Pyramiding genes for bacterial blight and blast resistance into an elite basmati rice restorer line (PRR78) through marker-assisted selection

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Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* and blast caused by *Magnaporthe oryzae* are major constraints that limit rice productivity. Marker-assisted backcross breeding was used to incorporate BB resistance genes (*xa13* and *Xa21*) and blast resistance genes (*Piz5* and *Pi54)* into the genetic background of an elite basmati restorer line, PRR78, which resulted in the development of Pusa1601 (PRR78 + *xa13* + *Xa21*) and Pusa 1609 (PRR78 + *Piz5* + *Pi54*), respectively. Furthermore, in order to combine the genes *xa13, Xa21, Piz5,* and *Pi54* into the genetic background of PRR78, Pusa 1601 and Pusa 1609 were intercrossed to develop Pusa 1790, and foreground marker-verified true F_1 plants were selfed to generate an F_2 population of 1,509 individuals. Stringent phenotypic selection was used to identify 400 plants resembling PRR78, which underwent foreground analysis. A total of three, five and 16 plants homozygous for four, three and two genes in different combinations, respectively, were identified and advanced to F_3. In F_3, evaluation for agronomic performance, disease reactions under artificial inoculations, and grain and cooking quality traits was performed, leading to the development of improved lines of PRR78. These lines, designated with the prefix Pusa 1790, carry genes *xa13+ Xa21* for BB resistance and *Piz5+Pi54* for blast resistance. Hybrids using improved restorer lines have been generated to evaluate their heterotic potential.

Keywords: bacterial blight, blast, gene pyramiding, hybrid rice, molecular markers, marker-assisted selection

Pusa RH10 is a superfine-grain aromatic rice hybrid combining high yield and short duration and thus very high per day productivity (Siddiq et al 2009). Despite being very popular, this hybrid is highly susceptible to two of the most dreaded diseases of rice, bacterial blight (BB) caused by *Xanthomonas oryzae* and blast caused by *Magnaporthe oryzae*. Presently, BB and blast diseases are managed mainly through the application of fungicide/antibiotics, which is costly as well as unsustainable. Despite the adoption of these control measures, under epidemic conditions, BB causes yield losses up to 70% (Mew and Vera Cruz 2001). Likewise, blast disease causes upto a

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50% yield loss in rice globally (Scardaci et al 1997). The development of resistant cultivars is considered to be the most effective method to counteract the pathogen. However, cultivars undergo rapid breakdown in their resistance mainly caused by the emergence of new pathotypes, due to the high instability in the genome of the pathogen (Dean et al 2005). Therefore, bringing together multiple genes conferring resistance to more than one pathotype into one genetic background is necessary for durable resistance.

To date, 39 BB resistance genes and 100 blast resistance genes have been identified, out of which 9 BB and 19 blast resistance genes have been cloned. The availability of such a large number of mapped resistance genes makes it possible to integrate two or more of them into a genotype, called ''gene pyramiding'' (Bonman et al 1992). This approach is considered a powerful tool to build up broad and durable resistance in a variety (Hittalmani et al 2000, Dangl and Jones 2001, Michelmore 2003, Werner et al 2005). The use of molecular markers is essential in gene pyramiding, which aids in tracking specific loci in segregating populations, which is a substitution for phenotypic screening (Francia et al 2005).

Marker-assisted selection (MAS) offers a simple, more efficient, and accurate way of breeding that is handy in breeding for disease resistance compared with selection only based on phenotype. A large repertoire of molecular markers is available in rice, which is quite valuable for marker-assisted selection. Marker-assisted pyramiding of major genes/QTLs has helped in tackling susceptibility for major diseases and insects such as bacterial blight (Huang et al 1997, Singh et al 2001, Zhang et al 2006, Liu and Anderson 2003, Joseph et al 2004,Gopalakrishnan et al 2008, Sundaram et al 2008, Basavaraj et al 2010, Hari et al 2011, Bhatia et al 2011, Suh et al 2011), blast (Hittalmani et al 2000, Zhou et al 2011, Singh et al 2011, Singh et al 2012a,b), sheath blight (Wang et al 2011, Singh et al 2012b), brown planthopper (Suh et al 2011, Hu et al 2012), and gall midge (Katiyar et al 2001).

