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Avirulence Gene Diversity of *Xanthomonas axonopodis* pv. *glycines* Isolated in Korea

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The hybridization patterns with the *avrBs3* gene that is known to determine the recognition of host specificity were used to study the diversity of *Xanthomonas axonopodis* pv. *glycines* causing bacterial leaf pustule in soybean. A total of 155 strains were isolated from diverse tissues of soybean cultivars collected in Korea and were classified into six different type strains of OcsF, SL1017, SL1018, SL1045, SL1157, and SL2098 according to the patterns of *avrBs3*-homologous bands. When these type strains were inoculated on various cultivars, most of the Korean strains mildly induced disease symptoms on the resistant CNS1 cultivars. Unlike other type strains, strain SL2098, which appeared not to contain any *avrBs3* homolog, induced only a few pustules on even highly susceptible cultivars. When a plasmid carrying the 3.7-kb *avrBs3*-homologous gene from strain SL1045 was introduced into SL2098, the transformant could not recover the pathogenicity in susceptible host plants. However, when *avrBs3*-homologous genes of strain SL1018 were mutated by transposon mutagenesis, one of the mutants in which a 5.2-kb chromosomal band homologous to *avrBs3* was disrupted could not induce the hypersensitive response on resistant cultivars such as William82 or CNS2. Our results suggest that the *avrBs3* homologs may play important roles in the pathogenicity of *Xanthomonas axonopodis* pv. *glycines* and the recognition of soybean cultivars.

Keywords: *Xanthomonas axonopodis* pv. *glycines*, avirulence gene

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A Gram-negative bacterial phytopathogen, *Xanthomonas axonopodis* pv. *glycines*, previously known as *X. campestris* pv. *glycines*, causes bacterial leaf pustule (BP) disease in soybean. Early symptoms are characterized by small, yellow to brown lesions with a raised pustule in the center. The lesion progressively merges to form large necrotic areas, which cause premature defoliation and subsequently a huge loss of grain yield.

The mechanism used by phytopathogenic bacteria to invade plants is very complex and poorly understood. Physiological studies to identify factors involved in diseases caused by *X. campestris* pathovars yielded largely inconclusive or negative results. It was reported that proteases, cellulases, and pectinases do not play a direct role in the disease caused by *X. campestris* pv. *campestris* [8]. In some cases, plant hormones such as auxin play a major role in the development of the disease. *X. axonopodis* pv. *glycines* is capable of producing indole-3-acetic acid from tryptophan; however, the hormone is produced *in vitro* by both pathogenic and nonpathogenic strains [19].

Most plants have a basal defense system that is triggered by functionally indispensable and structurally conserved microbial structures. Despite the presence of the basal defense system, phytopathogens are capable of multiplying in certain host plants to which they are adapted. Bacterial pathogenicity depends on a specialized type III secretion system (TTSS), which secretes some helper and accessory proteins to support the injection of the effector proteins, the major substrate class of the TTS system, into the host cell. In some cases, the effectors that suppress the basal defense trigger a second superimposed defense layer. The genes that encode these effectors have been functionally defined as avirulence (*avr*) genes. Recognition of microbial *avr* gene products is dependent on the simultaneous expression of corresponding R(resistance) gene products in the plant host and is often associated with a hypersensitive response (HR) [9].

Pathogen *avr* genes were first identified on the basis of their ability to direct specific recognition, leading to disease resistance in gene-for-gene plant microbe interactions Later, *avr* genes were shown to have another function to control components of pathogen fitness, including aggressiveness (the amount of disease) and symptom expression in the host pathogen interaction. Recent advances reveal that phytopathogenic bacteria use type III effectors, including *avr* gene product, toxins, and other factors, to inhibit host defences [1, 10, 11, 23, 32]. For example, the *Xanthomonas campestris* pv. *vesicatoria avrBs2* gene, which confers resistance in the *Bs2*-gene-containing pepper, also contributes to aggressiveness in pepper [17]. Genes homologous to *avrBs2* found in other xanthomonads also affect aggressiveness; mutations in the *avrBs2* homologs reduced the pathogen's ability to multiply in its respective host plant [17].

The infection of susceptible plants leads to an AvrBs3-dependent hypertrophy of the mesophyll tissue [22]. Delivery of *avrBs3* by *X. campestris* pv. *vesicatoria* into susceptible plants induces hypertrophy. Citrus canker is due to cell proliferation and hypertrophy caused by the *X. citri pthA* gene [29].

Plasmid bearing *avr* genes was previously cloned from strain XcmH of the cotton pathogen *X. campestris* pv. *malvacearum* [7]. All six *avr* genes have now been localized all six *avr* genes on the cloned fragments by subcloning and Tn5 insertional mutagenesis. None of these *avr* genes appeared to exclusively exhibit gene-for-gene patterns of interactions with cotton *R* genes, and *avrBs4* was demonstrated to confer *avr* gene-for-gene lines carrying either of two different resistance loci, *B1* or *B4*. Furthermore, the *B1* locus appeared to confer *R* gene to *avr* genes resistance to cotton against isogenic *X. campestris* pv. *malvacearum* strains carrying any one to three *avr* genes; *avrBs4*, *avrB6*, or *avrB102*. Restriction enzyme, Southern blot hybridization, and DNA sequence analyses showed that the XcmH *avr* genes are all highly similar to each other, to *avrBs3* and *avrBsp* from the pepper pathogen *X. campestris* pv. *vesicatoria*, and to the host-specific virulence genes that are different primarily in the multiplicity of portions of the genes, repeating 10 to 23 times in the members of this gene family.

