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Inheritance and identification of RAPD markers for resistance to spot blotch of barley caused by *Cochliobolus sativus* L.

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Abstract

Spot blotch of barley caused by *Cochliobolus sativus* is an important foliar disease of barley causing considerable yield loss every year. The present study was undertaken to identify molecular markers for the locus controlling spot blotch resistance in the accession IBON 18, using a set of 110 recombinant inbred lines (RILs). The screening of individual RILs using an isolate highly virulent on the popular Indian cultivar 'RD 2508' revealed the presence of a major locus for spot blotch resistance in IBON 18. Based on the screening of 360 RAPD primers employing Bulk Segregant Analysis (BSA), 75 (20.83%) primers gave polymorphic bands in between parents. Out of these, two RAPD markers OPM04 625 and OPB01520 were found to be linked to the spot blotch resistance locus with a map distance of 6.5 and 10.9 cM, respectively. The linked markers appear to be useful in incorporating spot blotch resistance gene into barley breeding lines.

Key words: *Hordeum vulgare*, bulked segregant analysis, spot blotch, resistance, random amplified polymorphic DNA marker, barley

Introduction

Spot blotch caused by *Cochliobolus sativus* (Ito and kurib.) Drechsl. ex Dastur *Bipolaris sorokiniana* (Sacc in sorok.) Shoem. *Helminthosporium sativum* Pamm, King and Bakke is responsible for yield and quality reduction in many parts of the world [1-2]. In susceptible barley cultivars, average yield losses of 16-33% have been reported [3]. Van Leur [4] reported around 40% yield loss in barley due to infection by *C. sativus*.

The importance of molecular markers in plant breeding is now well recognized [5]. With the development of molecular markers, it has become possible to locate genes for important agronomic traits precisely on the linkage maps in several crop plants [5]. Identification of markers linked to resistance gene facilitates marker-assisted selection of the gene in breeding populations as more resistance genes are identified. Polymerase chain reaction (PCR) based marker, such as random amplified polymorphic DNA (RAPD) [6], can reduce the cost of identifying genetic markers and allow large scale genotyping of individual at any locus. High-density genetic maps have been constructed for barley (*Hordeum vulgare* L.) in the last decade using RFLPs, RAPDs and microsatellites markers [7].

Till date, there is only one report [2] on molecular markers for spot blotch resistance in Indian barley lines. In view of the importance of molecular markers for resistance and paucity of such reports in Indian barley lines, the present study was initiated with an objective to find a suitable RAPD marker for resistance to an important disease like spot blotch. In this communication, the identification of flanking markers for a spot blotch resistant locus in cv. IBON 18, employing RAPD markers and recombinant inbred lines (RILs) of barley is reported.

Materials and methods

Plant material: The plant material used in present study included a popular cultivar, RD 2508, of Indian barley, which is highly susceptible to spot blotch, a resistant exotic collection IBON 18 and a set of 110 F_6 generation RILs obtained by selfing the F_2 progenies of the cross between RD 2508 and IBON 18. Resistant parent was crossed with the susceptible to obtain F_1 seeds. The

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progenies of the cross were advanced to the F_6 generations.

F_3 lines were obtained from around 120 randomly chosen space planted F_2 plants grown in the crop season 2002-03 at Varanasi. The F_3 lines were evaluated under induced epiphytotic condition during crop season 2003-04. Generation advance was performed using off-season nursery, Wellington, Tamil Nadu. The 110 families of F_6 lines were evaluated under induced epiphytotic conditions during crop season 2005-06. The plots of each line consisted of a single 3 m row with 30 cm space between the plots and approximately 40-50 plants per row. Sowing was done during the first fortnight of November in order to allow the post anthesis stage to coincide with the relatively warm temperature occurring in March and considered congenial for the development of spot blotch.

Inoculation procedure and disease assessment

Spot blotch disease was induced by inoculating spreader rows using a pure culture of the locally most aggressive isolates of *B. sorokiniana* (Isolates No. RCBHUBR1857) identified at this center [8]. A spore suspension (10^4 spores/ml) was uniformly sprayed at three stages: tillering, flag leaf emergence and anthesis during the evening hours, following the method of Chaurasia *et al.* [9]. The homozygous resistant and susceptible RILs were identified on the basis of disease severity (%) using a modified version of 0-9 scale of Saari and Prescott [10]. Spot blotch level was assessed three times [11-12]; at growth stages 65 (anthesis half complete), 73 (Early milk) and 77 (late milk) [13]. The genotypes that scored less than 30 were considered homozygous resistant and those having higher than 80 as homozygous susceptible in Fe generation. Area under disease progress curve (AUDPC) was also considered for the evaluation of resistance of RILs [14]. The lines that showed AUDPC (<500) were considered resistant while those with AUDPC (>2000) were considered susceptible. DNA from the resistant and susceptible RILs was pooled and two bulks were prepared.

