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Improvement of Dubraj and Safri-17 varieties for conferring resistance against bacterial leaf blight through marker assisted selection approach

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Abstract
Marker assisted selection was employed to pyramid three bacterial blight resistance genes Xa21, xa13 and xa5 into high yielding susceptible local varieties Dubraj and Safri-17. Homozygous and heterozygous genotypes were identified in Dubraj X RP-Bio-226 and Safri17 X RP-Bio-226 crosses with the help of PCR markers and goodness of fit was tested. We tried to introgress multiple resistance genes responsible for BLB resistance (*xa5, xa13, Xa21*) in local variety Dubraj and Safri17. Dubraj is local scented variety while Safri 17 is high yielding variety of Chhattisgarh. Both the varieties have premium cooking quality. We screened the host plants for resistance against BLB by inoculating the leaf with the strain of *Xanthomonas oryzae* at Raipur (Chhattisgarh) through clip inoculation method. Genotyping with SSR primers was done to select lines introgressed with multiple resistance genes for BLB as well as blast. Populations introgressed with resistance genes for both BLB and blast, including 20 lines of Dubraj, 15 lines of Safri17 in F3 generation, 8 and 22 lines of Dubraj and Safri in F5 generation respectively and 45 lines of Dubraj and 60 lines of Safri 17 in F6 generation were selected on the basis of genotyping and phenotyping. Genotypes carrying resistance genes in different combinations were identified, selected lines were backcrossed with susceptible parents and generation advancement was done for others. The pyramided lines showed a wider spectrum and higher level of resistance against Xoo isolates under field conditions.

Keywords: Bacterial leaf blight, marker assisted selection, gene pyramiding, *Xanthomonas oryzae*

Introduction
Plant breeding is the major tool for advancement of crop abilities such as diseases resistance, resistance against abiotic stresses and plays a pivotal role in increased production as well. Bacterial Leaf blight (BLB) is the most devastating disease affecting entire rice acreages and cause severe yield losses up to 80%. Dubraj is a very popular scented variety in Chhattisgarh state and people prefer Dubraj more than any other variety. But the problem is, it is highly susceptible to BLB. Seeds are long slender in shape, translucent, plant is tall and has good cooking quality. Safri-17 is another traditional variety of Chhattisgarh, suitable for low lying areas but susceptible to BLB.

Ttraditional breeding approaches for developing resistance are laborious, take long time and requires more number of breeding cycles and also have plenty of physiological, environmental and ecological constraints. So, to overcome these setbacks, there is an urgent need to bring new approaches like Marker Assisted Selection (MAS) combined with high throughput and precision phenotyping. MAS gives a platform for boosting the progress in selection of positive progenies precisely in fewer selection rounds. Use of markers in selecting plants or progenies containing desired trait referred as MAS. Molecular markers are DNA sequences situated at specific locations of the genome and transmitted by the standard laws of inheritance from one generation to next. Pyramiding multiple resistant genes in a single line confers wide-spectrum and durable resistance. Tightly linked DNA markers have been developed for several Bacterial Leaf Blight resistance genes. The BB resistance genes, Xa1, xa5, xa13, Xa21, Xa26 and Xa27 have been cloned and used for breeding program. With the exception of xa5 and xa13, the BB resistance genes are dominant in nature and the markers developed from the sequencing information of these genes are widely used in MAS (Song *et al*. 1995; Gu *et al*. 2005; Chu *et al*. 2006a) [7, 2, 1].

The variety containing single resistance gene proved susceptible due to horizontal resistance developed. To overcome this problem pyramiding of more than one resistance gene can be beneficial. Therefore, incorporation of multiple Bacterial Leaf Blight resistant genes combination was taken up in the popular variety Dubraj and Safri 17 background by integrating marker assisted backcrossing simultaneously with phenotypic selection for development of pyramided lines.

Materials and Methods

Experimental/Parental materials

RP-Bio-226 which is a high yielding variety and possess premium grain quality obtained by Directorate of Rice Research (DRR) Hyderabad, India was selected as a donor parent as it possess three BLB resistance genes Xa21, xa13 and xa5. Dubraj is an aromatic short to medium grain rice which is a traditional Indian variety while Safri 17 is high yielding variety is with good cooking quality and both are most commonly used in Madhya Pradesh and Chhattisgarh were used as recurrent parent for introgression of BLB resistance gene(s) from donor.

