Evidence of Genetic Exchange by Parasexual Recombination and Genetic Analysis of Pathogenicity and Mating Type of Parasexual Recombinants in Rice Blast Fungus, *Magnaporthe oryzae*

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ABSTRACT

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A selectable marker gene conferring resistance to bialaphos (BI) was introduced into rice blast isolate Y90-71BI and another conferring resistance to blasticidin S (BS) into isolate 3514-R-2BS of *Magnaporthe oryzae* to demonstrate exchange of DNA. Colonies obtained from co-cultures of these two isolates were resistant to both BI and BS and had both resistance genes as shown by Southern blot analysis of their genomic

DNA. Conidia from these BI-BS-resistant isolates had only one nucleus per cell after staining with 4',6-diamidino-2-phenylindole (DAPI). Using flow cytometry, however, these BI-BS-resistant isolates were found to be haploid. Segregation of BI-BS-resistant isolates for pathogenicity (avirulence to virulence) on rice line K59-1 was consistent with a 1:1 ratio, as was segregation for mating type. These BI-BS-resistant isolates were thus apparently derived from parasexual exchange of DNA and the segregation of pathogenicity and of mating type of the parasexual recombinants might correspond to that of the progeny of the offspring of the sexual cross.

Additional keywords: anastomosis, heterokaryosis, *Oryza sativa*.

Magnaporthe oryzae B. Couch (anamorph *Pyricularia oryzae* Cavara), previously known as *M. grisea* (Hebert) Barr (6), causes rice blast disease, which limits rice production worldwide. Resistant cultivars and fungicides usually control the disease. A vast number of breeders have developed numerous cultivars resistant to rice blast. Recently, multilines and cultivar mixtures have been used as biological and ecological methods to eliminate or reduce the need for chemicals to control many cereal diseases (1,3,4,7,14,16–19,21,22,27,28,30,38). In Japan, many multilines of rice varieties with blast resistance have been developed, and fields planted with multilines are increasing year after year (26). However, the potential demise of the stability of the multiline system is a concern because of the potential for new pathogenic variants of the fungus to develop.

Pathogenic variants may arise via sexual mating, mutation, or parasexual recombination. Within Japan, mutation and parasexual recombination seem to have been the two major causes of variation in the pathogenicity of *M. oryzae*, because until now, ascospore production from a cross between Japanese field isolates had never been reported. Multilines, which have diverse host genotypes, are considered to produce more diverse pathogen populations than do a single line (16). Because of the benefits of induced resistance mechanism and stabilizing selection, some researchers presumed that the components of resistant and susceptible hosts toward pathogen were more durable than that of only resistant hosts in the multiline system (3,15,22). Indeed, some multilines that contained resistant and susceptible hosts have been grown

under field conditions in Japan. The multilines which include common hosts of different races in the fields may present an opportunity for the fungus to acquire new virulence genes through parasexual recombination.

Heterokaryosis and parasexual cycle have been reported in the rice blast fungus and other filamentous fungi (8–10,24,25,29,32,33,37). Three processes were observed in parasexual recombination. The first process involves heterokaryosis, and heterozygous diploids occur in the second process. Heterozygous diploids are detected by the size of conidia, nuclear size, and DNA content of a nuclei. The final process is characterized by segregation and recombination during mitosis. After transient diploidy, migrating nuclei in fused cells are divided by mitotic recombination and new segregants occur. Hyphal anastomosis is a prerequisite for the establishment and development of heterokaryons. It consists of fusion between vegetatively compatible hyphae, translocation of one or more nuclei into fused cells, and compatible heterokaryotic state. Auxotrophic mutants were used to study vegetative compatibility, heterokaryosis, and parasexuality in *P. oryzae* (5,8,9). However, direct evidence of the genetic exchange of DNA through parasexual recombination has not been demonstrated. Many researchers presumed that pathogenicity changes in the rice blast fungus were caused by parasexual recombination (24,32), but the pathogenicity of parasexual recombinants of *M. oryzae* had not been analyzed genetically.

