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Construction of a high-resolution linkage map of a rice brown planthopper (*Nilaparvata lugens* Stål) resistance gene *bph2*

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Abstract The brown planthopper (BPH), *Nilaparvata lugens* Stål, is a significant insect pest of rice (*Oryza sativa* L.). *bph2* is one of the 12 major BPH resistance genes so far identified in several *indica* cultivars and two wild relatives. We have constructed a high-resolution linkage map as a foundation for map-based cloning of the *bph2* locus. An advanced mapping population derived from a cross of 'Tsukushibare' (a susceptible *japonica* cultivar) with 'Norin-PL4' (an authentic *bph2*-introgression line) was used. Segregation analysis by the mass seedling test showed that *bph2* behaved as a single dominant gene. Through bulked segregant analysis and linkage analysis, *bph2* was located within a 3.2-cM region containing eight AFLP markers. One marker (KAM4) showed complete co-segregation with *bph2*, and *bph2* was mapped within a 1.0-cM region delimited by KAM3 and KAM5, two flanking markers. KAM4 was converted into a PCR-based sequence-tagged-site (STS) marker and its co-segregation with *bph2* was validated.

Keywords Brown planthopper (BPH) · BPH resistance gene · *bph2* · AFLP and STS markers · Linkage map · Rice (*Oryza sativa* L.)

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Introduction

Insect pests are the major biotic constraints in crop production. The brown planthopper (BPH), *Nilaparvata lugens* Stål (Homoptera: Delphacidae), is the most-significant insect pest of rice (*Oryza sativa* L.) throughout rice-growing countries. BPH causes severe damage on rice plants either directly by feeding on phloem sap or indirectly by acting as vectors for tungro, grassy stunt and rugged stunt virus diseases (Heinrichs 1979). So far only one natural insect resistance gene has been cloned; a tomato gene *Mi-1* conferring resistance to root-knot nematodes simultaneously confers resistance to potato aphids (Rossi et al. 1998; Vos et al. 1998). Although several organic chemical substances have been proposed to be involved in BPH resistance (Shigematsu et al. 1982; Kim et al. 1994), little is known about its molecular mechanism. Map-based cloning represents one possible approach to isolating BPH resistance genes and elucidating the BPH resistance mechanism in rice.

Twelve major BPH resistance genes have been identified in *indica* rice cultivars and two wild relatives, *Oryza australiensis* and *Oryza officinalis*. *Bph10* from *O. australiensis* was the first BPH resistance gene identified in the wild relatives, and the gene was assigned to rice chromosome 12 (Ishii et al. 1994). *Bph1* from the cultivars 'Mudgo' and 'TKM6' and *bph2* from 'IR1154–243' were also mapped on the long arm of rice chromosome 12 (Hirabayashi and Ogawa 1995; Murata et al. 1997, 1998). Until now, four additional genes [*bph4*, *Bph9*, *bph11*(*t*), and *bph12*(*t*)] have been assigned to, or mapped on, rice chromosomes 6, 12, 3 and 4, respectively, using RFLP and microsatellite markers (Hirabayashi et al. 1998, 1999; Murata et al. 2000; Kawaguchi et al. 2001). These linkage maps, however, are not fine enough for map-based cloning.

To achieve map-based cloning, construction of a high-resolution linkage map with DNA markers is required. For this, we applied a modified version of the amplified fragment length polymorphism (AFLP) marker system, HEGS/AFLP (Kawasaki et al. 2000).

This system enabled both efficient marker selection and mapping. We now report the construction of a high-resolution linkage map of *bph2*. One co-segregating AFLP marker was converted into a PCR-based STS marker.

