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ORIGINAL PAPER

Characterization of two recessive genes controlling resistance to all races of bacterial spot in peppers

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Abstract Bacterial spot, one of the most damaging diseases of pepper, is caused by *Xanthomonas euvesicatoria*. This pathogen has worldwide distribution and it is particularly devastating in tropical and sub-tropical regions where high temperatures and frequent precipitation provide ideal conditions for disease development. Three dominant resistance genes have been deployed singly and in combination in commercial cultivars, but have been rendered ineffectual by the high mutation rate or deletion of the corresponding cognate effector genes. These genes are missing in race P6, and their absence makes this race virulent on all commercial pepper cultivars. The breeding line ECW12346 is the only source of resistance to race P6 in *Capsicum annuum*, and displays a non-hypersensitive type of resistance. Characterization of this resistance has identified two recessive genes: *bs5* and *bs6*. Individual analysis of these genes revealed that *bs5* confers a greater level of resistance than *bs6* at 25°C, but in combination they confer full resistance to P6 indicating at least additive gene action. Tests carried out at 30°C showed that both resistances are compromised to a significant extent, but in combination they provide almost full resistance to race P6 indicating a positive epistatic interaction at high temperatures. A scan of the pepper genome with restriction fragment length polymorphism and AFLP markers led to the identification of a set of AFLP markers for *bs5*. Allele-specific primers for a PCR-based *bs5*-marker have been developed to facilitate the genetic manipulation of this gene.

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Introduction

Xanthomonas euvesicatoria (*X. campestris* pv. *vesicatoria*; Jones et al. 2004) is the causal agent of bacterial spot in pepper. This disease can exert significant damages to pepper production in tropical and sub-tropical regions around the world where high temperatures and frequent precipitation provide ideal conditions for disease development. This pathogen can be transmitted mechanically through farm equipment or by seed, and can persist in the field from season to season on volunteer plants (Jones et al. 1986; Pohronezny et al. 1990). Despite cultural practices, such as the use of certified seed and weed control, the pathogen has continued to spread. Chemical control of this pathogen has also proven inadequate as natural populations have developed resistance to streptomycin (Stall and Thayer 1962; Ritchie and Dittapongpitch 1991), and copper (Basim et al. 2005; Marco and Stall 1983). These problems have led to the use of genetically controlled resistances as an alternative method of disease control.

Three monogenic dominant resistances controlling specific hypersensitive reactions to pepper strains of *X. euvesicatoria* have been characterized: *Bs1* from *Capsicum annuum* PI 163192 (Cook and Stall 1963); *Bs2* from *C. chacoense* PI 260435 (Cook and Guevara 1984), and *Bs3* from *C. annuum* PI 271322 (Kim and Hartmann 1985). Another hypersensitive reaction (HR)-mediated resistance has been identified in *C. pubescens* PI 235047 (Sahin and Miller 1998). An analysis of the virulence spectrum of a collection of *X. euvesicatoria* strains with each one of these resistances has led to the identification of 11 (P0–P10) pepper races (Kousik and Ritchie 1999; Sahin and Miller 1998).

The resistance genes *Bs1*, *Bs2* and *Bs3* have been effectively deployed, singly or in different combinations, in several commercial pepper cultivars with satisfactory results for a short period of time. Unfortunately, these resistances have been rendered useless by the relatively high rate of mutation in the cognate bacterial effectors *avrBs1* (Dahlbeck and Stall 1979) and *avrBs2* (Gassmann et al. 2000; Swords et al. 1996), and the proclivity of the plasmid carrying *avrBs3* to be lost (Minsavage et al. 1990). These three effectors are absent in P6, making this race virulent on all commercial pepper cultivars (Sahin and Miller 1998).

Two sources of resistance to race P6 have been identified in recent years. One of them is *C. pubescens* PI 235047 which carries HR-mediated resistance to P6, but efforts to introgress this resistance into *C. annuum* have been hindered by inter-specific hybridization barriers (Sahin and Miller 1998). The other source is the breeding line ECW12346 that was generated by backcrossing recessive resistances from the breeding line Pep13 and the accession PI 271322 to the breeding line Early Calwonder 123 (ECW123) (Jones et al. 2002). Segregation analysis of various progenies obtained with F_2-derived F_3 families, from the cross [ECW123 × ECW12346], revealed the presence of two recessive resistance genes with additive gene action. We have searched the *Capsicum* genome via restriction fragment length polymorphism (RFLP) and AFLP analysis to identify molecular markers for these resistance genes.

