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FUNGAL DISEASES

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Pi34-AVRPi34: a new gene-for-gene interaction for partial resistance in rice to blast caused by *Magnaporthe grisea*

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Abstract The japonica rice (*Oryza sativa*) cultivar Chubu 32 has a high level of partial resistance to blast, which is mainly controlled by a dominant resistance gene located on chromosome 11. The partial resistance to the rice blast fungus (*Magnaporthe grisea*) in Chubu 32 has isolate specificity; isolate IBOS8-1-1 is more aggressive on Chubu 32 than are other isolates. We hypothesized that the gene-for-gene relationship fits this case of a partial resistance gene in Chubu 32 against the avirulence gene in the pathogen. The partial resistance gene in Chubu 32 was mapped between DNA markers C1172 (and three other co-segregated markers) and E2021 and was designated *Pi34*. In the 32 F_3 lines from the cross between a chromosome segment substitution line (*Pi34*$^-$) from Koshihikari/Kasalath and Chubu 32, the lines with high levels of partial resistance to the *M. grisea* isolate Y93-245c-2 corresponded to the presence of *Pi34* estimated by graphic genotyping. This indicated that *Pi34* has partial resistance to isolate Y93-245c-2 in compatible interactions. The 69 blast isolates from the F_1 progeny produced by the cross between Y93-245c-2 and IBOS8-1-1 were tested for aggressiveness on Chubu 32 and rice cultivar Koshihikari (*Pi34*$^-$). The progeny segregated at a 1:1 ratio for strong to weak aggressiveness on Chubu 32. The results suggested that Y93-245c-2 has one gene encoding avirulence to *Pi34* (*AVRPi34*), and IBOS8-1-1 is extremely aggressive on Chubu 32 because of the absence of *AVRPi34*. This is the first report of a gene-for-gene relationship between a fungal disease resistance gene associated with severity of disease and pathogen aggressiveness.

Key words *Oryza sativa* · *Magnaporthe grisea* · Partial resistance gene · Avirulence gene · Gene-for-gene relationship

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Introduction

Blast caused by *Magnaporthe grisea* (Hebert) Barr [anamorph *Pyricularia grisea* (Cooke) Sacc.] (Rossman et al. 1990; Valent and Chumley 1991) continues to be a widespread disease and causes severe yield losses of rice (*Oryza sativa* L.) worldwide despite the availability of dozens of genes encoding complete resistance to the disease.

Complete resistance (termed "true resistance" by Japanese researchers) to blast is qualitative (prevents infection by the pathogen) and race-specific and is expressed as an incompatible interaction between the host and pathogenic isolates (Bonman and Mackill 1988; Lee et al. 1989). However, complete resistance in many rice cultivars has been broken down within a few years with the appearance of new pathogenic blast races (Kiyosawa 1982; Ou 1979; Watanabe 1980; Yunoki et al. 1969). There do not seem to be any examples of complete blast resistance that have proved durable. The relationship between complete resistance in rice and the rice blast isolate fits a gene-for-gene model (Berruyer et al. 2003; Chauhan et al. 2002; Orbach et al. 2000; Wang et al. 1994), in which incompatibility between the host and the pathogen is a result of the interaction between a dominant resistance (*R*) gene in the plant and a corresponding dominant avirulence (*avr*) gene in the pathogen (Flor 1971). To date, various *R* genes and corresponding *avr* genes have been characterized and cloned. In rice diseases, several *avr* genes associated with the *avrBs*3 gene family have been identified from a bacterial blight pathogen (*Xanthomonas oryzae* pv. *oryzae*). Bai et al. (2000) reported that some of these genes contribute to pathogen aggressiveness on the susceptible host and that the contributions are quantitatively different. Two *avr* genes, *AVR-*

Pita (Orbach et al. 2000) and *AVR1-Co39* (Farman and Leong 1998), were identified from *M. grisea*. Jia et al. (2000) demonstrated that the AVR-Pita$_{176}$ protein encoded by *AVR-Pita* binds directly to the Pi-ta LRD region inside the plant cell to initiate a *Pi-ta*-mediated defense response.

