AvrAC_{Xcc8004}, a Type III Effector with a Leucine-Rich Repeat Domain from *Xanthomonas campestris* Pathovar *campestris* Confers Avirulence in Vascular Tissues of *Arabidopsis thaliana* Ecotype Col-0[†]

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Xanthomonas campestris pathovar *campestris* causes black rot, a vascular disease on cruciferous plants, including *Arabidopsis thaliana*. The gene XC1553 from *X. campestris* pv. *campestris* strain 8004 encodes a protein containing leucine-rich repeats (LRRs) and appears to be restricted to strains of *X. campestris* pv. *campestris*. LRRs are found in a number of type III-secreted effectors in plant and animal pathogens. These prompted us to investigate the role of the XC1553 gene in the interaction between *X. campestris* pv. *campestris* and *A. thaliana*. Translocation assays using the hypersensitive-reaction-inducing domain of *X. campestris* pv. *campestris* AvrBs1 as a reporter revealed that XC1553 is a type III effector. Infiltration of *Arabidopsis* leaf mesophyll with bacterial suspensions showed no differences between the wild-type strain and an XC1553 gene mutant; both strains induced disease symptoms on Kashmir and Col-0 ecotypes. However, a clear difference was observed when bacteria were introduced into the vascular system by piercing the central vein of leaves. In this case, the wild-type strain 8004 caused disease on the Kashmir ecotype, but not on ecotype Col-0; the XC1553 gene mutant became virulent on the Col-0 ecotype and still induced disease on the Kashmir ecotype. Altogether, these data show that the XC1553 gene, which was renamed *avrAC*_{Xcc8004}, functions as an avirulence gene whose product seems to be recognized in vascular tissues.

Flor's gene-for-gene theory suggested that an incompatible interaction between microbial pathogens and plants is governed by an avirulence (*avr*) gene of a pathogen and the cognate resistance (*R*) gene of a host (19). Disease resistance occurs only if an *R* gene in a plant is matched by a cognate *avr* gene in a pathogen. The activated plant disease resistance is often associated with the hypersensitive reaction (HR), a type of rapid, localized, and programmed cell death at the infection sites of plants (31).

A number of genes involved in the HR and pathogenicity (*hrp*) in many gram-negative phytopathogenic bacteria have been identified and characterized. The *hrp* genes have been demonstrated to be required for the pathogens to cause disease in susceptible host plants and to induce HR in resistant host and nonhost plants (44). Most *hrp* genes encode components of the type III secretion system (T3SS) (6, 12). The T3SS

of plant-pathogenic bacteria translocates effector proteins directly into the plant cells (6). The effectors can act to activate or suppress plant defense signal transduction (1, 11, 21, 50). T3SS effectors have a modular structure, and the targeting signal generally resides in the N-terminal 50 or 100 amino acids (22, 51, 63). Many Avr proteins of plant-pathogenic bacteria have been shown to be translocated effectors (1, 11, 21, 50).

The availability of the genome sequences of major phytopathogenic bacteria facilitates the comprehensive identification of T3SS effector genes. Large-scale identification of effectors has been achieved in *Pseudomonas syringae* pv. *maculicola* and *Xanthomonas campestris* pv. *vesicatoria* by detecting the translocation of transposon-generated Avr effector domain-reporter protein fusions into plant cells (24, 59). Candidate genes for encoding effectors can be proposed by bioinformatic analysis of the genome sequence or by experimental approaches based on the following criteria: homologues to known T3SS effectors in other bacterial pathogens, the presence of sequence patterns associated with *hrp* promoters and T3SS targeting domains, genes flanking the *hrp* gene cluster, genes with similar regulation by *hrp* regulatory genes, and gene products exhibiting typical eukaryotic protein domains or motifs (1, 7, 13, 42, 73). The candidates can be functionally validated as effectors by translational fusion of 5' coding regions of candidate effector genes with a reporter. The calmodulin-dependent adenylate cyclase domain (Cya) of the *Bordetella pertussis* cyclolysin has been extensively used as a reporter. Adenylate cyclase activity (production of cyclic AMP) in infected

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leaves indicates the fusion protein was translocated into plant cells (8, 47, 63). The HR-inducing effector regions of Avr proteins, such as AvrRpt2 ($\Delta 79$ AvrRpt2) from *P. syringae* (10) and AvrBs3 (AvrBs3 $\Delta 2$) from *X. campestris* pv. *vesicatoria* (54), have also been employed as reporters. The elicitation of T3SS-dependent and *R* gene-dependent HR in plants revealed the presence of a functional translocation signal in the N-terminal region of the candidate gene product (22).

The leucine-rich repeat (LRR) motif is a typical protein motif commonly observed in eukaryotic proteins and appears to be involved in the mediation of protein-protein interactions (35, 37). LRR-containing proteins have been shown to be involved in the host defense systems of both plants and mammals, and many plant *R* genes identified to date encode proteins possessing LRR domains (31, 37). LRR-containing proteins have also been found in diverse groups of bacteria, and some of them have been shown to be T3SS effectors in pathogenic bacteria. In animal bacterial pathogens, some T3SS effector proteins with LRR motifs have been shown to be involved in virulence. These effector proteins include the IpaH9.8 protein of *Shigella flexneri* (57), the YopM proteins of *Yersinia* (71), and the SspH1 and SspH2 proteins of *Salmonella enterica* serovar Typhimurium (49). In plant-pathogenic bacteria, several LRR proteins have been identified. Examples are PopC (23), LrpE/Hpx5/RSp0842, and GALAs from *Ralstonia solanacearum* (3, 52, 60), HpaF from *Xanthomonas oryzae* pv. *oryzae* (65) and *Xanthomonas axonopodis* pv. *glycines* (36), and HpaG/XCV0408 from *X. campestris* pv. *vesicatoria* (55, 67). LrpE of *R. solanacearum* is probably not an effector, since it was shown to be an intracellular protein controlling Hrp pilus production and virulence (52). The functions of HpaF and HpaG are not clear yet. HpaF of *X. oryzae* pv. *oryzae* and HpaG of *X. campestris* pv. *vesicatoria* were found not to be required for virulence (55, 65), whereas HpaF of *X. axonopodis* pv. *glycines* was shown to control pathogenicity (36). Among these LRR proteins, only GALAs and PopC from *R. solanacearum* have been shown to be T3SS effectors (3, 13, 23, 52). However, only the specific function of GALAs has been analyzed in detail. These LRR-containing proteins also contain an F box-like domain, which is important in promoting disease on host plants. It was proposed that these effectors act by hijacking the host SCF-type E3 ubiquitin ligases to interfere with the ubiquitin/proteasome pathway (3). The role of the LRR domain in these effectors has yet to be characterized.

