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Coupling- and Repulsion-Phase RAPDs for Tagging of Brown Planthopper Resistance Genes in the F_2s of IR50XPtb33 of Rice

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Abstract: Brown planthopper (BPH), *Nilaparvata lugens* Stål is one of the most threatening pests, often significantly reducing the rice yield. Breeding of resistant cultivars has been one of the most effective ways for controlling the pest. Recent advancements in DNA marker technology together with the concept of marker-assisted selection provide new solutions for selection of more durable BPH resistant genotypes in rice. F_2s of a cross between IR50 and Ptb33 and their susceptible and resistant parents were used. In bulked segregant analysis, RAPD primers, OPC7 ($^{5'}$GTCCCGACGA$^{3'}$) and OPAG14 ($^{5'}$GGGAACGTGT$^{3'}$) showed co-dominant banding pattern, generated polymorphic DNA fragments, of which, $OPC7_{697}$ (697 bp) and $OPAG14_{680}$ (680 bp) were associated in coupling phase to the resistant allele, while $OPC7_{846}$ (846 bp) and $OPAG14_{650}$ (650 bp) were linked in repulsion phase. The $OPC7_{697}$ and $OPAG14_{680}$ RAPD markers could be used in a cost effective way for marker-assisted selection of BPH resistant rice genotypes.
Key words: random amplified polymorphic DNA marker; brown planthopper; bulked segregant analysis; marker-assisted selection; *Oryza sativa*

Rice (*Oryza sativa* L.) is one of the most important cereal crops of the world. Brown planthopper (BPH, *Nilaparvata lugens* Stål) is one of the most destructive insect pests of rice in rice growing areas. Outbreaks of BPH are closely associated with the misuse of insecticides, especially at the early crop stages. These insecticide sprays disrupt the natural biological control and favor the BPH development as a secondary pest (Pathak and Khan 1994). Both BPH nymphs and adults insert their sucking mouthparts into the plant tissue to remove plant sap from phloem cells, resulting in a severe damage symptom known as 'hopper burn' besides transmitting rice grassy stunt virus and ragged stunt virus as vectors (Rivera et al, 1966; Heinrichs, 1979).

Incorporating resistance gene(s) from wild species into cultivated species can be an alternative approach to develop BPH resistance in susceptible commercial cultivars (Rahman et al, 2009). Studies conducted by many researchers have investigated the genetics of resistance in rice to BPH. In map construction, F_2 segregating populations are the result of selfing F_1s of two homozygous inbred lines. Many molecular maps to date were based on segregation data from F_2 progenies (Wu et al, 2002). A novel BPH resistance gene has been introduced into cultivated rice lines from a distantly related species of *Oryza* and the gene has been mapped with a DNA marker by random amplified polymorphic DNA (RAPD) and bulked segregant analysis method (Jeon et al, 1999). Bulked segregant analysis is a rapid procedure for identifying molecular markers in specific regions of the genome, in which two pools contrasting for a trait are analyzed to identify markers that distinguish them (Michelmore et al, 1991). Molecular markers of restriction fragment length polymorphism (RFLP) and RAPD were used to develop detailed genetic maps in rice (Huang et al, 1997; McCouch et al, 1997; Jeon et al, 1999). RAPD markers were also used in optimizing hybrid vigor in japonica rice (Cho et al, 2004) and assessment of genetic relationships (Wakui et al, 2009). The PCR-based RAPD markers were used for tagging agronomic traits in several crops (Martin et al, 1991; Nair et al, 1995; Ford et al, 1999; Manninen, 2000) and for diversity analysis in the non-basmati scented rice collection (Mathure et al, 2010). RFLPs are often co-dominant but are restricted to regions with low or single copy sequences. Moreover, it requires large amount of DNA with high purity, specific probes and time consuming. RAPD relies on the differential enzyme amplification of small DNA fragments using PCR with arbitrary oligonucleotide primers (usually 10 mers). *Bph1* was tagged on rice chromosome using bulked segregant analysis with 520 RAPD primers to identify markers linked to the BPH resistance gene (Kim and Sohn, 2005). In the present study, 170 RAPD primers were used for identification of coupling and repulsion phase markers in the F_2 progeny of IR50× Ptb33. The selected RAPD markers could be used for marker-assisted selection (MAS) of BPH resistant lines.

