Mol Breeding (2018) 38:100
https://doi.org/10.1007/s11032-018-0853-7

Improved versions of rice maintainer line, APMS 6B, possessing two resistance genes, *Xa21* and *Xa38*, exhibit high level of resistance to bacterial blight disease

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Received: 14 February 2018 / Accepted: 29 June 2018

Abstract APMS 6B is the stable maintainer of the CMS line APMS 6A, which is the female parent of the popular Indian rice hybrid DRRH 3. APMS 6B has good combining ability and plant stature but is highly susceptible to bacterial blight (BB) disease. In order to improve the BB resistance of APMS 6B, we pyramided two major, dominant BB resistance genes, *Xa21* and *Xa38*, through marker-assisted backcross breeding (MABB). Improved Samba Mahsuri (ISM) was used as the donor for *Xa21* while PR 114 (Xa38) served as the donor for *Xa38*. Individual crosses [APMS 6B/ISM and APMS 6B/PR 114 (Xa38)] were performed, and true F_1 plants were then backcrossed with APMS 6B and the MABB process was continued till BC_3. A single positive BC_3F_1 plant identified from both the crosses with maximum genotypic and phenotypic similarity with APMS 6B was selfed to generate BC_3F_2s. At BC_3F_2 generation, plants homozygous for either *Xa21* or *Xa38* were identified and further confirmed for the absence of two major fertility restorer genes, *Rf3* and *Rf4*. A single such homozygous BC_3F_2 plant, each from both the crosses, was then inter-mated to generate ICF_1s (inter-cross F_1s). Selected ICF_1 plants possessing both the BB resistance genes were selfed to generate ICF_2s. A total of 42 ICF_2 plants homozygous for both *Xa21* and *Xa38* were identified and screened with parental polymorphic SSR markers to identify the best F_2 plants having the maximum recurrent parent genome recovery. Twelve best ICF_2 plants were advanced up to ICF_5. The ICF_5 lines displayed very high level of BB resistance and were similar to APMS 6B in terms of agro-morphological characters. Further, most of these lines also showed complete maintenance ability and such lines are being advanced for conversion to WA-CMS lines.

Keywords Bacterial blight · Resistance · Marker-assisted backcross breeding · Fertility restoration · Hybrid rice

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11032-018-0853-7) contains supplementary material, which is available to authorized users.

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Introduction

Rice is the principal food crop in many Asian countries including India, and there is a constant challenge of enhancing the rice production to meet the ever-growing human population. One of the most viable options to enhance the rice productivity in a sustainable

Published online: 20 July 2018

manner is the cultivation of rice hybrids, which gives a yield advantage of 15–20% over the inbred varieties (Hussain et al. 2010; Zhou et al. 2011). Rice hybrids are being commercially cultivated in many rice-growing countries especially in China where it occupies more than 50% rice area (Spielman et al. 2013; Yuan 2014). Despite clear yield gain through heterosis, the pace of adoption of hybrid rice has been sluggish in the rest of Asia. In India, hybrid rice presently occupies an area of 3 million hectares and contributes an additional 3–4 million tons of rice (Hariprasad et al. 2018). Among various reasons, one primary reason for its slow adoption is the high susceptibility of hybrids to different biotic stresses (Zhou et al. 2011; Spielman et al. 2013; Hariprasad et al. 2018).

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a major production constraint of rice in most of the rice-growing countries of Asia, including India, especially in irrigated and rainfed lowland ecosystems (Devadath 1992; Win et al. 2013). It is primarily a disease of monsoon (wet) season and causes significant grain yield loss in varieties and hybrids grown under heavy nitrogen fertilization (Laha et al. 2017). Yield loss due to the disease may be as high as 50% or more depending on the plant growth stage at the time of infection, weather condition, varieties, and extent of nitrogenous fertilizers used (Mew et al. 1993). In India, major BB epidemic occurred in Punjab, Haryana, and western Uttar Pradesh during 1979 and 1980 when severe "kresek" was observed and total crop failure was reported (Mew 1987). The epidemic occurrence of the disease was reported repeatedly from several rice-growing regions in south India during 1998, 2010, and 2013 (Priyadarisini and Gnanaminckam 1999; Yugander et al. 2014). Analyses of disease survey data over the years revealed that BB increased significantly both in terms of its intensity and geographical distribution (Laha et al. 2016). Chemical control for BB has not been very successful in India (Laha et al. 2017). Though different cultural and seed treatment practices are recommended, host resistance has been the most desirable, economical, and environmental friendly approach for managing the disease. Till date, at least 41 BB resistance genes have been identified from diverse sources including several wild species of *Oryza* (Verdier et al. 2012; Kim et al. 2015; Ellur et al. 2016). However, the effectiveness of these genes may vary in different rice-growing regions depending on the pathogen population structure. Analysis of *Xoo* population from India and elsewhere indicated a significant amount of pathogenic and genetic diversity in the pathogen (Adhikari et al. 1999; Shanti et al. 2001; Lore et al. 2011; Mishra et al. 2013; Yugander et al. 2017). BB resistance genes like *Xa4*, *xa5*, *Xa7*, *xa13*, *Xa21*, *Xa23*, *Xa27*, *Xa33*, and *Xa38* are being used for improvement of BB resistance in several cultivars and a few hybrid rice parental lines through marker-assisted selection (MAS) (Laha et al. 2017). Several studies have indicated that single BB resistance genes do not provide broad-spectrum resistance (Mishra et al. 2013; Yugander et al. 2017). The most practical way to ensure the durability of resistance in a cultivar is through pyramiding two or more BB resistance genes through MAS (Sundaram et al. 2008; Singh et al. 2011).

BB is a serious constraint in hybrid rice production as the commonly deployed parental lines are highly susceptible to the pathogen (Zhang et al. 1998; Balachiranjeevi et al. 2015). Various workers have introgressed different BB resistance genes in the parental lines in order to improve the BB resistance in the hybrids. Broad-spectrum BB resistance gene, *Xa21*, or combination of *Xa21* and *Xa7* was introgressed into Minghui 63, the restorer line of a widely cultivated hybrid "Shanyou 63" in China (Chen et al. 2000; Zhang et al. 2006) to ensure broad-spectrum BB resistance in the hybrids. Few attempts have also been made in India to improve the BB resistance in the parental lines of different rice hybrids by introgressing different BB resistance genes (Hari et al. 2011, 2013; Basavaraj et al. 2010; Balachiranjeevi et al. 2015). Ni et al. (2015) pyramided blast resistance gene *Pi9* and BB resistance gene *Xa23* into the genetic background of Guangzhan 63S, an elite photoperiodic and thermo-sensitive male sterile line and the improved line, and its derived hybrid exhibited high level of BB and blast resistance. Abhilash Kumar et al. (2016) pyramided two BB resistance genes (*Xa21* and *Xa33*) and two blast resistance genes (*Pi2* and *Pi54*) in the genetic background of RPHR 1005, the restorer line of rice hybrid variety, DRRH 3. Luo et al. (2016) developed restorer line Wanhui 6725 by introgressing two disease resistance genes (*Xa27* and *Pi9*), submergence tolerance gene *Sub1A*, and aromatic fragrance gene *badh2.1* in the genetic background of WH421.