X. campestris pathovar *campestris* is the causal agent of black rot of cruciferous plants, including the model plant *Arabidopsis thaliana* (2). The bacterium is a xylem-colonizing systemic pathogen that generally invades plant leaves through hydathodes and multiplies in vascular tissues. The whole-genome sequences of the *X. campestris* pv. *campestris* strains 8004 and ATCC 33913 have been determined (15, 58). Two genes, i.e., XC1553 (8004)-XCC2565 (ATCC 33913) and XC4273 (8004)-XCC4186 (ATCC 33913), were predicted to encode proteins containing LRRs in both strains (15, 58). In this paper, we demonstrate that XC1553 is translocated via the T3SS by using the HR-inducing domain of *X. campestris* pv. *campestris* AvrBs1 as a reporter and that it is an avirulence protein, which seems to be recognized in the vascular tissues of *A. thaliana* ecotype Col-0.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* and *Agrobacterium tumefaciens* EHA105 (28) were cultivated in LB medium at 37°C and 28°C, respectively. *Xanthomonas* strains were grown in NYG medium (14) or XVM2 medium (75) at 28°C. Antibiotics were added to the media in the following final concentrations: rifampin (Rif), 50 µg/ml; kanamycin (Kan), 25 µg/ml; tetracycline (Tc), 15 µg/ml for *E. coli* and 5 µg/ml for *Xanthomonas* and *Agrobacterium*; gentamicin (Gm), 10 µg/ml; spectinomycin (Spc), 100 µg/ml.

DNA and RNA manipulations. DNA manipulations followed the procedures described by Sambrook and associates (61). Plasmids were introduced into *E. coli* by electroporation and into *Xanthomonas* strains by triparental conjugation, as described by Turner et al. (69).

Overnight cultures of the wild-type strain 8004 and the XC1553 gene transposon insertional mutant 151H08 grown in XVM2 medium were used to extract the total RNAs with a total-RNA extraction kit (Promega, Shanghai, China), and the reverse transcription (RT) was performed using a cDNA synthesis kit (Fermentas Co.). Each kit was used according to the manufacturer's instructions.

The analysis of transcriptional products of the XC1552 and XC1553 genes in the *X. campestris* pv. *campestris* wild-type strain 8004 and the XC1553 gene Tn5gus45 insertional mutant 151H08 was carried out by RT-PCR. The synthesized cDNAs from the extracted total RNAs of the wild-type strain 8004 and the mutant 151H08 were used as templates to amplify the internal sequences of the XC1552 and XC1553 genes and a presumed sequence spanning from 5' terminal of XC1552 to 3' terminal of XC1553 with the primer sets 1552F/1552R, 1553F/1553R, and 1553-52F/1553-52R (see Table S1 in the supplemental material), respectively. Simultaneously, PCR with the total DNA or total RNA without the addition of reverse transcriptase was performed as a positive or negative control. The amplified 16S rRNA interval sequence was normalized as a control of the expression level. The RT-PCR products corresponding to transcribed internal sequences of XC1552 and XC1553 and the presumed sequence spanning from the 3' end of XC1553 to the 5' end of XC1552 were detected by 1% agarose gel electrophoresis. The predicted sizes of the products for XC1552 and XC1553 and the sequence spanning XC1552 and XC1553 were 477 bp, 302 bp, and 418 bp, respectively. No RT-PCR product was detected for the presumed sequence spanning XC1552 and XC1553. The RT-PCR product for XC1552 in 151H08 could still be detected.

Mutant construction and complementation. The Tn5gus45 insertional mutants of XC1553, *hrcV*, and *hrpF* were from the insertional mutant library of the *X. campestris* pv. *campestris* strain 8004 in the authors' laboratory in Nanning, China. The mutants were selected by mating the *X. campestris* pv. *campestris* strain 8005/pPH1J1 (69) with the strain 8004/pLAFR1::Tn5gus45 and by plating them on NYG agar containing Rif, Kan, Gm, and Spc. The Rif-Kan-Gm-Spc-resistant but Tc-sensitive individual colonies were picked as candidate mutants. The genomic positions of the transposon in the mutants were determined by thermal asymmetric interlaced PCR (45) and subsequent sequencing for comparison with the whole genome sequence of strain 8004 (58). The insertional site of Tn5gus45 was further confirmed by PCR using the primers on the transposon and on the gene upstream or downstream of the disrupted gene.

Using primer set IG1553F/IG1553R (see Table S1 in the supplemental material), the internal sequence of the XC1553 gene (from positions 24 to 560) was amplified from genomic DNA of the wild-type strain 8004 by PCR and cloned into the suicide plasmid pK18mob (62), generating a recombinant plasmid named pK1553. The recombinant plasmid was subsequently used for constructing a plasmid integrational mutant of XC1553 as described previously for *X. campestris* pv. *campestris* (16), which was confirmed by PCR and designated NK1553 (Table 1). A 2,045-bp DNA fragment containing the XC1553 gene coding region and extending 280 bp at the 5' end and 153 bp at the 3' end was amplified by PCR using the total DNA of strain 8004 as a template and primer set C1553F/C1553R (see Table S1 in the supplemental material) as primers. The amplified DNA fragment was cloned into pLAFR3 to generate recombinant plasmid pXC1553. This plasmid was transferred into the XC1553 mutants 151H08 and NK1553 by triparental conjugation, and transconjugants were selected on NYG medium supplemented with Rif, Kan, and Tc. The resulting complementary strains for 151H08 and NK1553 were designated C151H08 and CNK1553, respectively (Table 1).

To generate a deletion mutant of *avrBs1*, a 3,271-bp fragment containing the *avrBs1* coding region and its flanking sequence was amplified by PCR with the primer set DS1F/DS1R (see Table S1 in the supplemental material) and cloned into pT18mobH, a derivative of the suicide plasmid pT18mob (Table 1) in which the HindIII restriction site was disrupted, generating pTH2081. A 1,547-bp DNA sequence containing the *avrBs1* coding region on pTH2081 was replaced by a

