Collection of Blast Fungus from Resistant Rice Varieties and Their Virulence Analysis

Shigehisa Kiyosawa^{1)a}, Yasushi Okinaka^{1)b}, Donna Purba^{2)c} and Takashi Furutani²⁾

¹⁾ Tsukuba Research Laboratories, Kyowa Hakko Co., Tsukuba, Ibaraki 305, Japan

²⁾ Tsukuba International Agricultural Training Centre, Tsukuba, Ibaraki 305, Japan

Summary

Improvement of differential varieties is necessary, as the number of resistance genes used for breeding of blast resistance in rice increased. Establishment of new differential system is essential for population genetics and molecular genetics on host pathogen relationship. Blast fungus was collected from the ear necks of resistant varieties in several prefectures. Varieties from which the fungus was isolated were Kagahikari ("*Pi-i Pi-k*") and Notohikari ("*Pi-z*") in Ishikawa Prefecture, Niigatawase ("*Pi-z*" or "*Pi-a Pi-z*") in Niigata Prefecture, Hatsuboshi ("*Pi-i*") in Fukushima Prefecture, Akiyutaka ("*Pi-k Pi-z*") and Hanahikari ("*Pi-i*") in Yamagata Prefecture, and Yamahikari ("*Pi-ta*") in Mie, Gifu and Nagano Prefectures. These isolates were tested for pathogenicity using twelve differential varieties, and 66 races (genotypes) were obtained. When plural non-random associations in interactions between avirulence loci were recognized in a field, two types of interactions (cross-type and noncross-type) were always found.

Key words : *Oryza sativa*, rice, *Pyricularia oryzae*, blast, nonrandom association, virulence analysis.

Introduction

In Japan, blast is a major disease of rice. Gene analyses of blast resistance of rice varieties were greatly advanced by the systematic studies of Yamasaki and Kiyosawa (1966) and Kiyosawa (1974, 1976). Later, a gene *Pi-sh* was added (Inbe and Matsumoto 1985). To date, 14 resistance genes have been found in varieties in Japan and other countries (Kiyosawa and Ando 1990). On the other hand, the systematical studies on the pathological speciation of blast fungus were begun by Goto *et al.* (1961, 1964) and Kozaka *et al.* (1972). First, a set of twelve differential varieties was selected (Goto *et al.* 1964) and then secondary sets of nine or twelve differential varieties with single resistance genes were selected by Yamada *et al.* (1976) and Kiyosawa (1984b), respectively. These studies brought the development of population genetic studies on host-pathogen relation-

ships, for identification of genotypes of fungal isolates became possible by the application of the gene-for-gene theory.

Seven fungus strains were selected to classify rice varieties based on their resistance patterns (Yamasaki and Kiyosawa 1966). These fungus strains are, however, not sufficient to classify rice varieties based on their genotype for blast resistance, because many resistance genes were introduced to Japanese commercial varieties.

The recent progress in molecular genetics is brilliant, and is approaching the ability to clone disease resistance genes. For this purpose, many preparations are, however, necessary. One of them is the accumulation of resistance genes in a hybrid population of the host, which decreases the labor to cultivate the hybrid population, and to treat DNA for restriction fragment length polymorphism (RFLP) analysis. For example, when an analysis is carried out to know linkage relationships of four genes (A, B, C and D), the minimum number of plants or lines required for molecular genetic studies decreases to about 1/6 in one population including four genes in comparison with six populations including A and B, A and C, A and D, B and C, B and D, or C and D, respectively. This means decrease of labors for harvesting F₂ and F₃ seeds and for extraction of DNA to about 1/6. The method, however, requires many races to recognize four genes individually in a hybrid population.

For these two objectives in population genetics and molecular genetics, many isolates were collected from resistant varieties in several districts. Not only many new races were collected, but also new phenomena were found by tests for pathogenicity of these collections and virulence analysis by Wolfe *et al.* (1975, 1976).

