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ORIGINAL PAPER

# Physical delimitation of the pepper *Bs3* resistance gene specifying recognition of the AvrBs3 protein from *Xanthomonas campestris* pv. *vesicatoria*

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**Abstract** The pepper (*Capsicum annuum*) *Bs3* gene confers resistance to *avrBs3*-expressing strains of the bacterial spot pathogen *Xanthomonas campestris* pv. *vesicatoria*. To physically delimit *Bs3*, a pepper YAC library was screened with two flanking DNA markers that are separated from *Bs3* by 1.0 and 1.2 cM, respectively resulting in the identification of three YAC clones. Genetic mapping of the corresponding YACends revealed however, that these YACs do not cover *Bs3* and subsequent screens with newly developed YACend markers failed to identify new YAC clones. Marker saturation at the *Bs3* locus was carried out by amplified fragment length polymorphism (AFLP). The analysis of 1,024 primer combinations resulted in the identification of 47 new *Bs3*-linked AFLPs. High-resolution linkage mapping of *Bs3* was accomplished by inspecting more than 4,000 $F_2$ segregants resulting in a genetic resolution of 0.01 cM. Using tightly *Bs3*-linked YACend- and AFLP-derived markers we established a *Bs3*-spanning BAC contig and physically delimited the target gene within one BAC clone. The analysis of the *Bs3*-containing genomic region revealed substantial local variation in the correlation of genetic and physical distances.

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## Introduction

Gram-negative bacteria cause a multitude of diseases in crop plants. Their capability to infect plants depends on a molecular syringe, the so-called type III secretion system that delivers bacterial effector proteins into the host cytoplasm (Büttner and Bonas 2002). During plant-pathogen coevolution, plants evolved resistance (R) proteins that mediate recognition of individual bacterial effector proteins, also termed avirulence (Avr) proteins (Chisholm et al. 2006; da Cunha et al. 2006). Recognition of Avr proteins by matching plant R proteins is often accompanied by the occurrence of rapid host cell

death (so-called hypersensitive response; HR), which is concomitant with halt of pathogen spread (Lam 2004). Since R protein-mediated resistance occurs only if matching plant *R* and pathogen *avr* genes are expressed simultaneously, this kind of defense has been coined "gene-for-gene" resistance.

We study virulence and avirulence of type III effectors from *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), the causal agent of bacterial spot disease in pepper and tomato (Schornack et al. 2006). *Xcv* has become a model organism for the analysis of bacterial effectors (Gürlebeck et al. 2006) and is also of economical importance since bacterial spot disease results in leaf lesions, defoliation, fruit lesions, and ultimately yield loss of marketable fruit. Especially in regions with a warm and humid climate infection by *Xcv* can be devastating to commercial production of peppers and tomatoes (Stall 1995). In pepper, three resistance loci, *Bs1*, *Bs2,* and *Bs3* are known to confer resistance to particular strains of *Xanthomonas* (Cook and Guevara 1984; Cook and Stall 1982; Kim and Hartmann 1985). The first commercial *Xcv* resistant pepper varieties contained preferentially the *Bs2* gene. *Bs2*-mediated resistance was believed to be extraordinarily stable as AvrBs2 is an important pathogenicity factor of *Xcv* (Kearney and Staskawicz 1990). However, *Bs2* mediated resistance was eventually broken by *Xcv* strains that carried mutated variants of AvrBs2 (Gassmann et al. 2000). Subsequently, breeders generated pepper varieties that contained the *Bs1* and *Bs2* resistance genes. In the last two years *Xcv* strain 4 (P4) has defeated the *Bs1* and *Bs2* mediated resistances along the entire east coast of the US (W. Lindeman, personal communication). The pepper *Bs3* gene provides resistance to P4 and so far few, if any, commercial hybrids with *Bs3* resistance have been released (W. Lindeman, personal communication). Thus, the pepper *Bs3* gene is becoming increasingly important as a resistance resource against infection with *Xcv*.

Genetic and molecular studies have shown that *Xcv* strains expressing the *avrBs3* gene trigger *Bs3*-mediated resistance (Bonas et al. 1989). *avrBs3* encodes the type member of the AvrBs3-family, a large family of bacterial effectors that share 80–99% sequence identity (Schornack et al. 2006). The most striking structural feature of AvrBs3 and homologous proteins is the central repeat domain that consists of 5.5–25.5 nearly identical, tandemly arranged copies of a 34 amino-acid (aa) repeat unit (Gürlebeck et al. 2006; Schornack et al. 2006). AvrBs3 like proteins contain nuclear localization signals (NLSs) and a transcriptional activation domain (AD) in their C-terminus that are essential for their virulence activity (Ponciano et al. 2003; Szurek et al. 2002; White et al. 2000). Although AvrBs3 family members exhibit high amino acid identity, recognition of these proteins by cognate plant *R* genes is highly specific. This is illustrated by the pepper *Bs3* and the tomato *Bs4* gene that mediate specific recognition of the 96.6% identical *Xcv* AvrBs3 and AvrBs4 proteins, respectively (Ballvora et al. 2001; Schornack et al. 2005). Comparative analysis of pepper Bs3 and tomato Bs4 should provide valuable information on how plants distinguish between nearly identical microbial Avr proteins. While the pepper *Bs3* gene remains to be cloned, the tomato *Bs4* gene has been isolated already. Tomato *Bs4* encodes a nucleotide binding site (NB) leucine-rich repeat (LRR) protein that is homologous to the tobacco N protein, which confers resistance to tobacco mosaic virus (Schornack et al. 2004).

