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Chromosome landing at the tomato *Bs4* locus

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Abstract The tomato (*Lycopersicon esculentum*) *Bs4* gene confers resistance to strains of *Xanthomonas campestris* pathovar *vesicatoria* that express the avirulence protein AvrBs4. As part of a map-based cloning strategy for the isolation of *Bs4*, we converted *Bs4*-linked amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) markers into locus-specific sequence-tagged-site (STS) markers. The use of these markers for the analysis of 1972 meiotic events allowed high-resolution genetic mapping within a 1.2-cM interval containing the target gene. Two tomato yeast artificial chromosome (YAC) clones, each harboring inserts of approximately 250 kb, were identified using the marker most closely linked to *Bs4*. YAC end-specific markers were established and employed to construct a local YAC contig. The ratio of physical to genetic distance at *Bs4* was calculated to be 280 kb/cM, revealing that recombination rates in this region are about three times higher than the genome-wide average. Mapping of YAC end-derived markers demonstrated that the *Bs4* locus maps within a region of 250 kb, corresponding to a genetic interval of 0.9 cM.

Keywords *avrBs3* · *avrBs3-2* · Sequence-tagged-site (STS) marker · Bacterial spot disease · Cleaved amplified polymorphic sequence (CAPS)

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Introduction

Plant disease resistance can often be explained by the gene-for-gene hypothesis, which predicts that the product of an avirulence (*avr*) gene of the pathogen is recognized by a corresponding plant disease resistance (*R*) gene product (Flor 1971). Recognition is generally accompanied by rapid cell death at the site of attempted infection, termed the hypersensitive response (HR) (Klement and Goodman 1967), which restricts further spread of the infection (Richberg et al. 1998; Shirasu and Schulze-Lefert 2000). Plant *R* genes are envisaged to encode receptors that specifically bind the corresponding *avr*-encoded ligand and subsequently activate a coordinated defense response (Keen 1990). Cloning of plant *R* genes and complementary pathogen *avr* genes is required to test the proposed receptor-ligand model and has motivated plant pathologists to establish the necessary molecular tools. Since tagging systems are not available for many agronomically important plant species, positional (map-based) cloning remains the key option for the isolation of most *R* genes. The positional cloning approach can be subdivided into three stages: (1) genetic mapping, (2) physical mapping, and (3) complementation analysis. The speed and ease of genetic mapping was revolutionized by PCR-based marker technologies like sequence-tagged sites (STS) (Olson et al. 1989) or amplified fragment length polymorphisms (AFLPs) (Vos

et al. 1995). These technological advances allow efficient marker saturation and precise marker placing at a given target locus, thereby facilitating direct isolation of large-insert genomic clones that span the target gene, without need for chromosome walking (Tanksley et al. 1995). This concept has been termed "chromosome landing" and has become the new paradigm for map-based cloning in plants (Tanksley et al. 1995).

Isolation of *R* genes has revealed that the vast majority of them encode proteins containing a putative nucleotide binding site (NBS) and leucine-rich repeats (LRRs) (Ellis et al. 2000; Dangl and Jones 2001; Holub 2001; Jones 2001). By contrast, most Avr proteins show little or no homology to one another. However, members of the *Xanthomonas* spp. *avrBs3* gene family (named for its first sequenced member *avrBs3*), to which *avrBs4* belongs (Bonas et al. 1989, 1993), are notable exceptions. More than 15 *avrBs3*-like genes have been isolated so far; all show more than 90% sequence identity at the amino acid level (Vivian and Arnold 2000). Members of the AvrBs3 family contain, in their central portions, nearly perfect copies of a 34-amino acid repeat motif (Hopkins et al. 1992; Gabriel 1999) that determines recognition specificity (Herbers et al. 1992; Zhu et al. 1998; Yang et al. 2000; Ballvora et al. 2001). Another structural hallmark of AvrBs3-like proteins is the presence of functional nuclear localization signals (NLSs, Yang and Gabriel 1995; Van den Ackerveken et al. 1996). Deletion of the NLSs in most cases prevents recognition by the plant surveillance system (Zhu et al. 1998; Zhu et al. 1999; Yang et al. 2000), suggesting a nuclear localization for the cognate R proteins (Lahaye and Bonas 2001).

