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ORIGINAL ARTICLE

Pyramiding resistance genes for bacterial leaf blight (*Xanthomonas oryzae* pv. *Oryzae*) into the popular rice variety, *Pratikshya* through marker assisted backcrossing

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Abstract

Background Bacterial leaf blight (BLB) is one of the major biotic stress in rice cultivation. Management techniques, such as the development of BLB-resistant cultivars, are required to lessen the severity of the disease attack and yield losses. *Pratikshya* was selected in the present investigation as the recipient parent, as it is one of the popular high-yielding rice varieties of Odisha, India, which is having excellent grain as well as cooking quality. However, *Pratikshya* is highly susceptible to BLB which is prevalent in Eastern Indian region.

Methods and results Three major BLB resistance genes *xa5*, *xa13*, and *Xa21* from the donor source Swarna MAS (CR Dhan 800) were attempted to introduce into *Pratikshya* through a marker-assisted backcross breeding program. Those markers closely linked to the target genes were employed for foreground selection in the segregating generations till BC_2F_3. In each backcross generation, progenies containing all three targeted resistance genes and phenotypically more similar to the recipient parent, *Pratikshya* were selected and backcrossed. Screening of 1,598 plants of the BC_2F_2 population was conducted against BLB using *Xoo* inoculum and 35 resistant plants similar to *Pratikshya* were carried forward to the next generation. In the BC_2F_3 generation, 31 plants were found to possess all the three resistance genes. For background selection of plants carrying resistance genes 45 polymorphic SSR markers were employed. Evaluation of the pyramided lines at BC_2F_4 generation exhibited that, most pyramided lines were similar to *Pratikshya* in terms of morphological features and yield parameters, and some lines were superior to the recurrent parent in terms of morphological features and yield parameters.

Conclusion The three-gene pyramided lines showed a high level of resistance to BLB infection and are anticipated to offer a significant yield advantage over the recipient parent *Pratikshya*. The pyramided lines can further be used for multi-location trial, so as to be released as a variety or can be used as a potential donor for BLB resistance genes.

Keywords Bacterial leaf blight · Gene pyramiding · Marker-assisted backcrossing · Molecular markers · Rice

Introduction

Rice (*Oryza sativa* L.) is one of the most important cereals, serving as a principal source of nutrition to half of the global population, and particularly in India, it accounts a major portion of total food grain production [1]. India must produce 120 million tonnes of rice by 2030 to maintain self-sufficiency and to fulfil future food demands, which must be accomplished with limited land, water, labour, and using minimum amount of chemicals (i.e., fertilizers and pesticides etc.), as well as a continuous fight against developing disease, pests and the possible harmful consequences of climate change [1, 2]. Biotic stresses like disease and insect pests cause major yield loss in rice varieties across the globe

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[3]. Among many biotic stresses that affect rice production, bacterial leaf blight (BLB) is one of the most dreadful disease, causing yield reduction upto 74–81% depending on weather, location, and rice cultivar used for cultivation [4]. In both tropical and temperate rice-growing zones, the gram-negative bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causes bacterial leaf blight (BLB) disease [5]. During the maximum tillering stage, the BLB infection occurs, resulting in water-soaked lesions on the leaves that steadily enlarge and finally cause the rice plant to wilt [5]. Chemical pesticides and antibiotic sprays do not work against BLB [6], however antibiotics are utilized to control bacterial disease such as *Pseudomonas* spp., and *Xanthomonas campestris* [7, 8]. Therefore, host plant resistance represents the most practical and cost-effective strategy for disease management [9–13]. Till date, 47 genes have been identified, that provide resistance from BLB, of which many of them have been incorporated into high-yielding, popular rice cultivars across the globe [14–16]. The deployment of resistant cultivars with a single major resistance gene has been proven significant due to the development of novel strain of *Xoo* due to mutation [17].

Hence, the development of varieties with several resistance genes offer a viable alternative for broad-spectrum, long-term resistance against BLB in rice [18]. To achieve the goal, multiple resistance genes can be stacked or pyramided into the elite genetic background as a breeding strategy [19]. Meanwhile, marker-assisted selection (MAS) is a more effective and simpler approach for gene introgression than conventional breeding [19, 20]. The utilisation of MAS holds significant potential in assisting plant breeders to accomplish their objectives. However, its influence on the development of plant varieties has been limited [20]. In order to fully exploit the potential of MAS, it is crucial to establish a higher level of integration between MAS and breeding programmes. Additionally, it is important to understand the existing barriers and develop suitable methods to overcome them. The utilisation of the benefits of MAS in comparison to traditional breeding methods has the potential to significantly influence the improvement of cereal crops [20, 21].

Therefore, the present experiment was conducted to transfer valuable genes in rice variety *Pratikshya* for BLB resistance using MAS with the objectives: validation of parental lines for BLB resistance genes by using molecular markers, development of F_1 and the backcross populations and marker-assisted selection of lines possessing BLB resistance genes *xa5*, *xa13*, and *Xa21* in the populations F_1, BC_1F_1, BC_2F_1, BC_2F_3 (foreground selection), phenotypic screening of segregating population (BC_2F_2) for BLB resistance through artificial inoculation, marker-assisted selection of lines possessing background of recurrent parent *Pratikshya* in the population BC_2F_3 (background selection), and evaluation of pyramided lines for yield and agro-morphological characters in BC_2F_4 population.

Materials and methods

Plant materials

Pratikshya is a popular high-yielding rice variety of Odisha, India with good grain and cooking quality released by Odisha University of Agriculture and Technology (OUAT), Bhubaneswar, which is suitable for late sown rainfed medium land, moderately resistance to brown spot, sheath rot, sheath blight, leaf folder, white backed plant hopper, gall midge, stem borer, but highly susceptible to BLB. The donor parent for BLB resistance employed in the current crossing program was Swarna MAS (CR Dhan 800) released by ICAR-National Rice Research Institute (ICAR-NRRI), Cuttack, India that carries three BLB resistance genes, *i.e., xa5*, *xa13*, *Xa21* in the genetic background of Swarna variety [22].

Hybridization and marker-assisted selection

A step-by-step marker-assisted backcross breeding approach was implemented as shown in Fig. 1 for the effective introgression of *xa5*, *xa13*, and *Xa21* genes into *Pratikshya*. In *Kharif* season, 2017, F_1 seeds were successfully generated by crossing recipient parent *Pratikshya* with donor parent Swarna MAS. In the shallow pots located within the net house, F_1 seeds were planted in *Rabi* season 2017. True hybridity was checked in the F_1 generation plants using the three SSR markers (Table 1) linked to the *xa5*, *xa13*, and *Xa21* genes. The selected true F_1 plants were hybridized with the recurrent parent, *Pratikshya*, during *Rabi* season 2017 to produce BC_1F_1 seeds. The BC_1F_1 plants were then subjected to both foreground selection in *Kharif* season 2018 using MAS and phenotypic selection for agronomic similarity with *Pratikshya*.

Backcrossed seeds from selected BC_1F_1 plants were grown in the next generation as BC_2F_1 plants in *Rabi* season 2018. Selected plants from BC_2F_1 carrying the *xa5*, *xa13*, and *Xa21* genes were allowed to self-pollinate and then carried forward to BC_2F_2 generation during *Kharif* season, 2019. Resistant plants from BC_2F_2 generation were selected by phenotypic screening using clip inoculation technique of *Xoo* inoculum (*Xanthomonas oryzae* pv. *oryzae* brought from Crop Protection Division, National Rice Research Institute, Cuttack, India) by following a standard method [23], and by assessing the similarity of agronomic characteristics to *Pratikshya*. During *Rabi* season, 2019, plants from the BC_2F_3 generation were used for foreground

