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Marker-Assisted Pyramiding of Genes Conferring Resistance Against Bacterial Blight and Blast Diseases into Indian Rice Variety MTU1010

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Abstract: Two major bacterial blight (BB) resistance genes (*Xa21* and *xa13*) and a major gene for blast resistance (*Pi54*) were introgressed into an Indian rice variety MTU1010 through marker-assisted backcross breeding. Improved Samba Mahsuri (possessing *Xa21* and *xa13*) and NLR145 (possessing *Pi54*) were used as donor parents. Marker-assisted backcrossing was continued till BC_2 generation 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. Selected BC_2F_1 plants from both crosses, having the highest recoveries of MTU1010 genome (90% and 92%, respectively), were intercrossed to obtain intercross F_1 (ICF_1) plants, which were then selfed to generate 880 ICF_2 plants possessing different combinations of the BB and blast resistance genes. Among the ICF_2 plants, seven triple homozygous plants (*xa13xa13Xa21Xa21Pi54Pi54*) with recurrent parent genome recovery ranging from 82% to 92% were identified. All the seven ICF_2 plants showed high resistance against the bacterial blight disease with a lesion lengths of only 0.53–2.28 cm, 1%–5% disease leaf areas and disease scoring values of '1' or '3'. The seven ICF_2 plants were selfed to generate ICF_3, which were then screened for blast resistance, and all were observed to be highly resistant to the diseases. Several ICF_3 lines possessing high level of resistance against BB and blast, coupled with yield, grain quality and plant type on par with MTU1010 were identified and advanced for further selection and evaluation.

Key words: gene pyramiding; bacterial blight resistance; blast resistance; rice; marker-assisted backcross breeding

Rice is the principal staple food crop of the world and rice production has so far kept pace with the growing population, principally due to cultivation of high-yielding, high-input demanding, and semi-dwarf varieties (Gnanamanickam, 2009). However, the introduction of semi-dwarf rice varieties and the large-scale use of inputs like fertilizers and insecticides have changed the dynamics of pests and diseases of rice, increasing their incidence significantly in the recent years. Bacterial blight (BB) and rice blast are the two most important diseases causing significant yield loss in rice (Zhang et al, 2015), and they are endemic to several rice growing states of India (Production Oriented Survey, DRR, 2008). In Andhra Pradesh of

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India (including the newly created state of Telangana), the yield loss is very severe due to BB and blast (Rajarajeswari and Muralidharan, 2006; Sundaram et al, 2008). To minimize these problems, development of durable, broad-spectrum resistant varieties has been advocated (Jena and Mackill, 2008; Kumar et al, 2014; Sundaram et al, 2014). At least 40 genes conferring BB resistance have been identified (Sundaram et al, 2014) and many of them have been fine-mapped and cloned (Natrajkumar et al, 2012). To date, 101 blast-resistant genes (Rajashekara et al, 2014) and 350 quantitative trait loci (QTLs) have been identified (Sharma et al, 2012), with many fine-mapped and a few cloned. Closely linked or functional markers are available for many BB and blast resistance genes (Sundaram et al, 2014).

MTU1010 (Cottondora Sannalu), a short duration rice variety released in 2000 derived from the cross Krishnaveni/IR64, is extremely popular with farmers and has been planted for many years on a minimum of one million hectares. This variety possesses brown planthopper tolerance with long slender grains. However, MTU1010 is highly susceptible to both BB and blast diseases, which limits its spread to areas where the two diseases are endemic. As the availability of several resistance genes to BB and blast, pyramiding multiple genes into MTU1010 is considered as an ideal strategy to improve its resistance to these major diseases. Breeding for host-plant resistance is considered as the most economical and eco-friendly strategy for management of pests and diseases of crop plants and achieving yield stability. Molecular markers can accelerate resistance breeding efforts, as segregating plants can be selected on the basis of molecular marker alleles instead of their phenotypes and introgression of multiple resistance genes or gene pyramiding can be tracked easily in a population (Sundaram et al, 2014).

