

The *Xanthomonas* Type III Effector Protein AvrBs3 Modulates Plant Gene Expression and Induces Cell Hypertrophy in the Susceptible Host

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Xanthomonas campestris pv. *vesicatoria* bacteria expressing the type III effector protein AvrBs3 induce a hypersensitive response in pepper plants carrying the resistance gene *Bs3*. Here, we report that infection of susceptible pepper and tomato plants leads to an AvrBs3-dependent hypertrophy of the mesophyll tissue. *Agrobacterium*-mediated transient expression of the *avrBs3* gene in tobacco and potato plants resulted in a similar phenotype. Induction of hypertrophy was shown to depend on the repeat region, nuclear localization signals, and acidic transcription activation domain (AAD) of AvrBs3, suggesting that the effector modulates the host's transcriptome. To search for host genes regulated by AvrBs3 in an AAD-dependent manner, we performed a cDNA-amplified fragment length polymorphism analysis of pepper mRNA populations. Thirteen AvrBs3-induced transcripts were identified and confirmed by reverse transcriptase-polymerase chain reaction. Sequence analysis revealed homologies to auxin-induced and expansinlike genes, which play a role in cell enlargement. These results suggest that some of the AvrBs3-induced genes may be involved in hypertrophy development and that xanthomonads possess type III effectors that steer host gene expression.

Additional keywords: avirulence, bacterial spot disease, *SAUR* (small auxin up RNA), transcription factor, *upa* (upregulated by AvrBs3).

Phytopathogenic bacteria are responsible for a great variety of diseases in plants, causing important agricultural losses. Many gram-negative plant and animal pathogenic bacteria share a common mechanism to attack and exploit their eukaryotic hosts: the type III secretion system, which is required to deliver bacterial proteins into host cells. The delivered proteins, termed type III effectors, are thought to be involved in virulence by targeting specific steps of the host cell metabolism for the benefit of the bacterial invader (Cornelis and van Gijsegem 2000). In plant pathogens, one class of type III effectors are avirulence proteins (Bonas and Van den Ackerveken 1999). The term "avirulence" (*avr*) defines bacterial genes that determine specific recognition of the bacteria by plants possessing a matching resistance (*R*) gene. Plant *R* gene-mediated

recognition of an Avr protein leads to the induction of plant defense reactions that generally include the hypersensitive response (HR), a rapid localized cell death associated with the arrest of pathogen ingress (Morel and Dangl 1997). Thus, Avr proteins restrict the pathogen's host range, an effect that is deleterious to the bacteria and is probably not their primary function. In fact, a growing number of Avr proteins appear to play a role in bacterial virulence (i.e., bacterial growth and symptom formation) in susceptible host plants (Bai et al. 2000; Chen et al. 2000; Kearney and Staskawicz 1990; Kjemtrup et al. 2000; Lorang et al. 1994; Ritter and Dangl 1995; Shan et al. 2000; Tsiamis et al. 2000; Yang et al. 1994, 1996, 2000).

We study the gram-negative bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot disease of pepper and tomato plants. The ability of *X. campestris* pv. *vesicatoria* to cause disease depends on the type III protein secretion system encoded by the *hrp* gene cluster (Bonas et al. 1991; Rossier et al. 1999). A number of avirulence genes have been isolated from *X. campestris* pv. *vesicatoria* (Jones et al. 1998), including *avrBs3* (Bonas et al. 1989). Pepper plants carrying the *Bs3* resistance gene are able to recognize *X. campestris* pv. *vesicatoria* strains expressing *avrBs3* (Bonas et al. 1989; Minsavage et al. 1990). The AvrBs3 protein, which is secreted in a type III-dependent manner (Rossier et al. 1999), is recognized inside the plant cell (Van den Ackerveken et al. 1996), suggesting that it is translocated into the plant cell by the *X. campestris* pv. *vesicatoria* type III secretion system. AvrBs3 is a member of a large family of highly related proteins found in many *Xanthomonas* spp., the AvrBs3 family (Gabriel 1999; Vivian and Arnold 2000). In addition to an avirulence activity, some family members are involved in disease symptom formation (e.g., PthA from *X. citri* [citrus canker; Swarup et al. 1991], Avr6 from *X. campestris* pv. *malvacearum* [increased watersoaking of cotton leaves; Yang et al. 1996] and AvrXa7 from *X. oryzae* [leaf lesion length in rice; Bai et al. 2000]). In the case of AvrBs3 from *X. campestris* pv. *vesicatoria*, an activity in susceptible plants has not been reported.

The most striking feature of members of the AvrBs3 protein family is their central region composed of 12.5 to 25.5 nearly identical tandem repeats of a 34-amino-acid (aa) motif. Domain swapping experiments have shown that the repeat region determines both virulence and avirulence specificities (Yang et al. 2000). The 17.5 repeats of AvrBs3 were found to be essential for recognition by the *Bs3* resistance gene, because AvrBs3 repeat deletion mutants no longer were recognized by *Bs3* but unmasked new resistance genes in other pepper and tomato genotypes (Herbers et al. 1992). The N- and C-terminal protein regions are highly conserved among AvrBs3 family members and are functionally interchangeable (Ballvora et al. 2001; Zhu et al. 1998).

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GenBank accession numbers for *upa1* to *upa11* are AF492625 to AF492635.

The C-terminus of the proteins contains functional nuclear localization signals (NLSs) and an acidic transcription activation domain (AAD) (Van den Ackerveken et al. 1996; Yang and Gabriel 1995b; Zhu et al. 1998). Both types of motifs are required for activity (Szurek et al. 2001; Van den Ackerveken et al. 1996; Yang et al. 2000; Yang and Gabriel 1995b; Zhu et al. 1998), and the AvrBs3 NLSs interact in yeast and in vitro with pepper importin α (Szurek et al. 2001). Studies of AvrXa7, an AvrBs3 family member from *X. oryzae*, have suggested a direct interaction of the protein with AT-rich DNA sequences (Yang et al. 2000).

Here, we describe AvrBs3-induced mesophyll cell hypertrophy in different solanaceous plants. To investigate the role of

AvrBs3 as a modulator of host gene transcription, we studied pepper gene expression using the cDNA-amplified fragment length polymorphism (AFLP) technique. Many of the identified induced genes appear to be related to cell expansion.

RESULTS

AvrBs3 delivered by *X. campestris* pv. *vesicatoria* induces hypertrophy of mesophyll cells in susceptible plants.

