Marker-assisted pyramiding of brown planthopper (*Nilaparvata lugens* Stål) resistance genes *Bph1* and *Bph2* on rice chromosome 12

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Brown planthopper (BPH) (*Nilaparvata lugens* Stål) is a significant insect pest of rice (*Oryza sativa* L.). We constructed a gene-pyramided *japonica* line, in which two BPH resistance genes *Bph1* and *Bph2* on the long arm of chromosome 12 independently derived from two *indica* resistance lines were combined through the recombinant selection. The gene-pyramiding was achieved based on the previously constructed high-resolution linkage maps of the two genes. Two co-dominant and four dominant PCR-based markers flanking the loci were used to select for a homozygous recombinant line in a segregating population that was derived from a cross between the parental homozygous single-gene introgression lines. BPH bioassay showed that the resistance level of the pyramided line was equivalent to that of the *Bph1*-single introgression line, which showed a higher level of resistance than the *Bph2*-single introgression line. The pyramid line should provide a useful experimental means for studying the fine structure of the chromosomal region covering these two major BPH resistance genes.

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Brown planthopper (BPH), *Nilaparvata lugens* Stål (Homoptera: Delphacidae), is a significant sap-feeding insect pest of rice (*Oryza sativa* L.). Natural resistance systems has long been exploited as an environment-friendly BPH control measure that can be effectively utilized in the integrated pest management program. Twelve major BPH resistance genes have so far been identified in *indica* rice cultivars and two wild relatives, *O. australiensis* and *O. officinalis* (Sharma et al. 2003a) together with several quantitative trait loci (QTLs) controlling BPH resistance (Alam and Cohen 1998; Xu et al. 2002). A breeding program aiming at BPH control was initiated during the late 1960s in the International Rice Research Institute (IRRI), Philippines, after discovery of BPH resistant rice varieties (Pathak et al. 1969). Athwal et al. (1971) were the first to identify two major BPH resistance genes *Bph1* and *bph2* in this breeding program. A dominant gene *Bph1*, which was identified from an Indian cultivar 'Mudgo' and two other *indica* breeding lines 'CO22' and 'MTU15', was mapped on rice chromosome 12 by linkage analyses using restriction fragment length polymorphism (RFLP) markers (Hirabayashi and Ogawa 1995; Murata et al. 1997). A high resolution linkage map of *Bph1* was further constructed using a new mapping population and amplified fragment length polymorphism (AFLP) and RFLP markers (Sharma et al. 2003b). A recessive gene *bph2* that was first identified in an *indica* breeding line 'Karsamba Red ASD7' was originally suggested to be either allelic or tightly linked to *Bph1* (Athwal et al. 1971). The gene was later shown to behave as a dominant gene when introgressed into a *japonica* genetic background (Murata et al. 1997) and mapped at a locus different from the *Bph1* locus on the long arm of chromosome 12 using RFLP and AFLP markers (Murata et al. 1998; Murai et al. 2001). Based on the comparison of these two independently constructed linkage maps with the standard rice linkage map (Harushima et al. 1998), a genetic distance between the two BPH resistance genes was estimated to be ca 10-cM (Sharma et al. 2003b).

Occurrence of new virulent biotypes has been a serious problem in rice protection against BPH (Pathak and Heinrichs 1982). BPH biotypes are defined as BPH populations that have ability to feed on rice plants having monogenic resistance genes. Gene-pyramiding that combines more than two independent resistance genes into a particular line can offer a possible means to cope with the occurrence of such virulent BPH biotypes. In case of *Bph1* and *Bph2*, however, they can no longer serve as effective germplasm sources for this purpose because their

resistance was already broken down in many Asian countries (Feuer 1976; Irri 1982). Despite this fact, we attempted to construct a gene-pyramided line based on the expectation that such pyramid line should provide a powerful means for studying a fine structure of the chromosomal region covering the two independent genes, which are still effective against the biotype 1, prior to the final goal of map-based cloning. New virulent BPH biotypes can be experimentally selected by continuous rearing of BPH on resistant lines. Using this methodology, we in fact previously selected new virulent biotypes against each of the two resistance genes *bph8* and *Bph9* (Ketipearachchi et al. 1998). We hoped that the gene-pyramiding should also serve for evaluating its durability against BPH attack and possibly can provide useful means for studying BPH virulence/resistance mechanisms.

