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Pectate lyase from *Xanthomonas axonopodis* pv. *glycines* 12-2 and Associated Pili Transporter Constitute a Key Virulence Factor

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ABSTRACT

A recent study has demonstrated that XagP in *xagP* of *Xanthomonas axonopodis* pv. *glycines* 12-2)Xag 12-2), the causal agent of soybean bacterial pustule, is required to induce a hypersensitive response (HR) in tobacco (*Nicotiana rustica*). Until now, its role in virulence on soybean was unknown and whether the general secretion pathway (GSP) is different from other bacteria. To understand the possible relationship between functions, *xagP* and T4P operon-corresponding to XagP secretion and type IV pili of Xag 12-2, respectively- were mutated using the site-directed method. XagP secretion into media associated with Δ*xagP* and ΔT4Pmutants was reduced by 100 and 24.8% respectively. Pili mutants were less reduced in the Pel secretion than by other GSPs which might be necessary for an XagP transporter. Both mutants elicited HR-like typically necrotic symptoms which were the same as the wildtype, that is, within 48 hr after infiltration on susceptible soybean cv. Spencer. On this same host plant, disease severity induced by Δ*xagP* and ΔT4P mutants was highly reduced by 82.8 and 55.9%, respectively. It is also interesting to note that the HR and disease symptoms produced by the mutants were different from the wildtype. The mutants neither expressed haloes surrounding the HR nor scattered lesions over spray-inoculated leaves but revealed pustules around the leaf margin at hydathode pores. Complemented mutants showed enhanced secretion, transportation, HR and disease severity similar to the wildtype. This is the first report of a co-regulation mechanism of *xagP* and type IV pili secretion pathway in Xag 12-2 with bacterial pustule disease in soybean. Details involved in these interactions are discussed. The minimal translocation of XagP that is less coincident with the pili transporter should be further elucidated to determine if it is more dependent on other GSPs and can suppress secretion output of other virulence factors associated with Xag 12-2 pathogenesis.

Keywords: Bacterial pustule disease, disease severity, hypersensitive response, Δ*xagP*, ΔT4P

INTRODUCTION

Xanthomonas axonopodis pv. *glycines* (Xag) causes bacterial pustule disease in soybean that results in significant yield reductions from susceptible cultivars (Prathuangwong and Khandej, 1998). Strains of Xag were found to differ in virulence on soybean as well as in their ability to induce a hypersensitive response on a range of non-host plants (Kaewnum *et al.,* 2006). A 29 kb pathogenicity island in Xag includes a type III secretion system comprised of *hrp* and *hrc* genes

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required for a non-specific hypersensitive response (HR) on certain plant species but not on others. A component of the genes was also required for full virulence on soybean (Kim *et al.,* 2003). In addition, a pectate lyase in Xag encoded by *xagP* was also found to be required for induction of a non-specific HR on several plant species (Kaewnum *et al.,* 2006); however its role in virulence on soybean was not determined. Pectate lyase (pel) isozymes cleave α-1,4-galacturonosyl linkages in plant cell wall pectic polymers, resulting in tissue maceration and cell death due to osmotic fragility (Collmer and Keen, 1986). Pels have been shown to play significant roles in pathogenesis of certain bacterial pathogens; however, the role of pectate lyase homolog (*xagP)* in pathogenesis by Xag has not been elucidated (Kaewnum *et al.,* 2006). A general secretion pathway (GSP) allows protein secretion across the bacterial outer membrane. Six pathways for extracellular secretion have been identified to date. Exoenzymes such as proteases, cellulases, pectinases, phospholipases and pectate lyase almost exclusively translocate via the type I or type III pathway across the inner and outer membrane simultaneously, bypassing the periplasmic compartment (Cornelis and Van Gijsegem, 2000). The type II pathway involves two steps in which proteins containing an N terminal signal peptide are first translocated across the cytoplasmic membrane via the *sec* machinery. Then, following removal of their signal peptides and release into the periplasm, the mature proteins cross the outer membrane in a separate step (Sandkvist, 2001). Proteins released by Gram-negative microorganisms often require a specialized export apparatus distinct from the normal secretion machinery used for membrane or periplasmic localization (Cornelis and Van Gijsegem, 2000). Bacterial pili are also coupled to export the protein subunits across the bacterial membranes and subsequent incorporation into an organelle that protrudes away from the bacterial cell (Mattick, 2002). Type I pili are involved in biofilm development and type IV pili facilitate twitching motility (Van Houdt and Michiels, 2005). The external ends of the pili adhere to a solid substrate, either the surface to which the bacteria are attached or to other bacteria. Following attachment, the pilus contracts pulling the bacteria forward. Movement produced by type IV pili is typically non-smooth and distinct from other forms of bacterial motility, such as swimming motility (Mattick, 2002). This study assessed a contribution of XagP and its secreted function for transportation and virulence on soybean plant.

