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Identification and Validation of a Microsatellite Marker for the Seedling Resistance Gene *Lr24* in Bread Wheat

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Abstract

The background of PBW343, the high yielding and widely cultivated bread wheat cultivar of the Indian sub-continent was utilized. We were able to identify specific microsatellite markers for *Agropyron elongatum* derived seedling resistance gene *Lr24*. The two markers, Xgwm114 and Xbarc71 were mapped at a distance of 2.4 cM from *Lr24* locus. They can be unquestionably utilized as landmarks for identification of these genes. An F_2 population segregating for *Lr24* and *Lr48* in the background of PBW343 was utilized for this study. Though phenotypic reaction of the plants of the progeny populations to leaf rust infection was recorded in the seedling stage, it was difficult to perform the same in the adult plant stage as more than one gene effective against the same pathogen act mutually thus making it difficult to interpret and differentiate the resistance reaction of each of the two different genes. This is a major aspect of concern for many plant breeders in various gene pyramiding experiments since differentiating virulences of pathogens for each and every gene utilized cannot be available within all geographic locations. Molecular markers play a significant role in all such cases.

Keywords: Microsatellite markers; *Lr24*; Seedling resistance; Bread wheat

Introduction

Puccinia triticina, the causative of leaf rust, is a considerable pathogen in wheat which results in substantial amount of losses by decreasing the yield in almost all wheat growing areas of the world. Deployment of rust resistance genes into the cultivar is being used to provide resistance against the locally prevalent pathogen races as an economical, enduring and eco-friendly measure [1]. Diversity for resistance to leaf rust is available in the germplasm of related wheat genera and there are many affirmative reports which assure the effectiveness of genes originating from wild relatives of the cultivated wheat in conferring long lasting rust resistance [2]. So far more than 60 *Lr* genes have been identified in various wheat backgrounds [3]. *Lr24* is one such resistance gene transferred into bread wheat from *Agropyron elongatum* which confers resistance right from the seedling stage all through the life of the plant (seedling resistance). *Lr24* is being used in major wheat breeding and pyramiding programmes as a means to provide resistance to otherwise susceptible cultivars [4,5]. However, many of the seedling resistance genes when incorporated singly tend to become ineffective due the constantly evolving physiological races of the pathogen. To suppress such reviving pathogenesis, an approach to stack more than one gene into the same background has been suggested and is pursued in most of the rust resistance initiatives [6]. In this study, we have employed one such F_2 population which segregates for two *Lr* genes. One of them is *Lr24* and the other is a hypersensitive recessive adult plant resistance (APR) gene, *Lr48* which confers resistance to the plant only from the time the plant reaches its booting stage and in a way decreases the selection pressure on the pathogen thus inhibiting the development of new races [2]. Differentiating the phenotypic resistance reaction of two discrete *Lr* genes existing in the same cultivar is practically impossible in the absence of individual *Lr* gene specific pathogen virulences. In such cases, the presence of exclusive DNA based markers which act as indices for each *Lr* gene will be valuable. Molecular markers are utilized on a huge scale to reduce cumbersomeness and enable rapid detection of specific *Lr* genes. Codominant molecular markers are useful in breeding programmes as only they are efficient in differentiating the heterozygous and homozygous status in plants exhibiting resistance to the pathogen infection since only the latter are significant to forward for further generations. To enable the early selection of homozygosity at the adult plant rust resistance locus, two RAPD markers $S3_{450}$ and $S336_{775}$ have been utilized as a co-dominant marker system [7]. The SSR marker polymorphic for *Lr24* identified in our lab will be useful in wheat breeding populations and can help in fixing the genes by the F_2 population level itself without any further investment till F_5/F_6 generations.

The findings presented in this paper are a result of the work performed in N.P.F., I.A.R.I., New Delhi, India during the period 2007 to 2010.