In contrast, partial resistance is characterized by a decrease in the extent of pathogen reproduction in the compatible interaction. Parlevliet (1979) described partial resistance as follows: the reduction of spore production even though the plant is susceptible to infection, a form of incomplete resistance, which refers to all resistances that allow some spore production. In the case of rice blast, Bonman et al. (1989) defined partial resistance as a synonym of the term field resistance used in Japan and used it for rice cultivars having typical sporulating blast lesions but with less diseased leaf area than fully susceptible cultivars.

The partial resistance to blast is commonly non-race-specific and genetically polygenic (Bonman and Mackill 1988; Lee et al. 1989). However, some exceptions to partial resistance are controlled by single major genes such as *Pif* (Toriyama et al. 1968) and *Pb1* (Fujii et al. 2000). Rice cultivars St. No. 1 and Chugoku 31 with the *Pif* gene were partially resistant to races of *M. grisea* in Japan at the time of their release, but their resistance later broke down because new races evolved (Toriyama 1975). In contrast, the partial resistance against panicle blast by *Pb1* has not been broken down during the more than 15 years since the commercial cultivation of rice cultivars with *Pb1* began in Japan (Fujii et al. 1999). Numerous classical segregation analyses of the resistance genes involved in partial resistance to diseases have been conducted for more than 40 years, and quantitative trait locus (QTL) analyses with molecular tools recently have been carried out. However, the aggressiveness of a fungal pathogen in relation to partial resistance has not been genetically analyzed, although the analysis is important to ensure the stability of the partial resistance as a control measure.

We reported that partial resistance in rice cultivar Chubu 32 is mainly controlled by one QTL (Zenbayashi et al. 2002), and the partial resistance is probably controlled by a major gene, *Pi34*. Koizumi and Fuji (1995) reported that Chubu 32 is severely infected with a few isolates of *M. grisea* and suggested that the partial resistance in Chubu 32 is isolate-specific. On the basis of these results, we hypothesized that the interaction between the partial resistance gene *Pi34* and a corresponding avirulence gene follows the gene-for-gene model.

This study aimed to map the partial resistance gene *Pi34* derived from Chubu 32 with recombinant inbred lines (RILs) from a cross between Chubu 32 and Norin 29 and with F_3 lines (F_3's) from a cross between Chubu 32 and a chromosome segment substitution line (CSSL) from Koshihikari/Kasalath and to determine genetically that the rice blast fungus isolate has an avirulence gene corresponded to the partial resistance gene in Chubu 32.

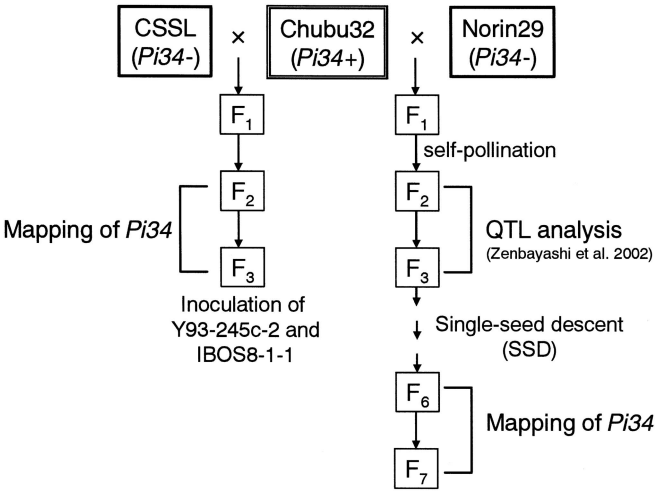

Fig. 1. Strategy for mapping *Pi34* and confirmation of the effectiveness of *Pi34* against *Magnaporthe grisea* isolate Y93-245c-2

Materials and methods

Plant materials and crossing

Chubu 32 is a japonica rice cultivar with the complete resistance genes *Pik-s* and *Pia*; it has a high level of partial resistance to most rice blast isolates in Japan (Koizumi and Fuji 1995). The cultivar was used as a donor parent for partial resistance in a cross with Norin 29, a japonica rice cultivar with a low level of partial resistance to blast. RILs comprised of 139 F_7 lines were produced from the F_3 lines from a cross between Chubu 32 and Norin 29 by single seed descent (Fig. 1). We also crossed Chubu 32 with the CSSL from Koshihikari/Kasalath and produced F_3's constituted of 32 lines. Most of the genome of the CSSL is derived from a japonica rice cultivar Koshihikari; only two-thirds of the region of chromosome 11 was substituted in the genome of an indica cultivar Kasalath. CSSL has the complete resistance gene *Pik-s* and a low level of blast partial resistance to field isolates of *M. grisea* in Japan.