Our laboratory studies *Xanthomonas campestris* pathovar *vesicatoria* (*Xcv*), the causal agent of bacterial spot disease of pepper (*Capsicum* spp.) and tomato (*Lycopersicon* spp.). In particular, we are interested in *R* gene-mediated perception of the *Xcv* effector proteins AvrBs3 and AvrBs4 (previously designated AvrBs3-2) (Bonas et al. 1993), two members of the AvrBs3 family that share 97% sequence identity at the amino acid level (Bonas et al. 1989, 1993). In spite of their pronounced similarities, AvrBs3 and AvrBs4 are recognized specifically by the pepper *Bs3* and the tomato *Bs4* gene products, respectively (Pierre et al. 2000; Ballvora et al. 2001). Among the known *R* gene products that detect AvrBs3-like proteins, tomato Bs4 is unique in that recognition of the Avr protein is independent NLS within AvrBs4 (Ballvora et al. 2001), which may indicate that Bs4 is a cytoplasmic protein (Lahaye and Bonas 2001).

Previously we reported the functional analysis and genetic mapping of the tomato *Bs4* locus (Ballvora et al. 2001). In this study, we describe the development of a high-resolution genetic map and report chromosome landing at the tomato *Bs4* locus as a foundation for our ultimate goal, the molecular isolation of *Bs4*.

Materials and methods

Bacterial inoculations

Tomato plants were inoculated with the *Xcv* strains 56 and 75-3 (Bonas et al. 1989; Minsavage et al. 1990; Stall et al. 1994) carrying or lacking the *avrBs4* gene (on pLAT211) (Bonas et al. 1993). Plants were grown and inoculated as described previously (Bonas et al. 1989, 1993). The concentration of the inoculum was approximately 5×10^8 colony-forming units/ml ($OD_{600}\approx0.4$) in 1 mM $MgCl_2$. Symptoms were scored over a period of 2–5 days post inoculation.

Screen for recombination events in the region of the *Bs4* locus

Inheritance of AvrBs4 recognition in tomato was studied using a cross between the *L. esculentum* cultivar (cv.) Moneymaker (resistant parent) and *L. pennellii* LA 2963 (susceptible parent). F_1 plants were backcrossed (BC) to the susceptible parent and 720 (BC_1F_1) descendants were analyzed for possible recombination events within the TG432–P11M6 interval. The recombinant screen was extended to an additional 626 F_2 individuals. Hence, the total number of meiotic events analyzed was 1972 ($720+626\times2$). Disease scoring was performed on BC_1F_1 plants or marker-selected homozygous F_3 individuals, respectively. Extraction of tomato DNA for PCR-based genotyping was performed as described by Edwards et al. (1991).

Development of PCR-based genetic markers

The sequences of previously cloned AFLP markers (Ballvora et al. 2001) and *Bs4*-linked RFLP markers were determined by dye terminator sequencing (Perkin Elmer, Norwalk, Conn.). Primers, cycling conditions and restriction enzymes used for PCR-based genotyping are summarized in Table 1. The locus-specific amplicons P11M6, P15M1, TG598, CT242 and TG441 derived from both the susceptible and resistant parents were scanned for potential polymorphisms on 2% (w/v) agarose gels using the restriction endonucleases *Aci*I, *Alu*I, *Bfa*I, *Cfo*I, *Dde*I, *Eco*32I, *Hae*III, *Hpa*II, *Mae*II, *Mae*III, *Mse*I, *Mvn*I, *Nla*III, *Rsa*I, *Sau*3A, *Sau*96I, *Taq*I or *Tsp*509I. Yeast artificial chromosome (YAC) end-specific amplicons of Y45R, Y316L and Y316R derived from the susceptible and resistant parents, respectively, were purified using the QIAEX II gel extraction kit (Qiagen, Düsseldorf, Germany) and sequenced for detection of potential polymorphisms.

YAC library screen

Using the *Bs4*-linked marker TG432, we screened two libraries according to Pillen et al. (1996). One library was derived from the *L. esculentum* cultivars VFNT Cherry and Rio Grande-PtoR (Martin et al. 1992). The other library (Bonnema et al. 1996) was derived from the *L. esculentum* cultivars VFNT Cherry and Moneymaker-*Cf4*.

