Mol Breeding (2015) 35:61
DOI 10.1007/s11032-015-0234-4

Molecular mapping and validation of the microsatellite markers linked to the *Secale cereale*-derived leaf rust resistance gene *Lr45* in wheat

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Received: 25 March 2014 / Accepted: 1 November 2014 / Published online: 29 January 2015
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Abstract The mode of inheritance of wheat leaf rust resistance gene *Lr45* was studied at seedling stage under greenhouse conditions against leaf rust race 77-5 in two F_2 populations derived from the crosses between Thatcher (Tc)+Lr45 and two susceptible cultivars Agra Local and NI5439. The genetic analysis in $F_{2:3}$ progeny validated the F_2 results which unambiguously showed segregation for a single dominant gene. Genetic analysis in F_2 and BC_1F_1 generations against five other leaf rust races confirmed the single dominant gene inheritance of *Lr45*. Mapping was carried out with 92 microsatellite markers specific to chromosome 2A on the F_2 population of the cross Agra Local × Tc+Lr45. Out of seven markers linked to the gene, four (*gwm372*, *gwm275*, *gpw3167* and *gwm122*) were co-dominant and the other three (*cfd168*, *cfd6* and *gwm249)* showed dominance, amplifying the allele only in the susceptible parent. The genetic map of 13.1 cM was constructed based on the results in 140 homozygous resistant and homozygous susceptible plants. *cfd168* was the closest marker linked to *Lr45*, followed by *gwm372*. These markers were validated on the NI5439 × Tc+Lr45 F_2 population, 12 different backcross lines carrying *Lr45* and near-isogenic lines, mostly in Tc background isogenic for 46 different *Lr* genes belonging to both native and alien species. The marker *gwm122* was found to be monomorphic. The closest co-dominant marker *gwm372* showed reduced polymorphism. Two sequence-based primer pairs, $G372_{94}$ and $G372_{185}$, were designed and validated. Hence, the markers $G372_{94}$ and $G372_{185}$ closely linked to the gene can serve as robust co-dominant markers for utilization of *Lr45* in wheat improvement.

Keywords Bread wheat · *Triticum aestivum* · SSR marker · *Puccinia triticina* · Molecular mapping · Genetic map

Introduction

Globally, wheat (*Triticum aestivum* L.) rusts occupy a prominent place among the fungal diseases, causing severe damage to the crop and leading to huge losses in grain production. Leaf rust caused by *Puccinia*

Electronic supplementary material The online version of this article (doi:10.1007/s11032-015-0234-4) contains supplementary material, which is available to authorized users.

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triticina Eriks. is present wherever wheat is grown in the world. Although rusts can be controlled by fungicide sprays, the use of genetic resistance in cultivars is a more cost-effective and environment friendly method.

Of the 71 documented leaf rust resistance (*Lr*) genes, approximately 38 have been transferred to bread wheat from related and distantly related species and genera, including *Aegilops*, *Agropyron*, *Secale cereale*, *Triticum*, *Thinopyrum* and *Elymus*. Several of them have been deployed to protect crops in different wheat-growing regions of the world (McIntosh et al. 2011; Herrera-Foessel et al. 2012; Singh et al. 2012; Tomar et al. 2014). Rye (*S. cereale* L.) is one of the important donors of disease and pest resistance to wheat. Among the many genes transferred from rye, only those on the short arm of chromosome 1R have been widely exploited. The 1BL.1RS wheat–rye translocation carrying the resistance genes for leaf, stem and stripe rusts and powdery mildew has contributed substantially to world wheat production. However, many of the alien genes, including those carried by IBL.1RS translocation, no longer confer resistance due to the appearance of new virulent races of *P. triticina.* This therefore necessitates the search for new resistance genes.

In this context, the wheat–rye translocation lines developed by Mukade et al. (1970) for rust resistance through spontaneous and X-ray irradiation assume great importance. One of the derivatives, a putative spontaneous translocation stock ST-1, showed a high degree of leaf rust resistance. Later it was subjected to cytogenetic analysis, C-banding and in situ hybridization studies by McIntosh et al. (1995) and the structure of the translocation chromosome was defined as *T2AS-2RS.2RL*. This translocation appeared to be carrying a new gene for leaf rust resistance and it was designated *Lr45*. However, the monosomic analysis of the F_2 population for leaf rust resistance showed deviation from the monogenic inheritance and an excess of susceptible segregants was observed. The gene *Lr45* confers a high degree of seedling and adult plant resistance to leaf rust but to date has not been utilized (Friebe et al. 1996; Tomar and Menon 2001; Bhardwaj et al. 2010) in wheat improvement. Hence, it is important to understand the mode of inheritance of *Lr45* before its utilization in breeding programmes.

