

Mapping of a QTL for Field Resistance to Blast (*Pyricularia oryzae* Cavara) in Inggoppor-tinawon, a Rice (*Oryza sativa* L.) Landrace from the Philippines

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Abstract

Rice blast, caused by *Pyricularia oryzae* Cavara (*Magnaporthe grisea* (Hebert) Barr), is one of the most severe global rice diseases. The Philippine rice landrace ‘Inggoppor-tinawon’ (IT) displays not only high field resistance to rice blast but also high tolerance to lodging, despite its very long culms. These characteristics make IT suitable for use in breeding animal feed varieties as whole-crop silage (WCS). To characterize the blast resistance of IT, quantitative trait locus (QTL) analysis of the resistance using simple sequence repeat (SSR) markers was conducted in F₃ progenies derived from crossing IT with a susceptible lowland variety, ‘Koshihikari’. A QTL for blast field resistance (*qBFR4*) was identified on chromosome 4, and the resistance allele was derived from IT. The QTL explained 73.5% of the phenotypic variation for blast resistance. *qBFR4* mapped to almost the same position as that of the blast field-resistance gene *Pi39*. Inoculation assays revealed that IT (containing *qBFR4*) showed resistance characteristics that differed from those of Mineharuka (containing *Pi39*), making it very likely that resistance derived from IT is distinct from *Pi39*. IT showed broad resistance to Japanese isolates and race-specific resistance to some Philippine isolates.

Discipline: Plant breeding/Plant disease

Additional key words: rice breeding, SSR marker, quantitative trait loci

Introduction

Rice blast caused by the filamentous ascomycete *Pyricularia oryzae* Cavara (*Magnaporthe grisea* (Hebert) Barr), is one of the most severe global rice diseases (Miah et al. 2013). In Japan, the blast epidemic area in 2011 was 590,133 ha, while the yield loss by blast constituted 45.9% of the total loss induced by all diseases (MAFF 2012). Therefore, one of the most effective ways to increase yield is to reduce the incidence of rice blast. To develop blast-resistant varieties, researchers have sought rice blast-resistant genes, more

than 90 of which have been reported (Miah et al. 2013). Japanese upland rice varieties are known to be potential sources of blast resistance, and several quantitative trait loci (QTLs) have been identified from such varieties, including *pi21* and *qBR4-2* from ‘Owarihatamochi’ (Fukuoka & Okuno 2001, Fukuoka et al. 2012), *Pi34* in ‘Chubu 32’ from ‘Sensho’ (Zenbayashi et al. 2002, Zenbayashi-Sawata et al. 2005, Zenbayashi-Sawata et al. 2007) and other QTLs from ‘Kahei’ (Xu et al. 2008, Miyamoto et al. 2001), ‘Sensho’ (Kato et al. 2002) and ‘Upland Rice Variety Norin 12’ (Sato et al. 2006). Recently, *pi21* has been cloned by map-based

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Received 11 December 2013; accepted 26 February 2014.

cloning (Fukuoka et al. 2009). Many *indica* varieties are also useful donors for blast resistance; for example, *Pb1* (*Panicle blast 1*) from the *indica* rice variety ‘Modan’ was identified (Hayashi et al. 2010) and has been widely used for breeding in Japan. To provide a tool for the systematic analysis of blast resistance genes, the International Rice Research Institute (IRRI)-Japan Collaborative Research Project has established 31 rice monogenic lines as a set of differential varieties containing 24 major resistance genes (Kobayashi et al. 2007, Telebanco-Yanoria et al. 2008, Tsunematsu et al. 2000). Moreover, Telebanco-Yanoria et al. (2008) selected standard differential blast isolates from the Philippines, and established a differential system for rice blast-resistant study.

Among global rice cultivation areas, the rice terrace of Banaue in the Philippines is famous for over two millennia of cultivation and was registered as a World Heritage Site in 1995. Because rice terraces in tropical regions are generally susceptible to blast disease, we expected that landraces cultivated in Banaue would be highly blast-resistant. We obtained seeds of landraces in Banaue from the International Rice Germplasm Collection (IRGC) of IRRI and performed inoculation assays, whereupon we found that some were resistant. Among them, ‘Inggoppor-tinawon’ (IT) showed resistance to many Japanese blast isolates, indicating broad-spectrum resistance. Moreover, IT not only displays high resistance to rice blast but also high tolerance to lodging, despite its very long-growing culms in paddy fields. Therefore, IT seems to be suitable breeding material for blast resistance, particularly for animal feed varieties as whole-crop silage (WCS). Despite the usefulness of blast resistance, this type of resistance has not yet been genetically analyzed. The objective of this study was to identify and characterize the genomic regions controlling field resistance to rice blast in IT to use it as a source of resistance for new rice varieties in future breeding programs.

