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ORIGINAL PAPER

Development of PCR-based allele-specific and InDel marker sets for nine rice blast resistance genes

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Abstract Blast resistance is one of the most important traits in rice breeding, and application of molecular markers for blast resistance breeding is likely to allow the rapid screening for the trait during early growth stages, without the need for inoculation of pathogen and phenotyping. Allele-specific PCR markers and insertion/deletion (InDel) markers, which genotype single-nucleotide polymorphisms and InDel polymorphisms, respectively, are useful tools for marker-assisted selections. We developed sets of allele-specific PCR and InDel markers for nine rice blast resistance genes—*Piz, Piz-t, Pit, Pik, Pik-m, Pik-p, Pita, Pita-2*, and *Pib*—which are commonly used in Japanese blast resistance rice breeding programs. For each resistance gene, we used the segregation information from thousands of progeny in several crosses or published gene locations to generate a marker that cosegregated with the gene and markers that closely flanked the gene on either side. The developed cosegregating markers uniquely discriminated among each of the lines with the individual resistance genes (except for *Pita* and *Pita-2*). Therefore, these markers will likely facilitate the development of multiline cultivars carrying one or a combination of these nine blast resistance genes. In addition, the systems we developed may be valuable tools in the quality control of seed production from blast-resistant multiline cultivars.

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Introduction

Blast disease, caused by *Magnaporthe grisea*, is one of the most devastating worldwide diseases of rice. Introgression of rice blast resistance genes into improved cultivars has been considered a cost-effective and environmentally beneficial means of minimizing crop losses due to the disease. However, when the blast resistance of a bred cultivar is based on a single resistance gene, it can rapidly be overcome by the emergence of compatible races of the pathogen (Kiyosawa 1974). To prevent disease development without such breakdown in disease resistance, the use of multiline cultivars, which are groups of near-isogenic lines (NILs) harboring various individual resistance genes in a common background, is considered a useful strategy for disease control in breeding systems (Zhu et al. 2000; Koizumi 2001; Mundt 2002; Koizumi et al. 2004).

Differential varieties, each of which retains one of the following blast resistance genes, *Pia, Pii, Pik* and its possible alleles *Pik-s, Pik-p* and *Pik-m, Piz* and its possible allele *Piz-t, Pita* and its possible allele *Pita-2, Pib,* and *Pit*, have been developed for pathogenicity testing (Yamada et al. 1976; Kiyosawa 1984). These varieties have also been used as blast resistance gene donors for the development of blast-resistant cultivars including NILs (Kiyosawa and Ling 2001; Koizumi 2001). During breeding processes, it is often difficult to monitor the presence of individual resistance genes in breeding

lines, because the phenotype-based screening frequently is affected by the developmental stage and environmental conditions. In contrast, DNA markers tightly linked to resistance genes offer an efficient and rapid means to select for many types of blast resistance genes without performing phenotype-based screening.

Several types of DNA markers have been developed for marker-assisted selections (Mohan et al. 1997; Kumar 1999). The prototypes of DNA markers, restriction fragment length polymorphisms (RFLPs), are laborious to use in a breeding program. To improve efficiency, PCR-based cleaved amplified polymorphic sequences and simple sequence repeat (SSR) markers have been devised, but each of these still has its drawbacks. In particular, it is often difficult to develop these kinds of markers within a small target gene region because of a shortage of appropriate polymorphisms.

Recently, genome sequencing projects revealed that single-nucleotide polymorphisms (SNPs) and small insertions/deletions (InDels) are highly abundant and distributed throughout the genome in rice (Nasu et al. 2002; Yu et al. 2002, 2005; Feltus et al. 2004). The abundance of these polymorphisms makes the SNP marker system a more attractive tool than any other marker system, especially during attempts to develop markers for many target genes simultaneously—investigators can easily find SNPs between the genomes of a blast-resistant donor and a susceptible recipient cultivar and then generate a sufficient number of SNP markers within target gene regions.

In a previous study, we demonstrated that the modified allele-specific PCR marker system offers a simple and efficient SNP genotyping method (Hayashi et al. 2004). By using allele-specific PCR primers, the genotype of an SNP can be assessed according to the presence or absence of PCR-amplified products on standard agarose gels. This technique simplifies the genotyping procedure, when combined with a rapid method of template DNA extraction (Hayashi et al. 2004).

In the present study, we used our PCR-based SNP and InDel marker system to develop DNA markers for introducing rice blast resistance genes at the *Piz, Piz-t, Pit, Pik, Pik-m, Pik-p, Pita, Pita-2*, and *Pib loci*, which have all been used frequently in blast resistance rice breeding in Japan, into the leading Japanese rice cultivar Koshihikari and other elite Japanese cultivars. To this end, we defined the precise genetic locus of each gene through fine-structure mapping by using large-scale F_2 segregating populations or previously reported information on gene location. The markers developed in this way reliably assayed the genotypes of the SNPs or InDels tightly linked to each of the resistance genes. In addition, we developed another set of markers that are specifically used for discriminating an individual line among a group of blast-resistant NILs. The markers we developed are valuable tools for introgression and pyramiding of the blast resistance genes into rice cultivars as well as for monitoring the seed quality of blast-resistant multilines.

