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SSRs for Marker-Assisted Selection for Blast Resistance in Rice (*Oryza sativa* L.)

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Abstract Rice blast caused by the fungus *Magnaporthe oryzae* is one of the most devastating diseases of rice in nearly all rice growing areas of the world including Malaysia. To develop cultivars with resistance against different races of *M. oryzae*, availability of molecular markers along with marker-assisted selection strategies are essential. In this study, 11 polymorphic simple sequence repeat (SSR) markers with good fit of 1:2:1 ratio for single gene model in F_2 population derived from the cross of Pongsu seribu 2 (Resistant) and Mahsuri (Susceptible) rice cultivars were analysed in 296 F_3 families derived from individual F_2 plants to investigate association with *Pi* gene conferring resistance to *M. oryzae* pathotype. Parents and progeny were grouped into two phenotypic classes based on their blast reactions. Chi-square test for the segregation of resistance and susceptibility in F_3 generation fitted a ratio of approximately 3:1. Association of SSR markers with phenotypic trait in F_3 families was identified by statistical analysis. Four SSR markers (RM413, RM5961, RM1233 and RM8225) were significantly associated with blast resistance to pathotype 7.2 of *M. oryzae* in rice ($p \leq 0.01$). These four markers accounted for about 20% of total phenotypic variation. So, these markers were confirmed as suitable markers for use in marker-assisted selection and confirmation of blast resistance genes to develop rice cultivars with durable blast resistance in Malaysian rice breeding programmes.

Keywords Rice (*Oryza sativa* L.) · Rice blast (*Magnaporthe oryzae*) · Simple sequence repeats (SSRs) markers · F_2 and F_3 population · Marker-assisted selection · Resistance gene

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Introduction

Rice as a staple food crop is grown more than 90% of the world's and consumed in Asia, and considerably, more rice production is anticipated due to the rapid population growth in this part of the world (Khush 2005). Three billion people depend on it as a major source of their subsistence diet (Cantrell and Reeves 2002). Rice blast caused by *Magnaporthe oryzae* is one of the most destructive diseases of this crop production in different part of the world. The disease causes considerably large grain yield losses in almost all major ecosystems where it is grown (Talbot 2003). DNA markers that co-segregate with the gene are a powerful method to develop a resistant cultivar. The availability of different molecular markers allows characterization of genes of interest. Simple sequence repeat (SSR) markers are extensively used in diversity analyses (Baraket et al. 2011; Swapna et al. 2010), marker-assisted selection (Jena and Mackill 2008; Zhu et al. 2009) and inheritance studies (Campoy et al. 2011). It is also applied to identify markers tightly linked to blast resistance genes and to detect genes and QTLs on rice chromosomes (Fjellstrom et al. 2004a, b; Zhu et al. 2004; Liu et al. 2005). Molecular breeding

Table 1 List of the SSR markers

Marker	Forward primer (5′–3′)	Reverse primer (5′–3′)	Chromosome no.	Product size (bp)
RM104	GGAAGAGGAGAGAAAGATGTGTGTCG	TCAACAGACACACCGCCACCGC	1	222
RM244	CCGACTGTTCGTCCTTATCA	CTGCTCTCGGGTGAACGT	10	163
RM6836	TGTTGCATATGGTGCTATTTGA	GATACGGCTTCTAGGCCAAA	6	240
RM8225	ATGCGTGTTCAGAAATTAGG	TTGTTGTATACCTCATCGACAG	6	221
RM206	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG	11	147
RM1233	GTGTAAATCATGGGCACGTG	AGATTGGCTCCTGAAGAAGG	11	175
RM5961	GTATGCTCCTCCTCACCTGC	ACATGCGACGTGATGTGAAC	11	129
RM168	TGCTGCTTGCCTGCTTCCTTT	GAAACGAATCAATCCACGGC	3	116
RM413	GGCGATTCTTGGATGAAGAG	TCCCCACCAATCTTGTCTTC	5	79
RM350	TGATCGTCGCGATTCCCGGC	CCCCACCCTGCGCCTCTCCC	8	208
RM1359	CTCGCGAGGAAGAAGACAAC	CGCCGGCTGGTTAATTAATC	4	170

