Theor Appl Genet (2000) 101:255–263

M. Pierre · L. Noël · T. Lahaye · A. Ballvora
J. Veuskens · M. Ganal · U. Bonas

High-resolution genetic mapping of the pepper resistance locus *Bs3* governing recognition of the *Xanthomonas campestris* pv *vesicatora* AvrBs3 protein

Received: 15 October 1999 / Accepted: 29 November 1999

Abstract The pepper (*Capsicum annuum*) *Bs3* gene confers resistance to *Xanthomonas campestris* pv *vesicatoria* strains expressing the avirulence protein AvrBs3. Using amplified fragment length polymorphism (AFLP) and bulked DNA templates from resistant and susceptible plants we identified markers linked to *Bs3* and defined a 2.1-cM interval containing the target gene. *Bs3*-linked AFLP fragments were cloned and conformity of isolated PCR products with the desired markers was determined by hybridisation to membrane-bound AFLP reactions. AFLP markers flanking the target gene were converted into locus-specific PCR-based markers. These markers were employed for the analysis of 790 plants segregating for *Bs3*, resulting in a linkage map with a genetic resolution of 0.13 cM. Mapping of *Bs3*-linked markers in tomato placed them to a syntenic interval on tomato chromosome 2.

Communicated by F. Salamini

M. Pierre · L. Noël · A. Ballvora · J. Veuskens · U. Bonas (✉)
Centre National de la Recherche Scientifique,
Institute des Sciences Végétales, Avenue de la Terrasse,
91190 Gif-sur-Yvette Cedex, France
e-mail: bonas@genetik.uni-halle.de

T. Lahaye
Institut für Genetik, Martin-Luther Universität, Weinbergweg 22,
D-06099 Halle (Saale), Germany

M. Ganal
Institute for Plant Genetics and Crop Plant Research (IPK),
Corrensstrasse 3, D-06466 Gatersleben, Germany

Present addresses:
L. Noël, U. Bonas, Institut für Genetik, Martin-Luther Universität,
Weinbergweg 22, D-06099 Halle (Saale), Germany
e-mail: lahaye@genetik.uni-halle.de

A. Ballvora, Max-Planck Institut für Züchtungsforschung,
Carl-von-Linné-Weg 10, D-50829 Köln, Germany

B.J. Veuskens, Institute of Molecular Cell Biology,
University of Amsterdam, 1098 SM Amsterdam, The Netherlands

Key words Map-based cloning · Positional cloning · Bacterial spot · *Capsicum* · Bulked-segregant analysis · Reverse AFLP

Introduction

Bacterial spot of pepper (*Capsicum* spp.) is caused by the gram-negative bacterium *Xanthomonas campestris* pv *vesicatora* (*Xcv*) and is a severe disease in cultivated areas where heavy rainfall and high temperatures are prevalent (Stall 1993). Compatible interactions result in bacteria multiplying to a high density in the leaf apoplast causing water-soaked lesions that later become necrotic. In contrast, incompatible interactions result in a hypersensitive response (HR), a localised defence reaction, which restricts bacterial growth. In pepper, three dominant, non-allelic resistance (*R*) genes, *Bs1*, *Bs2* and *Bs3*, have been identified that confer resistance to *Xcv* according to the gene-for-gene hypothesis (Hibberd et al. 1987).

We are studying the interaction between pepper plants expressing *Bs3* and *Xcv* strains carrying the corresponding avirulence gene *avrBs3*. The *avrBs3* gene contains 17.5 direct repeats of 102 bp each, sharing 91–100% sequence identity (Bonas et al. 1989). Deletion of repeats induces changes in the recognition specificity of the *avrBs3* gene when tested on different pepper genotypes (Herbers et al. 1992). The most striking example is the *avrBs3* deletion derivative *avrBs3*Δrep-16 that is no longer recognised by the pepper genotype *Bs3*, but instead by the genotype *bs3*, thereby converting a compatible into an incompatible interaction. The fact that the repeat region of *avrBs3* determines recognition-specificity suggests that the AvrBs3 protein itself is the signal recognised by the host. This is consistent with the finding that the AvrBs3 protein is secreted by the *Xcv* type-III secretion system (Rossier et al. 1999) and that recognition of the AvrBs3 protein occurs inside the plant cell (Van den Ackerveken et al. 1996). To gain deeper insights into the molecular mechanism of AvrBs3 recognition we intend to isolate the pepper *Bs3* locus.

Isolation of *R*-genes from different plant species, each recognising a different and taxonomically unrelated pathogen, revealed that the encoded R proteins show common structural motifs (Hammond-Kosack and Jones 1997; Parker and Coleman 1997). The majority of the R proteins carry nucleotide-binding-site and leucine-rich-repeat (NBS-LRR) motifs (Van der Biezen and Jones 1998). Conservation within these domains has been employed to isolate NBS-LRR sequence homologues from lettuce (Shen et al. 1998), potato (Leister et al. 1996; Hämäläinen et al. 1998), soybean (Kanazin et al. 1996; Yu et al. 1996), *Arabidopsis* (Aarts et al. 1998; Speulman et al. 1998), rice (Leister et al. 1999; Mago et al. 1999), maize (Collins et al. 1998) and barley (Leister et al. 1999). Such a homology-based approach could be used to isolate *Bs3*-candidate genes. However, since it is uncertain if *Bs3* belongs to the NBS-LRR *R* gene class, a cloning approach based exclusively on this assumption is risky. In contrast, transposon tagging (Walbot 1992) and positional cloning (Collins 1992) permit gene isolation based solely on a recognisable phenotype. Since pepper lacks a well-defined insertional-mutagenesis system, we are using a map-based cloning strategy to isolate the *Bs3* gene.

In this study, we describe the development of a high-resolution genetic map at the pepper *Bs3* locus. Using AFLP-based mapping a marker-interval containing the *Bs3* gene was defined as a foundation for a positional cloning approach.

Materials and methods

Plant material and bacterial inoculations

Plants were grown and inoculated as described (Bonas et al. 1989). The following *Capsicum* varieties have been tested for resistance: Ben Xi, CM 334, Early Calwonder 30R (ECW-30R), H 3, HAD 103, HAD 160, Perennial, PI 195299, PI 197409, PM 687, SC 81, Vania, Vat, Yolo wonder (all *Capsicum annuum*), Chi 7, Chi 8 (both *Capsicum chinense*) and PM 1156 (*Capsicum frutescens*). The *Xcv* strains used were strain 85-10, which lacks the *avrBs3* gene, and 85-10 (pD36), which expresses constitutively the AvrBs3 avirulence protein (Brown et al. 1993). The concentration of the inoculum was approximately 10^8 colony forming units/ml in 1 mM $MgCl_2$. Symptoms were scored 2–5 days post inoculation.

