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Marker-assisted pyramiding of bacterial blight and gall midge resistance genes into RPHR-1005, the restorer line of the popular rice hybrid DRRH-3

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Abstract Bacterial blight (BB) of rice caused by the pathogen *Xanthomonas oryzae* pv. *oryzae* and the insect gall midge (GM) (*Orseolia oryzae*) are two major constraints of rice production. The present study was carried out to improve RPHR-1005, a stable restorer line of the fine-grain-type rice hybrid DRRH-3, for BB and GM resistance through marker-assisted backcross breeding (MABB). Two major GM resistance genes, *Gm4* and *Gm8*, and a major BB resistance gene, *Xa21*, were selected as target genes for transfer to RPHR-1005. Two sets of backcrosses were carried out to combine either *Xa21* + *Gm4* or *Xa21*+ *Gm8* into RPHR-1005 using breeding lines in the genetic background of ISM possessing either *Gm4* or *Gm8* along with *Xa21*. Foreground selection was performed for *Xa21*, *Gm4*, *Gm8*, and the major fertility restorer genes *Rf3* and *Rf4* using gene-specific markers, while 61 polymorphic simple sequence repeat (SSR) markers were used for background selection and marker-assisted backcrossing was continued until BC_2 generation. A promising homozygous backcross-derived plant at the BC_2F_2 generation possessing *Xa21* + *Gm4*, and another possessing *Xa21* + *Gm8*, were intercrossed to stack the target resistance genes. At ICF_4 (inter-crossed F_4) , three promising lines possessing the three target resistance genes in a homozygous condition along with fine-grain type, complete fertility restoration, and better panicle exsertion than RPHR-1005 have been identified. Among these, a single line, # RPIC-16-65-125, showed better yield, was highly resistant to BB and GM, was of medium–slender grain type, and had complete fertility restoration along with better panicle exsertion and taller plant type than RPHR-1005. This is the first report of combining resistance against BB and GM in the genetic background of a hybrid rice parental line.

Keywords *Oryza sativa* (L.) · Bacterial blight resistance (*Xa21*) · Gall midge resistance (*Gm4* and *Gm8*) · Fertility restorers (*Rf3* and *Rf4*) · Marker-assisted backcross breeding (MABB)

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Introduction

Hybrid rice cultivation is one of the proven technologies for increasing rice production and productivity. Through good management, a yield advantage of 1.0–1.5 t/ha can be obtained by cultivation of hybrids compared with the inbred varieties. India is the second country (after China) to adopt hybrid rice technology and presently ~2.5 million ha are under hybrid rice cultivation (Abhilash et al. 2016a, b). One of the major problems encountered in hybrid rice cultivation is the susceptibility of many of the popular hybrids to various pests and diseases. For stable performance of hybrids across various locations, it is necessary that they possess resistance/tolerance to major biotic stresses such as bacterial blight (BB), blast, gall midge (GM), stem borer, brown plant hopper (BPH), and white-backed plant hopper (WBPH).

Among the 75 rice hybrids released so far in India, DRRH-3, which was developed and released by the ICAR-Indian Institute of Rice Research (ICAR-IIRR), Hyderabad, India, is the first that possesses the highly preferred, medium-slender, fine-grain type. The hybrid is becoming increasingly popular and has been recommended for cultivation in states such as Andhra Pradesh, Madhya Pradesh, Orissa, Uttar Pradesh, and Gujarat by the Government of India as a national/central release. The grain quality traits of the hybrid are similar to those of the very popular inbred variety Samba Mahsuri (BPT-5204), which has premium grain and cooking quality features. DRRH-3 has high yield (5776 kg/ha), milling (>71%), and head rice recovery (>60%), a desirable length/breadth (L/B) ratio (2.61), intermediate amylose content (24%), gel consistency of 63 mm, strong culm, superior performance even under lower levels of nitrogen (40 kg N/ha), indicating its higher nitrogen use efficiency, and produces about 23–30% more yield than BPT-5204 with comparable quality features. However, despite its popularity, DRRH-3 and its parental lines APMS6A (female parent) and RPHR-1005 (male parent) are highly susceptible to diseases such as BB and blast and pests such as GM and BPH, which limit its adoption and widespread cultivation (Viraktamath et al. 2010). Hence, it is desirable to incorporate at least one or more genes conferring resistance against major pests and diseases of rice in the restorer parent of the elite hybrid, so that not only DRRH-3 but also any other hybrid developed using improved versions of RPHR-1005 can be resistant to various biotic stresses such as BB and GM.

Improving host plant resistance is considered to be one of the best eco-friendly and sustainable strategies to tackle various biotic stresses affecting rice. There is a need to develop rice cultivars and hybrids possessing resistance/tolerance against multiple biotic stresses. Breeding for multiple pest/disease resistance is not a new concept, but the advent of reliable polymerase chain reaction (PCR)-based markers has facilitated rapid and easier combining of genes (i.e., gene pyramiding) through marker aided selection (MAS).

BB caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a serious threat to rice crops in irrigated and rain-fed areas of the world (Mew 1987). Numerous studies have been carried out regarding the diagnosis, management, and control of the disease. Enhancement of genetic resistance in rice has proven to be the most effective method for controlling the disease (Khan et al. 2014). To date, at least 40 BB resistance genes (both dominant and recessive) have been identified (Bhasin et al. 2012; Natrajkumar et al. 2012) and they have been designated in a series from *Xa1* to *Xa40* (Yang et al. 1998; Sun et al. 2003; Gu et al. 2005; Cheema et al. 2008; Kim et al. 2015). Of these, *Xa21*, a major resistance gene originally introgressed from *Oryza longistaminata*, was observed to confer resistance to most Indian isolates of the bacterial pathogen, and a highly efficient PCR-based marker called pTA248, developed by Ronald et al. (1992), is available for marker-assisted selection of *Xa21*. The gene has also been reported to confer durable resistance to the pathogen across many parts of the world, including India (Sundaram et al. 2014).