Fungal isolates and crossing

Y93-245c-2 (MAT1-1, race 137.0) is a highly fertile field isolate of *M. grisea* from Yunnan Province in China and is virulent to rice cultivars Chubu 32, Norin 29, CSSL, and Koshihikari. The isolate was provided by Ms. N. Yasuda (National Agricultural Research Center). IBOS-8-1-1 (MAT1-2, race 003.0) is a field isolate from upland rice in Ibaraki, Japan and was provided by Dr. N. Hayashi (National Institute of Agrobiological Sciences). IBOS8-1-1 is also virulent to all four cultivars and is characterized by strong aggressiveness against Chubu 32. Norin 29 and Koshihikari have low levels of partial resistance to isolates Y93-245c-2 and IBOS8-1-1, whereas Chubu 32 holds a high level of partial resistance to Y93-245c-2 but a low level of

resistance to IBOS8-1-1. Both isolates were put on the same plate oatmeal agar (30 g ground oatmeal, 5 g sucrose, 15 g agar in 1 l of water) and cultured in the dark at 22°C for 1 week and thereafter at 20°C for 3 weeks under fluorescent light. The perithecia produced at the junction of the mycelia from both of the isolates were picked up, and only asci with ascospores were picked from the perithecia and transferred to an agar plate. Single, germinated ascospores were randomly isolated and stored as F_1 progeny isolates.

Evaluation of partial resistance

To evaluate partial resistance in the RILs and F_3's to blast, upland nursery trials were carried out during 2001 and 2002, respectively, at the National Agricultural Research Center for Tohoku Region in Omagari, Akita. The method of evaluation followed that of Zenbayashi et al. (2002). Because partial resistance to blast can be detected only in the compatible combinations between complete resistance and blast isolates, we confirmed that the blast isolates in the field were virulent to the RILs, F_3's and parental cultivars before the evaluation of partial resistance in each line (data not shown). In the field trials, the disease severity of each line was estimated on the basis of the percentage of diseased leaf area (%DLA) according to the scale of Asaga (1981). After assessing disease three times from the middle to the end of July, we classified the lines as resistant (slightly infected: Chubu 32), susceptible (severely damaged: Norin 29 and Koshihikari) or moderate (segregating disease severity in the line or moderately damaged).

The partial resistance of F_3's against Y93-245c-2 was evaluated as follows: Ten seedlings of each line and the cultivars Chubu 32 and Koshihikari (as a substitute for CSSL) were grown until the 6th-leaf stage in seedling soil with chemical fertilizer containing 12/15/10 g of NPK in 20 kg in a greenhouse. The average temperature during the growing period was 25°C. Both blast isolates Y93-245c-2 and IBOS8-1-1 were incubated on oatmeal agar plates in the dark at 22°C for 2 weeks. The plates were then exposed to fluorescent light for 3 days after the hyphae were removed from the agar surface by brushing. Conidia were then detached by gently rubbing the agar surface with a paintbrush after adding sterilized water containing 0.02% Tween 20 (ICN Pharmaceuticals, Costa Mesa, CA, USA) to prepare a conidial suspension (1.6×10^5 conidia/ml). The seedlings were inoculated by spraying 2.4 ml of the conidial suspension per seedling. The inoculated seedlings were immediately placed in a growth chamber with 100% humidity at 25°C for 16 h to promote infection and then grown in a greenhouse at 20°C. Ten days after the inoculation, the %DLA was measured, and each F_3 line was evaluated as resistant, susceptible, or moderate according to the criteria described. The segregation data of the RILs or F_3's were analyzed for Mendelian inheritance using the chi-squared method.