MATERIALS AND METHODS

Pathogenicity test

*X.axonopodis*pv. *glycines* (Xag)12-2 kept at -80 °C was recovered on nutrient glucose agar and tested for pathogenicity on 15 d-old soybean cv. Spencer (susceptible) using previously described quantitative methods (Athinuwat *et al.,* 2009). Briefly, bacterial suspensions at cell densities of about 1×10^8 colony forming units (CFU). mL^{-1} were determined using a spectrophotometer (CE1011 1000 Series; Cecil Instruments Limited, Cambridge, UK). Disease severity was assessed using a scoring method (Prathuangwong and Khandej, 1998) 7 d after the soybean leaves were infiltrated. The results were statistically analyzed using the SAS software package (version 9.1.2; SAS Institute Inc.; Cary, NC, USA).

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown on Luria-Bertani (LB) agar or broth at 37 °C. Xag12-2 was grown on nutrient glucose agar (NGA) at 28 °C overnight (Kaewnum *et al.,* 2006). Media were amended with ampicillin (50 $\mu g.mL^{-1}$), kanamycin (50 $\mu g.mL^{-1}$), gentamicin (25 $\mu g.mL^{-1}$), and spectinomycin (100$\mu g.mL^{-1}$) for selection where required.

Cloning for mutant generation

Targeted mutations of the type IV pilli operon (PilQPONM) (T4P) and *xagP* in strain 12-2 were accomplished via gene deletion (Chuaboon *et al.*, 2012). The flanking sequences of targeted genes were amplified using primer sets derived from flanking sequences of the T4P operon and *xagP* previously identified in *X. axonopodis* pv. *citri* accession no. AY725200 and no.NC_003919, respectively (Table 2). Fragments containing the upstream and downstream regions of these target genes were ligated individually into pUC19 and transformed by electroporation into *E. coli* Top10 (Invitrogen; Carlsbad, CA, USA) as previously described by Athinuwat *et al.* (2009).

Clones were selected on LB agar amended with antibiotic markers (Table 1). The target sequences were visualized by gel electrophoresis. Confirmation of the constructs was done using polymerase chain reaction (PCR) and sequencing. The presence of antibiotic resistant marker genes was identified using PCR with primers xagP-*BamHI*-A and xagP-*SacI*-D; T4P-*EcoRI*-A and T4P-*EcoRI*-D for *xagP* and T4P mutants, respectively (Table 2).

Mutagenesis

Transformation into strain 12-2 was performed using electroporation as previously described by Athinuwat *et al.* (2009). Clones were verified for antibiotic marker fragment insertions by PCR with specific primers of *xagP* and the T4P operon (Table 1). Individual colonies of putative mutants were screened for motility, bioflim formation, virulence factors secretion (exoenzyme) and pathogenicity on soybean.

Sequencing and evaluation

The sequences of antibiotic resistant clones were identified using PCR amplification with primers xagP-*BamHI*-A and xagP-*SacI*-D; T4P-*EcoRI*-A and T4P-*EcoRI*-D corresponding to *xagP* and the T4P operon, respectively (Table 2). Analysis of sequences was conducted using

Table 1 Bacterial strains and plasmids.

Bacterial strain or plasmid	Relevant characteristic	Reference or source
Escherichia coli		
TOP10	lacZΔM15, endA1, recA1, *hsd*R, mcrA	Invitrogen (Carlsbad, CA, USA)
JM109	*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 ($r_K^-m_k^+$), e14$^-$ (*mcrA*$^-$), *sup*E44, *rel*A1, Δ(*lac-proAB*)/F' [*traD*36, *proAB*$^+$, *lac I*^{q}, *lacZ*ΔM15]	Promega (New York, NY, USA)
TOP10 (pUC19-XAGP)	*E. coli* TOP10 carrying pUC19-XAGP	This study
TOP10 (pUC19-T4P)	*E. coli* TOP10 carrying pUC19-T4P	This study
TOP10 (pBBR-XAGP)	*E. coli* TOP10 carrying pBBR-XAGP	This study
TOP10 (pBBR-T4P)	*E. coli* TOP10 carrying pBBR-T4P	This study
X. axonopodis pv. *glycines*		
Strain 12-2	Virulence strain, race 3	Thowthampitak *et al.* (2008)
12-2 xagP:: Gen^r	*xagP*:: Gm^r of 12-2 by gene deletion	This study
12-2 T4P::Km^r	T4P::Spec^r of 12-2 by gene deletion	This study
xagP:: Gm^r (pPZP201-XAGP)	*xagP*:: Gm^r of 12-2 carrying pPZP201-FLIC	This study
T4P::Km^r (pBBR-T4P	T4P::Km^r of 12-2 carrying pBBR-T4P ::Specr	This study
Plasmid		
pUC19	*E. Coli*; lacZ, Ampr	Promega
pBBR1MCS-5	Broad host range cloning vector, lacZ and Gm^r	Kovach *et al.* (1995)
pPZP201-XAGP	1.4 kb sequence of *xagP* in the pPZP201, Spec^r	This study
pBBR-T4P	4.5-kb sequence of T4P operon in the pBBR1MCS-5, Km^r and Gm^r	This study

