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Note

Quantitative trait loci analysis of blast resistance in *Oryza sativa* L. 'Hokuriku 193'

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To investigate the genetic background responsible for blast resistance in *Oryza sativa* L. 'Hokuriku 193', QTL analysis was conducted using the F_3 lines from the cross [ms-bo] Nekken 2 × Hokuriku 193 that were artificially infected with rice blast fungus (*Magnaporthe grisea*). QTLs were detected on chromosomes 1, 4, 6 and 12 that correlated with greater blast resistance in the Hokuriku 193-type lines. Notably, the QTL on chromosome 12 had a major effect and localized to the same region where *Pi20*(t), a broad-spectrum blast resistance gene, is positioned, suggesting strongly that the blast resistance of Hokuriku 193 was controlled by *Pi20*(t). Also, QTL analysis of the lines found to have no *Pi20*(t) detected two QTLs on chromosome 4 (*qBR4-1* and *qBR4-2*) and one QTL on chromosome 6 (*qBR6*), of which *qBR4-2* and *qBR6* correlated with higher percentages of resistant plants in the Hokuriku 193-type lines. The blast susceptibility of BR_NIL (a NIL of Hokuriku 193 from which *Pi20*(t) was eliminated) was greater than that of Hokuriku 193, suggesting that elimination of *Pi20*(t) may markedly increase blast susceptibility. The disease severity of BR_NIL was mild, which might be the effect of *qBR4-2* and/or *qBR6*.

Key Words: blast resistance, Hokuriku 193, near isogenic line (NIL), quantitative trait loci (QTL), rice.

Introduction

In recent years, rice (*Oryza sativa* L.) utilization has diversified from simple consumption as a staple food to commercial, processing and animal feed uses. Reducing rice production costs is essential for producers because price differences between rice and the other grain crops such as wheat and corn influence whether rice is used for these other value-added applications. One of the most effective cost reduction measures is to increase yield through the development of high-yielding cultivars.

On the other hand, rice blast disease caused by the fungus *Magnaporthe grisea* is an important disease in rice and conferring blast resistance is among the most important aims for rice breeding. Blast resistance can be roughly classified into two types (Ezuka 1972): complete resistance (plants do not get infected at all) and partial resistance (plants can get infected although the severity is mild). Complete resistance to blast is controlled by a single gene and is very effective against a particular blast race; however, once a cultivar with a new complete resistance gene is introduced, blast races capable of overcoming the resistance occur and spread, with eventual loss of resistance (Ashizawa 2007). Once blast resistance has broken down and if the cultivar's partial resistance is insufficient, subsequent cultivation of the cultivar will become difficult and paddy rice cultivation in the surrounding areas can also be negatively impacted. Therefore, it is very important in rice cultivar breeding that cultivars be given not only complete resistance but also strong partial resistance to blast so that even if infection occurs, the growth and other characteristics of rice are not negatively affected.

Although several blast resistance genes have been reported, many are complete resistance genes identified in cultivars grown in countries other than Japan (Koide *et al.* 2009). Most high-yielding rice cultivars bred in Japan in recent years obtained their high yielding characteristics from foreign *indica* rice cultivars; therefore, many of these new cultivars have complete resistance genes and/or unknown resistance genes. For example, 'Bekoaoba' (Nakagomi *et al.* 2006) has *Pita-2* (Nakamura *et al.* 1997) and 'Natsuaoba'

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(Nagaoka *et al.* 2013) has *Pib* (Wang *et al.* 1999), but the resistance genes of 'Momiroman' are unknown (Hirabayashi *et al.* 2010). These cultivars do not generally get infected with blast races that exist in Japan as of now. Thus, it is very difficult to evaluate the strength of partial resistance that is judged by disease severity and such cultivars are always at risk of losing resistance. Such is the case with Hokuriku 193 (Goto *et al.* 2009), a high-yielding *indica* rice cultivar, whose resistance to blast has not been previously investigated.

