Tagging of Brown Planthopper Resistance Genes in F₂s of IR50 × Ptb33 of Rice by Using Bulked Segregant Analysis

Venkateswarlu YADAVALLI¹,², Gajendra P. NARWANE³, M. S. R. KRISHNA³, Nagarajan POTHI², Bharathi MUTHUSAMY⁴
(¹Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, Andhra Pradesh, India-500046; ²Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamilnadu, India-641003; ³Biotechnology Unit, Acharya N. G. Ranga Agricultural University, Rajendranagar, Hyderabad, Andhra Pradesh, India-500030; ⁴Department of Entomology, Tamil Nadu Agricultural University, Coimbatore, Tamilnadu, India-641003)

Abstract: Brown planthopper (Nilaparvata lugens Stål) is one of the most damaging pests causing hopper burn in rice, and thereby reducing the productivity and also the quality of the product. The effective management strategy to control this pest is the identification and transfer of desirable genes to local rice cultivars. The most important approach for developing resistant cultivars is the identification of markers, which can help in marker-assisted selection of more durable resistant genotype. The susceptible parent IR50 and the resistant parent Ptb33, and their F₂ populations were used in bulked segregant analysis for identification of resistant genes with random amplified polymorphic DNA marker (RAPD) primers. The primers OPC7 and OPAG14 showed both dominant and susceptible specific banding pattern so called co-dominant markers. Moreover, OPC7 and OPAG14 showed resistant specific bands and thus being in coupling phase, whereas OPC7 and OPAG14 showed susceptible specific genotypic bands in bulked segregant analysis. Therefore, the coupling phase markers, OPC7 and OPAG14, are considered to be more useful in marker-assisted selection of rice genotypes in crop improvement.

Key words: random amplified polymorphic DNA marker; brown planthopper; bulked segregant analysis; marker-assisted selection; Oryza sativa

Rice (Oryza sativa L.) is the staple food over half of the population in Asian countries., but the diseases and insect pests affected its production and productivity. Among the pests, brown planthopper (BPH, Nilaparvata lugens Stål) noted as the most destructive insect pest in rice growing areas. There are several reports about the outbreak of BPH, which are closely associated with the indiscriminate use of pesticides. This causes imbalance in the natural biological control and thereby favoring the development of BPH (Pathak and Khan, 1994). The damage caused by both nymphs and adults of BPH by inserting their sucking mouthparts into the plant tissues and sucking up the sap from phloem cells. This results in burning symptom, popularly known as ‘hopper burn’. BPH also transmits rice grassy stunt virus and ragged stunt virus by acting as a vector (Rivera et al, 1966; Heinrichs, 1979).

Recent biotechnological approaches involving transfer of resistance gene(s) from wild species into cultivated species found to be an important alternative strategy to develop resistance to BPH (Rahman et al, 2009). This led to the study of BPH resistance genes identification, for which sufficient number of mapping populations is essential. Many linkage maps have been developed based on F₂ progeny, F₃ progeny, recombinant inbred lines, double haploids, etc. F₂ segregating population is the result of selfed progeny of F₁. By using F₂ population, several linkage maps have also been constructed (Wu et al, 2002). BPH resistance genes from distantly relative species of rice have been introduced into the cultivated species of rice by mapping with random amplified polymorphic DNA (RAPD) and bulked segregant analysis (BSA) method (Jeon et al, 1999). Compared with other mapping methods, BSA provides a rapid procedure for identifying resistance genes or alleles in the genome, where two pools or bulks of their respective progeny contrasting for a trait can be analyzed to identify resistant markers that can distinguish resistant genotypes from susceptible ones (Michelmore et al, 1991). In rice, molecular markers such as restriction fragment length polymorphism (RFLP) and RAPD were used to develop complete genetic maps for various traits (Huang et al, 1997; McCouch et al, 1997; Jeon et al, 1999). Besides, RAPD markers are also useful in evaluation of hybrid vigor (Cho et al, 2004) and genetic relationships (Wakui et al, 2009). There are several reports available for tagging of agronomic traits in various crops by using RAPD markers (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). RFLP markers have certain limitations because they are restricted to some regions with low or single copy sequences even though they are co-dominant markers. Moreover, it requires large amount of DNA with high purity, specific probes and laborious time. Thus, RAPD provides simple approach for tagging resistance genes besides it saves time and cost. The principle of RPAD relies on the differential enzyme amplification of small DNA fragments using polymerase chain reaction (PCR) with arbitrary oligonucleotide primers, usually decamers. One of the most successful approaches of RAPD markers in case of rice for tagging BPH resistance genes is the identification of Bph1 gene
on rice chromosome using the bulked segregant analysis (Kim and Sohn, 2005). Similarly, one RAPD marker i.e., OPA111111 has been successfully tagged with RAPD marker in the F2 of IR50 × CO46 for brown planthopper resistance (Yadavalli et al, 2011). In the present study, RAPD marker as a tool for identification of coupling phase markers, which are linked to the BPH resistance allele in the F2 progeny of IR50 × Ptb33. Such coupling phase markers could be used for marker-assisted selection of BPH resistant genotypes in the plant breeding.