Gene pyramiding through conventional phenotype-based screening is considered to be difficult and often impossible, due to the dominance and epistasis effects of genes governing disease resistance and also due to limitations related to screening against the two diseases across the year (Sundaram et al, 2009). The availability of molecular markers, closely linked to or located within the resistance genes (i.e. functional markers), makes the task of gene pyramiding easier (Singh et al, 2001; Sundaram et al, 2008; Shanti et al, 2010; Zhao et al, 2014). Functional markers are developed from polymorphic sites within genes that casually affect target trait variation i.e. based on functional characterization of polymorphism. Hence, they are more reliable to be used in marker-assisted backcross breeding, circumventing the recombination issue there by getting rid of false positives. Among the BB resistance genes identified so far, the dominant gene, *Xa21*, originally discovered from an accession of the wild rice, *Oryza longistaminata*, confers broad spectrum resistance to many *Xoo* isolates in India and elsewhere. The gene has been cloned and fine-mapped on the long arm of rice chromosome 11, and a gene-specific functional marker, named pTA248 (Ronald et al, 1992), is available for marker-assisted breeding. BB resistance gene *xa13* was first discovered in the rice variety BJ1, and mapped on the long arm of rice chromosome 8 (Ogawa et al, 1987; Zhang et al, 1996; Sanchez et al, 1999) and very tightly linked markers are available for the gene (Sundaram et al, 2014). The BB resistance gene combination, *Xa21* + *xa13*, is known to be very effective across India (Joseph et al, 2004; Gopalakrishnan et al, 2008). Among the major blast resistance genes, *Pi54* exhibits resistance to predominant isolates of the blast pathogen in India (Sharma et al, 2002, 2010) and is considered to be an ideal choice for introgression. In this study, we aimed to transfer two major BB resistance genes (*Xa21* and *xa13*) and one major blast resistance gene (*Pi54*) into MTU1010 through marker-assisted backcross breeding.

MATERIALS AND METHODS

Rice materials

Improved Samba Mahsuri (ISM), with high-yielding, fine-grain type and BB resistant, released by ICAR-Indian Institute of Rice Research (ICAR-IIRR), Hyderabad, Indian, possessing *xa5*, *xa13* and *Xa21* (Sundaram et al, 2008), and NLR145 (Swarnamukhi), obtained from the parentage CICA4/IR625-23-3-1// Tetep, a popular long slender and long duration variety released from Agricultural Research Station, Nellore, Indian, possessing *Pi54*, were used as the donor parents for BB and blast resistance, respectively. MTU1010 was used as the recurrent parent.

Marker-assisted selection for BB and blast resistance

For targeted introgression of *xa13*, *Xa21* and *Pi54* into MTU1010, a simultaneous and stepwise marker-assisted backcross breeding strategy as illustrated in Supplemental Fig. 1 was adopted. Two separate backcrosses were carried out wherein the BB resistance genes *Xa21* and *xa13* from ISM, as well as

the blast resistance gene *Pi54* from NLR145, were introgressed into MTU1010, respectively. The F_1 plants derived were confirmed for their hybridity by MTU1010/ISM (i.e. heterozygosity) using the co-dominant markers, pTA248 (specific for *Xa21*; Ronald et al, 1992) and *xa13*-prom (specific for *xa13*; Sundaram et al, 2011), while *Pi54* gene-specific co-dominant marker, *Pi54*-MAS (Ramkumar et al, 2011), was used for the hybridity by MTU1010/NLR145. The 'true' F_1 plants were backcrossed with MTU1010. BC_1F_1 plants (ISM/MTU1010//MTU1010) were screened with pTA248 and *xa13*-prom markers to identify plants heterozygous for *Xa21* and *xa13*, respectively. Backcross plants of NLR145/MTU1010//MTU1010 were screened with the marker *Pi54*-MAS to identify plants heterozygous for *Pi54*. The primer sequence information is presented in Supplemental Table 1. The positive plants identified from the two BC_1F_1s were then screened with a set of parental polymorphic SSR markers (Supplemental Table 2) to identify the recovery of MTU1010 genome. Marker-assisted backcrossing was done till BC_2 generation, after which the backcross plants (BC_2F_1) derived from the two crosses possessing the maximum recurrent parent genome recovery were intercrossed for pyramiding all the three resistance genes into MTU1010. The intercross F_1s were confirmed for their heterozygosity as described earlier using pTA248, *xa13*-prom and *Pi54*-MAS, and 'true' intercross F_1 with the maximum recurrent parent genome recovery were then selfed to generate intercross F_2 (ICF_2) plants. These were then screened with the three target-gene specific markers to identify 'gene' positive plants in homozygous condition, which were later screened using parental polymorphic SSR markers to identify the 'best' ICF_2 plants. From ICF_3 generation onwards, pedigree-based selection was carried out to identify the best homozygous lines. For marker-assisted selection, DNA was isolated from the parents, backcross plants and intercrossed plants/lines according to Zheng et al (1995). PCR and gel electrophoresis protocols recommended by Sundaram et al (2008) and Ramkumar et al (2011) were adopted for marker-assisted selection of *Xa21*, *xa13* and *Pi54*, respectively, while the background selection protocol recommended by Sundaram et al (2008) was adopted to identify backcross and intercross F_2 plants possessing the maximum recurrent parent genome recovery by Graphical genotype version 2.0 (van Berloo, 2008).