APMS 6B is an elite, stable maintainer line of APMS 6A, which is the cytoplasmic male sterile line (CMS) or A line of the popular, high-yielding rice hybrid DRRH 3 possessing the highly desirable medium slender grain

type. The hybrid has a yield advantage of 23–30% than Samba Mahsuri (a very popular and elite rice variety with medium slender grain type, valued for its grain and cooking quality). The maintainer line APMS 6B has good combining ability and plant stature but highly susceptible to BB. The BB resistance gene *Xa21*, originally identified from an accession of the wild species of rice, *Oryza longistaminata*, has been widely used for improving BB resistance in both inbred varieties and parental lines. The dominant BB resistance gene *Xa38* has been recently identified from an accession of another wild rice *Oryza nivara* and has been reported to provide broad-spectrum resistance to BB (Cheema et al. 2008; Bhasin et al. 2012). The present work describes our efforts in pyramiding of *Xa21* and *Xa38* into the genetic background of APMS 6B in order to provide in-built, broad-spectrum, durable resistance against BB.

Materials and methods

Plant materials

The BB-resistant rice variety, Improved Samba Mahsuri (ISM), possessing three major BB resistance genes (*Xa21*, *xa13*, and *xa5*) and medium slender grain type was used as the donor for *Xa21*. ISM was developed through MABB by introgressing the three BB resistance genes into the genetic background of Samba Mahsuri, a popular rice variety in South India (Sundaram et al. 2008). The line PR 114 (Xa38) (an introgression line derived from a cross between PR 114 and *Oryza nivara*) possessing new BB resistance gene, *Xa38*, was used as another donor (Cheema et al. 2008). APMS 6B, the stable maintainer line of APMS 6A, was used as the recurrent parent. The rice restorer line KMR 3 was used as a positive check for major fertility restorer genes, *Rf3* and *Rf4*. In addition to the above mentioned lines, Taichung Native 1 (TN1) (susceptible check), IRBB21 (*Xa21*), and IRBB59 (*xa5* + *xa13* + *Xa21*) were also included in the study during resistance evaluation.

Xoo strains, phenotyping, and disease scoring

The parents, backcross-derived lines (BDLs), and checks were raised in plastic trays (60 × 40 × 7 cm) in glasshouse following previously reported procedures (Laha et al. 2007). The *Xoo* strains used were IX-002 (Faizabad, Uttar Pradesh), IX-015 (Aduthurai, Tamil Nadu), IX-020 (Hyderabad, Telangana), IX-116 (Panvel, Maharashtra), IX-133 (Raipur, Chhattishgarh), IX-234 (Ludhiana, Punjab), IX-244 (Pantnagar, Uttarakhand), and IX-279 (Tanuku, Andhra Pradesh). These *Xoo* strains belonged to different pathotypes (Yugander et al. 2017). Individual *Xoo* strains were multiplied on modified Wakimoto's agar (MWA) (Yugander et al. 2014), and bacterial suspensions (ca. $10^{8–9}$ cfu/ml) were prepared using 3-day old cultures. When the plants were 40 days old, each tray was inoculated with individual *Xoo* strains following clip-inoculation method (Kauffman et al. 1973). For each genotype/*Xoo* strain combination, 12–15 fully grown leaves were clip-inoculated and observations were taken 15 days after inoculation by measuring the lesion length. Average lesion length up to 3 cm was taken as resistant (R), 3–6 cm as moderately resistant (MR), 6–9 cm as moderately susceptible (MS), and more than 9 cm as susceptible (S) (Chen et al. 2000).

Marker-assisted pyramiding of *Xa21* and *Xa38* into APMS 6B

Two independent crosses viz., APMS 6B × ISM (cross I) and APMS 6B × PR 114 (Xa38) (cross II), were made to individually transfer the BB resistance genes *Xa21* and *Xa38*, respectively, in the genetic background of APMS 6B. The resultant F_1 plants from both the crosses were then analyzed by using gene-specific markers to identify "true" F_1 plants. The true F_1 plants derived from the independent crosses (cross I and II) were individually backcrossed with the recurrent parent, APMS 6B, to generate two sets of BC_1F_1s. They were subjected to foreground selection using the gene-specific markers and background selection using a set of parental polymorphic SSR markers. The positive BC_1F_1 plants having the target genes (either *Xa21* or *Xa38*) along with maximum recurrent parent genome (RPG) recovery were then backcrossed with APMS 6B to generate BC_2F_1s. MABB as described above was continued till BC_3 generation (Supplementary Fig. S1). At BC_3F_1, plants from the two crosses which had either *Xa21* or *Xa38* along with maximum introgression of the RPG were identified and selfed. At BC_3F_2, plants homozygous for either *Xa21* or *Xa38* were identified with the help of gene-specific markers. Individual homozygous BC_3F_2 plants were further checked for the absence of restorer alleles with respect to two major fertility

restorer genes, *Rf3* and *Rf4*, using gene-specific markers. A single BC_3F_2 homozygous plant from both the crosses, phenotypically similar to APMS 6B and homozygous negative for *Rf3* and *Rf4*, was inter-mated to generate ICF_1s (inter-cross F_1s). True ICF_1 plants possessing both the target genes (*Xa21* and *Xa38*) in heterozygous state were identified with the help of gene-specific markers and selfed to generate ICF_2s. They were then genotyped to identify the plants having both *Xa21* and *Xa38* in homozygous condition. Such plants were subjected to background selection, in order to identify plants which have a maximum recovery of the RPG and also look phenotypically similar to APMS 6B. The selected plants were advanced further up to ICF_5 generation for further evaluation.

For marker-assisted foreground and background selection, genomic DNA was isolated from the parents and BDLs by following the protocol described in Zheng et al. (1995). Presence of the target genes, i.e., *Xa21* and *Xa38*, in the respective parents [*Xa21* in ISM and *Xa38* in PR 114 (Xa38)] and in the BDLs was confirmed by PCR using gene-specific markers pTA248 for *Xa21* (Ronald et al. 1992) and Os04g53050-1 for *Xa38* (Bhasin et al. 2012). PCR and gel electrophoresis protocols recommended by Sundaram et al. (2008) and Bhasin et al. (2012) were followed for detection of BB resistance genes *Xa21* and *Xa38*, respectively. Detection of the major fertility restorer genes *Rf3* and *Rf4* was done using the primers DRRM-RF3-10 and DRCG-RF4-14, respectively (Balaji Suresh et al. 2012). For background selection, SSR loci that are polymorphic between the donors [ISM and PR 114 (Xa38)] and the recurrent parent (APMS 6B) were identified by screening 366 rice SSR markers spread across the 12 chromosomes of rice as per the procedure described by Sundaram et al. (2008). A total of 62 (APMS 6B/ISM) and 57 [APMS 6B/PR 114 (Xa38)] parental polymorphic SSR markers, which were fairly well distributed throughout the 12 chromosomes of rice (i.e., ~3–5 polymorphic markers per chromosome), were identified. These polymorphic markers were used to genotype foreground selected plants at each backcross generation to identify a single plant having maximum RPG recovery in the respective crosses. More number of polymorphic SSR markers were used on target chromosomes (i.e., 20 in chr.11, where *Xa21* is located and 15 in chr. 4 where *Xa38* is located) in order to reduce the linkage drag in the genomic region around the target resistance genes. A total of 83 parental polymorphic markers (pooled from both the cross combinations) were used to genotype the homozygous ICF_2 plants to identify the best plants having the maximum RPG recovery. Using the polymorphic marker data, a schematic map depicting the genomic contribution of donor and recurrent parents was prepared using Graphical Genotype (GGT) version 2.0 software (Van Berloo 1999) to identify BDLs (ICF_2) possessing maximum recovery of RPG.