Earlier, the gene *Lr45* was mapped using amplified fragment length polymorphism (Zhang et al. 2006) and sequence characterized amplified region (Fein et al. 2009) markers. However, the unavailability of complete information along with the dominant nature of inheritance, high costs and labour involved restrict the use of these markers in marker-assisted breeding. Therefore, simple sequence repeat (SSR) markers are used more frequently than other markers due to advantages associated with co-dominance, accuracy, high repeatability, high level of polymorphism, chromosome specificity and ease of manipulation. The present investigation was therefore undertaken to study the mode of inheritance and to map the leaf rust resistance gene *Lr45* using SSR markers.

Here we report the mode of inheritance of *Lr45* in different segregating populations against multiple races of leaf rust pathogen. This study also reports the identification of SSR markers linked to the gene *Lr45*, and the development of new markers from the locus *gwm372* to provide ease of selection for marker-assisted breeding.

Materials and methods

Plant materials

A near-isogenic line (NIL) of Thatcher (Thatcher*7/ST-1 = RL6144) carrying leaf rust resistance gene *Lr45* (Tc+Lr45) and two susceptible cultivars, Agra Local and NI5439, were used to study the mode of inheritance and mapping of *Lr45*. The line Tc+Lr45 was crossed with Agra Local and NI5439. The F_1 plants were selfed and harvested separately to get the F_2 seed. The F_1 (Agra Local × Tc+Lr45) was backcrossed to Agra Local to get BC_1F_1 seeds. For screening the F_3 families, single selfed spikes with at least 20 seeds were utilized from each resistant and susceptible F_2 plant to confirm their genotype. The inheritance of *Lr45* was studied in both F_2 and BC_1F_1 generations, whereas the molecular mapping was done with the F_2 population from the cross Agra Local × Tc+Lr45.

Validation of the markers linked to *Lr45* was performed on a subset of the population comprising 92 F_2 plants from the cross NI5439 × Tc+Lr45. The efficiency of the linked markers was checked on backcross lines of wheat carrying leaf rust resistance gene *Lr45* in the genetic background of 12 different wheat cultivars. The specificity of the markers was

also tested on a set of 46 NILs of leaf rust resistance genes, mostly in Thatcher background carrying 23 leaf rust resistance genes derived from related/alien species and 23 leaf rust resistance genes derived from bread wheat.

Leaf rust races

The pure inoculum of the six different races of *P. triticina*, i.e. 77-5 (syn. 121R63-1), 12-1 (syn. 5R37), 162 (syn. 93R7), 104-2 (syn. 21R31), 107(45R3) and 108 (syn. 13R27), was obtained from the Directorate of Wheat Research, Regional Station, Flowerdale, Shimla, India. The races were maintained and multiplied on susceptible cultivar Agra Local under greenhouse conditions at the Division of Genetics, IARI, New Delhi, India. Spores were sprayed as a suspension in water with a drop of Tween20 (0.75 µl/ml). The parents, F_1, F_2 and F_3 populations from both the crosses were tested for infection type (IT) against the most virulent race 77-5 of *P. triticina*. The seedlings of F_2 and BC_1F_1 populations, along with the parents, were also screened against five other races mentioned above in addition to race 77-5.

Screening the population for rust resistance

The seeds were sown in trays containing soil mixed with one fifth of farmyard manure. About 10-day-old seedlings were inoculated by spraying with hand sprayer and were incubated for 48 h in humid glass chambers. After incubation, the seedlings were shifted to greenhouse benches at temperatures ranging between 20 and 25 °C under ambient light and relative humidity conditions. Later, the individual seedlings were scored for the rust reaction (IT) at 12 days after inoculation, using the 0–4 scale as described by Stakman et al. (1962).

Genomic DNA isolation

The leaf samples were collected from 40 to 45-day-old plants of the above mentioned plant material. The fresh leaf samples were utilized for DNA isolation using the CTAB method (Murray and Thompson 1980) with minor modifications (10 % of CTAB was used in extraction buffer and CsCl density gradient was not measured). Purified DNA was checked for quality and quantity using agarose gel electrophoresis and diluted to a final concentration of 20 ng/µl for PCR analysis.