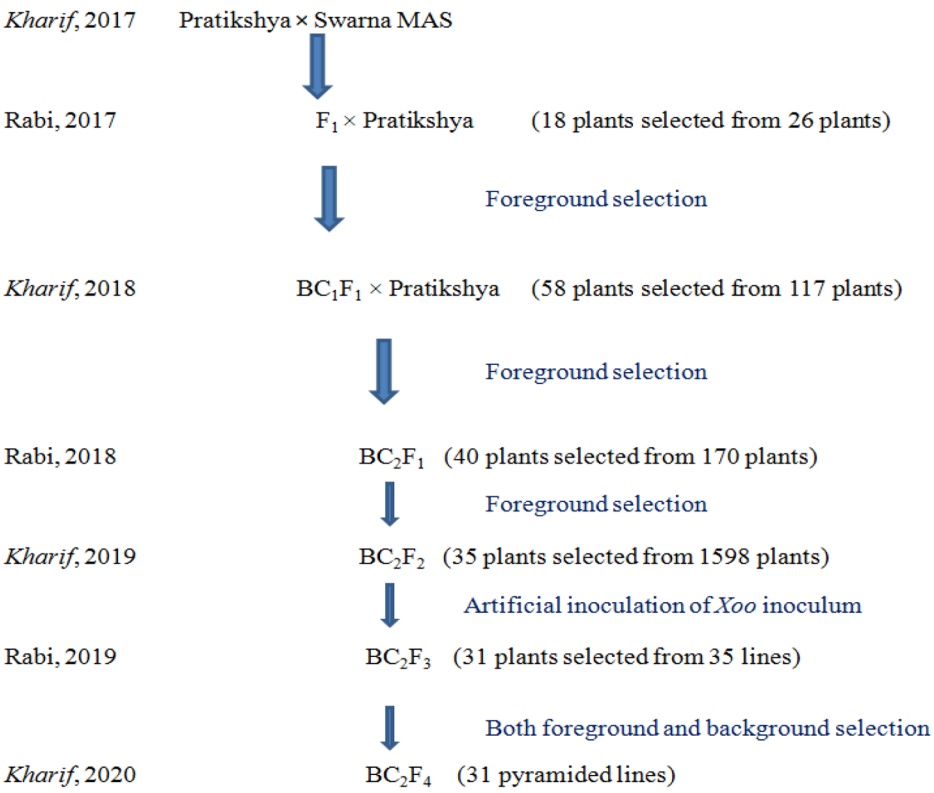

Fig. 1 Steps in pyramiding bacterial leaf blight resistance genes into the recipient variety, *Pratikshya* via Marker-assisted backcross breeding

Table 1 Markers used for foreground selection of bacterial leaf blight resistant line in rice variety *Pratikshya*

Gene	Chr No.	Marker	Resistant reaction	Primer sequences used for gene detection		Expected size (bp)	Reference
				Forward (5'- 3')	Reverse (5'- 3')		
xa5	5	RM122	Recessive	GAGTCGATGTAATGTCATCAGTGC	GAAGGAGGTATCGCTTTGTTGGAC	200 bp	Ashiba et al. 2020
xa13	8	*Xa13* prom	Recessive	GGCCATGGCTCAGTGTTTAT	GAGCTCCAGCTCTCCAAATG	500 bp	Singh et al. 2011
Xa21	11	pTA248	Dominant	AGACGCGGAAGGGTGGTTCCC-GGA	AGACGCGGTAATCGAAGATGAAA	1000 bp	Huang et al. 1997

selection and selected plants bearing all three resistance genes were subjected to background selection with markers showing parental polymorphism, and phenotypic selection for agronomic similarity with *Pratikshya*. Plants having all three resistance genes and good recipient parent genome recovery percentage were carried forward to BC_2F_4 generation during *Kharif* season, 2020 for agronomic trials.

Molecular characterization and SSR markers analysis

Total genomic DNA was isolated by the modified CTAB method of DNA extraction for rice [24], and quantified on Nanodrop - Spectrophotometer (NANODROP 2000c), by diluting in 1X TE buffer to a final concentration of around 50 ng/µl. The PCR reaction mixture for foreground selection of *xa5*, *xa13*, and *Xa21* consisted of 50 ng of genomic DNA, 10 mM of each primer, 10 mM of each dNTP, 10x PCR buffer, 3U Taq polymerase in a volume of 10 µl. The amplified products were subjected to electrophoresis on a 1.5% agarose gel for *xa13* prom primer (for *xa13* gene), pTA248 primer (for *Xa21* gene), 3% for RM122 primer (for *xa5* gene) (Table 1), and visualized on a gel documentation system (Bio-Rad Laboratories Inc., USA). To confirm the presence of the target genes, foreground selection was conducted till BC_2F_3 generation. However, 45 polymorphic SSR markers after the parental survey (Table 2) were employed for background selection to detect parental genome recovery in the pyramided lines [25].

The SSR bands from the gel photograph of BC_2F_3 generation were manually scored as 1 (if band present) and 0 (if band absent) to obtain the binary data for molecular analysis. Missing data were verified twice by repeating the genotyping process. The genetic distance between pyramided lines was calculated through the construction of the distance matrix using Jaccard's similarity coefficient from

Table 2 List of background primers used in the present study

	Primer	Forward Sequence (5' – 3')	Reverse Sequence (5' – 3')	No. of Alleles	Range	PIC value
1	RM10009	GATGCTCCGGAATAACTAGATTGG	GGAATTACAGCTGTCTTGGAAGG	2	110–120	0.91
2	RM10047	CAAGCAGTGATCATACAGCCTTCC	GCCATGGCTGAGAACAGAGAGC	3	140–170	0.68
3	RM11070	TCCCTACTCACTCTTTCTTCTGC	TGTACGGAGTGTGTAAGAGAAGC	3	190–210	0.50
4	RM11764	CACTGTCATCGTCGCCAAACG	GTTAATGAGCTACTCCCTCCGTCTCC	3	230–260	0.28
5	RM8085	TGCGTTTCGATTTCTTTTTA	GGAAAGTTGTGTTCTTTGGC	3	280–295	0.82
6	RM12061	GTCGGTTTGGGAATTTGACTAGTAGG	TAATTGTGGACTGCTCGTTTCTGG	3	350–370	0.69
7	RM6321	GGCTCTACCTCGCTGTTGTC	ACGAATATAACCTGCGGCAG	2	380–390	0.53
8	RM6842	TAAATCGAAGGAGGGGGAAG	GGAAGAAGGAGGAGGAGGTG	2	80–85	0.49
9	RM12548	AGTTAGGGAAGCTGGTGCATGG	ATTATATCGCGAACGAGCAAGAGG	2	100–110	0.36
10	RM12941	TTATGCCATGTGGTCCAATCAGC	ATTTGAACCATTTGGGCCTTGG	3	120–130	0.72
11	RM13366	GAATGGACGACATGTACGACACC	GGATGACGGACGAAAGCTAAGG	2	140–150	0.64
12	RM1497	TCCTCTTCACCTATGGGTGG	GCCAGTGCTAGGAGAGTTGG	2	160–170	0.80
13	RM14272	AAGAAGAGGAAGCTGTGGGTCAGG	ATGTGATGGGAAATGGAGAAGACG	3	190–210	0.78
14	RM15838	CGATGTCATTCGGTAGAAACAAGC	CCTAGTCAAGGCATGGTCAATCC	2	230–240	0.71
15	RM16153	TGGTTGTGGTATAGCACGGTAAGC	TGACCCAAGGAGATACTAGGTTGC	3	250–260	0.74
16	RM16577	GGTGAATTCTACTAAGACGGATCG	AGCCTTATTAGTCTCACCTCGTAACC	2	110–120	0.66
17	RM17611	GAGCAAATCCAGACCAGAAGTGC	ACACCTGGCAGCCAAGATATGG	2	140–150	0.52
18	RM17780	GGCTGATCTACACCGTCTATTGG	TATATTGCGGCCGTTAGTTAGG	2	250–260	0.83
19	RM3853	ATGTGCCCTTATACAAGGCC	GTGAGCTCATAGAGCAGCCC	2	270–275	0.94
20	RM18004	CTCGAAGCTATTAGCCGGGATCG	ATCTTCTTCCTCGCCGTCTTCC	3	280–310	0.43
21	RM18384	GCAGCAGAAAGGGAGAGAGTATGG	CAGCAACGTACGTACCAACAGG	2	340–350	0.76
22	RM19183	CATAAGCTAAGCACACCCACTCG	GCTTCATCGACGTCAACTACACG	3	360–375	0.42
23	RM7329	CAGAATTGCGAGCAACTGAG	GCCTGTGTGCATAGGATATG	3	260–270	0.54
24	RM20158	ACTCACCGTACGAACTCGATGC	ATCTGTCCTGAACCCGATACTGC	2	310–320	0.66
25	RM20834	GATATGGTTCCACTTCACCATGC	TTAGAAACTCGCCTTCAGAACTGC	2	240–250	0.48
26	RM8035	AATAAAAGGGTGTACATACA	TATATAACGCCATTAGAGAC	2	280–285	0.38
27	RM2966	GCTCCCATATATATACACAT	GTTGAGATTAATTAGCTGTC	3	300–310	0.35
28	RM22175	CCTTCCCAAATCAGTTCACAACC	TGTTGTTGGCTTGATGATGAGC	2	340–350	0.61
29	RM22459	ACCACCGCGACTTCAGTTCTCC	CGGAGGTGTTGGTGGAAGAGG	2	140–150	0.56
30	RM22720	ACTGCGTTGCGTAGTTTAGAGC	AAACAGCTGTAGCGAGAGATAGC	3	180–190	0.39
31	RM22905	CACTGCTCACTGCTGCCTTGC	CACGGGAGCTTCTGTCAGTGG	2	220–230	0.66
32	RM23345	GAGATCCTGCACATCTTTGAGACC	TGTGCCACGAAACAAATCTAGGC	3	250–260	0.41
33	RM23645	CATACAGCATGCTCACAGTTGATCG	CATCAGCATCTGGGACCTCTCC	2	280–290	0.52
34	RM5899	AGCGTTGTTTAACCGTGGTC	TCCACTAAAGCCACCTCGAG	2	120–130	0.60
35	RM24037	AGGAGATGCTGGAGGAAAGAAATGG	TGTCAAACGGACGTGCTCTATATCC	3	150–165	0.48
36	RM24071	TACTGAAGGCCAAGGAAGAGGTAGC	GAGACTATGGTGTGGCGTCAATGG	2	190–195	0.58
37	RM6364	GTTCATTTCGTCCTTCTCGG	TCTCGATTCTTCCTTCTCCG	3	210–230	0.48
38	RM25735	AGGCAGGCAAGCAGTAGTTTCG	ATCAAGATCAGGAGCCGCAAGG	3	280–290	0.46
39	RM3863	ATTGATCCCGTGCAAGTAGG	GCATTCTGCGTAGGTTTTGC	2	150–160	0.44
40	RM26885	ATCAGGTGGTGAGCTACAAAGG	GAAAGACCATGTGCATGTATCC	2	180–190	0.64
41	RM27180	AAGAAGAGAAGGGATGGGATCTGG	TCTAAACAGGGCCTCAAACTGTATCC	3	210–230	0.40
42	RM27446	GACAAGCCTAGGGCTCATGTCTCC	TAATACACCCTGGATGCGGTTTAGC	2	140–150	0.59
43	RM27900	CAAATATAACCGCATGGAGACACG	AGCAGTACTCCCTCCCTCCTTCC	3	180–200	0.39
44	RM28070	AAGGCACCAGGAATATGACAAGC	GGGATGTGGGATTTGGAGAGG	3	250–265	0.57
45	RM28616	CACCGGAGTTCCCTCAACTTACC	TACGTATGGCCAATTCAGACTGG	2	310–315	0.40

