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Genetic mapping of leaf blast resistance gene in landrace rice cultivar 'GS19769'

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ABSTRACT: The rice blast fungus, *Magnaporthe oryzae,* is highly varied and therefore overcomes resistance in a few years. Rice cultivars with the ability to resist different blast races are therefore required. In this study, a resistance gene of landrace rice cultivar 'GS19769' is identified using a mix of 19 blast isolates collected from several epidemic areas in Thailand. The selected cultivar was fertilised with the blast susceptible variety, Khao Dok Mali 105 (KDML105), to generate the mapping population. Segregation analysis in the F_2 population shows that 'GS19769' contains more than 1 resistance gene, as the Chi-square test for segregation of resistance and susceptibility does not fit the ratio of 3:1. The bulk segregant analysis by simple sequence repeat (SSR) markers shows that the identified resistance gene is linked to the SSR markers RM224 and RM144 on chromosome 11. Analysis of 15 F_2 plants susceptible to the blast was conducted. The gene was mapped to RM224 and RM144 at the same distance of 20 cM.

Key words: genetic mapping, rice blast, landrace rice, *Magnaporthe oryzae*, SSR markers

INTRODUCTION

Rice blast is one of the most devastating diseases for rice-growing countries worldwide. The causal fungal pathogen, *Magnaporthe oryzae*, has the ability to infect several parts of the plant at all 3 growth stages, i.e. seedling, tillering and panicle stages. The fungus can survive over seasons on many gramineous hosts other than rice. Moreover, the diversity of the fungus occurs as it easily adapts to different environmental conditions across seasons [1]. In Thailand, a high diversity has been confirmed by the determination of 623 pathotypes collected during 2002-2005 on 18 near

isogenic lines [2]. The disease dispersal in 1992 damaged over 650,000 tons or 60% of the total yield of rice in Thailand [3].

Developing blast-resistant rice cultivars is an effective approach to controlling the blast disease. The blast resistance in rice is controlled by a resistance (R) gene corresponding to an avirulence gene in blast fungal isolates and its reaction follows the gene-for-gene hypothesis [4]. Many blast-resistant cultivars have been generated by molecular-assisted breeding programmes, where an identified resistance gene was introduced to the cultivars. However, the new cultivars developed often lost their resistance after a few years. Blast isolates could break resistant cultivars, especially when a single resistance gene is present. The goal of the rice breeder is to develop a rice cultivar with a broad-spectrum-resistance phenotype against the infection of several blast isolates [5]. This type of resistant cultivar consists of a resistance (R) gene obtained from disease-resistant varieties. A number of broad-spectrum R genes have been identified from resistance genes in non-cultivated varieties. As summarised by Yang et al. [6], over 80 resistance genes have been identified and are distributed on 11 rice chromosomes, with the exception of chromosome 3. Among these, *Pi2*, *Pi9*, *Piz-t*, *Pigm* and *Pi50(t)* from the *Pi2/9* locus on chromosome 6 confer broad-spectrum resistance to blast isolates [5, 7-11].

Generally, R genes are identified in landraces, cultivars or wild rice collections [12]. Recent work on the identification of blast-resistant varieties from landrace rice using 29 blast isolates revealed that 4 out of 263 landrace rice varieties showed broad-spectrum resistance with no symptoms of disease after inoculation with a mix of blast isolates collected from disease outbreak areas in Thailand [13]. The objectives of this study are to locate the resistance gene and identify simple sequence repeats (SSR) markers closely linked to a broad-spectrum blast resistance gene of a landrace rice cultivar 'GS19769'. The marker obtained, linked to the resistant phenotype, would be useful for the marker-assisted selection of a blast-resistance trait in a rice breeding programme.

MATERIALS AND METHODS

Rice Variety

The landrace rice cultivar 'GS19769' used in this study was derived from screening of the landrace rice cultivars for a blast-resistant phenotype using 29 blast isolates collected from the disease epidemic areas in Thailand, as described in Salih et al. [13]. The F_1 obtained from the crossing between GS19769 and Khao Dok Mali 105 (KDML105, obtained from Rice Department, Ministry of Agriculture and Cooperatives, Thailand), a well-known aromatic rice variety which is susceptible to leaf and neck blast diseases, were grown for F_2 seed production. The resistant Jao Hom Nin (JHN) and susceptible KDML105 were used as standard check varieties in all experiments.