Km^r = Kanamycin resistance, Am^r = Ampicillin resistance, Gm^r = Gentamicin resistance, Spec^r = Spectinomycin resistance.

the Basic Local Alignment Search Tool (BLAST) and the Lasergene software package (DNASTAR Inc.; Madison, WI, USA). The MegAlign program of the Lasergene package was used for sequence alignment using CLUSTALW (Athinuwat *et al.*, 2009). MegAlignment was then calculated for the *xagP* and T4P operon sequences as available from GenBank (Athinuwat *et al.*, 2009) for selected *Xanthomonas* spp.

Complementation

PCR-amplified *xagP* (1.4 kb) and type IV pili operon (4.5 kb) in pUC19 plasmid were digested with *KpnI* and *SacI*; *EcoRI* and *Sac I*, respectively, and ligated into the multiple cloning site vector pPZP201 (pPZP-XAGP) and pBBR1MCS-5 (pBBR-T4P). They were individually mobilized into *xagP* and T4P mutants, respectively, using electroporation (Athinuwat *et al.*, 2009). Selection was made on nutrient yeast extract agar media amended with antibiotic markers (Table 1). They were verified by PCR and tested for motility, bioflim formation, virulence factors secretion (exoenzyme) and pathogenicity on soybean.

Functional characterization of mutants

Growth curve

Bacterial strains were grown overnight in NGA and then were inoculated into nutrient glucose broth (NGB) to a final optical density at 600 nm of 0.1. Aliquots of 150 µL were used to fill the different wells of a 96-well polystyrene plate and incubated at 28 °C for different periods (0, 1, 3, 6, 12, 24, 48, 60, 72, 84, 96, and 108 hrs). To confirm similar bacteria growth, optical density at 600 nm was measure using spectrophotometry.

Pectate lyase assay

The strains were also assayed for production of pectate lyase using an activity stain overlay technique (Reid and Collmer, 1985). One percent agarose plus 0.1% polygalacturonic acid that was dissolved by boiling in either 50 mM potassium acetate (pH 4.5), 100 mM potassium phosphate (pH 6.5), or 50 mMTris hydrochloride (pH 8.5) were poured into Petri dishes. Bacterial suspensions were in wells on the surfaces of the gels, incubated overnight and then washed from the gels with a stream of water. The surface of the gels was flooded with 0.05% (weight per volume, w/v) ruthenium red for 20 min and rinsed in water. Pectolytic activity was observed as clear zones at points where the bacteria were dropped.

Motility analysis

All mutants and complemented strains were tested for their motility compared to strain 12-2. The motility medium assay was modified from Das *et al.* (2002). The motility medium agar plates were stab-inoculated with bacteria from overnight cultures and incubated at 28 °C for 24 hr. Motility was observed as bacterial cells moving out from the bacterial stab. Strains were tested at least three times.

Biofilm formation

All bacterial strains were grown in yeast minimal medium broth with 6.7 g yeast nitrogen

Table 2 Polymerase chain reaction primers.

Primer	Sequence	Description
T4P-*EcoRI*-A	5′ TTCGAATTCAGCGGTAGTGGTCCT TGTT 3′	Amplification of 484-bp of
T4P-*AscI*-B	5′ TTGGCGCGCCGGCCGACGTGGCAGTTA 3′	upstream T4P
T4P-*AscI*-C	5′ TTGGCGCGCCATGCCCCGTATTCGCCG 3′	Amplification of 475-bp of
T4P-*EcoRI*-D	5′ TTGAATTCGCTCACTTTCAATCTTGCGC 3′	downstreamT4P
xagP-*BamHI*-A	5′ ATGGATCCCAGCCTTGGACGGATTGTTGA 3′	Amplification of 500-bp of
xagP- *HinDIII*-B	5′ TAA AGCTTGATAGGCCTTACTTGTATCGGC 3′	upstream *xagP*
xagP-*BamHI*-C	5′ ATGGATCCCAACGCTTACGTATTTCGTC 3′	Amplification of 500-bp of
xagP-*SacI*-D	5′ TAGAGCTCGTCAACAATCCGTCCAAGGC 3′	downstream *xagP*

base without amino acids and 20 g dextrose in 1 L distilled water. Samples (150 mL) of the bacterial suspensions were placed in the wells of a 96-well polyvinyl chloride plate. After incubation at 28 °C for 4 d, surface-attached cells were stained with 1% w/v Crystal violet. The attached dye was solubilized with 70% ethanol and quantified by absorbance at 570 nm (O'Toole and Kolter, 1998).

