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Marker Assisted Introgression of Blast Resistance Genes in to Rice Varieties IR-64, KMP-175 and KCP-1

H. B. Manoj Kumar and K. M. Harinikumar
Department of Plant Biotechnology, College of Agriculture, UAS, GKVK, Bengaluru-560 065
Email : manojmandya.17@gmail.com

Abstract

Blast is among the most widespread and damaging diseases of rice in upland and rainfed areas causing more than 50 per cent losses in yield. The present study was focused on introgression of the broad spectrum blast resistance genes that include *Pi1, Pi2, Pi9* and *Pi54* into high yielding but blast susceptible rice varieties IR-64, KMP 175 and KCP-1. In this context, in the present study, improved varieties, *i.e.,* IR-64, KMP 175 and KCP-1 have been selected for introgression with blast resistance genes in different combination *viz., Pi1, Pi2, Pi9* and *Pi54* through marker assisted selection and also marker-assisted backcrossing is under progress wherein PCR based functional markers specific for the resistance genes were used for foreground selection and a set of parental polymorphic microsatellite markers were used for background selection at each stage of backcrossing. Finally, developed breeding materials have to be evaluated for resistance to blast disease continuously over locations and cropping seasons.

Keywords : Introgression, blast resistance / suceptable, genes

.Improving disease resistance in crops is crucial for stable food production. Although the use of race specific resistance genes (*R*-genes) is a major strategy for disease control, these genes are vulnerable to counter evolution of pathogens. New resistance genes are then needed, thus continuing a cycle referred to as an evolutionary "arms race" between crops and pathogens. Quantitative trait loci (QTLs), which usually have smaller individual effects than *R*-genes but confer broad spectrum or non race specific resistance, can contribute to durable disease resistance (DR) (Kou and Wang., 2010) .

Marker assisted selection (MAS) is an approach that has enabled efficient and precise transfer of genes / QTL(s) in many crop species and offers a fast and efficient alternative to conventional breeding and selection methods. Marker assisted selection has been shown to be highly efficient for the precise transfer of major genes for BB resistance (Basavaraj *et al.,* 2010), blast resistance (Singh *et al.,* 2012) and QTLs for ShB resistance (Wang *et al.,* 2012) in rice.

To date, more than 100 rice blast resistance genes have been identified in rice (Sharma *et al.,* 2012), and 20 major R genes (*Pi1, Pi2, Pi5, Pi9, Pid3, Pi25, Pi36, Pi37, Pi54, Pia/Pi-Co39, Pib, Pid2, Pikm, Pikp, Pik, Pish, Pit, Pita* and *Pizt*) (Wongsaprom *et al.,* 2010, Kim *et al.,* 2013, Jiang *et al.,* 2012 and Singh *et al.,* 2013) and two partial resistance genes (*pi21* and *Pb1*) (Fukuoka *et al.,* 2009) have been cloned and characterized. All of the cloned R genes (except Pid2 and pi21) belong to the nucleotide-binding site and leucine-rich repeat (NBS–LRR) class of R genes and some of these R genes such as Pi5, Pia / Pi-Co39, Pikm, Pik and Pikp require two adjacent NBS-LRR class genes for full functionality (Lee *et al.,* 2009; Cesari *et al.,* 2013 and Okuyama *et al.,* 2011).

However, *Pid2* encodes a B-lectin receptor kinase, while *Pi21* encodes a proline rich protein that contained a putative heavy metal binding domain and putative protein-protein interaction motifs (Fukuoka *et al.,* 2009). Although characterization of these R genes has advanced our understanding of the molecular basis of blast resistance in rice, we know little about how these cloned R genes are distributed in modern cultivated rice varieties. Additionally, information both on the resistance effects of R genes in different genetic backgrounds and on which genes are more effective in breeding practice is also missing. Further, more, there are more than 100 R genes in the rice genome, which means that a single germplasm may harbor several R genes in various combinations, but

the fact that resistance reactions show significant differences between germplasms indicates that these variations could be caused by different R gene combinations. Therefore, the question arises of which R genes could be combined to provide a favorable resistance effect across multiple backgrounds. Addressing this question will be helpful in the improvement of blast resistance breeding programs. Accurate identification of a particular R gene in diverse elite germplasm is the first step for utilization of R genes in rice breeding programs. The conventional methodology to identify allelic variation based on phenotype is limited by large workload and time-consuming, as well as the strong dependence on the environmental conditions. However, for R genes that have been isolated, it is now possible to replace phenotypes with molecular markers as the basis for defining alleles (Hua *et al.,* 2012).

