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Recognition of the Bacterial Avirulence Protein AvrBs3 Occurs inside the Host Plant Cell

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Summary

The molecular mechanism by which bacterial avirulence genes mediate recognition by resistant host plants has been enigmatic for more than a decade. In this paper we provide evidence that the Xanthomonas campestris pv. vesicatoria avirulence protein AvrBs3 is recognized inside the plant cell. Transient expression of *avrBs3* in pepper leaves, using Agrobacterium tumefaciens for gene delivery, results in hypersensitive cell death, specifically on plants carrying the resistance gene *Bs3*. In addition, for its intracellular recognition, AvrBs3 requires nuclear localization signals that are present in the C-terminal region of the protein. We propose that AvrBs3 is translocated into plant cells via the Xanthomonas Hrp type III secretion system and that nuclear factors are involved in AvrBs3 perception.

Introduction

When a plant is attacked by a pathogen, it can in most cases fend off the infection by mounting a battery of defense responses (Lindsay et al., 1993). Activation of plant defense occurs upon pathogen recognition and results in the halt of pathogen ingress. In many interactions between plants and biotrophic pathogens, recognition involves pairs of complementary genes in both organisms: resistance genes in the plant and avirulence genes (*avr* genes) in the pathogen (Staskawicz et al., 1995). Such host–pathogen recognition does not occur in the absence of either the resistance gene or the corresponding *avr* gene. The term "avirulence" is commonly used in plant pathology to genetically define the inability of a pathogen to cause disease on a resistant host plant. A pathogen carrying a given avirulence gene is not impaired in its pathogenicity as it still causes disease on a host plant that lacks the corresponding resistance gene. The phenomenon of recognition is thought to have arisen during evolution as the host plant acquired the ability to specifically detect molecules from the pathogen. Obviously, it is not the primary function of *avr* genes to activate plant defense responses. Their "real" function for the pathogen, however, is unknown in most cases, although several *avr* genes have been found to be important for pathogen fitness and/or symptom formation (for review, see Dangl, 1994).

The mode of action by which bacterial avirulence genes confer host specificity has remained enigmatic since the isolation of the first *avr* gene (Staskawicz et al., 1984). At least 30 *avr* genes have been characterized genetically among plant-pathogenic gram-negative bacteria (Dangl, 1994). In almost all cases *avr* genes encode hydrophilic proteins with no obvious transmembrane domain or cleavable signal sequence. So far, no Avr protein has been found to be secreted by the bacterium. Only for the *avrD* gene of Pseudomonas syringae pathovar (pv.) tomato has a mode of action been described: *avrD* mediates the production of syringolide (Keen et al., 1990), a specific elicitor that is recognized by soybean lines carrying the resistance gene *Rpg4* (Keen and Buzzell, 1991).

We study the interaction between pepper plants and the bacterial pathogen Xanthomonas campestris pv. vesicatoria. A single gene in the pathogen, *avrBs3* (Bonas et al., 1989), mediates specific resistance of pepper genotype *Bs3*, which leads to the cessation of bacterial growth as a result of plant defense activation. The induced hypersensitive reaction (HR; Klement, 1982) is part of the resistance response and is characterized by a programmed cell death (Mittler and Lam, 1996) that develops into a clearly visible necrosis after inoculation of the leaf with a high density bacterial suspension. *avrBs3* is especially intriguing as genetic data have pointed to a direct recognition of the Avr protein by resistant pepper plants (Herbers et al., 1992; Bonas et al., 1993). The internal region of the 122 kDa AvrBs3 protein is composed of 17.5 nearly identical repeats of 34 amino acids, which determine avirulence gene–specificity. Precise deletion of a certain number of repeats results in changes in specificity of the *avr* gene when tested on different pepper and tomato genotypes. The most striking example is a four-repeat deletion (Δrep-16) that is no longer recognized by pepper genotype *Bs3* but is now recognized instead by pepper genotype *bs3* (Herbers et al., 1992).

The fact that changes in the repeat region of *avrBs3* can give rise to different specificities strongly suggests that the AvrBs3 protein is the actual elicitor molecule that is being recognized by the plant, rather than, for example, functioning in the bacterium as an enzyme required for the production of elicitor molecules. A first indication that members of the Xanthomonas AvrBs3 family of avirulence proteins might be recognized inside the plant cell was provided by Yang and Gabriel (1995), who described the finding of functional nuclear localization signals (NLSs) in the AvrBs3 homologous protein PthA. However, biochemical fractionation studies (Knoop et al., 1991) and immunoelectronmicroscopy (Brown et al., 1993) have shown that AvrBs3 localizes to the bacterial cytoplasm and is not secreted into the leaf intercellular space; the protein is generally hydrophilic and does not carry an obvious signal peptide sequence.

In this paper, we show that transient expression of *avrBs3* within cells of pepper plants carrying the corresponding resistance gene *Bs3* induces hypersensitive cell death. AvrBs3 was found to require functional NLSs for eliciting the hypersensitive reaction, suggesting that

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nuclear factors are involved in AvrBs3 perception by the plant. Based on these findings and recent advances made in the field of bacterial type III secretion, we propose that AvrBs3 is translocated from the bacterium directly into the plant cell.

Results

Agrobacterium-Mediated Transient Expression

Genetic data (Herbers et al., 1992; Bonas et al., 1993) strongly suggest that the AvrBs3 protein is the molecule that mediates the recognition of Xanthomonas by pepper plants carrying the resistance gene *Bs3*. However, there is no evidence for AvrBs3 secretion by Xanthomonas, nor does the AvrBs3 protein induce the HR when infiltrated into the intercellular space of pepper leaves (G. V.d. A. and U. B., unpublished data). We therefore tested the hypothesis that AvrBs3-protein recognition occurs inside the plant cell by expressing the *avrBs3* gene in pepper. As pepper is highly recalcitrant to regeneration following transformation, we developed a transient expression assay using A. tumefaciens (Rossi et al., 1993). A. tumefaciens is a plant pathogenic bacterium that delivers a well-defined piece of DNA, the transferred DNA (T-DNA), into the plant cell (for review, see Zupan and Zambryski, 1995). Transient expression in leaf cells of intact pepper plants was developed and optimized using the intron-containing reporter-gene *uidA* (encoding β-glucuronidase, GUS) present on the T-DNA of plasmid p35S GUS INT (Vancanneyt et al., 1990). This construct allowed testing for efficient transfer and expression of the gene in the plant cell, as the intron does not allow the synthesis of GUS in A. tumefaciens. Conditions as outlined in Experimental Procedures resulted in reproducible, transient GUS expression (Figure 1A).

