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Original Article

Marker-Assisted Introgression of Bacterial Blight and Blast Resistance Genes into Mega Rice Cultivar BRRI dhan28

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Abstract *- Two major bacterial resistance (BB) genes (Xa21 and xa13) and two blast resistance genes (Pi54 and Pi1) were transferred to the popular mega rice cultivar BRRI dhan28 by marker-assisted breeding. ISM (and Xa21 and xa13) and NLR145 (Pi54 and Pi1) were used as donor parents. Maker-assisted backcrossing was performed using PCR-based gene-specific markers for target genes and a set of 60 polymorphic SSR primers for background selection. The BC1F1 plants from two crosses had 73 and 72% gene recovery of BRRI dhan28, respectively, and were crossed to produce ICF1 and, after that, selfed to produce 120 ICF2 plants. Among ICF2 plants, seven homozygous plants (xa13xa13Xa21Xa21Pi54Pi54Pi1Pi1) were identified with 80% recovery of the parental genome. Seven ICF2 showed high resistance to blast and bacterial blight diseases. Several ICF2 lines with high levels of resistance to BB and blast with yield, seed quality and plant type similar to BRRI dhan28 were identified and advanced for evaluation and further selection.*

Keywords *- Gene pyramiding, Bacterial blight, Blast resistance, Marker-assisted breeding, Rice.*

1. Introduction

Rice is a staple food that is included in the diet of many people around the world. It is an important food and income product in Bangladesh. The rice crop is threatened by more than 40 diseases that damage the rice harvest in Bangladesh. The potential for biotic stress in rice production has increased at an alarming rate since climate change (Pandy et al. 2017). New rice cultivars that are resistant to biotic stresses or that combine some resistance genes in high-yielding local species can be beneficial for food security (Roychodori et al. 2012). However, it is difficult to incorporate multiple resistance genes through conventional breeding methods due to dominance and epistatic effects and linkage disequilibrium of genes controlling such resistance (Huang et al. 1997). Marker-assisted selection (MAS) has a unique advantage in creating pyramidal lines that can directly impart durability by overcoming the limitations of conventional breeding. Bacterial blight by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) reduces the yield of rice by reducing the level of photosynthesis (Pradhan et al. 2015), causing more than 50% yield loss in recent years (Yugander et al. 2018). BB contamination could not be completely controlled by any of the chemicals and antibiotics treatment (Laha et al. 2009). The most efficient and sustainable way to do this is to develop BB-resistant rice varieties. To date, more than 42 BB resistance genes have been identified, 9 of which have been cloned. Among them, the combination *Xa21 + xa13* has been shown to be better in Bangladesh (Yugander et al. 2018). Another important fungal disease is rice blast caused by the fungus *Magnaporthe oryzae* (Ou 1985). Estimates of crop loss due to outbreaks can be 50% or more in epidemics (Babujee and Gnanamanickam 2000) and as high as 60-100% in extreme cases (Joseph et al. 2013). Currently, more than 100 blast-resistant genes have been identified (Devi et al. 2020), and 21 of these genes have been cloned (Liu et al. 2014). Among them, two critical resistance genes (*Pi54* and *Pi1*) have been identified to confer resistance to the most pathogenic strains of blast in Bangladesh (Khan et al. 2018). There is high genetic diversity among BB and rice blast pathogens across geographic regions (Vasudevan et al. 2014). Previous studies have shown that plants with a single resistance gene do not show general resistance (Yugander et al. 2017). Therefore, targeting BB genes and blasting to create high-yielding rice cultivars through marker-assisted breeding (MAB) is an

important tool for developing long-lasting and effective resistance to rice fields. The ability of MAB to induce BB resistance (Huang et al. 1997, Chen et al. 2001, Sundaram et al. 2009) and blast resistance genes (Hittalmani et al. 2000, Arunakanthi et al. 2008) were successful. To our knowledge, there are no reports of simultaneous induction of bacterial resistance and blast resistance in the popular BRRI dhan28 megaris strain. BRRI dhan28 is an early maturing variety with a maturity of 140-145 days and is growing as a popular variety due to its cooking quality and seed size.

However, BB and blasts in the field were frequent during the 2023 Boro season and were difficult to control with aggressive herbicide sprays. Therefore, the country's decision-makers have already decided to remove this cultivar gradually from the farmer's farm. Therefore, by improving BRRI dhan28 by pyramiding effective BB and blast resistance genes, this species can be maintained in the breeding chain for a long time. Some research groups in Bangladesh have successfully introduced one or two BB or blast resistance genes into popular varieties but have not attempted to improve BRRI dhan28. Considering all these, this study was designed for pyramidal genes *Xa21*, *xa13*, *Pi54* and *Pi1* into BRRI dhan28.

1. Materials and methods

2.1. Plant Material

The parent is BRRI dhan28 (BR28), a very good and popular rice variety of the Boro period, released in 1994-1995 by the Bangladesh Rice Research Institute (BRRI), Gazipur. Improved Samba Mahsuri (ISM, containing *Xa21* and *xa13*) (Sundaram et al. 2009) and NLR145 (Swarna mukhi, containing *Pi54* and *Pi1*) (Vanisree et al. 2012) were selected as the main donors of the resistant genes, respectively. Both donors were collected from the Indian Institute of Rice Research (IIRR), Hyderabad, Telangana, India. Crossing and backcrossing programs were carried out according to the plan mentioned in Figure 1.

