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REVIEW
Parasexual Recombination in *Magnaporthe oryzae*

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Abstract

Parasexual recombination is thought to be one of the causes of variation in the pathogenicity of rice blast fungus, *Magnaporthe oryzae*, which breaks down resistant rice varieties. In this study, the virulence and fitness of parasexual recombinants of *M. oryzae* were examined *in vitro* and in the field. According to the results of the genetic analysis of the pathogenicity of parasexual recombinants of the fungus, the segregation ratios of avirulence and virulence among recombinants on rice cultivar Hattan 3 and line K59-1 were consistent with those of the sexual progeny of the fungus. This indicated that the avirulent genes of the parasexual recombinants were segregated in a manner similar to that in sexual reproduction. The fitness of parasexual recombinants derived from co-cultures of parental isolates was also investigated. The recombinants produced intermediate-sized lesions and intermediate numbers of spores between the parents on the host. Plants inoculated with the recombinant also showed intermediate disease severities between the parents in the field. Parasexual recombinants can attack a rice multiline system because their virulence and fitness is inherited from the parents.

Discipline: Plant disease
Additional key words: aggressiveness, fitness, *Pyricularia oryzae*

Introduction

Magnaporthe oryzae B. Couch (anamorph: *Pyricularia oryzae* Cavara), previously known as *M. grisea* (Hebert) Barr[10], causes rice blast disease, which is a serious problem in rice-producing regions. Resistant cultivars and fungicides normally control the disease; however, the emergence of fungicide-tolerant strains of the fungus and concern for environmental pollution by fungicides have made the development of resistant cultivars a priority. Breeders have developed numerous rice blast-resistant cultivars; however, the extensive use of these cultivars has led to the subsequent breakdown of their resistance as new pathogenic variants arise. Several groups have utilized resistant multiline cultivars or near-isogenic lines, mixtures of lines that are genetically uniform except for their disease resistance, to manage cereal diseases, including rice blast[1,5,8,11,26,29,31-35,54,59,60,62,68]. These methods have helped stabilize yield and lowered costs while reducing both disease and pest pressure. However, the usefulness of multilines may be limited when new variants of the pathogen arise.

The gene-for-gene hypothesis is that plants contain single dominant resistance (R) genes, which confer resistance to pathogens with the corresponding avirulence (AVR) gene[17]. R genes in rice blast have been reported and several R genes, such as *Pi-b*, *Pi-ta*, and *Pi9*, have been molecularly cloned[6,51,58]. *M. oryzae* AVR genes have been studied, and some of them have been molecularly characterized[15]. Products of avirulence genes can be recognized by cultivars carrying the corresponding R gene, and changes in the AVR gene that affect the transcription or function of AVR gene products disrupt the resistance of rice cultivars carrying the corresponding R gene. The *AVR-Pita* encoded a zinc-metalloprotease-like protein and expressed during infection and colonization in rice[45]. The protein was important for creating a signal recognized by the host. The gene is located near telomere, and the loss of the end of a chromosome is one mechanism for the appearance of a pathogenic variant[45].

Variation in pathogenicity may be generated by sexual mating, mutation, or parasexual recombination. In Japan, mutation and parasexual recombination are presumed to

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have been the major causes of variation in the pathogenicity of *M. oryzae* because, until now, ascospore production from a cross between Japanese rice field isolates has not been reported.

In this paper, we present analyses of parasexual recombination in *M. oryzae.* Based on our results, we have developed a number of guidelines for preventing the emergence of pathogenic variants in rice fields.

