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Introgression of *Pi 54* gene through marker assisted backcross breeding for development of blast resistant genetic stocks in rice

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Abstract
Blast is the most common biotic stress leading to the reduction of rice yield in many rice-growing areas of the world. Improvement of rice varieties for resistance to blast disease is one of the most important objectives of rice breeding programmes. Present study was undertaken to introgress the broad spectrum resistance gene *Pi54* into rice variety K 343 through marker assisted backcross breeding in order to avoid the losses caused by the fungus *M. oryzae*. Forground selection of 42 BC_2F_1 plants (K 343*^{3} /DHMAS) with marker RM 206 led to identification of 30 plants positive for *Pi54* gene. Background selection of gene positive BC_2F_1 plants with polymorphic SSR markers identified three plants (P1, P3, P17) which had recurrent parent genome recovery more than 83 percent and broader similarity with recurrent parent with respect to agro-morphological traits. Screening with PLP-1 strain depicted that these plants showed highly resistant reaction.

Keywords: Rice, blast disease, *Pi54* gene, foreground selection, background selection

Introduction
Rice (*Oryza sativa*), one of the most important staple crops, feeds more than half of the world's population. Asia contributes significantly with 90 percent of global rice production and its consumption [1]. Rice is especially important in many highly populated countries, including China and India. Rice blast, caused by the fungus *Magnaporthe oryzae*, is arguably the most devastating fungal disease of rice [2], is a major restriction on rice production in both tropical and temperate rice growing regions of the world [3]. This is a polycystic disease spread by asexual spores (conidia) that infect above ground tissues of rice plants [4, 5, 6]. Rice production has widely increased after the green revolution, but the yield of superior varieties is still not increasing as farmers expect due to the influence of biotic and abiotic factors. These stress conditions affect leaf length and width (flag-surface area), tillering, length of panicle, filled grains number/panicle, 1000 grain weight (seed quality), thereby resulting in low yield.
The disease is a serious production constraint for rice in North Western Himalayan region of India comprising the Union Territory Jammu and Kashmir, Uttrakhand and Himachal Pradesh [7]. Most of the popular rice varieties under cultivation in the hills of Jammu and Kashmir show variable reaction to blast varying from moderately resistant to highly susceptible response [8]. Blast frequently affects coarse grain Kashmiri *Japonica*/*Indica* rice cultivars. Disease severity varies with weather, location, crop growth stage and the innate level of partial resistance of cultivars [9]. Rice production can be managed by introducing new varieties possessing strong resistance against abiotic and biotic factors. Use of resistant varieties is the most economical and environment friendly method to manage this disease [10]. Currently, DNA marker technology has immensely contributed to genetic improvement through the selection of desirable traits, such as disease resistance. Molecular markers are a valuable resource in marker-assisted backcross (MABC) breeding to monitor the disease resistance genes. Thus the present study was planned to introgress *Pi54* gene into well adapted K 343 rice variety using marker assisted selection.

Materials and methods
Plant material
The plant material consisted of one *indica* rice donor genotype DHMAS and one *indica* rice recipient cultivar K 343. BC_2F_1 populations were developed (January to June, 2016) by crossing the recurrent parent (K 343) as a male with *Pi54* positive agronomically superior BC_1F_1 genetic stocks (K 343*^{2} /DHMAS) which were used as female plants.

Genotyping of research material generated

Genomic DNA was isolated from leaves of rice with slight modifications [11]. For foreground selection of *Pi54* gene SSR marker RM206 (0.7 cM) was used in BC_2F_1 population based on earlier studies [12, 13, 14]. 50 SSR markers which had shown parental polymorphism between the parents K 343 and DHMAS were used for background selection of the BC_2F_1 population (K 343*[3] / DHMAS). It was done to assess the recovery of recurrent parent genome and to select only those plants having maximum recovery of recurrent parent genome.

Amplification of DNA was carried out PCR tubes with total volume of master mix 10µl containing 5.3µl of nuclease free water, 2.2 µl 5X PCR buffer with 15mM (MgCl2), 0.3 µl of 2.5 mM/ µl dNTP, 0.5 µl of each forward and reverse primers, 5 U of Taq polymerase. An initial denaturation step (94°C) of 5 min was programmed in the thermo Cycler, followed by a loop of 35 cycles each consisting of denaturation (94°C for 30 sec), annealing (55°C – 58°C for 30 sec depending on the marker used) and extension (72°C for 30 sec). The final extension was performed at 72°C for 7 min.

