Xanthomonas campestris pv. *vesicatoria* Effector AvrBsT Induces Cell Death in Pepper, but Suppresses Defense Responses in Tomato

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A type III effector protein, AvrBsT, is secreted into plant cells from *Xanthomonas campestris* pv. *vesicatoria* Bv5-4a, which causes bacterial spot disease on pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*). To define the function and recognition of AvrBsT in the two host plants, *avrBsT* was introduced into the virulent pepper strain *X. campestris* pv. *vesicatoria* Ds1. Expression of AvrBsT in Ds1 rendered the strain avirulent to pepper plants. Infection of pepper leaves with Ds1 (*avrBsT*) expressing AvrBsT but not with near-isogenic control strains triggered a hypersensitive response (HR) accompanied by strong H₂O₂ generation, callose deposition, and defense-marker gene expressions. Mutation of *avrBsT*, however, compromised HR induction by *X. campestris* pv. *vesicatoria* Bv5-4a, suggesting its avirulence function in pepper plants. In contrast, AvrBsT acted as a virulence factor in tomato plants. Growth of strains Ds1 (*avrBsT*) and Bv5-4a Δ *avrBsT* was significantly enhanced and reduced, respectively, in tomato leaves. *X. campestris* pv. *vesicatoria*-expressed AvrBsT also significantly compromised callose deposition and defense-marker gene expression in tomato plants. Together, these results suggest that the *X. campestris* pv. *vesicatoria* type III effector AvrBsT is differentially recognized by pepper and tomato plants.

Plants perceive invading microorganisms using a sophisticated immune system that has been established through intimate but antagonistic coevolution. The plant immune system consists of basal defense or pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and resistance (*R*) gene-mediated defense or effector-triggered immunity (ETI) (Jones and Dangl 2006). In PTI, PAMPs, which are conserved microbial components, act as ligands for the plant's pattern-recognition receptor proteins (Göhre and Robatzek 2008). These proteins activate signaling pathways such as mitogen-activated protein kinase (MAPK) cascades to confer resistance (Asai et al. 2002). In ETI, pathogen effector proteins that are injected into the host cell via the type III secretion system

(TTSS) are recognized by specific *R* proteins. Upon recognition of some effector proteins, host cells undergo rapid programmed cell death known as the hypersensitive response (HR) (Greenberg and Yao 2004). The effector proteins causing hypersensitive cell death in host plants are proposed to be avirulence proteins.

Some effector proteins have been shown to possess virulence functions when cognate *R* genes are absent in host plants. Effector proteins target both the PTI and ETI systems (Boller and He 2009). The HopM1 effector protein from *Pseudomonas syringae* pv. *tomato* suppresses the deposition of callose in plants (DebRoy et al. 2004). The *P. syringae* pv. *tomato* HopA1 effector, which functions as a phosphothreonine lyase, inhibits phosphorylation of the MAPK 3 (MPK3) and MAPK 6 (MPK6) (Zhang et al. 2007). Host signaling components are directly or indirectly degraded by effector proteins. AvrRpt2 is a cysteine protease that cleaves the RPM1-interacting protein 4 (RIN4) protein of *Arabidopsis*, which may be involved in AvrRpm1 and AvrB recognition during ETI (Desveaux et al. 2007; Göhre and Robatzek 2008). *Arabidopsis* chitin elicitor receptor kinase 1 (CERK1) is ubiquitinated by AvrPtoB to promote bacterial virulence in plants (Gimenez-Ibanez et al. 2009). *P. syringae* pv. *tomato* type III effectors such as HopPtoE, AvrPphE_{Pto}, and AvrPtoB have been shown to suppress programmed cell death in plants (Jamir et al. 2004).

Xanthomonas campestris pv. *vesicatoria* causes bacterial spot disease on pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*) plants. Some *X. campestris* pv. *vesicatoria* strains are virulent in both pepper and tomato plants (the pepper/tomato group), although most are virulent on only one of the two plants. *X. campestris* pv. *vesicatoria* strains that are virulent only to tomato (tomato group) cause HR-like cell death in pepper, and, conversely, *X. campestris* pv. *vesicatoria* strains that are virulent only to pepper (pepper group) are avirulent to tomato (Jones and Stall 1998). The principle of avirulence in the pepper or tomato groups was found to be controlled by the *avrBs4* and *avrBsT* genes, respectively (Minsavage et al. 1990; Ciesielka et al. 1999). *X. campestris* pv. *vesicatoria* Bv5-4a, which belongs to the tomato group, contains the effector protein AvrBsT (Jones and Stall 1998). Inoculation with strain Bv5-4a produces a strong HR-like cell death in pepper leaves (Lee and Hwang 2003, 2005). Cell death in pepper leaves is accompanied by strong induction of the salicylic acid (SA)-dependent gene *CaBPR1* (pepper basic pathogenesis-related protein) and the accumulation of H₂O₂ (Lee and Hwang 2005; Choi et al. 2007). Transient *avrBsT* expression in pepper and *Nicotiana benthamiana* induces cell death (Orth et al. 2000; Escobar et al. 2001).

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Nucleotide sequence data of *avrBsT* from this article can be found in the GenBank data libraries under accession number GQ266402.

*The e-Xtra logo stands for "electronic extra" and indicates that three supplementary figures are published online. Figures 5 and 8 also appear in color online.

AvrBsT contains a putative YopJ-like serine/threonine acetyl-transferase domain. Disruption of a conserved catalytic triad consisting of His, Glu, and Cys abolishes the cell death response in *N. benthamiana* (Orth et al. 2000). Thus, the enzymatic activity of AvrBsT seems to be closely related to the cell death phenotype. The AvrBsT orthologue YopJ was identified in *Yersinia pestis*, the causal agent of bubonic plague. YopJ has been shown to exhibit acetyl-transferase activity and inhibit MAPK and the nuclear factor κ B signaling pathway, thus suppressing animal innate immunity (Orth et al. 2000; Mukherjee et al. 2006). However, the *X. campestris* pv. *vesicatoria* effector AvrXv4, which also has significant homology to YopJ, has been demonstrated to function as a small ubiquitin-like modifier (SUMO) protease (Roden et al. 2004). Therefore, the precise enzymatic function of AvrBsT is yet to be determined.

In this study, we aimed to further characterize the function of the effector protein AvrBsT in pepper and tomato plants. We first cloned *avrBsT* from the *X. campestris* pv. *vesicatoria* Bv5-4a and introduced it into the *X. campestris* pv. *vesicatoria* Ds1, which is highly virulent to pepper plants (Hwang et al. 1995). Introduction of *avrBsT* rendered the virulent strain Ds1 avirulent to pepper. Strain Ds1 (*avrBsT*) expressing AvrBsT induced an HR-like cell death in pepper leaves, which was accompanied by H₂O₂ accumulation and increased expression of defense marker genes. In contrast, AvrBsT suppressed basal defense and enhanced bacterial growth in tomato leaves, indicating that AvrBsT acts as a virulence factor in tomato plants. Our further analyses of *avrBsT* mutation in *X. campestris* pv. *vesicatoria* Bv5-4a also supported its avirulence and virulence functions in pepper and tomato plants, respectively. Diverse growth of *X. campestris* pv. *vesicatoria* strains Ds1 (*avrBsT*) and Bv5-4a Δ *avrBsT* was suggestive of distinct functions of AvrBsT in both host plants. Recognition of AvrBsT by host plants required the N- and C-terminal domains of the protein. Together, these results identified AvrBsT as a key effector protein that induces or suppresses cell death and defense responses in pepper or tomato plants, respectively.