Extracellular enzyme secretion assay

All the bacterial strains were tested for secretion of alpha-amylase, cellulase, endoglucanase, pectate lyases and for protease activity.

Alpha-amylase activity

The assay was modified from Rick and Stegbauer (1974). Bacterial cultures were centrifuged at 8,000 revolutions per minute (rpm) for 5 min and supernatant with the secreted enzyme (0.5 ml) was transferred to 0.5 mL of 1.0% soluble starch solution in a test tube. The mixture was incubated at 60 °C for 10 min and then 1.0 mL of 3,5-Dinitrosalicylic acid (DNS) was added to each tube. The test tubes were placed in boiling water for 5 min and cooled at room temperature. Then, each solution was diluted by adding 10 mL distilled water. The absorbance was determined at 546 nm using the spectrophotometer.

Cellulase activity

The assay was performed according to Ghose (1987). Briefly, bacterial cultures were centrifuged at 8,000 rpm for 5 min to collect the supernatant with the secreted enzyme. A sample (0.5 mL) of supernatant was added to 1 mL of 50 mM citric acid buffer (pH 4.8) and incubated at 50 °C for 60 min with the filter paper substrate (Whatman No. 1 filter paper 1 cm × 6 cm). Then 3 mL of dinitrosalicylic acid reagent (1% 3,5-dinitrosalycilic acid (Sigma; St. Louis, MO, USA.), 1.2% NaOH, 0.05% sodium sulfate and 20% potassium sodium tartrate tetrahydrorate) were added to the enzyme solution to stop the reaction and the mixture was boiled for 5 min. The reaction mixture was transferred to an ice-cold water bath and subsequently the cellulose activity was measured as absorbance at 540 nm with the spectrophotometer.

Endoglucanase activity

The endoglucanase activity assay was modified from Bernfeld (1955). Bacterial culture was centrifuged at 8,000 rpm for 5 min to collect the supernatant with the secreted enzyme; 0.5 mL was transferred to 0.5 mL carboxymethyl cellulose (1% volume per volume) in 100 mm Borax-NaOH buffer, pH 11 and incubated at 50 °C for 30 min. The reaction was stopped by the addition of 2 mL of 3, 5-dinitrosalicylic acid reagent. The absorbance was determined at 546 nm using the spectrophotometer.

Pectate lyases activity

Pectate lyase activity was assessed as described by Dow *et al.* (1987). Briefly, the mutant strains were grown overnight in minimal medium (Daniels *et al.*, 1984) supplemented with casamino acids (1.5 $g.L^{-1}$) and 0.25% (w/v) polygalacturonic acid, and cells were harvested by centrifugation. Cells were suspended in a solution containing 0.05M Tris-HCl buffer, pH 8.5, 1 mM $CaCl_2$, and 0.25% (w/w) polygalacturonic acid, and absorbance at 235 nm was assessed hourly (Nasuno and Starr, 1967).

Protease activity

Protease activity was determined using the method of Satake *et al.* (1963). Briefly, 0.1 g casein was added into 10 mL 200 mMMes buffer, pH 7.0. The solution was incubated at 60 °C for 30 min, centrifuged at 10,000 rpm for 15 min to collect the supernatant used as the protease substrate. A 20 µL trypsin solution was used as the positive control by mixing with the 180 µL casein solution mentioned above and incubated at 37 °C for 15 min. Subsequently, 400 µL of 5% trichloroacetic acid (TCA) was added to end the enzymatic reaction. The reaction was mixture and centrifugation at 10,000 rpm for 15 min. Protease activity was read at 280 nm using an ultraviolet spectrophotometer (Keay and Wildi, 1970).

Plant pathogenicity assay

The virulence of wild type12-2, mutants and complemented mutants were assessed on soybean cultivars (cv. Spencer) using spray inoculation techniques (Kaewnum *et al.,* 2006). Briefly, aqueous cell suspensions densities of about 1×10^8 CFU.mL^{-1} were sprayed on the leaves of plants maintained in the greenhouse. At 7-10 d after inoculation, disease severity was assessed using a scoring method (Prathuangwong and Khandej, 1998) based on the number of sections having at least one pustule observed in nine 1 cm^2 diameter sections per leaf. Three trifoliate leaves, collected from the top, middle and basal portion of three plants, were evaluated for each strain. The results were statistically analyzed using the SAS software package.

Plant HR assay

The virulence of the wild type 12-2, mutants and complemented mutants were assessed on soybean cultivars (cv. Spencer) by infiltration inoculation techniques (Athinuwat *et al.,* 2009). Briefly, a leaf infiltration technique under greenhouse conditions was used to assess the virulence on soybean leaves. Infiltrations were done using a completely randomized design. Bacterial suspensions (50 µL) at cell densities of about 1×10^8 CFU.ml^{-1} were infiltrated into the leaf mesophyll through a pin puncture using a 1 mL syringe without a needle. Nine leaves on different plants were inoculated for each strain. Infiltration assays were monitored for their ability to cause HR (necrosis) or disease (spreading infection with yellow halo) at 48 hr post-infiltration. The ability of all bacterial strains to induce an HR was assayed on a nonhost (tobacco, *N. rustica*) as previously described (Kaewnum *et al.,* 2006). The experiments were repeated three times.