Considering the economic impact of Pusa RH10 and concerns about its susceptibility to BB and blast, the incorporation of multiple BB and blast resistance genes into its restorer line, PRR78, was perceived as an essential requirement. Since Pusa RH10 is a superfine-grain aromatic rice hybrid, retention of grain quality traits in the parental line and the hybrid is of utmost importance during improvement of parental lines for these diseases. With this background, our investigation was undertaken with the objective of pyramiding genes for BB and blast resistance into the background of PRR78 without losing its heterotic potential with Pusa 6A, the female parent of the hybrid Pusa RH10, and its grain and cooking quality.

Materials and methods

Plant materials and the development of improved lines

PRR78, the restorer line of the hybrid Pusa RH10, was used as the recurrent parent, while Improved Pusa Basmati 1 (IPB1, carrying BB resistance genes *xa13+Xa21*), Tetep (carrying blast resistance gene *Pi54*), and C101A51 (carrying blast resistance gene *Piz5*) were used as the donor parents for the BB and blast resistance genes, respectively.

Marker-assisted backcross breeding

Three independent crossing programs were started for the development of BB- and blast-resistant lines using PRR78 as the female and three donor parents (IPB1, C101A51, and Tetep) as males, and the resultant crosses were designated as Pusa 1601, Pusa 1602, and Pusa 1603, respectively. In each case, true F_1s plants were identified based on the respective gene-based/linked markers and were backcrossed with PRR78 to produce BC_1F_1 seeds. In BC_1F_1, the plants heterozygous for the target locus underwent stringent phenotypic selection for identifying the plants possessing maximum similarity to the recurrent parent phenotype. The selected plants were used to generate BC_2F_1 seeds. For the development of Pusa1601, BC_2F_1 were selfed to generate a BC_2F_2 population, and the desirable plants were selfed through the pedigree method of selection upto the BC_2F_5 generation. However, BC_2F_1 plants possessing desirable alleles and with maximum recovery of the recurrent parent phenome (RPP) of Pusa 1602 and Pusa1603 series were intercrossed to generate Pusa 1609 (*Piz5* and *Pi54*). The plants homozygous for both genes (*Piz5* and *Pi54*) were identified in the F_2 generation and advanced upto the F_4 generation through pedigree selection. The elite lines of Pusa 1601 (*xa13+Xa21* and Pusa1609 (*Piz5* and *Pi54*) were intercrossed to develop a BB- and blast-resistant restorer line, which was designated as Pusa 1790 (carrying *xa13+Xa21* and *Piz5+Pi54*). The schematic representation of marker-assisted backcross breeding (MABB) appears in Figure 1.

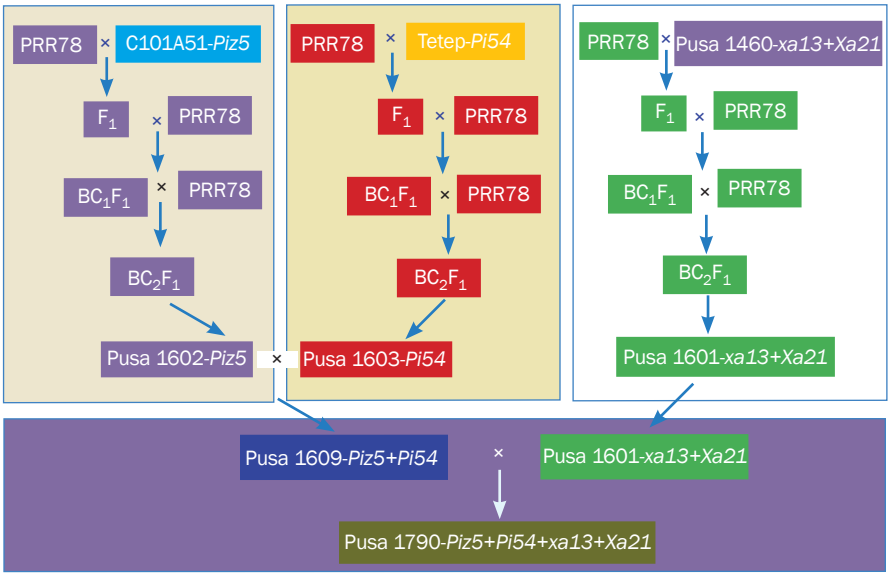

Fig. 1. Breeding scheme for the development of Pusa 1790 through marker-assisted introgression of BB and blast resistance genes into the genetic background of PRR78.

