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Genetic and structural characterization of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria*

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Summary. The avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria* was cloned and found to be localized on a self-transmissable plasmid. Genetic analysis of an *avrBs3* insertion mutation revealed that *avrBs3* constitutes a single locus, specifying the resistant phenotype on pepper plants. Southern blot experiments showed that no DNA sequences homologous to *avrBs3* were present in other races of *X. c.* pv. *vesicatoria*, which are unable to induce a hypersensitive reaction on ECW-30R. However, the DNA of several different pathovars of *X. campestris* hybridized to the *avrBs3* probe. A deletion analysis defined a region of 3.6–3.7 kb essential for *avrBs3* activity. The nucleotide sequence of this region was determined. A 3561 nucleotide open reading frame (ORF1), encoding a 125000 dalton protein, was found in the 3.7 kb region that was sufficient for *avrBs3* activity. A second long ORF (2351 nucleotides) was identified on the other strand. A remarkable feature of both ORFs is the presence of 17 direct repeats of 102 bp which share 91%–100% homology with each other.

Key words: *Xanthomonas campestris* – Avirulence gene – Disease resistance – DNA sequence – Deletion analysis

Introduction

Although many examples of disease resistance have been found for plant-microbe interactions (Day 1974) the molecular mechanism of disease resistance remains to be elucidated.

Xanthomonas campestris pathovar (pv.) *vesicatoria* is the causal agent of bacterial leaf spot disease on pepper and tomato. In the compatible interaction, i.e. when the plant is susceptible, the infection with *X. c.* pv. *vesicatoria* gives rise to lesions on leaves which at first appear watersoaked and later necrotic. Resistance to *X. c.* pv. *vesicatoria* depends on the presence of two different loci: a resistance locus in a particular cultivar of pepper and an avirulence locus in a particular race of *X. c.* pv. *vesicatoria*. The phenotype of this reaction is a hypersensitive response (HR), which is a defense reaction accompanied by rapid necrosis of the infected plant tissue (Maclan et al. 1974; Sequeira 1984). Induction of an HR prevents the accumulation of bacteria in the intercellular spaces of leaves. Several dominant, non-allelic resistance genes (e.g. *Bs1*, *Bs2*, *Bs3*) mediating an HR toward particular races of *X. c.* pv. *vesicatoria* have been identified in pepper (Cook and Stall 1963; Kim and Hartmann 1985; Hibberd et al. 1987) and have been introduced into the susceptible cultivar Early Cal Wonder (ECW; R.E. Stall, unpublished results). The ability to distinguish bacterial genotypes by their reactions on different nearly isogenic cultivars of pepper allows the isolation and characterization of the bacterial genes involved.

Several avirulence genes from bacterial pathogens such as *Pseudomonas syringae* pv. *glycinea* (Staskawicz et al. 1984, 1987; Napoli and Staskawicz 1987), *X. c.* pv. *malvacearum* (Gabriel et al. 1986) and *X. c.* pv. *vesicatoria* (Swanson et al. 1988; Stall et al., in preparation) have been cloned. Despite extensive molecular characterization, the biochemical function of the gene products remains an open and important question for our understanding of specific resistance.

This paper describes the isolation and the genetic and structural characterization of the *avrBs3* locus from *X. c.* pv. *vesicatoria* race 1.

Materials and methods

Bacterial strains, plasmids and media. The bacterial strains used are listed in Table 1. A spontaneous chloramphenicol-resistant (Cm^r) mutant of *X. campestris* strain T55 was isolated by plating T55 at 10^8 cells/ml on chloramphenicol-containing plates and selecting a resistant colony.

Escherichia coli strains were routinely cultured in LB medium (Miller 1972). *Xanthomonas* strains were grown in NYG broth (Daniels et al. 1984) or on NYG agar (1.5%). The following concentrations of antibiotics were used: ampicillin (Ap), 100 μg/ml; chloramphenicol (Cm), 10 μg/ml; kanamycin (Km), 50 μg/ml; nalidixic acid (Nal), 50 μg/ml; spectinomycin (Sp), 10 μg/ml; tetracycline (Tc), 10 μg/ml; rifampicin (Rif), 100 μg/ml.

Cloning procedures and bacterial conjugation. Plasmid DNA of *X. c.* pv. *vesicatoria* race 1 strain 71-21 was isolated for construction of the DNA library (Birnboim and Doly 1979), purified twice on CsCl gradients, partially digested with *Sau*3A and size-fractionated on a low-melting agarose gel. The purified DNA fragments of 20–30 kb were ligated into the *Bam*HI site of pLAFR3 (Staskawicz et al. 1987).

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Table 1. Bacterial strains and plasmids

Designation	Relevant characteristics	Source or reference
Xanthomonas campestris		
Strain T55	Rif^r, saprophyte	R.E. Stall
X. c. pv. *vesicatoria*		
Race 1		
Strain 71-21	Rif^r, carries pXV11	R.E. Stall
Strain 82-8	Rif^r, carries pXV11 and pXV12	R.E. Stall
Race 2		
Strain 85-10	Rif^r	R.E. Stall
Strain 81-23	Rif^r	R.E. Stall
Tomato race 1		
Strain 75-3	Rif^r	R.E. Stall
Escherichia coli		
DH5α	F$^-$ *recA Φ80dlacZΔM15*	Bethesda Research Laboratories
MV1193	*Δ(lac-proAB)*Tc^r Thi$^-$ [F′(*proAB,lacI^{q}ZΔM15*)]	M. Volkert, unpublished
C2110	Nal^r *polA*$^-$	E. Nester
HB101	F$^-$ *recA*	Boyer and Roulland-Dussoix (1969)
Plasmids		
pLAFR3	pLAFR1 containing *Hae*II fragment of pUC8	Staskawicz et al. (1987)
pLAFR6	pLAFR1 derivative containing *trp* terminators	B. Staskawicz, unpublished
pUC118	IG region of M13 in pUC18	Vieira and Messing (1987)
pRK2013	Km^r Tra$^+$ Mob$^+$; ColE1 replicon	Figurski and Helinski (1979)
pHoKmGus	Km^r Ap^r *tnpA*$^-$; promoterless β-glucuronidase gene	B. Staskawicz, unpublished
pSShe	Cm^r *tnpA*	Stachel et al. (1985)

The recombinant clones were screened for *avrBs3* activity by conjugation (see below) into *X. c.* pv. *vesicatoria* race 2 strain 85-10 and inoculation of the transconjugants into ECW, ECW-10R and ECW-30R plants. The first *avrBs3*-containing clone isolated, named Ec83, contained an insert of 29 kb which was shown to be non-contiguous. Subcloning of pEC83 revealed that *avrBs3* was located on a fragment smaller than 5 kb. A new DNA library from *X. c.* pv. *vesicatoria* race 1 was constructed as described above except that DNA fragments in the size range of 4–6 kb were ligated into pLAFR3. *E. coli* strain DH5α was used as host for the transformation.

