Genetic Mapping and Functional Analysis of the Tomato *Bs4* Locus Governing Recognition of the *Xanthomonas campestris* pv. *vesicatoria* AvrBs4 Protein

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Xanthomonas campestris pv. *vesicatoria* is the causal agent of bacterial spot disease on pepper (*Capsicum* spp.) and tomato (*Lycopersicon* spp.). Analysis of 17 different *Lycopersicon* accessions with *avrBs4*-expressing *X. campestris* pv. *vesicatoria* strains identified 15 resistant and two susceptible tomato genotypes. Genetic analysis revealed that AvrBs4 recognition in tomato is governed by a single locus, designated *Bs4* (bacterial spot resistance locus no. 4). Amplified fragment length polymorphism and bulked DNA templates from resistant and susceptible plants were used to define a 2.6-cM interval containing the *Bs4* locus. A standard tomato mapping population was employed to localize *Bs4*-linked markers on the short arm of chromosome 5. Investigation of *X. campestris* pv. *vesicatoria* *hrp* mutant strains revealed that AvrBs4 secretion and avirulence activity are *hrp* dependent. Agrobacterium-based delivery of the *avrBs4* gene into tomato triggered a plant response that phenotypically resembled the hypersensitive response induced by *avrBs4*-expressing *X. campestris* pv. *vesicatoria* strains, suggesting symplastic perception of the avirulence protein. Mutations in the *avrBs4* C-terminal nuclear localization signals (NLSs) showed that NLSs are dispensable for *Bs4*-mediated recognition. Our data suggest that tomato *Bs4* and pepper *Bs3* employ different recognition modes for detection of the highly homolo-

gous *X. campestris* pv. *vesicatoria* avirulence proteins AvrBs4 and AvrBs3.

Additional keywords: *avrBs3-2*, *avrBsP*, bulked segregant, gene-for-gene, positional cloning, type III secretion.

Plants must defend themselves against a broad array of parasitic organisms, including fungi, viruses, nematodes, and bacteria (McDowell and Dangl 2000). Resistance to invaders often is determined by the presence of a resistance (*R*) gene in the plant and a corresponding avirulence (*avr*) gene in the pathogen (Flor 1971). A biochemical interpretation of this gene-for-gene model is the receptor–ligand model in which the specific binding of the pathogen Avr product to the plant *R* protein represents the decisive step that activates a defense signaling pathway (Keen 1990).

Molecular isolation of plant *R* genes conferring resistance to bacterial pathogens revealed that the vast majority of the corresponding proteins are predicted to be intracellular. How intracellular *R* receptors interact with Avr ligands from extracellular bacterial pathogens was puzzling initially, but became clearer with the discovery of the bacterial type III secretion machinery (Alfano and Collmer 1997; Collmer 1998; Galán and Collmer 1999; He 1998; Lindgren 1997). The hypersensitive response and pathogenicity (*hrp*) gene-encoded proteins secrete and probably deliver bacterial effector proteins into the plant cell (Galán and Collmer 1999). Consistent with this concept is substantial evidence that Avr proteins are perceived by *R* genes when expressed in the host cell (Bonas and Van den Ackerveken 1997). Whether Avr and *R* proteins generally interact directly to induce *R*-dependent responses remains an open question. Some Avr proteins have been shown to interact with their cognate *R* gene products and provide supporting evidence for the receptor–ligand model (Jia et al. 2000; Leister and Katagiri 2000; Scofield et al. 1996; Tang et al. 1996). In spite of substantial efforts, however, physical interaction has not been observed for the majority of matching Avr–*R* protein pairs.

We study tomato and pepper bacterial spot disease, which is caused by the gram-negative bacterium *Xanthomonas campe-*

