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Development of a PCR-based marker to identify rice blast resistance gene, *Pi-2(t)*, in a segregating population

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Abstract The genomic clone RG64, which is tightly linked to the blast resistance gene *Pi-2(t)* in rice, provides means to perform marker-aided selection in a rice breeding program. The objective of this study was to investigate the possibility of generating a polymerase chain reaction (PCR)-based polymorphic marker that can distinguish the blast resistant gene, *Pi-2(t)*, and susceptible genotypes within cultivated rice. RG64 was sequenced, and the sequence data was used to design pairs of specific primers for (PCR) amplification of genomic DNA from rice varieties differing in their blast disease responsiveness. The amplified products, known as sequenced-tagged-sites (STSs), were not polymorphic between the three varieties examined. However, cleavage of the amplified products with the restriction enzyme *Hae*III generated a polymorphic fragment, known as specific amplicon polymorphism (SAP), between the resistant and the susceptible genotypes. To examine the power of the identified SAP marker in predicting the genotype of the *Pi-2(t)* locus, we determined the genotypes of the F_2 individuals at this locus by performing progeny testing for the disease response in the F_3 generation. The results indicated an accuracy of more than 95% in identifying the resistant plants, which was similar to that using RG64 as the hybridization probe. The identification of the resistant homozygous plants increased to 100% when the markers flanking the genes were considered simultaneously. These results demonstrate the utility of SAP markers as simple and yet reliable landmarks for use in marker-assisted selection and breeding within cultivated rice.

Key words Molecular markers · RFLP · PCR · SAPs · DNA sequence · Marker-assisted selection

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Introduction

Rice blast disease, caused by *Pyricularia grisea* (*or Magnaportha grisea*) Cav., is one of the most devastating diseases of rice throughout the world. The breeding of resistant varieties is an effective approach to eliminate the use of pesticides and minimize crop losses due to this disease. However, past experience has shown that resistant cultivars often break down within a few years after they are released for cultivation. To breed rice varieties with more durable blast resistance, multiple resistance genes must be incorporated into individual varieties. This necessitates the exploration of more efficient selection and breeding strategies than those currently exist. Recent advancements in DNA marker technology together with the concept of marker-assisted selection (MAS) may provide new solutions for selecting and maintaining more durable resistant genotypes in rice. In contrast to the traditional selection based on phenotypic screening, molecular markers are refractory to environmental variation. Upon identification of molecular markers closely linked to desirable trait(s), marker-assisted selection can be performed for multiple resistance in early segregating generations and at early stages of plant development.

Breeding disease-resistant genotypes using marker-assisted selection requires that (1) the resistance gene(s) be tagged by closely linked molecular markers, (2) the linkage be stable across generations and population and (3) an efficient way of screening large populations for molecular markers be available. Considerable progress has been made towards mapping blast resistance genes in rice. Yu et al. (1991) reported the mapping of two rice blast resistance genes using restriction fragment length

polymorphism (RFLP) markers from a set of near-isogenic lines developed by Mackill and Bonman (1992). These two genes, *Pi-2(t)* and *Pi-4(t)*, were mapped to chromosomes 6 and 12, respectively. A third blast resistance gene, *Pi-zh*, was mapped with a random amplified polymorphic DNA (RAPD) marker using double-haploid lines, and the linked RAPD marker was later mapped to rice chromosome 8 (Zhu et al. 1993). Furthermore, two major genes, *Pi-5(t)*, *Pi-7(t)*, and nine quantitative trait loci (QTLs) contributing to rice blast resistance were located on rice chromosomes using RFLP analysis of approximately 300 recombinant inbred lines (Wang et al. 1994).

One of the blast resistance genes mapped in rice, *Pi-2(t)* on chromosome 6, is located 2.8 cM distant from the RFLP marker RG64 (Yu et al. 1991), thereby providing an opportunity to initiate marker-aided selection. In the present article, we report the development of a PCR-based DNA marker based on the RG64 clone. PCR-based polymorphism was generated to distinguish between resistant and susceptible rice plants. The individual plants carrying *Pi-2(t)* were selected on the basis of the PCR marker, and the selection result was then compared to that of progeny testing. We also compared the effectiveness of using flanking markers for predicting the presence of *Pi-2(t)* in a segregating population.