DNA fragments were subcloned from pLAFR3 into pUC118 (Vieira and Messing 1987). The 4.3 kb *Eco*RI fragment from pL3XV1–6 (see Results) was cloned into pUC118 in both orientations to give rise to pUXV1006 and pUXV1009. To obtain single-stranded DNA by superinfection with the helper phage M13KO7, plasmids of selected subclones were transformed into *E. coli* MV1193.

Conjugation between *E. coli* and the appropriate *Xanthomonas* strain was performed by triparental mating using HB101 (pRK2013) as helper strain (Figurski and Helinski 1979; Ditta et al. 1980). The presence of the respective pLAFR derivative in transconjugants was verified by isolating and transforming plasmid DNA back into *E. coli*, followed by restriction enzyme analysis.

Insertion mutagenesis. For insertion mutagenesis of *avrBs3* a Tn*3*HoHo derivative (Stachel et al. 1985), pHoKmGus, (D. Dahlbeck and B. Staskawicz, unpublished) was used. This plasmid harbors a promoterless β-glucuronidase gene instead of β-galactosidase between the inverted repeats of Tn*3* (the so-called Gus-poson) which is irrelevant for the experiments described here. The insertion mutagenesis was performed as follows. HB101 (pHoKmGus, pSShe) was transformed with pL3XV1–6 DNA. Independent transformants (Ap^r, Cm^r, Km^r, Tc^r) were mated with *E. coli* C2110 using pRK2013 as helper plasmid. The bacterial cells were then grown in L broth (Nal, Tc, Km) for plasmid DNA isolation. After transformation into DH5α and selection on L agar (Tc, Km) the obtained insertion derivatives were analyzed by restriction enzyme analysis and transferred into *X. c.* pv. *vesicatoria* race 2 strain 85-10.

Site-directed gene replacement using the omega fragment (Ω; Prentki and Krisch 1984) was carried out as described (Staskawicz et al. 1987).

Plant material and inoculation. Pepper lines ECW, ECW-10R and ECW-30R were established by R.E.Stall (unpublished results). ECW-10R and ECW-30R carry the dominant resistance genes *Bs1* and *Bs3* respectively. Both cultivars are nearly isogenic to ECW. The plants were grown and inoculated with bacteria as described previously (Staskawicz et al. 1984; Swanson et al. 1988). The concentration of the inoculum was approximately 10^8 colony forming units (cfu)/ml 1 mM $MgCl_2$.

DNA hybridization experiments. Total genomic DNA from *Xanthomonas* cells grown in liquid culture was isolated as described (Staskawicz et al. 1984). The DNA was digested and separated on agarose gels. Transfer to Nytrane membrane (Schleicher & Schüll) was carried out as described by Southern (1975) and Wahl et al. (1979). As hybridization probe the 3.3 kb *Bam*HI fragment (see Fig. 1) from *avrBs3* was used, which was ^{32}P-labeled by the random priming method (Feinberg and Vogelstein 1983). Hybridizations

were performed for 16–20 h at 65° C in 3 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% SDS and 0.05% salmon sperm DNA. The filters were washed at 68°–72° C in 0.1 × SSPE, 0.1% SDS.

DNase I deletion analysis. For deletion analysis, plasmid DNA of the *avrBs3* subclones pUXV1006 and pUXV1009 was digested with DNase I in the presence of Mn^{2+} (Anderson 1981). The population of linear DNA molecules was isolated from a low-melting agarose gel, purified and then digested with *Sal*I which cuts only in the vector polylinker. The ends of the DNA fragments were filled in using Klenow fragment. After size fractionation of the deletion products on a low-melting agarose gel the DNA was purified and religated. Before transformation into DH5α the ligated DNA was digested with *Sma*I to eliminate deletion products which had been cut by DNase I in vector sequences and not in the insert. The resulting deletion clones of both pUXV1006 and pUXV1009 were digested with *Eco*RI and *Hin*dIII to determine their insert size. Several clones were chosen for subcloning into pLAFR3 and pLAFR6, conjugated into *X. c.* pv. *vesicatoria* race 2 strain 85-10 and inoculated into ECW and ECW-30R to check for the ability to induce HR on ECW-30R.

DNA sequence analysis. The nucleotide sequence of deletion subclones of pUXV1006 and pUXV1009 was determined by the dideoxy chain termination method (Sanger et al. 1977). Single-stranded template DNAs were isolated from the pUC118 derivatives according to the protocol of Vieira and Messing (1987). The sequencing reactions were carried out at 50° C using Klenow fragment because of the high GC content. Additionally several restriction fragments of the full-size or deletion clones were sequenced as described by Maxam and Gilbert (1980). The products of the sequence reactions were analyzed on 5% or 6% polyacrylamide gels which in some cases contained 40% formamide to overcome secondary structure problems (Martin 1987).

The sequences obtained were analyzed using the following computer programs and data banks: Microgenie (Beckman), Sequence analysis software (Genetics Computer Group, University of Wisconsin) EMBL Nucleotide Sequence Data Bank 1988. NBRF Nucleic Acid and Protein Sequence Data Bank 1988 and GenBank.