Rice GM (*Orseolia oryzae*) is a serious insect pest prevalent in India, China, and South-East Asia, while a closely related species, *Orseolia oryzivora*, is prevalent in Africa. In India, GM infestation is most prevalent in Madhya Pradesh, Chhattisgarh, Bihar, Jharkhand, Orissa, Andhra Pradesh, Maharashtra, Kerala, and the north-eastern states (Bentur et al. 2003). Breeding and cultivation of resistant varieties has been a viable and ecologically acceptable approach for management of the pest (Bentur et al. 2003). To date, seven distinct biotypes of GM (GMB1 through to GMB6, and GMB4M) and 11 non-allelic GM resistance genes (*Gm1* through to *Gm11*) have been reported (Vijayalaxmi et al. 2006; Himabindu et al. 2010). Eight of the 11 resistance genes (*Gm1*, *Gm2*, *gm3*, *Gm4*, *Gm6*, *Gm7*, *Gm8*, and *Gm11*) have been mapped (Yasala et al. 2012; Sama et al. 2014). *Gm5* has been tagged but not mapped. Resistance genes show two distinct types of resistance

mechanism. The *Gm2*, *gm3*, *Gm4*, *Gm5*, *Gm6*, *Gm7*, and *Gm11* genes confer hypersensitive reaction-associated (HR+ type) resistance wherein host cell death occurs at the site of insect attack. Interestingly, two genes, *Gm1* and *Gm8*, show hypersensitive reaction-independent (HR– type) resistance with no cell death at the site of infestation in the host (Bentur et al. 2003). None of the genes show resistance against all the seven biotypes (Bentur et al. 2011), and it will be desirable to pyramid two or more genes, possessing divergent mechanisms of resistance in the genetic background of elite varieties and parental lines through MAS, so that the spectrum and durability of resistance can be enhanced. The resistance gene *Gm4* identified in the Abhaya cultivar (Srivastava et al. 1993) has been tagged and mapped using random amplified polymorphic DNA (RAPD) (Nair et al. 1996) and restriction fragment length polymorphism (RFLP) (Mohan et al. 1997) markers on chromosome 8. Recently, a gene encoding a leucine-rich repeat (LRR) domain containing protein was identified to be candidate for the gene and a functional marker, LRR-del was developed for the detection of the gene (Divya et al. 2015a). *Gm4* confers resistance against GM biotypes 1, 2, 3, 4, and 4 M and displays an HR+ type of resistance mechanism. Similarly, another resistance gene, *Gm8*, present in the cultivar Aganni possesses HR– type of resistance and confers resistance to GM biotypes 1, 2, 3, 4, and 4 M (Bentur et al. 2011). *Gm8* has been tagged and fine-mapped within a 0.43 Mb region on chromosome 8 of rice (Sama et al. 2012). A functional marker, PRP, has been developed for *Gm8* and validated (Divya et al. 2013). Based on these points, the present study was conceptualized and carried out with an objective to introgress dominant resistance genes conferring resistance against BB (i.e., *Xa21*) and GM (i.e., *Gm4* and *Gm8*) into the genetic background of RPHR-1005 through a marker-assisted backcross breeding (MABB) strategy.

Material and methods

Plant material and crossing scheme

Two introgression lines in the genetic background of Improved Samba Mahsuri (ISM)—SM1, possessing *Xa21* + *Gm4* (Sama et al. 2012), and SM2, possessing *Xa21* + *Gm8* (Himabindu 2009) with a medium slender (MS) grain type—derived from the crosses of ISM possessing BB resistance gene *Xa21* with Abhaya (possessing *Gm4*) and Aganni (possessing *Gm8*), respectively, were used as donors. *Gm4* confers HR+ type resistance (Srivastava et al. 1993), while *Gm8* confers HR– type resistance (Kumar et al. 2000). Two separate crosses were made: (i) RPHR-1005 X SM1 (Cross I); and (ii) RPHR-1005 X SM2 (Cross II). Hybridity of the F_1s derived from the two crosses was confirmed with gene-specific markers and the 'true' F_1s were crossed with RPHR-1005 to generate BC_1F_1s. They were then grown and screened with the gene-specific markers to identify the BC_1F_1 plants which carry the gene combinations *Xa21* + *Gm4* (i.e., from Cross I) and *Xa21* + *Gm8* (i.e., from Cross II) in a heterozygous condition and the presence of fertility-linked alleles with respect to the major fertility restorer genes *Rf3* and *Rf4* using the gene-specific markers DRRM-RF3-10 and DRCG-RF4-14, respectively (Balaji et al. 2012). As plants homozygous for both *Rf4* and *Rf3* were selected at BC_1F_1 generation from both the crosses, no further selection was carried out in subsequent generations for the two fertility restorer genes. A single positive BC_1F_1 plant each from Cross I and Cross II that resembled the recurrent parent based on morphological features was then backcrossed with RPHR-1005 to generate BC_2F_1s. Marker-assisted identification of 'positive' plants was continued at BC_2F_1 as described earlier and a solitary BC_2F_1 plant from each of Cross I and Cross II were selfed to generate BC_2F_2. Plants homozygous for *Xa21* + *Gm4* (from Cross I) and *Xa21* + *Gm8* (from Cross II) were identified with the help of gene-specific markers and the positive, homozygous plants were then screened with a set of polymorphic simple sequence repeat (SSR) markers to assess the extent of recovery of recurrent parent genome (RPG). A solitary BC_2F_2 plant from each cross, possessing maximum recovery of RPG, was identified from Cross I and Cross II and then intercrossed to combine all the three genes, viz., *Xa21*, *Gm4*, and *Gm8*, in the genetic background of RPHR-1005. The presence of the three resistance genes in the ICF_1s (inter-crossed F_1) was confirmed using gene-specific markers and they were then selfed to generate ICF_2s. Among these, those that carried the gene combination *Xa21* + *Gm4* + *Gm8* were identified with the help of markers, and promising homozygous ICF_2 plants possessing maximum contribution of RPG (using polymorphic SSR markers) were identified and advanced in further generations, with selection at ICF_4 for resistance against BB and GM and also for key agro-morphological traits. The

homozygous BC_2F_2 plants were also selfed and forwarded further to BC_2F_6 generation through the pedigree method of selection. The methodology of MABB adopted in the study is depicted in Fig. 1.