In the *X. axonopodis* pv. *glycines* and soybean interaction, field resistance to BP has only been identified in CNS that was found to be nearly immune to the disease. Hartwig and Lehman [12] determined that the resistance of soybean to bacterial pustule pathogen in CNS is controlled by a single recessive gene, *rxp*. Hwang and Lim [16] reported that soybean cultivars with resistance derived from CNS exhibit no symptoms after separated inoculations with 20 different isolates of *X. campestris* pv. *glycines*. The *rxp* gene also conditions resistance to wildfire, which is caused by the bacterium *Pseudomonas tabaci* and a disease often associated with BP because of the pathogen's propensity to utilize BP lesions as an infection court.

In this study, the diversity of avirulence genes among Korean strains of *X. axonopodis* pv. *glycines*, the bacterial leaf pustules pathogen, was revealed by avirulence gene homology. Korean strains of *X. axonopodis* pv. *glycines* contained at least 3 copies of *avrBs3* gene family members, which were born from plasmid or chromosomal DNA. Intriguingly, the chromosomal avirulence gene induced hypersensitive response against resistant cultivars, when comparing the responses of wild type with those of disrupted-*avr* mutants. Herein, we suggest that one of the chromosomal *avrBs3* gene family members of *X. axonopodis* pv. *glycines* plays important key roles in interactions with soybean in that some of these genes contribute to the recognition of *X. axonopodis* pv. *glycines* to soybean.

MATERIALS ANS METHODS

Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. All strains of *X. axonopodis* pv. *glycines* used in this study were collected from farmers' fields in Korea from 1999 to 2002. All strains collected were first tested for pathogenicity using nonhost plant tomato for HR and susceptible cultivar Pella. All strains were subjected to FAME analysis [25] and the Biolog identification system (Bio-Rad) according to the manufacturer's instructions. Pure culture of all strains was maintained at the Plant Pathology Division, National Institute of Agricultural Science and Technology, RDA, Suwon, Korea. Bacterial strains were preserved in Luria-Bertani (LB) broth with sterile 15% glycerol at −80°C for long-term storage, retrieved from the stock, and were not transferred more than 2 or 3 times before testing of pathogenicity. Unless otherwise noted, the *E. coli* strain DH5α was used. The concentrations of antibiotics in media were rifampicin (Rif), 50 μg/ml; kanamycin (Kam), 50 μg/ml; tetracycline (Tet), 15 μg/ml; ampicillin (Amp), 50 μg/ml.

Plant Inoculation and Bacterial Multiplication *In Planta*

Seeds of soybean (*Glycine max* L.) of American and Korean varieties were obtained from S. K. Lee (Seoul National University), and they were germinated on moist filter paper in Petri dishes for 2 and 3 days in the dark. Plants were grown in the greenhouse for evaluation of resistance at the seedling stage. In the field, plants were transplanted 30 days after seedling by spraying. Inoculum was prepared by suspending *X. axonopodis* pv. *glycines* that had been grown on peptone-sucrose agar (PSA) for 2 to 3 days at 28°C [30]. Inoculum density was adjusted to 0.5 of optical density at 600 nm (OD_{600}) (5×10^8 CFU/ml) and then serially diluted to 10^6 CFU/ml in sterilized 10 mM $MgCl_2$ solution. Plant inoculation was performed to the fully expanded leaf by infiltration with a syringe and by spraying with an automizer into the intercellular space. All plant inoculation experiments were repeated 3 to 4 times, and each replicate consisted of three leaves. The index of disease severity was determined as numbers of pustules in one leaflet: 0, no pustule; 1, 1–25 pustules; 2, 26–50 pustules; 3, 51–75 pustules; 4, 76–100 pustules; 5, more than 100 pustules per leaflet.

Bacterial multiplication *in planta* was measured by viable cell counts at different time intervals after leaf infiltration and spraying inoculation. Ten leaf disks (6-mm diameter) were taken at differente time intervals after inoculation and ground in 1 ml of 10 mM $MgCl_2$ solution. Each suspension was appropriately serial-diluted and 10-μl aliquots were spotted onto a LB plate supplemented with proper antibiotics [24].

Table 1. Bacterial strains and plasmids used in this study.