Materials and Methods

Plant material

An F_2 population developed from the cross between the most widely cultivated and successful Indian wheat cultivar PBW343 carrying the gene *Lr24* (PBW343-*Lr24*) developed at IARI, India and the Australian cultivar Condor derived CSP44 line (with WW80/2*WW1511/Kalyansona parentage) carrying the gene *Lr48* (CSP44-*Lr48*) was used for the study. *Lr24* is a seedling resistance gene thus conferring resistance in all stages of the plant and *Lr48* is an adult plant resistance gene, effective only from the time the plant reaches booting stage. The zygosity of each of the F_2 individual plants was established both by F_3 progeny testing and co-dominant molecular marker analysis. A set of

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30 plants per each F_2 family were sown to erect the F_3 population. The experiments were conducted in the controlled conditions of National Phytotron Facility, IARI, New Delhi.

Pathotype of the fungal pathogen

The inoculum of the most virulent *Puccinia recondita* pathotype, 77-5 (121R63-1) was obtained from the Directorate of Wheat Research, Regional Station, Flowerdale, Shimla. Inoculation of the spores of the pathotype was done by spraying inoculum suspended in water fortified with Tween-20® (0.75 µl/ml) at an average concentration of 20 urediospores/microscopic field (10x × 10x).

DNA extraction

Young leaves from parents and individuals of the segregating population were collected, lyophilized and ground in liquid nitrogen using a pestle and mortar. DNA extraction was performed by the micro-extraction method described by Prabhu et al. [8]. Final concentration of DNA samples was maintained at 10 µg/µl for PCR reactions.

Seedling test

After sampling for DNA extraction, seedlings 8-10 days old at decimal code DC 11 stage were inoculated during the evening hours [9]. Prior to inoculation, the plants were sprayed with water to provide a uniform layer of moisture on the leaf surface. After inoculation, the seedlings were incubated for 36 h in humid glass chambers at a temperature of 23 ± 2°C and more than 85% relative humidity after which, the pots were shifted to muslin cloth chambers in the same green house. The disease reaction was recorded 12-14 days after inoculation, using the scoring method described by Stakman et al. [10].

PCR amplification using molecular markers

Twenty-four SSR markers specific to the 3D chromosome were selected from published data [11,12].The SSR markers (custom synthesized at Biobasic Inc, Canada) were used to screen the parents (PBW343-*Lr24* and CSP44-*Lr48*), F_2 population (comprising homozygous resistant, homozygous susceptible and heterozygous plants) and bulks (resistant and susceptible).

The PCR reactions with SSR markers were performed in a 20 µl volume which consisted of 10 mM Tris HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each dNTP (MBI Fermentas, Germany), 40 ng of each of the forward and reverse primers, 0.75 U Taq DNA polymerase (Banglore Genei Pvt. Ltd., India) and 50 ng template DNA. PCR amplifications for RAPD markers were done following the protocol developed by Williams et al. [13] in 20 µl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each dNTP (MBI Fermentas, Germany), 0.2 µM of primer, 0.75 U Taq DNA Polymerase (Bangalore Genei Pvt. Ltd., India) and 10-15 ng of genomic DNA. The amplification reactions were carried in a PTC-200 thermal cycler (MJ Research, Las Vegas, NV, USA) with the following thermal profile – initial denaturation of 94°C for 10 min followed by 44 cycles of 94°C for 1 min (denaturation), 60°C, 55°C and 36°C (for Xgwm114, Xbarc71 and RAPD markers respectively) for 1 min (annealing), 72°C (extension) and a final extension step of 72°C for 10 min. This was followed by 4°C for 10 min. The amplified products from SSR markers and RAPD markers were separated on a 3% Metaphor® agarose gel and 2% Agarose gel respectively, in 1X TAE buffer at 80 V for 3 hrs to separate the fragments. The gels were later stained with 10 mg/ml ethidium bromide and viewed in a digital gel documentation system (Alpha Innotech, San Leandro, CA, USA).

