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The tomato resistance protein Bs4 is a predicted non-nuclear TIR-NB-LRR protein that mediates defense responses to severely truncated derivatives of AvrBs4 and overexpressed AvrBs3

Sebastian Schornack[1,‡], Agim Ballvora[2,†,‡], Doreen Gürlebeck[1], Jack Peart[3], Martin Ganal[4], Barbara Baker[5], Ulla Bonas[1,2] and Thomas Lahaye[1,*]

[1]*Institut für Genetik, Martin-Luther-Universität Halle-Wittenberg, Weinbergweg 10, D-06120 Halle (Saale), Germany,*
[2]*Centre National de la Recherche Scientifique, Institut des Sciences Végétales, 91198 Gif-sur-Yvette, France,*
[3]*The Sainsbury Laboratory, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK,*
[4]*Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Corrensstrasse 3, D-06466 Gatersleben, Germany, and*
[5]*Plant Gene Expression Center, US Department of Agriculture, Albany, CA 94710, USA*

Received 31 July 2003; revised 16 September 2003; accepted 23 September 2003.
*For correspondence (fax +49 345 55 27151; e-mail lahaye@genetik.uni-halle.de).
†Current address: Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, D-50829 Köln, Germany.
‡These authors contributed equally to this work.

Summary

The *Lycopersicon esculentum Bs4* resistance (*R*) gene specifies recognition of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) strains that express the cognate AvrBs4 avirulence protein. *Bs4* was isolated by positional cloning and is predicted to encode a nucleotide-binding leucine-rich repeat (NB-LRR) protein that is homologous to tobacco N and potato Y-1 resistance proteins. *Xcv* infection tests demonstrate that *Bs4* confers perception of AvrBs4 but not the 97% identical AvrBs3 protein. However, when delivered via *Agrobacterium* T-DNA transfer, both, *avrBs4* and *avrBs3* trigger a *Bs4*-dependent hypersensitive response, indicating that naturally occurring AvrBs3-homologues provide a unique experimental platform for molecular dissection of recognition specificity. Transcript studies revealed intron retention in *Bs4* transcripts. Yet, an intron-deprived *Bs4* derivative still mediates AvrBs4 detection, suggesting that the identified splice variants are not crucial to resistance. The *L. pennellii bs4* allele, which is >98% identical to *L. esculentum Bs4*, has a *Bs4*-like exon-intron structure with exception of a splice polymorphism in intron 2 that causes truncation of the predicted bs4 protein. To test if the receptor-ligand model is a valid molecular description of *Bs4*-mediated AvrBs4 perception, we conducted yeast two-hybrid studies. However, a direct interaction was not observed. Defense signaling of the *Bs4*-governed reaction was studied in *Nicotiana benthamiana* by virus-induced gene silencing and showed that *Bs4*-mediated resistance is *EDS1*- and *SGT1*-dependent.

Keywords: ***Xanthomonas campestris* pv. *vesicatoria*, gene-for-gene interaction, type III effector, disease resistance gene, tomato, map-based cloning.**

Introduction

Plants are a nutritious habitat for phytopathogenic microbes and thus have had to evolve countermeasures that minimize assimilate plundering. Cells of resistant plant genotypes challenged by pathogens frequently respond with a controlled suicide program, termed the hypersensitive response (HR; Klement and Goodman, 1967). Genetic analysis of plant–microbe interactions has shown that perception of microbial invaders is often determined by complementary pairs of plant resistance (*R*) and pathogen avirulence (*avr*) genes. The receptor-ligand model is one biochemical interpretation of gene-for-gene resistance and predicts that the Avr ligand binds directly to an R receptor that subsequently activates a defense reaction (Gabriel and Rolfe, 1990; Keen, 1990). Analysis of two gene-for-gene interactions have provided experimental support for this model (Jia *et al.*, 2000; Kim *et al.*, 2002; Scofield *et al.*, 1996;

Tang *et al*., 1996). An alternative biochemical interpretation of gene-for-gene resistance known as the guard model (Van der Biezen and Jones, 1998b) postulates that the R protein (the 'guard') detects Avr-triggered changes of a plant virulence target (the 'guardee'). Identification and analysis of the *Arabidopsis* RIN4 protein provided experimental support for this model as RIN4 interacts physically with both the *Arabidopsis* RPM1 R protein and its cognate Avr proteins AvrRpm1 and AvrB (Mackey *et al*., 2002).

The majority of *R* genes cloned to date encode putatively cytoplasmic proteins with nucleotide-binding leucine-rich repeat (NB-LRR) domains (Dangl and Jones, 2001; Ellis *et al*., 2000b; Holub, 2001; Martin *et al*., 2003; Young, 2000), and this structure–function relationship has inspired homology-based approaches aimed at identification of potential candidate *R* genes (Pflieger *et al*., 2001). NB-LRR proteins differ structurally in their N-termini, comprising either a Toll/interleukin-1-receptor (TIR)-homologous region or a coiled-coil (CC) domain. Genetic dissection of defense signaling in *Arabidopsis* has shown that TIR-NB-LRR proteins signal through EDS1, whereas CC-NB-LRR proteins signal predominantly through NDR1 (reviewed in Feys and Parker, 2000; Glazebrook, 2001). By contrast, analysis of *Arabidopsis* Rar1 and SGT1 showed that these signaling components are engaged by both structural NB-LRR subtypes. Mutational studies of the *Nicotiana benthamiana* homologs of Rar1, EDS1 and SGT1, as well as the barley homologs of Rar1 and SGT1 indicate that these components are functionally conserved in *Arabidopsis*, *N. benthamiana* and barley (reviewed by Shirasu and Schulze-Lefert, 2003).

Genetic screens initially identified bacterial *avr* genes as mediators of gene-for-gene resistance (Staskawicz *et al*., 1984). The ability of many avirulent bacteria to elicit HR also depends upon the hypersensitive response and pathogenicity (*hrp*) genes. As the name implies, these genes are also required for bacteria to cause disease on susceptible plants. *hrp* genes encode a type III secretion system that injects bacterial Avr proteins into the host cell (Alfano and Collmer, 1997). This would allow these proteins to interact with NB-LRR type R proteins, which have an intracellular location. The existence of this sophisticated injection machinery implies that the primary function of Avr proteins is in virulence rather than avirulence. Indeed increasing evidence suggests a dual role of Avr proteins as recognition determinants in resistant and virulence determinants in susceptible hosts (Gabriel, 1999a; Luderer and Joosten, 2001; Van't Slot and Knogge, 2002).

Xanthomonas AvrBs3, the founder of a large protein family, exemplifies a well-studied Avr protein that has a documented contribution to both virulence and avirulence (reviewed in Büttner and Bonas, 2002). AvrBs3-like proteins share 90–97% sequence identity to each other and contain in the center of their polypeptide chain nearly perfect copies of a 34-amino-acid (aa) repeat motif that determines recognition specificity (reviewed by Gabriel, 1999b; Lahaye and Bonas, 2001; Leach *et al*., 2001; White *et al*., 2000). Other structural hallmarks of AvrBs3-homologous proteins are nuclear localization signals (NLSs) and an acidic transcriptional activation domain (AAD). Functional studies have shown that NLS and AADs are essential to nuclear import (Szurek *et al*., 2001) and for transcriptional activation of host genes (Marois *et al*., 2002), respectively. Mutational studies of multiple AvrBs3 family members revealed that NLSs and AADs are not only crucial for virulence but also for their avirulence function (Van den Ackerveken *et al*., 1996; Yang and Gabriel, 1995b; Yang *et al*., 2000; Zhu *et al*., 1998, 1999).

We study tomato and pepper bacterial spot disease, which is caused by the Gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*). In particular, our interest is in the *R* gene-mediated perception of AvrBs3 and AvrBs4 – two members of the *Xanthomonas* AvrBs3 family that share 97% sequence identity (Bonas *et al*., 1993). AvrBs3 and AvrBs4 are recognized specifically by the corresponding pepper *Bs3* and tomato *Bs4 R* genes, respectively (Ballvora *et al*., 2001a). We have shown that perception mediated by pepper *Bs3* but not by tomato *Bs4* depends on functional NLSs in the corresponding Avr proteins, which suggests different recognition principles for the detection of almost identical avirulence proteins in pepper and tomato (Ballvora *et al*., 2001a). The abundance and highly conserved structure of AvrBs3 family members make them a useful experimental system in which to study the specificity of *R* gene-mediated Avr protein perception. However, the molecular isolation of a corresponding *R* gene for any of the family members has not yet been reported.

Previously, we presented the genetic mapping and physical delimitation of the tomato *Bs4* locus (Ballvora *et al*., 2001a,b). Here, we report the isolation and functional analysis of the tomato *Bs4* gene. *Bs4* encodes a TIR-NB-LRR protein with most similarity to the tobacco N (Whitham *et al*., 1994) and potato Y-1 proteins (Vidal *et al*., 2002). Transcript studies uncovered *Bs4* splice variants, which, however, appear to be not required for *Bs4*-mediated HR. Analysis of recognition specificity indicates that Bs4 has the ability to mediate not only detection of AvrBs4 but also other AvrBs3-like proteins. Yeast two-hybrid (Y2H) studies suggest that Bs4 does not directly interact with AvrBs4. Furthermore, we demonstrate that *Bs4* is functional in *Solanum tuberosum* and *Nicotiana* species and that its function is *EDS1*- and *SGT1*-dependent.

