Visualization of novel virulence activities of the *Xanthomonas* type III effectors AvrBs1, AvrBs3 and AvrBs4

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SUMMARY

Xanthomonas campestris pv. *vesicatoria* secretes at least 20 effector proteins through the type III secretion system directly into plant cells. In this study, we uncovered virulence activities of the effector proteins AvrBs1, AvrBs3 and AvrBs4 using *Agrobacterium*-mediated transient expression of the corresponding genes in *Nicotiana benthamiana*, followed by microscopic analyses. We showed that, in addition to the nuclear-localized AvrBs3, the effector AvrBs1, which localizes to the plant cell cytoplasm, also induces a morphological change in mesophyll cells. Comparative analyses revealed that *avrBs3*-expressing plant cells contain highly active nuclei. Furthermore, plant cells expressing *avrBs3* or *avrBs1* show a decrease in the starch content in chloroplasts and an increased number of vesicles, indicating an enlargement of the central vacuole and the cell wall. Both AvrBs1 and AvrBs3 cause an increased ion efflux when expressed in *N. benthamiana*. By contrast, expression of the *avrBs3* homologue *avrBs4* leads to large catalase crystals in peroxisomes, suggesting a possible virulence function of AvrBs4 in the suppression of the plant defence responses. Taken together, our data show that microscopic inspection can uncover subtle and novel virulence activities of type III effector proteins.

INTRODUCTION

The pathogenicity of most Gram-negative plant pathogenic bacteria depends on the type III secretion (T3S) system that injects a battery of effector proteins into plant cells (Cornelis and Van Gijsegem, 2000; Tampakaki *et al.*, 2004). Historically, many

type III effectors have been identified by their avirulence (avr) activity, i.e. by betraying the presence of the bacterium to the plant's immune system. In this case, the Avr protein is recognized by a corresponding resistance (R) protein in the plant (Flor, 1971; Gabriel and Rolfe, 1990; Keen, 1990). Specific recognition often induces the hypersensitive response (HR), a rapid local plant cell death reaction that is concomitant with the halt of bacterial growth (Hammond-Kosack and Jones, 1996; Klement, 1982; Staskawicz, 2001). However, functional studies of type III effector proteins have revealed that the suicidal HR elicitation activity arises through plant evolution, and that their primary role in susceptible plants is in virulence. There, effector proteins modify the cellular metabolism to benefit bacterial growth, for example by suppressing host defence (Abramovitch and Martin, 2004; Grant *et al.*, 2006), or to cause bacterial dissemination (Wichmann and Bergelson, 2004). The list of effector proteins with a demonstrated virulence function has grown rapidly in recent years and includes, for example, PthA from *Xanthomonas axonopodis* pv. *citri*, which induces citrus canker and enhances the release of bacteria to the surface of infected leaves, thus promoting the spread of the pathogen to new infection sites (Swarup *et al.*, 1991, 1992). Similar effects have been observed for AvrB6 from *Xanthomonas campestris* pv. *malvacearum* (Yang *et al.*, 1994). Recent studies have suggested that the suppression or delay of plant defence reactions is one of the major virulence functions of type III effectors. The latter has been shown mainly for effectors from subspecies of *Pseudomonas syringae*, which, for example, suppress callose deposition at infection sites and cell death in non-host plants, or inhibit the avr activity of a particular effector protein in resistant plants (Abbo *et al.*, 1995; da Cunha *et al.*, 2007; Kim *et al.*, 2005; Mackey *et al.*, 2003; Nomura *et al.*, 2005; Oguiza and Asensio, 2005). In addition, several effector proteins from *Xanthomonas* species have also been found to suppress plant defence reactions (Brown *et al.*, 1995; Fujikawa *et al.*, 2006; Makino *et al.*, 2006; Metz *et al.*, 2005). Interestingly, there are indications that members of the AvrBs3 family can suppress cell death reactions (Fujikawa *et al.*, 2006; Makino *et al.*, 2006).