In this paper, selectable antibiotic-resistance genes were used to demonstrate genetic exchange of *M. oryzae* through parasexual recombination. We determined the ploidy and the number of nuclei per cell in a parasexual recombinant using flow cytometry and 4',6-diamine-2-phenylindole (DAPI)-staining of nuclei to examine if nuclei of parasexual recombinants were stable. We also genetically studied segregation of both the pathogenicity and mating type in the parasexual recombinants.

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MATERIALS AND METHODS

***M. oryzae* isolates.** We introduced plasmid pBARKS1, a bialaphos (BI)-resistance vector containing Ignite/blast-resistance (*bar*) gene (2), into Y90-71 (*Mat 1-1*, race 102), a field isolate collected from Yunnan Province in China in 1990 and produced a BI-resistant transformant, Y90-71BI. Plasmid pBF101, a blastidicin S (BS)-resistance vector containing *BSD* (12), was introduced into laboratory isolate 3514-R-2 (*Mat 1-2*, race 136), a progeny of a cross between a field isolate, CHINOS37-1-3, collected from a lowland rice from Yunnan Province in China and identified as race 136 by N. Hayashi and field isolate FR-10, race 136, collected from South America by H. Kato, and produced a BS-resistant transformant, 3514-R-2BS. Plasmid pBF101 was provided by T. Kamakura. Y93-164a-1 (*Mat1-1*, race 132) and Y93-245c-2 (*Mat1-2*, race 137), collected from Yunnan Province in China in 1993, were used as tester isolates to determine mating type. Y90-71, 3514-R-2, Y93-164a-1, and Y93-245c-2 were hermaphroditic, female fertile, and pathogenic to rice.

Plant materials. For genetic analysis of avirulence in paraxial recombinants, one rice cultivar and one rice line were used in this study; Hattan 3 is a Japanese rice cultivar and line K59-1 is one of the F₃ lines from the cross between K59 and Norin 3 (34). Norin 3 is considered to lack the resistance genes contributing to the resistance against the parental isolates used in this study. Line K59-1 has a rice blast resistance gene (*Pit*).

Transformation. Protoplasts of *M. oryzae* were prepared by using Novozyme 234 as described by Yelton et al. (36). Transformation of the fungus followed the method of Kimura et al. (12) with modifications. Plasmid pBARKS1 was linearized at the *Eco*RI recognition site, and plasmid pBF101 was linearized at the *Hind*III recognition site, before the transformation. To select stable transformants, 5, 10, or 20 μ l of the protoplast suspension was then added to 10 ml of fresh YG1/2SC medium in collagen-coated dishes (100 mm diameter) and then incubated at 28°C. After 3 h of incubation, the selective reagent was added to the medium (BI at 800 μ g/ml for isolate Y90-71 or BS at 100 μ g/ml for isolate 3514-R-2) and further incubated for 2 days. The incubation medium was replaced every day with fresh medium with a higher concentration of the antibiotic, placing surviving colonies under the most appropriate selective pressure (isolate Y90-71 was incubated in YG medium containing BI at 200 to 800 μ g/ml, and isolate 3514-R-2 was incubated in YG medium containing BS at 50 to 100 μ g/ml). BI- or BS-resistant colonies thus formed were picked and transferred to potato dextrose agar (PDA) plates containing either BI (800 μ g/ml) for isolate Y90-71 or BS (100 μ g/ml) for isolate 3514-R-2 using forceps and further analyzed.

Coculturing. Y90-71BI and 3514-R-2BS were cocultured in 30 ml of liquid yeast extract medium (5 g of yeast extract and 1 liter of distilled water) in 100-ml flasks for 7 days, and mycelia developed were then transferred to oatmeal agar plates (50 g of oatmeal, 20 g of sucrose, 15 g of agar, and 1 liter of distilled water). After incubation for 7 days at 25°C, hyphal fragments were collected from each mycelial mat and transferred to PDA plates containing both BI (800 μ g/ml) and BS (100 μ g/ml). A single colony was selected from each PDA plate. A single monoklonal isolate was then isolated from each double antibiotic-resistant colony.