Pathogen Isolates

The 19 blast isolates were collected from disease epidemic areas across the rice planting region of Thailand (Table 1). The fungi (a single spore each) isolated from leaf or neck with blast symptoms were cultured on rice-flour agar medium (20 g/L rice flour, 20 g/L agar, 2 g/L yeast extract and 40 mg/L streptomycin) at 25°C and stored as dried mycelium on filter paper at -20°C, as described by Sirithunya et al. [14]. These were used for further experiments.

Table 1. Nineteen isolates of *M. oryzae* used in this phenotypic analysis and their collection locations

Area	Province	Number of fungal isolates
North	Chiang Rai, Phitsanulok	5
North-east	Chaiyaphum, Khon Kaen, Nong Khai, Ubon Ratchathani, Surin	8
Middle	Bangkok, Chachoengsao, Ratchaburi	5
South	Phatthalung	1

Inoculation and Disease Assessment

F_2 seeds obtained from F_1 plants were sown individually in plastic trays (33 x 11 x 11 cm) half-filled with soil and fertilised with ammonium sulphate (5 g). JHN and KDML105 were planted at both sides of the end rows as resistant and susceptible check varieties respectively. Ammonium sulphate (1 g) was added to each tray 3 days prior to inoculation. Inoculation of the blast isolates was performed following the method described by Sreewongchai et al. [15]. All 29 isolates were grown on rice-flour agar medium and incubated at 25°C. Sporulation was induced by scraping 8- to 10-day-old mycelium from each plate and allowing growth for another 2 days. Spores were harvested and the concentration of spores was adjusted to 10^5 spores/mL in 0.5% gelatine. Inocula were sprayed onto 14-day-old seedlings using an air-brush pressure pump. The inoculated seedlings were placed in a high-humidity chamber for 18 hr at 25°C and were then transferred to a greenhouse. The degree of infection of each seedling was evaluated 7 days after inoculation by a standard reference scale for rice blast, scoring 0 (resistant) to 6 (susceptible) [16].

DNA Preparation, Amplification and Bulk Segregant Analysis

Genomic DNA of ten F_2 plants from each resistant group, susceptible group and two parental lines was extracted from leaves of seedlings by the cetyltrimethylammonium bromide method described by Doyle and Doyle [17]. The DNA quality was compared with known concentrations of DNA by electrophoresis on 0.8% agarose gel and staining with ethidium bromide.

The parents were used to screen the 270 SSR markers with known position and wide distribution on 12 rice chromosomes for polymorphism and possible association with blast resistance. These markers are reported online [18]. The polymerase chain reaction (PCR) mixture (10 µL) contained 5.9 µL of sterile distilled water, 1 µL of 10xPCR buffer, 1 µL of $MgCl_2$ (25 mM), 0.5 µL of dNTPs solution mix (2.5 mM), 0.1 µL of *Taq* DNA polymerase (5 units/µL) (Fermentas, USA), 0.5 µL of SSR primer pair (0.25 µM each) and 1 µL of rice genomic DNA (10 ng). The PCR was conducted in a thermal cycler as follows: initial denaturation step at 95°C for 10 min., followed by 40 cycles of 95°C for 20 sec., 57°C for 15 sec., 72°C for 30 sec., and a final extension at 72°C for 5 min. PCR products were observed by 6% polyacrylamide gel electrophoresis and made visible by silver staining [19].

The bulk segregant analysis (BSA) was arranged into 2 bulk sets according to the two F_1 plants obtained. The analysis resulted in a number of SSR markers that sufficiently distinguished the genotype of the two bulks. Two resistant bulk sets (B1) were comprised of 8 resistant F_2 plants from F_1 plant no. 1 (B1-1) and 7 resistant F_2 plants from F_1 plant no. 2 (B1-2), while 2 susceptible

bulk sets (B2) were comprised of 8 susceptible F_2 plants from F_1 plant no. 1 (B2-1) and 7 susceptible F_2 plants from F_1 plant no. 2 (B2-2). The DNA from each plant was extracted and the DNA pool was prepared for each bulk by mixing DNA of the respective F_2 DNA samples in equal quantity. The bulks were used to analyse the SSR markers obtained from parental screening (Table 4). The PCR profile was conducted and the PCR products were analysed as mentioned above.