Bulked segregant analyses were done to identify the markers' linkage to the dominant resistance gene. Ten randomly selected plants from the homozygous resistant and homozygous susceptible F_2 plants were used to prepare bulks [14]. The bulks differentiated for the presence and absence of the leaf rust resistance gene *Lr24* (Figure 1).

Statistical analysis

Segregation ratios were analyzed using a chi-square test. The individuals from the crosses that were scored as resistant and susceptible in the progeny populations were subjected to chi-square test for goodness of fit to test the deviation from the theoretically expected Mendelian segregation ratios. The linkage analysis was carried out using Mapmaker version 3.0 [15].

Results

Phenotypic reaction

The parent PBW343+*Lr24* showed resistance to rust infection and recorded infection type (IT) of ; to 1 while the other parent CSP44 showed high level of susceptibility (IT of 33^+) at seedling stage. At adult plant stage the parent PBW343+*Lr24* remained resistant while the other seedling susceptible parent showed resistance by recording a ; reaction type. All the F_1 plants remained resistant to the rust infection recording an infection type of ; to 1.

46 seedlings of the F_2 population showed susceptibility to the leaf rust infection while the remaining 136 plants remained resistant by expressing the seedling resistance conferred by the dominant resistance allele of the *Lr24* locus and the population followed a monogenic segregation ratio. All the susceptible F_2 derived F_3 families remained susceptible whereas only 41 out of the 136 resistant F_2 derived F_3 families were homozygous for resistance. The remaining 95 families were heterozygous thus distributing the F_2 genotypes into 1R:2R:1S monogenic segregation ratio (Table 1). The phenotypic expression of adult plant resistance could not be examined due to the interference of the dominant seedling resistance gene *Lr24* in the same genetic background.

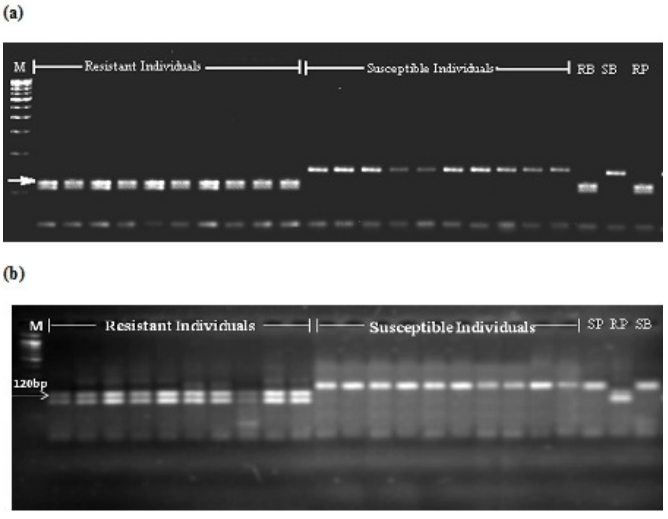


Figure 1: Screening of the SSR markers (a) Xgwm114 and (b) Xbarc71 on the bulked DNA constituent F_2 plants of the cross PBW343 + *Lr24* x CSP44*Lr48* for genetic linkage analysis.

F_1	F_2 reaction*				
R	Total tested	R	S	$\chi^2_{3:1}$	P
16	182	136	46	0.0072	0.9323

No. of F_2 families for F_3 testing		R in F_2				S in F_2	
R	S	NSeg	Seg	$\chi^2_{1:2}$	P	NSeg	Seg
136	46	41	95	0.6208	0.7331	46	0

*R: Leaf Rust Resistant; S: leaf rust susceptible, #NSeg: Non-Segregating Family for Leaf Rust; Seg: Segregating Family for Leaf Rust.

Table 1: Reaction of wheat plants in F_1, F_2 and F_3 generations of the cross Pbw343+*Lr24* X CSP44+*Lr48* at seedling stage (DC 11) to infection by the leaf rust pathotype 77-5 under controlled conditions.

