Molecular mapping of the *Aegilops speltoides*-derived leaf rust resistance gene *Lr36* in common wheat (*Triticum aestivum*)

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Research Article

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Abstract

Leaf rust caused by *Puccinia triticina* Eriks. is the most prevalent wheat rust worldwide and occurs annually wherever wheat is grown. The most economical and environmentally friendly method to control this fungal disease is genetic resistance, which is achieved through deploying effective resistance genes. Tightly-linked molecular markers facilitate gene tagging and their deployment with other resistance genes which in turn contribute to durable leaf rust resistance. The leaf rust resistance gene *Lr36* derived from *Aegilops speltoides* Taush and introgressed into hexaploid wheat by backcrossing, is located on chromosome 6BS. Despite detection of low frequencies of virulence for this gene, no molecular markers are available for marker-assisted selection in disease resistance breeding. Therefore, this research aimed at analysis of simple sequence repeat (SSR) markers linked with *Lr36* in an F₂ population from a cross between the *Lr36*-carrying line (ER84018) and the susceptible cultivar; Boolani. Of 36 primer pairs on chromosome 6BS tested for polymorphism in parents and resistant and susceptible bulks, eight were polymorphic of which the markers *Xgwm88* and *Xcfd13* flanked *Lr36* by 3.8 and 5.2 cM, respectively. Evaluation of these markers on 35 genotypes from different backgrounds including few Australian wheat leaf rust differential sets, Iranian landraces and cultivars, and several cultivars and/or breeding lines from Switzerland and Afghanistan, confirmed that they can be used in selection for *Lr36* in wheat breeding programs.

Introduction

Wheat (*Triticum aestivum*) is a strategic crop all over the world. However, its production is often threatened by biotic and abiotic stresses (Arzani and Ashraf 2017). Among biotic stresses, fungal diseases including the three rusts; leaf, stripe, and stem rusts, result in significant annual yield losses and detrimental effects on seed quality (McIntosh 1998). Leaf rust caused by *Puccinia triticina* Eriks. is the most prevalent wheat rust worldwide and occurs annually wherever wheat is grown (Kolmer 2013). The most economical and environmentally friendly method to control this disease is genetic resistance. However, rust pathotypes can mutate to virulence on wheat genotypes with previously effective resistance genes. Therefore, breeding for rust resistance can be a never-ending task that has to be supported by ongoing pathogenicity surveys to confirm continued resistance gene effectiveness.

Stacking multiple resistance genes is often difficult due to the unavailability of appropriate pathotype and/or problems concerning their presence in combination with other resistance genes conferring lower infection types. Recent advances in molecular biology have facilitated indirect selection of individual genes based on closely linked markers (Gupta et al. 1999), a procedure known as marker-assisted selection (MAS; Qureshi et al. 2018). Combinations of effective resistance genes not only increase the durability but, in many cases, also increase the degree of resistance. Simple sequence repeats (SSRs) or microsatellites are polymorphic tandem repeats of up to six base pairs (Tautz and Renz 1984) that can be identified and traced in genetic analysis or in selection. As markers, SSRs are highly polymorphic, low cost to phenotype, and their analysis can be automated (Hayden et al. 2006). Microsatellite consensus maps enable high density maps of the wheat genome. More than 80% of primer sets are genome-specific

and detect only a single locus in one of the three sub-genomes of bread wheat (A, B, or D). Publicly available databases provide opportunities to predict allele sizes in breeding populations and develop molecular and genomics strategies in gene mapping (Röder et al. 1998; Somers et al. 2004).

So far, more than 80 leaf rust resistance genes have been catalogued in wheat and its relatives (McIntosh et al. 2017; Qureshi et al. 2018), many of which have been mapped using molecular markers. These include but are not limited to *Lr18*, *Lr23*, *Lr48*, *Lr53*, *Lr65*, *Lr70*, *Lr71*, *Lr73* and *Lr80* (Aliakbari Sadeghabad et al. 2017; Chhetri et al. 2017; Dadkhodaie et al. 2011; Hiebert et al. 2014; Kumar et al. 2021; Mohler et al. 2012; Nsabiyaera et al. 2016; Park et al. 2014; Singh et al. 2013).