DNA extraction and RAPD analysis

DNA of parental type F_6 progeny rows was extracted by a scaled down modification of the Cetyltrimethyl ammonium bromide (CTAB) procedure [15]. DNA was isolated for each line from 15 days old plants grown in green house. DNA was diluted to a final concentration of 25 mg μl^{-1} using $T_{10}E_1$ (10 mM Tris-HCl and 0.1 mM EDTA) buffer. Equal amounts of DNA from five to six highly resistant RI lines were pooled to constitute the resistant bulk (RB). Similarly, DNA from five to six highly susceptible RILs were pooled to get the susceptible bulk (SB) for carrying out the bulk segregant analysis (BSA) [15]. Each polymorphic primer was tested at least three times to determine if both the polymorphism and banding pattern were reproducible. The bulks were then screened along with the parents against only those primers that gave rise to strong and reproducible polymorphism between the parents. If the same polymorphism appeared in the bulks as appeared in the parents (i.e., putative linkage), each of the individuals from the segregating population was individually screened for the polymorphism. RAPD profiles were generated by using single primer obtained from Operon Technologies, Alameda, USA, in polymerase chain reaction (PCR) following the standard protocol [16] with minor modification. PCR reactions were performed in the volume of 25 ul, containing 30-50 ng templates DNA, 1 unit of *Taq* DNA polymerase (Banglore Genei, India), 0.2 (µM of each primer, 200 µM of each dNTPs in 1X PCR buffer. Amplification was performed in Thermal cycler (Techgene, Cambridge, UK). After initial denaturation at 95°C for 30 second, 36°C for 60 seconds and 72°C for 120 seconds. Final extension was performed at 72°C for 10 min. The amplified products were separated by electrophoresis on 1.5% agarose gels in 1x TAE buffer (0.04m Tris-acetate, 0.01m EDTA for 4 h at 55 V). To determine the size of polymorphic fragment, a 100 bp ladder DNA marker and Lamda DNA/ Eco RI + Hind III Marker, 3 (MBI Fermentas, Vilnins, Lithuania) was used as size standard. DNA fragments ware visualized by staining gel using ethidium bromide and photographed using Gel Documentation systems.

Statistical analysis

To estimate the number of segregating genes in a cross, F_3 lines were grouped into three classes [12]. These classes were: (i) homozygous for the resistant parental response, (ii) homozygous for the susceptible parental response, and (iii) either segregating or homozygous for scores higher than the resistant, but less than the susceptible parent. The observed and expected distributions of F_3, lines in disease severity categories were tested by χ^2 analysis.

In the F_6 generation the RILs were grouped into two categories i.e. resistance and susceptible and analyzed in Mapmaker. The χ^2 distribution was used to test for goodness of fit of the RAPD markers and the spot blotch resistant gene. Mapmaker/Exp V3.0 [17] was used to calculate the genetic distance between markers and the spot blotch resistant gene with a minimum logarithm of odds ratio of 3.0 based on the Kosambi mapping function.

Results and discussion

Parental lines IBON 18 and RD 2508 differed significantly in the level of resistance to spot blotch. Disease severity and AUDPC were considerable high in the year 2003-04 (25.42 ± 4.80, 411.36 ± 87.75) for the resistant parent and 89.03 ± 4.97, 2205.83 ± 210.03 for susceptible parent RD2508. Disease severity and AUDPC were considerable low (20.01 ± 3.29, 390.00 ± 77.45) in the year 2002-03 for the resistant parent IBON 18, but high (84.33 ± 4.89, 2001.30 ±195.45) for susceptible parent RD 2508. The correlation between year and disease incidence (r = 0.77, p-value <0.0001) for spot blotch resistance was moderately high for the cross IBON 18 x RD 2508. This suggested that the phenotype and experimental designs followed in the experiment were appropriate.

In the F_3 generation, a continuous type of phenotypic distribution was observed for spot blotch reaction with few progeny rows displaying similarly to the parental lines (Fig. 1 and Table 1). This suggested that the resistance was under a polygenic control. The heritability estimate for disease severity was found moderately high (0.76). The disease score in the RILs of F_6 generation ranged from 18.30 to 90.00 with mean value of 54.15.