PCR-based approaches aimed at identification of *Bs4*-like sequences in pepper that represent potential *Bs3* candidate genes failed (T. Jordan and T. Lahaye, unpublished). Thus, we employed a map-based cloning strategy in order to isolate pepper *Bs3*. Previously we defined a 2.1 cM genetic target interval harboring the pepper *Bs3* gene (Pierre et al. 2000). In this study we report the next step: the physical delimitation of *Bs3* in a set of overlapping YAC and BAC clones.

## Materials and methods

### Plant material, bacterial strains, and resistance scoring

Parental lines and crosses that were used for genetic mapping of *Bs3* have been described previously (Pierre et al. 2000). Plants were grown and inoculated as described in Bonas et al. (1989). Scoring of disease resistance was performed on $F_3$ recombinants with *Xcv* strains that either contain (85–10 pDS300F and 82–8) or lack *avrBs3* (85-10 and 82-8 $avrBs2^{-},3^{-}$; Minsavage et al. 1990; Van den Ackerveken et al. 1996). Resistance, indicated by an HR, was scored over a period of 2–3 days post inoculation. The concentration of the inoculum was approximately $10^8$ CFU/ml in 1 mM $MgCl_2$.

### YAC library screen

The pepper ECW-123R YAC library has been described previously (Tai and Staskawicz 1999). The PCR-based screen was conducted on 47 pools each consisting of 384 individual clones. Positive pool individuals were identified by colony PCR. YACends were isolated by plasmid rescue (Bronson et al. 1991).

## AFLP analysis

AFLP analysis (Vos et al. 1995) was carried out on bulked DNA samples of nine resistant and nine susceptible plants, respectively (Giovannoni et al. 1991; Michelmore et al. 1991) using the hexacutter *Sac*I in combination with the tetracutter *Taq*I. After a *Sac*I + 1 and *Taq*I + 1 preamplification selective amplifications were carried out with $^{33}$P-labelled *Sac*I + 2 and non-labeled *Taq*I + 3 primers. The AFLP reaction products were resolved on 5% sequencing gels, dried, and exposed to X-ray film (Eastman Kodak, Rochester, NY, USA). Differential fragments were excised from the dried gel, eluted for 16 h in 200 μl water, reamplified by PCR, cloned into pCR 2.1-Topo vector (Invitrogen, Karlsruhe, Germany) and sequenced using an ABIPrism 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Reverse AFLP was carried out as described previously (Pierre et al. 2000).

## CAPS analysis

CAPS analysis of the segregating population was carried out on 5 μl (1:5 diluted) of miniprep DNA (Edwards et al. 1991) in a 20 μl PCR reaction with 200 μM dNTPs, 30 ng of each primer, 2 U *Taq* polymerase in 1× PCR reaction buffer (10× reaction buffer: 0.5 M KCl, 0.1 M Tris–HCl pH 8.3, 0.02 M $MgCl_2$, 1% (v/v) Triton X-100, 0.1% (w/v) gelatine). PCR conditions, primer sequences, and restriction enzymes that produce RFLPs are listed in Table 1. PCR conditions and primer sequences of BACends that have not been mapped genetically are available upon request.

## BAC library screen and BAC clone characterization

Two large-insert BAC libraries, constructed from the *C.annuum* cultivar HD208 (*bs3*) and the *Bs3*-resistant