**MATERIALS AND METHODS**

**Phenotypic screening for brown planthopper resistance**

Standard seed box screening method (Heinrichs and Mochida, 1984) was employed for screening BPH resistance in the greenhouse, at the Paddy Breeding Station, Coimbatore, India. All F2 seeds of the cross IR50 × Ptb33 were sown in rows along with their susceptible parent, IR50, and resistant parent, Ptb33. At the 3-leaf stage of rice seedlings (approximately 7 d after sowing), the 2nd–3rd instar nymphs of BPH were infested at the rate of 3-leaf stage of rice seedlings (approximately 7 d after sowing), amplification cycle has one denaturation at 94 °C for 1 min, 30 amplification cycles were programmed, in which each first step is initial denaturation at 95 °C for 3 min. In second step program was set up for amplification of DNA fragments. The polymerase (Invitrogen) and 1×PCR buffer. The following 100 pmol of primers, 0.5 mmol/L each dNTP, 0.5 U of Taq polymerase (Invitrogen) and 1×PCR buffer. The following primer were used in each PCR reaction. The final step is the extension of any unamplified products of earlier cycles which was set at 72 °C for 3 min. All the above PCR were carried out in a thermal cycler (Eppendorf, USA). The products of PCR were resolved on 1.2% agarose gels containing 0.2 μg/mL ethidium bromide in a standard horizontal gel electrophoresis unit (Broviga, Chennai, India) with tris borate EDTA (TBE) buffer (90 mmol/L Tris-borate, 1 mmol/L EDTA, pH 8.0). After running, DNA bands were visualized in a gel documentation system (AlphaImager) by exposing to the UV light. Initially, all 73 decamer primers were used for screening IR50 and Ptb33. The primers which shown more polymorphism between the parents than in resistant bulk (pooled DNA of ten resistant F2 seedlings) and susceptible bulk (pooled DNA of ten susceptible F2 seedlings) were selected along with IR50 and Ptb33. Finally, those primers, which shown consistent polymorphism in IR50, Ptb33, resistant bulk and susceptible bulk, were advanced to test all selected ten resistant F2 seedlings and ten susceptible F2 seedlings according to the method described by Michelmore et al (1991).

**RESULTS**

**Segregation of BPH resistance genes in F2 population**

All the F2 plants were tested for BPH resistance based on the standard evaluation system of rice from brown planthopper damage (International Rice Research Institute, 1996). Among the 170 F2 lines scored for BPH damage, 130 were resistant to BPH, which showed a damage score of 1, 3 or 5, and 40 susceptible to BPH, which showed a damage score of 7 or 9. These data were tested statistically with $\chi^2$. The expected ratio 3:1 fitted well to the observed ratio of the resistant seedlings to the susceptible seedlings ($\chi^2 = 0.19, P = 0.97–0.99$) (Fig. 1).