2.2. Foreground Selection

A parental polymorphism study between BRRI dhan28 and ISM was performed for BB target genes *Xa21* and *xa13* using pTA248 (Ronald et al. 1992) and functional marker xa13 (Sundaram et al. 2009). Similarly, the functional markers Pi-54MAS (Ramkumar et al. 2011) and RM224 (Hittalmani et al. 2000) were used in *Pi54* and *Pi1*, respectively.

2.3. Background Selection

A set of 100 SSR markers, spread over the entire rice genome, was used to investigate the polymorphism between the recurrent parents and the donor parents to identify plants with high recovery of the parental genome. Primer sequences of SSR markers were obtained from Gramene SSR Marker Resources (www.gramene.org). The recovery of the dominant gene (RPG) was estimated using polymorphic SSR markers using Graphical Genotype software (GGT) version 2.0.

2.4. MAB Schemes for Introgression of BB and Blast Genes into BRRI dhan28

A simultaneous stepwise transfer approach was used to introduce all four genes into a single genetic background. To develop resistant lines, two separate backcross programs were initiated using BRRI dhan28 as females and two donor parents (ISM and NLR145) as males: BRRI dhan28 × ISM (Cross-I) and BRRI dhan28 × NLR145 (Cross). In the first crossing program, ISM carrying *Xa21* and *xa13* genes were crossed with BRRI dhan28 and 50 F1 seeds were harvested. From there, 40 F1 plants were confirmed as true F1 using *Xa21* and *xa13* gene-specific markers by PCR, and they were used as the female parent in crossing back with recurrent parent BRRI dhan28.

In the second crossing program, Swarna mukhi carrying *Pi54* and *Pi1* were crossed with BRRI dhan28 and 30 F1 seeds were harvested. From there, 20 F1 plants were confirmed as true F1 using *Pi54* and *Pi1* gene-specific markers by PCR, and they were used as female parents in crossing back with recurrent parent BRRI dhan28. Figure 1 shows the Marker-Assisted Backcross (MAB) method used in the study. Background markers were used to identify BC1F1 using molecular markers of *Xa21* and *xa13* (Cross I) and *Pi54* and *Pi1* (Cross II) in PCR. From two crosses, a total of 250 BC1F1 plants were obtained, and four plants from each BC1F1 crossing program were confirmed as homozygous for *Xa21* and *xa13* (Cross I) and *Pi54* and *Pi1* (Cross II) genes. A total of 100 polymorphic SSR markers were used to identify two independent BC1F1 lines from each cross and 4 plats were identified, which resulted in in>70% recovery of the BRRI dhan28 genome. To combine the resistance genes of *Xa21*, *xa13*, *Pi54* and *Pi1* in a single plant, these lines were crossed to produce intercross F1s (ICF1). After screening with specific markers/links to resistant genes (background markers), 3 "true" ICF1 hybrids were selected and selfed to produce 220 ICF2 plants (Figure 1).

2.5. DNA Isolation and PCR Amplification

A standard protocol was used for plants to isolate genomic DNA from the leaf samples. The quality and quantity of the isolated DNA samples were evaluated using 0.8% agarose gel electrophoresis and nanodrop (Thermo Electronic Corporation, USA), respectively, before the PCR reactions were set. One µl of 50 ng template DNA, 10 picoM of forward and reverse primers, 200 M dNTPs, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$), and 0.5 U of *Taq* DNA polymerase were included in the PCR reaction mixture. The reaction volume was 10 µl. The PCR conditions used as one cycle of 95°C for 5 minutes of denaturation, 35 cycles of denaturation 95°C for 45 seconds, annealing 55°C for 45 seconds and extension 72°C for 1 minute, and one cycle of 72°C for 10 minutes of extension. The final PCR products were separated by electrophoresis on 2% agarose gel, documented with the help of the gel documentation system (BIO-RAD), and the images were saved and scored.