Molecular marker analysis

Foreground selection. Gene-based markers *xa13*-prom (Singh et al 2011) and pTA248 (Ronald et al 1992), were used for BB resistance genes*xa13* and *Xa21*, respectively. The SSR marker RM206 linked (0.6cM) to the blast resistance gene *Pi54* (Sharma et al 2005) and another SSR marker, AP5930, linked (0.1cM) to *Piz5* (Fjellstrom et al 2006), were used for foreground selection in segregating generations. Since the recurrent parent is a restorer line, foreground selection for fertility restorer gene *Rf1* was essential, which was done using *Rf1* gene-linked marker RM6100 (Singh et al 2011). The details of the genes, markers, and genetic materials used in the study are presented in Table 1.

Background selection. To help identify plants with a maximum recurrent parent genome, a set of 435 SSR markers spanning the 12 chromosomes was used to identify genome-wide polymorphic SSR markers in thee parental combinations, namely, PRR78 with IPB1, C101A51, and Tetep. These polymorphic SSR markers were used to estimate the RPG recovery in the backcross-derived line and, with the help of Graphical Genotypes (GGT) Version 2.0 (Van Berloo 1999) software, the genomic contribution of parents in selected elite lines was analyzed.

DNA extraction and PCR amplification

Total genomic DNA was extracted using the micro-extraction protocol of Prabhu et al (1998). Polymerase chain reaction was performed in a Thermal Cycler (G Storm, UK) using a total volume of 10 mL reaction containing 30 ng of template DNA, 5 pmole of each primer (synthesized from Sigma Tec., Bangalore), 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs (MBI Fermentas, Vilnius, Lithuania), and 0.5U of *Taq* polymerase (Bangalore Genei, Bangalore). The PCR condition was with one cycle of denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 30s, 55°C for 30s, and 72°C for 1 min, with a final extension of 72°C for 7 minutes. The amplified products were resolved in 3.5% Metaphor^{TM} gel (Lonza, Rockland, ME, USA) containing 0.1 µg/mL of ethidium bromide (Amresco, USA) and documented in the Gel documentation system (Biorad, USA).

Screening for BB resistance

The backcross-derived lines carrying BB resistance genes were inoculated with a bacterial suspension of 10^9 cells/mL at maximum tillering stage using the most virulent

Table 1. The markers used for foreground selection.[a]

Gene	Recurrent parent	Donor parent	Marker	LG	MD (cM)	Reference
xa13	PRR78	IPB-1	xa13-prom	8	GB	Singh et al (2011)
Xa21	PRR78	IPB-1	pTA248	11	GB	Ronald et al (1992)
Piz5	PRR78	C101A51	AP5930	6	0.10	Fjellstrom et al (2006)
Pi54	PRR78	Tetep	RM206	11	0.60	Sharma et al (2005)
Rf1	PRR78	–	RM6100	10	6.50	Singh et al (2011)

[a] IPB1 (Improved Pusa Basmati 1); LG = linkage group; MD = marker distance; GB = gene-based.

Kaul isolates of *Xanthomonas oryzae* pv. *oryzae* (Joseph et al 2004). The leaf clipping method of Kauffman et al (1973) was used to inoculate five young leaves in each plant and the disease reaction was recorded after 21 days. Plants exhibiting average lesion length of up to 6 cm were considered as resistant and those with lesion lengths of >6 cm were scored as susceptible.

Screening for blast resistance

The seedlings of selected advanced families were grown in plastic trays, and 21-day-old seedlings were inoculated with the four most virulent blast isolates collected from the basmati rice-growing regions of northern India as reported by Singh et al (2012a). The inocula were prepared as per the procedure of Bonman et al (1986) and the plants in each tray were sprayed with 50 mL of inoculum with a conidial density of 5×10^4 per mL and kept for 24 h at 25°C in a dew chamber. Subsequently, these plants were maintained for 1 wk at 25°C in the dew chamber before scoring. Seven days after inoculation, the plants from the families were scored for blast resistance on a 0−5 scale as per Bonman et al (1986). The plants exhibiting a reaction score of 0−3 were considered as resistant while those showing a score of 4−5 were considered as susceptible.