As for the *Bph2* locus, a co-segregating AFLP marker was previously converted into a sequence-tagged-site (STS) marker (Murai et al. 2001). In this study we further attempted to convert seven other AFLP markers linked to *Bph2* into PCR-based markers, aiming at selection of co-dominant markers useful in gene pyramiding with *Bph1*. For the *Bph1* locus, one co-dominant marker was already identified (Sharma et al. 2003b). We herein report the marker-assisted pyramiding of these two major BPH resistance genes. The resistance level of the pyramid line against BPH infestation was compared with that in the parental single-gene introgression lines.

MATERIAL AND METHODS

Plant materials and conversion of Bph2-linked AFLP markers to PCR-based markers

Total DNA was extracted from leaves of 10 resistant (R) and 10 susceptible (S) homozygotes to prepare R-DNA bulk and S-DNA bulk, respectively. The R and S homozygotes were identified in F_5 progenies derived from a cross between 'Tsukushibare' (abbreviated as TSU) and 'Norin-PL4' (PL4). TSU is a susceptible *japonica* cultivar and PL4 is a registered BPH resistant *japonica* line, in which *Bph2* was introgressed from an *indica* donor line 'IR1154-243' (Kaneda et al. 1986). The DNA bulks were used together with the parental DNA in the following analysis. DNA was digested with *Eco*RI and *Mse*I, and three selective nucleotides were added at the 3' ends of both *Eco*RI and *Mse*I adapter primers for selective amplification. PCR conditions were according to Murai et al. (2001). Amplified products were fractionated by electrophoresis through 13% non-denaturing polyacrylamide gels and stained using ethidium bromide (EtBr) or a silver-staining kit, Sil-Best Stain (Nakalai tesque, Japan). AFLP fragments specifically associated with the R-DNA bulk or the S-DNA bulk were dissected from dried polyacrylamide gels and rehydrated in TE buffer. The DNA-containing supernatant was used as template for PCR with the same primer sets as once used in the selective amplification. The amplified AFLP fragments were cloned into pGEM-T vector (Promega, USA). The inserts were identified by re-amplification with the same primer sets, followed by electrophoresis through 13% non-denaturing polyacrylamide gels and silver-staining.

After purification of the plasmid DNA, nucleotide sequences of the inserts were determined by the automated fluorescent dye deoxy terminator cycle sequencing method using ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, USA). Specific primers for the construction of STS markers were designed by the OLIGO 4.0 software. When PCR products with an identical size were amplified from both R- and S-DNA bulks and DNA from the parental lines, single nucleotide polymorphisms (SNPs) were searched for using a DNASIS software (Hitachi, Japan) based on the nucleotide sequences of the PCR products. A cleaved amplified polymorphic sequence (CAPS) marker was then developed by digestion with restriction enzymes recognizing the SNPs. Sequence homologies of these PCR-based markers were searched for with the BLAST algorithm (Karlin and Altschul 1993).

Linkage analysis and analysis of marker distribution in rice germplasms

Linkage analysis was performed for the *Bph2* locus using the PCR-based markers in 224 F_4 individuals derived from a cross between TSU and PL4. BPH genotypes of individual F_4 plants were previously determined by bioassay of the corresponding F_5 lines (Murai et al. 2001). For PCR amplification with the designed specific primer sets, template DNA (10 ng) and 1 pmol each of primers were mixed with 1.2 mM $MgCl_2$, 0.2 mM of each nucleotide and 1 unit of rTaq polymerase (TOYOBO, Japan) in a total volume of 20 μl. Thirty-five cycles of PCR were performed in a programmed temperature control system (9700, PE Applied Biosystems, USA) according to Murai et al. (2001). The DNA fragments generated for both STS and CAPS markers were analysed by EtBr-staining after 0.8% or 2.0% agarose or 13% non-denaturing polyacrylamide gel electrophoresis. 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 Kosambi function (Kosambi 1944).

To study genomic distribution of the selected markers, total DNA was extracted from seedling leaves of TSU, PL4 and R- and S-DNA bulks, and digested separately with seven restriction enzymes, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Kpn*I. The digested DNA was fractionated by electrophoresis through 0.8% agarose gels and blotted onto nylon membranes, HybondN^+ (Amersham). Southern hybridization using these markers as probes and their signal detection were made by ECL^{TM} Direct Nucleic Acid Labelling and Detection System (Amersham, USA). The *Bph2*-linked markers were further used for the assessment of their genomic distribution by PCR analysis using 24 accessions including BPH resistant and susceptible *japonica* and *indica* rice.

