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Research Paper

Rapid DNA-genotyping system targeting ten loci for resistance to blast disease in rice

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The fungal pathogen *Pyricularia oryzae* causes blast, a severe disease of rice (*Oryza sativa* L.). Improving blast resistance is important in rice breeding programs. Inoculation tests have been used to select for resistance genotypes, with DNA marker-based selection becoming an efficient alternative. No comprehensive DNA marker system for race-specific resistance alleles in the Japanese rice breeding program has been developed because some loci contain multiple resistance alleles. Here, we used the Fluidigm SNP genotyping platform to determine a set of 96 single nucleotide polymorphism (SNP) markers for 10 loci with race-specific resistance. The markers were then used to evaluate the presence or absence of 24 resistance alleles in 369 cultivars; results were 93.5%consistent with reported inoculation test-based genotypes in *japonica* varieties. The evaluation system was successfully applied to high-yield varieties with *indica* genetic backgrounds. The system includes polymorphisms that distinguish the resistant alleles at the tightly linked *Pita* and *Pita-2* loci, thereby confirming that all the tested cultivars with *Pita-2* allele carry *Pita* allele. We also developed and validated insertion/deletion (InDel) markers for ten resistance loci. Combining SNP and InDel markers is an accurate and efficient strategy for selection for race-specific resistance to blast in breeding programs.

Key Words: blast resistance, race-specific resistance, DNA marker, SNP, marker-assisted selection, *Oryza sativa* L.

Introduction

Rice blast caused by the fungal pathogen *Pyricularia oryzae* (syn. *Magnaporthe oryzae*) is a devastating disease of rice (*Oryza sativa* L.). Use of cultivars that carry resistance to this disease is a cost-effective and environmentally friendly means to control the pathogen. Therefore, breeders and researchers worldwide have made extensive efforts to explore genes for blast resistance and incorporate them in breeding programs. Since more than 100 loci for blast resistance have been reported (Ashkani *et al.* 2016, Koide *et al.* 2009), their characterization and the establishment of systems for selecting desirable resistance alleles are indispensable for enhancing breeding programs.

Blast resistance genotypes are conventionally determined by inoculating with differential pathogen strains to test sample rice varieties against reference varieties for respective resistance alleles (Hayashi 2015). Such conventional differential systems used in the Japanese rice breeding program have discriminated 12 resistance alleles (*Pik-s*, *Pia*, *Pii*, *Pik*, *Pik-m*, *Piz*, *Pita*, *Pita-2*, *Piz-t*, *Pik-p*, *Pib*, and *Pit*) (Kiyosawa 1984, Yamada *et al.* 1976). Recent breeding efforts to introduce beneficial agricultural traits from exotic genetic resources have increased the diversity in allelic combinations for blast resistance (Yonemaru *et al.* 2014); this has meant that determination of blast resistance genotypes has become difficult, especially in varieties with the *indica* genetic background (Hayashi *et al.* 2014, Hirabayashi *et al.* 2010, Kato 2008). To overcome this problem, new differential systems have been proposed (Hayashi and Fukuta 2009, Hayashi *et al.* 2014, Kobayashi *et al.* 2007, Telebanco-Yanoria *et al.* 2010, Tsunematsu *et al.* 2000). Such new systems are readily acceptable when (a) results are compatible with those obtained by conventional systems, and (b) the pathogen strains used give distinctive

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resistant or susceptible phenotypes against a wide range of rice varieties. The differential system reported by Hayashi and Fukuta (2009) meets these requirements and has successfully determined the presence or absence of 23 resistance alleles (*Pia*, *Pish*, *Pib*, *Pit*, *Pii*, *Pi3*, *Pi5*, *Pik-s*, *Pik*, *Pik-p*, *Pi7*, *Pik-m*, *Pi1*, *Pik-h*, *Piz*, *Piz-5*, *Piz-t*, *Pi9*, *Pi19*, *Pi20*, *Pita*, *Pita-2*, and *Pi12*) in 10 varieties with the *indica* genetic background. Such improved differential systems require more labor and expertise compared with conventional systems, which restricts their use. Another limitation of inoculation-based differential systems is the difficulty in updating the system, because the numbers of differential fungus strains and reference varieties increases proportionally to the number of alleles to be discriminated.

Genetic mapping of genes for blast resistance has contributed to the development of DNA markers for several resistance alleles (Hayashi *et al.* 2004, 2006, 2010a, Koide *et al.* 2009, Nonoue *et al.* 2018, Tian *et al.* 2016, Wu *et al.* 2015). Since DNA markers allow us to readily and reproducibly determine sample genotypes (i.e., without influence of environmental factors), they have been used to efficiently introduce resistance in the Japanese rice breeding program (Ashkani *et al.* 2015, Hasan *et al.* 2015, Miah *et al.* 2013). The utility of a DNA marker depends on the degree of association between its genotype and the resistant/susceptible phenotype that is conditioned by that locus when it is tested against diverse cross combinations. If a certain polymorphism is distant from the target resistance locus or is in the resistance gene but does not determine the resistant/susceptible phenotype, that marker would work in limited cross combinations and/or its reliability would be low (Hayashi *et al.* 2010a). In addition, for multiple resistance alleles, researchers need to select multiple polymorphisms carefully to identify respective alleles (Wu *et al.* 2015, Yadav *et al.* 2017, Zhai *et al.* 2011). Finding polymorphisms that condition phenotypic differences, i.e., "functional polymorphisms" is one solution for the development of reliable markers (Hayashi *et al.* 2010a); however, design of such markers can be difficult in cases where (a) combinations of multiple polymorphisms determine phenotype (Fukuoka *et al.* 2014, Su *et al.* 2015, Xu *et al.* 2014b); or (b) the resistance gene contains highly conserved DNA sequences or resides in a genome region that has undergone frequent rearrangement events (Hayashi *et al.* 2010b, Takahashi *et al.* 2010, Wu *et al.* 2012, Zhou *et al.* 2007).

Associating phenotypic variation with haplotypes defined by a small number of marker loci is a practical approach for developing DNA markers for breeding in cases where marker loci are tightly linked with each other and predict the presence or absence of the resistance gene. This approach, termed haplotype-based association, is widely used for detecting genes associated with agricultural traits in crop plants (Contreras-Soto *et al.* 2017, Lorenz *et al.* 2010). In this approach, markers do not need to be based on functional polymorphisms when association between haplotypes and phenotype is validated in diverse genetic resources. Hence, researchers can find DNA markers without identifying functional polymorphisms and can use DNA markers in genomic regions where amplification and allelic discrimination are stable. However, to conduct genotyping at a comparable high-throughput level to that of the differential system developed by Hayashi *et al.* (2014), which targets 23 alleles, a set of 46 markers or more would need to be analyzed. Since the haplotype-based approach uses multiple markers per locus, reliable discrimination of blast resistance alleles requires a large number of markers. Use of a 96.96 Dynamic Array IFC (96.96 IFC) chip provided by Fluidigm Inc. (South San Francisco, CA, USA) solves this issue, because genotypes at 96 SNP marker loci for 96 samples can be obtained in one or two days (excluding DNA preparation time) (Thomson 2014, Wang *et al.* 2009). Under these circumstances, SNP haplotype-based genotyping is a promising alternative to determine blast resistance alleles in the Japanese rice breeding program.

Here, we undertook to use the Fluidigm SNP genotyping platform to develop a rapid instant DNA genotyping system for 10 race-specific blast resistance loci that include all the known resistance alleles used in the Japanese breeding program. The system was developed to include insertion/deletion (InDel) markers to select for certain resistance alleles at these loci, which could be useful in breeding programs. To assess the utility of the system, the genotypes of varieties with the *indica* genetic background, including those undetermined by inoculation-based differential systems, were determined.

