Available online at http://www.journalijdr.com

IJDR

International Journal of
DEVELOPMENT RESEARCH

ISSN: 2230-9926

International Journal of Development Research
Vol. 06, Issue, 08, pp. 9194-9198, August, 2016

Full Length Research Article

ENHANCEMENT OF LEAF BLAST RESISTANCE IN RICE CULTIVAR 'SWARNA' BY MARKER ASSISTED BACKCROSS BREEDING

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ARTICLE INFO

Article History:

Received 17th May, 2016
Received in revised form
01st June, 2016
Accepted 26th July, 2016
Published online 30th August, 2016

Key Words:

Rice blast,
Swarna, Tetep (*Pi54*),
M.oryzae and Marker Assisted
Backcross Breeding (MABB).

ABSTRACT

Swarna is most popular indica rice variety cultivated in India. However, Swarna is highly susceptible to blast disease. As genes that confer effective resistance to blast are available along with gene-specific markers, the present study was carried out to introgress a major blast resistance gene, *Pi54* into the genetic background of Swarna through marker-assisted backcross breeding. Rice line Tetep possessing *Pi54* gene served as the donor. Marker-assisted backcross breeding strategy was used for introgression of the resistance gene into Swarna. This involved two rounds of backcrossing at each backcross generation, foreground selection was carried out using PCR based molecular marker specific for *Pi54* (i.e. Pi54 MAS) and background selection was done using a set of 52 parental polymorphic SSR markers spread across the rice genome. At BC_2F_2, a single plant possessing the targeted gene along with maximum recurrent parent genome recovery (~90.3%; plant ST-15-2-30-85) was selected and advanced further through selfing and pedigree-based selection for morphological traits. At BC_2F_4, four lines, viz, ST-15-2-30-85-62-9, ST-15-2-30-85-62-57, ST-15-2-30-85-62-72 and ST-15-3-85-62-175 possessing high level of resistance against blast and all the features identical to Swarna were identified.

INTRODUCTION

The fungus *Magnaporthe oryzae* is the causal agent of rice blast disease and belongs to phylum, Ascomycota and family Magnaporthaceae. It is one of the most devastating diseases in at least 85 countries worldwide. The disease often results in a significant yield loss, as high as 70-80% during an epidemic (Ou, 1985). Hence there is an urgent need to improve this variety by incorporating resistance genes of blast. As on date, 100 rice blast major resistance genes (R-genes) have been identified (Sharma *et al*., 2012) and among the major blast resistance genes, *Pi-k*^{h}, which has been recently renamed as *Pi54* (Sharma *et al*. 2010), exhibited resistance to predominant races of the pathogen in India (Sharma *et al.*, 2002). *Pi54* gene was originally identified from Tetep, a Vietnam indica source and mapped on cchromosome 11L with two tightly linked simple sequence repeat (SSR) markers TRS26 and TRS33 has been cloned (Sharma *et al*., 2005).

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Genotyping by these linked markers requires analysis through Poly Acrylamide Gel Electrophoresis (PAGE), which is cumbersome and time-consuming. Hence, marker-assisted introgression of *Pi54* into susceptible rice varieties is being achieved through another linked SSR marker, RM206, which can be resolved through agarose gel electrophoresis (Srinivasarao *et al*. 2008 and Srinivasarao *et al*. 2009).

However, the marker is not very close to the gene and this might result in some recombinants in MAS. (Ramkumar *et al*. 2011) developed PCR based functional marker Pi54MAS and it was observed to perfectly co-segregate with no recombinants. The rice cultivar 'Tetep' has been found to be resistant to most of the pathogenic races occurring in India (Padmanabhan *et al*. 1979) With this background, the present study was initiated with an objective to introgress a major, dominant resistance gene for blast (i.e. *Pi54*) into the genetic background of Swarna through maker-assisted backcross breeding (MABB).

