

Full Length Research Paper

Identification of coupling- and repulsion-phase markers in rice for brown planthopper resistance genes using F₂S of IR 50 X CO 46

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Accepted 15 December, 2010

Brown planthopper, *Nilaparvata lugens* Stål (Homoptera: Delphacidae) is one of the most threatening pests, often significantly reducing the rice yield. The breeding of resistant cultivar has been the most effective way of controlling this pest. Recent advancements in DNA marker technology together with the concept of marker-assisted selection (MAS) provide new solutions for selection of more durable brown planthopper (BPH) resistant genotypes in rice. F₂s of a cross between IR 50 and CO 46 and their susceptible and resistant parents were used for the present study. In bulk segregant analysis, random amplification of polymorphic DNA (RAPD) primer, OPC 11 (5'AAAGCTGCGG 3') showed co-dominant banding pattern, which amplified a susceptible phenotype specific marker OPC11₈₅₆ associated with repulsion phase. It also amplified resistant phenotype specific markers, OPC 11₈₁₇ which are associated in coupling phase to the resistant allele. The OPA11₈₁₇ RAPD marker could be used in a cost effective way for marker assisted selection of BPH resistant rice genotypes.

Key words: RAPD markers, brown planthopper, bulk segregant analysis, hopper burn, *Oryza sativa*.

INTRODUCTION

Rice (*Oryza sativa* L.) is the most important cereal crop of the world. Outbreaks of the insect pests are closely associated with insecticide misuse, especially during the early crop stages. These insecticide sprays, usually directed at leaf feeding insects, disrupt the natural biological control, which favour the brown planthopper (BPH) development as a secondary pest. Both the nymphs and adults of the brown planthopper 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 (Heinrichs, 1979; Rivera et al., 1966).

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 brown planthopper. To date, 21 genes have been reported out of which eleven resistance loci reported so far in rice have been identified from wild species. These are *Bph20(t)* on chromosome 4 and *Bph21(t)* on chromosome 12 (Rahman et al., 2009), *Bph10* on the long arm of chromosome 12 from *Ovalipes australiensis* (Ishii et al., 1994), *Bph12(t)* on the short arm of chromosome 4 from *Oryza latifolia* (Yang et al., 2002), *Bph13(t)* on the long arm of chromosome 2 from *Otto eichingeri* (Liu et al., 2001), another *Bph13(t)* against BPH biotype 4 on the short arm of chromosome 3 from *Oryza officinalis* (Renganayaki et al., 2002), *Bph14(Qbp1)* and *Bph15(Qbp2)* on the long arm of chromosome 3 and the short arm of chromosome 4, respectively, from *O. officinalis* (Huang et al., 2001),

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Bph18(t) on the long arm of chromosome 12 from *O. australiensis* (Jena et al., 2006), and *bph11(t)* and *bph12(t)* on the long arm of chromosome 3 and chromosome 4 respectively, from *O. officinalis* (Hirabayashi et al., 1999).

Dominant gene, *Bph₁*, governing resistance in 'Mudgo', 'MTU15', 'Co22', and 'MGL2' (Athwal et al., 1971), where as a single recessive gene, *bph₂*, conveys resistance in 'ASD7' and 'Ptb18'. Sri Lankan cultivar Rathu Heenati has a dominant gene for resistance, which is non-allelic to, and independent of, *Bph₁*, and was designated as *Bph₃* (Lakshminarayana and Khush, 1977). Another Sri Lankan cultivar, Babawee, has a recessive gene for resistance (Lakshminarayana and Khush, 1977), this gene is independent of *bph₂* and is designated as *bph₄*. Later *bph₅* in 'ARC10550', *Bph₆* in 'Swarnalatha' and *bph₇* in 'T12', by means of BPH biotypes from Bangladesh (Kabir and Khush, 1988; Bharathi and Chelliah, 1991). Another recessive gene, *bph₈*, identified in 'Thai Col.5', 'Thai Col 11' and 'Chin Saba'. In the Sri Lankan local cultivars, Pokkali, Balamawee, and Kaharamana a dominant gene, *Bph₉*, identified by Nemato et al. (1989).