Method

The recurrent parent Dubraj was crossed as female with donor RP-Bio-226 in kharif 2016. Resulting progenies were phenotypically and genotypically screened for resistance towards BLB. The plants were selected on genotypic well as phenotypic basis and were subsequently used for advancement of generation during next cropping season(s) summer 2017 and kharif 2017. Similarly, Safri 17 variety was crossed with donor RP Bio 226 and the resulting progenies were genotypically and phenotypically screened (Fig.1). The progenies which gave similar results in both genotype and phenotype were then selected for further research.

Phenotyping

Phenotyping for BLB resistance was done at Raipur using bacterial culture of *Xanthomonas oryzae pv oryzae (*Dhamtari isolates). The *Xoo* strain was cultured on Wakimoto agar media and bacterial suspension were artificially inoculated (10^9cfu/mL) through clip inoculation method at maximum tillering stage. Approximately 5-7 leafs per plant were inoculated and disease score was measured 21 days after inoculation as per standard evaluation system IRRI 2002.

Targeted genotyping

Marker assisted selection for BLB resistance

For the targeted introgression of Xa21, xa13, xa5 into Dubraj and Safri 17 stepwise marker assisted breeding was adopted. The crossing was carried out wherein the BLB resistance genes Xa21, xa13, xa5 from RP Bio-226 were introgressed into Dubraj and Safri 17. The derived plants were confirmed for hybridity using co dominant markers. The tightly linked SSR markers to xa5, xa13 and Xa21 genes (xa5R, xa5S, RM13, xa13 Pro, Xa21, PT-248-1) were used for foreground selection (Table 1). DNA from selected lines was extracted using modified CTAB method and used for PCR amplification at initial denaturation of 95 ^{0}C for 5 minutes with denaturation of 30 seconds, annealing at 55 ^{0}C for 30 s and extension at 72 ^{0}C for 1min with 30 cycles and final extension at 72 ^{0}C for 7 minutes. Amplified products will be resolved on 5% PAGE.

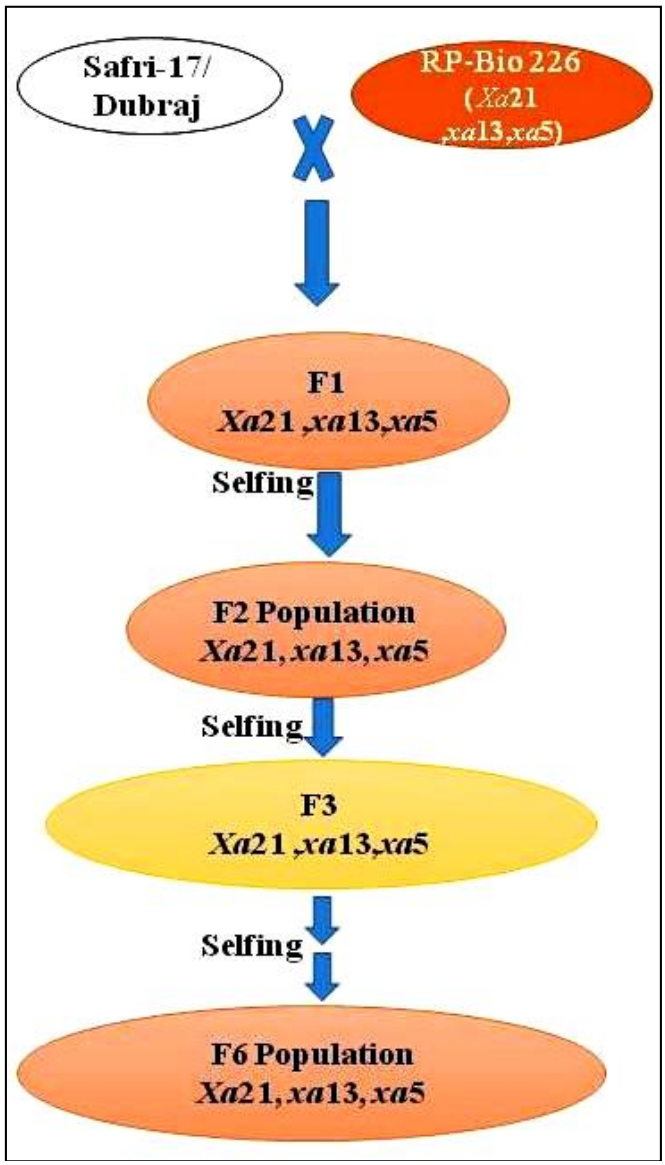

Fig 1: Steps involved for developing multiple resistance in Dubraj and Safri variety