Materials and methods

1. Plant materials

Inggoppor-tinawon (IT; IRGC-Acc. 8172) is a lowland landrace originated from the Philippines, while Koshihikari is a lowland variety originated from Japan. Both varieties are classified as *Oryza sativa* L. F₂ plants and F₃ lines were developed from crossing IT (resistant) and Koshihikari (susceptible). One hundred and twenty-four F₂ plants were grown at the Yawara Lowland Experimental Station of the National Agricultural Research Center (NARC). The F₂ plants were self-pollinated, and F₃ seeds of each plant were harvested separately. Total DNA was extracted from the leaf blades of each F₃ line (at least eight individuals per line) based on the CTAB method (Murray & Thompson 1980).

2. Evaluation of blast field resistance

Field resistance was assessed in the blast nursery at the Kannonдай Upland Experimental Field of the National Institute of Agrobiological Sciences (NIAS) in 2008. The 124 F₃ lines, the parents and the susceptible spreader variety Inabawase were planted by drilling on June 3. The block design of the blast nursery followed the conventional method (Asaga 1981) and the mean value of the disease score with three replications was used for analysis. Fifty seeds were sown in single-row plots (30×2×1 cm) with 10-cm spacing between rows. Nitrogen was applied at 140 kg ha⁻¹ as a basal fertilizer and top-dressed at 100 kg ha⁻¹ on July 18 and 25. Artificial inoculation with the blast isolate AI 79-142 (race 037.3), which has virulence to *Pish*, *Pii*, *Pia*, *Pik*, *Pik-m*, *Pik-p* and *Pib*, was conducted on July 14. Culture of the mycelia and inoculation by spraying with conidial suspension (2×10⁵ conidia ml⁻¹) were conducted following the *Rice Breeding Manual* (Tamura et al. 1995). The beds were wrapped with a 0.05-mm-thick polyethylene film (Silver polyto®, TOKAN KOSAN CO., LTD, Tokyo, Japan) for 14 h after inoculation. The level of disease in each plot was visually scored on August 13, using a 0-10 scale (Asaga 1981), in which 0 indicated no infection and 10 indicated dead plants.

3. Inoculation assay

We performed additional inoculation assays of IT, Mineharuka and Koshihikari using Japanese and Philippine blast isolates. Eleven blast isolates, which were categorized into different races as 001.2, 017.1, 003.0, 005.0, 007.0, 037.3, 077.1, 303.0, 337.3, 477.1 and 777.3, respectively, from Japan, were kindly provided by Dr. N. Hayashi of NIAS. Seedlings were grown in a greenhouse in 15×5×10-cm trays filled with sterilized soil (Bonsol No. 2, Sumitomo Kagaku Kougyo, Osaka, Japan) until the 5th-leaf stage. Fifteen seeds per cultivar were sown in a row in each tray. At the 5th-leaf stage, seedlings were sprayed with 30 ml of a spore suspension adjusted to 10⁵ spores ml⁻¹. Seven days after inoculation, the lesions on the 5th and 4th leaves were counted (Yaegashi 1995) and an inoculation assay using Japanese isolates was conducted at NIAS. Four isolates (CA41, C923-49, M64-1-3-9-1 and V850256) collected from the Philippines (Telebanco-Yanoria et al. 2008) were used for an inoculation assay performed in an isolated greenhouse at the Japan International Research Center for Agricultural Sciences (JIRCAS). The method for the Philippine isolates resembled that described for the Japanese isolates. To compare the resistance of IT and Mineharuka with that of Koshihikari, we used Dunnett’s test provided by JMP version 9.0 software (SAS Institute, Cary, NC, USA).