Materials and methods

Plant materials

To map target genes and to evaluate the linkage between the developed markers and the genes, we used the F_2 segregating plants from crosses between blast-resistant cultivars (donor parents) and blast-susceptible cultivars (recipient parents). We used the following nine differential varieties (Yamada et al. 1976; Kiyosawa 1984) as donor cultivars: Fukunishiki (*Piz*$^+$), Toride 1 (*Piz-t*$^+$), K59 (*Pit*$^+$), Kanto 51 (*Pik*$^+$), Tsuyuake (*Pik-m*$^+$), K60 (*Pik-p*$^+$), BL 1 (*Pib*$^+$), Yashiromochi (*Pita*$^+$), and Pi No.4 (*Pita-2*$^+$). As the blast-susceptible cultivar, we used the Japanese *japonica* cultivar Koshihikari. For the fine-structure mapping of *Pik* and *Pik-m*, we used two blast-susceptible cultivars, 99SL-44 and OISL235 (Ebitani et al. 2005), as the parents of the crosses. These are Nipponbare (99SL-44)- and Koshihikari (OSIL235)-based chromosomal substitution lines, in which a segment from chromosome 11 in each line is replaced with the corresponding segment from the Kasalath (an *indica* cultivar) genome. Information about the F_2 segregating plants that we used is shown in Table 1.

In addition to the previously mentioned cultivars, we included two blast-resistant cultivars, K3 (*Pik-h*$^+$) (Kiyosawa 1969) and Shin 2 (*Pik-s*$^+$) (Kiyosawa 1984), and two groups of blast-resistant NILs, Hokuriku ILs and Sasanishiki BLs, to evaluate the markers developed in this study. Hokuriku ILs are a group of four Kinuhikari-based NILs, comprising Hokuriku IL1 (*Pita-2*$^+$), IL2 (*Piz-t*$^+$), IL3 (*Pib*$^+$), and IL4 (*Piz*$^+$), whereas Sasanishiki BLs are a group of six Sasanishiki-based NILs, comprising Sasanishiki BL1 (*Pik*$^+$), BL2 (*Pik-m*$^+$), BL3 (*Piz*$^+$), BL4 (*Piz-t*$^+$), BL5 (*Pita-2*$^+$), and BL6 (*Pita*$^+$).

Seeds of the rice cultivars Shin 2, Kanto 51, Tsuyuake, Fukunishiki, Yashiromochi, Pi No.4, Toride 1, K60, BL1, K59, and the six Sasanishiki BLs were obtained from the gene bank at the National Institute of Agrobiological Sciences, Japan. Seeds of 99SL-44 and OISL235 were provided by Dr. M. Yano of the National Institute of Agrobiological Sciences, and the

Table 1 F_2 segregating populations used for mapping blast resistance genes and for confirming linkage between the developed markers and their target genes

Gene	Blast-resistant parent	Blast-susceptible parent	Number of individuals comprising the population
Piz	Fukunushiki	Koshihikari	2,680
Piz-t	Toride 1	Koshihikari	2,665
Pit	K59	Koshihikari	2,833
Pik-m	Tsuyuake	99SL-44	2,118[a]
	Tsuyuake	Koshihikari	390[b]
Pik	Kanto 51	OSIL 235	744[a]
	Kanto 51	Koshihikari	180[b]
Pik-p	K60	Koshihikari	309[b]
Pib	BL1	Koshihikari	42[b]
Pita	Yashiromochi	Nipponbare	42[b]
Pita-2	Pi No.4	Koshihikari	50[b]

[a] These populations were used only for mapping genes

[b] These populations were used for checking linkage between the developed markers and their target genes

four Hokuriku ILs were provided by the Laboratory of Rice Breeding, National Agricultural Research Center, Japan.

Phenotypic analysis

To score the phenotypes of the F_2 individuals, the blast resistance of ca. 35 F_3 progenies of each F_2 individual was evaluated by using the method described in Hayashi et al. (2004). Seedlings of the examined plants were inoculated with *Magnaporthe grisea* race 007.0. The inoculated plants were evaluated as resistant or susceptible by the scoring disease reaction.

DNA sequences

Nucleotide sequences from the examined cultivars were determined by the direct sequencing of PCR-amplified fragments as described (Hayashi et al. 2004). To design PCR primers, we used genome sequence data of the cultivar Nipponbare from the DDBJ/GeneBank/EMBL database. The corresponding locations for *Pita, Pita-2*, and *Pib* in the Nipponbare genome were deduced using the Rice Genome Automated Annotation System (RiceGAAS; http://www.ricegaas.dna.affrc.go.jp/) (Sakata et al. 2002).

PCR-based markers to genotype SNPs and InDels

An allele-specific PCR marker assays the genotype of a SNP (homozygous for allele A, allele B, or heterozygous) by examining the presence or absence of PCR amplification from a pair of amplification reactions—one with a primer pair specific to allele A and the other specific to allele B. The allele A-specific primer pair amplifies a product only from template DNA having allele A at the SNP site, whereas the allele B-specific primer does so only from the DNA having allele B at the SNP. An InDel marker assays the genotype of an InDel polymorphism in light of the difference in the size of the PCR products from amplification of an InDel-containing DNA fragment by using flanking markers. We used the rapid method for isolating rice DNA used in the SNP and InDel genotyping, as well as the development and PCR amplification schemes for these markers described in Hayashi et al. (2004).

For a multiplex assay, the 20-μl PCR reaction contained 2 μl rice DNA, 0.5U Hot Start *Taq* polymerase (Takara, Osaka, Japan), 2 μl 10× PCR buffer (supplied with the enzyme), and 1.6 μl 2.5 mM dNTPs. The primers used in the multiplex reaction were: for set 1, 0.6 μl each of a resistance gene-specific primer (25 μM) and reverse primer (25 μM) for the markers k39575 and ta5, 0.32 μl each of the two primers for the marker zt56591, and 1.3 μl each of the two primers for the marker k644. For set 2, the primers were 0.5 μl each of a resistance gene-specific primer (25 μM) and reverse primer (25 μM) for the markers z565962, b213, and t256, and 0.16 μl each of the two primers for the marker k6415. The amplification scheme was the same as that used for the dominant allele-specific markers.