Materials and methods

Plant material

The following pepper genotypes were used to screen AFLP markers for polymorphisms: Early Calwonder (ECW), ECW123, ECW134 LF-1, Pep13, ECW12346, 15-2 and 35-3. ECW is an old pepper cultivar which is susceptible to all races of *X. euvesicatoria*. ECW123 is an ECW-derived introgression line containing the *Bs1*, *Bs2* and *Bs3* resistance genes. ECW134 LF-1 is a breeding line that carries *Bs1*, *Bs3* and partial resistance from PI 271322. Pep13 is a breeding line carrying partial resistance to bacterial spot derived from PI163192 (McCarter 1992). ECW12346 is a breeding line carrying *Bs1*, *Bs2*, *Bs3* and two recessive resistances derived from Pep13 and PI 271322. Lines 15-2 and 35-3 are partially resistant selections derived from F_2 segregants of the cross [ECW123 × ECW12346], each carrying one of the recessive resistance genes in a homozygous state. The segregation and linkage analyses of resistance and molecular markers were performed in four F_2 populations obtained from the following crosses: Pop1 [ECW123 × 15-2], Pop2 [ECW12346 × 15-2], Pop3 [ECW123 × 35-3] and Pop4 [ECW × 15-2]. Lines 50R and 60R were derived from an F_2 population from the cross [ECW × ECW12346]. Approximately 2,700 seedlings were screened for the presence of the dominant resistance genes *Bs1*, *Bs2*, or *Bs3* by inoculation with strains carrying *avrBs1*, *avrBs2* and *avrBs3*. Seedlings that displayed a hypersensitive reaction were eliminated, and the remaining seedlings were inoculated with race P4. The 50R and 60R lines were obtained from single segregants that displayed inoculation phenotypes similar to those of lines 15-2 and 35-3, respectively. The selected segregants were selfed for 2 additional generations, and 24 seedlings were screened with race P4 in each generation to ensure homozygosity.

The marker for *bs5* was mapped in the AC99 F_2 population which was generated from a cross between the *C. annuum* cultivar 'NuMex R Naky' (Nakayama and Matta 1985) and the *C. chinense* accession PI 159234. F_2 seed was collected from a single F_1 plant. DNA was extracted from 100 F_2 seedlings, as described by Prince et al. (1997), which were genotyped with 424 RFLP and SSR markers (Ben-Chaim et al. 2006; Livingstone et al. 1999). The genetic map of this population comprises 15 linkage groups and spans over 1,304.8 cM; this map can be found in the Solanaceae Genomics Network at http://sgn.cornell.edu/cview/map.pl?map_id=11. A subset of 75 individuals from the AC99 population was used in the linkage analysis of *PepC2*.

Bacterial inoculations and evaluation of disease resistance

The *X. euvesicatoria* pepper strain XV157 of race 6 (P6) used in our screens was obtained from S. A. Miller (Ohio State University, Wooster). The inoculum was prepared by growing the bacterium cells overnight in nutrient broth (BBL, Cockersville, MD) at 28°C, pelleting the cells by centrifugation and resuspending them in sterile tap water at a concentration of 10^5 cfu/ml.

Young seedlings of the segregating progenies, along with those of parental genotypes, were transplanted into Farfard mix (Farfard, Inc., Greely, CO) in 4″ pots, and were grown in the greenhouse for approximately 3 weeks prior to

inoculation. The bacterial inoculum was infiltrated into the intercellular spaces on the abaxial side of the first true leaves with a syringe fitted with a 26-gauge hypodermic needle. The inoculated plants were incubated under greenhouse conditions for three weeks before evaluation. The rating scale to evaluate the level of resistance among segregants was as follows: 1 = no disease symptoms, 2 = slight to moderate yellowing and slight necrosis, 3 = extensive yellowing and moderate necrosis, and 4 = complete necrosis. A second evaluation was performed a week later to confirm results from the first rating. F_2 individuals were then grown to maturity to obtain seed. The F_2-derived F_3 families were also evaluated in the same manner as the previous generation to deduce the exact genotype of F_2 individuals at the resistance loci.

RFLP and AFLP analyses

DNA extractions were carried out essentially as described by Vallejos et al. (1992), or by Vallejos (2007). Genomic DNA samples were digested with restriction enzymes as indicated by the manufacturer (Invitrogen Corporation, Carlsbad, CA). Agarose gel electrophoresis, DNA blot hybridizations and autoradiography were as previously described (Vallejos et al. 1992). The high degree of synteny and sequence similarity between tomato and pepper (Livingstone et al. 1999; Prince et al. 1993) permitted the use of previously mapped markers from the former species as a source of potential markers for the resistance genes. Thus, genomic blots of pepper DNA were hybridized with tomato RFLP probes generously provided by Dr. Steve Tanksley from Cornell University (Tanksley et al. 1992; supplementary Table 1S). To allow for probe–target mismatches, hybridizations and washes of DNA blots were carried out at 60°C. A total of 136 tomato markers were screened via Southern hybridization of NILs DNAs digested with six restriction enzymes (*Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I). Twelve of these markers detected polymorphisms between ECW123 and ECW12346. However, only ten of them detected allelic differences between ECW123 and lines 15-2 or 35-3.

AFLP analysis (Vos et al. 1995) was conducted with the AFLP® Analysis System I as recommended by the manufacturer (Invitrogen Corporation, Carlsbad, CA). *Eco*RI primers were labeled with [γ-^{33}P]dATP (3,000 Ci/mmol) (MP Biomedicals, Solon, OH). The final 20-µl PCR reaction was carried out in a Perkin–Elmer GeneAmp96 thermocycler under the protocol recommended by the manufacturer. *Taq* DNA polymerase was used in all AFLP amplification reactions. The amplification products were denatured by the addition of 12 µl of denaturation solution (98% formamide, 5 mM EDTA, 0.025% bromophenol, and 0.025% xylene cyanole FF), followed by heating at 95°C for 5 min, and immediate quenching on ice for 10 min; 3-µl aliquots were resolved by urea–polyacrylamide gel electrophoresis in 0.4-mm thick gels prepared with 6% Long Ranger® (BMA Bioproducts, Lowell, MA), 7 M urea, and 0.5× TBE. Samples were electrophoresed for 1.5 h at a constant power of 50 W. Gels were dried onto filter paper, and labeled DNA sequences were visualized by autoradiography on Kodak X-Omat film. A total of 64 primer-pair combinations, from AFLP® Analysis System I, were screened for informative polymorphisms. These primer pairs generated between 100 and 140 AFLP fragments that were represented by bands of varying intensities with lengths between 100 and 600 nucleotides. Approximately, a total of 7,680 AFLP fragments were detected in this survey.