4. QTL analysis

A total of 94 polymorphic rice simple sequence repeat (SSR) markers from previous studies (IRGSP 2005, Akagi et al. 1996a, McCouch et al. 2002, Akagi et al. 1996b) were

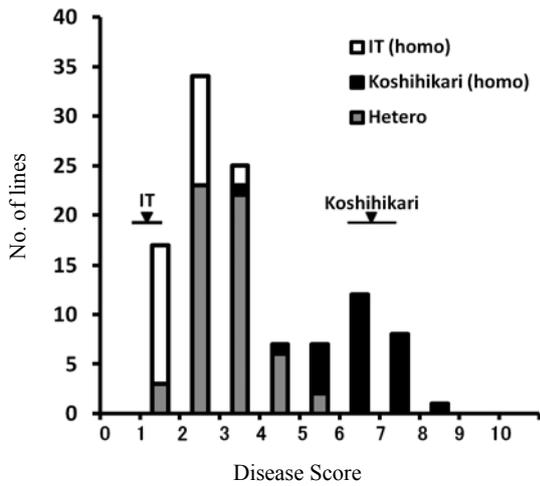


Fig. 1. Frequency distribution of disease scores in F₃ lines derived from crossing Inggoppor-tinawon (IT) and Koshihikari

The blast resistance was evaluated by inoculating with AI 79-142 (race 037.3), which has virulence to *Pish*, *Pii*, *Pia*, *Pik*, *Pik-m*, *Pik-p* and *Pib*, in a blast nursery. Genotypes were characterized using the SSR marker RM3843, which emerged to be linked to *qBFR4*. The x-axis labels indicate the maximum disease score in each bin. Genotypes of RM3843 are represented as *white bars* (homozygous for IT allele), *gray bars* (heterozygous) and *black bars* (homozygous for Koshihikari allele). *Arrowheads* indicate the mean values for IT and Koshihikari; *horizontal lines* across the arrowheads indicate the standard deviations.