RESULTS

X. campestris pv. *vesicatoria* Bv5-4a harbors a functional *avrBsT* gene.

The *X. campestris* pv. *vesicatoria* Ds1 that is highly virulent to pepper plants was isolated in 1991 from diseased pepper leaves in Korea (Hwang et al. 1995). Strain Bv5-4a, which induces HR in pepper plants, was generously provided by R. E. Stall. Interestingly, Bv5-4a triggered HR in all the pepper cultivars tested (Lee et al. 1994; Hwang et al. 1995). In our previous studies, we successfully isolated defense-related genes from pepper leaves infected by the avirulent strain Bv5-4a (Jung and Hwang 2000; Do et al. 2003; Lee and Hwang 2003, 2005; Choi et al. 2007, 2008, 2009; An et al. 2008a,b; Lee et al. 2008). Thus, we used DNA from Bv5-4a cells as a template to amplify the *avrBsT* open reading frame

(ORF) (Supplementary Fig. 1A). Sequence analysis of the amplicon revealed that *avrBsT* of Bv5-4a contained a minor variation compared with that of strain 75-3. However, this sequence difference did not interfere with the ability of AvrBsT to elicit cell death (Supplementary Fig. 3). The near-isogenic Ds1 strains expressing empty vector (EV) and *avrBsT*, referred to as Ds1 (EV) and Ds1 (*avrBsT*), respectively, were further generated to identify the function of AvrBsT in planta. Immunoblot analyses also were performed to determine whether AvrBsT proteins expressed in *X. campestris* pv. *vesicatoria* are secreted by the TTSS (Supplementary Fig. 2). AvrBsT:cMyc fusion proteins were detected in whole-cell lysates from *X. campestris* pv. *vesicatoria* strains Ds1 (*avrBsT*:cMyc) and Ds1 Δ *hrcV* (*avrBsT*:cMyc). However, the negative control strain Ds1 Δ *hrcV* (*avrBsT*:cMyc) failed to secrete the protein into the culture medium. The *hrcV* gene encodes an inner membrane protein of the TTSS and its mutation abolished the secretion of effector proteins from *X. campestris* pv. *vesicatoria* (Rossier et al. 1999). These data indicated that AvrBsT of *X. campestris* pv. *vesicatoria* is secreted in a TTSS-dependent manner.

AvrBsT attenuates the virulence of *X. campestris* pv. *vesicatoria* Ds1 in pepper leaves.

To examine in planta function of AvrBsT, pepper leaves were infiltrated with a low titer (5×10^4 CFU ml⁻¹) of the *X. campestris* pv. *vesicatoria* strains Ds1, Ds1 (EV), Ds1 (*avrBsT*), and Bv5-4a (Fig. 1A). Seventeen days after inoculation, Ds1 and Ds1 (EV) induced severe chlorosis and Ds1 (*avrBsT*) induced mild chlorosis, whereas Bv5-4a did not produce any visible symptoms on pepper leaves. When observed under UV light, chlorosis was apparent only in pepper leaves inoculated with Ds1 and Ds1 (EV) (Fig. 1A). Bacterial growth was measured 0, 3, 6, and 9 days after inoculation. The growth of Ds1 (*avrBsT*) was significantly retarded 10-fold compared with that of Ds1 and Ds1 (EV) but was 10-fold higher than that of Bv5-4a (Fig. 1B). Ds1 and Ds1 (EV) continued to grow 6 days after inoculation but the growth of Ds1 (*avrBsT*) was arrested, similar to that of the avirulent strain Bv5-4a.

Pepper leaves infiltrated with a high titer (10^8 CFU ml⁻¹) of Ds1 (*avrBsT*) and Bv5-4a exhibited a typical HR, whereas Ds1- and Ds1 (EV)-infiltrated leaves did not show any visible symptoms 2 days after inoculation. UV light illumination revealed that leaves infiltrated with Ds1 (*avrBsT*) and Bv5-4a accumulated defense-related fluorescent phenolics at the site of infiltration (Fig. 1C) (Choi et al. 2009). Plant cells undergoing programmed cell death release electrolytes into extracellular spaces. Thus, ion leakage measurement has been used as an indicator of plant cell death (Baker et al. 1991). When pepper leaves were challenged with a high titer (5×10^7 CFU ml⁻¹) of *X. campestris* pv. *vesicatoria*, Bv5-4a triggered a drastic increase in the level of electrolyte leakage 12, 24, and 35 h after inoculation (Fig. 1D). Induction of electrolyte leakage by Ds1 (*avrBsT*) infection was not as strong as that by Bv5-4a infection; however, its induction level was significantly higher

Fig. 1. AvrBsT attenuates the virulence of the *Xanthomonas campestris* pv. *vesicatoria* Ds1 in pepper leaves. **A**, Disease symptoms of pepper leaves caused by *X. campestris* pv. *vesicatoria* strains. Pepper leaves were inoculated with the indicated strains (5×10^4 CFU ml⁻¹) and photographed 17 days later. **B**, Measurement of bacterial growth. Pepper leaves were inoculated with the indicated strains (5×10^4 CFU ml⁻¹). Bacterial growth in leaves was measured at different time points after inoculation. Different letters indicate statistically significant differences (least significant difference [LSD]; $P < 0.05$). **C**, Response of pepper leaves to high bacterial titers. Pepper leaves were inoculated with the indicated strains (10^8 CFU ml⁻¹) and photographed 40 h later. **D**, Measurement of electrolyte leakage. Pepper leaves were inoculated with indicated strains (5×10^7 CFU ml⁻¹). Electrolyte leakage caused by cell death was monitored at the indicated time points after inoculation. Different letters indicate statistically significant differences (LSD; $P < 0.05$). **E**, Histochemical analyses. Pepper leaves were inoculated with the indicated strains (5×10^7 CFU ml⁻¹). Leaves were stained with aniline blue, 3,3'-diaminobenzidine (DAB), and trypan blue 12, 24, and 36 h after inoculation, respectively. Bars = 250 μ m (aniline blue), 500 μ m (DAB), and 500 μ m (trypan blue). Experiments were repeated three times with similar results. Data in B and D are means $\pm$ standard deviations from three independent experiments.

than Ds1- or Ds1 (EV)-induced ones. Electrolyte leakage in Ds1- and Ds1 (EV)-infiltrated leaves slightly increased at 24 h but returned to a basal level 36 h after inoculation.

