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NAME: Mr. Dusit Athinuwat

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Associate Professor Sutruedee Prathuangwong, Ph.D.)

COMMITTEE MEMBER

(Professor Thomas J. Burr)

COMMITTEE MEMBER

(Associate Professor Arinthip Thamchaipenet, Ph.D.)

COMMITTEE MEMBER

(Assistance Professor Choosak Jompuk, Ph.D.)

DEPARTMENT HEAD

(Associate Professor Narong Singburaudom, M.A.)

APPROVED BY THE GRADUATE SCHOOL ON _____

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

SPECIFICITY OF AVIRULENCE GENES OF *Xanthomonas axonopodis* pv. *glycines* ON DIFFERENT SOYBEAN CULTIVARS

DUSIT ATHINUWAT

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Three races of *Xanthomonas axonopodis* pv. *glycines* (*Xag*) strains were identified on pustule disease resistance (Williams82) and susceptibility (SJ4, Spencer, and PI 520733) soybean cultivars based on virulence phenotype. Race 1 induced hypersensitive response: HR within 48 h and Race 2 induced disease on all cultivars tested. Race 3 elicited HR on specific pustule-resistant cultivar (Williams82). In Race 3, strain KU-P-SW005, an *avrBs3* homolog, *avrXg1* new-named by this study was carried on plasmid DNA. An *avrXg1* conferred resistance expressed as HR on resistant cultivar Williams82. Mutations in two predicted functional domains, the 4th central repeat and an acidic activation domain, of *avrXg1* resulted in enhanced virulence and bacterial population on resistant and susceptible cultivars. Expression of *avrXg1* in Race 1, that was predicted to confer a non-specific HR, led to virulence on susceptible cultivars. This *avrXg1* was also expressed in Race 2 resulting in increased virulence and additive pathogen fitness on resistant and susceptible cultivars. Race 2 was shown to carry *avrBs3*-like genes but apparently not *avrXg1*. The results demonstrate multi-functions for *avrXg1* dependent on pathogen and plant genetic backgrounds.

The proteome of 15-day old soybean cultivar Williams82 inoculated with KU-P-SW005 in Race 3 expressed various defense related proteins including catalase, lipoxygenase-4, and phenylalanine ammonia-lyase. Williams82 also showed enhanced expression of PR-2, PR-4, PR-6, PR-10, and lipoxygenase for HR induction following inoculation with KU-P-SW005. This is the first study to examine the interaction between soybean and *Xag* based on a gene-for-gene relationship. It provides insight into breeding strategies for pustule resistance.

To elucidate the mechanism by which the *avrXg1* mutant was more efficient than the wildtype strain in virulent initiation, genes affected pathogenicity and virulence, including *flgC*, *flgK*, and *pilD* deletion were constructed. These gene mutations produced a dominant-negative effect on *Xag* virulence on soybean. Deletion of *flgC* resulted in complete loss of disease initiation, where *flgK* and *pilD* mutants displayed a significant reduction in disease development on soybean. This result particularly confirmed that swimming and twitching motility functioned by bacterial flagellum and pillus was required for full expression of disease and severity initiation. Moreover, a regulatory cascade initiated by *avrXg1* that its mutant generated stronger pustule severity than wildtype led to activation of the downstream *flgC*, *flgK*, and *pilD* genes via *hrp* cluster, was found in this study. The swarming ability of *Xag* therefore, seemingly depended on a functional *avrXg1* system that is a key contributor to pathogenicity and virulence in bacterial pustule pathogen. A new aspect of *avrXg1* identification is a significant advancement in studying *pilD* that found to mediate a twitching role for virulence and biofilm formation on soybean in this study, is the first report and lays the foundation for *Xag* motility during pathogenicity.

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Student's signature Thesis Advisor's signature

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Dusit Athinuwat

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LIST OF ABBREVIATONS

AAD	=	acidic activation domain
avr	=	avirulence genes
CC	=	coiled-coil
CC*	=	putative leucine zipper domain
CFU	=	colony forming units
DTT	=	dithiothreitol
EDTA	=	ethylenediaminetetraacetic acid
GSP	=	general secretory pathway
HR	=	hypersensitive reaction
hrp	=	*h*ypersensitive *r*eaction and *p*athogenicity
JA	=	jasmonic acid
LG	=	linkage group
LOX	=	lipoxygenase
LRR	=	leucine-rich repeat
Mdh	=	malate dehydrogrnase
NBS	=	nucleotide binding site
NLSs	=	nuclear localization signals
PAI	=	pathogenicity islands
PAL	=	phenylalanine ammonia-lyase
PAMPs	=	pathogen-associated molecular patterns
PK	=	protein kinase
PMF	=	peptide mass fingerprint
PMSF	=	phenylmethylsulfonyl fluoride
POX	=	peroxidase
PR	=	pathogenesis-related
pthA	=	avirulence and pathogenicity
R	=	resistance genes
SDW	=	sterile distilled water
TIR	=	toll and the mammalian interleukin-1 receptor
TM	=	transmembrane domain

SPECIFICITY OF AVIRULENCE GENES OF *Xanthomonas axonopodis* pv. *glycines* ON DIFFERENT SOYBEAN CULTIVARS

INTRODUCTION

Xanthomonas axonopodis pv. *glycines*, a motile Gram-negative, causes a serious disease, bacterial pustule, on soybean that results in reduced yield and crop quality (Prathuangwong and Khandej, 1998). Virulence of *X. axonopodis* pv. *glycines* is associated with different genetic determinants including a pathogenicity island that includes nine *hrp*, nine *hrc*, and eight *hpa* genes that are regulated by HrpG and HrpX. This cluster includes genes comprising a Type III Secretion System (TTSS) that is essential for pathogenesis and for induction of a hypersensitive reaction (HR). The HR is characterized by rapid necrosis of plant cells in proximity to the invading pathogen and by restricted multiplication and spread of the pathogen from the site of infection (Klement, 1982). The HrpG effector was shown to be essential for induction of a non-specific HR on different nonhost plant (Kim *et al.,* 2003). Recently another gene, *xagP*, that encodes a pectate lyase in *X. axonopodis* pv. *glycines* was also shown to be essential for induction of HR on tobacco and pepper but not on cucumber, sesame, and tomato (Kaewnum *et al.,* 2006). Together these studies, it can be demonstrated that *X. axonopodis* pv. *glycines* is a highly heterogeneous bacterial species which possesses several genes that are associated with induction of disease and HR.

Several plant pathogenic bacteria express host-specific virulence that is often confined to members of a single plant species or cultivar. Such scenarios often involve avirulence (*avr*) genes encoding proteins that react with specific resistance (R) receptors, produced in plants leading to reduction in pathogen growth and plant cell death (HR). The interaction between Avr proteins and R-protein receptors was first described in the well-known gene-for-gene hypothesis (Flor, 1942). It has also been shown that *avr* genes may contribute to pathogen aggressiveness (Kearney and Staskawicz, 1990; Bai *et al.,* 2000) and in some cases inhibit development of non-specific HR (Alfano and Collmer, 1996; Ritter and Dangl, 1996; Vivian and Gibbon, 1997; Chen *et al.,* 2000). In contrast to the Avr-R incompatible HR, a compatible reaction (where Avr or *R* are not present), results

with the pathogen multiplying for a prolonged time period, development of water-soaked lesions, and subsequently infected tissue that becomes necrotic and dies (Staskawicz *et al.,* 1987). Avr and other effector proteins are transported via the TTSS across the bacterial membrane and into plant cells (Barras *et al.,* 1994; Alfano and Collmer, 2004). Bacterial *avr* genes were characterized into two groups: group 1 includes members of the *avrBs3/pthA* (avirulence and pathogenicity) family from *X. axonopodis* pv. *vesicatoria* and group 2 from *Pseudomonas syringae*, *X. campestris,* and *Ralstonia solanacearum* (Vivian and Gibbon, 1997).

Members of the *avrBs3/pthA* family have unique structural features including repeats of 102-bp in their central portion, three nuclear localization signals (NLSs), and an acidic activation domain (AAD) in the C-terminal region (Saijo and Paul, 2008). Some *Xanthomonas* spp. contains multiple homologs of *avrBs3/pthA*. For example in *X. axonopodis* pv. *malvacearum*, multiple *avrBs3/pthA* genes contribute additively to the water-soaking on cotton (Yang *et al.,* 1996; Shiotani *et al.,* 2000). *X. axonopodis* pv. *vesicatoria* carries *avrBs3* and *avrBs4* that affect HR and disease on different pepper cultivars (Bonas *et al.,* 1989). *X. axonopodis* pv. *citri* (Hasse) (Vauterin *et al.,* 1995) also contains at least three *avrBs3/pthA* homologs (Kanamori and Tsuyumu, 1998) and all strains of *X. oryzae* pv. *oryzae* contain more than 15 *avrBs3/pthA* homologs, that are major pathogenicity genes required for full virulence in rice (Yang and White, 2004). Recently in *X. axonopodis* pv. *glycines*, two plasmids pAG1 and pXAG81 were found to carry *avrBs3* gene homologs, but their possible roles in HR and disease were not determined (Kim *et al.,* 2006).

In addition to swarming, swimming, and twitching motility, cell attachment, and biofilm formation are thought to be important factors in pathogen virulence. Flagella are complex organelles whose synthesis depends upon approximately 50 gene products (Wolfe and Visick, 2008). In several animal host systems, bacterial motility is an important virulence factor mediating adhesion, invasion, and colonization (Pfund *et al.,* 2004). Moreover, evidence is beginning to accumulate that component of the flagella apparatus participate in the export of virulence determinants in several pathogens. For example, experiments by Young *et al.* (1999) demonstrated that *Yersinia* secretes flagellar outer proteins (Fops) via the flagella apparatus. More recently, secretion of

virulence associated proteins from *Bacillus thuringiensis* has been found to be dependent on *flhA*, an essential component of the flagella export apparatus (Ghelardi *et al.*, 2002). A little was known about the genes governing *Xanthomonas* sp. flagella biosynthesis and movement. The *X. axonopodis* pv. *citri* flagella gene cluster (*flgABCDEFGHIJKLM*) encodes the protein subunits of a polar flagellum as well as several auxiliary proteins involved in the regulation of flagella assembly and swimming motility (Khater *et al.,* 2007). Such as *flgI*; flagella basal body P-ring protein, *flgA*; flagella basal body P-ring biosynthesis protein FlgA, *flgH*; flagella basal body L-ring protein, *flgB*; flagella basal body rod protein FlgB, *flgC*; flagella basal body rod protein FlgC, *flgD*; flagella basal body rod modification protein, *flgF*; flagella basal body rod protein FlgF, *flgG*; flagella basal body rod protein FlgG, *flgJ*; peptidoglycan hydrolase, *flgE*; flagella hook protein FlgE, *flgK*; flagella hook-associated protein FlgK, *flgL*; flagella hook-associated protein FlgL. *fliC* encode flagellin is a non-specific elicitor that candidate pathogen associated molecular pattern elicit a non-specific HR also (Pfund *et al.,* 2004). Shen *et al*. (2001) isolated a flagella operon region from *X. oryzae* pv. *oryzae*. The sequenced region contains six genes, among which a gene homologous to *flhF* from *B. subtilis* was further characterized. Mutations of *X. oryzae* pv. *oryzae flhF* retard but do not abolish bacterial motility. The mutants still produce normal flagella on swarm medium. *X. oryzae* pv. *oryzae* FlhF encoded by *flhF* interacts with XA21 and a *X. oryzae* pv. *oryzae* protein similar to PilL, a twitching motility protein from *P. aeruginosa*, in the yeast two-hybrid system, which suggests that FlhF plays a role in other cell functions. FlgN, a secretion chaperone for hook-associated proteins FlgK and FlgL, was identified in *X. axonopodis* pv. *citri* (Khater *et al.,* 2007). However, there are all genes that related to the flagella in *X. axonopodis* pv. *glycines* are not understood.

Type IV pili are long fibers, with a diameter of 6 to 8 nm and a length of up to 20 μm. They are produced by Gram-negative bacteria such as *P. aeruginosa, Neisseria gonorrhoeae, Myxococcus xanthus*, *Vibrio cholerae,* and *Salmonella enterica* serovar *Typhi.* These pili participate in various bacterial processes including adhesion to prokaryotic and eukaryotic cells and abiotic surfaces, target cell specificity, twitching motility, social gliding motility, biofilm formation, DNA uptake, and bacteriophage adsorption (Strom and Lory, 1993; Mattick, 2002; Nudleman and Kaiser, 2004). A genes cluster required for pilus assembly, retraction, and twitching movement in *P. aeruginosa*

and *Xylella fastidiosa*, e.g. *pilA, pilB, pilC, pilD, pilF, pilM, pilN, pilO, pilP, pilR, pilS, pilQ, pilT, pilZ,* and *pilU* (Li *et al.,* 2007). Nonpathogenic mutants of *R. solanacearum* are significantly reduced in virulence on tomato plant (Tans-Kersten *et al.,* 2001). The *pilA* mutants (pilin deficient) of *P. aeruginosa* have reduced virulence (Li *et al.,* 2007), and that *pilA, pilT,* and *pilQ* mutants of *R. solanacearum* cause slower disease development, and less severe wilting symptoms, in tomato plants (Kang *et al.,* 2002; Liu *et al.,* 2001). Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *P. aeruginosa* pili (Nunn *et al.,* 1990). Also, that mutations in genes for twitching motility resulted in reduction of virulence in *X. oryzae* pv. *oryzae* (Wang *et al.,* 2008). The *pilA* gene, which encodes the major structure of pili, is required for infection of *X. axonopodis* pv. *citri* by the filamentous bacteriophage Cf. *pilR* mutant, resistant to the infection of phage Cf, was unable to synthesize PilA protein. The *pilS* mutant, however, was susceptible to Cf infection, and the level of *pilA* expression in this mutant was similar to that of wildtype cells (Yang *et al.,* 2004). *pilD* of *X. campestris* pv. *campestris* encode type IV pre-pilin leader peptidase was demonstrated to be required for protein secretion, in addition to its involvement in biogenesis of type IV pill. *pilO* gene of *X. oryzae* pv. *oryzae* play a critical role in pathogenicity, twitching motility, and biofilm formation. Growth of the *pilQ* mutant was similar to that of wildtype (Lim *et al.,* 2008). Together these studies, it can be demonstrated that *pilD* is an important gene for pilus assembly and may play a role in twitching motility and pathogenicity that have not yet reported in *X. axonopodis* pv. *glycines*.

In this study, races of *X. axonopodis* pv. *glycines* from Thailand were described based on the expression of differential virulence on soybean cultivars that is determined by the presence of an avirulent gene and how it interacts with resistant cultivars to induce HR and defense response. A new member of the *avrBs3/pthA* family was described when introduced to Race 1 and Race 2 of *X. axonopodis* pv. *glycines* confers a virulence phenotype on a normally resistant and susceptible soybean cultivar. In addition, the molecular cloning and characterization of the *flgC, flgK*, and *pilD* that are associated with swarming, swimming, and twitching motility, biofilm formation, and pathogenicity of bacterial pustule disease on soybean have described.

OBJECTIVES

The objectives of this research were as follows:

1. To identify Race of *Xanthomonas axonopodis* pv. *glycines* Thai strains on different soybean cultivars.

2. To identify and characterize the avirulence genes of *Xanthomonas axonopodis* pv. *glycines*.

3. To evaluate the soybean protein response to avirulence genes of *Xanthomonas axonopodis* pv. *glycines* using proteomics approach what proteins involved in host defense mechanism.

4. To identify novel motility and pathogenicity associated genes in *Xanthomonas axonopodis* pv. *glycines* that may lead to development of strategies for control of pustule disease.

5. To evaluate the twitching motility of *Xanthomonas axonopodis* pv. *glycines* during pathogenesis on soybean.

LITERATURE REVIEW

1. Importance of soybean in Thailand

Soybean (*Glycine max* L.) is a legume that grows in tropical, subtropical, and temperate climates. Soybean is an important source of high quality, inexpensive protein, and oil. Approximately half of the world's soybeans are produced in the developing world, and the other half in the developed world. Originally domesticated in China around 1700-1100 B.C., soybean is now cultivated throughout East and Southeast Asia where people depend on it for food, animal feed, and medicine (Hartman *et al.,* 1999). During 1995 and 1996, soybean was grown in 47 countries; annual production is 124 million tons. The major producers include the United States (47%), Brazil (19%), The People's Republic of China (11%), and Argentina (10%) (Hartman *et al.,* 1999). Then, soybean can play a dominant role in the world agriculture.

In 1983, Thailand started growing soybeans in order to satisfy its poultry industry with raw materials for feed ingredients. The important soybean growing area is mainly in the north and northeast regions of the country including Chiang Mai, Chiang Rai, Lumpang, and Nakhorn Sawan with three growing seasons for planting, during May to August, June to November, and December to April. However, soybean product in Thailand is not enough for both human consumption and use in animal feed materials. Since then, the country has increased its soybean import volume every year in response to the demand from its poultry industry, whose exports grew over 85% between 1983 and 1993. However, because the imported soybeans have higher quality than domestically produced soybeans, Thailand increases its soybean imports every year. Thailand has imported over 61% or about 660,000 tons in 2000. In terms of import value, Thailand imported an average of over 8,000 million baht between 1997 and 2000. The trend will continue to increase, as Thailand continues to increase its soybean imports (TheBioenergySite, 2008).

Soybean production in recent years has been hovering around 200,000 tons mainly because of low productivity, near zero growth in planting area, and also several pathogens including fungi, bacteria, nematode, and virus are mainly problem to soybean production

(Prathuangwong and Amnuaykit, 1989; Prathuangwong *et al.,* 1996). Soybean production in during 2008-2009 is forecast at 180,000 tons, as compared with 210,000 tons in during 2007-2008. However, soybean growing area is expected to decline as soybeans are more labor intensive with prices relatively less attractive to other crops. As a result, soybean farmers have been shifting portions of their land to corn and paddy. Average soybean yields in Thailand are only 220-240 kgs/rai (about 1.38-1.50 tons/hectare), and are nearly identical to average yields from the previous decade. Corn, an alternate crop, has seen its productivity increase tremendously from about 400 kgs/rai (2.5 tons/hectare) in the early 1990's to currently 600-650 kgs/rai (3.75-4.06 tons/hectare). Thailand is a growing market for imported soybeans as demand continues to outpace domestic production (TheBioenergySite, 2008).

2. Soybean diseases

During the 1994-1995 growing season, a conservation estimate of worldwide losses to all soybean diseases was about 15 million tons, or about 11% of the amount produced. Agents that can be transmitted from an infected plant to a healthy plant cause biotic diseases. Soybean production in the major producing countries, of more than 100 pathogens that affect on soybean, about 35 are important economically. Important disease of soybean in Thailand include (1) Sudden death syndrome caused by *Fusarium solani*, (2) Anthracnose (*Colletotrichum truncatum*), (3) Rust (*Phakopsora pachyrhizi*), (4) Downy mildew (*Peronospora manshurica*), (5) Bacterial pustule (*X. axonopodis* pv. *glycines*), (6) Crack stem (*Fusarium* spp., *Phialophora* sp., and *Rhizoctonia* sp.), (7) Phyllosticta leaf spot (*Phyllosticta* sp.), (8) Myrothecium leaf spot (*Myrothecium* sp.), (9) Rhizoctonia aerial blight (*Rhizoctonia* sp.), (10) Yellow orange leaf spot (unidentified), (11) Basidiomycete rot (Basidiomycete fungus), (12) other fungal foliar disease (*Alternaria* sp., *Cercospora* sp., *Curvularia* sp., and Septoria sp.), (13) seedling disease (*Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Stylopage* sp., and *Rhizoctonia* sp.), (14) unidentified diseases, and (15) Other disease (symptom like physiogenic, mineral deficiency, mutative disease, virus, and phytoplasma) (Prathuangwong *et al.,* 1996, Prathuangwong and Khandej, 1998). All plant parts are susceptible to a number of pathogens that reduce the quality and quantity of yields. The extent of losses depends upon the pathogen or condition involved the developmental stage and health of the plants

when infection occurs, the severity of disease on individual plants, and the number of plants affected. Many pathogens can initiate an epidemic only under rather specific environmental conditions. Thus, the extent and severity of diseases also depend on the degree of compatibility between the host and the pathogen and on the influence of the environment on this association (Hartman *et al.,* 1999).

3. Bacterial pustule

Bacterial pustule caused by *X. axonopodis* pv. *glycines* is one of the most prevalent bacterial diseases in most soybean growing areas of the world with warm temperatures and sufficient rainfall (Moffett and Croft, 1983; Bradbury, 1986). The disease may cause premature defoliation, which may decrease yield by reducing seed size and number. In Thailand, Prathuangwong (1983) reported that yield losses due to natural dissemination in the susceptible soybean cultivar SJ4 were 20-35%.

X. axonopodis pv. *glycines* is a motile Gram-negative rod within 0.5-0.9 x 1.4-2.3 micrometer, motile by a single polar flagellum. Colonies on beef infusion agar, nutrient glucose agar (NGA), and Wakimoto' s agar are pale yellow, circular, and smooth with an entire margin (Fig. 3C). The pathogen produces abundant, slimy, yellow growth on sugar containing media (Hokawat, 1978). Optimum temperature for growth on potato dextrose agar is 25-30°C, maximum 35°C, and minimum 10°C (Bhatt and Patel, 1954). The bacterium overseasons in seeds, in surface crop residue, and in the rhizosphere of wheat roots. The bacterium spreads via splashing water or windblown rain and during cultivation when foliage is wet.

The semi-selective medium MXG of Khundet (1989) gave green convex-shaped colonies, 3-6 mm diameter with smooth margin and fluidal character after 3 days, and was used to detect the pathogen in infected seeds by using iodine solution to demonstrate the clear zone formation around infected seeds.

Early symptom of the disease is small pale green spots (Fig. 3A), yellow-to-brown lesions with raised pustules in the center (Weber *et al.,* 1966). Spots of bacterial pustule vary from minute specks to large, irregular, mottled brown areas that arise when smaller

lesion coalesce. In later stages, dried, broken remnants of pustules seen on small brown necrotic are surrounded by narrow yellowing haloes (Fig. 3B and 3D). The symptoms may develop on stems and pods of susceptible varieties. Those symptoms are sometimes confused with those of soybean rust. However, pustule lesions are characterized visually by small pustules surrounded by yellowing haloes, whereas rust disease forms tan or brown lesion in which uredospores are formed and released through a central pore. In previously reported, there has been research and development of soybean cultivars for resistance to disease such as Sukhothai1 (ST1), Sukhothai2 (ST2), and Chiang-Mai60 (CM60) (Prathuangwong and Amnuaykit, 1989). Symptoms on resistant soybean cultivars have also been reported, such as occasional small chlorotic spots, but no well-defined pustules (Hartwig and Lehman, 1951), or light green chlorosis and slight browning in greenhouse trials (Fett, 1984), or fewer and smaller pustules (Groth and Braun, 1986).

Under a moderate artificial infection the average seed yield reduction in the susceptible soybean lines was 4.3% of which 86% was attributed to reduction in seed number and 14% to seed size. Prathuangwog (1985) reported yield losses due to natural dissemination in the susceptible soybean cultivar (SJ4) were 20 - 35%. The most susceptible age of SJ4 was between 6 - 7 weeks after emergence.

Choethana (1992) classified resistant level on 3 soybean cultivars including SJ4, Doikham, and NS1 to susceptible, moderately susceptible, and highest resistant by disease severity with 18.45%, 12.45%, and 7.77% respectively. Moreover, the strains causing the greatest lesion size and highest colony forming unit of causal pathogen/lesion on soybean were the most aggressive while strains causing smallest lesion size and lowest CFU/lesion were the least aggressive. Strains of the pathogen showed large differences in their virulence (Hokawat and Rudolph, 1991). Several factors involved with the virulence of *X. axonopodis* pv. *glycines* have been described, such as production of indoleacetic acid and cytokinin (Millar, 1955; Fett and Dunn, 1987), extracellular polysaccharides (Jones and Fett, 1985), toxin (Hokawat and Rudolph, 1993), bacteriocins (Fett *et al.*, 1987) or cellulase and protopectinase (Hokawat and Rudolph, 1993).

Saisangthong (1999) analyzed the *X. axonopodis* pv. *glycines* genome with regard to plasmid and virulence diversities using random amplified polymorphic DNA (RAPD). The results showed a strong relationship between disease severity and RAPD patterns but less relation between disease severity and plasmid profile and not correlated with soybean growing area which *X. axonopodis* pv. *glycines* strains were obtained. The assays of symptom expression and RAPD were capable of distinguish strongly-from weakly aggressive strains where the correlation between the presence of plasmid and the variability of pathogenicity remain to be determined.

Ketsuwan (2003) classified 199 strains of *X. axonopodis* pv. *glycines* collected from different regions in Thailand using rep-PCR and disease severity on soybean. Several major rep-PCR products distinguished weakly from highly isolates into two major genotype groups, Group 1 (weakly) and 2 (virulent). These results suggested that the BOX and ERIC-PCR method are useful for the identification of *X. axonopodis* pv. *glycines* but not for determination of geographic origin of the strains. They concluded that *X. axonopodis* pv. *glycines* collected from soybean are genetically heterogeneous and therefore weakly and virulent isolates can be distinguished using rep-PCR.

Kaewnum *et al.* (2006) described role of the pectate lyase homolog, *xagP* 1.4-kb in twenty six *X. axonopodis* pv. *glycines* strains was highly correlated with their ability to induce an HR on tobacco which is the first study indicating a role for a functional pectate lyase in induction of a plant HR.

Athinuwat *et al*. (2006) classified 120 isolates of *X. axonopodis* pv. *glycines* collected from different regions in Thailand into two major genotype groups by using BOX-PCR , Group 1 (weakly) and 2 (virulent). BOX-PCR was more relatively unique, stable, and reproducible in that it could yield 650-bp fragment in all *X. axonopodis* pv. *glycines* strains tested and the band of 840-bp was only detected in high virulence strains by BOX primer. Their observations emphasized the need for selecting strains to use in inoculation studies that include a wide range of pathogenic capabilities representative of strain variability in the native population. They also, determined their pathogenicity on different soybean cultivars including SJ4, SJ5, NS1, CM60, and AGS292. Susceptible and resistant reactions to most *X. axonopodis* pv. *glycines* strains was typified by number of

infected leaf area with 61.3, 33.3, 17.8, 15.5, and 2.4% infection of SJ4, AGS292, SJ5, NS1, and CM60 respectively.

Thowthampitak *et al.* (2008) reported *rpfF* encoding for the biosynthesis of the extracellular diffusible factor (DSF) in *X. axonopodis* pv. *glycines* related to a well characterized quorum sensing molecule. The *rpfF* mutants exhibited reduction in virulence on soybean and production of extracellular polysaccharide and the extracellular enzymes including carboxymethylcellulase, protease, endo- β-1,4-mannanase, and pectate lyase were less than that of wild type.

Recently, Park *et al.* (2008) classified 155 strains of *X. axonopodis* pv. *glycines* into six different groups according to the patterns of *avrBs3* homologous bands from Southern blot analysis which is not race classification.

4. Elicitors and plant resistance

Elicitor or signal molecules from plant pathogen trigger host defense have been reported. One of the earliest characterized elicitors is a specifically 1,3-1,6-branched hepta-β-glucan from cell walls of the soybean pathogen *Phytophthora sojae* (Sharp *et al.*, 1984). Glucan perception appears to vary in different plant species, was not active in parsley (Parker *et al.*, 1988) and rice, whereas a 1,3-1,6-branched tetra-glucan from cell walls of the rice pathogen *Magnaporthe grisea* is active in rice, but not in soybean (Yamaguchi *et al.*, 2000). Mishra *et al.* (2008) identified a new proteinaceous elicitor from *P. colocasiae* effects on taro plant to induce the following responses of a typical HR: (1) induction of rapid plant cell death, (2) induction of defense responses, and (3) production of endogenous signals that are known to participate in the triggering of defense responses. *P. sojae* and *P. megasperma* were the source of another extracellular elicitor, the 42 and 32 kDa glycoprotein which induce defense reactions in the nonhost plants parsley and tobacco, respectively (Baillieul *et al*., 1995). Its gene was found in all *Phytophthora* species analyzed and the deduced proteins show at least two invariant domains essential for enzyme activity which the plant evolved a recognition system targeting a crucial portion of the microbial enzyme (Mishra *et al.*, 2008).

A similar strategy, the synthetic peptides from bacterial flagellin domain act as elicitors of defense reactions in cells of tomato and several other plant species (Felix *et al.*, 1999) which this peptide from *Agrobacterium tumefaciens* and *Rhizobium meliloti* did not act as elicitors (Mishra *et al.*, 2008). In *Arabidopsis thaliana* a region of flavonol synthase1, *FLS1* was determined flagellin sensitivity (Gomez-Gomez *et al.*, 1999). Moreover, *FLS2*, contains a ubiquitously expressed gene encoding a receptor kinase with an extracellular leucine rich repeat (LRR) domain and an intracellular serine/threonine protein kinase domain which also sensitive to flagellin (Gomez-Gomez and Boller, 2002). This receptor kinase shows structural homology with plant *R* gene products (Table 1), in particular with the product of the rice *R* gene, *Xa21* (Song *et al.*, 1995). Therefore, flagellin and other conserved microbial products, which are invariant among diverse groups of microorganisms, such as bacterial lipopolysaccharide, bacterial peptidoglycan or fungal constituents such as chitin, glucan, lipids or proteins have been recently referred to as pathogen-associated molecular patterns (PAMPs). It appears that PAMP recognition is mediated through phylogenetically conserved Toll-like receptors (Hayashi *et al.*, 2001) that activate plant species (nonhost) or basic resistance mechanisms (Slot, 2002).

PAMPs are virulent factors that have enabled the pathogen strains to overcome plant species resistance. Therefore, these factors have driven the co-evolution of plant *R* genes and, thus, the development of phylogenetically more recent cultivar-specific disease resistance to specific pathogen races. The processes of interactions between pathogen *avr* genes and host *R* genes can be summarized as incompatible and compatible as defined by the gene-for-gene hypothesis (Flor, 1971). Incompatible interaction is for every dominant *R* allele in the host cultivar there is a complementary dominant gene for *avr* gene in the pathogen. A compatible interaction result when there is a recessive allele at either or both loci. Therefore, individual *avr-R* pairs determine host resistance in specific race-cultivar interaction. The phenotype of host resistance associated with incompatible host-pathogen interactions is typified by a localized defense called the HR. Elicitation of the HR by bacteria is observed 12 to 24 h post inoculation when leaves are infiltrated with bacteria suspension of $>10^6$ cells per ml (Staskawicz *et al.,* 1984). In the compatible interaction, where HR is not elicited, the bacteria multiply for a prolonged period, water-soaked lesions appear 3 days or more after inoculation and the infected tissue subsequently becomes necrotic and dies (Staskawicz *et al*., 1987). HR is therefore

considered a generalized expression of plant resistance both by resistant cultivars of susceptible species and by nonhost species.

Hartwig and Lehman (1951) identified a single recessive gene, *rxp* conditioned bacterial pustule resistance in CNS cultivar. Subsequently, Hwang and Kim (1987) reported that CNS exhibited no symptoms on soybean after individual inoculations with 20 different *X. axonopodis* pv. *glycines* strains. The *AvrRxv* inhibits disease development by induced HR. Plamer *et al.* (1992) evaluated *Rxp* gene was liked to the malate dehydrogrnase (*Mdh*) locus with a recombination frequency of approximately 16%. This association defined linkage group (LG) 20 of the classical soybean genetic map, which consisted of isozyme, morphological, pigmentation, and pest-resistance loci (Narvel *et al.*, 2001). Disease resistance gene clusters have been identified in soybean (Kanazin *et al.*, 1996) such as *Rps2* and *Rps3* (*Phytopthora* root rot and stem rot), *Rmd* (powdery mildew), and *R12* locus (*Bradyrhizobium japonicum* - mediated nodulation), *Rsv1* (soybean mosaic virus) and *Rpv1* (peanut mottle virus), and *Rpg*1 (bacterial blight).

Hwang and Lim (1998) evaluated 63 *X. axonopodis* pv. *glycines* strains for their pathogenicity tested on 11 soybean cultivars including Pella, Ware, Chippewa, Flambeau, Norchief, Mandarin, Peking, Harosoy, Mukden, Manchu, and Williams. Based on different reaction of the cultivars, the strains are classified to Races 1, 2, 3, 4, and 5. Moreover, five cultivars, Chippewa, Harosoy, Mukden, Pella, and Williams differentiated these five races.

To date, avirulence determinants have been identified in viruses, bacteria, fungi and Oomycetes, and a candidate gene was recently isolated from a nematode. In addition, genes related to *avrBs3/pthA* genes of pathogens have been identified in *Rhizobium* species, suggesting their involvement in symbiotic interactions as well (Baron and Zambriski, 1995; Viprey *et al.*, 1998; Ciesiolka *et al.*, 1999; Slot, 2002).

Table 1 Isolate plant resistance genes (modified after Baker *et al*., 1997)[1/]

Class	*R* gene	Plant species	Pathogen	*avr* gene	Structure of R protein	Reference
1-I	*N*	tobacco	Tobacco mosaic virus	Replicase	TIR-NBS-LRR	Whitham *et al*., 1994
	RPS4	*A. thaliana*	*P. syringae* pv. *tomato*	*avrRps4*	TIR-NBS-LRR	Gassmann *et al*., 1999
	RPP1,10,14	*A. thaliana*	*Peronaspora parasitica*	?	TIR-NBS-LRR	Botella *et al*., 1998
	RPP5	*A. thaliana*	*Peronaspora parasitica*	(*avrRpp5*)	TIR-NBS-LRR	Parker *et al*., 1997
	L6,L1-12	flax	*Melampsora lini*	(*AL6*)	TIR-NBS-LRR	Lawrence *et al*., 1995
	M	flax	*Melampsora lini*	(*AM*)	TIR-NBS-LRR	Anderson *et al*., 1999
1-II	*RPS2*	*A. thaliana*	*P. syringae* pv. *tomato*	*avrRpt2*	CC*-NBS-LRR	Bent *et al*., 1996
	RPS5	*A. thaliana*	*P. syringae* pv. *tomato*	*avrPphB*	CC*-NBS-LRR	Warren *et al*., 1998
	RPM1	*A. thaliana*	*P. syringae* pv. *maculicula*	*avrRpm1;avrB*	CC*-NBS-LRR	Grant *et al*., 2006
	RPP8	*A. thaliana*	*Peronospora* partasitica	*(avrRpp8)*	CC*-NBS-LRR	McDowell *et al*., 1998
	HRT	*A. thaliana*	Turnip crinkle virus	Coat protein	CC*-NBS-LRR	Cooley *et al*., 2000
	Prf	tomato	*P. syringae* pv. *tomato*	*avrPto*	CC*-NBS-LRR	Salmeron *et al*., 1994
	Mi-1	tomato	*Meloidogyne incognita;* *Marcosiphum euphorbiae*	?(nematode) ?(aphid)	CC*-NBS-LRR	Milligan *et al*., 1998; Rossi *et al*., 1998; Vos *et al*., 1998
	I2	tomato	*Fusarium oxysporum*	?	CC*-NBS-LRR	Simons *et al*., 1998
	Rx1	potato	Potato virus X	Coat protein	CC*-NBS-LRR	Bendahmane *et al*., 1999
	Rx2	potato	Potato virus X	Coat protein	CC*-NBS-LRR	Bendahmane *et al*., 2000
	Gpa2	potato	*Globodera pallid*	?	CC*-NBS-LRR	van der Voort *et al*., 1999
	Dm3	lettuce	*Bremia lactucae*	?	CC-NBS-LRR	Meyers *et al*., 1998
	Bs2	pepper	*X. axonopodis* pv. *vesicatoria*	*avrBs2*	CC-NBS-LRR	Tai *et al*., 1999
	Sw-5	tomato	Tospovirus	?	CC-NBS-LRR	Brommonschenkel *et al*., 2000; Spassova *et al*., 2001
	Xa1	rice	*X. oryzae* pv. *oryzae*	?	CC-NBS-LRR	Yoshimura *et al*., 1998
	Pib	rice	*Magnaporthe grisea*	?	CC-NBS-LRR	Wang *et al*., 1999
	Pi-ta	rice	*Magnaporthe grisea*	*AVR-Pita*	CC-NBS-LRR	Bryan *et al.,* 2000
	Cre3	wheat	*Heterodera avenae*	?	CC-NBS-LRR	Lagudah *et al*., 1997
	Mla1	barley	*B. graminis* f.sp.*hordei*	(*AvrMla1*)	CC-NBS-LRR	Zhou *et al*., 2001
	Mla6	barley	*B. graminis* f.sp.*hordei*	(*AvrMal6*)	CC-NBS-LRR	Halterman *et al*., 2001
	Rp1-D	maize	*Puccinia sorghi*	?	CC-NBS-LRR	Collins *et al*., 1999
2	*Pto*	tomato	*P. syringae* pv. *tomato*	*avrPto*	PK	Martin *et al*., 1993
3	*Cf-9*	tomato	*Cladosporium fulvum*	*Avr9*	LRR-TM	Jones *et al*., 1994
	Cf-4	tomato	*Cladosporium fulvum*	*Avr4*	LRR-TM	Thomas *et al*., 1997
	Cf-2	tomato	*Cladosporium fulvum*	*(Avr2)*	LRR-TM	Dixon *et al*., 1996
	Cf-5	tomato	*Cladosporium fulvum*	*(Avr5)*	LRR-TM	Dixon *et al*., 1998
	Hcr9-4E	tomato	*Cladosporium fulvum*	*(Avr4E)*	LRR-TM	Takken and Joosten, 2000
	$Hs1^{pro-1}$	tomato	*Heterodera schachtii*	?	LRR-TM	Cai *et al*.,1997
4	*Xa21*	rice	*X.oryzae* pv. *oryzae*	?	LRR-TM-PK	Song *et al*., 1995
5	*Hml*	maize	*Cochlioblus carbonum*	-	HCT reductase	Johal and Briggs,1992
6	*Mlo*	barley	*B. graminis* f.sp. *hordei*	?	7 TM protein	Buschges *et al*., 1997

[1/]*avr* genes in brackets have not been cloned to date. CC; coiled coil domain, CC*; putative leucine zipper domain, NBS; nucleotide binding site, LRR; leucine rich repeats, PK; protein kinase, and TM; transmembrane domain.

5. Bacterial elicitors

Since 1984, the first bacterial *avr* gene was cloned from *Pseudomonas syringae* (Staskawicz *et al.*, 1984), subsequently, more than 40 *avr* genes from Gram-negative pathogens of the genera *Pseudomonas*, *Xanthomonas*, *Erwinia,* and *Ralstonia* (Dangl, 1994; Leach and White, 1996; Vivian and Gibbon, 1997). Mutation of several *avr* genes resulted not only in loss of virulence in the presence of the respective plant *R* genes but simultaneously in a reduction of virulence on susceptible host plants (White *et al.*,2000) and also decreased bacterial population and pathogens fitness (Vivian and Gibbon, 1997). Furthermore, several *avr* genes when promoted virulence on the host lacking the corresponding *R* gene induced more disease symptoms (Kjemtrup *et al.*, 2000).

5.1 The Type III Secretion System (TTSS)

The finding that almost all plant *R* genes acted against bacteria encoded by putative cytoplasmic proteins prompted the question that whether the bacterial signal enters the plant cells for direct interaction with the R protein. TTSS has been known that Gram-negative bacterial pathogens require *hrp* (*h*ypersensitive *r*eaction and *p*athogenicity) genes for HR triggering and pathogenicity. The *hrp* gene encoded proteins that control the production of the TTSS (Alfano and Collmer, 1996; Bonas and van den Ackerveken, 1997; Mudgett and Staskawicz, 1998; Galán and Collmer, 1999; Cornelis and van Gijsegem, 2000). Considerable homology has been identified between *hrp* genes and components of the type III protein secretion pathway in animal pathogens of the genera *Yersinia*, *Shigella,* and *Salmonella* (Fenselau and Bonas, 1995). Delivery of effector proteins into host cells appears to be mediated by a filamentous surface appendage, called the Hrp pilus (Roine *et al.*, 1997). Components of the TTSS are related to those of the bacterial flagella biogenesis complex indicating an evolutionary adaptation of the flagella export apparatus to secrete virulence factors (Van Gijsegem *et al.*, 1995).

Alfano and Collmer (1996) classified two main groups of bacteria that can be distinguished in relation to their Hrp pathways: group I contains *P. syringae* and *E. amylovora* and group II, *R. solanacearum* and *X. axonopodis* pv. *vesicatoria*. The six to eight complementation groups in each cluster encode a TTSS, genes which regulate the

expression of *hrp* and *avr* genes, and a proteinaceous elicitor of HR, called harpin (Bonas *et al.*, 1993). Fujikawa *et al.* (2006) reported *P. fluorescence* 55 required *hrp* gene cluster from *P. syringae* pv. *syringae* for encode *ap11, avrXa7* and *avrXa10*. Therefore, *hrp* gene cluster control protein secretions of plant pathogenic bacteria via TTSS.

5.2 The AVR proteins from *Pseudomonas*

A characteristic of the gene in this genus is the absence of any recognizable features such as membrane spanning domains or signal sequences (Vivian and Gibbon, 1997). *P. syringae avr* genes are located elsewhere in the genome or on plasmids (Leach and White, 1996). All *avr* genes of this group and their target in host cell were identified (Table 2). The first *avr* gene, *avrPto* was identified from *P. syringae* pv. *tomato*, the causal agent of the tomato speck disease. AvrPto, is a 164-amino acid, hydrophilic protein (Ronald *et al.*, 1992) interact with the corresponding *R* gene product, Pto in tomato (Scofield *et al.*, 1996; Tang *et al.*, 1996). *Pto* encodes a serine/threonine-specific protein kinase that specifically phosphorylates a second serine/threonine kinase, Pti1, (Zhou *et al.*, 1995). Moreover, Pto interacts with three putative transcription factors, Pti4, Pti5 and Pti6, which bind to the 'PR box' present in the promoter region of a large number of genes encoding pathogenesis-related (PR) proteins (Jia and Martin, 1999; Zhou *et al.*, 1997), the DNA binding of Pti4 being enhanced upon specific phosphorylation by Pto (Gu *et al.*, 2000). Therefore, Pto mediates resistance in tomato by a phosphorylation cascade that is triggered by the bacterial AvrPto protein (Slot, 2002). Additional *avrPto* play a role that beneficial for the pathogen, the gene enhances the virulence of *P. syringae* pv. *tomato* in a strain-dependent manner in tomato plants lacking *Pto* (Chang *et al.*, 2000) correlated with a small increase in bacterial growth.

For many bacteria, *avr* genes showed a homologous with other *avr* gene that required for pathogenicity. An *avrRpm1* from *P. syringae* pv. *maculicola* and *avrPpiA* from *P. syringae* pv. *pisi* secreted 97% homologous protein (Dangl *et al.*, 1992). Only *avrRpm1* involved bacterial virulence on susceptible *A. thaliana* (Ritter and Dangl, 1995) whereas *avrPpiA* did not (Gibbon *et al.*, 1997). Interestingly, an *avrEF* from *P. syringae* pv. *glycinea* is homologous to the *dspEF* (syn. *dspAB*) that is required for pathogenicity of *E. amylovora* (Bogdanove *et al.*, 1998; Gaudriault *et al.*, 1997). *dspE* and *avrE* encode

hydrophilic proteins of 198 kDa and 195 kDa, respectively that function as chaperones for virulence factors secreted by TTSS (Wattiau *et al.*, 1996). *dspE* required avirulence on soybean when expressed in *P. syringae* pv. *glycinea*. *avrE* could function in *E. amylovora* after transformed this gene into *E. amylovora dspE* mutants on pear (Bogdanove *et al.*, 1998).

Together with AvrB, AvrC, AvrPto and a proteolytic cleavage product of AvrPphB, the AvrRpm1 protein belongs to a subset of AVR proteins containing N-terminal fatty acid acylation motifs (myristoylation and palmitoylation sites). These covalent modifications that occur on a wide variety of cellular signaling proteins such as protein kinases, G proteins, and transmembrane receptors have been shown to promote plasma membrane targeting and binding and to influence protein-protein interactions and cellular signal transduction (Slot, 2002). For AvrRpm1 and AvrB the consensus myristoylation sites were shown to be required for maximal function in virulence and avirulence (Nimchuk *et al.*, 2000). In addition, an epitope-tagged RPM1 that recognizes both AVR proteins was found to be a peripheral membrane protein that likely resides on the cytoplasmic face of the plasma membrane (Boyes *et al.*, 1998). Interestingly, the corresponding R protein Pto also contains a myristoylation site that has been suggested to play a role in signaling (Martin *et al.*, 1993; Slot, 2002).

In bacterial pathogens of animals *vir* genes are frequently part of gene clusters termed pathogenicity islands (PAI) (Hacker *et al.*, 1997). PAIs occupy up to 200-kb of genomic DNA, but have also been found on plasmids (Hu *et al.*, 1998). One of the *vir* genes in the PAI, *virPphA*, was isolated and shown to partially restore virulence towards bean of the plasmid-cured strains. This gene provides an excellent example for a link between *avr* and *vir* gene function because its product was demonstrated to act as an HR activator in soybean (Jackson *et al.*, 1999).

5.3 The AVR proteins from *Xanthomonas*

The key feature of a large family of homologous *avr* genes identified in *Xanthomonas* spp. is a unique structural features including repeats of 102-bp in their central portion, three nuclear localization signals (NLSs), and an acidic activation domain (AAD) in the C-terminal region (Saijo and Paul, 2008).The first *avr* genes identified from

this group is *avrBs2* from *X. axonopodis* pv. *vesicatoria* (Kearney and Staskawicz, 1990). The *avrBs2* gene encodes an 80-kDa protein with homology to the agrocinopine synthase from *A. tumefaciens* and to the glycerophosphoryl diester phosphodiesterase (UgpQ) from *E. coli*. Both enzymes are involved in the synthesis or hydrolysis of phosphodiester linkages suggesting a possible enzymatic function as a phosphodiesterase for the AvrBs2 protein (Swords *et al.*, 1996). This gene matched with *R* gene *Bs2* in pepper (*Capsicum annuum*) that encodes a member of the CC-NBS-LRR-class of R proteins (Tai *et al.*, 1999). Mutation of *avrBs2* gene reduced bacterial growth on susceptible plants. This gene restored *Bs2*-specific resistance and enhanced virulence to *avrBs2* mutants of *X. axonopodis* pv. *vesicatoria* demonstrating functional conservation (Swords *et al.*, 1996). In addition, sequences related to *avrBs2* occur in all strains of *X. axonopodis* pv. *vesicatoria* (Minsavage *et al.*, 1990) and in many other pathovars of *X. axonopodis* (Kearney and Staskawicz, 1990) and *X. oryzae* pv. *oryzae* (Mazzola *et al.*, 1994).