Evaluation of agronomic performance and grain quality analysis

The advanced backcross-derived lines along with parental lines were planted at 15 × 15 cm spacing in a randomized complete block design with three replications and evaluated for agronomic traits during kharif 2012 at the experimental farm of the Division of Genetics, IARI, New Delhi, India. Data for five plants were taken to determine the following agronomic traits: plant height (PH), number of tillers per plant (NT), panicle length (PL), filled grains per panicle (FG/P), spikelet fertility (SF), 1,000-grain weight (GW), and yield per plant (Y/P). Grain quality traits were analyzed according to grain size, kernel length before cooking (KLBC), kernel length after cooking (KLAC), kernel breadth before cooking (KBBC), kernel breadth after cooking (KBAC), length/breadth ratio (L/B), elongation ratio (ER), alkali spreading value (ASV), and aroma as described in Basavaraj et al (2010).

Results

Marker-assisted backcross breeding comprising foreground and background selection was employed to develop BB- and blast-resistant restorer lines in the genetic background of basmati rice restorer line PRR78. The improved versions of PRR78, namely, Pusa1601 (carrying BB resistance genes *xa13* and *Xa21*), Pusa 1602 (carrying blast resistance gene *Piz5*), Pusa 1603 (carrying blast resistance gene *Pi54*), Pusa 1609 (carrying two blast resistance genes, *Piz5* and *Pi54*), and Pusa1790 (carrying two BB resistance genes, *xa13* and *Xa21,* and two blast resistance genes, *Piz5* and *Pi54*), were developed and the significant findings are presented as follows.

The development of Pusa 1601 (PRR78+*xa13*+*Xa21*)

Pusa 1601 was developed by crossing PRR78 with IPB 1 as the donor for BB resistance genes, *xa13* and *Xa21* in our lab earlier (Basavaraj et al 2010). The BC_2F_5 plants of

the backcross series were highly resistant to bacterial blight disease, showing lesion length of <2.0 cM. The agronomic performance and grain and cooking quality attributes of Pusa 1601 lines in both backcross series were similar to those of the respective recurrent parents. However, some of the selections in both populations were superior to the recurrent parents. Improved lines of PRR78 showed a yield advantage up to 5.23%, respectively, under disease-free conditions, which could be due to the presence of donor parent segments with a positive effect in other genomic regions. Pusa 1601 (PRR78+*xa13*+*Xa21*) lines were crossed with CMS line Pusa6A, the female parent of the original hybrid Pusa RH10, to generate a new set of hybrids. The newly developed hybrids showed heterosis similar to that of Pusa RH10 with resistance to bacterial blight.

The development of Pusa 1602 (PRR78+*Piz5*) and Pusa 1603 (PRR78+*Pi54*)

In an earlier study in our lab, two major broad-spectrum genes, namely, *Piz5* and *Pi54,* conferring resistance to blast disease were transferred into PRR78 from C101A51 and Tetep, respectively, in two separate backcross series and named Pusa1602 and Pusa 1603, respectively (Singh et al 2012a). Marker-assisted foreground and background selection was also integrated with phenotypic selection for agronomic and grain and cooking quality traits, to accelerate recovery of the recurrent parent genome and phenome. The best BC_2F_1 plants from each backcross breeding program were selfed to produce F_2 populations and MAS was used to identify plants homozygous for each gene; the selected plants were advanced to the BC_2F_5 generation through pedigree selection to develop improved versions of PRR78 with blast resistance. Based on the background analysis using a genome-wide SSR marker,Pusa 1602-06-30-1-51 showed 89.01% and Pusa 1603-06-10-2-12 showed 87.88% recurrent parent genome recovery (RPG). The disease reaction of improved lines and their recurrent and donor parents was variable under artificial inoculation with different isolates (Fig. 2A and 2B). The hybrids produced by crossing Pusa 6A with improved lines of PRR78 with blast resistance were found to be on a par with hybrid Pusa RH10 in yield and grain and cooking quality traits, with an advantage of blast resistance.

The development of Pusa 1609 (PRR78+*Piz5*+*Pi54*)

The pyramiding of disease resistance genes into an elite genetic background is an effective approach for managing important diseases in crops. In yet another study carried out in our lab earlier (Singh et al 2013), amarker-assisted simultaneous but stepwise backcross breeding (MASS-BB) approach was used for pyramiding two blast resistance genes from two different donors into PRR78to develop Pusa 1609 in our lab. Improved versions of Pusa RH10 developed by crossing Pusa6A, the female parent of Pusa RH10, with different lines of Pusa 1609 (PRR78+*Piz5*+*Pi54*) performed on a par with the original Pusa RH10 and showed resistance to blast disease both under artificial screening and at hot-spot locations.

