIP at the beginning of the experiment; **Xf**, grapevines were inoculated with *Xf* after week 4; **PBS**, grapevines were inoculated with PBS as negative control.

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Appendix A: Symbiotic Control of Pierce's Disease

Abstract

Pierce's Disease (PD) of grapevines is caused by specific strain of the gram-negative, xylem-inhabited bacteria, *Xylella fastidiosa*. The most important vectors of *X. fastidiosa* in California is the xylem-feeding sharpshooter leafhopper, *Homalodisca vitripennis*. We investigated the symbiotic bacterium, *Paenibacillus* 47V1 1AC1, as a possible biological control agent to protect grapevines from the pathogen causing PD. *Paenibacillus* 47V1 1AC1 was found in grapevines of a commercial vineyard in Temecula that showed apparent resistance to PD. We tested the fate of PB by inoculating Chardonnay grapevines. PB colonized the xylem of grapevines. Moreover PB-inoculated grapevines showed no conclusive PD-resistance in the greenhouse. In Spring 2008, we planted 180 grapevines of these same three varieties (60 each) in the 9F field at Agricultural Operations (AgOps) at UC Riverside. Half of the grapevines were inoculated with PB. Only 10% of all grapevines have tested positive for Xf by early summer of 2009. There was no significant difference between PB-inoculated and the control group.

Introduction

Pierce's Disease

Pierce's Disease (PD) of grapevines is caused by the xylem-inhabiting, Gram-negative bacterium, *Xylella fastidiosa* (Purcell et al., 1979; Purcell and Hopkins, 1996). Symptoms of PD in infected vines include leaf drying, or "scorching". As part of this

process, the leaves become yellow. Later, the dead leaves fall off, leaving the petioles attached to the cane and leading to the formation of what are known as “matchsticks”. The mechanism by which *X. fastidiosa* blocks the xylem vessels of the plant remains unknown.

Glassy-Winged Sharpshooter (GWSS)

During the early 1990s, a major vector of *X. fastidiosa*, the glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*) (Takiya et al., 2006), invaded Southern California. GWSS is an aggressive flyer and feeder in the leafhopper family. It transmits *X. fastidiosa* to a number of plants such as citrus, almond, oleander and grapevine (Purcell and Saunders, 1995; Purcell and Saunders, 1999; Purcell et al., 1999). *X. fastidiosa* are expelled from the GWSS foregut into the xylem where they aggregate and occlude the water system causing PD of grapevine (Backus et al., 2005; Leopold et al., 2003)

Paenibacillus

Paenibacillus 47V1 1AC1 was found in apparently PD-resistant grapevines of a vineyard in Temecula, CA. The objectives of this study are to (i) identify in planta interaction between *Paenibacillus* 47V1 1AC1 and *X. fastidiosa* and (ii) evaluate the use of *Paenibacillus* 47V1 1AC1 for the symbiotic control of PD in grapevines.

Materials and Methods

ELISA

Grapevine samples were tested to determine the presence of *Xylella fastidiosa* using Enzyme-Linked Immunosorbent Assay (ELISA) with a Pathoscreen XF kit (Agdia Incorporated, IN) according to the manufacturers' instructions. Leaves from the same plant were combined together and macerated in an Agdia bag. 800 μ L of Extraction Buffer was mixed with the plant liquid. 100 μ L aliquots of liquid mixture were pipetted into Pathoscreen 96-well plates and incubated at the room temperature for two hours or overnight in a refrigerator at 4° C. Then each 96-well plate was washed with sterile phosphate buffered saline with tween 20 (PBST), 6-8 times. 100 μ l of Anti-*X. fastidiosa* peroxidase conjugated from Pathoscreen were added into each well and incubated for two hours followed by washing the plate with PBST, 6-8 times. 100 μ L of Tetramethylbenzidine (TMB) peroxidase were added to each well and incubated for 20 min. Blue color signaled presence of *X. fastidiosa*. Plates were scanned at 650 nm using a microplate reader and data was normalized and analyzed using Microsoft Excel. Samples twice as high as background were considered positive

Quantitative real-time PCR (rtPCR)

To verify the presence of the PD strain of *X. fastidiosa* in each sample, DNA was extracted using GeneClean DNA extraction kit (catalogue number 1101-600). The real-time PCR (rtPCR) was performed on a Roto-Gene machine (Corbett research). The Xf primers and TaqMan probe were forward primer 5'-AAAAATCGCCAACATAAACCC

A-3', reverse primer 5'-CCAGGCGTCCTCAC AAGTTAC-3', and probe 5'-FAM-ACCTATGCCAACATCAAACCCTGAATGCA-BHQ-1 3'. The 20- μ L reaction consisted of 10 μ L iQ SuperMix (Bio-Rad), which contained 50 mM KCl, 20 mM Tris HCl, 0.8 mM dNTP, 3 mM MgCl₂, 0.5 units of iTaq polymerase (catalogue number 170-8862), 0.4 μ L forward primer, 0.2 μ L reverse primer, and 0.2 μ L probe.

