Regulation of eight *avr* genes by *hrpG* and *hrpX* in *Xanthomonas campestris* pv. *campestris* and their role in pathogenicity*

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Abstract Eight putative avirulence genes in *Xanthomonas campestris* pv. *campestris* (*Xcc*) strain 8004 were characterized by Tn5*gus*A5 mutagenesis and gene expression analysis. The virulence test of mutants on Chinese radish showed that all mutants in individual *avr* genes except *avrBs2* mutant were not significantly different from the wild type in virulence. The *avrBs2* mutant showed reduced virulence and bacterial growth *in planta*. Gene expression analysis using β-glucuronidase as reporter indicated that *avrBs1.1*, *avrBs1*, *avrXccB*, *avrXccC*, *avrXccE1* were regulated by *hrpG*, whereas *avrXccA1*, *avrXccA2* and *avrBs2* were not. RT-PCR analysis showed that all *hrpG*-regulated genes except *avrBs1* were also regulated by *hrpX*. In addition, it was demonstrated that *avrBs1* was responsible for elicitation of a type III dependent hypersensitive reaction (HR) on nonhost plant pepper ECW-10R, and wild type *Xcc* 8004 was unable to cause HR on pepper ECW-20R.

Keywords: ***Xanthomonas campestris* pv. *campestris*, avirulence gene, virulence, expression analysis.**

Many Gram-negative pathogenic bacteria have evolved a specialized apparatus type III secretion system (TTSS) that transports so-called effector proteins across the bacterial membranes and directly into the host cell[1]. It is clear that the TTSS of phytopathogenic bacteria such as *Pseudomonas syringae*, *Xanthomonas* subsp., *Ralstonia solanacearum* and *Erwinia amylovora*, is encoded by *hrp* (hypersensitive reaction and pathogenicity) genes, in which nine members are renamed as *hrc* genes and are highly conserved in plant and animal pathogens. *hrp* gene mutant is unable to cause disease symptoms on the host plant and fails to induce the hypersensitive reaction (HR) on resistant host and nonhost plants[2]. It seems that the secreted proteins of plant pathogens are extremely diverse and mainly include proteins essential for the function of the TTSS machinery, pathogenicity proteins, as well as harpins and avirulence proteins[3]. The avirulence genes were originally defined by their HR inducing activity in plants that expressed a corresponding disease resistance gene (*R* gene). Once the *avr* or the *R* gene or both are absent, no recognition takes place between a pathogen and its host, and disease occurs[4]. However, many avirulence genes have been validated to have dual functions, a role in virulence as well as avirulence, after *avrBs2* from *X. campestris* pv. *vesicatoria* (*Xcv*) was found to have dual functions[5,6]. In *Xanthomonas*, the expression of *hrp* genes is regulated by both the AraC family regulatory protein HrpX and the response regulator HrpG of two-component regulatory system[7,8]. In *Xcv*, many avirulence proteins were characterized as TTSS effectors, e.g. AvrXv3, AvrBsT, AvrBs1, AvrBs2, and the expression of *avrBsT* and *avrBs1* was independent of *hrpG* and *hrpX*[9], but the expression of *avrXv3* was plant inducible and controlled by *hrpG* and *hrpX*[10].

Xanthomonas campestris pv. *campestris* (*Xcc*) is the causal agent of black rot disease of cruciferious plants. The genome of *Xcc* strain 8004 had been sequenced and eight *avr* genes were annotated[11]. Most of these *avr* genes are functionally unknown. Only AvrBs2 is highly conserved in four sequenced *Xanthomonas* species[12—14], and was well studied as type III effector, sharing homology with both agrocinopine synthase of *Agrobacterium tumefaciens* and glycerophosphoryl-diester phosphodiesterase UgpQ of *E. coli*[15]. Eight *avr* genes were also annotated in

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the genome of *X. campestris* pv. *campestris* ATCC 33913, which are almost the same as those in *Xcc* 8004 (homology 99% or above)[12]. Castañeda et al. had shown that mutagenesis of all eight *avr* genes in *Xcc* ATCC 33913 (528^T) had no detected effect on virulence on host plant and HR on nonhost plant pepper[16]. The purpose of this work is to study the potential role in pathogenicity of all eight annotated *avr* genes of *Xcc* 8004 and to analyze the regulation of their expression by *hrpG* and *hrpX*. The results showed that *avrBs2* is required for the full virulence of *Xcc* 8004 and five of eight *avr* genes are regulated by *hrpG*, and four of the five *hrpG*-regulated genes are also regulated by *hrpX*. In addition, we also demonstrated that *avrBs1* was responsible for eliciting a typical HR on nonhost plant pepper ECW-10R in a type III dependent manner.

