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Pyramiding of two bacterial blight resistance and a semidwarfing gene in Type 3 Basmati using marker-assisted selection

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Abstract A traditional Type 3 Basmati rice cultivar grown in India is tall and lodges even under low nitrogen fertilizer dose. In addition to lodging, it is highly susceptible to several diseases and pests including bacterial blight (BB). BB resistance genes (*Xa21* and *xa13*) and a semidwarfing gene (*sd-1*) were pyramided in Type 3 Basmati from a rice cultivar PR106-P2 using marker-assisted selection (MAS). Foreground selection for BB resistance genes, *Xa21* and *xa13* and reduced plant height gene, *sd-1* was carried on the basis of linked molecular markers pTA248, RG136 and 'h', respectively. The BC_2F_3 progenies with both the BB resistance genes were highly resistant with lower lesion length than either of the genes individually. Background profiling of the selected 16 BC_2F_3 progenies was done using 95 anchored SSR and 12 ISSR markers. Among the selected 16 BC_2F_3 progenies, 38-5-2 and 38-5-36 closely clustered along with the recipient parent Type 3 Basmati showing above 85% genetic similarity with the same. Further selection was continued till F_5 generation for higher recovery for Type 3 Basmati characteristics. The desirable alleles of intermediate amylose content (*wx*) and aroma (*fgr*) loci of Type 3 Basmati were also tracked using the linked SSR markers. The BC_2F_5 pyramid lines T3-4, T3-5, T3-6 and T3-7 homozygous for the three target genes *Xa21*, *xa13* and *sd-1* from the donor parent with *wx* and *fgr* alleles of Type 3 Basmati had excellent cooking quality and strong aroma.

Keywords Basmati rice · Bacterial blight · Resistance gene · *Xanthomonas oryzae* pv. *oryzae* · MAS · Background selection · Pyramiding · Semidwarf

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Introduction

Basmati, the unique aromatic quality rice, is a nature's gift to Indian sub-continent which has been

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grown in the foothills of the Himalayas for hundreds of years. The traditional tall basmati varieties, Taroari followed by Basmati 370 and Type 3 Basmati (Dehraduni) have maintained supremacy over other varieties, in the export markets, due to their exquisite quality characteristics. Type 3 Basmati with short-day photosensitive growth habit, flowers in the second week of October and matures in the third week of November in northern India thus maintaining its strong aroma and excellent cooking quality. Type 3 Basmati possesses long, slender, translucent grains with strong and pleasant aroma. Cooked rice is almost of double the length of uncooked raw milled rice, non-sticky, moist and tender to eat with excellent palatability. Traditional basmati varieties including Type 3 Basmati grown in India are, however, low yielding, tall, and susceptible to lodging even under low nitrogen fertilizer dose. In addition to lodging, all the traditional basmati varieties are highly susceptible to bacterial blight (BB), thus making basmati rice a high-risk crop for farmers. In rice, about 31 bacterial blight resistance (R) genes (22 dominant and 9 recessive) have been identified and mapped (Chu et al. 2006; Wang et al. 2009). The recessive R gene, *xa13* was first characterized in the rice variety BJ1 and fine-mapped to a genomic region <4 cM on the long arm of rice chromosome 8 (Zhang et al. 1996; Sanchez et al. 1999). The broad spectrum bacterial blight resistance gene *Xa21* was introgressed from a wild species *O. longistaminata* onto *O. sativa* chromosome 11 (Khush et al. 1989). BB resistance dominant gene *Xa21*, has been found to confer resistance to diverse BB pathotypes (Khush et al. 1990; Ikeda et al. 1991). A RFLP marker RG103 was found to be tightly linked to *Xa21* at a distance of 1.2 cM. Based on RG103 marker, a PCR-based STS marker pTA248 was developed, which can be used efficiently in marker-assisted selection (Ronald et al. 1992). Marker-assisted selection allows the selection of plants with multiple resistance genes hitherto difficult with the conventional breeding approaches.

The dwarfing *sd-1* gene was first identified in the Chinese variety Dee-geo-woo-gen (DGWG) which was crossed in the early 1960s with Peta (tall) to develop a semidwarf 'Green Revolution' cultivar IR8 (IRRI 1967). The *sd-1* gene confers semidwarf stature, improves lodging tolerance (Pinthus 1973) and harvest index (Walcott and Laing 1976). The semidwarf gene *sd-1* has been mapped on chromosome 1 of rice and cloned (Monna et al. 2002; Sasaki et al. 2002) and a perfect PCR based marker 'h' has been developed (Ellis and Spielmeyer 2002). A locus *fgr* with a major effect on aroma has been mapped on chromosome 8 of rice (Lorieux et al. 1996; Garland et al. 2000) and a gene controlling intermediate *amylose* content on chromosome 6 (Ayres et al. 1997).

The conventional breeding methods to improve traditional basmati cultivars have not met with much success. Marker-assisted selection for pyramiding important genes along with rapid background recovery of the recurrent parent (Xu and Crouch 2008), while maintaining the exquisite quality characteristics of basmati rice could be more effective approach for its improvement. Huang et al. (1997) developed near isogenic and pyramid lines for four bacterial leaf resistance genes *Xa4*, *xa5*, *xa13* and *Xa21* in IR24 rice line using marker assisted selection and found that the pyramid lines had wider spectrum and higher level of resistance than lines with individual genes. Singh et al. (2001) pyramided three BB resistance genes *xa5*, *xa13* and *Xa21* in PR106 cultivar using MAS and during testing with 17 *Xanthomosnas oryzae* pv. *oryzae* (*Xoo*) isolates under artificial inoculation and field conditions in Punjab found that the combination of genes provided wider spectrum of resistance to the pathogen populations prevalent in the region. The extent of background recovery in the BC_2F_3 lines of PR106 using molecular markers was, however, not established. Joseph et al. (2004) were able to recover 80.4–86.7% recurrent parent background of Pusa Basmati 1 in BC_1F_3 with two BB resistance genes, *xa13* and *Xa21* along with grain and cooking quality characteristics and desirable agronomic features by a combination of phenotypic and molecular marker aided selection. Sundaram et al. (2008) were able to pyramid three BB resistance genes *xa5*, *xa13* and *Xa21* using marker assisted backcross breeding in an elite rice variety Samba Mahsuri along with its nearly 97% background recovery by BC_4F_1 through foreground and background selection during each backcross generation.

The article reports marker aided pyramiding of bacterial leaf blight resistance genes, *Xa21* and *xa13* along with the semidwarfing gene *sd-1* in the traditional Indian basmati rice cultivar Type 3 Basmati and marker assisted background profiling of selected BC_2F_3 progenies using rice SSR and ISSR markers.