Polymerase chain reaction for foreground and background selection

Mini-scale DNA isolation of parents and backcross and intercross derived lines was carried out from 25-day-old seedlings following the procedure of Zheng et al. (1995). The PCR protocols adopted for marker-assisted selection of *Xa21*, *Gm4*, *Gm8*, *Rf3*, and *Rf4* were those described by the studies in Table 1. PCR was performed using 1 U of Taq DNA polymerase (Bangalore Genei, Bangalore, India) and 1X PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, 1.8 mM MgCl, and 0.01 mg/ml gelatin), 5 picomoles of each primer, 0.05 mM dNTPs, and 50 ng template DNA in 25 μl reaction volume with a thermal profile of 94 °C for 5 min (initial denaturation), followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and a final extension of 7 min at 72 °C. Products were resolved in a 1.5% Agarose gel and the gel images were documented in an Alpha Imager gel documentation system (Alpha Innotech Inc., San Leandro, CA, USA). At ICF_2, the extent of recovery of the RPG was assessed using a set of 61 polymorphic SSR markers using the procedure described in Sundaram et al. (2008). Using the data from polymorphic SSR markers, a schematic map illustrating the genomic contribution of donor and recurrent parents at ICF_2 was prepared using Graphical Genotype (GGT) Version 2.0. (Van Berloo 1999).

Screening for resistance against bacterial blight (BB)

The recurrent parent, RPHR-1005, three selected ICF_4 lines of improved RPHR-1005, along with TN1 (the susceptible check), Abhaya (possessing *Gm4*), Aganni (possessing *Gm8*), and ISM (resistant check) were screened for their resistance against BB disease through the artificial clip inoculation method (Kauffman et al. 1973) under glasshouse conditions during the 2015 wet season at ICAR-IIRR. DX-020, a virulent isolate of *Xanthomonas oryzae* pv. *oryzae* collected from

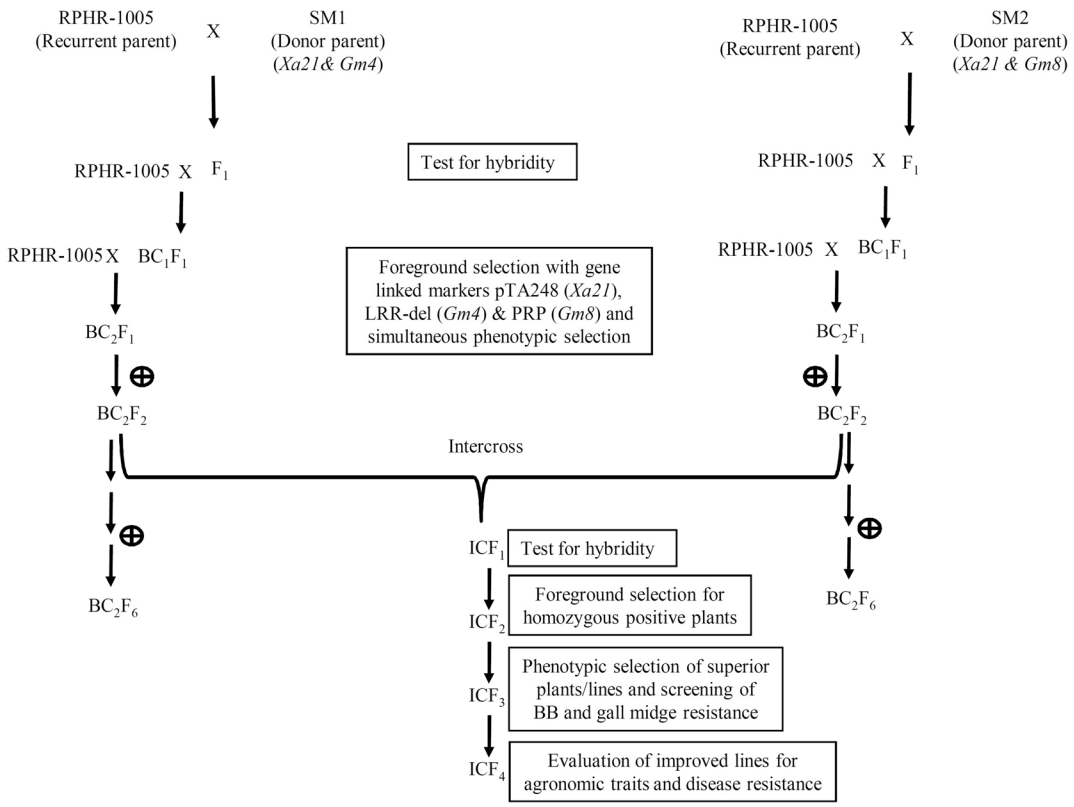

Fig. 1 Scheme for the development of *Xa21*, *Gm4*, and *Gm8* resistance genes into RPHR-1005

Table 1 Markers used in the foreground selection (*Xa21*, *Gm4*, *Gm8*, *Rf3* and *Rf4*) and their sequence information

Gene	Chromosome no.	Primer name	Primer sequence	Reference
Xa21	11	pTA248F pTA248R	AGACGCGGAAGGGTGGTTCCCGGA AGACGCGGTAATCGAAAGATGAAA	Ronald et al. 1992
Gm4	8	LRR-del F LRR-del R	GTGGATCGAGAGAAGACAAG CTTGAGGACGATATTCAAGC	Divya et al. 2015a
Gm8	8	PRP F PRP R	TCATGTTGTGCAGATCAACC AGCCATATGAAAACCACCAA	Divya et al. 2013
Rf3	1	DRRM-Rf3–10- F DRRM-Rf3–10- R	GCAATGCTTGTATTCAGCAAA TCCAGCTGTAAATCCGTCAA	Balaji et al. 2012
Rf4	10	DRCG-RF4–14-F DRCG-RF4–14-R	TCACCTCTTCCTGCTTCGAC CTCCACCAGTGCAGGTTTTT	

Hyderabad (Andhra Pradesh, India) was cultured and maintained as explained in Laha et al. (2009) and used for inoculation of the rice lines as described in Sundaram et al. (2009). The inoculated plants were scored by the IRRI-SES (International Rice Research Institute–Standard Evaluation System) scale (IRRI 1996) 15 days after inoculation.