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tris pv. *vesicatoria* (Bonas et al. 2000). In particular, our interest is in the *R* gene-mediated perception of *avrBs3* and *avrBs4* (previously designated *avrBs3-2*) (Bonas et al. 1993) and *avrBsP* (Canteros et al. 1991), two members of the *Xanthomonas avrBs3* family, sharing 97% sequence identity on the amino acid level (Bonas et al. 1993). Recognition triggers the hypersensitive response (HR), a rapid cell death reaction that coincides with the arrest of bacterial growth. In spite of their pronounced similarities, AvrBs3 and AvrBs4 are recognized specifically by corresponding host surveillance systems in pepper and tomato, respectively (Bonas et al. 1993; Pierre et al. 2000; Van den Ackerveken et al. 1996). Members of the *Xanthomonas* spp. *avrBs3* gene family, named after its first sequenced member (Bonas et al. 1989), show a 90 to 97% sequence identity on the amino acid level and contain, in their central portion, nearly perfect copies of a 34 amino acid repeat motif (Gabriel 1999; Hopkins et al. 1992). Variability within the *avrBs3* gene family is most pronounced in the repeat region (Gabriel 1999). Exchanging the repeat domain alters the avirulence specificity of the protein to the specificity of the gene from which the domain was derived (Yang et al. 1994; Zhu et al. 1998). Likewise, deletions within the repeat domain can give rise to new specificities (Herbers et al. 1992). This indicates that the repeats determine recognition specificity. The repeat composition suggests a structural rather than enzymatic function for these proteins, supporting the hypothesis that they may interact directly with R proteins (Herbers et al. 1992). Their abundance and highly conserved structure should make *avrBs3* family members a fruitful object in which to study the specificity of *R* gene-mediated Avr protein perception. Indeed, *R* gene-based recognition of several *avrBs3* homologs has been analyzed (Vivian and Gibbon 1997). The molecular isolation of a corresponding *R* gene, however, has not yet been reported.

Previously, we found that *avrBs4* triggers the HR on tomato (Bonas et al. 1993), although the genetic basis of this resis-

tance was not investigated. Here, we describe the genetic mapping of a tomato *R* gene, *Bs4*, conferring specific recognition of AvrBs4. Comparison of tomato *Bs4* and pepper *Bs3* with the use of *avrBs3*- and *avrBs4*-deletion derivatives suggests different *R* gene-mediated recognition modes in tomato and pepper.

RESULTS

Resistance to *avrBs4* expressing *Xanthomonas* spp. strains is inherited as a dominant, monogenic trait.

Previous analysis of the interaction between *X. campestris* pv. *vesicatoria* strains 75-3 and 87-7 and their host plant, tomato, revealed that expression of the avirulence gene *avrBs4* triggers the HR, which is indicative of resistance, in the *Lycopersicon* (*L.*) *esculentum* cultivars Bonny Best and Money-maker (Bonas et al. 1993; Canteros et al. 1991). Because genetic dissection of plant disease resistance requires resistant and susceptible hosts, different accessions of the *Lycopersicon* species (*L. esculentum*, *L. cheesmanii*, *L. peruvianum*, *L. pimpinellifolium*, and *L. pennellii*) were tested for the absence and/or presence of the HR upon infection with *avrBs4* expressing *X. campestris* pv. *vesicatoria* strains (Fig. 1). The appearance of water-soaked lesions, characteristic for the compatible interaction (Rudolph 1993), was detected only in the *L. pennellii* accessions LA2963 and LA1282. Inheritance of AvrBs4-induced HR in tomato was subsequently studied in a cross between Money-maker (resistant parent) and LA 2963 (susceptible parent). F₁ descendants displayed a resistant phenotype. The corresponding F₂ progeny segregated into resistant and susceptible individuals by 3:1 (Table 1), indicating the presence of a dominant monogenic resistance gene. As expected for the inheritance of a monogenic dominant trait, 1:1 segregation was observed for descendants of a cross between an F₁ plant and the susceptible parent (Table 1). Analogous to the previously characterized gene-for-gene interaction

Fig. 1. Phenotype of different *Lycopersicon* spp. accessions after *Xanthomonas campestris* pv. *vesicatoria* infiltration. Leaves of tomato accessions **A**, *L. pennellii* LA 2963; **B**, *L. esculentum* cv. Money-maker; and **C**, MM^{bs4}-BC4 were inoculated with the wild-type strain *X. campestris* pv. *vesicatoria* 75-3 (–) and *X. campestris* pv. *vesicatoria* 75-3 expressing *avrBs4* (+). Photographs were taken 3 days after inoculation.

Table 1. Segregation ratios for *Bs4*-mediated resistance

Progeny type	No. of plants	Phenotype	Observed	Expected	χ^2 /degree of freedom
F ₂	132	Resistant	92	99	1.97/1
		Susceptible	40	33	
Backcross individuals	92	Resistant	51	46	1.08/1
		Susceptible	41	46	

between *Xanthomonas* spp. *avrBs3* and pepper *Bs3* (Minsavage et al. 1990; Pierre et al. 2000), the tomato *R* gene, which confers recognition of the *avrBs4* encoded protein, was designated *Bs4* (bacterial spot *R* gene no. 4).

Amplified fragment length polymorphism (AFLP) markers genetically linked to the *Bs4* locus.

