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Development of PCR-based SNP markers for rice blast resistance genes at the *Piz* locus

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Abstract We assessed the utility of single-nucleotide polymorphisms (SNPs) and small insertion/deletion polymorphisms (InDels) as DNA markers in genetic analysis and breeding of rice. Toward this end, we surveyed SNPs and InDels in the chromosomal region containing the *Piz* and *Piz-t* rice blast resistance genes and developed PCR-based markers for typing the SNPs. Analysis of sequences from a blast-susceptible Japanese cultivar and two cultivars each containing one of these genes revealed that SNPs are abundant in the *Piz* and *Piz-t* regions (on average, one SNP every 248 bp), but the number of InDels was much lower. The dense distribution of SNPs facilitated the generation of SNP markers in the vicinity of the genes. For typing these SNPs, we used a modified allele-specific PCR method. Of the 49 candidate allele-specific markers, 33 unambiguously and reproducibly discriminated between the two alleles. We used the markers for mapping the *Piz* and *Piz-t* genes and evaluating the size of DNA segments introgressed from the *Piz* donor cultivar in Japanese near-isogenic lines containing *Piz*. Our findings suggest that, because of its ability to generate numerous markers within a target region and its simplicity in assaying genotypes, SNP genotyping with allele-specific PCR is a valuable tool for gene mapping, map-based cloning, and marker-assisted selection in crops, especially rice.

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Introduction

Molecular markers are essential for mapping genes of interest, marker-assisted breeding, and cloning genes by map-based cloning strategies. Commonly used molecular markers include restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs), and cleaved amplified polymorphic sequences (CAPS) (reviewed by Mohan et al. 1997). Among these, PCR-based markers are preferable to RFLPs because they are efficient and cost-effective: they require only a small amount of genomic DNA for typing and thus are suitable for selection at early seedling stages. However, each of the PCR-based markers has its drawbacks. For example, genotyping with CAPSs requires treating amplified DNA with a restriction enzyme. In SSR analysis, the size differences between the products amplified from each allele are usually small, complicating reliable scoring by standard agarose gel electrophoresis.

Recently, public accessibility to the genome sequences of several organisms has enabled the study of sequence variations between individuals, cultivars, and subspecies. These studies revealed that single-nucleotide polymorphisms (SNPs) and insertions and deletions (InDels) are highly abundant and distributed throughout the genome in various species including plants (Garg et al. 1999; Drenkard et al. 2000; Nasu et al. 2002; Batley et al. 2003a). By comparing sequences from a *japonica* rice cultivar to those from an *indica* cultivar, Yu et al. (2002) identified, on average, one SNP every 170 bp and one InDel every 540 bp in rice. Nasu et al. (2002) reported almost the same frequency of rice SNPs. The abundance of these polymorphisms in plant genomes makes the SNP marker system an attractive tool for marker-assisted breeding and map-based cloning (Gupta et al. 2001; Rafalski 2002; Batley et al. 2003b).

Several techniques have been developed to genotype SNPs, including pyrosequencing (Ahmadian et al. 2000; Alderborn et al. 2000), *Taq*Man (Livak 1999), and

fluorescence energy transfer (Chen et al. 1998) methods. These techniques require specialized detection equipment and/or labeled PCR primers, which cost more than standard primers. A method for rapid, simple, inexpensive, and reliable genotyping of SNPs is desirable. The allele-specific PCR method meets these requirements (Newton et al. 1989). In principle, SNPs can be detected simply by using allele-specific PCR primers designed so that the 3′ terminal nucleotide of a primer corresponds to the site of the SNPs. An allele-specific primer matches perfectly with one allele (the specific allele) or has a 3′ mismatch with a nonspecific allele. Because mismatched 3′ termini are extended by *Taq* polymerases with much lower efficiency than correctly matched termini, the allele-specific primer preferentially amplifies the specific allele. SNPs can easily be typed by the presence or absence of PCR-amplified products on standard agarose gels. Unfortunately, this allele-specific PCR method has not been used widely to assay SNPs, because a single-base mismatch at the 3′ end of the nonspecific allele is usually not sufficient for reliable discrimination between the two alleles. To overcome this problem, modified methods for designing allele-specific primers with increased specificity and reliable discrimination between alleles have been suggested (Kwok et al. 1990; Drenkard et al. 2000). Drenkard et al. (2000) demonstrated that their modified allele-specific PCR procedure enabled reliable assay of SNPs in the *Arabidopsis thaliana* genome.