approach involving DNA markers, QTLs mapping, marker-aided selection (MAS) and genetic transformation for resistance against blast or other traits has been used in developing a new improved cultivated rice by many rice researchers and breeders (Narayanan et al. 2002; Wang et al. 2010; Mallikarjuna and Sarla 2010; Zhao et al. 2010). PCR-based DNA markers now are used as a low-cost, high-throughput alternative to conventional phenotypic screening for direct detection of disease resistance genes, allowing rapid introgression of genes into susceptible varieties as well as the incorporation of multiple genes into individual lines for more durable blast resistance. DNA markers are neutral to environmental variation, and therefore, can be selected accurately (McCouch et al. 2001). MAS has become an important tool for durable resistance screening, minimising the time and cost to make progress in breeding programmes. MAS in rice is being practised for blast disease (Liu et al. 2002; Toojinda et al. 2005). The model markers used in MAS are based on PCR, and SSR is taken into increasing account by virtue of more polymorphism and better repetition over other marker systems. In particular, SSR markers are abundant in plants, which would be more conducive to crop genetic improvement (McCouch et al. 2001). These markers are believed to have an important role in MAS, especially for selection of resistance genes (Liu et al. 2003). SSR markers linked to the *Pi* gene reported, which indicated these markers were useful for selection of resistance genes at the *Pi* gene locus in rice germplasm (Fjellstrom et al. 2006). Many useful DNA markers related to major genes conferring race-specific resistance of blast have been identified (Fjellstrom et al. 2004a, b). Fjellstrom et al. (2004a, b) developed and then demonstrated that DNA markers tightly linked to three resistant genes, *Pi-b*, *Pi-k* and *Pi-ta*, respectively, were suitable for MAS for these three genes in various segregating populations. Sharma et al. (2005) identified two SSR markers, TRS26 and TRS33, and both were closely associated with the resistant genes, and therefore, can be used in MAS for the resistant genes. The objectives of the present investigation were to determine suitable SSR markers for marker-assisted selection of the genes conferring resistance to *M. oryzae* pathotype, 7.2 in F_3 families derived from cross of Pongsu seribu 2 (resistant cultivar) and a susceptible rice cultivar, Mahsuri.

Material and Methods

Plant Materials

Ten seeds per plant of F_2 population derived from a cross between resistant rice variety, Pongsu seribu 2 and a susceptible cultivar, Mahsuri, were used to produce F_3 families under greenhouse conditions in 2010 at Malaysian Rice Research Centre, MARDI. Pongsu seribu 2 is a local resistant variety that possesses broad-spectrum resistance to blast fungal isolates originating from Malaysian Rice Research Centre, MARDI (Akama 1978). Mahsuri is very

Table 2 Segregation in the F_3 population obtained from cross between the rice cultivars Pongsu seribu 2 × Mahsuri inoculated with pathotype 7.2 of *M. oryzae*

Total no. of seedlings	Resistant (R)	Susceptible (S)	Expected ratio	χ^2	*p* value
296	208	88	3:1	3.52	0.0701

According to a model on a single dominant gene, (S): Susceptible and (R): Resistant. df=1.0; X^2 (0.05, 1)=3.84

Table 3 Chi-square test for two independent genes (9:3:3:1) and epistasis effect (15:1) for blast resistance in F3 population derived from cross between the rice cultivars Pongsu seribu 2 × Mahsuri inoculated with pathotype 7.2 of *Magnaporthe oryzae*

Gene models	Total no. of F_3 seedlings	Observed ratio				Expected ratio	χ^2	*p*-value
		R	MR	MS	S			
Independent genes[a]	296	128	80	60	28	9:3:3:1	24.9*	<0.0001
Epistasis effect[b]	296	268	–	–	28	15:1	4.7*	0.030

[a] χ^2 0.05,3 = 7.81, df=3; [b] χ^2 0.05,1 = 3.84 ,df=1; * significant at 5% level