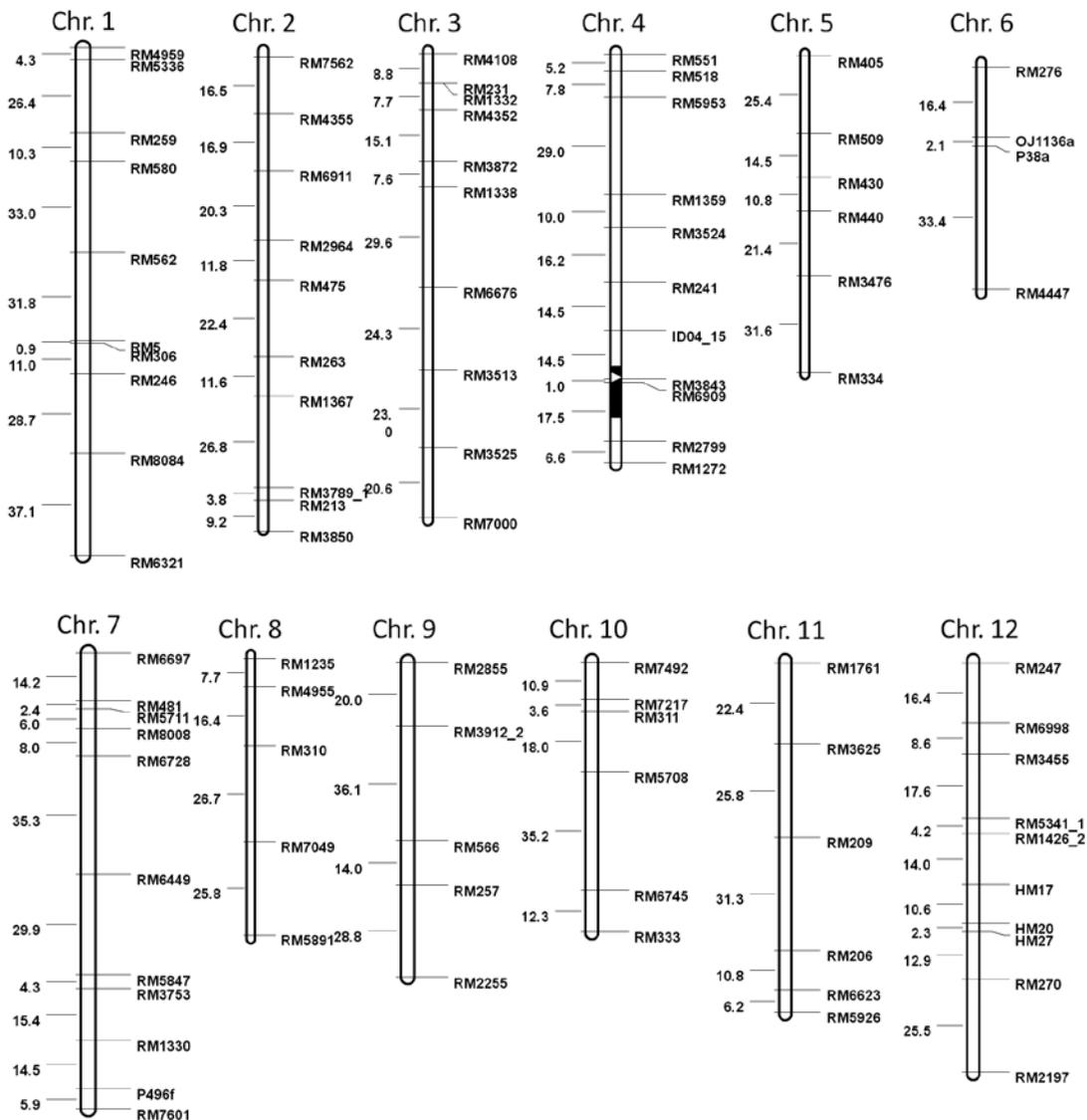


Fig. 2. Linkage map and position of blast resistance QTL

The numbers on the left-hand side of each chromosome indicate the map distances between markers, obtained using the Kosambi function. Marker names are shown on the right-hand side of each chromosome. The *white arrowhead* and *black box* on chromosome 4 (Chr. 4) represent the LOD peak of the putative QTL and its 1.5-LOD support interval, respectively.

used to construct a linkage map. SSR analysis was also performed (Kono et al. 2000) with the following modification: the amplified product was separated in a 3% agarose gel (Type1-A, Low EEO, Sigma-Aldrich Co., St. Louis, Missouri, USA) at 150 V for 120 min. Linkage groups and the order of markers were determined using MAPMAKER/EXP ver. 3.0 (Lander et al. 1987). Map units (in cM) were calculated using the Kosambi function. Composite interval mapping (CIM) was performed using QTL Cartographer 2.5 (Wang et al. 2005). The threshold for CIM was based on the results of 1000 permutation tests at the 5% level of significance (Churchill & Doerge 1994) and a LOD threshold of 2.5 was employed to identify QTLs. A putative QTL was assumed to be located in the vicinity of the peak of the LOD score, and the additive effects and phenotypic variance explained by each QTL (R^2) were estimated at the peak of the LOD score. 1.5-LOD support interval was employed (Dupuis & Siegmund 1999) and a Microsoft Excel macro (Liu & Meng 2003) was used to draw genetic linkage maps based on the linkage data obtained.

Results

1. Variation of blast resistance in F_3 lines

Evaluation of blast resistance was conducted in a blast nursery by inoculation with AI 79-142 (race 037.3), which has virulence to *Pish*, *Pii*, *Pia*, *Pik*, *Pik-m*, *Pik-p* and *Pib*. A significant difference in blast resistance emerged between IT and Koshihikari (Fig. 1). The average disease scores were 1.2 in IT and 6.7 in Koshihikari, while the disease scores of

the 124 F_3 lines ranged from 1.2 to 8.3 and showed bimodal distribution. This distribution indicated that the resistance found in IT is controlled by a single major resistance gene.

2. Linkage map construction and QTL analysis

We found 94 markers showing polymorphism between IT and Koshihikari, and constructed a linkage map based on 124 F_2 individuals derived from crossing IT and Koshihikari (Fig. 2 and Table 1). The markers were grouped into 12 linkage groups corresponding to the 12 chromosomes of rice. The total map distance was 1337.4 cM, corresponding to 86.9% of a previously published rice genetic map constructed using SSR markers (McCouch et al. 2002).

