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Development and Characterization of Blast Resistant Genetic Stocks in Rice for *Pi 9* Gene through Backcross Breeding

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A B S T R A C T

A major issue in rice production is the control of *Magnaporthe oryzae*, the causal agent of rice blast. K 343 being a well adapted variety in the hill zone of Jammu and Kashmir region is susceptible to blast. Present study was undertaken to introgress the broad spectrum resistance gene *Pi9* through marker assisted backcross breeding in order to avoid the losses due to this fungus. Foreground selection *Pi9* gene in BC_2F_1 (K 343*3/RML 22) population with marker AP5930 identified 30 target gene positive plants. Background selection for analysis of recurrent parent genome in target gene positive plant using genome wide polymorphic SSR markers led to identification of three plants three plants (P3, P11, P28) in BC_2F_1 which had recurrent parent genome recovery more than 85 percent. These plants exhibited broader similarity with recurrent parent with respect to agro-morphological traits and resistant to highly resistant reaction to PLP-1 strain of rice blast fungus *M. oryzae.* It indicates the effectiveness of resistance provided *Pi9* gene. These plants would serve as genetic stocks for development of blast resistant lines/varieties or donor for development of blast resistant varieties.

Introduction

Rice (*Oryza sativa* L.) is a self-pollinated diploid plant (2n=24) with a genome size of 430 Mb and is the most important staple cereal food crop in world. Asia accounts for 90 percent of global rice consumption, and total rice demand continues to rise. To ensure food security, the efficient way is the continuous improvement of rice yield (Liang *et al.*, 2014) and the current level of production needs to be increased to 130 million tonnes by the year 2025. Inspite of being an important staple crop, rice is affected by many biotic and abiotic stresses. Of all the biotic stresses, blast caused by the heterothallic ascomycete fungus *Magnaporthe oryzae* (Hebert), is a major restriction on rice production in both tropical and temperate rice growing regions of the world (Raghu *et al.*, 2018). This is a polycystic disease spread by asexual spores (conidia) that infect above ground tissues of rice plants (Ou *et al.*, 1985; Talbot and Wilson, 2009; Pennisi, 2010).

Several rice blast epidemics have occurred in different parts of the world, resulting in heavy yield losses upto 90 percent (He *et al.,* 2012; Singh *et al.,* 2015; Li *et al.,* 2019) The incidence of the disease has been reported in 85 countries, particularly in the irrigated and rainfed lowlands of temperate and subtropical Asia, Latin America and Africa (Sharma *et al.,* 2012). 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 (Sharma *et al.,* 2002). 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 (Ali *et al.,* 2009). 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 (Anwar *et al.,* 2009). The disease can be managed through agronomic practices, use of fungicides, planting resistant cultivars and biotechnological interventions (Ribot *et al.,* 2008). The excessive and indiscriminate use of fungicides prompts the evolution of resistance in the fungus, which in turn leads to disease resurgence, hence, breeding for host resistance is the most reliable, simple, economical and eco-friendly approach for the management of this disease (Khush and Jena, 2009). Thus, the present study was planned to introgress *Pi9* blast resistance gene into well adapted rice variety K 343 using marker assisted selection.

Materials and Methods

Plant material

The plant material consisted of one *indica* rice donor genotype RML22 and one *indica* rice recipient cultivar K 343. BC_2F_1 population was developed (January to June, 2016) by crossing the recurrent parent (K 343) as a male with BC_1F_1 genetic stocks (K 343*^{2} /RML 22) which were used as female plants.

Genotyping of research material generated

Genomic DNA was isolated following Doyle and Doyle (1990) method, with slight modifications. For foreground selection of *Pi9* gene with SSR marker AP5930 (0.05cM) was donw in BC_2F_1 population based on earlier studies (Sharma *et al.,* 2005; Fjellstrom *et al.,* 2006; Hangloo, 2018). A total of 51 SSR markers which had shown parental polymorphism between the parents K 343 and RML 22 were used for background selection of the BC_2F_1 population (K 343*^{3} /RML 22). 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 in 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) developed by Van Berloo (1999) at Wageningen University, The Netherlands. 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 RML 22 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 design-II design in the field. Observations on single plants were recorded as per the DUS guidelines of IIRR, Hyderabad (Rani *et al.*, 2006).