To evaluate the potential of SNP markers as DNA markers for genomic studies and practical breeding in rice, we developed PCR-based SNP markers for rice blast resistance genes at the *Piz* locus. This locus is composed of the dominant genes *Piz*, *Piz-t*, *Pi2(t)*, and *Pi9(t)* (Yokoo and Kiyosawa 1970; Inukai et al. 1992) and was reported to be positioned near the centromere of chromosome 6, close to marker P130 (Yu et al. 1991; Mew et al. 1994; Hashimoto et al. 1998; Jiang and Wang 2002; Liu et al. 2002). The genes *Piz-t* and *Pi2(t)* were considered to have originated in *indica* cultivars (Nagai et al. 1970; Mackill and Bonman 1992), whereas *Pi9(t)* originated in a wild rice (Amante-Bordeos et al. 1992). Both *Piz* and *Piz-t* genes have been used for conferring blast resistance to Japanese cultivars. Because of their importance in rice breeding in Japan, in the present study, we focused on these two genes. We first surveyed the frequency of SNPs in the chromosomal regions *Piz* and *Piz-t* and assessed the feasibility of generating a number of SNP markers within the targeted gene regions. We then developed allele-specific PCR markers. Finally, we evaluated the utility of the developed markers and procedure by performing fine mapping of *Piz* and *Piz-t*. Our results demonstrate that SNPs are a valuable tool for gene mapping studies, especially because these markers offer the potential to generate fine-structure mapping.

Materials and methods

Plant materials

To survey SNPs and InDels, we used the American cultivar Zenith as a *Piz*-containing cultivar, the Japanese cultivar Toride 1 (Nagai et al. 1970) as a *Piz-t* containing cultivar, and the Japanese cultivar Nipponbare as a blast-susceptible cultivar. Each of the F_2 populations used for mapping of *Piz* and *Piz-t* was derived from a cross between the blast-susceptible Japanese *japonica* cultivar Koshihikari and the *Piz*-containing cultivar Niigatawase and a cross between Koshihikari and the *Piz-t* containing cultivar Toride 1, respectively. In addition to these two populations, we developed another segregating population from a cross between Koshihikari and the *Piz*-containing cultivar Fukunishiki. This population was used for optimizing allele-specific PCR procedures. Seeds of the rice cultivars Toride 1, Zenith, Fukunishiki, Fukuhikari, Yamatenishiki, Oou 244, Urumamochi, Hukei 64, Fujihikari, Katsurawase, Hanaechizen, and Sasanishiki BL3 were obtained from the gene bank at the National Institute of Agrobiological Sciences, Japan. Information about breeding these cultivars is described in Sasaki et al. (1994) for Sasanishi BL3, Nagai et al. (1970) for Toride 1, and Watanabe (1980) for other Japanese *Piz*-containing near-isogenic lines. Seeds of Hokuriku IL4 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 35 F_3 progenies of each F_2 individual was evaluated. Seedlings at the fourth- to fifth-leaf stage were grown in trays and inoculated with *Pyricularia grisea* race 007.0, which was provided by the Plant Disease Control Laboratory, National Agricultural Research Center, Japan. For the inoculation, the plants were sprayed with a blast spore suspension (5×10^5 spores/ml). The inoculated plants were placed in darkness in a dew chamber for 24 h at 25°C and subsequently transferred to a moist vinyl tunnel in a greenhouse at 25°C. The disease reactions of the inoculated plants were scored 7–10 days after inoculation by using the method of Yamaguchi (1980) as follows: in case an inoculated plant had no evidence of infection, small brown specks, or roundish to elliptical lesions with brown margins, the plant was considered resistant, and where typical spindle-shaped blast lesions were observed on leaves of a plant or its leaves were partially killed by coalescence of lesions, the plant was considered susceptible.

DNA extraction and Southern analysis

A large-scale method was used for isolating highly purified DNA from rice leaves for Southern analysis. Briefly, rice leaves (ca. 2 g) were ground in liquid nitrogen, mixed with 20 ml extraction buffer (0.1 M Tris-HCl, 50 mM EDTA, 0.5 M NaCl, 1.25% SDS, pH 8.0), and incubated for 15 min with shaking at 65°C. Afterwards 3 ml of 5 M potassium acetate was added, and the solution was incubated at 0°C for 20 min. The DNA in the mixture was extracted twice with 20 ml chloroform-isoamyl alcohol, treated with RNase A, and precipitated with isopropyl alcohol. The DNA was dissolved in 0.4 ml of 0.1× TE (10 mM Tris-HCl, 1 mM EDTA) buffer (pH 8.0). A rapid method of DNA isolation was used for isolating a small amount of crude DNA from the tip of a rice leaf. We used the procedure described by Monna et al. (2002) with modifications: The tip of rice leaves (ca. 0.02 g) was soaked in 0.45 ml isolation buffer (100 mM Tris-HCl, 10 mM EDTA, 1 M KCl, pH 8.0) and ground with the Multibeadshocker (Yasui Kikai, Osaka, Japan) for 6 s at room temperature. After rice-leaf debris was removed by centrifugation, the supernatant was collected. DNA in the supernatant was precipitated by adding an equal volume of isopropyl alcohol, recovered by centrifugation for 15 min, and finally dissolved in 60 μl of TE buffer (pH 8.0).

Southern hybridization was performed according to the following procedure: Rice DNA (ca. 3 μg) was digested with an appropriate restriction enzyme, separated on a 0.8% agarose gel, and blotted onto a positively charged nylon membrane (Roche Diagnostics, Basel, Switzerland). For probe labeling and detection of signals, we used the ECL Direct Labelling and Detection System (Amersham Pharmacia, N.J., USA).