Analysis of yeast clones

Individual YAC clones were grown for 2 days at 30°C in 100 ml of synthetic dextrose minimal medium (SD) lacking uracil and tryptophan (Rose et al. 1990). YAC DNA for PCR analysis was prepared as described by Green and Olson (1990). Terminal sequences of YAC inserts were isolated by inverse PCR (IPCR) according to Silverman et al. (1989). The observed amplicons were size-fractionated on agarose gels, excised, purified using the QIAEX II gel extraction kit (Qiagen) and sequenced. High-molecular-weight yeast DNA was prepared in low-melting-point agarose as described by Carle and Olson (1985). Yeast chromosomes were separated by pulsed-field gel electrophoresis (PFGE)

Table 1 PCR-based markers linked to the *Bs4* locus

Marker[a]	Primer sequence (5′→3′)	PCR conditions[b]	Amplicon size (bp)	Restriction enzyme
P15M1 (A)[c]	CACTCAAAATTNGAATGGTGTCATTTGTAAC/ GCGGAATCTTGAAACAGAGCGTG	10 s at 94°C, 10 s at 60°C, 20 s at 72°C	250	–
P15M1 (B)[c]	CACTCAAAATTNGAATGGTGTCATTTGTAAC/ CTGAGAAACATATCCATGAATVTGAACTCTG	10 s at 94°C, 10 s at 60°C, 20 s at 72°C	200	*Tsp*509I
P11M6	GAGGTAGGACTTAGAAAACATA/ AATCAACACCACTAAATGCAGA	10 s at 94°C, 20 s at 55°C, 1 min at 72°C	700	*Eco*32I
CT242	GTACCAATGAGCTAACAAGAG/ GATGGAAATGGACAGAGCGT	10 s at 94°C, 20 s at 50°C, 1min at 72°C	500	–
TG432[d]	GCTGCTTTAATTGTAAGCCCTTRTACTAC/ CAYTTGWAGATTGTAATCACTTCTTGCC	10 s at 94°C, 5 s at 55°C, 20 s at 72°C	175[e]/210[f]	–
TG441	TCCAAGCCTGCTCTGAGGTAA/ CAGCTTGAACTGTGTCATGTAAC	10 s at 94°C, 20 s at 50°C, 1 min at 72°C	1000	–
TG598	TCTGCTTTGATTGGCTGTAGT/ CCAGAAAGTTGGAGTAGTCTA	10 s at 94°C, 20 s at 50°C, 1 min at 72°C	1000	*Rsa*I, *Sau*3A
Y45L[g]	TCCTCATCAATCCACCGTTCCT/ AATGATAACTAAGTTACGACT	10 s at 94°C, 20 s at 55°C, 1min at 72°C	550	–
Y45R	CAGTAGACACGTCTTATAGTTAG/ CTTGTGTAATTATTATTATTGTTGC	10 s at 94°C, 10 s at 50°C, 1 min at 72°C	600	*Eco*32I
Y316L	TCAATTTTATATTTCATTCTCCCTTTTTCATC/ TCTAAAGTTTGCGGGGATCCTATTACC	10 s at 94°C, 10 s at 45°C, 90 s at 72°	500	–
Y316R	GAATTCTAAGCTCTTTTCCCAATGC/ TCCTTTTTCTATCTGAACTATCACCATC	10 s at 94°C, 10 s at 50°C, 30 s at 72°C	400	*Sau*3A

[a]For each marker the sequences of the primer pairs, the PCR conditions, and the restriction enzyme that detects polymorphism are shown
[b]Each PCR was preceded by an initial denaturation step at 94°C for 2 min
[c]The primer pairs P15M1 (A) and P15M1 (B) were used consecutively for specific amplification of the marker locus P15M1
[d]Amplicons corresponding to marker TG432 had different sizes in the susceptible and resistant parents, allowing allele–specific discrimination of PCR products without endonuclease digestion
[e,f]Amplicon sizes in resistant and susceptible parents, respectively
[g]Primers corresponding to Y45L resulted in amplification only in the resistant but not the susceptible parent

using a 1.2% (w/v) agarose gel (Seakem LE; FMC BioProducts, Rockland, Me.) using a CHEF (contour-clamped hexagonal electric field) DR II apparatus (BioRad, Richmond, Calif.). Electrophoretic conditions were: 200 V, a constant 60-s switch time for 15 h, followed by a constant 90-s switch time for 6–8 h, at 14°C in 0.5×TBE buffer. The marker TG432 was used as a probe for Southern hybridization to determine the YAC insert sizes. Probes were labeled with digoxigenin (Roche Diagnostics, Mannheim, Germany) and Southern hybridization was performed as described by Lahaye et al. (1996).

