DEVELOPMENT OF STS MARKERS TO INDENTIFY BROWN PLANTHOPPER RESISTANCE IN A SEGREGATING POPULATION

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ABSTRACT

This was done with an informative RFLP marker RG457, which showed the closest linkage to gene Bph-10. Based on the sequence data of sequence tagged site (STS), new marker technologies: STS marker analysis of the introgression lines for BPH resistance have been detected from O. autraliensis and O. sativa. Two introgression lines (IR65482-4-136, and IR65482-17-511) resistant to BPH biotypes 1, 3 were selected from BC2F4 of the cross between O. sativa (IR31917-45-3-2) and O. australiensis (accession 100882). Two polymorphic markers were amplified with

RG457FL : GCAGTGGCAGATGGGATCGT
RG457RL: GCTCCGAAATCCCAAGCGAT and
RG457RB: TGAGCTGATGGTTTGCATGG combined to RG457FL

Two primers RG457FL/RL and RG457FL/RGRB allow heterozygotes to be distinguished from the two homozygotes. To increase the predicting power of the DNA markers on Bph gene, we conducted the fine mapping of Bph using F2 population from IR31917/ IR54742. The two markers were mapped to Bph10 with distances of 1.7 cM. These PCR-based markers will also be useful as genetic markers for plant breeding applications such as marker-aided selection. In addition, PCR-based markers provided information on the basis of STS polymorphisms in rice and on the nature of the STS primer sites.

Key words: marker, brown planthopper, resistance

INTRODUCTION

A new class of PCR based markers are being exploited. Among these are sequence tagged microsatellites (Wu et al., 1989), sequence characterized amplified regions (SCAR's) (Paran and Michelmore, 1993) and sequence tagged sites (STS's) (Tragoonrung et al.,1992). RFLP markers are converted to STS by sequencing their termini so that a suitable pair of primers can be designed for each marker and synthesized for PCR use (Williams et al. 1991, Robeniol et al. 1996). If the PCR products of STS primers prove monomorphic, these products could be digested with appropriate restriction enzymes to reveal polymorphism. Such polymorphism are referred to as PCR-based RFLP (PBR) (Ghareyazie et al. 1994) or specific amplicon length polymorphism (SAP) (Hittalmani et al., 1995), or post amplification restriction polymorphism (PARA) (Penner et al., 1995).

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The concept of marker-assisted selection (MAS) in plant breeding programs has been practiced for many years. Breeders use MAS when an easily scorable character (considered as the marker trait) is linked to an agronomically important character that is difficult or costly to select. For example, in rice, the purple coleoptile of some traditional varieties grown in Northeast India is linked to a gene for resistance to brown planthopper (*bph*-2). More than 95% correlation had been reported between a resistant F_2 plant and purple coleoptile (IRRI 1993). In a two-line hybrid seed production, MAS was used to eliminate seed contamination resulting from incomplete male sterility of thermosensitive genic male sterile (TGMS) lines (Lang et al 1997).

According to the concept of DNA marker-assisted selection, the target genes can be identified in a segregating population at any plant growth stage based on linked molecular marker.(RFLP) analysis was carried out to tag the alien genes for brown planthopper (BPH) resistance from wild species *Oryza autraliensis* into cultivated rice. The gene for BPH resistance is linked with RG457 of chromosome 12 at distance of 3.68+1.29 cM (Ishi et al 1994). In this study, the strategy was prepared DNA sequencing designed primers and synthesize primers. The PCR- based polymorphism was generated to distinguish resistance and susceptible.

PCR-based markers: The amplification of molecular markers RG457 linked to the resistance gene using polymerase chain reaction (PCR), methodology provides a more effective means of performing marker-aided selection than screening by the RFLP. PCR analysis is less expensive than RFLP analysis because of the lower cost of reagents and the reduction of time and labor required (Penner et al. 1995).