Screening for resistance against gall midge

Plants of test entries along with those of the standard resistant check varieties, i.e., Abhaya (possessing *Gm4*), Aganni (possessing *Gm8*), and the susceptible checks, TN1 and ISM, were screened against biotype 1 of GM in the screening facility (i.e., glasshouse) at ICAR-IIRR by planting one row of 20 hills per variety/culture. Fertilizers were applied according to local recommended practice for obtaining optimum yield at each location. At 30 and 50 days after transplanting (DAT), all plants were observed to identify the number of GM damaged/infested plants. Entries were scored for reaction in terms of percentage plant damage in two replications. The test was considered valid if the susceptible checks showed more than 80% plant damage. Test entries with 0–10% plant damage (i.e., presence of galls) were considered resistant, while those with >80% plant damage were considered susceptible.

Evaluation of agro-morphological characters

Thirty-day-old seedlings of selected ICF_4 lines were transplanted in the experimental field of ICAR-IIRR during the 2015 wet season along with the donor and recurrent parents. Standard agronomic practices were followed as recommended in Hari et al. (2013). The following agronomic traits were recorded, as described in Abhilash et al. (2016a, b) and Balachiranjeevi et al. (2015), in three replications and five plants per replication: days to 50% flowering (DFF), mean days to maturity, mean plant height (cm), number of productive tillers per plant, panicle weight (g), standard heterosis for grain yield (%), panicle length (cm), grain yield per plant (g), 1000-grain weight (g), and grain type. The data were tabulated and analyzed statistically for various agro-morphological traits with the help of standard techniques as per Gomez and Gomez (1984). Coefficient of variation (CV) and least significant Difference (LSD) values were calculated using standard errors of mean (SEM) at a 5% level of significance using the MS Excel® package (Micosoft Corp., Richmond, WA, USA). Statistical analysis was performed with SAS® version 9.2 (SAS Institute Inc., Cary, NC, USA). The PROC GLM procedure of SAS® was used to conduct analysis of variance (ANOVA) to determine the significant variation between the lines.

Results

Marker-assisted introgression of *Xa21* and *Gm4* into RPHR-1005

RPHR-1005 was initially crossed with SM1 and 'true' F_1 plants were identified with the help of *Xa21* specific co-dominant marker, pTA248 (Ronald et al. 1992) and *Gm4*-specific functional co-dominant marker LRR-del (Divya et al. 2015a). They were then backcrossed with RPHR-1005. Foreground analysis of 314 BC_1F_1 plants

with gene-specific markers revealed that 19 plants were heterozygous for both of the target genes (i.e., *Xa21* and *Gm4*). Among these, four were identified to be homozygous for *Rf3* and *Rf4* through marker analysis (i.e., positive for the target resistance genes and fertility restorer genes). As plants homozygous for both *Rf4* and *Rf3* were selected at BC_1F_1 generation, no further selection was carried out in subsequent generations for the two fertility restorer genes. A solitary BC_1F_1 plant (# RPBC-74) was identified through background selection as possessing maximum RPG (~73.7%). Foreground selection among 234 BC_2F_1 plants revealed a total of 14 plants possessing *Xa21* and *Gm4* in heterozygous condition. A single BC_2F_1 (# RPBC-74-121) plant with maximum RPG (~85.2%) was then identified through background selection. Marker-assisted screening of 582 BC_2F_2s helped in identification of 36 double-positive homozygous plants (i.e., positive for *Xa21* + *Gm4*). Among these, a single plant (# RPBC-74-121-201) with maximum RPG (93.4%) was identified through background selection and used for intercrossing.

Marker-assisted introgression of *Xa21* and *Gm8* into RPHR-1005

RPHR-1005 was initially crossed with SM2 and 'true' F_1 plants were identified with the help of gene-specific co-dominant markers as described earlier and then backcrossed with RPHR-1005. A total of 22 BC_1F_1s were identified to be heterozygous for both the target genes (i.e., *Xa21* and *Gm8*) after screening 365 BC_1F_1 plants. Five of these were identified to be homozygous for *Rf3* and *Rf4* through marker analysis. As plants homozygous for both *Rf4* and *Rf3* were selected at BC_1F_1 generation, no further selection was carried out in subsequent generations for the two fertility restorer genes. Among these, a solitary BC_1F_1 plant (# RPBC-18) was identified through background selection to possess maximum RPG (~76%). Foreground selection among 293 BC_2F_1 plants revealed a total of 18 plants with *Xa21* and *Gm8* in heterozygous condition. A single BC_2F_1 (# RPBC-18-94) plant with maximum RPG (~89%) was then identified through background selection and then selfed. Marker-assisted screening of 608 BC_2F_2s resulted in identification of 38 double-positive homozygous plants (i.e., positive for *Xa21* and *Gm8*). Among these, a single plant (# RPBC-18-94-138) possessing maximum RPG (94.5%) was identified through background selection.

Pyramiding of BB and gall midge resistance genes into RPHR-1005

A solitary homozygous plant identified from each of the two crosses (viz., plant # RPBC-74-121-201Cross I and plant # RPBC-18-94-138Cross II) that possessed maximum RPG recovery were intercrossed. Forty-five 'true' heterozygous ICF_1 plants (Fig. 2) were identified among 56 plants screened. Of 856 ICF_2 plants, 53 were identified based on visual selection for agro-morphological traits specific for the recurrent parent RPHR-1005 and, among these, a single plant, viz. # RPIC-16-65, possessing a maximum introgression (i.e., ~93%) of RPHR-1005 genome was identified through background selection. This plant was then further examined to determine the extent of donor parent genome introgression around the three target resistance genes, viz., *Xa21*, *Gm4*, and *Gm8*. Analysis of genome introgression associated with BB resistance gene *Xa21* and GM resistance genes *Gm4* and *Gm8* on chromosomes 11 and 8, respectively, in the improved lines of RPHR-1005 indicated that the donor segment introgression was limited to ~2.0 Mb. With respect to *Xa21*, a segment of 0.6 Mb was introgressed at the proximal side in the best ICF_2 plant (i.e., plant # RPIC-16-65-125), while at the distal side a segment of 0.4 Mb was introgressed. Thus, in total, a segment of 1.0 Mb was introgressed from the donor parent with respect to *Xa21*. With respect to *Gm4*, a segment of 0.2 Mb was introgressed both at the proximal and distal sides (totaling 0.4 Mb) in plant # RPIC-16-65-125, and a segment of 0.3 Mb was introgressed both at the proximal and distal sides of *Gm8* (totaling 0.6 Mb). The position of the polymorphic SSR markers in Mb on chromosomes 11 and 8 is given adjacent to each marker (Fig. 3). This plant (# RPIC-16-65-125) was then advanced through pedigree-based phenotypic selection and, finally, three promising, advanced intercross-derived lines were identified at ICF_4. They were subjected to phenotypic evaluation for disease resistance, yield, fertility restoration, and other agro-morphological parameters.