Fig 2: Clip inoculation of *Xanthomonas oryzae*

Table 1: Markers used for foreground selection of three bacterial blight resistance genes in marker-assisted breeding

S. No.	Marker		Sequence	Resistance gene	Reference
1.	Xa 21	Forward	AGACGCGGAAGGGTGGTTTCCCGGA	Xa21	Huang *et al* 1997 [3]
		Reverse	AGACGCGGTAATCGAAAGATGAAA		
2.	PT248-1	Forward	AGACGCGAAGGGTGGTTCCCGA	Xa21	
		Reverse	AGACGCGGTAATCGAAGATGAAA		
3.	xa5R	Forward	AGCTCGCCATTCAAGTTCTTGAG	xa5	Sundaram *et al* 2011
		Reverse	TGACTTGGTTCTCCAAGGCTT		
4.	xa5S	Forward	GTCTGGAATTTGCTCGCGTTCG	xa5	
		Reverse	TGGTAAAGTAGATACCTTATCAAACTGGA		
5.	RM13	Forward	TCCAACATGGCAAGAGAGAG	xa5	
		Reverse	GGTGGCATTCGATTCCAG		
6.	xa13Pro	Forward	GGCCATGGCTCAGTGTTTAT	xa13	
		Reverse	GAGCTCCAGCTCTCCAAATG		

Results

The present study was proceed with the aim to improve Bacterial Blight resistance in safri-17 and Dubraj variety through MAS. Xa 21, PTA248-1, xa5R, xa5S, RM13 and xa13Pro were used for resistance genes viz., Xa21, xa5 and xa13 respectively. Aassessment of parental polymorphism is a prerequisite to commence marker assisted selection. Unless the parents are polymorphic for the traits of interest, the further selection of plants carrying the traits of interest is not achievable in the progenies.

Pyramiding of Resistance genes into Safri-17 and Dubarj background

The recurrent parent Safri-17 and Dubraj were crossed as female with donor RP-Bio-226**.** The progenies were confirmed for the presence of resistance gene(s) with PCR based linked markers. For Xa21, pT-248-1 and Xa21 an STS marker amplified resistant parent with 1200bp fragment while 300bp fragment was obtain with xa13 linked primer xa13 pro. Selection for xa5 gene was done with RM 13 with 140bp band for resistance parent and xa5R marker (presence of 160bp band only in resistant). In the current investigation the donor parent RP-Bio 226 possess all the three bacterial blight genes (Xa21, xa13 & xa5). Polymorphism was very clear in parents for the targeted genes and these markers are suitable for the selection of progenies.

The inoculated plants were screened for resistance against bacterial leaf blight according to the standard evaluation system IRRI 2002. It was observed that in Dubraj cross [(DxDPB)x(DxPR122)] out of 20 lines consisting of 60 plants, only 2 lines with 8 plants were selected with multiple resistance gene combination. Similarly in Safri 17 cross [(S17xRPB)x(S17xPR122)] out of 150 plants, 16 were selected in Kharif 2016 season. The presence of genes were determined by respective molecular marker. These markers are linked to resistance genes and scoring was done based on the banding pattern as per their respective parents. Those bands likely to be that of resistant and susceptible parents were score as R and S respectively. While plants showing bands form both the parents were scored as Heterozygous (H). Seeds of selected 8 plants of [(DxDPB)x(DxPR122)] cross from Kharif 2016 were then further sown as 8 lines (with 170 plants). Out of 170 progenies, 47 plants were selected in summer 2017 through genotypic scoring of banding pattern with linked marker. These selected 47 plants were further sown for generation advancement in Kharif 2017 (Table 2,3). Similarly in Safri-17 cross [(S17xRPB)x(S17xPR122)] out of 480 plants 56 were selected with marker banding pattern during summer 2017. Selected 56 progenies were used for further generation advancement.