**Table 1** PCR-based genetic markers at the *Bs3* locus

| Marker locus | Source | Primer sequence | PCR conditions[a] | | | Endonuclease that generates RFLP |
|---|---|---|---|---|---|---|
| Y65-1 | YAC | -GGCATAACGTAAAAATGGCTTCCTGATCC<br>-CAGAGGCACATCCCATCCGAGAGGTGTC | 94°C, 10 s | 55°C, 10 s | 72°C, 30 s | *Rsa*I[c] |
| Y65-2 | YAC | -GAGCGACACATACTTGTACTCCGTGTGTG<br>-CTACGGGCGACTGAATACTTGAACCTC | 94°C, 10 s | 60°C, 5 s | 72°C, 60 s | *Pvu*II[b] |
| Y110-1 | YAC | -AGTGAATAAATCTTAATAGGAGTGGACCAC<br>-GCTATATATGTTCATTGTAGATAAGTAATCC | 94°C, 10 s | 55°C, 20 s | 72°C, 60 s | *Cvi*RI[b] |
| Y110-2 | YAC | -CTTACCTTAAGTGACACTCTAGGCCAG<br>-GGGCATATGGAGCTACGCTTGCAC | 94°C, 10 s | 55°C, 10 s | 72°C, 20 s | *Dra*II[b] |
| Y152-1 | YAC | -GGCATAACGTAAAAATGGCTTCCTGATCC<br>-CAGAGGCACATCCCATCCGAGAGGTGTC | 94°C, 10 s | 55°C, 10 s | 72°C, 30 s | *Sty*I[c] |
| Y152-2 | YAC | -AGTGTTAGTATCCGTGGCATCAGTTAGTCC<br>-GCTTAGTGTCCGATTATGAATACGTTGC | 94°C, 10 s | 55°C, 10 s | 72°C, 50 s | *Mbo*II[c] |
| S1464 | AFLP | -ATTCGAGAGAGTGGTCTCTTTCTTGGTCC<br>-TACCCCTATCCCACTCCTACTCACG | 94°C, 10 s | 55°C, 10 s | 72°C, 30 s | *Taq*I[b] |
| S2245 | AFLP | -CCCATGTTGGCCTTGTCAATARACGTTGTG GAGGC<br>-GATCCTAGTCAAGCTAGCTTCCT | 94°C, 10 s | 60°C, 5 s | 72°C, 60 s | *Eco*147I[c] |
| B3T7 | BAC | -CTTGTGTTGAGATATGGTAC<br>-TGACTGTTAGTTGGCGTGTGAAG | 94°C, 10 s | 50°C, 20 s | 72°C, 40 s | *Hin*dIII[b] |
| B14T7 | BAC | -CTATCATGTAATTTACCATAG<br>-CCTAATAGAATTCTTTCCAATATTG | 94°C, 10 s | 49°C, 10 s | 72°C, 20 s | *Bcu*I[c] |
| B30T7 | BAC | -TCAAGAGTTTAAGCTTCCCATG<br>-AGAACATAACATGGGAATTGC | 94°C, 10 s | 50°C, 20 s | 72°C, 35 s | *Mbo*II[b] |
| B103T7 | BAC | -GAAGAATCTACTTCAACCGATATGC<br>-ACTTTAACGCCTTATATTCCCTCCT | 94°C, 10 s | 62°C, 20 s | 72°C, 40 s | *Rsa*I[b] |
| B103SP6 | BAC | -AGGTGGAAGTACGGGGTATT<br>-TTCCCTCAACGCCAGTGTAT | 94°C, 10 s | 66°C, 20 s | 72°C, 40 s | *Alu*I[b] |
| B104SP6 | BAC | -TAATGTTGGGCTCATGGTGA<br>-AATAAATCGGGACCGGAGTC | 94°C, 10 s | 60°C, 20 s | 72°C, 40 s | *Hph*I[b] |
| B128T7 | BAC | -TGGAAAGAAAGATTGTGTGCTC<br>-ATCGGAATTGTGGGATGGTA | 94°C, 10 s | 63°C, 20 s | 72°C, 35 s | *Mun*I[b] |

[a] All PCRs started with a denaturing step of 3 min at 94°C, followed by 35 cycles of amplification performed under the conditions indicated

[b] and [c] indicate whether ECW-30R-(*Bs3*) or PI 197409- (*bs3*) derived PCR products are cleaved by a given endonuclease, respectively

pepper line ECW-30R (Ruffel et al. 2002; T. Jordan and T. Lahaye, unpublished) were employed for physical mapping of *Bs3*. For BAC sizing 500 ng of BAC DNA was digested with 10 U *Not*I and fractionated by pulsed-field gel electrophoresis (PFGE) using a Chef Mapper system (Bio-Rad, California, USA) in 1% agarose gel (6 V/cm, switch time 1–50 s, included angle 120°, 20 h run time, 14°C, 0.5× TBE). The insert size was determined by comparison with a size standard (Mid Range I PFG marker, New England Biolabs, Frankfurt, Germany). For BAC fingerprinting 500 ng DNA was digested with 20 U *Eco*RI, *Hin*dIII and *Bam*HI and fractionated on 0.8% agarose gels.

### BACend isolation, sizing of BAC inserts, and contig assembly

Sequences flanking the cloning site of the BAC vector pIndigoBAC-5 (Epicentre, Madison, WI, USA) were designated T7 and SP6. The T7 end was defined according to presence of the T7 promoter primer in pIndigoBAC-5 (311–330), while the opposite side was defined as the SP6 end. Insert ends of BAC clones were determined via direct sequencing. Sequencing was performed with an ABIPrism 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The sequence information was used to design BACend specific primers and to define the overlap relationship within BAC contigs. The BACends were also used as hybridization probes on *Hin*dIII-digested BAC DNA to confirm the PCR results. For sizing, BAC inserts were released by *Not*I digest and PFGE-fractionated. Subsequently BAC insert sizes were determined by comparison with a size standard (Mid Range II PFG marker, New England Biolabs, Frankfurt, Germany). The conditions for PFGE were switch time ramping from 5 to 10 s, temperature 14°C, 6 V/cm, pulse angle 120° using 0.5× TBE buffer for 20 h.