The seedling stage leaf rust resistance gene, *Lr36*, which was transferred from *Aegilops speltoides* Taush ($2n = 14$) into hexaploid wheat by backcrossing, is located on the short arm of chromosome 6B (Dvořák and Knott 1990). This gene has been rarely deployed in wheat cultivars despite having no linkage to undesirable quality or agronomic characters (Dvorak and Knott 1990) and being effective in the USA, China, Poland, Kazakhstan, Pakistan, Egypt, and Iran (Aktar-Uz-Zaman et al. 2017; Ali et al. 2018; Czajowski and Czembor 2016; Koyshybaev 2019; Li et al. 2016; Safavi and Afshari 2013; Zhang et al. 2019). Hence, it could be deployed with other rust resistance genes in gene pyramids or gene cassettes. However, linked molecular markers that benefit its deployment in wheat breeding programs have not been reported (McIntosh et al. 2017). Therefore, this study describes the molecular mapping of *Lr36* with SSR markers in a bi-parental population and assesses the efficiency of closely linked markers in different genetic backgrounds.

Materials And Methods

Plant materials and leaf rust inoculation

The line carrying the leaf rust resistance gene *Lr36* (ER84018; Dvorak and Knott 1990) was crossed with 'Boolani', the Iranian susceptible cultivar, to produce F_1 seeds. Subsequently, the resulting F_2 population with 171 individuals and their F_3 progenies were used for phenotypic and genetic analysis. Parental genotypes and all F_2 plants were grown in 10 cm diameter pots in a temperature-controlled greenhouse (18 ± 2 °C).

The *P. triticina* pathotype FHTQQ (isolate no. 92-23; virulent for *2c*, *3a*, *3bg*, *3ka*, *10*, *11*, *14b*, *16*, *17*, *26*, *30*, *B*), which produced infection types '0;1⁺N' and '33⁺' on ER84018 and Boolani (Table 1), respectively, was multiplied on a susceptible line. Fresh urediniospores were then mixed with talcum powder in a ratio of 1:4 and used to inoculate the test populations at the two-leaf stage. The inoculated plants were kept in a plastic-covered container at 100% humidity, 18°C, and dark condition for 24 hours prior to moving them to microclimate rooms at 18-24 °C. Infection types (ITs) were recorded according to the 1-4 scale described by McIntosh et al. (1995) approximately 10-12 days post inoculation (dpi) when the susceptible cultivar Boolani showed an IT of 33⁺. Plants with ITs less than 2 were considered resistant and those with IT 3 and higher were classified as susceptible.

After rust scoring, all 171 F₂ plants were transplanted to 20 cm diameter pots and individually harvested. Ten to 12 seedlings from F₃ family and both parents were inoculated as described previously and phenotypes were recorded as homozygous resistant, segregating heterozygous and homozygous susceptible. The segregation ratio from F₂ population was confirmed by testing F₃ families with the same pathotype as described before.

Marker analysis

Rust-free leaf tissue from each F₂ individual and both parents was used to isolate DNA using the CTAB method (Yu et al., 2017). A Nanodrop ND-1000 (Wilmington, USA) and 1% (w/v) agarose gel were used to evaluate the quality and quantity of DNA. Working solutions for both genomic DNA and primers were prepared in 200 µl volumes at a concentration of 50 ng µl⁻¹. As described by Michelmore et al. (1991), two DNA bulks (resistant and susceptible) were made from equal amounts of DNA from 20 most resistant and 20 most susceptible F₂ individuals.