Virulence among parasexual recombinants of *M. oryzae*

Parasexual recombinants of fungi are produced by a parasexual cycle involving the following three steps: first, anastomosis (hyphal fusion) to produce a heterokaryon; secondly karyogamy; and thirdly somatic recombination generating recombinants. Heterokaryosis and parasexuality have been reported in filamentous fungi[12,28,48,56,61,65]. The possibility of parasexual recombination in *M. oryzae* was first suggested by Yamasaki and Niizeki[64], who observed nuclear behavior in anastomosis and obtained variants by pairing two different auxotrophic strains. Genovesi and Magill[20] showed that auxotrophic recombinants could be produced by pairing different auxotrophic parental isolates. Fatemi and Nelson[16] also paired different isolates and recovered recombinants. Namai and Yamanaka[36,37] obtained pathogenic variants by the pairing-inoculation of different pathogenic isolates on both agar media and rice leaves, indicating that the variants were produced by the parasexual cycle. Thus, most researchers presumed that changes in the pathogenicity of rice blast fungus were caused by parasexual recombination[36,37,46]. However, the pathogenicity of parasexual recombinants of *M. oryzae* has not been investigated genetically.

Noguchi et al.[40,42] examined the segregation of parasexual recombinants for pathogenicity (avirulence or virulence) on a rice cultivar and line. Two rice blast isolates, a field isolate Y90-71 (*AvrHattan3, Mat1-1*, race 102), which was collected from Yunnan Province in China in 1990, and a laboratory isolate 3514-R-2 (*AvrPit*, *Mat1*-2, race 136), were used as parental isolates. A bialaphos (BI)-tolerant plasmid was introduced into Y90-71 to produce BI-tolerant transformant Y90-71BI[23]. A blasticidin S (BS)-tolerant plasmid was introduced into 3514-R-2 to produce BS-tolerant transformant 3514-R-2BS. Parasexual recombinants were obtained by co-culture with Y90-71BI and 3514-R-2BS in a liquid yeast extract medium. After one week of incubation at 25°C, aerial mycelia were collected and transferred to potato dextrose agar containing BI and BS. Monoconidial isolates tolerant to both antibiotics were isolated and named BI-BS-tolerant isolates. In the results of the examination of the BI-BS- tolerant isolates for the presence of both the antibiotic- tolerance genes by southern blotting using the probes of BI and BS-tolerant genes, forty-nine of the BI-BS-tolerant isolates were hybridized with both probes. The isolates were further analyzed for virulence on the japonica-type cultivar Hattan 3, which has *Piks* as a resistant gene, and line K59-1, which is an F3 line from the cross between K59 and Norin 3[66] possessing resistance gene *Pit* (Table 1). Y90-71 with *AvrHattan3* was avirulent on cv. Hattan 3 but virulent on line K59-1 carrying *Pit*. In contrast, 3514-R-2 carrying *AvrPit* was virulent on Hattan 3 but avirulent on line K59-1. The 49 BI-BS-tolerant isolates that were produced as parasexual recombinants showed a segregation ratio of 12 avirulent to 37 virulent isolates in the results of the inoculation to Hattan 3. In comparison, the segregation ratio was 24 avirulent to 25 virulent isolates on line K59-1. Yasuda et al.[66] 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 fit a 1:1 ratio. The

Table 1. *Magnaporthe oryzae* isolates and plant materials used in this study

Isolates, plant materials	Relevalent properties	Source of references
Magnaporthe oryzae isolates		
Y90-71BI	*BI*^{r}, *AvrHattan 3*, *Mat 1-1*, race 102	40
3514-R-2BS	*BS*^{r}, *AvrPit*, *Mat 1-2*, race 136	40
NAO-02	race 133.1	41
TH77-1	race 047.0	41
KZB	race 177.1	41
Plant materials		
Hattan 3	Japonica-type cultivar, *Piks*	66
line K59-1	F3 line from the cross between K59 and Norin 3, *Pit*	66
Aichiasahi	Japonica-type cultivar, *Pia*	
Kanto 51	Japonica-type cultivar, *Pik*	
Fukunishiki	Japonica-type cultivar, *Piz*	
Akiyutaka	Japonica-type cultivar, *Pik*, *Piz*	

Table 2. Segregation of avirulence of the sexual progenies and parasexual isolates in Hattan 3 and K59-1