Results

Isolation and identification of avrBs3

Previous experiments revealed that spontaneous loss of the ability to induce an HR on the pepper cultivar ECW-30R was associated with the loss of a 45 kb plasmid in *X. c.* pv. *vesicatoria* race 1 strain 71-21 (data not shown). This suggested that *avrBs3* was plasmid borne. To isolate *avrBs3* a DNA library from *X. c.* pv. *vesicatoria* race 1 strain 71-21 plasmid DNA was constructed in pLAFR3 (see Materials and methods). Recombinant plasmids were conjugated into *X. c.* pv. *vesicatoria* race 2 and the resulting transconjugants tested for their reaction on different pepper cultivars (Table 2). *X. c.* pv. *vesicatoria* race 2 causes a compatible reaction on both ECW and ECW-30R. However, transconjugants carrying an active *avrBs3* gene induce an HR on ECW-30R, i.e. cause an incompatible reaction. Three different clones were found to induce HR on ECW-30R in race 2 transconjugants. The *avrBs3*-clone pL3XV1–6, with an insert of about 5 kb, was chosen for detailed analyses (Fig. 1).

Table 2. Phenotypes of near-isogenic cultivars of pepper inoculated with different strains of *Xanthomonas campestris* pv. *vesicatoria*

X. c. pv. *vesicatoria*[a]	Pepper cultivars		
	ECW	ECW-30R (*Bs3*/*Bs3*)[d]	ECW-10R (*Bs1*/*Bs1*)
Race 1 strain 71-21	C[e]	I	C
Race 1 strain 82-8	C	I	C
Race 2 strain 85-10	C	C	I
Strain 85-10 (pL3XV1–6)[b]	C	I	I
Strain 82-8::Ω1[c]	C	C	C

[a] Bacteria were inoculated at 10^8 cells/ml into the underside of leaves
[b] 85-10 (pL3XV1–6) is a transconjugant carrying *avrBs3*
[c] 82-8::Ω1 is an insertion mutant carrying the Ω fragment in *avrBs3*
[d] *Bs3* and *Bs1* are resistance loci conferring resistance to bacterial spot caused by *X. c.* pv. *vesicatoria* race 1 and 2 respectively
[e] C, compatible; I, incompatible interaction

To obtain further evidence that the cloned fragment contained a gene required for HR induction in ECW-30R, we tried to inactivate the gene by insertion mutagenesis of pL3XV1–6 (see Materials and methods). The positions of independent insertions by a transposon (Gus-poson) and a site-specific insertion by the Ω fragment are illustrated in Fig. 1B. Except for Gus-poson-insertion no. 1 all insertion mutants showed a compatible reaction on pepper cultivars ECW and ECW-30R. This result suggested that the function of the *avrBs3* gene in induction of HR on ECW-30R had been destroyed by the insertions. An Ω insertion mutant was generated by marker gene replacement of *avrBs3* in *X. c.* pv. *vesicatoria* race 1 wild-type strain 82-8. This mutant strain, designated 82-8::Ω1, was compatible with ECW-30R and could be complemented for avirulence activity (i.e. induction of HR on ECW-30R) by any of the three different active *avrBs3* clones (data not shown). Subsequently the mutant *X. c.* pv. *vesicatoria* 82-8::Ω1 was tested for the ability to grow in NYG broth and in planta. In NYG broth the growth curves of *X. c.* pv. *vesicatoria* 82-8::Ω1 and the wild-type strain *X. c.* pv. *vesicatoria* 82-8 were identical (data not shown). When *X. c.* pv. *vesicatoria* race 1 wild-type strain 82-8 was inoculated at a concentration of 10^5 cfu/ml into leaves of pepper cultivars ECW and ECW-30R respectively the growth rate and the final population size were, as expected, smaller in ECW-30R than in ECW (factor 10^2–10^3). However, the *avrBs3* mutant strain 82-8::Ω1 showed equal growth rates and population size in both ECW and ECW-30R. The final population size of these compatible interactions was 10^2- to 10^3-fold higher compared with the incompatible interaction between *X. c.* pv. *vesicatoria* strain 82-8 (or 71-21) and ECW-30R (data not shown). These results confirm that induction of HR is concomitant with stopping of bacterial growth in the plant and that comparison of in planta growth curves reflects the type of interaction taking place. This has been demonstrated earlier by others (e.g. Stall and Cook 1966).