In order to investigate blast resistance in Hokuriku 193, it is indispensable to gain knowledge of the genetic background, especially about complete resistance. In this study, quantitative trait loci (QTL) analysis was conducted to characterize the genetic background responsible for blast resistance in Hokuriku 193. Also, a near isogenic line (NIL) were created by eliminating major complete resistance from Hokuriku 193, and the blast resistance of the NIL were evaluated.

Materials and Methods

Plant materials for QTL analysis

In 2009, the F_2 population of the cross [ms-bo] Nekken 2 × Hokuriku 193 was cultivated in a paddy field at the Central Region Agricultural Research Center, NARO (Joetsu City, Niigata Prefecture, 37°6′59″ N, 138°16′14″ E). '[ms-bo] Nekken 2' is a *japonica* rice cultivar developed by conferring cytoplasmic male sterility to 'Nekken 2' that was bred by introducing *S-5*^{n}, a high affinity gene that reduces *japonica*/*indica* hybrid sterility, to 'Akihikari' (Ikehashi 2009). There was concern that hybrid sterility may occur in the cross between Hokuriku 193 and *japonica* rice, thereby reducing population homogeneity and affecting our evaluation. Therefore, Nekken 2 was selected for the cross with Hokuriku 193 because it has a high affinity gene *S-5*^{n}. Sowing and transplantation were carried out on 22 April and 20 May, respectively. Nitrogen, P_2O_5 and K_2O (5 g/m^2 each) were applied as a basal fertilizer and no additional fertilizer was applied. Following maturity, 248 plants were randomly selected.

Evaluation of blast resistance

Resistance to blast was tested by the upland nursery trial (Higashi and Kowata 1995, Nguyen *et al.* 2006). From the F_2 plants of the cross [ms-bo] Nekken 2 × Hokuriku 193 obtained in 2009, 234 plants with sufficient numbers of seeds were evaluated in the trial. On 8 June 2010, clumps of approximately 100 seeds of the F_3 lines were sown in an upland field at the Center at an interval of 10 cm. Nitrogen (20 g/m^2) was applied as a basal fertilizer. After 20 days of growth, the leaves of 'Wataboushi' (Sasaki *et al.* 1996) artificially infected with *Magnaporthe grisea*, blast race 037.1 were evenly spread over the young plants. To promote the onset of disease symptoms, additional fertilizer (nitrogen [20 g/m^2]) was applied one day prior to the spread of the infected leaves, and the plants were watered for 10 minutes in the morning and late afternoon thereafter. About four weeks later, the plants were harvested and the numbers of infected and resistant plants were determined for each line. Rice plants having elongated lesions on their leaf blades were considered infected. The percentage of resistant plants relative to the total number of tested plants was used as an indicator of blast resistance.

QTL analysis

In accordance with the method of Monna *et al.* (2002), DNA was extracted from each F_2 plant and their genotypes were determined using 115 single sequence repeat (SSR) markers covering the entire genome (IRGSP 2005, McCouch *et al.* 2002). The linkage map was created using MAPMAKER/EXP 3.0 (Lander *et al.* 1987). As a mapping function for estimating distances between markers, the Kosambi mapping function (Kosambi 1944) was used. QTL analysis was performed using CIM with Windows QTL Cartographer 2.5 (Wang *et al.* 2006). The thresholds corresponding to the 5% significance level were estimated by performing 1,000 permutations. A QTL was detected when the logarithm of the odds (LOD) score exceeded the threshold. For analysis, the arcsine transformed ratio of infected to resistant plants was used to standardize the distribution.

