Incorporation of Bacterial Blight Resistance Genes Into Lowland Rice Cultivar Through Marker-Assisted Backcross Breeding

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Accepted for publication 4 March 2016.

ABSTRACT

Pradhan, S. K., Nayak, D. K., Pandit, E., Behera, L., Anandan, A., Mukherjee, A. K., Lenka, S., and Barik, D. P. 2016. Incorporation of bacterial blight resistance genes into lowland rice cultivar through marker-assisted backcross breeding. *Phytopathology* 106:710-718.

Bacterial blight (BB) of rice caused by *Xanthomonas oryzae* pv. *oryzae* is a major disease of rice in many rice growing countries. Pyramided lines carrying two BB resistance gene combinations (*Xa21+xa13* and *Xa21+xa5*) were developed in a lowland cultivar Jalmagna background through backcross breeding by integrating molecular markers. In each backcross generation, markers closely linked to the disease resistance genes were used to select plants possessing the target genes. Background selection was continued in those plants carrying resistant genes until BC₃ generation. Plants having the maximum contribution from the recurrent parent genome were selected in each generation and hybridized with the recipient parent.

The BB-pyramided line having the maximum recipient parent genome recovery of 95% was selected among BC₃F₁ plants and selfed to isolate homozygous BC₃F₂ plants with different combinations of BB resistance genes. Twenty pyramided lines with two resistance gene combinations exhibited high levels of tolerance against the BB pathogen. In order to confirm the resistance, the pyramided lines were inoculated with different *X. oryzae* pv. *oryzae* strains of Odisha for bioassay. The genotypes with combination of two BB resistance genes conferred high levels of resistance to the predominant *X. oryzae* pv. *oryzae* isolates prevalent in the region. The pyramided lines showed similarity with the recipient parent with respect to major agro-morphologic traits.

Additional keywords: foreground selection, gene pyramiding, marker-assisted selection.

Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae*, is one of the most destructive diseases of rice in the major rice-growing countries of the world. BB causes major losses in the rain-fed lowland rice area that covers around 16 million ha in India of which majority are located in the eastern region of the country, exhibiting a lower productivity level (Ismail et al. 2013). The country needs additional rice production of around 2 million tons annually and a total of 135 to 140 million tons by 2030 to maintain self-sufficiency. This production increase needs to be obtained from less land, less water, less labor, and fewer chemicals under the constant threat of new emerging pathogens and pests and possible adverse effects of climate change (Khush 2005). BB is a serious problem of lowland rice, which gets further triggered by application of high dosages of nitrogenous fertilizer for higher yield. Yield losses in rice due to BB varied from 20 to 80% depending on location, season, and stage of infection (Khush et al. 1989; Pradhan et al. 2015a, b; Singh et al. 2001; Sonti 1998; Suh et al. 2013). Therefore, breeding for disease resistance was considered to be the most effective, environmental friendly, and economical means to manage BB of rice.

Globally, 39 BB resistance genes have been identified from diverse sources (Zhang et al. 2014). Many of these genes have been tagged by closely linked molecular markers (Gu et al. 2008; Rao et al. 2002; Sonti 1998; Yoshimura et al. 1995). Among them, 11 are recessive in nature (*xa5*, *xa5(t)*, *xa8*, *xa13*, *xa15*, *xa19*, *xa20*, *xa24*, *xa28*, *xa31*, and *xa32*) and the remaining 28 are dominant genes. Nine resistance genes (*Xa1*, *Xa3*, *xa5*, *Xa10*, *xa13*, *Xa21*, *Xa25*,

Xa27, and *Xa38*) have been cloned, following a map-based cloning approach (Bhasin et al. 2012; Chu et al. 2006; Han et al. 2014; Singh et al. 2001). Several germplasm donors carrying diverse genes for BB resistance have been used to develop BB resistant varieties (Pradhan et al. 2015b).

Large-scale and long-term cultivation of varieties to the single gene may enable the pathogen to overcome BB resistance. However, this can be delayed by pyramiding multiple resistance genes into a popular variety susceptible to BB. The probability of simultaneous transfer of more than one gene through conventional breeding approach is difficult. The availability of molecular markers closely linked with resistance gene help to identify plants with multiple resistance genes. The chance of pathogen mutation to overcome two or more BB resistant genes is much lower than that for a single BB resistant gene present in a rice cultivar. Pyramiding of two BB resistance gene combinations in a single line confers wide-spectrum durable resistance. Tightly linked DNA markers have been developed for several BB resistance genes and are widely used in marker-assisted selection (Bharatkumar et al. 2008; Dokku et al. 2013; Hu et al. 2008; Huang et al. 1997; Joseph et al. 2004; Perez et al. 2008; Pha and Lang 2004; Pradhan et al. 2015b; Rajpurohit et al. 2011; Sanchez et al. 2000; Shanti et al. 2001; Singh et al. 2001; Suh et al. 2013; Sundaram et al. 2008). We report here the successful pyramiding of two BB resistance genes, *Xa21* with *xa5* and *xa13* through marker-assisted selection in a lowland BB-susceptible rice cultivar Jalmagna.