Hypersensitive Cell Death Induced by *avrBs3* Expression in the Plant

To obtain efficient expression of *avrBs3* in the plant but not in A. tumefaciens, we used the 35S* promoter from pKEx4tr (Mindrinos et al., 1994) in our constructs. This promoter contains a bacterial transcription terminator, resulting in very low expression in bacteria. Using A. tumefaciens for gene delivery, transient expression of *avrBs3* in pepper leaves led to hypersensitive cell death on the resistant pepper genotype *Bs3* but not on the susceptible genotype *bs3* (Table 1). The cell death induced by transient expression of *avrBs3* in genotype *Bs3* (Figure 1B, [3], and 1C, [1]) phenotypically resembled the HR induced by Xanthomonas expressing *avrBs3* (Figure 1B, [1]). In the absence of the *avrBs3* gene the HR is not induced (Figure 1B, [2] and [4]). Inoculated leaf areas of pepper genotype *bs3* showed only slight yellowing (Figure 1C, [1]), a nonspecific response induced by A. tumefaciens (Figure 1C, [3]). Transient expression of the *avrBs3* deletion derivative Δrep-16 (Herbers et al., 1992) in pepper resulted in the expected change in specificity, i.e., strong HR induction on genotype *bs3* but not on genotype *Bs3* (Figure 1C, [4], Table 1). A slight necrosis appeared on genotype

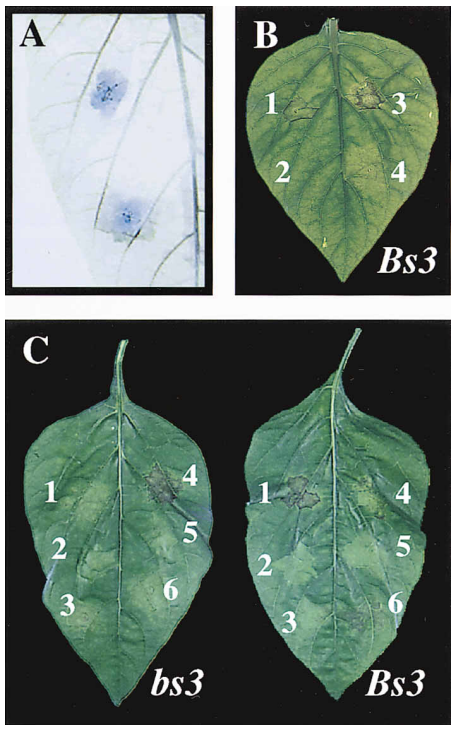

Figure 1. A. tumefaciens–Mediated Transient Expression in Pepper Leaves

A. tumefaciens transconjugants carrying different constructs were grown under *vir* gene–inducing conditions and inoculated into the intercellular space of pepper leaves. Symptoms were photographed 40 hr (for [A] and [C]) or 32 hr (for [B]) after plants were shifted from 20°C to 28°C (see Experimental Procedures).

(A) GUS activity at two inoculation sites as a result of A. tumefaciens–mediated expression of the *uidA*-intron gene by strain C58C1 (pGV2260, p35S GUS INT).

(B) Early HR of pepper genotype *Bs3* to Xanthomonas strain 85E (pDS300F) expressing *avrBs3* ([1], inoculated when plants were shifted from 20°C to 28°C) and A. tumefaciens strain C58C1 (pGV2260, pVS300F) containing *avrBs3* on its T-DNA (3). In the absence of *avrBs3*, Xanthomonas (2) and Agrobacterium (4) do not induce the HR.

(C) Response of pepper genotypes *bs3* and *Bs3* to A. tumefaciens strain C58C1 carrying the following plasmids: (1), pGV2260+pVS300F (*vir*$^+$, T-DNA::p35S*-*avrBs3*); (2), pVS300F (*vir*$^-$, T-DNA::p35S*-*avrBs3*); (3), pGV2260 (*vir*$^+$); (4), pGV2260+pVS316F (*vir*$^+$, T-DNA::p35S*-Δrep-16); (5), pGV2260+pDS300F (*vir*$^+$, plac-*avrBs3*); and (6), pGV2260+pVS3Δ1-3 (*vir*$^+$, T-DNA::p35S*-*avrBs3*Δ1-3). Further details are given in the text.

Bs3 (Figure 1C, [4]), which is not observed when Δrep-16 is expressed in Xanthomonas and could be due to overexpression from the strong 35S* promoter in the plant.

To rule out the possibility that HR induction was due to some residual *avrBs3* expression in A. tumefaciens, two control experiments were conducted (outlined in Table 1). First, the plasmid pVS300F containing the *avrBs3* gene was introduced into A. tumefaciens strain C58C1, which is cured of the *vir* gene–containing Ti-plasmid. In the absence of *vir* genes, A. tumefaciens is unable to transfer the *avrBs3*-containing T-DNA to the plant. Strain C58C1 (pVS300F) indeed failed to elicit the HR on pepper genotype *Bs3* (Figure 1C, [2]). As a second control, the expression plasmid pDS300F, lacking T-DNA border sequences and therefore not transferable

Table 1. A tumefaciens-Mediated Transient Expression of *avrBs3* in Pepper Genotype *Bs3* Induces the HR

Plasmids[a]	*vir* Genes	T-DNA	AvrBs3 in A. tumefaciens[b]	HR on Genotype[c] *bs3*	*Bs3*
None	−	−	−	−	−
pGV2260	+	−	−	−	−
pVS300F	−	+	−	−	−
pGV2260+pVS300F	+	+	−	−	+
pGV2260+pVS316F	+	+	−	+	−
pGV2260+pDS300F	+	−	+	−	−
pGV2260+pVS3Δ1-3	+	+	−	−	−[d]
pGV2260+pVS3Δ1-3::SV	+	+	−	−	+