Materials and methods

Genetic materials and disease evaluation

The near-isogenic line, C101A51, carrying the *Pi-2(t)* resistance gene was crossed to its recurrent susceptible parent, 'CO39', and F_1 seeds were generated. One hundred and nineteen F_2 progeny, resulting from self-pollination of F_1 individuals, were grown for DNA isolation and RFLP and specific amplicon polymorphism (SAP) genotyping. The F_2 individuals were self-pollinated to generate F_3 family seeds. Twenty individuals of each F_3 family were grown in a plastic tray under standard greenhouse conditions for phenotypic disease scoring. The parental resistant and susceptible cultivars were also included as controls. Twenty-one-day-old seedlings (about four-leaf stage) were inoculated with blast isolates, IK81-3 and V89013, in two separate trials. The inoculum was prepared as described by Bonman and Mackill (1988). The rice seedlings were sprayed with 50 ml of blast inoculum suspension per tray (5×10^4 conidia/ml) and incubated for 24 h in a controlled dew chamber maintained at 25 °C. The seedlings were then transferred to a temperature- and humidity-controlled chamber. Plants were monitored for blast disease symptoms for 1 week after inoculation and scored for resistance and susceptibility when the typical blast lesions developed on the susceptible parental cultivar 'CO39'. This phenotypic evaluation was repeated twice.

Plant DNA isolation and Southern blotting

Total genomic DNA from both the F_2 plants and the parental isogenic lines was extracted according to the method of Dellaporta et al. (1983) using fresh frozen leaf tissue. The quality and quantity of the DNA was determined spectrophotometrically. Southern blotting, probe labeling and hybridization were done following the standard protocols. The restriction enzymes tested included *Bam*H1, *Dra*I *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Hin*fI, *Msp*I, *Mva*I, *Pst*I, *Rsa*I, *Sti*I, *Sac*I, *Taq*I, *Xba*I and *Xho*I. Blots of the parents and of the F_2 progeny were probed by RG64 and RG456, RFLP markers flanking the *Pi-2(t)* gene.

DNA sequencing of rice genomic clone RG64

A plasmid vector containing the RFLP marker RG64 was obtained from Steve Tanksley, Cornell University, USA, and used to transform *E. coli* strain JM109. In preparation for DNA sequencing, the RG64 insert was subcloned into pBluescript KS- (Stratagene Corp) and subjected to unidirectional exonuclease treatment according to the procedure of Henikoff (1988). Single-stranded DNA was isolated and used as template for the dideoxy chain-termination sequencing method (Sanger et al. 1977) as modified by Biggin et al. (1993). The DNA sequence has been submitted to GenBank under the accession number L32142.

PCR primers

The nucleotide sequence of RG64 was used to design and synthesize four oligonucleotide primers (named nos. 426, 427, 431 and 432) that range in length from 23 to 28 nucleotides. These primers were synthesized using a Cyclone Plus DNA synthesizer (Milligen/Biosearch). The nucleotide sequence, length, orientation and location of these primers relative to the sequence of the RG64 are shown in Fig. 1.

PCR amplification conditions

The genomic DNA of the resistant donor parent (5173), recurrent susceptible parent ('CO39'), the near-isogenic resistant line (C101A51) and F_2 progeny of the cross between 'CO39' and C101A51 were subjected to PCR amplification using the synthesized primers. The PCR reaction mixture consisted of 50 ng template DNA, 20 ng of each of the primers, 0.1 m*M* dNTP's, 1 × PCR buffer (10 m*M* Tris pH 8.0, 50 m*M* KCl, 1.8 m*M* $MgCl_2$ and 0.01 mg/ml gelatin) and 1 unit of *Taq* polymerase in a volume of 25 µl. Template DNA was initially denatured at 93 °C for 5 mn, followed by 40 cycles of PCR amplification under the following parameters: 1 min denaturation at 93 °C, 1 min primer annealing at 60 °C and 2 min primer extension at 72 °C. A final 5 min-incubation at 72 °C was allowed for completion of primer extension on a Perkin Elmer Cetus thermal cycler. The amplified products were electrophoretically resolved on a 1% agarose plus 1% NuSieve gel using 1 × TAE buffer.

