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Validation of Molecular Marker associated with major Spot blotch resistance QTLs

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Abstract:

Resistance to spot blotch in wheat is quantitative (Joshi *et al*. 2004) and results in retarded growth and reproduction of the pathogen (Bashyal*et al*. 2011). The expression of resistance to spot blotch in wheat may also be influenced by growth stages. And if avoid it, early maturing genotypes will appear more susceptible than late maturing ones on a particular date (Joshi and Chand, 2002). This often leads to wrong judgment while screening for resistance and early maturing genotypes are considered susceptible compared to the late maturing ones which invite disease much later after attaining susceptible growth stage only. In this study, resistance in early maturing RILs showing phenological background of susceptible parent sonalika were used to understand the function of different components of resistance. Duveiller and Gilchrist (1994) had suggested that tolerance or the slower rate of foliar blight development in wheat was often associated with late maturity. Duveiller*et al*. (1998) reported that early maturing wheats showed higher levels of Helminthosporium blight compared with late-maturing genotypes. However, Joshi and Chand (2004) found no association of susceptibility or resistance with maturity duration when growth stage was taken in account.

INTRODUCTION:

Wheat is world's most widely cultivated food crop which belongs to the family Graminae (Poaceae) and the genus *Triticum*. It is the most important food grain in the world that ranks second in total production

as a cereal crop, behind maize and ahead of rice. Wheat is a temperate crop, but still sustains well under wider agro climatic conditions. Major wheat production is concentrated between 30º and 60ºN and 27º and 40ºS latitudes (Nuttenson, 1955). It is still being grown beyond these limits successfully due to its wider adoptability of diverse species, which has lead to the harvesting of this crop in one or the other parts of the world throughout the year.

It is believed that wheat developed from a type of wild grass native to the arid lands of Asia Minor. Cultivation of wheat is thought to have originated in the Euphrates Valley as early as 10,000 B.C., making it one of the world's oldest cereal crops. In the Mediterranean region, centuries before recorded history, wheat was an important food. The central Asia, Near East, Mediterranean and Ethopian regions are the world most important centre of diversity of wheat and its related species (Kundu and Nagarajaan, 1996; Perrino and Porcedo, 1990). Hindukush area is the centre of diversity of hexaploid wheat (Kundu and Nagarajan, 1996). In India, the majority of the cultivated wheat varieties belong to three main species of the genus *Triticum*,the hexaploid,*T.aestivum* L. (bread wheat),the tetraploid,*T. durum* Desf and the diploid, *T.diococcum* and *T.monococcum* Schrank; Schulb.

Spot blotch disease, caused by fugal pathogen *Bipolaris sorokiniana* (Sacc) Shoem. [Syn.*Helminthosporium sativum* telomorph (*Cochliobolous sativus*)] have emerged as a major constraint to wheat (*Triticum aestivum* L.) production in warmer and humid region of the world particularly in South Asia. For a long time, spot blotch was considered as a part of Helminthosporium Leaf Blight (HLB) which was understood to be a complex of many pathogenic fungi occurring simultaneously at different growth stages of the wheat. However, recently it has been established that leaf blight observed in the North-Eastern Plain Zone of India is actually spot blotch caused by *B. sorokiniana* (Sacc.) shoem syn. *Drechslera sorokiniana* (Sacc.) Subrm and Jain (syn. *Helminthosporium sativum*, teleomorph *Cochliobolus sativus*) (Chaurasia et al. 2000). Due to wide spread losses, this disease is considered as the most significant disease of wheat in North-Eastern Plain Zone of India (Saari 1998; Joshi et al. 2007b). Globally, an estimated 25 million hectares of wheat cultivated land is affected by spot blotch disease (van Ginkel and Rajaram 1998). Indian sub-continent has 10 million ha of affected land out of which India alone has 9 million ha,

most of which is in rice-wheat cropping system (Nagarajan and Kumar, 1998). In *e*arly symptoms, lesions on the leaves start as dark brown lesions of a few mm that extend as elongated dark brown spots greater than 1 cm. A yellowing due to toxin production is sometimes observed extending from the lesion. As the disease progresses the spots join together forming large blotches that cover the leaves and eventually killing it. Fruiting structures develop readily under humid conditions and are generally easily observed on old lesions. If spikelet is affected, it can result in shriveled grain and black point, a dark staining of the embryo end of the seed.