For two independent genes (R): Resistant; (MR): Moderately Resistant; (MS): ModeratelySusceptible, and (S): Susceptible and for epistasis effect R, MR and MS considered as R and S as susceptible

susceptible to blast, and currently, it is used as a check variety for blast screening. The F_3 seeds were pre-germinated by soaking in water at 25°C for 48 h and were grown in a plastic tray (36 cm×23 cm×10 cm) in a green house at 25–30°C for 2–4 weeks, until they reached the four-leaf stage (Filippi and Prahbu 2001). Twenty-two-day-old plants, with three or four fully expanded leaves, were used as rice materials for the resistance spectrum tests. For DNA extraction, the young leaves samples of F_3 families and parents were collected from 30-day-old seedlings.

Inoculation Methods and Disease Assessment

For screening of resistant and susceptible parents and their F_3 progenies, the most virulent *M. oryzae* pathotype 7.2 obtained from Malaysian rice research centre, MARDI, were used to test for their disease reaction. Standard Seedling Evaluation System (SES) of International Rice Research Institute (IRRI, 1996) was used to evaluate plants for their disease resistance reaction of blast lesion degree (BLD), using a scale from 0 to 9 as follows: 0=no evidence of infection, 1=brown specking indicative of an relative humidity (RH) response (<0.5 mm in diameter), 3=brown lesions of 0.5–1 mm wide, 5=round to elliptical lesions of 1–3 mm in diameter (or length) with a grey centre, 7= spindle shaped lesions with a grey centre, and 9=coalesced type 4 lesions across the majority of the leaf. Plants scored with a reaction of less than 3 were rated as resistant, whereas plants with a reaction greater than 3 were rated as susceptible (IRRI, 1996; Mackill and Bonman 1992). The individual plants of the F_3 families were used to study the genetic inheritance of blast resistance and SSR markers' segregation. For segregation analysis of a single dominant gene model, plants with disease reaction score of 0, 1 and 3 were considered as resistant, while disease reaction scores of 5, 7 and 9 were considered as susceptible. Two-gene model was also analysed by classification of resistance to pathotype P7.2 as resistance (R), moderately resistance (MR), moderately susceptible (MS) and susceptible (S). The plants with lesion score 0 to 1 were considered R, 3 as MR, 5 as MS and 7 to 9 as S (IRRI, 1996). According to the Mendelian principle, the phenotypic segregation for a two-gene model is 9:3:3:1 for R:MR:MS:S, respectively. Test of epistasis was carried out by analysing F_3 populations to determine whether it segregates with 15(R):1(S) ratio or not. When only recessive alleles are present in the homozygous condition, it forms susceptible plants. Therefore, plants with score of 5 (moderately susceptible) were also considered as resistant in epistasis effect analysis. The plants with disease reaction score of 0, 1, 3 and 5 were considered as R, while disease reaction scores of 7 and 9 were considered as S.

DNA Extraction

Leaves samples of five plants from each 296 F_3 family were harvested, and DNA extraction was done. Bulked DNA of five plants from each family was subjected to SSR analysis (Ronald and Vasil 1994). DNA extractions were done using the modified CTAB extraction protocol described by McCouch et al. (1988). Quality of DNA was observed by running on 1% agarose gel electrophoresis by staining with 0.1 μg/ml ethidium bromide using 1× TAE buffer at a constant voltage of 72 V for 30 min and visualised under UV light. DNA samples were diluted with sterile-distilled

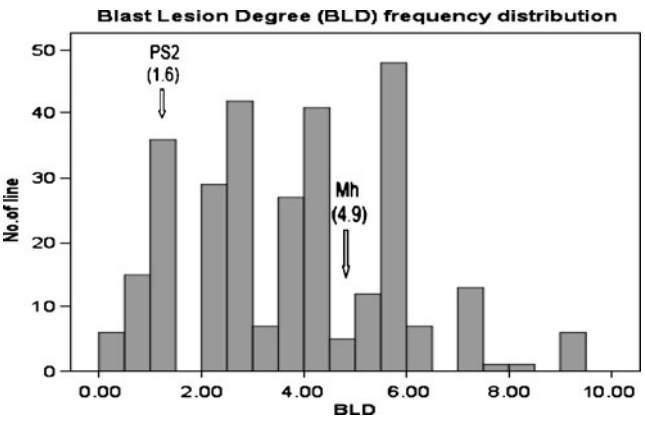

Fig. 1 Distribution of blast lesion degree (*BLD*) in the F3 families inoculated with rice blast pathotype, 7.2