## Results

### Screening of a pepper YAC library

In order to obtain large insert genomic clones covering the *Bs3* locus, we screened a pepper YAC library with the *Bs3*-linked markers P23-70 and P22-3 that are located on either side of *Bs3*, separated by 1.0 and 1.2 cM, respectively (Fig. 1a; Pierre et al. 2000). The PCR-based library screen yielded the YAC clones Y65 (identified with P23-70) and Y110 (identified with P22-3). The orientation of the YACs with respect to *Bs3* was defined by genetic mapping of their ends (Fig. 1a). For this, we isolated both ends of Y65 and Y110 by plasmid rescue, determined their sequences, derived YACend-specific oligonucleotides and PCR-amplified the corresponding loci from the parental genotypes of the mapping population (PI-197409, [*bs3*] and ECW-30R [*Bs3*]). Comparative sequence analysis revealed restriction site polymorphisms (RFLPs) for each YACend facilitating the development of corresponding CAPS (cleaved amplified polymorphic sequences; Konieczny and Ausubel 1993) markers (Table 1). Analysis of 790 $F_2$ backcross plants revealed that Y65-2 and Y110-2 were the most closely *Bs3*-linked YACend markers on either side of the target gene and that none of the YACs covers the *Bs3* locus (Fig. 1a). PCR analysis of the YACend markers on YAC template DNA indicated that Y65 and Y110 do not overlap, which is in agreement with the linkage mapping that placed Y65- and Y110-derived markers on opposite sides of the *Bs3* locus. In order to identify YAC clones that potentially cover the *Bs3* locus we performed a second library screen with Y65-2 resulting in the identification of YAC clone Y152. By contrast, a screen with Y110-2 only led to the re-identification of YAC clone Y110. We isolated both ends of Y152 and established corresponding CAPS markers (Table 1). Linkage mapping placed Y152-1 between Y65-2 and *Bs3*, separated from *Bs3* by 0.6 cM (Fig. 1a). Furthermore, PCR analysis showed that the marker loci Y152-1 and Y152-2 couldn't be amplified from Y110-derived template DNA. Thus YACs Y152 and Y110 do not overlap and YAC Y152 does not cover the *Bs3* locus. Given that Y152-1 was the most closely *Bs3*-linked marker, we initiated a new YAC library screen. However, we did not identify any new YAC clones with the Y152-1 YACend marker.

In summary, we were not able to physically delimit *Bs3* by chromosome walking, which might be due to a gap in the pepper YAC library. However, linkage mapping of the YACends allowed narrowing down the target interval from 2.1 to 1.7 cM with Y152-1 at a distance of 0.6 cM being the most closely *Bs3*-linked marker.

### Marker-assisted construction of DNA pools for the identification of *Bs3*-linked AFLPs

Since pepper BAC libraries have been established recently (Ruffel et al. 2002; Yoo et al. 2001, 2003) we considered to physically delimit the *Bs3* gene via a BAC-based chromosome walk. However, in pepper 1 cM equals approximately 1,200–2,500 kb (Lefebvre et al. 1995). Therefore the genetically most closely linked *Bs3* marker Y152-1 (0.6 cM apart from *Bs3*;