Results

Bs4 *mediates recognition of AvrBs4-deletion derivatives*

Analysis of F_2 segregants derived from a cross between *Lycopersicon esculentum* cultivar (cv.) Moneymaker (MM)

Figure 1. The tomato *Bs4* gene mediates perception of AvrBs4 and deletion derivatives.
Inheritance of AvrBs4-induced HR was studied in F_2 segregants derived from a cross between *L. esculentum* cv. MM (*Bs4*) and *L. pennellii* LA2963 (*bs4*). Tomato plants were infiltrated with *Xcv* transconjugants delivering the depicted AvrBs4 derivative. Infection phenotypes were scored 48 h after inoculation. a, Boxed areas represent repeat units and white and black diamonds represent nuclear localization signals and the transcriptional activation domain, respectively; b, +/– indicates presence/absence of HR, respectively; and c, described previously by Bonas *et al.* (1993).

AvrBs4 derivatives		Infection phenotype[b]		
		Plant genotype		
Avr protein (vector designation)	protein structure[a]	*MM*[c]	*Bs4/—*	*bs4/bs4*
AvrBs4 (pLAT211)	1 17	+	+	–
AvrBs4 Δ215 (pLAT215)	1 17	+	+	–
AvrBs4 Δ218 (pLAT218)	1 17	+	+	–
AvrBs4 Δ221 (pLAT221)	1 13	+	+	–
AvrBs4 Δ227 (pLAT227)	1 5	+	+	–
AvrBs4 Δ230 (pLAT230)	1 3	+	+	–
AvrBs4 Δ233 (pLAT233)	1	–	–	–

and *L. pennellii* LA2963 has shown that recognition of *Xcv* strains that express AvrBs4 is mediated by the *L. esculentum* cv. MM *Bs4* locus (Ballvora *et al.*, 2001a). Earlier studies have shown that not only full-length AvrBs4 but also AvrBs4-deletion derivatives trigger an HR on *L. esculentum* cv. MM (Figure 1; Bonas *et al.*, 1993). Yet, it remained unclear, if *Bs4* or other unlinked *R* loci govern recognition of these AvrBs4-deletion derivatives. We therefore analyzed six different C-terminal AvrBs4-deletion derivatives on a set of 20 F_2 progenies derived from a cross between *L. esculentum* cv. MM (*Bs4*) and *L. pennellii* LA2963 (*bs4*) (Figure 1). The allele configuration of these F_2 segregants at the *Bs4* locus was determined with molecular markers that flank *Bs4* on either side of the locus (see Methods for details). *Xcv* infection tests showed that C-terminal AvrBs4-deletion derivatives, which contain 3.5 or more repeat units, triggered an HR in *Bs4/–* segregants and the *L. esculentum* cv. MM but not in *bs4/bs4* segregants (Figure 1). On the contrary, an AvrBs4 derivative that contains 26 residues of the first repeat unit (AvrBs4 Δ233) did not induce an HR in any of the tested tomato genotypes. Notably, all tested *Bs4/–* segregants and *L. esculentum* cv. MM showed identical infection phenotypes with AvrBs4 and its deletion derivatives. These findings indicate that the previously determined avirulence activity of AvrBs4-deletion derivatives (Bonas *et al.*, 1993) was because of *Bs4*-dependent recognition.

Degenerate PCR and high-resolution mapping uncovers a Bs4 *candidate gene*

A PCR-based strategy with degenerate primers targeting the conserved TIR motif of TIR-NB-LRR-encoding *R* genes was applied to isolate *Bs4* candidate genes from tomato genomic DNA. The chromosomal positions of the cloned PCR products were assessed by RFLP mapping in a standard tomato mapping population (Tanksley *et al.*, 1992) and placed one of the isolated fragments (T26 RGC) in the vicinity of the *Bs4*-linked RFLP marker TG432 (Figure 2; Ballvora *et al.*, 2001b). T26 RGC was converted into a PCR-based RFLP marker and employed for the analysis of a mapping population that segregates for *Bs4* resistance. Linkage analysis of 1972 meiotic events revealed that T26 RGC fragments diagnostic for the *Bs4*-parental genotype *L. esculentum* cv. MM co-segregated with an HR phenotype that was visible 48 h after infiltration of *avrBs4*-expressing *Xcv*. Furthermore, analysis of two yeast artificial chromosome (YAC) clones, that were shown previously to span the

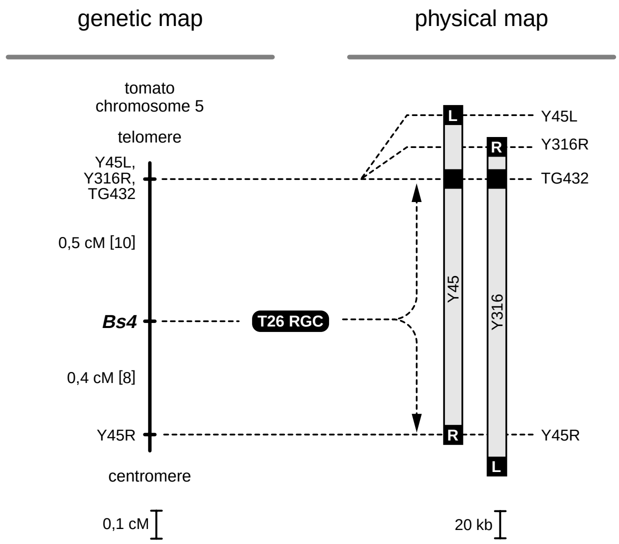

Figure 2. Integrated genetic/physical map of the *Bs4* locus.
Based on the analysis of 1972 meiotic events, T26 RGC shows complete linkage to the *Bs4* target locus. Physical mapping placed T26 RGC between TG432 and Y45R. Arrows indicate the area within which T26 RGC was physically delimited. Marker loci are shown as horizontal, dashed lines. Numbers in square brackets denote the number of recombinants identified. YAC inserts are displayed to scale as bars with their respective right (R) and left (L) ends. Note that spaces between markers in the YAC insert do not represent defined physical distances.

Bs4 locus (Ballvora *et al.*, 2001b), demonstrated that both YACs contain the T26 RGC marker locus. In summary, high-resolution genetic and physical mapping supported the notion that T26 RGC was part of a potential *Bs4* candidate gene.

A TIR-NB-LRR-encoding Bs4 *candidate gene mediates specific recognition of AvrBs4*

To determine if the YAC insert DNA that was used for physical delimitation of *Bs4* could be employed for complementation studies, we performed infection tests with *L. esculentum* cv. VFNT Cherry, the DNA source for YAC library construction (Bonnema *et al.*, 1996; Martin *et al.*, 1992). Inoculation tests showed that *avrBs4*- but not *avrBs3*-expressing *Xcv* triggers an HR, indicating that the *L. esculentum* cv. VFNT Cherry contains the *Bs4* gene.

In order to isolate T26 RGC-flanking sequences, we generated a cosmid library of YAC clone Y45, which was shown previously to span the *Bs4* locus (Ballvora *et al.*, 2001b). Sequence analysis of T26 RGC-containing cosmids revealed a putative TIR-NB-LRR-encoding candidate gene (termed *Bs4*^{c}). Amplification and sequence analysis of the *Bs4*^{c} homolog from *L. esculentum* cv. MM, the genotype that was used for high-resolution linkage mapping of *Bs4*, revealed that the nucleotide sequence of *Bs4*^{c} is identical in VFNT Cherry and MM. Southern analysis of *L. esculentum* cv. MM and YAC Y45 DNA indicated that *Bs4*^{c} is a single-copy gene (data not shown).

To determine whether *Bs4*^{c} mediates AvrBs4 recognition, the previously established *L. esculentum bs4* backcross line MM^{bs4}–BC4 (Ballvora *et al.*, 2001a) was transformed with a binary vector (pVTSB1) in which the *Bs4*^{c} genomic fragment is under transcriptional control of the CaMV 35S promoter. *Xcv* infection tests showed that 16 out of 22 transgenic tomato plants displayed an AvrBs4-dependent HR (Figure 3). Analysis of several independent T_1 and T_2 lines showed that inheritance of AvrBs4-responsiveness was strictly dependent on the presence of *Bs4*^{c}, indicating that *Bs4*^{c} is indeed the *Bs4* gene.

Recognition specificity of the *Bs4*^{c}-transgenic plants was tested with *Xcv* strains that deliver (i) AvrBs4, (ii) AvrBs4 Δ227 (C-terminal deletion derivative of AvrBs4), (iii) AvrBs3, or (iv) AvrBs1 (no sequence homology with AvrBs4; Ronald and Staskawicz, 1988), respectively (Figure 3). Infiltration of the *Bs4*^{c}-transgenic lines with xanthomonads that express *avrBs4* or its deletion derivative *avrBs4 Δ227* triggered a rapid HR. By contrast, *avrBs3*- and *avrBs1*-expressing *Xcv* did not induce an HR. Notably, all *avr* derivatives produced identical infection phenotypes in *Bs4*^{c}-transgenic lines and the *Bs4*-containing genotype *L. esculentum* cv. MM and did not trigger HR in the *bs4* genotype MM^{bs4}–BC4 (Figure 3). Taken together, our complementation studies led us to conclude that *Bs4*^{c} is indeed the tomato *Bs4* gene.

