Indian J. Genet., 71(4): 304-311 (2011)

Molecular marker assisted pyramiding of leaf rust resistance genes *Lr19* and *Lr28* in bread wheat (*Triticum aestivum* L.) variety HD2687

Kailash B. Bhawar, Vinod*, J. B. Sharma, A. K. Singh, M. Sivasamy[1], Mona Singh, K. V. Prabhu, S. M. S. Tomar, T. R. Sharma[2] and B. Singh

Division of Genetics, Indian Agricultural Research Institute, New Delhi 110012; [1]IARI Regional Station, Wellington, The Nilgiris 643 231; [2]National Research Centre on Plant Biotechnology, IARI Campus, New Delhi 110 012

(Received: July 2011; Revised: October 2011; Accepted: November 2011)

Abstract

Backcross lines of high yielding cultivar HD2687 developed earlier carrying leaf rust resistance genes *Lr19* and *Lr28* were utilized to pyramid the two genes using marker assisted selection. Background analysis using 602 SSR markers was done to compare the genotypic background of backcrossed lines with recipient variety HD2687 as well as the donor genotypes Cook*6/C80-1 and CS2A/2M#4/2. Backcross lines of HD2687 with *Lr19* and *Lr28* i.e. HD2687+*Lr19* and HD2687+ *Lr28* showed 89 and 92.1 percent genomic similarity with HD2687. The two backcross lines were crossed and plants carrying both *Lr19* and *Lr28* were identified in F_2 generation. Background analysis of 51 plants carrying both *Lr19* and *Lr28* was undertaken using polymorphic SSR markers to identify plants with better recovery of HD2687 background. Although, *Lr19* and *Lr28* individually are ineffective against leaf rust pathotypes 77-8 and 77-10 but pyramided lines are expected to provide resistance against all the races.

Key words: Leaf rust, *Lr19*, *Lr28*, molecular markers, marker assisted selection, gene pyramiding, wheat

Introduction

The leaf rust (*Puccinia triticina* Eriks. Syn. *Puccinia recondita tritici* Rob. Ex Desm.) infection has been kept under control, mainly by judicious deployment of effective resistance genes in different wheat zones in India. Although several rust resistance gene(s) have been postulated in present day Indian wheat cultivars but many of them are rendered ineffective by virulent pathotypes of rust. Genes originating from alien sources exhibit resistance to leaf rust and stem rust but a few of them have been reported to have yield penalty [1]. However, not all the resistant genes are having deleterious effect, rather a few of them enhances yield potential as well, e.g., 1BL.1RS (*Lr26*/*Sr31*/*Yr9*/*Pm8*) translocation from *Secale cereale*. Singh *et al*. [2] observed that 7D.7Ag segment with *Lr19*/*Sr25* increases grain yield by 10-15% in a range of genotypes. Similarly the alien segment *Lr24/Sr24* does impose any deleterious effect on yield [3]. The linked genes *Lr19/Sr25* have been combined in an Australian line Cook with another DNA segment carrying *Sr36/Pm6* from *Triticum timopheevi*.

Most of the genes conferring resistance against Indian pathotypes of leaf rust are of alien origin but virulent races have evolved against several of them reducing their utility. However, pyramiding of genes can prolong the effectiveness of these resistance genes. Resistance genes can be combined in a single genetic background if discriminating races are available. Nowadays with the availability of robust molecular markers linked to various rust resistance genes [4, 5], two or more genes can be pyramided efficiently in a relatively short time even in the absence of virulence. Several rust resistance genes have been transferred in the genetic background of popular Indian cultivars using conventional backcross breeding [6, 7]. Though, only a few of these backcross lines such as HW2004 (C306+*Lr24*), HW2044 (PBW226+*Lr24*), HW2045 (HD2285+*Lr24*) and HW2034 (C306+*Lr28*) could be released as a cultivar, nevertheless, these backcross lines are useful genetic resources providing rust resistance genes in the improved and diverse genetic backgrounds. These backcross lines can also be utilized

