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Resistance in tomato to *Xanthomonas campestris* pv *vesicatoria* is determined by alleles of the pepper-specific avirulence gene *avrBs3*

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Abstract. Bacterial spot disease of tomato and pepper caused by *Xanthomonas campestris* pv *vesicatoria* is prevented by resistance genes in the plant that match genes for avirulence in the bacterium. Based on DNA homology to the avirulence gene *avrBs3*, which induces the resistance response on pepper, we have isolated another avirulence gene from *X. c. vesicatoria*, designated *avrBs3-2*. This gene differs in specificity from *avrBs3* in inducing the hypersensitive response on tomato but not on pepper. Sequence analysis of the *avrBs3-2* gene revealed a high degree of conservation: the 3480 bp open reading frame contains an internal region of 17.5 nearly identical 102 bp repeat units that differ in their order from those present in the *avrBs3* gene. The coding region is 97% identical to *avrBs3* and expresses constitutively a 122 kDa protein, thus representing a natural allele of this gene. The previously isolated 1.7 kb *avrBsP* gene from *X. c. vesicatoria* is 100% identical to the corresponding *avrBs3-2* sequence, indicating that these genes might be identical. Interestingly, derivatives of *avrBs3-2* lacking the C-terminal region and part of the repetitive region are still able to confer incompatibility in tomato. The *avrBs3-2* gene is compared with the sequence of *avrBs3* derivatives generated by deletion of repeat units that also have avirulence activity on tomato. Both genes, *avrBs3* and *avrBs3-2*, are flanked by a 62 bp long inverted repeat, which prompts speculations about the origin of the members of the *avrBs3* gene family.

Key words: Avirulence gene – *avrBs3* – Disease resistance – Tomato – *Xanthomonas campestris* pv *vesicatoria*

Introduction

Resistance of plants to microbial infection is often determined by gene-for-gene relationships. Genes for resistance in the plant are matched by corresponding genes for avirulence in particular races of the pathogen (race-specific resistance). Generally, the result of such an incompatible interaction is induction of the hypersensitive response (HR) in the challenged plant cells. The HR is a local defense reaction accompanied by rapid necrosis of the infected tissue which in bacterial diseases prevents multiplication of the pathogen in the infected region (Klement 1982). In the absence of dominant alleles for resistance in the host or for avirulence in the pathogen, plant tissues are susceptible to colonization.

Understanding the mechanisms underlying plant disease resistance is a major goal in the study of plant-microbe interactions. Characterization of the avirulence genes involved is a possible key to unravelling the processes of cell signalling and recognition leading to the induction of race-specific resistance. Progress has been made over recent years by the characterization of a number of avirulence genes from plant pathogenic bacteria. Examples include genes from pathovars of *Xanthomonas campestris* (recently reviewed by Daniels and Leach 1992) and *Pseudomonas syringae* (see reviews by Keen 1990, 1992); however, their biochemical function as well as the nature of the corresponding plant resistance genes are unknown. The only exception so far is the *avrD* gene from *P. s. tomato* which leads to the production of a race-specific elicitor, suggesting an enzymatic function of the *avrD* gene in elicitor production (Keen et al. 1990).

Xanthomonas campestris pv *vesicatoria* is the causative agent of bacterial spot disease of pepper (*Capsicum annuum*) and tomato (*Lycopersicon esculentum*). In the compatible interaction, the bacteria multiply to a high density in the intercellular spaces of leaves causing water-soaked lesions that later become necrotic, whereas in an incompatible interaction the HR is induced, leading to restriction of bacterial growth.

The avirulence gene *avrBs3* from *X. c. vesicatoria*, which specifies incompatibility in the pepper cultivar ECW-30R to infection with *X. c. vesicatoria*, is being studied in our laboratory. This gene is particularly interesting because its internal part consists of a tandemly

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repeated, nearly identical motif of 102 bp, present in 17.5 copies (Bonas et al. 1989). We showed by generating deletions that the repeat units determine the specificity of the avirulence gene and that new alleles that differ in specificity with respect to the plant genotype can be found (Herbers et al. 1992). Furthermore, the presence of homologous sequences in some, but not all, strains of *X. c. vesicatoria* and in other pathovars of *Xanthomonas* is intriguing (Bonas et al. 1989; Canteros et al. 1991; Knoop et al. 1991; Swarup et al. 1992). This raises the question whether these sequences represent functional alleles of the *avrBs3* gene with differing specificities. Recently, the characterization of an *avrBs3*-homologous gene from *X. c. vesicatoria*, *avrBsP*, has been reported (Canteros et al. 1991). The *avrBsP* gene is present on a 1.7 kb fragment and confers avirulence activity towards tomato.

Here we describe the isolation and characterization of the *avrBs3-2* gene which determines incompatibility on tomato but not on pepper. Sequence analysis and plant assays show that this gene is indeed a functional allele of *avrBs3*.

Materials and methods

Bacterial strains, plasmids and media. The *X. c. vesicatoria* strains used were: strains 71-21 and 82-8 (pepper race 1), strain 85-10 (pepper race 2), strain 75-3 (tomato race 1) (Bonas et al. 1989; Minsavage et al. 1990), and strain 87-7 (Canteros et al. 1991). Strain 82-8L was kindly provided by Dr. R.E. Stall. The following plasmid vectors were used: the broad host range vectors pLAFR3 (Staskawicz et al. 1987) and pLAFR6 in which the multicloning site is flanked by transcriptional terminators (Bonas et al. 1989), and pBluescript II KS (Stratagene; La Jolla, Calif.). Plasmids pL3XV1-6 and pUXV1009 carry *avrBs3* (Bonas et al. 1989); the *avrBs3Δrep* derivatives were described previously (Herbers et al. 1992).

Plasmids were introduced into *Escherichia coli* DH5α (Bethesda Research Laboratories) by electroporation and into *Xanthomonas* by conjugation, using pRK2013 as a helper plasmid in triparental matings (Figurski and Helinski 1979; Ditta et al. 1980).

