Evidence that *ppsA* in *Xanthomonas axonopodis* pv. *glycines* Affects Carbon Utilization and Secretion of Virulence Factors

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Abstract

Mutans of *Xanthomonas axonopodis* pv. *glycines* that were defective in pathogenesis were produced by Tn5 mutagenesis. One mutant KUMNTP2 that was affected in virulence factor secretion, was reduced in disease production on soybean cultivar SJ4. Sequence analysis showed the Tn5 inserted into a phosphoenal pyruvatesyntase (*ppsA*) gene having a highly level of homology to the gene in *X. axonopodis* pv. *citri*. KUMNTP2 was unable to grow on minimal media without sugar or medium amended with NaAC. KUMNTP2 showed delayed growth in soybean plants that could be complemented by adding 10% glucose. Furthermore, the production of extracellular polysaccharide and extracellular cellulose was significantly decreased but not amylase or protease. By complementation it was shown that enhanced cellulose secretion requires induction of *ppsA* in a feedback mechanism that modulates *ppsA* expression. The results also indicate that secretion of cellulose may be dependent on *ppsA*. We further demonstrated that KUMNTP2 failed to induce a hypersensitive response on tobacco but still induced the response on tomato. Taken together, our date suggest that *ppsA* effects either directly or indirectly the secretion of virulence factors that affect the development of pustule disease.

Keywords: bacterial pustule disease, transposon mutagenesis, exoenzyme secretion, EPS production, pathogenesis reduction

Introduction

Bacterial pustule caused by *X. axonopodis* pv. *glycines* (Xag) is an important disease of soybean grown in tropical countries including Thailand (Vauterin, et al.,1995). The favorable conditions for disease development are warm and high humidity (Prathuangwong et. al., 1990; Prathuangwong et. al., 1996). The ability of plant pathogenic bacteria to cause disease in particular host plant depends on several factors including optimum condition of environmental factors, physiological state of plant and the expression of pathogenicity and virulence factors by the bacterial cells (Sigee, 1993). The interaction between plant and pathogen is a dynamic process that involves the exchange of signals. An important subset of pathogenicity genes performs regulatory functions that are likely required for the bacteria to adapt to the environmental and physiological changes encountered during infection. Plant-pathogen interaction including HR and disease induction are often affected by bacterial Type III-dependent effector proteins that include harpin, avirulence protein (Leach and White, 1996) and pectate lyase (Kaewnum et al., 2006). The expression of pathogenicity or pathogenicity related genes is regulated by environmental factors, plant signals (carbon sources, organic nitrogen, and phosphate), and by catabolite repression (Rahme et al., 1992; Schulte and Bonus, 1992). The products of pathogenicity- or virulent related factors may function as signal molecule indicating that the environment is suitable for pathogen growth and initial pathogenesis (Sigee, 1993). Phytopathogenic bacteria utilize several strategies to access the nutritional in the intracellular spaces of plant tissues. They enter their hosts through natural openings or wounds on the plant surface (James and Collmer, 1996), where the environment is less harsh and rich in nutrients that include glucose, fructose, xylose, sucrose, and galactose (Padmanbhan et al., 1974). Higher plants contain potentially a lot of nutrients sources for many bacterial species within the cells. Most bacteria are small enough to enter from stomata and other natural openings into the apoplast as known. Surprisingly, few bacteria raid the nutrient stores of living plant cells, apparently because the metabolic involved in parasitism (James and Collmer 1996). Most phyto-pathogenic bacteria are necrotrophic and thus have requirements to degrade for their metabolic use a certain amount of plant tissues. It is the apoplastic colonizers that are the common pathogens that produce the rots, spots, wilts, cankers, and blights afflicting virtually all crop plants, and their relationship with the host is defined by two features. HR (hypersensitivity response) is a form of programmed cell death and is commonly considered as an efficient defense mechanism developed by plant to restrict pathogen growth to the immediate vicinity of the infected region (Sigee, 1993). HR could be triggered by entire microorganisms or by microbe associated products, referred to as elicitors. harpin is a protein encoded by hypersensitive reaction and pathogenicity genes (*hrp*) that secreted by most plant pathogenic bacteria via their type III secretion system into the plant intercellular space (Wilis, *et al.,* 1991) caused disease (compatible interaction) and HR (incompatible interaction). In the terms of pathogenicity of bacterial pathogen, the ability to acquired nutrients from the host is essential for a pathogen to establish and infection. Bacteria occupy a wide variety of habitats and perform many ecological functions including nutrient or carbon utilization. Bacteria may use the gluconeogenesis pathway to synthesize glucose from non sugar C2 or C3 compounds or the inter-mediates of the tricarboxylic acid (TCA) cycle (Inui et al., 1999; Osteras et al., 1997; Osteras et al., 1995). There are many reports shown that gluconeogenesis is required for virulence of a number of animal pathogens such as *Salmonella enterica* serovar Typhimurium (Allen et al**.,** 2000), *Mycobacterium bovis* (Collins et al., 2003), *M. tuberculosis* (McKinney et al., 2000), and *Candida albicans* (Lorenz and Fink, 2001), but there is a few information involved in the pathogenicity of phytopathogenic bacteria especially in Xag.

In this study, we isolated pathogenicity-deficient mutants using transposon insertion and marker exchange technique, and demonstrated that one of the mutants, in which a phosphoenal pyruvate synthase (*ppsA*) genes was disrupted, could not grow in synthetic (M9) medium supplemented with non-sugar substance and showed no deficiency in cellulase secretion and EPS production.

Materials and Methods

Bacterial Strains, Plasmid, Media and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Strains of *X. axonopodis* pv. *glycines* (Xag) were grown routine on nutrient yeast extract agar (NYA) at room temperature (28-30°C). *Escherichia coli* were grown on Luria Bertani (LB) agar or broth and incubated at 37°C. Media were amended with appropriate antibiotics at the following concentrations: kanamycin 50 µg mL^{-1}, ampicillin 100 µg mL^{-1}, and tetracycline 25 µg mL^{-1} for selected Xag mutant, *E. coli* carrying plasmid vector, and complementation strains respectively.

Transposon Mutagenesis

The suicide plasmid carrying the transposon Tn5 fragment, pJB4JI was introduced into Xag strain No.12-2 chromosome by triparental conjugation mating. The donor (*E. coli* with pJB4JI) and helper (*E. coli* HB101 with pRK2013) strains cultured in LB containing kanamycin and incubated overnight at 37°C at logarithm phase were mixed with Xag with a ratio 1:1:1. Mixed cell suspension was spreaded on yeast-extracted peptone agar (YPA) supplemented with 50 µg mL^{-1} kanamycin and incubated at room temperature for 1-3 days.

Table 1 Bacterial strains and plasmids used in this experiment.