Molecular analysis

Since the gene *Lr45* has been reported to be located on chromosome 2A, a parental polymorphism survey was conducted on parental lines Tc+Lr45 and Agra Local using a total of 92 SSR markers which were specific to chromosome 2A. Genomic SSR markers (comprising CFD, GWM, CFA, WMC, GDM and BARC series) were tested for polymorphism and used in bulked segregant analysis (BSA). The primer sequences of all the SSR markers used in this study were obtained from the GrainGenes website http://www.graingenes.pw.usda.gov. The SSR marker analyses were carried out in 10-µl reaction volumes containing 4 mM Tris- HCl (pH 8.0), 20 mM KCl, 0.8 mM $MgCl_2$, 200 µM of each dNTP (MBI Fermentas, Germany), 1.0 unit *Taq* DNA polymerase (Bangalore Genei Pvt Ltd, India), 5 pM each of forward and reverse primers and 20 ng of genomic DNA. The polymerase chain reaction (PCR) was carried out in 96-well PCR plates with thermal seal in an Eppendorf thermal cycler (model Mastercycler pro S, Hamburg, Germany; www.eppendorf.com) with the following thermal profile: initial denaturation step of 94 °C for 4 min, followed by 45 cycles of 94 °C for 1 min (denaturation), 50–60 °C for 1 min (primer annealing) and 72 °C for 1 min (primer extension), with a final extension of 72 °C for 10 min. MetaPhor™ (Lonza) agarose gel (3.5 %) was used to resolve the amplified PCR products in 1X TBE buffer and they were visualized by ethidium bromide staining. Gel photographs were documented using Syngene G:Box Geldoc system (www.syngene.com).

Bulked segregant analysis

Bulked segregant analysis (Michelmore et al. 1991) was employed to identify SSR markers linked to the leaf rust resistance gene *Lr45*. The homozygous resistant and homozygous susceptible F_2 plants identified based on F_3 progeny testing were used for BSA. Equal amount of DNA from 10 homozygous resistant and 10 homozygous susceptible F_2 plants was pooled to constitute the contrasting resistant and susceptible bulks, respectively. The resistant and susceptible bulks, along with parents, were tested

with polymorphic SSR markers to identify putatively linked SSR markers.

Development of sequence-specific markers

To provide an easily detectable polymorphic marker, the most tightly linked co-dominant SSR marker *gwm372* band was excised from agarose gel and purified using Qiagen Gel Extraction Kit. The purified PCR product was sequenced using the forward primer of *gwm372* at Saf Lab Private Limited (www.saflabs.com) and Xcelris Private Limited (www.xcelrisgenomics.com) using the Sanger sequencing protocol. Later, these sequences were used to develop multiple sequence alignment (MSA) using BioEdit and ClustalW programs (http://www.ebi.ac.uk/tools/clustalw2/index.html). The MSA files were analyzed for the presence of deletions and a pair of 20-mer oligonucleotide primers was designed flanking the major deletion from within the sequence information of the marker fragment. Amplification of genomic DNA was done using all combinations of newly designed forward and reverse primers along with the primer pair of *gwm372* at various annealing temperatures. The PCR reaction and amplified product separation was carried out as mentioned earlier.

Linkage analysis

The putative markers identified in BSA were used to genotype the entire F_2 population to determine the number of recombinants produced by each marker, whereas, for linkage analysis involving both co-dominant and null allele dominant SSR markers, only homozygous resistant and homozygous susceptible F_2 plants were used, as described by Gupta et al. (2006). Linkage analysis was done using the software MAPMAKER version 3.0 (Lander et al. 1987). The recombination frequencies were converted to map distances in centimorgans using the option of Kosambi's function in the software.

Statistical analysis

The segregation ratios in F_2 and BC_1F_1 plants scored as resistant and susceptible were subjected to Chi-squared (χ^2) analysis to test the goodness of fit to the theoretically expected Mendelian segregation ratios. In F_3 analysis, the number of segregating and non-segregating families were also subjected to Chi-square test to confirm the F_2 genetic ratio, using $\chi^2 = \sum(O - E)^2/E$, where O = observed number of individuals and E = expected number of individuals.

Results

Genetics of leaf rust resistance gene *Lr45*

The parental plants of Tc+Lr45 were distinguishable by resistance characterized by zero fleck (IT 0;) seedling reaction from susceptible response (IT 33^+ to 4) of Agra Local and NI5439 against the leaf rust race 77-5. The rust reaction on F_1 plants of the crosses Agra Local × Tc+Lr45 and NI5439 × Tc+Lr45 also exhibited IT 0; indicating the dominant nature of the gene *Lr45*. Out of the 323 F_2 seedlings of the cross Agra Local × Tc+Lr45, 251 seedlings were resistant and 72 susceptible. Similarly, in the population NI5439 × Tc+Lr45, out of 335 F_2 seedlings 260 showed resistant reaction and 75 seedlings were susceptible (Table 1). The segregation showed a goodness of fit to a 3:1 resistant:susceptible ratio with a P value of 0.2608 at $\chi^2_{3:1} = 1.26$ in the cross Agra Local × Tc+Lr45, whereas in the cross NI5439 × Tc+Lr45 a $\chi^2_{3:1}$ value of 1.22 was obtained with P-value of 0.2505. This indicated that the resistance conferred by *Lr45* is dominant and monogenic. The F_3 segregation ratio of 1:2:1 (non-segregating resistant:segregating resistant:non-segregating susceptible) validated the results of F_2 analysis (Table 1).