Strains of *E. coli* DH5α were cultivated in Luria-Bertani (LB) medium (Miller 1972). *Xanthomonas* strains were routinely grown at 28° C in NYG broth (Daniels et al. 1984) or on NYG 1.5% agar. Antibiotics were added to the media at the following final concentrations: 100 μg/ml ampicillin; 50 μg/ml kanamycin; 10 μg/ml tetracycline; 100 μg/ml rifampicin.

Plant material and plant inoculations. Descriptions of the nearly isogenic pepper cultivars ECW and ECW-30R have been given by Minsavage et al. (1990). The tomato cultivars used were Bonny Best and Money Maker. The leaves of plants were inoculated with bacterial suspensions by infiltrating the bacteria into the intercellular spaces of fully expanded leaves using a plastic syringe (Staskawicz et al. 1984). The concentration of the inoculum was approximately 10^8 cfu/ml in 1 mM $MgCl_2$. Reactions were scored over a period of several days. To determine bacterial growth in the plant the inoculum used was approximately 5×10^5 cfu/ml in 1 mM $MgCl_2$. After various incubation times 0.5 cm disks were cut out and macerated in 1 mM $MgCl_2$; bacterial cell numbers were determined by plating appropriate serial dilutions.

Cloning procedures. Standard molecular techniques were used (Maniatis et al. 1982). For the isolation of the *avrBs3-2* gene, a 15 kb *Eco*RI and a 10 kb *Bam*HI fragment of plasmid DNA of *X. c. vesicatoria* strain 82-8, which both hybridized to the *avrBs3* gene (see Results), were isolated from an agarose gel. The fragments were ligated into the *Eco*RI site of pLAFR6 (pLE15) or the *Bam*HI site of pLAFR3 (pLB10). For sequence analysis an internal 7.5 kb *Spe*I(filled-in)-*Pvu*II fragment of pE15 was subcloned in both orientations into the *Hin*cII site of the polylinker region in pBluescript to generate pAT200 and pATR200. Deletion derivatives of both plasmids were generated by DNase I as described previously (Bonas et al. 1989). To test for avirulence activity, the inserts of deletion derivatives of pAT200 were isolated, ligated with *Hin*dIII linkers and cloned into pLAFR6.

DNA sequence analysis. The nucleotide sequence of overlapping deletion subclones of pAT200 and pATR200 was determined by the dideoxy chain-termination method (Sanger et al. 1977). Double-stranded template DNA was sequenced using commercial primers (Stratagene; La Jolla, Calif.) or custom primers and T7 polymerase (Pharmacia; Uppsala, Sweden). Derivatives of the *avrBs3* gene were sequenced using custom primers specific for sequences upstream and downstream of the repeat region. The sequence was analyzed using the University of Wisconsin GCG package (Version 7.1; Devereux et al. 1984). Pairwise alignments were done by BESTFIT (Devereux et al. 1984). The DNA sequence of *avrBs3-2* was submitted to EMBL (accession number X68781 XCAVIR).

Protein analysis. Protein extraction and analysis by Western blotting were performed as previously described (Knoop et al. 1991).

Results

Isolation of avrBs3-2

Total genomic DNA (including plasmids) of different strains of *X. c. vesicatoria*, digested with *Eco*RI or *Bam*HI, was hybridized in Southern blot experiments using an internal 3.3 kb *Bam*HI fragment of the *avrBs3* gene as probe. A 26 kb *Eco*RI fragment and a 3.3 kb *Bam*HI fragment corresponding to the *avrBs3* gene hybridize to this probe in DNA from strain 71-21, from which the gene was originally isolated (Bonas et al. 1989; Fig. 1A and B, lanes 1). Both fragments are also detected in DNA of other *X. c. vesicatoria* race 1 strains. In addition, 15 kb *Eco*RI and 10 kb *Bam*HI DNA fragments

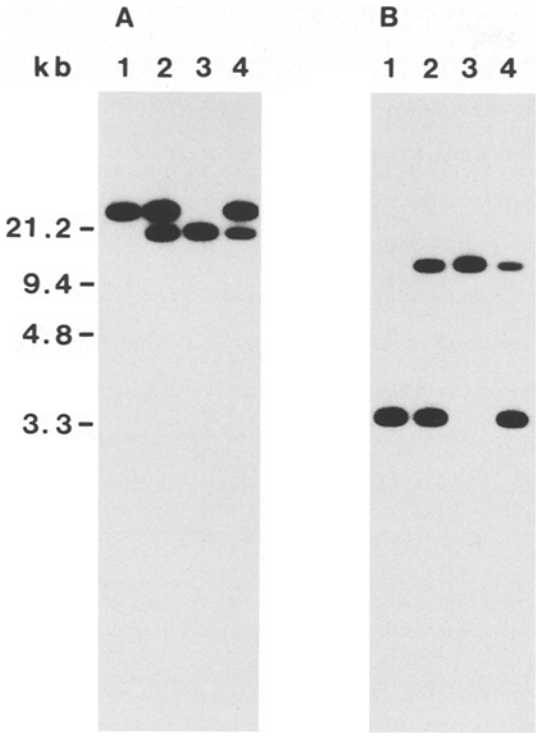

Fig. 1A, B. *AvrBs3*-homologous DNA fragments present in different strains of *Xanthomonas campestris* pv. *vesicatoria*. The Southern blot of total genomic DNA (1.5 µg per lane; separated in a 0.7% agarose gel) was hybridized to the internal 3.3 kb *Bam*HI fragment of the *avrBs3* isolated from pUXV1009 (Bonas et al. 1989). **A** *Eco*RI digested DNA. **B** *Bam*HI digested DNA. The strains used were: 71-21 (lane 1); 82-8 (lane 2); 82-8L (lane 3) and 87-7 (lane 4)

with homology to *avrBs3* are present in a number of strains, for example, in *X. c. vesicatoria* strains 82-8 and 87-7 (Fig. 1, lanes 2 and 4). Hybridization studies using plasmid DNA revealed that the *avrBs3*-homologous region observed is located on an endogenous plasmid, designated pXV12. By contrast, the *avrBs3* gene is carried by a different endogenous plasmid, pXV11 (Bonas et al. 1989). Strain 82-8L is a derivative of strain 82-8 lacking pXV11 (Fig. 1, lanes 3), and thus is virulent on pepper cultivar ECW-30R.