Screening for BB resistance

A virulent isolate of the bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), collected from Rajendranagar farm of Indian Institute of Rice Research, DRR *Xanthomonas* collection-022 (DX-022), was used to screen the donor and recurrent parents along with ICF_2 plants for bacterial blight resistance under both glasshouse and field conditions. In the greenhouse, disease severity is assessed based on lesion length measurement or estimation of diseased leaf area. Due to the large amount of breeding lines assessed in the field, disease severity is usually measured in diseased leaf area. The *Xoo* strain was cultured and stored as described by Laha et al (2009). The rice plants were clip-inoculated with a bacterial suspension of 10^9 cfu/mL at the maximum tillering stage (50 d after transplanting) according to the method of Kauffman et al (1973). Approximately 10 leaves per plant were inoculated, and disease reaction was scored 14 d after inoculation. BB lesion length was measured and the disease score was calculated as per IRRI standard evaluation system (IRRI-SES) scale (IRRI, 1996).

Screening for blast resistance

Two highly virulent *Magnaporthe oryzae* fungal isolates collected from Agriculture Research Station (ARS), Nellore and Andhra Pradesh Rice Research Institute (APRRI), Maruteru, Andhra Pradesh, India were used to screen the donor and recurrent parents along with ICF_3 lines for blast resistance under *in vivo* conditions following uniform blast nursery method at ARS and APRRI. The pathogen strains were cultured and stored as described by Prasad et al (2011). The young seedlings at the four-leaf stage were inoculated with the fungal conidial suspension at a concentration of 1×10^5 cfu/mL, and high relative humidity was maintained for disease development. Inoculated seedlings were monitored for the development of blast lesions one week after inoculation. The plants were scored and evaluated on a 0–9 scale as per IRRI-SES scale (IRRI, 1996).

Evaluation of agro-morphological characters

Thirty-day-old seedlings of the selected ICF_3 lines were transplanted in the main field at a spacing of 20 cm × 15 cm along with the donor and recurrent parents. Standard agronomic practices were followed to raise a healthy crop and the progenies were

evaluated during the Rabi season, 2012–2013. Days to 50% flowering, days to maturity, plant height (cm), number of productive panicles per plant, panicle weight (g), panicle length (cm), grain yield per plant (g), 1000-grain weight (g) and grain type were recorded in three replications and the replicated data was calculated for the mean, coefficient of variation (CV) and critical difference (CD).

RESULTS

Confirmation of marker polymorphism for gene-specific markers and identification of parental polymorphic markers

The DNAs from the recurrent parent MTU1010 and the donor parents B95-1/ISM (for *Xa21* and *xa13*) and NLR145 (for *Pi54*) were used to determine marker polymorphism. The primer pair pTA248 amplified fragments of 900 bp in the resistant parent (ISM), while that from the susceptible parent MTU1010 was 650 bp. With respect to the primer pair, *xa13*-prom, ISM amplified a 500 bp fragment, while MTU1010 amplified a 250 bp. Similarly, with respect to the marker *Pi54*-MAS, a fragment of 210 bp was amplified in NLR145, while a 350 bp fragment was amplified in MTU1010. Thus, all the markers were able to distinguish resistant lines from susceptible ones in a co-dominant fashion.