Designation	Relevant characteristics	Reference or source
E. coli		
DH5α	F-, *endA1, hsdR17(rk-mk+), supE44, thi-1,recA1, gyrA, relA1, Φ80dlacZ, ΔM15, Δ(lacZYA-argF)U169*	Life Technologies
X. axonopodis pv. *glycines*		
OcsF	Typical Korean strain isolated in field, Rif^R	This work
SL1017	Typical Korean strain isolated in field, Rif^R	This work
SL1017 PM	SL1017 mutated at plasmid-borne *avr* gene by recombination with pTn5avrXa10, Rif^R, Kan^R	This work
SL1017 CM	SL1017 mutated at chromosome-borne *avr* gene by recombination with pTn5avrXa10, Rif^R, Kan^R	This work
SL1018	Typical Korean strain isolated in field, Rif^R	This work
SL1018 PM	SL1018 mutated at plasmid-borne *avr* gene by recombination with pTn5avrXa10, Rif^R, Kan^R	This work
SL1018 CM	SL1018 mutated at chromosome-borne *avr* gene by recombination with pTn5avrXa10, Rif^R, Kan^R	This work
SL1045	Typical Korean strain isolated in field, Rif^R	This work
SL1045 PM	SL1045 mutated at plasmid-borne *avr* gene by recombination with pTn5avrXa10, Rif^R, Kan^R	This work
SL1045 CM	SL1045 mutated at chromosome-borne *avr* gene by recombination with pTn5avrXa10, Rif^R, Kan^R	This work
SL1157	Typical Korean strain isolated in field, Rif^R	This work
SL1157 PM	SL1157 mutated at plasmid-borne *avr* gene by recombination with pTn5avrXa10, Rif^R, Kan^R	This work
SL1157 CM	SL1157 mutated at chromosome-borne *avr* gene by recombination with pTn5avrXa10, Rif^R, Kan^R	This work
SL2098	Typical Korean strain isolated in field, Rif^R	This work
SL2098 (pXag33)	SL2098 with pXcg33, Rif^R, Tet^R	This work
SL2098 (pTMxag33)	SL2098 with pTn5xcg33, Rif^R, Tet^R, Kan^R	This work
Plamids		
pLAFR3	Broad-host-range cosmid vector, Tet^R	[28]
pBluescript II KS+	Phagemid sequencing vector, Amp^R	Stratagene
pBSavrXa10	*avrXa10* clone in pBluescript II KS+, Amp^R	[15]
pTn5avrXa10	pBSavrXa10 with Tn5-disrupted *avr*Xa10, Amp^R, Kan^R	This work
pXag33	cDNA clone with *avrBs3* homologous family member from SL1045 in pLAFR3, Tet^R	This work
pTMxag33	pXcg33 with Tn5-disrupted *avrBs3* homologous gene, Tet^R, Kan^R	This work

Amp^R, ampicillin resistant; Kan^R, kanamycin resistant; Rif^R, rifampicin resistant; Tet^R, tetracycline resistant.

After incubation for 48 h at 28°C, the number of colonies formed on LB plates was counted. Each treatment was replicated six times.

Analysis of Genomic DNA

Genomic DNA of *X. axonopodis* pv. *glycines* was isolated by standard methods as previously described [26] and plasmid DNA was extracted with the Plasmid Midi Kit (Qiagen, Valemcoa, U.S.A.) as described by the manufacturer. Restriction enzymes were purchased from Roche and reactions were carried out according to the supplier's instructions. Electrophoretic separation and transfer to nylon membranes were performed as previously described [26]. DNA probes were labeled with $\alpha^{32}P$ dCTP with the Megaprime DNA labeling kit (Amersham Biosciences, Piscataway, U.S.A.) and probed against DNA immobilized on a Hybond N+. Membrane hybridization and subsequent washing were carried out and then exposed to X-ray film or BAS film.

Transposon Mutagenesis

Using an EZ::Tn Km transposon kit (EPI Centre), pBSavrXa10 containing the avirulence gene internal to pBluescript II KS+ was mutated. From in vitro mutation, thousands of putative mutants were selected on LB medium supplemented with ampicillin and kanamycin. Among these mutants, the plasmid interrupted by the integration of

Table 2. Numbers of Southern blotted bands and distribution ratio of *Xanthomonas axonopodis* pv. *glycines* type strains isolated in Korea.

		Type strains					
		OcsF	SL1017	SL1018	SL1045	SL1157	SL2098
Number of bands homologous to	*avrBs1*	0	0	0	0	0	0
	avrBs2	1	1	1	1	1	1
	avrBs3	5	6	3	5	4	0
	avrRxv	0	0	0	0	0	0
Distribution ratio (%)		82.0	3.2	7.1	1.9	1.9	1.3

transposon to *avrXa10* was chosen after confirmation by sequence analysis. The resultant plasmid was transformated into 6 typical strains of *X. axonopodis* pv. *glycines* by *E. coli* Pulser (Bio-Rad), allowing integration by double crossover. Transformants were selected on LB agar supplemented with rifampicin, kanamycin, and ampicillin. To verify that double crossover had occurred in marker exchange, DNA blot analysis of each mutant was used to confirm changes in sizes of DNA fragments digested with BamHI.

Construction of Genomic DNA Library

Genomic DNA from the SL1045 strain of *X. axonopodis* pv. *glycines* was partially digested with Sau3AI and fractionated by sucrose gradient density. Fragments between 20 and 25 kb were extracted. The cosmid vector pLAFR3 was digested to completion with BamHI and treated with calf intestinal alkaline phosphatase. The *X. axonopodis* pv. *glycines* genomic DNA and vector DNA fragments were ligated, packaged into lambda phage *in vitro*, and transduced into *E. coli* DH5α. Colonies were selected for resistance to tetracycline. Restriction enzyme analysis of random selected clones revealed an average DNA insert size of 20 to 25 kb. On the basis of an insert size of 20 kb, about 2,000 colonies needed to represent each gene at least once with a probability of 99% were screened [18].

DNA Sequencing

Nested deletions of pXag33 were generated in pBluescript II clones as previously described [2]. Sequence was also obtained from pBluescript subclones containing 8.5, 4.6, 0.6, 6.5, and 2.5 kb BamHI fragments adjacent to the 3.7 kb BamHI insert of pXag33. Single-stranded template was prepared and primed with either M13 forward, M13 reverse, or synthetic primers. The dideoxy sequencing method [27] was performed with the Big Dye Terminator sequencing reaction according to the manufacturer's protocols (Applied Biosystems, Foster City, U.S.A.). Samples wer purified and analyzed with the ABI310 Genetic Analyzer (Applied Biosystems, Foster City, U.S.A.).