the binary data to deduce the genetic relationships between the pyramided lines and two parents, by constructing a dendrogram by following sequential UPGMA (unweighted pair group method with arithmetic mean) using software package TASSEL 5.0 [26].

Screening for bacterial blight resistance and morphological characterization

During *Kharif* season, 2017, field screening for BLB resistance was conducted by utilizing the inoculum of *Xanthomonas oryzae* pv. *oryzae*. Each Plant artificially inoculated by the leaf clip inoculation method, in which, the

top leaves of each plant were clipped at the maximum tillering stage and then inoculated [23]. The symptoms started to develop five to six days after the inoculation, and the observation period was between 14 and 21 days after inoculation. The plants with a score of 1 were regarded as resistant, those with a score of 3 as moderately resistants, those with a score of 7 as susceptible, and a score of 9 as highly susceptible [27]. The observations were recorded for percentage of diseased leaf area (DLA) followed by a standard evaluation system [27]. However, a multi-location trail was conducted at Rice Research Station, OUAT, Bhubaneswar, India (GPS co-ordinates: latitude NL 20° 15′ 55″, longitude of EL 85° 48′ 33″), and at agricultural farm, OUAT, Bhubaneswar, India (GPS co-ordinates: latitude of 20° 16' 09.3'' N, longitude of 85° 47' 29.0'' E), for proper validation of the screening.

Thirty-days-old seedlings of BC_2F_4 generation carrying BLB resistance genes were transplanted along with the donor and recipient parents in the main field of Rice research station, OUAT, Bhubaneswar with 20 cm × 15 cm spacing. The crop was successfully grown using conventional agronomic practices in *Kharif* season, 2020. The phenotypic trait observations were recorded for 10 plants in three replications and the replicated data were used to calculate mean, coefficient of variation (CV), and critical difference (CD). The observations were recorded for yield component characters *viz.,* days to 50% flowering, plant height (cm), total number of tillers per plant, number of productive tillers per plant, panicle length (cm), filled grains/panicle, 1000-grain weight (g), and seed yield per plant (g). Data analyses were carried out with the use of the software, Grapes version 1.0.0 [28].

Results

Molecular validation of parental lines

The resistant parent (Swarna MAS) and susceptible parent (*Pratikshya*) were validated for the presence of BLB resistance genes *xa5*, *xa13*, and *Xa21* with help of gene-linked markers RM122, xa13 prom, and pTA248, respectively. Genomic DNA from both parents was amplified using the above-mentioned three SSR markers and then parental polymorphism was revealed from gel electrophoresis. The resistant allele of *xa5* showed a band at 200 bp using the RM122 marker. The resistant allele of the *xa13* gene generated a fragment at 500 bp, whereas the resistant allele of the *Xa21* gene was found in the resistant parent Swarna MAS at 1000 bp (Fig. 2).

Pyramiding of bacterial leaf blight resistance genes

During each generation from F_1 to BC_2F_3 (except for BC_2F_2, due to more number of population and use of clip inoculation technique), foreground selection was conducted to select plants having all three resistance genes (positive plants) and only those plants were advanced to the next generation. The hybridity of F_1 plants was assessed using molecular markers, and it was determined that out of a total of 26 F_1 plants, 18 exhibited characteristics consistent with

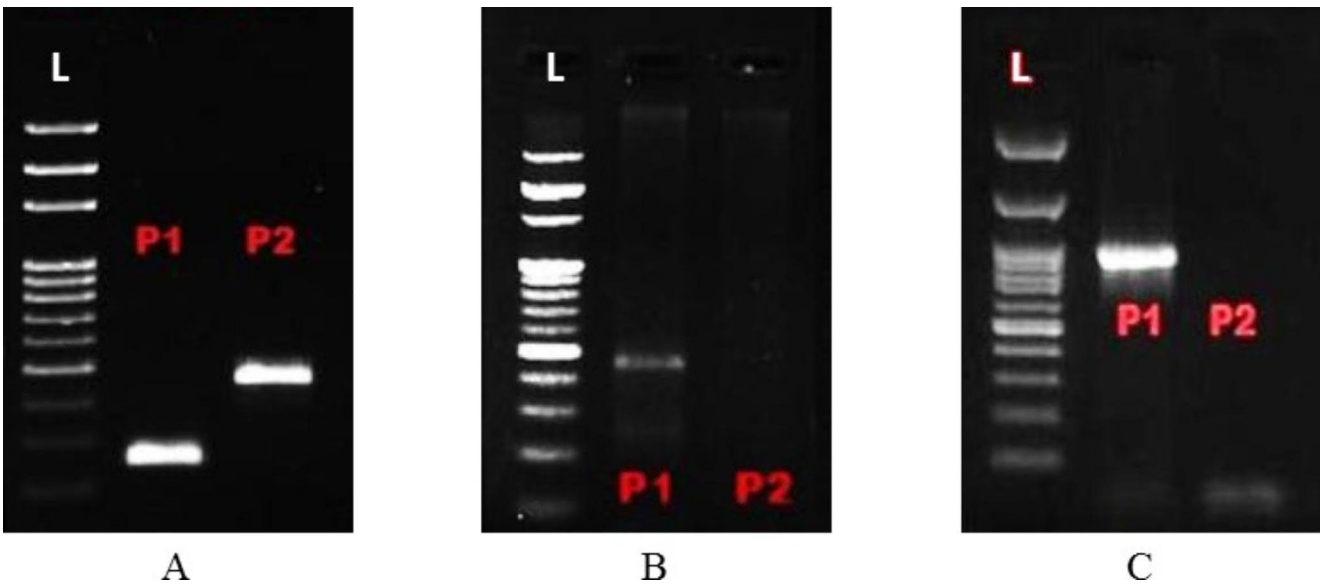

Fig. 2 Gel photographs of parents indicate **(A)** presence of expected base pair specific band for *xa5* (200 bp), **(B)** specific band for *xa13* (500 bp), **(C)** specific band for *Xa21* (1000 bp). Lane 1 represents DNA ladders (100 bp); Lane 2: P1 - Swarna MAS; Lane 3: P2 – Pratikshya, and L represents 100 bp ladder