Evaluation of recurrent parent genome recovery in BC_2F_1 using GGT 2.0 software

The SSR bands for all the plants in BC_2F_1 populations were counted and scored manually as "A'' for their resemblance with the one parent, "B'' for its resemblance with the other parent, "H'' if both the bands were present i.e. resembled with both the parents and "-'' if no band was present. The sizes of the bands were estimated by comparing them with 100bp standard marker along with the both the parents. The graphical representation of molecular marker data was done using computer programme GGT 2.0 (an acronym for Graphical GenoTypes) [15]. GGT 2.0 software is able to graphically represent chromosome wise and overall recovery of recurrent parent genome and also gives numerical representation of recurrent parent genome recovery (%) of each plant genotyped.

Phenotyping for agro-morphological traits in BC_2F_1 gene positive plants

The BC_2F_1 population along with parents K 343 and DMAS were evaluated at Experimental Research Farm and Greenhouse at School of Biotechnology, SKUAST-Jammu during *Kharif* seasons of 2017. The 25 days old selected plants were transplanted with spacing of 15 × 20 cm in augmented-II design in the field. Observations on single plants were recorded as per the DUS guidelines of DRR, Hyderabad [16].

To test the significance of variations among different genotypes evaluated in the study, data with respect to blocks and treatments (including checks and test genotypes) were subjected to analysis of variance as per augmented design-II [17] to obtain adjusted trait values for checks as well as test genotypes.

Pathotyping of BC_2F_1 populations for blast symptoms

The pathotypic screening of the BC_2F_1 plants population was done using the PLP-1 strain of *M. oryzae*, which is the predominant biotype in the North Western Himalayan region. All BC_2F_1 plants along with parents were inoculated with PLP-1 using spray under greenhouse at School of Biotechnology (Plate 3.5). The seedlings were inoculated with conidial suspension (1×10^5 spores/ml) of *Magnaporthe oryzae* at the three to four leaf stages (Sharma *et al.*, 2005b). The inoculated plants were then placed in dark at high relative humidity (> 90%) for 24 h, and subsequently transferred to a polyhouse, under a regime of 16 h light/8 h dark at 80 per cent relative humidity. Day and night temperatures were maintained at 35± 2°C and 21± 2°C, respectively.

Disease reactions of inoculated plants were recorded on a scale of 0–5 [18], 6–7 days after inoculation. The plants exhibiting reactions that scored 0-2 were considered resistant while those showing reactions that scored 3-5 were categorized as susceptible.

Results and discussion

Rice blast is considered as the major disease of rice because of its wide distribution and extent of destruction under favorable conditions. In Jammu and Kashmir, it is the most devastating disease in hill and temperate ecologies where rice is grown in hundred percent irrigated and cool night ecology of *Kharif* season [8] which aids in blast build up and subsequently widely occurring blast epidemics in rice in the Union territory. Using major resistance genes for rice blast resistance improvement is considered to be an efficient and technically feasible approach to achieve optimal grain yield.

Foreground and background selections in backcross progenies

Identified *Pi54* gene positive agronomically superior BC_1F_1 plants were backcrossed with the recurrent parent K 343 to develop BC_2F_1 population. Backcrossing is done to further increase the recovery of recurrent parent genome in upcoming generations while the foreground selection is done to track a particular trait like disease resistance using marker assisted selection (MAS). In foreground selection homozygous plants were selected as they do not segregate during crossing over in the process of recombination and hence are stable. BC_2F_1 population was subjected to foreground selection to track the presence of *Pi54* gene in the population and to ensure it is not lost during the process of recombination.

A total of 42 BC_2F_1 (K 343*[3] /DHMAS) plants were grown and screened for the presence of *Pi54* gene by using closely linked marker RM206. Out of these, 30 plants were found positive for *Pi54* gene through foreground selection (Plate1). They were subjected to background selection to identify the plants with maximum percentage of recurrent parent genome. Similar studies have carried out earlier [19, 20, 21].