A molecular marker has provided some edge to the resistance breeding. Molecular markers are superior to morphological and protein markers. They are neutral, occur throughout the genome, not influenced by the environment, co-dominant, and monitored in any tissue and stage of the plant and often follow expected Mendelian segregation. Several molecular marker types are available and they each have their advantages and disadvantages. Restriction fragment length polymorphisms (RFLPs) were the first to be developed (some 15 years) and have been widely and successfully used to construct linkage maps of various species, including wheat. With the development of the polymerase chain reaction (PCR) technology, several marker types emerged. The first of those were random amplified polymorphic DNA (RAPD), which quickly gained popularity over RFLPs due to the simplicity and decreased costs of the assay. However, most researchers now realize the weaknesses of RAPDs and use them with much less frequency. The amplified fragment length polymorphism (AFLP) approach takes advantage of the PCR technique to selectively amplify DNA fragments previously digested with one or two restriction enzymes (Hosington et al., FAO Document Repository). Microsatellite markers or SSRs (Simple Sequence Repeats) take advantage over RAPD and RFLP. Microsatellite markers or simple sequence repeats (SSRs) combine the power of RFLPs (co-dominant markers, reliable, specific genome location) with the ease of RAPDs and have the advantage of detecting higher levels of polymorphism. Playing with the number of selective bases of the primers and considering the number of amplification products per primer pair, this approach is certainly the most powerful in terms of polymorphisms identified per reaction. SSR markers were used to map QTLs for Spot blotch resistance in wheat lines Yangmai 6

(Kumar et al., 2009), Ning 8201 and Chirya 3 (Kumar et al., 2010).

Marker assisted selection (MAS) can offer an effective and efficient breeding tool for detecting, tracking, retaining, combining, and pyramiding disease resistance genes (Kelly and Miklas, 1998 and 1999). Marker assisted breeding can improve the efficiency of conventional breeding especially in the case of low heritable and recessive traits, where phenotypic selection is difficult, expensive, lack accuracy or precision. Identification of resistant or susceptible lines at seedling stage is possible, when MAS is employed. Linkage drag is also one of the serious problems while transferring resistance from unadapted wild and weedy germplasm into elite lines and it can be dissected out through tightly linked markers. It can help in the introgression of resistance from wild relatives and fastest recovery of the recurrent parent genome can be achieved by using foreground and background selection approach. In foreground selection, flanking markers around a target gene are used to guide selection whereas in background selection, markers dispersed throughout the genome are used to recover the RP genotype more efficiently than by phenotypic selection. The present study was undertaken with the following objectives:

- To validate the molecular markers associated with major spot blotch resistance QTLs in a RIL population derived from cross Yangmai 6 (resistant parent) and Sonalika (susceptible parent).
- Utilization of the validated markers for foreground selection in backcross population (BC_1F_1) derived from cross (HUW-234 x 2nd CSISA 6713) x HUW-234.

Fig. 3.1. Spot blotch (caused by *Bipolaris sorokiniana*) disease severity on flag leaf of **(A)** Yangamai 6 **(B)** Chirya 3 and **(C)** Sonalika.

MATERIALS AND METHOD:

The field trials were performed at Agricultural Research Farm, Institute of Agriculture Sciences, Banaras Hindu University, Varanasi during the Rabi season 2011-12. The Agriculture Research Farm is situated in South- Eastern part of Varanasi city at 25°15' North latitude and 83°03' East longitude at an elevation of 129.23 m above the mean sea level. The molecular biology experiments were performed in the laboratory of "Niche Area of Excellence", Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, B.H.U., Varanasi. Varanasi is subjected to extremes of weather conditions i.e., extremely hot summers and cold winters. Although temperature begins to rise from mid-February and attain a maximum in May-June (Mean maxi. temp. is about 43.6°C), it decreases from July onwards reaching an average minimum of 5.0°C in December-January. The average rainfall in Varanasi region is about 1150 mm and mean relative humidity is 68%. Most of the precipitation is usually received from the South-West monsoon.

3.1. Plant Material

The plant material constituted by two resistant parental genotypes i.e., Yangamai 6 and Chirya 3, and one susceptible parental genotype Sonalika (Fig. 3.1). The recombinant inbred lines (RILs) derived from the cross between Yangmai 6 (resistant to spot blotch) and Sonalika (susceptible to spot blotch) was used to validate the markers linked to QTLs for spot blotch resistance. The validated markers were utilized for foreground analysis in BC_1F_1 population of the cross HUW 234 (recurrent parent) × CSISA 6713 (donor parent).

3.2. Methodology

3.2.1. Isolation of Plant genomic DNA

Young leaves were collected from 20-25 days old wheat seedlings and immediately stored in -20°C till further processing. The DNA was extracted following CTAB extraction method (Doyle and Doyle, 1987).

Table 3.1. Extraction buffer used for genomic DNA isolation.

Chemical	Stock concentration	Final concentration	Final Volume (100ml)
Tris (pH 7.5)	1 M	100 mM	10
Na CI	5 M	1.4 M	28
EDTA (pH 8.0)	0.5 M	20 mM	4
CTAB	10 %	2%	20
β-Mercaptoethanol	0.02M	0.08mM	0.4
Distilled H_2O	-	-	37.6

3.2.2. DNA quality estimation

Analysis of UV absorption by the nucleotides provides a simple and accurate estimation of the concentration of nucleic acids in a sample. Purines and pyrimidines in nucleic acid show absorption maxima around 260 nm (eg., dATP: 259nm; dCTP: 272nm; dTTP: 247nm) if the DNA sample is pure without significant contamination from proteins or organic solvents. The ratio of OD at 260 and 280 nm should be determined to assess the purity of the sample. The DNA quality estimation was done using Biophotometer plus (Eppendorf, USA).