The SSR markers found to be polymorphic among the bulks were used for the co-segregation study of F_2 progenies. Leaves of each F_2 plant and its parents were excised to give pieces 0.5 mm in diameter and 5 pieces were added into the PCR plate containing 10 μL of KAPA plant PCR mix (KAPA Biosystems, USA) for genetic analysis. The PCR products were analysed by electrophoresis as described previously.

Data Analysis

The clearly detected amplicons of SSR were scored manually as A for the susceptible parent allele, B for the resistant parent allele and H for alleles from both parents. The data sheet was generated and scored. A Chi-square (X^2) test for goodness of fit of the F_2 population for the phenotype and marker data was performed by CropStat 7.2 computer software [20]. The SSR markers surrounding the resistance gene on the specific region of the chromosome were identified. The phenotype and SSR data were combined for linkage analysis by using MAPMAKER/EXP3.0 program [21]. In addition, the frequency of recombination was calculated using the genotype of markers and the resistant phenotype of F_2 plants that showed susceptible phenotype to fungal infection.

RESULTS AND DISCUSSION

Phenotype Distributions and Correlation

A population of 250 F_2 seedlings plus two check varieties were inoculated with a mix of 19 blast isolates. The blast fungi were isolated from naturally infected rice leaf and leaf sheath from different regions of a paddy field in Thailand. They were found to be highly genetically diverse when analysed using *M. grisea* microsatellite markers, as described in Tansian et al. [22]. The score of each seedling was evaluated after inoculation and used for further analysis. Among the 250 seedlings tested, 235 were characterised as resistant while only 15 were found to be susceptible to fungal isolates (Table 2). Chi-square tests of the data obtained from the segregation of resistance are shown in Tables 2 and 3. Analysis of a two-independent-gene model or two loci interactions shows that the expected number of resistant and susceptible plants in the segregation ratio for the model of more than one dominant gene is in accordance with the 9:3:3:1 segregation ratio at the $0.90 < p < 0.95$ level of significance. The one-dominant-gene model (3:1) is also considered. However, the Chi-square data obtained is 48.13, with a p value of less than 0.0005 ($p < 0.0005$) (Table 2). The Chi-square data on the segregation analysis suggest that the resistant phenotype of the GS19769 cultivar against blast disease is controlled by more than one gene.

Table 2. Segregation of F_2 population obtained from the cross between KDML105 and GS19769 rice cultivars and inoculated with 19 isolates of *M. oryzae*

Total no. of seedlings	Resistant (R)	Susceptible (S)	Expected ratio	χ^2	p value
250	235	15	3:1	48.133	0.000

Note: X^2 (0.05, 1) = 3.84, df =1.0

Table 3. Chi-square tests of two independent genes (9:3:3:1) and epistatic effect (15:1) for blast resistance in F_2 population derived from the cross between KDML105 and GS19769 rice cultivars inoculated with 19 isolates of *M. oryzae*

Gene model	Total no. of F_2 seedlings	Observed ratio				Expected ratio	χ^2	*p* value
		R	MR	MS	S			
Independent genes [a]	250	134	59	42	15	9:3:3:1	3.980	0.264
Epistatic effect [b]	250	235	-	-	15	15:1	0.027	0.870

[a] χ^2 0.5, 3 = 2.38, df=3; χ^2 0.25, 3 = 4.11, df=3
[b] χ^2 0.75, 1 = 0.10, df=1; χ^2 0.90, 1 = 0.02, df=1
R = resistant; MR = moderately resistant; MS = moderately susceptible; S = susceptible