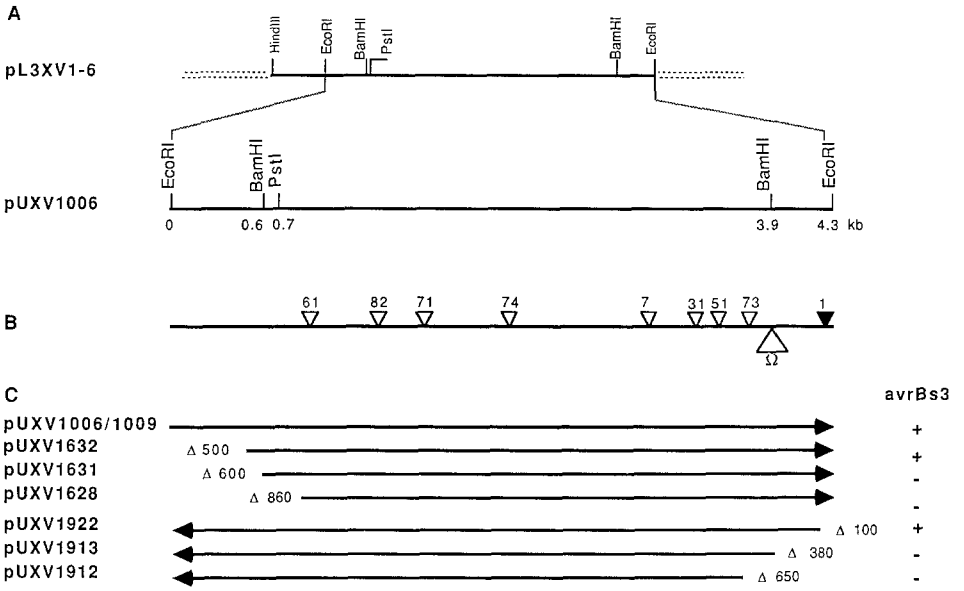

Fig. 1. A Restriction map of the DNA region containing *avrBs3* activity. Clone pL3XV1–6 contains a 5 kb *Sau*3A fragment, cloned into the *Bam*HI site of pLAFR3. The insert is flanked by a *Hin*dIII and an *Eco*RI site originating from the vector; the *dashed lines* represent vector sequences. The 4.3 kb *Eco*RI fragment from clone pL3XV1–6 was subcloned in both orientations into pUC118. The positions of independent insertions into *avrBs3* are indicated in **B** by *triangles*. The *Ω* fragment is inserted into the *Bam*HI site at 3.9 kb. The *triangles* and *numbers* above he line refer to the insertion of the Gus-poson from pHoKmGus. Only no. 1 leaves the *avrBs3* gene intact. **C** Several deletion subclones from pUXV1006 and pUXV1009 were tested for avirulence gene activity; the extent of the deletions is indicated by *Δ* and the adjacent *numbers*. +, active; −, inactive *avrBs3* as defined by the reaction on ECW-30R

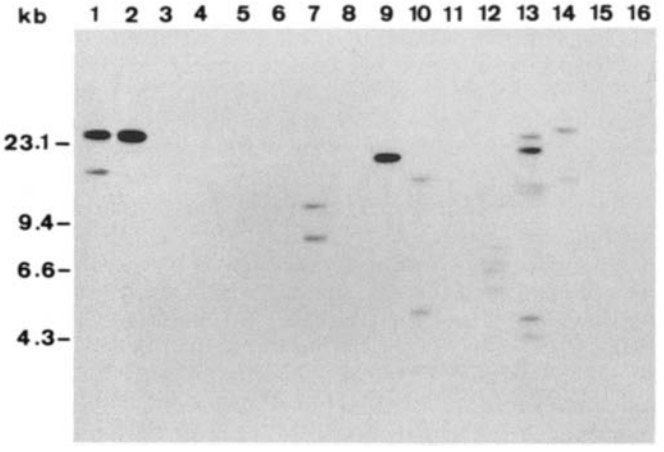

Fig. 2. Genomic Southern blot of total DNAs isolated from different *Xanthomonas* strains. Approximately 2 µg of *Eco*RI-digested DNA was loaded per lane and separated on a 0.7% agarose gel. The blot was hybridized to the 3.3 kb *Bam*HI fragment (see text). Lanes 1–5, *Xanthomonas campestris* pv. *vesicatoria* strains 82-8 (1), 71-21 (2), 85-10 (3), 81-23 (4), 75-3 (5). Lane 6, *X. campestris* T55; lanes 7–16, *X. c.* pv. *alfalfae* (7); *begoniae* (8); *campestris* (9); *carotae* (10); *fragariae* (11); *glycines* (12); *malvacearum* (13); *phaseoli* (14); *vignicola* (15); *vitians* (16)

The plasmid harboring avrBs3 *is self-transmissable*

Southern hybridization experiments using *avrBs3* as a probe confirmed that the gene is localized on the endogenous plasmid pXV11 (data now shown). To determine if this plasmid is self-transmissable *X. c.* pv. *vesicatoria* race 1 mutant strain 82-8::*Ω*1 (Sp^r) was grown together with the plasmid-free *X. campestris* strain T55 (Cm^r) which is non-pathogenic on pepper. We obtained T55 cells which were Cm^r and Sp^r. DNA analysis and hybridization experiments showed that the transconjugants carried the 45 kb plasmid from *X. c.* pv. *vesicatoria* race 1 (data not shown). This illustrates that *avrBs3* resides on a conjugative plasmid. The T55 transconjugants did not elicit any reaction on the pepper lines described nor did T55 transconjugants containing the plasmids pLAFR3 or pL3XV1–6.

DNA homology of avrBs3 *to other* X. campestris *pathovars*

Total genomic DNA of different races of *X. c.* pv. *vesicatoria* and of different *X. campestris* pathovars was digested with *Eco*RI, separated on an agarose gel and hybridized in a Southern blot experiment to the 3.3 kb *Bam*HI fragment which is an internal fragment of *avrBs3* (Figs. 1, 2). Figure 2 shows that the *avrBs3* gene is located on a 26 kb *Eco*RI fragment in *X. c.* pv. *vesicatoria* race 1. The second hybridizing fragment in race 1 strain 82-8 (lane 1) is due to homology with an additional endogenous plasmid, pXV12, which is absent in race 1 strain 71-21 (U. Bonas, unpublished). Since *avrBs3* is inactive in the mutant strain 82-8::*Ω*1 the second hybridizing fragment cannot replace the *avrBs3* gene. No hybridization of the *avrBs3* probe to strains of other *X. c.* pv. *vesicatoria* races tested was evident (Fig. 2). DNA from strains of the *X. c.* pathovars *begoniae*, *fragariae*, *holcicola*, *pruni* and *vignicola* also did not hybridize. Homologous DNA sequences were detected in the *X. c.* pathovars *alfalfae*, *campestris*, *carotae*, *glycines*, *malvacearum* and *phaseoli*. Only in *X. c.* pv. *malvacearum* was one of the hybridizing *Eco*RI or *Bam*HI fragments (data not shown) of the same size as in *X. c.* pv. *vesicatoria* race 1. The pathovars containing *avrBs3* homologous sequences induced an HR on pepper cultivar ECW-30R; however, the