Table 4 Marker analysed in the F_3 population derived from cross between the rice cultivars Pongsu seribu 2 × Mahsuri indicates that the observed F_3 segregation ratios of this population were not significantly different from the expected 1:2:1 single dominant gene segregation

According to a model on a single dominant gene, (A): Resistant; (B): Susceptible; and (AB or SG): Segregant. df=1.0; χ^2 (0.05, 1)=3.84; χ^2 (0.01, 1) =6.63

Markers	Marker segregation analysis (no. of lines observed)			χ^2 (1:2:1)	*p* value
	A	AB=SG	B		
RM104	90	110	96	19.76	<0.0001
RM168	84	129	83	4.89	0.0867
RM1359	115	98	83	40.7	<0.0001
RM413	81	136	79	1.97	0.3734
RM8225	81	135	80	2.29	0.3182
RM6836	88	125	83	7.32	0.0257
RM350	120	95	81	48.24	<0.0001
RM244	99	96	100	35.16	<0.0001
RM1233	84	134	78	2.89	0.2357
RM5961	79	138	79	1.35	0.5092
RM206	96	122	78	11.32	0.0035

water to a concentration of 20 ng and kept in a refrigerator of−20°C for PCR analysis.

Microsatellite Analysis

Primer sets of 120 microsatellites with known chromosomal positions distributed on rice chromosomes were selected from the Gramene database (www.gramene.org) related to blast resistance genes (*Pi* genes) which have been mapped by Akagi et al. (1996), Temnykh et al. (2000) and McCouch et al. (2002a, b). The two parental lines were used to screen SSR marker polymorphism. Twenty-three of polymorphic SSR markers were used in genetic evaluation and segregation analysis of F_2 individuals derived from cross of two rice cultivars, Pongsu seribu 2 and Mahsuri. Eleven polymorphic microsatellites (Table 1) that showed good fit of 1:2:1 ratio for single gene model in F_2 population were used for analysis in the F_3 generation. PCR reactions were performed in a total volume of 12.5 µl as described in McCouch et al. (2002a, b). Final concentrations of the components were 1× PCR buffer (10 mM Tris–HCl, 100 µM of each dNTP (0.2 mM each of dATP, dTTP, dGTP and dCTP), 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 1.0 µM of forward and reverse primers, and 0.2 U *Taq* polymerase and 40 ng template DNA. PCR amplification was carried out in a GeneAmp System 9700 (Applied Biosystems, Foster City, CA). After an initial denaturing step for 94°C for 5 min, 35 cycles of the polymerization reaction were performed with 30 s at 94°C, 30 s at either 48°C, 51°C, 55°C or 60°C (depending on the primer pair), and 30 s at 72°C, followed by a final extension step at 72°C for 5 min by rapid cooling to 4°C prior to analysis. Amplified PCR products were resolved on a 3.0% agarose gel stained with 0.1 µg of ethidium bromide per microlitre to detect the amplicons in 1× TBE buffer (0.05 M Tris, 0.05 M boric acid, 1 mM EDTA, and pH 8.0). The gel was run at a constant voltage of 90 V for 1 h and were illuminated using UV light and analysed using Bio-Imaging System (Chemi-Genius, USA).

