Fungicide Sensitivity and Genetics of IBP-Resistant Mutants of *Pyricularia oryzae*

M. Taga, T. Waki, M. Tsuda, and A. Ueyama

Pesticide Research Institute, Kyoto University, Kyoto, 606, Japan.
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ABSTRACT

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Mutants resistant to IBP (*S*-benzyl diisopropyl phosphorothiolate) were obtained from wild-type strains of *Pyricularia oryzae* isolated from finger millet. They were grouped in two classes, highly resistant (HR) and moderately resistant (MR). HR strains grew at 0.2 mM IBP and showed cross-resistance to isoprothiolane (diisopropyl 1,3-dithiolane-2-ylidenemalonate). In contrast, MR strains did not grow at 0.2 mM IBP, and were sensitive to isoprothiolane. The genetic nature of these strains was studied in crosses with sensitive strains. Random ascospore progenies from crosses with HR strains segregated 1:1 for resistance:sensitivity, indicating that a single major gene controls IBP-resistance in HR strains. Tests for allelism among HR strains indicated the existence of a common resistance locus designated as *ibp*. Data from crosses with MR strains suggested that MR is not controlled by a single factor.

Additional key words: fungal genetics, fungicide resistance.

IBP (*S*-benzyl diisopropyl phosphorothiolate, Kitazin P®) is widely used to control rice blast in Japan. It is an organophosphorus thiolate compound having a P-S-C linkage in its chemical structure.

IBP-resistant strains of the blast fungus, *Pyricularia oryzae* Cavara, were first reported by Uesugi et al (8), who obtained resistant mutants in the laboratory by selection among conidia of wild-type isolates. Naturally occurring resistant strains were later isolated from paddy fields in Niigata and Toyama prefecture (1,4); the damage caused by them was not so severe as that caused by kasugamycin-resistant strains of *Pyricularia oryzae*.

Characteristics of these mutants, such as sensitivity to fungicides, biochemical mechanisms of resistance, pathogenicity, etc, have been studied (4,7–9). Their genetic basis, however, has remained unknown, because the methods for genetical analyses in *P. oryzae* were not developed until recently.

Previously, we identified three loci for kasugamycin-resistance by analyzing ascospore isolates from sexual crosses of isolates of *P. oryzae* from finger millet (5,6). Finger millet isolates are much more fertile than rice isolates, and thus are better suited for crossing experiments.

In this paper we report the results of studies on fungicide sensitivity of IBP-resistant mutants obtained from cultures originally isolated from finger millet and the genetic basis of their IBP-resistance.

MATERIALS AND METHODS

Fungicides. The fungicides used were IBP from Kumiai Chemical Industry Co., Ltd., and isoprothiolane (diisopropyl 1,3-dithiolane-2-ylidenemalonate, Fuji-One®) from Nihon Nohyaku Co., Ltd. They were dissolved in ethanol and added to still molten culture medium at the rate of 0.5 ml of ethanol solution to 200 ml of medium. At this concentration, the effects of ethanol on fungal growth were negligible.

Fungal strains. Wild-type strains were either monoconidial isolates from finger millet, *Eleusine coracana* (L.) Gaertner, or monoascospore isolates from crosses between those isolates. IBP-resistant mutants were obtained from wild-type strains in two ways. In the first method, a large number of conidia were mixed with selection medium (Difco potato extract 4 g, sucrose 20 g, and agar 15 g per liter of medium) containing IBP at a concentration of 0.1 or 0.15 mM. After several days of incubation at 27 C, mycelium from each colony growing on the medium was transferred to fresh medium containing the same concentration of IBP to confirm the resistance. Hyphal tips were taken from the resulting colonies to establish stock cultures of IBP-resistant mutants. Of the mutants selected at 0.1 mM IBP, only those that grew at 0.1 mM, but not at more than 0.15 mM, were retained for further study. In the second method, mycelial disks 4 mm in diameter were cut from 1-wk-old colonies on Takahashi-A medium (peptone 10 g, NaCl 5 g, sucrose 10 g, EBIOS® [EBIOS Chemical Industry Co., Ltd., Tokyo] five tablets, agar 15 g per liter of medium), and inoculated on the selection medium amended with 0.2 mM IBP. After incubation for 10 or more days, resistant mutants were obtained from each fast-growing sector as described for the first method. Mutants selected from conidia on 0.1 and 0.15 mM IBP are referred to as M-IBP and C-IBP strains, respectively, and those from sectors as S-IBP strains. In addition, six kasugamycin-resistant mutants, two each of the following genotypes (*kas-1*,+,+), (+,*kas-2*,+), and (+,+,*kas-3*), were used to evaluate fungicide sensitivity. All strains were cultured and maintained on Misato-Hara medium (soluble starch 10 g, yeast extract 2 g, agar 15 g in 1L of medium) at room temperature.

