Identification of Three Different Loci Controlling Kasugamycin Resistance in *Pyricularia oryzae*

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ABSTRACT

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Two kasugamycin resistant strains of *Pyricularia oryzae* were obtained in the laboratory from finger millet isolates of the fungus. In each of these strains resistance is controlled by a single gene. Allelism tests, in which a previously studied strain was included, revealed the existence of three loci, *kas-1*, *kas-2*, and *kas-3*, for kasugamycin resistance in *P. oryzae*. None of these three loci appears to be linked to either of the remaining two or to the mating type. Mutation at the *kas-3* locus appears also to be responsible for resistance to blasticidin S.

Additional key words: rice blast fungus, antifungal antibiotic, fungal genetics, fungicide resistance.

Fungicide resistance in *Pyricularia oryzae* Cavara poses a threat to rice blast control: naturally occurring resistant strains have caused rice crop failure in paddies treated solely with kasugamycin (8). Up to the present time, resistance of *P. oryzae* to several fungicides has been reported. Kasugamycin (4,8,9,11,12,16), blasticidin S (3,10,13,16), *S*-benzyl diisopropyl phosphorothiolate (IBP, Kitazin P) (6,16), *S*-benzyl *O,O*-diethyl phosphorothiolate (EBP, Kitazin) (16), edifenphos (ethyl *S,S*-diphenyl phosphorodithiolate, EDDP, Hinosan) (16), and isoprothiolane (diisopropyl 1,3-dithiolane-2-ylidenemalonate, Fuji-One) (6) are among these fungicides. Genetic studies of sexual crosses have not been reported because only recently has culture of the perfect state of *P. oryzae* (7,17,19), and ascospore analysis, become possible. In this case, however, strains isolated from rice plants ("rice isolates") are self incompatible even if strains of opposite mating types are paired (18). On the other hand, strains isolated from finger millet ("finger millet isolates") are compatible with their opposite mating types and those of rice isolates. Fertility, especially mature ascospore formation, of crosses between rice isolates and finger millet isolates is much lower than that of crosses between finger millet isolates. From this point of view, finger millet isolates are thought to be more suitable for genetic studies of *P. oryzae* than are rice isolates.

In a previous study (15), we artificially obtained kasugamycin-resistant mutants from a large number of conidia of finger millet isolates, and proved that resistance of a certain mutant was controlled by a single major gene. In the present paper, further studies on genetical control of kasugamycin resistance in *P. oryzae* will be presented with special reference to identification of three different resistance loci.

MATERIALS AND METHODS

Fungal strains. All the wild-type strains used in this study were monoascosporic isolates derived from crosses between compatible finger millet strains. Antibiotic resistant strains were obtained from wild-type strains without the aid of mutagens. Of these, SC-2 and B1-100-4 were obtained by hyphal tip isolations from fast-growing sectors produced when wild-type strains were grown on medium containing 100 μg/ml kasugamycin and blasticidin S, respectively. Although B1-100-4 showed resistance to both kasugamycin and blasticidin S, it was more resistant to kasugamycin than to the latter (Table 1). The third kasugamycin resistant strain, Ta-1-5, was obtained by selection from a large number of conidia. That resistance of Ta-1-5 is controlled by a single major gene was reported in the previous paper (15). All strains were cultured on Misato-Hara medium at room temperature unless otherwise mentioned.

Crossing of strains and isolation of ascospores. Crossing was done in 9 cm diameter petri dishes by the methods of Ueyama and Tsuda (17). Small pieces of mycelia of the compatible strains to be crossed were inoculated on both sides of a sterilized rice straw put on Sachs' agar medium. Then the dish incubated at 24 C under intermittent fluorescent light. Thirty days of incubation was sufficient for the formation of mature perithecia. The unordered ascospore tetrads and random ascospores were isolated aseptically with a micromanipulator from several perithecia, which had been crushed in water. Each ascospore was allowed to germinate on a small agar block (3 × 3 mm) at 27 C and cultured on Misato-Hara medium at room temperature.

Testing antibiotic resistance of ascospore isolates. Kasugamycin resistance of ascospore isolates was tested using 1-wk-old cultures. Mycelial disks 4 mm in diameter were cut from the slant culture and placed upside down on the test medium containing 100 μg/ml of kasugamycin. Rice decoction agar adjusted to pH 5.0 with McIlvaine buffer was used as the basic medium. Resistance or sensitivity of the isolates to kasugamycin was determined after 6 days of incubation at 27 C on the basis of mycelial growth. Ascospore progenies of B1-100-4 × C-41 were tested similarly for resistance to blasticidin S (100 μg/ml).

Mating types of ascospore isolates. Two tester strains of opposite mating types were used to determine the mating type of each of the ascospore isolates. Crossing was done as mentioned above.