1 Materials and methods

1.1 Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used are listed in Table 1. *E. coli* strains were cultivated at 37℃ in LB medium[17], and *Xanthomonas* strains were grown in NYG[18] or XVM2[19] at 28℃. Antibiotics were used as described by Daniels et al.[18]

Table 1. Bacterial strains and plasmids used in this work

Strains/plasmids	Relevant characteristics	Source/reference
E. coli		
JM109	*RecA1*, *endA1*, *gyrA96*, *thi*, *supE44*, *relA1* △(*lac-proAB*)/*F'* [*traD36*, *lacIq*, *lacZ* △*M15*]	Yanisch-Perron et al.[20]
X. campestris pv. *campestris*		
8004	Wild-type, Rif^r	Daniels et al.[21]
8004△*hrpG*	*hrpG* deletion mutant of 8004, Rif^r, Km^r, Spc^r, Gm^r	Our lab's collection
8004△*hrpX*	*hrpX* deletion mutant of 8004, Rif^r, Km^r, Spc^r, Gm^r	Our lab's collection
8004△*avrBs1*	*avrBs1* deletion mutant of 8004, Rif^r, Gm^r	Our lab's collection
050B12	*hrcV* Tn5 insertion mutant of 8004, Rif^r, Km^r, Spc^r, Gm^r	Our lab's collection
121D06	*hrpF* Tn5 insertion mutant of 8004, Rif^r, Km^r, Spc^r, Gm^r	Our lab's collection
047A01	*avrBs1* Tn5 insertion mutant of 8004, Rif^r, Km^r, Spc^r, Gm^r	Our lab's collection
011C06	*avrBs1.1* Tn5 insertion mutant of 8004, Rif^r, Km^r, Spc^r, Gm^r	Our lab's collection
208H07	*avrBs2* Tn5 insertion mutant of 8004, Rif^r, Km^r, Spc^r, Gm^r	Our lab's collection
153H12	*avrXccA1* Tn5 insertion mutant of 8004, Rif^r, Km^r, Spc^r, Gm^r	Our lab's collection
003C12	*avrXccA2* Tn5 insertion mutant of 8004, Rif^r, Km^r, Spc^r, Gm^r	Our lab's collection
122A06	*avrXccB* Tn5 insertion mutant of 8004, Rif^r, Km^r, Spc^r, Gm^r	Our lab's collection
051F05	*avrXccC* Tn5 insertion mutant of 8004, Rif^r, Km^r, Spc^r, Gm^r	Our lab's collection
206A12	*avrXccE1* Tn5 insertion mutant of 8004, Rif^r, Km^r, Spc^r, Gm^r	Our lab's collection
ZH0052	*avrBs2* single crossover mutant of 8004, Rif^r, Km^r	This work
DM047G	047A01, but *hrpG*::pT18mob, Rif^r, Km^r, Spc^r, Gm^r, Tc^r	This work
DM011G	011C06, but *hrpG*::pT18mob, Rif^r, Km^r, Spc^r, Gm^r, Tc^r	This work
DM208G	208H07, but *hrpG*::pT18mob, Rif^r, Km^r, Spc^r, Gm^r, Tc^r	This work
DM153G	153H12, but *hrpG*::pT18mob, Rif^r, Km^r, Spc^r, Gm^r, Tc^r	This work
DM003G	003C12, but *hrpG*::pT18mob, Rif^r, Km^r, Spc^r, Gm^r, Tc^r	This work
DM122G	122A06, but *hrpG*::pT18mob, Rif^r, Km^r, Spc^r, Gm^r, Tc^r	This work
DM051G	051F05, but *hrpG*::pT18mob, Rif^r, Km^r, Spc^r, Gm^r, Tc^r	This work
DM206G	206A12, but *hrpG*::pT18mob, Rif^r, Km^r, Spc^r, Gm^r, Tc^r	This work
CLBs1	8004△*avrBs1* harboring pLBs1, Rif^r, Gm^r, Tc^r	This work
CL047	047A01 harboring pLBs1, Rif^r, Km^r, Tc^r	This work
Plasmids		
pK18mob	Suicide plasmid in *Xcc*, Tra^-, Mob^+, Km^r	Schafer et al.[22]
pT18mob	A tetracycline resistant derivative of pK18mob, Tc^r	Our lab's collection
pRK2073	Helper plasmid, Tra^+, Mob^+, ColE1, Spc^r	Leong et al.[23]
pLAFR3	Broad host range cloning vector, RK2 replicon, Tra^-, Mob^+, Tc^r	Staskawicz et al.[24]
pLAFR6	Broad host range cloning vector, Tc^r	Huynh et al.[25]
pPH1JI	Tra^+, Mob^+, IncP replicon, Spc^r, Gm^r	Hirsch et al.[26]
pTG2081	pT18mob carrying *avrBs1* flanking sequences and Gm^r gene, Tc^r	This work
pLBs1	pLAFR6 containing only entire *avrBs1* gene, Tc^r	This work
pT18J26	pT18mob containing a 400 bp internal fragment of *hrpG*, Tc^r	This work

