Research Paper

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Marker-assisted pyramiding of bacterial blight and gall midge resistance genes in Samba Mahsuri and study of their interactions

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KEYWORDS : MAS, gene pyramiding, gene interaction, Gm1, Gm4, Xa21, xa13, Bacterial blight, gall midge

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ABSTRACT

Bacterial blight (BB) of rice caused by Xanthomonas oryzae pv. oryzae and gall midge (Orseolia oryzae) are two major constraints in rice production. In order to combine BB and GM resistance genes against two constraints, B277-SM2113 (a NIL of Samba Mahsuri) possessing BB resistance genes xa13 and Xa21 was individually crossed with two GM resistant varieties viz., Kavya possessing Gm1 (conferring HR- type resistance) and Abhaya possessing Gm4 (conferring HR+ type resistance). True F1s from the individual crosses were again crossed to combine all 4 resistance genes. The resulting IF1s possessing all the 4 resistance genes in heterozygous condition were selfed to generate IF_2 plants. A total of 35 IF_2 plants were obtained. All the 35 IF_2 plants were vegetatively multiplied through tiller separation technique, genotyped with gene specific markers for all 4 resistance genes and phenotypically evaluated against both BB and gall midge. Out of 35 IF_2 plants tested, a single plant showed the presence of two BB and two gall midge resistance genes in homozygous condition. Four plants carrying only BB resistance genes exhibited gall midge resistance in addition to BB resistance, while nine plants carrying only gall midge resistance genes exhibited BB resistance in addition to gall midge resistance indicating pleiotropic effect of the BB/GM resistance genes. Majority of the plants (82%) possessing both gall midge resistance genes i.e., Gm1 and Gm4 showed HR+ type resistance indicating preponderance of HR+ reaction over HR- type reaction.

Introduction

Pyramiding of two or more resistance genes through Marker Assisted Selection (MAS) has been a successful approach to ensure multiple and durable host plant resistance against a wide spectrum of biotic stresses (Yoshimura *et al.,* 1995). Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the serious diseases of rice across countries and can cause yield losses as high as 74 to 81% (Srinivasan and Gnanamanickam, 2005). Till date, at least 38 BB resistance (R) genes have been identified (Sundaram *et al*., 2014), of which 19 genes have been tagged and mapped with closely linked markers. Using MAS breeding approaches three or more BB genes, like *xa5, xa13, Xa21* have been successfully pyramided in diverse elite rice varieties like IR64, PR106, Pusa Basmati 1, Lalat, Tapaswini, Swarna, IR64 and Samba Mahsuri (Sundaram *et al*., 2014). One of these cultivars called Improved Samba Mahsuri (ISM) has been released for commercial cultivation and several breeding lines in the genetic background of Samba Mahsuri possessing different combination of BB resistance genes have been developed (Sundaram *et al*., 2008). The Asian rice gall midge (GM), *Orseolia oryzae* (Wood-Mason) is economically third most important pest of rice in India causing average annual yield loss of about US $80 million (Sundaram, 2007). Host resistance against this insect is reported to be conferred by 11 GM genes of which eight have been tagged and mapped (Yasala *et al*., 2012). Here we report pyramiding of two each of major GM and BB resistance genes (*Xa21* and *xa13* for BB resistance and *Gm1* and *Gm4* for gall midge resistance) in the genetic background of Samba Mahsuri through marker assisted selection and the interaction of BB and GM resistance genes.

Material and methods

Plant material and crossing scheme

The plant materials consisted of two gall midge resistant rice varieties viz., Kavya possessing *Gm1* which confers HR- type resistance (Sundaram, 2007) and Abhaya possessing *Gm4* conferring HR+ type resistance (Divya *et al*., 2015) and a BB resistant line, B277-SM2113 (NIL of Samba Mahsuri) possessing two BB resistance genes, *xa13* & *Xa21* (Sundaram *et al*., 2008). Two separate crosses were made between B277-SM2113 X Kavya and B277-SM2113 X Abhaya. Hybridity of the F_1s was confirmed with gene linked markers. True F_1s from both the crosses were then intercrossed to combine all the four genes viz., *Xa21*, *xa13*, *Gm1* and *Gm4*. The presence of 4 resistance genes in heterozygous condition in the IF_1s (inter-crossed F_1) was carried out with gene specific markers. The true IF_1s possessing all 4 resistance genes (in heterozygous condition) was selfed to generate IF_2s. The IF_2 plants were individually genotyped and phenotyped for their reaction to BB and GM. (multiple plants were generated using tillers separation technique).