*Corresponding author's e-mail: vinod.genetics@gmail.com
Published by Indian Society of Genetics & Plant Breeding, F2, First Floor, NASC Complex, PB#11312, IARI, New Delhi 110 012
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for pyramiding of two or more rust resistance genes. Moreover, with the availability of SSR markers covering all the chromosomes and chromosome arms in wheat, marker assisted background analysis for recovering the recurring parental genotype becomes an attractive proposition. In the present communication, we report the pyramiding of leaf rust resistance genes *Lr19* and *Lr28* using backcross lines of HD2687 carrying these genes individually. Marker assisted background analysis has also been done to compare the genomic similarity between original donors of *Lr19* and *Lr28* as well as backcross lines of HD2687 carrying these genes individually. Virulence of the race 77-8 (253R31) has been reported against *Lr19* [8] and 77-10 (377R60-1) against *Lr28* [9] in India, though these genes have not yet been deployed commercially on large scale, the pyramiding of these two genes is expected to provide resistance against all existing Indian races of leaf rust in the genetic background of high yielding cultivar HD2687.

Materials and methods

Plant material

The plant material comprised donor genotypes Cook*6/ C80-1 carrying *Lr*19 (CLr19), CS2A/2M#4/2 carrying *Lr28* and backcross lines of cultivar HD2687 carrying leaf rust resistance genes *Lr19* and *Lr28* individually and HD2687. These lines were developed through conventional backcross breeding and identity of genes was confirmed earlier using molecular markers [7, 10]. Crosses were made between backcross lines of HD2687 carrying leaf rust resistance genes *Lr19* and *Lr28* during *rabi* 2008-09. The F_1 plants were grown in the off-season during 2009 in National Phytotron Facility, Indian Agricultural Research Institute, New Delhi. Each F_1 plant was selfed by bagging with butter paper bags to produce F_2 seed. All the 51 selected F_2 plants carrying both *Lr19* and *Lr28* were advanced to F_3 generation for further morphological observations and identification of homozygous families with combination of both the genes.

Rust inoculum

Highly virulent and commonly prevalent Indian pathotype 77-5 (121R63-1) of leaf rust (*Puccinia triticina* f. sp. tritici) was used for leaf rust resistance in parents, F_1 and F_2 population. The inoculum was obtained from Directorate of Wheat Research, Regional Station, Flowerdale, Shimla and was multiplied in isolation in glass house.

DNA extraction

Leaf tissues for DNA isolation were collected from either 7-10 day old seedlings or 3-4 week old plants. Genomic DNA was isolated by using CTAB method [11]. DNA samples were quantified by comparison with 100ng/ 200ng of λ uncut DNA on 0.8% agarose gel. The DNA was diluted in TE to 25ng/µl before amplification.

Molecular markers

For foreground selection of targeted genes, one SSR marker *Xwmc*221 and two SCAR markers SCS253 and SCS265 [12] were used for screening of gene *Lr19* whereas only one marker SCS421 [13] was used for *Lr28*. For background analysis of backcross lines of HD2687 carrying *Lr19* and *Lr28* and F_2 population, a total of 602 SSR markers spanning all chromosome arms were used.

PCR amplification and electrophoresis

PCR amplification was performed in 20 µl reaction volume containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each dNTP (MBI Fermentas, Germany), 1 unit Taq DNA Polymerase (Bangalore Genei Pvt Ltd, India), 0.2 µM of primer and 25-30 ng of genomic DNA. PCR amplification was achieved in Eppendorf's thermal cycler with the following thermal profile: one cycle of 5 min at 95°C (initial denaturation), followed by 35 cycles of 1min at 94°C (denaturation), 1min at 60°C (primer annealing) and 1min at 72°C and 7 min at 72°C (a final extension). PCR products were resolved on 3% metaphor gel for SSR marker and on 2% agarose gel for SCAR markers at 120 V for 3.5h.