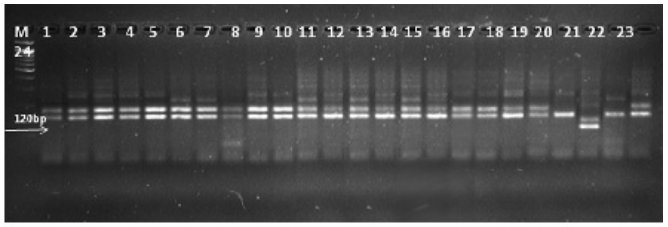
Molecular marker study

Out of twenty-four SSR markers specific to the 4AL chromosome, only two markers, Xgwm114 (F: 5' ACAAACAGAAAATCAAAACCCG 3' R: 5'ATCCATCGCCATTGGAGTG3') with annealing temperature of 60°C and Xbarc71 (F:5'GCGCTTGTTCCTCACCTGCTCATA3' R: 5'GCGTATATTCTCTCGTCTTCTTGTTGGTT3') with annealing temperature of 55°C were identified to be polymorphic between the parents. 10 randomly selected samples were taken from the resistant and susceptible plants to prepare bulks for bulk segregant analysis (Figure 1). The markers were found to be putatively linked to the *Lr24* locus. The polymorphic SSR markers were analyzed on the 182 F_2 plants for linkage analysis with the *Lr24* locus. Both the markers were associated with the *Lr24* locus and located at a distance of 2.4 cM from it (Table 2). The PBW343-*Lr24* resistance allele linked SSR marker allele amplified a 120 bp fragment and the CSP44 susceptibility allele linked marker allele amplified a 151 bp fragment. The marker Xgwm114 differentiated the population into 45 homozygous resistant, 89 heterozygous resistant and 48 homozygous susceptible plants. The 120 bp fragment was specific to the *Lr24* resistance allele and did not amplify in other *Lr* genes carrying lines from other native and alien sources (Figure 2).

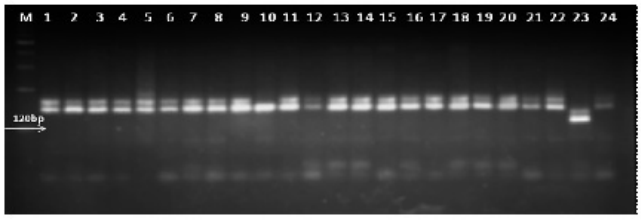
By employing the flanking RAPD markers $S3_{450}$ (5'CATCCCCTG3') and $S336_{775}$ (5'TCCCCATCAC3') linked respectively to the recessive resistance allele and dominant susceptible allele of the *Lr48* locus; plants which were homozygous for recessive APR gene *Lr48* were identified, as these two markers served as one co-dominant marker system capable of identifying both dominant and recessive alleles of heterozygous plants. 10 F_2 plants were found to be the homozygous at both the dominant seedling resistance locus of *Lr24* and the recessive adult plant resistance locus of *Lr48* (Table 3).

Discussion

The *Lr24* gene transferred from *Agropyron elongatum* is important to wheat since there are reports of its locus being linked to the stem rust resistance gene *Sr24* [16]. The *Agropyron* chromosome segment is located on the satellite of chromosome 1B and the translocation chromosome designated as T1BL·1BS-3Ae#1L. T1BL·1BS-3Ae#1L was inherited from Teewon wheat and carries resistance genes to stem rust (*Sr24*) and leaf rust (*Lr24*). *Sr24* is highly effective against TTKS (Ug99), a recently emerged race with virulence to *Sr31* that is considered to be a serious threat to wheat crop produce all over the world [17]. Though Xgwm114 has not been testified in populations segregating for stem rust resistance in this experiment, an assumption can be made that the identification of presence of *Lr24* through this marker also suggests the existence of stem rust resistance. Such a marker will thus be economically important in wheat breeding programmes. Pathotypes virulent on *Lr24* have been reported from North America , South America and South Africa [5,18-21]. This requires that *Lr24* should be used only in combinations with other *Lr* genes. Worldwide, no virulence has been reported on the combination *Lr9* and *Lr24* [6].