Of these 360 random primers, 75 (20.83%) RAPD primers detected reproducible polymorphism between the two parental genotypes differing for spot blotch resistance. However, only 10 of the 75 primers distinguished the resistant bulk from the susceptible bulk for resistance. Following this, the available 110 DNA samples with desirable DNA quality from the SSD F_6 plants were screened in order to determine the linkage between the RAPD markers and the spot blotch gene. Of ten primers, only two *viz.*, OPM04 (GGC GOT TGT C) and OPB01 (GTT TCG CTC C) were found to be linked to the locus conditioning spot blotch resistance. The primer OPM04 generated a RAPD marker in an estimated band size of 620 bp, designated as OPM04620, this band was present in the resistant parent IBON 18 and the resistant bulk, but absent in the susceptible parent RD 2508 and susceptible bulk (Fig. 2). The banding pattern generated by this marker OPM04620 was present in the five resistant lines but

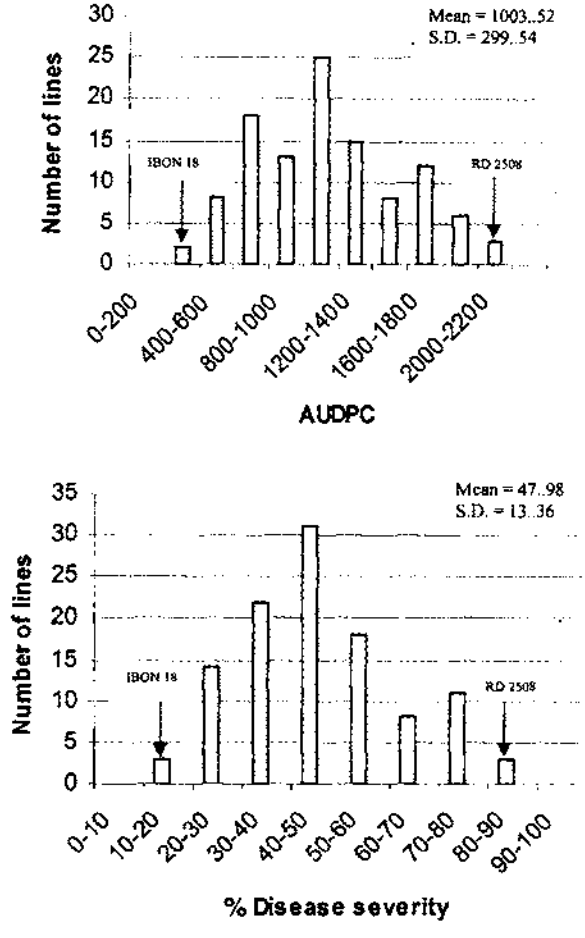

Fig. 1. Distribution of AUDPC and % disease severity of F_3 lines of cross between IBON 18 x RD2508 for spot blotch in barley

Table 1. Goodness of fit ratios observed and hypothesized class frequencies for F_3 lines from the cross between IBON 18 and RD 2508

Generation	Observed ratio in F_3						
	HRPT[a]	Seg[b]	HSPT[c]	Hypoth. ratio	χ^2 value	P value	Gene number
F_3	2	105	3	1:62:1	1.02	0.60	3

[a]Homozygous for resistant parental type (homozygous for all resistance alleles); [b]Segregating for disease levels higher than that of resistant parent but less than or equivalent to that of susceptible parent (homozygous for at least one resistant allele of heterozygous for at least one locus and homozygous for susceptibility alleles at other loci); [c]Homozygous for susceptible parental type (homozygous, lacking all resistance alleles).

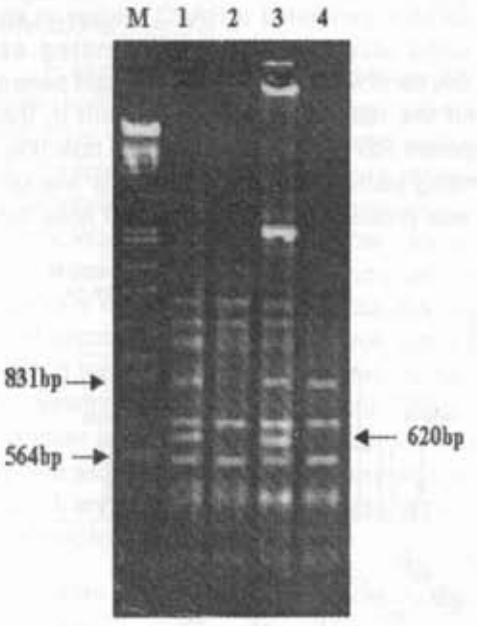

Fig. 2. Bulk segregant analysis of RILs (representing extreme group) with OPM04 RAPD primer. Lambda DNA/EcoRI +Hind III Marker, 3; 1,2,: resistant (IBON 18) and susceptible (RD 2508); 3, 4: bulked segregants for resistant and susceptible for spot blotch of barley