Materials and methods

Plant materials

A cross was made between 'Tsukushibare', a BPH-susceptible *japonica* line, and 'Norin-PL4', an authentic *japonica bph2*-introgression line. The pedigree of 'Norin-PL4' (Kaneda et al. 1986) is 'Asominori'*3/'IR1154–243', BC_2F_{11}. 'IR1154–243' is the *indica* donor line of *bph2* (Martinez and Khush 1974). One heterozygous F_2 plant that possessed a 9.8-cM segment introgressed from 'IR1154–243' was selected to obtain F_3 plants during the course of the mapping study. F_4 and F_5 progenies were derived from two F_3 individuals that showed identical marker genotypes within this chromosomal region. These F_4-F_5 families were used in a segregation analysis of BPH resistance and a mapping study of *bph2*. 'Tsukushibare' and 'Norin-PL4' were used as susceptible and resistant controls.

BPH bioassay

The BPH colony used in the following bioassay was a mixture of two independent colonies from Kyushu Agricultural Experiment Station and Hyogo Agricultural Research Center, Japan. Conditions for the maintenance of the mixed colony were according to Murata et al. (1998). Genotypes of BPH response (RR: homozygous resistant, RS: segregating heterozygous, SS: homozygous susceptible) of 224 F_4 individuals were determined by assaying the phenotypes (either R or S) of the corresponding F_5 progenies. The bioassay was done by the mass seedling test (Kaneda et al. 1981), which is a modified version of the original bulked seedling test (Pathak et al. 1969). Seventeen seedlings from each F_5 family were planted in a 12 cm-row plot with a row spacing of 2.2 cm and with two replications in a tray (27.5×27.5×2.5 cm). Two rows each of the resistant and susceptible parents were grown as controls, together with two rows each of eight test seedlings in a tray. The tray was placed in a ventilated cage and incubated in a growth chamber under a 16-h photoperiod and day/night temperatures of 30–25°C. At the 1st leaf stage, the 2nd to 4th instar nymphs were released for infestation at a density of 10–12 nymphs per seedling. Between the 8th and 10th day of infestation, damage on the test seedlings was assessed daily by comparing damage on the control seedlings to determine their phenotypes.

Bulked segregant analysis

DNA was extracted and bulked from ten RR and ten SS F_4 individuals. The DNA bulks were used together with the parental DNA in the following bulked segregant analysis (Michelmore et al. 1991). A high-efficiency AFLP genome-scanning system (HEGS/AFLP) (Kawasaki et al. 2000), which is a modified version of the original AFLP method (Vos et al. 1995), was applied for the marker selection. An apparatus for non-denaturing polyacrylamide-gel electrophoresis was equipped with four compact glass plates (18×18 cm in size); each accommodating a gel with 66 lanes (Nippon Eido, Japan). This system enables an analysis of 264 samples in a single run with four lanes (containing parental R, bulked R, bulked S and parental S DNA) for each of the 64 primer combinations in the case of the bulked segregant analysis. Twenty cycles of PCR were performed for pre-amplification; a denaturation step at 94°C for 30 s, an annealing step at 56°C for 1 min, and an extension step at 72°C for 1 min. *Eco*RI and *Mse*I primers, which do not contain an additional nucleotide at the 3′ end, were used for the pre-amplification. Before starting the first cycle, a 2-min DNA denaturation step was performed at 94°C. Thirty nine cycles of PCR were performed for the selective amplification with the following three phases. The first phase was for one cycle; 30 s at 94°C, 30 s at 68°C, and 1 min at 72°C. The second touch-down amplification phase was for 16 cycles with the annealing temperature decreasing stepwise from 67.3°C to 58.5°C. The last phase was for 22 cycles: 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C. Amplified products were fractionated by electrophoresis through 13% non-denaturing polyacrylamide gels and stained using a silver-staining kit, Sil-Best Stain, for protein/PAGE (nacalai tesque, Japan).

Construction of the linkage map

Linkage analysis was performed by the HEGS/AFLP system using 224 F_4 individuals. Recombination values were calculated by MAPMAKER Version 2.0 (Lander et al. 1987) and a genetic linkage map was constructed based on LOD scores greater than 3.0. Map distances were calculated using the Kosambi function (Kosambi 1944).