A large family of homologous *avr* genes was identified in *Xanthomonas* spp. (Leach and White, 1996; Vivian and Gibbon, 1997) and named after its first member, *avrBs3*, from *X. axonopodis* pv. *vesicatoria* (Bonas *et al.*, 1989). Although most members of this family were identified based on their avirulence function, several genes encode pathogenicity/virulence factors (Table 3). The central portion was conserved region and comprises varying numbers of repeats in the different proteins specifies resistance on the host plants (Ballvora *et al.*, 2001; Bonas *et al.*, 1993; Bonas *et al.*, 1989; Canteros *et al.*, 1991; De Feyter *et al.*, 1993; Gabriel *et al.*, 1986; Hopkins *et al.*, 1992; Swarup *et al.*, 1991; Yang and Gabriel, 1995). Deletion of repeats results in changes in specificity, although deletion variants of the same length have different specificities suggesting that it is the positions and sequences of the deleted repeats rather than overall length that critically determine the avirulence function (Herbers *et al.*, 1992). In the C-terminal regions of *avrBs3* the predict proteins were identified similar to leucine zippers and three NLSs (van den Ackerveken *et al.*, 1996; Yang and Gabriel, 1995; Zhu *et al.*, 1998). Mutations in all three NLS sequences of *avrXa10* caused a loss in avirulence and virulence activities on rice (Yang *et al.*, 2000; Zhu *et al.*, 1998). Deletion of the putative activation domain in AvrXa10, AvrBs3, and AvrXa7 without removal of the NLSs resulted in the loss of avirulence activity. Therefore, the products of the *avrBs3* gene family are virulence factors that are targeted to host cell nuclei. In contrast, the NLSs of

AvrBs4 are not required for HR elicitation in the tomato host suggesting that it is recognized before reaching the nucleus and, hence, two different pathways are mediating HR induction and virulence (Ballvora *et al.*, 2001).

Moreover, *pthA* from *X. axonopodis* pv. *citri* was identified as *avr* gene and enhanced virulent of bacteria (Swarup *et al.*, 1991). From *X. axonopodis* pv. *vesicatoria* four other *avr* genes, *avrRxv* (Whalen *et al.*, 1993), *avrBsT* (Minsavage *et al.*, 1990), *avrXv3* (Astua-Monge *et al.*, 2000b), and *avrXv4* (Astua-Monge *et al.*, 2000a), were isolated that do not belong to the *avrBs3* family, also fall into this group.

Table 2 Bacterial *avr* genes with demonstrated or presumed virulence activity

Pathogen	Gene	Avr function	Vir function	Reference
P. s. pv. *tomato*	*avr Pto*	HR in Pto tomato plants that also carry Prf	- enhanced growth and necrosis in susceptible tomato lines carrying *Prf*, necrosis expression in tomato	Chang *et al*., 2000; Ronald *et al*., 1992
	avrA	HR elicitor	- small reduction in virulence in tomato	Lorang and Keen, 1995
	avrD	enzyme catalyzing synthesis of HR-eliciting syringolides in RPG4 soybean	- highly conserved non-functional alleles in virulent pathogen races	Lorang *et al*., 1994 Kobayashi *et al*., 1990
	avrE	HR elicitor in soybean and tobacco, functional homolog of dspE	- restores pathogenicity of *dspE*-deficient *E. amylovora,* necrosis upon expression in *A. thaliana*	Kjemtrup *et al*., 2000; Lorang and Keen, 1995; Lorang *et al*., 1994
	avrF		- functional homolog of *dspF*	Bogdanove *et al*., 1998
	avrrRpt2	HR elicitor in RPS2 soybean and RPS2A. thaliana	- growth promotion in *A. thaiana* lacking *RPS2*,more severe disease symtoms	Bent *et al*., 1996; Chen *et al*., 2000; McNellis *et al*., 1998
P. s. pv. *glycinea*	*avrA*	HR elicitor in RPG2 soybean		Staskawicz *et al.,* 1987
	avrB	HR elicitor in RPG1 soybean and RPM1 *A.thaliana*	- chlorosis and browning upon expression in *A. thaliana*	Kjemtrup *et al*., 2000; McNellis *et al*., 1998; Staskawicz *et al*., 1987
	avrC	HR elicitor in RPG3 soybean		Staskawicz *et al.,* 1987
P. s. pv. *maculicola*	*avrRpm1*	HR elicitor in RPM1 *A.thaliana* and RPG1 soybean	- neither growth nor generation of disease symptoms by avrRpm1 mutants on *A.thalina*	Ritter and Dang,1995
P. s. pv. *phaseolicola*	*avrPphB*	HR elicitor in R3 bean and RPS5 *A.thaliana*	- weak browning upon expression in bean	Puri *et al.,* 1997; Stevens *et al*., 1998
	avrPphC	HR elicitor in soybean (avrC homolog)		Yucle *et al*., 1994
	avrPphE	HR elicitor in R2 bean	- highly conserved non-functional alleles in virulent pathogen races, weak browning upon expression bean	Mansfield *et al*., 1994; Stevens *et al*., 1998
	avrPphF	HR elicitor in R1 bean	- confers virulence in bean and soybean	Jackson *et al*., 1999
	virPphA	HR elicitor in soybean	- quantitative contribution to virulence in bean	Jackson *et al*., 1999
P. s. pv. *pisi*	*avrPpiA*	HR in R2 pea	- loss of pathogenicity towards susceptible *A. thaliana* ecotypes	Vivian *et al*., 1989
P. s. pv. *syringae*	*hopPsyA (*syn.*hrmA)*	HR in tobacco	- part of a pathogenicity island	van Dijk *et al*., 1999
Erwinia amylovora	*dspEF (*syn.*dspAB)*	quantitative contribution to HR elicitation in tobacco ,converts *P. syringae* pv. *glycinea* to avirulence in soybean	- required for pathogenicity on pea, apple, cotoneaster	Bogdanove *et al*., 1998; Gaudriault *et al*., 1997
X. a. pv. *vesicatoria*	*avrBs1*	HR in Bs1 pepper	- non-functional allele improves bacterial survival in the soil	Ronald and Staskawicz, 1988
	avrBs2	HR in Bs2 pepper	- strains lacking the gene show reduced virulence	Kearney and Staskawicz,1990
	avrRxv	HR in Rxv bean and in tomato	- member of the YopJ gene family	Ciesiolka *et al*., 1999
	avrXv3	HR in Xv3 tomato	- member of the YopJ gene family	Astua-Monge *et al*., 2000a
	avrXv4	HR in Xv4 tomato	- member of the YopJ gene family	Astua-Monge *et al*., 2000b
	avrBsT	HR in pepper	- member of the YopJ gene family	Ciesiolka *et al*., 1999; Minsavage *et al*., 1990

Table 3 The *avr/pth* gene family from *Xanthomonas* sp.

Pathogen	Gene	Avr function	Refence
X. a. pv. vesicatoria	*avrBs3*	in Bs3 pepper	Bonas *et al*., 1989; Herbers *et al*., 1992
	avrBs4 (syn.avrBs3-2,avrBsP)	in Bs4 tomato	Ballvora *et al*., 2001; Bonas *et al*., 1993; Canteros *et al*., 1991
X. a. pv. *malvacearum*	*avrBn*	in cotton	Gabriel *et al*., 1986
	avrB4	in B1and B4 cotton	De Feyter and Gabriel, 1991
	avrb6	in B1 cotton	De Feyter and Gabriel, 1991
	avrb7	in cotton	De Feyter and Gabriel, 1991
	avrBIn	in cotton	De Feyter and Gabriel, 1991
	avrB101	in cotton	De Feyter and Gabriel, 1991
	avrB102	in B1 cotton	De Feyter and Gabriel, 1991
	avrB103	in cotton	Yang *et al*., 1996
	avrB104	in cotton	Yang *et al*., 1996
	avrB5	in cotton	Yang *et al*., 1996
	pthN		Chakrabarty *et al*., 1997
	pthN2		Chakrabarty *et al*., 1997
X. a. pv. *aurantifolii*	*pthB*		Gabriel *et al*., 1996
	pthB2		Gabriel *et al*., 1996
	pthC		Gabriel *et al*., 1996
	pthC2		Gabriel *et al*., 1996
X. oryzae pv. *oryzae*	*avrxa5*	in xa-5 rice	Hopkins *et al*., 1992
	avrXa7	in Xa-7 rice	Hopkins *et al*., 1992
	avrXa10	in Xa-10 rice	Hopkins *et al*., 1992
X. a. pv. *citri*	*pthA*	in bean and cotton	Gabriel *et al.,* 1986
	avrXc1		Gabriel *et al*., 1986
	avrXc2		Gabriel *et al*., 1986
	avrXc3		Gabriel *et al*., 1986
X. phaseoli	*avrXp1*		Gabriel *et al.,* 1986

5.4 Harpins

Harpins is the bacterial effector proteins trigger plant defense by HR induction. They are heat stable, rich in glycine and/or serine, lack cysteine, and differ in their primary sequences. They lack an N-terminal signal peptide and are secreted via the TTSS, presumably into the extracellular compartment, not into the host cytoplasm (Slot, 2002). The first harpins was identified in *E. carotorova*, the causal agent of fire-blight disease on many rosaceous plants, such as apple and pear (Wei *et al.*, 1992). The *hrpNEa* is located within the *hrp* gene cluster of *E. carotorova* encoding harpinEa and also a homologous gene, *hrpNEch* was identified in the soft-rot pathogen *E. chrysanthemi* (Bauer *et al.*, 1995). The *hrpNEa* mutants were nonpathogenic to pear (Wei *et al.*, 1992) and the *hrpNEch* mutants were reduced ability to infections in witloof chicory leaves. Therefore, it can be concluded that both genes involved in pathogenicity or virulence of bacteria. In contrast, *popA* gene in the *R. solanacearum* tomato pathogen secreted PopA1 and its degradation derivative PopA3 are structurally dissimilar to the *Erwinia* spp. *hrpN* products (Arlat *et al.*, 1994). Also, *popA* mutant did not effect with pathogenicity, indicating that this gene is not essential for pathogenicity. In *E. amylovora* and *P. syringae* pv. *tomato* a second harpin was identified that is encoded by the *hrpW* gene (Charkowski *et al.*, 1998; Kim and Beer, 1998). The HrpW proteins are composed of two domains, an N-terminal harpin-like domain with sequence similarity to HrpN, HrpZ, and PopA and a C-terminal domain homologous to pectate lyases from plant pathogenic fungi and from *E. carotorova* (Kim and Beer, 1998). In the presence of Ca^{2+} HrpW was shown to bind to pectate. However, no enzyme activity could be detected and the pectate lyase domain is not required for HR elicitation (Charkowski *et al.*, 1998). Nevertheless, these findings support the notion that this type of harpin has a site of action in the plant cell walls (Hoyos *et al.*, 1996), possibly helping the Hrp pilus (Roine *et al.*, 1997) to pass through (Slot, 2002). Kim *et al.* (2003) sequenced 29-kb region including *hrp* and *hrc* (*hrp* and conserved) genes from *X. axonopodis* pv. *glycines*. The *hrp* and *hrc* mutants failed to induce HR in pepper plant but induced HR in tomato plant tested. Moreover, HpaG acting as a TTSS effector protein elicited HR in pepper, tobacco, and *Arabidopsis thaliana* ecotype Cvi-0 has been found.

6. Plant resistance genes

The elicitor-receptor model has been employed to explain the recognition of a pathogen by a host plant based on the *Avr-R* gene interaction (Keen, 1990; Lamb, 1996; De Wit, 1997). This model predicts that microbial *Avr* gene products directly or indirectly interact with receptors encoded by host *R* genes (Slot, 2002). The phenotype of the plant secreted R protein response to AVR signal from bacterial pathogen is characterized by a HR. Other typical resistance reactions include a burst of active oxygen species, the stimulation of ion fluxes, changes in protein phosphorylation, and the synthesis of pathogenesis-related proteins (Ebel and Mithöfer, 1998; Hutcheson, 1998; Bolwell, 1999; Grant and Loake, 2000; Sessa *et al.,* 2000; Muthukrishnan *et al.*, 2001; Slot, 2002).

In 1992, the first *R* gene was isolated encoding an enzyme that inactivates a host-specific toxin (Johal and Briggs, 1992). Since then more than thirty *R* genes have been cloned from various plants (Bent, 1996; Hammond-Kosack and Jones, 1997; Martin, 1999; Takken and Joosten, 2000). The structure of *R* gene products show remarkable similarities although effective against a variety of different pathogens such as viruses, bacteria, Oomycetes, fungi, nematodes and even aphids, most of them containing a leucine-rich repeat (LRR) domain along with a small number of other putative signaling domains (Ellis and Jones, 1998; Jones and Jones, 1997; Martin, 1999; Michelmore and Meyers, 1998; Takken and Joosten, 2000; van der Biezen and Jones, 1998b) (Table 1). In R proteins they are believed to directly interact with AVR factors, thereby determining recognition specificity (Bent, 1996; Jones and Jones, 1997; Martin, 1999). Ligand-induced conformational changes in the R proteins may enable the cleavage of ATP or GTP, which then allows the N-terminal domains to activate downstream effectors (van der Biezen and Jones, 1998a).

The majority of *R* genes cloned encodes cytoplasmic proteins of the NBS-LRR type (Table 1). These can be divided into two major groups (Pan *et al.*, 2000a). Group I NBS domains are always linked with an N-terminal domain with similarity to the cytoplasmic portion of the *Drosophila* Toll and the mammalian interleukin-1 receptor (TIR). The latter proteins are involved in innate immunity in the respective organisms suggesting an evolutionary relationship between plant and animal basic related systems

(Aderem and Ulevitch, 2000; Aravind *et al.*, 1999; Cohn *et al.*, 2001). The N-terminus of group II NBS domain proteins potentially adopts coiled-coil (CC) structures, i.e., bundles of α-helices showing a distinctive packing of amino acid side chains at their interfaces. A subset of the CC domains represents putative leucine zipper domains. This suggests the presence of at least two distinctive basic signaling pathways mediated by the TIR and CC domains, respectively (Aarts *et al.*, 1998; Parker *et al.*, 2000). Group II genes were detected in mono- and dicotyledonous species. In contrast, group I genes appear to lack from the genomes of major cereal species, indicating divergent evolution of R genes in the major groups of land plants (Pan *et al.*, 2000b). One of the first cloned *R* genes, *Pto*, encodes a protein deviating from most R proteins by the absence of LRRs (Martin *et al.*, 1993). Instead, the gene product is a serine/threonine-specific protein kinase that is part of a plant defense-signaling cascade. Only relatively few *R* genes isolated to date encode transmembrane proteins with extracellular LRR domains. Of these, however, only the product of the rice *Xa21* gene, a transmembrane receptor protein kinase, complies with the original expectation of a surface-localized AVR receptor with a cytoplasmic signal transduction domain (Song *et al.*, 1995; Slot, 2002).

7. Bacterial motility genes

7.1 Swimming and flagella associated genes

Swimming motility mediated by extracellular flagella that responsible for motility and early interactions with hosts is an example of a motor functioning at the molecular level (Vande Broek and Vanderleyden, 1995; Moens and Vanderleyden, 1996; Finley and Falkow, 1997; Minamino and Namba, 2004; Honko and Mizel, 2005; Journet *et al.*, 2005; Khater *et al*., 2007). The structural component of flagella consists of three basic parts: a basal body, a hook, and a filament (Schuster and Khan, 1994). The flagellum exports its own extracellular component proteins and assembles them at a distant tip and, together with its cognate sensory transduction system, receives sensory information from the cytoplasm which controls the direction of its high-speed rotation, a process critical for chemotaxis (Macnab, 2003; Khater *et al*., 2007). The filament of a flagellum is a tubular structure made up of 11 protofilaments, which are nearly longitudinal helical arrays of many hundred; 45-kDa flagellin molecules. The long helical

filament of the flagellum is formed by about 30,000 subunits of flagellin (FliC) and is capped at its extremity by a hook-associated protein, FliD, which acts as a nucleation point for FliC monomers to polymerize into the growing filament (Vonderviszt *et al*., 1998). The filament is attached to a flexible hook (FlgE), extending from the cell surface (Aizawa, 1996) by two hook-associated proteins FlgK and FlgL (Jones *et al*., 1990; Fahrner *et al*., 1994). Flagellum assembly is sequential: FlgK and FlgL rings must be present before FliD can polymerize, and the FliD cap must be in place before FliC can be incorporated (Kubori *et al*., 1992). During assembly of the flagellum on the bacterial cell surface, structural proteins are exported via a flagellum specific export pathway and polymerize to form contiguous substructures (Moens and Vanderleyden 1996; Journet *et al*., 2005). In addition to its role in motility, flagellin is a known elicitor of animal innate immune responses mediated by toll-like receptors (Pfund *et a*l., 2004). Flagellins are a PAMP that is recognizable by the innate immune systems of plants and animals (Gomez-Gomez and Boller, 2002; Gomez-Gomez, 2004; Nurnberger *et al*., 2004; Ramos *et al*., 2004). Although most flagellin is assembled into flagella, flagellin can also leak into the bacterial environment during the construction of flagella (Komoriya *et al*., 1999), and flagellin is a component of the detritus associated with a bacterial colony. However, nonmotile pathogens can still caused disease symptoms, flagella motility is essential for the overall pathogenicity of bacterial plant pathogens (Panopoulos and Schroth, 1974; Haefele and Lindow, 1987).

In *Arabidopsis* seedlings, *flg22* peptide can elicit rapid defense-associated responses such callose deposition, an oxidative burst, and induction of PR proteins gene expression (Felix *et al*., 1999; Gomez-Gomez *et al*., 1999). Some bacterial species produce a flagellin that is not recognized by host flagellin detection systems. Although the flg22 region of bacterial flagellins is highly conserved, there are a few variable positions, and the plant-associated bacteria *A. tumefaciens*, *Sinorhizobium meliloti*, and *R. solanacearum* diverge at a sufficient number of flg22 residues that peptides based on the flagellins from these species fail to elicit plant responses (Felix *et al*., 1999; Pfund *et al*., 2004).

7.2 Type IV pilus assembly and twitching motility genes

Infection is initiated with bacterial attachment to and colonization of host tissues via surface structures and appendages. Specifically, type IV pili may contribute to bacterial pathogenesis by affecting adhesion, twitching mobility on a solid surface, and secretion or interaction with host tissues (Craig *et al*., 2004). Twitching motility is a means of flagella-independent bacterial movement over moist surfaces (Li *et al*., 2007). Twitching motility plays an important role in host colonization by several animal pathogens (Mattick, 2002). Approximately 40 genes have been identified that are involved in the biogenesis and function of type IV pili in *P. aeruginosa* (Mattick, 2002), including the genes that encode the major structural protein (PilA), and those that encode the minor proteins involved in formation of the base and/or tip of the pilus, e.g. PilE, PilV, PilW, PilX, PilY1, PilY2 and FimT. A number of other proteins are required for pilus assembly and retraction, e.g. PilB, PilC, PilD, PilF, PilM, PilN, PilO, PilP, PilQ, PilT and PilU (Li *et al*., 2007). Genomic annotation revealed that at least 26 genes are related to type IV pili assembly and are highly conserved in *X. campestris* pv. *campestris* 8004 and *Xcc* ATCC 33913 genomes (Qian *et al*., 2008). *X. axonopodis* pv. *citri* has four gene clusters and two separately located genes that are predicted to be involved in type IV pilus biosynthesis and regulation. Two genes encoding proteins called fimbrillins, FimA, gi|21243966 and gi|21243967 (85% similar in predicted amino acid sequence), are located within one of the clusters, and a gene designated *pilA* elsewhere in the genome is similar to type II pilin (PilE) from *Neisseria meningitidis*. The sequence of the two *fimA* genes is similar to *pilA* from other bacteria and they are located in a cluster of genes containing other type IV pilus genes *pilB*, *pilC*, *pilD*, *pilR,* and *pilS*. The gene products of *pilS* and *pilR* are homologous to a two-component sensor protein and its corresponding regulatory protein, respectively, and control the expression of *pilA* (Hobbs *et al*., 1993; Wu and Kaiser, 1997). The major subunit of the type IV pilin is first exported by the general secretory pathway (GSP). It has a short, basic N-terminal signal sequence, unique to type IV pilus proteins. PilD is a specific leader sequence peptidase that removes the signal sequence of PilA and other type IV pilus biosynthesis proteins, and methylates the new N-terminus (Finlay and Falkow, 1997; Russel, 1998). Mature, translocated pilin polymerizes at the plasma membrane, and the pilus is pushed through the central cavity of the outer membrane secretin (Russel, 1998). The pilus biogenesis machinery and

assembly is highly conserved in bacteria (Hultgren *et al*., 1993). Their assembly genes are similar to type II secretion genes, but the N-terminal signal sequences are different (Russel, 1998). Type IV pili (also called fimbriae) have been proposed to attach bacterial pathogens to the host cell wall (Farinha *et al.,* 1994; Kang *et al.,* 2002) and retract (Skerker and Berg, 2001; Wall and Kaiser, 1999), pulling the bacterium closer to the host cell (Wall and Kaiser, 1999).

In addition to twitching motility, cell attachment and biofilm formation are thought to be important factors in pathogen virulence (Li *et al*., 2007). Type IV pili have been shown to be important for the virulence of *R. solanacearum* (Kang *et al*., 2002) and *P. aeruginosa* (Hahn, 1997). Mutations were identified in two of these pili assembly genes, *pilB* and *pilC*, which encode multidomain proteins driving energy-prone polymerization of pilin (Qian *et al*., 2008). Disruption of *pilY1* in *Xylella fastidiosa* caused a reduction, but not a complete loss, of type IV pili and twitching motility (Li *et al.,* 2007). Mutants of *pilQ* and *pilM*, respectively, resulted reside at either end of an operon conserved across several species and required for pilus assembly, twitching motility, and phage sensitivity (Rumszauer *et al.*, 2006). PilZ is a predicted receptor for the secondary messenger bis-(3'-5')-cyclic dimeric GMP, which regulates processes such as biofilm formation, twitching motility, photosynthesis, and virulence (Amikam and Galperin, 2006; Brouillette *et al*., 2005; Galperin *et al*., 2001). *pilT* encodes a putative hexameric ATPase required for type IV pilus retraction (Aukema *et al.,* 2005). This gene cluster involved, whether attachment, motility, biofilm formation, or some combination, remains to be determined (Wang *et al*., 2008).

MATERIALS AND METHODS

1. Bacterial strains, plasmids, and recombinant techniques

Strains of *X. axonopodis* pv. *glycines* and *Escherichia coli*, and plasmids used in this study were listed in Table 4 and 6. Thirty four strains of *X. axonopodis* pv. *glycines* were initially isolated and characterized from infected soybean plants collected in various Thai provinces by Saiseangthong (1999) except strains KU-P-34040, KU-P-34069, KU-P-34072, KU-P-34093, and ST-015 which were derived from Tusanasarit (1995). The rest strains of *X. axonopodis* pv. *glycines* were collected from important soybean growing area in Thailand during 2005-2007. Briefly, infected leaves were washed with 95% ethanol for 5 min and 3 times of sterile distilled water (SDW) for 3 min per each time. After rinsing in SDW, the tissue was ground in a droplet of SDW in a pertri plate and streaked onto nutrient glucose agar (NGA) (3.0 g of beef extract, 5.0 g of bacto peptone, 2.5 g of glucose, and 15.0 g of agar) with a wire loop and incubated at 28°C for 24 - 48 h. A yellow-pigmented bacterium of single colony was streaked again onto new NGA or nutrient yeast extract (NBY) agar (8.0 g of nutrient broth, 2.0 g of yeast extract, 2.0 g of K_2HPO_4, 0.5 g of KH_2PO_4, 2.5 g of glucose, and 15.0 g of agar, after autoclaving, add 1.0 ml of a sterile solution of 1 M $MgSO_4.7H_2O$) (White and Gonzalez, 1995) at 28°C for pathogenicity proof on SJ4 cultivar of susceptible soybean (Prathuangwong and Khandej, 1998) or for next experiment. *E. coli*, used as the cloning host, was cultured in Luria Bertani (LB) agar (10.0 g of bacto typtone, 5.0 g of yeast extract, 10.0 g of NaCl, and 15.0 g of agar) at 37°C. The cultures were stored in 50% glycerol solution at -80°C and used for all studies. Antibiotics were added to media at concentrations: ampicillin, 100 μg/ml; kanamycin, 15 or 50 μg/ml; gentamicin, 25 or 40 μg/ml; tetracycline 15 μg/ml. All DNA manipulations, including the DNA isolation, plasmid extractions, restriction digests, ligations, and gel electrophoresis were performed as described previously (Sambrook *et al*., 1989).

Table 4 Bacterial strains and plasmids

Bacterial strain or plasmid	**Relevant characteristic** [1/]	**Reference or source**
Escherichia coli DH5α	F′ 80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1/F′ *pro*AB+ *lac*IqZΔM15 zzf::Tn*5*, Km^r	Invitrogen
E. coli TOP10	lacZΔM15, endA1, recA1, *hsd*R, mcrA	Invitrogen
E. coli 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}, *lac*ZΔM15]	Promega
E. coli BL21	*E. coli* B F^- *dcm* omp^T *hsdS* (r_B^- m_B^-) *gal* λ(DE3) [Cam]	Invitrogen
E. coli BL21 (pET-AVR)	*E. coli* BL21 carrying pET-AVR	This study
E. coli BL21 (pRSETB-AVR)	*E. coli* BL21 carrying pRSETB-AVR	This study
E. coli TOP10 (pUC19-AVR)	*E. coli* TOP10 carrying pUC19-AVR	This study
E. coli TOP10 (pUC19-FLGC)	*E. coli* TOP10 carrying pUC19-FLGC	This study
E. coli TOP10 (pUC19-FLGK)	*E. coli* TOP10 carrying pUC19-FLGK	This study
E. coli TOP10 (pUC19-PILD)	*E. coli* TOP10 carrying pUC19-PILD	This study
E. coli TOP10 (pBBR-AVR)	*E. coli* TOP10 carrying pBBR-AVR	This study
E. coli TOP10 (pBBR-FLGC)	*E. coli* TOP10 carrying pBBR-FLGC	This study
E. coli TOP10 (pBBR-FLGK)	*E. coli* TOP10 carrying pBBR-FLGK	This study
E. coli TOP10 (pBBR-PILD)	*E. coli* TOP10 carrying pBBR-PILD	This study
X. axonopodis pv. *glycines*		
KU-P-SW005	Race 3	Nakhon Rachasima
KU-P-SW005 *avrXg1*::Tn5AAD	*avrXg1*::Tn5 of KU-P-SW005 by random mutagenesis	This study
avrXg1::Tn5AAD (pBBR-AVR)	*avrXg1*::Tn5 of KU-P-SW005 carrying pBBR-AVR	This study
KU-P-SW005 *avrXg1*::Tn5REP	*avrXg1*::Tn5 of KU-P-SW005 by site-directed mutagenesis	This study
avrXg1::Tn5REP (pBBR-AVR)	*avrXg1*::Tn5 of KU-P-SW005 carrying pBBR-AVR	This study
KU-P-34070	Race 1	Sukhothai
KU-P-34070 (pBBR-AVR)	Race 1 carrying pBBR-AVR	This study
KU-P-KPS06	Race 2	Nakhon Prathom

Table 4 (Continued)

Bacterial strain or plasmid	**Relevant characteristic** [1/]	**Reference or source**
KU-P-KPS06 (pBBR-AVR)	Race 2 carrying pBBR-AVR	This study
KU-P-SW005 *flgC*::Tn5	*flgC*::Tn5 of KU-P-SW005 by site-directed mutagenesis	This study
flgC::Tn5 (pBBR-FLGC)	*flgC*::Tn5 of KU-P-SW005 carrying pBBR-FLGC	This study
KU-P-SW005 *flgK*::Tn5	*flgK*::Tn5 of KU-P-SW005 by site-directed mutagenesis	This study
flgK::Tn5 (pBBR-FLGK)	*flgK*::Tn5 of KU-P-SW005 carrying pBBR-FLGK	This study
KU-P-SW005 *pilD*::Tn5	*pilD*::Tn5 of KU-P-SW005 by site-directed mutagenesis	This study
pilD::Tn5 (pBBR-PILD)	*pilD*::Tn5 of KU-P-SW005 carrying pBBR-PILD	This study
KU-P-SW005 *flgC-pilD*::Tn5	*flgC* and *pilD* ::Tn5 of KU-P-SW005 by site-directed mutagenesis	This study
Plasmids		
pBBR1MCS-5	Broad host range cloning vector, *lacZ*, Gm^r	Kovach *et al.*, 1995
pUC19	ColE1; *lacZ*, Amp^r	Promega
pGEM-T easy	PCR cloning vector, *lacZ*, Amp^r	Promega
pET160/GW/D-TOPO	Expression plasmid	Invitrogen
pRSETB	Expression plasmid	Invitrogen
pRSETB-AVR	3.8-kb *avrXg1* fragment from KU-P-SW005 cloned in pRSETB, Amp^r	This study
pET-AVR	3.8-kb *avrXg1* fragment from KU-P-SW005 cloned in pET160/GW/D-TOPO, Amp^r	This study
pUC19-AVR	5.0-kb *avrXg1* fragment from KU-P-SW005 carrying Tn5 insertion cloned in pUC19, Km^r Amp^r	This study
pUC19-FLGC	1.6-kb *flgC* fragment from KU-P-SW005 carrying Tn5 insertion cloned in pUC19, Km^r Amp^r	This study

Table 4 (Continued)

Bacterial strain or plasmid	**Relevant characteristic** [1/]	**Reference or source**
pUC19-FLGK	3.1-kb *flgK* fragment from KU-P-SW005 carrying Tn5 insertion cloned in pUC19, Km^r Amp^r	This study
pUC19-PILD	2.5-kb *pilD* fragment from KU-P-SW005 carrying Tn5 insertion cloned in pUC19, Tet^r Amp^r	This study
pBBR-AVR	3.8-kb sequence of *avrXg1* in the pBBR1MCS-5, Km^r Gm^r	This study
pBBR-FLGC	408-bp sequence of *flgC* in the pBBR1MCS-5, Km^r Gm^r	This study
pBBR-FLGK	1.87-kb sequence of *flgK* in the pBBR1MCS-5, Km^r Gm^r	This study
pBBR-PILD	863-bp sequence of *pilD* in the pBBR1MCS-5, Tet^r Gm^r	This study

[1/] Km^r, kanamycin resistance; Amp^r, ampicillin resistance; Gm^r, gentamycin resistance; Tet^r, tetracycline resistance.

2. Race-cultivar specificity

All strains of *X. axonopodis* pv. *glycines* were evaluated for pathogenicity on 15-day old soybean cultivars including Williams82 (resistant), Spencer (susceptible), PI 520733 (susceptible) (Kanazin *et al*., 1996; Hwang and Lim, 1998; Goradia *et al*., 2004), and SJ4 (Prathuangwong and Khandej, 1998). The resistance gene, *rxp*, for bacterial pustule was originally identified in CNS (resistant) and subsequently transferred to other commercial cultivars including Williams82 (Kanazin *et al*., 1996). A leaf infiltration technique under greenhouse conditions was used to assess virulence on soybean leaves. Infiltrations were done in a completely randomized design (CRD). Fifty µl of a bacterial suspension (OD_{600} = 0.2, ca. 10^8 cfu per ml) was infiltrated into the leaf mesophyll through a pin puncture with 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 h post-infiltration. The experiments were repeated three times.

3. Identification of *avr* genes in *X. axonopodis* pv. *glycines*

3.1 Random mutagenesis

Random mutation of *X. axonopodis* pv. *glycines* was made by using the EZ-Tn5™<KAN-2>Tnp Transposome™ (Epicentre, WI, USA). The EZ-Tn5™ <KAN-2>Tnp Transposome™ was the stable complex formed between the EZ-Tn5 transposase enzyme and the EZ-Tn5 <KAN-2> transposon. The EZ-Tn5 <KAN-2> transposon contains the Tn903 kanamycin resistance gene (Kan^R) that is functional in *E. coli*, flanked by hyperactive 19-bp mosaic end EZ-Tn5 transposase recognition sequences. The EZ-Tn5 transposome can be electroporated into living cells where the EZ-Tn5 transposase is activated by Mg^{2+} in the host's cellular environment resulting in random insertion of the EZ-Tn5 transposon into the host genomic DNA. The primers provided in the kits were used for sequencing. The primers can be used for bi-directional DNA sequencing or mapping of transposon insertion sites in target genomic DNA, including direct sequencing from genomic DNA without cloning (Fig. 1).

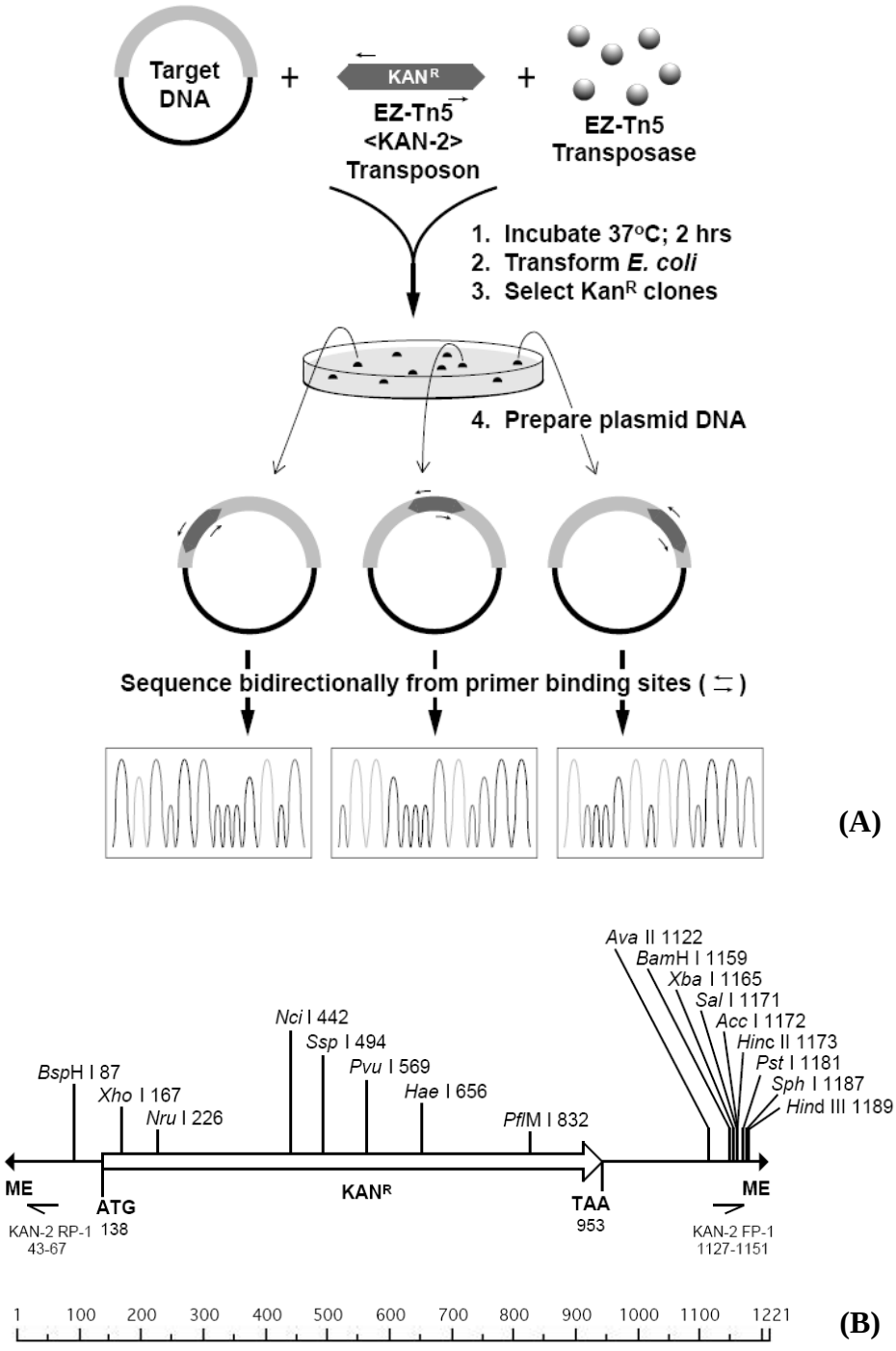

Figure 1 The protocol of EZ-Tn5^{TM}<KAN-2>Tnp Transposome^{TM} (Epicentre, WI, USA) generated the *Xanthomonas axonopodis* pv. *glycines* mutants (A) and EZ-Tn5 Transposon map (B).

3.1.1 *X. axonopodis* pv. *glycines* electrocompetent cell preparation

An electrocompetent cell of wildtype strain KU-P-SW005 from Race 3 was modified as previously described by Kaewnum *et al.* (2006). Briefly, KU-P-SW005 was grown on NBY plates at 28°C for 24 h and suspended in 55 ml of NBY broth without glucose or $MgSO_4$ and incubated at 28°C on a rotary shaker for 16 - 18 h at 225 rpm to obtain a cell density of OD_{600} = 0.50 - 0.65. Subsequently, cells were concentrated by centrifugation at 4,000 g at 4°C for 20 min. The bacterial pellet was washed in 0.5 and 0.25 volume respectively (25 ml and 12.5 ml respectively) of cold SDW and vortexes well to resuspend cells before pelleting again by centrifugation at 4,000 g at 4°C for 20 min. Cells were resuspended in 10 ml of cold 10% sterile glycerol, concentrated by centrifugation at 4,000 g at 4°C for 10 min, and resuspended in 10 ml of cold 10% sterile glycerol and concentrated again. The cells were resuspended in 500 µl of cold 10% sterile glycerol and transferred to a sterile microcentrifuge tube to yield a final cell concentration of 50 µl per tube and kept on ice. The competent cells were used directly for electroporation or stored at -80°C for future use.

3.1.2 Electroporation

Random mutagenesis of KU-P-SW005 was performed as described previously by Kaewnum *et al*. (2006). Briefly, 1 µl of EZ-Tn5™<KAN-2>Tnp Transposome™ was added to 50 µl of electrocompetent cells, and 0.5 µl of TypeOne™ restriction inhibitor (Epicentre, WI, USA); mixtures were placed in a 0.25 cm electroporation cuvette on ice. The cells were electroporated with a BioRad GenePluser Xcell electroporation system (Hercules, CA, USA) at 2.5 kV, 25 µF, and 200 Ω and were then added to fresh 1 ml of NBY broth and incubated at 28°C for 1 h. Cells were then plated on NBY medium supplemented with kanamycin 50 µg/ml. Resistant clones were screened for their ability to cause the disease or HR on different soybean cultivars as described above and were subsequently verified for EN-Tn5 insertion by PCR with KAN-1-FP and KAN-1-RP (Table 5).

3.2 Site-directed mutagenesis

A targeted mutation in the *avrBs3* homolog in KU-P-SW005 was accomplished by using the EZ-Tn5™ <KAN-2> Insertion Kit (Epicentre, WI, USA) according to the supplier's direction (Fig. 1). First a 3.8-kb fragment was amplified from KU-P-SW005 by using primers AVR-FP 5' ATGGATCCCATTCGTTCG 3' and AVR-RP 5' TCACTGAGGCAATAGCTCCA 3' that are based on the sequence of the *avrBs3* homolog (AY780632.1) previously identified in *X. axonopodis* pv. *glycines* strain AG1 (Kim *et al*., 2006).

3.2.1 Chromosomal DNA extraction

Total genomic DNA of wildtype strain KU-P-SW005 was extracted by UltraClean™ Microbial DNA Isolation Kit Components (MO BIO, CA, USA). Briefly, 1.8 ml of bacterial culture was added to a microcentrifuge tube and centrifuged at 10,000 g for 30 sec at room temperature (28±2°C). The supernatant was decanted and the tubes were spun at 10,000 g for 30 sec at room temperature and the media supernatant was removed completely with a pipette tip. The cell pellet was resuspended in 300 µl of MicroBead solution and gently vortexed to mix. Resuspended cells were transferred to MicroBead tube. Fifty µl of solution MD1 was added to the MicroBead tube. The tubes were centrifuged at 10,000 g for 30 sec at room temperature. The supernatant was transferred to a clean microcentrifuge tube. A hundred µl of solution MD2 was added to the supernatant and vortexed 5 sec. Then incubated at 4°C for 5 min and the tubes were centrifuged at room temperature for 1 min at 10,000 g. The entire volume of supernatant was transferred to a clean microcentrifuge tube. Nine hundred µl of solution MD3 was added to the supernatant and vortexed 5 sec. About 700 µl of the supernatant was loaded into the spin filter and centrifuged at 10,000 g for 30 sec at room temperature. The flow through was discarded, and the remaining supernatant was added to the spin filter, and centrifuged at 10,000 g for 30 sec at room temperature. A total of 2 to 3 loads for each sample processed are required. All flow through liquid was discarded. Three hundred µl of solution MD4 was added and centrifuged at room temperature for 30 sec at 10,000 g. The flow through was discarded and centrifuged at room temperature for 1 min at 10,000 g. Being careful not to splash liquid on the spin filter basket, spin filter was placed in a

new microcentrifuge tube. Fifty µl of solution MD5 was added to the center of the white filter membrane. Centrifuged at room temperature for 30 sec at 10,000 g. Discarded spin filter. The DNA in the tube is now ready for any downstream application. The DNA was recommend to store in DNA frozen (-20°C).

3.2.2 PCR analysis *avrBs3* homolog in *X. axonopodis* pv. *glycines*

Genomic DNA of KU-P-SW005 was amplified with AVR-FP 5' ATGGATCCCATTCGTTCG 3' and AVR-RP 5' TCACTGAGGCAATAGCTCCA 3' (Table 5). DNA was amplified in a total volume of 50 µl containing 5 µl of AccuTaq LA 10 X buffer (500 mM Tris-HCl, 150 mM ammonium sulfate (pH 9.3, adjusted with KOH), 25 mM $MgCl_2$ 1% Tween 20), 1 µl of 10 pmol of each primer, 2.5 µl of dNTP mixed, 0.5 µl of 100 ng of purified total bacterial DNA, 37.5 µl of SDW, and 2.5 µl of REDAccuTaq LA DNA Polymerase Mix (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM ethylenediaminetetraacetic acid: EDTA, 1 mM dithiothreitol: DTT, 0.5% Tween® 20, 0.5% IGEPAL® CA-630, 50% glycerol, inert dye) (Sigma, USA). A total of 30 amplification cycles were performed in an automated thermocycler (P-100). Each cycle consisted of 20 sec of denaturation at 95°C, 20 sec of annealing at 58°C, 6 min of extension at 65°C, and the last extension step at 68°C was extended to 20 min. Amplified DNA was determined by agarose gel electrophoresis on a horizontal, 0.7% agarose gels in Tris/Borate/EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) at 70 volts constant for 4 h using 1X TBE as a running buffer. The gel was stained with 0.5 µg of ethidium bromide per ml for 10 min and briefly then destined in water before visualizing under UV light and photographed over a transilluminator (GDS 800, Complete Gel Documentation Analysis System).

3.2.3 Cloning and construction of *avrBs3* homolog

The 3.8-kb fragment containing the coding region of *avrBs3* homolog was cloned into pGEM-T easy vector (Promega, WI, USA). Briefly, 5 µl of 2X rapid ligation buffer, 1 µl of pGEM-T easy vector (50 ng), 3 µl of PCR product, and 1 µl of T4 DNA ligase were mixed by pipetting and the reaction was incubated overnight at 4°C. Two µl of mixture reaction was added to 50 µl of *E. coli* JM109 competent cell (Promega,

WI, USA) and gently mixed and chilled on ice for 20 min. The cell was heat shock for 45 sec in a water bath at exactly 42°C. The tubes were returned on ice immediately for 2 min. Nine hundred and fifty µl room-temperature SOC medium [2.0 g of Bacto®-tryptone, 0.5 g of Bacto®-yeast extract, 1 ml of 1 M NaCl, 0.25 ml of 1 M KCl, 1 ml of 2 M Mg^{2+} stock (filtersterilized), 1 ml of 2 M glucose (filtersterilized)] was added to the tubes containing cells transformed with ligation reactions. The cells were incubated for 1.5 h at 37°C with shaking at 225 rpm. Then plated onto the LB amended ampicillin 100 µg/ml, IPTG (100 µg/ml), and X-gal (40 µg/ml). The transformed colonies with white color were selected for further determined.

The recombinant plasmids from the white colonies were extracted by UltraClean™ 6 Minute Mini Plasmid Prep Kit (MO BIO, CA, USA). Briefly, bacterial cells were grown in liquid LB amended ampicillin 100 µg/ml at 37°C overnight. Up to 2 ml of culture was added in microcentrifuge tube, cells were spun, the supernatant was discarded, and more cultures were added to the same tubes and spun again. The reaction was repeated until 5 ml worth of cells have been processed and centrifuged for 1 min at 15,000 rpm. The supernatant was decanted by inverting the tube and pouring into a waste container and then centrifuged 5 sec at 15,000 rpm. All visible liquid was removed with a narrow pipette tip. Fifty µl of solution 1 was added to each cell pellet tube and the tubes were closed and the tube tip was held on the vortex head for 10 sec. A hundred µl solution 2 was added to the cell suspension and all tubes were closed. The tubes were inverted gently once to mix, 325 µl solution 3 was added and closed the tubes and gently inverted once to mix and centrifuged for 1 min at 15,000 rpm. All of the clear liquid supernatant was transferred to a spin filter and centrifuged full speed 30 sec. The plastic filter basket was lift out from the collection tube. Three hundred µl of solution 4 was added to the spin filter and centrifuged 30 sec at 15,000 rpm. Flow through liquid was discarded from the collection tube, and centrifuged again for 5 sec; spin filter basket was placed in a new collection tube. Fifty µl of solution 5 or SDW was added directly in the middle of the white spin filter membrane and centrifuged 30 sec at 15,000 rpm. Filter basket was removed and tube lid was closed. Plasmid DNA in the collection tube is now ready to use for any application.