Evaluation of agro-morphological characters of BDLs

For evaluation of different agro-morphological characters, 30-day-old seedlings of the 12 selected BDLs along with the parents were transplanted in 3×2 m^2 mini-plots in the fields (in three replications) following a spacing of 15×20 cm during the wet season of 2016. Fertilizers were applied @ 120 kg N/ha, 60 kg P_2O_5/ha and 40 kg K_2O/ha. The plots were irrigated as and when required. Observations were recorded on different morphological parameters like mean plant height (cm), number of panicles/hill, days to 50% flowering (DFF), number of grains/panicle, panicle length (cm), grain weight (g)/plant, 1000 grain weight (g), and grain type as explained by Hari et al. (2013). The data were subjected to statistical analysis following Gomez and Gomez (1984).

Assessment of maintenance ability of the selected BDLs

In order to confirm the sterility maintenance ability of the improved two-gene pyramid lines of APMS 6B, pollens collected from the gene-pyramid lines were dusted on APMS 6A to generate F_1s. The F_1 plants were then grown in single-plant progenies during the wet season of 2016 to study the pollen sterility. Pollen sterility was assessed following standard protocol using 1% iodine-potassium iodide (IKI) stain (Lee et al. 2005). F_1 plants which displayed complete unstained and withered sterile pollens were considered as true maintainers. Apart from the pollen study, seed setting percentage of all the F_1 plants was also evaluated by covering individual panicles with butter paper cover at the time of heading.

Results

BB resistance of parental lines

The level and spectrum of BB resistance of the donors, recurrent parent, and various checks was determined by

inoculating them with eight different *Xoo* strains before starting the crossing program. The reaction pattern of the parents and checks is presented in Supplementary Fig. S2. The donor parent, ISM, possessing three BB resistance genes (*Xa21*, *xa13* and *xa5*), showed a high level of resistance to all the *Xoo* strains tested with lesion length less than 3 cm. Individually, BB resistance gene *Xa21* (IRBB21) showed a moderate BB reaction with lesion length ranging from 4.7 to 11.1 cm. Donor PR 114 (Xa38) showed high level of resistance to all the *Xoo* strains except IX-116, to which it showed susceptibility with a lesion length of 10.8 cm (Supplementary Fig. S2). The recurrent parent APMS 6B showed very high degree of susceptibility to all the *Xoo* strains with lesion length ranging from 16.4 to 24.5 cm. The results indicate that it would be highly useful to improve the BB resistance of APMS 6B by pyramiding two important dominant BB resistance genes, *Xa21* and *Xa38*.

Marker-assisted pyramiding of *Xa21* and *Xa38* in APMS 6B

The donors ISM (donor for *Xa21*) and PR 114 (Xa38) (donor for *Xa38*) were individually crossed with the recurrent parent, APMS 6B (Supplementary Fig. S1), and the F_1 plants derived from these two crosses were screened with the gene-specific markers (pTA248 for *Xa21* and Os04g53050-1 for *Xa38*) to identify true F_1 plants possessing the target genes in heterozygous condition. Out of 76 F_1 plants derived from the cross-I (APMS 6B/ISM), a total of 69 plants were identified to possess *Xa21* gene in heterozygous condition (i.e., *Xa21xa21*). Similarly, out of 58 F_1 plants derived from cross II [APMS 6B/ PR 114 (Xa38)], a total of 53 plants possessed *Xa38* gene in heterozygous condition (i.e., *Xa38xa38*). The true F_1 plants derived from both the crosses were individually backcrossed with APMS 6B.

Individual backcrossing was continued till BC_3, and in each generation, the F_1 plants were checked for the presence of respective BB resistance genes using gene-specific markers. At the BC_1F_1 stage, 36 out of 62 plants were found to be positive for *Xa21* in cross I while in cross II, 33 out of 62 plants were found to be positive for *Xa38*. The recovery of RPG among the gene-positive BC_1F_1 plants ranged from 75.4 to 77.4%, and a single plant from each cross possessing maximum RPG (no. GSY-1-27-16 with RPG recovery of 77.4% in cross I and no. GSY-2-19-36 with RPG recovery of 77.1% in cross II) was then backcrossed with APMS 6B to generate BC_2F_1 seeds. Among the BC_2F_1s, 42 plants (out of 81 plants) were found to be positive for *Xa21* in cross I while 42 plants (out of 85 plants) were found to be positive for *Xa38* in cross II. The RPG in BC_2F_1 plants from both the crosses ranged between 87.7 and 90.3%. A single selected BC_2F_1 plant each from both the crosses having maximum RPG (no. GSY-1-27-16-41 with RPG recovery of 90.3% in cross I and no. GSY-2-19-36-17 with RPG recovery of 89.4% in cross II) and recurrent parent phenotype and also BB resistance was then backcrossed with APMS 6B to generate BC_3F_1. In cross I, out of 66 BC_3F_1 plants, 37 were positive for *Xa21* while in cross II, out of 48 plants, 25 were found positive for *Xa38* and the RPG in the selected BC_3F_1 plants ranged between 92.9 and 95.1%. A single BC_3F_1 plant each from both the individual crosses (no. GSY-1-27-16-41-35 with RPG recovery of 95.1% in cross I and no. GSY-2-19-36-17-48 with RPG recovery of 94.7% in cross II) confirmed to possess respective BB resistance genes (either *Xa21* or *Xa38* in heterozygous state), having maximum genotypic and phenotypic similarity with the recurrent parent and which showed BB resistance was then selfed to generate BC_3F_2s (~ 500 seeds). BC_3F_2 plants each from both the crosses were raised and screened with the gene-specific marker(s) to identify homozygous plants. A total of 46 (out of 200) plants from cross I and 41 (out of 200) plants from cross II were identified as homozygous positive for *Xa21* and *Xa38*, respectively. These homozygous BC_3F_2 plants were further checked for the absence of two major fertility restorer genes, *Rf3* and *Rf4*, using gene-specific markers. Out of 46 BC_3F_2 plants homozygous for *Xa21*, 41 plants were found to be homozygous negative for both the fertility restorer genes in cross I. Similarly, in cross II, out of 41 BC_3F_2 plants homozygous for *Xa38*, 38 plants were found to be homozygous negative for both the fertility restorer genes.

