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

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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 tightly-linked marker is available in disease resistance breeding. Therefore, this research aimed at analysis of simple sequence repeats (SSR) markers linked with *Lr36* in 171 individuals of an F_2 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. The identified markers were validated on 35 genotypes with different genetic backgrounds including few Australian wheat leaf rust differential sets, Iranian landraces and cultivars, and several cultivars and/or breeding lines from Switzerland and Afghanistan, which confirmed that they can be used in selection for *Lr36* in wheat breeding programs.

Keywords Brown rust · Bulked segregant analysis · Marker-assisted selection · Microsatellite markers · Resistance genes · Wheat

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 have 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

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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 pathotypes and/or problems concerning their presence in combination with other resistance genes conferring lower infection types. Recent advances in molecular biology have facilitated the 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. Though more comprehensive marker systems have been developed, SSR markers still provide valuable information for MAS in rust resistance programs because they are highly polymorphic, low cost to phenotype, and their analysis can be automated (Hayden et al. 2006). In addition, microsatellite consensus maps enable high-density maps of the wheat genome. In this context, 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). Moreover, 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; Nsabiyera 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 pyramided and deployed with other rust resistance genes. However, linked molecular markers that benefit its deployment in wheat breeding programs have not been reported (McIntosh et al. 2017). Therefore, this study describes mapping the gene *Lr36* using SSR markers in a bi-parental population and validates the efficiency of closely-linked markers in different genetic backgrounds.

Materials and methods

Plant materials and leaf rust inoculation

The wheat line ER84018, carrying the leaf rust resistance gene *Lr36* (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 h 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 above were classified as susceptible.

Table 1 Leaf rust responses of *Lr36*-carrying genotype 'ER84018' and *Lr36*-lacking cultivar 'Boolani' to *Puccninia triticina* pathotype FHTQQ (isolate no. 92-23) at the seedling stage

Parent	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

After rust scoring, all 171 F_2 plants were transplanted to 20 cm diameter pots and individually harvested. The segregation ratio from F_2 population was confirmed by testing F_3 families with the same pathotype as described before. Ten to 12 seedlings from each F_3 family and both parents were inoculated as described previously, and phenotypes were recorded as homozygous resistant, segregating heterozygous, and homozygous susceptible.

Marker analysis

Rust-free leaf tissue from each F_2 plant 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), resistant and susceptible DNA bulks were made from equal amounts of DNA from 20 most resistant and 20 most susceptible F_2 individuals.

Thirty-six SSR primer pairs 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, each polymorphic marker (Table 2) was amplified on the entire F_2 population to determine the number of recombinants between the marker and leaf rust response locus. Polymerase chain reactions (PCR) were conducted in final volumes of 20 µl, comprising 1 µl DNA (50 ng μl^{-1}) as a template, 1 µl of 50 ng μl^{-1} each pair of forward and reverse primers (Metabion, Germany), 5 µl Taq DNA Polymerase 2×Master Mix Red (5 U μl^{-1}, Ampliqon, Denmark), and 12 µl ddH_2O. The PCR conditions were incubation at 94 °C for 5 min, followed by 35 cycles of 94 °C for one minute,

Table 2 Sequences and annealing temperatures for polymorphic primer sets used to map *Lr36* 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)
barc198	CGCTGAAAAGAAGTGCCGCATTATGA CGCTGCCTTTTCTGGATTGCTTGTCA	59
wmc105	AATGTCATGCGTGTAGTAGCCA AAGCGCACTTAACAGAAGAGGG	61.5
gwm193	CTTTGTGCACCTCTCTCTCC AATTGTGTTGATGATTTGGGG	58
gwm88	CACTACAACTATGCGCTCGC TCCATTGGCTTCTCTCTCAA	59
cfd13	CCACTAACCAAGCTGCCATT TTTTTGGCATTGATCTGCTG	56.5
gwm518	AATCACAACAAGGCGTGACA CAGGGTGGTGCATGCAT	58
barc101	GCTCCTCTCACGATCACGCAAAG GCGAGTCGATCACACTATGAGCCAATG	63.5
wmc486	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

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 min. 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 BioImaging, 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 F_3 families were used to establish the genotypes of each F_2 individual. The χ^2 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 linked markers to select *Lr36*-carrying genotypes was evaluated in 35 wheat genotypes with different genetic backgrounds. The validating genotypes included two groups; the first group comprised of Australian cultivars with known resistance genes used as differential testers in leaf rust studies. The second group included Iranian cultivars and landraces, Swiss cultivars and breeding lines, and Afghan genotypes. The genotypes in this group were classified as carrying/lacking *Lr36* based on their pedigrees or rust response in the greenhouse. The DNA extraction protocol and PCR conditions were the same as mentioned earlier.