Materials and Methods

Plant materials

To design and validate DNA markers for 24 blast resistance alleles, we used the rice varieties and lines listed in **Table 1**. They include donor varieties used for cloning of resistance genes (Ashkani *et al.* 2016, Koide *et al.* 2009), differential varieties for blast resistance (Kiyosawa 1984, Kobayashi *et al.* 2007, Telebanco-Yanoria *et al.* 2010, Tsunematsu *et al.* 2000, Yamada *et al.* 1976), and blast-resistant modern cultivars. To determine SNP and InDel marker haplotypes in the vicinity of resistance loci and to associate them with allelic variations at those loci, we used a set of rice varieties representing the wide range of accessions used in the Japanese rice breeding program (i.e., varieties used for staple food, rice cake, sake rice, and animal feed, including those categorized as upland rice, landraces, and cultivars of foreign origin) (**Supplemental Table 1**). To determine the resistance allele at the *Piz* locus in an *indica* cultivar 'Nona Bokra', two chromosome segment substitution lines (CSSLs), SL519 and SL521, were used. Both lines have a chromosome segment containing the *Piz* locus introduced from 'Nona Bokra' in the 'Koshihikari' genetic background (Takai *et al.* 2007). As a control for *Piz-5* and *Piz-t* alleles (at the *Piz* locus), two monogenic lines ('IRBLz5-CA' and 'IRBLzt-T-19F') (Tsunematsu *et al.*

Table 1. Blast resistance alleles targeted in this study

Locus	Allele	Cloned/ Mapped	Chr.	Map position (bp)	RAP[d] (Os ID)	MSU[e] (LOC_Os ID)	Donor and cultivar or line carrying the allele	Reference	Note[f]
Pit	*Pit*	Cloned	1	2682019–2684988	Os01g0149500	LOC_Os01g05620.1	K59, Deng Pao Zhai	Hayashi and Yoshida 2009	*
Pish	*Pish*	Cloned	1	33141127–33144999	Os01g0782100	LOC_Os01g57340.1	Nipponbare, Koshihikari	Takahashi *et al.* 2010	–
	Pi35[a]	Cloned			As above	As above	Hokkai 188	Fukuoka *et al.* 2014	–
Pib	*Pib*	Cloned	2	35108842–35115834	Os02g0818450	LOC_Os02g57305.1	BL1	Wang *et al.* 1999	*
Piz	*Piz*	Mapped	6	10372676–10471201	Not available	Not available	Fukunishiki, Hanaetizen	Hayashi *et al.* 2006	*
	Piz-t	Cloned			Os06g0287000	LOC_Os06g17920.1	Toride 1, IRBLzt-T	Zhou *et al.* 2006	*
	Piz-5(Pi2)	Cloned			As above	As above	IRBLz5-CA[LT], IRBLz5-TA[LT]	Zhou *et al.* 2006	–
	Pi9	Cloned			As above	As above	IRBL9-W[LT], IRBL9-W[US], Koshihikari Kanto BL1	Qu *et al.* 2006	*
Pi13	*Pi13*	Mapped	6	11677578–19614430[b]	Not available	Not available	Kasalath, Koshihikari Toyama BL7	Ebitani *et al.* 2011	*
Pii	*Pii*	Cloned	9	9667216–9674617	Os09g0327600	LOC_Os09g15840.1	Fujisaka 5, Ishikarishiroke	Takagi *et al.* 2013	*
	Pi5	Cloned			As above	As above	IRBL5-M[LT], IRBL5-M	Lee *et al.* 2009	–
	Pi3	Mapped			Not available	Not available	IRBL3-CP4[LT], IRBL3-CP4	Jeon *et al.* 2003	–
Pia	*Pia*	Cloned	11	6542301–6561363	Os11g0225100 Os11g0225300	LOC_Os11g11790.1 LOC_Os11g11810.1	Sasanishiki, Aichiasahi	Okuyama *et al.* 2011	*
Pik	*Pik*	Cloned	11	27978523–27988874	no hit Os11g0689100	LOC_Os11g46200.1 LOC_Os11g46210.1	Kusabue, Kanto 51	Zhai *et al.* 2011, Ashikawa *et al.* 2012	*
	Pik-m	Cloned			As above	As above	Tsuyuake, Hinohikari Kanto BL2	Ashikawa *et al.* 2008	*
	Pik-p	Cloned			As above	As above	K60, IRBLkp-K60	Yuan *et al.* 2011	*
	Pik-s	Mapped			Not available	Not available	Shin 2, IRBLks-B40[LT], IRBLks-Zh[LT]	Fjellstrom *et al.* 2004	*
	Pik-h	Cloned			As above	As above	IRBLkh-K3, IRBLkh-K3[LT]	Zhai *et al.* 2014	*
	Pi7	Mapped			Not available	Not available	IRBL7-M[LT]	Campbell *et al.* 2004	–
	Pi1	Cloned			As above	As above	IRBL1-CL[LT]	Hua *et al.* 2012	–
Pita	*Pita*	Cloned	12	10607519–10611770	Os12g0281300	LOC_Os12g18360.2	Yashiromochi, IRBLta-CP1	Bryan *et al.* 2000	*
Pita-2	*Pita-2*	Cloned	12	10824087–10833494	Os12g0285100	LOC_Os12g18729.2	PiNo.4, Ikuhikari	Takahashi *et al.* 2017	*
	Pi19	Cloned			As above	As above	Nipponbare, Koshihikari	Takahashi *et al.* 2017	–
Pi20	*Pi20*	Mapped	12	10081105–14181731[c]	Not available	Not available	IR 24	Li *et al.* 2008	–

Chr., chromosome number.

[a] Quantitative resistance allele at the *Pish* locus.

[b] The interval delimited by marker loci *R2123* and *RM20155* in rough mapping.

[c] The interval delimited by marker loci *OSR32* and *RM28050* in rough mapping.

[d] Rice Annotation Project: http://rapdb.dna.affrc.go.jp/.

[e] Michigan State University Rice Genome Annotation Project: http://rice.plantbiology.msu.edu/.

[f] The alleles with asterisks (*) are required in the application for variety registration in Japan.

2000) and their recurrent variety 'Lijiangxintuanheigu' were used. Similarly, to determine resistance or susceptibility alleles for several SNP haplotypes at *Pish*, *Pib*, *Piz*, *Pii*, *Pia*, *Pik*, *Pita*, or *Pita-2* loci, we used 6 to 14 CSSLs that each carry a target chromosomal region from diverse donors in a well-characterized genetic background ('Koshihikari') (Abe *et al.* 2013, Ebitani *et al.* 2005, Hori *et al.* 2015, Nagata *et al.* 2015a, 2015b, Takai *et al.* 2007, 2014). To discriminate alleles at the *Pik* locus, further inoculation tests were conducted by using 17 lines for *Pik* alleles and 19 blast isolates. For the direct sequencing of the *Pita-2* locus, we used six varieties, which included 'Ikuhikari' carrying the *Pita-2* allele; 'Nipponbare' carrying the *Pi19* allele, which is allelic to *Pita-2*; and two cultivars ('Yashiromochi' and 'Sasanishiki BL6') and two monogenic lines ('IRBLta-CP1' and 'IRBLta-CT2') for the resistance allele at the *Pita* locus, which is located close to *Pita-2*.

Detection and extraction of SNPs for design of genotyping assay

To detect candidates for the SNP genotyping assay for 10 loci with race-specific resistance to blast (**Fig. 1**), we used two methods *in silico*. In the first method, genomic or cDNA sequences of the resistance genes in the resistant or susceptible cultivars in the public databases, DDBJ (http://www.ddbj.nig.ac.jp/index-j.html) and NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/), were aligned against the corresponding sequences in the *japonica* reference 'Nipponbare' IRGSP-1.0 reference genome sequence (Kawahara *et al.* 2013) and *indica* reference 'IR64' *de novo* assembly sequence (Schatz *et al.* 2014) by using ClustalW version 2.1 (http://clustalw.ddbj.nig.ac.jp/index.php?lang=ja) or Kalign (https://www.ebi.ac.uk/Tools/msa/kalign/) software. In the second method, whole genome re-sequencing data (mainly from NCBI SRA database; https://www.ncbi.nlm.nih.gov/sra/, but also our unpublished data) were mapped to the 'Nipponbare' IRGSP-1.0 sequence by using CLC Genomics Workbench v8.0 software (Qiagen, Hilden, Germany) with default parameter settings; only uniquely mapped reads with a mapping quality score of ≥20 were used in mapping. SNPs that were informative (i.e., could discriminate resistant and susceptible varieties) were extracted.

To distinguish *Piz-t* and *Piz-5* (also known as *Pi2*), we used three SNPs in the *Pi2* locus (Zhou *et al.* 2006) and SNPs between re-sequencing data of 'Nona Bokra' (Yonemaru *et al.* 2015), which had a similar haplotype to that of *Piz-t*–carrying 'Toride 1' variety at the *Piz* locus (**Supplemental Table 1**) and was confirmed to carry *Piz-t* by an inoculation test using CSSLs (**Supplemental Table 2**), and the corresponding sequences of *Piz-5*–carrying 'C101A51' (DQ352453).

Recently *Pi19*, which is allelic to *Pita-2*, was cloned (Takahashi *et al.* 2017). Here, we screened for polymorphisms in cDNA sequences of *Pi19* among six varieties, a subset of which harbor alleles at the *Pita-2* locus, by direct sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and ABI 3730xl DNA analyzer (Thermo Fisher Scientific). Primers are listed in **Supplemental Table 3**. Sequences were assembled by using Sequencher 5.0.1 software (Gene Codes Corp., Ann Arbor, MI, USA) and aligned by using ClustalW 2.1.

Finally, 338 SNP genotyping assays based on allele-specific polymerase chain reaction (PCR) on the Fluidigm dynamic array platform were designed and oligonucleotides were synthesized by Fluidigm Inc. (**Table 2**).