A single homozygous plant from both the crosses (GSY-1-27-16-41-35-71 in cross I and GSY-2-19-36-17-48-124 in cross II) and homozygous negative for both the fertility restorer genes was inter-mated to generate ICF_1s (123 seeds). Out of 67 ICF_1 plants raised, 54 plants were found to be positive for both the BB resistance genes, *Xa21* and *Xa38* (in heterozygous state). A single true ICF_1 plant was selfed to generate ICF_2s (~ 1200 seeds). Out of 727 ICF_2 plants analyzed, 42 plants showed the presence of both the BB resistance genes, *Xa21* and *Xa38* (in homozygous state), and were also found to be homozygous negative for both the fertility

restorer genes, *Rf3* and *Rf4* (Fig. 1). These plants had RPG range of 92.7–95.1% and also closely resembled with the recurrent parent APMS 6B in terms of most of the phenotypic attributes. At ICF_2, 12 best BDLs which showed RPG recovery more than 93% and similar to APMS 6B were selected, advanced up to ICF_5 generation, and evaluated for different agro-morphological characters and BB resistance. When these lines were analyzed for the linkage drag in the vicinity of the target resistance genes, majority of them were observed to possess an introgression of 0.9 Mb segment (0.6 Mb at the proximal end and 0.3 Mb at the distal end) with respect to *Xa21* and ~ 0.2 Mb segment with respect to *Xa38* (0.1 Mb at either side) of the donor parent genomes (Fig. 2).

BB resistance of backcross-derived two-gene pyramid lines of APMS 6B

Twelve best BDLs (having maximum RPG and phenotypic similarity with APMS 6B) at the ICF_5 stage were evaluated for BB resistance using eight different *Xoo* strains under glasshouse condition. The details of the reactions of the BDLs and the parents are presented in Table 1. All the BDLs possessing both the BB resistance genes exhibited a very high degree of resistance to BB with lesion length ranging from 0.2 to 0.5 cm (Table 1; Fig. 3) when compared to the recurrent parent APMS 6B which exhibited a lesion length of 16.6–23.9 cm.

Agro-morphological characters of the two-gene pyramid lines of APMS 6B

Mean plant height of the BDLs ranged from 76.3 to 82.3 cm and was statistically on par with the recurrent parent, APMS 6B (Table 2). Mean number of panicles/plant ranged from 13 to 15 among the different two-gene pyramid BDLs and was statistically similar to the recurrent parent APMS 6B. Days to 50% flowering of the BDLs varied from 91.7 to 96 days while in the case of APMS 6B, it was 96 days. Some of the BDLs, viz. GSY-141 and GSY-279, were significantly earlier than APMS 6B by about 5–6 days. Average number of grains/panicle of the BDLs (134.7–147.7 grains/panicle) was also on par with the recurrent parent, APMS 6B (135.7 grains/panicle) (Table 2). Two BDLs, viz. GSY-4 and GSY-308, were observed to be promising with comparatively more number of grains/panicle. Mean panicle length of the BDLs was equivalent to that of the recurrent parent, APMS 6B. The average weight of 1000 grains of the improved lines did not differ significantly from that of the recurrent parent except the lines GSY-411 and GSY-542. Mean grain weight/plant of the improved lines ranged from 19.4 to 24.2 g/plant while in the case of APMS 6B, it was 20.4 g/plant. All the BDLs showed medium slender grain type similar to that of APMS 6B.

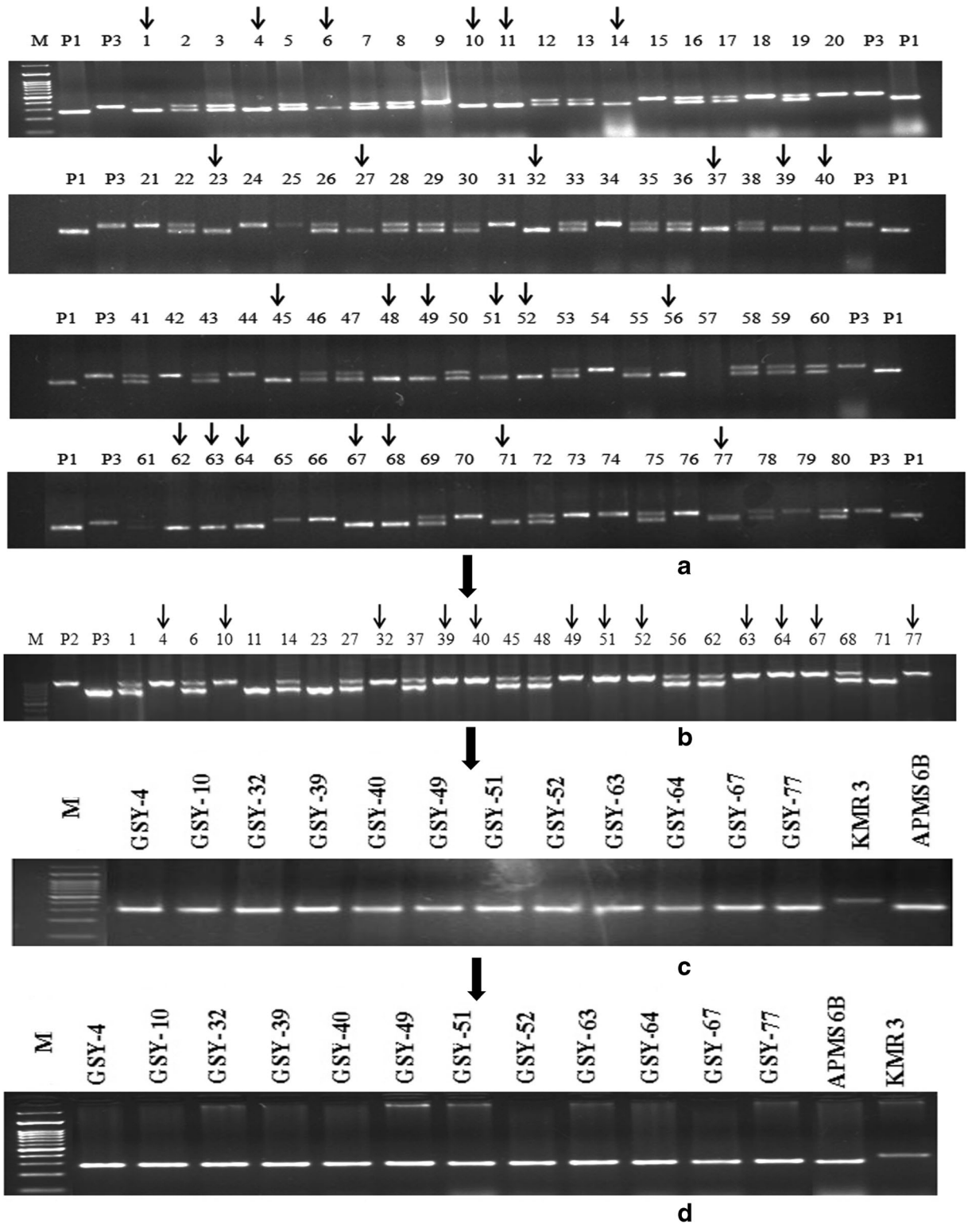
Fig. 1 Molecular detection of *Xa38*, *Xa21*, *Rf4*, and *Rf3* in the inter-cross F_2 plants through PCR using gene-linked markers Os04g53050-1, pTA248, DRCG-RF4-14, and DRRM-RF3-10, respectively. The numbers shown on the top of the each gel represents the inter-cross F_2 plant numbers. **a** Screening of plants using *Xa38*-linked marker Os04g53050-1. **b** Screening of plants using *Xa21*-linked marker pTA248. **c** Screening of plants using *Rf4*-linked marker DRCG-RF4-14. **d** Screening of plants using *Rf3*-linked marker DRRM-RF3-10. Lanes M: 100 bp molecular weight ladder. P1—Donor parent PR 114 (Xa38); P2—Donor parent (ISM-*Xa21*); P3—Recurrent parent (APMS 6B); KMR 3—Positive check for *Rf3* and *Rf4*; arrow indicates homozygous positive plants