To study the region homologous to *avrBs3* in more detail, the 15 kb *Eco*RI and 10 kb *Bam*HI fragments were isolated from strain 82-8 plasmid DNA and cloned into the broad host-range vectors pLAFR6 and pLAFR3, resulting in plasmids pLE15 and pLB10, respectively. Recombinant plasmids were transferred into *X. c. vesicatoria* strain 85-10 by conjugation, and the resulting transconjugants were tested on pepper and tomato cultivars. As shown in Table 1, *X. c. vesicatoria* strain 85-10 is virulent on pepper cultivar ECW-30R and on tomato, giving rise to water-soaked lesions after infection. Transconjugants carrying pLE15 induced a hypersensitive response on tomato whereas pLB10 did not. Both 85-10 transconjugants were unaffected in their interaction with the pepper cultivars tested. These results indicated the presence of a resistance locus in tomato and a corresponding tomato-specific avirulence gene

Table 1. Plant reactions after inoculation with different strains of *Xanthomonas campestris* pv *vesicatoria*

X. c. vesicatoria strain[a]	Pepper ECW-30R	Tomato Bonny Best
82-8	R[b]	R
85-10	S[c]	S
85-10 (pL3XV1–6)[d]	R	S
85-10 (pLE15)	S	R
85-10 (pLB10)	S	S

[a] Bacteria were inoculated at 10^8 cfu/ml
[b] R, resistant
[c] S, susceptible
[d] Contains the *avrBs3* gene (Bonas et al. 1989)

within the cloned 15 kb *Eco*RI fragment, whereas the cloned 10 kb *Bam*HI fragment did not contain this activity. The *avrBs3*-homologous gene was designated *avrBs3-2*.

DNA sequence analysis of avrBs3-2

For a more detailed study, the 15 kb *Eco*RI fragment was cloned into pBluescript, resulting in plasmid pAT15. DNA heteroduplex studies using the cloned 15 kb *Eco*RI fragment and a plasmid containing the *avrBs3* gene indicated that a region of 3.8–3.9 kb in the cloned 15 kb *Eco*RI fragment is homologous to the *avrBs3* gene, starting near the *Bam*HI site at approximately 5 kb from the left end of this clone (Fig. 2). This homology was confirmed by Southern hybridization experiments.

For sequence analysis, the internal 7.5 kb *Spe*I-*Pvu*II fragment of pAT15 (Fig. 2) was subcloned in both orientations into pBluescript, to yield pAT200 and pATR200. The sequence of the *avrBs3-2* gene is shown in Fig. 3. As predicted, a region of approximately 3900 bp, including some non-coding sequences, was found to be highly homologous to *avrBs3* (Bonas et al. 1989; Knoop et al.

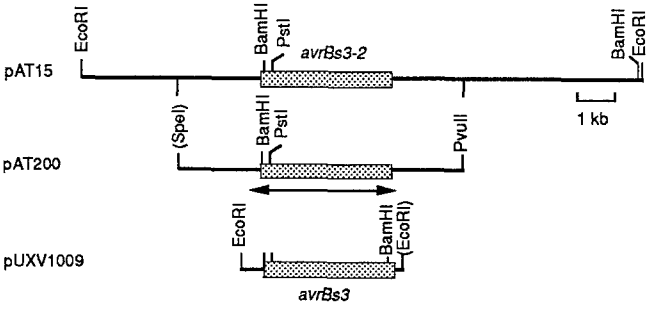

Fig. 2. Overall structure of *avrBs3-2*. Restriction map of the 15 kb *Eco*RI fragment carrying the *avrBs3-2* gene. The 10 kb *Bam*HI fragment (clone pLB10) is contained within the *Eco*RI fragment. A 7.5 kb *Spe*I (filled-in)-*Pvu*II fragment was subcloned into the *Hinc*II site of pBluescript. For comparison, the 4.3 kb *Eco*RI fragment carrying *avrBs3* is shown. Note that the second internal *Bam*HI site is deleted in *avrBs3-2*. The *shaded boxes* represent the highly homologous open reading frames. The *double-headed arrow* indicates the region of pAT200 which was sequenced; the second strand was sequenced using deletion clones of pATR200