Restriction digestion of PCR products

When combinations of primer pairs nos. 431 and 432 were used to amplify the DNA of the parent 'CO39' and the near-isogenic resistant line (C101A51), the resulting PCR products were monomorphic (Fig. 3), therefore, restriction enzymes were used to generate SAP fragments (as described by Williams et al. 1991 and Kleinhofs et al. 1993). The amount and quality of PCR amplification was first monitored by running 5-µl aliquots of the reaction mixture on an agarose gel. Restriction enzyme digestion was then carried out directly in the PCR tube without separating the PCR product from the oil overlay. The enzyme mix [3 × restriction enzyme buffer, 2 units of enzyme, 1.5 µl of 80 m*M* spermidine (1.5 ×) and sterile water] was added to a final volume of 30 µl. After a brief spin in a microfuge and 3–4 h of incubation at 37 °C, the digested products were run on an agarose/NuSieve gel to resolve digested PCR fragments.

Statistical analysis

Chi-square tests were performed to examine the goodness-of-fit between expected Mendelian ratio and the RFLP, SAP and phenotypic data for RG64 and RG456 and the disease screening data based on F_3 family progeny (Table 1).

Results

RG64 DNA sequence and PCR primer

The nucleotide sequence of RG64 (1557 bp, Fig. 1) was determined on the basis of both orientations to ensure

Fig. 1 The complete DNA sequence of the RG64 RFLP marker. The short sequences *below* the long DNA sequences are the four primers synthesized for PCR analysis. Restriction sites in the primer sequences are *underlined*. The DNA sequence was submitted to GenBank under the accession number L32142

