Xanthomonas axonopodis pv. *glycines* Soybean Cultivar Virulence Specificity Is Determined by *avrBs3* Homolog *avrXg1*

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ABSTRACT

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Three races of *Xanthomonas axonopodis* pv. *glycines* were identified on pustule disease resistant and susceptible soybean cultivars based on virulence phenotype. For race 3, an *avrBs3* homolog, *avrXg1* was identified that conferred resistance expressed as a hypersensitive response on resistant cultivar Williams 82. Mutations in two predicted functional

domains of *avrXg1* resulted in gained virulence on Williams 82 and an increase in bacterial population number on susceptible cultivars. Expression of *avrXg1* in race 1, that is predicted to confer a nonspecific HR, led to virulence on susceptible cultivars Spencer and PI 520733. Expression of *avrXg1* in race 2, that is predicted of carrying *avrBs3*-like genes, resulted in gained virulence and fitness of pathogen on both resistant and susceptible cultivars. The results demonstrate multifunctions for *avrXg1* dependent on pathogen and plant genetic backgrounds.

Additional keywords: avirulence gene, bacterial pustule, soybean.

Xanthomonas axonopodis pv. *glycines*, causes a serious disease, bacterial pustule, on soybean that results in reduced yield and crop quality (31). 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 composing a type III secretion system (TTSS) that is essential for pathogenesis and for induction of a hypersensitive reaction (HR) on some plant species (30). The HrpG effector was shown to be essential for induction of a nonspecific HR on different nonhost plants (27). Mutation of HrpG demonstrated that it plays critical roles in HR on tobacco (26). 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 (20). Together, these studies demonstrated that *X. axonopodis* pv. *glycines* 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 reduced pathogen growth and plant cell death (HR). The interaction between Avr proteins and *R*-gene receptors was first described in the well-known gene-for-gene hypothesis (10). It has also been shown that *avr* genes may contribute to pathogen aggressiveness (4,24) and, in some cases, inhibit development of nonspecific HR (2,8,11,33,47).

In contrast to the dominant Avr-R incompatible HR, a compatible reaction (where Avr or R proteins 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 (41). Avirulence and other effector proteins are transported via the TTSS across the bacterial membrane and into plant cells (3,5). 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* (47).

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 (34). Some *Xanthomonas* spp. contain multiple homologs of *avrBs3/pthA*. For example, in *X. axonopodis* pv. *malvacearum*, multiple *avrBs3/pthA* genes contribute additively to the water soaking on cotton (39,52). *X. axonopodis* pv. *vesicatoria* carries *avrBs3* and *avrBs4* that affect HR and disease on different pepper cultivars (7). *X. axonopodis* pv. *citri* (Hasse) (46) also contains at least three *avrBs3/pthA* homologs (21) and all strains of *X. oryzae* pv. *oryzae* contain more than 15 *avrBs3/pthA* homologs, which are major pathogenicity genes required for full virulence in rice (51). 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 (25,40).

R gene clusters within soybean associated with pustule disease resistance have also been identified (22). The resistance gene *rx* for bacterial pustule was originally identified in resistance cultivar CNS and subsequently transferred to other commercial cultivars, including Williams 82 (22); cultivars carrying the *Rxp* allele are susceptible (44).

In this study, we describe races of *X. axonopodis* pv. *glycines* from Thailand that express differential virulence on soybean cultivars that is determined by the presence of an *avr* gene. A new member of the *avrBs3/pthA* family is described that, when intro-

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duced to race 1 and 2 of *X. axonopodis* pv. *glycines*, confers a virulence phenotype and additive fitness of pathogen on normally resistant and susceptible soybean cultivars.

MATERIALS AND METHODS

Bacterial strains, plasmids, and recombinant techniques.

Strains of *X. axonopodis* pv. *glycines* and *Escherichia coli* and plasmids used in this study are listed in Table 1. *X. axonopodis* pv. *glycines* strains were initially isolated and characterized from infected soybean plants collected in various Thai provinces by Saiseangthong (35). All strains were shown to cause pustule disease on soybean cv. SJ4. They were cultured in nutrient glu-

cose agar (NGA) or nutrient yeast extract (NBY) agar (49) at 28°C. *E. coli*, used as the cloning host, was cultured in Luria-Bertani (LB) agar at 37°C. Antibiotics were added to media at the following concentrations: ampicillin, 100 µg/ml; kanamycin, 15 or 50 µg/ml; and gentamicin, 25 or 40 µg/ml. All DNA manipulations, including the DNA isolation, plasmid extractions, restriction digests, ligations, and gel electrophoresis, were performed as described previously (36).