The pUC19 vector (Promega, WI, USA) and the 3.8-kb sequence comprising *avrXg1* in pGEM-T easy vector were digested with *Eco*RI (no restriction site in *avrBs3* homolog gene). Total volume 20 µl mixed of 3 µl of recombinant plasmids or pUC19, 1 µl of *Eco*RI, 2 µl of *Eco*RI buffers, and 14 µl of SDW was incubated in water bath for 2 h at 37°C. The 3.8-kb of *avrBs3* homolog and pGEM-T easy vector was separated by gel electrophoresis. An *avrBs3* homolog fragments with size in the vicinity of 3.8-kb were recovered from agarose gel by GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Biosciences, NJ, USA). Briefly, the gel slices was added to 10 µl of capture buffer for each 10 mg of gel and mixed by vortex vigorously and incubated at 60°C until the agarose is compleately dissolved (5-15 min). The sample was transferred to the GFX column and centrifuged in a microcentrifuge at full speed for 30 sec. The flow-through was discarded by emptying the collection tube. The GFX column was placed back inside the collection tube and added 500 µl of wash buffer to the column and centrifuged at full speed for 30 sec. The collection tube was discarded and transferred the GFX column to a fresh 1.5 ml microcentrifuge tube. Fifty µl of elution buffer (10 mM Tris-HCl pH 8.0, TE pH 8.0) or SDW was applied directly to the top of the glass fiber matrix in the GFX column. The sample was incubated at room temperature for 1 min and centrifuged at full speed for 1 min to recover the purified DNA for use in the next step. The pUC19 digested by *Eco*RI were purified by the same kit and dephosphorylated with a restriction enzyme, calf intestinal alkaline phosphatase (CIAP) catalyzes. Briefly, 30 µl of DNA, 8 µl of CIAP 10X reaction buffer, 1 µl of CIAP, and 41 µl of SDW were mixed and incubated at 37°C for 1 h. After that 1 µl of CIAP was added and continued incubation at 37°C for an additional 30 min. DNA was purified from solution again. An *avrBs3* homolog 3.8-kb fragment and pUC19 vector digested with *Eco*RI and purified were separated by gel electrophoresis to confirm that correct size.

The target fragment size 3.8-kb of *avrBs3* homolog was ligated into *Eco*RI restriction site of pUC19 by DNA Ligation Kit <Mighty Mix> (Takara Bio Inc., USA). Briefly, 4 µl of pUC19 *Eco*RI digested, 6 µl of *avrBs3* homolog 3.8-kb fragment, and 10 µl of ligation mix were mixed by pipetting and incubated the reaction for 30 min at 16°C. Then the reaction mixture was transformed to *E. coli* TOP10 (Invitrogen, CA, USA) by heat shock technique, and recombinant plasmid was extracted as same as described above and sequenced. Subsequently an EN-Tn5 targeted insertion in *avrBs3* homolog was made

in vitro following to the supplier's directions. Briefly, 1 µl of pUC19 containing the *avrBs3* homolog was mixed with 0.5 µl of EZ-Tn5 <KAN-2> Transposon, 1 µl of EZ-Tn5 10X reaction buffer, 1 µl of EZ-Tn5 Transposase, and 6.5 µl of SDW; the mixture was transformed by heat shock into *E. coli* TOP10 again. Clones were selected on LB agar amended with kanamycin 50 µl/ml and ampicillin 100 µl/ml. Selection of the pUC19-AVR with single EN-Tn5 insertion in the middle of the target gene was shown by plasmid extraction and digestion with *Eco*RI and separation by gel electrophoresis as described above. The pUC19-AVR construction was confirmed by PCR with primers AVR-FP 5' ATG GAT CCC ATT CGT TCG 3' and AVR-RP 5' TCA CTG AGG CAA TAG CTC CA 3' as described above. Sequencing of pUC19-AVR was done at the Cornell Life Sciences Core Laboratories Center.

Table 5 Polymerase chain reaction primers

Primer	Sequences	Description	Reference
AVR-FP	5’ ATGGATCCCATTCGTTCG 3'	Amplification of 3.8-kb of	AY780632.1
AVR-RP	5’ TCACTGAGGCAATAGCTCCA 3'	*avrBs3* homolog	
AVR-1-FP	5’ AATATTGGCGGCAAGCAGGC 3'	Amplification of 863-bp	This study
AVR-1-RP	5’ CGCCTGCTTGCCACCAATATT 3'	fragment of *avrXg1*	
AVR-2-FP	5’ AATATTGGTGGCAAGCAGGCG 3’	Amplification of 1.0-kb	This study
AVR-2-RP	5’ TTGCCACCAATATTGCTGGCGAT 3’	fragment of *avrXg1*	
AVR-3-FP	5’ AGCCACTTGTAGAACGTG 3’	Amplification of 4.1-kb	AY780632.1
AVR-3-RP	5’ GCTAACTCGCTGTCAGTA 3’	*avrBs3* homolog and flanking sequences	
KAN-1-FP	5’ AAGCCGATGCGCCAGAGTT 3’	Amplification of kanamycin	Epicentre,
KAN-1-RP	5’ CGCCGTCCCGTCAAGTCAG 3’	gene (800-bp)	USA
KAN-2-FP	5’ ACCTACAACAAAGCTCTCATCAACC 3’	For sequencing	Epicentre,
KAN-2-RP	5’GCAATGTAACATCAGAGATTTTGAG 3’		USA
M13-F	5’ TGTAAAACGACGGCCAGT 3’	Universal primers	Cornell Life
M13-R	5’AACAGCTATGACCATG 3’		Sciences Core Laboratories Center
PET-FP	5’ CACCAGCCACTTGTAGAACG 3'	Amplification of 3.8-kb of	AY780632.1
PET-RP	5’ CTGAGGCAATAGCTCCATCA 3'	*avrBs3* homolog	
PR-1aF	5’ TGATGTTGCCTACGCTCAAG 3’	Amplification of 365-bp of	Graham *et al.*,
PR-1aR	5’ ATCCAAGACGCACCGAGTTA 3’	PR-1	2003
PR-2F	5’ CCTAGCATCTAGCCAAGACA 3’	Amplification of 434-bp of	Graham *et al.*,
PR-2R	5’ GTGAACCATCTTGCACTACC 3’	PR-2	2003
PR-4F	5’ CTCGTGGCCGTGATTCTTGT 3’	Amplification of 246-bp of	Graham *et al.*,
PR-4R	5’ GAGCATCGAGGATGGAGAGT 3’	PR-4	2003
PR-6F	5’ GCCTTCACCACCTCATACCT 3’	Amplification of 457-bp of	Graham *et al.*,
PR-6R	5’ CAGCTTGCTGTGGACAGAAC 3’	PR-6	2003

Table 5 (Continued)

Primer	Sequences	Description	Reference
PR-10F PR-10R	5’ AGTTACAGATGCCGACAACG 3’ 5’ CCTCAATGGCCTTGAAGAGA 3’	Amplification of 377-bp of PR-10	Graham *et al.,* 2003
PAL1-F PAL1-R	5’ CCAAGGAACCCCTATTGG 3’ 5’ CCATTCCACTCCCCAAGG 3’	Amplification of 992-bp of phenylalanine ammonia-lyase	Estabrook and Sengupta-Gopalan, 1991
LOX-F LOX-R	5’ CCGAGAGCATCCAAATACAA 3’ 5’ GCTCTATTATCGTTTGGACA 3’	Amplification of 350-bp of lipoxygenase	Moy *et al.,* 2004
POX-F POX-R	5’ ACACTTACGTTTACGGCA 3’ 5’ CCGGTGGTCACACAAT 3’	Amplification of 290-bp of peroxidase	Moy *et al.,* 2004
18SrRNAF 18SrRNAR	5’ CTGGCGACGCATCATTC 3’ 5’ GAATTACCGCGGCTGCT 3’	Amplification of 270-bp of 18S ribosomal RNA	Cadle-Davidson and Jahn, 2005
FlgC-F FlgC-R	5’ GAATTCATGAGCAATCTTCCCATCTTCG 3’ 5’ GAATTCTCAGCGACCCATGGTGAG 3'	Amplification of 408-bp of *flgC* homolog	NP_642311.1
FlgK-F FlgK-R	5’ TAAGCTTATGTCCATCATGTCCACCGG 3’ 5’ TGAATTCTCAGCGTACGGCACCCAG 3’	Amplification of 1.87-kb of *flgK* homolog	NP_642303.1
PILD-F PILD-R	5’ TAAGCTTATGGCATTTCTCGACCAGCATC 3’ 5’ AGAGCTCTCAACGCAGGCCTGCGAAGT 3’	Amplification of 863-bp of *pilD* homolog	NP_643551.1
TET-F TET-R	5’ TAGGCTTGGTTATGCCGGTA 3” 5’ TTCTCGCCGAAACGTTTG 3’	Amplification of tetracycline gene (800-bp)	This study

3.2.4 Electroporation

Transformation pUC19-AVR into KU-P-SW005 was performed by electroporation as described above. Briefly, 2 µl of pUC19-AVR and 0.5 µl of TypeOne^{TM} restriction inhibitor were add to 50 µl of KU-P-SW005 electrocompetent cells and subjected to electroporation in a 0.25 cm cuvette at 2.5 kV, 25 µF, and 200 Ω. Electroporated cells were added to fresh NBY broth and incubated at 28°C for 1 h. Transformants were selected on NBY agar containing appropriate antibiotics. Resistant clones were screened for their ability to cause the disease or HR on different soybean cultivars as described above and were subsequently verified for EN-Tn5 insertion by PCR.

4. Analysis of gene abolished HR on Williams82

4.1 Determined EZ-Tn5 insertion

The *avr* mutants, KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP that gene abolished HR on Williams82 were verified for EZ-Tn5 insertion by PCR. Kan^{R} was amplified by PCR from the *avr* mutants (KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP), KU-P-SW005, and pUC19-AVR used for unknown, negative control, and positive control, respectively using the forward primers KAN-1-FP 5’ AAG CCG ATG CGC CAG AGT T 3’ and reverse primer KAN-1-RP 5’ CGC CGT CCC GTC AAG TCA G 3’ (Table 5) to obtain EZ-Tn5 inserted in *X. axonopodis* pv. *glycines* genome. Total genomic DNA of wildtype strain KU-P-SW005 and *avr* mutants were extracted by UltraClean™ Microbial DNA Isolation Kit Components as described above. DNA was amplified in a total volume of 50 µl. The reaction mixture contained 5 µl of 10 X ThermoPol rection buffer (100 mM KCl, 10 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% NP-40, and 50% glycerol), 1 µl of 10 pmol of each primer, 2.0 µl of dNTP mixed, 1.0 µl of 100 ng of purified total bacterial DNA, 39.5 µl of SDW, and 0.5 µl of *Taq* DNA Polymerase (BioLabs, USA). A total of 30 amplification cycles were performed in an automated thermocycler (P-100). Each cycle consisted of 15 sec of denaturation at 94°C, 30 sec of annealing at 55°C,1 min of extension at 72°C, and the last extension step at 72°C was

extended to 5 min. Amplified DNA was determined by agarose gel electrophoresis as described above.

4.2 PCR analysis of the DNA flanking EZ-Tn5 insertion

Total DNA of EN-Tn5 mutant KU-P-SW005 *avrXg1*::Tn5AAD, which gained virulence function on soybean cultivar Williams82, was extracted, partially digested with *Eco*RI and *Sac*I (no restriction site in transposon) and cloned into pUC19 and transformed by heat shock into *E. coli* DH5α (Invitrogen, CA, USA). Clones were selected on LB agar amended with kanamycin 50 µl/ml and ampicillin 100 µl/ml. Sequences of clones carrying EZ-Tn5 transposon were identified by PCR amplification with M13F 5' TGT AAA ACG ACG GCC AGT 3' and M13R 5' AAC AGC TAT GAC CAT G 3' universal primers (Table 5).

Genomic sequences flanking the EZ-Tn5 transposon insertions in KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP were identified by PCR amplification of the disrupted gene with primers KAN-2-FP 5' ACC TAC AAC AAA GCT CTC ATC AAC C3' and KAN-2-RP 5'GCA ATG TAA CAT CAG AGA TTT TGA G 3' (Epicentre, WI, USA). DNA sequencing was done at the Cornell Life Sciences Core Laboratories Center.

Also, genomic DNA of the mutants KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP were identified the size of *avrBs3* homolog EN-Tn5 inserted compared with KU-P-SW005 (no inserted) by PCR with AVR-FP 5' ATG GAT CCC ATT CGT TCG 3' and AVR-RP 5' TCA CTG AGG CAA TAG CTC CA 3' (Table 5). Amplified DNA was determined by agarose gel electrophoresis as described above.

5. Analysis of the *avrXg1* mutant and wildtype

5.1 HR induction

The *avrXg1* mutants, KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP, were tested for their ability to induce HR on three species of tobacco

(*Nicotiana tabacum* cv. Xanthi, *N. glauca*, and *N. rustica*) and on tomato (*Lycopersicon esculentum* cv. Seeda 4) compared with wildtype and *E. coli* carrying pBBR-AVR. A hundred μl of each bacterial suspensions (OD_{600} = 1.5, ca. 5 X 10^9 cfu per ml) was infiltrated through a pin puncture into the leaf mesophyll using a 1-ml hypodermic syringe without a needle (Wei *et al.*, 1992). Tested plant leaves were inoculated by picking the leaves with a pin and then pressing the leaves with a tuberculin syringe against the hole in the top of the leaf while closing the hole and supporting the bottom of the leaf with a finger. Infiltrated areas were monitored for development of tissue collapse and necrosis for 48 h post- infiltration. For all experiments, at least three leaf panels per three plants for each treatment were infiltrated and experiments were repeated three times.

5.2 Enzyme assays

Relative levels of exoenzyme production were assessed by radial diffusion assays. Race 3 wildtype KU-P-SW005 and *avrXg1* mutants, KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP, were grown in LB at 28°C overnight. Ten μl of a bacterial suspensions at OD_{600} = 0.2 was placed in a 0.5-cm well, made using a cork borer, in an agar medium in a pertri plate. The medium composition, incubation conditions for assay plates, and detection conditions for each assay were specified later. Enzyme activity was estimated from the diameter of zones surrounding the culture supernatants of each strain. All experiments were done three times with three replicate plates.

5.2.1 Cellulase

Inoculated plates containing an assay medium (0.1% carboxymethyl cellulose (CMC), 25 mM sodium phosphate pH 7.0, and 0.8% agarose) (Andro *et al.*, 1984) were incubated at room temperature overnight, stained with 0.1% congo red for 20 min, and washed twice with 1 M NaCl. Carboxymethyl cellulase (CMCase) activity was visualized as while halos surrounding the wells.

5.2.2 α-Amylase

Inoculated plates containing α-amylase assay medium (0.5% yeast extract, 1.0% tryptone, 0.25% NaCl, 0.2% soluble starch, and 0.8% agarose) were incubated at room temperature overnight and stained with potassium iodine for 10 min as described by Ray *et al*. (2000). Levels of α-amylase were measured by radius of clear zones.

5.2.3 Protease

Inoculated plates containing nutrient yeast glucose agar (NYGA) supplemented with 0.5% skimmed milk were incubated at room temperature for 48 h as described by Barber *et al*. (1991). Extracellular protease production was detected visually as clear halos surrounding the wells.

5.2.4 Pectate lyase

Bacterial cells were spread on the surface of potato slices; incubated on moist filter paper at 28°C in petri plate. The development of soft rot was evaluated after 24 - 48 h (Kaewnum *et al.,* 2006).

5.2.5 Endoglucanase

Bacterial cells were grown in NYGA supplemented with 0.125% CMC (Barber *et al*., 1991). Inoculated plate was stained with 0.1% congo red for 30 min, rinsed with water, and washed twice with 1M NaCl. Endoglucanase activity was observed by a pale yellow zone of clearing contrast a red background.

5.3 Motility test

An *avrXg1* mutants (KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP); Race 1, strain KU-P-34070; Race 2, strain KU-P-KPS06; and Race 3, strain KU-P-SW005 were tested for swarming motility on semi-solid medium surface,

NGA solidified with 0.4% agar. Bacteria were grown on NGA for 24 h and bacterial cell from NGA was picked with a sterile toothpick, and gently spotted onto NGA 0.4% agar surfaces. The plates were incubated at 28°C for 2 days, after which the bacterial motility was examined.

6. Presence of the *avrBs3* homolog in *X. axonopodis* pv. *glycines* races

Thirty four represented *X. axonopodis* pv. *glycines* strains (Tusanasarit, 1995; Saiseangthong, 1999) were evaluated for the presence of the *avrBs3* 3.8-kb region amplified by primers AVR-FP 5' ATG GAT CCC ATT CGT TCG 3' and AVR-RP 5' TCA CTG AGG CAA TAG CTC CA 3' using REDAccuTaq LA DNA Polymerase Mix as described above. PCR products were separated by gel electrophoresis. The presence of the PCR product was correlated with strain-cultivar pathogenicity determinations.

7. Sequencing and evaluation

The 3.8-kb region of *avrXg1* amplified from KU-P-SW005 was cloned into the pGEM-T easy vector. All primer sequences (AVR-1-FP and AVR-1-RP and AVR-2-FP and AVR-2-RP) were listed in Table 5. Primer walking and PCR were employed for amplification and sequencing of the entire gene. DNA sequencing was done as described above. Analysis of sequences was conducted by using BLAST and the DNASTAR Lasergene software package (DNASTAR, Wl, USA). The MegAlign program of the DNASTAR package was used for sequence alignment using CLUSTALW. MegAlignment was then calculated for the *avr* gene sequences as available from GenBank for selected *Xanthomonas* spp. EMBOSS Transeq, ScanSite pI/Mw, and BPROM were used to translate DNA to deduced proteins, compute the theoretical isoelectric point (pI) and molecular weight (Mw) of proteins, and for prediction of bacterial promoters respectively.

8. Genome location of *avrXg1*

Total genomic and plasmid DNA of wildtype Race 3 KU-P-SW005; *avrXg1* mutants, KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP; and

wildtype Race 1 KU-P-34070 were extracted by UltraClean™ Microbial DNA Isolation Kit Components and UltraClean™ 6 Minute Mini Plasmid Prep Kit, respectively. The plasmids DNA were size-fractionated on a low-melting 0.5% agarose gel. Purified fragments of 15 to 30-kb were used as the PCR template. Primers KAN-1-FP 5' AAG CC GAT GCG CCA GAG TT 3' and KAN-1-RP 5' CGC CGT CCC GTC AAG TCA G 3' were used to amplify an 800-bp product including the kan^R from the total genomic and plasmid DNA. PCR products were separated by gel electrophoresis as described above.

The genome location of *avrXg1* was further analyzed by PCR amplification of regions flanking the gene (274-bp upstream and 70-bp downstream) with primers AVR-3-FP 5' AGC CAC TTG TAG AAC GTG 3' and AVR-3-RP 5' GCT AAC TCG CTG TCA GTA 3' (Table 5). PCR products were cloned in pGEM-T easy vector. DNA sequencing was identified by PCR amplification of the *avrXg1* fragment with primers AVR-3-FP 5' AGC CAC TTG TAG AAC GTG 3' and AVR-3-RP 5' GCT AAC TCG CTG TCA GTA 3' and with M13F and M13R universal primers.

9. Transformation of *avrXg1* mutants, Race 1, and Race 2 with *avrXg1*

To complement KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP, the 3.8-kb sequence comprising *avrXg1* in pGEM-T easy vector was digested with *Eco*RI and ligated into the multiple cloning site vector pBBR1MCS-5 (pBBR-AVR) as same as method of the other vector that described above. Plasmid pBBR-AVR was introduced into *avrXg1* mutants KU-P-SW005 *avrXg1*::Tn5AAD, KU-P-SW005 *avrXg1*::Tn5REP, wildtype Race 1 KU-P-34070 (induces HR on all cultivars tested), and wildtype Race 2 KU-P-KPS06 (caused disease on all cultivars tested) by electroporation according to the method described above. Selection was made on NBY with kanamycin 50 µl/ml and gentamicin 25 µl/ml for complemented KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP, and with gentamicin 25 µl/ml for the mutants KU-P-34070 and KU-P-KPS06 transformed with the pBBR-AVR. They were verified by their ability to cause HR on Williams82 and disease on Spencer, PI 520733, and SJ4 as described above.

10. Virulence and bacterial growth on soybean

Virulence of KU-P-SW005, KU-P-SW005 *avrXg1*::Tn5AAD, KU-P-SW005 *avrXg1*::Tn5REP, *avrXg1*::Tn5AAD (pBBR-AVR), *avrXg1*::Tn5REP (pBBR-AVR), KU-P-34070, KU-P-34070 (pBBR-AVR), KU-P-KPS06, and KU-P-KPS06 (pBBR-AVR) were assessed on soybean cultivars Spencer and Williams 82, using previously described quantitative methods (Kaewnum *et al.,* 2005). Briefly, aqueous cell suspensions (OD_{600} = 0.2, ca. 10^8 cfu per ml of each) were sprayed on leaves of plants maintained in the greenhouse. At 7-10 days 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.

Bacterial growth was also assessed within the infiltrated zone. Leaf discs, 5 mm in diameter, were cut from the center of the infiltrated zone at 1, 2, 3, and 4 days post-infiltration (three discs for each times). Samples were placed individually in 1 ml sterile water, triturated, and dilutions plated on NGA. Leaf surface inoculation and infiltration experiments were repeated three times. The results were statistically analyzed using SAS 9.1.2 (SAS Institute Inc., Cary, NC, USA).

11. Expression of *avrXg1*

An *avrXg1* 3.8-kb fragment was amplified from KU-P-SW005 by using primers PET-FP 5’ CAC CAG CCA CTT GTA GAA CG 3' and PET-RP 5’ CTG AGG CAA TAG CTC CAT CA 3' (Table 5). Expression of *avrXg1* was expressed in two different expression vectors including pET160/GW/D-TOPO$^{(R)}$ (Invitrogen, CA, USA) and pRSETB (Invitrogen, CA, USA). First, the target fragment was ligated into pET160/GW/D-TOPO$^{(R)}$ according to the supplier’s direction. Briefly, 3 µl of fresh PCR product, 1 µl of salt solution, 1 µl SDW, and 1 µl TOPO$^{®}$ vector were mixed and incubated for 5 min at room temperature. The reaction was placed on ice and proceeded to transform by heat shock into *E. coli* TOP10 as described above. The second, target fragment, *avrXg1* 3.8-kb in pGEM-T easy vector was digested with *Eco*RI and then

ligated into the *Eco*RI sites of pRSETB by DNA Ligation Kit <Mighty Mix> according to the supplier's direction and transformed by heat shock into *E. coli* TOP10 as described above.

Selection pET-AVR and pRSETB-AVR was made on LB with amplicilin 100 µl/ml and was shown by plasmid extraction and digestion with *Tsp*509I and *Eco*RI, respectively as described above. DNA sequencing was done at the Cornell Life Sciences Core Laboratories Center. *E. coli* BL21 (Invitrogen, CA, USA) were transformed with each expression plasmids as described above and grown at 37°C in LB medium containing ampicillin 100 µg/ml. Expression of the protein was induced with 1 mM IPTG at 28°C and 37°C for 0, 1, 2, 3, 4, 7, 10, 12, and 24 h.

Protein sample from pET-AVR was detected by Lumio^{TM}Green Detection Kit (Invitrogen, CA, USA). The cell pellets of each sample were resuspended in 50 µl of lysis buffer (30 ml of 5 M NaCl, 10 ml of NP-40 (Igepal CA-630, Sigma #I-3021), 1 M Tris pH 8.0, and 110 ml of SDW). Sample was centrifuged at full speed in a microcentrifuge for 5 min at 4°C to pellet insoluble proteins. Supernatant was transferred to a fresh tube and stored on ice. Pellets were washed once with lysis buffer to remove any residual soluble proteins. The pellets were resuspended in 50 µl of 8 M urea. Fifteen µl proteins samples were used and 5 µl of 4X Lumio^{TM} Gel Sample Buffer and 0.2 µl of Lumio^{TM} Green Detection Reagent were added and then incubated at 70°C for 10 min. The sample was allowed to cool about 2 min and centrifuged briefly at full speed in a microcentrifuge. Two µl of Lumio^{TM} In-Gel Detection Enhancer was added to the sample. Then sample was mixed well and incubated at room temperature for 5 min and 20 µl of the sample was loaded into each well for sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). The SDS-PAGE was performed using the Bio-Rad Mini-Protein III system. The gel system in SDS-PAGE was composed of separating and stacking gels. The separating gel was consisted of 3% crosslinker, 12% gel, 0.375 M Tris-HCl (pH 8.8), and 0.1% SDS. The stacking gel contained 3% crosslinker, 5% gel, 0.125 M Tris-HCl (pH 6.8), and 0.1% SDS. The gel was run in Tris-glycine buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS). Electrophoresis was performed with constant ampere of 100 V/gel at room temperature. After electrophoresis complete, the gel was removed from the cassette and placed on UV transilluminator equipped with a standard camera.

Protein sample from pRSETB-AVR was prepared by mixing the sample with 4X loading buffer (60 mM Tris-HCl pH 7.5, 2% of SDS, 10% glycerol, 0.025% bromophenol blue, and 100 mM DTT) in 3:1 (v/v) ratio and boiled at 100°C for 5 min. The heated samples were vigorously mixed using vortex and centrifuged at 10,000 rpm for 10 min to precipitate any insoluble materials. Supernatant equivalent to 0.1 OD_{600} of the cell culture was loaded into each well for SDS-PAGE analysis and gel electrophoresis as described above. After electrophoresis, the protein band on the gel was visualized by 20 min soaking in staining solution containing 50% methanol, 10% glacial acetic acid, and 0.1% Coomassie brilliant blue R-250 in H_2O. The gel was then soaked in destaining solution (10% methanol and 10% glacial acetic acid) overnight or until the background clears.

12. Proteomic analysis of soybean response to *X. axonopodis* pv. *glycines* Race 3 and *avrXg1* mutant

The 15-day old soybean leaves cultivar Williams82 (resistant) and Spencer (susceptible) were sprayed with 20 ml of bacterial suspension of KU-P-SW005 (wildtype) and KU-P-SW005 *avrXg1*::Tn5REP (*avrXg1* mutant) at a concentration of 1 x 10^8 cfu per ml of each. Twenty ml of SDW was also inoculated into the soybean leaves as a control. The inoculated plants were grown in a greenhouse under the temperature at 28°C and 70% humidity. Soybean leaves inoculated were collected at 0, 1, and 2 days after spray inoculation for determination protein profile of resistance and susceptible soybean response to KU-P-SW005 and KU-P-SW005 *avrXg1*::Tn5REP inoculation involved in mechanisms of host defense and disease induction, respectively. The experiment was done with CRD and three repetitions per treatment were used for each protein analyze. Soybean leaves samples were stored immediately at -80°C until extraction. Soybean leaves samples were homogenized at 4°C in 2 ml of extraction buffer modified from Sarma *et al*. (2008) consisting of 0.1 M Tris-HCl pH 8.8 containing 50 mM DTT, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) per 2 g tissue quickly in prechilled (-20°C) mortar and -pestle. The homogenate was centrifuged at 4000 *g* for 20 min at 4°C. The resulting supernatant was stored at -80°C until use. The protein samples were loaded into each well of SDS-PAGE analysis, gel electrophoresis, and gel staining prepared according to the methods described above. The unique protein band was identified by mass spectrometry analysis. Amino acid sequencing of the unique band was done at the

Cornell Life Sciences Core Laboratories Center. Database searching, peptide mass fingerprint (PMF) data were generated from raw MALDI-TOF-MS data using the SNAP algorithm (Bruker) with a signal/noise threshold. The PMF data were used to screen the NCBInr database (http://129.93.41.90/mascot/cgi/master_results.pl?file=../data/20080219/F011009.dat) and using local MASCOT (MASCOT 2.1.0, Matrix Science).

13. Determination of pathogenesis-related (PR) protein and defense enzyme genes expression in soybean using semiquantitative reverse transcription (sqRT)-PCR

Fifteen-day old soybean leaves of resistance cultivar Williams82 inoculated with 20 ml of KU-P-SW005, KU-P-SW005 *avrXg1*::Tn5REP, and SDW were collected at 0, 24, and 48 h after inoculation as described above. Three repetitions per treatment with CRD were used for each total RNA analyzes. Total RNA was then isolated from 0.5 g of each tissue sample with 2 ml of TRIzol® Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA samples were treated with DNase I (Promega, WI, USA) and calculated the concentration of RNA in each sample by Thermo Scientific NanoDrop™ 1000 Spectrophotometer (NanoDrop, USA) according to the manufacturer's instructions. Fifty µl of DNase-treated RNA samples was synthesized cDNA into the 20 µl reaction mixture supplied by Bio-Rad Laboratories (CA, USA) containing 4 µl of 5X iScript reaction mix, 1 µl of iScript reverse transcriptase, 10.5 - 9.5 µl of nuclease-free water (up to the concentration of the RNA), and 4.5 - 6.5 µl of RNA template (up to needed the cDNA final concentration). The reaction mixture was incubated at 25°C for 5 min, at 42°C for 30 min followed by heat at 85°C for 5 min, and hold at 4°C. The resulting first-strand cDNA was amplified using forward and reverse primers PR-1aF 5' TGA TGT TGC CTA CGC TCA AG 3' and PR-1aR 5' ATC CAA GAC GCA CCG AGT TA 3', PR-2F 5' CCT AGC ATC TAG CCA AGA CA 3' and PR-2R 5' GTG AAC CAT CTT GCA CTA CC 3', PR-4F 5' CTC GTG GCC GTG ATT CTT GT 3' and PR-4R 5' GAG CAT CGA GGA TGG AGA GT 3', PR-6F 5' GCC TTC ACC ACC TCA TAC CT 3' and PR-6R 5' CAG CTT GCT GTG GAC AGA AC 3', PR-10F 5' AGT TAC AGA TGC CGA CAA CG 3' and PR-10R 5' CCT CAA TGG CCT TGA AGA GA 3', PAL1-F 5' CCA AGG AAC CCC TAT TGG 3' and PAL1-R 5' CCA TTC CAC TCC CCA AGG 3', LOX-F 5' CCG AGA GCA TCC AAA TAC AA 3' and LOX-R 5' GCT

CTA TTA TCG TTT GGA CA 3', and POX-F 5' ACA CTT ACG TTT ACG GCA 3'and POX-R 5' CCG GTG GTC ACA CAA T 3' (Table 5) to amplify 365, 434, 246, 457, 377, 992, 350, and 290-bp respectively that involved PR-proteins and defense enzyme genes in soybean. The expression levels of 18S ribosomal RNA (rRNA) gene were used as internal standards, and fragments were amplified using primers designed from the transcribed region of the soybean 18S rRNA gene (Cadle-Davidson and Jahn, 2005). cDNA was amplified in a total volume of 50 µl. The reaction mixture contained 5 µl of 10 X ThermoPol rection buffer (100 mM KCl, 10 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% NP-40, and 50% glycerol), 1 µl of 10 pmol of each primer, 2.0 µl of dNTP mixed, 1.0 µl of 100 ng of purified total bacterial DNA, 39.5 µl of SDW, and 0.5 µl of *Taq* DNA Polymerase (BioLabs, USA). A total of 30 amplification cycles were performed in an automated thermocycler (P-100). Each cycle consisted of 15 sec of denaturation at 94°C, 30 sec of annealing at 55°C (for all genes),1 min of extension at 72°C and the last extension step at 72°C was extended to 5 min. Amplified DNA was determined by agarose gel electrophoresis as described above. In addition, all PCR primer combinations were tested using total RNA as a negative control. The quantity and base pair size of the PCR generated DNA fragments were estimated relative to DNA ladder standards.

14. Identification of motility associated *g*enes, *flgC, flgK,* and *pilD* in *X. axonopodis* pv. *glycines*

Bacterial flagella (*flg*) and pilus (*pil*) genes associated with flagella and pilus biosynthesis that controlled swimming, swarming, and twitching motility respectively, cell attachment, and biofilm formation are thought to be important factors in pathogen virulence. Then, the association of specific genes with motility and biofilm formation in motile bacteria strain KU-P-SW005 were determined.

14.1 Site-directed mutagenesis

A targeted mutation in the *flgC* and *flgK* homolog in KU-P-SW005 was selected to study and accomplished using the EZ-Tn5™ <KAN-2> Insertion Kit. The *pilD* was selected to study and accomplished using the EZ-Tn5™ <TET-1> Insertion Kit

(Epicentre, WI, USA) according to the supplier's directions (Fig. 2). First a 408-bp, 1.87-kb, and 863-bp fragment of *flgC*, *flgK*, and *pilD* homolog including restriction sites for enzymes *Eco*RI, *Hind*III and *Eco*RI, and *Hind*III and *Sac*I respectively were amplified from KU-P-SW005 using primers FlgC-F 5' GAA TTC ATG AGC AAT CTT CCC ATC TTC G 3' and FlgC-R 5' GAA TTC TCA GCG ACC CAT GGT GAG 3', FlgK-F 5' TAA GCT TAT GTC CAT CAT GTC CAC CGG 3' and FlgK-R 5' TGA ATT CTC AGC GTA CGG CAC CCA G 3', and PILD-F 5' TAA GCT TAT GGC ATT TCT CGA CCA GCA TC 3' and PILD-R 5' AGA GCT CTC AAC GCA GGC CTG CGA AGT 3' that are based on the sequence of the *flgC*, *flgK*, and *pilD* homolog (NP_642311.1, NP_642303.1, and NP_643551.1 respectively) previously identified in *X. axonopodis* pv. *citri* strain 306 (da Silva *et al.,* 2002).

14.1.1 Cloning and construction of *flgC*, *flgK*, and *pilD*

Genomic DNA of *flgC*, *flgK*, and *pilD* genes from KU-P-SW005 was amplified with specific primer FlgC-F and FlgC-R, FlgK-F and FlgK-R, and PILD-F PILD-R respectively (Table 5). DNA was amplified in a total volume of 50 µl. The reaction mixture contained 5 µl of AccuTaq LA 10 X buffer (500 mM Tris-HCl, 150 mM ammonium sulfate (pH 9.3, adjusted with KOH), 25 mM $MgCl_2$ 1% Tween 20), 1 µl of 10 pmol of each primer, 2.5 µl of dNTP mixed, 0.5 µl of 100 ng of purified total bacterial DNA, 37.5 µl of SDW, and 2.5 µl of REDAccuTaq LA DNA Polymerase Mix (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween® 20, 0.5% IGEPAL® CA-630, 50% glycerol, inert dye) (Sigma, USA). A total of 30 amplification cycles were performed in an automated thermocycler (P-100). Each cycle consisted of 20 sec of denaturation at 95°C, 20 sec of annealing at 55°C (for all genes), 2 min of extension at 65°C, and the last extension step at 68°C was extended to 20 min. Amplified DNA was determined by agarose gel electrophoresis as described above at 80 volts constant for 1.5 h using. The gel was stained with 0.5 µg of ethidium bromide per ml for 10 min and briefly then destined in water before visualizing under UV light and photographed over a transilluminator (GDS 800, Complete Gel Documentation Analysis System). The PCR product was cloned into pUC19 vector as described above and sequenced.

Subsequently an EN-Tn5 targeted insertion in *flgC*, *flgK*, and *pilD* genes in pUC19 vector were made in vitro following to the supplier's directions as described above. Selection of the pUC19-FLGC, pUC19-FLGK, and pUC19-PILD with single EN-Tn5 insertion in the middle of the target gene was shown by plasmid extraction and digestion with the same restriction sites for enzymes on each construct and separated the target genes and pUC19 by gel electrophoresis as described above. Construction of the pUC19-FLGC, pUC19-FLGK, and pUC19-PILD was confirmed by PCR with the same primers that using amplifies *flgC*, *flgK*, and *pilD* genes from wildtype to amplify the gene that EN-Tn5 inserted compared the PCR product size with the original size of each gene. Sequencing of pUC19-FLGC, pUC19-FLGK, and pUC19-PILD was done at the Cornell Life Sciences Core Laboratories Center.

14.1.2 Electroporation

Transformation pUC19-FLGC, pUC19-FLGK, and pUC19-PILD into KU-P-SW005 were performed by electroporation as described above. Transformants were selected on NBY agar containing appropriate 50 μg/ml of kanamycin for *flgC* and *flgK* mutations. Transformants of *pilD* was selected on NBY agar containing appropriate 15 μg/ml of tetracycline. Individual colonies of putative mutants were verified by PCR for EN-Tn5 insertions with FlgC-F and FlgC-R, FlgK-F and FlgK-R, PILD-F and PILD-R, KAN-1-FP and KAN-1-RP, and TET-F and TET-R (Table 5) as described above. Resistant clones were screened for ability of swimming motility in the water flow under light microscope (100X) observation.

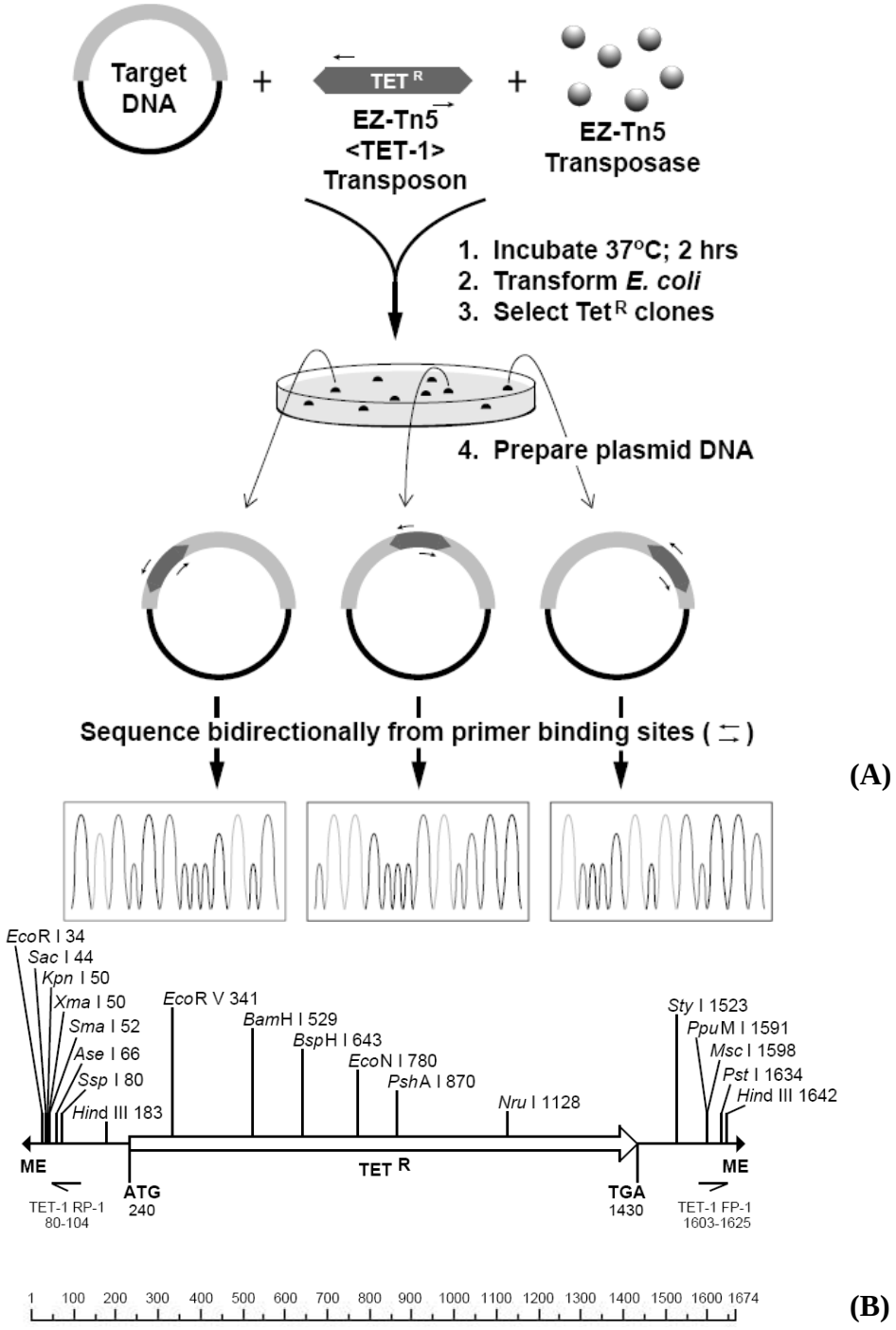

Figure 2 The protocol of EZ-Tn5™ <TET-1> Insertion Kit (Epicentre, WI, USA) generated the *Xanthomonas axonopodis* pv. *glycines* mutants (A) and EZ-Tn5 Transposon map (B).

14.2 Double mutation in *pilD* and *flgC*

Transformation pUC19-FLGC into KU-P-SW005 *pilD*::Tn5 was performed by electroporation as described above. Transformants were selected on NBY agar containing appropriate kanamycin 50 μg/ml and tetracycline 15 μg/ml. Individual colonies of putative mutants were screened for ability of swimming motility in the water flow under light microscope (100X) observation and verified by PCR for EN-Tn5 as described above.

15. Sequencing and evaluation

The 408-bp, 1.87-kb, and 863-bp of *flgC, flgK,* and *pilD* fragments, respectively amplified from KU-P-SW005 were cloned into the pUC19 vector as described above. Primer walking and PCR were employed for amplification and sequencing of the entire each gene. All primer sequences were listed in Table 5. Analysis of sequences was conducted by using BLAST and the DNASTAR Lasergene software package (DNASTAR, Wl, USA). The MegAlign program of the DNASTAR package was used for sequence alignment using CLUSTALW. MegAlignment was then calculated for the *flgC, flgK,* and *pilD* sequences as available from GenBank for selected *Xanthomonas* spp. EMBOSS Transeq, ScanSite pI/Mw, and BPROM were used to translate DNA to deduced proteins, compute the pI and Mw of proteins, and for prediction of bacterial promoters respectively.

16. Transformation of mutants with *flgC, flgK,* and *pilD*

To complement KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, and KU-P-SW005 *pilD*::Tn5, the 408-bp, 1.87-kb, and 863-bp sequence comprising *flgC*, *flgK*, and *pilD* in pUC19 vector was digested with *Eco*RI, *Hind*III and *Eco*RI, and *Hind*III and *Sac*I, respectively and ligated into the multiple cloning site vector pBBR1MCS-5. The constructed of pBBR- FLGC, pBBR- FLGK, and pBBR- PILD were introduced to KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, and KU-P-SW005 *pilD*::Tn5, respectively by electroporation according to the method described above. Selection was made on NBY, with kanamycin 50 μl/ml and gentamicin 25 μl/ml for complemented KU-P-SW005

flgC::Tn5 and KU-P-SW005 *flgK*::Tn5. Selection was made on NBY, with tetracycline 15 µl/ml and gentamicin 25 µl/ml for complemented KU-P-SW005 *pilD*::Tn5. They were verified by their ability of motility in the water under microscopic observation as described above.

17. Motility and microscopy image analysis

Mutants (KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, KU-P-SW005 *pilD*::Tn5, and KU-P-SW005 *flgC-pilD*::Tn5) and complemented strains, *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD) were tested for swarming, swimming, and twitching motility compared with wildtype KU-P-SW005.

Swimming motility tested all bacterial strains were grown on NGA for 24 h and bacterial cell from NGA was picked with a sterile needle, and gently stabbed onto NGA solidified with 0.4% agar tube. The tubes were incubated at 28°C for 2 days. Swimming positive was observed by the bacterial cell moved from the stabbed center. Bacterial strain lacking moved from the stabbed center or just grown in the stabbed center was checked several times to verify the swimming motility-minus phenotype.

Microscopic observation of KU-P-SW005, KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, KU-P-SW005 *pilD*::Tn5, and KU-P-SW005 *flgC-pilD*::Tn5 activities were assessed on NGA solidified with 1.2% agar on an inverted Olympus IMT-2 microscope using 40X phase-contrast optics. Time-lapse images were recorded with a SPOT-RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) controlled with MetaMorph Image software (Universal Imaging Corp., Downington, PA). The movies were made from each picture for observed the swimming motility of bacterial cells.

To investigate the twitching motility of *X. axonopodis* pv. *glycines* cells, the bacterial cells of wildtype strain KU-P-SW005, mutants (KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, KU-P-SW005 *pilD*::Tn5, and KU-P-SW005 *flgC-pilD*::Tn5) and complemented strains, *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD) were spotted on the surface of water agar (WA) that were air

dried prior to spotting by removing the pertri dish lid on a clean bench. Following incubation at 28°C for 12 h, the edge morphology of the colonies was observed under a light microscope (100X). Colonies with a marginal fringe were designated as having a wildtype twitching phenotype, while colonies lacking a marginal fringe were designated as no having a twitching phenotype (Meng *et al.,* 2005).

Swarming motility tested all bacterial strains were grown on NGA for 24 h and bacterial cell from NGA was picked with a sterile needle, and gently stabbed onto NGA solidified with 0.4% agar plate. The plates were incubated at 28°C for 2 days. Swarming positive was observed by the bacterial cell moved from the stabbed center. Bacterial strain lacking moved from the stabbed center or just grown in the stabbed center was checked several times to verify the swarming motility-minus phenotype.

18. Biofilm formation

Cells of mutants (KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, KU-P-SW005 *pilD*::Tn5, and KU-P-SW005 *flgC-pilD*::Tn5), wildtype (KU-P-SW005), and complemented strains, *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD) taken from cultures grown on NGA for 24 h were suspended in liquid LB, PD2, NBY, and NGB and adjusted to an OD_{600} of 0.1. A 5 ml cell suspension of each strain in various medium was added to glass tubes, and grown at 28°C for 7 days, with agitation at 150 rpm. The cell density of 7-day old culture was determined at regular intervals by measuring OD_{600}, the contents of the tube were removed. Subsequently, biofilm development was assessed on the inner surface of glass tubes by staining with 6 ml of 0.1% crystal violet for 20 min, and rinsed three times with SDW. The presence of a biofilm was visualized as a purple ring on the tube side wall, usually at the air–medium interface (O'Toole *et al.,* 1999). Following assessment of biofilm development of the tubes was removed, and the integrity of the side wall biofilm was determined by adding 50 ml of acetone: ethanol with 6: 4, and swirling vigorously to disperse cells that were not tightly bound to the tubes. The disperse cells in 0.1% crystal violet was determined at regular intervals by measuring OD_{600} again.

19. Pathogenicity test and bacterial growth on soybean

Virulence of wildtype KU-P-SW005, mutants KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, KU-P-SW005 *pilD*::Tn5, KU-P-SW005 *flgC-pilD*::Tn5, complemented strains *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD) were assessed on soybean cultivars Spencer by infiltration and spray inoculation technique as described above. Disease severity was assessed using a scoring method (Prathuangwong and Khandej, 1998). Bacterial growth was also assessed within the infiltrated zone. Leaf discs, 5 mm in diameter, were cut from the center of the infiltrated zone at 1, 2, 3, 4, and 10 days post-infiltration. Samples were placed individually in 1 ml sterile water, triturated, and dilutions plated on NGA. Ten leaves of ten plants were evaluated for each strain. Moreover, diameter of infiltrated zone and the number of pustules surround the infiltrated zone were observed also. The experiment was repeated three times. The results were statistically analyzed using SAS 9.1.2 (SAS Institute Inc., Cary, NC, USA).