MATERIALS AND METHODS

Plant materials. The donor parent CRMAS 2232-85, a derivative of a cross Swarna × IRBB 60 containing BB resistance genes *xa5*, *xa13*, and *Xa21* in the background of a mega variety Swarna,

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<http://dx.doi.org/10.1094/PHYTO-09-15-0226-R>
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was developed at the National Rice Research Institute (NRRI), Cuttack, India (Pradhan et al. 2015a, b). Jalmagna, the recurrent parent, is a popular variety of the water-logged ecosystem of India but highly susceptible to BB. Jalmagna was hybridized with CRMAS 2232-85 during the wet season in 2010 and the true F₁ plants were backcrossed with recipient parent Jalmagna in the dry season in 2011 under rapid generation advancement (RGA) facility. Around 360 BC₁F₁ seeds were generated and they were subjected to foreground selection with markers of three resistance genes (Table 1), and the positive plants were employed for background selection. BC₁F₁ line with maximum recurrent genome content was hybridized with recurrent parent to generate BC₂F₁. A Total of 122 BC₂F₁ plants were developed during the wet season in 2011. Similarly, 285 BC₃F₁ seeds were generated during the dry season in 2012 under RGA facility. The foreground selection positive plant possessing maximum recipient genome content was selfed to produce BC₃F₂ generation seeds during the wet season in 2012. Foreground selection was again continued in BC₃F₃ to confirm homozygous lines for the target gene combinations. The RGA facility was used during the dry season because Jalmagna is a strongly photo-sensitive and long-duration variety.

DNA isolation and PCR amplification. A mini-scale standard DNA preparation isolation protocol was followed (Dellaporta and Wood 1983). The PCR reaction mixture contained 50 ng of templates DNA, 5 picomole of each of the primers, 200 µM dNTPs, 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and gelatin at 0.01 mg/ml), and 0.6 unit of *Taq* DNA polymerase in a volume of 20 µl and amplification of target sequences were as per earlier reports (Table 1). The PCR products of sequence-tagged site marker RG136 were digested with restriction enzyme *Hinf*I as per manufacturer's instructions. The PCR products and the DNA fragments produced by restriction digestions were separated by gel

electrophoresis and gel images were captured by the gel documentation system (SynGene, Germany).

Marker analysis. The publicly available, three tightly linked markers of target genes were used for foreground selection (Table 1). Among the 236 SSR markers used for parental polymorphism survey, 120 were found to be polymorphic between the parents, and 60 were used (four to six per chromosome) for background selection. Data were analyzed and similarity matrix was constructed from binary data with Jaccard's coefficients. The dendrogram was generated with unweighted pair group method arithmetic average (UPGMA) algorithm, using FreeTree software (Hampl et al. 2001; Pavalice et al. 1999), and the dendrograms were visualized by Treeview 32 software (Page 1996). Graphical Geno Types (GGT) Version 2.0 (Van Berloo 1999) software program was used for the assessment of the genomic contribution of the parent in the selected recombinants based on SSR marker data.

Bioassay. Forty-day-old pyramided plants carrying BB resistant genes in combinations, along with the control, were inoculated with eight isolates of *X. oryzae* pv. *oryzae* (Kauffman et al. 1973). Eight highly virulent isolates of *X. oryzae* pv. *oryzae* identified based on their reaction against near isogenic line differentials carrying resistance genes *Xa3*, *Xa4*, *xa5*, *Xa7*, *Xa10*, and *Xa21* maintained at National Rice Research Institute, Cuttack, Odisha, India were used for bioassay. The *X. oryzae* pv. *oryzae* isolates were prepared by suspending the bacterial mass in sterile water to a concentration of approximately 10⁹ cells ml⁻¹ (Kauffman et al. 1973). Four leaves from four different plants of each entry were clip-inoculated at the maximum tillering stage and lesion lengths (LL) were recorded after 15 days. The disease symptoms were scored as resistant (R, LL ≤ 3.0 cm), moderately resistant (MR, LL 3.0 cm ≤ 6.0 cm), moderately susceptible (MS, LL 6.0 cm ≤ 9.0 cm), or susceptible

TABLE 1. Markers used for foreground selection for three bacterial blight resistance genes in marker-assisted backcross breeding

Resistance gene	Chromosome number	Marker	Primer sequences used for gene detection		Expected size (bp)	Band type	Reference
			Forward (5'-3')	Reverse (5'-3')			
<i>xa5</i>	5	<i>xa5S</i> (multiplex)	GTCTGGAATTTG CTCGCGTTTC	TGGTAAAGTAGATAC CTTATCAAACCTGGA	410 bp, 310 bp, 180 bp	STS	Sundaram et al. 2011 Pradhan et al. 2015b
		<i>xa5SR/R</i> (multiplex)	AGCTCGCCATTC AAGTTCTTGAG	TGACTTGGTTCT CCAAGGCTT			
<i>xa13</i>	8	RG136	TCCCAGAAAGCT ACTACAGC	GCAGACTCCAGT TTGACTTC	530 bp, 490 bp	STS	Huang et al. 1997
<i>Xa21</i>	11	pTA248	AGACGCGGAAGG GTGGTTCCCGGA	AGACGCGGTAATC GAAGATGAAA	1,000 bp	STS	Huang et al. 1997

TABLE 2. Microsatellite markers are polymorphic between Jalmagna and CRMAS 2232-85

Chromosome	Number of markers analyzed	Total number of polymorphic markers	Name of the polymorphic markers
1	25	11	RM23, RM48, RM212, RM272, RM575, RM428, RM488, SSR09, SSR 31, SSR 60, SSR 71
2	25	12	RM154, RM211, RM233A, RM263, RM475, RM45, RM530, SSR11, SSR 14, SSR 44, SSR 71, SSR 85
3	21	10	RM16, RM130, RM218, RM203, SSR 06, SSR 13, SSR 18, SSR 45, SSR 85, SSR 93
4	18	12	RM241, RM307, RM401, RM55, RM471, RM518, RM470, SSR 04, SSR 10, SSR 19, SSR 32, SSR 40
5	19	12	RM164, RM592, RM440, SSR 05, SSR 13, SSR 21, SSR 27, SSR 34, SSR 37, SSR 43, SSR 50, SSR 59
6	20	10	RM225, RM276, RM340, RM402, RM586, RM589, RM588, SSR 21, SSR 31, SSR 54
7	21	9	RM10, RM336, RM560, RM432, RM346, SSR 28, SSR 37, SSR 41, SSR 44
8	20	10	RM223, RM241, RM407, RM3395, RM6208, RM22550, RM22506, RM8271, SSR 14, SSR 48
9	16	7	RM219, RM242, RM257, RM410, RM3555, SSR 40, SSR 42
10	17	9	RM171, RM216, RM333, RM330, SSR 03, SSR 06, SSR 11, SSR 25, SSR 30
11	18	11	RM21, RM144, M202, RM206, RM209, RM260, RM287, SSR 3, SSR 4, SSR 11, SSR 27
12	17	7	RM17, RM195, RM415, RM23, SSR 23, SSR 26, SSR 36
Total	236	120	

(S, LL > 9.0 cm) (Amante-Bordeos et al. 1992). The standard error for lesion length measurements were estimated using Microsoft excel software.

