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A Trial for the Identification of Brown Planthopper (*Nilaparvata lugens* Stål) Resistance Alleles in Two Rice Lines, Norin-PL3 and Norin-PL4, Using Next-generation Sequencing and Simple Graphical Genotyping Methods

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Abstract

Norin-PL3 and Norin-PL4 are rice (*Oryza sativa* L.) breeding lines that carry a brown planthopper (BPH) resistance gene. Norin-PL3 was bred by introgressing *BPH1* derived from the Indica Group variety Mudgo into Japonica Group rice, whereas Norin-PL4 was bred by introgressing *BPH2* derived from the Indica Group variety IR 1154-243 into Japonica Group rice. To detect the alleles derived from resistance donor varieties in these lines, we developed a simple method to create graphical genotypes using a part of the InDel information obtained by a short-read next-generation sequencing system. In Norin-PL3, chromosomal regions derived from Mudgo were found on chromosome 12 and two other locations on the short arm of chromosome 1. In Norin-PL4, the chromosomal regions derived from IR 1145-243 were found on chromosomes 5 and 12, the short arm of chromosome 4, and chromosome 8. Among these regions derived from resistant donors, chromosomal regions contributing to resistance were identified. This method could also assist in creating graphical genotypes of the other rice lines with both Japonica Group and Indica Group varieties as parents. Moreover, the results of the graphical genotype and DNA marker information of Norin-PL3 and Norin-PL4 will be useful for rice breeding research on BPH resistance.

Discipline: Crop Science
Additional key words: *BPH1*, *BPH2*, insertion–deletion (InDel), resistance gene

Introduction

A graphical genotype portrays the parental origin and allelic composition of a chromosome (Young & Tanksley 1989). This information is important for the use of breeding lines for functional genetic analyses and further breeding. Moreover, when performing gene expression analysis using RNA-seq data, graphical genotype information would be useful for mapping genes accurately on the chromosome. Furthermore, when creating a knockout transformant using CRISPR/Cas9 to analyze gene function, it would be useful to predict the allele type of a target gene by referring to its graphical genotype. In general, DNA markers have been used to create graphical genotypes. Restriction-fragment length polymorphism (RFLP) markers have been used to create early graphical genotypes for gene mapping (Tamura et al. 1999, Murata et al. 1998). DNA markers in which polymorphisms can be easily analyzed through polymerase chain reaction (PCR) and electrophoresis, such as simple sequence repeat (SSR) markers, have been subsequently developed (McCouch et al. 2002, International Rice Genome Sequencing Project 2005); they have been used to create graphical genotypes (Takai

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et al. 2007, Okada et al. 2018).

In recent years, whole-genome sequencing has been conducted much more easily given the progress in short-read next-generation sequencing (NGS) technology (Shendure & Ji 2008). In rice, a high-quality reference genome sequence of Nipponbare was generated by the International Rice Genome Sequencing Project (International Rice Genome Sequencing Project 2005) and Kawahara et al. (2013). Therefore, single nucleotide polymorphism (SNP) and insertion–deletion (InDel) polymorphism information is easy to obtain with the whole-genome sequencing of some rice varieties using NGS and mapping of the short-sequencing reads to the reference genome sequence of Nipponbare (Yamamoto et al. 2010, Arai-Kichise et al. 2011, 2014, Takano et al. 2014, Yonemaru et al. 2015). If the polymorphism information, such as SNPs and InDels, of a breeding line and its donor varieties are compared with those of Nipponbare using NGS data, then the chromosomal regions from each donor in the breeding line can be detected by searching for polymorphisms that also exist in the donor variety. Therefore, it is possible to create a graphical genotype that portrays chromosomal regions derived from each donor variety throughout the whole chromosome. Moreover, with polymorphism information such as SNPs and InDels obtained with NGS, it is possible to obtain more polymorphism information from chromosomal regions where conventional SSR markers do not exist. Furthermore, it will be possible to create new PCR-based InDel markers and cleaved amplified polymorphic sequences (CAPS) markers using InDel and SNP information. These markers enable the systematic elimination of undesirable chromosomal introgressions from a donor variety in the chromosomes of the breeding line.

The brown planthopper (BPH; *Nilaparvata lugens* Stål) is an important pest of rice (*Oryza sativa* L.) and is widely distributed from tropical to temperate regions of Asia. The BPH withers rice plants by sucking phloem sap, and in Southeast Asia, it also mediates viral diseases, such as grassy rice stunt and rice ragged stunt disease (Catindig et al. 2009). Attempts to prevent damage from BPH by using resistant varieties have been encouraged since the 1970s at the International Rice Research Institute, and many resistant varieties have been bred and cultivated in Southeast Asia (Brar et al. 2009). However, continual use of the same single gene often results in the outbreak of harmful biotypes of BPH. In Southeast Asia, the number of BPHs dramatically decreased immediately following the release of the resistant variety carrying the resistance gene *BPH1*. However, the incidence of the harmful biotype of BPH has increased, and the resistant variety has been rendered ineffective a few years after its release (Sogawa 1982). The same phenomenon was observed following the use of a variety carrying the BPH resistance gene *BPH2* (Brar et al. 2009).

Many BPH resistance genes, including *BPH1*, *BPH2*, *BPH9*, *BPH10*, *BPH18*, *BPH21*, and *BPH26*, have been identified and mapped to the long arm of chromosome 12 (Fujita et al. 2013). Some of these genes have been cloned and eventually identified to be the same, and thus have been classified as alleles (Zhao et al. 2016). For instance, *BPH1* and *BPH18* (Zhao et al. 2016) as well as *BPH2* and *BPH26* (Tamura et al. 2014) are the same alleles. *BPH26* induces phloem sucking inhibition against BPH (Tamura et al. 2014). The details of the mechanism underlying sucking inhibition, such as the signal transduction system leading to sucking inhibition and the causative substances of the inhibition, have not been elucidated. If the mechanism of sucking inhibition induced by the resistance genes *BPH1* and *BPH2* is clarified, then helpful information will be obtained regarding the adaptive mechanism of virulent biotypes and the sustainable use of the resistance genes.

Norin-PL3 and Norin-PL4 are Japonica Group breeding lines, harboring the BPH resistance genes originating from Indica Group rice varieties. These are conserved at the Genetic Resources Center, NARO. Norin-PL3 carries *BPH1*, which was introgressed from an Indica Group variety, Mudgo, and Norin-PL4 carries *BPH2,* which was introgressed from an Indica Group variety, IR 1154-243 (Kaneda et al. 1985, Kaneda et al. 1986). Norin-PL3 and Norin-PL4 were developed by backcross breeding with Japonica Group rice varieties, Tsukushibare and Asominori, respectively, as recurrent parents. Most of the chromosomes of both varieties seem to have been derived from these recurrent parents. The genetic background of Norin-PL3 is common with that of Tsukushibare, and the effect of *BPH1* is easy to evaluate by comparing these two lines. Moreover, the effect of *BPH2* can be evaluated easily by comparing Norin-PL4 and Asominori.

Regarding Norin-PL3, no graphical genotype has been reported yet, and it is unclear how much the chromosome introgressions from Mudgo remained in the genetic background of Norin-PL3. Regarding Norin-PL4, although an example of a graphical genotype using RFLP markers was created (Murata 1998), RFLP markers are limited in number and not widely used. Therefore, the development of PCR-based DNA markers based on InDel information detected by NGS analyses can provide useful information for Norin-PL3 and Norin-PL4 for DNA marker-assisted breeding.

In this study, we devised a method to easily detect

chromosomal regions from the resistant donor variety in a breeding line by selecting a part of the InDel information from a large number of InDels distributed throughout the whole genome. With this newly devised and simple analysis method, we created graphical genotypes of Norin-PL3 and Norin-PL4 in which the chromosomal regions derived from each resistant donor variety were portrayed on the chromosomes. Moreover, we aimed to identify chromosomal introgressions from resistant donor varieties that contribute to BPH resistance. Furthermore, we developed PCR-based DNA markers that can easily detect chromosomal regions derived from resistant donor varieties.

Sharma et al. (2004) reported that *BPH1* and *BPH2* on chromosome 12 were on different loci. However, Zhao et al. (2016) reported that *BPH1* and *BPH2* were different alleles on the same locus. In this paper, the resistance gene derived from Mudgo was described as *BPH1*, and the resistance gene derived from IR 1154-243 was described as *BPH2* by precedent. Moreover, following the new gene nomenclature system for rice (McCouch & CGSNL 2008), the abbreviation of the gene full name such as BPH is written in italics and all capital letters as "*BPH*."