AFLP analysis (Vos et al. 1995) of bulked segregant pools (Giovannoni et al. 1991; Michelmore et al. 1991) was used to identify *Bs4*-linked DNA markers. AFLP fragments were compared between the resistant parent and four pools, each consisting of ten susceptible F_2 segregants. The utilization of four DNA bulks rather than one was chosen in order to minimize the size of the target interval and avoid a time-consuming analysis of non-tightly linked AFLP loci. *Bs4*-linked AFLP markers in the resistant parent were indicated by the absence of the corresponding polymerase chain reaction fragment in the susceptible pools (Fig. 2). Employing the enzyme combination *Pst*I–*Mse*I, 243 primer combinations (PCs) were analyzed, generating an average of 80 scorable AFLP fragments per PC and displaying a genome-wide polymorphism of 35% between the parental lines Moneymaker and LA 2963. The experimental setup allowed identification only of the markers linked in *cis* with *Bs4*, thereby reducing the amount of scorable fragments by 50%. Considering each AFLP fragment as one genetic locus and, neglecting possible allelism between fragments, approximately 3,400 ($243 \times 80 \times 0.35 \times 0.5$) polymorphic loci have been inspected. We detected six fragments in the resistant parent that were absent from all four susceptible bulks, indicating linkage to the *Bs4* locus (Fig. 2). Analysis of these AFLP fragments on the pool individuals revealed recombination events, enabling us to position the DNA markers with respect to *Bs4* (Fig. 3A). Linkage of the observed markers were 1.3 (P11M6, P12M3, and P15M1) to 3.9 cM (P11M8, P15M2, and P15M8), and a 2.6-cM target interval containing the *Bs4* locus (P11M6 to P12M3 and P15M1) could be defined.

Integration of *Bs4*-linked AFLP markers in a genome-wide tomato linkage map.

The most closely *Bs4*-linked AFLP markers on either side of the target gene (P11M6, P12M8, and P15M1) were cloned, and the identity of cloned DNA fragments with desired AFLP markers was confirmed by reverse AFLP, as previously described (Pierre et al. 2000). Hybridization-based linkage analysis was employed to localize the *Bs4* locus in a genome-wide restriction fragment length polymorphism (RFLP) map. Linkage mapping was performed on 42 F_2 individuals of a standard tomato mapping population (Tanksley et al. 1992) (Fig. 3B). Marker P11M6 was located between the RFLP markers TG432 and CT16. The AFLP locus P12M3 was positioned distal to P11M6, cosegregating with the RFLP marker CT53. Mapping of the AFLP marker P15M1 revealed two cross-hybridizing polymorphic loci in the tomato genome. One copy of P15M1, P15M1(A), was located distal to P11M6 and cosegregated with CT242. The second copy of P15M1, P15M1(B), was located proximal to P11M6 and cosegregated with TG96. This position is not in agreement with the AFLP map (Fig. 3A), indicating that P15M1(B) does not correspond to AFLP locus P15M1. Taken together, the linkage mapping indicates that the *Bs4* gene is located distally on the short arm of chromosome 5.

RFLP markers genetically linked to the *Bs4* locus.

Positional cloning of the *Bs4* locus, our long-term goal, requires marker saturation in the respective chromosome segment. To increase marker density in the vicinity of *Bs4*, we selected RFLP probes out of the high-density tomato linkage map (Tanksley et al. 1992) within the marker interval P12M3–P11M6 (CT242, TG598, TG441, and TG432) and mapped them with respect to *Bs4* (Fig. 3C). RFLP probes CT242, TG441, and TG598 were separated from *Bs4* by one recombination event within 40 F_2 segregants, cosegregating with AFLP markers P12M3 and P15M1 on the basis of *Eco*RV, *Dra*I, and *Hae*III polymorphisms, respectively. RFLP marker TG432 cosegregated with *Bs4* based on *Eco*RV-generated polymorphisms.

Introgression of the *bs4* allele into Moneymaker.