Race-cultivar specificity. Thirty-four strains of *X. axonopodis* pv. *glycines* were evaluated for pathogenicity on 15-day-old soybean cultivars, including Williams 82 (resistant), Spencer (susceptible), and PI 520733 (susceptible) (13,17,22). A leaf infiltration technique under greenhouse conditions was used to assess

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid ^y	Relevant characteristic ^z	Reference or source (Thai province)
<i>Escherichia coli</i> TOP10	lacZΔM15, endA1, recA1, hsdR, mcrA	Invitrogen
<i>E. coli</i> JM109	recA1, endA1, gyrA96, thi-1, hsdR17(rK ⁻ mk ⁺), e14 ⁻ (mcrA ⁻), supE44, relA1, Δ(lac-proAB)/F' [traD36, proAB ⁺ , lac I ^q , lacZΔM15]	Promega
<i>E. coli</i> TOP10 (pUC19-AVR)	<i>E. coli</i> TOP10 carrying pUC19-AVR	This study
<i>E. coli</i> TOP10 (pBBR-AVR)	<i>E. coli</i> TOP10 carrying pBBR-AVR	This study
<i>E. coli</i> DH5α	F' 80dlacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rK ⁻ , mk ⁺) phoA supE44 λ ⁻ thi-1 gyrA96 relA1/F' proAB+ lacIqZΔM15 zff::Tn5, Km ^r	Invitrogen
<i>Xanthomonas axonopodis</i> pv. <i>glycines</i>		
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 (43)
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 (43)
KU-P-34071	Race 1	Loei
KU-P-34072	Race 1	Tusanasarit (43)
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 (43)
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
ST015	Race 2	Tusanasarit (43)
KU-P-SW008	Race 3	Nakhon Rachasima
KU-P-SW005	Race 3	Nakhon Rachasima
KU-P-SW005 <i>avrXgI</i> ::Tn5AAD	Random Tn5 insertion in <i>avrXgI</i> acidic activation domain	This study
<i>avrXgI</i> ::Tn5AAD (pBBR-AVR)	Complemented KU-P-SW005 <i>avrXgI</i> ::Tn5	This study
KU-P-SW005 <i>avrXgI</i> ::Tn5REP	Targeted Tn5 insertion in <i>avrXgI</i> central repeat region	This study
<i>avrXgI</i> ::Tn5REP (pBBR-AVR)	Complemented KU-P-SW005 <i>avrXgI</i> ::Tn5REP	This study
KU-P-34070	Race 1	Sukhothai
KU-P-34070 (pBBR-AVR)	Race 1 transformed with <i>avrXgI</i>	This study
KU-P-KPS06	Race 2	Nakhon Prathom
KU-P-KPS06 (pBBR-AVR)	Race 2 transformed with <i>avrXgI</i>	This study
Plasmids		
pBBR1MCS-5	Broad host range cloning vector, lacZ, Gm ^r	Kovach et al. (28)
pUC19	ColE1; lacZ Amp ^r	Promega
pGEM-T easy	Polymerase chain reaction cloning vector, lacZ, Amp ^r	Promega
pUC19-AVR	5-kb <i>avrXgI</i> fragment from KU-P-SW005 carrying Tn5 insertion cloned in pUC19, Km ^r Amp ^r	This study
pBBR-AVR	3.8-kb Sequence of <i>avrXgI</i> in the pBBR1MCS-5, Km ^r Gm ^r	This study

^y ADD, acidic activation domain; REP, central repeat region.

^z Km^r, Amp^r, and Gm^r indicate resistance to kanamycin, ampicillin, and gentamicin, respectively.

virulence on soybean leaves. Infiltrations were done in a completely randomized design. Bacterial suspensions (50 µl, optical density at 600 nm [OD₆₀₀] = 0.2, ≈10⁸ CFU/ml) were 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) 48 h post inoculation. The experiment was repeated three times.