Thirty-six pairs of SSR primers specific to chromosome 6BS (GrainGenes database, <http://wheat.pw.usda.gov>) were selected for evaluation of polymorphism on both resistant and susceptible parents and the corresponding bulks. Thereafter, the polymorphic markers (Table 2) were amplified on the entire F₂ population to determine the numbers of recombinants between the marker and leaf rust response loci. Polymerase chain reactions (PCR) were conducted in final volumes of 20 µl, comprising 1 µl DNA (50 ng µl⁻¹) as template, 1 µl of 50 ng µl⁻¹ each pair of forward and reverse primers (Metabion, Germany), 5 µl Taq DNA Polymerase 2x Master Mix Red (5 U µl⁻¹, Ampliqon, Denmark), and 12 µl ddH₂O. The PCR conditions were incubation at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for one minute, 56-64 °C for one minute depending on the primer pair, 72 °C for one minute, and the final extension at 72 °C of 10 minutes. The amplified products were separated in 2% agarose gels containing 1×TAE buffer (54 g Tris-borate, 27.5 boric acid, 200 ml EDTA) and stained with DNA Safe Stain (SinaClon BioScience, Iran). The bands were visualized and photographed in a Gel documentation system (Gene Flash, Syngene Biolmaging, Vaughan, Canada) under UV light. The band size of each amplicon was estimated using a DNA marker of 100 bp (DNA Ladder Plus, MBI Fermentas).

Statistical and linkage analyses:

Alleles for the SSR markers were scored A, B, and H for the resistant, susceptible and heterozygous, respectively, and ‘–’ was used for missing values (PCR failure). The phenotypic data of F₃ families were used to establish the genotypes of each F₂ individual. The c² test was used to evaluate the goodness of fit of observed and predicted ratios. Linkage analysis was conducted using JoinMap v0.5 (Van Ooijen 2019) with a threshold LOD ≥ 3.0 and the Kosambi mapping function (1943) based on the recombination frequencies.

Marker validation:

The efficiency of the closely linked markers to select *Lr36* was evaluated on 35 wheat genotypes from Australia, Afghanistan, Iran and Switzerland. The DNA extraction protocol and PCR conditions were the same as mentioned earlier. The materials from Australia were cultivars with known resistance genes used as differential testers in leaf rust studies. The other genotypes included Iranian cultivars and landraces, cultivars and breeding lines from Switzerland and/or Afghanistan genotypes, classified for the presence/absence of *Lr36* based on their pedigrees rust response in greenhouse.

Results

Leaf rust phenotyping

The F_2 population of 171 progenies segregated into 137 individuals with IT '0;1+2N' and 34 plants with IT '33+' fitting a 3:1 ratio ($\chi^2 = 2.38$, $P_{df=1} = 0.122$). Since eight F_2 plants did not set seed, the remaining 163 F_3 families were used for progeny testing, of which 50, 80 and 33 were homozygous resistant (*Lr36Lr36*), heterozygous (*Lr36lr36*), and homozygous susceptible (*lr36lr36*) (Fig. 1) and conformed to the expected 1:2:1 ratio for a single locus ($\chi^2 = 3.60$, $P_{df=2} = 0.165$; Table 3).

Genotyping and linkage map

Of the 36 pairs of SSR primer sets located on chromosome 6B, eight showed polymorphisms between the parents and bulks and hence, were used to genotype the whole F_2 population (Fig. 2). Based on PCR amplifications, the primer *barc198* produced a band of 130 bp in ER84018 while it amplified a 150 bp band in Boolani. The primer set *wmc105* amplified alleles of 220/350 bp in the resistant parent whereas amplicons of 200/320 bp were produced in Boolani. For the *gwm193* primer set, a band of 190 bp was observed in the resistant genotype while PCR produced a 175 bp fragment in the susceptible parent. The corresponding bands in the resistant and susceptible parents for *gwm88* were 160 bp and 140 bp, respectively. PCR amplification of *cfd13* detected a band of 480 bp in the resistant parent and 220 bp in the susceptible parent. Similar to *wmc105*, the *gwm518* primer produced two alleles in each parent; 160/210 bp and 170/220 bp in the resistant and susceptible genotypes, respectively. The *barc101* primer pair produced fragments of 170 and 160 bp in ER84018 and Boolani, respectively. Finally, the primer pair *wmc486* amplified a fragment of 200 bp in the susceptible cultivar; Boolani, the susceptible progenies and the heterozygotes while no band was amplified in the resistant parent and the homozygous resistant offsprings. The χ^2 tests for segregation of these loci, indicated that all primers except *wmc486* comply with the expected Mendelian ratio of 1:2:1 for co-dominant inheritance, whereas the *wmc486* primer showed a 3:1 ratio for dominance (Table 4) and at $LOD \geq 3$, showed a significant distortion, and was consequently categorized in a separate linkage group.