The partial resistance of Chubu 32 and Koshihikari against Y93-245c-2, IBOS8-1-1, and F_1 progeny was evaluated as follows: Five seedlings of Chubu 32 and Koshihikari at the 7.0–7.5-leaf age were sprayed with 10 ml conidial suspension (2.0×10^5 conidia/ml) of F_1 progeny. Seven days after inoculation, the mean %DLA (%MDLA) of test cultivars was caluculated for each F_1 progeny. The progeny isolates were classified into two virulence types (I-type, Y-type) on the basis of the disease reactions of the plants. The I-type has almost the same degree of strong aggressiveness on both cultivars, similar to IBOS8-1-1, whereas the Y-type is strongly aggressive only on Koshihikari and is weakly aggressive on Chubu 32, similar to Y93-245c-2. We considered that the isolates with a significant difference between the %MDLA on the two cultivars in a *t*-test ($P = 0.05$) were the Y-type, and those with no significant difference were the I-type. The segregation data of the isolates were also analyzed for Mendelian inheritance using the chi-squared method.

Construction of genetic map

The two genetic maps were constructed using the RILs and F_3's. We mapped *Pi34* on each genetic map and examined whether *Pi34* was located in the same region. The two genetic maps on chromosome 11 were composed of restriction fragment length polymorphism (RFLP) and single sequence repeat (SSR) markers. Plant DNA was extracted from fresh or frozen leaf tissues of individual plants according to a cetyltrimethyl-ammonium bromide (CTAB) method (Murray and Thompson 1980). Electrophoresis, Southern blotting, probe labeling, detection of RFLP, polymerase chain reaction (PCR), and electrophoresis for detecting SSR markers were performed as previously reported (Zenbayashi et al. 2002). The cDNA clones for RFLP probes (Harushima et al. 1998) were provided by the Rice Genome Research Program and Cornell University (McCouch et al. 1988), SSR primer-pair sequence information was obtained from the genetic map of Cornell (Temnykh et al. 2000). MAPMAKER/EXP version 3.0 (Lander et al. 1987) was used to establish the genetic maps and to locate *Pi34* on them.

Results

Mapping of *Pi34* on the genetic map

If the partial resistance in Chubu 32 is controlled by a single major gene (*Pi34*), the expected segregation ratio is 63:2:63 (resistant:segregating:susceptible) in the 139 RILs and 1:2:1 in the 32 F_3's. In the field trial, 70 partially resistant, 66 susceptible, and 2 segregating lines were observed in the RILs, and the F_3's were classified into 10 partially resistant, 12 susceptible, and 10 segregating lines. The calculated chi-squared probabilities for the RILs and F_3's with segregation ratios were both above 0.05, indicating good fits to the expected ratios (Table 1).

Mapping the partial resistance gene *Pi34* on the two genetic maps indicated that *Pi34* is located between markers C1172 (and three co-segregating markers RG702,

Table 1. Segregation for partial resistance in recombinant inbred lines from the cross between Chubu 32 and Norin 29 and F_3's in upland nursery trials

Population	No. of lines			Expected segregation ratio	χ^2	*P*
	Resistant	Segregating[a]	Susceptible			
RILs	70	2	66	63:2:63	0.14	0.93
F_3's	10	10	12	1:2:1	4.75	0.09

RILs, recombinant inbred lines; F_3's, F_3 lines from a cross between Chubu 32 and a chromosome segment substitution line