Characterization for agro-morphological traits. Thirty-day-old seedlings of the BC₃F₃ pyramided lines and both the parents were transplanted in five rows with 20 plants per row per entry at 15 × 20 cm spacing under a randomized complete block design with two replications at the experimental farm of NRRI, Cuttack, during the wet season in 2014. Data were recorded on 10 plants from each entry and replication for agronomic traits (plant height [cm], tillers/plant, panicle length [cm], number of filled grains/panicle, 1,000-grain weight [g], spikelet fertility [%], grain length [mm], grain breadth [mm], flag leaf length [cm], and flag leaf breadth [cm]), while days to 50% flowering was recorded on whole plot basis. Analysis of variance for various agro-morphologic traits and principal component analysis (PCA) were performed using SAS statistical software (SAS Institute Inc. 2010).

RESULTS

Pyramiding of BB resistance genes. The molecular markers used in the foreground selection (Table 1) for selecting the genotypes

with the target genes *Xa21*, *xa13*, and *xa5* were first validated by confirming the polymorphism obtained with pTA248, RG136 and *xa5S*, R (multiplex) in the donor (CRMAS 2232-85) and recurrent parent (Jalmagna). In addition, the parents were screened with 236 rice microsatellite markers (Table 2) of which 120 were polymorphic and 60 among them were used for background selection.

Marker-assisted breeding program was integrated in the conventional backcross breeding until BC₃F₃ generation. After hybridization, foreground selection was followed in F₁ hybrids to check true hybrids carrying donor segments. The selection in BC₁ to BC₃ generations for target trait was carried out to select the plants containing resistance alleles of the target genes and only progenies having the resistance alleles were advanced up to BC₃F₃ generation (Fig. 1). Background selection was started from BC₁F₁ and continued up to BC₃F₁ generation. In each backcross generation, genotype possessing maximum genome content of the recipient parent was selected to hybridize with recipient parent for next backcross. A representative electrophoregram for background selection in the backcross progenies is depicted in Figure 2. A total of 650 F₁ plants were produced and 150 F₁ plants were tested for the hybridity and 143 hybrid plants were confirmed by their heterozygosity for the resistance gene linked markers. The true F₁s were backcrossed

Fig. 1. PCR amplification of markers linked to resistance genes **A**, *Xa21* and *xa13* and **B**, *Xa21* and *xa5* using primers pAT248, RG136, and *xa5S* and *xa5R* for resistance genes *Xa21*, *xa13*, and *xa5*, respectively, in BC₃F₃ pyramided lines. Molecular weight marker for *Xa21* and *xa13* (100-bp ladder) and *xa5* (50-bp ladder).

with the recipient parent to get BC₁F₁ generation seeds. These crossed seeds were raised for further backcrossing with recurrent parent. Ninety-three plants showed the presence of *Xa21* resistance gene-specific bands (1,000 bp), 113 plants for the presence of *xa5* resistance gene-specific bands (160 bp), while 91 plants showed the presence of *xa13* resistance gene-specific bands (490 and 530 bp) in the BC₁F₁ generation. Based on the amplification of resistance specific bands, 31 BC₁F₁ plants showed the presence of *Xa21* and *xa13* resistance genes, while 42 BC₁F₁ plants showed the presence of *Xa21* and *xa5* resistance genes. Forty-six BC₁F₁ plants showed the presence of *xa13* and *xa5* resistance genes. Only 14 plants showed the presence of three BB resistance genes (*Xa21*, *xa13*, and *xa5*). Out of these 14 BC₁F₁ progenies, the plant showing 77.5% of recurrent genome (plant no. 53) was backcrossed with recurrent parent Jalmagna.

The foreground selection in 122 BC₂F₁ generation progenies revealed the presence of 21, 33, and 30 progenies containing resistance genes *Xa21*, *xa13*, and *xa5*, respectively. Based on the genotyping data, 11 BC₂F₁ plants showed the presence of *Xa21* and *xa13* resistance genes while 13 BC₂F₁ plants showed the presence of *Xa21* and *xa5* resistance genes. Twenty-three BC₂F₁ plants showed the presence of *xa13* and *xa5* resistance genes. From the genotyping analysis, nine plants exhibited the presence of three resistance genes (*Xa21*, *xa13*, and *xa5*). The background selection of these nine BC₂F₁ plants with 60 polymorphic SSR markers exhibited the presence of 88.13 to 91.82% with an average of 90.95% of recipient parent genome content. The plant containing 91.82% genome content of Jalmagna (plant no. 53-21) was used for backcrossing.

Fig. 2. Representative electrophoregram showing background selection in 20 BC₃F₃ lines pyramided with two bacterial blight resistant gene combinations (*Xa21*+*xa13* and *Xa21*+*xa5*) along with parents using microsatellite markers.