Evaluation of fungicide sensitivity. Sensitivity of the strains to IBP and isoprothiolane was evaluated three ways: by measuring minimum inhibitory concentration (MIC), radial growth of the colony, and percent germination of conidia on the test media. MIC and radial growth of the colony were measured by the plate dilution method. In either case, mycelial disks 5 mm in diameter were cut with a sterilized corkborer from the periphery of 8-day-old colonies growing on Takahashi-A medium at 27 C. Each disk was placed upside down in the center of a 9-cm-diameter petri dish containing 15 ml of rice decoction agar (rice decoction 750 ml [100 g dried rice plant per liter of water], 1/15 M McIlvaine buffer [pH 5.0] 250 ml, sucrose 5 g, and agar 15 g per liter of medium). MIC was estimated at 3 days after inoculation by observing mycelial growth on the medium with 0.1, 0.2, 0.3, 0.4, and 0.5 mM fungicide. Radial growth of colonies was measured at 0.1 and 0.2 mM IBP and 0.1 mM isoprothiolane 4 and 6 days after inoculation. The mean values of two replicates were used to compare the degree of resistance among strains according to the formula: ([diameter of the colony in the test plate − 5 mm] × 100)/([diameter of the colony in the control plate] − 5 mm). Resistance of conidia to IBP was tested in

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9-cm-diameter petri dishes containing 15 ml of test medium (glucose 5 g, agar 10 g per liter of medium) by spreading a drop of conidial suspension ($1 - 10^6$ conidia per 1 ml of deionized water) and then assessing percent germination after 24 hr of incubation at 27 C. More than 500 conidia were counted in each test.

Crossing and ascospore analysis. Crosses and ascospore isolation were carried out according to the methods previously reported (5). Monoascospore isolates were cultured on Misato-Hara slants at room temperature. IBP-resistance of progenies derived from S- and C-IBP strains (except C-IBP-12) was tested at 0.15 mM. Mycelial disks 4 mm in diameter were cut from 1-wk-old slant cultures, and placed upside down on test medium (rice decoction agar). Isolates were classified as resistant or sensitive to IBP on the basis of mycelial growth at 5 days after inoculation. Progenies of M-IBP strains and C-IBP-12 were scored for response to IBP at 0.1, 0.125, and 0.15 mM. Inoculation to the test medium was as described above except that the inoculum was a mycelial disk 5 mm in diameter from a 1-wk-old colony newly cultured on Misato-Hara agar (15 ml/9-cm-diameter petri dish). Mycelial growth was assessed at 5 days after inoculation.

RESULTS

Fungicide sensitivity. A total of 36 resistant mutants were tested for MIC and radial growth. The MIC values of C- and S-IBP strains ranged from 0.2 to 0.4 mM of either fungicide (Table 1). On the other hand, M-IBP, wild types, and kasugamycin-resistant strains could not grow at 0.2 mM of either fungicide, and therefore were distinguishable from C- and S-IBP strains. From the measurements of radial growth it was evident that C- and S-IBP strains were much more resistant to IBP and isoprothiolane than M-IBP and control strains (Fig. 1). With the exception of a strain C-IBP-12, C- and S-IBP strains showed similar resistance. The growth of C-IBP-12 was significantly suppressed by fungicides as compared with other C-IBP strains. Although M-IBP strains, as a whole, were a little more resistant to 0.1 mM IBP than the control strains, the ranges of their growth rates overlapped. M-IBP and control strains were equally sensitive to isoprothiolane. None of the kasugamycin-resistance genes conferred resistance to either IBP or isoprothiolane.