To define the defense response of pepper plants at the cellular level, various histochemical analyses were performed on pepper leaves infiltrated with high titers of *X. campestris* pv. *vesicatoria* strains (5×10^7 CFU ml⁻¹) (Fig. 1E). Infection with Ds1 (*avrBsT*) and Bv5-4a but not with Ds1 and Ds1 (EV) triggered callose deposition in pepper leaves. Leaves inoculated with Ds1 (*avrBsT*) and Bv5-4a strongly accumulated H₂O₂ compared with Ds1 and Ds1 (EV) when detected by 3,3'-diaminobenzidine (DAB, a histochemical reagent for H₂O₂) staining. Trypan blue staining of pepper leaves also revealed the induction of HR-like cell death by Ds1 (*avrBsT*) and Bv5-4a but not by Ds1 and Ds1 (EV). These findings suggest that the trans-

formation of *avrBsT* into Ds1 rendered this strain avirulent in pepper plants.

AvrBsT functions as a key avirulence factor of the *X. campestris* pv. *vesicatoria* Bv5-4a in pepper leaves.

To determine whether AvrBsT functions as an avirulence factor in plants, the deletion mutants were generated from strain Bv5-4a by using site-directed mutagenesis (Lee et al. 2009). Bv5-4a Δ *avrBsT* but not Bv5-4a and Bv5-4a Δ *avrBsT* (*avrBsT*) induced severe brownish bacterial spots on pepper leaves 14 days after infiltration with a low titer (5×10^4 CFU ml⁻¹) of *X. campestris* pv. *vesicatoria* (Fig. 2A). Bacterial growth also was measured 0, 3, 6, and 9 days after inoculation (Fig. 2B). The growth of Bv5-4a Δ *avrBsT* significantly increased 10-fold higher than that of Bv5-4a and Bv5-4a Δ *avrBsT* (*avrBsT*) dur-

Fig. 2. AvrBsT functions as a key avirulence factor of the *Xanthomonas campestris* pv. *vesicatoria* Bv5-4a in pepper leaves. **A**, Disease symptoms of pepper leaves caused by *X. campestris* pv. *vesicatoria* strains. Pepper leaves were inoculated with the indicated strains (5×10^4 CFU ml⁻¹) and photographed 14 days later. **B**, Measurement of bacterial growth. Pepper leaves were inoculated with the indicated strains (5×10^4 CFU ml⁻¹). Bacterial growth in leaves was measured on indicated days after inoculation. Different letters indicate statistically significant differences (least significant difference [LSD]; $P < 0.05$). **C**, Response of pepper leaves to high bacterial titers. Pepper leaves were inoculated with the indicated strains (10^8 CFU ml⁻¹) and photographed 36 h later. **D**, Measurement of electrolyte leakage. Pepper leaves were inoculated with indicated strains (5×10^7 CFU ml⁻¹). Electrolyte leakage caused by cell death was monitored at the indicated time points after inoculation. Different letters indicate statistically significant differences (LSD; $P < 0.05$).

ing infection. The increased growth of *avrBsT*-mutated Bv5-4a supported the susceptible lesions caused by this *X. campestris* pv. *vesicatoria* mutant.

Pepper leaves infiltrated with a high titer (10^8 CFU ml⁻¹) of Bv5-4a and Bv5-4a Δ *avrBsT* (*avrBsT*) exhibited a typical HR, whereas Bv5-4a Δ *avrBsT* did not show any visible symptoms 2 days after inoculation. UV light illumination also showed the compromised accumulation of defense-related fluorescent phenolics at the site of infiltration with Bv5-4a Δ *avrBsT* (Fig. 2C). Measurements of electrolyte leakage supported significantly attenuated cell death in pepper leaf tissues inoculated with Bv5-4a Δ *avrBsT* compared with Bv5-4a and Bv5-4a Δ *avrBsT* (*avrBsT*) (Fig. 2D). Together, these findings strongly suggest that the deletion of *avrBsT* from Bv5-4a rendered this strain virulent in pepper plants.

AvrBsT induces defense-related gene expression in pepper leaves.

To determine whether the reduced virulence of strain Ds1 (*avrBsT*) is due to the altered defense response in pepper

plants, we further analyzed the transcript levels of some defense-related genes (*CaBPR1*, *CaPO2*, *CaSAR82A*, and *CaDEF1*) in pepper leaves infected with *X. campestris* pv. *vesicatoria* (Fig. 3) (Lee and Hwang 2005; Choi et al. 2007). Strain Bv5-4a and Ds1 (*avrBsT*) infection drastically induced *CaBPR1* and *CaPO2* expression in pepper leaves compared with the virulent Ds1 and Ds1 (EV) infection. Notably, increase in *CaBPR1* transcript was strikingly strong in pepper leaves at an early time, 12 h after inoculation with Ds1 (*avrBsT*) and Bv5-4a. The transcript level of *CaPO2* in pepper leaves infected with Bv5-4a and Ds1 (*avrBsT*) was approximately 10,000-fold higher than in those infected by Ds1 and Ds1 (EV) 12 h after inoculation (Fig. 3). The pepper defense-related *CaSAR82A* gene was also rapidly induced in pepper leaves 12 h after inoculation with Bv5-4a and Ds1 (*avrBsT*) (Fig. 3). *CaDEF1* was sevenfold higher in pepper leaves inoculated with Ds1 (*avrBsT*) compared with Ds1 and Ds1 (EV). These data suggest that AvrBsT elicits defense-related gene expression in pepper plants, thus rendering Ds1 (*avrBsT*) avirulent in pepper plants.

Fig. 3. AvrBsT induces pathogenesis-related (PR) gene expression in pepper leaves. Total RNA was isolated from pepper leaves inoculated with *Xanthomonas campestris* pv. *vesicatoria* strains Ds1, Ds1 (empty vector [EV]), Ds1 (*avrBsT*), and Bv5-4a (5×10^7 CFU ml⁻¹). Quantitative real-time polymerase chain reaction was performed for pepper PR genes *CaBPR1* (basic PR1), *CaPO2* (peroxidase 2), *CaSAR82A* (SAR8.2), and *CaDEF1* (defensin). Abundance of mRNA 12 and 36 h after inoculation are shown. mRNA levels of 18S rRNA were used for normalization of mRNA abundance in each sample. Different letters indicate statistically significant differences (least significant difference; $P < 0.05$). Experiments were repeated three times with similar results. Data are means $\pm$ standard deviations from three independent experiments.