Populations of BPH were categorized into five biotypes on the basis of their differential reactions to a set of reference cultivars (Chelliah and Bharathi, 1993). In most of the map construction, F₂ segregating populations are the result of selfing F₁s of two homozygous inbred lines. Most of the molecular maps to date are based on segregation data from F₂ progenies (Jena and Khush, 1992; 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 RAPD and bulked segregant analysis method (Jena and Khush, 1992; Jeon et al., 1999). Bulk segregant analysis (BSA) is a rapid procedure for identifying 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). Two types of molecular markers have been used to develop detailed genetic maps in, RFLPs and RAPDs in rice (Huang et al., 1997; McCouch et al., 1997; Jeon et al., 1999). PCR based RAPD markers have been used for tagging agronomic traits in several crops (Martin et al., 1991; Nair et al., 1995; Ford et al., 1999; Manninen, 2000). RFLPs are often co-dominant but are restricted to regions with low or single copy sequences, moreover it requires large amount of highly pure DNA, 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). Polymorphisms result from either chromosomal changes in the amplified regions or base changes that alter primer binding. The procedure is rapid, requires only small quantity of DNA, which need not be of high quality, and involves no radioactivity. As no southern hybridization is involved, polymorphisms can be detected in fragments containing highly repeated sequences; this provides

markers in regions of the genome previously inaccessible to analysis. Due to its simplicity, inexpensiveness still using for marker assisted selection in *Pisum sativum* for nematode resistance (Burrow et al., 1996; Garcia et al., 1996), scab resistance in *Malus domestica* (Yang et al., 1997), sclerotinia rot resistance in *Trifolium pratense* (Page et al., 1997), brown planthopper resistance in F₃ population of interspecific cross between *O. sativa* and *O. officinalis* (Jena et al., 2002), anthracnose resistance and angular leaf spot resistance in *Phaseolus vulgaris* (Caixeta et al., 2003; Martin and Menarim, 2000), soybean mosaic virus resistance in *Glycine max* (Zheng et al., 2003), rust resistance in *Vicia faba* (Avila et al., 2003), rust resistance in *P. sativum* (Vijayalakshmi et al., 2005), Fusarium wilt resistance in *Cajanus cajan* (Kotresh et al., 2006), karnal bunt resistance in *Triticum aestivum* (Kumar et al., 2006), anthracnose resistance gene in sorghum (Singh et al., 2006), *Arachis hypogaea* for rust resistance (Mondal et al., 2007; He and Prakash, 1997). *Bph 1* has been tagged in rice by using bulked segregant analyses, 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 tagging of bph resistance genes in the F₂s of IR 50 x CO 46.

MATERIALS AND METHODS

Screening for BPH resistance

Screening for BPH resistance was done in the green house, paddy breeding station (PBS), coimbatore by 'standard seed box screening' test (Heinrichs and Mochida, 1984). The F₂s of the cross IR 50 X CO 46 were tested along with the susceptible check (TN1) and the resistant check (CO 46) for their resistance to BPH infestation. Seven days after sowing, when the seedlings were at three-leaf stage they were infected with second and third instar nymphs (8 nymphs of BPH 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 Jena et al. (2002) with slight modifications. To extract DNA from the parents, the F₂s 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 to 20 days so as to extract sufficient quantity of DNA.

RAPD and bulk segregant analysis

The RAPD analysis was performed following the method recommended by Saiki et al. (1988) with required modifications. A total of seventy-three decamer primers obtained from Operon Technologies Inc., California, USA were used in this study. 10 ng of template DNA was used for PCR amplification, which is carried out