Materials and methods

Plant material

Type 3 Basmati cultivar also called as Dehraduni Basmati was taken as the recurrent parent. *O. sativa* line PR106-P2 was used as the donor parent for two bacterial blight resistance genes *xa13* and *Xa21* and a semidwarfing gene *sd-1*. The semidwarf PR106-P2 line with the genes was developed through MAS at the Punjab Agricultural University, Ludhiana (Singh et al. 2001). Crosses were made between Type 3 Basmati (tall and BB susceptible cultivar) and PR106-P2 and the F_1 plants were backcrossed with Type 3 Basmati. Among the BC_1F_1 plants, polymerase chain reaction (PCR)-based molecular markers linked to *xa13*, *Xa21* and *sd-1* were used to select plants with BB resistance and semidwarfing alleles of the donor line. A similar strategy was used in BC_2F_1 to obtain BC_2F_2 generation from which lines with *xa13*, *Xa21* and *sd-1* genes were selected using the linked molecular markers. The BC_2F_2 plants were selfed to obtain BC_2F_3 progenies. The BC_2F_3 progenies homozygous for one or more of the target genes (BB resistance and semidwarfing) were identified on the basis of molecular marker analysis and BB incidence under artificial inoculation conditions, photosensitivity and plant height. Location and year wise details of development of plant material, advancement of generations, foreground and background selection leading to the development of pyramid lines are given in schematic diagram (Fig. 1).

Screening for BB resistance

Seven *X. oryzae* pv. *oryzae* (*Xoo*) isolates, each representing seven pathotype groups of northern India, used for testing the resistance in cultivars and segregating materials, were maintained on potato sucrose peptone agar (PSPA) at 4°C. Plants selected on the basis of molecular marker analysis from the F_1, BC_1F_1, BC_2F_1, BC_2F_2 and BC_2F_3 generations were inoculated with a mixture of seven predominant *Xoo* isolates from northern India. The plants were clip inoculated at maximum tillering stage with the *Xoo*

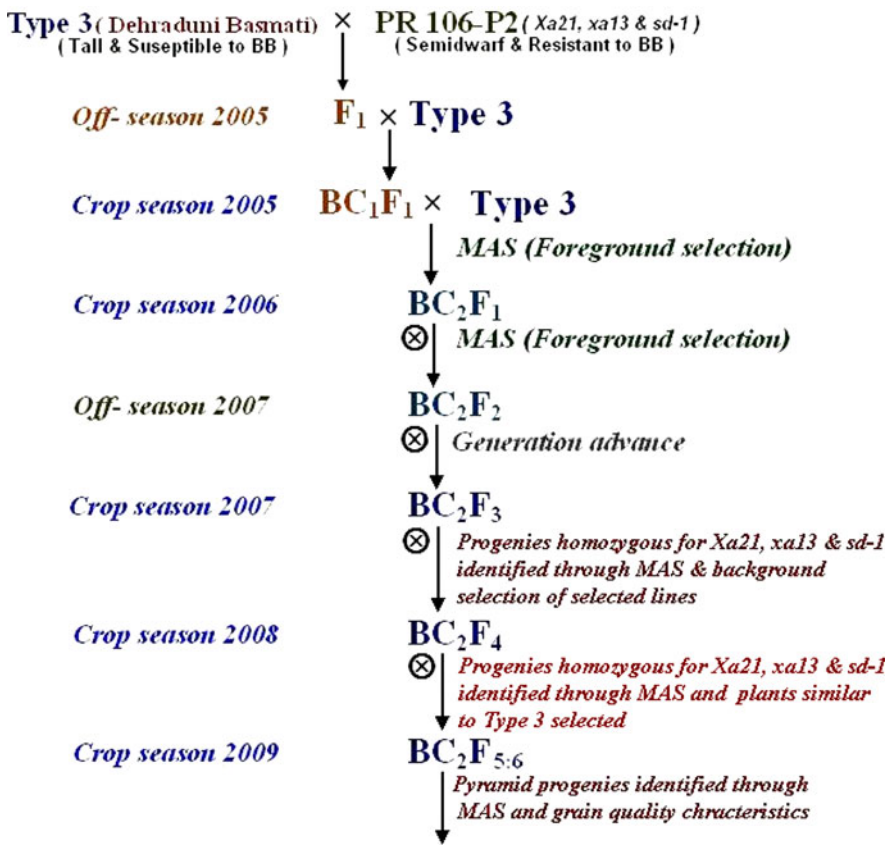


Fig. 1 Schematic diagram for pyramiding bacterial blight resistance genes into Type 3 Basmati cultivar through MAS

isolates (Kauffman et al. 1973). On an average five leaves per plant were inoculated and the lesion length was measured 14 days after inoculation.

Marker-assisted selection

Three STS markers pTA248 and RG136 (Huang et al. 1997) tightly linked to BB resistance genes *Xa21* and *xa13*, respectively and 'h' (Monna et al. 2002) tightly linked to semidwarfing gene were used to monitor the presence of each gene and their different combinations. BC_2F_3 progenies were analyzed and classified for markers representing two or three gene combinations (Table 1). The plants homozygous for all three genes, and their homozygous and heterozygous combinations were identified and inoculated with *Xoo* isolates collected from northern India. In addition to this, two rice SSR markers RM190 (Temnykh et al. 2000) and RM42 (Jain et al. 2006) linked to *wx* gene for intermediate amylose content and fragrance gene, *fgr* for aroma, respectively, were used to screen BC_2F_3, BC_2F_4 and $BC_2F_{5:6}$ progenies (Table 1).

DNA extraction and PCR amplification

Midiscale DNA isolation for PCR analysis of the parents and backcross progenies was carried out following the procedure described by Murray and Thompson (1980). The PCR reaction mixture contained 50 ng template DNA, 0.25 µM of each primer, 0.05 mM dNTPS, 1× PCR buffer (10 mM Tris pH 8.4, 50 mM KCl, 1.8 mM $MgCl_2$ and 0.01 mg/ml gelatine) and 1 U Taq DNA polymerase in a volume of 20 µl. Template DNA was initially denatured at 94°C for 5 min followed by 30 cycles of PCR amplification with the following parameters for *Xa21*, *xa13*, *sd-1*and SSRs; each cycle with 1 min denaturation at 94°C, 1 min primer annealing at 55°C and 2 min of primer extension at 72°C. The amplified product was resolved on 1% agarose in 1× TAE buffer. For identification of amplification of *xa13* gene initially, 5 µl of PCR product was used for gel electrophoresis. The remaining PCR product was used for restriction digests. The reaction mixture for restriction of PCR amplicon consisted of 3.2 µl sterile distilled water, 1.5 µl restriction buffer (10×), 0.3 µl restriction enzyme *Hinf1* (10 U/µl) and 15 µl of PCR product. Incubation of the reaction mixture varied from 4 h to overnight at 37°C. The DNA fragments produced by restriction were separated by gel electrophoresis on 2.5% agarose and visualized under UV light after staining with ethidium bromide.