MATERIALS AND METHODS:

Plant materials

The material consisted of an elite breeding line of rice (IR31917-45-3-2) , wild species (*O. autraliensis*, accession 100882), and introgression line(IR65482-4-136-2-2) into which gene for resistance to BPH and early maturity had been introgressed (Table 1) The F2 and F3 population were produced from the cross of IR54742 (introgression line from *O. officinalis*) with the recurrent parent IR31917-45-3-2

Table 1. Reaction to BPH in the recurrent parent and introgression line

(R: resistant S: susceptible)

Material	Reaction to biotype		
	1	2	3
IR31917-45-3-2 (recurrent parent)	S	S	S
O.*autraliensis*	R	R	R
IR65482-4-136-22	R	R	R
IR65482-7-216	R	R	S
IR65482-17-511	R	S	R

IR65482-13-539	R	S	S

Line development

Pairwise crosses between each recurrent parent and donor line were made. F1 hybrids were produced by crossing three elite breeding lines of *O. sativa* - all susceptible to biotypes 1, 2 and 3 of BPH - as female parents with four accessions of *O. autraliensis.* The pollinated panicles were sprayed twice a days with a mixture of the growth hormones gibberellic acid + naphthalene acetic acid and kinetin in then proportion of 100, 25, and 5 mg/l, respectively. Fourfeen days after pollination , hybrid embryos were excised and cultured on 1/4 MS medium.The cultured embryos were incubated in darkness at 25^{o}C until germination and subsequently transferred to light. The young seedling at the three-leaf stage were transferred to light. The young seedlings at the three-leaf stage were transferred to a liquid nutrient solution and were grown in a phytotron. After 10 days, the seedlings were transplanted into soil. The F_1 plants were grown and self-pollinating to produce the F_2 populations for fine mapping

DNA extraction for PCR analysis

DNA suitable for PCR analysis was prepared using a simplified miniscale procedure (Kangle et al.1995). A piece of young rice leaf (2 cm) was collected and placed in a labeled 1.5 ml centrifuge tube in ice. The leaf was ground using a polished glass rod in a well of a Spot Test Plate (Thomas Scientific) after adding 400 µl of extraction buffer (50 mM Tris-HCl pH 8.0, 25mM EDTA, 300mM NaCl and 1% SDS). Grinding was done until the buffer turned green which is an indication of cell breakage and release of chloroplasts and cell contents. Another 400 µl of the extraction buffer was added and mixed into the well by pipetting. Around 400 µl of the lysate was transferred to the original tube of the leaf sample. The lysate was deproteinized using 400 µl of chloroform. The aqueous supernatant was transferred to a new 1.5 ml tube and DNA precipitated using absolute ethanol. DNA was air-dried and resuspended in 50 µl of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). An aliquot of 1 µl is sufficient for PCR analysis. The remaining DNA was stored in -20^{o}C for any later use.

PCR amplification

PCR amplification components and conditions were done based on the method used by Kangle et al. (1995). The PCR reaction mixture contained 20-50 nanogram (ng) template DNA, 50ng of each primers, 0.05 mM dNTP's, 1X PCR buffer (10mM Tris pH 8.4, 50mM KCl, 1.8mM $MgCl_2$ and 0.01 mg/ml gelatin) and 1 unit of *Taq* polymerase in a total volume of 20 µl. Template DNA was initially denatured at 94^{o}C for 5 minutes followed by 30 cycles of PCR amplification using the following parameters: denaturation of 94^{o}C (30 sec), primer annealing at 55^{o}C or 60^{o}C (30 sec), and primer extension at 72^{o}C (1 min). Completion of primer extension was alllowed by a final 5 min incubation at 72^{o}C.

An aliquot of 10 µl of the PCR product was routinely taken for gel electrophoresis to determine if amplification was successful. When the

primers detected an amplicon length polymorphism, the samples were readily scored. The remaining 10 µl of PCR products was used for restriction digestion to detect PBR polymorphism in case of other primers. The digestion reaction normally contained 3.2 µl sterile distilled water, 1.5 µl restriction buffer (10X), 0.3 µl restriction enzyme (10 U/µl) and 10.0 µl of PCR products in a total volume of 15 µl. The digestion reaction was incubated for 4 hours to overnight at appropriate incubation temperature for the enzyme used.

The PCR products or the DNA fragments produced by restriction digestion were resolved electrophoretically on 1% agarose gel in 1X TAE buffer.

Development of additional STS markers

The available PCR marker for BPH is based on the linked RFLP marker RG457, The primers were designed based on DNA sequences derived by manual sequencing from both ends of RFLP clones RG457. Both these markers reveal PCR-based RFLP. RG457 primer PCR products needed to be digested with *Hin*fI and *Alu*I.

Designing STS primers.

The *BPH-10* linked to marker RG457. DNA sequencing was done using the flourescent dye Cy^{5TM} (Pharmacia-Cy^{5TM} Autoread^{TM} Sequencing Kit) for the ALF^{TM} Express Automated Sequencing Machine. The method uses the dideoxy-chain terminating DNA sequencing principle (Sanger et al. 1977; Vos et al. 1993).