RESULTS

Isolation of *X. axonopodis* pv. *glycines* Strains

In order to investigate the genetic diversity of *X. axonopodis* pv. *glycines* in Korea, 155 *X. axonopodis* pv. *glycines* strains were isolated from diverse tissues such as leaves, pods, and stems of 27 soybean cultivars from 40 regions of 8 different provinces of Korea (Table 2). DNA blot analysis of the 155 bacterial strains was performed by hybridization of BamHI-digested genomic DNA with the well-known avirulence gene of *X. campestris* pv. *vesicatoria*, *avrBs1*, *avrBs2*, *avrBs3*, and *avrRxv* probe. In Korean *X. axonopodis* pv. *glycines* strains, 3 to 7 fragments of genomic DNA were hybridized with the *avrBs3* gene, whereas no hybridization was observed with the *avrBs1* and *avrRxv* probe and only a single fragment was hybridized with the *avrBs2* probe (Table 2 and Fig. 1). The multiple fragments hybridized with the *avrBs3* were chosen for further in-depth investigation. Since the Southern

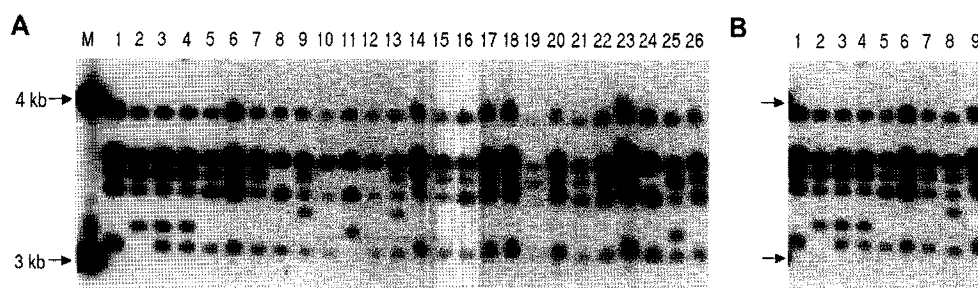

Fig. 1. Avirulence gene profiles of Korean strains of *X. axonopodis* pv. *glycines* isolated from diverse cultivars (**A**) and tissues (**B**) nationwide.

EcoRI digested genomic DNA of *X. axonopodis* pv. *glycines* was hybridized with BamHI fragments of *avrBs3* isolated from *X. campestris* pv. *vesicatoria*. **A.** Strains of *X. axonopodis* pv. *glycines* were isolated in different cultivars of leaves: Jangyeop (lanes 1, 2, & 26), Daewon (lanes 3 & 4), Jangwon (lanes 5, 6, and 7), Hwanggeum (lane 8 and 25), Dawon (lane 9), Sunheuk (lane 10), Manri (lane 11), Jinyeul (lane 12), Sowon (lanes 13 & 14), Danbaek (lane 15), Sodam (lane 16), Jangsu (lane 17), Bogwang (lane 18), Jinpum (lanes 19 and 20), Geumjeong1 (lane 21), Geumjeong2 (lanes 22), Gwanggyo (lane 23), Taegwang (lane 24), respectively. Lane M, DNA sizes in kb at the left. **B.** Strains of *X. a.* pv. *glycines* were isolated from different tissues of cultivars: leaves and pods of Jangyeop (lanes 1 and 2), leaves and pods of Daewon (lanes 3 and 4), leaves, pods, and stems of Jangwon (lanes 5, 6, and 7), leaves and pods of Sowon (lanes 8 and 9), respectively.

Fig. 2. Southern blot analysis of type strain *X. axonopodis* pv. *glycines*.

Blots of genomic DNA (**A**) and plasmid DNA (**B**) digested by BamHI were hybridized with pBSavrXa10. Lane M, DNA sizes in kb at the left; lane 1, OcsF; lane 2, SL1017; lane 3, SL1018; lane 4, SL1045; lane 5, SL1157; lane 6, SL2098.

hybridization showed several different homology patterns with BamHI digested genomic DNA, other restriction enzymes such as EcoRI and HindIII were used to confirm the different homology patterns of the 155 bacterial strains. Specifically, *avrBs3* genes are known to control the host specificity, and restriction enzyme BamHI treatment makes unique DNA fragments for the genes [4, 21]. DNA blot analysis of the 155 bacterial strains was performed by hybridization of genomic DNA with the 3.2-kb BamHI fragment isolated from plasmid pBSavrXa10 (data not shown). Based on the *avrBs3* homologous band patterns with three different restriction enzymes EcoRI, BamHI, and HindIII, the 155 bacterial strains isolated were divided into 6 groups, and OcsF, SL1017, SL1018, SL1045, SL1157, and SL2098 were selected as type strains (Fig. 2A). Differences among strains were obvious in the fragments between 3 and 4 kb (Fig. 1). Furthermore, there was a difference in the intensities of *avrBs3* homologous bands. Since different intensities of *avrBs3* homologous bands might result from the different copy numbers or origin of genes, native plasmids from *X. axonopodis* pv. *glycines* were isolated. Native plasmid was isolated from 5 type strains, except SL2098, and the plasmid showed homology with the *avrBs3* avirulence gene (Fig. 2B). These data suggest that the dark intense bands may be due to the *avrBs3* gene located on the plasmid.

The distributions of these 6 groups are not uniform: the OcsF group was predominant among *X. axonopodis* pv. *glycines* isolated in Korea (Table 2). The majority of isolated

Table 3. Disease severity of the bacterial leaf pustule of 6 type strains of *X. axonopodis* pv. *glycines* in 8 universal soybean cultivars and 14 soybean cultivars of Korea.

