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A novel blast resistance locus in a rice (*Oryza sativa* L.) cultivar, Chumroo, of Bhutan

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Abstract The rice cultivar 'Chumroo' is commonly cultivated in the mid- and high-altitude areas of Bhutan. This cultivar has shown durable blast resistance in that area, without evidence of breakdown, for over 20 years. Chumroo was inoculated with 22 blast isolates selected from the race differential standard set of Japan. The cultivar showed resistance to all the isolates. To identify the resistance gene(s), Chumroo was crossed with a susceptible rice cultivar, Koshihikari. The F_1 plants of the cross showed resistance. Segregation analyses of 300 F_3 family lines fitted the segregation ratio of 1:2:1 and indicated that a single dominant gene controls the resistance to a blast isolate Ao 92-06-2 (race 337.1). The Chumroo resistance locus (termed *Pi46*(t)) was mapped between two SSR markers, *RM6748* and *RM5473*, on the terminal region of the long arm of chromosome 4, using linkage analysis with SSR markers. The nearest marker, *RM5473*, was linked to the putative resistance locus at a map distance of 3.2 cM. At the chromosomal region, no true resistance genes were identified, whereas two field resistance genes were present. Therefore, we designated *Pi46*(t) as a novel blast resistance locus.

Keywords Rice (*Oryza sativa* L.) · Rice blast fungus (*Magnaporthe oryzae*) · Resistance gene · Genetic mapping · SSR marker

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Introduction

Magnaporthe oryzae (anamorph, *Pyricularia oryzae*) is the causal agent of blast disease in rice (*Oryza sativa* L.) (Couch and Kohn 2002). Blast resistance is the most effective means of controlling the disease without chemical applications. Race–cultivar specificity between the rice and *M. oryzae* complies with the gene-for-gene hypothesis (Flor 1956; Silué et al. 1992), which states that for every race-specific resistance gene in the host, a corresponding avirulence gene in the pathogen causes an incompatible response. More than 40 resistance genes, and several avirulence genes,

conferring blast resistance have been identified in rice and the blast pathogen, respectively (Chen et al. 1999; Yasuda et al. 2004, 2005).

The extensive use of blast-resistant rice cultivars with an introduced resistance gene has engendered the development of new blast races that are virulent to the resistant cultivars, leading to subsequent breakdown of that resistance (Kiyosawa 1982; Bonman 1992). Based on the gene-for-gene hypothesis, the International Rice Research Institute (IRRI)-Japan collaborative research project is successfully constructing an international differential system of blast resistance genes by developing near-isogenic lines and monogenic lines with the genetic backgrounds of three blast susceptible varieties (Kobayashi et al. 2007; Fukuta et al. 2009; Hayashi and Fukuta 2009). This differential system will improve our understanding of the characteristics of blast disease and facilitate the development of blast-resistant rice breeding (Telebanco-Yanoria et al. 2008; Kobayashi et al. 2009).

The rice cultivar 'Chumroo' was selected from eight Bhutanese cultivars using the race differential standard set of Japan and identified as a potential donor of a novel resistance gene (Thinlay et al. 2000a). Chumroo is cultivated mostly in the mid- and high-altitude rice growing areas in Bhutan (Thinlay et al. 2000b). Chumroo can be grown under irrigated or upland conditions; the area under cultivation for this one cultivar is estimated to be about 20–30% of the high altitude rice area in Bhutan. Chumroo shows high resistance to Bhutanese blast fungus isolates; no evidence of breakdown has been found for over 20 years after its introduction from Nepal (Thinlay, pers. commun.). Chumroo was the only variety that was unaffected during the 1995/1996 rice blast epidemic in Bhutan (Thinlay, pers. commun.).

This study examines this remarkable blast resistance of Chumroo, by inoculating different Japanese isolates onto Chumroo and by linkage mapping of a locus that contributes to this resistance.

Materials and methods

Plant materials

The blast resistant rice cultivar from Bhutan, Chumroo, was used in this study. This cultivar is a Japonica-type with a bold grain and a red pericarp. A lowland rice cultivar, Koshihikari, harboring a blast resistance gene, *Pik-s*, was used as a susceptible cultivar. A cross between a female Koshihikari and pollen from Chumroo was conducted. For dominance analysis, 30 F_1 individuals were used. An F_1 plant was cultivated and self-fertilized, and 300 F_2 plants were self-fertilized to develop 300 F_3 family lines. This F_3 line group was used for F_2 segregation analysis and linkage analysis.