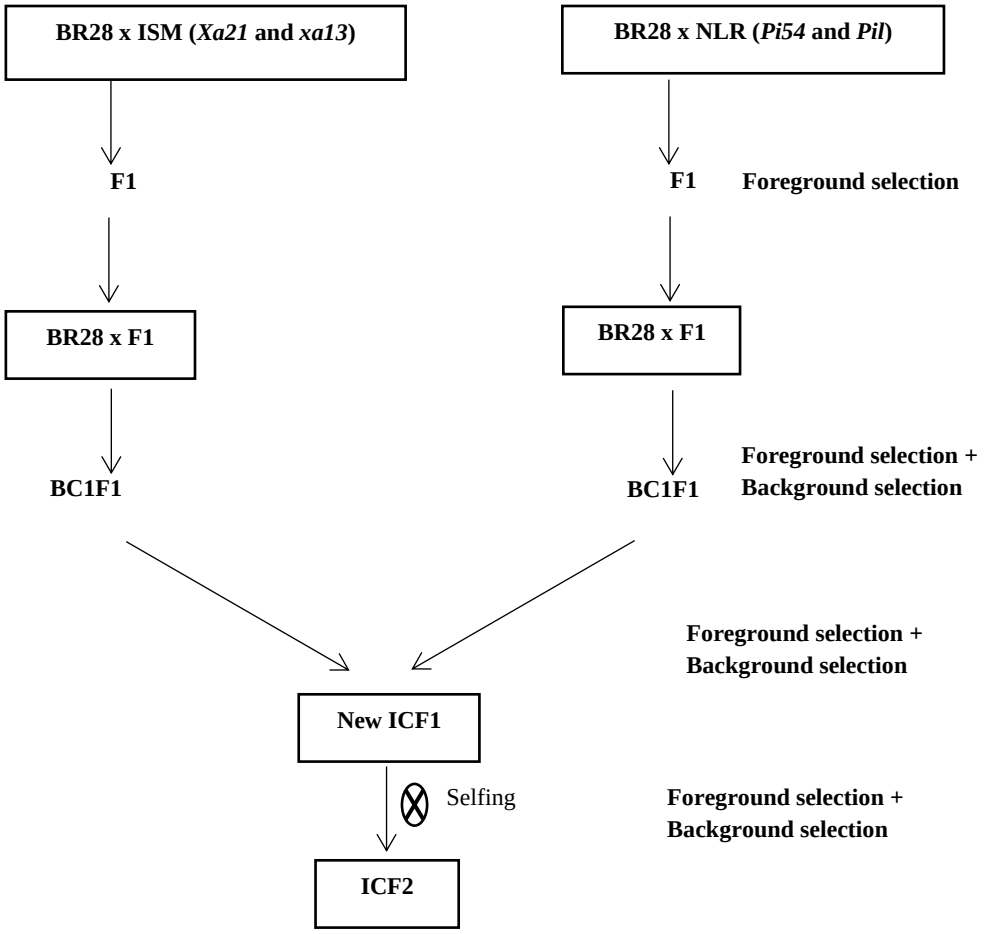

Fig. 1 Marker-assisted backcrossing scheme for pyramiding two Bacterial blight and two Blast resistance genes in BRRI dhan28 background

2.6. Screening for BB Resistance

One hundred and fifty selected lines of ICF2, carrying BB resistance genes, were transferred with parents to the main plot at a distance of 15-20 cm. At the age of 45 days, the seedlings were inoculated with a bacterial suspension culture of two virulent isolates, C5 and P6 of *Xanthomonas oryzae* pv. *Oryzae* has a concentration of 108 cfu/ml on sunny days. Five infected leaves from each plant were collected 21 days after inoculation, and immune response was determined using visual indices according to the IRRI Standard Evaluation System (SES) 1996. Then, the infected leaf area was averaged from five leaves.

2.7. Screening for Blast Resistance

One hundred and twenty ICF2 lines and their parents were planted after 30 days of germination in the field in rows 20 cm and 15 cm apart for plants during the Boro season (Jan-May) 2022. In the fourth leaf stage of the plants, blast pathogen inocula of two highly invasive isolates of *Magnaporthe oryzae* species collected from two locations in Bangladesh were sprayed. To promote infection, young plants were inoculated with a specific fungal suspension at a concentration of 1 x 10^5 spores/ml. After one week of inoculation, the plants were examined for rice blast disease. Plants were examined for the presence of blast lesions, and plants were scored on a scale of 0 to 9 according to IRRI-SES (IRRI, 1996).

2.8. Evaluation of Agronomic Performance

30-day-old screened ICF2 lines and parents were transplanted in the main field at a distance of 15-20 cm in 3 replicates at GPB Research Farm, Bangladesh Agricultural University, during Boro 2023. Two seedlings were planted on each hill. For the cultivation of healthy crops, common agricultural methods were followed. The crop was harvested in mid-April 2024. After harvest, data on agro-morphological characteristics were collected from 10 randomly selected plants and averaged.

2.9. Data Analysis

The software package Graphical Genotype (GGT) version 2.0 was used to analyze the genetic data to determine the percentage of recovery of the chromosomal segment of the recurrent parent in the selected backcross population. Agro-morphological data were subjected to compute Analysis of Variance (ANOVA), separation of means (Tukey's test), Coefficient of Variation (CV%) and significant differences (CD) using a computer statistical software MINITAB 17.1.0 (Minitab, available at: https://www.minitab.com).

3. Results and Discussion

3.1. Confirmation of Polymorphism for Gene-Specific Markers

DNA from recurrent parent BRRI dhan28 and donor parent ISM (*Xa21* and *xa13*) and NLR145 (*Pi54* and *Pi1*) were used to determine genetic polymorphisms using molecular markers in PCR. The first pair of pTA248 amplified fragments of 900 bp and 500 bp, respectively, in the resistant parent (ISM) and the susceptible parent BRRI dhan28 produced a band of 650 bp. In the case of the first pair of xa13-prom, ISM amplified a 500-bp fragment, while BRRI dhan28 amplified a 250 bp fragment. Similarly, for *Pi54* and *Pi1* markers, a 210 bp and 450 bp segment was amplified in NLR145, respectively, while a 550 bp segment was amplified in BRRI dhan28. Therefore, all markers can distinguish resistant lines from susceptible lines (Figure 2).