A total of 285 BC₃F₁ backcross derivatives were generated by hybridizing the plant carrying 91.82% of recurrent genome with the recipient parent. Genotyping analysis of BC₃F₁ plants exhibited a total of 28 plants positive for *Xa21*, 35 for *xa5*, and 14 for *xa13*. Eighteen BC₃F₁ plants showed the presence of *Xa21* and *xa13* resistance genes while 14 plants showed the presence of *Xa21* and *xa5* resistance genes. These BC₃F₁ plants showed recipient parent genome content ranging from 91.7 to 95% with an average of 93.2%. During BC₃F₂ genotyping analysis, plants homozygous for two BB resistance gene combinations were selected and advanced to next generation, BC₃F₃. *Xa21* and *xa5* gene combination was noticed in six lines, while 14 plants exhibited combination of *Xa21* and *xa13* resistance genes. The BC₃F₃ derivatives carrying gene combination of *Xa21+xa13* and showing 95% recipient genome content were observed to be SPJ 53-21-77-13-1, SPJ 53-21-77-25-4, and SPJ 53-21-77-56-8 (Table 3). Pyramided lines with *Xa21+xa5* gene combination also exhibited 95% genome contribution from

recipient parent in SPJ 53-21-77-51-2 and SPJ 53-21-77-51-4 (Table 3).

Bioassays. The resistance and susceptibility reaction of the pyramided lines, donor and recipient parents to BB lesions were analyzed under artificial inoculation condition for confirmation of the results. The donor parent showed low average lesion lengths (2.1 to 2.8 cm) while the recurrent parent Jalmagna had longer lesion lengths (9.4 to 12.8 cm) (Table 4). The phenotypic results indicated that the pyramided lines were more resistant than the recurrent parent. The lesion lengths observed from the lines containing *Xa21+xa13* gene combination varied from 3.1 to 5.9 cm, while *Xa21+xa5* gene combination showed a range of 3.9 to 6.0 cm. Though all the gene combinations tested did not show any susceptible reaction to any of the eight isolates, the gene pyramids with *Xa21+xa13* and *Xa21+xa5* genes displayed higher levels of disease resistance with shorter lesion lengths against all BB isolates. Results indicated that the degree of severity of the disease from the data, the

TABLE 3. Percentage of recurrent parent's genome contribution and similarity coefficient in 20 pyramided lines containing two bacterial blight resistant genes

Sl number	Pyramided lines	Gene combination	Recurrent parent's genome contribution (%)	Similarity coefficient with recurrent parent
1	SPJ 53-21-77-20-5	<i>Xa21+xa13</i>	93.3	0.93
2	SPJ 53-21-77-20-2	<i>Xa21+xa13</i>	93.3	0.93
3	SPJ 53-21-77-20-1	<i>Xa21+xa13</i>	93.3	0.95
4	SPJ 53-21-77-13-1	<i>Xa21+xa13</i>	95.0	0.93
5	SPJ 53-21-77-15-1	<i>Xa21+xa13</i>	93.3	0.95
6	SPJ 53-21-77-12-1	<i>Xa21+xa13</i>	91.7	0.95
7	SPJ 53-21-77-25-8	<i>Xa21+xa13</i>	91.7	0.93
8	SPJ 53-21-77-25-7	<i>Xa21+xa13</i>	91.7	0.92
9	SPJ 53-21-77-25-6	<i>Xa21+xa13</i>	91.7	0.92
10	SPJ 53-21-77-25-4	<i>Xa21+xa13</i>	95.0	0.92
11	SPJ 53-21-77-27-4	<i>Xa21+xa13</i>	91.7	0.92
12	SPJ 53-21-77-35-2	<i>Xa21+xa13</i>	91.7	0.95
13	SPJ 53-21-77-56-8	<i>Xa21+xa13</i>	95.0	0.92
14	SPJ 53-21-77-35-3	<i>Xa21+xa13</i>	93.3	0.92
15	SPJ 53-21-77-51-5	<i>Xa21+xa5</i>	91.7	0.93
16	SPJ 53-21-77-51-4	<i>Xa21+xa5</i>	95.0	0.95
17	SPJ 53-21-77-51-3	<i>Xa21+xa5</i>	93.3	0.93
18	SPJ 53-21-77-51-8	<i>Xa21+xa5</i>	93.3	0.93
19	SPJ 53-21-77-51-7	<i>Xa21+xa5</i>	93.3	0.92
20	SPJ 53-21-77-51-2	<i>Xa21+xa5</i>	95.0	0.95
21	CRMAS 2232-85	<i>Xa13+Xa21+xa5</i>	0.0	0.00
22	Jalmagna	—	100.0	100.00

TABLE 4. Bacterial blight disease reaction of parental and BC₃F₃ pyramided lines against different *Xanthomonas oryzae* pv. *oryzae* strains

