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Introgression of bacterial leaf blight resistance and aroma genes using functional marker-assisted selection in rice (*Oryza sativa* L.)

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Abstract Functional markers reduce the risk of false selection in marker-assisted selection (MAS), and recombination between the molecular marker and the gene of interest. The present study was conducted with the objective of combining the important basmati quality traits with resistance to bacterial leaf blight (BLB) by a combination of phenotypic selection and MAS. Screening of 29 recombinants for BLB resistant genes along with aroma (*fgr*) gene was performed using a suite of different markers. Twenty genotypes were found in homozygous condition for *Xa21* and *xa13* genes. Restriction digestion analysis with *Bsr*I, gave nine resistant recombinants. Out of nine resistant recombinants, seven were in homozygous and two were in heterozygous conditions. Recombinants viz., IRS 5441-2-21, IRS 5441-2-79, IRS 5441-2-81, IRS 5441-2-85 and IRS 5441-2-91 possessed all the three BLB resistance genes and *fgr* in the homozygous condition. Recombinants with enhanced resistance to BLB, basmati quality and desirable agronomic traits were identified. This germplasm can be directly developed into commercial varieties or used as immediate potential donors of BLB resistance in basmati breeding programs.

Keywords Functional markers · Bacterial leaf blight · Markers assisted selection · Gene pyramiding · *Oryza sativa* L.

Abbreviations

BLB	Bacterial leaf blight
FM	Functional marker
Xoo	*Xanthomonas oryzae* pv. *oryzae*
MAS	Marker-assisted selection
CD	Critical difference
SE (m)	Standard error mean
SE (d)	Standard error difference
KOH	Potassium hydroxide

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Introduction

Basmati rice is desirable in the international market for its quality attributes, such as a distinct pleasant aroma, fluffy texture of cooked rice, and high volume expansion during cooking, which is characterized by linear kernel elongation with minimum breadth wise swelling, palatability, easy digestibility and long shelf life. Bacterial leaf blight (BLB) of rice caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a major

pathogen that negatively impacts rice production, especially in irrigated and rainfed lowland agricultural production systems (Mew et al. 1992). The bacterium invades the xylem tissue, either through wounds or stomata, leading to systemic infection. Host resistance has been shown to be the only reliable, economical and environmental-friendly method to control this disease; genetically coming from non-basmati sources. The indica varieties have been found to be generally more susceptible to BLB than japonica varieties. There is no known source of BLB resistance in the available basmati rice germplasm.

To date, 21 resistance genes including *Xa4*, *xa5*, *xa13* and *Xa21* have been identified (Wang et al. 2007; Ram et al. 2010), which have been deployed by traditional breeding methods to control the disease. In the basmati germplasm, however, no cultivar has been reported to have resistance to BLB, which has necessitated the transfer of resistance genes from non-basmati sources. However, transfer of a single resistance gene is not likely to be effective because large scale and long term cultivation of such resistant varieties may result in significant shifts in the virulence pattern of the pathogen population leading to breakdown of resistance (Mew et al. 1992). The deployment of rice cultivars that have multiple BLB resistance genes is expected to lead to more durable resistance. Studies conducted to identify the best gene combinations conferring broad spectrum resistance showed that a four gene (i.e., *Xa4* + *xa5* + *xa13* + *Xa21*) combination was the most effective and did not show any sign of breakdown of resistance to various strains of the pathogen (Shanti and Shenoy 2005; Nayak et al. 2008).

Pyramiding multiple resistance genes in a single rice variety is suggested as a strategy to prevent or delay the breakdown of resistance. The probability of simultaneous pathogen mutations for virulence to overcome the resistance conferred by two or more effective genes is much lower than for a single gene (Mundt 1990). Practical examples of gene pyramiding for BLB resistance were not available until recently because of the difficulty in identifying lines containing this many genes using conventional breeding methods. For instance, it is difficult to identify plants having more than one resistance gene if there is a major dominant gene such as *Xa21*, which confers resistance against many races. Availability of tightly linked molecular markers makes it possible to identify plants with multiple resistance genes. Many BLB resistance genes have been mapped relative to molecular markers (Ronald et al. 1992) that have been used for gene pyramiding in rice (Huang et al. 1997; Sanchez et al. 2000; Singh et al. 2001).