Construction of a PCR-based STS marker

One AFLP marker that showed complete co-segregation with *bph2* in the linkage analysis was cloned into a pGEM-T vector (Promega, USA). Conformity of the cloned fragment with the AFLP marker was determined in the following manner. A nucleotide sequence of the cloned fragment was first determined by the automated fluorescent dye deoxy terminator cycle sequencing system using a ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, USA). The fragment was then converted into a PCR-based sequence-tagged-site (STS) marker using the following site-specific oligonucleotide primer sets: 5′-TAACTGGTGTTAGTGCGAATGC-3′ (KAM4-F) and 5′-AATTCACGGCATGTGAAGCCCTAG-3′ (KAM4-R). The locus-specific amplification of the STS marker was determined by PCR using the bulked R and S DNA and the parental R and S DNA as templates. Southern hybridization was also conducted against the AFLP products blotted on nylon membranes (Boehringer Mannheim), using the cloned marker as a probe. The membranes were washed in 2×SSC, air-dried and baked. Southern hybridization and signal detection were conducted by the ECL Direct Nucleic Acid Labelling and Detection System (Amersham). Co-segregation of the PCR-based STS marker with *bph2* was determined using the same mapping population employed for the linkage analysis.

Results

BPH bioassay

Segregation of BPH resistance conferred by *bph2* was studied using 224 F_4–F_5 families derived from the cross

Table 1 Segregation of BPH resistance in the mapping population derived from the cross 'Tsukushibare'/'Norin-PL4' (*bph2*)

F_4 genotype	Number of F_4 individuals[a]	Number of F_5 progenies
RR	51	3,014
RS	120	6,456[b]
SS	53	3,065
Total	224	12,535

[a] χ^2 value for 1RR:2RS:1SS is 1.18 (p=0.55)
[b] χ^2 value for 3R (4,294):1S (2,162) is 248 (p<0.001) in the F_5 progenies from the heterozygous F_4 individuals