The ratio (OD_{260}/OD_{280} ratio) thus obtained was used to estimate the nucleic acid purity in the different DNA samples. A ratio of 1.8-2.0 denotes that the absorption in the UV range is due to nucleic acids. A ratio lower than 1.8 indicates the presence of proteins and/or other UV absorbers. A ratio higher than 2.0 indicates that the samples may be contaminated with chloroform or phenol. In either case (<1.8 or >2.0) samples were re-precipitated to purify the DNA.

3.2.3. SSR markers linked to spot blotch

A total of 22 SSR primers associated with spot blotch resistance in genotypes Yangmai 6, Ning 8201 and Chirya 3 were selected from Kumar et al., 2009 and 2010 for the validation. The polymorphic markers were then used in marker aided selection. The details of the primers are given in table 3.2.

3.2.4. Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed to selectively amplify *in vitro* a specific segment of the total genomic DNA to a billion fold (Mullis et al., 1986). The most essential requirement of PCR is the availability of a pair of short (typically 20-25 nucleotides) primers having sequence complementary to either end of the target DNA segment (called template DNA) to be synthesized in large amount. The PCR conditions standardized for the present experiment are presented in the table 3.3.

The components of the PCR reaction were first added in a sterilised 1.5ml microcentrifuge tube thoroughly in a sequence as mentioned in table 3 and then mixed thoroughly by vortexing. To each PCR tubes (0.2 ml), 14 µl of reaction mixture was distributed, and finally template DNA of individual wheat genotypes was added. The tubes containing reaction mixture were placed in the wells of the thermal cycler block and amplification reaction was carried out with the thermalcycler programme summarised in table 3.4.

For PCR programming all the steps were kept as such except the annealing

temperature. For adjustment of concentration of various chemicals, amount of $MgCl_2$ was changed keeping other PCR components as constant. Annealing temperature was determined based on the GC content of the primer using the formula given below:

$$Tm = [2 \times (A+T) + 4 \times (G+C)] - 4$$

This formula gave preliminary information but not the exact annealing temperature. Therefore, the correct annealing temperature was determined based on best PCR amplification. All the amplifications were performed in the Eppendorf Thermo-cycler (USA). After the completion of the PCR, the products were stored at -20ºC until the gel electrophoresis was done.

Table 3.2. Details of the SSR primers associated with spot blotch resistance QTLs.

S. No.	Locus	Left primer (5` to 3`)	Right primer (5` to 3`)	Tm	Chromosome
1.	Xbarc91	TTCCCATAACGCCGATAGTA	GCGTTTAATATTAGCTTCAAGATCAT	50	2B
2.	Xbarc159	CGCAATTTATTATCGGTTTTAGGAA	CGCCCGATAGTTTTTCTAATTTCTGA	50	2A
3.	Xbarc175	GCGTAACAGAAGCGGAGAAAGC	GCGAATCATTTAGTGTTAGGTGGCAGTG	55	6D
4.	Xbarc353	GAAGTTCCCAAAATGCCTCTGTC	GCGGATCGAAGACCTAAGAAAAG	55	2D
5.	Xgwm67	ACCACACAAACAAGGTAAGCG	CAACCCTCTTAATTTTGTTGGG	60	5B
6.	Xgwm111	TCTGTAGGCTCTCTCCGACTG	ACCTGATCAGATCCCACTCG	55	7D
7.	Xgwm129-1	TCAGTGGGCAAGCTACACAG	AAAACTTAGTAGCCGCGT	50	2B
8.	Xgwm148	GTGAGGCAGCAAGAGAGAAA	CAAAGCTTGACTCAGACCAAA	60	2B
9.	Xgwm213	TGCCTGGCTCGTTCTATCTC	CTAGCTTAGCACTGTCGCCC	60	5B
10.	Xgwm255	?	?	55	7B
11.	Xgwm263	?	?	55	7B?
12.	Xgwm371	GACCAAGATATTCAAACTGGCC	AGCTCAGCTTGCTTGGTACC	60	5B
13.	Xgwm374	ATAGTGTGTTGCATGCTGTGTG	TCTAATTAGCGTTGGCTGCC	60	2B
14.	Xgwm425	GAGCCCACAAGCTGGCA	TCGTTCTCCCAAGGCTTG	60	2A
15	Xgwm4	TTTGTTGGGGGTTAGGAT	CCTTAACACTTGCTGGTAGTG	55	2A

.	45	TAG	A		
16.	Xgwm455	ATTCGGTTCGCTAGCTACCA	ACGGAGAGCAACCTGCC	55	2D
17.	Xgwm533	AAGGCGAATCAAACGGAATA	GTTGCTTTAGGGGAAAAGCC	60	3B
18.	Xgwm732	?	?	60	3B
19.	Xgwm815	?	?	60	7D
20.	Xgwm1037	?	?	55	3B
21.	Xgwm1168	?	?	60	7D
22.	Xswm008	GCTCTTGAACTTAGTCTCATCAAGG	CTCTCCCGCTGCAGTGTCTC	55	7D

? Primer sequence not available in public domain, sequences obtained from Dr. Uttam Kumar by personal communication.