Creation and evaluation of a NIL

Individual selection was carried out in 2010 using the BC_2F_2 population created by back crossing Hokuriku 193 with [ms-bo] Nekken 2 twice, and starting in 2011 individual plants were genotyped based on the marker information. In 2015, for the BC_2F_7 generation, genotype analysis was performed on the entire genome using 325 single nucleotide polymorphism (SNP) markers (Ebana *et al.* 2010, Nagasaki *et al.* 2010). A single NIL "BR (Blast Resistance) _NIL" in which most regions are of the Hokuriku 193 type except the regions where major blast complete resistance genes are positioned was selected and its blast resistance was evaluated in 2015 and 2016. The disease severity of leaf blast was evaluated by the upland nursery trial. For comparison, Hokuriku 193 and Akihikari, which is the genetic background of Nekken 2, were used. Seeds were sown on 10 June in 2015 and 8 June in 2016, respectively, and the infected leaves were spread on 29 June in both years. Disease progression was evaluated on 13 and 21 July in 2015, and 20 and 27 July in 2016, respectively, in accordance with the method of Asaga *et al.* (1981), where plants are evaluated on a 11-point grading scale from 0 (no infection) to 10 (all leaves dead). The percentage of resistant plants was calculated by the method previously described after evaluation of disease progression.

Results

QTL analysis of blast resistance

The frequency distribution of blast resistance percentages in the F_3 lines of the cross [ms-bo] Nekken 2 × Hokuriku

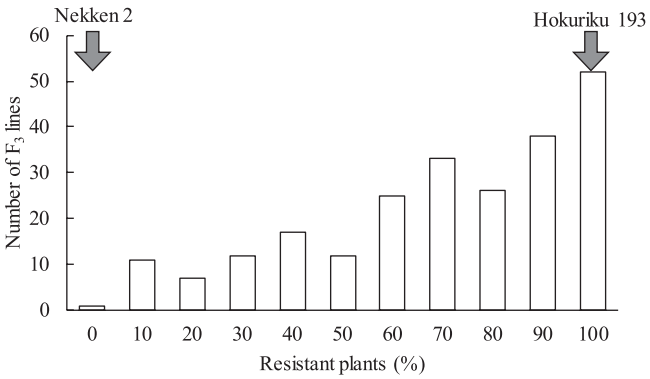

Fig. 1. Frequency distribution of blast resistant plants in the F_3 lines of the cross [ms-bo] Nekken 2 × Hokuriku 193.

193 is shown in **Fig. 1**. The distribution was continuous but disproportionately higher toward the higher percentages of resistance. The results of the QTL analysis of blast resistance in the F_3 lines are shown in **Fig. 2** and **Table 1**. In the CIM analysis, the LOD threshold was 3.50 at the 5% significance level estimated by 1,000 permutations, and QTLs were detected at five locations, one of which had two LOD peaks. Among these QTLs, the QTLs on chromosomes 1, 6, 12 and on the long arm of chromosome 4 correlated with a high percentage of resistance in the Hokuriku 193-type lines. Most notably, the QTL detected on chromosome 12 contributed significantly (52.4%) to resistance.

Eighty lines in which the SSR marker RM7102_1, where the LOD peak for the QTL detected on chromosome 12 was positioned, was homozygous for the Nekken 2-type regions were selected and QTL analysis was repeated. The LOD threshold was 3.76, corresponding to the 5% significance level estimated by 1,000 permutations, and QTL were detected at three locations (**Fig. 2**, **Table 1**). These putative QTL were detected at the same positions as in the first QTL analysis where all 234 lines were tested and were named *qBR* (*Blast Resistance*) *4-1*, *qBR4-2* and *qBR6*. *qBR4-2* and *qBR6* were QTL that increased the resistance in the Hokuriku 193-type lines, whereas *qBR4-1* was a QTL that decreased the resistance in the Hokuriku 193-type lines