Materials and methods

1. Plant materials

We used the following rice varieties, which are conserved at the Genetic Resources Center, National Agriculture and Food and Research Organization (NGRC, NARO), Tsukuba, Japan: Suitou Chuukanbohon Nou 3 (Norin-PL3; also named Kantou PL 4; JP7693), Suitou Chuukanbohon Nou 4 (Norin-PL4; also named Kantou PL 5; JP7694), Mudgo (JP12491), IR 1154-243 (JP12439), Tadukan (JP12485), Tsukushibare (JP11283), Asominori (JP11299), Saikai 190 (JP68142), and Nipponbare (JP229579). The rice variety Nankai 133 was originally developed at the Miyazaki Agricultural Experiment Station, Japan, and has been conserved at the Institute of Agrobiological Sciences, NARO, since 2010. Figure 1 shows the genealogies of Norin-PL3, Norin-PL4, Asominori, Saikai 190, and Nankai 133. Norin-PL3 and Saikai 190 are resistant lines carring *BPH1* derived from crosses among Mudgo as a donor and several Japonica Group varieties. Norin-PL4 is a resistant line carring *BPH2* derived from crosses among IR 1154-243 as a donor and several Japonica Group varieties. Nankai 133 is a Japonica Group rice line that was bred by crossing Norin-PL4 with Japonica Group rice varieties twice. The rice used for the DNA extraction experiments was cultivated in a greenhouse for approximately 1 month under long-day conditions at 25°C.

2. Detection of InDels in Norin-PL3 and Norin-PL4 by Illumina short-read sequencing

DNA was extracted using the CTAB method (Murray & Thompson 1980) from rice leaves of each variety. Illumina HiseqX (Illumina Inc., CA, USA) was used to obtain short-read sequences covering the whole genomes of Norin-PL3, Norin-PL4, Mudgo, and IR 1154-243. The paired reads were discarded when either read contained adapter contamination when uncertain nucleotides (N) constituted more than 10% of either read and when low-quality nucleotides (base quality less than 5, $Q \leq 5$) constituted more than 50% of either read. The effective sequencing data were aligned with the Nipponbare reference sequence (Os-Nipponbare-Reference-IRGSP-1.0; https://rapdb.dna.affrc.go.jp/download/irgsp1.html) using BWA (Li & Durbin 2009) software (parameters: mem -t 4 -k 32 -M). The duplicate sequences were removed using SAMTOOLS (Li et al. 2009). InDels refer to the insertion or deletion of ≤ 50 bp sequences in the DNA. SAMTOOLS (Li et al. 2009) with the parameter "mpileup -m 2 -F 0.002 -d 1,000" was used to detect InDels, and annotation was performed using ANNOVAR (Wang et al. 2010). Analysis of short reads using Illumina HiseqX, mapping to the Nipponbare reference genome, and detection of InDels were performed by Novogene Corporation, Inc. Illumina short-read sequences covering the whole genomes of Mudgo, IR 1154-243, Norin-PL3, and Norin-PL4 are available in the DDBJ Sequenced Read Archive under the accession numbers DRX209249, DRX209250, DRX209251, and DRX209252, respectively.

3. Analysis of polymorphism between rice varieties

(1) InDel polymorphism information used for genotype analyses of two breeding lines, Norin-PL3 and Norin-PL4

The chromosomal regions derived from Mudgo contained in Norin-PL3 were detected by comparing the InDel polymorphisms between Nipponbare and Norin-PL3 with those between Nipponbare and Mudgo. The chromosomal regions derived from IR 1154-243 contained in Norin-PL4 were detected by comparing the InDel polymorphisms of Nipponbare and Norin-PL4 with those of Nipponbare and IR 1154-243.

(2) The whole-genome survey at every 1 Mbp on chromosomes of the breeding lines, Norin-PL3 and Norin-PL4

We determined whether the same polymorphism on the exon observed between Nipponbare and the resistant donor variety also existed in Nipponbare and the breeding

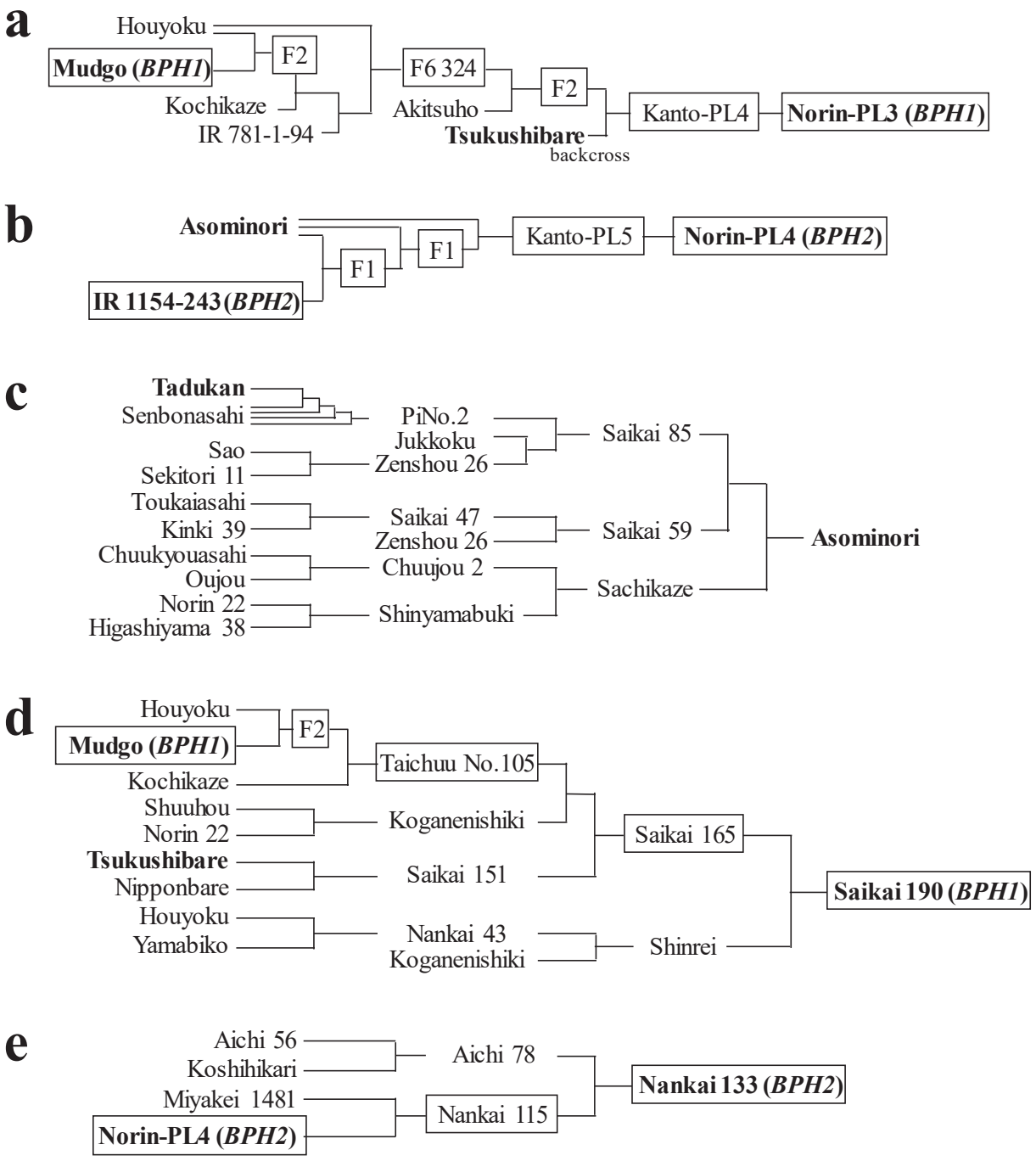

Fig. 1. The genealogies of Norin-PL3, Norin-PL4, Asominori, Saikai 190, and Nankai 133 used in this experiment
a: Genealogy of Norin-PL3 (Kaneda et al. 1985)
b: Genealogy of Norin-PL4 (Kaneda et al. 1986)
c: Genealogy of Asominori (database on rice varieties) used for backcrossing in the breeding of Norin-PL4
d: Genealogy of Saikai 190 (database on rice varieties)
e: Genealogy of Nankai 133 (database on rice varieties)
The rice lines enclosed by rectangles indicate BPH resistance.

line by setting a reference point at every 1 Mbp on the chromosomes of the breeding line. To increase the accuracy of the analysis, we selected InDel polymorphisms between Nipponbare and the resistant donor variety in three different locations within the range of ± 0.2 Mb across the reference points. The reference points, where donor alleles were observed in all three selected InDels, were judged as the Indica Group genotype. Three polymorphisms in the analysis area (reference point ± 0.2 Mb) were selected in the order of the length of InDels; if there were numerous polymorphisms with the same length, the polymorphism closest to the reference point was selected. When three InDel polymorphisms were not found in the analysis area (reference point ± 0.2 Mb), InDel polymorphisms other than those in exons were added to the analysis targets for InDels.