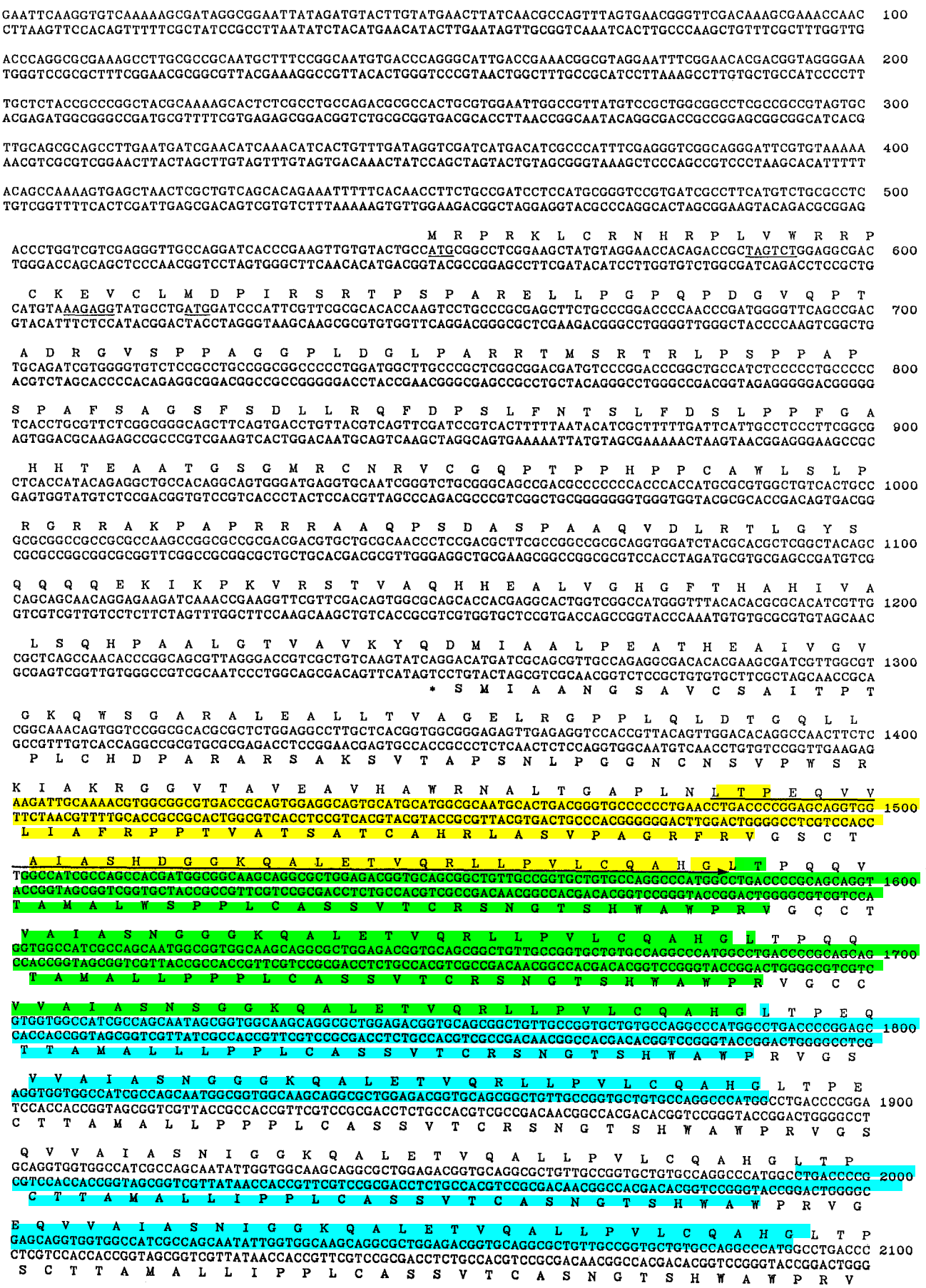

Fig. 3

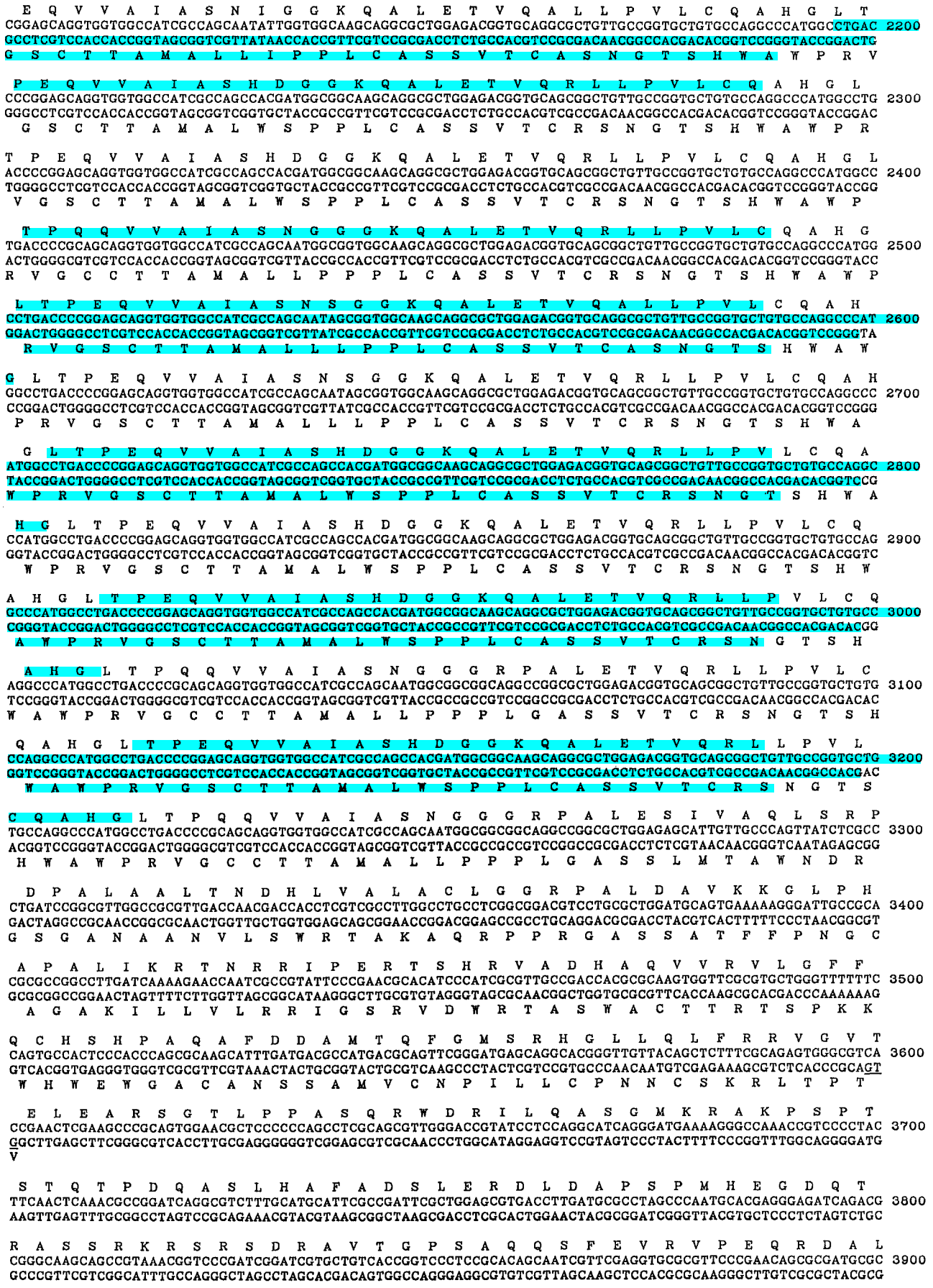

Fig. 3 (continued)

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  H  L  P  L  S  W  R  V  K  R  P  R  T  S  I  G  G  G  L  P  D  P  G  T  P  T  A  A  D  L  A  A  S
TGCATTTGCCCCTCAGTTGGAGGGTAAAACGCCCGCGTACCAGTATCGGGGGCGGCCTCCCGGATCCTGGTACGCCCACGGCTGCCGACCTGGCAGCGTC 4000
ACGTAAACGGGGAGTCAACCTCCCATTTTGCGGGCGCATGGTCATAGCCCCCGCCGGAGGGCCTAGGACCATGCGGGTGCCGACGGCTGGACCGTCGCAG

  S  T  V  M  R  E  Q  D  E  D  P  F  A  G  A  A  D  D  F  P  A  F  N  E  E  E  L  A  W  L  M  E  L
CAGCACCGTGATGCGGGAACAAGATGAGGACCCCTTCGCAGGGGCAGCGGATGATTTCCCGGCATTCAACGAAGAGGAGCTCGCATGGTTGATGGAGCTA 4100
GTCGTGGCACTACGCCCTTGTTCTACTCCTGGGGAAGCGTCCCCGTCGCCTACTAAAGGGCCGTAAGTTGCTTCTCCTCGAGCGTACCAACTACCTCGAT

  L  P  Q  *
TTGCCTCAGTGAGGCTCAGTCGGTGACTACCTGAGCGTCGGCAGGGATTGGTGTAAGTAACCTTTACTGACAGCGAGTTAGCCCACTTTTGGCTGTTTTT 4200
AACGGAGTCACTCCGAGTCAGCCACTGATGGACTCGCAGCCGTCCCTAACCACATTCATTGGAAATGACTGTCGCTCAATCGGGTGAAAACCGACAAAAA

TACACAAATCCCTGCCTCCCCTCTGGTTGCACCACACCCGTACACCAAGCGCGGCGGCGAAGCAGGCACCGAGTGGTTCCGCTGCGGTGTTGCGTTCCCT 4300
ATGTGTTTAGGGACGGAGGGGAGACCAACGTGGTGTGGGCATGTGGTTCGCGCCGCCGCTTCGTCCGTGGCTCACCAAGGCGACGCCACAACGCAAGGGA

AACCAGGGCGGTGGCTATACGCTCAAGCTGCGCACCGTCCCGGTGGCGATCGACGACGAAATG
TTGGTCCCGCCACCGATATGCGAGTTCGACGCGTGGCAGGGCCACCGCTAGCTGCTGCTTTAC
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Fig. 3. Complete nucleotide sequence of the *Eco*RI fragment from clone pXV1–6 containing *avrBs3*, presented in the 5′-3′ direction. The translation of ORF1 and ORF2 into amino acids is shown in the one letter code; translational stop codons are indicated by *asterisks*. ATG codons 1 and 2 of ORF1 are *underlined*. Putative recognition sequences [−10 box at position 585, Shine-Dalgarno (1974) at position 607] are *underlined*. The 1st of 17 repeat units in the 5′-3′ direction starts at position 1482 and is marked by an *arrow*