DNA sequencing

Nucleotide sequences from Nipponbare, Zenith, and Toride 1 were determined by direct sequencing of PCR-amplified fragments, typically 500 bp long, with a CEQ 2000XL DNA Analysis System (Beckman Coulter, Calif., USA). To verify sequence variants, each PCR product was sequenced in both directions. To design the PCR primers, we used genome sequence data for the cultivar Nipponbare from the DDBJ/GenBank/EMBL database.

PCR-based markers to genotype SNPs and InDels

InDels were typed by amplifying an InDel-containing DNA fragment with two flanking primers. The genotype was then determined in light of the difference in size of the PCR products from the two alleles on agarose gels.

To assay SNPs, we used the allele-specific PCR method. Information about an SNP genotype was provided by the presence or absence of a PCR amplification product from allele-specific primers. To improve the specificity of traditional allele-specific PCR amplification, we basically followed a modified method (Kwok et al. 1990; Drenkard et al. 2000), in which an artificial base pair mismatch was introduced within four nucleotides of the 3′ end of the primer in addition to the 3′ mismatch with the nonspecific allele (Fig. 2A). Assaying a single SNP with a dominant allele-specific marker was done by a pair of PCR amplifications, one with a primer specific to one allele and another with a primer specific to the other allele. With a codominant allele-specific marker, a single PCR amplification allows assaying an SNP. The codominant markers were developed by combining two independent allele-specific markers (Fig. 3A), one specific to allele 1 at SNP1 and another specific to allele 2 at SNP2, and performing the PCR amplifications (with a common reverse primer) in the same tube. The difference in size of the products was adjusted to about 100 bp. If the difference is too large, the shorter product is amplified preferentially, and if the two products are too similar in size, unambiguous discrimination on agarose gels is compromised.

PCR amplification reactions (volume: 20 μl) of dominant allele-specific markers each contained 3 μl (ca. 25 ng/μl) rice genomic DNA isolated by either the large-scale or the rapid method, 0.5 U of Hot Start *Taq* polymerase (Takara, Osaka, Japan), 2 μl 10× PCR buffer, 1.6 μl 2.5 mM dNTPs, and 0.5 μl each of an allele-specific primer (10 μM) and a reverse primer (10 μM). The amplification scheme consisted of 30 cycles of 96°C for 30 s, 60°C for 30 s, and 72°C for 30 s. For codominant allele-specific markers, the reactions (volume: 20 μl) each contained 3 μl rice genomic DNA, 0.5 units of Ampli*Taq* Gold polymerase (PE Applied Biosystems, Calif., USA), 2 μl 10× PCR buffer, 2 μl 2.0 mM dNTPs, and 0.5 μl each of 10 μM allele 1-specific primer, 10 μM allele 2-specific primer, and 10 μM reverse primer. For InDel markers, the reaction solution contained the same constituents as that for codominant allele-specific markers, except that a pair of 10 μM primers (5 μl each) flanking an InDel were used instead of allele-specific primers. The amplification program was 94°C for 10 min for initial denaturation followed by 36 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. All the PCR reactions were performed in a PCR Thermal Cycler SP (Takara). PCR products were visualized by gel electrophoresis on 2% (w/v) agarose.

Results

Survey for SNPs and InDels in the *Piz* and *Piz-t* gene regions

To evaluate the frequencies of SNPs and InDels in the *Piz* and *Piz-t* regions, we sequenced DNA fragments from Nipponbare, Zenith, and Toride 1. We sequenced 132 fragments near the *Piz* region in the Zenith genome and 44 fragments around the *Piz-t* region in the Toride 1 genome. These sequenced fragments were selected randomly and, thus, may contain both coding and non-coding regions. We obtained a total of 60,781 bp of DNA sequences from Zenith, 18,414 bp from Toride 1, and DNA sequences corresponding to these regions from Nipponbare. To detect sequence polymorphisms among the three cultivars, we aligned the sequences, which revealed a total of 156 SNPs (1 per 390 bp DNA) and 21 InDels (1 per 2,894 bp DNA) between Nipponbare and Zenith and 106 SNPs (1 per 173 bp) and 12 InDels (1 per 1,535 bp) between Nipponbare and Toride 1 (Table 1). These results indicate that SNPs are abundantly distributed in the *Piz* and *Piz-t* genome regions. This high rate of SNPs also occurred between Zenith and Toride 1; a total of 132 SNPs (1 per 140 bp) were identified in this region. In contrast, the InDel levels among the three cultivars

Table 1 SNPs and InDels identified between the genomes of Nipponbare, Zenith, and Toride 1 in the gene regions of *Piz* and *Piz-t*

	Number of read (bp)	Genotype						
		A/G	A/T	A/C	C/T	C/G	G/T	Total
SNPs number Nipponbare [SNPs rate (%)]	60,781	60	15	7	52	7	15	156 (0.26)
Zenith InDels[a] number [InDels rate (%)]	60,781							21 (0.034)
SNPs number Nipponbare [SNPs rate (%)]	18,414	31	10	8	49	4	4	106 (0.58)
Toride 1 InDels number [InDels rate (%)]	18,414							12 (0.065)
SNPs number Zenith [SNPs rate (%)]	18,414	41	6	8	61	5	11	132 (0.72)
Toride 1 InDels number [InDels rate (%)]	18,414							19 (0.10)