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MATERIALS AND METHODS

Screening for BPH resistance

Screening for BPH resistance was done using the standard seed box screening test in a green house, Paddy Breeding Station, Coimbatore, India (Heinrichs and Mochida, 1984). The F_2s from the cross of IR50× Ptb33 were tested for their resistance to BPH infestation. Taichung Native 1 (TN1) and Ptb33 served as the susceptible and resistant check, respectively. Rice seedlings at three-leaf (7 days after sowing) were infested with the 2^{nd}–3^{rd} instar nymphs at 8 nymphs per seedling. When the seedlings of the susceptible check were almost completely dead, the test entries were rated according to the damage rating of the Standard Evaluation System for rice (International Rice Research Institute, 1996) (Table 1).

Isolation of genomic DNA

Isolation of genomic DNA was done following the method recommended by Wu et al (2002) with slight modifications. To extract DNA from the parents, the F_2s from the scored plants, the surviving plants immediately after screening were freed from the insects and were planted in separate clay pots in order to grow those to grown up stage for about 15–20 days so as to extract sufficient quantity of DNA.

RAPD and bulked segregant analysis

RAPD analysis was performed following the method described by Saiki et al (1988). A total of 73 decamer primers obtained from Operon Technologies Inc., California, USA, were used in this study. PCR was carried out in a total volume of 20 μL containing 10 ng of template DNA, 100 pmol primers, 0.5 mmol/L each of dNTPs, 0.5 U of *Taq* polymerase (Invitrogen) and 1×PCR buffer containing 1.5 mmol/L $MgCl_2$. PCR was setup for initial denaturation at 95°C for 3 min, and then each amplification cycle contained one denaturation step at 94°C for 1 min, annealing step at 36°C for 40 s, one extension step at 72°C and final extension is for three min at 72°C in a thermal cycler (Eppendorf, USA). PCR amplification products were run on 1.2% agarose gels containing 0.2 μg/mL ethidium bromide in a standard horizontal gel electrophoresis unit (Broviga, Chennai, India) with TBE buffer (90 mmol/L Tris-borate, 1 mmol/L EDTA, pH 8.0). The DNA bands were photographed in a gel documentation system (AlphaImager). Initially, all 73 decamer primers were used for parental screening on BPH-susceptible IR50 and resistant Ptb33. Polymorphic primers were tested in two DNA bulks and parents following Michelmore et al (1991).

Table 1. Standard evaluation system for rice brown planthopper damage.

Scale	Criteria	Category
0	None	
1	Very slight damage	Highly resistant
3	First and second leaves with orange tips; slight stunting	Resistant
5	More than half the leaves with yellow - orange tips; pronounced stunting	Moderately resistant
7	More than half of the plants wilting or dead and remaining plants severely stunted or drying	Moderately susceptible
9	All plants dead	Susceptible

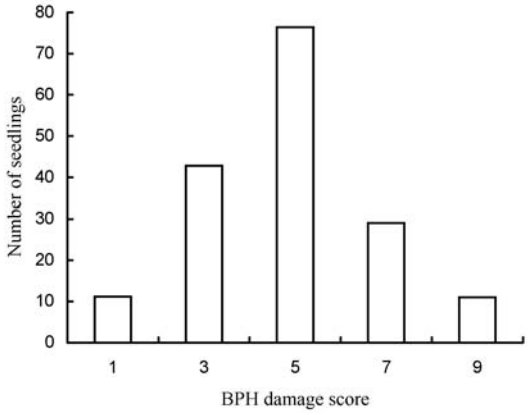

Fig. 1. Frequency distribution of score ratings infested with *N. lugens* on 170 F_2 seedlings of IR50/Ptb33 based on the standard evaluation system.

