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# Identification of RAPD markers associated with Helminthosporium leaf blight (HLB) disease resistance in wheat

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**Abstract**

**To detect a set of RAPD markers associated with spot blotch/ Helminthosporium leaf blight (HLB) resistance in wheat, $F_2$ population of a cross, WH 147 x Chirya 3, comprising 332 individuals was analyzed by Bulked segregant analysis (BSA). Four putative polymorphic and reproducible RAPD markers; $OPK06_{791}$, $OPA10_{1040}$, $OPN15_{765}$ and $OPA12_{464}$ were identified. The markers, $OPK06_{791}$ and $OPA10_{1040}$ were associated with resistance in repulsion phase while, $OPN15_{765}$ and $OPA12_{464}$ in coupling phase. Together, these markers determined a significant proportion of total phenotypic variation (25.05%). Regression analysis revealed three markers, $OPK06_{791}$, $OPA10_{1040}$ and $OPN15_{765}$ to be significantly associated with disease phenotype.**

**Key words**: Helminthosporium leaf blight, spot blotch, RAPD markers, bulked segregant analysis, disease resistance

In recent years, Spot blotch or Helminthosporium leaf blight (HLB) caused by *Bipolaris sorokiniana* (Sacc.) Shoem (syn. *Helminthosporium sativum* Pamm, King and Bakke.) has emerged as a serious concern for wheat cultivation in nontraditional, warm and humid regions, limiting the productivity [1, 2]. Yield losses due to spot blotch disease is variable but considered to be significant [3]. Modern elite cultivars possess low to moderate levels of in-built resistance against the spot blotch disease [4, 5, 6 and 7]. Breeding for durable host resistance in commercial cultivars offers safer, economical and sustainable means to combat spot blotch disease. Novel sources of resistance to spot blotch were reported to be available in the synthetic hexaploid wheats using different sources of *Aegilops tauschii* (syn. *Triticum tauschii*) and durum wheats [8, 9]. Concurrently, the wide crossing programme at CIMMYT (International Maize and Wheat Improvement Center) produced wheat lines containing germplasm from *Thinopyrum curvifolium* that had high levels of spot blotch resistance [10]. We had identified Chirya 3, one of those CIMMYT germplasm wheat lines, available at a hotspot in India at Pusa, Bihar, as a potential source of spot blotch resistance while screening the wheat lines [11]. The genetic analysis of spot blotch resistance in Chirya 3 revealed digenic complementary and recessive nature of resistance [12]. In yet another study analyzing monosomic lines, we have located the genes that governed HLB disease resistance in Chirya 3. These two genes were located one on each of the two chromosomes 7B and 7D in hexaploid wheat [13]. Leaf tip necrosis (Ltn) linked to Lr 34 which is present in 7D has also been reported to be associated with resistance to spot blotch [1]. The efficient transfer of the identified potential resistance from Chirya 3 into elite cultivars would be facilitated by identifying a set of molecular markers associated with the disease resistance locus/ loci.

Molecular markers associated with disease resistance enable effective and early selection of resistant genotypes, despite lacking favorable environment. Random Amplified Polymorphic DNA (RAPD) markers are polymerase chain reaction (PCR) based markers [14] that can be used as markers specific to a genotype. The present findings elucidate the identification of RAPD markers associated with spot blotch disease resistance in Chirya 3.

## Materials and methods

Plant material comprised of parents, $F_1$ and $F_2$ population of the cross WH 147 x Chirya 3. In $F_2$,

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individual plants were analyzed for spot blotch reaction and marker association. The surface sterilized seeds were sown at the rate of four seeds/ pot in sterilized media made of decomposed agropeat, sand and vermiculite in the ratio of 1:1:1 in a growth chamber at National Phytotron Facility, New Delhi. A 10 ½ h photoperiod, at 280 µ Em-2/s light intensity and an 18-25$^0$C night and day temperature regime was maintained throughout the crop growth period.

