Mutagenesis of all Eight *avr* Genes in *Xanthomonas campestris* pv. *campestris* Had No Detected Effect on Pathogenicity, But One *avr* Gene Affected Race Specificity

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Suppression subtractive hybridization (SSH) was used to identify genes present in the systemic crucifer black rot pathogen *Xanthomonas campestris* pv. *campestris* 528^T but missing from the nonsystemic crucifer leaf spot pathogen, *X. campestris* pv. *armoraciae* 417. Among the DNA fragments unique to 528^T was Xcc2109, one of eight putative *avr* genes identified in the published 528^T genome (NC_003902). Individual and sequential deletion, insertion mutations, or both of all eight 528^T *avr* gene loci were made, but no change in pathogenicity was observed with any combination of *avr* mutations, including a strain with all eight *avr* genes deleted. However, insertion or deletion mutants affecting the Xcc2109 locus lost avirulence (i.e., became virulent) on Florida Mustard, an *X. campestris* pv. *campestris* race-determining, differential host. The Xcc2109 open reading frame as annotated was cloned and found to be nonfunctional. A longer gene, encompassing Xcc2109 and here designated *avrXccFM*, was cloned and found to complement the Xcc2109 mutants and to confer avirulence to two additional wild-type *X. campestris* pv. *campestris* strains, thereby changing their races. Resistance in Florida Mustard to 528^T strains carrying *avrXccFM* occurred without a typical hypersensitive response (HR) on leaves, although a vascular HR was observed in seedlings.

Additional keywords: chaperones, effectors, gene-for-gene, *hrp*, horizontal transfer, PCR, splice-overlap, type III secretion.

Xanthomonas campestris pv. *campestris* is a xylem-colonizing systemic pathogen that causes black rot of crucifers, a devastating disease worldwide. *Xanthomonas campestris* pv. *armoraciae* (McCulloch 1929) Dye 1978b also infects crucifers but not systemically and causes only localized leaf spots in the mesophyll and also hydathode necrosis (Black and Machmud 1983). Genes contributing to systemic movement of *X. campestris* pv. *campestris* or limiting systemic movement of *X. campestris* pv. *armoraciae* have not yet been identified. As with other xanthomonads, hypersensitive response and pathogenicity (*hrp*) gene mutations in *X. campestris* pv. *campestris* result in a loss of both the ability to induce a hypersensitive response (HR) on nonhosts and pathogenicity on hosts (Daniels et al. 1988; Kamoun and Kado 1990), indicating that type III secretion (Alfano and Collmer 2004; Jin and He 2001) is criti-

cal for pathogenicity of *X. campestris* pv. *campestris*. However, no type III effectors needed for pathogenicity of *X. campestris* pv. *campestris* have yet been identified. Three type III effectors of the AvrBs3 family have been shown to contribute additively to the pathogenicity of *X. campestris* pv. *armoraciae* (Kay et al. 2005).

Specialized methods have been developed to identify type III effectors (Buttner et al. 2003; Nomura and He 2005), including reporter gene fusions (Cunnac et al. 2004; Guttman et al. 2002; Roden et al. 2004a), genomic analyses for plant inducible-promoter (PIP) consensus signatures (Mukaihara et al. 2004) and promoter-trap assays (Losada et al. 2004). More general methods have also been effectively used to identify bacterial genes contributing to pathogenicity, whether type III effectors or not, including chemical and transposon mutagenesis (Daniels et al. 1988; Kingsley et al. 1993), pathogenicity enhancement assays (Chen et al. 1994; Swarup et al. 1991), and suppression subtractive hybridization (SSH) (Golubov et al. 2003; Harakava and Gabriel 2003).

The purpose of this study was to identify *X. campestris* pv. *campestris* genes that contribute to either systemic movement or pathogenicity or both, whether by type III secretion or otherwise. When SSH was applied using *X. campestris* pv. *campestris* 528^T as tester and *X. campestris* pv. *armoraciae* 417 as driver, a putative avirulence (*avr*) gene was found in 528^T but not 417. A search of the annotated genome of 528^T (NC_003902; *Xanthomonas*-ONSA FAPESP network) revealed a total of seven additional putative *avr* genes (Xcc052, Xcc1629, Xcc2099, Xcc2100, Xcc2109, Xcc2396, Xcc3731, and Xcc4229). Since several studies have shown that bacterial *avr* genes encode type III effectors that often function for pathogenicity (Badel et al. 2003; Lin and Martin 2005; Lorang et al. 1994; Wichmann and Bergelson 2004; Yang et al. 1996), this study was focused on the potential role of all annotated 528^T *avr* genes in pathogenicity. Individual and collective mutagenesis of all eight putative *avr* genes in 528^T was performed by using splice overlap polymerase chain reaction (PCR) and marker-interruption techniques.

RESULTS

Suppression subtractive hybridization revealed an *X. campestris* pv. *campestris* *avr* gene.

In an initial screen to find genes present in (systemic) *X. campestris* pv. *campestris* but absent in (nonsystemic) *X. campestris* pv. *armoraciae*, SSH was performed using *X. campestris* pv. *campestris* 528^T as tester against *X. campestris* pv. *armora-*

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ciae 417 as driver. Dot-blot analyses showed that about half of the 528^T clones identified in the SSH screen were not present in 417 (data not shown). A total of 226 clones found in 528^T but not in 417 after dot-blot analyses were sequenced. From these 528^T fragments, 119 corresponded to hypothetical proteins, 11 to enzymatic proteins, 2 to two-component system proteins, and 1 to a predicted Avr protein (Xcc2109), among others. No other potential plant effectors were identified. A total of 21 transposable elements and related genes were also found in 528^T and not in 417. Genes of particular interest were also confirmed as present in 528^T and missing in 417 by Southern hybridization (data not shown).

No evidence of pathogenicity function by any individual 528^T *avr* gene or *XopD*.

To test if any putative *avr* gene in 528^T functioned for pathogenicity, all eight *avr* genes and a *xopD* homolog, Xcc2896, were individually mutated using marker interruption (Fig. 1A, Table 1). Mutagenesis was confirmed by PCR and Southern hybridization (data not shown), and mutants were inoculated on cabbage, white turnip, Seven Top turnip, and radish at two concentrations. No differences in pathogenicity were observed among the mutant and wild-type strains at either of the concentrations tested.

No evidence of collective pathogenicity function by all annotated 528^T *avr* genes.

Collective mutagenesis of all eight annotated *avr* genes in 528^T was performed primarily by using splice overlap PCR-mediated gene deletion (Fig. 1C). This technique proved to be very efficient in 528^T; new mutations were usually made and verified within two weeks. Two different series of sequential, additive mutations in the 528^T *avr* genes were constructed, using the X21 and X37.2 mutants as starting strains to perform successive rounds of mutations (Table 1). The mutations were confirmed by PCR and Southern hybridization (Fig. 2). All mutants were screened initially on cabbage plants and later on white turnip, Seven Top turnip, and radish. No mutant strains, including X8.8, which has mutational deletions or insertions in all eight putative 528^T *avr* genes, showed any alteration in pathogenicity when compared with the wild type. Growth kinetic studies in planta using wild-type 528^T and strain X8.8 revealed that population dynamics in white turnip of the two

strains was not significantly different up to 8 days postinoculation (Fig. 3).

avrXccFM determines avirulence in 528^T on Florida Mustard without a typical HR.