Marker-assisted selection of a recombinant pyramided line

A F_3 line homozygous for the *Bph1* locus was obtained from a cross between TSU and 'Norin-PL3' (PL3) (Sharma et al. 2003b). PL3 is a registered *japonica* introgression line, in which a dominant *Bph1* gene was introgressed from an *indica* donor cultivar 'Mudgo' (Kaneda et al. 1985). The *Bph1*-introgressed homozygous line was crossed as a female parent with the *Bph2*-introgressed homozygous line as a male parent. From this cross, a single F_1 plant heterozygous for the markers was selected and F_2, F_3 and F_4 populations were obtained. Marker genotypes of individual plants in these generations were determined to select for a homozygous recombinant line.

Bioassay for evaluating the BPH resistance level of the pyramided line

A mixture of two independent BPH colonies (biotype 1) provided by Kyushu Agricultural Experiment Station and Hyogo Agricultural Research Center, Japan, were used in the following BPH bioassay. The bioassay was conducted to compare the levels of BPH resistance among PL3, PL4 and the pyramid line. TSU was used as a susceptible control. Resistance levels of the test lines were measured by directly comparing the differences of growth (plant height) between the BPH-infested and non-infested seedlings or indirectly by comparing resistance indexes (Ri). Ri values were calculated according to the following formula: $Ri = (Hi/Hn) \times 100$, where Hi = average plant height of infested seedlings and Hn = average plant height of non-infested seedlings. For this bioassay, 5 seedlings from each line were separately grown in five glass tubes (2×30 cm) containing soils with water and each seedling was infested with 15 BPH nymphs at the 2nd and 3rd instar stages. In a control experiment, 5 seedlings from each test line were separately grown in 5 test tubes without BPH infestation. At the beginning of the bioassay, plants of ca 3.5 cm in height were selected for adjustment of growth stage. The test seedlings were incubated in a growth chamber under a 16-h photoperiod and day-night temperature of 30–25°C. Plant height of test seedlings was measured at the 8th day of incubation. The whole experiment was repeated at least three times and the mean values were statistically evaluated by Duncan's test.

RESULTS AND DISCUSSION

Conversion of Bph2-linked AFLP markers into STS and CAPS markers, linkage analysis

Five *Bph2*-linked AFLP markers that were previously designated as KAM markers (Murai et al. 2001) were converted into STS or CAPS markers (KPM markers) (Table 1). KPM1, 3 and 8 were found to be simple dominant STS markers that could be defined either by S-associated (KPM1) or R-associated (KPM3 and KPM8) single fragments (data not shown). A nested PCR was required for conversion of KAM5 into R-associated KPM5. In case of KAM2, same-sized PCR fragments were amplified with the specific primer set from all of the R- and S-line DNA and R- and S-DNA bulks. The nucleotide sequences of the PCR fragments were compared between the R- and S-line DNA to reveal 9 single-nucleotide polymorphisms (SNPs) among them, and one SNP at a *Hin*fI site was unique in the R-line DNA. As expected, *Hin*fI digestion of the PCR products generated different sized fragments, i.e. an S-associated fragment of 161-bp and R-associated fragments of 105-bp and 56-bp, and thus KAM2 was converted into a co-dominant CAPS marker, KPM2. Two other AFLP markers, KAM6 and KAM7, could not be converted into PCR-based markers. A nucleotide sequence of the KAM7-derived PCR fragment showed high homology to rice *gypsy*-type retrotransposon *RIRE3* (Kumekawa et al. 1999). No homology was detected for the other marker sequences.