The differential rice varieties presented in Table 1 were supplied from the National Agricultural Research Center for Tohoku Region (Yamada et al. 1976; Kiyosawa 1984; Noda et al. 1999; Koizumi et al. 2007). Each variety possesses several resistance genes, chosen from *Pik-s*, *Pish*, *Pia*, *Pii*, *Pik*, *Pik-m*, *Piz*, *Pita*, *Pita-2*, *Piz-t*, *Pik-p*, *Pib*, *Pit*, and *Pi19*(t).

Fungal isolates, inoculation, and disease rating

Table 1 shows the races of the *Magnaporthe oryzae* isolates used for this study. All blast isolates were selected from the race differential standard set of Japan (Hayashi 2005). The race number of each fungus isolate was estimated according to the differential varieties (Yamada et al. 1976; Kiyosawa 1984; Noda et al. 1999). These fungus isolates are compatible with several resistance genes (*Pik-s*, *Pish*, *Pia*, *Pii*, *Pik*, *Pik-m*, *Piz*, *Pita*, *Pita-2*, *Piz-t*, *Pik-p*, *Pib*, *Pit*, and *Pi19*(t)).

For inoculation, fungal isolates were grown on oatmeal agar medium at 26°C for 11–12 days. Aerial mycelia of the agar culture were then washed off gently with a water-soaked paintbrush. They were placed at 21°C for 3–4 days under continuous illumination with fluorescent light to induce sporulation. To prepare a spore suspension, the mycelia were scraped and flooded with water containing 0.05% Tween 20. The suspension was filtered through a gauze mesh and adjusted to 1×10^5 spores/ml. Simultaneously, approximately 30 rice seeds were sown in a plastic tray (15 × 5 × 10 cm) and grown in a greenhouse at 20–30°C. Rice seedlings were inoculated at the 3.8-leaf to 5.0-leaf stages by spraying with 50 ml of the spore suspension described above. The inoculated seedlings were placed immediately in a dark chamber with a moisture-saturated atmosphere at 25°C for 20 h and then transferred to a greenhouse at 20–28°C.

Approximately 7 days after inoculation, the plants were scored for resistance to the blast fungus isolates according to the classification of Hayashi et al.

Table 1 Reactions of Japanese differential cultivars and Chumroo to 22 *Magnaporthe oryzae* isolates from Japan with different pathogenicities

Isolate	Race[b]	Reaction to cultivar (cultivar/*Pi* gene[a])														
		Shin 2 *Pik-s*, *Pish*	Aichi Asahi *Pia* *Pi19*(t)	Ishikari Shiroke *Pii*, *Pik-s*	Kanto 51 *Pik*	Tsuyuake *Pik-m*	Fukunishiki *Piz*, *Pish*	Yashiro-mochi *Pita*	Pi No. 4 *Pita-2*, *Pish*	Toride 1 *Piz-t*, *Pish*	BL1 *Pib*, *Pish*	K59 *Pit*, *Pik-s*	K60 *Pik-p*, *Pish*	AA/S2-3 *Pish*	AA/S2-75 *Pik-s*	Chumroo *Pi46*(t)
Mu-95	001.2	S	R	R	R	R	R	R	R	R	S	R	R	S	S	R
Kyu89-246	003.0	S	S	R	R	R	R	R	R	R	R	R	R	S	S	R
95Mu-29	003.2	S	S	R	R	R	R	R	R	R	S	R	R	S	S	R
Shin 83-34	005.0	S	R	S	R	R	R	R	R	R	R	R	R	S	S	R
Ina 86-137	007.0	S	S	S	R	R	R	R	R	R	R	R	R	S	S	R
Kyu92-22	017.1	S	S	S	S	R	R	R	R	R	R	R	S	S	S	R
1804-4	031.1	S	R	R	S	S	R	R	R	R	R	R	S	S	S	R
Ina 72	031.1	S	R	R	S	S	R	R	R	R	R	R	S	S	S	R
TH68-126	033.1	S	S	R	S	S	R	M	R	R	R	R	S	S	S	R
24-22-1-1	037.1	S	S	S	S	S	R	R	R	R	R	R	S	S	S	R
Ai79-142	037.3	S	S	S	S	S	R	R	R	R	S	R	S	S	S	R
TH2000-53	037.7	S	S	S	S	S	R	R	R	R	S	S	S	S	S	R
Kyu9439013	047.0	S	S	S	R	R	S	R	R	R	R	R	R	S	S	R
TH69-8	071.1	S	R	R	S	S	S	R	R	R	R	R	S	S	S	R
Sasamori 121	077.1	S	S	S	S	S	S	R	R	R	R	R	S	S	S	R
Ina 168	101.0	S	R	R	R	R	R	S	R	R	R	R	R	S	S	R
GFOS8-1-1	303.0	S	S	R	R	R	R	S	S	R	R	R	R	S	S	R
P-2b	303.1	S	S	R	R	R	R	S	S	R	R	R	S	S	S	R
0528-2	333.1	S	S	R	S	S	R	S	S	R	R	R	S	S	S	R
Ao92-06-2	337.1	S	S	S	S	S	R	S	S	R	R	R	S	S	S	R
IW81-04	437.1	S	S	S	S	S	R	R	R	S	R	R	S	S	S	R
Ai74-134	477.1	S	S	S	S	S	S	M	R	S	R	R	S	S	S	R