Moreover, all mutants (KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, KU-P-SW005 *pilD*::Tn5, and KU-P-SW005 *flgC-pilD*::Tn5) and complemented strains, *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD) were tested for their ability to induce an HR on four species of tobacco (*N. tabacum* cv. Xanthi, *N. glauca*, *N. rustica* and *N. bentamina*) and on tomato (*L. esculentum* cv. Seeda 4) by using the method as described above. For all experiments, at least three leaf panels per three plants for each treatment were infiltrated and experiments were repeated three times.

RESULTS AND DISCUSSION

Results

1. Strains of *X. axonopodis* pv. *glycines* and pathogenicity test

Two hundred strains of *X. axonopodis* pv. *glycines* originated from soybean production areas of Thailand during 2005-2007 were isolated in this studies and shown to cause pustule disease on susceptible soybean cultivar SJ4. Symptoms were small, yellow-green spots with reddish-brown centers on the upper leaf surface at 3 - 5 days after inoculation (Fig. 3A). The central portion of each spot appeared slightly raised and developed into a small pustule, especially on the underside of the leaves. Several infections on the same leaf produced a large, yellow to brown area with small, and dark brown spots. The brown, necrotic areas on older leaves might break up and caused a ragged appearance (Fig. 3B and 3D).

2. Race-cultivar specificity

Three races of *X. axonopodis* pv. *glycines* were identified from 234 strains collected in Thailand (Table 4 and 6) based on their ability to cause disease or HR on four soybean cultivars (Fig.4). Race 1 (23 strains) induced HR within 48 h and Race 2 (137 strains) caused disease on all cultivars tested. Race 3 (74 strains) elicited HR on the resistant cultivar (Williams82) and caused pustule disease on pustule-susceptible cultivars SJ4, Spencer, and PI 520733. 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 7 days after inoculation (Fig. 4).

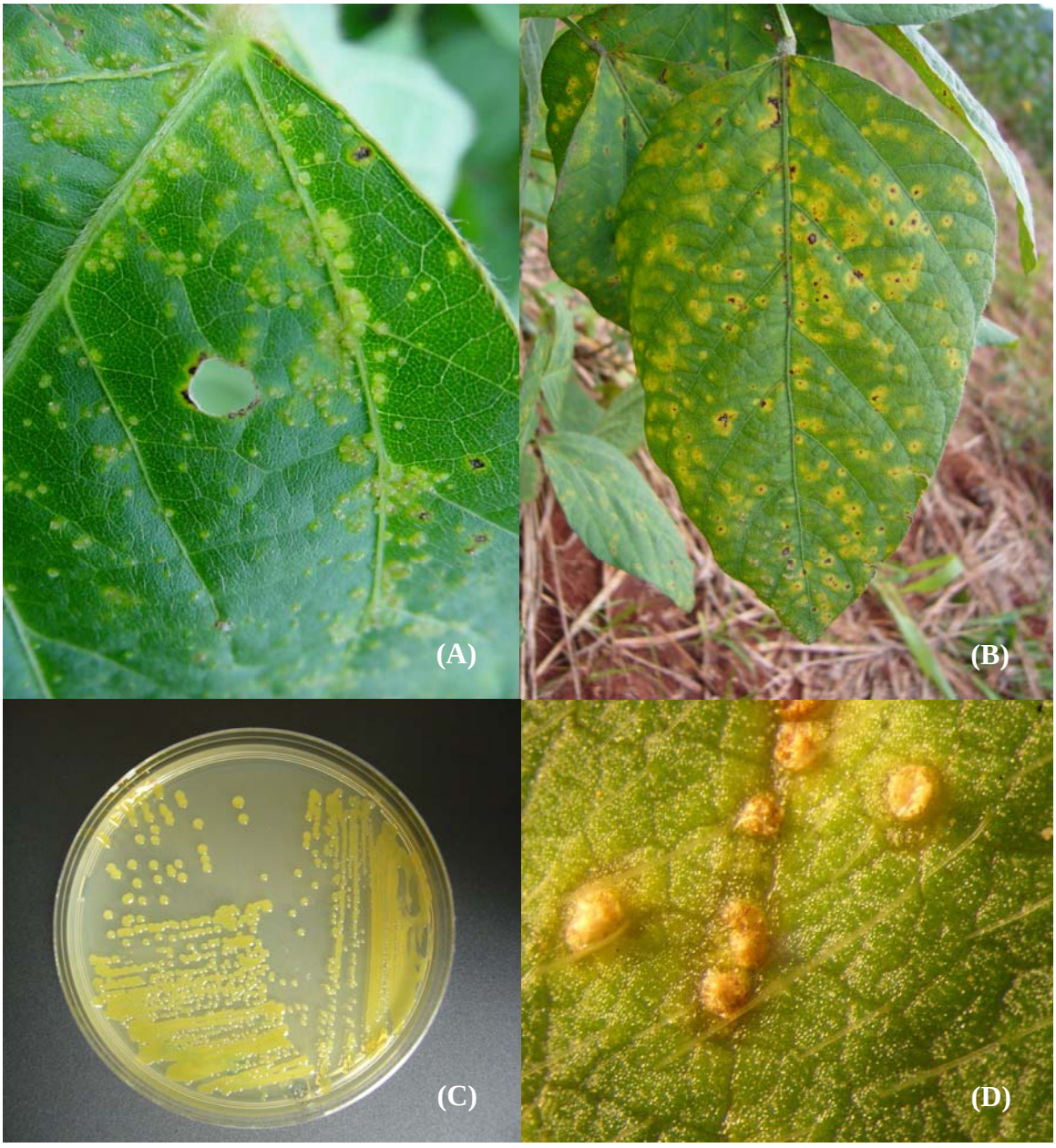

Figure 3 Bacterial pustule with pale green spots with elevated pustules (A), small necrotic lesions on soybean leaves (B and D) caused by *Xanthomonas axonopodis* pv. *glycines* with colonies on nutrient glucose agar (C).

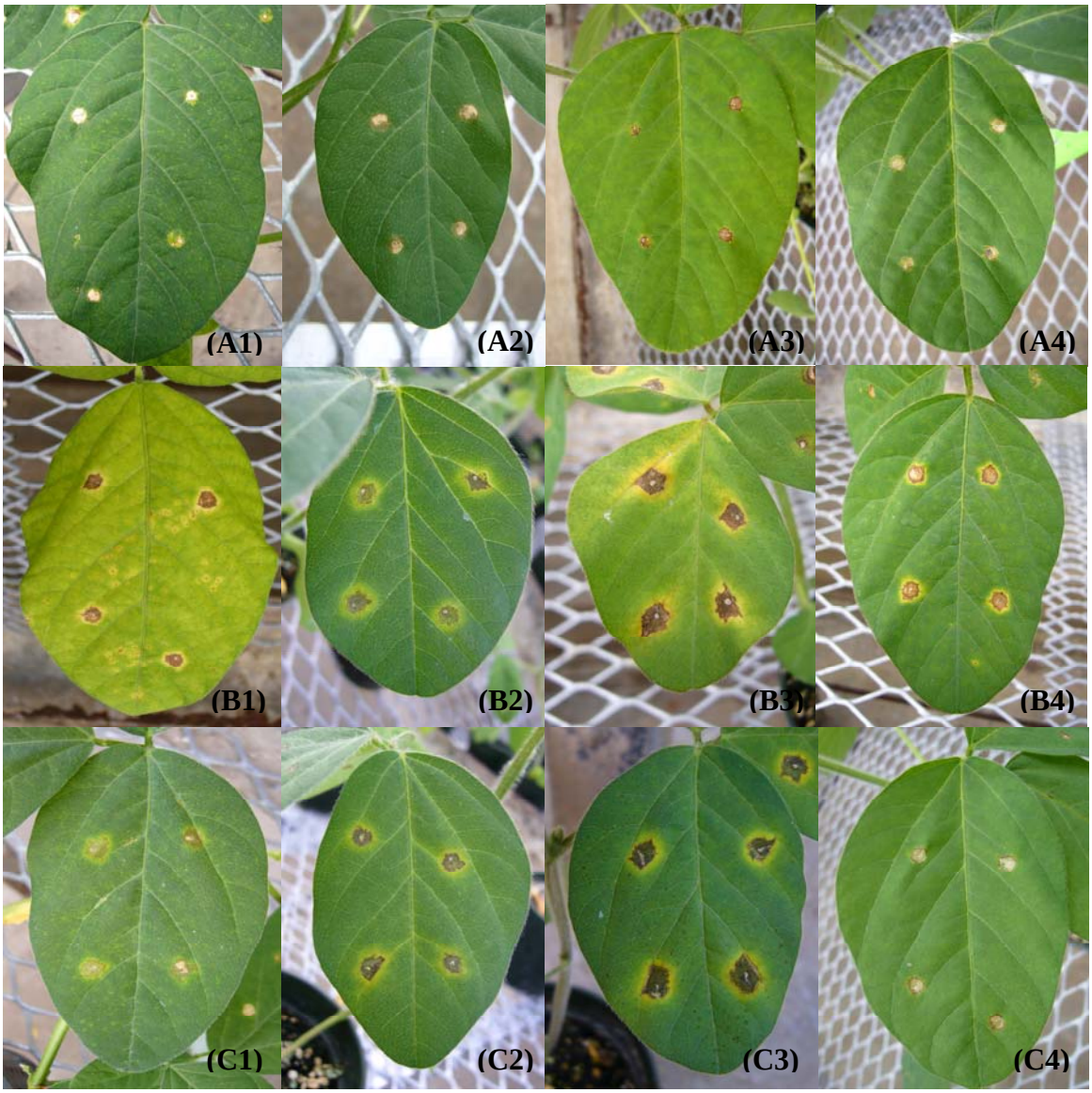

Figure 4 Compatibility phenotypes of *Xanthomonas axonopodis* pv. *glycines*-soybean interactions. Hypersensitive response (HR) resulted in necrosis. Disease showed necrosis with surrounding yellow halo and pustules formed on inoculated leaves at 7 days after infiltration assay. Race 1 (A) induced HR within 48 h and Race 2 (B) caused disease on all cultivars tested. Race 3 (C) caused disease on PI 520733 (1), Spencer (2), and SJ4 (3) and induced HR on Williams82 (4).

Table 6 Phenotypes of *Xanthomonas axonopodis* pv. *glycines* - soybean interactions

Bacterial strain	Relevant characteristic [1/]	Reference or source (Thai province)
KU-P-34001	Race 1	Nakhon Ratchasima
KU-P-34008	Race 2	Chiang Mai
KU-P-34009	Race 1	Khonkaen
KU-P-34016	Race 2	Chainat
KU-P-34016a	Race 1	Uttaradit
KU-P-34017	Race 1	Nakhon Sawan
KU-P-34021	Race 1	Nakhon Ratchasima
KU-P-34028	Race 1	Lop Buri
KU-P-34031	Race 1	Uthai Thani
KU-P-34032	Race 1	Sukhothai
KU-P-34040	Race 1	Tusanasarit, 1995
KU-P-34042	Race 2	Lop Buri
KU-K-34043	Race 1	Uthai Thani
KU-P-34054	Race 1	Phrae
KU-P-34062	Race 1	Chiang Rai
KU-P-34064	Race 1	Sukhothai
KU-P-34069	Race 1	Tusanasarit, 1995
KU-P-34071	Race 1	Loei
KU-P-34072	Race 1	Tusanasarit, 1995
KU-P-34075	Race 1	Prachin Buri
KU-P-34083	Race 1	Tak
KU-P-34085	Race 1	Khonkaen
KU-P-34089	Race 2	Phitsanulok
KU-P-34093	Race 1	Tusanasarit, 1995
KU-P-34096	Race 1	Kamphaeng Phet
KU-P-34098	Race 2	Loei
KU-P-340100	Race 1	Phetchabun
KU-P-KPS01	Race 3	Nakhon Prathom
KU-P-KPS04	Race 3	Nakhon Prathom
KU-P-SW008	Race 3	Nakhon Rachasima
ST015	Race 2	Tusanasarit, 1995
KU-P-48001	Race 2	Nakhon Prathom
KU-P-48002	Race 2	Nakhon Prathom
KU-P-48003	Race 2	Nakhon Prathom
KU-P-48004	Race 2	Nakhon Prathom
KU-P-48005	Race 2	Nakhon Prathom

Table 6 (Continued)

Bacterial strain	Relevant characteristic [1/]	Reference or source (Thai province)
KU-P-48006	Race 2	Nakhon Prathom
KU-P-48007	Race 2	Nakhon Prathom
KU-P-48008	Race 2	Nakhon Prathom
KU-P-48009	Race 2	Nakhon Prathom
KU-P-48010	Race 2	Nakhon Prathom
KU-P-48011	Race 3	Nakhon Prathom
KU-P-48012	Race 2	Nakhon Prathom
KU-P-48013	Race 2	Nakhon Prathom
KU-P-48014	Race 2	Nakhon Prathom
KU-P-48015	Race 2	Nakhon Prathom
KU-P-48016	Race 2	Nakhon Prathom
KU-P-48017	Race 2	Nakhon Prathom
KU-P-48018	Race 2	Nakhon Prathom
KU-P-48019	Race 2	Nakhon Prathom
KU-P-48020	Race 3	Nakhon Prathom
KU-P-48021	Race 3	Nakhon Prathom
KU-P-48022	Race 2	Nakhon Prathom
KU-P-48023	Race 2	Nakhon Prathom
KU-P-48024	Race 3	Nakhon Prathom
KU-P-48025	Race 2	Nakhon Prathom
KU-P-48026	Race 2	Nakhon Prathom
KU-P-48027	Race 2	Nakhon Prathom
KU-P-48028	Race 2	Nakhon Prathom
KU-P-48029	Race 3	Nakhon Prathom
KU-P-48030	Race 2	Nakhon Prathom
KU-P-48031	Race 3	Nakhon Prathom
KU-P-48032	Race 2	Nakhon Prathom
KU-P-48033	Race 2	Nakhon Prathom
KU-P-48034	Race 2	Nakhon Prathom
KU-P-48035	Race 3	Nakhon Prathom
KU-P-48036	Race 3	Nakhon Prathom
KU-P-48037	Race 2	Nakhon Prathom
KU-P-48038	Race 2	Nakhon Prathom
KU-P-48045	Race 3	Chiang Rai
KU-P-48046	Race 3	Chiang Mai
KU-P-48047	Race 2	Chiang Mai

Table 6 (Continued)

Bacterial strain	Relevant characteristic [1/]	Reference or source (Thai province)
KU-P-48050	Race 3	Chiang Rai
KU-P-48053	Race 2	Chiang Rai
KU-P-48054	Race 2	Chiang Mai
KU-P-48055	Race 3	Chiang Mai
KU-P-48056	Race 2	Chiang Rai
KU-P-48057	Race 2	Chiang Mai
KU-P-48058	Race 3	Chiang Mai
KU-P-48059	Race 3	Chiang Rai
KU-P-48060	Race 2	Chiang Mai
KU-P-48063	Race 2	Chiang Rai
KU-P-48064	Race 3	Chiang Rai
KU-P-48066	Race 3	Chiang Rai
KU-P-48067	Race 3	Lop Buri
KU-P-48068	Race 3	Lop Buri
KU-P-48069	Race 2	Lop Buri
KU-P-48070	Race 2	Lop Buri
KU-P-48071	Race 2	Lop Buri
KU-P-48073	Race 3	Lop Buri
KU-P-48074	Race 2	Lop Buri
KU-P-48075	Race 2	Lop Buri
KU-P-48088	Race 3	Chiang Mai
KU-P-48089	Race 2	Chiang Mai
KU-P-48090	Race 2	Chiang Mai
KU-P-48091	Race 3	Chiang Mai
KU-P-48092	Race 2	Chiang Mai
KU-P-48093	Race 3	Chiang Mai
KU-P-48094	Race 2	Chiang Mai
KU-P-48095	Race 3	Chiang Mai
KU-P-48096	Race 2	Lop Buri
KU-P-48097	Race 2	Lop Buri
KU-P-48098	Race 3	Chiang Mai
KU-P-48101	Race 2	Chiang Mai
KU-P-48104	Race 2	Chiang Mai
KU-P-48105	Race 3	Lop Buri
KU-P-48107	Race 2	Nakhon Ratchasima
KU-P-48109	Race 3	Nakhon Ratchasima

Table 6 (Continued)

Bacterial strain	Relevant characteristic [1/]	Reference or source (Thai province)
KU-P-48111	Race 2	Nakhon Ratchasima
KU-P-48112	Race 3	Lop Buri
KU-P-48114	Race 2	Nakhon Ratchasima
KU-P-48115	Race 2	Nakhon Ratchasima
KU-P-48118	Race 3	Lop Buri
KU-P-48131	Race 2	Nakhon Ratchasima
KU-P-48135	Race 2	Nakhon Ratchasima
KU-P-48136	Race 2	Nakhon Ratchasima
KU-P-48143	Race 3	Nakhon Ratchasima
KU-P-48145	Race 2	Nakhon Ratchasima
KU-P-48148	Race 2	Nakhon Ratchasima
KU-P-48162	Race 2	Lop Buri
KU-P-48167	Race 2	Nakhon Ratchasima
KU-P-49001	Race 3	Nakhon Ratchasima
KU-P-49003	Race 2	Nakhon Ratchasima
KU-P-49004	Race 2	Nakhon Ratchasima
KU-P-49005	Race 3	Nakhon Ratchasima
KU-P-49008	Race 2	Nakhon Ratchasima
KU-P-49009	Race 2	Nakhon Ratchasima
KU-P-49012	Race 2	Nakhon Ratchasima
KU-P-49039	Race 3	Nakhon Ratchasima
KU-P-49045	Race 2	Nakhon Ratchasima
KU-P-49047	Race 2	Nakhon Ratchasima
KU-P-49051	Race 3	Nakhon Ratchasima
KU-P-49052	Race 2	Nakhon Ratchasima
KU-P-49053	Race 2	Nakhon Ratchasima
KU-P-49056	Race 2	Nakhon Ratchasima
KU-P-49058	Race 2	Nakhon Ratchasima
KU-P-49075	Race 3	Nakhon Ratchasima
KU-P-49076	Race 2	Nakhon Ratchasima
KU-P-49077	Race 3	Nakhon Ratchasima
KU-P-49078	Race 3	Nakhon Ratchasima
KU-P-49079	Race 2	Nakhon Ratchasima
KU-P-49081	Race 3	Nakhon Ratchasima
KU-P-49084	Race 2	Nakhon Ratchasima
KU-P-49085	Race 3	Nakhon Ratchasima

Table 6 (Continued)

Bacterial strain	Relevant characteristic [1/]	Reference or source (Thai province)
KU-P-49087	Race 2	Nakhon Ratchasima
KU-P-49089	Race 2	Nakhon Ratchasima
KU-P-49135	Race 3	Nakhon Ratchasima
KU-P-49235	Race 2	Nakhon Ratchasima
KU-P-49244	Race 2	Nakhon Ratchasima
KU-P-49248	Race 3	Nakhon Prathom
KU-P-49357	Race 2	Nakhon Prathom
KU-P-49444	Race 2	Nakhon Prathom
KU-P-49445	Race 2	Nakhon Prathom
KU-P-49446	Race 2	Nakhon Prathom
KU-P-49459	Race 2	Nakhon Prathom
KU-P-49473	Race 3	Nakhon Prathom
KU-P-49474	Race 2	Nakhon Prathom
KU-P-49475	Race 3	Nakhon Prathom
KU-P-49476	Race 3	Nakhon Prathom
KU-P-49483	Race 3	Nakhon Prathom
KU-P-49487	Race 2	Nakhon Prathom
KU-P-49498	Race 2	Nakhon Prathom
KU-P-49512	Race 2	Nakhon Prathom
KU-P-49515	Race 3	Nakhon Prathom
KU-P-49519	Race 3	Nakhon Prathom
KU-P-49522	Race 2	Nakhon Ratchasima
KU-P-49523	Race 2	Nakhon Ratchasima
KU-P-49526	Race 2	Nakhon Ratchasima
KU-P-50034	Race 2	Nakhon Ratchasima
KU-P-50047	Race 2	Nakhon Ratchasima
KU-P-50048	Race 2	Nakhon Ratchasima
KU-P-50049	Race 3	Nakhon Ratchasima
KU-P-50056	Race 2	Nakhon Prathom
KU-P-50057	Race 2	Nakhon Prathom
KU-P-50058	Race 2	Nakhon Prathom
KU-P-50061	Race 3	Nakhon Prathom
KU-P-50064	Race 3	Nakhon Prathom
KU-P-50065	Race 2	Nakhon Prathom
KU-P-50066	Race 3	Nakhon Prathom
KU-P-50074	Race 2	Nakhon Prathom

Table 6 (Continued)

Bacterial strain	Relevant characteristic [1/]	Reference or source (Thai province)
KU-P-50075	Race 2	Nakhon Prathom
KU-P-50076	Race 2	Nakhon Prathom
KU-P-50087	Race 3	Nakhon Prathom
KU-P-50089	Race 3	Nakhon Prathom
KU-P-50090	Race 2	Nakhon Prathom
KU-P-50094	Race 3	Nakhon Prathom
KU-P-50097	Race 2	Chiang Mai
KU-P-50099	Race 2	Chiang Mai
KU-P-50127	Race 3	Chiang Mai
KU-P-50128	Race 3	Chiang Mai
KU-P-50134	Race 2	Chiang Mai
KU-P-50137	Race 2	Chiang Mai
KU-P-50138	Race 2	Chiang Mai
KU-P-50139	Race 2	Chiang Mai
KU-P-50143	Race 2	Chiang Mai
KU-P-50146	Race 2	Chiang Mai
KU-P-50147	Race 3	Chiang Mai
KU-P-50148	Race 3	Chiang Mai
KU-P-50149	Race 2	Chiang Mai
KU-P-50156	Race 2	Chiang Mai
KU-P-50158	Race 2	Chiang Mai
KU-P-50159	Race 3	Chiang Mai
KU-P-50161	Race 2	Nakhon Prathom
KU-P-50164	Race 2	Chiang Mai
KU-P-50165	Race 2	Chiang Mai
KU-P-50166	Race 3	Nakhon Prathom
KU-P-50171	Race 2	Phetchabun
KU-P-50174	Race 2	Phetchabun
KU-P-50177	Race 3	Phetchabun
KU-P-50179	Race 2	Phetchabun
KU-P-50196	Race 3	Phetchabun
KU-P-50197	Race 2	Nakhon Prathom
KU-P-50198	Race 2	Nakhon Prathom
KU-P-50231	Race 3	Phetchabun
KU-P-50232	Race 3	Phetchabun
KU-P-50233	Race 3	Phetchabun

Table 6 (Continued)

Bacterial strain	Relevant characteristic [1/]	Reference or source (Thai province)
KU-P-50234	Race 3	Phetchabun
KU-P-50235	Race 3	Phetchabun
KU-P-50236	Race 2	Phetchabun
KU-P-50239	Race 2	Phetchabun
KU-P-50241	Race 3	Phetchabun
KU-P-50252	Race 2	Phetchabun
KU-P-50256	Race 3	Phetchabun
KU-P-50257	Race 2	Phetchabun
KU-P-50259	Race 3	Phetchabun
KU-P-50264	Race 3	Phetchabun
KU-P-50265	Race 2	Phetchabun
KU-P-50268	Race 2	Phetchabun
KU-P-50269	Race 2	Phetchabun
KU-P-50270	Race 2	Phetchabun
KU-P-50275	Race 2	Phetchabun

[1/] Race 1 induced hypersensitive response (HR) on SJ4, Spencer, PI 520733, and Williams82, Race 2 caused disease on all cultivars tested, and Race 3 caused disease on susceptible cultivars (SJ4, Spencer, and PI 520733) and induced HR on resistant cultivar (Williams82).

3. Screening and characterization of *avrXg1* mutants

Of 3,336 random Tn5 mutants generated from strain KU-P-SW005, one designated KU-P-SW005 *avrXg1*::Tn5AAD caused disease on all four soybean cultivars including Williams82 (wildtype causes HR) (Fig. 5). It was therefore hypothesized that an avirulence factor was knocked out making the mutant virulent on Williams82. The region surrounding the Tn5 insertion in KU-P-SW005 *avrXg1*::Tn5AAD was sequenced and showed identity (100% at nucleotide level) within a deduced ADD domain in the C-terminal region (Fig. 14) of an *avrBs3* gene homolog in *X. axonopodis* pv. *glycines* strains AG1 and 8ra (AY780631.1 and AY780632.1 respectively) (Kim *et al.,* 2006), *X. axonopodis* pv. *malvacearum* (L06634.1), *X. axonopodis* pv. *vesicatoria* (X68781.1 and X16130.1), and *X. axonopodis* pv. *citri* (AB021365.1, AB206389.1, AB206387.1, U28802.1, AE008925.1, AE008924.1, AB021363.1, AB021364.1, and AB175482.1). This strongly suggested the gene mutated was the member of the *avrBs3* gene family.

To confirm that the virulence phenotype observed on Williams82 resulted from disruption of *avrXg1,* pUC19-AVR construct was made as described in the materials and methods. The 3.8-kb fragement of *avrBs3* homolog was amplified from KU-P-SW005, ligated with pUC19, inserted Tn5 in the middle of the target gene in pUC19 (Fig. 6 and 7). The construct was confirmed by *EcoRI* digestion, PCR, and DNA sequencing. A targeted mutation was made at the 4th repeat region within the central portion of the gene (Fig. 14). The resulting mutant, KU-P-SW005 *avrXg1*::Tn5REP, displayed the same phenotypes as KU-P-SW005 *avrXg1*::Tn5AAD (Fig. 5).

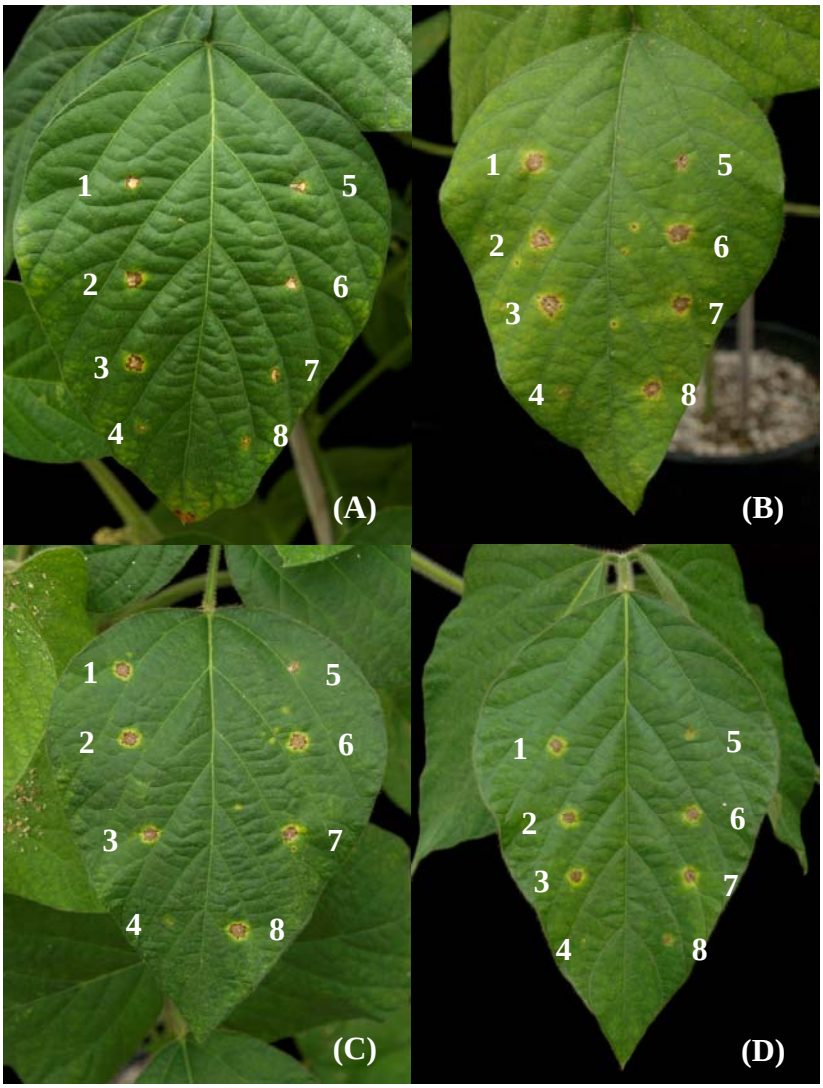

Figure 5 Comparison of compatible and incompatible reactions on Williams82 (A), Spencer (B), PI 520733 (C), and SJ4 (D) by wildtype KU-P-SW005 (1), *avrXg1* Tn5 mutant; KU-P-SW005 *avrXg1*::Tn5AAD (2), *avrXg1* targeted mutant; KU-P-SW005 *avrXg1*::Tn5REP (3), *Escherichia coli* TOP10 (pBBR-AVR) (4), Race 1 wildtype KU-P-34070 (5), *avrXg1*::Tn5AAD (pBBR-AVR) (6), *avrXg1*::Tn5REP (pBBR-AVR) (7), and KU-P-34070 (pBBR-AVR) (8). Bacteria were infiltrated into soybean leaves as described in text. Symptoms were recorded at 7 days after infiltration.

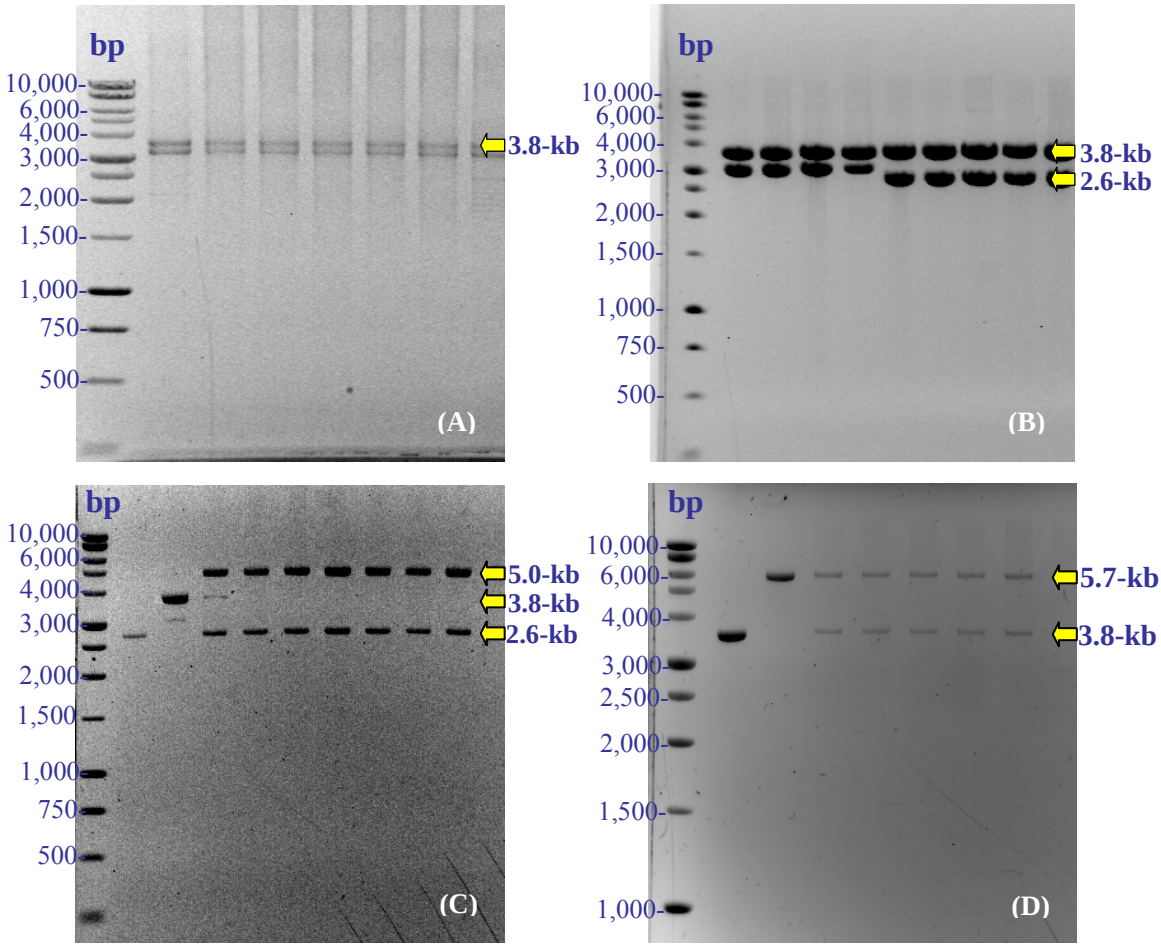

Figure 6 An *avrBs3* homolog 3.8-kb product was detected in *Xanthomonas axonopodis* pv. *glycines* KU-P-SW005 (A). The 3.8-kb sequence in pUC19 vector was digested with *Eco*RI showed an *avrBs3* homolog 3.8-kb fragment and pUC19 2.6-kb (B). Digestion of pUC19-AVR and pBBR-AVR with *Eco*RI showed 5.0-kb of *avrBs3* homolog that Tn5 inserted and 2.6-kb of pUC19 (C); 3.8-kb of *avrBs3* homolog and 5.7-kb of pBBR1MCS-5 (D), respectively.

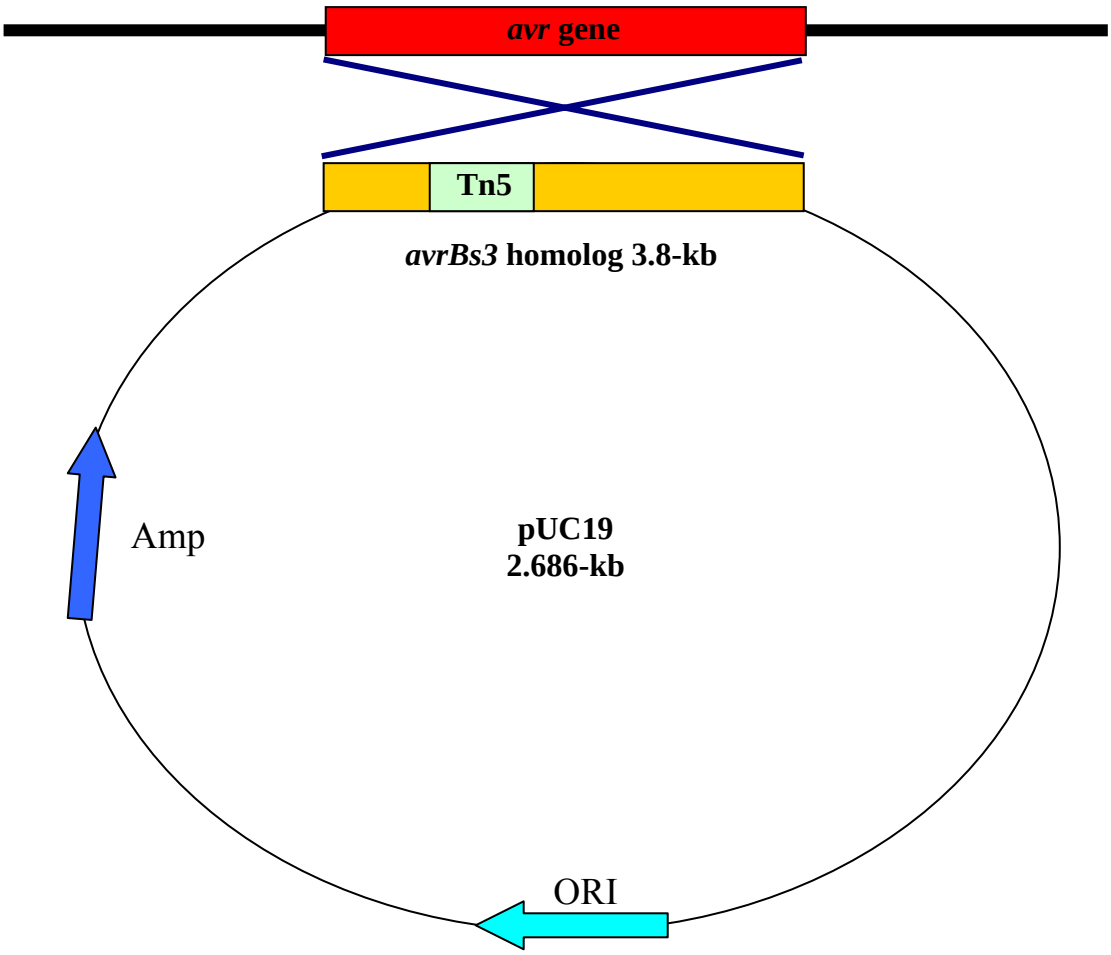

Figure 7 Site-directed mutation and schematic of pUC-AVR 5.0-kb *avrBs3* homolog fragment containing transposon insertion from KU-P-SW005 in pUC19, Km^r Amp^r.

4. Analysis of gene abolished HR on Williams82

X. axonopodis pv. *glycines* mutants, KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP were selected to amplify the Tn5 fragment by the primer KAN-1-FP and KAN-1-RP compared to wild type strain. The 800-bp of Kan^R were detected from both *X. axonopodis* pv. *glycines* mutants. In contrast, the wild type could not amplify DNA product of PCR analysis (Fig. 8A). Also, an *avrBs3* homolog Tn5 insertion showed size 5.0-kb which detected by PCR with primers AVR-FP and AVR-RP whereas 3.8-kb of *avrBs3* homolog gene was detected in the wild type. It was determined that the transposon insertion occurred in the *avrBs3* homolog of wildtype strain (Fig. 8B).

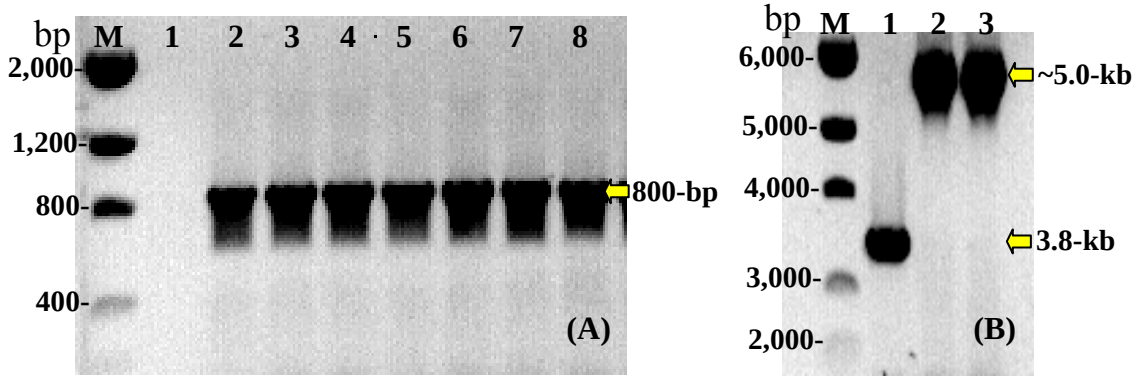

Figure 8 Polymerase chain reaction amplification of kanamycin resistant marker 800-bp in EZ-TN^{TM} <KAN-2> Transposon inserted of *Xanthomonas axonopodis* pv. *glycines* mutants with primer KAN-1-FP and KAN-1-RP (A). A 5.0-kb of *avrBs3* homolog Tn5 insertion detected by PCR with AVR-FP and AVR-RP compared with 3.8-kb original *avrBs3* homolog from wildtype (B). The PCR products were separated by electrophoresis on a 0.7% agarose gel.

5. Analysis of the *avrXg1* mutant and wildtype

5.1 HR induction

The *avrXg1* mutants, KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP; Race 3 wildtype, KU-P-SW005; Race 1 wildtype, KU-P-34070; Race 1 transformed with 3.8-kb *avrXg1*, KU-P-34070 (pBBR-AVR); complemented strains, *avrXg1*::Tn5AAD (pBBR-AVR) and *avrXg1*::Tn5REP (pBBR-AVR); Race 2 wildtype, KU-P-KPS06; and Race 2 transformed with *avrXg1,* KU-P-KPS06 (pBBR-AVR) induced HR which produced a typical HR on all plant species tested whereas *Escherichia coli* TOP10 carrying pBBR-AVR did not cause HR on plant species tested (Table 7 and Fig. 9). *X. axonopodis* pv. *glycines* strains were differed with respect to their expression of HR on three species of tobacco and also tomato including their degree of virulence on soybean. The results showed symptoms were not different between all of strains and it could be suggested that the *avrXg1* gene mutants did not effect HR induction on tobacco and tomato.

5.2 Enzyme assays

The virulence factors including cellulase, pectate lyase, endoglucanase, protease, mannanase, and polygalacturonate lyase of *X. axonopodis* pv. *glycines* were reported (Thowthampitak *et al.*, 2008). Also, *avr* gene was secreted virulence factors via TTSS (Vivian and Gibbon, 1997). We compared *avrXg1* mutants (KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP) and wildtype (KU-P-SW005) derivatives for exoenzyme activity. The *avrXg1* mutants were more efficient in producing cellulase and pectate lyase compared with wildtype (Fig. 10). There was no difference in α-amylase, protease, and endoglucanase production. In the same trend the virulence *X. axonopodis* pv. *malvacearum* strain HVS GSPB 2388, GSPB 2389 (isolate from Sudan), and ATCC 49294 showed higher cellulase activity than GSPB 1386 (Race 18) and GSPB 2385 (Race 11) (Huang and Rudolph, 2008). It can be suggested that *avrXg1* mutation may involve other genes inside the bacterial cell such as cellulase and pectate lyase.

Table 7 Morphotype strains of *Xanthomonas axonopodis* pv. *glycines* that induced the hypersensitive response on three-species of tobacco and on tomato

Bacterial strain[1/]	**Tobacco plant specie**[2/]			**Tomato**[2/]
	***Nicotiana tabacum* cv. Xanthi**	***N. glauca***	***N. rustica***	
KU-P-SW005	+	+	+	+
KU-P-SW005 *avrXg1*::Tn5AAD	+	+	+	+
avrXg1::Tn5AAD (pBBR-AVR)	+	+	+	+
KU-P-SW005 *avrXg1*::Tn5REP	+	+	+	+
avrXg1::Tn5REP (pBBR-AVR)	+	+	+	+
KU-P-34070	+	+	+	+
KU-P-34070 (pBBR-AVR)	+	+	+	+
KU-P-KPS06	+	+	+	+
KU-P-KPS06 (pBBR-AVR)	+	+	+	+
E. coli TOP10 (pBBR-AVR)	-	-	-	-

[1/]Comparison of hypersensitive response on three species of tobacco leaf including *Nicotiana tabacum* cv. Xanthi, *N. glauca,* and *N. rustica* and on tomato leaf by *Xanthomonas axonopodis* pv. *glycines* Race 3 wildtype KU-P-SW005, *avrXg1* Tn5 mutant; KU-P-SW005 *avrXg1*::Tn5AAD, complemented strain; *avrXg1*::Tn5AAD (pBBR-AVR), *avrXg1* targeted mutant; KU-P-SW005 *avrXg1*::Tn5REP, complemented strain; *avrXg1*::Tn5REP (pBBR-AVR), Race 1 wildtype KU-P-34070, Race 1 transformed with *avrXg1*; KU-P-34070 (pBBR-AVR), Race 2 wildtype KU-P-KPS06, Race 2 transformed with *avrXg1*; KU-P-KPS06 (pBBR-AVR), and *Escherichia coli* TOP10(pBBR-AVR).

[2/] A positive response (+) resulted in complete collapse and necrosis of the infiltrated area. A negative response (-) may show slight yellowing but no visible collapse or necrosis of infiltrated zone. Infiltrated areas were monitored for development of tissue collapse and necrosis for 48 h post inoculation.

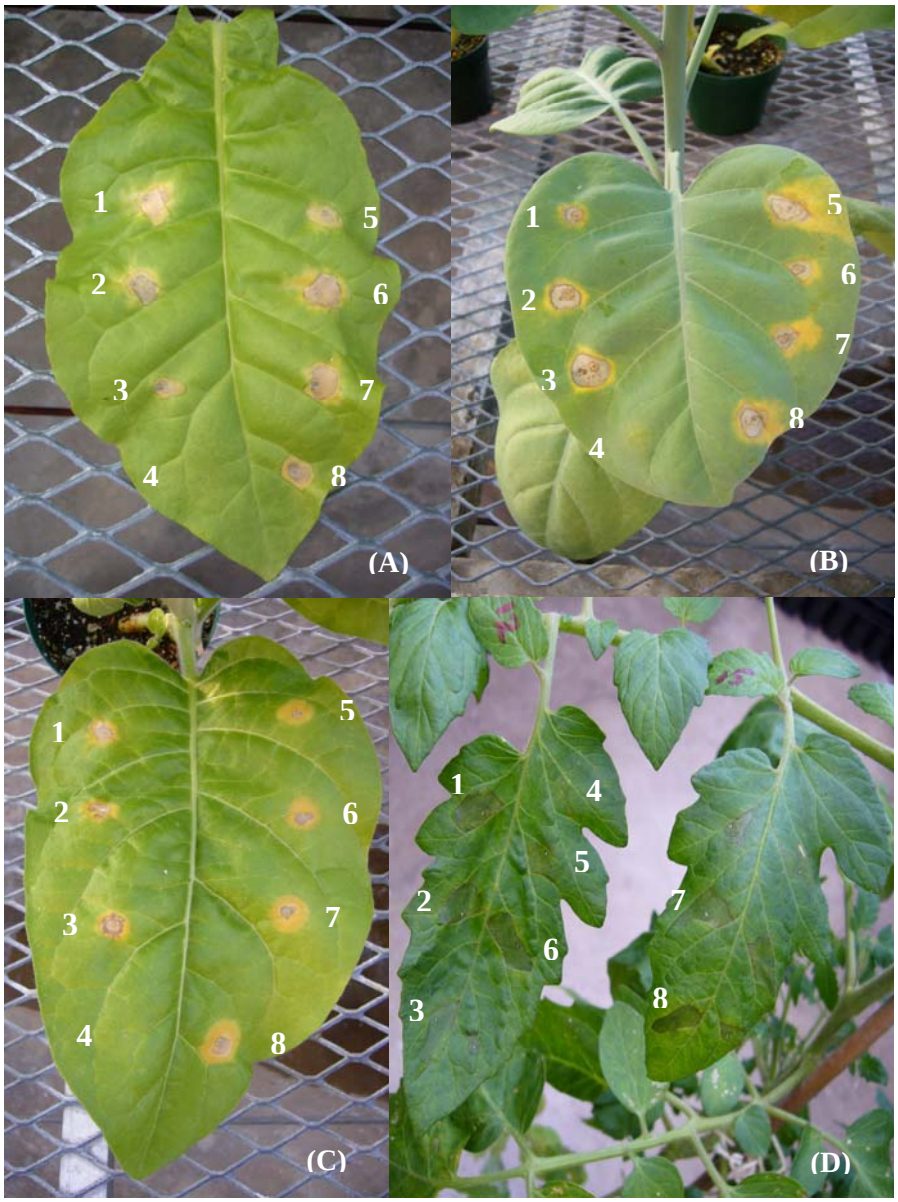

Figure 9 Hypersensitive reaction elicited by *Xanthomonas axonopodis* pv. *glycines* Race 3 wildtype KU-P-SW005 (1), *avrXg1* Tn5 mutant; KU-P-SW005 *avrXg1*::Tn5AAD (2), *avrXg1* targeted mutant; KU-P-SW005 *avrXg1*::Tn5REP (3), *Escherichia coli* TOP10 (pBBR-AVR) (4), Race 1 wildtype KU-P-34070 (5), *avrXg1*::Tn5AAD (pBBR-AVR) (6), *avrXg1*::Tn5REP (pBBR-AVR) (7), and KU-P-34070 (pBBR-AVR) (8) on three species of tobacco leaf; *Nicotiana tabacum* cv. Xanthi (A), *N. glauca* (B), and *N. rustica* (C), and tomato (D).