[a] *InDels* insertion/deletion polymorphisms

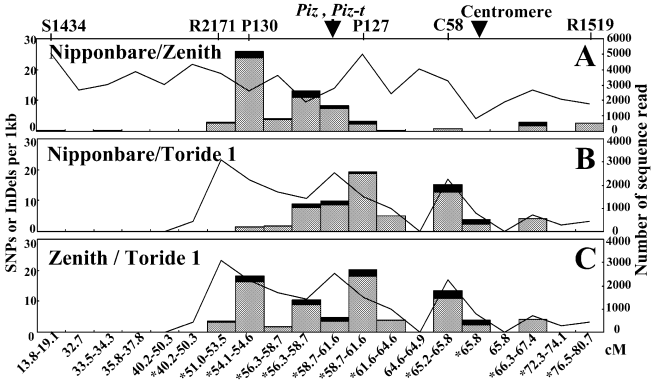

Fig. 1A–C Frequency of distribution of SNPs (*gray bars*) and InDels (*black bars*) near *Piz* and *Piz-t* on chromosome 6. The numbers of these polymorphisms per kb between the genomes of **A** Nipponbare and Zenith (*Piz* donor), **B** Nipponbare and Toride 1 (*Piz-t* donor), and **C** Zenith and Toride 1 are shown. At the *top* of figure, the positions of some known markers are shown. The locations of the polymorphisms are given in centiMorgans from the terminus of the short arm of this chromosome (http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/chr06.html). The number of sequences read for each position is shown also (*solid lines*)

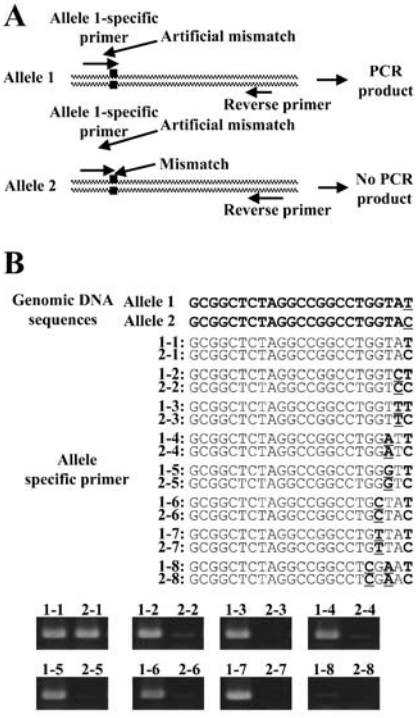

Fig. 2 A Schematic representation of the modified allele-specific PCR method. The allele *1*-specific primer has an artificial mismatch within four bases of the 3′ terminus as well as the mismatch to the allele *2* genomic sequence at the 3′ terminus. **B** An example of the test examining the effect of incorporating mismatches within four nucleotides of the 3′ terminus on the specificity of allele-specific PCR. In this test, we tried to generate allele-specific PCR primers to discriminate between the Nipponbare and Zenith (*Piz*) genomes at the SNP z3943 locus. The mismatched bases are *underlined* in the sequences of the primers used in the test. For PCR amplification, allele *1* DNA (Nipponbare) was used as the template. The presence or absence of each PCR product was analyzed on 2% agarose gels (*bottom*)

were only about one-eighth of the SNP levels. Of the 52 InDels identified, 42 (81%) were small, comprising insertions or deletions of one to five nucleotides; only four were larger than 40 bp.

The majority (72% for Nipponbare-Zenith and 75% for Nipponbare-Toride 1) of the changes in nucleotides at the SNP sites were due to transitions (A-G or C-T), whereas transversions (A-T, A-C, C-G, or G-T) accounted for only one-fourth of the detected nucleotide polymorphisms. Of the SNPs, only two were triallelic polymorphisms; the remaining SNPs were diallelic polymorphisms.

The SNPs and InDels were not evenly distributed around the *Piz* and *Piz-t* regions (Fig. 1). The SNPs between Nipponbare and Zenith were most dense near marker P130. In contrast, the SNPs between Nipponbare and Toride 1 were most abundant in the region of marker P127, which was about 7 cM away from P130. The profile of the InDel distribution on the chromosome was similar to that of the SNP distribution; most InDels occurred in genome regions with high SNP rates.

Development of PCR-based SNP and InDel markers

The high density of SNPs and InDels in the *Piz* and *Piz-t* genome regions suggested the possibility of easily generating a large number of markers within the targeted gene regions. Unfortunately, only one InDel marker, z4794, that discriminated the genome of blast-susceptible Nipponbare from those of *Piz*-containing Zenith and *Piz-t*-containing Toride 1 was generated, because most of the InDels were small and therefore, the two alleles could not be unambiguously discriminated on agarose gels.