Table 3.3. Standardized concentration of the PCR components used in the present study.

PCR Component	Stock concentration	Final concentration	Volume used for 15 µl reaction
Primer(F + R)	10 pM	0.7 pM	1 µl
Taq DNA Polymerase	5U/ µl	1U	0.2 µl
$Mgcl_2$	25 mM	0.3 mM	0.2 µl
Taq Assay Buffer	10x	1x	1.5 µl
dNTPs	10 mM	0.14 mM	0.2 µl
Genomic DNA	50 -100 ng/ µl	50 ng / 15 µl reaction volume	1 µl
HPLC water	-	-	9.9 µl

Table 3.4. The thermalcycler programme for PCR used in this study.

Cycle	Temperature	Duration	Objective
First cycle	94°C	4 minutes	Initial denaturation
The next 39 cycles	94°C	45 second	Denaturation
All 40 cycles	T_m °C*	30 second	Annealing
All 40 cycles	72°C	30 second	Extension
At the end of the 40^{th} cycle	72°C	7 minutes	Final extension
Hold	15°C	∞	Hold

*T_m depend on the annealing temperature of the primer used.

3.2.5. Visualization of amplification products

The amplified DNA fragments generated through SSR primers were resolved through electrophoresis in 2.5 % agarose gel prepared in TAE [242 g Tris-base; 57.1ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) bring final volume to 1000 ml] buffer. Ethidium bromide solution at a final concentration of 0.03 ng/µl was added to the agarose solution.

For electrophoresis, 15 µl of the PCR product was mixed with 2 µl of 6X loading dye (0.25% bromophenol blue in 30% glycerol) and loaded in the slot of the agarose gel. In order to determine the molecular size of the amplified products, each gel was also loaded with 1 µg DNA of a 100 bp DNA size marker (Fermentas, USA). Gel electrophoresis was performed at a constant voltage of 65 V for about 3.5 hours. Finally, the gels were visualized under a UV light source in a gel documentation system (Gel Doc™ XR+, BIO-RAD, USA) and the images of amplification products were captured and stored in a computer for further analysis and future use.

3.2.6. Band scoring: The amplified fragments were scored as either resistant parent types or susceptible parent types in RIL population. While in backcross populations, bands were scored either as heterozygote (presence of resistant and susceptible parent type bands) or homozygote for susceptible band.

Selection of BC_1F_1 plants for back cross

Plants showing heterozygous for both the marker alleles corresponding to resistant and susceptible parents were selected through foreground selection and will be further utilized for background analysis and future back crossing with recurrent parent.

Disease scoring for spot blotch: A pure culture of the most aggressive isolate of *B. Sorokiniana* was used for the creation of artificial epiphytotic condition in the experimental field. The isolate was multiplied on wheat grains and a spore suspension was adjusted to 10^4 spores/ml of water. The spore suspension was uniformly sprayed during evening hours. The field was irrigated immediately after inoculation and a total of six to eight irrigations were given in the entire crop period to provide a favourable environment for the development of spot blotch disease. The disease severity was recorded when it appears on the susceptible check i.e., Sonalika following 0-9 scale (Saari and Prescott, 1975). Spot blotch disease severity was recorded at different growth stages **(**Zadoks et al., 1974)

to calculate area under disease progress curve (AUDPC) using following formula (Van der Plank, 1963):

$$AUDPC = \sum_{i=1}\left[\frac{Y_i + Y_{i+1}}{2} \times t_{i+1} + t_i\right]$$

Where, Y_i = disease level at time t_i and ($t_{(i+1)} - t_i$) = time (days) between two disease scores.

RESULTS AND DISCUSSION:

Wheat (*Triticum aestivum*) is one of the most widely cultivated food crops belonging to the family Graminae (Poaceae). Spot blotch disease, caused by fugal pathogen *Bipolaris sorokiniana* have emerged as a major constraint to wheat production in warmer and humid region of the world particularly in South Asia. Marker assisted selection (MAS) can offer an effective and efficient breeding tool for detecting, tracking, retaining, combining, and pyramiding disease resistance genes (Kelly and Miklas, 1998 and 1999). Marker assisted breeding can improve the efficiency of conventional breeding especially in the case of low heritable and recessive traits, where phenotypic selection is difficult, expensive, lack accuracy or precision. Identification of resistant or susceptible lines at seedling stage is possible, when MAS is employed.

Linkage drag is also one of the serious problems while transferring resistance from unadapted wild and weedy germplasm into elite lines and it can be dissected out through tightly linked markers. It can help in the introgression of resistance from wild relatives and fastest recovery of the recurrent parent genome can be achieved by using foreground and background selection approach. In foreground selection, flanking markers around a target gene are used to guide selection whereas in background selection, markers dispersed throughout the genome are used to recover the recurrent parent genotype more efficiently than by phenotypic selection.