Transposon mutagenesis. Random mutagenesis of wild-type race 3 strain KU-P-SW005 was performed as described previously by Kaewnum and associates (20). Briefly, 1 µl of EZ-Tn5 <KAN-2>Tnp Transposome (Epicentre, WI) was added to 50 µl of electrocompetent cells and 0.5 µl of TypeOne restriction inhibitor (Epicentre); 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) 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. Resistant clones were screened for ability to cause HR and disease on the soybean cultivars as described above and were subsequently verified for Tn5 insertion by polymerase chain reaction (PCR).

Total DNA of Tn5 mutant KU-P-SW005 *avrXg1::Tn5AAD*, which gained virulence function on soybean cv. Williams 82, was extracted, partially digested with *EcoRI* and *SacI* (no restriction site in transposon), cloned into pUC19 (Promega Corp., Madison, WI) and transformed by heat shock into *E. coli* DH5α (Invitrogen, CA). Clones were selected on LB agar amended with kanamycin and ampicillin and verified by PCR amplification of an 800-bp product using the EZ-Tn5 derived primers KAN-1-FP and KAN-1-RP (Table 2). Sequences of clones carrying EZ-Tn5 transposon were identified by PCR amplification with M13F and M13R universal primers.

Genomic sequences flanking the EZ-Tn5 transposon insertions in KU-P-SW005 *avrXg1::Tn5AAD* were identified by PCR amplification of the disrupted gene with primers KAN-2-FP and KAN-2-RP (Epicentre). DNA sequencing was done at the Cornell Life Sciences Core Laboratories Center.

Site-directed mutagenesis. A targeted mutation in the *avrBs3* homolog in KU-P-SW005 was accomplished using the EZ-Tn5 <KAN-2> Insertion Kit (Epicentre) according to the supplier's directions. First, a 3.8-kb fragment was amplified from KU-P-SW005 using primers AVR-FP and AVR-RP that were based on the sequence of the *avrBs3* homolog (AY780632.1) previously identified in *X. axonopodis* pv. *glycines* strain AG1 (25).

The 3.8-kb fragment containing the coding region of the *avrBs3* homolog was cloned into pGEM-T easy vector (Promega Corp.) and digested with *EcoRI* (no restriction site in *avrBs3* homolog gene), and the target fragment was ligated into pUC19. Subsequently, a Tn5 targeted insertion in the gene was made in

vitro following the supplier's directions. Briefly, 1 µl of pUC19 DNA containing the *avrBs3* homolog was mixed with 0.5 µl of EZ-Tn5 <KAN-2> transposon, 1 µl of EZ-Tn5 10× reaction buffer, 1 µl of EZ-Tn5 transposase, and 6.5 µl of sterile water; the mixture was transformed by heat shock into *E. coli* TOP10 (Invitrogen). Clones were selected on LB agar amended with kanamycin and ampicillin. Selection of plasmid pUC19-AVR carrying a single Tn5 insertion in the middle of the target gene was shown by digestion with *EcoRI* and separation by gel electrophoresis. Confirmation of the pUC19-AVR construct was by PCR with primers AVR-FP and AVR-RP. Sequencing of pUC19-AVR was done at the Cornell Life Sciences Core Laboratories Center.

Transformation into KU-P-SW005 was performed by electroporation as described above. Briefly, 2 µl of pUC19-AVR and 0.5 µl of TypeOne restriction inhibitor were added 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 ability to cause HR and disease on the soybean cultivars as described above and were subsequently verified for Tn5 insertion by PCR.

Presence of the *avrBs3* homolog in *X. axonopodis* pv. *glycines* races. All 34 strains were evaluated for the presence of the *avrBs3* 3.8-kb region amplified by primers AVR-FP and AVR-RP (Table 2). PCR products were separated by gel electrophoresis. The presence of the PCR product was correlated with strain-cultivar pathogenicity reactions.

Sequencing and evaluation. The 3.8-kb region amplified from KU-P-SW005 was cloned into the pGEM-T easy vector. Primer walking and PCR were employed for amplification and sequencing of the entire gene. All primer sequences are listed in Table 2. The PCR products were cloned into pGEM-T easy vector. DNA sequencing was done as described above. Analysis of sequences was conducted using BLAST and the DNASTAR Lasergene software package (DNASTAR, Inc., WI). 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.