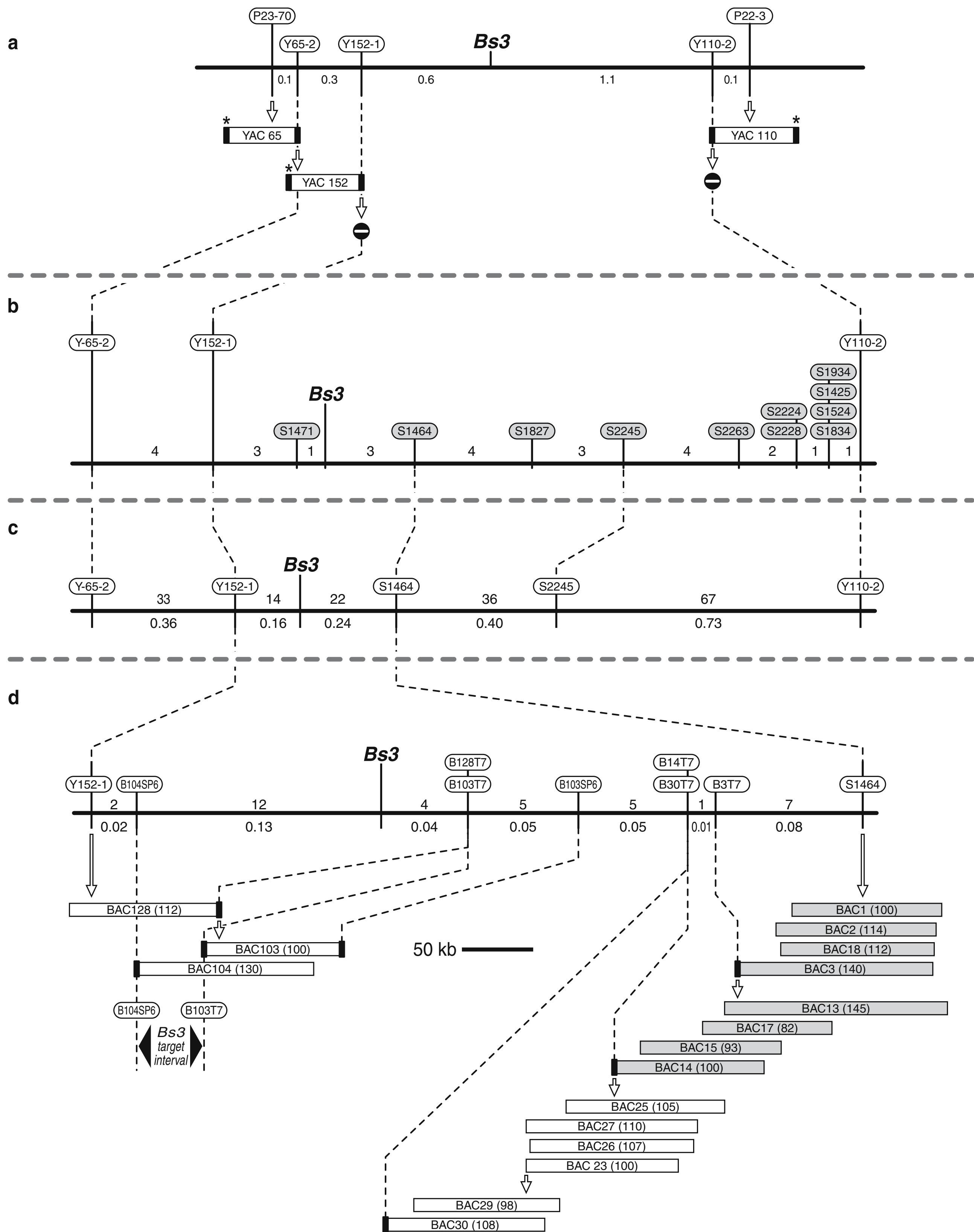


Fig. 1a) should be 700–1,500 kb away from *Bs3*. Because this physical distance seemed unsuitable for a BAC-based walking strategy, we decided to first saturate the *Bs3* locus with DNA markers by an AFLP bulked-segregant approach (Michelmore et al. 1991; Vos et al. 1995). To avoid identification of AFLP loci

◀**Fig. 1** Successive stages of the high-resolution genetic and physical mapping of the pepper *Bs3* locus. *Horizontal lines* represent the chromosome segment at the *Bs3* locus. *Vertical lines* attached to rounded boxes show the name and location of DNA-based markers. *Markers highlighted* in *white* have been mapped genetically as co-dominant CAPS-markers and markers highlighted in *gray* have been mapped genetically as dominant AFLP-markers. The genetic distance (in cM) between two given markers is indicated below the horizontal line and the number of recombinants between these two markers is indicated above the horizontal line. **a** YAC clones at the *Bs3* locus. *Vertical arrows* indicate library screens with a given marker. *Open bars* represent isolated YAC clones. *Short, bold vertical lines* indicate genetically mapped YACends. The genetic position of YACends marked with an *asterisk* has been determined with a small subset of the mapping population and thus has not been integrated into the map. Linkage mapping of the other YACends is based on the analysis of 790 $F_2$ backcross progenies. **b** Linkage map of newly identified *Bs3*-linked AFLP markers. Map positions were determined by analysis of 26 recombinants selected out of 790 $F_2$ backcross progenies. Genetic distances are given as the number of recombination events with respect to the adjacent marker. **c** High-resolution genetic map of the *Bs3* locus. Map positions are based on the analysis of 9,226 meiotic events (790 $F_2$ backcross progeny and 4218 $F_2$ plants). **d** A BAC contig covering the pepper *Bs3* locus. Pepper HD208- and pepper ECW-30R-derived BAC clones are displayed as *gray* and *white boxes*, respectively. The designation and size of each BAC (in kb) is indicated. *Short, bold vertical lines* indicate genetically mapped BACends. *Vertical arrows* indicate library screens with a given marker. The minimum genetic target interval that covers *Bs3* is indicated by *arrowheads*

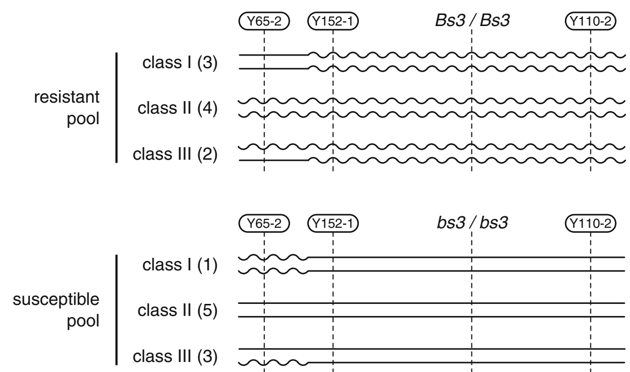


**Fig. 2** Graphical genotypes of the bulked segregant pools for the targeted AFLP marker screen. The DNA pools consist of three different classes: (I) homozygous recombinant within the marker interval Y65-2/Y152-1, (II) without detectable recombination event within the marker interval Y65-2/Y110-2 and (III) heterozygous recombinant in the marker interval Y65-2/Y152-1. Chromosome fragments derived from the resistant and susceptible parent are displayed as *curved* and *straight horizontal lines*, respectively. *Vertical lines* and *rounded boxes* denote the location and the designation of DNA-based markers, respectively. The number of used plants for each class is given in *brackets*

not tightly linked to *Bs3*, we used marker-assisted selection of segregants for the construction of DNA pools. Both the resistant (R) and the susceptible (S) pool each contained nine plants representing three different classes of recombinants (Fig. 2). The pools contained homozygous and heterozygous $F_3$ individuals with a recombination event between Y65-2 and Y152-1 and segregants without a detectable recombination event in the Y65-2–Y110-2 marker interval (Fig. 2). Since all pooled individuals were homozygous in the Y152-1–Y110-2 marker interval, *Bs3*-linked AFLP markers could be detected in both the resistant and the susceptible pools.

