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Simple Sequence Repeat (SSR) markers linked to the blast resistance gene *Pi-1* in rice for marker-assisted selection (MAS)

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Introduction

Rice blast caused by *Pyricularia grisea* (Cooke) Sacc., is the most limitant biotic factor for rice production in the world. Although the use of resistance genes is the most important method for blast control, resistance is rarely effective for more than 2-3 years (Correa-Victoria and Zeigler, 1993). Strategies aiming at breeding for durable rice blast resistance have recently focused on the possibility of using a lineage exclusion strategy to target resistance gene combinations that are likely to provide an effective barrier to the fungus (Zeigler *et al.*, 1995). In addition, molecular marker technologies, such as development of closely linked molecular markers, have made it possible to pyramid bacterial blight and blast resistance genes into one rice genotype (Huang *et al.*, 1997; Hittalmani *et al.*, 2000). It has been also indicated, that marker-assisted selection could be particularly useful to improve disease resistance in commercial rice cultivars (Mohan *et al.*, 1997; Sánchez *et al.*, 2000; Chen *et al.*, 2000). Here we report six new markers for the blast resistance gene *Pi-1*, which were identified using SSR sequences available in public database. Three of these SSR markers resulted highly linked to the resistance gene at the end of rice chromosome 11, therefore, they can be potentially used in MAS to introduce *Pi-1* into blast susceptible varieties and to pyramid blast resistant genes to develop commercial varieties with more durable blast resistance.

Materials and Methods

Plant material and disease evaluation

The near-isogenic lines C101LAC (resistant line) and C101A51 (susceptible line) developed at IRRI were crossed and F_1 seeds generated. The F_2 progeny, resulting from self-pollination of F_1 individuals, were self-pollinated to generate CT13432 F_3 lines. Blast disease evaluation was performed according to CIAT's rice pathology laboratory manuals. The inoculum was prepared as described by Correa-Victoria and Zeigler (1993). Plants were evaluated 15 days (two life cycles of the pathogen) after inoculation and scored for resistance and susceptibility (Figure 1) in two replications.

Figure 1. Typical symptoms: leaf blast (a) and neck blast (b). Lesion type scale: Resistant (0, 1 and 2); Susceptible (3 and 4).

DNA extraction and PCR assay

The DNA extraction was conducted following the procedure described by Dellaporta *et al.*, (1983). Polymerase chain reaction (PCR) was conducted according to CIAT standard procedures. The amplification product were loaded on high-resolution agarose gels prepared mixing 1.5 % Sinergel (DiversifieD Biotech), 0.7 % agarose molecular grade (Invitrogen Life Technologies), and containing 0.5mg/mL of ethidium bromide.

SSR polymorphism analysis

Sequences of twenty-six SSR markers were selected from the Gramene database (www.gramene.org) considering their relative proximity to *Pi*-1 gene (located no more than 10cM of the corresponding gene) in the current rice genetic map. The isogenic lines C101LAC and C101A51 and their common genetic background, the susceptible recurrent parent CO39, were used to identify SSR polymorphisms associated to the blast resistance gene. Polymorphic SSR markers identified above were assayed by bulked segregant analysis. DNA bulks were prepared from resistant and susceptible lines within the CT13432 F_3 families inoculated with the isolate Oryzica Yacu 9-19-1. The diagnostic potential of the SSR markers associated to *Pi-1* gene was also evaluated on DNA obtained from fifteen elite commercial rice varieties grown in Latin America. Comparing with phenotypic evaluation obtained as indicated above, the veracity of the assay was corroborated.

Genetic and Linkage analyses

Genetic analysis of the resistance was conducted measuring the goodness of fit to the expected ratio for a single gene model using a chi-square test. For this purpose, we used F_3 and F_4 segregating populations derived from single F_2 plants with no selection. Putative molecular markers linked to the *Pi*-1 gene were used in linkage progeny analysis. Associations between SSR markers and the resistance *Pi-1* gene were demonstrated using a chi-square test. Linkage analysis was performed using MAPMAKER software on the segregation data obtained from SSR markers and blast resistance scoring of the CT13432 F_3 population. Distances between markers were expressed in Kosambi centiMorgans (cM).