Overall, seven primer sets were mapped to one linkage group (with an LOD of 3 and greater) covering an interval of approximately 37.7 cM on the chromosome 6BS. The closest markers were *Xgwm88* and *Xcfd13* flanking *Lr36* at a distance of 3.8 and 5.2 cM, respectively. The markers *Xgwm518*, *Xgwm193*,

and *Xwmc105* were located 9.1, 10 and 15.2 cM from this gene, respectively. The marker *Xbarc101* was mapped 17.5 cM proximally while the marker *Xbarc198* was placed 20.2 cM distal to *Lr36* (Fig. 3).

Marker validation

To validate their usefulness, the closely linked markers; *Xgwm88* and *Xcfd13* were used to evaluate 35 wheat genotypes from Australia, Afghanistan, Iran and Switzerland. The markers *Xgwm88* and *Xcfd13* produced 220 and 140 bp amplicons, respectively, in all genotypes that lacked *Lr36* (Supplementary Table 1).

Discussion

Wheat relatives are valuable resources for resistance breeding to biotic stresses especially rust diseases. Though the *Aegilops speltoides*-derived leaf rust resistance gene, *Lr36*, has been mapped on the short arm of 6B (Dvorak and Knott 1990), it has not been deployed in wheat cultivars likely due to the unavailability of a diagnostic marker for its selection. Therefore, phenotyping and genotyping were performed on an F_2 population and its F_3 progenies to map it using SSR marker.

In the current study, *P. triticina* pathotype FHTQQ (isolate no. 92-23) showing a very low IT on *Lr36*-carrying genotype and a high IT on Boolani, was used to phenotype the F_2 and F_3 populations derived from the cross between these two genotypes. Most F_2 individuals were resistant and showed a segregation ratio of 3:1 indicating that the resistance in ER84018 was controlled by a single dominant gene which was confirmed by segregation in the F_3 generation (1:2:1 ratio).

To map *Lr36*, SSR primers specific to chromosome 6B, were evaluated for polymorphism between the two bulks of resistant and susceptible, of which eight revealed clear polymorphisms. As generally expected with SSR markers, all tested markers displayed co-dominant inheritance except *Xwmc486* that fitted to a 3:1 genotypic ratio. This marker showed null allele in the resistant plants (amplified only in Boolani, the susceptible lines and the heterozygotes) and therefore, could not differentiate heterozygotes from the susceptible homozygotes. This is likely due to altering of the annealing site, which has led to the loss of the amplicon at the breakage point, resulting in null alleles (Naik Vinod et al. 2015).

The closest markers to *Lr36* were the flanking markers; *Xgwm88* and *Xcfd13*, with distances of 3.8 and 5.2 cM followed by *Xgwm518* and *Xwmc105* at 9.1 and 10 cM, respectively. The order of markers was slightly different from that of the SSR consensus map developed by Somers (2004). This is likely due to the translocation of *Lr36* from *Aegilops speltoides* to wheat, which has led to different recombination frequencies depending on the distance from the breakage points. Additionally, the type and sample size of tested population might have contributed to the altered genetic distances (Liu et al. 2013).

Similar to *Lr53* and *Yr35*, the gene *Lr36* was conclusively mapped on the short arm of 6B. As Dadkhodaie et al. (2011) reported the two tightly linked leaf and yellow rust resistance genes (*Lr53* and *Yr35*) are located on the short arm of chromosome 6B and segregate independently from the gene *Lr36*. According

to their findings, the marker *Xbarc198* was distal to *Lr53* at an approximate distance of 28 cM while our results located this marker at a distance of 20.2 cM from *Lr36*. Therefore, it could be inferred that the gene *Lr36* is located distal to *Lr53*.