Results

Leaf rust phenotyping

The F_2 population of 171 progenies segregated into 137 individuals with IT "0;1", "1^+2 N" and 34 plants with IT "33^+" fitting a 3:1 ratio ($P_{df=1} = 0.122$). As eight F_2 plants failed to 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 ($P_{df=2} = 0.165$; Table 3).

Genotyping and linkage map

Of the 36 pairs of SSR primer sets located on chromosome 6B, eight showed polymorphisms between

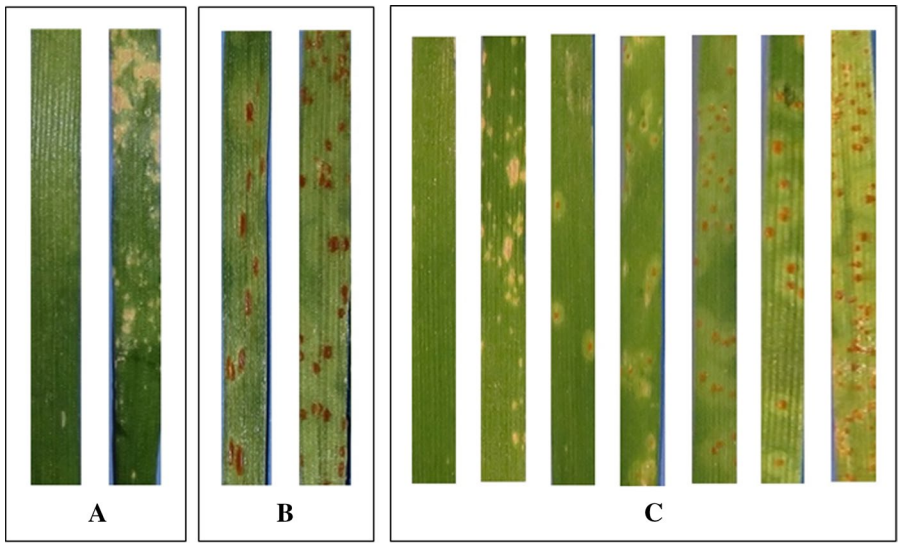

Fig. 1 Response of **A** the leaf rust resistant line carrying *Lr36*; 'ER84018', **B** the susceptible cultivar 'Boolani' and **C** selected F_3 plants from the cross between the resistant and susceptible genotypes at the seedling stage to *Puccinia triticina* pathotype FHTQQ (isolate no. 92-23)

Table 3 Frequencies of different phenotypes in F_2 and F_3 populations from the cross between the leaf rust resistant line carrying *Lr36*; '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	*P* value
	R	Seg	S			
F_2 plants	137	–	34	3:1	2.38[c]	0.122
F_3 families	50	80	33	1:2:1	3.60[d]	0.165

[a]The total number of F_3 differs from that of F_2 because some of them did not set seed

[b]R and S represent resistant and susceptible phenotypes, respectively in F_2. R, Seg and S indicate resistant (*Lr36Lr36*), segregant (*Lr36lr36*) and susceptible (*lr36lr36*) in F_3, respectively. The resistant individuals showed resistance ITs "0;1" and "1^+2 N", the susceptible showed the ITs "33^+" and the segregating 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 $p = 0.01$ ($df = 2$)

the parents and bulks and hence, were used to genotype the whole F_2 population (Fig. 2). Based on PCR amplifications, the primer *gwm88* produced a band of 160 bp in ER84018 while it amplified a 140 bp band in Boolani. The corresponding bands in the resistant and susceptible parents for *cfd13* were 480 bp and 220 bp, respectively. 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 remaining markers produced amplicons as presented in Table 4. 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 fitted a 3:1 ratio for dominance (Table 4) and at LOD ≥ 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 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 to *Lr36* while the marker *Xbarc198* was placed 20.2 cM distal to it (Fig. 3).