These six PCR-based markers were applied to reconstruct the *Bph2* linkage map. A few recombinant chromosomes were identified, which were unrecognized in our previous study (Murai et al. 2001). The marker genotypes of all the recombinants were confirmed by the AFLP analysis using the eight AFLP markers. Because of these recombinant chromosomes the marker order and distance were slightly changed: map-position of KAM3 was proximal to the *Bph2* locus in the previous map but KPM3 was now mapped distal to the *Bph2* locus with a distance of 0.4 cM

Table 1. *A list of AFLP markers linked with the Bph1 locus and PCR-based Bph2-linked STS-markers (KPMs) converted from AFLP markers.*

Marker name	Amplified fragment length (bp)	Primer sequence[e]
Bph1-linked		
em24G[b]	200 (RR), 195 (r r)	E-GACTGCGTACCAATTCACA M-GATGAGTCCTGAGTAACTG
em5814N[c]	252	E-GACTGCGTACCAATTCCTG M-GATGAGTCCTGAGTAAACG
em32G[c]	175	E-GACTGCGTACCAATTCACT M-GATGAGTCCTGAGTAACTT
Bph2-linked		
KPM1[a]	341	5′- TAAGGACGATGAAGGATATCA -3′ 5′- AATTCGCCATCACCCAAGTGAAGT -3′
KPM2[b]	105 & 56(RR), 161(r r)	5′- TAAGATTGCCAAGAGAAATGTC -3′ 5′- AATTCCGGCTTGGGCTGAGAAATG -3′
KPM3[c]	116	5′- TAAAGAGAAAAAGAACAGTCCA -3′ 5′- ATCCAGGGAAAATGGTACGG -3′
KPM4[c,d]	300	5′- TAACTGGTGTTAGTGCGAATG -3′ 5′- AATTCACGGCATGTGAAGCCCTAG -3′
KPM5[c] (primary)		5′- TAAGATCTTGTGGAGGTACAAG -3′ 5′- AATTCCTAGATGAAGTGGGAATCA -3′
(nested)	153	5′- CATGACATGCGGTACTCACGGC -3′ 5′- ATCAAGCTAGAGTTCTCCGCCC -3′
KPM8[c]	149	5′- TAAATCCACCACACAAACAACG -3′ 5′- AATTCCCACAAGGATTCGAACTCC -3′

[a] susceptible-associated marker.
[b] co-dominant CAPS marker. A susceptible-associated fragment (161-bp) and resistance-associated fragments (105- and 56-bp) were generated after digestion with *Hin*fI.
[c] resistance-associated marker.
[d] after MURAI et al. (2001).
[e] E and M indicate the *Eco*RI and *Mse*I primer, respectively.

(Fig. 1). KPM4 generated from KAM4 showed complete co-segregation with *Bph2*, and the *Bph2* locus was mapped within a 1.1-cM region between the two flanking markers, KAM2 and KAM3.

Determination of copy number of KPM markers in the rice genome and their distribution among rice varieties

DNA markers must be unique singletons in the genome if they can be effectively used in gene-pyramiding, physical mapping and map-based cloning. To examine copy numbers of the converted PCR markers in the rice genome, genomic Southern blot analysis was conducted using the R- and S-DNA bulks and their parental DNA. PCR fragments amplified with KPM1 and KPM5 primers showed multiple bands (data not shown), indicating that the rice genome contain several copies of the KPM1 and KPM5 sequences at homologous loci. PCR products of KPM2 and KPM3 showed hybridisation to low-copy bands and that of KPM8 to two distinct bands. KPM4 showed hybridisation to one major band with a few minor bands after digestion with all seven restriction enzymes.

To examine the specificity of the converted PCR markers for the *Bph2* locus, distribution of the six KPM markers was analyzed in 24 rice accessions with or without known BPH resistance genes. All three accessions possessing the *Bph2* gene showed R-type alleles at all of the loci (Table 2). All but one BPH-resistant accessions having *Bph1*, *Bph3*, *bph4*, *bph8* or *Bph9* contained R-type alleles at some but not all KPM loci. Three of eight susceptible cultivars had one or two R-type alleles of the two KPM loci (KPM1 and KPM2). These KPM marker-possessing susceptible cultivars were all *indica* types, whereas all susceptible *japonica*-type cultivars did not have any of these markers. 'Rathu Heenati', which is a Sri Lankan local cultivar possessing the *Bph3* gene on chromosome 4, showed R-type alleles at five out of the six KPM loci including the co-segregating KPM4 locus. Only KPM3 allele of 'Rathu Heenati' was S-type. A precise reason for this remains unknown, but the observation might indicate that the chromosomal region of 'Rathu Heenati' containing *Bph3* is homologous to the segment including KPM4 locus.