S Susceptible, *M* intermediate, *R* resistant

[a] Hayashi et al. (1998) and Noda et al. (1999)

[b] According to Kiyosawa (1984)

(2009). The classification comprises: 0 = no visible evidence of infection; 1 = uniform or scattered brown specks of smaller than 0.5 mm in diameter, no sporulation; 2 = small lesions with distinct tan centers surrounded by a darker brown margins approximately 1 mm in diameter, no sporulation; 3 = small eyespot lesions less than one and a half times the interval between thin veins or less than 1.5 mm in diameter surrounded by dark brown, lesions capable of sporulation; 4 = intermediate-size eyespot lesions less than twice the distance between thin veins or less than 2 mm diameter; and 5 = large eyespot lesions more than twice the interval between thin veins or more than 2 mm in diameter. Rice classified as type 0–2 is said to be resistant, and rice classified as type 3–5 is considered susceptible.

Segregation analysis and dominance analysis

A blast isolate, Ao 92-06-2 (race 337.1) was used for genetic analysis due to its stable virulence and high sporogenesis ability. In addition, compared to other isolates, it is compatible with many resistance genes shown in Table 1. The F_3 plants were inoculated with Ao 92-06-2 to analyze the segregation of resistance in their parental F_2 plants. The F_3 family lines were classified into three phenotypes: resistant homozygote, segregating heterozygote, and susceptible homozygote, respectively. The reactions of 10 F_1 plants were also confirmed using the same blast isolate.

Linkage mapping using SSR markers

Whole genomic DNA was extracted from leaves of each F_2 plant according to the modified CTAB method (Murray and Thompson 1980). Polymerase chain reaction (PCR) was performed in a 20 µl reaction mixture containing 10 ng of DNA, 1 pmol of each primer, and 0.4 unit of *Taq* polymerase. Thermal cycling was conducted using a GeneAmp PCR System 9700 (PerkinElmer Inc., Boston, USA) programmed for 9 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C; and 5 min at 72°C for the final extension. The PCR products were separated on 4% polyacrylamide denaturing gels and banding patterns were visualized using the silver staining method, as described by Panaud et al. (1996).

To identify the locus associated with resistance in Chumroo, bulk segregant analysis was conducted. Polymorphisms between Koshihikari and Chumroo were surveyed using 283 SSR markers covering all rice chromosomes (Temnykh et al. 2000; McCouch et al. 2002). Polymorphic markers were subjected to bulk segregant analysis in the F_3 family lines. First, the map location of the resistant locus was roughly estimated in 94 randomly selected F_3 lines. To detect the linkage with these SSR markers, we used an χ^2 test (Ise 1996) and the program MAPMAKER/EXP 3.0, based on the Kosambi function (Lander et al. 1987). For further detailed mapping, we selected 16 SSR markers on chromosome 4 and 143 F_3 lines with a clear response to inoculation. The program MAPMAKER/EXP 3.0, based on the Kosambi function, was used to build the linkage map.