To confirm the results obtained in both the crosses against race 77-5, the F_2 and BC_1F_1 populations of the cross Agra Local × Tc+Lr45 were also screened against five other races, 12-1, 162, 104-2, 107 and 108. For all the races tested on F_2 and BC_1F_1 populations, monogenic ratios of 3:1 and 1:1, respectively, were obtained, confirming the segregation of a single dominant gene for resistance (Table 1).

Identification of SSR markers linked to *Lr45*

Out of 92 SSR markers specific to chromosome 2A, 34 were polymorphic between the parents Agra Local and Tc+Lr45. However, only seven markers located on the short arm were found putatively associated with

Table 1 Segregation of crosses of Tc+Lr45 with susceptible parents Agra Local (*AL*) and NI5439 (*NI*) in F_1, F_2, F_3 and BC_1F_1 generations against different leaf rust races

Leaf rust races	Parents/crosses	Generations	Number of plants				Expected ratio (*R*:*S*)	χ^2	*P* value
			R	Seg	*S*	Total			
77-5	Tc+Lr45	P1	10	–	–	10	–	–	–
	AL	P2	–	–	10	10	–	–	–
	NI	P3	–	–	10	10	–	–	–
	AL/Tc+Lr45	F_1	10	–	–	10	–	–	–
	NI/Tc+Lr45	F_1	10	–	–	10	–	–	–
	AL/Tc+Lr45	F_2	251		72	323	3:1	1.26	0.2608
	NI/Tc+Lr45	F_2	260		75	335	3:1	1.22	0.2505
Homogeneity χ^2								0.0008	
	AL/Tc+Lr45	F_3	77	174	72	323	1:2:1	0.79	0.3720
	NI/Tc+Lr45	F_3	84	176	75	335	1:2:1	0.12	0.9403
Homogeneity χ^2								0.05	
	AL/Tc+Lr45//AL	BC_1F_1	54	–	43	97	1:1	1.24	0.2640
164	AL/Tc+Lr45	F_2	41	–	14	55	3:1	0.01	0.9379
	AL/Tc+Lr45//AL	BC_1F_1	22	–	17	39	1:1	0.64	0.4233
12-1	AL/Tc+Lr45	F_2	42	–	12	51	3:1	1.47	0.2252
	AL/Tc+Lr45//AL	BC_1F_1	24	–	32	56	1:1	1.14	0.285
104-2	AL/Tc+Lr45	F_2	43	–	16	59	3:1	0.14	0.707
	AL/Tc+Lr45//AL	BC_1F_1	16	–	22	38	1:1	0.94	0.3303
107	AL/Tc+Lr45	F_2	58	–	16	74	3:1	0.45	0.5021
	AL/Tc+Lr45//AL	BC_1F_1	31	–	28	59	1:1	0.15	0.6961
108	AL/Tc+Lr45	F_2	49	–	13	62	3:1	0.53	0.4634
	AL/Tc+Lr45//AL	BC_1F_1	28	–	17	45	1:1	2.68	0.1010

R resistant, *Seg* segregating, *S* susceptible

Lr45 in the bulked segregant analysis (Table 2). Four markers, namely *gwm372*, *gwm122*, *gwm275* and *gpw3167*, behaved as co-dominant, while three markers, namely *cfd168*, *cfd6* and *gwm249*, were dominant, amplifying the marker fragment only in susceptible plants. The seven SSR markers differentiating the two contrasting bulks were screened for the respective marker fragment on the entire F_2 population. In the case of co-dominant SSR markers, the lowest number of recombinants was observed for *gwm372* (6/323) (Supplementary Table S1). Of the three null allele markers, *cfd168* showed only one recombinant out of 323 F_2 plants tested (Supplementary Table S2). Both the gene and the markers in combination deviated significantly from the expected independent assortment ratio, suggesting that the two loci involved were tightly linked to each other. The χ^2 test for linkage also confirmed that the leaf rust resistance gene *Lr45* and the other markers were linked ($P = 0.00$ in each case) (Supplementary Table S1 and Table S2).

Development of genetic linkage map of *Lr45* locus

Three-point and multi-point analysis using MAPMAKER on 70 homozygous resistant and 70 homozygous susceptible plants fixed the seven SSR markers within a distance of 13.1 cM from the gene *Lr45* (Fig. 1). No recombinant was observed for the marker *cfd168* in a mapping population of 140 homozygous individuals and it was placed on the gene *Lr45* with a logarithm of odds (LOD) score of 84.5. The closest co-dominant marker *gwm372* was located at 0.7 cM distant from the gene *Lr45* with a LOD score of 80.32. A linkage map of *Lr45* was synthesized, where all the markers linked to the gene were aligned towards one side of the locus *Lr45* (Fig. 1).