Legend (a): Lanes 1-20, NILs carrying different non-alien *Lr* genes in the following order: *Lr1*, *Lr2a*, *Lr3*, *Lr3ka*, *Lr10*, *Lr11*, *Lr12*, *Lr13*, *Lr14a*, *Lr14b*, *Lr14ab*, *Lr15*, *Lr16*, *Lr17*, *Lr18*, *Lr20*, *Lr22b*, *Lr27+Lr31*, *Lr30*, *Lr33;* lane 21: CSP44*Lr48*, 22: *Lr24*, 23: *Lr49*, 24: Agra Local, M: 100-bp DNA ladder.



(b) Lanes 1-20, NILs carrying different alien *Lr* genes in the following order: *Lr9*, *Lr19*, *Lr21*, *Lr22a*, *Lr23*, *Lr24*, *Lr25*, *Lr26*, *Lr27*, *Lr28*, *Lr29*, *Lr32*, *Lr35*, *Lr36*, *Lr37*, *Lr39*, *Lr40*, *Lr41*, *Lr42*, *Lr43* ; lane 21: CSP44*Lr48*, 22: *Lr44*, 23: *Lr24*, 24: Agra Local, M: 100-bp DNA ladder.

Figure 2: Validation of the SSR marker Xgwm114 linked to leaf rust resistance gene *Lr24* on a set of 21 (a) non-alien and (b) alien *Lr* genes in wheat.

Loci	Segregants									Marker Fragment		*Lr24* gene & marker fragment		Linkage	
	++/R	-+/R	--/R	++/H	-+/H	--/H	++/S	-+/S	--/S	$\chi^2_{1:2:1}$	P	$\chi^2_{1:2:1:2:4:2:1:2:1}$	P	χ^2	P
***Lr24*-Xgwm11**	41	0	0	4	89	2	0	0	46	0.1866	0.9109	326.87	0.00	329.68	0.00
***Lr24*-Xbarc71**	41	0	0	2	93	0	2	0	44	0.0987	0.9518	326.69	0.00	336.70	0.00

R: Homozygous Resistant; H: Heterozygous; S: Homozygous Susceptible
Marker Fragment
++: Homozygous Resistant
+-: Heterozygous
--: Homozygous Susceptible

Table 2: Test of linkage between the leaf rust resistance gene *Lr24* and SSR markers (Xgwm114 and Xbarc71) in the F_2 population of the cross PBW343+*Lr24* X CSP44-*Lr48* of wheat.

F_2 plant No.	$S3_{450}$ SCAR	$S336_{775}$ SCAR	Genes Carried (*Lr*)	F_2 No.	$S3_{450}$ SCAR	$S336_{775}$ SCAR	Genes Carried (*Lr*)
1	-	+	24,	31	-	+	24
2	-	+	24	32*	+	-	24,48
3 *	+	-	24,48	33	-	+	24
4	-	+	24	34	-	+	24
5	+	+	24,48	35*	+	-	24,48
6	-	+	24,	36	-	+	24
7 *	+	-	24,48	37	+	+	24,48
8	-	+	24	38	-	+	24
9	-	+	24	39*	+	-	24,48
10*	+	-	24,48	40	-	+	24
11	-	+	24	41	-	+	24
12	-	+	24				
13	+	+	24,48				
14	-	+	24				
15	-	+	24				
16	+	+	24,48				
17	-	+	24				
18*	+	-	24,48				
19	-	+	24				
20*	+	-	24,48				
21	-	+	24				
22*	+	-	24,48				
23	-	+	24				
24	-	+	24				
25	+	+	24,48				
26	-	+	24				
27	-	+	24				
28*	+	-	24,48				
29	-	+	24				
30	-	+	24				

Table 3: Marker assisted selection in segregating F_2 progeny of a cross *Lr24* and *Lr48* for *Lr48* in PBW343 background. Only 41 plants with homozygous bands for *Lr24* locus were screened with $S3_{450}$ and $S336_{775}$ RAPD markers. *homozygous for *Lr48*.