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Reference or source
<i>X. campestris</i> pv. <i>campestris</i>		
8004	Wild type; Rif ^r	14
Xcc568	Wild type; Rif ^r strain, derivative of <i>X. campestris</i> pv. <i>campestris</i> LMG568/ATCC33913	48
121D06	As 8004, but <i>hrpF</i> ::Tn5 <i>gusA5</i> Rif ^r Kan ^r	This work
050B12	As 8004, but <i>hrcV</i> ::Tn5 <i>gusA5</i> Rif ^r Kan ^r	This work
151H08	As 8004, but XC1553::Tn5 <i>gusA5</i> Rif ^r Kan ^r	This work
8004Δ <i>hrpX</i>	<i>hrpX</i> deletion mutant of 8004; Rif ^r Kan ^r	This work
8004Δ <i>hrpG</i>	<i>hrpG</i> deletion mutant of 8004; Rif ^r Kan ^r	This work
8004Δ <i>avrBs1</i>	<i>avrBs1</i> deletion mutant of 8004; Rif ^r Gm ^r	This work
8004Δ <i>avrBs1</i> /pLAFR6	8004Δ <i>avrBs1</i> harboring plasmid pLAFR6; Rif ^r Gm ^r Tc ^r	This work
8004Δ <i>avrBs1</i> /pLBs1	8004Δ <i>avrBs1</i> harboring plasmid pLBs1; Rif ^r Gm ^r Tc ^r	This work
8004Δ <i>avrBs1</i> /pLBs1 _{59-445P}	8004Δ <i>avrBs1</i> harboring plasmid pLBs1 _{59-445P} ; Rif ^r Gm ^r Tc ^r	This work
8004Δ <i>avrBs1</i> /pLTXC1553	8004Δ <i>avrBs1</i> harboring plasmid pLTXC1553; Rif ^r Gm ^r Tc ^r	This work
121D06/pLTXC1553	121D06 harboring plasmid pLTXC1553; Rif ^r Kan ^r Tc ^r	This work
8004/pLG1553	8004 harboring plasmid pLG1553; Rif ^r Tc ^r	This work
8004Δ <i>hrpG</i> /pLG1553	8004Δ <i>hrpG</i> harboring plasmid pLG1553; Rif ^r Kan ^r Tc ^r	This work
8004Δ <i>hrpX</i> /pLG1553	8004Δ <i>hrpX</i> harboring plasmid pLG1553; Rif ^r Kan ^r Tc ^r	This work
NK1553	As 8004, but XC1553::pK18mob Rif ^r Kan ^r	This work
CNK1553	NK1553 harboring plasmid pXC1553; Rif ^r Kan ^r Tc ^r	This work
C151H08	151H08 harboring plasmid pXC1553; Rif ^r Kan ^r Tc ^r	This work
<i>A. tumefaciens</i>		
EHA105	Agropine strain; Rif ^r	28
EHA105/pBI121	EHA105 harboring plasmid pBI121; Rif ^r Kan ^r	This work
EHA105/pBBs1 _{59-445P}	EHA105 harboring plasmid pBBs1 _{59-445P} ; Rif ^r Kan ^r	This work
EHA105/pBBs1	EHA105 harboring plasmid pBBs1; Rif ^r Kan ^r	This work
<i>E. coli</i>		
JM109	<i>recA1 endA1 gyrA96 thi supE44 relA1 Δ(lac-proAB)/F' [traD36 lacI^q lacZΔM15]</i>	78
ED8767/pRK2093	Helper strain harboring pRK2073; <i>recA met</i> Spc ^r	53
Plasmids		
pLAFR3	Broad-host-range IncP cloning cosmid; Mob ⁺ Tra ⁻ ; contains <i>plac</i> ; Tc ^r	64
pLAFR6	Broad-host-range cloning vector; Tc ^r	29
pRK2073	Helper plasmid; Tra ⁺ Mob ⁺ ColE1 Spc ^r	41
pPH1JI	Tra ⁺ Mob ⁺ IncP replicon; Spc ^r Gm ^r	27
pBI121	Plant expression vector; Kan ^r	30
pK18mob	Suicide plasmid in <i>X. campestris</i> pv. <i>campestris</i> ; Mob ⁺ Tra ⁻ Kan ^r	62
pT18mob	Tetracycline-resistant derivative of pK18mob; Tc ^r	66
pT18mobH	pT18mob but HindIII restriction site was destroyed; Tc ^r	This work
pK1553	pK18mob containing a 537-bp internal fragment of XC1553; Kan ^r	This work
pTH2081	pT18mobH containing <i>avrBs1</i> ORF and its flanking sequences; Tc ^r	This work
pTG2081	pTH2081 but <i>avrBs1</i> ORF was replaced by Gm ^r gene; Gm ^r Tc ^r	This work
pLBs1 _{59-445P}	pLAFR6 containing <i>avrBs1</i> promoter plus <i>avrBs1</i> ₅₉₋₄₄₅ fusion; Tc ^r	This work
pBBs1 _{59-445P}	pBI121 containing <i>avrBs1</i> promoter plus <i>avrBs1</i> ₅₉₋₄₄₅ fusion; Kan ^r	This work
pLBs1	pLAFR6 containing entire <i>avrBs1</i> gene; Tc ^r	This work
pBBs1	pBI121 containing entire <i>avrBs1</i> gene; Kan ^r	This work
pXC1553	pLAFR3 containing entire XC1553 gene; Tc ^r	This work
pLG1553	pLAFR6 containing fused XC1553 promoter and promoterless <i>gus</i> ; Tc ^r	This work
pLTXC1553	pLAFR6 containing XC1553 translocation fusion fragment; Tc ^r	This work

DNA fragment containing a Gm resistance gene amplified from plasmid pPH1JI with the primer set GmF/GmR (see Table S1 in the supplemental material), generating the recombinant plasmid pTG2081. The plasmid pTG2081 was confirmed by sequencing and then introduced from *E. coli* into strain 8004 by triparental conjugation, and an *avrBs1* deletion mutant, named 8004Δ*avrBs1*, was selected on plates containing Rif and Gm. The mutant was confirmed by PCR and complementation.

To complement the *avrBs1* deletion mutant, a 1,543-bp DNA sequence containing the entire coding region of *avrBs1* and its 205-bp upstream sequence was amplified by PCR from the total DNA of the strain 8004 with the primer set PS1F-F/CS1R-R (see Table S1 in the supplemental material), confirmed by sequencing, and ligated into the XbaI and BamHI sites of pLAFR6, generating the recombinant plasmid pLBs1. The plasmid pLBs1 was introduced into the *avrBs1* deletion mutant 8004Δ*avrBs1* by triparental conjugation.

To construct an *hrpG* deletion mutant, the upstream and downstream frag-

ments flanking *hrpG* were amplified with the primer sets DG-LF/DG-LR and DG-RF/DG-RR (see Table S1 in the supplemental material), respectively, using the total DNA of strain 8004 as a template. Simultaneously, the Kan-resistant fragment was amplified with the primer sets Km-F/Km-R (see Table S1 in the supplemental material), using pLAFR1::Tn5*gusA5* as a template. These three fragments were cloned into the BamHI-XbaI-SalI-SphI sites of the suicide vector pGEM-3Zf(+) one by one, yielding the recombinant plasmid pGDG. After confirmation by sequencing, the plasmid pGDG was ligated into the BamHI site of the cosmid pLAFR3, yielding the recombinant plasmid pLGDG. The plasmid pLGDG was transferred into the wild-type strain 8004 by triparental conjugation, and Kan-resistant transconjugants were screened. The plasmid pPH1JI, which is incompatible with pLAFR3, was then introduced by mating from the *X. campestris* pv. *campestris* strain 8005/pPH1JI (69). The *hrpG* deletion mutant was selected on NYG medium supplemented with Rif, Kan, Gm, and Spc simultaneously. The mutant colonies were then checked for Tc sensitivity and were

further confirmed by PCR. The mutant was named 8004 Δ *hrpG*. An *hrpX* deletion mutant was constructed by the same strategy. The primer sets used, DX-LF/DX-LR and DX-RF/DX-RR, are listed in Table S1 in the supplemental material, and the mutant obtained was named 8004 Δ *hrpX*.