This experiment was conducted at Zonal Agricultural Research Station (ZARS), V.C. Farm, Mandya, Karnataka, during 2015-2016 and molecular work was done at MAS laboratory ZARS, AICRP on Rice, V.C. Farm, Mandya and Department of Plant Biotechnology, UAS, GKVK, Bengaluru.

Material and Methods

The plant material selected, the procedural details of field work carried out, laboratory techniques employed, the method followed for the selection are described below:

Rice material

Recurrent parents

IR-64 (obtained from the parentage IR 5657-35-2-1/IR 2061465-1-5-5): with high-yielding, fine-grain type, good cooking quality and water logged resistance, released by IRRI, Philippines, possessing *Pita, Pikm, Pik* and *Pib* (Asif *et al.,* 2014), but susceptible to blast disease.

KMP-175 crossed between Thanu and IET 30864: High yielding, medium size grain type, Water saving upto 60 per cent and tolerant to moisture stress.

KCP-1: medium duration with high yielding and semi tall variety. It produces long panicles with 180-200 spikelets per plant.

Cultural management of hybridization nursery

Complete N: P: K fertilizers were applied at recommended doses. Urea was applied both at transplanting day and 30 Days after transplanting. Weeding and rouging were done carefully. Other cultural management practices were carried out as required.

Crossing programme

Hybridization : Crosses were obtained involving recipient parents and donor parents. Three staggered sowings of the parents (females and males) were undertaken at an interval of ten days to ensure synchronous flowering to produce adequate seed of the crosses. Healthy plants of the female lines with just emerged panicles were uprooted and potted in the evening hours of the day into plastic buckets filled with mud and were transferred to the green house. Productive tillers with healthy panicles were selected. Further, florets that had completed anthesis (at the top) and young florets at the bottom of the panicle were also removed. Florets due to flower on the next day alone were used for crossing. Top 1/3rd of each floret was clipped with scissors and the florets were carefully emasculated by removing anthers without damaging stigmas in any way. Emasculated florets were covered with butter paper bags and labeled properly. The emasculation process was carried out during early morning hours, 6 AM. – 9 AM.

Pollination : On the same day of emasculation, panicles ready for anthesis were selected from healthy male parents and were brought to the crossing chamber in which temperature, relative humidity and light conductive for anthesis was maintained. When the male parents were ready for dehiscence the female parent was brought inside the crossing chamber. Butter paper bags covering emasculated panicles of the female parents were removed. Further, the panicles were gently shaken so that the dry extruded anthers fell off. Panicles of male parents were then gently shaken over the female parents until adequate pollen was deposited on the stigmas of the emasculated spikelets. The pollinated spikelets were then covered with fresh butter paper bags, duly labeled and tied with the pins. The process of pollination was continued upto 12 PM depending the pollen dehiscence of male

parents. Crossed seeds were collected after three to four weeks from the plants maintained in the pots.

Isolation of genomic DNA : DNA was extracted from the frozen leaf sample (at –80 °C) using the standard CTAB protocol.

Sample preparation : Already collected and stored tender, fully expanded leaves (20-25 days old) were surface sterilized with ethanol (75%) before extraction.

DNA extraction and SSR analysis : 20-25 days rice leaves were used to extract DNA with extraction buffer (2% CTAB, 100 mM Tris, 20 Mm EDTA, 1.4 M NaCl) preheated at 60 °C. DNA quantification and purity was checked by measuring the O.D at 260 nm and 280 nm using a UV visible spectrophotometer.

Polymerase Chain Reaction (PCR)

The polymerase chain reaction was carried out in applied biosystem thermal cycler using three gene targeted primers (Table I).

- ✓ The PCR reaction mix includes the following: DNA 2µl of 10 ng/µl; 5µl of 2X Takara Mix, 2µl of DDW and 1µl of 10 µM each of forward and reverse primers.
- ✓ The PCR profile starts with Initial denaturation at 94 °C for 5 min, followed by denaturation, 94°C for 30 sec, primer annealing at 55 °C for 30 sec, extension 72 °C for 1 min, final extension 72 °C for 10 min, followed by cooling 4 °C for infinite time. These steps were repeated for 34 cycles for amplification of DNA. After completion of amplification, PCR products were stored at –20°C.
- ✓ The amplified products were analyzed by electrophoresis using 3 per cent agarose gels. Ethidium bromide was added while pouring the gel, so that the DNA fluoresces when gel was exposed to UV light. The DNA fragments was then visualized under UV transilluminator and the banding pattern was observed and recorded using gel documentation unit (Gene flash) which was stored for further scoring and permanent records.