The development of Pusa 1790 (PRR78+*xa13*+*Xa21*+*Piz5*+*Pi54*)

The near-isogenic lines of PRR78 (Pusa1601 and Pusa 1609) were intercrossed to develop Pusa 1790. Gene-based markers were used for confirming the hybridity and

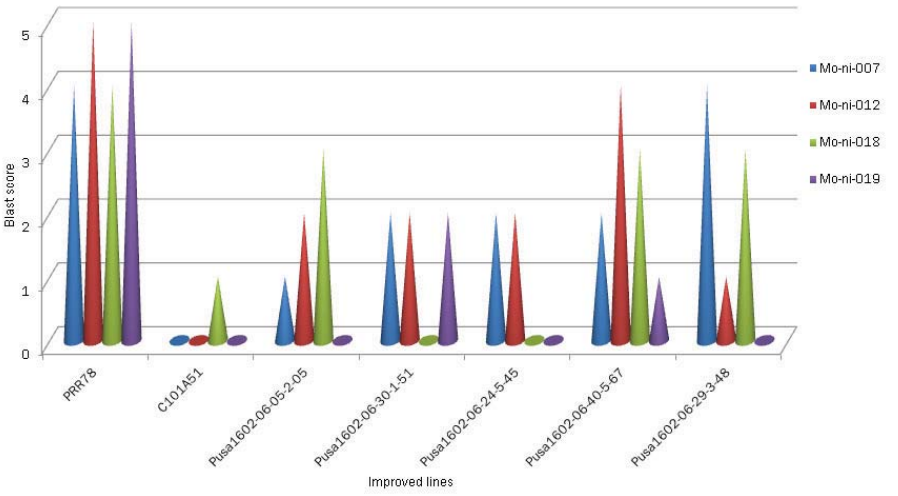

Fig. 2a. Graphical representation of recurrent parent (PRR78), donor parent (C101A51), and near-isogenic lines of PRR78 (Pusa 1602) carrying gene *Piz5* in artificial inoculation condition. Different color bars represent the disease reaction score for four different virulent isolates collected from different location of Basmati-growing regions of India.

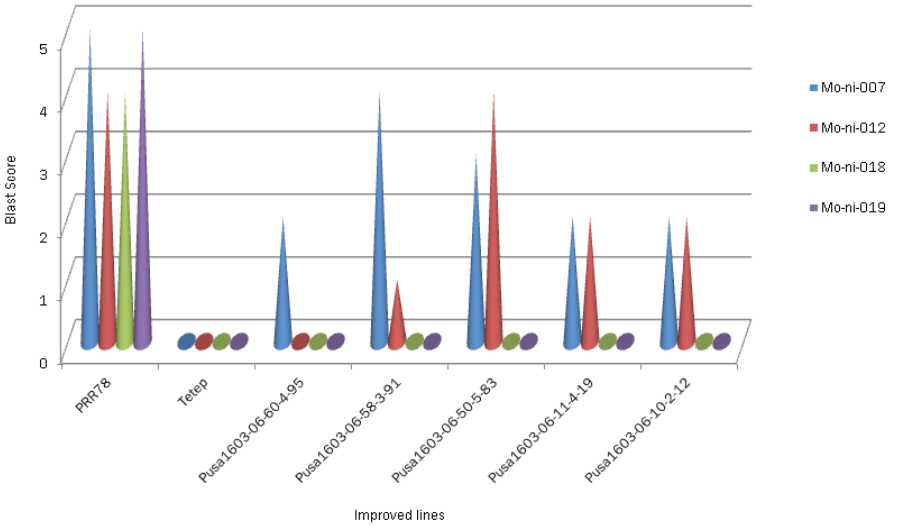

Fig. 2b. Graphical representation of recurrent parent (PRR78), donor parent (Tetep), and near-isogenic lines of PRR78 (Pusa 1603) carrying gene *Pi54* in artificial inoculation condition. Different color bars represent the disease reaction score for four different virulent isolates collected from different location of Basmati-growing regions of India.

true F_1 plants were selfed to generate an F_2 population of 1,509 individuals. On the basis of phenotypic selection for agro-morphological characters in the recurrent parent, 400 plants were selected and subjected to foreground analysis. Marker-assisted

foreground selection was carried out for identifying plants carrying a combination of two, three, and four genes using the respective markers, as mentioned above. A total of three, seven, and 16 plants homozygous for four, three, and two genes in different combinations, respectively, were identified and advanced to the F_5 generation (Table 2). In F_5, phenotypic selection for agronomic performance, disease reactions under artificial inoculation, and grain and cooking quality traits was performed. These promising lines are now being used in generating testcross hybrids to evaluate their heterotic potential.