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                    20                  40                  60
                    *                   *                   *
CTGCAGAAACCTTCTATGATGCATACTCCAATCTCTAGATCTTTGGGATGATGCCCAAAA
                          5'CCAATCTCTAGATCTTTGGGATGATGCC 3'
                            #426 XbaI
                    80                  100                 120
                    *                   *                   *
GCTTAGATAGGCAACATCTCAATTAGATCAAGAGTTGCACATGGAGTTGTGTAAAGTTAA
                   140                 160                 180
                    *                   *                   *
TTAAACTGCTGGTAGAGGAGCACACTGAGCTCGCCAATACTAATTAACATCAGTGGTGTA
                   200                 220                 240
                    *                   *                   *
TGTTTGCTCTTATACATGACGGTGAGAGATATGCGTGCCTCACATACCATTCACCTCACT
                   260                 280                 300
                    *                   *                   *
CGCCATACGTGCCTTGCAACTCTTTCATGAAAAGTTAAATGTTGAGTTCACCTCAACACA
                   320                 340                 360
                    *                   *                   *
TTAACGGCAATCGAGCGAGCAACATCCACCTTGCTGAGTAGCCGATGGATATCGTCTAAG
                   380                 400                 420
                    *                   *                   *
ACCACGGCATGGCTTGTACGTAGCCATATCATATCAGTATTCGTTGCTTCTGGCATGCAG
                                              3'AACGAAGACCGTACGTC
                   440                 460      #427 Sph-1 480
                    *                   *                   *
GTCGAGTGGTTTTTGCTAGCTAGATAGGGCAGATAAGAAGATGTTGTTGTGAACATTGTG
CAGCTC 5'           500                 520                 540
                    *                   *                   *
TTGCCTGGTTGTTTGAGCCCTCCAATGCCTGTTCTTATACGTAGTACTCCCTCCATTCCA
     5'GTTGTTTGAGCTCTCCAATGCCTGTTC 3'
       #431   Sst 1                  580                 600
                    *                   *                   *
AAATATATGATGCCGTTGACTTTTCGTATAATGTTTGATTACTTGTCTTTTACAAATAAA
                   620                 640                 660
                    *                   *                   *
ATTATATAAATATTATTTATTTTTTATGACTTATTTGATTATCAAATAGTTGTACATAT
                   680                 700                 720
                    *                   *                   *
TTATACAAATTCGTTTTTAATAAGATAAATCGTCAAATGTTACGAGAAAAATCAACGGTG
                   740                 760                 780
                    *                   *                   *
TTATATATTTTGGAACGGAGGTAGTACTACATAAGCACAAACTAAAATTTGTTTATATGA
                   800                 820                 840
                    *                   *                   *
GAATGTGCAGCCGTCACCATAGATATGATTCGGTGCTATGGCGATATTAGCACCATATAA
                   860                 880                 900
                    *                   *                   *
ATATTACAATCAAACATCTTCTGAACAACACACCAATTTATTTTCTTTGATAAAATCGCA
                   920                 940                 960
                    *                   *                   *
CACAAATTATTTGCTCGACTATGCTGTAGCAGAGATCTATGCCTCTCTGCAAAGTATCTT
                   980                1000                1020
                    *                   *                   *
GTCACTATTCTGACAAGAGAGTAAAGCCTTAAATATAGCCTGGCATATATAATTATGTTC
                  1040                1060                1080
                    *                   *                   *
TTGCTATTCACTGTATGCACGCGCACTAATTAGTTATGAAAACGCGTGTGTATGCTAAGA
                  1100                1120                1140
                    *                   *                   *
GCTTGTACTGTATTTCTAACCAAAAAAAAAATGTTAACAAACCTAACATATATGCTAACT
                  1160                1180                1200
                    *                   *                   *
TGTGCCAACTCAACAGCCCCTACGAGATGACATGGTTTCCCATGCTCCACAGCATGTACC
                  1220                1240                1260
                    *                   *                   *
ACGCCTAGAGCTAGAGGTAACCATAAGATGCAGGCCGTTGTGTGTACCTGACCTTGTGAG
                  1280                1300                1320
                    *                   *                   *
TGTTGTGTTGATCGATCACCATAGCATGTGCTCCACCCTGCCTCCATCACCATCTCTACT
                  1340                1360                1380
                    *                   *                   *
CTGAAGCTGACATGACCAACTGTTACACATGCGGCATCACTAGAGCTGAGGTGACCATCT
                  1400                1420                1440
                    *                   *                   *
GACCAAAGCTGATAGCCTGCCTTGGTGCGAACGCCTTTTGGACAGCATCCATGGACTCTG
                  1460                1480                1500
                    *                   *                   *
TGTTGGTACAGAAGTGGTTGCTCATACTTCTTGTTAGTGCAAGTAACTTCTATTGGCAGA
                  1520                1540                1557
                    *                   *                   *
ATGTATTTTTGTTCTCCGATTGATGCCTCCTAGCCCTGGCCGTACATTGCACTGCAG
                                  3'GGACCGGCATGTAACGTGACGTC 5'
                                                #432   Pst 1
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accuracy. The present G+C content of the clone was determined to be only 40%, suggesting that RG64 sequence corresponds to an intron or intergenic region in the rice genome (see Simmons et al. 1992). A stem-loop like structure was identified in the RG64 DNA sequence that is highly homologous to the Stowaway transposable element (Bureau and Wessler, 1994). This transposable element is named Stowaway-Os8 (R. Bureau, personal communication).

When RG64 was used as a DNA template, three PCR products were generated from the pair combinations of the four primers. The primer combination nos. 426 and 427 gave rise to a 399-bp PCR product, nos 431 and 432 produced a 1155-bp product and primer nos. 426 and and 432 produced a 1530-bp product (data not shown). The PCR products were of the sizes expected from the DNA sequence of RG64.