	Sexual progenies		Parasexual recombinants [a]			
Rice plants	A[b]	V	A	V	χ^2 value[c]	P value[d]
Hattan 3	21	49	12	37	0.47	0.70-0.50
K59-1	43	27	24	25	1.82	0.20-0.10

[a]: Sexual progenies and parasexual isolates were produced by crossing and co-culture of the isolates Y90-71BI and 3514-R-2BS, respectively.
[b]: A=avirulence,V=virulence. Pathogenicity was estimated 6 to 7 days after spraying conidial suspension on Hattan 3 and K59-1 seedlings.
[c,d]: expected ratio; the ratio of avirulent to virulent in parasexual isolates on Hattan 3 and K59-1 was comparable with that in sexual progeny.

Table 3. Length of lesions produced on leaves of rice cultivars by the parents and the variant

		Lesion length(mm)[a]			
Isolates	(race)	Aichiasahi (*Pia*)	Kanto 51 (*Pik*)	Fukunishiki (*Piz*)	Akiyutaka (*Pik,Piz*)
NAO-02	(133.1)	24.8±7.9a[b]	22.4±5.7a	nd[c]	nd
TH77-1	(047.0)	13.6±3.9b	nd	11.7±3.3a	nd
KZB	(177.1)	18.8±4.8ab	12.5±5.2b	12.8±5.7a	16.8±5.5

[a]: Inoculation at the 6 th-leaf stages with the blast fungus was performed with press-injuring method. After 21days of inoculation, lesion length was measured[38].
[b]: The mean and standard deviation (mean±SD)were calculated for 4 -12 lesions of each isolate.
Within columns, values followed by the same letter are not significantly different (Tukey's test; P<0.05).
[c]: nd, not determined due to incompatible combination.

reason why the segregation of avir/vir of the BI-BS-tolerant isolates on Hattan 3 did not match a 1:1 ratio should be the linkage of the antibiotic-tolerance genes and virulence genes as a result of the transformation. To examine whether the segregation of the virulence of parasexual recombinants matched that of sexual progeny, Y90-71BI and 3514-R-2BS were crossed as described by Yaegashi and Kobayashi[63] to produce BI-BS-tolerant sexual progeny. In the results, the avr/vir segregation ratios of the virulence of 70 BI-BS-tolerant sexual progeny on Hattan 3 and K59-1 were 21:49 and 43:27, respectively. Chi-square analysis indicated that the avr/vir ratios of the parasexual isolates on Hattan 3 and line K59-1 were almost consistent with those of the sexual progeny, although the P value was low in K59-1 (Table 2). Thus, our results suggest that variation in pathogenesis among the parasexual recombinants of rice blast fungus corresponds with that among sexual progeny.

Fitness of the parasexual recombinants

The success of new pathogenic variants in the field depends on their fitness compared to the prevalent races in the area. Many researchers have postulated that changes in pathogenicity occur through parasexual recombination; yet few reports have dealt with the fitness of parasexual recombinants in the field[38,39]. Fitness was defined by Crow[13] as the combined ability of an organism to survive and reproduce. The fitness of phytopathogens can be estimated using characteristics such as reproductive rate, infection efficiency, lesion size, efficiency of sporulation, and extent of disease (i.e., aggressiveness)[22].