[a] Resistant, susceptible, and moderate infected plants are mixed in the lines

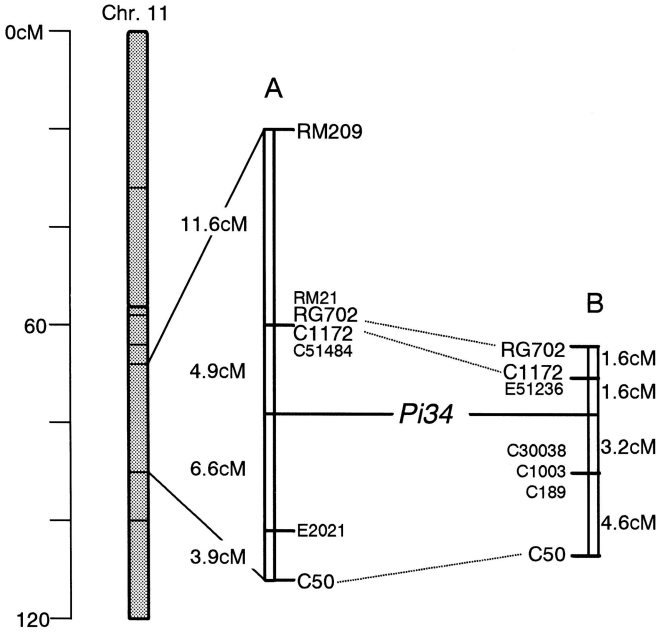

Fig. 2. Two genetic maps of the *Pi34* locus determined by MAPMAKER/EXP version 3.0 on the long arm of rice chromosome 11. The full-length chromosomal genetic map (*left*) is based on a high-resolution genetic map previously reported (Harushima et al. 1998). Maps A and B were constructed with the segregation data for markers in Norin 29/Chubu 32 RILs and in F_3 lines from the CSSL/Chubu 32 cross, respectively. Markers denoted by *C* or *G* numbers are restriction fragment length polymorphism (RFLP) markers obtained from RGP, and *RG702* was obtained from Cornell University. Microsatellite markers are designated *RM*

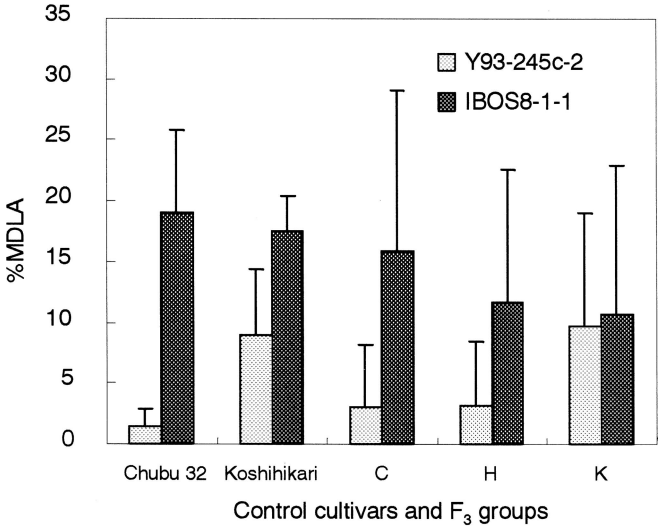

Fig. 3. Partial resistance of the three groups of F_3 lines from the cross between CSSL and Chubu 32 classified by the genotypes of the region between the markers C1172 and C189 to *Magnaporthe grisea* isolates Y93-245c-2 and IBOS8-1-1. *C*, *H*, and *K* indicate genotypes Chubu 32 homozygous, heterozygous, and Kasalath homozygous, respectively. *Vertical bars* indicate standard deviations

C51484, and RM21) and E2021 on the RILs map (Fig. 2A), and between C1172 (and co-segregating marker E51236) and C30038 (C1003, C189, and C6) on the map of the F_3's (Fig. 2B). This result indicates that the QTL conferring the partial resistance to blast in Chubu 32 can be detected and mapped as one gene, *Pi34*, even if different populations are used. Not enough markers were used to map *Pi34* precisely, but each genetic map seemed to be fairly accurate because the chromosomal location and order of the markers are consistent with those of the high-resolution genetic maps reported by Harushima et al. (1998) and McCouch et al. (1988).

Correlation between genotypes and phenotypes in the F_3 lines to M. grisea isolates Y93-245c-2 and IBOS8-1-1

In the F_3's, the genotype of the region including *Pi34* is known because we already analyzed them while mapping *Pi34*. We tested whether the genotypes of the *Pi34* region of the F_3's are correlated with their phenotypes (resistant, susceptible, or moderate as described earlier) with Y93-245c-2 and IBOS8-1-1. If *Pi34* works as the resistance gene to Y93-245c-2, the F_3's with *Pi34* would be resistant to Y93-245c-2 and the lines without it would be susceptible. In contrast, regardless of whether each line has *Pi34*, all the F_3's are expected to be susceptible to IBOS8-1-1. The F_3's were classified into three groups – Chubu 32-type ($Pi34^+$, $n = 11$), Kasalath-type ($Pi34^-$, $n = 12$), heterozygous ($n = 9$) – according to the genotypes between the markers C1172 and C189. When the lines were inoculated with *M. grisea* isolate Y93-245c-2, the Chubu 32-type group had lower %MDLA than did the Kasalath-type (Fig. 3). Although individual seedlings in the line heterozygotes varied in %DLA, the heterozygotes had almost the same %MDLA (3.16 ± 5.22)

as the Chubu 32 type. A similar result was obtained in the same population in an upland nursery trial (data not shown). In contrast, the %MDLA and the genotypes in the lines were not significantly correlated when they were inoculated with isolate IBOS8-1-1 (Fig. 3). The result suggests that *Pi34* works as a partial resistance gene against Y93-245c-2 but not against IBOS8-1-1.