***Paenibacillus* detection**

Primers were designed using the Primer3 web-based software (<http://frodo.wi.mit.edu/primer3>) for use in PCR-based assays for the presence of *Paenibacillus*. The reaction was performed in a Rotor-Gene 3000 machine (Corbett Robotics Inc., San Francisco, CA). Designed primers PB16S-4L and PB16S-4R were able to amplified 16S DNA of PB that were inoculated into grape. Product size was estimated 178 kb.

Field plot

In April 2008, 60 Chardonnays, 60 Cabernets, and 60 Orange Muscats were planted in the experimental vineyard (9F) at Agricultural Operations (Ag Ops), the University of California, Riverside (Figure 5.1). Half of them were inoculated with PB in the greenhouse before transferred into 9F.

Results

***Paenibacillus* localization**

We tested the localization of PB on Chardonnay grapes. This experiment was done in a greenhouse. PB was prepared in Luria-Bertani (LB) medium and measured concentration to OD600 = 0.1. The 20 μ L PB was inoculated into each of 3 plants of 6-week grown Chardonnay grapevine at 5-cm above the rootstock's cane. Phosphate buffered saline (PBS) inoculation was used for negative control. The leaf samples were collected at one month after inoculation, and extracted for DNA using GeneClean DNA extraction kit. Positive control was done by add PB solution into leaf samples before DNA extraction. The PCR with PB16S-4L and PB16S-4R primers was performed. The result of gel electroporation was shown that samples from inoculation point were positive for PB and the rest of samples were negative. The positive control was positive (data not shown).

For further experiment, to determine the localization of PB in xylem of grapevine. PB was prepared in LB medium and measured concentration to OD600 = 0.1. The 20 μ L PB was inoculated into each of 9 plants of 6-week grown Chardonnay grapevine at 5-cm above the rootstock's cane. PBS inoculation was used for negative control. The stem samples were collected at one month after inoculation. Pressure bomb was used to extract xylem fluid from grapevine samples. Fluid was extracted for DNA using GeneClean DNA extraction kit. The PCR with PB16S-4L and PB16S-4R primers was performed. The result of gel electroporation was shown that 5 out of 9 samples from inoculation

point were positive for PB and the rest of samples were negative (Figure 5.2). The positive control was positive.

ELISA result of field survey

The leaf and stem samples were randomly collected from each plant from 9F field at AgOps. Half of the samples were tested with ELISA for the presence of Xf . ELISA result was shown in the table 5.1. Some of the plants were found positive for Xf but all plants were not showing visible PD symptom (as of September 2008). The other half of the samples were tested with rtPCR. Due to the high sensitivity of PCR detection, contamination and human error, most of the rtPCR results were discarded from the experiment and did not continue in 2009.

Discussion

PB was evaluated for ability to colonize grapevines. We injected rootstock test Chardonnay grapevines with large amounts of PB 47V1 1AC1 from laboratory colonies. Our results showed that PB colonized mostly at the inoculation point (A) and small amounts a few centimeter above the inoculation site (B and C) . We did not find PB in non-inoculated branches of grapevines (D and E). In addition, PB formed small colonies in the xylem of 5 out of 9 inoculated grapevines. Moreover PB colonies declines 2 months post-inoculation. Since PB was originally isolated from grapevines in the PD-prone area of Temecula, CA, we could not explain our inability to duplicate the natural condition.

PB may be appropriate as a delivery vehicle for symbiotic control of PD, but it would have to do so without becoming highly mobilized in the grapevines if delivered by needle inoculation. We did not test the ability of the vector insect, *Homalodisca vitripennis*, to transmit PB between grapevines. Field trials to duplicate the apparent protection of grapevines against PD are in progress.