The risk of recombination between the molecular marker and the gene of interest has led to a false selection in marker-assisted selection (MAS), whereas it was overcome by the use of functional markers (FMs) (Ingvardsen et al. 2008). FMs were successfully designed for contexts within coding sequences of different resistance genes, as exemplified by the L locus, conferring rust resistance in flax (Hausner et al. 1999), *pvr1* gene for potyvirus resistance in *Capsicum* sp. (Yeam et al. 2005) and *Pm3* gene for powdery mildew resistance in bread wheat (*Triticum aestivum*) (Tommasini et al. 2006). Cloning some of the identified BLB resistance genes (*Xa1*, *xa5, xa13, Xa21, Xa26* and *Xa27*) (Song et al. 1995; Yoshimura et al. 1998; Iyer and McCouch 2004; Chu et al. 2006) made it possible to develop FMs. Recently, a FM for recessive resistance gene *xa5* has been developed (Iyer and McCouch 2007). In the case of *xa13* gene, markers reflecting promoter level variations between resistant and susceptible parents were identified (Chu et al. 2007). FM for *Xa21* was developed based on the coding sequence of both the alleles (*Xa21* and *xa21*) reported by Song et al. (1995, 1997). This FM is expected to enhance the reliability of MAS, as it helps in direct selection of genes involved in BLB resistance. Here we report pyramiding of three BLB resistance genes, *xa5*, *xa13* and *Xa21* along with grain and cooking quality characteristics and desirable agronomic features of the leading rice variety, IRS 5441-2, by a combination of phenotypic and FM-aided selection. The introgression of resistance genes into cultivars would be the most economical and effective method to control this disease (Ogawa et al. 1991). Some genes were incorporated into elite rice varieties by using conventional breeding method and then were pyramided into breeding lines by using MAS (Khush et al. 1989; Huang et al. 1997).

Through marker-assisted gene pyramiding, two or more resistance genes, such as *Xa21*, *xa13, xa5* and *Xa4,* have been incorporated in the genetic background of elite varieties (Huang et al. 1997; Sanchez et al. 2000; Singh et al. 2001; Joseph et al. 2004; Sundaram et al. 2008). Even though gene pyramid combinations, such as, *Xa21* + *xa13* + *xa5* or *Xa21* + *xa5* or *Xa21* + *xa13,* have been observed to possess high level of resistance against multiple isolates of *Xoo*, the

durability of resistance in such gene pyramid lines has not been validated so far because of continuously evolution of new pathogenic races of *Xoo*. In order to enhance the durability of resistance, it is desirable to identify and characterize new genes from wild relatives of rice so that they could be deployed along with *Xa21* or *xa13* or *xa5* in elite rice varieties.

The present study was designed to identify desirable recombinants that combine BLB resistance with basmati quality traits in BC_1F_3 generation derived from the cross between basmati breeding line IRS 5441-2 and non-basmati donor of BLB resistance, IRBLB59 developed earlier by Joseph et al. (2004). We have used marker-assisted backcross breeding to introgress the *xa5, xa13* and *Xa21* resistance genes into the basmati breeding line. MAS is particularly useful for this purpose because *xa13* and *xa5* are recessive resistance genes. In the absence of markers, identifying backcross plants that have these recessive genes would require progeny testing, which causes a time delay of an additional generation. Therefore, we would not able to distinguish, through phenotypic screens, rice lines that have only *Xa21* or *xa13* from those having both genes. Background analysis using simple sequence repeat (SSR) markers was integrated with foreground selection for BLB resistance with maximum recovery of the basmati genome along with the quality traits and minimum non-targeted genomic introgression of the donor chromosomes. The present work is the first report of combining three genes for BLB resistance and basmati quality characteristics together using FMs. The study also provides evidence for the accuracy and consistency with which the methodology can be applied on large scale in MAS.

Materials and methods

Plants

IRS 5441-2 is a secondary selection from INGER nursery from International Rice Research Institute, Philippines (IRRI), and a medium short duration (135 days), medium tall, sturdy stemmed and lengthy panicled aromatic breeding line. IRBB59, the donor parent, carries three BLB resistance genes namely, *xa5, xa13* and *Xa21* which were introgressed from wild species in the background of IR24, a widely grown rice variety developed at IRRI. IRBB59 which harbors three resistance genes, *xa5, xa13* and *Xa21,* was used as male donor and crossed with IRS 5441-2 to obtain F_1s. Fifteen F_1s plants were backcrossed to IRS 5441-2 to obtain BC_1F_1 genotypes. About 120 BC_1F_1 plants were self-fertilized to obtain BC_1F_2 generation and were screened for basmati quality characteristics along with BLB resistance. Four genotypes of BC_1F_2 population showed complete basmati quality characteristics along with BLB resistance and were self-fertilized to obtain BC_1F_3 generation. Twenty nine phenotypically superior individuals were selected and further characterized for basmati quality traits and BLB resistance through molecular markers.