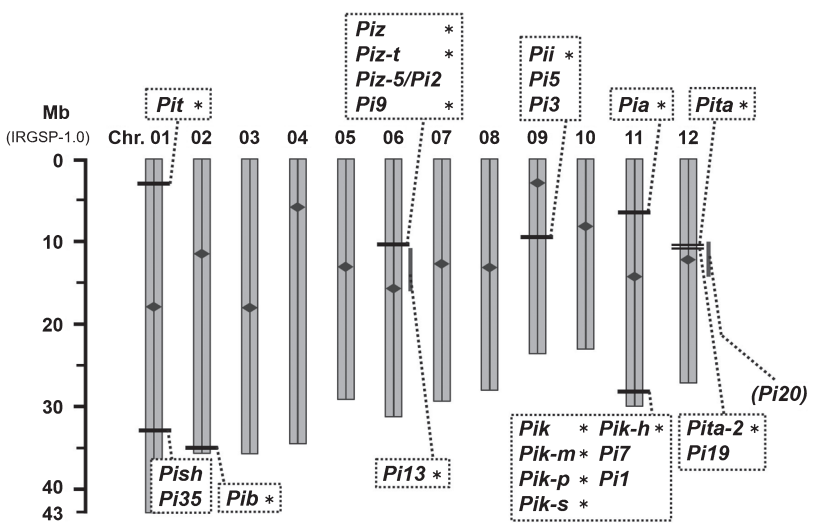

Fig. 1. Positions of the 24 blast resistance alleles used for SNP genotyping assays and PCR-based markers on the rice chromosomes. Scale in Mb ('Nipponbare' IRGSP-1.0.) is indicated on the left. Positions of the resistance loci are indicated by horizontal bars; ranges for roughly mapped loci are indicated by vertical gray bars. For each chromosome (Chr.), the centromere is indicated by a rhombus. Alleles with asterisks (*) are required in the application for variety registration in Japan.

Table 2. Summary of the SNP genotyping assays designed in this study

Locus[a]	Chr.	Number of alleles	Number of extracted SNPs	Number of qualified SNPs	Selected (Tested) assays			
					Number	Distribution range (kb)	Distance (kb)	
							from 5′ end of CDS	from 3′ end of CDS
Pit	1	1	88	46	6 (34)	8.8 (26.5)	2.0 (12.1)	3.8 (11.4)
Pish	1	2	179	153	11 (42)	189.1 (196.3)	155.4 (155.4)	29.8 (37.0)
Pib	2	1	62	47	9 (26)	49.0 (49.7)	19.2 (20.0)	22.7 (22.7)
Piz	6	4	160	74	12 (38)	66.5 (76.6)	11.5 (11.5)	–43.4 (–33.4)[c]
Pi13	6	1	80	58	9 (37)	144.7 (165.8)	64.4 (85.5)	77.8 (77.8)
Pii	9	3	81	74	9 (29)	202.6 (208.0)	9.8 (15.3)	185.3 (185.3)
Pia	11	1	51	44	8 (24)	185.7 (196.8)	20.9 (32.0)	145.7 (145.7)
Pik	11	7	119	106	20 (67)	188.4 (314.2)	160.6 (281.3)	17.4 (22.5)
Pita	12	1	75	44	6 (24)	28.0 (62.9)	0.0 (34.9)	23.7 (23.7)
Pita-2	12	2	87[b]	79	6 (17)	51.8 (54.5)	22.8 (22.8)	19.6 (22.3)
Total		23	982	725	**96** (338)			

Chr., chromosome number; CDS, coding sequence.

[a] Genotyping assays for *Pi20* are included in those for *Pita* and *Pita-2* loci.

[b] Includes one insertion–deletion polymorphism in the *Pita-2* gene.

[c] Negative values indicate that the assay at the 3′ end of the target region is located upstream of the CDS (see **Supplemental Fig. 4**).

DNA preparation

Genomic DNA was prepared from fresh leaves by one of two methods. In the first method, 1- to 3-cm leaf sections of 2- to 3-week-old seedlings were disrupted in 30 µL 0.5 M NaOH by using a Multi-Beads Shocker (Yasuikikai, Osaka, Japan), and then added to 120 µL 1 M Tris-HCl (pH 8.0) for neutralization. After centrifugation (1500 rpm for 1 min), the supernatant was directly used as a template. In the second method, a minor modification of the method reported by Monna *et al.* (2002), 3- to 4-cm leaf sections of a 2- to 3-week-old seedling were disrupted in 300 µL TPS buffer (100 mM Tris-HCl, 1 M KCl, 10 mM EDTA, pH 8.0) by using a Malti-Beads Shocker, and then centrifuged. From the resulting supernatant, DNA was precipitated by addition of 2-propanol, washed with 70% ethanol, and dissolved in 50 µL 0.1× TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). DNA samples prepared by the first method were used for InDel markers, and those prepared by the second method were used for SNP genotyping.

SNP genotyping

SNP genotypes were determined by using the 96.96 Dynamic Array IFC (96.96 IFC) chip according to the 'SNPtype 96x96 v1' protocol except that the number of additional cycles after touchdown PCR was reduced from 34 to 30. Scanned data obtained with an EP1 reader (Fluidigm Inc.) were analyzed with SNP genotype analysis software (Fluidigm Inc.) and converted to scatter plot diagrams and allele data. SNP genotyping assays that gave clear and stable plot diagrams were selected to produce the set of 96 SNPs used in further experiments (**Table 2**).

Assignment of resistance alleles in tested varieties

Resistance alleles at respective resistance loci in tested varieties were determined based on the identity between their SNP haplotypes and those of differential varieties. Public information on resistance alleles at race-specific resistance loci in Japanese commercial cultivars, mostly in a *japonica* genetic background (Plant variety protection database of the Ministry of Agriculture, Forestry and Fisheries, http://www.hinshu2.maff.go.jp/; Rice variety database of the Institute of Crop Science, National Agriculture and Food Research Organization, http://ineweb.narcc.affrc.go.jp/), was used to confirm the validity of the respective SNP haplotypes. The resistance alleles of the SNP haplotypes that were not found in the differential varieties were assigned by using public information pertaining to blast resistance of the varieties or by conducting inoculation tests. The resistance alleles for SNP haplotypes that lack information on resistance phenotypes, i.e., 5 SNP haplotypes at *Pish* locus; 10 at *Pib* locus; 8 at *Piz* locus; 6 at *Pii* locus; 10 at *Pia* locus; 9 at *Pik* locus; 3 at *Pita* locus; and 4 at *Pita-2* locus were determined by inoculation tests using CSSLs carrying respective SNP haplotypes and reference varieties (**Supplemental Table 4**). Presence or absence of resistance alleles at the various loci listed below were determined from the results of the challenge by differential blast isolates: Kyu77-07A for *Pish*; Ina86-137 for *Pib*; IW81-04, Ai74-134, Ken54-20, Ina86-137, Ken54-04, 24-22-1-1, Ina91-10, K59, and H97-227-1 for *Piz*; Ken54-20 for *Pii*; Mu-95 for *Pia*; V850196, P-2b, H05-99-1, and the other 17 isolates for *Pik* (**Supplemental Table 5**); and CHNOS58-3-1, C08, CHNOS121-2-4, and PH77-111-1 for *Pita-2*, *Pita*, and *Pi20*.

Design of InDel markers and genotyping

To facilitate discrimination by electrophoresis using a 4% (w/v) agarose gel, InDel variations larger than 10 bp and less than 500 bp were selected by the procedures outlined above for SNP detection. Primer pairs for each InDel were designed using Primer 3 software (Rozen and Skaletsky 2000) with the default settings except for the following: optimum product size, 85 to120 bp (range, 85 to 500); optimum primer melting temperature, 59°C or 63°C (range, 50°C to 70°C); optimum primer size, 22-mer (range, 18- to

28-mer); and optimum primer GC%, 50 (range, 20 to 80). To distinguish *Pita* from other alleles, two SNPs in the *Pita* gene (Bryan *et al.* 2000) were used as SNP markers. PCR with confronting two-pair primers (PCR-CTPP) was used to develop markers for *Pita*. All marker information is listed in **Supplemental Table 6**.

Each PCR was conducted in a 10 µL reaction mixture containing 0.5 µL DNA, 0.4 µM primers, 1× GoTaq Green Master Mix (Promega, Madison, WI, USA) with initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 15 s, 55°C or 60°C for 30 s, and 68°C for 1 min, and a final extension at 68°C for 5 min, using a thermal cycler ProFlex PCR system with 2× 384-well blocks (Applied Biosystems, Foster City, CA, USA). The PCR products were separated on 4% agarose gels with a 3:1 ratio of agarose Type I-A (Sigma-Aldrich, St. Louis, MO, USA): Metaphor agarose (Lonza, Basel, Switzerland) in 0.5× TBE buffer; the gels were stained with ethidium bromide and photographed under a transilluminator with a UV lamp.