(3) Detection of a donor allele on the chromosome of the breeding line using large InDel information

We extracted large InDels—with a length of 10 bp or more—among InDel polymorphisms between Nipponbare and the breeding line. We confirmed whether the InDel polymorphisms matched the polymorphisms between the Nipponbare and donor varieties. The chromosomal area where large InDels with donor alleles were observed in three or more locations within 1 Mbp on the chromosome of a breeding line was determined to be a highly probable area from the Indica Group variety.

(4) Determination of chromosomal regions derived from the donor variety in breeding lines

Additional detailed analyses were conducted at the candidate area from the donor variety in experiments (2) and (3) to survey the area's limits. The genotypes of InDel polymorphisms around the donor allele detected by experiments (2) and (3) were examined at intervals of approximately 50 kbp on both sides of the donor-type allele toward the short arm end and the long arm end. The polymorphism analyses ended when all non-donor-type alleles appeared over 500 kbp in succession. InDels (≥ 1 bp) not only in exons but also in non-coding regions were used for these polymorphism analyses. The ranges wherein donor-type alleles were detected over 150 kbp by this analysis were determined to be candidate regions derived from an Indica Group variety.

(5) Genotype verification using DNA markers

The genotype of the chromosomal candidate regions from an Indica Group variety in the analysis of (4) was reexamined using a DNA marker. First, we searched for effective markers to detect a donor allele among the SSR markers reported in the International Rice Genome Sequencing Project (2005). Next, InDel markers were created in the chromosomal regions where no effective SSR markers were found by using InDel information on short-read NGS. InDel detected by direct sequencing of Sanger Sequencing (3730xl DNA Analyzer; Applied Biosystems/HITACHI) using a primer designed based on the sequence of Nipponbare was also used to create InDel markers. Multiplex PCR Assay Kits (TaKaRa Bio, Inc.) were used for PCR. The polymorphisms of the PCR products were confirmed by electrophoresis, and polymorphism analyses of base sequence information were performed using a 3730xl DNA Analyzer (Applied Biosystems/HITACHI). The genotype of each marker for Norin-PL3 was primarily determined by comparison with the electrophoresis pattern of the Tsukushibare (Japonica Group repetitive parent of Norin-PL3) and Mudgo. The genotype of each marker of Norin-PL4 was primarily determined by comparison with the electrophoresis pattern of Asominori (Japonica Group repetitive parent of Norin-PL4) and IR 1154-243, with supplementary use of Nipponbare and Indica Group rice variety Tadukan.

(6) Creations of graphical genotypes of the two breeding lines

The regions on chromosomes wherein the donor-type allele was confirmed by both analyses of the NGS (4) and the DNA markers (5) were determined as the chromosomal regions introgressed by the Indica Group variety, and a graphical genotype was created. An outline of the procedure for creating the graphical genotype is presented in Figure 2.

4. Bioassays

BPH, which was collected in Osaka Prefecture, Japan, in 1973 and has been reared at our institute, was

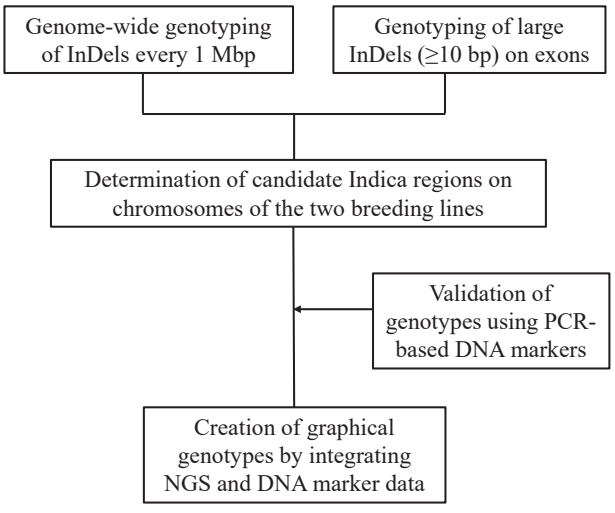

Fig. 2. Outline for creating graphical genotypes using short-read NGS data for Norin-PL3 and Norin-PL4

used for bioassays (Tamura et al. 2014). The rice varieties Saikai 190 and Nankai 133 were used to evaluate BPH resistance. The rice variety Tsukushibare, which is susceptible to BPH, was used as a control. Four germinating seedlings were placed in a test tube (diameter, 1 cm; height, 18 cm) with absorbent cotton moistened with a liquid fertilizer; and the test tube was secured with a cotton plug and incubated for 13 days at 25°C under a 16:8 light/dark cycle. Four individual BPHs (two males and two females) that were one or two days old after emergence were released from each test tube. The number of live BPH was recorded 7 days after the release. The tests were repeated 10 times, and the average number of live BPH per test tube was calculated. The data were analyzed using Tukey–Kramer test.

Results

1. Analyses of InDel polymorphisms by short-read NGS

As many as 102-122 M short-read sequences were obtained from the analyses by Illumina short-read NGS. The mapping rate ranged from approximately 97%-99%, and the average depth ranged from approximately 35%-42% (Table 1). Coverage (%) tended to be smaller for Indica Group rice varieties (Mudgo and IR 1154-243) than for the two breeding lines (Norin-PL3 and Norin-PL4). Table 2 shows the number of InDel polymorphisms detected when comparing the two breeding lines and the two Indica Group varieties with Nipponbare. The total number of InDels between Nipponbare and Mudgo was approximately 14 times higher than that between Nipponbare and Norin-PL3. Among the InDel polymorphisms in exons, the number of large InDels (≥ 10 bp) was 1,807 for Mudgo and 106 for Norin-PL3; Mudgo had approximately 17 times more InDels (≥ 10 bp) compared with Norin-PL3. Likewise, the total number of InDels between Nipponbare and IR 1154-243 was greater than that between Nipponbare and Norin-PL4, and among InDels in exons, the number of large InDels (≥ 10 bp) for IR 1154-243 was approximately 7.8 times greater than that for Norin-PL4 (Table 2).

2. Identification of chromosomal regions introgressed from Mudgo in Norin-PL3

First, a whole-genome survey of the Mudgo alleles in Norin-PL3 was performed using InDel polymorphisms in exons. Figure 3a shows the genotype of Norin-PL3 by examining equal intervals of every 1 Mbp. The same alleles as Mudgo were detected around 8 Mbp from the short arm end of chromosome 1 and 23-25 Mbp from the short arm end of chromosome 12. Among the 367 sites surveyed at equal intervals of every 1 Mbp across the genome, four sites were found to have the same alleles as Mudgo (Fig. 3a).

Next, we examined alleles showing the Mudgo genotype in 106 large InDels (≥ 10 bp) in exons contained in Norin-PL3 to efficiently detect chromosome introgressions derived from Mudgo in Norin-PL3 (Fig. 3b). There were 56 Mudgo alleles and 50 alleles of other varieties among the 106 large InDels. Fifty-six of the 106 large InDels in exons in Norin-PL3 (53%) were Mudgo alleles. The total number of large InDels (≥ 10 bp) in the exons of Mudgo were 1,807 (Table 2), although the number of those in exons of Norin-PL3 was only 106.

Table 2. The number of InDels detected while comparing each variety with the Nipponbare reference genome sequence (Nipponbare-IRGSP-1.0)

Name	InDels (total)	InDels (on exons)	Large InDels on exons (≥10bp)
Mudgo	383,687	9,596	1,807
Norin-PL3	27,983	1,038	106
IR 1154-243	380,324	9,530	1,770
Norin-PL4	55,826	1,597	228

Table 1. Information of short-read sequences analyzed by an Illumina sequencer

Name	Mapped reads	Total reads	Mapping rate (%)	Average depth ($\bar{x}$)	Coverage (%)
Mudgo	98,197,668	101,652,766	96.60	34.87	91.67
Norin-PL3	103,770,060	104,432,850	99.37	35.22	98.74
IR 1154-243	118,359,324	121,992,194	97.02	41.78	92.21
Norin-PL4	106,480,542	107,462,624	99.09	36.79	97.59

Mapped reads: The number of clean reads mapped to the reference assembly, including both single-end reads and reads in pairs
Total reads: Total number of effective reads in clean data
Mapping rate: The ratio of the reference genome assembly mapped reads to the total sequenced clean reads
Average depth: The average depth of mapped reads at each site, calculated by the total number of bases in the mapped reads divided by the size of the assembled genome
Coverage: The percentage of the assembled genome with more than one read at each site