Artificial screening for BLB resistance

Thirty-day old seedlings of the parental lines (IRS 5441-2 and IRBB59) and BC_1F_2 generation genotypes pyramided with three BLB resistance genes in different combinations were evaluated for BLB resistance under greenhouse conditions with BXO1 strain (a widely distributed pathotype of *Xoo*) in the Division of Plant Breeding & Genetics, Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu, India. This material was grown in plastic pots with a mixture of soil and farmyard manure (3:1 ratio). The cultures of these isolates were grown in nutrient agar liquid medium with agitation at room temperature for 56 h. After adjusting the optical density to 0.3 at 600 nm (approximately 1.2×10^9 CFU/ml) by distilled water, the cultures were used for screening the rice plants for BLB resistance. The leaf blades were inoculated by clipping with scissors at 3 cm below the leaf tips (Kauffman et al. 1973). Lesion length for BLB was scored 14 days after inoculation (Fang et al. 1990). The resistant plants were self-fertilized to produce BC_1F_3 generation genotypes.

Evaluation of agronomic traits

Agronomic traits of plant height, effective tillers per plant, panicle length, grains per panicle, 1,000 grain weight and grain yield were recorded in the rice lines pyramided with three resistance genes in BC_1F_3 generation. The pyramided lines were raised in a randomized block design with three replications during the wet season of 2010 at the Division of Plant Breeding & Genetics, Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu, India.

Each line was raised in five rows, each row containing 15 plants. The agronomic traits were recorded from five randomly selected plants in each line in each replication.

Grain quality characteristics of pyramided lines

Seeds harvested from individual plants in the BC_1F_3 generation were analysed for physico-chemical characters such as milled kernel dimensions, grain shape and aroma. To determine the kernel length/breadth ratio, five fully developed whole milled rice kernels were measured for their length and breadth. The grains were classified into different types based on their dimensions according to IRRI (1988). For testing aroma, 1 g milled rice kernels were soaked in 10 ml of 1.7% KOH at room temperature in covered Petri plates for 10 min (Sood and Siddiq 1978). Coded samples were subjectively evaluated by a panel of five experts who have rich experience in basmati rice breeding and quality evaluation. IRBLB59, the non-aromatic parent and basmati 370, a highly aromatic traditional basmati variety were used as standards. The samples were scored on 0–3 scale with 0, 1, 2 and 3 corresponding to absence of aroma, mildly aromatic, strongly aromatic and very strongly aromatic, respectively. The score for a sample was recorded based on consensus among the majority of experts. The statistical analysis of the data performed using MSTAT-C (http://www.msu.edu/’breed/mstat.htm).

DNA marker analysis

The total genomic DNA of the two parents and the individual progeny plants was isolated from 3-week-old plants using the standard cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990). All DNA samples were diluted to a working concentration of 50 ng/μl with distilled water. After adjusting the final DNA concentrations to 50 ng/μl, the DNA samples were stored at −20°C. Sequence tagged sites (STS) markers, RG556, RG136 and pTA248 closely linked to the BLB resistance genes, *xa5*, *xa13* and *Xa21*, respectively (Huang et al. 1997) were used to confirm the presence of the resistance gene. The RG556 marker is for *xa5* (Iyer and McCouch 2007), the RG136 marker for *xa13* (Chu et al. 2007) and the pTA248 marker for *Xa21* (Ronald et al. 1992). The PCR reaction was performed in a 25 μl reaction mixture containing 50 ng of template DNA, 5 pmol of each forward and reverse primer, 10 mM dNTPs, 10× PCR buffer, 25 mM $MgCl_2$, and 5 U of *Taq* DNA polymerase. The PCR reaction was initiated by denaturation at 94°C for 5 min followed by 35 cycles of PCR amplification with the following parameters: 30 s of denaturation at 94°C, 1 min at 55°C for *Xa21* and *xa5*, and 59°C for *xa13* respectively, for primer annealing, and 1 min of primer extension at 72°C. A final 7 min incubation at 72°C was allowed for the completion of primer extension. The amplified PCR product of pTA248 was electrophoretically resolved on a 1.2% agarose gel containing 0.5 μg/ml of ethidium bromide in 1× TBE buffer. The PCR products were visualized using a gel documentation system. A 100 bp DNA ladder was used to estimate allele sizes in base pairs (bp) for gel. Polymorphisms in the DNA profiles were scored visually by comparison with two parents and a standard DNA ladder. For the amplified product of *xa5*, 5 μl of PCR product was used for gel electrophoresis to determine whether PCR was successful. The remaining PCR product was used for restriction digestion. The reaction mixture consisted of 0.4 μl (10 U/μl) of restriction enzyme *Bsr*I for RG556 amplicon, 2 μl of 10× restriction buffer, 7 μl PCR product and adjusted to 20 μl with sterile distilled water (Perumalsamy et al. 2010). The reaction mixture was incubated for 4 h at 37°C and the products of the restriction digestion were separated by gel electrophoresis (1.5% agarose) and visualized using a gel documentation system. MAS was also carried out for *fgr* gene located on the short arm of rice chromosome 8 using primer RM515 (Temnykh et al. 2001).