Molecular Marker Analysis

Two hundred and seventy SSR markers were screened for polymorphism between the parents KDML105 and GS19769. The 66 markers which showed polymorphism between the parents and were widely distributed along 12 rice chromosomes were then used in the BSA. Markers with polymorphic bands that showed distinct alleles between resistant and susceptible plants, either A:B:B:A or A:B:H:A for KDML105: GS19769: B1: B2, were selected (Table 4). In this experiment, SSR markers that scored H (heterozygous) for the resistant phenotype were also considered as this indicated that the resistant phenotype in this BSA might be controlled by a dominant allele. Out of the 66 SSR markers, 4 markers that fit the genotype patterns were selected and used in the analysis of the individual F_2 population. These markers were: RM125 (Chr.7), RM201 (Chr.9), RM144 (Chr.11) and RM247 (Chr.12). Their genotype patterns (KDML105: GS19769: B1-1: B2-1: B1-2: B2-2, where B1-1 and B2-1 were progenies from F_1 plant number 1, and B1-2 and B2-2 were from F_1 plant number 2) were ABAABA, ABAAHA, ABHAHA and ABAAHA respectively (Figure 1). The segregation analysis of F_2 plants revealed that the resistant allele(s) associated with markers RM125 and RM144 had the Chi-square results of 1.20 (1:2:1 at p = 0.549, where df = 2) and 6.02 (*p* = 0.049, where df = 2) respectively (Table 5). These Chi-square data confirmed that the analysis of the F_2 population according to marker RM125 and RM144 fitted Mendel's segregation theory of 1:2:1. Moreover, the data indicated that the population segregation was normal. The results suggest that the resistant allele(s) might link either to RM125 on chromosome 7 or to RM144 on chromosome 11. Interestingly, marker RM144 is the only one that provides a distinguishable genotype between resistance and susceptibility in the BSA. However, the segregation analysis of RM125 in individual F_2 plants results in over 50 per cent recombination (Table 6), suggesting that the position of RM125 is not linked to the resistant allele.

In order to define the location of the blast resistant allele, additional markers from chromosomes 7, 9, 11 and 12 were selected to define which chromosome harbours the resistant allele (Table 4). Among these, RM224 and RM287, located 3.1 and 54.6 cM respectively above RM144 on chromosome 11, were found in the same F_2 population. RM224 has a Chi-square of 3.91 with *p* = 0.14, while the value for RM287 is 1.60 with *p* = 0.44, which fits the 1:2:1 segregation and represent the normal segregation of this population. Thus, the results confirm the location of the resistant allele on chromosome 11 and link its location to those 3 markers.

Table 4. List of SSR markers used in BSA

| Marker	Forward primer (5′–3′)	Reverse primer (5′–3′)	Chromosome no.	Product size (bp)
RM125	ATCAGCAGCCATGGCAGCGACC	AGGGGATCATGTGCCGAAGGCC	7	127
RM11	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG	7	140
RM1132	ATCACCTGAGAAACATCCGG	CTCCTCCCACGTCAAGGTC	7	93
RM5122	CTCGCAATTTATACGTAATC	CTCACGAAATAAAATGAGTG	9	161
RM201	CTCGTTTATTACCTACAGTACC	CTACCTCCTTTCTAGACCGATA	9	158
RM205	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG	9	122
RM287	TTCCCTGTTAAGAGAGAAATC	GTGTATTTGGTGAAAGCAAC	11	118
RM224	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	11	157
RM144	TGCCCTGGCGCAAATTTGATCC	GCTAGAGGAGATCAGATGGTAGTGCATG	11	237
RM247	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG	12	131
RM1261	GTCCATGCCCAAGACACAAC	GTTACATCATGGGTGACCCC	12	168
RM235	AGAAGCTAGGGCTAACGAAC	TCACCTGGTCAGCCTCTTTC	12	124
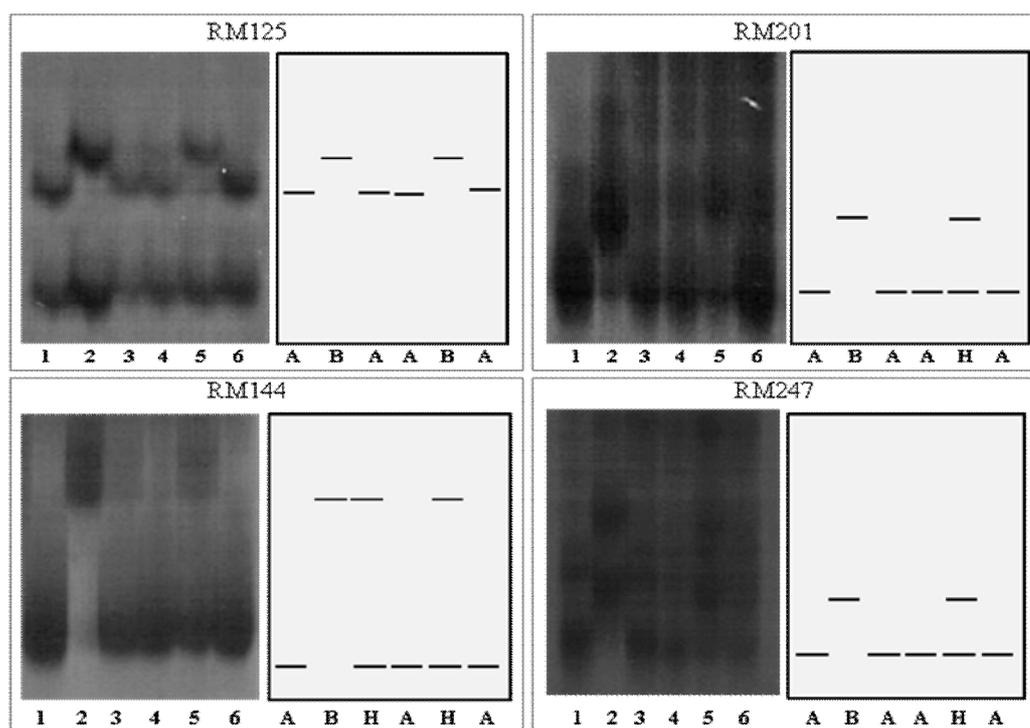
Figure 1. Results BSA by using susceptible parent KDML105 (1) and resistant parent GS19769 (2) and their respective bulks: resistant bulks B1-1 (3) and B1-2 (5); susceptible bulks B2-1 (4) and B2-2 (6), with SSR markers RM125, RM201, RM144 and RM 247