Phenotyping for BB and gall midge resistance

BB resistance

The recurrent parent RPHR-1005 was observed to be highly susceptible to BB disease with a lesion length of 19 cm, while the resistant checks SM1 and SM2 were

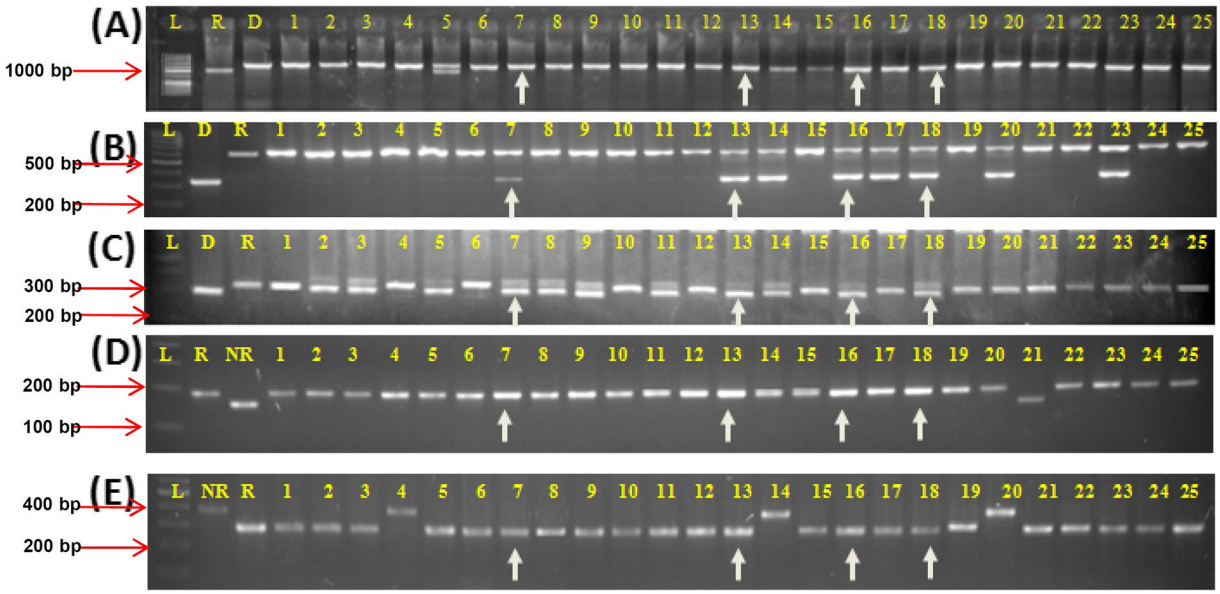

Fig. 2 Marker-assisted foreground selection at ICF_1 (intercrossed F_1) generation for *Xa21* (A), *Gm4* (B), *Gm8* (C), *Rf3* (D), and *Rf4* (E). While heterozygous plants were selected for *Xa21*, *Gm4*, and *Gm8* based on gene-specific markers, plants homozygous for the restorer allele (determined based on marker analysis) were selected with respect to *Rf3* and *Rf4*. *Arrows* indicate 'positive plants'. Plants # 7, 13, 16, and 18 were positive for all the five genes. *1–25* ICF_1 plants, *D* donor parent [(A & B): SM1, (C):: SM2, (D) and (E): RPHR-1005], *NR* Non-restorers, *L* 100 bp molecular weight ladder, *R* recurrent parent/restorer (RPHR1005)

observed to be highly resistant with a lesion length ranging from 1.2 to 1.5 cm (Table 2). All the three improved ICF_4 lines showed high level of resistance to BB, with a lesion length of 1.0–1.3 cm indicating successful introgression of *Xa21* in these lines.

Gall midge resistance

TN1, the susceptible check, ISM, and the recurrent parent RPHR-1005 showed complete damage (100% incidence), while the three improved ICF_4 lines were observed to show complete resistance (0%) against biotype 1 of gall midge (similar to the resistant checks, Abhaya and Aganni, possessing *Gm4* and *Gm8*, respectively) (Table 2).

Evaluation of agro-morphological characters of the improved RPHR-1005 lines

The DFF of the recurrent parent RPHR-1005 ranged from 101 to 103 days and for the donors (i.e., SM1 and SM2) ranged from 96 to 98 days. The DFF of the selected intercross F_4 lines of RPHR-1005 (possessing *Xa21* + *Gm4* + *Gm8*) ranged from 91 to 101 days (Table 3). While most of the improved derivative lines of RPHR-1005 were observed to flower similar to the original recurrent parent (i.e., RPHR-1005), a single line, ICF_4 line # RPIC-16-65-125, flowered significantly earlier (i.e., 11–12 days) than the recurrent parent RPHR-1005. Significant difference was noticed in terms of plant height among the three improved lines; a single plant, # RPIC-16-65-125, was observed to be taller than the recurrent parent RPHR-1005 (Table 3). The mean values for grain yield per plant of the selected ICF_4 lines ranged from 21.0 ± 0.7 to 25.5 ± 0.5 g. A single ICF_4 line, # RPIC-16-65-125, was observed to be superior in terms of grain yield per plant compared with the recurrent parent RPHR-1005. Even though no significant variation was observed with respect to the number of productive tillers/plant, panicle weight, panicle length, and thousand grain weight in the above-mentioned plant, it had a higher number of grains per panicle and better panicle exertion (i.e., fully exerted) compared with RPHR-1005 (which had only partial exertion of the panicle), which could be responsible for increased grain yield in the plant.