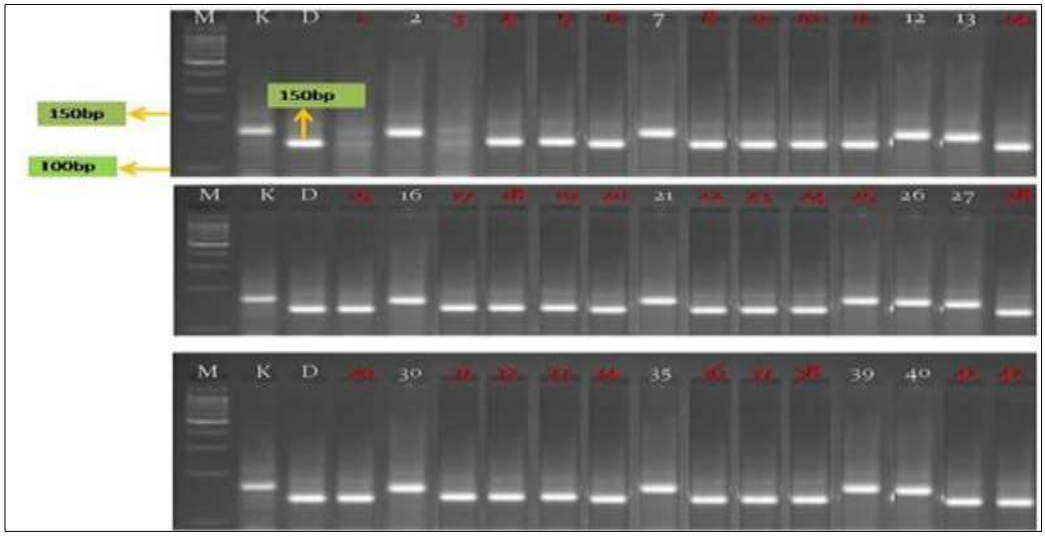

Plate 1: Foreground selection of *Pi54* gene in BC_2F_1 generation using RM206 marker (K = K 343; D =DHMAS; 1-42=BC_2F_1 plants)

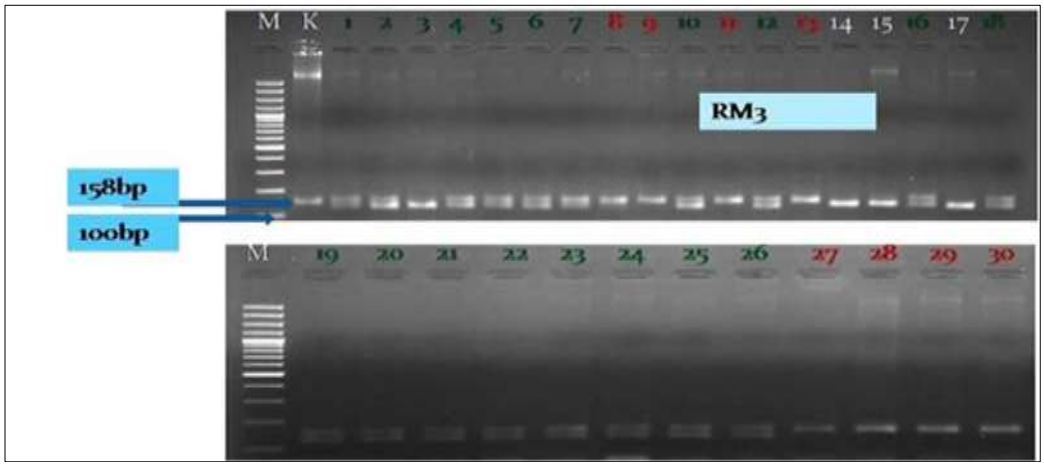

Plate 2: Band amplification pattern of SSR marker RM3 (K= K 343; 1 to 30 = BC_2F_1 plants)