Construction of a promoter-reporting plasmid for XC1553. A promoter-reporting plasmid for the XC1553 gene was constructed by fusing a 400-bp DNA sequence upstream of the start codon of XC1553 with promoterless *gus*. The XC1553 promoter region was amplified from the total DNA of strain 8004 by using a specific primer set, PV1553F/PV1553R (see Table S1 in the supplemental material), producing a DNA fragment with a 16-bp sequence at the 3' end overlapping with the 5' end of the *gus* open reading frame (ORF), and the *gus* coding region was amplified by PCR with the primer set *gusF/gusR* (see Table S1 in the supplemental material), using transposon Tn5*gusA5* as a template. The two PCR products were fused by fusion PCR using *Pfu* DNA polymerase (Fermentas) without adding any other primers. The fused fragment was cloned into pLAFR6 to generate the recombinant plasmid pLG1553. The plasmid pLG1553 was confirmed by sequencing and then introduced into the wild-type strain 8004 and the deletion mutants 8004 Δ *hrpG* and 8004 Δ *hrpX* by triparental conjugation.

Transient expression of the HR-inducing domain of AvrBs1 in pepper. The 205-bp DNA sequence upstream of the start codon of *avrBs1* and the 1,164-bp DNA sequence encoding the C-terminal amino acids 59 to 445 of AvrBs1 (AvrBs1₅₉₋₄₄₅) were amplified with primer sets Bs1P-F/Bs1P-R and F59aa-F/F445aa-R (see Table S1 in the supplemental material), respectively, using the total DNA of strain 8004 as a template. The two amplified PCR products were fused by recombination PCR. The digested PCR product was cloned into the XbaI and BamHI sites of pBI121, generating recombinant plasmid pBBs1_{59-445P}.

The 1,543-bp DNA sequence containing the entire coding region of *avrBs1* and the 205-bp sequence upstream of the ORF was amplified by PCR from the total DNA of strain 8004 with the primer set PS1F-F/CS1R-R (see Table S1 in the supplemental material), and the digested PCR product was ligated into the XbaI and BamHI sites of pBI121, generating recombinant plasmid pBBs1.

The plasmids pBBs1_{59-445P} and pBBs1 and the vector pBI121 were introduced into *A. tumefaciens* strain EHA105 by triparental conjugation. The *A. tumefaciens* strain EHA105 carrying pBBs1_{59-445P} or pBBs1 or the vector pBI121 was grown in LB medium supplemented with Kan at 28°C for 17 h. The bacterial cells were washed, resuspended in the inducing medium (10 mM MES [morpholineethanesulfonic acid], pH 5.6, 10 mM MgCl₂, 150 μ M acetosyringone [Sigma]) and incubated at 28°C for 2 h just before use (51). Pepper seedlings were grown in a greenhouse with 12-h day and night cycle illumination by fluorescent lamps at temperatures of 25 to 28°C. A bacterial suspension at an optical density at 600 nm of 0.5 in the inducing medium was introduced into the leaves of pepper (cultivar ECW-10R) at the stage of four fully expanded leaves by infiltration. After infiltration, the plants were grown at 28°C with a 16-h photoperiod per day and 80% relative humidity.

Detection of the XC1553 translocation signal. A 1,170-bp DNA segment containing a 501-bp DNA sequence upstream of the start codon of XC1553 and a 651-bp sequence at the 5' end of the XC1553 ORF was amplified by PCR using the total DNA of strain 8004 as a template with the primer set T1553F/T1553R (see Table S1 in the supplemental material). The sequence encoding AvrBs1₅₉₋₄₄₅ was amplified with the primer set 59aa-F/F445aa-R. The two amplified fragments were digested with EcoRI/XbaI and XbaI/PstI, respectively, and ligated with the EcoRI/PstI-digested pUC19. After confirmation by sequencing, the verified recombinant fragment was cloned into the EcoRI/PstI site of pLAFR6 to generate the recombinant plasmid pLTXC1553. The plasmid pLTXC1553 was introduced into 8004 Δ *avrBs1* and the *hrpF* mutant 121D06 by triparental conjugation. The transconjugants were tested on pepper cultivar ECW-10R or ECW-20R as described below. As a control, the *avrBs1* promoter region (205 bp upstream of the *avrBs1* ORF) amplified with primer set Bs1P-F/Bs1P-R (see Table S1 in the supplemental material) was fused to the 5' end of *avrBs1*₅₉₋₄₄₅, and the resulting fragment was cloned into pLAFR6 to generate recombinant plasmid pLBs1_{59-445P}. The plasmid pLBs1_{59-445P} was transferred into 8004 Δ *avrBs1* by triparental conjugation, and the resulting strain was similarly infiltrated into the leaves of cultivar ECW-10R or ECW-20R.

Plant assay. The virulence of *X. campestris* pv. *campestris* strains was assayed on the *A. thaliana* ecotypes Columbia (Col-0) and Kashmir by piercing inoculation or classical mesophyll infiltration as described by Meyer and associates (48). For in planta growth of bacteria, two holes were pierced at the leaf base, close to the petiole. At least four 4-week-old plants were inoculated for each strain tested. Symptoms were recorded as previously described (48). Each strain was tested in at least three separate experiments. For the measurement of in planta growth of bacteria, three leaf discs from different inoculated leaves of one inoculated plant were sampled using a cork borer (diameter, 0.65 cm; surface area, 0.33 cm²) at days 0, 3, and 5 after inoculation and were ground with a pestle

in 500 μ l sterile water. Discs collected at the inoculation sites (on the leaf base, close to the petiole) and at the tips of the inoculated leaves were designated "proximal sample" and "distal sample," respectively (see Fig. 6). The homogenates were serially diluted, and 5- μ l drops were spotted three times for each dilution with a multichannel pipette on well-dried plates supplemented with Rif. The plates were incubated at 30°C for 24 h and then at 4°C for 17 h. Colonies were counted in spots containing 1 to 30 colonies. Bacterial densities in leaves were calculated and were given in log CFU/cm².

The abilities of the *X. campestris* pv. *campestris* strains to elicit HR on pepper were assayed as previously described (4).

Determination of GUS activity. β -Glucuronidase (GUS) activity was measured by using *p*-nitrophenyl- β -D-glucuronide as a substrate, as described by Henderson and associates (25), after growth of the *X. campestris* pv. *campestris* strains in XVM2 for 18 h or in NYG for 16 h.

Light microscopy protocol. Plants (*Arabidopsis* ecotype Sf-2) were inoculated by the piercing inoculation method with the wild-type strain Xcc568 (ATCC 33913) as described above. The infected plants were fixed, and sections were prepared as previously described (70).

RESULTS

XC1553 codes for a protein with LRRs and seems to be specific to *X. campestris* pv. *campestris*. Two genes, i.e., XCC4186 and XCC2565, were initially annotated to encode proteins with LRRs, which were postulated to be putative effector/avirulence proteins in the genome of the *X. campestris* pv. *campestris* strain ATCC 33913 (15). The XC4273 protein of *X. campestris* pv. *campestris* strain 8004 (504 amino acids) is identical to XCC4186 of strain ATCC 33913. XC1553 in strain 8004 and the protein XCC2565 in ATCC 33913 are both 536 amino acids in length and differ in one residue, i.e., R70 in XC1553 is replaced by S70 in XCC2565.