528^T is a race 1 *X. campestris* pv. *campestris* strain that is avirulent on Florida Indian Mustard (Florida Mustard), one of four differential host cultivars (in three different species) used to determine races of *X. campestris* pv. *campestris* (Kamoun et

Fig. 1. Mutagenesis strategies used. **A**, Marker interruption. A DNA fragment internal (hatched) to the target open reading frame (ORF) (vertical bars denote start and stop codons) was cloned in a suicide vector and was introduced into the target strain. Following homologous recombination at the target site, a duplication of the cloned region occurred, and the vector became integrated between incomplete copies of the target gene. **B**, *flp* recognition target (FRT) interruption. A DNA fragment internal to the target ORF was cloned in a suicide vector between FRT sites. Following introduction into the target strain and homologous recombination as for marker interruption, the vector was deleted by addition of a second plasmid, encoding flippase (FLP) recombinase, into the target strain. FLP recombinase catalyzes recombination between two FRT sites, and this resulted in a single FRT "scar" in place of the vector. **C**, Splice-overlap polymerase chain reaction (PCR) (Horton et al. 1989). A region upstream and a region downstream of the target ORF were PCR-amplified and then fused to form a PCR product that carried both ends of the target ORF but not the target region. The PCR product was cloned into a suicide vector containing the *sacB* gene and was introduced into the target strain. Following homologous recombination at one of the target sites, a second homologous recombination event was selected on medium containing sucrose. Since only strains that have lost *sacB* survive on sucrose, double recombination was selected and, as a result, a deletion in the target gene was obtained. Arrows represent primers. Filled arrows represent FRT sites. Regions of homology are shown in boxes with vertical lines.

Table 1. Bacterial strains and plasmids

Strains	Relevant characteristics ^a	Reference or source
<i>E. coli</i>		
<i>E. coli</i> DH5 α	F ⁻ , <i>endA1</i> , <i>hsdR17</i> (rk-mk-), <i>supE44</i> , <i>thi-1</i> , <i>recA1</i> <i>gyrA</i> , <i>relA1</i> , f80d <i>lacZDM15</i> , Δ (<i>lacZYA-argF</i>) U169	Invitrogen Corp. Carlsbad, CA, U.S.A.
<i>E. coli</i> DH5 α -T1	F Φ 80, <i>lacZDM15</i> , Δ (<i>lacZYA-argF</i>) U169, <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (τ_k^- , m_k^+), <i>phoA</i> , <i>supE44</i> ⁻ , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	Invitrogen Corp.
<i>Xanthomonas</i> spp.		
95-2	<i>Xanthomonas axonopodis</i> pv. <i>vesicatoria</i> , wild-type, <i>avrBs3+</i>	J. Jones, University of Florida
417	<i>Xanthomonas campestris</i> pv. <i>armoraciae</i> wild-type	Alvarez et al. 1994
3849A	<i>Xanthomonas campestris</i> pv. <i>campestris</i> wild-type Race 2	Kamoun and Kado 1992
6181	<i>Xanthomonas campestris</i> pv. <i>campestris</i> wild-type Race 0	Vicente et al. 2001
528 ^T	<i>Xanthomonas campestris</i> pv. <i>campestris</i> wild-type, Race 1, type strain of species, synonym: ATCL33913	Alvarez et al. 1994
X54	3849A/pAC19 (<i>avrXccFM</i> in pUFR070), Gm ^r	This work
X55	3849A/pAC10 (Xcc2109 in pUFR070), Gm ^r	This work
X83	6181/pAC19 (<i>avrXccFM</i> in pUFR070), Gm ^r	This work
X84	6181/pAC10 (Xcc2109 in pUFR070), Gm ^r	This work
528 ^T derivatives ^b		
X05	Xcc0052::pAC7, Rif ^r , Kn ^r	This work
X16	Xcc1629::pUFR12 Rif ^r , Kn ^r	This work
X99	Xcc2099::pAC7 Rif ^r , Kn ^r	This work
X100	Xcc2100::pAC7 Rif ^r , Kn ^r	This work
X109	Xcc2109::pAC7 Rif ^r , Kn ^r	This work
X23	Xcc2396::pAC7 Rif ^r , Kn ^r	This work
X37.1	Xcc3731::pAC7 Rif ^r , Kn ^r	This work
X42	Xcc4229::pAC7 Rif ^r , Kn ^r	This work
X48	Δ Xcc2109 Rif ^r	This work
X37.2	Xcc3731::FRT Rif ^r , Kn ^r	This work
X44	Xcc2896::TOPO Rif ^r , Kn ^r	This work
X49	Δ Xcc2099, Xcc3731::pAC3.1, Rif ^r , Kn ^r	This work
X21	Δ Xcc2099, Δ Xcc2100, Rif ^r	This work
X4.4	Δ Xcc0052, Xcc3731::FRT, Rif ^r	This work
X4.1	Xcc3731::FRT, Δ Xcc2099, Δ Xcc2100, Rif ^r	This work
X5.1	Δ Xcc2099, Δ Xcc2100, Δ Xcc2109, Rif ^r	This work
X6.1	Δ Xcc0052, Δ Xcc2099, Δ Xcc2100, Rif ^r	This work
X12.1	Xcc3731::FRT, Δ Xcc2099, Xcc2100, Δ Xcc2109, Rif ^r	This work
X2.1	Δ Xcc0052, Δ Xcc2099, Δ Xcc2100, Δ Xcc2109, Rif ^r	This work
X1.1	Δ Xcc0052, Δ Xcc2099, Δ Xcc2100, Δ Xcc2109, Xcc3731::FRT, Rif ^r	This work
X5.5	Δ Xcc0052, Δ Xcc2099, Δ Xcc2100, Δ Xcc2109, Δ Xcc2396, Rif ^r	This work
X6.6	Δ Xcc0052, Δ Xcc2099, Δ Xcc2100, Δ Xcc2109, Δ Xcc2396, Xcc3731::FRT, Rif ^r	This work
X6.7	Δ Xcc0052, Δ Xcc2099, Δ Xcc2100, Δ Xcc2109, Δ Xcc2396, Δ Xcc4229, Rif ^r	This work
X7.7	Δ Xcc0052, Δ Xcc2099, Δ Xcc2100, Δ Xcc2109, Δ Xcc2396, Xcc3731::FRT, Δ Xcc4229, Rif ^r	This work
X7.8	Δ Xcc0052, Δ Xcc2099, Δ Xcc2100, Δ Xcc2109, Δ Xcc2396, Δ Xcc3731, Δ Xcc4229, Rif ^r	This work
X8.8	Δ Xcc0052, 1629::pUFR12, Δ Xcc2099, Δ Xcc2100, Δ Xcc2109, Δ Xcc2396, Xcc3731::FRT, Δ Xcc4229, Rif ^r , Kn ^r	This work
X8.10	Δ Xcc0052, Δ Xcc2099, Δ Xcc2100, Δ Xcc2109, Δ Xcc2396, Xcc2896::TOPO, Δ Xcc3731, Δ Xcc4229, Rif ^r , Kn ^r	This work
X50	X48/pAC10 (Xcc2109), Rif ^r , Gm ^r	This work
X52	X48/pAC19 (<i>avrXccFM</i>), Rif ^r , Gm ^r	This work
X111	X109/pAC10 (Xcc2109), Rif ^r , Kn ^r , Gm ^r	This work
X113	X109/pAC19 (<i>avrXccFM</i>), Rif ^r , Kn ^r , Gm ^r	This work
Plasmids		
TOPO	PCR cloning vector, Ap ^r , Kn ^r	Invitrogen Corp.
pGEMTez	PCR cloning vector, Ap ^r	Promega, Madison, WI, U.S.A.
pUFR004	ColE1, Mob+, <i>lacZα+</i> , Cm ^r	De Feyter et al. 1990
pUFR012	ColE1, Mob+, <i>lacZα+</i> , Cm ^r , Kn ^r	This work
pUFR047	IncW, Mob+, <i>lacZα+</i> , Par+, Gm ^r , Ap ^r	De Feyter et al. 1993
pUFR070	IncW, Mob+, <i>lacZα+</i> , Par+, Gm ^r , Cm ^r	This work
pUFR080	<i>SacB</i> gene cloned into <i>Dra</i> III site of pAC3.1. ColE1, Mob+, <i>lacZα+</i> , <i>SacB</i> , Cm ^r , Kn ^r	This work
pAC3.1	Derivative of pUFR004. ColE1, Mob+, <i>lacZα+</i> , Cm ^r , Kn ^r	This work
pAC7	Derivative of pUFR004. ColE1, Mob+, Cm ^r , Kn ^r	This work
pUC118	ColE1, M13, <i>lacZα+</i> , Ap ^r	Yanisch-Perron et al. 1985
pFLP2	ColE1, <i>sacB</i> ⁺ , <i>flp</i> ⁺ (Cro promoter), Ap ^r	Hoang et al. 1998
pJR4	<i>sacB</i> ⁺ and <i>flp</i> ⁺ (Cro promoter) from pFLP2 cloned into pUFR47. IncW, Mob+, <i>lacZα+</i> , Par+, <i>sacB</i> ⁺ , <i>flp</i> ⁺ (Cro promoter), Gm ^r , Ap ^r	This work
pKD4	FRT-Kn ^r -FRT, oriR6K, Ap ^r , Kn ^r	Datsenko and Wanner 2000
pKLN66	Kn ^r gene in pGEMT-ez derivative of pKLN56	Newman et al. 2003
pBY17.1	FRT sites from pKD4 recombined into pAC3.1. Cm ^r , Kn ^r	This work
pAC10	Xcc2109 ORF (2492436..2493431 of NC_003902) cloned into pUFR70. Gm ^r , Cm ^r	This work
pAC19	<i>avrXccFM</i> (2492436..2493431 of NC_003902) cloned into pUFR070. Gm ^r , Cm ^r	This work