Because the availability of congenic lines is advantageous for in-depth studies of a given trait, the generation of *Bs4* and *bs4* containing near-isogenic lines was initiated. Furthermore, because LA 2963 (susceptible parent) generally produces small quantities of seeds, Moneymaker (resistant parent) was chosen as the recurrent parent. Introgression of the *bs4* allele into the resistant parent was performed as follows: descendants derived from a cross between Moneymaker (*Bs4/Bs4*) and LA 2963 (*bs4/bs4*) were selfed and susceptible F_2 offspring were backcrossed to the resistant parent. Selfing, selection of susceptible offspring, and backcrossing to the recurrent parent was repeated four times and resulted in the susceptible line MM^{*bs4*}–BC4, which displayed the morphological features

Fig. 2. Identification of *Bs4*-linked amplified fragment length polymorphism (AFLP) markers. Autoradiograph shows the AFLP fragments of resistant parent (P_R) and four susceptible pools ($Pools_S$) with the *Pst*I–*Mse*I AFLP primer combination P11M6 and P15M1. *Bs4*-linked AFLP fragments are indicated by arrows.

of the resistant parent (Fig. 1C). The *bs4/bs4* introgression in MM^{*bs4*}-BC4 plants was confirmed by fingerprint analysis with the *Bs4*-linked AFLP markers P11M6 and P15M1.

***Bs4*-mediated recognition of AvrBs4 occurs inside the host plant cell.**

Most bacterial *avr* genes, including *avrBs3*, require *hrp* genes to induce the HR on their respective host plants (Knoop et al. 1991; Leach and White 1996). To test whether AvrBs4-triggered HR is *hrp* dependent, tomato plants were inoculated with a *X. campestris* pv. *vesicatoria* *hrp* mutant strain (828Δ*hrcV*) (Rossier et al. 1999) and the corresponding isogenic wild-type strain (82-8) carrying *avrBs4* on an endogenous plasmid (Minsavage et al. 1990). In contrast to the wild type, the *hrp* mutant did not elicit the HR on tomato genotype *Bs4* (data not shown). These results show that functional *hrp* genes are required for avirulence activity of AvrBs4.

Because *X. campestris* pv. *vesicatoria*-*hrp* genes encode a type III secretion machinery, a recently established in vitro secretion system (Rossier et al. 1999) was employed to test

for AvrBs4 secretion. Western blot analysis was performed on the type III mutant strain 85*Δ*hrcV*(pDS200F) and the corresponding isogenic wild-type strain 85*(pDS200F), both expressing *avrBs4* from a plasmid. The intracellular HrcN protein (Rossier et al. 1999) was used as a control for the absence of lysis (data not shown). AvrBs4 was detected in the culture supernatant of the wild type but not the *hrp* mutant (Fig. 4), indicating that AvrBs4 secretion is *hrp* dependent.

To determine whether *Bs4*-mediated AvrBs4 perception occurs inside the plant cell, *Agrobacterium tumefaciens*-based gene delivery was employed. Transient expression of *avrBs4* in tomato leaves induced the HR on genotype *Bs4* (Fig. 5). In contrast, no HR was observed with the isogenic *A. tumefaciens* strain lacking *avrBs4* (Fig. 5) or on tomato genotype *bs4* (data not shown).

Functional analysis of *Bs4*-mediated resistance.

AvrBs4 and AvrBs3 are highly similar avirulence proteins (97% identity on amino acid level) (Bonas et al. 1993), triggering *Bs4*- and *Bs3*-specific HR induction, respectively. The

Fig. 3. Linkage mapping at the *Bs4* locus. **A**, Amplified fragment length polymorphism (AFLP) map displaying the position of *Bs4*-linked AFLP loci. Figures in square brackets correspond to the number of identified informative recombinants. **B**, Integration of *Bs4*-linked AFLP markers into a genome-wide tomato linkage map. P15M1(A) and P15M1(B) refer to two different copies of AFLP marker P15M1. **C**, Map positions of tomato restriction fragment length polymorphism markers with respect to *Bs4*. All maps are drawn to scale. Horizontal lines indicate the positions of respective marker loci. Employed mapping populations are indicated.

central region of both avirulence proteins is composed of 17.5 nearly identical copies of a 34 amino acid sequence. Differences between AvrBs4 and AvrBs3 reside exclusively in the repeat region, with the exception of a four amino acid deletions in the C terminus of AvrBs4, with respect to AvrBs3 (Fig. 6) (Bonas et al. 1993). Striking similarities between AvrBs3 and AvrBs4 led us to consider whether the mode of recognition by the corresponding plant *R* genes, tomato *Bs4*, and pepper *Bs3* is functionally conserved. This was addressed by the use of congenic resistant and susceptible lines in combination with *X. campestris* pv. *vesicatoria* strains expressing different *avrBs4* and *avrBs3* derivatives.