Background selection in BC_2F_1 stocks

Background selection is the process of using markers to minimize the length of the donor segment around a target locus to accelerate the recovery of recurrent parent genome during backcrossing. Background selection in target gene (*Pi54*) positive plants in the genetic stock (K 343*^{3}/DHMAS) led to estimation of percent recurrent parent genome recovery using about 50 genome wide polymorphic SSR markers. Genotypic data when analyzed using GGT 2.0 software [15] identified 3 plants (P1=86.4%, P17= 83.65% and P3= 83.40) which had recurrent parent genome recovery more than 83 percent in the genetic stock K 343*^{3}/ DHMAS with chromosomes 1 and 2 showing more than 90 percent recovery in most of the plants (Table 1)**.** Thus marker assisted background selection is a potential tool to identify the plants among the large population having more than average recurrent parent genome recovery and thus accelerates the pace of selection and development of varieties in comparison to conventional breeding approaches of selection. Integration of foreground, background and /or phenotypic selection to achieve high recovery of recurrent parent genome and phenome has been practiced in various studies [22, 23, 24, 19, 26, 27, 20, 21].

Table 1: Recurrent parent genome recovery in BC_2F_1 population (K 343*^{3}/DHMAS)

Plant	A% (Recurrent parent genome)	B% (Donor parent genome)
P1	86.40	13.60
P2	36.95	63.05
P3	83.40	16.60
P4	33.10	66.90
P5	40.80	59.20
P6	35.60	64.40
P7	33.75	66.25
P8	38.30	61.80
P9	40.00	59.90
P10	32.20	67.80
P11	43.30	56.70
P12	35.90	64.20
P13	75.30	24.70

P14	31.20	68.80
P15	30.25	69.75
P16	33.65	66.35
P17	83.65	16.35
P18	42.10	57.90
P19	47.95	52.05
P20	29.75	70.25
P21	40.85	59.25
P22	32.40	67.60
P23	68.15	31.85
P24	36.80	63.10
P25	79.15	20.95
P26	72.40	27.60
P27	34.10	66.00
P28	43.40	56.50
P29	42.05	57.95
P30	45.85	54.65

A graphical representation of all the individual plants for all the chromosomes of the selected genetic stocks for blast resistance is shown in (Figure 1). The red coloured regions represent the homozygous regions of the recipient genome and the maximum recovery of recurrent parent genome was observed for chromosome number 1 and 2. The blue coloured regions represent genome of donor parent. Most of the residual segments from donor genome were distributed on chromosomes 3, 4, 5, 6, 8, 9, 10, 11 and 12, while the light green coloured regions indicate heterozygous regions.

Analysis of variance for morphological/agronomical traits in BC_2F_1 population of gene positive plants exhibited non-significant variations for most of the agro-morphological traits except for plant height, panicle length, number of effective tillers and grain length which gave indication about uniformity of traits in genetic stocks (Table 2). Most of the test entries were similar in various morpho-physiological traits like the recipient parent, K 343. Maximum grain yield was recorded in case of P8 and P5 (29.1g) while minimum grain yield (22.40g) was observed in P2.The average grain yield per plant was recorded as 26.10g with the range varying from 22.40 g to 29.10 g. (Table 3) In case of plant height, the maximum plant height was recorded in P8 (131.90cm) followed by P23 (131.30cm), P20 (131.3cm) and P22 (130.10cm) where as P4 recorded a minimum plant height i.e. 120.20cm. The range of plant height in BC_2F_1 population was between 120.20- 131.90 cm with an average of 126.66 cm. The number of effective tillers per plant ranged between 8-10 with an average of 9 tillers per plant. The maximum numbers of effective tillers per plant were recorded in P1, P3, P7, P10, P13, P14, P15, P21 (10) followed by P2 (9), where as the minimum numbers of effective tillers per plant were recorded in P3, P5 P16 P22, P29 (8). In case of panicle length, the maximum value was recorded in P1 and P20 (23.50cm) followed by P14 (23.3cm), P15(22.9cm) and P29 (22.7cm) whereas the minimum value was recorded in P22 (18.60cm). The panicle length had a range varying from 18.6 to 23.5cm with an average value of 21.17 cm. Highest value of 1000-grain weight was observed in P8 and P21 (29.20g), followed by P19 (28.50g), P17 (28.3g) and P13 (28.2g) whereas the lowest 1000- grain weight was recorded in P2 (21.70g).The mean value of 1000-grain weight recorded was 26.10 g and ranged between and 21.70 g to 29.20 g. In case of days to 50 percent flowering the BC_2F_1 which took maximum days to flowering were P10 (94 days) where as the minimum number of days to 50 percent flowering were recorded in P2 (88) and ranged between 88-94 with an average value of 92 days. Duration of grain filling in the BC_2F_1 ranged from 35-39 days with an average value of 35.86 days. P21 took maximum duration of grain filling (39days) whereas the P1 took minimum number of duration of grain filling (35 days).

