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High-resolution genetic and physical mapping of the region containing the *Bs2* resistance gene of pepper

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Abstract The *Bs2* resistance gene of pepper confers resistance against the bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria*. As a first step toward isolation of the *Bs2* gene, molecular markers tightly linked to the gene were identified by randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) analysis of near-isogenic lines. Markers flanking the locus were identified and a high-resolution linkage map of the region was developed. One AFLP marker, A2, was found to cosegregate with the locus, while two others, F1 and B3, flank the locus and are within 0.6 cM. Physical mapping of the A2 and F1 markers indicates that these markers may be within 150 kb of each other. Together, these results indicate that the *Bs2* region may be cloned either by chromosome walker or landing. The linked markers were also used to characterize gamma-irradiation-induced mutants at the *Bs2* locus.

Key words *Bs2* resistance gene · Pepper · RAPD · AFLP · Positional cloning

Introduction

Bacterial spot of tomato and pepper, caused by *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), can be a devastating disease in tomato- and pepper-growing regions of the world. While control of this disease is based primarily on the application of pesticides, genetic resistance has been identified in both hosts (Cook and Stall 1963; Cook and Guevara 1984; Kim and Hartmann 1985; Jones and Scott 1986). In pepper, three dominant loci (*Bs1*, *Bs2* and *Bs3*) have been shown to confer resistance to *Xcv* in a gene-for-gene manner (Hibberd et al. 1987). The corresponding avirulence genes (*avrBs1, avrBs2* and *avrB3*) from *Xcv* have been cloned (Swanson et al. 1988; Bonas et al. 1989; Minsavage et al. 1990).

Characterization of these avirulence genes has provided insight into the interactions between *Xcv* and pepper. While the *avrBs1* gene encodes a novel protein (Ronald and Staskawicz 1988) whose function has yet to be determined, the *avrBs2* gene has been shown to contribute to the fitness of *Xcv* and is widespread among several races of *Xcv* as well as other pathovars of *X. campestris* (Kearney and Staskawicz 1990). Recent analysis indicates that *avrBs2* may encode a phosphodiesterase (Swords et al. 1996). Involvement in pathogen fitness has not been demonstrated for AvrBs3; however, this protein is a member of a family of avirulence genes identified in various *Xanthomonas* species, some of which have been implicated in pathogenicity (Gabriel 1997). Furthermore, AvrBs3 appears to be delivered into the host-cell cytoplasm where it may localize to the nucleus (Van den Ackerveken et al. 1996). The actual function of these avirulence genes in triggering a resistance response awaits the cloning and characterization of the cognate resistance genes.

Bs2 resistance, recognizing strains of *Xcv* containing *avrBs2*, was identified in a wild species of pepper, *Capsicum chacoense,* and introduced into *Capsicum annuum* over 15 years ago (Cook 1984; Cook and Guevara 1984). Among the pepper R genes conferring resistance to bacterial spot, the *Bs2* gene has proven to be effective in the field against the widest array of *Xcv* races (Kousik and Ritchie 1996). The effectiveness of the *Bs2* gene appears to be due to the involvement of AvrBs2 in pathogen fitness. In addition, the widespread distribution of the

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Table 1 Recombinants identified by analysis with SCAR markers

Population	Size	Number of recombinants between the *Bs2* locus and		
		S2	S45	DCO[b]
TC1	333	8	2	1
TC2	420	7	1	0
F2.1	476[a]	11	4	0
F2.2	348	3	7	6
Totals	1577	29	14	7

[a] Total population was 1672. Only susceptible individuals (476) were screened with molecular markers
[b] DCO – double crossovers. The high number of double crossovers from the F2.2 population is probably due to incorrect scoring. These plants were not used for further analysis

avrBs2 gene suggests that the *Bs2* gene may serve as a source for resistance against a number of other *X. campestris* pathovars.

Given its origin and the availability of near-isogenic lines (Minsavage et al. 1990), it should be possible to efficiently identify useful molecular markers. Identification of linked molecular markers provides a valuable starting point for positional cloning of the *Bs2* resistance gene. A number of plant resistance genes have been isolated in this manner (Baker et al. 1997; Hammond-Kosack and Jones 1997). Using PCR-based approaches, tightly linked markers were identified and used to develop a high-resolution genetic map of the locus. These markers will be useful for isolation of the *Bs2* resistance gene by positional cloning.