Fig. 4. AvrBsT promotes virulence of the *Xanthomonas campestris* pv. *vesicatoria* Ds1 in tomato leaves. **A**, Disease symptoms of tomato leaves caused by *X. campestris* pv. *vesicatoria* strains. Tomato leaves were inoculated with the indicated strains (5×10^4 CFU ml⁻¹) and photographed 2 weeks later. **B**, Measurement of bacterial growth. Tomato leaves were inoculated with the indicated strains (5×10^4 CFU ml⁻¹). Bacterial growth in leaves was measured at different time points after inoculation. Different letters indicate statistically significant differences (least significant difference [LSD]; $P < 0.05$). **C**, Response of tomato leaves to a high bacterial titer. Tomato plants were inoculated with the indicated strains (5×10^7 CFU ml⁻¹) and were photographed 48 h later. **D**, Electrolyte leakage measurement. Tomato plants were inoculated with indicated strains (5×10^7 CFU ml⁻¹). Electrolyte leakage caused by cell death was monitored at indicated time points after inoculation. Different letters indicate statistically significant differences (LSD; $P < 0.05$). **E**, Histochemical analyses. Tomato leaves were inoculated with the indicated strains (5×10^7 CFU ml⁻¹). Leaves were stained with aniline blue, 3,3'-diaminobenzidine, and trypan blue 12, 24, and 36 h after inoculation, respectively. Bars = 500 μ m. Experiments were repeated three times with similar results. Data in B and D are means $\pm$ standard deviations from three independent experiments.

Fig. 5. AvrBsT functions as an important virulence factor of the *Xanthomonas campestris* pv. *vesicatoria* Bv5-4a in tomato leaves. **A**, Disease symptoms of tomato leaves caused by *X. campestris* pv. *vesicatoria* strains. Tomato leaves were inoculated with the indicated strains (5×10^4 CFU ml⁻¹) and photographed 12 days later. **B**, Measurement of bacterial growth. Tomato leaves were inoculated with the indicated strains (5×10^4 CFU ml⁻¹). Bacterial growth in leaves was measured at different time points after inoculation. Different letters indicate statistically significant differences (least significant difference [LSD]; $P < 0.05$). **C**, Response of tomato leaves to high bacterial titers. Tomato leaves were inoculated with the indicated strains (5×10^7 CFU ml⁻¹) and photographed 36 h later. **D**, Measurement of electrolyte leakage. Tomato leaves were inoculated with indicated strains (5×10^7 CFU ml⁻¹). Electrolyte leakage caused by cell death was monitored at the indicated time points after inoculation. Different letters indicate statistically significant differences (LSD; $P < 0.05$).

AvrBsT promotes virulence of *X. campestris* pv. *vesicatoria* Ds1 in tomato leaves.

To examine virulence function of AvrBsT in tomato, we inoculated tomato leaves with a low titer (5×10^4 CFU ml⁻¹) of *X. campestris* pv. *vesicatoria* strains Ds1, Ds1 (EV), Ds1 (*avrBsT*), and Bv5-4a. Fourteen days after inoculation, Ds1 (*avrBsT*) and Bv5-4a produced mild and severe susceptible chloroses, respectively, in tomato leaves whereas tomato leaves infected with Ds1 and Ds1 (EV) did not show any visible symptoms (Fig. 4A). Under UV illumination, a yellowish coloration was visible only in leaves inoculated with Bv5-4a and Ds1 (*avrBsT*). Bacterial growth in leaf tissues was measured over 9 days after inoculation (Fig. 4B). The growth of Ds1 (*avrBsT*) in tomato leaves was significantly promoted compared with that of Ds1 and Ds1 (EV). Ds1 (*avrBsT*) exhibited 10-fold higher bacterial growth in leaf tissues than did Ds1 and Ds1 (EV) during infection.

Two days after infiltration with a high titer (5×10^7 CFU ml⁻¹) of *X. campestris* pv. *vesicatoria* strains, no visible symp-

toms were produced in tomato leaves (Fig. 4C). However, UV illumination revealed that Ds1 and Ds1 (EV) elicited a stronger accumulation of fluorescent phenolics at leaf inoculation sites than did Ds1 (*avrBsT*) and Bv5-4a (Fig. 4C). Infection with Ds1 and Ds1 (EV) significantly induced electrolyte leakage from tomato leaf tissues 72 h after infiltration compared with Bv5-4a- and Ds1 (*avrBsT*)-infiltrated leaves (Fig. 4D).

To examine the effects of AvrBsT on the early response of plant cells to *X. campestris* pv. *vesicatoria* infection, histochemical analyses were performed on tomato leaves inoculated with a high titer (5×10^7 CFU ml⁻¹) (Fig. 4E). At 12 h after inoculation, Ds1 and Ds1 (EV) triggered significantly higher callose deposition in leaf tissues than did Ds1 (*avrBsT*) and Bv5-4a. However, neither distinct DAB nor trypan blue pigmentation was detectable on tomato leaves 24 and 36 h, respectively, after inoculation with any of the *X. campestris* pv. *vesicatoria* strains tested (Fig. 4E). This supports the notion that strain Ds1 is relatively less virulent than Bv5-4a but not avirulent to tomato plants. Together, these results suggest that

Fig. 6. AvrBsT inhibits induction of defense-related genes in tomato leaves. Total RNA was isolated from tomato leaves inoculated with the *Xanthomonas campestris* pv. *vesicatoria* strains Ds1, Ds1 (empty vector [EV]), Ds1 (*avrBsT*), and Bv5-4a (5×10^7 CFU ml⁻¹). Quantitative real-time polymerase chain reaction was performed for tomato defense-related genes, including *Senu4* (PR1), *Chi17* (PR3), *Cevi16* (peroxidase), and *Tgas118* (defensin). mRNA levels of the tomato actin gene were used for normalization of mRNA abundance of the tested genes. Different letters indicate statistically significant differences (least significant difference; $P < 0.05$). Experiments were repeated three times with similar results. Data are means $\pm$ standard deviations from three independent experiments.

expression of *avrBsT* in Ds1 confers enhanced virulence to tomato plants.

AvrBsT is an important virulence factor

of *X. campestris* pv. *vesicatoria* Bv5-4a in tomato leaves.

To determine the effect of *avrBsT* deletion from strain Bv5-4a, tomato leaves were infiltrated with a low titer (5×10^4 CFU ml⁻¹) of the strains Bv5-4a, Bv5-4a Δ *avrBsT*, and Bv5-4a Δ *avrBsT* (*avrBsT*) (Fig. 5A). Twelve days after inoculation, Bv5-4a and Bv5-4a Δ *avrBsT* (*avrBsT*) induced severe bacterial chlorosis, whereas Bv5-4a Δ *avrBsT* produced mild symptoms on tomato leaves. Bacterial growth also was measured 0, 3, 6, and 9 days after inoculation (Fig. 5B). The growth of Bv5-4a Δ *avrBsT* significantly decreased 10-fold lower than that of Bv5-4a and Bv5-4a Δ *avrBsT* (*avrBsT*) during infection. This indicates that the deletion of *avrBsT* from Bv5-4a significantly reduced its virulence in tomato plants.

Infiltration with a high titer (10^8 CFU ml⁻¹) of Bv5-4a, Bv5-4a Δ *avrBsT*, and Bv5-4a Δ *avrBsT* (*avrBsT*) neither exhibited HR nor accumulated fluorescent phenolics at the sites of infiltration in tomato leaves (Fig. 5C). Tomato leaves also were challenged with a high titer (5×10^7 CFU ml⁻¹) of these *X. campestris* pv. *vesicatoria* strains (Fig. 5D). Only Bv5-4a Δ *avrBsT* infection significantly induced increased electrolyte leakage from the leaves 72 h after infiltration. However, tomato leaves infiltrated with Bv5-4a and Bv5-4a Δ *avrBsT* (*avrBsT*) exhibited gradually increased electrolyte leakage during infection.