4.1. Polymorphism of SSR markers associated with spot blotch resistance

Initially, parental polymorphism survey were performed among parental genotypes Yangmai 6, Chirya 3 and Sonalika using 22 SRRs to validate the SSR markers linked to spot blotch resistance. In both resistant genotypes (Yangmai 6 and Chirya 3), different QTLs are associated with spot blotch resistance, except *QSb.bhu-2B* (Kumar et al., 2009 and 2010). Out of 22 SSRs, only two SSRs produced reproducible and polymorphic bands which are shown in figure 4.1. Xgwm 148 produced 170 bp band in resistant parent, while Xgwm 111

amplified 150 bp band in the resistant parent. SSR marker Xgwm148 is linked with QTL, *QSb.bhu-2B and* Xgwm111 is linked with *QSb.bhu-7D* (Table 4.1). Rest SSR markers did not show polymorphism in agarose gel electrophoresis in both the resistant parent and Sonalika. This may be due to low resolving power of agarose as compared to polyacrylamide gel electrophoresis (PAGE) and automated gel electrophoresis systems. The electrophoretic banding patterns of SSRs are shown in figure 4.1 and 4.2.

In wheat, hundreds of microsatellites developed by several workers (Roder et al., 1995; Pestova et al., 2000; Gupta et al., 2002). These markers are publicly available and are being used for gene tagging, mapping and phylogenetic studies. Kumar et al. (2009) reported four quantitative trait loci (QTL) on the chromosomes 2AL, 2BS, 5BL and 6DL, for resistance to spot blotch in wheat from a RILs populaton developed from a cross between a Chinese source of resistance (Yangmai 6) and a spot blotch susceptible cultivar (Sonalika). These QTLs were designated as *QSb.bhu-2A, QSb.bhu-2B, QSb.bhu-5B* and *QSb.bhu-6D*, respectively. All QTL alleles for resistance were derived from the resistant parent 'Yangmai 6'. Kumar et al. (2010) identified QTLs for spot blotch resistance in other sources of resistance (Ning 8201 and Chirya 3). Four QTLs were mapped in 'Ning 8201' on the Chromosomes 2AS, 2BS, 5BL, and 7DS and five QTLs on the chromosomes 2BS, 2DS, 3BS, 7BS, and 7DS in 'Chirya'.

Table 4.1. Details of different QTLs associated with spot blotch resistance in wheat.

S. No.	QTL	Marker interval	Interval size (cM)	Chromosome	*R^2 (%)
Yongmai 6 × Sonalika					
1.	*QSb.bhu-2A*	Xbarc353-Xgwm445	37.4	2AL	14.80
2.	*QSb.bhu-2B*	Xgwm148-Xgwm374	15.0	2BS	20.50
3.	*QSb.bhu-5B*	Xgwm067-Xgwm371	13.2	5BL	38.62
4.	*QSb.bhu-6D*	Xbarc175-Xgwm732	30.1	6DL	22.50
Chirya 3 × Sonalika					
1.	*QSb.bhu-2B*	Xgwm148-Xgwm129	15	2BS	13.1
2.	*QSb.bhu-2D*	Xgwm455-Xgwm815	9	2DS	10.7
3.	*QSb.bhu-3B*	Xgwm533-Xgwm1037	8	3BS	9.7
4.	*QSb.bhu-7B*	Xgwm263-Xgwm255	5	7BS	10.2
5.	*QSb.bhu-7D*	Xgwm111-Xswm008	25	7DS	11.9

*R^2 (%) is the phenotypic variance of the QTL.

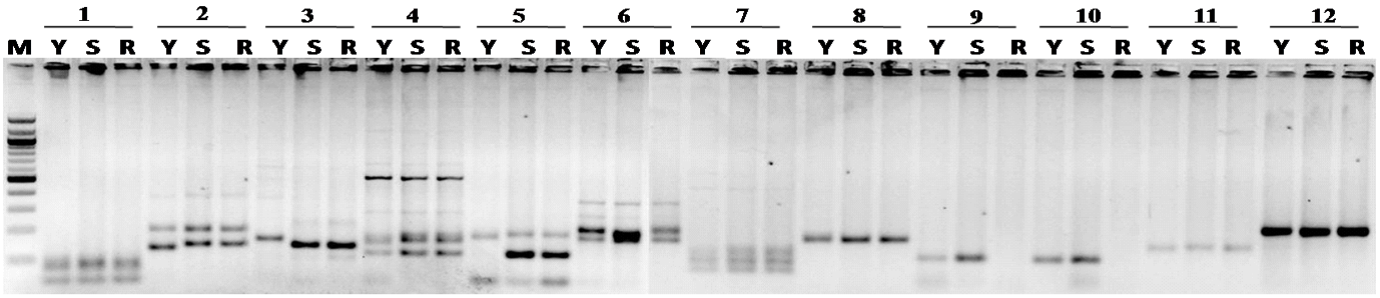
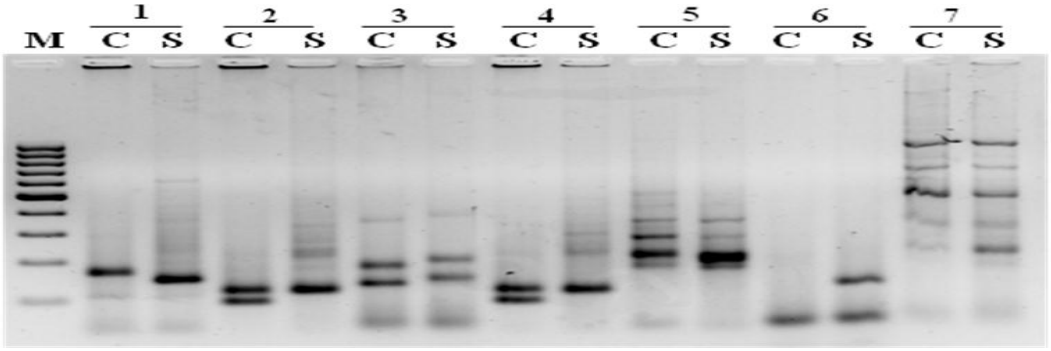