## Identification of AFLP markers closely linked to *Bs3*

1,024 AFLP primer combinations (*Taq*I + 3/*Sac*I + 2) were tested on pool and parental DNA samples, generating 100 AFLP loci on average. The parental lines showed a genome-wide polymorphism of 16%. Thus, we analyzed approximately 16,400 (1,024 × 100 × 0.16) loci for *Bs3* linkage from which 23 and 24 were specific to R and S bulks, respectively. Analysis of the pool individuals revealed that 29 AFLP loci were separated from *Bs3* by at least one recombination event while 18 fragments showed complete linkage to *Bs3* within the individuals of each bulk. To position the 18 new AFLP markers with respect to *Bs3*, 26 additional segregants that carry recombination events within the Y65-2–Y110-2 marker interval were analyzed. Eleven out of the 18 new *Bs3*-linked AFLP markers mapped between Y65-2 and Y110-2 (Fig. 1b). S1471, is located between Y152-1 and *Bs3* while the others are located between Y110-2 and *Bs3*. Seven out of 18 newly identified AFLPs mapped outside the Y65-2–Y110-2 interval, which is presumably a consequence of the design of the bulks that were used in this study (see Discussion).

## Conversion of dominant AFLP into co-dominant CAPS markers

Since large-scale recombinant screens require robust, co-dominant markers we aimed to convert dominant AFLPs into co-dominant CAPS markers. The four most closely *Bs3*-linked AFLP loci (S1471, S1464, S1827, and S2245; Fig. 1b) were 200–700 bp in size and thus suitable in principle for the conversion into CAPS markers. For this, the AFLP fragments were excised from the polyacrylamide gel, eluted, reamplified, cloned and sequenced. For each AFLP fragment multiple corresponding clones were analyzed. With the exception of S2245, we found multiple clones that were not sequence identical for all excised AFLP fragments. To identify the genuine sequence for each AFLP locus we analyzed the cloned fragments by “reverse AFLP”

(Pierre et al. 2000). For this, AFLP reactions on S and R bulks were separated by polyacrylamide gel-electrophoresis (PAGE) electro-blotted to a nylon membrane and probed with the cloned AFLP fragments. For all AFLP loci we identified at least one cloned fragment that generated differential hybridization patterns on S and R bulks and thus represented the desired AFLP locus (data not shown). BLAST analysis of the corresponding DNA sequences uncovered no obvious sequence similarity with repetitive sequences and so we proceeded by designing primer pairs for each marker fragment. PCR amplifications with primers corresponding to the markers S1471 and S1827 gave rise to multiple bands indicating that these fragments are not single copy loci. Therefore they were not suitable for conversion into CAPS markers. By contrast, amplifications with primers corresponding to the marker loci S1464 and S2245 produced uniformly sized fragments indicating that these AFLP loci represent single-copy loci. Since the PCR products of resistant (ECW-30R) and susceptible (PI-197409) parents were identical in size for each marker locus, the amplification products were sequenced to determine potential single nucleotide polymorphisms (SNPs). We did not find SNPs that affect restriction enzyme recognition sites. Yet, based on a SNP in marker locus S2245 we designed mismatch primers that generate an *Eco*147I recognition site in the resistant but not the susceptible parental genotype (Table 1). Since marker S1464 showed no SNP between the parental lines the sequence was extended beyond the flanking *Taq*I and *Sac*I sites of this AFLP locus. For this, a corresponding large-insert clone from a pepper BAC library (Ruffel et al. 2002) was identified and subcloned into a high-copy vector. S1464-containing clones were identified by Southern hybridization. Sequence information obtained from the BAC-derived fragments was used to amplify an extended AFLP-locus from the susceptible and resistant parents. Sequence analysis of the differential S1464 AFLP fragment revealed a *Taq*I polymorphism, which could be used to generate a corresponding CAPS marker. Taken together, two out of four *Bs3*-linked AFLP markers were successfully converted into CAPS markers (Table 1).

### Recombinant screen at the *Bs3* locus

Previously, 790 backcross plants were analyzed to identify recombination events at the *Bs3* locus (Pierre et al. 2000). To increase the genetic resolution in the vicinity of the target locus, we have analyzed here 4,218 additional $F_2$ segregants. The screen was carried out with CAPS markers Y65-2 and Y110-2 and resulted in the identification of 172 plants that showed a recombination event within this marker interval (Fig. 3a shows the identification of some recombinant plants). Recombinant plants were subsequently analyzed using the CAPS markers Y152-1, S1464 and S2245 to define the location of the recombination events more precisely (Fig. 1c). To test whether the recombinant individuals are susceptible or resistant, we performed infection tests with *avrBs3*-expressing *Xcv* strains on marker-selected, homozygous $F_3$ individuals. In summary, we established a genetic map based on the analysis of 9,226 gametes (790 backcross and 4,218 F2 plants), which corresponds to a genetic resolution of 0.01 cM.

### Identification of pepper BAC clones that cover the pepper *Bs3* locus

In order to generate a physical contig spanning *Bs3*, we exploited an available BAC library that was prepared from the *C.annuum bs3*-genotype HD208 (Ruffel et al. 2002) (Fig. 1d; HD208-derived BACs are displayed in gray color). We conducted PCR-based library screens with S1464 and Y152-1, two markers that were located on opposite sides of *Bs3*. Marker S1464 yielded three positive clones (BAC1, BAC2, and BAC3) while marker Y152-1 yielded no positive clones. The lack of Y152-1 containing clones was unexpected given that the pepper HD208 BAC library represents ten genome equivalents (Ruffel et al. 2002). In addition, it is unlikely that the pepper line HD208 lacks the Y152-1 locus entirely, because a PCR with corresponding primers amplified a fragment of expected size from plant genomic DNA of line HD208 (data not shown).