To assess the relative contribution of the two parental genomes to the segregants and to identify selections with greater genetic similarity with the recurrent parent, microsatellite markers were used. For background selection, genomic DNA was isolated from 35-day old plants using CTAB method (Doyle and Doyle 1990). The original source, repeat motifs, primer sequences and chromosomal positions for these markers can be found in rice genome database (http://www.gramene.org). Preference was given to those SSRs that have been extensively used for the analysis of basmati rice. The parental polymorphic SSR markers were used to genotype foreground selection in BC_1F_3 generation.

Results

Screening of pyramided lines for BLB resistance genes

In BC_1F_3 population, plants with single resistance gene and with combinations of two and three resistance genes were identified. The presence of BLB resistance genes viz., *Xa21*, *xa13* and *xa5* in the BC_1F_3 generation recombinants were determined by using respective linked marker (Fig. 1). For *Xa21*, the STS marker pTA248 amplified a resistant parent specific 1376 bp fragment in 20 recombinants (Fig. 1a). In case of *xa13* recessive gene, STS marker RG136 also amplified a 699 bp fragment in 20 resistant recombinants (Fig. 1b). These results confirmed the presence of both *xa13* and *Xa21* in the homozygous condition in identified recombinants. Restriction digestion of 952 bp amplified product from the *xa5*-linked marker RG556 with *Bsr*I, gave nine resistant recombinants (Fig. 1c). Out of nine resistant recombinants, seven were found in the homozygous and two were found in heterozygous conditions. These results thus confirmed the presence of *Xa21*, *xa13* and *xa5* in the homozygous condition in six selected recombinants. Six recombinants possessed all three BLB resistance genes in homozygous conditions. The molecular analysis with aroma *fgr* gene linked to RM 515 marker produced a 210 bp allele in 15 recombinants, whereas, non-basmati aromatic genotypes did not have this allele (Fig. 1d). Out of six BLB resistant genotypes, five genotypes were found to be homozygous for all the three BLB resistance and *fgr* genes.