MATERIALS AND METHODS

Plant materials and Breeding strategy

Swarna was used as recurrent parent, while Tetep carrying (*Pi54*) resistance gene was used as donor parent. F_1 seeds were developed from the normal hybridization between Swarna and Tetep. Selected F_1 plant was then backcrossed with Swarna to produce BC_1F_1 seeds. Selected plant carrying resistance gene with highest background parent genome recovery and maximum phenotypic similarity to the recurrent parent were backcrossed with Swarna to generate BC_2F_1 seeds. Foreground and background selection were carried out to select the elite plant from each backcross generation. The BC_2F_1 plants were also subjected to foreground selection followed by phenotypic selection to identify plants homozygous for *Pi54* gene with maximum recovery for RPG. These plants were then selfed to generate BC_2F_2 populations. In the BC_2F_2 generation, plants homozygous for *Pi54* gene were identified and then advanced to the BC_2F_4 generation through the pedigree method of selection.

DNA extraction and PCR Amplification

Mini scale DNA isolation of parents and backcross derived lines was carried out from 25-day old seedlings following the procedure of Zheng *et al*. (1995). The PCR protocols recommended for marker-assisted backcross breeding of *Pi54* gene (Sundaram *et al*. (2008) and Ramkumar *et al*. (2011). The PCR based functional marker Pi54 MAS wsa used for PCR amplification of *Pi54* gene. PCR reactions were performed on thermal cycler (AB Bio systems). Each 10 µl PCR reaction mixture contained 50 ng genomic DNA, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl2, 2 mM dNTPs, 10 µM each of the primer pair and 1 unit Taq DNA polymerase. Template DNA was initially denatured at 94°C for 5 min prior to 35 cycles of denaturation at 94°C (30s), annealing at 55°C (30s), and extension at 72°C (1 min). At the final step, the reaction mixture was incubated at 72°C for 10 min before the completion. The amplified products were then electrophoretically resolved on a 3 % agarose gel in 1 × TAE buffer. Background selection was done using 52 parental polymorphic SSR markers following the procedure described in Sundaram *et al*. (2008).

Screening of backcross derived lines against Blast

The local a virulent fungal isolate (SPI-28) of *Magnaporthae oryzae,* from Indian Institute of Rice Research (IIRR), Hyderabad, India (Madhan Mohan *et al*. 2011), was used to screen the donor and recurrent parent along with backcross derived lines of Swarna for blast resistance under *in vivo* conditions following uniform blast nursery (UBN) method at Indian Institute of Rice Research, Hyderabad, India. In inoculation test, this isolate was found to be highly virulent on rice cultivar HR12 and other blast differentials. The pathogen strain was cultured and stored (Prasad *et al*., 2011). The young seedlings at four-leaf stage were inoculated with the fungal conidial suspension at a concentration of 1 x 10^5 conidia/ml with the help of hemocytometer. The parents along with the improved lines of BC_2F_4 population were evaluated for their reaction to blast disease. The plants were sown in rows and were surrounded with the densely sown spreader rows of susceptible cultivar HR12.The seedlings at four-leaf stage were sprayed with spore suspension of a highly virulent isolate of *M. oryzae (*SPI-28). High relative humidity was maintained for disease development. Data were recorded three times using a scale of 0-9 (IRRI, 1996) at 10 days intervals starting from 30 days after sowing. The lines with scores of 0-3 were considered as resistant, 4-5 as moderately resistant, 6 as moderately susceptible and 7-9 as susceptible.

Agro-morphological characters evaluation

Thirty-days-old seedlings of the selected introgressed lines at BC_2F_4 were transplanted in the field along with the donor and recurrent parents. Standard agronomic practices were followed to develop promising lines, which were evaluated during the wet season (Kharif) in 2014. Data were recorded for various agronomic traits viz,days to 50 % flowering (DFF), Days to maturity (DM), plant height (PH), number of tillers (NT), panicle length (PL), Grain per panicle (GP), 1000-grain weight (TW) and yield per plant (Y/P). These traits were recorded from all of the best selected lines of BC2F4 along with the recurrent parent. The procedures for measurement of these traits have been followed by (abhilash kumar *et al*. 2015).