Cloning of polymorphic amplicons

Selected AFLP fragments were cloned as described by Vallejos et al. (2000). Briefly, the target band was cut out of the dried gel after aligning it with the X-ray film, eluted in TE buffer after 10 min imbibition in 150 µl of TE buffer, and boiled for 15 min. After eliminating the debris by centrifugation, the eluted DNA was precipitated with three volumes of ethanol in the presence of 500 µg/ml molecular biology grade glycogen. The DNA was dissolved in 40 µl of water and a 5-µl aliquot was used for PCR amplification with the AFLP kit and Turbo *Pfu*I DNA polymerase (Stratagene, La Jolla, CA). If the amplification resulted in a low yield, then a second amplification was carried out using 1/10 of the volume of the first reaction as a template. The amplification product was cloned into *Eco*RV digested pBlueScript plasmid following standard procedures.

Because it is practically impossible to isolate a pure fragment from the gel, it was necessary to screen multiple white colonies via AFLP analysis to select the desired clone. Ten single white colonies were grown overnight in 2 ml of LB liquid medium. A 200 µl aliquot of each culture was pelleted and the cells were resuspended in 200 µl of sterile water, subjected to a freeze/thaw cycle, and the cellular debris was pelleted at 13,000 rpm for 15 min at 4°C. The supernatants were used as templates for PCR amplification with the appropriate ^{33}P-labeled-*Eco*RI and *Mse*I primer pair. The PCR reactions from the bacterial colonies and those from the plus and minus genomic DNA samples were resolved side by side in urea–PAGE and visualized by autoradiography to identify the correct clone.

Segregation and linkage analysis

The linkage between the resistance loci and the DNA markers was examined in each of the F_2 progenies with MAPMAKER/EXP 3.0 (Lander et al. 1987; Lincoln et al. 1992). Map distances were calculated using the Kosambi function.

Results

Genetic characterization of the recessive resistance

Previous analysis of an F_2 family, from the cross [ECW123 (P6-susceptible) × ECW12346 (P6-resistant)], detected four phenotypic classes after inoculation with race P6 of *X. euvesicatoria*. These classes were found in a 9:3:3:1 ratio corresponding to susceptibility (score = 4), low intermediate resistance (score = 3), high intermediate resistance (score = 2), and full resistance (score = 1), respectively (Jones et al. 2002). These results indicated the presence of two recessive resistances with additive gene action. Several steps were taken to further test both the mode of inheritance and gene action of these resistance genes. First, F_2-derived F_3 families from the intermediate resistance classes were screened by inoculation with race P6 to identify those that were fixed at both putative resistance loci. Among these, the F_3 families from F_2 plants #15 (score = 2) and #35 (score = 3) were selected for crosses and further analysis. F_1 individuals from the cross between members of these two families were fully susceptible to race P6. These results confirmed the recessive and additive nature of these two resistance genes.

Four different F_2 populations were generated to evaluate independently the segregation of the resistance genes, and also to use them for linkage analysis with selected molecular markers. The strongest of the two resistances was designated *bs5* (line 15-2 with a score = 2), and was analyzed in the following two populations: Pop1 (ECW123 × Line 15-2), and Pop4 (ECW × Line 15-2). F_2-derived F_3 families from Pop1 revealed 18 resistant, 51 heterozygous susceptible, and 19 homozygous susceptible [χ^2 (1:2:1) = 2.25, $P = 0.32$], while F_2 individuals from Pop4 yielded 44 susceptible and 16 resistant individuals [χ^2 (3:1) = 0.09, $P = 0.76$]. Thus, goodness-of-fit tests in these populations clearly indicated that *bs5* displayed monogenic segregation.

The second resistance, designated *bs6*, was analyzed with Pop2 [ECW12346 (resistant, score = 1) × Line 15-2 (intermediate, score = 2)], and Pop3 [ECW123 (susceptible, score = 4) × Line 35-3 (intermediate, score = 3)]. Pop2 progeny tests with F_2-derived F_3 families revealed 18 resistant, 54 heterozygous susceptible, and 20 homozygous susceptible [χ^2 (1:2:1) = 2.87, $P = 0.24$]. In addition, progeny tests of Pop3 revealed 22 resistant, 49 heterozygous susceptible, and 21 homozygous susceptible individuals [χ^2 (1:2:1) = 0.41, $P = 0.81$]. These segregation analyses clearly confirmed that each one of these resistances is controlled by a single locus.

Detection of polymorphic candidate markers

A search for polymorphic informative markers can be conducted globally using a segregating progeny to test the linkage relationships between the target locus and a set of segregating markers distributed throughout the genome. Alternatively, the search can focus on the target locus using either bulked segregant analysis (Michelmore et al. 1991), or near isogenic lines (NILs; Young et al. 1988). Because *bs5* and *bs6* were introgressed into the P6-susceptible ECW123 line via eight backcross cycles with continuous selection to generate the P6-resistant ECW12346 line, these lines were considered to be NILs and suitable for searching informative markers for these resistances. Also, since progeny derived from lines 15-2 and 35-3 can also be considered NILs and specific for each of the genes, these lines were included in the screening of AFLP markers and previously mapped RFLP markers from tomato.

