

GENETIC AND PHYSICAL MAPS OF GENE *Bph-10* CONTROLLING BROWN PLANT HOPPER RESISTANCE IN RICE (*Oryza sativa* L.)

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ABSTRACT

Fine mapping and physical map were conducted initial steps in an effort to isolate gene Bph-10 which control the resistance to brown plant hopper (biotype 2+3). Advanced mapping population derived from a cross of IR31917-45-3-2 / IR54742 in which recurrent line is female parent, and IR54742 is a derivative line from Oryza officinalis, as a donor. Through bulked segregant and linkage analyses, Bph-10 was detected within 4.6 cM region containing 11 microsatellites and RFLPs in chromosome 12. One RFLP was converted into a PCR-based sequence tagged site (STS) marker which co-segregated with Bph-10.

Key Words: BPH, Gene cloning, Microsatellite, Physical mapping, PCR, RFLP, STS.

INTRODUCTION

The brown planthopper (BPH), *Nilaparvata lugens* Stal has been one of the most devastating pests to rice crop in Vietnam and Asia, and yield loss can be up to 60% from its hopper burn (Khush 1979, Panda and Khush 1995).

Twelve major BPH resistance genes have been identified in indica rice cultivars and two wild relatives, *Oryza australiensis* and *Oryza officinalis*. Breeding resistant cultivars has proven to be one of the most efficient ways to control BPH (Pathak 1969, Pathak and Saxena 1980). Two of these BPH resistance genes, *Bph-1* and *Bph-10(t)* were found to be closely linked with two RFLP markers, C185 and RG457 on rice chromosome 12 (Jena et al. 1992, Ishii et al. 1994, Hirabayashi and Ogawa 1996). Recent advances in DNA marker technology have greatly facilitated studies to understand the genetic basis of complex phenotypes.

Positional cloning provides a promising method for isolating a gene based only on its phenotype and genomic location. To achieve map-based cloning, construction of a linkage marker with DNA markers is required. A high resolution map for a specific genomic region is commonly obtained by resolving the order of a number of markers tightly flanking the target gene using a relatively large mapping population. The physical distance

between the available markers could be covered using a large insert genomic library (BAC, PCA or YAC). The construction of genetic map around the BPH resistance gene using the map-based cloning approach was reported in this paper as a first result of screening rice BAC library with RG 457 marker. Gene *Bph-10* is linked with RG457 on chromosome 12 at distance of 3.68+1.29 cM (Ishii et al. 1994). In this study, DNA sequencing, primer designing and synthesizing were prepared. The PCR-based polymorphism was generated to distinguish resistant from susceptible genotypes. Mapping of *Bph-10(t)* for BPH resistance: A gene conferring resistance to three BPH biotypes from Philippine was introgressed from *O. australiensis* into rice cultivar. The recurrent parent (IR31917-45-3-2) is susceptible to all three biotypes whereas *O. australiensis* is resistant. The introgression line (IR65482-4-136-2-2) derived from this cross was also resistant to three biotypes of BPH. The F₁ of IR65482-4-136-2-2 and IR31917-45-3-2 was resistant to BPH biotype 1, and the segregation data showed that a single dominant gene confers BPH resistance (Lang et al. 1999).

MATERIALS & METHODS

An elite breeding line of rice IR31917-45-3-2 was used as a recurrent parent, then IR54742, derivative line from *Oryza officinalis* was used as a donor. The F₂ and F₃ populations

were produced from the cross IR31917-45-3-2 / IR54742.

DNA extraction for PCR analysis

DNAs for PCR analysis were prepared using a simplified miniscale procedure (Lang 2002). 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 μ l 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 μ l of the extraction buffer was added and mixed into the well by pipetting. Around 400 μ l of the lysate was transferred to the original tube of the leaf sample. The lysate was deproteinized using 400 μ l 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 μ l of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). An aliquot of 1 μ l is sufficient for PCR analysis. The remaining DNA was stored in -20°C for any later use.