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In our laboratory, we have studied *X. campestris* pv. *vesicatoria*, the causal agent of bacterial spot disease in pepper and tomato. The pathogenicity depends on the T3S system, which translocates at least 20 effectors into the plant cell (Thieme *et al.*, 2005; F. Thieme and U. Bonas, unpublished data). A large number of *avr* genes have been cloned from *X. campestris* pv. *vesicatoria* (Gürlebeck *et al.*, 2006; Minsavage *et al.*, 1990; van't Slot and Knogge, 2002), three of which, *avrBs1*, *avrBs3* and *avrBs4*, are the focus of this study. The *avrBs3* gene encodes a 122-kDa protein with a central domain composed of 17.5 nearly identical repeats of 34 amino acids (Bonas *et al.*, 1989; Knoop *et al.*, 1991). Analyses of AvrBs3 and homologues have shown that the number and order of the repeats determine *R* gene specificity (Bonas *et al.*, 1993; Herbers *et al.*, 1992). AvrBs3 is translocated from *X. campestris* pv. *vesicatoria* via the T3S system into the host cells, where it dimerizes and localizes to the nucleus (Gürlebeck *et al.*, 2005, 2006; Szurek *et al.*, 2002; Van den Ackerveken *et al.*, 1996). The induction of HR in resistant pepper plants depends on the presence of at least one nuclear localization signal (NLS) and the acidic activation domain (AAD), both located in the C-terminal region of the AvrBs3 protein (Van den Ackerveken *et al.*, 1996), and is accompanied by a rapid macroscopic cell death. Yeast two-hybrid, *in vitro* and *in planta* bimolecular fluorescence complementation studies have shown that importin α binds to the AvrBs3-NLSs to mediate nuclear import inside the plant cell (Szurek *et al.*, 2001, 2002; D. Gürlebeck and U. Bonas, unpublished data). In susceptible plants, AvrBs3-delivering *X. campestris* pv. *vesicatoria* strains induce hypertrophy, which is an enlargement of mesophyll cells (Bonas *et al.*, 1989; Marois *et al.*, 2002). Macroscopically, this hypertrophy leads to pustules on the lower surface of infected leaves. As the NLSs and AAD of AvrBs3 are required for both HR and hypertrophy, we postulated that AvrBs3 acts as a transcriptional regulator in the nucleus (Gürlebeck *et al.*, 2005, 2006; Marois *et al.*, 2002; Szurek *et al.*, 2001, 2002). Indeed, AvrBs3 induces the expression of plant genes necessary for the induction of cell enlargement (Kay *et al.*, 2007; Marois *et al.*, 2002).

Ongoing studies have revealed a diversity of molecular methods of action of type III effector proteins from phytopathogens, e.g. as E3-ligases, cysteine proteases, phosphatases or transcriptional regulators (for a review, see Gürlebeck *et al.*, 2006; Mudgett, 2005). However, in contrast with effectors from mammalian pathogens, effector gene knockout mutants of phytopathogenic bacteria rarely result in a visible decrease in virulence. Hence, the function of most *X. campestris* pv. *vesicatoria* effector proteins is still mysterious. For example, AvrBs1 lacks sequence homologies that suggest a biochemical function (Napoli and Staskawicz, 1987; Ronald and Staskawicz, 1988). Furthermore, despite its 97% amino acid identity to AvrBs3, the virulence function of AvrBs4 (Bonas *et al.*, 1993; Schornack *et al.*, 2004) has so far escaped observation.

In this article, we present our investigations to uncover the potential virulence functions of the *X. campestris* pv. *vesicatoria* type III effector proteins AvrBs1, AvrBs3 and AvrBs4. A combination of light and electron microscopy, immunohistochemistry and physiological analyses has revealed that the three type III effector proteins under study induce changes in plant cellular morphology and ion leakage, suggesting novel virulence activities for these *Xanthomonas* effectors.

RESULTS

AvrBs1 and AvrBs3 cause chlorotic symptoms in the plant

To unravel the possible virulence functions of the individual effector proteins AvrBs1, AvrBs3 and AvrBs4 in the plant, we transiently expressed the corresponding genes or derivatives thereof for all performed studies in *Nicotiana benthamiana* under the control of the constitutive 35S promoter, mediated by *Agrobacterium tumefaciens*. As a negative control, we used an *Agrobacterium* strain containing an empty vector or expressed the *Escherichia coli* gene *uidA* for β -glucuronidase (GUS) in *N. benthamiana*. *N. benthamiana* was used because it lacks the corresponding resistance genes and, in contrast with pepper and tomato, does not display basal defence reactions to *Agrobacterium* at early time points after infection. As shown in earlier studies, the three effector genes, as well as *uidA*, are well expressed *in planta* using *Agrobacterium*-mediated transient expression (Ballvora *et al.*, 2001; Escobar *et al.*, 2001; Kay *et al.*, 2005; Marois *et al.*, 2002; Schornack *et al.*, 2004; Szurek *et al.*, 2002; Van den Ackerveken *et al.*, 1996).

First, we observed the phenotypic reactions induced in *N. benthamiana* after *Agrobacterium*-mediated expression of *avrBs1*, *avrBs3*, *avrBs4* or *uidA*, which were monitored over a period of 5 days. Tissue reactions started to be visible at 4 days post-infiltration (dpi) and increased within the next 24 h. Figure 1 shows macroscopically visible reactions induced by the effectors under study 5 dpi of the *Agrobacterium* strains. A swelling of the upper epidermis was observed in leaf areas that contained AvrBs1 or AvrBs3. In contrast, AvrBs4 and GUS did not induce swelling of the tissue. As expected, AvrBs3 caused hypertrophy on the lower surface of the leaf (Marois *et al.*, 2002). In the case of AvrBs1, AvrBs4 and GUS, there was no visible pustule formation.

In addition, the expression of *avrBs1* and *avrBs3* led to chlorosis in *N. benthamiana* (Fig. 1B,D). Interestingly, AvrBs4, a close homologue of AvrBs3, caused very little chlorosis (Fig. 1A). Furthermore, the expression of *avrBs1* resulted in weak necrotic reactions of the infiltrated tissue (Fig. 1B). The expression of *uidA* did not result in an altered plant phenotype (Fig. 1C), showing that the reactions caused by AvrBs1, AvrBs3 and AvrBs4 are specific and not caused by infection with *Agrobacterium*.