Field screening for leaf rust resistance

The parents, F_1 and F_2 population were tested for leaf rust resistance at adult plant stage under artificially inoculated conditions. Parental rows were inoculated at boot leaf stage with urediospore suspension of leaf rust pathotype 77-5. Spreader rows were planted after every ten rows. Sufficient humidity was maintained in the field for uniform spread of rust. Individual plants were scored either as susceptible or resistant depending on infection type (IT). Rust reaction was recorded as per the modified Cobb's scale and estimated on the basis of percent area covered with pustules [14].

Marker assisted background analysis

The software Graphical GenoTypes (GGT) Version 2.0 was used for marker assisted background analysis.

Genotyping data were scored as allele from HD2687 as 'A', HD2687+*Lr19* as 'B', HD2687+*Lr28* as 'C', common allele from HD2687+*Lr19* and HD2687+*Lr28* as 'D', while individual carrying one allele from HD2687 and other allele from HD2687+*Lr19* or HD2687+*Lr28* was scored as 'H'.

Results and discussion

Gene pyramiding of two or more race specific genes in a single genetic background can be an effective strategy to enhance the durability of major resistance genes. The present study was an attempt to pyramid the race specific leaf rust resistance genes *Lr19* and *Lr28* in the genetic background of high yielding but susceptible cultivar HD2687. The F_1 plants were screened with molecular markers *Xgwm221* and SCS421 specific for *Lr19* and *Lr28* respectively to confirm the hybridity of each plant. The results showed that all the plants in F_1 carried both the resistance genes. Marker assisted selection was practised in F_2 to identify plants carrying both *Lr19* and *Lr28.*

MAS in F_2 generation

F_2 plants were screened with three molecular markers linked with leaf rust resistance gene *Lr19* and one marker linked with *Lr28*. SSR marker *Xwmc221* and two SCAR markers *viz*., SCS265 and SCS253 were used for screening of F_2 plants for leaf rust resistance gene *Lr19*. While SSR marker *Xwmc221* is a co-dominant marker, the two SCAR markers SCS265 and SCS253 are dominant markers with SCS265 linked with gene *Lr19* in coupling phase and SCS253 in repulsion phase. The two markers were used in a multiplex PCR reaction and thus homozygous and heterozygous plants could be distinguished. The results obtained were also confirmed by *Xwmc221*. F_2 plants were also screened with SCAR marker SCS421 [13] linked to leaf rust resistance gene *Lr28* in coupling phase. The use of molecular markers linked to resistance gene(s) is one of the best strategies for achieving selection for different gene combinations in suitable genetic background [15, 16].

Marker segregation

The segregation of molecular marker alleles is shown in Table 1. Out of 288 plants in F_2 population, 63 were homozygous for resistance allele, 140 plants were heterozygous while remaining 85 were homozygous for susceptible allele for co-dominant marker *Xwmc221* (Fig. 1). The observed frequency of F_2 plants fits well with the expected 1:2:1 ratio with non significant chi-square value of 3.58 (P-value 0.16).

Repulsion phase marker SCS253 produced no amplification in 65 plants whereas 223 plants showed amplification to produce marker band of 736 bp size. The observed frequency of this repulsion phase marker fits well in the expected ratio of 1:3 with a chi-square value of 0.91 and P-value of 0.34. Dominant marker SCS265 which is linked with *Lr19* in coupling phase amplified marker allele of 512bp size in 204 plants whereas no amplification was observed in 84 plants fitting well in the expected ratio of 3:1 with a non significant chi square value of 2.67 (P-value 0.10). Similarly, SCAR marker SCS421 segregated into 3:1 ratio with 212 plants showing presence of marker allele while remaining plants showed absence of the allele with chi square and P-value of 0.29 and 0.59 respectively (Table 1, Fig. 1).