Results

High-resolution genetic mapping at the *Bs4* locus

The tomato *Bs4* locus was previously localized to tomato chromosome 5, employing AFLP and restriction fragment length polymorphism (RFLP) markers (Fig. 1A) (Ballvora et al. 2001). Since application of AFLP and RFLP is costly and labor-intensive, these marker systems are of limited use for large-scale, locus-specific applications. Therefore our aim was to convert the *Bs4*-linked AFLP markers (P11M6, P15M1) and RFLP markers (CT242, TG432, TG441, TG598) into locus-specific PCR-based markers. Sequencing and subsequent BLAST analysis (Altschul et al. 1990) of P11M6 and P15M1 did not reveal significant homologies to repetitive sequence motifs. Therefore the AFLP marker-derived sequences, like the RFLP markers, were suitable for conversion into locus-specific PCR markers.

We designed oligonucleotides for the amplification of all the *Bs4*-linked marker loci (Table 1) and performed PCR on the resistant (*L. esculentum* cv. Moneymaker) and susceptible (*L. pennellii* accession LA2963) parental lines. Size fractionation by agarose gel electrophoresis indicated that all primer combinations, except those corresponding to P15M1, generated uniformly sized PCR products, thereby indicating the amplification of single-copy loci. Oligonucleotides corresponding to P15M1 generated multiple amplicons of different sizes, which is consistent with the fact that this AFLP fragment detects more than one genetic locus when used as a hybridization probe on genomic blots (Ballvora et al. 2001). However, using a nested-primer approach (Table 1), a uniformly sized amplicon population for P15M1 was generated. Sequencing revealed that these PCR products were identical in sequence to the AFLP source fragment.

All primer pairs generated amplicons of the same apparent size in both parental genotypes (data not shown), with the exception of the TG432-specific oligonucleotides. TG432-derived amplicons displayed different sizes in the susceptible and resistant parents, allowing allele-specific discrimination of PCR products without endonuclease digestion (Fig. 2). Monomorphic PCR products were inspected for possible sequence polymorphisms using a set of endonucleases (see Materials and methods for details). Polymorphic endonuclease recognition sites were identified in P11M6 (*Eco*32I), P15M1 (*Tsp*509I), and TG598 (*Rsa*I, *Sau*3A),

