

Identification, by selective genotyping, of quantitative trait loci conferring resistance to *Cochliobolus sativus* in barley line TR 251

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ABSTRACT

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Spot blotch, caused by *Cochliobolus sativus*, is one of the most damaging foliar diseases of barley around the world. Although chemical control of plant diseases is an effective tool to reduce pathogen damage, the most efficient and environmentally sound means of spot blotch control is through the deployment of resistant cultivars. The aim of this study was to elucidate the genetic basis of spot blotch resistance in barley line TR 251, a Canadian breeding line with a high level of spot blotch resistance. Infection responses induced by *C. sativus* isolate WRS1938 in 226 doubled haploid lines derived from the cross TR 251 × CDC Bold were analyzed, and DNA samples from the parents and bulks of 11 resistance and 11 susceptible lines were tested using bulked segregant analysis (BSA) with 376 SSR markers and 256 combinations of amplified fragment length polymorphism (AFLP) markers. Identification of the quantitative trait loci (QTL)-linked markers was done using a selective genotyping approach by both binomial distribution and hypergeometric distribution tests. Four putative loci on chromosomes 1H, 3H, 5H, and 7H were found to be associated with spot blotch resistance in line TR 251; of these, the two loci located on chromosomes 1H and 5H had not been reported in previous studies. Both of these loci are likely unique and presumably contribute to the superior *C. sativus* resistance of line TR 251.

Keywords: doubled haploid line, foliar disease, infection response, QTL analysis

INTRODUCTION

Spot blotch caused by *Cochliobolus sativus* (Ito and Kuribayashi) Drechs. ex Dastur. [(anamorph *Bipolaris sorokiniana* (Sacc.) Shoemaker, syn. *Helminthosporium sativum* Pamm. King and Bakke.)] is one of the most important foliar diseases of barley worldwide. The pathogen can attack any organ of a barley plant and causes destructive diseases of barley such as common root rot and seedling blight (Kumar *et al.*, 2002). Infection of heads can result in dark seed discoloration termed black point or smudge (Bailey *et al.*, 2003).

Yield losses due to spot blotch depend greatly on climatic conditions. Under epidemic conditions, yield reductions of up to 30% have been reported for susceptible barley cultivars in Canada (Clark, 1979; Dostaler *et al.*, 1987). Although fungicides can be used to reduce spot blotch damage, a more economical and environmentally desirable means of control is through the deployment of resistant cultivars. Six-rowed malting barley cultivars developed and grown in the state of North Dakota (ND), USA, have remained resistant to all

pathotypes of *C. sativus* in ND for more than 40 years. This durable resistance is mostly derived from line ND B112, which has improved levels of spot blotch resistance (Valjavec-Gratian and Steffenson, 1997a). In contrast, two-rowed barley genotypes developed in ND generally possess a lower level of resistance to *C. sativus* than six-rowed types, as indicated in both greenhouse and field trials (Fetch and Steffenson, 1994).

Studies on the inheritance of spot blotch resistance in barley have indicated that both monogenic and oligogenic as well as polygenic resistance have been reported in barley genotypes. Valjavec-Gratian and Steffenson (1997b) reported that a single recessive gene controlled the resistance to isolate ND90Pr (ND pathotype '2') in line ND 5883, showing gene-for-gene interaction. Monogenic inheritance of spot blotch resistance to isolate ND90Pr was recently confirmed when Bilgic *et al.* (2006) identified a single gene (designated *Rcs6*) on chromosome 1H of line Calicuchimasib/Bowman-BC. Gonzalez Cenicerros (1990) identified two resistance genes to isolate ND85F

(ND pathotype '1') in cv. Bowman. Monogenic and oligogenic (mostly 2 genes) inheritance of genes for resistance to *C. sativus* isolates has also been frequently reported in wheat (Ragiba *et al.*, 2004a,b; Mikhailova *et al.*, 2004; Bhushan *et al.*, 2002; Adlakha *et al.*, 1984). However, recent studies have indicated that resistance to pathogenic isolates of *C. sativus* with higher virulence (i.e., isolate ND85F, representative of ND pathotype '1') is based on more complex inheritance in barley cultivars showing durable resistance. Several genes/QTLs have been identified in cv. Morex that contribute resistance to isolate ND85F (Bilgic *et al.*, 2005; Steffenson *et al.*, 1996; Steffenson and Smith, 2004). This suggests that the genetics of spot blotch resistance in this line is polygenic.

Quantitative trait loci are defined as regions of the genome affecting polygenic traits that are measurable and show continuous variation (Geldermann, 1975). Several statistical techniques such as t-test, ANOVA, linear regression, and maximum likelihood were used previously to estimate the position and impact of QTLs on the genome of different organisms (Liu, 1998). However, these techniques were gradually replaced by more sophisticated biometrical methods such as interval mapping (Lander and Botstein, 1989), composite interval mapping (Zeng, 1994) and multiple interval mapping (Kao and Zeng, 1997; Kao *et al.*, 1999). An alternative QTL analysis based on 'selective genotyping' can be employed when QTL mapping for a particular trait within a large population is desired (Lebowitz *et al.*, 1987; Lander and Botstein, 1989).