Materials and Methods

Collections were carried out for three years from 1989 to 1991 from fields of resistant varieties. Varieties and locations from which fungus was collected are shown in Table 1 and Fig. 1.

When fungus is collected from varieties with true resistance, virulent races to the varieties are naturally expected to be isolated. In these collections, as many infected necks of ears as possible were collected from the same variety in order to collect many more races.

The isolation of fungus from the collected ear necks was carried out using a special type of microscope

Present Address a: Tsukuba International Agricultural Training Centre, Tsukuba, Ibaraki, 305 Japan

b: Hokkaido University, Sapporo, Hokkaido, 060 Japan

c: Directorate of Food Crops Production Development, JI.AUP 3, Pasar Minggu, Jakarta 12550 Indonesia

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Table 1. Varieties and locations of collection and major helpers

Year	Variety (Gene)	Location	Major helper
1989	Kagahikari ("Pi-i Pi-k") ¹⁾ Notohikari ("Pi-z") ²⁾	Anamizu, Fugeshi, Ishikawa	Norihiro Matsumoto Ishikawa Agr. Expt. Sta.
	Niigatawase ("Pi-a Pi-z") or ("Pi-z") ^{3, 4, 5)}	Shitada, Minamikanbara, Niigata	Yuichi Fujimaki Niigata Agr. Expt. Sta.
1990	Hatsuboshi ("Pi-i") ⁶⁾	Hanawa, Higashishirakawa, Fukushima Ten-ei, Iwase, Fukushima	Akira Hashimoto Fukushima Agr. Expt. Sta.
	Niigatawase ("Pi-a Pi-z") or ("Pi-z") ^{3, 4, 5)}	Deyu, Sasakami, Niigata Imaita, Sasakami, Niigata Kitakanbara, Niigata Yamaya, Kamihayashi, Niigata Iwafune, Niigata Monzen, Murakami, Niigata	Ryoei Harasawa Niigata Agr. Expt. Sta.
	Akiyutaka ("Pi-k Pi-z") ⁷⁾	Sasakami, Mogami, Yamagata Myojin, Mogami, Yamagata Higashimurayama, Yamagata	Yasuhisa Fujita Yamagata Agr. Expt. Sta.
	Hanahikari ("Pi-i") ⁸⁾	Myojin, Mogami, Yamagata	Yasuhisa Fujita Yamagata Agr. Expt. Sta.
1991	Yamahikari ("Pi-ta") ⁹⁾	Karato, Miyagawa, Mie Ema, Miyagawa, Mie Taki, Miyagawa, Mie	Naoaki Tachibana Mie Agr. Expt. Sta.
	Fukuhikari ("Pi-z") ⁹⁾	Kaminoho, Gifu	Kazuhiro Sugimoto Gifu Agr. Expt. Sta.
	Yamahikari ("Pi-ta") ⁹⁾	Mugi, Gifu Ena, Gifu	Kazuhiro Sugimoto Gifu Agr. Expt. Sta.
	Yamahikari ("Pi-ta") ⁹⁾	Kamisato, Shimoina, Nagano Yamamoto, Iida, Nagano	Hidekazu Yamaguchi Nanshin Branch, Nagano Agr. Expt. Sta.

¹⁾ Kiyosawa *et al.* 1978 ; ²⁾ Matsumoto *et al.* 1985 ; ³⁾ Ichikawa *et al.* 1981 ; ⁴⁾ Terasawa and Iijima 1984 ; ⁵⁾ Yaegashi *et al.* 1983 ; ⁶⁾ Komura *et al.* 1977 ; ⁷⁾ Watanabe *et al.* 1981 ; ⁸⁾ Kamei *et al.* 1980 ; ⁹⁾ Mizuno *et al.* 1978.

Fig. 1. Location (prefectures) where fungus was collected.