Marker validation

To validate their usefulness, the closely linked markers; *Xgwm88* and *Xcfd13* were used to evaluate 35 wheat genotypes from Australia, Iran, Switzerland

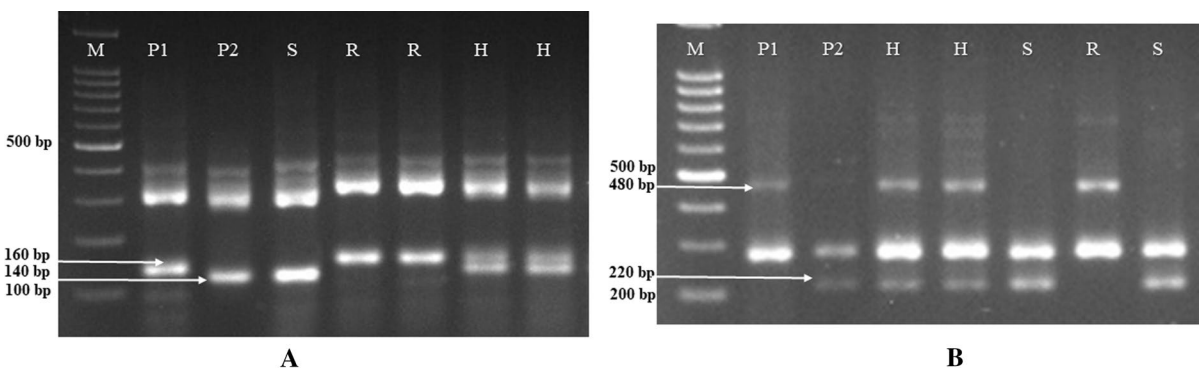

Fig. 2 Polymorphic markers on 2% agarose gel. **A** The *Xgwm88*, **B** *Xcfd13*. M shows 100 bp DNA ladder. P_1; leaf rust resistant parent (ER84018), P_2; susceptible parent (Boolani), R; resistant line, S; susceptible line and H; segregating in F_2 population from a cross between the two parents when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

Table 4 Segregation of SSR primers in F_2 plants from the cross between the leaf rust resistant line carrying *Lr36*; 'ER84018' and the susceptible cultivar 'Boolani' on wheat 6BS chromosome

Marker	Product size (bp)		Ratio[a]		χ^2	*P*-value
	Resistant[a]	Susceptible[b]	Observed[c,d]	Expected		
Xbarc198	150	160	32:87:34	1:2:1	2.93 [ns]	0.231
Xwmc105	220/350	200/320	36:80:39		0.28 [ns]	0.869
Xgwm193	190	175	42:77:30		2.10 [ns]	0.349
Xgwm88	160	140	45:86:27		5.34 [ns]	0.069
Xcfd13	480	220	36:89:38		1.43 [ns]	0.489
Xgwm518	160/210	180/220	33:99:29		8.70*	0.012
Xbarc101	170	160	48:86:27		6.23*	0.044
Xwmc486	–[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^{+}2 N" to *Puccinia triticina* pathotype FHTQQ

[b]The susceptible individuals showed high ITs of "33^{+}" to the same pathotype

[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) is some individuals

[e]No band was amplified

[ns]Non-significant

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

and Afghanistan. The markers *Xgwm88* and *Xcfd13* amplified 140 and 220 bp amplicons, respectively, in all the 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 markers.

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 a segregation ratio of 1:2:1 in the F_3 generation.

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 a 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. Altering of the annealing site has likely led to the loss of the amplicon, resulting in null alleles (Naik Vinod et al. 2015).

The most closely-linked markers to *Lr36* were the flanking markers; *Xgwm88* and *Xcfd13,* at 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 et al. (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