Conidial germination of C-IBP-6 and C-IBP-7 was not affected on the IBP-containing media, but that of the other strains was suppressed (Fig. 2). C-IBP-12, which showed an intermediate degree of resistance in mycelial growth, also was intermediate with respect to conidial germination. Wild-type strains were clearly more susceptible than M-IBP strains; germination of the wild type S-38 was completely inhibited at 0.1 mM. These results indicate that germination rates on the test media reflect the degree of resistance observed in tests of mycelial growth. Based on these tests, C- and S-IBP strains will be referred to as highly resistant (HR) strains, and M-IBP strains and C-IBP-12 as moderately resistant (MR) strains.

TABLE 1. Minimum inhibitory concentration (MIC) of IBP and isoprothiolane on the test medium inoculated with IBP-resistant mutants of *Pyricularia oryzae* (finger millet isolates)

Strains	Total (no.)	MIC of IBP (mM)[a]					MIC of isoprothiolane (mM)[a]				
		<0.2	0.2	0.3	0.4	>0.5	<0.2	0.2	0.3	0.4	>0.5
C-IBP	14	0	1	5	8	0	0	5	7	2	0
S-IBP	12	0	1	1	10	0	0	2	8	2	0
M-IBP	10	10	0	0	0	0	10	0	0	0	0
Wild type	2	2	0	0	0	0	2	0	0	0	0
KSM-R[b]	6	6	0	0	0	0	6	0	0	0	0

[a] Estimated at 3 days after inoculation on the test medium (rice decoction agar).
[b] Kasugamycin-resistant mutants.

