AA	seq.RII/mammals	PHE	ASP	ARG	ARG	VAL	SER	VAL	CYS	ALA	GLU
AA	seq.RI/mammals	ARG	ARG	ARG	ARG	GLY	ALA	ILE	SER	ALA	GLU
AA	seq.RD/NP2	ARG	LYS	ARG	ARG	GLY	ALA	ILE	SER	SER	GLU
AA	seq.RD/HTY217	ARG	LYS	ARG	ARG	GLY	**THR**	ILE	SER	SER	GLU
DNA	R/NP2	CGA	AAA	AGA	AGA	GGT	GCA	ATT	AGT	AGT	GAA
DNA	R/HTY217	CGA	AAA	AGA	AGA	GGT	**A**CA	ATT	AGT	AGT	GAA

FIG. 3 The regulatory subunit of HTY217 is mutated in the sequence essential for inhibition of the activity of the catalytic subunit. The sequence of the pseudo-substrate region from a number of R subunits of pk-A is compared. This region of the protein is believed to bind into the active site of the C subunit and so inhibit its activity. The amino acids (AA) of the consensus sequence are underlined. RI and RII are from the bovine isozymes[22,23], RD from *Dictyostelium*, with NP2 being the parental strain and HTY217 the *rde*C mutant. HTY217 has a G→A transition, resulting in a mutation of Ala to Thr (bold type). PCR amplification[24] was made from minipreps of total DNA[25]. The mutation was detected in two independent experiments by direct sequencing of the PCR products[24] from NP2 and HTY217 DNA. The absence of additional mutations was confirmed by sequencing the entire PCR products, after cloning in M13 phage.

less than 1% in its transformant. The transformant also developed at a slower rate and produced a proportion of normal fruiting bodies, though many still showed various degrees of mutant morphology. This incomplete correction of the mutant morphology is probably due to our enforced use of a heterologous promoter to drive R subunit expression, though secondary mutations introduced in the isolation of strain HTY217 may also have contributed.

Because three independent alleles of *rde*C are affected in the R subunit (two null alleles, one with a point mutation) and the phenotype of an *rde*C strain can largely be reversed by transformation with a wild-type R subunit-coding region, we conclude that the *rde*C locus encodes the R subunit of pk-A. As a result of their lack of an effective R subunit, *rde*C mutants are expected to have a constitutively active C subunit. They are characterized by unrestrained spore formation: mutant prespore cells transform into spores shortly after they appear in normal development and in submerged monolayers they form spores where wild-type cells do not[7,10]. These results therefore provide strong genetic evidence that spore maturation is normally triggered by the activation of pk-A[7]. Because stalk cell differentiation is also premature in mutant development, it may also be triggered by activation of pk-A. The pk-A is on the signal transduction pathway leading from extracellular cAMP and cAMP levels are known to rise strongly as spores mature during normal development[5,17]. It therefore seems that the cAMP-signalling pathway ultimately controls spore and, possibly, stalk cell maturation. The cAMP signal transduction pathway bifurcates after the cell surface cAMP receptors with one branch using intracellular cAMP to control terminal cell differentiation, as we have argued. The other branch uses inositol(1,4,5)triphosphate (IP3)/cGMP and is believed to control cell motility and presumably morphogenesis[18,19]. It is this branching of the second messenger pathways that allows mutants like *rde*C to be heterochronic, because one branch can be mutated independently of the other. Finally, the very low cAMP levels in *rde*C mutants[5] suggests that cAMP levels are under negative feedback control from pk-A. This feedback could also be involved in the adaptation of cells to continuous stimulation by extracellular cAMP[20]. It is likely that other heterochronic mutants, such as *rde*A, will also be affected in the cAMP signal-transduction pathway. □

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Race-specificity of plant resistance to bacterial spot disease determined by repetitive motifs in a bacterial avirulence protein

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ELUCIDATION of the genetic and molecular basis of plant disease resistance is a major objective in the investigation of plant–microbial interactions. *Xanthomonas campestris* pathovar *vesicatoria* (*Xcv*), the causal agent of bacterial spot disease of pepper and tomato, has been developed as a model host–pathogen system to study the genetic interactions that specify the expression of plant disease resistance[1–6]. Several plant resistance genes (*Bs1, Bs2, Bs3*) have been genetically characterized from pepper (*Capsicum annuum*) that determine resistance to particular races of the pathogen carrying specific avirulence genes[7–9]. For example, pepper plants carrying the resistance locus *Bs3* are resistant to *Xcv* strains expressing the avirulence gene *avrBs3*. Nucleotide sequence analysis of the *avrBs3* gene revealed that the internal portion of the predicted protein product consists of a nearly identical 34 amino acid repeat unit, present in 17.5 copies[4]. We report here that the repetitive region of the *avrBs3* gene determines race-specificity and that deletions of repeat units generate new avirulence specificities and unmask undiscovered resistance genes in pepper and tomato.