Parental polymorphism survey was carried out using 617 SSR markers (Supplemental Table 2). Among them, 82 markers showed polymorphism between MTU1010 and ISM, while 83 markers showed polymorphism between MTU1010 and NLR145. Parental polymorphism ranged from 5 markers on chromosome 9 to 12 markers on chromosome 8 (Supplemental Table 2). The average physical distance between each polymorphic marker was 4.1 Mb.

Marker-assisted introgression of BB resistance into MTU1010

F_1s generated from the cross C1 were screened for the presence of *Xa21* and *xa13* using pTA248 and *xa13*-prom to identify the 'true' F_1s showing heterozygous amplification pattern (Table 1). Of 125 F_1s screened, 101 were identified to be true heterozygotes and further used as male parent and backcrossed with MTU1010 to generate BC_1F_1. Out of a total of 293 BC_1F_1 plants generated, 55 were identified to be positive for *Xa21*, 68 were positive for *xa13* and 8 were double positive for both *Xa21* and *xa13*. These heterozygous plants were then subjected for background selection using 82 SSR markers, which were earlier identified to be polymorphic between MTU1010 and ISM. A solitary 'positive' BC_1F_1 plant (C1-BC_1F_1-34) possessing the maximum recovery of recurrent parent (MTU1010) genome (72%) was selected and after that backcrossed with MTU1010 to generate BC_2F_1s. A similar marker-assisted selection procedure was followed for selection of BC_2F_1 (534) plants and a solitary 'positive' BC_2F_1 plant (C1-BC_2F_1-23) possessing the maximum recovery of recurrent parent (MTU10101) genome (90%) was selected and utilized for intercrossing with the selected BC_2F_1 plant from the cross ISM/MTU1010// MTU1010///MTU1010.

Marker-assisted introgression of blast resistance into MTU1010

The F_1s generated from the cross C2 were screened for the presence of the target resistance gene, *Pi54* using the functional marker *Pi54*-MAS to identify the 'true' F_1s showing heterozygous amplification pattern (Table 1). Of 110 F_1s, 74 plants were observed to possess target resistance gene in heterozygous (*Pi54pi54*) condition, which were then used as male parent and backcrossed with MTU1010 to generate

Table 1. Details of 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
MTU1010 × ISM (C1)	C1-F_1	125	101	*Xa13xa13Xa21xa21*
MTU1010 × C1-F_1	C1-BC_1F_1	293	8	*Xa13xa13Xa21xa21*
MTU1010 × C1-BC_1F_1	C1-BC_2F_1	534	11	*Xa13xa13Xa21xa21*
MTU1010 × NLR145 (C2)	C2-F_1	110	74	*Pi54pi54*
MTU1010 × C2-F_1	C2-BC_1F_1	80	35	*Pi54pi54*
MTU1010 × C2-BC_1F_1	C2-BC_2F_1	268	17	*Pi54pi54*
C1-BC_2F_1 × C2-BC_2F_1	ICF_1	360	4	*Xa13xa13Xa21xa21Pi54pi54*
Selfed progeny of selected ICF_1 plant	ICF_2	880	7	*xa13xa13Xa21Xa21Pi54Pi54*
Selfed progeny of ICF_2	ICF_3	–	7	*xa13xa13Xa21Xa21Pi54Pi54*

BC_1F_1 plants. Of the 80 BC_1F_1 plants screened, a total of 35 were identified to be positive (i.e. heterozygous), when screened with *Pi54*-MAS and they were then subjected for background selection using 83 parental polymorphic SSR markers. A single 'positive' BC_1F_1 plant (C2-BC_1F_1-17) possessing the maximum recovery of recurrent parent genome (79%) was selected and then backcrossed with MTU1010 to generate BC_2F_1 plants. A similar marker-assisted selection procedure was followed for selection of BC_2F_1 (268) plants, wherein a single 'positive' BC_2F_1 plant (C2-BC_2F_1-4) possessing 92% recurrent parent genome was selected and intercrossed with the best backcross plant generated from the cross NLR/MTU1010//MTU1010///MTU1010.