AvrBsT inhibits induction

of defense-related gene expression in tomato leaves.

To determine whether the enhanced virulence of strain Ds1 (*avrBsT*) is due to the altered defense response of tomato plants, we analyzed the transcript levels of defense-related genes *Senu4* (PR1) (Cunnac et al. 2007), *Chi17* (PR3) (Cunnac et al. 2007), *Cevi16* (peroxidase) (Gadea et al. 1996), and *Tgas118* (defensin) (van den Heuvel et al. 2001) in tomato leaves infected with different *X. campestris* pv. *vesicatoria* strains (Fig. 6). Expression of *avrBsT* in Ds1 significantly decreased the induction of *PR1* and *PR3* in tomato leaves during *X. campestris* pv. *vesicatoria* infection compared with that with Ds1 and Ds1 (EV). In particular, *PR3* transcript levels were strikingly low in tomato leaves 48 and 72 h after inoculation with Ds1 (*avrBsT*) and Bv5-4a. The tomato peroxidase gene *Cevi16* is induced by viroid infection (Gadea et al. 1996). Transcription levels of *Cevi16* drastically declined in tomato leaves 48 h after inoculation with Ds1 (*avrBsT*) and Bv5-4a. However, *avrBsT* did not significantly affect the transcript levels of the tomato defensin gene *Tgas118*. These data indicate that *avrBsT* promotes virulence of the transformed Ds1 strain, leading to the downregulation of defense marker genes in tomato plants.

Transient expression

of *avrBsT* in pepper and tomato plants.

The cell-death-inducing activity of *avrBsT* was evaluated by the *Agrobacterium* spp.-mediated transient expression of *avrBsT* in pepper leaves. As expected, cell death was developed around the sites infiltrated with *Agrobacterium* spp. carrying the p35S:*avrBsT*:8Myc (35S:*avrBsT*) construct but not the p35S:8Myc construct (35S:00) (Fig. 7A). *avrBsT* used for transient expression was tagged with the cMyc epitope, and expression of *avrBsT* in pepper leaves was confirmed by Western blotting with an anti-cMyc antibody (Fig. 7C). Fluorescent pigments were also strongly induced around the *avrBsT*-expressing regions (Fig. 7A, UV). Electrolyte leakage measurement and histochemical DAB staining revealed significantly

strengthened cell death and H₂O₂ burst, respectively, in *avrBsT*-expressing pepper leaf tissues (Fig. 7B and D). These results indicate that expression of *avrBsT* alone was able to trigger the HR-like cell death response in pepper leaves.

The activity of *avrBsT* in tomato leaves was also evaluated. *Agrobacterium* spp.-mediated transient expression of *avrBsT* did not induce cell death and reactive oxygen species (ROS) burst in tomato leaves (Fig. 7E through H). Electrolyte leakage was measured to quantify cell death response. Infiltration with *Agrobacterium* spp. did not significantly induce electrolyte leakage from tomato leaf tissues (Fig. 7F). Transient expression of *avrBsT* in tomato leaves was confirmed by Western blotting with the anti-cMyc antibody (Fig. 7G). In contrast to the response of pepper leaves, tomato leaves did not produce H₂O₂, as revealed by DAB staining (Fig. 7H). These results indicate that the expression of *avrBsT* did not trigger the cell death response in tomato leaves, unlike in pepper leaves.

Full-length *avrBsT* is required for plant cell death.

To characterize the function of *avrBsT* during cell death, a series of N-terminal and C-terminal deletion mutants was tested using the *Agrobacterium tumefaciens* GV3101 (Fig. 8). When transiently expressed, full-length *avrBsT* induced cell death in *N. benthamiana* leaves. However, all of the deletion mutants failed to elicit cell death (Fig. 8), indicating that *avrBsT* requires the enzymatic domain and the N-terminal and C-terminal regions to trigger cell death.

DISCUSSION

Pathogenic gram-negative bacteria have various virulence factors, such as effector proteins, toxins, and degradative enzymes, which allow them to invade plants (Barras et al. 1994; Wolpert et al. 2002; Göhre and Robatzek 2008). The TTSS, which delivers effector proteins into host cells, is critical during pathogenesis. Mutant bacteria lacking a TTSS are unable to secrete effector proteins into host cells and, thus, are avirulent. Some effectors trigger the HR in host plants that express cognate *R* genes; however, hosts lacking *R* genes fail to recognize the effectors and become diseased (Göhre and Robatzek 2008). *avrBsT*, a type III effector of *X. campestris* pv. *vesicatoria*, is secreted via a TTSS and delivered into plant cells during infection (Escobar et al. 2001). To define the in planta function of *avrBsT*, we cloned *avrBsT* from *X. campestris* pv. *vesicatoria* Bv5-4a and introduced it into *X. campestris* pv. *vesicatoria* Ds1, which is highly virulent to pepper plants. This transformant, Ds1 (*avrBsT*), induced cell death in pepper leaves. The virulence of Ds1 (*avrBsT*) was compromised by *avrBsT* expression; however, this strain was more virulent in pepper leaves than was the avirulent strain Bv5-4a. These data suggest that the avirulent strain Bv5-4a may contain other unidentified avirulence factors in addition to *avrBsT*. As expected, the decreased virulence of Ds1 (*avrBsT*) may result from *avrBsT*-induced cell death of pepper plants. Ds1 (*avrBsT*) induced more severe and rapid cell death in pepper leaves than did wild-type Ds1, although not to the level caused by the Bv5-4a, the origin of *avrBsT*. Early defense indicators, such as callose deposition and generation of ROS, were also induced in pepper leaves during Ds1 (*avrBsT*) infection, which suggests that *avrBsT* triggers HR-like cell death responses in pepper leaves. Decreased HR-like cell death induced by Bv5-4a Δ *avrBsT* infection in pepper leaves also supports this notion. Defense responses elicited by *avrBsT* seem likely to be closely related to ROS generation and the SA- and jasmonic acid-mediated signaling pathways. Infection with Ds1 (*avrBsT*) and Bv5-4a led to a strong induction of SA-induced pepper defense marker genes, *CaBPR1* (PR1) and *CaSAR82A* (SAR8.2), 12 and 36 h

