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Molecular Mapping of Resistant Genes to Brown Planthopper, *Bph1 and bph2*, in Rice

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ABSTRACT

This study was carried out to map *Bph1* and *bph2* gene in Mudgo and Sangju13 (*Oryza sativa* L.) respectively conferring resistance to brown planthopper (BPH) and to establish the marker-assisted selection (MAS) system. Bulked seedling (grown for 20 days) test was conducted with the 73 F4 lines derived from a cross between Nagdongbyeo and Mudgo for *Bph1* and with 53 BC3F5 lines derived from the Milyang95/Sangju13 cross for *bph2*. *Bph1* was mapped between RG413 and RG901 on chromosome 12 at a distance of 7.5 cM from RG413 and 8.4 cM from RG901. A recessive gene *bph2* was located near RZ76 on chromosome 12 at a distance of 14.4 cM. *Bph1* and *bph2* were linked to each other with a distance of about 30 cM. An RFLP marker, RG413 linked to *Bph1*, was converted to an STS marker to facilitate the marker-assisted selection. BPH resistant genotypes could be selected with 92% accuracy in a population derived from a line of NM47-B-B.

Keywords : rice, *Oryza sativa* L., brown planthopper, *Bph1*, *bph2*, RFLP, STS marker, marker-assisted selection.

Brown planthopper (BPH) is one of the most devastating insects in rice. Its high infestation gives rice plants to die off, called 'hopper burn'. The control against BPH almost depends on agricultural chemicals. The abuse of the chemicals brings the imbalance of the whole ecosystem by decreasing natural enemies or predators and deteriorates human health. Breeding of resistance varieties may be safer, more economical and more ecologically sound than any other ways (Heu, 1983). Many resistant varieties such as Milyang30, Hangangchalbyeo, Cheongcheongbyeo, Samgangbyeo, Gayabyeo, Hwacheongbyeo, Jangseongbyeo and Chilseongbyeo were developed through crossing with resistant varieties in Korea for the past two decades.

So far, 10 resistance genes to BPH have been discovered in many Indica varieties; *Bph1*, *bph2* in Mudgo and ASD7 (Athwal et al., 1971), *Bph3*, *bph4* in Rathu Heenati and Babawee (Lakshminarayana et al., 1977), *bph5*, *Bph6*, *bph7* in ARC10550, Swarnalata and ARC15831(b) (Khush et al., 1985), *bph8*, *Bph9* in Col. 5 Thailand and Balamawee (Nemoto et al., 1989), *Bph10*(t) in *O. australiensis* (Ishii et al., 1994). *Bph1* is known to be either allelic or closely linked with *bph2* (Athwal et al., 1971) through allelism tests. Later, Ikeda & Kaneda (1983), however, reported different results from those. *Bph1* is known to be located on chromosome 12 with RFLP and RAPD techniques (Hirabayashi & Ogawa, 1995; Chun et al., 1997; Murata et al., 1998). *bph2* and *Bph9* were also reported to be mapped on chromosome 12 (Yeo et al., 1997; Murata et al., 1998).

MAS (marker-assisted selection) is being used as a powerful tool to increase the selection efficiency for developing new varieties with resistance to disease and insects, better quality, and higher yield potential. Genes of agronomic importance have already been mapped with DNA markers (Yu et al., 1991; Messeguer et al., 1991) and the markers are currently being used for crop breeding. DNA markers will be more useful when one has difficulties in preparing pathogens and in phenotyping. This study aimed at tagging *Bph1* and *bph2* genes and facilitating marker-assisted selection in resistance breeding programs. Also linked markers may be employed as a starting point for cloning the gene to verify the resistance mechanism.

MATERIALS AND METHODS

Plant materials and screening for BPH resistance

Seventy-three F3 lines, derived from a cross between Nagdongbyeo and Mudgo, were used to construct a framework map. Mudgo is an indica variety harboring *Bph1* resistance gene to BPH biotype I (Pathak et al., 1969). Nagdongbyeo is a japonica variety susceptible to BPH. An F4 population, derived from the cross between Nagdongbyeo and Mudgo, was used for phenotyping with insects. Fifty-three

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BC3F5 lines, derived from a cross between Milyang95 and Sangju13, were used to locate *bph2* gene on a molecular map.

Bulked seedling test (Athwal et al., 1971) was carried out in a greenhouse at the National Crop Experiment Station in Suwon. Seedlings with 3 or 4 leaves were infested with a density of around 10 insects per plant, which are the 2nd-3th instar nymphs of BPH biotype I. When the susceptible check variety, Nagdongbyeo, died (5~7 days after infestation), genotype of each line was determined by counting the number of plants alive.