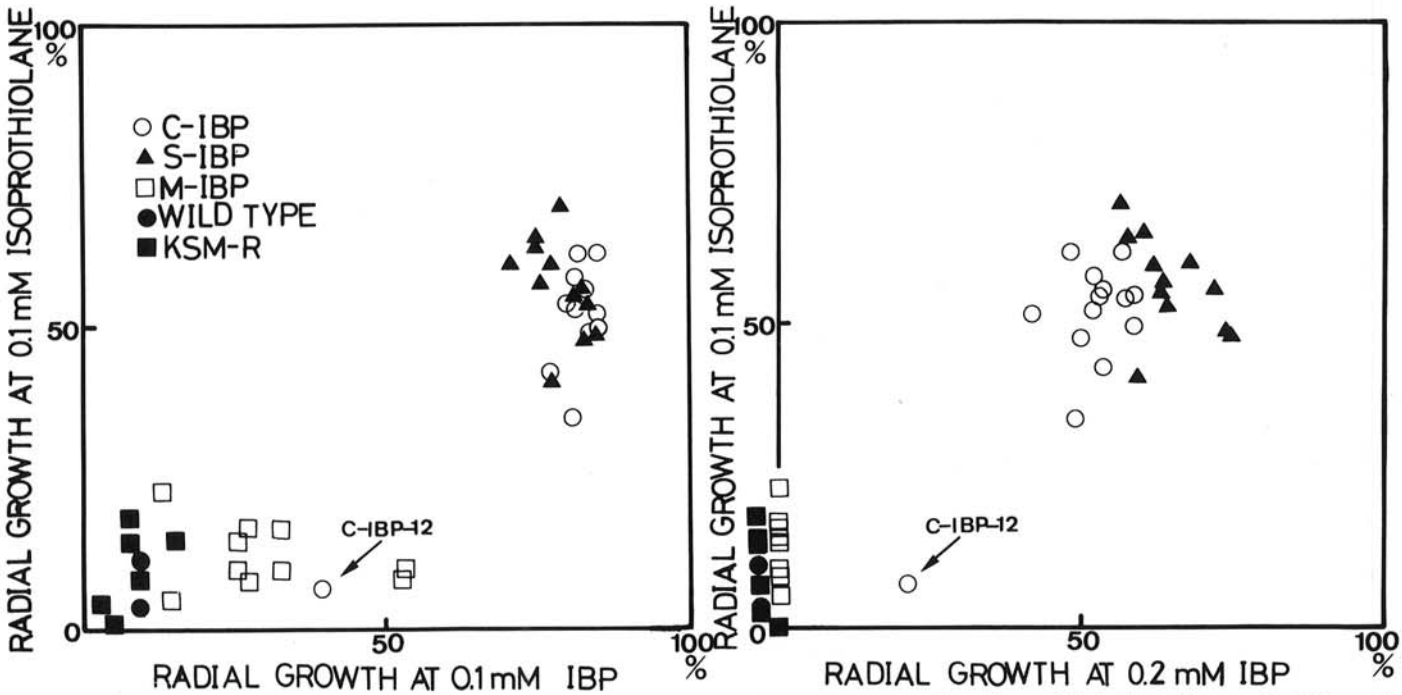

Fig. 1. Effects of IBP and isoprothiolane on radial growth of IBP-resistant mutants of *Pyricularia oryzae* from finger millet isolates. Data are: (radial growth on the test medium) × 100/(radial growth on the control medium). Values are means of two replications measured at 4 days after inoculation on rice decoction agar at 27 C.

Crosses between HR strains and sensitive strains. Each of the C-and S-IBP strains was crossed with a compatible IBP-sensitive strain and ascospore progenies were analyzed. For random ascospores, all crosses gave a 1:1 segregation of resistant:sensitive (Table 2). Tetrad analyses were done with 15 asci, of which six were obtained from S-IBP-4 × G-17 and nine from S-IBP-12 × G-17. In every ascus, 4:4 segregation for resistance and sensitivity was observed (Fig. 3). We concluded from these results that IBP-resistance in HR strains is controlled by a single major gene. These tetrads were also tested for resistance to isoprothiolane at 0.1 mM. IBP-resistance was always linked to isoprothiolane-resistance (Fig. 3). This fact suggests that the cross-resistance between IBP and isoprothiolane is also controlled by the same gene.

Test for allelism in HR strains. Crosses among HR strains were done to test allelic relationships among resistance genes of HR strains. In cases in which strains to be crossed were of the same mating type, an ascospore progeny of opposite mating type was substituted for its parent. All crosses yielded only progenies resistant to IBP (Table 3). This indicates that the resistance genes of all sixteen strains are at the same locus, that is, they are allelic. We named the locus *ibp*.

Crosses between MR strains and sensitive strains. Three MR strains, C-IBP-12, M-IBP-5, and M-IBP-9, were crossed with IBP-sensitive strains, and four tetrads were analyzed for each cross (Table 4). In these analyses, responses to IBP were observed in more than in analyses for HR strains, since the classification of resistance or sensitivity of each isolate was difficult. Segregation ratios of resistant:sensitive in tetrads varied according to the concentration of IBP or the asci tested. This suggests that IBP-resistance in MR strains is not controlled by a single major gene, though the nature of inheritance cannot be clearly determined from this experiment.

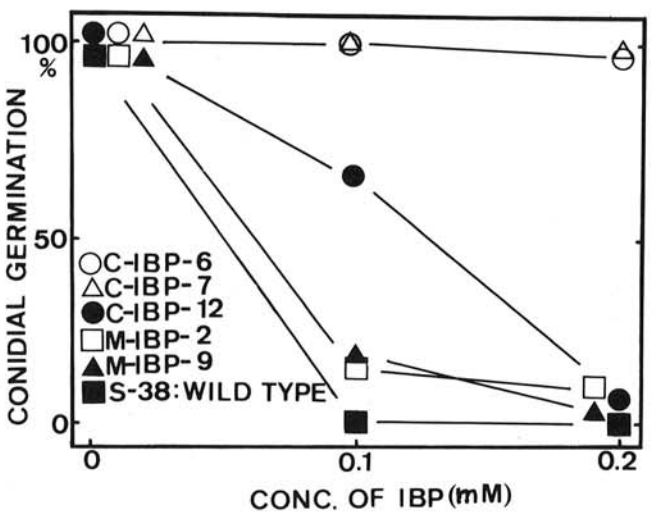

Fig. 2. Effects of IBP on the conidial germination of IBP-resistant mutants of *Pyricularia oryzae* (finger millet isolates). Percent germination was assessed by examining more than 500 conidia after 20 hr of incubation at 27 C.

