ISSN: 0975-833X

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

International Journal of Current Research
Vol. 8, Issue, 05, pp.30270-30275, May, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

MARKER ASSISTED INTROGRESSION OF DURABLE BLAST RESISTANCE GENE *PI-1* INTO POPULAR *INDICA* VARIETY

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ARTICLE INFO

Article History:
Received xxxxxxxxxx, 2016
Received in revised form
xxxxxxxxxxxxxxxx, 2016
Accepted xxxxxxxxxx, 2016
Published online xxxxxxx, 2016

Key words:
Swarna,
Blast Resistance gene (*Pi-1*),
Marker-Assisted Backcrossing (MABC),
Magnaporthe oryzae.

ABSTRACT

Swarna is high yielding and widely grown *indica* rice cultivar covering a substantial portion of rice area in India and in the Asian countries such as Bangladesh, Philippines, Thailand and Indonesia, but it is highly susceptible to blast disease. The donor selected for the resistant gene is C101LAC carrying blast resistance gene *Pi-1* derived from LAC23. The *Pi-1* gene was done introgressed into Swarna by using Marker assisted backcross breeding method. Foreground selection was using RM224 linked marker to identify plants possessing resistance alleles in the segregating generations along with stringent phenotypic selection for faster recovery of the recurrent parent genome (RPG). Foreground selection coupled with stringent phenotypic selection identified plants homozygous for *Pi-1*, which was advanced to BC_2F_4 through pedigree selection. Marker-assisted selection for *Pi-1* in BC_2F_4 using flanking markers identified five homozygous families. Background analysis with parental SSR markers was used to estimate the recovery of RPG in improved lines and it revealed that RPG recovery was up to 94.7% (# SL-10-3-7-63-45-7). Screening with highly virulent isolate SPI-28 showed that the improved lines were resistant to blast disease and were on a par with swarna for yield, and grain quality. These introgressed lines provide valuable material for resistant breeding program.

Citation: Rambabu, R., Vijay kumar, S., Abhilash Kumar, V., Madhavi, K. R., Aruna, J., Madhav, M. S. and *Srinivas Prasad, M, 2016. "Marker assisted introgression of durable blast resistance gene pi-1 into popular Indica variety", *International Journal of Current Research*, 8, (05), 30270-30275.

INTRODUCTION

Rice blast, caused by the fungal pathogen *Magnaporthe oryzae* (Hebert) Barr. (Anamorph= *Pyricularia grisea*), is devastating disease that occurs in rice-growing areas of the world (Ou *et al*., 1985). It is the most important fungal disease ranks first because of its wide distribution and high incidence under favorable conditions. The fungus *M.oryzae* is known for its tremendous genetic diversity in natural environments, causing a breakdown in the resistance of elite varieties soon after they are released, due either to the emergence of new pathogen races or the selection of a rare component of the pathogen population that is already virulent (Bonman *et al*., 1992). Commercial chemical fungicides are effective in controlling the important blast disease when applied in sufficient doses and at correct time. Use of chemical fungicides disturb the rice ecosystem, induce development of resistant mutants of the pathogen, and pollute the environment in contrast to eco-friendly nature of plant-derived products.

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Genetic resistance is the most economical and environmentally appropriate strategy for blast disease management in rice. Deployment of resistance by introducing new resistant rice cultivars has been the preferred means for managing the disease. To date, more than 100 major blast resistance genes from japonica (45%), indica (51%) and other (4%) genotypes have been identified and documented (Ballini *et al*., 2008), (Chen *et al*., 2002), (Huang *et al*., 2010), (Liu *et al*., 2004), (Liu *et al*., 2005) and (Sharma *et al*., 2012). Out of them, 22 have been cloned namely Pib**,** Pita**,** Pik-h**,** Pi9**,** Pi2**,** Piz-t**,** Pid2**,** Pi36**,** Pi37**,** Pik-m, Pit, Pi5, Pid3, pi21, Pb1, Pish, Pik, Pik-p, Pia, NLS1, Pi25 and Pi54rh (Bryan *et al*., 2000), (Chen *et al*., 2011), (Das *et al*., 2012), (Sharma *et al*., 2005), (Wang *et al*., 1999) and (Zhou *et al*., 2006). Among these, the blast-resistance gene *Pi-1* (chromosome-11) was first identified in the cultivar 'LAC23' (Mackill and Bonman *et al*., 1992), an upland cultivar from Liberia, which had a broad spectrum of resistance against Chinese blast isolates (Chen *et al*., 2001), as well as isolates of southern India (Srinivasachary *et al*., 2002). In the mapping studies conducted by (Fuentes *et al*., 2008) from intercross of C101LAC/C101A51, markers RM1233*I, RM5926 and RM224 were mapped to the same position (0.0

cM) with the *Pi-1* gene and recognized as valuable for introgression and pyramiding of *Pi-1* gene into blast-susceptible rice cultivars (Liu *et al*., 2003) and (Prasad *et al*., 2009). The main objective of this work was to convert widely cultivated variety swarna to blast resistant variety through MABC.