Days to maturity in the BC_2F_1 ranged from and 128 to 131 days with an average value of 128.3 days. P10 took maximum days to mature (131days) whereas the P2 took minimum number of days to mature (128 days). The grain quality attributes like grain length showed an average value of 5.77mm with a range varying from 5.11-6.91mm. The maximum value for grain length was recorded in P4 (6.91mm) followed by P15 (6.33mm), P23 (6.29mm), P12 (6.20) whereas the minimum grain length was observed in (5.11mm). The grain quality attributes like grain breadth showed an average value of 2.53 mm with a range varying from 2.30- 2.72mm. The maximum breadth of grains was recorded in P23 (2.72mm) followed by P3 (2.68mm) and P15 (2.65mm) whereas the minimum grain breadth was recorded in P2 (2.22mm).

Table 2: Analysis of Variance of genotypes BC_2F_1 (K 343*[3]/DHMAS) for yield and yield contributing traits

Source of variation	DF	Plant height (cm)	Days to 50% flowering	Days to maturity	Duration of grain filling	Panicle length (cm)	Effective tillers	Grain length (mm)	Grain breadth (mm)	Grain yield /plant (g)	1000 grain weight (g)
Mean sum squares											
Blocks	2	0.17	2.16	3.50	10.66	2.85*	0.00*	0.001*	0.0004	1.68	2.13
Treatment	31	46.90*	3.65	4.37	1.89	1.65*	0.45*	0.21*	0.018	2.20	2.75
Tests	29	48.79*	1.45	0.93	1.88	1.41*	0.41*	0.17*	0.007	1.38	2.14
Checks	1	13.05*	32.66*	54.00	2.66	9.15*	1.50*	1.46*	0.12*	9.15*	5.41
Test v/s checks	1	25.80*	38.27*	54.45	1.42	1.05*	0.67*	0.02*	0.22*	18.7*	17.75
Error	2	0.11	1.16	3.50	0.66	0.001	0.00	0.001	0.005	0.32	1.35

* - Significant at 5% (level of significance opted by user), NS - Non Significant
p-Value < 0.05 - Significant at 5%, p-Value < 0.01 - Significant at 1%

Table 3: Mean performance of genotypes BC_2F_1 (K 343*[3]/DHMAS) for yield and yield contributing traits