In linkage analyses, the genotype evaluation of an SNP or an InDel in an F_2 individual was carried out using DNA isolated from a seedling leaf of the F_2 plant or from ca. ten seeds harvested from the plant.

The sequences of markers developed and the sizes of the products PCR-amplified with the markers are listed in Table 2.

Results

Strategy for developing allele-specific and InDel markers for blast resistance genes

We developed allele-specific and InDel markers for the blast resistance genes *Piz, Piz-t, Pit, Pik, Pik-m, Pik-p, Pita, Pita-2*, and *Pib*. We generated one group of markers for each gene, comprising one (or more) marker that cosegregated with the gene and markers that closely flanked the gene on both sides. Because the chromosomal locations of *Piz, Piz-t, Pit, Pik, Pik-m*, and *Pik-p* had not been precisely determined, we used large-scale F_2 segregating populations to map their

Table 2 Primer sequences of the developed SNP and InDel markers

Marker	Target genes	Koshihikari-specific primer	Blast resistance gene-specific primer	Reverse primer	Size of products (bp)
z4794[a]	*Piz, Piz-t*	cacgccacccttcaatggagact		tgaatgtgagaggttgactgtgg	139, 194
z60510	*Piz, Piz-t*	ggagttggtgcgacggtgccgttat	ggagttggtgcgacggtgccgttac	gcgcggaccggccagctaggtgac	356
z5765	*Piz, Piz-t*	aatgtgaaattggatgagccggata	aatgtgaaattggatgagccggatg	ttaccgatgttcgtcgctctcagg	344
z56592	*Piz*	ggacccgcgttttccacgtgtac	ggacccgcgttttccacgtgtaa	aggaatctattgctaagcatgac	292
z565962	*Piz*	aagaaataatatttttgaaacatggcaaat	aagaaataatatttttgaaacatggcaaag	ccatggtggtaactggtatgtg	267
zt56591	*Piz-t*	ttgctgagccattgttaaacg	ttgctgagccattgttaaaca	atctcttcatatatatgaaggccac	257
zt5659	*Piz-t*	ggacccgcgttttccacgtgtac	ggacccgcgttttccacgtgtaa	catccacgggctctcggacatc	196
t311	*Pit*	cgtgaacccaaggcaccagtattc	cgtgaacccaaggcaccagtatta	catgtagttctggatgttgtagctactc	287
t256	*Pit*	ggatagcagaagaacttgagactg	ggatagcagaagaacttgagacta	catgtctttcaacataagaagttctc	322
t8042	*Pit*	ctcaagattgtatcgtcgacgacta	ctcaagattgtatcgtcgacgactc	gagaggtttgcagccagaccagg	155
k6816[a]	*Pik, Pik-m*	tcgccgatgcggttgatttactc		cgtattttgtgttgttaggagataagg	339, ca. 380
k2167[a]	*Pik, Pik-m*	cgtgctgtcgcctgaatctg		cacgaacaagagtgtgtcgg	619, ca. 300
k641	*Pik-m, Pik-p*	ggctggaacaccaacatccatgg	gctgggacaccaacatccatgc	gcgctggacttggaactagtgc	387
k6441	*Pik-m*	tgtaaaatactttctatgcgcaggt	tgtaaaatactttctatgcgcaggc	gtttatggagagagtagtcgctg	404
k4731	*Pik-m*	gcagatgcatcagccagtgagtg	gcagatgcatcagccagtgagtt	gtgcaggaccggcacgcag	171
k7237	*Pik-m*	agtgtgcctcgttgccctgttctg	agtgtgcctcgttgccctgttcta	tatagcttgcattagatcctctctgttga	246
k6438	*Pik*	gcgaccctgtctttggactgc	gcgaccctgtctttggactgg	gaatgatgaggagagaaggctgtcg	226
k6415	*Pik*	ctaatggaattaaacggttgagctg	ctaatggaattaaacggttgagcta	atcccgatgtcatcgatcac	140
k8823	*Pik*	gttgtgggttcctctatacaact	gttgtgggttcctctatacaaca	gcatgacagatggaagtgtagatgg	168
k8824[b]	*Pik*	ccacgctcctagctaccccg	gttgtgggttcctctatacaaca	Acaagggaacccagaaactc atcgcagcgactgtatgtgc	555, 222
k3951	*Pik*	aagtaacaacatggtcaatagtac	aagtaacaacatggtcaatagtaa	ccagaatttacaggctctgg	192
k39512	*Pik*	gccacatcaatggctacaacgtc	gccacatcaatggctacaacgtt	ccagaatttacaggctctgg	112
k39575	*Pik-p*	ggtgtttgggaacctgaaccctg	ggtgtttgggaacctgaacccta	tttctgttcgtcggatgctc	158
k403	*Pik-p*	catcttgacgacaacgacaccattagtta	cttgacgacgacgacaccattagttg	ccaaaatgaacaaaccgattcgac	350
k3957	*Pik-p*	atagttgaatgaatggaatggaac	atagttgaatgaatggaatggaat	ctgcgccaagcaataaagtc	148
b213	*Pib*	gcattagatagtgatgaaagccga	gcattagatagtgatgaaagccgg	tgttcatccaggcaattggc	218
b28	*Pib*	gactcggtcgaccaattcgca	gactcggtcgaccaattcgcc	atcaggccaggccagatttg	388
b2	*Pib*	gcattagatagtgatgaaagcata	gcattagatagtgatgaaagccgg	aatggactggtgttcatccaggc	215
b3989	*PIb*	tgtaagcgcgggatatccga	tgtaagcgcgggatatccgg	ttgtgagctttgccactccac	464
ta642	*Pita, Pita-2*	ggtcaaacatgaagtgagatggg	ggtcaaacatgaagtgagatgga	ctgcatcacacttctgatgaac	306
ta801	*Pita, Pita-2*	caagccaaatctgaatcttaccaa	caagccaaatctgaatcttaccat	tatggaaatgttgccccaatctg	139
ta3	*Pita, Pita-2*	ggagtacgtgtctttttccatgtata	ggagtacgtgtctttttccatgcatt	cttggtcctacctgtcatacacac	173
ta577	*Pita, Pita-2*	atgaacaccacagcctaaacg	atgaacaccacagcctaaacc	cagacccgaaacaacactagg	303
ta5	*Pita, Pita-2*	cagcgaactccttcgcatacgcg	cagcgaactccttcgcatacgca	cgaaaggtgtatgcactatagtatcc	515