Strain or plasmid	Relevant characteristic[1/]	Reference source[2/]
Escherichia coli DH 5α	F^- ϕ 80d*lacZ*ΔMΔ15 (*lacZYA-argF*) *U169 endA1 deoR recA hsdR17* ($r_k^- m_k^+$) *phoA supE44 λ- thi-l gyrA96 relAl*	Invitrogen
X. axonopodis pv. *Glycines* Strain No.12-2	Laboratory wild type, spontaneous rifampicin resistance	KU/This study
X. axonopodis pv. *Glycines* mutant strain (KUMNTP2)	Pathogenicity deficient: Tn5-Km^r	KU/This study
Plasmids		
pJB4JI	$mob,^+$ Mu,Tn5	Shizuoka University
pUC18	Amp ^{r} cloning vector	Takara company
pRK2013	Helper vector: Km^r	Shizuoka University
pLAFR3	Derivative of pLAFR1, 23 kb, polylinker from pUC8, $lacZa^+$, Tc^r	Shizuoka University
pBluscript II S/K (-)	High copy number cloning vector: Amp^r	Stratagene

[1/] Amp^r = ampicillin resistance; Km^r = kanamycin resistance, Tc^r = tetracycline resistance, Gm^r=gentamycin resistance, Cm^r= Chloramphinical resistance.
[2/] KU = Kasetsart University.

Colonies were restreaked several times onto the same media before tests for pathogenicity and for hypersensitive response (HR) assays. The clones deficient in developing in pathogenicity and/or HR were selected for further study.

Analysis of Tn5 Insertion, Gene Cloning and Sequencing

Total genomic DNA of mutant strain was isolated as standard protocol (Sambrook et al., 2001). The transposon inserted fragment in mutant chromosome was detected by PCR and southern hybridization (Sambrook et al., 2001). The primers were designed from the sequence of the transposon fragment of Tn5 F1 5' TGA GCT GTA ACA GCC TGA CCG C3' and Tn 5 R1 5' CAC CAC ATT CCG CAC CGT AG 3' were used. Chromosomal DNA of mutant strain containing Tn5 fragment was digested with *Sac*I and cloned into pBluescript SK(+). The flanking region of Tn5 insertions was sequenced with primer specific (M13F and M13R) by capillary automatic sequencer (Beckman). Computer base homology search was performed with BLAST, and EMBL program for identify the genes concerned. The target gene was subcloned from Xag wild type strain No.12-2 into plasmid vector pLAFR3 and introduced into mutant strain (Sambrook et al., 2001) to be used for complement-tation test.

Plant Materials and Plant Inoculation

Susceptible soybean cultivar (SJ4) was grown in a greenhouse. For disease induction assays, the puncture wounds were introduced per 1-cm^2 area of soybean cotyledon with a standard 12-gauge needle. Bacterial suspension prepared from overnight culture of each strain in YP broth, with potassium phosphate buffer (10 mM, pH 7) at a density of 0.2 OD. ($A_{600\ nm}$) which is correspond to 10^8 cfu mL^{-1} were applied in 10-ml droplets directly onto the puncture wound sites on each cotyledon (Rukayadi et al., 2000). The non-pathogenicity mutants were observed and identified by absence of chlorotic symptoms around the inoculation site within 2 to 3 days after inoculation.

Relative pathogenicity of leaf bioassay was assayed as method described by Prathuangwong and Khandej (1998). The bacterial inocula were sprayed onto the foliage of 30-day old soybean plant. Wild type strain of Xag and potassium phosphate buffer were served as a positve and negative control in each trial, respectively. The inoculated plants were observed at 7 days after inoculation and evaluated for the severity of disease symptom as reported by Prathuangwong et al. (1993). Severity was calculated based on the numbers of pustules observed in nine 1 cm^2 diameter sections per leaf. For each isolate at least three leaves, collected from the middle and basal portion of four plants, were evaluated. The results were statistically analysed using spss version 11 (SPSS Inc.).

Bacterial Growth and Cell Density on Disease Induction

To investigate the growth of Xag strains under the restricted carbon concentration, all Xag wild type, mutant, and complementation strains cultured at log phase were prepared as cell suspension at 1.0 OD ($A_{600\ nm}$). The 1.5 mL of each bacterial suspension was added into 200 mL of minimal medium (M9) supplemented with 0.5% glucose (sugar), 0.25 % sodium acetate (NaAC: non-sugar) and only M9 withut carbon source served as control, incubated at room temperature on incubator shaker at 200 rpm. Population density of each bacterial strain was measured at 0, 3, 6, 9, 12, 18, 24, 36, 48, 60, 72, 84, 96, and 120 h post incubation with spectro-photometer ($A_{600\ nm}$).

The 30-day old soybean plant (cv. SJ4) grown under greenhouse conditions was tested for In *planta* bacterial growth by tissue infiltration methods (Tom et al., 1995). The ten µL of each bacterial cell suspension at 10^8 cfu mL^{-1} was infiltrated into mesophyll of soybean leaves using a 1 mL hypodermic syringe. Growth of wild type, mutant, and complementation strains was monitored after infiltration. Leaf discs of 5 mm diameter tissue samples were cut with a cork borer from the center of the infiltrated zone at 0, 6, 12, 24, 36, 48, 72 and 96 h post infiltration to determine total populations. Samples were macerated and placed individually in 1 mL of sterile distilled water, triturated, and isolated by plating for individual colonies on YP medium containing the appropriate antibiotics including 50 µg mL^{-1} rifampicin, 50 µg mL^{-1} kanamycin, and 50 µg mL^{-1} of tetracycline for wild type, mutant, and complementation strains respectively. All 3 strains of No.12-2, KUMNTP2, and the complementation were tested for their ability with varied cell density to induce disease symptom on leave of most susceptible SJ4 cultivar by same infiltration method. Each bacterial culture were prepared for cell density at 10^8, 10^9, and 10^{11} cfu mL and individually infiltrated into mesophyll of 10-day soybean leaves. Initial symptom as necrotic lesions was observed on infiltrated leaves at 0, 12, 24 36, 48, 72, 96 and 120 h post infiltration. The lesion sizes were also measured as the judgement of their virulence.

Co-Inoculation of Xag and Glucose

In order to confirm whether glucose allowed growth of Xag and affected disease induction in host plans, cell suspension of each bacterial strain was adjusted by sterilized distilled water at 10^8 cfu mL^{-1}. Bacterial suspension was induced by added with 10% (v/v) glucose and their mixture was inoculated onto soybean plants with two methods of infiltration and foliar spray as the methods described above. The population density of each bacterial strain was evaluated at interval of 0, 6, 12, 24, 48, 72, and 96 h after inoculation with infiltrated assay with dilution plate count method. The development of disease symptom in which infiltration test was detected at 48 to 96 h post infiltration as previously described. The disease severity along with foliar spray inoculation was evaluated after inoculation at 4-10 days with the methods described by (Prathuangwong and Khandej, 1998; Prathuangwong et al., 1993).

Assays of Extracellular Enzymes and Measurement of EPS

The phenotypic defects of Xag Mutant NUMBER in the secretion of extracellular enzyme and production of extracellular polysaccharide (EPS) were examined. Cultured media of Xag wild type, mutant and complementation strains in M9 supplemented with 0.5% glucose at 72 h were centrifuged at 8000 rpm for 10 min. The supernatant was taken as the extracellular fraction as method described by Hu et al. (1992). The pellet was washed twice with equal volumes of water and then treated on ice for 2 h with lysozyme (200 mg mL^{-1}). The lysozyme treated cells were pelleted by centrifigation at 10000 rpm for 10 min. The supernatant was collected as the periplasmic fraction. The cell pellet was resuspended in 10 mM Tris-HCl (pH 8.0), and centrifuged at 12000 rpm for 15 min. The supernatant was collected as the cytoplasmic fraction. Each of the above fraction was precipitated by 50% (w/v) ammonium sulfate and centrifuged at 8500 for 10 min. The pellets were dissolved in one-tenth of the original volume of acetate buffer and assayed for enzyme activity. The production of extracellular enzymes included cellulases, proteases and alpha-amylase were assayed basically as method described by Ray (2000), Lory (1998) and Andro et al. (1984), respectively. Activities of exoenzyme in different cellular fractions were assayed according to the procedure described by Biely et al. (1988). One mL of fraction was in test tube in ice box (4^{o}C). Mixed 3 mL of dinitrosalicylic acid (DNS) reagent into 3 mL of glucose solution sample in a loosely capped test tube was studied. To avoid the loss of liquid due to evaporation, cover the test tube with a piece of paraffin film if a plain test tube is used. Then, each sample tube was heated at 80ºC for 15 min to develop the red-brown color. One mL of a 40% potassium sodium tartrate (Rochelle salt) solution was added to stop the reaction. After cooling to room temperature in a cold water bath, the absorbance at 575 nm was read with a spectrophotometer.