Plant DNA isolation

Due to the high amount of polysaccharides in pepper leaves, genomic DNA for AFLP analysis was extracted from nuclei according to a modified protocol of Vallejos et al. (1986). Frozen leaf material of 3-week-old plants (400 mg) was ground and mixed with 300 µl of extraction buffer [0.35 M Sorbitol, 0.1 M Tris-HCl pH 7.5, 5 mM EDTA, 1% (v/v) ß-mercaptoethanol]. The homogenate was filtered through Miracloth (Calbiochem Corporation, Darmstadt, Germany) and centrifuged. The plant nuclei were re-suspended in 200 µl of extraction buffer and lysed by adding 200 µl of lysis buffer [0.2 M Tris-HCl pH 7.5, EDTA 50 mM, NaCl 2 M, CTAB 55 mM, 5% (w/v) sarkosyl]. After incubation at 65°C for 20 min and extraction with chloroform/isoamyl alcohol, the DNA was precipitated by isopropanol. The DNA pellet was washed with 70% (v/v) ethanol and re-suspended in distilled water. For PCR analysis, based on locus-specific oligonucleotides, extraction of pepper DNA was performed as described (Edwards et al. 1991).

AFLP analysis

AFLP analysis (Vos et al. 1995) was carried out on bulked DNA samples of ten resistant and susceptible plants, respectively (Giovannoni et al. 1991; Michelmore et al. 1991) using the hexacutters *Hin*dIII, *Eco*RI and *Pst*I in combination with the tetracutter *Mse*I. Selective amplification was performed with ^{33}P-labelled hexacutter primers (*Pst*I+2, *Hin*dIII+3 and *Eco*RI+3) and non-labelled tetracutter (*Mse*I+3) primers. AFLP reaction products were denatured and size-fractionated on 5% polyacrylamide sequencing gels. Gels were transferred to Whatman 3MM paper, dried and exposed to X-ray films.

Cloning and analysis of AFLP fragments

X-ray films were aligned with the dried acrylamide gels and AFLP products of interest were excised. DNA was eluted in 200 µl of sterile water overnight. Re-amplification was performed using 2 µl of the eluate employing the same cycling conditions as for selective PCR. The re-amplified amplicons were separated on an agarose gel, excised and purified using the Qiaex gel-extraction kit (Qiagen, Hilden, Germany). The PCR products were ligated into a pBluescript (Stratagene, Amsterdam, The Netherlands) derivative which was modified according to Marchuk et al. (1991). Ligated products were transformed into *Escherichia coli* DH5α by electroporation (Dower et al. 1988). AFLP gels were transferred to Hybond N^+ (Amersham Pharmacia Biotech, Germany) by electroblotting (Gebhardt et al. 1989). The DNA was covalently bound to the membrane by UV irradiation. Subsequent exposure to X-ray film was employed to visualise the AFLP pattern and the efficiency of the transfer. Hybridisations with ^{32}P-labelled cloned AFLP fragments were performed using standard conditions (Sambrook et al. 1989).

Syntenic mapping in tomato

A population of 67 F_2 plants derived from the interspecific cross of *Lycopersicon esculentum* cultivar (cv) VF36-*Tm2a* × *Lycopersicon pennellii* LA716 served as the mapping population for tomato. Plant material and genetic mapping have been described elsewhere (Tanksley et al. 1992).

Locus-specific amplifications

The following cycling conditions were used for locus-specific amplification of H6-4, H17-6, H17-52 and H19-47: 94°C for 3 min, and 40 cycles of 94°C for 30 s, 50°C for 45 s, 72°C for 60 s, and generated a presence/absence polymorphism for H6-4. All PCR products, except H6-4, were analysed on 2% (w/v) agarose gels using the following restriction endonucleases: *Aci*I, *Alu*I, *Bfa*I, *Cfo*I, *Dde*I, *Hae*III, *Hpa*II, *Mae*II, *Mae*III, *Mse*I, *Mvn*I, *Nla*III, *Rsa*I, *Sau*3A, *Sau*96I and *Taq*I. A visible *Sau*3A polymorphism was revealed for H19-47. For PCR-based genotyping of the segregating population 2 µl of miniprep DNA were generally used in a 20-µl PCR reaction. The following oligonucleotides were employed for locus-specific amplification: H6-4, 5'-AAGCTTAAC ACATCTTTC-3' and 5'-TTAAACGGTGGCTGTTAC-3'; H17-6, 5'-CAACAAGGAATTTCAGTGG-3' and 5'-AAGCGGATATGT AGTTGG-3'; H17-52, 5'-CTTATATAATAACAGAGGCG-3' and 5'-GTAATGAGCCACACTAAC-3'; H19-47, 5'-AAGCTTCGTA CTTTAGTTAG-3' and 5'-TTAACAACATGGATTTACAA-3'.

Results

Analysis of *Bs3*-mediated resistance in different pepper varieties

Seventeen different *Capsicum* varieties (see Materials and methods) were tested for resistance to *Xanthomonas* strains expressing the AvrBs3 avirulence protein. The hypersensitive response (HR), indicative of resistance, was induced only in pepper cultivar ECW-30R. Earlier studies have shown that resistance in this line is based on a single dominant *R* gene (Minsavage et al. 1990), designated *Bs3* (Kim and Hartmann 1985). ECW-30R was crossed with different susceptible pepper cultivars to generate a population segregating for *Bs3*-mediated resistance. Viable seeds were only obtained for susceptible parental lines CM 334, PI-197409, SC 81 and Chi 8. ECW-30R × PI-197409 was the most-fertile combination, and 196 F_2 progeny derived from this cross segregated for resistant and susceptible plants in a 3:1 ratio, confirming that *Bs3*-mediated resistance was inherited as a single dominant trait.

