RAPD markers linked to brown planthopper *Nilaparvatha lugens* resistance locus in rice

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Brown planthopper, a major pest in rice causes “hopper burn” in the field. The resistance gene for brown planthopper was mapped by using 20 recombinant inbred lines (RIL’s) derived from a cross between resistant line *Oryza officinalis* derivative (IR 54742-2-12-17-6) and a susceptible rice cultivar ASD 16 using bulked segregant analysis. On an average of 4 loci were amplified and two RAPD primers amplified loci that co-segregated with resistance/susceptibility. The segregating RAPD loci were mapped using Mapmaker programme into 13 groups. The expected and the 95% confidence level were found to be 15.2 and 47.7 eM respectively, confirming the location of the brown planthopper resistant gene on the region of chromosome 4. These RAPD markers will accelerate breeding programme for brown planthopper resistance.

Brown planthopper, *Nilaparvatha lugens* is a serious pest in rice crop and is a problem in many rice growing areas of Asian countries. Plant hoppers damage the rice crop by sucking plant sap resulting in “hopper burn” in the field. A total of nine biotypes have been reported in brown planthopper by Khush et al. The genes resistant to Bph-3 and Bph-4 biotype and Bph-1 and Bph-2 biotype were located on chromosome 10 and 4 respectively. Wild species of rice are rich source of resistant genes for brown planthopper. Introggression of the resistant genes from the wild species to the cultivated varieties has become priority in breeding programme and has resulted in the identification of resistant genes.

The pace of DNA-based mapping and DNA-based selection have been accelerated in the last few years by the advent of polymerase chain reaction (PCR) with a thermostable DNA polymerase and related approaches such as randomly amplified polymorphic DNA’s (RAPD’s). Michelmore et al. described an application of the RAPD technology termed bulked segregant analysis (BSA) to identify molecular markers linked to a trait of interest. The insect and pest resistant traits are generally governed by single/double dominant gene/s and by use of molecular markers the characters can be linked to the rice genome. RAPD markers were used for tagging genes of agronomic importance in rice. Against bacterial blight, gall midge, blast. In the present study recombinant inbred lines have been used to identify RAPD markers linked to brown planthopper resistance genes in rice.

Recombinant inbred lines (F2) were derived from a cross between brown planthopper resistant introgressed line of *Oryza officinalis* (IR 54742-2-12-17-6) and the susceptible cultivar (ASD 16). F2 populations (250) were screened for brown planthopper and the scoring indicated that the resistance was controlled by two dominant complementary genes showing 9:7 segregation ratio (X^2 = 0.005). The brown planthopper insects were reared with Taichung-Native (TN1) as the susceptible host following the method of International Rice Research Institute. The F2’s were forwarded till F6.

Brown planthopper resistance was measured under glasshouse condition in arrays of 20 seedlings each of recombinant inbred lines’s (F6) along with the parents interspersed with the susceptible check TN-1 and resistant check PTB 33 (5). The seedlings were scored consistently for resistant and susceptible.

**DNA Extraction** — Rice seeds were germinated in dark for two weeks. The shoots were harvested for DNA extraction. The quantity of the DNA was measured at 260nm in the Beckman Spectrophotometer DU-64. The quality of the DNA was checked by Agarose Gel Electrophoresis using TBE (Tris-Boric acid EDTA) Buffer.

**Bulk segregant analysis** — The DNA was pooled from 10 resistant recombinant inbred lines and 10
susceptible lines. The two bulks were used as target DNA for RAPD analysis.

**PCR analysis**—PCR amplification reaction contains 50 ng template DNA, 1 unit Taq DNA polymerase, 1X PCR buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris HCl, pH 9) 100 mM each of dNTPs and 200 mM primer. The random primer was obtained from Operon technologies, Alameda, California. The Taq polymerase and dNTPs were obtained from Bangalore Geneti Private Ltd. Bangalore. In order to prevent the evaporation the samples were overlaid with mineral oil. The PCR reaction samples were subjected to 45 cycles in a MJ Research Thermal Cycler under following conditions: 4 min at 94°C before the cycle starts, 1 min at 92°C, 3 min at 35°C, 2 min at 72°C and at end of the reaction 8 min at 72°C.

Data analysis—Linkage analysis was conducted as per Lander et al. using the software Mapmaker/Exp 3.1b. Markers were ordered with a minimum of the odds ratio (LOD) score (3.0) and maximum distance (50.0). Recombination fractions were converted into centiMorgans by applying the Haldane function.

The expected minimum distance was calculated using the formula \( c/2 (n \times +1) \), where \( c \) = genome size in cM (1491 for rice) \( n \) = number of primers (12), \( x \) = average number of PCR products per primer (4). The distance at a 95% confidence level = \( (c/2)(1-0.05) \) as described by Martin et al.