Fig. 4.1. Parental polymorphism survey among Yangami 6 (resistant parent), Sonalika (susceptible parent) and a recombinant inbred line No. 4 derived from the cross Yangami 6 × Sonalika, where M = 100 bp DNA size marker, Y = Yangmai 6, S = Sonalika and R = RIL-4; 1-12 are SSR markers as given below:

• Xgwm067	• Xgwm111	• Xgwm148	• Xgwm213
• Xgwm371	• Xgwm374	• Xgwm425	• Xgwm445
• Xgwm533	• Xgwm008	• Xbarc353-2A	• Xbarc159

Fig. 4.2. Parental polymorphism survey among Chirya 3 (resistant) and Sonalika (susceptible) with SSR primers (1) Xgwm148 (2) Xgwm371 (3) Xgwm111 (4) Xgwm008 (5) Xgwm455 (6) Xgwm1037 and (7) Xbarc263, where M = 100 bp DNA size marker, C = Chirya 3, S = Sonalika.



4.2. Validation of SSR markers associated with spot blotch resistance

The two polymorphic SSR markers, Xgwm 148 and Xgwm 111 were further validated in a RIL population derived from the cross Yangmai 6 and Sonalika. The phenotypic data in the form of disease severity (%) of individual RILs was available through previous experiments. Each line was characterized for their level of resistance to spot blotch on the basis of area under disease progress curve (AUDPC). SSR marker *Xgwm148* linked with *QSb.bhu-2B* was validated in the RILs (Fig. 4.3). In RIL population, marker *Xgwm148* amplified a polymorphic product of approximately

180 and 170 bp in resistant (Yangmai-6) and susceptible parent (Sonalika), respectively. In most of the resistant and moderately resistant lines the corresponding resistant band was amplified in RIL population which indicated the association of this marker to spot blotch resistance. The banding pattern shown in figure is in accordance to the report of Kumar et al. (2009). The validation of this marker suggests that it can be used for marker assisted selection (MAS) of spot blotch resistance.

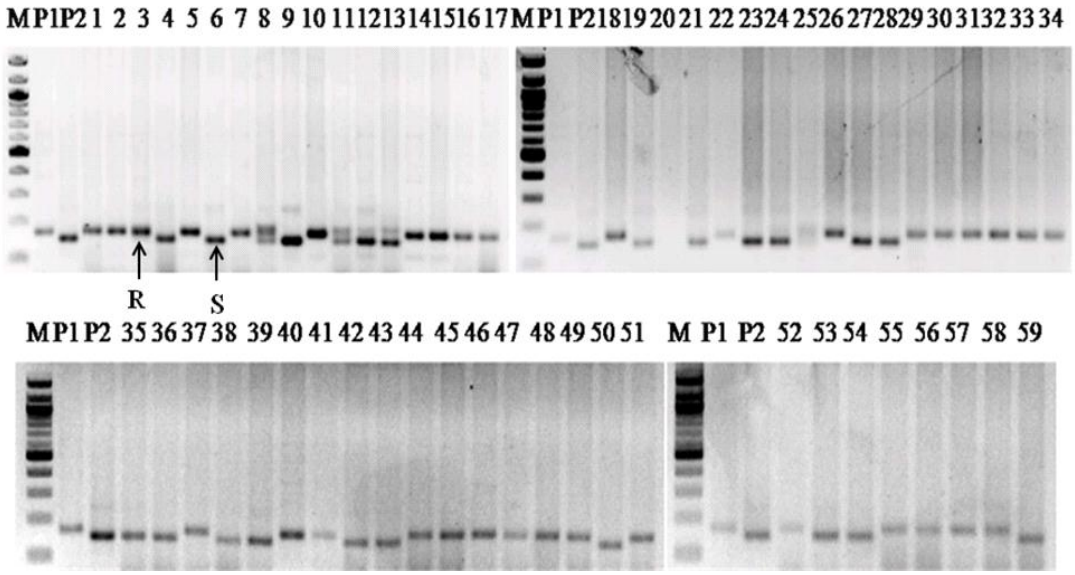


Fig. 4.3. Validation of SSR marker *Xgwm148* linked with spot blotch resistance QTL *QSB.bhu2B* in the RIL population (1-59 lines) derived from the cross Yangmai-6 (resistant; P1) x Sonalika (susceptible; P2). M is 100 bp DNA size marker.