Fig. 1 AvrBs1, AvrBs3 and AvrBs4 induce macroscopically visible reactions in *Nicotiana benthamiana*. Derivatives of *Agrobacterium* strain GV3101 delivering a T-DNA with (A) *avrBs4*, (B) *avrBs1*, (C) *uidA* and (D) *avrBs3* were inoculated into *N. benthamiana*. The upper surface of the leaf was photographed at 5 days post-inoculation (dpi). Similar reactions were obtained in at least three independent experiments.

AvrBs1 and AvrBs3 induce increased ion leakage from *N. benthamiana* cells

To investigate whether there are additional physiological changes that may be induced by the type III effectors under study, we analysed the ion leakage of *N. benthamiana* cells expressing *uidA*, *avrBs1*, *avrBs3* and *avrBs4*. Ion leakage is often used as a measure of cellular changes, e.g. cell death reactions after bacterial infection (Minsavage *et al.*, 1990). Leaf material was collected 1 and 4 dpi in deionized water and the relative conductivity was determined. In comparison with uninfected tissue, *uidA*- and *avrBs4*-expressing cells behaved similarly, whereas AvrBs3 and AvrBs1 induced a significant increase in ion leakage up to 4 dpi (Fig. 2).

AvrBs1 induces morphological changes in mesophyll cells

Next, we investigated the effects of the different effectors on *N. benthamiana* cells in more detail using light microscopy. The leaf tissue was analysed 1, 2, 3, 4 and 5 dpi. The first morphological changes were only observed at 4 dpi, which further increased within 24 h (5 dpi). Representative example photographs are shown in Fig. 3. Mesophyll cells expressing *uidA* appeared

Fig. 2 Determination of the ion leakage from plant cells following the expression of *uidA*, *avrBs1*, *avrBs3* and *avrBs4*. Leaves of five *Nicotiana benthamiana* plants were infiltrated with infiltration medium and with derivatives of *Agrobacterium* strain GV3101 delivering a T-DNA with *uidA*, *avrBs4*, *avrBs3* and *avrBs1*. At 1 and 4 days post-inoculation (dpi), leaf discs were harvested and the relative conductivity was determined. Columns represent the mean relative conductivity of five independent samples per treatment. Standard errors of the mean values are indicated as error bars. The experiment was repeated three times showing comparable results.

Fig. 4 Subcellular localization of AvrBs4 and AvrBs1 in the plant cell. (A) AvrBs4 localizes to the nucleus. Pepper Early Cal Wonder leaves were infected with the *Xcv* strains 85* and 85*Δ*hrcV*, both expressing *avrBs4*. Leaves were inoculated with bacterial suspensions of 10^9 colony-forming units (cfu)/mL and harvested at 12 h post-inoculation (hpi). Total cell extracts were fixed, immunostained for AvrBs4 and 4',6-diamidino-2-phenylindole (DAPI) stained for the nuclei. The arrowhead indicates an AvrBs4-containing nucleus among visible chloroplasts. (B) AvrBs1 localizes to the cytoplasm. For localization studies, *avrBs1-gfp* was transiently expressed in *Nicotiana benthamiana* using *Agrobacterium tumefaciens* strain GV3101 (*avrBs1-gfp*). To visualize the nuclei, the leaves were infiltrated at 48 h post-inoculation (hpi) with DAPI, harvested 1 h later, mounted in $1 \times$ phosphate-buffered saline (PBS) and viewed by a Zeiss confocal laser fluorescence microscope (Jena, Germany) using specific filter settings for enhanced green fluorescent protein (EGFP) and DAPI. In parallel, total protein extract from a leaf sample was separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto nitrocellulose and incubated with anti-GFP antibody (right panel).

morphologically comparable with uninfiltrated tissue of the same leaf. All inspected palisade cells were similar in size and shape and contained chloroplasts densely arranged to each other in the cytoplasm. The intercellular spaces were clearly visible. In contrast, tissue expressing *avrBs3* contained strongly enlarged and bloated mesophyll cells and therefore reduced intercellular spaces. In particular, the palisade cells were elongated and wider than the cells in the negative controls.

Interestingly, cells expressing *avrBs1* were also morphologically different from those of the negative controls. However, in contrast with *avrBs3*-expressing cells, the palisade cells were not elongated but only bloated, thus changing their shape and leading to a decrease in the intercellular spaces. We also detected dead cells in *avrBs1*-expressing tissue, which is in agreement with the macroscopically visible weak necrosis induced by AvrBs1 (Figs 1B, 3D). Cells expressing the *avrBs3* homologue *avrBs4* did not show substantial cellular changes (Fig. 3).