AFLP markers linked to the *Bs3* locus

For identification of DNA markers linked to *Bs3* the AFLP technology (Vos et al. 1995) was used in conjunction with a bulked-segregant approach (Michelmore et al. 1991). Employing the enzyme combination *Hin*dIII/*Mse*I, an average of 120 AFLP loci were amplified from each DNA pool, displaying a genome-wide polymorphism of 14% between the parental lines PI-197409 and ECW-30R. Ten susceptible (*bs3*/*bs3*) and ten resistant (*Bs3*/*Bs3* or *Bs3*/*bs3*) F_2 progeny, respectively, were mixed to generate susceptible (S) and resistant (R) DNA bulks. Since DNA from the susceptible parent (*bs3*) is also present in the R pool, only markers linked in *cis* with *Bs3* can be identified by this comparison, thereby reducing the amount of scorable fragments by 50%. In total, 750 *Hin*dIII/*Mse*I primer combinations were inspected in R and S pools. Considering each AFLP fragment as one genetic locus, and neglecting possible allelism between fragments, approximately 6300 (750 × 120 × 0.14 × 0.5) polymorphic loci have been analysed.

We detected 16 fragments displaying polymorphisms between the R and S DNA bulks (example in Fig. 1A). Analysis of these 16 AFLP markers on the individuals of each pool revealed that ten were separated from *Bs3* by at least one recombination event. Six AFLP fragments displayed complete linkage to *Bs3* within individuals of each pool and were subsequently used for the analysis of 80 additional F_2 segregants. Within these progeny lines, 19 recombination events were detected (example in Fig. 1B) enabling us to position the AFLP markers with respect to *Bs3* (Fig. 1C). Linkage of the observed markers ranged from 2 cM (H19-47) to 15 cM (H6-4). The genetic target interval (H19-47/H17-52) was defined to be 14 cM.

Cloning of AFLP markers linked to *Bs3*

Unlike RFLP probes, most AFLP markers are not transferable between different members of the *Solanaceae* family (Van der Voort et al. 1997) and are, therefore, less suitable for syntenic mapping. Furthermore, the labour-intensive, multi-step protocol limits the value of this marker type for the analysis of large segregating populations. To circumvent these drawbacks, AFLP fragments linked to *Bs3* were excised from the polyacrylamide gel, re-amplified and subsequently cloned. A potential risk of this procedure is the isolation of (1) PCR products from the gel which are adjacent to the desired linked fragments or (2) *Mse*I/*Mse*I-derived fragments which are not visible on the autoradiograph. To confirm the identity of cloned DNA fragments with desired AFLP markers, AFLP reactions on R and S DNA templates were performed with the appropriate primer combinations, separated by polyacrylamide gel-electrophoresis (PAGE) and transferred to a membrane. Immobilised ^{33}P-labelled PCR products were visualised by exposure to X-ray film (Fig. 2A). Subsequently, the membranes were probed with ^{32}P-labelled, cloned AFLP markers. Polymorphisms between S and R pool DNAs would indicate that the desired linked fragment has been isolated (Fig. 2B). This "reverse AFLP" analysis was performed for all six *Bs3*-linked *Hin*dIII/*Mse*I-based AFLP markers and confirmed that the desired markers were isolated.

Mapping of *Bs3*-linked AFLP markers in tomato

Comparative mapping in tomato and pepper has shown a high extent of colinearity between both genomes (Livingstone et al. 1999). Therefore, tomato RFLP markers provide a possible resource for marker saturation in a given syntenic chromosome segment in pepper. However, cross-hybridisation of *Bs3*-linked pepper probes is a necessity to define a syntenic chromosome segment in tomato. Hence, the cloned AFLP markers H2-4, H3-4 and H6-4 were tested for cross-hybridisation to tomato genomic DNA. H2-4 generated a smear, indicating the presence of repetitive sequences within this probe. In contrast, H3-4 and H6-4 revealed a more discrete banding pattern with polymorphism between the parental genotypes of the mapping population (Tanksley et al. 1992). H3-4 and H6-4 were mapped based on *Dra*I/*Eco*RV and *Eco*RI/*Eco*RV polymorphisms, respectively. These probes, which define a 19-cM interval in pepper, were both located on the short arm of tomato chromosome 2 (Fig. 3). Based on the analysis of 60 F_2 plants, the H6-4 probe cosegregated with the tomato RFLP marker TG33 (Fig. 3). Only 39 progeny could be evaluated for probe H3-4 and the observed cosegregation with both TG33 and TG31 was probably caused by the low mapping resolution. Genetic mapping of the tomato probes TG31 and TG33 in pepper could not be achieved due to the lack of TG31 cross-hybridisation to pepper genomic DNA and the