RESULTS AND DISCUSSION

Targeted introgression of *Pi54* gene into Swarna background through marker assisted backcross breeding (MABB)

A total of 94 F_1 seeds were generated from the cross Swarna/Tetep, 71 were identified to be 'true' F_1s (Table-1, Figure 1 (A) & 1 (B)) based on the analysis using gene specific marker/ gene linked marker, Pi54MAS.They were then backcrossed with swarna to generate 346 BC_1F_1 seeds. Foreground analysis of these plants with the gene-specific markers revealed that 87 plants were heterozygous for the target gene. Among these, one plant i.e., # ST -15-2 possessing maximum recurrent parent genome recovery (~ 71.1%; Table 2) was identified with the help of 52 parental polymorphic SSR markers through background selection and it was backcrossed with Swarna to produce a total of 140 BC_2F_1 plants. Foreground selection among BC_2F_1 plants revealed a total of 36 plants possessing *Pi54* in heterozygous condition, which were then subjected to background genome recovery analysis. A single BC_2F_1 plant (# ST-15-2-30) with maximum RPG (~ 84.6%) was identified and selfed to develop a total of 410 BC_2F_2s.

Marker-assisted screening of these plants identified 102 single positive plants (*Pi54*) and among these, a single plant (# 90.3% ST-15-2-30-85; Table 2) possessing maximum recurrent parent genome recovery (90.3%;) was identified through background selection. This plant was then selfed and these were then advanced through pedigree method involving morphological trait based selection and four promising advanced backcross derived lines were identified at BC_2F_3. They were then subjected for phenotypic evaluation for disease resistance, yield and other agro morphological parameters and forwarded for further advanced through pedigree method to identify best lines of Swarna possessing *Pi54*.

Table 1. Details of foreground and background selection among the backcross plants derived from the cross Swarna/Tetep

S. No	Generation	No. of plants screened	Foreground Selection	Background selection			Best plant selected based on background selection
			+ve for *Pi54*	SSRs used analyzed	polymorphic SSRs, homozygous for R' allele	(%) recovery of Recurrent parent genome	
1	F_1	94	71	-	-	-	-
2	BC_1F_1	346	87	52	37	71.1%	ST-15-2
3	BC_2F_1	140	36	15	7	84.6%	ST-15-2-30
4	BC_2F_2	410	102	6	3	90.3%	ST-15-2-30-85

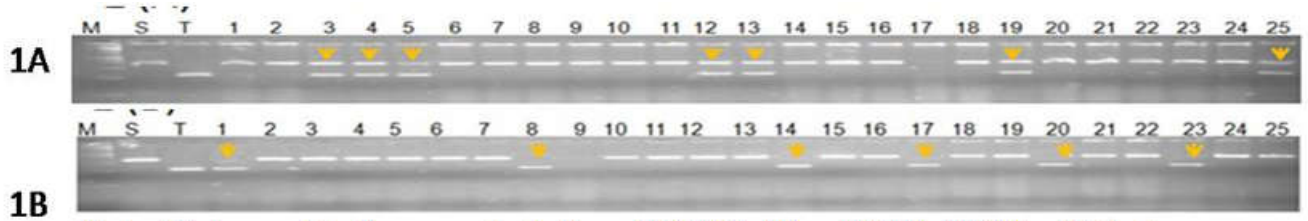

Fig 1: Marker assisted foreground selection at BC_2F_1 (1 (A) and BC_2F_2 (1(B) for *Pi54* using gene linked marker Pi54MAS, ,-100bp, S-Swarna, T-Tetep; 1-25 BC_2F_1 plants;arrows indicate heterozygote positive for(*Pi54*) gene and BC_2F_2 1-25 arrows indicate homozygous positives for (*Pi54*) gene (1(B).