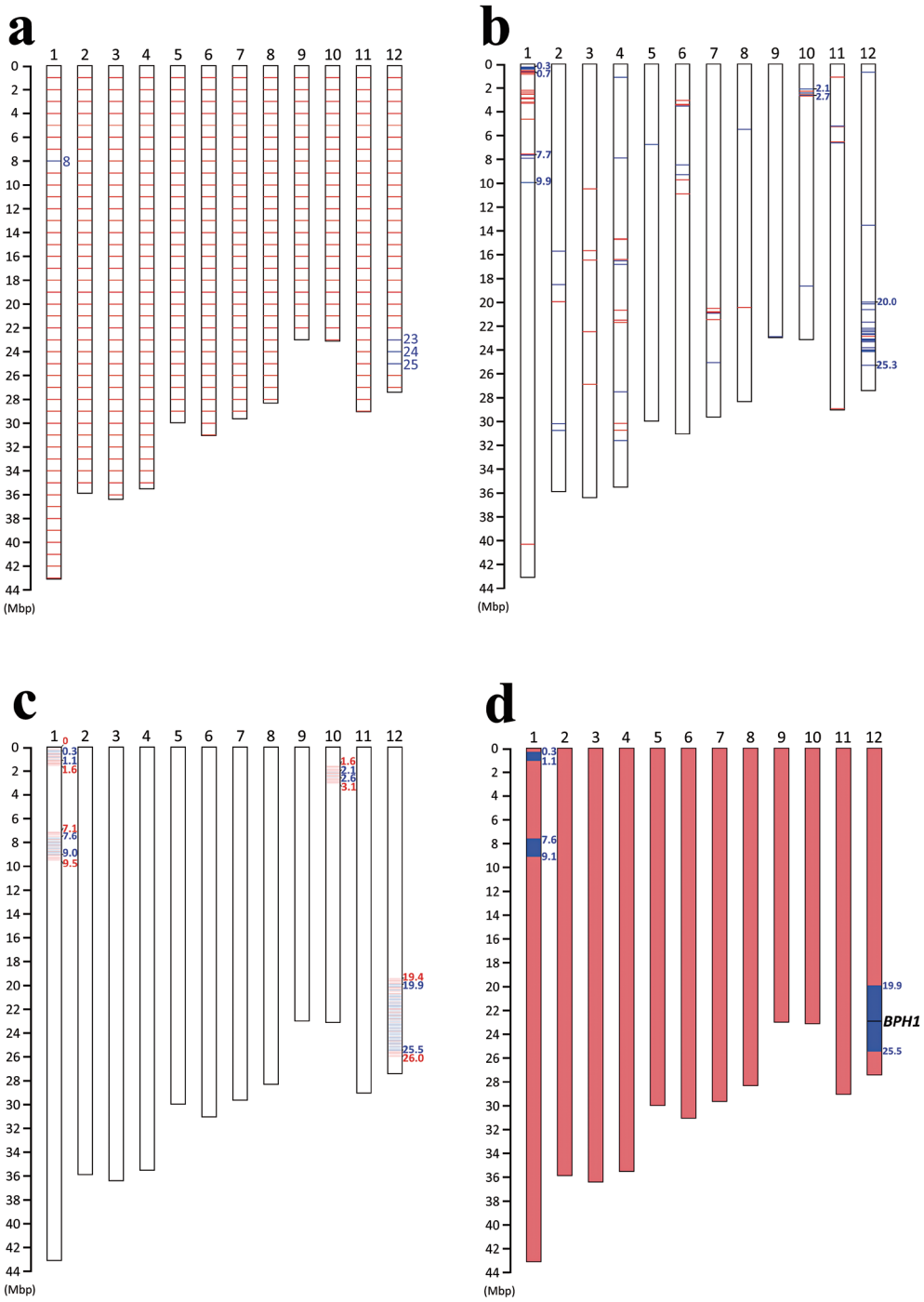

Fig. 3. Analyses of chromosomal introgressions from Mudgo in Norin-PL3

a: The genotypes of InDel polymorphisms in exons were analyzed for the whole genome of Norin-PL3 at every 1 Mbp. Mudgo alleles are shown as blue lines, and all other alleles are red lines.

b: Analyses of genotypes of large InDels (≥ 10 bp) in exons. Mudgo alleles are displayed in blue, and all other alleles are in red.

c: The ranges wherein Mudgo alleles were detected by detailed analyses of the genotypes of InDels (every 50 kbp) for the region predicted to be chromosomal regions derived from Mudgo in the analyses of a and b.

d: A graphical genotype of Norin-PL3 created by integrating the analysis results of NGS and PCR-based DNA markers. Chromosomal regions derived from Mudgo are displayed in blue, and other chromosomal regions are in red. *BPH1* locus is based on the report of Zhao et al. (2016).

Therefore, the number of large InDels (≥ 10 bp) in exons between Nipponbare and Japonica Group varieties used for breeding appeared to be considerably less compared with that between Nipponbare and Mudgo. There were several places where large InDels of Mudgo alleles existed concentrically on the chromosome of Norin-PL3 (Fig. 3b). Many Mudgo alleles were detected in the vicinity of 8 Mbp on chromosome 1 and the long arm of chromosome 12, as shown in Figure 3a. Furthermore, many Mudgo alleles were detected at the short arm end (0.3-0.7 Mbp) of chromosome 1 and in the vicinity of 2.5 Mbp on chromosome 10 (Fig. 3b). Moreover, large InDels were detected in the vicinity of 16 Mbp on chromosome 4, 3 Mbp on chromosome 6, and 21 Mbp on chromosome 7. Because the number of Mudgo alleles was lower than three within the range of 1 Mbp, these were excluded from the candidate chromosomal regions derived from Mudgo.

The genotypes of InDels surrounding the Mudgo alleles shown in Figure 3a and Figure 3b were analyzed in more detail (every 50 kbp) to determine the limits of the area of chromosomal introgression of Mudgo (Fig. 3c). Chromosomal introgressions from Mudgo were detected between 0.30 and 1.05 Mbp and between 7.60 and 9.05 Mbp on chromosome 1, between 2.10 and 2.55 Mbp on chromosome 10, and between 19.90 and 25.45 Mbp on chromosome 12.

The presence of candidate regions introgressed from Mudgo in Figure 3c was confirmed using DNA markers. Table 3 shows the primers' sequence information, and Figure 4a shows the positions of the markers on the chromosome. Mudgo alleles were detected between 0.30 and 1.05 Mbp on chromosome 1 with InDel markers of 1-371K and 1-382K, between 7.60 and 9.05 Mbp on chromosome 1 with InDel markers of 1-7689K and 1-7822K Figurs 4b). Moreover, Mudgo alleles were detected between 19.9 and 25.45 Mbp on chromosome 12, with markers RM28449S1, RM28466, 12-23906K, and 12-24110K (Fig. 4b). The CAPS marker BIM3, which has previously been reported to be linked to *BPH18* (Ji et al. 2016), and InDel marker ID-28L4, which has been reported to be linked to *BPH26* (Tamura et al. 2016), could also detect the Mudgo alleles (data not shown). In the region between 2.10 and 2.55 Mbp on chromosome