A purified monoconidial isolate of *Bipolaris sorokiniana* (KL-8) was obtained from the Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi. The spot blotch isolate was cultured and maintained following the method of Gilchrist [15]. The spore suspension contained 6x10$^4$spores/ml [17] and a surfactant, Tween-20 was added to have a uniform spray. Besides spraying of the inoculum, inoculation by injecting approximately 0.1 ml of spore suspension with a hypodermal syringe after four days of spraying into the mid-rib of leaves at boot leaf stage was followed to ensure consistent and uniform inoculation. The inoculated plants were maintained in undisturbed darkness for 24 hours at 22-24$^o$C with relative humidity of 95% in the normal day by night conditions. Thereafter, they were maintained at 22-24$^o$C with relative humidity of 80%. Disease reaction of individual plants was scored using the double-digit method [18], seven days after inoculation. Plants were scored as resistant (R), moderately resistant (MR), moderately susceptible (MS), susceptible (S) and highly susceptible (HS) based on the computation of percentage leaf area damage due to the disease on flag and flag-1 leaves. The percentage of diseased (necrosed) leaf area was converted into a disease score [12].

The leaves from individual $F_2$ plants that were four-five weeks old were harvested separately under aseptic conditions prior to the inoculation of disease. Leaf samples were labeled, lyophilized and stored at –80$^o$C. DNA was extracted from lyophilized leaves by micro-extraction method [19]. About 1 ml of the crude leaf extract homogenized in warm CTAB buffer and phenol was used to isolate DNA. Extracted DNA was quantified using spectrophotometer based on the UV absorbency at 260 nm wavelength. The diluted working DNA samples of 10-20 ng/ µl were prepared individually from the stock DNAs for PCR assay.

The resistant and susceptible bulked DNA was prepared by pooling DNA of ten plants from each of the highly resistant and susceptible group of plants separately [16]. The parents and bulks (resistant and susceptible) were screened with 100 decamer oligonucleotide random primers from Operon Technologies Inc. (Alameda, CA, USA) to detect polymorphism. After detecting polymorphism, individual $F_2$ plants were screened with putative polymorphic (primer) marker to analyze segregation of the putative marker with the disease phenotype. Polymerase Chain Reactions (PCR) were performed in 20µl reaction mixtures containing 10x PCR buffer (10 mM Tris HCl (pH 8.5 at 25$^o$C), 50 mM KCl, 0.1% Triton X-100), 0.2 mM (Operon) 10-mer primer, 2 mM $Mgcl_2$ (M/S Sigma), 200 µM each of the four dNTPs (M/S Sigma), 0.5 units of Taq polymerase (M/s Bangalore Genei) and 15-20 mg of DNA/sample. The RAPD protocol consisted of an initial denaturing step of 2 minutes at 95$^0$C followed by 40 cycles of 92$^o$C for 30 sec (denaturation), 36$^0$C for 1 minute (annealing) and 72$^o$ C for 1 minute (extension), with a final extension step at 72$^o$C for 5 minutes on a Perkin Elmer 9600 thermal cycler (Barnstead/ Thermolyne Cropa, Iowa, USA). Amplified fragments were separated by electrophoresis in 2% (w/v) agarose gels in 1X Tris-Acetic acid EDTA buffer. The gels were stained in ethidium bromide solution (35 µl of 10mg/ml Ethidium bromide solution in 500 ml of $H_2O$) for 20 minutes and visualized in UV light transilluminator and photographed using digital gel documentation system (Vilber Lourmat). The experiments were repeated twice to check for the reproducibility.

Phenotyping of disease reaction of individual plants were recorded as '+' or '-' for the resistant and susceptible plants respectively. Likewise, RAPD data was recorded as '+' or '-'for the presence and absence of bands. Analysis of the scored data was performed using simple and multiple linear regression analysis [20] using marker segregation data in all the 20 resistant and 316 susceptible $F_2$ individual plants screened for Helminthosporium leaf blight disease.

**Results and discussion**

In an earlier experiment carried under natural epiphytotic conditions against broad spectrum of natural flora of spot blotch pathogen in a hotspot, Pusa (Bihar) in India [11], Chirya 3 and WH 147, the parental lines of the cross were identified as highly resistant and susceptible genotypes respectively. The disease reaction of parents was confirmed to be consistent when inoculated against a pure monoconidial isolate, KL-8 (Karnal-8) under controlled environmental conditions in National Phytotron Facility, New Delhi [12]. $F_1$ was found to be susceptible, indicating that HLB resistance was recessive in nature. $F_2$ plants segregated in the ratio of

1:15 (resistant: susceptible) indicating digenic recessive complementary gene action. $F_2$ plants were scored quantitatively based on phenotype and scores were pooled into two distinct classes based on the segregation of RAPD marker i.e., presence or absence of polymorphic DNA band amplified in the individual $F_2$ plants. Our research findings deviate from the previously reported inheritance studies [21] studies due to differences in the isolates used in the study. The $F_3$ family segregation as $F_2$ progeny test resulting into the families from the resistant $F_2$ plants showed typical observation of resistance with no segregation within the family. All the $F_3$ families from the twenty resistant plants of the cross WH 147 x Chirya 3 remained resistant [12].