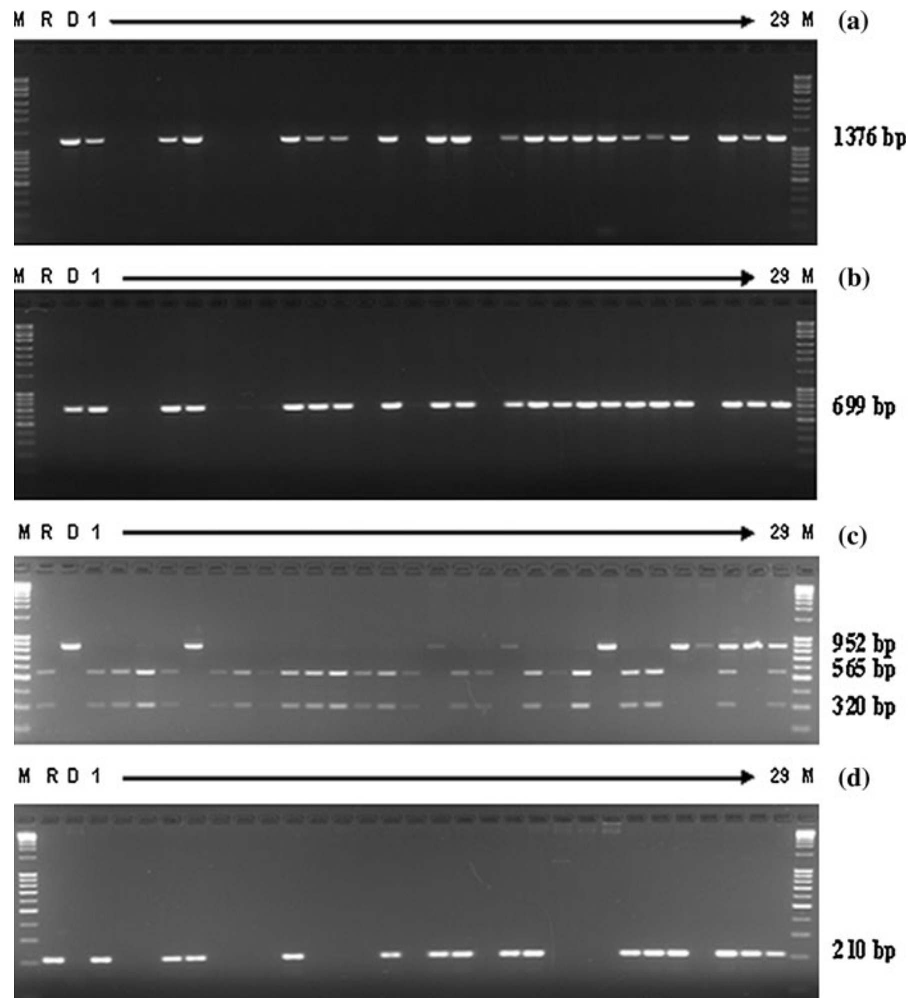

Fig. 1 PCR amplification of BC_1F_3 of IRS 5441-2 × IRBLB59 cross **a** with *Xa21F*, *Xa21R*, **b** with *Xa13F*, *Xa13R*, **c** PCR products obtained using *xa5F, xa5R* were restricted with the enzyme *Bsr*I to detect polymorphism which is evident from the difference in the fragment sizes between the pareds and progeny, **d** PCR products obtained using RM515 for fgr gene. *M, R, D*, indicates ladder marker, recurrent parent and donor parent, respectively

Distribution of *Xa/xa* and *fgr* gene combinations in potential lines

The distribution of different BLB resistance genes in different combinations along with aroma gene has been shown in Table 1. Out of 29 genotypes, five genotypes viz., IRS 5441-2-21, IRS 5441-2-79, IRS 5441-2-81, IRS 5441-2-85 and IRS 5441-2-91 were found to be homozygous for all the three BLB resistance genes and the *fgr* gene (*Xa21Xa21* + *xa13xa13* + *xa5xa5* + *fgrfgr*) whereas, genotypes IRS 5441-2-51, IRS 5441-2-60 and IRS 5441-2-74 possessed two BLB resistance and *fgr* gene (*Xa21Xa21* + *xa13xa13* +*fgrfgr*).

Disease reaction of pyramided rice lines for BLB isolate

The BC_1F_3 BLB resistant genotypes along with basmati quality characteristics selected from BC_1F_2 grown in the field were evaluated for disease response by artificial inoculation that revealed resistance reaction by 29 plants with an average lesion length of less than 2.0 cm. Mean lesion lengths of plants with different combination of resistance genes along with the fragrance gene in BC_1F_3 population is shown in Table 2. The plants with all the three resistance genes in the homozygous condition (*Xa21Xa21/xa13xa13/xa5xa5*) and those with two genes in homozygous condition showed higher levels of resistance with mean lesion length of <2.0 cm. The recombinants with two genes in homozygous condition (*xa5xa5*, *xa13xa13* and *Xa21Xa21*) showed moderate level resistance reaction against both the *Xoo* isolate.

Table 1 Distribution of *Xa/xa* and *fgr* gene combinations in BC_1F_3 plants

BC_1F_3 plant	*Xa/xa* and *fgr* gene (s) present
IRS 5441-2-21	*Xa21Xa21* + *xa13xa13* + *xa5xa5* + *fgrfgr*
IRS 5441-2-79	*Xa21Xa21* + *xa13xa13* + *xa5xa5* + *fgrfgr*
IRS 5441-2-85	*Xa21Xa21* + *xa13xa13* + *xa5xa5* + *fgrfgr*
IRS 5441-2-48	*Xa21Xa21* + *xa13xa13* + *fgrfgr*
IRS 5441-2-51	*Xa21Xa21* + *xa13xa13* + *fgrfgr*
IRS 5441-2-60	*Xa21Xa21* + *xa13xa13* + *fgrfgr*
IRS 5441-2-74	*Xa21Xa21* + *xa13xa13* + *fgrfgr*
IRS 5441-2-81	*Xa21Xa21* + *xa13xa13* + *Xa5xa5* + *fgrfgr*
IRS 5441-2-91	*Xa21Xa21* + *xa13xa13* + *Xa5xa5* + *fgrfgr*