Genotyping for presence of target genes

DNA was isolated from each individual IF_2 plants using CTAB method (Zheng *et al*., 1995). Polymerase chain reaction was carried out to detect the presence of two (GM) and two BB resistance genes using gene specific markers. The presence of *Gm1* gene was detected using flanking SSR markers RM23914 and RM23956 (Sundaram *et al*., 2014) and the presence of another gene, *Gm4* with the functional marker LRR-del (Divya *et al*., 2015). The IF_2 plants were also tested for the presence of BB resistance gene *xa13* using the functional marker, *xa13-* prom (Sundaram *et al*., 2014) and BB resistance gene *Xa21* with tightly linked marker pTA248 (Ronald *et al*., 1992). For both BB and GM genes, TN1 was used as negative check (Fig.1).

Gall midge phenotyping

Selected IF_2 plants were grown in earthen pots under glass house condition. At the tillering stage, two to three tillers were separated carefully from each plant and grown separately to generate multiple individuals of each IF_2 plant. One each of freshly emerged male and female gall midge (biotype 1) adult insects were released in a small plastic cup covered inside with moist filter paper for two days under room temperature for oviposition. On the fourth day, eggs were observed under microscope to detect fertile eggs with eye spots which were ready to hatch. Such eggs were used for infesting the isolated tillers of the test plants (Bentur *et al*., unpublished). After 10 days from day of infestation, the tillers were dissected under microscope

to check maggot survival and for HR reaction. Plants with dead maggot and with expression of HR or without HR were rated as resistant HR+ or HR- plants. Plants with live maggots were rated susceptible.

BB phenotyping

A virulent isolate of the bacterial blight pathogen, DX-020 was used for screening the test plants for BB resistance. The bacterium was grown on modified Wakimoto's mdium for 72-96 hours at 27°C. Plant leaves were clip-inoculated with a bacterial suspension (10^9cfu/ml) at maximum tillering stage (45-55 days after establishment) following the methodology of Kauffman *et al*., (1973). Approximately 3–6 leaves were inoculated per plant and disease reaction was recorded 14 days after inoculation. Lesion length of each inoculated leaf was measured and the disease severity was scored as per IRRI-standard evaluation system (IRRI, 2002). Plants with 0-3 score were rated as resistant (R), those with 3.1-5 were rated as moderately resistant (MR) and the plants with disease score >5 were considered susceptible.

Results

Detection of presence of GM and BB resistance genes through analysis with markers and phenotypic screening

Selfing of a true IF_1 having all 4 genes in heterozygous condition resulted in generation of 35 IF_2 plants. Genotyping of 35 IF_2 plants for the presence of the resistance genes for GM (*Gm1*, *Gm4*) and BB (*Xa21*, *xa13*) using the gene-specific/linked markers revealed that, a single plant had all the four genes viz., *Gm1, Gm4, Xa21* and *xa13* in homozygous condition (Table 1, genotyping details are given in supplementary Table 1). Twenty plants showed different combination of three resistance genes (i.e. *Gm1*, *Gm4* and *xa13*; *Gm1*, *Xa21* and *xa13*). Five plants showed the presence of different two-gene combinations (i.e. *Gm1* and *Gm4*; *Gm4* and *Xa21*; *Xa21* and *xa13*). Nine plants showed the presence of different single genes.

All the 35 IF_2 plants were subjected to phenotypic screening for both GM and BB separately. All the 35 IF_2 plants showed resistance to GM (either HR+ or HR- types). Of these, 20 plants showed HR+ type of reaction while rest 15 plants showed HR-ve type. For BB, all the plants showed resistance whether with a single gene or with the combination of both the genes in homozygous conditions.

Four plants (*i.e*., plant no: 19, 20, 21, 26), which possessed *Xa21* gene singly or in combination with *xa13* gene, but none of the GM resistance genes were present, (based on marker-analysis) were observed to be resistant to GM based on phenotypic screening. Out of these, two plants (*i.e*., plant no: 21, 26) revealed the presence of dead maggot inside the plants, when dissected, while another two (*i.e*., plant no: 19, 20) did not show the presence of dead maggots, indicating the possibility that these could be escapes. Interestingly, a similar observation was noticed with respect to cross resistance to BB conferred by GM resistance genes. Three plants (*i.e*., plant no: 7, 32, 34) did not possess either *Xa21* or *xa13,* but both the GM resistance genes showed resistance to BB. Another six plants which have either of the GM genes (*Gm1*/*Gm4*), without any of the BB genes (*Xa21*/*xa13*) showed resistance to BB when phenotyped. The above observations indicated that some kind of complementary effect of GM resistance genes to BB resistance and vice-versa.