Discussion

RPHR-1005, a stable restorer line of rice, was developed by ICAR-IIRR from the cross BPT5204 × SC_3

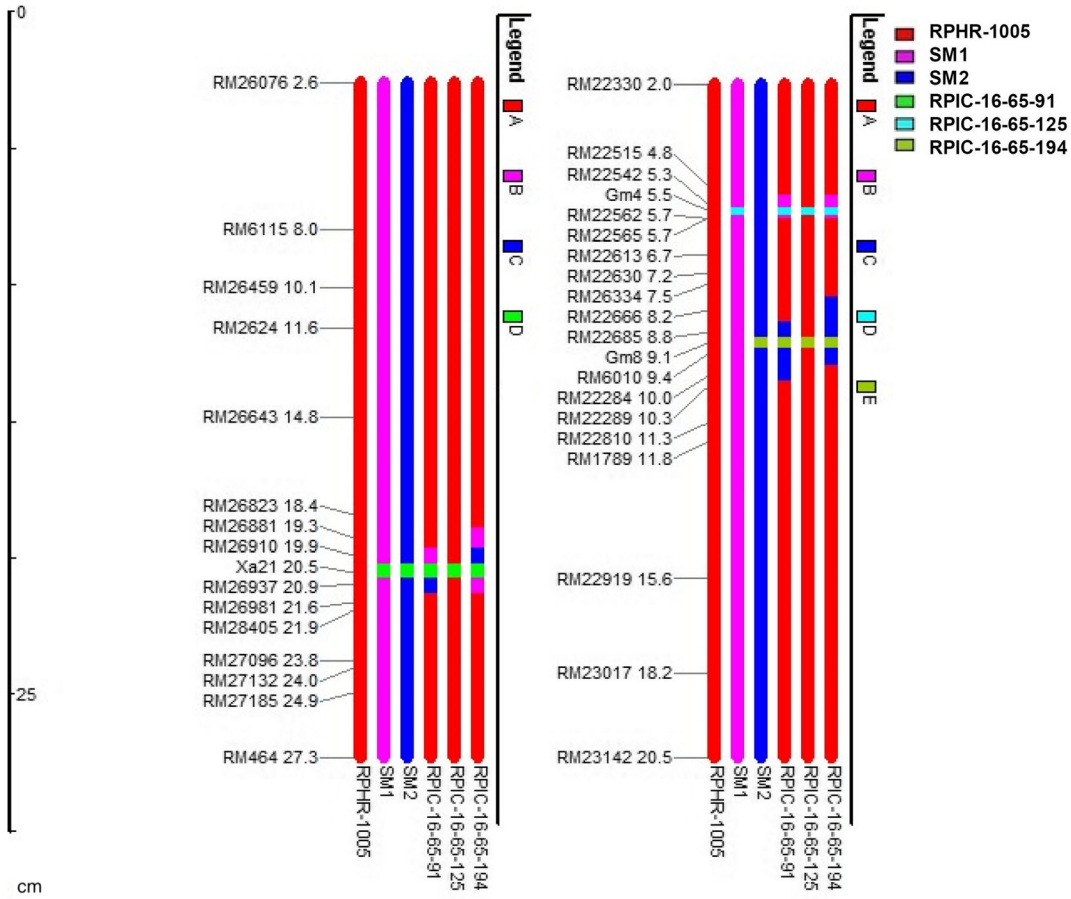

Fig. 3 Analysis of genome introgression associated with bacterial blight (BB) resistance gene *Xa21* and gall midge resistance genes *Gm4* and *Gm8* on chromosomes 11 and 8, respectively, in the improved lines of RPHR-1005 indicating the donor segment introgression limited to ~2.0 Mb. With respect to *Xa21*, a segment of 0.6 Mb was introgressed in the proximal side from the donor parent genome in the best ICF_2 (inter-crossed F_2) plant (i.e., plant # RPIC-16-65-125), while in the distal side a segment of 0.4 Mb was introgressed. Thus, in total, a segment of 1.0 Mb was introgressed from the donor parent with respect to the genomic region in the vicinity of *Xa21*. With respect to *Gm4*, a segment of 0.2 Mb was introgressed both in the proximal and distal side (totaling 0.4 Mb). The position of the polymorphic SSR markers in Mb on chromosomes 11 and 8 is given adjacent to each marker, while each marker has also been positioned with respect to each other in terms of cm scale

_126–3-2-4 (Ramesha et al. 2010). It completely restores fertility, is of medium duration, and has a highly desirable fine-grain type and semi-dwarf plant type. As a result of consistent and profound efforts, a hybrid, DRRH-3 (APMS6A × RPHR-1005), with grain type similar to the highly popular and elite rice variety Samba Mahsuri (also known as BPT5204; the check variety) was developed and released by ICAR-IIRR using RPHR-1005 as the male parent in the year 2009 for commercial cultivation in the states of Andhra Pradesh, Orissa, Gujarat, Madhya Pradesh, and Uttar Pradesh (AICRIP 2009–2010). DRRH-3, besides having MS grain type and very good grain quality features, matures earlier (by about 10 days) than Samba Mahsuri with a yield advantage of 20–25% over the elite mega-variety, Samba Mahsuri. Despite its superior grain and yield qualities, DRRH-3 and its parents RPHR-1005 and APMS6A are highly susceptible to two major stresses: BB and GM. The present study was therefore carried out with an objective to improve RPHR-1005 for durable resistance against BB and GM by targeted introgression of three major genes, conferring resistance against the two stresses through MABB coupled with phenotype-based selection. We selected dominant genes conferring

Table 2 Phenotyping of selected ICF_4 (inter-crossed F_4) lines of RPHR-1005 against gall midge biotype GMB1 and bacterial blight (BB) isolate DX-020 in a greenhouse