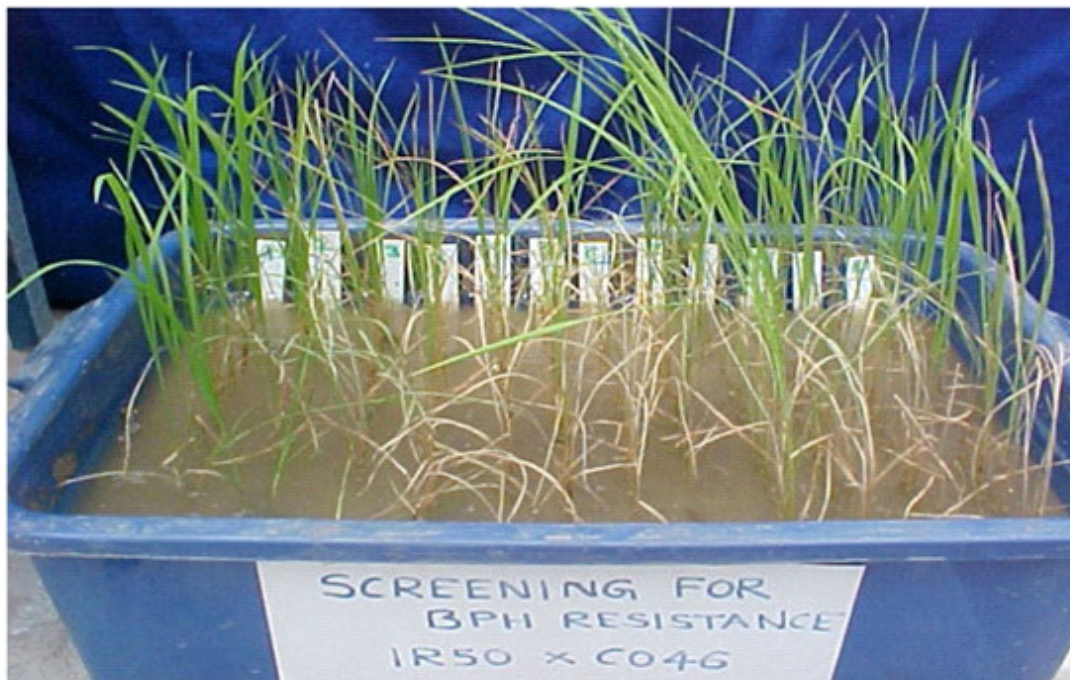


Figure 1. Artificial screening for BPH resistance in the F₂s. Each lane consists of 17 seedlings. First 10 lanes from left to right constitutes a total of 170 seedlings of the cross IR 50 x CO 46. 11th lane is the susceptible check (IR 50) and 12th lane is Resistant check (CO 46).

in a total volume of 20 μ L. The final concentrations are 100 pmol primers, 0.5 mM each of dGTP, dATP, dTTP, dCTP, one unit of Taq polymerase (Invitrogen) and 1X PCR buffer containing 1.5 mM MgCl₂. The programme for amplification was setup for initial denaturation at 95°C for 3 min, and then thirty amplification cycles each amplification cycle contained one denaturation step at 94°C for one minute, annealing step at 36°C for 40 s and one extension step at 72°C. Final extension was set 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 μ g/mL ethidium bromide in a standard horizontal gel electrophoresis unit (Broviga, Chennai, India) having TBE buffer (90 mM Tris-borate, 1 mM EDTA pH 8.0). The DNA bands were photographed in a gel documentation system.

Initially IR 50 (Susceptible) and Co 46 (resistant) parents were screened with all 73 decamer primers. Polymorphic primers were tested on two DNA bulks, as well as parents (Michelmore et al., 1991). Those primers, which show polymorphism between the parents were used to test for polymorphism in the F₂ population of resistant and susceptible populations each comprising ten samples. Resistant bulk comprises of the genomic DNA from all ten resistant populations in the same way susceptible bulk comprises of genomic DNA from all 10 susceptible populations which are used in this study.

EXPERIMENTAL RESULTS

Inheritance pattern of BPH resistance gene in the F₂ progeny

A total of 170 F₂s were categorized as resistant and

susceptible. The plants showing a damage score of 3 and 5 were grouped as resistant (124) and plants showing a damage score of 7 and 9 were grouped as susceptible (46). This data fitting well to the expected 3:1 ratio ($\chi^2 = 0.38$, P 0.50 to 0.75) (Figures 1, 2 and Table 1).