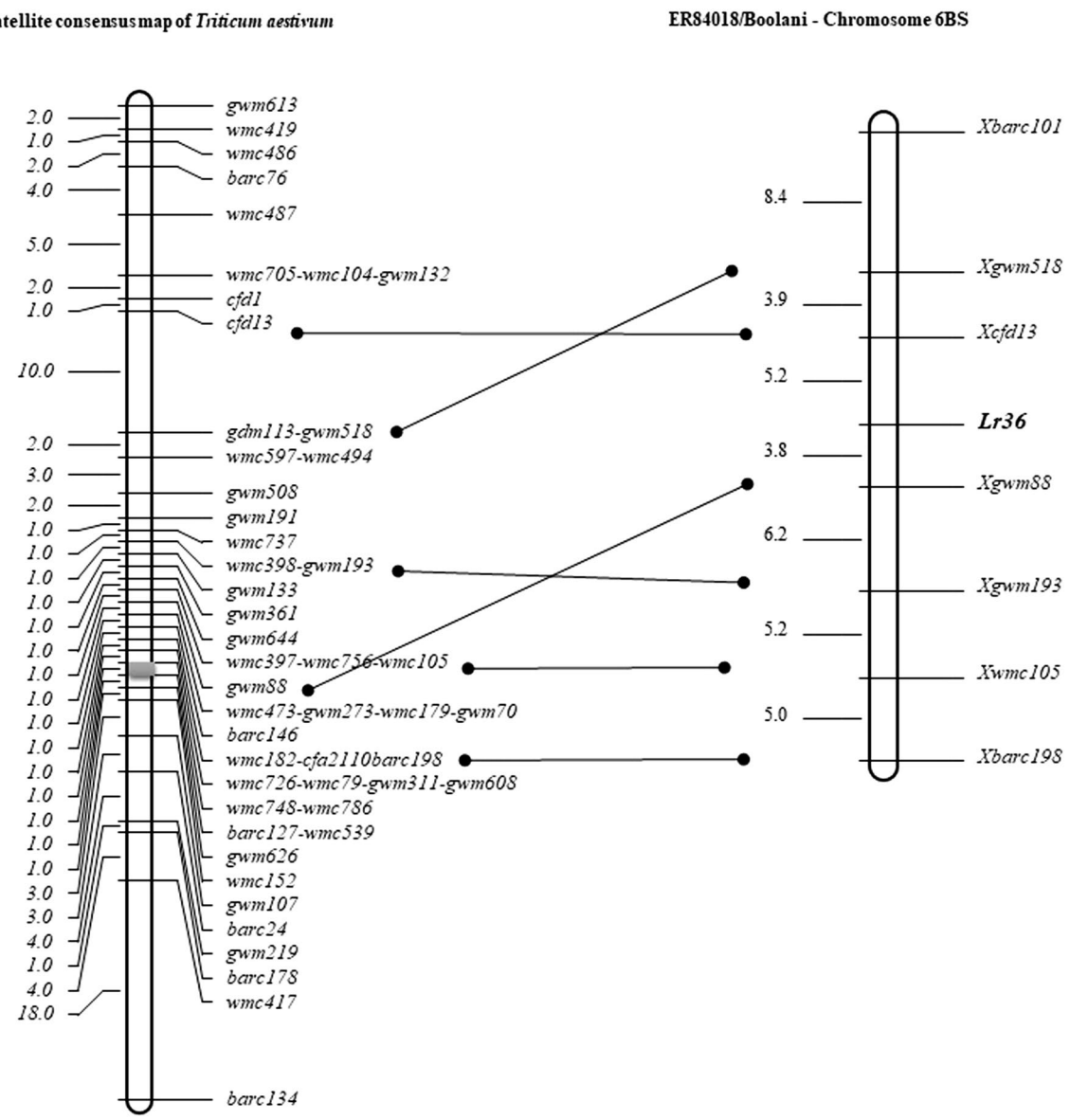

Fig. 3 Genetic mapping of the leaf rust resistance gene *Lr36* on chromosome 6BS based on analysis of an F_2 population derived from a cross between the resistant line 'ER84018', and the susceptible parent 'Boolani', and seven linked SSR markers. The 6B consensus map described by Somers et al. (2004) is also shown. Marker loci common to both maps are connected by solid lines. The genetic distances are indicated in cM on the left side of each map and the markers on the right side

size of the tested population might have contributed to the altered genetic distances (Liu et al. 2013).

The gene *Lr36* was conclusively mapped on the short arm of 6B similar to the two tightly linked leaf and yellow rust resistance genes *Lr53* and *Yr35* as reported by Dadkhodaie et al. (2011). According to their findings, these genes segregate independently from the gene *Lr36*, and 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. The Australian

cultivars are known to carry known resistance genes. The Iranian cultivars are mostly derived from CIMMYT germplasms, 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 were 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 clearly scorable bands and categorized resistant, susceptible, and heterozygotes. Furthermore, their application in tracking *Lr36* in diverse wheat lines and genotypes produced unambiguous and precise outcomes and confirmed the marker-trait association. Therefore, both markers 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.

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.

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

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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