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Research Paper

Microsatellite markers for the *Triticum timopheevi*-derived leaf rust resistance gene *Lr18* on wheat 5BL chromosome

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Leaf rust, caused by *Puccinia triticina*, is a common wheat disease worldwide. Developing resistant cultivars through deploying new or pyramiding resistance genes in a suitable line, is the most effective approach to control this disease. However, to stack genes in a genotype, efficient and reliable markers are required. In the present study, F_2 plants and their corresponding F_3 families from a cross between the resistant line; Thatcher (Tc) Lr18, and the susceptible cultivar 'Boolani' were used to map rust resistance gene, *Lr18* using SSR markers on chromosome 5BL of hexaploid wheat. The *P. triticina* pathotype no 15 was used to inoculate plants. Out of 20 primers tested, eight showed polymorphism between the two parents and were subsequently genotyped in the entire F_2 population. The markers *Xgpw7425* and *Xwmc75* flanked the locus at a distance of 0.3 and 1.2 cM, respectively. Analysis of 81 genotypes from different backgrounds with these two markers confirmed their usefulness in screening absence or presence of *Lr18*. Therefore, these markers can be used for gene postulation and marker-assisted selection (MAS) of this gene in wheat breeding programs in future.

Key Words: genetic mapping, leaf rust, marker-assisted selection, molecular markers, *Puccinia triticina*, simple sequence repeats, wheat.

Introduction

Leaf rust caused by the fungus *Puccinia triticina* is one of the most widespread wheat diseases and can cause yield losses exceeding 50% in wheat growing areas (McCallum *et al.* 2012). The causal pathogen can be disseminated thousands of kilometers by wind (Kolmer 2005) and adapt to different temperatures (Huerta-Espino *et al*. 2011).

Although fungicides have been applied effectively to combat this disease, producing resistant cultivars remains as a cost-effective and environmentally safe method (Goutam *et al*. 2015). Yet, the effectiveness of resistance genes deployed in wheat cultivars changes by occurrence of new pathotypes and hence, genes must be pyramided and/or new resistance genes must be incorporated into a suitable background.

International wheat breeding has given major emphasis on genetic control of this disease by introducing new resistance genes into elite commercial cultivars (Kole *et al*. 2015). So far, more than 100 leaf rust resistance genes have been documented in wheat, of which 76 have been formally named (McIntosh *et al.* 2014) and many have been transferred into wheat from wild/cultivated relatives. However, these genes singly could provide low levels of resistance. Conversely, enhanced levels of resistance could be achieved through the combination of race-specific resistance genes and/or race non-specific ones such as *Lr13*, *Lr16* and *Lr34* (Dadkhodaie 2008, German and Kolmer 1992). A hindrance to this strategy is lack of appropriate pathotypes or epistasis in which the effect of a gene is masked by the second one.

The use of tightly linked molecular markers would allow selection of resistance gene(s) in segregating populations even in the absence of disease infection and therefore, could accelerate gene pyramiding (Mandoulakani *et al.* 2015) and the introgression of alien genes to hexaploid wheat (Asiedu *et al*. 1989). Many molecular markers linked to rust resistance genes, have been developed. These include but are not limited to Sequence-Tagged Site (STS), Sequence Characterized Amplified Region (SCAR) and Cleaved Amplified Polymorphic Sequences (CAPS) markers for the genes *Lr24* (Schachermayr *et al*. 1995), *Lr34* (Lagudah *et al.* 2009), *Lr35* (Gold *et al*. 1999), *Lr47* (Helguera *et al.* 2000), *Lr53* (Dadkhodaie *et al.* 2011), *Lr67* (Hiebert *et al.* 2010) and *Lr70* (Hiebert *et al.* 2014). In a study, Zhou *et al*. (2013) developed the STS marker *Hbsf-1* to detect *LrZH84* and

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Lr26. Similarly, Ayala-Navarrete *et al*. (2007) developed and mapped STS markers for the linked genes *Lr19* and *Sr25* on chromosome 7DL.

