Avirulence Proteins AvrBs7 from *Xanthomonas gardneri* and AvrBs1.1 from *Xanthomonas euvesicatoria* Contribute to a Novel Gene-for-Gene Interaction in Pepper

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A novel hypersensitive resistance (HR) in *Capsicum baccatum* var. *pendulum* against the bacterial spot of pepper pathogen, *Xanthomonas gardneri*, was introgressed into C. annuum cv. Early Calwonder (ECW) to create the near-isogenic line designated as ECW-70R. A corresponding avirulence gene *avrBs7*, in *X. gardneri* elicited a strong HR in ECW-70R. A homolog of *avrBs7*, *avrBs1.1*, was found in *X. euvesicatoria* 85-10, which showed delayed HR on ECW-70R leaves. Genetic analysis confirmed the presence of a single dominant resistance gene, *Bs7*, corresponding to the two *avr* genes. Both AvrBs7 and AvrBs1.1 share a consensus protein tyrosine phosphatase (PTP) active site domain and can dephosphorylate para-nitrophenyl phosphate. Mutation of Cys(265) to Ser in the PTP domain and subsequent loss of enzymatic activity and HR activity indicated the importance of the PTP domain in the recognition of the Avr protein by the *Bs7* gene transcripts. Superpositioning of AvrBs7 and AvrBs1.1 homology models indicated variation in the geometry of the loops adjacent to the active sites. These predicted structural differences might be responsible for the differences in HR timing due to differential activation of the resistance gene. Mutating the PTP domain of AvrBs1.1 to match that of AvrBs7 failed to activate HR on ECW-70R, indicating the possibility of differential substrate specificities between AvrBs1.1 and AvrBs7.

Bacterial spot of tomato and pepper is a disease leading to significant yield loss (Pohronezny and Volin 1983). Based on the current classification, there are four genetically distinct groups of xanthomonads infecting tomato and pepper. These have been named as *Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri* (Jones et al. 2000, 2004). These groups are sufficiently different to be assigned to different species (Bouzar et al. 1999; Jones et al. 1998).

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Phytopathogenic xanthomonads possess a type III secretion system (T3SS), encoded by a hypersensitive response and pathogenicity (*hrp*) cluster (Bonas et al. 1991). The T3SS serves to secrete effector proteins that subsequently interfere with host immunity and manipulate host cellular processes (Buttner and He 2009; Grant et al. 2006). Avirulence (Avr) proteins are type III effectors that are recognized by resistance (*R*) gene products, in particular host genotypes. Many Avr proteins have been characterized, and in some cases, the mechanism of *R*-gene interaction has been studied. In the absence of the corresponding *R* gene, Avr proteins sometimes act as virulence factors contributing to susceptibility (Kjemtrup et al. 2000). Avirulence (*avr*) genes have also been studied for their contribution to pathogen evolution. Chemical control strategies such as sprays of copper or streptomycin have not significantly mitigated bacterial spot disease (Bouzar et al. 1999). Efforts have been focused on breeding for resistance in tomato and pepper cultivars. Five resistance genes in peppers (*Bs1*, *Bs2*, *Bs3*, *Bs4*, and *BsT*) that provide a hypersensitive type of resistance have been identified, and the corresponding *avr* genes from the pathogen have been characterized (Stall et al. 2009). Two recessive resistance genes, *bs5* and *bs6*, provide a non-hypersensitive or multigenic resistance that has been shown to be more durable (Stall et al. 2009). Screening for such novel resistance genes continues to be paramount, since the pathogen continues to evolve as a result of selective pressure and overcomes host resistance in the field (Gassmann et al. 2000; Swords et al. 1996; Wichmann and Bergelson 2004). While searching for resistance in pepper genotypes against bacterial spot pathogens, we identified a novel resistance in *Capsicum baccatum* var. *pendulum* against races of *X. euvesicatoria* and *X. gardneri*. Genetic segregation analysis in the F₂ population of introgression line ECW-70R confirmed the presence of a single dominant resistance gene conferring resistance against two *avr* gene homologs from *X. gardneri* and *X. euvesicatoria*. Here, we present an example of an *avr* gene possessing enzyme activity, which is essential for triggering a resistance pathway. In this paper, we also compare two *avr* genes with significant genotypic homology but with differences in the timing of activation of the phenotypic resistance response. Based on the results of the mutational analyses by catalytic site exchange and analysis of homology models generated for each protein, we speculate that the two Avr proteins recognize different substrates during *R*-gene activation.

RESULTS

Identification of resistance in pepper against bacterial spot xanthomonads and development of introgression lines carrying the resistance gene.

A *Capsicum* genotype from Brazil, designated 1556, was tested for hypersensitive resistance (HR) against races of *X. euvesicatoria* and against *X. gardneri* 444. A HR was observed in plants of line 1556 when inoculated with pepper races 1, 2, 3, and 6. This line was a member of *Capsicum baccatum* var. *pendulum*. The fruit of this line is of the elongated chili type and is pungent. Since this line appeared to have resistance to different races of the bacterial spot xanthomonads, crosses were made with *Capsicum annuum* cv. Early Calwonder (ECW) for the purpose of transferring the resistance to a plant with bell-shaped, nonpungent fruit.

The first cross was interspecific, and only a few seeds were obtained. Some of the seeds germinated and a few plants of an F_2 population were obtained following self-pollination of a F_1 plant. Segregation of resistance in the F_2 population seemed to occur, but the inheritance of the resistance could not be obtained because of the low number of plants. It was then decided to transfer the resistance via a backcross (BC) program to the recurrent susceptible parent, ECW (Fig. 1). Plants in each BCF_1 generation were screened for resistance to Xv444, which provided a strong HR in resistant plants. In a third backcross, F_1 plants were male sterile but female fertile. Instead of pollination of susceptible parent plants, as was done previously in the backcross procedure, pollination of resistant plants with pollen from a susceptible plant was necessary. In the next generation, a plant that was resistant to Xv444 and male fertile was identified. All future backcrosses were based on this plant and were always male fertile. Eventually, a population of plants in the seventh backcross was obtained that did not appear to have the fertility problems present in the early population of the interspecific cross and was uniform in resistance. A population in the seventh BCF_4 was used to determine the inheritance of

the resistance gene, designated as *Bs7*. A genotype containing *Bs7* and near isogenic to ECW was named ECW-70R.

AvrBs7 from Xv444 elicits HR in pepper cv. ECW-70R.

Among the different strains of the bacterial spot pathogens tested on pepper cv. ECW-70R, *X. gardneri* Xv444 was found to elicit a strong HR. An *avr* gene corresponding to the *R* gene (i. e., *Bs7*) was isolated by mobilizing pLAFR3 clones from a genomic DNA cosmid library of Xv444 into *X. gardneri* Xg5, which is virulent on ECW-70R. One cosmid (pXv444-352) was found to elicit a HR in ECW-70R. A 5-kb fragment, pXv444-352Bam8, from the above cosmid clone was further subcloned into pLAFR3, using *Bam*HI, *Pst*I, and *Eco*RI restriction enzymes. This resulted in three subclones of 1, 1.8, and 2 kb. None of the subclones elicited a HR in pepper cv. ECW-70R. Sequence analysis of the clone showed that there was one open reading frame (ORF) spanning the region cut by the restriction enzymes used to generate the subclones. This ORF with 500 bp of upstream sequence was cloned by polymerase chain reaction (PCR), using *avrBs7F* and *avrBs7R* primers, into the pGEMT-Easy vector, and the insert was confirmed by sequencing. The insert was then transferred to pLAFR3 and was conjugated into Xg51. Transconjugants carrying this insert exhibited a strong HR similar to the wild-type Xv444 by 24 h after infiltration (Fig. 2). This ORF was named *avrBs7*. It consists of 1,071 bp encoding a 356-amino-acid protein of 39.734 kDa. It shows 67% sequence identity at the amino-acid level to *avrBs1.1* (XCVd0105) from *X. euvesicatoria* 85-10, whose genome was sequenced previously (Thieme et al. 2005). The possibility that *avrBs1.1* from *X. euvesicatoria* 85-10 was responsible for HR when infiltrated into leaves of ECW-70R was pursued experimentally.