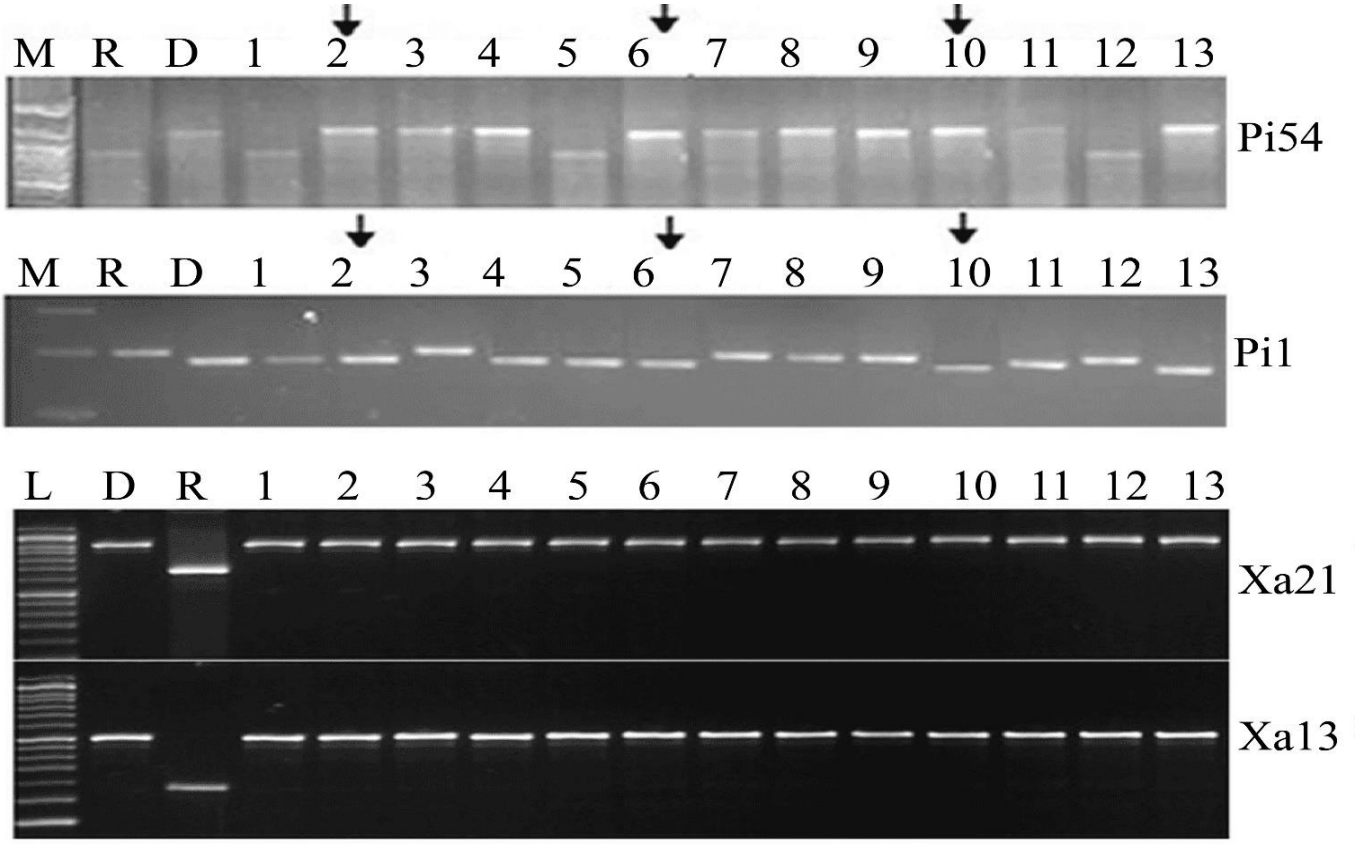

Fig. 2 Marker-based confirmation of F_1 plants for developing BC_1F_1. D, R, number and arrows are represented as donor parent, recipient parent, number of plants screened by PCR and plants were selected for using as backcross female parents, respectively.

3.2. Marker-Assisted Introgression of BB Resistance into BRRI dhan28

F_1s generated from the cross I was screened for the presence of *Xa21* and *xa13* using pTA248 and xa13-prom to identify "true" C1-F1s showing heterozygous amplification (*xa13xa13Xa21xa21*) (Table 1 and Figure 2). Of the 115 C1-F1 examined, 15 were identified as true heterozygous and were then used as the female parent and backcrossed with BRRI dhan28 to obtain C1-BC1F1. From a total of 185 C1-BC1F1 plants produced, 44 were detected positive for *Xa21*, 28 were positive for *xa13*, and 7 were double positive for both *Xa21* and *xa13* (Figure 3). These double-positive plants were then subjected to background selection using 60 SSR markers identified as polymorphic between BRRI dhan28 and ISM. One "positive" C1-BC1F1(10) plant with maximum recovery of the recurrent parental genome (BRRI dhan28) (73%) was selected for gene pyramid crossing.