TABLE 1. Kasugamycin resistant strains of *Pyricularia oryzae* (finger millet isolates) employed in breeding studies to identify the loci of resistance genes

Strains	Source	Sensitivity to: kasugamycin (100 μg/ml)	Sensitivity to: blasticidin S (100 μg/ml)	Mating type
Ta-1-5	conidium	91[a]	...	A
SC-2	sector	83	21	A
B1-100-4	sector	59	42	A

[a]The value indicates the rate of mycelial growth relative to untreated controls; ie,

$$\frac{\text{mycelial growth on the test medium}}{\text{mycelial growth on the control medium}} \times 100.$$

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RESULTS

Crossing between resistant and wild-type strains. Resistant strains, B1-100-4 and SC-2, were crossed with the wild-type strains, and random ascospore analysis was done (Table 2). On the test medium, resistant isolates were clearly distinguished from the wild-type isolates. No intermediate strains were obtained from any cross. The 1:1 segregation ratio in each cross showed that resistance of each of these strains is controlled by a single major gene. When each ascospore progeny of B1-100-4 × C-41 was tested for blasticidin S resistance, kasugamycin resistance always was linked to blasticidin S and sensitive progenies were sensitive to both chemicals.

Tests for allelism. As it became evident that resistance of three strains, one of which (Ta-1-5) had been reported in the previous paper, was controlled in each instance by a single major gene, an allelism test was done. In this fungus, mating ability declined rapidly during successive cultures on artificial media; therefore, ascospore progenies were substituted for parent strains. For example, E-80 and E-135 were the ascospore progenies of B1-100-4 × C-41, and each had the same resistance gene as B1-100-4. Similarly, B-26 and D-11 inherited the resistance gene of Ta-1-5, and G-46 the gene of SC-2.

The data for random ascospore and unordered tetrad analyses are shown in Table 3. In unordered tetrad analyses, the resistance/sensitivity ratios of parental ditype were 8:0, the nonparental ditype 4:4, and the tetratype 6:2 (Fig. 1). Only these three tetrad types were obtained in this study. When the cross between two resistant strains yields wild-type ascospore progenies, it should be concluded that the resistant genes of the two strains are not allelic. From both random and tetrad analysis data (Table 3) we interpret that resistance genes of these three strains were not allelic. We tentatively assigned *kas-1* to the resistance locus of Ta-1-5, *kas-2* to SC-2, and *kas-3* to B1-100-4, respectively (Fig. 2).

In random ascospore analyses, resistance/sensitivity segregation ratios in each cross did not significantly differ from 3:1, although the value of chi-square in goodness-of-fit tests for progeny of Ta-1-5 (B-26) × SC-2 (G-46) did not sufficiently fit to 3:1 in comparison with the other two crosses. This result indicates that none of these three loci is linked to either of the other two.

TABLE 2. Random ascospore analyses of the crosses between kasugamycin resistant and wild-type strains of *Pyricularia oryzae*

Cross			Ascospores tested	R[a] (no.)	S[a] (no.)	Chi-square (1:1)
Resistant	×	Wild-type				
B1-100-4	×	C-41	122	58	64	0.30
SC-2	×	C-42	47	26	21	0.53

[a]Abbreviations: R = resistant to kasugamycin, and S = sensitive to kasugamycin.

TABLE 3. Random and unordered tetrad analyses of the crosses among kasugamycin resistant strains of *Pyricularia oryzae*

Analysis methods		Crosses: SC-2 × B1-100-4 (E-80)	Ta-1-5(B-26) × SC-2 (G-46)	B1-100-4(E-135) × Ta-1-5 (D-11)
Tetrad analysis	PD[a]	4	2	4
	NPD[a]	0	7	5
	T[a]	3	8	0
Random analysis	R[b]	56	88	82
	S[b]	21	40	30
Chi-square (3:1)		0.28	2.67	0.19

[a]Abbreviations; PD = parental ditype, NPD = nonparental ditype, and T = tetratype.
[b]Abbreviations: R = resistant, and S = sensitive.

A double-resistant mutant selected from tetratype tetrad of SC-2 × B1-100-4 (E-80) was crossed with a wild-type strain (Table 4). Random ascospore analysis confirmed that no linkage relationships existed between *kas-2* and *kas-3* even though in the tetrad analyses the frequencies of the three tetrad types differed from that of SC-2 × B1-100-4 (E-80).