Results

Expected and observed segregation ratios for F_3 and F_4 populations are shown in Table 1. The F_3 population analysis showed a good fit to the expected segregation ratio (1:2:1) for a single gene model ($\chi^2 = 1.0$, $p < 0.05$). This segregation ratio was also confirmed in the F_4 population ($\chi^2 = 0.1$, $p < 0.01$). Correlation between F_3 and F_4 population ratios reached a value of 0.96 (Spearman range coefficient, $p < 0.0001$). These results confirmed the hypothesis of a single dominant gene for *Pi-1*.

Table 1. Segregation of F_3 and F_4 lines of the genetic cross between C101LAC (Pi-1)/C101A51 inoculated with the blast isolate Oryzica Yacu 9-19-1of *Pyricularia grisea*.

Population	Expected ratio†	No. of lines expected			No. of lines observed		
		S	SG	R	S	SG	R
F_3 Lines	1:02:01	71	141	71	76	133	74
F_4 Lines	1:02:01	71	141	71	72	139	72

(†) According to a model based on a single dominant gene as indicated in materials and methods; (S): Susceptible, (SG): Segregant; (R): Resistant

Of the twenty-six microsatellite sequences tested, eleven were polymorphic and seven (27 %) were linked to *Pi-1* (all χ^2 values were greater or equal to 128.99, $p < 0.0001$). Five SSR markers were not polymorphic, and ten, did not amplify with the primer pairs used.

Linkage between the markers and blast resistance was confirmed by screening 157 F_3 lines from the cross C101LAC/C101A51 segregating for *Pi-1*. Genetic distance between the markers and the *Pi-1* locus ranged from 0.0 (no recombination between the marker and resistance factor) to 23.8 cM (Figure 2). Among the SSR makers linked to *Pi-1* gene, three markers (RM1233*I, RM5926 and RM224), showing a codominant feature (Figure 3), mapped in the same position (0.0 cM) with the *Pi-1* gene. Other three dominant markers corresponding to the same genetic locus (RM7654) were located at 18.5 cM above the *Pi-1* gene, while marker RM6094 was identified at 23.8 cM below of the gene. This last SSR marker was characterized by the presence of a band in the susceptible genotype and by the absence of the band in the resistant lines, therefore being not potentially useful for MAS and excluded in further analysis.

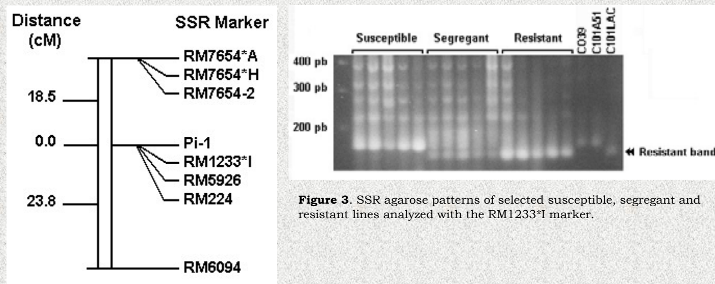

Figure 2. Chromosome 11 generated through linkage analysis. Relative positions of SSR markers to blast resistance gene *Pi-1* are indicated.

Figure 3. SSR agarose patterns of selected susceptible, segregant and resistant lines analyzed with the RM1233*I marker.

In order to determine the potential application of these SSR markers in rice breeding programs based on marker-assisted selection, different statistical indicators were calculated (Table 2). All SSR markers were highly sensible and specific, but three of them (codominant markers) also showed zero values of false positive (false resistant) and a predictive capacity of the resistance events (PPV) that reached 100 percent. For this reason, these three markers were selected as putative candidate markers to be used in MAS programs aiming at improving blast resistance in rice.

Table 2. Quality indicators for six molecular markers associated to the blast resistance gene *Pi-1* based on results of linkage progeny analysis.