Out of the 64 primer pairs, 35 detected potentially informative AFLP fragments: 24 detected 1 polymorphic fragment, 9 detected 2 polymorphic fragments, and 2 detected 3 polymorphic fragments. These 48 informative amplicons were selected for segregation and linkage analysis. On the other hand, five polymorphic RFLP marker loci were detected for line 15-2: TG14 (T2/P2*), TG194 (T11/P12), TG221(T6/P6*), TG259 (T1/P1*), and TG267 (T1/P1*); and seven for line 35-3: TG36 (T11/P11), TG123 (T4/P5), TG153 (T6/P5), TG221 (T6/P6*), TG267 (T1/P1*), TG269 (T1/P1), and TG 332 (T2/P2*). ECW12346 alleles for TG221 and TG267 were found in both lines. The alphanumerics in parenthesis indicate the chromosome association of the marker in the tomato (T) and pepper (P) genomes. The asterisks indicate that the chromosome assignment in pepper was deduced from the established syntenic relationships between the tomato and pepper genomes (Livingstone et al. 1999; Prince et al. 1993; Tanksley et al. 1992).

Linkage analysis

Linkage relationships between informative marker loci (AFLP and RFLP) and *bs5* and *bs6* were analyzed with Mapmaker 3.0 (Lander et al. 1987; Lincoln et al. 1992). Linkage between the strongest of the two resistances, *bs5*, and a group of AFLP marker loci was detected in Pop1 (87 individuals) using the Kosambi function. The linear order and map distances between the AFLP marker loci and *bs5* are depicted in Fig. 1. No linkage was detected between *bs5* and any of the segregating RFLP markers. Unfortunately, analysis of Pop2 and Pop3 did not detect linkage between *bs6* and any of the segregating AFLP fragments or the RFLP markers.

Development of PCR-based marker for *bs5*

AFLP fragments that were the most closely linked to *bs5* were eluted out of the gel, PCR amplified, cloned, sequenced, and the sequences were submitted to GenBank

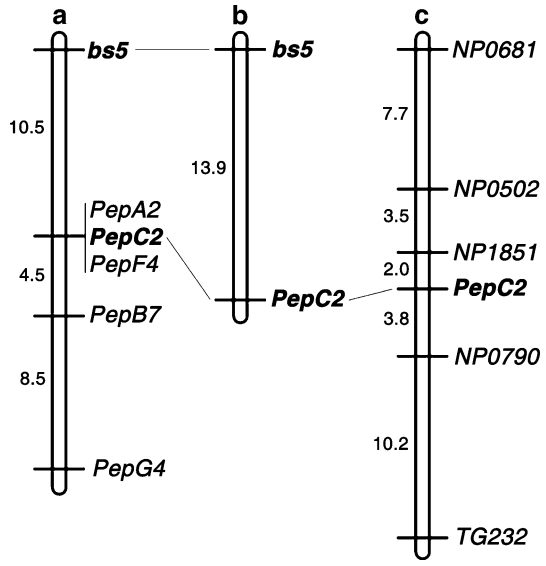

Fig. 1 Linkage maps. **a** Relative map position of *bs5* and the linked AFLP marker loci. **b** Relative position of the *PepC2* on chromosome 6 in the AC99 pepper map. **C** Relative position of *PepC2* on chromosome 6 of the COSII map

(Table 1). A similarity search of the non-redundant nucleotide collection from GenBank with the BLAST search engine (Altschul et al. 1997) detected significant similarities between the DNA sequences of *PepC2* and tomato BAC sequences from chromosomes 6 and 8 (*E* values = $4e^{-57}$–$4e^{-44}$). Furthermore, a search of the protein database revealed multiple hits with significant similarity to the *gag*-domain of retrotranposons.

Based on the sequence length of these markers, *PepC2* was targeted for the development of PCR-based allele-specific markers for *bs5*. In the first step, Primer 3 (Rozen and Skaletsky 2000) was used to design primers from the *PepC2* sequence to amplify allelic sequences from genomic DNA of ECW123 and line 15-2. The primers PepC2.F (5′-TTGAAAAGGTTATCAATCATGGAA-3′) and PepC2.R (5′-TCATCCCACAATCTTTTGAGC-3′) amplified a 357-bp fragment from the two genotypes. The allelic sequences were cloned and sequenced in both directions. Alignment of these sequences with ClustalX (Thompson et al. 1997) was used to identify possible base substitutions that could be exploited in the development of allele-specific primers. As expected, the sequence for the 15-2 allele had a perfect alignment with the sequence obtained from the AFLP fragments (FJ465512). In contrast, alignment of the 15-2 and the ECW123 (FJ589719) alleles revealed 7 base substitutions, two of which were contiguous and were included at the 3′ end of the following primers: PepC2-ECW.R1 (5′-CTTTGGCAATCCTGGAATATT-3′) and PepC2-15-2.R1 (5′-CTTTGGCAATCCTGGAATAGA-3′). Each of these primers in conjunction with C2.F amplified a 215 bp amplicon. Surprisingly, however, these primers showed quantitative differences in their ability to amplify each of the two alleles. When the PepC2-ECW.R primer was used, it yielded a robust amplification with the ECW123 template, and amplified the 15-2 template to a yield that was approximately 10–20% of that obtained with the ECW123 template. The opposite was observed with the PepC2-15-2.R primer. To overcome this problem and eliminate any ambiguities in the differential amplification of the alleles, new allele-specific primers were designed by introducing mismatches near the 3′ end. Certain mismatches near the 3′ end of a primer have been found to be more effective in reducing the yield of a PCR amplification reaction (Kwok et al. 1990). Accordingly, an A:A mismatch was introduced in the fourth base from the 3′ end of PepC2-ECW.R2 and a G:A mismatch at the same position in PepC2-15-2.R2 (Fig. 2). These mismatches imparted allele specificity to the primers because they did not significantly reduce the yield of the cognate sequences, and virtually eliminated the amplification of the alternative allele.