Inoculation of virulent *X. campestris* pv. *vesicatoria* strains into leaves of pepper (*Capsicum annuum*) and tomato (*Lycopersicon esculentum*) plants at high inoculum leads to the for-

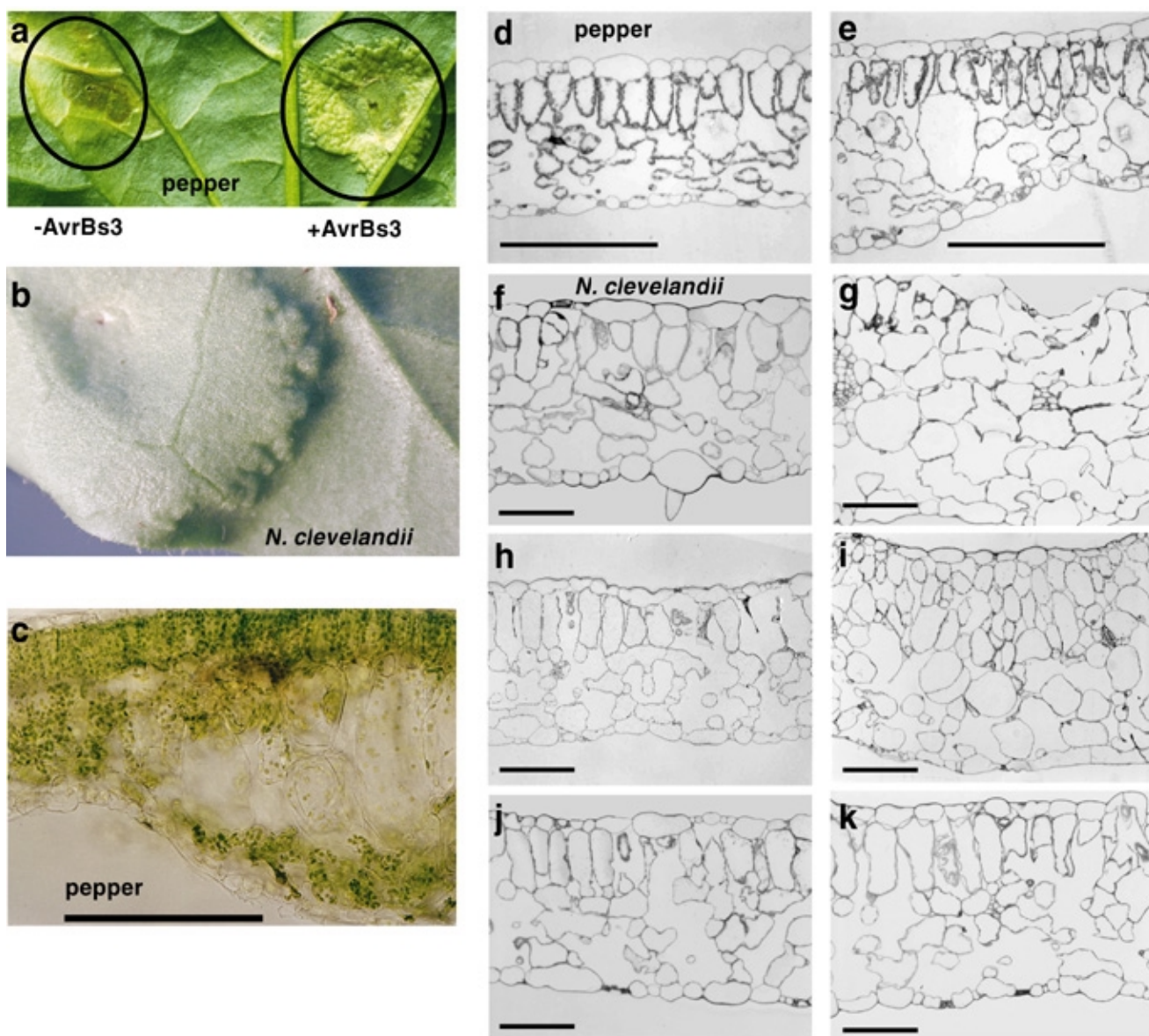


Fig. 1. AvrBs3 causes hypertrophy in plant leaves. **a**, A susceptible pepper leaf was inoculated with *Xanthomonas campestris* pv. *vesicatoria* strain I74A (empty pDSK602) ("–AvrBs3") and with *X. pv. vesicatoria* I74A expressing *avrBs3* from pDSF300 ("+AvrBs3"). Inoculation density was 5×10^8 cfu per ml. Only the *avrBs3*-expressing strain causes hypertrophy. The photograph was taken 4 days postinoculation (dpi). **b**, Transient expression of *avrBs3* in *Nicotiana clelandii*. *Agrobacterium* strain GV3101 (at 7 dpi, $OD_{600} = 0.8$) delivering the *avrBs3* gene from pVSF300. **c**, Leaf section through a susceptible pepper plant (ECW) leaf infected with *X. campestris* pv. *vesicatoria* strain I74A expressing *avrBs3*, observed with light microscopy. Spongy mesophyll cells appear to be vertically elongated in the infected area, pushing out the abaxial epidermis. Note the dilution of chloroplasts in the infected mesophyll due to cell hypertrophy. **d** and **e**, Sections at 4 dpi through pepper leaves infected with *X. campestris* pv. *vesicatoria* strain I74A expressing **d**, *avrBs3*ΔAAD (plasmid pDSF341) and **e**, functional *avrBs3* (plasmid pDSF340). **f** and **g**, Section at 7 dpi through *N. clelandii* leaf infected with *Agrobacterium* delivering **f**, an empty vector control (plasmid pVB60) and **g**, the *avrBs3* gene (pVSF300). **h** and **i**, Section at 10 dpi through *N. clelandii* leaf infected with *Agrobacterium* delivering **h**, the GUS gene used as negative control (plasmid p35SGUSINT) (Vancanneyt et al. 1990) and **i**, the *avrBs3* gene (pVSF300). **j** and **k**, Section at 7 dpi through *N. clelandii* leaf infected with *Agrobacterium* delivering **j**, the *avrBs4* gene and **k**, the *upa7a* gene (putative expansin). All genes delivered by *Agrobacterium* are expressed under the control of the 35S* promoter (Mindrinos et al. 1994). Bar represents 200 μ m.

mation of watersoaked lesions that later become necrotic (day 4 to 5). Growth curve experiments revealed that multiplication in planta of *X. campestris* pv. *vesicatoria* strains differing only in the presence or absence of the *avrBs3* gene is identical (Bonas et al. 1989). However, susceptible pepper plants infected with *X. campestris* pv. *vesicatoria* strains naturally containing *avrBs3* (e.g., strain 82-8) often develop pustules on the abaxial leaf surface, in a type III secretion-dependent manner. Deletion of the *avrBs3* gene abolished pustule induction (data not shown). Introduction of a plasmid-borne *avrBs3* copy into strains naturally lacking *avrBs3*, such as 85-10, resulted in pustule induction in pepper (Fig. 1a). Strains 75-3 and 85-10 ectopically expressing *avrBs3* also induced hypertrophy in *L. esculentum* and in the wild tomato species *L. pennellii* (S. Schornack and U. Bonas, unpublished data). Microscopic analysis revealed that pustules are the consequence of cell hypertrophy in the spongy mesophyll (Fig. 1c, d, e). Hypertrophy appears 4 days after bacterial inoculation and persists until the tissue collapses, which occurs from the center of the infiltrated area toward its margin, where the largest pustules are found. Reproducible observation of the *avrBs3*-induced hypertrophy in pepper and tomato is best with *X. campestris* pv. *vesicatoria* strains growing slowly in vitro and in planta, such as the 85-10 derivative I74A, which was used in this study. Fast-growing *X. campestris* pv. *vesicatoria* strains inoculated in laboratory conditions often caused tissue watersoaking and collapse before hypertrophy could develop.

Induction of pustules by transient expression of *avrBs3* within plant cells.