AvrBs1 localizes to the plant cell cytoplasm and AvrBs4 to the nucleus

To understand the mode of action of a certain effector, its localization within the host cell is of special interest. As shown in previous

studies, AvrBs3 localizes to the nucleus of plant cells (Szurek *et al.*, 2002). Because of its high similarity to AvrBs3, AvrBs4 was proposed to undergo the same subcellular localization. To prove this assumption, we performed a 'nuclear targeting assay' successfully used to show the nuclear localization of AvrBs3 (Szurek *et al.*, 2002). For this, leaves of susceptible pepper (Early Cal Wonder) plants were infiltrated with the *avrBs4*-expressing *Xanthomonas* strains *Xcv* 85* (pD200) and *Xcv* 85*Δ*hrcV* (pD200). The latter strain is a T3S mutant, which is unable to secrete and translocate effector proteins. Nuclei were isolated at 12 h post-inoculation (hpi), fixed on microscopy glass slides and incubated with the AvrBs3-specific antibody, which recognizes AvrBs3 and the 97% identical AvrBs4 protein. As expected, AvrBs4 protein was detected in the nuclei of plant cells infected with *Xcv* 85* (pD200), but not in plant cells infected with the T3S-deficient strain *Xcv* 85*Δ*hrcV* (pD200). 4',6-Diamidino-2-phenylindole (DAPI) staining confirmed the localization of AvrBs4 to the nucleus (Fig. 4A, bottom panels).

Next, we investigated the subcellular localization of AvrBs1 using *Agrobacterium*-mediated expression of *avrBs1-gfp* in *N. benthamiana*. For this, we cloned *avrBs1* into the binary vector pGWB5, which allows the expression of *avrBs1* as a translational fusion to green fluorescent protein (GFP). Two dpi of strain

Fig. 3 AvrBs1 and AvrBs3 cause morphological changes in mesophyll cells. Leaves of *Nicotiana benthamiana* with cells expressing *uidA* (B), *avrBs3* (C), *avrBs1* (D) or *avrBs4* (E) were analysed at 1–5 days post-inoculation (dpi) by light microscopy. (A) Untreated. Morphological changes were clearly visible at 5 dpi, shown here in representative example photographs of two magnifications as indicated. Size bars correspond to all photographs of the vertical panel. E, epidermis; P, palisade parenchyma; S, spongy parenchyma; V, vascular tissue. Areas containing visible chloroplasts are indicated by arrows, intercellular spaces by asterisks and dead cells by arrowheads.

GV3101 (*avrBs1-gfp*) into *N. benthamiana*, leaf material was analysed by fluorescence microscopy. As shown in Fig. 4B, AvrBs1-GFP localizes exclusively to the cytoplasm of the plant cells. Immunoblot analysis verified that the GFP signal in the cytoplasm was indeed caused by the fusion protein AvrBs1-GFP and not GFP alone (Fig. 4B, right panel).

Cell enlargement is accompanied by enlargement of the vacuole

To further analyse the cellular changes induced by the effectors under study, we also performed electron microscopy to inspect membrane structures and the different organelles. Figure 5A gives an overview of the ultrastructures visualized within a plant cell using electron microscopy.

As shown in Fig. 3, the enlargement of mesophyll cells expressing *avrBs3* or *avrBs1* does not appear to be the result of an excess production of cytoplasm, but rather of the enlargement of the central vacuole. This is supported by the detection of an increased amount of closed circular membrane structures within the vacuoles of cells expressing *avrBs1* or *avrBs3*, in comparison with *uidA*- and *avrBs4*-expressing cells (Fig. 5B-1). In addition, *avrBs1*- and *avrBs3*-expressing cells contained an increased number of dictyosomes and Golgi vesicles, which probably deliver components for cell wall synthesis (data not shown).

Fig. 5 Specific effects of AvrBs1, AvrBs3 and AvrBs4 in plant mesophyll cells. *Nicotiana benthamiana* plants were inoculated with *Agrobacterium* strains, leading to the expression of the genes as indicated. Leaf material was collected at 5 days post-inoculation (dpi) (or as indicated) and inspected by electron microscopy. (A) Cytoplasm of an uninfected mesophyll cell. The visible organelles are indicated as follows: ch, chloroplast; cy, cytoplasm; g, Golgi apparatus; m, mitochondrion; p, peroxisome; tm, thylakoid membrane; white arrow, cristae mitochondriales; black arrows, double membranes of organelles. (B) Ultrastructure of cell wall and plasma membrane (B-1), chloroplasts (B-2), mitochondria (B-3), dictyosomes (B-4), peroxisomes (B-5) and nuclei (B-6). n, nucleolus; p, plasma; s, starch grain; v, nucleolar vacuole. Size bars correspond to 1 μ m. (C) Analyses of peroxisomes of infected tissue. The two photographs on the left show the ultrastructure of peroxisomes of cells expressing *avrBs4* or *avrBs4* Δ 227. The diagram shows crystal formation in peroxisomes of tissue infected with *avrBs4*-delivering *Agrobacterium*. Crystals were counted daily in 100 peroxisomes and are given as a percentage of the number of crystal-containing peroxisomes. The right panel shows a crystal structure in a peroxisome immunolabelled by anti-catalase antibody (black dots correspond to gold particles coated with secondary antibody). (D) Statistical results of the size measurements of 69 nuclei and nucleoli per infiltration site after analyses of ultrathin sections. Displayed are the empirical mean values of the sizes of the nuclei and nucleoli. The error bars reflect the standard errors of the mean (see Supporting Information for detailed results of statistical analysis).