Table 2 Details of marker sequences and sequence specific primers designed from the unique adjacent regions of the simple sequence repeats

Marker	Primer sequence (5′–3′)	Allele size (bp)			Annealing temperature (°C)
		Tc+Lr45	Agra Local	NI5439	
gwm372	F-AATAGAGCCCTGGGACTGGG	307	285	311	60
	R-GAAGGACGACATTCCACCTG				
gwm122	F-GGGTGGGAGAAAGGAGATG	275	240	240	60
	R-AAACCATCCTCCATCCTGG				
gpw3167	F-TAGGCTTTTGCCCGAAGAC	120	110	110	60
	R-GTGGGTGTCATTTCCCCTC				
gwm275	F-AATTTTCTTCCTCACTTATTCT	108	120	120	50
	R- AACAAAAAATTAGGGCC				
cfd168	F-CTTCGCAAATCGAGGATGAT	–	235	235	60
	R-TTCACGCCCAGTATTAAGGC				
cfd6	F-ACTCTCCCCCTCGTTGCTAT	–	325	325	60
	R-ATTTAAGGGAGACATCGGGC				
gwm249	F-CAAATGGATCGAGAAAGGGA	–	275	55	55
	R-CTGCCATTTTTCTGGATCTACC				
$G372_{94}$	F-GACGTTAGCTCACGCAACCG	94	62	62	55
	R-CTCTTGAAACACAAAGCACA				
$G372_{185}$	F-AATAGAGCCCTGGGACTGGG	185	129	153	60
	R-CTCTTGAAACACAAAGCACA				

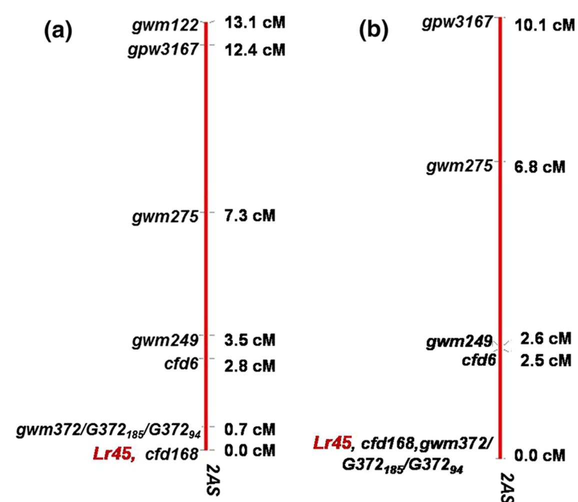

Fig. 1 Genetic linkage map of leaf rust resistance gene *Lr45* and relative distance to markers located on chromosome arm 2AS in the populations **a** Agra Local × Tc+Lr45 and **b** NI5439 × Tc+Lr45

Validation of the SSR markers

SSR markers linked to the gene *Lr45* in the F_2 population Agra Local × Tc+Lr45 were validated on 92 plants of the second F_2 population derived from the cross NI5439 × Tc+Lr45. One of the markers *gwm122*, however, was found monomorphic between NI5439 and Tc+Lr45. The polymorphic fragments produced by the rest of the markers were of the same size in NI5439 as found in the susceptible parent Agra Local, except for the marker *gwm372*. These five SSR markers were tested on 92 F_2 plants of the cross NI5439 × Tc+Lr45. The minimum number of recombinants was observed for null allele marker *cfd168* (1/92), followed by *cfd6* (6/92) and *gwm249* (7/92). However, co-dominant markers *gwm275* (8/90) and *gpw3167* (11/92) showed relatively higher frequencies of recombination between marker loci and *Lr45*. The marker *gwm372* was found to be the closest co-dominant marker linked to *Lr45*, producing a fragment of 285 bp in Agra Local and 307 bp in Tc+Lr45. However, the same marker amplified a fragment of approximately 311 bp in NI5439, thus making it difficult to distinguish it from the Tc+Lr45 fragment on a MetaPhor agarose gel (Supplementary Fig. S3). Hence, it was difficult to differentiate the alleles produced by *gwm372* in the parents Tc+Lr45 and NI5439.

Validation of SSR markers linked to *Lr45* on backcross lines of wheat cultivars carrying *Lr45* was also carried out. However, the marker *gwm372* failed to distinguish four backcross lines of *Lr45* from their respective recurrent parents, HD2329, Kalyansona, NI5439 and WH147 (Supplementary Fig. S4). The specificity of these SSR markers to the gene *Lr45* was confirmed on a set of NILs in Thatcher background carrying 46 different leaf rust resistance genes derived from alien and native wheat germplasm. Five SSR markers, *cfd168*, *cfd6*, *gwm249*, *gwm275* and *gpw3167*, amplified the expected marker fragments only in Tc+Lr45. However, the marker *gwm372* failed to distinguish the NILs carrying different *Lr* genes from *Lr45* (Supplementary Fig. S5).