Results

Inoculation of Japanese fungus isolates of *M. oryzae* onto Chumroo

Table 1 shows the reactions of Chumroo and differential rice cultivars to the 22 fungal isolates. Chumroo was resistant to all the blast isolates. Figure 1 shows the symptoms of Koshihikari and Chumroo inoculated with a blast isolate Ao 92-06-2. Chumroo showed eyespot symptoms surrounded by brown necrotic margins, which is classified as a true resistant response according to the standard of Hayashi et al. (2009). Koshihikari showed typical susceptible symptoms with no brown lesions, which is classified as a susceptible response.

A cross between Koshihikari and Chumroo was conducted and the F_1 individuals from the cross were inoculated with fungus isolate Ao 92-06-2. All the F_1 individuals displayed brown necrotic lesions (Fig. 1). The symptoms of the F_1 individuals were similar to those of Chumroo and were classified as a true resistant response.

Segregation analysis of F_2 by F_3 line group

Subsequently, 300 F_3 family lines were developed from the cross between Koshihikari and Chumroo. Segregation analyses of the 300 F_3 family lines showed that 86, 142, and 72 F_2 lines were resistant, segregating, and susceptible, respectively, to blast isolate Ao 92-06-2 (Table 2). The segregation ratio corresponded to the expected segregation ratio

Fig. 1 Symptoms appearing on the leaves of Koshihikari, Chumroo, and F_1 (Koshihikari × Chumroo) plants inoculated with *M. oryzae* isolate Ao 92-06-2

Table 2 Segregation of resistance to blast isolate Ao 92-06-2 in the F_3 family lines derived from a cross between Koshihikari and Chumroo

Response of F_3 family lines in nursery inoculation			χ^2	P
Resistant	Segregating	Susceptible		
86	142	72	2.16	0.3396

governed by a single dominant locus, i.e. 1:2:1 ($\chi^2 = 2.16$, $P = 0.3396$). This result suggests that at least one dominant locus contributes to the blast resistance of Chumroo.

Linkage mapping

The PCR screening and linkage analysis results are shown in Fig. 2. To analyze the locus containing the resistance gene, we surveyed polymorphisms between Chumroo and Koshihikari using 283 SSR markers covering all rice chromosomes. Polymorphisms were observed for 153 markers. Co-dominant

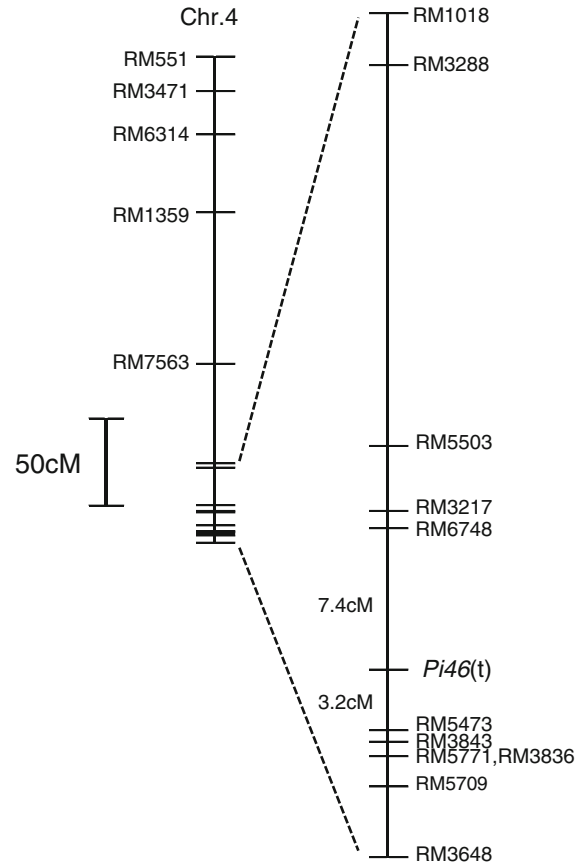

Fig. 2 Linkage map and position of *Pi46*(t) on chromosome 4, developed by SSR marker analysis of F_2 (Koshihikari × Chumroo) plants. *Vertical lines* represent the chromosome. *Horizontal lines* represent SSR markers

polymorphisms were observed for 114 markers. For linkage analysis, we randomly selected 94 F_3 lines and 39 co-dominant markers covering all rice chromosomes, where the map distance between each marker was at least 20 cM. Accordingly, linkage between the resistance locus of Chumroo and markers on chromosome 4 was detected (data not shown). Thus, for more detailed linkage mapping, we selected 16 co-dominant SSR markers on chromosome 4 and 143 F_3 lines with a clear response to inoculation. As a result, the resistance locus was mapped between markers *RM6748* and *RM5473* on the terminal region of the long arm of chromosome 4 (Fig. 2). The putative map distances of *RM6748* and *RM5473* to the resistance locus were 7.4 and 3.2 cM, respectively.