Only one QTL for blast field resistance (*qBFR4*) was identified on chromosome 4 near RM3843, based on an LOD threshold of 2.5 (Fig. 2 and Table 2). Allelic variation at *qBFR4* explained 73.5% of the total phenotypic variation, while the IT allele decreased the disease score by 2.3 points on a 10-point scale (Table 2). Based on the genotype at RM3843, we classified each line as homozygous for either the IT allele or Koshihikari allele or heterozygous (Fig. 1). Lines homozygous for the IT allele at RM3843 had disease scores from 1.2 to 3.7. Conversely, the disease score in lines homozygous or heterozygous for the Koshihikari allele ranged from 3.5 to 8.3 and from 1.7 to 5.2, respectively. Thus, plants in the class homozygous for the IT allele tended to show lower disease value scores than those in the class homozygous for the Koshihikari allele. These results clearly demonstrate the existence of a QTL for blast resistance on chromosome 4 near RM3843 (Fig. 2).

Table 1. Sequences of primers (except RM primers¹⁾) used in this study

Primer ²⁾	Chromosome ³⁾	Forward (5'–3')	Reverse (5'–3')
ID04_15	4	ATAGGCGAATGGTTGACAAGA	CGCTTGGAGAAGGTGACCTG
OJ1136a	6	AGAGGTGGCAGCTTATTTTA	TGGAAAAACAAAATCAAAC
P38a	6	AGGGTAAGACGTTTAACTTGTA	CGACAGGCCTTTAGTTAT
P496f	7	CTGGCGATAGAGATATTGAA	TCCGATAACTACAAGCAAAC
HM17	12	CGACATATTAATGACCCAT	CGCCGACCTCACAAC
HM20	12	AAAATAAGAGTAATCCACCAT	CAGCAACCAACAACTAC
HM27	12	ATGGCATCTGCTCAACTAAAC	GTGGGTTCCGGTGGTAAAT

¹⁾ RM primers were as in previous studies (IRGSP 2005; McCouch et al., 2002).

²⁾ Markers are based on the length polymorphism of PCR products.

³⁾ Map locations of the marker loci are indicated in Fig. 2.

Table 2. A putative QTL for blast field resistance in Inngoppor-tinawon (IT)

QTL	Chromosome	Marker interval ¹⁾	LOD score	Additive effect ²⁾	R^2 (%) ³⁾
<i>qBFR4</i>	4	ID04_15– <u>RM3843</u>	42	–2.3	73.5

¹⁾ The nearest marker to the QTL is underlined.

²⁾ The additive effect of the IT allele based on the disease score.

³⁾ The phenotypic variance explained by the QTL.

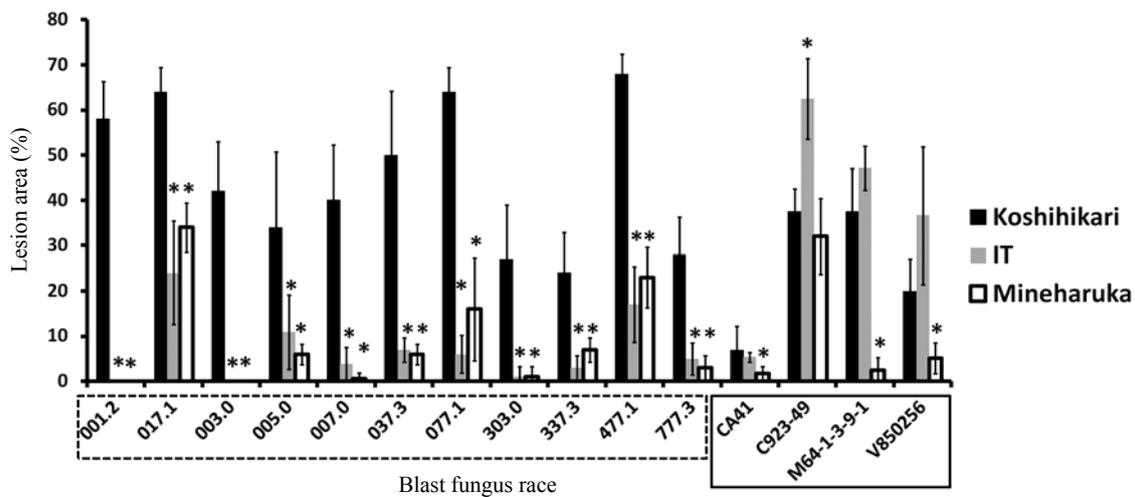


Fig. 3. Inoculation test of blast fungus races in Koshihikari, Inggoppor-tinawon (IT) and Mineharuka

Bars indicate means, and error bars indicate SD. Races in the dotted box originated from Japan and races in the solid box are from the Philippines. Asterisks (*) indicate significant differences from Koshihikari at the 5% level by Dunnett's test.