reaction was greener and lacked a sharp margin. Since this atypical HR was induced on the pepper cultivars ECW, ECW-10R and ECW-30R the hybridizing fragment either contains a non-functional or incomplete *avrBs3* gene, or other genes may be involved in inducing the plant reaction.

Deletion analysis of avrBs3

The *avrBs3* clone pL3XV1–6 contains an insert of approximately 5 kb. For further studies the 4.3 kb *Eco*RI fragment (see Fig. 1) was subcloned into pUC118 (Vieira and Messing 1987) giving rise to pUXV1006 and, in the inverted orientation, pUXV1009 (Fig. 1). This subfragment still confers *avrBs3* activity when cloned into pLAFR3 (Staskawicz et al. 1987) or pLAFR6 (D. Dahlbeck and B. Staskawicz, unpublished), conjugated into *X. c.* pv. *vesicatoria* race 2 strain 85-10 and inoculated on ECW-30R. To determine the minimal size of the DNA fragment required for *avrBs3* activity, a deletion analysis was performed. The *avrBs3* activity of several deletion clones was analyzed on ECW-30R. The reaction was either incompatible or compatible (Fig. 1). In no case was a phenotype intermediate between watersoaking and HR or a slower HR observed. These results indicate that a DNA region spanning 3.6–3.7 kb is necessary to confer *avrBs3* activity (Fig. 1). As pLAFR6 contains transcription termination signals flanking the insert DNA and the induction of the HR was found to be independent of the orientation of the subcloned insert, the cloned avirulence gene is probably expressed from its own promoter.