Marker-assisted background profiling

The background profiling of the BC_2F_3 progenies selected for target genes, reduced plant height and grain characteristics similar to Type 3 Basmati was done using anchored rice polymorphic SSR markers and ISSR markers. Out of 209 mapped rice markers, a set of 95 SSR markers found polymorphic between the two parents and uniformly spread over 12 rice linkage groups were selected as based on the linkage maps of Akagi et al. (1996), Temnykh et al. (2000,

Table 1 Various genes, their chromosome location, linked molecular markers and their PCR primers used for marker assisted selection

Gene (trait)	Chromosome	Linked marker	Primer pair	Reference
Xa21 (BB resistance)	11	pTA248	F: 5′-AGACGCGGAAGGGTGGTTCCCGGA-3′ R: 5′-AGACGCGGTAATCGAAGATGAAA-3′	Huang et al. (1997)
xa13 (BB resistance)	8	STS-RG136	F: 5′-TCCCAGAAAGCTACTACAGC-3′ R: 5′-GCAGACTCCAGTTTGACTTC-3′	Huang et al. (1997)
sd-1 (Semidwarfing)	1	'h'	F: 5′-GACTCAACAGGCCCTCCAAA-3′ R: 5′-CCACGCGGTTATTGCAAGTT-3′	Monna et al. (2002)
wx (Intermediate amylose content)	6	RM190	F: 5′-CTTTGTCTATCTCAAGACAC-3′ R: 5′-TTGCAGATGTTCTTCCTGATG-3′	Temnykh et al. (2000), Akagi et al. (1996)
fgr (Aroma)	8	RM42	F: 5′-ATCCTACCGCTGACCATGAG-3′ R: 5′-TTTGGTCTACGTGGCGTACA-3′	Jain et al. (2006), Temnykh et al. (2000)

2001), Garland et al. (2000) and McCouch et al. (2002) for background recovery. 12 ISSR markers (Nagaraju et al. 2002) were also used for background recovery of the selected progenies.

DNA amplicons obtained with all the above markers were scored visually for the presence (1) and absence (0) of parental alleles for all the 16 BC_2F_3 progenies and the parents. Genetic similarities between the genotypes were measured by the Dice similarity coefficient (Nei 1973) values based on the proportion of shared alleles using different modules of NTSYS ver 2.1 software package (Rohlf 2000). Sequential agglomerative hierarchical nested (SAHN) clustering was performed on the similarity matrix using Dice coefficients and the unweighted paired group method with arithmetic averages (UPGMA) subprogram of NTSYS ver 2.1 (Rohlf 2000). In BC_2F_3 progenies, the proportion of genome of the recipient parent was estimated using the formula of Sundaram et al. (2008):

$$G = [(X + 1/2Y) \times 100]/N$$

where N = total number of parental polymorphic markers screened, X = number of markers showing homozygosity for the recipient parent alleles, Y = number of markers showing heterozygosity for the parental alleles.

Analysis of quality characteristics

The amylose content of the selected progenies was estimated according to Juliano (1971). Aroma was analyzed from the polished and cooked kernels by panel of four persons including a specialized biochemist. Cooking quality was estimated by cooking 25 intact polished grains. The grains were soaked in 20 ml of distilled water for 30 min in test tubes. The tubes were placed in vigorously boiling water for 10 min and then cooled in cold water. The average length and width of 10 intact cooked kernels was measured in mm and the length/width (L/W) ratio calculated from the average.

Results

Validation of linked molecular markers

The transferability and polymorphism of the STS markers pTA248 (linked to *Xa21*), RG136 (linked to *xa13*) and '*h*' (linked to *sd-1*) was done using the donor and the recipient parents. The amplified product with marker pTA248 from the resistant donor line was of 1,000 bp while that from the susceptible parent Type 3 Basmati it was about 650 bp which could be easily resolved on 1% agarose (Fig. 2). Amplicon with CAPS marker RG136 linked to *xa13* was monomorphic (1,100 bp) in both the parents. Restriction digestion of the PCR product with *Hinf*1 generated polymorphism between the parents (Huang et al. 1997). When digested with *Hinf*1, the resistant donor PR106-P2 gave three fragments of approximately, 520, 450 and 130 bp (not visible) whereas the susceptible variety Type 3 gave only two fragments, 970 and 130 bp. The primer pair 'h' for *sd-1* gene gave amplification of approximately 850 bp in the dwarf parent while an amplicon of approximately 820 bp in the tall parent Type 3 Basmati. RM190 was used for monitoring intermediate amylose content. RM190 amplified 120 bp fragment in PR106-P2 and 130 bp in Type 3. For confirming the presence of *fgr* gene, the linked marker RM42 gave an amplification of 170 bp in PR106-P2 and 165 bp in Type 3 (Fig. 2). So all the markers reported to be linked to various target genes were validated for their transferability and polymorphism between the parents.

Pyramiding of BB resistance genes into Type 3 Basmati through MAS

The presence of molecular markers linked to BB resistance genes and semidwarfing gene was confirmed in F_1 plants which were backcrossed with Type 3 Basmati resulting in 310 BC_1F_1 seeds. Out of 212 BC_1F_1 plants established only 21 were found heterozygous for all the three target genes of the donor parent (*Xa21*, *xa13* and *sd-1*) using linked molecular markers and the same were backcrossed with Type 3 Basmati to get 956 BC_2F_1 seeds. The selected BC_1F_1 plants were also tested for resistance to BB under artificial inoculation with a mixture of *Xoo* isolates. Co-dominance of all the three markers linked to *xa13*, *Xa21* and *sd-1* helped to identify BC_1F_1 and BC_2F_1 plants heterozygous for a dominant *Xa21* and two recessive genes *xa13* and *sd-1* for effective selection of plants with all the three genes for further backcrossing. This is evident from the non-significant χ^2 values indicating goodness of fit of 1:1 ratio of homozygous Type 3 Basmati and heterozygous