Agrobacterium-mediated transient transformation of leaves has been a powerful tool to study the effect of individual pathogen proteins in plants (Bonas and Van den Ackerveken, 1999). Transient expression of *avrBs3* under the control of the 35S *Cauliflower mosaic virus* promoter in resistant *Bs3* pepper

leaves resulted in the HR (Van den Ackerveken et al. 1996), indicating that *AvrBs3* acts inside host cells. Transient expression of the same *avrBs3* construct in *Nicotiana clevelandii* (Fig. 1b), *N. benthamiana*, *N. tabacum*, and in potato (*Solanum tuberosum*) induced pustules 4 to 5 days postinoculation (dpi). *Agrobacterium* strains carrying an empty vector did not cause any visible change in *Nicotiana* spp. and potato leaves (not shown), whereas susceptible pepper and tomato leaves reacted with chlorosis and necrosis 4 to 5 dpi against *Agrobacterium* spp., which inhibited *avrBs3*-dependent effects in susceptible host plants. The pustules resulting from *avrBs3* expression in the nonhost plants were macroscopically similar to those triggered by *X. campestris* pv. *vesicatoria*-delivered *AvrBs3* in pepper and tomato leaves. Microscopic examination of the *N. clevelandii* tissue expressing *avrBs3* revealed enlarged mesophyll cells (Fig. 1f, g). Unlike pepper tissue infected with *X. campestris* pv. *vesicatoria*, the hypertrophied *N. clevelandii* tissue never became necrotic. At later time points (Fig. 1h, i) the number of cells in the affected tissue increased. This phenomenon was not observed in the case of type III-delivered *AvrBs3* into pepper leaves and might be due to overexpression of *avrBs3* under the control of the 35S promoter or to the longer survival time of *Agrobacterium*-infected *Nicotiana* cells, allowing them to develop novel *AvrBs3*-induced phenotypes. Different *Nicotiana* spp. (*N. tabacum*, *N. benthamiana*, and *N. clevelandii*) developed *avrBs3*-induced pustules to various extents, the largest being obtained in *N. clevelandii* (Fig. 1g, i). Transient expression of *avrBs3* in *Arabidopsis* spp. did not induce any visible cellular changes, suggesting restriction of *AvrBs3*-specific effects to solanaceous plants.

Table 1. Induction of hypertrophy and hypersensitive response (HR) by different *Xanthomonas campestris* pv. *vesicatoria* strains

Strain ^a	Presence of <i>avrBs3</i> or derivative thereof ^b	Hypertrophy ^c	HR ^d
85-10	...	-	-
85-10	<i>avrBs3</i>	±	+
85-10	<i>avrBs4</i>	-	-
75-3	...	-	-
75-3	<i>avrBs3</i>	+	+
82-8	<i>avrBs3</i> , <i>avrBs4</i>	±	+
82-8Δ <i>avrBs3</i>	<i>avrBs4</i>	-	-
82-8Δ <i>avrBs2</i> ^e	<i>avrBs3</i> , <i>avrBs4</i>	+	+
I74A	...	-	-
I74A	<i>avrBs3</i>	+	+
I74A	<i>avrBs3</i> Δ <i>rep16</i>	-	+
I74A	<i>avrBs3</i> Δ1-3 ^f	-	-
I74A	<i>avrBs3</i> Δ1-3SV40 ^f	+	+
I74A	<i>avrBs3</i> ΔAAD	-	-
I74A	<i>avrBs3</i> ΔAAD::VP16	-	±

^a 85-10, 82-8, and 75-3 are field isolates of *X. campestris* pv. *vesicatoria*. I74A is a derivative of strain 85-10.

^b Strains not endogenously expressing *avrBs3* or a derivative thereof were transformed with a pDSK602 derivative containing the gene.

^c Hypertrophy was visually scored on susceptible Early Cal Wonder (ECW) pepper leaves, except for strain 75-3 (on *Lycopersicon pennellii*); ± indicates occasional hypertrophy.

^d HR was recorded on *Bs3* pepper line ECW-30R, or on *bs3* line ECW in the case of Δ*rep16*. + and ± indicate a fully developed and a partial or delayed HR, respectively.

^e *avrBs2* is an avirulence gene unrelated to *avrBs3*, the deletion of which causes a fitness penalty (Kearney and Staskawicz 1990).

^f *avrBs3*Δ1-3 is a nuclear localization signal (NLS) deletion mutant, and *avrBs3*Δ1-3SV40 is the same mutant complemented by introduction of the SV40 NLS (Van den Ackerveken et al. 1996).

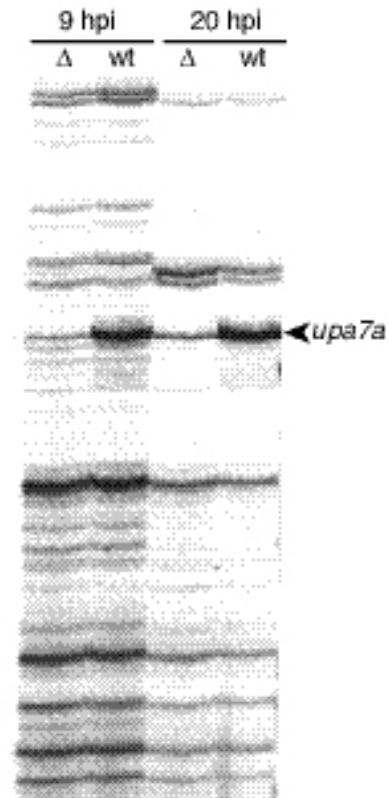


Fig. 2. Identification of differentially expressed cDNAs by amplified fragment length polymorphism (AFLP). Autoradiograph of an AFLP gel showing a fraction of the transcription profile for one primer pair. cDNA profiles in leaves of pepper cultivar ECW infected with *Xanthomonas campestris* pv. *vesicatoria* strain 85-10 expressing *avrBs3*ΔAAD (Δ) or wild-type *avrBs3* (wt) are compared, 9 and 20 h postinoculation (hpi). The differential band corresponds to *upa7a*.

The fact that transient *avrBs3* expression in plant cells could mimic the phenotype induced by AvrBs3 delivered by *X. campestris* pv. *vesicatoria* indicates that hypertrophy induction does not require *X. campestris* pv. *vesicatoria* effectors other than AvrBs3.

Induction of hypertrophy requires the AvrBs3 repeat region, NLSs, and AAD.

A collection of *avrBs3* mutant derivatives expressed by the virulent *X. campestris* pv. *vesicatoria* strain I74A was tested for hypertrophy induction in pepper (Table 1). We first tested whether the repeat deletion derivative *avrBs3Δrep16*, which renders *X. campestris* pv. *vesicatoria* avirulent in the *bs3* pepper line Early Cal Wonder (ECW) but virulent in *Bs3* lines, induces hypertrophy in *Bs3* plants. Neither *avrBs3Δrep16* nor other *avrBs3* repeat deletion derivatives (Herbers et al. 1992) were able to induce hypertrophy, indicating that hypertrophy depends on the wild-type AvrBs3 repeat region. Similarly, the AvrBs4 protein (previously designated AvrBs3-2; Bonas et al. 1993), which is 97% identical to AvrBs3 and differs from it mainly in the repeat region, did not induce hypertrophy in pepper, tomato, or when transiently expressed in *N. clevelandii* leaves (Fig. 1j).