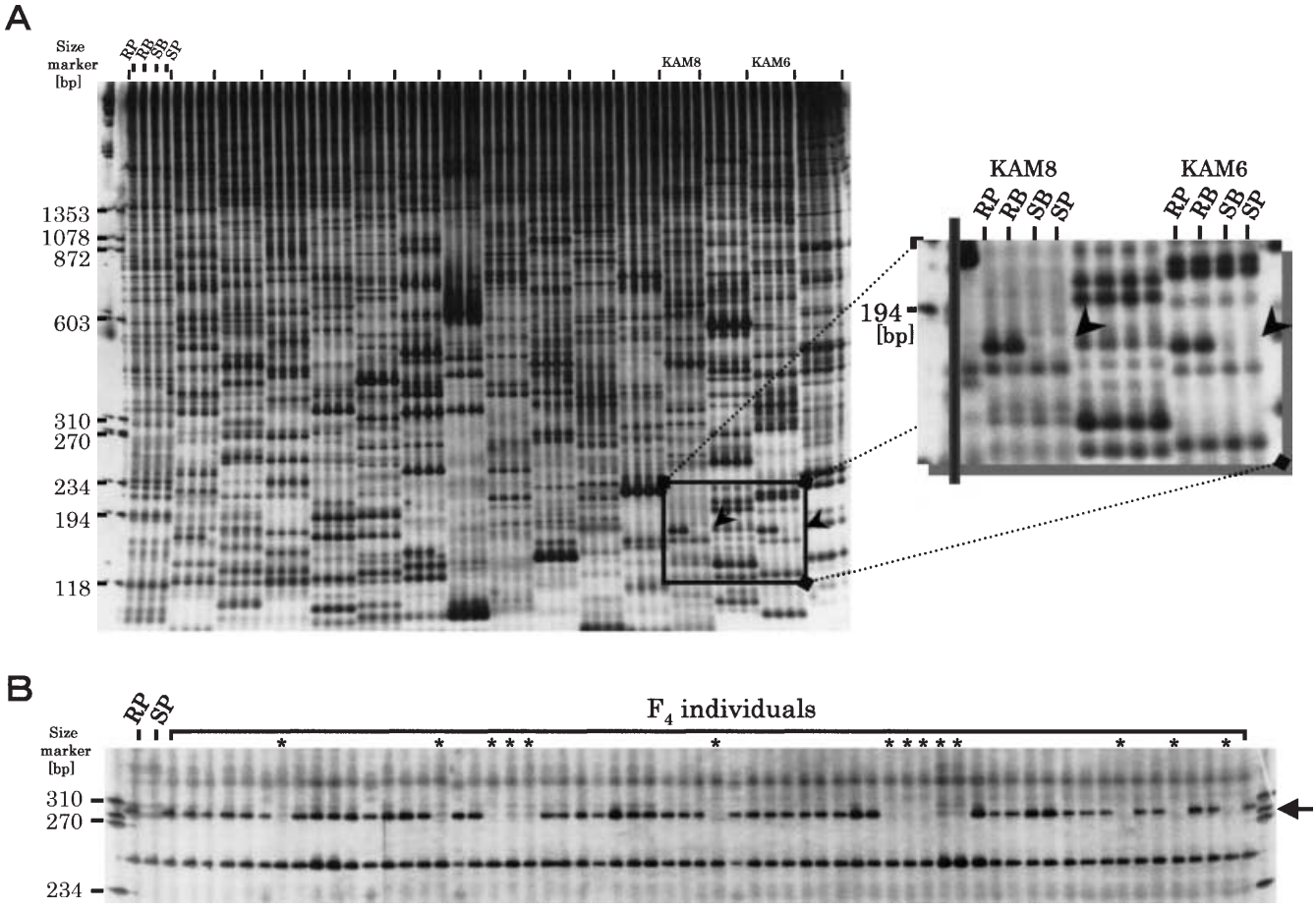

Fig. 1A, B Bulked segregant analysis and linkage analysis of *bph2*. **A** Bulked segregant analysis using a HEGS/AFLP system. Two AFLP markers (KAM6: 160-bp, KAM8: 160-bp) are associated with R. *RP* resistant parent 'Norin-PL4', *RB* resistant bulk, *SB* susceptible bulk, SP susceptible parent 'Tsukushibare', and *M* size markers (ϕ×174 *Hae*III digests). **B** Linkage analyses using the co-segregating AFLP marker, KAM4. The marker fragment indicated by an *arrow* shows complete co-segregation with *bph2* in the mapping population. F_4 individuals with * are SS, and all others are RR or RS

Table 2 A list of AFLP markers linked to *bph2* and their segregation in the mapping population

Marker[a]	Marker type[c]	Number of F_4s with genotypes[b]				*P*
		SS	RS	RR	χ^2 value[d]	
KAM1 (E-GCC/M-GGA; 330)	S	172		52	0.38	0.54
KAM2 (E-CGG/M-GAT; 220)	S	174		50	0.86	0.35
KAM3 (E-GCG/M-AGA; 170)	R	54	170		0.10	0.76
bph2		53	120	51	1.18	0.55
KAM4 (E-ACG/M-CTG; 300)	R	53	171		0.21	0.64
KAM5 (E-CTA/M-GAT; 230)	R	50	174		0.86	0.35
KAM6 (E-CCT/M-ACT; 160)	R	51	173		0.60	0.44
KAM7 (E-TAG/M-ACC; 300)	R	51	173		0.60	0.44
KAM8 (E-CCA/M-ATC; 160)	R	47	177		1.93	0.16

[a] Figures in parentheses after AFLP markers indicate selective amplification primers [*Eco*RI adapter (E) – three selective nucleotides/*Mse*I adapter (M) – three selective nucleotides] followed by marker sizes

[b] SS: 'Tsukushibare'-homozygous, RS: heterozygous, RR: 'Norin-PL4' homozygous. Numbers for dominant AFLP markers represent total numbers of SS+RS for S-associated markers and RR+RS for R-associated markers