MATERIALS AND METHODS

Plant materials and breeding procedure:

The indica variety swarna susceptible to blast was used as the recurrent parent, while the cultivar 'C101LAC' was used as donor for the blast resistance gene, *Pi-1*. A single F_1 plant was backcrossed with 'swarna' to generate the BC_1F_1s (Figure: 1).

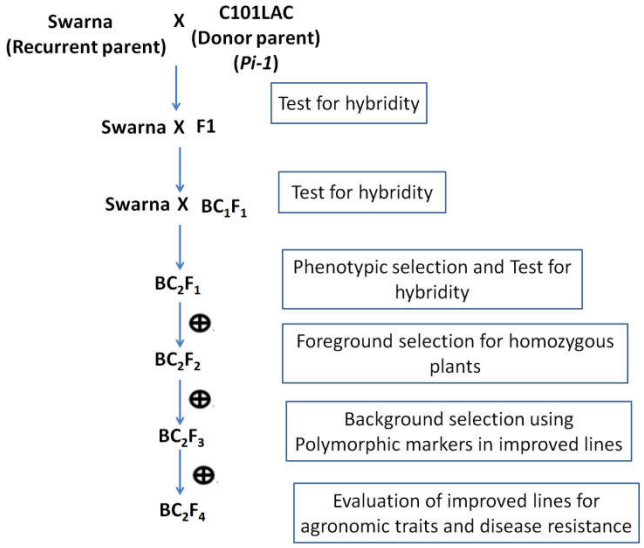

Fig. 1. Breeding strategy for introgression of *Pi-1* gene into swarna background

Marker-assisted foreground selection was employed using gene linked marker RM224 to identify the plant heterozygous for the *Pi-1* gene. Further, the selected plants were subjected to stringent phenotypic selection for agro-morphological attributes. The BC_1F_1 plant with maximum recovery (SL-10-3) of the recurrent parent genome (RPG) was backcrossed to develop the BC_2F_1 generation. The BC_2F_1 plants were also subjected to foreground selection followed by phenotypic selection to identify plants heterozygous for *Pi-1* with maximum recovery (SL-10-3-7) for RPG. These plants were then selfed to generate BC_2F_2 populations. In the BC_2F_2 generation, plants homozygous for *Pi-1* gene were identified and then advanced to the BC_2F_4 generation through the pedigree method and later studied for other agro-morphological traits.

DNA Extraction and PCR analysis

Total DNA was extracted according to the procedure of (Zheng *et al*., 1995). The PCR protocol recommended by (Sundaram *et al*., 2008). Adoption of MAS was facilitated by using simple sequence repeat (SSR) marker RM224 for *Pi-1* (Fuentes *et al*. 2008, Table 1).

PCR reactions were performed on thermal cycler (AB Bio-systems). Each 10 µl PCR reaction mixture contained 50 knag genomic DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl2, 2.5 mM dNTPs, 10 µM each of the primer pair and 3 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 m). 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.

Phenotypic evaluation of blast resistance

The most virulent isolate SPI-28 of *Magnaporthe oryzae* was used for blast screening Prasad and (Madhan Mohan *et al* 2011). The method of inoculum preparation and inoculation was the same as described by (Prasad *et al*., 2011). The introgressed lines, C101LAC (*Pi-1* gene donor), Swarna (recurrent parent) and HR12 (susceptible check) were grown in UBN (Uniform blast nursery) at ICAR-Indian Institute of Rice Research (IIRR), Rajendranagar, India. The young seedlings at four leaf stage were inoculated with the fungal conidial suspension at concentration of 1 X 10^{-5} conidia /ml. The inoculated plants were observed after 9 days of inoculation for blast disease lesions. The plants and blast lesion degrees (BLDs) were evaluated on the basis of 0-9 of the IRRI-SES scale (IRRI, 1996, Roumen *et al*., 1997).