Development of sequence-specific markers and validation

The marker fragment amplified by *gwm372* in Tc+Lr45, Agra Local and NI5439 was sequenced. Later, the multiple sequence alignment showed the presence of a large unique region of 185 bp adjacent to the simple sequence repeats in the resistant parent Tc+Lr45 (Fig. 2). A common deletion of 32 bp was found in both the susceptible parents Agra Local and NI5439, while an additional 26 bp deletion was specific to Agra Local in the unique flanking region. These deletions resulted in flanking regions of 165 and 141 bp in NI5439 and Agra Local, respectively. Marker $G372_{94}$ was designed by a pair of 20-mer oligonucleotide primers (forward GACGTTAGCTCACGCAACCG, reverse CTCTTGAAACACAAAGCACA) flanking the 32-bp deletion region which amplified a fragment of 94 bp in the resistant parent Tc+Lr45 lacking the deletion region and a fragment of 62 bp in both the susceptible parents Agra Local and NI5439. Another marker $G372_{185}$, developed by combining the reverse primer of $G372_{94}$ along with the forward primer of original marker *gwm372*, amplified the whole unique region of 185 bp adjacent to the repeat region in Tc+Lr45. It produced the expected amplicon of 153 bp in the parent NI5439 and a fragment of 127 bp in Agra Local which carries two deletions of 32 bp and 26 bp (Fig. 3).

When the two newly developed markers $G372_{94}$ and $G372_{185}$ were tested on 92 F_2 individuals of the cross NI5439 × Tc+Lr45, genotyping results for both the primer pairs were found to be same as that of *gwm372* (Supplementary Table S1). They were also validated on backcross lines of *Lr45* in Indian wheat cultivars (Supplementary Fig. S6) and also on NILs carrying different leaf rust resistance genes, mostly in Thatcher background (Supplementary Fig. S7). Results showed that markers $G372_{94}$ and $G372_{185}$ produced 94-bp and 185-bp alleles, respectively, only in the backcross lines carrying *Lr45* and not in the original recurrent parents or NILs of Thatcher carrying alien or native leaf rust resistance genes.

Discussion

A large number of alien rust resistance genes have been transferred into wheat (Friebe et al. 1996) but many of them have not been used in commercial cultivars. *S. cereale*-derived linked rust resistance genes *Lr26/Sr31/Yr9* were deployed globally, though the wheat–rye translocation 1BL.1RS carrying these genes involved the entire short arm of the rye chromosome (Zeller 1973). Thus, in some cases a large size of alien segment may not necessarily have an adverse effect. The structure of *Lr45* carrying translocation *T2AS-2RS.2RL* suggests that the translocation is a compensating type involving homoeologous chromosomes from group 2 only. However, the effect of this translocation on various agro-morphological traits needs to be studied. Genetic analysis of *Lr45* in F_2, F_3 and BC_1F_1 generations of two crosses involving susceptible cultivars Agra Local and NI5439 against the most virulent race 77-5 showed segregation for a single dominant gene. The present findings are in contrast to the earlier report (McIntosh et al. 1995) that gametes carrying *Lr45* had poor transmission resulting in segregation distortion as more susceptible plants were recorded in the F_2 generation. However, in the present study, the monogenic inheritance of *Lr45* was also confirmed against five other races of leaf rust, viz. 12-1, 162, 104-2, 107 and 108. Thus, like other alien genes, the *S. cereale*-derived resistance gene (*Lr45*) may also prove useful in wheat breeding.

Lr45-based resistance has not been utilized in marker-assisted selection because of the non-availability of markers. In the present study the leaf rust resistance gene *Lr45* was mapped using seven SSR markers spanning a distance of 13.1 cM (Fig. 1). The marker order is similar to the high-density SSR consensus map developed by Somers (2004), with one exception, that *cfd6* mapped proximal to *gwm372*