The overlap relationship of S1464-corresponding BAC clones (BAC1, BAC2, and BAC3) was determined by absence or presence of BACend-specific PCR products and confirmed by fingerprint analysis. Genetic mapping of the BACends revealed that the T7 end of BAC3 was the most closely *Bs3*-linked marker within the contig and thus defined the direction for subsequent chromosome walking towards *Bs3*. Using B3T7 as a probe, four additional BAC clones were identified from the HD208 BAC library (BAC13, BAC14, BAC15, and BAC17). Subsequently B14T7 was defined as the most closely *Bs3*-linked BACend within this contig. However, genetic mapping of B14T7 showed that the established BAC contig still did not cover *Bs3*.

Since the HD208 BAC library did not contain a BAC clone covering the Y152-1 locus and since insert DNA from the HD208 derived pepper BAC clones was not suitable for complementation studies, we constructed a new BAC library from the *Bs3*-resistant

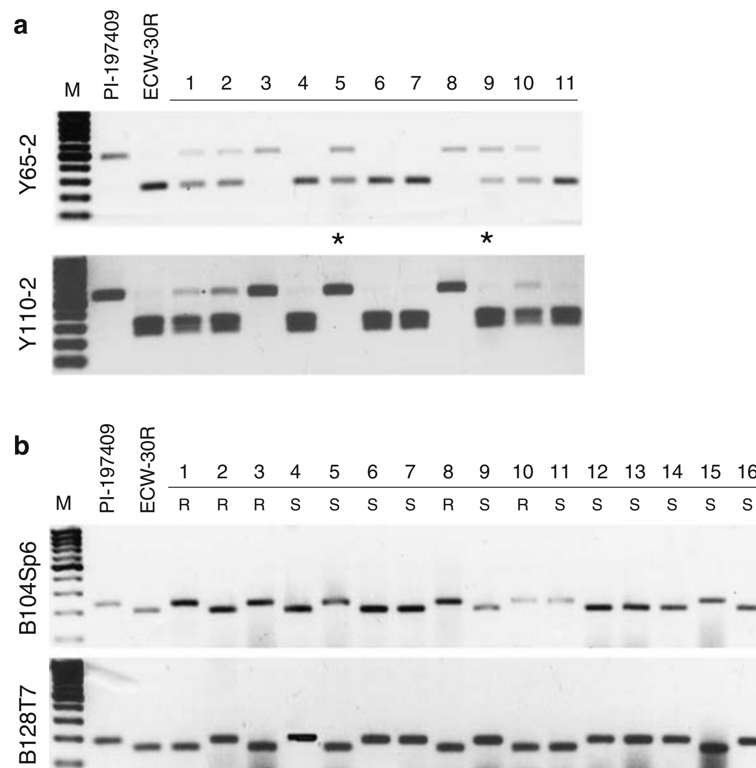


**Fig. 3** PCR-based analysis of segregants and recombinants. **a** CAPS marker-based identification of recombinants at *Bs3* using markers Y65-2 and Y110-2. *Numbers on top* represent different $F_2$ individuals of the mapping population. Segregants that showed a recombination event between Y65-2 and Y110-2 are marked by an *asterisk*. The *first two lines* show the fragment pattern of the susceptible (PI-197409) and the resistant (ECW-30R) parent, respectively. **b** Linkage analysis of the BAC-derived CAPS markers B104SP6 and B128T7. *Numbers on top* represent the 16 $F_3$ recombinants that are encompassed by marker interval B104SP6 / B128T7. *Upper case letters* represent the genotype at the *Bs3* locus, R, *Bs3*/*Bs3*; S, *bs3*/*bs3*. Ethidium bromide-stained cleaved PCR amplicons were resolved on 2.5% agarose gels. M, GeneRuler 100 bp ladder (MBI Fermentas, Vilnius, Lithuania)

pepper cultivar ECW-30R (details of the library construction and its characterization will be described elsewhere). A first screen of the ECW-30R BAC library with Y152-1 yielded one corresponding BAC clone (BAC128; Fig. 1d; ECW-30R-derived BACs are displayed in white). Linkage mapping revealed that the T7 end of BAC128 (B128T7) and Y152-1 are located on opposite sides of *Bs3*, separated by 4 and 14 recombinants, respectively. Thus BAC128 spans the *Bs3* locus by means of mutually exclusive recombination events. A successive library screen with B128T7 resulted in the identification of BAC103 and BAC104. Through genetic and physical mapping of the corresponding BACends *Bs3* could be narrowed down to the B104SP6–B103T7 minimal physical target interval (Fig. 1d).

We also used B14T7 as a starting point for two successive screens in the newly established ECW-30R BAC library. This resulted in a contig of 14 BAC clones, which, however, did not overlap with the *Bs3*-spanning contig (Fig. 1d).

In summary, mutually exclusive recombination events led us to conclude that the *Bs3* locus is located between the markers B104SP6 and B103T7, separated from the *Bs3* target gene by 12 and 4 recombination events, respectively (Fig. 3d). Given that both *Bs3*-flanking markers are present in BAC clones 128 and 104, we have physically delimited the pepper *Bs3* locus.