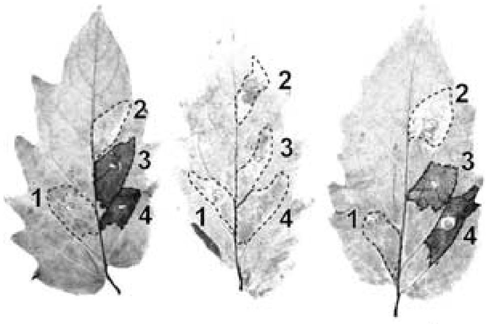

Figure 3. Functional analysis of the tomato *Bs4* candidate gene.
The tomato genotypes *L. esculentum* cv. MM (*Bs4*), the near isogenic line MM^{bs4}–BC4 (*bs4*) and a transgenic plant that contains the *Bs4* candidate (*Bs4*^{c}) gene were infiltrated with *Xcv* strains that deliver AvrBs1 (1), AvrBs3 (2), AvrBs4 Δ227 (3), and AvrBs4 (4), respectively. Leaves have been bleached by ethanol treatment for better visualization of the HR. Dashed lines indicate the infiltrated leaf areas. Photographs were taken 48 h post infection.

Agrobacterium tumefaciens-*mediated expression of* avrBs3 *triggers a* Bs4-*dependent HR*

Recognition specificity of the *L. esculentum* cv. MM *Bs4* allele was also studied by *A. tumefaciens*-mediated transient expression (agroinfiltration). In contrast to our *Xcv* infection tests we found that, when delivered by agroinfiltration, not only *avrBs4* and its deletion derivative *avrBs4 Δ227* but also *avrBs3* triggered an HR in *L. esculentum* cv. MM and the *Bs4* transgenic lines (Figure 4a). We did not observe an HR in the *bs4* genotype MM^{bs4}–BC4, indicating that agroinfiltrated *avrBs3* was detected in a *Bs4*-dependent manner. It is worth noting that the HR was indistinguishable in the *Bs4*-transgenic lines and *L. esculentum* cv. MM although the *Bs4* transgene is under control of the CaMV 35S promoter (Figure 4a).

Analysis of unchallenged tissue by real-time PCR revealed that the *Bs4* transcript levels in our 35S::*Bs4* transgenic tomato lines were approximately 100-fold higher when compared to *L. esculentum* cv. MM (*Bs4*) (data not shown). To examine whether *Bs4* overexpression influences the infection phenotypes, we cloned a genomic fragment containing the *Bs4* open-reading frame (ORF) and approximately 3.5-kbp upstream sequence into a promoterless binary vector (pVTSB3). Functionality of *Bs4* under transcriptional control of (i) its putative native promoter or (ii) the CaMV 35S promoter was compared by *Agrobacterium*-mediated co-expression with *avrBs4* (Figure 4b). In this assay, a *bs4* plant genotype was infiltrated with a mixture of two *Agrobacterium* cultures, one expressing the respective *avr* gene, and the other expressing *Bs4* under transcriptional control of either the CaMV 35S or the putative *Bs4* native promoter. Phenotypic inspection of the reactions mediated by the CaMV 35S and the native promoter constructs showed no

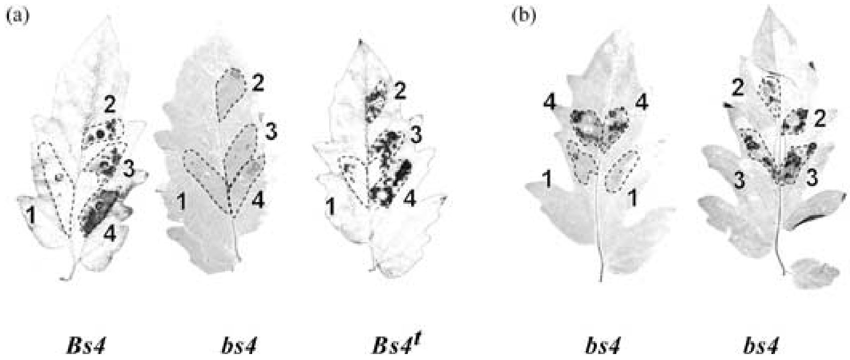

Figure 4. *Agrobacterium*-mediated delivery of *avrBs3* triggers a *Bs4*-dependent HR.
(a) Analysis of *Bs4* recognition specificity by *Agrobacterium*-mediated delivery of *avr* derivatives. *Agrobacterium* strains that mediate T-DNA-based delivery of *avrBs1* (1), *avrBs3* (2), *avrBs4* $\Delta 227$ (3), or *avrBs4* (4), respectively, were infiltrated into *L. esculentum* cv. MM (*Bs4*), the near isogenic line MM^{bs4}–BC4 (*bs4*) and *Bs4*-transgenic line (*Bs4*^{t}).
(b) *Agrobacterium*-mediated coexpression of the *Bs4* and *avr* derivatives. For transient co-expression, respective *avr*-containing *Agrobacterium* strains 1–4 (see a) were mixed with equal amounts of *Agrobacterium* strains that contain the *Bs4* candidate gene under transcriptional control of the CaMV 35S promoter (left side of the leaf) or its own promoter (right side of the leaf). Leaves have been bleached by ethanol treatment for better visualization of the HR. Dashed lines indicate the infiltrated leaf tissue. Photographs were taken 6 days post infection.

differences with respect to recognitional specificity, extent, timing, and intensity of the HR (Figure 4b).

The Bs4 *transcript undergoes alternative splicing*

Reverse transcriptase (RT)-PCR was used to determine the *Bs4* exon-intron structure (summarized in Figure 5), because attempts to isolate a *Bs4* full-length cDNA clone were unsuccessful. Based on rapid amplification of cDNA ends (RACE) analysis, the *Bs4* transcript contains 25 and 500 bp of 5′- and 3′-untranslated region (UTR), respectively. A comparison of the *Bs4* genomic sequence with the RACE products revealed the presence of a 90-bp intron in the 3′-UTR. Inspection of the *Bs4* ORF by means of intron finder algorithms suggested the presence of three introns in the *Bs4* ORF that could be confirmed by RT-PCR with primers flanking the predicted introns. In addition, we obtained *Bs4*-splice variants that still contained intron 2 or 3 but lacked intron 1. To determine the functional relevance of these splice variants, we removed introns 1–3 and used this intron-deprived *Bs4* derivative for agroinfiltration of the *bs4* genotype MM^{bs4}–BC4. We found, that the intron-deprived *Bs4* ORF mediates *avrBs4* recognition, indicating that the introns are not crucial to *Bs4* functionality. Likewise, we tested *Bs4* constructs lacking the 5′- or 3′-UTRs and found that UTRs are functionally dispensable.

The predicted Bs4 protein is highly related to the virus resistance proteins potato Y-1 and tobacco N

The *Bs4* transcript encodes a predicted protein of 1146 amino acids with a molecular weight of 131 kDa. Among

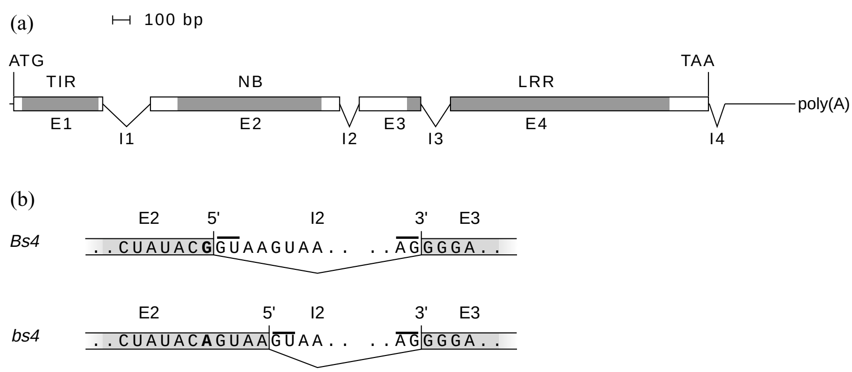

Figure 5. *Bs4* transcript structure.
(a) Schematic representation of the *Bs4* exon-intron structure. Putatively coding or intronic regions are depicted as boxes and angled lines, respectively. Exons (E) and introns (I) are numbered above and below the drawings. Shaded areas represent the TIR-, NB-, and LRR-encoding regions. Please note that the LRR region is encoded by sequence stretches in exon 3 (E3) and exon 4 (E4). The putative ATG start codon, TAA stop codon, and the poly(A) tail are indicated.
(b) Sequence comparison of *Bs4*- and *bs4*-derived cDNAs reveals a splice-site polymorphism at intron I2 that causes a frameshift in *bs4* transcripts. Boldface letters highlight a G/A polymorphism between the *Bs4* and *bs4* alleles. Thick horizontal lines highlight the 5′-splice donor and 3′-splice acceptor sites in *Bs4*/*bs4* alleles.