First, the C-terminal region of *avrBs3* was replaced with the corresponding region of *avrBs4* to create *avrBs3*-CT2 (Fig. 6). The chimeric protein triggered *Bs3*- but not *Bs4*-mediated HR induction, confirming the previous finding that recognition specificity resides within the repeat region (Herbers et al. 1992). In addition, avirulence activity of the chimeric protein, which carries a four amino acid deletion with respect to AvrBs3, indicates that the C-terminal parts of AvrBs3 and AvrBs4 are functionally interchangeable.

Previous analysis of *Bs3*-mediated resistance revealed that functional nuclear localization signals (NLSs) located in the C terminus of AvrBs3 are required for *Bs3*-specific HR induction (Fig. 6) (Van den Ackerveken et al. 1996). To determine whether NLSs in AvrBs4 are needed for induction of *Bs4*-mediated HR, the C-terminal portion of AvrBs4 was replaced with the corresponding region of AvrBs3 Δ 1-3 to create AvrBs4 Δ 1-3. Interestingly, the chimeric protein retained the ability to trigger *Bs4*-mediated HR, in contrast to AvrBs3 Δ 1-3, which no longer triggers a *Bs3*-specific HR (Fig. 6). The fact that HR induction by pepper *Bs3* and not tomato *Bs4* is dependent on functional NLSs in the corresponding avirulence protein suggests different recognition mechanisms for highly similar avirulence proteins in pepper and tomato.

DISCUSSION

Bs4 and *Bs3* are located in nonsyntenic genome segments.

Phenotypic analysis of F_2 descendants from the interspecific cross between Moneymaker and LA 2963 indicate that the

avrBs4-triggered HR in tomato is determined by a single dominant locus, designated *Bs4*. Employing a bulked-segregant approach, we identified *Bs4*-linked AFLP loci that were subsequently mapped to the short arm of tomato chromosome 5.

Tomato *Bs4* and pepper *Bs3* recognizing highly similar Avr proteins may be caused by the convergent evolution of unrelated plant receptors. Alternatively, both *R* genes represent variants of a common ancestor. If tomato *Bs4* and pepper *Bs3* are orthologous genes with functional analogy, they should be located in colinear chromosome segments, as shown previously for maize *Hm1* and *Hm2* (Multani et al. 1998) and Arabidopsis *GAI* (Peng et al. 1999). Moreover, comparative mapping on a genomewide scale has shown a high degree of colinearity between the tomato and pepper genomes (Livingstone et al. 1999), providing the necessary framework to analyze colinearity of potential orthologs. Yet, *Bs3*-linked probes were localized on tomato chromosome 2 (Pierre et al. 2000), unlinked to the tomato *Bs4* locus, suggesting that *Bs4* and *Bs3* are not derived from a common ancestor.

In accordance with our observation of two functionally related *R* genes in nonsyntenic genome segments, a case study at the *Sw-5* locus showed that phenotypically defined resistances to the same pathogen were not located in correlated positions in tomato and pepper (Jahn et al. 2000). Systematic analysis of phenotypically defined *R* genes in the *Solanaceae* genera tomato, potato, and pepper also failed to identify an *R* gene whose homolog cosegregated with resistance of similar pathogen specificity in another genus (Grube et al. 2000). A comparison of orthologous genome segments at the sequence level revealed that nonsyntenic gene order may pertain, in particular, to disease resistance genes (Devos and Gale 2000; Keller and Feuillet 2000; Michelmor 2000; Schmidt 2000). Thus, the lack of colinearity between the *Bs3*- and *Bs4*-containing marker intervals could be explained by a lack of colinearity on the microstructural level, as reported for the barley *Rpg1* locus (Kilian et al. 1997), or alternatively as an indication that these *R* genes are not orthologous. Elucidation of the evolutionary relationship between *Bs3* and *Bs4*, however, will require the isolation of both *R* genes.

Fig. 4. *hrp*-dependent secretion of AvrBs4. Total protein (T) and supernatants (SN) of *Xanthomonas campestris* pv. *vesicatoria* 85*(pDS200F) (lanes 1 and 4), *hrp*-mutant strain *X. campestris* pv. *vesicatoria* 85* Δ *hrpV*(pDS200F) (lanes 2 and 5) and *X. campestris* pv. *vesicatoria* 85*(pDSK602; vector control) (lane 3) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) and analyzed by immunoblotting with anti-AvrBs3 antibody. The AvrBs4-specific signal is indicated by an arrow.

Fig. 5. *Agrobacterium tumefaciens*-mediated transient expression of AvrBs4 in tomato. *A. tumefaciens* strain GV3101 (-) and the transconjugant GV3101 (pVS200F) (+) carrying *avrBs4* were infiltrated into *Lycopersicon esculentum* cv. Moneymaker leaves. Photograph was taken 3 days after inoculation.