Table 2. Segregation for Y and I infection types[a] in F_1 progeny from the cross between *Magnaporthe grisea* isolates Y93-245c-2 and IBOS8-1-1

Parameter	Result
F_1 progeny (n = 61)	
Y-type	37
I-type	24
Expected segregation ratio	1:1
χ^2	2.78
P	0.09

[a]Classification of the infection types of F_1 progeny was based on the result of the inoculation test shown in Fig. 4. The I-type has almost the same degree of strong aggressiveness on Koshihikari and Chubu 32 similar to IBOS8–1-1, whereas Y-type is strongly aggressive only on Koshihikari and is weakly aggressive on Chubu 32, similar to Y93–245c-2

Segregation analysis of avirulence in Y93-245c-2 on Chubu 32

We hypothesized that isolate Y93-245c-2 has a gene that confers weak aggressiveness on Chubu 32 because this isolate produce fewer lesions on Chubu 32 than on the susceptible cultivar Koshihikari. For convenience, the gene is called an avirulence gene in this article. To confirm this hypothesis, we investigated the avirulence to Chubu 32 in 61 F_1 progeny isolates from the cross between Y93-245c-2 and IBOS8-1-1. Seedlings of Chubu 32 and Koshihikari were inoculated with each of the 61 F_1 isolates. The inoculation assay revealed that the F_1 progeny consisted of 37 isolates of the Y-type and 24 of the I-type (Table 2). The segregation of the F_1 progeny isolates for the Y- and I-types fit the expected ratio (1:1) based on the hypothesis that Y93-245c-2 has a single avirulence gene against Chubu 32.

Discussion

The segregation analyses for the partial resistance to blast in the F_2 population from the cross between Norin 29 and Chubu 32 (Zenbayashi et al. 2002) and the RILs indicate that a single dominant gene *Pi34* affects the partial resistance to blast in Chubu 32. In this study, we identified the