Confirmation of stability of maintenance ability in the BDLs

The F_1 plants derived from 9 (GSY-4, GSY-10, GSY-32, GSY-112, GSY-222, GSY-279, GSY-308, GSY-411, and GSY-619) out of 12 sets of crosses displayed complete pollen sterility and were observed to be highly resistant to BB. Further, none of the F_1 plants derived from these nine crosses could set seeds at the time of maturity confirming the maintaining ability of these selected two-gene pyramid lines of APMS 6B.

Discussion

DRRH 3 is a very popular, high-yielding (6–6.5 t/ha) hybrid with a medium slender grain (with most grain quality features equivalent to elite rice variety, Samba Mahsuri) and was released for commercial cultivation in India in 2009. The hybrid can give about 23–30% more yield than Samba Mahsuri (Abhilash Kumar et al. 2016). However, the hybrid is highly susceptible to BB, a major rice disease in India. Development and deployment of host plant resistance is the most effective and environmentally safe method for managing this disease. Most of the gene pyramiding program involves use of few BB resistance genes like *Xa4*, *xa5*, *xa13*, and *Xa21* (Joseph et al. 2004; Sundaram et al. 2008, 2009; Basavaraj et al.


M P1 P3 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 P3 P1
P1 P3 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 P3 P1
P1 P3 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 P3 P1
P1 P3 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 P3 P1
a
M P2 P3 1 4 6 10 11 14 23 27 32 37 39 40 45 48 49 51 52 56 62 63 64 67 68 71 77
b
M GSY-4 GSY-10 GSY-32 GSY-39 GSY-40 GSY-49 GSY-51 GSY-52 GSY-63 GSY-64 GSY-67 GSY-77 KMR 3 APMS 6B
c
M GSY-4 GSY-10 GSY-32 GSY-39 GSY-40 GSY-49 GSY-51 GSY-52 GSY-63 GSY-64 GSY-67 GSY-77 APMS 6B KMR 3
d

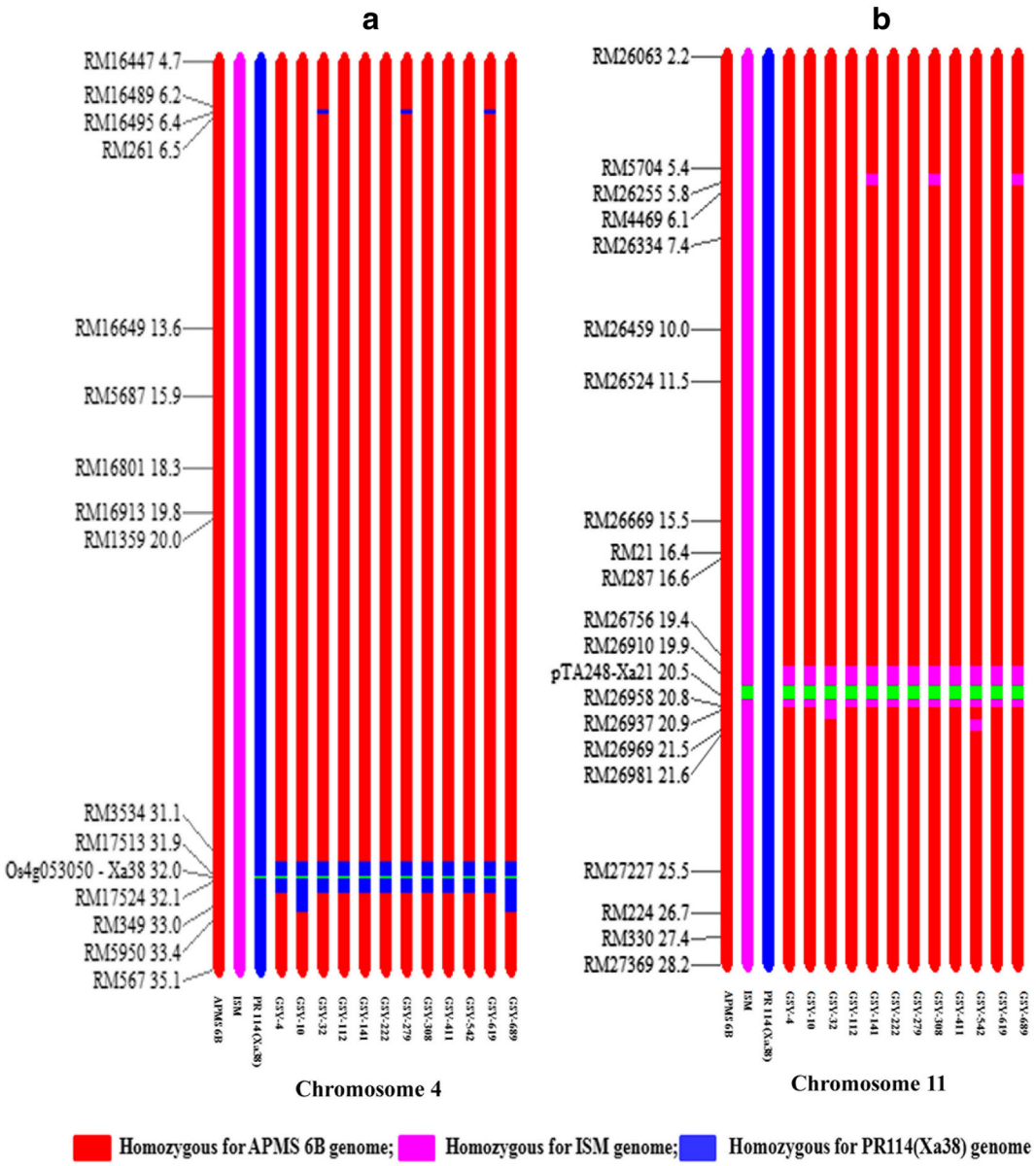


Fig. 2 Analysis of genome introgression associated with the BB resistance gene *Xa38* on chromosome 4 and *Xa21* on chromosome 11 in the 12 two-gene pyramid lines of APMS 6B. **a** Analysis of genome introgression associated with the *Xa38*: a genomic region limited to ~ 0.2 Mb has been only introgressed from the donor parent [PR 114 (Xa38)] in the best backcross plants. **b** Analysis of genome introgression associated with the *Xa21*; a genomic region limited to ~ 0.9 Mb has been only introgressed from the donor parent (ISM) in the best backcross plants. The position of each polymorphic SSR markers in Mb on Chr. 4 and 11 is given adjacent to each marker