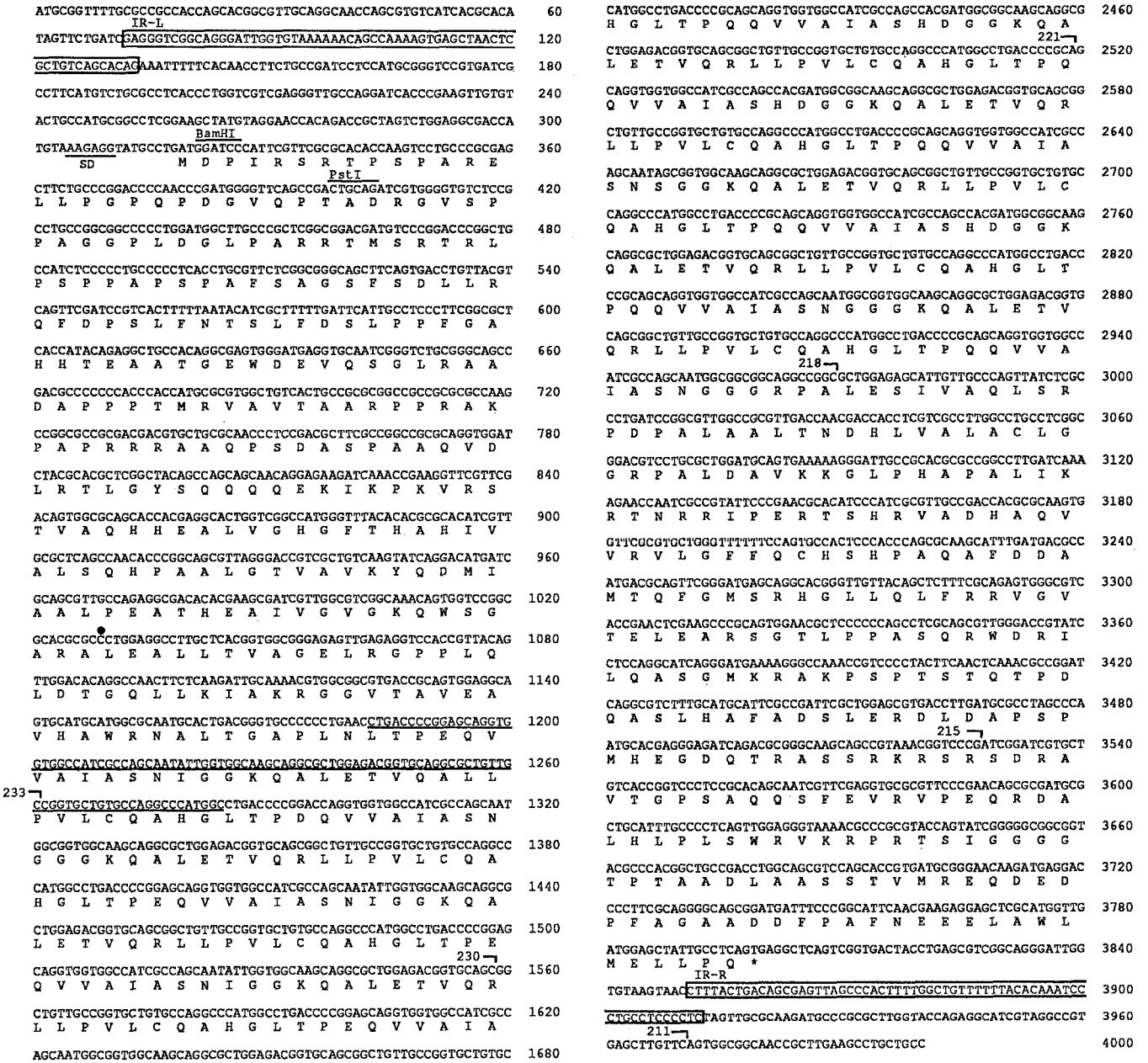

Fig. 3. Nucleotide sequence of the region containing the *avrBs3-2* gene. The sequence is shown in the 5′-3′ direction. The translation of the open reading frame into amino acids is shown in the one-letter code (ATG at position 319); the translational stop is indicated by an *asterisk*. The two inverted repeat sequences (IR-L, IR-R) are *boxed*. The putative Shine-Dalgarno sequence (SD; see Knoop et al. 1991) and the first unit of 17.5 repeat units which starts at position 1183, are *underlined*. For clarification, the *Bam*HI and *Pst*I restriction sites are indicated in the sequence (see Fig. 2). The nucleotide exchange, in comparison to *avrBs3*, at position 1029 is marked with ●. The last nucleotide of the insert of C-terminal deletion clones is indicated above the sequence; only the numbers of the pLAT plasmids are given (compare Table 2)

```
rep 1    LTPEQVVAIASNIGGKQALETVQALLPVLCQAHG   avrBs3-2
         ..........HD.........R.........   avrBs3

rep 2    LTPDQVVAIASNGGGKQALETVQRLLPVLCQAHG
         ...Q..............................

rep 3    LTPEQVVAIASNIGGKQALETVQRLLPVLCQAHG
         ...Q........S.....................

rep 4    LTPEQVVAIASNIGGKQALETVQRLLPVLCQAHG
         ...........G......................

rep 5    LTPEQVVAIASNGGGKQALETVQRLLPVLCQAHG
         ...........I.........A.........

rep 6    LTPEQVVAIASNGGGKQALETVQRLLPVLCQAHG
         ...........I.........A.........

rep 7    LIPQQVVAIASNIGGKQALETVQRLLPVLCQDHG
         .T.E..................A.......A..

rep 8    LTPQQVVAIASNSGGKQALETVQRLLPVLCQAHG
         ...E.......HD.....................

rep 9    LTPEQVVAIASNGGGKQALETVQRLLPVLCQAHG
         ..........HD......................

rep10    LTPQQVVAIASNIGGKQALETVQRLLPVLCQDHG
         ...........G..................A..

rep11    LTPQQVVAIASNSGGKQALETVQRLLPVLCQAHG
         ...E..................A.........

rep12    LTPQQVVAIASNGGGKQALETVQRLLPVLCQAHG
         ...E........S.....................

rep13    LTPQQVVAIASHDGGKQALETVQRLLPVLCQAHG
         ...E..............................

rep14    LTPQQVVAIASHDGGKQALETVQRLLPVLCQAHG
         ...E..............................

rep15    LTPQQVVAIASNSGGKQALETVQRLLPVLCQAHG
         ...E.......HD.....................

rep16    LTPQQVVAIASHDGGKQALETVQRLLPVLCQAHG
         ...........NG..RP.................

rep17    LTPQQVVAIASNGGGKQALETVQRLLPVLCQAHG
         ...E.......HD.....................

rep18    LTPQQVVAIASNGGGRPALESIVAQLSRPDPALA
         ................................
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Fig. 4. Comparison between the repetitive region of *avrBs3-2* and *avrBs3*. Both genes contain 17.5 repeat units, each 34 amino acids long. The first repeat starts at amino acid position 289 in both genes (see Fig. 3; Bonas et al. 1989). Only the differences in the sequences are indicated

1991). The predicted amino acid sequence of the AvrBs3-2 protein is 1160 amino acids long and is 97% identical to AvrBs3. The sequence of the promoter region is 100% identical to that of the *avrBs3* gene. The nucleotide exchange at position 1029 does not result in an amino acid exchange. A deletion of 12 bp (4 amino acids) was found between positions 3657 and 3658 (see Fig. 3). As described for *avrBs3* (Bonas et al. 1989), the internal region of the *avrBs3-2* gene consists of nearly identical 102 bp repeat units, present in 17.5 copies. Basically the same repeat motifs are present in both *avrBs3* and *avrBs3-2*, with a variable region at amino acid positions 12 and 13. As shown in Fig. 4, however, the organization of the repeats is slightly different. Due to a higher content of basic amino acids the isoelectric point of the predicted AvrBs3-2 protein is 8.5 (AvrBs3: 7.6).

There is 100% identity between the 5′ region of the *avrBs3-2* sequence and the 1.7 kb *avrBsP* sequence isolated from a different *X. c. vesicatoria* strain, 87-7 (see Fig. 1, lane 4). This gene also has avirulence activity on tomato and was described previously (Canteros et al. 1991). It should be noted that the C-terminal part of the open reading frame is probably missing from *avrBsP* because there is no translational stop codon (Canteros et al. 1991). We assume that *avrBs3-2* and *avrBsP* are identical genes. In addition, the *pthA* gene from *Xanthomonas citri*, which has been partially sequenced, seems to be homologous to *avrBs3-2* (Swarup et al. 1992). The promoter as well as the first 123 bp of the putative coding region of *pthA* are identical to *avrBs3-2*, *avrBsP* and *avrBs3* (Swarup et al. 1992). The *pthA* gene also contains repeat units, the first two of which are nearly identical to the first repeats in *avrBs3-2* and *avrBs3*. No other similarities to known sequences in the data banks were found.