Discussion

Pyramiding is the process of combining genes or QTLs in a single plant from different parents (Hittalmani *et al*., 2000). There is strong evidence that combining resistance genes can provide broad-spectrum and durable resistance (Yoshimura *et al*., 1995; Singh *et al*., 2001). Besides, such pyramids are good tools to study interactions among the R genes. Marker Assisted Selection (MAS) has made the task of pyramiding genes much easier. While development of multiple resistant elite varieties need more time and selection for the desirable agronomic traits, gene interaction studies can be done in very early stages as well. Normally, F_2 derived F_3 families are used for phenotypic evaluation of the segregating population for studies on genetics or tagging and mapping of desired gene/QTL. We used IF_2 plants for genotyping in the present study to identify plants possessing both GM and BB resistance, to study interaction between genes conferring resistance against the two biotic stresses and also to study the interaction between genes conferring resistance against GM through two diverse mechanisms (i.e. HR+ conferred by *Gm4* and HR- conferred by *Gm1*). Since separate tests against BB and gall midge could not have been done on a single plant simultaneously, we adopted tiller separation technique for vegetative propagation of the same plant for screening against gall midge and BB simultaneously and the technique was observed to give full-proof results without any false positives.

Our results showed that, at least in some of the plants, the presence of GM resistance genes (*Gm1, Gm4*) singly or in combination, resulted in resistance to both GM and BB, even without the presence of the BB resistance genes. Similarly the presence of the BB resistance gene (*Xa21, xa13*) singly or in combination resulted in resistance to BB and GM, without the presence of GM genes in them. These types of observations indicate complementary effects of *Gm1* and/or *Gm4* to BB resistance and complementary effect of *xa13* and/or *Xa21* towards gall midge resistance, possibly due to pleiotropic effect of the resistance gene(s). The GM resistance in plants carrying *xa13* and *Xa21* genes needed further confirmation through phenotyping, as the flanking markers for *Gm1* was only linked to the gene and not part of the gene.

Our study also revealed that most of the plants which showed amplification of the resistance linked allele for both the flanking markers for *Gm1* were also phenotypically resistant indicating the strategy of using flanking markers is the best one when the markers are linked and not part of the gene (Biradar *et al*., 2004; Himabindu *et al*., 2007). BB resistance in plants with only *Gm1* and *Gm4* genes was unambiguous. There were no qualitative or quantitative differences in BB resistance in plants that carried both of the BB resistance genes, either singly or in combination.

It is pertinent to note that, *Gm1* and *Gm4* genes confer resistance through two distinctly different modes HR+ type and HR- type (Sundaram, 2007). Interaction between these two genes, when pyramided together, was observed to show interesting results in our study. Majority of the plants carrying both these genes expressed HR+ type of resistance, indicating the preponderance or epistatic interaction of *Gm4* conferred HR+ reaction over the HR- reaction conferred by *Gm1*. In our earlier studies also, Rao *et al*., (2004) observed that HR+ type is epistatic over HR- type of resistance. A typical interaction among different BB resistance genes was reported by Sundaram *et al*., (2009) who found that in the genetic background of Triguna, the lines possessing gene combination *xa5* + *Xa21* showed significantly longer lesion length compared to the lines having only *Xa21* indicating a negative interaction between *xa5* and *Xa21* in the genetic background of rice variety Triguna.

Thus it is likely that *Gm4* gene action may be epistatic over the *Gm1* gene action. Through our study, we could validate all the gene-specific markers for *Gm1*, *Gm4*, *Xa21* and *xa13*. Our preliminary studies have highlighted some of the possible interactions among the resistance genes showing cross resistance when pyramided in a single plant, an aspect which requires further reconfirmation and elaboration.