Table 2 Average lesion length in BC_1F_3 recombinant lines

BC_1F_3 plant	Mean lesion length (cm)	Grain yield/ plant (g)	Aroma
IRS 5441-2-21	1.10	31	Strong
IRS 5441-2-79	1.20	30	Strong
IRS 5441-2-85	1.12	31	Strong
IRS 5441-2-48	1.56	31	Strong
IRS 5441-2-51	1.58	33	Strong
IRS 5441-2-60	2.00	30	Strong
IRS 5441-2-74	1.90	29	Strong
IRS 5441-2-81	1.76	33	Strong
IRS 5441-2-91	1.98	27	Very strong
CD (5% level)	0.649	3.27	–
SE(d)	0.283	1.41	
SE(m)	0.200	1.00	

Genotypes IRS 5441-2-81 and IRS 5441-2-91 are heterozygous for *xa5* gene (*Xa5xa5*) but showed complete resistance against the pathogen.

Assessment of agronomic performance and grain quality in BC_1F_3 plants

A total of 29 plants representing phenotypically superior individuals were selected and further characterized. Molecular marker analysis of these recombinants confirmed the presence of three BLB resistance genes *Xa21*, *xa13* and *xa5* along with *fgr* gene in the homozygous condition. The results on agronomic performance (Table 3) showed that the majority of the selections were superior to the popular high yielding basmati genotype IRS 5441-2. The plant height of the recombinants ranged from 88 cm in IRS 5441-2-71 to 114 cm IRS 5441-2-17. In comparison to IRS 5441-2, there was considerable reduction in plant height with as many as 11 recombinants having plant height of less than 100 cm. Out of 29 recombinants, two had shorter plant height than IRBB59. The effective tiller number per plant varied from 9 to 18. Seventeen individuals showed higher effective tiller number than that of IRS 5441-2. Number of grains per panicle varied from 140 in IRS 5441-2-10 to 189 in IRS 5441-2-64. Ten plants had higher number of grains per panicle than IRS 5441-2. Twenty two individuals had longer panicles than IRS 5441-2 parent. The 1,000 grain weight was highest in IRS 5441-2-26 (25 g) and lowest in IRS 5441-2-33 (18 g). Seventeen individuals had higher

Table 3 Agronomic and quality characteristics of the parents and the selected BC_1F_3 recombinants