3.3. Marker-Assisted Introgression of Blast Resistance into BRRI dhan28

The F1s generated from the cross C2 were screened for the presence of target resistance genes *Pi54* and *Pi1* using the functional markers Pi-54MAS and RM224 to identify "true" C2-F1s with heterozygous amplification (Table 1 and Fig. 2). Among 110 C2-F1, 100 plants were found to have the target resistance gene in the heterozygous state (*Pi54pi54Pi1pi1*), which were then used as female parents and backcrossed with BRRI dhan28 to produce C2-BC1F1 plants.

A total of 12 of the 120 C2-BC1F1 plants screened were identified as positive (Figure 3) when screened with Pi54-MAS and then subjected to background selection using 60 polymorphic SSR markers. One "positive" plant, C2-BC1F1(18), with the highest (72%) recovery of the recurrent parental genome, was selected as a gene pyramid cross.

Table 1. Number of plants generated and confirmed to be resistance gene positive through marker analysis in each generation

Cross combination	Particular of cross combination	No. of plants screened	No. of plants confirmed	Gene combination in the confirmed plants
BRRI dhan28 × ISM (C1)	C1-F1	115	15	*xa13xa13Xa21xa21*
C1-F1 × BRRI dhan28	C1-BC1F1	185	7	*xa13xa13Xa21Xa21*
BRRI dhan28 × NLR145 (C2)	C2-F1	100	14	*Pi54pi54Pi1pi1*
C2-F1 × BRRI dhan28	C2-BC1F1	125	12	*Pi54Pi54Pi1Pi1*
C1-BC1F1 × C2-BC1F1	ICF1	210	4	*xa13xa13Xa21xa21Pi54pi54Pi1pi1*

3.4. Marker-Assisted Introgression of BB and Blast Resistance Genes into BRRI dhan28

C1-BC1F1(10) with *xa13xa13Xa21Xa21* was used as a female parent and crossed with C2-BC1F1(18) homozygous *Pi54pi54 Pi1Pi1* to produce a set of 210 ICF1 seeds (Table 1). A total of four such homozygous positive ICF1 plants (*xa13xa13Xa21xa21Pi54pi54Pi1pi1*) were identified and then screened by polymorphic SSR markers.

One ICF1 plant (ICF1-16) with the highest percentage recovery of the recurrent parental genome (80%) was identified, and 150 ICF2 seeds were produced. They were then grown in the research field at the Bangladesh Agricultural University, Mymensingh. A total of 120 ICF2 plants were genotyped, and 7 plants with all target resistance genes in the homozygous state (*xa13xa13Xa21xa21Pi54pi54Pi1pi1*) were identified (Figure 4).

3.5. Agro-Morphological Evaluation of ICF2 Lines

The yield and agro-morphological traits of seven ICF2 lines were evaluated during the 2023 boro season at Bangladesh Agricultural University, Mymensingh (Table 2).

ICF2-16-12, ICF2-16-35 and ICF2-16-72 were identified as promising due to their high BB and blast resistance (Figure 5), slender grain type and high yield, which showed even higher grain yield compared to BRRI dhan28 (26.7 g/plant) with marginal differences. No significant difference in grain number/plant, 1000-grain weight, panicle length and grain yield/plant were observed compared to BRRI dhan28.