4.3. Marker assisted selection in a back cross population

The SSR markers Xgwm148 and Xgwm111 were further used for foreground analysis of individual plants of back cross population of (HUW-234 x 2nd CSISA 6713) x HUW-234. In BC_1F_1 population, two types of banding patterns were amplified, *i.e.*, homozygous susceptible types and heterozygous types broadly into 1:1 ratio. The individual plants which amplified heterozygous banding pattern were selected for making back crosses with HUW-234. Plant number 8, 9, 11, 14, 16, 27, 28, 29 were selected for back crossing since they showed heterozygotic condition for both the markers (figure 4.4).

Marker-assisted foreground selection was proposed by Tanksley (1983) and investigated in the context of introgression of resistance genes by Melchinger (1990). If in BC_1 generation more than one individual satisfying the strongest condition is found, selection between them can be performed on the basis of analysis of other marker loci

(located either on the carrier or on non-carrier chromosomes) to determine the most desirable individual for producing BC_2 (Tanksley et al., 1989).

The success of markers assisted backcross breeding (MAB) depends upon several factors, including the distance between the closest markers and the target gene, the number of target genes to be transferred, the genetic base of the trait, the number of individuals that can be analyzed and the genetic background in which the target gene has to be transferred, the type of molecular marker(s) used, and available technical facilities (Weeden et al., 1992; Francia et al., 2005). Identification of molecular markers that should co-segregate or be closely linked with the desired trait (if possible, physically located beside or within genes of interest) is a critical step for the success of MAB. The most favourable case for MAB is when the molecular marker is located directly within the gene of interest (direct markers). MAB conducted using direct markers is called gene assisted selection (Dekkers, 2003). Alternatively, the marker is genetically linked to the trait of interest. Before a breeder can utilize linkage-based associations between a trait and markers, the associations have to be assessed with a certain degree of accuracy so that marker genotypes can be used as indicators or predictors of trait genotypes and phenotypes.

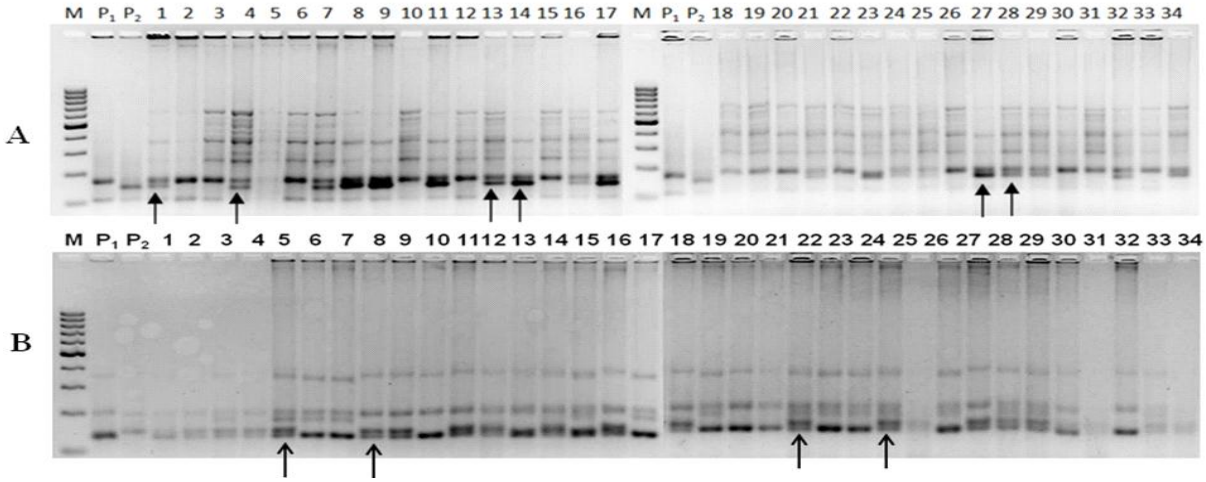


Fig. 4.4. Foreground analysis of BC_1F_1 population derived from the cross (HUW-234 x 2nd CSISA 6713) x HUW-234 with SSR markers (A) *Xgwm148* (B) Xgwm 111 associated with spot blotch resistance QTLs *QSb.bhu-2B* and *QSb.bhu-7D*, respectively. Lane M =100 bp DNA size marker; P_1 = HUW-234 (susceptible parent) and P_2 = CSISA 6713 (resistant parent) 1-34 are individual backcross plants. *Arrow* indicates some of the heterozygous BC_1F_1 individual plants selected through foreground selection.