For measurement of EPS production, Xag wild type, mutant, and complemented strain was separately cultured in tryptone-glucose yeast (TGY) broth containing (per liter of distilled water) 5 g tryptone, 5 g glucose, 3 g yeast extract, 700 mg K2HPO4 and 250 mg MgSO4·7H2O (Haynes et al., 1955). A loop of 48-h-grown culture of each bacterial strain was inoculated into 20 ml of TGY broth, and the culture was allowed to grow for 48 h at 28°C using a rotary shaker at 180 rpm. A 2% inoculum was then transferred into 250 ml of TGY broth and allowed to grow for 120 h at 28°C in a rotary shaker at 180 rpm. EPS was precipitated from culture supernatants by ethanol, dried and weighed as described by Tang et al. (1991). Cultured was centrifuged at 7,000 rpm for 10 min at 4°C to remove the cells. The supernatant was then mixed with a 10% (v/v) saturated KCl solution as an electrolyte, precipitated with an equal volume of 95% ethanol, and kept at 4°C overnight. The precipitate was removed by centrifugation at 85000 rpm for 15 min at 4°C, washed twice with 95% ethanol, air-dried, and measured quantity by weighting analysis.

Characterization of the HR Induction

The ability of Xag to induced an HR was assayed on two non-host plants, tobacco (*Nicotiana tabacum* cv. santhi) and tomato (*Lycopersicon esculentum* cv. sridatip-II) which is one of the nonhosts commonly used to test the HR of Xag (Kaewnum et al., 2005). Bacterial suspension was separate prepared from cell cultures of Xag mutant and wild type strains with sterile distilled water. Bacterial suspensions at cell density of about 2×10^8, 5×10^9 and 2×10^{11}cfu mL^{-1} were individually infiltrated into the mesophyll of each tested plants which 4 to 5 weeks old of tobacco, and 3 or 4 weeks old of tomato respectively by using a 1 mL hypodermic syringe without a needle (Wei et al., 1992). The inoculateted plants were maintain in greenhouse at 30^{o}C and high relative humidity. Infiltrated areas were monitored for development of tissue collapse and necrosis for 24-48 h post infiltration.

Results

Isolation and Characterization of the Nonpathogenic Mutant

The 2580 kanamycin resistant colonies obtained from transposon mutagenesis were selected and tested for their pathogenicity by inoculating into puncture wounds of soybean cotyledon and foliar spray inoculation on soybean plants. Inoculated leaves were examined over the 7-day period normally required for disease development. Typical symptoms of bacterial pustule of young greenish-pale spots caused by wildtype was occurred with in 4 days and showed clear symptoms of necrotic spot appeared slightly raised and developed into a small pustule on the underside of the leaves and produced a large, yellow to brown area with small, dark brown spots at 7 days after inoculation. The representative mutant strain KUMNTP2 was failed to induce disease symptoms on cotyledon and tested host plants in 7 days (Table 3). The KUMNTP2 was also inability to induce HR on tobacco where wildtype showed cell collapse within 48 h after infiltration at the cell concentration of 10^8 cfu mL^{-1}

Identification of the Mutated Gene

The insertion site of mutant strain KUMNTP2 could amplified by Km^r specific primer with fragment size of 750 bp. Southern blot analysis confirmed the presence of a single insertion of Tn5 in the mutant KUMNTP2 (data not shown). To clone the transposon-tagged gene, we screened a plasmid pBluscrip SK(+) prepared with KUMNTP2 DNA. One of the positive clones grown on NYA supplemented with ampicillin and kanamycin and used for gene sequencing. A 7.1 kb *Sac*I fragment containing a portion of Tn5 (5.8 kb) and Xag flanking DNA was isolated and sequenced with the synthetic oligonucleotide primer that corresponds to the Tn5 termini and primer specific M13F (5'-AGTCA CGACG TTGTA-3') and M13R 5'-CAGGA AACAG CTATG AC-3') and primer walking. However, sequences were found to be similar to those in phosphoenalpyruvate synthase (ppsA) genes of *X. axonopodis* pv. *citri* strain 360 (GenBank) with calculated homology at 99% (Figure 1). There was further explored with a BLAST search using possible translation products of larger complete fragment of the homologous among Xag and other *Xanthomonas* spp. The total gene that deficient pathogenicity phenotype KUMNTP2 occurred in a putative gene of 2374 bp (Figure 1).The sequences were identified and showed high significant homology with *ppsA* of *X. campestris* pv. *vesicatoria* (97%), *X. ory*zae pv. *oryzae* (94%), and *X. campestris* pv. *campestris* (91%) respectively. The DNA flanking Tn5 was used as a probe to isolate clones from library prepared with genomic DNA from Xag wildtype No.12-2. One of the resulting positive *E.coli* clones was selected for subcloning into pLARF3, a broad-host-range plasmid which low-copy-number, for its complementation studies.

Growth of Mutant in Medium Containing Sugar and in Planta

To determine the effect of the mutation *ppsA* on bacterial growth in culture by incubating KUMNTP2 in synthetic medium containing sugar (glucose) or nonsugar (NaAC) as the carbon source. The mean growth rate determined among Xag wildtype, mutant KUMNTP2, and complementation strains in M9 medium with glucose limitation (M9 supplemented with 0.5% glucose, M9 supplemented with 0.25% NaAC, and M9 alone) are presented in Figure 2. The study revealed that Xag wildtype and complementation strain were able grown in M9 supplemented with glucose or NaAC as the sole carbon source where the nonpathogenic mutant KUMNTP2, *ppsA* defective mutant, grew normally in only M9 supplemented with glucose, but it was unable grown in the that with NaAC. The results indicate that catabolism of NaAC is necessary for bacterial growth, and also consistent with previously report that glucose is catabolized mainly by the Entner-Doudroff pathway in *Xanthomonas campestris* (Tang et al., 2005). The lack of growth of mutant strain in the medium with NaAC as the sole carbon source might be explained that *ppsA* gene looses the function in gluconeogenesis pathway.