Results

Development of SNP genotyping assays

To design SNP genotyping assays for 10 blast resistance gene loci, 982 polymorphisms were extracted *in silico* (**Table 2**); these included one InDel to discriminate between *Pita-2* and *Pi19* at the *Pita-2* locus. Of these, we excluded 198 SNPs (20.2% of the total) that were located in multi-hit sequences in a BLASTN search against the 'Nipponbare' genome, and 59 SNPs (6.0%) that were judged unsuitable on the basis of proximal sequences (e.g., presence of other polymorphisms or lack of sequence information in some reference varieties). From the resultant 725 SNPs (73.8%), we selected representative SNPs that were closest to the resistance loci and gave identical allelic distribution patterns in blast differential varieties. Finally, we obtained 338 SNPs for SNP genotyping assays. The distribution of the SNP markers for resistance loci ranged from 26.5 kb (*Pit*) to 314.2 kb (*Pik*) (mean, 135.1 kb) (**Table 2**, **Supplemental Figs. 1–10**).

Validation and selection of representative genotyping assays by using blast differential varieties

To confirm the discriminating ability of SNP genotyping performed using the Fluidigm 96.96 IFC chip platform, the 338 assays were tested on DNA samples from blast differential varieties. On the basis of signal intensity, allelic signal balance of respective assays, and the discriminating ability of the SNP haplotypes, we selected a set of 96 genotyping assays designated as 'Blast resistance gene-assays version 1 (BRA1)'. These assays distinguished the 23 resistance alleles, except for *Pi5* vs. *Pi3* at the *Pii* locus (**Supplemental Table 7**). At the *Pik* locus, two genome types ("N-type" and "K-type") had extremely low sequence similarity to each other (Ashikawa *et al.* 2008 and **Supplemental Fig. 8C**). To discriminate seven resistance alleles in K-type genomes, the set of 96 assays included 8 assays (FA4710, FA4712, FA5375, FA4717, FA4718, FA4721, FA4723, and FA4726) that produced signals for K-type genomes but not N-type genomes. In these 8 assays, genotypes in about half of the tested varieties were judged as "No Call" (labeled "-" in allele type). The number of the assays per locus in BRA1 ranged from 6 (*Pit*, *Pita*, and *Pita-2*) to 20 (*Pik*) (**Table 2**, **Supplemental Figs. 1B–10B**). The distribution range of the assays for resistance loci in BRA1ranged from 8.8 kb (*Pit*) to 202.6 kb (*Pii*) (mean, 111.4 kb; **Table 2**, **Supplemental Figs. 1B**, **1C–10B**, **10C**). The distribution of the SNP markers differed among loci in terms of distance from the coding sequences of the resistance loci; in particular, at the *Pish*, *Pii*, *Pia*, and *Pik* loci, some SNPs were located more than 140-kb upstream or downstream of the resistance gene, owing to unsuitable genome structure (lower sequence similarity among varieties or abundance of multi-copy sequences) close to the locus (**Table 2**, **Supplemental Figs. 2**, **6**, **7**, **8**). The haplotypes for resistance alleles obtained by using BRA1 are listed for each locus in **Table 3**. At the *Pii* locus, two SNP haplotypes were detected among the varieties that carry *Pii* allele ('Fujisaka 5' and 'Ishikarishiroge' vs. 'Nerica 1'); these were discriminated by two assays, FA4527 and FA4533. Similarly, we identified seven SNP haplotypes for the *Pia* allele by assay FA4598, two SNP haplotypes for the *Pik* allele ('Kusabue' vs. 'Kanto 51') by assay FA4710, and seven SNP haplotypes for *Pik-s* allele by a combination of SNP assays. Further inoculation tests using additional differential rice lines and pathogen isolates to characterize SNP haplotypes at the *Pik* locus showed that the lines with the SNP haplotypes Pik_H12 and Pik_H04-3 had different blast response patterns from those of known resistance alleles, despite variations in response among certain differential lines that shared the same SNP haplotype (**Supplemental Table 5**).

The genotypes of the marker locus FA4541, which targets the functional polymorphism at the *Pita* locus, were identical between varieties carrying the *Pita-2* allele and those carrying the *Pita* allele. At the *Pita* and *Pita-2* loci, three SNP haplotypes were detected; the genotypes detected by FA5655 assay distinguished between the presence of the *Pita-2* allele and its absence (including presence of allelic *Pi19*) (**Fig. 2**). Another SNP haplotype (Pita_H02 and Pita-2_H04) was observed in a rice variety ('IR24') carrying the *Pi20* resistance allele whose locus has been roughly mapped to near the *Pita-2* locus (Li *et al.* 2008).

Application of BRA1 to diverse rice varieties

To test the selected genotyping assays in diverse rice samples, genotypes of a total of 369 varieties were determined with BRA1 (**Supplemental Table 1**). After preparation of sample DNA, we successfully determined genotypes of 192 varieties within 2 days; SNP haplotypes at 10 blast resistance loci were determined (**Table 3**, **Supplemental Table 4**); SNP haplotypes that are absent in **Table 3** were found mostly in cultivars of foreign origin, Japanese upland

Table 3. Haplotypes of each resistance locus in the donor varieties

Locus	Allele	Cultivar/Line name	Assay name	No.	IRGSP-1.0	*Pit* Chr. 1	*Pish* Chr. 1	*Pib* Chr. 2	*Piz* Chr. 6	*Pi13* Chr. 6
			FA4467	1	26800046	A	G			
			FA4453	2	26812102	T	G			
			FA4455	3	26826051	C	C			
			FA4461	4	26830651	A	A			
			FA4482	5	26885501	C	C			
			FA4483	6	26888281	A	G			
			FA4649	1	32985740		T	G		
			FA4645	2	33074934		G	C		
			FA4644	3	33075459		C	A		
			FA4636	4	33080208		A	C		
			FA4650	6	33146248		C	G		
			FA4651	7	33146996		A	A		
			FA4652	8	33148998		C	C		
			FA4654	9	33151594		G	G		
			FA4665	10	33174003		C	A		
			FA4667	11	33174848		G	A		
			FA4424	1	35089605			C		
			FA4422	2	35090234			G		
			FA4416	3	35092033			G		
			FA4408	4	35095702			G		
			FA4407	5	35095970			T		
			FA4404	6	35097776			G		
			FA4401	7	35111607			T		
			FA4439	8	35133756			G		
			FA4446	9	35138569			T		
Pit	*Pit*	K59, Deng Pao Zhai	FA5376	1	10361215			G	G	
Pish	*Pish*	Nipponbare, Koshihikari	FA4676	2	10379796		G	G	G	
	Pi35	Hokkai 188	FA4678	3	10382967		C	C	C	
Pib	*Pib*	BL1	FA4679	4	10384890		C	G	G	
Piz	*Piz*	Fukunishiki, Hanaechizen	FA4680	5	10385269		C	C	T	
	Piz-t	Toride 1, IRBLzt-T	FA5378	6	10389855		G	G	T	
	Piz-5(Pi2)	IRBLz5-CA[LT], IRBLz5-TA[LT]	FA4697	7	10421778		A	C	G	
	Pi9	IRBL9-W[LT], IRBL9-W[US], Koshihikari Kanto BL1	FA4698	8	10422671		G	C	T	
			FA4683	9	10422831		A	G	T	
			FA4702	10	10426588		C	G	T	
			FA4703	11	10427313		A	G	T	
			FA4704	12	10427757		G	T	T	
Pi13	*Pi13*	Kasalath, Koshihikari Toyama BL7	FA5269	1	17096200					G
			FA5270	2	17105485					G
			FA5271	3	17121368					G
			FA5280	4	17159082					G
			FA5294	5	17161116					A
			FA5283	6	17166899					G
			FA5288	7	17174653					T
			FA5292	8	17186714					G
			FA5293	9	17240935					G