DNA from resistant and susceptible recombinant inbred lines (10 each) and the parents were used as the template for the amplification of RAPD markers with a total of 37 random decamer oligonucleotide primers. Twelve primers amplified the template DNA and generated on an average 4 fragments per primer. Two primers, OPF 10 and OPC 19 generated amplification products that were present in resistant bulk and absent in the susceptible bulk. The polymorphism was confirmed by repeated amplification by comparing with the parents and individual resistant and susceptible lines.

Primers OPF 10 (Fig 1) and OPC 19 (Fig 2) each generated RAPD products, OPF 10 (1200 bp) and OPC 19 (1500 bp), OPC 19 (1300 bp) respectively, that was polymorphic and present in the resistant pool and resistant parent and not in the susceptible parent or susceptible lines.

The expected distance from the target gene is 15.2 cM and the upper 95% confident limit is 47.7 cM for brown planthopper resistant gene amplified by 12 primers proving the target locus on chromosome 4, calculated using the formula described by Martin et al.

The RAPD products exhibited 79 segregating loci.

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Fig. 1—RAPD products resolved on 1.5% agarose gel electrophoresis, a. For primer OPF 10 [Lane 1: Lambda marker (Eco RI and Hind III digest); 2: Susceptible parent; 3: Resistant parent; 4: Resistant bulk; 5: Susceptible bulk].

Fig. 2—RAPD products resolved on 1.5% agarose gel electrophoresis, b. For primer OPC 19 [Lane 1: Lambda marker (Eco RI and Hind III digest); 2 and 3: No DNA; 4: Resistant parent; 5: Susceptible parent; 6: Susceptible bulk; 7: Resistant bulk].
Fig 3—Linkage groups for RAPD markers [Map Scale is 5.0 cM/cm. Kosambi Mapping Function. Log-likelihood: -136.20]

*1cm=8.71cM
and they were analysed using the Map Maker programme. The linkage groups with RAPD markers (Fig 3) were numbered after the primer kit and designated as F 174 and so on. RAPD markers were linked into thirteen linkage groups out of which in linkage group I and IV many were clustered together and located apart from each other.

The potential benefits of a marker-assisted selection strategy have been discussed widely by Paterson et al. In this study, PCR-based marker has been linked to the brown planthopper resistant gene in rice which is as reliable as an RFLP marker. Bulk segregant analysis assumes that markers adjacent to the target gene will be in linkage equilibrium (i.e. recombination will not randomise the markers with respect to the gene) and as the linkage distance increases, more recombinants will be present in the bulk, culminating in 50% recombinants, no linkage disequilibrium and therefore no difference between the bulk. By following this in the present study we have identified RAPD markers linked to the bph resistant gene. In this study, through bulk segregant analysis we could detect RAPD markers OPP 10 1200 which is present in the resistant parent and in the resistant bulk. Primer OPC 19 detected two RAPD products OPC 19 1500 and OPC 19 1300 in the resistant bulk.

Recombinant inbred lines are a source of near isogenic lines (NILs) and in the present study the resistant and susceptible lines were pooled, since after six generations of selfing (F6 progeny) the genome of the selected individual consists of almost exclusively of that line into which the gene is being introgressed as the individual lines also produced polymorphism for the same primer as the bulk.

To screen markers linked to single traits with in bulks, RAPD markers provide useful alternative to RFLP analysis. Although the RAPD markers identified in the present study are sensitive to minor changes of conditions, the sequence of the RAPD products could be determined and a specific pair of this region could be generated to tag specific regions reliably as reported by Paran and Michelmore. RAPD amplification can likely be initiated from genomic sites that perfectly match primer sequence. An unpredicted decrease in the number of amplification products has been observed due to decrease in the primer length as discussed by Caeteno-Anolles et al. This study reveals that the GC rich primers bring about amplification. In the present study out of 37 primers only 12 primers (60-70% GC content) amplified the template DNA. The primers that had a GC content of 40-60% failed to amplify.

As discussed by Martin et al. the expected distance and 95% confidence level were calculated in the present study for the n primers (n=12), RAPD markers were linked into 15 linkage groups out of which in linkage group I and IV many were clustered together and located apart from each other showing the amplification may be from the same region of the chromosome and from different region proving the existence of polymorphism.

The RAPD markers identified in this study are the part of the resistant gene and will be helpfull for the breeders to screen the breeding material in the F2 generation itself to select the homozyogous resistant line and to develop into a cultivar instead of wasting time, land and labour until F7/F8 generation and even then instability occurs due to segregation.

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