Marker-assisted introgression of BB and blast resistance genes into MTU1010 through intercrossing

C1-BC_2F_1-23 possessing *Xa21xa21Xa13xa13* was used as a female parent and crossed with C2-BC_2F_1-4 possessing *Pi54* in heterozygous condition, and a set of 360 ICF_1 seeds were generated (Table 1). A total of four such 'triple heterozygous positive' ICF_1 plants (*Xa13xa13Xa21xa21Pi54pi54*) were identified and then screened with parental polymorphic SSR markers. A single ICF_1 plant (ICF_1-16), which possessed the maximum-percentage of recurrent parent genome recovery (90%) was identified and 2216 ICF_2 seeds were produced. They were then grown under field conditions at Professor Jayashankar Telangana State Agricultural University, Hyderabad, India, during wet season in 2012. A total of 880 ICF_2 plants were genotyped and 7 plants possessing all the three target resistance genes in homozygous condition (*xa13xa13 Xa21Xa21Pi54Pi54*) (Fig. 1) were identified.

Background genome analysis of backcross derived BB and blast resistant lines of MTU1010

Background analysis was carried out among the seven three-gene homozygous ICF_2 plants using the polymorphic SSR markers by GGT2 or Graphical

Fig. 1. Foreground selection for *xa13* (A), *Xa21* (B) and *Pi54* (C) among ICF_2 plants.
R, Recurrent parent (MTU1010); D, Donor parent (ISM); M, 50 bp ladder molecular weight marker; Lanes 9–104 represent ICF_2 plants. Arrow indicates a triple gene homozygous plant (ICF_2-16-59).

Genotyper software (van Berloo, 2008). The analysis revealed an average recovery of 87% of MTU1010 genome, with a residual heterozygosity of 5.95%. Four plants had a recovery of more than 85% of MTU1010 genome. Chromosome-wise analysis of the background showed complete recovery of chromosomes 3, 5, 6, 7, 9 and 10 from MTU1010 genome in all the recombinants. A single ICF_2-16-59 showed the highest recovery of MTU1010 genome (92%), while two ICF_2 plants, ICF_2-16-235 and ICF_2-16-521 plants scored the lowest recurrent genome recovery (82%). A small genomic region spanning 1.0 Mb around *xa13* on chromosome 8 and 3.5 Mb region around *Xa21* and *Pi54* on chromosome 11, to be introgressed from the donor parent. Interestingly, ICF_2-16-59 inherited chromosomes 1, 3, 5, 6, 7, 9 and 10 completely from the recurrent parent, and possessed only short segments from donor parent in the telomeric end of chromosomes 2, 4, 8 and 12, and relatively longer donor segments from the long arm of chromosome 11 (Supplemental Fig. 2).

Screening of ICF_2 lines for bacterial blight resistance

All the three-gene positive ICF_2 plants (those possessing *Xa21*, *xa13* and *Pi54* in homozygous condition) were screened for BB resistance. The donor genotype ISM showed an average lesion length of 0.77 cm with disease scoring scale value '1', while the recurrent parent MTU1010 possessed an average lesion length of 12.23 cm (90% diseased leaf area) with disease scoring scale of '9'. All the three-gene pyramid ICF_2 plants showed highly resistant against the disease with a lesion length of only 0.53–2.28 cm and 1%–5% diseased leaf area with disease scoring values of '1' or '3' (Fig. 2 and Table 2).

Screening of ICF_3 families for blast resistance

The seven ICF_2 plants possessing the three resistance genes in homozygous condition were selfed to generate ICF_3. ICF_3 lines were then screened during dry seasons in 2012 and 2013 for blast resistance under uniform blast nursery at APRRI and ARS, which are blast epidemic areas, along with the resistant parent NLR145, the susceptible parent MTU1010 and the susceptible check NLR34242. NLR145 showed high level of resistance against blast, with an average disease score of '3' and MTU1010

Fig. 2. Screening of selected improved lines of MTU1010 against bacterial blight (A) and blast (B) diseases under controlled conditions.

A, With respect to screening for bacterial blight resistance, the recurrent parent MTU1010 was highly susceptible, while the donor parent and the selected gene pyramided lines at ICF_2 generation (1, ICF_2-16-59; 2, ICF_2-16-231; 3, ICF_2-16-235; 4, ICF_2-16-282; 5, ICF_2-16-521; 6, ICF_2-16-786; 7, ICF_2-16-837) were highly resistant to the disease. B, When the selected ICF_3 plants were screened for blast resistance through uniform blast nursery method, the susceptible check NLR34242 and recurrent parent MTU1010 were highly susceptible to blast disease, while the resistant donor NLR145 along with gene pyramided line ICF3-16-59 showed high level of resistance.