Serial no.	Screening against gall midge biotype 1 (GMB1)			Screening against *Xoo* isolate DX-020	
	Rice genotypes	Plant damage (%)[a]	Rating	Average lesion length (cm)[b]	Rating
1	RPHR-1005 (recurrent parent)	100	S	19.4 ± 0.2	S
2	Improved Samba Mahsuri (*Xa21* + *xa13* + *xa5*)—resistant check for BB	100	S	1.0 ± 0.2	R
3	Abhaya (*Gm4*)	0	R	22.8 ± 0.2	S
4	Aganni (*Gm8*)	0	R	19.9 ± 0.3	S
5	RPIC-16-65-91	0	R	1.3 ± 0.3	R
6	RPIC-16-65-125	0	R	1.0 ± 0.3	R
7	RPIC-16-65-194	0	R	1.2 ± 0.2	R

R resistant, *S* susceptible

[a] A total of 20 seedlings were screened from each genotype in two replications

[b] Average of 3 plants per genotype were clip inoculated with *Xoo* (5 leaves per plant)

resistance against the two major biotic stress stresses for introgression in the present study as introgression of dominant genes ensures that the derived hybrids will also possess resistance (Hari et al. 2011). Further, introgression of dominant resistance genes into the male parent (i.e., restorer line RPHR-1005) accelerates the process of development of BB and GM-resistant hybrids as introgression of the genes into the female parent (i.e., the maintainer line or the cytoplasmic male sterile line) is cumbersome and involves several rounds of backcrosses to introgress the target genes first into the maintainer line and then later to the CMS line (Hari et al. 2013; Balachiranjeevi et al. 2015).

MABB has been demonstrated to be an efficient technique for precise transfer of one or few target genes into the genetic background of an elite variety or parental line. Earlier, Sundaram et al. (2008, 2009) and Hari et al. (2011, 2013) developed BB resistant versions of the varieties ISM and Triguna, the restorer line KMR-3R, and the maintainer line IR58025B, respectively through MABB. We adopted an approach similar to that adopted by Hari et al. (2013), wherein markers were used not only for introgression of target resistance genes but also for fertility restoration (for the major gene *Rf4* and the minor gene *Rf3*). However, in contrast to Hari et al. (2013), we adopted background selection as a strategy for accelerated recovery of the RPHR-1005 genome, thus limiting the number of backcrosses to just two. Adopting a similar approach, Sama et al. (2012) have improved Samba Mahsuri for resistance against BB and GM and Das and Rao (2015) have improved the *indica* rice genotype, Lalat, for resistance against the biotic stresses of BB, blast, and GM and abiotic stresses of submergence and salinity.

MABB strategy has proven to be the most effective, economical, and environmentally safe option for management of BB disease (Khush et al. 1989) and GM (Bentur et al. 1987). *Xa21* is a major, dominant resistance gene conferring resistance against BB and the gene is known to be very effective across India (Gopalakrishnan et al. 2008; Sundaram et al. 2008). Hence, it has been selected in this study for the targeted improvement of RPHR-1005. To date, nine of the 11 reported GM resistance genes have been mapped (Yasala et al. 2012; Sama et al. 2014). Rapid evolution of virulent biotypes against the resistant rice varieties carrying a single major gene during the 1980s and thereafter has necessitated a change in the breeding approach with respect to deployment of single resistance genes (Himabindu 2009) and it has been suggested that the combination of at least two genes can provide broad-spectrum durable resistance (Sundaram et al. 2014). Considering these points, in the present study, two major and GM resistance genes, *Gm4* and *Gm8*, which are known to be effective in India, were selected for introgression into the recurrent parent RPHR-1005.

The improved lines possessing these genes showed a significantly higher level of resistance against both BB

Table 3 Details of agronomic performance of the parents and improved lines of RPHR-1005 under field conditions

Serial no.	Plant identity	Days to 50% flowering (DFF)	Mean plant height (cm)	No. of productive tillers/plant (NTP)	Panicle weight (g)	Panicle length (cm)	Days to Maturity	Grain yield per plant (g)	1000 seed weight (g)	Panicle exsertion	Grain type
1	RPHR-1005	102.0 ± 0.6	89.0 ± 1.1	15.7 ± 0.7	1.8 ± 0.09	15.5 ± 0.2	132.0 ± 0.6	24.0 ± 0.6	16.5 ± 0.1	PE	MS
2	SM1 (ISM/Abhaya)	96.3 ± 1.5	80.6 ± 0.3	12.7 ± 0.7	1.5 ± 0.06	17.0 ± 0.3	126.3 ± 0.9	17.4 ± 0.5	12.1 ± 0.1	PE	MS
3	SM2 (ISM/Aganni)	98.7 ± 1.2	86.7 ± 1.1	12.3 ± 0.3	1.6 ± 0.07	15.6 ± 0.6	133.7 ± 0.9	16.1 ± 0.2	12.8 ± 0.1	PE	MS
Intercross (IC) possessing *Xa21* + *Gm4* + *Gm8*											
4	RPIC-16-65-91	100.7 ± 0.3	86.6 ± 0.7	15.3 ± 0.3	1.8 ± 0.07	13.9 ± 0.5	129.3 ± 0.3	21.0 ± 0.7	15.4 ± 0.6	FE	MS
5	RPIC-16-65-125	91.7 ± 1.2 [a]	94.1 ± 0.7[a]	17.0 ± 0.6[a]	1.9 ± 0.06[a]	16.4 ± 0.3[a]	124.3 ± 0.3[a]	25.5 ± 0.5[a]	16.9 ± 0.1[a]	FE	MS
6	RPIC-16-65-194	98.7 ± 0.6	89.1 ± 0.5	15.0 ± 0.6	1.5 ± 0.10	13.9 ± 0.5	127.0 ± 1.0	24.0 ± 0.1	16.7 ± 0.1	FE	MS
	F	19.01	30.92	11.1	5.65	8.76	24.49	37.79	64.56	–	–
	p-value	<0.0001	<0.0001	<0.0004	<0.0066	<0.0011	<0.0001	<0.0001	<0.0001	–	–
	CV (%)	1.48	1.56	6.42	7.69	4.89	0.96	5.08	2.99	–	–
	LSD ($p = 0.05$)	3.98	3.76	2.59	0.35	2.07	3.42	2.98	1.24	–	–