RESULTS AND DISCUSSION

Pathogenicity test

Symptoms caused by Xag strain 12-2 on soybean were small, yellow green spots with reddish-brown centers on the upper leaf surface at 5-7 d after inoculation (Athinuwat *et al.,*2009) (Figure 1). One aim of this research was to describe the ability to cause disease and to describe the level of disease. It was determined that strain 12-2 has the ability to cause pustule disease.

Functional characterization of mutant

Growth curve

The effect of *xagP* and *T4P* mutants on bacterial growth in culture was determined by incubating bacteria in NGB medium. The study revealed that the populations of mutants and the wild type were not different when observed in NGB (Figure 2). This result showed that the *xagP* and *T4P* genes are not involved with the primary metabolism of bacterial cells.

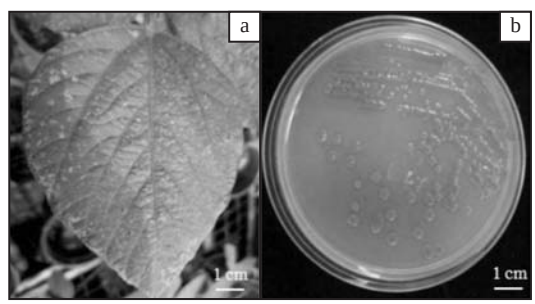

Figure 1 *Xanthomonas axonopodis* pv. *glycines* 12- 2: (a) Bacterial pustule symptoms; (b) Colony characteristics on nutrient glucose agar.

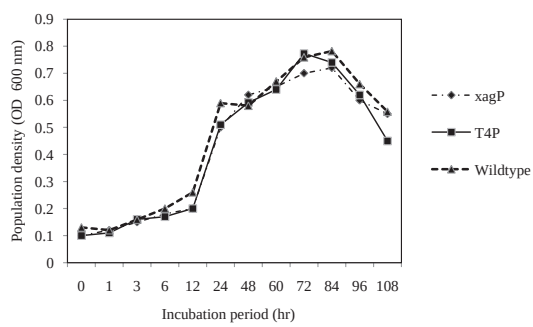

Figure 2 Growth rates of *xagP* and *T4P* mutants compared with wildtype12-2 in nutrient glucose broth medium.

Pectate lyase assay

The *xagP* mutant completely lost ability to produce pectate lyases whereas the T4P mutant expressed a reduction in pectate lyase secretion. Therefore T4P pili may be associated with secretion of pectate enzymes (Figure 3).

Motility analysis

The T4P mutant lost twitching ability when compared with the wildtype strain (12-2). Twitching is visualized as a thin, radial twitching zone approximately 1.5 cm in diameter around the point of inoculation after 24 hr of incubation (Figure 4). If the average Xag 12-2 cell is approximately 2 μM in length, this translates to around 312 cell lengths per hour, although the actual distance traveled is likely to be considerably farther, as the bacteria do not travel in a straight line (Burrows, 2012). The results present the direct observation of twitching motility by a plant-pathogenic bacterium. This phenomenon may very well explain how Xag 12-2 is able to spread within plant tissues. These findings on twitching-mediated motility present an observed phenomenon for cell movement on the soft-medium test.

Biofilm formation

Initiation of disease may depend on the attachment of bacteria to host cells and the subsequent development of biofilms on leaf surfaces. To examine the roles of type IV pili in biofilm formation, the ability of 12-2 and mutants to develop biofilms was assessed in 96-well polystyrene plates. When 12-2 was cultured in YMM it formed visible biofilms in wells after continuous agitation. In contrast, ΔT4P and Δ*xagP* exhibited significantly ($P < 0.05$) reduced biofilm formation (Figure 5).

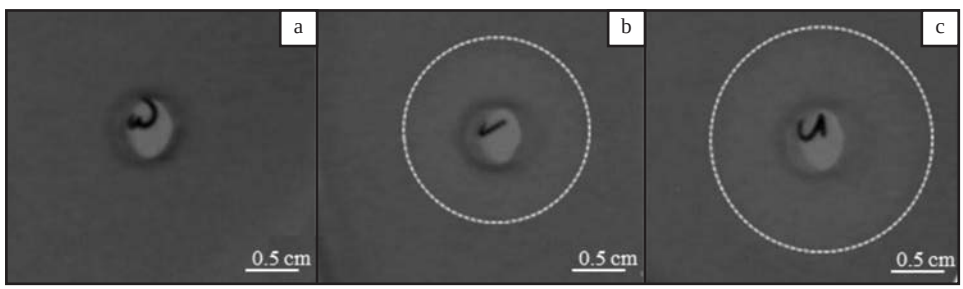

Figure 3 Pectate lyase activity (clear zone width in white dotted circle) of mutants of *X. axonopodis* pv. *glycines*12-2: (a) Δ*xagP*; (b) ΔT4P; (c) Wildtype 12-2. Filtrates from bacterial cultures were placed in wells in assay media to assess enzyme activity.