The $F_2$ population was screened for putative polymorphic markers associated with the disease by BSA. Screening of DNA of bulks and parents (resistant and susceptible) revealed seven putative polymorphic markers of which four markers – OPK06; OPA10; OPN15 and OPA12, showed repeated reproducibility. The markers amplified a fragment of DNA each that was polymorphic between the resistant and susceptible phenotypes. Two of the four markers – OPK06 (791bp) and OPA12 (464 bp) were associated with HLB resistance in repulsion phase (i.e., presence of band in the resistant genotype), while the other two RAPD markers – OPA10 (1040 bp) and OPN15 (765 bp) were associated with spot blotch disease resistance in coupling phase (i.e, absence of band in resistant genotype). This revealed that the RAPD markers $OPK06_{791}$ (Fig. 1) and $OPA12_{464}$ (Fig. 2) were associated with expression of resistance to HLB and RAPD markers; $OPA10_{1040}$ and $OPN15_{765}$ (Fig. 3) were associated with susceptibility expression of HLB disease. The polymorphism detected were analysed using regression approach [23] taking percent disease recorded on flag and flag-1 leaves as dependent variable while the expression of markers was considered as independent variable. Single factor analysis of the four markers for between and within marker class variation in disease expression showed significant variation for markers $OPK06_{791}$, $OPA10_{1040}$ and $OPN15_{765}$ (Table 1). The marker $OPA12_{464}$ proved to be independent of resistant loci. Simple regression analysis showed significant regression coefficient of the markers $OPK06_{791}$, $OPA10_{1040}$ and $OPN15_{765}$ on percent disease score, while the marker $OPA12_{464}$ showed non-significant regression coefficient with disease score as dependent variable (Table 2). The RAPD marker $OPA10_{1040}$ had the highest coefficient of determination ($R^2$) of 12.7% while $OPN15_{765}$ and $OPK06_{791}$ contributed 8.9% and 3.14% respectively of total observed variation.



**Table 1.** Single factor analysis of variance for spot blotch and RAPD marker classes in $F_2$ population of the wheat cross, WH 147 x Chirya 3 of wheat

| Source of Variation | Degrees of Freedom | $OPK06_{791}$ | | $OPA10_{1040}$ | | $OPA15_{765}$ | |
|---|---|---|---|---|---|---|---|
| | | MSS | F value | MSS | F value | MSS | F value |
| Between Marker classes | 1 | 36395339.8 | 11.449*** | 137691575.9 | 47.9458 | 96562143.18 | 32.225*** |
| Within marker classes | 330 | 3178812.53 | | 2871854.24 | | 2996488.88 | |

***Significant at p = 0.001

**Table 2.** Analysis of variation (ANOVA) for simple linear regression of spot blotch score as dependent variable and RAPD marker expression as independent variable in $F_2$ population of he cross, WH 147 x Chirya 3 of wheat

| Source of Variation | Degrees of Freedom | $OPK06_{791}$ | | $OPA10_{1040}$ | | $OPA15_{765}$ | |
|---|---|---|---|---|---|---|---|
| | | MSS | F value | MSS | F value | MSS | F value |
| Regression | 1 | 36395339.8 | 11.449*** | 37691575.9 | 47.9458 | 96562143.18 | 32.225*** |
| Residual | 330 | 3178812.53 | | 2871854.24 | | 2996488.88 | |
| Regression coefficient | | –703.43(0.00)*** | | 2124.1(0.00)*** | | 169.0(0.00)*** | |
| $R^2$ | | 3.40% | | 12.70% | | 8.90% | |

bp = base pairs; ***Significant at p = 0.001

Significant variation among markers was revealed by multiple regression analysis indicating that at least some of the markers were associated with spot blotch expression (Table 3). The correlation coefficients indicated association between the RAPD markers and spot blotch disease phenotype as well as among the RAPD markers. The cumulative $R^2$ over the 3 markers was 16.9% suggesting co-linearity among the markers (Table 4). The RAPD marker, $OPA10_{1040}$ had highest significant correlation of 0.36 with disease phenotype followed by marker $OPN15_{765}$ with 0.30. A negative significant correlation was observed for RAPD markers with disease repulsion phase. One of the four markers, $OPA12_{464}$ (Fig. 2) showed non-significant association with disease phenotype and other RAPD markers indicating that the marker is not associated with resistance.