Sl number	Pyramided lines/ <i>X. oryzae</i> pv. <i>oryzae</i> strains	Gene combination	Mean lesion length in cm (mean ± standard error)								Disease reaction ^a
			Xa-17	Xa-7	xa-2	xb-7	xc-4	xd-1	xa-1	xa-5	
1	SPJ 53-21-77-20-5	<i>Xa21+xa13</i>	3.6±0.44	3.4±1.12	3.5±1.12	3.1±0.65	3.4±0.74	3.7±0.46	3.6±1.20	3.7±0.63	MR
2	SPJ 53-21-77-20-2	<i>Xa21+xa13</i>	3.9±0.54	3.8±1.21	3.6±1.14	3.7±0.55	3.7±0.92	3.8±0.77	3.4±1.13	3.9±0.7	MR
3	SPJ 53-21-77-20-1	<i>Xa21+xa13</i>	4.1±0.46	4.3±0.64	3.9±0.83	3.6±0.46	3.8±0.65	3.6±0.52	4.1±0.39	3.9±0.65	MR
4	SPJ 53-21-77-13-1	<i>Xa21+xa13</i>	5.2±0.63	4.4±0.61	3.8±0.75	3.9±0.52	4.2±0.44	4.3±0.55	5.8±0.78	4.3±1.14	MR
5	SPJ 53-21-77-15-1	<i>Xa21+xa13</i>	4.5±0.72	3.1±0.42	3.4±0.61	4.2±0.44	4.1±0.53	4.5±0.61	5.6±0.48	4.6±0.88	MR
6	SPJ 53-21-77-12-1	<i>Xa21+xa13</i>	4.7±0.68	4.2±0.53	4.1±0.72	4.6±0.54	4.3±0.49	4.7±0.52	4.3±0.65	4.3±0.72	MR
7	SPJ 53-21-77-25-8	<i>Xa21+xa13</i>	4.2±0.51	4.7±0.62	4.3±0.67	4.5±0.57	4.1±0.57	4.2±0.68	4.5±0.57	4.4±0.66	MR
8	SPJ 53-21-77-25-7	<i>Xa21+xa13</i>	5.3±0.45	5.1±0.58	5.2±0.55	5.1±0.63	4.7±0.73	4.5±0.71	4.6±0.69	4.7±0.73	MR
9	SPJ 53-21-77-25-6	<i>Xa21+xa13</i>	4.9±0.53	5.4±0.46	5.4±0.53	5.5±0.88	5.1±0.92	5.0±0.81	4.8±0.77	4.9±0.55	MR
10	SPJ 53-21-77-25-4	<i>Xa21+xa13</i>	5.2±0.76	5.4±0.38	5.2±0.83	5.5±0.74	5.7±0.92	5.2±0.82	4.7±0.88	5.3±0.66	MR
11	SPJ 53-21-77-27-4	<i>Xa21+xa13</i>	5.3±0.36	5.3±0.75	5.2±0.77	5.4±0.68	5.3±0.51	5.4±0.67	5.7±0.36	5.3±0.59	MR
12	SPJ 53-21-77-35-2	<i>Xa21+xa13</i>	5.6±0.43	4.7±0.98	5.5±0.74	5.6±0.68	5.7±0.52	5.5±0.79	5.4±0.92	5.6±0.83	MR
13	SPJ 53-21-77-56-8	<i>Xa21+xa13</i>	5.8±0.65	5.3±1.21	5.7±0.86	5.6±0.91	5.5±0.95	5.8±0.55	5.7±0.66	5.8±0.75	MR
14	SPJ 53-21-77-35-3	<i>Xa21+xa13</i>	5.9±1.06	5.8±0.93	5.8±0.71	5.9±0.72	5.8±0.83	5.6±0.63	5.8±0.62	5.7±0.87	MR
15	SPJ 53-21-77-51-5	<i>Xa21+xa5</i>	4.8±0.57	4.7±1.4	4.9±0.72	4.5±0.63	4.7±1.2	4.3±0.72	4.2±0.48	3.9±0.73	MR
16	SPJ 53-21-77-51-4	<i>Xa21+xa5</i>	4.5±0.85	4.9±1.3	5.4±0.63	4.8±0.82	4.9±0.91	4.3±0.52	4.2±0.63	5.1±0.65	MR
17	SPJ 53-21-77-51-2	<i>Xa21+xa5</i>	4.9±0.55	4.8±0.63	5.1±0.56	5.4±0.71	5.1±0.92	5.3±0.60	5.5±0.69	5.2±0.43	MR
18	SPJ 53-21-77-51-8	<i>Xa21+xa5</i>	5.6±0.71	5.4±0.92	5.8±0.95	5.9±1.12	5.8±1.06	5.4±0.53	5.3±0.75	5.6±0.42	MR
19	SPJ 53-21-77-51-7	<i>Xa21+xa5</i>	5.7±1.1	5.3±0.8	5.8±0.6	5.9±0.7	5.5±1.3	5.3±0.49	5.7±0.72	4.9±0.53	MR
20	SPJ 53-21-77-51-3	<i>Xa21+xa5</i>	5.9±0.7	5.8±0.8	6.0±0.7	5.7±0.7	5.6±0.9	5.5±0.76	5.8±0.84	5.7±0.65	MR
21	CRMAS 2232-85	<i>Xa13+Xa21+xa5</i>	2.8±0.4	2.5±0.3	2.4±0.4	2.1±0.4	2.8±0.5	2.4±0.4	2.6±0.8	2.2±0.6	R
22	Jalmagna	—	12.6±1.7	11.4±1.4	12.8±1.5	9.4±1.2	11.6±1.6	9.8±1.8	10.2±1.7	11.6±1.8	S

^a MR, moderately resistant; R, resistant; and S, susceptible.

order of gene combinations in conferring resistance was in the trend of *xa13*+*Xa21* < *xa5*+*Xa21*.

Yield and agro-morphologic traits of the pyramided lines. The pyramided lines were evaluated along with parents to compare the yield, agro-morphologic and grain quality traits with the recipient variety. Twenty pyramid lines containing two BB resistance genes at BC₃F₃ generation along with the donor and recipient parents were evaluated during the wet season in 2014 at NRRI, Cuttack. The recipient parent Jalmagna exhibited the mean grain yield of 17.35 g/plant, while the donor parent (Swarna BB pyramided line) recorded 20.5 g/plant. All of the test entries with two BB resistance gene combinations showed grain yields higher than recurrent parent Jalmagna (Table 5). Many pyramided lines showed similar agro-morphologic traits like the recipient parent, Jalmagna (Table 5). A dendrogram generated on the basis of 12 agro-morphologic traits of 20 pyramided and two parental lines showed two clusters and all the pyramided lines belonged to a single cluster (cluster II) with the recipient parent, Jalmagna, while the cluster I was a monogenotypic cluster with the genotype CRMAS 2232-85 (Table 5; Fig. 3). The genotype-by-trait biplot generated using 12 agro-morphologic traits of 20 pyramided lines along with parents also showed clearly that the pyramided lines clustered near the origin remaining closer to each other, while the donor parent placed far apart (Fig. 4). Most of the pyramided lines were in the first quadrant along with the recipient parent Jalmagna. The first principal component explained 63.7% of variation, while the second component exhibited 15.5% of the total variability. Among the agro-morphologic traits studied, panicle length contributed maximum toward diversity followed by grain length (Fig. 4).