1.2 DNA manipulations

DNA manipulations followed the procedures described by Sambrook et al.[27]. Plasmids were introduced into *E. coli* strains by electroporation and into *Xanthomonas* strains by triparental mating using pRK2073 as the helper plasmid[28]. Restriction enzymes and DNA ligase were used in accordance with the manufacturer's (Promega Co.) instructions.

1.3 Mutant construction

We chose 8 insertional mutants of *Xcc* 8004 from our mutant library (unpublished), which have transposon Tn5*gus*A5 inserted in genes *avrBs1*, *avrBs1.1*, *avrBs2*, *avrXccA1*, *avrXccA2*, *avrXccB*, *avrXccC* and *avrXccE1*, respectively. All the mutants were confirmed by PCR using the primer sp1 on Tn5*gus*A5 and the primer on the gene upstream or downstream of the disrupted gene. With the method of gene inactivation by homologous plasmid integration[29] using pK18mob as the vector[22], we inactivated *avrBs2* gene using the primer set of ZHBs2F/ZHBs2R (5′-CCCGAATTCTTGCCCTTGACCCCGTA-3′/ 5′-GGGTCTAGTGATCTGTGCGCCTGCCTG-3′) and the resulting mutant was designated as ZH0052.

To inactivate *hrpG* in each Tn5*gus*A5 insertional mutant of *avr* genes, a 400 bp internal fragment of *hrpG* amplified using the total DNA of wild type *Xcc* 8004 as template and the primer set J26GF/J26GR (5′-GGTCTAGAGCGCTGCCTGCGCCACTCGCCGTGC-3′/ 5′-GGGTCGACCACGCCCGGATTCGAAAACAGCAGC-3′) as primers was cloned into pT18mob (Table 1) to create the recombinant plasmid pT18J26. The plasmid pT18J26 was introduced from *E. coli* JM109 into each of the eight mutant strains, respectively, by triparental mating. Transconjugants were screened on NYG agar supplemented with rifampicin, kanamycin and tetracycline. The obtained transconjugants with a mutation in *hrpG* gene were confirmed by PCR, which was performed by using the total DNA of the obtained transconjugants as template and the primer set PpK18mobCF/26Comple-R (5′-TTTCGTTCCACTGAGCGTCAGACCCC-3′/ 5′-GGAAGCTTCAATATTTCCGGTGTCGGCGATGCC-3′) as primers, and the PCR using the total DNA of corresponding mutant strain with only *avr* gene disrupted as template was used as negative control.