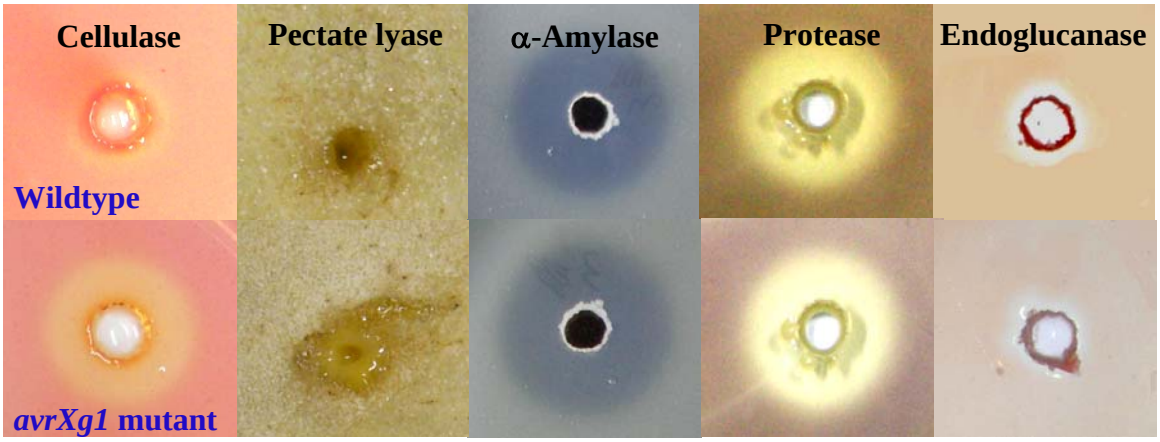

Figure 10 Exoenzyme assay revealed the *avrXg1* mutants, KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP were more efficient in producing cellulase and pectate lyase compared with wildtype (KU-P-SW005). There was no difference in α-amylase, protease, and endoglucanase production.

5.3 Motility test

Represented *X. axonopodis* pv. *glycines* from Race 3, strain KU-P-SW005 showed higher swarming efficacy than that of the other races. Interestingly, *avrXg1* mutants (KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP) showed greater swarming motility than wildtype (Fig. 11). This indicated that *avrXg1* essential for bacterial motilily that may involve with flagella or pili cluster expression is being investigated.

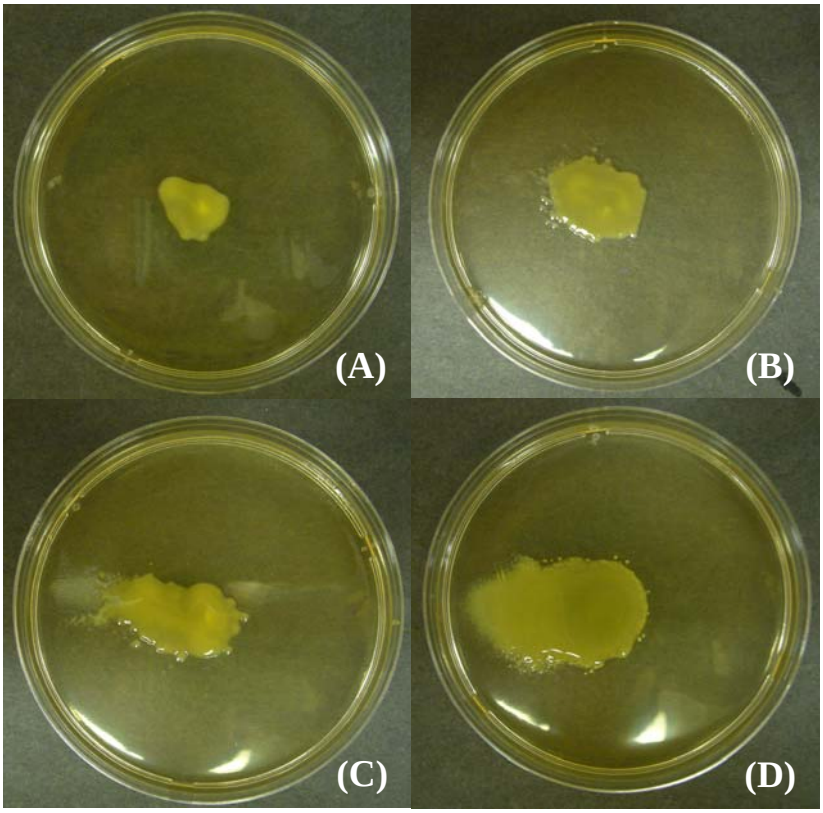

Figure 11 Swarming motility of *Xanthomonas axonopodis* pv. *glycines* Race 1, strain KU-P-34070 (A), Race 2, strain KU-P-KPS06 (B), Race 3, strain KU-P-SW005 (C), and *avrXg1* mutant; KU-P-SW005 *avrXg1*::Tn5REP (D) on a nutrient glucose agar solid with 0.4% agar surface for 2 days.

6. Presence of *avrBs3*-like genes in races of *X. axonopodis* pv. *glycines*

Further characterization of *X. axonopodis* pv. *glycines* races was accomplished by PCR analyses of genomic DNA with primers AVR-FP and AVR-RP. A 3.8-kb product was detected in Races 2 and 3 but not in Race 1 strains (Fig. 12). These results suggest that the non-specific HR phenotype of Race 1 is not associated with an *avrBs3*-like gene but is associated with a different mechanism of HR. The detection of the amplicon in Race 2 strains suggests the presence of *avrBs3*-like genes, however not *avrXg1* that reacts with a receptor in Williams82 to confer the host-specific HR phenotype.

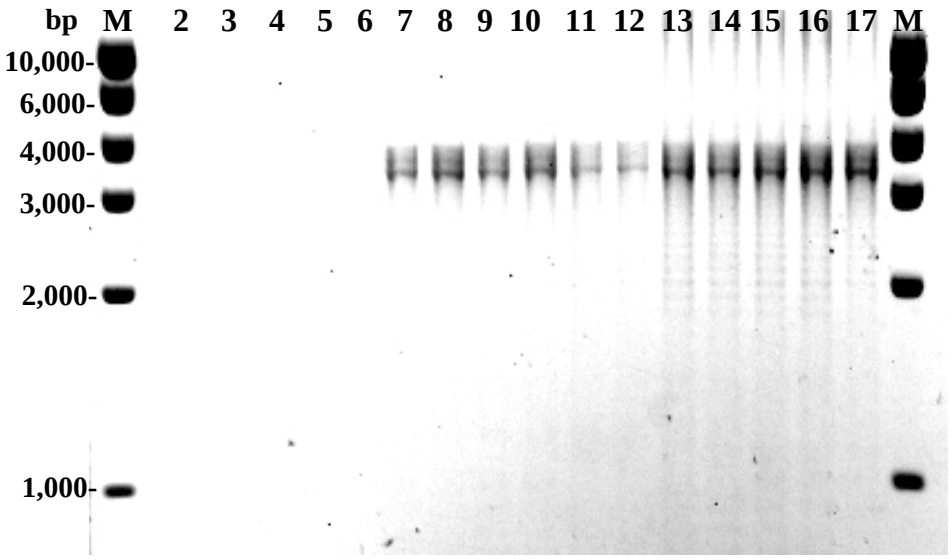

Figure 12 Presence of 3.8-kb coding region of *avrBs3* homolog gene in representative strains of Race 1, Race 2, and Race 3. Lanes 1 and 18 = 1-kb size marker, lanes 2-6 = Race 1; strains KU-P-34043, KU-P-34054, KU-P-34062, KU-P-34070, and KU-P-34071, lanes 7-13 = Race 2; strains KU-P-34008, KU-P-34016, KU-P-34042, KU-P-34089, KU-P-34098, KU-P-KPS06, and ST015, lanes 14-17 = Race 3; strains KU-P-KPS01, KU-P-KPS04, KU-P-SW005, and KU-P-SW008. The PCR products were separated by electrophoresis on a 0.7% agarose gel.

7. Analyses of *avrXg1*

The complete sequence of *avrXg1* was compared to the *avrBs3* homolog in *X. axonopodis* pv. *glycines* strain AG1 (AY780631.1) (Kim *et al.,* 2006). Primers for PCR amplification of gene fragments were described in the Table 5. The coding region of *avrXg1* gene was 3.8-kb in length with an overall G+C content of 67%. The central region of the gene was characterized by 20.5 copies of tandemly-arranged 102-bp direct repeats. The C-terminal region of the gene was defined by three NLSs and an AAD domain, characteristic of other sequenced members of the *avrBs3*/*pthA* gene family (Fig. 14).

Alignment analysis of *avrXg1* by MegAlign program (DNAstar inc) revealed the deduced protein shares identity with AvrBs3-like protein sequences (98% at amino acid levels) of *X. axonopodis* pv. *glycines* strain AG1 and 8ra (YP_001965982.1 and YP_001966011.1 respectively) and encodes a predicted 1,266 amino acid protein with a molecular mass of 132.8 kDa and pI of 8.8. The amino acid sequence of the deduced protein from *avrXg1* shows differences in 13 amino acid residues in the N-terminal region at positions 23, 76, 81, 82, 85, 89, 91, 92, 94, 95, 130, 134, and 197 respectively as compared to the *avrBs3* homologs from AG1 and 8ra (YP_001965982.1 and YP_001966011.1 respectively) (Kim *et al.,* 2006) (Fig. 13). The putative start codon of *avrXg1* is ATG, and a predicted promoter position, -35 and -10 boxes are located at position 171 and 190, -TTGCCA- and -TTGTGTACT- respectively upstream from the start codon. A signal peptide, MDPIRSRTPSPARELLPGPQPDRVQPTADR. is also predicted for AvrXg1 (Fig. 13 and 14).

Nucleotide sequence accession number, the complete DNA sequence obtained from *X. axonopodis* pv. *glycines* strain KU-P-SW005, referred to as *avrXg1*, was deposited as GenBank accession no. FJ439173.

Figure 13 Alignment of the deducted protein sequence of AvrXg1 and AvrBs3. Identical amino acids are represented in bold. Open boxes show non-conserved amino acids substitutions. Light hachured boxes show conserved amino acid substitutions and dark hachured boxes semi-conserved substitutions.

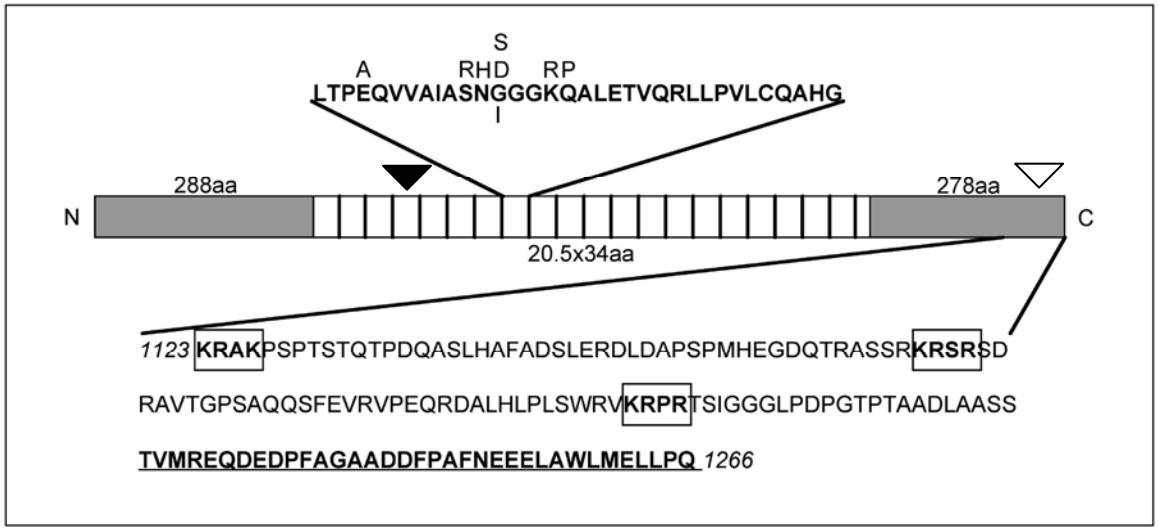

Figure 14 The deduced AvrXg1 protein (1,266 amino acids, 132 kDa) illustrating three major subdomains: the N-terminal domain of 288 amino acids, the internal domain consisting of 20.5 nearly identical repeats of 34 amino acids, and the C-terminal domain of 278 amino acids. Conserved sequence of the repeats is in bold with variances indicated. The C-terminal region (amino acids 1123-1266) contains three putative nuclear localization sequences (NLSs) and an acidic activation domain (AAD). The boxed and bold sequences fit the consensus for monopartite NLSs (K-K/R-X-K/R). The bold and underlined amino acid sequences indicate the proposed AAD. A targeted mutation was made at the 4th repeat region indicated by back arrow. Random Tn5 mutation was sequenced within a deduced ADD domain in the C-terminal region indicated by white arrow.

8. Location of *avrXg1* in KU-P-SW005 genome

It was determined that Race 3 wildtype KU-P-SW005 and *avrXg1* mutants (KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP) carry a 26-kb, 15-kb, and 1.3-kb native plasmids, whereas Race 1, strain KU-P-34070 (causes HR on all soybean cultivars) does not have the 26-kb and 15-kb plasmid (Fig. 15). The sequence of three native plasmids pAG1, pXAG81, and pXAG82 of sizes 15, 26, and 1.3-kb respectively were previously reported in *X. axonopodis* pv. *glycines* strains AG1 and 8ra and only pAG1 and pXAG81 contained *avrBs3* homologs (AY780631.1 and AY780632.1 respectively) (Kim *et al.,* 2006).

For *avrXg1* mutants KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP total genomic and plasmid DNAs were examined for the presence of the 800-bp kanamycin resistance marker. The amplicon was detected on both templates of mutants providing evidence that *avrXg1* was located on native plasmid of KU-P-SW005. Moreover, DNA sequencing of the putative coding region 274-bp upstream and 70-bp downstream of *avrXg1* from plasmid DNA of KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP showed identity (100% at nucleotide level) to regions upstream and downstream of an *avrBs3* homolog located on pAG1 and pXAG81 (Kim *et al.,* 2006) again indicating that *avrXg1* is plasmid-borne.

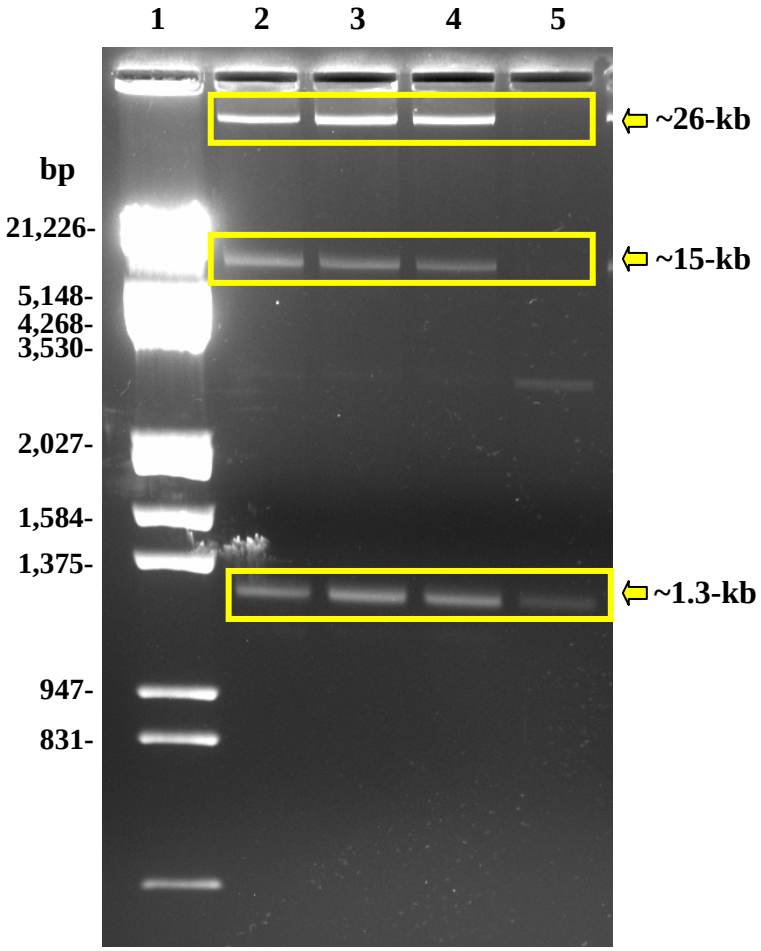

Figure 15 The native plasmid size 26, 15, and 1.3-kb were detected in Race3 wildtype KU-P-SW005 (lane 2), *avrXg1* Tn5 mutant; KU-P-SW005 *avrXg1*::Tn5AAD (lane 3), and *avrXg1* targeted mutant; KU-P-SW005 *avrXg1*::Tn5REP (lane 4), whereas detected plasmid size was 2.5 and 1.3-kb in Race 1 wildtype strain KU-P-34070 (lane 5). The PCR products were separated by electrophoresis on a 0.5% agarose gel and using lamda DNA + *Hind*III marker (lane 1).

9. Complementation and virulence assays on soybean

Complementation of KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP was accomplished by transformation with pBBR-AVR (Fig. 6D). This resulted in regained ability to induce a strong HR on Williams82 and pustule disease on SJ4, Spencer, and PI 520733 within 48 h. *E. coli* TOP10 harboring pBBR-AVR did not cause HR or disease on Williams82, SJ4, Spencer or PI 520733 (Fig. 5). Lesion expansion of KU-P-SW005, KU-P-SW005 *avrXg1*::Tn5AAD, KU-P-SW005 *avrXg1*::Tn5REP, *avrXg1*::Tn5AAD (pBBR-AVR), and *avrXg1*::Tn5REP (pBBR-AVR) on Williams82 and Spencer did not differ (Table 8). However, virulence of strains refers to disease severity rather than lesions size.

Function of *avrXg1* was further verified by expression of the gene in Race 1, strain KU-P-34070 transformed with pBBR-AVR resulting in HR on Williams82 within 48 h and disease on SJ4, Spencer, and PI 520733 within 5 days after infiltration. The surrounding yellow halo caused by transformed KU-P-34070 on the susceptible varieties however appeared smaller than for wildtype Race 3, KU-P-SW005 (Table 8 and Fig. 5). These results suggest that *avrXg1* in the Race 3 genetic background blocks expression of the non-specific HR on susceptible cultivars.

In addition, function of *avrXg1* was further verified by expression of the gene in Race 2, strain KU-P-KPS06 transformed with pBBR-AVR resulting in gained virulence on Williams82 and Spencer within 3 days after infiltration whereas Race 2, wildtype KU-P-KPS06 induced disease within 5 days after infiltration. The surrounding yellow halo by transformed KU-P-KPS06 on the both varieties did not differ with Race 3. These results suggested that *avrXg1* in the Race 3 effect pathogen aggressiveness also (Kearney and Staskawicz, 1990; Bai *et al.*, 2000).

Mutants KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP gave the highest virulence ratings; 51.02% and 49.79% on Spencer respectively; 11.11% and 12.34% on Williams 82 respectively. In similar trend, the bacterial virulent factors; cellulase and pectate lyase were increased on the *avrXg1* mutants leading to a 20%-increase in the rating of bacterial pustule on Spencer as compared to wildtype (Fig. 16).

In contrast, KU-P-SW005, *avrXg1*::Tn5AAD (pBBR-AVR), *avrXg1*::Tn5REP (pBBR-AVR), and KU-P-34070 (pBBR-AVR) gave disease severities of 29.62%, 26.33%, 25.51%, and 23.86% on Spencer respectively which were lower than both *avrXg1* mutants where Race 1, wildtype KU-P-34070 induced HR (Table 8). Interesting, Race 2 wildtype KU-P-KPS06 transformed with pBBR-AVR designed KU-P-KPS06 (pBBR-AVR) gave disease severities 57.61% and 58.01% on Williams82 and Spencer respectively, whereas wildtype KU-P-KPS06 gave disease severities 53.50% and 54.73% on Williams82 and Spencer respectively. Its mean KU-P-KPS06 (pBBR-AVR) was gained virulence to a 7.52% and 5.99%-increase in the rating of bacterial pustule on Williams82 and Spencer respectively as compared to wildtype.

Growth of mutants KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP and Race 2 wildtype transformed with *avrXg1,* KU-P-KPS06 (pBBR-AVR) on Spencer and Williams82 tissue were significantly increased ($P \leq 0.05$), reaching population numbers higher than wildtype strains (Fig. 17). Furthermore, KU-P-34070 tramsformed with *avrXg1*, KU-P-34070 (pBBR-AVR), grew in leaves and induced the disease on susceptible cultivars whereas wildtype KU-P-34070 caused HR and was not detected on Spencer. These results suggested that the central repeat and AAD regions are associated with ability of AvrXg1 to confer both additive fitness and redundancy of virulence functions.

Viable cells of KU-P-SW005, *avrXg1*::Tn5AAD (pBBR-AVR), *avrXg1*::Tn5REP (pBBR-AVR), KU-P-34070, and KU-P-34070 (pBBR-AVR) were not detected on Williams82 leaves at 48 h after infiltration and HR phenotype development.

Table 8 Lesion size and disease severity for wildtype, *avrXg1* mutants, and complemented mutants on cultivars Williams82 and Spencer[1/]

Strain[2/]	Lesion mean diam (mm)		Disease severity (%)[3/]	
	Williams82	**Spencer**	**Williams82**	**Spencer**
KU-P-SW005	HR	0.65a	HR	29.62b
KU-P-SW005 *avrXg1*::Tn5AAD	0.65a	0.64a	11.11b	51.02a
avrXg1::Tn5AAD (pBBR-AVR)	HR	0.64a	HR	26.33b
KU-P-SW005 *avrXg1*::Tn5REP	0.66a	0.65a	12.34b	49.79a
avrXg1::Tn5REP (pBBR-AVR)	HR	0.63a	HR	25.51b
KU-P-34070	HR	HR	HR	HR
KU-P-34070 (pBBR-AVR)	HR	0.52b	HR	23.86b
KU-P-KPS06	0.65a	0.66a	53.50a	54.73a
KU-P-KPS06 (pBBR-AVR)	0.67a	0.67a	57.61a	58.01a

[1/] Numbers followed by different letters show significance at $P \leq 0.05$.

[2/] KU-P-SW005, wildtype Race 3; KU-P-SW005 *avrXg1*::Tn5AAD, Tn5 *avrXg1* mutant; KU-P-SW005 *avrXg1*::Tn5REP, targeted *avrXg1* mutant; *avrXg1*::Tn5AAD (pBBR-AVR) and *avrXg1*::Tn5REP (pBBR-AVR), complemented mutants; KU-P-34070, Race 1; KU-P-KPS06, Race 2; KU-P-34070 (pBBR-AVR) and KU-P-KPS06 (pBBR-AVR), Race 1 and Race 2 transformed with *avrXg1*, respectively.

[3/] Disease severity was evaluated as described in text. For each strain at least three leaves, collected from the top, middle, and basal portion of three plants, were evaluated. HR = hypersensitive response.

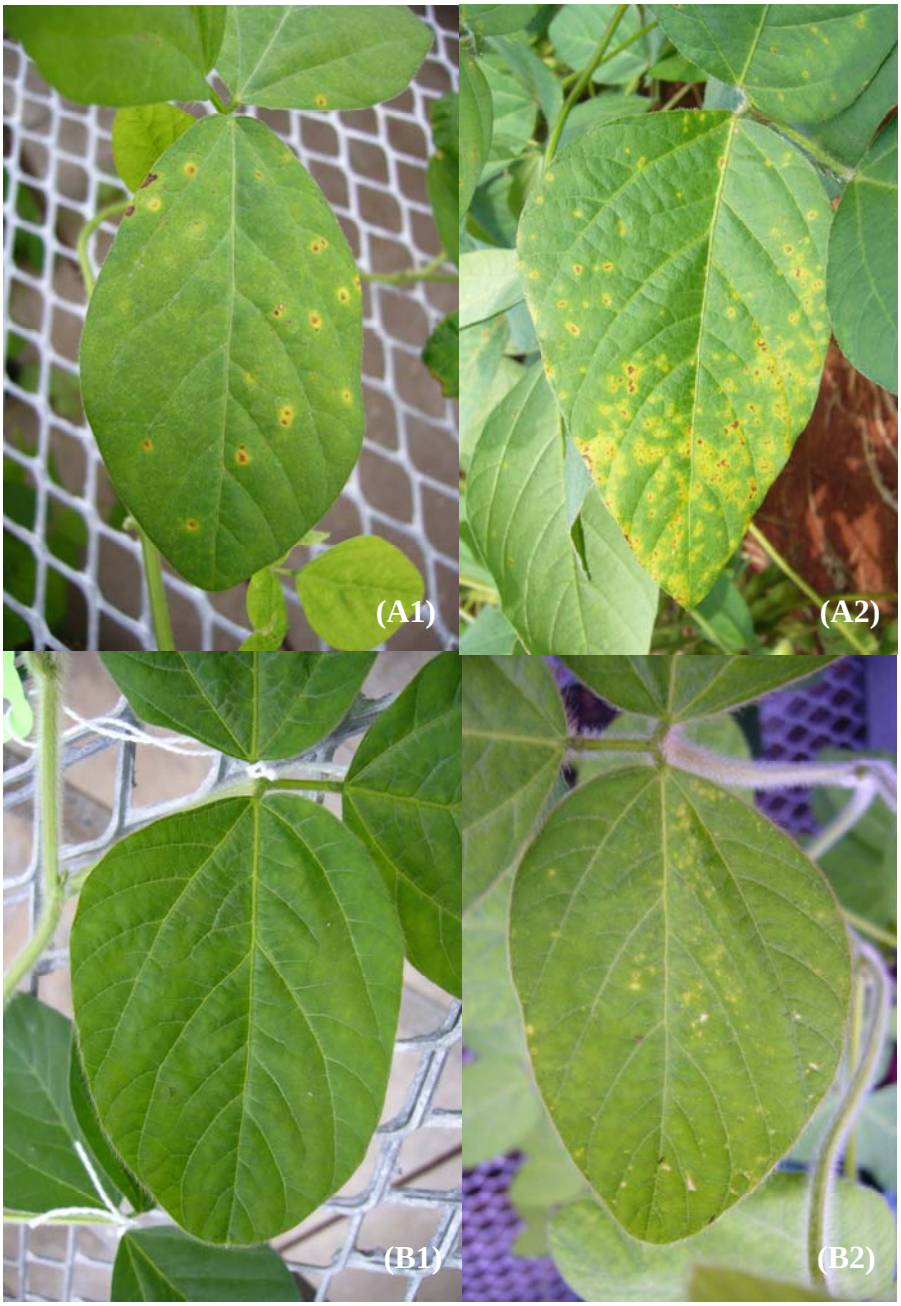

Figure 16 Disease induction on Spencer (A) and Williams82 (B) caused by wildtype Race 3 (1) and *avrXg1* mutants (KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP) (2) by spray inoculation at 10^8 cfu/ml. The *avrXg1* mutants induce the pustule symptoms that are smaller than the wildtype.

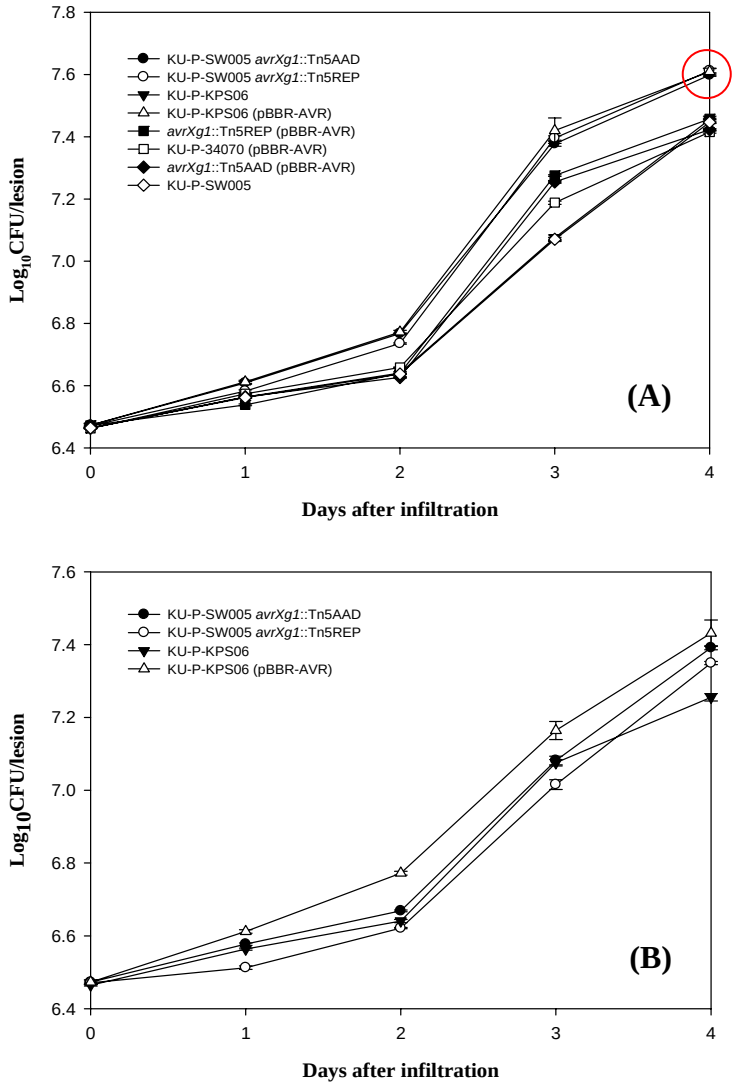

Figure 17 Time course of bacterial growth in soybean leaves Spencer (A) and Williams82 (B). Leaves were sampled at 1, 2, 3, and 4 days after infiltration. For Spencer KU-P-34070 caused HR and were undetectable in leaves within 48 h. For Williams82, KU-P-SW005, KU-P-34070, *avrXg1*::Tn5AAD (pBBR-AVR), *avrXg1*::Tn5REP (pBBR-AVR), and KU-P-34070 (pBBR-AVR) caused HR and were undetectable within 48 h. Data represents means from three repetitions, and vertical bars represents standard errors.

10. Expression of *avrXg1*

An *avrXg1* was ligated and expressed in two different expression vectors including pET160/GW/D-TOPO$^{(R)}$ and pRSETB. The construct DNA sequencing was done at the Cornell Life Sciences Core Laboratories Center. The results showed it was not expressed in both expression vectors in concentration of 1 mM IPTG at 28°C and 37°C (Fig. 18). This supported by *avr* genes may after all be virulence factors, introduced by the TTSS into plant cells (Vivian, and Gibbon, 1997). Moreover, the interactions may be involved in limiting the host range of a pathogen in addition to the general defenses of the plant. Kobayashi *et al*. (1990) found that *avr* genes from *P*. *syringae* pv. *tomato* exhibited cultivar-specificity on soybean when expressed in *P*. *syringae* pv. *glycinea*.

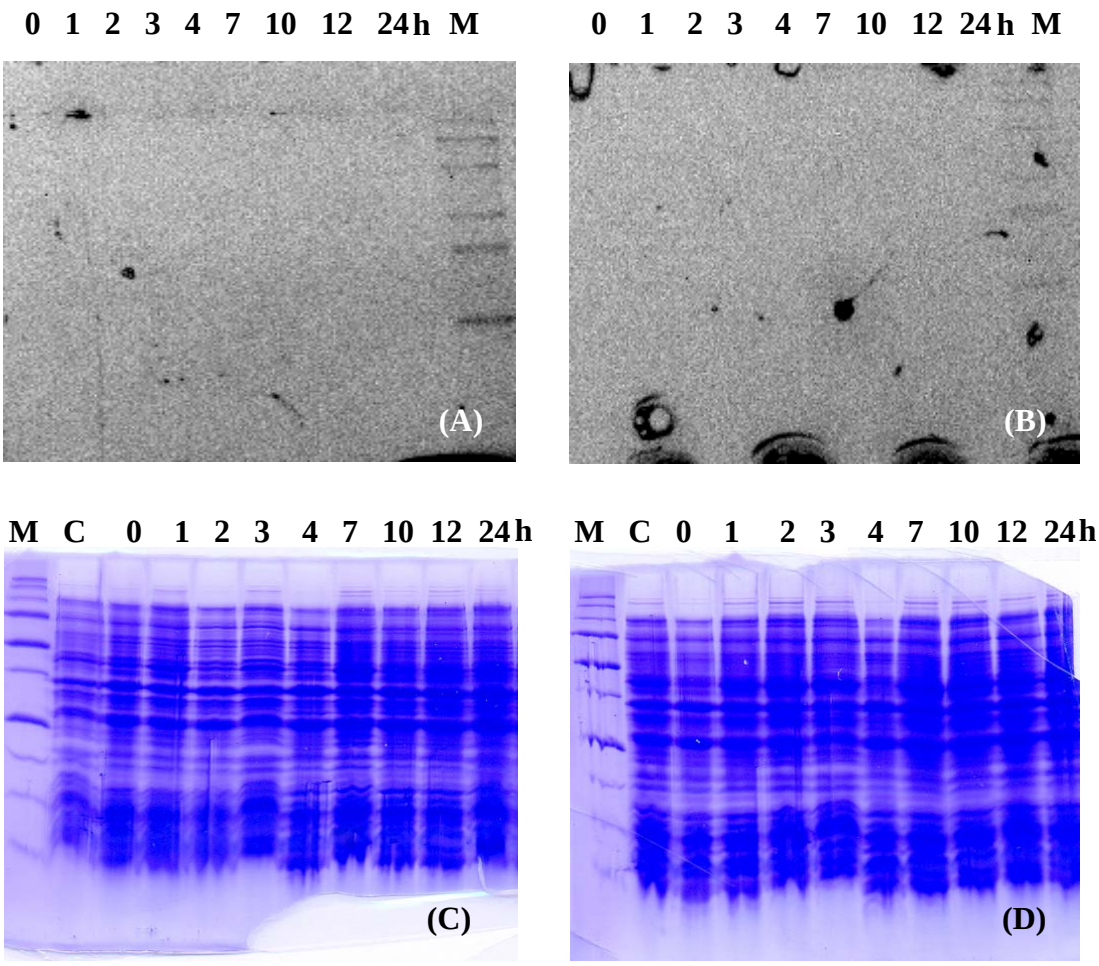

Figure 18 Expression of the *avrXg1* was induced with 1 mM IPTG at 28°C (A and C) and 37°C (B and D) for 0, 1, 2, 3, 4, 7, 10, 12, and 24 h. Protein sample detected by Lumio^{TM}Green Detection Kit (Invitrogen, CA, USA) (A and B) and Coomassie brilliant blue R-250 staining (C and D).

11. Proteomic analysis of soybean response to *X. axonopodis* pv. *glycines* Race 3 and *avrXg1* mutant

In this study, we used proteomics approach, mass spectrometry, and database searching to analyze the changes in the proteomes of soybean plants when inoculated *X. axonopodis* pv. *glycines* Race 3 wildtype KU-P-SW005 and *avrXg1* mutant (KU-P-SW005 *avrXg1*::Tn5REP) on susceptible (Spencer) and resistance (Williams82) cultivars. The total proteins of Spencer which inoculated with KU-P-SW005 and KU-P-SW005 *avrXg1*::Tn5REP were analysed with SDS-PAGE gel images. These results showed both of them induced disease symptoms and protein pattern at 0, 24, and 48 h were not different in this cultivar. Interestingly, the protein pattern was different in Williams82 inoculated with KU-P-SW005 that induced HR and KU-P-SW005 *avrXg1*::Tn5REP that caused the pustule disease. We found one unique band size 70 kDa was occured at 24 h after inoculation with KU-P-SW005 whereas did not find this band in total proteins of Williams82 inoculated with KU-P-SW005 *avrXg1*::Tn5REP and water (Fig. 19 and 20). The unique band was identified the proteins by mass spectrometry. The results showed 35 identified proteins were found to be upregulated when inoculation with KU-P-SW005. The number of peptide matches and the percentage of sequence coverage for each identified gene product are shown in Table 9. The identities of proteins were deduced by similarity to available plant sequences. The thirty-five proteins upregulated by KU-P-SW005 classified into twelve functional categories, ten of which are related to growth and development, one of which are involved disease and defense response, and the last categories is unknown proteins. These ten categories, attached to 26 proteins, included energy/ pentose phosphate; metabolism; ATP synthesis coupled proton transport; carbohydrate metabolism and polysaccharide degradation; Energy/ glycolysis/ glyoxylate cycle/ gluconeogenesis; protein polymerization; hydrolase activity; proteolysis; O-methyltransferase/ S-adenosylmethionine-dependent methyltransferase; and serine protease inhibitor. Three proteins, catalase, lipoxygenase-4 (LOX-4), and phenylalanine ammonia-lyase (PAL) were associated with disease and defense response. Moreover, six identified proteins are unknown which separated into the last groups. These results indicated that three proteins, catalase, LOX-4, and PAL were directly response to KU-P-SW005 and invovled HR induction on soybean.

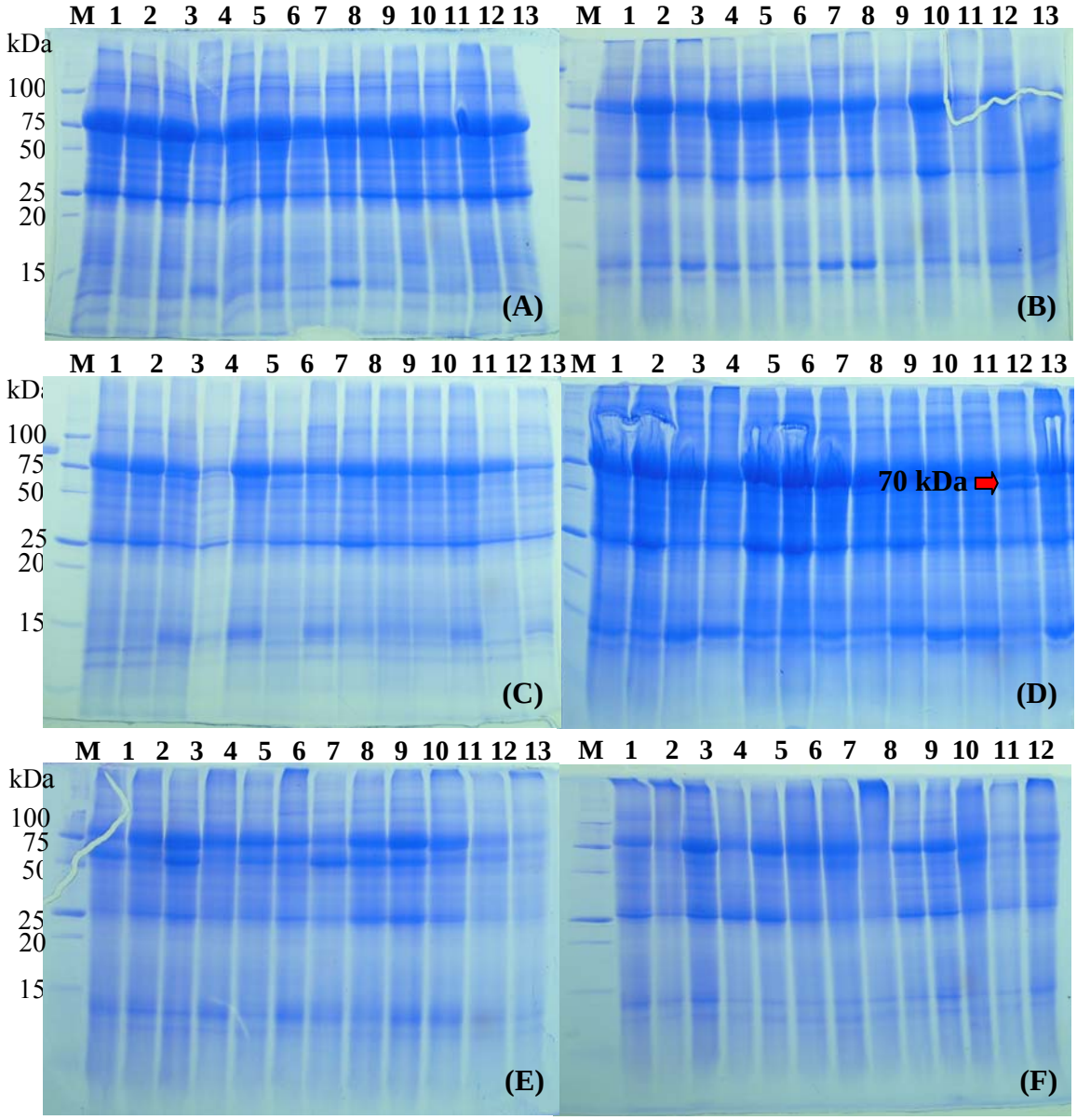

Figure 19 SDS-PAGE analysis total protein of Spencer at 0 (A), 24 (C), and 48 h (E) after inoculation; Williams82 at 0 (B), 24 (D), and 48 h (F) after inoculation. Lane M = protein standard; lanes 1-5 = proteins sample from soybean inoculated with water; lanes 6-11 = proteins sample from soybean inoculated with *avrXg1* mutants; and lanes 12-13 = proteins sample from soybean inoculated with wildtype. The 70 kDa band showed with red arrow.

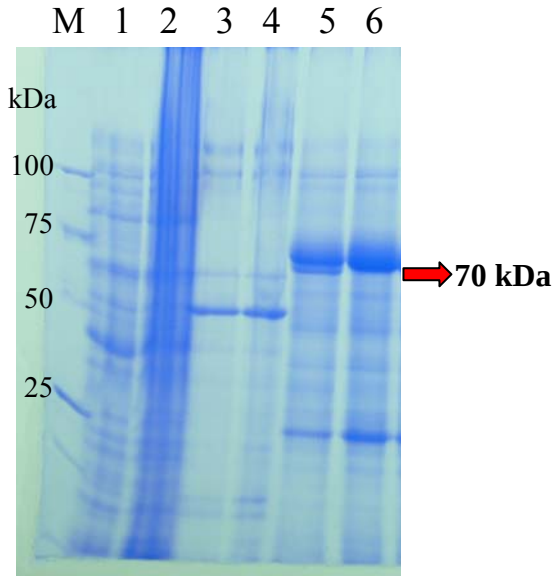

Figure 20 Comparisons of total protein of *Xanthomonas axonopodis* pv. *glycines* wildtype KU-P-SW005 (lane 1 and 2) and *avrXg1* mutants (lane 3 and 4) at 24 and 48 h respectively. Total proteins of Williams82 inoculated with wildtype (lane 5) and *avrXg1* mutant (lane 6) at 24 h after inoculation. Lane M = protein standard.