BlastP analysis revealed that XC4273 shows homology (around 42% identity and 59% similarity) over its full length to a number of leucine-rich proteins from *Xanthomonas* species or pathovars. Mutants of the XC4273 gene displayed the same reaction as the wild-type 8004 on both *A. thaliana* ecotypes Columbia (Col-0) and Kashmir (data not shown). Therefore, we focused our work on the XC1553 gene, which seems specific to pathovar *campestris* of *X. campestris*, since no significant homologues were detected in the genomes of *X. campestris* pv. *vesicatoria* strain 85-10 (67), *X. axonopodis* pv. *citri* strain 306 (15), or *X. oryzae* pv. *oryzae* strain KACC10331 (40). However, homology (around 29% identity and 43% similarity) was detected with two LRR-containing proteins, Aave1508 (accession number YP_969871) and Aave4631 (accession number YP_972940) of *Acidovorax avenae* subsp. *citulli* AAC 00-1, the causal agent of bacterial fruit blotch (76). The region of homology encompasses the LRRs and C-terminal parts of these proteins. XC1553 contains six LRRs. All LRRs can be divided into a highly conserved segment and a variable segment. The highly conserved segment consists of an 11-residue stretch, LXXLXLXXNXL, or a 12-residue stretch, LXXLXLXXCXXL, in which L is Leu, Ile, Val, or Phe; N is Asn, Thr, Ser, or Cys; and C is Cys or Ser (32). Seven classes of LRRs have been proposed, depending on the lengths and the consensus of the variable segments of repeats (17, 32, 46). In XC1553, the first LRR is imperfect, and the lengths of the other LRRs range from 23 to 24 amino acid residues. The 23-amino-acid LRRs of XC1553 match the 11-residue canonical conserved segment. However, the 24-amino-acid LRRs seem to have an unconventional sequence, since the conserved C position (Cys or Ser) is occupied by an asparagine residue (N) (Fig. 1), suggesting that XC1553 might have a unique structure.

(amino acids 1 to 144) and the sequence located C-terminal to the Fic domain (amino acids 496 to 536) do not share significant homology with any other proteins in the databases.

XC1553 is a monocistronic gene. The XC1553 ORF, which is 1,611 bp in length, is located in the circular chromosome of strain 8004 from positions 1867667 to 1869277 in the complementary strand (GenBank accession no. CP000050). The left-hand gene, XC1552, which encodes a putative carboxymethylenebutenolidease, has the same transcriptional orientation as

FIG. 2. Genetic organization and analysis of the XC1553 locus in the genome of *X. campestris* pv. *campestris* and comparison with syntenic regions in *X. campestris* pv. *vesicatoria* and *X. axonopodis* pv. *citri*. (A) Representation of XC1553 and its flanking genes and of the corresponding regions in the genomes of *X. campestris* pv. *vesicatoria* strain 85-10 and *X. axonopodis* pv. *citri* strain 306. The Arabic numbers represent the nucleotide positions in the genomic sequence of strain 8004 (accession no. NC_007086). The big arrows represent the predicted protein-coding genes. Orthologous genes in *X. campestris* pv. *campestris*, *X. campestris* pv. *vesicatoria*, and *X. axonopodis* pv. *citri* have the same color. The DNA regions showing significant sequence identity (above 85%) between *X. campestris* pv. *campestris*, *X. campestris* pv. *vesicatoria*, and *X. axonopodis* pv. *citri* are represented by boxes with the same motif, and colinear regions are delimited by gray boxes. The PIP and -10 box found upstream of the XC1553 coding sequences is indicated by three vertical bars. The DNA sequence displaying homologies with the IS1478 insertion sequence is indicated by a black box. Horizontal black bars indicate sequences used in plasmid construction. The small arrows above and below the coding sequences represent the positions and directions of the primers used for analysis of transcriptional products. The black triangle above the XC1553 ORF indicates the integration site of a pK18mob derivative in the mutant NK1553. The small flag above the XC1553 ORF stands for the direction and position of the transposon Tn5gusA5 in the mutant 151H08. (B) Analysis of transcriptional products of XC1552 and XC1553 in the *X. campestris* pv. *campestris* wild-type strain 8004 and the XC1553 Tn5gusA5 insertional mutant 151H08 by RT-PCR. The predicted sizes of the products for XC1552 and XC1553 and the sequence spanning XC1552 and XC1553 were 477 bp, 302 bp, and 418 bp, respectively.

XC1553. The right-hand gene, XC1554, which encodes a conserved hypothetical protein, is transcribed in the opposite direction to XC1553 (Fig. 2A). To determine whether XC1552 is transcribed in the same operon as XC1553, the transcriptional products of XC1552 and XC1553 were analyzed in the wild-type strain 8004 and in a Tn5gusA5 insertional mutant of XC1553, 151H08 (see below), by RT-PCR. No RT-PCR product spanning the 3' end of XC1553 to the 5' end of XC1552 could be detected in strain 8004. However, an RT-PCR product of XC1552 was amplified from the XC1553 mutant 151H08 (Fig. 2B), indicating that the disruption of XC1553 does not affect the transcription of XC1552. Therefore, XC1552 and XC1553 seem to be transcribed independently.

The expression of XC1553 is regulated by *hrpG* and *hrpX*. *hrpG* and *hrpX* are two key *hrp* regulatory genes in xanthomonads (56, 74, 75). The expression of *hrp* genes, including

the regulators *hrpG* and *hrpX* in *X. campestris*, has been demonstrated to be induced in minimal medium and repressed in rich medium (75). The two regulatory genes form a cascade in which *hrpG* regulates the expression of *hrpX*, which then regulates downstream *hrp* or effector genes. The regulation by HrpX is mediated by the binding of this regulator to a *cis*-regulatory element, the PIP box (TTCGC-N₁₅-TTCGC), present in the promoter region of HrpX-regulated genes (38). Recent studies have shown that imperfect PIP boxes can also drive expression by HrpX (20, 38, 68). The analysis of the XC1553 promoter region revealed an imperfect PIP box followed by a -10 box-like sequence (TTCAC-N₁₅-TTCGC-N₃₂-TACGTT) 30 nucleotides upstream of the start codon of XC1553 (Fig. 2A). To determine if XC1553 is transcriptionally regulated by *hrpG* or *hrpX*, the reporter plasmid pLG1553 carrying the fused 400-bp XC1553 promoter region and the

FIG. 3. XC1553 of *X. campestris* pv. *campestris* strain 8004 is transcriptionally regulated by *hrpG* and *hrpX*. GUS activities were determined after growth of *X. campestris* pv. *campestris* strains in the minimal medium XVM2 for 16 h or in the rich medium NYG for 12 h. The data are the means $\pm$ standard deviations of triplicate measurements.

promoterless *gus* gene was introduced into the wild-type strain 8004, the *hrpG* mutant 8004Δ*hrpG*, and the *hrpX* mutant 8004Δ*hrpX* by triparental conjugation, and the GUS activities of the obtained transconjugants grown in minimal medium (XVM2) or rich medium (NYG) were assayed. The results showed that XC1553 is not expressed in rich medium and is activated in XVM2 minimal medium. The expression level of XC1553 in 8004Δ*hrpG* or 8004Δ*hrpX* was significantly lower than that in the wild-type strain 8004 in XVM2 medium, showing that its expression is regulated by *hrpG* and *hrpX* (Fig. 3).