Marker Assisted Introgression of rice blast resistance genes : Sequences of PCR based SSR markers were selected from the Gramene database (www.gramene.org) and selected gene specific markers were employed in the Marker Assisted Selection programme. The SSR marker RM224 for *Pi-1* gene (Hittalmani *et al.,* 2000), AP56595 for *Pi2* gene (Fjellstrom *et al.,* 2004), NMSMPi9-1 for *Pi-9* gene (Qu *et al.,* 2006) and PIKHMAS for *Pi-54* gene (Ramkumar *et al.,* 2011) were used for the polymorphism study between the parents.

The markers which are clearly differentiating between the parents were employed for the confirmation and foreground selection of the target genes. The list of primers used for parental polymorphism and foreground selection is given in Table-I.

Donor parents

Monogenic differentials such as Monogenic-1, Monogenic-2, Monogenic-4 and Monogenic-7 possess *Pi1, Pi54*, *Pi9* and *Pi2* blast resistant genes, respectively, were used as the donor parents for blast resistance (Table II).

TABLE I

Markers list used for foreground selection

Gene	Marker	Forward Primer	Reverse Primer	Chr	Product Size
Pi-1	RM224	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	11	157
Pi2	AP56595	CTCCTTCAGCTGCTCCTC	TGATGACTTCCAAACGGTAG	6	288
Pi-9	NMSMPi9-1	CGAGAAGGACATCTGGTACG	GAGATGCTTGGATTTAGAAGAC	6	168
Pi54/Pikh	Pikh MAS	CAATCTCCAAAGTTTTCAGG	GCTTCAATCACTGCTAGACC	11	216

TABLE II

List of number of true F_1 plants identified from crosses

Name of the cross	Markers used for F_1 confirmation	Gene	Number of Plants produced	Number of true F_{1s} generated
IR 64 x MONOGENIC-1	RM 224	*Pi1*	28	19
IR 64 x MONOGENIC-7	AP56595	*Pi2*	63	49
IR 64 x MONOGENIC-4	NMSMPi9-1	*Pi9*	82	76
IR 64 x MONOGENIC-2	PIKH MAS	*Pi54*	32	28
KMP 175 x MONOGENIC-1	RM 224	*Pi1*	126	101
KMP 175 x MONOGENIC-7	AP56595	*Pi2*	135	98
KMP 175 x MONOGENIC-4	NMSMPi9-1	*Pi9*	93	71
KMP 175 x MONOGENIC-2	PIKH MAS	*Pi54*	41	26
KCP 1 x MONOGENIC-1	RM 224	*Pi1*	108	90
KCP 1 x MONOGENIC-7	AP56595	*Pi2*	65	56
KCP 1 x MONOGENIC-4	NMSMPi9-1	*Pi9*	84	81
KCP 1 x MONOGENIC-2	PIKH MAS	*Pi54*	61	39

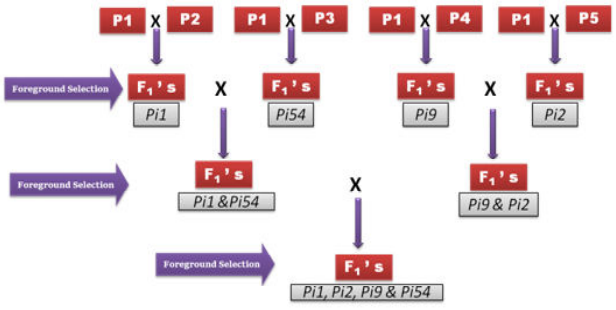


Fig 1: Schematic representation of crossing programme

Where,

P1 – Recipient Parents (IR64, KMP175 and KCP1), P2 – MONOGENIC LINE 1 (*Pi1*)

P3 - MONOGENIC LINE 2 (*Pi54*), P4 - MONOGENIC LINE 4 (*Pi9*),P5- MONOGENIC LINE 2 (*Pi2*)

RESULTS AND DISCUSSION

Parental polymorphism study : Nine gene specific SSR markers were used for surveying the parental polymorphism between improved varieties of IR64, KMP 175, KCP 1 and donor parents at molecular level. Out of nine markers used (RM1233*I and RM224 for *Pi1*, MSM1, AP4007and AP56595 for *Pi2*, NMSM Pi-9-1 for *Pi9*, TRS 26, RM206 and PIKHMAS for *Pi54*), four markers *viz.,* RM224 for *Pi1,* AP56595 for *Pi2*, NMSM Pi-9-1 for *Pi9*, PIKHMAS for *Pi54* were clearly differentiated between the recipient and donor parents at the selected genomic regions (Fig. 2).