PCR amplification

The PCR reaction mixture contained 20-50 nanogram (ng) template DNA, 50ng of each primers, 0.05 mM dNTP's, 1xPCR buffer (10mM Tris pH 8.4, 50mM KCl, 1.8mM MgCl_2 and 0.01 mg/ml gelatin) and 1 unit of *Taq* DNA polymerase in a total volume of 20 μ l. Template DNA was initially denatured at 94°C for 5 minutes followed by 30 cycles of PCR amplification using the following parameters: 30 sec denaturation of 94°C , 30 sec primer annealing at 55°C or 60°C and 1 min primer extension at 72°C . Completion of primer extension was allowed by a final 5 min incubation at 72°C .

An aliquot of 10 μ l 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 μ l of PCR products was used for restriction digestion to detect

PBR polymorphism in the case of other primers. The digestion reaction normally contained 3.2 μ l sterile distilled water, 1.5 μ l restriction buffer (10X), 0.3 μ l restriction enzyme (10 U/ μ l) and 10.0 μ l of PCR products in a total volume of 15 μ l. 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 1 X TAE buffer.

Development of additional STS markers

The available PCR marker for *Bph-10* 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 by *HinfI* and *AluI*

Physical mapping

A BAC library from IR64 nuclear DNAs was constructed at IRRI. A total of 18,432 clones corresponding to 3.28 rice genome equivalents were qualified and grown on 48 microtiter plates. Each plate is in an array of 384 wells with 16 rows and 24 columns. The insert size ranged from 37 to 364 kb with an average of 107kb.

Individual BAC clones were grown in 48 microtiter plates with 16 rows and 24 columns on each plate. Two levels of BAC DNA pools were prepared. The first level is on the entire library and the second level is on the individual plate. For the first level, three DNA pools were prepared, including 48 plate pools (384 clones each), 16 row pools (1152 clones each, 24x48), and 24 column pools (768 clones each, 16x48). Before DNA isolation, the BAC clones were grown to saturation before pooling. Thus there are a total of 88 first level DNA pools. For the second level, the bacterial cells from each row of a microtiter plate (24 clones) or each column of one plate (16 clones) were separately mixed together for DNA isolation. For each plate, there are 40 second level DNA pools (16 row pool and 24 column pool). For the entire BAC

library of 48 microtiter plates, a total of 1920 second level pools were prepared.

Isolation of BAC DNAs

The putative clones were picked from the library and inoculated to 2 ml LB containing 12.5% µg/ml chloramphenicol and incubated at 37°C overnight.

Southern hybridization of the overlapping clones

Individual BAC DNA was digested by *HindIII* which cut on the site of exogenous fragment and vector DNA. The generated products were separated on 0.7% agarose gel and transferred to Hybond-N⁺ (Amersham) nylon membrane by each overlapping group. The clone which produced more digestion fragments was further selected for the probe, labeling with (³²P-dCTP). Hybridization was done following the protocol of clone hybridization. After autoradiography, pairwise comparison of the banding patterns of putative overlapping clones was done.

RESULTS

BPH bioassay

The BPH colony used in the following bioassay was mixture of three independent colonies. Genotypes of BPH response (RR: homozygous resistant, RS: segregating heterozygous, SS: homozygous susceptible) of 227 F₂ individuals were determined by assaying the phenotypes (either R or S) of the corresponding F₃ progenies.

The reaction to BPH biotypes 1, 2, 3 in the recurrent parent and introgression line is shown in table 1

Table 1: Segregating of BPH resistance in the mapping population derived from the cross IR31917-45-3-2 / IR54742

F ₂ genotype	Number of F ₂ individual
RR	50
RS	117
SS	60
Total	227

Detection of STS marker

The nucleotide sequence of RG457 was determined on the basis of the orientation to ensure accuracy (Lang et al. 1999). We are performing STS analysis using the cross of IR31917-45-3-2 / IR54742. It is obviously in figure 1 that PCR can detect size of 900 bp and polymorphism at certain loci. For example, amplification of DNA from IR 54742 and IR31917-45-3-2, with the primer RG 457FL/RB gave rise to 850bp product for IR 54742 and 800bp for IR31917-45-3-2, then in case of primer RG 457FL/RL, amplification gave rise to 800 bp product for IR 54742 and 650 bp for IR31917-45-3-2.