^a Ap = ampicillin, Gm = gentamicin, Cm = chloramphenicol, Kn = kanamycin, Rif = rifampicin, ORF = open reading frame.

^b All marker-interruption mutations are indicated by novel joint fusions of the indicated plasmid and the indicated 528^T locus. All flippase (FLP) recombinase mutations are indicated by novel joint fusions of the indicated 528^T locus with the *flp* recognition target (FRT) scar. All deletion derivatives were made by splice-overlap polymerase chain reaction (PCR) at the indicated 528^T locus.

al. 1992). Strain 528^T elicited neither disease symptoms nor an HR on leaves of Florida Mustard, although it did elicit a vascular HR in seedlings of Florida Mustard (not shown). No significant electrolyte leakage was observed in leaves of Florida Mustard inoculated with 528^T even 120 h after inoculation (Fig. 4). By contrast, *X. campestris* pv. *armoraciae* 417, which elicited an HR on leaves, exhibited greatly increased electrolyte leakage beginning 24 h after inoculation.

By contrast with the wild type, all mutant strains carrying single or multiple mutations and affecting the Xcc2109 locus of 528^T, whether obtained by marker interruption (e.g., X48) or by splice overlap PCR (e.g., X109), caused systemic pathogenic symptoms on leaves of Florida Mustard, including progressive chlorosis and necrosis outside of the immediate inoculation zone or zones (Fig. 5; Table 2). These symptoms occurred as early as 4 days after inoculation, whether inoculated at high or low concentration. All Xcc2109 mutants also failed to induce the vascular HR on seedlings elicited by the wild-type 528^T strain (not shown). These mutations created a race change from the wild-type 528^T race 1 to race 0 (Table 3), indicating that Xcc2109 was in fact an *avr* gene locus.

da Silva and associates (2002), annotated the Xcc2109 open reading frame (ORF) as *avrXccC*, 996 nucleotides long and encoding 332 amino acids. pAC10, carrying this ORF translationally fused to a 5-amino acid LacZ leader, failed to complement X48 and X109. Further analysis of the Xcc2109 locus indicated that the functional gene was more likely 1,323 bp long, encoding 441 amino acids and starting 327 bp upstream from the annotated start site. This longer gene, here designated *avrXccFM* (avirulence of *X. campestris* pv. *campestris* on Florida Mustard), contains a Shine-Dalgarno (SD) region -11 bp from the start site (2493758 in NC_003902) and a putative PIP box in the promoter region -67 and -83 bp from the start site (TTCG...N16...TTCG). The 1,323-bp *avrXccF* with its native SD and driven by the *lacZ* promoter in pAC19 comple-

mented X48 and X109. Transformants of X48 and X109 carrying pAC19 (*avrXccFM*) were as avirulent on Florida Mustard leaves as the wild-type strain 528^T (Fig. 5). These transformants also restored the vascular HR on seedlings identical to 528^T (data not shown) and completely suppressed the electrolyte leakage otherwise elicited by X48 in inoculated leaves to levels identical to 528^T (Fig. 4).

***avrXccFM* confers race-specific avirulence to *X. campestris* pv. *campestris* strains of other races.**

Strain 6181 belongs to *X. campestris* pv. *campestris* race 0 and 3849A belongs to *X. campestris* pv. *campestris* race 2, based on the host differentials proposed by Kamoun and associates (1992). Southern blot analyses revealed that *avrXccFM* did not hybridize with DNA extracted from either of these strains (not shown). When pAC19 (*avrXccFM*) was introduced into strain 6181, the transformant elicited much

Fig. 3. Growth of wild-type 528^T and X8.8 (all eight *avr* genes interrupted or deleted) in white turnip cultivar Hakurei Hybrid.

Fig. 2. Southern blots of 528^T wild type and X8.8 (mutated in eight annotated *avr* genes) DNA, digested with *EcoRI*. On each blot, from left to right: λ *HindIII* marker, wild type 528^T, and mutant strain X8.8. Except for Xcc1629 (marker-interrupted) and Xcc3731 (*flp* recognition target [FRT] interrupted), all other *avr* gene loci (as annotated) were entirely deleted by splice-overlap polymerase chain reaction (PCR). Probes used were DNA fragments amplified with the following PCR primer sets or DNA fragments amplified and cloned in TOPO or pUFR080. **A**, 52C-52D1 to detect Xcc0052 deletion—loss of an *EcoRI* site results in a larger hybridizing fragment of expected size; **B**, AC5-AC-6 to detect Xcc1629 interruption—integration of the suicide vector introduces a new *EcoRI* site and results in two larger fragments of expected size; **C**, 2099A-2099D to detect simultaneous deletion of both Xcc2099 and Xcc2100, resulting in a smaller fragment of expected size; **D**, AC35-AC45 to detect deletion of Xcc2109, resulting in a smaller fragment of expected size; **E**, 2396A1-2396D to detect deletion of Xcc2396, resulting in a smaller fragment of expected size; **F**, AC32-AC48 to detect interruption of Xcc3731 by an FRT site, resulting in a larger fragment containing an internal region duplication and the FRT scar of expected size, and **G**, 4229A-4229D to detect deletion of Xcc4229, resulting in loss of an *EcoRI* site and creation of a single fragment of expected size.

smaller lesions in Florida Mustard compared with those in the 6181 wild type (Fig. 6), and the bacterial titer in planta was 10 to 100 times lower than found in the wild type (Fig. 7). Introduction of pAC19, therefore, caused a change of 6181 from race 0 to a new and previously uncharacterized race (Table 3). Similarly, when pAC19 was introduced into strain 3849A, the transformant elicited no lesions at all and became avirulent on Florida Mustard plants, resulting in a change of 3849A from race 2 to yet another new and previously uncharacterized race (Fig. 6; Table 3). These results confirmed that *avrXccFM* is an active, race-determining *avr* gene recognized by Florida Mustard plants. When pAC10 carrying the truncated *avrXccFM* (Xcc2109) was introduced into either strain 6181 or 3849A, no effect on virulence was observed on the set of host differentials. (The term “virulence” is used in this paper in a gene-for-gene sense as the antonym of avirulence [Gabriel 2001]).

No evidence of effect of 528^T *avr* mutants on two nonhosts.