We reported previously that AvrBs3 contains two functional NLSs (Van den Ackerveken et al. 1996). Induction of hypertrophy in susceptible pepper leaves had requirements for the NLSs identical to those for the HR (i.e. hypertrophy developed only when NLS2 or NLS3 were present) (Table 1). A mutant carrying an 83-aa deletion of the NLS region, ΔNLS1-3 (Van den Ackerveken et al. 1996) could be complemented for hypertrophy induction by the heterologous, 8-aa NLS from the large T-antigen of simian virus SV40. In addition, the deletion derivative of AvrBs3 deleted in the acidic activation domain (AvrBs3ΔAAD) (Szurek et al. 2001) was unable to induce hypertrophy. Introduction of the heterologous AAD from the *Herpes simplex* protein VP16 did not restore hypertrophy (Table 1), although HR in resistant plants was partially restored (Szurek et al. 2001). This is probably due to the lower activity of the VP16 constructs already evidenced by a weak complementation for HR induction (Szurek et al. 2001). Transient expression of *avrBs3* mutant derivatives in *Nicotiana* spp. showed that pustule induction had identical requirements for the AvrBs3 motifs as in *X. campestris* pv. *vesicatoria* infection experiments (data not shown). Taken together, these data indicate that all the AvrBs3 motifs found earlier to be needed for HR induction in *Bs3* pepper plants also are essential for hypertrophy induction.

AvrBs3-induced gene expression in pepper.

Our previous results suggest that AvrBs3 is transported from *X. campestris* pv. *vesicatoria* into the plant nucleus where it may function in modulating transcription. To identify plant genes whose expression is affected by the AAD of AvrBs3, we performed a cDNA-AFLP analysis (discussed below). PolyA⁺ mRNA from susceptible pepper plants infected with *X. campestris* pv. *vesicatoria* strain 85-10 expressing AvrBs3 or AvrBs3ΔAAD was isolated 9 and 20 h postinoculation (hpi) and used in the cDNA-AFLP procedure. All 256 possible primer combinations corresponding to the *ApoI/TaqI* enzyme pair were employed, and approximately 21,800 different cDNA fragments (on average, 85 per primer combination) were inspected. Thirty induced (Fig. 2) and two repressed transcripts were identified. AFLP fragments were 60 to 326 bp in length and were extended by polymerase chain reaction (PCR) using a cDNA library as template (discussed below). Sequence analysis revealed that the fragments corresponded to 22 different induced and 2 repressed genes, because the same *ApoI/TaqI* fragments sometimes were amplified by multiple primer combinations in spite of a mismatch at the second-but-last 3' nucleotide position of a primer. In one case, two different, noncontiguous *ApoI/TaqI* fragments belonged to the same gene. The majority of differential fragments could already be detected at 9 hpi, but generally were more abundant at 20 hpi. Out of the 24 differentially expressed genes identified by the AFLP screen, 13 could be reproducibly confirmed to be induced by *X. campestris* pv. *vesicatoria* expressing *avrBs3* (discussed below) using Reverse transcriptase-polymerase chain reaction (RT-PCR) and were studied further. These genes were designated *upa1* to *upa13* (upregulated by AvrBs3). The 11 other genes from the AFLP screen had highly variable or constitutive expression levels or proved difficult to amplify, and were not studied further.

Sequence analysis of AvrBs3-induced genes.

Five AvrBs3-induced genes (*upa1* to 5) are homologous to members of a family of auxin-induced genes, the SAUR family (small auxin up RNA) (McClure and Guilfoyle 1987) (Table 2). Three *upa* genes show high homology to α-expansin genes. Two of these, *upa7a* and *upa7b*, are 100% identical over 191 bp corresponding to the smaller AFLP fragment, containing an extra *ApoI* site which allowed its separate isolation. Screening a pepper cDNA library, we obtained a full-length cDNA clone corresponding to the larger fragment *upa7a*. Its deduced amino acid sequence is 89% identical to NtEXP1, an expansin from tobacco (Link and Cosgrove 1998). RT-PCR primers may not discriminate between the transcripts corresponding to *upa7a*

Table 2. AvrBs3-induced genes identified by cDNA-amplified fragment length polymorphism and confirmed by reverse transcriptase-polymerase chain reaction

cDNA ^a	Homology to	Most homologous sequence (plant)	Protein identity; similarity (%)	NAA inducibility	Cycloheximide sensitivity ^b
<i>upa1</i>	Auxin-induced protein (SAUR family)	pir1T17020 (apple tree)	73; 86	No	Yes
<i>upa2</i>	Auxin-induced protein (SAUR family)	pir1JQ1096 (soybean)	71; 84	Yes	Yes
<i>upa3</i>	Auxin-induced protein (SAUR family)	pir1T07798 (radish)	52; 59	Yes	Yes
<i>upa4</i>	Auxin-induced protein (SAUR family)	TGSAUR22 (tulip)	59; 73	Yes	Yes
<i>upa5</i>	Auxin-induced protein (SAUR family)	pir1JQ1098 (soybean)	32; 52	Yes	Yes
<i>upa6</i>	α-expansins	gblAAD13633.11 (tomato)	96; 96	Yes	Yes
<i>upa7a, 7b</i>	α-expansins	gblAAC96077.11 (tobacco)	89; 91	No	Yes
<i>upa8</i>	Pectate lyases	F22K18.20 (<i>Arabidopsis</i>)	85; 92	No	Yes
<i>upa9</i>	Hypothetical protein	AL132959 (<i>Arabidopsis</i>)	63; 79	No	Yes
<i>upa10</i>	Hypothetical protein	F22K18.80 (<i>Arabidopsis</i>)	49; 66	No	No
<i>upa11</i>	Anthocyanidin rhamnosyl transferases	Q43716 (<i>Petunia</i>)	28; 45	No	No

^a All cDNAs sequences contain a complete open reading frame with the exception of *upa3* (370 bp), *upa8* (1,196 bp), and *upa11* (790 bp), for which only part of the cDNA sequence is known.

^b Pepper leaves were inoculated with *Xanthomonas campestris* pv. *vesicatoria* expressing *avrBs3ΔAAD* or wild-type *avrBs3* from plasmids pDSF341 and pDSF340, respectively, without (–) or with (+) 50 μM cycloheximide. Samples for RNA isolation were collected 12 h postinoculation. Two samples of each inoculation are shown. The experiment was repeated three times with similar results.

and *upa7b*; therefore, the RT-PCR product designated *upa7* (Fig. 3) might represent several homologous expansinlike transcripts. The deduced amino acid sequence of *upa6* is 96% identical to tomato α -expansin Exp5, expressed in developing fruit (Brummell et al. 1999) and is 62% identical to Upa7a. Transient expression of *upa6* or *upa7a* under the control of the 35S promoter in *N. clevelandii* (Fig. 1k) or in pepper (data not shown) was not sufficient to trigger tissue alterations such as hypertrophy.

The deduced amino acid sequence of *upa8* shows homology to plant pectate lyases. Transcript *upa9* contains a 67-aa open reading frame that exhibits weak homology (GAP: 30% aa identity) to a 65-aa protein of cucumber (CRG16), whose mRNA levels are gibberellin responsive and increase during cucumber hypocotyl elongation (Chono et al. 1996). The *upa10*-encoded protein shows no significant homology to any known protein. Computer-based analysis indicated a probable membrane localization (PSORT, 70%), and two polygalacturonase motifs (BLOCKS) that might indicate involvement of Upa10 in the metabolism of cell wall polymers. The gene *upa11* is 45% similar on the amino acid level to anthocyanidin glucoside rhamnosyl transferases involved in the formation of purple pigmentation of flowers and stressed tissue (Brugliera et al. 1994). Both *upa12* and *upa13* encode putative transcription factors that will be described elsewhere.