**Table 3.** ANOVA for multiple regression of spot blotch score as dependent variable and 4 RAPD markers as independent variables variables in the $F_2$ population of cross WH 147 x Chirya 3 of wheat

| Source of variation | Degrees of freedom | Mean sum of squares | F value |
|---|---|---|---|
| Regression | 4 | 48542557.67 | 17.81*** |
| Residual | 327 | 2725483.93 | |

***Significant at p = 0.001

RAPD markers are fragments of DNA amplified from genomes of organisms [14, 22] and the RAPD technique employing random arbitrary oligonucleotide primers to amplify regions of template DNA is a simple, fast and feasible method to detect polymorphism for a trait with no clues of its molecular positioning and linkages with other known markers. We assorted to RAPD analysis in conjunction with BSA following the method given by Michelmore *et al*. [16] that allows identifying markers linked to trait of interest with greater probability even in a highly variable mapping population such as $F_2$. Three RAPD markers, $OPA10_{1040}$, $OPN15_{765}$ and $OPK06_{791}$ were identified to be associated with HLB resistance genes in Chirya 3. These associations could be useful in marker-facilitated programs to increase the efficiency of transferring Chirya 3 resistant genes into desirable breeding germplasm. Regression approach was resorted to establish the molecular marker association in a segregating population where individual gene effect is not distinguishable with a particular phenotype as in this case. For single marker approaches, statistical analysis such as regression method was recommended [23], where single marker is considered an independent variable.

The three markers identified influenced the phenotypic variation of 12.7% ($OPA10_{1040}$) followed by $OPN15_{763}$ with 8.95% and $OPK06_{791}$ with 3.4% (Table 2). Kumar and associates [24] also reported polygenic resistance controlled by four QTLs with a total phenotypic variation of 63.10%. Kutcher and associates [25], showed similar observations while associating RAPD markers with root rot and spot blotch caused by *Cochliobolus sativus* on barley and showed that markers together influenced phenotypic variation up to 20.2%. For powdery mildew resistance in Maize, Stuber *et al*. [26] showed similar results where a maximum of 11% variation in the phenotype was explained by the markers. Heun [27] showed a near estimated variation of 10.6 to 12% for powdery mildew resistance phenotype in barley.

When the resistance is recessive in nature, there is a need to look for a repulsion phase marker associated with it as there is a possibility of underestimating the plants, which are heterozygote at resistance locus also as recessive homozygote due to the presence of the

**Table 4.** Correlation matrix of markers $M_1$ - $M_3$ and spot blotch scores in $F_2$ population of the cross WH 147 x Chirya 3 of wheat

| | Marker $M_1$ $OPR06_{791}$ | Marker $M_2$ $OPA10_{1040}$ | Marker $M_3$ $OPA15_{765}$ | HLB disease score |
|---|---|---|---|---|
| Marker $M_1$ $OPR06_{791}$ | 1.000 | | | |
| Marker $M_2$ $OPA10_{1040}$ | 0.248*** | 1.000 | | |
| Marker $M_3$ $OPA12_{464}$- | 0.001 | –0.106 | | |
| Marker $M_4$ $OPN15_{765}$ | 0.169*** | 0.284*** | 1.000 | |
| HLB Disease Score | 0.183*** | 0.356*** | 0.298*** | 1.000 |

Coefficient of determination, $R^2$ of $M_1$, $M_2$ & $M_3$ as independent variables on disease score value = 16.9%
bp = base pairs; ***Significant at p = 0.001