Locus	Allele	Cultivar/Line name	Assay name	No.	IRGSP-1.0	*Pii* Chr. 9	*Pia* Chr. 11	*Pik* Chr. 11	*Pita* Chr. 12	*Pita-2* Chr. 12
			FA4515	1	9657397				FA4541	1
			FA4505	2	9663840				FA4544	2
			FA4494	3	9667330				FA4546	3
			FA4495	4	9668417				FA4553	4
			FA4501	5	9672424				FA4556	5
			FA4526	6	9787343				FA4569	6
			FA4527	7	9787450				FA4571	2
			FA4533	8	9834576				FA4572	3
			FA4536	9	9859957				FA4585	5
Pii	*Pii*	Fujisaka 5, Ishikarishiroge	FA4608	1	6521353	G	A	A	FA4588	6
	Pii	Nerica1	FA4605	2	6540957	G	A	A		
	Pi5	IRBL5-M[LT], IRBL5-M	FA4598	3	6543100	G	A	A		
	Pi3	IRBL3-CP4[LT], IRBL3-CP4	FA4615	4	6544401	G	A	A		
Pia	*Pia*	Aichiasahi, Sasanishiki	FA4617	5	6634911	G	A	A		
	Pia	Bozu, Ochikara	FA4620	6	6638905	G	A	A		
Pik	*Pik*	Kusabue	FA4624	7	6656541	G	A	A		
	Pik	Kanto 51	FA4625	8	6707030	G	A	A		
	Pik-m	Tsuyuake, Hinohikari Kanto BL2	FA4742	1	27817940	A	G	T	FA4580	1
	Pik-p	K60, IRBLkp-K60	FA4740	2	27818557	A	G	T	FA4571	2
	Pik-s	Shin 2	FA4737	3	27830870	A	G	T	FA4572	3
	Pik-s	IRBLks-B40[LT]	FA4735	4	27832073	A	G	T	FA5655[a]	4
	Pik-s	IRBLks-Zh[LT]	FA4734	5	27832722	A	G	T	FA4585	5
	Pik-h	IRBLkh-K3, IRBLkh-K3[LT]	FA4732	6	27828066	A	G	T	FA4588	6
	Pi7	IRBL7-M[LT]	FA5375	9	no hit	G	A	C		
	Pi1	IRBL1-CL[LT]	FA4712	8	no hit	G	A	C		
Pita	*Pita*	Yashiromochi, IRBLta-CP1	FA4710	7	no hit	G	A	C		
Pita-2	*Pita-2*	PiNo.4, Ikuhikari	FA4717	10	no hit	G	A	C		
	Pi19	Nipponbare, Koshihikari	FA4718	11	no hit	G	A	C		
	*Pi20**	IR 24	FA4721	12	no hit	G	A	C		
			FA4723	13	no hit	G	A	C		
			FA4726	14	no hit	G	A	C		
			FA4729	15	27992151	A	G	T	FA4541	1
			FA4750	16	27993468	A	G	T	FA4544	2
			FA4751	17	27995372	A	G	T	FA4546	3
			FA4752	18	27995713	A	G	T	FA4553	4
			FA4753	19	27996426	A	G	T	FA4556	5
			FA4758	20	28006310	A	G	T	FA4569	6
			FA4580	1	10801324				FA4571	2
			FA4571	2	10830872				FA4572	3
			FA4572	3	10833033				FA4585	5
			FA5655[a]	4	10833370–10833387				FA4588	6
			FA4585	5	10849598					
			FA4588	6	10853113					

Chr., chromosome number.
* The haplotype for the *Pi20* allele was tentatively defined by genotyping assays for *Pita* and *Pita-2* loci.
[a] At the FA5655 marker locus, the allele-type [A] indicates "AAAAACCAG" and [B] indicates "TG".

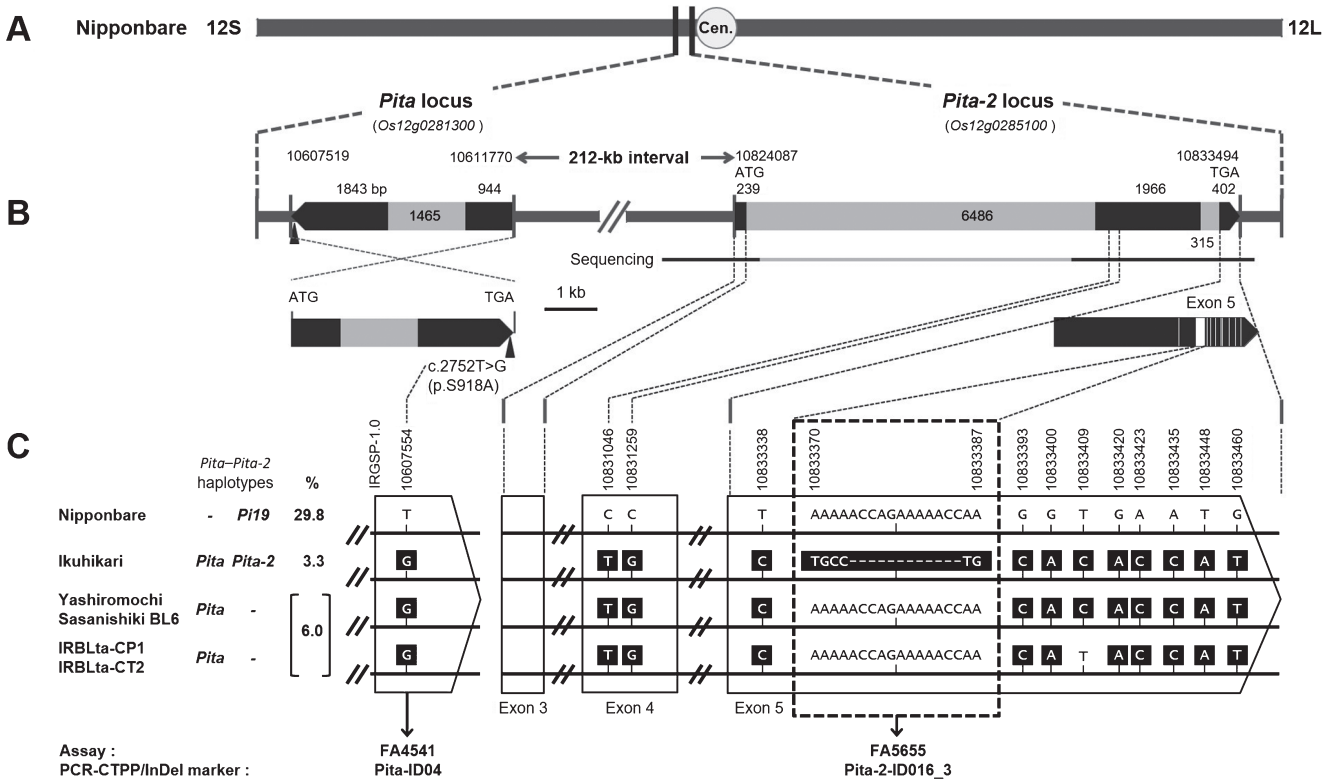

Fig. 2. Identification of polymorphisms to discriminate *Pita-2* from *Pi19* and frequency of various *Pita–Pita-2* haplotypes among Asian cultivated rice. (A) Positions of the *Pita* (*Os12g0281300*) and *Pita-2* (*Os12g0285100*) loci on chromosome 12 in 'Nipponbare' IRGSP-1.0. 12S, short arm side of chromosome 12; 12L, long arm side of chromosome 12; Cen., centromere. (B) Gene structures of *Pita* and *Pita-2* in 'Nipponbare'. Black, coding regions; gray, introns. The upward arrowhead on *Pita* represents the position of the functional nucleotide polymorphism described previously (Bryan *et al.* 2000). For *Pita-2*, the horizontal black lines labeled "Sequencing" indicate regions where the DNA sequences of reference varieties were determined by direct sequencing, and the regions in white within exon 5 represent sites containing polymorphisms between 'Nipponbare' (*Pi19*) and 'Ikuhikari' (*Pita-2*). (C) *Pita–Pita-2* haplotypes in the six cultivars, functional nucleotide polymorphisms of *Pita* (detected by allele-specific markers FA4541 and Pita-ID04) and polymorphisms that discriminate between *Pita-2* and *Pi19* (detected by allele-specific markers FA5655 and Pita-2-ID016_3). Frequency of each of the three DNA haplotypes among tested samples (**Supplemental Table 1**) is indicated as a percentage. The sequences within the dotted line box show variations that specifically discriminate the *Pita-2* allele ('Ikuhikari') from the other alleles (*Pi19* and susceptibility allele).

rice, landraces, or some *indica* varieties. The number of SNP haplotypes at each locus ranged from 7 (*Pit*) to 29 (*Pik*). Most SNP haplotypes were judged as a susceptibility allele based on previously available information on resistance gene genotypes of varieties; this was confirmed here by inoculation test (**Supplemental Table 4**). The rate of concordance of the genotypes for blast resistance between the present study and publicly available data in *japonica* varieties was 93.5% (**Supplemental Table 1**). To increase the high-throughput capabilities of the system for future studies, we selected a "core set" consisting of 35 assays from BRA1 (**Supplemental Table 4**, **Supplemental Figs. 1B–10B**). Although this core set was unable to discriminate all the SNP haplotypes detected in the present study, it was sufficient to determine the presence or absence of all the 24 resistance alleles used in the present study.