All *X. campestris* pv. *campestris* *avr* mutants and mutant combinations were inoculated on nonhost pepper plants ECW, ECW-10R, ECW-20R, and ECW-30R at four different concentrations. No change in intensity of the HR from that elicited by the wild type was observed after 48 h in any of the plants tested (Table 2). Similarly, all *X. campestris* pv. *campestris* *avr* mutants and mutant combinations were inoculated on *Arabidopsis thaliana* ecotypes Col-1 and Col-0 and no change from

Fig. 4. Timecourse of electrolyte leakage from leaves of Florida Mustard plants inoculated with *Xanthomonas campestris* pv. *campestris* wild-type strain 528^T, three of its mutants and *X. campestris* pv. *armoraciae* 417.

Fig. 5. Florida Mustard plants scissor-inoculated with wild-type 528^T and selected mutant strains at 10⁵ CFU/ml. Asterisks indicate the inoculation sites. **A**, 528^T, left; X48 (ΔXcc2109), right. **B**, 528^T, left; X109 (Xcc2109::pAC7), right. **C**, X48, left; X52 (X48/(*avrXccFM*)), right. **D**, X109, left; X113 (X109/*avrXccFM*), right. Photos taken 7 days after inoculation.

the null response elicited by the wild type was observed after 48 h (Table 2).

DISCUSSION

Genomic analysis of *X. campestris* pv. *campestris* 528^T showed that numerous horizontal gene-transfer events probably occurred in this strain; 109 genes related to transposable elements, as well as extended regions with low percent G+C content and uncommon codon usage were found (da Silva et al. 2002). Further analysis revealed at least 285 genes in 528^T that appear to have been horizontally acquired (Garcia-Vallvé et al. 2003), including three of the eight putative *avr* gene homologs investigated in this study.

It is often assumed that, when *avr* genes are acquired through horizontal gene transfer, they become fixed in the bacterial genome because they confer a selective advantage in terms of pathogenicity (Alfano and Collmer 2004; Rohmer et al. 2004). Pleiotropic pathogenicity effects are a common—but not universal—feature of *avr* genes and have been reported in several pathogen species (Abramovitch et al. 2003; Alfano and Collmer 2004; Badel et al. 2003; Bogdanove et al. 1998; Kearney and Staskawicz 1990; Lorang et al. 1994; Swarup et al. 1992; Wichmann and Bergelson 2004; Yang et al. 1996). Once fixed in the genome, the percent G+C content of an acquired gene with selective value may gradually evolve to match that of the recipient (Hacker and Kaper 2000; Lawrence and Ochman 1997). Since five of the eight putative 528^T *avr* genes studied were not identified as being horizontally transferred by Garcia-Vallvé and associates (2003), adaptation to 528^T and a selective value in terms of pathogenicity for these five might seem particularly likely.

Nevertheless, none of the eight putative *avr* gene loci, when deleted or interrupted in 528^T, affected pathogenicity on any of the six cruciferous host differential species tested in growth chambers. Furthermore, they did not appear to confer any pathogenic effect even in an additive way, since inoculations of strain X8.8 (all eight putative *avr* gene loci mutated) was as pathogenic as the wild type when inoculated onto five susceptible *Brassica* species. Growth kinetic studies confirmed no differences between wild type and X8.8 in infections of white turnip plants.

Some effector genes found in *Pseudomonas* spp., including *avr* genes, are known to suppress host defenses, as indicated by suppression of the HR (Abramovitch et al. 2003; Alfano and

Collmer 2004). Indeed, Kamoun and associates (1992) provided evidence for host-defense suppression by *X. campestris* pv. *campestris*; mutations affecting the entire *X. campestris* pv. *campestris* *hrp* system result in the appearance of a vascular HR in inoculated seedling stems that is not elicited by wild-type *X. campestris* pv. *campestris* strains. The one or more effectors involved have not been identified. However, none of

Fig. 6. Race changes observed with transformants of strains from two different *Xanthomonas campestris* pv. *campestris* races carrying *avrXccFM* scissor-inoculated at 10^5 CFU/ml. Asterisks indicate the inoculation sites. **A**, *X. campestris* pv. *campestris* race 0 strain 6181, left; 6181/*avrXccFM*, right. Photo taken 7 days after inoculation. **B**, *X. campestris* pv. *campestris* race 2 strain 3189A, left; 3189A/*avrXccFM*, right. Photo taken 14 days after inoculation.

the eight *avr* genes found in the 528^T genome and tested by mutational analysis in this study appeared to encode effectors that suppress this vascular HR, individually or collectively. Furthermore, none of the *avr* genes found in 528^T, singly or in

Fig. 7. Growth of *Xanthomonas campestris* pv. *campestris* 6181 and its transformant X83 (6181/*avrXccFM*) in Florida Mustard.

Table 2. Disease symptoms on five crucifer hosts and a nonhost species after inoculation with *Xanthomonas campestris* pv. *campestris* 528^T and mutant strains

<i>X. campestris</i> pv. <i>campestris</i> strains	<i>Arabidopsis thaliana</i> ^a	Cabbage	Turnip	Radish	Mustard	Pepper
528 ^T	Ø	+	+	+	Ø	HR
X05	ND ^b	+	+	+	Ø	HR
X16	ND	+	+	+	Ø	HR
X99	ND	+	+	+	Ø	HR
X100	ND	+	+	+	Ø	HR
X109 ^{*c}	ND	+	+	+	+	HR
X23	ND	+	+	+	Ø	HR
X3731	ND	+	+	+	Ø	HR
X42	ND	+	+	+	Ø	HR
X48*	Ø	+	+	+	+	HR
X37.2	Ø	+	+	+	Ø	HR
X44	ND	+	+	+	ND	ND
X49*	ND	ND	ND	ND	+	ND
X21	ND	+	+	+	Ø	HR
X5.1*	Ø	+	+	+	+	ND
X6.1	Ø	+	+	+	Ø	HR
X2.1*	Ø	+	+	+	+	ND
X5.5*	ND	+	+	+	+	HR
X6.7*	ND	ND	ND	ND	+	ND
X7.8*	ND	ND	ND	ND	+	ND
X8.8*	ND	+	ND	+	ND	HR
X8.10*	Ø	ND	+	ND	+	ND
X50* (X48/pAC10)	ND	ND	+	ND	+	ND
X52 (X48/pAC19)	ND	ND	+	ND	Ø	ND
X111* (X109/pAC10)	ND	ND	+	ND	+	ND
X113 (X109/pAC19)	ND	ND	+	ND	Ø	ND
3849A*	ND	+	+	ND	+	ND
X53* (3849A/pAC10)	ND	ND	+	ND	+	ND
X54 (3849A/pAC19)	ND	ND	+	ND	Ø	ND
6181*	ND	+	+	ND	+	ND
X82* (6181/pAC10)	ND	ND	+	ND	+	ND
X83 (6181/pAC19)	ND	ND	+	ND	(+)	ND

^a Plant cultivars used were: *Arabidopsis thaliana*, ecotypes Col-1 and Col-0; cabbage, Early Jersey Wakefield; turnip, Hakurei Hybrid; radish, Sparkler; Radish; mustard, Florida Broad Leaf Indian Mustard; and pepper, ECW, ECW10R, ECW20R, ECW30R.

^b ND = not determined; + = typical black rot symptoms; (+) = reduced black rot symptoms; Ø = null response; HR = hypersensitive response.

^c An asterisk (*) indicates strains in which the *Xcc2109* locus (*avrXccFM*) was deleted, interrupted, or absent.