Background selection. Background selection was carried out to accelerate the recovery of recurrent parent's genome content in progenies of various backcross generations. Background selection was performed in the genotypes that were observed to be positive for three target resistance genes using 60 SSR markers in BC₁F₁, BC₂F₁, and BC₃F₁ generations. The genotyping of BC₃F₁ derivatives showed a total of 120 alleles from 60 markers with similarity coefficient ranging from 0.85 to 1.0. A dendrogram generated using the SSR data grouped the 20 pyramid lines with two-gene combinations into two major clusters (Fig. 3B). Cluster I possessed donor parent CRMAS 2232-85 and the rest pyramided lines and the

recipient parent were accommodated in cluster II. Nearly all the entries in cluster II were very close to recipient parent Jalmagna with similar Jaccard's coefficient value. Among the 20 pyramided lines observed, 100% similarity was noticed in SPJ 53-21-77-51-5 with SPJ 53-21-77-25-6 and SPJ 53-21-77-56-8 with SPJ 53-21-77-20-2 on the basis of 60 background primers used (Fig. 3B).

Analysis of genome introgression on the carrier and noncarrier chromosomes. The genotyping of backcross derivatives using five microsatellite markers on each of three carrier chromosomes in the genomic region flanking to *xa5*, *xa13*, and *Xa21* were polymorphic. The genotypic results for the target genes in the 20 pyramided lines indicated that 14 of the pyramided lines showed homozygosity for the target segment of *xa13* and *Xa21*. No drag was observed in *xa13* carrying chromosome but a donor segment was observed between marker RM287 and pTA248 for *Xa21* carrier chromosome in all 14 pyramided lines (Fig. 5A). The genotyping results of six pyramided lines carrying target genes *Xa21* and *xa5* exhibited no drag in four lines (SPJ 53-21-77-51-4, SPJ 53-21-77-51-5, SPJ 53-21-77-51-7, and SPJ 53-21-77-51-8) for *xa5* carrier chromosome while a donor segment observed between marker HYV 5-27 and HYV 5-43 in the rest of two pyramids (SPJ 53-21-77-51-2 and SPJ 53-21-77-51-3) (Fig. 5B). In the introgression analysis of *Xa21* carrier chromosome, it was observed that five pyramided lines showed no introgression from donor segment while a chromosomal segment drag was observed between markers HYV11-3 and RM 287 in the pyramided line SPJ 53-21-77-51-7 (Fig. 5A).

DISCUSSION

Integration of molecular markers in the backcross breeding increases the precision in transferring the desired gene(s) to the recipient variety, as well as reduces the duration of selection cycle compared with conventional backcross breeding. Phenotypic selections in three backcross generations followed by two selfing generations were sufficient to transfer two BB resistance genes into the lowland cultivar Jalmagna background. Jalmagna is a popular lowland rice of about 160-day duration with strong photo-sensitivity. Control of BB using chemicals under waterlogged ecology is a relatively difficult task. Therefore, development of BB tolerant lines

TABLE 5. Agro-morphologic traits of parental and selected BC₃F₃ pyramided lines

Sl number	Pyramided lines	Plant height (cm)	Days to 50% flow	panicles/plant	Panicle length (cm)	Number of grains/panicle	Fertility %	1,000-seed weight (g)	Single plant yield (g)	Grain length (mm)	Grain Breadth (mm)	Flag leaf length (cm)	Flag leaf breadth (cm)
1	SPJ 53-21-77-20-5	152	128	10	23.95	205.5	88.2	23.15	19.15	0.75	0.865	32.0	1.6
2	SPJ 53-21-77-20-2	173	126	10.2	23.1	204.2	87.5	23.35	18.85	0.74	0.875	33.0	1.5
3	SPJ 53-21-77-20-1	163	125	9.8	24.25	205.5	83.6	22.62	17.95	0.74	0.885	30.0	1.6
4	SPJ 53-21-77-13-1	171	124	8.9	23.25	208.4	84.9	22.74	18.15	0.75	0.865	29.0	1.4
5	SPJ 53-21-77-15-1	158	130	9.4	24.2	207.5	83.2	22.61	20.15	0.74	0.885	35.0	1.7
6	SPJ 53-21-77-12-1	153	122	9.6	23.25	200.5	86.3	23.05	18.60	0.75	0.875	35.0	1.6
7	SPJ 53-21-77-25-8	152	129	8.4	22.8	199.3	84.6	22.85	19.65	0.75	0.875	37.5	1.5
8	SPJ 53-21-77-25-7	175	125	10.2	23.95	203.5	86.1	23.65	18.80	0.75	0.875	35.0	1.5
9	SPJ 53-21-77-25-6	153	123	10.1	23.95	208.5	85.2	21.95	18.95	0.74	0.865	37.0	1.4
10	SPJ 53-21-77-25-4	176	124	10.6	22.9	200.5	83.8	23.21	17.55	0.75	0.890	34.0	1.6
11	SPJ 53-21-77-27-4	176	130	9.4	23.95	203.2	85.5	21.85	17.85	0.74	0.865	36.0	1.7
12	SPJ 53-21-77-35-2	174	124	10.3	23.6	208.2	85.4	22.15	18.85	0.74	0.875	36.0	1.6
13	SPJ 53-21-77-56-8	178	123	9.8	25.15	205.5	84.6	21.75	17.55	0.74	0.885	38.0	1.5
14	SPJ 53-21-77-35-3	179	124	11.2	23.4	203.1	86.1	20.52	18.75	0.74	0.890	29.0	1.4
15	SPJ 53-21-77-51-5	162	130	10.3	24.25	200.5	85.5	21.8	17.95	0.72	0.885	32.0	1.5
16	SPJ 53-21-77-51-4	154	122	9.5	25.05	196.5	83.5	20.15	18.35	0.74	0.865	33.0	1.6
17	SPJ 53-21-77-51-2	166	127	10.1	25.05	196.5	87.2	22.25	18.15	0.72	0.890	34.0	1.7
18	SPJ 53-21-77-51-8	155	130	10.5	25.05	198.5	86.5	22.75	18.85	0.73	0.885	33.0	1.5
19	SPJ 53-21-77-51-7	171	124	10.9	25.15	204.5	88.1	20.95	18.25	0.74	0.875	34.0	1.5
20	SPJ 53-21-77-51-3	153	126	8.8	24.32	208.5	84.8	21.85	18.35	0.74	0.885	34.0	1.7
21	CRMAS 2232-85	105.5	105	12.0	24.15	218.4	84.5	18.85	20.50	0.72	0.840	34.0	2.0
22	Jalmagna	174	130	9.0	24.95	186.7	85.5	23.25	17.35	0.75	0.880	28.0	1.5
	LSD _{5%}	14.79		3.075	1.62	45.12	7.02	1.65	4.52	0.82	0.110	5.83	0.412
	CV%	4.45		14.1	3.28	10.86	4.12	3.95	12.39	5.39	6.34	7.28	12.2