AvrBs1.1 from *X. euvesicatoria* 85-10 elicits delayed HR on ECW-70R.

The *avrBs1.1* gene with 500 bp of upstream sequence was isolated by PCR using primers 8510-Bs1.1F and 8510-Bs1.1R,

Fig. 1. Backcross program for the development of introgression lines carrying the resistance gene *Bs7*.

was cloned into pLAFR3, and was conjugated in Xg51. Transconjugants carrying *avrBs1.1* produced a weak HR by 32 h postinoculation (hpi) and a complete necrosis by 48 hpi in leaves of ECW-70R (Fig. 2B). The susceptible reaction due to Xg51 was first observed at 72 h after infiltration.

Genetic analysis of *Bs7* resistance in ECW-70R.

Genetic segregation of resistance was analyzed by inoculating an F₂ population (ECW-70R × ECW) with Xg51 transconjugants carrying the *avrBs7* clone and with Xg51 transconjugants carrying the *avrBs1.1* clone in parallel. The two clones were also introduced into the race 6 strain *X. euvesicatoria* TED3 and were tested for their phenotype on the F₂ population.

A total of 166 F₂ plants were infiltrated with Xg51 transconjugants carrying the *avrBs7* clone. They were scored for HR or susceptibility at 48 hpi; 117 plants (70.5%) developed a HR and 49 (29.5%) exhibited a water-soaked phenotype (Table 1). An analysis of segregation ratio showed a fit of 3:1 ratio ($\chi^2 = 1.80$; *P* value between 0.2 and 0.1), confirming that the resistance trait was inherited as a single dominant resistance gene. However, when the *avrBs7* clone was introduced into *X. euvesicatoria* pepper race 6 strain TED3 and tested on another F₂ population, more susceptible plants were found than expected. Of a total 59 F₂ plants tested, 24 plants (40%) were susceptible. Analysis

revealed a $\chi^2 = 7.93$ and a *P* value between 0.01 and 0.001, with the closest fit to a Mendelian segregation ratio of 3:1. The *P* values for other segregation ratios were insignificant.

When Xg51 transconjugants carrying the *avrBs1.1* clone were infiltrated into an ECW-70R F₂ population in parallel with Xg51 transconjugants carrying the *avrBs7* clone, the F₂ population exhibited 71 resistant (66%) plants and 36 (34%) susceptible plants, giving a χ^2 value of 4.264 with the *P* value slightly lower than 0.05. The same *avr* gene, when introduced into *X. euvesicatoria* TED3 race 6 and inoculated in the leaves of the second F₂ population of 59 F₂ plants, yielded 40 resistant (68%) plants and 19 susceptible (32%) plants, with the χ^2 value of 1.63 and *P* value between 0.3 and 0.2. This indicated a good fit to a 3:1 Mendelian segregation ratio (Table 1).

In an inoculation of a backcross of an F₁ plant with ECW-70R, both transconjugants of *avrBs7* and *avrBs1.1* clones in *X. euvesicatoria* TED3 caused a HR. Hence, we can speculate that *AvrBs1.1* might react with the *Bs7* *R* gene transcript itself or possibly with another resistance gene closely linked to *Bs7*.

In-planta growth studies and electrolyte leakage.

In population experiments performed through 6 days postinoculation (dpi) of ECW-70R, the size of the Xg51 population carrying the *avrBs7* clone increased by approximately 3 log, while the Xg51 population carrying the *avrBs1.1* clone showed .5 log more growth than the *avrBs7* clone, after which growth for both transconjugants ceased to increase. The wild-type virulent strain Xg51 increased up to 4 log by 8 dpi (Fig. 3A). Wild-type Xv444 showed approximately a 2.5 log CFU/cm² increase in growth by 8 dpi in ECW-70R and stayed at the same level afterwards, whereas deletion mutant Xv444 Δ *avrBs7* grew approximately 2.5 log less compared with wild type by 8 dpi (Fig. 3C).

In electrolyte leakage experiments, there was a difference in the extent and speed of tissue damage between *avrBs7* and *avrBs1.1* clones on ECW-70R (Fig. 4A and B). The electrolyte leakage in tissue inoculated with Xg51 carrying *avrBs7* in-

Table 1. Genetic segregation analysis of F₂ population

Recipient strain	AvrBs7		AvrBs1.1	
	HR	W	HR	W
Xg51	117	49	71	36
	70.5%	29.5%	66%	34%
	$\chi^2 = 1.80$		$\chi^2 = 4.264$	
	<i>P</i> = 0.2 to 0.1		<i>P</i> = 0.05 to 0.01	
TED3 race 6	35	24	40	19
	60%	40%	68%	32%
	$\chi^2 = 7.93$		$\chi^2 = 1.63$	
	<i>P</i> = 0.01 to 0.001		<i>P</i> = 0.3 to 0.2

Fig. 2. Phenotype on ECW-70R **A**, 24 and **B**, 48 h postinfiltration by wild-type strains, transconjugants, and mutants. Order of inoculation as follows: 1, Xg51 (pLAFR3: *avrBs7*); 2, Xg51 (pLAFR3: *avrBs1.1*); 3, Xv444 Δ *avrBs7* mutant; and 4, Xg51.

creased significantly by 36 h, showing a peak value at 48 h. For Xg51 carrying *avrBs1.1*, electrolyte leakage started increasing slowly after 12 h, with a significant increase after 36 h, continuing to increase to 60 h after inoculation. Electrolyte leakage of Xv444 and Xv444 Δ *avrBs7* was similar after the first 12 h, but leakage increased dramatically for Xv444 in the next 24 h, showing a peak at 36 h after inoculation, while leakage caused by Xv444 Δ *avrBs7* remained almost unchanged at 36 hpi. In the last 12 h, tissue damage caused by Xv444 Δ *avrBs7* showed only a slight increase (Fig. 4C). In ECW, electrolyte leakage remained low for both strains over 60 h (Fig. 4D).

A catalytic tyrosine phosphatase domain is involved in recognition by the *BS7 R* gene product in ECW-70R.

Sequence analysis of both *avr* genes identified the presence of a tyrosine phosphatase domain in the C-terminal region. AvrBs7 belongs to classical pTyr-specific protein tyrosine phosphatases (PTP) (pfam family Y_phosphatase PF00102). AvrBs1.1 contains a dual specificity phosphatase domain (pfam family DSPc PF00782), indicating its ability to dephosphorylate Ser and Thr phosphate containing proteins in addition to Tyr phosphate-containing proteins.