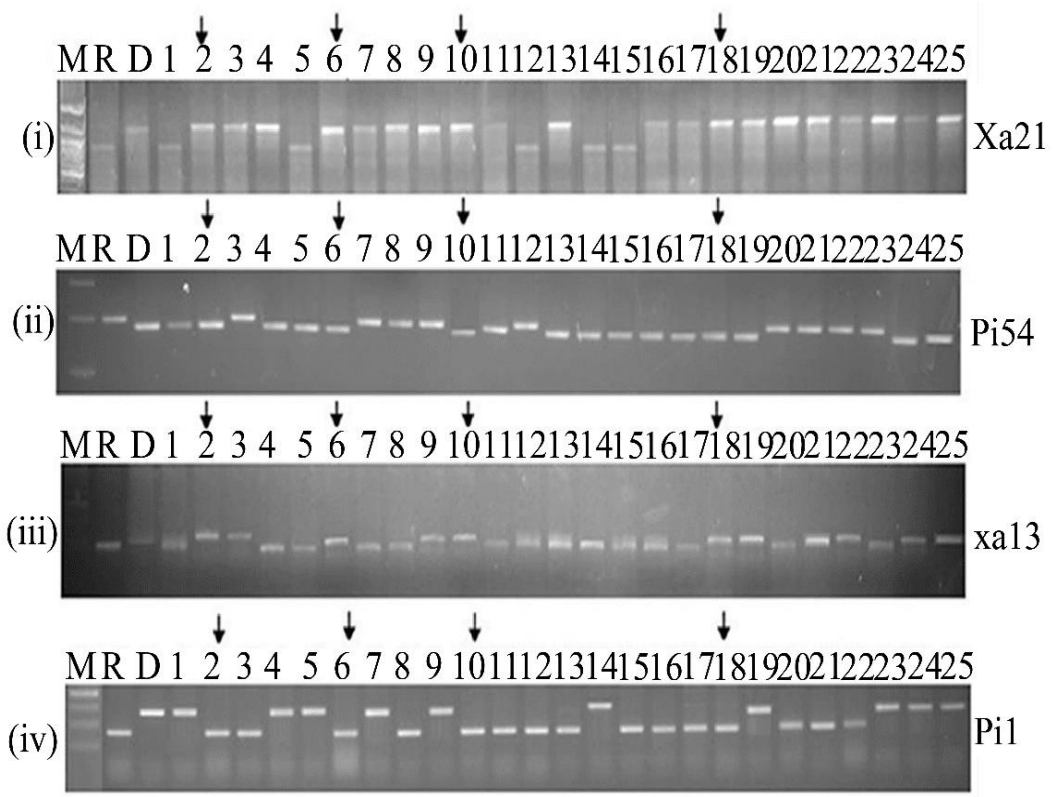

Fig. 3 Foreground selection of BC1F1 plants for *Xa21, Pi54, xa13* and *Pi1* Gel 1 represents the screening of BC1F1 plants through *Xa21* gene-linked marker, Gel 2 represents the screening of BC1F1 plants through *Pi54* gene-linked marker, Gel 3 represents the screening of BC1F1 plants through *xa13* gene-inked marker, while, Gel 4 represents the screening of BC1F1 plants through *Pi1* gene-linked marker. The numbers shown on top of the gel represents the BC1F1 plant numbers. M, molecular weight marker (100 bp ladder); D, donor parent; R, recipient parent. Arrow indicates the double positives for the *Xa21, Pi54, xa13* and *Pi1* genes in BC1F1 plants

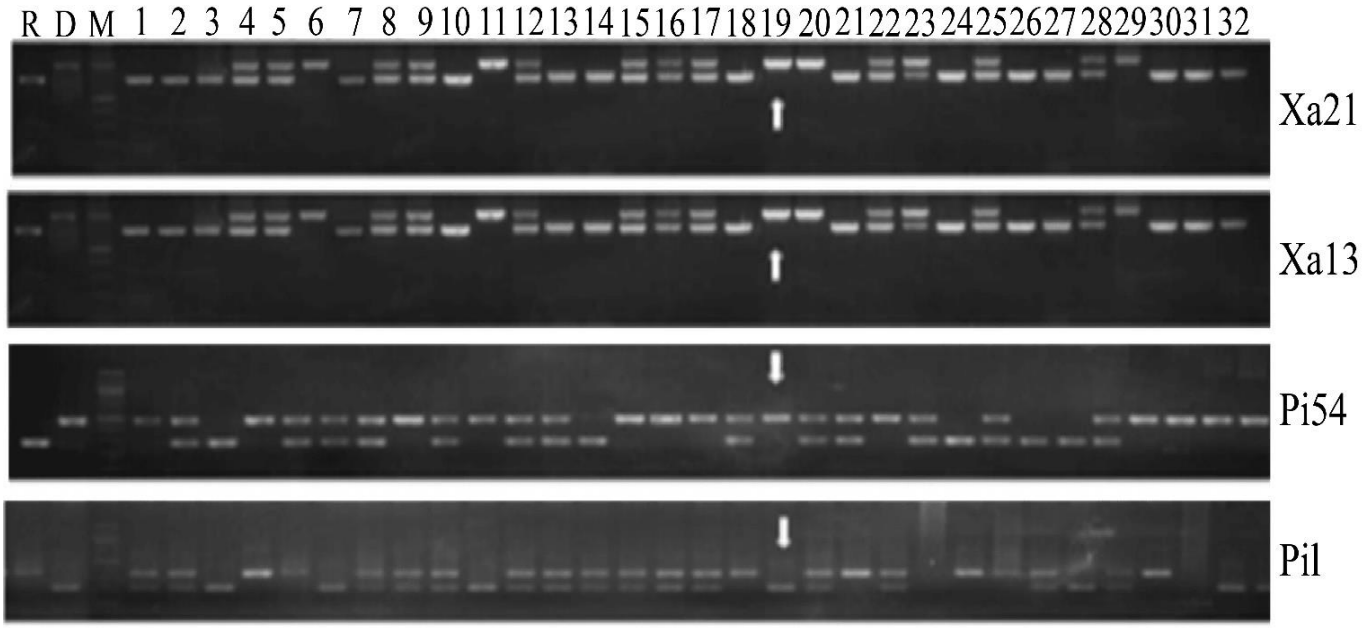

Fig. 4 Foreground selection for *Xa21*, *xa13*, *Pi54* and *Pi1* among ICF1 plants. R, Recurrent parent; D, Donor parent; and M, molecular weight marker (100 bp ladder). Arrow indicates a quadrille genes homozygous plant (ICF1-19).