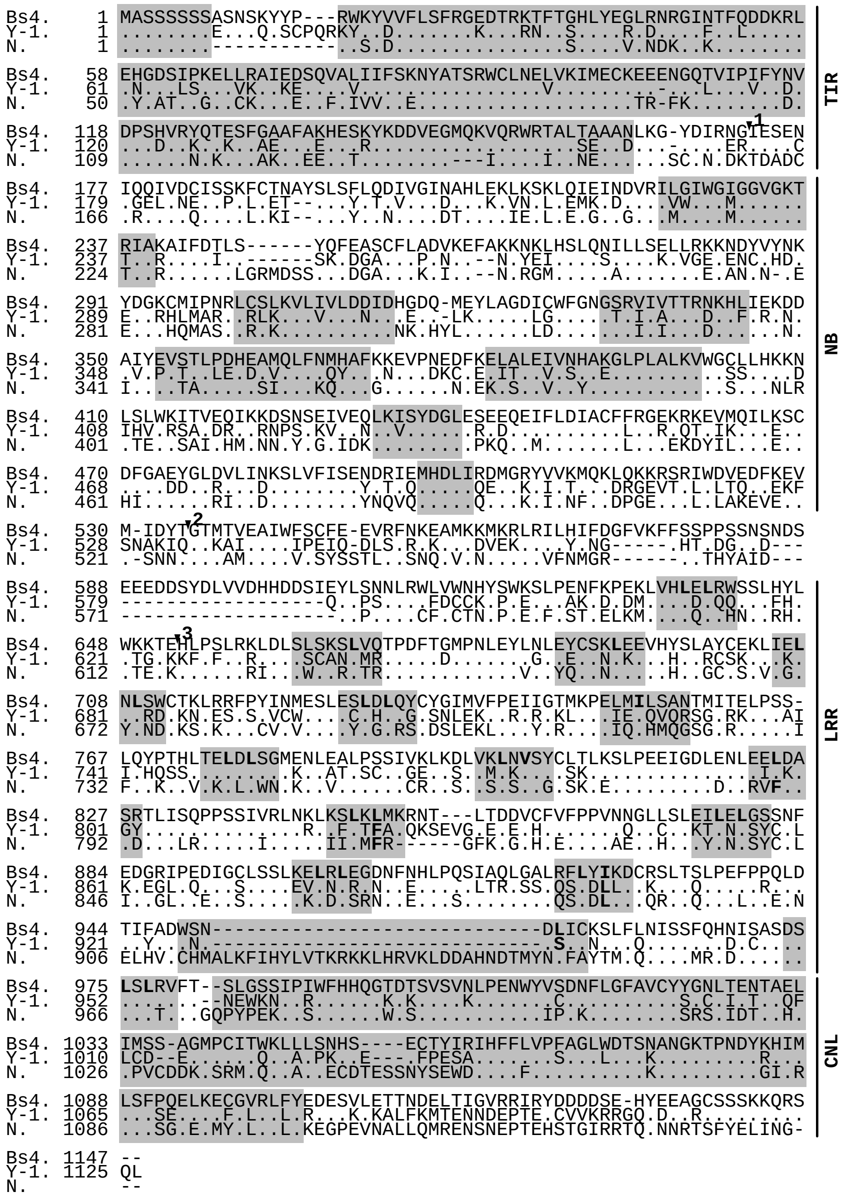

Figure 6. Bs4 is highly similar to the virus resistance proteins Y-1 and N.
Alignment of the deduced amino-acid sequences of tomato *Bs4*, potato *Y-1*, and tobacco *N*. TIR, NB, LRR, and C-terminal non-LRR (CNL) homology domains are indicated by gray boxes. Dots represent residues in Y-1 and N that are conserved with respect to Bs4. Sequence gaps inserted to maintain the alignment are indicated by dashes. Arrowheads mark the intron positions in *Bs4*. NB and CNL domains were defined according to Van der Biezen and Jones (1998a) and Dodds *et al.* (2001), respectively. Residues that form the structural backbone of the LRR units were defined according to Ellis *et al.* (2000a) and are shown in bold.

proteins with a known function, Bs4 most closely resembles the TIR-NB-LRR proteins potato Y-1 (57% identity, 71% similarity; Vidal *et al.*, 2002) and tobacco N (54% identity, 67% similarity; Whitham *et al.*, 1994) (Figure 6). The Bs4 protein displays at its far N-terminus (aa residues 1–8) a characteristic sequence motif (MASSSSSS), which is also present in potato Y-1 (Vidal *et al.*, 2002), tobacco N (Whitham *et al.*, 1994), *Arabidopsis* RPP4 (Van der Biezen *et al.*, 2002), *Arabidopsis* RPP5 (Parker *et al.*, 1997), and other putative TIR-NB-LRR proteins (Hehl *et al.*, 1999). Comparison of the Bs4 TIR (aa residues 17–162), NB (aa residues 224–500), and LRR (aa residues 626–987) regions with the corresponding domains of Y-1 and N shows that the TIR is the most conserved and the LRR the most divergent domain between these R proteins. The C-terminal region of Bs4 is composed of 15 repeat units with similarity to the cytoplasmic LRR consensus sequence (Jones and Jones, 1997). We found no apparent N-terminal signal sequences in Bs4, suggesting that it is a cytoplasmic protein. However, PROSITE motif search (http://www.expasy.ch/prosite) identified potential myristoylation sites in Bs4, which might mediate membrane anchoring of the protein.

bs4 *transcripts encode a truncated bs4 protein*

Sequence comparison of the *L. pennellii* LA2963 *bs4* allele with the *L. esculentum* cv. MM *Bs4* allele revealed in total 74 nucleotide polymorphisms (Figure 7). Analysis of

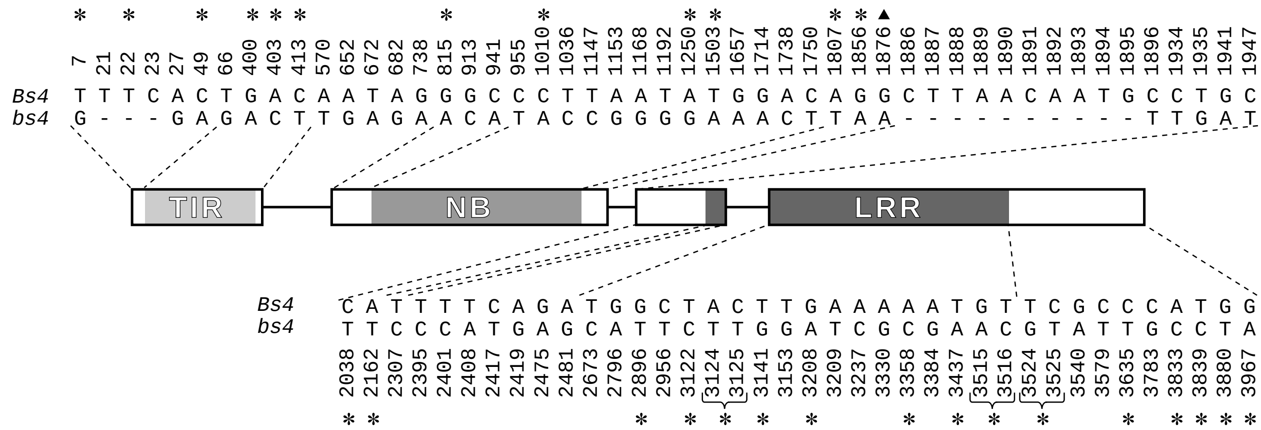

Figure 7. Sequence comparison between the *L. esculentum* cv. MM *Bs4* and *L. pennellii* LA2963 *bs4* alleles.
Exonic and intronic sequence are shown as boxes and lines, respectively. Dashes indicate deletions in *bs4* with respect to *Bs4*. Gray-boxed areas indicate TIR, NB and LRR regions. Asterisks mark nucleotide polymorphisms that cause amino-acid differences between the predicted Bs4 and bs4 proteins. Brackets indicate amino-acid differences between Bs4 and bs4 that are caused by two base pair differences within one codon. The triangle marks a G/A polymorphism that is located next to the *Bs4*-splice donor site of intron 2 and possibly causes a translational stop codon 21 bp downstream of this polymorphism. To compare *Bs4*- and *bs4*-encoded proteins downstream of intron 2, we assumed the presence of *bs4* transcripts with a *Bs4*-like exon-intron structure. Polymorphisms are numbered with respect to *Bs4*.

putative exons predicts that 28 out of 60 nucleotide polymorphisms would lead to aa differences between the *bs4*- and *Bs4*-encoded proteins. The predicted *bs4* exons do not contain an early stop codon that would unequivocally define *bs4* as a null allele. Transcript analysis showed that *bs4* and *Bs4* share an almost perfect conservation of exon–intron junctions with the exception of the 5′ splice site of intron 2 (Figure 7). This modified exon–intron boundary is predicted to cause a frame-shift and hence to encode a truncated protein that lacks the LRR region. This is confirmed by sequence analysis of 32 cloned RT-PCR fragments, which revealed no *bs4* transcripts that would encode an LRR region.