In this approach, after thoroughly phenotyping the entire population for the trait of interest, QTLs that affect the trait are identified by subjecting only selected individuals displaying extreme phenotypic values to genetic analysis. Theoretically, the allele frequency of genes/QTLs affecting a specific trait changes under directional selection for that trait (Falconer, 1989; Foolad *et al.*, 2001; Lander and Botstein, 1989). The 'hitchhiker' effects between such gene/QTL alleles and nearby marker alleles are expected to produce corresponding changes in the allele frequencies of linked markers (Lebowitz *et al.*, 1987). This allows limiting the number of the individuals used to detect QTLs having even a small effect on the trait, but is less useful for estimating the effects of these QTLs (Lynch and Walsh, 1998). Selective genotyping has been used in many studies to detect QTLs related to important quantitative traits in plant breeding research (Hu *et al.*, 1995; Foolad *et al.*, 1997, 2001; Miklas *et al.*, 1996; Ni *et al.*, 1998).

In several recent studies, the two-rowed malting line TR 251 (developed at the AAFC Brandon Research Centre, MB, Canada) showed a high level of spot blotch resistance against all pathotypes of *C. sativus* collected from Canada and other countries (Ghazvini and Tekauz, 2007, 2008). The objective of this study was to elucidate the genetics of the resistance present in line TR 251, and compare it to that found in other cultivars. This would facilitate the use of TR 251 as a source of spot blotch resistance in barley breeding programs across the world.

MATERIALS AND METHODS

Plant materials

A population of 226 doubled haploid (DH) lines generated from the cross between line TR 251 as a resistant parent (maternal) and cv. CDC Bold as a susceptible parent (paternal) was used in this study. A microspore culture protocol was used to develop the DH population. Microspore isolation and culture techniques were essentially based on the protocol of Kasha *et al.*, (2001).

Fungal isolates and resistance assessment

Fungal isolate WRS1938, representative of the common virulence found in Manitoba, Canada (Ghazvini and Tekauz, 2007), was used for spot blotch screening at the seedling stage. Parents and DH progenies were inoculated twice at the seedling stage. Mean infection responses (IRs) of the parents and DH lines were calculated within and between replications. Host plant preparation for inoculation, as well as inoculum preparation and application, were performed as described by Ghazvini and Tekauz (2007). The second leaves of seedlings were scored twice for their infection responses (IRs) 8 and 10 days after inoculation using the illustrated numerical scales of 1-9 for spot blotch assessment (Fetch and Steffenson, 1999). To evaluate whether resistance to spot blotch infection in the population behaves as a discrete (qualitative) or a continuous (quantitative) trait, a frequency histogram of the IRs of the DH lines was constructed.

DNA extraction and quantification

Genomic DNA was extracted from 5-6 fresh leaves of 2-week-old seedlings grown in pathogen-free greenhouse compartments. The detached leaf samples were immediately frozen in liquid nitrogen and stored at -80 °C before lyophilization. The DNA extraction procedure followed that described by Pallotta *et al.* (2003). Concentrations of extracted DNA were determined by fluorimetry using Hoechst 33258 stain.

Bulked segregant analysis (BSA)

Two bulks of DNA samples were prepared from either 11 resistant or 11 susceptible DH lines by pooling equal amounts of DNA from each of the selected lines and diluted them to a final concentration of 25 ng/μl for amplified fragment length polymorphism (AFLP) and 10 ng/μl for simple sequence repeat (SSR) analyses. A total of 376 SSR markers, which had been developed in earlier studies (Ramsay *et al.*, 2000; Becker and Heun, 1995; Liu *et al.*, 1996), were used to compare the parents and the two bulked DNA samples. The PCR conditions were performed in a 10-μl volume containing 1.5 mM MgCl₂, 50 mM KCl, 0.8 mM dNTPs, 0.2 pmol forward primer, 2 pmol reverse primer, 1.8 pmol M13 primer fluorescently labelled with 6-FAM, VIC, NED, or PET (Applied Biosystems), 0.5 U Taq DNA polymerase (Promega) and 25 ng of template DNA. Thermal cycling was according to optimized PCR conditions for each SSR primer pair described by Ramsay *et al.* (2000).

AFLP analysis essentially followed that of Vos *et al.* (1995). Briefly, 250 ng of genomic DNA was digested with *Pst*I and *Mse*I restriction endonucleases. The *Pst*I and *Mse*I adaptor pairs were then ligated to cohesive ends of restriction fragments. Pre-amplification was performed using primers with one selective nucleotide at the 3' end (*Pst*I-A and *Mse*I-C), followed by selective amplification by *Pst*I and *Mse*I selective primers, each with three selective bases at the 3' end. Pre-amplification PCR was performed in a thermal cycler programmed for 20 cycles at a profile of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min. The selective amplification PCR program consisted of 1 cycle at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min, then 12 cycles to reduce the annealing temperature by 0.7 °C each cycle, followed by 23 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. A total of 256 possible combinations of the selective primers was used to screen the parents and two bulked DNA samples. *Mse*I primers used in selective amplification were fluorescently labelled with 6-FAM, HEX and NED (Applied Biosystems). For both SSR and AFLP analyses, M13 tailing (Schuelke, 2000) and fluorescent capillary electrophoresis on an ABI3100 fragment analyzer (Applied Biosystems) were used to collect genotypic data.

Genescan-500 LIZ labelled size standard (Applied Biosystems) was used to estimate the size of the amplified fragments. Chromatograms were processed by GeneScan software (version 3.7; Applied Biosystems), and data were converted into a gel-like image using Genographer software

(customized version 1.6, CRC, AAFC). The nomenclature of the AFLP markers was based on the primer pairs as designated by KeyGene® (listed at <http://wheat.pw.usda.gov/ggpages/keygeneAFLPs.htm>) and their relative molecular weights.