**Bulked segregant analysis**

RAPD primers were screened for polymorphism between IR50 and Ptb33 with five series of operon primers and results are shown in Table 1. Among these highest polymorphism, 23.91% was found with OPAG series primers and the least level of parental polymorphism was shown by the OPE series, 15.00%. Furthermore, all these primers were screened for repeatable and the highest polymorphic nature in parents as well as in bulks. All these primers were tested for polymorphism between susceptible parent (IR50), resistant parent (Ptb33), susceptible bulk, resistant bulk, ten susceptible F2 seedlings and ten resistant F2 seedlings.

![Fig. 1. Frequency distribution of score ratings infested with N. lugens on 170 F2 seedlings of IR50 × Ptb33 based on the standard evaluation system.](image-url)
Of which, only two RAPD primers, OPC7 (5′-GTCCCGACG A-3′) and OPAG14 (5′-GGGAACGTGT-3′), showed co-dominant banding pattern with distinct, repeatable and high degree of polymorphism (Fig. 2 and Fig. 3). Fig. 2 showed the polymorphic nature among all resistant verse all susceptible with co-dominant banding pattern, i.e., 697 bp of OPC7 (OPC7<sub>697</sub>) was found in the resistant parent, resistant bulk and all ten resistant F<sub>2</sub> seedlings tested, similarly, 846 bp of OPC7 (OPC7<sub>846</sub>) was found in the susceptible parent, susceptible bulk and all ten susceptible F<sub>2</sub> seedlings tested. Thus, OPC7<sub>697</sub> can be associated with dominant type of genotypic marker and is said to be in coupling phase, whereas other allele OPC7<sub>846</sub> was associated with susceptible nature and is said to be in repulsion phase. We can easily identify the coupling phase markers in segregating population, thus coupling phase markers could be most effectively used in plant breeding program. Fig. 3 showed the co-dominant banding pattern, in which 680 bp of OPAG14 (OPAG14<sub>680</sub>) found to be associated with coupling phase to the resistant allele, whereas 650 bp of OPAG14 (OPAG14<sub>650</sub>) found to be in repulsion phase. In this case, OPAG14<sub>680</sub> could be used in marker-assisted selection in plant breeding program.

**DISCUSSION**

The results of our study showed that the performance of F<sub>2</sub> is of expected population. Thus, the screening of the parents, bulks and F<sub>2</sub> of all susceptible and resistant types revealed the consistency of the screening protocol of the standard evaluation system for brown planthopper (International Rice Research Institute, 1996). Marker-assisted selection is one of the important criteria for identifying resistance genes in plant breeding program. For genotypic characterization and mapping, amplification of DNA products with RAPD primers has been proposed as an alternative method in targeting DNA sequences

<table>
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<tr>
<th>Sl. No.</th>
<th>Polymorphic primer</th>
<th>IR50</th>
<th>Ptb33</th>
<th>Total</th>
<th>Monomorphic band</th>
<th>Polymorphic band</th>
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<td>56</td>
<td>89</td>
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<td>76.08</td>
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<td>426</td>
<td>442</td>
<td>868</td>
<td>704</td>
<td>153</td>
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**Table 1. Pattern of polymorphism between parents (IR50 and Ptb33) detected by RAPD analysis using operon primers.**

![Fig. 2. Co-segregation banding pattern of the RAPD primer OPC7 DNA fragment: OPC7<sub>697</sub> (697 bp) was associated in coupling phase to the resistant allele, whereas OPC7<sub>846</sub> (846 bp) was in repulsion phase.](image)

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<sub>680</sub> (680 bp) was associated in coupling phase to the resistant allele, whereas OPAG14<sub>650</sub> (650 bp) was in repulsion phase.](image)