[c] S: S-associated, R: R-associated

[d] χ^2 values for all AFLP markers are based on a 3:1 ratio

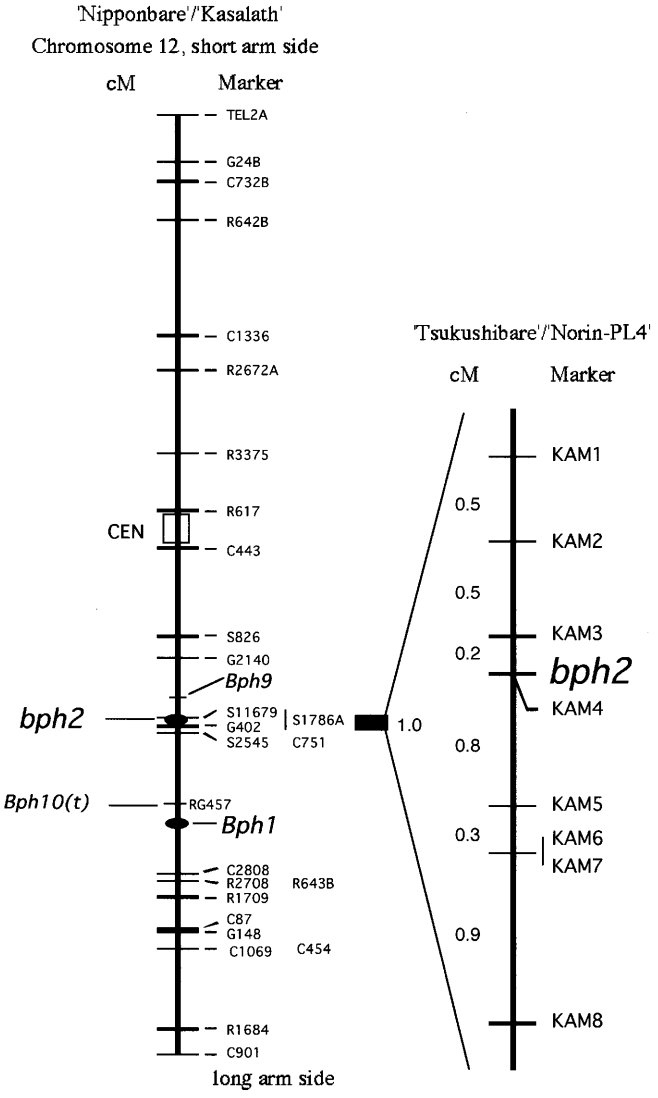

Fig. 2A linkage map of *bph2* and three other BPH resistance genes. Left is the standard 'Nipponbare'/'Kasalath' map of rice chromosome 12 (Harushima et al. 1998), and right is the map constructed for *bph2*. The map positions of *Bph1* (Murata et al. 1998), *Bph9* (Murata et al. 2000) and *Bph10* (Ishii et al. 1994) shown on the standard map are arbitrary because they were determined using different mapping populations