Cultivars		Isolates					
		OscF	SL1017	SL1018	SL1045	SL1157	SL2098
Universal	Peking	3.5	4.8	4.1	4.5	4.0	0
	CNS1	1.0	3.3	4.2	3.8	2.3	0
	CNS2	1.0	0.2	0.5	0.8	0.5	0
	Mukden	2.5	3.0	3.5	4.5	4.5	0
	Chippewa	3.0	2.8	4.5	2.8	3.7	0
	Williams82	0.8	2.2	0.5	0.2	0.2	0
	Harosoy	2.0	3.3	3.3	3.3	2.8	0
	Pella	2.0	3.2	2.2	2.8	1.3	0
Korean	Dawon	5.0	5.0	3.8	4.2	3.8	0
	Milyang78	3.0	5.0	4.0	3.5	3.5	0
	Jangyeop	2.5	4.7	4.0	3.3	3.7	0
	Gumjeoung1	2.0	4.2	4.7	2.7	2.7	0
	Sinpaldal1	1.0	1.3	0.8	0.3	1.3	0.2
	Moohan	1.5	2.7	3.8	4.7	2.5	0.1
	Suwon157	1.5	3.5	3.3	4.2	2.8	0
	Manri	4.0	1.8	3.7	3.7	3.2	0
	Sinpaldal2	3.0	3.2	1.0	3.2	3.3	0
	Milyang68	2.0	4.7	1.7	3.5	3.5	0
	Hannam	3.0	4.0	1.2	3.5	4.8	0
	Danbaek	1.0	3.7	3.5	1.3	3.3	0
	Gwangan	0.8	0.8	2.2	0.3	3.7	0.1
	Pureun	3.0	1.5	1.8	1.2	3.0	0.5

Number of pustules in one leaflet: 0, no pustule; 1, 1–25 pustules; 2, 26–50 pustules; 3, 51–75 pustules; 4, 76–100 pustules; 5, more than 100 pustules per leaflet.

X. axonopodis pv. *glycines* was of the OcsF group. of the 155 strains tested, 127 (82%) isolates belonged to OcsF group. In particular, two strains belonging to the SL2098 group showed no *avrBs3* homologous band at all (Figs. 2A and 2B).

To determine whether the patterns of the avirulence genes were related to the cultivars isolated, the avirulence genes of several strains isolated in the same cultivars and diverse tissues (leaves, pods, and stems) were compared with one another. However, there were no relationships between the cultivars and the avirulence gene patterns, because *avrBs3* patterns of strains isolated in the same cultivars and same tissue were different (Fig. 1).

Pathogenicity of Six Different *X. axonopodis* pv. *glycines* Type Strains

In order to investigate the relationships between the avirulence gene diversity and the host specificity, 22 different soybean cultivars were tested for the response to the 6 different type strains (Table 3). Among those cultivars, Williams82, CNS1, and CNS2 cultivars are known to carry the *rxp* resistance gene. CNS2 cultivars were very resistant to all 6 Korean type strains, and CNS1 showed different responses to Korean type strains: CNS1 was relatively resistant to the OcsF strain but was susceptible to other type strains except SL2098. Williams82 was resistant to most type strains but was relatively susceptible to SL1017. All Korean cultivars tested and selected, except Sinpaldal1, were susceptible to 5 type strains: OcsF, SL1017, SL1018, SL1045, and SL1157. Sinpaldal1 showed very strong resistance to all type strains. Several cultivars showed different response to different type strains that carry various *avrBs3* homology bands. Moohan and Suwon157 showed resistance to OcsF, and Manri showed resistance to SL1018. Sinpaldal2, Milyang68, and Hannam showed resistance to SL1018. It suggests that there may be a host-specific interaction between these type strains and these cultivars. Universally susceptible cultivars such as Peking, Chippewa, Mukden, and Harosoy and several Korean cultivars such as Dawon, Milyang 78, and Janyeop exhibited susceptibility to all types of *X. axonopodis* pv. *glycines* strains, except to SL2098, which has no *avrBs3* homologous band in Southern hybridization.

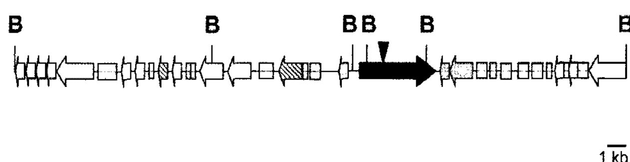

Fig. 3. Location of *avrBs3* homologous gene in relation to transposases (gray arrows), resolvases (shaded arrows), other ORFs (open arrows), and hypothetical proteins (boxes) in the 26.4 kb cDNA library clone identified in the SL1045 strain.

The insert cloned in pXag33 (vector pLAFR3) is shown; Physical map of clones with *avrBs3* homologous gene (dark arrow). Positions of transposon Tn5 insertions (dark triangle ▼) that inactivate the avirulence phenotypes are shown. Transposon insertions used for marker exchange and physiological studies are indicated. B, BamHI

Even when infection of SL2098 occurred on highly susceptible cultivars such as Dawon, Milyang78, Jangyeop, and Gumjeong1, in which other groups with *avrBs3* homologs induced hundreds of pustules, only a few small flecks and pustules were formed on young leaves. In order to confirm this strain as *X. axonopodis* pv. *glycines*, fatty acid analysis and Biolog identification were carried out and the SL2098 strain was found to be a kind of *X. axonopodis* pv. *glycines* strain (data not shown). It is possible that *avrBs3* homologous genes could influence the pathogenicity of *X. axonopodis* pv. *glycines* in soybean cultivars.