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

Transformation of mutants and race 1 with *avrXg1*. To complement KU-P-SW005 *avrXg1::Tn5AAD* and KU-P-SW005 *avrXg1::Tn5REP*, the 3.8-kb sequence comprising *avrXg1* in

TABLE 2. Polymerase chain reaction primers

Primer	Sequence	Description
AVR-FP	5' ATG GAT CCC ATT CGT TCG 3'	Amplification of 3.8-kb of <i>avrBs3</i> gene family
AVR-RP	5' TCA CTG AGG CAA TAG CTC CA 3'	...
AVR-1-FP	5' AAT ATT GGC GGC AAG CAG GC 3'	Amplification of 863-bp fragment of <i>avrXg1</i>
AVR-1-RP	5' CGC CTG CTT GCC ACC AAT ATT 3'	...
AVR-2-FP	5' AAT ATT GGT GGC AAG CAG GCG 3'	Amplification of 1.0-kb fragment of <i>avrXg1</i>
AVR-2-RP	5' TTG CCA CCA ATA TTG CTG GCG AT 3'	...
AVR-3-FP	5' AGC CAC TTG TAG AAC GTG 3'	Amplification of 4.1-kb <i>avrBs3</i> homolog and flanking sequences
AVR-3-RP	5' GCT AAC TCG CTG TCA GTA 3'	...
KAN-1-FP	5' AAG CC GAT GCG CCA GAG TT 3'	Amplification of kanamycin gene (800 bp)
KAN-1-RP	5' CGC CGT CCC GTC AAG TCA G 3'	...
KAN-2-FP	5' ACC TAC AAC AAA GCT CTC ATC AAC C 3'	For sequencing
KAN-2-RP	5' GCA ATG TAA CAT CAG AGA TTT TGA G 3'	...
M13F	5' TGT AAA ACG ACG GCC AGT 3'	Universal primers
M13R	5' AAC AGC TAT GAC CAT G 3'	...

pGEM-T easy vector was digested with *EcoRI* and ligated into the multiple cloning site vector pBBR1MCS-5 (pBBR-AVR). Plasmid pBBR-AVR was introduced into mutants KU-P-SW005 *avrXgI*::Tn5AAD, KU-P-SW005 *avrXgI*::Tn5REP, wild-type race 1 KU-P-34070, and wild-type race 2 KU-P-KPS06 by electroporation according to the method described above. Selections were made on NBY, with appropriate antibiotics. Clones were verified by PCR and evaluated for their ability to cause HR on Williams 82 and disease on Spencer and PI 520733.

Virulence and bacterial growth on soybean. Virulence of KU-P-SW005, KU-P-SW005 *avrXgI*::Tn5AAD, KU-P-SW005 *avrXgI*::Tn5REP, *avrXgI*::Tn5AAD (pBBR-AVR), *avrXgI*::Tn5REP (pBBR-AVR), KU-P-34070, KU-P-34070 (pBBR-AVR), KU-P-KPS06, and KU-P-KPS06 (pBBR-AVR) were assessed on soybean cvs. Spencer and Williams 82, using previously described quantitative methods (19). Briefly, aqueous cell suspensions ($OD_{600} = 0.2$, $\approx 10^8$ CFU/ml of each) were sprayed on leaves of plants maintained in the greenhouse. At 7 to 10 days after inoculation, disease severity was assessed using a scoring method (32) based on the number of sections having at least one pustule observed in nine 1-cm² 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 of sterile water and triturated, and dilutions were plated on NGA. Leaf surface inoculation and infiltration experiments were repeated three times. The results were statistically analyzed using SAS (version 9.1.2; SAS Institute Inc., Cary, NC).

Genome location of *avrXgI*. Total genomic and plasmid DNA of KU-P-SW005, KU-P-SW005 *avrXgI*::Tn5AAD, KU-P-SW005 *avrXgI*::Tn5REP, and KU-P-34070 were extracted by UltraClean Microbial DNA Isolation Kit Components and UltraClean 6 Minute Mini Plasmid Prep Kit (MO BIO, CA), respectively. The plasmid DNAs were size fractionated on a low-melting agarose gel. Purified fragments of 15 to 30 kb were used as the PCR template. KAN-1-FP and KAN-1-RP primers were used to amplify an 800-bp product, including the kanamycin

resistance marker from the total genomic and plasmid DNA. PCR products were separated by gel electrophoresis.

The genome location of *avrXgI* was analyzed further by PCR amplification of regions flanking the gene (274 bp upstream and 70 bp downstream) with primers AVR-3-FP and AVR-3-RP (Table 2). PCR products were cloned in pGEM-T easy vector. DNA sequence was identified by PCR amplification of the *avrXgI* fragment with primers AVR-3-FP and AVR-3-RP and with M13F and M13R universal primers.