being true F_1 plants. True F_1 plants were backcrossed with the recipient parent *Pratikshya* to generate BC_1F_1 seeds. In the BC_1F_1 generation, 117 plants were grown and out of these, 58 plants were identified to possess all three resistance genes. These 58 BC_1F_1 plants positive for resistance genes were backcrossed with recurrent parent *Pratikshya*. Of the 170 BC_2F_1 plants grown, 40 were detected to possess three-resistance genes. Thus only these 40 BC_2F_1 plants were allowed to self-pollinate and advanced to BC_2F_2 generation (Fig. 3).

In BC_2F_2 generation phenotypic screening procedures were followed instead of the use of molecular screening procedures to identify resistant plants. Selected 35 BC_2F_2 plants were grown as a total of 35 lines in BC_2F_3 generation and plants from those lines homozygous for three resistance genes combinations were identified (Fig. 2). Out of the 35 lines only thirty-one plants were found to possess all the three genes and hence were subjected to background selection. The background selection of these 31 BC_2F_3 plants with forty-five polymorphic SSR markers showed genome recovery of *Pratikshya* in the range of 64.44–93.33%. Out of 31 plants, 5 were showed genome recovery from 91.11 to 93.33% (Table 3; Fig. 4). Thereafter, thirty-one BC_2F_3 plants were allowed to self-pollinate to obtain plants for BC_2F_4 population.

Genetic similarity of the pyramided lines with the recipient parent using SSR markers

The dendrogram was constructed from SSR data grouped the 31 three-gene pyramided lines along with both parents into two major clusters with Swarna MAS in cluster I and the remaining 31 pyramided lines, including *Pratikshya*, in cluster II along with the similarity matrix of the pyramided

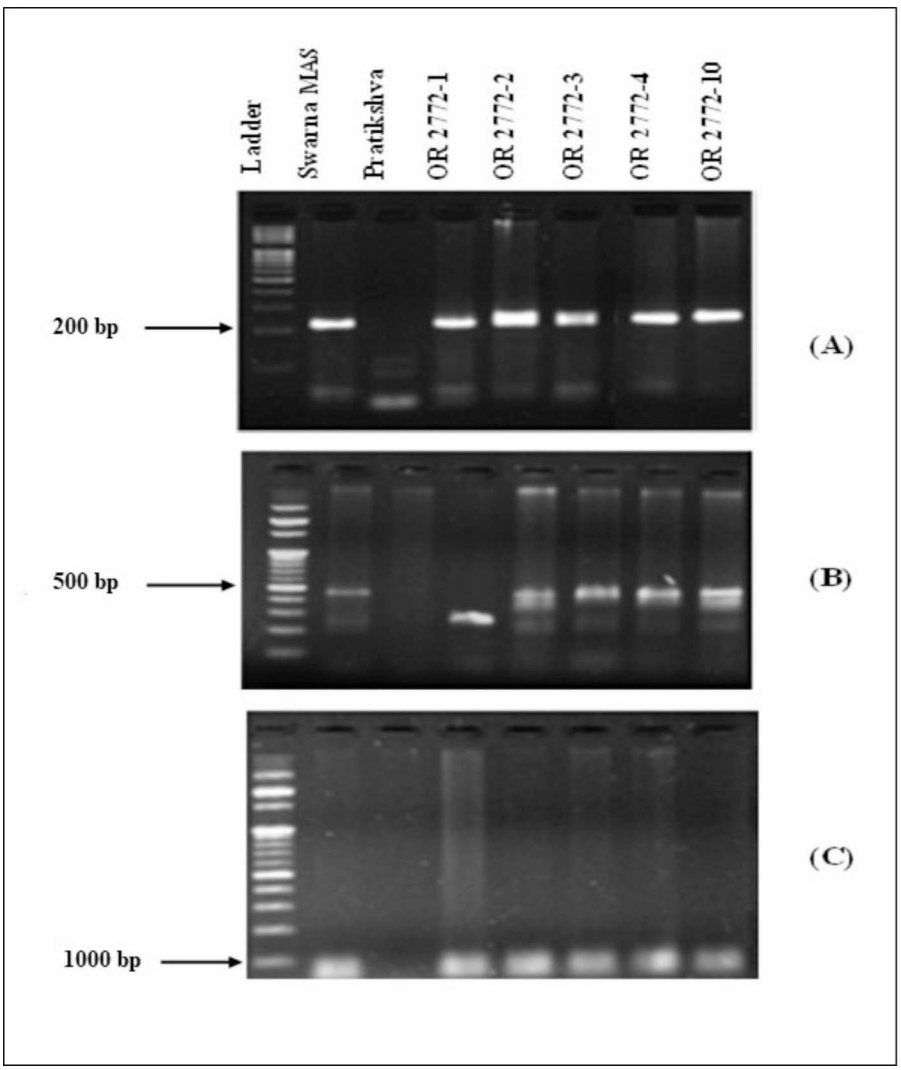

Fig. 3 Foreground selection of plants from BC_2F_3 generation, **(A)** for *xa5* gene with 100 bp ladder, **(B)** for *xa13* gene with 100 bp ladder, **(C)** for *Xa21* gene with 1 kb ladder

Table 3 Recurrent parent genome recovery in thirty-one pyramided lines for BLB resistance

Sl. No.	Line No.	Gene combination	Number of markers similar to the recurrent parent	Percent genome recovery (in %)
1	OR 2772-1	*xa5+xa13+Xa21*	35	77.77
2	OR 2772-2	*xa5+xa13+Xa21*	36	80.00
3	OR 2772-3	*xa5+xa13+Xa21*	40	88.88
4	OR 2772-4	*xa5+xa13+Xa21*	41	91.11
5	OR 2772-10	*xa5+xa13+Xa21*	29	64.44
6	OR 2772-13	*xa5+xa13+Xa21*	30	66.66
7	OR 2772-15	*xa5+xa13+Xa21*	38	84.44
8	OR 2772-16	*xa5+xa13+Xa21*	33	73.33
9	OR 2772-17	*xa5+xa13+Xa21*	40	88.88
10	OR 2772-18	*xa5+xa13+Xa21*	33	73.33
11	OR 2772-19	*xa5+xa13+Xa21*	39	86.66
12	OR 2772-20	*xa5+xa13+Xa21*	37	82.22
13	OR 2772-21	*xa5+xa13+Xa21*	38	84.44
14	OR 2772-27	*xa5+xa13+Xa21*	39	86.66
15	OR 2772-28	*xa5+xa13+Xa21*	38	84.44
16	OR 2772-30	*xa5+xa13+Xa21*	40	88.88
17	OR 2772-31	*xa5+xa13+Xa21*	39	86.66
18	OR 2772-32	*xa5+xa13+Xa21*	39	86.66
19	OR 2772-33	*xa5+xa13+Xa21*	39	86.66
20	OR 2772-51	*xa5+xa13+Xa21*	41	91.11
21	OR 2772-54	*xa5+xa13+Xa21*	42	93.33
22	OR 2772-62	*xa5+xa13+Xa21*	38	84.44
23	OR 2772-64	*xa5+xa13+Xa21*	40	86.00
24	OR 2772-65	*xa5+xa13+Xa21*	42	93.33
25	OR 2772-73	*xa5+xa13+Xa21*	40	88.88
26	OR 2772-74	*xa5+xa13+Xa21*	39	86.66
27	OR 2772-76	*xa5+xa13+Xa21*	35	77.77
28	OR 2772-77	*xa5+xa13+Xa21*	40	88.88
29	OR 2772-90	*xa5+xa13+Xa21*	39	86.66
30	OR 2772-92	*xa5+xa13+Xa21*	42	93.33
31	OR 2772-93	*xa5+xa13+Xa21*	38	84.44