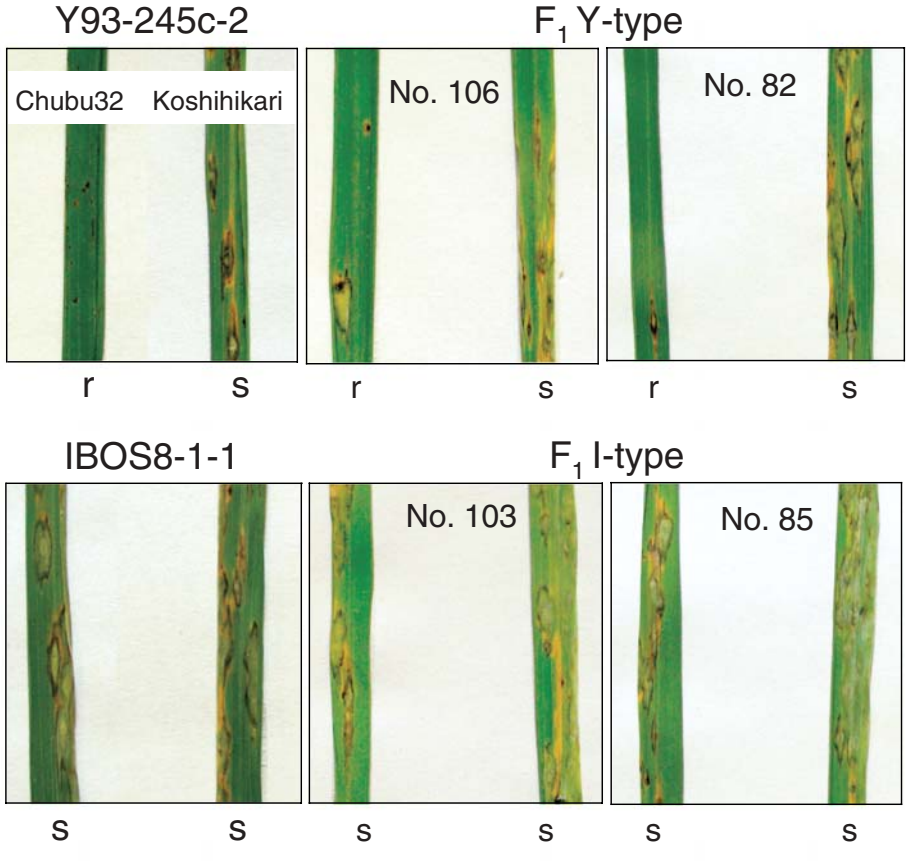

Fig. 4. Host plant interaction phenotypes of rice cultivars Chubu 32 and Koshihikari with *Magnaporthe grisea* isolates Y93-245c-2 and IBOS8-1-1, and their F_1 progeny at 7 days after inoculation. *r*, a few large, spindle-shaped lesions and/or pinpoint necrotic (hypersensitive response) lesions were observed on the inoculated leaves; *s*, a number of large, broad spindle-shaped, water-soaked lesions were observed; *Y-type*, an isolate strongly virulent only to Koshihikari as Y93-245c-2; *I-type*, an isolate aggressively virulent to both Koshihikari and Chubu 32 as IBOS8-1-1

avirulence (weak aggressiveness) gene in *M. grisea* associated with the partial resistance gene *Pi34* by the cross between a unique rice blast isolate with high virulence to *Pi34* and an isolate with high fertility. We found that the partial resistance gene in Chubu 32, *Pi34*, is effective against isolate Y93-245c-2, which has gene *AVR-Pi34* encoding avirulence to Chubu 32. IBOS8-1-1 apparently lost the function of *AVR-Pi34* for some reason. This is a new gene-for-gene relationship between a partial resistance gene in the host cultivar and a gene for aggressiveness in the fungal isolate.

In most genetic studies on partial resistance to blast in rice cultivars, the inheritance of resistance has been polygenic, and some QTL analyses have associated more than one locus with partial resistance (Lin 1986; Wang et al. 1994). However, molecular genetic analyses also have identified a partial resistance to blast that is controlled by single genes, such as *Pb1* and *Pi34*. Does the number of genes conferring partial resistance have some relevance to isolate specificity and durability? Most of the enduring partial resistance observed in the field seems to be polygenic. Whether polygenic partial resistance can be isolate-specific in the field is unknown because no epidemiological evidence has been reported, even though in the QTL analysis by Talukder et al. (2004) of partial resistance to blast after inoculation with race 3 of *M. grisea* most QTLs detected were race-specific (Talukder et al, 2004). In contrast, there are some reports that the partial resistance controlled by a "major" gene, such as in Chubu 32 (Koizumi and Fuji 1995) and rice cultivar St. No. 1 with partial resistance gene *Pif* (Yunoki et al. 1970), varies with the pathogenic isolate. Based on these results, we believe that any isolate-specificity relating to the durability of resistance is determined by the number of genes associated with resistance and has no relation to the nature of the resistance (complete or partial, qualitative or quantitative). Therefore, there is some possibility that *Pi34* breaks down.

The distribution of the blast isolates that possess *AVR-Pi34* is also interesting, but this research is difficult because these field isolates in Japan have low fertility. However, we think that most isolates in Japan have *AVR-Pi34* because rice cultivars with *Pi34* maintain their high levels of partial resistance to blast despite their long history of cultivation as mother plants for breeding in various regions. Why do rice blast isolates without *AVR-Pi34* rarely appear? We believe they are rare because cultivars with *Pi34* are not widely cultivated. The fact that IBOS8-1-1 was isolated from an upland rice cultivar suggests that *AVR-Pi34*$^-$ isolates of *M. grisea* may be present in fields in which the upland rice cultivar with *Pi34* is grown.

We are the first to report that the gene-for-gene relationship applies to a resistance gene associated with disease reduction and an avirulence gene controlling pathogen aggressiveness. The gene-for-gene relationship between host and pathogen is thought to operate not only for complete resistance expressed in incompatible combinations but also for partial resistance in compatible interactions. However, there are few examples so far that clearly show the molecular basis of the physical interaction between the resistance gene in the plant and the avirulence gene in the pathogen. The *Pi34* and *AVR-Pi34* relationship may be a promising system for elucidating the plant-microbe interaction in a resistance phenotype that differs from the hypersensitive response.

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