Marker Genotyping

All 296 F_3 families were genotyped with 11 SSR markers. These marker alleles were detected on 3% agarose gels. The allele size of individual was compared with allele size of the parents for the specific marker. A ladder was added with the first load, and was used to confirm the allele sizes observed in the parental and progeny survey. The

Table 5 Molecular markers statistics associated with blast resistance to pathotype 7.2 in the F_3 families

Trait	Marker	SMR (R^2)	SMR (F)	Percentage[a]	*p*[b]
BLD	RM413	0.081	13.05	8	0.00001
	RM1233	0.023	3.4	2	0.04493
	RM8225	0.022	3.36	2	0.03602
	RM5961	0.068	10.8	7	0.00004

BLD blast lesion degree, *SMR* single marker regression

[a] Proportion of the total phenotypic variance accounted for the markers

[b] The probability of an association

individuals, which showed the size of resistant parent alleles, were scored as "A," those with a banding pattern similar to the susceptible parent alleles were scored as "B," and the heterozygous or segregating plants were scored as ("AB or SG").

SSR Marker-Assisted Selection

Selection of resistant plants in F_3 segregating progenies carrying the known genes of blast resistance was carried out at seedling stage with specific DNA marker alleles as a diagnostic tool (Ribaut and Hoisington 1998). Only individuals representing allele sizes of markers tightly linked with resistant gene were selected in marker genotyping.

Statistical Analysis

Chi-square analysis for goodness of fit to 3:1, 15:1 and 9:3:3:1 ratios was calculated using the formula, $\chi^2=(O-$