Both markers were tested on a panel of 35 wheat genotypes from different backgrounds, known to lack *Lr36*. The Australian cultivars are known to carry known resistance genes. The Iranian cultivars are mostly derived from CIMMYT germplasm, which lack *Lr36* in their pedigrees. Previous phenotypic evaluation of these genotypes along with landraces had shown they do not carry this gene (A. Dadkhodaie, unpublished data). Similarly, phenotyping and pedigree information evidently showed the absence of this gene in Swiss and Afghan genotypes. Therefore, the results of genotyping with these markers was in agreement with those of previous studies confirming their reliability in screening for *Lr36*.

Overall, though sequence-based genotyping and single nucleotide polymorphism (SNP) markers have dominated in genomic selection, SSR markers could facilitate finding neighboring SNPs in gene regions and fine mapping. In the present study, we developed PCR-based molecular markers (SSR) for the leaf rust resistance gene *Lr36* for the first time. Both linked markers; *Xgwm88* and *Xcfd13* gave scorable bands clearly and categorized resistant, susceptible and heterozygotes. Furthermore, their application in tracking *Lr36* in diverse wheat lines and genotypes produced unambiguous and precise outcomes. These confirmed marker-trait association, demonstrating that they could be utilized for marker-assisted selection in breeding programs. Since this gene is still effective in many countries across the world, these markers not only enable researchers to combine it with other seedling and adult plant rust resistance genes but also lay the foundations for its map-based cloning and fine-mapping.

Declarations

Funding

Partial financial support was received from Shiraz University, Higher Education Office, to conduct this research.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Availability of data and material

The data that support the findings of this study are available on request from the corresponding author.

Code availability

Not applicable

Authors' contribution

ZP conducted the research and drafted the manuscript; AD designed the experiment, supervised the study, and revised the manuscript and AN helped to improve the manuscript.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

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Tables

Table 1 Leaf rust responses of parental genotypes to *Puccinia triticina* pathotype FHTQQ (isolate no. 92-23) at the seedling stage.

No.	Genotype	Leaf rust reaction	
		Infection Type ^a	response
1	ER84018	0;1 ⁺ N	resistant
2	Boolani	33 ⁺	susceptible

^a Infection types were scored based on a modified scale by McIntosh et al., 1995 where '0' = no visible uredinia; ';' = hypersensitive flecks; '1' = small uredinia with necrosis; '2' = small to moderate size uredinia with green islands surrounded by necrosis or chlorosis; '3' = moderate size uredinia with or without chlorosis; C = chlorosis; 'N' = necrosis; '-' and '+' denote smaller or larger uredinia.

Table 2 Sequences and annealing temperatures for polymorphic primer sets used in a wheat population from the cross between the leaf rust resistant line ER84018 and the susceptible cultivar Boolani.

Locus ^a	Sequence (5'-3')	Annealing temperature (°C)
<i>barc198</i>	CGCTGAAAAGAAGTGCCGCATTATGA CGCTGCCTTTTCTGGATTGCTTGTC	59
<i>wmc105</i>	AATGTCATGCGTGTAGTAGCCA AAGCGCACTTAACAGAAGAGGG	61.5
<i>gwm193</i>	CTTTGTGCACCTCTCTCTCC AATTGTGTTGATGATTTGGGG	58
<i>gwm88</i>	CACTACAACCTATGCGCTCGC TCCATTGGCTTCTCTCTCAA	59
<i>cf13</i>	CCACTAACCAAGCTGCCATT TTTTTGGCATTGATCTGCTG	56.5
<i>gwm518</i>	AATCACAACAAGGCGTGACA CAGGGTGGTGCATGCAT	58
<i>barc101</i>	GCTCCTCTCACGATCACGCAAAG GCGAGTCGATCACACTATGAGCCAATG	63.5
<i>wmc486</i>	CCGGTAGTGGGATGCATTTT ATGCATGCTGAATCCGGTAA	58.5

^a The loci and their primer sequences were obtained from GrainGenes at <http://wheat.pw.usda.gov/cgi-bin/GG3/browse.cgi>.