Kinetics and specificity of gene induction.

Using RT-PCR, we studied the time-course of *upa* induction, comparing the mRNA patterns in tissue infiltrated with *X. campestris* pv. *vesicatoria* expressing *avrBs3*, *avrBs3* Δ AAD, or 10 mM MgCl₂, from 4 to 20 hpi (Fig. 3). Although most genes were induced by wild-type AvrBs3 as early as 6 hpi, the five SAUR-like transcripts (*upa1-5*) only began to accumulate at 9 hpi, a delay suggesting an AvrBs3-dependent activation cascade. AvrBs3 Δ AAD also induced some of the identified genes (Figs. 3 and 4), albeit more weakly than AvrBs3. Induction of some genes (e.g., *upa11*) was absolutely dependent on the C-terminal AAD. Previous results in yeast suggested that the N-terminal region in AvrBs3 also bears transcriptional activation activity (Szurek et al. 2001; Zhu et al. 1998), which might be responsible for the residual activation of other genes (e.g., *upa10*) by AvrBs3 Δ AAD (Figs. 3 and 4).

We also tested whether plant gene activation by AvrBs3 is repeat-region specific using *X. campestris* pv. *vesicatoria* strains delivering AvrBs3 Δ rep16 or the AvrBs3 homologue AvrBs4 (Fig. 4). Both strains induced some but not all *upa* genes, and to different levels. For example, AvrBs4 activated *upa6* and *upa7*, but not *upa9*, *upa10*, or *upa11*, while AvrBs3 Δ rep16 activated *upa9*, but not *upa10* and *upa11*.

Induction of the expansinlike gene *upa7* by AvrBs4 varied from experiment to experiment (Fig. 4A and B), but *upa7* transcript levels usually were markedly higher with AvrBs3 than with AvrBs4. Environmental or developmental cues probably modulate the responsiveness of the *upa7* promoter, and AvrBs3 appears to overcome potential repressors more efficiently than AvrBs4.

Taken together, these results suggest a complex and specific host-gene activation spectrum for each AvrBs3-like protein.

Dependence of *upa* induction on *de novo* protein synthesis.

To investigate whether any of the *upa* genes might be directly induced by AvrBs3, we infiltrated cycloheximide together with the bacterial suspension to block plant protein synthesis, and analyzed *upa* profiles at 12 hpi by RT-PCR (Fig. 5; Table 2). Expression of ubiquitin, used as a control, was not affected by cycloheximide treatment in this time frame. In contrast, cycloheximide prevented induction of most AvrBs3-

induced genes. Induction of these genes, therefore, requires synthesis of additional proteinaceous components and may not be directly induced by AvrBs3. However, *upa10* and *upa11* still were induced in the presence of cycloheximide, indicating either direct activation by AvrBs3 or via an available transcription factor, in an AvrBs3 AAD-dependent manner. AvrBs3-mediated *upa11* transcript accumulation was markedly increased by cycloheximide treatment (Fig. 5), implying that a negative feedback loop involving protein synthesis normally limits accumulation of this transcript. This was also true, to a lesser extent, for *upa10*. Cycloheximide-treated tissue died 48 h after treatment; therefore, the dependency of hypertrophy induction on *de novo* protein synthesis could not be assessed. In summary, cycloheximide treatment revealed that AvrBs3 induces only a few transcripts directly and most *upa* genes indirectly.

Auxin treatment induces some of the *upa* genes.

To test whether the SAUR-like genes and other *upas* are induced upon auxin treatment, a solution of the synthetic auxin naphthalene acetic acid (NAA) (10 μ g/ml) was infiltrated into pepper leaves. RT-PCR (Fig. 6; Table 2) revealed that four of the five SAUR-like genes were induced by NAA, indicating that they are probably the pepper equivalent of known SAURs. Interestingly, *upa6* (expansinlike gene) also appeared to be induced upon NAA infiltration. Genes *upa7* to *upa11* did not show induction after NAA treatment (Fig. 6), even at later time points (data not shown). These results suggest that the AvrBs3-induced genes fall into several classes distinguished by their auxin responsiveness.

DISCUSSION

AvrBs3 affects host cell morphology.

Delivery of AvrBs3 by *X. campestris* pv. *vesicatoria* into susceptible pepper and tomato plants induces hypertrophy.

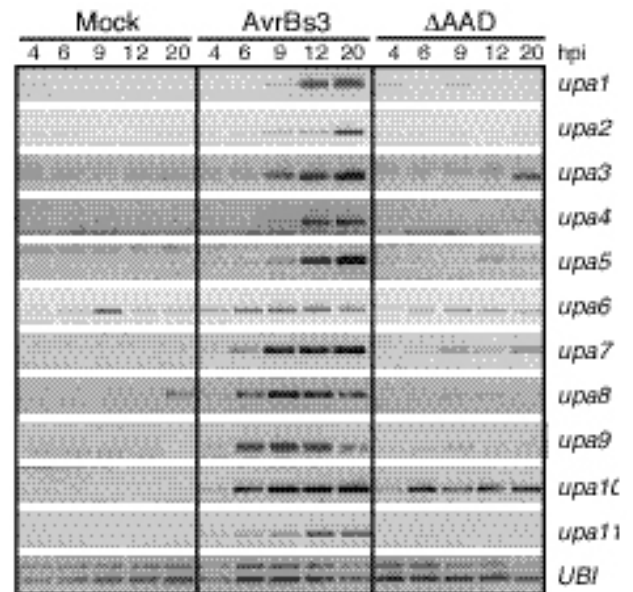


Fig. 3. Time course of pepper gene induction. The kinetics of *upa* (upregulated by AvrBs3) expression after infection with *Xanthomonas campestris* pv. *vesicatoria* was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Pepper cultivar ECW leaves were inoculated with 10 mM MgCl₂ (mock), *X. campestris* pv. *vesicatoria* strain 85-10 expressing *avrBs3* (from pDSF340), or *avrBs3* Δ AAD (from pDSF341). Tissue samples for RNA isolation were taken at 4, 6, 9, 12 and 20 h postinoculation. *UBI*: ubiquitin was used as control for a constitutively expressed gene (PCR yields two ubiquitin-specific bands). Sequence homologies are shown in Table 2.