AvrBs1, AvrBs3 and AvrBs4 induce different changes within organelles

Our electron microscopy studies of plant cells expressing *avrBs1*, *avrBs3* or *avrBs4* did not hint at any degradation in chloroplasts (Fig. 5B-2), mitochondria (Fig. 5B-3), Golgi apparatus (Fig. 5B-4), endoplasmic reticulum or peroxisomes (Fig. 5B-5) when compared with the ultrastructure of these organelles in *uidA*-expressing or uninfected cells. However, the chloroplasts of *avrBs3*- and *avrBs1*-expressing cells contained little or no starch grains relative to chloroplasts of *avrBs4*- or *uidA*-expressing tissue (Fig. 5B-2).

Intriguingly, all peroxisomes of *avrBs4*-expressing cells contained striking rhomboid structures (Fig. 5B-5,C). For quantification of the crystals, we inspected 100 peroxisomes per day over a period of 5 dpi. At 1 and 2 dpi, none of the investigated peroxisomes contained crystals. At 3 dpi 5%, at 4 dpi 32% and at 5 dpi 100% of the observed peroxisomes contained a crystal (see diagram, Fig. 5C). Interestingly, cells expressing the deletion derivative *avrBs4* Δ 227, lacking the NLSs and AAD, did not contain any rhomboid structures (Fig. 5C). In contrast with wild-type AvrBs4, expression of the highly homologous *avrBs3* gene also did not lead to crystals in peroxisomes (Fig. 5B-5).

Earlier studies have shown that such crystals within peroxisomes consist of the protein catalase (Frederick and Newcomb, 1969;

Fig. 5 continued.

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Huang and Beevers, 1973). To confirm this for the observed crystal structures in our studies, we immunolabelled ultrathin sections derived from *avrBs4*-expressing leaf material using a polyclonal anti-catalase antibody. As shown in Fig. 5C, the anti-catalase antibody bound to the crystal in the peroxisome. Positive immunolabelling was observed in all inspected peroxisome crystals. Thus, AvrBs4 wild-type protein leads to the accumulation of catalase in *N. benthamiana* cells.

AvrBs3 causes an increased transcriptional activity in plant cell nuclei

Next, we inspected the nuclei of plant cells after expression of the three effector genes under study. For each effector and

negative controls, we measured the sizes of 69 nuclei and their nucleoli followed by statistical analyses (Fig. 5D and Supporting Information). The nuclei of uninfected cells and of cells expressing *uidA*, *avrBs1* or *avrBs4* were significantly smaller in size (mean size, 60–110 μm^2) than the nuclei of *avrBs3*-expressing cells (mean size, 160 μm^2 ; Fig. 5D). Moreover, the nucleoli in these nuclei were significantly larger (mean size, 17 μm^2) than the nucleoli in control tissue, *uidA*-, *avrBs4*- and *avrBs1*-expressing tissue (mean size, 4–9 μm^2) (Fig. 5D). Strikingly, in 52% of the inspected nucleoli in *avrBs3*-expressing cells, a central, bright area was detectable (Fig. 5B–6). However, in cells expressing *avrBs1* or *avrBs4*, only 6% and 9%, respectively, of the nucleoli showed a bright area. Similar results were obtained for uninfected plant tissue (4%).

DISCUSSION

In this study, microscopic techniques allowed us to identify *X. campestris* pv. *vesicatoria* type III effector-induced plant phenotypes. Cellular changes induced by the type III effectors AvrBs1, AvrBs3 and AvrBs4 were examined in *N. benthamiana* after transient expression of single effector genes via *Agrobacterium*. This allows the study of single effectors, whereas *Xanthomonas campestris* pv. *vesicatoria* injects a large cocktail of different effectors, some of which may act similarly. Furthermore, *N. benthamiana* lacks the corresponding resistance genes (*Bs1*, *Bs3*, *Bs4*) and, in contrast with pepper and tomato, does not display basal defence reactions to *Agrobacterium*. Thus, the observed changes presumably represent specific virulence activities of the individual effectors.

AvrBs1 and AvrBs3 induce hypertrophy of mesophyll cells and increased ion leakage

Microscopy showed that AvrBs1 causes plant cell enlargement reminiscent of the effect induced by AvrBs3 (Marois *et al.*, 2002). This is interesting as the two proteins show no similarity on the amino acid level. Furthermore, the two proteins show different localization within plant cells. AvrBs3 localizes to the nucleus, whereas AvrBs1 localizes to the cytoplasm. Strikingly, the AvrBs3 homologue AvrBs4 induces almost no enlargement.

As described for plant cell growth in general (Cosgrove, 1993), expression of *avrBs1* and *avrBs3* also caused an expansion of the central vacuole and an enlargement of the cell wall. The number of chloroplasts did not appear to be affected, as degrading or dividing plastids were not observed. In addition to an increased number of dictyosomes and Golgi vesicles, which probably contain material for cell wall synthesis, membrane structures were observed within the central vacuole, which may be involved in the enlargement of the vacuole. Furthermore, chloroplasts of enlarged plant cells expressing *avrBs1* or *avrBs3* did not contain any starch grains, in contrast with cells expressing *uidA* or *avrBs4* and cells of untreated tissue. It is probable that little starch storage takes place because carbohydrates are used to generate energy for cell growth, and pre-existing starch grains are catabolized for energy generation.