Materials and methods

Plant material, bacterial inoculations, isolation of genomic DNA

Molecular markers were identified using the pepper (*C. annuum*) cultivar Early Calwonder (ECW; *bs1/bs1, bs2/bs2, Bs3/bs3*), Early Calwonder-20R (ECW-20R; *bs1/bs1, Bs2/Bs2, bs3/bs3*), and *C. chacoense* (PI 260435, wild species donor of the *Bs2* allele; *bs1/bs1*, *Bs2/Bs2, bs3/bs3*). Mapping populations were derived from crosses made between the female parent ECW and the male parent Early Calwonder-123R (ECW-123R; *Bs1/Bs1, Bs2/Bs2, Bs3/Bs3*). Segregation analysis was performed on two F_1 testcross (TC1 and TC2) and two F_2 populations (F2.1 and F2.2) as shown in Table 1. Plants were grown under standard greenhouse conditions. Disease resistance was determined by hand-infiltration of suspensions of *Xcv* strains expressing only *avrBs2* (race 69-1 or race 82-8 *avrB2⁻, 3⁻*p81538, Minsavage et al. 1990) at a concentration of $2–5 \times 10^8$ colony forming units per ml as described (Swanson et al. 1988). A hypersensitive response observed 24–36 h post-infiltration was indicative of resistance. Standard and miniprep isolation of pepper genomic DNA were performed as described (Tai and Tanksley 1990) with minor modifications (Tai 1995). DNA gel-blot analysis and othr molecular methods were carried out according to standard protocols unless otherwise indicated (Sambrook et al. 1989).

Identification and mapping of RAPD markers

RAPD markers were identified by screening genomic DNA of the ECW-20R (resistant) and ECW (susceptible) cultivars with approximately 300 primers (10-mers). RAPD-PCR reactions were performed according to estabished protocols (Welsh and McClelland 1990; Williams et al. 1990). Following cloning and partial sequencing, RAPD markers were converted into sequence-characterized amplified-region (SCAR) markers (Paran and Michelmore 1993). SCAR primers, as shown in Table 2, were used to screen the mapping populations to identify recombinants between the markers and the *Bs2* locus.

Identification and cloning of tightly linked AFLP markers

AFLP marker analysis was performed at Keygene n.v. (Wageningen, The Netherlands) as described in Zabeau and Vos (1993) and Vos et al. (1995). Genomic DNA from ECW, ECW-20R, *C. chacoense*, and a pool of five susceptible recombinants identified from the TC1 mapping population, was employed for selective restriction fragment amplification. The restriction enzymes *Pst*I and *Mse*I were used to prepare the DNA prior to selection and amplification. AFLP markers of interest were eluted from the polyacrylamide gel, re-amplified, and then cloned into the *Sma*I site of pUC19. Cloned fragments were sequenced to obtain primers for amplification of sequence-tagged-site (STS) markers.

Physical mapping of AFLP markers

High-molecular-weight genomic DNA (HMW DNA) was isolated from pepper as described (Ganal and Tanksley 1989) with minor modifications (Tai 1995). Following in-gel digestion with rare-cutting restriction enzymes, HMW DNA sampls were subjected to contour-clamped homogeneous electric field (CHEF) gel-electrophoresis in 1% agarose gels and 0.5 × TBE buffer using a CHEF DR II apparatus (BioRad, Richmond, Calif.). Electrophoresis conditions were as follows: 200 V, 5–120 s ramped switch time, 24–35 h, 10–14°C. DNA gel-blotting was performed essentially as described (Ganal and Tanksley 1989). Cloned AFLP markers were radiolabeled and used to probe DNA gel-blots as described (Tai 1995).

Molecular analysis of gamma-irradiation-induced mutants

Previously isolated gamma-irradiation-induced mutants at the *Bs2* locus (Stall et al. 1995) were characterized using the SCAR and AFLP markers. Molecular marker and DNA gel-blot analysis was carried out as described in previous sections.

Results

Identification of flanking RAPD markers and development of a genetic map of the *Bs2* region

To map the *Bs2* locus in pepper, genomic DNA of the near-isogenic lines ECW-20R and ECW were screened with approximately 300 RAPD primers. Three markers