Bs4 and AvrBs4 do not show interaction in the Y2H system

To test whether AvrBs4 interacts physically with Bs4, we performed Y2H studies. An AvrBs4 bait construct (N-terminal fusion of AvrBs4 to the LexA DNA-binding domain) was found to activate both the *leu2* and the *lacZ* reporter genes in the absence of the prey construct. To overcome this autoactivation problem, we constructed an N-terminal deletion derivative of AvrBs4 (AvrBs4 Δ152) lacking aa residues 1–152. AvrBs4 Δ152 no longer activates the yeast reporter genes but still triggers a *Bs4*-dependent HR when the corresponding gene is delivered by *Agrobacterium*. This finding suggests that the N-terminus of AvrBs4 is not required for *Bs4*-dependent recognition. Next, an intron-deprived *Bs4* gene, that lacks introns 1–3, and that was shown to be functional (see above) was cloned into the prey vector. We also generated reciprocal bait and prey constructs (AvrBs4 Δ152, prey vector; Bs4, bait vector) and confirmed by Western blot analyses for all constructs expression of appropriate-sized proteins in yeast (data not shown). A repression assay (Brent and Ptashne, 1984) showed that the LexA-AvrBs4 Δ152 and LexA-Bs4 fusion proteins can bind to nuclear-localized operator sequences, indicating that both proteins are suitable for Y2H interaction studies. Pepper importin α1 (Caimpα1), previously identified as an interactor of AvrBs3 (Szurek *et al.*, 2001), also interacts with AvrBs4 and was used as a positive control for our Y2H studies. The *Drosophila* bicoid protein was used as a control for non-specific interactions.

To study interaction of Bs4 and AvrBs4, we tested AvrBs4 Δ152 bait and Bs4 prey constructs and the reciprocal combination (AvrBs4 Δ152 prey; Bs4 bait). The *lacZ* reporter gene activity measured in AvrBs4 Δ152–Bs4 co-transformants in no case exceeded the levels of the negative controls (bicoid in combination with AvrBs4 Δ152 or Bs4) and was about 10 times lower than that for the positive control (Figure 8). The same yeast co-transformants lacked leucine prototrophy, indicating that AvrBs4 Δ152 and Bs4 proteins do not interact in yeast. We also cloned different Bs4 regions (TIR, NB-LRR, and LRR) into the prey vector and tested these for interaction with the AvrBs4 Δ152 bait protein. In accordance with the data observed for the full-length Bs4 protein, co-transformation of the separated Bs4 regions with AvrBs4 Δ152 (Figure 8) did not activate the *leu2* or the *lacZ* reporter gene, suggesting that a simple receptor-ligand model does not hold true for the *avrBs4*–*Bs4* gene-for-gene interaction.

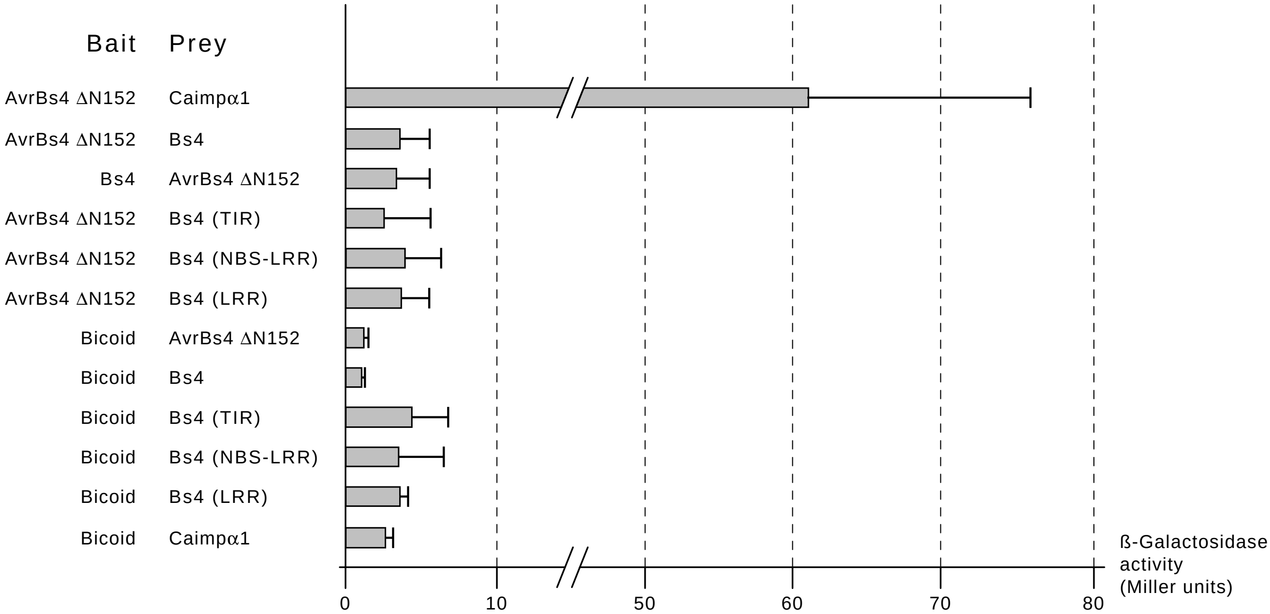

Figure 8. Y2H analysis of the Bs4–AvrBs4 interaction.
Reporter gene activation was determined by measuring β-galactosidase activity of yeast strains expressing the respective bait and prey proteins. The interaction of AvrBs4 Δ152 and Caimpα1 was used as positive control and *Drosophila* bicoid as negative control.

Tomato Bs4 *is functional in* S. tuberosum *and* Nicotiana *species*

To examine whether species other than tomato have all accessory proteins that are needed for a *Bs4*-mediated defense response, we performed agroinfiltration of the solanaceous plant species *N. tabacum*, *N. clevelandii*, *N. benthamiana*, *Capsicum annuum*, *C. frutescens*, *S. tuberosum*, and *Petunia hybrida*. Agroinfiltration of a binary vector carrying the β-glucuronidase (GUS) gene induced reporter activity in the infiltrated tissues, thereby indicating that all tested plant species are transformable by *Agrobacterium* (data not shown). Subsequently, we performed agroinfiltration of these different plant species using *Bs4* in combination with *avrBs4*, *avrBs4 Δ227*, *avrBs3*, and *avrBs1*. Co-expression of *Bs4* with *avrBs4*, *avrBs4 Δ227* and *avrBs3*, but not *avrBs1* triggered an HR in *N. tabacum*, *N. clevelandii*, *N. benthamiana*, and *S. tuberosum* (Figure 9; data not shown). No HR was observed in *P. hybrida*, *C. annuum*, and *C. frutescens*. We noted in *S. tuberosum* that agroinfiltration of *avrBs4*, but not *avrBs3*, induced a *Bs4*-independent HR, which is most likely because of an intrinsic *R* gene. However, as the *avrBs3*-triggered HR in *S. tuberosum* was only observed when co-expressed with *Bs4*, we conclude that *Bs4* is functional in *S. tuberosum*. In summary, our results indicate that *S. tuberosum* and *Nicotiana* species but not *Capsicum* or *Petunia* species contain the elements that are required for the *Bs4*-mediated HR.

Bs4*-mediated HR is* EDS1- *and* SGT1*-dependent*

As a first step towards elucidation of the signal components that mediate the *Bs4*-specified defense response we analysed known *R* gene pathway elements. To study the

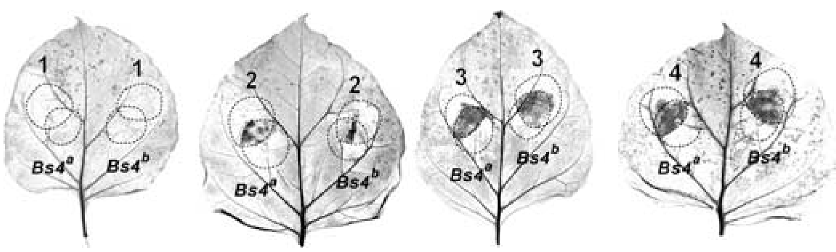

Figure 9. Tomato *Bs4* is functional in *N. benthamiana*.
Agrobacterium-based co-expression of *Bs4* and different *avr* genes. *Agrobacterium* strains containing *avrBs1* (1), *avrBs3* (2), *avrBs4 Δ227* (3), or *avrBs4* (4) were infiltrated into leaves of *N. benthamiana*. Subsequently, *Agrobacterium* strains that contain *Bs4* under transcriptional control of the CaMV 35S promoter (*Bs4*[a]) or *Bs4* under transcriptional control of its own promoter (*Bs4*[b]), were infiltrated. Leaves were bleached by ethanol treatment seven days after agroinfiltration for better visualization of the HR. Dashed lines indicate the infiltrated leaf tissue.