To determine if the two Avr proteins possess PTP activity, the purified proteins from culture filtrates of *Escherichia coli* BL21-DE3 transformants expressing His-tagged AvrBs7, AvrBs1.1 and CysmutAvrBs7 (*avrBs7* containing a single

amino-acid mutation at the PTP catalytic domain Cys265 to Ser) were tested for their ability to hydrolyze an artificial substrate, para-nitrophenyl phosphate (pNPP) (Howard et al. 1992). The purified proteins, AvrBs7 and AvrBs1.1, hydrolyzed a general substrate pNPP with the enzyme activity of 316 and 440.6 pmole/min, respectively, at pH 4.8 at 37°C. The calculated specific activities for purified AvrBs7 and AvrBs1.1 proteins were similar (402. pmole/min per milligram of protein for AvrBs7 and 496.8 pmole/min per milligram of protein for AvrBs1.1). The lack of PTP activity for CysmutAvrBs7 protein indicated that the Cys residue within the catalytic domain is critical for enzyme function.

Blastp searches using *avrBs7* and *avrBs1.1* as queries yielded matches with effectors from other plant pathogens. Interestingly, some effectors from other xanthomonads and other plant-pathogenic bacterial genera also possess tyrosine phosphatase activity, specifically dual-specificity phosphatase activity. *Xanthomonas campestris* pv. *campestris* 33913 along with *X. campestris* strains 8004 and B100 encode the AvrBs1.1 effector. Effector *avrBs1.1* has been classified as *xopH* according to the recent classification of Xop effectors (White et al. 2009). *Pseudomonas syringae* pv. *tomato* DC3000 secretes the HopAO1 effector (HopPtoD2), which has been shown to suppress programmed cell death in *Nicotiana benthamiana*, suggesting its role as a virulence factor interfering with a mitogen-activated protein kinase (MAPK) pathway and downstream defense path-

Fig. 3. Time course of bacterial population growth after infiltration of leaves of pepper genotypes Early Calwonder (ECW) and ECW-70R by Xg51 trans-conjugants and mutant strains.

ways (Espinosa et al. 2003). Despite a lack of significant sequence identity to AvrBs7, the effector HopAO1 also belongs to the Y-phosphatase pfam family. *Acidovorax citrulli* AAC00-1 contains another homolog, Aave_3502, with a dual-specificity phosphatase domain. The widespread distribution of phosphatase activities in phytopathogenic bacteria underscores their importance in HR and led us to determine if the tyrosine phosphatase domains in AvrBs7 and AvrBs1.1 are responsible for activating the resistance pathway in ECW-70R. Tyrosine phosphatases use an aspartic acid and a cysteine to serve as respective proton donor and nucleophile in the catalysis of the phosphatase reaction (Espinosa et al. 2003). Mutation of 10 amino acids (aa) in the catalytic domain (HCGVGQGRGTG to AAAAASAAAA) of *avrBs7* abolished HR activity of Xg51 carrying the *avrBs7* clone (Fig. 5A). Mutation of a single aa (Cys265 to Ser) in the same domain of *avrBs7* also failed to elicit a HR on ECW-70R (Fig. 5B). Although mutant AvrBs7 protein was produced, it lacked PTP activity and, in turn, could no longer be recognized by the *R* gene product. These data strongly support the hypothesis that this domain contributes to recognition of *Bs7* gene product inside the plant cell and that *avrBs7* encodes a tyrosine phosphatase.

Avr proteins AvrBs7 and AvrBs1.1 display similar tertiary protein structure based on homology modeling.

AvrBs7 shares 67% amino-acid identity to AvrBs1.1, and the lowest energy models generated for each protein via homology modeling show similar structural identities, despite using different input structure sets for the initial Modeller run (Fig. 6). As previously discussed, both proteins are most closely related to the PTP family of proteins based upon BLAST queries and mul-

tipule sequence alignments. PTP catalyze the hydrolysis of phosphate groups from phosphotyrosine in a reaction mechanism involving cysteine as a nucleophile, aspartic acid as a general acid, and histidine as a charge-stabilizing residue. The active sites of these proteins contain the phosphotyrosine binding loop (P-loop), in which the nucleophilic cysteine residue is found as well as a loop bearing the general acid moiety (WPD loop). Superpositioning of the lowest energy models generated for each Avr protein did not result in significant differences detected with respect to active site geometry, including both the P-loop and the WPD loop (Fig. 6C). The three distinguishable differences between the AvrBs7 and AvrBs1.1 models include loops adjacent to the active site spanning aa 190 to 213 loop and aa 304 to 315 as well as a longer segment of protein sequence spanning aa 1 to 83 that was predicted to adopt a different geometry in nearly every homology model obtained. These regions are highly variable in the predicted models, indicating the possibility of their involvement in either substrate binding, differentiation, or both. Electrostatic surfaces placed over the superpositioned models (Fig. 6A1 and A2), reveal that the AvrBs1.1 structure is predicted to have a more closed active site cleft, due to the encroachment of the 199 to 205 loop over the active site, further supporting the hypothesis that there might be differences in substrate binding sites between AvrBs7 and AvrBs1.1.

There is difference in the timing of HR elicitation by AvrBs7 and AvrBs1.1.

Xg51 transconjugants carrying *avrBs1.1* show a delayed HR compared with Xg51 transconjugants carrying the *avrBs7* clone (Fig. 2). This phenotype was also confirmed by higher

Fig. 4. Electrolyte leakage from pepper genotypes Early Calwonder (ECW) and ECW-70R after infiltration of leaves with Xg51 wild type, transconjugants, and mutant strains.

population growth by day 6 and slower tissue damage, in the case of Xg51 carrying *avrBs1.1*, compared with *avrBs7* (Figs. 3 and 4). We were interested in learning why AvrBs1.1 causes a delayed HR. As mentioned above, *avrBs7* encodes a conserved catalytic domain for tyrosine phosphatase (HCGVGGQRTG), whereas, *avrBs1.1* is predicted to encode a dual specificity phosphatase (HCGMGLGRTT), based on pfam domains. Our hypothesis was that the difference in the timings of tissue damage and HR is due to differences in the amino-acid residues at the catalytic domain. We aligned AvrBs7 and AvrBs1.1 using clustalw (Fig. 7). We fused the amino-terminal 262 aa of AvrBs7 (just upstream of the catalytic domain) to the carboxy-terminal 90 aa of AvrBs1.1 containing the catalytic domain of AvrBs1.1, resulting in the replacement of the catalytic domain of AvrBs7 with that of AvrBs1.1. This exchange abolished the hypersensitive response of AvrBs7 on ECW-70R (Fig. 8A). According to our hypothesis, if the difference between AvrBs7 and AvrBs1.1 was only at the catalytic domains, replacement of AvrBs7 catalytic domain to that of AvrBs1.1 should have altered the hypersensitive response of AvrBs7 by delaying it similarly to AvrBs1.1. The homology models of this fusion construct indicated that the fusion protein might have become inactive, due to

modification in tertiary structure (Supplementary Fig. 1). To rule out the possibility of disruption of tertiary structure, we created three single amino-acid changes within the catalytic domain of *avrBs1.1*, mutating residues to match those found in the catalytic domain of *avrBs7* (HCGMGLGRTT to HCGVGGQRTG) by using site-directed mutagenesis. If the mutated residues were important in determining the timings of HR and, in turn, activation of the appropriate *R* gene, exchange of catalytic domains of AvrBs1.1 with that of AvrBs7 should have resulted in stronger HR within 24 hpi. However, the site-directed mutant did not exhibit HR by either 24 or 48 hpi (Fig. 8B). This result is likely due to differences in substrate specificities for the two Avr proteins during the activation of the resistance response and differences caused by variations in the loops lining the active sites. Based on the models, the loop encompassing aa 190 to 213 is highly variable in the two Avr proteins, with the closest approach between it and the P-loop housing the nucleophilic cysteine predicted to be 1.25 nm in AvrBs7 and only .48 nm in AvrBs1.1 (Fig. 6A and B). This difference results in a much more closed active site geometry for AvrBs1.1, which would prevent the substrate that normally fits into the AvrBs7 active site from binding in the mutated protein.