Table 2. Reaction of introgressed lines of Swarna to blast

S.No	Rice line	Resistance genes genotyped by linked marker	Reaction against to blast	
		Pi54 (Pi54 MAS)	SPI-28 Score	R/S
1	Swarna	--	9	S
2	Tetep	++	0	R
3	HR12	--	9	S
4	ST-15-2-30-85-62- 9	++	2	R
5	ST-15-2-30-85-62-57	++	0	R
6	ST-15-2-30-85-62-72	++	1	R
7	ST-15-2-30-85-62-175	++	1	R

Table 3. Agronomical characters of selected four Swarna improved lines

S.No	DFF (Days)	DM (Days)	PH (Cm)	PN	PL (cm)	GP	TGW (gms)	Y/P	(RPG) % Recovery
Swarna	126.0 ± 1.00	156.3 ± 0.88	80.3 ± 0.27	10.0 ± 0.58	24.8 ± 0.79	169.0 ± 2.08	17.67 ± 0.24	21.7 ± 0.27	
Tetep	94.7 ± 1.45	128.3 ± 1.67	86.7 ± 0.72	8.7 ± 0.67	22.3 ± 0.44	150.3 ± 2.73	16.13 ± 0.18	18.4 ± 0.38	
ST-15-2-30-85-62- 9	124.0 ± 2.08	152.7 ± 3.93	80.0± 0.57	10.3 ± 0.33	24.9 ± 0.44	169.0 ± 1.2	17.90 ± 0.17	21.7 ± 0.12	93.5
ST-15-2-30-85-62-57	126.7 ± 1.67	154.7 ± 1.45	79.3 ± 0.33	9.3 ± 0.67	25.2 ± 0.32	170.0 ± 1.15	17.70 ± 0.21	21.5 ± 0.37	93.1
ST-15-2-30-85-62-72	125.0 ± 2.89	153.3 ± 2.40	79.7 ± 0.66	10.0 ± 1.0	25.3 ± 0.72	172 .0 ± 1.7	17.87 ± 0.15	22.0 ± 0.31	94.0
ST-15-2-30-85-62-175	123.3 ± 1.20	154.0 ± 1.00	80.3 ± 0.88	9.0 ± 0.58	24.7 ± 0.58	170.7 ± 0.88	17.67 ± 0.29	22.1 ± 0.35	93.9

DFF: Days to 50% flowering, DM: Days to maturity, PH: Mean plant height (cm), PN: No.of panicle per plant, PL: Panicle length (cm), TGW (gm): 1000 grain weight (gm), Y/P: Yield per plant (gm) and Recurrent parent genome recovery (%) (RPG).

Evaluation of blast Resistance

The selected four introgressed lines ST-15-2-30-85-62-9, ST-15-2-30-85-62-57, ST-15-2-30-85-62-72 and ST-15-3-85-62-175 possessing *Pi54* gene were evaluated for their resistance to blast in the Uniform Blast Nursery (UBN) beds (Table 2). The susceptible check, HR12 and the recurrent parent Swarna (Score-9) were highly susceptible to blast, while the resistant check, Tetep (Score-0) and all the introgressed lines were found to be highly resistant to the disease with a score of 1.

Agro-morphological traits evaluation

The selected four introgressed lines (ST-15-2-30-85-62-9, ST-15-2-30-85-62-57, ST-15-2-30-85-62-72 and ST-15-3-85-62-175) which exhibited high level of resistance to blast was evaluated for key agro-morphological traits *viz.* days to flowering, days to maturity, plant height, panicle number per plant , panicle length, no of grains per panicle, thousand grain weight and yield per plant (Table 3). The introgressed lines ST-15-2-30-85-62-72 and ST-15-2-30-85-62-175 displayed grain yield slightly higher than (22.0 ± 0.31 and 22.1 ± 0.35) respectively that of recurrent parent (i.e. Swarna 21.7 ± 0.27), while other introgressed lines (ST-15-2-30-85-62-9 and ST-15-2-30-85-62-57 with an RPG 93.5%, 93.1% respectively) displayed yield per plant equivalent to that of the recurrent parent. No significant variation was observed with respect to the, no. of panicles and panicle length, plant height, panicle length, no. of grains per panicle, thousand grain weight and yield per plant among the four introgressed lines as compared to Swarna. One introgressed line, i.e. ST-15-2-30-85-62-175 found to be better than that of the Swarna as it had better yield per plant (Table 3).