Marker-assisted selection of a recombinant pyramided line

To identify the recombinants between the *Bph1* and *Bph2* loci, six DNA markers tightly-linked to these

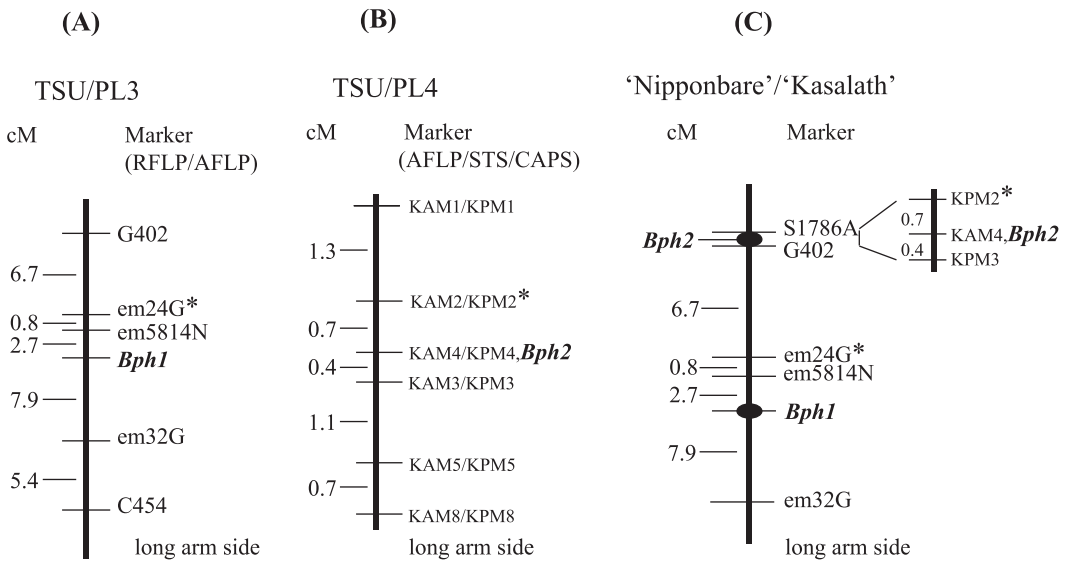

Fig. 1A–C. Map positions of the *Bph1*, *Bph2* and marker loci on the long arm of rice chromosome 12. (**A**) *Bph1*. (**B**) *Bph2*. (**C**) combined map of *Bph1* and *Bph2* on a standard map. Markers with asterisks indicate the co-dominant markers.

loci were selected based on their positions on the high-resolution linkage maps (Fig. 1). Table 2 shows a list of these six markers used in the marker-assisted screening together with the other *Bph2*-linked STS markers. One co-dominant AFLP marker (em24G) and two R-associated dominant AFLP markers (em5814N and em32G) were used for determining *Bph1* genotypes. Similarly, one co-dominant CAPS marker (KPM2) and two R-associated dominant markers (KAM4 and KPM3) were used for determining *Bph2* genotypes. We first confirmed that the *Bph1*-linked markers were absent in the single-*Bph2*-introgressed line (PL4), and conversely that the *Bph2*-linked markers were absent in the single-*Bph1*-

Table 2. *Assessment of chromosomal region containing the Bph2 locus using KPM markers in 24 rice accessions.*

Accession	BPH resistance gene	KPM1	KPM2	KPM4	KPM3	KPM5	KPM8
IR64	*Bph1*	R	S	S	S	S	R
KPL4	*Bph1*	S	S	S	S	R	S
Mudgo	*Bph1*	S	S	S	S	S	R
PL3	*Bph1*	S	S	S	S	S	R
PL7	*Bph1*	S	S	S	S	S	R
IR1154-243	*bph2*	R	R	R	R	R	R
KPL5	*bph2*	R	R	R	R	R	R
PL4	*bph2*	R	R	R	R	R	R
Muthumanikan	*Bph3*	S	R	S	S	S	S
PL10	*Bph3*	S	S	S	S	S	S
Rathu Heenati	*Bph3*	R	R	R	S	R	R
Babawee	*bph4*	S	R	S	S	S	S
Kalukuruwee	*bph4*	S	R	S	S	S	S
Vellai IIIankali	*bph4*	S	R	S	S	S	S
Chin Saba	*bph8*	S	R	S	S	S	S
Kaharamana	*Bph9*	S	S	S	S	S	R
Asominori	susceptible	S	S	S	S	S	S
IR24	susceptible	R	R	S	S	S	S
IR8	susceptible	S	R	S	S	S	S
Kasalath	susceptible	R	S	S	S	S	S
Lemont	susceptible	S	S	S	S	S	S
Nihonbare	susceptible	S	S	S	S	S	S
Tsukushibare	susceptible	S	S	S	S	S	S
Notohikari	susceptible	S	S	S	S	S	S