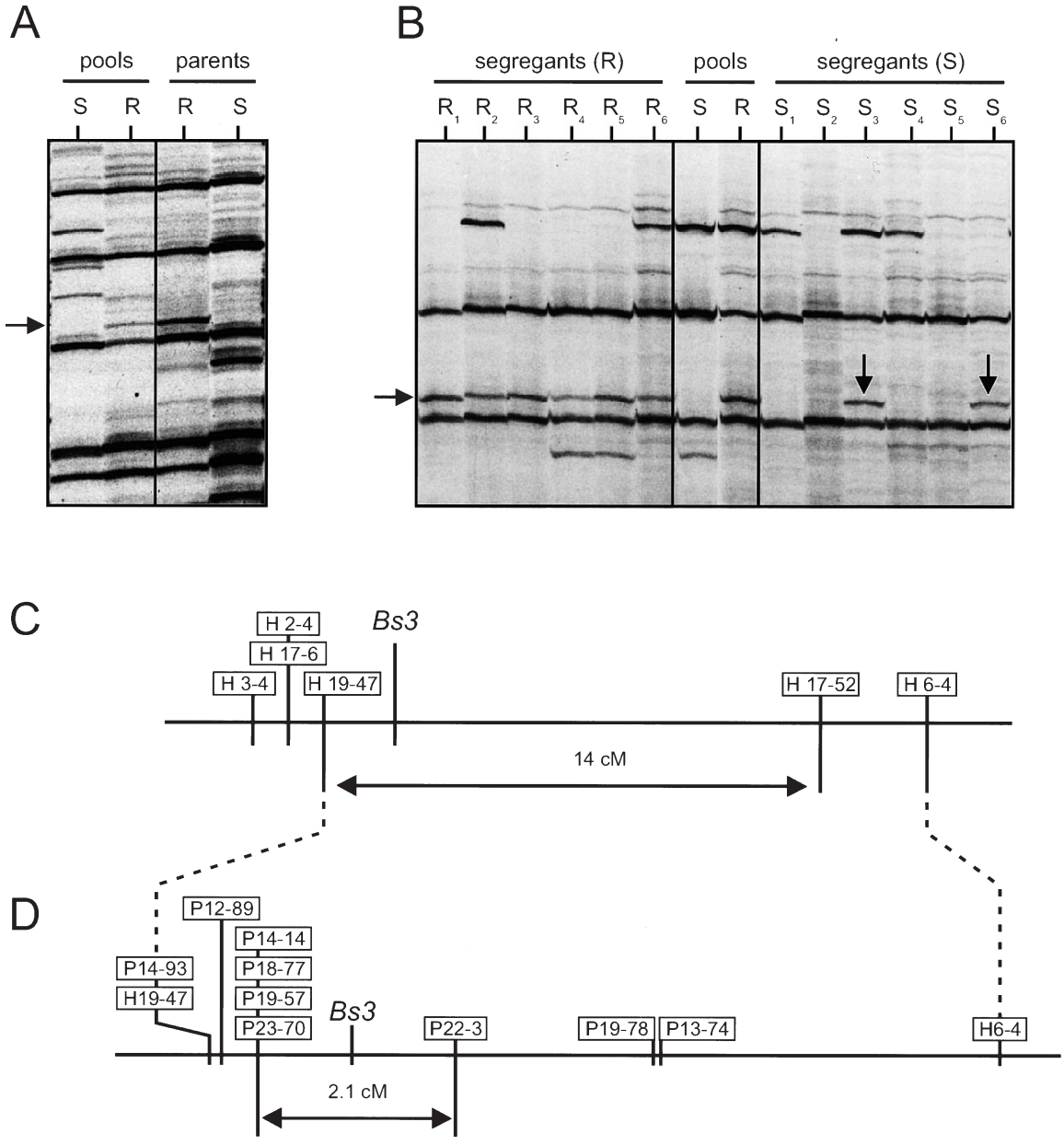

Fig. 1A-D DNA markers linked to *Bs3*. **A** The autoradiograph shows the pattern of AFLP marker H6-4 on DNA templates from susceptible (*S*) and resistant (*R*) parents and F_2 pools. One *Bs3*-linked AFLP fragment (H6-4) is marked by an *arrow*. **B** Segregation pattern of AFLP marker H6-4 in individual resistant (*R1–R6*) and susceptible (*S1–S6*) F_2 segregants and respective pools (*R*, *S*). The presence of the *R*-linked AFLP fragment in two of the susceptible individuals is marked by a *vertical arrow*, indicating recombination events between the AFLP locus H6-4 and *Bs3*. **C** Linkage map of the *Bs3* locus. Map positions are based on the analysis of 100 F_2 backcross progeny. **D** High-resolution genetic map at the *Bs3* locus. Map positions are based on the analysis of 790 F_2 backcross progeny. Map distances in (C) and (D) are drawn to scale. Genetic distances (cM) were calculated on the basis of two-point estimates. *Bs3*-containing target intervals are indicated by *double-headed arrows*. *Hin*dIII/*Mse*I-based (*H*) and *Pst*I/*Mse*I-based (*P*) AFLP markers are *boxed* and designated according to the number of the chosen selective oligonucleotides

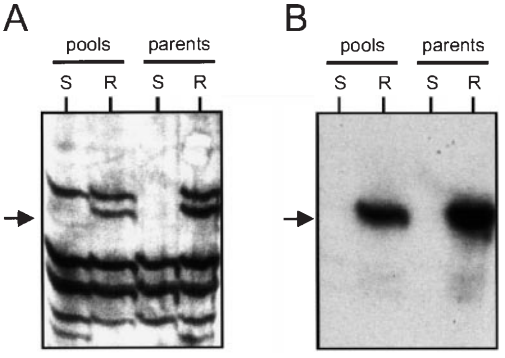

Fig. 2A, B Analysis of cloned AFLP fragments. **A** Autoradiograph showing the membrane-bound, ^{33}P-labelled AFLP marker *P12-89*. The fragment discriminating between the resistant (*R*) pools, the susceptible (*S*) pools and their *parents*, is indicated by the *arrow*. **B** Southern analysis of the membrane shown in (**A**) using the cloned AFLP marker P12-89 as a probe

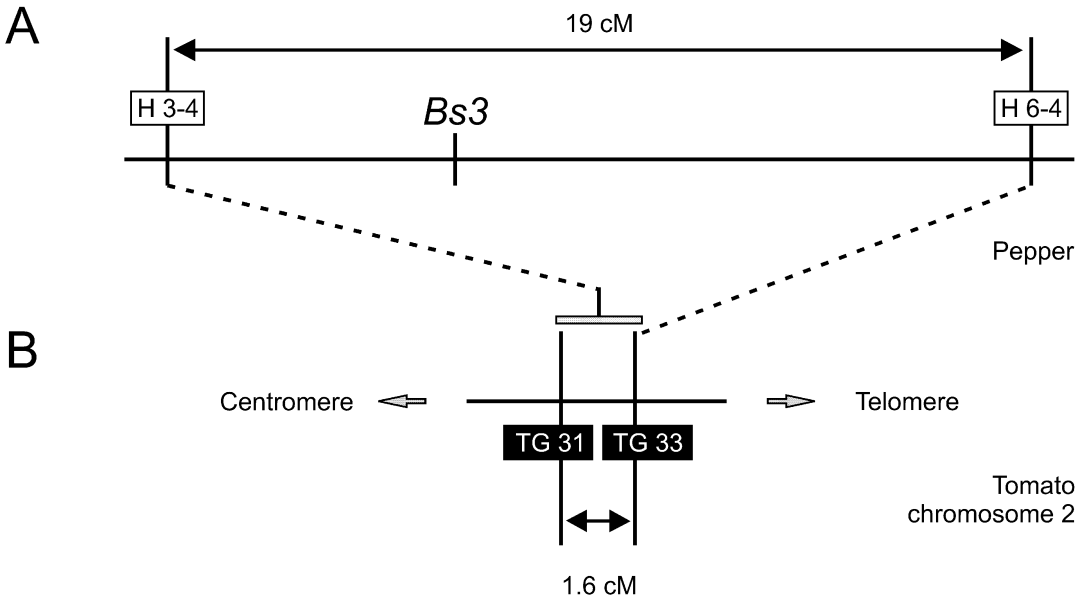

Fig. 3A, B Comparative mapping of *Bs3*-linked markers in pepper and tomato. *Horizontal lines* represent segments of pepper (**A**) and tomato chromosome 2 (**B**). Map positions of the pepper markers (*white boxes*) are connected via *dashed lines* with the tomato markers (*black boxes*) (Tanksley et al. 1992) which map to the same genetic position. H3-4 and H6-4 were mapped in tomato based on the analysis of 60 and 39 F_2 progeny respectively. Cosegregation of H3-4 with TG31 and TG33 is indicated by a *bar*

lack of TG33 polymorphism. Since syntenic mapping in tomato did not provide any additional genetic markers, high-resolution linkage mapping had to rely solely on pepper AFLP markers.