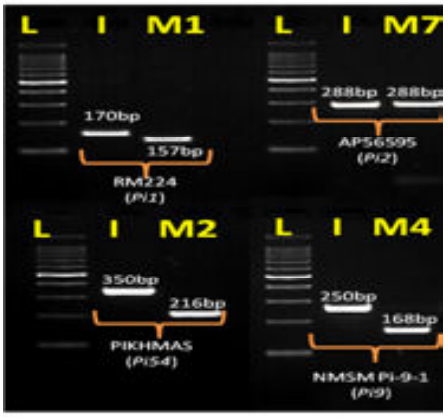
Generation and confirmation of F_1s : In the, first cross during *Kharif* 2015 IR-64, KMP-175 and KCP-1 were crossed with individual monogenic differentials, Monogenic-1 (donor for blast resistance genes *Pi1*), Monogenic-2 (donor for blast resistance genes *Pi54*) Monogenic-7 (donor for blast resistance genes *Pi2*), Monogenic-4 (donor for blast resistance genes *Pi9*) and the true F_1s possessing *Pi1, Pi2, Pi9* and *Pi54* individually were selected and crossed with each other (Table III). Finally, developed different set of gene combination plants with the selected varieties (IR-64, KMP-175 and KCP-1) background.

The new set of F_1s generated from these crosses were checked for presence of all the resistance genes *Pi1, Pi2, Pi9* and *Pi-54* using the molecular markers RM224, AP56595, NMSM-Pi-9-1 and PIKH MAS, respectively. Since the resistance and susceptibility specific amplicons produced by the markers RM224 (specific for *Pi-1*), AP56595 (specific for *Pi2*), NMSM-Pi-9-1 (specific for *Pi-9*) and PIKH MAS (specific for *Pi-54)* are in different size ranges, their

TABLE III

Number of plants harboring blast resistance genes in different combinations

Varieties		*Pi 1 + Pi2*	*Pi l + Pi9*	*Pi l + Pi54*	*Pi 2 + Pi9*	*Pi 2 + Pi54*	*Pi 9 +Pi54*	*Pi 1 Pi 2 + Pi9*	*Pi 1 Pi 2 + Pi54*	*Pi 1 Pi 9 + Pi54*	*Pi 2 Pi 9 + Pi54*	*Pi 1 Pi 2 + Pi9 + Pi54*
IR 64	Number of plants generated	72	63	81	102	57	26	28	33	28	17	8
KMP 175		63	105	62	56	44	61	59	21	33	34	19
KCP 1		49	101	132	38	41	54	36	39	42	32	14



Where, L – 100 bp ladder, I - IR 64, M1 - Monogenic line for *Pi1,* M7 - Monogenic line for *Pi2,* M2 - Monogenic line for *Pi54* and M4 - Monogenic line for *Pi9.*

Specific Markers were used to perform parental polymorphism; Marker RM 224 was used for identification of *Pi1* gene (A), Marker AP56595 was used for identification of *Pi2* gene (B), Marker PIKHMAS was used for identification of *Pi54* gene (C), Marker Nmsm Pi-9-1 was used for identification of *Pi9* gene (D). For all these markers, resistant allele size have mentioned in gene profiling Table I.

Fig. 2: Agarose gel showing polymorphism between the parents by gene specific markers