2010; Salgotra et al. 2012; Balachiranjeevi et al. 2015; Abhilash Kumar et al. 2016). Extensive use of these genes is leading to the development of strains of *Xoo* which can break the resistance conferred by these genes. BB pathogen is highly dynamic and a significant amount of pathogenic and genetic diversity in the pathogen has been reported by several workers in India and elsewhere (Adhikari et al. 1999; Shanti et al. 2001; Lore et al. 2011; Mishra et al. 2013; Mondal et al. 2014; Pandey et al. 2014; Yugander et al. 2017). As the single gene conferred resistance is often short lived, the best option to ensure the durability of resistance is to pyramid two or more BB resistance genes into a cultivar or hybrid rice parental lines using MAS. Pyramiding of different BB

Table 1 Mean lesion length of backcross-derived lines (BDLs) and parents to multiple strains of *Xanthomonas oryzae* pv. *oryzae*

Plant materials	*Xoo* strains							
	IX-002	IX-015	IX-020	IX-116	IX-133	IX-234	IX-244	IX-279
GSY-4	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1
GSY-10	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
GSY-32	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
GSY-112	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
GSY-141	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
GSY-222	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.1
GSY-279	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
GSY-308	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
GSY-411	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.1
GSY-542	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
GSY-619	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
GSY-689	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.1
Imp. Samba Mahsuri	2.1 ± 0.1	2.5 ± 0.2	1.5 ± 0.1	2.6 ± 0.2	0.3 ± 0.1	1.7 ± 0.2	0.6 ± 0.1	2.5 ± 0.2
PR 114 (Xa38)	2.7 ± 0.4	1.7 ± 0.2	1.3 ± 0.3	10.5 ± 0.1	4.8 ± 0.8	2.7 ± 0.3	0.2 ± 0.0	5.3 ± 0.8
APMS 6B	21.6 ± 0.7	19.6 ± 0.6	20.1 ± 1.0	22.1 ± 0.1	17.5 ± 0.8	23.9 ± 1.0	16.6 ± 0.9	23.1 ± 0.6

The BDLs (at ICF_5 stage), donors (ISM and PR 114 (Xa38)), and the recurrent parent APMS 6B were evaluated with eight *Xoo* strains in glass house under controlled conditions

resistance genes has been reported to provide broad-spectrum resistance to BB (Joseph et al. 2004; Zhang et al. 2006; Sundaram et al. 2008; Basavaraj et al. 2010; Abhilash Kumar et al. 2016). In the present study, we improved the BB resistance of APMS 6B by pyramiding two major, dominant BB resistance genes *Xa21* and *Xa38* through MABB strategy coupled with stringent phenotypic selection. The BB resistance gene *Xa21*, originally characterized from *O. longistaminata*, provides broad-spectrum resistance to multiple races of *Xoo* in India. Several studies have revealed that the gene is effective under Indian conditions and is considered

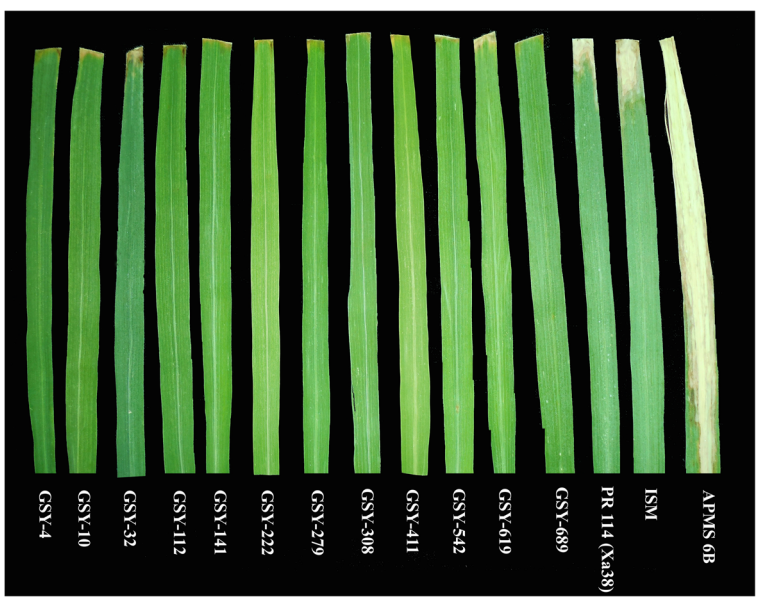


Fig. 3 Reaction of the selected ICF_5 two-gene pyramid lines against bacterial blight of rice. Forty-day-old plants were clip-inoculated using 3-day-old culture of IX-020 *Xoo* strain, and observations were taken by measuring the lesion length (cm) after 15 days of inoculation

Table 2 Agro-morphological characters of selected backcross-derived lines (BDLs) of APMS 6B along with parents under field conditions