HR induction on pepper genotypes *bs3* and *Bs3* following inoculation with A. tumefaciens strain C58C1 containing different plasmids. The presence or absence of functional *vir* genes on the Ti-plasmid, of the avirulence gene on the T-DNA, and of the AvrBs3 protein in A. tumefaciens is indicated.
[a]pGV2260, Ti-plasmid carrying *vir* genes; pVS300F, binary vector carrying p35S*-*avrBs3*; pVS316F, binary vector carrying p35S*-*avrBs3*Δrep-16; pDS300F, plasmid carrying plac-*avrBs3*; pVS3Δ1-3 and pVS3Δ1-3::SV, binary vectors carrying p35S*-*avrBs3*Δ1-3 and Δ1-3::SV, respectively.
[b]The presence of AvrBs3 in bacterial extracts was analyzed by Western blotting using the FLAG–M2 antibody (− not detectable; + abundant).
[c]Symptoms were scored 28 hr after shifting inoculated plants from 20°C to 28°C. Identical results were obtained in four independent experiments.
[d]HR becomes visible 20 hr later.

into the plant cell, was introduced into strain C58C1 (pGV2260). In A. tumefaciens, *avrBs3* is highly expressed from the lac promoter in pDS300F and not from the 35S* promoter in pVS300F, as shown by detection of the epitope-tagged AvrBs3 protein in bacterial extracts (Figure 2). Although A. tumefaciens C58C1 (pGV2260, pDS300F) expresses a high amount of AvrBs3 protein, the HR is not induced on genotype *Bs3* (Figure 1C, [5]). Hence, HR induction by A. tumefaciens–mediated transient expression only occurs if the *avr* gene can be transferred to and expressed in the plant cell.

AvrBs3 Carries Functional Nuclear Localization Signals

Comparison of short fragments of the AvrBs3 amino acid sequence with the Swiss/Prot protein database using BLAST (Altschul et al., 1990) revealed similarities

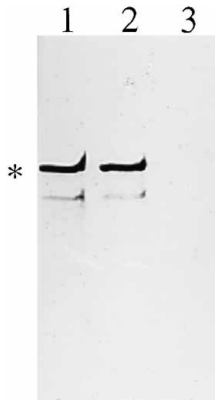

Figure 2. Expression of *avrBs3* in A. tumefaciens

Total protein extracts of A. tumefaciens C58C1 (pGV2260) transconjugants carrying plasmids pDS300F (plac-*avrBs3*, lane 2) and pVS300F (p35S*-*avrBs3*, lane 3) were separated on a 7% SDS–PAGE gel and analyzed by Western blotting using the FLAG–M2 monoclonal antibody. Lane 1 shows protein extract of Xanthomonas (pDS300F). The full-size AvrBs3 protein is indicated by the asterisk.

between part of the AvrBs3 C-terminus (amino acids 1001–1100) and a region of the A. tumefaciens VirD2 protein (amino acids 378–441) that carries a functional nuclear localization signal (Howard et al., 1992). Further inspection of the AvrBs3 sequence showed that the C-terminal region contains 3 sequences that fit the consensus for monopartite NLSs (Figure 3, boxed sequences; for review, see Garcia-Bustos et al., 1991). In addition, each of these signals is flanked by a putative casein kinase II phosphorylation site (Figure 3, underlined), a feature commonly associated with NLSs (Rihs et al., 1991).

To test whether the putative NLSs in the C-terminal region of AvrBs3 are able to target a reporter protein to the nucleus, AvrBs3-GUS fusions were constructed. The C-terminal region (amino acids 975–1164) of the *avrBs3* gene was translationally fused to the *uidA* gene in plant expression plasmid pKEx4tr-G (Mindrinos et al., 1994). The construct was introduced by particle bombardment into onion epidermal tissue, which is easily obtained and consists of a single layer of translucent cells. Transient expression of the reporter-gene fusion showed that GUS activity was localized in the nucleus of all transformed onion cells inspected (Figures 4B and 4C). In contrast, transient expression of the nonmodified *uidA* gene by bombardment with plasmid pKEx4tr-G resulted in cytoplasmic localization of GUS activity, as expected (Figure 4A). These results showed that the C-terminal region of AvrBs3 carries functional NLSs. Similar findings have recently been reported by Yang and Gabriel (1995) for the *pthA* gene and other members of the Xanthomonas *avrBs3* family.

AvrBs3 Requires NLSs for Induction of the HR

The importance of the AvrBs3 C-terminal NLSs for the induction of genotype-specific HR on pepper was tested by site-directed mutagenesis. As the putative NLSs in AvrBs3 resemble monopartite NLSs, we mutated the conserved lysine residue in position 1 of the sequence K-R-X-K/R to threonine in each putative NLS individually

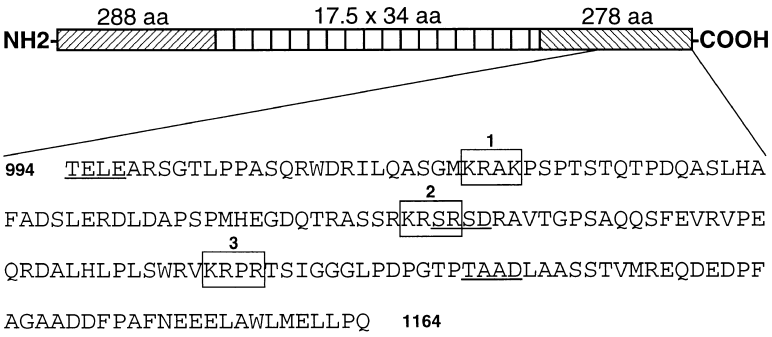

Figure 3. The AvrBs3 C-Terminal Region Contains Three Putative Nuclear Localization Signals

The AvrBs3 protein (1164 amino acids, 122 kDa) can be divided into three major subdomains: the N-terminal domain of 288 amino acids, the internal domain consisting of 17.5 nearly identical repeats of 34 amino acids, and the C-terminal domain of 278 amino acids. The C-terminal region (amino acids 994–1164 are indicated) contains three putative nuclear localization sequences (NLS) and casein kinase II phosphorylation sites (CKII). The boxed and underlined amino acid sequences fit the consensus for monopartite NLSs (K-K/R-X-K/R) and CKII sites (S/T-X-X-D/E), respectively.