Phenotyping of pyramided genotypes against BB pathogen

It was also found that sometimes there is differences in phenotypic and genotypic resistance (Table 3). In case of Dubraj cross [(DxDPB)x(DxPR122)], 90 progenies were found resistant in phenotypic screening in kharif 2016. It was found that Xa21 marker gene was also showing total 90 resistant progenies with xa5R marker gene absent and 84 progenies resistant when screened with xa13 marker. Out of 90 progenies in summer 2017 season, 76 were phenotypically resistant and 72 progenies were showing resistance due to Xa21, 80 and 6 progenies were showing resistance due to xa13 and xa5 respectively. The evaluation of kharif 2017 generations found that 54 progenies out of 90 were resistant phenotypically and rest were susceptible. Also, 28 progenies were found resistant with marker for Xa 21 gene and 69 progenies were showing absence of Xa 21 gene, 2 progenies were showing resistance due to xa5 gene and 35 were due to xa13 gene.

In kharif 2016 Safri 17 cross, 118 progenies showed phenotypic resistance (Fig. 3, 4) but Xa21 resistance gene was only present in 70 progenies and xa5R and xa13 was showing resistance in 14 and 100 progenies. And in summer 2017 we have 106 resistant phenotype, 35 resistant progenies from Xa21 and 34 from xa5R and 104 from xa13 marker gene. So now we have 94 progenies showing resistance in phenotypic screening but only 28 progenies were showing resistance in Xa21 marker with 60 and 23 progenies in xa 13 and xa5R in kharif 2017. As expected, parents i.e. Safri-17 & Dubraj recorded as susceptible and RP-Bio 226 were recorded as resistance disease reactions. As above result showed that when three gene present in homozygous condition (xa21 xa21/xa13 xa13/ xa5 xa5) show resistance disease reaction, While all these three gene in heterozygous condition (Xa21 xa21/xa13 xa13/ Xa5xa5) show MR reaction. Two gene combinations show more resistance reactions than solitary gene reactions.

Fig 3: BLB infected rice Plants

Fig 4: BLB infected rice Plants taken for scoring

Table 2: Selected lines after genotypic and phenotypic screening

Season	Cross	Lines	Plants
KH 2016	DxDPBxDxPR122 TOTAL	20	60
	DxDPBxDxPR122 SELECTED	2	8
	S17xRPBxS17xPR122 TOTAL	15	150
	S17xRPBxS17xPR122 SELECTED	6	16
S 2017	DxDPBxDxPR122 TOTAL	8	170
	DxDPBxDxPR122 SELECTED	7	47
	S17xRPBxS17xPR122 TOTAL	22	480
	S17xRPBxS17xPR122 SELECTED	16	56
KH 2017	DxDPBxDxPR122 TOTAL	45	130
	DxDPBxDxPR122 SELECTED		
	S17xRPBxS17xPR122 TOTAL	60	300
	S17xRPBxS17xPR122 SELECTED		