Noguchi et al.[41] examined the fitness of parasexual recombinants of *M. oryzae*. Recombinant isolates (KZA, KZB and KZC) were produced by co-culture with two isolates of *M.oryzae*, NAO-02 (race 133.1, Japan) and TH77-1 (race 047.0, Japan). NAO-02 was virulent on Aichiasahi (*Pia*) and Kanto 51 (*Pik*), but avirulent on Fukunishiki (*Piz*) and Akiyutaka (*Pik,Piz*), because it was carrying *AvrPiz* , but not carrying functional *AvrPia* and *AvrPik*. In contrast, TH77-1 was virulent on Aichiasahi and Fukunishiki, but avirulent on Kanto 51 and Akiyutaka, because it was carrying *AvrPik*, but not carrying functional *AvrPia* and *AvrPiz*. KZB (race 177.1) was virulent on all cultivars in the experiment, because it was not carrying functional *AvrPia*, *AvrPik,* and *AvrPiz*. Statistical analysis showed significant differences in lesion length on cultivar Aichiasahi of the parents, but those of KZB showed no differences to those of the parents 21 days after inoculation. The lesion length of KZB on Kanto 51 was smaller than that of NAO-02, but the lesion length of KZB on Fukunishiki was not statistically larger than that of TH77-1 (Table 3). The number of spores per lesion of the recombinants (KZA, KZB, and KZC) on cultivar Aichiasahi was between those of the parents (Table 4). Statistical analysis showed significant differences between the results for NAO-02 and TH77-1 but no significant differences between the parents and the recombinants, except for TH77-1 and KZA. The ability of lesion elongation and spore production in parasexual recombinants were thought to be inherited from the parents.

To examine the potential ability of parasexual recombinants to cause damage in nature, we investigated blast development caused by a recombinant and parents in the field. In a field cultivated with a single cultivar (Aichiasahi), NAO-02 caused the most severe epidemic, followed by the recombinant, KZB, and then TH77-1 (Table 5). This result corresponded with the experiment on the length of lesions

produced on Aichiasahi by the parent and the parasexual recombinant. While in the field of cultivar mixture, the recombinant caused more severe damage than the parents. This is presumably because the pathogenic recombinant produced lesions on Kanto 51 (*Pik*) and Fukunishiki (*Piz*), the cultivars that neither parent could attack, and the pathogenic recombinant had a similar fitness to the parents in lesion enlargement and spore production on the hosts. The study suggests that the recombinant caused severe damage in the multiline system because they could attack more rice cultivars than parents and had sufficient fitness to cause rice blast.

Mutation is another major mechanism for producing pathogenic variation, and some reports of pathogenic mutation have been published[24,25]. However, the mutants of rice blast fungus have been described to be less or equally aggressive than the original isolate[18]. In contrast, our results indicate that the parasexual recombinants had a level of aggressiveness that was intermediate of the parents; in other words, the aggressiveness of the recombinants was not less than that of the parents. Fujita and Suzuki[19] reported that pathogenic variants of rice blast in paddy fields increased in aggressiveness every year. Thus, new parasexual recombinants could potentially match the fitness of parents and may increase in fitness after several generations in paddy fields.

Table 4. Number of spores on a single lesion on leaves of rice cultivar Aichiasahi produced by the parents and the recombinants

Isolates		Number of spores [a] (x 10^3spores)
Parents	NAO-02	50.2±5.8a[b]
	TH77-1	5.9±1.2b
Recombinants	KZA	43.3±4.8a
	KZB	29.4±4.9ab
	KZC	29.6±8.1ab

[a]: Inoculation of the isolates was performed by press-injuring method on 6 th-leaf-stage plants. Seven days after inoculation, a lesion was cut out and incubated for 24h at 26°C. Spores were suspended in 1ml of sterile water and counted with a hematocytometer[38].

[b]: The mean and standard error (mean±SE)were calculated. Within columns, values followed by the same letter are not significantly different (Tukey's test; P <0.05).

Stability of parasexual recombinant

The stability of the pathogenicity of *M. oryzae* isolates affected successive prevalence in fields[39]. Ou and Ayad[46,47] reported significant pathogenic variation among single-spore isolates of *M. oryzae* from a single lesion and from monoconidial cultures; however, other studies have shown pathogenic stability (i.e., no variants in *M. oryzae*)[4,27]. To evaluate the stability of the pathogenicity of parasexual recombinants, successive inoculation of parasexual recombinant KZB derived from the co-culture of NAO-02 and TH77-1 was performed. After seven successive inoculations with the parasexual recombinants, monoconidial isolates were obtained from leaf lesions on the cultivars Akiyutaka and Aichiasahi, and their pathogenicity was determined. No change in pathogenicity (from race 177.1) was detected in the isolates, indicating that parasexual recombination should produce stable pathogenic variants of rice blast fungus.