Table 3. Race changes determined by *avrXccFM* addition or deletion in three *Xanthomonas campestris* pv. *campestris* strains^a

<i>Xanthomonas</i> strains	Early Jersey Wakefield	Just Right Turnip	Seven Top Turnip	Florida Broad Leaf Indian Mustard
528 ^T (<i>avrXccFM</i>) race 1	+	+	+	Ø
X48 (528 ^T Δ <i>avrXccFM</i>) race 0	+	+	+	+
X52 (528 ^T Δ <i>avrXccFM</i> / <i>avrXccFM</i>) race 1	+	+	+	Ø
6181 race 0	+	+	+	+
X83 (6181/ <i>avrXccFM</i>) race ?	+	+	+	(+)
3849A race 2	+	+	Ø	+
X54 (3849A/ <i>avrXccFM</i>) race ?	+	+	Ø	Ø

^a + = typical black rot symptoms; (+) = reduced symptoms; Ø = null response.

any combination, affected the nonhost HR elicited by 528^T on pepper plants (Table 2).

A few putative non-*avr* effector genes have been identified in 528^T, based on sequence similarity to *Ralstonia*, *Pseudomonas*, and *Xanthomonas* type III secreted proteins, but only one has some experimental support as an effector in *Xanthomonas* spp. This ORF, classified as *psvA* (Xcc2896) by da Silva and associates (2002), is 71% identical to *xopD* from *X. campestris* pv. *vesicatoria*. Since *xopD* was shown to be secreted by the type III system and also to have small ubiquitin-like modifier proteolytic activity (Noel et al. 2002; Roden et al. 2004b), this gene was also included in the present study. However, interruption of the *xopD* homolog in 528^T alone or in combination with up to seven of the *X. campestris* pv. *campestris* *avr* genes (Tables 1 and 2) also did not reveal any effect on 528^T pathogenicity.

avrXccFM functioned for avirulence. Mutations of this gene in 528^T, which is avirulent on Florida Mustard, enabled the mutants to become virulent on that differential host. *avrXccFM* is

among three *avr* genes identified in 528^T as likely acquired by horizontal gene transfer (Garcia-Vallvé et al. 2003). When *avrXccFM* was transferred to strains from two other races of *X. campestris* pv. *campestris* that are virulent on Florida Mustard, the bacteria became either completely avirulent, as in the case of race 2 strain 3849A, or somewhat avirulent, as in the case of race 0 strain 6181 (Table 3; Figs. 6 and 7). The visible although much smaller lesions observed in the latter case might be due to i) poor gene expression of *avrXccFM* in strain 6181; ii) a defense suppression mechanism specific to 6181, or iii) lack of a chaperone-like protein or an imperfectly functioning chaperone-like protein required for delivering *avrXccFM* into the host. Chaperone-like proteins have been identified as important secretion cofactors for some Avr effectors and can enhance their delivery to the host and the subsequent defense response (Alfano and Collmer 2004; Buttner et al. 2004).

Despite conferring avirulence to *X. campestris* pv. *campestris* strains on Florida Mustard, *avrXccFM* did not elicit an obvious

Table 4. Primers and conditions used in this study

Number	Sequence ^a	Time	Gene	Ex. time ^b
For marker interruption				
AC-5	<u>GGAATTC</u> <u>CAGAATAGGAA</u> CCTTCAATTCATGGGCAGGAAGCGCACTG	>72	1629	30 s
AC-6	GGGATCCAGTATAGGAACTTCTCTGAATCCGTTGTCTGTCCAG	>72	1629	30 s
AC-23	<u>GGATCC</u> ATGATGAGAGACTGCATGTAC	68	2099	30 s
AC-24	<u>GAGCTC</u> TGACCGTTCATTACGAAATTC	68	2099	30 s
AC-29	<u>GGATCC</u> ACGCTGCATGACATTGTC	66	2100	30 s
AC-30	<u>GAGCTC</u> ATTTCACGGATATGACTTCC	66	2100	30 s
AC-27	<u>GGATCC</u> AAGGAACTGCTACAACATATC	66	2109	30 s
AC-28	<u>GAGCTC</u> GCACATAATGGCATTATCATC	68	2109	30 s
AC-25	GGCATCCAGTTCTACAGCGGGCGG	68	2396	30 s
AC-26	<u>GAGCTC</u> AGCGGCGTCAACGG	68	2396	30 s
AC-19	<u>GTTAAC</u> GTGGAGCGGATCCATG	68	3731	30 s
AC-20	<u>GAGCTC</u> ACATAGAGCACGTCAGAG	70	3731	30 s
AC-17	CCACCTGGATCCGGGCTTCG	68	4229	30 s
AC-18	<u>GAGCTC</u> AGGGTCACGCTCCACG	68	4229	30 s
AC-C1	GGCGACGGCGTGTCCAGCGCC	>72	4229	30 s
AC-C2	GTGTAGTCCAGTTGACGTTGC	68	4229	30 s
AC-60	TGGCCGCGAATTCGACCTCAAC	68	2896	30 s
AC-61	CGACGACGAGCAATGACCAATGAAAGT	>72	2896	30 s
For interruption confirmation				
AC-13	GCAGGCGGCCTACCAGCTTG	68	1629	1 min
AC-14	TGCGCGGCGAAATGGGCTGC	68	1629	1 min
AC-15	GGTCATCATCTGCCCGCCATG	68	1629	1 min
AC-37	ATGTCAATGGAGCGGGAGATGG	68	2099	1 min
AC-43	TTATGCATTGTGGTCGAGCCATTC	70	2099	1 min
AC-49	GCGAGCGCGGCAGGACTAC	66	2099	1 min
AC-50	CCAGCAAGGTGGTGCAATCGG	68	2099	1 min
AC-34	ATGTCCGACATGAAAGTTAATTTCTG	70	2100	1 min
AC-41	TTACGCTTCTCCTGCATTGTGAAC	68	2100	1 min
AC-35	ATGTGGTCTCAGCCCGTATGG	66	2109	1 min
AC-39	TTAGGATAATCAGCCACAATTTGG	66	2109	1 min
AC-45	CTGCAGTTTTTGTACGAATCCCTACCGATC	68	2109	1 min
AC-36	GTGCTGGAGAGTGCCGATGGC	70	2396	1 min
AC-44	GTGAGACCACAGTGAATCGCC	66	2396	1 min
AC-32	GTGGTGGCGGCCGAGAATCAC	66	3731	1 min
AC-48	TTAGCTCCAGTACTCGGCGTC	66	3731	1 min
AC-33	TGCCCAGCGCCCTCATGC	66	4229	1 min
AC-42	AGTTCCAGATCGCCACGCACC	68	4229	1 min
FW-XopD	ATGGAATATATACCAAGATA	50	2896	1 min
RV-XopD	CTAGAACTTTTCCACCACTT	58	2896	1 min
For splice overlap PCR				
52-A1	CGCTGGCCGCGCAATGGATG	68	52	1 min
52-F	<u>TCATACGCGTTCAGATCTTA</u> CTGTTCTAGCGCAGGCGATG	60	52	1 min
52-C	<u>TAAGATCTGAACGCGTATGA</u> GCAGACCATTCGCACGATG	60	52	1 min
52-D1	CCGATGGATCTATTGTTCTTCG	64	52	1 min

(Continued on next page)

^a Underlined sequences indicate restriction enzyme sites or homology regions added for splice overlap PCR. Unless otherwise indicated, all primer sequences from 5' to 3'.

^b Polymerase chain reaction (PCR) extension time used.

^c NA = not applicable.

^d V = variable time.

HR in any *X. campestris* pv. *campestris* strain tested on Florida Mustard leaves. Florida Mustard leaves are capable of an obvious HR, since they exhibited a typical HR response to wild-type *X. campestris* pv. *armoraciae*, beginning 48 h after inoculation, that was coincident with electrolyte leakage (Fig. 4). Resistance to bacterial plant pathogens is not always associated with an obvious HR (Balague et al. 2003). More specifically, resistance of *Brassica* plants to *X. campestris* pv. *campestris* is not (usually) associated with an obvious HR (Buell 2002; Buell and Somerville 1997). Kamoun and associates (1992) proposed that resistance to *X. campestris* pv. *campestris* is not associated with a mesophyllic HR but is associated with a more difficult-to-detect vascular HR. Significantly, *avrXccFM* appeared responsible for eliciting the vascular HR in seedlings inoculated with 528^T, since mutants at the locus lost such ability and *avrXccFM* complemented the mutations. This observation is therefore consistent with the hypothesis that an HR occurs in leaves but is limited to cells surrounding vascular tissue (Bretschneider et al. 1989; Kamoun et al. 1992).