DNA sequence analysis

The DNA sequence of both strands of the 4.3 kb *Eco*RI fragment was determined (Fig. 3). The 4363 bp sequence has an overall GC content of 65% and reveals interesting structural features: a region of direct repeats is located in the middle of the fragment. These repeats, each 102 bp long, are present 17 times without a spacer sequence and are 91%–100% homologous to each other. A comparison of the repeated segments is depicted in Fig. 4. Furthermore many inverted repeat structures can be found throughout the whole sequence (data not shown). Several potential coding regions are contained within this DNA fragment (Fig. 5). The two longest open reading frames (ORFs) have been translated into amino acids in Fig. 3. Both ORFs reside within the 3.7 kb region which is required for *avrBs3* activity and lie on opposite strands (Fig. 1). ORF1 is in the upper strand, is 3561 nucleotides in size, and encodes a protein of 1187 amino acids (pep1). ORF2 is present in the lower strand, is 2351 nucleotides long and could encode a protein of 784 amino acids (pep2). The calculated molecular weights of pep1 and pep2 are 125.3 and 82 kDa respectively. There are also several shorter ORFs in both strands (see Fig. 5). All ORFs are calculated from the first ATG or GTG codon. ORF2 and ORF3 span the repetitive region in the same orientation but use different frames. The overall high GC content of the sequence is reflected in the codon usage. In ORF1 70% and in ORF2 84% of the codons have a G or C in the third position. Sequence comparisons revealed that the upstream regions of these ORFs do not show extended homology to typical prokaryotic promoters nor to the putative promoter of *avrBs1* from *X. c.* pv. *vesicatoria* race 2 (Ronald and Staskawicz 1988). Preliminary data on transcription of ORF1 of *avrBs3* indicate a −10 region around position 590 (TAGTCT; see Fig. 3) which would place the translational start at position 621.

Discussion

Here we describe the molecular and genetic analysis of the avirulence gene *avrBs3* from *X. c.* pv. *vesicatoria*. The genetic data show that the isolated *avrBs3* gene, residing on a 5 kb DNA fragment in clone pL3XV1–6, is responsible for the induction of the HR in the pepper cultivar ECW-30R. Insertion mutagenesis into a defined site as in *X. c.* pv. *vesicatoria* 82-8::Ω1 or randomly by a transposon into different sites spanning the 4.3 kb *Eco*RI fragment (Fig. 1) resulted in complete inactivation of this gene. In contrast to *X. c.* pv. *vesicatoria* wild-type race 1 strain 82-8 the mutant 82-8::Ω did not induce an HR on ECW-30R. Instead this *avrBs3* mutant strain lead to a compatible interaction on all pepper cultivars tested. All other *avrBs3* mutants, i.e. *X. c.* pv. *vesicatoria* race 2 transconjugants carrying insertion or deletion mutants of clone pL3XV1–6, also induced a compatible reaction on ECW and on ECW-30R. These results differ from those derived from studies of deletion mutants of the *avrBs1* gene from *X. c.* pv. *vesicatoria*

```
ORF1

           1                                                                                                102
rep  1     CTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTGCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCATGGC
rep  2     .........C............................C..T............................................................
rep  3     .........C..........................A.C..T............................................................
rep  4     ......................................C..T............................................................
rep  5     ....................................AT...T...........................GC...............................
rep  6     ....................................AT...T...........................GC...............................
rep  7     ....................................AT...T...........................GC...............................
rep  8     .................................C.C.A...C............................................................
rep  9     .................................C.C.A...C............................................................
rep 10     .........C............................C..T............................................................
rep 11     ....................................A.C..T...........................GC...............................
rep 12     ....................................A.C..T............................................................
rep 13     .................................C.C.A...C............................................................
rep 14     .................................C.C.A...C............................................................
rep 15     .................................C.C.A...C............................................................
rep 16     .........C............................C..C...G..C.....................................................
rep 17     .................................C.C.A...C............................................................
rep 18     .........C............................C..C...G..C............GCA.TGTTGCC.A...AT.TCGC.CTGAT.C...GTTG.C.

consensus  CTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATGGTGGYGGCAAGCAGGCGCTGGAGACGGTGCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCATGGC

aa         LeuThrProGluGlnValValAlaIleAlaSerHisAspGlyGlyLysGlnAlaLeuGluThrValGlnArgLeuLeuProValLeuCysGlnAlaHisGly
                    Gln                     AsnGly      ArgPro                  Ala
                                               Ser
                                               Ile
```

Fig. 4. Nucleotide and corresponding amino acid sequence of the repetitive DNA region in the sequence of *avrBs3* presented in Fig. 3. To demonstrate the homology between the direct repeats the nucleotide sequence of *avrBs3* from position 1482 to 3318 is shown in blocks of 102 nucleotides. In comparison to the sequence of the first repeat unit (rep 1) only the differences in the following repeats are indicated in rep 2 to rep 18. Rep 18 shows the end of the homologous region. The derived amino acid sequence of rep 1 is given in the last line. Different amino acids present in other repeat segments are indicated at their respective position but do not necessarily occur within the same repeat

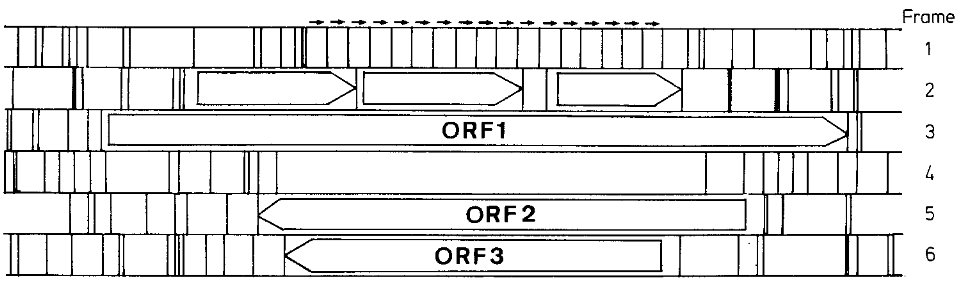

Fig. 5. The locations of the longest ORFs within the 4363 bp sequence (see Fig. 3) are shown by *large arrows*. The start codon is ATG in ORF1 and GTG in all other ORFs indicated. The positions of translational stop codons of all six frames are indicated by *vertical bars*. The *arrows* above frame 1 represent the direct repeats

race 2. In this case several *avrBs1* mutants induced an intermediate HR (Ronald and Staskawicz 1988). Growth of the *avrBs3* mutant strain 82-8::Ω1 in broth and in pepper cultivar ECW was identical to that of the wild-type but differed from wild-type strain *X. c.* pv. *vesicatoria* 82-8 when inoculated into ECW-30R. In ECW-30R the *avrBs3* mutant grew at the same rate as in the susceptible pepper cultivar ECW (data not shown). This result was anticipated since *X. c.* pv. *vesicatoria* strain 82-8::Ω1 leads to a compatible interaction in both cultivars. The fact that a mutation in *avrBs3* did not abolish growth of the mutant strain does not exclude that a mutated avirulence gene could have consequences for the free-living bacteria under other physiological conditions. The genetic data presented above prove that the race-specific induction of the HR in ECW-30R after inoculation with *X. c.* pv. *vesicatoria* race 1 is associated with the single locus *avrBs3*.