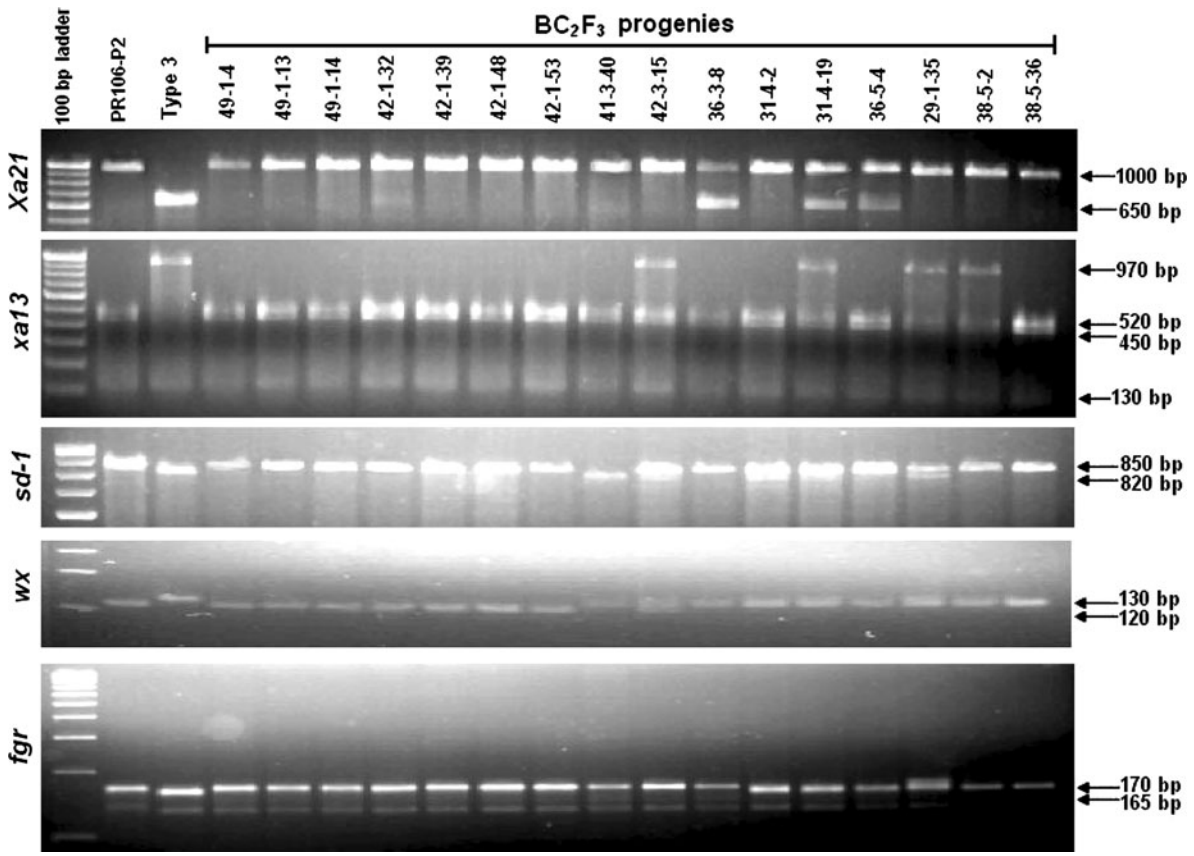


Fig. 2 PCR analysis of the parental lines and foreground selection in BC_2F_3 progenies. DNA amplified using pTA248 primers linked to *Xa21*, RG136 (digested with *Hinf*I) linked to *xa13* and 'h', RM190 and RM42 linked to semidwarfing *sd-1*, intermediate amylose content, *wx* and fragrance, *fgr* genes, respectively

genotypes in both the backcrosses (Table 2). 802 BC_2F_1 plants were established from 956 BC_2F_1 seeds. Out of 802 BC_2F_1 plants, 32 BC_2F_1 plants heterozygous for all the 3 genes of the donor parent were identified using linked molecular markers and screening for BB resistance. 200 seeds from each of the selected plants were grown at the Central Rice Research Institute, Cuttack, Orissa for generation advance and around 150 plants were transplanted giving a total of 4,852 BC_2F_2 plants. Individual ($BC_2F_{2:3}$) plants were harvested. The 4,852 BC_2F_3 progenies were grown at two locations viz., Indian Institute of Technology, Roorkee and Sardar Vallabh Bhai Patel University of Agriculture and Technology, Meerut in 2007. Marker assisted selection was done for *Xa21*, *xa13* and *sd-1* for BB resistance and semidwarfing genes (Fig. 2). Disease reaction was recorded in BC_2F_3 progenies containing individual *xa13* and *Xa21* genes or combination of both under artificial inoculation conditions**.** Only a part (40 progenies each) of the data on BB incidence among hundreds of BC_2F_3 progenies under artificial inoculation condition using mixture of seven *Xoo* isolates (Table 3) showed that the progenies homozygous for both the effective alleles *Xa21* and *xa13* had lower mean of BB lesion length than the individual allele at a time. Homozygous recessive *xa13xa13* was more effective than *Xa21Xa21* gene. Type 3 Basmati without any of the effective alleles is highly susceptible whereas the pyramid donor line PR106-P2 was highly resistant against the mixture of *Xoo* isolates used for inoculation.

Some of the BC_2F_3 progenies showed segregation for *sd-1* gene. 80 BC_2F_3 lines with all the 3 genes *Xa21*, *xa13* and *sd-1* mostly in homozygous condition were selected. Out of 80 progenies, 16 BC_2F_3 progenies with high similarity to Type 3 Basmati with respect to seed size and length/width ratio were

Table 2 Segregation of BC_1F_1 and BC_2F_1 plants at three marker loci linked to BB resistance and semidwarfing genes

Genes	Segregation in BC_1F_1			Segregation in BC_2F_1		
	Homozygous	Heterozygous	χ^2 (1:1)*	Homozygous	Heterozygous	χ^2 (1:1)*
Xa21	88	106	0.196	264	267	0.926
xa13	89	105	0.250	249	282	0.152
sd-1	91	103	0.388	252	279	0.241

*Data gave a good fit to 1:1 ratio at $P \leq 0.05$ and 1 df

Table 3 Range of lesion length in the BC_2F_3 progenies under field inoculation conditions with a mixture of seven *Xoo* isolates

Gene combination[a]	No. of progenies screened	Range		
		Lesion length	Mean	Standard error
Xa21Xa21/*Xa13Xa13*	40	1.5–6.5	3.36–4.69	0.14–0.39
xa21xa21/***xa13 xa13***	40	1.0–3.4	1.89–2.75	0.07–0.25
Xa21Xa21/xa13 xa13	40	0.5–3.0	1.35–2.27	0.08–0.24
Xa21Xa21/xa13 xa13	PR106-P2	0.9–1.6	1.36	0.07
xa21xa21/Xa13Xa13	Type 3	18.0–27.0	23.2	0.94

[a] Alleles of *Xa21* and *xa13* providing BB resistance are highlighted as *bold* font

selected for background profiling and further selection. Among 16 BC_2F_3 progenies, 49-1-4, 49-1-13, 42-1-14, 42-1-32, 42-1-39, 42-1-48, 42-1-53, 31-4-2 and 38-5-36 were homozygous for *Xa21*, *xa13* and *sd-1*, 41-3-40 was homozygous for *Xa21* and *xa13* only (Table 4). Progenies 36-3-8 and 36-5-4 were homozygous only for *xa13* and *sd-1* target genes. 42-3-15 and 38-5-2 were homozygous only for *Xa21* and *sd-1* genes. 31-4-19 was homozygous for *sd-1* while heterozygous for both the BB resistant genes. Two progenies 38-5-36 and 31-4-2 were homozygous for three genes *Xa21*, *xa13* and *sd-1* from the donor parent along with intermediate amylose content and aroma loci of Type 3 (Fig. 2; Table 4). However, in BC_2F_4 all these lines achieved homozygosity for *Xa21*, *xa13* and *sd-1*, except 31-4-19-3 and 42-3-15-7 without *xa13* and 41-3-40-4 in which *sd-1* was absent (Table 5).

Most of the BC_2F_3 progenies selected for generation advance and further selection for background recovery had short lesion length (Table 4) slightly longer than the donor parent PR106-P2 with three BB resistance genes *Xa21*, *xa13* and *xa5*. All the lines heterozygous for *xa13* had longer lesion length (3.05–3.71) as compared to the remaining lines homozygous for recessive allele *xa13xa13* and heterozygous for *Xa21*. All the selected progenies were photosensitive similar to the traditional Type 3 Basmati so that these could be planted late with monsoon on set for flowering in October and maturity in November facilitating superior grains and cooking quality characteristics. All of these homozygous progenies except 29-1-35 and 41-3-40 were still taller than the donor parent PR106-P2 with *sd-1* indicating that the selection for photosensitivity, controlling flowering during short days, probably led to their prolonged vegetative growth. Two lines, 41-3-40 and 29-1-35 without *sd-1* and heterozygous for *sd-1*, respectively were nearly as tall as that of Type 3 Basmati. Heterozygosity for the target traits and lower than the expected (87.5%) background recovery in some BC_2F_3 progenies suggests that continued MAS among progenies in subsequent selfed generations would not only lead to higher background recovery but also homozygosity for the target traits needed for stability and limited testing for their release.