Linkage analysis

Segregation data were utilized to anchor AFLP markers to specific chromosomes, based on their linkage to SSR markers with known chromosome positions. A partial genetic linkage map consisting of 26 markers (19 SSRs and 7 AFLPs) which showed association with spot blotch resistance was constructed using genotypic data obtained from 96 randomly selected DH lines. Linkage relationships among marker loci were estimated using the maximum likelihood approach implemented in the JOINMAP version 3.0 software package (Biometris, Wageningen, The Netherlands, <http://www.joinmap.nl>). Map distances in centiMorgans (cM) were calculated using the Kosambi mapping function (Kosambi, 1944).

Identifying QTL-linked markers using the binomial distribution test

All 226 DH lines used for phenotyping were ranked according to their mean IRs to *C. sativus* isolates at the seedling stage. Progenies with the highest and lowest mean IRs, located within 5, 10 and 15% of the population with extreme phenotypes (10, 20, and 30%, respectively, of all progenies), were used for selective genotyping. The genotypes of selected lines were determined for the SSR and AFLP markers identified through BSA. Genotype numbers of marker loci among selected lines were used to estimate marker allele frequency among resistant and susceptible groups. The variance of allele frequency for each marker locus was calculated as a binomial variance (Foolad *et al.*, 2001):

$$S^2_q = pq / 2N$$

where p and q are the allele frequencies at a given marker locus in the selected sample and N is the number of progenies genotyped at that locus (Falconer, 1989). For each marker locus, allele frequency differences between resistant and susceptible DH lines in each tail ($q_r - q_s$) were calculated, where q_r and q_s are the frequencies of the i th allele at the k th marker locus among the selected resistant and selected susceptible progenies, respectively (Foolad *et al.*, 1997, 2001). Allelic frequency differences were considered significant if

$$q_r - q_s \geq z_{(\alpha/2)} \sigma_q$$

where $z_{(\alpha/2)}$ is the value of z at a 0.001 significance level and σ_q is the standard error of the

difference between marker allele frequencies. The standard error of the difference between marker allele frequencies was calculated by the following equation:

$$\sigma_q = (p_r q_r / 2N_r + p_s q_s / 2N_s)^{1/2}$$

where N_r and N_s are the number of selected resistant and susceptible progenies, respectively, in each tail (Zhang *et al.*, 2003). At each marker locus, significant allele frequency differences between the spot-blotch-resistant and spot-blotch-susceptible classes were considered as associations of the marker locus with QTL(s) controlling spot blotch resistance (Foolad and Jones, 1993; Foolad *et al.*, 1997; Lander and Botstein, 1989).

Identifying QTL-linked markers by hypergeometric distribution

For each marker and each proportion of the population (10, 20, and 30%), the number of lines of each parental genotype within each selected tail was counted and a statistical test based on hypergeometric distribution was conducted (Steel *et al.*, 1997). This test assesses the probability that n individuals sampled without replacement from a population of N individuals will consist of no more than n_1 individuals of a given type (either resistant individuals in one tail or susceptible individuals in the other tail). Here, N represents the total population size (226 DH lines), n represents the total number of lines in the selected upper and lower tails of the distribution (e.g., 22 for genotyping of 10% of the population), and n_1 indicates the sum of the number of progenies with susceptible genotype (e.g., 'CDC Bold') in the lower extreme of the distribution, representative of resistant phenotype, and the number of lines with the resistant genotype (e.g., TR 251) in the upper extreme of the distribution, representative of susceptible phenotype. Data were analyzed with the "probypr" function of SAS (SAS Inst., 2001) to test the hypothesis that a sample of n lines drawn at random from a population of N lines without replacement would include no more than n_1 lines of a certain genotype (Ayoub and Mather, 2002).

RESULTS

Phenotypic data analysis (resistance assessment)

The difference in IRs of line TR 251 (resistant parent) and cv. 'CDC Bold' (susceptible parent), inoculated with isolate WRS1983, was relatively small (IRs of 3.5 and 6.0 for TR 251 and 'CDC Bold', respectively). Infection responses of the DH lines induced by isolate WRS1983 in two replications showed a significant correlation for IRs of the individuals ($r=0.85$) in two different tests. The

frequency distributions of the IRs of the DH lines demonstrated a continuous distribution, consistent with a complex trait (Fig. 1). The lack of discrete classes of resistance and susceptibility among the DH population indicated polygenic inheritance of spot blotch resistance in line TR 251 is controlled by more than one or two genes; therefore, appropriate quantitative approaches were used to identify the locations of the QTLs.

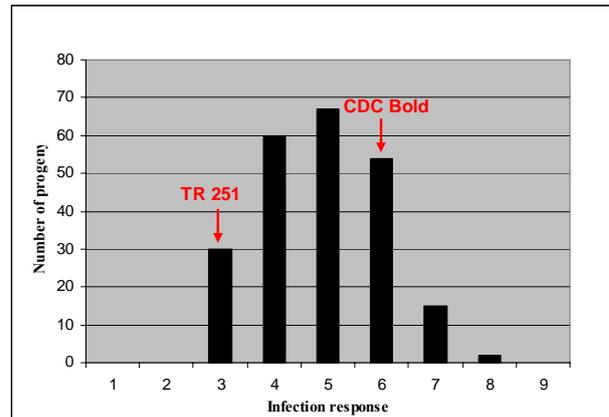


Fig. 1. Frequency distribution of the mean infection responses of the 226 DH lines of the cross TR 251 × CDC Bold and the parents inoculated with isolate WRS1938 of *Cochliobolus sativus*.