Linkage relationships between mating types and resistance. In our previous work (15), we showed that *kas-1* is not linked to the mating-type gene. The possibility of linkage between the mating-type gene and either *kas-2* or *kas-3* was investigated by determining the mating type of random ascospore isolates of the crosses, B1-100-4 × C-41 and SC-2 × C-42 (Table 5). In the cross, B1-100-4 × C-41, the segregation ratio of parental type to recombinant type was 1:1. Therefore the resistance locus of B1-100-4, *kas-3*, was not linked to the mating-type locus. The occurrence of the tetratype and the nonparental ditype tetrads in this cross showed that there existed recombinations between *kas-3* and centromere or between mating type locus and centromere. Although sufficient data for determining linkage relationships could not be obtained in the cross SC-2 × C-42, it is possible that *kas-2* also was not linked with mating type locus.

DISCUSSION

In *Nectria haematococca* (syn. *Hypomyces solani*), Georgopoulos and Panopoulos found five loci for resistance to chlorinated nitrobenzene (2), and Kappas and Georgopoulos (5) four loci to dodine. In plant pathogenic fungi other than *N. haematococca*, several instances are known in which more than one gene controls resistance to certain fungicides (1). It became evident that such a multi-genic system exists in the *P. oryzae* isolates resistant to kasugamycin. In the present study, we demonstrated that there are at least three kasugamycin resistance loci in *P. oryzae*, although only three resistant strains were employed. In addition to these three loci, it is very probable that new resistance loci will be revealed by further studies with more strains. The fact that strains with different resistant levels are obtainable from sectors also supports this view (14).

TABLE 4. Random and unordered tetrad analyses of the cross between a double resistant strain and a wild-type strain of *Pyricularia oryzae*

Tetrad analysis		Random analysis	
PD[a]	0	R[b]	80
NPD[a]	1	S[b]	34
T[a]	6		
	7		114
		Chi-square (3:1) = 1.28	

[a]Abbreviations: PD = parental ditype, NPD = nonparental ditype, and T = tetratype.
[b]Abbreviations: R = resistant, and S = sensitive.

TABLE 5. Linkage relationships between kasugamycin resistance and mating types in the crosses, B1-100-4 × C-41 and SC-2 × C-42 of *Pyricularia oryzae*

B1-100-4 × C-41					SC-2 × C-42		
Tetrad analysis		Random analysis			Random analysis		
		Parental type			Parental type		
PD[a]	5	RA[b]	24	61	RA	12	23
NPD[a]	1	Sa[b]	37		Sa	11	
T[a]	8	Recombinant type			Recombinant type		
		Ra[b]	34	61	Ra	8	13
	14						
		SA[b]	27		SA	5	

[a]Abbreviations: PD = parental ditype, NPD = nonparental ditype, and T = tetratype.
[b]Abbreviations: R = resistant, S = sensitive, A = mating type A, and a = mating type a.

Although double mutants (ie, strains having two resistance genes) were obtained from each allele test, the effect of doubling of resistance genes was not examined in this study. According to Kappas and Georgopoulos (5), the effects of dodine resistance genes of *N. haematococca* were additive. Of resistant strains used in our study, Ta-1-5 and SC-2 showed almost the same level of resistance, but B1-100-4 was slightly less resistant than the other two strains. When B1-100-4 and SC-2 were crossed, no ascospore progenies with a higher level of resistance than that of SC-2 were obtained based on mycelial growth. This fact suggests that there are no additive relationships between *kas-2* and *kas-3*. Further detailed data concerning interactions among resistance genes will be reported subsequently.

Some information about linkage relationships among resistance and mating-type loci was obtained by random ascospore analysis. The three resistance loci appeared to be unlinked to each other. In comparison with random ascospore analysis, unordered tetrad analysis was not powerful enough to define linkage relationships because the number of isolated tetrads was rather small. In the previous paper (15) we reported that resistance of naturally occurring resistant strains was chromosomally controlled. The finding by Ito and Yamaguchi (4) that both high and intermediate resistance strains existed among naturally occurring resistant strains strongly suggests the probability of multiple resistance loci (4). Whether there are common resistance loci between naturally occurring resistant strains and the strains selected in vitro is an interesting subject for subsequent study. We are now conducting allele tests using both kinds of resistant strains.