To assay SNPs, we used the modified allele-specific PCR method (Fig. 2A). To simplify the procedure for designing primers and test the feasibility of our improvement, we examined the effect of incorporating mismatches into the last four bases on the primer's specificity; we then tried to determine which base substitution and which location in the primer sequence retained the highest specificity. An example of the results of this analysis is shown in Fig. 2B. At the *z3943* locus, T in allele *1* was substituted with C in allele *2*. When used with a reverse primer, the allele-specific PCR primer containing only a mismatch at the 3′ terminus (primer 1-1/2-1) did not discriminate the two alleles, demonstrating that the traditional method did not work. Then, we incorporated a mismatch at various positions in both the specific and nonspecific primers and investigated the effects on specificity (Fig. 2B). In most cases, the specificity increased after we added an artificial mismatch to the primers. Among the seven examples in Fig. 2B, the primers 1-3/2-3, 1-5/2-5, and 1-7/2-7 showed favorable results: no PCR product was detected with the nonspecific

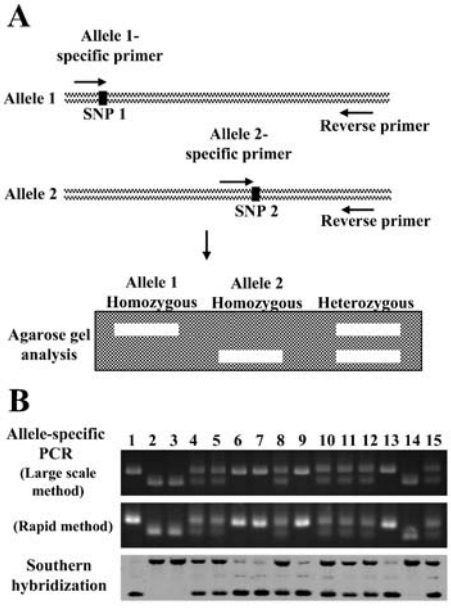

Fig. 3 A Schematic representation of converting a dominant allele-specific marker to a codominant one. PCR amplification with both an allele *1*-specific primer and an allele *2*-specific primer combined with a common reverse primer discriminated the allele *1* homozygous, allele *2* homozygous, and heterozygous genotypes. **B** An example of the use of codominant allele-specific PCR. The genotypes were assayed by allele-specific PCR with the z3542 marker using DNA isolated by the large-scale method (*upper*) and the rapid method (*middle*). Southern hybridization patterns (*bottom*) obtained with a probe to this SNP region yielded the same information as the allele-specific PCR. The DNAs used in this experiment were Koshihikari (blast-susceptible parent, *lane 1*), Zenith (*Piz* donor, *lane 2*), Fukunishiki (*Piz* donor, *lane 3*), F_1 progeny derived from a cross between Koshihikari and Fukunishiki (*lane 4*), and F_2 progenies derived from the cross Koshihikari × Fukunishiki (*lanes 5–15*)

primers, and the product was amplified with the specific primers. In the primer pair 1-5/2-5, the T at the third base from the 3′ terminus was changed to G. From the results of these tests, we devised a rule for easily designing allele-specific PCR primers: to optimize primer specificity, a mismatch should be incorporated at the third base from the 3′ terminus in both the specific and nonspecific primers. This substitution should be either of T-G or C-A. For the 49 SNPs we examined, our modified method had an overall success rate of 67% in generating primers able to reliably discriminate between two alleles.

Figure 3B shows one example of the application of our improved allele-specific PCR to determine the genotype of F_2 individuals derived from a cross between the blast-susceptible cultivar Koshihikari and the *Piz*-containing cultivar Fukunishiki. Allele-specific PCR analysis with codominant marker z3542, even when crude DNA isolated with the rapid method was used as a template (middle), provided the same information with regard to the genotype of each F_2 plant as was obtained by RFLP analysis (bottom).

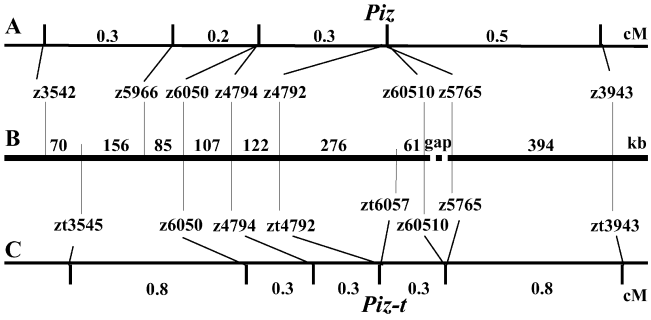

Fig. 4 A A genetic map of the *Piz* gene region constructed with the one InDel and seven SNP markers. **B** A physical map of the *Piz* and *Piz-t* gene region generated by using the published genome sequence data and the results of the genetic mapping with the SNP markers. **C** A genetic map of the *Piz-t* gene region constructed with the one InDel and seven SNP markers

Information on the allele-specific primers used for the subsequent gene mapping and for assessing the size of introgressed DNA segments is shown in Table 2.