Joint segregation of markers for Lr19 and Lr28

For identification of plants carrying *Lr19*, three molecular markers *viz*., *Xwmc221*, SCS265 and SCS253 were used. Since *Xwmc221* is a co-dominant marker it facilitated the identification of plants as homozygous or heterozygous for leaf rust resistance gene *Lr19*. A perusal of Table 1 shows that molecular marker *Xwmc221* identified 63 plants as homozygous resistant and 140 plants as heterozygous resistant carrying *Lr19*. SCAR marker SCS421 identified 212 plants positive for leaf rust resistance gene *Lr28* out of 288 plants studied. Since, SCS421 is a dominant marker, these 212 identified individuals included both homozygous and heterozygous resistant plants for *Lr28*. Joint segregation analysis of two markers *i.e.,* one co-dominant marker *Xwmc221* for *Lr19* and a dominant marker SCS421 for *Lr28* was undertaken to identify plants with different genotypic constitution with respect to the two rust

Table 1. Segregation of molecular markers in F_2 population[#]

Marker	*Lr*19		*Lr*28	
	*Xwmc*221	SCS253*	SCS265	SCS421
Homozygote(R)	63	65 (-)	204(+)	212(+)
Heterozygote(R^1)	140	223(+)		
Homozygote(S)	85		84(-)	76 (-)
Expected Ratio	1:2:1	1:3	3:1	3:1
χ^2 value	3.58	0.91	2.67	0.29
P-value	0.16	0.34	0.10	0.59

R^1: Resistance, S^2: Susceptible, *: Repulsion phase dominant marker; #: Total plants in F_2 = 288; + = allele present; – = allele absent