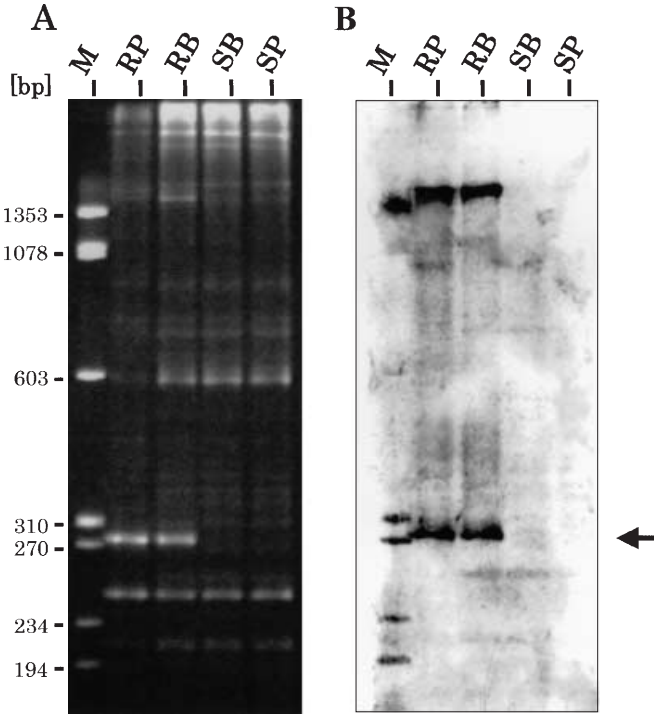

Fig. 3A, B Cloning of a co-segregating marker, KAM4. **A** AFLP products amplified by a primer combination of E-ACG/M-CTG. **B** Southern-blot hybridization of the AFLP products using the PCR-amplified KAM4 as a probe. An *arrow* indicates the fragment of KAM4, which distinguishes the resistant bulk (*RB*) and resistant parent (*RP*) DNA from the susceptible bulk (*SB*) and susceptible parent (*SP*) DNA. *M* size markers (ϕ×174 *Hae*III digests)

'Tsukushibare'/'Norin-PL4.' Genotypes of the BPH response of F_4 individuals were determined by assaying the phenotypes of the corresponding F_5 progenies. In this bioassay, susceptible seedlings either died or did not develop second leaves within the 10-day infestation period. The F_4 segregation did not deviate from the 1 RR:2 RS:1 SS ratio (Table 1). Segregation of R and S plants in the whole F_5 progenies, however, deviated from the 3 R:1S ratio, with more susceptible progenies than expected for the single dominant gene-control of resistance. This was most likely due to our stringent bioassay conditions, in which no escapes were allowed, but occasionally nearly 10% of the seedlings of the resistant parent showed a susceptible-like response. Homozygosity of resistance was thus judged based on more than 90% resistant progenies. The frequency of resistant F_5 progenies from the heterozygous F_4 s showed a normal distribution. The heterozygous F_4s, however, had a mode of 70–75% of resistant F_5 progenies, confirming that *bph2* was a dominant gene.

Bulked segregant analysis and linkage analysis

For the detection of linked markers and the mapping of *bph2*, the HEGS/AFLP system was employed (Kawasaki et al 2000). Using this modified AFLP marker-detection system, 1344 primer combinations were efficiently examined in the bulked segregant analysis. Twenty one primer combinations gave either R- or S-associated fragments. An example of two R-associated markers (KAM6 and KAM8) detected in a single gel is shown in Fig. 1A. The segregation of the 21 AFLP markers was next assessed in a preliminary experiment using 64 randomly selected F_4 individuals in a single gel. Eight markers were selected as closely linked candidates and they were further used to screen the remaining 160 F_4 individuals.

All eight AFLP markers were dominant and showed normal segregation in the mapping population (Table 2). Two markers were S-associated and six were R-associated. Linkage between the markers and the *bph2* locus