Table 9 Identification of difference proteins from Williams82 after inoculated with wildtype compared with *avrXg1* mutants and negative control at 24 h after inoculation

Protein number	Protein name	Accession number	Source	Molecular mass (Da)	Total
Functional category 1: Energy/ pentose phosphate					
1	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	gi|91214125 gi|88984670 gi|83595726	*Glycine max*	754.52	76.36
Functional category 2: Metabolism					
2	Aldehyde dehydrogenase 1 precursor	gi|29373073	*Lotus corniculatus*	1,096.50	4.94
3	Adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase) (AdoHcyase) (Cytokinin binding protein CBP57)	gi|78102508 gi|441217 gi|2588781 gi|1857024	*Nicotiana tabacum*	2,886.43	22.01
4	UDP-glucose pyrophosphorylase	gi|17026394	*Amorpha fruticosa*	2,467.23	13.16
5	Glycine hydroxymethyltransferase	gi|437995 gi|1346155	*Flaveria pringlei*	2,653.42	7.18
6	Putative glutamate decarboxylase	gi|32493114	*Glycine max*	1,624.63	2.84
Functional category 3: ATP synthesis coupled proton transport					
7	ATP synthase CF1 beta subunit	gi|91214126 gi|83595727	*Glycine max*	1,327.68	49.90
8	Mitochondrial F-1-ATPase subunit 2	gi|897618 gi|84028177	*Zea mays Oryza sativa*	2,171.10	16.91
9	ATP synthase CF1 alpha subunit	gi|91214148 gi|83595749	*Glycine max*	1,500.69	10.35
10	ATP synthase subunit alpha, mitochondrial; atpA	gi|5305369 gi|231585	*Glycine max*	1,025.55	9.85
11	H(+)-transporting ATP synthase	gi|4995858	*Tilia platyphyllos*	2,399.07	2.00

Table 9 (Continued)

Protein number	Protein name	Accession number	Source	Molecular mass (Da)	Total
Functional category 4: Carbohydrate metabolism and polysaccharide degradation					
12	Beta-amylase	gi|902938 gi|71673373	*Glycine max*	1,520.67	34.46
Functional category 5: Oxidation reduction					
13	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (Non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase) (Glyceraldehyde-3-phosphate dehydrogenase [NADP+]) (Triosephosphate dehydrogenase)	gi|2494076 gi|1842115	*Nicotiana plumbaginifolia*	1,343.70	15.36
14	Glutathione reductase	gi|6723469	*Betula pendula*	1,697.92	2.00
15	O-methyltransferase/ S-adenosylmethionine-dependent methyltransferase	gi|6899898 gi|20334816 gi|17979264 gi|15228652	*Arabidopsis thaliana*	2,308.48	1.4
Functional category 6: Energy/ glycolysis/ glyoxylate cycle/ gluconeogenesis					
16	Enolase	gi|42521309	*Glycine max*	1,937.71	12.36
Functional category 7: Protein polymerization					
17	Cell division protein FtsZ	gi|92872824	*Medicago truncatula*	1,971.82	4.02
18	Elongation factor Tu, domain 2; Translation elongation factor G	gi|92887257 gi|92887256	*Medicago truncatula*	2,257.07	2.00
19	Transposable element protein, putative	gi|77550043 gi|62733928	*Oryza sativa*	1,061.69	2.00
Functional category 8: Hydrolase activity					
20	Apyrase GS50	gi|11225135	*Glycine soja*	1,160.61	2.40

Table 9 (Continued)

Protein number	Protein name	Accession number	Source	Molecular mass (Da)	Total
Functional category 9: Defense response					
21	Catalase	gi|8050693 gi|79326317 gi|7302765 gi|7270460 gi|4336756 gi|4336754 gi|4336752 gi|2924519 gi|21280989 gi|18377696 gi|17865693 gi|15451166 gi|15236264 gi|1246399	*Raphanus sativus* *Brassica juncea* *Arabidopsis thaliana*	1,524.88	2.35
22	Lipoxygenase-4	gi|585418 gi|2160320 gi|1236949	*Glycine max*	1,927.08	2.15
23	Phenylalanine ammonia-lyase	gi|9367317	*Juglans nigra*	3,253.36	2.0
Functional category 10: Proteolysis					
24	Putative leucine aminopeptidase	gi|75261364	*Oryza sativa*	1,187.66	2.0
25	Putative alanine aminotransferase	gi|50510015	*Oryza sativa*	3,022.35	2.00
26	Putative alliinase	gi|28950959	*Arabidopsis thaliana*	3,871.60	2.00
27	Putative ornithine aminotransferase	gi|91680594	*Pinus sylvestris*	2,320.00	1.40
28	AlaT1	gi|71842524	*Vitis labrusca*	1,256.60	1.40
Functional category 11: Serine protease inhibitor					
29	Kunitz trypsin inhibitor	gi|96777258 gi|7638034	*Glycine soja* *Glycine max*	3,260.43	2.00

Table 9 (Continued)

Protein number	Protein name	Accession number	Source	Molecular mass (Da)	Total
Functional category 12: Unknown					
30	Unnamed protein product	gi|39923102	*Nicotiana tabacum*	1,095.60	2.00
31	unknown protein (Os05g0396900)	gi|50931341; gi|46981322	*Oryza sativa*	1,120.58	2.00
32	Os11g0455800	gi|11364506	*Oryza sativa*	1,617.72	1.57
33	Hypothetical protein L23H3.30	gi|7486768 gi|7270159 gi|4914452 gi|30580400 gi|18418034 gi|11141605	*Arabidopsis thaliana*	1,132.63	1.52
34	F22C12.1	gi|6692119	*Arabidopsis thaliana*	3,018.65	1.4
35	unknown protein Os06g0119100	gi|55775232 gi|55296918 gi|55296440 gi|11359467	*Oryza sativa*	1,489.72	1.4

Source: NCBI and SwissProt database

12. Determination of pathogenesis-related (PR) protein and defense enzyme genes expression in soybean using semiquantitative reverse transcription (RT)-PCR

The interaction of host and pathogen has been a useful association to elucidate various biochemical aspects of host-pathogen interactions. The secondary product pathways in soybean leading to the accumulation of the pterocarpan phytoalexins, the glyceollins, were among the first to be thoroughly characterized (Ebel, 1986) and it was also in soybean that the first comprehensive plant metabolic profiling protocols were developed (Graham, 1991). The PR proteins are thought to play an important role in induced and in some cases constitutive resistance. The systemic activation of certain PR protein genes has led to their association with various forms of systemic induced resistance in a number of plants (van Loon, 1997). This experiment was conducted to determine the PR-protein and defense enzyme genes expression after Race 3 wildtype KU-P-SW005 induced HR on Williams82 by sqRT-PCR. The results showed over expression of PR-2, PR-4, PR-6, PR-10, and LOX at 24 h after inoculated wildtype, that highest when estimated relative to the band from other treatment and DNA ladder standards, that amplified from the same concentration of cDNA. Subsequently, could not or slightly detect the band of these genes at 48 h after inoculation with *avrXg1* mutants and water, correlated with above results that the *avrXg1* mutants gained virulence 20% increased of disease severity on Spencer and caused pustule disease on Williams82 where wildtype induced HR on Williams82. In contrast, the band of PR-1, PAL, and peroxidase (POX) could not be detected (Fig. 21).

LOX are a family of iron-containing enzymes and found in plant, animal, and fungi that catalyse the dioxygenation of polyunsaturated fatty acids in lipids containing a cis,cis-1,4- pentadiene structure. These enzymes are most common in plants where they may be involved in a number of diverse aspects of plant physiology including growth and development, pest resistance, and senescence or responses to wounding (Needleman *et al.,* 1986). We found Williams82 inoculated with witdtype, LOX was increased expression at 0, 24, and 48 h after inoculation whereas Williams82 inoculated with *avrXg1* mutant, LOX was decreased at 24 h after inoculation and did not expressed at 48 h after inoculation compared with 18Sr RNA expression.

Endo-glucanases were among the earliest PR proteins to be studied for their involvement in defense and have been designated as the PR-2 class (Kombrink and Somssich, 1997; van Loon and van Strien, 1999). There are multiple distinct β-1, 3-glucanases in soybean that fit into the five classes established in tobacco (Jin *et al.,* 1999). Graham *et al.* (2003) electronic expression analysis of the soybean EST database, which includes several recently added pathogen infection related libraries, suggests that potentially only two of these β -1,3- glucanase genes are likely highly expressed in these soybean defense responses. In this study, it has been shown that PR-2 increased expression in plant inoculated with wildtype and that was decreased and no expression in plant inoculated with *avrXg1* mutant.

The PR-4 genes are somewhat divergent, but may contain one or more elements with homology to chitin-binding domains, wound-induced (WIN) proteins of potato, and several plant lectins (Kombrink and Somssich, 1997) but the WIN like protein genes is notably activated by wounding (Stanford *et al.,* 1989). In this study, it has ben shown that PR-4 increased expression in plant inoculated with wildtype at 0, 24, and 48 h after inoculation. PR-4 was decreased expression in plant inoculated with *avrXg1* mutant at 0, 24, and 48 h after inoculation.

Protease inhibitors have recently been categorized as PR-6 proteins (Kombrink and Somssich, 1997; van Loon and van Strien, 1999). The soybean Kunitz trypsin inhibitor (KTI) was originally isolated as a seed storage protein with potential anti-feeding and anti-nutritional effects on herbivores and humans. In this study, it has been shown that PR-6 increased expression in plant inoculated with wildtype at 0, 24, and 48 h after inoculation. PR-6 was decreased expression in plant inoculated with *avrXg1* mutant at 0, 24, and 48 h after inoculation. Interestingly, PR-6 also was not expressed in plant inoculated with water. These suggest that PR-6 gene may pay a direct role of host-specific with *avrXg1* against bacterial pustule pathogen.

Parsley ‘PR1’ was an early and well-studied prototypic PR-10 gene (Somssich *et al.,* 1986; 1988). Homologs of this class of genes were found in many plants in which they have been associated with pathogenesis, general stress, development or as pollen allergens (Kombrink and Somssich, 1997; van Loon and van Strien, 1999). It deviates

from the original, classically defined PR protein genes in that it encodes an intracellular product. In soybean, an earlier cDNA clone encoding a general stress protein (Starvation Associated Messages 22, SAM22) (Crowell *et al.,* 1992) turns out to be homologous to the PR-10 family. Our results showed PR-10 increased expression in plant inoculated with wildtype at 0, 24, and 48 h after inoculation. PR-10 was decreased expression in plant inoculated with *avrXg1* mutant at 0, 24, and 48 h after inoculation.

This suggested that Williams82 produced PR-2, PR-4, PR-6, PR-10, and LOX are as very rapid spatial signaling process in defense mechanism pathway against *X. axonopodis* pv. *glycines*.

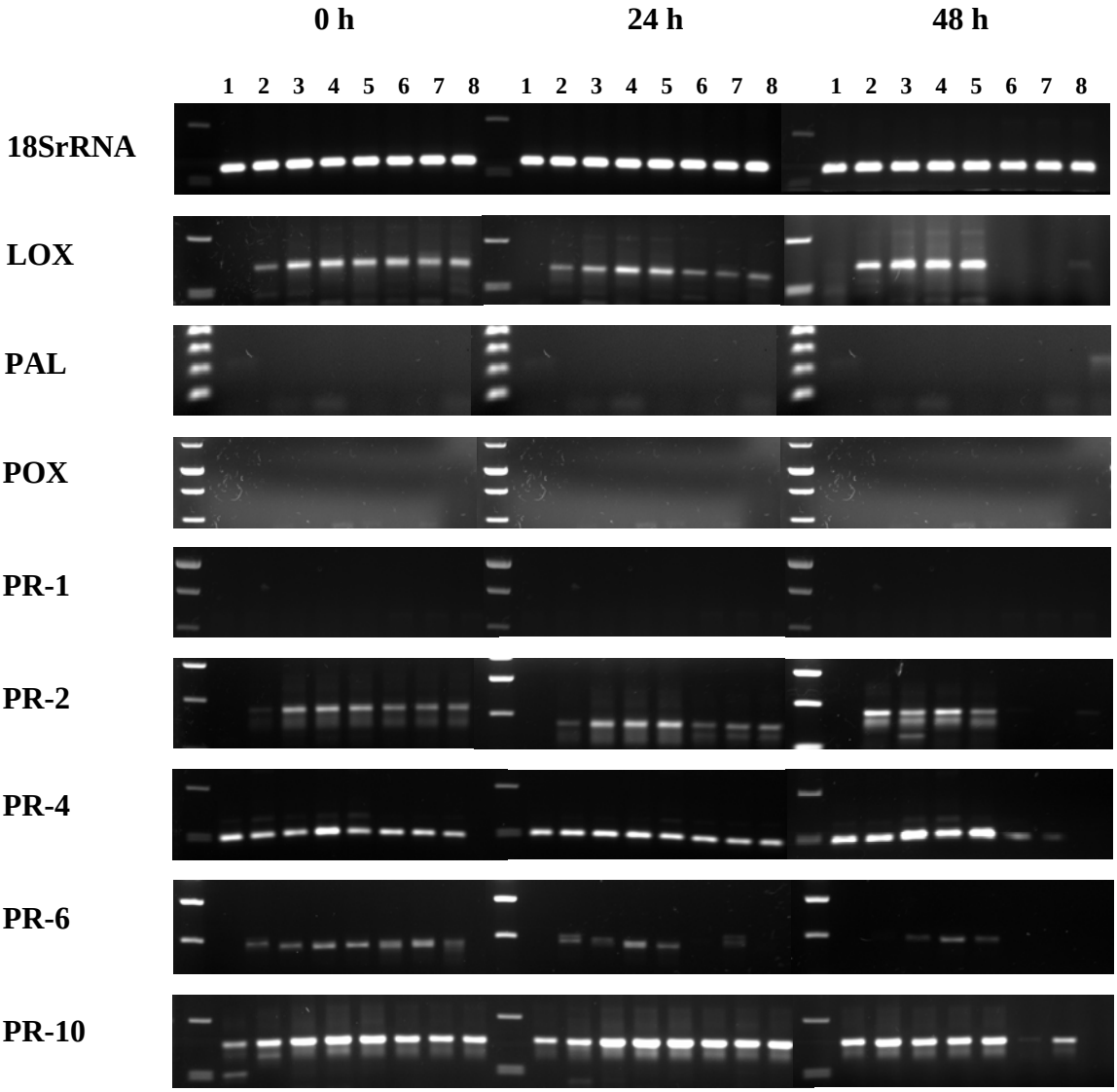

Figure 21 Wildtype and *avrXg1* mutant induced gene expression of PR-1, PR-2, PR-4, PR-6, PR-10, phenylalanine ammonia-lyase (PAL), lipoxygenase (LOX), and peroxidase (POX) which 18SrRNA used as internal standards at 0, 24, and 48 h after inoculation. Lanes 1-2 =water; lanes 3-5 = Race 3 wildtype KU-P-SW005; lanes 6-8 = *avrXg1* mutant.

13. Identification of motility associated *g*enes, *flgC, flgK,* and *pilD* in *X. axonopodis* pv. *glycines*

The *flgC, flgK,* and *pilD* fragment size 408-bp, 1.87-kb, and 864-bp respectively were detected in *X. axonopodis* pv. *glycines* strain KU-P-SW005 and cloned into restriction site of pUC19 vector. Digestion of individual constructs with restriction enzyme *Eco*RI, *Hind*III and *Eco*RI, and *Hind*III and *Sac*I, respectively found the target size 408-bp, 1.87-kb, and 864-bp of *flgC, flgK,* and *pilD* genes, respectively and pUC19 size around 2.6-kb by gel electrophoresis (Fig. 22A, 22B, and 22C). The pUC19-FLGC, pUC19-FLGK, and pUC19-PILD were made by Tn5 insertion in the middle of individual gene by electroporation. Digestion pUC19-FLGC with *Eco*RI showed two different bands size including 1.6-kb band of *flgC* gene Tn5 inserted and 2.6-kb of pUC19 vector (Fig. 22D). Digestion of pUC19-FLGK with *Hind*III and *Eco*RI showed two different bands including 3.1-kb band of *flgK* gene Tn5 inserted and 2.6-kb band of pUC19 (Fig. 22F). Digestion of pUC19-PILD with *Hind*III and *Sac*I showed two different bands including 2.5-kb and 2.6-kb bands of *pilD* gene Tn5 inserted and pUC19 respectively (Fig. 22E).

The target mutation in *flgC*, *flgK*, and *pilD* genes in *X. axonopodis* pv. *glycines* were made by transformed pUC19-FLGC, pUC19-FLGK, and pUC19-PILD (Table 4) into wildtype KU-P-SW005 by electroporation (Fig. 23 and 24). Transposon insertion (fragment size 800-bp) was detected in all mutants tested by PCR, but was not presented in the wildtype *X. axonopodis* pv. *glycines* strain (Fig. 25A). The transposon insertion into the genes of the mutants were further confirmed by PCR amplification using a primer set that amplified the *flgC*, *flgK*, and *pilD* genes of wildtype. As shown in Fig. 25B, 25D, and 25C, the size of PCR product 1.6, 3.1, and 2.5- kb of *flgC*, *flgK*, and *pilD* genes respectively from mutants KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, and KU-P-SW005 *pilD*::Tn5, respectively were compared to 400-bp, 1.87-kb, and 864-bp PCR products of wildtype strains, confirming the transposon (Kan^R1.2 and Tet^R1.6-kb) insertion in the *flgC*, *flgK*, and *pilD,* respectively. For identifying transposon target sequence, the 1.6, 3.1, and 2.5- kb PCR product were cloned. The sequencing analysis showed a transposon is inserted in sequence of *flgC*, *flgK*, and *pilD* genes (data not shown).

Putative motility-minus mutants were selected by the presence of swimming motility in the water under microscopic observation. The *flgC* mutant and *flgK* mutant could not be swimming in the water where *pilD* mutant was showed swimming motility. This indicated that *flgC* and *flgK* affects with flagella and swimming motility of *X. axonopodis* pv. *glycines* but not for *pilD*. Comfirmation *flgC* are important for swimming and flagella assembly by made double mutation in the KU-P-SW005 *pilD*::Tn5. The result showed a double mutant is nonmotile bacteria. Therefore, the *pilD* functions are being investigated in next experiment.

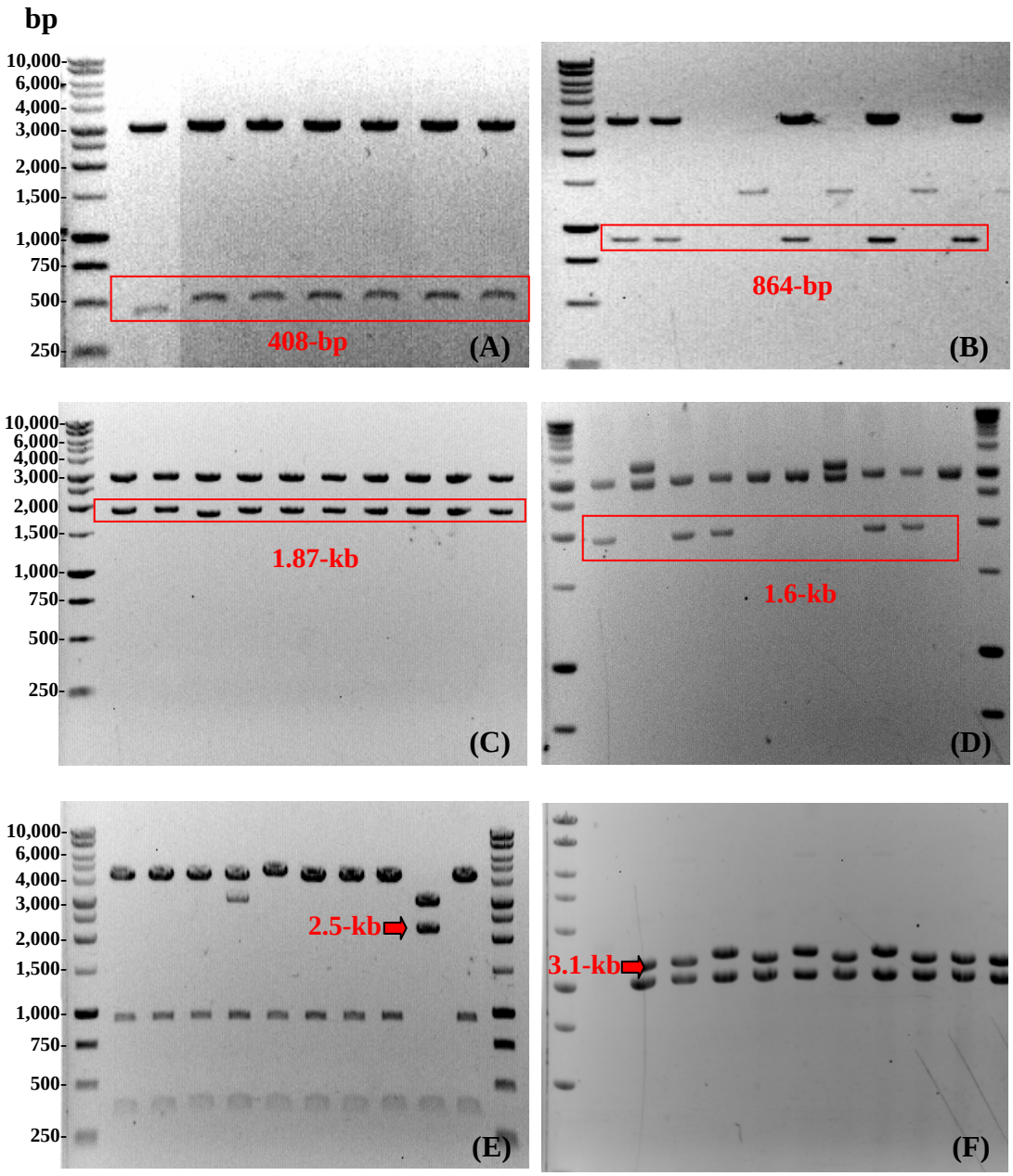

Figure 22 The *flgC, pilD,* and *flgK* fragment 408-bp (A), 864-bp (B), and 1.87-kb (C) respectively were detected in *Xanthomonas axonopodis* pv. *glycines* KU-P-SW005 and cloned into pUC19. Digestion pUC19-FLGC (D), pUC19-PILD (E), and pUC19-FLGK (F) with restriction enzymes showed pUC19 and the target gene Tn5 inserted size 1.6, 2.5, and 3.1-kb respectively.

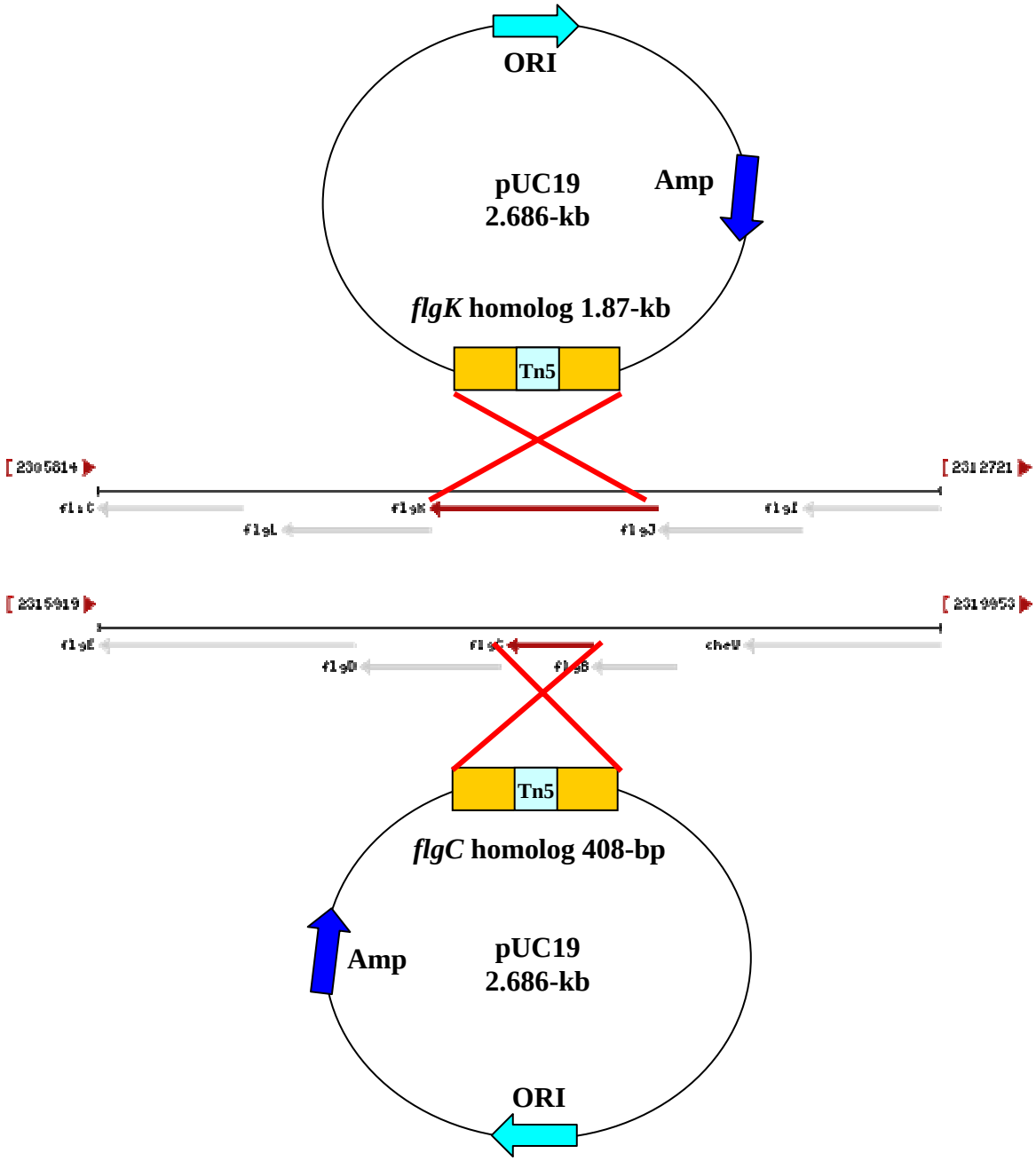

Figure 23 Genomic organization of genes flanking Tn5 insertions in *Xanthomonas axonopodis* pv. *glycines*. Flagella gene cluster of *Xanthomonas axonopodis* pv. *citri*; grey arrows and gene disrupted in this study; red arrows. Source: http://www.ncbi.nlm.nih.gov/.

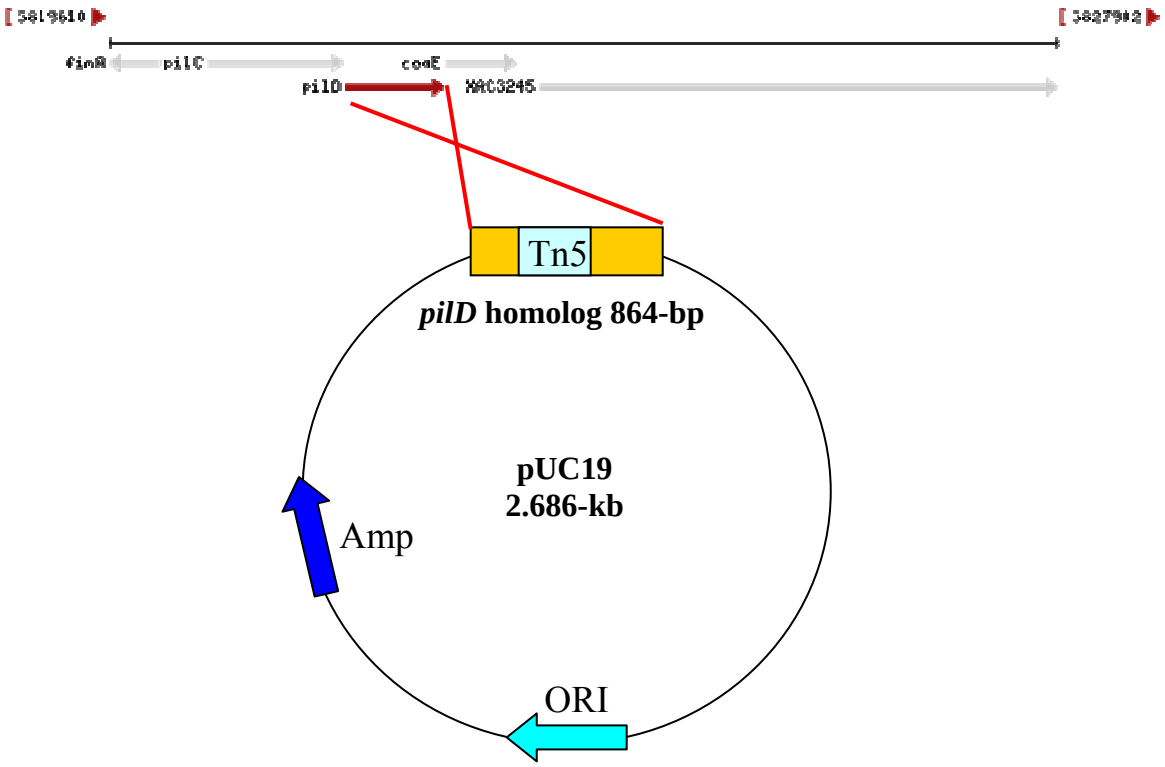

Figure 24 Genomic organization of genes flanking Tn5 insertions in *Xanthomonas axonopodis* pv. *glycines*. Pili gene cluster of *Xanthomonas axonopodis* pv. *citri*; grey arrows and gene disrupted in this study; red arrows. Source: http://www.ncbi.nlm.nih.gov/.

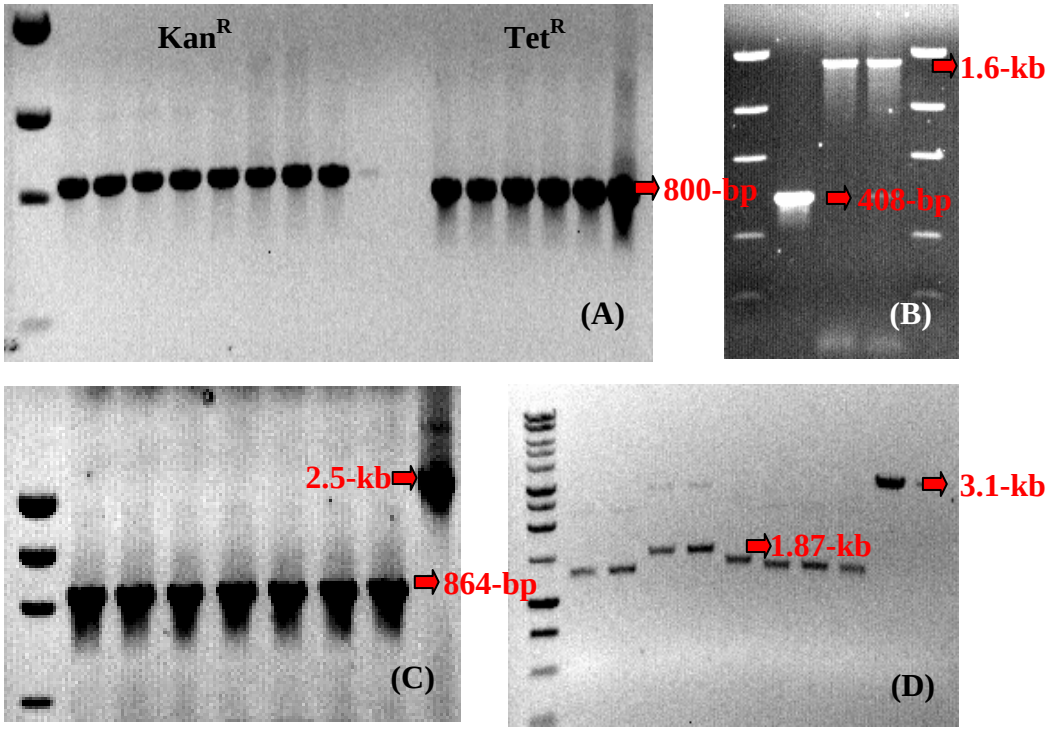

Figure 25 Polymerase chain reaction amplification of kanamycin (Kan^R) and tetracycline (Tet^R) resistant marker 800-bp in transposon inserted of *Xanthomonas axonopodis* pv. *glycines* mutants with primer KAN-1-FP and KAN-1-RP; and TET-F and TET-R, respectively (A). A 1.6, 2.5, and 3.1-kb of *flgC* (B), *pilD* (C), and *flgK* (D) Tn5 insertion were detected by PCR whereas detected 408-bp, 864-bp, and 1.87-kb in wildtype. The PCR products were separated by electrophoresis on a 0.7% agarose gel.

14. Sequencing and evaluation

The complete sequence of *flgC, flgK,* and *pilD* of *X. axonopodis* pv. *glycines* were compared to the *flgC, flgK,* and *pilD* homolog in *X. axonopodis* pv. *citri* strain 306 (NP_642311.1, NP_642303.1, and NP_643551.1 respectively) (da Silva *et al.*, 2002). The coding region of *flgC, flgK,* and *pilD* gene are 408-bp, 1.87-kb, and 864-bp respectively in length with an overall G+C content of 64%, 65%, and 64% respectively.

Alignment analysis of *flgC, flgK,* and *pilD* of *X. axonopodis* pv. *glycines* by MegAlign program (DNAstar inc) revealed the deduced protein shares identity with *flgC, flgK,* and *pilD* homolog protein sequences (100%, 99%, and 99% at amino acid levels) of *X. axonopodis* pv. *citri* strain 306 (NP_642311.1, NP_642303.1, and NP_643551.1 respectively) and encodes a predicted 136, 625, and 287 amino acid protein with a molecular mass of 34.2, 152.6, and 71.5 kDa and pI of 5.2, 4.8, and 5.0 respectively. The putative start codon of *flgC, flgK,* and *pilD* is ATG. A signal peptide, MSNLPIFDVAGSALH, LEDGVGGRDHLCGGL, and MAFLDQHPGLGFPAA are also predicted for FlgC, FlgK, and PilD respectively.

Nucleotide sequence accession number, the complete DNA sequence of *flgC, flgK,* and *pilD* obtained from *X. axonopodis* pv. *glycines* strain KU-P-SW005, were deposited as GenBank accession no. FJ825660, FJ825661, and FJ825662 respectively.

15. Complementation and motility analysis

The pBBR-FLGC, pBBR-FLGK, and pBBR-PILD were constructed and digested with restriction enzyme *Eco*RI and analyzed by gel electrophoresis. Analysis pBBR-FLGC digestion showed two different bands site including 408-bp band of *flgC* gene and 5.7-kb of pBBR1MCS-5 broad host range cloning vector (Fig. 26A). Digestion of pBBR-FLGK with *Eco*RI showed three different bands including 875-bp and 1.0-kb band of *flgK* gene because this gene size 1.87-kb was occurred in one position *Eco*RI restriction site at 1.0-kb on *flgK* gene of *X. axonopodis* pv. *glycines* wildtype, therefore *Eco*RI was digested the gene into two fragment and another one band is 5.7-kb of pBBR1MCS-5

(Fig. 26B). Digestion of pBBR-PILD with *Eco*RI showed two different bands including 864-bp and 5.7-kb bands of *pilD* gene and pBBR1MCS-5 respectively (Fig. 26C).

Complementation of KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, and KU-P-SW005 *pilD*::Tn5 were accomplished by transformation with pBBR-FLGC, pBBR-FLGK, and pBBR-PILD respectively by electroporation. This resulted in restored ability of motility in complementary strain *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD), respectively the same with wildtype (Fig. 27).

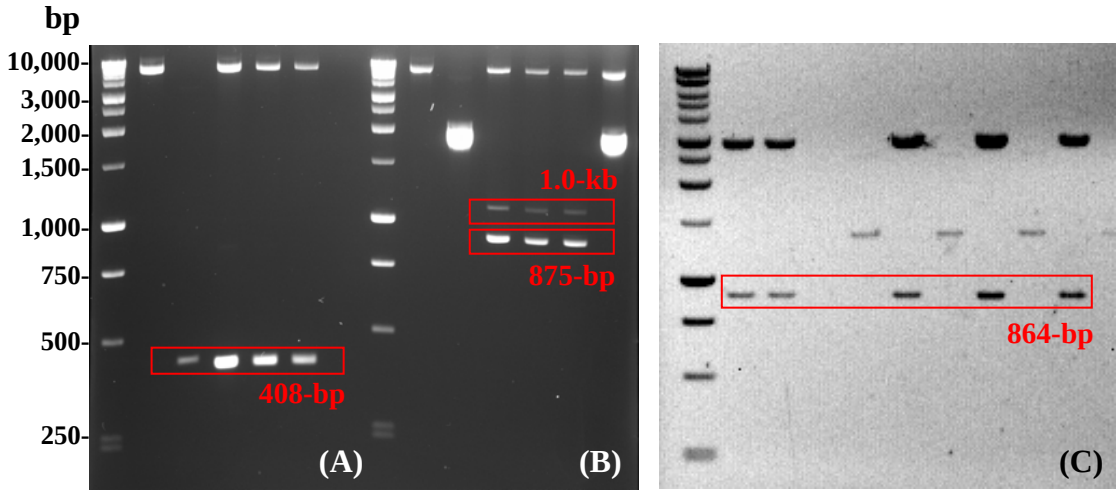

Figure 26 Digestion of pBBR-FLGC (A), pBBR-FLGK (B), and pBBR-PILD (C) with *Eco*RI showed the target band 408-bp and 864-bp of *flgC* and *pilD*. For pBBR-FLGK, *Eco*RI digested *flgK* into fragment are 1.0-kb and 875-bp.

16. Motility and microscopy image analysis

Putative motility-minus mutants, KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, KU-P-SW005 *pilD*::Tn5, and KU-P-SW005 *pilD-flgC*::Tn5 were tested by the presence of swimming motility within NGA solidified with 0.4% agar tube (Fig. 27). KU-P-SW005 *flgC*::Tn5 and KU-P-SW005 *flgK*::Tn5 were lost swimming in the agar, predicting bacteria were lost the flagella for swimming motility (Fig. 27B and 27C). The *pilD* mutant (KU-P-SW005 *pilD*::Tn5) showed different phenotype with KU-P-SW005 *flgC*::Tn5 and KU-P-SW005 *flgk*::Tn5, it could be swimming movement in the agar (Fig. 27D), predicting bacterial have flagella for swimming motility because *pilD* did not involved the flagella assembly but encode type IV pre-pilin leader peptidase was demonstrated to be required for protein secretion, in addition to its involvement in biogenesis of type IV pili. Type IV pili were involved twitching motility. Also, the motility movies were made from time-lapse technique showed *flgC* mutant and *flgK* mutant did not moved, the cells just grown and spread them out from the colonies. For *pilD* mutant was showed swimming motility the same with wildtype (data not shown). Therefore, twitching motility by *X. axonopodis* pv. *glycines* need to be proof and demonstrated in the experiment. In addition to a *pilD* mutants, a second round of mutagenesis into the previously described mutant KU-P-SW005 *pilD*::Tn5, which was may deficient for type IV pili (Meng *et al.*, 2005), was performed to create a 'double' mutant that may lack both type IV pili (*pilD*) and flagella (*flgC*). The nonmotility double mutants were obtained herein (Fig. 27E). The results indicated the *flgC* and *flgK* functions control swimming motility of *X. axonopodis* pv. *glycines* and *pilD* may play a role of twitching motility that need the very good technique to prove such as transmission microscopy, time-lapse technique, and culture in growth chamber. However, the swimming motility was restored in all complemented strains, *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD) as showed in Fig. 27F, 27G, and 27H respectively.

Twitching motility of *pilD* was further verified by comparison between efficacy of motility of wildtype, all mutants, and complemented strains on solid surface. It has been reported that type IV pili is required for pathogenesis and a form of surface-associated movement known as twitching motility. Motility is a consequence of the extension and

retraction of type IV pili, which promotes bacteria across a surface by an undescribed mechanism. In different Gram negative bacteria, it has been reported that *pilB* and *pilQ* genes involved in type IV pili are closely related to twitching motility (Meng *et al.,* 2005). Therefore, we assessed the twitching motility phenotype of the mutant KU-P-SW005 *pilD*::Tn5 carrying *pilD* mutated. The bacterial colonies of mutants; KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, KU-P-SW005 *pilD*::Tn5, complemented strains; *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD), and wildtype were spotted on WA plate and incubated for 12 h. The twitching motility was assessed by light microscopy (100X). Under the microscope, the edge of the colonies of strains that were proficient in twitching motility was highly irregular. This kind of marginal fringe was observed on the wildtype, mutants; KU-P-SW005 *flgC*::Tn5 and KU-P-SW005 *flgK*::Tn5, and complemented strains; *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-FLGD), while the smooth-colony edge phenotype was observed on the mutants KU-P-SW005 *pilD*::Tn5 and KU-P-SW005 *flgC-pilD*::Tn5 (Fig. 28). This is considered a consequence of the surface movement associated with type IV pili. The finding suggests that *X. axonopodis* pv. *glycines* cells actively migrate to other plant tissue via twitching motility that have not seen before in this pathogen.

Mutants, KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, and KU-P-SW005 *flgC-pilD*::Tn5 did not show swarming motility on NGA solidified with 0.4% agar (Fig. 29B, 29C, and 29E respectively) where KU-P-SW005 *pilD*::Tn5 did swarming (Fig. 29D) as same as with the wildtype (Fig. 29A). This suggests that *flgC* and *flgK* play the role of swarming also. From this observation, *X. axonopodis* pv. *glycines* exhibited swimming and twitching motility by flagella and pili respectively. On NGA solidified with 0.4% agar is the semi-solid surface and wet condition *X. axonopodis* pv. *glycines* played the swarming movement but on WA which is the solid surface, low nutrient, and dry condition, *X. axonopodis* pv. *glycines* played the twitching movement. Therefore, *flgC, flgK,* and *pilD* genes can be activated involved the environment response. The flagellum is the major component for swarming motility.

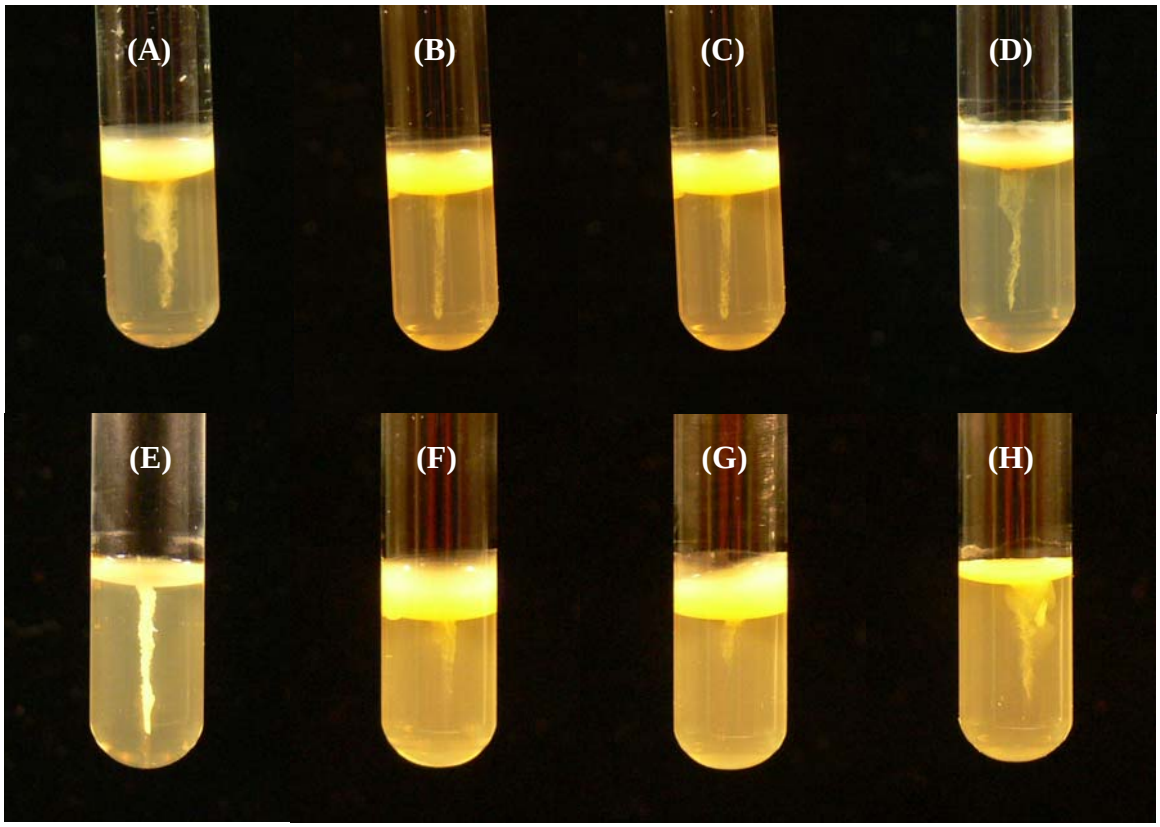

Figure 27 Motility phenotypes of KU-P-SW005, wildtype (A), KU-P-SW005 *flgC*::Tn5, *flgC* mutant (B), KU-P-SW005 *flgK*::Tn5, *flgK* mutant (C); KU-P-SW005 *pilD*::Tn5, *pilD* mutant (D), KU-P-SW005 *flgC-pilD*::Tn5, *pilD* and *flgC* double mutants (E), and *flgC*::Tn5 (pBBR-FLGC) (F), *flgK*::Tn5 (pBBR-FLGK) (G), and *pilD*::Tn5 (pBBR-PILD) (H), complemented mutants in NGA solid with 0.4% agar.

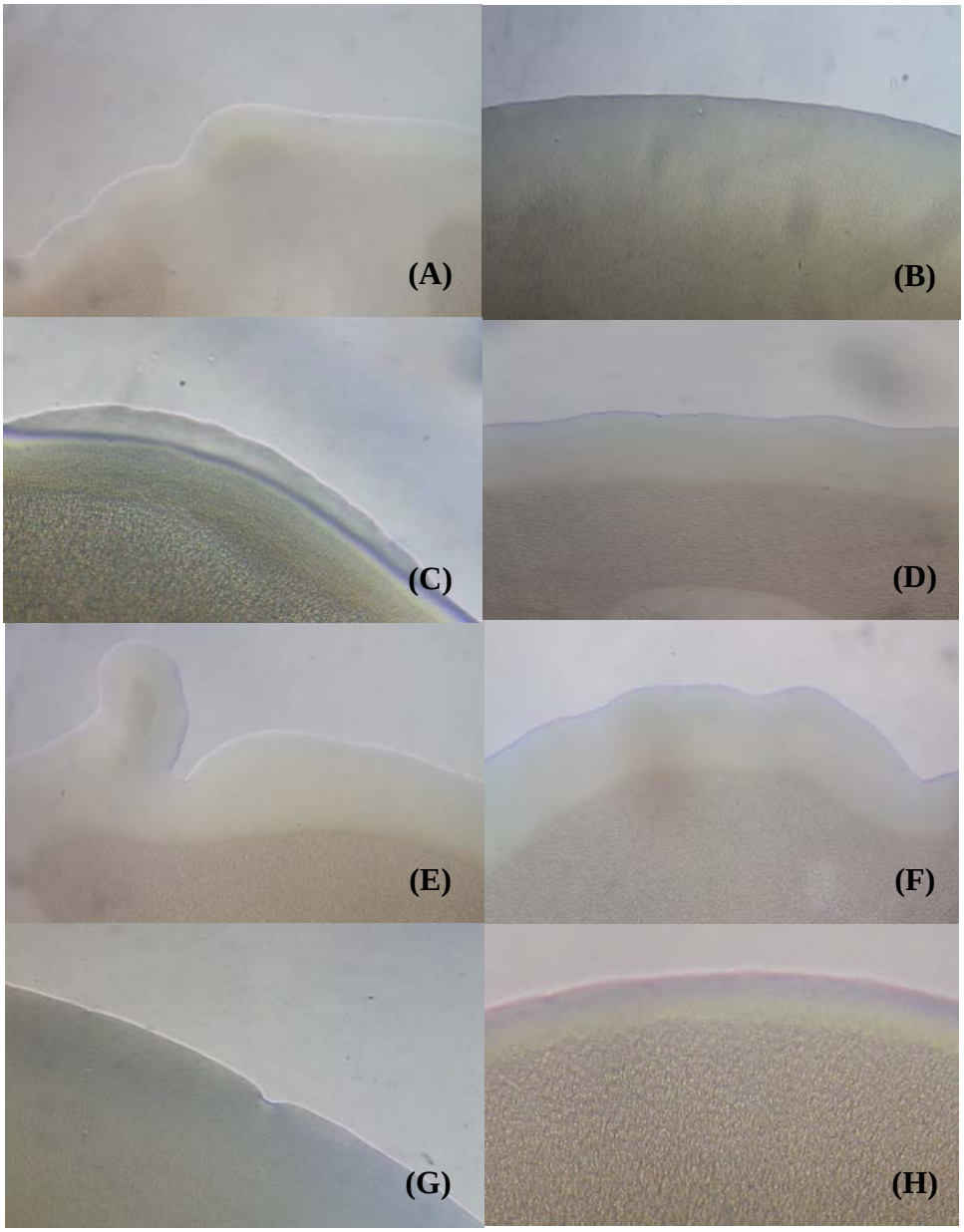

Figure 28 Colony morphologies of wildtype, KU-P-SW005 (A); *pilD* and *flgC* double mutants, KU-P-SW005 *flgC-pilD*::Tn5 (B); *flgC* mutant, KU-P-SW005 *flgC*::Tn5 (C); *flgC* complemented strain, *flgC*::Tn5 (pBBR-FLGC) (D); *flgK* mutant, KU-P-SW005 *flgK*::Tn5 (E); *flgK* complemented strain, *flgK*::Tn5 (pBBR-FLGK) (F); *pilD* mutant, KU-P-SW005 *pilD*::Tn5 (G); and *pilD* complemented strain, *pilD*::Tn5 (pBBR-PILD) (H) grown on a water agar solid surface for 12 h.