Fig. 5. Tyrosine phosphatase domain is essential for hypersensitive resistance (HR) elicitation on ECW-70R. **A**, Alanine mutagenesis of catalytic domain of *avrBs7* abolished HR on ECW-70R. 1 and 2, Xg51 transconjugants carrying the AlaBs7::pLAFR3 clone with alanine mutagenesis within the catalytic domain of *avrBs7*; 3, Xg51 transconjugants carrying the AvrBs7::pLAFR3 clone; and 4, Xg51 carrying the pLAFR3 clone. **B**, Mutation of Cys to Ser within catalytic domain of *avrBs7* loses HR on ECW-70R. 1, Xg51 transconjugants carrying the cysBs7::pLAFR3 clone mutating Cys to Ser within catalytic domain of *avrBs7*; 2, TED3 race 6 transconjugants carrying the cysBs7::pLAFR3 clone mutating Cys to Ser within catalytic domain of *avrBs7*; and 3, Xg51 transconjugants carrying the AvrBs7::pLAFR3 clone.

Both the Avr proteins are partially dependent on global chaperone HpaB for translocation.

Translocation of the two Avr proteins by a race 6 *X. euvesicatoria* strain, TED3, Δ HpaB mutant was tested to confirm if either of the Avr proteins are dependent on chaperone for translocation and if chaperone dependency is the reason for the differences in the timings of HR by the two Avr proteins. Both *X. euvesicatoria* TED3 race 6 Δ HpaB transconjugants carrying *avrBs7* and *avrBs1.1* showed slower HR development, with visible HR 48 hpi for *avrBs7* and 72 hpi for *avrBs1.1*. This indicates that both the Avr proteins can be translocated in the absence of HpaB, albeit, in reduced amounts. The chaperone does not seem to have an effect on relative differences in the timing and nature of HR by the two Avr proteins.

Host specificity of bacterial spot strains.

Strains belonging to groups A, B, and D of bacterial spot xanthomonads were tested for their phenotype on ECW-70R (Table 2). HR was classified into two categories—strong HR observed after 24 h (as seen for *avrBs7* containing transconju-

gants) and weak HR observed after 48 h (as seen for *avrBs1.1* containing transconjugants).

We also developed *avrBs7*- and *avrBs1.1*-specific primers to identify the type of *avr* gene present in strains listed in Table 2. PCR amplification results correlated well with the phenotype observed after infiltration, thus classifying strains into those carrying *avrBs7* and those carrying *avrBs1.1*. The *avr* gene *avrBs7* was exclusively limited to *X. gardneri* strains (D group). Gene *avrBs1.1* was found in *X. euvesicatoria* (A group) and *X. vesicatoria* (B group) strains. *X. vesicatoria* Xv1111 (B group strain), the draft genome, which has been already sequenced (Potnis et al. 2011), also has *avrBs1.1* but with an internal stop codon. The internal stop codon was confirmed by sequencing the PCR product. Thus, the *avr* gene in this strain is inactive and the strain seems to escape detection by the *R* gene in ECW-70R. Xv1111 encodes the AvrBsT protein, which elicits HR on all pepper cultivars. Therefore, whether the inactive form of AvrBs1.1 helps the pathogen to escape detection by the *R* gene could not be tested experimentally in pepper. There were strains in each group that did not contain either *avr* gene.

Fig. 6. Electrostatic surface rendering of the avirulence superpositioned models (A1: AvrBs7, B1: AvrBs1.1). The positive-charge density at the core of the protein corresponds to the active site pocket of the protein. The active site cleft of AvrBs1.1 is obscured by a protruding loop spanning amino acids 199 to 205 in the sequence. Surfaces were calculated using APBS with the superpositioned models of each protein generated by Theseus as input. A2 and B2 represent superposition of the lowest energy homology models generated for AvrBs7 and AvrBs1.1, respectively. Amino acids 1 to 83 could not be modeled due to a lack of suitable templates in the Protein Data Bank and, as such, are not included in the figure. The catalytic amino acids (AvrBs7: Cys²⁶⁵, Asp²³⁶ and Arg²⁷¹; AvrBs1.1: Cys²⁶⁷, Asp²³⁸ and Arg²⁷³) are shown as sticks. For the models of AvrBs7 and AvrBs1.1, very little variation is seen in the catalytic region of the protein, but the surrounding loops show significant variation, which may reflect the differing substrate specificities of the two proteins. Superposition of the lowest energy models for *avrBs7* (red), *avrBs1.1* (blue). The catalytic amino acids for the proteins are shown as sticks. The phosphotyrosine binding loop (P loop) and the loop containing the general acid moiety (WPD loop) are shown using the *avrBs7* amino acid numbering.

Avr genes *avrBs7* and *avrBs1.1* are encoded on large transmissible plasmids.

The *avrHah1* gene has already been identified from Xv444 to be a plasmid-borne gene (Schornack et al. 2008). The J1 mutant of Xv444, a strain cured of the plasmid encoding AvrHah1, showed a water-soaking phenotype by 3 days after infiltration of ECW-70R (data not shown). This indicates that two *avr* genes, *avrBs7* and *avrHah1*, might be encoded on the same plasmid in Xv444.

Gene *avrBs1.1* from *X. euvesicatoria* 85-10 is encoded on plasmid pXCV183 (not copper resistant), upstream of *avr* gene *avrBs1* (Thieme et al. 2005). Some of the *X. euvesicatoria* strains, tested for phenotype on ECW-70R, are also known to carry a copper-resistance plasmid similar to pXCV183. There was a correlation noted between copper resistance and the *avrBs1.1* phenotype (Table 2).