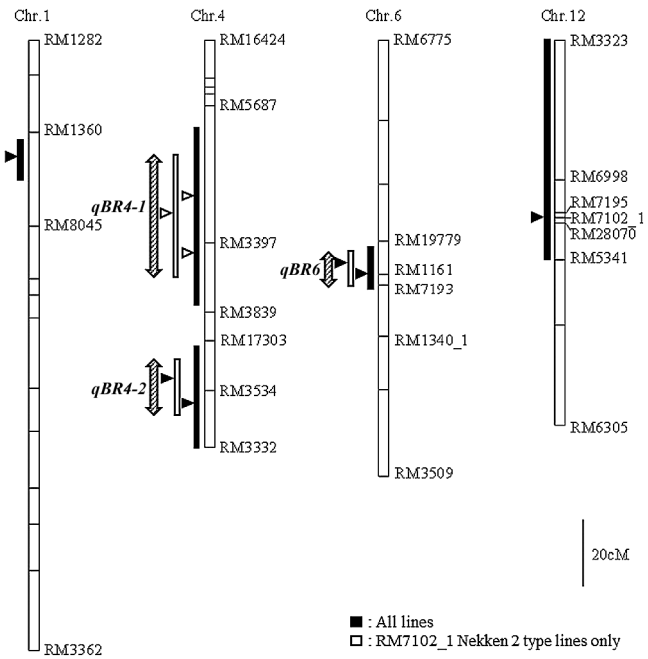

Fig. 2. Regions where QTL associated with blast resistance exist in Hokuriku 193. Main SSR markers are shown on the right of each chromosome. Bars on the left of each chromosome indicate intervals in which the LOD threshold is exceeded and triangles indicate LOD peaks for the intervals. Dark and white triangles indicate QTL peaks at which the percentage of resistant plants decreases in the Nekken 2 type and the Hokuriku 193 type, respectively. Shaded arrows indicate regions where QTL are estimated to exist. Chromosomes where QTL were not detected are not shown.

(**Table 1**). The QTL on chromosome 1, detected when all 234 lines were tested, was not detected by the re-analysis of the 80 sampled lines.

Creation and evaluation of a NIL

The graphical genotype of BR_NIL in which significantly contributing QTL regions detected on chromosome 12 were substituted with the Nekken 2-type region is shown in **Fig. 3**. Although much of chromosome 12 was substituted with the Nekken 2-type region and chromosome 1 retained

Table 1. QTL associated with blast resistance in Hokuriku 193 detected by CIM analysis

	Chr.	Marker interval	Nearest marker	Peak (cM)	LOD	Additive effect[a]	PVE[b]
All lines	1	RM1360–RM8045	RM1360	35.9	3.84	–5.17	5.1
	4	RM5687–RM3839	RM3397	47.1	10.67	12.09	18.4
			RM3397	64.2	9.01	9.30	9.8
	4	RM17303–RM3332	RM3534	109.9	6.70	–7.68	7.2
	6	RM19779–RM7193	RM1161	70.7	5.09	–5.36	4.7
	12	RM3323–RM5341	RM7102_1	53.1	44.56	–20.71	52.4
RM7102_1 Nekken 2 type lines only	4	RM5687–RM3839	RM3397	56.4	6.17	12.43	32.6
	4	RM17303–RM3332	RM3534	109.5	4.60	–11.39	19.2
	6	RM19779–RM7193	RM1161	62.0	4.10	–8.64	15.6

[a] The additive effect was positive in Nekken 2-type genotypes.
[b] Percentage of phenotypic variation explained by each QTL.