A significant number of leaf rust resistance genes originate from *Triticum aestivum* cultivars, but some were initially introgressed into common wheat cultivars from other species. The genes *Lr18* (McIntosh 1983) and *Lr50* (Brown-Guidera *et al*. 2003) have been transferred from *Triticum timopheevii* to bread wheat. *Lr50* is linked to the microsatellite markers *Xgwm382* and *Xgdm87* on 2BS chromosome (Brown-Guidera *et al*. 2003). The gene *Lr18* located on 5BL chromosome, confers resistance at seedling stage. Despite having the potential to be employed in breeding programs in some geographical regions (McIntosh *et al.* 1995), it has not been utilized extensively in wheat breeding programs. In addition, there is no marker available for this gene (Aoun *et al.* 2016, McCallum *et al.* 2012), and its recessive mode of action adds further to the complexity of selection because breeders can only select genes in homozygote status in the absence of molecular markers. These factors reiterate the need for a marker that could benefit the deployment of this gene in breeding programs. Therefore, the objective of this study was to identify markers for *Lr18* and validate their usefulness for marker-assisted selection (MAS).

Materials and Methods

Thatcher near-isogenic line (NIL) carrying the leaf rust resistance gene *Lr18* (TcLr18; RL6009) and the Iranian susceptible cultivar, 'Boolani' were crossed to produce F_1 and F_2 plants. The F_2 plants were rust tested and tagged as either resistant or susceptible, transplanted and harvested individually to produce F_3 families. Twelve seeds from each F_3 family were inoculated to identify the genotypes of F_2 plants, which were classified as resistant, segregating and susceptible. Goodness of fit for genetic ratios in each generation was assessed using the Chi-squared (χ^2) test.

Seedlings' response to leaf rust

The *P. triticina* pathotype no 15 with the avirulence/virulence formula (*Lr1*, *2a*, *2b*, *9*, *10*, *11*, *14a*, *16*, *17*, *18*, *19*, *20*, *24*, *25*, *27+31*/*Lr2c*, *3*, *3bg, 3ka*, *13*, *15*, *21, 26*, *28*, *30*, *35*, *37*) was multiplied on the susceptible cultivar 'Boolani' and used to inoculate plants. Both parents and all F_2, F_3 plants and the NILs were grown in 10 cm pots at 22 ± 2°C and inoculated with the above mentioned pathotype when second leaf emerged.

Briefly, urediniospores were mixed with Talcum powder (1:4) and rubbed off against upper surfaces of seedling leaves using a small duster. The plants were kept in a dark room (18°C and nearly 100% relative humidity) for 24 h and were subsequently moved to microclimate rooms maintained at 19–22°C. Infection types (ITs) were recorded 12 days after inoculation following the method of McIntosh *et al.* (1995) where plants with ITs '2' and lower were considered as resistant while those with ITs '3' and higher were classified as susceptible.

Marker genotyping

Genomic DNA was extracted from leaf tissues of parents and F_2 progenies using a modified CTAB method (Murray and Thompson 1980). Following extraction, DNA pellets were dried at 56°C for 5 minutes and dissolved in TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA) overnight at 4°C. A Nanodrop ND-1000 (Wilmington, USA) was used to measure DNA quality and quantity. The working solutions for both genomic DNA and primers were prepared in 200 µl volume at a concentration of 50 ng μl^{-1}.

Twenty genomic SSR primers specific for chromosome 5BL were selected from the GrainGenes website (http://wheat.pw.usda.gov/cgi-bin/GG3/browse.cgi.) to test polymorphism between parents and contrasting resistant and susceptible progenies.

Equal amounts of genomic DNA from 10 susceptible and 10 resistant F_2 individuals from the cross TcLr18 × Boolani were bulked according to the method of Michelmore *et al.* (1991). The polymerase chain reaction assays (PCR) were performed in 20 µl volumes on parents and bulk in an Eppendorf Mastercycler (ep Gradient 5341, Germany) following the conditions mentioned in **Table 1**. The reaction mixture included 1 µl of 50 ng μl^{-1} DNA template, 8 µl ddH_2O, 0.5 µl of 50 ng μl^{-1} for forward and reverse primers (Metabion, Germany) and 10 µl *Taq* DNA Polymerase 2× Master Mix Red (5 U μl^{-1}, Ampliqon, Denmark). Products were separated on a 3% agrose gel in 1× Tris borate buffer (54 g tris-borate, 27.5 boric acid, 200 ml EDTA). Gels were stained with ethidium bromide, visualized and photographed using Gel documentation system (Gene Flash, Syngene BioImaging) under UV light. DNA marker of 100 bp (DNA Ladder Plus, MBI Fermentas) was used to estimate the band size of each amplicon. The entire F_2 population was tested with polymorphic primers to determine the number of recombinants.

Data analysis

The mapping software Mapmaker/Exp ver. 3.0 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) was used to determine linkage between *Lr18* and polymorphic primers using threshold LOD score of 3.0, with a maximum Haldane distance of 50 cM. Genetic distances (cM) were calculated based on recombination frequencies using the Kosambi mapping function (1943). Linkage groups and LOD bars were drawn with MapChart v2.2 (Voorrips 2002).