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

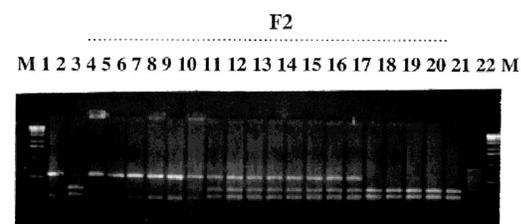


Figure 1: The Polymorphic pattern detected in rice DNA from F₂ and parents IR31917-45-3-2 / IR54742 using STS marker RG457-L-L and digested with *HinfI*

ANALYSIS OF THE F₂ POPULATION

DNA genome from the F₂ population of IR31917 / IR54742 was assessed through PCR amplification using primer RG457 FL/RL. The PCR products were spliced out by double enzymes digestion with *HinfI* and *AluI*. Fragments were resolved on agarose gels, then 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 bp fragment, homozygote for IR31917 type marker bp fragment, and heterozygotes [displaying both fragments IR54742 and IR31917]. Similarly, when combinations of primer pair RG457L-B 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 bp fragment, homozygote for IR31917 type marker bp fragment, and heterozygotes [displaying both fragments IR54742 and IR31917].

Recombination values were estimated using the maximum likelihood equation and the recombination values were converted into genetic map distances (cM) using the Kosambi function.

Bulked segregant and linkage analyses

Twelve primer pairs were efficiently examined in the bulked segregant analysis. RG 457B-L detected the target gene in F₂ population of IR31917 / IR54742 (figure 2)

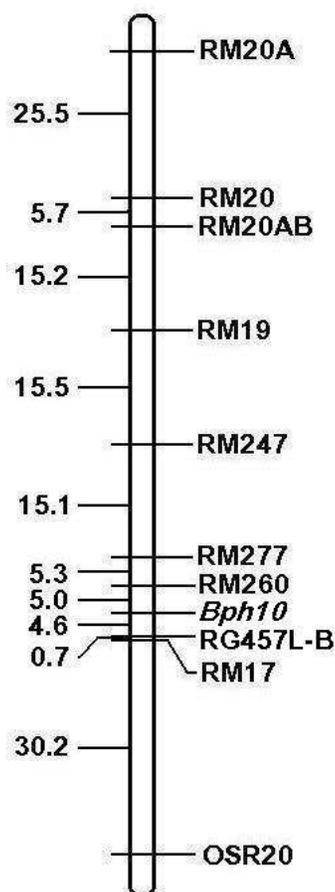


Figure 2: The molecular map of the *Bph10* region on rice chromosome 12. Map was derived from plant of the cross between IR31917-45-3-2 / IR54742

Fine mapping of the *Bph* region

The parental lines and the F₂ individuals of the mapping population clearly exhibited distinct reactions to BPH.

Figure 2 shows the genetic map containing *Bph-10* and all the selected DNA markers constructed in mapping population. The STS marker RG 457 was mapped to *Bph-10* at a distance of 4.6cM in chromosome 12

Screening of the rice BAC library with RG457

Screening of the BAC library with RG 457 marker led to the identification of two positive clones. All two BAC inserts have a *HindIII* restriction site. This indicates that the maximum distance between RG457 and *HindIII* restriction site is 100kb, which is the size of the smaller BAC insert (figure 3)