DISCUSSION

Capsicum baccatum is the most commonly grown chile pepper in South America and can be found from southern Brazil to Bolivia, Ecuador, Peru, and Chile. A wild form denominated *C. baccatum* var. *baccatum* and a domesticated form (*C. baccatum* var. *pendulum*) exist (De Witt and Bosland 2009). Diversity for pod shape and size can be found on domesticated forms, with fruits ranging from short, pointed pods borne erect to long and pendant pods. In Brazil, *C. baccatum* var. *pendulum* is one of the most popular chile peppers and at least two fruit types are very common and largely used in field production, i.e., lady finger fruit type (“dedo-de-moça”) and the “cambuci” type, introduced in the United States from Brazil and known as ‘Christmas Bell’. Brazil is also considered a genetic diversity center of *Capsicum* spp., and at least 16 species of this genus already described are found in Brazil (Barboza and Bianchetti 2005; Moscone et al. 2007). The multiple and diverse ways to use

these fruits are related to multicultural and ethnic aspects, and it is probably the result of the genetic diversity observed in each region of the country (Sudré et al. 2010). There are some expectations that new species (Barboza and Bianchetti 2005) and new genes for breeding peppers can be found in this important genetic resource that remains unexploited. In the present work, the specific accession *Capsicum baccatum* var. *pendulum* coded as 1556 was used as a new resistance source for bacterial spot resistance, for introgressing a resistance gene into *C. annuum* in order to obtain recombinant inbred lines. The interspecific crosses have been carried out in the past for introgressing bacterial spot resistance gene transfer (Astua-Monge et al. 2000; Cook and Guevara 1984) but not using *C. baccatum*. We isolated corresponding *avr* genes from Xv444 and *X. euvesicatoria* 85-10. The *avr* gene from Xv444 has been designated as *avrBs7*, while the *X. euvesicatoria* 85-10 gene was previously referred to as *avrBs1.1* (Thieme et al. 2005).

Segregation analysis of an F₂ population from the cross ECW-70R × ECW identified a gene-for-gene interaction for the *avr* gene *avrBs7* from *X. gardneri* Xv444 and a corresponding *R* gene *Bs7* from pepper cv. ECW-70R, similar to the gene-for-gene model explained by Flor (1971). The segregation of resistance in an F₂ population was identical after inoculation with *avrBs7* containing transconjugants and *avrBs1.1* containing transconjugants, indicating that both *avr* genes possibly interact with the same *R* gene or a linked *R* gene. Two Avr proteins interacting with the same *R* gene transcript has been reported previously. For instance, the two homologous Avr proteins AvrBs3 and AvrHah1 both interact with the promoter of the *Bs3* gene in pepper (Schornack et al. 2008). Two different Avr proteins, AvrB and AvrRpm1, from *Pseudomonas syringae* strains also have been shown to interact with the RPS3 and RPM1 resistance product from *Arabidopsis* (Bisgrove et al. 1994). The two Avr proteins AvrBs7 and AvrBs1.1 show differences in the elicitation of the resistance response on

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avrBs1.1      MPNKISGSIAPSASSDAMKSADCAENIKEEVVSKHVHQAVPAELADLPSRQPPRSKTA-L
avrBs7        MPNPVSRSSSTSSVSGKGSDDADVADIKQEAVVEPGNQSTPHGLEGLA----PRSKTARD
               ***  : *  *  : . * . . . . . * . . : * : * . * : : * : . *  * . * . *****

avrBs1.1      YQVIQKFRDPLPLPPPPTSHPVLAYDRDLGSS-DNFRSSDEFDLPESLNPTGWKNLHVSG
avrBs7        LSLIKKFSNPLPLPQRPTEIPVLQYDRSPRSSSDNFRSSDDFDLPESCNP TGWKDLHVSG
               . : * : * * : * * * * * * * . * . * * * * * . * * * * * * * : * * * * * : * * * * *

avrBs1.1      SGSIASIGQITRLRPSKERPVVLDAREESHAIVGGYPGTWRTPNNWGNAGKSRDEALAD
avrBs7        SGSIASISQITRLNPSRDRPVIVLDVREESHAIVGGYPATWRAPNNWANVGKSRREEVLAD
               * * * * * . * * * * . * * : : * * : * * . * * * * * * * * * . * * : * * * * . * * *

avrBs1.1      EQQRIQALKSQETVHIFHRKDVKSEARNPRGATLSKPLIFSEEELVRAAGAKYVRLTVTD
avrBs7        EHEKIRAIKSQETVQILHRKDVKNGFPNPRSVKLSNPSIFSEEELVRNAGA EYLRLTVTD
               * : : : * : * * * * * : * : * * * * . * * * . . * : * * * * * * * * * : * : * * * * *

avrBs1.1      HLSPRADDIDAFIAMEREMAHDERLHVHCGMGLGRTTIFIVMHDILRNAAMLSFDDIIER
avrBs7        HLGPRADDIDAFVRMERNMAPHERLHVHCGVGQGRGTGFIAMHDILRNAHIISFEDIKR
               * . * * * * * * * : * * : * * . * * * * * : * * * * * . * * * * * : : * * : * * : *

avrBs1.1      QRKFNPGRSLDNNKDVSDKGRSEFRNERSEFLPLFYEYAKQNPKGQPLLWSEWLDHNA--
avrBs7        QLAFNPGRALDFNKDVSHEGRSDFRNDRLLELISLFYEYAKSNPNQGQPSLWSEWLRAANKT
               * * * * * : * * * * * . : * * : * * : * * : : * * * * * . * * : * * * * *

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Fig. 7. Alignment of *avrBs1.1* and *avrBs7* amino acid sequences using clustalw.

ECW-70R. The *avrBs1.1* containing transconjugants elicited a delayed HR on ECW-70R compared with *avrBs7* containing transconjugants. Further experiments, such as bacterial population dynamics and electrolyte leakage (Cook and Stall 1968), corroborated the differences in the timing of resistance-gene activation by the two *avr* genes on ECW-70R.

Avr genes with putative enzyme activity have been characterized in xanthomonads (Kearney and Staskawicz 1990; Mudgett et al. 2000; Roden et al. 2004). Here, we present evidence of another *avr* gene possessing enzyme activity that is required for elicitation of HR. Although *avrBs7* and *avrBs1.1* share only 67% identity at the amino-acid level, they have a common characteristic; both belong to the tyrosine phosphatase superfamily. The carboxy-terminus of both *Avr* proteins contains a consensus PTP active site domain (HCGVVGQGRGTG for *avrBs7* and HCG MGLGRIT for *avrBs1.1*) along with a possible general acid motif (TVTDH) 24 aa upstream. Both *Avr* proteins can dephosphorylate artificial substrate pNPP, confirming their phosphatase activity. Superpositioning the lowest energy models of each *Avr* protein resulted in low RMSD values for the main chains of the PTP domain and the general acid motif (Fig. 6C), with nearly identical positions observed for the amino-acid side chains of the two proteins. Alanine mutagenesis of the entire *avrBs7* PTP domain as well as a single Cys265 to Ser mutation within the PTP domain failed to elicit a HR on ECW-70R, indicating that the PTP domain appears to be important in recognition of the *Avr* protein by *R* gene transcripts. The characteristic phosphotyrosine recognition region (Fauman and Saper 1996) was located in the two *Avr* protein sequences. Sequence YDR, at positions

81, 82, and 83 of *AvrBs7* and at positions 84, 85, and 86 of *AvrBs1.1*, represents a possible recognition loop of PTP.

Tyrosine phosphatases have been found as type III effectors in animal as well as plant pathogens. They have been shown to interfere with the host signal transduction pathways, thus functioning as virulence factors. *Yersinia pseudotuberculosis* secretes the type III effector YopH, a classic PTPase family protein. YopH, in activated form, dephosphorylates p130^{Cas} and FAK substrates and, thus, resists its uptake by the host mammalian cells (Persson et al. 1997). Similarly, *Salmonella* type III effector SptP possesses tyrosine phosphatase activity, which increases pathogen replication by dephosphorylating host AAA+ ATPase valosin-containing proteins (Humphreys et al. 2009). *Pseudomonas syringae* pv. *tomato* DC3000 contains HopPtoD2 (i.e., HopAO1 type III effectors), which is a chimeric protein with an N terminal region similar to *avrPphD* hooked onto a tyrosine phosphatase containing a C terminal region. *AvrPphD* from *Pseudomonas syringae* pv. *phaseolicola* elicits a nonhost HR on pea (Arnold et al. 2001). Various hopPtoD alleles have been found in pseudomonads. In contrast to *AvrPphD*, HopPtoD2 acts as a virulence factor, targeting proteins downstream or independent of MAPK, suppressing plant innate immunity (Bretz et al. 2003; Underwood et al. 2007).