Primers were designed based on sequences obtained from the automated sequencing experiments. Generally, 20-mer primers were designed containing 50% G+C in the sequence. Two forward primers were designed from sequences obtained using the forward sequencing primer. Two reverse primers were designed from the sequence obtained using the reverse sequencing primer. Thus, four primer combinations can be made for one clone. The primers were synthesized by commercial companies (Life Technologies) and diluted to 50ng/µl concentration for standard PCR use.table

Validation of primers.

Initial PCR reaction using the designed primers was carried out at 55^{o}C annealing temperature using the resistant and susceptible parental DNA as PCR templates. Ten µl of the PCR products were generally used to check if amplification is successful. If multiple bands are obtained, the annealing temperature was raised up to 60^{o}C or less amount of the thermostable *Taq* DNA polymerase was used to reduce the number of bands. When monomorphic bands were obtained, several restriction enzymes were tried on the PCR products to generate a PCR based RFLP (PBR).

Evaluation of accuracy of marker-aided selection

The accuracy of MAS was evaluated based on its ability to predict the genotype of the F_2 plants for the resistance locus. The genotypes of the F_2 for the resistance locus was inferred from the F_3 progeny test. The actual disease reaction

RESULTS

Reaction of parents to BPH biotypes

The reaction to BPH biotypes 1,2,3 in the recurrent parent and introgression line is shown in Table 2. Four lines, IR65482-4-136-22, IR65482-7-216, IR65482-17-511, IR65482-13-539 showed moderate resistance to BPH biotypes 1, and 2 as compared to IR65482-4-136-22, IR65482-7-216. Resistance to BPH biotype 3 were noticed in IR65482-4-136-22, IR65482-17-511.

Detection of STS markers

RG457 DNA sequence and PCR primer: The nucleotide sequence of RG457 was determined on the basis of the orientation to ensure accuracy (fig 1). We are performing STS analysis on a cross between IR54742 and IR31917-45-3-2. It is clear from fig 2 that PCR can detect size and polymorphism at certain loci. For example, amplification of DNA from IR54742 and IR31917-45-3-2 with the primer RG457FL/RB gave rise to 800-bp product for IR54742 and 750-bp for IR31917-45-3-2. Primer RG457FL/RL gave rise to 750-bp product for IR54742, and 700-bp for IR31917-45-3-2.

Digestion of PCR products with restriction endonuclease has been found to increase the level of polymorphism as well as the number of alleles in rice (Willians et al 1991). We are performing STS analysis on a cross between IR54742 and IR31917-45-3-2. It is clear that PCR can detected size for polymorphism at certain loci (fig 2). For example, amplification of DNA from IR54742 and IR31917-45-3-2 with the primer RG457140FB/RL gave rise to 500-bp product for IR54742, and 300-bp for IR31917-45-3-2.

In case of RG457FL/RL, two bands are present in PCR product of IR 31917-45-3-2 with the size of 500, 200bp . Three restriction sites for *Hin*fI are present in PCR product of IR 54742 (300, 250, and 200 bp).This polymorphism can be exploited as markers to distinguish BPH resistant from susceptible lines, because all loci were scored as codominant.

Germplasm survey of polymorphism

The potential of using a PCR-based approach to detect DNA polymorphism for rice germplasm classification was analysed. Genomic DNA of the various genotypes was evaluated.

Introgression from *O. australiensis* into *O.sativa* was detected throught RFLP analysis (Ishi et al 1994). RG457 supports the occurrence of crossing over in transferring the segments of wild species chromosomes to *O. sativa.* DNA of the various genotypes IR65482-4-136-3, IR65482-7-216, IR65482-17-511, IR65482-13-539, and IR31917, were tested with primers RG457FL / RL. Restriction site for *Hin*fI is present in PCR product for IR65482-4-136-3, , IR65482-17-511, with the size of 300, 250 and 200 bp and for IR65482-7-216, IR65482-13-539, IR31917 with the size of 500, 200bp.

With primers RG457FL / RB, restriction sites for *Hin*fI is present in PCR product for IR65482-4-136-3, IR65482-17-511 with the size of 500, 300 bp, and for IR65482-7-216, IR65482-13-539, IR31917 with the size of 550, 200bp.