XC1553 is a translocated T3SS effector. To determine whether XC1553 is a T3SS effector translocated into plant cells, we designed a new HR-inducing Avr reporter system. Many Avr proteins have been demonstrated to be translocated T3SS effectors (1, 11, 21, 50). *avrBs1* of *X. campestris* pv. *vesicatoria* strain 85-10 is responsible for the elicitation of HR on the pepper cultivar ECW-10R, which contains the cognate resistance gene *Bs1* (26). Interestingly, the *X. campestris* pv. *campestris* strain 8004 carries a copy of the *avrBs1* gene (XC2081), which codes for a protein that is identical to AvrBs1 of the *X. campestris* pv. *vesicatoria* strain 85-10 (67). Strain 8004 can cause HR on the nonhost plant pepper cultivar ECW-10R (Fig. 4A). The deletion mutant of *avrBs1*, 8004Δ*avrBs1*, completely lost the ability to elicit HR on pepper cultivar ECW-10R (Fig. 4A). The complemented strain 8004Δ*avrBs1*/pLBs1, harboring the wild-type *avrBs1* gene provided in *trans* in pLAFR6 (pLBs1), caused a normal HR on pepper cultivar ECW-10R (Fig. 4A). Furthermore, the *hrcV*-Tn5*gusA5* mutant and the *hrpF*-Tn5*gusA5* mutant carrying or not carrying pLBs1 could not induce HR on pepper cultivar ECW-10R (Fig. 4A). These results indicate that AvrBs1 of strain 8004 is responsible for the pathogen's elicitation of HR on pepper cultivar ECW-10R in a T3SS-dependent manner.

T3SS effectors generally have a modular structure, and the

FIG. 4. XC1553 of *X. campestris* pv. *campestris* strain 8004 is a T3SS translocated effector. (A) AvrBs1 of *X. campestris* pv. *campestris* strain 8004 is responsible for its elicitation of HR on the nonhost plant pepper cultivar ECW-10R. Spot 1, sterilized water; spot 2, *hrcV* Tn5*gusA5* insertional mutant 050B12; spot 3, wild-type strain 8004; spot 4, 8004Δ*avrBs1*; spot 5, *hrpF* mutant 121D06/pLBs1; spot 6, 8004Δ*avrBs1*/pLBs1. The photograph was taken 48 h postinoculation. (B) Amino acids 59 to 445 of *X. campestris* pv. *campestris* AvrBs1 contain functional domains to induce HR on pepper cultivar ECW-10R. Bacterial cells were infiltrated into pepper leaves by using a needleless syringe. The photographs were taken 3 days postinfiltration. (C) XC1553 of the *X. campestris* pv. *campestris* strain 8004 is a T3SS translocated effector. Bacterial cells of *X. campestris* pv. *campestris* strains were introduced into leaves of pepper cultivar ECW-10R by infiltration. a, sterilized water; b, 050B12 (*hrcV* Tn5*gusA5* insertional mutant); c, 121D06 (*hrpF* Tn5*gusA5* insertional mutant); d, wild-type strain 8004; e, 8004Δ*avrBs1*; f, 8004Δ*avrBs1*/pLBs1_{59-445P}; g, 8004Δ*avrBs1*/pLTXC1553; h, 121D06/pLTXC1553. The photographs were taken 48 h postinfiltration.

targeting signal generally resides in the N-terminal 50 or 100 amino acids (51, 63). The *A. tumefaciens* strain EHA105 containing pBBs1_{59-445P}, which contains a copy of *avrBs1* lacking the sequence including the first 58 codons, could cause HR on pepper cultivar ECW-10R, as did EHA105/pBBs1, which har-

FIG. 5. Symptoms caused by *X. campestris* pv. *campestris* strains after infiltration into the mesophyll of *A. thaliana* Columbia (Col-0) (A) or Kashmir (B) leaves. Image 1, wild-type strain 8004; image 2, 151H08 (*avrAC_{Xcc8004}::Tn5gusA5*); image 3, C151H08 (151H08 mutant carrying the pXC1553 plasmid, which contains the entire *avrAC_{Xcc8004}* gene). The photographs were taken 5 days after inoculation. (C) In planta bacterial growth of *X. campestris* pv. *campestris* strains after infiltration of Columbia (Col-0) or Kashmir mesophyll tissues. Each data point represents the mean and standard deviation calculated from four replicates. The experiments were repeated at least twice with equivalent results.

bors a derivative of pBI121 carrying the *avrBs1* gene (Fig. 4B). The strain EHA105 containing the vector pBI121 could not elicit an HR (Fig. 4B). As a control, the *avrBs1* deletion mutant of strain 8004 containing pLBs1_{59-445P} could not cause an HR on pepper cultivar ECW-10R (Fig. 4B). These results indicate that the sequence containing amino acids 59 to 445 of *AvrBs1* possesses the functional HR-inducing domain but does not enable its translocation into plant cells.

The 1,170-bp DNA sequence containing the 651-bp sequence upstream of the start codon of the XC1553 ORF and the 501-bp sequence at the 5' end of its coding region was amplified by PCR. After confirmation by sequencing, the fragment was fused to the 5' end of *avrBs1*₅₉₋₄₄₅, giving an in-frame fusion between the 167 N-terminal amino acids of XC1553 and the truncated version of *AvrBs1* lacking its 58 N-terminal amino acids. The resulting fused sequence was cloned into pLAFR6 to generate the recombinant plasmid pLTXC1553. This plasmid was introduced into 8004Δ*avrBs1* and 121D06 (8004 *hrpF::Tn5gusA5*), a mutant affected in the *X. campestris* pv. *campestris* T3SS translocon (47). The resulting transconjugants, 8004Δ*avrBs1*/pLTXC1553 and 121D06/pLTXC1553, were infiltrated into leaves of different pepper plants. 8004Δ*avrBs1*/pLTXC1553 induced an HR in the *Bs1*-expressing pepper cultivar ECW-10R (Fig. 4C) but not in cultivar ECW-20R, which does not possess a functional *BS1* resistance gene (data not shown). Strain 121D06 (*hrpF*)/pLTXC1553 did not induce an HR on cultivar ECW-10R or ECW-20R. These data indicate that XC1553 carries a translocation signal in its N-terminal region and might thus be considered a T3SS effector.

The XC1553 gene confers avirulence on *X. campestris* pv. *campestris* strain 8004 on *A. thaliana* ecotype Col-0. A

Tn5gusA5 insertional mutant of XC1553, named 151H08, was obtained from the mutant collection of strain 8004 in the authors' laboratory in Nanning, China (Table 1). The transposon was inserted at the 143rd nucleotide of the XC1553 ORF in this mutant (Tang et al., unpublished). A mutant carrying a single-crossover insertion in the XC1553 gene was also constructed using the suicide plasmid pK18mob (62) harboring an internal sequence of XC1553 (from positions 24 to 560) and was designated NK1553.