M, Marker; RP, Resistant parent; RB, Resistant bulk; SP, Susceptible parent; SB, Susceptible bulk.
(Williams et al, 1990). The data from Table 1 showed high number of amplified products per primer. Such high number of amplified loci is essential for tagging of resistance genes (Welsh and McClelland, 1990). The next step is the screening of the entire population either by probes or with primers. This is the most time consuming step for DNA marker development. The alternative to this step is the bulked segregant analysis, proposed by Michelmore et al (1991). In the bulked segregant analysis, the minimum size of either resistant or susceptible bulk depends on the frequency of linked loci that is detected as polymorphic DNA fragments between the bulked samples. Fig. 2 and Fig. 3 showed the polymorphic DNA fragments of OPC7 and OPAG14, respectively. Of which, OPC7697 (697 bp) and OPAG14680 (680 bp) were associated in coupling phase to the resistant allele in resistant parent, resistant bulk and all ten resistant F2 seedlings, whereas OPC7586 (846 bp) and OPAG14650 (650 bp) were linked in repulsion phase. Thus, OPC7 and OPAG14 are considered to be co-dominant primers, and such type of co-dominant markers would provide more information than either dominant or recessive type of markers in marker-assisted selection. Here, the two pools or bulks contrasting for BPH resistance were distinguished by two co-dominant markers. For linkage analysis, co-dominant markers provide the maximum linkage information per individual in the mapping population under the study. Thereby, co-dominant markers can easily distinguish between recombinant homozygotes and recombinant heterozygotes (Williams et al, 1990; Mohan et al, 1997; Semagn et al, 2006). Several such co-dominant RAPD markers have been successfully developed for marker-assisted selection in several crops (Mondal et al, 2007). We have shown a clear cut polymorphism between susceptible individuals and resistant ones in respect of BPH resistance in the bulked segregant analysis. Thus, bulked segregant analysis with RAPD markers provides the marker-assisted selection in the F2 population of IR50 and Ptb33. Poulson et al (1995) showed that the bulked segregant analysis is the straight forward method to population of IR50 and Ptb33. Poulson et al (1995) showed that a clear cut polymorphism between susceptible individuals and markers have been successfully developed for marker-assisted and recombinant heterozygotes (Williams et al, 1990; Mohan et al, 1997). Such high number of amplified products per primer. Such high number of amplified loci is essential for tagging of resistance genes (Welsh and McClelland, 1990). The next step is the screening of the entire population either by probes or with primers. This is the most time consuming step for DNA marker development. The alternative to this step is the bulked segregant analysis, proposed by Michelmore et al (1991). In the bulked segregant analysis, the minimum size of either resistant or susceptible bulk depends on the frequency of linked loci that is detected as polymorphic DNA fragments between the bulked samples. Fig. 2 and Fig. 3 showed the polymorphic DNA fragments of OPC7 and OPAG14, respectively. Of which, OPC7697 (697 bp) and OPAG14680 (680 bp) were associated in coupling phase to the resistant allele in resistant parent, resistant bulk and all ten resistant F2 seedlings, whereas OPC7586 (846 bp) and OPAG14650 (650 bp) were linked in repulsion phase. Thus, OPC7 and OPAG14 are considered to be co-dominant primers, and such type of co-dominant markers would provide more information than either dominant or recessive type of markers in marker-assisted selection. Here, the two pools or bulks contrasting for BPH resistance were distinguished by two co-dominant markers. For linkage analysis, co-dominant markers provide the maximum linkage information per individual in the mapping population under the study. Thereby, co-dominant markers can easily distinguish between recombinant homozygotes and recombinant heterozygotes (Williams et al, 1990; Mohan et al, 1997; Semagn et al, 2006). Several such co-dominant RAPD markers have been successfully developed for marker-assisted selection in several crops (Mondal et al, 2007). We have shown a clear cut polymorphism between susceptible individuals and resistant ones in respect of BPH resistance in the bulked segregant analysis. Thus, bulked segregant analysis with RAPD markers provides the marker-assisted selection in the F2 population of IR50 and Ptb33. Poulson et al (1995) showed that the bulked segregant analysis is the straight forward method to cope with the low levels of phenotypic misclassification, especially with bulks constructed with sufficient number of individuals.

In summary, the co-dominant RAPD markers OPC7697 and OPAG14680, which are associated with coupling phase to the resistant allele of brown planthopper resistance, could be used in marker-assisted selection in plant breeding program as well as in identifying genes at any stage of the crop growth period to save time and cost of traditional breeding programs.

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REFERENCES