(Figure 5, constructs M1, M2, and M3), thereby creating a MluI restriction site. In addition, the residue in position 3 of the consensus sequence was changed to alanine in M2 and M3. To test for HR-inducing activity, the mutated genes were cloned behind the triple lacUV5 promoter in plasmid pDSK602 and introduced into Xanthomonas strain 85E, which is virulent on pepper genotypes *bs3* and *Bs3*. As shown in Table 2, individual mutations in the putative NLSs did not affect AvrBs3 activity.

Combinations of mutations were generated as indicated in Figure 5 (see Experimental Procedures). Mutation of NLS1 together with that of NLS2 or NLS3 (M12 and M13) did not affect AvrBs3 activity (Table 2). In contrast, mutation of both NLS2 and NLS3 (M23) resulted in reduced AvrBs3 activity, i.e., the HR on pepper genotype *Bs3* appeared one to two days later and was reduced in intensity. AvrBs3 activity was further diminished by mutation of all three putative NLSs (M123).

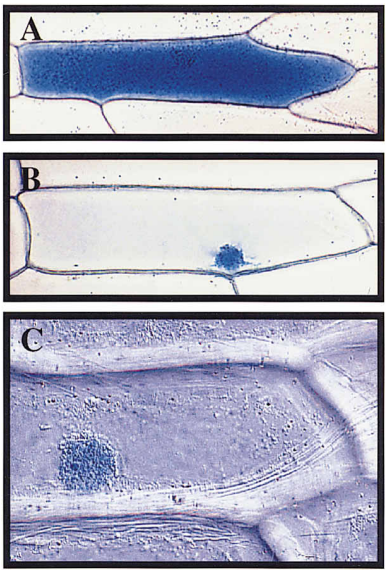

Figure 4. The C-Terminal Region of AvrBs3 Contains NLSs That Function in the Plant

Histochemical localization of GUS activity following bombardment of onion epidermal cell layers with DNA constructs expressing either GUS (A) or a fusion of the AvrBs3 C-terminal region (amino acids 975–1164) to GUS (B and C) is shown. GUS activity was revealed 24 hr after bombardment. For (C), Nomarski optics were used.

These data suggest that the presence of either an intact NLS2 or NLS3 is required for full AvrBs3 activity. Furthermore, the importance of the putative NLS1 appears to be minor, as mutation of NLS1 only showed a slight additional reduction of AvrBs3 activity when combined with mutations in NLS2 and NLS3.

In addition, in-frame deletions were created in the AvrBs3 C-terminal domain by making use of the unique MluI sites in the mutated NLSs (see Figure 5). Deletion of the region between mutated NLS1 and NLS2 (46 amino acids, Δ1-2) did not affect AvrBs3 activity, whereas deletion of the region between NLS2 and NLS3 (37 amino acids, Δ2-3) as well as that between NLS1 and NLS3 (83 amino acids, Δ1-3) resulted in complete loss of AvrBs3 activity (Table 2).

Finally, we replaced the NLS region of AvrBs3 by the simian virus 40 (SV40) large T-antigen NLS (Kalderon et al., 1984). A synthetic adapter was designed that allows the in-frame insertion of the SV40 NLS (amino acid sequence PKKKRKVS) into the MluI site present in each mutated NLS (Figure 5). Insertion of the SV40 adapter into Δ1-2, which was fully active, showed that the introduced sequence does not have a negative effect on AvrBs3 activity (Table 2). Strikingly, insertion of the SV40 adapter into Δ2-3 and Δ1-3, which were both inactive, resulted in gain of AvrBs3 activity (Table 2). Introduction of the adapter in reverse orientation (amino acid sequence PRLLLALD, Figure 5) did not restore activity (Table 2), indicating that the NLS sequence restores AvrBs3 activity and not the mere introduction of eight amino acids. It should be noted that disease symptoms caused by Xanthomonas 85E on the susceptible pepper genotype *bs3* were not altered by expression of these *avrBs3* constructs.

avrBs3 construct Δ1-3, from which the NLS region is deleted, was also transiently expressed in pepper, using A. tumefaciens as described above. A delayed and reduced HR on genotype *Bs3* was visible one to two days later than that obtained by transient expression of *avrBs3* (Table 1; Figure 1C, [6]). The weak HR-inducing activity might be due to overexpression from the strong 35S* promoter, as it is not observed when Δ1-3 is expressed in Xanthomonas and tested on pepper. Transient expression of Δ1-3::SV, in which the deleted NLS-region is replaced by the eight–amino acid NLS from SV40, resulted in appearance of the HR at the same time as with *avrBs3* (Table 1), confirming that a foreign NLS restores AvrBs3 activity.

Table 2. Nuclear Localization Signals in the AvrBs3 C-Terminal Region Are Required for HR Induction on Pepper Genotype *Bs3*

avrBs3 Construct	Intact NLS	HR on Pepper Genotype *Bs3*[a]	Localization of GUS Activity[b]
WT	1, 2, 3	+++	N
M1	2, 3	+++	nd
M2	1, 3	+++	nd
M3	1, 2	+++	nd
M12	3	+++	nd
M13	2	+++	N
M23	1	+	nd
M123	−	+/−	C>N
Δ1-2	3	+++	N>C
Δ2-3	1	−	C
Δ1-3	−	−	C
Δ1-3::SV	c	++	N
Δ1-3::VS	−	−	nd
Δ1-2::SV	3[c]	+++	nd
Δ2-3::SV	1[c]	++	nd

avrBs3 constructs, mutated in the NLS region, were introduced into Xanthomonas strain 85E and tested for HR induction on pepper genotype *Bs3*. The C-terminal domains of a selected number of constructs were amplified by PCR, fused to the reporter gene *uidA* in pKEx4tr-G, and transiently expressed in onion epidermal cells. Localization of GUS activity was determined by histochemical analysis.

[a]+++, strong HR, starting 24 hr after inoculation; ++, slightly reduced HR, visible at 48 hr after inoculation; +, reduced HR, visible at 72 hr after inoculation; +/−, weak necrosis, visible at 72 hr after inoculation; −, no HR (disease).