Fig. 7. Transient expression of AvrBsT in pepper leaves. **A**, Induction of the cell death phenotype by AvrBsT 5 days after infiltration with *Agrobacterium* spp. carrying the indicated constructs at titers of an optical density at 600 nm (OD_{600}) = 1, 0.5, and 0.1 into pepper leaves. **B**, Electrolyte leakage measurement. Pepper leaves were infiltrated with *Agrobacterium* spp. carrying 35S:8Myc (empty vector control) or 35S:avrBsT:8Myc at the titer of OD_{600} = 1.0. Electrolyte leakage caused by cell death was monitored at the indicated time points after infiltration. **C**, Protein was extracted from *Agrobacterium* spp.-infiltrated pepper leaves and subjected to Western blotting. AvrBsT was detected using an anti-cMyc antibody that binds to the cMyc-epitope tag introduced for vector construction. **D**, Reactive oxygen species (ROS) formation in pepper leaves. Leaves harvested 3 days after agroinfiltration were stained with 3,3'-diaminobenzidine (DAB). Bars = 250 μm . **E**, Expression of AvrBsT in tomato leaves infiltrated with *Agrobacterium* spp. at the titers of OD_{600} = 1, 0.5, and 0.1 into tomato leaves. Pictures were taken 5 days after infiltration. **F**, Electrolyte leakage measurement. Tomato plants were infiltrated with *Agrobacterium* spp. carrying 35S:8Myc (empty vector control) or 35S:avrBsT:8Myc at the titer of OD_{600} = 1.0. Electrolyte leakage was monitored at the indicated time points after infiltration. Asterisks indicate statistically significant differences (*t* test; $P < 0.05$). **G**, Protein was extracted from *Agrobacterium* spp.-infiltrated tomato leaves and subjected to Western blotting. AvrBsT was detected using the anti-cMyc antibody. **H**, ROS formation in tomato leaves. Leaves harvested 3 days after agroinfiltration were stained with DAB. Bars = 250 μm . Experiments were repeated three times with similar results. Data in B and F are means $\pm$ standard deviations from three independent experiments.

after inoculation. Surprisingly, Ds1 (*avrBsT*) induced higher transcript levels of these defense marker genes than did strain Bv5-4a at early stages of infection. Expression of the peroxidase gene *CaPO2* regulates ROS generation and cell death induction in pepper leaves (Choi et al. 2007). Infection with Ds1 (*avrBsT*) also induced significantly higher levels of *CaPO2* mRNA than did Ds1 and Ds1 (EV), suggesting the involvement of *CaPO2*-dependent ROS generation and cell death regulation upon recognition of *AvrBsT* by pepper plants. The decrease in mRNA abundance of *CaPO2* from 12 to 36 h by infection with the virulent strains Ds1 and Ds1 (EV) supports the notion that Ds1 possesses unknown virulent factors responsible for lowering *CaPO2* transcript accumulation and the cell death response. Collectively, *AvrBsT* in pepper plants may function as an avirulent factor that causes HR-like cell death, accompanied by callose deposition, ROS generation, and the induction of defense-related genes.

In contrast to the interactions of *X. campestris* pv. *vesicatoria* with pepper plants, *X. campestris* pv. *vesicatoria* strains Ds1, Ds1 (EV), Ds1 (*avrBsT*), and Bv5-4a were able to colonize tomato plants and cause susceptible symptoms. All four strains inoculated into tomato leaves restricted early ROS generation during the infection process. However, Ds1 (*avrBsT*) grew well and caused susceptible responses in tomato leaves compared with Ds1 or Ds1 (EV). These results suggest that *AvrBsT* does not function as an avirulence factor in tomato plants. During infection with different *X. campestris* pv. *vesicatoria* strains at high titers, *AvrBsT* seemed to delay the induction of cell death in tomato leaves. Cell death induction was monitored by measuring electrolyte leakage from leaves inoculated with strains Ds1, Ds1 (EV), Ds1 (*avrBsT*), and Bv5-4a. At 72 h after inoculation, *X. campestris* pv. *vesicatoria* strains lacking *avrBsT* began to induce cell death in tomato plants. The electrolyte leakage induced by Ds1 (*avrBsT*) was similar to that of Bv5-4a. The early defense responses to these *X. campestris* pv. *vesicatoria* strains were evaluated in tomato plants as in pepper plants. Neither ROS nor cell death generation was detectable in tomato leaves inoculated with strains Ds1, Ds1 (EV), Ds1 (*avrBsT*), and Bv5-4a. However, callose deposition profiles were significantly different for the inoculated strains. Bv5-4a induced the lowest level of callose deposition in tomato leaves. Callose deposition was slightly higher in leaves inoculated with Ds1 (*avrBsT*) compared with leaves inoculated with Bv5-4a but significantly lower than those inoculated with Ds1 and Ds1 (EV). These results support the notion that *AvrBsT* suppresses basal defense of tomato plants. The growth of Bv5-4a Δ *avrBsT*, in which *avrBsT* was deleted, was retarded in tomato plants compared with that of the wild-type strain Bv5-4a, suggesting that *AvrBsT* functions as an

important virulence factor in tomato plants. Notably, abundance of mRNAs of *PR1* and *PR3* in tomato leaves was greatly reduced by infection with Ds1 (*avrBsT*) and Bv5-4a. *Cevi16* (peroxidase), which is induced in viroid-infected tomato (Gadea et al. 1996), was also drastically downregulated 48 h after inoculation with Ds1 (*avrBsT*) and Bv5-4a. Together, these data suggest that *AvrBsT* functions as a virulence factor in tomato plants to suppress early defense signaling.

AvrBsT cloned from the strain Bv5-4a differed in two amino acid residues compared with the sequence previously reported by Ciesiolka and associates (1999). However, transient expression of *AvrBsT* in pepper plants induced cell death similar to that observed by Escobar and associates (2001). Cell death induction by *AvrBsT* has been shown to require a functional catalytic domain (Orth et al. 2000). However, the central domain of *AvrBsT*, which is collinear with the YopJ serine/threonine acetyltransferase, may be responsible for enzymatic activity but might not induce cell death in plants. The N- and C-terminal regions are required to induce cell death in *N. benthamiana*, raising the possibility that these domains may be essential for binding of *AvrBsT* to host target proteins. Individual domains or regions may not be sufficient to trigger cell death in plants. In contrast, expression of *AvrBsT* in tomato leaves did not induce hypersensitive cell death, suggesting the absence of the receptor components necessary to bind *AvrBsT* in tomato cells. Therefore, the cell death response in *N. benthamiana* leaves caused by *AvrBsT* may result from a host-species-dependent recognition process.

Many effector proteins that function as plant immunity and disease susceptibility factors have been identified in the interactions of pathogens with host species (Göhre and Robatzek 2008). In this study, we show that *AvrBsT* from strain Bv5-4a triggers HR in pepper and *N. benthamiana* plants. This suggests that pepper and *N. benthamiana* plants may contain certain *AvrBsT* recognition factors which are lacking in tomato plants. Certain host factors, such as a conserved carboxylesterase, a suppressor of *AvrBsT*-elicited resistance 1 (SOBER1) in *Arabidopsis*, could be involved in HR triggered by *AvrBsT* in both pepper and *N. benthamiana*, as observed in *Arabidopsis* plants (Cunnac et al. 2007). However, in tomato plants, *AvrBsT* not only suppresses basal defense but also significantly enhances *X. campestris* pv. *vesicatoria* growth. In further studies, it will be very interesting to identify virulence targets of *AvrBsT* from tomato plants. Taken together, our findings suggest that *AvrBsT* is differentially recognized as avirulence and virulence factors by pepper and tomato plants, respectively. Finding and identifying the signaling mechanisms underlying the recognition of *AvrBsT* will be pivotal for understanding the evolutionary machinery governing the effector-defense complex.