Application of BRA1 to Japanese high-yielding varieties

To comprehensively summarize the resistance genotypes of Japanese high-yielding varieties with mainly *indica* genetic backgrounds, we used BRA1 to genotype 23 varieties selected across the country from north to south (**Table 4**). Our data include information on four alleles (*Pish*, *Pik-s*, *Pi19*, and a new allele represented by the SNP haplotype Pik_H04-3) that are absent from public databases for variety registration (http://www.hinshu2.maff.go.jp/, http://ineweb.narcc.affrc.go.jp/). The number of estimated resistance alleles at 10 loci among these varieties ranged from 2 (e.g., 'Kitaaoba' and 'Kusahonami') to 5 ('Iwaidawara') (mean, 3.9). The resistance genotypes estimated in our study were compared with those determined by inoculation tests (**Table 4**). When we compared our data to those in the public databases, in which the genotypes of three varieties were undetermined, the rate of concordance in resistance loci ranged from 45% (*Pia*) to 100% (*Pit*, *Pib*, and *Piz*) (mean, 88%; **Table 4**). When we compared our data to an advanced differential system that allows estimation of the genotypes for *Pish*, *Pik-s*, *Pi19*, and *Pi20* (Hayashi *et al.*

Table 4. Estimation of race-specific resistance alleles in Japanese high-yielding varieties

Cultivar name	Alleles estimated by SNP genotyping assays	Discrepancy in genotype estimate betweeen present study and public databases[a]								Discrepancy in genotype estimate betweeen present study and Hayashi *et al.* (2014)								
		Pit	*Pib*	*Piz*	*Pii*	*Pia*	*Pik*	*Pita*	*Pita-2*	*Pit*	*Pish*	*Pib*	*Piz*	*Pii*	*Pia*	*Pik*	*Pita*	*Pita-2*
Kitaaoba	*Pish Pi19*									ND	ND	ND	ND	ND	ND	ND	ND	ND
Kitamizuho	*Pish Pia Pik Pi19*									ND	ND	ND	ND	ND	ND	ND	ND	ND
Tachijobu	*Pii Pia Pi19*									ND	ND	ND	ND	ND	ND	ND	ND	ND
Bekogonomi	*Pish Pib Pia Pik Pi19*					*pia*				ND	ND	ND	ND	ND	ND	ND	ND	ND
Natsuaoba	*Pish Pib Pia Pik-s Pi19*					*pia*				ND	ND	ND	ND	ND	ND	ND	ND	ND
Iwaidawara	*Pish Pib Pia Pik Pi19*					*pia*				ND	ND	ND	ND	ND	ND	ND	ND	ND
Fukuhibiki	*Pish Pib Pia Pi19*																	
Bekoaoba	*Pia Pik-s Pita Pita-2*					*pia*				ND	ND	ND	ND	ND	ND	ND	ND	ND
Yumeaoba	*Pish Pib Pia Pik-s Pi20*					*pia*			*Pita-2*	ND	ND	ND	ND	ND	ND	ND	ND	ND
Kusayutaka	*Pish Pia Pik-m Pi19*						*Pik*			ND	ND	ND	ND	ND	ND	ND	ND	ND
Tachisugata	*Pib Pii Pia Pik-s Pi19*				*pii*	*pia*												
Takanari	*Pib Pia Pik-s Pi20*	ND	ND	ND	ND	ND	ND	ND	ND									
Hoshiaoba	*Pish Pib Pia Pik-m Pi20*					*pia*	*pik-m*		*Pita-2*	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mochidawara	*Pib Pi5/Pi3 Pia New gene (Pik_H04-3) Pi20*	ND	ND	ND	ND	ND	ND	ND	ND						*pia*			
Hokuriku 193	*Pi5/Pi3 Pia Pik-s Pi20*				*Pii*	*pia*									*pia*			*Pi19*
Momiroman	*Pish Pib Pia Pik-s Pi20*					*pia*		*Pita*							*pia*	*pik-s*		*Pi19*
Nishiaoba	*Pish Pia Pik-m Pi19*									ND	ND	ND	ND	ND	ND	ND	ND	ND
Kusahonami	*Pik-s Pi20*	ND	ND	ND	ND	ND	ND	ND	ND							*Pik or Pk-p*		
Kusanohoshi	*Pib Pia Pi20*					*pia*		*Pita*		ND	ND	ND	ND	ND	ND	ND	ND	ND
Hamasari	*Pish Pia Pi19*														*pia*			*pi19*
Leafstar	*Pish Pi19*					*Pia*									*Pia*			
Tachisuzuka	*Pib Pi20*							*Pita*		ND	ND	ND	ND	ND	ND	ND	ND	ND
Tachiaoba	*Pish Pii Pia Pi19*									ND	ND	ND	ND	ND	ND	ND	ND	ND
	Match rate (%)	100	100	100	90	45	90	85	90	100	100	100	100	100	44	78	100	67

ND, not determined through lack of information in the public databases.

[a] Alleles in public databases that differ from those in the present study; genotypes beginning with a lowercase letter indicate susceptibility alleles.

2014), the rate of concordance in resistance loci ranged from 44% (*Pia*) to 100% (*Pit*, *Pish*, *Pib*, *Piz*, *Pii*, and *Pita*), (mean, 88%; **Table 4**); the second highest number of mismatches was at the *Pita-2* locus.

Development of InDel markers for genotyping 10 resistance loci

To establish a genotyping workflow for practical breeding programs, we developed a total of 172 InDel markers for 10 loci, containing two PCR-CTPP markers (Pita-ID03 and Pita-ID04) targeting the two SNPs in the *Pita* gene. The number of the designed markers per locus ranged from 6 (*Pia*) to 38 (*Piz*) (mean, 17.2). Based on the SNP haplotypes obtained by BRA1 (**Supplemental Table 4**), a total of 22 or 23 varieties were genotyped for each locus. Finally, 155 out of 172 markers that gave stable amplification were selected (**Supplemental Table 6**). The number of the selected markers per locus ranged from 6 (*Pia*) to 27 (*Piz*) (mean, 15.5). In the case of *Pit*, the markers were located in the target gene and adjacent regions. In other loci, markers were located in chromosomal regions that ranged in length from 121.9 kb (*Piz*) to 385.8 kb (*Pi13*) (mean, 231.5 kb). The product size of the InDel markers in the 'Nipponbare' reference genome IRGSP-1.0 ranged from 80 bp to 779 bp (mean, 133.7 bp) The variation in amplicon size for different alleles at the same locus ranged from 6 bp to 430 bp (mean, 33.7 bp). These variations were readily distinguishable by 4% agarose gel electrophoresis. The number of alleles per marker locus ranged from 2 to 5, including alleles that were not amplified by PCR (**Supplemental Table 6**). Genotypes obtained by using the markers in the selected varieties are shown together with the SNP haplotypes obtained by SNP genotyping assays in **Supplemental Fig. 11-1A–11-10A**.

Selection of an InDel marker set to discriminate the resistance alleles

To select a set of InDel markers that efficiently discriminate resistance alleles, haplotypes at resistance gene loci obtained by using InDel markers in 22 or 23 varieties were compared with SNP haplotypes at the corresponding resistance loci (**Supplemental Fig. 11-1A–11-10A**). At the *Pit* locus, all 7 SNP haplotypes (Pit_H01 to H07) were discriminated by 16 InDel markers (**Supplemental Fig. 11-1A**). At the *Pish* locus, 7 out of 16 SNP haplotypes (exceptions being Pish_H05, H08, and H10 to H16) were discriminated by 14 InDel markers (**Supplemental Fig. 11-2A**). Similarly, at the other resistance loci, SNP haplotypes for resistance alleles were discriminated from other resistance alleles at the same locus or from major SNP haplotypes that represent susceptibility alleles. Importantly, the haplotypes for a certain resistance allele were distinguished from those for the susceptibility allele or other resistance alleles at the same locus by using a single marker or a combination of 2 to 5 markers at each of the 10 loci: e.g., at the *Pib* locus (**Supplemental Fig. 11-3A**), the resistance allele represented by haplotype Pib_H01 was distinguished from the others by the marker Pib-ID11 alone. With respect to the *Pita* and *Pita-2* resistance alleles, which lie at a distance of 212 kb from each other, the markers for both alleles (Pita-ID04 and Pita-2-ID016_3) are required to identify varieties having *Pita* allele but not *Pita-2* allele (**Fig. 2**). Finally, from the 155 markers described above, we selected 23 markers, including 22 InDel and 1 PCR-CTPP, that clearly and efficiently discriminated the resistance alleles by electrophoresis in 4% agarose gel (**Table 5**, **Supplemental Fig. 11-1B–11-10B**). The number of the selected markers per locus ranged from 1 (*Pit*, *Pib*, *Pi13*, and *Pia*) to 5 (*Pik*). We note that these 23 markers could not distinguish between the following resistance alleles: *Piz-t* vs. *Piz-5*, *Pik-m* vs. *Pik-s*, and *Pik-h* vs. *Pi7*.

Discussion

Here we demonstrate the use of SNP haplotypes of regions containing race-specific blast resistance loci to estimate the presence or absence of resistance alleles among diverse rice varieties. Assays for a set of 96 SNPs (BRA1) selected for analysis by using Fluidigm 96.96 IFC chip could efficiently determine 24 resistance alleles at 10 loci in one round of experiment. Additionally, we developed InDel markers for 10 resistance loci that distinguish 20 resistance alleles from respective susceptibility alleles. These InDel marker sets would be useful for selecting for resistance alleles in a wide range of breeding populations after the resistance alleles of the parents were determined by using BRA1. Thus, our DNA-based marker system could be used to comprehensively and rapidly identify genotypes of blast resistance loci, and provides a cost-effective genotyping workflow for practical breeding programs.