Inoculated on Chinese radish Manshenghong by the leaf-clipping method, XC1553 mutants induced disease symptoms similar to those provoked by the wild-type strain 8004 (data not shown). These mutants and the wild-type strain 8004 were then tested on *A. thaliana* ecotypes Col-0 and Kashmir by piercing inoculation or by leaf infiltration (48). As observed for the strain Xcc568 (ATCC 33913) (48), strain 8004 induced typical disease symptoms on both Col-0 and Kashmir ecotypes after infiltration of bacterial cells into the leaf mesophyll (Fig. 5). The 151H08 and NK1553 mutants gave similar results by this method of inoculation (Fig. 5), whereas *hrp* mutants did not show any symptoms on both ecotypes (data not shown) (48). The growth of both mutants was similar to that of the wild-type strain on both ecotypes. However, we observed that 5 days after inoculation, the growth of *X. campestris* pv. *campestris* strains was significantly lower in the Col-0 ecotype than in the Kashmir ecotype. However, this differential behavior was not dependent on the XC1553 gene (Fig. 5C).

We then performed inoculation by the piercing method, which seems to allow the delivery of bacterial cells into xylem tissues. Using this method, we observed active propagation of *X. campestris* pv. *campestris* cells in or along vascular tissues of susceptible *Arabidopsis* ecotypes (Sf-2 and Kashmir) (48). This

FIG. 6. Analysis of the interaction phenotypes of plant and bacterial strains after piercing inoculation on *A. thaliana* Columbia (Col-0) or Kashmir leaves. Four fully expanded leaves of each plant were inoculated by piercing three holes in the central vein with a needle dipped in a bacterial suspension. At least 4 plants were inoculated for each strain tested. These experiments were repeated at least three times and gave similar results. Disease indexes were determined as described by Meyer et al. (48): 0, no symptoms; 1, chlorosis surrounding the wound site; 2, strong V-shaped chlorosis; 3, developing necrosis; 4, leaf death. The average disease index scores and the standard deviations were calculated from the values of four plants with four inoculated leaves per plant. (A) Symptoms caused by *X. campestris* pv. *campestris* strains on Col-0-inoculated leaves. Image 1, wild-type strain 8004; image 2, 151H08 (*avrAC*_{Xcc8004}::*Tn5gusA5*); image 3, C151H08 (151H08 mutant carrying the pXC1553 plasmid, which contains the entire *avrAC*_{Xcc8004} gene). The photographs were taken 5 days after wound inoculation. (B) Disease progress on Col-0. (C) Symptoms caused by *X. campestris* pv. *campestris* strains on the inoculated Kashmir leaves. The photographs were taken 7 days after wound inoculation. Image 1, wild-type strain 8004; image 2, 151H08; image 3, C151H08. (D) Disease progress on Kashmir.

observation was made by using the Xcc568 (ATCC 33913) reporter strain carrying the LUX operon of *Photobacterium luminescens* (48). We also performed a microscopic study to analyze bacterial propagation (Xcc568) in the susceptible *Arabidopsis* Sf-2 ecotype after piercing inoculation. Cross sections of infected leaves made at various times showed that bacteria mainly colonize and multiply in xylem vessels (see Fig. S2 in the supplemental material).

Inoculated by the piercing method, the wild-type strain 8004 induced typical black rot disease symptoms on the Kashmir ecotype but caused no symptoms on Col-0 (Fig. 6). Such differential behavior had already been observed for strain Xcc568 (ATCC 33913) (48). Interestingly, XC1553 mutants became virulent on the Col-0 ecotype, inducing typical V-shaped lesions (Fig. 6A). The complemented strains of XC1553 mutants, C151H08 and CNK1553, which correspond to mutants 151H08 and NK1553, respectively, carrying the recombinant plasmid pXC1553, which contains the entire XC1553 gene, were then tested on *Arabidopsis*. C151H08 and CNK1553 caused no symptoms on Col-0, as observed for the wild-type strain (Fig. 6), prov-

ing that the phenotype of 151H08 and NK1553 mutants is due to the mutation in XC1553 and confirming that the gene is monocistronic.

Internal growth curve assays showed that the growth of the wild-type and C151H08 complemented strains seemed to stop or slow down in the Col-0 ecotype 3 days after piercing inoculation (Fig. 7), whereas XC1553 mutants continued to grow, reaching population levels significantly higher than those of the wild-type strain at both the inoculation site (proximal) (Fig. 7A) and the tip of the leaf (distal) (Fig. 7B). It is worth noting that the growth rate of the C151H08 complemented strain was significantly lower than that of the wild-type strain, suggesting an effect of the XC1553 gene copy number. In contrast to these effects, the wild-type strain 8004, the mutant 151H08, and the complemented strain C151H08 could grow equally well in *A. thaliana* ecotype Kashmir (Fig. 7C and D).

Altogether, these results reveal that XC1553 behaves as an avirulence gene on the *A. thaliana* ecotype Col-0 and that either wounding or initial vascular colonization is important for its recognition. Thus, the XC1553 gene was renamed

FIG. 7. In planta bacterial growth of *X. campestris* pv. *campestris* strains after piercing inoculation of *A. thaliana* leaves. (A) Bacterial growth at the inoculation sites (proximal samples) of Columbia (Col-0) leaves. (B) Bacterial growth at the tips of the Col-0-inoculated leaves (distal samples). (C) Bacterial growth at the inoculation sites (proximal samples) of Kashmir leaves. (D) Bacterial growth at the tips of the Kashmir-inoculated leaves (distal samples). (E) Diagram of an *Arabidopsis* leaf showing the proximal and distal zones used for growth curve assays. Each data point represents the mean and standard deviation calculated from four replicates. The experiments were repeated at least twice with equivalent results.

*avrAC*_{Xcc8004} following the unified nomenclature proposed by Lindeberg and colleagues (43).

DISCUSSION

AvrAC_{Xcc8004} is a novel effector translocated into plant cells by the *X. campestris* pv. *campestris* T3SS. Using the HR-inducing domain of the AvrBs1 protein of the *X. campestris* pv. *campestris* strain 8004 as a reporter, we provide evidence for the T3SS-dependent translocation of AvrAC_{Xcc8004}. Analysis of the N-terminal 90 amino acids of AvrAC_{Xcc8004} revealed that this portion contains 26.7% serine and proline, having the features of positive serine and proline bias in the N-terminal region of T3SS effectors of gram-negative plant and animal bacterial pathogens (22, 24).

The expression of *avrAC*_{Xcc8004} is regulated by HrpG and HrpX regulatory proteins. An imperfect PIP box was identified in the promoter region of *avrAC*_{Xcc8004}. Recently, it has been demonstrated that HrpX binds to the PIP box (perfect or

imperfect) present in the promoter region of four *hrp* operons of *X. campestris* pv. *vesicatoria* strain 85-10, suggesting that HrpX directly activates most HrpX-regulated genes via binding to corresponding PIP boxes (38). Base substitution experiments showed that point mutations in the PIP boxes may diminish or abolish the binding of HrpX. However, a study of the *hrpC* operon in *X. oryzae* pv. *oryzae* showed that a base substitution(s) in the PIP box did not always reduce promoter activity and could even confer considerable activities (68). Moreover, several genes having imperfect PIP boxes were shown to be regulated by HrpX (20, 68). Therefore, it is probable that *avrAC*_{Xcc8004} expression is directly activated by HrpX.