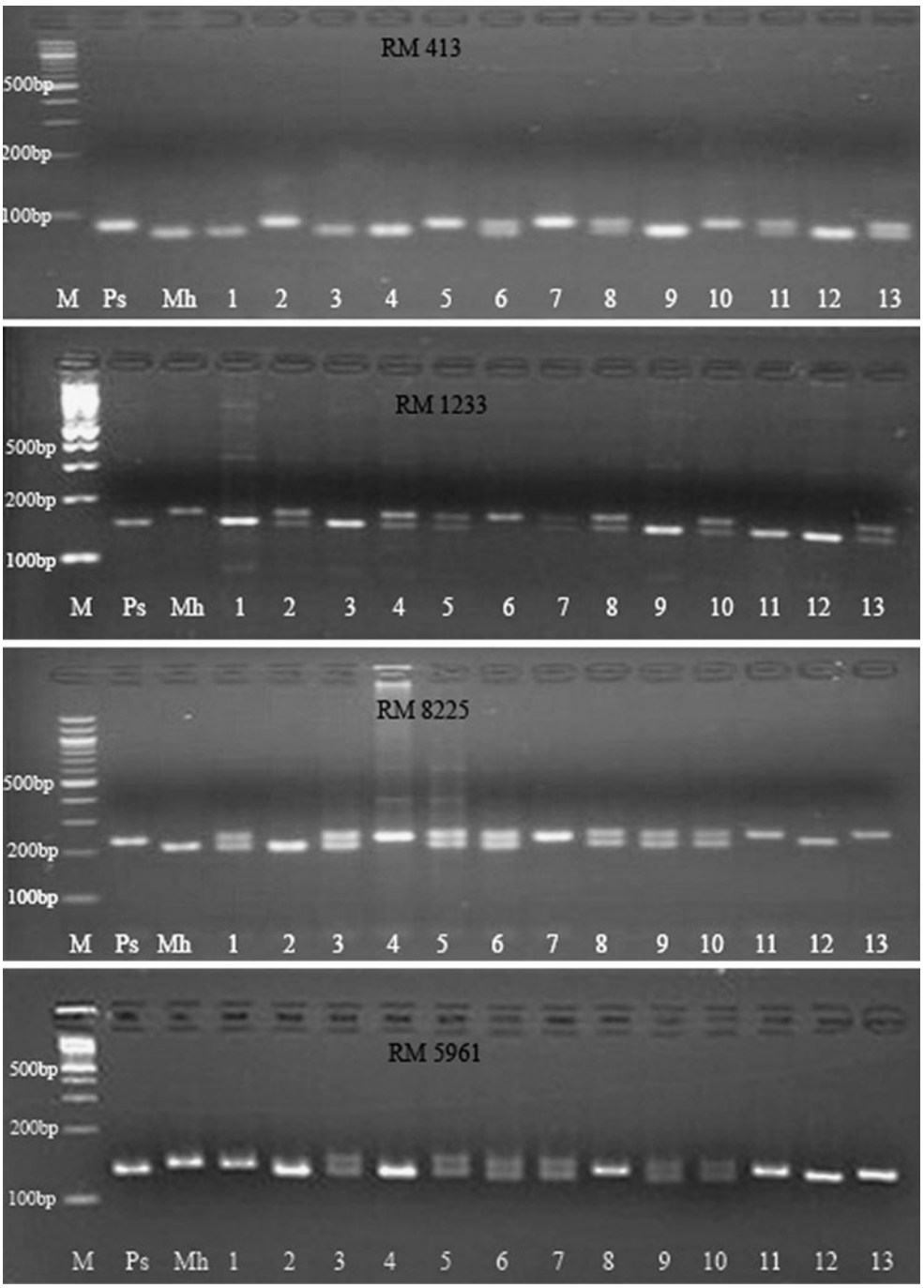

Fig. 2 PCR products for genotyping with markers RM413, RM1233, RM8225 and RM5961, linked to blast resistance genes in F_3 population of rice derived from Pongsu seribu 2 (*Ps*) × Mahsuri (*Mh*). Running on 3% Agarose gel stained with ethidium bromide, only 13 samples plus the two parents for each markers are shown (*M*=100 bp ladder)

$E)^2/E$, where O is an observed value and E is the expected value. For each locus in this research, the observed segregation data were also tested by chi-square analysis against the expected Mendelian ratio (1:2:1). Simple regression analysis was conducted using the General Linear Model (glm) procedure in SAS (SAS Institute 1989) for test of association between gene markers and phenotypes with a threshold significance level of $p \leq 0.05$ and 0.01.

Results

Phenotypic Data Analysis

Two hundred and ninety-six F_3 families, including two parents in three replicates, were inoculated with pathotype, P7.2. The score of five plants from each F_3 families was averaged, and the mean scores in replicates were used in analysis. The results of blast disease evaluation of F_3 families are shown in Tables 2 and 3. Chi-square tests of data obtained from segregation of resistance of different gene models, i.e. two-independent-gene model and/or two locus interactions, showed that the expected number of resistant and susceptible plants in the segregation ratio for a single dominant gene model was not significantly different from the number of observed resistance and susceptible plants and fitted the 3:1 segregation ratio at the $p \leq 0.05$ significance level.

Trait Frequency Distribution

Frequency distribution of blast disease evaluation for trait, BLD (blast lesion degree), is shown in Fig. 1. The pathotype 7.2 was virulent on Mahsuri with average BLD scores of 4.9 compared to 1.6 for this trait in Pongsu seribu 2. About 27% of the F_3 families showed average scores below 3.

Molecular Marker Assays

Eleven SSR markers were analysed for the alleles existing in F_3 plants. All of these markers exhibited visible polymorphic bands and showed consistent band sizes between resistant or susceptible plants. Segregation ratios for used markers are shown in Table 4. The segregation analysis revealed that the Pongsu seribu 2 carried resistant genes associated with SSR markers, RM413, RM1233, RM8225 and RM5961. As indicated in Table 5, these markers showed a good fit to the expected marker segregation ratio (1:2:1) in a Mendelian fashion (df=1.0, $p \leq 0.05$). PCR products for SSR markers RM413, RM1233, RM8225 and RM5961 with presence of differing SSR alleles are shown in Fig. 2.

Effects of Markers on Disease Severity

Marker-trait analysis in segregating F_3 population showed significant association between genotypes of four SSR markers RM413, RM1233, RM8225 and RM5961 at the $p \leq 0.01$ level of significance. These markers conformed large resistance effect about 20% for phenotypic variation to a given pathotype. Single-point analysis of variance or regression on a marker that reveals significant ($p \leq 0.05$) associations between markers and blast resistance is shown Table 5.

Marker-Assisted Selection

For specific alleles of four SSR markers RM413, RM1233, RM8225 and RM5961, F_3 families were analysed. Parental bands that were amplified as controls along with the F_3 individuals were used in screening. For plants that possessed the resistant gene alleles of linked markers, they were retained in programme as resistant plants and otherwise were eliminated. Figure 3 represents resistant plant screening using RM5961 based on resistant parent Pongsu seribu 2 (PS) allele (129 bp).