Isolation and Characterization of an *avrBs3* Homologous Gene from *X. axonopodis* pv. *glycines*

In order to identify the function of *avrBs3* homologous genes in the pathogenicity, *avrBs3* homologous clones were screened from the *X. axonopodis* pv. *glycines* genomic library. Twenty clones from a genomic library of strain SL1045 hybridized with the 3.2 kb *avrXa10* BamHI fragment in approximately 2,000 clones by colony hybridization. Based on the RFLP of cosmid DNA, a final six clones were selected and subcloned with pBluescript KS+ for DNA sequencing. Sequence analysis of the pXag30 clone revealed a single open reading frame (ORF) of AvrBs3-like protein in about 30 hypothetical proteins such as transposases, resolvases, and conjugal transfer proteins (Fig. 3). Sequence homology search of the pXag33 clone containing the avirulence gene yielded significant homology to native plasmid pXAG81 of *X. axonopodis* pv. *glycines* 8ra [20].

To investigate the effects of *avrBs3* gene family members on the aggressiveness of *X. axonopodis* pv. *glycines*, the

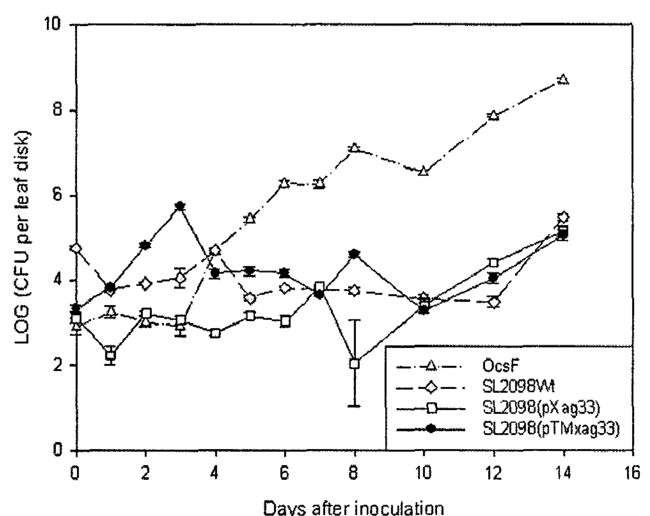

Fig. 4. Time course of bacterial growth in leaves of soybean susceptible cultivars Jangyeop.

Leaves were sprayed with bacterial suspension and sampled for 14 days after inoculation. Jangyeop cultivars were inoculated with *X. axonopodis* pv. *glycines* OcsF (open triangles), SL2098 (gray diamonds), SL2098 (pXag33) (open boxes), and SL2098 (pTMxag33) (closed circles). Values represent means from six repetitions, and vertical bars represent standard deviation.

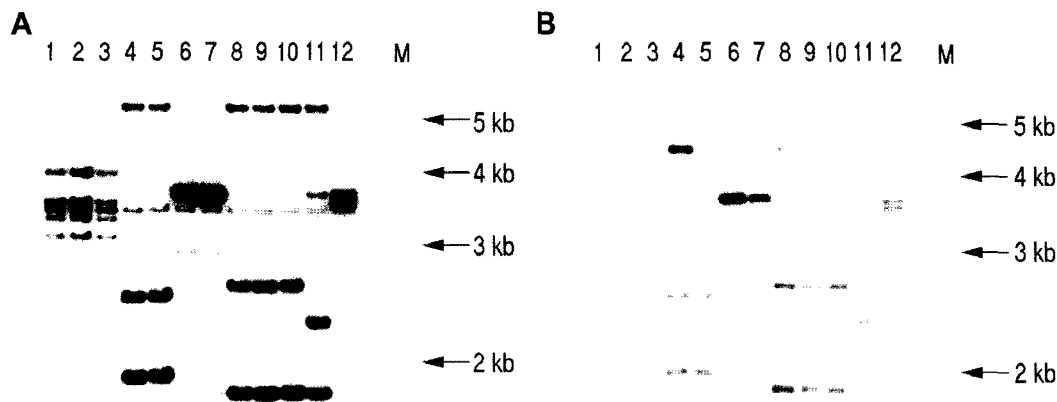

Fig. 5. Southern blot analysis of marker exchange mutants of *X. axonopodis* pv. *glycines*.
Blots of genomic DNA (**A**) and plasmid DNA (**B**) digested by BamHI were hybridized with pBSavrXa10. SL1017 PM and SL1018 PM lack the 3.7 kb BamHI fragment of genomic and plasmid DNA of wild types. SL1018 CM and SL1157 CM show same band pattern of wild type in plasmid DNA, but lack 5.2 and 4.0 kb BamHI fragment, respectively. Lanes 1 to 12, the following marker exchange mutants of wild type: SL1017 PM1, SL1017 PM2, SL1017 PM3, SL1018 PM, SL1018 PM2, SL1018 CM, SL1018 CM2, SL1018 PM3, SL1018 PM4, SL1018 PM5, SL1018 PM6, and SL1157 CM, respectively.

constructs pXag33 and pTMxag33 were introduced into the avirulent *X. axonopodis* pv. *glycines* strain SL2098 by electroporation. Highly virulent OcsF strain, avirulent SL2098 strain, SL2098 (pXag33) mutant, and SL2098 (pTMxag33) mutant were inoculated into the susceptible cultivars Jangyeop. The numbers of SL2098 strain and its mutants increased about 10-fold and then remained constant for 14 days, whereas OcsF increased 10^4-fold (Fig. 4). It seems that, unlike virulent OcsF strain, the plasmid-borne *avrBs3* gene might have only a minor role in the recovery of aggressiveness of avirulent strain because the growth patterns of SL2098 (pXag33) and SL2098 (pTMxag33) mutants were similar to that of wild-type SL2098 in the susceptible host Jangyeop (Fig. 4).