DNA isolation and Southern blot analysis. DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method (20) with modifications (35). DNA samples were digested with *Eco*RI (Takara Shuzo Co., Ltd., Otsu, Japan), fractionated in 0.7% agarose gel, and transferred to a Hybond-N+ membrane (Amersham, Buckinghamshire, UK). Labeling of probe DNA and hybridization were performed with the enhanced chemiluminescence (ECL) direct nucleic acid labeling/detection system (ECL system: Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's recommendations and instructions.

***Pot-2* repetitive sequence based-polymerase chain reaction (rep-PCR).** *Pot-2* rep-PCR was carried out as described by George et al. (11). Amplified PCR products were separated by gel electrophoresis in 1.0 % gels in 0.5× Tris-borate-EDTA buffer.

Number of nuclei per cell. Mycelia and conidia were collected by washing a culture growing on oatmeal agar. The mycelia or a spore suspension were collected by centrifugation (3,000 × *g*, 10 min). Conidial suspension was adjusted to 10⁷ conidia per ml. A 1- μ l sample of the suspension was stained with DAPI (final concentration of 1 μ g/ml in Vectashield [Vector Laboratories, Burlingame, CA]). Nuclei were observed with fluorescence microscopy using ultraviolet light.

Ploidy. Protoplasts of parental isolates and BI-BS-resistant isolates were suspended in buffer A of the Partec high resolution staining kit for plant DNA analysis (Partec GmbH, Germany). After we added 0.2 ml of buffer A, the suspension with nuclei was filtered through a 30- μ m mesh filter. About 1.5 ml of buffer B from the kit was added, and the fluorescence intensity of the nuclei was immediately measured with the Partec PA flow cytometer.

Pathogenicity. *M. oryzae* isolates were cultivated on oatmeal agar plates at 26°C. After 11 to 12 days of subculture, aerial mycelia were washed off by gentle rubbing with a water-soaked paintbrush. The colonies were then placed under near-ultraviolet light (360 nm, 18 W) at 21°C for 3 to 4 days to induce sporulation. The conidia were scrapped and suspended in water containing 0.01% Tween 20. The suspension was filtered through three layers of gauze mesh and adjusted to 1 × 10⁵ conidia per ml. Hattan 3 and line K59-1 were inoculated at the fourth-leaf stage by spraying 30 ml of a spore suspension (10⁵ conidia per ml) of the fungus with Diaspray (FURUPLA Co., Tokyo, Japan). Inoculated plants were immediately placed in a dew chamber at 24.5°C for 20 h and then transferred to a greenhouse at 26 to 28°C. Six to seven days after inoculation, the pathogenicity of *M. oryzae* isolates was determined.

Mating type. Mating type was determined by crossing according to the procedure described by Yaegashi (31), using the testers.

RESULTS

Parasexual exchange. A total of 302 colonies resistant to both antibiotics were obtained. All grew on BI-BS PDA (Fig. 1).

The presence of the two antibiotic-resistance genes in BI-BS-resistant isolates was then confirmed by Southern blot analysis. Forty-nine BI-BS-resistant isolates hybridized with both the probe *bar* gene and probe *BSD* (Fig. 2), and 253 with either the probe *bar* gene or probe *BSD*.

***Pot-2* rep-PCR of BI-BS-resistant isolates.** Amplified bands ranged from more than 0.6 to 4.8 kb in length in the parental isolates and BI-BS-resistant isolates (Fig. 3). *Pot-2* rep-PCR fingerprints distinguished the parental isolates and fingerprint patterns of BI-BS-resistant isolates composed of bands from parental isolates.

Number of nuclei per cell. Hyphal cells were too small to distinguish each cell and we were unable to determine the number of nuclei per cell. DAPI staining showed the presence of three nuclei in each conidium of parental isolates and BI-BS-resistant isolates. Conidia of the rice blast fungus usually consisted of three cells. The conidia of parental isolates and BI-BS-resistant isolates were mostly uninucleate (Fig. 4). Some had two nuclei, but these seemed to be in the process of cell division.