Recombinant screen at the *Bs3* locus

The characteristics of AFLP markers (dominant, costly and labour-intensive) determine their limited value for large-scale, locus-specific applications. Therefore, we converted *Bs3*-linked AFLPs into locus-specific PCR-based markers. BLAST analysis (Altschul et al. 1990) of the isolated *Hin*dIII/*Mse*I AFLP fragments did not reveal significant homologies to repetitive sequence motifs, indicating that these sequences were suitable for conversion into locus-specific markers. We defined oligonucleotides for the amplification of all *Hin*dIII/*Mse*I-derived AFLP loci with the exception of the short fragments H2-4 and H3-4. Primers corresponding to H6-4 generated a PCR product only in the resistant (ECW-30R) and not the susceptible (PI-197409) parent (Fig. 4). In contrast, oligonucleotides corresponding to H17-6, H17-52 and H19-47 generated amplification products of identical size in both parental genotypes. These PCR products, monomorphic between ECW-30R and PI-197409, were, therefore, inspected for polymorphisms with a set of endonucleases (see Materials and methods). The *Sau*3AI-endonuclease digest revealed a polymorphism between the parental genotypes for H19-47 (Fig. 4). However, the

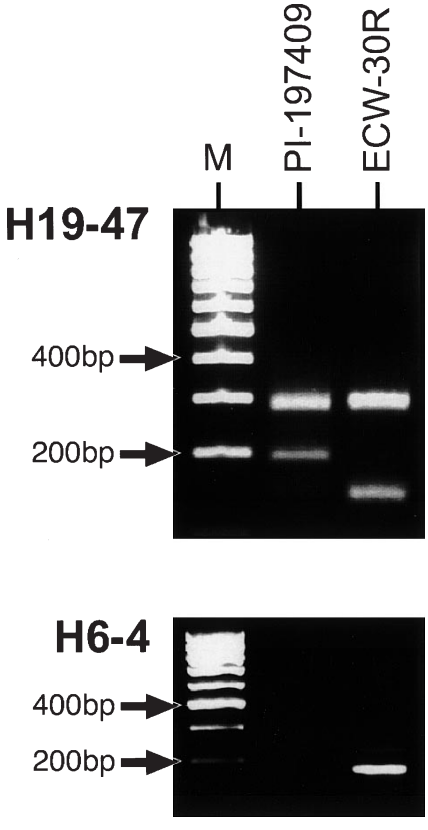

Fig. 4 PCR-based markers linked to *Bs3*. PCR was carried out on the parental pepper lines *PI-197409* (susceptible) and *ECW-30R* (resistant). Amplification products of the marker locus *H19-47* were digested by the *Sau*3AI-endonuclease to reveal a fragment length polymorphism. Oligonucleotides corresponding to the AFLP locus *H6-4* generated an absence/presence polymorphism on the parental genotypes. PCR products were resolved on 2% (w/v) agarose gels and stained by ethidium-bromide. *M* size marker; *bp* base pairs

chosen set of restriction enzymes did not detect polymorphisms in the PCR products corresponding to H17-6 and H17-52. Therefore, we used DNA markers H6-4 and H19-47 for the analysis of 790 F_2 backcross (BC) plants, and identified 61 crossover events. Results of the bacterial infection tests placed 50 recombination events between H6-4 and *Bs3*, and 11 into the H19-47/*Bs3* interval.

*Pst*I/*Mse*I- and *Eco*RI/*Mse*I-based AFLP marker screens

To increase the marker density in the vicinity of *Bs3*, 660 *Pst*I/*Mse*I and 160 *Eco*RI/*Mse*I AFLP primer combinations were used to detect polymorphisms between the R and S DNA pools. The *Pst*I/*Mse*I and *Eco*RI/*Mse*I enzyme combinations generated on average 80 and 120 fragments, displaying a genome-wide polymorphism of 10% and 14%, respectively. In conclusion, 2600 ($660 \times 80 \times 0.1 \times 0.5$) *Pst*I/*Mse*I and 1350 ($160 \times 120 \times 0.14 \times 0.5$) *Eco*RI/*Mse*I PCR products were inspected for linkage to *Bs3*. AFLP fragments displaying polymorphisms between the DNA bulks were subsequently scored on plants carrying recombination events in the marker interval H6-4/H19-47 and were placed with respect to *Bs3* (Fig. 1D). Nine *Pst*I/*Mse*I AFLP markers were placed within the H6-4/H19-47 target interval. In contrast, none of the tested *Eco*RI/*Mse*I primer combinations uncovered a *Bs3*-linked AFLP fragment. Based on the most-tightly linked AFLP markers, a 2.1-cM genomic target interval encompassing the *Bs3* locus was defined (Fig. 1D). With the exception of P14-14, all *Pst*I/*Mse*I-derived AFLP markers were cloned and subjected to BLAST analysis. AFLP markers P13-74, P14-93, P18-77 and P19-57 showed high similarity to different transposable elements indicative of the repetitive character of these sequences. *Bs3* is separated from P23-70 and P22-3 by only 1 cM and 1.1 cM, respectively (Fig. 1D). These are the most tightly linked and potentially non-repetitive AFLP fragments found for the *Bs3* locus and should be valuable tools for the physical delimitation of *Bs3*, the next step in our map-based cloning approach.

Discussion

Non-random distribution of AFLP rare-cutter recognition sites

We have established a high-resolution linkage map as a foundation for the map-based cloning of the *Bs3* locus. DNA-marker saturation of the target interval was accomplished by application of the AFLP technology, employing the frequent cutter *Mse*I in combination with three different rare-cutting restriction enzymes (*Eco*RI, *Hin*dIII and *Pst*I). In combination with *Mse*I, different rare-cutter enzymes revealed significant differences between each other with respect to the degree of polymorphism detected and the distribution of their respective markers on the genetic map. Comparison of different hexacutters revealed that *Pst*I (a GC-rich recognition sequence) uncovers less polymorphic sites than *Hin*dIII and *Eco*RI (both AT-rich recognition sequences). This is in agreement with results observed in *Arabidopsis*, where AFLP fragments derived from *Sac*I (a GC-rich recognition sequence) detected a lower level of polymorphism than *Eco*RI based PCR products (Cnops et al. 1996).