Tm of the PCR reactions for all the markers is 60°C

[a] InDel marker

[b] Two reverse primers are necessary for this allele-specific PCR

locations. We first developed two markers that flanked a target gene on both sides by using a subpopulation (typically 200 plants) randomly selected from an F_2 population. Hereafter, we will refer to these two markers as initial markers. Then, using these initial markers, we selected individuals that harbored chromosomal recombination in the regions targeted by using the initial markers from the F_2 population. These selected individuals were grouped as a subset of the mapping population and used for further linkage analysis. Subsequently, we screened SNPs and InDels by sequencing DNA segments in the target gene region from blast-resistant donor and recipient parents, developed allele-specific or InDel markers that genotyped the identified SNPs or InDels, and finally analyzed the linkage between these developed markers and the target gene by using the subset of F_2 individuals.

Because the precise locations of *Pita, Pita-s*, and *Pib* have already been determined (Wang et al. 1999; Bryan et al. 2000), we needed only to survey the SNPs and InDels around each gene region and generate markers. Through linkage analyses using small-scale F_2 populations, we confirmed that the generated markers were mapped to the target gene regions.

Developing markers for *Piz* and *Piz-t*

In our previous study, using F_2 populations comprising ca. 190 individuals, we mapped *Piz* and *Piz-t* to a ca. 500 kb segment between the markers z4794 and z5765 on chromosome 6 (Fig. 1) (Hayashi et al. 2004). In the present study, to define the location of *Piz* more precisely, we used 2,680 F_2 segregating plants derived from the cross of blast-susceptible Koshihikari × Fukunishiki

(*Piz*$^+$); to fine-map *Piz-t*, we used 2,665 F_2 plants derived from the cross Toride 1(*Piz-t*$^+$) × Koshihikari. As subsets of these F_2 populations, we selected 24 individuals for *Piz* and 31 for *Piz-t* that harbored recombination in the gene region by using the initial flanking markers z4794 and z5765. Through sequencing a total of ca. 4,000 bp DNA of the region between these two markers, we identified 21 SNPs and 5 InDels between Fukunishiki (*Piz*$^+$) and Koshihikari and 11 SNPs and 2 InDels between Toride 1 (*Piz-t*$^+$) and Koshihikari. PCR-based markers in the target gene region were generated using these polymorphisms. By analyzing the linkage between the developed markers and the target genes, we developed the allele-specific PCR marker z56592 as the cosegregating marker for *Piz* and marker zt5659 as that for *Piz-t*. As flanking markers for both genes, we established z4794 on one side and the markers z60510 and z5765 on the other side (Fig. 1).

Developing markers for *Pit*

The gene *Pit* was reported to be located on the short arm of chromosome 1, close to the RFLP marker R1613 (Kaji et al. 1997). To develop initial flanking markers for the *Pit* locus, we generated several markers randomly around the R1613 locus and analyzed the linkage between these markers and *Pit* by using a small subpopulation (280 individuals) of the F_2 population derived from the cross K59 (*Pit*$^+$) × Koshihikari. Two markers, t311 and t8042, were selected as the initial flanking markers of *Pit* at distances of 0.89 and 0.53 cM, respectively, on either side of the gene (Fig. 1). Using these two markers, we selected a subset of 41 individuals from the 2,833 F_2 segregating plants. We then identified 57 SNPs and 14 InDels between the cultivars K59 and Koshihikari from a total of 6,900 bp of the sequence located between the two initial markers and we developed PCR-based markers. Finally, by analyzing the linkage between the developed markers and *Pit* by using the subset, we established the marker t256 as the cosegregating marker for *Pit* and t311 and t8042 as the two closely flanking markers on either side of the gene (Fig. 1).

Developing markers for *Pik, Pik-m*, and *Pik-p*

Pik, Pik-m, and *Pik-p* genes are retained in the cultivars Kanto 51, Tsuyuake, and K60, respectively. These genes are believed to constitute multiple alleles and were reported to be located on the long arm of chromosome 11, close to the telomere (Inukai et al. 1994; Kiyosawa and Ling 2001). To efficiently develop markers for these genes, we first determined the location of one of these genes, *Pik-m*, and then applied information about its chromosomal location to the mapping of *Pik* and *Pik-p* on the assumption that these three genes are truly allelic, i.e., they are located at the same chromosomal position.