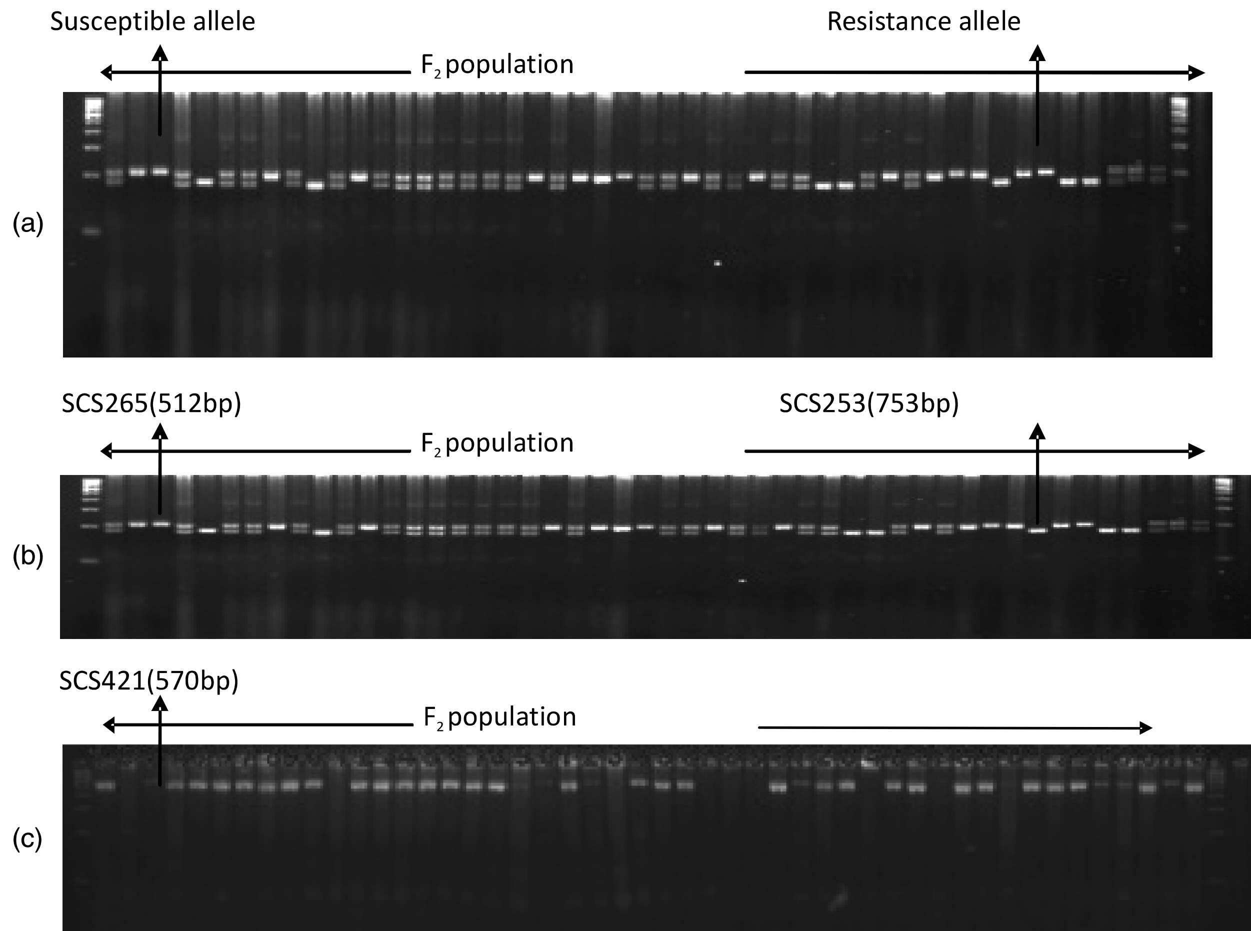

Fig. 1. Screening of F_2 population for leaf rust resistance genes *Lr*19 and *Lr*28 with (a) SSR marker *Xwmc*221(b) SCAR markers SCS253 (Repulsion phase marker) and SCS265 (Coupling phase marker) and (c) SCAR marker SCS421 in F_2 population

resistance genes. The expected and observed frequencies of different genotypic classes are shown in Table 2. Out of 63 plants identified as homozygous resistant for *Lr19*, 51 were observed to carry leaf rust resistance gene *Lr28* as well, either in homozygous or in heterozygous condition. Remaining 12 plants, homozygous for *Lr19* did not carry *Lr28* gene. Out of 140 plants identified as carrying *Lr19* in heterozygous state, 103 also carried *Lr28* in homozygous or heterozygous condition as against expected frequency of 108. Thirty seven plants were observed to carry only *Lr19* in heterozygous condition as against an expectation of 36 plants. Fifty eight plants were observed to carry only *Lr28*, either in homozygous or heterozygous state. Out of 288 plants 27 did not carry either *Lr19* or *Lr28* as against expected value of 18. The six categories of genotypic classes expected from segregation of a dominant and a co-dominant marker are shown in Table 2. A perusal of Table 2 showed that

Table 2. Genotypic frequencies in F_2 population based on joint segregation of two markers *Xwmc*221 and SCS 421

Genotype frequency	Expected	No. of plants		χ^2
		Expected	Observed	
AAB-	3/16	54	51	0.146
AAbb	1/16	18	12	1.96
AaB-	6/16	108	103	0.198
Aabb	2/16	36	37	0.0356
aaB-	3/16	54	58	0.326
aabb	1/16	18	27	4.589
Total		288	288	7.25
Genotypic ratio	3:1:6:2:3:1	χ^2=7.25, P-value = 0.20		

A= *Lr19*, B= *Lr28*, '-' = either homozygous or heterozygous for marker allele

the observed values of six genotypic classes fits well with the expected ratio of 3:1:6:2:3:1 with a non significant chi square value of 7.25 (P- value 0.20).

Marker assisted background analysis

A total of 602 SSR markers spanning all the chromosomes and chromosome arms were selected for background analysis. Out of these SSR markers, 149 were found polymorphic between HD2687 and CLr19, of these, only 48 showed polymorphism between HD2687 and HD2687+*Lr19.* Similarly, 129 SSR markers were polymorphic between HD2687 and CS2A/2M#4/2, out of which only 37 SSR markers were polymorphic between HD2687 and HD2687+*Lr28*. Analysis of data using GGT software showed that HD2687 shared 65 per cent genomic similarity with CLr19 and 89% with backcross line HD2687+*Lr19*. Surprisingly, HD2687 showed greater genomic similarity of 73.5 percent with CS2A/2M#4/2 which morphologically appears much diverse than *CLr19*. However, the backcross line HD2687+*Lr28* had slightly better genomic recovery with 92.1 percent similarity with cultivar HD2687. Thus, the two backcross lines of HD2687 carrying *Lr19* and *Lr28* individually, which were developed by conventional backcross breeding [7] showed good recovery of HD2687 genotypic background. However, the backcross lines still showed about ten percent divergence with the recurrent parent HD2687 which is probably due to limited number of backcrosses.