Back cross-derived lines	Plant height (cm)	No. of panicles/hill	Days to 50% flowering	No. of grains/panicle	Panicle length (cm)	1000 grain weight (g)	Grain weight (g)/plant	Grain type	Genome recovery
GSY-4	80.7 ± 0.9	14.3 ± 0.3	94.3 ± 0.3	144 ± 2.1	23.7 ± 0.2	10.9 ± 0.3	22.7 ± 0.4	MS	93.9
GSY-10	77.3 ± 1.5	14 ± 0.6	93.7 ± 0.9	141 ± 2.1	22.0 ± 0.1	11.5 ± 0.1	21.6 ± 0.4	MS	95.1
GSY-32	76.3 ± 1.5	13.7 ± 0.9	93.0 ± 1.1	138 ± 4.9	23.1 ± 0.2	11.1 ± 0.3	20.7 ± 0.6	MS	95.1
GSY-112	80.3 ± 1.2	14.3 ± 0.3	96.0 ± 0.6	141 ± 2.6	22.7 ± 0.2	10.4 ± 0.3	22.4 ± 0.3	MS	95.1
GSY-141	76.7 ± 1.2	13 ± 0.6	91.7 ± 0.3	136 ± 3.1	21.5 ± 0.2	11.8 ± 0.2	19.5 ± 0.3	MS	93.9
GSY-222	81.7 ± 0.9	13.7 ± 0.9	93.0 ± 0.6	135.7 ± 2.9	23.1 ± 0.1	11.0 ± 0.3	20.3 ± 0.3	MS	95.1
GSY-279	80.3 ± 0.7	13 ± 0.6	91.7 ± 0.3	134.7 ± 1.8	22.1 ± 0.2	11.6 ± 0.3	19.4 ± 0.5	MS	93.9
GSY-308	82.3 ± 1.5	15 ± 0.6	93.0 ± 0.6	147.7 ± 1.4	21.5 ± 0.2	11.1 ± 0.7	24.2 ± 0.2	MS	95.1
GSY-411	80.7 ± 1.5	14 ± 0.6	92.0 ± 0.6	140 ± 2.9	21.3 ± 0.1	12.4 ± 0.1	23.5 ± 0.3	MS	95.1
GSY-542	78.3 ± 1.2	13.7 ± 0.3	92.3 ± 0.3	137.3 ± 4.3	21.6 ± 0.2	12.6 ± 0.1	21.4 ± 0.2	MS	93.9
GSY-619	79.0 ± 1.1	14 ± 0.0	94.7 ± 0.9	139.3 ± 2.9	21.9 ± 0.1	11.5 ± 0.2	21.5 ± 0.2	MS	95.1
GSY-689	81.0 ± 1.5	14 ± 0.6	92.0 ± 1.1	138.7 ± 2.7	22.0 ± 0.3	10.4 ± 0.1	21.3 ± 0.2	MS	93.9
APMS 6B	80.3 ± 1.2	13.7 ± 0.3	96.0 ± 0.6	135.7 ± 4.7	21.7 ± 0.1	10.6 ± 0.3	20.4 ± 0.3	MS	–
ISM	76.0 ± 0.6	11.3 ± 0.3	99.3 ± 1.8	105.3 ± 2.6	21.2 ± 0.3	11.3 ± 0.2	13 ± 0.1	MS	–
PR 114 (Xa38)	95.0 ± 1.1	10.7 ± 0.3	114.0 ± 0.8	95.7 ± 3.5	22.4 ± 0.2	15.0 ± 0.4	15.3 ± 0.3	LS	–
CV (%)	2.6	6.13	1.41	3.75	1.5	4.4	2.76		
LSD (*P* = *0.05*)	3.5	1.3	2.24	8.3	0.5	0.8	0.94		

APMS 6B—recurrent parent; improved Samba Mahsuri and PR 114 (Xa38)—donor parents; ±—Standard error and values given are mean of three replications

MS medium slender, *CV* coefficient of variance, *LSD* least significant difference at 5% probability level

very important in Indian resistance gene-pyramiding program (Gopalakrishnan et al. 2008; Sundaram et al. 2008, 2009; Hari et al. 2013). Hence, *Xa21* was an obvious choice for pyramiding in the genetic background of APMS 6B in the present study. The novel BB resistance gene *Xa38* was identified from *Oryza nivara* (Cheema et al. 2008), later fine-mapped on the long arm of chromosome 4 (Bhasin et al. 2012) and demonstrated to confer broad-spectrum resistance (Ellur et al. 2016). Hence, this gene was also selected for pyramiding into APMS 6B in the present study.

Backcross breeding method is a commonly used strategy for improving one or few traits in elite crop varieties and hybrids (Stoskopf et al. 1993), and it has already been demonstrated through several studies that application of molecular markers can accelerate the process of backcross breeding and add efficiency and precision to the breeding process (Sundaram et al. 2008; Balachiranjeevi et al. 2015; Abhilash Kumar et al. 2016; Ellur et al. 2016). With respect to hybrid parental lines where major changes cannot be executed in their genome, MABB is the right approach as adoption of other breeding strategies may alter the maintenance/restoration ability of the parental lines and important characters of the hybrid (Zhou et al. 2011). Simultaneous deployment of markers for both foreground and background selection has limited the number of backcross to just 3–4 as reported in a few earlier studies (Sundaram et al. 2008; Gopalakrishnan et al. 2008). Several studies have reported successful improvement of biotic stress resistance in rice by pyramiding multiple resistance genes (Joseph et al. 2004; Sundaram et al. 2008, 2009; Basavaraj et al. 2010; Salgotra et al. 2012; Balachiranjeevi et al. 2015; Abhilash Kumar et al. 2016). Popular rice varieties, Samba Mahsuri and Triguna, were improved for broad-spectrum BB resistance by incorporating three BB resistance genes, *Xa21*, *xa13* and *xa5* (Sundaram et al. 2008, 2009). Basavaraj et al. (2010) improved the BB resistance of Pusa6B and PRR78, the parental lines of basmati quality rice hybrid Pusa RH 10, by incorporating two BB resistance genes, *Xa21* and *xa13*, into both the parents. In a similar approach, DRR 17B (maintainer line) and RPHR 1005 (restorer line) were improved for both BB and blast resistance by incorporating different combinations of BB and blast resistance genes (Balachiranjeevi et al.

2015; Abhilash Kumar et al. 2016). The BB resistance gene *Xa21* or combination of *Xa21* and *Xa7* was introgressed into Minghui 63, the restorer line of a widely cultivated hybrid "Shanyou 63" in China (Chen et al. 2000; Zhang et al. 2006) to provide broad-spectrum BB resistance. Based on these success stories, we also adopted MABB strategy for improvement of BB resistance of APMS 6B.

For pyramiding *Xa21* and *Xa38* in APMS 6B, the elite rice variety ISM was utilized as the donor for *Xa21*, while PR 114 (Xa38) was used as the donor for the other BB resistance gene, *Xa38*. Individually, both *Xa21* (IRBB21) and *Xa38* [PR 114 (Xa38)] showed a high level of resistance to most of the *Xoo* strains (Supplementary Fig. S2), justifying the selection of these two genes for pyramiding into APMS 6B. We deployed two PCR-based markers, viz., pTA248 and Os04g53050-1, for foreground selection of BB resistance genes, *Xa21* and *Xa38*, respectively. Precise foreground selection is vital for the success of MABB (Hospital et al. 1997). The marker pTA248 is a functional marker for *Xa21* (Hajira et al. 2016) and Os04g53050-1 is a highly reliable marker for the BB resistance gene, *Xa38* (Bhasin et al. 2012). In addition to foreground selection of the target BB resistance genes, we also conducted background selection using a reasonable number of polymorphic SSR markers (no. 62 in cross I and 57 in cross II) in order to identify the plants (BCF_1s) having the maximum RPG recovery at each stage of backcrossing. The number of polymorphic SSRs was more (nos. 15–20) on target chromosomes (Chr. 4 and 11) as compared to the non-target chromosomes (nos. 3–5). The strategy of accelerated genome recovery of the recurrent parent using polymorphic SSRs has been used by several workers during target trait introgression through MABB (Joseph et al. 2004; Sundaram et al. 2008; Abhilash Kumar et al. 2016; Ellur et al. 2016).