Genetic and molecular data revealed that *avrBs3* is plasmid borne. Mating experiments showed that this plasmid can be transferred from *X. c.* pv. *vesicatoria* to *X. campestris* T55. Self-transmissability may allow for natural spreading of the *avrBs3* gene. The T55 wild-type strain as well as transconjugants carrying the mutated plasmid from *X. c.* pv. *vesicatoria* race 1, pXV11:Ω, or the recombinant plasmid pL3XV1 did not elicit any reaction when inoculated into the pepper cultivars ECW and ECW-30R. The nature of other loci residing on pXV11 is not known.

Other *X. c.* pv. *vesicatoria* races, which according to their reaction on ECW-30R are lacking a functional *avrBs3* gene, do not contain sequences homologous to *avrBs3* (Fig. 2). Interestingly the repetitive sequence within the *avrBs3* gene seems to be unique for *X. c.* pv. *vesicatoria* race 1 and the *avrBs3* gene itself. It is not present in other *X. c.* pv. *vesicatoria* races. However we did find homologous sequences in strains of other pathovars of *X. campestris*, e.g. in *alfalfae*, *campestris* and *malvacearum* (see Fig. 2). The structure of homologous fragments and the extent of homology (e.g. is the repeat unit present and in how many

copies?) in these other pathovars is not known but might be interesting for evolutionary studies since *avrBs3* is located on a self-transmissable plasmid. When inoculated into pepper cultivars ECW, ECW-10R and ECW-30R, the *X. campestris* pathovars *alfalfae*, *campestris*, *carotae*, *begoniae* and *malvacearum* induced a greener, only light-brown HR. The observations that the HR was different from that normally associated with *avrBs3* and the absence of distinguishable phenotypes on the different peppers suggest that there is no functional *avrBs3* participating in the reaction. The observed light-brown HR may be due to the expression of non-host resistance which in several cases has been shown to be induced by avirulence genes (Whalen et al. 1988).

The DNA sequence of *avrBs3* reveals interesting features (Fig. 3). The presence of several long ORFs is striking (Fig. 5). It seems very likely that ORF1 (3.4 kb) is important for AvrBs3 activity. This conclusion is drawn from deletion analyses showing that a 3.6–3.7 kb DNA fragment is required for AvrBs3 activity (Fig. 1). This size and the length of ORF1 correspond. If ORF2 confers AvrBs3 activity this should allow deletions extending further without loss of the capability to induce HR on ECW-30R. However, it cannot be ruled out at the present time that other ORFs in this region besides ORF1 encode proteins which are required for function of *AvrBs3*. Mutational analyses should help to resolve this question.

A 1.7 kb DNA region consisting of 17 direct repeats of 102 bp, each corresponding to 34 amino acids, is remarkable (Fig. 4). The repeat motif is highly conserved, i.e. differences in the nucleotide and amino acid sequences of repeats occur in 3–5 positions at the most. The amino acid changes are summarized in Fig. 4. The significance of these repeats for the function of AvrBs3 is at present unclear. A homology search in the sequence data bank did not reveal any properties in common with known proteins. However the repetitive structure is reminiscent of a microtubule-associated protein in *Trypanosome brucei* which contains at least 7 repeats with 38 amino acids per block (Schneider et al. 1988). Other examples of proteins with direct repeats are bacterial ice nucleation protein (Green and Warren 1985), haemolysin from *E. coli* (Ludwig et al. 1988), erythrocyte spectrin (Speicher and Marchesi 1984) and extensin from plant cell walls (Chen and Varner 1985). All these proteins are associated with membranes or with structural components of the cell. The subcellular location of the presumptive AvrBs3 protein(s) is not known. The localization of the AvrBs3 protein is important for an understanding of the mechanism of induction of hypersensitivity. Both pep1 and pep2 reveal large hydrophobic segments. Hydropathy analysis of pep1 applying the Chou and Fasman (1974) method predicts a hydrophobic internal region (repeats) flanked by a hydrophilic N- and C-terminus. Pep2 could be a hydrophobic protein with short hydrophilic regions throughout the whole sequence. However, the codon usage of pep2 differs completely from the codon usage of pep1 and proteins of e.g. *E. coli* or *Pseudomonas syringae* (Aota et al. 1988). This could indicate that pep2 is less likely to be expressed. Secondary structure predictions for both pep1 and pep2 display a regular helix-turn-helix structure for the region spanning the repeats (not shown) suggesting a possible localization of these putative proteins in the cell wall. Further studies on the expression of the different ORFs are necessary to resolve this question.

Since the induction of the HR in different gene-for-gene systems has been shown to involve a functional avirulence gene in the bacteria one might postulate sequence homology between different avirulence genes. Therefore it was interesting to compare the sequence of *avrBs3* (both strands) with sequences of other avirulence genes, i.e. *avrBs1* from *X. c.* pv. *vesicatoria* race 2 (Ronald and Staskawicz 1988) and *avrA*, *avrB* and *avrC* from *P. syringae* pv. *glycinea* (Napoli and Staskawicz 1987; Tamaki et al. 1988). Surprisingly, no significant sequence homology at the nucleic acid or amino acid level was detected. At the present time the products of these various avirulence genes have not been localized or assigned a biochemical function. It will be interesting to learn if they share at least functional analogy when interacting with their respective counterparts, the resistance genes of their respective host plants.

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