Interestingly, slowly growing *avrBs1*-expressing *X. campestris* pv. *vesicatoria* strains (which do not carry AvrBs3) induced some pustules in pepper (data not shown), which could be caused by AvrBs1. Thus, the observed change in *N. benthamiana* is likely to be a virulence effect of the effector rather than an *avr* effect. However, as *X. campestris* pv. *vesicatoria* secretes a large number of effectors (Thieme *et al.*, 2005), we cannot exclude the possibility that another, yet unidentified, effector is responsible for cell enlargement.

An open question is how *X. campestris* pv. *vesicatoria* benefits from the induction of hypertrophy. One possibility is that plant cell growth generates squeezing forces that release the bacteria onto the leaf surface from where they can infect other parts of the plant. Indeed, two members of the AvrBs3 family (Avrb6 from *X. campestris* pv. *malvacearum* and PthA from *X. citri*) have been shown to enhance bacterial release to the plant surface (Yang *et al.*, 1994). However, Avrb6 and PthA induce cell proliferation (hyperplasia) resulting in rupture of the tissue (Brunings and Gabriel, 2003; Gürlbeck *et al.*, 2006; Swarup *et al.*, 1991), which was not observed in our microscopic studies. Another possibility is that cellular growth accompanied by a decrease in the intercellular spaces expels the bacteria through stomata. This could explain the enhanced fitness and distribution of *X. campestris* pv. *vesicatoria* strains expressing *avrBs1* and *avrBs3* in field studies (Wichmann and Bergelson, 2004).

Interestingly, the nematode *Meloidogyne javanica* also induces enlarged plant cells, termed giant cells, in its host plant tomato (Gal *et al.*, 2006). The growth of giant cells depends on expansins, which cause a loosening of the cell wall (Gal *et al.*, 2006; McQueen-Mason, 1997). This is reminiscent of the finding that AvrBs3 induces the expression of expansins in susceptible pepper plants (Marois *et al.*, 2002). The giant cells represent a feeding site, providing the nematode with nutrients (Williamson and Hussey, 1996). Thus, it is possible that AvrBs3- and AvrBs1-induced cell enlargement improves the nutrient supply of *X. campestris* pv. *vesicatoria*. In contrast with the nematode-induced polynucleated giant cells, the *X. campestris* pv. *vesicatoria* effectors studied here did not induce nuclear divisions in mesophyll cells.

The expression of *avrBs1* and *avrBs3* in *N. benthamiana* caused an increased ion leakage, which is normally measured during HR (Minsavage *et al.*, 1990). Indeed, expression of *avrBs1* led to macroscopically and microscopically visible cell death reactions. However, in *avrBs3*-expressing tissue, dead cells were observed only rarely, although the ion leakage was comparable with that induced by AvrBs1. These data suggest that the ion efflux caused by AvrBs1 and AvrBs3 is induced independent of cell death. Hence, the induction of ion leakage may represent another virulence activity of AvrBs1 and AvrBs3. It is also possible that ion leakage occurs as a result of hypertrophy and reflects an enhanced release of nutrients, which would be of advantage for the pathogen.

AvrBs3 induces a stronger nuclear activity than the similar AvrBs4 protein

AvrBs3 and AvrBs4 localize to the plant cell nucleus, where both may act as transcriptional regulators (Kay *et al.*, 2007; Marois *et al.*, 2002; Szurek *et al.*, 2002; this study). Strikingly, the nuclei and nucleoli of cells expressing *avrBs3* were significantly enlarged compared with the nuclei of *avrBs4*-expressing cells or

untreated cells. Furthermore, the nucleoli of *avrBs3*-expressing cells displayed electron-transparent regions, termed 'nucleolar vacuoles'. The expansion of the nucleoli and the formation of nucleolar vacuoles point to an increased need for ribosomes, and thus to an enhanced transcriptional activity (Gonzalez-Camacho and Medina, 2006; Jordan, 1984; Shaw and Doonan, 2005). Previously, it has been shown that AvrBs3 and other family members induce the transcription of plant genes (Gu *et al.*, 2005; Kay *et al.*, 2007; Marois *et al.*, 2002; Sugio *et al.*, 2007; Szurek *et al.*, 2002; Yang *et al.*, 2006). Interestingly, our results suggest that AvrBs4 induces less transcription than AvrBs3. As AvrBs1 localizes to the plant cell cytoplasm and only a few nucleoli were enlarged or contained a nuclear vacuole in *avrBs1*-expressing cells, AvrBs1 probably uses a different, indirect molecular mechanism compared with AvrBs3 to induce hypertrophy.

In addition to our effector studies, it is interesting to note that *uidA*, which is often used as a negative control or reporter gene, has effects in the plant cell. First, it seems to have a positive effect on starch grain storage. Secondly, it leads to larger nuclei. Thus, in future studies, it must be kept in mind that a reporter gene may have effects, which we simply do not see.