After three generations of backcrossing, the recovery of RPG (in both the cross) was in the range of 92.9–95.1%, which is slightly higher than the theoretical expected value. This was possible because, in the present study, we carried out background selection with more number of parental polymorphic SSR markers covering the entire genome at each backcross generation particularly focusing on the target chromosomes, coupled with a stringent phenotypic selection. This resulted in accelerated recovery of the genome of APMS 6B within three backcross generations similar to earlier reports (Sundaram et al. 2008; Abhilash Kumar et al. 2016). A single BC_3F_2 homozygous plant from both the crosses, having maximum similarity with APMS 6B and homozygous negative for the two major fertility restorer genes, *Rf3* and *Rf4*, was inter-mated to generate ICF_1 plants. A true ICF_1 plant having maximum similarity with APMS 6B was selfed to generate ICF_2 seeds. Out of 727 ICF_2 plants genotyped, 42 plants were identified to possess BB resistance genes, *Xa21* and *Xa38*, in the homozygous state which was very close to the expected value. All of these homozygous plants were also homozygous negative for both the major fertility restorer genes, *Rf3* and *Rf4*. The presence of fertility non-restoring alleles of major fertility restorer genes, *Rf3* and *Rf4*, is an important criterion for selection of a maintainer line. The introgression of the donor chromosomal segment in most of the 12 selected ICF_2 lines was observed to be limited on either side of the target genes to a small region of ~ 0.9 Mb in the case of *Xa21* and 0.2 Mb in the case of *Xa38* (Fig. 2).

Twelve BDLs at ICF_2 generation possessing both the BB resistance genes (*Xa21* and *Xa38*) in homozygous condition and non-restoring alleles of major fertility restorer genes, *Rf3* and *Rf4*, and close resemblance with the recurrent parent were selected for further evaluation of phenotypic and agro-morphological traits. The selected BDLs were advanced to ICF_5, and strict phenotypic selection was followed in each generation while advancing the lines from ICF_3 to ICF_5 generation. The selected lines were also subjected for marker-assisted assessment of RPG recovery. All the 12 selected advanced BDLs showed a very high level of BB resistance against all the eight *Xoo* strains used, demonstrating broad-spectrum resistance conferred by the combination of two BB resistance genes. Individually, BB resistance gene *Xa21* (IRBB21) showed a moderate reaction to some of the *Xoo* strains used. BB resistance gene, *Xa38*, showed a resistant reaction to all the *Xoo* strains except IX-116 to which it showed a lesion length more than 10 cm. However, the average lesion length of the BDLs was below 0.5 cm indicating a synergistic effect of these two BB resistance genes. The recurrent parent APMS 6B showed a very high degree of susceptibility with lesion length ranging from 16.6 to 23.9 cm (Table 1; Fig. 3). The data indicate that combination of BB resistance genes, *Xa21* and *Xa38*, provides high degree of resistance to all the *Xoo* strains used in the present study. BB resistance gene *Xa21* provides broad-spectrum resistance to majority of the pathotypes in India and is widely used in gene pyramiding program (Gopalakrishnan et al.

2008; Sundaram et al. 2008, 2009; Basavaraj et al. 2010; Hari et al. 2011; Abhilash Kumar et al. 2016). However, there are reports of existence of *Xa21*-breaking strains of *Xoo* in India (Mishra et al. 2013; Yugander et al. 2017). In our study also, two *Xoo* strains (IX-133 and IX-234) exhibited susceptibility to *Xa21*. The other gene deployed in this study, *Xa38*, provides a high level of resistance to BB against most of the prevailing races of *Xoo* from Punjab state of India (Cheema et al. 2008). In our study, the gene has exhibited wide-spectrum resistance to all the strains of *Xoo* except IX-116 (Supplementary Fig. S2). Ellur et al. (2016) also reported that *Xa38* showed a susceptible reaction to race 4 of BB pathogen. The combination of *Xa21* and *Xa38* in the selected advanced BDLs exhibited broad-spectrum BB resistance as compared to the parental lines containing only *Xa21* developed in many earlier studies (Chen et al. 2000; Hari et al. 2011), and this is also evident in our study (Table 1).

Retention or improvement of the agro-morphological characters and yield in the advanced backcross-derived improved lines is very essential for the success of a MAS program. Many marker-assisted breeding programs aim at introgression of target traits using gene-specific markers and also involve strong phenotypic selection for retaining or improvement of agro-morphological characters and yield of the recurrent parent (Joseph et al. 2004; Sundaram et al. 2008; Basavaraj et al. 2010; Hari et al. 2011, 2013; Balachiranjeevi et al. 2015; Abhilash Kumar et al. 2016; Ellur et al. 2016). When the 12 selected BDLs were evaluated for different agro-morphological characters, they were observed to be on par with APMS 6B or better with respect to some of the plant characters like number of grains/panicle and mean grain weight/plant. Identification of such two-gene pyramid lines with higher grain yield was possible in the present study because of the stringent phenotypic selection followed during advancement of elite homozygous breeding lines from ICF_2 generation onwards, and similar observations have been reported in other studies involving MABB coupled with phenotypic selection (Balachiranjeevi et al. 2015; Abhilash Kumar et al. 2016). The BDLs, GSY nos. 4, 308, and 411, were observed to be promising with respect to higher grain weight/plant. Few of the BDLs, viz. GSY nos. 141 and 279, matured early by 5–6 days, and these lines may help in developing elite hybrids with duration shorter as compared to DRRH 3. All the BDLs showed more than 93% recovery of the RPG and retained the highly preferred, medium slender grain type of APMS 6B. Assessment of maintenance ability of the improved maintainer lines is vital before their actual conversion to WA-CMS lines. A total of 12 advanced improved lines were test crossed with CMS line, APMS 6A, to test the sterility of the generated F_1 plants. The F_1 plants derived from nine BDLs showed complete sterility indicating the maintaining ability of these two-gene pyramid lines. They are being crossed with APMS 6A for line conversion using MAS as described by Hari et al. (2013) and Balachiranjeevi et al. (2015). Three of the 12 BDLs did not show complete pollen sterility in our study despite possessing non-restorer alleles at both *Rf3* and *Rf4* (as inferred from marker analysis). This could be due to presence of other minor QTLs which are known to be involved in the fertility restoration in WA-CMS lines (Balaji Suresh et al. 2012; Viraktamath et al. 2013).

Bacterial blight of rice is a major production constraint in India especially during wet season. In the present study, we have developed improved versions of the stable maintainer line, APMS 6B, which possess a very high level of BB resistance (conferred by two major, dominant BB resistance genes *Xa21* and *Xa38*) and marginally higher yields as compared to the recurrent parent. Furthermore, most of the improved lines also retained the complete sterility maintenance ability, and all of them possessed medium slender grain type like APMS 6B. The improved two-gene pyramid lines of APMS 6B, after their line conversion, will be helpful in development of disease-resistant and high-yielding versions of the elite rice hybrid, DRRH 3, and thus benefitting the farmers and rice consumers.

Acknowledgements The authors are thankful to the Director, ICAR-IIRR, for providing the research facilities.

Funding information Funding support was provided by Indian Council of Agricultural Research (ICAR) and Department of Biotechnology (DBT) (Grant No. BT/AB/FG-2 (PH-II)/2009), New Delhi, India.

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