Table 3. Primer information used for genotyping of Norin-PL3 and Norin-PL4

Ch	Primer name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Purpose
1	1-371K	CAA ACC ATA TCA TTT TCG GCT A	TGC CTT CTC TTT CAC CCT TT	InDel marker
1	1-382K	GCG TTT CGC CGA TAT TTA C	ATG CAG AAG CAG CTT AGA CCT	InDel marker
1	1-2587K	ATC AAT CAT GGG GAC CAA AA	TAC CTG CAG CTT GAG CAA TG	InDel marker
1	1-3337K	GCA GCT GAT GAT TGT CTA ATG C	GCT GGC ACC GTG TTT CTA TT	InDel marker
1	1-7689K	CAT GGG GTG ATT TCA TTG TG	TGC GGC AGC TAC CTA AAT AAA	InDel marker
1	1-7822K	CGG AAA AGA GGG GAA GAA AC	CCA CCG TGA AAA ACA GTC CT	InDel marker
4	4-846K	CTG GAG CCA CGT AAT GGT TT	GTC ATC AAG TGG AGG GTC GT	InDel marker
4	4-31467K	CAG GAC GTA CGG GTA GTG GT	ATG GTT CTT TGG GAC AGT GG	InDel marker
5	5-7336K	GCA AAC CGT GAG ACG AAT CT	CGC CAA GGA ATT AAG GTC AA	InDel marker
5	5-7893K	CGT CAC ACC AAG CAA TGA AC	TTA AAA TGT GCA CCA GGA TCA	InDel marker
5	5-8456K	GCT TAT GGG CAG GAT TTC AA	AAC TAC TCT CTC CGT TCC CAT A	InDel marker
5	5-13923K	AAC GTG AAT TTG CTC GAT GA	AAG CCG CTC GCA ACA AGT	InDel marker
8	8-15061K	AAC ACC GAT GAG GTT TCA CC	AGA AGC AGC AGC CCT AAC AA	InDel marker
10	10-2471K	TGG GGC TTC TAC TTG TCA GC	GAG GTC AGG CCT GCT GTA TC	InDel marker
10	10-2514K	CTC GAG CTG CCT AAT TCT GG	TGC GTA AAC AGA TCC TCT GC	InDel marker
12	12-2413K	AAC CCA CAG GAT TGG AAC TG	CGA AGA GAC ATC CTC GGA AC	InDel marker
12	RM7102	GGG CGT TCG GTT TAC TTG GTT ACT CG	GGC GGC ATA GGA GTG TTT AGA GTG C	SSR marker
12	RM28449S1	TAG TGA TAT TCA CGT TCT TG	CTC TGG CTG AAA GAG TGG CG	SSR marker
12	12-22719K	CAG TGC GCC TAG GAA GTA GG	ATT CAC GTC TTT CGG GTG TC	InDel marker
12	RM28466	CCG ACG AAG AAG ACG AGG AGT AGC C	AGG CCG GAG AGC AAT CAT GTC G	SSR marker
12	12-23906K	CCT TTC CCC CTT TGT CTA GG	CTT GCC CTT TTG GAC CAC TA	InDel marker
12	12-24110K	AAG GGA ACG GGA GAC TAG GA	CTT GGC CCA ATT AAC CCA TA	InDel marker
12	ID-28L4	GAAGGGAAATGGAAGCATGA	TACACCCGACAAGGAACACA	InDel marker
12	BIM3	TCTCCCTAGCATTGCTATGC	TCCTGGGAGAAATTCTAGCC	CAPS marker

Ch, Chromosome number. SSR, simple sequence repeat. InDel, insertion—deletion.

10 where the percentage of the Mudgo allele was low, Tsukubare alleles were detected with InDel markers of 10-2471K and 10-2514K (Fig. 4b).

Therefore, chromosomal regions from Mudgo were determined to exist between 0.30 and 1.05 Mbp as well as 7.60 and 9.05 Mbp on chromosome 1 and between 19.90 and 25.45 on chromosome 12, based on the analyses with short-read NGS and DNA markers. These three regions are shown in blue as chromosomal introgressions from Mudgo, and other regions are shown in red in the graphical genotype in Figure 3d.

3. Identification of chromosomal regions introgressed from IR 1154-243 in Norin-PL4

First, a whole-genome survey of the IR 1154-243 alleles in Norin-PL4 was performed using the InDel polymorphisms in exons as in the case of Norin-PL3. The results of the genotype of Norin-PL4 at equal intervals every 1 Mbp is shown in Figure 5a. Next, the genotypes of 228 large InDels (≥ 10 bp) in exons in Norin-PL4 were examined to detect chromosomal introgressions derived from IR 1154-243 (Fig. 5b). There were 138 IR 1154-243 alleles among 228 large InDels in exons, which indicated that approximately 61% of large InDels in exons in Norin-PL4 were IR 1154-243 alleles. There were several places where large InDels of IR 1154-243 alleles were concentrated (Fig. 5b). Many IR 1154-243 alleles were detected in the vicinity of 8-9 and 14 Mbp on chromosome 5, 5 Mbp on chromosome 7, 15 Mbp on chromosome 8, and in a wide range of 2-23 Mbp on chromosome 12, similar to the results in Figure 5a. Moreover, many IR 1154-243 alleles were detected in the vicinity of 2.6-2.8 Mbp on chromosome 1 and 0.6-1.1 Mbp as well as 30.8-31.8 Mbp on chromosome 4 (Fig. 5b).

The genotypes of InDels around IR 1154-243 alleles shown in Figure 5a and Figure 5b were analyzed in more detail (every 50 kbp) to determine the limits of chromosomal introgression of IR 1154-243 in Norin-PL4 (Fig. 5c). As a result, chromosomal introgressions derived from IR 1154-243 were detected between 2.50 and 3.50

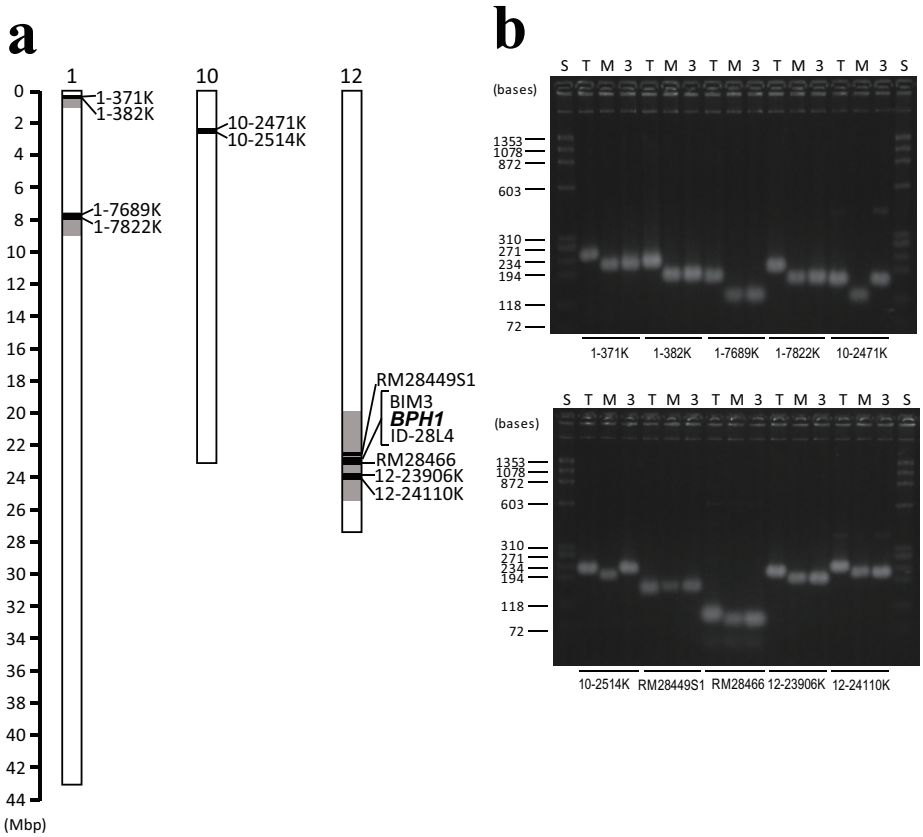

Fig. 4. Analyses of genotypes of Norin-691 PL3 by PCR-based DNA markers

a: Names of the developed DNA markers and positions on chromosomes. *BPH1* locus is based on the report of Zhao et al. (2016). CAPS marker BIM3 is linked to *BPH18* (Ji et al. 2016), and InDel marker ID-28L4 is linked to the *BPH26* (Tamura et al. 2014). b: Gel band patterns amplified by DNA markers. S above the gel indicates a size marker, T indicates the Japonica Group rice variety Tsukushibare, M indicates the Indica Group rice variety Mudgo, and 3 indicates Norin-PL3. The lower part of the gel shows marker names.