Screening for Agro-morphological traits

Agronomical characteristics and yield performance of lines having maximum recovery of recurrent parent along with target gene and morphological similarity with the recurrent parent were evaluated in replicated trials conducted in the rice fields of Indian institute of Rice Research, Rajendranagar in the Kharif season of 2014. Agronomical traits were compared with Random block design with three replications for each line was arranged and 3 rows with total 30 individuals each replicate were planted with spacing 15 cm x 20 cm. 6 individuals was sampled in each plot for phenotyping. Trait measurements included were days to 50% flowering (DFF), days to maturity (DM), plant height (PH), panicles per plant (PP), panicle length (PL), grains per panicle (GP), weight of 1000 grains (TGW) and yield per plant (YP) (Hari *et al*., 2013).

RESULTS AND DISCUSSION

Foreground selection for Blast Resistance

A total of 26 'true' F_1s were identified and backcrossed with Swarna to generate 260 BC_1F_1 seeds. Foreground analysis of the BC_1F_1 plants using the gene-specific marker RM224 (for *Pi-1*) revealed that 65 plants were heterozygous for *Pi-1* gene (Figure 2a and 2b; Table 2). Among these, a single plant (# SL-10-3) was selected for further backcrossing as it was observed to possess a maximum recovery of recurrent parent genome (~ 72.5%); as inferred through background selection using 51 polymorphic SSR markers) and a total of 134 BC_2F_1 plants were thus produced.

Table 1. Marker Details used for introgression

Trait	Gene	Marker	LG	MD (Cm)	Forward sequence	Reverse sequence	Reference
Blast	Pi-1	RM224	11	0	TTCGTTTTCCTTGGTTAGTG	ATTGGCTCCTGAAGAAGG	Fuentes *et al.* (2008)

LG: linkage group, MD: map distance

Table 2. Details of foreground and background selection among the backcross plants derived from the cross Swarna/C101LAC

S. No	Generation	No. of plants screened	Foreground Selection	Background selection			Best plant selected based on background selection
			+ve for Pi-1	SSRs used analyzed	polymorphic SSRs, homozygous for R' allele	(%) recovery of Recurrent parent genome	
1	F_1	105	26	-	-	-	-
2	BC_1F_1	260	65	51	37	72.5%	SL-10-3
3	BC_2F_1	134	33	14	8	86.0%	SL-10-3-7
4	BC_2F_2	321	80	8	3	89.1%	SL-10-3-7-63

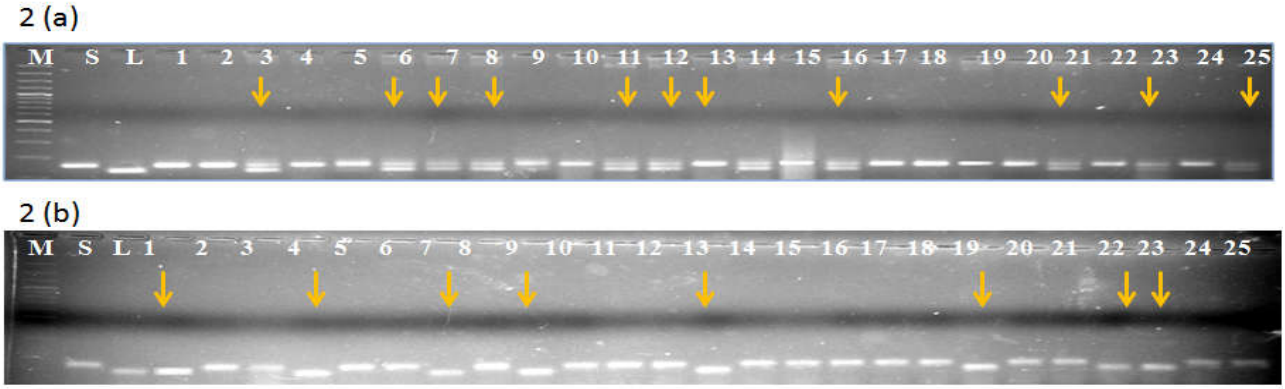

Fig-2: Marker-assisted foreground selection at BC_2F_1 (2 (a)) and BC_2F_2 (2 (b)) for *Pi-1* gene Using gene linked marker RM224. M-100 bp ladder; S- Swarna; L- C101LAC; 1-25 BC_2F_1 Plants ; arrows indicate heterozygote positives for (*Pi-1*) gene (2 (b)) and 1-25 BC_2F_2Plants ; arrows indicate homozygous positives for (*Pi-1*) gene (2 (b).