This phenomenon is reminiscent of various pustule and canker symptoms induced by several *Xanthomonas* spp. in their hosts, including *X. campestris* pv. *glycines* in soybean, *X. citri* in citrus trees, and *X. populi* in poplar (Swings and Civerolo 1993). Pustules induced in soybean by *X. campestris* pv. *glycines* have been associated with mesophyll cell hypertrophy (Jones and Fett 1987), but the bacterial effectors responsible for this symptom have not been studied. Citrus canker is due to cell proliferation caused by the *X. citri pthA* gene (Swarup et al. 1992), which encodes a protein 96% identical to AvrBs3. When transiently expressed in *Citrus* spp. using particle bombardment or *Agrobacterium* spp., *pthA* triggered cell hypertrophy and proliferation (Duan et al. 1999). Transient expression of *avrBs3* in leaves of *Nicotiana* spp. and potato plants also resulted in cell hypertrophy and, later, in cell division. However, *pthA* expression in *Citrus* spp. leads to eruption of the mesophyll tissue through the abaxial leaf epidermis and to cell death (Duan et al. 1999), which was not observed with *avrBs3* in solanaceous plants. As the hypertrophied *Nicotiana* spp. tissue ages, cell division also occurs, which was not observed in pepper. Possible explanations for this difference include: (i) AvrBs3 sharing with PthA a capability to induce cell division if the infected cells survive long enough, which is not the case in *X. campestris* pv. *vesicatoria*-infected pepper tissue; (ii) dif-

ferent reactions to AvrBs3 depending on the plant species; and (iii) cell division resulting from AvrBs3 overexpression under the control of the 35S promoter.

For growth of *X. campestris* pv. *vesicatoria* in pepper leaves in laboratory conditions, hypertrophy is not a prerequisite. This is in contrast to the PthA-induced canker on citrus trees, which appears to provide an ecological niche necessary for bacterial growth (Swarup et al. 1991). Hypertrophy also could play a role in bacterial dispersal by decreasing the intercellular space volume at the end of the bacterial growth phase, resulting in a pressure that might expulse the bacteria out of the leaves, a dissemination mechanism proposed for PthA (Gabriel 1999). Another member of the *avrBs3* family, *avrB6* from *X. campestris* pv. *malvacearum*, was shown to enhance water-soaking of the infected tissue and promote release of the pathogen to the surface of cotton leaves (Yang et al. 1994). In field conditions, these effects might also apply for AvrBs3.

Testing *avrBs3* repeat-, NLS-, and AAD-mutant derivatives for hypertrophy-inducing activity largely confirmed the information on the functional regions in AvrBs3 based on HR tests. The requirement for these regions in both HR and hypertrophy induction implies that the two pathways share a similar beginning, probably up to gene activation. *upa* genes indeed are activated in resistant *Bs3* plants before the onset of the HR

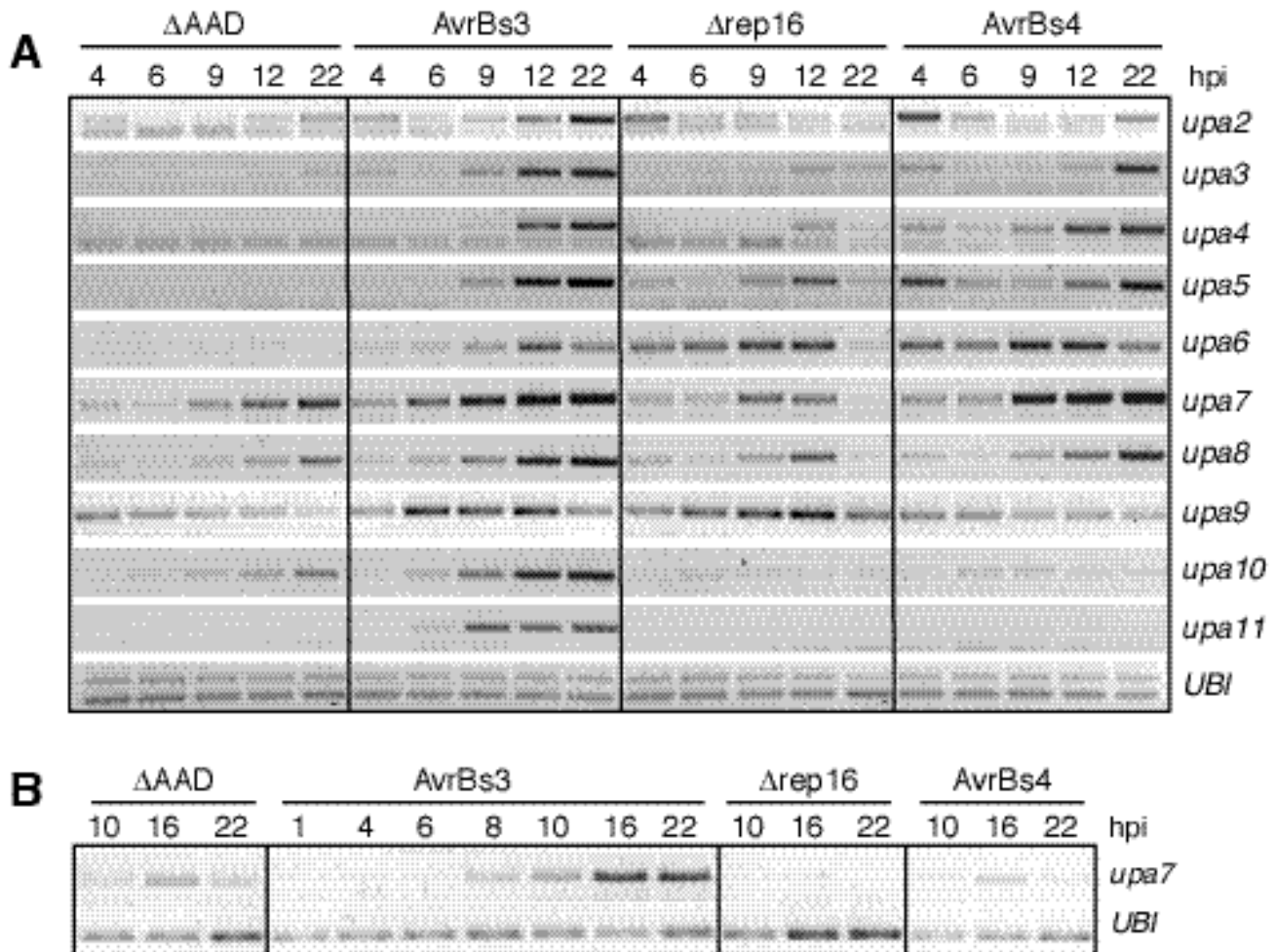


Fig. 4. Effect of AvrBs3, AvrBs3 Δ rep16, and AvrBs4 on *upa* induction. **A**, Leaves from pepper cultivar ECW were infiltrated with *Xanthomonas campestris* pv. *vesicatoria* strain 85-10 expressing *avrBs3* Δ AAD, *avrBs3*, *avrBs3* Δ rep16, and *avrBs4* from plasmids pDSF341, pDSF340, pDSF316, and pDSF200, respectively. Pepper cultivar ECW leaves were inoculated with 10 mM MgCl₂ (mock), *X. campestris* pv. *vesicatoria* strain 85-10 expressing *avrBs3* (from pDSF340), or *avrBs3* Δ AAD (from pDSF341). Tissue samples for RNA isolation and reverse transcriptase-polymerase chain reaction analysis were taken at 4, 6, 9, 12 and 20 h postinoculation. Note that for *avrBs3* Δ rep16, the 22 h time point corresponds to the onset of the hypersensitive response. In this experiment, *upa1* could not be amplified. **B**, Independent repeat of this experiment showing the expression pattern of *upa7*.