Identification of RAPD marker linked to BPH resistance

Out of the total 73 RAPD primers initially screened on the parental lines of IR 50 and CO 46, 18 primers (24.6%) showed amplification in both parents. Among these 18 primers, 7 primers did not show any polymorphism and the remaining 11 primers showed reproducible polymorphism between parents. Among these 11 primers, 4 primers showed co-dominant banding pattern of polymorphism between parents. This could be useful for distinguishing heterozygotes from homozygotes. Three primers produced dominant amplicons specific to resistant parent and four primers shown susceptible parent specific amplicons (Table 2). Out of four co-dominant RAPD primers, one primer that is, OPC 11 shown distinct, repeatable and high degree of polymorphism in the resistant parent, resistant bulk, susceptible parent and susceptible bulks. OPC 11 generated polymorphic DNA fragments of OPC 11₈₁₇ (817 bp) and OPC 11₈₅₆ (856 bp). Out of these two markers identified, OPC 11₈₁₇ was associated in coupling

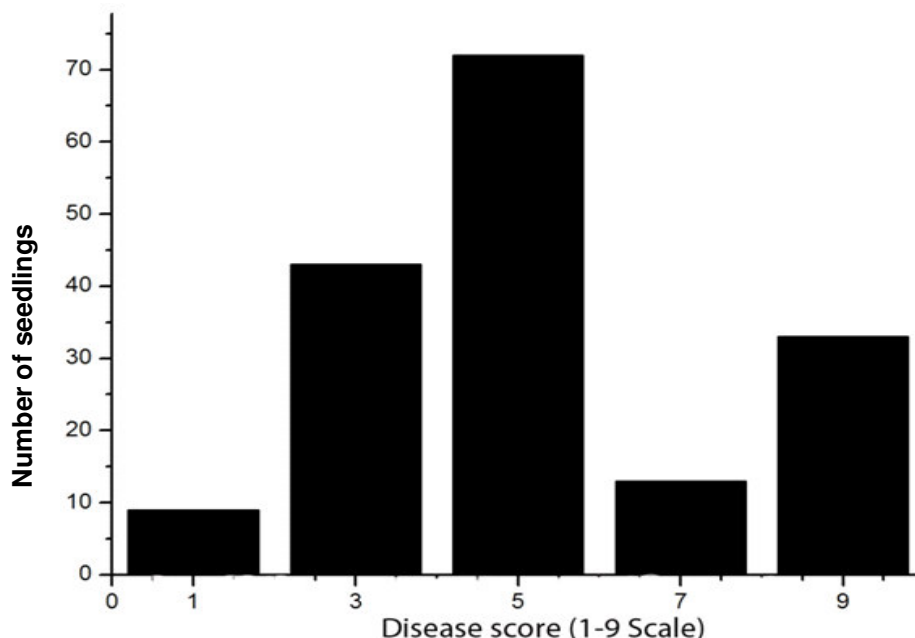


Figure 2. Hopper burn severity score of 170 F₂ seedlings of the cross IR 50 x CO 46 based on standard evaluation system.

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.

Table 2. Pattern of polymorphism between parents (IR 50 and CO 46) detected by RAPD analysis using Operon primers.

Polymorphic type	Primers (No)	Polymorphism (%)
Co-dominant	4	5.4
Dominant (Resistant specific band)	3	3.8
Recessive (Susceptible specific band)	4	5.19
Monomophic	7	9.09
No amplification	55	71.42
Total	73	100

phase to the resistant allele, while OPC 11₈₅₆ was linked in repulsion phase (Figure 3).

DISCUSSION

The performance of the parents IR 50, CO 46 and the F₂s

during screening reveals the consistency of the screening protocols for BPH resistance in this study. DNA amplified products obtained from PCR analysis using random primers have 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