RESULTS

Race-cultivar specificity. Three races of *X. axonopodis* pv. *glycines* were identified from strains collected in Thailand based on their ability to cause disease or an HR on three soybean cultivars. Race 1 (23 strains) induced HR within 48 h and Race 2 (7 strains) caused disease on all three cultivars. Race 3 (four strains) elicited HR on the resistant cultivar (Williams 82) and caused pustule disease on pustule-susceptible cvs. Spencer and PI 520733 (Table 1; Fig. 1). 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 (not shown).

Screening and characterization of mutants. Of 3,336 random Tn5 mutants generated from strain KU-P-SW005, 1, designated KU-P-SW005 *avrXgI*::Tn5AAD (Tn5 insertion in putative AAD domain of *AvrXgI*), caused disease on all three soybean cultivars, including Williams 82 (wild-type causes HR). Therefore, it was hypothesized that an avirulence factor was knocked out, making the mutant virulent on Williams 82. The region surrounding the Tn5 insertion in KU-P-SW005 *avrXgI*::Tn5AAD was sequenced and showed identity (100% at nucleotide level) within a deduced ADD domain in the C-terminal region of an *avrBs3* gene homolog in *X. axonopodis* pv. *glycines* strains AG1 and 8ra (AY780631.1 and AY780632.1, respectively) (25), *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

Fig. 1. Comparison of compatible and incompatible reactions on soybean cvs. **A**, Williams 82; **B**, Spencer; and **C**, PI 520733 following leaf infiltration with KU-P-SW005 (1), KU-P-SW005 *avrXgI*::Tn5AAD (2), KU-P-SW005 *avrXgI*::Tn5REP (3), *Escherichia coli* TOP10 (pBBR-AVR) (4), race 1 KU-P-34070 (5), complemented mutant *avrXgI*::Tn5AAD (pBBR-AVR) (6), and *avrXgI*::Tn5REP (pBBR-AVR) (7), and KU-P-34070 (pBBR-AVR) (8). Bacteria were infiltrated into soybean leaves as described in text. Symptoms were recorded 7 days after infiltration.

strongly suggested that the gene mutated was a member of the *avrBs3* gene family that hereafter is referred to as *avrXg1*.

To confirm that the virulence phenotype observed on Williams 82 resulted from disruption of *avrXg1*, a targeted mutation was made at the fourth repeat region within the central portion of the gene. The resulting mutant, KU-P-SW005 *avrXg1*::Tn5REP, displayed the same phenotypes as KU-P-SW005 *avrXg1*::Tn5AAD (Fig. 1).

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 (not shown). These results suggest that the nonspecific 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 demonstrates the presence of one or more genes in the *avrBs3/pthA* family but not the presence of *avrXg1* specifically.

Analyses of *avrXg1*. The complete sequence of *avrXg1* was compared with the *avrBs3* homolog in *X. axonopodis* pv. *glycines* strain AG1 (AY780631.1) (25). 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 (Fig. 2), characteristic of other sequenced members of the *avrBs3/pthA* gene family.

Alignment analysis of *avrXg1* by MegAlign program (DNASTar, Inc.) revealed that 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* showed 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 compared with the *avrBs3* homologs from AG1 and 8ra (YP_001965982.1 and YP_001966011.1, respectively) (25). The putative start codon of *avrXg1* is ATG, and predicted promoter positions, -35 and -10 boxes, are located at position 171 and 190, -TTGCCA- and -TTGTGTACT-, respectively, upstream from the start codon. A signal peptide, MDPIR-SRTSPARELLPGPQPDRTVQPTADR, is also predicted for *AvrXg1*.

Complementation and virulence assays on soybean. Complementation of KU-P-SW005 *avrXg1*::Tn5AAD and KU-P-SW005 *avrXg1*::Tn5REP were accomplished by transformation with pBBR-AVR. This resulted in regained ability to induce a strong HR on Williams 82 and pustule disease on Spencer and PI 520733 within 48 h and 5 days after infiltration, respectively. *E. coli* TOP10 harboring pBBR-AVR did not cause HR or disease on Williams 82, Spencer, or PI 520733. 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 Williams 82 and Spencer did not differ (Table 3). However, virulence of strains refers to disease severity rather than lesions size.