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 (Federer, 1956) 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-1isolate 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 as standardized by Bonman *et al.,* (1986) under greenhouse at School of Biotechnology. The seedlings were inoculated with conidial suspension (1×10^5 spores/ml) of *Magnaporthe oryzae* at the three to four leaf stages as described by (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$\pm$ 2°C and 21$\pm$ 2°C, respectively.

Disease reactions of inoculated plants were recorded on a scale of 0–5 (Bonman *et al.*, 1986), 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

K 343 being a predominant rice variety in the hill zone of Jammu region shows moderate to susceptible response to blast fungus over the years depending upon the prevailing weather conditions. With the sequencing of whole rice genome closely linked/gene derived markers and potential donors for the genes are available. Thus, these genes can be easily deployed for generating resistance response in susceptible temperate rice variety (K 343). The marker assisted backcross breeding approach coupled with phenotypic selection helped in improving the elite genotype with blast resistance genes.

Foreground and background selections in backcross progenies

Advances in rice genomics and sequencing of whole rice genome have enabled the use of

DNA marker system mainly to track the trait of interest like disease resistance in segregating generations (Jena and Mackill, 2008). In the present investigation 42 BC_2F_1 plants were grown and screened for the presence of *Pi9* gene by using closely linked marker AP5930. Out of the 42 BC_2F_1 plants (K 343*^{3} /RML 22), 30 plants were found positive for *Pi9* gene using foreground selection (Plate1).

The purpose of background selection was to know the recovery of recurrent parent's genome content in the backcross derived lines. Background selection was performed in 30 foreground positive BC_2F_1 plants using 51 polymorphic SSR markers (Plate 2).

The BC_2F_1 plants confirmed positive for the gene *Pi54* and *Pi9* were subjected to background selection to identify the plants with maximum percentage of recurrent parent genome. Foreground selection is often followed by recombinant selection process to select for recurrent parent alleles at markers flanking target regions with the aim of reducing linkage drag. Similar studies have carried out earlier by Singh *et al.*, (2012a); Patroti *et al.*, (2019) and Sagar *et al.*, (2020)

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 (*Pi9*) positive plants in each of the genetic stock K 343*^{3}/RML 22 led to estimation of percent recurrent parent genome recovery using about 51 genome wide polymorphic SSR markers. Genotypic data when analyzed using GGT 2.0 software (Van Berloo, 1999) identified 3 plants as P3 (86.4%), P11 (85.8%) and P28 (93.25%) with chromosomes 1, 2 and 10 showing 85-90 percent of recovery in most of the plants in the stock population (Table 1), (Figure 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 Neeraja *et al.,* (2005); Sundaram *et al.,* (2008); Gopala Krishnan *et al.,* (2008); Singh *et al.,* (2012a); Divya *et al.,* (2014) and Miah *et al.,* (2014); Patroti *et al.,* (2019) and Sagar *et al.,* (2020).

Analysis of variance for morphological/agronomical traits in both BC_2F_1 populations exhibited non-significant variations for most of the agro-morphological traits except for plant height, number of effective tillers and grain length which gave indication about uniformity of traits in genetic stocks (Table 2). Many test entries with the target genes showed grain yield slightly higher than the recurrent parent, K 343 (Table 3). Most of the test entries were similar in various morpho-physiological traits like the recipient parent, K 343. Maximum grain yield was recorded in P12 (27g) followed by P25 (26.9g), P11 (26.5g) and P13 (26.3g) whereas a minimum grain yield of 24g was recorded in P30. The average grain yield per plant was recorded as 25.60g with the range varying from 24g- 27g (Table 3). In case of plant height, the maximum value was recorded in P2 (133.1cm), followed by P21 (132.1cm) and P23 (131.7) where as P29 recorded a minimum plant height i.e. 121.21cm. The range for plant height in the BC_2F_1 population was between 121.21- 133.10cm with an average of 128.77cm. The number of effective tillers per plant ranged between 8-9

with an average of 8.83. The maximum numbers of effective tillers per plant were recorded in P1 (9) where as the minimum numbers of effective tillers per plan were recorded in P4 (8). In case of panicle length, the maximum value was recorded in P3 (26.20cm) followed by P12 (25cm) and P13 (24.4cm) whereas the minimum value was recorded in P16 (18.90cm). The panicle length had a range varying from 18.90 - 26.20cm with an average value of 22.12 cm. Highest value of 1000-grain weight was observed in P9 (28.50g), followed by P15 (28.10g) and P17 (28g) whereas the lowest 1000- grain weight was recorded in P25 (23.60g).