RESULTS

Inheritance pattern of BPH resistance gene in F_2 progeny

In a total of 170 F_2s tested, the plants rated a damage score of 1, 3 or 5 were grouped as resistant (130) and ones rated a damage score of 7 or 9 were grouped as susceptible (40). This data fitted well to the expected ratio of resistant against susceptible at 3:1 (χ^2 =0.19, P =0.97–0.99) (Fig. 1).

Identification of RAPD markers linked to BPH resistance

In parental screening, out of 73 primers used, 66 exhibited DNA amplification. Out of five kits of RAPD operon primers that were tested for parental polymorphism, OPAG series primers showed maximum polymorphism (23.91%) between the parents, IR50 and Ptb33 (Table 2). The polymorphism showed by the RAPD primer series, OPC, OPE, OPAL and OPM were found at 20.36%, 15.00%, 15.20% and 15.92%, respectively in the parents IR50 and Ptb33. Among all these primers, two co-dominant RAPD primers of OPC7 and OPAG14 were shown distinct, repeatable and high degree of polymorphism in the parents and bulks. OPC7 and OPAG14 were generated polymorphic DNA fragments of which, $OPC7_{697}$ (697 bp) and $OPAG14_{680}$ (680 bp) were associated with coupling phase to the resistant allele, while $OPC7_{846}$ (846 bp) and $OPAG14_{650}$ (650 bp) were linked to repulsion phase (Fig. 2 and Fig. 3).

DISCUSSION

The performance of the parents IR50, Ptb33 and the F_2s during screening revealed the consistency of the screening

Table 2. Pattern of polymorphism between parents (IR50 and Ptb33) detected by RAPD analysis using operon primers.

Sl. No.	Polymorphic primer	No. of bands			Monomorphic band		Polymorphic band	
		IR50	Ptb33	Total	No.	%	No.	%
1	OPC series	113	108	221	178	80.54	45	20.36
2	OPE series	141	130	271	238	85.00	42	15.00
3	OPAL series	62	61	128	104	83.20	19	15.20
4	OPM series	72	87	159	114	87.69	25	15.92
5	OPAG series	33	56	89	70	76.08	22	23.91
	Total	426	442	868	704		153	

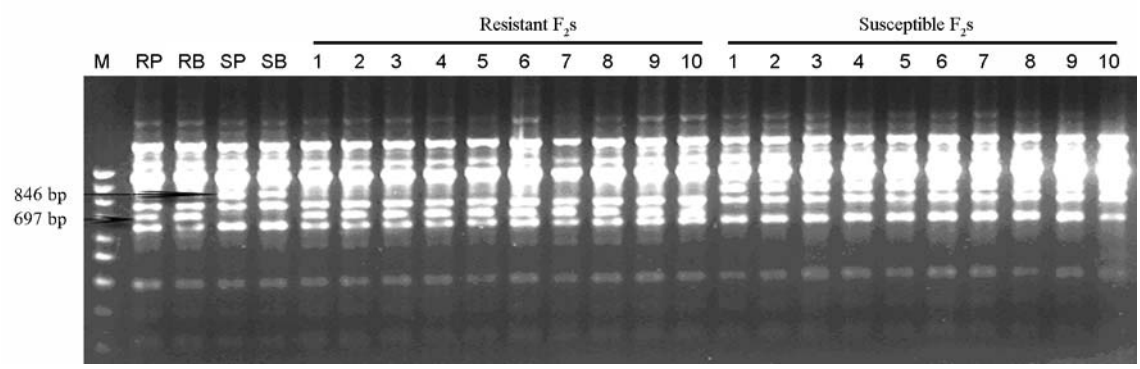

Fig. 2. Co-segregation banding pattern of the RAPD primer OPC7 DNA fragment, $OPC7_{697}$ (697 bp) was associated in coupling phase to the resistant allele, while $OPC7_{846}$ (846 bp) was in repulsion phase.
M, Marker; RP, Resistant parent; RB, Resistant bulk; SP, Susceptible parent; SB, Susceptible bulk.