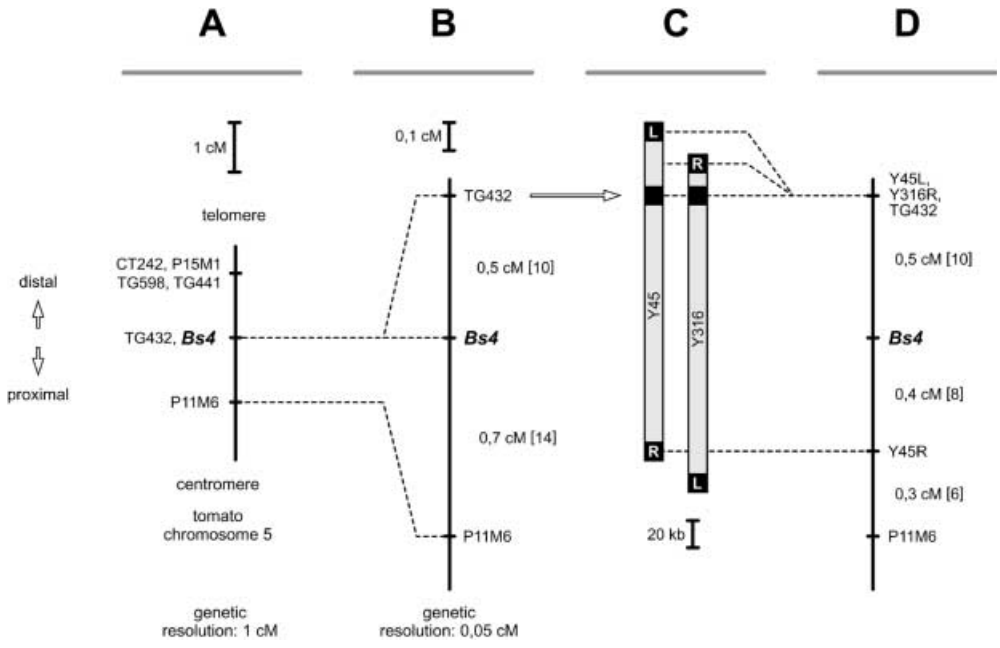

Fig. 1A–D Successive stages of the high-resolution genetic and physical mapping of the *Bs4* locus. **A** Initial AFLP/RFLP map, based on the use of 40 F_2 plants (Ballvora et al. 2001). **B** High-resolution genetic map based on the analysis of 1972 meiotic events. The numbers in *brackets* indicate the numbers of recombinants identified. **C** Physical map of the region encompassing the *Bs4* locus. YAC inserts are displayed to scale as *boxes*, with their respective right (R) and left (L) ends indicated. Note that spaces between markers in the YAC insert do not represent defined physical distances. **D** Genetic mapping of YAC end-derived markers. Numbers in *brackets* indicate the numbers of recombinants identified

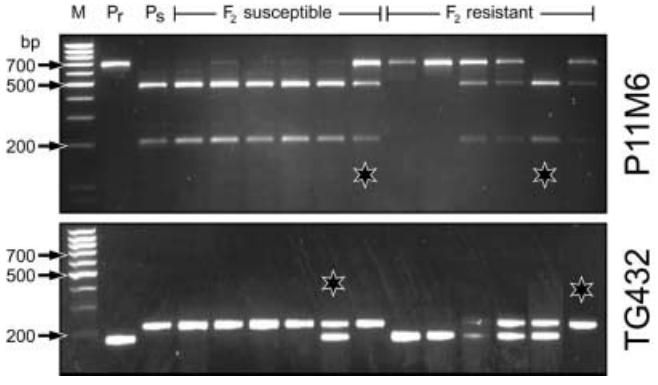

Fig. 2 PCR-based screen for recombinants at the *Bs4* locus. Linkage of the markers P11M6 (*upper panel*) and TG432 (*lower panel*) is shown by the profiles generated from the resistant parent (Pr), the susceptible parent (Ps) and susceptible/resistant F_2 progeny. Diagnostic amplicon profiles, indicating recombination events between *Bs4* and the respective DNA marker, are indicated by the *stars*. M, GeneRuler 100 bp DNA ladder plus (MBI Fermentas, Vilnius, Lithuania)

but not in CT242 or TG441 (summarized in Table 1). The PCR-based STS marker TG432 and the cleaved amplified polymorphic sequence (CAPS) markers P11M6, P15M1 and TG598 were subsequently employed for the analysis of approximately 100 F_2 segregants. Recombinants identified between *Bs4* and TG432 placed the STS marker distally with respect to the centromere (Fig. 1B). Thus, the minimal genetic target interval encompassing the *Bs4* locus was delimited distally by TG432 and proximally by P11M6.

The CAPS marker P11M6 and the STS marker TG432 were subsequently used for a large-scale screen for recombinants in the vicinity of the *Bs4* locus. A total of 1972 meiotic events were analyzed (see Materials and methods for details), corresponding to a genetic resolution of 0.05 cM. High-resolution genetic mapping reduced the size of the target interval to 1.2 cM and placed the nearest markers TG432 and P11M6 on opposite sides of the *Bs4* locus at a distance of 0.5 and 0.7 cM, respectively (Fig. 1B).

YAC contig assembly

Using TG432, the marker most closely linked to *Bs4*, two tomato YAC clones were identified (see Materials and methods). Size fractionation by PFGE and Southern analysis of the TG432-harboring yeast clones Y45 and Y316, revealed that both YAC inserts were approximately 250 kb in size (Fig. 3). Neither Y45 nor Y316 contained P11M6, which is the marker closest to *Bs4* on the centromere-proximal side. Thus, genetic mapping of the YAC termini was necessary to clarify whether Y45 and/or Y316 spanned the target locus. To establish a physical contig based on the two identified YACs, we isolated their left (L) and right (R) ends by IPCR (Silverman et al. 1989). YAC end-specific oligonucleotides were designed (Table 1) and employed to determine the presence or absence of these sequences in the respective yeast clones. While Y316R, Y45R and TG432 were present on both yeast clones, Y45L and Y316L were only amplified from the yeast clones from