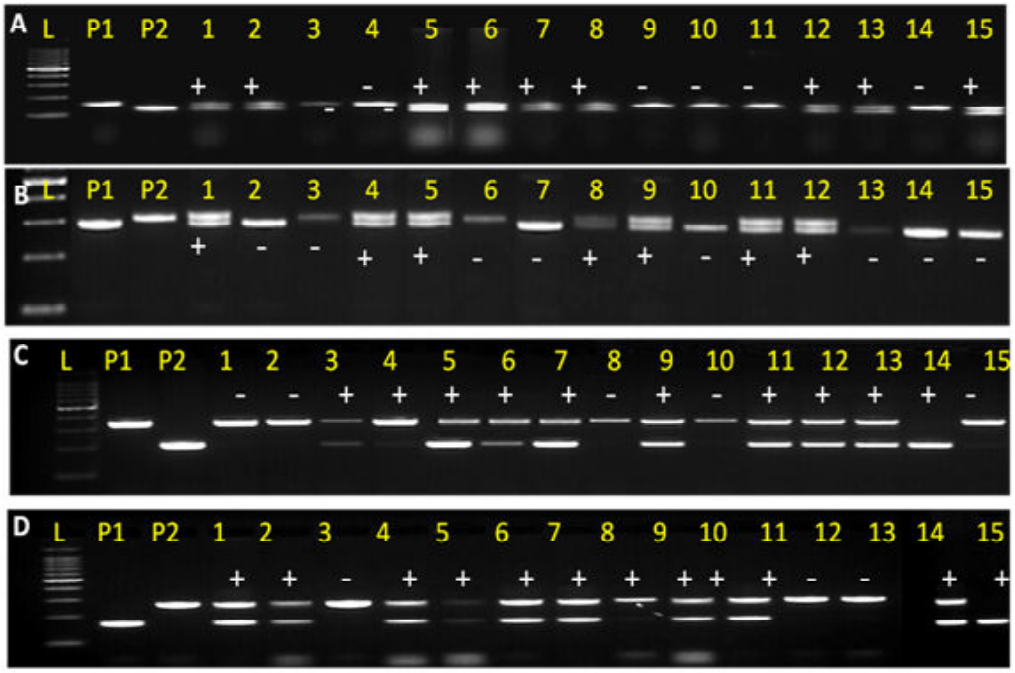


Fig. 3: Hybridity confirmation in crossed materials using gene specific makers.

+ve Denotes – Crossed seeds; -ve Denotes – Self seeds

A. Hybridity confirmation study for the IR64 X MONOGENIC 1 cross - (P1: IR 64; P2: MONOGENIC 1; 1-15: F1s)

B. Hybridity confirmation study for the IKCP-1 X MONOGENIC 7 cross- (P1: MONOGENIC 7; P2: KCP-1; 1-15: F1s)

C. Hybridity confirmation study for the IR64 X MONOGENIC 2 cross- (P1: IR 64; P2: MONOGENIC 2; 1-15: F1s)

D.Hybridity confirmation study for the IR64 X MONOGENIC 4 cross- (P1: MONOGENIC 4; P2: IR 64; 1-15: F1s)

primer sequences do not have any significant homologies, have identical Tm values and their sizes do not overlap.

Molecular markers particularly the co-dominant markers like SSR's are ideal for identification of 'true' F_1's since they are environmentally stable, easier to handle, rapid, as a result of which both dominant and recessive alleles could be identified in heterozygous condition. Hence, it is always a better choice to confirm F_1's using molecular markers.

The plants showing all the four genes like *Pi1+Pi2+Pi9+Pi54*, three genes like *Pi1+Pi2, +Pi9, Pi1+Pi2+Pi54, Pi2+Pi9+Pi54* and *Pi1+Pi9+Pi54,* two genes like Pi1+*Pi2, Pi1+ Pi9, Pi1+Pi54, Pi2+Pi9, Pi2+Pi54*and *Pi9+Pi54*, will be used for backcrossing with the respective recurrent parents to development of Marker Assisted Backcrossing (MABC) material. After generation of the BC_3F_1 backcrossing material, these lines need to be screened in hotspots with differential isolates in order to confirm their stability and durability of resistance.

Introgression of rice blast resistance genes *viz., Pi1, Pi2, Pi9* and *Pi54* into rice varieties (IR-64, KMP-175 and KCP-1) in different combination

The polymorphic SSR markers selected (RM224 for *Pi1*, AP56595 for *Pi2*, NMSM Pi-9-1 for *Pi9* and PIKH MAS for *Pi54*) are differentiating the parents with the expected resistant amplicon product sizes of 157, 288, 168 and 216 bp, respectively, (Hittalmani *et al.,* 2000; Fjellstrom *et al.,* 2004, Qu *et al.,* 2006 and Ramkumar *et al.*, 2011). Hence, the choice of the markers for the selection / screening of the generated material is appropriate.

Scientist have introgressed two dominant, broad-spectrum blast-resistance genes, *viz., Pi1* and *Piz5*, into PRR78 from the donor parent Pusa RH-10 pyramid using marker-assisted backcrossing (MABC). Microsatellite markers RM5926 and AP5659-5 tightly linked to *Pi1* and *Piz5* genes, respectively, were used for foreground selection to derive introgression lines (Gouda *et al.,* 2013).

The present research outcome in one of the successful implications of Marker Assisted Selection and its efficient utilization. The combination genes will be serves as valuable genetic stocks to meet furure blast disease resistance breeding needs.

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