When genomic DNA of the various *indica* genotypes ('CO39', 5173 and 'IR48') were PCR-amplified using the above primer combinations, no polymorphisms were revealed (see Figs. 2 and 3). Cleavage of the PCR products, however, generated certain polymorphic bands. For example, digestion of the 1155-bp PCR product by the *Hae*III enzyme distinguished between 'CO39' and 'IR48' (Fig. 2), and between 'CO39' and C101A51 (Fig. 3). Two restriction sites for *Hae*III are present in the PCR product of 'CO39', whereas only one is present in 'IR48' and C101A51) (Fig. 2 and 3). Therefore, a PCR-based RFLP (SAP) linked to the *Pi-2(t)* resistance gene is generated between the two *indica* varieties. No polymorphism was identified between the *indica* varieties when the amplified product was digested with restriction enzymes *Hin*fI and *Rsa*I (Figs. 2 and 3).

In contrast to the absence of polymorphism within *indica* varieties, polymorphisms can be found between *indica* and *japonica* varieties when genomic DNA is amplified with the specific primers. Digestion of the amplified fragments with restriction enzymes *Hae*III, *Hin*fI and *Rsa*I generated additional polymorphisms (Fig. 2).

Generation of SAP between C101A51 and 'CO39'

Electrophoretic analysis of PCR products derived from C101A51 and 'CO39' genomic DNA using various

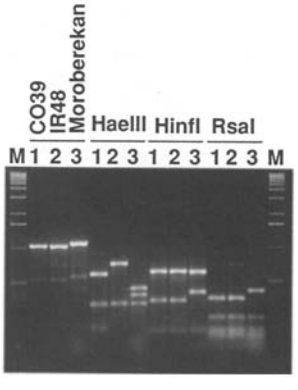

Fig. 2 Agarose gel electrophoresis of PCR products amplified with DNA from three rice varieties, 'CO39' (*1*), 'IR48' (*2*) and 'Moroberekan' (*3*) with primers 431 and 432. The PCR products were analyzed before or after digestion with restriction enzymes *Hae*III, *Hin*fI and *Rsa*I. *M* Molecular weight marker

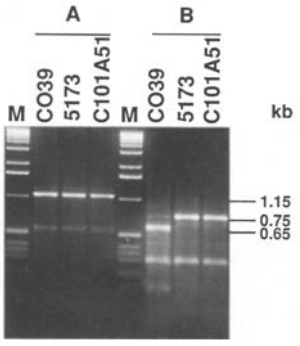

Fig. 3 The generation of sequence-specific amplicon polymorphism between resistant (C101A51) and susceptible ('CO39') rice varieties for marker-aided selection. **A** Gel analysis of PCR products before restriction digestion, **B** Gel analysis of PCR products after digestion with restriction enzyme *Hae*III. The molecular weight of PCR products as banding patterns are indicated in kb (kilobases). *M* Molecular weight marker (kb ladder)

primer combinations showed no polymorphism (data not shown). Restriction digestion of the PCR products, produced from primers nos. 431 and 432, however, did generate polymorphic markers. Of the 16 restriction enzymes used, only the *Hae*III digestion revealed a polymorphism between 'CO39' and C101A51. This polymorphism can, therefore, be used as a marker to distinguish between the *Pi-2(t)* resistance gene and its allelomorph conferring susceptibility. The 750 bp band corresponds to an allele from the resistant parent, whereas the 650 bp band represents the susceptible parent 'CO39' (Fig. 3).