[b]For each construct, at least 50 transformed onion cells were scored for localization of GUS activity. N, GUS activity in nucleus; C, GUS activity in cytoplasm; nd, not done.

[c]Contains the SV40 NLS.

Nuclear Localization and AvrBs3 Activity Are Correlated

To investigate whether NLS mutations in AvrBs3 affect nuclear localization activity, a number of appropriate fusions between the AvrBs3 C-terminal region (from M975) and GUS were constructed as described above and tested by transient expression in onion epidermal cells. Constructs with mutations that did not affect AvrBs3 activity (M13, Δ1-2, and Δ1-3::SV) still localized GUS to the nucleus (Table 2). In contrast, mutations that resulted in loss or strong reduction of AvrBs3 activity (M123, Δ2-3, and Δ1-3) were also impaired in their ability to target GUS to the nucleus. Hence, there is a positive correlation between nuclear localization activity and HR-inducing activity of AvrBs3.

To rule out the possibility that loss of activity was due to protein instability, Xanthomonas transconjugants expressing *avrBs3* constructs with different NLS mutations were analyzed by Western blotting. As shown in Figure 6, the antibody, specific for the epitope introduced at the C-terminus of AvrBs3, detected proteins of the expected size in all bacterial extracts. The signal strength obtained for the different constructs was identical to that obtained for the wild-type AvrBs3 protein.

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           NLS1                                          NLS2                                          NLS3
WT         MKRAKPSPTSTQTPDQASLHAFADSLERDLDAPSPMHEGDQTRASSRKRSRSDRAVTGPSAQQSFEVRVPEQRDALHLPLSWRVKRPRTSI
            *                                             *                                    *

M1         .T.........................................................................................
M2         ...............................................T.A.........................................
M3         ....................................................................................T.A....

M12        .T.............................................T.A.........................................
M13        .T..................................................................................T.A....
M23        ...............................................T.A.....................................T.A....
M123       .T.............................................T.A.....................................T.A....

Δ1-2       .T----------------46 aa deletion----------------.A.........................................
Δ2-3       ...............................................T----------37 aa deletion-------------.A....
Δ1-3       .T----------------------------------------83 aa deletion-----------------------------.A....

Δ1-3::SV   .T----------------------83 aa replaced by RPKKKRKVS (SV40 NLS)-----------------------.A....
Δ1-3::VS   .T----------------------83 aa replaced by RDLALLLRP (SV40 INV)-----------------------.A....
Δ1-2::SV   .T----------46 aa replaced by SV40 NLS----------.A.........................................
Δ2-3::SV   ...............................................T-------37 aa replaced by SV40--------.A....
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Figure 5. Mutations in the AvrBs3 C-Terminal Region

Site-directed mutations were created by PCR and combined by exchange of restriction fragments. The nomenclature indicated on the left is used throughout the paper (WT = sequence of the wild-type AvrBs3 protein from amino acids 1020 to 1110). Unaltered amino acids are indicated by the dots; substitutions, by standard one-letter code; and deletions, by the dashes. The conserved lysine residue in position 1 of each putative NLS (indicated by the asterisk) was substituted for threonine. In NLS2 and NLS3, an additional amino acid was exchanged, resulting in an alanine residue in position 3 of each NLS. For details, see Experimental Procedures.

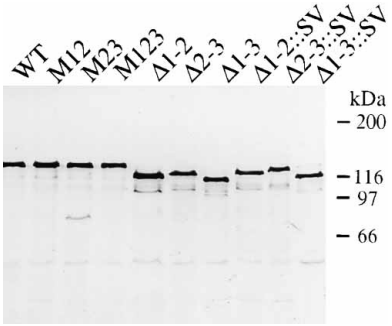

Figure 6. NLS Mutations Do Not Affect AvrBs3 Stability in Xanthomonas

Total protein extracts of Xanthomonas transconjugants expressing different *avrBs3* constructs from the triple lacUV5 promoter of pDSK602 (wild-type, WT and NLS mutations using the nomenclature as for Figure 5) were separated on a 7% SDS–PAGE gel and analyzed by Western blotting. The FLAG–M2 monoclonal antibody reacts only with the full-size AvrBs3 protein carrying the C-terminal FLAG-epitope.

The NLS mutations and deletions therefore do not affect AvrBs3 protein stability in the bacterium.

Discussion

AvrBs3 Acts inside the Plant Cell to Elicit Hypersensitive Cell Death

Expression of *avrBs3* in pepper cells, using A. tumefaciens for gene delivery, resulted in induction of hypersensitive cell death specifically on genotype *Bs3*. The response resembled phenotypically the HR induced by the pathogen Xanthomonas campestris pv. vesicatoria expressing *avrBs3*. Transient expression of *avrBs3* deletion–derivative Δrep-16 in pepper led to the expected change in specificity: HR on genotype *bs3*. These results clearly demonstrate that the two avirulence proteins (AvrBs3 and Δrep-16) act inside the plant cell. These novel data add a key finding to the understanding of the so far elusive mechanism by which bacterial pathogens are recognized by the resistant host plant.

Recently, Gopalan et al. (1996) have shown that expression of the P. syringae *avrB* gene in transgenic Arabidopsis thaliana plants containing the resistance gene *RPM1* elicits hypersensitive cell death. However, in this case, HR induction was obtained when AvrB was expressed with a signal peptide. It is therefore not entirely clear whether recognition occurs within the plant cell in which *avrB* is expressed, or whether the protein needs to be exported. Nevertheless, these data show the direct role of a bacterial avirulence protein in triggering host cell death.

AvrBs3 Nuclear Localization Signals

The AvrBs3 C-terminal region was shown to be able to direct the cytoplasmic reporter-protein GUS to the plant cell nucleus, thereby confirming the results obtained by Yang and Gabriel (1995) for the *avrBs3* homologous gene *pthA*, which contains an identical C-terminal sequence. Here we describe the site-directed mutagenesis of three putative monopartite NLSs, present in the AvrBs3 C-terminus. The presence of NLS2 or NLS3 appeared to be important for full AvrBs3 activity, i.e., specific induction of HR on pepper carrying the resistance gene Bs3, and for nuclear localization activity. We do not rule out, however, that NLS2 and NLS3 function as one bipartite NLS, although the spacing between the two basic regions (33 amino acids) is much larger than the consensus distance of 10 amino acids proposed for bipartite NLSs (Dingwall and Laskey, 1991). Furthermore, the activity of a deletion derivative lacking the NLS region could be restored by the introduction of the NLS from SV40 T-antigen, which was previously shown to be functional in plants (Van der Krol and Chua, 1991). This result is particularly informative, as it indicates that the NLS region in AvrBs3 does not have an essential function other than nuclear targeting.