Quality Indicators	Molecular marker analyzed					
	RM1233*I	RM7654*A	RM7654*H	RM7654-2	RM5926	RM224
F3 population	Probability qualification (%)					
Test sensibility†	**99.18**	98.37	97.52	97.58	**98.33**	**99.18**
Test specificity‡	**100.00**	97.14	94.29	97.14	**100.00**	**100.00**
False positive (FP)	**0.00**	2.86	5.71	2.86	**0.00**	**0.00**
False negative (FN)	**0.82**	1.63	2.48	2.44	**1.67**	**0.82**
Positive predictive value (PPV)	**100.00**	99.18	98.33	99.17	**100.00**	**100.00**
Negative predictive value (NPV)	**97.22**	94.44	91.67	91.89	**94.59**	**97.22**

(†) Probability that the SRR test were positive (presence of band) if the line was resistant in the pathogenicity test. (‡) Probability that the SSR test were negative if the line was susceptible in the pathogenicity test. (FP) Probability that the SSR test were positive given that the line was susceptible. (FN) Probability that the SSR test were negative given that the line was resistant. (PPV) Probability that the line were resistant given that the SSR test was positive. (NPV) Probability that a line were susceptible given that the SSR test was negative.

Table 3. Analysis of the predictive capacity of blast resistance in commercial rice varieties using SSR markers linked to the resistant *Pi-1* gene.

Variety	Country	PA	Molecular marker analyzed				
			RM1233*I	RM7654*A	RM7654*H	RM7654-2	RM224
CO-39†	Philippines	S	-	-	-	-	-
C101LAC‡	Philippines	R	+	+	+	+	+
Cica-8	Colombia	R	+	+	+	+	+
Fedearroz	Colombia	R	-	-	-	-	-
Colombia XX1	Colombia	S	-	-	-	-	-
Oryzica 1	Colombia	S	-	-	-	-	-
Fedearroz 50	Colombia	R	-	-	-	-	-
Epagri 108	Brazil (irrigated)	S	-	-	-	-	-
BRIRGA409	Brazil (irrigated)	R	+	+	+	+	+
Primavera	Brazil (upland)	R	-	-	-	-	-
Bonanza	Brazil (upland)	R	-	-	-	-	-
El Paso 144	Uruguay, Argentina	R	+	+	+	+	+
Cimarron	Venezuela	R	-	-	-	-	-
Capirona	Peru	S	-	-	-	-	-
Panamá 1048	Panama	R	+	+	+	-	+
CR 1113	Costa Rica	R	+	+	+	+	+
Jucarito-104	Cuba	R	-	-	-	-	-

(†) Susceptible control. (‡) Resistant control; **PA**:Results of the pathogenicity assay, **R**: resistant genotype, **S**: susceptible genotype, (+) presence of resistant allele, (-) absence of resistant allele

To examine whether the markers identified would be of general utility on a wider range of rice germplasm characteristic of applied breeding programs in Latin American, the resistant alleles of five markers were examined in elite rice varieties and compared to the reported inheritance of *Pi-1* (Table 3). For this purpose, we used known sources of blast resistance as positive controls and considered as predictive criteria of the resistance, the amplification in each variety of the specific SSR alleles for *Pi-1* gene. Comparing with phenotypic data on blast resistance, our results showed that all known sources of resistance (C101LAC, Cica 8, BR IRGA 409, CR 1113, El Paso 144 and Panama 1048) present the specific *Pi-1* allele. In addition, four susceptible varieties (Colombia XXI, Epagri 108, Capirona and Oryzica 1) and the negative control (CO-39) had not the resistant allele. On the other hand, other seven varieties (Jucarito-104, Fedearroz 2000, CR 1821, Primavera, Cimarón, Bonanza and Fedearroz 50), which were resistant in the pathogenicity assay, did not show the allele characteristic of the *Pi-1* gene, most probably due to the interaction of other resistance genes in these cultivars and the presence of other avirulence genes different to avr-Pi-1 in the blast isolate used in the pathogenecity tests.

Conclusions

Near isogenic lines are very useful for identifying highly linked molecular markers to blast resistance genes in rice. Three markers (RM1233*I, RM5926 and RM224) are closely linked (0 cM) to the resistance gene *Pi-1*. These markers showed a highly sensible and specific, zero value of false positives, and a predictive capacity of the resistance events (PPV) that reached 100 percent. These markers can be used for the selection of resistant sources carrying the *Pi-1* resistance gene and to eliminate susceptible germplasm. However, the use of these markers as a diagnostic tool for determining the presence of the resistance gene *Pi-1* in a wider range of rice germplasm require additional studies for further confirmation of the results reported here. The speed, simplicity and reliability of PCR based approaches using SSR markers, together with the availability of nucleotide sequence database (**www.gramene.org**), make SSR analysis an attractive tool for MAS in rice breeding programs aiming at developing durable rice blast resistant cultivars.

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