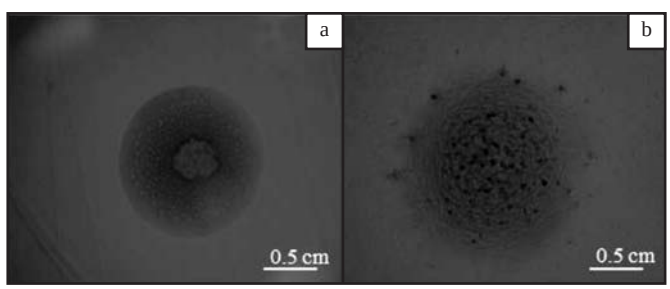

Figure 4 Twitching motility of T4P mutant of *X. axonopodis* pv. g*lycines*: (a) Compared with wildtype strain 12-2; (b) Stab-inoculated on motility medium and grown for 24 h at 28 °C. To analyze the borders of the migration zones, the plates were observed under compound microscope (10× magnification).

These results indicate that specific T4P and *xagP* genes play significant roles in biofilm formation. The type IV pili appear to be typically associated with the ability of bacteria to colonize surfaces and in most cases these bacteria are intercellular. Xag 12-2 is also known to form biofilms (Chatnaparat *et al.,* 2011), and mutations in the T4P operon and *xagP* gene affect biofilm formation (Figure 4). Thus, the defect in biofilm formation in the mutants could be due to a weakened outer membrane (Sandkvist *et al.,* 1997). Genes affecting twitching motility have been shown to be important for infection by *P. aeruginosa* (Argenio *et al.,* 2001), as well as for biofilm formation which appears to be involved in disease infections (Whitchurch *et al.,* 2002).

Extracellular enzyme secretion assay

Strain 12-2 and type IV pili operon deficient mutants were compared for expression of different exoenzymes. The *xagP* and T4P mutant strains exhibited reduced production of extracellular enzyme compared to strain 12-2 (Figure 6).

The results show that the *xagP* and T4P mutant strains were significantly reduced in pectate lyase production and secretion by 100 and 24.8%, respectively, when compared to Xag 12-2. The results suggest that the T4P mutant produces the enzyme but is impaired in secretion possibly due to lack of pili. Enzyme secretion was related with disease severity; the Δ*xagP* and ΔT4P showed reduced disease severity of 82.8 and 55.9%, respectively. The results suggest that type IV pili operon is associated with secretion of extracellular enzymes especially pectate lyase. This is the first report where the pili export apparatus has been shown to function also as a secretion system for the transport of pectate lyase in the pathogenic bacteria. Previous reporting of the expression of most proteins (proteases, cellulases, pectinases, phospholipases, lipases and toxins) secreted via the type II pathway has been growth-phase dependent or strictly regulated by environmental signals (Sandkvist, 2001). The current studies indicate the pectate lyase is a significant virulence factor of Xag 12-2, and export by the pili export apparatus influences bacterial-host interactions and disease development of Xag 12-2.

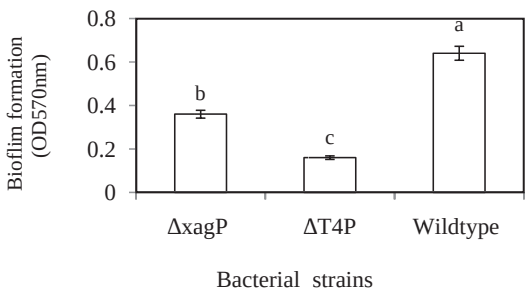

Figure 5 Bioflim formation of *X. axonopodis* pv. *glycines* strain 12-2 and mutant strains was cultured in yeast minimal medium. Error bars show ±5% percentage value.

Plant hypersensitive response and pathogenicity assay

The *xagP* and T4P mutants expressed reduced disease ratings of 82.8 and 55.9% disease reduction, respectively, at 10 d after inoculation on soybean (cv. Spencer) compared to the wildtype (Figure 7). Moreover the *xagP* and T4P mutants developed an HR-like necrosis on soybean within 48 hr after infiltration. The HR was visualized as a localized necrosis in the leaf infiltration zone whereas disease was expressed as necrosis with a surrounding yellow halo. Characteristic pustules developed beyond the infiltration zone 10 d after inoculation (not shown). In contrast, the wildtype and all complemented mutants showed typical progressive symptoms that included a yellow halo around the inoculated area within 48 hr after inoculation (Figure 8). On tobacco, ΔT4P caused an HR within 48 hr following infiltration and Δ*xagP* did not cause an HR (Figure 9). These results indicate a role for type IV pili in the secretion of pectate lyase encoded by *xagP* in Xag 12-2. The type IV pili operons are essential structures in the pathogenic potential of bacteria as they provide motility, colonization or increasing adhesion and secretion of the virulence factors or

virulence factor translocation such as exoenzyme for the development of soybean bacterial pustule disease.