Fig. 2. 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 sequences of the repeats are in bold with variances indicated. The C-terminal region (amino acids 1,123–1,266) 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.

TABLE 3. Lesion size and disease severity caused by *X. axonopodis* pv. *glycines* strains, *avrXg1* mutants, and complemented mutants on cvs. Williams 82 and Spencer^x

Strain ^y	Lesion mean diameter (mm)		Disease severity (%) ^z	
	Williams 82	Spencer	Williams 82	Spencer
KU-P-SW005	HR	0.65 a	HR	29.62 b
KU-P-SW005 <i>avrXg1</i> ::Tn5AAD	0.65 a	0.64 a	11.11 b	51.02 a
<i>avrXg1</i> ::Tn5AAD (pBBR-AVR)	HR	0.64 a	HR	26.33 b
KU-P-SW005 <i>avrXg1</i> ::Tn5REP	0.66 a	0.65 a	12.34 b	49.79 a
<i>avrXg1</i> ::Tn5REP (pBBR-AVR)	HR	0.63 a	HR	25.51 b
KU-P-34070	HR	HR	HR	HR
KU-P-34070 (pBBR-AVR)	HR	0.52 b	HR	23.86 b
KU-P-KPS06	0.65 a	0.66 a	53.50 a	54.73 a
KU-P-KPS06 (pBBR-AVR)	0.67 a	0.67 a	57.61 a	58.01 a

^x Numbers followed by different letters show significance at $P \leq 0.05$. HR = hypersensitive response.
^y KU-P-SW005, race 3; KU-P-34070, race 1; KU-P-KPS06, race 2; and derivatives as described in text. ADD, acidic activation domain; REP, central repeat region.
^z 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.

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 Williams 82 within 48 h and disease on Spencer and PI 520733 within 5 days after infiltration. The surrounding yellow halo caused by transformed KU-P-34070 on the susceptible cultivars, however, appeared smaller than for wild-type race 3 KU-P-SW005 (Table 3). These results suggest that *avrXg1* in the race 3 genetic background blocks expression of the nonspecific HR on susceptible cultivars.

Wild-type race 2 strain KU-P-KPS06 was also transformed with pBBR-AVR, resulting in more rapid and increased disease on Williams 82 and Spencer (i.e., disease observed in 3 days compared with 5 days for wild-type). Lesion expansion of KU-P-SW005, KU-P-KPS06, and KU-P-KPS06 (pBBR-AVR) on Williams 82 and Spencer did not differ (Table 3). These results suggest that *avrXg1* can also induce gain of virulence on soybean.

Mutants KU-P-SW005 *avrXg1*::Tn5AAD, and KU-P-SW005 *avrXg1*::Tn5REP; wild-type race 2 KU-P-KPS06; and transformed race 2 KU-P-KPS06 (pBBR-AVR) gave the highest virulence ratings on Spencer and Williams 82 (Table 3). In contrast, wild-type race 3 KU-P-SW005, complemented strains *avrXg1*::Tn5AAD (pBBR-AVR) and *avrXg1*::Tn5REP (pBBR-AVR), and transformed race 1 KU-P-34070 (pBBR-AVR) gave disease severities on Spencer that were lower than both mutants and wild-type race 2 (Table 3).

Growth of mutants KU-P-SW005 *avrXg1*::Tn5AAD, KU-P-SW005 *avrXg1*::Tn5REP, and KU-P-KPS06 (pBBR-AVR) in Spencer and Williams 82 tissue were significantly increased ($P \leq 0.05$), reaching population numbers higher than wild-type strains (Fig. 3). Furthermore, KU-P-34070 (pBBR-AVR) grew in leaves and induced the disease on susceptible cultivars, whereas wild-type KU-P-34070 caused HR and was not detected on Spencer. These results suggest that the central repeat and AAD regions are associated with the 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 in the HR-expressing tissues of Williams 82 leaves 48 h after.

Location of *avrXg1* in KU-P-SW005 genome. It was determined that KU-P-SW005 carries 26-, 15-, and 1.3-kb native plasmids, whereas race 1 strain KU-P-34070 does not have the 26- and 15-kb plasmids (not shown). The sequence of three native plasmids pAG1, pXAG81, and pXAG82 of sizes 26, 15, 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) (25).