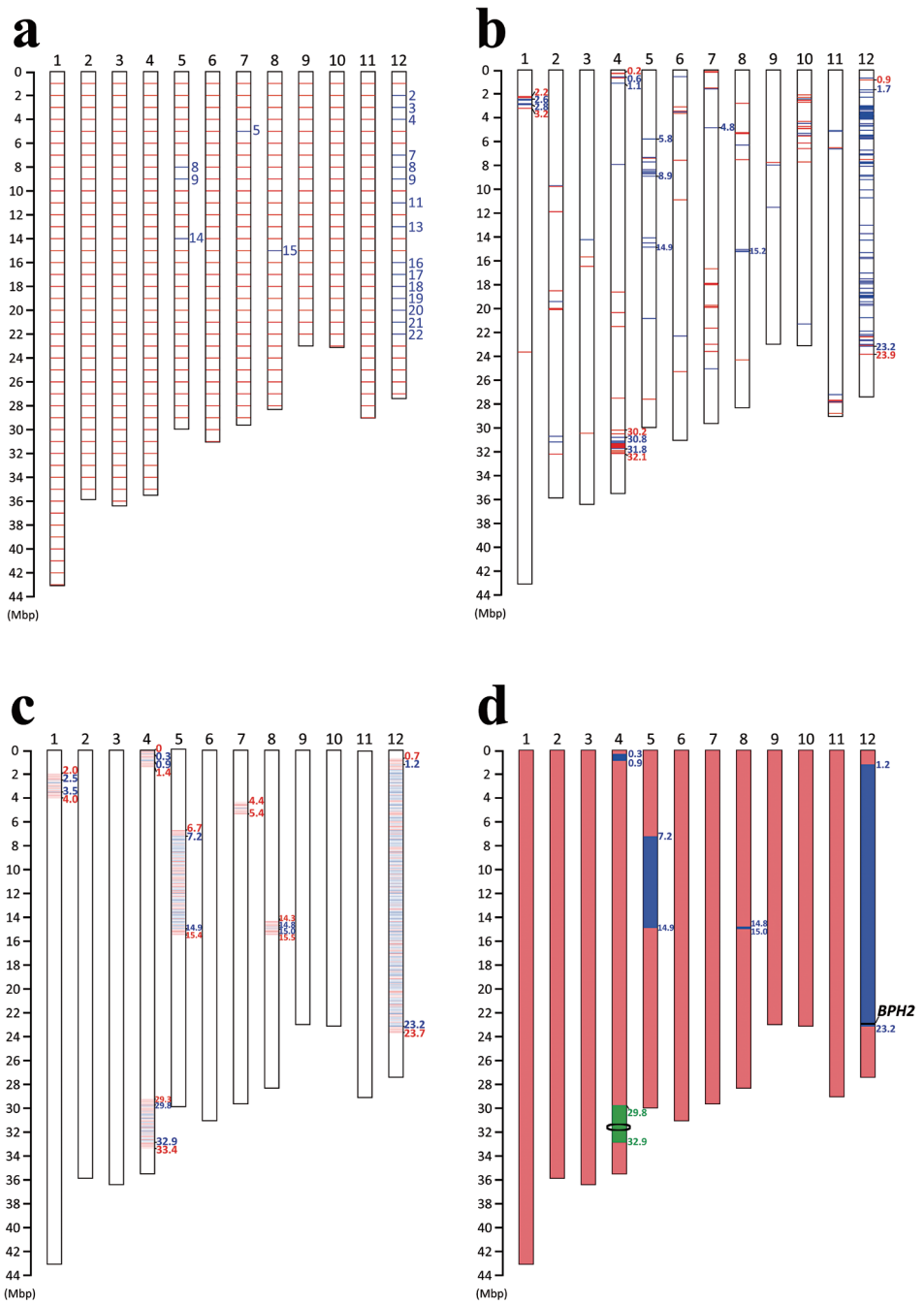

Fig. 5. Analyses of chromosomal introgressions from IR 1154-243 in Norin-PL4

a: The genotypes of InDel polymorphism in exons were analyzed for the whole genome of Norin-PL4 at every 1 Mbp. IR 1154-243 alleles are displayed as blue lines, and all other alleles are red lines.

b: Analyses of genotypes of large InDels (≥ 10 bp) in exons. IR 1154-243 alleles are displayed in blue, and all other alleles are in red.

c: The ranges wherein IR 1154-243 alleles were detected by detailed analyses of the genotypes of InDels (every 50 kbp) for the region predicted to be chromosomal regions from IR 1154-243 in the analyses of a and b.

d: A graphical genotype of Norin-PL4 created by integrating the analysis results of NGS and PCR-based DNA markers. Chromosomal regions derived from IR 1154-243 are displayed in blue, from Tadukan is in green, and others are in red. *BPH2* locus is based on the report of Zhao et al. (2016). A circle on chromosome 4 indicates the candidate region of bacterial leaf blight (BLB) resistance gene *Xa1-as*(t) (Endo et al. 2012).

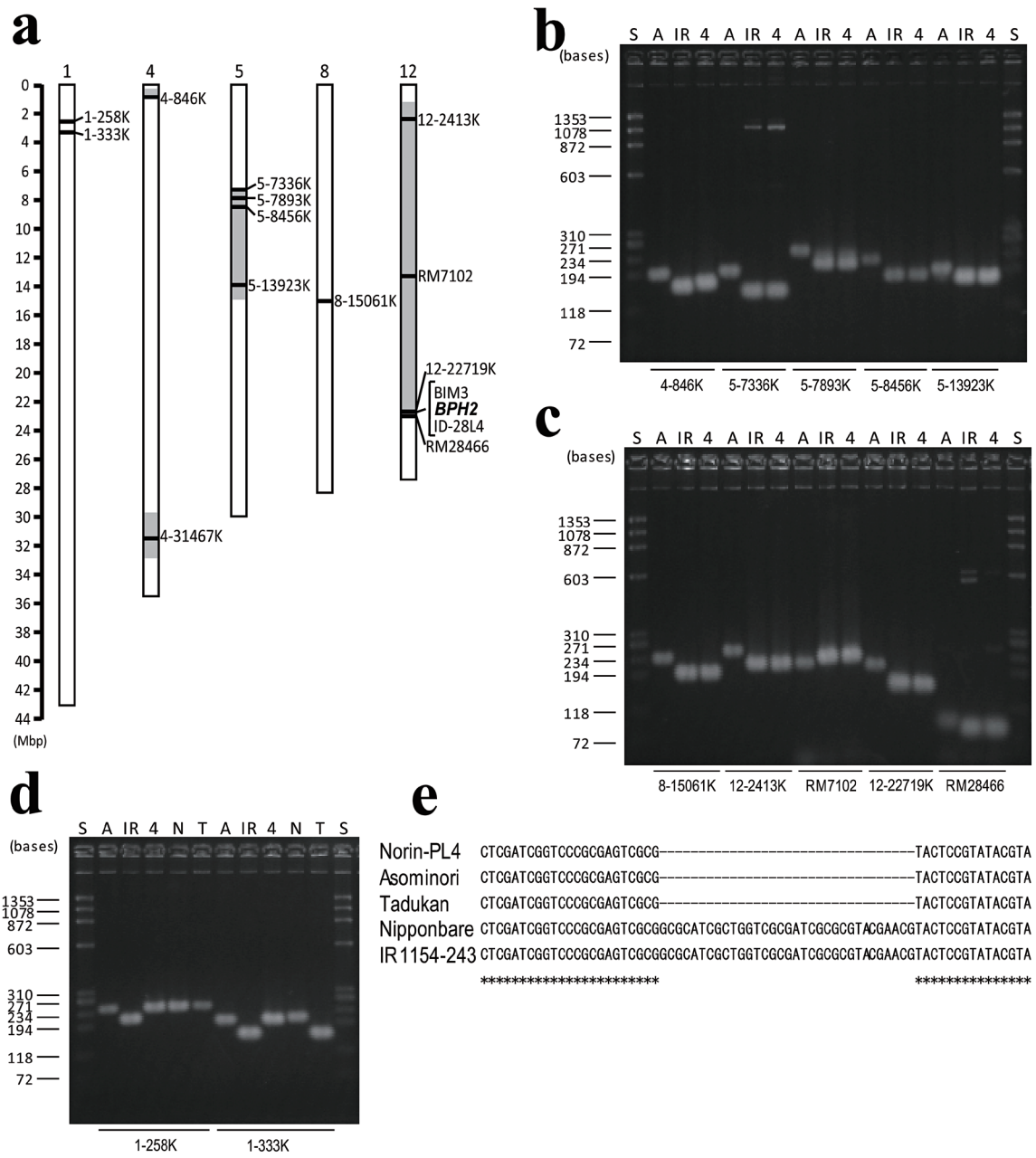

Fig. 6. Analysis of genotypes of Norin-716 PL4 by PCR-based DNA markers

a: Names of the developed DNA markers and positions on chromosomes. *BPH2* locus is based on the report of Zhao et al. (2016). CAPS marker BIM3 is linked to *BPH18* (Ji et al. 2016), and InDel marker ID-28L4 is linked to the *BPH26* (Tamura et al. 2014).

b: Gel band patterns amplified by DNA markers on the short arms of chromosomes 4 and 5. S above the gel indicates a size marker, A indicates the Japonica Group rice variety Asominori, IR indicates the Indica Group rice variety IR 1154-243, and 4 indicates Norin-PL4. The lower part of the gel shows marker names.

c: Gel band patterns amplified by DNA markers on chromosomes 8 and 12. The ellipsis above and under the gel has the same meanings as that in Figure 6b.

d: Gel band pattern of electrophoresis with DNA marker on chromosome 1. In the upper part of the gel, S indicates a size marker, A indicates the Japonica Group rice variety Asominori, IR indicates the Indica Group rice variety IR 1154-243, 4 indicates Norin- PL4, and N indicates Nipponbare. The lower part of the gel shows marker names.

e: Comparison of nucleotide sequences of DNA amplified by the 4-31467K marker on chromosome 4. The base sequence corresponds to the region from 31,468,146 bp to 31,468,216 bp on chromosome 4 of Nipponbare. Norin-PL4, Tadukan, and Asominori have the same base sequences; however, IR 1154-243 has a different base sequence.