Another striking feature of the *avrBs3-2* sequence was found outside of the gene: *avrBs3-2* is flanked by nearly perfect inverted repeats (IR-L and IR-R), each 62 bp long (see Fig. 3). These IR are also present in the *avrBs3* sequence (Bonas et al. 1989); their possible significance, however, now becomes more evident. Interestingly, the IR-L is also present upstream of the *pthA* gene from *X. citri*; there is no sequence information on the right portion of this gene (Swarup et al. 1992). The two sets of IR sequences in the *avrBs3-2* and the *avrBs3* genes are identical, with the exception of one nucleotide in the IR-L. To test whether more copies of these IRs are present, e.g. as part of a larger transposon in the flanking region of the avirulence genes or integrated somewhere else in the genome, we performed Southern hybridization experiments using the IR sequence as a probe. No additional DNA fragments homologous to the IR were found to be genetically linked to the *avrBs3* or *avrBs3-2* gene, i.e. on the respective plasmids. There are, however, homologous DNA sequences present in the chromosomal DNA of various *X. c. vesicatoria* strains, including strain 85-10, which does not carry sequences homologous to the internal region of *avrBs3* (data not shown).

Analysis of avrBs3-2 activity

Various fragments originating from the subclone pAT15 and deleted in the C-terminal portion of the gene were cloned into pLAFR6 and transferred into *X. c. vesicatoria* strain 75-3. The bacteria were inoculated into the tomato cultivars Bonny Best and Money Maker. Identical results were obtained irrespective of the tomato cultivar used, as summarized in Table 2. Plasmid pLAT211, which contains the complete gene, has avirulence activity on tomato. Surprisingly, C-terminal deletions of up to 2240 bp into the *avrBs3-2* coding sequence did not abolish gene activity. Three deletion derivatives (pLAT215, pLAT218 and pLAT221), lacking between 250 bp and

Table 2. Activity of *avrBs3-2* derivatives on tomato cultivars Bonny Best and Money Maker

X. c. vesicatoria strain[a]	Length of open reading frame (nucleotides)	Response
75-3	–	S[b]
75-3 (pLAT211)	3480	R
75-3 (pLAT215)	3208	S/R
75-3 (pLAT218)	2677	S/R
75-3 (pLAT221)	2200	S/R
75-3 (pLAT227)	1428	R
75-3 (pLAT230)	1239	R
75-3 (pLAT233)	942	S

[a] Bacteria were inoculated at 10^8 cfu/ml; plasmids see text and Fig. 3

[b] S, susceptible; R, resistant; S/R, intermediate response

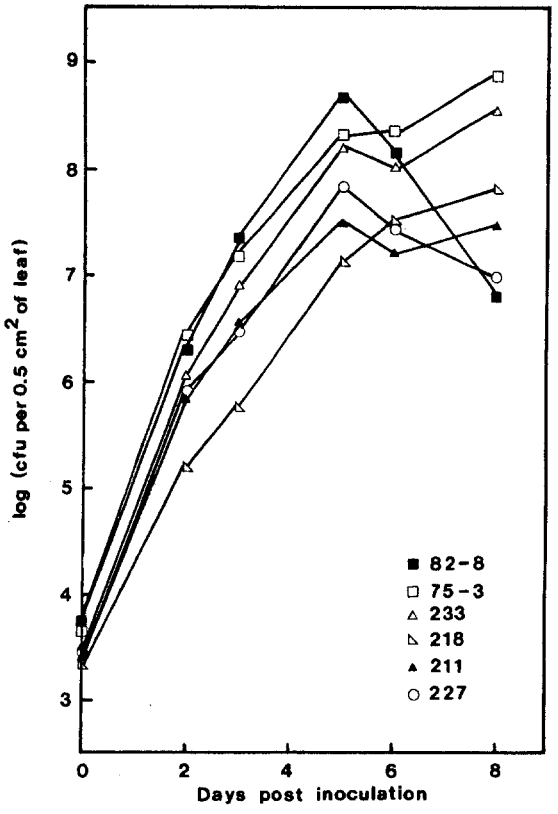

Fig. 5. Time course of multiplication of *X. c. vesicatoria* wild-type strains and transconjugants in the tomato cultivar Bonny Best. The following strains were used: strain 82-8 (source of *avrBs3-2*), strain 75-3 (tomato-race) and 75-3 transconjugants carrying pLAT211 (complete *avrBs3-2* gene), and the C-terminal deletion derivatives of *avrBs3-2* on pLAT218, pLAT227 and pLAT233. Compare Table 2 for phenotypic reactions. Bacteria were inoculated into tomato leaves at 5×10^5 cfu/ml in 1 mM $MgCl_2$. Values are the mean of three samples taken at each time point; one representative experiment is shown

1279 bp of the open reading frame (ORF), induced a delayed/intermediate HR. Using a tenfold higher inoculum of pLAT218 resulted in a full HR phenotype. Further deletion in the same direction, however, led to a recovery of full avirulence activity, with pLAT227 eliciting a stronger response than the original *avrBs3-2* allele. All smaller derivatives tested that lost more than 2408 bp of the ORF were inactive. This means that a truncated gene in which the ORF is only 1239 bp long (pLAT230) is sufficient for avirulence activity. The latter results might explain why the *avrBsP* gene mentioned above was active. A 1.7 kb *Sau*3A fragment carrying *avrBsP* was isolated by screening small subclones from a large, active cosmid clone for avirulence activity (Canteros et al. 1991). The ORF present in *avrBsP* contains six repeats and is 1490 bp long; the sites of termination of transcription and translation are not known (Canteros et al. 1991). None of the *avrBs3-2* derivatives tested was found to elicit an HR on the nearly isogenic pepper cultivars ECW or ECW-30R.