Genotypes	Plant height(cm)	Days to 50% Flowering	Days to maturity	Duration of grain filling	Panicle length (cm)	No. of effective tillers/ plant	Grain length (mm)	Grain breadth (mm)	Grain yield /plant (g)	1000 grain weight (g)
P1	122.23	93	128	35	23.50	10	6.25	2.42	25.02	24.60
P2	125.71	88	130	33	21.10	9	5.22	2.22	22.40	21.70
P3	125.14	93	128	35	21.10	10	6.05	2.68	28.40	24.50
P4	120.20	93	128	35	20.80	9	6.01	2.57	25.50	25.20
P5	130.00	93	128	35	21.51	8	5.22	2.53	29.10	24.30
P6	125.00	93	128	35	20.70	9	6.91	2.60	25.23	25.00
P7	125.21	93	128	35	20.31	10	5.21	2.30	26.90	23.60
P8	131.90	93	128	35	20.00	9	5.11	2.52	29.10	29.20
P9	125.31	93	128	35	21.20	9	5.21	2.45	25.50	25.00
P10	127.87	94	131	37	20.21	10	5.44	2.45	25.80	26.30
P11	126.21	93	128	35	20.90	9	5.70	2.48	25.30	27.40
P12	126.56	93	128	35	21.20	9	6.20	2.54	25.70	25.34
P13	125.21	93	128	35	22.13	10	6.20	2.52	26.00	28.20
P14	126.62	93	128	35	23.30	10	5.92	2.63	26.20	27.40
P15	121.71	93	128	35	22.90	10	6.33	2.67	26.00	28.10
P16	121.51	93	128	35	21.70	8	6.19	2.60	25.61	25.40
P17	125.52	93	128	35	21.20	9	6.11	2.45	25.43	28.30
P18	126.26	93	128	35	21.00	10	5.22	2.47	26.13	27.50
P19	121.21	93	128	35	21.60	9	5.19	2.52	26.21	28.50
P20	130.30	93	128	35	23.50	9	5.61	2.61	25.60	24.30
P21	126.20	89	128	39	19.80	10	5.30	2.50	25.1	29.20
P22	130.10	89	128	39	18.60	8	5.91	2.52	27.00	27.60
P23	131.30	89	128	39	19.30	9	6.29	2.72	24.20	26.20
P24	128.70	89	128	39	22.60	9	5.90	2.61	27.10	25.20
P25	126.10	89	128	39	21.60	9	5.90	2.60	25.30	25.50
P26	124.20	93	128	35	21.20	9	5.88	2.51	26.00	26.20
P27	123.60	93	128	35	22.30	8	5.72	2.53	26.10	25.40
P28	125.10	93	128	35	20.90	9	6.02	2.60	26.00	24.10
P29	124.50	93	128	35	22.70	8	5.81	2.41	25.41	25.40
P30	121.20	93	128	35	20.20	9	5.88	2.54	25.43	26.30
K 343 (C)	130.02	93	128	35	23.50	10	6.25	2.42	24.60	25.02
DHMAS(C)	127.50	87	120	33	19.00	9	5.21	2.21	24.50	23.00
Mean	126.60	92.43	128.30	35.80	21.10	9.10	5.70	2.53	26.10	26.10
CV (%)	3.55	2.00	1.30	2.55	1.80	4.00	2.10	3.00	2.25	5.15
SE(m)	0.50	0.10	0.20	0.10	0.60	0.22	0.40	0.04	0.10	0.10
CD (5%)	4.00	6.50	3.10	4.50	3.60	1.50	1.30	0.50	7.70	7.55

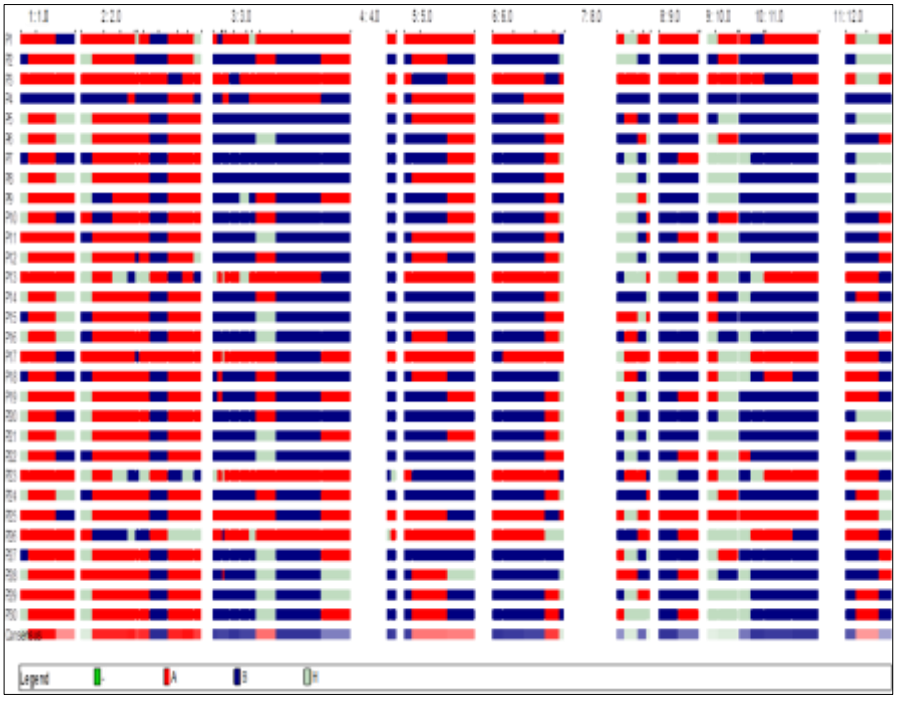

Fig 1: Genome introgression of 30 BC_2F_1 (K 343*[3]/ DHMAS) introgressed lines using software Graphical GenoTypes (GGT 2.0) (Van Berloo, 1999)