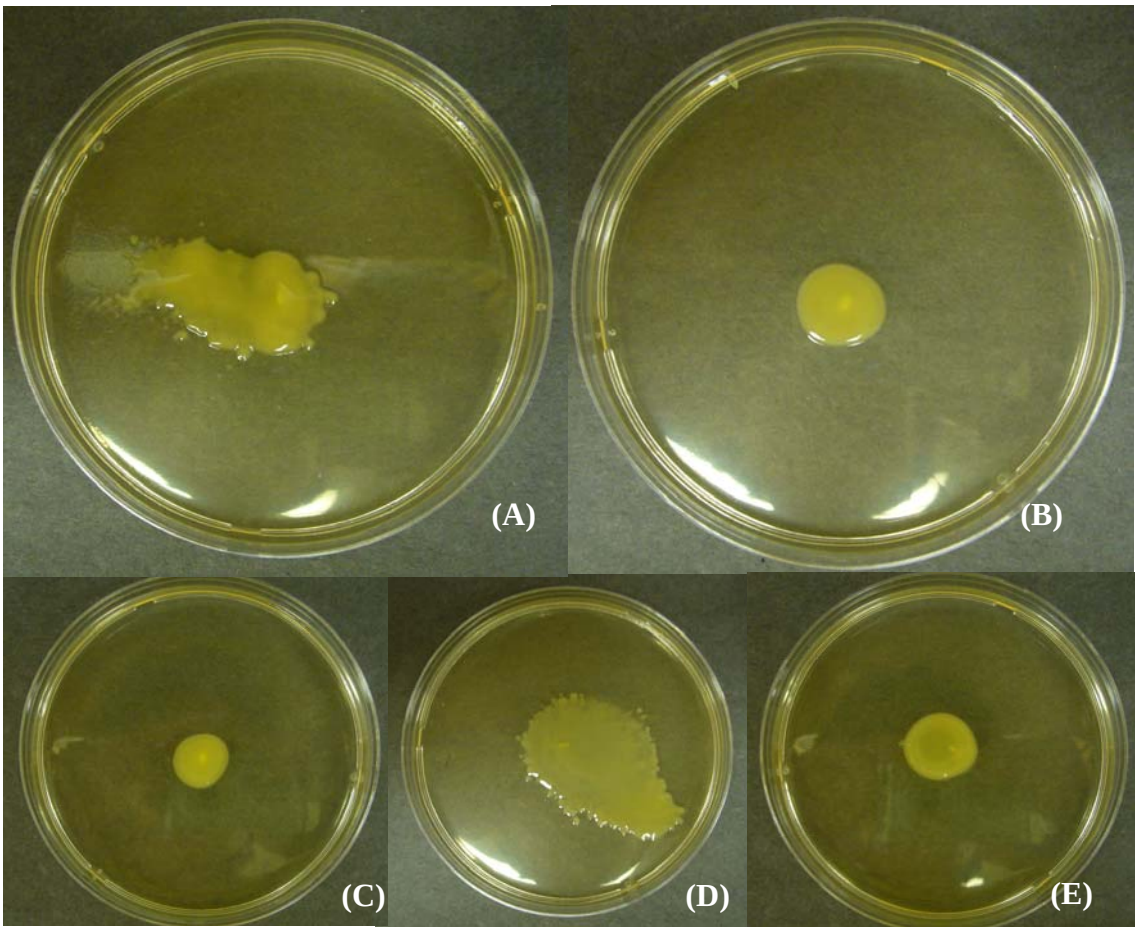

Figure 29 Swarming motility of wildtype, KU-P-SW005 (A); *flgC* mutant, KU-P-SW005 *flgC*::Tn5 (B); *flgK* mutant, KU-P-SW005 *flgK*::Tn5 (C); and *pilD* mutant, KU-P-SW005 *pilD*::Tn5 (D); and *pilD* and *flgC* double mutants, KU-P-SW005 *flgC-pilD*::Tn5 (E) on a nutrient glucose agar solid with 0.4% agar surface for 2 days.

17. Biofilm formation

The initial disease induction may depend on bacterial population that attached and development of biofilm on leaf surface. To examine the roles of flagella and pilus in the involvement of biofilm information in bacterial pustule disease, the ability of *X. axonopodis* pv. *glycines* to develop a biofilm has been investigeted by plate count technique. We further characterized the development of biofilms in newly generated flagella and pilus mutants on different surfaces and media. When *X. axonopodis* pv. *glycines* wildtype KU-P-SW005 was cultured in LB, PD2, NBY, and NGB, they formed visible biofilms on glass tube surfaces with continuous agitation. In contrast, the flagella mutants KU-P-SW005 *flgC*::Tn5 and KU-P-SW005 *flgK*::Tn5, pili mutant KU-P-SW005 *pilD*::Tn5, and double mutations, nonmotile strain KU-P-SW005 *flgC-pilD*::Tn5 were exhibited significantly reduced biofilms on similar surfaces (Fig. 30). The flagella mutants formed more robust biofilms on glass tube surface than pili mutants and nonmotile bacteria. However, no differences were observed on glass tube with these same mutants, suggesting that the surface material greatly affects attachment of the bacteria. Furthermore, it was noted that the biofilms formed on glass tube were easily removed from the glass surfaces by swirling in acetone: ethanol with 6: 4. Interestingly, the resulting biofilm was also more strongly attached to glass surfaces, especially in PD2, as assayed by crystal violet staining. A comparison of the subpopulations of attached and planktonic cells showed the highest 2 - 3 times when grown in PD2 were significantly attached cells in other medium (Fig. 31). Biofilm formation were also tested by the wildtype, and mutants KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, KU-P-SW005 *pilD*::Tn5, and KU-P-SW005 *flgC-pilD*::Tn5 cultured in polystyrene and polypropylene surfaces with continuous agitation. However, the biofilm information could not be detected by all tested strains (data not shown). These results suggest that flagella and pilus play an important role in biofilm formation by causing more cells to clump together, and forming larger biofilm.

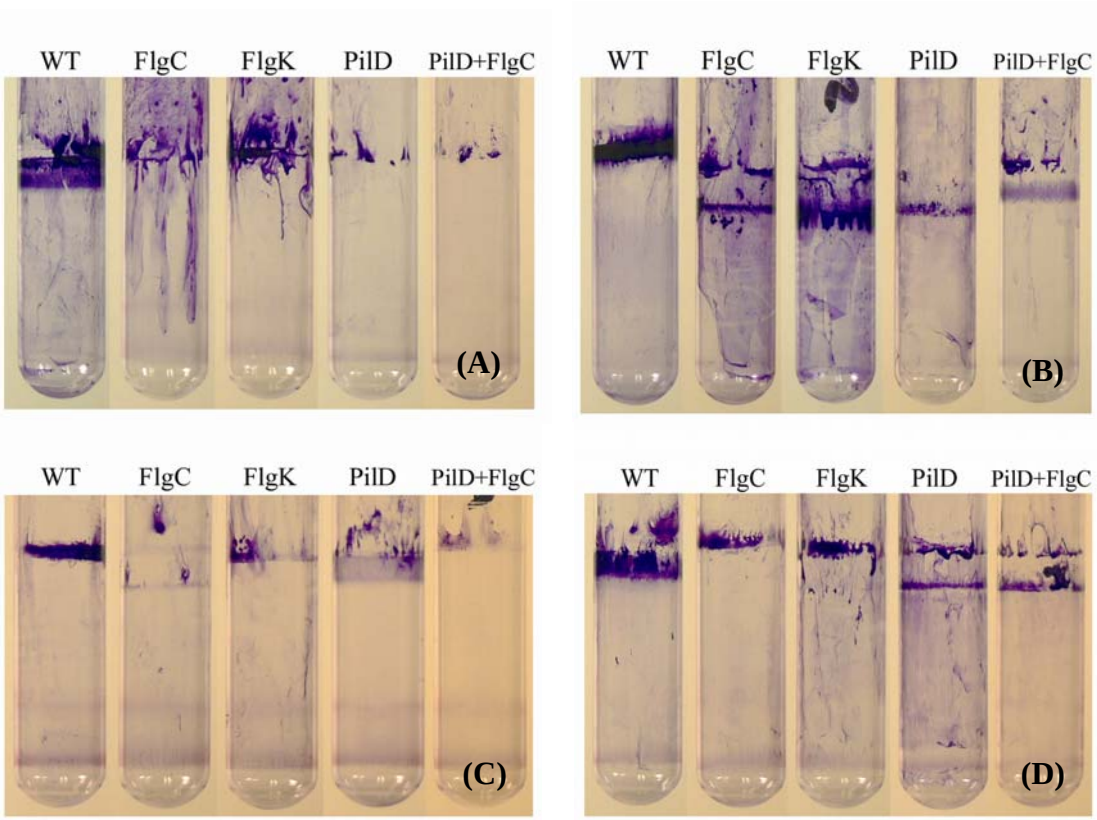

Figure 30 Biofilm formation by *Xanthomonas axonopodis* pv. *glycines* wildtype and various mutants on glass surfaces following 7 days of growth in Luria Bertani broth (A), PD2 (B), nutrient yeast extract broth (C), and nutrient glucose broth (D) at 28ºC and 150 rpm, and staining with crystal violet.

Table 10 Comparison of the total OD_{600} of suspension of wildtype, various motility mutants, and complemented mutants on different media at 28°C for 7 days[1/]

Bacterial strain[2/]	**Medium**[3/]			
	LB	**PD2**	**NBY**	**NGB**
KU-P-SW005	0.41b	0.34c	0.45ab	0.45bc
KU-P-SW005 *flgC*::Tn5	0.58a	0.57a	0.54ab	0.52a
flgC::Tn5 (pBBR-FLGC)	0.42b	0.33c	0.43b	0.43bc
KU-P-SW005 *flgK*::Tn5	0.42b	0.46b	0.40b	0.53a
flgK::Tn5 (pBBR-FLGK)	0.43b	0.34c	0.44ab	0.41c
KU-P-SW005 *pilD*::Tn5	0.64a	0.59a	0.54a	0.43bc
pilD::Tn5 (pBBR-PILD)	0.42b	0.32c	0.45ab	0.48b
KU-P-SW005 *flgC-pilD*::Tn5	0.44b	0.49b	0.44ab	0.44bc

[1/] Numbers followed by different letters show significance at $P \leq 0.05$.

[2/] KU-P-SW005, wildtype; KU-P-SW005 *flgC*::Tn5, *flgC* mutant; KU-P-SW005 *flgK*::Tn5, *flgK* mutant; KU-P-SW005 *pilD*::Tn5, *pilD* mutant; KU-P-SW005 *flgC-pilD*::Tn5, *pilD* and *flgC* double mutants; *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD), complemented mutants.

[3/] Kind of liquid medium, LB = Luria Bertani, PD2 = Pierce's disease, NBY = nutrient yeast extract, and NGB = nutrient glucose broth.

Table 11 Comparison of biofilm formations between wildtype, various motility mutants, and complemented mutants on different media[1/]

Bacterial strain[2/]	**Medium**[3/]			
	LB	**PD2**	**NBY**	**NGB**
KU-P-SW005	0.52a	0.60a	0.57a	0.48a
KU-P-SW005 *flgC*::Tn5	0.31c	0.33b	0.46c	0.28b
flgC::Tn5 (pBBR-FLGC)	0.48a	0.57a	0.53b	0.49a
KU-P-SW005 *flgK*::Tn5	0.38b	0.37b	0.35d	0.28b
flgK::Tn5 (pBBR-FLGC)	0.50a	0.52a	0.51b	0.51a
KU-P-SW005 *pilD*::Tn5	0.2d	0.30b	0.27e	0.25bc
pilD::Tn5 (pBBR-FLGC)	0.50a	0.58a	0.52b	0.50a
KU-P-SW005 *flgC-pilD*::Tn5	0.12e	0.18c	0.27e	0.24bc

[1/] Numbers followed by different letters show significance at $P \leq 0.05$.

[2/] KU-P-SW005, wildtype; KU-P-SW005 *flgC*::Tn5, *flgC* mutant; KU-P-SW005 *flgK*::Tn5, *flgK* mutant; KU-P-SW005 *pilD*::Tn5, *pilD* mutant; KU-P-SW005 *flgC-pilD*::Tn5, *pilD* and *flgC* double mutants; *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD), complemented mutants.

[3/] Kind of liquid medium, LB = Luria Bertani, PD2 = Pierce's disease, NBY = nutrient yeast extract, and NGB = nutrient glucose broth.

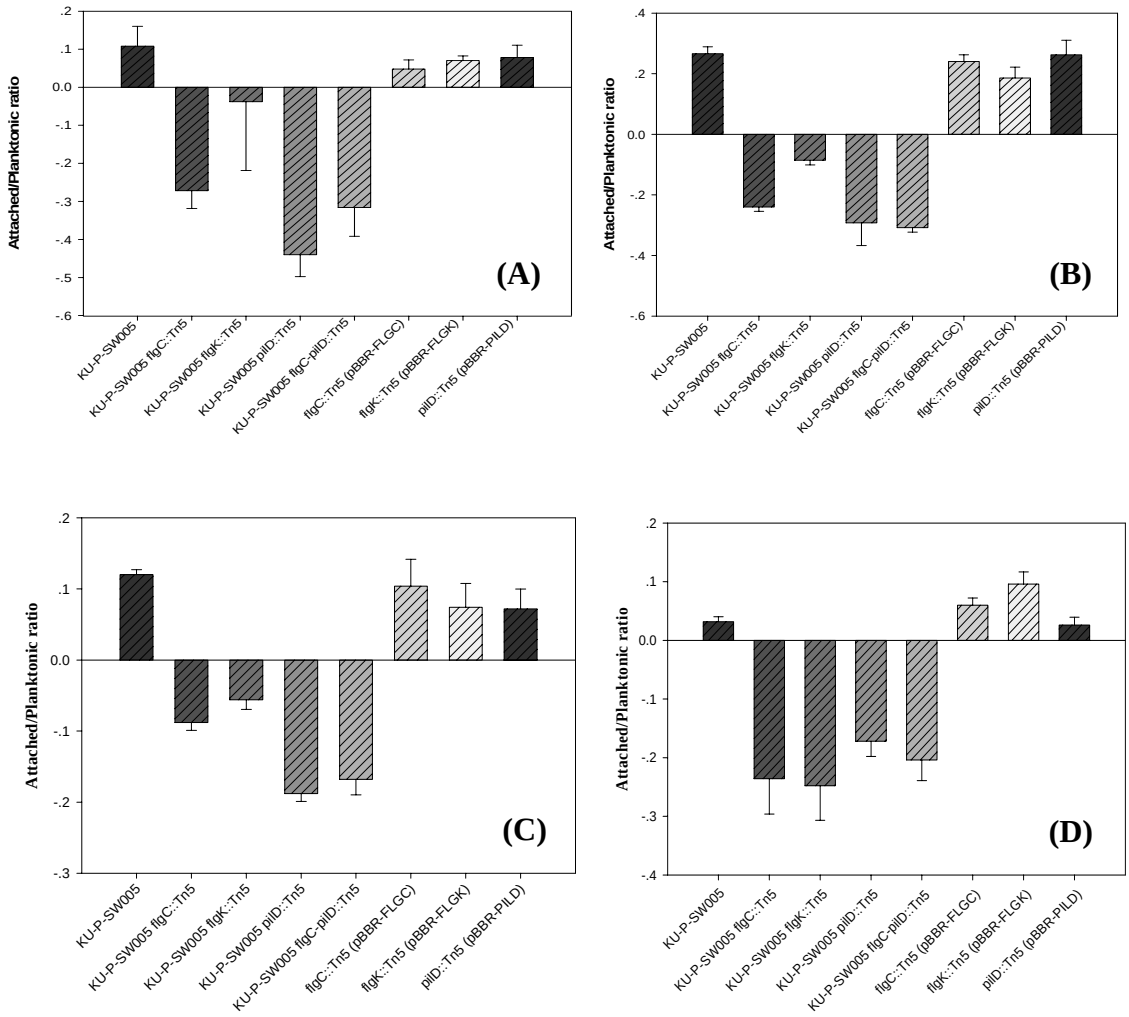

Figure 31 Effect of medium on biofilm formation on glass surfaces by *Xanthomonas axonopodis* pv. *glycines* and various mutants. After 7 days of growth in Luria Bertani broth (A), PD2 (B), nutrient yeast extracts broth (C), and nutrient glucose broth (D) at 28ºC and 150 rpm, optical density of planktonic and attached cells was measured.

18. Pathogenicity test and bacterial growth on soybean

The flagella mutants, KU-P-SW005 *flgK*::Tn5 and pili mutant, KU-P-SW005 *pilD*::Tn5 gave virulence ratings 11.11% and 37.44% whereas wildtype KU-P-SW005 gave the highest virulence rating 51.98% at 10 days after inoculation that KU-P-SW005 *flgK*::Tn5 and pili mutant, KU-P-SW005 *pilD*::Tn5 were reduced disease severity 78.63% and 27.97% respectively (Fig. 32). Interestingly, the flagella mutants, KU-P-SW005 *flgC*::Tn5 and nonmotile double mutations, KU-P-SW005 *flgC-pilD*::Tn5 were no found disease symptomps on Spencer cultivars (susceptible) seem like they were lost pathogenesis and were nonpathogenic strains (Fig. 32B and Fig. 32E). Lesion expansion of KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, KU-P-SW005 *pilD*::Tn5, and KU-P-SW005 *flgC-pilD*::Tn5 were significantly smaller than wildtype (Table 12 and Fig 33). In addition, the lesion of KU-P-SW005 *flgC*::Tn5 and KU-P-SW005 *flgC-pilD*::Tn5 showed the necrosis region seem like HR at 10 days after inoculation and were no surrounding yellow halo after keep in the greenhouse for 1 month (Fig. 33B and 33E). Moreover, the pustules were occurred surrounding the infiltrated zone of wildtype, KU-P-SW005 *flgK*::Tn5 and KU-P-SW005 *pilD*::Tn5 (Fig. 34). Number of pustule induction by wildtype was the highest with 58.56 pustule/ lesion whereas KU-P-SW005 *flgK*::Tn5 and KU-P-SW005 *pilD*::Tn5 showed the pustule with 6.56 and 12.56 pustule/ lesion respectively observed at 10 days after infiltration. KU-P-SW005 *flgC*::Tn5 and KU-P-SW005 *flgC-pilD*::Tn5 were not pustule occur, observed in the same time. The complemented strains of *flgC*, *flgK*, and *pilD* mutants, *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD) respectively were showed the pathogenicity phenotype similar with wildtype (Table 12). Also, wildtype, mutants KU-P-SW005 *flgC*::Tn5, KU-P-SW005 *flgK*::Tn5, KU-P-SW005 *pilD*::Tn5, and KU-P-SW005 *flgC-pilD*::Tn5, and complemented strains *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD) induced HR on four species of tobacco (*N. tabacum* cv. Xanthi, *N. glauca*, *N. rustica* and *N. benthamiana*) and tomato (*L. esculentum* cv. Seeda 4) within 48 h (Table 13 and Fig. 35).

Growth of wildtype, mutants KU-P-SW005 *flgK*::Tn5 and KU-P-SW005 *pilD*::Tn5 and complemented strains *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD) were not different when observed on Spencer leaves at at 1, 2, 3,

4, and 10 days after infiltration. Except the population of mutants KU-P-SW005 *flgC*::Tn5 and KU-P-SW005 *flgC-pilD*::Tn5 on Spencer were significantly decreased ($P \leq 0.05$), reaching population numbers lower than wildtype strains (Fig. 36).

Table 12 Lesion size, disease severity, and number of pustule induction for wildtype, flagella and pilus associated gene mutants, and complemented mutants on cultivar Spencer[1/]

Bacterial strain[2/]	**Lesion mean diam (mm)**	**Disease severity (%)[3/]**	**Number of pustule induction (pustule)[4/]**
KU-P-SW005	0.83a	51.98a	58.56a
KU-P-SW005 *flgC*::Tn5	0.50b	0	0
flgC::Tn5 (pBBR-FLGC)	0.74ab	49.79ab	43.67b
KU-P-SW005 *flgK*::Tn5	0.56b	11.11d	6.56d
flgK::Tn5 (pBBR-FLGK)	0.76ab	47.73ab	47.89
KU-P-SW005 *pilD*::Tn5	0.56b	37.44c	12.56c
pilD::Tn5 (pBBR-PILD)	0.76ab	49.79ab	48.11b
KU-P-SW005 *flgC-pilD*::Tn5	0.50b	0	0

[1/] Numbers followed by different letters show significance at $P \leq 0.05$.

[2/] KU-P-SW005, wildtype; KU-P-SW005 *flgC*::Tn5, *flgC* mutant; KU-P-SW005 *flgK*::Tn5, *flgK* mutant; KU-P-SW005 *pilD*::Tn5, *pilD* mutant; KU-P-SW005 *flgC-pilD*::Tn5, *pilD* and *flgC* double mutants; *flgC*::Tn5 (pBBR-FLGC), *flgK*::Tn5 (pBBR-FLGK), and *pilD*::Tn5 (pBBR-PILD), complemented mutants.

[3/] Disease severity was evaluated as described in text. For each strain at least three leaves, collected from the top, middle, and basal portion of sthree plants, were evaluated.

[4/] Number of pustules were calculated by total pustule surrounding the infiltration zone 10 days after infiltration.

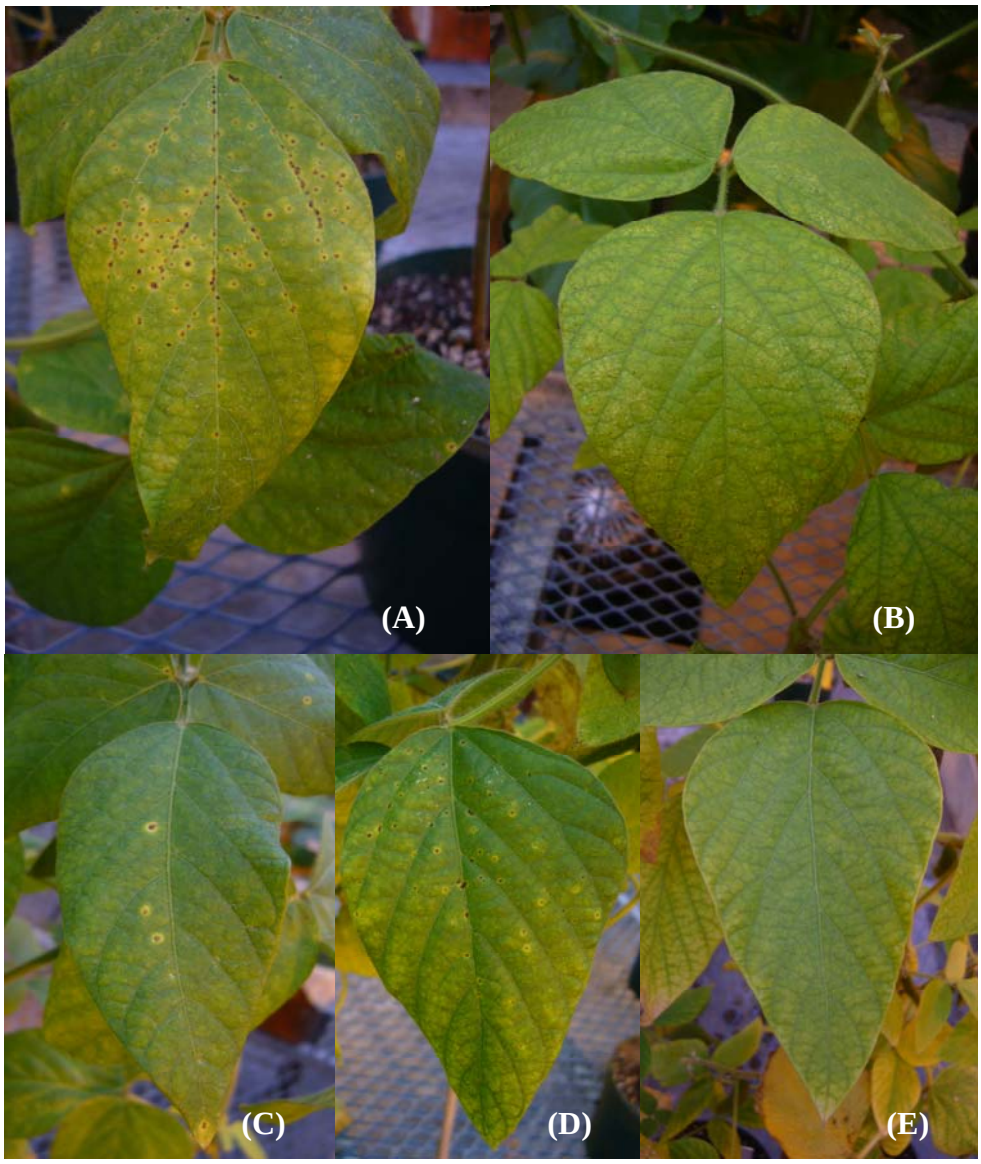

Figure 32 Comparison of disease severity on Spencer by wildtype KU-P-SW005 (A), *flgC* mutant KU-P-SW005 *flgC*::Tn5 (B), *flgK* mutant KU-P-SW005 *flgK*::Tn5 (C), *pilD* mutant KU-P-SW005 *pilD*::Tn5 (D), and *pilD* and *flgC* double mutants KU-P-SW005 *flgC-pilD*::Tn5 (E). Bacteria suspension was sprayed into soybean leaves as described in text. Symptoms were recorded 10 days after infiltration.

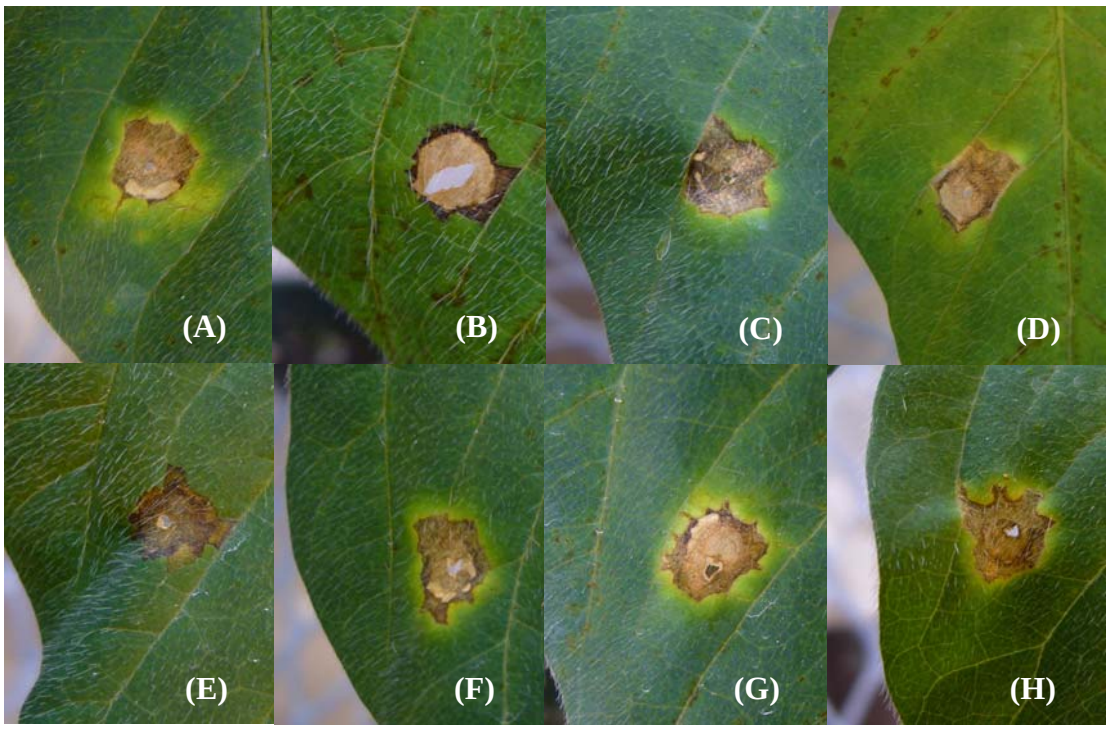

Figure 33 Comparison of disease severity on Spencer by wildtype KU-P-SW005 (A), *flgC* mutant KU-P-SW005 *flgC*::Tn5 (B), *flgK* mutant KU-P-SW005 *flgK*::Tn5 (C), *pilD* mutant KU-P-SW005 *pilD*::Tn5 (D), *pilD* and *flgC* double mutant KU-P-SW005 *flgC-pilD*::Tn5 (E), *flgC* complemented mutants *flgC*::Tn5 (pBBR-FLGC) (F), *flgK* complemented mutants *flgK*::Tn5 (pBBR-FLGK) (G), and *pilD* complemented mutants *pilD*::Tn5 (pBBR-PILD) (H). Bacteria were infiltrated into soybean leaves as described in text. Symptoms were recorded 2 days after infiltration.

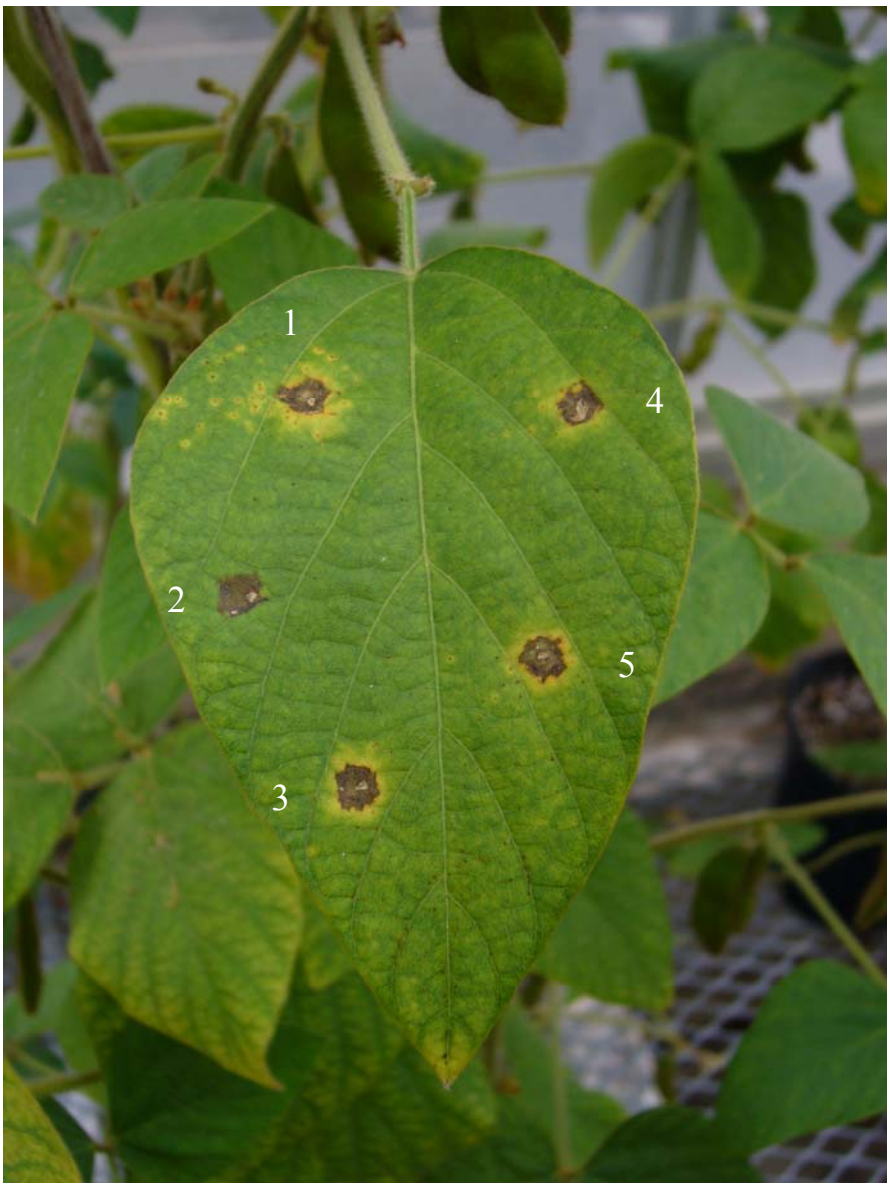

Figure 34 Comparison of number of pustule induction on Spencer by wildtype KU-P-SW005 (1), *flgC* mutant KU-P-SW005 *flgC*::Tn5 (2), *flgK* mutant KU-P-SW005 *flgK*::Tn5 (3), *pilD* mutant KU-P-SW005 *pilD*::Tn5 (4 and 5). Bacteria were infiltrated into soybean leaves as described in text. Symptoms were recorded 10 days after infiltration.

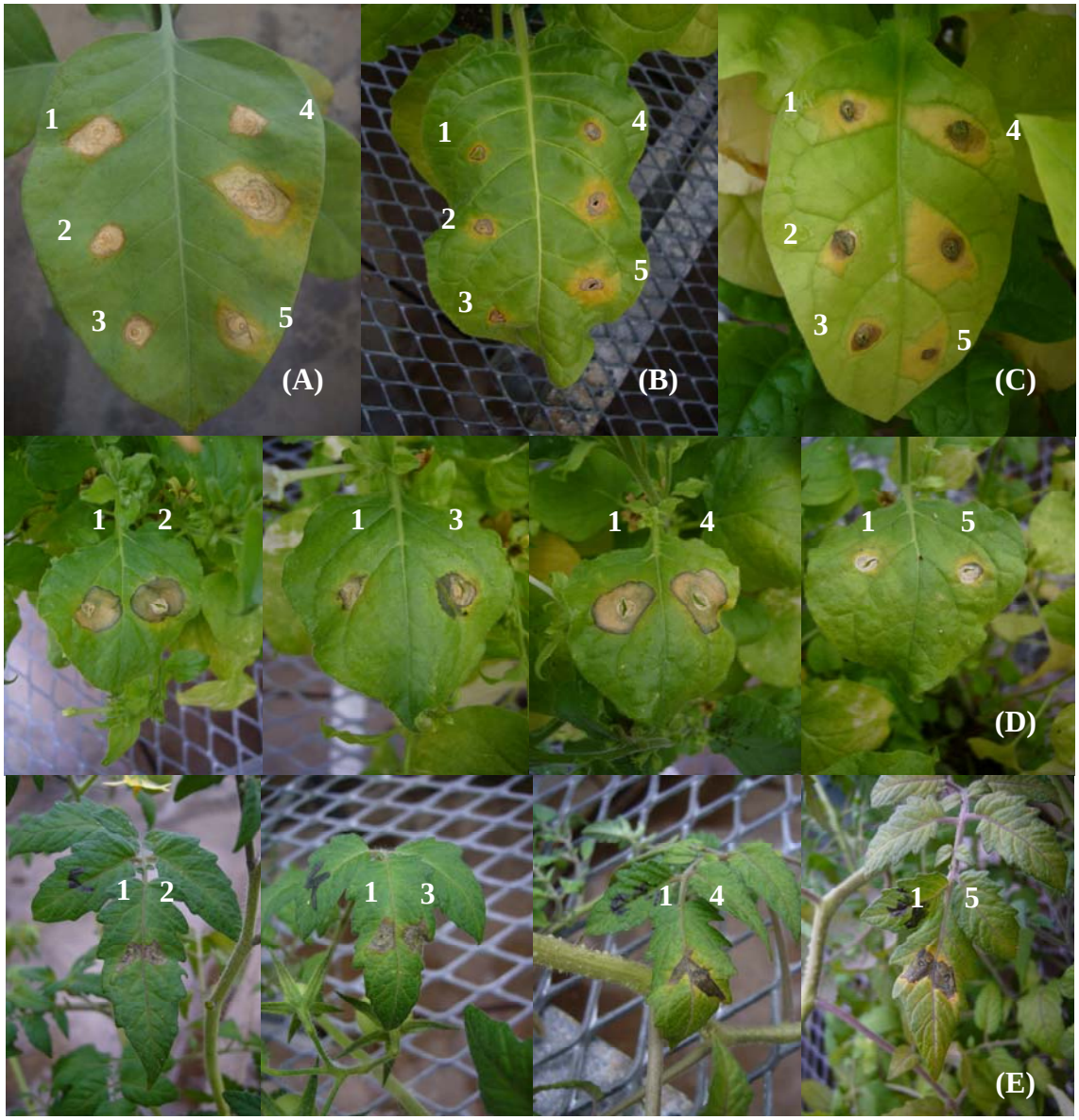

Figure 35 Hypersensitive reaction elicited by *Xanthomonas axonopodis* pv. *glycines* wildtype KU-P-SW005 (1), *flgC* mutant KU-P-SW005 *flgC*::Tn5 (2), *flgK* mutant KU-P-SW005 *flgK*::Tn5 (3), *pilD* mutant KU-P-SW005 *pilD*::Tn5 (4), and *pilD* and *flgC* double mutant KU-P-SW005 *flgC-pilD*::Tn5 (5) on four species of tobacco leaf; *Nicotiana glauca* (A), *N. rustica* (B), *N. tabacum* cv. Xanthi (C), *N. benthamiana* (D), and tomato (E).

Table 13 Morphotype of *Xanthomonas axonopodis* pv. *glycines* wildtype and various mutants that induced the hypersensitive response on four species of tobacco and on tomato

Bacterial strain[1/]	**Tobacco plant specie**[2/]				**Tomato**[2/]
	***Nicotiana tabacum* cv. Xanthi**	***N. benthamiana***	***N. glauca***	***N. rustica***	
KU-P-SW005	+	+	+	+	+
KU-P-SW005 *flgC*::Tn5	+	+	+	+	+
KU-P-SW005 *flgK*::Tn5	+	+	+	+	+
KU-P-SW005 *pilD*::Tn5	+	+	+	+	+
KU-P-SW005 *flgC-pilD*::Tn5	+	+	+	+	+
flgC::Tn5 (pBBR-FLGC)	+	+	+	+	+
flgK::Tn5 (pBBR-FLGK)	+	+	+	+	+
pilD::Tn5 (pBBR-PILD)	+	+	+	+	+

[1/]Comparison of hypersensitive response on three species of tobacco leaf including *Nicotiana tabacum* cv. Xanthi, *N. glauca, N. benthamiana,* and *N. rustica* and on tomato leaf by *Xanthomonas axonopodis* pv. *glycines* wildtype KU-P-SW005, *flgC* mutant KU-P-SW005 *flgC*::Tn5, *flgK* mutant KU-P-SW005 *flgK*::Tn5, *pilD* mutant KU-P-SW005 *pilD*::Tn5, *pilD* and *flgC* double mutant KU-P-SW005 *flgC-pilD*::Tn5, *flgC* complemented mutants *flgC*::Tn5 (pBBR-FLGC), *flgK* complemented mutants *flgK*::Tn5 (pBBR-FLGK), and *pilD* complemented mutants *pilD*::Tn5 (pBBR-PILD).

[2/] A positive response (+) resulted in complete collapse and necrosis of the infiltrated area. A negative response (-) may show slight yellowing but no visible collapse or necrosis of infiltrated zone. Infiltrated areas were monitored for development of tissue collapse and necrosis for 48 h post inoculation.

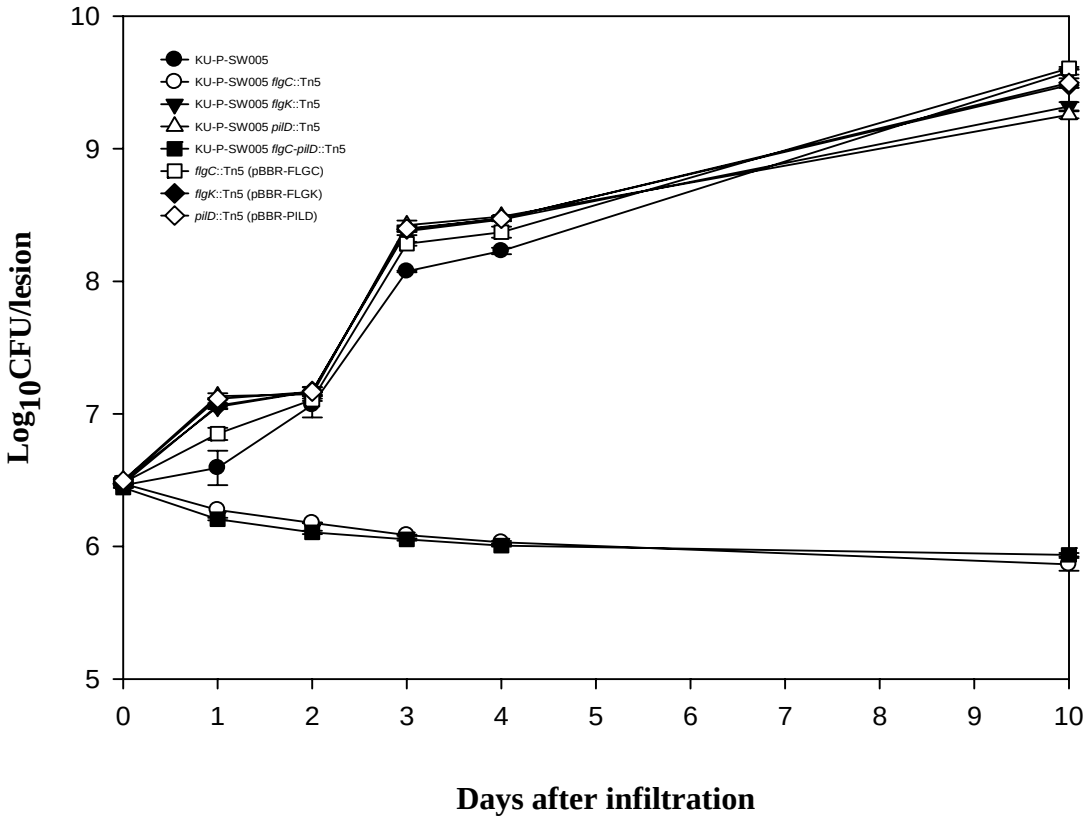

Figure 36 Time course of bacterial growth in soybean leaves Spencer. Leaves were sampled 1, 2, 3, 4, and 10 days after infiltration. Data represents means from three repetitions, and vertical bars represents standard errors.

Discussion

Bacterial pustule disease is a limiting factor in soybean production in Thailand caused by *X. axonopodis* pv. *glycines*. Bacterial pathogenesis then, depends on the TTSS encoded by *hrp* genes and *avr* gene products delivered to plant cells by the *hrp* system. Besides, the productions of xanthan and the extracellular enzyme, including protease, pectinase, and cellulase are collectively required for pathogenicity (Denny, 1995; Thowthampitak *et al.,* 2008). RAPD analysis was correlated with genetic variation of *X. axonopodis* pv. *glycines*, plasmid profile, and virulence of these pathogen (Saisangthong, 1999). Rep-PCR and BOX-PCR analyses were also correlated with virulence of the bacterial pustule pathogen and disease severity. However, the genetic diversity was not correlated with the geographic origin of the strains (Ketsuwan, 2003; Athinuwat *et al.,* 2006). Recent research has significantly advanced our more understanding of the heterogeneity of *X. axonopodis* pv. *glycines* and how it interacts with different plant species (Kim *et al.,* 2003; Kaewnum *et al.,* 2005). Race structure of *X. axonopodis* pv. *glycines* from the USA was initially determined based on reactions with five soybean cultivars including Williams82 that determined to be resistant to all five races (Hwang and Lim, 1998). Recently, Park *et al.* (2008) characterized *X. axonopodis* pv. *glycines* strains from Korea into six groups based on the predicted number of copies of *avrBs3*-like genes within each strain. When strains were probed with a 3.6-kb internal fragment of an *avrBs3* homolog, three to eight copies of the gene were detected in digestibility of total genomic and plasmid DNA suggesting the presence of multiple copies of *avrBs3*-like genes. Pathogenicity of representative strains from each group however did not show specificity to soybean cultivars. Previous work also demonstrated the presence of an *avrBs3* homolog carried on plasmids in Korean strains of *X. axonopodis* pv. *glycines* (Kim *et al.,* 2006). It was shown that *X. axonopodis* pv. *glycines* strains from pustule infected soybean carried one of two related plasmids that include an *avrBs3* homolog. Function for the putative gene however, was not determined. We have characterized strains from Thailand into three races based on their interactions with susceptible and resistant soybean cultivars and have demonstrated the presence of a multi-functional member of the *avrBs3* family, *avrXg1* and it is also provided evidence that is carried on plasmid DNA.

The discovery that resistance of plants to infection by bacterial pathogens can be mediated in a gene-for-gene manner that has provided significant insight into mechanisms of bacterial pathogenesis and towards the development of strategies for disease control. The function of bacterial *avr* genes were demonstrated following their transfer from avirulent to virulent races of different bacteria (Staskawicz *et al.,* 1984). When bacterial Avr proteins and corresponding plant R receptor proteins interact in plant cells, an incompatible hypersensitive response may develop if either component is missing, recognition of the invading pathogen fails resulting in disease (Alfano and Collmer, 2004). Moreover, *avr* genes have shown diverse functions that contribute to additive fitness and increased growth of the pathogen sometimes resulting in increased disease on susceptible host plants (Vivian and Gibbon, 1997; Chen *et al.,* 2000). Bai *et al.* (2000) reported that not all *avr* family members contribute to pathogen aggressiveness and that the contributions are quantitatively different.

Phytopathogenic bacteria are known to produce several effector proteins that include Avr proteins, toxins, and other factors, that can suppress host defense responses (Abramovitch and Martin, 2004; Grant *et al.,* 2006; Gurlebeck *et al.,* 2006). For example, the suppression of HR and defense gene expression was more efficient in the transformants with *apl1* and *avrXa7* than the transformant with *avrXa10* (Fujikawa *et al.,* 2006). The importance of the TTSS in delivery of effectors to plant cells has been demonstrated for several pathogens and mutation of *hrp* genes comprising the TTSS abolishes *avr* gene function (Pirhonen *et al.,* 1996). Fujikawa *et al.* (2006) reported AvrBs3-like proteins are predicted to be delivered into the plant cell via TTSS where they would affect HR or disease, depending on the presence of other effectors and plant *R* genes. The importance of the TTSS (Kim *et al.,* 2003) and a pectate lyase (Kaewnum *et al.,* 2006) have also been shown to play roles in *X. axonopodis* pv. *glycines*- plant interactions including non-specific HR on different plant species.