To determine how the phenotypic responses of the various avirulence derivatives correlated with bacterial growth in the plant leaf tissue, we inoculated some of the strains at low density into tomato cultivar Bonny Best and followed their multiplication. Strain 75-3 is more aggressive on tomato than 85-10 and was therefore used as recipient for the *avrBs3-2* derivatives. As shown in Fig. 5, bacterial growth of strain 82-8 (source of *avrBs3-2*) and of strain 75-3 transconjugants carrying pLAT211 or pLAT227 was clearly reduced by a factor of 100–500 as compared to strain 75-3. Strain 75-3 carrying pLAT218 was also reduced in its growth in tomato but could grow five to tenfold better than the other avirulent strains. This confirms the observation that some derivatives display partial avirulence activity. Strain 75-3 (pLAT233) which does not induce the HR in tomato, grows like the virulent parent strain.

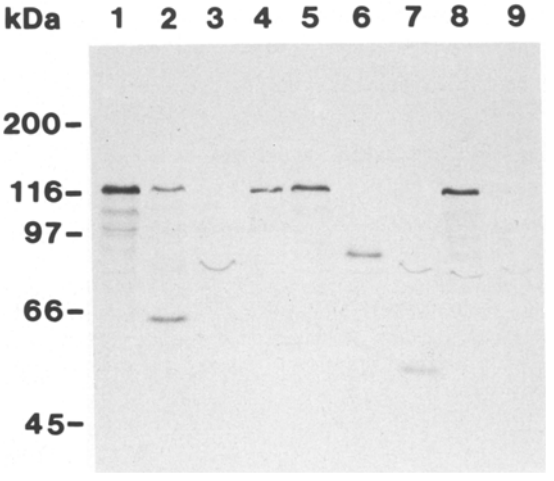

Fig. 6. Western blot analysis of AvrBs3 homologous proteins in *X. c. vesicatoria*. The following strains were tested: strain 82-8 (lane 1), 82-8::Tn*3-gus*74, an *avrBs3*-mutant (Bonas et al. 1989; lane 2), strain 85-10 (lane 3), 85-10 transconjugant harboring *avrBs3-2* on pLE15 (lane 4) and 75-3 transconjugants carrying pLAT211, pLAT218, and pLAT227 (lanes 5–7), 75-3 (pL3XV1–6) (lane 8) and 75-3 (lane 9). Total protein extracts of cells grown in NYG broth from different strains were separated in a 9% polyacrylamide gel and electrophoretically transferred to nitrocellulose. The blot was reacted with affinity-purified antiserum directed against AvrBs3 (1:15000 dilution). Bound antibody was visualized by reaction with alkaline phosphatase-conjugated secondary antibody. The faint band present in all lanes is due to unspecific binding of the antibody

Expression of the AvrBs3-2 protein

Since the upstream region of the *avrBs3-2* gene is identical to the promoter region of *avrBs3* we assumed that expression of *avrBs3-2* is also constitutive. This was confirmed by Western blot analysis of total protein extracts isolated from different *X. c. vesicatoria* strains after growth in complex medium, using an AvrBs3-specific antibody. As shown in Fig. 6 (lane 1), strain 82-8 expresses proteins of approximately 125 kDa that react with the antibody. Since strain 82-8 also expresses the AvrBs3 protein, the AvrBs3-2 protein band is masked and only apparent in mutant strains like strain 82-8::Tn*3-gus*74 in which the *avrBs3* allele was inactivated by a Tn*3-gus* insertion, resulting in a low molecular weight protein (Fig. 6, lane 2). The concentration of the protein expressed by the *avrBs3-2* allele in strain 82-8 is two to threefold lower than that of AvrBs3, which correlates with a lower copy number of the endogenous plasmid carrying this allele (U. Bonas, unpublished results). The same protein is detectable in transconjugants of *X. c. vesicatoria* carrying pLE15 (Fig. 6, lane 4). The subclone pLAT211 also expresses the full-size protein whereas the sizes of the cross-reacting proteins produced by pLAT218 and pLAT227 (Fig. 6, lanes 6, 7) are reduced in accordance with the sizes of the C-terminal deletions. The two transconjugants carrying pLAT211 and pLAT227, the latter leading to a faster HR phenotype in the plant, seem to express the protein in comparable amounts.

Comparison of avrBs3 *alleles with avirulence activity on tomato*

Derivatives of the pepper-specific *avrBs3* gene which were obtained by internal deletion of repeat units and display new avirulence specificities, were described previously (Herbers et al. 1992). Interestingly, some of the new avirulence derivatives confer resistance to the tomato cultivars Bonny Best and Money Maker when introduced into tomato-virulent *X. c. vesicatoria* strains, whereas the original *avrBs3* gene has no avirulence activity on tomato. Besides a clear phenotypic induction of the HR by derivatives *avrBs3Δrep*-27, -118, -40, -21, and -33, there is a group of derivatives inducing an intermediate response between water-soaking and HR (e.g. *avrBs3Δrep*-32, -29, -119, -120; Fig. 7). We wanted to know whether the tomato-specific derivatives contain repeats that are in the same order as in the *avrBs3-2* gene.

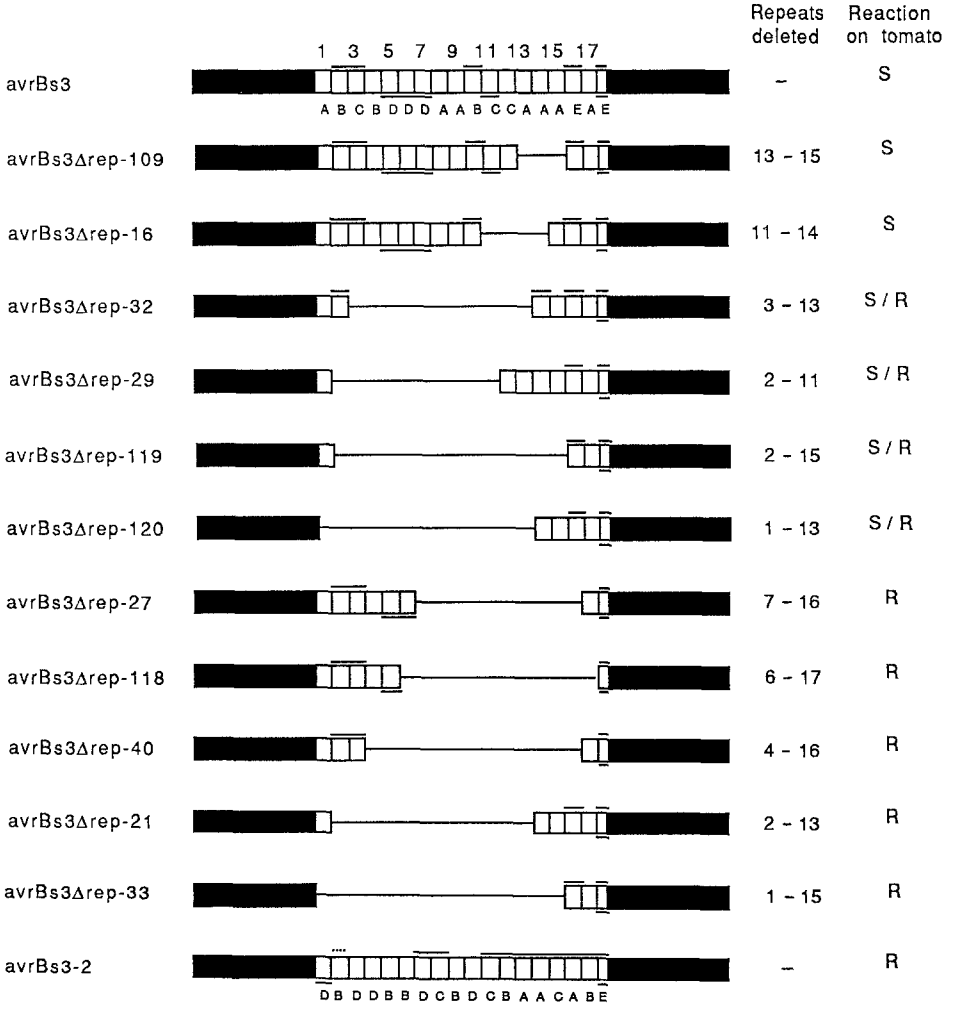