Table 2 Molecular markers linked to the *Bs2* resistance locus in pepper

Marker	RAPD/AFLP primer	SCAR/STS primers	Marker size
S2	5′CTTTGGCCCT3′	5′GCGATGAACTTTGACAACTG3′ 5′CGTGATTTATTGGTCATCTG3′	~800 bp
S45	5′CTGGAGAGCA3′	5′CTGGAGAGCACGATATCATAGTTG3′ 5′CTGGAGAGCAGCCAATAGATAC3′	~900 bp
S19	5′CTGACCAGCC3′	5′CTGACCAGCCAAACATGG3′ 5′CTGACCAGCCCTTTCAACTTAC3′	~240 bp / ~ 220 bp[a]
A2	*Pst*I: 91T23(GT) *Mse*I: 92G42(TTG)	5′GCCATCCAGCCAACTCAAAG3′ 5′GCAGGTGTTCTCCAATCAGG3′	528 bp
B3	*Pst*I: 92E11(TG) *Mse*I: (92G35)TGG	5′TAATGGGTATGCCAAGTAAA3′ 5′GCCTCTATTCACAGAATGTT3′	213 bp
E1	*Pst*I: 91I02(GG) *Mse*I: 92G43(TTC)	5′AGACTTGCTACAACGAAGGT3′ 5′GCTGTTTCTACTGCCTCCAT3′	230 bp
F1	Pst*I: 91I09(CAG)* *Mse*I: 92G18(GCT)	5′TTAAGCTTGGCAGGCTACTACA3′ 5′CTGCAGCAGACCAGTGGAAGTT3′	106 bp
G1	Pst*I: 91I09(CAG)* *Mse*I: 92G18(GCT)	5′CGGCGGATGCTACCCAAGTT3′ 5′CAGGTAAACAGCACGTTAGT3′	253 bp

[a] Co-dominant marker (resistant allele/susceptible allele)

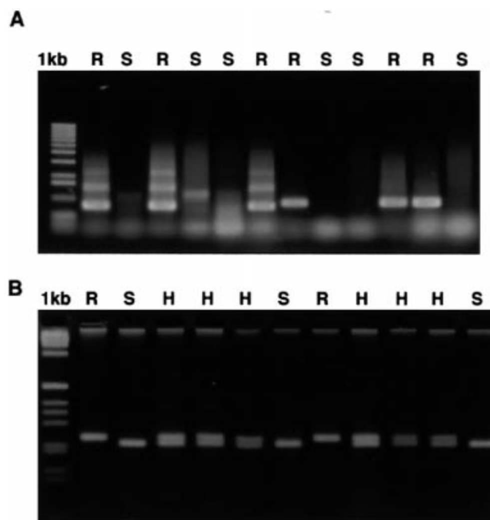

Fig. 1 A, B Scar markers linked to the *Bs2* locus. **A** SCAR markers S2 (*lanes 1–6*) and S45 (*lanes 7–12*): *R*=resistant, *S*=susceptible. **B** SCAR marker S19: *R*=ECW123R allele, *S*=ECW allele, *H*=heterozygote. 1 kb = 1-kb ladder (Gibco BRL, Gaithersburg, Md.)

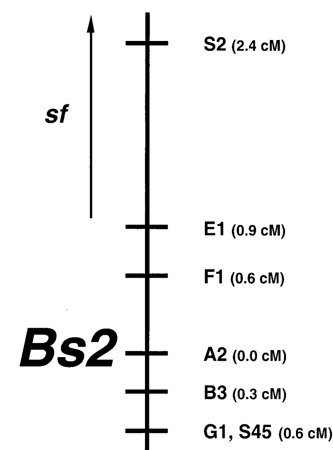

Fig. 2 High-resolution genetic map of the *Bs2* resistance locus of pepper. Map distances, indicated in *parentheses*, are relative to the *Bs2* gene and are based on the TC1 mapping population (333 individuals). Data from additional populations were in agreement. S2 and S45 are SCAR markers identified via RAPD analysis. A2, B3, E1, F1, and G1 are AFLP-based markers. *sf* is the small-fruit locus

(P2, P19 and P45) linked to the *Bs2* locus were identified. These markers were cloned and sequenced, and primers were designed for the amplification of SCARs designated as S2, S19 and S45 (Table 2, Fig. 1). Two of the SCAR markers, S2 and S45, were linked in coupling with the *Bs2* allele. The third marker, S19, was co-dominant and amplified fragments in the resistant and the susceptible parent (Fig. 1). Initial mapping of these markers using a testcross population (TC1, see Table 1) of 333 individuals indicated that S2 and S45 flank the locus as shown in Fig. 2. Based on this TC1 population, S2 maps approximately 2.4 cM from the *Bs2* locus and S45 about 0.6 cM away. The S19 marker is located approximately 7 cM from the locus on the S45 side.