Mbp on chromosome 1, between 0.30 and 0.90 Mbp as well as 29.75 and 32.85 Mbp on chromosome 4, between 7.20 and 14.90 Mbp on chromosome 5, between 14.80 and 14.95 Mbp on chromosome 8, and between 1.21 and 23.15 Mbp on chromosome 12. Regarding the area at 4.80 Mbp on chromosome 7, the range where IR 1154-243 alleles were detected was so narrow (within 100 kbp) that the area was excluded from the candidate chromosomal regions derived from IR 1154-243.

The presence of candidate regions introgressed from IR 1154-243 shown in Figure 5c was also confirmed using DNA markers. Table 3 shows the sequence information of the primers used in this study, and Figure 6a shows the markers on chromosomes. IR 1154-243 alleles were detected between 0.30 and 0.90 Mbp on chromosome 4 with the InDel markers 4-846K and between 7.20 and 14.90 Mbp on chromosome 5 with InDel markers 5-7336K, 5-7893K, 5-8456K, and 5-13923K (Fig. 6b). Moreover, IR 1154-243 alleles were detected in the vicinity of 15.0 Mbp on chromosome 8 with the InDel markers 8-15061K (Fig. 6c). Furthermore, the IR 1154-243 alleles were confirmed between 1.21 and 23.15 Mbp on chromosome 12 with markers 12-2413K, RM7102, 12-22719K, and RM28466 (Fig. 6c). The CAPS marker BIM3 and InDel marker ID-28L4 could also detect the IR 1154-243 alleles (data not shown). The genotype analyzed by the InDel markers 1-2587K and 1-3337K between 2.50 and 3.50 Mbp on chromosome 1 was identical to that of Asominori and Nipponbare (Fig. 6d). Therefore, this region was probably not an IR 1154-243 allele. The genotype analyzed by the InDel markers of 4-31467K between 29.75 and 32.85 Mbp on chromosome 4 was identical to that of Asominori and Indica Group rice variety Tadukan, and different from that of Nipponbare and IR 1154-243 (data not shown). This PCR product was sequenced, and the nucleotide sequences were compared. The sequence of Norin-PL4 was identical to Asominori and Tadukan, which is one of the parents of Asominori (Fig. 1c, Fig. 6e).

Therefore, the chromosomal regions derived from IR 1154-243 existed between 0.30 and 0.90 Mbp on chromosome 4, between 7.20 and 14.90 Mbp on chromosome 5, between 14.80 and14.95 Mbp on chromosome 8, and between 1.21 and 23.15 Mbp on chromosome 12, by analyses using short-read NGS and DNA markers. Furthermore, the chromosome region between 29.75 and 32.85 Mbp on chromosome 4 was determined to be a chromosomal region derived from Tadukan contained in Asominori. Based on these, a graphical genotype of Norin-PL4 was created (Fig. 5d). Chromosomal introgressions from IR 1154-243 are shown in blue, the chromosomal introgression from Tadukan is shown in green, and the other regions are shown in red.

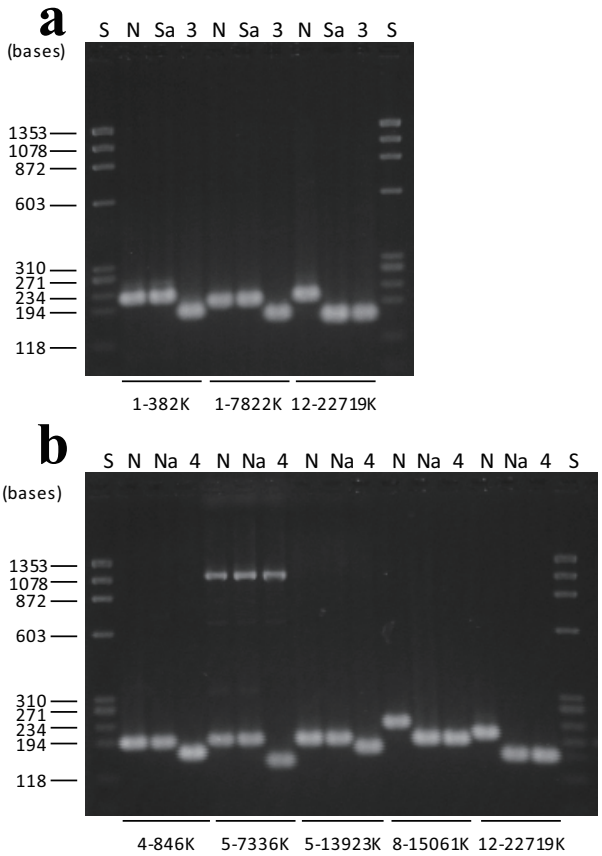

Fig. 7. Analyses of Saikai 190 and Nankai 133 genotypes 734 using PCR-based DNA markers

a: Gel band patterns amplified by DNA markers on chromosomes 1 and 12. Above the gel, S indicates a size marker, N indicates the Japonica Group rice variety Nipponbare, Sa indicates the Japonica Group rice line Saikai 190, and 3 indicates Norin-PL3. The lower part of the gel shows marker names.

b: Gel band patterns amplified by DNA markers on chromosomes 4, 5, 8, and 12. Above the gel, S indicates a size marker, N indicates the Japonica Group rice variety Nipponbare, Sa indicates the Japonica Group rice line Saikai 190, and 4 indicates Norin-PL4. The lower part of the gel shows marker names.

4. Candidate DNA markers linked with BPH resistance genes

To confirm whether each of the chromosomal introgressions from Mudgo on chromosomes 1 and 12 in Norin-PL3 contributes to resistance, we examined whether Saikai 190 (Fig. 1d) also harbors the chromosomal regions from Mudgo. The genotypes of two DNA markers—1-382K and 1-7822K—on chromosome 1 of Saikai 190 were Japonica-type, and the genotype of 12-22719K located near *BPH1* was the same as that of Norin-

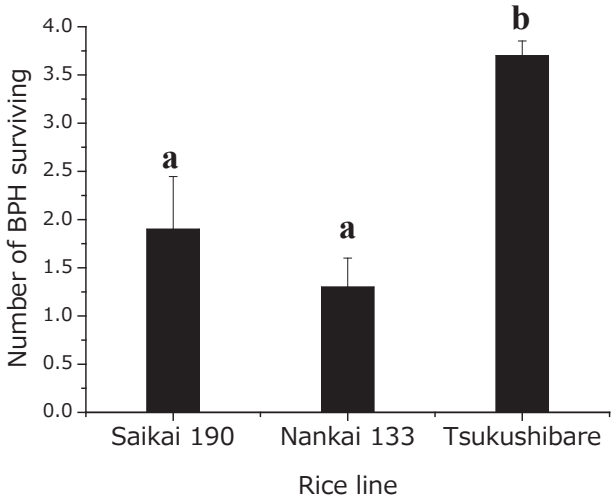

Fig. 8. Evaluation of resistance of Saikai 190 and Nankai 133 to brown planthopper

a Number of survived BPH in a test tube on the three rice lines. Four adult BPHs were released in the test tube, and the number of live BPHs was recorded 7 days after their release. Values represent mean and SE (n = 10). Means labeled with the same letters are not significantly different based on the Tukey–Kramer test ($P < 0.05$). Tsukushibare is the susceptible variety against BPH, which was used as a control.

PL3 (Fig. 7a). Saikai 190 showed BPH resistance even though it did not have chromosomal regions from Mudgo on chromosome 1 (Fig. 8). Next, to confirm whether each of the chromosome introgressions from IR1154-243 on chromosomes 4, 5, 8, and 12 in Norin-PL4 contributes to resistance, we examined whether Nankai 133 (Fig. 1e) also harbors the chromosomal regions from IR1154-243. The genotypes of the chromosomal regions on chromosomes 4, 5, 8, and 12 in Nankai 133 were analyzed using five DNA markers: 4-846K, 5-7336K, 5-13923K, 8-15061K, and 12-22719K. The genotypes of 4-846K, 5-7336K, and 5-13923K of Nankai 133 were Japonica-type, and genotypes of 8-15061K and 12-22719K were the same as Norin-PL4 (Fig. 7b). The introgressions from IR 1154-243 were confirmed on chromosomes 8 and 12, but not on chromosomes 4 and 5 in Nankai 133. Nankai 133 showed BPH resistance even though it did not have chromosomal regions from IR 1154-243 on chromosomes 4 and 5 (Fig. 8).