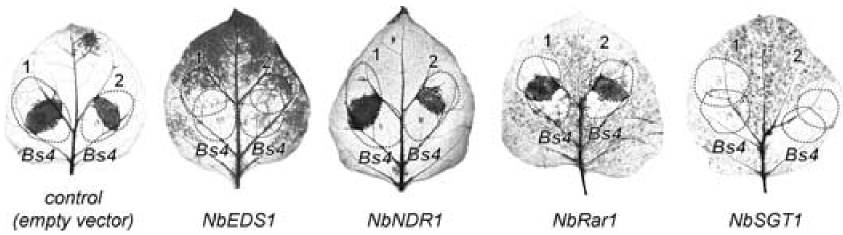

Figure 10. Virus-induced gene silencing of *NbEDS1* or *NbSGT1* suppresses *Bs4* function in *N. benthamiana*.
Plants were infected with TRV derivatives of *NbEDS1, NbNDR1, NbSGT1, NbRar1*, or an empty TRV vector. Twenty-one days later *Agrobacterium* strains that deliver *Bs4*, *avrBs3* (1), or *avrBs4* (2), respectively, were infiltrated. HR symptoms are diagnostic for functionality of the *Bs4* gene. Seven days after agroinfiltration, the leaves were bleached by ethanol treatment to visualize HR. Dashed lines indicate infiltrated leaf areas.

functional relevance of EDS1, NDR1, RAR1, and SGT1 in the *Bs4*-mediated HR, we employed virus-induced gene silencing (VIGS), a well-established tool for transient gene silencing in *N. benthamiana* (Baulcombe, 1999). As VIGS requires a high degree of sequence similarity between the recombinant virus and the target RNA (Mueller *et al*., 1995), it was necessary to use the *N. benthamiana* orthologs of *EDS1* (*NbEDS1*), *NDR1* (*NbNDR1*), *Rar1* (*NbRar1*), and *SGT1* (*NbSGT1*). The *NbEDS1* and *NbSGT1* genes have been described before (Peart *et al*., 2002a,b) and for isolation of *NbNDR1* and *NbRar1*, we employed a homology-based approach (see Experimental procedures for details). VIGS was initiated by inoculation of tobacco rattle virus (TRV) vector derivatives carrying a fragment of *NbEDS1*, *NbNDR1*, *NbRar1*, or *NbSGT1*, respectively. Approximately 21 days after the first inoculation, *Bs4* was agroinfiltrated in combination with *avrBs4* or *avrBs3*, respectively. Plants that were infiltrated with an empty virus vector showed an HR in the leaf area, in which *Bs4* was co-expressed with *avrBs4* or *avrBs3* (Figure 10). This indicates that the viral infection did not interfere with the *Bs4*-mediated HR. However, in *NbEDS1*- and *NbSGT1*-silenced plants co-expression of *Bs4* with *avrBs4* or *avrBs3* (Figure 10) triggered no HR, indicating that both genes are crucial to *Bs4*-governed HR execution. On the contrary, *Bs4*-mediated HR was not affected in *NbNDR1* and *NbRar1*-silenced plants.

Discussion

A putatively cytoplasmic R protein mediates recognition of a nuclear-targeted Avr protein

A positional candidate approach was used to isolate the tomato *Bs4* gene, which encodes a novel member of the TIR-NB-LRR class of R proteins. Tomato *Bs4* was shown to mediate recognition of the *Xanthomonas* AvrBs4 protein, which is a member of the well-characterized AvrBs3 family (Lahaye and Bonas, 2001). AvrBs3-like proteins contain NLS sequences that are indicative for nuclear targeting (Gabriel, 1997; Leach and White, 1996; White *et al*., 2000), and recent immunocytochemical studies indeed demonstrated that AvrBs3 and AvrBs4 are localized to the plant nucleus (Szurek *et al*., 2002; Szurek and Bonas, unpublished results). Molecular analysis of several gene-for-gene interactions suggests that R-protein localization is generally dictated by the Avr protein destination (reviewed by Bonas and Lahaye, 2002; Martin *et al*., 2003) and thus *Bs4* would be predicted to encode a nuclear protein. However, tomato *Bs4* has no apparent sequence signatures that would indicate nuclear localization. This prediction is in agreement with the fact that *Bs4* mediates recognition of NLS-deprived AvrBs4-deletion derivatives (this study and Ballvora *et al*., 2001a) and supports our previously proposed working model that *Bs4*-mediated perception of AvrBs4 occurs in the cytoplasm before AvrBs4 has reached its final destination, the nucleus (Ballvora *et al*., 2001a). However, it needs to be considered that computer-based predictions are error-prone as exemplified by the RPM1 R protein that was found to be membrane bound although it was predicted to be cytoplasmic (Boyes *et al*., 1998). Hence, localization studies are needed to clarify the subcellular localization of Bs4.

Agrobacterium-*mediated* avrBs3 *expression triggers a* Bs4-*dependent HR*

Xanthomonas infection tests demonstrated that *Bs4* mediates recognition of AvrBs4 and its C-terminal deletion derivatives, but not the 97% identical AvrBs3 protein (Figure 3). However, when the corresponding genes are delivered via *Agrobacterium*, both AvrBs4 and AvrBs3 but not AvrBs1 trigger a *Bs4*-dependent HR (Figure 4). This finding indicates that *Agrobacterium*-mediated delivery causes a partial loss of recognition specificity. The reasons for this are not fully understood.

One possibility is that increased AvrBs3 expression levels generate the observed loss of Bs4 recognition specificity as transgene expression in the binary vector is under control of the CaMV 35S promoter. Thus, AvrBs3 levels in the *Agrobacterium*-mediated expression system might exceed the quantity that is delivered by the *Xanthomonas*-type III secretion system, possibly causing loss of Bs4 recognition specificity. However, in the converse experiment, the pepper *Bs3 R* gene that confers perception of AvrBs3 in an NLS-dependent fashion (Van den Ackerveken *et al*., 1996) does not mediate recognition of agroinfiltrated AvrBs4 (S. Schornack and T. Lahaye, unpublished results). The seemingly different degrees of specificity in *Bs3*- and *Bs4*-mediated resistance in agroinfiltration assays might be related to the fact that *Bs3* confers only recognition of NLS-bearing AvrBs3 derivatives (Ballvora *et al*., 2001a; Van den Ackerveken *et al*., 1996). Previous studies have shown that AvrBs3 recruits the host's nuclear import machinery in order to reach the plant nucleus (Szurek *et al*., 2001). Assuming that the host's nucleocytoplasmic shuttle system has only a limited transfer capacity, it might be possible that in agroinfiltration assays, only a small fraction of NLS-bearing AvrBs3-like proteins are actually transferred into the nuclear compartment where it can interact with Bs3. In contrast, the putatively cytoplasmic tomato Bs4 might be confronted with high levels of AvrBs3-like proteins. Hence, the apparently different degrees of recognition specificity of Bs3 and Bs4 may reflect their subcellular localization rather than intrinsic properties of the proteins.

A second possibility is that 'relaxed' recognition specificity is an intrinsic property of Bs4. This hypothesis is supported by the identification of two novel AvrBs3-like proteins that are distinct from AvrBs4, which are both recognized in a *Bs4*-dependent manner when delivered by *Xanthomonas* (S. Kay and U. Bonas, unpublished results). Considering that several *Xanthomonas* strains contain multiple AvrBs3-like proteins (Leach *et al*., 2001; Van't Slot and Knogge, 2002) and given that genes encoding AvrBs3-like proteins can rapidly change their structure by intra- and inter-repeat recombination (Yang and Gabriel, 1995a), it seems economically sensible to employ one R protein with a 'relaxed' recognition specificity rather than expressing multiple, highly specific R proteins.

Intron-deprived Bs4 *derivatives mediate AvrBs4 detection*

Molecular analysis of transcripts encoding TIR-NB-LRR class R proteins has revealed many cases of alternative splicing (Anderson *et al*., 1997; Ayliffe *et al*., 1999; Dinesh-Kumar and Baker, 2000; Gassmann *et al*., 1999; Lawrence *et al*., 1995; Whitham *et al*., 1994). Recent studies of tobacco *N* and *Arabidopsis RPS4*, both encoding TIR-NB-LRR proteins, showed that intron-deprived genes have no or only reduced activity, suggesting that alternative splicing is crucial to these defense-signaling pathways (reviewed by Jordan *et al*., 2002).

The *Bs4* exon-intron structure is characteristic of an *R* gene transcript that encodes an TIR-NB-LRR protein (reviewed by Jordan *et al*., 2002) and has three conserved introns that are located between the TIR-, NB-, and LRR-encoding regions. RT-PCR uncovered in several *Bs4* transcripts retention of introns 2 and 3, which has also been described for the *Arabidopsis RPS4* (Gassmann *et al*., 1999) and the flax *L6* gene (Ayliffe *et al*., 1999). Complementation analysis showed that intronless *RPS4* derivatives mediate no or only a reduced defense response in comparison to genomic *RPS4* constructs (Zhang and Gassmann, 2003). In contrast to the findings observed for *RPS4*, we did not observe obvious functional differences between genomic and intron-deprived constructs. Nonetheless, one cannot exclude the possibility that these *Bs4* splice variants confer subtle biological effects.

Sequence analysis of the bs4 *allele provides no unequivocal evidence for a complete-loss-of-function (null) allele*

Analysis of 74 DNA polymorphisms between the *L. pennellii* LA2963 *bs4* and *L. esculentum* cv. MM *Bs4* genomic sequences (Figure 6) failed to uncover mutations that would unequivocally classify *bs4* as a null allele. RT-PCR revealed that *bs4* and *Bs4* transcripts differ with respect to the 5′ splice site of intron 2 (Figure 4b). Usage of an alternative GT splice donor site in *bs4* transcripts generates a frame-shift and is predicted to encode truncated bs4 proteins that lack the LRR region. However, we analyzed only 32 cloned RT-PCR fragments and it might well be possible that *bs4*-derived transcripts that encode a functional, full-length TIR-NB-LRR protein remained undetected. Phenotypic inspection of *L. pennellii* LA2963 and segregants that harbor the corresponding *bs4* allele revealed a delayed HR in *bs4* genotypes that appears about 10 days after *Xanthomonas* infection (data not shown; *Bs4* mediates HR 48 h after infection). Notably, only *avrBs4*-expressing xanthomonads trigger this delayed HR, indicating that the *L. pennellii* LA2963 *bs4* allele still has residual function with respect to AvrBs4 perception. In summary, the lack of mutations that would clearly classify *L. pennellii bs4* as a null allele and the delayed AvrBs4-dependent HR in *bs4* genotypes supports the hypothesis that *bs4* is not a non-functional allele but rather an allele with reduced activity.