To finely map *Pik-m*, we first planned to use an F_2 segregating population derived from the cross Tsuyuake (*Pik-m*$^+$) × Koshihikari. Unfortunately, in the presumed *Pik-m* region, the SNP level between these two parents was too low to develop a sufficient number of markers. To identify a number of SNPs for developing markers in this region, we prepared another F_2 population, in which the chromosome segment substitution line 99SL-44 was used instead of Koshihikari as the blast-susceptible parent. Because the chromosomal segment of the *Pik-m* region of 99SL-44 is derived from an *indica* rice cultivar, Kasalath, we expected to obtain sufficient polymorphisms between the two parents. To map *Pik-m*, we first developed two initial flanking markers, k6816 and k403, by using ca. 200 plants from the 2,118 F_2 segregating plants. Then, we selected 44 individuals as the subset of the F_2 population. As expected, between the two parents we found numerous polymorphisms in the *Pik-m* region: 10 SNPs and 5 InDels in a total of 1,045 bp segments. By using the subset of the F2 population to analyze the linkage between the markers developed to genotype these detected polymorphisms and the gene *Pik-m*, we generated a genetic map of *Pik-m* (Fig. 1). *Pik-m* was revealed to cosegregate with the markers k2167 and k4731. According to the Nipponbare DNA genome sequence data (http://www.rgp.dna.affrc.go.jp/cgi-bin/statusdb/status.pl), the physical distance between these markers was estimated to be as large as 203 kb, thus indicating that recombination in this region was highly suppressed.

We applied this positional information to the mapping of *Pik*. Using the two initial flanking markers used for *Pik-m*, we selected 23 F_2 individuals as the subset of the mapping population from 744 F_2 segregating plants derived from the cross Kanto 51 (*Pik*$^+$) × OISL235, a blast-susceptible chromosome segment substitution line. Analysis of linkage between three markers in this region—k2167, k3951, and k8824—and *Pik* revealed that the markers k2167 and k3951 both cosegregated with *Pik* (Fig. 1). Both *Pik* and *Pik-m* cosegregated to marker k2167, indicating that these genes were in fact located at the same position, or at least close to each other.

The markers developed above for *Pik* and *Pik-m* were not used directly as selection markers, because they were specific for *indica* rather than *japonica*