The requirement of NLSs for AvrBs3 recognition suggests that the protein is imported into the nucleus. However, AvrBs3 could not be detected in nuclei isolated from Xanthomonas-infected pepper leaves of both genotype *bs3* and *Bs3*, as examined by immunofluorescence microscopy (G. V. d. A., unpublished data). This negative result could be due to a low amount of AvrBs3 protein in the nuclei (below the detection limit) or masking of the AvrBs3 epitopes that are recognized by the polyclonal antibody available.

To date, only a few bacterial proteins that carry NLSs have been shown to be targeted to the nucleus of the eukaryotic host cell. In A. tumefaciens, the VirD2 (Howard et al., 1992) and VirE2 (Citovsky et al., 1992) proteins are involved in the processing and transport of the T-DNA from the bacterium into the plant cell nucleus where T-DNA integration takes place (for review, see Zupan and Zambryski, 1995). The transport of the NLS-containing VirD2 and VirE2 proteins into the plant occurs in the form of a complex with the T-DNA. The IgA1 protease–associated α-protein of the mammalian pathogen Neisseria was found to enter human cells via an endocytic route (Pohlner et al., 1995). Inside the host cell, NLSs direct the α-protein to the nucleus, where it is suggested to have a regulatory function.

Hrp Type III Secretion Is Required for AvrBs3 Activity in Xanthomonas

The fact that expression of *avrBs3* in the plant is sufficient to induce the HR indicates that other bacterial genes are not required for avirulence protein recognition. However, the Xanthomonas *hrp* (hypersensitive reaction and pathogenicity) genes (Bonas et al., 1991) are indispensable for the recognition of the bacterium by pepper genotype *Bs3* (Knoop et al., 1991). The discovery of homology between a number of Xanthomonas Hrp proteins and proteins from bacterial pathogens of mammals involved in type III secretion (Fenselau et al., 1992) suggested that Hrp proteins might constitute a specialized transport system. Intriguingly, Yersinia, a pathogenic bacterium of mammals, has recently been shown to translocate proteins into the host cell via the type III secretion system (Rosqvist et al., 1994; Sory and Cornelis, 1994; Persson et al., 1995; Hakansson et al., 1996).

In plant pathogenic bacteria, Hrp-dependent secretion of elicitor proteins has been demonstrated for Erwinia amylovora (Wei et al., 1992), Pseudomonas syringae (He et al., 1993), and Ralstonia solanacearum (Arlat

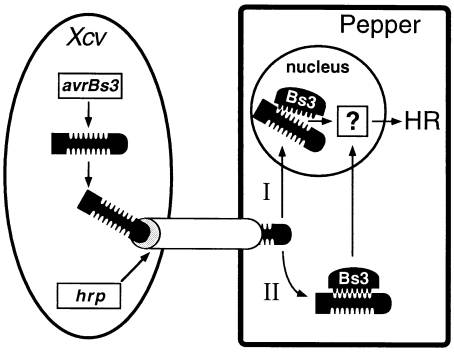

Figure 7. Model for the Polarized Transport of the Xanthomonas campestris pv. vesicatoria (*Xcv*) AvrBs3 Protein and Its Recognition in Pepper Cells Containing the Resistance Gene *Bs3*

See text for further details.

et al., 1994). However, AvrBs3 is not secreted by Xanthomonas, nor does the AvrBs3 protein induce the HR when infiltrated into the intercellular space of pepper leaves (G. V. d. A. and U. B., unpublished data). We therefore propose that AvrBs3 is translocated directly into the host plant cell via the Hrp type III secretion system (as depicted in Figure 7). Other bacterial Avr proteins are likely transported to the plant in a similar way, as most of them depend on the Hrp type III secretion system for recognition (Dangl, 1994). However, the bacterium must overcome the plant cell wall to deliver such proteins into the host cytoplasm. It is conceivable that contact to the host cell occurs by means of a pilus-like structure that is sufficiently thin to pass between the cell wall polymers, possibly with the help of bacterial hydrolytic enzymes. This idea is reinforced by the fact that pili are required for bacterial conjugation and Ti-mediated T-DNA transfer (Lessl and Lanka, 1994; Fullner et al., 1996). There is no evidence for DNA transfer from Xanthomonas to the plant cell, as sequences flanking the *avrBs3* coding region are dispensable for HR induction.

Model for Avirulence Protein AvrBs3 Recognition

Following the Hrp-dependent translocation of AvrBs3 into the host plant cell (as discussed above), we believe that AvrBs3 interacts with the product of the resistance gene *Bs3* (Figure 7). The interaction is thought to be mediated by the internal region of AvrBs3, containing 17.5 nearly identical repeats of 34 amino acids, which confer the resistance gene specificity (Herbers et al., 1992; Bonas et al., 1993). We envisage two alternative routes leading to induction of hypersensitive cell death. First (Figure 7, I), the AvrBs3 protein is targeted to the nucleus where it interacts with the Bs3 protein, thereby initiating a signal-transduction cascade which leads to hypersensitive cell death. In the absence of functional NLSs, the AvrBs3 protein is not targeted to the nucleus and is therefore not recognized. The second possibility (Figure 7, II) is that the AvrBs3–Bs3 interaction occurs in the cytoplasm. The Bs3 protein is then transported "piggyback" with AvrBs3 into the nucleus. This complex or yet another interacting component then initiates signal transduction. In the absence of functional NLSs, the interaction with the Bs3 protein can still take place, but the complex is no longer targeted to the nucleus and therefore does not lead to HR induction. There are several examples of piggyback transport of cytoplasmic proteins by NLS-containing proteins; for example, the hsp90 protein from chicken was found to be transported to the nucleus via its interaction with the rabbit progesterone receptor that is essentially nuclear when expressed in COS cells (Kang et al., 1994). Also, the simian virus 40 structural proteins Vp1 and Vp2/3 were shown to interact in the cytoplasm and to be able to transport each other piggyback to the nucleus (Ishii et al., 1994).