R: resistant-type allele, S: susceptible-type allele

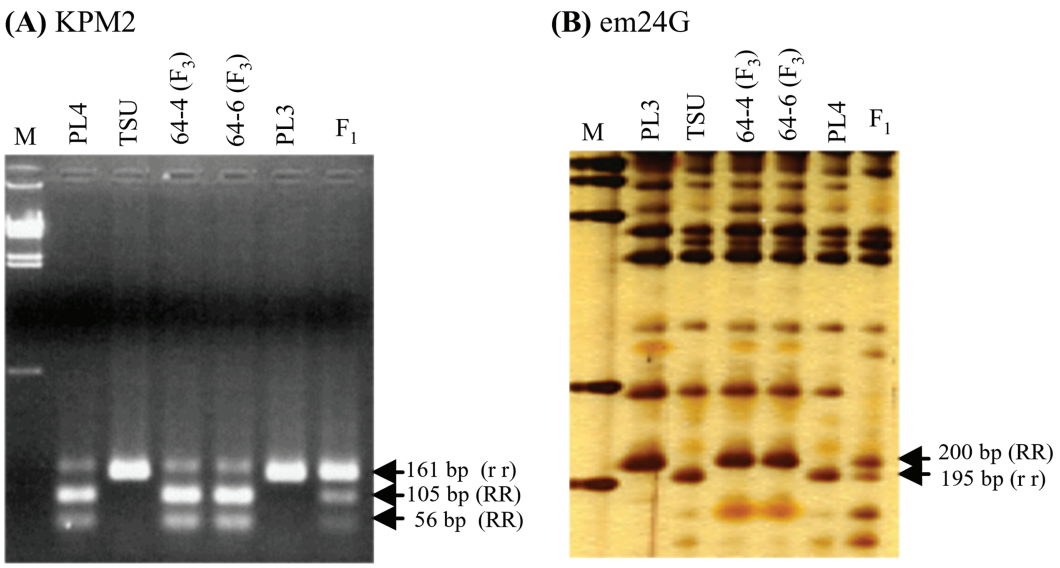

Fig. 2. Marker specificity to the *Bph1* and *Bph2* loci. (**A**) *Bph2*-linked co-dominant marker PKM2. (**B**) *Bph1*-linked co-dominant marker em24G. m: *Hin*dIII digests of fx174 DNA. For map positions of the markers, see Fig. 3.

introgressed line (PL3). Results of using a *Bph2*-linked co-dominant marker KPM2 and a *Bph1*-linked co-dominant marker em24G are shown in Fig. 2. DNA extracted from several F_1 plants obtained from the crosses between the *Bph1*- and *Bph2*-introgressed lines were then analyzed for the presence of these markers. The F_1 plants possessed all of the markers in heterozygous conditions as judged by the two co-dominant markers. From a single F_1 plant 150 F_2 plants were grown, from which DNA was extracted and analyzed with the markers. Out of these F_2 plants, one plant was identified as a recombinant that was found to be homozygous for the *Bph1*-linked markers and heterozygous for the *Bph2*-linked markers. The observed recombination frequency was thus 0.3% in the meiosis. This value apparently was much smaller than the value expected based on the map distance of ca 10 cM according to the standard rice map. The result suggested that recombination was restricted in the chromosomal region due to a low sequence homology between the *japonica* and *indica* segments involved. We could not specify the recombination point on the map, because no markers were available on the chromosomal region between KPM2 and em24G (Fig. 1C). Ten F_3 plants were then grown from this single F_2 recombinant, and two were identified as gene pyramided lines possessing both *Bph1*-linked and *Bph2*-linked markers in homozygous conditions (Fig. 2). Ten F_4 plants from one F_3 pyramided line were further analyzed to confirm their homozygosity for the *Bph1* and *Bph2* loci (Fig. 3).