Table 2. Screening of three-gene positive ICF_2 and ICF_3 plants for resistance against bacterial blight (BB) disease and blast disease.

Plant identity	Allelic status of *xa13*, *Xa21* and *Pi54*	Disease scoring scale for BB in ICF_2	Disease scoring scale for rice blast in ICF_3	Background genome recovery (%)
MTU1010	*Xa13Xa13xa21xa21pi54pi54*	9	7	–
Improved Samba Mahsuri	*xa13xa13Xa21Xa21*	1	–	–
NLR145	*Pi54Pi54*	–	3	–
$ICF_{2:3}$-16-59	*xa13xa13Xa21Xa21Pi54Pi54*	1	1	92
$ICF_{2:3}$-16-231	*xa13xa13Xa21Xa21Pi54Pi54*	1	2	85
$ICF_{2:3}$-16-235	*xa13xa13Xa21Xa21Pi54Pi54*	1	2	82
$ICF_{2:3}$-16-282	*xa13xa13Xa21Xa21Pi54Pi54*	1	2	83
$ICF_{2:3}$-16-521	*xa13xa13Xa21Xa21Pi54Pi54*	1	1	82
$ICF_{2:3}$-16-786	*xa13xa13Xa21Xa21Pi54Pi54*	3	2	83
$ICF_{2:3}$-16-837	*xa13xa13Xa21Xa21Pi54Pi54*	1	2	88

showed presence of disease lesions in more than 50% leaf area with an average disease scoring scale '7' and NLR34242 showed presence of disease lesions in more than 75% leaf area with an average disease scoring scale '9'. Most of the improved rice breeding lines derived from homozygous ICF_2 lines displayed a high level of resistance for rice blast. ICF_3-16-59 and ICF_3-16-521 showed small brown specks of pinhead size without sporulating center on the leaves and with a disease scoring scale of '1' similar to NLR145 (Fig. 2 and Table 2). The remaining lines showed small roundish to slightly elongated necrotic grey spots, which were about 1–2 mm in diameter with a distinct brown margin, with lesions mostly found on the lower leaves with a disease score of '2' and categorized as resistant.

Agro-morphological evaluation of seven ICF_3 lines

Seven ICF_3 lines were evaluated for yield and agro-morphological characters during dry seasons in 2012 and 2013 at APRRI (Table 3). ICF_3-16-59, ICF_3-16-521 and ICF_3-16-235 were identified to be promising based on their high level of resistance to both blast and BB, long-slender grain type, high yield, and were further evaluated in wet season, 2014. ICF_3-16-59 and ICF_3-16-521 exhibited grain yield on par with MTU1010 (0.62 kg/m^2) with marginal differences. No significant variation was observed among the plants of the two elite lines with respect to the number of productive panicles per plant, panicle weight, panicle length and grain yield per plant when compared with MTU1010. Interestingly, ICF_3-16-235 exhibited higher yield (0.64 kg/m^2) as compared to MTU1010.

DISCUSSION

In addition to the use of markers, we also deployed phenotypic selection for agro-morphological traits in the later generations to identify backcross resistant plants which are not only the closest to MTU1010, but also better than the elite mega-variety. ISM whose cultivation area is steadily increasing due to its BB resistance and fine-grain type (Sundaram et al, 2014) and NLR145 possessing *Pi54* were used as the donor

Table 3. Mean values of agro-morphological characters of three resistant gene pyramided ICF_3 lines.

Line	Days to heading (d)	Plant height (cm)	No. of productive panicles per plant	Panicle length (cm)	No. of filled grains per panicle	Grain yield per plant (g)	1000-grain weight (g)	Grain type
ICF_3-16-59	87.3	106.0	15.7	31.3*	118.3	24.0	16.3	LS
ICF_3-16-231	83.6	104.0	17.0	30.3*	111.7	24.7	16.5	LS
ICF_3-16-235	86.0	105.7	18.7	29.0*	130.0*	27.0*	17.1	LS
ICF_3-16-282	85.3	107.3	17.7	28.7	114.7	24.3	16.5	LS
ICF_3-16-521	85.7	106.3	14.7	27.7	110.3	24.0	16.4	LS
ICF_3-16-786	87.7	107.0	13.3	28.0	101.7	23.3	16.8	LS
ICF_3-16-837	88.0	103.7	16.7	26.7	108.0	23.7	16.7	LS
MTU1010	86.7	107.3	16.7	26.0	117.7	23.7	16.4	LS
SD	1.4	1.4	1.7	1.6	8.4	1.1	0.3	
CV (%)	1.7	1.3	10.4	6.2	7.4	4.4	1.6	
CD (5%)	1.4	2.7	2.0	2.0	2.6	1.0	0.1	