In *planta* experiment, cell numbers of mutant KUMNTP2 was significantly lower than that of wildtype and complementation strains in all tested periods from 0 to 120 h after inoculation (Figure 3). Population density of wildtype and complementation strains showed rapidly multiplied from the initiate inoculation source of 1×10^8 to 5.2×10^8 and 2.5×10^9 cfu mL^{-1} at 18 and 120 h post inoculation, respectively. The mutant KUMNTP2 showed lower level of population density at all incubation periods. The cell number of mutant was maintained at the range of $1x10^8$ to 2.7×10^8 cfu mL^{-1} at 0 to18 h, and slowly increased at 5.1×10^8, 5.5×10^8, 6.7×10^8, and 7.4×10^8 cfu mL^{-1} at 48, 72, 96 and 120 h post inoculation (Figure 3). The cell number of the *ppsA* mutant increased slightly during the first 24 h post inoculation and remained lower than that of the wild type throughout the experiment. These data suggest that *ppsA* is essential for the early stage of pathogen multiplication and critical for persistence in the later stage of infections. Also, the results indicated that an intact gluconeogenic pathway is required for full virulence and the reduced virulence is probably related to the reduced bacterial numbers of the mutant in plant tissues.

As the results described, the multiplication of Xag mutant KUMNTP2 was deficient and may be related to the development of epiphytic fitness to pathogenicity phase. KUMNTP2 could neither induce any disease symptoms nor necrotic lesion in the area of infiltration site at 10^8 cfu mL^{-1} concentration, where wildtype and complementation strains showed clearly necrotic lesions around infiltration area within 72 h (the initial symptom could be detected at 48 h) (Table 2). The initiate symptoms have shown with the water soaking spot, the symptoms developed to dried and brown colors

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481  cgcgaacgccttggtgaaggagggatcgacataacggctggcgccgcggaagccgatcat  541
      R  E  R  L  G  E  G  G  I  D  I  T  A  G  A  A  E  A  D  H
541  cgggtttcttcatgcggctcgtaacgcgaaccgccgatcaggttggcgtattcgttgga  601
      R  V  F  F  M  R  L  V  T  R  T  A  D  Q  V  G  V  F  V  G
601  cttgaagtccgacagacggacgatcaccgtattgggcgcaaccgacgcggtcagcgtggc  661
      L  E  V  R  Q  T  D  D  H  R  I  G  R  N  R  R  G  Q  R  G
661  gataccttcggccaggcgattgacgtagaagctcaccggatcgccgtaaccggcaatctt  721
      D  T  F  G  Q  A  I  D  V  E  A  H  R  I  A  V  T  G  N  L
721  ggcgtcgatcttcttgaggacgtcggcgtcctgcttgtcgtattccagcagcgcgttcgg  781
      G  V  D  L  L  E  D  V  G  V  L  L  V  V  F  Q  Q  R  V  R
781  gtggatgccgatgtgcgcggcgatgatcatctcaagacgcgcaagaccaatgccggcatt  841
      V  D  A  D  V  R  G  D  D  H  L  K  T  R  K  T  N  A  G  I
841  gggcaactggccgaagtcgaaggcccgctccgggtttgccacgttcatcatgatcttgag  901
      G  Q  L  A  E  V  E  G  P  L  R  V  C  H  V  H  H  D  L  E
901  cggggcaggcggcatgttgcccagatcggtggtggtgcgctcgaacggcagcaggccatc  961
      R  G  R  R  H  V  A  Q  I  G  G  G  A  L  E  R  Q  Q  A  I
961  gtagatgaagccggtgtcgccttcggcgcaactgaccgtcacttcctggccgtcgctgag  1021
      V  D  E  A  G  V  A  F  G  A  T  D  R  H  F  L  A  V  A  E
1021 cacgtcggtggcattgcccgagcccaccaccgccggcacgccgagctcacgcgcgatgat  1081
      H  V  G  G  I  A  R  A  H  H  R  R  H  A  E  L  T  R  D  D
1081 cgctgcatggcaggtgcggccgccgcggttggtgacgatggcagaggcgcgcttcatcac  1141
      R  C  M  A  G  A  A  A  V  G  D  D  G  R  G  A  L  H  H
1141 cggctcccaatcggggtcggtcatgtccgcgatcaacacatcgccagcctggacgcgatt  1201
      R  L  P  I  G  V  G  H  V  R  D  Q  H  I  A  S  L  D  A  I
1201 catgtcgtccagcgagcgcaccacgcgtgccacgccgctaccgatcttggcaccgacggc  1261
     H  V  V  Q  R  A  H  H  A  C  H  A  A  T  D  L  G  T  D  G
1261 gcggccttcggccaggatcttggcgcccttggcttccagcgcaaaccgttcgatctgggt  1321
      A  A  F  G  Q  D  L  G  A  L  G  F  Q  R  K  P  F  D  L  G
1321 cgcatgactgcgcgacttcaccgtctccggacgcgcctgcacgatgaacagcttgccgct  1381
      R  M  T  A  R  L  H  R  L  R  T  R  L  H  D  E  Q  L  A  A
1381 gaccccgtccttggcccactcgatgtccatcgggcggccgtaatgcttttcgatcaccag  1441
      D  P  V  L  G  P  L  D  V  H  R  A  A  V  M  L  F  D  H  Q
1441 tgcctgcttggacagttcctgcacgtcttcgtcgctgatcgaaaaggtgctgcgcagttc  1501
      C  L  L  G  Q  F  L  H  V  F  V  A  D  R  K  G  A  A  Q  F
1501 caccggcgtgtcttcggtgcgcacccgttcgccgggcacatccgaatagaccatgcgaat  1561
      H  R  R  V  F  G  A  H  P  F  A  G  H  I  R  I  D  H  A  N
1561 ggccttgctgccgagcgagcggcgcaggatcgccggcttgcctgcagtgagcgtgggctt  1621
      G  L  A  A  E  R  A  A  Q  D  R  R  L  A  C  S  E  R  G  L
1621 gtagacatagaactcgtccgggttgaccgcgccctgcacgaccatttcgcccaggccgaa  1681
      V  D  I  E  L  V  R  V  D  R  A  L  H  D  H  F  A  Q  A  E
1681 gctcgatgtgacgaacaccacgtcgcggaaaccggattcggtgtccagcgtgaacaacac  1741
      A  R  C  D  E  H  H  V  A  E  T  G  F  G  V  Q  R  E  Q  H
1741 gcctgccgcgcctacgcccgagcgcaccatcaactgcacgccggccgacaggaacacgtc  1801
      A  C  R  A  Y  A  R  A  H  H  Q  L  H  A  G  R  Q  E  H  V
1801 ttcatgcttgaagccgtgatgcacgcgataggcaatcgcacgatcgttgtagaggctggc  1861
      F  M  L  E  A  V  M  H  A  I  G  N  R  T  I  V  V  E  A  G
1861 gaacacttccttgaccttgtgcaccacatcgtcggcgccggtcacattgaggaaggtctc  1921
      E  H  F  L  D  L  V  H  H  I  V  G  A  G  H  I  E  E  G  L
1921 ctgctggcctgcgaacgaggcgtccggaaggtcctcggcggttgccgaggagcgcacggc  1981
      L  L  A  C  E  R  G  V  R  K  V  L  G  G  C  R  G  A  H  G
1981 cacggcaacgtcgccgccgccgttctcggcgcacaactgggcgtaggcgctgcggatgtc  2041
      H  G  N  V  A  A  A  V  L  G  A  Q  L  G  V  G  A  A  D  V
2041 gcggtccaggtccggctgcagtggggcatcgatcacccagccgcggatttccttgccggc  2101
      A  V  Q  V  R  L  Q  W  G  I  D  H  P  A  A  D  F  L  A  G
2101 cagcgtgagtgcattgacgtcttcgacatccagcgttgccagcctgtcgaagatgcgctt  2161
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Figure 1 Partial Nucleotide sequences of *ppsA* genes of *X. axonopodis pv. glycines* with 2,374 bp. Deduced amino acid shown by single letter codes below. Potential ribosome-binding site (S/D) preceding one open reading frames (ORF) is underlined, horizontal arrows indicate orientation of transcription**.**