All mutants altered the production of extracellular enzymes and significantly ($P < 0.05$) reduced disease reduction on soybean (cv. Spencer) 10 d after inoculation. Barras *et al*. (1994) reported pectolytic and cellulolytic enzymes are predominantly secreted via the type II pathway. Pectolytic and cellulolytic enzymes degrade components of the plant cell wall are main virulence factors (Cornelis and Gijsegem, 2000). Endoglucanases (carboxymethyl cellulases) that hydrolyze cellulose are abundantly produced by *Xanthomonas* spp (Barras *et al.*, 1994). Pectic polymers of the primary plant cell wall and middle lamella are cleaved at α-1,4-polygalaturonyl linkages by hydrolysis (polygalacturonases) or β-elimination (pectin and pectate lyases), whereas pectin methylesturonases remove methoxy groups on galacturonate residues of the chain (Boch and Bonas, 2001). For some bacteria the primary virulence characteristic is the coordinated production of large amounts of multiple, secreted, plant-cell-wall-degrading enzymes, which leads to the breakdown of plant tissue (Toth *et al.*, 2003). Some well-studied pectinases include those of *Erwinia carotovora* subsp. *carotovora*-primarily multiple isoforms of pectate lyase (Pel), together with isoforms of polygalacturonase, pectin

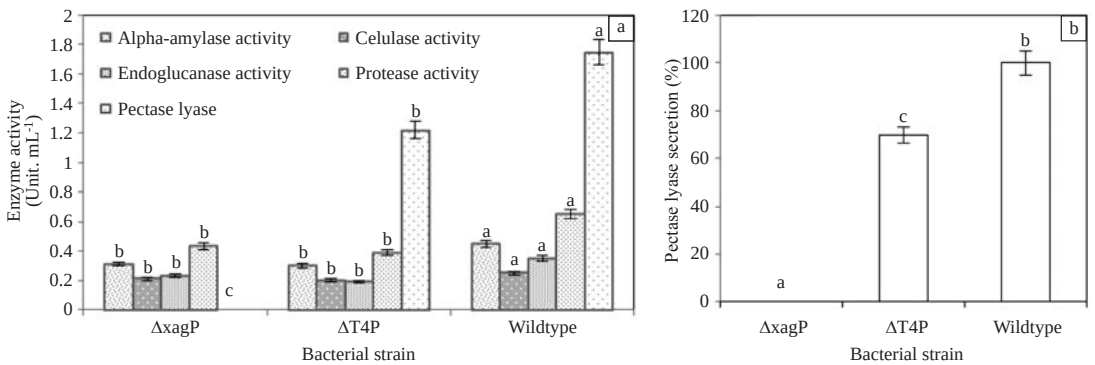

Figure 6 Type IV pili operon (T4P) and *xagP* mutants exhibited: (a) Reduced production of alpha-amylase, cellulose, endoglucanase, protease, and pectate lyase activities compared to wildtype strain 12-2; (b) Pectate lyase secretion compared to wildtype strain. Error bars show ±5% percentage value.

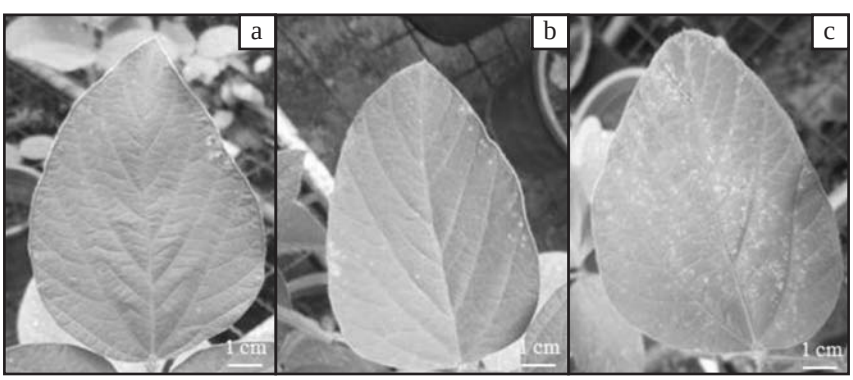

Figure 7 Disease severity on soybean leaves (cv. Spencer) sprayed with mutant strains 10 days after inoculation: (a) Δ*xagP*; (b) ΔT4P; (c) Wildtype.

methylesterase, and pectin lyase (Thomson *et al.*, 1999). The current study found that the *xagP* mutant resulted in lost pectate lyase production when compared with the wildtype 12-2. Although Pels have been shown to play significant roles in the pathogenesis of certain bacterial pathogens (Collmer and Keen, 1986), the *xagP* in Xag was found to be required for HR induction on several plant species (Kaewnum *et al.*, 2006). With ΔT4P, the current study found that the mutant strain still had pectolytic activity but there was reduced possibility because of reduced secretion due to the lack of pili. In addition, the mutant strain was greatly impaired in disease development.