Table 3. Background analysis showing recovery of recurrent parent genome and percentage of donor genome in each F_2 plant carrying both *Lr19* and *Lr28*

S No.	Plant No.	? (%)	A (%)	B (%)	C (%)	D (%)	H (%)	Total A%
1	101	0.1	90.2	4.5	0.9	1.6	2.7	92.45
2	105	0	88.0	4.9	2.7	0.6	3.8	89.90
3	110	0.1	92.6	3.9	0.8	0.9	1.7	93.45
4	115	0	88.1	4.3	0.7	1.6	5.3	90.75
5	12	0.6	93.9	3.0	0.3	0.8	1.3	94.55
6	122	0.3	88.6	4.1	3.1	0.6	3.3	90.25
7	123	0	88.7	4.6	1.5	0.9	4.4	90.90
8	13	0	91.9	2.9	0.3	1.4	3.6	93.70
9	135	0.3	88.9	4.2	3.0	0.6	2.9	90.35
10	140	0	88.7	5.3	0.8	2.1	3.1	90.25
11	143	0	92.1	3.9	1.0	1.5	1.6	92.90
12	144	0.4	91.1	4.2	0.5	1.5	2.4	92.30
13	149	0	89.4	4.9	1.5	1.5	2.7	90.75
14	16	0	93.6	3.4	0.1	1.9	1.0	94.10
15	161	0.6	90.9	4.0	1.3	0.9	2.3	92.05
16	169	0.1	88.8	4.3	2.3	0.9	3.6	90.60
17	177	1.9	88.9	4.0	1.3	0.9	3.1	90.45
18	180	1.6	86.2	4.1	1.4	0.9	5.7	89.05
19	187	0	90.3	4.3	1.6	1.0	2.8	91.70
20	195	0	89.3	4.8	1.1	0.9	3.8	91.20
21	202	0	90.3	4.3	1.3	1.1	3.0	91.80
22	203	0	88.3	5.3	1.2	0.9	4.3	90.45
23	205	0	90.4	3.9	0.7	1.6	3.4	92.10
24	206	0	89.1	5.3	1.8	0.9	2.8	90.50
25	208	0	90.1	4.4	0.8	1.5	3.2	91.70
26	210	0.2	89.5	4.1	0.6	1.7	3.8	91.40
27	211	0	89.3	4.8	1.5	1.3	3.1	90.85
28	232	0	88.7	4.1	0.5	1.8	4.9	91.15
29	236	0.3	87.2	4.9	3.0	0.9	3.8	89.10
30	242	0	88.7	4.3	0.5	1.7	4.7	91.05
31	246	0	88.4	4.2	3.1	1.4	2.9	89.85
32	25	0.1	90.0	3.9	0.6	2.0	3.5	91.75
33	258	0.4	87.9	4.0	1.1	1.7	4.9	90.35
34	26	0	90.9	4.4	1.9	1.6	1.3	91.55
35	27	0	91.8	3.9	0.6	0.8	2.9	93.25
36	271	0	88.2	4.3	0.8	1.7	5.0	90.70
37	273	0	88.9	5.1	1.2	1.3	3.5	90.65
38	276	0	90.1	4.3	0.9	1.2	3.5	91.85
39	278	0	89.8	5.2	1.2	1.1	2.6	91.10
40	280	0	88.6	3.9	3.3	1.5	2.7	89.95
41	283	0.2	90.2	5.0	2.2	1.0	1.5	90.95
42	44	0	92.2	3.2	0.5	1.7	2.4	93.40
43	46	0.6	93.4	2.7	1.8	0.3	1.2	94.00
44	53	0	90.8	4.1	2.2	1.2	1.7	91.65
45	55	0	86.9	5.6	3.4	0.6	3.4	88.60
46	61	0	88.3	4.4	1.8	0.9	4.5	90.55
47	71	0	87.9	4.1	0.6	0.9	6.4	91.10
48	78	0	88.9	4.2	0.6	1.0	5.3	91.55
49	81	0	86.6	4.3	2.7	0.8	5.6	89.40
50	94	0.3	89.8	5.0	1.9	0.6	2.4	91.00
51	99	0	87.8	5.1	1.2	2.3	3.7	89.65