RFLP and isozyme analysis

For the analysis of restriction fragment length polymorphism (RFLP), 5 grams of three-week old leaf tissue were harvested from 20 seedlings and bulked for DNA extraction. DNA was extracted with 20~25 ml of the extraction buffer (0.5M NaCl, 0.1M Tris-Cl, 0.05M EDTA, 1.25% SDS, 0.38 g/100 ml of sodium bisulfite added before use, pH 7.8~8.0) based on the technique described by Causse et al. (1994). Five micrograms of genomic DNA were digested with seven enzymes (*Xba*I, *Sca*I, *Dra*I, *Hind*III, *Eco*RI, *Bam*HI, *Eco*RV). Southern blotting and hybridization procedures were similar to those described in Causse et al. (1994).

For the analysis of isozyme *Sdh-1*, seeds were germinated in 9-cm petri dish for 5 days and ground in 100 μl of distilled water, followed by brief centrifugation. Ten μl of supernatant was transferred onto the wicks (5×5 mm^2) made of electrode strip for isoelectric focusing (IEF). The wicks with the sample solution were inserted in the middle of 8.6% starch gel (0.002M citric acid, pH 6.5). The gel was run in a buffer tray (0.04M citric acid, pH 6.1) with 40mA at 4℃ for 6 hours. And then, the gel was incubated in staining buffer (0.1M Tris-Cl pH 7.5, 1% MTT, 1% NADP, 1% PMS, 1mg/ml Shikimic acid) for 2 hours. The band of shikimate dehydrogenase (*Sdh*-1) was immediately scored for linkage analysis.

Linkage analysis

Linkage analysis was conducted on the segregation data obtained from DNA markers, an isozyme *Sdh*-1, *Bph1* and *bph2* using Mapmaker Macintosh V.2.0 (Lander et al., 1987). Markers were first divided into several groups using "two-point/group" command with LOD ≥ 3.0 and recombination fraction (θ) = 0.40. Secondly the most likely order among DNA markers was determined using three-point / "order" and "compare" commands. And the "ripple" command was used to verify the order. A framework map was constructed using a few markers within a group. Additional markers were located on the framework map using the "try" command when they met the criteria of LOD ≥ 2 and recombination fraction θ = 0.4. Map distances between markers were represented in centimorgans (cM) using Kosambi function (Kosambi, 1944).

Conversion of RFLP makers to PCR markers

RFLP markers linked to *Bph1* were sequenced with an automated sequencer (ABI 377, Perkin Elmer). Forward and reverse primers (21-mer long) were designed from the sequence (Fig. 1). The primers were used to select lines or plants carrying *Bph1* with PCR profile (denaturing - 95℃ /1 min, annealing - 55℃/1 min, extending - 72℃/2 min, with 35 cycles).

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RG413; F: 5′- CAT TGT TTC GTC CAT GTT TAG-3′
       R: 5′- ACC AAG TCT GTT ACA CAT AAA-3′
RG901; F: 5′- GTG ATA TAC GAC TTT GTT GTG-3
       R: 5′- AAG CAG ACC ATG TAT TTG GAG-3′
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Fig. 1. Primers designed on the basis of sequences of RFLP markers, RG413 and RG901.

RESULTS

Reaction of plants to BPH

Seventy-three F4 lines derived from a cross between Nagdongbyeo and Mudgo (*Bph1*) were segregated into 25 homozygous resistant: 39 segregating: 9 homozygous susceptible (χ^2 = 7.36, $P < 0.05$). The segregation did not agree with the expected ratio of 1:2:1 at the 5% level. *Bph1* in Mudgo, however, has been known to inherit in monogenic mode (Athwal et al., 1971; Heu et al., 1974; Martinez & Khush, 1974). Based on the previous studies, it was thought that the distribution of *Bph1* in this population was distorted to the resistance (61.0% frequency of Mudgo allele).

Linkage analysis of *Bph1* and *bph2*

The linkage map of chromosome 12 consisted of 10 DNA markers and 1 isozyme marker. Five of the markers deviated from the expected ratio of monogenic mode (1:2:1) in the F3 population from a cross of Nagdongbyeo with Mudgo at the 5% level (data not shown). Shikimate dehydrogenase (*Sdh-1*) showed a pair of alleles on starch electrophoresis in the F3 population; one in Mudgo is slower in mobility than the other in Nagdongbyeo (Fig. 2). The isozyme was segregated as 19 homozygotes for Mudgo genotype : 46 heterzygotes : 9 homozygotes for Nagdongbyeo genotype and its segregation was also significantly different from the expected ratio of 1: 2: 1 (χ^2 = 7.08,

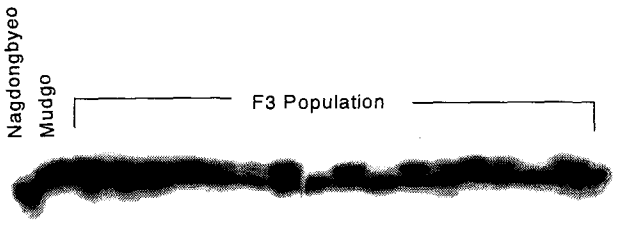

Fig. 2. Evaluation of shikimate dehydrogenases (*Sdh-1*) among Mudgo, Nagdongbyeo and their F3 population.