To understand the differences between the two *avr* genes in terms of their mechanism of differential resistance gene activation, we carried out several mutational analyses. The results of the mutational analyses were supported by three-dimensional homology modeling. Superpositioning of the *AvrBs7* and *AvrBs1.1* homology models did not show significant structural

Table 2. List of bacterial strains and plasmids used in this study

Strain designation	Relevant characteristics ^a	Source or reference ^b
<i>Xanthomonas euvesicatoria</i>		
85-10	Pepper race 2, tomato race 1, Rif ^r	Minsavage et al. 1990
TED3	Pepper race 6	
TED3 ΔHpaB	Pepper race 6 deletion mutant for <i>hpaB</i> gene	N. Potnis, unpublished
E3	Cu ^r , HR(+) ECW 70R, <i>avrBs1.1</i> (+)	R. E. Stall (Florida, 1960) unpublished
Xv718	Cu ^r , HR(+) ECW 70R, <i>avrBs1.1</i> (+)	J. B. Jones (Puerto Rico 1991) unpublished
Xv881	Cu ^s , HR(-) ECW 70R, <i>avrBs1.1</i> (-)	J. B. Jones (Mexico, 1992) unpublished
Xv669	Cu ^s , HR(-) ECW 70R, <i>avrBs1.1</i> (-)	J. B. Jones unpublished
Xv1025	Cu ^s , HR(-) ECW 70R, <i>avrBs1.1</i> (-)	J. B. Jones (Mexico, 1992) unpublished
Xv818	Cu ^s , HR(-) ECW 70R, <i>avrBs1.1</i> (-)	J. B. Jones unpublished
ENA4135	Cu ^s , HR(-) ECW 70R, <i>avrBs1.1</i> (-)	R. Rodrigues, unpublished
<i>Xanthomonas gardneri</i>		
Xg51	HR(-) ECW 70R, <i>avrBs7</i> (-)	G. Minsavage, unpublished
Xv444	HR(+) ECW 70R, <i>avrBs7</i> (+)	Jones et al. 2004
Xv444 Δ <i>avrBs7</i>	Xv444, <i>avrBs7</i> deletion mutant	This study
J1	Mutant strain of Xv444 cured of the plasmid carrying <i>avrHah1</i>	Schornack et al. 2008
XV1927 (BSX104A)	HR(+) ECW 70R, <i>avrBs7</i> (+)	D. Cuppels, AAFC, London, ON, Canada
01T46A	HR(+) ECW 70R, <i>avrBs7</i> (+)	D. Cuppels, AAFC
02T1A	HR(+) ECW 70R, <i>avrBs7</i> (+)	D. Cuppels, AAFC
04T5	HR(+) ECW 70R, <i>avrBs7</i> (+)	D. Cuppels, AAFC
98T3A	HR(-) ECW 70R, <i>avrBs7</i> (-)	D. Cuppels, AAFC
00T12B	HR(-) ECW 70R, <i>avrBs7</i> (-)	D. Cuppels, AAFC
99T4A	HR(-) ECW 70R, <i>avrBs7</i> (-)	D. Cuppels, AAFC
1782	HR(+) ECW 70R, <i>avrBs7</i> (+)	Phytopathology Culture Collection of Instituto Biológico, CEIB, Campinas, SP, Brazil
1783	HR(+) ECW 70R, <i>avrBs7</i> (+)	Phytopathology Culture Collection of Instituto Biológico
Furman 3	HR(+) ECW 70R, <i>avrBs7</i> (+)	G. Minsavage, unpublished
XV451	HR(+) ECW 70R, <i>avrBs7</i> (+)	J. B. Jones, unpublished
<i>Escherichia coli</i>		
DH5α	F ⁻ <i>recA</i> Φ80 <i>lacZ</i> ΔM15	Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.
λPIR	Host for pOK	Huguet et al. 1998
Plasmids		
pLAFR3	Tc ^r <i>rlx</i> ⁺ RK2 replicon, Tc ^r	Staskawicz et al. 1987
pBlueScript II KS +/-	Phagemid sequencing vector, Ap ^r	Stratagene, La Jolla, CA, U.S.A.
pRK2073	Sp ^r Tra ⁺ , helper plasmid	Figurski and Helinski 1979

^a Rif^r, Tc^r, and Sp^r, = rifampicin-, tetracycline-, and spectinomycin resistant; Cu^r and Cu^s = copper resistant or sensitive, respectively.

^b AAFC = Agriculture and Agri-Food Canada; CEIB = Centro Experimental Central do Instituto Biológico.

differences in the active site region of the proteins. However, there was some variation in the geometry of the loops adjacent to the active sites. In the lowest energy homology models of AvrBs1.1, the loop spanning aa 199 to 213 protrudes above the active site and masks it, a placement that is not seen for the same loop in the lowest energy models of AvrBs7. In fact, the same loop in AvrBs7 is predicted to be nearly three times farther away from the P-loop at closest approach, resulting in a much more open active site cleft. Given the disparate nature of the predicted structure of these loops, these results indicate that significant differences may exist in the actual structures of these two proteins. Interestingly, none of the computational methods used could reliably predict the structures of the initial 83 aa of either protein. All models shown start with aa 84, which lies within 0.7 nm of the P-loop in models of the proteins, and may indicate a role for some of the initial 83 aa in substrate recognition. Though there are few aa differences within and immediately around the catalytic site (Fig. 7), these aa differences are likely the reason for the differences in HR timing due to differential activation of *R* gene. We constructed a fusion protein containing 262 N-terminal aa of AvrBs7 fused to 90 C-terminal aa of AvrBs1.1, thereby exchanging the AvrBs7 catalytic site with the AvrBs1.1 catalytic site. Exchanging catalytic do-

main did not change the timing of HR elicitation. HR activity was completely lost when the fusion construct was expressed in ECW-70R leaves. This loss of activity is likely due to closure of the active site by loop regions adjacent to it. The results of the homology modeling protocols consistently predicted the binding site cleft of AvrBs7 to be much more open than that of AvrBs1.1. Whereas it is inherently difficult to obtain reliable models for the loop regions of proteins, the consistency seen within the lowest energy structures of each protein coupled with the significant predicted structural differences between AvrBs7 and AvrBs1.1 in the loops adjacent to the putative active sites allow us to hypothesize that the two proteins bind to different substrates within the plant cell and eventually activate the *R* gene transcripts by a different pathway or they might be activating different linked resistance proteins. We also mutated three individual aa within the catalytic domain of AvrBs1.1 to those corresponding in AvrBs7, thereby changing the catalytic domain of AvrBs1.1 to that of AvrBs7, with the remainder of the protein being AvrBs1.1 sequence. However, this mutant did not elicit a HR on ECW-70R. This difference may be due to differential substrate specificities between AvrBs1.1 and AvrBs7. The predicted three-dimensional structures for the two proteins have provided insights into the

Fig. 8. A, Fusion protein containing the N terminal of *avrBs7* and C terminal of *avrBs1.1* does not elicit hypersensitive resistance on ECW-70R. Picture was taken 48 h after infiltration. Order of inoculation : 1, Xg51; 2, Xg51 transconjugant carrying the fusion clone; 3, Xg51 transconjugant carrying the *avrBs7* clone; and 4, Xg51 transconjugant carrying the *avrBs1.1* clone. **B,** Site-directed mutagenesis within catalytic domain of AvrBs1.1 mutating it to AvrBs7. 1, Xg51; 2, Xg51 transconjugant carrying the site-directed mutant clone; and 3, Xg51 transconjugant carrying the *avrBs7* clone; and 4, Xg51 transconjugant carrying the *avrBs1.1* clone.

mechanisms used by the two *avr* genes in activation of *R* genes.