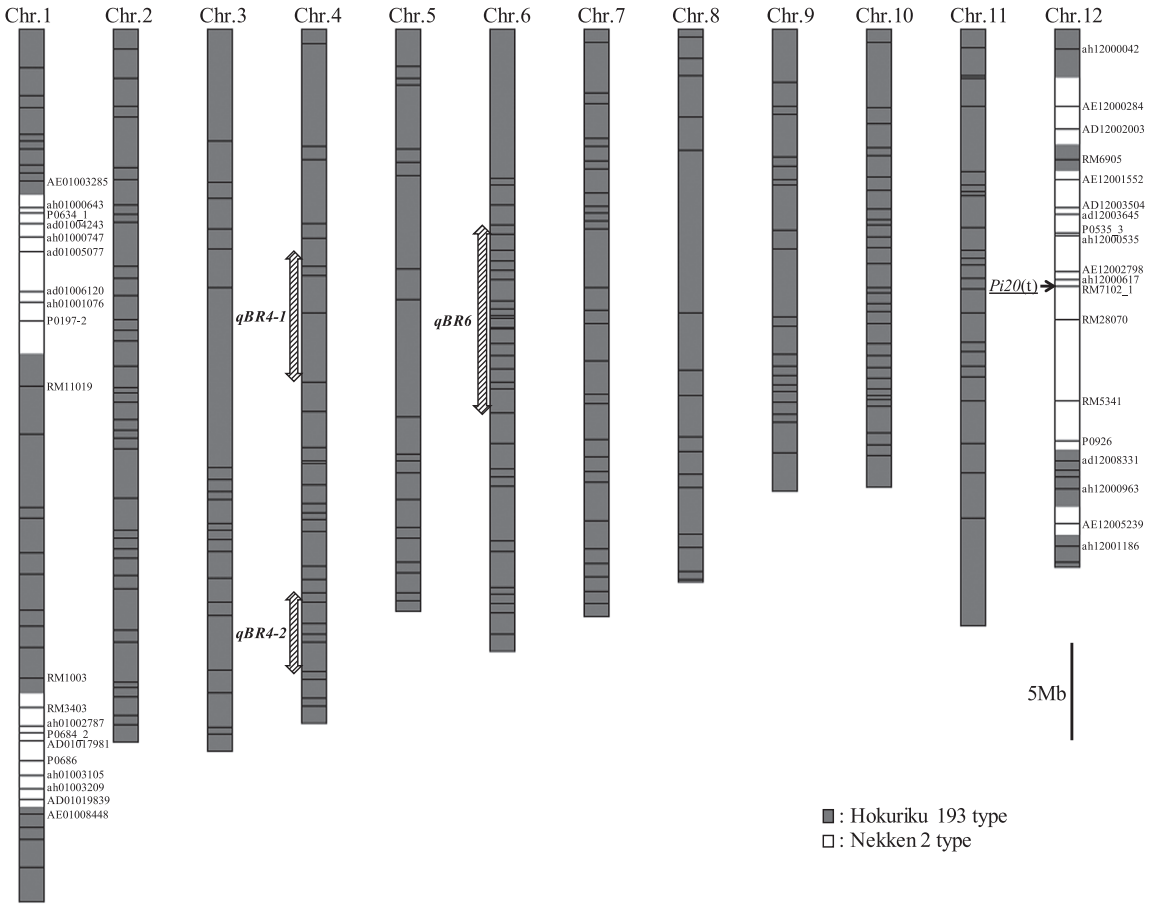

Fig. 3. Graphical genotype of BR_NIL. Main markers are shown on the right of each chromosome. The positions of *qBR4-1*, *qBR4-2*, *qBR6* and *Pi20*(t) are indicated on the left of each chromosome. Shaded arrows indicate the regions where QTL are estimated to exist. The physical positions of markers are based on IRGSP Ver. 1.0.

Table 2. Blast resistance property of BR_NIL

Year	Cultivar	Disease severity (0–10)	Resistant plants (%)
2015	BR_NIL	1.3 b	37.1 b
	Hokuriku 193	0.0 a	98.8 a
	Akihikari	4.0 c	0.0 c
2016	BR_NIL	2.8 b	38.3 b
	Hokuriku 193	0.0 a	99.2 a
	Akihikari	4.4 c	0.0 c
ANOVA	Cultivar (A)	***	***
	Year (B)	ns	ns
	A × B	ns	ns

***: Significant at 0.1% level. ns: not significant at a level of 5%.
No significant difference between values with the same alphabetical letter (5% level, Tukey test).

some regions derived from Nekken 2, all other regions including *qBR4-1*, *qBR4-2* and *qBR6* were of the Hokuriku 193-type.

The blast resisting properties of BR_NIL are shown in **Table 2**. This line's susceptibility to blast was significantly higher compared with Hokuriku 193 but significantly lower compared with Akihikari with blast severity being mild.

Discussion

The genetic background responsible for the blast resistance of Hokuriku 193 was investigated by carrying out QTL analysis, creating a NIL in which the major complete resistance was eliminated and evaluating the NIL for their blast resistance properties.