Evaluation of BPH resistance in the pyramid line by BPH bioassay

Successful marker-assisted pyramiding of disease resistance genes have already been reported in rice, including four bacterial blight resistance genes, *Xa4*, *xa5*, *xa13* and *Xa21* (Huang et al. 1997; Sanchez et al. 2000; Singh et al. 2001) and three blast resistance genes, *Pi1*, *Piz-5* and *Pita* (Hittalmani et al. 2000). Recently, production of transgene-pyramided rice lines resistant against bacterial blight, yellow stem borer and sheath blight has been reported (Datta et al. 2002). Importantly, these pyramided lines showed broader-spectrum resistance than the original single-gene resistant lines. We therefore evaluated resistance levels of the pyramid line (*Bph1*/*Bph2*), TSU, PL3 (*Bph1*) and PL4 (*Bph2*) by directly comparing the growth differences (plant height) between the BPH-infested and non-infested seedlings (Fig. 4A). The growth differences measured during the tested 8-day-period were much smaller in the pyramided line (#64-4 and #64-6) and the parental single gene introgression lines (PL3 and PL4) than in the susceptible parent (TSU). The pyramid line showed an equivalent level of resistance against BPH infestation to that of the *Bph1*-single introgression line, which was more resistant than the *Bph*2-single introgression line. Resistance levels of the test lines was also evaluated by comparing the resistance indexes (Ri) of the pyramid line and the single gene-introgression lines. Ri values represent relative growth in plant height observed during the test period under non-infestation vs infestation. The