In summary, we have identified a gene-for-gene interaction in the *Xanthomonas*-pepper system in addition to five already identified gene-for-gene interactions (Stall et al. 2009). Future research on differences in the activation of the *R* gene by *avrBs1.1* and *avrBs7* will contribute to our understanding of the mechanism of activation of this resistance.

MATERIALS AND METHODS

Plant material and plant inoculations.

Several *Capsicum* genotypes were collected by R. Rodrigues in Brazil and were screened for resistance against races of the bacterial spot *xanthomonads*. One accession of *C. baccatum* var. *pendulum* was identified as resistant to *xanthomonads* race 1, 2, 3, and 6 with typical hypersensitive response.

Pepper (*Capsicum annuum* L.) cv. ECW, its near-isogenic line ECW-70R, F₁ cross (ECW-70R × ECW), 225 F₂ plants, and backcrosses [(ECW-70R × ECW) × ECW-70R; (ECW-70R × ECW) × ECW] were grown in the greenhouse. Inoculated plants were kept in a greenhouse under a 26°C 12-h-light and 16°C 12-h-dark cycle. Plants were observed for 48 h following inoculations for development of HR watersoaking symptoms.

A 4- to 5-cm² area of fully expanded leaves were infiltrated using a hypodermic needle and syringe with bacterial suspension adjusted to 10⁸ CFU/ml (Hibberd et al. 1987). All experiments were carried out in three replicates.

Bacterial strains, plasmids and media.

Bacterial strains and plasmids used and developed in this study are listed in Table 2. All *Xanthomonas* strains were grown at 28°C on nutrient agar (NA) plates. The cultures were suspended in sterile tap water and the optical density at 600 nm (OD₆₀₀) was adjusted to 0.3 with a spectrophotometer (Spectronic 20, Spectronic UNICAM, Rochester, NY, U.S.A.) for plant inoculations. *E. coli* strains or transformants were grown at 37°C on Luria-Bertani agar or broth (Maniatis et al. 1982) amended with appropriate antibiotics. Protocols for ligation of plasmids and transformation into *E. coli* DH5α were according to Maniatis and associates (1982). These plasmids were mobilized from *E. coli* into *Xanthomonas* recipient strains by triparental mating with the aid of pRK2073 helper plasmid (Ditta et al. 1980; Figurski and Helinski 1979). After incubating the matings on nutrient yeast glycerol agar at 28°C overnight (Daniels et al. 1984), the growth was suspended in 2 ml of sterile tapwater and was plated on NA plates containing appropriate antibiotics. The resulting transconjugants were then grown for pure culture. Any necessary antibiotics were used in the following final concentrations: ampicillin, 125 µg/ml; kanamycin, 50 µg/ml; rifampicin, 100 µg/ml; spectinomycin, 50 µg/ml; tetracycline 12.5 µg/ml. All strains and constructs were stored as 20% glycerol stocks at -80°C.

Library preparation and isolation of clone with *avr* activity.

A genomic library of *X. gardneri* Xv444 was constructed in *E. coli*, using the cosmid vector pLAFR3 following the protocol as described earlier (Minsavage et al. 1990; Staskawicz et al. 1987). Individual clones were mobilized by triparental mating into recipient *X. gardneri* Xg51, a strain virulent on pepper cv. ECW-70R. Transconjugants were inoculated into the leaves of pepper cv. ECW-70R to screen for the clone eliciting a hypersensitive response. A cosmid clone, pXv444-352, containing a 20-kb insert, gave a HR on ECW-70R. This clone was selected for further subcloning. A 5-kb *Bam*HI-*Bam*HI DNA fragment

from the pXv444-352 clone was transferred into pBlueScript (Stratagene, La Jolla, CA, U.S.A.). This 5-kb subclone pXv444-352Bam8 was sequenced at the Interdisciplinary Center for Biotechnology Research sequencing facility (University of Florida, Gainesville, FL, U.S.A.) with the Applied Biosystems (Foster City, CA, U.S.A.) model 373 system. Further subcloning of the pXv444-352Bam8 clone was achieved using restriction enzymes *Bam*HI, *Eco*RI, and *Pst*I. The digestion products were tested for HR in leaves of pepper cv. ECW-70R by cloning them into pLAFR3 and by mobilization into recipient strain Xg51. For cloning *avrBs7* with endogenous promoter region, primers *avrBs7F* (5' CAAGGTGGTGATGGACATGG 3') and *avrBs7R* (5' GTTGTCACCGCCGACAAGTT 3') were used for amplification and were purified. PCR product was then cloned in the pGEMT-Easy vector. Gene *avrBs1.1* was cloned in pGEMT-Easy vector following amplification of the gene with the upstream 500-bp region, using primers 8510-Bs1.1F (5' CGTTTCTACGACAGCACCAA 3') and 8510-Bs1.1R (5' CCTCTTGGGGGTTTGAATAAT 3'). Primers specific for *avrBs7* and *avrBs1.1* were designed to screen different bacterial spot strains as follows: *avrBs7spF1* (5' CCTTGAAGGTTTGCC 3') and *avrBs7spR1* (5' CGGACCATAAAGAGGG 3'); *avrBs1.1spF1* (5' CGATAATTTCCGCTCTT 3') and *avrBs1.1spR1* (5' TCTCACAAGCTCTTCTTC 3').

Deletion mutant construction.

An in-frame deletion of the coding region of *avrBs7* in the vector pGEMT-Easy was carried out. PCR primers were designed in the outward directions to create the deletion and to add *Bam*HI restriction enzyme sites. The PCR product was purified and digested with *Bam*HI. After religation and transformation into *E. coli* DH5α, a clone lacking the complete ORF of *avrBs7* and containing only flanking regions of *avrBs7* was chosen. The deletion was confirmed by sequencing the insert in the pGEMT-Easy vector. The ORF-deleted fragment of the gene with flanking regions was excised from pGEMT-Easy and was cloned into the suicide vector pOK1. This deletion mutant gene was conjugated into Xv444 using homologous recombination, as previously described, and mutants were identified by PCR (Huguet et al. 1998).

Bacterial population dynamics in infiltrated leaf tissue.