Fig. 7. *avrBs3* alleles with and without avirulence activity for tomato. The sequences of the original *avrBs3* gene and the *avrBs3-2* gene are compared with internal deletion derivatives of *avrBs3* lacking repeat units. The lettering of the repeat units refers to the variable positions 12 and 13 in each unit; *bars* above and underneath the *boxes* represent the E/Q and R/A exchanges, respectively (see Fig. 4). *Dots* above the second repeat of *avrBs3-2* indicate the amino acid D (all other cases E or Q). The activity of the alleles was tested in transconjugants of strain 75-3 on tomato cultivars Bonny Best and Money Maker which both gave identical results. Bacteria were inoculated at 10^8 cfu/ml. S, susceptible; R, resistant (HR); S/R indicates an intermediate response

The sequence of the repetitive region of nine tomato-specific *avrBs3* alleles, containing 7.5 repeats or less was determined. A comparison to *avrBs3* and *avrBs3-2* is schematically shown in Fig. 7. For clarification we have designated the different repeat motifs with letters A, B, C, D, and E, referring to the variable positions found in each repeat unit (see Fig. 4). The following points have emerged: first, most of the new tomato-specific *avrBs3* alleles are small, i.e. they have 11.5 or fewer repeats; and second, no other *avrBs3* alleles analyzed so far, except for the original allele and two *avrΔrep* alleles that are active on pepper (Herbers et al. 1992), contain repeat motif D three times in tandem. Thirdly, there are no obvious tomato-specific repeat units or blocks of repeats.

Discussion

We have characterized the avirulence gene *avrBs3-2* from *X. c. vesicatoria* which is responsible for the induction of a hypersensitive resistance response in tomato. Our results demonstrate that *avrBs3-2* is a natural allele of the avirulence gene *avrBs3* from *X. c. vesicatoria* (Bonas et al. 1989). In contrast to *avrBs3*, *avrBs3-2* recognizes tomato, but not pepper. The *avrBs3-2* genes encodes a protein of 1160 amino acids, the sequence of which is 97% identical to the AvrBs3 protein (1164 amino acids). The fact that expression of the AvrBs3-2 protein occurs in complex medium was expected because the promoter region of *avrBs3-2* is identical to that of the *avrBs3* gene (see Fig. 3; Knoop et al. 1991).

Among the different bacterial avirulence genes isolated so far there are a few other examples of genes that share significant sequence homology with each other but differ in specificity. Examples are the *avrB* and *avrC* genes from *Pseudomonas syringae* pv *glycinea* that induce resistance on different soybean cultivars (42% amino acid identity; Tamaki et al. 1988, 1991). A gene from the same pathogen is a recessive allele of the *avrD* gene from *P. s. tomato* (Kobayashi et al. 1990). In contrast, there appears to be a large family of *avrBs3*-homologous genes, present in *X. c. vesicatoria* and in other pathovars of *Xanthomonas* as has been postulated earlier (Knoop et al. 1991). The isolation of an *avrBs3*-related avirulence gene, *avrBsP*, from *X. c. vesicatoria* was reported recently (Canteros et al. 1991). We assume that *avrBsP* is part of the *avrBs3-2* gene described here. The 1.7 kb sequence of *avrBsP* is identical to the corresponding region in *avrBs3-2*, both genes are localized on an endogenous plasmid that is of similar size in the strains from which they were isolated, and both display avirulence activity on different tomato cultivars. Another example of an *avrBs3*-related gene is the *pthA* gene from *X. citri* (Swarup et al. 1992). When the *pthA* gene is introduced into *Xanthomonas* pathovars *phaseoli* and *malvacearum*, it induces an HR on bean and on cotton, respectively, indicating that a corresponding resistance gene might be present in different plant species. In contrast to the *avrBs3* alleles, a mutated *pthA* gene seems to affect pathogenicity of *X. citri* (Swarup et al. 1991). Swarup and coworkers (1992) reported that several cotton-specific avirulence genes, isolated from *X. c.* pv *malvacearum* (De Feyter and Gabriel 1991), also appear to be homologous to *avrBs3*, however, no information on the sequence of these genes is published. In addition, *avrBs3*-related avirulence genes were isolated from *X. c.* pv *oryzae* that correspond to resistance genes in a monocot (rice) (Daniels and Leach 1992).