Parasexual recombination in nature

Studies of the population structure of this fungus are useful for the breeding of blast-resistant cultivars and have revealed sexual or parasexual recombination events[9]. Several reports on rice blast populations have relied on DNA fingerprinting[7,14,30,49,52]. Population structure studies in China and the Himalayan area, from where the land races of rice derive, indicated genetic diversity, suggesting sexual or parasexual reproduction[67]. This indicates that divergence among host genotypes may be related to the sexual or parasexual reproduction of this fungus. In areas of the United States and Europe, in contrast, simple population structures that appear to reflect clonal reproduction have been observed[43,50]. Don et al.[14] reported the existence of five lineages among rice blast isolates collected before 1960 in

Table 5. Leaf blast severities of a single cultivar and cultivar mixtures in the experimental fields[a]

	The number of leaf blast lesions per a hill (Percentages of diseased hills)[b]	
Spreader Isolates	Aichiasahi	Cultivars mixture
NAO-02	129.7±130.9[c] (100)	5.2±9.6 (51.8)
TH77-1	26.5±9.0 (100)	0.4±1.52 (4.2)
KZB	90.7±33.0 (100)	19.1±23.2 (86.6)

[a]: Trial fields were planted with Aichiasahi or cultivars mixture (Aichiasahi: Kanto 51: Fukunishiki=1:1:1) on 13 May in 1998 and infested with either NAO-02, TH77-1 or KZB on 1 June 1998.

[b]: Severities (the number of leaf blast lesions per hill and percentages of diseased hills) was recorded on 6 July 1998.

[c]: The mean and standard deviation (mean±SD) were calculated.

Japan, whereas only two lineages were detected between 1972 and 1993. Sone et al.[55] classified Japanese *M. oryaze* isolates into five clonal lineages by phylogenetic analysis based on DNA fingerprinting using MGR 586 and pMG6015 microsatellite markers. No relationship could be detected between the lineages and pathotypes. In Japan, asexual reproduction dominates under field conditions because a small numbers of lineages have occurred. However, several multilines and near-isogenic lines have recently been developed and cultivated for rice blast control in some Japanese prefectures[53]. A rice multiline with diverse host genotypes (different resistance genes corresponding to the pathogenicity of the blast pathogen) may lead to different parasitic genotypes and races of rice blast pathogen.

Complex races that can attack various resistance genes have been assumed to be unable to increase in a cultivar mixture because of the costs associated with a lack of avirulence genes[57]. AVR gene products were thought to be important for infection and aggressiveness[15]. For example, the *Ace1* avirulence gene, which confers avirulence towards rice cultivars carrying Pi33, encodes a polyketide synthase/nonribosomal peptide synthetase fusion protein, which is expected to produce a secondary metabolite relating to the penetration of the host.

However, Noguchi et al.[41] suggested that complex races can overcome multiple host genotypes in a multiline system by producing variants that can infect resistant lines through parasexual recombination. The variants had a more complex virulence than the parents and exhibited a level of fitness equal to that of the parent. In particular, in a cultivar mixture, leaf blast caused by the variants was more severe than that caused by either parent. These results suggest that parasexual recombination not only alters pathogenicity but also enhances fitness, such as lesion enlargement and spore production.

For durable usages of rice multilines, the parasexual recombination of different pathogenic genotypes should be prevented. Monitoring of blast race frequency in a multiline cultivated field[3,21,44] were helpful to avoid severe blast development caused by parasexual recombination. Simulation models of leaf and panicle blast development and frequency of races in multilines[2] were useful to determine the time to rotate multiline cultivars. In addition , if the ratio of pathogenic parasexual recombinants in rice fields could be obtained, it should be useful for determining the time for rotation. However, it is difficult to estimate the parasexual recombination rate, even *in vitro*, because of the lack of efficient methods for detecting the parasexual recombinants of the pathogen. Thus, molecular and cell biological techniques should be developed for estimating the parasexual recombination rate in the future.

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