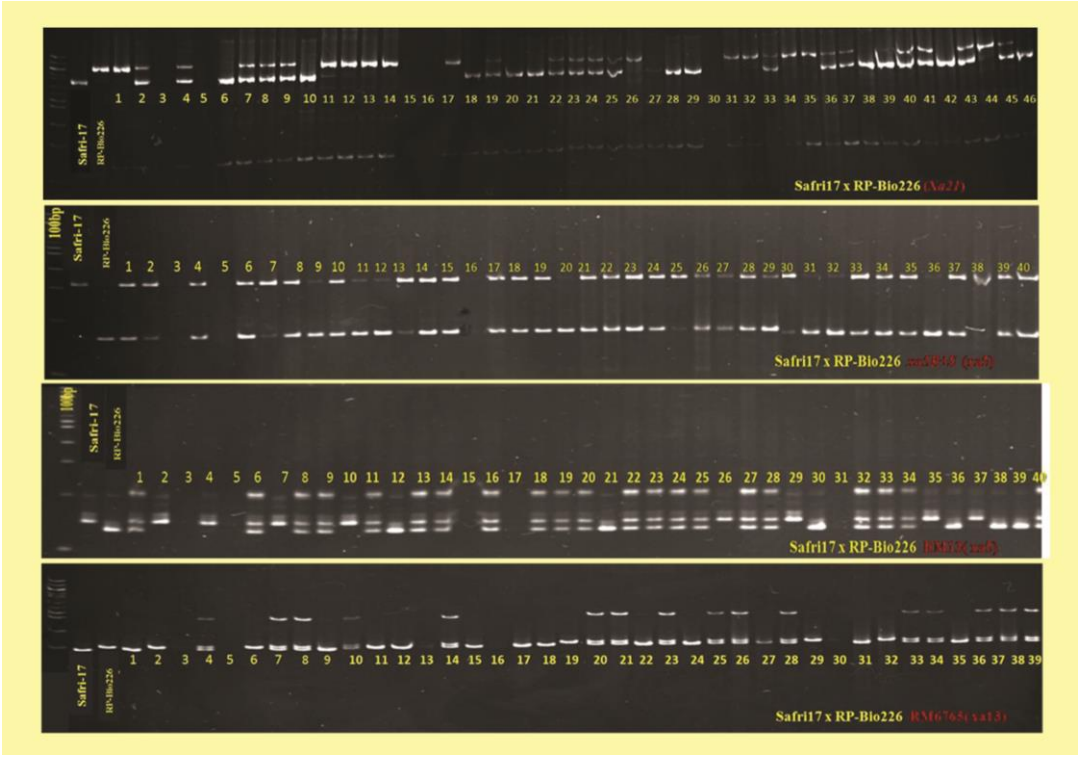

Fig 5: PCR amplification of population derived from Safri17x RP-Bio226 using linked markers for Xa21, Xa13and Xa5 genes

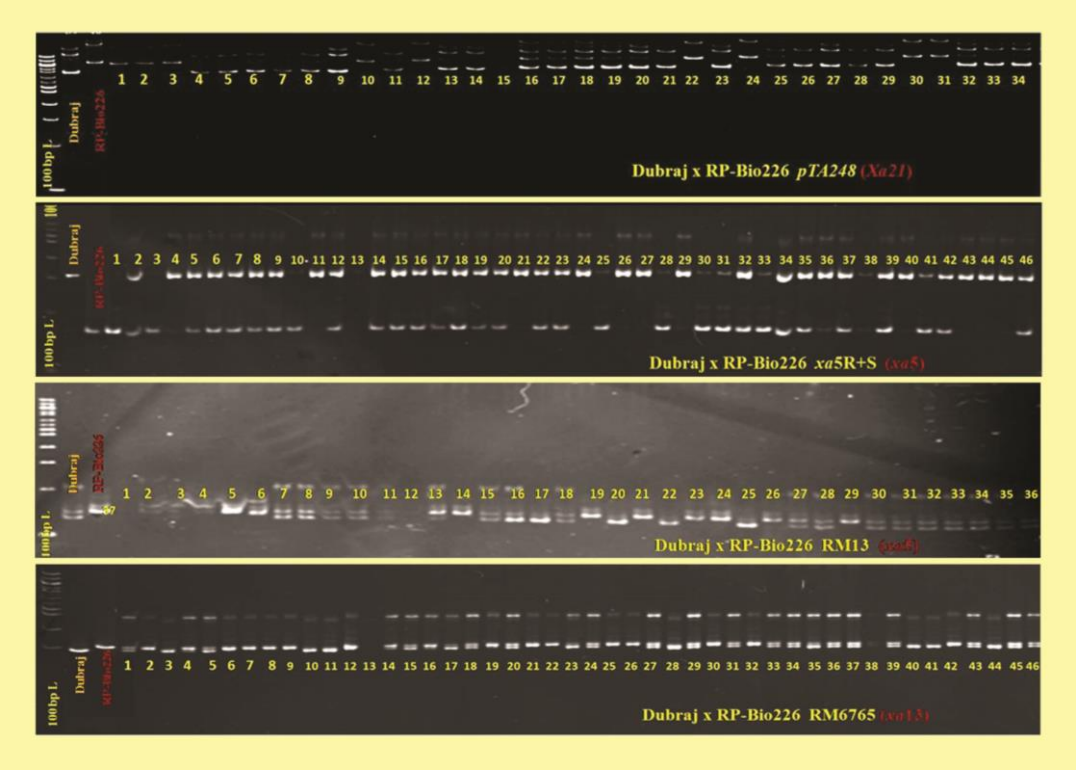

Fig 6: PCR amplification of population derived from Dubraj x RP-Bio226 using linked markers for Xa21, Xa13and Xa5 genes