Reliability and efficiency of identifying plants carrying desirable resistance alleles determines the efficiency of breeding. Notably, five (*Pish*, *Piz*, *Pii*, *Pik*, and *Pita-2*) of the ten loci tested in our study have multiple resistance alleles; discrimination of such alleles from each other by using inoculation testing or by DNA markers requires more labor than for loci with single resistance alleles (Ashikawa *et al.* 2008, 2012, Fukuoka *et al.* 2014, Hua *et al.* 2012, Lee *et al.* 2009, Qu *et al.* 2006, Su *et al.* 2015, Takagi *et al.* 2013, Takahashi *et al.* 2010, 2017, Yuan *et al.* 2011, Zhai *et al.* 2011, 2014, Zhou *et al.* 2006). The PCR-based (SNP or InDel) markers previously developed for *Piz* and *Pik* resistance loci discriminated two to six resistance alleles (Hayashi *et al.* 2006, Wu *et al.* 2015, Yadav *et al.* 2017, Zhai *et al.* 2011). Those studies selected markers that discriminate respective resistance alleles in pairs of donor varieties, but did not confirm that the marker loci selected were suitable for discrimination of alleles among diverse genetic resources. This potential lack of reliability would limit the use of these markers in a wide range of breeding programs. A recent study has validated the use of four markers (i.e., for alleles *Pia*, *Pii*, *Pik*, and *Pik-m*) among diverse varieties or breeding lines

Table 5. Selected markers for 21 allele types at the 10 blast resistance loci

Locus	Marker name	Primer sequences (5′ to 3′)		Marker type	Temp. (°C)[b]	Gel (%)[b]	Genotype A ('Nipponbare' type)[c]		Genotype B[c]		Genotype C[c]		Allele types distinguishable by marker combinations
		Forward	Reverse				Size (bp)	Allele type	Size (bp)	Allele type	Size (bp)	Allele type	
Pit	Pit-ID003	CCTGAAACACATTATCTATGTTG	AGAAAGAAGCATAAGTTTAAAATAGA	InDel	55	4	160		190	*Pit*			1) *Pit*
	Pish-ID007	TACACCGCTCGGCTTTTCACC	ATGCCCCTCGTTGCAGCC	InDel	↓	↓	100	*Pish* or *Pi35*	87				
Pish	Pish-ID010	TGCTACATATATATGATAATTGTCGAGG	TCAATCTACACCGTTAGATCAT	InDel	50	↓	94	*Pish*	81	*Pi35*			1) *Pish* 2) *Pi35*
	Pish-ID011	AGCACTTGACACTCCACAGCAG	GGCAAAACCCGTGTTTCTGACG	InDel	55	↓	102	*Pish*	116	*Pi35*			
Pib	Pib-ID11	AGAGTGGTTGGTTGGAGGTG	GCCCATATTGCTTTGCTCCAAA	InDel	↓	↓	103		92	*Pib*			1) *Pib*
	Piz-ID18	CTGCTGCTACCGTTTGGAAGTCA	CTCCTGGCCCCACGCGTC	InDel	↓	↓	98	*Piz-t*, *Piz-5*, or *Pi9*	84	*Piz*			1) *Piz*
Piz	Piz-ID22	ATGTGGGGTTTCTGATTCCAAT	CTTGATTAGTGAGATCCATTGTTCC	InDel	↓	↓	127	*Piz*	100	*Piz-t* or *Piz-5*	118	*Pi9*	2) *Piz-t* or *Piz-5* 3) *Pi9*
	Piz-ID31	CCAATTTCAGGCTTAGTTTGAT	AGCTATTTATTAAGCTGATTTCTCA	InDel	↓	↓	107	*Piz* or *Pi9*	84	*Piz-t* or *Piz-5*			
Pi13	Pi13-ID008	GTGAGCTGGAATACTAGATCGA	GTCAAAGTTCCTGCAATTTTGTGA	InDel	↓	↓	114		88	*Pi13*			1) *Pi13*
	Pii-ID07	TTCGGTCATTAGCCGGTGCT	GGCGGCAGGTATGGTACTTCA	InDel	↓	3 or 4	450		289	*Pii*, *Pii**, *Pi5*, or *Pi3*	493		1) *Pii*
Pii	Pii-ID21	AAGCGAACGACTCTAGCTAGAA	TCTCCATATGTATGTATAACTGGCTT	InDel	↓	4	84	*Pii**	96	*Pii*, *Pi5*, or *Pi3*			2) *Pii** 3) *Pi5* or *Pi3*
	Pii-ID24	ATGAGGAGATGACAACGAGGAG	GAAGAGGGGAACGCCGAG	InDel	↓	↓	100	*Pii**, *Pi5*, or *Pi3*	88	*Pii*			
Pia	Pia-ID01_2	ACGGTAGAGCAATTTAGAAGCAGTGA	AGTGCGACTGACACTTTCAATAGCA	InDel	55	↓	195		152	*Pia*			1) *Pia*
	Pik-ID001	CTTCTTAGCCTCCAGATTTGCA	TCATGTGCATCAAAATGGGCTA	InDel	↓	↓	100	*Pik*, *Pik-h*, *Pi7*, or *Pi1*	88	*Pik-m*, *Pik-p*, or *Pik-s*			
	Pik-ID007	AACGAATATTTATGACTAAAGAAAGT	AGAAGCTTGACTCCGTTAG	InDel	↓	↓	120	*Pik*	406	*Pik-m*, *Pik-p*, *Pik-s*, *Pik-h*, *Pi7*, or *Pi1*			1) *Pik* 2) *Pik-m* or *Pik-s*
Pik	Pik-ID011	GGTTAAATAGGACTCCCTCTA	GCATCCAATAGAATCAGAGA	InDel	↓	↓	169	*Pik*, *Pik-m*, *Pik-s*, or *Pi1*	150	*Pik-p*, *Pik-h*, or *Pi7*			3) *Pik-p* 4) *Pi1*
	Pik-ID014	TTCTTCTTTATCCCGTCTTCTT	ATGAGGAAAACGAAGATGAGAG	InDel	↓	↓	344	*Pik*, *Pik-p*, *Pik-h*, or *Pi7*	149, 150	*Pik-m*, *Pik-s*, or *Pi1*			5) *Pik-h* or *Pi7*
	Pik-ID018	ATCCTCTGTGTCTGAAGCCAT	AGGCTTCTCTGCTCCTATAACA	InDel	↓	↓	110	*Pik*, *Pik-m*, *Pik-p*, *Pik-s*, *Pik-h*, *Pi7*, or *Pi1*	100				
	Pita-ID13	AGGCAAGAGTACAATGGAAAC	TGCCCTCTGAAAATAAAGTTT	InDel	↓	↓	100	*Pita*	112	*(Pi20)*			
Pita	Pita-ID04[a]	CGTGAAGAGGATTCCGGTAGCA	TGCCGTGGCTTCTATCTTTACgTT	PCR-CTPP	↓	↓	137	*(Pi20)*	216	*Pita*			1) *Pita* 2) *(Pi20)*
		CAAGTCAGGTTGAAGATGCATtGC	CCGACGCCGAGCACTCTTAT										
	Pita-2-ID009	CACATAGCATTAGAGCACTAAACT	AACCATCATACCGGCCTATTTA	InDel	↓	↓	102	*Pi19* or *Pita-2*	114	*(Pi20)*			1) *Pita-2*
Pita-2	Pita-2-ID016_3	AAAGTTCATCGCATCGAATTTA	ACACGCGTTGGGATCTTCCTC	InDel	50	↓	135	*Pi19* or *(Pi20)*	123	*Pita-2*			2) *Pi19*
	Pita-2-ID011	TGCAATTAGTCTGGTGTTGTTGA	ACATGAATCAGTCTGACGTCAT	InDel	55	↓	115	*Pi19*	85	*Pita-2*	104	*(Pi20)*	3) *(Pi20)*

* *Pii* allele type with an asterisk ('Nerica 1') differs in marker genotypes from that in the reference varieties for *Pii* ('Fujisaka 5' and 'Ishikarishiroke') despite its typical reaction against differential pathogen races.

[a] Lowercase letters in primer sequences indicate mismatch bases against target complementary sequences. PCR-CTPP, polymerase chain reaction with confronting two-pair primers to distinguish co-dominant markers (Hamajima *et al.* 2000). PCR conditions for this marker: 95°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min 30 s, 72°C for 1 min, and finally 68°C for 5 min.

[b] The condition with a downward arrow is the same as the one above. Temp., annealing temperature; Gel, agarose gel.

[c] Genotype A represents the 'Nipponbare'-type, and B and C represent genotypes that differ from each other and from genotype A in terms of product size.

The genotype A obtained by the three markers (Pik-ID007, Pik-ID011, and Pik-ID014) represents the genotype of 'Kusabue' (HM048900) or 'Kanto 51' (AB616659), whose product sizes are indicated in gray boxes.

The genotype B obtained by the marker Pik-ID014 includes amplicons of two sizes that could not be clearly discriminated in an 4% agarose gel.