SSR and ISSR markers based background recovery among BC_2F_3 progenies

Out of 209 rice SSR markers tested, a set of 95 markers uniformly distributed over 12 rice linkage maps showed polymorphism between the parents. These markers along with 12 ISSR markers (Nagaraju et al. 2002) were

Table 4 Various genes and genotypes of their linked markers, BB incidence and plant height of 16 BC_2F_3 progenies of pyramid lines of Type 3 Basmati selected for background profiling

S. no.	Parents and pyramid lines	*Xa21* pTA248	*xa13* RG136	*sd-1* 'h'	*wx* RM190	*fgr* RM42	Lesion length	Plant height	% Background recovery[a]
1.	PR106-P2	+	+	+	−	−	1.36 ± 0.21	60.6 ± 1.58	–
2.	Type 3	−	−	−	+	+	23.2 ± 2.97	158.1 ± 2.23	–
3.	29-1-35	+	H	H	+	+	3.23 ± 0.39	150.6 ± 3.69	92.10
4.	31-4-2	+	+	+	+	+	2.22 ± 0.38	119.4 ± 2.41	91.05
5.	31-4-19	H	H	+	+	+	3.25 ± 0.35	104.1 ± 4.01	89.47
6.	36-3-8	H	+	+	+	−	2.65 ± 0.22	106.7 ± 2.50	87.89
7.	36-5-4	H	+	+	+	H	2.56 ± 0.25	110.1 ± 2.81	87.89
8.	38-5-2	+	H	+	+	+	3.05 ± 0.39	106.2 ± 2.97	85.78
9.	38-5-36	+	+	+	+	+	2.11 ± 0.65	96.6 ± 2.50	88.42
10.	41-3-40	+	+	−	+	−	1.69 ± 0.50	154.8 ± 1.69	81.57
11.	42-1-14	+	+	+	−	−	1.98 ± 0.51	97.5 ± 4.48	84.73
12.	42-1-32	+	+	+	−	−	2.27 ± 0.52	105.8 ± 3.01	86.31
13.	42-1-39	+	+	+	−	−	1.77 ± 0.43	107.4 ± 4.01	85.78
14.	42-1-48	+	+	+	−	−	2.07 ± 0.45	94.8 ± 2.53	85.78
15.	42-1-53	+	+	+	−	−	1.82 ± 0.38	95.3 ± 2.36	85.78
16.	42-3-15	+	H	+	H	−	3.71 ± 0.60	104.4 ± 3.31	90.00
17.	49-1-4	+	+	+	−	−	2.24 ± 0.48	97.8 ± 3.85	84.73
18.	49-1-13	+	+	+	−	−	2.12 ± 0.53	104.4 ± 1.58	84.73

+ Homozygous; − absent

H heterozygous

[a] Background recovery based on SSR markers

used for background recovery. 16 BC_2F_3 progenies with nearly Type 3 Basmati seeds were finally selected for background profiling. The PCR profiles of seven representative SSR markers in parents and selected progenies used for background recovery are given in Fig. 3. On the basis of SSR markers, these lines showed background recovery from 81.57% (41-3-40) to 92.10% (29-1-35). The genetic similarities among different lines obtained from both the marker systems were analyzed to find out the clustering of progenies among each other and the parents. The maximum genetic similarity coefficient (0.99) between any two genotypes was detected by combined marker data between 49-1-4 with 49-1-13 and 49-1-4 with 49-1-14 progenies. The minimum genetic similarity coefficient (0.65) between any two progenies was detected between 31-4-19 and PR106 with the combined SSR and ISSR marker data. All the major clusters were supported by reasonably high bootstrapping values indicating a good fit of the data for diversity analysis. All the 16 progenies and the recipient parent Type 3 Basmati fell in one group with two major clusters (Fig. 4). Cluster I consisted of recipient parent Type 3 and two progenies 38-5-2 and 38-5-36 with high boot strap value of 86%. Cluster II consisted of the remaining 14 progenies derived mainly from six BC_1F_1 plants. As expected the donor parent PR106-P2 remained as a separate group. Out of 16 BC_2F_3 progenies, 31-4-2 and 38-5-36 were homozygous for BB resistance, semidwarfing, intermediate amylose content and fragrance genes along with 91.05 and 88.42% background recovery. The only line 41-3-40 without *sd-1* allele had least background recovery of 81.57%. Graphical genotypes of all the selected BC_2F_3 progenies based on SSR markers indicated higher linkage drag on the carrier chromosomes around the target genes *Xa21*, *xa13* and *sd-1* (Supplementary Fig. 1) responsible mainly for reduced background recovery.

Five best plants were selected from each of the 16 selected BC_2F_3 progenies on visual observation of paddy grain length and width and plant to row BC_2F_4

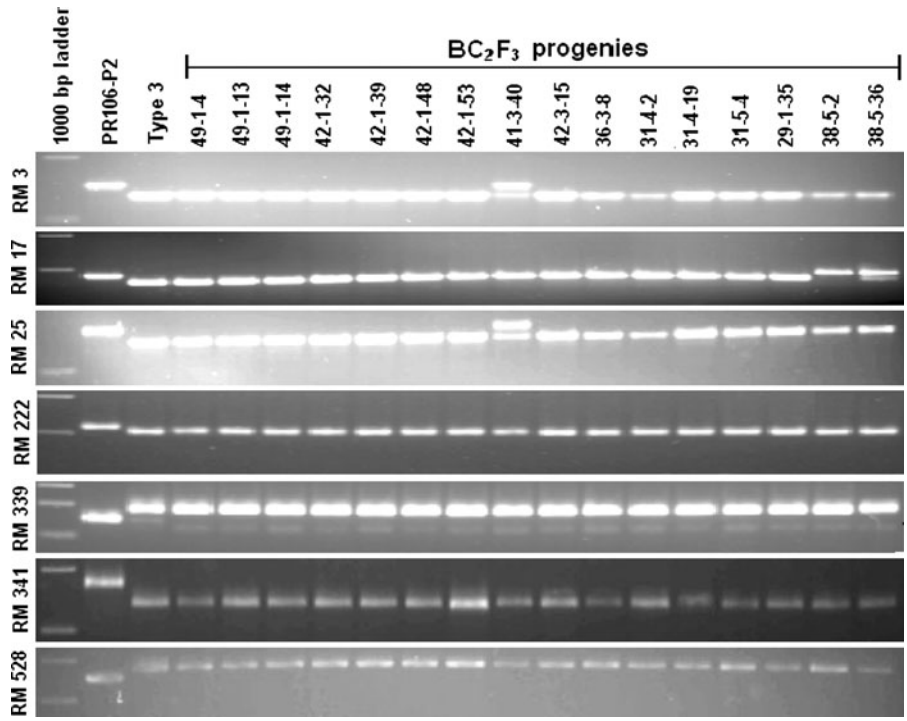
Table 5 Genes, linked marker genotypes, days to flowering and seed length/width ratio of parents and selected BC_2F_4 progenies of pyramid lines of Type 3 Basmati