Table 3. Phenotypic evaluation of selected BC_2F_4 improved lines reaction with blast pathogen SPI-28

S.No	Rice line	Resistance genes genotyped by linked marker Pi-1 (RM224)	Reaction against to blast SPI-28 Score	R/S
1	Swarna	--	9	S
2	C101LAC	++	0	R
3	HR12	--	9	S
4	SL-10-3-7-63-45- 7	++	2	R
5	SL10-3-7-63-45-81	++	0	R
6	SL-10-3-7-63-45-97	++	1	R
7	SL-10-3-7-63-45-125	++	1	R
8	SL-10-3-7-63-45-151	++	0	R

* ++ possessing homozygous, trait-specific positive allele at the particular gene as inferred through analysis with gene-specific marker,
- - possessing homozygous, trait-specific negative allele at the particular gene as inferred through analysis with gene-specific marker
R, Resistant; S, Susceptible

Fig-3: Phenotypic evaluation of BC_2F_4 Selected improved lines against blast disease following by UBN Method. Swarna (Recurrent parent), C101LAC (Donor parent), HR12 (Susceptible parent) and BC_2F_4 Selected lines (IL-1 , SL-10-3-7-63-45-7), (IL-2, SL-10-3-7-63-45-81), (IL-3, SL-10-3-7-63-45-97), (IL-4, SL-10-3-7-63-45-125) and (IL-5, SL-10-3-7-63-45-151)

Table 3. Agronomical characters of selected five Swarna improved lines

S.No	DFF (Days)	DM (Days)	PH (cm)	PN	PL (cm)	TGW (gms)	YP	RPG (%)
Swarna	125.7 ± 0.6	146.7 ± 1.6	76.3 ± 0.8	8.33±0.8	25.4 ± 0.22	18.1 ± 0.0	20.9 ± 0.15	---
C101LAC	90.0 ± 1.1	115.0 ± 0.0	81.0 ± 0.5	7.0 ± 0.5	24.6 ± 0.23	17.0 ± 0.1	19.8 ± 0.30	---
SL-10-3-7-63-45- 7	125.7 ± 1.5	146.0 ± 1.0	76.8 ± 0.5	9.0 ± 0.3	25.4 ± 0.22	18.6 ± 0.1	21.6 ± 0.24	94.7
SL10-3-7-63-45-81,	110.0 ± 0.5	135.7 ± 1.6	77.0 ± 1.1	8.1 ± 0.8	25.4 ± 0.00	18.1 ± 0.2	21.1 ± 0.32	94.2
SL-10-3-7-63-45-97	125.0 ± 2.8	145.5 ± 0.5	76.3 ± 0.3	8.3 ± 0.8	25.3 ± 0.07	18.2 ± 0.3	21.0 ± 0.12	93.5
SL-10-3-7-63-45-125	125.0 ± 2.8	145.5 ± 0.5	76.2 ± 0.3	8.23 ± 0.5	25.1 ± 0.15	18.4 ± 0.3	20.7 ± 0.12	93.0
SL-10-3-7-63-45-151	126.7 ± 1.6	147.0 ± 0.0	76.0 ± 0.5	8.2 ± 0.5	25.0 ± 0.12	18.2 ± 0.3	20.6 ± 0.38	93.5

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 RPG: Reccurent parent genome.

Foreground selection among BC_2F_1 plants revealed a total of 33 plants possessing *Pi-1* in heterozygous condition, which were then subjected to background genome recovery analysis. A single BC_2F_1 plant with maximum RPG (~ 86.0%) was identified and selfed to develop a total of 321 BC_2F_2s. Marker-assisted screening of these plants showed 80 positive homozygous plants (*Pi-1*) and among these, a single plant possessing maximum RPG (~89.1%) was identified and forwarded by selfing through pedigree method involving morphological trait-based selection till BC_2F_4, wherein five promising advanced backcross lines were identified viz., SL-10-3-7-63-45- 7, SL10-3-7-63-45-81, SL-10-3-7-63-45-97, SL-10-3-7-63-45-125 and SL-10-3-7-63-45-151. They were then subjected for phenotypic evaluation for disease resistance and agro morphological parameters as given below.

Evaluation of introgressed lines of the Swarna

Leaf blast resistance of improved lines (IL) and the two parents were assessed using SPI-28 isolate of *M.oryzae* from IIRR on UBN nursery. The donor parent C101LAC (score-0) containing *Pi1* gene, showed durable resistance to rice blast with high resistance frequency and the recurrent parent swarna (score-9) was found susceptible to rice blast with less resistance frequency. The HR-12 blast susceptible check was showed susceptible reaction to blast (scores-9). The improved backcrossed lines were observed to be highly resistant to the disease with a score of 1 (Table-3).