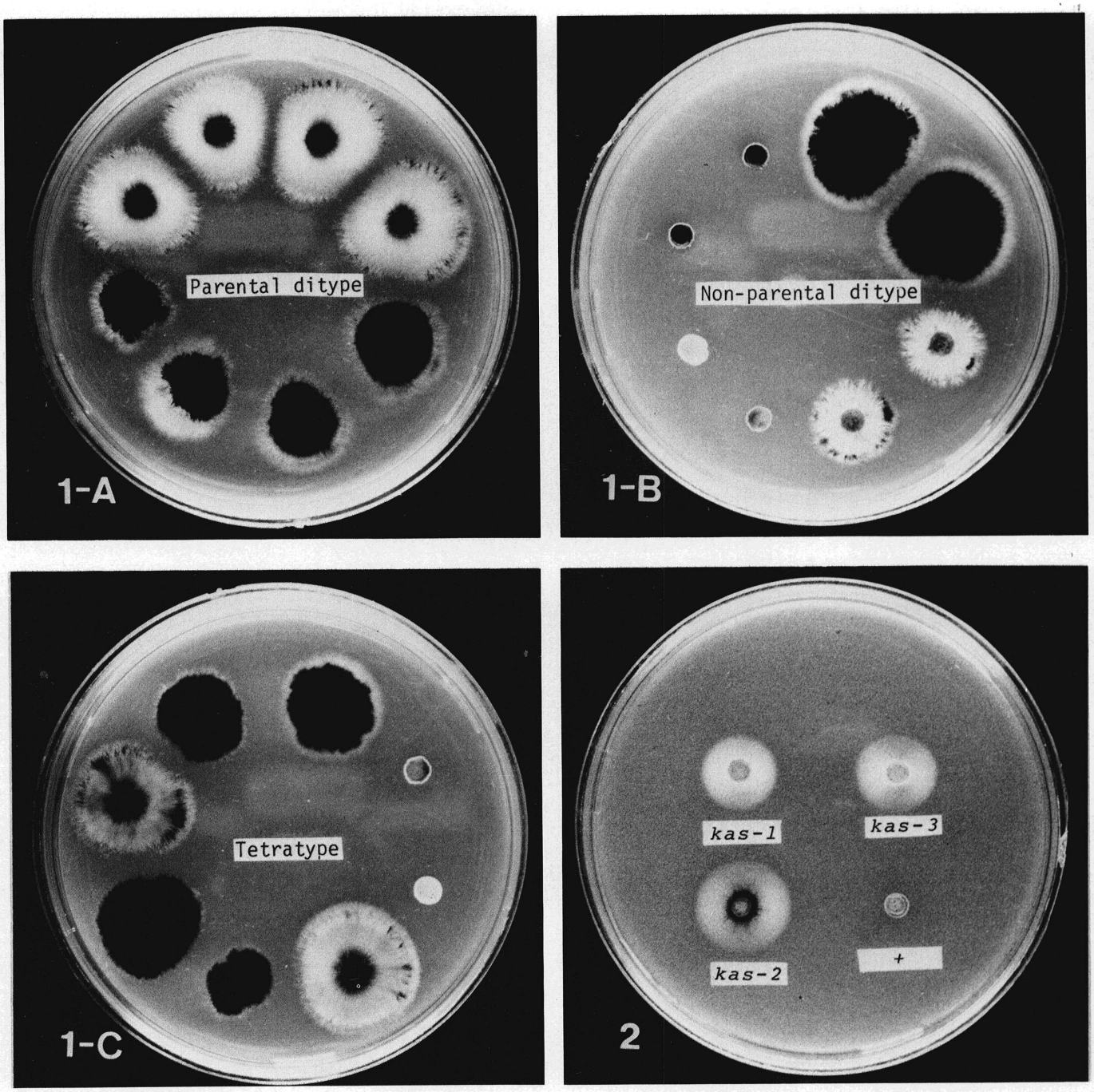

Fig. 1-2. Colony morphologies of *Pyricularia oryzae* parents and progenies used to investigate the inheritance of resistance to kasugamycin. **1**, six-day-old colonies of eight ascospore progenies of three kinds of tetrads obtained from the cross, Ta-1-5 (B-26) × SC-2 (G-46). **A**) parental ditype (ratio resistant:sensitive = 8:0) **B**) nonparental ditype (ratio resistant:sensitive = 4:4) **C**) tetratype (ratio resistant:sensitive = 6:2). The medium was rice decoction agar containing 100 μg/ml kasugamycin. **2**, Four-day-old colonies of the strains of *P. oryzae* having each resistant gene on kasugamycin (100 μg/ml)-containing medium.

Sakurai et al (12) reported that kasugamycin resistant strains of *P. oryzae* showed cross-resistance to blasticidin S and polyoxin D. Conversely, Hwuang and Chung (3) obtained a blasticidin S resistant strain which showed cross-resistance to kasugamycin. It was shown by Katagiri and Uesugi (6) that IBP resistant strains of *P. oryzae* were resistant to isoprothiolane at the same time. In the strictly genetic sense of the word, the term "cross-resistance" should be used only when a single gene controls resistance to several fungicides. For this reason, the case mentioned above cannot definitely be accepted as proof of cross-resistance until the crossing experiments are completed. In our study, each kasugamycin resistant ascospore progeny of B1-100-4 × C-41 also was without exception resistant to blasticidin S. This fact strongly suggests that resistance of B1-100-4 to these two fungicides is controlled by a single gene; by cross resistance.

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