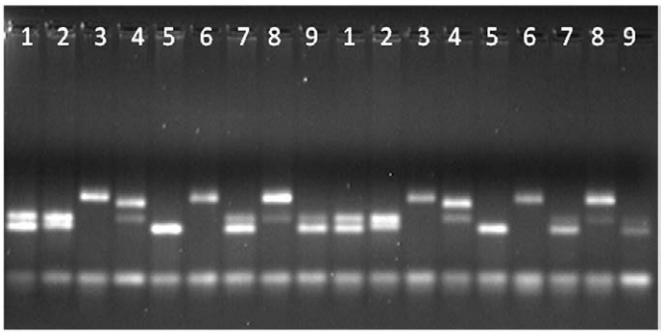


Figure 3: A gel depicting the extent of similarity in segregation between Xgwm114 and Xbarc71 in a segregating F_2 population.

Lr24 still continues to be highly effective in India and three cultivars Vidisha, Vaishali (DL784-3) and HW2004 carrying both *Lr24*/*Sr24* have been released for commercial cultivation in India.

Several markers showing a dominant inheritance pattern have been reported to be linked to the *Lr24* resistance locus. A SCAR marker developed by Cherukuri et al., [22] in the same laboratory is currently being successfully employed to track the transfer and establishment of this gene in different genetic background. A PCR-based DNA-STS marker, six RFLP markers completely linked to *Lr24* - one inherited as a codominant marker (PSR1205), one in coupling phase (PSR1203), and four in repulsion phase (PSR388, PSR904, PSR931, PSR1067) were reported to be inherited with *Lr24*. A RAPD marker, OPJ-09 also was shown to be in complete linkage to the *Lr24* resistance gene [23]. The markers have been used in wheat breeding experiments employing MAS [24]. There are other reports of plymorphic RAPD and SCAR markers for *Lr24* by Dedryver et al., [25].

Simple sequence repeats DNA called microsatellites are ubiquitously distributed within the eukaryotic genome, and SSR is more polymorphic than other marker systems [12,26]. The genetic map constructed by Roder [26] uses microsatellite markers located on seven chromosome groups and Xgwm114 was located on chromosome arm 3B and 3D. Xgwm114 is reported to be associated with powdery mildew resistance in wheat [27]. Three microsatellite markers, Xgwm247, Xgwm181 and Xgwm114 located on chromosome 3BL, were shown to be associated with the stem solidness locus and with sawfly cutting in durum wheat [28]. McIntosh [29] reports the location of *Lr24* on the long arm of 3D and the current experiment shows the linkage of Xgwm114 with the locus of *Lr24*.

Xbarc71 is reported to be sharing the same position on the long arm of 3D chromosome along with Xgwm114 in the chromosome map developed by Torada et al., [11]. This was reconfirmed by the pattern of segregation shown by both the markers in the F_2 population (Figure 3). However, the same markers are placed considerably far apart in the chromosome map developed by Somers et al., [30,31]. In the present experiment, both the markers were able to differentiate the presence of *Lr24* in segregating populations almost with equal precision and here we report that they can be used interchangeably to identify homozygous *Lr24* locus.

Such codominant SSR markers will be extremely useful in large scale wheat breeding programmes where selection of homozygous resistant plants which potentially carry the resistance genes will be achieved at very early generations. A segregating F_2 population will suffice to select plants in which the gene is fixed.

In this experiment, the pair of the RAPD markers also was valuable only because they could be employed as a codominant marker system. $S3_{450}$ was linked to the recessive adult plant resistance allele and $S336_{775}$ was linked to the dominant susceptibility linked allele of the *Lr48* locus. Since both the alleles are easily differentiated, we could select those plants homozygous for the recessive resistance allele linked adult plant resistance at the *Lr48* locus.

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