Pepper plants harbouring the *Bs3* locus, for example, cultivar ECW-30R, are resistant to *Xcv* race 1 strains. When ECW-30R (*Bs3*/*Bs3*) is inoculated with *Xcv* race 1, a hypersensitive response[10] is induced and only limited growth of the bacteria is observed in the region of infection[4,5]. Inoculation of ECW-30R with *Xcv* race 2 gives rise to water-soaked lesions and permits growth of the bacteria to high levels[4]. The genetic locus responsible for induction of a hypersensitive response on ECW-30R is the bacterial avirulence gene *avrBs3*. On the basis of the gene-for-gene hypothesis[11] such a race-specific resistance is thought to be the result of the recognition between the product of a particular plant resistance gene and an elicitor that is produced by a corresponding avirulence gene product[12].

The *avrBs3* gene encodes a protein of relative molecular mass 122,000, AvrBs3 (ref. 13). To investigate whether all of the almost identical repeat units in AvrBs3 are necessary for hypersensitive

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TABLE 1 Reactions of pepper and tomato caused by *Xcv* wild-type strains and race 2 transconjugants expressing *avrBs3* and representative *avrBs3Δrep* alleles

Xcv strains*	Number of repeats deleted	Pepper ECW	Pepper ECW-30R	Tomato Bonny Best	Group
71-21		S†	R‡	R§	
75-3		R§	R§	S	
85-10		S	S	S	
85-10 (*avrBs3*)‖		S	R	S	
85-10 (*avrBs3Δrep*-109)¶	3	S	R	S	I
85-10 (*avrBs3Δrep*-110)	3	S	R	S	I
85-10 (*avrBs3Δrep*-16)	4	R	S	R	II
85-10 (*avrBs3Δrep*-99)	4	R	S	S	II
85-10 (*avrBs3Δrep*-15)	6	R	R	R	III
85-10 (*avrBs3Δrep*-32)	10	R	R	R	III
85-10 (*avrBs3Δrep*-21)	12	R	R	R	III
85-10 (*avrBs3Δrep*-14)	1	S	S	S	IV
85-10 (*avrBs3Δrep*-9)	3	S	S	S	IV
85-10 (*avrBs3Δrep*-13)	6	S	S	R	IV
85-10 (*avrBs3Δrep*-111)	9	S	S	R	IV
85-10 (*avrBs3Δrep*-27)	10	S	S	R	IV
85-10 (*avrBs3Δrep*-121)	15	S	S	R	IV
85-10 (*avrBs3Δrep*-45)	17	S	S	S	IV

* Bacteria were inoculated at 10^8 c.f.u. ml^{-1} as described[15]. Only 14 out of 30 *avrBs3Δrep* strains are shown.
† S, susceptible (water-soaked lesion).
‡ R, resistant (hypersensitive response).
§ The hypersensitive response in these interactions is caused by avirulence genes that are not related to *avrBs3*.
‖ 85-10 (*avrBs3*) carries the *avrBs3* clone pL3XV1-6 (ref. 4).
¶ 85-10 (*avrBs3Δrep*) are deletion derivatives of *avrBs3*, subcloned into the broad host range vector pLAFR3 (ref. 15) and introduced into strain 85-10 by triparental mating (refs 16, 17).

response induction on ECW-30R, we generated random deletion derivatives differing in the number of repeat units. The restriction enzyme *Bal*I that recognizes one site per repeat unit (102 base pairs (bp)) was used for this purpose. Plasmids containing the deletion derivatives (*avrBs3Δrep*) were introduced into a *Xcv* race 2 strain. In all *avrBs3Δrep* strains a protein of the expected size could be identified by western blotting (data not shown). According to the induced plant reactions on pepper the *avrBs3Δrep* mutants were classified into four groups, representatives of which are shown in Table 1. Group 1 *avrBs3Δrep* mutants retained the ability to induce a hypersensitive response on ECW-30R whereas most mutants tested (24 out of 30; group II+IV) had lost this ability. When tested on ECW which is nearly isogenic to ECW-30R, differing only at the *Bs3* locus, some *avrBs3Δrep* mutants could induce a hypersensitive response (group II). The group III *avrBs3Δrep* mutants caused