Table 5. Markers analysed in F$_2$ population derived from the cross between KDML105 and GS19769 cultivars

Marker	Marker segregation analysis (no. of lines observed)			χ^2 (1:2:1)	*p* value
	A	AB=SG	B		
RM144	46	133	71	6.024	0.049
RM224	49	135	66	3.912	0.141
RM287	54	129	67	1.608	0.448
RM125	55	130	65	1.200	0.549

Note: df=2, χ^2 0.025=7.38, χ^2 0.05= 5.99, χ^2 0.1=4.61, χ^2 0.25=2.77, χ^2 0.5=1.39
A = resistant; B = susceptible; AB or SG = segregant, according to model with single dominant gene

Table 6. Resistant genotype and phenotype of markers showing frequency of recombination occurring in F$_2$ plants that appear to be phenotypes susceptible to *M. oryzae* infection

Chr.	Marker	Genotype																	% Recombination
		Parents		F2 population															
		P1	P2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
7	RM125	A	B	H	H	A	H	A	H	A	H	H	A	A	H	H	A	H	60
7	RM11	A	B	H	B	A	B	A	H	B	H	H	A	H	H	H	A	H	73.33
7	RM1132	A	B	H	B	A	H	A	B	H	H	H	H	H	H	H	A	B	80
9	RM5122	A	B	H	H	A	H	H	H	A	B	A	H	H	H	H	A	H	73.33
9	RM201	A	B	H	A	A	H	B	H	A	B	H	H	B	A	H	A	H	66.66
9	RM205	A	B	H	A	A	H	B	B	H	B	H	A	B	A	H	A	A	60
11	RM287	A	B	A	A	B	B	A	H	H	B	B	A	B	A	H	H	A	60
11	RM224	A	B	A	B	A	A	A	A	H	H	A	A	A	A	A	A	A	20
11	RM144	A	B	A	B	A	A	A	A	H	H	A	A	A	A	A	A	A	20
12	RM247	A	B	A	H	A	H	H	H	H	B	H	B	H	A	B	H	A	73.33
12	RM1261	A	B	A	A	A	A	A	A	H	B	H	H	A	H	B	H	H	53.33
12	RM235	A	B	A	B	A	A	B	B	B	B	B	B	A	B	B	B	A	66.66
		Phenotype																	
		5	0	5	5	5	5	5	5	5	5	5	5	6	5	5	5	6	

Note: A = susceptible parent allele, B = resistant parent allele, H = alleles from both parents