AvrBs4—a microbial defence against plant defence?

It is notable that AvrBs4, despite its high degree of identity to AvrBs3, induces different plant reactions. What could be the virulence activity of AvrBs4? The work presented here revealed that the expression of *avrBs4* in *N. benthamiana* leads to large rhomboid structures in peroxisomes. Such structures have been shown in earlier studies to be a result of the accumulation of catalase (Frederick and Newcomb, 1969; Huang and Beevers, 1973), which was confirmed in our experiments. Catalase detoxifies hydrogen peroxide (H_2O_2), which is generated in peroxisomes during oxidative processes and the disproportionation of reactive oxygen species (ROS; del Rio *et al.*, 2006). Pathogen attack induces an enhanced production of H_2O_2 (del Rio *et al.*, 2006), which is toxic for pathogens and acts as a signal molecule triggering plant defence reactions (Alvarez *et al.*, 1998; Apostol *et al.*, 1989; Gechev and Hille, 2005; Montillet *et al.*, 2005; Willekens *et al.*, 1995). Interestingly, the application of catalase prevents necrotic reactions during pathogen infection and inhibits ROS formation (Devlin and Gustine, 1992; Masuta *et al.*, 1991; Willekens *et al.*, 1995). In addition, a reduced catalase content leads to an increased resistance of the plant against pathogens (Chamnongpol *et al.*, 1998; Mittler *et al.*, 1999). Furthermore, signal molecules, which induce plant defence reactions, inhibit the catalase activity in tobacco (Conrath *et al.*, 1995). Taken together, the present knowledge about plant catalases suggests that the AvrBs4-induced increase in the catalase content in plant peroxisomes may reflect a virulence function of AvrBs4, i.e. in the inhibition of plant defences.

Consistent with this, there are a growing number of type III effectors from *Pseudomonas* and *Xanthomonas* which suppress basal and specific plant defence reactions (Abramovitch and Martin, 2004; Abramovitch *et al.*, 2006; Bretz *et al.*, 2003; DebRoy *et al.*, 2004; Espinosa *et al.*, 2003; Fujikawa *et al.*, 2006; Keshavarzi *et al.*, 2004; Kim *et al.*, 2005; Makino *et al.*, 2006; Metz *et al.*, 2005). Future studies must show whether the AvrBs4-induced increase in the catalase content in the plant is a control point of plant defence reactions.

In conclusion, our studies demonstrate that, in addition to screening for cell death induction and suppression by effectors, microscopic analysis is an excellent technique to uncover potential virulence activities of type III effector proteins.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The *A. tumefaciens* strains used in this study were GV3101 (*uidA*), GV3101 (*avrBs3*), GV3101 (*avrBs1*), GV3101 (*avrBs4*) and GV3101 (*avrBs4Δ227*) [designated GV3101 (p35SGUSint), GV3101 (pVSF300), GV3101 (pVS100), GV3101 (pVS200F) and GV3101 (pVS227) in earlier studies; Ballvora *et al.*, 2001; Escobar *et al.*, 2001; Gürlebeck *et al.*, 2005; Marois *et al.*, 2002; Schornack *et al.*, 2004; Szurek *et al.*, 2001; Van den Ackerveken *et al.*, 1996]. For localization studies of AvrBs1, the *avrBs1* gene was amplified from strain Xcv 85-10 using the oligonucleotides aBs1-fe (CACCATGTCCGACATGAAAGTTAATTTC) and aBs1-rsl (CGCT-TCTCTGCATTGTAAACATG), and cloned into pENTR/D TOPO (Invitrogen GmbH, Karlsruhe, Germany), resulting in plasmid pENTR(*avrBs1*-sl). Subsequently, the gene was recombined via GATEWAY technology (Invitrogen GmbH) into pGWB5 (C-terminal GFP tag; Nakagawa *et al.*, 2007). *A. tumefaciens* strain GV3101 was transformed with pGWB5::*avrBs1* by electroporation, resulting in strain GV3101 (*avrBs1-gfp*).

For nuclear targeting assays, the *X. campestris* pv. *vesicatoria* (also designated as *Xanthomonas euvesicatoria*; Jones *et al.*, 2004) strains Xcv 85* (pDSK200) and Xcv 85* Δ *hrcV* (pDSK200), which both express *avrBs4*, were used (Ballvora *et al.*, 2001). *A. tumefaciens* cells were cultivated at 30 °C in yeast extract broth (YEB) medium (Van den Ackerveken *et al.*, 1996) and Xcv in nutrient yeast glycerol (NYG) medium (Daniels *et al.*, 1984). Antibiotics were added to the media at the following final concentrations: kanamycin, 25 µg/mL; rifampicin, 100 µg/mL; spectinomycin, 50 µg/mL.

Plant material and inoculations

Pepper (*Capsicum annuum*) plants of cultivar Early Cal Wonder and *N. benthamiana* plants were grown as described previously (Bonas *et al.*, 1989; Marois *et al.*, 2002; Szurek *et al.*, 2002).