TABLE 2. Ascospore analyses of IBP-resistance in crosses between highly resistant × sensitive strains of *Pyricularia oryzae* (finger millet isolates)

Crosses (resistant × sensitive)	Ascospores tested	Ratio R[a]:S[a]	χ^2 values[b] (1:1)
S-IBP-1 × F-36	26	14:12	0.15
S-IBP-4 × G-17	107	60:47	1.58
S-IBP-6 × E-144	33	13:20	1.48
S-IBP-7 × F-92	8	7:1	3.13[c]
S-IBP-8 × G-17	19	7:12	1.32
S-IBP-10 × G-17	56	32:24	1.14
S-IBP-11 × I-34	42	17:25	1.52
S-IBP-12 × G-17	140	66:74	0.46
C-IBP-1 × P-94	62	27:35	1.03
C-IBP-4 × M-69	74	36:38	0.02
C-IBP-5 × M-69	90	42:48	0.40
C-IBP-6 × M-69	96	55:41	1.02

[a] R = resistant and S = sensitive; tested 5 days after inoculation on the medium containing 0.15 mM IBP.
[b] Critical value at $P = 0.05$ is 3.84.
[c] Yates' continuity correction.

DISCUSSION

Results of this study revealed that IBP-resistant mutants could be grouped in two classes on the basis of mycelial growth at 0.2 mM IBP. A similar result has been reported by Katagiri et al (4), who studied the fungicide sensitivity of naturally occurring resistant strains as well as that of mutants selected in the laboratory. However, the occurrence of such a strain as C-IBP-12 in this study indicates that there are more than two classes of resistant strains, because the degree of resistance of C-IBP-12, assessed both as mycelial growth and conidial germination, was intermediate between HR and other MR strains.

This study also showed that the degree of resistance is only four or five times higher than that of sensitive strains when MIC was

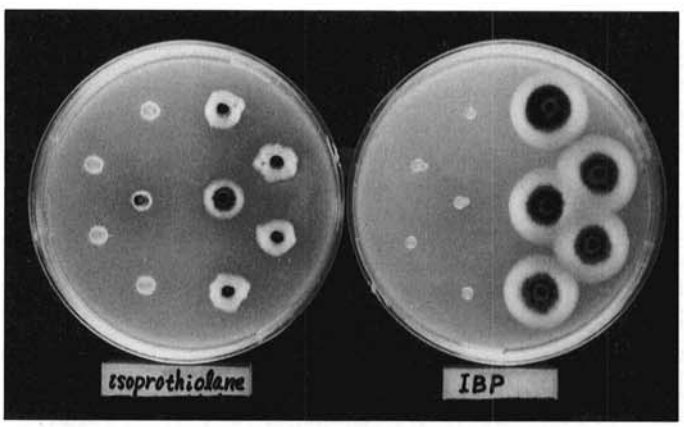

Fig. 3. Resistance to IBP and isoprothiolane of eight ascospores isolated from an ascus in the cross between highly resistant × sensitive strains (S-IBP-12 × G-17). The right plate is amended with 0.15 mM IBP, and the left with 0.1 mM isoprothiolane. Each ascospore isolate is inoculated at a similar position in both plates. The two strains in the center are the parents in the cross: on the right, S-IBP-12; and on the left, G-17.

TABLE 3. Tests for allelism of the genes controlling high IBP-resistance in *Pyricularia oryzae* isolated from finger millet

Crosses	Ascospores tested (no.)	R[a]:S[a]
S-IBP-12 × S-IBP-1	78	78:0
S-IBP-4	72	72:0
S-IBP-6	112	112:0
S-IBP-7	54	54:0
S-IBP-10	62	62:0
S-IBP-11	18	18:0
C-IBP-1	47	47:0
C-IBP-4	28	28:0
C-IBP-5	66	66:0
C-IBP-6	77	77:0
C-IBP-7[b]	64	64:0
C-IBP-8[b]	54	54:0
C-IBP-9[b]	64	64:0
C-IBP-10[b]	55	55:0
S-IBP-4 × S-IBP-6	29	29:0
S-IBP-10	68	68:0
S-IBP-1 × S-IBP-10	182	182:0
S-IBP-8 × S-IBP-11	24	24:0

[a] R = resistant and S = sensitive; tested at 5 days after inoculation on the medium containing 0.15 mM IBP.
[b] Not analyzed in Table 2.