Discussion

There is a great sequence diversity between Indica Group and Japonica Group rice varieties (Garris et al. 2005, Londo et al. 2006), in contrast with the little sequence diversity among Japonica Group rice varieties (Gao & Innan 2008, Yamamoto et al. 2010). Accordingly, InDel polymorphisms are also more frequently observed between Indica Group and Japonica Group rice varieties compared with among Japanese rice varieties (Yonemaru et al. 2015). Both Indica Group varieties, Mudgo and IR 1154-243, which are resistant donors of Norin-PL3 and Norin-PL4, had approximately 380,000 InDels compared to Nipponbare (Table 2). The coverage by short reads from Mudgo and IR 1154-243 when referring to the genome sequence of Nipponbare was approximately 92%, which was lower than that of the breeding lines (approximately 98%-99%; Table 1). This means that there is a high sequence diversity in the nucleotide sequences of Mudgo and IR 1154-243 when compared to Nipponbare. Assembly errors are likely to occur in the portion of the existing sequence diversity between the two genome sequences when mapping the short reads obtained by NGS (Lin et al. 2011). It is difficult to eliminate assembly errors from the 380,000 InDel polymorphisms. Therefore, in this analysis, only the locations where plural Indica-type alleles were detected were set as candidate regions derived from Indica Group rice to reduce the influence of assembly error. Furthermore, the presence of chromosomal introgressions from donors was validated using PCR-based DNA markers. Thus, our method minimized the influence of assembly errors in the graphical genotype.

In this study, the genotypes of 367 points on chromosomes of the breeding line at regular intervals of 1 Mbp were analyzed using a part of the large amount of InDel polymorphism information. Through this analysis, wide Indica Group rice chromosomal introgressions, such as 1 Mbp or more, were detected in both Norin-PL3 and Norin-PL4. However, short Indica chromosomal introgressions of less than 1 Mbp were not always detected. There were fewer large InDels (≥ 10 bp) in exons among Japonica Group rice varieties, and more than half of the large InDels in exons in the breeding lines showed Indica Group genotypes. Examining the genotype of large InDels in exons in the two breeding lines enabled the efficient detection of donor-type alleles, including short introgressions less than 1 Mbp, such as that in the short arm of chromosome 1 in Norin-PL3 and in chromosomes 4 and 8 in Norin-PL4. In this study, it was shown that the chromosomal regions derived from the Indica Group varieties could be efficiently detected by combining the method for examining the genotypes at regular intervals of every 1 Mbp and the method of examining the genotypes for large InDels in exons on the chromosomes of the breeding lines.

A wide chromosomal introgression from Mudgo existed in the long arm of chromosome 12, and two narrow introgressions from Mudgo also existed in the

short arm of chromosome 1 in Norin-PL3. It is known that *BPH1* is located on chromosome 12 (Hirabayashi & Ogawa 1995, Ji et al. 2016, Zhao et al. 2016). Genotypes of two DNA markers, 1-382K and 1-7822K on chromosome 1 in Saikai 190, introduced for BPH resistance from Mudgo, were Japonica-type. These results suggest that the two chromosomal regions derived from Mudgo on chromosome 1 of Norin-PL3 were not essential for BPH resistance. *BPH1* on chromosome 12 from Mudgo was harbored in Norin-PL3 and Saikai 190. In contrast, chromosomal regions derived from IR 1154-243 were found on chromosomes 4, 5, 8, and 12 in Norin-PL4. Nankai 133 harbored chromosomal introgressions from IR 1154-243 on chromosomes 8 and 12, but not on chromosomes 4 and 5. These results suggest that two introgressions from IR 1154-243 on chromosomes 4 and 5 of Norin-PL4 were not essential for BPH resistance. A common introgressed region on chromosome 12, located in *BPH2*, was found in Norin-PL4 and Nankai 133.

Tamura et al. (2014) reported that the InDel marker ID-28L4 was linked to *BPH26*, and Ji et al. (2016) reported that the CAPS marker BIM3 was linked to *BPH18*. ID-28L4 and BIM3 were useful for detecting the Mudgo and IR 1154-243 alleles, respectively (data not shown). Therefore, these markers are thought to be useful as markers linked to *BPH1* and *BPH2*. Because these two markers were confirmed only as polymorphisms between the resistance donor and the recurrent parent, it is necessary to confirm polymorphism with the other materials and co-segregation when introducing the resistance gene into rice varieties with different genetic backgrounds.

A graphical genotype of Norin-PL4 reported by Murata et al. (1998) using 99 RFLP markers also suggested that chromosomal regions derived from IR 1154-243 were present on chromosomes 4, 8, and 12. The region derived from IR 1154-243 on chromosome 5 was detected in this study. However, the RFLP markers did not detect the region. In contrast, it has been reported that IR 1154-243 alleles were detected on the long arm of chromosomes 1, 2, and 11 in the RFLP analyses. The number of InDel polymorphisms in the corresponding regions on chromosomes 1, 2, and 11 of Norin-PL4 was counted using InDel polymorphisms not only in exons but also on non-coding regions. The survey suggested that two regions, a 42.283- to 42.538-Mbp region on chromosome 1 and a 27.822- to 28.099-Mbp region on chromosome 11, were rich in InDel polymorphisms (data not shown). Comparing the positions of these InDel polymorphisms with those of IR 1154-243 showed that the concordance rates of the InDel polymorphisms were as low as approximately 70% on the corresponding regions on chromosomes 1 and 11. Thus, we concluded that these regions were not derived from IR 1154-243. Likewise, we concluded that the chromosomal region of chromosome 2, where a lot of InDel polymorphisms identical with those of IR 1154-243 were not found, was not derived from IR 1154-243 (data not shown).

Furthermore, this study suggested that the long arm of chromosome 4 in Norin-PL4 contains a chromosomal region derived from Tadukan, which was used for breeding Asominori (Fig. 1c). Endo et al. (2012) reported that both Tadukan and Asominori showed resistance to bacterial leaf blight (BLB), and the BLB resistance gene in Asominori (*Xa1-as*(t)) was located on chromosome 4. Kaneda et al. (1986) reported that Norin-PL4 showed resistance to BLB. The graphical genotype indicated that the candidate region of *Xa1-as*(t) was located on the chromosomal region derived from Tadukan (Fig. 5d). It is hypothesized that the chromosomal region from Tadukan may be important for the BLB resistance of Asominori.

We aimed to identify the chromosomal regions introgressed from an Indica Group-resistant donor in Norin-PL3 and Norin-PL4 and to develop DNA markers to detect these regions. DNA polymorphisms obtained from NGS provide a detailed view for this purpose. Because most breeding lines are not available for NGS analysis, we used two resistant donor varieties (Mudgo and IR 1154-243) and two breeding lines (Norin-PL3 and Norin-PL4) and precisely detected chromosomal regions in the breeding lines from respective donors. Thus, this method for creating graphical genotypes is considered useful for analyzing the chromosomal regions from the Indica Group rice varieties in the breeding lines that had both Japonica Group and Indica Group varieties as parents, by using part of a large amount of NGS data obtained with low effort and cost. Regarding genotyping using SSR markers, the difference in InDel size between the two alleles must be large enough to be detected by electrophoresis. However, in genotyping using NGS data, InDel, which differs in size by only one base, can be used to identify alleles. In this study, the number of InDels detected between Japonica Group rice varieties and Indica Group rice varieties was as high as approximately 380,000 (Table 2). Therefore, the number of InDels to be analyzed was reduced by limiting the chromosome locations (at every 1 Mbp) and the size of InDel (≥ 10) in exons. We used small InDel (≥ 1) information almost exclusively to determine the precise ranges of the donor-type alleles. Because a large amount of InDel polymorphism information can be obtained via NGS, this information would be useful in creating graphical genotypes not only for the breeding lines that have parents with many comparative polymorphisms (i.e.,

polymorphisms between the parents), such as Japanese Group and Indica Group rice varieties, but also for breeding lines that have parents with fewer comparative polymorphisms. By adjusting the number of InDels by adding small InDels to the analysis target, one could adapt this method to create graphical genotypes of breeding lines that have parents with fewer comparative polymorphisms. At present, in addition to Nipponbare, reference genome sequences of Indica Group rice varieties have been released (Tanaka et al. 2020). Therefore, it is important to obtain accurate polymorphism information to select an appropriate reference genome according to the target varieties in the analysis.

The graphical genotypes created in this study provided an overview of the locations and proportions of chromosomal regions introgressed from resistant donors in two breeding lines. These graphical genotypes and DNA markers are useful in performing functional analyses of the BPH resistance genes *BPH1* and *BPH2* by using these breeding lines or by removing unnecessary Indica-type alleles in the two breeding lines marker-assisted selection of rice breeding.

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