Genotypic data analysis

Marker (SSR and AFLP) and bulked segregant analyses

Of the 376 SSR markers tested, 279 were monomorphic in the parents. Fourteen SSR primers were not able to amplify any fragments using the optimized PCR profile for these primers (Ramsay *et al.*, 2000). Bulk segregant analysis indicated that of the 83 SSR primers that generated polymorphic fragments in parents, 64 were not related to spot blotch resistance loci in line TR 251. This speculation was based on the strong amplification of both parental alleles among the resistant and susceptible bulks (Fig. 2). However, the banding pattern of pooled DNA in resistant and susceptible bulks was in concordance with those of the corresponding parents when the parents and bulks were screened with the other 19 SSR markers (Fig. 2). Of these markers, 11, 5, 2 and 1 were located on chromosomes 3H, 7H, 1H and 5H, respectively.

All 11 SSR markers on chromosome 3H in both resistant and susceptible bulks demonstrated high association with the corresponding markers in the parents. However, based on the small distances among some of these markers, only eight SSR markers that were distributed in different parts of chromosome 3H were chosen for selective genotyping analysis (Table 1). Of several

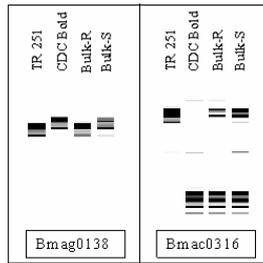


Fig. 2. Bulked segregant analysis of resistant bulk (Bulk-R), susceptible bulk (Bulk-S) and the corresponding resistant (TR 251) and susceptible (CDC Bold) parents for a QTL-linked marker on chromosome 3H (Bmag0138) and a QTL-unlinked marker on chromosome 6H (Bmac0316).

polymorphic SSR markers on chromosome 7H, only three SSR markers on chromosome 7HS (EBmag0794, Bmag0007, and AF022725A) and two on 7HL (EBmac0755 and Bmac0156) showed to be associated with spot blotch resistance in this chromosome. However, three other SSR markers on chromosome 7H that show no clear association with spot blotch resistance were added to these markers for further linkage analysis and as the control

markers for selective genotyping (Fig. 3 and Table 1). In total, 19 SSR markers, including 8, 8, 2 and 1 on chromosomes 3H, 7H, 1H and 5H, respectively, were chosen for selective genotyping (Fig. 3).

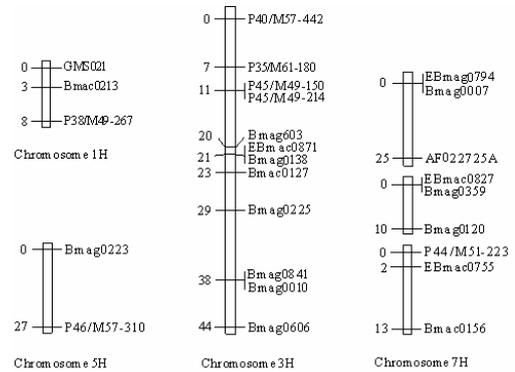


Fig. 3. Partial linkage map of chromosomes 1H, 3H, 5H and 7H constructed based on the segregation data of 96 randomly selected progenies of the TR 251 x CDC Bold DH population. Map distances were calculated using the Kosambi mapping function.

Table 1. Monogenic segregation of SSR and AFLP markers that exhibited association with spot blotch resistance loci at the seedling stage in a DH population of a cross between barley line TR 251 (disease resistant) and CDC Bold (disease susceptible). Marker-QTL association was tested by a binomial distribution-based selective genotyping analysis, using 5, 10, and 15% of the selected individuals with extreme phenotypes in each tail of the population.