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

F2 population analysis

DNA genome from the F2 population, the cross IR 54742/IR31917 was assessed through PCR amplifcation using primer pair RG457 FL/RL. The PCR products were spliced out by double enzymes digestion with *Hin*fI and *Alu*I. Fragments were resolved on agarose gels and the banding patterns were scored with reference to those of the parents. The banding pattern of the 37 of F2s individuals could be classified into homozygote for the IR54742 type marker with the size of 300, 250 and 200 bp fragments, homozygote for IR31917 type marker with the size of 500, 200bp fragments, and heterozygotes, displaying both fragments IR54742 and IR31917 (fig 3). Similarly, when combinations of primer pair RG457FL / RB, fragments were resolved on agarose gels and the banding patterns were scored with reference to those of the parents. The banding pattern of the F2' s individuals could be classified into homozygote for the IR54742 type marker with the size of 500, 300 bp fragments, homozygote for IR31917 type marker with the size of 550, 200bp fragments, and heterozygotes displaying both fragments IR54742 and IR31917. All loci scored as codominant values were determind using maximum likelihood algorithm in the MAPMARKER V20 program (Lander et al 1987). The gene *Bph*-10 is found to be located at chromosome 12, linked to RG457, with a distance of 1.7 cM (the cross of IR54742/IR31917).

PCR allows the amplification of specific regions of DNA from complex sequence using short oligonucleotide primers and thermostable DNA polymerse. Thus, markers detected by PCR are termed amplicon length polymorphism (ALP). ALP is detected by standard PCR analysis of multiple individuals. Variation in the amplified region (amplicon) is revealed by gel electrophoresis of the PCR products. Advantages of ALP over RFLP are: (1) it is rapid, (2) no radioisotope is required, (3) it is low cost, and (4) only small quantity of DNA is required (Innis et al. 1990).

DISCUSSION

Marker-aided selection (MAS) refers to the selection of target genes based on linked DNA markers. MAS offers the opportunity to expedite selection and transfer of desirable traits as it can be performed for multiple traits in early segregating generations and at early stages of plant development. Practical application of MAS requires that markers would be identified with a high level of accuracy and efficiency, be effective and be easy to use (Huang et al. 1997). PCR-based markers are the most practical markers for MAS because the PCR approach is much faster and easier, less laborious and time-consuming than Southern analysis. To date, however, actual examples of the application of MAS are very few.

The success of MAS is highly dependent on the tight linkage of the molecular marker with the target gene. The relationships of mapping DNA markers among different populations need to be clarified. It is important that variation in

genetic distance among different populations would be non-significant. That will allow the use of information gained from a population for selection of the same gene in a different genetic background. Thus, it will ensure the efficient selection of genotypes across different genetic backgrounds.

The accuracy of marker-aided selection for BPH was verified through F_3 progeny tests. The F_2 genotype at the resistance locus was inferred from the F_3 progeny testing. The accuracy of predicting homozygous resistant genotypes based on flanking marker data was 100% for BPH using a single marker. The discrepancy between the marker data prediction and F_3 progeny tests could be due to recombination between the resistance locus and the marker. A single marker for a target gene could thus be as accurate as two flanking markers provided the single marker is sufficiently close such that the target gene and the marker behaves meiotically as a single block.

The accuracy of marker-aided selection for BPH was verified through F2, F3 progeny tests. The accuracy of predicting homozygous resistant genotypes based on flanking marker data was 100% for BPH using a single marker. The successful PCR based markers for *Bph*-10 have made it possible to use these markers in future MAS program for the transfer of BPH into elite breeding lines of rice.

Acknowledgements

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Table 2. Sequence of 20-mer oligonucleotide primers derived from RFLP markers linked o gene BPH-10

Primer	Sequence
RG457FL	5' GCAGTGGCAGATGGGATCGT 3'
RG457RL	5' GCTCCGAAATCCCAAGCGAT 3'
RG457FB	5' CGTTATCCTCAGTTCCTAGG 3'
RG457RB	5' TGAGCTGATGGTTTGCATGG 3'

Table 3. BPH analysis of F2 through progeny test and PCR from IR54742 / IR31917

Phenotype test		PCR analysis			Accuracy(%)
Genotype	Number of Plant	RR	RS	SS	
RR	8	8			100.00
RS	20		19		95.00
SS	9			9	100.00