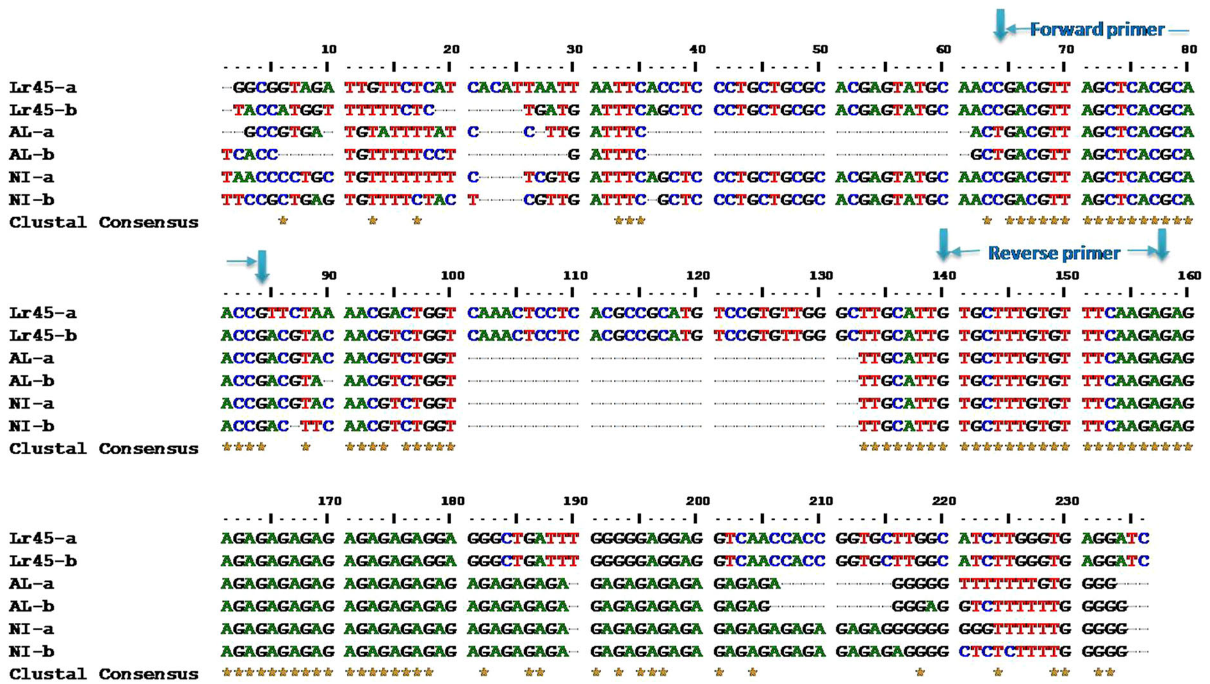

Fig. 2 Multiple sequence alignment using nucleotide sequence of marker *gwm372* from Tc+Lr45, Agra Local and NI5439 indicating the forward and reverse primers of $G372_{94}$. *Lr45-a*, *AL-a* and *NI-a* are sequencing results of Tc+Lr45, Agra Local and NI5439, respectively, from Saf Lab Pvt Ltd and *Lr45-b*, *AL-b* and *NI–b* are sequencing results of Tc+Lr45, Agra Local and NI5439, respectively, from Xcelris Pvt Ltd

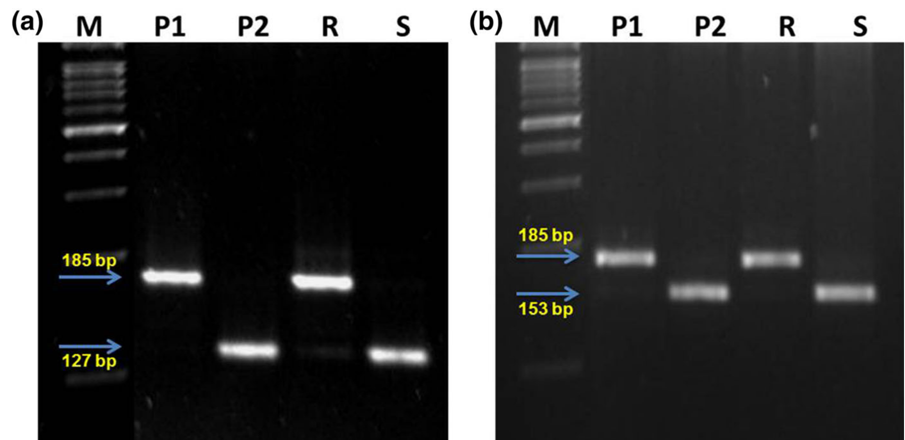

Fig. 3 Screening of the resistant parent (*P1*), susceptible parent (*P2*), resistant F_2 plant (*R*) and susceptible F_2 plant (*S*) of the cross **a** Agra Local × Tc+Lr45 and **b** NI5439 × Tc+Lr45 with a co-dominant marker $G372_{185}$

on the map by Somers (2004), whereas *cfd6* was located distal to *gwm372* in our map. Even though the SSR markers are expected to generally inherit as co-dominant loci, in the present study three SSR markers inherited as dominant loci amplifying only in susceptible plants and showing null allele in the resistant plants. The *T2AS-2RS.2RL* translocation, being alien to the *T. aestivum* genomic region, could have caused disturbances in the primer annealing sites of these SSR primers at the breakage point, resulting in the null alleles as reported in other alien gene-transferred wheat lines (Liu et al.2002; Brown-Guerdira et al. 2003; Malik et al. 2003; Adhikari et al. 2004; Vikal et al. 2004; Gupta et al. 2006; Singh et al. 2011).