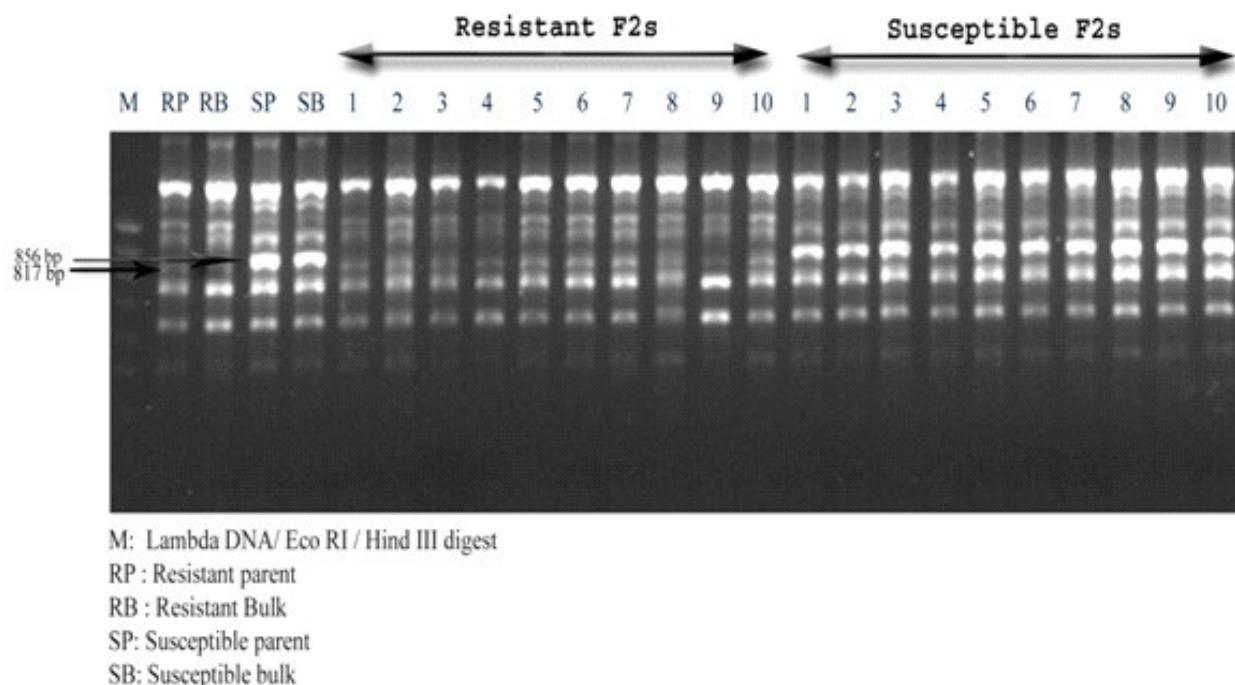


Figure 3. Co-segregation banding pattern of the RAPD primer OPC 11. OPC 11₈₁₇ was associated in coupling phase to the resistant allele, while OPC 11₈₅₆ was linked in repulsion phase.

primer were 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 bulk segregant analysis (BSA). The minimum size of the bulk is determined by the frequency with which linked loci might be detected as polymorphic between the bulked samples. For a dominant RAPD marker, the probability of a bulk of 'n' individuals having band and a second bulk of equal number of individuals not having a band will be $2(1-1/4)^n(1/4)^n$, when a locus is linked to the target gene (Michlemore et al., 1991). Of the four primers (OPC 10, OPC 11, OPE 14 and OPM 13) that were tested in the BSA with the resistant and susceptible bulks along with the resistant parent and susceptible parent and their F₂s, only one primer, that is, OPC 11 showed co dominant phenotype specific banding pattern. Two pools contrasting for a trait, that is, resistant and susceptible to BPH were analyzed to identify markers that distinguish them. Markers that are polymorphic between the bulks were genetically linked to the loci that determine the trait was used to construct the pools (Michlemore et al., 1991). Results obtained in F₂ seedlings indicate that RAPDs are co-dominant, highly polymorphic and informative in nature. These co-dominant RAPD markers are comparatively rare. Similar to other kinds of co-dominant markers, these co-dominant RAPDs 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 provides easy discrimination between recombinant homozygotes to recombinant heterozygotes (Williams et al., 1990; Mohan et al., 1997; Semagn et al., 2006). RAPD markers which show co-dominant nature were successfully employed in marker assisted selection (MAS) in various crops (Jena et al., 2002; Mondal et al., 2007).

Phenotypic evaluation should be performed with more reliable methods to avoid false positives in further MAS (Mackill and Ni, 2001). A clear polymorphism between the bulks comparable to that between the parents was observed. Poulson et al. (1995) suggested that when bulks are constructed from enough individuals, the BSA is sufficiently robust to cope with the low level of phenotypic misclassification. Bulk segregant analysis by using RAPD markers were successfully used in the development of linked molecular markers. Thus, OPA11₈₁₇ RAPD marker could be used in a cost effective way for marker assisted selection of BPH resistant rice genotypes

ACKNOWLEDGEMENTS

The authors sincerely acknowledge DBT and the Government of India, for providing financial assistance to the study. Also, they acknowledge Mr. K. Kalyana Babu, Scientist, VKPAS, Almora (UK), India and Mr. M.S.R Krishna for their critical suggestions on the preparation of the manuscript.

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