$P < 0.05$).

Bph1 was mapped in the interval between RG413 and RG901 at a distance of 7.5 cM and 8.5 cM, respectively (Fig. 3). *Sdh-1* was located in a distal region away from *Bph1*. This agreed with the results of the previous reports (Hirabayashi & Ogawa, 1995; Murata et al., 1998).

Ikeda & Kaneda (1981) reported that a recessive gene *bph2* is closely linked or allelic to *Bph1* through allelism test. We used 53 BC3F5 lines derived from a cross between Milyang95 and Sangju13 to investigate the linkage between the two genes. The *bph2* gene was located at a distance of 14.4 cM from a RFLP marker RZ76 (Fig. 4). On the basis of the same DNA markers mapped on chromosome 12 in the two populations, *bph2* was linked to *Bph1* with a distance of about 30 cM and this is consistent with the previous results (Ikeda & Keneda, 1981; Murata et al., 1998).

Conversion RFLP markers to STS markers

For raising utility of DNA markers in marker-assisted selection (MAS), RFLP-based markers are converted to PCR-based markers because of the efficiency of PCR technology (Kangle et al., 1995). This STS (Sequence Tagged Site) marker system can detect only a short stretch of targeted sequence with PCR.

RFLP markers RG413 and RG901, which were closely linked to *Bph1*, were sequenced. Two sets of forward and reverse primers were designed from the sequences. The primers from RG901 produced two pairs of bands in equal size from the two parents. Even when amplified DNAs were digested with 22 restriction enzymes (*Bam*HI, *Bst*E II, 4 *Bst*O I, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hind* III, *Hae* III, *Kpn*I, *Nco*I, *Nde*I, *Nru* I, *Pvu*II, *Rsa*I, *Sac*II, *Stu*I, *Sal*I, *Sca*I, *Taq*I, *Xba*I, *Xho*I), no polymorphism was found (data not shown). On the contrary, a pair of primers from RG413 produced bands different in the two parents. The segregation of bands was identical to that in the RFLP analysis of the F3 generation (Fig. 5). Therefore, RFLP analysis could be replaced with PCR analysis using a pair of the primers from RG413.