similar to the kind of popular variety Jalmagna by integrating molecular markers in the breeding program is highly desirable as that can be achieved in relatively short time period compared with conventional breeding. Further, utilization of the RGA facility hasten the duration of breeding cycle in case of long duration genotype. In this experiment, lines with pyramided genes exhibited higher levels of tolerance against the tested virulent *X. oryzae* pv. *oryzae* isolates. Development of pyramided lines through marker-assisted selection successfully have been reported earlier illustrating less duration, more precision and more environment friendly (Dokku et al. 2013; Huang et al. 1997; Narayanan et al. 2004; Perez et al. 2008; Pradhan et al. 2015a, b; Rajpurohit et al. 2011; Sanchez et al. 2000; Singh et al. 2001; Suh et al. 2013; Sundaram et al. 2008). The pyramided lines with two gene combinations expressed higher levels of resistance in comparison with the susceptible cultivar Jalmagna. The results suggest that two gene combinations with *Xa21*+*xa13* pyramided lines had higher resistance level with shorter lesions lengths followed by *Xa21*+*xa5* containing lines than the recipient variety. Lines with *Xa21* in combination with either *xa13* or *xa5* had shown better tolerance disease reaction. The utility of *Xa21* in achieving higher levels of resistance in rice has been reported earlier (Huang et al. 1997; Sanchez et al. 2000; Singh et al. 2001; Sridhar et al. 1999) and the synergistic action and/or quantitative complementation between the resistant genes might result in enhanced levels of resistance (Sanchez et al. 2000).

The recovery of higher percentage of recurrent parent genome with less drag in BC₃F₁ was achieved using 60 polymorphic

primers. This is very much in line with earlier marker-assisted selection product development reports (Dokku et al. 2013; Perez et al. 2008; Pradhan et al. 2015a, b; Rajpurohit et al. 2011; Suh et al. 2013; Sundaram et al. 2008). Following this approach, after three backcross generations, it was possible to identify plants carrying the recurrent parent genome of more than 95% in the pyramided lines. In the BC₃F₃ generation, 20 pyramided lines homozygous for both *xa13* plus *Xa21*, and *xa5* plus *Xa21* were obtained exhibiting maximum recurrent parent genome recovery. There is a variation in the theoretically expected value of contribution from the recurrent parent genome to the BC₁F₁ plants and in other backcross generations. As per reports of Sundaram et al. (2008), there may be a “pull” for introgression of the *Xa21*, *xa13*, and *xa5* genes during selection which favors inheritance of additional unlinked loci from the donor genome in BC₁F₁ plants and BC₂F₁ generation. However, we found no pull effect during the transfer of *Xa21*, *xa13*, and *xa5* genes in different backcross generations.

Complete transfer of the agro-morphologic, grain quality and yield traits of the recipient variety to the pyramided lines containing BB tolerance genes from donor was the target in gene pyramiding work through marker-assisted backcross breeding approach. The results of recurrent parent genome contribution indicated that genotypes SPJ 53-21-77-13-1, SPJ 53-21-77-25-4, and SPJ 53-21-77-56-8 possessing *Xa21* and *xa13* resistance genes combination and SPJ 53-21-77-51-4 and SPJ 53-21-77-51-2 containing *Xa21* and *xa5* gene pyramids had 95% of the recurrent parent genome content (Table 3). Further, graphical genotyping data of the pyramided lines also indicated that no linkage drag was observed in chromosome 8 carrying *xa13* (Fig. 5A). Besides, no drag was found in four lines (SPJ 53-21-77-51-4, SPJ 53-21-77-51-5, SPJ 53-21-77-51-7, and SPJ 53-21-77-51-8) for *xa5* carrier chromosome in *Xa21*+*xa5* pyramided lines (Fig. 5B). Similar results were obtained in earlier studies with accelerated recovery of recurrent parent genome and less donor segment drag suggesting the importance of more number of background markers (Dokku et al. 2013; Pradhan et al. 2015b; Suh et al. 2013; Sundaram et al. 2008). No genetic linkage drag was observed for the transfer of genes *Xa21*, *xa13*, and *xa5* (Fig. 5) as the donor parent was a derivative of mega variety Swarna, which has less undesirable effects than using a wild or landrace type of donor source for BB resistance genes. The mega variety Swarna is

Fig. 3. Dendrogram illustrating the genetic relationship between parents and 20 pyramided lines based on **A**, 12 agro-morphological traits using Euclidean type of test by Furthest Neighbor method. **B**, Genotype numbers used are listed in Table 3 and dendrogram is based on 60 microsatellite markers.