Inspection of a 900-kb contigous DNA sequence in *Arabidopsis* showed that *Sac*I recognition sites were more often located within coding sequences than *Eco*RI recognition sites (Breyne et al. 1999). Due to selection pressure, sequence conservation within coding sequences should be higher than in non-coding areas, thus providing an explanation for the lower frequency of polymorphism observed for *Sac*I in *Arabidopsis*. Although prevalence of *Sac*I recognition sites in gene-rich areas was shown for *Arabidopsis*, there is currently no proof that pepper *Pst*I recognition sites are primarily adjacent to, or within, coding sequences. However, *Pst*I cleavage is inhibited by methylation of the recognition-sequence (McClelland et al. 1994) and transcribed plant DNA is generally hypo-methylated (Antequera and Bird 1988). Therefore, *Pst*I probably cleaves predominantly in gene-rich chromosome segments. Consequently, the high level of sequence conservation within coding sequences would explain the lower degree of polymorphism observed with *Pst*I-based primer combinations.

Syntenic relationships between tomato and pepper

Pepper and tomato are phylogenetically closely related, which is consistent with the finding that: (1) tomato single-copy probes commonly cross-hybridise to pepper DNA (Tanksley et al. 1988), and (2) both genomes reveal a high degree of colinearity (Livingstone et al. 1999). The genome-wide tomato RFLP map comprises more than 1000 markers and represents probably the most advanced map within the *Solanaceae* species (Tanksley et al. 1992). This indicates the potential of tomato RFLP markers as a resource for marker saturation within the *Bs3* target interval in pepper. However, a prerequisite for exploitation of these resources is the integration of *Bs3*-linked AFLP markers into the tomato RFLP map.

Syntenic mapping of the pepper AFLP markers H3-4 and H6-4, which encompass a 19-cM interval in pepper, placed the *Bs3*-linked probes at the upper end of tomato chromosome 2, separated by a maximum genetic distance of 1.6 cM (Fig. 3). Genetic maps of tomato and pepper cover 1246 and 1276 map units respectively (Tanksley et al. 1992; Livingstone et al. 1999; both RFLP maps derived from interspecific crosses). Due to the comparable genetic length of these genomes, genetic distances for any syntenic interval should be similar between any two given markers. The more than ten-

fold size difference indicates that the H3-4/H6-4 interval is either highly prone to recombination in pepper or that recombination is suppressed in the syntenic tomato chromosome segments. Indeed, inspection of the syntenic tomato interval reveals a clustering of RFLP markers (Tanksley et al. 1992), which is indicative of a suppression of recombination in this region. The occurrence of meiotic nodules is an alternative measure of crossover events (Carpenter 1975; Sherman and Stack 1995; Anderson et al. 1997) and has suggested a lack of recombination in the vicinity of tomato telomeres (Sherman and Stack 1995). The fact that *Bs3*-linked markers map in the proximity of a tomato telomere supports the idea that the syntenic tomato chromosome segment has a reduced recombination frequency, in comparison to the respective interval in pepper. The observed clustering of RFLP markers in the syntenic tomato interval makes these probes less attractive for high-resolution mapping of *Bs3*, since their exact location within our pepper high-resolution map is unpredictable.

The possibility that *Bs3* might resemble an NBS-LRR type of *R* gene is another potential drawback in the application of synteny, since such loci have been found at a high frequency at non-syntenic locations (Leister et al. 1998). Therefore, our cloning approach should not rely on colinearity between pepper and tomato, but instead focus on generating a high-resolution map based on pepper-derived AFLP markers.

"Reverse AFLP" as a tool for the analysis of cloned AFLP markers

Despite the reported use of the AFLP technique in various applications, little information is available concerning the cloning of AFLP fragments for conversion to other marker types and the associated problems. The conversion protocol includes excision of the AFLP fragment from a dried polyacrylamide gel using the superimposed autoradiograph and can result in the isolation of a non-homogeneous amplicon mixture (Reamon-Büttner et al. 1998; Shan et al. 1999). Therefore, re-investigation of a cloned PCR product is necessary to prove that the desired AFLP marker has been isolated. We have used a "reverse-AFLP" strategy to analyse the individual cloned fragments. Alternative protocols involve genetic mapping of cloned PCR products to prove conformity of AFLP markers and isolated DNA fragments (Meksem et al. 1995; Bendahmane et al. 1997; Brigneti et al. 1997; De Jong et al. 1997; Lu et al. 1999; Schwarz et al. 1999). However, genetic mapping of cloned AFLP fragments is time consuming and sometimes difficult due to their repetitive nature, lack of polymorphism or small size (Meksem et al. 1995; Kasuga et al. 1997; Schwarz et al. 1999). In contrast, the "reverse-AFLP" strategy was applicable for all cloned fragments investigated, including those *Pst*I/*Mse*I markers with significant homology to repetitive-sequence motifs.

Tightly-linked AFLP markers as a foundation for a chromosome landing

Positional cloning involves the identification of genetically linked DNA markers (genetic mapping) and use of these markers to isolate genomic clones that span the target locus (physical mapping; Collins 1992). If the closest markers are physically far from the target locus, long-range walking techniques (chromosome walking) become necessary (Stubbs 1992). Chromosome walking in complex plant genomes is hampered by the high frequency of repetitive DNA and unfavourable relationships between genetic and physical distances. Advances in both DNA-marker technology (Williams et al. 1990; Vos et al. 1995) and genetic-screening methods (Young et al. 1988; Michelmore et al. 1991) avoid these problems by identifying molecular markers so close to the target locus that chromosome walking is unnecessary. This concept has been termed "chromosome landing" (Tanksley et al. 1995) and is now a paradigm for map-based cloning in plants with large genomes.

In this paper, we describe the construction of a high-resolution genetic map at the *Bs3* locus, which is an essential step of the chromosome-landing approach. Linkage mapping was performed on an intraspecific cross between two *C. annuum* varieties. Based on the genome size of pepper (2702–3420 Mb; Arumuganathan and Earle 1991) and the estimated total length of an intraspecific pepper map (2500 cM; Palloix, personal communication), we calculated that 1 cM corresponds on average to a physical distance of 1–1.5 Mb. The analysis of 790 chromosomes, which corresponds to a genetic resolution of 0.13 cM, should therefore yield one crossover event every 150 kb. Given that a pepper YAC library is available, with an average insert size of 500 kb (Tai and Staskawicz 1999), the density of recombination events should be sufficient to prove physical delimitation of the target locus on a respective YAC clone.