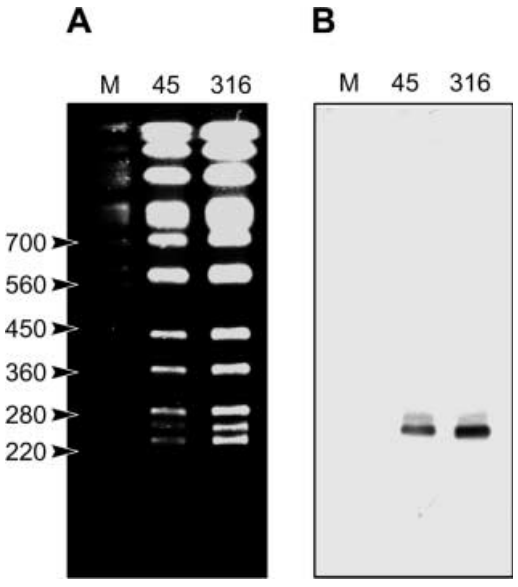

Fig. 3A, B PFGE and Southern analysis of *Bs4*-spanning YAC clones. **A** Ethidium bromide-stained agarose gel of PFGE-resolved yeast chromosomes. M, PFGE II marker (Roche Diagnostics). Marker fragments of defined size (in kb) are indicated by corresponding arrows. **B** Southern analysis of YAC clones. The agarose gel shown in **A** was blotted to a nylon membrane and probed with the marker TG432

Table 2 YAC overlap analysis

Tomato YAC	*Bs4*-linked markers[a]					
	Y45L	Y45R	Y316L	Y316R	TG432	P11M6
Y316	–	+	+	+	+	–
Y45	+	+	–	+	+	–

[a] "+" indicates that the YAC was shown by PCR analysis to contain the respective marker. Absence of a marker is indicated by –

which they were derived (Table 2). Thus, we concluded that Y45L and Y316L determine the outer limits of the YAC contig (Fig. 1C). In addition, the presence of Y316R on Y45 and Y45R on Y316 indicates that the corresponding YAC inserts are most probably non-chimeric and colinear with the source tomato DNA.

Genetic mapping of YAC end-derived markers

A PCR-based approach was used to search for possible sequence polymorphisms within YAC end-specific amplicons derived from the resistant and susceptible parents, respectively. Oligonucleotides corresponding to Y45L amplified DNA fragments from the resistant, but not the susceptible, parent (data not shown). PCR products corresponding to markers Y45R, Y316L and Y316R yielded amplicons of identical size in the resistant and susceptible parents and were analyzed for DNA polymorphisms by direct sequencing. Comparative sequence analysis revealed polymorphic restriction sites in the case of Y45R and Y316R (Table 2), whereas no DNA polymorphism was detected for Y316L. The presence/absence polymorphism of Y45L did not allow codominant scoring of F_2 segregants. To overcome this problem, we used the co-dominant markers TG432 and P11M6 for the selection of homozygous-recombinant F_3 individuals. Genotyping of the homozygous F_3 recombinants revealed cosegregation of Y45L, Y316R and TG432, and placed these markers centromere-distal, separated by 0.5 cM from the *Bs4* locus (Fig. 1D). Cosegregation of Y45L, Y316R and TG432 is in agreement with the YAC overlap analysis. However, the recombinant screen was performed within the TG432–P11M6 interval and since physical mapping has indicated that Y45L and Y316R are located distally to TG432 (Fig. 1C), we could not precisely determine the genetic distance between TG432 and the YAC end markers Y45L and Y316R. Linkage mapping of Y45R was performed on marker-selected F_3 recombinants (see above) and positioned this YAC end centromere-proximal, separated from the target locus by 0.4 cM (Fig. 1D). In summary, the genomic region containing *Bs4* was genetically and physically delimited to an interval of 0.9 cM and 250 kb, flanked by the markers Y45R (centromere-proximal) and TG432 (centromere-distal).