Marker validation

The two tightly linked markers were used to analyze their validity on 37 isogenic lines for leaf rust resistance genes including the resistant parent, 19 Iranian commercial wheat cultivars, 14 wheat lines from CIMMYT, eight F_3 genotypes, two genotypes obtained from Switzerland and

Table 1. Sequences and thermocycle temperature profiles for polymorphic primer sets used in the present study

Locus	Sequence (5′-3′)	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Final extension
wmc75	GTCCGCCGCACACATCTTACTA						
	GTTTGATCCTGCGACTCCCTT G	94 (180)[a]	94 (45)	63 (45)	72 (45)	45	72 (600)
gpw7425	CTGAACTCGAAGAAGGCCAA						
	CCTCGATAGGCTCTGTCCTG	94 (180)	94 (45)	63 (45)	72 (45)	45	72 (600)
gwm499	ACTTGTATGCTCCATTGATTGG						
	GGGGAGTGGAAACTGCATAA	94 (180)	94 (45)	62 (45)	72 (45)	45	72 (600)
wmc118	AGAATTAGCCCTTGAGTTGGTC						
	CTCCCATCGCTAAAGATGGTAT	94 (180)	94 (45)	63 (45)	72 (45)	45	72 (600)
gwm118	GATGTTGCCACTTGAGCATG						
	GATTAGTCAAATGGAACACCCC	94 (180)	94 (45)	59 (45)	72 (45)	45	72 (600)
wmc783	AGGTTGGAGATGCAGGTGGG						
	TCTTCCTTCTCCTGCCGCTA	94 (180)	94 (60)	62 (45)	72 (45)	45	72 (600)
barc243	CGCAAAATCGAAATTAAAAATGGAAA						
	GATCCTCCTTTCAGCTGGCCTATTA	94 (180)	94 (45)	60 (45)	72 (45)	35	72 (600)

[a] Temperature "°C" (time "s").

the susceptible parent. The DNA extraction approach and PCR conditions were the same as before.

Results

The genotype TcLr18 inoculated with *Puccinia triticina* (*Pt*) pathotype no 15 displayed a very low IT (;1N) whereas 'Boolani' showed an IT of '3^{+}'. In F_2 and F_3 populations, ITs varied from highly resistant ';N' to highly susceptible '3^{+}' for different lines. Most susceptible plants showed ITs ≥ 3 similar to that of 'Boolani' with the exception of a few displaying ITs '2^{+}'. Based on these IT patterns, 101 F_2 plants were classified as susceptible and 29 as resistant which fitted as a 1:3 ratio ($\chi^2 = 0.5$, $P > 0.05$; **Table 2**, **Fig. 1**, **Supplemental Fig. 1**) indicating the recessive action of this gene. The segregation ratio of F_3 families conformed to 1 resistant: 2 segregating: 1 susceptible ($\chi^2 = 1.77$, $P > 0.05$; **Table 2**), confirming the F_2 results for one gene segregation. The number of F_3 families was slightly lower than that of F_2 because some plants did not set seeds.

Of 20 SSR primer sets located on chromosome 2B, eight showed polymorphism between two parents, resistant and susceptible bulks on 3% agarose gel. Six loci, namely wmc75, wmc783, wmc118, gwm118, gpw7425 and barc243 behaved as co-dominant, while the locus gwm499 was dominant amplifying only a fragment of 180 bp in the susceptible plant; 'Boolani'. This primer set showed a segregation ratio of 1:3 (**Table 3**). The primer set gpw7425 amplified a fragment of 180 bp in TcLr18 while a fragment of 300 bp was produced in Boolani (**Fig. 2A**). Similarly, primer sets wmc75, wmc118 and barc1032 amplified two fragments; 180 bp in TcLr18, and 200 bp in Boolani (**Fig. 2B**). However, barc1032 showed significant segregation distortion and was removed from the linkage group (**Table 3**). The gwm118 primer set produced an allele of 200 and 250 in TcLr18 and 'Boolani', respectively. The corresponding bands in resistant parent were 400 and 150 bp for wmc783 and barc243 while bands of 350 and 200 bp were produced