To generate a deletion mutant of *avrBs1*, the 3271 bp fragment including *avrBs1* coding region and its flanking region was amplified by PCR with the primer set dS1F/dS1R (5′-GCGTCTAGAATCGCATTTCGTTTCGAGGCCGC-3′/5′-GCGGAATTCAACACGTTCATCAAGCGGTTCCC-3′) and was cloned into pT18mobH, a derivative of suicide plasmid pT18mob in which *Hin*dIII restriction site was disrupted, generating pTH2081. A 1547 bp fragment including *avrBs1* coding region on pTH2081 was replaced by another fragment containing Gentamycin resistance gene amplified from plasmid pPH1JI with the primer set GmF/GmR (5′-CCAAGCTTAATTGACATAAGCCTGTTCGGTTCG-3′/5′-CCAAGCTTTGACAATTTACCGAACAACTCCGC-3′), creating the recombinant plasmid pTG2081. This plasmid was introduced into wild type *Xcc* 8004. Tetracycline sensitive transconjugants were screened out and confirmed by PCR with primers dS1F and dS1R as the deletion mutant 8004 △*avrBs1*.

1.4 Complementation of *avrBs1* mutant

A 1542 bp DNA fragment including the *avrBs1* coding region and extending 205 bp at the 5′ terminal was amplified by PCR using the total DNA of *Xcc* 8004 as template and the primer set CBs1F (5′-GCTCTAGACTCAGAATTTCGTAATGAACGG-3′), CBs1R (5′-GCGGATCCTTACGCTT CTCCTGCATTTGTAAC-3′) as primers. After confirmed by sequencing analysis, the fragment was cloned into pLAFR6 to generate plasmid pLBs1. This recombinant plasmid was transferred into the mutants 8004 △*avrBs1* and 047A01 by triparental conjugation (Table 1).

1.5 Reverse transcriptional PCR (RT-PCR)

Overnight cultures of wild type *Xcc* 8004 and 8004△*hrpX* in liquid medium XVM2 were used to extract the total RNA with total RNA extraction kit (Promega Co.) according to the procedure recommended by the manufacturer. The reverse transcription was performed by using the cDNA synthesis kit (Fermentas Co.) following the instructions. Using the cDNA of wild type *Xcc* 8004 and 8004△*hrpX* as template, the 200—500 bp (according to the ORF size) internal fragments of the *avr* genes were amplified by PCR. And the PCR with the total DNA and total RNA without reverse transcriptase as templates were used as positive and negative controls, respectively. The internal fragment of 16S rDNA was am-

plified simultaneously as the control of expression level.

1.6 Plant assays

Tests on virulence and the bacterial growth *in planta* were performed on potted Chinese radish Manshenhong (*Raphanus sativus* L. var. radiculus Pers.) and cabbage (*Brassica. oleracae* cultivar Jingfeng No. 1) with leaf clipping method as described by Tang et al.[30], but cell concentration was adjusted to an optical density of 0.01 at 600 nm.

The near-isogenic lines of pepper (*Capsicum annuum*) ECW, ECW-10R and ECW-20R were used for testing HR of wild type *Xcc* 8004 and mutant strains. Bacterial cells were infiltrated into pepper leaves at an optical density of 0.3 at 600 nm by diluting overnight cultures with sterilized water.

1.7 GUS activity assay

The β-glucuronidase (GUS) activity was determined after growth of *Xcc* strains in XVM2 for 16 h to an optical density of 0.5 at 600 nm by measuring the OD_{415} using ρ-nitrophenyl β-D-glucuronide as substrate as described by Henderson et al.[31].

2 Results

2.1 Mutants of all putative *avr* genes of *Xcc* 8004

In the genome of *Xcc* 8004, eight ORFs were annotated to be putative avirulence genes. Mutants with a Tn5*gus*A5 transposon inserted near N-terminal of the *avr* gene and the promoterless *gus* in the same transcriptional direction as the *avr* gene were chosen from our mutant library of *Xcc* 8004. They were designated as 047A01, 011C06, 208H07, 153H12, 003C12, 122A06, 051F05 and 206A12 for the mutant of *avrBs1*, *avrBs1.1*, *avrBs2*, *avrXccA1*, *avrXccA2*, *avrXccB*, *avrXccC* and *avrXccE1*, respectively. All these mutants were confirmed by PCR and were validated as unique insertion by Southern hybridization (data not shown).