Selection	Agronomic traits						Quality traits				
	Plant height (cm)	Effective tillers/ plant (no.)	Grains/ panicle (no.)	Panicle length (cm)	1,000 grain weight (g)	Grain yield/ plant (g)	Grain length (cm)	Grain breadth (cm)	L/B ratio	Grain shape	Aroma[a]
IRS 5441-2	110	12	175	27	20	27	7.1	1.8	3.9	LS	Strong
IRBLB 59	90	11	156	22	20	29	6.1	2.0	3.1	LM	Absent
IRS 5441-2-10	112	11	140	26	24	33	7.2	1.7	4.2	LS	Mild
IRS 5441-2-14	100	15	168	30	19	31	7.0	1.8	3.9	LS	Strong
IRS 5441-2-17	114	14	155	35	20	30	6.7	1.7	3.9	LS	Mild
IRS 5441-2-20	98	17	145	34	22	34	7.1	1.8	3.9	LS	Mild
IRS 5441-2-21	105	16	187	31	23	32	7.0	1.6	4.4	LS	Strong
IRS 5441-2-24	95	14	170	27	21	31	7.0	1.9	3.7	LS	Strong
IRS 5441-2-26	100	12	158	34	25	30	7.0	1.7	4.1	LS	Mild
IRS 5441-2-29	110	11	166	36	20	29	6.7	1.9	3.5	LS	Strong
IRS 5441-2-31	89	16	154	23	19	27	6.9	1.8	3.8	LS	Strong
IRS 5441-2-33	96	13	165	35	18	28	7.2	1.7	4.2	LS	Very strong
IRS 5441-2-39	104	10	188	34	23	32	7.1	1.8	3.9	LS	Mild
IRS 5441-2-42	100	10	157	28	21	31	6.9	1.9	3.6	LS	Strong
IRS 5441-2-43	109	11	145	26	19	28	7.4	1.7	4.4	ELS	Mild
IRS 5441-2-45	94	9	171	34	20	30	7.0	1.6	4.3	LS	Strong
IRS 5441-2-48	104	18	189	36	22	31	7.5	1.9	3.9	ELS	Strong
IRS 5441-2-51	98	11	158	34	22	33	7.0	1.7	4.1	LS	Strong
IRS 5441-2-55	102	16	177	34	23	31	7.6	1.9	4.0	ELS	Mild
IRS 5441-2-60	96	16	188	33	22	30	7.2	1.8	4.0	LS	Strong
IRS 5441-2-62	112	14	174	26	20	30	7.3	1.7	4.3	LS	Mild
IRS 5441-2-64	104	16	189	24	24	34	7.5	1.8	4.2	ELS	Strong
IRS 5441-2-66	99	12	158	33	21	32	7.0	1.9	3.7	LS	Strong
IRS 5441-2-68	101	10	155	31	20	29	7.4	1.7	4.4	ELS	Strong
IRS 5441-2-71	88	16	145	36	19	27	7.3	1.7	4.3	LS	Mild
IRS 5441-2-74	108	13	169	25	23	29	7.0	1.8	3.9	LS	Strong
IRS 5441-2-79	111	12	188	29	21	30	7.5	1.9	3.9	ELS	Strong
IRS 5441-2-80	102	12	158	33	20	28	7.0	1.7	4.1	LS	Mild
IRS 5441-2-81	99	15	178	28	23	33	7.2	1.6	4.5	LS	Strong

Table 3 continued

Selection	Agronomic traits						Quality traits				
	Plant height (cm)	Effective tillers/ plant (no.)	Grains/ panicle (no.)	Panicle length (cm)	1,000 grain weight (g)	Grain yield/ plant (g)	Grain length (cm)	Grain breadth (cm)	L/B ratio	Grain shape	Aroma[a]
IRS 5441-2-85	102	17	179	34	22	31	7.3	1.9	3.8	LS	Strong
IRS 5441-2-91	102	14	187	28	19	27	7.5	1.7	4.4	ELS	Very strong
CD	7.30	4.90	16.36	4.90	3.27	3.27	0.32	0.16	0.20	–	–
SE(d)	3.53	1.41	3.53	2.12	1.41	1.41	1.06	0.53	0.70	–	–
SE(m)	2.50	1.00	2.50	1.50	1.00	1.00	0.75	0.37	0.50	–	–

[a] The test for aroma was conducted by a panel of five experts engaged in basmati quality evaluation. *LS* = Long slender, *MS* = Medium slender and *ELS* = Extra long slender

1,000 grain weight than IRS 5441-2. Most of the selections were found to meet the basmati grain quality standard. Twenty five genotypes had a desirable milled rice kernel length of more than 7 mm. The L/B ratio was highest in IRS 5441-2-81 (4.5) and lowest in IRS 5441-2-29 (3.5). The aroma was mild to very strong in these selections. With respect to grain shape, the recombinants having long slender to extra long slender grain shape which did not differ significantly from recurrent parent. The selection IRS 5441-2-21, IRS 5441-2-79 and IRS 5441-2-85 were found to be superior to IRS 5441-2 both in agronomic performance and grain quality characteristics.

Discussion

The main objective of this study was to combine basmati quality traits with BLB resistance. High level of susceptibility of basmati rice to BLB, caused by the bacterium *Xoo*, is a serious constraint to basmati rice production, which results in major yield loss. In addition, the grain and cooking quality of the basmati rice is severely affected by this disease. Hence, in the present study, an attempt was made to develop high yielding BLB resistant gene pyramided genotypes through MAS approach. The three gene pyramid line IRBB59 was found very effective against BLB isolates from the rice growing region of the world. Earlier reports reveal that this line is resistant to all the Philippine races and the Punjab isolates of *Xoo* (Huang et al. 1997; Singh et al. 2001).