We have shown here that a Xanthomonas avirulence protein acts within the host plant cell to elicit hypersensitive cell death. The requirement of NLSs for induction of the HR suggests that recognition and/or signaling occurs in the nucleus. However, none of the products of plant resistance genes cloned so far is predicted to be localized in the nucleus (for review, see Staskawicz et al., 1995). Isolation of the pepper resistance gene *Bs3*, which is in progress, will enable us to determine whether the Bs3 and AvrBs3 proteins interact, and if so, where this interaction occurs.

It is conceivable that not only avirulence proteins but also pathogenicity factors are translocated directly from the bacterium into the plant cell. Such proteins could interfere with signal-transduction pathways, like the Yersinia Ser/Thr kinase YpkA (Hakansson et al., 1996) and the Tyr phosphatase YopH (Persson et al., 1995), and/or modulate metabolic processes in the plant cell to create a favorable niche for bacterial multiplication. Protein translocation into the eukaryotic cell is emerging as a widespread mechanism by which bacterial pathogens conquer the host. Having acquired the ability to recognize certain translocated bacterial proteins, plants are able to counteract and resist pathogen infection.

Experimental Procedures

Bacterial Strains and Plasmids

The Escherichia coli strain DH5α (Gibco BRL), the A. tumefaciens strains C58C1 (Van Larebeke et al., 1974) and C58C1 (pGV2260) (Deblaere et al., 1985), and the X. c. pv. vesicatoria strain 85E (Wengelnik et al., 1996) have been described. Plasmids used were pBluescript KS (Stratagene), pUC119 (Vieira and Messing, 1987), pDSK602 (Murillo et al., 1994), and pKEx4tr and pKEx4tr-G (Mindrinos et al., 1994). The binary vector pCP60 is a modified pBIN19 plasmid (Bevan, 1984) and was kindly provided by C. Corronado and P. Ratet. pD36 is pDSK602 containing a translational fusion of *avrBs3* from pUXV1922 (Bonas et al., 1989) under control of a triple lacUV5 promoter, which is constitutive in Xanthomonas. Plasmids were introduced into E. coli by electrotransformation and into Xanthomonas and A. tumefaciens by conjugation, using pRK2013 as a helper plasmid in triparental matings (Figurski and Helinski, 1979).

DNA and Protein Methods

Standard methods were used (Sambrook et al., 1989; Ausubel et al., 1996) unless otherwise stated. PCR mutagenesis was performed using the *Pfu* enzyme (Stratagene) according to the manufacturer's instruction. All mutations were verified by DNA sequencing. Bacterial protein extracts were separated on 7% SDS–polyacrylamide gels and blotted to nitrocellulose (Schleicher and Schuell). AvrBs3 was detected using the FLAG–M2 monoclonal antibody (IBI/Kodak), following the manufacturer's instructions, and an alkaline phosphatase-coupled secondary antibody (Promega). Equal loading of protein samples was checked by staining with Coomassie Brilliant Blue G-250 (BioRad).

avrBs3 Activity Assay on Pepper

Pepper (Capsicum annuum) plants of cultivar Early Cal Wonder (ECW, genotype *bs3*) and its near-isogenic line ECW-30R, containing the resistance gene *Bs3* (genotype *Bs3*), were grown as described (Bonas et al., 1989). Leaves were inoculated by hand with bacterial suspensions in water of 5×10^8 cfu/ml using a needleless syringe. Symptoms were scored at 24, 48, 72, and 96 hr after inoculation.

avrBs3 Gene Modifications

The FLAG epitope (IBI/Kodak) was engineered at the C-terminus of *avrBs3* by PCR, resulting in the replacement of the stop codon by a serine codon followed by the FLAG coding sequence (encoding DYKDDDDK), a stop codon, and a HindIII restriction site. For this, a 330 bp BamHI/HindIII fragment containing the coding sequence for the 49 C-terminal amino acids of AvrBs3 was subcloned from pD36 into pBluescript KS. Following PCR amplification using the primers T7 and avrBs3–FLAG (5′-CCCAAGCTTCACTTGTCATCGT CGTCCTTGTAGTCCGACTGAGGCAATAGCTCC-3′) and digestion of the PCR products by BamHI and HindIII (underlined in primer sequence), the 183 bp fragment was cloned into pBluescript KS. The 68 bp SacI/HindIII fragment containing the epitope tag was recloned into pD36, resulting in pD36FLAG.

EcoRI and ClaI restriction sites were introduced directly upstream of the start codon of *avrBs3*, which was optimized for translation initiation according to Kozak (1989). For this, the 83 bp BamHI/PstI fragment encoding amino acids 2–28 of *avrBs3* was cloned into pBluescript KS. Following PCR amplification using the primers T3 and 117 (5′-GGAATTCATCGAT**ATG**GATCCCATTCGTTCG-3′) and digestion of the PCR product by PstI and EcoRI (underlined in primer sequence), the 90 bp fragment was cloned into EcoRI/PstI digested pUC119. The resulting plasmid was digested with PstI and HindIII, and the *avrBs3* coding sequence downstream of the PstI restriction site was added as a PstI/HindIII fragment from pD36FLAG, resulting in plasmid pUS300F. The 3.5 kb EcoRI/HindIII fragment from pUS300F was cloned into pDSK602, resulting in plasmid pDS300F, thereby creating a translational fusion with eight additional amino acids (MKGKEFID) upstream of the methionine residue of AvrBs3. The 3.1 kb PstI/SstI fragment from *avrBs3* deletion-derivative Δrep-16 (Herbers et al., 1992), which lacks 4 repeats, was ligated into PstI/SstI digested pUS300F, resulting in plasmid pUS316F. None of the above-described modifications affected *avr* gene activity.