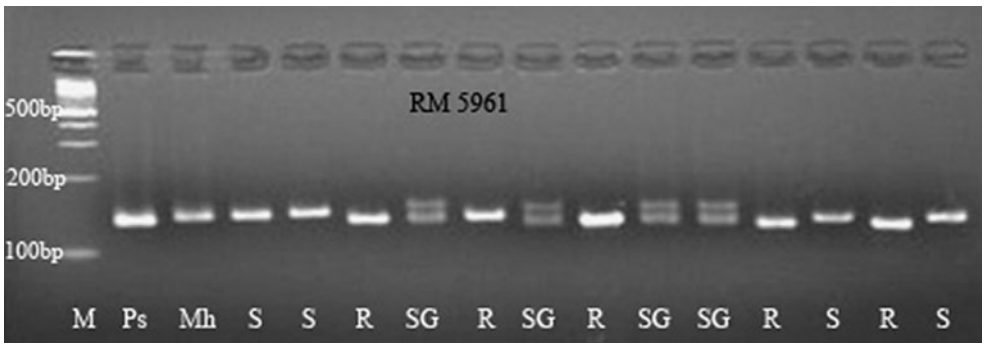

Fig. 3 Examples of marker-assisted selection in F_3 population derived from Pongsu seribu 2 (*PS*) × Mahsuri (*Mh*) rice cultivars for SSR markers RM5961 linked to blast resistance genes. Running on 3% agarose gel stained with ethidium bromide, only 13 samples plus their corresponding parents as example are shown (*M*=100 bp ladder)

Discussion

The plants resistant to blast pathotype P7.2 from F_3 population of Pongsu seribu 2 × Mahsuri that showed a (3:1) ratio for single dominant gene model were linked to genotypes of four SSR markers RM413, RM1233, RM8225 and RM5961 with observed segregation ratio of (1:2:1). These markers were distributed on three different chromosomes in four different positions and were closely linked with single dominant gene. These findings are likely to show that the observed segregation ratios in F_3 in the greenhouse screening for pathotype P7.2 were likely consistent with a single dominant gene model. Segregation ratio (3:1) suggests a single specific resistance gene segregating against pathotype 7.2 in the F_3 families. These results also suggest that the same locus presence in resistant parent and resistant F3 families controls the resistance to pathotype P7.2. The durable resistance of the cultivar Pongsu seribu 2 could be the result of the complement of race-specific resistant genes. Alternatively, the resistance could be in part due to the accumulation of genes with smaller but potentially non-specific effects. From the previous studies, also, we can infer that resistance to blast may be governed either by single gene or polygenic, depending on the genotypes or cultivars, as well as their specificity to *M. oryzae* isolates, where resistance to blast disease is host-specific and effective against only one specific strains of *M. oryzae* (Zhou et al. 2004, 2007). The report of MAS application in rice shows that the target genes can be identified more efficiently in a segregating population at any plant growth stage with the use of tightly linked DNA markers (Huang et al. 1997; Hittalmani et al. 2000). Analysis of our selected SSRs markers in F_3 segregating population indicated that these markers were linked with blast resistance genes with the confirmation of large resistance effect about 20% for phenotypic variation to a given pathotype. Because these markers had high selection accuracy for resistant plant sources, they can be used in MAS for the resistant gene. Some families, which were resistant in pathogenicity assay, did not show the allele characteristic of link *pi* gene markers, most probably due to interaction of other resistance genes in this line and the presence of other avirulent genes in the blast pathotype used in the pathogenicity assay. The deployment of major gene resistance will minimise selection pressure and thereby prevent evolution of resistance in the pathogen population (Bonman et al. 1992). This approach will help breeders to expedite breeding research in crops by enabling selection based on the genotype rather than on the phenotype. The markers reported here provide rice breeders and geneticists a valuable tool for marker-aided selection of the disease resistance gene.

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