RG457 F

1 GTGACACTAT AGAATACGAA TTCGAGCTCG GTACCGGGG ATCCTCTAGA

51 GTCGACCTGC AGACAGGAGG AGTTTGATAG ATCGGCAGCTGGAAGAGCAG

101 CAAAAGCACA AATGAAGGCT ATGAAGGAAG CCAAGACATC ATCAAACCAA

151 GGAGAACCAG TTCTTAAGGT ATCGTTATCC TCAGTTCCTA GGTTAAAAGT

201 AAATGTTTAA ACCTGATACC ATCCAATTTG TTTGTTGAGA GTATTTATCA

251 CTTTAATCTC TTGATTGATG CAGTGGCAGA TGGGATCGTA AATTCTTCAT

301 TAGGTAGTTG ACCCTTAAAG AAAAAAAAAA AAATTACAAA AAA

RG457 R

1 AAAAACCAAG CTCTAATACG ACTCACTATA GGGAGACAAG CTTGCATGAC

51 CTGCAGGAGG TGCTTTATAC AAGATATGAT TTGACAAATT TCTGTAGAAT

101 TCAGTACCAG TAGTGTTACT ACTCTACTAC TTCTCTATGA GCTGATGGTT

151 TGCATGGGGG GAGAAAATAA ATCAAGCTCC GAAATCCCAA GCGATTGTAC

201 CAAAACGAGA GAAAAAAAAA ATACTATACA AAACTACTAC AGAAATGTAC

251 ATGATCTGTT CCAACAAACA CACAAGCTTG GACAGATTTA ATTCTTGGAA

301 TTTATCGTTG GTTGGCCGCA AGCCCATTGG AACCAAGGGG GGGAATTTTC

351 CAAGCCCAAT CCCCGCAGCC TTTTCGAAGG GGCGCGCC CCCGCCCGGG

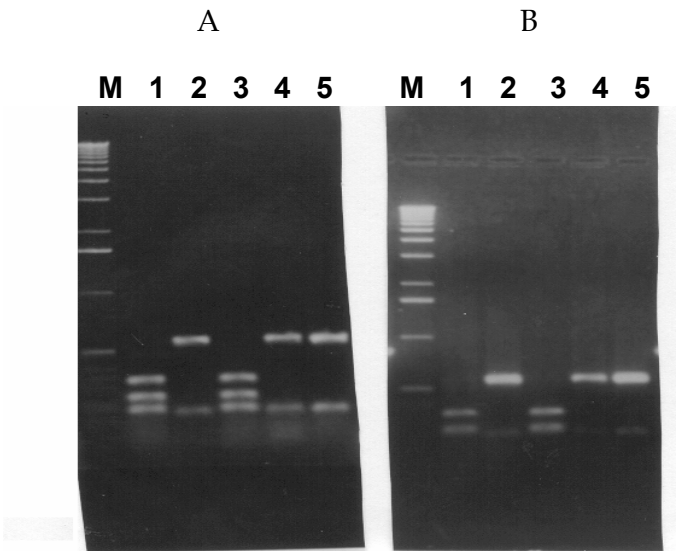

Figure 1: DNA sequencing of RG457

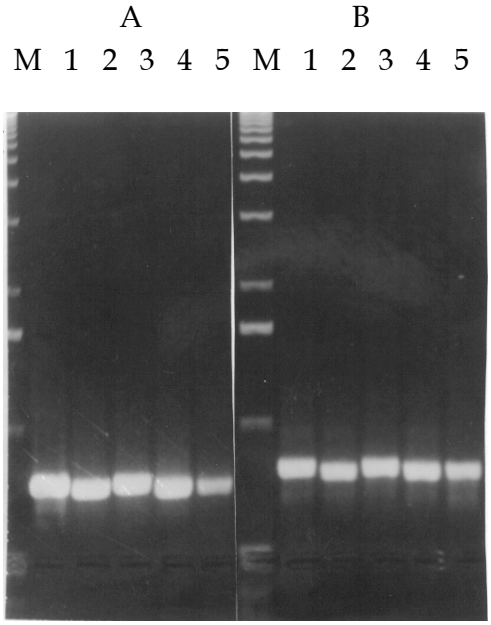

Figure 2. Agarose gel electrophoresis of PCR products amplified with DNA from varieties before digestion A:with primers RG457FL/RL M: 1kb, Lane 1: IR54742 (750 bp) lane 2: IR31917-45-3-2 (700bp). 3: IR65482-4-136-2, 4:: IR 65482-7-216, 5: IR65482-13-539.
B: With primer RG457FL/RB, M: 1kb, Lane 1: IR54742 (800 bp) lane 2: IR31917-45-3-2 (750bp). 3: IR65482-4-136-2, 4:: IR 65482-7-216, 5: IR65482-13-539.