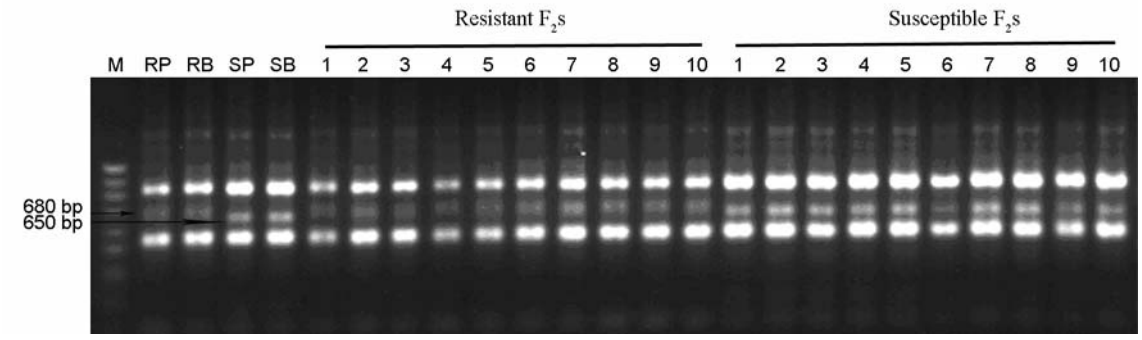

Fig. 3. Co-segregation banding pattern of the RAPD primer OPAG14 DNA fragment $OPAG14_{680}$ (680 bp) were associated in coupling phase to the resistant allele, while $OPAG14_{650}$ (650 bp) was in repulsion phase.
M, Marker; RP, Resistant parent; RB, Resistant bulk; SP, Susceptible parent; SB, Susceptible bulk.

protocols (SES, 1996) for BPH resistance in this study. Phenotypic evaluation should be performed with more reliable methods to avoid false positives in further MAS (Machill and Ni, 2001). DNA amplification products obtained from PCR analysis using random primers has been proposed as an alternative method in targeting DNA sequences for genetic characterization and mapping (Williams et al, 1990). Relatively higher number of amplified products per primer was found in rice, when compared to other plants, like maize (Welsh and McClelland, 1990). One of the most time consuming requirements of DNA marker development, is the need to screen entire mapping populations, with every probe or primer and this has been removed by the bulked segregant analysis. The minimum size of a bulk is determined by the frequency with which linked loci might be detected as polymorphic between the bulked samples. OPC7 and OPAG14 were generated polymorphic DNA fragments of which, $OPC7_{697}$ (697 bp) and $OPAG14_{680}$ (680 bp) were associated in coupling phase to the resistant allele, while $OPC7_{846}$ (846 bp) and $OPAG14_{650}$ (650 bp) were linked in repulsion phase (Fig. 2 and Fig. 3). Two pools contrasting for a trait of BPH susceptibility were analyzed to identify markers that distinguish them. The polymorphic markers between the pools will be genetically linked to the loci that determine the trait was used to construct the pools (Michelmore et al, 1991). Results obtained in F_2 seedlings indicate that RAPD markers are co-dominant, highly polymorphic and informative in nature. These co-dominant RAPD markers are comparatively unique. Similar to other kinds of co-dominant markers, these co-dominant RAPD markers can be of particular value for the purpose of linkage analysis because they provide maximum linkage information per individual in the segregating populations. Co-dominant markers provide easy discrimination between

recombinant homozygotes and recombinant heterozygotes (Williams et al, 1990; Mohan et al, 1997; Semagn et al, 2006). So far, co-dominant RAPD markers have been successfully employed in MAS in various crops (Mondal et al, 2007).

A clear polymorphism between the bulks in comparison with the parents was observed. Poulson et al (1995) suggested that bulked segregant analysis can be robust to cope with the low level of phenotypic misclassification when bulks are constructed with good size of individuals. Bulked segregant analysis using RPAD markers were successfully used in the development of linked molecular markers. Thus, RAPD markers $OPC7_{697}$ and $OPAG14_{680}$ could be used in a cost effective way for MSA of BPH-resistant rice genotypes.

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