(data not shown). Interestingly, none of the repeat deletion derivatives of AvrBs3 that retained or gained avirulence activity on different pepper and tomato lines (Herbers et al. 1992) displays hypertrophy-inducing activity. In contrast, a number of repeat deletion derivatives of PthA could still induce canker (Yang and Gabriel 1995a). Although the AvrBs3 homologue AvrBs4 induced some *upa* genes (e.g., the expansinlike transcripts), we never observed any AvrBs4-induced hypertrophy. AvrBs4 probably fails to induce hypertrophy because genes are not induced to a sufficient level or induction of key hypertrophy-related transcripts is missing. Consistent with the idea that hypertrophy may result from the cooperative action of many induced genes, overexpression of the *upa7a* expansinlike cDNA alone did not induce hypertrophy in pepper or *N. clevelandii* (Fig. 1k and data not shown).

AvrBs3 induces host genes in an AAD-dependent manner.

In the experimental design of our cDNA-AFLP screen, differences in gene expression are due only to a 36-aa deletion in the AAD of the *X. campestris* pv. *vesicatoria* effector AvrBs3. In this case, the number of differential genes is expected to be lower than when compatible and incompatible interactions are compared (Durrant et al. 2000) and the identified genes should reflect the specific activity of AvrBs3 in susceptible plants. The *ApoI*-*TaqI* enzyme pair was chosen to maximize the number of appropriate cDNA fragments for the AFLP technique. Based on the restriction site frequency in 33 known pepper cDNAs from the GenBank database, our screen is estimated to cover 30 to 40% of all expressed pepper genes, hence of the total number of AvrBs3-induced genes.

The success in identifying *upa* genes whose induction is dependent on the AvrBs3 C-terminal activation domain is a strong argument in favor of the hypothesis that AvrBs3 acts as a transcription factor within the plant cell. This makes AvrBs3 the first known bacterial type III effector reported to target directly the host's genome. The results of cycloheximide treatment experiments suggest a model in which AvrBs3 induces a few genes directly, whereas most *upa* genes are probably activated subsequently by AvrBs3-induced transcription factors.

Presumed role of the induced genes.

It is striking that many of the AvrBs3-induced genes show homology to genes involved in cell expansion, such as the expansinlike transcripts *upa6*, *upa7a*, and *upa7b*. The association of expansins with cell enlargement is well documented (Cosgrove 2000) and several expansin transcripts have been identified as auxin-induced (Catala et al. 2000; Civello et al. 1999; Hutchison et al. 1999), which was observed as well for *upa6* (Fig. 6). The presence of *SAUR* transcripts shortly before cell enlargement has been reported (McClure and Guilfoyle 1989). Rapid induction of *SAUR* transcripts (McClure and Guilfoyle 1987) also was observed for *upa2* to *upa5*. The small *SAUR*-encoded proteins, recently found to have calmodulin-binding activity, are hypothesized to be involved in the auxin signal transduction pathway (Yang and Poovaiah 2000).

Several families of early auxin-induced genes are known (Abel and Theologis 1996). Among these, our cDNA-AFLP screen identified only *SAUR* genes, which suggests action of AvrBs3 downstream of auxin rather than in stimulating plant auxin synthesis. In preliminary experiments to address whether AvrBs3 stimulates the synthesis of auxin, auxin concentration in *X. campestris* pv. *vesicatoria*-infected pepper leaves appeared not to be correlated with presence or absence of *avrBs3* (data not shown).

Consistent with the AvrBs3-induced cellular phenotype, *upa8* encodes a putative pectate-lyase. Hydrolysis of wall polymers by such enzymes has been hypothesized to facilitate

cell expansion (Carpita and Gibeaut 1993; Domingo et al. 1998; Inouhe and Nevins 1991), whereby they might function in synergy with expansins that promote "polymer creep" (Cosgrove 2000).

More puzzling is the AvrBs3-induced expression of *upa11*, 45% similar on the amino acid level to plant anthocyanidin glucoside rhamnosyl transferases. Members of this gene family are involved in anthocyanin synthesis in flowers and stressed tissue. The fact that cycloheximide does not block *upa11* induction suggests that the *upa11* promoter might contain a consensus sequence required for AvrBs3 induction.

Mechanism of AvrBs3-mediated gene induction.

Our results suggest that different AvrBs3 family members activate distinct sets of plant genes. In support of this hypothesis,

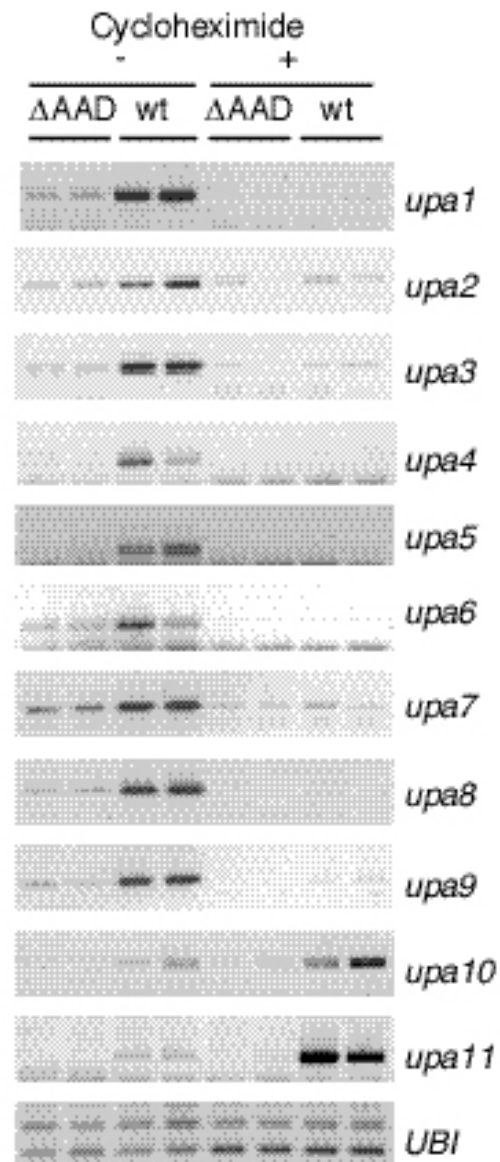


Fig. 5. Effect of cycloheximide treatment on *upa* induction. Reverse transcriptase-polymerase chain reaction analysis of *upa* gene induction in the presence of the eukaryotic protein synthesis inhibitor cycloheximide. Pepper leaves were inoculated with *Xanthomonas campestris* pv. *vesicatoria* expressing *avrBs3ΔAAD* or wild-type *avrBs3* from plasmids pDSF341 and pDSF340, respectively, without (–) or with (+) 50 μM cycloheximide. Samples for RNA isolation were collected 12 h postinoculation. Two samples of each inoculation are shown. The experiment was repeated three times with similar results.

the many AvrBs3 family members present simultaneously in certain *Xanthomonas* strains contribute to virulence symptoms through distinct pathways rather than in an additive manner (Bai et al. 2000; Yang et al. 1996). It is not yet understood how subtle amino acid differences between the repeat regions of different AvrBs3 family members account for their different specificities. A central question concerning the likely role of AvrBs3 as a transcription factor is whether it binds to target promoter sequences or to host nuclear proteins which, themselves, make contact with regulatory DNA sequences. Our study provides the basis for the identification of AvrBs3-responsive sequences in the plant that will allow to test this hypothesis.

MATERIALS AND METHODS

Bacterial strains and plasmids, plant inoculations.