2.2 The *avrBs2* is required for full virulence of *X. campestris* pv. *campestris*

To study the potential role of *avr* genes in the virulence of *Xcc*, the virulence of all eight mutants in individual *avr* gene was tested on Chinese radish and cabbage by leaf clipping inoculation. It turned out that 208H07, the mutant of *avrBs2*, showed a significant reduction in virulence (Table 2), whereas all the other 7 mutants did not show any significant changes in virulence compared with the wild type *Xcc* 8004 (data not shown).

Table 2. The virulence of *X. campestris* pv. *campestris* strains on Chinese radish and cabbage*

	Average lesion length (mm) ± standard deviation	
	Chinese radish (Manshenhong)	Cabbage (Jingfeng No. 1)
Wild-type strains *Xcc* 8004	14.73±0.32a	9.2±0.21a
208H07 (Tn5*gus*A5 insertion mutant)	11.48±0.59b	6.8±0.40b
ZH0052 (Integration mutant)	10.83±0.31b	6.6±0.53b

* Mean and standard deviation of 30 replicate measurements of lesion length are given. Similar results were obtained in three independent experiments. Different letters in each data column indicate significant differences at $p = 0.05$.

To further confirm the role of *avrBs2* in virulence of *Xcc* 8004, a single crossover integrational mutant of *avrBs2* was constructed and designated as ZH0052. The mutant was tested for virulence and also showed reduced virulence on Chinese radish and cabbage (Table 2).

To test the role of *avrBs2* in bacterial growth of *Xcc* 8004 in the host, the growth kinetics of 208H07, ZH0052 and 8004 in radish leaves was measured. The bacterial number of 208H07 and ZH0052 recovered from the infected leaves were respectively lower than that of the wild-type strain (Fig. 1).

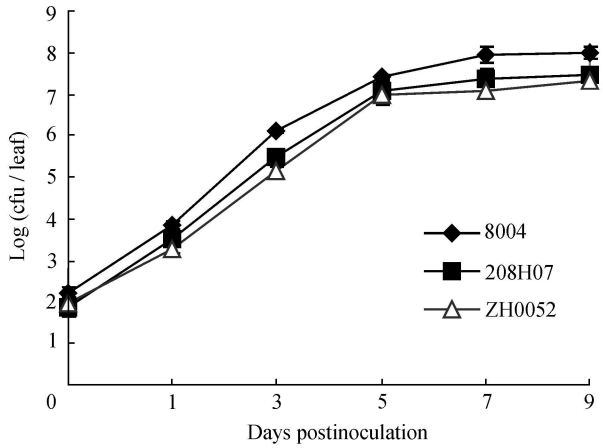

Fig. 1. Growth of *Xanthomonas campestris* pv. *campestris* strains in Chinese radish leaves. Five inoculated leaves for each strain were taken at a two-day interval and were homogenized in sterile water. Diluted homogenates were plated on rich medium supplemented with rifampicin (for the wild-type strain) and rifampicin plus kanamycin (for *avrBs2* mutants). Bacterial colonies were counted after incubation at 28℃ for 2 days and were expressed as colony forming unit (CFU) per leaf. Data are the mean ± standard deviation from three repeats.

2.3 Five *avr* genes are regulated by *hrpG* or *hrpX*

To study whether the eight *avr* genes are regulated by *hrpG*, the *hrpG* gene in each Tn5*gus*A5 insertional mutant of the *avr* gene was disrupted by single crossover integration. The resulting double mutants of *hrpG avr* were named respectively as DM047G, DM011G, DM208G, DM153G, DM003G, DM122G, DM051G and DM206G for *avrBs1*, *avrBs1.1*, *avrBs2*, *avrXccA1*, *avrXccA2*, *avrXccB*, *avrXccC* and *avrXccE1* genes.

GUS activity of double mutant grown in XVM2 was assayed using the corresponding Tn5*gus*A5 mutant as a control. The results showed that the GUS activities of five double mutants, DM047G, DM011G, DM122G, DM051G and DM206G, were 6.9—18.7 folds lower respectively than that of their corresponding Tn5*gus*A5 mutants (Table 3). These indicated that the expression of *avrBs1.1*, *avrBs1*, *avrXccB*, *avrXccC* or *avrXccE1* is regulated by *hrpG*.