Fig. 4. Genotype-by-trait biplot analysis of 20 pyramided lines containing two gene combinations (*Xa21*+*xa13* and *Xa21*+*xa5*) along with parents for first two principal components. The numbers in the figure represent the serial number of the genotypes listed in Table 3. DFF, days to 50% flowering; PH, plant height (cm); FLL, flag leaf length (cm); FLB, flag leaf breadth; PN, panicles/plant; PL, panicle length; GL, grain length; GB, grain breadth; TW, seed test weight; SF, spikelet fertility; SN, spikelets/panicle; and GY, grain yield.

a highly adapted variety for the favorable ecology. Results indicated that a broad-based highly adapted variety as a source of donor might give better performance and less drag compared with the wild and land races as donor (Pradhan et al. 2015b). It is expected that all the favorable genes are accumulated in the mega variety and subsequently transfer of these genes would help in further improving the background of the pyramided lines. The approach used in the study ensured the realization of the major objective resulting in development of a cultivar with enhanced resistance to BB and accelerated recovery of recurrent genome with better yield.

Evaluation of 20 pyramided lines along with their parents for yield and agro-morphologic traits exhibited excellent features of recurrent parent and also yielding ability of recipient parent with tolerance to BB resistance in the developed pyramided lines. In genotype-trait biplot analysis, the donor parent is placed away from the origin, indicating that lesser genetic drag segment recovered from donor and majority of the traits are from recipient parent (Fig. 4). As the recipient parent is not much away from the center, the pyramided lines with similar in phenotype are expected to be near the origin. On the other hand, the genotypes located near the origin indicated their better stability compared with the distant ones. The high yielding capacity of the pyramided lines even more than recipient parent might have achieved by the use of donor parent of mega variety Swarna derivative (CRMAS 2232-85) which might have contributed some yield traits or QTLs. Consequently, the complete recovery of yield and grain quality traits of Jalmagna along with more grain yield and exhibiting tolerance to BB disease is a very significant achievement. Utilization of sufficient number of background polymorphic markers in the breeding program helped to recover very high recipient parent genome content. The genotyping results and its further analysis indicated that the contribution of

recurrent genome was higher in few backcross derivatives than the theoretical expected value of 75% in BC₁F₁ (Table 3) and 87% in BC₂F₁ generation. In BC₃F₃, the genome content of Jalmagna in selected derivatives (SPJ 53-21-77-13-1, SPJ 53-21-77-25-4, SPJ 53-21-77-56-8, SPJ 53-21-77-51-2, and SPJ 53-21-77-51-4) was as high as 95%. The background selections with many molecular markers accelerated the recovery of recurrent genome content in a backcross breeding program and further indicate that selection for resistance gene combinations of *Xa21* with *xa13* and *xa5* genes may not give antagonistic effects for yield and other traits (Dokku et al. 2013; Pradhan et al. 2015b; Suh et al. 2013).

The performance evaluation of BC₃F₃ pyramided lines showed that, all the pyramided lines had registered higher grain yield than the recipient parent Jalmagna with similar agro-morphological and grain quality features of recipient parent. The higher level of tolerance to BB disease and the absence of any yield penalty due to accumulation of resistance genes in the pyramids offer confidence in integrating application of molecular markers in classical breeding for selection of the desired trait(s) and recovery of the recurrent parental genome. Deployment of a two gene combination like *xa21*+*Xa13* and *Xa21*+*xa5* can achieve higher level of tolerance in many BB prone rice growing areas of the country. The study clearly establishes the success of marker-assisted selection in precise breeding of recessive and dominant genes for resistance and providing higher level of tolerance to the most destructive diseases of rice in a long-duration, photosensitive, lowland rice cultivar.

Performance of the pyramided lines indicated that using functional markers in marker-assisted backcross program is a reliable approach for pyramiding BB resistance genes in rice. We were successful in identifying superior BB pyramided lines with higher level of resistance to the disease in a long-duration,

Fig. 5. Analysis of genome introgression of 20 pyramided lines containing two bacterial blight resistant genes **A**, *Xa21* on chromosome 11 and *xa13* on chromosome 8 in 14 BC₃F₃ pyramided lines and **B**, *Xa21* on chromosome 11 and *xa5* on chromosome 5 in six BC₃F₃ pyramided lines developed from backcross breeding of Jalmagna and CRMAS 2232-85.

photosensitive, lowland rice cultivar. These BB pyramided breeding lines may provide a solution in the BB endemic areas of the country. These lines can be further nominated for multilocation testing for their performance and release as varieties in the country or can be used as potential BB resistance donors. Development of two gene combination resistance compared with single gene deployment against the bacterial disease in the Indian subcontinent is an important achievement as the chance of breakdown of resistance in single gene deployment is very high. The rich diversity of various agro-climatic zones of the country maintains number of genetically distinct virulent *X. oryzae* pv. *oryzae* strains. Therefore, these BB pyramided lines are expected to have a high impact on the yield stability and sustainability of lowland rice ecology particularly in the eastern region of the country where the majority of the rice areas are rain-fed lowlands.

ACKNOWLEDGMENTS

We thank R. Thakur, former scientist, ICRISAT, and P. Nayak, former Editor-In-Chief, Oryza for their valuable suggestions for improvement of the manuscript. We also thank Director and Head Crop Improvement Division, National Rice Research Institute for providing all the necessary facilities. The work was supported by a grant from Department of Biotechnology, Government of India.

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