lines (Fig. 5; Table 4). Cluster II was further divided into two sub-groups, cluster II-A and cluster II-B (Table 5). Cluster II-A was further subcategorized into two sub-groups, cluster II-Aa, and cluster II-Ab. Cluster II-Aa consists of pyramided lines OR 2772-4, OR 2772-28, OR 2772-73, OR 2772-31, OR 2772-2, OR 2772-20, OR 2772-15, OR 2772-74, OR 2772-77, OR 2772-92, OR 2772-1, OR 2772-17, OR 2772-32, OR 2772-90, OR 2772-16, OR 2772-3, OR 2772-19, OR 2772-21, OR 2772-27 and OR 2772-30, OR 2772-33, OR 2772-54, OR 2772-64, OR 2772-51, OR 2772-65, OR 2772-62, OR 2772-10, and OR 2772-93, while Cluster II-Ab contains OR 2772-13 and OR 2772-18. Cluster II-B consists of OR 2772-76.

Phenotypic screening of the BC_2F_2 against bacterial leaf blight

The recipient parent *Pratikshya* showed a disease score of 7 indicating a greater level of susceptibility to BLB. Swarna MAS displayed a high level of resistance to the infection with a disease score of 0. The disease score of BC_2F_2 plants ranged from 0 to 1 showing resistance to the disease. Out of all the plants of the BC_2F_2 population (1598 plants), 1412 were observed to be resistant and 186 were susceptible, across the multi-location trail.

Evaluation of pyramided lines for agro-morphological traits in BC_2F_4 generation

The mean values of eight agronomic traits *viz.,* days to 50% flowering, plant height (cm), number of tillers per plant, number of productive tillers per plant, panicle length (cm),

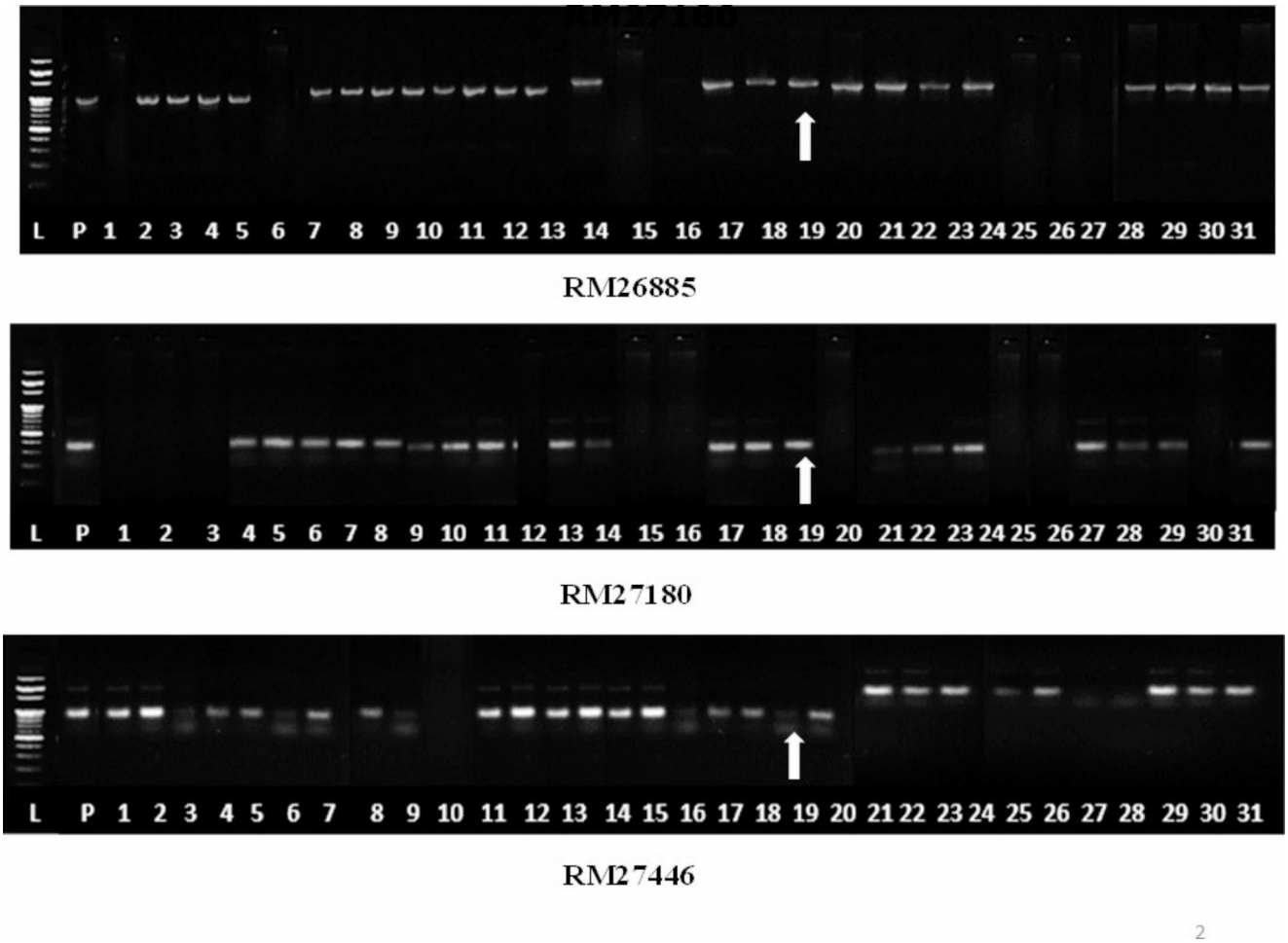

Fig. 4 Background selection of plants from BC_2F_3generationwith different polymorphic SSR markers. The amplified fragments with respect to marker RM26885 is present in *Pratikshya*, plant no. 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 17, 18, 19, 20, 21, 22, 23, 24, 28, 29, 30, 31. The amplified fragments with respect to marker RM27180 is present in *Pratikshya*, plant no, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 17, 18, 19, 21, 22, 23, 27, 28, 29, 31.The amplified fragments with respect to marker RM27446 is present in *Pratikshya*, plant no 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31. Lane 1- 100 bp ladder, Lane 2- *Pratikshya* (P). However, the best improved line no. 19 - OR 2772-19 gave a seed yield/plant of 65.5 g

number of filled grains per panicle, seed yield per plant (g) and 1000 grain weight (g) are presented in Table 6. From the statistical analysis, it was noticed that a significant difference was observed between the pyramided lines and both of the parents for all the characters except for total tillers, though, the difference was very less. The pyramided line OR 2772-19 displayed the highest seed yield per plant of 65.5 g. The average seed yield per plant of pyramided lines in the field was found to be 42.37 g, and the yield is varying due to the change in their genotyping composition after introgression.

The recurrent parent, *Pratikshya*, recorded a mean seed yield of 44 g/plant, while the donor parent (Swarna MAS) recorded 49.4 g/plant. Among pyramided lines, ten lines attained more seed yield per plant than *Pratikshya*. For panicle length, among the pyramided lines, OR 2772-21 had the longest panicle length (34.7 cm) followed by OR 2772-20 (28.9 cm). The parent *Pratikshya* displayed a panicle length of 24.5 cm. Out of thirty-one pyramided lines, nineteen lines produced longer panicles, while ten lines produced smaller panicles than *Pratikshya*. The highest plant height of 109.6 cm was observed in OR 2772-76 followed by 107 cm in OR 2772-27, while the shortest plant height of 88.3 cm was observed in OR 2772-13. Majority of the pyramided lines attained more grain weight than the *Pratikshya,* while OR 2772-92 showed a similar grain weight with the recurrent parent. From these observations, it was concluded that agro-morphological features play a significant part in the yield of the plant.