Values are given as the mean of three replications at 5 plants per replication ± standard error

CV coefficient of variance, *FE* full exertion; *LSD* least significant difference at 5% probability level, *MS* medium slender, *PE* partial exertion, *RPHR-1005* recurrent parent, *SM1 and SM2* donor parents

[a] Better than the recurrent parent

and blast (Table 2), indicating that the choice of resistance genes selected in this study (*Xa21* + *Gm4* + *Gm8*) was indeed appropriate. Although there are a few previous reports regarding the breakdown of resistance conferred by a single BB resistance gene in rice (Mew et al. 1992; Khush et al. 1989), to date there has been no report on the large-scale breakdown of resistance conferred by *Xa21* in any country. The present study is also unique with respect to the choice of GM resistance genes. *Gm4* is reported to possess HR+ resistance (Srivastava et al. 1993), while *Gm8* is known to possess HR– resistance (Kumar et al. 2000). Pyramiding of two or more *R* genes with contrasting mechanisms of resistance is considered an effective strategy to enhance durability of resistance and delay breakdown of resistance (Bentur et al. 2015). Such gene combinations are also known to display complementation, wherein the presence of multiple genes has an additive effect on the overall level of resistance (Sama et al. 2012; Das and Rao 2015; Divya et al. 2015b). There are a few reports wherein breeders have improved hybrid rice parental lines for resistance against BB alone (Chen et al. 2001; Shanti et al. 2010; Hari et al. 2011), but ours is the first report on improvement of both BB and blast resistance in a hybrid rice parental line by stacking three major genes (*Xa21* + *Gm4* + *Gm8*) through marker-assisted breeding. It was observed that the donor genome segment is limited to ~2.0 Mb on either side of the target resistance genes in the best backcross plant (possessing all three target resistance genes), ensuring that the intercross derived plants are unlikely to have adverse linkage drag from the donor parent. Through stringent MAS in the initial stages, coupled with phenotype-based pedigree selection in the later stages, we were able to recover the good grain type traits of RPHR-1005 in the advanced backcross lines developed in this study. Further, we identified one intercrossed line (RPIC-16-65-125) which possessed higher yield than RPHR-1005 (Table 3).

Tall plant stature is an extremely important characteristic for restorer lines in the three-line system of hybrid rice breeding (Virmani and Kumar 2004). As the plant height of RPHR-1005R is short (89.0 ± 1.1 cm), we focused on identification of intercross-derived plants possessing higher plant height, in addition to plants possessing good culm strength (to prevent lodging). The intercrossed line, RPIC-16-65-125 (94.1 ± 0.7 cm), was observed to be taller than RPHR-1005 (89.0 ± 1.1 cm) and possessed a strong stem in addition to being a complete fertility restorer, indicating that this line could be a better restorer parent than RPHR-1005. Another major impediment to RPHR-1005 serving as an excellent restorer is its incomplete panicle exertion, resulting in decreased pollen production and a reduced seed-set in the CMS parent. In this study, through careful phenotype-based selection, we identified backcross-derived intercross lines possessing better panicle exertion than RPHR-1005. In fact, all the three promising BB- and GM-resistant, backcross-derived lines of RPHR-1005 displayed better panicle exertion than RPHR-1005. Among the improved lines of RPHR-1005, RPIC-16-65-125 was identified as the best line as it possessed all of the good phenotypic traits such as high yield, greater plant height (94.1 ± 0.7 cm), and near-complete panicle exertion. With respect to other parameters also, it was marginally better than RPHR-1005 as it flowered early. We are assessing its potential for developing superior hybrids by crossing it with multiple wild-abortive cytoplasmic male sterility (WA-CMS) lines.

For any restorer parent, possession of a complete set of fertility restorer genes is imperative. In the present study, emphasis was laid on selection through the deployment of molecular markers linked to fertility restorer genes along with morphological and visual selection for tall plant stature, right from the first backcross generation. As the fertility restoration of CMS-WA lines is controlled mainly by two independent and dominant nuclear fertility restoring genes, *Rf3* and *Rf4* (Zhang et al. 2002), it is necessary to retain these two genes in a homozygous condition (i.e., *Rf3Rf3*, *Rf4Rf4*) while improving the restorer parental line RPHR-1005 for BB and GM resistance. Since the donor parents SM1 and SM2 did not have *Rf4* and *Rf3* genes, homozygous plants with respect to the two genes controlling fertility restoration were identified at BC_1 generation, thus ensuring that the plants of subsequent generations are complete fertility restorers. Recently, Basavaraj et al. (2010) also followed a similar strategy of MAS for screening of fertility restorer genes *Rf3* and *Rf4*.

All the three triple-gene-positive (i.e., *Xa21* + *Gm4* + *Gm8*) promising lines with complete fertility restoration (i.e., positive for *Rf3* and *Rf4*) were analyzed for the recovery of the RPG (i.e., RPHR-1005) using a set of 61 parental polymorphic SSR markers and were observed to have >92% recovery of RPHR-1005 genome with very minimal segments introgressed from the donor parent genome in the vicinity of the all three target resistance genes (Fig. 3). The near-complete recovery of yield attributes and grain quality characters of the

recurrent parent in the improved versions of RPHR-1005 lines along with BB and GM resistance and some improvement with respect to plant type are significant achievements of this study. This is particularly so because yield and grain quality characters are multigenic traits encoded by loci that are distributed across the rice genome (Sundaram et al. 2009). With Indian farmers and consumers increasingly preferring rice varieties and hybrids with fine-grain type and high yield, the improved versions of RPHR-1005 developed in the present study can be expected to replace RPHR-1005 for development as they have better fine-grain type, BB and GM resistance, and are expect to give yield levels equivalent to or slightly higher than RPHR-1005 under BB- and GM-free conditions or significantly higher than RPHR-1005 under BB and GM infection/infestation, along with taller plant type. In conclusion, in the present study we have developed improved versions of the elite restorer line RPHR-1005 that possess resistance against BB and GM and have better panicle exsertion along with complete fertility restoration and MS grain type.

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