(Nonoue *et al.* 2018); however, this limited number of markers would not be sufficient to comprehensively determine required resistance alleles for variety registration. Here, we successfully developed SNP markers for 24 resistance alleles at 10 loci and validated their ability to discriminate all the multiple resistance alleles from each other, except for *Pi3* vs. *Pi5* (**Table 3**). Since *Pi3* and *Pi5* have not been used in the Japanese rice breeding program, our system covers all the resistance alleles that are required for cultivar registration in Japan. In addition, we developed 155 PCR-based markers (153 InDel and 2 PCR-CTPP markers); a subset of 23 of these PCR-based markers was sufficient to discriminate almost all resistance alleles from the counterpart susceptibility alleles. These markers are useful for selecting plants based on single resistance alleles even in large-scale breeding populations (**Table 5**, **Supplemental Table 6**, **Supplemental Fig. 11-1–11-10**). After analysis of parental lines by BRA1, loci that are segregating in respective populations can be selected by PCR-based markers. Therefore, these two estimation systems are a precise and efficient means for selecting genes for blast resistance from early generations onwards in breeding programs.

Haplotype-based association is a widely used approach for gene detection among crop plant varieties (Contreras-Soto *et al.* 2017, Gawenda *et al.* 2015, Lorenz *et al.* 2010). We applied this approach to resistance alleles by using multiple SNP markers around respective resistance gene loci and confirmed its applicability among the varieties tested (**Supplemental Table 1**). Our study successfully discriminated even among resistance alleles with highly similar DNA sequences that were found in the "K-type" genome at the *Pik* locus (**Supplemental Fig. 8D**). We carefully selected SNPs to discriminate resistance alleles located in a region containing multiple NBS-LRR (nucleotide-binding site–leucine-rich repeat) protein encoding genes, which were highly variable in number owing to genome rearrangement events in that region among diverse rice varieties. Key to the effective discrimination of the alleles at the *Piz* locus was the identification of combinations of allele-specific SNPs in single copy sequences that were conserved enough to among varieties to enable the design of genotyping assays or primers (**Supplemental Fig. 4C**). As mentioned above, DNA markers previously reported for the *Piz* and *Pik* loci have not been validated among diverse varieties; so, the user would need to confirm the utility of respective markers in their own cross combinations. We extended our evaluation system from the discrimination of reference varieties to the validation of haplotypes found in diverse rice varieties. Users can readily select suitable marker combinations from these haplotypes, which are listed in **Supplemental Table 4**. An important observation from our study is the identification of varieties that show the same response as reference varieties for certain alleles against a set of differential pathogen strains but have different SNP haplotypes at the *Pii*, *Pia*, and *Pik* loci. Since varieties that differ in SNP haplotypes are considered to have distinctive origins, the resistance alleles therein might have functional variations that have not been identified by conventional differential strain systems. Hence, the systematic identification of haplotypes suggests candidate varieties that require further pathogenic characterization (e.g., 'Nerica 1' for the *Pii* locus and 'Kanto 51' for the *Pik* locus); such trials would contribute to further improvement of differential systems. We validated the resistance genotypes by inoculation testing using experimental lines (here, CSSLs) that each carry a chromosomal region harboring certain resistance allele(s) in a well-characterized genetic background. This procedure was even effective for characterization of SNP haplotypes that lack information on resistance. These examples reinforce the idea that the haplotype-based approach, in combination with use of experimental lines, can enhance the development of selective markers for a wide range of varieties.

DNA markers designed around the *Pita* and *Pita-2* loci in previous studies (Hayashi *et al.* 2006, Jia *et al.* 2002, 2004) and our preliminary survey were unable to discriminate reference varieties for *Pita* and *Pita-2*, even though they differed in their responses to differential isolates. Since all the tested varieties carrying *Pita-2* also have *Pita*, researchers speculated that the broader resistance spectrum of *Pita-2* compared with *Pita* is due to the combined effect of *Pita* and *Pita-2* (Bryan *et al.* 2000, Jia *et al.* 2003). Thus, we assumed that donor varieties for these two resistance alleles are closely related and a loss-of-function or gain-of-function mutation at the *Pita-2* locus in one of the donors resulted in the allelic difference at this locus. Recently, *Pi19* was identified as an allele of the *Pita-2* locus, which is located 212 kb from *Pita* (Takahashi *et al.* 2017). Accordingly, we sequenced the *Pita-2* coding region in reference varieties for *Pita* and *Pita-2* to identify polymorphisms that could distinguish the presence or absence of *Pita-2*. We successfully developed two allele-specific markers (FA5655 and Pita-2-ID016_3) that target polymorphisms in *Pita-2* locus (**Fig. 2C**). This observation reminds us that rapid evolution of disease resistance genes should be taken into account when using the haplotype-based approach. Accumulating whole genome sequence data in multiple rice varieties will allow researchers to search for DNA variations in resistance genes to increase the number of DNA markers for blast resistance.

The recent increase in the use of high-yield varieties with an *indica* genetic background in Japan (Kato 2008, Yonemaru *et al.* 2014) has made it difficult to determine the genotypes for blast resistance by using conventional differential systems because such varieties harbor multiple resistance alleles and there is a lack of pathogen isolates to discriminate such alleles. The genotypes for some of these varieties are undetermined, as shown in the variety registration databases (http://www.hinshu2.maff.go.jp/, http://ineweb.narcc.affrc.go.jp/). From the viewpoint of reducing the use of agricultural chemicals and environmental burden when using such varieties, enhancement of blast resistance is a high-priority breeding objective. Thus, determining

unknown resistance genotypes and setting up marker-assisted selection systems for these varieties is of paramount importance. For example, the presence or absence of *Pi20*, which has a wide blast resistance spectrum (Li *et al.* 2008), cannot be determined by a conventional evaluation system in these varieties. To overcome this issue, an improved inoculation-based differential system surveying 23 alleles including *Pi20* was developed to characterize 10 varieties, mostly in *indica* genetic background (Hayashi *et al.* 2014). Concordance between the two systems was high except for *Pia* and *Pita-2* loci, confirming the discriminatory ability of our system (**Table 4**). Among the 23 alleles, only *Pia* (at *Pia* locus) and *Pi19* (at *Pita-2* locus) have extremely narrow resistance spectra and lack fungus strains that selectively identify them. This might be the reason for the difficulty in estimating these alleles by inoculation testing, and thus we believe that the DNA-based estimation of this locus in the current study is more reliable than that provided by inoculation test-based estimation in the previous study (Hayashi *et al.* 2014). Furthermore, the phenotypes of some differential strains previously used to discriminate between *Pita*-2 and *Pi20* are unstable, being influenced by environmental factors or unidentified resistance alleles in the genetic background. By contrast, DNA genotyping in our study clearly discriminated these alleles based on SNP haplotype. We were unable to discriminate between *Pi3* and *Pi5* (at *Pii* locus), as was the case for the inoculation-based system (Hayashi *et al.* 2014); we need to confirm whether the reference lines that we used for *Pi3* and *Pi5* are identical or not. Collectively, the results indicate that our DNA marker-based genotyping system is an efficient and reliable alternative for determining the resistance genotypes of varieties or breeding lines with *indica* genetic background.

Continual updating of the evaluation system is important to meet breeders' demands for new resistance alleles and to improve discriminability. The differential system by Hayashi *et al.* (2014) uses many differential experimental lines and pathogenic races obtained from large-scale screening and establishment of lines, whose improvement requires much effort and expertise. By contrast, the DNA-based system is readily updated by addition or replacement of markers based on accumulated SNP-haplotype information including newly-identified resistance loci. Thus, we propose that our system can be used as a standard procedure for selection of blast resistance alleles in the Japanese rice breeding program. As discussed above for the case of *Pita-2*, recent mutation events that cause loss or change of resistance spectrum might be undetectable in the SNP haplotype-based approach; mutant alleles with extremely low frequency may also be undetectable by this approach. In case of the *Pik* locus, where more than eight resistance alleles have been reported, we found that some monogenic lines for a resistance allele differed in response type according to the pathogen isolate used (**Supplemental Table 5**). Unidentified resistance alleles in the genetic background and/or recent variation at the *Pik* locus could explain the observations; the former is more likely when the lines carry the same SNP haplotype. To distinguish between these possibilities and to precisely determine resistance genotype for variety registration, it is desirable to determine the entire coding sequence of each sample with reference resistance alleles. Alternatively, the resistance alleles identified by BRA1 could be confirmed by inoculation testing. Breeders or pathologists would be able to reduce the number of inoculation tests because they could select appropriate differential strains according to the BRA1 results. Trials to clarify the reasons for any disagreements between the two methods would contribute to the identification of new resistance alleles at known or unidentified loci, thus improving the system.

Our method of developing a genotyping system for blast resistance could also be applied to the development of markers for genes involved in quantitative resistance rather than race-specific resistance (e.g., *pi21*, *Pb1*, *Pi39*, *Pi34*, *Pi63*; Fukuoka *et al.* 2009, Hayashi *et al.* 2010b, Terashima *et al.* 2008, Xu *et al.* 2014a, Zenbayashi-Sawata *et al.* 2007). Furthermore, based on breeders' requests, our procedures could be expanded to set up genotyping systems for several agronomic traits, such as days to heading, yield-related traits, grain quality, and resistance to other diseases and pests in breeding programs. Establishment of such systems will further enhance marker-assisted breeding in rice.

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