Tagging the *Piz* and *Piz-t* genes with allele-specific PCR markers

To evaluate the feasibility of using our allele-specific PCR markers for mapping genes, we applied these markers to localizing two dominant rice blast resistance genes, *Piz* and *Piz-t*. For this mapping, we used one InDel and 11 SNP markers. Of these, four detected polymorphism between Koshihikari, a blast-susceptible cultivar, and both Niigatawase, a resistant donor cultivar of *Piz*, and Toride 1, a resistant donor cultivar of *Piz-t*; another four discriminated between Koshihikari and Niigatawase, and the remaining four discriminated between Koshihikari and Toride 1. To locate *Piz* and *Piz-t*, we performed linkage analysis with 186 F_2 segregating plants for *Piz* and 195 F_2 segregating plants for *Piz-t*. The *Piz* gene cosegregated with markers z4792, z60510, and z5765 and was flanked on one side by markers z6050 and z4794 (with one recombination) and on the other side by marker z3943 (with two recombinations) (Fig. 4A). The *Piz-t* gene mapped close to *Piz*, cosegregated with zt4792 and zt6057, and was flanked on one side by z4794 (with one recombination) and on the other side by z60510 and z5765 (with one recombination) (Fig. 4C). These mapping results confirm that these two genes are allelic or close to each other.

As mentioned, we used published genome sequence data to design the markers for this genetic mapping project. Therefore, the physical locations of the markers relative to each other, and sometimes relative to the candidate genes, could be directly deduced at the DNA sequence level (Fig. 4B) For *Piz-t*, the genetic mapping defined the candidate gene region to a 459-kb interval delimited by markers z60510 and z4792 on the Nipponbare sequence map. Unfortunately, in *Piz*, the presence of

Table 2 Allele-specific and InDel markers used to map the resistance genes and estimate the size of introgressed DNAs

Maker name	Discriminated genes	Nipponbare-specific primer	Zenith- or Toride 1-specific primer	Reverse primer
z2143	*Piz*	GTGTTGATTTCTGAAGTCTTTAGCTGTTCC	GTGTTGATTTCTGAAGTCTTTAGCTGTTCT	GTCACTGCAAATACTGTAAGCACACTG
z2174[a]	*Piz*	CTTAGGAAAAGAGCACGAATGGAGGG	CCACTCCACGATATAGCGCGTTGCA	CGTTAGTCCCGGTTGGTAACACC
z1671	*Piz*	TCTGGACAGAGAGCTTGTTCAGATTCTGAA	TCTGGACAGAGAGCTTGTTCAGATTCTGAG	GCCTCCACCAATGCGCCGCCCAC
z3542[a]	*Piz*	GATGAATAAAGGCTCTTGCGAATTCCCATT	TGTCGTGCTGCTGGGTTCTCCCA	TAGGGTGTTACAGGCTGCCTGCG
zt3545[a]	*Piz-t*	GGGCTATGAAAAAAGCTCAGAGATC	CAAGCCTAGCGAGCTCGAGCGCC	AAGCCTCTGCAGCTTCTCTGGTG
z5966	*Piz*	CCATTCAAGATGCTACCGCGAGTACCACAG	CATTCAAGATGCTACCGCGAGTACCAACAT	GGCAACTCGATCTACTGTACGAG
z4792[a]	*Piz*	TATATTTTGGGGCGGAGGTAGGAT	CGCACGCTTTCCGAACTACTCCCGCT	AGTGTTGTGCGGCACGTGCTTTG
zt4792	*Piz-t*	TATATTTTGGGGCGGAGGTAGGAT	TATATTTTGGGGCGGAGGTAGGAC	AGTGTTGTGCGGCACGTGCTTTG
z4794[c]	*Piz, Piz-t*	TGAATGTGAGAGGTTGACTGTGG		CACGCCACCCTTCAATGGAGACT
z6050	*Piz, Piz-t*	GGTCATGGGACTCTTCTGGGGCA	GGTCATGGGACTCTTCTGGGGCG	ATCTCTCCGGCACGACCGAAGC
z60510	*Piz, Piz-t*	GGAGTTGGTGCGACGGTGCCGTTAT	GGAGTTGGTGCGACGGTGCCGTTAC	GCGCGGACCGGCCAGCTAGTTGAC
z5765	*Piz, Piz-t*	AATGTGAAATTGGATGAGCCGGATA	AATGTGAAATTGGATGAGCCGGATG	TTACCGATGTTCGTCGCTCTCAGG
z1671	*Piz*	CCGGCGCGAGAGGCTGGATCACC	CCGGCGCGAGAGGCTGGATCACT	CCCGTCAGAACTGCCGAACAACC
z3943[b]	*Piz*	CACTCCGTGCAGCTGCCCGATAC	GCGGCTCTAGGCCGGCCTGGGAC	GCTGGTGCTATAGCTGTTATCCTC GCCAACCACCATCTTCGCCTCGC
zt3943	*Piz-t*	GCGGCTCTAGGCCGGCCTGGAAT	GCGGCTCTAGGCCGGCCTGGAAC	GCTGGTGCTATAGCTGTTATCCTC
z5361[b]	*Piz*	GCTGGCATGAATATGTCATTCATTATGTGAAG	GGCTACTTCATCTGATTCACCTGTTCAGGC	TTCCCGACACATCTGAGGTCTAC ACCCGTGCGATTTCTCTCAGAGC
z2052	*Piz*	GTGCAGCAAAAAAGTCCGATTTCTATAGT	GTGCAGCAAAAAAGTCCGATTTCTATAGA	ACCCTATTCTTCTGGTCCGCGAG
z21423	*Piz*	ACTTACTATCCATGGATCACAATTCATAAG	ACTTACTATCCATGGATCACAATTCTTAAC	TGTCGGCGTGAACCGCCCGGTGC
zt6057	*Piz-t*	GGAAGCTCAAACTAGGAACGTGACGA	GAAGCTCAAACTAGGAACGTGACGC	ACTGGAAGTCCCTCTATATGCCC