Discussion

Recombination frequencies at the *Bs4* locus

The molecular isolation of genes based solely on their map location (map-based or positional cloning; Collins 1992) is applicable to any gene whose phenotypic effect can be followed in populations segregating for natural variants or mutant forms of the gene. Despite its universal potential, map-based cloning is challenging in plant species with large genomes, and can be considered routine only in the model species *Arabidopsis*, which has a small genome (Lukowitz et al. 2000). The successful outcome of this approach depends to a large extent on the local relationship between physical and genetic distances. Unfortunately, meiotic stability is by no means constant and varies with respect to the chromosomal location (Frary et al. 1996), as well as with respect to the degree of sequence polymorphism (Dooner and Martinez Ferez 1997; Schnable et al. 1998; Petes 2001).

We have delimited the *Bs4* locus to an 0.9-cM interval bracketed by the markers Y45R and TG432, which are separated by a maximum physical distance of 250 kb. The deduced ratio of physical to genetic distance at *Bs4* is therefore 280 kb/cM. For positional cloning this is approximately three times more favorable than the genome-wide estimate of 750 kb/cM (Tanksley et al. 1992). The increased recombination frequency at the target locus is in agreement with its telomeric location, which can be deduced from the chromosomal location of

Bs4-linked markers within a genome-wide tomato linkage map (Tanksley et al. 1992). In contrast, both the barley *Mla* and lettuce *Dm3* *R*-gene clusters show reduced recombination frequencies, in spite of their telomeric location (Wei et al. 1999; Chin et al. 2001). It has been speculated that biased recombination frequencies at the *Mla* and *Dm3* loci are due to structural heterozygosity between haplotypes. In agreement with this hypothesis, comparative sequence analysis of the *Arabidopsis RPP5* locus and the tomato *Cf-4/9* locus has shown a pronounced lack of haplotype synteny in conjunction with low recombination frequency (Parniske et al. 1997; Noël et al. 1999). Taken together, these data suggest that the genetic behavior of a resistance locus reflects its structure and level of complexity. The fact that the *Bs4*-containing target interval does not show reduced, but rather increased, recombination frequencies, might indicate that *Bs4* represents a non-complex *R*-gene locus.

The minimum physical target interval containing *Bs4*

By defining a YAC contig spanning *Bs4* we have achieved a major step in our positional cloning approach. Our next steps will be the assembly of a cosmid contig and complementation analysis. Can we make any predictions with respect to the size of the minimal tiling path that can be defined based on available recombinants? The analysis of 1972 meiotic events at *Bs4* has revealed 18 recombination breakpoints within a physical interval of 250 kb. Assuming that meiotic recombination breakpoints are evenly distributed within this target interval, *Bs4*-bordering crossover events should encompass a sequence stretch of less than 15 kb (250 kb/18). However, detailed investigations in maize have shown that even within a physical interval of 140 kb, recombination frequencies can vary up to seven-fold (Civardi et al. 1994). Moreover, genetic dissection of a quantitative trait locus on tomato chromosome 9 revealed a recombination hotspot responsible for 13 recombinants within 943 bp, compared with only 15 for the rest of the surrounding interval of 100 kb (Fridman et al. 2000). These studies indicate that meiotic stability, even within short chromosomal stretches, is not necessarily homogeneous, and our calculations for the *Bs4* target interval must therefore be treated with caution.

A candidate gene approach designed to isolate *Bs4*

The molecular isolation of more than 30 plant disease *R* genes from a range of monocot and dicot plant species (Takken and Joosten 2000; Dangl and Jones 2001; Holub 2001; Jones 2001) revealed that the majority of these genes encode a putative NBS and LRR region. Conserved amino acid motifs in and around the putative NBS of NBS-LRR resistance proteins have been used extensively for PCR-based amplification of *R*-gene-like (RGL) sequences from plant genomes (for recent examples, see Hayes and Saghai Maroof 2000; Timmerman -Vaughan et al. 2000; Noir et al. 2001; Vicente and King 2001). The combination of such an homology-based approach and positional cloning has been successfully employed for the isolation of the maize *Rp1-D* *R*-locus (Collins et al. 1999) and appears also suitable for the identification of *Bs4* candidate genes. Using this "candidate gene approach" (Collins 1992), we have recently isolated an RGL sequence, which is located on the isolated YAC inserts (B. Baker, unpublished). Mapping and complementation experiments are currently underway to determine if the identified RGL sequence is indeed part of the *Bs4* gene.

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