? = Unknown allele, A= HD2687 allele, B= COOK*6/C80-1, C= CS2A/2M#4/2, D=Common allele of COOK*6/C80-1 (CLr19) and CS2A/2M#4/2, H= Heterozygous for HD2687 allele and either of donor.

Fig. 2. Genomic constitution of F_2 plant with highest percentage of genome (94.55%) from HD2687. A = HD2687 allele, B= COOK*6/C80-1, C= CS2A/2M#4/2, D = Common allele of COOK*6/C80-1 (CLr19) and CS2A/2M#4/2, H= Heterozygous for HD2687 allele and either of donor.

In the F_2 population of the cross HD2687+*Lr19*/ HD2687+*Lr28*, out of 288 plants, 51 were homozygous for *Lr19* based on three molecular markers used, and also carried *Lr28* either in a homozygous or heterozygous condition. These 51 F_2 individuals were genotyped using sixty eight markers polymorphic either between HD2687 and HD2687+*Lr19* or HD2687 and HD2687+*Lr28* or between HD2687 and both of

HD2687+*Lr19* and HD2687+*Lr28*. Analysis of data indicated that recovery of HD2687 genome varied from 88.4% to 94.55% with an average of 91.12% (Table 3). The plant with highest genome recovery of 94.55 percent showed 100% recovery of 3 chromosomes. The Chromosomes 6A, 6B and 2D showed 100% recovery of recurrent parent HD2687 allele (Fig. 2). Though not much information is available about efficiency of marker assisted background selection in wheat, both foreground and background selection has been effectively used in rice improvement [17-21].

Phenotypic screening of F_2 population for leaf rust resistance

The F_2 population was screened against commonly prevalent leaf rust pathotype 77-5 under artificial inoculated conditions. Out of 288 plants, 263 showed resistance to leaf rust pathotype 77-5, while 25 plants showed susceptibility. Since two dominant leaf rust resistance genes *Lr19* and *Lr28* are segregating in the F_2 population, resistant and susceptible plants are expected to segregate in 15:1 ratio. The observed values of 263 resistant and 25 susceptible plants fits well into expected 15:1 ratio with non-significant chi square value of 2.90 (P-value 0.09).

All the plants identified to carry leaf rust resistance gene(s) *Lr19* or *Lr28*, individually or in combination were observed resistant with very few exceptions, validating the results obtained from molecular markers. Two plants (Plant No. 222 and 227) showed presence of marker allele for *Lr28* but were found to be susceptible. Similarly, two plants (Plant no. 2 and 282) showed absence of marker band for *Lr28* but were found to be resistant under artificial inoculated conditions.

The pyramided F_3 lines exhibited a high degree of adult plant resistance against leaf rust pathotype 77-5. The interaction of two or more than two genes in a single genetic background may or may not work profitably as indicated in one of the earlier studies that the association of *Sr36* and *Pm6* is not very strong and therefore, they poorly recombine in some of the genotypes, e.g., Maris Huntsman and Combination III a rare recombinant of *Sr9e* and *Sr36* (20cM) [22]. However, the gene *Lr19* combined with *Lr28* in present study seems to have no negative effect on phenotype based on visual observations. The successful marker-assisted pyramiding of disease resistance genes has already been reported in wheat with respect to three leaf rust genes *Lr13*, *Lr34* and *Lr37* [23] and three powdery mildew genes Pm3, Pm4a and Pm21. Samsampour et al [24] also successfully pyramided *Lr24* and *Lr*48 in the commercial Indian wheat variety. The combination of rust resistance genes *Lr19* and *Lr28* available in genetic background of commercial wheat variety HD2687 is likely to provide durability of resistance which can be strategically deployed after testing the yield potential.

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