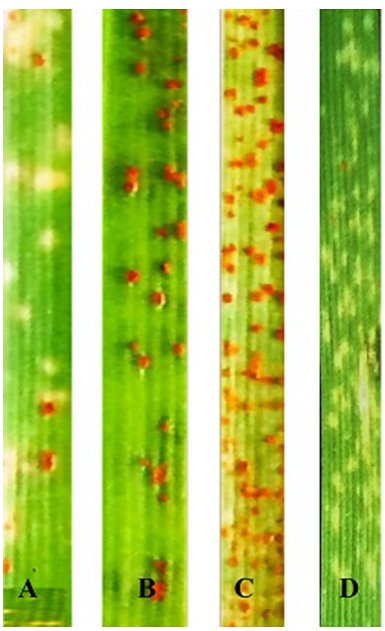

Fig. 1. Responses of wheat genotypes to *Puccinia triticina* pathotype no 15. A: Resistant parent Thatcher (Tc) Lr18, B: Susceptible parent (Boolani), C: susceptible F_2 plant, D: resistant F_2 plant.

Table 2. Frequencies of different phenotypes in F_2 and F_3 populations of wheat from a cross between the near isogenic line 'TcLr18' and 'Boolani' cultivar when inoculated with *Puccinia triticina* Eriks. pathotype no 15 at seedling stage

Generation	Total	Phenotype [a]			Expected ratio	Chi-square tests
		R	S	Seg		
F_2	134	29	105	–	1:3	$0.5 < \chi^2_{0.05}$
F_3	127	28	61	38	1:2:1	$1.77 < \chi^2_{0.05}$

[a] F_2 ratio is R:S; F_3 ratio is R:Seg:S.
R, Seg and S indicate resistant, segregating and susceptible, respectively.

Table 3. Segregation of SSR primers in F_2 lines from the cross between the near isogenic line 'TcLr18' and 'Boolani' cultivar on wheat 5BL chromosome

Marker	Product size (bp)		Ratio [a]		χ^2	*P*-value
	Resistant	Susceptible	bserved	Expected		
wmc75	180	200	45:60:29	1:2:1	5.28	0.01
gpw7425	180	300	42:63:29	1:2:1	3	0.05
wmc118	180	200	45:63:26	1:2:1	5.8	0.01
gwm118	200	250	45:63:26	1:2:1	5.8	0.01
wmc783	400	350	37:68:29	1:2:1	0.98	0.05
barc243	150	200	27:75:32	1:2:1	2.28	0.05
gwm499	–	180	28:106	1:3	1.2	0.05

[a] Ratio is for the marker alleles associated with *Lr18* alleles for homozygous resistance, heterozygous and homozygous susceptible, respectively.

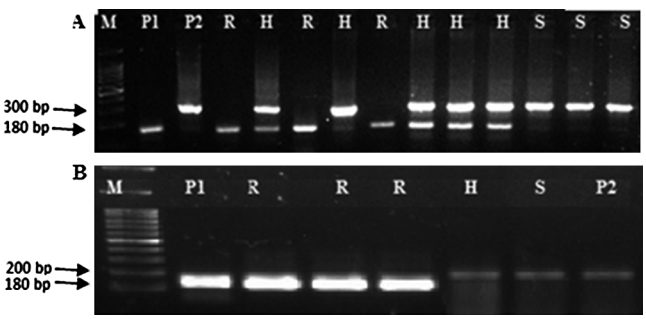

Fig. 2. Polymorphic markers on 3% agarose gel (A) The gpw7425 and (B) wmc75. The wheat genotypes from left to right include P_1; resistant parent, P_2; susceptible parent, R; resistant line, S; susceptible line and H; segregating line to *Puccinia triticina* pathotype no 15. M shows 100 bp DNA ladder.

in the susceptible parent, respectively. The χ^2 test for co-dominant loci indicated they fit the expected Mendelian ratio of 1:2:1 (**Table 3**).

These polymorphic primer sets were used to analyze all individuals in the F_2 population derived from the cross between TcLr18 and 'Boolani' (**Supplemental Table 2**). Based on marker segregation, a linkage group was constructed with seven SSRs at LOD 3.0, representing the 5BL chromosome (**Fig. 3**). The markers *Xgwm499* and *Xwmc75* were located distal to the gene while the remaining markers aligned proximal. The closest markers flanking the *Lr18* locus, were *gpw7425* and *wmc75* at a distance of 0.3 cM and 1.2 cM. These were followed by *gwm499* (2.3 cM). The remainder of markers i.e. *Xwmc783* (13.3 cM), *Xbarc243* (16.7 cM), *Xwmc118* (21.2 cM) and *Xgwm118* (28.8 cM) showed relatively higher frequencies of recombination with *Lr18* (**Fig. 3**).