AvrAC_{Xcc8004} is an effector protein displaying LRR and Fic domains. In plant-pathogenic bacteria, the number of effector proteins containing LRRs is rather limited, and the function of only one of them, i.e., the GALA family of *R. solanacearum*, has been studied in some detail (3, 52). AvrAC_{Xcc8004} is clearly different from GALAs, since it does not contain an F-box

domain. AvrAC_{Xcc8004} possesses a Fic-like domain (Pfam database no. PF02661). Proteins displaying a Fic domain are widely distributed in bacteria but are also found in archaea and eukaryota in the Pfam database (18). However, surprisingly, although this domain is found in fungi and numerous animals, it was not detected in proteins or proteomes of plants. The functions of the proteins carrying the PF02661 domain are not known.

The *X. campestris* pv. *campestris* strain 8004 possesses three proteins with the PF02661 domain (i.e., XC0055, XC0223, and AvrAC_{Xcc8004}). Interestingly, the other *Xanthomonas* species (*X. oryzae* pv. *oryzae*, *X. axonopodis* pv. *citri*, and *X. campestris* pv. *vesicatoria*) represented in the Pfam database carry only one protein with a Fic domain, which is the ortholog of XC0223. Thus, the two proteins XC0055 and AvrAC_{Xcc8004} seem specific to *X. campestris* pv. *campestris* strains. These two proteins are not highly conserved with one another; they display similarities only in their Fic regions (25% identity and 40% similarity). It is also worth noting that the central highly conserved motif, HPFXXGNG (where H stands for histidine, P for proline, F for phenylalanine, G for glycine, N for asparagine, and X for any amino acid residue), found in Fic proteins is not perfectly conserved in AvrAC_{Xcc8004}, since a glycine residue is replaced by an alanine residue (A) (see Fig. S1 in the supplemental material). Whether this difference has any effect on the function of this domain is not known.

The DNA region encompassing *avrAC*_{Xcc8004} coding sequences appears to be unique to *X. campestris* pv. *campestris* strains (Fig. 2). Moreover, the G+C content of this region is 57% and thus differs significantly from the average value of 64.94% of the whole genome of the *X. campestris* pv. *campestris* strain 8004. This suggests that AvrAC_{Xcc8004} might have been acquired by horizontal gene transfer.

Is AvrAC_{Xcc8004} an avirulence effector recognized in vascular tissues? Using the piercing inoculation method, we observed differential behavior of strain 8004 toward Col-0 and Kashmir ecotypes, since the strain is avirulent on Col-0 whereas it induces disease symptoms on Kashmir. When the strain was infiltrated into the mesophyll using the leaf infiltration method, we did not observe such differential behavior between these two ecotypes, as both were susceptible to strain 8004. The *avrAC*_{Xcc8004} mutant became virulent on Col-0 after inoculation by the piercing method, whereas we did not detect any changes in virulence when this mutant was infiltrated into the leaf mesophyll of the Col-0 or Kashmir ecotype. How can we explain the difference observed between these two modes of inoculation? Our microscopic observations clearly show that the piercing inoculation method allows the colonization of xylem vessels (see Fig. S2 in the supplemental material). Thus, although this method bypasses the normal entry route via hydathodes, it allows the delivery of this vascular pathogen into xylem tissues. Therefore, our data could suggest the existence of specific recognition of AvrAC_{Xcc8004} in xylem tissues of Col-0. However, we cannot totally exclude the possibility that wounding induced by the piercing inoculation plays a role in the recognition of AvrAC_{Xcc8004} and the resistance of Col-0. The fact that *X. campestris* pv. *campestris* is recognized as a vascular pathogen prompts us to favor the hypothesis suggesting that AvrAC_{Xcc8004} induces vascular resistance. However, further work is necessary to show that this is really the case.

Interestingly, the phenotype of the *avrAC*_{Xcc8004} mutant on a resistant host can be compared with those of the *avrXccFM* mutant of *X. campestris* pv. *campestris* strain 528^T and the *avrXa10* mutant of *X. oryzae* pv. *oryzae*, a vascular pathogen of rice, on their corresponding resistant host plants. *X. campestris* pv. *campestris* strain 528^T elicited neither disease symptoms nor a typical HR on the leaves of the host plant Florida mustard. The *avrXccFM* mutant of 528^T became virulent on Florida mustard but was as virulent as the wild-type strain, 528^T, on susceptible host plants (9). Similarly, the *avrXa10* mutant of *X. oryzae* pv. *oryzae* became pathogenic on a resistant rice cultivar but was as virulent as the wild-type strain on a susceptible rice cultivar (5). However, our work brings a new dimension to these studies by demonstrating that AvrAC_{Xcc8004} is not recognized in mesophyll tissues but shows its avirulence function after wounding of vascular tissues. Further studies are necessary to clearly establish the molecular and physiological bases of this recognition.

Is the resistance response triggered by this avirulence gene similar to those already described in mesophyll tissues and resulting in HR elicitation? Are these resistance mechanisms present or expressed only in vascular tissues and absent in the mesophyll? Previous studies have shown that resistance of *Brassica* plants to *X. campestris* pv. *campestris* is not usually associated with a typical mesophyll HR (9) but is rather proposed to be associated with a vascular HR (VHR). This VHR shows some resemblance to the mesophyll HR but is more difficult to observe (34). Although we did not determine whether the vascular/wounding resistance of Col-0 is associated with a VHR, it is highly probable that the resistance observed in this work is typical of *X. campestris* pv. *campestris*/*Brassica* incompatible interactions. It will now be interesting to compare the behavior of the wild type and the *avrAC*_{Xcc8004} mutant on the set of natural *Brassica* hosts used to propose the existence of six races of *X. campestris* pv. *campestris* (72). The characterization of the *avrAC*_{Xcc8004} gene, which seems to behave as a typical avirulence gene in vascular tissues, may be a step forward in understanding vascular resistance. The further study of *avrAC*_{Xcc8004} and the characterization of the putative corresponding *R* gene in Col-0, which is now under way, will undoubtedly increase our understanding of the molecular basis of this specificity.

Related questions are whether AvrAC_{Xcc8004} has a function in virulence in vascular tissues and what its potential targets are inside plant cells. Our experiments did not reveal a major contribution of this effector protein to disease induction in Kashmir and Col-0, but other *Arabidopsis* ecotypes have to be tested. We also tested the virulence of the *avrAC*_{Xcc8004} mutants by the leaf-clipping method (16) on Chinese radish Manshenghong and did not observe any difference with the wild-type strain (data not shown). It is well established that the contributions of most *avr* genes of *X. campestris* pv. *campestris* to virulence in susceptible hosts can be very small (9, 77). In addition, as reported for other phytopathogenic bacteria, it seems that the effects of individual effectors for disease induction on susceptible host plants is difficult to assess. This might be due to the existence of functional redundancy and/or host specificity (21).

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