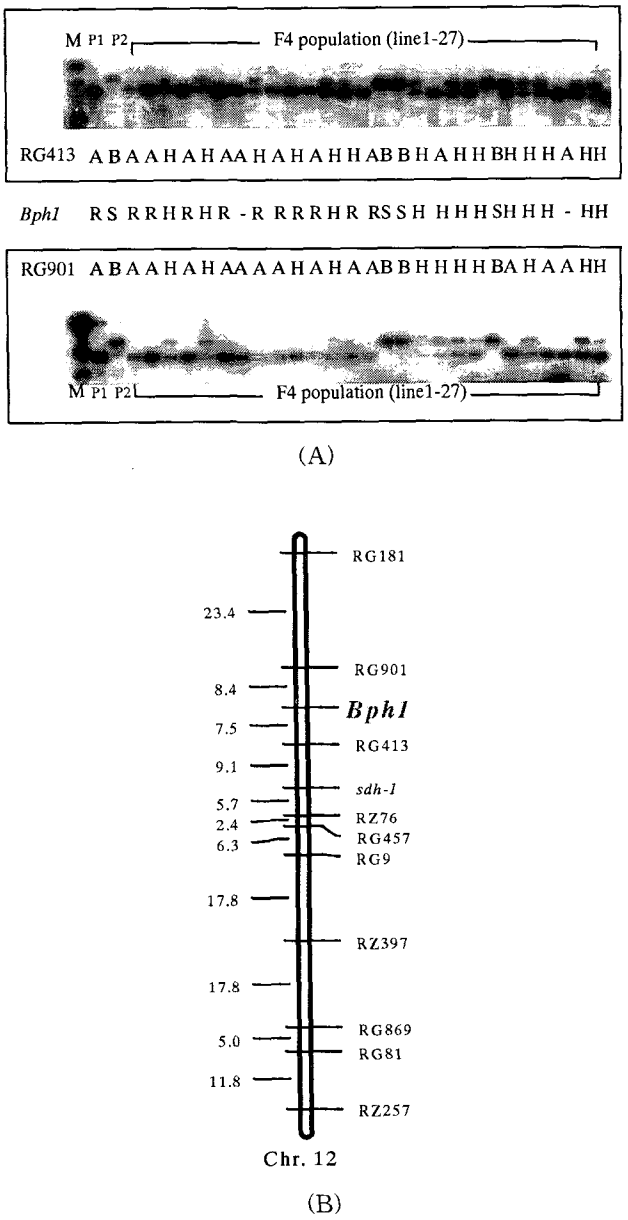

Fig. 3. (A) Segregation of *Bph1* and two DNA markers, RG413 and RG901, in the F3 and F4 population derived from a cross between Nagdongbyeo and Mugdo. M= λ DNA/*Hind*-III fragments, P1=Mudgo (resistant), P2= Nagdongbyeo (susceptible). Their progenies were classified into 3 types (A: homozygous genotype for Mudgo, B: homozygous genotype for Nagdongbyeo and H: heterozygous genotype for each locus). (B) Molecular map of *Bph1* on chromosome 12.

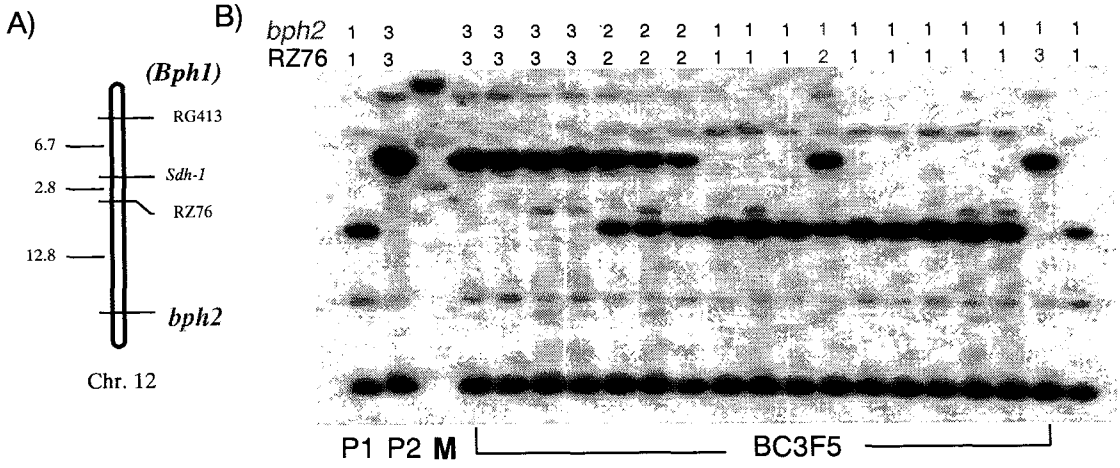

Fig. 4. (A) Molecular map of *bph2* gene. (B) Segregation of *bph2* and RZ76 in 53 BC3F5 lines derived from a cross of Milyang95 with Sangju13.

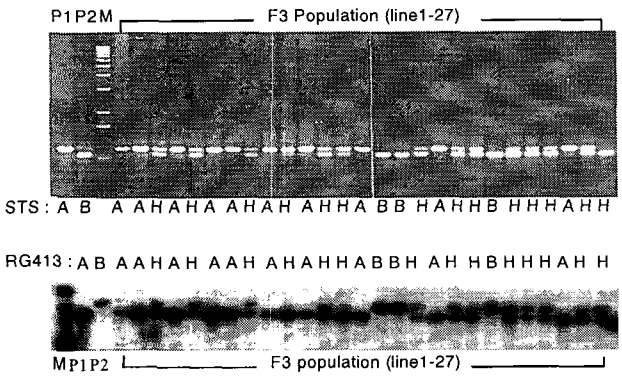

Fig. 5. STS marker RG413 showing segregation identical to the RFLP marker RG413 in the F3 lines (P1 = Mudgo, P2 = Nagdongbyeo).

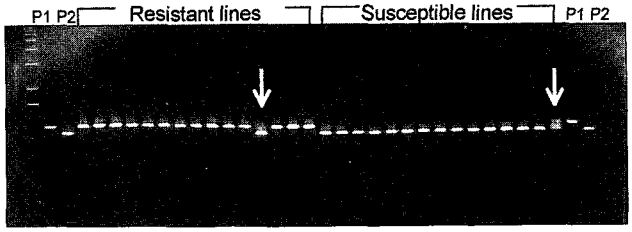

Fig. 6. Coincidence of *Bph1* with bands amplified with RG413 primer set. The PCR bands (ca. 1.4 kbp) were produced with DNA from 15 resistant lines and 15 susceptible lines selected through bulked seedling test. The arrows in the picture designate recombinants whose reactions to BPH do not accord with the pattern of the bands. P1 = Mudgo, P2 = Nagdongbyeo.