Availability of tightly linked markers represents another crucial pre-requisite for the proposed chromosome-landing approach. The most tightly linked AFLP markers we have identified so far are separated from *Bs3* by 1 cM which corresponds, on average, to a physical distance of 1–1.5 Mb. These calculations suggest that the physical linkage of the identified markers is not yet sufficient to identify a single YAC clone covering the target locus. However, in theory our analysis of 10250 AFLP loci should have provided at least three markers per Mb (10250 AFLP loci / 2702–3420 Mb). Therefore our results (about one marker per cM) indicate that the *Bs3*-containing interval shows favourable relationships between physical and genetic distances.

Future experiments towards the isolation of *Bs3* will focus on the physical delimitation of the target locus within large insert clones. The successful outcome of this approach will depend to a large extent on the local relationship between physical and genetic distances.

Acknowledgements We thank our colleagues Desmond Bradley, Dirk Nennstiel and Eric Marois for helpful suggestions on the manuscript. This work was in part funded by grants from CNRS (ATIPE) and INRA (AIP Gènes de Résistance) to U.B; A.B. was supported by a long-term EMBO fellowship.

References

Aarts MGM, Hekkert BT, Holub EB, Beynon JL, Stiekema WJ, Pereira A (1998) Identification of *R*-gene homologous DNA fragments genetically linked to disease resistance loci in *Arabidopsis thaliana*. Mol Plant-Microbe Interact 11: 251–258

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410

Anderson LK, Offenberg HH, Verkuijlen WMHC, Heyting C (1997) RecA-like proteins are components of early meiotic nodules in lily. Proc Natl Acad Sci USA 94: 6868–6873

Antequera F, Bird AP (1988) Unmethylated CpG islands associated with genes in higher plant DNA. EMBO J 7: 2295–2299

Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9: 208–219

Bendahmane A, Kanyuka K, Baulcombe DC (1997) High-resolution genetical and physical mapping of the *Rx* gene for extreme resitance to potato virus X in tetraploid potato. Theor Appl Genet 95: 153–162

Bonas U, Stall RE, Staskawicz B (1989) Genetic and structural characterization of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv *vesicatoria*. Mol Gen Genet 218: 127–136

Breyne P, Rombaut D, Van Gysel A, Van Montagu M, Gerats T (1999) AFLP analysis of genetic diversity within and between *Arabidopsis thaliana* ecotypes. Mol Gen Genet 261: 627–634

Brigneti G, Garcia-Mas J, Baulcombe DC (1997) Molecular mapping of the potato virus Y resistance gene Ry_{sto} in potato. Theor Appl Genet 94: 198–203

Brown I, Mansfield J, Irlam I, Conrads-Strauch J, Bonas U (1993) Ultrastructure of interactions between *Xanthomonas campestris* pv. *vesicatoria* and pepper, including immunocytochemical localization of extracellular polysaccharides and the AvrBs3 protein. Mol Plant-Microbe Interact 6: 376–386

Carpenter ATC (1975) Electron microscopy of meiosis in *Drosophila melanogaster* females. II. The recombination nodule – a recombination-associated structure at pachytene? Proc Natl Acad Sci USA 72: 3186–3189

Cnops G, Den Boer B, Gerats A, Van Montagu M, Van Lijsebettens M (1996) Chromosome landing at the *Arabidopsis TORNADO1* locus using an AFLP-based strategy. Mol Gen Genet 253: 32–41

Collins FA (1992) Positional cloning: let's not call it reverse anymore. Nature Genet 1: 3–6

Collins NC, Webb CA, Seah S, Ellis JG, Hulbert SH, Pryor A (1998) The isolation and mapping of disease resistance gene analogs in maize. Mol Plant-Microbe Interact 11: 968–978

De Jong W, Forsyth A, Leister D, Gebhardt C, Baulcombe DC (1997) A potato hypersensitive resistance gene against potato virus X maps to a resistance gene cluster on chromosome 5. Theor Appl Genet 95: 246–252

Dower WJ, Miller JF, and Ragsdale CW (1988) High-efficiency transformation of *E.coli* by high-voltage electroporation. Nucleic Acids Res 16: 6127–6145

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19: 1349

Gebhardt C, Ritter E, Debener T, Schachtschabel U, Walkemeier B, Uhrig H, Salamini F (1989) RFLP analysis and linkage mapping in *Solanum tuberosum*. Theor Appl Genet 78: 65–75

Giovannoni JJ, Wing RA, Ganal MW, Tanksley SD (1991) Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. Nucleic Acids Res 19: 6553–6558

Hämäläinen JH, Sorri VA, Watanabe KN, Gebhardt C, Valkonen JPT (1998) Molecular examination of a chromosome region that controls resistance to potato Y and A potyviruses in potato. Theor Appl Genet 96: 1036–1043

Hammond-Kosack KE, Jones JDG (1997) Plant disease resistance genes. Annu Rev Plant Physiol Plant Mol Biol 48: 575–607

Herbers K, Conrads-Strauch J, Bonas U (1992) Race-specificity of plant resistance to bacterial spot disease determined by repetitive motifs in a bacterial avirulence protein. Nature 356: 172–174

Hibberd AM, Bassett MJ, Stall RE (1987) Allelism tests of three dominant genes for hypersensitive resistance to bacterial spot of pepper. Phytopathology 77: 1304–1307

Kanazin V, Marek LF, Shoemaker RC (1996) Resistance gene analogs are conserved and clustered in soybean. Proc Natl Acad Sci USA 93: 11746–11750

Kasuga T, Salimath SS, Shi JR, Gijzen M, Buzzell RI, Bhattacharyya MK (1997) High-resolution genetic and physical mapping of molecular markers linked to the *Phytophthora* resistance gene *Rps1*-k in soybean. Mol Plant-Microbe Interact 10: 1035–1044

Kim BS, Hartmann RW (1985) Inheritance of a gene (Bs_3) conferring hypersensitive resistance to *Xanthomonas campestris* pv *vesicatoria* in pepper (*Capsicum annuum*). Plant Dis 69: 233–235

Leister D, Ballvora A, Salamini F, Gebhardt C (1996) A PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. Nature Genet 14: 421–429