1. Mie. 2. Ishikawa. 3. Gifu. 4. Nagano. 5. Niigata. 6. Saitama. 7. Ibaraki. 8. Chiba. 9. Yamagata. 10. Fukushima.

Fig. 2. Single spore isolation under a microscope.

The right arm of the slide holder of the mechanical stage was cut off, and a cork elbow (b) was set there. Into the cork elbow, Nichrome wire (c) with a flat tip was inserted. Before use, Nichrome wire is sterilized by fire, and an agar piece (d) with a sharp tip which is cut away from sterilized potato decoction sucrose agar in a test tube is put on the tip of the Nichrome wire. An infected neck (e) mounted on a slide is placed on a cylinder (f) set on the condenser. A single spore on the neck is looked for by moving the condenser adjustment knob and then the condenser is brought down for a while. Then, the agar tip is focused by the coarse adjustment knob on that side of the microscopic field, and a single spore is focused on by the condenser adjustment knob on this side of the microscopic field. By moving the mechanical stage, the agar tip is moved toward the spore, and when both touch, the single spore sticks to the agar tip. This agar tip is transferred back on the culture medium in the test tube.

with a movable condenser and a large central hole (Fig. 2).

Pathogenicity of the isolates was tested and evaluated using Kiyosawa's differential varieties which include twelve varieties with single different resistance genes by a spray method at four to five leaf stages (Kiyosawa 1984 b).

Results and Discussion

1. Pathogenicity test

Results of pathogenicity tests are shown in Tables 2 to 10. Among them, many of isolates collected from the Fukuhikari field did not attack Fukuhikari ("Pi-z" differential variety) (Table 2). It is thought that this field was contaminated by other variety or varieties, because some morphologically different plants were found in the

field.

As a whole, the following points were noticed from these results:

1) Fungal isolates which are not virulent to a differential variety having the same gene as varieties from which the fungus isolated were collected, although most isolates were naturally virulent.

Especially, such isolates were harvested from *Pi-z* varieties^{a)}, Notohikari (Table 4), Niigatawase (Table 5) and Akiyutaka (Table 6). This suggests that expression of the gene *Pi-z* is incomplete on the ear.

2) Predominant races collected from varieties with the same genotypes for blast resistance do not always have the same genotype for pathogenicity.

Races, 041.0, and 047.0 and 043.0, were collected from "*Pi-z*" (Notohikari, Table 4) and "*Pi-a Pi-z*" (Niigatawase, Table 5) varieties. From Yamahikari ("*Pi-ta*²" variety, Table 7), 301.0 (Mie, Gifu and Nagano Prefectures) and 303.0 (Gifu and Nagano Prefectures) were collected as predominant races. This indicates that races from which virulent mutants originated are different among prefectures and even within the prefectures.

a): "*Pi-z*" variety means a variety having only *Pi-z* among genes found to date. *Pi-z* variety means a variety having at least *Pi-z* among genes found to date, in spite of the presence or absence of other genes. The same differentiation is also given to fungal genotypes.

Table 2. Pathogenicity tests of isolates collected "Fukuhikari" (Unknown)

Location	Race									Total
	042.0	043.0	101.0	201.0	300.0	301.0	302.0	303.0	342.1	
Kaminoho	3	14	3	1	5	26	1	2	1	56

Table 3. Pathogenicity tests of isolates collected from Kagahikari ("Pi-i Pi-k")

Location	Race			Total
	017.1	037.1	077.1	
Anamizu	1	28	1	30

Table 4. Pathogenicity tests of isolates collected from Notohikari ("Pi-z")

Location	Race				Total
	003.0	041.0	043.0	077.1	
Anamizu	2	201	4	1	208

Table 5. Pathogenicity tests of isolates collected from Niigatawase ("Pi-a Pi-z")