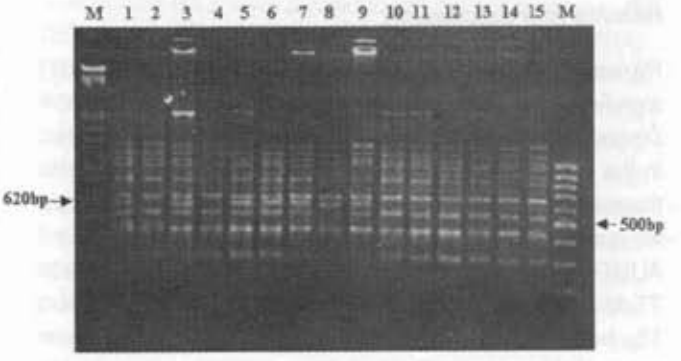

Fig. 3. Selective genotyping of RILs (representing extreme group) with OPM04, RAPD primer. Lane M: Lambda DNA/Eco RI = Hind III Marker and 100-bp ladder marker. 1,2: resistant (IBON18) and susceptible (RD2508); 3,4: bulk segregants; 5-9: RILs with low disease severity; 10-15: RILs with high disease severity

absent in the six susceptible (Fig. 3). In addition, the primer OPB01 designated as OPB01525, produced a DNA fragment of 525bp in the resistant parent IBON 18 and the resistant bulk of the DNA but not in the susceptible parent RD 2508 and susceptible bulk (Fig. 4), indicating that this DNA marker was associated with spot blotch resistant gene. The banding pattern generated by the RAPD primer OPB01 for 16 of the selective individual lines shown in Fig. 5 confirmed this association.

The marker produced by OPM04620 was found to be linked to resistance at the map distance of 6.5 cM based on the 110 F_6 RILs screened, whereas the one generated by OPB01525 was loosely linked to spot blotch resistance at a map distance of 10.9 cM (Table 2). The random primers reported to be used earlier [18] to map spot blotch resistance gene, failed to distinguish the two parental lines, RD 2508 and IBON 18.

The present study on identification of RAPD markers for spot blotch resistance in barley was undertaken because of the importance of highly linked flanking markers in designing marker-assisted selection schemes for the efficient selection of resistant lines. Identification of resistance locus conferring resistance to the spot blotch pathogen *Cochliobolus sativus* present in the accession IBON 18 of barley was accomplished by employing RAPD markers in conjunction with bulked segregant analysis of the F6 generation RILs. The favorable weather and epiphytotic condition in the field facilitated proper evaluation of RILs for disease reactions. A total of 360 random 10-mer primers were tried for detecting polymorphism between spot blotch resistant parent (IBON 18) and susceptible parent (RD 2508)

Table 2. Linkage analysis between two random amplified polymorphic DNA (RAPD) markers $OPM04_{620}$ and $OPB01_{525}$ and the spot blotch resistance gene in barley cross between IBON 18 and RD 2508

Markers	Genotypes[1]				Test of linkage	Genetic distance[2] (cM)	LOD score
	R/+	R/-	S/+	S/-			
$OPM04_{625}$	49	7	5	49	P<0.000	6.5	16.6
$OPB01_{520}$	47	9	9	45	P<0.000	10.9	11.79

R: Resistant; S: Susceptible;'+' Presence of marker fragment;'-' Absence of marker fragment; [1]Based on the spot blotch reaction of the parents and the RILs of F_6 generation; [2]Map distance was carried out using Mapmaker/Exp V3.0 (Lincoln *et al.* 1993) with a minimum logarithm of odds ratio of 3.0 based on the Kosambi mapping function.