Genetic marker	5% selection intensity				10% selection intensity				15% selection intensity			
	q_r^a	q_s^b	σ_p^c	$q_r - q_s^d$	q_r	q_s	σ_p	$q_r - q_s$	q_r	q_s	σ_p	$q_r - q_s$
Chromosome 3H												
P40/M57-442	1.00	0.18	0.08	0.82 ***	1.00	0.13	0.05	0.87 ***	0.94	0.15	0.05	0.79 ***
P35/M61-180	0.91	0.27	0.11	0.64 ***	0.96	0.30	0.07	0.65 ***	0.82	0.29	0.07	0.53 ***
P45/M49-214	0.91	0.27	0.11	0.64 ***	0.87	0.26	0.08	0.61 ***	0.79	0.26	0.07	0.53 ***
P45/M49-150	0.91	0.27	0.11	0.64 ***	0.87	0.26	0.08	0.61 ***	0.79	0.26	0.07	0.53 ***
Bmag0603	1.00	0.27	0.09	0.73 ***	1.00	0.35	0.07	0.65 ***	0.91	0.38	0.07	0.53 ***
EBmac0871	1.00	0.27	0.09	0.73 ***	1.00	0.35	0.07	0.65 ***	0.91	0.38	0.07	0.53 ***
Bmag0138	1.00	0.27	0.09	0.73 ***	1.00	0.35	0.07	0.65 ***	0.91	0.38	0.07	0.53 ***
Bmac0127	1.00	0.27	0.09	0.73 ***	1.00	0.30	0.07	0.70 ***	0.91	0.35	0.07	0.56 ***
Bmag0225	0.91	0.36	0.12	0.55 ***	0.91	0.35	0.08	0.57 ***	0.88	0.38	0.07	0.50 ***
Bmag0841	0.91	0.36	0.12	0.55 ***	0.87	0.48	0.09	0.39 ***	0.88	0.47	0.07	0.41 ***
Bmag0010	0.91	0.36	0.12	0.55 ***	0.87	0.48	0.09	0.39 ***	0.88	0.47	0.07	0.41 ***
Bmag0606	1.00	0.45	0.11	0.55 ***	0.96	0.57	0.08	0.39 ***	0.91	0.53	0.07	0.38 ***
Chromosome 7H												
EBmag0794	0.91	0.00	0.06	0.91 ***	0.65	0.17	0.09	0.48 ***	0.62	0.21	0.08	0.41 ***
Bmag0007	0.91	0.00	0.06	0.91 ***	0.65	0.22	0.09	0.43 ***	0.62	0.24	0.08	0.38 ***
AF022725A	0.64	0.00	0.10	0.64 ***	0.35	0.09	0.08	0.26	0.38	0.15	0.07	0.24
EBmac0827	0.18	0.18	0.12	0.00	0.30	0.17	0.09	0.13	0.35	0.21	0.08	0.15
Bmag0359	0.18	0.18	0.12	0.00	0.30	0.17	0.09	0.13	0.35	0.21	0.08	0.15
Bmag0120	0.18	0.36	0.13	-0.18	0.30	0.39	0.10	-0.09	0.35	0.35	0.08	0.00
EBmac0755	0.73	0.36	0.14	0.36	0.70	0.35	0.10	0.35 ***	0.62	0.35	0.08	0.26
P44/M51-223	0.73	0.27	0.13	0.45 ***	0.70	0.30	0.10	0.39 ***	0.62	0.29	0.08	0.32 ***
Bmac0156	0.73	0.36	0.14	0.36	0.70	0.48	0.10	0.22	0.62	0.47	0.08	0.15
Chromosome 1H												
GMS021	1.00	0.55	0.11	0.45 ***	0.96	0.52	0.08	0.43 ***	0.91	0.53	0.07	0.38 ***
Bmac0213	1.00	0.45	0.11	0.55 ***	0.96	0.48	0.08	0.48 ***	0.91	0.50	0.07	0.41 ***
P38/M49-267	0.91	0.36	0.12	0.55 ***	0.91	0.43	0.08	0.48 ***	0.88	0.44	0.07	0.44 ***
Chromosome 5H												
Bmag0223	0.73	0.00	0.09	0.73 ***	0.74	0.17	0.09	0.57 ***	0.65	0.21	0.08	0.44 ***
P46/M57-310	0.82	0.00	0.08	0.82 ***	0.65	0.17	0.09	0.48 ***	0.59	0.18	0.08	0.41 ***

^a q_r represents TR 251 (spot blotch resistant parent) allele frequency among the selected resistant group.

^b q_s represents TR 251 (spot blotch resistant parent) allele frequency among the selected susceptible group.

^c σ_p is the standard error of the difference between marker allele frequencies.

^d $q_r - q_s$ represents allele frequency difference between the selected resistant and susceptible groups. A negative sign shows that allele frequency changed in the opposite direction to the parental phenotype.

^e *** represents significance at the 0.001 probability level.

Of the 256 combinations of AFLP primers, 16 failed to generate DNA fragments. DNA fragments generated from 240 different primer combinations were screened to find AFLPs demonstrating similar banding patterns between resistant and susceptible parents and their corresponding bulked groups. AFLP bands in six combinations of primer pairs, including seven AFLPs, appeared to be linked to the spot blotch resistance in line TR 251 (Table 1). These were added to 19 SSR markers for further linkage analysis and selective genotyping.

Linkage analysis

A partial linkage map based on segregation data of 96 of the progenies was constructed to assign the AFLP markers to specific chromosomes, and to estimate relative distances between markers tested by selective genotyping analyses. Of seven AFLP markers screened by BSA, four were linked to the SSR markers on chromosome 3H (Fig. 3). Each of the three remaining AFLP markers was linked to the SSR markers located on chromosomes 7H, 1H and 5H (Fig. 3). All eight SSR markers that had previously been assigned to chromosome 3H (Ramsay *et al.*, 2000) were syntenic to the SSR-based linkage map of barley developed in SCRI (Fig. 3). These, together with the four linked AFLP markers, formed a unique linkage group with a total map length of 44 cM. In contrast, eight SSR markers previously mapped on chromosome 7H (Ramsay *et al.*, 2000), together with a linked AFLP marker, formed three separate linkage groups, each with three markers (Fig. 3). Because only two and one SSR markers were identified on chromosomes 1H and 5H, respectively, orientation of the AFLP markers could not be determined on these chromosomes.

Identifying QTL-linked markers by the binomial distribution test

To evaluate the significance of the association between targeted markers with spot blotch resistance loci identified through BSA, differences in the allele frequencies of those marker loci were analyzed using a binomial distribution test. Testing resistant and susceptible progenies at three different selected intensities, all eight SSR markers and four AFLPs on chromosome 3H displayed significant changes in their allele frequencies (Table 1). An AFLP marker (P40/M57-442) located in the distal region of 3HS (Figure 3) and an SSR marker (Bmag0127) located in the proximal regions of 3HL (Ramsay *et al.*, 2000) had the highest allele frequency differences using all three proportions of the selected individuals (Table 1).