Table.1 Recurrent parent genome recovery in BC_2F_1 population (K 343*^{3}/RML 22)

Genotypes	A%	B%
P1	84.85	**15.15**
P2	55.80	**44.10**
P3	86.40	**13.50**
P4	80.80	**19.20**
P5	70.50	**29.50**
P6	59.05	**40.95**
P7	70.05	**29.95**
P8	79.50	**20.50**
P9	70.50	**29.50**
P10	69.20	**30.70**
P11	85.80	**14.20**
P12	63.90	**36.10**
P13	74.35	**25.65**
P14	68.55	**31.45**
P15	61.60	**38.40**
P16	65.30	**34.70**
P17	29.80	**70.20**
P18	33.50	**66.50**
P19	60.95	**39.05**
P20	73.30	**26.70**
P21	56.75	**43.25**
P22	64.40	**35.60**
P23	58.75	**41.25**
P24	63.60	**36.40**
P25	71.70	**28.20**
P26	65.50	**34.50**
P27	65.40	**34.60**
P28	93.25	**6.75**
P29	74.65	**25.35**
P30	**64.35**	**35.65**

Table.2 Analysis of Variance of genotypes BC_2F_1 (K 343*^{3}/RML 22) 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	0.071	2.666	.000	2.66	4.80	0.000*	0.025	0.0034	1.349	**2.101**
Treatment	31	7.906*	6.282	7.322	0.561	3.47	0.198*	0.073*	0.0353	1.654	**1.538**
Tests	29	7.870*	2.107	0.868	0.355	2.86	0.141*	0.066*	0.0189	0.538	**0.975**
Checks	1	11.76*	60.16*	96.00	4.16	8.16*	1.50*	0.20*	0.17*	12.24*	**6.82**
Test v/s checks	1	5.08*	73.47*	105.80	2.93	16.56*	0.55*	0.16*	0.374*	23.42*	**12.55**
Error	**2**	**0.01**	**0.66**	**0.00**	**0.66**	**0.41**	**0.00**	**0.003**	**0.005**	**0.37**	**0.22**

Table.3 Mean performance of genotypes BC_2F_1 (K 343*^{3}/RML 22) 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)
K 343	129.2	93	128	35	19.5	9	5.44	2.47	26.1	**25.8**
RML 22	126.5	86	120	34	18	8	5.11	2.01	22.51	**24**
P1	129.20	93	128	35	20.90	9	5.70	2.48	25.30	**27.40**
P2	133.10	93	128	35	21.20	9	5.31	2.54	25.70	**25.10**
P3	133.10	93	128	35	26.20	9	5.21	2.52	26.00	**25.50**
P4	125.30	93	128	35	23.30	8	5.92	2.63	26.20	**27.40**
P5	133.10	93	128	35	22.90	9	5.44	2.67	26.00	**25.20**

P6	129.30	93	128	35	21.70	8	5.64	2.60	26.10	**26.60**
P7	130.30	93	128	35	21.20	9	5.55	2.45	26.21	**26.30**
P8	129.10	93	128	35	21.00	9	5.22	2.47	25.00	**27.50**
P9	127.10	93	128	35	21.60	9	5.19	2.52	26.20	**28.50**
P10	130.30	93	128	35	23.50	9	5.61	2.61	25.60	**25.30**
P11	128.90	94	131	37	22.20	9	5.39	2.53	26.50	**26.40**
P12	126.30	94	131	37	25.00	8	5.59	2.37	27.00	**26.40**
P13	127.30	94	131	37	24.40	9	5.99	2.70	26.30	**26.50**
P14	127.30	94	131	37	19.50	9	5.25	2.18	26.20	**25.70**
P15	131.30	94	131	37	22.90	9	5.57	2.65	25.50	**28.10**
P16	129.10	94	131	37	18.90	9	5.01	2.36	26.10	**25.30**
P17	130.10	89	128	39	21.30	9	5.95	2.43	25.20	**28.00**
P18	130.90	89	128	39	21.60	8	5.34	2.58	25.60	**26.60**
P19	129.80	89	128	39	22.90	9	5.25	2.45	26.10	**25.40**
P20	129.30	89	128	39	22.30	9	5.87	2.65	25.20	**25.20**
P21	132.10	93	128	35	21.10	9	5.43	2.68	25.20	**25.50**
P22	127.30	93	128	35	20.80	9	5.21	2.57	25.50	**25.20**
P23	131.70	93	128	35	24.30	9	5.22	2.53	25.11	**24.30**
P24	129.20	93	128	35	20.70	9	5.55	2.60	24.20	**25.00**
P25	129.00	93	128	35	23.20	9	5.21	2.30	26.90	**23.60**
P26	121.51	93	128	35	21.70	8	5.56	2.60	25.61	**25.40**
P27	125.52	93	128	35	21.20	9	5.42	2.45	25.43	**26.21**
P28	125.20	93	128	35	21.00	9	5.22	2.47	24.21	**24.12**
P29	121.21	93	128	35	21.60	9	5.19	2.21	24.01	**25.51**
P30	130.30	93	128	35	23.50	9	5.61	2.21	24.00	**24.30**
Mean	128.77	92.66	128.60	35.93	22.12	8.83	5.45	2.50	25.60	**25.91**
CV	0	1	0	2	3	0	1	3	2	**2**
SE(m)	**0**	**0.17**	**0**	**0.17**	**0.17**	**0**	**0**	**0**	**0.17**	**0**