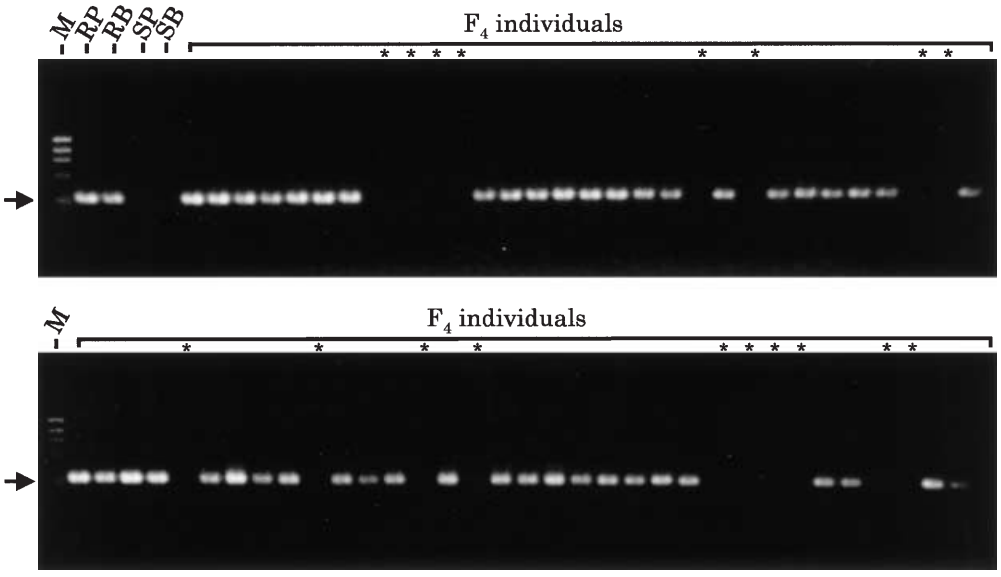

Fig. 4 Conversion of KAM4 into a PCR-based STS marker and confirmation of its co-segregation with *bph2*. PCR was conducted using the marker-specific primer set. The amplified product was resolved by electrophoresis through a 1.2% agarose gel and stained by ethidium bromide. The upper left four lanes represent DNA from the resistant parent (*RP*), resistant bulk (*RB*), susceptible bulk (*SB*) and the susceptible parent (*SP*), respectively, and all other lanes represent DNA from segregating F_4 individuals. F_4 individuals with * are SS, and all others are RR or RS. An *arrow* indicates the co-segregating STS marker. *M* size markers (ϕ×174 *Hae*III digests)

was evaluated based on LOD scores greater than 3.0, and the markers were located within a 3.2-cM region on the long arm of chromosome 12 (Fig. 2). This region corresponds to a map interval of 1.0 cM between two RFLP markers, S11679 and G402, on the standard 'Nipponbare'/'Kasalath' map (Harushima et al. 1998). Three markers (KAM1–3) were located proximal to the *bph2* locus, with map distances of 0.2 to 1.2 cM. The other four markers (KRM5–8) were located distal to the *bph2* locus with map distances of 0.8 to 2.0 cM. One marker (KAM4) showed complete co-segregation with *bph2* (Fig. 1B), and the *bph2* locus was mapped within a 1.0-cM region between two flanking markers (KAM3 and KAM5).

Construction of a PCR-based STS marker

AFLP markers can not be used as probes for the selection of positive clones from a large-insert library. To convert the co-segregating AFLP marker, KAM4, into a more convenient marker-type, the following experiments were conducted. After several cycles of PCR amplification and selection, KAM4 was cloned into the pGEM-T vector. The insert (324 bp) was amplified using the selective primer set, and was used as a probe against a membrane in which whole AFLP products from the R and S bulk DNA and the parental R and S DNA were immobilized (Fig. 3). A fragment corresponding to KAM4 was detected only in the R DNA. Larger positive fragments were also detected in the R DNA. Although the nature of these fragments was unclear, they might represent artifacts produced by the non-denaturing gel electrophoresis. We next tried to convert KAM4 into a PCR-based STS marker in the following way. The cloned KAM4 marker was sequenced and an oligonucleotide primer set was designed for the marker locus. After PCR, a single product with 300 bp unique to the R DNA was obtained using the parental and bulked R and S DNA as templates (Fig. 4). Co-segregation of this STS marker with *bph2* was confirmed by PCR using the same mapping population as that used for the linkage analysis.

Discussion

In our previous mapping study of *bph2* using F_2–F_3 families, only six RFLP markers were detected within a 29-cM region of the long arm of rice chromosome 12 that was introgressed from the donor line 'IR1154–243' into the *japonica* introgression line 'Norin-PL4' (Murata et al. 1998). To construct a high-resolution linkage map, we employed the HEGS/AFLP system, which is an efficient method for bulked segregant analysis and for analyzing a large number of the segregating population (Kawasaki et al. 2000). We also used an advanced mapping population (F_4–F_5 families) that was derived after recombination near the *bph2* locus. In the bulked segregant analysis, eight AFLP markers were detected within a 3.2-cM region encompassing the *bph2* locus (Fig. 2). The efficiency of the adapted marker-detection system is obvious when we consider the significant number of polymorphic markers within this short region. The *bph2* locus was mapped within a 1.0-cM region delimited by two flanking markers. The 3.2-cM region of the present map corresponds to the 1.0-cM region on the standard 'Nipponbare'/'Kasalath' map (Harushima et al. 1998). The difference in the two map distances might be due to the use of different mapping populations, in which the local relationship might differ between physical and genetic distances.