## Discussion

### Identification of tightly *Bs3*-linked AFLP markers

A crucial step in map-based gene cloning from complex plant genomes is the generation of a marker-saturated genetic map. In this study we employed the AFLP technology (Vos et al. 1995) in conjunction with bulked segregant analysis (Giovannoni et al. 1991; Michelmore et al. 1991) to enrich the *Bs3* containing genomic region with DNA markers. To minimize detection of AFLP markers that are not tightly linked

to *Bs3*, we used a targeted marker screen (Lahaye et al. 1998). In this approach the DNA bulks contain marker-selected segregants that display a recombination event in vicinity of the target locus. Integration of such recombinant plants minimizes the genetic target interval that is defined by the sum of the pooled individuals and thus excludes markers that are not tightly linked to a given target gene. We integrated individuals in our pools that contain a recombination event between Y65-2 and Y152-1. To delimit the target interval on the other side of the *Bs3* we could have included recombinants between *Bs3* and Y110-2. However, we avoided these sorts of recombinants, as well as, recombinants between *Bs3* and Y152-1 because we would have possibly integrated one or more segregants that by chance carry a recombination event that is only a few kilobases away from *Bs3*. Integration of such a recombinant plant into a bulk would drastically reduce the genetic target interval and would make it almost impossible to detect *Bs3* linked markers at this side of the target gene where the recombination event is located. However, as a consequence of our pool design, we were not able to exclude such AFLPs that were located towards Y110-2 but further away from *Bs3* than Y110-2 itself. Indeed, we identified seven AFLPs that were further away from *Bs3* than Y110-2. Nevertheless, our marker-based selection of bulk individuals allowed us to efficiently identify new, tightly *Bs3*-linked AFLP markers.

## Recombination frequency at the *Bs3* locus

The most closely *Bs3*-linked markers S1464 and Y152-1 served as starting points to establish two corresponding BAC contigs (defined as S1464- and Y152-1-contigs). The Y152-1-contig consists of three BAC clones, covers 23 recombination events and contains the *Bs3* gene. By contrast, the S1464-contig, which consists of 14 BAC clones, covers only eight recombination events. Although we have not determined the exact physical size of both BAC contigs, fingerprint analysis indicates that the S1464-contig is at least twice as long as the Y152-1-contig. Thus, there is significant variation in the recombination frequencies in both BAC contigs.

We have also observed significant variation in recombination frequencies within the S1464-contig, since BAC3 (140 kb) covers seven recombination events while BAC30 (108 kb) covers none. The different recombination frequencies that are observed in BAC3 and BAC30 demonstrate that even within a relatively small genomic segment, recombination frequencies can differ substantially. These observations are consistent with studies in maize, where recombination frequencies were found to vary up to seven-fold even within a physical interval of only 140 kb (Civardi et al. 1994).

How do the observed recombination frequencies within the different BAC clones relate to the genome-wide average? BAC3, which is approximately 140 kb in size, covers a genetic distance of at least 0.08 cM. Hence, 1 cM in this genome segment corresponds to a maximum physical distance of 1,750 kb. Given that in pepper 1 cM equals on average 1200-2500 kb (Lefebvre et al. 1995) the region covered by BAC3 shows a recombination frequency within the expected range. However, the situation is significantly different within BAC128, which was shown to cover *Bs3*. BAC128 (112 kb) covers a 0.19 cM genetic interval that is bracketed by Y152-1 and B128T7 (Fig. 1d). Thus the relationship between genetic and physical distance within the genomic stretch covered by BAC128 is maximally 600 kb/cM. Such a high recombination frequency is remarkable since genomic areas that contain resistance genes are usually located within recombination "cold spots" (Van der Hoorn et al. 2002). The occurrence of recombination cold and hot spots has been extensively studied in yeast (Petes 2001). It has been observed that high recombination frequencies are generally indicative for gene-rich regions. Thus, the relatively high recombination frequency observed within BAC128 might indicate the presence of a gene-island in the *Bs3*-containing genomic region.

## Pepper *Bs3* does not share sequence homology with known *R* genes

Tomato *Bs4*, rice *Xa27,* and rice *xa5* represent *R* genes that have been isolated most recently and that, like pepper *Bs3*, mediate recognition of a matching AvrBs3-like protein (Gu et al. 2005; Iyer and McCouch 2004; Schornack et al. 2004). Tomato *Bs4* encodes an NB-LRR type R protein like the majority of plant *R* genes (Schornack et al. 2004). By contrast, rice *Xa27* and *xa5* encode proteins that neither share sequence homology to each other nor to other known plant R proteins (Gu et al. 2005; Iyer and McCouch 2004). Thus the small repertoire of *R* genes that mediate recognition of AvrBs3-like proteins is structurally and functionally surprisingly divergent although the corresponding Avr proteins share high sequence similarity.

The identity of the pepper *Bs3* gene remains to be clarified. To identify potential candidate genes the *Bs3* containing BAC clone 128 has been shotgun-cloned and sequenced with an average of six-fold redundancy (P. Römer, T. Jordan and T. Lahaye, unpublished).

BLAST analysis (Altschul et al. 1990) of these shotgun clones uncovered neither NB-LRR encoding genes nor genes encoding proteins with homology to Xa27 or xa5. Thus it seems likely that pepper Bs3 employs recognition principles that are mechanistically different to Bs4, Xa27 or xa5.

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