Table 3 Frequencies of different phenotypes in F₂ and F₃ populations from the cross between the resistant line ER84018 and the susceptible cultivar Boolani when infected with *Puccinia triticina* pathotype FHTQQ (isolate no. 92-23).

Generation ^a	Observed ^b			Expected ratio	Chi-square	R-value
	R	Seg	S			
F ₂	137	-	34	3:1	2.38 ^c	0.122
F ₃	50	80	33	1:2:1	3.60 ^d	0.165

^a The total number of F₃ differs from that of F₂ because some of them did not set seed.

^b R and S represent resistant and susceptible phenotypes, respectively in F₂. R, Seg and S indicate resistant (*Lr36Lr36*), segregant (*Lr36lr36*) and susceptible (*lr36lr36*) in F₃, respectively. The resistant individuals showed resistance ITs “0;1” and “1+2N”, the susceptible showed the ITs ‘33+’ and the segregated families represented a combination of the above-mentioned ITs to the *Puccinia triticina* pathotype FHTQQ.

^c Non-significant at $p=0.01$ (df=1)

^d Non-significant at $p=0.01$ (df=2)

Table 4 Segregation of SSR primers in F₂ lines from the cross between the leaf rust resistant line carrying *Lr36*; ‘ER84018’ and the susceptible cultivar ‘Boolani’ on wheat 6B chromosome.

Marker	Product size (bp)		Ratio ^a		c ²	R-value
	Resistant ^a	Susceptible ^b	Observed ^{c,d}	Expected		
<i>Xbarc198</i>	150	160	32:87:34	1:2:1	2.93 ^{ns}	0.231
<i>Xwmc105</i>	220/350	200/320	36:80:39		0.28 ^{ns}	0.869
<i>Xgwm193</i>	190	175	42:77:30		2.10 ^{ns}	0.349
<i>Xgwm88</i>	160	140	45:86:27		5.34 ^{ns}	0.069
<i>Xcfd13</i>	480	220	36:89:38		1.43 ^{ns}	0.489
<i>Xgwm518</i>	160/210	180/220	33:99:29		8.70*	0.012
<i>Xbarc101</i>	170	160	48:86:27		6.23*	0.044
<i>Xwmc486</i>	- ^e	200	42:121	1:3	0.05 ^{ns}	0.823

^a The resistant individuals displayed a range of ITs i.e. “0;1” and “1+2N” to *Puccinia triticina* pathotype FHTQQ.

^b The susceptible individuals showed high ITs of ‘33+’ to leaf rust pathotype FHTQQ.

^c The ratios represent the number of individuals in which bands corresponding to homozygous resistant, heterozygous and homozygous susceptible were amplified, respectively.

^d Differences in total number of observed genotypes are due to the non-amplification of the corresponding band(s) in some individuals.

^e No band was amplified.

^{ns} Non-significant

* Significant at $p=0.05$ (df=2)

Supplementary Tables

Supplementary Table 1 was not included with this version.

Figures

Image not available with this version

Figure 1

Response of (A); the resistant line ER84018, (B); the susceptible cultivar Boolani and (C); selected F_3 plants from the cross between the resistant and susceptible genotypes at the seedling stage to *Puccinia triticina* pathotype FHTQQ (isolate no. 92-23)

Image not available with this version

Figure 2

Polymorphic markers on 2% agarose gel. (A) The *Xgwm88*, (B) *Xcfd13*. M shows 100 bp DNA ladder. P_1 ; resistant parent (ER84018), P_2 ; susceptible parent (Boolani), R; resistant line, S; susceptible line and H; segregating in F_2 population from a cross between the two parents when tested with pathotype FHTQQ (isolate no. 92-23)

Image not available with this version

Figure 3

Genetic linkage map of chromosome 6BS based on analysis of an F_2 population derived from a cross between the resistant line (ER84018), and the susceptible parent (Boolani) and seven linked SSR markers on wheat chromosome 6BS. The genetic distances are indicated in cM on the left side of the map and the markers on the right side