For 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* is located on the 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 (25), again indicating that *avrXg1* is plasmid-borne.

DISCUSSION

Recent research has significantly advanced our understanding of the heterogeneity of *X. axonopodis* pv. *glycines* and how it interacts with different plant species (19,27). Race structure of *X. axonopodis* pv. *glycines* from the United States was initially determined based on reactions on five soybean cultivars with

Williams 82 determined to be resistant to all five races (17). Recently, Park et al. (29) 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 digests of total genomic and plasmid DNA, suggesting the presence of multiple copies of *avrBs3*-like genes. However, pathogenicity of representative strains from each group 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* (25). It was shown that *X. axonopodis* pv. *glycines* strains from pustule-infected soybean leaves 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 multifunctional member of the *avrBs3/pthA* family, *avrXg1*, and provide evidence that it also is carried on plasmid DNA.

The *avrBs3/pthA* gene family was originally identified in *X. axonopodis* pv. *vesicatoria* (7) and is 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; and a C-terminal region consisting of imperfect heptad leucine zipper (LZ) repeats, NLSs, and AAD (34). 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 (23,48). As indicated, the number of repeats in *avrBs3* genes varies considerably between bacterial species (6,7,9,16,42,50). In addition, *AvrBs3* requires a functional NLS for transport into the nucleus of the plant cell (23). Some avirulence effectors such as *AvrXa7* from *X. oryzae* pv. *oryzae* also require an AAD domain for virulence (48). 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, and an AAD in the C-terminal region (7,12,45,53).

It has been demonstrated that *avr* genes may have diverse functions that contribute to additive fitness and increased growth of the pathogen, sometimes resulting in increased disease on susceptible host plants (8,47). Inactivation of *avrBs3/pthA* genes by mutation generally results in loss of avirulence but also may lead to reduced virulence and growth rate in the host plant (47). 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 pepper cultivars 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 (45). Mutation in the *hssB3.0* gene in *X. axonopodis* pv. *citri* resulted in increased aggressiveness on *Citrus grandis* cv. Otachibana (38). Also, *pthA* isolated from *X. axonopodis* pv. *citri* confers an enhanced ability to induce cankers in the related *X. axonopodis* pv. *citrumelo* (47). 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 was involved as a virulence factor in the production of water-soaking symptoms and the release of greater numbers of bacteria to the leaf surface (47). 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 (45). Our results indicate that the central repeat regions of *avrXg1* are important for recognition by the Williams 82 receptor and that the C-terminal ADD domain is also required, presumably for virulence (14,15). In addition to virulence, we show that *avrXg1* affects increased establishment of bacterial population in soybean leaves.

avrXg1 was also expressed in *X. axonopodis* pv. *glycines* race 1 and race 2 strains. Race 1 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 nonspecific, 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

Fig. 3. Time course of bacterial growth in leaves of soybean **A**, Spencer and **B**, Williams 82. Leaves were sampled 1, 2, 3, and 4 days after infiltration. For Spencer, KU-P-34070 caused a hypersensitive reaction (HR) and viable cells were undetectable in leaves within 48 h. For Williams 82, 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 represent means from three repetitions, and vertical bars represent standard errors.

formation on susceptible cvs. Spencer and PI 520733 and HR on Williams 82, thereby suppressing nonspecific HR in the susceptible but not in resistant cultivars (1,11). Further research on interactions of the AvrXg1 in these cultivars is needed to clarify further this interesting interaction demonstrating the multiple functions that Avr proteins 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. Additional research is required to determine the specific protein structure of *avrBs3* open reading frames in race 2 and how they compare with the protein encoded by *avrXg1*. Expression of *avrXg1* in race 2 led to increased virulence and fitness, another phenotype attributed to *avr* genes in other bacteria (4,24,47).

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 (8). Ritter and Dangl (33) reported that *avrRpt2* of *P. syringae* may promote pathogenesis and interfere with induction of *avrRpm1/Rpm1*-mediated resistance. Chen et al. (8) provided a hypothesis for *avrRpt2* function promotion of virulence via two different mechanisms. The first states that *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 *xrp*, *avrXg1* functions to promote virulence and bacterial populations of *X. axonopodis* pv. *glycines* on 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 (18,33,37).

avrXg1 was discovered and its functional domains were successfully identified based on its ability to confer avirulence on cv. Williams 82 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 development of useful methods for pustule disease control.

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