Agronomical performance of selected improved lines

The selected five BC_2F_4 improved Swarna lines showing resistance against blast disease were evaluated for their agronomic traits as compared to recurrent parent Swarna during Kharif 2014. The results (Table 3) showed non-significant differences for the days to flowering, number of panicles per plant, 1000-grain weight, panicle length between the newly developed (SL-10-3-7-63-45-97, SL-10-3-7-63-45-125, and SL-10-3-7-63-45-151) lines and Swarna. The single plant (#SL 10-3-7-63-45-81 with an RPG 94.2%) showed days to flowering and days to maturity were significantly early by 15 days than swarna. No significant variation was observed with respect to the no. of panicles and panicle length with as compared to Swarna. The number of panicles per plant and Yield per plant of improved line (#SL-10-3-7-63-45-7 with an RPG 94.7%) was higher than Swarna by 0.5 gms and 0.7 gms respectively, and found to be the best plant in terms of yield advantage and other agro-morphological traits were no significant difference to recurrent parent swarna

DISCUSSION

Swarna is one of the most popular rice varieties in India producing a high yield, good grain quality and requires 25% less nitrogen as widely claimed by the farmers released in Andhrapradesh in 1982, its spread across the subcontinent and into Bangladesh, where it was never officially released (P.K Agrawal *et al*., 2015). Despite, its superior grain and yield qualities swarna are highly susceptible to blast disease (Saha *et al*., 2008) which is significantly reduce the yields of rice varieties. Therefore, in the present study carried out with an aim to improve the swarna for resistance against blast, while retaining the premium grain, cooking quality and other good features of swarna through MABC with stringent phenotypic selection. We selected dominant resistance gene *Pi-1* for introgression in the present study.

In the current study focused to improve the blast resistance of mega variety swarna through MABC breeding approach along with phenotypic selection for Agro-morphological traits. Earlier studies, the Luhui17 Restorer line was improved for blast resistance coupled with phenotypic selection for agro morphological traits similar to our study (Wen *et al*., 2012). (Narayanan *et al*., 2002) introgressed *Piz-5* blast resistance gene into rice cultivar IR50 and improved the blast resistance. (Madhavi and Prasad 2012), Hasan *et al*., 2015) introgressed the blast resistance gene *Pi-kh* into Improved Samba Mahsuri and Malaysian Cultivar, MR264 Respectively from donor parent Tetep. This is the first report in swarna for developing blast resistance and introgression of blast resistance gene *Pi-1* into swarna through MABC breeding coupled with phenotypic selection for agro morphological traits.

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 *Pi-1* from India or abroad. Further, as per a recent report (DRR annual report, 2008-14), Rice line C101LAC possessing *Pi-1* displayed resistance across multiple locations in India. This was evident, when the improved lines of Swarna were screened under UBN using SPI-28 isolate; Most of the plants derived from homozygous BC_2F_4 lines displayed a high level of resistance to blast disease. The selected improved lines, viz. SL-10-3-7-63-45- 7, SL10-3-7-63-45-81, SL-10-3-7-63-45-97, SL-10-3-7-63-45-125 and SL-10-3-7-63-45-151) displayed a high level of blast resistance.

In the present study, the PCR based marker specific for *Pi-1* (RM224) were deployed for foreground selection. The marker was observed to be highly efficient in identification of blast resistant lines, respectively and no-false positives were observed (Table 1). In addition to this marker, we also deployed a modest number of (i.e. 51) of parental polymorphic SSR markers for background selection and background genome analysis. At BC_2F_4 generation, the background genome recovery varied from 93.0% to 94.7% among the five selected plants and all of them were identical or slightly better than Swarna in most of the agro-morphological features and grain type. Among the improved lines of Swarna (Table-3), no apparent yield penalty associated with the presence of blast (*Pi-1*) resistance gene was noticed. This indicates that cultivation of blast resistant, improved lines would be of great advantage in blast endemic areas. Among the improved lines of Swarna, SL-10-3-7-63-45-7 with 94.7% RPG recovery (Table-3), was identified as best line and is being used a potential parent for future breeding programmes. The improved lines of Swarna (possessing blast resistance along with yield advantage) being further evaluated for their agronomic performance at IIRR, Hyderabad, India.

Conclusion

Rice blast is one of the most important disease worldwide troubles rice production. Recently, it has become more serious and outspread, which urgently requires the development of rice blast resistant varieties. We effectively introgressed a broad-spectrum rice blast resistance gene *Pi-1* into an mega variety swarna to develop five blast resistance Swarna lines by using a dual selection strategy of phenotypic and genotypic selection along with background selection to separate improved breeding lines. These developed Swarna lines will be valuable for further future blast resistance breeding programmes.

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