Analysis of the F_2 population

To examine the validity of SAPs as genetic markers, genomic DNAs from the 119 F_2 individuals of the cross between the susceptible recurrent parent ('CO39') and the resistant isogenic line (C101A51) were PCR-amplified using primers nos. 431 and 432, and the resultant PCR products were digested with *Hae*III. Restriction fragments were resolved on agarose gels, and the banding patterns were scored with reference to those of the parents (Fig. 4). The banding patterns of the F_2 individuals could be classified either as homozygous for the 'CO39'-type marker (650-bp fragment), homozygote for the C101A51-type marker (750-bp fragment) or as heterozygotes (displaying both fragments; Fig. 4). In order to compare the segregation pattern of the SAP marker with that of the RFLP marker, Southern blots of the F_2 population were probed with radiolabeled RG64. The segregation pattern of the RFLP marker was the same as that of the SAP marker, indicating that the same locus was detected by both procedures (data not shown). Furthermore, Chi-square test of goodness-of-fit suggested close agreement of the SAP and RFLP markers segregation with that of the expected Mendelian 1:2:1 ratio (Table 1).

The marker genotypes were then used to predict the presence of the *Pi-2(t)* gene in the F_2 individuals. Since RG64 is located 2.8 cM from the *Pi-2(t)* gene, we predicted that those F_2 individuals carrying only the 750-bp fragment were mostly homozygotes for the *Pi-2(t)* locus. Therefore, such individuals were classified as homozygote resistant (RR), whereas those carrying only the 650-bp fragment were classified as homozygote susceptible (rr). Similarly, those F_2 individuals carrying both the 750-bp and the 650-bp fragments were classified as heterozygote resistant (Rr).

To compare this prediction with the data derived from the actual disease scoring, we inoculated F_3 fami-

Fig. 4 Segregation of the RG64 locus as determined by SAP analysis. PCR products of the entire segregating population were generated by DNA amplification with primers nos. 431 and 432. With reference to the banding patterns of the parents, 'CO39' (*S* susceptible) and C101A51 (*R* resistant), the genotypes of each F_2 individual was given the designation *S*, *R* or *Rr* based on the banding pattern of the RG64 locus. *M* Molecular weight marker (kb ladder)

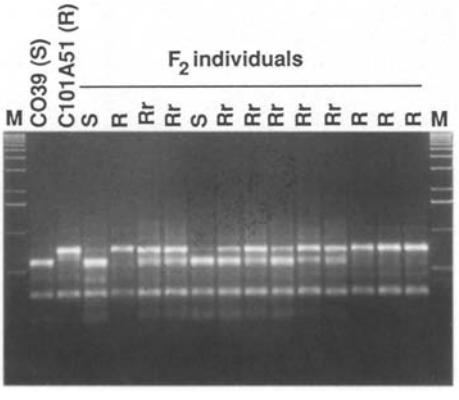

Table 1 Chi-square test for the segregation of blast resistance and the two flanking markers, RG64 (RFLP/SAP) and RG456 (RFLP)

F_2 individuals					
Marker	RR	Rr	rr	Total	$\chi^2(1:2:1)$
RG64 RFLP/SAP	31	58	30	119	0.316
RG456 RFLP	31	53	25	109	1.622
F_3 progeny test	33	55	31	119	0.738

Table 2 Comparison of *Pi-2(t)* genotype of F_2 plants based on SAP/RFLP analysis of the RG64 locus and F_3 progeny testing

Number of plants			
Genotype	RFLP/SAP	F_3 progeny	Match (%)
RR	31	30	96.8
Rr		1	
rr		0	
RR		3	
Rr	58	54	93.1
rr		1	
RR		0	
Rr		0	
rr	30	30	100.0

Table 3 Prediction of genotypes of F_2 plants based on the flanking RFLP markers (RG456 and RG64) for the *Pi-2(t)* blast resistance locus

Prediction		Progeny testing			Accuracy of prediction
RG456	RG64	RR	Rr	rr	
RR (31[a])	RR (25[a])	25			100%
	Rr (6)	2	4		
	RR (4)	4			
Rr (53)	Rr (44)	1	43		97.7%
	rr (5)			5	
	Rr (1)		1		
rr (25)	rr (24)			24	100%