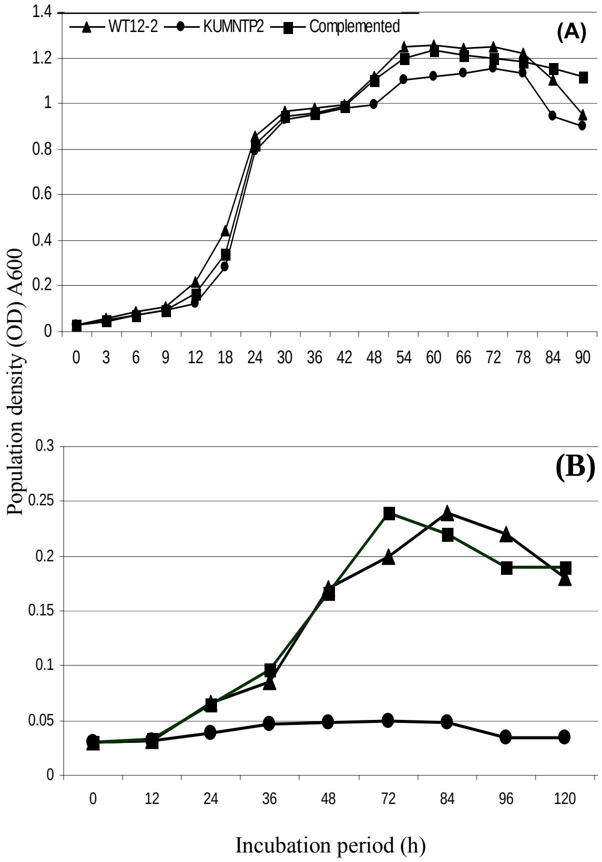

Figure 2 Growth rated of *X. axonopodis* pv. *glycines*, wildtype (Wt), KUMNTP2 and complemented mutant (Cp) in minimal medium (M9) containing with 0.5 % glucose (A) and 0.25 % sodium acetate (NaAC) (B).

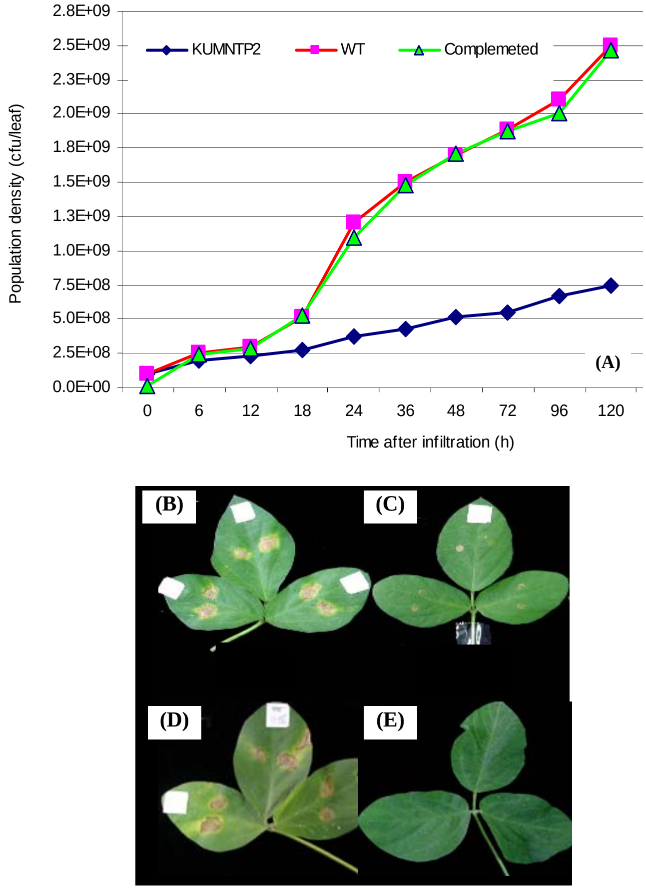

Figure 3 Population density of *X. axonopodis* pv. *glycines* wild type (WT), KUMNTP2 and complemented mutant in infected leaves of soybean at various incubation periods (A). Disease symptoms on soybean leaves at 120 h post inoculated with each bacterial suspension were demonstrated from (B) to (E). Wild type and KUMNTP2 complementation showed clearly aggressive symptom (B and D), whereas mutant could not induce disease on tested plants (C), including negative control infiltrated with distilled water (E).

surrounded with halo yellow. The size of lesions caused by infiltrated inoculation was also increased as the incubation period increased.

Quantification of ppsA that Required for Virulence

The potential for disease induction and virulence of Xag mutant strain was studied comparing with wildtype and complementation strains by co-inoculated with 10% (v/v) glucose by infiltration and spray inoculation. The results of infiltration assay showed that the mutant KUMNTP2 recovered disease induction with a small necrotic lesion in the infiltrated area at 3 days after addition of glucose mixture, where as wildtype and complementation strains showed initiate necrotic lesion around infiltration area within 36 h and the size of lesions was increased as the incubation periods increased from 2 to 5 days (Table 3).

In the foliar spray inoculation, soybean plant inoculated with wildtype and complemented strains showed disease incidence within 4 days after inoculation with disease severity of 25 and 12% (Table 5). The disease severity induced by wildtype showed highest severity with 44, 62 and 84% at 5, 7, and 10 days after inculcation respectively. While mutant strain exhibited the pathogenicity recovered with disease incidence at 7 and 10 days after cell suspension mixed with 10% glucose at the level of disease severity of 14 and 24% respectively. The *ppsA* mutant seemed to produce some secreted protein effectors when applied with exogenous glucose, which suggests that mutant do not produce enough glucose to result in visible disease induction.

Table 2 Induction of bacterial pustule by *X. axonopodis* pv. *glycines*, KUMNTP2 mutant and complemented mutant on soybean SJ4.

Incubation period (h)	Cell-concentration ($cfu\ mL^{-1}$)	Disease incidence[1/] Bacterial strain: Wild type	KUMNTP2	Complement
48	10^8	-/+	-	-
	10^9	+	-	-/+
	10^{11}	+	-	+
72	10^8	+	-	+
	10^9	++	+	+
	10^{11}	+++	+	++
96	10^8	++	-	+
	10^9	+++	+	++
	10^{11}	+++	++	++
108	10^8	++	-	++
	10^9	+++	++	++
	10^{11}	+++	++	++
120	10^8	++	-	+++
	10^9	+++	++	++
	10^{11}	++++	++	+++

[1/] Tissues are infiltrated and monitored for development of tissue collapse and necrosis from 48 to 120 h post inoculation in which + = slightly necrotic spots visiblein infiltrated area; ++ = moderated necrosis at margins of infiltrated area; +++ = clear necrosis of the entire infiltrated area with brown tissue; ++++ = completely dried at infiltrated area; and - = no visible spot was observed.

Table 3 Efficacy of glucose on induction of bacterial pustule by *X. axonopodis* pv. *glycines* KUMNTP2 mutant and complemented mutant on soybean SJ4.