X. campestris pv. *campestris* was proposed to be comprised of five races, based on disease reactions using four differential varieties in three species: Early Jersey Wakefield cabbage (*B. oleracea*), Just Right turnip and Seven Top Green turnip (*B. rapa*), and Florida Broadleaf Indian Mustard (*B. juncea*) (Kamoun et al. 1992). Vicente and associates (2001) proposed a new race structure for *X. campestris* pv. *campestris* defined by two additional host differentials, PI199947 (*B. carinata*) and Miracle F1 (*B. oleracea*). However, we could not reproduce the proposed race reactions on these additional differentials and therefore used the original system of Kamoun and associates (1992). It is possible that some varietal variation exists within PI199947 and Miracle F1.

Ignatov and associates (2003) suggested that an *avrBs2* homolog determines avirulence of *X. campestris* pv. *campestris* 512/2 on *Brassica* plants with the B genome, specifically, both Florida Mustard and PI199947, and was also responsible for a mesophyllic HR on leaves of both Florida Mustard and nonhost pepper plants carrying *Bs2*. However, our analy-

Table 4. (Continued from preceding page)

Number	Sequence ^a	Time	Gene	Ex. time ^b
For splice-overlap PCR (Continued)				
2099-A	GGAATTCTCGATGACCTGCTCCAACG	>72	2099, 2100	1 min
2099-B	TCATACGCGTTCAGATCTTACATCTATGGGGCCTGTTCG	60	2099, 2100	1 min
2099-C	TAAGATCTGAACGCGTATGAAGAGAAGAAGTATCCGCCAC	60	2099, 210	1 min
2099-D	GGAATTCGGACGAACCTCGCCAGCC	>72	2099, 2100	1 min
2109-A	TTTGTCGAGCGAGCGTCAC	60	2109	1 min
2109-B	TCATACGCGTTCAGATCTTACCAATTTGTGGCTGATTATCC	60	2109	1 min
2109-E	TAAGATCTGAACGCGTATGAGTCCGAAATCTGGTGAAGAG	60	2109	1 min
2109-F	GTGAGTTCGGCCTACAACCA	62	2109	1 min
2396-A1	GCTGATCTGGAAGTTGTAGG	60	2396	1 min
2396-B	TCATACGCGTTCAGATCTTATACCTGCTGATGCACATGTC	60	2396	1 min
2396-C	TAAGATCTGAACGCGTATGAGGTCGTGCAAGTGGGCAGTGG	60	2396	1 min
2396-D	GCAGTGCGGATGGCAGCC	62	2396	1 min
4229-A	GGCGTTTTCCATGCTGATGTAC	66	4229	1 min
4229-B	TCATACGCGTTCAGATCTTAGCAGGCGGCGGGGCAATGCAGGC	60	4229	1 min
4229-C	TAAGATCTGAACGCGTATGAGCCGATCAACAGCCTGCGCTC	60	4229	1 min
4229-D	CGCGTGGTCTGACTGACAACG	66	4229	1 min
For deletion confirmation				
52-E	GCTGGATCTGATCCGAGT	60	52	1 min 30 s
52-H	CCTGGGTGGACACGATGTG	66	52	1 min 30 s
2099-A1	GTGCTGCGCTGATGTATTCGG	66	2099, 2100	2 min
2099-D1	AAGACAAGAGCGACCAACACC	64	2099, 2100	2 min
2109-A1	GCAATCGAGGTATCGTCATG	60	2109	1 min 30 s
2109-F	GTGAGTTCGGCCTACAACCA	62	2109	1 min 30 s
2396-A1	GCTGATCTGGAAGTTGTAGG	60	2396	1 min 30 s
2396-D1	CCGTACTACCGCCATGCC	60	2396	1 min 30 s
4229-A1	AATCGGCGAACTCGTTGTTG	60	4229	1 min 30 s
4229-D1	CTCCGGGCGCACCATCCAGATC	74	4229	1 min 30 s
For <i>flp</i> recognition target (FRT) plasmid construction				
FRTKn-F	GAATTCGCTGCTTCGAAGTTCCTATAC	62	NA ^c	1 min 30 s
FRTKn-R	AAGCTTATCCTCCTTAGTTCCAATTCC	60	NA	1 min 30 s
For complementation assays				
9.03	GAATTCATGGGTCTATGCGCTTCAAAACC	68	2109	1 min 30 s
9.04	GAATTCAGGAGATCGACATGGGTCTATG	70	2109	1 min 30 s
AC-35	ATGTGGTCTCAGCCCGTATGG	66	2109	1 min 30 s
AC-45	CTGCAGTTTTGTACGAATCCCTACCGATC	68	2109	1 min 30 s
For SacB cloning				
SAC-C2	CACGTA GTGATATGGATGACGGATG	70	SacB	1 min
SAC-D2	CACTACGTGAAATGAGATATTATGATATTTTC	70	SacB	1 min
For suppression subtractive hybridization (SSH)				
Adaptor 1	CTAATACGACTCACTATAGGGCTCGAGCGGCCGCGGAGGT3'- GGCCCGTCCA-5	SSH	NA	NA
Adaptor 2R	CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT3'GCCGGC TCCA5'	SSH	NA	NA
Primer 1	CTAATACGACTCACTATAGGGC	SSH	NA	
Nested primer 1R	TCGAGCGGCCGCGGCGGAGGT	SSH	NA	NA
Nested primer 2R	AGCGTGGTTCGCGGCCGAGGT	SSH	NA	NA
Vector-based primers				
M13R(-48)	AGCGGATAACAATTTACACAGGA	64	Vector based	V ^d
M13(-47)	GCCAGGGTTTTCCAGTCACG	64	Vector based	V
JR2	ACGATCGGTTTCGCCAGCCAGGAC	54	Gm ^f	1 min

sis showed that deletion of the entire putative *avrBs2* homolog Xcc0052 (Fig. 2; Table 2) in *X. campestris* pv. *campestris* 528^T i) had no effect on avirulence in Florida Mustard (i.e., all Xcc0052 knockout strains remained avirulent) and ii) had no effect on the nonhost HR in any of the four different pepper cultivars inoculated (i.e., all Xcc0052 knockout strains elicited an HR), including ECW-20R (carrying *Bs2*). Furthermore, wild-type 528^T was virulent on PI199947 (data not shown), and we did not detect a mesophylllic HR in leaves of Florida Mustard either visually or using electrolyte leakage, using wild-type 528^T. It is possible that strain 512/2 carries an active *avrBs2* homolog and 528^T did not. If so, by gene-for-gene theory, Florida Mustard would carry at least two resistance genes against *X. campestris* pv. *campestris* (*FM* and *Bs2* or functional equivalent) and 512/2 would not carry *avrXccFM*. This hypothesis is testable.

Of the eight putative 528^T *avr* genes and putative *xopD* effector gene experimentally investigated here, only *avrXccFM* appeared to be phenotypically functional. There are a number of possible explanations for this. First, it is possible that some or all of the *avr* effectors are pseudogenes or poorly expressed. Second, these genes may encode proteins with defective secretion signals and so fail to be recognized by the type III secretion system or by a required chaperone; at least some effectors appear to require chaperones for secretion (Alfano and Collmer 2004). Third, it is possible that some of these genes were horizontally acquired without their chaperones and so fail to be secreted. However, these explanations seem generally unlikely for all eight *avr* genes.