Marker validation

The two tightly linked markers for *Lr18* (*Xgpw7425* and *Xwmc75*) amplified fragments of 300 and 200 bp respectively, in 72 wheat genotypes tested while both markers amplified fragments of 180 bp in seven genotypes thereby predicting the absence/presence of *Lr18* (**Supplemental Table 1**). The remaining two genotypes had bands similar to both parents and therefore were segregating for this gene. The marker *gwm499* was not validated because it amplified a band only in the susceptible parent.

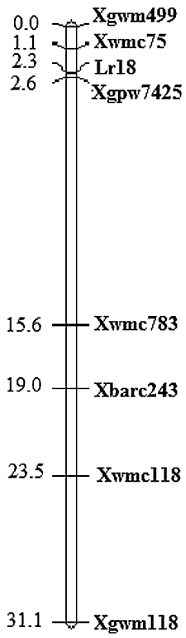

Fig. 3. The position of *Lr18* on the genetic linkage map of wheat chromosome 5BL in the F_2 population of TcL18/Boolani.

Discussion

Wild germplasms have contributed to wheat improvement as sources of new rust resistance genes (Friebe *et al.* 1996). However, many of these genes have not been extensively used in breeding programs due to the absence of appropriate DNA markers or pathotypes that facilitate their selection in segregating populations. For example, marker-assisted selection has not been utilized for the gene *Lr18* because markers have not been available yet. This gene has originated from *T. timopheevii* and is an all-growth-stage resistance gene (Dyck and Samborski 1968) indicating its potential use in wheat breeding programs.

In a study by Aliakbari *et al.* (2016), all Iranian pathotypes that were used to evaluate near-isogenic lines, had an incompatible interaction with *Lr18*. Of them, *Pt* 15 clearly differentiated the *Lr18*-carrying isogenic line from the susceptible genotype; 'Boolani'. Therefore, this pathotype was used to study phenotypes of F_2 and F_3 populations generated from the cross TcLr18/Boolani. The results of F_2 phenotyping, confirmed a 1:3 segregation ratio in F_2, and 1:2:1 in F_3 generation suggesting that this gene is controlled by a single recessive gene.

In this study, the leaf rust resistance gene *Lr18* was mapped to the terminal region of 5BL using seven SSR markers spanning a distance of 25.7 cM (**Fig. 3**). The only other leaf rust resistance gene located on chromosome 5B is

Lr52 which is associated with markers *gwm443* (Hiebert *et al.* 2005) and *gwm234* (Singh *et al.* 2010). The markers *Xwmc75* and *Xgpw7425* co-segregated for *Lr18* and flanked the gene at 1.2 and 0.3 cM distance, respectively. Both these markers were able to distinguish heterozygotes from homozygote susceptible. Though the latter was more closely associated with the gene, their combined use could be valuable in screening genotypes for the presence of this gene because they flank the gene. Moreover, both markers amplified one fragment in each parent and allele sizes are different that can be easily resolved and scored on agarose gel. Therefore, *Xgpw7425* and *Xwmc75* identified in this study are potential candidates for MAS. The marker *gwm499* was mapped about 2.3 cM distal to *Lr18*. However, this marker could not differentiate heterozygotes from susceptible homozygotes and therefore, could have contributed to a higher distance. Typically, SSR markers are co-dominant and observing dominant alleles could suggest changes in SSR primers at breakage points resulting in null alleles (Naik Vinod *et al*. 2015).

These linked markers were positioned on chromosome 5BL with a slight difference from previously reported linkage maps (Somers *et al*. 2004). The *wmc783* marker was distal to *wmc118* but here we reported it as proximal. The likely reason is possible amplification of a band different from the 144-bp locus mapped by Somers *et al.* (2004).

The markers reported in this study to be linked with *Lr18*, can be effectively utilized for MAS, as indicated by validation in different wheat genotypes. In the study by Aliakbari *et al.* (2016), none of the tested Iranian cultivars had *Lr18* and here, we have confirmed their results by both markers. Similarly, the agreement between these markers and the isogenic lines was high. The presence of *Lr18* was uncertain in CIMMYT-derived lines tested in this study. The marker analysis showed none of these genotypes had this gene while its presence was confirmed in nine genotypes. These results demonstrated the ability of these markers to predict absence/presence of this gene in high accuracy.

Despite the presence of virulent pathotypes for this gene, it still continues to provide levels of resistance in the world. The markers identified here can be used for pyramiding *Lr18* with other race nonspecific rust resistance genes and also be used for postulating this gene in wheat genotypes.

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