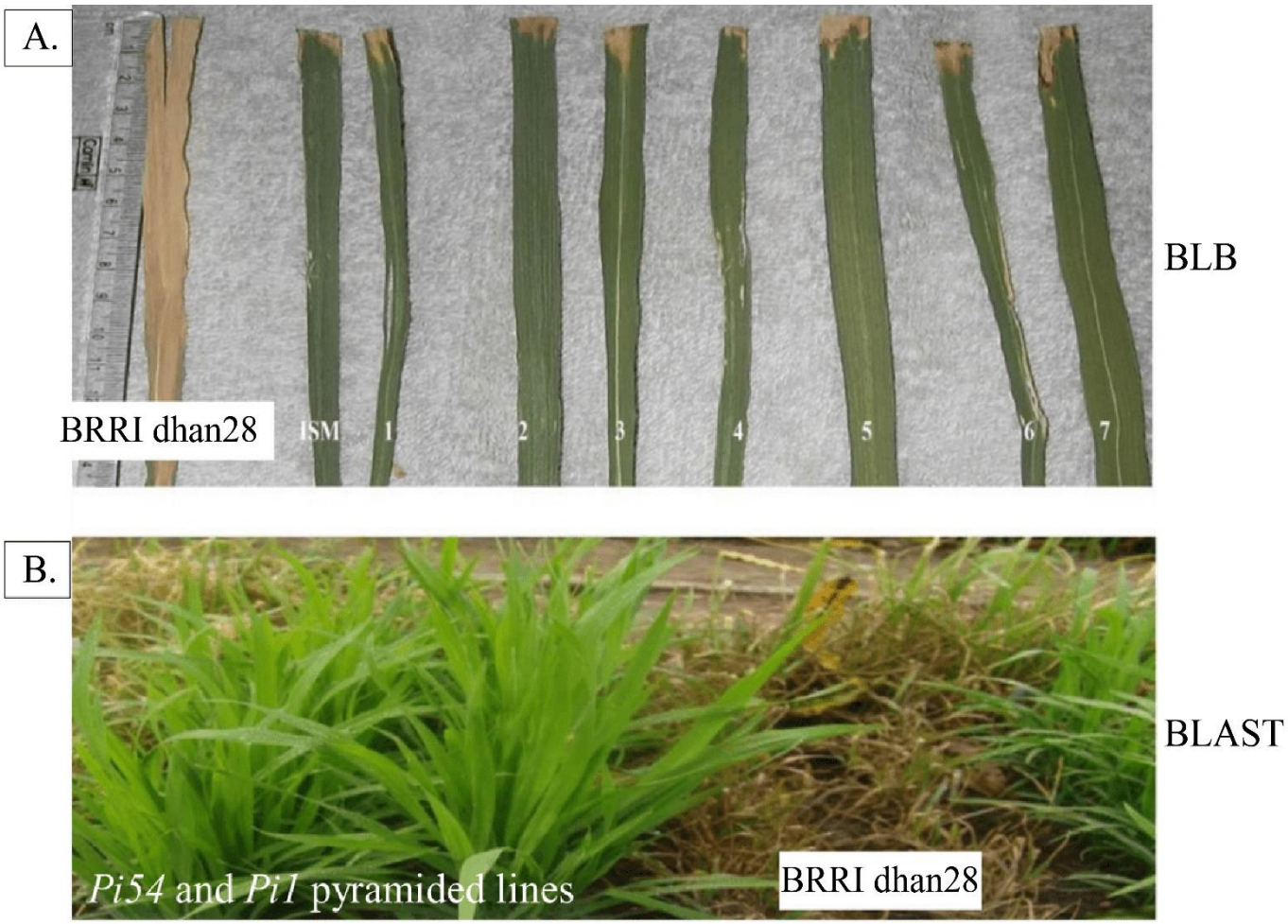

Fig. 5 Screening of selected BC_1F_2 plants compared to BRRI dhan28 against bacterial blight (A) and blast (B) diseases under natural conditions.

Table 2. Mean values of agro-morphological characters of four resistant genes pyramided ICF2 lines

Line	Days to heading	Plant height (cm)	No. of panicles/plant	Panicle length (cm)	No. of filled grains/panicles	1000-grain weight (g)	Grain yield/ plant (g)	Grain type
ICF-16-12	87.3	93.0	16.7	27.3	118.3	19.8	27.0	Slender
ICF-16-23	83.6	94.0	17.0	26.3	117.7	19.5	26.7	Slender
ICF-16-35	82.0	92.2	18.7	27.0	119.0	20.1	27.0	Slender
ICF-16-58	85.3	92.3	17.7	26.7	119.7	19.5	26.3	Slender
ICF-16-72	85.7	91.3	16.7	26.2	118.3	20.4	27.0	Slender
ICF-16-86	87.7	92.0	16.3	27.0	116.7	19.8	26.3	Slender
ICF-16-111	88.0	93.7	16.7	26.4	118.2	19.7	25.7	Slender
BRRI dhan28	82.7	92.3	16.7	26.0	117.7	20.4	26.7	Slender
CV (%)	3.2	5.4	2.6	4.2	9.3	1.8	3.6	
CD $_{(0.05)}$	1.5	2.8	2.1	2.1	2.9	0.8	1.4	