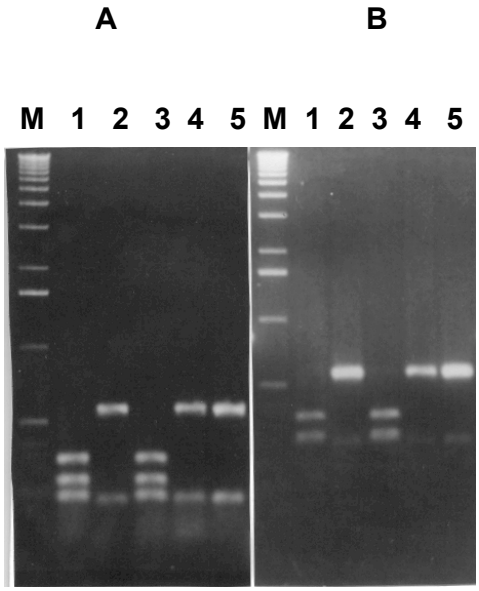

Figure 3. Agarose gel electrophoresis of PCR products amplified with DNA from varieties A:After digestion with HinfI for primers RG457FL/RL M: 1kb, Lane 1: IR54742 (300,250,200 bp) lane 2: IR31917-45-3-2 (500,200bp). 3: IR65482-4-136-2, 4:: IR 65482-7-216, 5: IR65482-13-539.
B: With primer RG457FL/RB ,after digestion with HinfI M: 1kb, Lane 1: IR54742, M: 1kb, Lane 1: IR54742 (500,300 bp) lane 2: IR31917-45-3-2(550,200bp). 3: IR65482-4-136-2, 4:: IR 65482-7-216, 5: IR65482-13-539.

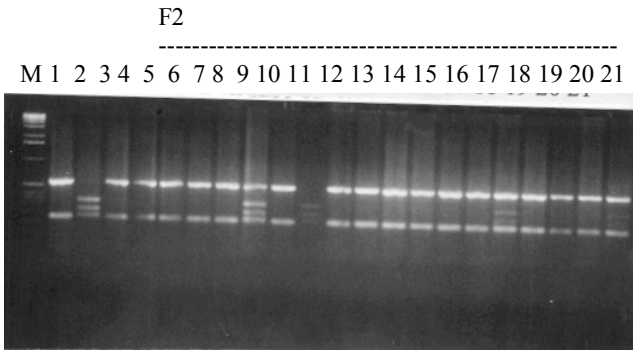

Figure 4. Segregation of the RG457L-L locus determine by SAP analysis. PCR products segregating population were generated by DNA amplification with primer RG457L-L with reference to the banding pattern of parents, IR 54742/IR31917 and genotypes of each F2 individual.
Lane 1: IR31917(susceptible) , lane 2 :IR 54742 (resistant) .

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Áp dụng sts marker để chọn lọc dòng kháng rầy nâu trong quần thể đang phân ly

Chuỗi ký tự của RG457 (RFLP marker) đã được thiết kế thành STS marker thành hai cặp primer tương ứng RF457FL, RG457RL, và RG457FB, RG457RB. Liên kết giữa marker RG457 với gen kháng rầy nâu *Bph-10* đã được công bố trước đó tại Viện Lúa Quốc tế (IRRI). Chúng tôi sử dụng STS marker để phân tích sự du nhập gen kháng rầy nâu từ loài hoang dại *O. australiensis* vào giống lúa trồng *O. sativa* (IR31917-45-3-2). Hai dòng IR65482-4-136 và IR65482-17-511 là kết qủa của cặp lai giữa lúa hoang với lúa trồng theo cách này, được ghi nhận kháng với rầy nâu biotype 2 và 3. Quần thể BC2F4 của chúng đã được chọn lọc dòng kháng rầy nhờ STS marker. Đa hình đã được ghi nhận trong cặp primer RG457FL/R**L** và RG457FL/R**B.** Hai cặp primer này cho phép phân biệt các dòng dị hợp tử với các dòng đồng hợp tử. Chúng tôi đã thực hiện "fine mapping" trên quần thể F2 của tổ hợp lai IR31917 / IR54742. Marker này có giá trị liên kết với gen kháng *Bph-10* là 1,7 cM. Đây là một trong những ví dụ khá rõ về chọn lọc giống nhờ marker (MAS) tại Viện Lúa Đồng Bằng Sông Cửu Long.