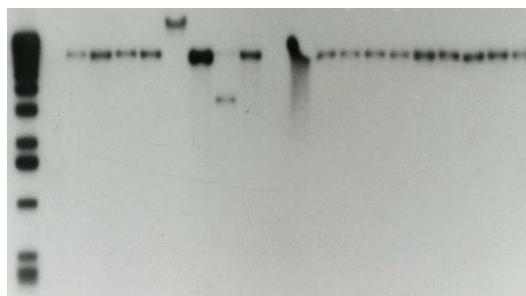


Figure 3: Integration of BAC clones into contig using *HindIII* digested

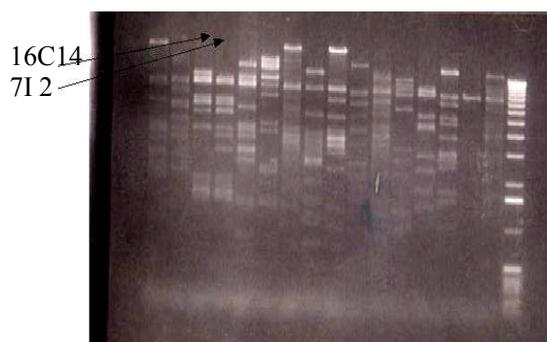


Figure 4: Identification of overlapping BAC clones with BAC-Clone DNA samples were digested with *HindIII*

Identification and analyses of BAC clones nearly located to *Bph-10*

RG457 was used to screen the IR64 BAC library in order to identify overlapping clone nearly located to *Bph-10*. RG457 detected two clones namely 16-C₁₄ and 07-I₂ (figure 4).

The orientation of the clones in individual contigs was determined based on the hybridization pattern of BAC end probes. The resulting alignment of overlapping hybridization patterns using the digested clones as probes (figure4)

Physical mapping of *Bph-10* region

The physical relationship of BAC clones with the RFLP markers was confirmed by Southern hybridization of the RFLP markers to the clones of the contig marker RG457

Marker RG457 was contained in a 1.5kb band to two clones : 16-C₁₄, 07-I₂

Figure 5 shows the genetic map aligned with the physical map of the BAC contig encompassing the *Bph* locus.

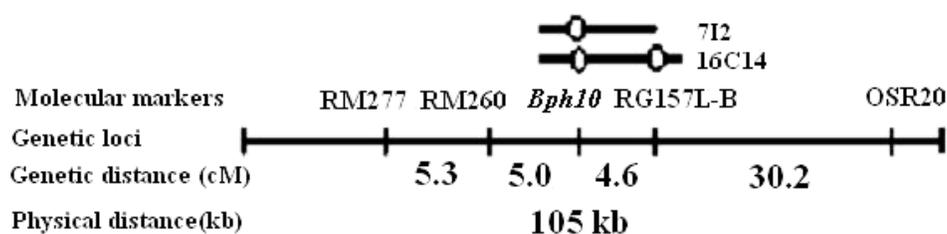


Figure 5 : Alignment of the genetic and physical maps of *Bph-10* region.

DISCUSSION

Positional cloning provides a promising method for isolating a gene based only on its phenotype and genomic location. Fine genetic mapping and physical mapping were

conducted initial steps in effort to isolate the gene. The RG457 contig consisting of two overlapping clones 16-C₁₄ and 07-I₂. Genetic mapping indicated that the *Bph-10* locus was contained in the RG457 contig.

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SUMMARY IN VIETNAMESE

Bản đồ di truyền và bản đồ vật lý gen kháng rầy nâu *Bph-10*.

Rầy nâu là đối tượng gây hại rất nghiêm trọng ở Việt Nam, quần thể rầy nâu ở Đồng bằng sông Cửu Long (ĐBSCL) thay đổi loại hình rất phức tạp. Gen kháng rầy nâu *Bph-10* có nguồn gốc từ lúa hoang có khả năng kháng rầy nâu biotype 2 và 3 đã được khai thác thành công ở ĐBSCL. Kỹ thuật cloning được thực hiện thông qua vectơ BAC, khai thác nguồn dữ liệu của thư viện genome của quần thể DH thuộc tổ hợp lai IR64 x Azucena. Kết quả RG457 phân lập được hai clone 07-I₂ và 16-C₁₄. Bên cạnh đó việc thực hiện bản đồ chi tiết phục vụ chọn giống (fine map) được tiến hành trên quần thể RIL thuộc tổ hợp lai IR31917-45-3-2 x IR54742. Hai cặp primer được thiết kế từ RG457, và những microsatellite marker sau đây đã được khuyến cáo sử dụng trong qui trình MAS: RM227, RM260