S. no.	Genotypes	*Xa21*	*xa13*	*sd-1*	Days to flowering	L/W ratio of paddy rice		
		pTA248	RG136	'h'		Length	Width	Ratio
1.	PR106-P2	+	+	+	100	9.0	2.6	3.46
2.	Type 3	−	−	−	118	9.7	2.3	4.21
3.	29-1-35-9	+	+	+	118	10.5	2.6	4.03
4.	31-4-2-5	+	+	+	116	10.4	2.6	4.00
5.	31-4-2-6	+	+	+	119	9.8	2.6	3.76
6.	31-4-19-3	+	−	+	104	12.7	2.4	5.29
7.	36-3-8-5	+	+	+	119	10.6	2.5	4.24
8.	36-5-4-2	+	+	+	118	10.3	2.4	4.29
9.	38-5-2-5	+	+	+	116	10.6	2.5	4.24
10.	38-5-2-6	+	+	+	120	10.2	2.3	4.43
11.	38-5-36-2	+	+	+	118	10.7	2.6	4.11
12.	41-3-40-4	+	+	−	118	10.6	2.3	4.60
13.	42-1-14-4	+	+	+	110	10.5	2.5	4.20
14.	42-1-32-6	+	+	+	110	10.5	2.6	4.03
15.	42-1-32-9	+	+	+	108	10.5	2.6	4.03
16.	42-1-39-2	+	+	+	109	10.9	2.6	4.19
17.	42-1-39-5	+	+	+	109	11.4	2.5	4.56
18.	42-1-48-8	+	+	+	109	11.0	2.7	4.07
19.	42-1-53-5	+	+	+	116	11.2	2.7	4.14
20.	42-1-53-9	+	+	+	115	10.3	2.6	3.96
21.	42-3-15-2	+	+	+	115	10.2	2.5	4.08
22.	42-3-15-3	+	+	+	115	10.0	2.4	4.16
23.	42-3-15-7	+	−	+	130	10.5	2.4	4.37
24.	42-3-15-8	+	+	+	130	10.2	2.5	4.08
25.	49-1-4-1	+	+	+	110	10.3	2.5	4.12
26.	49-1-4-3	+	+	+	115	10.5	2.6	4.03
27.	49-1-13-1	+	+	+	120	10.5	2.6	4.03
28.	49-1-13-9	+	+	+	120	10.4	2.6	4.00

+, Homozygous; −, absent

progenies (about 50 plants per row) were grown at two locations in 2008. All the BC_2F_4 progenies were again tested on the basis of bulked DNA per progeny for the three linked markers for the BB resistance genes *Xa21* and *xa13* and one semidwarfing gene *sd-1*. Only the homozygous progenies or plants were selected for BC_2F_5. Some uniform progenies were bulked. The marker data given in Table 5 indicated that all the selected progenies except three, 31-4-19-3, 41-3-40-4 and 42-3-15-7 were homozygous for all the three markers/genes. Two of the progenies did not have favorable *xa13* allele and one did not carry semidwarfing gene *sd-1*. All the selected BC_2F_4 progenies had medium to high photosensitivity and hence flowered under short-day conditions around the flowering time of Type 3 Basmati. Most of these had paddy seed length/width ratio greater than 3.75 considered to be the threshold limit for basmati rice.

Seventeen uniform dwarf and photosensitive BC_2F_5 progenies with grains similar to Type 3 Basmati were selected for linked markers analysis of both donor and recipient parents' traits, morphological and grain quality characteristics (Fig. 5; Table 6). As expected for BC_2F_4, marker genotype



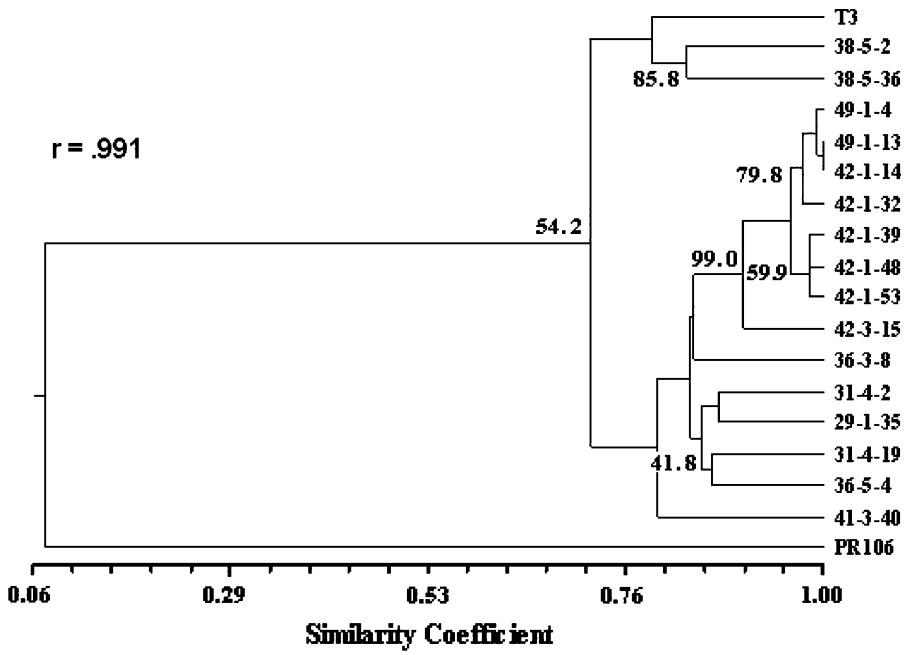


Fig. 3 Genotypes of seven representatives SSR markers in parents and selected BC_2F_3 progenies used for background profiling

Fig. 4 Dendrogram of parents and selected BC_2F_3 progenies showing similarities among themselves and parents using SSR and ISSR markers

of pyramid line T3-8 (41-3-40-4-8) lacked dwarfing gene while T3-15 (42-3-15-7-7) lacked *xa13* gene. All the other selected pyramid lines had the three genes transferred from PR106-P2 into Type 3 Basmati through MAS. In addition to those three genes, two genes of Type 3 Basmati known for basmati