VIGS of NbEDS1 *and* NbSGT1 *suppresses* Bs4*-mediated HR execution*

Genetic dissection has shown that *EDS1* and *NDR1* define defense-signaling pathways that are differentially employed by TIR-NB-LRR and CC-NB-LRR proteins, respectively (reviewed by Feys and Parker, 2000; Glazebrook, 2001). In agreement with this postulate, we found that functionality of the TIR-NB-LRR protein Bs4 was compromised by *EDS1*- but not by *NDR1*-silencing.

Unlike *EDS1* and *NDR1*, the pathway elements *Rar1* and *SGT1* were shown to be engaged by both TIR–NB-LRR and CC-NB-LRR subtypes (reviewed by Dodds and Schwechheimer, 2002). Noteworthy, activity of the tobacco N protein, which shares 54% sequence identity with Bs4, is impaired by VIGS of *Rar1* and *SGT1* (Liu *et al.*, 2002a,b). By contrast, our studies show that *Bs4*-mediated HR execution is only impaired by *SGT1*- but not by *Rar1*-silencing. This is somewhat surprising, given that Bs4 and N share extensive sequence homology. However, previous studies of the barley MLA1, MLA6, and MLA12 mildew R proteins, which share approximately 90% sequence identity (Halterman *et al.*, 2001; Shen *et al.*, 2003; Zhou *et al.*, 2001), have shown that, despite their pronounced sequence homology, only MLA6 and MLA12 require RAR1 and SGT1 for execution of a defense response (Halterman *et al.*, 2001; Shen *et al.*, 2003; Zhou *et al.*, 2001). Domain swaps between the *Rar1/SGT1*-independent *Mla1* and the *Rar1/SGT1*-dependent *Mla6* alleles generated *Rar1/SGT1*-independent chimeras with *Mla6* recognition specificity (Shen *et al.*, 2003), thereby demonstrating that recognitional specificity and *Rar1*/*SGT1* requirements are defined by distinct protein regions. Analysis of the barley *Mla* alleles demonstrates also that highly similar R proteins that recruit distinct signaling elements provide a functional tool for allocation of protein regions that specify dependency on certain downstream components. In analogy to the MLA variants, Bs4 and N represent highly similar R proteins, which however differ only with respect to their *Rar1* but not with respect to their *SGT1* dependency. Hence, domain swaps between Bs4 and N might allow allocation of domains that define *Rar1*-dependency.

However, there is a caveat to our VIGS-based *Rar1* and *NDR1* knockdown assays as gene-silencing does not generally facilitate complete elimination of the targeted transcripts. Thus, we cannot exclude that Bs4 acts in a *NDR1*- and *Rar1*-dependent manner and that residual amounts of the corresponding transcripts are sufficient for *Bs4*-mediated HR execution.

No direct interaction between Bs4 and AvrBs4?

Resistance proteins are thought to detect Avr proteins either directly by physical interaction or indirectly because of virulence-associated Avr actions (Bonas and Lahaye, 2002; Dangl and Jones, 2001; Martin *et al.*, 2003; Van der Biezen and Jones, 1998b). Thus far, no virulence function has been assigned to AvrBs4. However, as AvrBs4 has been maintained in nature, despite the fact that it exerts negative selective pressure in the interaction with most tomato hosts, it is likely to have a virulence function. Conceivably, AvrBs4 employs molecular virulence strategies similar to those of the 97% identical AvrBs3 protein, and hence it seems likely that AvrBs4-deletion derivatives that lack C-terminal AAD and NLS domains have no or only reduced virulence activity. We showed by analysis of deletion constructs that an AvrBs4-derivative (AvrBs4 Δ230), which contains only 3.5 out of 17.5 repeat units and lacks its AAD and NLS regions was still able to trigger a *Bs4*-dependent HR. This might be interpreted as an argument against the guard model as recognition in the conceptual framework of the guard model requires biologically active, disease-promoting Avr effectors. Taken together, genetic analysis of the *avrBs4–Bs4* gene-for-gene interaction favors a receptor-ligand rather than a guard model. However, AvrBs4 and Bs4 seem not to interact *in yeast*, despite the fact that Western analyses and repression assays showed that AvrBs4 and Bs4 derivatives are in principle suitable for Y2H studies. How can we explain these seemingly contradictory findings? Recent biochemical studies suggest that R proteins are part of multiprotein complexes (Bogdanove, 2002; Ellis *et al.*, 2002; Leister and Katagiri, 2000), and it may well be possible that R proteins fulfill their Avr-receptor function only in the context of this multicomponent recognition complex. Further clarification of the molecular principles that govern the AvrBs4-Bs4 interplay will therefore foreseeable require techniques that allow analysis of proteins in their natural environment.

Experimental procedures

avr *derivatives and* Xcv *inoculations*

Plants were grown and inoculated as described previously by Bonas *et al.*, (1989, 1993). Analysis of *avrBs4*-deletion derivatives in pLAFR6 (description of constructs by Bonas *et al.*, 1993) was conducted with respective *Xcv* transconjugants. All other *avr* genes were assayed in *Xcv* transconjugants that carry pDSK602 constructs of *avrBs1* (pDS100, Escolar *et al.*, 2001), *avrBs3* (pDS300F, Van den Ackerveken *et al.*, 1996), *avrBs4* (pDS200F, this study) and *avrBs4 Δ227* (pDS227, this study). *avrBs4* and *avrBs4 Δ227* were cloned into pDSK602 (Murillo *et al.*, 1994) as follows. An *Eco*RI/*Hin*dIII fragment from pUS200F, bearing *avrBs4* (Ballvora *et al.*, 2001a), was cloned into pDSK602, resulting in plasmid pDS200F. AvrBs4 Δ227 (pAT227) is a C-terminal *avrBs4*-deletion derivative that was generated by DNAse I digest of an *Eco*RI-linearized pAT200 (Bonas *et al.*, 1993) and released from pAT227 by *Bam*HI digest and replaced the corresponding *avrBs3* fragment in pDSK602-36 giving rise to pDS227. pDSK602-36 was generated by cloning a *Bam*HI–*Nde*I–*Bam*HI linker into the *Bam*HI site of pUXV1006 (Bonas *et al.*, 1989) and ligating the *Nde*I–*Hin*dIII fragment with the *avrBs3*-ORF into *Eco*RI/*Hin*dIII sites of pDSK602.

Plant material

Inoculation tests of *avrBs4*-deletion derivatives were performed on 10 *bs4/bs4* and 10 *Bs4/–* F_2 segregants of a cross between the *L. esculentum* cv. MM (*Bs4*) and *L. pennellii* LA 2963 (*bs4*) (Ballvora *et al.*, 2001b). The genotype of F_2 plants in the *Bs4* locus was determined with the *Bs4*-flanking markers TG432 and P11M6 (Ballvora *et al.*, 2001a,b).

PCR with degenerate oligonucleotide primers targeting the TIR motif

Primer pairs based on the conserved TIR motif of NB-LRR-encoding *R* genes were used for amplification of *Bs4* candidate genes from VF36 tomato DNA. Their sequences were: RD5: GT(T/G)TT(T/C)TT(A/G)AGTTT(C/T)AG(A/G)GG; and RD10: GGATCCAC(A/C)(T/A)(C/T)ATA(G/A)AA(A/T)AT(A/C)GG. Amplification was carried out with the following program: 5 cycles of 94°C for 45 sec, 42°C for 45 sec, and 72°C for 90 sec; 30 cycles of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 90 sec. PCR products were mapped on a population of 47 F_2 plants from *L. esculentum* × *L. pennellii* (Tanksley *et al.*, 1992) using MAPMAKER (Lander *et al.*, 1987).

Genetic mapping of a Bs4 *candidate gene*

We developed two PCR-based RFLP markers within the *Bs4* candidate gene; RGA2 (primers: Bs4-A02: CTACCATCATCTCTTCAGTACCAACTC; and Bs4-B02: GAAATTGGAGGAACCGAGCTCCAG; *Msp*I-polymorphism); and RGA3 (primers: Bs4-A03: GGGTTGGAGTCCGAAGAGCAGG; and Bs4-B03: GACTAACCAACGCAAGTTATTGGACAGG; *Rsa*I-polymorphism). Inheritance of AvrBs4 recognition in tomato was studied using a cross between the *L. esculentum* cv. MM (resistant parent) and *L. pennellii* LA 2963 (susceptible parent; Ballvora *et al.*, 2001b).