Figures and Tables

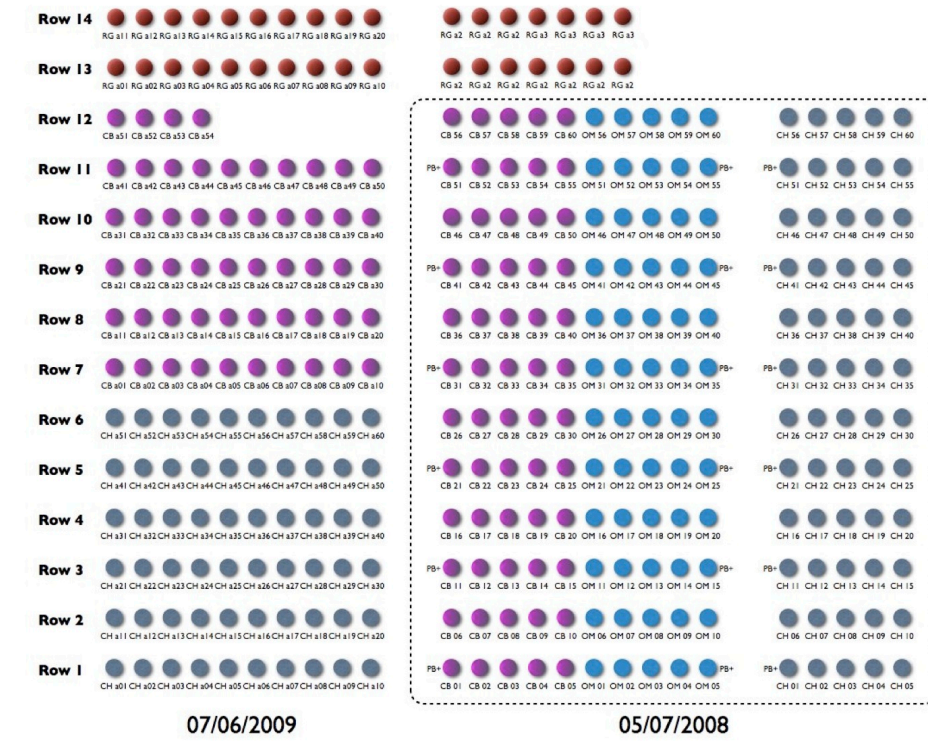


Figure 5.1: Map of 9F vineyard. 9F vineyard was at Agricultural Operations, the University of California, Riverside. Gray color, Chardonnays; blue color, Orange Muscat; purple color, Cabernet; red color, Red Globe.

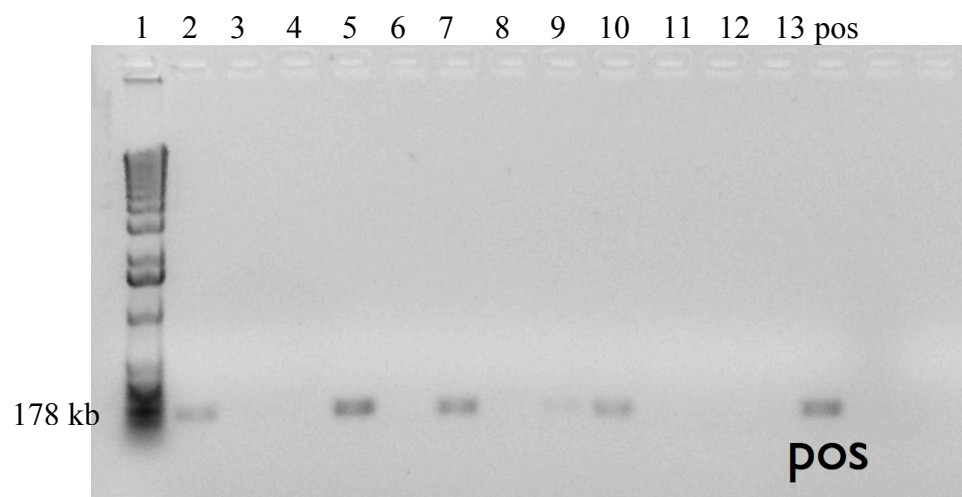


Figure 5.2: Detection of *Paenibacillus*. The reaction was performed in a Rotor-Gene 3000 machine (Corbett Robotics Inc., San Francisco, CA). Designed primers PB16S-4L and PB16S-4R were able to amplified 16S DNA of PB. Lane 1, DNA ladder; lane 2-13, grapevines were inoculated with PB. Product size was estimated 178 kb.

Varieties	PB	05/2008	08/2008	10/2008	05/2009	06/2009	07/2009	08/2009
Chardonnay	+	0	6	8	0	4	5	N/A
	-	0	6	7	0	1	6	N/A
Orange Muscat	+	0	1	6	0	0	17	N/A
	-	0	2	7	0	0	14	N/A
Cabernet	+	0	0	6	0	0	3	N/A
	-	0	0	5	0	0	3	N/A

Table 5.1: Number of infected plants. Infected plants were found positive of *X. fastidiosa* by ELISA test.

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