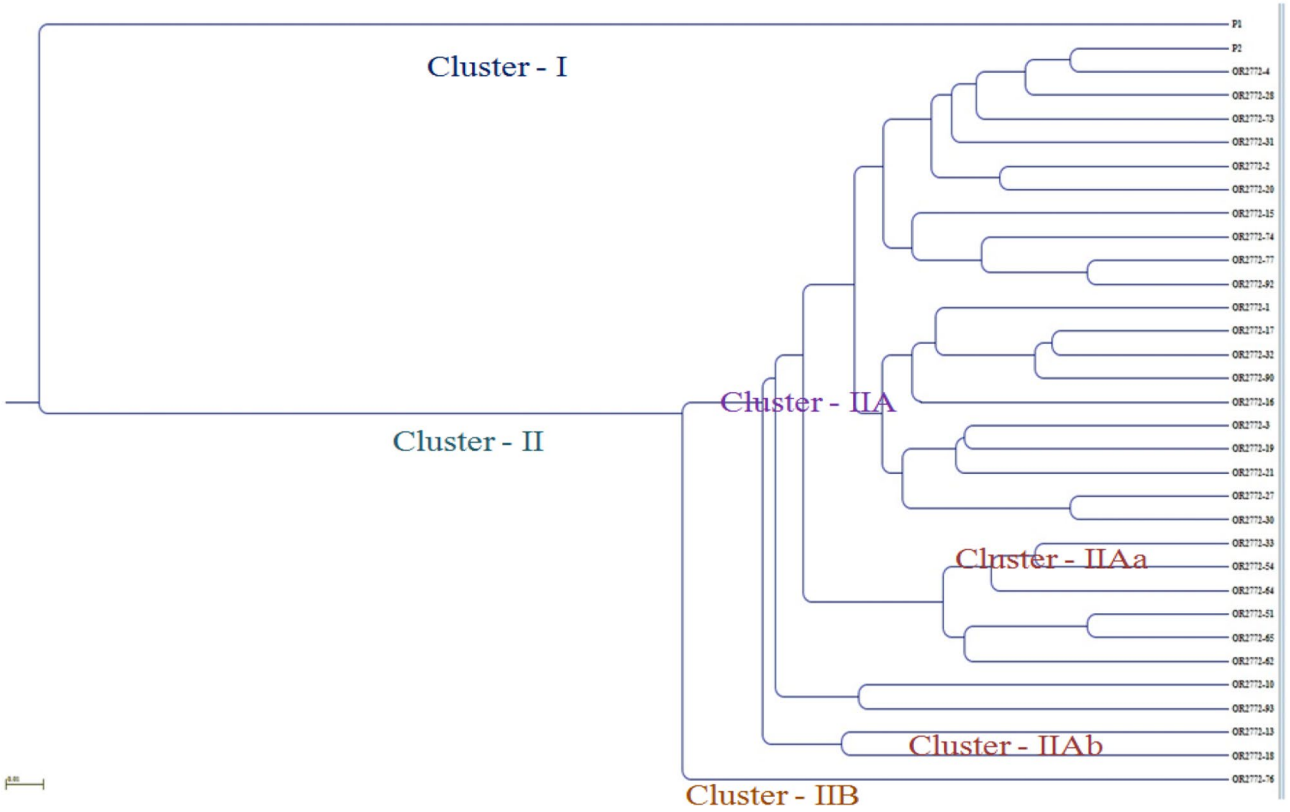

Fig. 5 Dendrogram representing the genetic relationship between pyramided lines of the BC_2F_3 generation. The donor parent Swarna MAS (P1) is in cluster I, whereas the recipient parent (*Pratikshya* (P2) and the other 31 pyramided lines are clustered into a separate cluster II.

Discussion

Pratikshya (Parentage: *Swarna* × *IR64*) is late-maturing, popular high-yielding rice variety in Eastern India [25]. It can be harvested in 146 days, and has a high degree of adaptability, and it creates opportunities for cropping system intensification in the coastal zone of India by earlier establishment of *Rabi* season crops [29]. However, the main limitation of *Pratikshya* is its susceptibility to BLB, which significantly reduces yield, despite the fact that it has potential for high yield with a desirable plant type. BLB acts as a biotic constraint in the decline of productivity in South-East Asian countries. The extent of severity of 10-20% annual reduction in rice production worldwide caused by BLB in rice demands the development of effective management strategies [30]. However, in order to prevent the spread of BLB, it has been found that host plant resistance is the most efficient environmental friendly method [31]. The incorporation of resistance genes in combination remains as an effective strategy for managing BLB and providing durable resistance [32]. In contrast to conventional phenotypic evaluation, marker-assisted backcross breeding permits for the selection of recessive alleles, selection of desirable plants at the seedling stage before the formation of a visible phenotype, and the pyramiding of important traits into a single genetic background. The application of molecular markers allow for the genetic categorization of progeny at each generation and speeds up the selection process [33].

Pyramiding multiple resistance genes with potential characteristics into a single genotype through MAS can improve the efficiency of generating new crop varieties exhibiting disease resistance, as well as other desirable traits, however, it is crucial for the maintenance of yield stability in the rice variety *Pratikshya* [34]. Meanwhile, in several studies, this approach has been successfully employed to introduce resistance genes into the elite genetic background [11, 30, 31, 34]. The BLB resistance genes *xa5*, *xa13*, and *Xa21* have been successfully incorporated into different genetic backgrounds of rice [11, 35].

Hence, these three resistance alleles covering three chromosome regions were selected to transfer into the popular variety *Pratikshya* in the present investigation. Out of 46 identified BLB resistance genes, three major R genes *xa5*, *xa13*, and *Xa21* have been used in the current study. However, previous research has successfully cloned and described all three of these resistances, *i.e., Xa21* resistance