The mutants did not express haloes of HR nor scattered lesions over spray-inoculated leaves as an indication of disease development, but they did cause pustule formation around the leaf margin at hydathode pores. It is proposed that Xag can directly enter leaves through hydrathodes, whereas the T4P mutant does not cause disease at stomata as it can invade leaves directly at hydrathodes and cause disease. However bacteria cannot directly penetrate the leaf epidermis and must enter leaf tissues through natural openings or wounds on the leaf surface. Stomata dominate the natural openings on the aerial part of the plant. Other natural openings include hydathodes in leaf margins, nectarthodes in flowers, and lenticels in stems and roots (Huang, 1986). However, recent evidence suggests that entry of bacteria into leaf tissue is more complex and dynamic than the simple act of swimming into the leaf through passive openings (Melotto *et al.*, 2006).

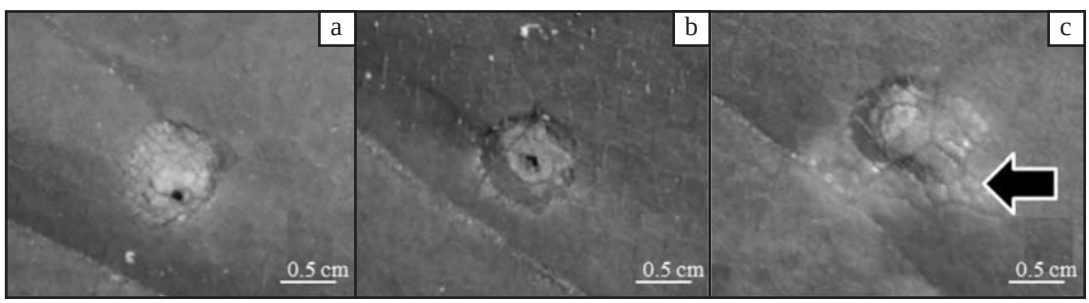

Figure 8 Hypersensitive response induced on soybean (cv. Spencer) leaves infiltrated with mutant strains *of X. axonopodis* pv. *glycines*: (a) Δ*xagP*; (b) ΔT4P; (c) Wildtype 12-2, showing typical progressive symptoms that include a yellow halo around the inoculation point (black arrow) within 48 hr after inoculation.

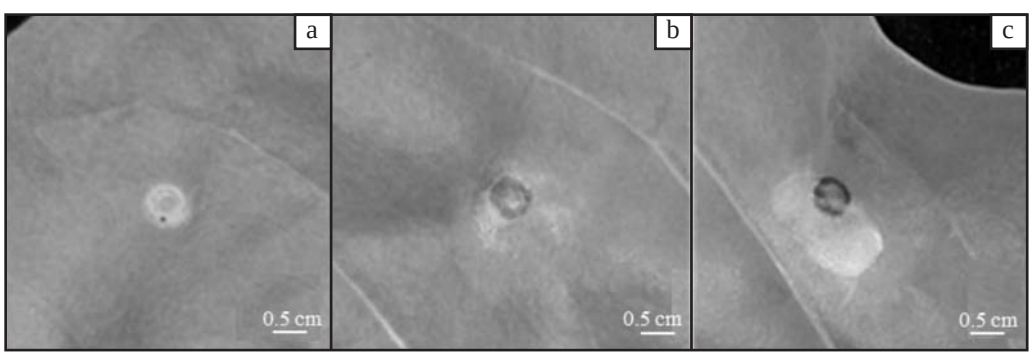

Figure 9 Hypersensitive response on tobacco (*N. rustica*) leaves showing HR development 48 hrs after infiltration with mutant strains of *X. axonopodis* pv. *glycines*: (a) Δ*xagP*; (b) ΔT4P; (c) Wildtype 12-2.

CONCLUSION

This work reports a multifaceted analysis of the pathogenicity of Xag-secreted XagP (or Pel) transported coincident with type IV pili. The results demonstrated that while XagP is required for full virulence on soybean plants, type IV pili also play a role in the secretion of enzymes such as pectate lyase. This study has also reported an unusual symptom induced by x*agP* and T4P mutants with the lack of haloes and the produced lesion pattern around leaf margins. In addition to affecting pathogenicity, the *xagP* and T4P mutants also were defective in secretion/production of alpha-amylase, cellulase, endoglucanase, pectate lyases and protease. Future experiments are in progress to determine if other organelles such as flagella also affect enzyme secretion.

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