[a] Number of plants predicted to carry the indicated genotype

lies with the blast fungus and scored for blast resistance. The F_2 genotype at the resistance locus was then inferred from the F_3 progeny testing. The results of F_2 genotype prediction based on marker genotypes and progeny testing are compared in Table 2. Of the 31 individual plants that were scored as homozygous resistant based on the SAP and RFLP markers, 30 were found to be homozygous and 1 heterozygous upon progeny testing. This gives an accuracy of 96.8% (Table 2). Of the 58 plants that were scored as heterozygous resistant based on the SAP and RFLP markers, 54 plants were found to be heterozygous upon progeny testing. This gives an accuracy of 93.1% (Table 2). An accuracy of 100% was obtained when identifying homozygous susceptible genotypes. The small discrepancies between scoring based on the marker genotypes (RFLP and SAP) for the homozygous and heterozygous resistant classes and that based on the progeny testing could be due to recombination events between the RG64 and the *Pi-2(t)* locus (Yu et al. 1991).

It is expected that the accuracy of marker-assisted selection can be improved if selectable flanking markers are available on both sides of the gene of interest. To examine this possibility, another RFLP marker (RG456) flanking the *Pi-2(t)* gene was used. RG456 has a genetic distance of 5.0 cM from *Pi-2(t)* gene (Tanksley et al. 1992). The simultaneous use of RG64 and RG456 segregation data allowed us to correctly identify all of the 25 homozygous resistant F_2 plants, which were confirmed to be homozygous at the *Pi-2(t)* resistance gene on the basis of F_3 progeny testing (Table 3). This is the most important category when selecting the resistant individuals in a breeding program. Therefore, the probability of selecting the homozygous resistant genotypes increased when both flanking markers were considered simultaneously.

Discussion

The potential benefits of marker-assisted selection strategy have been widely discussed (see Paterson et al. 1991 for a review), but actual examples of the application of this approach are scant. In this study, we have developed a PCR-based marker linked to the blast resistance gene *Pi-2(t)* in rice, which is as reliable as an RFLP marker. This genetic marker segregates as a co-dominant marker. We used the PCR marker to identify *Pi-2(t)* in an F_2 segregating population of a cross between a susceptible inbred line ('CO39') and its resistant isoline (C101A51).

To examine if the PCR marker could distinguish genotypes within closely related varieties, two resistant cultivars, 'IR48' (*indica*) and 'Moroberekan' (*japonica*), were compared with our susceptible *indica* variety 'CO39'. The results indicated that the identified SAP marker clearly distinguished between these varieties, suggesting the usefulness of this technology in a breeding program exploiting intra-subspecific as well as inter-subspecific variation. Furthermore, the speed, simplicity and reliability of this technology, together with the increasing worldwide availability of nucleotide sequence data, make SAP analysis as attractive alternative to other molecular marker analysis. This is especially true in terms of using SAP markers for marker-assisted selection in the breeding of disease-resistant varieties. SAP analysis requires only small amounts of DNA that can be obtained from a half seed (Chunwongse et al. 1992) or leaf tissue without extraction procedure (Abenes et al. 1993). Efforts to sequence the ends of a number of rice RFLP markers (J. Bennett, personal communication) will provide the sequence information needed to make SAP analysis a choice for marker-aided selection.

The accuracy of identifying individuals carrying the resistance gene by selecting for the linked marker was examined by genotyping F_2 individuals through progeny testing of their F_3 families for the blast disease responsiveness. Comparison of identifying a resistant plant with *Pi-2(t)* gene based on the genotype of the linked RG64 marker indicated a matching of up to 97%. Furthermore, the accuracy of identifying a resistant genotype can be improved to 100% when two markers flanking the *Pi-2(t)* resistance gene were used simultaneously. These results demonstrate the usefulness of marker-assisted selection to precisely identify the genotype of a linked target gene in a segregating population. Identification of homozygotes for the *Pi-2(t)* resistance gene is of critical importance in early stages of plant development and in early segregating generations, especially when the selected plants are to be used for further crosses. We are currently using marker-assisted gene identification to pyramid the major blast-resistant genes with the ultimate goal of producing rice varieties with more durable blast disease resistance.

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