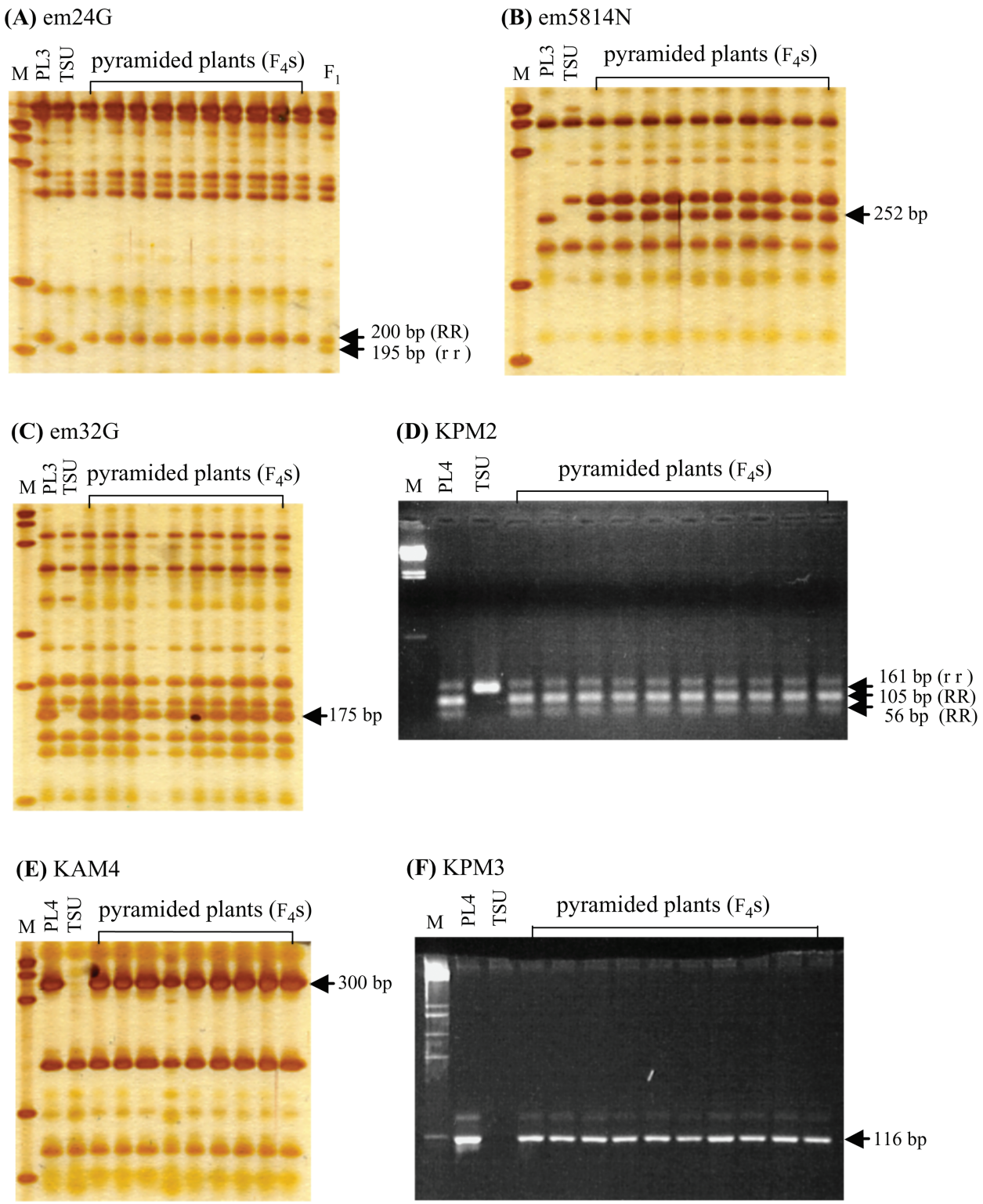

Fig. 3A–F. Marker-assisted selection of the *Bph1*/*Bph2* pyramided line. (**A**) *Bph1*-linked co-dominant marker em24G. (**B**) *Bph1*-linked dominant marker em5814N. (**C**) *Bph1*-linked dominant marker em32G. (**D**) *Bph2*-linked co-dominant marker KPM2. (**E**) *Bph2*-linked dominant marker KAM4. (**F**) *Bph2*-linked dominant marker KPM3. m: *Hae*III digests of fx174 DNA. For map positions of the markers, see Fig. 3.

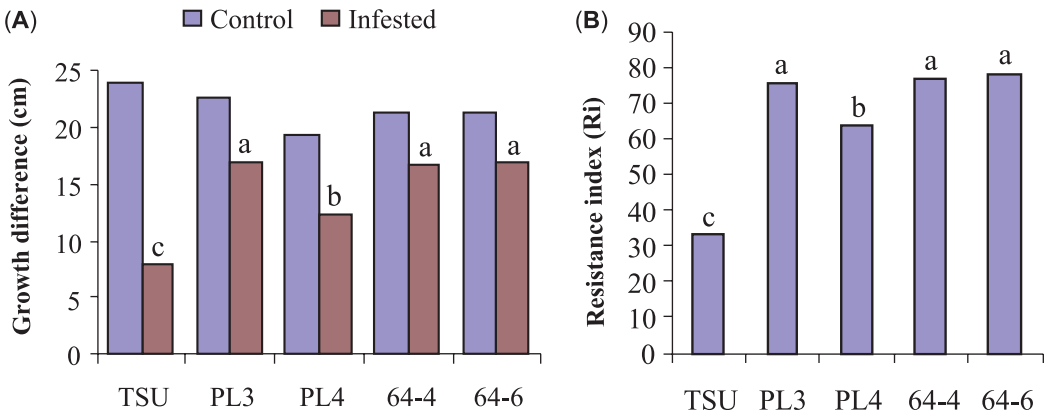

Fig. 4A–B. Evaluation of the BPH resistance level of the *Bph1*/*Bph2* pyramided line by BPH bioassay. (A) test for growth differences. (B) test for resistance indexes (Ri). Means with different alphabets are significantly different at the 1% level according to Duncan's test.

result showed that the effect of pyramiding was not additive and the resistance level of the pyramided line was similar to that of the *Bph1*-single introgressed line (Fig. 4B).

Bph1 and *Bph2* genes have already been broken down by the appearance of virulent BPH biotypes in many Asian countries (FEUER 1976; IRRI 1982). Therefore they can no longer serve as valuable resistance sources in the practical breeding for BPH resistance. However, these two major resistance genes are functional in the BPH bioassay using the biotype 1 as we showed in Fig. 4. An important point drawn from this fact is that they can still serve as a useful experimental means for achieving a final goal of map-based cloning, which depends on the critical phenotyping and available DNA markers. Prior to this goal, a fine structure of the chromosomal region covering the two major BPH resistance genes can effectively be studied using the pyramided line constructed in the present study.

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