Discussion

We surveyed the reactions of Chumroo to blast fungus isolates selected from the differential standard set of Japan. Blast resistance is classified into two classes: true (vertical, complete) resistance comprising incompatible, hypersensitive symptoms; and field (horizontal, partial) resistance, which is a reduction in the degree of compatible symptoms (Ezuka 1972; Parlevliet 1979; Asaga 1981). Chumroo showed signs of true resistance to all fungal isolates presented in Table 1. F_1 individuals from the cross of Koshihikari and Chumroo showed symptoms similar to those of Chumroo (Fig. 1). The estimated segregation ratio of the F_2 generation of the cross between Koshihikari and Chumroo fitted well to the expected segregation ratio governed by a single dominant locus, suggesting that a dominant locus contributes to Chumroo's resistance to Ao 92-06-2. Subsequently, we conducted linkage analysis to investigate relationships between this locus conferring resistance of Chumroo and known resistance genes. The locus of Chumroo was mapped on the terminal part of the long arm of chromosome 4. We tentatively designated this resistance locus in chromosome 4 of Chumroo as *Pi46*(t), under the international agreement of nomenclature (Kinoshita and Rothschild 1995). In future studies, one or several genes responsible for conferring resistance of Chumroo will be identified in this locus.

There is no report of a true resistance gene in this region, whereas the presence of two field resistance genes has been reported. *Pi39*(t) and *Pikahei-1*(t) are located on the terminal part of the long arm of chromosome 4 and these are tightly linked with *RM5473* (Terashima et al. 2008; Xu et al. 2008). Rice variety Mineharuka, with *Pi39*(t), was susceptible to some blast races and developed the same type of blast lesions as susceptible cultivars (Terashima et al. 2008). Likewise, *Pikahei-1*(t) was described as a field resistance gene (Xu et al. 2008). Generally, blast resistance genes show isolate (race) specificity. There are numerous reports for multiple resistance genes, each detected from a separate resource and located in a single locus (Fukuta et al. 2009). Whereas, there is no report on the specificity of *Pi46*(t), *Pi39*(t) and *Pikahei-1*(t). To investigate this point, a near isogenic line (NIL), into which the resistance gene will be inserted in a susceptible variety's genetic background, should be developed for accurate differentiation. The NIL will be challenged with various blast isolates, e.g. those shown in Table 1, to confirm that the incompatible response to these isolate are controlled by *Pi46*(t).

Variations in race-specificity have posed a problem in the breeding of blast-resistant cultivars. One strategy for developing a rice cultivar with durable blast resistance is the multiline concept. Jensen (1952) and Borlaug (1953) proposed multiline concepts for disease control in oat (*Avena sativa* L.) and wheat (*Triticum aestivum* L.), respectively. Thereafter, multiline rice cultivars were bred in Japan to suppress damage caused by rice blast disease. At present, three multiline cultivars—Sasanishiki BL (Abe 2004), Koshihikari Toyama BL (Kojima et al. 2003), and Koshihikari Niigata BL (Ishizaki 2007)—are used in practical cultivation by farmers. In Japan, 10 resistance genes (*Pia*, *Pii*, *Piz*, *Piz-t*, *Pita*, *Pita-2*, *Pib*, *Pik*, *Pik-m*, and *Pik-p*) were introduced into the multiline cultivars. However, the existence of blast isolates compatible with each gene or of multiple genes of these 10 genes, coupled with the breakdown of resistance of several genes, has led to fears of the appearance of a multi-compatible fungus isolate that infects all the multilines. Consequently, for sustainable use of multiline cultivars, it is important to introduce new resistance genes into the cultivar. Therefore, *Pi46*(t), when introduced into multiline cultivars, is potentially very useful.

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