Additional mapping populations analyzed using S2 and S45 indicated a genetic distance of about 1.2 to 2.4 cM between S2 and the *Bs2* locus, and 0.4–0.9 cM between S45 and the locus. Segregation analysis of the various populations resulted in the identification of 14 recombinants between S45 and the locus, and 29 recombinants between S2 and the locus (Table 1).

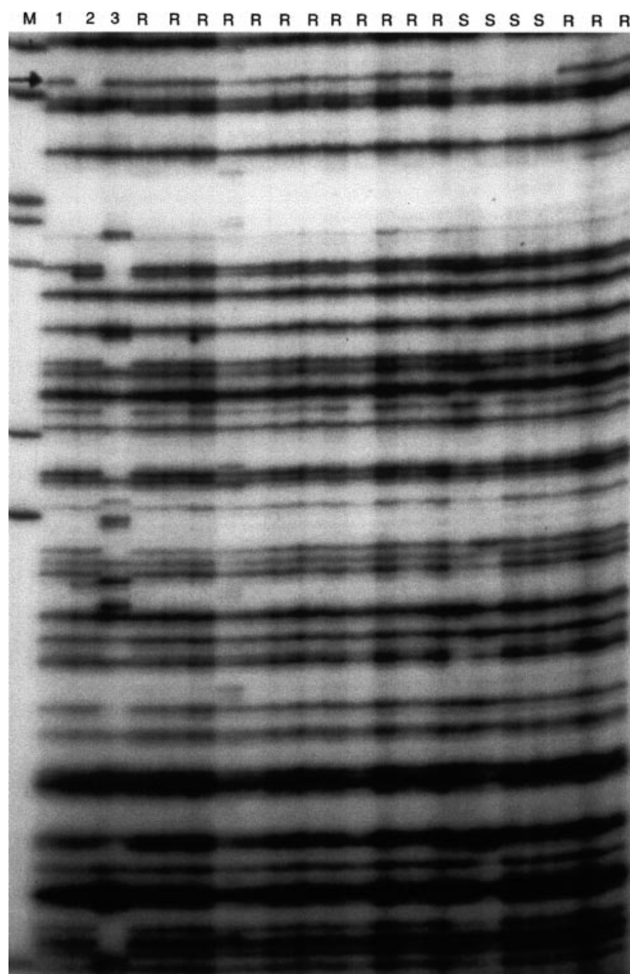

Fig. 3 AFLP autoradiogram of A2 marker. The A2 marker (*arrow*) co-segregates with the *Bs2* resistance gene. *Lanes: 1* ECW-20R (*Bs2/Bs2*); *2* ECW (*bs2/bs2*); *3 C. Chacoense* (*Bs2/Bs2*); *4 through 22 R*=resistant, *S*=susceptible

Identification and mapping of tightly linked AFLP markers

AFLP markers linked to the *Bs2* locus were identified by the analysis of DNA from ECW-20R (*Bs2/Bs2*), ECW (*bs2/bs2*), *C. chacoense* (the wild species donor of *Bs2*), and a pool of susceptible recombinants identified in the initial mapping population described in the previous section (Fig. 3). A total of 319 double-primer combinations (equivalent to 638 primer combinations) were used. On average, 73 fragments were detected per primer combination for a total of approximately 23300 loci screened. Five markers linked in coupling with the *Bs2* allele (A2, B3, E1, F1 and G1, Table 2) were identified and mapped using the additional recombinants described earlier. The A2 marker co-segregates with the *Bs2* locus while, with the exception of G1 which maps to the same location as S45, the other AFLP markers flank the locus and are more tightly linked than the S2 and S45 markers (Fig. 2).

The AFLP markers were cloned and sequenced to develop primers for use as STS markers (Table 2). Preliminary Southern-blot analysis indicated that the A2, B3, and F1 markers were low- to single-copy in the genome. RFLP mapping of the A2 and F1 markers confirmed the placement of these markers with respect to the *Bs2* locus (T. Tai, unpublished results). Although the B3 marker initially appeared to be a low-copy number probe, repeated attempts at RFLP mapping were unsuccessful due to a high, non-specific background (T. Tai, data not shown).

Physical mapping of AFLP markers tightly linked to the *Bs2* locus

Physical mapping of the *Bs2* region was performed by using the A2 and F1 markers to probe DNA gel-blots of HMW DNA digested with various rare-cutting restriction enzymes and separated by CHEF gel-electrophoresis. Hybridization of a gel-blot of *Cla*I-digested DNA with the A2 marker revealed a fragment of approximately 150 kb. Upon stripping the blot and re-probing with the F1 marker, a signal was observed in the same region of the blot. It is likely that the A2 and F1 markers are hybridizing to the same *Cla*I fragment, thus suggesting that these markers are, at most, 150 kb apart (data not shown). Based on the physical and genetic mapping data, one cM is about 250 kb in this region. Other markers in the region were too highly repetitive to be used for physical mapping.