Use of markers in resistance breeding

To reconfirm the locus of *Bph1* and to evaluate utility of linked markers in MAS, we selected one of 73 F4 lines, NM47-B-B, which had heterozygous genotypes in the markers mapped on chromosome 12 in this study (Fig. 3-B). Two hundred seeds from the line were planted in a greenhouse and then harvested by individuals. They were used in screening reaction to BPH. 135 lines, showing clear reaction, were used for Southern analysis and linkage analysis. *Bph1* was mapped in the same location as previously analyzed, but the map distances were a little changed {RG901-(12.1)-*Bph1*-(6.7 cM)-RG413-(2.6 cM)-RZ76 -(1.8 cM)-RG457-(0.4 cM)-RG9-(10.7 cM)-*Sdh1*}.

When PCR was conducted with the RG413 primer set on 135 lines, the pattern of amplified bands showed considerable accordance with their phenotypes (Fig. 6) Only 7 lines showed difference between genotypes and phenotypes (8%). This almost coincided with the expected one from the recombination value in the F3 population.

DISCUSSION

Two resistance genes to BPH, *Bph1* and *bph2*, were mapped on chromosome 12 and these results agreed with Murata et al.'s reports (1998). However their exact positions on chromosome 12 could not be directly compared with that in Murata et al.'s report (1998) because of the different markers used. Integration between JRGP's map (Rice Genome Program, Japan) and CU's (Cornell University) are being attempted (S. R. McCouch, Cornell University, personal communication), and comparison of the locations of the two genes would be possible in the near future.

Bph1 was located in the interval of RG413 and RG901 with map distances of 7.5 cM and 8.4 cM, respectively. The map distance between the RFLP

markers in this study is much larger than those in the interspecific map (Causse et al., 1994). When estimated on basis of the same markers mapped on chromosome 12, the map distance between *Bph1* and *bph2* was about 30 cM. This agreed with the estimated distance on the standard 'Nipponbare/Kasalath' map in Murata et al.'s results (1998). They also reported that it was much shorter than that obtained from two different populations (Nipponbare/Norin-PL7 and Nipponbare/Norin-PN4). Therefore, their actual distance will have to be measured through physical mapping in the region because it depends on the kinds of population tested.

Loci associated with skewed allele frequencies were observed on several chromosomes (data not shown). Such was especially remarkable in the loci between RG901 and RZ76 harboring *Bph1* (Fig. 3-B). Skewed loci mapped on chromosome 12 were found on others' maps (McCouch et al., 1988; Tsunematsu et al., 1996) and located on both ends of the chromosome. The region shown skewness on chromosome 12 in this study was also a distal part of the chromosome. In addition, Tsunematsu et al. (1996) suggested the possibility of relatedness with gametophyte gene. Therefore, the cause by which the segregation of *Bph1* gene was distorted from 1:2:1 ratio at 5%($\chi^2 = 7.36$, $P < 0.05$) was thought to be due not to its different mode of inheritance but to unequal distribution of its alleles in the population from a cross between Nagdongbyeo and Mudgo.

Two RFLP markers linked to *Bph1* were converted to STS markers to facilitate MAS in the resistance breeding program. Primers designed from RG413 produced two bands, whose segregation was the same as that in RFLP analysis in the F3 population from the Nagdongbyeo/Mudgo cross. On the contrary, primers designed from RG901 did not produce polymorphism between the parents even when amplified DNAs were cleaved with 22 restriction enzymes. So only the primers from RG413 were used as a useful STS marker. Practically, its utility was evaluated using 135 lines derived from one of the 73 F4 lines. As 8% of them showed discrepancy between genotypes and phenotypes, genotypes of *Bph1* in this population could be selected with 92% accuracy. Moreover, if two markers RG413 and RG901 are used simultaneously for breeding resistant varieties with *Bph1*, the selection efficiency will be much higher.

More closely linked DNA markers will be developed to introgress only the resistance genes from *indica* varieties into *japonica* varieties. So we are finding DNA markers closely linked to *Bph1* and *bph2* using techniques such as AFLP (Amplified Fragment Length Polymorphism) or SSLP (Simple Sequence Length Polymorphism) with combination of BSA (bulked segregant analysis).

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