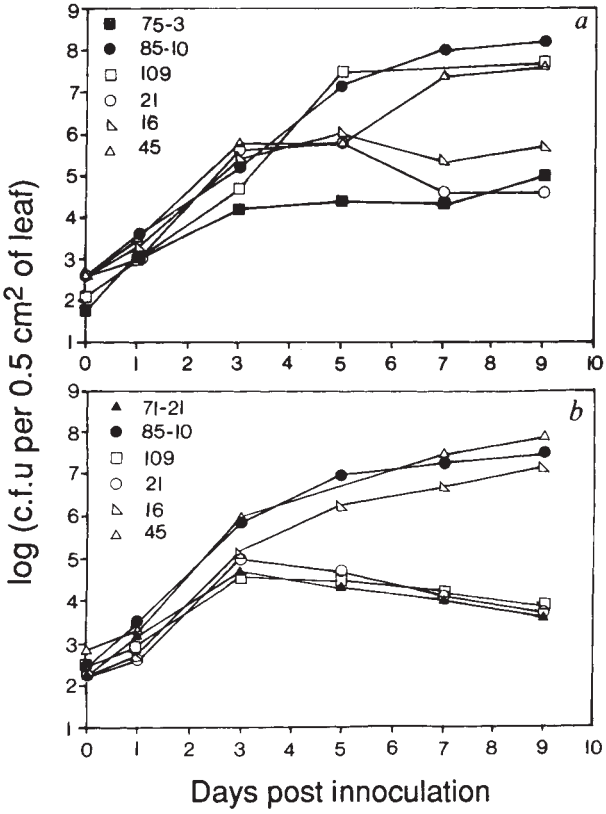

FIG. 1 Time course of multiplication of wild-type *Xcv* strains and transconjugants in ECW (*a*) and ECW-30R (*b*) pepper cultivars. The following *Xcv* strains were used: race 1 strain 71-21 (source of *avrBs3*), race 2 strain 85-10, tomato strain 75-3 and strain 85-10 transconjugants carrying *avrBs3Δrep* derivatives Δrep-109 (group I), Δrep-21 (group III), Δrep-16 (group II) and Δrep-45 (group IV) (see also Table 1 for phenotypic reactions). METHODS. Bacteria were inoculated into pepper leaves at 5×10^4 c.f.u. ml^{-1} in 1 mM $MgCl_2$ as described in ref. 15. Values are the means of three samples taken at each time point. For description of the deletion derivatives see Fig. 2.

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an intermediate reaction, between hypersensitive response and water-soaked lesions, on both ECW and ECW-30R.

To determine how the hypersensitive response or water-soaked lesion phenotype correlated with the *in planta* growth of the respective bacteria, we followed the multiplication of one representative strain of each group in both pepper cultivars (Fig. 1). Those *avrBs3Δrep* strains that were associated with water-soaked lesions multiplied to a high density differing from that of strain 85-10 by less than a factor of 2. The hypersensitive response inducing wild-type strains 71-21 (on ECW-30R) and 75-3 (on ECW), as well as hypersensitive response-inducing *avrBs3Δrep* strains, including strain carrying *avrBs3Δrep*-21 (intermediate hypersensitive response), multiplied as expected; after 9 days their density was at least two orders of magnitude lower than that of strain 85-10. These results prove that the *avrBs3* deletion derivatives that induce a novel hypersensitive response on pepper are functional alleles of this avirulence gene.

Two putative plant resistance loci recognized by different *avrBs3Δrep* alleles were characterized genetically by inoculation of F_2 progeny (332 plants) from a cross between ECW and ECW-30R (*Bs3/Bs3*, *Bs3/bs3*, *bs3/bs3*; 1:2:1 segregation) (Table 2). Derivative *avrBs3Δrep*-109 induced a hypersensitive response only on plants that were either homozygous or heterozygous for *Bs3*, whereas water-soaked lesions occurred on *bs3/bs3* plants. This suggests that derivative *avrBs3Δrep*-109 recognizes *Bs3* or a dominant locus closely linked to *Bs3*. The inoculation tests of mutant *avrBs3Δrep*-16 on the F_2 progeny plants indicate that the plant resistance locus recognized might be identical or linked to *bs3*. The former possibility is intriguing because the *bs3* allele in ECW and in heterozygous *Bs3/bs3* plants might behave like a functional, dominant resistance allele as does *Bs3* with *avrBs3*.