3. Inoculation assay with additional blast isolates

The position of *qBFR4* was almost the same as that of the blast field-resistance gene *Pi39*, detected in the rice variety 'Mineharuka' (Terashima et al. 2008). To evaluate the usefulness of resistance derived from IT, we conducted an inoculation assay with many blast isolates and compared the reaction patterns of IT (*qBFR4*) with those of Mineharuka (*Pi39* and *Pii*) (Terashima et al. 2008) and Koshihikari (*Pish*) (Imbe & Matsumoto 1985, Fukuta et al. 2004). Both IT and Mineharuka were significantly more resistant to all the Japanese races than the susceptible variety Koshihikari, and IT showed no race-specific resistance against the examined Japanese races (Fig. 3). In general, resistance controlled by QTLs is considered durable, i.e. without race specificity or gene-for-gene interaction (Ezuka 1972, Parlevliet 1979). However, the field resistance controlled by *Pi34* reported varied with the pathogenic isolate tested (Koizumi & Fujii 1995, Zenbayashi-Sawata et al. 2005), and several Philippine races were found to be virulent to several resistance genes that were not thought to have race specificity (Fukuta, unpublished data). Therefore, we conducted an inoculation assay with four Philippine isolates (CA41, M64-1-3-9-1, V850256 and C923-49) that were virulent to *Pii* (Telebanco-Yanoria et al. 2008) and some partial-resistance genes (unpublished data). Koshihikari showed resistance to CA41; this resistance is probably conferred by *Pish*, which is present in Koshihikari and confers resistance against many Philippine races. Although Mineharuka was resistant to both M64-1-3-9-1 and V850256, IT was susceptible to these races. As for C923-49, both Mineharuka and IT were infected, but IT had a significantly larger lesion area than Mineharuka. From these results, IT (containing *qBFR4*) showed different reac-

tion patterns from those of Mineharuka (containing *Pi39*). Thus, there is the possibility that resistance derived from IT is distinct from *Pi39*.

Discussion

It is necessary to discover and characterize novel genes for blast resistance to breed resistant varieties for sustainable rice production. We found a QTL, *qBFR4*, which plays a major role in expressing blast resistance in IT. Because IT showed broad resistance to Japanese races, IT has the potential to be a useful blast resistance donor in breeding programs. IT displays not only resistance to rice blast but also high tolerance to lodging, despite having very long culms. Therefore, IT was crossed with several WCS varieties and breeding is underway. However, it is necessary to characterize *qBFR4* in greater detail, because IT showed race-specific resistance when tested with several Philippine isolates. Because we selected four Philippine isolates that were virulent to some partial-resistance genes in the previous study (unpublished data), it is unknown whether other Philippine isolates are also virulent to IT. IT has been widely cultivated in the Philippines, so it is possible that some Philippine blast isolates have lost the avirulence gene associated with resistance of IT. Recently, *Pi34*, which was known to be resistant to most of Japanese races, was reportedly susceptible to an isolate originated from an upland rice variety (Zenbayashi-Sawata et al. 2005). Because *Pi34* was identified from an upland rice variety, 'Sensho', *AVR-Pi34* isolates of *Magnaporthe grisea* may be present in fields in which the upland rice variety with *Pi34* is grown (Zenbayashi-Sawata et al. 2005). Therefore, there is some possibility that the resistance of IT will break

down if varieties with *qBFR4* are widely cultivated in Japan. However, unlike insects such as rice brown planthoppers, transfer of blast isolates from foreign countries is quite rare. In this experiment, inoculation tests by Philippines isolates were performed in an isolated greenhouse at JIRCAS to prevent disease from spreading in fields. In conclusion, we think that resistance derived from IT is useful for breeding in Japan by monitoring isolates in cultivating area of varieties with *qBFR4*.