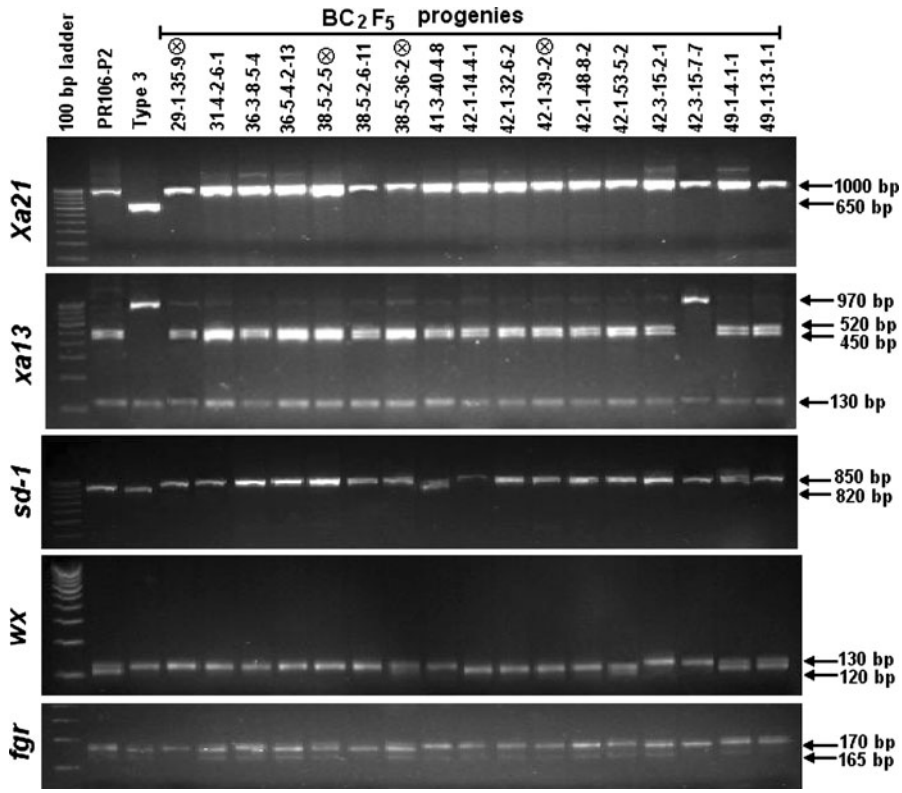


Fig. 5 PCR analysis of the parental lines and foreground selection in BC_2F_5 progenies. DNA amplified using pTA248 primers linked to *Xa21*, RG136 (digested with *Hinf*I) linked to *xa13* and 'h', RM190 and RM42 linked to semidwarfing *sd-1*, intermediate amylose content, *wx* and fragrance, *fgr* genes, respectively

characteristics viz., intermediate amylose content, *wx* (RM190) and aroma, *fgr* (RM42) were also monitored through their linked markers. Only 7/17 BC_2F_5 progenies derived from those BC_2F_3 progenies were homozygous for both the Type 3 markers linked to the quality traits. Almost, all the progenies having *sd-1* except T3-8 had plant height intermediate between the two parents and were not as dwarf as that of PR106-P2, the donor of *sd-1*. Most of the progenies being photosensitive flowered during the same time as that of Type 3 Basmati while a few with medium photosensitivity flowered a few days earlier than Type 3 Basmati. There was only one pyramid line T3-10 (42-1-32-6-2) with length/width ratio of milled rice at par with Type 3 Basmati while 9/17 had better length/width ratio of cooked rice than that of Type 3 Basmati. All of them had superior length/width ratio of milled as well as cooked rice than the donor parent PR106-P2. There was hardly any association between the presence of *wx* loci (RM190) and intermediate amylose content and length/width ratio of cooked kernels as some lines with higher (T3-13) or lower (T3-2) amylose content also gave better length/width ratio of cooked kernels.

Discussion

All the five co-dominant molecular markers linked to the target genes *Xa21*, *xa13*, *sd-1*, *wx* and *fgr* used for MAS and pyramiding (Singh et al. 2001; Sharma et al. 2001) were found to be transferable and polymorphic between the donor parent PR106-P2 and recurrent parent Type 3 Basmati. The validated markers thus could be successfully used to pyramid one dominant and two recessive genes from the donor parent simultaneously and monitor the quality characteristics of the recurrent parent in the pyramid lines. Such a task of pyramiding a dominant and two recessive genes could be accomplished only through MAS. The conventional backcross breeding requiring selfing after every backcross for selection of recessive genes could have taken twice as longer time.

On the basis of comprehensive foreground selection, phenotypic selection for morphological and quality traits, and background profiling, four BC_2F_5 pyramid lines T3-4, T3-5, T3-6 and T3-7 homozygous for all the three target genes from donor parent and two genes for quality characteristics of the recipient parent with equivalent or superior cooking

Table 6 Genotypes of various genes and their linked markers along with morphological and grain quality characteristics of $BC_2F_{5:6}$ selected pyramid lines of Type 3 Basmati at IIT Roorkee, 2009

Pyramid lines	Pedigree	*Xa21*	*xa13*	*sd-1*	*wx*	*fgr*	Plant height	Tiller no. per plant	Days to flowering	L/W ratio of milled rice			L/W ratio of cooked rice			Polished rice	
		pTA248	RG136	'h'	RM190	RM42				Length	Width	Ratio	Length	Width	Ratio	% Amylose	Aroma
	PR106-P2	+	+	+	−	−	86.80	11.2	111	6.70	2.0	3.35	10.4	2.5	4.16	24.88	None
	Type 3	−	−	−	+	+	187.60	11.4	134	7.40	1.8	4.11	12.7	2.5	5.08	21.73	Strong
T3-1	29-1-35-9⊗	+	+	+	+	−	149.20	15.6	127	7.70	2.1	3.67	14.1	2.5	5.64	12.69	Mild
T3-2	31-4-2-6-1	+	+	+	+	+	146.00	11.2	125	7.50	2.3	3.26	14.2	3.0	4.73	10.00	Mild
T3-3	36-3-8-5-4	+	+	+	+	−	125.30	10.0	134	7.60	1.9	4.00	13.0	2.6	5.00	25.38	None
T3-4	36-5-4-2-13	+	+	+	+	+	125.80	6.8	134	7.50	2.0	3.75	13.8	2.8	4.93	22.38	Strong
T3-5	38-5-2-5⊗	+	+	+	+	+	121.00	10.6	137	7.30	1.8	4.06	15.0	2.9	5.17	22.76	Strong
T3-6	38-5-2-6-11	+	+	+	+	+	143.80	14.6	143	7.10	1.9	3.74	12.5	2.2	5.68	22.07	Strong
T3-7	38-5-36-2⊗	+	+	+	+	+	145.60	14.8	134	7.80	2.0	3.90	15.1	2.6	5.81	19.23	Strong
T3-8	41-3-40-4-8	+	+	−	+	−	157.90	11.4	130	7.80	2.1	3.71	13.8	2.8	4.93	18.76	None
T3-9	42-1-14-4-1	+	+	+	−	−	132.80	15.6	117	8.00	2.1	3.81	14.9	2.6	5.73	22.23	None
T3-10	42-1-32-6-2	+	+	+	−	−	130.80	9.8	117	7.80	1.9	4.11	12.7	2.4	5.29	16.07	None
T3-11	42-1-39-2⊗	+	+	+	−	−	138.40	19.2	117	8.10	2.0	4.05	13.3	2.8	4.75	20.61	None
T3-12	42-1-48-8-2	+	+	+	−	−	128.20	12.4	115	8.00	2.2	3.64	12.8	2.9	4.41	27.23	None
T3-13	42-1-53-5-2	+	+	+	−	−	130.80	9.4	122	8.10	2.2	3.68	13.2	2.2	6.00	26.15	None
T3-14	42-3-15-2-1	+	+	+	+	+	137.80	12.2	134	7.20	2.1	3.43	12.6	2.8	4.50	22.15	None
T3-15	42-3-15-7-7	+	−	+	+	+	141.40	18.6	138	7.50	2.0	3.75	12.1	2.4	5.04	22.46	Mild
T3-16	49-1-4-1-1	+	+	+	−	−	120.60	10.2	122	8.00	2.2	3.64	13.1	2.5	5.24	26.15	None
T3-17	49-1-13-1-1	+	+	+	−	−	124.40	14.6	144	7.90	2.1	3.76	12.7	2.4	5.29	25.07	Mild

+, Homozygous; − absent

and aroma characteristics were selected for field testing and possible release. Their progenitor BC_2F_3 progenies had nearly the average expected (87.5%) background recovery. The four pyramid lines selected for homozygosity for various traits and the recurrent parent Type 3 Basmati photosensitivity, grain length/width ratio, cooking quality and aroma during two generations of selfing and selection might have further led to their higher background recovery than their parental BC_2F_3 progenies. Joseph et al. (2004) reported to have recovered favorable characteristics of Pusa Basmati 1 with two BB resistance genes through MAS just in BC_1 due to stringent phenotypic selection without any background selection during segregating generations. Sundaram et al. (2008) developed BC_4 pyramid lines of Sambha Mahsuri with three BB resistance genes *xa5*, *xa13* and *Xa21* through simultaneous foreground and background selection and recovered 97% recurrent parent background exhibiting a broad spectrum resistance against multiple *Xoo* isolates.