Most bacterial strains and plasmids were described earlier (Szurek et al. 2001; Van den Ackerveken et al. 1996). Stability of all AvrBs3 derivatives was verified by immunoblotting. Strain I74A is a derivative of strain 85-10 with a wild-type *Hrp* phenotype but slower growth than the wild type in culture medium and in planta. For plant inoculations, *X. campestris*

pv. vesicatoria was resuspended at an optical density at 600 nm (OD_{600}) of 0.4 (5×10^8 CFU/ml) in 10 mM $MgCl_2$ and inoculated with a needleless syringe into the intercellular space of the abaxial leaf surface. HR was scored 24 to 48 hpi, water-soaking or hypertrophy 4 to 6 dpi. *Agrobacterium*-mediated transient expression assays were performed with *A. tumefaciens* strain GV3101 as described (Van den Ackerveken et al. 1996) with the following modifications: incubation of bacteria in induction medium for 5 to 7 h and inoculation at an OD_{600} of 0.5 to 1 in infiltration medium (10 mM $MgCl_2$, 5 mM MES, pH 5.3, 150 μ M acetosyringone). Binary vector constructs have been described (Van den Ackerveken et al. 1996). For cycloheximide treatment, leaf tissue was inoculated with a bacterial suspension as above, containing 50 μ M cycloheximide. Bacterial suspensions without cycloheximide were used as controls.

Plant material.

Pepper (*Capsicum annuum*) plants of cultivar ECW and the near-isogenic line ECW-30R containing the resistance gene *Bs3* (Minsavage et al. 1990), tomato cultivar MoneyMaker, and *Nicotiana* spp. were grown in greenhouse conditions. Six-week old plants were used for bacterial inoculations. Inoculated plants were transferred to a Percival growth chamber (Percival Scientific, Perry, IA, U.S.A.) at 28°C/24°C, 16 h of light; *Nicotiana* and tomato plants were transferred to a walk-in chamber (Vötsch Industrietechnik, Balingen-Frommern, Germany) at 25°C/22°C and 16 h of light.

Pepper cDNA library.

A cDNA library was constructed from pepper line ECW using the λ ZAP cDNA kit (Stratagene, La Jolla, CA, U.S.A.). RNA was isolated from a mix of healthy leaves and leaves infected with *avrBs3*-containing *X. campestris pv. vesicatoria* strain 85-10 (pDSF340) collected 6, 9, and 20 hpi.

cDNA-AFLP analysis.

cDNA-AFLP was performed as described (Bachem et al. 1996; Durrant et al. 2000). To minimize the identification of false positive transcripts due to leaf-to-leaf variability, one half of each leaf was infected with one strain and the other half with the second strain. For each time point, nine infected leaf halves from three different ECW plants were pooled for RNA extraction using standard protocols. PolyA⁺ RNA was isolated using Oligo(dT)-coupled DynaBeads (DYNAL, Oslo, Norway), and cDNA was produced using Expand Reverse-Transcriptase (Roche, Mannheim, Germany) according to the manufacturer's instructions. AFLP reactions were carried out with ³³P-labeled *ApoI* primers and resolved on 5% sequencing gels which were dried and exposed to X-ray film (Eastman Kodak, Rochester, NY, U.S.A.). After visual inspection of the autoradiographs, differential fragments were excised from the gel, eluted for 16 h in water, reamplified by PCR, and sequenced using an ABIPrism 377 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). For each primer combination yielding a differential fragment in the first AFLP screening, the AFLP procedure was repeated three times with cDNA isolated from independent inoculation experiments. The full-length sequence of most identified cDNAs was obtained by carrying out PCR reactions on the pepper cDNA library described above using primers internal to the AFLP fragment, in combination with M13 primers binding in the vector. Homology searches were performed with the BLAST and BLASTX programs (Altschul et al. 1990).

Semiquantitative RT-PCR experiments.

RT-PCR experiments were performed using primers yielding a 350- to 450-bp product for each *upa* gene. RT-PCR tem-

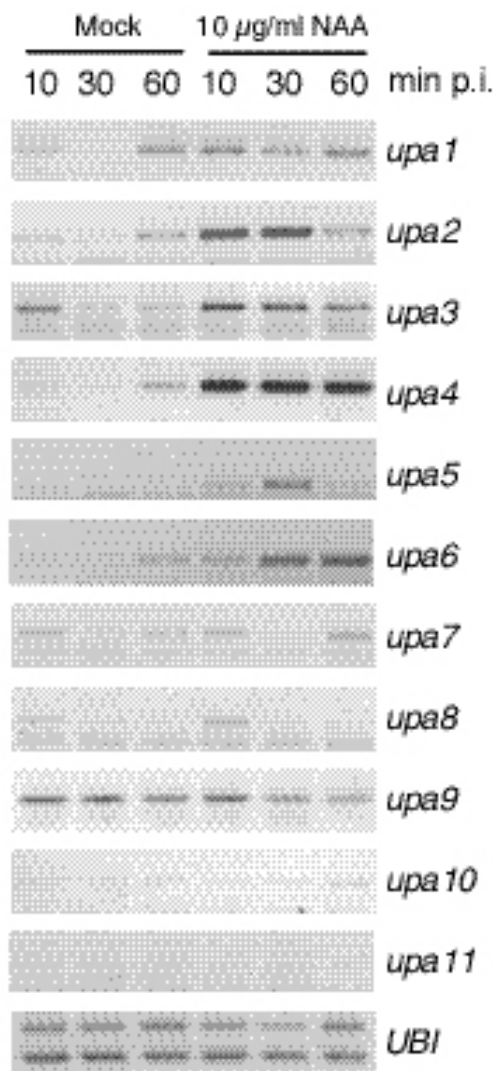


Fig. 6. Effect of auxin on *upa* induction. Reverse transcriptase-polymerase chain reaction analysis of *upa* gene expression was performed after infiltration of 10 μ g of the synthetic auxin naphthalene acetic acid (in 2 mM NaOH) or 2 mM NaOH alone (mock) per ml into pepper leaves. RNA was isolated from tissue collected 10, 30, and 60 min after infiltration.

plates were produced as follows: four leaf discs (1.3-cm diameter) from different pepper plants infected with the tested strains were pooled for RNA isolation. First-strand cDNA was synthesized from 4 µg of total RNA, with 200 pmol oligo-(dT)₂₀ and 200 units MMuLV-Reverse Transcriptase (Roche), according to the manufacturer's instructions. Reactions were diluted 10 times and used as a template for PCR. Specific primers amplifying the ubiquitin transcript were used as control. Appropriate PCR cycle numbers specific to each gene and to the primer combination were determined by testing a wide range of cycle numbers and choosing one for which DNA amplification was still in the exponential phase. PCR conditions appropriate for each gene and the corresponding primer pair sequences are available upon request.

Microscopy.

Sections of plant leaf material (Fig. 1c) were made in agarose-embedded tissue maintained in styrofoam using a vibratome. For semithin sections (Fig. 1d-k), leaf segments were fixed for 3 h with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and dehydrated in a graded ethanol series. Ethanol was substituted by epoxy resin (Spurr 1969) and samples were polymerized at 70°C. Sections (1 µm) were made with a Ultracut-S ultramicrotome (LEICA, Reichert, Vienna) and stained with toluidine blue.

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