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Table 1: Phenotypic and Genotypic evaluation of IF_2 plants with different resistance gene combinations against BB and gall midge

	Allelic status of GM R genes based on linked marker analysis		Allelic status of BB R genes based on linked marker analysis		Phenotypic analysis		No. of Plants	HR+	HR -
S.No	Gm1	Gm4	Xa21	xa13*	GM	BB			
1	RR	RR	RR	rr	R	R	1	1	0
2	RR	RR	rr	rr	R	R	18	15	3
3	RR	rr	RR	rr	R	MR	2	0	2
4	RR	RR	rr	RR	R	R	3	1	2
5	rr	rr	RR	rr	R	MR	1	0	1
6	rr	RR	RR	RR	R	NI#	1	1	0
7	rr	RR	rr	RR	R	R	1	1	0
8	rr	rr	RR	RR	R	MR	3	0	1
9	RR/ Rr	rr	rr	RR	R	R	5	0	4

Not Inoculated, RR- Homozygous Dominant, rr – Homozygous Recessive, Rr – Heterozygous, R – Resistant, MR – Moderately Resistant, HR+ - Hypersensitive type of reaction, HR - - Non Hypersensitive type of reaction

* With respect to *xa13* gene, as the gene is recessive in its inheritance, RR indicates that the dominant allele is present in homozygous condition and the gene will not express (i.e. the plant will be susceptible to BB), while Rr indicates that both the dominant and recessive alleles are present in heterozygous condition and the gene will not express. Only those plants possessing the recessive allele in homozygous condition (i.e. rr) will be resistant to BB

Figure 1:

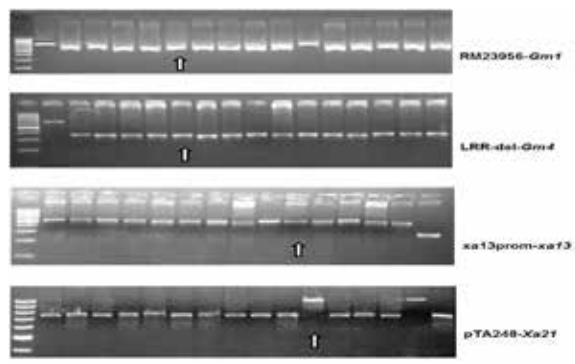

Fig. 1 Amplification pattern of two GM genes and two BB genes with each individual IF_2 plants along with their respective resistant and susceptible parents and 100bp ladder. Arrows indicate the positive plant for all the four genes.

Supplementary Table: 1
Total Phenotypic and Genotypic evaluation of IF_2 plants.

	Allelic status of GM R genes based on linked marker analysis		Allelic status of BB R genes based on linked marker analysis		Phenotypic analysis		GM resistance type
Pl.No	Gm1	Gm4	Xa21	xa13	GM	BB	HR+/-
1	RR	RR	rr	rr	R	R	HR+
2	RR	RR	rr	rr	R	R	HR+
3	RR	RR	rr	rr	R	R	HR+
4	RR	RR	RR	rr	R	R	HR+
5	RR	RR	rr	rr	R	R	HR-
6	RR	RR	rr	rr	R	R	HR+
7	RR	RR	rr	RR	R	R	HR+
8	RR	RR	rr	rr	R	R	HR-
9	rr	RR	rr	RR	R	R	HR+
10	RR	RR	rr	rr	R	R	HR+
11	RR	RR	rr	rr	R	Not inoculated	HR+
12	RR	RR	rr	rr	R	R	HR+
13	RR	RR	rr	rr	R	R	HR+
14	RR	RR	rr	rr	R	R	HR-
15	RR	RR	rr	rr	R	R	HR+
16	RR	RR	rr	rr	R	R	HR+
17	RR	RR	rr	rr	R	R	HR+
18	RR	RR	rr	rr	R	Not inoculated	HR+
19	rr	rr	RR	RR	Escape	MR	
20	rr	rr	RR	RR	Escape	MR	
21	rr	rr	RR	RR	R	MR	HR-

	Allelic status of GM R genes based on linked marker analysis		Allelic status of BB R genes based on linked marker analysis		Phenotypic analysis		GM resistance type
Pl.No	Gm1	Gm4	Xa21	xa13	GM	BB	HR+/-
22	Rr	rr	rr	RR	R	R	HR-
23	Rr	rr	rr	RR	R	MR	HR-
24	RR	rr	rr	RR	R	MR	HR-
25	RR	rr	rr	RR	Escape	MR	
26	rr	rr	RR	rr	R	MR	HR-
27	RR	rr	rr	RR	R	MR	HR-
28	RR	rr	RR	rr	R	MR	HR-
29	RR	RR	rr	rr	R	R	HR+
30	RR	RR	rr	rr	R	R	HR+
31	RR	RR	rr	rr	R	R	HR+
32	RR	RR	rr	RR	R	R	HR-
33	Rr	rr	RR	rr	R	Not inoculated	HR-
34	RR	RR	rr	RR	R	R	HR+
35	rr	RR	RR	RR	R	Not inoculated	HR+

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