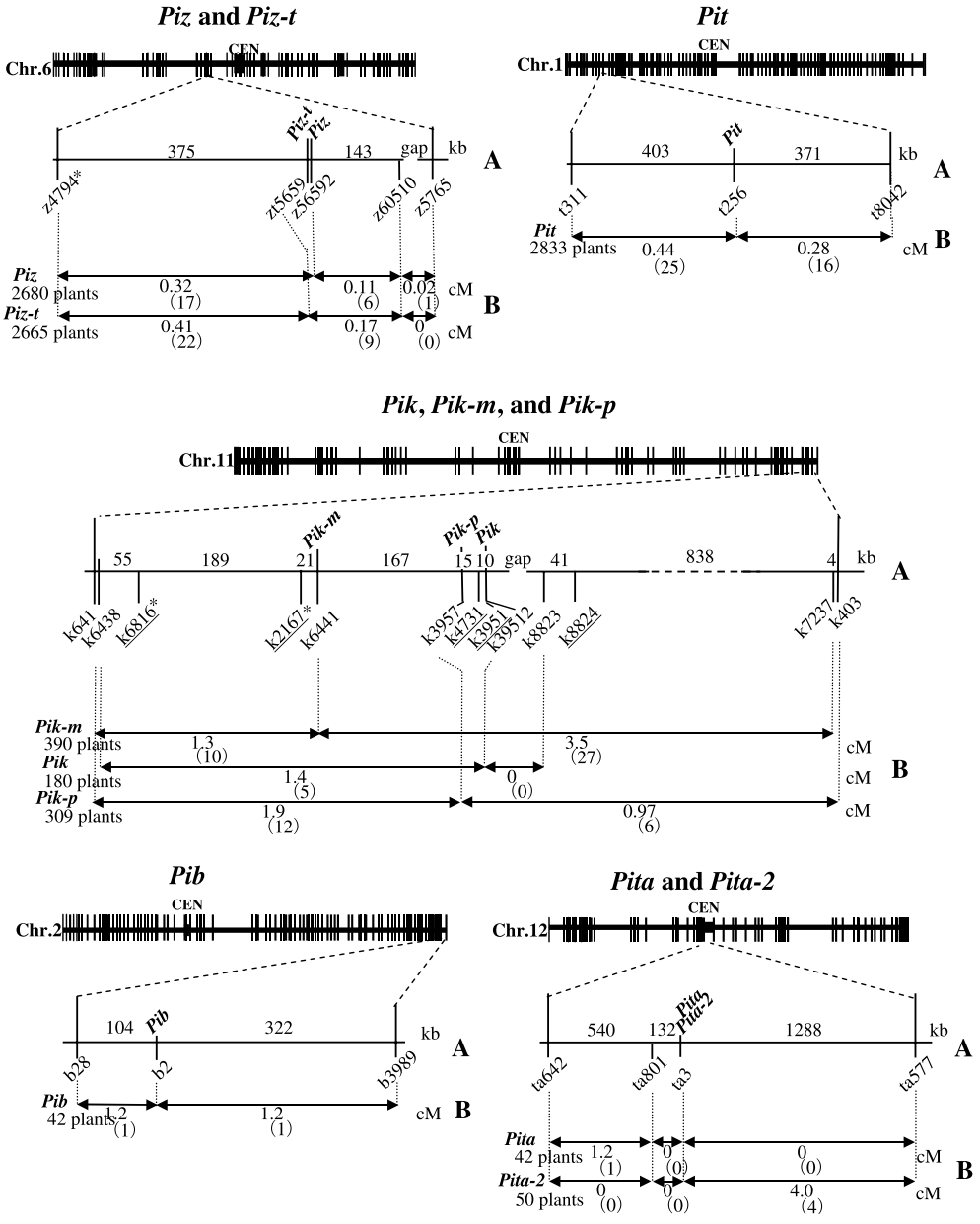

Fig. 1 Genetic and physical locations of the SNP and InDel markers in the *Piz*, *Piz-t*, *Pit*, *Pik, Pik-m*, *Pik-p*, *Pib*, *Pita*, and *Pita-2* regions. *A* Physical maps for these genes. The physical distances in the figure were calculated by using the published genome sequence data of Nipponbare (http://www. rgp.dna. affrc.go.jp/). *B* Genetic maps for these genes. Each of these maps was constructed on the basis of the F_2 segregation analysis. *Asterisks* indicate InDel markers. *Underlined markers* were used only for mapping gene regions. The numbers of F_2 individuals analyzed in this study are indicated at the *left side* of each genetic map and the numbers of recombinants detected between the markers are shown in *parentheses*

sequences. As a next step, using the positional information obtained we developed selection markers for the *japonica* forms of these genes. We searched for polymorphisms in a total of ca. 4,763 bp DNA in the regions between the markers k2167and k4731 of Koshihikari and Kanto 51 (*Pik*$^+$) and between k2167 and k3951 of Koshihikari and Tsuyuake (*Pik-m*$^+$). A total of 12 SNPs and 2 InDels were identified between Kanto 51 and Koshihikari, whereas we found only two SNPs between Tsuyuake and Koshihikari. We assigned two markers, k39512 and k6441, as the cosegregating selection markers for *Pik* and *Pik-m*, respectively (Fig. 1). As flanking markers, we generated the markers k6438 and k8823 for *Pik* and k641 and k7237 for *Pik-m* (Fig. 1). The relative locations of these markers to each gene were evaluated by linkage analyses using the F_2 populations comprising 180 segregating plants from the cross Kanto 51 × Koshihikari for *Pik* and 390

segregating plants from the cross Tsuyuake × Koshihikari for *Pik-m* (Fig. 1).

We also applied the positional information of *Pik* and *Pik-m* to the developing markers for *Pik-p*. Comparing a total of 2,731 bp of the sequence from the k6816—k403 region between the cultivar K60 (*Pik-p*$^+$) and Koshihikari, we identified 8 SNPs and 5 InDels. Through the linkage analysis of markers developed with these polymorphisms and *Pik-p* by using the 309 F_2 segregation plants from the cross K60 × Koshihikari, we assigned k3957 as the cosegregating marker and k641 and k403 as flanking markers (Fig. 1). This mapping showed that *Pik-p* was located within the *Pik*—*Pik*-m region, thus suggesting that these three genes actually were allelic.

Developing markers for *Pib, Pita*, and *Pita-2*

The gene *Pib* has been localized to a position close to the telomere of chromosome 2 (Wang et al. 1999). We used this information to develop PCR-based selection markers for this gene. We compared the genomic sequence around the *Pib* locus between the *Pib*-containing cultivar BL1 and Koshihikari. The high frequency of polymorphism (i.e., 78 SNPs and 11 InDels) detected in a total of 1,575 bp DNA segments adjacent to the 5′-untranslated region of *Pib* allowed us to develop b2, a candidate tightly-linked selection marker (Fig. 1). In contrast, the SNP and InDel levels were quite low in regions flanking the gene. Nevertheless, we identified three SNPs in a total of 5,404 bp segments on one side of the gene and developed a candidate flanking marker, b28. On the other side of the gene, we identified four SNPs and two InDels in a total of 1,300 bp of the sequence and developed another candidate marker, b3989. Through the linkage analysis of these markers with *Pib* by using 42 F_2 segregating plants derived from the cross Koshihikari × BL1, we confirmed the linkage between these markers and *Pib* (Fig. 1).

Pita is located at a position in the centromere of chromosome 12 (Bryan et al. 2000). Its possible allele *Pita-2* is tightly linked to *Pita* (Wang et al. 2002). To develop markers for *Pita*, we searched for polymorphisms by comparing the DNA sequence in the gene region from Koshihikari with that from the *Pita*-containing cultivar Yashiromochi. For *Pita-2*, we compared the sequence in the gene region from Koshihikari and that from the *Pita-2*-containing cultivar Pi No. 4. Between Koshihikari and Yashiromochi, eight SNPs were identified in the region adjacent to *Pita* and four and two SNPs in the regions flanking the gene on either side, respectively. We established a marker, ta3, as the cosegregating marker for *Pita* and ta642, ta801, and ta577 as the flanking markers. We could not find any polymorphism between Yashiromochi and Pi No.4. Thus, the 4 markers developed for *Pita* (ta642, ta801, ta3, and ta577) could not discriminate between *Pita* and *Pita-2*. Through the analyses of linkage between these markers and *Pita* and *Pita-2* with 42 F_2 segregating plants from the cross Nipponbare × Yashiromochi (*Pita*$^+$) and 50 F_2 plants from the cross Koshihikari × Pi No.4 (*Pita-2*$^+$), we confirmed that these markers were positioned in the *Pita*–*Pita-2* region (Fig. 1).