Ploidy. The flow cytometry histograms of BI-BS-resistant isolates and the parental isolates were nearly identical. They had three peaks, but the peak near zero resulted from debris in the nuclear DNA. The peaks represented intensity of fluorescence. The peak positions corresponded to the ploidy level; the first peaks represented the haploid and the second represented the diploid. The position of the first peak was about 65.2 in Y90-71. The first peaks were located at 67.58, 61.73, 61.85, and 64.93 in

3514-R-2BS, BI-BS-resistant isolates (BIS1, BIS132, and BIS209). The second peaks were located at 121.85, 123.93, 118.25, 117.03, and 119.27 in Y90-71BI, 3514-R-2BS, BIS1, BIS132, and BIS209. The second peak was located about the same distance from the first peak in each pattern, indicating that cells were in the G2 period in the cell cycle. From our results, the ploidy of BI-BS-resistant isolates was haploid, as was the case with the parental isolates.

Segregation of avirulence/virulence in BI-BS-resistant isolates. Y90-71 has *AvrHattan3* and was avirulent on cv. Hattan 3, but it was virulent on line K59-1 carrying *Pit*. Isolate 3514-R-2 has *AvrPit* and was virulent on Hattan 3, but it was avirulent on line K59-1. Inoculation of Hattan 3 with the 49 BI-BS-resistant isolates revealed that 12 isolates were avirulent and 37 were virulent. A chi-square value of 12.76 showed that the segregation was not consistent with a 1:1 ratio (Table 1). Inoculation of line K59-1 with the 49 BI-BS-resistant isolates yielded a segregation ratio of 24 avirulent to 25 virulent isolates. A chi-square value of 0.02 indicated that the segregation was consistent with a 1:1 ratio.

Segregation of mating. A total of 21 of the 49 BI-BS-resistant isolates were of *Mat1-1* and 28 were of *Mat1-2*. A chi-square

value of 1.00 indicated that segregation was consistent with a 1:1 ratio ($P = 0.3$ to 0.5).

DISCUSSION

The generation of genetic diversity in plant pathogens is a critical problem for the control of plant diseases. One of the significances of parasexuality in asexual filamentous fungi is to produce genetic diversity (37). We have provided the first direct evidence on genetic exchange of DNA through parasexual recombination in *M. oryzae* by using two different antibiotic-resistance

Fig. 3. Agarose gel electrophoresis of *Pot-2* rep-PCR fingerprint patterns from genomic DNA of parental isolates and BI-BS-resistant isolates. BI, Y90-71BI (parent); BS, 3514-R-2BS (parent); the lanes 1 to 6, BI-BS-resistant isolates (lane 1, BIS71; 2, BIS110; 3, BIS112; 4, BIS116; 5, BIS120; and 6, BIS122).

Fig. 1. Colonies of parental isolates and BI-BS-resistant isolates on potato dextrose agar (PDA) and selective medium. **A**, PDA; **B**, PDA with BI (800 µg/ml); **C**, PDA with BS (100 µg/ml); and **D**, PDA with BI (800 µg/ml) and BS (100 µg/ml). 1, Y90-71BI (parent); 2, 3514-R-2BS (parent); and 3 and 4, BI-BS-resistant isolates (BIS1, BIS2).

Fig. 4. 4',6-Diamidino-2-phenylindole (DAPI) staining of conidia of *Magnaporthe oryzae*. Y90-71BI (parent), 3514-R-2BS (parent), and BIS2 (BI-BS resistant isolate). Scale bar indicates 10 µm.

Fig. 2. Southern blot analysis of parental isolates and BI-BS-resistant isolates. Total genomic DNA was digested with *EcoRI*. Electrophoresed DNA digests were hybridized with the **A**, probe *bar* gene **B**, probe *BSD*. BI, Y90-71BI (parent); BS, 3514-R-2BS (parent); and BI-BS-resistant isolates (BIS1, BIS2, BIS3).

genes. BI-BS-resistant isolates proved to have different phenotypes from the parental isolates in virulence and mating type.

A majority (85%) of BI-BS-resistant isolates had, however, either of the two antibiotic-resistance genes; they did not originate from parasexual recombination. They seem to have acquired resistance through either mutation or cross feeding. BS is used as a fungicide to control rice blast, and BS-resistant mutants have been known in nature. We considered that the isolates were able to acquire resistance to this fungicide. BI is used as a herbicide, and in nature, BI-resistant mutants have not been known in this fungus. However, the fungus could have acquired resistance to the herbicide. Even though mutation to pesticides is considered to be involved in BI-BS-resistant isolates, its rate seems too high. Cross feeding possibly contributed to the phenomenon also.