Small-fruit phenotype maps on the S2 side of the *Bs2* locus

In addition to the recombinants identified using the SCAR markers, a single plant expressing *Bs2* resistance and a large-fruit phenotype was obtained from an unrelated study (R. Stall, unpublished). This recombinant was analyzed using the SCAR and AFLP markers. All of the markers were found to be present except S2, thus indicating that the recombination event responsible for this individual is located distal to the E1 marker (Fig. 2). Based on these results, the locus controlling the small-fruit phenotype associated with *Bs2* resistance is located distal to the E1 marker on the S2 side of the region.

Characterization of the gamma mutants did not reveal any large lesions

In previous work, three ECW-123R pepper mutants (B-7, E-14 and AE-2) no longer expressing *Bs2* resistance due to lesions at the locus were identified (Stall et al. 1995). Southern analysis of *Nru*I-digested HMW DNA from the mutants was performed using the S2 marker as a probe (data not shown). The S2 marker does not hybridize to the DNA of the susceptible ECW cultivar. While hybridization was observed with the DNA from the mutants, the pattern of hybridizing restriction

fragments was altered from that of the resistant parent ECW-123R from which the mutants were derived. These mutants were also subjected to PCR analysis using primers for the A2, B3 and F1 markers. All three markers were present in each of the mutants indicating that large deletions were not likely to be responsible for the susceptible phenotype observed in these mutants. DNA gel-blot analysis using the A2 and F1 markers as probes did not reveal alterations in the mutants. As noted above, the B3 marker was too repetitive to be used as a probe.

Discussion

Positional cloning requires the identification of molecular markers tightly linked to the gene of interest. The availability of high-density molecular genetic maps for systems such as tomato, potato, rice and *Arabidopsis* (Tanksley et al. 1992; Causse et al. 1994; Chang et al. 1988) makes these plants ideal for positional or map-based cloning strategies. However, although pepper is one of the world's major vegetable crops, there are few well-developed molecular resources currently available for it. Fortunately, several methods such as RAPD (Welsh and McClelland 1990; Williams et al. 1990) and AFLP (Zabeau and Vos 1993) analysis have been developed. The PCR-based analysis of near-isogenic lines or bulked segregants has facilitated the rapid and efficient identification of molecular markers linked to genes of interest, thus circumventing the construction of high-density molecular maps for the purpose of gene isolation (Giovannoni et al. 1991; Martin et al. 1991; Michelmore et al. 1991; Paran et al. 1991).

In the present study, RAPD analysis was performed on near-isogenic lines (NILs) of pepper, *C. annuum*, which differ by the presence of introgressed *C. chacoense* DNA containing the *Bs2* resistance gene. This resulted in the identification of three linked markers. These RAPD markers were converted into more robust SCAR markers and used to develop of genetic map of the locus. Additional markers were generated by AFLP analysis of the NILs. A fine-structure genetic map of the *Bs2* region was then developed by mapping the AFLP markers using the recombinants previously identified with the SCAR markers.

The development of a high-resolution genetic map of the *Bs2* locus also facilitated the genetic mapping of a locus conferring a small-fruit phenotype. Small fruit is a highly undesirable trait with regard to commercial pepper production and its association with the resistance locus has limited the use of the *Bs2* gene for resistance against bacterial spot (R. Stall, personal communication). The identification of markers very tightly linked to the *Bs2* locus should be very useful for marker-assisted breeding efforts to deploy *Bs2* resistance while eliminating linkage drag (i.e., small-fruit phenotype).

Identification of several tightly linked markers flanking the *Bs2* locus fulfills the first step of the positional cloning strategy. Physical mapping of the region using the AFLP markers A2, which co-segregates with the *Bs2* gene, and the F1 AFLP marker, one of the closest flanking markers, suggests that this segment of the locus is ≤150 kb. This result supports the need for the development of a large-insert genomic library of pepper and also suggests that it may be possible to clone the *Bs2* gene by chromosome landing (Tanksley et al. 1995). Based on an analysis of susceptible pepper mutants using these newly developed molecular markers, it appears that the mutant phenotype may be due to subtle lesions at the *Bs2* locus. These mutants may provide valuable information for the identification and functional characterization of the *Bs2* gene in future studies.

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