The BC_1F_3 recombinants derived in this study from the cross IRS 5441-2 × IRBB59, were found equally effective against the most virulent BLB isolate, as the donor line IRBB59. The increased level of resistance of pyramid lines expressed as reduced lesion length has also been reported in all previous gene pyramiding programs on BLB resistance (Huang et al. 1997; Sanchez et al. 2000; Singh et al. 2001; Sundaram et al. 2008). This could be the result of synergistic action and/or complementation between the resistant genes used in the pyramid lines. The increased level of resistance conferred by more than one gene governing resistance to a single pathogen race has been described as quantitative complementation (Sanchez et al. 2000).

Gene pyramiding has been successfully applied in several crop breeding programs to evolve high level of

resistance using multiple resistance genes (Huang et al. 1997; Samis et al. 2002). In soybean, pyramided lines with three resistance genes (*Rsv1*, *Rsv3*, and *Rsv4*) exhibited high level of resistance against soybean mosaic virus (Shi et al. 2009). Four BLB resistance genes (*Xa4*, *xa5*, *xa13*, and *Xa21*) pyramided in rice showed a wider level of resistance against *Xoo* strains (Bharathkumar et al. 2008).

Whereas there was increased lesion length after 21 days of inoculation in two-gene pyramid lines, there was no such expansion in the lesion length in the three-gene pyramid lines. A similar observation was made in the study by Sundaram et al. (2008), indicating there could be a "critical mass" of genetic resistance inherent in such systems. This hints on the importance of pyramiding more than two genes for conferring durable resistance against pathogens such as *Xoo*. The higher level of resistance may be the result of gene interaction or quantitative complementation between resistant genes (Yoshimura et al. 1995; Huang et al. 1997; Sanchez et al. 2000).

Molecular marker-assisted background analysis of the segregants/recombinants is useful in determining the relative contribution of the progenitor parents. Molecular marker analysis with SSR gives quick evaluation of the genetic background of the recombinants. In the present study, we resorted to a single backcross followed by pedigree selection in order to derive maximum genetic background of IRS 5441-2 with the desirable features such as BLB resistance, grain number per panicle from IRBLB59. The background analysis in BC_1F_3 recombinants revealed the maximum recovery of the recurrent parent alleles. In a related study, Sundaram et al. (2008) used SSR markers to select for the genetic background of the recurrent parent while incorporating *Xa21, xa13* and *xa5* in Samba Mahsuri, an elite line.

The present work is the first report of combining three genes for BLB resistance and basmati quality characteristics together using functional markers. This is a successful example of an applying an integrated approach to plant breeding. MAS was used for identifying plants with multiple BLB resistance genes and conventional phenotypic selection for recovering basmati quality-aroma traits. In addition, SSR based background analysis helped in determining the extent of IRS 5441-2 alleles in the recombinants that possessed desirable traits from IRBB59, such as BLB resistance and increased number of grains per panicle. This approach provided three distinct advantages compared to either conventional breeding or MAS alone. First, BC_1F_3 generation helped to reduce the cost of MAS through the progressive reduction in the number of individuals compared to F_2, F_3, or BC_1F_2 genotypes subjected to final marker analysis by stepwise phenotypic screening of resistant genotypes for basmati quality characteristics. Otherwise, screening of genotypes in F_2, F_3, or BC_1F_2 through MAS require higher cost compared to screening an advanced generation i.e., BC_1F_3. Second, phenotypic selection along with MAS reduced the time period (3–4 years) required for the recovery of desirable recombinants to a considerable extent. It takes at least 8–9 years to allow for transfer of these resistance genes through strictly conventional methods. Third, restricted backcrossing followed by pedigree selection also simplifies the breeding procedure, as a large number of crosses are not required unlike more backcrossing which needs extensive hand emasculation and pollination.

Conclusions

MAS using functional markers reduce the risk of false selection in MAS, and recombination between the molecular marker and the gene of interest. We were successful in identifying superior recombinants for three BB resistance genes (*Xa21, xa13* and *xa5*) along with *fgr* in the homozygous condition. Therefore, the homozygous resistant obtained genotypes in the BC_1F_3 generation can serve as immediate sources of BLB resistance in basmati breeding programs. The identified genotypes can be further improved through phenotypic selection as well as molecular markers for the development of potential BLB resistance donors. Since these genotypes also have desirable basmati fragrance, their use in basmati rice breeding will be advantageous compared to the use of non-basmati BLB donors. Advanced basmati breeding lines, which will be derived through MAS and phenotypic selection from these genotypes will therefore be of practical value in providing durable bacterial blight resistance in the basmati growing region and are expected to have a high impact on the yield stability and sustainability of basmati rice production.

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