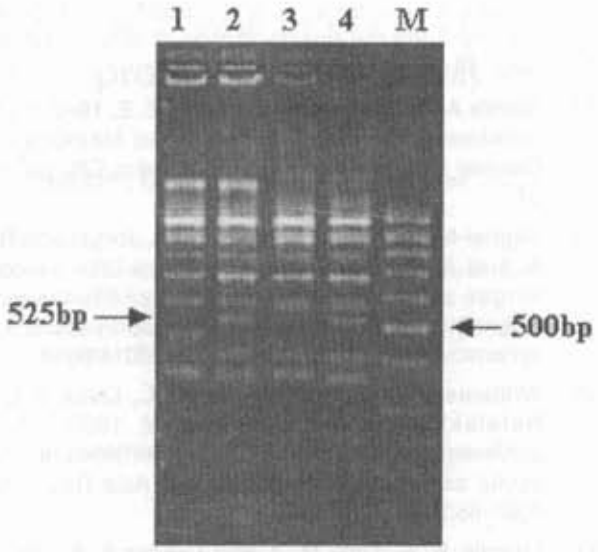

Fig. 4. Bulk segregant analysis of RILs (representing extreme group) with OPB01 RAPD primer. 100-bp ladder marker; 1, 2: susceptible (RD2508) and resistant (IBON 18); 3, 4: bulked segregants for susceptible and resistant for spot blotch disease of barley

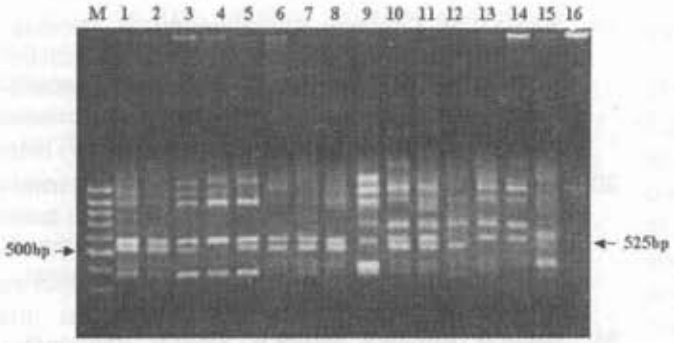

Fig. 5. Selective genotyping of RILs (representing extreme group) of a cross (IBON18 x RD2508) of barley with OPB01 RAPD primer. Lane M: 100-bp ladder marker. 1-3, 5-8 and 10-12: RILs with low disease severity; 4, 9, 13, 14 and 16; RILs with high disease severity

through the BSA approach using 110 F_6 RILs. Bulked segregant analysis can be used to identifying marker linked to a gene of interest [19]. This process is highly efficient because it detects only a small percentage of polymorphisms in F_2 individual progeny tests. BSA has been used to identify RAPD marker linked to genes controlling resistance to many pathogens [18, 19-20].

Of 360 random primers, only two RAPD markers OPM04620 and OPB01525 were found to be linked to spot blotch resistance. Kutcher *et al.* [18] screened 186 RAPD primers and found that three RAPD markers were associated with common root rot and spot blotch reaction in barley Pellio *et al.* [21] screened 1,200 RAPD primers and found 144 RAPD primers differentiating between the resistant and susceptible pools; out of these, two markers OP-AF18H971 and OP-AH12H550, closely linked to the Rym5 locus, were identified.

In the present study, the marker OPM04620 generated a band of 620 bp in the resistant parent and resistant progeny lines, which was absent in the susceptible parent as well as in the susceptible progeny lines. The marker OPB01525 amplified a fragment of 525 bp in the resistant parent and resistant progeny lines, which was absent in susceptible parent as well as in the susceptible progeny lines. Segregation of the markers in the RILs revealed that the marker OPM04620 was linked to the spot blotch resistance gene with a map distance of 6.5 cM. Whereas, the marker OPB01525 was found to be linked to the resistance gene with a map distance of 10.9 cM. Komatsuda *et al.* [22] reported that RAPD fragment, CMNA-38700 was linked to the v locus a recombination frequency of zero, while OPJ- 09850 and OPP-02700 were linked to the v locus at a map distance of 1.4 cM in barley. Nissan-Azzouz *et al.* [23] identified two RAPD markers, OP-AQ11H250 and OP-AU03H350, flanking rym11 locus in barley at a distance of 12.0 cM and 13.7 cM.

The RAPD markers identified for the spot blotch resistance in this study were found reproducible in our laboratory conditions. Hence, the results of the present study appear to suggest that RILs used in the present study can be effectively employed for efficient identification of spot blotch resistance and use of the markers OPM04620 and OPB01525 will aid identification of resistance homozygotes. RAPD pattern, however, are reported to be influenced by factors that include primer and DNA concentrations, Thermal cycler used, concentration on Taq DNA polymerase etc. [23]. Attempts are, therefore, being made to convert the flanking RAPD markers into sequence characterized amplified region (SCAR) markers. Microsatellites markers are also being employed to identify additional markers more tightly linked to the gene. This would facilitate routine use of the DNA markers in marker-assisted selection for spot blotch resistance and eventually, isolation of the target gene.

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