In our bioassay, *bph2* behaved as a dominant gene (Table 1). This result was consistent with the previous one obtained using F_2 progenies from the same cross combination (Murata et al. 1998). *bph2* was originally identified as a recessive gene in an *indica* breeding line,

'Karsamba Red ASD7' (Athwal et al. 1971). The study of resistance segregation in a large number of F_2 and F_3 progenies from a cross between 'Taichung Native 1' (an *indica* susceptible cultivar) and 'Karsamba Red ADS7' clearly showed the recessive behavior of *bph2*. In our present and previous study, we used 'Norin-PL4', which was an authentic *bph2*-introgression line derived from an IRRI *indica* line 'IR1154–243' (Kaneda et al. 1986). Graphical genotyping of 'Norin-PL4' showed the presence of a large segment (29 cM) that was derived from 'IR1154–243' (Murata et al. 1998). 'IR1154–243' was a derivative from a cross involving two BPH susceptible *indica* cultivars, 'IR8' and 'Zenith' (Martinez and Khush 1974). Because of the susceptibility of both parents, the presence of a dominant inhibitor gene was postulated, perhaps in 'Zenith', which was segregated out to result in 'IR1154–243.' A similar situation was found in the case of 'TKM6', which was shown to be a latent carrier of *Bph1* (Martinez and Khush 1974). No such inhibitor or modifier gene(s) was (were) detected in the present cross combination involving 'Norin-PL4.' It is known that recessive resistance genes behave as dominant genes under different genetic backgrounds and with different pathotypes (races) or biotypes. Because of this, the Catalogue of Gene Symbols for wheat, for example, recommends the use of capital letters to designate all resistance genes irrespective of dominance or recessiveness (McIntosh 1988). A possible explanation might be that the dominance/recessiveness of *bph2* depends on the interaction between BPH biotypes with different virulence properties and different genetic backgrounds for the host rice plants. Three sympatric biotypes were identified from Southeast Asian BPH populations and designated as biotypes 1, 2 and 3 (IRRI 1976). Biotype 3 can infest *bph2*-carriers and cultivars susceptible to biotype 1 that have the ability of infesting rice cultivars lacking BPH resistance genes. These and other BPH biotypes have also been selected under laboratory conditions through continuous rearing on BPH-resistant lines (Pathak and Heinrichs 1982; Ketipearachchi et al. 1998). Interaction between the genetically defined biotype 3 and the heterozygous F_1s must be studied for a critical test of the dominance/recessiveness of *bph2*.

So far four functional BPH resistance genes (*Bph1*, *bph2*, *Bph9* and *Bph10*) have been mapped on the long arm of rice chromosome 12 (Ishii et al. 1994; Hirabayashi and Ogawa 1995; Murata et al. 1997, 1998, 2000). The map locations of these genes were determined independently using different mapping populations. Nevertheless, they are located in the vicinity (about 25 cM) of the *bph2* locus on the standard map (Fig. 2). A similar linkage block, i.e. a block of six resistance genes, was reported for Hessian fly resistance in wheat (Ohm et al. 1995). The size of the linkage block, however, was over 100 cM when estimated by test crosses. The apparently much-closer clustering of functional BPH resistance genes on the long arm of rice chromosome 12 is notable. This would provide a favorable condition for gene pyramiding which combines *bph2* with the other nearby BPH resistance genes.

In conclusion, we were able to construct a high-resolution linkage map of a major BPH resistance gene, *bph2*. One AFLP marker, KAM4, which showed complete co-segregation, was further converted into a PCR-based STS marker. This marker should provide a useful means for marker-assisted selection in BPH resistance breeding programs. In map-based cloning, closely linked DNA markers are used to isolate large-insert genomic clones that span the target locus (Collins 1992). Conversion of seven other closely linked AFLP markers into STS/RFLP markers is now underway for use in contig construction of the introgressed region.

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