[a] Codominant marker: two forward primers and one reverse primer are necessary for allele-specific PCR
[b] Codominant marker: two forward primers and two reverse primers are necessary for allele-specific PCR
[c] InDel marker

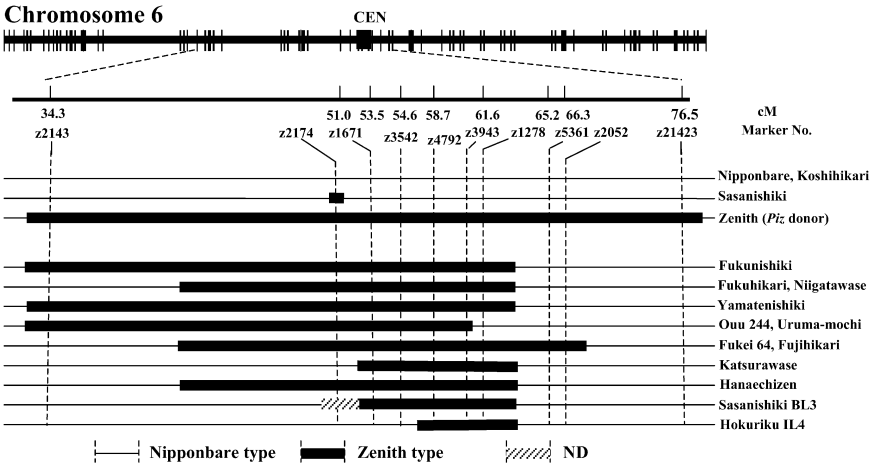

Fig. 5 Evaluation of the size of DNA segments introgressed from Zenith into 12 Japanese, *Piz*-resistant near-isogenic lines. The sizes of introduced DNAs were deduced from the genotypes of SNPs revealed with the ten allele-specific markers. Marker z2174 failed to determine whether Sasanishiki BL3 had the Zenith-type genome, because its recurrent parent (Sasanishiki) happened to have the Zenith-type nucleotide at the SNP locus rather than the Nipponbare-type nucleotide

a gap in the sequence data between markers z60510 and z5765 precluded defining the candidate gene region.

Application of the SNP markers to evaluating the size of the introgressed DNAs

To confer resistance to blast infection to Japanese elite rice cultivars, efforts have been made to introgress blast-resistance genes into these cultivars by backcrossing. Currently, several Japanese near-isogenic lines, in which blast-resistance genes were introduced from genetically distant donor cultivars, have been bred. For *Piz*, the cultivar Zenith has been used as a blast-resistant parent. Using the SNP markers developed across the *Piz* region, we evaluated the sizes of the DNA segments introgressed from Zenith into these blast-resistant, Japanese near-isogenic lines. We assayed the genotype of each of ten SNPs in 12 *Piz*-containing lines. Because Japanese rice cultivars are quite similar genetically, most of the cultivars have the same nucleotide as Nipponbare at each SNP site. Of the ten SNP markers we chose, only one (z2174) could not be used for genotyping all the lines. At this SNP locus, Sasanishiki has the Zenith-type nucleotide rather than the Nipponbare type. Therefore, the genotype of this SNP in Sasanishiki BL3, which was bred from Sasanishiki as a recurrent parent, could not be determined with this marker. Otherwise, the SNPs in the examined lines were directly typed with these markers.

Figure 5 shows the size of the DNA segments introgressed from Zenith in each of the lines examined. Because of the absence of an SNP between the markers z2143 and z2174, we could not develop a marker in this region; therefore, in some cultivars there might be an ambiguity in defining the left margin of the introgressed fragments. In Fukunishiki and Yamatenishi, the introgressed DNAs were as long as 30 cM. The smallest fragment was detected in Hokuriku IL4, in which about 3 cM Zenith DNA was revealed to have been introduced into Kinuhikari, the recurrent parent of Hokuriku IL4.