Table 3: Result of Genotyping and Phenotyping

(S17 x RPB) X (S17 x PR-122)																			
	xa13				xa5R				xa 5 S				Xa 21				Phenotype		
	R	S	H	AB	R	S	H	AB	R	S	H	AB	R	S	H	AB	R	S	Total
KH 16	100	14	4	-	14	-	-	104	-	84	-	34	70	28	20	-	118	-	118
KH 17	60	12	20	26	23	-	-	95	-	85	-	33	28	17	4	69	94	24	118
S 17	104	12	-	2	34	-	-	84	-	94	-	24	35	20	6	57	106	12	118

(D x RPB) X(D x PR-122)																			
	xa13				xa5R				xa 5 S				Xa 21				Phenotype		
	R	S	H	AB	R	S	H	AB	R	S	H	AB	R	S	H	AB	R	S	Total
KH 16	84	4	2	-	-	-	-	90	-	90	-	-	90	-	-	-	90	-	90
KH 17	35	7	40	8	2	-	-	88	-	81	-	9	73	12	-	5	54	36	90
S 17	80	10	-	-	6	-	-	84		84	-	6	72	16	2	-	76	14	90

Note:- R represents Resistant progeny, S- susceptible, H- Hybrid, AB- Absent, KH 16- Kharif 2016, KH 17- Kharif 2017, S17- Summer 2017, D- Dubraj, RPB- RP Bio, S17- Safri 17

Discussion

Phenotypic selections in three selfing generations coupled with SSR based selection was sufficient for transfer of Xa21, xa13 and xa5 genes into Dubraj and Safri 17 background. The three gene combination pyramided lines expressed higher levels of resistance in comparison to parental lines, two and single gene combination. The results suggest that two gene combinations with Xa21 + xa13 was most effective with shorter lesions lengths followed by Xa21 + xa5 while lines with xa13 + xa5 were relatively less effective. Lines with Xa21 in combination with either xa5, xa13, or both have shown promise advocating the utility of Xa21 in achieving higher levels of resistance in rice as reported earlier (Singh *et al*. 2001; Sanchez *et al*. 2000; Huang *et al*. 1997) [6, 5, 3] suggesting that synergistic action and/or quantitative complementation between the resistant genes might result in enhanced levels of resistance (Sanchez *et al*. 2000) [5]. All the three resistance genes that have been considered in the present work have been cloned and characterized. Xa21 is a dominant resistance gene that encodes a receptor kinase containing NBS-LRR domains (Song *et al*. 1995) [7], while xa5 is a recessive resistance gene and encodes a variant form of transcription factor cIIa (Iyer and McCouch 2004) [4]. The xa13 resistance gene is also recessive in nature and has been shown to be a mutation in the promoter region of a gene that is a homolog of the nodulin MtN3 (Chu *et al*. 2006) [1]. In rice lines containing the dominant (susceptibility) allele of the gene, the expression of the nodulin homolog is up regulated upon infection with Xoo. It appears that the increased expression of this gene is necessary for Xoo to grow on rice. This up regulation does not occur in rice lines containing the resistance (recessive) xa13 allele. The apparently different modes of action of the three resistance genes used in this work might contribute to make the resistance in the three-gene pyramid lines quite durable.

The high levels of resistance to BB and the absence of any yield penalty due to accumulation of resistance genes in the pyramids provides us a successful example of the integrated approach of selection at both molecular and phenotypic levels for transfer of the desired trait(s) and recovery of the recurrent parental genome. Development of broad-spectrum resistance against BB in the Indian subcontinent is a major challenge due to the rich diversity of the agroclimatic zones where rice is cultivated, as well as the presence of a number of

genetically distinct virulent Xoo strains in different geographical areas of India. Deployment of a three gene combination like xa5 + xa13 + Xa21 can achieve durable and broad-spectrum resistance in many BB prone rice growing areas in India including the deepwater ecosystem. The study clearly establishes the utility of MAS in pyramiding recessive genes like xa5 and xa13, and dominant gene Xa21 to present a multiple gene barrier against one of the most destructive diseases of rice in a long duration, photosensitive and deepwater rice.

Conclusion

We were successful in identifying superior recombination's for three BB resistance genes (Xa21, xa13 and xa5) in homozygous as well as in heterozygous condition. The pyramided genotypes can be further be used for multi-location testing to be released as variety in the country or be used as potential BB resistance donors. The BB pyramided deepwater breeding lines, which are developed through MAS and phenotypic selection, will be of practical value in providing durable bacterial blight resistance in the growing region where control through chemicals was less effective. These BB pyramided lines are expected to have a high impact on the yield stability and sustainability of rice production.

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