Three SSR markers located on chromosome 7HS (AF022725A, Bmag0007, and EBmag0794) had significant changes in allele frequency when 5% of the DH lines in each tail were tested (Table 1). The difference in the allele frequency of two of these markers (Bmag0007 and EBmag0794) was also significant when 10% and 15% of the selected progenies were tested. Significant changes in the allele frequency of all three different proportions of the population were also found when selected individuals were screened with AFLP marker P44/M51-223, located on chromosome 7HL. Of the two SSR markers (EBmac0755 and Bmac0156) that exhibited association with resistance through BSA and showed close linkage to AFLP marker P44/M51-223 (Fig. 3), only EBmac0755 showed a significant difference in allele frequency when 20% of the population (10% in each tail) was tested with this marker. However, differences in the allele frequency of these markers were lower than those of other markers on chromosome 7HS (or other chromosomes), using allele frequency data at different selection intensities (Table 1).

The significant change in marker allele frequency was detected when the selected lines were screened with the two SSR markers and a linked AFLP marker located on chromosome 1HS. Among these, SSR marker Bmac0213 and AFLP marker P38/M49-267 had higher allele frequency differences than SSR marker GMS021, when these markers were screened with different proportions of the population. A significant allele frequency difference was also detected for SSR marker Bmag0223 and a linked AFLP marker P46/M57-310 in the centromeric regions of chromosome 5H, using all the different selection intensities (Table 1).

Identifying QTL-linked markers by the hypergeometric distribution test

The hypergeometric distribution test was used as a complementary tool to evaluate the significance of QTL-linked markers detected by BSA and the binomial distribution test. The significant P-values (< 0.0005) of the differences in allele frequency between selected resistant and susceptible lines in each tail were used as a measure for detecting QTL-linked markers (Table 2). This analysis confirmed most of the results achieved through BSA and the binomial distribution test. Significant associations were detected between spot blotch resistance QTLs and most of the identified marker loci on chromosomes 3H, 5H and those on 7HS, using allele frequency data in all three proportions of the selected individuals.

None of the SSR markers located in the distal

Table 2. Monogenic segregation of SSR and AFLP markers that exhibited association with spot blotch resistance loci at the seedling stage in a DH population of a cross between barley line TR 251 (disease resistant) and CDC Bold (disease susceptible). Marker-QTL association was tested by a hypergeometric distribution-based selective genotyping analysis, using 5, 10, and 15% of the selected individuals with extreme phenotypes in each tail of the population.

Genetic marker	5% selection intensity				10% selection intensity				15% selection intensity			
	n_s^a	n_r^b	n_1^c	P-value	n_s	n_r	n_1	P-value	n_s	n_r	n_1	P-value
Chromosome 3H												
P40/M57-442	0	2	2	0.0000 *** ^d	0	3	3	0.0000 ***	2	5	7	0.0000 ***
P35/M61-180	1	3	4	0.0014 ***	1	7	8	0.0000 ***	6	10	16	0.0000 ***
P45/M49-214	1	3	4	0.0014 ***	3	6	9	0.0000 ***	7	9	16	0.0000 ***
P45/M49-150	1	3	4	0.0014 ***	3	6	9	0.0000 ***	7	9	16	0.0000 ***
Bmag0603	0	3	3	0.0002 ***	0	8	8	0.0000 ***	3	13	16	0.0000 ***
EBmac0871	0	3	3	0.0002 ***	0	8	8	0.0000 ***	3	13	16	0.0000 ***
Bmag0138	0	3	3	0.0002 ***	0	8	8	0.0000 ***	3	13	16	0.0000 ***
Bmac0127	0	3	3	0.0002 ***	0	7	7	0.0000 ***	3	12	15	0.0000 ***
Bmag0225	1	4	5	0.0060	2	8	10	0.0000 ***	4	13	17	0.0000 ***
Bmag0841	1	4	5	0.0060	3	11	14	0.0023	4	16	20	0.0000 ***
Bmag0010	1	4	5	0.0060	3	11	14	0.0023	4	16	20	0.0000 ***
Bmag0606	0	5	5	0.0060	1	13	14	0.0023	3	18	21	0.0001 ***
Chromosome 7H												
EBmag0794	1	0	1	0.0000 ***	8	4	12	0.0002 ***	13	7	20	0.0000 ***
Bmag0007	1	0	1	0.0000 ***	8	5	13	0.0008	13	8	21	0.0001 ***
AF022725A	4	0	4	0.0014	15	2	17	0.0342	21	5	26	0.0146
EBmac0827	9	2	11	0.5885	16	4	20	0.2045	22	7	29	0.0958
Bmag0359	9	2	11	0.5885	16	4	20	0.2045	22	7	29	0.0958
Bmag0120	9	4	13	0.8692	16	9	25	0.7955	22	12	34	0.5576
EBmac0755	3	4	7	0.0573	7	8	15	0.0063	13	12	25	0.0067
P44/M51-223	3	3	6	0.0207	7	7	14	0.0023	13	10	23	0.0011
BmaC0156	3	4	7	0.0573	7	11	18	0.0683	13	16	29	0.0958
Chromosome 1H												
GMS021	0	6	6	0.0207	1	12	13	0.0008	3	18	21	0.0001 ***
Bmac0213	0	5	5	0.0060	1	11	12	0.0002 ***	3	17	20	0.0000 ***
P38/M49-267	1	4	5	0.0060	2	10	12	0.0002 ***	4	15	19	0.0000 ***
Chromosome 5H												
Bmag0223	3	0	3	0.0002 ***	6	4	10	0.0000 ***	12	7	19	0.0000 ***
P46/M57-310	2	0	2	0.0000 ***	8	4	12	0.0002 ***	14	6	20	0.0000 ***

^a n_s represents the number of lines with a susceptible genotype (e.g., CDC Bold) in the lower extreme of the distribution, representative of a resistant phenotype.