All *avr* genes' expression level in wild type *Xcc* 8004 and in 8004△*hrpX* was measured by RT-PCR. The results indicated that all *hrpG*-regulated genes except *avrBs1* were also regulated by *hrpX* (Fig. 2).

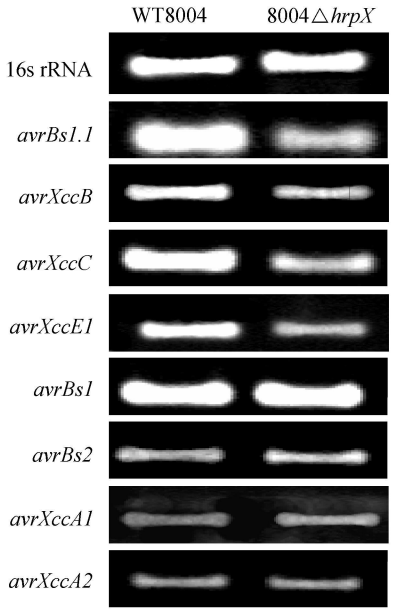

Fig. 2. The expression level of *avr* genes in wild type *Xcc* 8004 and *hrpX* deletion mutant 8004△*hrpX*. Each expressed *avr* gene in wild type *Xcc* 8004 and 8004△*hrpX* was detected by RT-PCR. The detection of expressed 16S rRNA in each strain was included as control. The negative control was the reaction adding no reverse transcriptase and no any bands appeared (pictures not shown).

Table 3. GUS activity assay of *hrpG avr* mutants

Gene name	Mutants	GUS activity[a] (mg/min. OD. ml)	Ratio[b]
avrBs1	047A01	0.0910±0.00043	7.3
	DM047G	0.0012±0.00016	
avrBs1.1	011C06	0.0107±0.00138	6.9
	DM011G	0.0016±0.00041	
avrXccB	122A06	0.0217±0.00262	11.3
	DM122G	0.0019±0.00049	
avrXccC	051F05	0.0182±0.00039	18.7
	DM051G	0.0010±0.00035	
avrXccE1	206A12	0.0072±0.00049	6.9
	DM206G	0.0011±0.00016	
avrBs2	208H07	0.0066±0.00064	0.8
	DM208G	0.0080±0.00155	
avrXccA1	153H12	0.0041±0.00157	0.8
	DM153G	0.0050±0.00161	
avrXccA2	003C12	0.0088±0.00107	0.9
	DM003G	0.0096±0.00217	

a) β-glucuronidase (GUS) activity was assayed after growth of *X. campestris* pv. *campestris* strains in minimium medium XVM2 to OD_{600} 0.5. Data are the mean ± standard deviation of triplicate measurements; b) Data are the ratio of GUS activity value of Tn5*gus*A5 mutant to that of the corresponding double mutant.

2.4 The *avrBs1* is functional in eliciting HR on nonhost plant pepper ECW-10R

All *avr* mutants and wild type *Xcc* 8004 were inoculated onto nonhost plant pepper ECW, ECW-10R and ECW-20R. *Xcc* 8004 could elicit typical HR on ECW-10R (carrying the resistance gene *Bs1*), but not on ECW and ECW-20R (carrying the resistance gene *Bs2*). All mutants except the *avrBs1* mutant and *avrBs1.1* mutant gave the same phenotype as that elicited by the wild type *Xcc* 8004 on ECW-10R. The *avrBs1.1* mutant gave a trace HR and the *avrBs1* mutant completely failed to give an HR on ECW-10R (Fig. 3). The HR inducing ability of *avrBs1* mutants 8004△*avrBs1* and 047A01 could be complemented by complete *avrBs1* gene *in trans* (Fig. 3). At the same time, the *hrcV* or *hrpF* mutant of *Xcc* 8004, 050B12 (*hrcV* mutant) and 121D06 (*hrpF* mutant) in our mutant collection, could not cause an HR on ECW-10R (Fig. 3). These data indicated that *avrBs1* is essential for *Xcc* 8004 to elicit HR on pepper ECW-10R in a type III dependent manner.