Time after inoculation (day/h)	Disease incidence[1/]/Disease severity (%)[2/] — Application treatment: Individual cell suspension inoculation[3/] — Infiltration: Wt	Mt	Cp	Foliar spray: Wt	Mt	Cp	Mixed inoculation with 10% glucose (v/v)[3/] — Infiltration: Wt	Mt	Cp	Foliar spray: Wt	Mt	Cp
2/48	-	-	-	-	-	-	++	-	+	-	-	-
3/72	+	-	-	-	-	-	++	+	+	-	-	-
4/96	++	-	+	10	-	-	+++	+	+	25	-	12
5/120	++	-	++	25	-	12	++++	+	++	44	-	23
7/168	+++	-	++	57	-	36	++++	++	++	62	14	38
10/240	++++	+	+++	78	-	65	++++	++	+++	84	24	67

[1/] Disease incidence subjected to infiltration method monitored for development of tissue collapse and necrosis that + = slightly necrotic spots visible in infiltrated area; ++ = moderated necrosis at margins of infiltrated area; +++ = clear necrosis of the entire infiltrated area with brown tissue; ++++ = completely dried at infiltrated area; and - = no visible spot was observed.

[2/] Diseases severity subjected to foliar spray method expressed as percent leaf area infection (Prathuangwong et al., 1993).

[3/] Bacterial strains including, wild type (Wt), mutant (Mt) and complement (Cp).

Production of Eextracellular Enzyme and EPS

Enzyme plate assay for detection of extracellular enzymes secreted by Xag strains revealed that wildtype and complementation strains secreted cellulase in tested plates (Figure 4), where as the mutant strain showed decreased level of production and secretion of cellulase. All tested wild type strains, mutant and complemented strains could produce equal amount of protease. Mutation of the *ppsA* had no effect on the production of alpha amylase. These results indicate that cellulase secretion requires induction from *ppsA*. Cellulase secretion as one of type II protein secretion system may be dependent on *ppsA* homolog that many Gram negative plant pathogenic bacteria employ type II to deliver effector proteins differently into the host cell during infection with consistent to the reports that several secreted proteins including auxin, cytokinin, toxin, EPS, protease, and celluase are the virulent factors of Xag (Fett and Dunn, 1987).

The activity of cellulase was assayed quantitatively in extracellular, periplasmic and cytoplasmic fractions. Most of the cellulase activity were detected in the periplasmic fraction and very few in the extracellular fraction of mutant strain (Figure 5). Wildtype strain showed high activity of its extracellular expression, indicates that the secretion of cellulase to the extracellular space was affected by the mutation in *ppsA* gene.

To determine whether the reduced virulence of Xag mutant was affected from its deficient cellulase production, the efficacy of cellulase for enhanced disease severity was defined by conducting phytotoxic activity tested. In soybean plant experiment, the results shown the typical symptom as necrotic lesions around the infiltration site on tested soybean leaves were different exhibited. The necrotic lesions were variable with color and size of symptoms depended on concentration and incubation period. Among concentration of 100% cellulase, and diluted concentration of 1:1 and 1:2 however, they showed aggressiveness to induce cell necrotic within the same period of 3 days (Table 4). The initiate symptom observed have shown with the small yellow lesion area around the infiltrated site, the symptom was expanded to adjacent area with brown color occurred in the middle of lesions surrounded with halo yellow. The size of lesions was increased as the incubation periods increased.

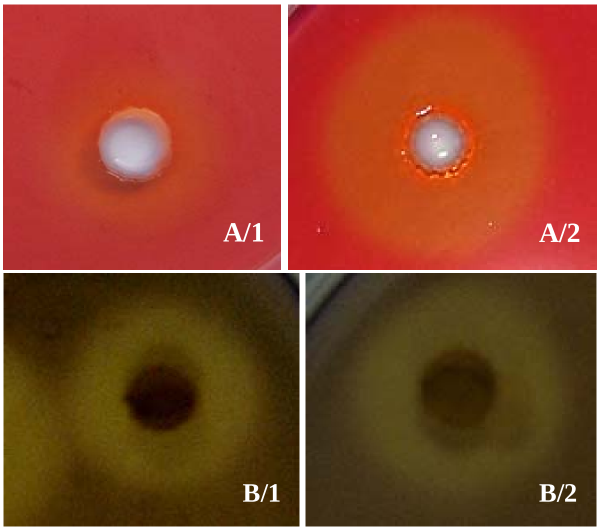

Figure 4 Extracellular enzyme plate assay revealed the nonpathogenic mutant strain KUMNTP2 (A1) was deficient production cellulase as compared to wild type (A2). No differences were observed for amylase production (B1 and B2).

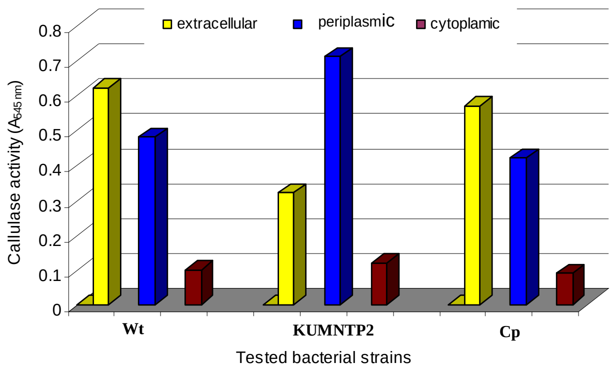

Figure 5 Distribution of cellulase activity in various cellular fractions, extracellar, periplasmic, and cytoplasmic membrane of *X. axonopodis* pv. *glycines* wildtype No.12-2 (Wt), KUMNTP2 mutant and complemented mutant (Cp).

In lower concentrations, initiate lesions showed slowly. This result could be confirmed that cellulase played a virulent factor for Xag to enhance disease severity an full virulence.

Maximum levels of EPS production by the three strains were detected in shaked culture media with the method described (Poplawsky and Chun, 1997). The 72-h cell-free culture of Xag mutant was lower contained EPS production comparing with wildtype and complementation strains. Wildtype showed a

Table 4 Effect of cellulose concentration on induction of necrotic lesions on infiltrated soybean leaves.

Dilution of cellulase: H_20	Development of necrotic lesion (DAI)[1/]					
	1	2	3	4	5	6
1:1	-	-	+	++	++	+++
1:2	-	-	+	+	++	+++
1:3	-	-	+/-	+	++	++
1:4	-	-	-	+/-	+	++
1:5	-	-	-	+	+	+
100% Cellulase	-	+	++	++	+++	++++
dH_20	-	-	-	-	-	-

[1/] Infiltrated areas were monitored for development of tissue collapse and necrosis from 1-6 days post inoculation in which + = slightly necrotic spot visible in infiltrated area; ++ = moderated necrosis at margins of infiltrated area; +++ = clear necrosis of the entire infiltrated area with brown tissue; ++++ = completely dried at infiltrated area; and - = no visible spot was observed; and DAI = days after infiltration.

higher amount of EPS production of 32 µg mL^{-1} followed by complementation strain with a weight of 30 µg mL^{-1}. The amount of EPS produced by the *ppsA* mutant was six fold lower than that of wildtype cells that it could be detected at 5 µg mL^{-1}. Culture supernatants of *ppsA* mutant restored EPS production in the complementation strain to the level seen in wildtype. This suggests that reduction of EPS can compensate for absence of some signalling protein in EPS synthesis and supports the idea that *ppsA* affects the production of EPS via the lower cell multiplication and cell-cell communication.