Mapping of Resistant Allele

The localisation of the gene of interest is useful for further operations such as map-based cloning, identification of a resistance gene and breeding of a disease-resistant variety. The determination of the frequency of recombination on rice chromosomes would provide an estimated position of the resistant allele. Chromosome 11 was speculated for gene location and was analysed by SSR and additional markers. Fifteen F$_2$ plants that showed a susceptible phenotype (score 5 and 6) to blast fungi were analysed. As they were phenotypically confirmed, using 15 susceptible plants was sufficient and enabled the percentage of recombination on the chromosome of those plants to be analysed. As shown in Table 6, markers RM224 and RM144 have a similar recombination of 20%. This can be explained by a map of SSR markers [18] as shown on Figure 2, which shows a close proximity of both markers, with a distance of 3.1 cM. RM287 shows 60% recombination, thus indicating that the marker is not linked to the resistant allele. In addition, SSR markers on other

chromosomes such as 7, 9 and 12 were also analysed to clarify the gene location. The results show that the recombination percentage of each detected marker is higher than 50% (Table 5).
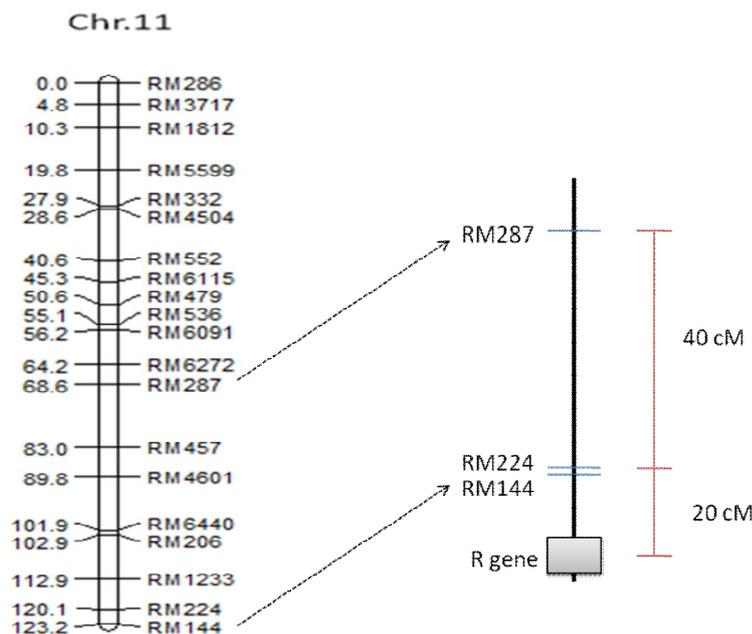

Figure 2. SSR map of resistance gene on rice chromosome 11. The linkage map was obtained from analysis using a KDML105 x GS19769 F_2 population. Location and distance in cM of known SSR markers are shown according to the comparative map available online [18]. The resistance gene is located close to RM224 and RM144.

A number of resistance genes have been identified by SSR markers on chromosome 11, including *Pi34^b*, *Pi38*, *PiCO39*, *Pik-h*, *Pik-s* and *Pi-1* [5, 23-24]. Among these, the *Pik-h*, *Pik-s* and *Pi-1* genes were reported to link with RM224 at the distance of 0.0 cM. A previous study mapped *Pi-1* gene at a distance of 6.8 cM away from RM144 [23]. However, the resistance gene identified in this study is mapped downstream of RM224 and RM144 with a distance of 20 cM. Therefore, it is possible that it is a new blast-resistance gene candidate.

CONCLUSIONS

Analysis of leaf blast resistance genes reveals a dominant inheritance pattern controlling the resistant phenotype in the landrace rice cultivar GS19769. The identified resistance gene is found to be a broad-spectrum blast resistance gene as it is resistant to a mixture of 19 blast isolates. From the segregation analysis, the GS19769 cultivar contains more than 1 resistance gene loci. From bulk segregant analysis, the gene is found to be on chromosome 11 and is tightly linked to the SSR markers RM144, being mapped downstream of the markers at a distance of 20 cM. Locating of the resistance gene and the linked SSR markers should help to narrow down the position of the resistance gene in this landrace cultivar.

ACKNOWLEDGEMENTS

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