Discussion

Sequence information produced by recent genome sequencing projects has enabled the rapid identification of SNPs in various genome regions of interest. In the present study, we examined the abundance and localization of SNPs in the regions of the rice blast-resistance genes *Piz* and *Piz-t* (Fig. 1, Table 1) in the genomes of the susceptible Japanese *japonica* cultivar Nipponbare and each of the *Piz* and *Piz-t* donor cultivars. We found an abundant distribution of SNPs, one SNP every 390 bp in the *Piz* gene region and one every 173 bp in the *Piz-t* region. Between the genomes of the *Piz* and *Piz-t* donor cultivars, we also identified a high level of SNPs, which suggests that these two resistance genes have distinct evolutionary backgrounds. As has been reported for other organisms (Garg et al. 1999), the SNPs we detected were mostly due to transitions rather than transversions. This finding implies that methylated cytosines in CpG dinucleotides changed into thymines during the genesis of the rice SNPs (Tsaftaris and Polidoros 2000). A similar abundance of SNPs has been reported for maize (one every 48 bp in noncoding DNA regions) (Batley et al. 2003a).

Compared to SNPs, InDels in the *Piz* and *Piz-t* regions were much less frequent. In the maize genome, the frequency of InDels is also much lower than that of SNPs (Batley et al. 2003a). In *A. thaliana*, however, the levels of InDels (one every 6.1 kb) and SNPs (one every 3.3 kb) between the genomes of Columbia and Landsberg *erecta* differ by a factor of only 2 (Drenkard et al. 2000). These results suggest that, for fine mapping of a gene in rice or

maize, the use of SNPs rather than InDels as markers appears to be a good choice.

As we described earlier, one advantage of SNP markers over traditional marker systems is that a large number of SNP markers can be easily generated within a small target genome region. To take advantage of this merit of SNPs in gene mapping or marker-assisted breeding, an SNP typing procedure that is rapid, inexpensive, and reliable is beneficial. Several SNP genotyping methods have already been developed (Chen et al. 1998; Livak 1999; Ahmadian et al. 2000; Alderborn et al. 2000). Although some of these methods allow high-throughput genotyping of SNPs, they require special instruments or sophisticated methodologies. In contrast, typing SNPs by using allele-specific PCR requires only a simple electrophoresis-based assay. Therefore, it is accessible to any molecular biology laboratory or any plant-breeding group.

In the present study, we devised a simple method for designing allele-specific PCR primers. With this method, 67% of the primers initially generated were confirmed to have sufficient specificity in discriminating alleles. These markers enabled reliable SNP genotyping. In both the mapping of *Piz* and *Piz-t* and the evaluation of the size of introgressed DNA segments in nine Japanese *Piz*-containing near-isogenic lines, the developed markers clearly discriminated either *Piz* or *Piz-t*-containing alleles from blast-susceptible alleles. We did not detect any inconsistency between the genotypes analyzed by the markers and the phenotypes revealed by the blast spore inoculation tests. This type of marker, when used with the DNA templates extracted according to the rapid method, enabled rapid and reliable genotyping. The use of codominant allele-specific markers further facilitated the entire genotyping process (Fig. 3B).

We assessed the feasibility of applying the SNP markers we generated to genetic studies and breeding in rice by mapping two rice blast-resistance genes. This study demonstrated several advantages inherent in SNPs as DNA markers. First, as repeatedly described, several cosegregating and closely linked markers for discriminating the blast-resistant *Piz* and *Piz-t* alleles from the blast-susceptible *piz* and *piz-t* alleles were generated within a short period of time. In our breeding program, we are currently using these PCR-based, SNP markers for introgressing *Piz* and *Piz-t* into the Japanese elite cultivars Koshiibuki, Gohyakumangoku, and Koganemochi. Second, because we developed these markers using genome sequence data of rice, the location of each marker could be directly mapped onto the genome sequence, which facilitates the identification of a candidate gene on the sequence map. In our mapping of *Piz-t*, although we analyzed only 195 F_2 individuals, we defined the candidate gene region within a 459-kb region. Within this region, we identified a stretch of sequence that appears to be a candidate gene region for *Piz-t*. They were among a cluster of genes having the nucleotide binding site motif (data not shown) that is characteristic of plant resistance genes, including the rice blast-resistance genes that have been cloned (Wang et al. 1999; Bryan et al. 2000). Thus, in this *Piz-t* mapping, the physical location of each marker relative to the candidate *Piz-t* gene locus was also directly deduced. Third, because the mutation rate of SNPs is moderately low, closely related cultivars, such as most Japanese rice cultivars, contain the same genotype at most SNP positions. Thus, an SNP marker developed for discriminating between an allele from a foreign cultivar containing a useful trait and one from a Japanese cultivar can be used for introgressing the trait into most Japanese cultivars. In our study to assess the size of DNA segments introgressed from Zenith into nine Japanese, *Piz*-resistant near-isogenic lines (Fig. 5), nine of the ten SNP markers evaluated could be used for typing SNPs in all of the lines. Together, our results demonstrate the potential of SNPs as the next generation of genetic markers for rapid and effective genotyping for genetic mapping, marker-assisted breeding, and map-based cloning.

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