Developing markers for the unique discrimination of each of the rice blast resistance genes

Once a group of blast-resistant multiline cultivars has been developed in a common background, a technique that discriminates each member of the multiline is essential for quality control. A set of DNA markers that uniquely identifies each member of the NILs should afford an efficient and reliable means of discrimination. Of the markers we developed that cosegregate with their respective genes, some were not necessarily specific to their target genes: the markers z56592, k39512, k3957, and ta3, which are the cosegregating markers for *Piz*, *Pik*, *Pik-m*, and *Pita/Pita*-2, respectively, amplify PCR products from cultivars containing blast resistance genes other than the ones for which they were developed. To convert these markers to the ones unique to their respective genes, we searched for SNPs specific to a single blast resistance gene. Unfortunately in the case of *Pita* and *Pita-2*, as previously, we failed to find an SNP that distinguishes between these genes. However, we successfully identified SNPs unique to *Piz, Pik*, *Pik-p*, and *Pita/Pita-2*. Using these SNPs, we developed additional markers—z565962, k6415, k39575, and ta5—that specifically discriminated the respective blast resistance genes. Although the new marker set failed to distinguish *Pita* from *Pita-2*, they showed amplification bands unique to each NIL (Fig. 2).

Developing a multiplex detection system

To discriminate all of the rice blast resistance genes by the use of our markers, eight independent PCR amplifications were needed. A multiplex PCR would greatly reduce the number of PCR amplifications necessary for discrimination, allowing efficient and unique identification of the resistance genes. In a multiplex PCR, instead of a single primer pair, a mixture of several primer pairs is used to amplify several distinct genome regions, enabling the simultaneous genotyping of several distinct polymorphic loci. To develop a multiplex PCR system, we modified markers zt5659 and b2 into zt56591 and

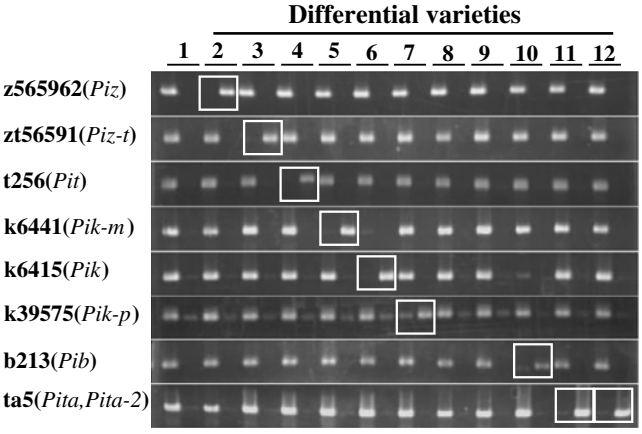

Fig. 2 PCR amplification patterns of the eight SNP markers that discriminate each of the nine blast resistance genes (except for *Pita* and *Pita-2*) and a blast-susceptible cultivar, Koshihikari. DNAs from the cultivars (*1*) Koshihikari, (*2*) Fukunishiki (*Piz*$^+$), (*3*) Toride 1 (*Piz-t*$^+$), (*4*) K 59(*Pit*$^+$), (*5*) Tsuyuake (*Pik-m*$^+$), (*6*) Kanto 51(*Pik*$^+$), (*7*) K60 (*Pik-p*$^+$), (*8*) Shin 2 (*Pik-s*$^+$), (*9*) K3 (*Pik-h*$^+$), (*10*) BL1 (*Pib*$^+$), (*11*) Yashiromochi (*Pita*$^+$), and (*12*) Pi No.4 (*Pita-2*$^+$) were used as templates for PCR amplification. In each pair of amplification patterns, the *left lane* indicates the amplification products obtained with the Koshihikari-specific primer and the *right lane* that of the blast-resistant cultivar-specific primer. *Boxes* indicate PCR patterns obtained with a pair of the DNA template and the primer specific to the cultivar. PCR amplification with the Koshihikari-specific primer k6415 was performed at the annealing temperature of 53°C; all the others were annealed at 60°C

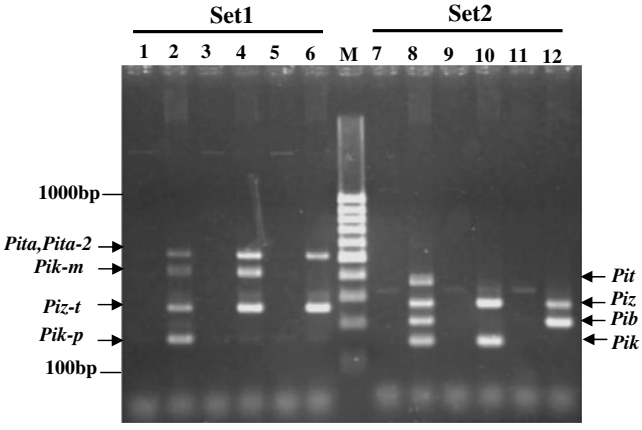

Fig. 3 PCR product patterns obtained for two NIL populations amplified with the two sets of multiplex markers: set 1 comprising the markers ta5, k6441, zt56591, and k39575, and set 2 comprising markers t256, z565962, b213, and k6415. The templates were from Koshihikari (*lanes 1, 7*), the mixture of the nine blast-resistant donor cultivars (*2, 8*), Sasanishiki (*3, 9*), the mixture of six Sasanishiki BLs [Sasanishiki BL1 (*Pik*), BL2 (*Pik-m*), BL3 (*Piz*), BL4 (*Piz-t*), BL5 (*Pita-2*), and BL6 (*Pita*)] (*4, 10*), Kinuhikari (*5, 11*), and the mixture of the four Hokuriku ILs [Hokuriku IL1 (*Pita-2*), IL2 (*Piz-t*), IL3 (*Pib*), and IL4 (*Piz*)] (*6, 12*). *M* indicates the 100 bp-ladder