To analyse whether resistance determinants corresponding to *avrBs3* deletion derivatives can be unmasked in a different plant species as well, all transconjugants were tested for their phenotype on tomato cultivar Bonny Best. Surprisingly, most of the different *avrBs3Δrep* strains caused a hypersensitive response on this tomato cultivar. Among these were 16 *avrBs3*Δrep derivatives that were non-functional on pepper (Table 1).

What is the distinctive feature of the *avrBs3Δrep* alleles that gives rise to their avirulence specificity? Because a number of deletion derivatives, despite being of identical size, belong to different reaction groups, the length of the repetitive region cannot be the relevant parameter for the function of these genes.

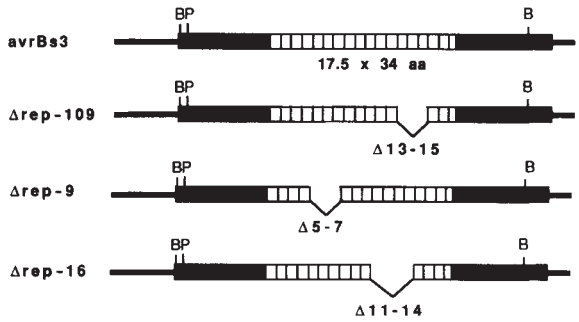

FIG. 2 Overall structure of the *avrBs3* gene and sequenced deletion derivatives. The thick bar indicates the ORF in pUXV1006 that expresses AvrBs3 (refs 4, 13). Each repeat unit (102 bp) is represented by an open box. The DNA sequences of the derivatives Δrep-109, 9 and 16 were identical to the *avrBs3* sequence[4] except for the deletion of repeat units at the positions indicated. B, *Bam*HI site; P, PstI site.

METHODS. The deletion derivatives of *avrBs3* were generated by partial digestion of pUXV1006 with *Ball* that recognizes one site per repeat unit. After size fractionation the DNA was religated and transformed into *E. coli* DH5α. The sequence was determined for the entire gene using avrBs3-specific primers for the unique regions and unidirectional deletion clones for the repetitive region as described in ref. 4.

TABLE 2 Reactions of a segregating F_2 population of an ECW-30R×ECW cross

Genotype	Number of F_2 plants	*avrBs3* allele in *Xcv* 85-10		
		avrBs3	Δrep-109	Δrep-16
Bs3/Bs3*	72	R	R	S
Bs3/bs3	163	R	R	R
bs3/bs3	97	S	S	R

A heterozygous *Bs3/bs3* plant from a cross of the nearly isogenic pepper cultivars ECW-30R (*Bs3/Bs3*)×ECW (*bs3/bs3*) was selfed. A total of 332 plants segregating for *Bs3* were analysed. The strains used are described in Table 1. Bacteria were inoculated at 10^8 c.f.u. ml^{-1} as described[15]. S, susceptible; R, resistant (hypersensitive response).

* The (*Bs3/Bs3*) genotype was confirmed by testing 14 populations (about 15 plants each) of different selfed F_2 plants that were susceptible for Δrep-16.

For three derivatives the sequence has been determined (Fig. 2). The sequences are identical to the original *avrBs3* except for the length of the repetitive region. In each derivative the deletion occurred at a different position. The distinct functions of *avrBs3Δrep*-9 (non-functional on pepper) and *avrBs3Δrep*-109 (hypersensitive response on ECW-30R) must therefore be due to the position of a repeat in the repetitive region and the type(s) of repeat units present or absent. This is also true for *avrBs3Δrep*-16 which has gained a new specificity. Thus a single or a few amino acid differences in the internal region of the protein determine its avirulence function.

The finding of unknown resistance determinants in pepper and tomato leads us to speculate that plant resistance genes complementary to *avrBs3* and its alleles may be widespread in the plant kingdom. Reciprocally, proteins homologous to AvrBs3 that may have an avirulence function occur naturally in certain strains of *Xcv* and in different pathovars of *Xanthomonas* (ref. 13 and I. Balbo, J.C.-S. and U.B., manuscript in preparation). This suggests that *avrBs3* may be one member of a family of homologous avirulence genes.

To account for the great diversity of avirulence specificity we favour a direct interaction between the avirulence gene product and the corresponding resistance gene product, possibly mediated by the repetitive structure of the avrBs3 protein and its allelic derivatives. This model is supported by the observation of intermediate hypersensitive response that could be due to a weaker binding of the molecules involved. The only example for a postulated direct interaction of an avirulence gene product with the plant is the fungal avirulence gene *avr9* from *Cladosporium fulvum*[14]. □

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