Fig. 8. *AvrBsT* induces cell death in leaves of *Nicotiana benthamiana*. **A**, Transient expression of *AvrBsT* deletion mutants. An *Agrobacterium* sp. carrying the indicated constructs at a titer of optical density at 600 nm = 0.5 was infiltrated into *N. benthamiana* leaves, which were photographed 48 h later. **B**, Immunoblot analyses of *AvrBsT* deletion mutants. Proteins were extracted from *N. benthamiana* leaves infiltrated with an *Agrobacterium* sp. carrying the indicated constructs, followed by Western blotting. *AvrBsT* was detected using the anti-cMyc antibody. Numbers above the Western blot image represent the constructs indicated in A.

MATERIALS AND METHODS

Plant materials and growth conditions.

Pepper (*C. annuum* L., cv. Nockwang), tomato (*Lycopersicon esculentum*, cv. Sunmyung), and *N. benthamiana* were planted in plastic pots (8 cm in diameter) containing a soil mix (loam soil/perlite/vermiculite, 3:1:1, vol/vol/vol). Plants were grown at 28°C with a long-day scheme (16 h of light and 8 h of darkness) at a light intensity of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Pathogenic bacterial strains.

Strains Ds1 and Bv5-4a of *X. campestris* pv. *vesicatoria* were used. Strain Ds1 was isolated in 1991 from pepper plants in Korea (Hwang et al. 1995). Strain Bv5-4a was generously provided by R. E. Stall, Department of Plant Pathology, University of Florida, Gainesville.

Plasmid construction.

To clone *avrBsT* into the TOPcloner Blunt V2 vector (Enzymatics, Daejeon, Korea) for use as a template, the *avrBsT* ORF was amplified from *X. campestris* pv. *vesicatoria* Bv5-4a using *Pyrococcus furiosus* DNA polymerase (Enzymatics) and the primers ATGAAGAATTTTATGCGTTCACTTG (F, forward) and TTATGATTCAATAGTTTTCCTAATTTT (R, reverse). The amplicon generated with primers F, GAGACTCGAGATGAAGAATTTTATGCGTTCACTT and R, GAGAGGATCCTTATGATTCAATAGTTTTCCTAATTTT, which contains *XhoI*/*Bam*HI sites flanking the *avrBsT* ORF, was digested with *XhoI*/*Bam*HI and ligated into the vector pBBR1MCS-2 (Kovach et al. 1995). A new vector, p35S-8myc, was constructed to generate myc-tagged *avrBsT*. The DNAs coding for 4 $\times$ myc with *KpnI*/*NdeI* sites (F primer, GCGGTACCAAAGCTATGGAGCAAAAGC and R primer, GCCATATGGCCCAAGCTCTCCATTTT) or *NdeI*/*SacI* (F primer, GCCATATGAAAGCTATGGAGCAAAAGC and R primer, GCGAGCTCTAGCCCAAGCTCTCCATTT) were amplified from the vector myc-pBA and ligated at the *NdeI* site with the other myc DNA to generate an 8 $\times$ myc fragment. pSPYNE-35S (Walter et al. 2004) was digested with *KpnI*/*SacI* to remove a yellow fluorescent protein fragment and ligated to the 8 $\times$ myc fragment to produce p35S-8myc. *AvrBsT* with a deletion of the termination codon and incorporation of *SpeI*/*XhoI* sites (F primer, GAGAACTAGTATGAAGAATTTTATGCGTTCACTTG and R primer, GAGACTCGAGTGATTCAATAGTTTTCCTAATTTTCC) was amplified and ligated to p35S-8myc. Primers F, GAGACTCGAGATGAAGAATTTTATGCGTTCACTT and R, GAGAGGATCCCTAGCCCAAGCTCTCCATTT, encompassing *avrBsT* and 8 $\times$ myc, were used to amplify an *avrBsT*:8 $\times$ myc fusion construct (*XhoI*/*Bam*HI), which was ligated to pBBR1MCS-2. Various deletion mutants of *avrBsT* (*SpeI*/*XhoI*) were generated by polymerase chain reaction (PCR) and subcloned into p35S-8myc using the primers GAGAACTAGTATGAAGAATTTTATGCGTTCACTTG (F1), GAGAACTAGTATGGCTATTGCGCTGGAAGAATCGCT (F2), GAGACTCGAGATTTGAACTATTCAGCGATTCTTCC (R1), GAGACTCGAGTCGAACGTTTCTTCTTAAACAATC (R2), and GAGACTCGAGTGATTCAATAGTTTTCCTAATTTTCC (R3). Amplified DNA fragments were confirmed by sequencing. For generation of the *X. campestris* pv. *vesicatoria* Bv5-4a Δ *avrBsT* mutant, the 475-bp fragment of *avrBsT* was amplified by PCR using *P. furiosus* DNA polymerase and the following primer pairs: knock-out forward (5'-GAATTCGAAATGCGGATGTATGCGGA-3') and knock-out reverse (5'-GAATTCTAAGCGCACAGAGCCATATT-3'). The amplified fragment of *avrBsT* was digested with *EcoRI* and cloned into the pVIT321 suicide vector. The resulting construct was used for

biparental mating to generate Bv5-4a Δ *avrBsT* (Lee et al. 2009). For generation of the Ds1 Δ *hrcV* mutant, the 617-bp fragment of *hrcV* was amplified by PCR using *P. furiosus* DNA polymerase and the following primer pairs: knock-out forward (5'-GAATTCGCCGCGTCATGATCACC-3') and knock-out reverse (5'-GAATTCTCCTGGATGCCGACGAACA-3'). The amplified fragment of *hrcV* was digested with *EcoRI* and cloned into the pVIT321 vector. The resulting construct was used for biparental mating to generate Ds1 Δ *hrcV*.

Bacterial transformation.

X. campestris pv. *vesicatoria* strains Ds1 and Bv5-4a were grown overnight in yeast nutrient (YN) media (5 g of yeast extract and nutrient broth at 8 g liter⁻¹) at 28°C. Ds1 and Bv5-4a cultures were centrifuged and washed twice with sterile water. Plasmids pBBR1MCS-2, pBBR1MCS-2:*avrBsT* or pBBR1MCS-2:*avrBsT*:8 $\times$ myc were introduced into competent *X. campestris* pv. *vesicatoria* cells using a MicroPulser (Bio-Rad, Hercules, CA, U.S.A.), which were then grown at 28°C on YN agar containing kanamycin (Kan) at 50 mg liter⁻¹. Various *avrBsT* constructs were also transformed into competent *A. tumefaciens* GV3101 and grown at 28°C on Luria-Bertani agar containing rifampicin (Rif) at 50 mg liter⁻¹ rifampicin (Rif) and Kan at 50 mg liter⁻¹.