Briefly, 6-week-old plants were used for bacterial inoculations. Inoculated *N. benthamiana* plants were kept in a Percival growth chamber (Percival Scientific, Perry, IA, USA) at 22 °C, 16 h of light and 70% humidity. For transient expression assays, *N. benthamiana* leaves were co-infiltrated with *Agrobacterium* strain C58CI (pCH32/pBin6lp19; Voinnet, 2001), leading to the expression of the silencing inhibitor p19, and strain GV3101, delivering the T-DNA with the appropriate gene. Bacteria were resuspended at an optical density at 600 nm (OD₆₀₀) of 1.0 (1.25×10^9) in infiltration medium [5 mM 2-(*N*-morpholino)ethanesulphonic acid (MES), pH 5.3, 10 mM MgCl₂, 150 mM acetosyringone], and inoculated with a needle-less syringe into the intercellular spaces of abaxial leaf surfaces. Plant reactions were scored over 7 days. For AvrBs1 localization studies, *N. benthamiana* leaves were infiltrated with *Agrobacterium* strain GV3101 (avrBs1-gfp), leading to the expression of AvrBs1-GFP. The strain was resuspended to an OD₆₀₀ of 0.5 prior to infiltration. *Xanthomonas* strains were inoculated as described previously (Szurek *et al.*, 2002).

Microscopy

For localization studies by fluorescence microscopy, *Agrobacterium*-infected *N. benthamiana* leaves were infiltrated 2 dpi with DAPI, harvested 1 h later, mounted in 1 × phosphate-buffered saline (PBS), and viewed by a Zeiss confocal laser fluorescence microscope LSM510 (Jena, Germany) using specific filter settings for enhanced green fluorescent protein (EGFP) and DAPI.

The nuclear targeting assay was performed as described by Szurek *et al.* (2002).

For ultrastructural studies, leaf segments were collected at 1, 2, 3, 4 and 5 dpi, fixed with 3% glutaraldehyde in sodium cacodylate buffer (SCB), pH 7.2, for 3 h at room temperature, washed with SCB, post-fixed with 1% osmium tetroxide in SCB, dehydrated in a graded ethanol series, and embedded in epoxy resin (Spurr, 1969). The material was sectioned with an ultramicrotome S (Leica, Bensheim, Germany). Semi-thin sections (1 µm) were stained with 1% toluidine blue and observed with an Axioskop 20 light microscope (Zeiss). Ultra-thin sections (80 nm) were transferred to coated copper grids, stained with uranyl acetate and lead citrate, and inspected with an EM 900 transmission electron microscope (Zeiss SMT, Oberkochen, Germany) at an acceleration voltage of 80 kV. Electron micrographs were taken with a slow scan camera (Variospeed SSCCD camera SM-1k-120, TRS, Moorenweis, Germany). For morphometric measurements, images of mesophyll nuclei containing a nucleolus were used to avoid the analysis of peripheral sections. Measurements were carried out using analySIS Software (SIS, Münster, Germany).

For immunocytochemistry studies of the ultrastructural localization of catalase, 2-mm leaf discs were high-pressure frozen, freeze-substituted and embedded in HM20 (Polysciences Europe, Eppelheim, Germany), as described previously (Thieme *et al.*,

2007). Ultrathin sections were immunolabelled using a polyclonal α-catalase antibody (Biotrend, Cologne, Germany) and donkey α-sheep immunoglobulin G (IgG) conjugated to 6-nm gold particles (Biotrend, Cologne, Germany). After staining with uranyl acetate and lead citrate, sections were analysed as described above.

Statistical analyses

Statistical analyses were performed using SPSS12 software (Brosius, 2004). For all tests, the significance level was 0.05. Normal distribution was ensured using the Kolmogorov–Smirnov test. The mean values were compared using an analysis of variance (ANOVA) test including a *post hoc* test (Tamhane).

Electrolyte leakage measurement

At 1 and 4 days after *Agrobacterium* infiltration, two leaf discs ($d = 9$ mm) per infiltrated area were harvested and incubated for 15 h in 6 mL of deionized water in a 15-mL plastic tube (Greiner BIO-one GmbH, Frickenhausen, Germany) by head-over-head inversion. The conductivity was measured using a conductometer 703 (Knick, Berlin, Germany). Samples were boiled for 5 min, cooled to 22 °C, and the total conductivity was determined. The relative conductivity reflecting the ion leakage from plant tissue into water was calculated as follows: sample conductivity/total conductivity × 100%. Experiments were repeated three times with five independent samples for each strain.

Immunoblot analyses

Protein samples were separated by 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and blotted on to a nitrocellulose membrane. Western blots were reacted with polyclonal anti-GFP antibody (Invitrogen GmbH) and anti-rabbit secondary antibody (Sigma-Aldrich, Hamburg, Germany), followed by enhanced chemiluminescence.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Descriptive statistics for size of nuclei for several treatments.

Table S2 ANOVA test.

Table S3 Tamhane test as *post hoc* test.

Table S4 Descriptive statistics for size of nucleoli for several treatments.

Table S5 ANOVA test.

Table S6 Tamhane test as *post hoc* test.

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