The hyphal cells of filamentous fungi are either uninucleate or multinucleate. It was reported that hyphal and conidial cells of *P. oryzae* are predominately uninucleate and haploid (H. Saito and S. Kato, unpublished data). Yamasaki and Niizeki (32) reported that the majority of cells in conidia, germ tubes, mycelia, and conidiophores of *P. oryzae* are uninuclear, based on results of nuclear staining with Giemsa's solution, basic fuchsin, acetoorcein, or aceto-carmin.

The ploidy level in fungal cells has in the past been determined by quantifying the DNA with a colorimetric assay such as the Feulgen reaction, by physical methods such as meiotic chromosome counts, and by spore size. Flow cytometry is a convenient method for estimating the amount of DNA in a nucleus and for determining ploidy in fungi, plant, and mammal cells. To our knowledge, this study is the first report on the determination of ploidy of parasexual recombinants in *M. oryzae* by flow cytometry. The conidia of the parental isolates and of BI-BS-resistant isolates were almost always uninucleate and had the same ploidy. These results suggested that the isolates derived from parasexual recombination were stable as were the parental isolates.

Yasuda et al. (35) reported that the segregation of avr/vir in the progeny derived from crossing Y90-71 and 3514-R-2 on Hattan 3 and on line K59-1 (data not shown) fitted a 1:1 ratio. The discrepancy in the segregation of virulence to avirulence of BI-BS-resistant isolates on Hattan 3 from the 1:1 ratio was assumed that the antibiotic-resistance genes and genes for virulence to Hattan 3 might be linked as a result of the transformation. Another reason was that the segregation of virulence of the parasexual recombinants in the rice blast fungus was not corresponding to that of sexual progeny. However, from the results of the segregation in virulence on K59-1 and in mating type, the genetic characteristics of parasexual recombinants were shown to segregate as does the sexual progeny. Genetic studies of parasexual recombinants are further needed.

Heterokaryon and parasexuality have been known to occur in laboratory cultures of the rice blast fungus (8–10), and studies on lineage of isolates from the Indian Himalayans and Philippines (13,37) suggested the occurrence of parasexual recombination in nature indirectly, but parasexuality under field condition has never been observed. The potential of hyphal fusion was considered to be the reason for possible parasexual recombination. Hyphal anastomosis between isolates from the same locality was reported in the rice blast fungus (5,37).

TABLE 1. Segregation of avirulence (Avr) to virulence (Vir) in BI-BS-resistant isolates derived from co-culturing Y90-71BI and 3514-R-2BS

Rice	Parental isolates ^a		Isolates		P value
	Y90-71BI	3514-R-2BS	Avr:Vir	χ^2 1:1	
Hattan 3	R	S	12:37	12.76	<0.01
Line K59-1	S	R	24:25	0.02	0.80–0.90

^a R, resistant reaction; S, susceptible reaction.

Frequency of parasexual recombination is a critical issue, but its exact estimate has not been reported even in laboratory experiments. Methods for efficient production of parasexual recombinants and their selection need to be developed to examine the rate of parasexual recombination in the laboratory. Characteristics concerning the stability and fitness of parasexual recombinants are important for their survive under field conditions. Results from nuclei observation and ploidy determination imply the stability of parasexual recombinants in nature. Their behavior in the field further remains to be studied even though a few reports have dealt with the fitness of parasexual recombinants (23). Parasexuality is thought to be useful for genetic diversification in rice blast fungus populations lacking sexual production. Genetic exchange through parasexual recombination would occur and contribute to the survival under severe condition, in addition to generating a broad pathogenic spectrum against rice cultivars.

A rice multiline having diverse host genotypes in blast resistance gene may support different parasitic genotypes (different races) (16). Our results suggest the occurrence of complex races of the rice blast fungus through parasexual exchange in multiline fields. Their fitness should further be compared in different paddy fields planted under different multiline systems so that the system may operate effectively to control rice blast.

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