Characterization of HR induction

The activity of HR induction was related between bacterium cell and host plant. To determine the effect of cell-concentration of Xag wildtype, mutant, and complementation strains on HR induction was investigated on tobacco and tomato leaves as methods described. The results revealed that Xag wildtype and complementation strains could induce HR lesions on tomato and tobacco leaves with all of the cell concentrations tested. The minimum concentration of Xag wildtype and complementation strains that consist entry caused complete collapse of infiltration tissues after 24 h was about 2×10^8 cfu mL^{-1} (Table 5). For the mutant KUMNTP2, it was failed to induce visible HR on tobacco but still induced cell death on tomato. The HR did not develop when a cell concentration of mutant at 2×10^8 cfu mL^{-1} was injected, and the mild chlorotic spot was occurred within the infiltrated zone when the cell concentration was increased to 5×10^9 cfu mL^{-1}. However, the clear HR induction was restored when the cell concentration of mutant was

Table 5 Effect of *X. axonopodis* pv. *glycines* cell concentrations on induction of a hypersensitive response on tobacco and tomato leaves.

X. axonopodis pv. *glycines* strain	Concentration (cfu mL^{-1})	HR induction[1/]	
		Tobacco	Tomato
Wildtype No. 12-2	2×10^8	+	+
	5×10^9	+	+
	2×10^{11}	+	+
Mutant KUMNTP2	2×10^8	-	+
	5×10^9	-	+
	2×10^{11}	+	+
Complementation strain	2×10^8	+	+
	5×10^9	+	+
	2×10^{11}	+	+

[1/] HR= hypersensitive response; + = positive reaction, - = negative reaction, infiltrated areas were monitored for development of tissue collapse and necrosis for 48 h post inoculation. The negative reaction is no visible nercrosis. The positive reaction is complete collapse and necrosis of the entire infiltrated area.

increased to 2×10^{11} cfu mL^{-1} with completed tissue collapse and dryness within 24 h after infiltration (Table 5). The results indicate that *ppsA* might be not affected to induce the same signalling pathways between these nonhost plants, tobacco and tomato (Figure 6). Number of cell concentration and type of tested plants are the one factor for HR induction. Bacterial cells sense their population density through a cell-cell communication system with production of signalling and receptors molecule and trigger expression of particular genes when the density reaches a threshold including exoenzymes, EPS, and *hrp* genes. The interfere of *ppsA* was related to carbon source utilization and cell multiplication of Xag on host plants and might be direct or indirect effect to quorum sensing or biofilm formation (Poplawsky and Chun, 1997; Poplawsky et al., 1998). These results suggest that *ppsA* disrupted was also effected to the quorum sensing of Xag which an important roles in plant-bacterial interaction.

Discussion

Mutagenesis in *ppsA* gene gives an affected phenotype of Xag mutant strain KUMNTP2 with reduced cell multiplication, extracellular enzyme secretion, EPS production, and affected secretion protein related to HR induction. This study also demonstrates that *ppsA* was required for gluconeogenesis and related to virulence of Xag for induced bacterial pustule disease and HR on host plant, soybean and non host plant tobacco respectively. Mutant strain KUMNTP2 showed deficient pathogenicity when foliar spray inoculation on susceptible soybean (SJ4) cultivar with normal cell concentration (10^8 cfu mL^{-1}) was investigated and also exhibited the deficient HR induction on tobacco when infiltrated with the same concentration, but was able to induce HR on tomato. The pathogenicity of mutant strain was recovered when the cell concentration was increased or mixed with 10% exogenous glucose (v/v). The results indicate that the *ppsA* is required for physiology and virulence, and may indirect effect to hypersensitive response and pathogenicity *(hrp)* genes in Xag.

The first intimation revealed the growth of mutant KUMNTP2 in a synthetic medium (M9) supplemented with glucose was normally but was unable to grow in M9 medium supplemented with NaAc, whereas wild type and complementation strains were able to grow well in these two media. Indicating that the deficient *ppsA* function was related to carbon source or carbohydrate utilization

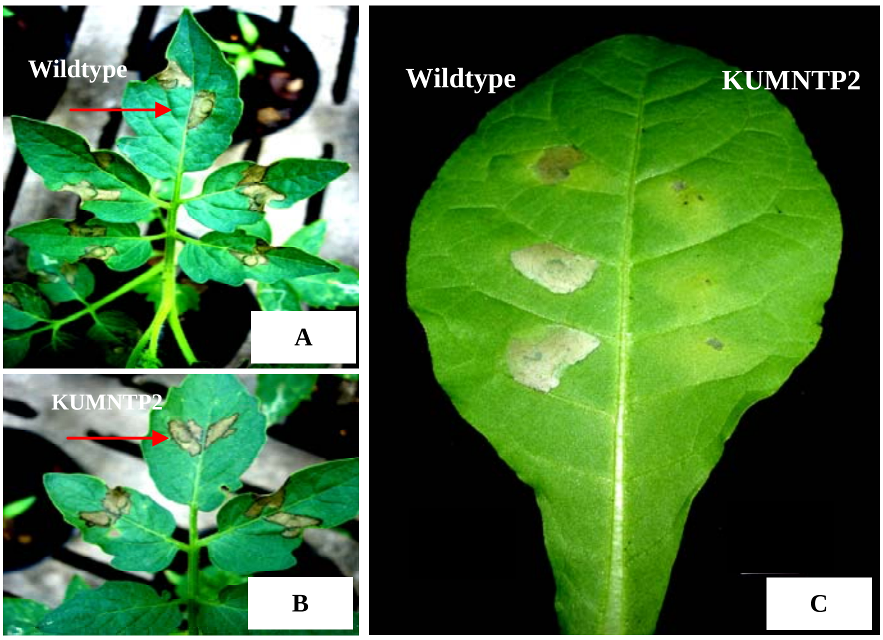

Figure 6 Hypersensitive response (HR) tested, wildtype strain No.12-2 show clearly typical HR both on tobacco (A) and (C) tomato all of cell concentration , whereas the KUMNTP2 mutant retained the ability to induce an HR on tomato but not on tobacco at cell concentration of 10^8 cfu mL^{-1} (B) and (C).

and cell multiplication since the ability to acquire nutrients from the host is the one essential factor for a pathogen to establish the next an infection process. Phytopathogenic bacteria utilize an apparent strategies to access the nutritional haven afforded them in the intracellular spaces of plant tissues (Dangl, 1995). In *planta* experiment, the multiplication level of Xag mutant was significantly lower than wildtype and complementation strains at all tested periods. The multiplication of mutant strain on host plant was corresponded to result of bacterial growth in *vitro* and the secreted effector accumulation affected to severity reduction of bacterial pustule on host plant. Taken data suggest that gluconeogenic pathway encoded by *ppsA* is required for virulence and related to the reduced bacterial numbers of the mutant in plants. The results correspond to previous report that the severity reduction of black rot disease of cruciferous crops caused by *X. campestris* pv. *campestris* was effected by the deficient of ability to uptake nutrients from host plants (Tang et al., 2005). Thus, the ability to acquire nutrients from the apoplast is critically important for it to cause disease. Disruption of the gluconeogenic pathway resulted in significant reduction both in multiplication in plant tissue and virulence of bacterial pathogen (Tang et al., 2005). However, there are no report before that the carbon utilization ability depended *ppaA* was effected to pathogenicity and virulence of Xag. Our phenotypic characterization of Xag *ppsA*-null mutant showed that a type II protein secretion system is an important virulence determinant in this bacterium. This is demonstrated cellulase activity as a virulence factor in Xag. In the absence of *ppsA*, the extent of lesion development was reduced on all methods/techniques investigated for Xag pathogenicity. Exogenous glucose added to the bacterial suspensions restored the full virulence of the *ppsA*-null mutant, further suggesting that phosphoenal pyruvate is the cause of the reduced lesion development in this mutant. The data were consistent with a role for *ppsA* in the pre-and post-penetration stages of the infection process that allowed the colonization of the host. One hypothesis that could be considered is that *ppsA* mutant develops more slowly in the host soybean plants tested because of stress imposed by insufficient gluconeogenesis supplied that impaired growth rate and amount of secreted protein effectors then, the pathogenicity impact.