It is argued that assays conducted in growth chambers may not be sensitive enough to detect additive *avr* gene contributions to pathogenicity or not done in the right context to detect *avr* gene effects on ecological fitness (Chang et al. 2004). For example, although mutation of *avrBs2* in *X. axonopodis* pv. *vesicatoria* had a major effect on pathogenicity and the mutation of three additional *avr* genes in the same strain had no detected effect on pathogenicity in growth-chamber tests, field tests revealed statistically significant, additive effects of the three additional *avr* genes on ecological fitness (Wichmann and Belgersen 2004). However, the converse is also true; pathogenicity assays in growth chambers may not be sensitive enough to detect negative effects on pathogenicity or on ecological fitness, such as the reduced survival in soil conferred by *avrBs1* to *X. axonopodis* pv. *vesicatoria* (O'Garro et al. 1997). In the experiments reported here, all eight of the putative *avr* genes identified in the *X. campestris* pv. *campestris* genome were disrupted or removed, including *avrBs2*, leaving none behind. While it is possible that some of these genes may contribute to reduced or increased ecological fitness in the field, they had no detected effect, even additively, on in planta growth or pathogenicity.

Clearly, since *hrp* system knockouts in *X. campestris* pv. *campestris* are completely nonpathogenic in growth chamber tests (Kamoun et al. 1992), the type III effectors primarily responsible for *X. campestris* pv. *campestris* systemic pathogenicity are not among the eight *avr* gene homologs identified to date. Besides the eight *avr* effectors and *xopD* examined in this study, eight additional putative effector genes were identified in 528^T that remain to be functionally tested. These include two leucine-rich proteins having PIP boxes (Xcc2565 and Xcc4186; da Silva et al. 2002) and six genes identified as type III-secreted (Xcc1072, Xcc1089, Xcc1246, Xcc1247, Xcc3258, and Xcc3600; Roden et al. 2004a; Rohmer et al. 2004; Genin and Boucher 2004).

Since the type III secretion machine is indiscriminate, secreting all available effectors with a type III signal into both host and nonhost cells alike (Jin et al. 2003; Rossier et al. 1999;

Silhavy 1997), horizontal transfer of effector genes should result in the accumulation in any given pathogenic strain of genes or gene relics annotated as effector homologs. Given the high degree of host specificity of type III effectors, relatively recent horizontal transfer events should result in gratuitous, out-of-context effectors (Gabriel 1999). Unadapted type III effectors should be either generally detrimental (O'Garro et al. 1997) or detrimental in specific contexts, as are all avirulence genes, by definition. Adaptation, if any, of detrimental genes to new host or pathogen contexts should often result simply in loss-of-function mutations, which may have occurred with some or all of the remaining seven of the annotated 528^T *avr* gene homologs investigated in this study.

MATERIALS AND METHODS

Plasmids, bacterial strains, and culture conditions.

Bacterial strains and plasmids used or constructed in this study are listed in Table 1. *X. campestris* pv. *campestris* strains were grown in PYGM at 30°C (De Feyter et al. 1990), and *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium (Maniatis et al. 1982). When appropriate, antibiotics were used at the following concentrations (μg/ml): ampicillin (Ap), 50; gentamicin (Gm), 5; kanamycin (Kn), 12.5 for xanthomonads or 20 for *E. coli*; rifampicin (Rif), 75; and chloramphenicol (Cm), 35. When needed, sucrose was added to a final concentration of 5%.

SSH.

The PCR select bacterial genome subtraction kit, (Clontech Laboratories, Inc., Palo Alto, CA, U.S.A.) was used according to the manufacturer's instructions, with the following modifications. 528^T and 417 genomic DNA were extracted using the Genomic prep kit (Amersham Biosciences, Piscataway, NJ, U.S.A.). Approximately 2 μg of both 528^T and 417 genomic DNA were digested with 15 U of *RsaI* for 16 h. Half of the DNA from 528^T was ligated to adaptor 1 and the other half to adaptor 2R as recommended. Digested 417 DNA was mixed with each of the 528^T ligated adaptor DNAs in separate tubes, and the samples were hybridized at 63°C. Amplification of the SSH product was performed with nested primer 1 (Table 4) at 61°C for 35 cycles, and the second round of amplification was performed with nested primer 2R at 61°C for 25 cycles.

DNA from the second PCR amplification mix (3 μl) was ligated into pGEMTeasy (Promega Co., Madison, WI, U.S.A.) according to the manufacturer's instructions, were transformed into DH5α cells, and were plated on LB plus Ap.

Xanthomonas electroporation.

Overnight liquid *Xanthomonas* broth cultures in PYGM medium were grown at 28°C to mid-exponential growth phase (optical density at 600 nm (OD₆₀₀) = 0.5 to 0.6). Cells were incubated on ice for 30 min and were collected by centrifugation at 4,000 × g at 4°C. Cell pellets were washed three times with approximately 10 ml of chilled sterile distilled water and were collected as before. Cells were then resuspended in 160 μl of sterile, ice-cold 10% glycerol, and 40 μl of cells were electroporated immediately or were aliquoted and stored at –80°C. Approximately 250 ng of plasmid DNA was electroporated into 40 μl of electrocompetent *Xanthomonas* cells at 18 kV/cm, using an Eppendorf 2510 electroporator (Westbury, NY, U.S.A.). Following electroporation, 960 μl of liquid PYGM was added to the mix, the culture was grown at 30°C for 2 to 3 h at 120 rpm, and the entire amount was spread onto PYGM plates containing an appropriate antibiotic for selection of the introduced plasmid.

Site directed and serial mutagenesis

Marker interruption, *flp* recognition target (FRT) interruption, and splice-overlap PCR (marker deletion) mutagenic methods (Fig. 1) were used to create individual and serial mutations in the 528^T genome following electroporation. Primers used were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.) and are listed in Table 4.

For marker interruption (Fig. 1A), an internal region of the target gene was PCR-amplified, using a single colony touched with a toothpick and swirled into the PCR mix as the source of template DNA. Reactions (25 µl) were performed using Invitrogen (Carlsbad, CA, U.S.A.) *Taq* polymerase and PCR buffer according to instructions, with each primer at a final concentration of 0.4 µM. The initial denaturation step was at 95°C for 3 min, followed by 35 cycles of 30 s at 95°C, 30 s at the specified annealing temperature, 70°C for the specified extension time, with a final 10 min extension at 70°C. The PCR reaction (1 µl) was used for cloning into TOPO vector (Invitrogen Corp., Carlsbad, CA, U.S.A.), using the manufacturer's protocol and, except for the *XopD* homolog, in which the internal region was cloned into TOPO and used directly for interruption, the insert was recloned into pAC7, pAC3.1, or pUFR12 and was used for electroporation into 528^T or mutant derivatives. Colonies that grew on PYGM plus Kn were transferred to PYGM plus Cm plates to confirm vector integration. Those that were resistant to both antibiotics were then tested by PCR and Southern hybridization to verify that the target gene was disrupted. For marker interruption using TOPO, the colonies that grew on PYGM plus Kn were PCR-tested immediately.

For FRT interruption (Fig. 1B), a simplified variation on the method of Hoang and associates (1998) was used. An internal region of the target gene was PCR-amplified using the appropriate primers (Table 4) and was cloned into TOPO, recloned into pBY17.1, and electroporated into 528^T. Marker interrupted mutant strains of 528^T were first selected on PYGM plus Kn, followed by screening on PYGM plus Cm, and then were analyzed by PCR using both vector- and fragment-based primers. Positive colonies were selected and then were again electroporated with 50 ng of pJR4, were plated onto PYGM plus Gm, and then, were grown in sucrose to evict the integrated suicide vector. The resulting colonies were replica-plated onto PYGM plus sucrose to induce curing of pJR4, and colonies growing only on sucrose without Kn were selected for screening by PCR and Southern hybridization.