b213, respectively, so that their PCR products do not overlap those from the other markers on agarose gels. Then, we grouped the eight cosegregating markers into two sets: set 1 contained the markers ta5, k6441, zt56591, and k39575, and set 2 contained the markers t256, z565962, b213, and k6415. In these sets, the concentration of each primer was adjusted so that bands of equal intensities could be obtained on agarose gels (see Materials and methods). With these marker sets, PCR amplifications with two reaction tubes discriminated the blast resistance genes. Figure 3 shows one example of the results, in which the templates were a mixture of DNAs from the four Hokuriku ILs (*Pita-2*, *Piz-t*, *Pib*, and *Piz*) and another mixture of DNAs from six Sasanishiki BLs (*Pik, Pik-m, Piz, Piz-t, Pita-2*, and *Pita*). Set 1 and set 2 markers yielded the expected products for both of the mixed DNA solutions, although the use of set 2 identified some minor non-specific bands.

Discussion

To develop molecular markers that can be used reliably in marker-assisted selection, the recombination frequency between the markers and their target genes must be as low as possible. This goal can be achieved by producing markers tightly linked to the gene. Our approach makes maximum use of identified polymorphisms, both SNPs and InDels, which allowed the successful development of selection markers for the nine rice blast resistance genes. Among the 329 polymorphisms identified between Koshihikari and the 9 differential cultivars, 274 SNPs and 44 InDels could be considered candidate polymorphisms for the development of allele-specific PCR markers (data not shown). If 67% of these SNPs were available during the development of markers (as for markers at the *Piz* locus) (Hayashi et al. 2004), one SNP marker could be generated every 250 bp, on average. This estimation indicates that a large number of SNP markers can be generated within a small target genome region by using our method. In addition, this method is effective for developing markers in genome regions with only a few polymorphisms between the two parent cultivars, as we showed in the current study when developing markers in the *Pik-m* region.

An annotation analysis of genes predicted from the Nipponbare genome sequence information is useful to assess the reliability of mapping and developed markers. The Nipponbare genome carries approximately 500 genes having the nucleotide binding site–leucine-rich repeats (NBS–LRR) motif, such as *Pib* and *Pita* homologues, and these are considered candidate resistance genes (Monosi et al 2004; Zhou et al 2004).

By using RiceGAAS (Sakata et al. 2002) to analyze the Nipponbare DNA sequences in the mapped gene regions of the *Piz* family, *Pik* family, and *Pit* we found that all these regions contained clusters of the NBS–LRR gene homologues (data not shown). This result suggests that our mappings were performed properly and tagged disease resistance gene regions.

For the *Pib*, *Pita*, and *Pita-2* genes, Jia et al. (2002) and Fjellstrom et al. (2004) reported the development of PCR-based SSR or STS markers by using the positional information for these genes. In addition to these previous markers, we developed our markers for these genes, because we consider that having a group of allele-specific PCR-type markers that identified most of the commonly used blast resistance genes would greatly benefit blast-resistant multiline breeding.

Although our marker set failed to discriminate *Pita* from *Pita-2*, it uniquely discriminated each of the other blast resistance genes. We consider these markers to be an excellent tool for pyramiding resistance genes. It is often difficult to monitor the presence of individual resistance genes and pyramid these in breeding lines by using traditional phenotypic screening because the action of one resistance gene may mask the action of others (Hittalmani et al. 2000). Our markers, because of their specificity for each gene, enabled the introgression of additional blast resistance genes into a line in which several had already been introduced. Furthermore, these unique markers enabled us to devise a multiplex detection system to improve the efficiency of genotyping multilines. Allele-specific PCR markers are suitable for developing multiplex PCR systems, in which several resistance gene-specific markers are mixed, because the size of the PCR product of each marker can be easily adjusted so that the bands from the various primer pairs used in a multiplex PCR marker analysis do not overlap each other in agarose gels. Our multiplex PCR system offers one of the simplest, most reliable, and lowest cost methods for identifying which blast resistance gene is harbored by a particular line from a group of blast-resistant multilines.

We aimed to develop markers for introgressing blast resistance genes from various differential cultivars into the Japanese cultivar Koshihikari. To this end, we designed PCR-based markers that would discriminate between SNPs or InDels identified in each of the blast-resistant differential cultivars and Koshihikari. Because the genetic backgrounds of most of the Japanese elite cultivars resemble that of Koshihikari, we expect that the markers we developed can be applied to the introgression of blast resistance genes into other representative Japanese elite cultivars. In fact, we have confirmed that our markers specifically discriminated between the genomes of the blast-resistant donor cultivars and those of the blast-susceptible Japanese elite cultivars Sasanishiki, Kinuhikari, Hitomebore, Akitakomachi, and Hinohikari (data not shown). We suppose that our markers can be used for developing blast-resistant NILs in most modern Japanese cultivars. We have not evaluated whether our markers can be applied directly to blast resistance breeding of the non-Japanese rice cultivars. However, we expect that the information we presented here—regarding the precise chromosomal locations of the blast resistance genes *Piz, Piz-t, Pit, Pik, Pik-m*, and *Pik-p*; the SNPs and InDels for each resistance gene; and the strategy for developing markers for these genes—can be applied to produce markers for use in non-Japanese cultivars (e.g. *indica*).

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