The lower the genetic distance between the marker and the gene, the more reliable is the application of the marker in MAB because only in few cases will the selected marker allele be separated from the desired trait by a recombination event. The presence of a tight linkage between desirable trait(s) and a molecular marker(s) may be useful in MAB to increase gain from selection. Based on studies by Lee (1995) and Ribaut et al. (2002), it could be generalized that whenever a target gene is introduced for the first time from either wild or unadapted germplasm, flanking markers as close as 2 cM is considered an ideal option, while in the transfer of the same target gene in subsequent phases from elite into elite lines, positioning the flanking markers at 12 cM might be effective in reducing the required size of the backcross population.

MAB has generated a good deal of expectations, which in some cases has led to over-optimism and in others to disappointment because many of the expectations have not yet been realized. Although documentation is limited, the current impact of MAB on products delivered to farmers farmers seems small (for the reasons given above, it is indirectly present in many F_1 hybrids). New developments and improvements in marker technology, the integration of functional genomics with QTL mapping, and the availability of more high-density maps are the other factors that will greatly affect the efficiency and effectiveness of QTL mapping and MAB in the future. The development of high-density maps that incorporate new marker types, such as single nucleotide polymorphisms (SNPs) and expressed sequence tags (EST) will provide researchers with a greater arsenal of tools for QTL mapping and MAB. The number of EST and genomic sequences available in databases is growing rapidly (especially from genome sequencing projects), and the accumulation of these sequences will be extremely useful for the discovery of SNPs and data mining for new markers in the future (Gupta et al., 2001; Kantety et al., 2002). It is expected that the development of high resolution maps will also facilitate the isolation of actual genes (rather than markers) via ‘map based cloning’ (also ‘positional cloning’), which involves the use of tightly linked markers to isolate target genes

The BC_1F_1 plants selected in this study on the basis of foreground selection will be further subjected to backcrossing with the recurrent parent (HUW-234) and/or background selection. Thus, selected plants will finally be tested under detailed

agronomic trails for identification of such plants which are similar to recurrent parent in yield and other traits but resistant to spot blotch.

SUMMARY AND CONCLUSION

Spot blotch caused by *Bipolaris sorokiniana* is a destructive disease of wheat in warm and humid wheat growing regions of the world. Several QTLs have already been identified for spot blotch resistance, including three resistant sources viz., Ning 8201, Yangmai 6 and Chirya 3 and common susceptible cultivar 'Sonalika'. However, more information with respect to the identification of QTLs in different genetic background is required for better understanding of the allelic relationships and to make an effective breeding program. Therefore, the present study entitled "Validation and utility of SSR markers associated with spot blotch disease resistance in wheat (*Triticum aestivum* L.)" was undertaken to validate the efficacy of the markers closely associated with the spot blotch resistance QTLs and their further utilization in marker assisted backcross breeding programme.

Parental polymorphism survey was firstly performed among parental genotypes Yangmai 6, Chirya 3 (resistant parents) and Sonalika (susceptible parent) using 22 SSR markers to validate these markers linked to spot blotch resistance. Out of 22 SSRs, only two markers produced reproducible and polymorphic bands. Xgwm 148 produced 170 bp band in resistant parent, while Xgwm 111 amplified 150 bp band in the resistant parent. SSR marker Xgwm148 is linked with QTL, *QSb.bhu-2B and* Xgwm111 is linked with *QSb.bhu-7D*. Rest SSR markers did not show polymorphism in agarose gel electrophoresis in both the resistant parent and Sonalika. This may be due to low resolving power of agarose as compared to polyacrylamide gel electrophoresis (PAGE) and automated gel electrophoresis systems.

Further, the two polymorphic SSR markers, Xgwm 148 and Xgwm 111 were validated in a RIL population derived from the cross Yangmai 6 and Sonalika. The phenotypic data in the form of disease severity (%) of individual RILs was compared with the genotypic data thus generated. Each line was characterized for their level of resistance to spot blotch on the basis of area under disease progress curve (AUDPC). In the RIL population, marker *Xgwm148* amplified a polymorphic product of approximately 180 and 170 bp in resistant (Yangmai-6) and susceptible parent (Sonalika), respectively. In most of the resistant and moderately resistant lines the corresponding resistant band was amplified

in RIL population which indicated the association of this marker to spot blotch resistance.

The SSR markers Xgwm148 and Xgwm111 were further used for foreground analysis of individual plants of back cross population of (HUW-234 x 2nd CSISA 6713) x HUW-234. In BC_1F_1 population, two types of banding patterns were amplified, *i.e.*, homozygous susceptible types and heterozygous types broadly into 1:1 ratio. The individual plants which amplified heterozygous banding pattern were selected for making back crosses with HUW-234. Plant number 8, 9, 11, 14, 16, 27, 28, 29 were selected for back crossing since they showed heterozygotic condition for both the markers.

The closely linked markers Xgwm148 to the QTL on chromosome 2B and Xgwm111 to the QTL on chromosome 7D are potentially diagnostic markers for spot blotch resistance. The BC_1F_1 plants selected in this study on the basis of these two markers will be further subjected to backcrossing with the recurrent parent (HUW-234) and/or background selection. Thus, selected plants will finally be tested under detailed agronomic trails for identification of such plants which are similar to recurrent parent in yield and other traits but resistant to spot blotch.

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