To test the reliability of these primer pairs, and evaluate their effectiveness as markers, they were used to amplify the ECW and 15-2 alleles of Pop1 ($n = 96$) and Pop4 ($n = 60$) segregants, and estimate the distance between the *PepC2* marker locus and *bs5*. The results from the differential amplifications are shown in Fig. 3. A distance of 13.9 cM (LOD = 12.47) for Pop1, and 13.8 cM (LOD = 6.42) for Pop4 were detected with the new primers (Fig. 1). These results were congruent with those observed previously with the AFLP markers. The difference was related to the fact that the AFLP marker behaved as dominant and the derived PCR markers were able to detect all three genotypes. At this distance, a total of four plants with the *PepC2*

Table 1 Characterization of *bs5*-linked AFLP markers

AFLP marker	*Eco*RI/*Mse*I extensions	Sequence length (bp)	GenBank ID	BLAST search results
PepA2	AAC/CAC	73	FJ589718	None
PepC2	AAG/CAC	358	FJ465512	Retrotransposon *gag*-domain[a]
PepF4	ACT/CAT	179	FJ589717	None

[a] BLASTn (Altschul et al. 1997) detected similarities to BAC sequences from chromosomes 6 and 8 of tomato ($E = 4e^{-57}$–$4e^{-47}$), and tBLASTx detected similarities to dbjlBAD99219.1, a polypeptide with a *gag*-domain from *Petunia* × *hybrida* ($E = 5e^{-34}$)

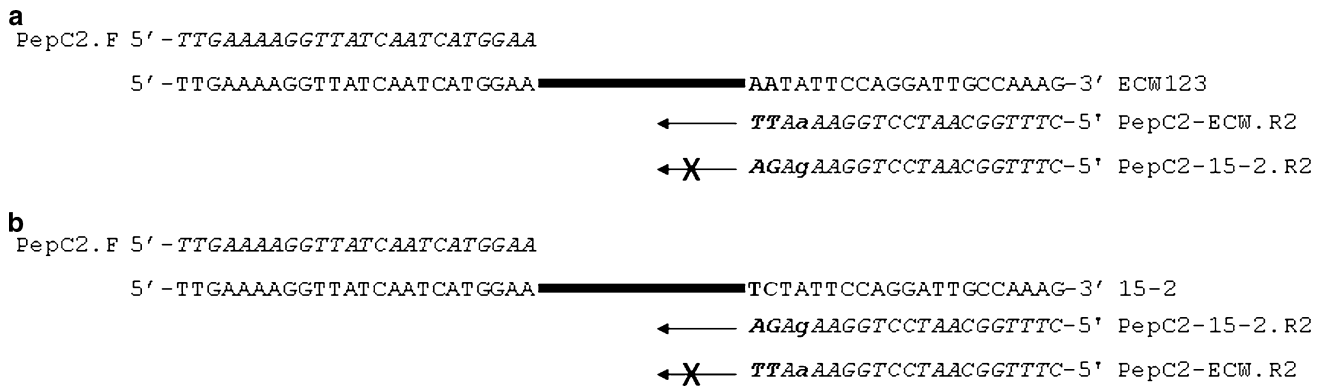

Fig. 2 Polymorphic sites in **a** ECW123 and **b** line 15-2 used to design the allele-specific primers are shown in *bold letters*, primer sequences in *italics* and mismatches in *lowercase bold letters*

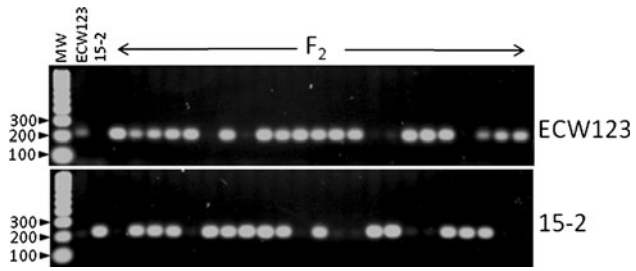

Fig. 3 Differential amplification of PCR marker for *bs5*. **a** Amplification of the ECW123 *PepC2* allele in *trans* with *bs5*. **b** Amplification of the 15-2 *Pep*C2 allele in *cis* with *bs5*. Individual F_2 segregants that amplify with a single primer pair are homozygotes and those that amplify with both primer pairs are considered heterozygotes

marker allele from line 15-2 are required to have a 99.9% probability of co-selecting *bs5* in a backcross population.