***Bs4*-cosegregating marker TG432 detects a major resistance gene cluster in potato.**

R gene classification by function can sometimes present a misleading argument against orthology because the tomato *Mi* locus confers resistance to pathogens with no apparent taxonomic relationship such as nematodes and aphids (Rossi et al. 1998; Vos et al. 1998). Hence, tomato-*Bs4* orthologs in other plant genera might confer resistance to pathogens other than *Xanthomonas* spp. In particular, the *Bs4*-linked probe TG432 is linked to a major *R* gene cluster in potato, conferring resistance to fungi, viruses, and nematodes (De Jong et al. 1997; Grube et al. 2000; Rouppe van der Voort et al. 1998; Rouppe van der Voort et al. 1999). Potato *Rx2* and *Gpa2* represent two cloned *R* genes within this cluster (Bendahmane et al. 2000; Van der Vossen et al. 2000) and, similar to *Bs4*, show linkage with DNA marker TG432 (De Jong et al. 1997; Rouppe van der Voort et al. 1999). Given that *Rx2*, *Gpa2*, and *Bs4* reside in colinear genome segments in potato and tomato, respectively, it is possible that these genes emerged from a common ancestor.

AvrBs4 acts inside the plant cell to elicit hypersensitive cell death.

Avirulence activity of the vast majority of bacterial *avr* genes is dependent on functional *hrp* genes (Holt et al. 2000; Kjemtrup et al. 2000; Leach and White 1996). We found that the AvrBs4-triggered HR in *Bs4* genotypes is *hrp* dependent, which is in agreement with findings for other *avrBs3* family members (Knoop et al. 1991; Yang and Gabriel 1995).

Expression of AvrBs4 in tomato genotype *Bs4* with *A. tumefaciens* for gene delivery resulted in a plant response that phe-

notypically resembled the HR induced by *X. campestris* pv. *vesicatoria* expressing *avrBs4*. This suggests that the perception of AvrBs4 by *Bs4* occurs inside the host cell. Agrobacterium-mediated expression of other AvrBs3 family members in pepper (Van den Ackerveken et al. 1996) and cotton (De Feyter et al. 1998) also resulted in gene-for-gene-based HR induction. Symplastic perception is probably not a peculiarity of AvrBs3 homologs but rather appears to be a universal principle in *R* gene-mediated recognition of bacterial Avr determinants (Bonas and Van den Ackerveken 1997). The leucine-rich repeat domain of rice Xa21 conferring resistance to the *Xanthomonas oryzae* pv. *oryzae*, however, is predicted to be extracellular (Song et al. 1995), suggesting that the perception of bacterial Avr determinants generally is not intracellular.

Requirement of NLSs in the recognition of AvrBs3 homologous proteins.

All members of the *avrBs3* gene family contain monopartite NLSs in their C terminus (Gabriel 1997; Gabriel 1999). Functionality of the NLSs has been demonstrated for AvrBs3 family members AvrBs3, PthA, and AvrB6 (Van den Ackerveken et al. 1996; Yang and Gabriel 1995). Further analysis of AvrBs3 revealed a direct correlation between nuclear localization activity of various *avrBs3* mutant alleles and their capacity to induce the HR in pepper plants containing the *Bs3* resistance gene (Van den Ackerveken et al. 1996). Therefore, our finding that the C-terminal regions of AvrBs3 and AvrBs4 are functionally interchangeable with respect to *Bs3*-mediated resistance suggests that the NLSs in AvrBs4 are functional and that AvrBs4 is targeted to the plant nucleus.

<i>avrBs3</i> / <i>avrBs4</i> derivatives		<i>R</i> gene	
designation	structure	<i>Bs3</i> ^a	<i>Bs4</i> ^b
		HR induction	
<i>avrBs3</i>		+	-
<i>avrBs3Δ1-3</i>		-	-
<i>avrBs3-CT2</i>		+	-
<i>avrBs4</i>		-	+
<i>avrBs4Δ1-3</i>		-	+