Biparental mating was used to generate *X. campestris* pv. *vesicatoria* Bv5-4a Δ *avrBsT* and Ds1 Δ *hrcV* (Lee et al. 2009). The donor *Escherichia coli* 17-1 λ -pir harboring pVIT321:*avrBsT* or pVIT321:*hrcV* was mixed with Bv5-4a or Ds1, respectively, and then spread onto YN-agar media without antibiotics. Overnight culture was collected and transformants were selected by spreading on YN agar containing tetracycline (Tc) at 5 mg liter⁻¹ and Rif at 50 mg liter⁻¹. To express *avrBsT* in *X. campestris* pv. *vesicatoria* strains, the plasmid pBBR1MCS-2:*avrBsT* was introduced into Bv5-4a Δ *avrBsT* or Ds1 Δ *hrcV* by electroporation. Transformants were then selected on the YN agar supplemented with Tc at 5 mg liter⁻¹, Rif at 50 mg liter⁻¹, and Kan at 50 mg liter⁻¹.

Bacterial inoculation.

X. campestris pv. *vesicatoria* strains Ds1, Ds1 (EV), Ds1 (*avrBsT*), and Bv5-4a were cultured overnight. The bacteria were harvested and resuspended in 10 mM MgCl₂ solution, which was infiltrated into pepper and tomato leaves using a needleless syringe (Kim et al. 2007). Bacterial growth in leaves was monitored 0 and 3 days after inoculation with *X. campestris* pv. *vesicatoria* (5 $\times$ 10⁴ CFU ml⁻¹). Three leaf discs (1.0 cm in diameter) were ground in 1 ml of 10 mM MgCl₂ sterile solution. The suspension was serially diluted and spread on YN agar. For transient expression of various *avrBsT* constructs, *Agrobacterium* cultures were grown overnight and resuspended in medium (10 mM morpholine-ethanesulfonic acid [pH 5.7], 10 mM MgCl₂, and 0.2 mM acetosyringone) to infiltrate into *N. benthamiana*, pepper, and tomato leaves.

Ion leakage assay.

Leaves of pepper and tomato plants were harvested at various time points after inoculation with strains Ds1, Ds1 (EV), Ds1 (*avrBsT*), and Bv5-4a. Leaf discs (0.5 cm in diameter) were removed with a cork borer and washed in 10 ml of sterile double-distilled water for 30 min with gentle agitation. Washed leaf discs were transferred to 15 ml of sterile double-distilled water and incubated for 3 h at room temperature with gentle agitation. The conductivity of the leaf samples was measured using a Sension 7 conductivity meter (Hach Co., Loveland, CO, U.S.A.). Experiments were carried out three times with similar results.

Histochemistry.

Callose deposition was visualized by depigmenting the inoculated leaves in 95% ethanol and staining with 150 mM K_2HPO_4 solution (pH 9.5) containing 0.01% aniline blue for 30 min. H_2O_2 production was visualized by placing the inoculated leaves in DAB solution at 1 mg ml^{-1} (Sigma-Aldrich, St. Louis) for 15 h (Thordal-Christensen et al. 1997). Chlorophyll was cleared from the stained leaves by boiling in 95% ethanol. Cell death was monitored by trypan blue staining of healthy or inoculated leaves (Koch and Slusarenko 1990). Leaves were stained with lactophenol-trypan blue solution (10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, and 10 mg of trypan blue, dissolved in 10 ml of distilled water) and destained in chloral hydrate solution (chloral hydrate at 2.5 g ml^{-1}). The samples were photographed using a light microscope mounted with a digital camera (Olympus, Tokyo). Chlorosis, cell death, or phenolic compound accumulation (Park 2005) in leaves was visualized using a hand-held UV lamp (UVP, Upland, CA, U.S.A.).

Quantitative reverse-transcription PCR.

Total RNA was isolated from leaves inoculated with *X. campestris* pv. *vesicatoria* strains using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). RNA (2 μ g) was used in a reverse-transcription reaction with MMLV reverse transcriptase (Enzymatics, Seoul, Korea). Pepper gene-specific primers for *CaBPR1* (F, CAGGATGCAACACTCTGGTGG; R, ATCAAAGGCCGGTTGGTC), *CaPO2* (F, ATGAGTATTTCAAACAACTCTTTT; R, TTAATTTATAGCTCCACACACAGT), *CaSAR82A* (F, CAGGGAGATGAATTCTGAGGC; R, CATATGAACCTCTATGGATTCTG), *CaDEF1* (F, CAAGGGAGTATGTGCTAGTGAGAC; R, TGCACAGCACTATCATTGCATAC), and 18S rRNA (F, CCGGTCCGCCTATGGTGTGCACCGTCGTC; R, GCAGTTGTTTCGTCCTTCATAAATCCAAGAA) were used. Tomato gene-specific primers for *Actin* (F, GAGCGTGGTTACTCGTTCA; R, CTAATATCCACGTCACATTTTCA), *Senu4* (F, CTGGTGCTGGGGAGAATC; R, GTCCGATCCAGTTGCCTACA), *Chil17* (F, AGGCAATCAAATGGGAAGTG; R, CAATCCGATCCTCCACTTGT) (Cunnac et al. 2007), *Cevi16* (F, GTTTTACTGAATCTGTTGCTGCTC; R, TCAGTTGAATGCAGAGCAAACCTT), and *Tgas118* (F, ATGGCAAACCTCCATGCGTTTA; R, TTATTCATTCATGAGTATTTCCGAT) were used. Real-time PCR was performed using iQ SYBR Green Supermix and iCycler iQ (Bio-Rad). The 18S rRNA transcript level was used to normalize the transcript level of each gene. Relative expression levels were determined by comparing the values with that of the uninoculated control.

Protein extraction and Western blots.

Leaves infiltrated with *Agrobacterium* spp. were harvested and homogenized in liquid nitrogen. Protein extraction buffer (2 ml) containing 50 mM Tris-Cl (pH 8.8), 50 mM NaCl, 10 mM EDTA, 0.2% Triton X-100, and 1 $\times$ protease inhibitor cocktail (Roche, Indianapolis, IN, U.S.A.) was added to 1 g of each homogenized sample. The samples were centrifuged at 13,000 $\times$ g at 4°C for 30 min. Supernatants were resolved on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Western blot analysis was performed using an anti-cMyc-peroxidase conjugate (Sigma-Aldrich).

Type III secretion assay.

Type III secretion experiments were carried out in M9 minimal medium (Rossier et al. 1999). After incubation for 4 h at 28°C, cells were pelleted by centrifugation and the supernatant was filtered through a 0.2- μ m syringe filter. Cellular and super-

natant proteins were precipitated according to Rossier and associates (1999), resolved in 12% SDS-PAGE gels, and transferred to PVDF membranes for Western blot analysis using anti-cMyc (Sigma-Aldrich) and anti-NptII (Abcam, Tokyo) antibodies.

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