The nearest SSR marker, *cfd168*, behaved as a dominant marker amplifying an allele specific to the

susceptible parent. Although it produced one recombinant out of 323 F_2 plants, no recombinant was identified in 140 homozygous individuals used for mapping analysis. Another marker, *gwm372*, behaved as co-dominant, but the allele produced in susceptible parent NI5439 was difficult to distinguish from the allele linked to *Lr45*. Hence, an effort was made to convert the less polymorphic *gwm372* into a clearly distinguishable polymorphic marker by sequencing the PCR product, and designing the primers from the unique adjacent regions of the repeat sequences. One of the markers designed, $G372_{94}$, produced a fragment difference of 32 bp between the resistant gentoype and all the genotypes devoid of the gene *Lr45*. Another primer marker $G372_{185}$ amplified the complete adjacent unique region of 185 bp in Tc+Lr45. The genetic map of *Lr45* involving six molecular markers on the 40 homozygous resistant and susceptible F_2 plants of the population NI5439 × Tc+Lr45 covered a distance of 10.1 cM (Fig. 1), and was almost similar to the genetic map of Agra Local × Tc+Lr45. The order of the markers remained the same and the difference found between the two genetic maps was very small, which shows that tight linkage existed between the marker and the gene *Lr45*. This small difference between the two maps may be because of the difference in the size of the F_2 populations.

Marker validation, a process of examining the behavior of markers and the associated polymorphism, is necessary before applying a marker in breeding (Langridge and Chalmers 1998). Gupta et al. (1999) suggested that the validity of a molecular marker linked with any trait should be examined in crosses other than the ones in which the marker was developed. One of the SSR markers, *gwm122*, turned out to be monomorphic in the second F2 population used for validation. The validation study conducted by Sharp et al. (2001) showed that the STS marker developed by Naik et al. (1998) linked to *Lr28* was ineffective in marker-assisted breeding due to the poor linkage of marker to gene. A SCAR marker developed for leaf rust resistance gene *Lr24* by Schachermayr et al. (1995) and Dedryver et al. (1996) was found to be monomorphic in Indian sources, as they have a shortened alien segment relative to the original source, Agent, and have therefore been found to be ineffective in marker-assisted selection (Prabhu et al. 2004).

Both the sequence-specific markers developed were tested on two different sources that were used for validation of other linked markers. When validated on the 12 resistant backcross lines carrying *Lr45* and their susceptible recurrent parents, the allele specific to Tc+Lr45, i.e. 94 bp in the case of $G372_{94}$ and 185 bp in the case of $G372_{185}$, was amplified in all the 12 NILs carrying gene *Lr45*. In 12 recurrent parental genotypes, an allele of 64 bp was produced by the marker $G372_{94}$. In the case of $G372_{185}$, recurrent parents HD2329, Kalyansona, NI5439 and WH147 produced the allele of 153 bp and the rest produced the allele of 127 bp. Although the marker $G372_{185}$ produced two distinct alleles in susceptible recurrent parents, the allele size is sufficient to differentiate them easily from the allele of the resistant parent, indicating that both the sequence-specific markers are highly specific to gene *Lr45* and can be effectively used for the selection of this gene in different genetic backgrounds. These markers were also validated for their uniqueness and specificity to *Lr45*, because they amplified the resistance allele only in the Thatcher NIL carrying gene *Lr45* and not in the Thatcher NILs carrying other 46 alien or native *Lr* genes in wheat.

Molecular markers can predict the presence of a specific gene with very high probability without the need for disease evaluation, and thus aid the transfer of several resistance genes into adapted materials to pyramid genes in one plant. The markers reported in this study can be effectively utilized for the selection of this gene in different genetic backgrounds, as indicated by successful validation on divergent *Lr* genes and wheat cultivars. The newly designed co-dominant markers $G372_{94}$ *and* $G372_{185}$ were able to identify the heterozygotes, and would serve as an important tool to rapidly transfer this gene into other wheat cultivars.

Acknowledgments The senior author is grateful to Post Graduate School, Indian Agricultural Research Institute and the Indian Council of Agricultural Research, New Delhi, India for financial assistance given as SRF during the course of study. The authors are grateful to the Directorate of Wheat Research, Regional Station, Flowerdale, Shimla, India, for providing pure inoculum of leaf rust pathotypes and the Indian Agricultural Research Institute, New Delhi for facilitating the experiments.

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