Constructs for *avrBs3* Expression in Plants

For Agrobacterium-mediated transient expression a new binary vector, pVB60, was constructed by removing the 35S promoter and polylinker from pCP60 by digestion with HindIII and SstI and replacing it with the modified 35S promoter (35S*) and polylinker from pKEx4tr (Mindrinos et al., 1994). The *avrBs3* gene was isolated from pUS300F by digestion with EcoRI and HindIII and cloned into pBluescript KS, to yield pBS300F. The insert was isolated from pBS300F by digestion with EcoRI and XhoI (downstream of HindIII) and cloned into pVB60, resulting in plasmid pVS300F. The *avrBs3* derivatives Δrep-16 and Δ1-3 were cloned from pUS316F and pUS3Δ1-3 into pVB60, resulting in pVS316F and pVS3Δ1-3, respectively. The specificity of the *avr* genes in plant expression plasmids was verified by recloning the EcoRI/HindIII fragments into pDSK602, conjugation into Xanthomonas 85E, and plant inoculation.

Agrobacterium-Mediated Transient Expression

A. tumefaciens strain C58C1 containing the Ti-plasmid pGV2260 (Deblaere et al., 1985) or individual transconjugants thereof were grown in 2 ml YEB medium containing the appropriate antibiotics. Following overnight growth at 28°C, bacteria were pelleted from 400 μl culture by centrifugation and resuspended in 2 ml induction medium according to Bundock et al. (1995) with minor modifications. The medium contained 10.5 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 1.0 g/l $(NH_4)_2SO_4$, 0.5 g/l NaCitrate·$2H_2O$, 1 mM $MgSO_4 \cdot H_20$, 0.2% glucose, 0.5% glycerol, 50 μM acetosyringone (Aldrich), and 10 mM N-morpholino-ethanesulfonic acid (MES) (pH 5.6). After overnight incubation at 28°C, at $OD_{600} = 0.5$–0.6, bacteria were pelleted by centrifugation, washed with Murashige and Skoog's medium (MS, Sigma) with 10 mM MES (pH 5.6), and resuspended to $OD_{600} = 0.5$ in MS–MES medium with 150 μM acetosyringone. Bacterial suspensions were infiltrated into young but fully expanded leaves of 6- to 8-week-old pepper plants using a needleless syringe. After infiltration, plants were immediately covered with transparent plastic bags and placed at 20°C (reported optimal for T-DNA transfer; Fullner and Nester, 1996). After 40–48 hr, plastic bags were removed and plants placed at 28°C, 16 hr light/8 hr dark, and 80% humidity to allow symptoms to develop.

Nuclear Localization Assay

For the construction of a fusion between GUS and the AvrBs3 C-terminus, the *avrBs3* C-terminal region of 570 bp (from aa M975 to Q1164) was amplified from pDS300F and its derivatives carrying NLS mutations using primers FUSGUS-A (5′-CTCTAGAGCCAT GACGCAGTTC-3′) and FUSGUS-B (5′-CAGATCTCTGAGGCAA TAGCTC-3′) as described (Yang and Gabriel, 1995). The PCR products were digested with XbaI and BglII and cloned into XbaI/BamHI–digested pKEx4tr-G (contains the 35S* promoter; Mindrinos et al., 1994), resulting in translational fusion of the AvrBs3 C-terminal region to the N-terminus of GUS.

avrBs3–GUS constructs were transiently expressed in onion epidermal cells by using the BioRad Biolistic PDS-1000/He system essentially as described by Varagona et al. (1992). Briefly, inner epidermal layers obtained from onions (purchased at a local supermarket) were placed on MS basal medium with 30 g/l sucrose, 2% agar, 2.5 μg/ml amphotericin B (Sigma), and 5 μg/ml chloramphenicol. DNA-coated gold particles (1.6 μm gold, BioRad) were briefly sonicated before bombardment. Purified plasmid DNA (0.83 μg) was bombarded onto each sample at a pressure of 1100 lb/in^2 and a target distance of 9 cm. Petri dishes were sealed with parafilm and incubated for 24 hr at 28°C in the dark. GUS activity was determined by histochemical staining at 37°C in X-glcUA solution (50 mM $NaPO_4$ [pH 7.0], 0.5 mM sodium ferro/ferri cyanide, 0.05% Triton X-100, and 0.5 mg/ml 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid, cyclohexylammonium salt, Biosynth AG).

NLS Mutagenesis

Amino acid substitutions in the putative NLSs of *avrBs3* were obtained by PCR and resulted in the creation of MluI restriction sites (underlined in primer sequence). Primers used were:
NLS1A, 5′-GCACGCGTCATCCCTGATGCCTG-3′, NLS1B, 5′-GG ACGCGTGCCAAACCGTCCCC-3′, NLS2A, 5′-GCACGCGTACGGCT GCTTGCCC-3′, NLS2B, 5′-GGACGCGTGCCCGATCGGATCGTG-3′, NLS3A, 5′-GCACGCGTTACCCTCCAACTGAG-3′, and NLS3B, 5′-GGACGCGTGCGCGTACCAGTATCG-3′. The 823 bp HincII/HindIII fragment from pUS300F, containing the *avrBs3* C-terminal region, was cloned into pBluescript KS. Following amplification using primer T3 and one of the primers A, the PCR products were digested with XhoI and MluI. Following amplification using primer T7 and one of the primers B, the PCR products were digested with HindIII and MluI. Digested PCR fragments 1A and 1B, 2A and 2B, and 3A and 3B were cloned in a three-step ligation into XhoI/HindIII–digested pBluescript and recloned into HincII/HindIII–digested pUS300F. The EcoRI/HindIII inserts carrying the NLS mutations were then cloned into pDSK602 and conjugated into Xanthomonas. Combinations of multiple mutated NLSs were created by exchange of domains, making use of an SphI restriction site between NLS1 and NLS2 and an AgeI restriction site between NLS2 and NLS3. Internal in-frame deletions were created by MluI digestion and religation of clones containing two mutated NLSs. The SV40 NLS was introduced into the MluI site of deletion clones by ligation of an adapter composed of the following oligonucleotides: SV40-A, 5′-CGCGGCCGAAGAA GAAGCGCAAGGTCT-3′; and SV40-B, 5′-CGCGAGACCTTGCGCT TCTTCTTCGGC-3′.

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