Table 4 Similarity matrix of the pyramided lines used in the present study

	P1	P2	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24	G25	G26	G27	G28	G29	G30	G31
P1	0.000																																
P2	0.651	0.000																															
G1	**0.642**	**0.174**	**0.000**																														
G2	**0.651**	**0.147**	**0.248**	**0.000**																													
G3	**0.624**	**0.138**	**0.239**	**0.211**	**0.000**																												
G4	**0.624**	**0.083**	**0.202**	**0.138**	**0.183**	**0.000**																											
G5	**0.633**	**0.220**	**0.266**	**0.257**	**0.229**	**0.248**	**0.000**																										
G6	**0.624**	**0.229**	**0.239**	**0.266**	**0.202**	**0.202**	**0.266**	**0.000**																									
G7	**0.661**	**0.138**	**0.257**	**0.174**	**0.183**	**0.165**	**0.229**	**0.257**	**0.000**																								
G8	**0.670**	**0.202**	**0.174**	**0.275**	**0.248**	**0.229**	**0.202**	**0.229**	**0.211**	**0.000**																							
G9	**0.642**	**0.119**	**0.147**	**0.193**	**0.165**	**0.183**	**0.284**	**0.202**	**0.183**	**0.174**	**0.000**																						
G10	**0.569**	**0.193**	**0.220**	**0.284**	**0.275**	**0.257**	**0.303**	**0.202**	**0.257**	**0.229**	**0.165**	**0.000**																					
G11	**0.615**	**0.147**	**0.211**	**0.239**	**0.138**	**0.174**	**0.257**	**0.229**	**0.174**	**0.220**	**0.138**	**0.193**	**0.000**																				
G12	**0.661**	**0.156**	**0.257**	**0.119**	**0.239**	**0.110**	**0.229**	**0.202**	**0.183**	**0.211**	**0.183**	**0.239**	**0.229**	**0.000**																			
G13	**0.615**	**0.147**	**0.174**	**0.239**	**0.138**	**0.174**	**0.183**	**0.211**	**0.174**	**0.165**	**0.156**	**0.211**	**0.147**	**0.248**	**0.000**																		
G14	**0.633**	**0.147**	**0.211**	**0.183**	**0.174**	**0.174**	**0.275**	**0.266**	**0.174**	**0.275**	**0.174**	**0.211**	**0.202**	**0.211**	**0.202**	**0.000**																	
G15	**0.642**	**0.101**	**0.165**	**0.156**	**0.202**	**0.110**	**0.248**	**0.220**	**0.220**	**0.229**	**0.183**	**0.257**	**0.193**	**0.165**	**0.193**	**0.211**	**0.000**																
G16	**0.624**	**0.119**	**0.183**	**0.193**	**0.128**	**0.165**	**0.229**	**0.239**	**0.165**	**0.229**	**0.147**	**0.202**	**0.156**	**0.220**	**0.156**	**0.083**	**0.202**	**0.000**															
G17	**0.606**	**0.119**	**0.239**	**0.174**	**0.183**	**0.165**	**0.284**	**0.202**	**0.183**	**0.229**	**0.128**	**0.202**	**0.174**	**0.165**	**0.229**	**0.193**	**0.147**	**0.202**	**0.000**														
G18	**0.661**	**0.138**	**0.147**	**0.229**	**0.183**	**0.183**	**0.284**	**0.257**	**0.165**	**0.156**	**0.092**	**0.202**	**0.138**	**0.220**	**0.138**	**0.156**	**0.202**	**0.147**	**0.183**	**0.000**													
G19	**0.532**	**0.193**	**0.275**	**0.211**	**0.220**	**0.202**	**0.303**	**0.257**	**0.275**	**0.303**	**0.183**	**0.239**	**0.211**	**0.220**	**0.266**	**0.211**	**0.202**	**0.220**	**0.165**	**0.239**	**0.000**												
G20	**0.569**	**0.211**	**0.275**	**0.266**	**0.165**	**0.220**	**0.229**	**0.257**	**0.239**	**0.229**	**0.220**	**0.257**	**0.174**	**0.257**	**0.138**	**0.211**	**0.239**	**0.165**	**0.257**	**0.202**	**0.165**	**0.000**											
G21	**0.560**	**0.183**	**0.303**	**0.220**	**0.193**	**0.211**	**0.312**	**0.303**	**0.248**	**0.294**	**0.174**	**0.266**	**0.220**	**0.229**	**0.220**	**0.183**	**0.211**	**0.174**	**0.211**	**0.229**	**0.101**	**0.101**	**0.000**										
G22	**0.606**	**0.193**	**0.257**	**0.284**	**0.202**	**0.239**	**0.321**	**0.294**	**0.239**	**0.266**	**0.202**	**0.257**	**0.193**	**0.257**	**0.229**	**0.193**	**0.220**	**0.183**	**0.239**	**0.165**	**0.183**	**0.128**	**0.101**	**0.000**									
G23	**0.560**	**0.183**	**0.211**	**0.239**	**0.229**	**0.211**	**0.294**	**0.248**	**0.248**	**0.239**	**0.174**	**0.248**	**0.239**	**0.229**	**0.220**	**0.239**	**0.193**	**0.193**	**0.229**	**0.211**	**0.138**	**0.138**	**0.110**	**0.174**	**0.000**								
G24	**0.550**	**0.229**	**0.275**	**0.266**	**0.220**	**0.257**	**0.284**	**0.294**	**0.294**	**0.284**	**0.239**	**0.275**	**0.248**	**0.257**	**0.211**	**0.211**	**0.257**	**0.202**	**0.257**	**0.239**	**0.183**	**0.073**	**0.138**	**0.147**	**0.156**	**0.000**							
G25	**0.642**	**0.138**	**0.239**	**0.193**	**0.202**	**0.092**	**0.303**	**0.202**	**0.165**	**0.248**	**0.183**	**0.239**	**0.156**	**0.147**	**0.211**	**0.193**	**0.165**	**0.165**	**0.147**	**0.183**	**0.183**	**0.202**	**0.193**	**0.220**	**0.193**	**0.239**	**0.000**						
G26	**0.606**	**0.138**	**0.275**	**0.229**	**0.220**	**0.202**	**0.303**	**0.275**	**0.165**	**0.248**	**0.165**	**0.257**	**0.211**	**0.220**	**0.248**	**0.229**	**0.202**	**0.239**	**0.147**	**0.183**	**0.257**	**0.275**	**0.229**	**0.220**	**0.248**	**0.239**	**0.220**	**0.000**					
G27	**0.624**	**0.284**	**0.294**	**0.248**	**0.294**	**0.239**	**0.303**	**0.330**	**0.239**	**0.284**	**0.239**	**0.385**	**0.248**	**0.257**	**0.303**	**0.303**	**0.330**	**0.294**	**0.294**	**0.239**	**0.294**	**0.312**	**0.321**	**0.312**	**0.321**	**0.349**	**0.220**	**0.294**	**0.000**				
G28	**0.606**	**0.119**	**0.257**	**0.248**	**0.202**	**0.165**	**0.303**	**0.294**	**0.165**	**0.266**	**0.165**	**0.239**	**0.174**	**0.220**	**0.229**	**0.211**	**0.183**	**0.183**	**0.165**	**0.202**	**0.239**	**0.220**	**0.193**	**0.220**	**0.248**	**0.202**	**0.183**	**0.110**	**0.330**	**0.000**			
G29	**0.679**	**0.138**	**0.165**	**0.156**	**0.165**	**0.202**	**0.266**	**0.239**	**0.147**	**0.156**	**0.092**	**0.257**	**0.138**	**0.165**	**0.193**	**0.174**	**0.202**	**0.147**	**0.147**	**0.110**	**0.220**	**0.220**	**0.193**	**0.183**	**0.193**	**0.239**	**0.183**	**0.147**	**0.202**	**0.202**	**0.000**		
G30	**0.624**	**0.119**	**0.257**	**0.229**	**0.165**	**0.128**	**0.266**	**0.275**	**0.165**	**0.229**	**0.183**	**0.257**	**0.156**	**0.202**	**0.174**	**0.174**	**0.202**	**0.147**	**0.165**	**0.165**	**0.220**	**0.183**	**0.174**	**0.183**	**0.211**	**0.165**	**0.128**	**0.147**	**0.275**	**0.073**	**0.183**	**0.000**	
G31	**0.661**	**0.174**	**0.202**	**0.248**	**0.220**	**0.183**	**0.193**	**0.239**	**0.202**	**0.193**	**0.183**	**0.312**	**0.193**	**0.220**	**0.211**	**0.229**	**0.220**	**0.183**	**0.220**	**0.165**	**0.257**	**0.220**	**0.266**	**0.257**	**0.193**	**0.239**	**0.165**	**0.220**	**0.202**	**0.239**	**0.165**	**0.183**	**0.000**

P1 - Swarna MAS; P2 – Pratikshya; G1 – G31: pyramided lines

Table 5 Clustering of the pyramided lines into different major clusters and sub-clusters

Sl No.	Major cluster	Total no. of lines	Sub-cluster	Sub-sub-cluster	Lines
1	Cluster I	1			Swarna MAS
2	Cluster II	32	Cluster II-A	Cluster II-Aa	*Pratikshya*, OR 2772-4, OR 2772-28, OR 2772-73, OR 2772-31, OR 2772-2, OR 2772-20, OR 2772-15, OR 2772-74, OR 2772-77, OR 2772-92, OR 2772-1, OR 2772-17, OR 2772-32, OR 2772-90, OR 2772-16, OR 2772-3, OR 2772-19, OR 2772-21, OR 2772-27 and OR 2772-30, OR 2772-33, OR 2772-54, OR 2772-64, OR 2772-51, OR 2772-65, OR 2772-62, OR 2772-10 and OR 2772-93
				Cluster II-Ab	OR 2772-13 and OR 2772-18
			Cluster II-B		OR 2772-76

gene is dominant and has been cloned with a high level of resistance from many *Xoo* strains [36]. The gene *xa5* is in the recessive gene category and codes an alternative form of transcription factor cIIa [37]. The *xa13* is a recessive gene that originated because of a mutation in the promoter region of a gene which is homolog to nodulin MtN3 [38].

The pyramided lines carrying these three genes in the present study exhibited higher degrees of resistance than both of the parents. This aligns with the study of some scientists elucidating the contribution of synergistic action of resistance genes in achieving increased levels of resistance [39, 40]. These three R genes are resistant to all the prevalent races of BLB pathogen in the coastal region of East India. However, previously many workers have used these three genes for the transfer of BLB resistance to many traditional varieties of Odisha, India, as well as in other parts of the country [22, 41].