Table.4 Pathotyping of BC_2F_1 (K 343*[3]/RML 22) plants for blast symptoms

S. No.	Genotype	Score	Disease reaction
1	K 343	3	**Susceptible**
2	RML 22	0	**Resistant**
3	P1	0	**Highly Resistant**
4	P2	1	**Resistant**
5	P3	0	**Highly Resistant**
6	P4	0	**Highly Resistant**
7	P5	2	**Moderately Resistant**
8	P6	2	**Moderately Resistant**
9	P7	2	**Moderately Resistant**
10	P8	0	**Highly Resistant**
11	P9	2	**Moderately Resistant**
12	P10	1	**Resistant**
13	P11	2	**Moderately Resistant**
14	P12	2	**Moderately Resistant**
15	P13	2	**Moderately Resistant**
16	P14	2	**Moderately Resistant**
17	P15	2	**Moderately Resistant**
18	P16	2	**Moderately Resistant**
19	P17	0	**Highly Resistant**
20	P18	1	**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	2	**Moderately Resistant**
28	P26	2	**Moderately Resistant**
29	P27	2	**Moderately Resistant**
30	**P28**	**0**	**Highly Resistant**

Table.5 Agronomical and pathological status of genetic stocks K 343*[3]/RML 22 with maximum RPG recovery

K 343*[3]/RML 22					
Gene positive plants *Pi9*	**RML 22**	**K 343**	**P 3**	**P 11**	**P 28**
RPG (%)			86.4	85.8	**93.2**
Disease score	0	3	0	0	**0**
Plant height (cm)	126.5	129.2	133.1	128.9	**125.2**
Days to 50 percent flowering	86	93	93	94	**93**
Days to maturity	120	128	128	131	**128**
Duration of grain filling	34	35	35	37	**35**
Panicle length (cm)	18	19.5	26.2	22.2	**21**
Effective tillers	8	9	9	9	**9**
Grain length (mm)	5.1	5.4	5.2	5.3	**5.2**
Grain breadth (mm)	2.0	2.4	2.5	2.5	**2.4**
Yield per plant (g)	22.5	26.1	26	26.5	**24.2**
1000 grain weight (g)	**24**	**25.8**	**25.5**	**26.4**	**24.1**

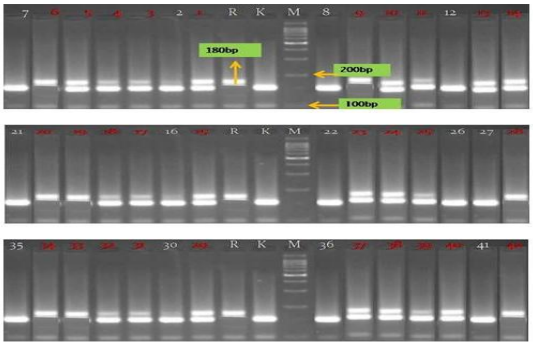


Plate 1: Foreground selection of *Pi9* gene in BC_2F_1 generation using AP5930 marker (K = K 343; R =RML 22; 1-42=BC_2F_1 plants)