Fig. 6. Recognition of AvrBs3 and AvrBs4 derivatives. Plants were infiltrated with *Xanthomonas campestris* pv. *vesicatoria* transconjugants expressing the depicted *avrBs4* or *avrBs3* derivatives. +/– indicates presence/absence of hypersensitive response (HR) induction, respectively. Hatched areas refer to regions of identical amino acid sequence within AvrBs3 and AvrBs4. White-black color indicates AvrBs3- or AvrBs4-derived stretches, respectively. Boxed areas in the central part of the protein represent the repeat region. Diamonds represent previously described nuclear localization signal motifs (Van den Ackerveken et al. 1996). Triangle depicts a deletion of four amino acids present in AvrBs4 with respect to AvrBs3. ^a*Bs3*-specific HR induction on pepper was determined on ECW-30R (*Bs3*) and the near-isogenic line (NIL) ECW (*bs3*). ^b*Bs4*-specific HR induction was tested by analyzing *Lycopersicon esculentum* cv. Moneymaker (*Bs4*) and the NIL MM^{bs4}–BC4 (*bs4*). ^cDescribed previously in Van den Ackerveken et al. (1996).

Mutational studies of the AvrBs3 family members AvrBs3, AvrXa7, AvrXa10, and AvrB6 revealed that their gene-for-gene-based recognition by cognate *R* gene products depends upon functional NLSs (Gabriel et al. 1996; Van den Ackerveken et al. 1996; Yang et al. 2000; Zhu et al. 1999). These findings indicate that the destination of bacterial Avr proteins within the host cell and the site of molecular recognition by the corresponding *R* gene product coincide, as previously suggested by Parker and Coleman (1997). The most recent support for spatial interdependency of Avr and *R* gene-encoded proteins is provided by the *Pseudomonas* spp. avirulence proteins AvrB and AvrRpm1. Both Avr proteins have been detected in the plant plasma membrane (Nimchuk et al. 2000) as their corresponding Arabidopsis *R* protein, RPM1 (Boyce et al. 1998). Our data suggest, however, that *Bs4*-mediated resistance does not comply with the model that *R*-protein localization is dictated by the Avr protein destination on the basis that NLS-deletion derivatives of AvrBs4, which probably do not localize to the nucleus, are still able to induce *Bs4*-mediated resistance. We hypothesize, therefore, that AvrBs4 recognition occurs before the protein has reached the nucleus. It is necessary to emphasize that the NLS-independent recognition mode is a feature of the *Bs4*-encoded *R* protein and not a peculiarity of AvrBs4. This is supported by a detailed investigation of the avirulence activity of AvrB6, a well-studied member of the AvrBs3 family. Mutations in the NLSs of AvrB6 abolished HR in cotton but did not affect recognition on bean (Gabriel et al. 1996). We predict two *R* gene classes recognizing AvrBs3 homologous proteins that are either NLS dependent ("*Bs3* type") or NLS independent ("*Bs4* type"). Whether proteins of the two categories are, in fact, localized in different cell compartments has yet to be determined.

MATERIALS AND METHODS

avrBs3/avrBs4 domain swaps.

The *avrBs3-2* gene (Bonas et al. 1993) was designated *avrBs4* when correspondence with the tomato *Bs4* locus was demonstrated. A 3,386-bp *PstI*–*SacI* fragment carrying almost the entire *avrBs4* coding sequence was released from pAT200 (Bonas et al. 1993) and transferred into pUS300F (Van den Ackerveken et al. 1996). *avrBs3* sequences were removed from pUS300F, resulting in pUS200F. The differences between pUS200F and pUS300F are exclusively within the exchanged fragment. The NLS modifications, previously engineered into *avrBs3* (Van den Ackerveken et al. 1996), were introduced into *avrBs4* by exchanging the *HincII*–*HindIII* fragments from pUS300FΔ1-3 into pUS200F. The *EcoRI*–*HindIII* insert from the resulting plasmid pUS200Δ1-3 was cloned into the broad-host expression vector pDSK602 (Murillo et al. 1994), resulting in pDSK200Δ1-3. To construct *avrBs3*–CT2, the *HincII*–*HindIII* fragment from *avrBs4* (containing the region after the repeats) was excised from pBS200F and cloned in *HincII*–*HindIII*-digested pBS300F, yielding pDSK300CT2. The *avrBs3*-derivatives pDSK300 (*avrBs3*) and pDSK300Δ1-3 (*avrBs3*Δ1-3), have been described previously (Van den Ackerveken et al. 1996). Plasmids were introduced from *Escherichia coli* into *X. campestris* pv. *vesicatoria* strains 75-3, 56, and 85-10 by triparental mating, as described (Bonas et al. 1989).

Plant material and *X. campestris* pv. *vesicatoria* inoculations.