4. Discussion

In addition to markers, phenotypic selection for agro-morphological traits was done to identify backcross resistant plants that were not close to BRRI dhan28 but were superior to better mega varieties. ISM and BB resistance (Sundaram et al. 2014) and NLR145 harboring *Pi54* and *Pi1* genes were used as donor genes for rice blast resistance. Although the donor parent, ISM carries three BB resistance genes (*xa5*, *xa13* and *Xa21*), only two (xa13 and Xa21) genes were tried to transfer to BRRI dhan28 because *xa5* gene is known to display partial dominance and additive to the avirulent races and possesses only relatively small but significant residual effects (Lee et al. 2001) and has been reported to show negative effects in introgressed lines (Sundaram et al. 2009).

However, the *xa13* is very effective against many races of *Xoo*. Similarly, *Xa21* shows complete dominance against the avirulent *Xoo* races (Li et al. 2001). NLR145 was used as a donor of *Pi54* and *Pi1* because it is a popular and high-yielding cultivar with many favorable agro-morphological traits in India. PCR markers xa13-prom, pTA248, Pi-54MAS and RM224 were able to identify positive plants (*xa13*, *Xa21*, *Pi54* and *Pi1*) with no false positive results (Ramkumar et al. 2011; Sundaram et al. 2011). Therefore, we can use these markers for the MAS without phenotype-based selection of BB and blast disease resistance. In addition to background selection, polymorphic SSR markers were used to recover the recurrent parental genetic makeup in a very short period of crossings, as suggested by Hospital and Charcosset (1997). Also, they estimated the proportion of parental genome at each level of backcrossing. In this study, the number of backcrosses was limited to only one, and up to 60 main polymorphic SSR markers (polymorphic between BRRI dhan28 and the donor parents) were included to accelerate recovery of the background genome, and the success in identifying plants in the back cross generation with more than 70 percent of BRRI dhan28 genome. Importantly, when selecting the background, we focused on the introduction of many polymorphic SSR markers on chromosomes 8 and 11, where the target genes are distributed. This is because the carrier chromosomes should be of particular interest in backcrossing programs; they have large selection pressure on the donor allele of the target genes (*xa13*, *Xa21*, *Pi54* and *Pi1*) with a lower rate of recovery to the recipient genotype on the target chromosomes compared to non-carrier chromosomes (Hospital 2001). An elite line ICF1-16 was identified that recovered the maximum percentage (80%) of the parent genome. All lines obtained from the selfing of heterozygous ICF1 plants showed strong resistance to blast and BB diseases. In particular, ICF2-16-12, ICF2-16-23, ICF2-16-35, ICF2-16-58, ICF2-16-72, ICF2-16-86 and ICF2-16-111 showed significantly high levels of resistance. Regarding bacterial blight, the level of BB resistance is higher in improved versions of BRRI dhan28 compared to BRRI dhan28. Biswas et al. (2021) pyramided effective BB resistance genes (*Xa* genes) into background varieties. The population of the four BB resistance genes *Xa4*, *xa5*, *xa13* and *Xa21* was analyzed. By scoring the phenotype against three virulent bacterial strains, C5, P6, and V, promising recombinants were selected from the F_6 generation.

Marker-assisted selection (MAS) using gene-specific markers confirmed the pyramiding of BB resistance genes in Recombinant Introgressed Lines (RILs) with broad-spectrum resistance against BB. Most importantly, the yield levels of the seven selected lines were not significantly different from those of the parent BRRI dhan28 (Table 2), indicating that the presence of resistance genes is not related to the amount of yield. A similar observation was noted by Shanti et al. (2010) and Sundaram et al. (2009). Interestingly, ICF2-16-35 (*xa13xa13Xa21Xa21Pi54Pi54Pi1Pi1*) recorded significantly better in the number of filled grains, panicle length and grain yield/plant, plant height and duration compared to the recurrent parent BRRI dhan28. Thus, ICF2-16-35 can be considered a transgressive segregant for some yield-related traits. This was possible because, while we selected strictly for traits similar to BRRI dhan28 in the early backcross generation following phenotypic selection under field conditions, we also selected backcross-derived lines superior to BRRI dhan28 in later generations, starting with the ICF1 generation.

5. Conclusion

This study reports the successful pyramiding of two BB and two blast resistance genes into the popular short-duration rice variety BRRI dhan28. No negative interactions were observed between blight and blast resistance genes. In addition, no negative effects due to the presence of genes were observed in any of the gene pyramided lines, as both donors were improved cultivars with desired agro-morphological and grain quality characteristics. By adopting phenotype-based selection combined with marker-assisted selection, we were able to recover the desired plant type and grain type in the improved version of BRRI dhan28. Improved versions of the BRRI dhan28 are expected to replace the BRRI dhan28 after being evaluated through multi-location trials or could be used as good source materials for pyramiding of BLB and blast resistance genes in other popular mega rice cultivars.

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