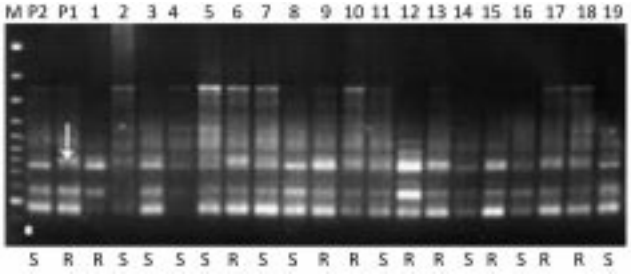


**Fig. 1. Screening of the $F_2$ plants of the cross, WH147 x Chirya 3 for polymorphism with the repulsion phase RAPD marker, OPK06 (791bp). The arrow mark shows the presence of amplified band in the resistant parent ($P_1$) and the absence in susceptible parent ($P_2$). A random sample of segregants (lanes, 1-19) were presented that were scored for RAPD marker and the spot blotch disease phenotype as resistant (R) and susceptible (S) in response to spot blotch disease. Lane, M: 1 Kb DNA ladder.**

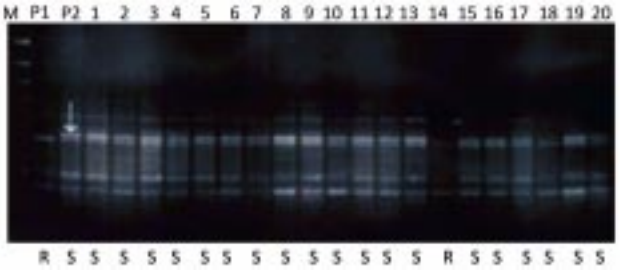


**Fig. 3. Screening of the $F_2$ plants of the cross, WH147 x Chirya 3 for polymorphism with the coupling phase RAPD marker, OPN15 (765bp). The arrow mark shows the presence of amplified band in the susceptible parent ($P_2$) and the absence in resistant parent ($P_1$). A random sample of segregants (lanes, 1-20) were presented that were scored for RAPD marker and the spot blotch disease phenotype as resistant (R) and susceptible (S) in response to spot blotch disease. Lane, M: 1 Kb DNA ladder.**

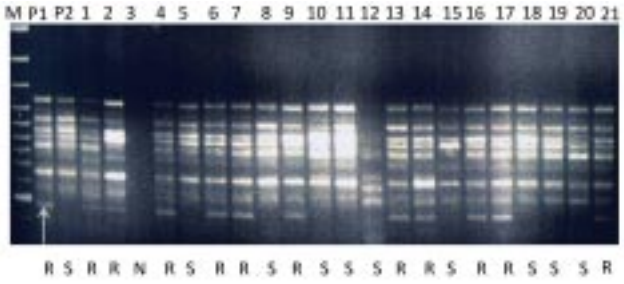


**Fig. 2. Screening of the $F_2$ plants of the cross, WH147 x Chirya 3 for polymorphism with repulsion phase RAPD marker, OPA12 (464bp). The arrow mark shows the presence of amplified band in the resistant parent ($P_1$) and the absence in susceptible parent ($P_2$). A random sample of segregants (lanes, 1-21) were presented that were scored for RAPD marker and the spot blotch disease phenotype as resistant (R) and susceptible (S) in response to spot blotch disease. Lane, M: 1 Kb DNA ladder & lane3: not applicable to score.**

marker in the absence of phenotypic data. In this study, although a marker, $OPK06_{791}$ was found to be associated with resistance, the low association of 3.7% is obviously an indication of the maker band showing up in most of the heterozygote, which are phenotypically scored as susceptible. Similar underestimation of $R_2$ with the molecular markers associated with the trait has also been observed when there are duplicate effects of homo-alleles in different genomic areas [20]. In the present case the situation is analogous because the marker is expressed in heterozygous locus also like the homozygous ones. However, screening by pooling (resistant) repulsion phase RAPD marker and a coupling phase RAPD marker could be advantageous when marking the recessive resistance genes. In addition, absence of a coupling phase marker and presence of repulsion phase marker gives identity to those plants carrying homozygous alleles for resistance. The above analogy has to be assessed jointly with $R_2$ values so that those markers having maximum association with resistant and susceptible phenotype can be employed in selection with high degree of precision. For this purpose, an analysis of correlation coefficients between the markers and the phenotype has to be significant. In our results, the marker $OPN15_{765}$, a coupling phase marker had a maximum correlation with susceptibility of 0.3 and the repulsion marker had a significant but negative correlation with susceptibility. Because of the overlapping phenotypic status with RAPD markers superimposed by the two recessive genes in $F_2$ population of cross WH 147 x Chirya 3, it was not possible to map each of the two recessive genes to a particular marker. Nonetheless, considering the disease data as a quantitative expression, the three markers were found to be associated with spot blotch resistance loci in Chirya 3.

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