Internal bacterial populations were determined at selected time intervals after inoculation. *Xanthomonas* culture suspensions (wild type, mutant, recipient carrying *avr* clones) were diluted to a concentration of 10⁵ CFU/ml, using sterile tap water, and were then infiltrated into the leaves of the parental lines of pepper cultivars ECW-70R and ECW. The inoculated plants were incubated in the greenhouse, maintained at 16 to 26°C (12-h dark and 12-h light period) for 8 days. Leaves were sampled every 48 h. An area of 1 cm² of leaf tissue was cut from the infiltrated area using a sterile cork-borer. Using sterile forceps, the leaf disk was then placed into a sterile tube containing 1 ml of sterile tap water and was triturated. Standard 10-fold dilution plating onto NA plates was carried out, and plates were incubated at 28°C for 3 days. Colonies were then counted and the bacterial populations were calculated as CFU/cm² of leaf area.

Determination of electrolyte leakage from infiltrated leaf tissue.

Tissue necrosis after inoculation of leaves with the different *Xanthomonas* strains was assessed by quantifying electrolyte leakage as described previously (Cook and Stall 1968). Leaves were infiltrated with bacterial suspensions adjusted to 10⁸ CFU/ml (OD₆₀₀ = 0.3). Inoculated plants were kept in the greenhouse at 26°C during a 12-h light period and 16°C during

a 12-h dark period. Electrolyte leakage was expressed as the increase in conductivity per hour at 28°C.

Site-directed mutagenesis of *avrBs7*.

Catalytic residues of *avrBs7* were mutated to alanine by a PCR-mutagenesis approach using primers designed as follows: Avr1.1AlaF (5' GCGCTAGCGCCGCCGACGCCACATGC AGCCT 3') and Avr1.1AlaR (5' GCGCTAGCGCAGCTGCAG CCATCTTCATTGCT 3'). Both primers had *NheI* overhangs at the 5' end. pGEMT-Easy:*avrBs7* was used as template for mutagenesis PCR. The amplified PCR product was purified using the Qiagen (Valencia, CA, U.S.A.) spin kit and was subsequently digested with *NheI* enzyme. The digested product was religated and transformed into *E. coli* DH5 α . The mutated gene construct was then moved to the *Xanthomonas*-compatible plasmid pLAFR3. The pLAFR3 clone was then mobilized into two recipients, Xg51 and *X. euvesicatoria* TED3, by triparental mating. Stratagene's QuikChange II mutagenesis kit was used for constructing site-directed mutants. Primers used for the single aa mutation (Cys to Ser) within the catalytic site of *avrBs7* were CysmutF1 (5' GCGGTGCACTCAGGGGTCG GCCA 3') and CysmutR1 (5' GCGGTGCACATGCAGCCTC TCAT 3'). For mutation of three aa from the catalytic domain of *avrBs1.1* to *avrBs7* (HCGMGLGRIT to HCGVQGRTG), primers 1.1to7F (5' GTACATTGTGGTGTGGGCCAAGGC CGTACGGGAATATTATTGTC 3') and 1.1to7R (5' GACA ATAAATATTCCTGACGGCCTTGGCCACACCACAATG TAC 3') were used.

Phosphatase assay.

The coding sequences of *avrBs7* and *avrBs1.1* were cloned individually into the expression vector pET21a-His to generate His-tagged constructs. Primers used to amplify the coding region of the respective genes were as follows: *avrBs7*EF1 (5' CCGCTCGAGCGGATGCCTAATCCTGTCTCCAG 3') and *avrBs7*ER1 (5' CCGCTCGAGCGGCGTTTGTGGCCGCTC TAA 3'), and *avrBs1.1*EF1 (5' CCGCTCGAGCGGATGCCGA ACAAATCTCCG 3') and *avrBs1.1*ER1 (5' CCGCTCGAGC GGCTGCAATGTGGTCGAGCCAT 3'). The pET21-a vectors were then transformed into *E. coli* BL21-DE3 expression cells. The pure cultures of *E. coli* BL21-DE3 carrying *avrBs7*-pET-21a and *avrBs1.1*-pET-21a were inoculated in 5 ml of LB (with 5 μ l of 100 μ g/ml ampicillin, 20 μ l of 50% glucose) and were incubated at 37°C overnight. Overnight bacterial cultures (500 μ l) were transferred to 50 ml of LB (with 50 μ l of 100 μ g/ml ampicillin, 200 μ l of 50% glucose) and were cultured at 37°C until OD₆₀₀ = 0.6 to 0.8. For induction, 25 μ l of 1 M isopropyl- β -D-thiogalactoside was added to each culture and incubation was continued at 37°C for 3 h. The cells were pelleted at 6,000 rpm using an Eppendorf Centrifuge 5810R (Westbury, NY, U.S.A.), 10 min at 4°C. The His-tagged AvrBs7 and AvrBs1.1 proteins were purified using the His-Link spin protein purification system (Promega, Madison, WI, U.S.A.). Protein estimation in the purified His-tagged AvrBs7 and AvrBs1.1 protein samples was carried out using the Bradford protein assay (Bradford 1976). The PTP activity was measured using the pNPP assay with 10 mM p-nitrophenyl phosphate as substrate in citrate buffer, pH 4.8, as described by Howard and associates (1992).

Sequence analysis and protein homology modeling.

Sequence analysis was carried out using several programs including BLAST (Altschul et al. 1997) and Pfam (Finn et al. 2010). Homology models were created using four different software programs. Automated homology modeling was performed on the Phyre2 (Kelley and Sternberg 2009) and iTasser (Roy et al. 2010) servers. The Phyre2 server returned a single

lowest energy structure for each protein and the iTasser server returned the five lowest energy structures for each protein sequence submitted. Modeling was performed on local computational resources using Modeller (Eswar et al. 2006) and Rosetta (Leaver-Fay et al. 2011). Local modeling was performed using template structures identified from the Protein Data Bank (PDB) by a BLAST search of the PDB with the amino-acid sequences of each Avr protein used as a query. For AvrBs7, six unique proteins of known structure were identified with *E* values less than 0.1 (PDB entries 3F41, 1U24, 2GJT, 2G59, 2C46, and 1I9T). For AvrBs1.1, PDB entries 3F41 and 1U24 were identified in addition to four other proteins with *E* values less than 0.1 (PDB entries 2IMG, 1OHC, 2NLK, and 2FH7). Using similar *E* value criteria, a BLAST search of the PDB with the amino-acid sequence of the Avr fusion protein identified six homologous proteins that were used as template structures (PDB entries 3F41, 1U24, 2IMG, 2PQ5, 2JJD, and 2B30). For each Avr protein, a ClustalW multiple sequence alignment was performed with all homologues identified from the PDB and the resulting output was used in conjunction with the PDB coordinate files by Modeller to generate core packing models of the proteins. The loop model class in Modeller was used to generate initial models refined in fast mode and the final loop-rebuilt models were subjected to slow refinement before program completion. The resulting output models were ranked by energy, and the lowest energy model was subjected to *ab initio* loop rebuilding using Rosetta. Fragment files required for the *ab initio* loop modeling of each protein were generated and downloaded from the Robetta server. Following loop rebuilding, the final models were scored using the scoring module of Rosetta and the top five models were evaluated using the validation tools of Phenix (Adams et al. 2010). The best models for each protein were superposed with the program THESEUS (Theobald and Wuttke, 2006), which generated a c-alpha trace of the average model for each protein ensemble as well as a PDB file containing the superposed models.

Models were visualized and figures were generated with Chimera (Pettersen et al. 2004).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Robetta server: rosetta.bakerlab.org