Inoculation of *Lycopersicon* accessions with *avrBs4*-expressing *X. campestris* pv. *vesicatoria* strains revealed a rapid HR induction in the genotypes Bonny Best (NSL 5791); Moneymaker (NSL 27107); Lyc1714, T1101, T1102, T91, Lyc476, T1534, T999 (all *L. esculentum*); T666 (*L. peruvianum*); T641, T1127, T1041, T97 (all *L. pimpinellifolium*); T675 (*L. cheesmanii*); and LA716 (*L. pennellii*). No HR induction was observed on LA2963 and LA1282 (both *L. pennellii*).

Plants were grown and inoculated as described previously (Bonas et al. 1989; Bonas et al. 1993). The concentration of the inoculum was approximately 10⁸ CFU/ml in 1 mM MgCl₂. Symptoms were scored over a period of 2 to 5 days postinoculation. *X. campestris* pv. *vesicatoria* strains 75-3 and 56 (Bonas et al. 1989; Minsavage et al. 1990; Stall et al. 1994), carrying and/or lacking the *avrBs4* gene, were inoculated into tomato. 82-8Δ*hrcV* (Rossier et al. 1999) is a *hrp* mutant derivative of 82-8 (Minsavage et al. 1990). Inoculations on pepper were performed with *X. campestris* pv. *vesicatoria* 85-10 (Minsavage et al. 1990). *avrBs3* and *avrBs4* derivatives were introduced into *X. campestris* pv. *vesicatoria* the described pDSK602 derivatives.

Agrobacterium-mediated transient expression.

The binary vector pVB60 (Van den Ackerveken et al. 1996) was used for Agrobacterium-mediated transient expression assays. The *avrBs4* gene was isolated from pUS200F by digestion with *EcoRI* and *HindIII* and cloned into pBluescript KS to yield pBS200F. The *EcoRI*–*XhoI* fragment was cloned into pVB60, yielding pVS200F. The plasmid was conjugated into *A. tumefaciens* GV3101 (Van Larebeke et al. 1974). Infiltration of plants was performed as described previously (Van den Ackerveken et al. 1996).

Plant DNA isolation and nucleic acid hybridization.

Tomato genomic DNA was extracted as described previously (Bernatzky and Tanksley 1986). For Southern hybridization, approximately 4 μg of DNA was digested with *DraI* (New England Biolabs, Frankfurt, Germany), *HaeIII*, or *EcoRV* (Life Technologies, Karlsruhe, Germany), according to the supplier's instructions. Electrophoretic separation of DNA fragments and transfer to membranes were performed as described (Sambrook et al. 1989). Hybridization conditions were according to Church and Gilbert (1984). The probe was labeled with α³²P dCTP (ICN Biomedicals, Costa Mesa, CA, U.S.A.) and the Megaprime DNA labeling kit (Amersham, Freiburg, Germany). Filters were washed twice with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) (15 min. at 65°C), once with 0.1× SSC and 0.1% SDS (15 min. at 65°C), and exposed to X-ray film.

Construction of DNA pools, AFLP analysis, and cloning AFLP markers.

Four susceptible (S1, S2, S3, and S4) DNA pools were constructed. Each contained bulked DNA samples from ten susceptible F₂ individuals, respectively. AFLP analysis was performed, essentially as described by Vos et al. (1995), with the enzyme combination *PstI*–*MseI*. Endonuclease-digested tomato genomic DNA was ligated to *PstI*–*MseI* adapters and

amplified with (*MseI*+1) and (*PstI*+0) primers. Selective amplification was performed with nonlabeled *MseI*+3 and ³³P-labeled *PstI*+2 oligonucleotides. Visualization and cloning of AFLP products were performed as described previously (Pirre et al. 2000). Identified AFLP markers have been designated according to the selective oligonucleotides. *MseI*+3 primer M1 (–AAT), M2 (–ACA), M3 (–ACC), M6 (–AGC), and M8 (–AGA). *PstI*+2 primer P11 (–AA), P12 (–AC), P15 and (–CA). The genomewide polymorphism between the parental lines MoneyMaker and *L. pennellii* LA 2963 was determined to be 35% on the basis of the analysis of ten randomly chosen PCs.

Secretion assays.

Secretion assays were performed as described previously (Rossier et al. 1999) with *X. campestris* pv. *vesicatoria* 85* (Wengelnik et al. 1999) and the nonpolar *hrp* mutant *X. campestris* pv. *vesicatoria* 85* Δ *hrcV* (Rossier et al. 2000), both expressing *hrp* genes constitutively as a result of a mutation in the regulatory gene *hrpG*.

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