Leister D, Kurth J, Laurie DA, Yano M, Sasaki T, Devos K, Graner A, Schulze-Lefert P (1998) Rapid reorganization of resistance gene homologues in cereal genomes. Proc Natl Acad Sci USA 95: 370–375

Leister D, Kurth J, Laurie DA, Yano M, Sasaki T, Graner A, Schulze-Lefert P (1999) RFLP- and physical-mapping of resistance gene homologues in rice (*O. sativa*) and barley (*H. vulgare*). Theor Appl Genet 98: 509–520

Livingstone KD, Lackney VK, Blauth JR, Van Wijk R, Kyle Jahn M (1999) Genome mapping in *Capsicum* and the evolution of genome structure in the *Solanaceae*. Genetics 152: 1183–1202

Lu ZX, Sossey-Alaoui K, Reighard GL, Baird WV, Abbott AG (1999) Development and characterization of a codominant marker linked to root-knot nematode resistance, and its application to peach rootstock breeding. Theor Appl Genet 99: 115–222

Mago R, Nair S, Mohan M (1999) Resistance gene analogues from rice: cloning, sequencing and mapping. Theor Appl Genet 99: 50–57

Marchuk D, Drumm M, Saulino A, Collins FS (1991) Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res 19: 1154

McClelland M, Nelson M, Raschke E (1994) Effect of site-specific modification on restriction endonucleases and DNA modification methyltransferases. Nucleic Acids Res 22: 3640–3659

Meksem K, Leister D, Peleman J, Zabeau M, Salamini F, Gebhardt C (1995) A high-resolution map of the vicinity of the *R1* locus on chromosome V of potato based on RFLP and AFLP markers. Mol Gen Genet 249: 74–81

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88: 9828–9832

Minsavage GV, Dahlbeck D, Whalen MC, Kearny B, Bonas U, Staskawicz BJ, Stall RE (1990) Gene-for-gene relationships specifying disease resistance in *Xanthomonas campestris* pv *vesicatoria* - pepper interactions. Mol Plant-Microbe Interact 3: 41–47

Parker JE, Coleman MJ (1997) Molecular intimacy between proteins specifying plant-pathogen recognition. Trends Biochem Sci 22: 291–296

Reamon-Büttner SM, Schondelmaier J, Jung C (1998) AFLP markers tightly linked to the sex locus in *Asparagus officinalis* L. Mol Breed 4: 91–98

Rossier O, Wengelnik K, Hahn K, and Bonas U (1999) The *Xanthomonas* Hrp type-III system secretes proteins from plant and mammalian pathogens. Proc Natl Acad Sci USA 96: 9368–9373

Sambrook J, Fritsch EF, Maniatis T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Schwarz G, Michalek W, Mohler V, Wenzel G, Jahoor A (1999) Chromosome landing at the *Mla* locus in barley (*Hordeum vulgare* L.) by means of high-resolution mapping with AFLP markers. Theor Appl Genet 98: 521–530

Shan X, Blake TK, Talbert LE (1999) Conversion of AFLP markers to sequence-specific PCR markers in barley and wheat. Theor Appl Genet 98: 1072–1078

Shen KA, Meyers BC, IslamFaridi MN, Chin DB, Stelly DM, Michelmore RW (1998) Resistance gene candidates identified by PCR with degenerate oligonucleotide primers map to clusters of resistance genes in lettuce. Mol Plant-Microbe Interact 11: 815–823

Sherman JD, Stack SM (1995) Two-dimensional spreads of synaptonemal complexes from solanaceous plants. VI. High-resolution recombination nodule map for tomato (*Lycopersicon esculentum*). Genetics 141: 683–708

Speulman E, Bouchez D, Holub EB, Beynon JL (1998) Disease resistance gene homologs correlate with disease resistance loci of *Arabidopsis thaliana*. Plant J 14: 467–474

Stall RE (1993) *Xanthomonas campestris* pv *vesicatora*: cause of bacterial spot of tomato and pepper. In: Swings JG, Civerolo EL (eds) *Xanthomonas*. Chapman and Hall, London, UK, pp 57–60

Stubbs L (1992) Long-range walking techniques in positional cloning strategies. Mammal Genome 3: 127–142

Tai T, Staskawicz BJ (2000) Construction of a yeast artificial chromosome library of pepper (*Capsicum annuum* L.) and identification of clones from the *Bs2* resistance locus. Theor Appl Genet 100:112–117

Tanksley SD, Bernatzky R, Lapitan NL, Prince JP (1988) Conservation of gene repertoire but not gene order in pepper and tomato. Proc Natl Acad Sci USA 85: 6419–6423

Tanksley SD, Ganal MW, Prince JP, Devicente MC, Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB, Messeguer R, Miller JC, Miller L, Paterson AH, Pineda O, Roder MS, Wing RA, Wu W, Young ND (1992) High-density molecular linkage maps of the tomato and potato genomes. Genetics 132: 1141–1160

Tanksley SD, Ganal MW, Martin GB (1995) Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes. Trends Genet 11: 63–68

Vallejos CE, Tanksley SD, Bernatzky R (1986) Localization in the tomato genome of DNA restriction fragments containing sequences homologous to the rRNA (45 S), the major chlorophyll-a/b binding polypeptide and the ribulose bisphosphate carboxylase genes. Genetics 112: 93–105

Van den Ackerveken G, Marois E, Bonas U (1996) Recognition of the bacterial avirulence protein AvrBs3 occurs inside the host plant cell. Cell 87: 1307–1316

Van der Biezen EA, Jones JDG (1998) Plant disease-resistance proteins and the gene-for-gene concept. Trends Biochem Sci 23: 454–456

Van der Voort JNAMR, Van Zandvoort P, Van Eck HJ, Folkertsma RT, Hutten RCB, Draaistra J, Gommers FJ, Jacobsen E, Helder J, Bakker J (1997) Use of allele specificity of comigrating AFLP markers to align genetic maps from different potato genotypes. Mol Gen Genet 255: 438–447

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23: 4407–4414

Walbot V (1992) Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertional mutagenesis. Annu Rev Plant Physiol Plant Mol Biol 43: 49–82

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18: 6531–6535

Young ND, Zamir D, Ganal MW, Tanksley SD (1988) Use of isogenic lines and simultaneous probing to identify DNA markers tightly linked to the *Tm-2a* gene in tomato. Genetics 120: 579–585

Yu YG, Buss GR, Maroof MAS (1996) Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. Proc Natl Acad Sci USA 93: 11751–11756