SD, Standard deviation; *CV*, Coefficient of variation; *CD*, Critical difference; LS, Long slender grain type.
*, Values significantly different from MTU1010 at the 0.05 level.

parents for bacterial blight and blast resistances, respectively. Though the donor parent, ISM contained three BB resistance genes (*xa5*, *xa13* and *Xa21*), we attempted to transfer only two (*xa13* and *Xa21*) of the three genes into MTU1010 since *xa5* is known to display partial dominance and additivity to the avirulent races and possesses only relatively small but significant residual effects (Li et al, 2001). Further, *xa5* is also known to confer only moderate resistance to Indian races (Li et al, 2001) and has been reported to display negative effects in gene pyramid lines of the Indian rice variety Triguna (Sundaram et al, 2009). In contrast, *xa13*, is completely recessive, has no residual effects against the virulent races, and is known to show more pronounced race specificity and considered effective against many Indian races of *Xoo*. Similarly, *Xa21* shows complete dominance against the avirulent *Xoo* races and has large residual effects against virulent ones and acts independently and cumulatively along with other genes (Li et al, 2001) and has a mode of action which is distinct from *xa13* (Sundaram et al, 2008). NLR145 was used as the donor for *Pi54* instead of Tetep, the original source of *Pi54*. This is because Tetep is a tall, very low-yielding Vietnamese landrace and has partially photosensitivity and hence may lead to linkage drag even if a small donor segment is introgressed into MTU1010, while NLR145 is a popular, high yielding variety with many desirable agromorphological features.

Three PCR-based markers *xa13*-prom, pTA248 and *Pi54*-MAS were able to identify the triple-positive (*xa13*, *Xa21* and *Pi54*) plants precisely without any false positives as they are located within *xa13*, *Xa21* and *Pi54* (Ronald et al, 1992; Ramkumar et al, 2011; Sundaram et al, 2011), respectively. Hence, we can confidently use these markers for MAS without deploying phenotype-based selection for both BB and blast resistance. In addition to markers for foreground selection, polymorphic SSR markers were utilized for background selection in order to recover the recurrent parent genome in the shortest number of backcross generations as recommended by Hospital and Charcosset (1997) and also for estimation of the recurrent parent genome contribution at each generation of backcrossing. Sundaram et al (2008) concluded that background selection with a limited number of polymorphic SSR markers (approximately 50), in conjunction with four backcrosses is sufficient to recover the yield and quality characteristics of the recurrent parent while introgressing the target trait. While introgressing BB resistance genes into Pusa Basmati-1 genetic background through marker-assisted backcross breeding, Gopalakrishnan et al (2008) used 69 polymorphic SSR markers for assessment of the recovery of the recurrent parent genome and the individual selected at BC_1F_5, named Pusa 1460-01-32-6-7-67 (possessing *xa13* and *Xa21*), possesses a 86.9% recovery of Pusa Basmati-1 genome. In this study, we limited the number of backcrosses to only two and deployed a maximum 109 parental polymorphic SSR markers (polymorphic between MTU1010 and the donor parents) for accelerating background genome recovery, and by the second backcross generation, we managed to identify plants possessing more than 90% recovery of MTU1010 genome. Significantly, while carrying out background selection, we gave special emphasis deploying many polymorphic SSR markers on chromosomes 8 and 11, on which target genes are located. This is because, the carrier chromosomes deserve special consideration in backcross programs, as they have greater selection pressure for the donor parent allele at the target gene (*xa13*, *Xa21* and *Pi54*) in each backcross generation, with the rate of return to recipient genotype on target chromosomes slower than on non-carrier chromosomes (Hospital, 2001). Hence, we deployed 12 polymorphic markers with respect to chromosome 8, while 7 polymorphic markers were deployed with respect to chromosome 11 for background selection. Significantly, at least two of these polymorphic markers each on chromosomes 8 and 11 were flanking *xa13*, *Xa21* and *Pi54*, respectively, and we were able to select backcross plants possessing the recurrent parent allele with respect to the polymorphic SSR markers by the second backcross generation. We identified an elite intercross plant ICF_2-16-59 in which a maximum percent of recurrent parent genome was recovered (92%). Further, in the selected plants, the donor genome segment was observed to be limited to only 1.0 Mb in the vicinity of *xa13* and 3.5 Mb in the vicinity of *Xa21* and *Pi54*. The recovery of recombination event in the interval as close as 0.6 Mb observed in the *xa13* flanking region in two of the selections also revealed that there exists a possibility of reducing the linked genomic regions to even lesser degree.