The map position of *PepC2* was obtained by analyzing the linkage relationships between this marker and those of previously mapped markers in a *C. annuum* × *C. chinense* F_2 population (AC99). The linkage map for this population can be found at the SOL Genomics Network website (SGN; http://sgn.cornell.edu; Mueller et al. 2005). The parental lines showed a plus/minus polymorphism after amplification with the primer pair PepC2.F/PepC2-15-2.R2. Only the *C. annuum* line yielded an amplicon that was of the same size as that produced by line 15-2. Linkage analysis with this progeny placed this marker on the centromeric region of chromosome 6 as shown in Fig. 1. This finding is in agreement with the observation that the BLAST search of GenBank with the PepC2 sequence detected a similarity with a segment of a tomato BAC sequence assigned to chromosome 6 of tomato, a chromosome that is syntenic with chromosome 6 of pepper (Livingstone et al. 1999; Wu et al. 2009).

An attempt was made to determine the map position of *bs5* in reference to COSII marker loci on chromosome 6 (Wu et al. 2009). The following markers covering a span of 21 cM in the centromeric region of chromosome 6 were used: C2At2g30100 (41.2), C2At2g29630 (47.6), Pd3g56130 (48.5), C2At3g56040 (49.5), C2At1g06110 (55.9), and C2At1g44760 (62.2). Primer pairs listed by Wu et al. (2009) for these markers were used to amplify ECW123 and line 15-2 alleles from genomic DNA samples. No size polymorphisms were detected between the allelic amplicons. Allelic amplicons for C2At1g44760 were cloned and sequenced, but no polymorphisms were detected either. Next, we used the *Arabidopsis* sequences of the first three COSII markers to search the EST database of GenBank to identify *C. annuum* sequences with a high degree of similarity. The goal of this effort was to obtain pepper sequences that lead to the design of primers that would amplify longer sequences than those obtained with the primers listed by Wu et al. (2009). The following matches were detected: gb|CA517016.1 with At2g30100, gb|GD054092.1 with At2g29630, and gb|GD125562 with Pd3g56130. The pepper EST sequences were aligned with the genomic *Arabidopsis* sequences to deduce the potential location of introns in genomic DNA. Primer sequences from intron-flanking regions were selected and genomic DNA from ECW123 and line 15-2 were amplified. The amplicons were of approximately 2,550, 1,100, and 1,400 bp, respectively. Allelic amplicons were completely sequenced and no polymorphisms were detected. The search for polymorphisms was discontinued at this point.

Functional analysis of the two recessive resistances

To determine the *in planta* effect of each resistance gene on bacterial growth and the extent to which visual scores correlate with bacterial population levels, each resistance gene was transferred to the 'Early Calwonder' susceptible background—ECW50R (*bs5*) and ECW60R (*bs6*). The growth of bacterial populations of race P6 was monitored for 8 days after inoculation and incubation at 25 and 30°C. ECW123 and ECW12346 were used as negative and positive controls, respectively. Bacterial growth curves *in planta* (Fig. 4) showed that bacterial populations increased

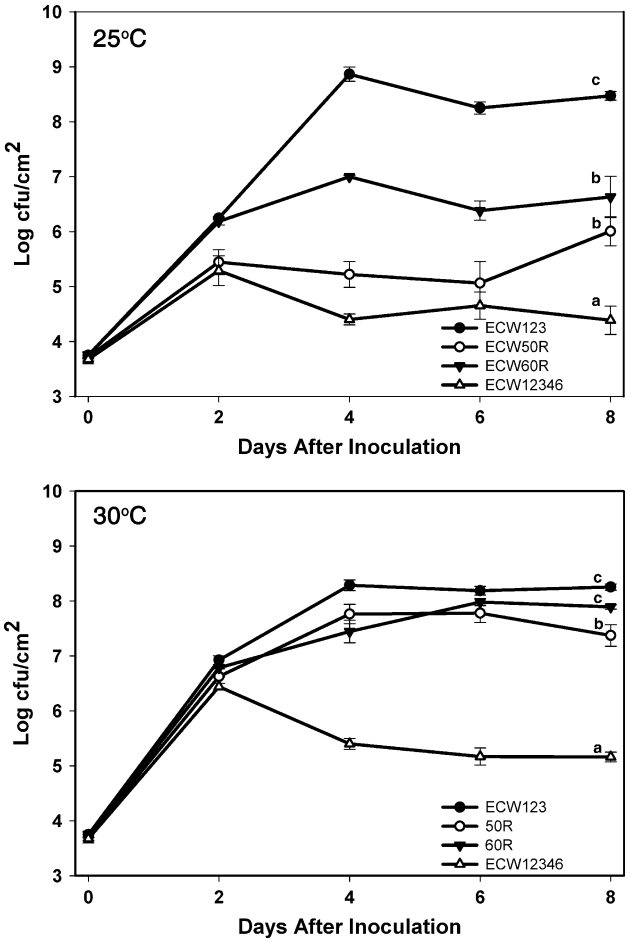

Fig. 4 Bacterial population growth in inoculated leaves of different genotypes at 25 and 30°C. ECW123: a parental line fully susceptible to race P6. ECW12346: a breeding line carrying *bs5* and *bs6*. ECW50R: a breeding line carrying *bs5*. ECW60R: breeding line carrying *bs6*. Population means with the *same letter* are not significantly different from each other (Holm–Sidak method)

approximately 4–5 log units in 4 days before leveling off in the susceptible genotype ECW123, and that temperature had no significant effect on final population levels. In contrast, ECW12346, which carries both resistance genes, restricted bacterial growth at both temperatures. At 25°C, the population only rose by <1 log unit and at 30°C, the increase was <2 log units. Although the susceptible ECW123 showed advanced necrosis in the inoculated area, ECW12346 had hardly any noticeable symptoms (Fig. 5).