Transposon Mutagenesis of *avrBS3* Homologous Genes
Marker-exchange derivative strains, SL1017 PM (designated plasmid-borne *avr* gene mutant), SL1018 PM, SL1018 CM (designated chromosome-borne *avr* gene mutant), and SL1157 CM were identified by DNA blot analysis (Fig. 3). In Southern blot analysis, SL1017, SL1018, and SL1157 contained several different-sized BamHI fragments that hybridize to *avrXa10* (Fig. 5). Most of the fragments were located between 3 kb and 5 kb in the genomic DNA of 6 groups of *X. axonopodis* pv. *glycines* (Fig. 5A). Among these fragments, the 3.7 kb BamHI fragments were present in the majority of the coding region of the plasmid-borne avirulence gene family (Fig. 5B). SL1017 PM, which contains a Tn5 insertion in the plasmid-borne avirulence gene, was lacking of the 3.7 kb BamHI fragment that hybridizes to *avrXa10*, whereas keeping up others of the chromosome-borne avirulence gene. The exchange of the 1.9 kb and 2.6 kb fragments with Tn5-disrupted copy was confirmed by the absence of the 3.7 kb BamHI fragment in mutant SL1018 PM. Although there was no change of plasmid gene pattern, the 5.2 kb and 4.0 kb chromosome-borne avirulence fragments were lost in SL1018 CM and SL1157 CM, respectively (Fig. 5).

To investigate the relationship between the pathogenicity and the mutation of *avrBs3* homologous gene of *X. axonopodis* pv. *glycines*, SL1018 PM (3.7 kb plasmid-borne *avr* gene mutant) and SL1018 CM (5.2 kb chromosome-borne *avr* gene mutant) were selected among these mutants. According to the gene-for-gene theory, *X. axonopodis* pv. *glycines* strains harboring functional *avr* genes elicit a hypersensitive response (HR) after infiltration of soybean leaves with corresponding resistance genes. Wild-type SL1018 caused hypersensitive necrosis response on resistant cultivars, Williams82 and CNS2 twenty-four hours after infiltration inoculation (Fig. 6).

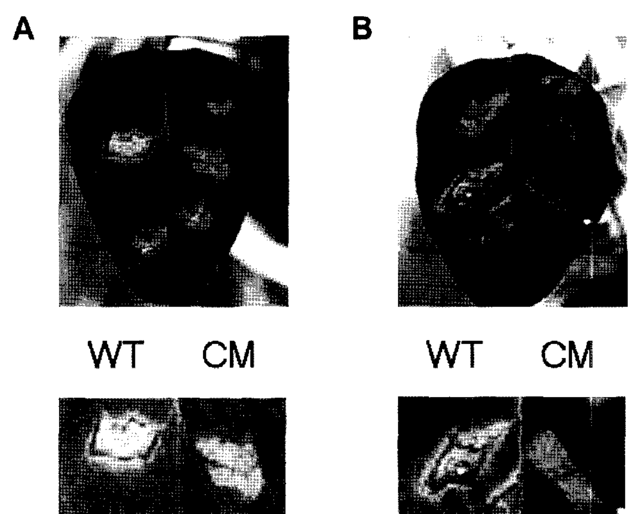

Fig. 6. Phenotype of different soybean cultivars after *X. axonopodis* pv. *glycines* inoculation.
Williams82 cultivars (**A**) and CNS2 cultivars (**B**) were infiltrated with the wild-type SL1018 (WT) and SL1018 CM (CM) of *X. axonopodis* pv. *glycines*. Photographs were taken 2 days after inoculation to allow for expression of resistant symptomology.

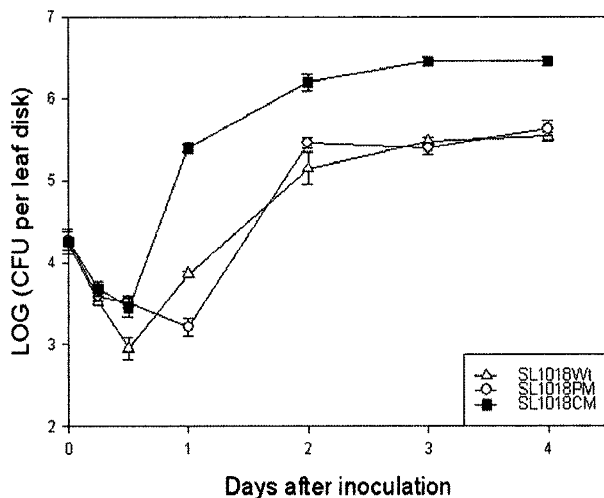

Fig. 7. Growth patterns of *Xanthomonas axonopodis* pv. *glycines* SL1018 and its mutants in leaves of resistant soybean cultivars Williams82.

Leaves were inoculated with 10^6 CFU/ml of each bacterial strain SL1018 (open triangles), SL1018PM (gray circles), and SL1018CM (dark boxes). Multiplication assays were performed by grinding each leaf disk (6 mm diameter) in 1 ml of 10 mM $MgCl_2$ solution, followed by serial dilution and plating of the samples onto agar plates with appropriate antibiotics. The values reported are the means of six replicates. Error bars indicate the standard deviation.