Meanwhile, due to the masking effect of dominant genes over recessive genes, combining them at the same time might be a challenge via phenotypic selection. In such cases, molecular markers for both recessive and dominant resistance genes can assist in the identification and selection of desirable plants with multiple resistance genes [41, 42]. However, pyramided lines with the highest recovery of the recipient parent genome were found with the help of marker-assisted background selection, in the present study. In the BC_2F_3 generation, the highest genome recovery rate was 91.11–93.33% in five pyramided lines. The low recovery of the background of recurrent parent observed in a few lines can be attributed to linkage drag, referring to the reduction in fitness in cultivars due to deleterious genes introduced along with the beneficial gene during backcrossing [42]. It is possible that the functional portion of the genome is not recovered since SSR markers are often used to target non-coding and heterochromatic areas of chromosomes [43].

The phenotypic selection was also used in each generation to identify plants that are similar to *Pratikshya* in terms of agronomic characteristics, but also better from it. However, the functionally expressed area of the genome is an indirect target of phenotypic selection and helps to accelerate the recovery of the recurrent parent phenotype [41, 42]. Meanwhile, the linkage drag is kept to a minimum in this study by using genetically related parents in the crossing program. Swarna MAS (CR Dhan 800) is a derivative of the variety Swarna, which is a highly adaptable variety. Previous researchers concluded that using a highly adaptable variety as a donor parent results in better performance and less linkage drag than using a wild species or landrace as a donor parent [42]. However, the parentage of the recipient parent is Swarna and IR64, and the shared ancestry of *Pratikshya* with donor parent Swarna MAS speeds up the recovery of the recipient parent genome [42, 43].

Yield and agro-morphological parameters from thirty-one pyramided lines, *Pratikshya* and Swarna MAS showed that the pyramided lines have the outstanding yielding ability of recipient parent and resistance to BLB in the present study. The analysis of variance for yield and agro-morphological traits displayed that the mean for all characters were at par with *Pratikshya* in most of the lines, many lines performed to a greater degree in terms of seed yield per plant, and the majority of the lines were close to the recurrent parent, in the present study. However, various studies have revealed that the high seed yield of some pyramided lines may be attributable to the transmission of yield features from the donor source to the recipient parent [30, 34]. Meanwhile, in the background of *Pratikshya*, pyramided lines had no

Table 6 Agro-morphological and yield characters of thirty-one bacterial leaf blight resistance genes pyramided lines

Sl No.	Line no.	BLB score	Days to 50% flowering	Plant height (cm)	No. of tillers/Plant	No. of productive Tillers	Panicle length (cm)	No. of Filled Grains	1000 grain weight (g)	Seed yield/ plant (g)
1	*Pratikshya*	9	110	102.0	16	10	24.5	148	22.2	44.0
2	Swarna MAS	1	106	96.5	15	10	23.3	163	26.0	49.4
3	OR 2772-1	1	105	103.3	17	10	26.2	149	23.6	45.1
4	OR 2772-2	1	109	98.1	13	11	24.0	149	25.2	40.1
5	OR 2772-3	1	112	103.0	19	10	25.9	152	22.4	44.5
6	OR 2772-4	1	113	98.7	16	10	25.7	147	25.4	41.5
7	OR 2772-10	1	111	104.1	14	9	25.4	146	25.8	44.5
8	OR 2772-13	1	108	88.3	15	9	24.8	147	22.7	44.9
9	OR 2772-15	1	107	104.5	17	10	25.5	148	23.5	45.8
10	OR 2772-16	1	98	103.8	15	9	23.1	149	23.9	45.6
11	OR 2772-17	1	113	102.7	15	10	23.1	148	26.4	46.0
12	OR 2772-18	1	109	102.5	18	7	22.4	151	24.8	51.0
13	OR 2772-19	1	112	104.7	16	11	25.3	150	25.6	65.5
14	OR 2772-20	1	111	106.0	17	9	28.9	147	24.5	47.0
15	OR 2772-21	1	110	103.2	15	8	34.7	148	23.8	41.9
16	OR 2772-27	1	110	107.0	18	9	21.9	153	22.4	43.8
17	OR 2772-28	1	106	101.2	17	13	25.3	150	22.7	44.3
18	OR 2772-30	1	111	105.4	16	8	24.7	164	23.4	43.3
19	OR 2772-31	1	109	102.0	15	9	24.9	150	25.0	43.6
20	OR 2772-32	1	110	101.5	15	12	27.6	152	21.2	44.8
21	OR 2772-33	1	110	106.7	16	11	24.7	149	23.8	42.3
22	OR 2772-51	1	112	103.4	16	10	24.8	148	23.1	43.2
23	OR 2772-54	1	96	105.6	17	9	26.4	149	23.3	35.6
24	OR 2772-62	1	106	103.5	16	10	22.3	150	23.2	44.9
25	OR 2772-64	1	114	104.0	18	9	24.7	149	23.6	32.6
26	OR 2772-65	1	108	88.5	16	10	22.8	152	23.3	42.3
27	OR 2772-73	1	113	104.6	15	9	24.3	149	21.6	43.6
28	OR 2772-74	1	111	105.8	15	9	26.0	150	23.25	36.7
29	OR 2772-76	1	111	109.6	15	9	24.2	150	21.9	34.4
30	OR 2772-77	1	106	103.8	15	9	29.0	147	22.9	41.1
31	OR 2772-90	1	92	106.2	16	10	23.2	148	24.4	42.6
32	OR 2772-92	1	112	91.9	12	9	26.0	148	22.2	44.2
33	OR 2772-93	1	109	106.1	17	9	23.1	147	23.1	45.4
	Mean	1	108	102.4	16	9	25.1	150	23.6	42.4
	$CD_{0.05}$		4.0	3.1	NS	1.5	1.5	1.3	1.8	11.8
	CV (%)		1.8	1.5	10.8	7.7	3.1	3.8	3.8	10.3

antagonistic effect due to the introgression of resistance genes. High degrees of resistance to BLB, the lack of a penalty on yield, and good recovery of recurrent parent genome point towards a successful selection approach at both the molecular and phenotypic levels in the preset study. However, some scientists also found similar results from their study [11, 22, 34].

Due to the inadequacy of a single resistance gene in providing resistance against different pathogen races of BLB in Odisha, India, a wide range of resistance against BLB is critical. However, some scientists, reached a similar conclusion, observing a broad spectrum of resistance against bacterial leaf blight when multiple genes were introgressed into an elite genetic background rather than a single gene [44]. However, in the present study, pyramiding three resistance genes in a single genetic background was found to be an effective barrier against prevalent pathogen races of BLB in Odisha, India conditions, and expected to provide durable resistance.

Conclusion

Marker-assisted foreground and background selection along with phenotypic selection was found to be the best way to breed resistant varieties, making the breeding program

much more effective. The current research concludes that, the marker-assisted backcross breeding program was successful in introducing three BLB resistance genes (*xa5*, *xa13*, and *Xa21*) into the high-yielding rice variety *Pratikshya*. The resulting pyramided lines exhibited a high level of resistance to BLB infection and were similar to *Pratikshya* in terms of morphological features and yield parameters. This study suggests that, the three-gene pyramided lines could offer a significant yield advantage over the recipient parent *Pratikshya*. The pyramided lines can further be used for testing at multilocation, so as to be released as a variety or can be used as a potential donor for BLB resistance genes. The present study also suggests the deployment of multiple resistance genes can be more effective than the use of a single resistance gene. The BLB pyramided lines are proven to be successful in providing long-lasting and durable resistance in the genetic background of *Pratikshya*, which is an accomplishment for developing BLB-resistant pyramided lines bearing three resistance genes.

Author contributions DB, KCS supervised the work. MP, DB developed the genetic materials. MP, DB, KCS, MD, and JPS performed and supported the molecular analyses. MP, DB performed the field evaluations. MP, DB, and JPS performed and supported the analyses of data. MP, DB, KCS, MD, and JPS contributed in writing and critically revised the manuscript. All the authors read and approved the final manuscript.

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Data Availability All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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