Location	Race																								Total
	000.0	003.0	006.0	007.0	017.0	033.1	037.1	041.0	043.0	043.1	046.0	047.0	047.1	047.4	053.0	057.1	063.0	067.0	073.1	077.0	077.1	141.0	143.0	147.0	
Shitada I				1				1				17					1								20
Shitada II		2	1	4				11	9		1	110	2					1		1	2	1		1	146
Yamaya				2				3				161						1						4	171
Monzen				1						1	33		1	1											37
Imaita					2	1	3	10	1		2	1							1	6					27
Deyu	1	1					1	34				12				1				10		1			61

Table 6. Pathogenicity tests of isolates collected from Akiyutaka ("Pi-k Pi-z")

Location	R a c e															Total
	007.0	017.0	032.0	033.0	033.1	037.1	057.1	072.0	072.1	073.0	073.1	075.1	076.1	077.1	573.1	
Sasamori						3		1	2	1	13	7	1	96		124
Myojin A					1						21			1		23
Myojin B	1	1		1							2			1	1	7
Myojin C			1		1						1	7		4		14
Myojin D	8						1					1		21		31

Table 7. Pathogenicity tests of isolates collected from Yamahikari ("Pi-ta")

Location	Race							Total
	003.0	103.0	107.0	201.0	300.0	301.0	303.0	
Ema		1		11	1	68		81
Karato						16		16
Ena 1							16	16
Ena 2							14	14
Kamisato				1			20	21
Yamamoto		1	1			6	3	11
Kaminoho	1				2	95	9	107

Table 8. Pathogenicity tests of isolates collected from Hanahikari ("Pi-i")

Location	Race			Total
	002.0	007.0	017.0	
Myojin H	2	12	1	15

Different predominant races were collected from fields where located in adjacent or nearby places (in the same administrative unit, Mogamimachi) (Table 6).

As a whole, the number of races identified is 66.

2. Virulence analysis

It is considered that virulent races predominate by mutation from avirulent races or selective advantage of the virulent strains after cultivation of resistant varieties over a few years. In other words, the frequency of virulent races is largely due to the frequency of resistant varieties. If mutation of an avirulence gene occurs independently of other avirulence genes, the frequency of a genotype having two virulence genes, a^+ and

Table 9. Pathogenicity tests of isolates collected from Hatsuboshi ("Pi-r")

Location	Race									
	003.2	005.0	006.0	007.0	007.2	036.0	047.0	077.1	707.6	Total
Ten-ei	2		3	68	1	1	1	1	1	78
Hanawa		1	1	23						25

Table 10. Pathogenicity tests of isolates collected from the Kanto District (genotypes : unknown)

Location	Race																											Total
	001.0	003.0	003.2	005.0	007.0	007.1	007.2	011.1	013.0	013.1	013.2	017.0	017.2	031.0	031.1	033.1	035.1	037.4	101.0	107.0	107.4	107.6	131.1	137.0	150.0	307.6		
Yoshikawa		3														2											5	
Ichihara	3	5			1	1										1											11	
Kamikoike 1		5	2		2		1																				10	
Kamikoike 2	4	33			1								1														39	
Shimone	1	4							2	1						3			2								13	
Okami	13	15	1		3		1				1	2				2											38	
Hoyodai	6	11	2		3								1	1	2												26	
Nakayama 3	4	20		1	11			1	1			1		2	11	3	1	1		2	1	2	3	1	1	1	68	
Wakakuri		3			4						1																8	

Table 11. Cases in which nonrandom association was observed in virulence analysis