DISCUSSION

Swarna was released in Andhrapradesh in year 1982 by Maruteru (AP-ARI)) research station and many other states of India (West Bengal, Kerala, Karnataka and Tamilnadu) cultivating in large portion. Swarna Parentage was (Vasista X Mahsuri), matures in 155 days with short plant height, profuse tillering, with short bold grain, requires 25% less nitrogen. High level of susceptibility of Swarna to Blast Saha *et al*. (2008), caused by the fungus *Magnoporthe oryzae*, is a serious constraint to rice production, which results in major yield loss. Hence, in the present study, an attempt was made to develop high yielding resistant Swarna through MABB approach. Hence a selected dominant resistance gene *Pi54* was selected for introgression into Swarna in the present study.

Earlier, through MAS blast resistant version were developed in the background of Improve Samba Mahsuri (Madhavi *et al*., 2012), Zhenshan 97A (Liu *et al*. 2003), the restorer line Luhui17 (Wen *et al*. 2011), the TGMS line, C815S (Jiefeng *et al*. 2015), Swarna (Rambabu *et al*., 2016) through MABB. Introgression of *Pi-kh* Resistance gene into a Malaysian Cultivar, MR264 using Marker-Assisted Backcrossing (MABC) by Hasan *et al*. (2015) by implementing an approach similar to that used in the present study. Thus through this study , four introgressed lines of Swarna possessing good grain quality, high yielding and excellent resistance against blast along with short bold grain type were developed. This is the first report on introgression of blast resistance gene *Pi54* into Swarna through MABB breeding coupled with phenotypic selection. The *Pi54* gene shows a wide-spectrum of leaf blast resistance. Even though there are few previous reports about breakdown of resistance conferred by a single blast resistance gene (Khush *et al*., 1989) in rice, till date there is no report about large-scale breakdown of resistance conferred by *Pi54* from India or abroad. Further, as per a recent reports (DRR annual report, 2008-14), rice line Tetep possessing *Pi54* displayed resistance across multiple locations in India. PCR-based DNA marker Pi54 MAS was used in the present study tightly linked with the Pi54 gene (Ramkumar *et al*., 2011). The Pi54 MAS marker presents on chromosome 11 below the centromere. This marker is highly polymorphic and can be detected very easily and therefore have great potential to serve as an important tool to introgress Pi54 blast resistant gene into blast susceptible rice varieties. The importance and benefit of using tightly linked markers for gene pyramiding have been discussed earlier by Hittalmani *et al*. (2000) for blast disease screening. However, the success of marker-assisted selection heavily depends upon the strong linkage between the marker and target gene. Thus, from the blast disease screening results, four selected lines, ST-15-2-30-85-62-9, ST-15-2-30-85-62-57, ST-15-2-30-85-62-72 and ST-15-3-85-62-175 showed strong resistance against virulent isolate SPI-28 similar to the donor parent.

The results of the phenotypic screening against blast disease reaction of the introgressed lines carrying the *Pi54* gene with a background of the recurrent parent Swarna conferred complete resistance to the highly virulent isolate SPI-28, indicating the strong bond between this marker with the trait. The donor parent Tetep and the recurrent parent Swarna showed significantly different agro-morphological traits. However, in the blast resistant, improved lines of Swarna, no apparent yield penalty was observed. Therefore, the cultivation of introgressed blast resistant lines would be of great advantage to reduce the yield losses in blast disease endemic areas. Improved blast resistant Swarna lines showed a similar agro-morphological performance in the field as a par recurrent parent Swarna. The mean value of blast resistance lines carrying the Pi54 for all morphological characters were mostly similar with the recipient parent Swarna, indicating that the performance of introgression lines is similar with Swarna for such traits. The present results strongly support that our phenotypic selection practice was efficient.

Conclusion

The present study suggests that DNA marker for blast resistance *Pi54* gene are reliable for marker-assisted selection of blast resistance in rice breeding. The recovery of the recurrent parent along with the intogression of blast resistance gene with MABB breeding was much faster than that with conventional breeding. Four introgressed blast resistance lines were produced from a backcross between the rice variety Swarna and Tetep. These introgressed blast resistant lines could be utilized as a source of genetic material for blast resistance breeding with a high yielding background of rice varieties.

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