bs5 and *bs6* had different temperature responses. At 25°C, the bacterial population grew by approximately 2.3 log units in ECW50R (*bs5*) and by 2.9 log units in ECW60R (*bs*6). These results indicate that the visual scores obtained at 25°C correlated well with the extent of bacterial growth in the inoculated area. ECW50R barely shows some discoloration in the inoculated area 8 days after inoculation; in contrast, ECW60R shows more noticeable yellowing (Fig. 5). At 30°C, both resistance genes had limited capacity to restrict the bacterial growth. Although individually these resistances perform poorly at high temperatures, results obtained at 30°C with ECW12346, the positive control, clearly indicate that these resistances have a synergistic effect that is very noticeable at high temperatures. An analysis of variance and pairwise multiple comparisons (Holm–Sidak method; Supplemental Table 2) of population levels detected no significant differences between ECW123 and ECW60R on day 8 at 30°C, but significant differences between these two and ECW50R. ECW12346 had a significantly lower population level than any of the other three genotypes (Fig. 4). A recently produced line carrying both bs5 and bs6 in the absence of the dominant resistances has shown the same post-inoculation phenotype as ECW12356 at elevated temperatures. The effect of temperature on each genotype can be examined in supplementary Fig. 1S.

Discussion

The results presented here confirm a previous report (Jones et al. 2002) that the ECW12346 resistance to race P6 of *X. euvesicatoria* is controlled by two recessive genes. The original two recessive genes hypothesis was initially supported by the 9 (susceptible):3 (resistant intermediate-high):3 (resistant intermediate-low):1 (resistant) ratio, and the fact that lines 15-2 and 35.3 with intermediate resistance produced completely susceptible progeny. In this work, we have analyzed the segregation ratios of various F_2 progenies obtained between these two lines and the susceptible genotypes ECW and ECW123. In all cases, Mendelian phenotypic ratios of 3 susceptible:1 resistant were detected.

Seasonal variation in the resistance phenotypes of different lines and progenies prompted an investigation about the temperature stability of these resistance genes. This was accomplished through a comparative analysis of bacterial population growth in inoculated leaves of the different genotypes. These studies demonstrated first that the symptoms observed in line 15-2 carrying *bs5* and line 35-3 carrying *bs6* correlate with the bacterial populations observed at 25°C—*bs5* being the strongest of the resistances. However, both resistance genes allowed similar bacterial population growth at 30°C, but in either case they were marginally lower than that of the susceptible control. Of the two genes, *bs5* was the most dramatically affected by high temperature. The most interesting aspect of these results was, however, the epistatic interaction between *bs5* and *bs6* which can be observed at two levels: in symptom expression and temperature response. Bacterial growth was very similar in both ECW12346 (*bs5, bs6*) and ECW50R (*bs5*), yet the symptoms in the former were not noticeable at all, whereas the latter consistently displayed mild symptoms. Of greater

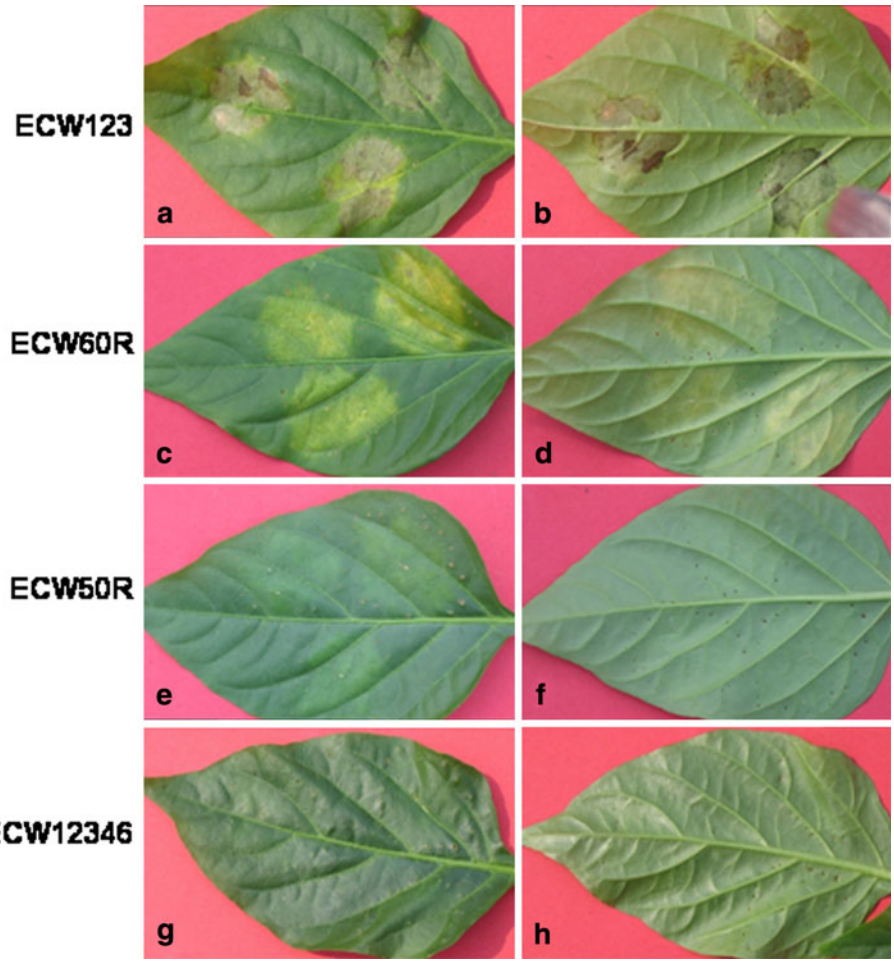

Fig. 5 Symptoms of bacterial spot 8 days after inoculation with race P6 in ECW123 (*Bs5Bs5Bs6Bs6*), ECW60R (*Bs5Bs5bs6bs6*), ECW50R (*bs5bs5Bs6Bs6*), and ECW12346 (*bs5bs5bs6bs6*). Adaxial (**a**, **c**, **e**, **g**) and abaxial (**b**, **d**, **g**, **h**) views