The *avrBs3/pthA* gene family was originally identified in the *X*. *axonopodis* pv. *vesicatoria* (Bonas *et al.,* 1989) and are now known to play diverse roles in interactions between *Xanthomonas* spp. and plants. They share unique structural features including an N-terminal secretion signal peptide essential for delivery via the bacterial TTSS; a central repeat region consisting of 1.5 to 28.8 nearly identical tandem repeats of 34 or 35 amino

acids (102-bp); and a C-terminal region consisting of imperfect heptad leucine zipper (LZ) repeats, NLSs, and AAD (Saijo and Paul, 2008). Bacterial proteins normally do not carry such signals because bacterial cells are devoid of nuclei. All of these regions affect function of the effectors in plant cells. For example, the highly conserved central repeat region determines host-specific pathogenicity and avirulence functions and may interact with conserved targets in diverse host species (White *et al.,* 2000). Deletions in repeats result in changes in protein specificity inferring it is the position and specific sequence of the repeats that determines the avirulence function (Kay *et al.,* 2007). The numbers of repeats in *avrBs3* genes also varies considerably. For example *avrBs3* and *avrBs3-2* in *X. axonopodis* pv. *vesicatoria* both contain 17.5 copies of 102-bp direct repeats (Bonas *et al.,* 1989; 1993), *avrB6*, *avrB4*, *avrB7*, *avrBIn*, and *avrB102* in *X. axonopodis* pv. *malvacearum* contain 14, 19, 19, 21, and 18 copies of 102-bp direct repeats respectively (De Feyter *et al.,* 1993). *X. oryzae* pv. *oryzae* carries *avrXa7* and *avrXa10* contain 25 and 15.5 copies of 102-bp direct repeats, respectively (Hopkins *et al.,* 1992) whereas strain of *X. oryzae* pv. *oryzae* carries only 1.5 copies of 102-bp direct repeats (Wu *et al.,* 2007) and *pthA* in *X. axonopodis* pv. *citri* also contains 17.5 copies of 102-bp direct repeats (Swarup *et al.,* 1992). In addition AvrBs3 requires a functional NLS, for transport into the nucleus of the plant cell (Kay *et al.,* 2007). Some Avr effectors such as AvrXa7 from *X. oryzae* pv. *oryzae* also require an AAD domain for virulence (White *et al.,* 2000). Biochemical analyses of AvrBs3 of *X. axonopodis* pv. *vesicatoria* Race 1 showed it to be predominantly a soluble intracellular protein and the central repeats of 102-bp are less hydrophilic than the N-terminal and the C-terminal regions (Bonas *et al.,* 1993). The deduced protein encoded by *avrXg1* is typical of the *Xanthomonas avrBs3*/*pthA* family members. It has a central domain containing a series of 20.5 copies of 102-bp direct repeats, three NLSs, an AAD in the C-terminal region (Bonas *et al.,* 1989; Van den Ackerveken *et al.,* 1996; Zhu *et al.,* 1998; Gabriel, 1999). The sequence at positions 171 to 190 is located -10 and -35 bp upstream of the ATG codon in ORF1 in the assumed promoter region indicating translation of AvrXg1 starts at position 1 and ends at position 3,801, giving rise to a 132.8 kDa protein of 1,266 amino acids. However, AvrXg1 was not expressed by expression vectors tested. The elicitation of a genotype-specific HR requires the production of a functional TTSS in a single bacterial cell, together with the HrpZ harpin and an *avr* gene product that matches the resistance genotype (Vivian and Gibbon, 1997). Therefore, AvrBs3 would not be transported within cell grown in vitro

(Bonas *et al.,* 1989). In *Yersinia*, the YopD protein is required for entry of virulence factors into animal host cells (Rosqvist *et al*., 1994), but no YopD homologue has been identified in phytopathogenic bacteria. Innes (1996) has suggested that harpin may serve an analogous role to YopD for the transfer of Avr products into plant cells.

Inactivation of *avrBs3*/*pthA* genes by mutation generally results in loss of avirulence but also may lead to reduce virulence and growth rate in the host plant (Vivian and Gibbon, 1997). Deletion of four-repeats of *avrBs3* in *X. axonopodis* pv. *vesicatoria* resulted in loss of recognition by receptor protein Bs3 in pepper cultivars carrying *Bs3* with gain of recognition in peppers carrying *bs3*. Deletion of the region between NLS2 and NLS3 (37 amino acids) as well as that between NLS1 and NLS3 (83 amino acids) of *avrBs3* in *X. axonopodis* pv. *vesicatoria* resulted in complete loss of activity (Van den Ackerveken *et al.,* 1996). Mutation in the *hssB3.0* gene in *X. axonopodis* pv. *citri* resulted in increased aggressiveness on *Citrus grandis* cv. Otachibana (Shiotani *et al.,* 2007). Also, *pthA* isolated from *X. axonopodis* pv. *citri*, confers an enhanced ability to induce cankers in the related *X*. *axonopodis* pv. *citrumelo* (Vivian and Gibbon, 1997). Mutations in *avrBs2* were shown to alter both virulence and avirulence in *X*. *axonopodis* pv. *vesicatoria*. Mutation of *avrb6* in *X*. *axonopodis* pv. *malvacearum* did not affect bacterial growth in planta, but are involved as virulence factors in the production of water-soaking symptoms and the release of greater numbers of bacteria to the leaf surface (Vivian and Gibbon, 1997). We provide evidence that mutation of the central repeat and C-terminal regions of *avrXg1* affects cultivar-specific responses. The requirement of the NLS and AAD for both hypertrophy induction and disease resistance is consistent with a role of AvrBs3 as transcriptional activator in the host nucleus (Van den Ackerveken *et al.,* 1996). Our results indicated that the central repeat regions of *avrXg1* are important for recognition by the Williams82 receptor and that the C-terminal ADD domain is also required presumably for virulence. The *avrXg1* mutants were more efficient in producing virulence factors, cellulase and pectate lyase for giving high disease severity when compared with wildtype. In contrast, *rpf*F mutants exhibited reduced virulence on soybean and produced lower than wildtype of extracellular polysaccharide and the extracellular enzymes protease, cellulase, endo-β-1,4-mannanase, and polygalacturonate lyase and pectolytic activity (Thowthampitak *et al.,* 2008). The *avrXg1* mutants produced no visible change in the HR on the nonhost (Ritter and Dangl, 1995). In addition to

virulence, it has been shown that *avrXg1* is plasmid-borne affects increased epiphytic fitness including swimming motility and establishment of bacterial population in soybean leaves. In contrast, the report of Park *et al* (2008) which revealed that the interaction with host plant is depended on the specific chromosome-borne *avr* gene combination but not on plasmid-borne.

avrXg1 was also expressed in *X. axonopodis* pv. *glycines* Race 1 which normally causes HR on susceptible and resistant soybean cultivars but does not appear to carry *avrBs3* homologous genes. Therefore, we predict that the HR induced by Race 1 is non-specific, similar to that associated with harpins and presence of *xagP* in several strains of *X. axonopodis* pv. *glycines*. Interestingly, expression of *avrXg1* in Race 1 resulted in disease formation on susceptible cultivars Spencer, SJ4, and PI 520733 and HR on Williams82 thereby suppressing non-specific HR in the susceptible but not in resistant cultivars. This supported by previously reported that some *avr* gene suppressed the HR or plant defense response induction (Fujikawa *et al.,* 2006) such as AvrRpt2 act inside the plant cell to suppress host defense response (Vivian and Gibbon, 1997). Further research on interaction of AvrXg1 with these cultivars is needed to shed light on demonstration of multiple functions of Avr proteins that can have in plants.

Race 2 of *X. axonopodis* pv. *glycines* caused disease on resistant and susceptible cultivars. PCR analyses indicated the presence of *avrBs3* homologous genes in Race 2 however their sequence and function are yet to be determined. In addition *avrXg1* was also expressed in *X. axonopodis* pv. *glycines* Race 2. Therefore, we predict AvrXg1 has not been overcome *avrBs3* homologous genes in Race 2. Race 2 transformant with the *avrXg1* was increased virulence and bacterial population on both Spencer and Williams82 cultivars. This confirm that *avrXg1* enhanced bacterial fitness, virulence, and aggressive of *X. axonopodis* pv. *glycines* (Bai *et al.,* 2000). Additional research is required to determine the specific protein structure of *avrBs3* ORFs in Race 2 and how they compare to the protein encoded by *avrXg1*.

Several bacterial *avr* genes have been shown to contribute to virulence on susceptible plants lacking the corresponding *R* gene. The mechanisms by which *avr* genes promote parasitism and disease, however, are not well understood (Chen *et al.,* 2000).

Ritter and Dangl (1996) reported that *avrRpt2* of *P. syringae* may promote pathogenesis and interfere with induction of *avrRpm1/Rpm1*-mediated resistance. Chen *et al.* (2000) provided a hypothesis for *avrRpt2* function promotion of virulence via two different mechanisms. The first states *avrRpt2* interferes with host defense and results in complete inhibition of resistance. The second, involves modification or inhibition of a common component of the defense response, rather than interfering specifically with known *R*-gene-mediated resistance and results in an increase in growth of *P. syringae* pv. *tomato* strain DC3000. We have also shown that in the absence of a functional of *rxp*, *avrXg1* functions to promote virulence and bacterial population of *X. axonopodis* pv. *glycines* on a susceptible Spencer. Therefore, *avrXg1* should be added to the list of *avr* genes that also function as virulence (*vir*) factors on susceptible hosts lacking the corresponding *R* gene (Ritter and Dangl, 1996; Jackson *et al.,* 1999; Chen *et al.,* 2000). In spite of an *avrXg1* function in different host cultivars that lacking or occurring the corresponding *R* gene, these observations could imply that the gene may have a host-specific role in pathogenicity as a pathogenicity factor also.

Signal pathways in host defense response that are conserved in both plant and animal cells may be responsible for the broad membership of the *avrBs3/yopJ* gene family. YopJ has been shown to bind MAPKK1 and IKKb (an inhibitor of NFkb kinase kinase), which are involved in the activation of NFkb during an inflammatory response to infection by *Yersinia* (Orth *et al.,* 1999). It is tempting to speculate therefore, that AvrRxv and related proteins from plant pathogenic bacteria interact with MAP kinases that are otherwise activated by general defense signaling mechanisms. Previous analyses, increasing information has tended to prove that metabolites generated by the LOX pathway play a role in the reactions of defense of higher plants to pathogen attack. LOX activity was found to be stimulated during pathogen attack of tomato (Katoetal, 1992), Arabidopsis (Peng *et al.,* 1994), rice (Melan *et al.,* 1993) or during treatment of plant cell cultures by fungal elicitors (Fournier *et al.,* 1993). Estabrook and Sengupta-Gopalan (1991) reported the symbiosis specific PAL and chalcone synthase (CHS) genes expression in soybean during nodule development. Graham *et al.* (2003) reported *Phytophthora sojae* cell wall glucan elicitor (WGE) induced PR-1a, PR-2 (elicitor-releasing endoglucanase; GLU), PR-4 (a WIN-like protein; WIN), PR-6 (a Kunitz trypsin inhibitor; KTI), and PR-10 families in soybean. Buensanteai *et al.* (2008) reported a

pattern of induced systemic resitance (ISR) of soybean associated with the accumulation of phenolic content and defense related enzyme of increased β-1,3-glucanase and POX activities in plant. Recently, a very detailed analysis of the effects of natural and synthetic analogs of jasmonic acid (JA) on phytoalexin and phenylpropanoid enzyme gene activation has been reported (Fliegmann *et al.,* 2003). It is thus apparent that JA has effects on both secondary product and PR gene defense responses. It was hypothesized that PR proteins might play a role in this form of induced resistance. PR proteins have been studied in many systems in the context of infection or elicitation. We have observed that the soybean response was similarly effective in stimulating PR-2, PR-4, PR-6, PR-10, LOX, PAL, and catalase associated with defense response in Williams82 at 24 h after inoculation with wildtype Race 3. PR-1a and POX were not expressed in this study. In terms of response to wounding or elicitor, PR-1a gene expression is essentially induced only by WGE (Graham *et al.,* 2003). This indicated that Race 3 can induce soybean defense response therefore, *X. axonopodis* pv. *glycines* Race 3 can be used for screening technique for resistant cultivars in breeding program.

This is surprising in that genes for members of the PR-2, PR-4, PR-6, and PR-10 families and LOX genes are expressed over a similar time in Williams82 inoculated with wildtype. PR-2 and PR-4 families are known to be wound or ethylene activated (Yoshikawa *et al.,* 1990) and PR-6 families are known to be protease inhibitors. PR-10 has been associated with pathogenesis, general stress, development or as pollen allergens (Kombrink and Somssich, 1997; van Loon and van Strien, 1999). While more detailed analyses are needed, the patterns of activation of the PR protein genes described here suggest that GLU, WIN and KTI are potential candidates for involvement. Interestingly, the expression of PR-6 (KTI) genes showed a different pattern from all of the other genes in their activation in Williams82 inoculated with wildtype at 0, 24, and 48 h after inoculation whereas that was not expressed in Williams82 inoculated with the *avrXg1* mutants and minimal-wound water control at 48 h after inoculation. This may suggest a PR-6 different mechanism and specific defense response to wildtype carrying *avrXg1* gene. Our finding suggest that AvrXg1 elicitor from *X. axonopodis* pv. *glycines* Race 3 are found to be associated with induces both parsley phenylpropanoid phytoalexins and PR proteins in soybean that induced by *X. axonopodis* pv. *glycines* in a TTSS dependent manner (Parker *et al.,* 1991; Nuernberger *et al.,* 1994). Therefore, it was considered that

the *avrXg1* gene participate in diverse functions associated directly or indirectly with this main pathogenicity related genes required by receptor proteins from soybean. This is the first study of host-parasite interaction between soybean-*X. axonopodis* pv. *glycines* based on gene-for-gene concept leading to find out source of *R* in soybean for development of resistant cultivar to overcome bacterial pustule pathogen.

Discovery of *avrXg1* and its functional domains were successfully identified based on its ability to confer avirulence on Williams82 cultivar carrying a predicted *R* gene for bacterial pustule resistance. Further characterization of other *avr* genes in *X. axonopodis pv. glycines* and subsequent specific receptors in soybean will provide further insight into mechanisms of virulence and plant defense and will lead to the development of useful methods for pustule disease control.

Inaddition, *avrXg1* mutant exhibited higher virulence and swarming motility than wildtype. The results indicated that the *avrXg1* plays a specific role in its virulence and avirulence activities. Among the secreted mutant Hrp proteins, there was a general correlation between loss of avirulence and virulence activities that no mutations were found to abolish one without affecting the other (Vera Cruz *et al.,* 2000). This suggests that the virulence and avirulence activities of *avrXg1* may not be functionally separable.

The pathogen-plant relationship in the *X. axonopodis pv. glycines* system follows a gene-for-gene pattern. The pathogenicity island (PAI) of *X. axonopodis* pv. *glycines* was composed of nine *hrp*, nine *hrc*, and eight *hpa* genes with seven plant-inducible promoter boxes (Kim *et al.,* 2003). The avirulence genes, *avrBs3* homolog, *avrXg1* charecterized in this study and some virulence genes such as *xagP, ppsA,* and *rpf*F also have been isolated (Kaewnum *et al.,* 2006; Kasem and Prathuangwong, 2006; Kim *et al.,* 2006; Thowthampitak *et al.,* 2008). Although significant progress has been made in the characterization of host and bacterial genes governing the interaction, little is known about the genes governing *X. axonopodis pv. glycines* flagella and pili biosynthesis, their functions and involvement in bacterial pathogenesis.

Bacterial flagella are important virulence factors for pathogenesis of animal and plant (Moens and Vanderleyden, 1996; Finley and Falkow, 1997). In some plant-

pathogen systems, flagella-driven chemotaxis plays a role in the early interactions with host plants (Vande Broek and Vanderleyden, 1995). Swimming motility enables foliar pathogens to reach internal sites in the leaves (Beattie and Lindow, 1995). Bacterial flagella are complex structures. Their biosynthesis and locomotion require many genes (Macnab, 1992). For example, the entire *S. typhimurium* flagella system requires at least 40 genes (Jones and Macnab, 1990). The *X. axonopodis* pv. *citri* genome was showed 14 *flg* genes (*flgABCDEFGHIJKLMN*) involved flagella assembly, FlgN is a secretion chaperone for the hook-associated proteins FlgK and FlgL and a translational regulator of the anti-sigma^{28} factor FlgM (Kutsukake *et al.,* 1994; Fraser *et al.,* 1999; Bennett and Hughes, 2000; Aldridge *et al.,* 2003; Blocker *et al.,* 2003; Minamino and Namba, 2004). Search on databases of the *X. campestris* pv. *campestris* ATCC33913 and Xc17 genomes revealed that both had a cluster of 17 genes involved in flagellum biosynthesis, 14 upstream *flg* genes, and 3 downstream *fli* genes (*fliC–fliD–fliS*) (Lee *et al.,* 2003). Mutation in *fliC* was nonmotile (Lee *et al.,* 2003). Bennett and Hughes (2000) showed that FlgN is found as a homodimer species in *S. typhimurium* and interacts with FlgK in a 2:1 stoichiometry. The dimerization interface of FlgN has been suggested to be a leucine zipper-like region localized in the N-terminus (Bennett and Hughes, 2000). The analysis of all 23 preys derived from FlgK revealed that its C-terminus is sufficient for interaction with *X. axonopodis* pv. *citri* FlgN. The primary structure of *X. axonopodis* pv. *citri* FlgK indicates that the FlgN-interacting domain corresponds almost exactly to a conserved domain frequently found in the C-terminus of hook-associated proteins. Fraser *et al.* (1999) showed that removal of the C-terminal 42 amino acids of FlgK from *S. typhimurium* completely abolished binding of FlgN, and that a polypeptide comprising the C-terminal 69 amino acid residues of FlgK binds to FlgN, the FlgK C-terminal domain seems to be specific to flagellar basal-body rod and flagellar hook proteins. However, there have not reported on gene coding for the positive regulators in *X. axonopodis* pv. *glycines*. We have characterized that *flgC* and *flgK* mutants were lost swimming and swarming motility, it may be probably due to lost of flagella. FlgC and FlgK proteins act as positive regulators of the flagella reglon, i.e., controlling the expression of all other flagella genes. One of those, *flgC* located at the^{5th} level of the cascade is the flagella basal body rod protein FlgC. Another one, *flgK* located at the 13th level of the cascade which codes for a flagella hook-associated protein of the flagella apparatus (Khater *et al.,* 2007). We conclude that *flgC* and *flgK* were the important genes

for flagella biosynthesis and played a role of swimming and swarming motility. It was previously reported that *flgC* and *flgK* in *P. aeruginosa* LESB58 were function as cell to cell communication and motility control. The finding together with the results that lost of flagella and motility in the *flgC* and *flgK* mutants suggests that *X. axonopodis* pv. *glycines* has a single *flgC* and *flgK* genes. Even though the flagella were lost in function and swimming and swarming motility, they have interestingly shown that the fringe surrounding the colonies of *flgC* and *flgK* mutants on solid surface where *pilD* mutant exhibited smooth colonies. This can be considered that PilD was important in pilus assembly and a consequence of the surface movement associated with type IV pili. Such a marginal fringe can be referred to as twitching motility, because it closely resembles that of TFP-dependent characteristics in well-studied strains of *R. solanacearum* and *X. fastidiosa* (Meng *et al.,* 2005). This finding suggests that *X. axonopodis* pv. *glycines* cells actively migrate to other plant tissue via twitching motility.

Type IV pili are flexible, filamentous surface appendages which adhere to host epithelial cells and invade on the solid surface, called twitching motility. They are also involved in biofilm formation, natural DNA uptake, auto-aggregation of cells (Wall and Kaiser, 1999) and infection of bacteria by F-specific phages (Romantschuk, 1992). It was revealed that type IV pili promote the attachment of bacterial pathogens to the specific receptors of host cells during colonization (Bieber *et al.,* 1998). Such attachment is an essential event for the initiation of infection. Limited to colonizing the water-conducting xylem vessels of plants, phytopathogenic bacteria develop biofilms that contribute to the blockage of sap flow resulting in plant stress and disease (Maxwell *et al.,* 2003; Meng *et al.,* 2005). A cluster of at least 30 *pili* genes required for type IV pilus biogenesis, have been identified in many bacteria (Mattick *et al.,* 1996). Two *pilA* genes, *pilA1* and *pilA2*, arranged in tandem encode the major structural subunits or pre-pilin. Three genes, *pilD, pilC,* and *pilB*, located upstream and downstream, respectively, of *pilA*, are involved in the biogenesis of type IV pilus. *pilB* and *pilC* code for proteins that are associated with cytoplasmic membrane and are required for the assembly of surface pili. *pilD* encodes a bifunctional enzyme, which is responsible for the cleavage and methylation of the *pilA* subunit (Strom *et al.,* 1993). Mutation in *P. aeruginosa pilD* caused significant decrease *in vitro* peptidase (Strom *et al.,* 1993). Recently, the twitching motility of *X. fastidiosa* cells has been directly observed in situ (Meng *et al.,* 2005). *pilD* of *X. campestris* pv.

campestris encode type IV pre-pilin leader peptidase was demonstrated to be required for protein secretion, in addition to its involvement in biogenesis of type IV pili. It is conceivable that mutations to glycine or serine in any one of the four cysteines of PilD may have caused changes in its secondary or tertiary structure, which in turn may affect the efficiency of the enzyme activities of PilD (Hu *et al.,* 1995). Although the existence of type IV pili among *Xanthomonas* had previously been reported (Van Doorn *et al.,* 1994; Ojanen-Reuhs *et al.,* 1997), the mechanism for assembly and their expression of *Xanthomonas* pili was not identified. Our result showed *pilD* mutant lost twitching motility but also can be swum by flagella. It have previously shown that *pilD* mutant of *P. aeruginosa* PAK are unable to process pre-pilin to the mature form (Nunn *et al.,* 1990). The high level of enzymatic activity of the purified PilD on the purified pre-pilin substrate demonstrated that PilD was the pre-pilin leader peptidase and does not require any additional protein cofactors. PilD evidently acts on components of an excretion apparatus for these proteins, rather than on the excreted proteins themselves (Nunn *et al.,* 1990). This suggests *pilD* gene is important in the pili regulon for pilus biosynthesis. In confirmation, the double mutant of *pilD* and *flgC* was lost twitching and became nonmotile bacteria. Therefore, it can be concluded that *flgC, flgK*, and *pilD* of *X. axonopodis* pv. *glycines* play a major role in swimming and swarming and twitching motility and that may be the first report here.

The importance of flagella and pili for bacterial pathogens infecting plants has remained controversial, in part due to the lack of well-characterized bacterial adhesins. In this communication, we report the characterization of a novel gene, *flgC, flgK,* and *pilD* encoding bundle-forming flagella and pili respectively, biofilm formation, and cell aggregation of the plant pathogen *X. axonopodis* pv. *glycines* that infects soybean plants. The amino acid sequences deduced from *flgC, flgK,* and *pilD* genes in *X. axonopodis* pv. *glycines* were compared with other protein sequences from different bacterial species in GenBank. Consequently, *flgC, flgK,* and *pilD* of *X. axonopodis* pv. *glycines* showed high identities more than 98% to amino acids of FlgC, FlgK, and PilD from *Xanthomonas* species, showing a closely related genetic relationship among them. Sequence identity to *Pseudomonas* sp.; and *E. coli* were 43%, 32%, and 51%; and 50, 30, and 33% respectively. This result suggests that *flgC, flgK,* and *pilD* are conserved in *Xanthomonas* species, but not in other bacterial species.

A biofilm is a complex aggregation of microorganisms marked by the excretion of a protective and adhesive matrix. Biofilms are also often characterized by surface attachment, structural heterogeneity, genetic diversity, complex community interactions, and an extracellular matrix of polymeric substances (Costerton *et al.,* 1995). In phytopathogenic bacteria, biofilm formation plays an important role in cell to cell communication between pathogen and host plant and consequently is associated with pathogenicity (Marques *et al.,* 2002; Meng *et al.,* 2005). Timmer *et al.* (1987) described that *X. axonopodis* pv. *vesicatoria* colonizes tomato leaf surfaces in bacterial aggregates. Our demonstration that *X. axonopodis* pv. *glycines* KU-P-SW005 forms compact aggregates, or biofilm formation, on glass surfaces is an agreement with those findings. Furthermore, our results indicate that *flgC, flgK,* and *pilD* of *X. axonopodis* pv. *glycines* are involved in determining the cell-to-cell aggregation. We observed that the *flgC, flgK,* and *pilD* mutants failed to form the biofilm that were formed efficiently by wildtype *in vitro*. The adhesion and the initial colonization of soybean leaves by wildtype were significantly affected by the *flgC, flgK,* and *pilD* mutations, it can be suggested that FlgC, FlgK, and PilD are a primary determinant of the initial colonization including quorum sensing on soybean leaf surface. It was reported that type IV pili-mediated twitching motility in *P. aeruginosa* may be necessary for cells to migrate along the surface to form muti-cell aggregates that lead eventually to the formatiom of biofilm (O'Toole *et al.,* 1999). Mutations in *fimA* gene of *X. fastidiosa* resulted in the cell phenotype with short pili, causing a decrease in biofilm formation (Meng *et al.,* 2005). Although the requirement for flagella motility in the early stages of biofilm formation remains controversial (Meng *et al.,* 2005), different reports show that flagella might not be required within a mature biofilm. Accordingly, genes encoding components of the flagellum are repressed soon after the bacteria reach the surface (O'Toole *et al.,* 1999). Therefore, the repression of flagella gene expression might be one of the first and most thoroughly documented examples of genetic 'reprogramming' that leads to a sessile lifestyle. Moreover, it is important to note that the strain defective in *flgC, flgK,* and *pilD* genes expresses neither swimming and swarming and twitching motility reduces biofilm formation. It is reasonably assumed that flagella and type IV pili play a direct role in stabilizing interactions with the abiotic surface and forming cell-to-cell interactions required to form biofilm. This can be suggested that *flgC*, *flgK,* and *pilD* play an important role in biofilm formation of bacterial pustule pathogen.

The cell surface component of the pathogen may play a role in systemic colonization during interactions between host and pathogen. Many biofilm matrix polysaccharidic components have been identified recently. In addition to the PIA/PNAG (polysaccharide intercellular adhesion/poly-N-acetylglucosamine) polymer encoded by the *icaABCD* locus in *Staphylococcus aureus* and *S. epidermidis*, Gram-negative bacteria components, such as colanic acid (*E. coli*), alginate, glucose and mannose-rich Pel and Pls matrix components (*P. aeruginosa*), cellulose and b-1,6-GlcNac polymer (*Salmonella* and *E. coli*), have been reported to play important roles for biofilm formation. These extracellular polysaccharides are key elements that shape and provide structural support for bacterial biofilms. However, most of the questions regarding the temporal and spatial regulation of exopolysaccharide production remain unanswered (Beloin and Ghigo, 2005). An *avrXg1* mutant increased cellulase and pectate lyase activities and biofilm formation also. It has mainly been focused on extracellular polysaccharides (EPS), extracellular enzyme and lipopolysaccharides (LPS) biosynthesis related genes as candidates in *X. axonopodis* pv. *glycines*. Therefore, expression of *avrXg1* in the *flgC* mutant was significantly reduced. It has been demonstrated that *avrXg1* encoded enzymes (cellulase and pectate lyase activities) contributed to bacterial aggregation disposal. Also, aggregation of bacterial cells on the plant leaf surface is a mechanism to protect epiphytic bateria against environment stress, i.e., factors such as UV light or desiccation (Wilson and Lindow, 1994), showing good agreement with our result that *avrXg1* mutant produced a lot of biofilm (data not shown) and exhibited high population desities on soybean leaves. This indicates that the reduction of cell aggregation of the *flgC*, *flgK,* and *pilD* mutants may be due to the reducibility in the expression of *avrXg1*. However, how *flgC, flgK,* and *pilD* affect the expression of *avrXg1* remains to be further investigated.

Flagella and pili were used for movement in liquid and on solid surfaces, respectively (Mattick, 2002). The movement of the bacterial cell is required for expanding the disease in plant tissue. It was previously shown that the type IV pilus subunit protein is essential for virulence, although it was not clear if the effects on virulence resulted from reduced flagella and pili-mediated cell adherence, loss of swarming, swimming, and twitching motility, or reduced some proteins secretion. For example, *R. solanacearum* and *X. fastidiosa*, the causal agent of vascular wilt disease of plants, possess twitching-mediated movement since they were previously shown to

exhibit colony morphological characteristic of twitching behavior (Marques *et al.*, 2002; Meng *et al.*, 2005). The results presented in this study provided evidence that flagella and type IV pili mediated swimming, swarming, and twitching motility, respectively are essential for *X. axonopodis* pv. *glycines* cells to cause bacterial pustule disease. Interestingly, a flagella assembly-related *flgC* and *flgK* genes were also identified as a novel pathogenicity gene of bacterial pustule pathogen. Virulence studies on soybean showed that one independently derived *flgC* mutants and double mutaions of *pilD* and *flgC* failed to cause disease, two independently derived *flgK* mutants and a *pilD* mutant all significantly reduced disease induction on soybean, unlike the wildtype strain. It has been reported that mutations in *pilQ* genes for twitching motility and biofilm formation resulted in reduced virulence in *X. oryzae* pv. *oryzae* (Wang *et al.*, 2008), showing consistent with our result. Furthermore, complementation of the *flgC, flgK,* and *pilD* mutants restored the ability to cause virulent disease. The *flgC* and *flgK* mutants had lost swimming and swarming motility and reduced biofilm formation and to adhere to epithelial cells, *pilD* mutant had lost twitching motility and reduced biofilm formation, but these parameters could not have been responsible for the loss of virulence in these mutants since the *flgC, flgK,* and *pilD* mutants had unaltered secretion and adhesion phenotypes. The common feature of the *avrXg1* mutant was their ability to enhance virulence factors and swarming motility and *flgC, flgK,* and *pilD* mutants was their inability to undergo swarming and twitching motility and they could not produce flagella and type IV pili on the surface and reduced disease initiation. It is possible that a bacterial virulence factors i.e. secretion of *avrXg1* and *flgC* are able to overcome the host defense. We postulate that the role of swimming, swarming, and twitching motility in the disease process is to enable *X. axonopodis* pv. *glycines* cells to move, communicate, and the ability to quorum sensing in a cell density-dependent manner in suitable location within the developing lesion, thereby increasing the probability that the cells will find a more suitable environment for growth and EPS and LPS productions.

The *flgC* activated flagella genes that contributed to biofilm formation, symptom development, and activity of pectate lyase and cellulase including increased growth rate. Therefore, disease and severity initiation are correlated with bacterial growth and swarming. Swarming, biofilm formation, and cell motility also associated with quorum sensing has been found in *X. campestris* pv. *campestris*, and increased ability of bacterial

cells to interact with their prospective host cells. This ability, termed quorum sensing, functions through the secretion and detection of autoinducer molecules, which accumulate in a cell density-dependent manner. Traits under quorum sensing control include surface attachment (Dunne, 2002), extracellular polymer production (Sakuragi and Kolter, 2007), biosurfactant synthesis (Schuster, 2006), sporulation (Grossman, 1995), competence (Li *et al.,* 2001), bioluminescence (Miller and Bassler, 2001), and the secretion of nutrient-sequestering compounds and virulence factors (Hentzer *et al.,* 2003). Quorum sensing and biofilm formation are often closely linked, and it is likely that their interaction is central to the pathogenesis of many bacterial infections (Sakuragi and Kolter, 2007). Regulation of bacterial motility appears to be a complex system involving multiple processes that genes responsible for quorum sensing, production of EPS, amino acid and polyamine metabolism, and production of flagella are required for swimming and swarming motility. Furthermore, it is likely that additional processes are to be involved in motility, because various races or strains of *X. axonopodis* pv. *glycines* display different motility behaviors, possibly due to unidentified environmental factors (Wilson and Lindow, 1994). In *X. campestris* pv. *campestris*, the quorum sensing system also regulates the biofilm formation and the synthesis of EPS and extracellular enzymes (amylase, endonuclease, protease) (Barber *et al.,* 1991; Thowthampitak *et al.,* 2008). The overlap function of *flgC* and biofilm formation system suggests that *flgC* also might directly regulate the production of extracellular products via modulating the biofilm formation system in *X. axonopodis* pv. *glycines.*

Although most flagellin is assembled into flagella, flagellin can also leak to the bacterial environment during the construction of flagella (Komoriya *et al.,* 1999), and flagellin is a component of the detritus associated with a bacterial colony. Stray or partially degraded flagellin monomers can be recognized by plant cells (Felix *et al.,* 1999). The discovery of flagellin perception by plants originated from the observation that many phytopathogenic bacteria can trigger an oxidative burst in the host when added to suspension cultured plant cells (Felix *et al.,* 1999). Bacterial flagellins have been portrayed as a relatively invariant pathogen-associated molecular pattern (PAMP) that was recognizable by the innate immune systems of plants and animals (Gomez-Gomez and Boller, 2000). An impact of flagellin detection on plant disease resistance has been demonstrated only for *P. syringae* pv. *tomato* strain DC3000 (Zipfel *et al.,* 2004). Crude

flagellin extracts from *A. tumefaciens*, *Sinorhizobium meliloti*, *X. axonopodis* pv. *vesicatoria*, and *X. axonopodis juglandis* were reported to be inactive for defense elicitation in tomato (Felix *et al.,* 1999), and *R. solanacearum* strain K60 bacteria are not detected by the *Arabidopsis* FLS2 system (Pfund *et al.,* 2004). Flagellin glycosylation apparently can also serve as a specific determinant of compatibility between phytopathogenic bacteria and plants (Takeuchi *et al.,* 2003). The hydrogen bonding of Asp-43 may play a central role in receptor elicitation (Sun *et al.,* 2006). It remained possible that FLS2 receptors may instead sense flagellin indirectly by monitoring changes that flagellin elicits in other host molecules, as occurs with those *R* gene products that sense Avr proteins via a guard mechanism (Nurnberger *et al.,* 2004). However, the empact of flagellin expression on bacterial virulence and the efficacy of the plant defenses activated following glagellin recognition remain unclear. The differential glycosylation of flagellin may provide understanding the phenomenon of compatible or incompatible interaction that determining (glycosylation sites and carbohydrate structures) *flgC* secretion is important for future research.

Some proteins that have the classical type III secretory apparatus show amino acid sequence similarity with flagella structural proteins (Mecsas, and Strauss, 1996; Macnab, 1999). Moreover, evidence is beginning to accumulate that component of the flagella apparatus participate in the export of virulence determinants in several pathogens. For example, experiments by Young *et al.* (1999) demonstrated that *Yersinia* secretes flagella outer proteins (Fops) via the flagellar apparatus. More recently, secretion of virulence associated proteins from *Bacillus thuringiensis* has been found to be dependent on *flhA*, an essential component of the flagella export apparatus (Ghelardi, 2002). Consistent with the notion that component of the flagella export apparatus can play a role in the export of virulence-associated proteins in some organisms. Konkel *et al.* (2004) reported mutations of five genes encoding three structural components of the flagella, the flagella basal body (*flgB* and *flgC*), hook (*flgE2*), and filament (*flaA* and *flaB*) genes, as well as in genes whose products are essential for flagella protein export (*flhB* and *fliI*). While mutations that affected filament assembly were found to be nonmotile and did not secrete Cia proteins. Also, some *Pseudomonas* species, including *P. syringae* and *P. putida*, are known to penetrate their host via the pili. Similar infection patterns can also be found in the closely related plant pathogenic *Xanthomonas* species, in which type IV pili play a role in the

entry of *X. axonopodis* pv. *hyacinthi* and *X. axonopodis* pv. *vesicatoria* to their host plant (van Doorn *et al.,* 1994). It has been postulated that in *Dichelobacter nodus* extracellular proteases primarily were exported from the cell by type IV pili system, which also acts as the sole type II secretion system in this bacterium (Han *et al.,* 2008). Our result was similar trend, the *flgK* and *pilD* mutants showed attenuated pathogenicity on the susceptible Spencer when compared with the wildtype KU-P-SW005. Moreover, *flgC* mutant and double mutant of *flgC* and *pilD* showed no symptom on Spencer. This result indicated that the *flgC, flgK,* and *pilD* genes are associated directly with pathogenicity and might penetrate their host via the pili (type II secretion) or type III apparatus. It was not previously reported in Xanthomonads. However, *flgC, flgK,* and *pilD* expression and secretion are important for future research. Furthermore, the bacterial population of the *flgK* and *pilD* mutants were assayed on Spencer leaves at different time intervals by comparing it with that of wildtype. It was revealed that the growth rate and population of the mutants are similar to that of the wildtype and complemented strains, indicating the *flgK* and *pilD* genes are not caused to reduce the bacterial growth of *X. axonopodis* pv. *glycines*. In contrast, *flgC* mutant and double mutant *flgC* and *pilD* bacterial population were significantly decreased day by day. Moreover, the number of pustule surrounded the infiltration zone were different significantly between wildtype and all mutants. The *flgK* and *pilD* mutants were reduced disease severity and number of pustule where *flgC* mutant and double mutant *flgC* and *pilD* could not induce disease symptoms, the infiltration lesion was necrosis seem to be HR. These suggest that the *flgC* mutant and double mutant *flgC* and *pilD* are nonpathogenic bacteria and *flgC* effect directly with pathogenecity and this was not previously reported in Xanthomonads and other plant pathogenic bacteria. It can be indicated that *flgC, flgK,* and *pilD* of *X. axonopodis* pv. *glycines* play important roles in adhesion, biofilm formation and cell to cell communication, swarming, swimming, and twitching motility, and pathogenesis of *X. axonopodis* pv. *glycines*.

CONCLUSION

From the experiment results and discussion of the study, the conclusion can be drawn as follow:

1. Two hundred and thirty four strains of *X. axonopodis* pv. *glycines* were classified based on their interactions on soybean cultivars into three races. Race1 induced HR (incompatibility) within 48 h and Race 2 induced diseases (compatibility) on all cultivars tested. Race 3 elicited HR on specific pustule-resistant cultivar (Williams82).

2. Ramdom and site-directed mutations in *avrXg1* of Race 3 wildtype KU-P-SW005 showed the same phenotype, increased virulence and caused disease on all four soybean cultivars including Williams82 (wildtype causes HR). Complemented strains are restored ability of HR induction on Williams82 and also all characterization tested same with the wildtype.

3. The *avrXg1* mutants, KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP gave the highest virulence ratings on Spencer and Williams82 whereas wildtype induced HR on Williams82.

4. The bacterial virulence factors; cellulase and pectate lyase were increased on the *avrXg1* mutants leading to a 20%-increase in the rating of bacterial pustule as compared to wildtype. There was no difference in α-amylase, protease, and endoglucanase production.

5. The *avrXg1* mutants did not effect HR induction on tobacco and tomato.

6. A 3.8-kb product of *avrBs3* like-gene was detected in Races 2 and 3 but not in Race 1 strains, suggest that the non-specific HR phenotype of Race 1 is not associated with and *avrBs3*-like gene but is associated with a different mechanism of HR.

7. The complete sequence of *avrXg1* was cloned from *X. axonopodis* pv. *glycines*. The coding region of *avrXg1* gene is 3.8-kb in length with an overall G+C content of

67%. The central region of the gene was characterized by 20.5 copies of tandemly-arranged 102-bp direct repeats. The C-terminal region of the gene was defined by three NLSs and an AAD domain, characteristic of other sequenced members of the *avrBs3*/*pthA* gene family.

8. An *avrXg1* encodes a predicted 1,266 amino acid protein with a molecular mass of 132.8 kDa and pI of 8.8. The amino acid sequence of the deduced protein from *avrXg1* shows differences in 13 amino acid residues in the N-terminal region at positions 23, 76, 81, 82, 85, 89, 91, 92, 94, 95, 130, 134, and 197 respectively as compared to the *avrBs3* homologs from AG1 and 8ra (YP_001965982.1 and YP_001966011.1 respectively).

9. The putative start codon of *avrXg1* is ATG, and a predicted promoter position, -35 and -10 boxes are located at position 171 and 190, -TTGCCA- and -TTGTGTACT- respectively upstream from the start codon. A signal peptide, MDPIRSRTPSPARELLPGPQPDRVQPTADR. is also predicted for AvrXg1.

10. An *avrXg1* is plasmid-borne recognized by Williams82 receptors. An *avrXg1* was no expressed in expression vectors.

11. Function of *avrXg1* was further verified by expression of the gene in Race 1, strain KU-P-34070 transformed with pBBR-AVR resulting in HR on Williams82 within 48 h and disease on SJ4, Spencer, and PI 520733 within 5 days after infiltration.

12. Function of *avrXg1* was further verified by expression of the gene in Race 2, strain KU-P-KPS06 transformed with pBBR-AVR resulting in gained virulence and bacterial population on Williams82 and Spencer.

13. We concluded the central repeat and AAD regions are associated with ability of AvrXg1 to confer both additive fitness and redundancy of virulence functions.

14. The complete DNA sequence obtained from *X. axonopodis* pv. *glycines* strain KU-P-SW005, referred to as *avrXg1*, was deposited as GenBank accession no. FJ439173.

15. The thirty five proteins of Wiliams82 upregulated by Race 3 wildtype classified into twelve functional categories, ten of which are related to growth and development, one of which are involved defense response, and the last categories is unknown proteins.

16. Three proteins of Williams82 were identified including catalase, LOX-4, and PAL were associated with disease and defense response.

17. Williams82 produced PR-2, PR-4, PR-6, PR-10, and LOX is as very rapid spatial signaling process in defense mechanism pathway against *X. axonopodis* pv. *glycines* Race 3 carrying AvrXg1. In contrast, PR-1, PAL, and POX were not expressed after inoculated with wildtype Race 3.

18. This is the first study to examine the interaction between soybean and *X. axonopodis* pv. *glycines* based on a gene-for-gene relationship. It provides insight into breeding strategies for pustule resistance.

19. Putative *flgC* and *flgK* mutants are swarming and swimming-minus. A double mutation of *pilD* and *flgC* is nonmotile bacteria. The *pilD* play a role of twitching motility in *X. axonopodis* pv. *glycines* is movement independent of flagella that occurs by extension, tethering, and retraction of type IV pili. Complemented strains are restored ability of motility both swimming and twitching movement.

20. The complete sequence of *flgC, flgK,* and *pilD* were cloned from *X. axonopodis* pv. *glycines* strain KU-P-SW005. The coding region of *flgC, flgK,* and *pilD* genes are 408-bp, 1.87-kb, and 864-bp in length with an overall G+C content of 64%, 65%, and 64% respectively that showed identity with *flgC, flgK,* and *pilD* homolog protein sequences (100%, 99%, and 99% at amino acid levels) of *X. axonopodis* pv. *citri* strain 306 (NP_642311.1, NP_642303.1, and NP_643551.1 respectively).

21. The *flgC, flgK,* and *pilD* encode a predicted 136, 625, and 287 amino acid proteins with a molecular mass of 34.2, 152.6, and 71.5 kDa and pI of 5.2, 4.8, and 5.0,

respectively. A signal peptide, MSNLPIFDVAGSALH, LEDGVGGRDHLCGGL, and MAFLDQHPGLGFPAA are also predicted for FlgC, FlgK, and PilD, respectively.

22. *X. axonopodis* pv. *glycines* wildtype KU-P-SW005 was cultured in LB, PD2, NBY, and NGB, they formed visible biofilms on glass tube surfaces with continuous agitation. In contrast, the *flgC*, *flgK, pilD* mutants and the *flgC* and *pilD* double mutations, were exhibited significantly reduced biofilms on similar surfaces.

23. *X. axonopodis* pv. *glycines* develop the highest biofilm on glass surface when grown in PD2 are significantly attached cells in other medium. *X. axonopodis* pv. *glycines* develop the better biofilm on glass surface than polystyrene and polypropylene surfaces.

24. The *flgK* and *pilD* mutants were reduced disease severity comparing wildtype. Interestingly, the *flgC* mutants and double mutations are nonpathogenic strains.

25. The *flgC, flgK,* and *pilD* mutants did not effect HR induction on tobacco species and tomato.

26. The *flgK* and *pilD* mutants did not affect population number of bacteria on Spencer, except the population of *flgC* mutant and double mutants on Spencer were significantly effected ($P \leq 0.05$), reaching population numbers lower than wildtype.

27. The results demonstrate functions for *flgC, flgK,* and *pilD* lead to develop monoclonal antibodies for *X. axonopodis* pv. *glycines* flagellum and pili.

28. Nucleotide sequence accession number, the complete DNA sequence of *flgC, flgK,* and *pilD* obtained from *X. axonopodis* pv. *glycines* strain KU-P-SW005, were deposited as GenBank accession no. FJ825660, FJ825661, and FJ825662 respectively.

29. This finding is the first study indicating a function for *flgC, flgK,* and *pilD* genes of *X. axonopodis* pv. *glycines* that are associated directly with swarming, swimming, and twitching motility and disease induction on soybean. This is the first reported *flgC, flgk,* and *pilD* genes among xanthomonads and thire functions.

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APPENDIX

Recipes of media in this study

1. Nutrient glucose agar (NGA) and nutrient broth (NGB)*

	per L
Beef extract	3.0 g
Bacto peptone	5.0 g
Glucose	2.5 g
Agar	15.0 g

Nutrient agar or nutrient broth may be purchased in dehydrated form Difco.

* Do not add agar if nutrient broth is desired.

2. Nutrient-broth yeast extract agar (NBY) and nutrient-broth yeast extract broth*

	per L
Nutrient broth	8.0 g
Yeast extract	2.0 g
K_2HPO_4	2.0 g
KH_2PO_4	0.5 g
Glucose	2.5 g
Agar	15.0 g

After autoclaving, add 1.0 ml of a sterile solution of 1M $MgSO_4$. $7H_2O_2$

*Do not add agar if nutrient broth is desired.

3. Luria bertani (LB) agar and Luria bertani broth *

	per L
Bacto typtone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Agar	15.0 g

*****Do not add agar if nutrient broth is desired.

CIRRICULUM VITAE

NAME: : Mr. Dusit Athinuwat

DATE OF BIRTH : 26 April 1983

PLACE OF BIRTH : Chachoengsao, THAILAND

ADDRESS:

12 Thepkunakron 2 Rd., Mueng, Chachoengsao, THAILAND 24000, Phone : (66) 8-1578-8377

INSTITUTION ATTENDED:

1. Kasetsart University, Khampangsang, Thailand, 2001-2004, Bachelor Degree of Science (Plant Pathology)
2. Kasetsart University, Bangkok, Thailand, 2004-2009, Doctor of Philosophy (Plant Pathology)

AWARDS AND SCHOLARSHIPS:

1. The Royal Golden Jubilee Ph.D. Scholarship, from the Thailand Research Fund, 2004-2009.
2. The first place award of oral presentation of The 45th Kasetsart University Annual Conference 2007.
3. The first honorable mention award of article and oral presentation of The 46th Kasetsart University Annual Conference 2008.
4. The first honorable mention award of article and poster presentation of The 46th Kasetsart University Annual Conference 2008.
5. The first honorable award of photo on insect's majority of The 8th National Plant Protection Conference 2008.