Construction of binary vectors carrying Bs4

For construction of a cosmid library, YAC Y45 was partially digested with *Sau*3A, and cloned into the *Bam*HI site of the binary vector pCLD04541 (Jones *et al.*, 1992). The T26 RGC-bearing cosmid T2-2 was identified by colony hybridization and confirmed by PCR. A T2-2-derived genomic fragment containing *Bs4* and 1.2 kbp upstream of the ATG was transferred into the *Sma*I site of pCP60 (kindly provided by C. Coronado and P. Ratet; *Bs4* transgene is under control of CaMV 35S promoter) yielding pVTSB1. We removed the 35S* promoter from the binary vector pVB60 (Van den Ackerveken *et al.*, 1996) yielding pVB61. Next, we cloned *Bs4* and 3.5 kbp upstream sequence into the *Sal*I–*Not*I sites of pVB61 yielding pVTSB3.

Construction of binary vectors containing avrBs4 *derivatives*

The binary vector pVB60 (Van den Ackerveken *et al.*, 1996) was used for *Agrobacterium*-mediated transient expression of *avr* derivatives. We assayed *avrBs4* (pVS200F, Ballvora *et al.*, 2001a), *avrBs4 Δ227* (pVS227, this study), and *avrBs4 Δ152* (pVS256F, this study). pVS227 and pVS256F originate from pAT227 (avrBs4 Δ227 in pUC118; Bonas *et al.*, 1993) and pUS256 (this study, see below for description), respectively. *Eco*RI/*Hin*dIII inserts from pAT227 and pUS256 were cloned into pBluescript KS yielding pBS227F and pBS256F, respectively. Subsequently *Eco*RI/*Xho*I fragments from pBS227F and pBS256F were transferred into pVB60 creating pVS227F and pVS256F, respectively.

Complementation studies

The binary vector pVTSB1, containing *Bs4* under transcriptional control of the CaMV 35S promoter, was transferred into *A. tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) and transformed into the previously described *bs4/bs4 L. esculentum* genotype MM^{bs4}–BC4 (Ballvora *et al.*, 2001a). Transformation and plant regeneration was performed as described by Ling *et al.* (1998). Transgenic plants were confirmed by PCR with primers for the neomycin phosphotransferase (*npt*II) gene and primers that distinguished between the transgenic *Bs4* and endogenous *bs4* sequences.

RACE

Total RNA was isolated using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and enriched for poly(A) RNA using the Oligotex mRNA Mini kit (Qiagen). RACE PCR was carried out using the SMART RACE cDNA Amplification Kit (Clontech, Heidelberg, Germany). Amplicons were cloned into pCR2.1 (Invitrogen, Karlsruhe, Germany) and sequenced using vector-specific primers.

Construction of an intron-deprived Bs4*-derivative*

An intron-deprived *Bs4* derivative was generated by amplification of exonic regions. Restriction sites present at the end of Bs4 exons were used to fuse PCR-amplified exons. Briefly, an *Xba*I – *Not*I fragment from pVTSB1 containing the *Bs4* genomic sequence was subcloned into pBluescript SK II (Stratagene, La Jolla, CA, USA) to yield pBlue:Bs4. A *Xba*I–*Kpn*I fragment of pBlue:Bs4 that contained all introns to be removed was subcloned into pUC119 (Vieira and Messing, 1987) to yield pUCSB6. A *Bs4* clone with pBluescript backbone lacking *Eco*RV, *Eco*RI, and *Pst*I was generated by *Eco*RV–*Sma*I digest, religation, and *Xba*I–*Not*I introduction of *Bs4* from pBlue:Bs4 into this vector to yield pBSB3. Primers I1fwd-*Eco*RV, I2rev-*Bst*XI, and I3rev-*Bgl*II and the corresponding reverse primers derived from the genomic *Bs4* sequence were used to amplify exonic regions from pBlue:Bs4 and introduce exon–exon junctions instead of introns. These amplicons replaced corresponding restriction fragments in pUCSB6 by *Eco*RV–*Sac*II, *Sac*II–*Bst*XI, and *Bst*XI–*Bgl*II digest, respectively, to create pUCSB6 Δ123. A *Xba*I–*Pst*I fragment from pUCSB6 Δ123 was then cloned into pBSB3, thus replacing the corresponding genomic *Bs4* fragment and creating pBSB5. pBSB5 represents an intron-deprived *Bs4* fragment containing flanking genomic sequences. A *Xba*I–*Not*I fragment from pBSB5 was subsequently cloned into the binary vector pCP60 for *in planta* assays.

Bs4 *pathway dissection by VIGS*

Expressed sequence tag (EST) database searches using *Arabidopsis* NDR1 (AI776252.1) as input returned a closely related tomato EST (EST257352). Based on this EST, we designed primers in regions of highest homology to *Arabidopsis* NDR1 (JP NDR 2: TATAATCTCGTCGTAACGAACACCTTTGTC and JP NDR 1: ACTGCAGGCTTAACAGCTCTCTTTATCTGG) and performed RT-PCR on *N. benthamiana* cDNA. We observed a 198-bp PCR fragment that was cloned into pGEM-T Easy vector (Promega, Mannheim, Germany). The 5′ and 3′ ends of *NbNDR1* were determined by RACE, using the MARATHON cDNA amplification kit (Clontech). A 480-bp *NbNDR1* fragment used to produce TRV:NbNDR1 was generated by PCR on a plasmid containing *NbNDR1* cDNA, using primers JP NDR 4: (TACCTGGCTTTTACCAAGGTCATGAC) and JP NDR 5: (CCAACTACAGTCAGTTTGCACCGA). We designed primers for amplification of *NbRar1* based on the *N. tabacum Rar1* mRNA sequence (AF480487). A 585-bp *NbRar1* fragment used to produce TRV:NbRar1 was generated by RT-PCR on *N. benthamiana* cDNA, using primers Rar1-f2 (TGCACTTATCACGAATCCGG) and Rar1-r2 (TGGGCTGGCGTTGTGCCATCCTG). Construction of TRV:EDS1 and TRV:SGT1 has been described before (Peart *et al.*, 2002a,b). Infection of plants with TRV derivatives was performed by agroinfiltration as described previously by Peart *et al.* (2002a).

Sequence analysis of the L. esculentum *cv. MM* Bs4 *and* L. pennellii *LA2963* bs4 *genes*

The *Bs4* sequence from *L. esculentum* cv. VFNT Cherry was obtained from cosmid T2-2. Corresponding sequences from *L. esculentum* cv. MM (*Bs4*), and *L. pennellii* LA2963 (*bs4*) were determined by sequencing of respective PCR products. Sequences were obtained using the BigDye Terminator Kit (PE Biosystems, Foster City, CA, USA) and analyzed using Sequencher 4.0 (Gene Codes Corp., Ann Arbor, MI, USA).

Y2H interaction studies

For Y2H studies, the yeast interaction trap was used (Gyuris *et al.*, 1993). Bait and prey plasmids were co-transformed into yeast strain EGY48 (Estojak *et al.*, 1995) containing the *lacZ*-reporter plasmid pSH18-34. Transformants grown on selective glucose medium were transferred on galactose medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to determine β-galactosidase (*LacZ*) activity, and on galactose plates lacking leucine to measure *Leu2* expression. Quantitative assays were performed on liquid cultures. Three individual yeast transformant colonies for each construct were inoculated into liquid glucose medium and incubated overnight (o.n.) at 30°C. The culture was diluted in galactose medium inducing the expression of the prey and grown until an OD_{600} of 0.5–1.0. β-galactosidase activity was assayed using *o*-Nitrophenyl β-D-galactopyranoside as described previously by Ausubel *et al.* (1996). For Y2H analysis, *avrBs4* and *Bs4* were cloned into bait (pYB) and prey (pYP) vectors. Full-length AvrBs4 autoactivated transcription in yeast and to overcome this problem, we cloned the N-terminal deletion derivative AvrBs4 Δ152 (lacking aa residues 1–152) into bait (pYB256) and prey (pYP256) vectors, respectively. The N-terminal deletion was generated by replacing the *Stu*I/*Hin*dIII fragment of pUS356 (contains N-terminal deletion of avrBs3, Szurek *et al.*, 2001) with the corresponding fragment of pAT200 (contains avrBs4, Bonas *et al.*, 1993), yielding pUS256 (*avrBs4 Δ152* in pUC118). The *Eco*RI/*Xho*I fragment from pUS256 was transferred into pEG202 and pJG4-5 (OriGene Technologies Inc., Rockville, MD, USA) yielding pYB256 and pYP256. *Bs4* domains were amplified from cDNA using primers annealing adjacent to TIR (Bs4-TIR-fwd, Bs4-TIR-rev), NB (Bs4-NB-fwd, Bs4-NB-rev) and LRR (Bs4-LRR-fwd, Bs4-LRR-rev) or combinations, all containing *Mun*I/*Sal*I sites that allowed cloning into *Eco*RI/*Xho*I of pEG202 and pJG4-5. The previously described Caimpα1 (Szurek *et al.*, 2001) was used as a positive control for Y2H studies. In all cases, expression and stability of fusion proteins was confirmed by immunoblotting using a monoclonal anti-LexA antibody (Clontech Laboratories) and monoclonal anti-HA antibody (3F10, Roche, Mannheim, Germany), respectively.

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The GenBank accession number for the *N. benthamiana NDR1* and *Rar1* sequence is AY438029 and AY438026, respectively. The GenBank accession number for *L. esculentum* cv. MM *Bs4* and *L. pennellii* LA2963 *bs4* is AY438027 and AY438028, respectively.