Unlike wild type, SL1018 CM was not able to cause resistant responses (Fig. 6). The authenticity of the resistance reactions was further corroborated by the analysis of *in planta* bacterial growth. Bacterial numbers of SL1018 wild type and SL1018 PM increased steadily to approximately 10^5 CFU/leaf disk until 2 days after inoculation and the rate of multiplication decreased thereafter, and bacterial numbers did not substantially increase in Williams82 resistance cultivars (Fig. 7). In contrast, the number of SL1018 CM *in planta* increased by about 10-fold from 3 days after inoculation and remained constant (Fig. 7), suggesting that the resistant cultivars Williams82 may have the cryptic resistance gene counterpart of the 5.2 kb avirulence gene and recognize the 5.2 kb chromosome-borne avirulence gene of *X. axonopodis* pv. *glycines* for eliciting the HR.

DISCUSSION

In this study, *X. axonopodis* pv. *glycines* was isolated from various tissues, such as leaves, pods, and stems, of soybean showing symptoms of bacterial pustules disease, and tissue specificity was not observed. DNA blot analysis of the avirulence diversity of *X. axonopodis* pv. *glycines* strains showed that Korean strains of *X. axonopodis* pv. *glycines* contain several copies of members of the *avrBs3* avirulence gene family in the chromosomal and plasmid DNA. Since no difference was detected in the patterns of *avrBs1*, *avrBs2*, and *avrRxv* homologous genes, this may indicate that the diversity of the *X. axonopodis* pv. *glycines* depends on the *avrBs3* homolog. Based on the *avrBs3* homology patterns, the *X. axonopodis pv. glycines* collected in Korea were classified into 6 groups.

Diverse members of the *avrBs3* gene family are involved in the host specificity [3], and repeated region of the *avrBs3* determines race specificity [14]. Based on the gene-for-gene hypothesis, a direct interaction occurs between a pathogen race-specificity gene (*avr* gene) product and the product of the complementary disease resistance gene (*R* gene) in the plant. The results of disease severity of representative strains on various soybean cultivars indicated that the difference of *avrBs3* homolog patterns was related to the responses of soybean hosts that have the corresponding resistance gene.

From the relationship between the diversity of *avrBs3* homologous genes and pathogenicity of the bacteria, we believe that *avrBs3* gene regions play important roles in the pathogenicity and avirulence of numerous *X. axonopodis* pv. *glycines* strains. Some *avrBs3* family members have a dual function, including aiding pathogenicity and becoming avirulence factors, that trigger defense [5]. First, five typical groups (OcsF, SL1017, SL1018, SL1045, and SL1157) of *X. axonopodis* pv. *glycines*, which are representatives of genetic diversity of Korean strains, had three to seven DNA bands homologous to the 3.2 kb *avrBs3* fragment and induced severe disease symptoms in susceptible cultivars, whereas SL2098 having no avrBs3 homologous fragment did not induce virulence in soybean cultivars at all (Table 2). Band intensities in the genomic DNA and the plasmid DNA indicated that the plasmid-derived fragments were clearly present at higher copy number in total *X. axonopodis* pv. *glycines* DNA than the other fragments, suggesting that the former groups are borne in plasmid DNA. The information of avirulence gene suggested that the avirulence gene of our clone is located in a pathogenic native plasmid of *X. axonopodis* pv. *glycines*. *X. axonopodis* pv. *citri* contains four copies of *avrBs3/pth*, located in the plasmid, but *X. campestris* pv. *campestris* lacks *avrBs3/pth* [6]. However, we demonstrated that the timing of the interaction with host, when the plasmid-borne *avrBs3* gene was in the genetic background [SL2098(pXag33) mutant], was the same as that observed in the wild-type strain (SL2098) (Fig. 4). We, therefore, conclude that the interaction with host plant is dependent on the specific chromosome-borne avirulence resistance gene combination, not the plasmid-borne.

Second, many plant pathogenic bacteria possess avirulence genes that betray the parasite to the resistance gene encoded surveillance system of plants [5]. The resistance response is accompanied by the hypersensitive reaction that is a rapid localized programmed cell death by the arrest of bacterial growth within the infested plant tissue [31]. Although wild-type SL1018 created the HR in resistant soybean, the Tn5 mutant that had a single transposon insertion within the small region in the 5.2 kb BamHI fragment of the chromosome-borne avirulence gene never gave rise to HR in resistant

cultivars such as Williams82 and CNS2 (Fig. 4). These results strongly support the possibility that the avirulence gene, chromosome-borne of the *avrBs3* homologous gene, of SL1018 is responsible for the recognition of soybean cultivars Williams82.

In the present study, we also described virulence of Korean stains of *X. axonopodis* pv. *glycines* to soybean carrying *rxp* resistance genes [12, 13]. Soybean resistance by *rxp* has proven to be effective in controlling bacterial leaf pustule. However, when we characterized a large number of these strains by pathogenicity tests and genotypic analysis, *X. axonopodis* pv. *glycines* strains virulent to *rxp* were distributed over a wide region of Korea. Based on the gene-for-gene concept, overcoming resistance of the *rxp* gene should result from the absence or inactivation of a bacterial gene (*avr rxp*). Because Korea is one of the soybean origins, soybean pathogens as well as soybeans have experienced several evolutions. Genetic diversity of soybeans may accelerate the phytopathogen's adaptation to their host. Within bacteria, selection pressure may result in selection for the entire bacterial genome harboring the genes that could avoid the plant defense mechanism.

Additional studies on the resistance gene of diverse soybean cultivars are necessary to demonstrate whether avirulence genes of *X. axonopodis* pv. *glycines* truly interact with incompatible resistance genes. Plants and pathogenic bacteria have coevolved during a long period of coexistence and interaction. Further understanding of the mechanisms between phytopathogenic invasion and plant defense could decrease crop losses attributed to plant disease.

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