The inoculation assay revealed that IT (*qBFR4*) showed different reaction patterns from those of Mineharuka (*Pi39*). When we began this experiment, the dominant gene *Pi39* was the most effective and well-characterized of the resistance QTLs located near *qBFR4* (Xu et al. 2008, Terashima et al. 2008, Fukuoka & Okuno 2001, Miyamoto et al. 2001, Kato et al. 2002). Recently, a QTL derived from a wild relative (*Oryza rufipogon*) was also detected in a similar position (Hirabayashi et al. 2010). Analysis of *qBR4-2* (Fukuoka et al. 2012), a QTL found in Owarihatamochi and located at the similar position of *qBFR4*, revealed that *qBR4-2* comprises three loci, designated *qBR4-2a*, *qBR4-2b* and *qBR4-2c*. The predicted protein products of both *qBR4-2a* and *qBR4-2b* resembled previously reported disease resistance proteins containing a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs) (McHale et al. 2006). Two adjacent NBS-LRR class genes were also reportedly required to confer *Pik-m*-specific resistance (Ashikawa et al. 2008). Therefore, these results suggest that allelic variation in one or more NBS-LRR genes is responsible for differences in blast resistance in rice (Fukuoka et al. 2012). It is unknown whether *qBFR4* is allelic to one of the three loci in *qBR4-2*; to determine this, it will be necessary to conduct inoculation assays of *qBFR4* and *qBR4-2* in matching genetic backgrounds.

The genes for blast resistance have been extensively studied and classified as two types of resistance, complete resistance and field resistance (Parlevliet 1979, Ezuka 1972). Field resistance was known to be non-race specific and partial (incomplete) resistance previously (Parlevliet 1979, Ezuka 1972). However, some exceptions such as resistance derived from IT and *Pi34* have been found to show race-specificity, despite partial (incomplete) resistance (Zenbayashi-Sawata et al. 2005, Zenbayashi-Sawata et al. 2007). Conversely, several complete resistance genes such as *Pik-m*, *Pia* and *Pit* were known as NBS-LRR genes (Ashikawa et al. 2008, Okuyama et al. 2011, Hayashi & Yoshida 2009, Hayashi, Yasuda et al. 2010). However, some genes detected as field resistance such as *Pb1*, *qBR4-2a* and *qBR4-2b* have been recently reported as NBS-LRR genes (Hayashi, Inoue et al. 2010, Fukuoka et al. 2012). Therefore, it does not matter when distinguishing complete resistance from field resistance. For the systematic analysis of blast resistance genes, the IRRI-Japan Collaborative Research Project established rice monogenic lines as a differential variety set containing

major resistance genes and the standard differential blast isolates from Japan and the Philippines (Kobayashi et al. 2007, Telebanco-Yanoria et al. 2008, Tsunematsu et al. 2000). In future, it will be necessary to make a monogenic line from IT and compare its response to the standard isolates with those of other monogenic lines.

There are two possible strategies for using resistance of IT as a source of blast-resistant rice. One is gene pyramiding, i.e. producing varieties containing multiple resistance genes; the other is producing multiple lines, each with a single resistance gene. In Japan, multiple lines for blast resistance were bred, and some resistance genes such as *Pia*, *Pii*, *Pita2*, *Piz*, *Pik*, *Pik-m*, *Piz-t* and *Pib* were used (Ishizaki et al. 2005). However, continuous use of the same limited set of genes increases the likelihood of resistance breakdown. Based on simulation modeling, Ashizawa reported that at least an eight-line mixture was needed to effectively control blast (Ashizawa 2007); hence the importance of increasing the number of available resistance genes. A backcrossing program is therefore underway to introduce *qBFR4* into Koshihikari of the susceptible background and develop the isogenic line for *qBFR4*. A line containing *qBFR4* in the Koshihikari genetic background would complement Koshihikari lines containing other resistance genes.

Acknowledgements

We thank Dr. N. Hayashi (NIAS) for providing the blast isolates which originated from Japan. We also thank Drs. T. Imbe (NARO), I. Ando (NARO) and M. Yano (NARO) for their valuable discussion and the staff of the technical support section of the NIAS for their field management and experimental support. This research was supported by grants from the Ministry of Agriculture, Forestry and Fisheries of Japan (QTL2002 and LCT13).

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