Pathotyping of BC_2F_1 population (K 343*^{3} /DHMAS)

All the 30 gene positive plants carrying *Pi54* gene in the background of K 343 in BC_2F_1 generation along with the donor and recipient parents were inoculated with PLP-1 strain of *M. oryzae*. These plants showed 0-2 score depicting resistant reaction while the recipient parent K 343 showed susceptible reaction with the score 3 (Table 4).

The genetic stocks of K 343*^{3}/DHMAS with maximum recovery of recurrent parent genome were compared agronomically and pathologically with the recurrent parent (Table 5). The maximum recovered recurrent parent genome in plant numbers P1, P3 and P17 had broader agronomical similarity to the recurrent parent and pathologically related to the donor parent. The results confirmed the accuracy of marker assisted selection (MAS) for the gene *Pi54* using the corresponding marker RM206. These plants would serve as genetic stocks for development of blast resistant lines/varieties or donor for development of blast resistant varieties [12, 27].

Table 4: Pathotyping of BC_2F_1 (K 343*^{3}/DHMAS) plants for blast symptoms

S. No.	Genotype	Score	Disease reaction
1	K 343	3	Susceptible
2	DHMAS	1	Highly resistant
3	P1	0	Highly resistant
4	P2	0	Highly resistant
5	P3	0	Highly resistant
6	P4	2	Moderately resistant
7	P5	2	Moderately resistant
8	P6	2	Moderately resistant
9	P7	1	Resistant
10	P8	2	Moderately resistant
11	P9	2	Moderately resistant
12	P10	2	Moderately resistant
13	P11	2	Moderately resistant
14	P12	2	Moderately resistant
15	P13	0	Highly resistant
16	P14	2	Moderately resistant
17	P15	2	Moderately resistant
18	P16	1	Resistant
19	P17	0	Highly resistant
20	P18	2	Moderately resistant
21	P19	2	Moderately resistant
22	P20	2	Moderately resistant
23	P21	2	Moderately resistant
24	P22	2	Moderately resistant
25	P23	2	Moderately resistant
26	P24	2	Moderately resistant
27	P25	1	Resistant
28	P26	2	Moderately resistant
29	P27	2	Moderately resistant
30	P28	2	Moderately resistant

Table 5: Agronomical and pathological status of genetic stock K343*^{3}/DHMAS with maximum RPG recovery

K 343*^{3}/DHMAS					
Gene positive plants *Pi54*	**DHMAS**	**K 343**	**P1**	**P3**	**P17**
RPG (%)			86.40	83.40	83.65
Disease score	0	3	0	0	0
Plant height (cm)	127.30	130.00	122.20	125.10	125.52
Days to 50 percent flowering	88	93	93	93	93
Days to maturity	121	128	128	128	128
Duration of grain filling	35	35	35	35	35
Panicle length (cm)	21.10	23.50	21.10	21.51	21.20
Effective tillers	9	10	10	8	9
Grain length (mm)	5.22	6.25	6.05	5.22	6.11
Grain breadth (mm)	2.22	2.42	2.68	2.53	2.45
Yield per plant (g)	22.40	25.02	28.40	29.10	25.43
1000 grain weight (g)	21.70	24.60	24.50	24.30	28.30

Conclusion

K 343 being a well adapted variety but susceptible to blast, needed to be introgressed with the broad spectrum resistance gene like *Pi54* in order to avoid the losses due to the fungus *M. oryzae*. The *Pi54* positive genetic stocks of K 343*^{3}/DHMAS with maximum recovery of recurrent parent genome had broader agronomical similarity to the recurrent parent and pathologically related to the donor parent. The results confirmed the accuracy of marker assisted selection (MAS) for the gene *Pi54* using the corresponding marker RM206. These plants would serve as genetic stocks for development of blast resistant lines/varieties or donor for development of blast resistant varieties [12, 27].

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