Incidentally, two of the pyramid lines in a cluster with recurrent parent Type 3 Basmati had strong aroma under various conditions while the donor parent had no aroma. Only four of the seven pyramid lines with marker RM42 linked to aroma gene, *fgr* had strong aroma in cooked kernels while two had mild aroma and one with none suggests that effective MAS with some certainty could be done for aroma using the linked marker RM42 in only a few lines.

BB resistance genes *Xa21 and xa13* and semidwarf gene *sd-1* have been pyramided through marker-assisted backcross breeding into a rice cultivar Type 3 Basmati from a non-basmati rice cultivar along with the retention of intermediate amylose content, *wx*, and aroma, *fgr*, genes of the recipient parent. Four pyramid lines T3-4, T3-5, T3-6 and T3-7 were found homozygous for all the five traits as based on foreground selection along with 85.75–88.42% background recovery in their parental BC_2F_3 progenies. In addition to the marker-aided selection, photosensitive BC_2F_5 progenies were selected to maintain the exquisite quality characteristic of Type 3 Basmati. In rice, successful marker-assisted pyramiding of two to three disease resistance genes *xa5*, *xa13* and *Xa21* into various rice cultivars including PR106, BPT 5204, Triguna, Pusa Basmati 1, Sambha Mahsuri has been done with and without background selection (Singh et al. 2001; Sharma et al. 2001; Joseph et al. 2004; Sundaram et al. 2008) exhibiting a broad spectrum resistance against multiple *Xoo* isolates.

16 BC_2F_3 progenies mostly homozygous for the target traits as based on MAS with high tiller number, photoperiod sensitivity and high level of resistance against bacterial blight were selected for background recovery analysis. The background recovery varied from 81.57% (41-3-40) to 92.10% (29-1-35). Theoretically with two backcrosses average background recovery should be 87.5% but in our study higher background recovery in some lines may be attributed to directional selection for photosensitivity and grain quality characteristics of recurrent parent Type 3 Basmati. Reduced background recovery in some lines is largely due to linkage drag of the donor genotype on the carrier chromosomes around three target genes *Xa21*, *xa13* and *sd-1*. Randhawa et al. (2009) reported only 82% recovery of background of recurrent wheat parent 'Zak' in BC_4F_7 with phenotypic selection without marker assisted background selection (MABS) during introgression of stripe rust resistance gene *Yr 15*. However, they could recover 97% background in $BC_2F_{2:3}$ plants using foreground selection of target traits, background selection for flanking markers, non-carrier chromosome markers and whole marker screen during two successive backcrosses using large backcross population. Similar strategy of simultaneous foreground and background selection should be followed for higher background recovery in two backcrosses.

The graphical genotype of various BC_2F_3 progenies for the markers used for background recovery showed that the background recovery for various carrier chromosomes 1, 6 and 8 was less as compared to the non-carrier chromosomes. The bacterial blight resistance gene *xa13* and a recessive gene betaine aldehyde dehydrogenase (*BAD2*) controlling aroma, linked to RG136 and RM42 markers, respectively are on the opposite arms of chromosome 8 of rice nearer to the centromere. The failure to recover the aroma gene *BAD2* of the recurrent parent Type 3 Basmati in 8/17 BC_2F_5 lines involving two backcrosses with the same is, however, not very clear. During an attempt to transfer *Tm-2* gene for tobacco mosaic virus resistance through backcross breeding in tomato, the linkage drag of 51 cM of the donor chromatin around the gene continued even after 11 backcross generations (Young and Tanksley 1989). The linkage drag could be reduced to 7–8 cM in some tomato varieties

through recombination of flanking markers tightly linked to the gene. In the present study, genotyping for the fragrance gene and background recovery was done only at the end among selected homozygous BC_2F_3 lines. An additional backcross with the recurrent parent Type 3 and strict MAS will be required to recover the effective fragrance gene in the remaining deficient lines.

Different strategies have been followed for MAS (i) each target gene is transferred separately and then the plants carrying different genes in the same background are crossed to pyramid the genes and (ii) all the genes are pyramided through simultaneous foreground and background selection. Using the second approach in this attempt it was possible to pyramid one dominant (*Xa21*) and two recessive genes (*xa13* and *sd-1*) simultaneously. The first strategy could have taken more time for pyramiding the same set of genes. However, due to lack of large backcross population required for the second approach and background profiling after two backcrosses higher background recovery, than the expected, could not be achieved. Keeping in view the progress of MAS, the best strategy to pyramid multiple genes and recover maximum background in the shortest time will be to take up their transfer simultaneously, attempt large BC_1 population, select the target genes through foreground selection and recurrent parent's flanking markers nearest to one side of the target gene. Large BC_2 population should be generated using the selected plants. Select the target genes through foreground selection and the recurrent parent's flanking markers nearest to the opposite side of the target genes to reduce the persistent linkage drag to the minimum possible. The expensive, cumbersome and time consuming background selection can be avoided and substituted by another backcross with the recurrent parent, if necessary.

Traditional basmati varieties including Basmati 370, Type 3 Basmati and Tarori etc. are highly photosensitive and flower only under short-day conditions. These were adapted primarily for rainfed cultivation during monsoon season in low lying areas of river beds of northern India. Their nursery is grown with the first monsoon shower in mid-June followed by transplanting with the onset of monsoon in mid-July. Due to inherent photosensitivity, their flowering occurs under short-day conditions in October followed by maturity in early November under cooler day temperature, thus maintaining their highly volatile aroma, prized high head rice yield and exquisite cooking quality. Utmost care was taken to select only the photosensitive progenies with all the three genes so that the exquisite quality characteristics of Type 3 Basmati could be maintained to meet consumer preferences and export quality requirements. The release and cultivation of such lines suited for late sowing could also save valuable underground water in rice growing areas which is being overexploited during rice cultivation in May to June before the onset of monsoon. Based on homozygosity for all the target and basmati traits four pyramid lines T3-4, T3-5, T3-6 and T3-7 with superior length/width ratio, excellent cooking quality and aroma have been bulked for testing for yield and disease resistance in multi-location and replicated trials for their possible release.

The emergence of *Xoo* isolates virulent on *Xa21* and *xa13* have been reported from India and abroad indicating that the deployment of rice pyramid lines with these two genes may not provide the durable resistance against bacterial blight for a longer time. Novel BB resistance genes such as *Xa30* transferred recently from *Oryza nivara* (Cheema et al. 2008) should be used for MAS in combination with *Xa21* and *xa13*. Traditional basmati varieties are also highly susceptible to blast (*Magnaporthe grisea*) for which a number of effective genes have been tagged and cloned (Sharma et al. 2005). The well characterized pyramid lines developed here can be used for pyramiding the effective blast resistance genes to develop basmati varieties resistant to both the devastating diseases.

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