^b n_r represents the number of lines with a resistant genotype (e.g., TR 251) in the upper extreme of the distribution, representative of a susceptible phenotype.

^c n_1 represents the sum of n_s and n_r .

^d *** represents significance at the 0.001 probability level.

region of chromosome 3HL had significant P-values when differences in the allele frequency were assessed using 5% selection intensity, whereas other SSRs and AFLPs located on the short arm and proximal regions of the long arm had significant P-values, indicating their positions are closer to the resistance QTL(s) on this chromosome. Of the three markers on chromosome 7HS, significant allele frequency difference at all the different selection intensities was detected only for SSR marker EBmag0794. Of the two other SSR markers in this region, only Bmag0007 had significant P-values when differences in allele frequency were assessed using 5 and 15% selection intensity. Two QTL-linked markers on chromosome 5H showed significant allele frequency differences between resistant and susceptible lines in phenotypic distribution tails using all three different selection intensities (Table 2).

This analysis did not indicate significant effects for the three markers positioned near a QTL on chromosome 1H, using allele frequency data at 5%

selection intensity. However, when 10 and 20% of the individuals in each tail were subjected to this analysis, two and then all three of these markers, respectively, were found to be associated to a resistance QTL on chromosome 1H. In contrast, significant P-values of allele frequency differences were not detected for three markers on chromosome 7HL, using any of the three proportions of the population (Table 2).

DISCUSSION

Bulked segregant analysis (Michelmore *et al.*, 1991) was applied to target the genomic regions affecting the spot blotch resistance of line TR 251. Using this screening analysis, all polymorphic markers on chromosome 3H and a few on chromosomes 7H, 1H and 5H were found to be associated with spot blotch resistance QTL regions of line TR 251. To verify the results found through BSA, marker loci screened by BSA were subjected to selective genotyping analyses. Both selective genotyping approaches indicated that selection for

spot blotch resistance/susceptibility resulted in significant changes in allele frequency between the two selected groups for most of the identified marker loci. This confirmed the power of BSA to detect QTL-linked markers. Selective genotyping has also been reported to be useful for confirming the results of BSA in other studies (Prasad *et al.*, 1999; Roy *et al.*, 1999; Wingbermuehle *et al.*, 2004).

The association between spot blotch resistance and all polymorphic SSR markers previously mapped on both the long and short arms of chromosome 3H strongly supports the presence of QTL regions on this chromosome. The greatest difference in the allele frequencies between resistant and susceptible lines in all three proportions (5, 10, 15%) of the selected individuals was found for an AFLP marker (P40/M57-442) mapped on chromosome 3HS. However, several SSR markers located farther away in the proximal regions of 3HL also indicated a high allele difference. Steffenson and Smith (2004) reported that two QTLs on the short and long arms of chromosome 3H contribute to overall spot blotch resistance to isolate ND85F at the adult plant stage in cv. Morex. Subsequently, Bilgic *et al.* (2005) reported two QTLs on the short and long arms of chromosome 3H of cv. Morex that explained 19% of the phenotypic variations in Steptoe/Morex and 4% of the variations in Dicktoo/Morex populations, respectively.

Both selective genotyping approaches found the three SSR markers previously mapped on chromosome 7HS (Ramsay *et al.*, 2000). Based on allele frequency differences found by both analytical approaches, EBmag0794 is likely the most closely linked marker to a putative QTL detected in this region. Steffenson *et al.* (1996) were the first to report mapping of a spot blotch resistance gene at the seedling stage and a QTL with a minor effect ($r^2 = 0.09$) at the adult plant stage, both located on chromosome 7HS in cv. Morex. The presence of this gene/QTL was further confirmed when Bilgic *et al.* (2005) mapped resistance QTLs with major effects at the same location on chromosome 7HS using four different mapping populations.

Yun *et al.* (2005) constructed a linkage map of the cross between *Hordeum vulgare* subsp. *spontaneum* and cv. Harrington, and mapped a resistance QTL with a major effect in a similar genomic region of chromosome 7H that explained approximately 25-42% of phenotypic variation. Interestingly, SSR marker Bmag0007, which showed close linkage to the resistance QTL in our study, was also located in the vicinity of the resistance QTL detected by Yun *et al.* (2005). This finding suggests the possibility of allelism between

the spot blotch resistance loci detected on chromosome 7HS in this study and those reported previously (Steffenson *et al.*, 1996; Bilgic *et al.*, 2005). However, further work is needed to clarify this possible allelism.

The BSA and subsequent selective genotyping analyses used in this study were able to detect three markers linked to a resistance QTL on chromosome 1HS. These markers were located in regions of the barley genome that are not well saturated with SSR markers (Ramsay *et al.*, 2000). Based on a loose correlation with morphological marker *v3* (six row, intermedium), Gonzalez Cenicerros (1990) identified a spot blotch resistance gene on chromosome 1H that confers resistance at the seedling stage in cv. Bowman. The *v3* locus is located on chromosome 1H, near the centromeric region (Jensen and Jørgensen, 1975; Persson, 1969). Subsequently, a resistance QTL with a major effect that explains 62% of the phenotypic variation for spot blotch resistance at the adult plant stage, was mapped on chromosome 1H of cv. Morex (Steffenson *et al.*, 1996).