All the lines derived from homozygous ICF_2 plants displayed a high level of resistance to both blast and BB diseases. Particularly, ICF_2-16-59, ICF_2-16-231, ICF_2-16-235, ICF_2-16-282, ICF_2-16-521, ICF_2-16-786 and ICF_2-16-837 displayed a high level of blast resistance. With respect to bacterial blight, the level of

BB resistance in the improved versions of MTU1010 was observed to be higher as compared to MTU1010 (lesion length ranging from 22.23 cm ± 0.26 cm) in all the seven selected improved versions of MTU1010. Most importantly, the yield levels of the seven lines were not significantly different from that of the parent MTU1010 (Table 3), indicating that there is no yield penalty associated with the presence of the resistance genes. Similar observation was noticed by Shanti et al (2010) and Sundaram et al (2009). ICF_3-16-59 (*xa13xa13Xa21Xa21Pi54Pi54*) exhibited yield and yield related characters on par with MTU1010 with maximum amount of recurrent parent genome recovery, carrying donor segment of 1.0 Mb on chromosome 8 and 3.5 Mb donor segment on chromosome 11 and also showed high resistances to BB and blast. These lines along with other lines are being advanced for further evaluation in multi-locations.

Interestingly, ICF_3-16-235 (*xa13xa13Xa21Xa21Pi54 Pi54*) recorded significant superiority over MTU1010 with respect to number of filled grains, panicle length and grain yield per plant, with plant height and duration being similar to the recurrent parent. ICF_3-16-235, hence, can be considered as a transgressive segregant for some of the yield and yield-related traits. This was possible, because, while selecting strictly for traits similar to that of MTU1010 in the early backcross generations, in the later generations, starting from ICF_2 generation, we also selected for backcross derived lines which were better than MTU1010, through a stringent phenotype-based selection under field conditions.

In this study, no positive or negative interactions were observed between genes conferring resistance against bacterial blight and blast. Similar results were found by Sundaram et al (2008), Hari et al (2013) and Balachiranjeevi et al (2015). Further, negative effects due to presence of the genes were not observed in any of the three-gene pyramid lines, since both the two donors were improved varieties with desirable agro-morphological and grain quality characters. Through deployment of phenotype-based selection coupled with marker-assisted selection, we were able to recover the desirable plant type and grain type in the improved version of MTU1010. The improved versions of MTU1010 (ICF_3-16-59, ICF_3-16-231, ICF_3-16-282, ICF_3-16-521, ICF_3-16-786, ICF_3-16-837 and ICF_3-16-235) can be expected to replace MTU1010 (after their stringent evaluation through multi-location testing).

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SUPPLEMENTAL DATA

The following materials are available in the online version of this article at http://www.sciencedirect.com/science/journal/16726308; http://www.ricescience.org.

Supplemental Fig. 1. Schematic illustration of crossing programme for marker assisted introgression of *xa13*, *Xa21* and *Pi54* into MTU1010.

Supplemental Fig. 2. Representation of genomic constitution of the elite bacterial blight and blast resistant backcross derived line of MTU1010, ICF_2-16-59 through graphical genotyping based on analysis with parental polymoprhic simple sequence repeat marker.

Supplemental Table 1. Sequence of primer pairs used for foreground selection.

Supplemental Table 2. List of markers and their polymorphism between parents.

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