The most intriguing feature shared by the internal portion of the *avrBs3-2* gene, and typical of all *avrBs3*-related genes, is a 102 bp repeat motif, present in 17.5 copies in *avrBs3-2*. This means that both *avrBs3-2* and *avrBs3* contain the same number of nearly identical repeat units. We believe that the difference in specificity of these genes is due to the different organization of the repeat units and thus to single or a few amino acid exchanges. This has clearly been demonstrated to be important for *avrBs3* specificity. A number of *avrBs3* alleles lacking a block of several repeat units have not only lost *avrBs3* activity but in some cases have gained new avirulence specificities (Herbers et al. 1992). The aforementioned *pthA* gene from *X. citri* is closely related to the *avrBs3* gene family: it appears to contain 17.5 copies of the basic repeat unit as well, with the first repeat being identical to *avrBs3-2* and *avrBsP*; the second repeat of *pthA* is different. There is no sequence information on the other repeats (Swarup et al. 1992). We tried to address the possibility that there are tomato- and pepper-specific repeat units. The comparison between a number of *avrBs3*-derived tomato-specific alleles with *avrBs3* and *avrBs3-2* (see Fig. 7) has not given a clear answer. It remains to be tested if the single amino acid exchanges (glu/gln; arg/ala; asp/ala), present outside of the variable region within the repeat unit, are important for avirulence specificity.

To our surprise, shorter derivatives of *avrBs3-2*, lacking up to 2240 bp of the C-terminal portion of the coding region, also display avirulence activity on tomato. Analogous *avrBs3* derivatives do not have avirulence activity on either pepper or tomato, nor do the shorter *avrBs3-2* derivatives have any effect on the interaction of virulent *X. c. vesicatoria* with pepper. These results help to explain the somewhat puzzling observation that *avrBsP* is active on tomato although the coding region is only 1.4 kb long (Canteros et al. 1991). Even more surprising than the fact that clone pLAT227 has avirulence activity on tomato is that the HR induced is much stronger than with the longer versions of the gene, and conversely, that clones pLAT215, 218 and 221 induce an intermediate HR. This could indicate differences in the ultimate, tertiary structure of the AvrBs3-2 protein that might affect binding of an as yet unknown reaction partner. Intermediate phenotypes were also observed with several *avrBs3* derivatives and their reaction on pepper (Herbers et al. 1992). It cannot be ruled out at present, however, that the corresponding plant resistance gene in tomato, which is responsible for the HR induction by pLAT227, is not identical to the hypothetical *Bs3-2* resistance gene postulated to interact with the *avrBs3-2* gene. All tomato cultivars tested (10 different cultivars and more than 19 wild lines) react to *avrBsP* with HR (Canteros et al. 1991).

The origin of the *avrBs3* gene family remains speculative. Considering the high sequence conservation, these genes might have evolved only recently. Do they originate from insertion elements, transposons or viruses? The sequence comparison between *avrBs3-2* and *avrBs3* revealed the presence of long IR sequences, flanking each gene on both sides. It is remarkable that the homology is restricted to the region in between the two IR sequences. One copy of this IR sequence is also present upstream of the *pthA* gene, there is no sequence downstream of the gene available (Swarup et al. 1992). Obviously, the gene and the IR sequences are genetically linked in the members of the *avrBs3* gene family. Furthermore, it could very well be that the members of this gene family are spread by plasmids. Both *avrBs3-2* and *avrBs3* genes are encoded by different endogenous plasmids that can occur within the same strain; the plasmid carrying *avrBs3* was shown to be self-transmissable (Bonas et al. 1989).

The function of the *avrBs3-2* and related genes is still elusive. We hypothesize that there might be a direct interaction between the avirulence gene product and a plant molecule. Our recent findings that the putative products of the *X. c. vesicatoria* pathogenicity genes (*hrp* genes) might be part of a transport and secretion apparatus support this idea (Fenselau et al. 1992).

Based on our findings it is tempting to speculate that not only the members of the *avrBs3* avirulence gene family are highly related, differing in specificity, but that the same might be true for the corresponding resistance genes. The resistance genes recognized by members of the *avrBs3* gene family appear to be widespread in the plant kingdom, including important crop plants such as pepper, tomato, bean, cotton, and rice.

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