Based on a consensus linkage map of barley (Langridge *et al.*, 1995), marker ABG494 mapped in the vicinity of this QTL was located in the proximal regions of chromosome 1HL, and in the lower stream of the *Ica1* locus mapped on the centromeric region. Based on the consensus map of barley (Karakousis *et al.*, 2003), the resistance QTL on chromosome 1H identified in this study is located on the short arm, near the telomeric region, and appears to be different from those reported previously.

In addition, Bilgic *et al.* (2006) identified a single gene (designated as *Rcs6*) on chromosome 1H of line Calicuchima-sib using the Calicuchima-sib/Bowman-BC DH population, which confers spot blotch resistance to pathotype '2' at the seedling and adult plant stages. This major resistance gene is positioned near the telomeric regions of chromosome 1HS, in the upper stream of SSR marker Bmac0213. Surprisingly, SSR marker Bmac0213 is also located in the vicinity of the resistance QTL on chromosome 1H detected in our study. However, this coincidence is more likely due to the close linkage between two different loci rather than to alternate alleles at the same locus. This assumption is based on the diverse origin of line TR 251 compared to line Calicuchima-sib, which has the pedigree LBIran/UNA8271//Gloria/Comanche (Hayes *et al.*, 2000), as well as the different responses of the two populations in question to different pathotypes of *C. sativus*.

In this study, two markers with significant effects were identified to be linked to a resistance QTL

located near the centromeric region of chromosome 5HL. The SSR marker Bmag0223, previously mapped on chromosome 5HL (Ramsay *et al.*, 2000), was used to assign AFLP marker P46/M57-310 to this chromosome. Griffee (1925) reported the position of a spot blotch resistance gene on chromosome 5H in cv. Svanhals, based on a loose correlation between morphological marker *Blp* (formerly *B/b*) and spot blotch resistance at the adult plant stage. Bilgic *et al.* (2005) identified a resistance QTL with minor effect on chromosome 5H of cv. Harrington, which explains 5% of phenotypic variation for adult-plant resistance. To date, there has been no report of a gene/QTL on chromosome 5HL that is responsible for spot blotch resistance at the seedling stage. Therefore, this resistance QTL is likely unique and responsible, in part, for the superior resistance of line TR 251 to *C. sativus*.

Based on the different marker systems used in this study and those employed in previous studies, it is difficult to determine with certainty whether the QTLs identified here are allelic to previously reported loci. However, based on pedigree comparisons, it can be speculated that the QTLs detected on chromosomes 7H and 3H may be allelic to those in cv. Morex (a ND B112-derived cultivar) previously mapped on these chromosomes (Bilgic *et al.*, 2005; Steffenson *et al.*, 1996). The other two QTLs detected on chromosomes 1HS and 5HL are unique and presumably contribute to the superior resistance of line TR 251. The presence of these loci in resistant barley cultivars from the upper Midwest US has not been reported in any of several genetic studies for spot blotch resistance.

The identification of several QTLs, each contributing a portion of the spot blotch resistance in line TR 251, together with the fact that differences in the IRs of the parents of the DH population used were not too distinct (TR 251= 3.0; CDC Bold= 6.3), was problematic for the precise scoring of mid-parent progenies, and for subsequently finding an appropriate approach to analyze the data with the least error. Therefore, we sacrificed individual QTL effects, in favor of the number and locations of the QTLs. We employed 'selective genotyping', an alternative QTL analysis, to determine the chromosome locations of spot blotch resistance QTLs in line TR 251 using individuals with distinct resistant or susceptible reactions to *C. sativus*.

Although this analysis is less able to determine the effects of each individual QTL, it is thought to be more efficient than the standard marker-based analysis at detecting locations of the QTLs (Tanksley, 1993). In a comparative study, selective

genotyping reliably detected almost all of the mapped QTLs identified through interval mapping, often with only 10% of the population genotyped (Ayoub and Mather, 2002). However, using 0.01 significance thresholds (probabilities below 0.005 in a two-tailed statistical test) in hypergeometric distribution-based selective genotyping, Ayoub and Mather (2002) identified the presence of several spurious QTLs in regions of the genome where interval mapping had given no evidence of QTL effects.

To be more stringent, in this study we used a significance level of 0.001 (probabilities below 0.0005) to test the linkage between marker loci and corresponding linked QTLs in hypergeometric distribution-based selective genotyping. Moreover, for additional stringency of results, we also used a significance level of 0.001 (z value of 3.29) to test probabilities below 0.0005 in the binomial distribution-based test. To test the association between the marker and a QTL in the binomial distribution-based selective genotyping analysis, Foolad *et al.* (1997) and Zhang *et al.* (2003) used a z value of 3 to test the association between the marker and a QTL. The estimation of the linkage between a marker locus and a QTL has been tested by an even lower z value of 2 (Foolad *et al.*, 2001). Using more stringent threshold values in both selective genotyping approaches in this study, the type I error was reduced to 0.001 on a per-marker basis. This reduced the probability of detecting false-positive QTLs and provided more robustness to the spot blotch resistance QTLs identified in line TR 251.

Although the chromosome locations of several spot blotch resistance QTLs in line TR 251 were determined in this study, further work is needed to elucidate the contribution of each of the identified QTLs. To provide more reliable phenotypic averages, particularly for those lines with intermediate IRs, additional replications could be used to minimize the experimental error in a marker-based QTL analysis. The use of isolates with greater differential virulence on the parents could also expand the range of IRs among progenies. If feasible, genotyping the whole population using markers in the targeted regions detected in this study could be done to estimate the contribution of each spot blotch resistance QTL to phenotypic variation.

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