For splice overlap PCR (Fig. 1C; Horton et al. 1989), two regions approximately 1 kb long, upstream and downstream of the target gene, were independently PCR-amplified with their respective primer sets a, b and c, d using Fail Safe PCR premix D (Epicenter Technologies, Madison, WI, U.S.A.) and PCR conditions as described above. Both PCR products were diluted 1:25, 1 µl of each were mixed together, and a fill-in reaction was done in the same buffer (95°C for 3 min, 50°C for 1 min, and 70°C for 5 min) using *Taq* polymerase. External primers (Fig. 1C, a and d) were then added at 0.4 µM each to the reaction, and 35 cycles of PCR were performed as before. This reaction (1 µl) was cloned directly into TOPO or the desired band was gel-purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA, U.S.A.) and was cloned into TOPO. The resulting plasmids were transformed in DH5α cells. Inserts of the correct size were recloned into pUFR080 and were sequenced and electroporated into 528^T or mutant derivatives. Colonies with plasmid integration at one of the 528^T target sites were selected on PYGM plus Kn plates and were subsequently confirmed on PYGM plus Cm plates. These were grown in liquid PYGM overnight, the OD₆₀₀ was adjusted to 0.13, and 20 µl were spread onto

PYGM containing sucrose and Rif. Colonies that grew on sucrose were replica-plated onto PYGM plus Kn and sucrose, and colonies growing only on sucrose plates without Kn were selected and analyzed by PCR and Southern hybridization. Probes used for Southern hybridizations were PCR products cloned in TOPO or pUFR080.

DNA sequencing and analysis.

All DNA sequencing was performed by the ICBR (Interdisciplinary Center for Biotechnology Research) DNA Sequencing Core Facility at the University of Florida. All 528^T DNA clones resulting from SSH were sequenced. To confirm that the cloned DNA fragments were unique to 528^T, the SSH clones were PCR-amplified with vector-based primers M13R (-48) and M13 (-47). Each PCR amplification product (2 µl) was spotted twice with a replicator (VP408S2a; V & P Scientific Inc., San Diego, CA, U.S.A.) on two different nylon membranes and also was run on 1% agarose gels to confirm that the amplified products were uniform in concentration.

Total *RsaI*-digested 528^T and 417 DNA (500 ng) were separately labeled with ³²P, using the Random primer kit II (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's protocol. The two labeled DNA samples were used as a probe with each of the corresponding membranes. Clones of interest were further confirmed as 528^T specific by Southern hybridization analyses.

Plant inoculation assays.

All plants were grown under greenhouse conditions. Leaves from 3-week-old cabbage Early Jersey Wakefield (*B. oleracea* var. *capitata*; Sawan Seed Co., Pelham, GA, U.S.A.), Seven Top Turnip (*B. rapa*; Sieger Seed Co., Zeeland, MI, U.S.A.), Sparkler Radish (*R. sativa*; Excel Seeds, Camp Point, IL, U.S.A.), Florida Broadleaf Indian Mustard (*B. juncea*; Sieger Seed Co.), white turnip Hakurei Hybrid (*B. rapa*; Ferry-Morse Seed Co. Phoenix, AZ, U.S.A.), PI199947 (*B. carinata*), cauliflower Miracle F1 (*B. oleracea* var. *botrytis*), and *A. thaliana* ecotypes Coimbra-1 and Columbia-0 were inoculated at two concentrations.

Xanthomonas cultures were grown overnight in liquid PYGM, and then, were centrifuged at 5,000 × *g* for 5 min, were washed with sterile tap water, and were adjusted to OD₆₀₀ = 0.3 (approximately 10⁸ CFU/ml). A 1:1,000 dilution of this suspension was made for low-concentration inoculations (approximately 10⁵ CFU/ml). For each inoculation, 2 cm on each side of each leaf was cut with scissors that had been dipped in the appropriate bacterial suspension. Scissors were flame-sterilized between inoculations. For HR screening, cultures at an OD₆₀₀ = 0.3 were pressure-infiltrated using the blunt end of a tuberculin syringe into the abaxial leaf surface. After inoculation, plants were maintained in a standard growth chamber equipped with fluorescent and incandescent lights for 16 h at 28°C, followed by 8 h of dark at 27°C for up to 14 days. Pathogenic symptoms were scored daily. HR symptoms were scored every hour, from 12 to 36 h after inoculation. At least three plants were inoculated in each experiment. All experiments were reproduced at least two times.

For seedling assays, Florida Mustard seeds were grown under continuous light in growth chambers at 29°C. Seven-day-old plants were inoculated by pricking the hypocotyls with a 25-gauge syringe needle that was dipped into a bacterial colony (Kamoun et al. 1992). Plants were observed starting at 16 h and for up to 5 days.

For the nonhost HR, pepper plants ECW, ECW-10R, ECW-20R, and ECW-30R (*Capsicum annuum*) were inoculated by pressure infiltration, using the blunt end of a tuberculin syringe into the abaxial leaf surface at four serially diluted concentra-

tions (10^8 , 10^7 , 10^6 , and 10^5 CFU/ml) and were observed at 18, 24, 36, and 48 h after inoculation. HR symptoms were scored every hour, from 12 to 36 h after inoculation. At least three plants were inoculated in each experiment, and each experiment was repeated at least two times.

Electrolyte leakage assays.

Bacterial suspensions were diluted to a concentration of 1×10^5 CFU/ml in sterile tap water, and measurements were carried out exactly as described previously (Hibberd et al. 1987). Essentially, for each sample, six 0.5-cm^2 leaf disks were removed with a #7 cork borer, were submerged in 3 ml of distilled water, and were vacuum infiltrated, and the net leakage after 1 h was measured with a conductivity meter. Two samples were taken for each measurement in each experiment; the experiments were repeated three times.

Growth kinetics.

The abaxial leaf surface of fully expanded leaves from white turnip and Florida Mustard plants were syringe-infiltrated with 1×10^5 CFU/ml of inoculum. Each inoculation site was flooded to achieve a zone of approximately 0.5 cm in diameter. Samples were taken in triplicate with a #14 cork-borer (2.2-cm diameter) to encompass the entire inoculation site starting at day 0 and then every two days until day 8. The leaf tissue samples were macerated in 1 ml of 100 μ l of Silwet L-77 (Osi Specialties Inc., Friendly, WV, U.S.A.) plus saturated CaCO_3 in water, were serially diluted, and were plated on PYGM plates. Colonies were counted two days after plating.

Complementation assays.

Primers AC-35 and AC-45 (Table 4) were used to amplify ORF Xcc2109 (*Xanthomonas*-ONSA FAPESP network). Primers 09-04 and AC-45 were used to amplify a longer ORF that includes Xcc2109 and 327 bp upstream (*avrXccFM*), including the SD region of the gene. The two DNA sequences were cloned into TOPO and were recombined into pUFR070 to form pAC10 and pAC19, respectively. Correct orientation of both clones for expression from the *lacZ* promoter on pUFR070 was confirmed by sequencing, and approximately 50 ng of DNA was electroporated into the individual Xcc2109 knockout mutants X48 and X109 (Table 1). Colonies appearing on appropriate selection media were grown in liquid PYGM supplemented with antibiotics. The cultures were centrifuged and washed with tap water, the concentration was adjusted to 10^5 CFU/ml, and the suspension was inoculated into Florida Mustard leaves using scissors as described. Plants were scored one week after inoculation.

Race specificity change.

X. campestris pv. *campestris* 6181 and 3849A, representing races 0 and 2, respectively (Kamoun et al. 1992), were electroporated using pAC19 and pAC10 DNA and were selected on the appropriate antibiotics. Cultures were grown and inoculated in Florida Mustard, Early Jersey Wakefield cabbage, Just Right turnip, and Seven Top turnip plants as described for other strains of *X. campestris* pv. *campestris*. At least three plants were inoculated in each experiment, and each experiment was repeated at least twice.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Xanthomonas-ONSA FAPESP network:
cancer.lbi.ic.unicamp.br/xanthomonas