Fig. 3. Response of pepper ECW-10R to different *X. campestris* pv. *campestris* strains. Bacterial cells in sterilized water were infiltrated into pepper leaves at concentration of OD_{600} 0.3 with needleless syringe and the picture was taken 24 h post inoculation. 1, 050B12 (*hrcV* mutant); 2, 121D06 (*hrpF* mutant); 3, wild type *Xcc* 8004; 4, sterilized water; 5, CLBs1 (8004△*avrBs1* complementary strain); 6, CL047 (047A01 complementary strain); 7, 8004△*avrBs1*; 8, 047A01.

3 Discussion

We have a Tn5*gus*A5 random insertional mutant library of *Xcc* 8004 with the positions of transposon well localized by TAIL-PCR, which makes mutants of interesting genes readily available. The Tn5*gus*A5 insertion site in the mutant of each *avr* gene used in this study was confirmed by PCR and Southern hybridization. Seven of the eight *avr* genes' mutants of *Xcc* 8004 were not observed to give an altered virulence compared with that of wild type *Xcc* 8004. The *avrBs2* mutant of *Xcc* 8004 showed a significant reduction in virulence when tested on both Chinese radish and cabbage in several independent experiments. But for *Xcc* strain ATCC 33913 (528^T), mutagenesis of all eight *avr* genes had no detected effect on pathogenicity[16]. The *avrBs2* distributed widely in *Xanthomonas*[6, 11—14] and was required for full pathogen fitness and virulence in *Xcv*[6, 32]. Even though, we still cannot say that the other seven *avr* genes do not play any roles in pathogenicity. This is partly because redundant roles of effectors in pathogenicity. Mutants with two or more *avr* genes simultaneously mutated should be constructed and tested.

The regulation of expression of all *avr* genes in *Xcc* by *hrpG* and *hrpX* was first analyzed in this work. It turned out that *avrBs1.1*, *avrXccB*, *avrXccC* and *avrXccE1* were regulated by both *hrpG* and *hrpX*, and *avrBs1* was probably only regulated by *hrpG*. In *Xcv*, the expression of *avrBs1* was independent of *hrpG* or *hrpX*[9].

The elicitation of HR on resistant host and nonhost is thought to be the main function of avirulence genes. Pepper is the host plant of *Xcv*, but not the host of *Xcc*. Sequence analysis revealed that AvrBs1 of *Xcc* 8004 was identical to AvrBs1 of *Xcv* 85—10 and AvrBs1 of *Xcc* ATCC 33913 (528^T). We tested the wild type *Xcc* 8004 and all the mutants on nonhost plant pepper ECW, ECW-10R and ECW-20R. The mutant of *avrBs1* failed to elicit HR on pepper ECW-10R that contains the corresponding *R* gene *Bs1*[33] and could be restored to wild type by entire *avrBs1* provided *in trans*. This differs from the observation for *Xcc* ATCC 33913 (528^T) that *avrBs1* mutant did not show altered HR on nonhost pepper[16]. Furthermore, a mutant of *hrcV* (essential for the TTSS machinery) and a mutant of *hrpF* (essential for effector translocation) failed to give an HR on pepper ECW-10R. These data suggested that AvrBs1 of *Xcc* 8004 is responsible for eliciting HR on pepper ECW-10R that is dependent on type III secretion system.

Xcc 8004 could not induce an HR on pepper ECW-20R. The AvrBs2 of *Xcc* 8004 has 19 amino acid residues different from AvrRxc1/3 of *Xcc* 512/2, which could provide the avirulence activity of *Xcc* 512/2 on pepper ECW-20R[34]. Gassmann and colleagues reported that the strains of *Xcv* with 2 point mutations in AvrBs2 isolated from the natural field lost the HR eliciting ability on pepper ECW-20R[35]. Sequence alignment revealed that the two point mutations occurred in AvrBs2 in *Xcv* field isolates could not be found in AvrBs2 sequence of *Xcc* 8004 and *Xcc* 512/2. However, it was found that 7 amino acid residues of AvrBs2 in *Xcc* 8004 out of the 19 different amino acid residues between *Xcc* 8004 AvrBs2 and *Xcc* 512/2 AvrRxc1/3 were different from those of AvrBs2 in *Xcv* 85-10. It is probable that these corresponding 7 amino acid residues in AvrBs2 of *Xcv* 85-10, which are altered in AvrBs2 of *Xcc* 8004, are essential for the protein function in inducing HR.

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