TABLE 4. Tests for IBP-resistance of unordered tetrads obtained from crosses between moderately resistant × sensitive strains of *Pyricularia oryzae* (finger millet isolates)

Ascus	Ascospores	C-IBP-12 × P-94			M-IBP-5 × P-94			M-IBP-9 × P-94		
		0.1	0.125	0.15	0.1	0.125	0.15	0.1	0.125	0.15
1	1	+[a]	±	±	+	+	±	±	±	±
	2	+	±	±	+	+	±	±	±	−
	3	++	+	±	−	−	−	±	−	−
	4	++	+	±	−	−	−	+	±	±
	5	++	+	±	±	±	−	±	±	−
	6	++	+	±	±	±	±	±	−	−
	7	++	+	+	++	+	+	+	±	−
	8	++	+	+	++	+	+	±	±	−
2	1	++	+	+	±	−	−	+	±	±
	2	++	+	+	±	−	−	+	±	±
	3	++	++	++	++	+	+	+	±	−
	4	++	++	++	++	+	+	+	±	−
	5	++	+	+	+	±	±	+	±	+
	6	++	+	+	+	+	+	+	±	±
	7	±	−	−	+	+	+	±	±	−
	8	±	±	−	+	±	±	±	±	−
3	1	++	+	±	±	±	−	+	±	−
	2	++	+	±	±	±	−	±	−	−
	3	++	±	−	++	++	±	±	−	−
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	8	++	+	±	+	+	−	−	−	−
4	1	++	−	−	++	++	++	+	±	−
	2	+	−	−	+	±	±	+	−	−
	3	++	+	±	+	±	±	−	−	−
	4	++	+	±	++	++	+	±	−	−
	5	++	++	+	±	−	−	−	−	−
	6	++	++	+	±	−	−	−	−	−
	7	+	±	±	++	+	+	−	−	−
	8	+	±	±	++	+	+	−	−	−

[a] Mycelial growth from the inoculum was observed 5 days after inoculation on media with 0.1, 0.125, or 0.15 mM IBP; − no growth, ± very little growth, + little growth, and ++ good growth.

compared. This is also the case with resistance to isoprothiolane, but mutants of *P. oryzae* resistant to kasugamycin and blasticidin S are usually over 100 times more resistant with the wild type. This contrast in levels of resistance of mutants may be due to differences in mechanisms of action between these fungicides.

Katagiri and Uesugi (2) had found cross-resistance between IBP and isoprothiolane with mutants obtained from conidia. Results of this study also showed it to be true of mutants from sectors (S-IBP strain), as well as from conidia.

The sensitivity test based on conidial germination gave results similar to those based on mycelial growth, but we regard the former test to be more conclusive than the latter because conidial germination of the wild type was completely inhibited at 0.1 mM IBP, while some mycelial growth occurred at 0.1 mM. Therefore, measurement of the rate of germination may be the best method to distinguish wild types from resistant mutants, especially from M-IBP strains.

Crosses of resistant mutants with wild types and allelism tests among resistant mutants revealed that IBP-resistance of each HR strain was controlled by a single major gene at the same locus. This result contrasts with the case of kasugamycin-resistance, in which three different loci were identified according to the methods used for mutant selection. As to why only one locus, *ibp,* was identified in this study, we suppose that no other loci for IBP-resistance exist in the genome of this fungus, or that the mutation rates of other loci were too low to be detected. The single locus for IBP-resistance in comparison with three loci for kasugamycin-resistance is consistent with the observation of Katagiri and Uesugi (3) that the frequency of kasugamycin-resistant mutants among conidia of wild type is higher than that of IBP-resistant mutants.

Analyses of progeny from crosses of MR strains indicate that moderate resistance is genetically controlled, but the manner of inheritance remains uncertain. The fact that the degree of resistance varied among progenies suggests that resistance is not controlled by a single gene, but that a polygenic system may be involved. If resistance in MR strains is under polygenic control, an appropriate test based on quantitative assessment of resistance must be employed in ascospore analysis.

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