As the results have been shown, the effective potential of Xag, wild type and complementation strains for uptake some available carbon sources on tested soybean leaves might be higher than the mutant and could be enhanced development of bacterial cell from the epiphytic phase to pathogenic phase (Sigee, 1993). This results suggest that the mutant strain was deficient to use some carbon source on host plant and carbon diet of a pathogen inside the host is correlated to the composition of the available carbon source in the infection site. From evolutionary point of view, the nutritional requirements of a pathogen during infection and the molecular mechanism by which this pathogen acquires nutrients from the host may be the result of co-evolution of the pathogens with their hosts (Sigee, 1993).

The several factors which may contribute to the virulence of plant pathogenic bacteria including Xag have been reported, such as production of indole acetic acid and cytokinin (Fett and Dunn, 1987), extracellular polysaccharides (EPS), toxin, bacteriocins or cellulase, xylanase and protopectinases (Sun et al., 2005; Hokawat and Rudolph, 1993). In this study, the results showed that mutant KUMNTP2 was deficient to produce the cellulase and EPS that is the one of virulence factors of Xag. The lack of enzyme secretion and EPS production might involve in its virulence that effected by defective *ppsA* genes and quorum sensing. The results of this study could be considered the *pps*A genes are probable roles or involved in the virulence of Xag by effect to carbon utilization on host plant and capability to multiply in the infection areas, and involved in the virulent factor production such as cellulase and EPS. The disrupted *ppsA* function might be also direct or indirect effects for enzyme secretion channel or protein transport membrane. In this study, the decrease of cellulase production was effected from some problems of secretion pathway. The level of cellulase detected in periplasmic space of Xag mutant strain however, higher amount than that wildtype and complementation strains. The accumulation of cellulase in periplasmic space was relating to the transport membrane not function, and indicated that *ppsA* might be involved in protein transport membrane. In generally, type II secretion pathway is encoded by at least 12 genes and specifically supported the transport of a group of seemingly unrelated proteins across the outer membrane (Sandkvist, 2001). Proteins secreted by the type II pathway include proteases, cellulases, pectinases, phospholipases, lipases, and toxins. These proteins are associated with destruction of various tissues, which contributed to cell damage and disease severity. The previous report have shown that regulating the production of extracellular enzymes and EPS themselves are under quorum sensing control or are strictly regulated by the environment at the site of colonization (Poplawsky et. al., 1998). However, *ppsA* in this study remains to determine whether it links to *rpf* encoded DS or DSF function of Quorum sensing in Xag. Additionally, the EPS and EPS-encoding genes of genus *Xanthomonas* have been well characterized (Harding et al., 1987; Reinhard et al., 1992; Chou et al., 1997). Polysaccharides of *X. campestris* pathovars were found to contain sugars such as glucose, mannose and glucuronic acid with pyruvate and acetate subunits (Coplin and Cook, 1990). Although the correlation between EPS production and virulence has been studied recently in *Pseudomonas* and *Xanthomonas* pathogens (Sutton and Williams 1970; Coplin and Cook 1990; Katzen et al., 1998; Dharmapuri et al., 1999), but not yet in Xag on host plants soybean. We showed this type of regulation results in controlled secretion of virulence factors of Xag that will only occur when the bacteria have reached their correct location and obtained a critical mass for success infection that compromised with previous report (Sandkvist, 2001).

Our experiment also found that *ppsA* could participate to Hrp effect that triggered cell death in different nonhosts. Xag mutant failed to induce HR on tobacco, but was able to induce HR on tomato at the cell concentration of 10^8 cfu mL^{-1}. The mutant strain recovered to induce HR on tobacco when the cell concentration was increased to 2×10^{11}. The result suggests that bacterial density was effected in their ability to HR induction. Previous research on the HR elicited by Xag reported that this bacterium did not induce the HR on tobacco but efficiently causes the response on pepper and, in particular on tomato (Park and Hwang, 1999; Kim et al., 2003; Hwang et al., 1992). In contrast, Kaewnum et al. (2005) reported that some strains of Xag isolated from Thailand showed aggressive ability to cause HR on various tobacco cultivars when used cell concentration of 10^9-10^{11} cfu mL^{-1}. The ability of Xag to induction of HR on tobacco was related on the function of pactate lyase in which a one type of exoenzyme secreted by Xag (Kaewnum et al., 2006). In this study however, we also demonstrated that Xag wildtype No.12-2 succeeded in HR elicitation on tobacco that was correlated with protein secretion affected by *ppsA* null-mutant.

Consistent to these information, our result indicated that ppsA might be affected to HR induction on tobacco base on protein secretions involved in the number of cell multiplication. Interfere of *ppsA* was related to carbon source utilization and cell multiplication of Xag on tobacco and might be direct or indirect effect to expression of *hrp*-or *hrp* associated genes that depended on quorum sensing or biofilm formation (Poplawsky and Chun, 1997; Poplawsky et al., 1998). Plant pathogenic bacterial cells sense their population density through a cell-cell communication system or quorum sensing with production of signaling and receptor molecules and triggered expression of particular genes when the density reached a threshold including exoenzymes, EPS, and *hrp* genes. (Tang et al., 1991; Barber et al., 1997). These results suggest that *ppsA* disrupted may also be effected to the quorum sensing of Xag which an important roles in plant-bacterial interaction that remains to further study.

The mutant KUMNTP2 strain was deficient to secrete cellulase that is one of Xag virulent factor. The reduction of cellulase concentration was related to full virulence of Xag mutant. One possibility could be considered that the lower level of cellulase secreted by mutant strain might be played the roles of plant defense elicitors. Previous report revealed that some exoenzyme secreted by plant pathogenic bacteria such as Erwinia carotovora and fluorescent Pseudomonad, which secreted plant cell-wall degrading enzymes (CDEs) including pectate lyas, polygalacturonase, cellulase, and protease played significant rules on host-pathogen interaction as plant defense elicitor and HR induction when applied with optimum concentration. Pectinase and cellulase are able to induce a defense response both locally and systemically on host plants and nonhost plants (Vidal et al., 1997, 1998) by a signal transduction (Vidal et al., 1997). However, details of plant defense hypothesis involved with Xag *ppsA* mutant, have been reported in the separated paper (data not shown).

Thus, all of discussion could indicate that the disrupted *ppsA* gene of Xag, the causal agent of soybean bacterial pustule was effected to its virulence ability. Detected characterization of *ppsA* signalling with linked gene components and their relationship with each other is under scrutiny. Also, we did not characterize genes for virulence roles, especially cellulase genes of Xag that remains to be determined.

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