In our QTL analysis, blast resistance was evaluated in the F_3 lines of the cross [ms-bo] Nekken 2 × Hokuriku 193. Blast resistance showed continuous distributions, but the distribution was biased toward highly resistant plants (**Fig. 1**). This finding suggested that blast resistance in Hokuriku 193 was controlled by a small number of genetic factors with major effects.

In genetically unfixed lines, complete resistance is regarded as a quantitative trait because blast resistance percentages become continuous. QTL analysis of the F_3 lines detected one QTL on chromosome 12 that contributed significantly to blast resistance (**Fig. 2**, **Table 1**). Recently, Hayashi *et al.* (2014) reported that Hokuriku 193 had four complete resistance genes: *Pii* (Ise 1991), *Pik-s* (Fjellstrom *et al.* 2004), *Pi19*(t) (Hayashi *et al.* 1998) and *Pi20*(t) (Imbe *et al.* 1997). Although both *Pi19*(t) and *Pi20*(t) are

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positioned in the QTL region detected on chromosome 12 (Miah *et al.* 2013), considering that *Pi19*(t) gets infected with almost all blast races that exist in Japan including 037.1, whereas *Pi20*(t) does not (Hayashi *et al.* 1998), and *Pi20*(t) is closely linked to the SSR marker RM7102_1 (Li *et al.* 2008), our results strongly suggested that the blast resistance in Hokuriku 193 was controlled by *Pi20*(t).

On the other hand, the blast resistance of Hokuriku 193 may involve genetic factors other than *Pi20*(t) that are hypothesized to exist in *qBR4-2* and *qBR6*, the QTL correlated with the higher blast resistance in the Hokuriku 193-type genotypes (**Fig. 2**, **Table 1**). For example, *Pi46*(t) (Matsushita *et al.* 2011) and *Pigm*(t) (Deng *et al.* 2006) were reported to be present in these regions in a broad-spectrum resistant Chinese cultivar, but genes present in Hokuriku 193 at similar locations have not been determined. Also, only the analysis using all 234 F_3 lines detected a high resistance QTL on chromosome 1 (**Fig. 2**, **Table 1**). Considering that the analysis using 80 lines did not detect such a QTL, the action of this QTL might be very minor.

In contrast, QTL *qBR4-1* correlated with lower resistance in the Hokuriku 193-type lines (**Fig. 2**, **Table 1**). Although *pi21* was reported to be present in this region in upland *japonica* rice (Fukuoka and Okuno 2001), no other blast resistance genes have been reported. Unknown resistance genes might also exist in this region. Certainly, with its significant contribution to blast resistance, continued analysis of *qBR4-1* is warranted**.**

Although it was not possible to identify unknown blast resistance genes in Hokuriku 193 based on the results of this study, *Pi20*(t) should be eliminated in order to make it possible to evaluate the strength of partial resistance and remove the possible loss of resistance. Therefore, we created BR_NIL, a NIL of Hokuriku 193 in which a region containing *Pi20*(t) was substituted with a *japonica*-type region. BR_NIL, including *qBR4-1*, *qBR4-2* and *qBR6*, had a Hokuriku 193-type genotype except for much of chromosome 12 that included *Pi20*(t) and small parts of chromosome 1 that was substituted with a Nekken 2-type region (**Fig. 3**). BR_NIL's resistance to blast was significantly lower compared with Hokuriku 193 with elongated lesions on leaf blades (**Table 2**). This result suggested that elimination of *Pi20*(t) in Hokuriku 193 would make blast susceptibility greatly higher in Japan. Also, BR_NIL's resistance to blast was significantly higher compared with Akihikari with the disease severity being minor (**Table 2**). This result suggested that Hokuriku 193 had genetic factors other than *Pi20*(t) that increase blast resistance, likely *qBR4-2* and/or *qBR6*. To gain further knowledge, *qBR4-2* and *qBR6* must be characterized in more detail; we will continue our investigation by narrowing down regions through the evaluation of NILs while simultaneously carrying out inoculation tests.

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