The development of improved Pusa RH10

Several testcross combinationswere generated using the improved lines of Pusa 1601 and Pusa 1609 with Pusa6A (CMS line of Pusa RH10). The promising hybrid combinationsare being multiplied on a large scale for multilocation testing. Only one BB-resistant hybrid (Pusa RH 10-01-03) was found to be on a par with the original Pusa RH10. However, blast-resistant hybrids showed a yield advantage of 23.53% in Pusa RH 10-09-14 to 37.06% in Pusa RH 10-09-16 in comparison with the original Pusa RH10 (Table 3).

Table 2. Promising lines of Pusa 1790 with different gene combinations and superior grain quality.

Plant number	Generation	Gene status (gene-based/linked markers)			
		xa13 (xa13-prom)	*Xa21* (pTA248)	*Piz5* (AP5930)	*Pi54* (RM206)
Pusa 1790-10-638	F_5	++	++	++	++
Pusa 1790-10-651	F_5	++	++	++	++
Pusa 1790-10-705	F_5	++	++	++	++
Pusa 1790-10-539	F_5	++	–	++	–
Pusa 1790-10-646	F_5	++	–	–	++

Table 3. Yield advantage of BB- and blast-resistant hybrids.

Hybrids	Details	Yield in (kg) plot of 3.2 m^2	% advantage over Pusa RH 10
Pusa RH 10-01-03	Pusa6A/Pusa 1601-5	3.10	2.94
Pusa RH 10-09-14	Pusa6A/Pusa 1609-6	3.80	23.53
Pusa RH 10-09-15	Pusa6A/Pusa 1609-3	3.96	28.24
Pusa RH 10-09-16	Pusa6A/Pusa 1609-D	4.26	37.06
Imp. restorer line	Pusa 1601 (R)	2.60	–
Imp. restorer line	Pusa 1609 (R)	2.30	–
Original restorer line	PRR78	2.20	–
Original hybrid	Pusa RH10	3.00	–

Conclusions

Our study was undertaken with a view to improve basmati quality restorer line PRR78, the male parent of a superfine-grain aromatic rice hybrid, Pusa RH10, for resistance to BB and blast diseases, with an ultimate goal to develop rice hybrids with built-in resistance to BB and blast. To achieve this goal, marker-assisted foreground selection was successfully combined with phenotypic selection for yield and its components and grain and cooking quality traits, followed by background analysis to develop an improved version of PRR78 with resistance to BB and blast. The use of MAS with robust STMS markers has been very effective in improving PRR78. The improved versions of PRR78, namely, Pusa 1601 (PRR78+*xa13*+*Xa21*), Pusa 1602 (PRR78+*Piz5*), Pusa 1603 (PRR78+*Pi54*), Pusa 1609 (PRR78+*Piz5*+*Pi54*), and Pusa 1790 (PRR78+*xa13*+*Xa21*+*Piz5*+*Pi54*) carrying BB and blast resistance genes in different combinations were either on a par with or superior to recurrent parent PRR78 in agronomic performance and grain and cooking quality traits, with an added advantage of BB and blast resistance. The improved lines Pusa 1601 and Pusa 1609 were crossed with Pusa6A, and the resulting hybrids were either on a par with or better than Pusa RH10 in yield. The newly developed parental lines and hybrids can be used to replace Pusa RH10, after required testing. Commercial cultivation of BB- and blast-resistant varieties and hybrids will reduce the cost in managing these diseases, thereby increasing the profitability of farmers.In the future, the development of high-throughput markers such as single nucleotide polymorphisms (Gopala Krishnan et al 2012) and high-throughput genotyping facilities (Henry et al 2012) will enable plant breeders to use MAS to expedite the process of improving parental lines and variety development.

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Notes

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