Collection			The number of isolates					χ^2 (Degree of freedom)
Variety	Location	(Gene combination)	ab ^a	a+	+b	++	Total	
1. Niigatawase	Shitada II	Exp.	1.9	10.1	21.1	112.9	146	69.9**
	(Av-a, Av-i) ^b	Obs.	12	0	11	123	146	(3)
2. Niigatawase	Imaita	Exp.	6.7	6.3	7.3	6.7	27	10.8*
	(Av-i, Av-k)	Obs.	11	2	3	11	27	(3)
3. Niigatawase	Imaita	Exp.	3.1	12.0	2.9	10.0	27	8.3*
	(Av-k, Av-z)	Obs.	0	14	6	7	27	(3)
4. Niigatawase	Deyu	Exp.	28.8	7.2	19.2	4.8	60	22.5**
	(Av-i, Av-k)	Obs.	36	0	12	12	60	(3)
5. Akiyutaka	Myojin D	Exp.	2.1	5.9	5.9	17.1	31	31.0**
	(Av-k, Av-z)	Obs.	8	0	0	23	31	(3)
6. Fukuhikari	Kaminoho	Exp.	23.8	11.3	14.3	6.8	56	44.2**
	(Av-a, Av-z)	Obs.	35	0	3	18	56	(3)
7. Fukuhikari	Kaminoho	Exp.	10.6	24.4	6.4	14.6	56	40.7**
	(Av-a, Av-ta)	Obs.	0	35	17	4	56	(3)
8. Fukuhikari	Kaminoho	Exp.	11.5	26.5	5.5	12.5	56	51.5**
	(Av-z, Av-ta)	Obs.	0	38	17	1	56	(3)
9. Unknown	Nakayama 3	Exp.	16.2	7.8	29.8	14.2	68	9.8**
	(Av-a, Av-i)	Obs.	22	2	24	20	68	(3)
10. Unknown	Nakayama 3	Exp.	14.8	9.2	27.2	16.8	68	26.3**
	(Av-a, Av-k)	Obs.	5	19	37	7	68	(3)

a : a is an avirulence at left-hand in () of each row, and b is one at right-hand. + is a virulence, respectively.
b : a and b are avirulence genes in question.

b⁺, should be a⁺ × b⁺. Other genotypes are (1 - a⁺) × (1 - b⁺), (1 - a⁺) × b⁺, and a⁺ × (1 - b⁺) for genotypes, ab, ab⁺ and a⁺b, respectively. Using this principle, Wolfe *et al.* (1975, 1976) examined deviation from inde-

pendent occurrences of double mutations. This analytical method was used in Japan, and various nonrandom associations among virulence genes were found (Kiyosawa 1980, Kiyosawa *et al.* 1993). On the other hand,

Roelfs and Groth (1980) used the distribution of the numbers of virulence genes in propagules to describe the structure of pathogen populations under the assumption that binomial distribution of virulence genes is expected from random association of virulence genes. Thus, they showed various patterns in the distribution that deviated from binomial distribution. Later, Kiyosawa (1984 a) compared both methods, and postulated that the latter is more sensitive to know the deviation from random association, but the former is easier to know the reason of nonrandom association. Therefore, distribution of genotype frequencies in pathogen population on various resistance genotypes were examined by the Wolfe's method (Wolfe *et al.* 1975, 1976).

As shown in Table 11, some nonrandom associations were observed on some resistant varieties; only two types of nonrandom associations were found. One is a pattern which shows larger observed frequencies of genotypes which have only one resistance gene in comparison with expected frequencies from random association (observed a^+b and ab^+ > expected a^+b and ab^+), and the other is the case where a reverse relationship (observed ab and $++$ > expected ab and $++$) was found. Here, a and b are avirulence genes, and a^+ and b^+ are virulence genes. The former pattern was named as cross-type of nonrandom association, because SR/RS reactions were observed on the varieties (genotypes) with corresponding single resistance genes, AB^+ and A^+B . Here, A and B are resistance genes and A^+ and B^+ are susceptibility genes. The other pattern is noncross-type of nonrandom association in which the RR/SS reaction pattern on corresponding host genotypes, AB and A^+B^+ , appears.

Only cases where statistically significant differences were obtained between observed and expected frequencies at the levels of 1 % or 5 %, are shown in Table 11. It was characteristic that both cross- and noncross-types of nonrandom associations were found, when two or more significant differences were observed in the same fields.

In the next paper, we will search for the reason for the appearance of these two types of nonrandom association.

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