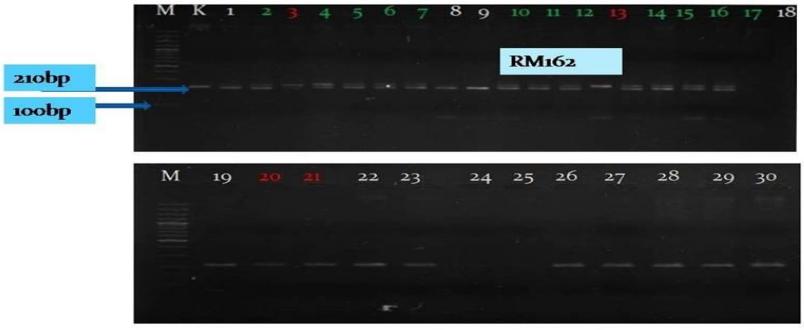
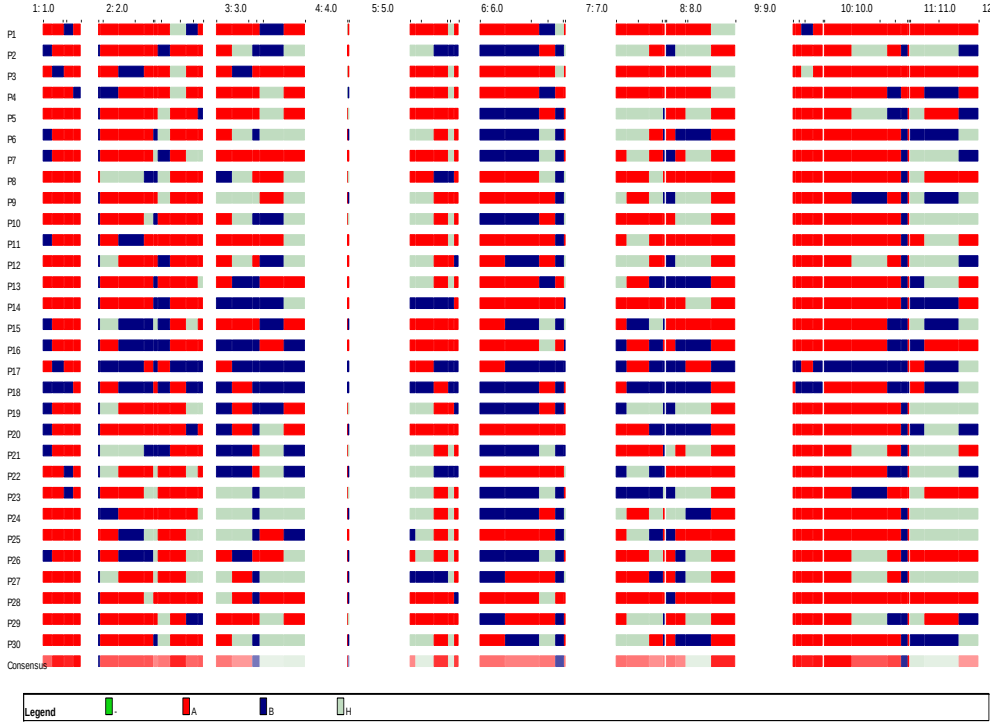


Plate 2: Band amplification pattern of SSR markers RM162

Fig.1 Genome introgression of 30 BC_2F_1 (K 343*^{3}/ RML 22) introgressed lines using software Graphical GenoTypes (GGT 2.0) (Van Berloo, 1999)



The mean value of 1000-grain weight recorded was 25.91g and ranged between and 23.60-28.50g respectively. In case of days to 50 percent flowering the BC_2F_1 which took maximum days to flowering were P11 (94 days) where as the minimum number of days to 50 percent flowering were recorded in P17 (89) and ranged between 89-94 with an average value of 92.66 days. Duration of grain filling in the BC_2F_1 ranged from 35-39 with an average value of 35.93 days. P17 took maximum duration of grain filling (39days) followed by P11 (37days) whereas the P1 took minimum number of duration of grain filling (35 days).

Pathotyping of BC_2F_1 population (K 343*^{3} /RML 22)

All the 30 gene positive plants carrying *Pi9* 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}/RML 22 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 P3, P11 and P28 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 *Pi9* using the corresponding marker AP5930. These results revalidate the findings of Sharma *et al.,* (2005a) and Rathour *et al.,* (2008). These plants would serve as genetic stocks for development of blast resistant lines/varieties or donor for development of blast resistant varieties.

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