interest, and practical application, however, is the high temperature response of ECW12346. The presence of both genes kept the bacterial populations three log units below those observed in the susceptible control, while in the lines carrying one of those genes the population levels were kept only close to one log unit below the control. The observation that each gene alone has an effect on resistance supports the idea of additive action, but their combined effect on symptom development and performance at high temperature indicates that the epistatic effect on resistance is greater than the additive effect. Further evidence of epistatic interaction between these genes comes from the observation that only in combination these genes confer resistance to some new Brazilian strains of *X. gardneri*, as evidenced by a visible reduction in disease symptoms (Jones and Stall, unpublished results).

Previous experience with the relatively short-lived effectiveness of the dominant resistances (*Bs1*, *Bs2* and *Bs3*) due to mutation or plasmid loss (Dahlbeck and Stall 1979; Minsavage et al. 1990; Swords et al. 1996) raises the question about the potential durability of *bs5* and *bs6*. However, several lines of evidence suggest the bs5 and bs6 will have greater durability than the one displayed by the dominant resistances. First, there are examples of recessive resistances with durability; these include several virus resistances (Diaz-Pendon et al. 2004), *mlo*-controlled resistance to powdery mildew in barley (Jørgensen 1992), and resistance to leaf bacterial blight in rice (Iyer-Pascuzzi and McCouch 2007). Resistance controlled by *bs5* and *bs6* is not associated with a hypersensitive reaction, and for this reason, no specific effector or avirulence factor appears to be involved in interactions with a host gene product to produce a resistance phenotype. In fact, examination of recently isolated recessive resistance genes suggests that it is the lack of interaction that confers resistance. At least three of these resistances are encoded by genes with well established normal cell functions and are not involved in defense. For instance, resistance to potyviruses in lettuce and pepper is mediated by eIF4E, a translation initiation factor (Kang et al. 2005; Nicaise et al. 2003; Ruffel et al. 2002), and in rice two recessive genes, *xa5* and *xa13*, control resistance to different strains of *X. oryzae* pv. *oryzae* and encode TFIIAγ, a transcription factor II A gamma subunit (Iyer and McCouch 2004), and a nodulin-like protein essential for pollen development (Chu et al. 2006), respectively. The model that is emerging from these recessive

resistances is one that invokes a passive role. Pathogens like *X. euvesicatoria* deliver a set of effector proteins into the host cytoplasm to subvert defenses and alter host metabolism to facilitate colonization of the extracellular space (Grant et al. 2006). Because the recessive resistance genes are expressed and the corresponding proteins carry out their normal functions, then it appears that the variation that leads to resistance involves lack of recognition by the pathogen-encoded cognate protein as appears to be the case for *xa5* (Iyer and McCouch 2004), or that the level of expression is not high enough to support the demand of the pathogen as observed in *xa13* (Chu et al. 2006). The pepper genes *bs5* or *bs6* may represent these types of variants.

Furthermore, a successful strategy used by bacterial pathogens is the functional redundancy of some sequence unrelated effectors (Jones and Dangl 2006). Then, one possible explanation for the epistatic interactions between *bs5* and *bs6* is a case in which the encoded gene products interfere with a single aspect of the bacterial mechanisms which is essential for growth, especially at elevated temperatures. In general, deployment of the two resistance genes can lower the probability for the pathogen to overcome the resistance provided by these two genes and lengthen their effectiveness. In fact, attempts to generate race P6 mutants that can overcome *bs5*- or *bs6*-controlled resistance have not been successful so far (Minsavage et al. unpublished results).

It was disappointing not being able to detect an informative marker for *bs6*, especially after examining 7,500 AFLP fragments and 135 RFLP markers, or additional markers from the COSII pepper map. However, these results are what should be expected based on previous assessments of polymorphisms in *Capsicum*. For instance, Prince et al. (1993) estimated that most of the RFLPs detected between *C. annuum* and *C. chinense* were probably due to DNA rearrangements rather than base substitutions. More recently, Min et al. (2008) have reported that only 19% of 850 SSR markers detected polymorphisms between these two species, but <4.5% within *C. annuum*. In this work, comparisons of partial genomic sequences from four COSII markers which included a substantial proportion of intronic sequences and covered over 5.5 kb revealed no polymorphisms. The fact that the *PepC2* marker was derived from a low copy retroelement suggests that future informative markers from this region of the pepper genome are likely to emerge from low copy intergenic sequences. Nevertheless, the PCR marker developed in this project for *bs5*, the stronger of the two resistances, can expedite the transfer of this recessive gene to new pepper cultivars using a backcross program. Given the epistatic interactions between bs5 and bs6, particularly at high temperature, deploying this gene combination appears to be the best strategy against bacterial spot in tropical and subtropical environments.

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