SUPPRESSION OF THE KILLER PHENOTYPE IN *USTILAGO MAYDIS*

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ABSTRACT

Nineteen sensitive cell lines of *U. maydis* were crossed with three killer strains and sample progenies were screened for killer segregation patterns. Crosses involving 11 lines gave killer frequencies ranging from 71%–100% of the progeny and 4:0 segregations in tetrads. Segregations in some crosses involving each of the remaining 8 lines gave killer frequencies from 0%–58% and mixed tetrads containing both non-killer and killer meiotic products. Many of the killers were unstable on further culture. Killer suppression showed varying degrees of specificity, appeared to be cytoplasmically determined for at least one strain, and was associated with possession of dsRNA in this strain and one other. No dsRNA was detected in two other suppressive strains. There was no evidence for segregation of nuclear maintainer genes for any of the killer determinants.

KILLER strains of *Ustilago maydis* (PUHALLA 1968) possess virus-like particles (VLPs) that contain double-stranded RNA (dsRNA) (WOOD and BOZARTH 1973; KOLTIN and DAY 1976). Immunity to killer is associated with the presence of dsRNA and, like the killer property, is inherited as a cytoplasmic trait. Three killer specificities, designated P1, P4, and P6, are known in *U. maydis*. The relationship between killer function, immunity, and possession of dsRNA is similar for all three. Crosses and heterokaryons between different killer strains showed that there are both unilateral and mutual restriction systems that prevent inclusion of two killer specificities in the same cell (KOLTIN and DAY 1976).

The role of nuclear genes in the replication, maintenance, and expression of cytoplasmic entities is well documented (JINKS 1964; SAGER 1972). A killer system similar to that of *U. maydis* is known also in the yeast *Saccharomyces cerevisiae* (SOMERS and BEVAN 1969; BEVAN, HERRING and MITCHELL 1973; VODKIN and FINK 1973). Nuclear genes that affect the maintenance of the killer function in yeast were reported by BEVAN, SOMERS and THEIVENDIRARAJAH (1969) and by NESTEROVA and ZEKNOV (1973). SOMERS (1973) also reported suppressive sensitive mutants of killer yeast that were isolated following treatment with a chemical mutagen. In spite of the similarity between the killer phenomena in yeast and Ustilago, there are no indications that nuclear genes are involved in the maintenance of killer in Ustilago.

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The incidence of killers among natural isolates of *U. maydis* is low. The three that are known were each recovered once from about 60 samples from North America. None was found among 30 samples from Poland (SILVA 1972) and 10 from Mexico (KOLTIN and DAY, unpublished). A low frequency of maintainer genes in the population could account for the low incidence of killer among natural isolates. We undertook a search for nuclear genes involved in the replication and maintenance of killer among 19 known sensitive strains. The study revealed various types of suppression of the killer phenotype—some that are cytoplasmically transmitted and, in two instances, related to the presence of unpackaged dsRNA in sensitive strains.

MATERIALS AND METHODS

Strains: The killer strains used were listed in KOLTIN and DAY (1976). The sensitive strains and their origins are listed in Table 1.

Analytical procedures: Media, crosses, teliospore germination, isolation of spores and tetrads are described in STEVENS (1974). Cultures were grown on Ustilago complete medium and double-strength complete medium was used for determination of mating types.

Identification of phenotypes: Killers and nonkillers were distinguished by replication to a lawn of a sensitive strain. The plates were incubated at 25° and scored 24 to 36 hours later. Killers were surrounded by a halo of inhibited sensitive cells, whereas nonkillers had no effect.

TABLE 1

Sensitive strains of U. maydis *used in the study**

Strain number	Mating type	Source
1	a2 bA	England†
5	a1 bC	Conn., U.S.A.
7	a1 bE	Canada
12	a2 bB	England
14	a2 bA	England
18	a2 bG	Conn., U.S.A.
20	a1 bH	Conn., U.S.A.
36	a2 bI	Conn., U.S.A.
39	a1 b–	Conn., U.S.A.
49	a2 bK	Conn., U.S.A.
54	a2 bH	Conn., U.S.A.
60	a1 bL	Penn., U.S.A.
66	a2 bF	Ill., U.S.A.
70	a2 bI	Ohio, U.S.A.
79	a1 bI	Ontario, Canada
88	a2 b–	Ga., U.S.A.
127	a2 bA	‡
1003	a2 bI	Conn., U.S.A.
1168	a1 bS	Poland†

* The strains were either sensitive to all three killer specificities or carried nuclear genes for resistance (KOLTIN and DAY 1976). None was immune as determined by genetic analysis.

† The strains from England are from the laboratory of R. HOLLIDAY, Medical Research Council, Mill Hill. The strain from Poland was provided by SILVA (1972).

‡ Derived from a cross between strain 1 and a P1 strain carrying the nuclear gene for resistance for P1.

Immunity was tested either by using the unknown as a lawn upon which a killer strain was spotted or by streaking cells of unknowns along a pregrown streak of a killer strain. Growth was examined after 24 hours' incubation at 25°.

Extraction of dsRNA and isolation of the VLPs: The procedure for extraction of dsRNA was described by VODKIN, KATTERMAN and FINK (1974). Modifications of this procedure used in studies of the Ustilago VLPs were described by KOLTIN and DAY (1976).

RESULTS AND DISCUSSION

In previous studies (PUHALLA 1968; KOLTIN and DAY 1976) crosses between killer strains with P1, P4 and P6 specificities and sensitive strains led to segregation patterns among the progenies typical of cytoplasmic inheritance. Cytoplasmic inheritance of the killer phenotype was confirmed by the demonstration that the killer phenotype is transmitted to sensitive cells by cytoplasmic mixing (DAY and ANAGNOSTAKIS 1973; KOLTIN and DAY 1976). If a nuclear gene determines replication or maintenance of the VLPs and this gene is not carried by the sensitive parent in a cross with a killer, then instead of a 4:0 segregation for killer in tetrads a 2:2 segregation should be detected. In a search for this pattern of segregation 19 sensitive strains were crossed with each of the 3 killers. The first tests were for deviation from the expected 4:0 segregation in crosses with P4 and P6. Where a deviation was detected all three crosses involving that sensitive strain were examined. Inviability of the teliospores of some crosses sometimes confined analysis to one or two of the three crosses of a sensitive strain.

TABLE 2

Cytoplasmic inheritance of P4 and P6 killer phenotypes

Parents		Random progeny		Tetrads*				Diploids		
NK ×	K	Killers	Total	NK	K	SEG	Total	NK	K	Total
1	P4	63 (87.5)†	72	—	—	—	—	—	—	—
1	P6	101 (89.3)	113	—	—	—	—	—	—	—
5	P6	718 (95.0)	755	1	20 (95.2)†	0	21	0	9	9
7	P6	93 (81.0)	115	—	—	—	—	—	—	—
12	P4	—	—	1	9 (90.0)	0	10	—	—	—
12	P6	—	—	2	10 (83.3)	0	12	—	—	—
20	P6	86 (76.8)	112	—	—	—	—	0	3	3
60	P4	96 (86.0)	108	—	—	—	—	—	—	—
60	P6	59 (71.0)	83	—	—	—	—	—	—	—
79	P6	—	—	3	36 (92.3)	0	39	1	2	3
88	P4	—	—	1	15 (93.7)	0	16	—	—	—
127	P4	124(100.0)	124	0	19(100.0)	0	19	—	—	—
127	P6	21 (72.4)	29	—	—	—	—	—	—	—
1003	P4	502 (86.1)	583	3	10 (76.9)	0	13	0	18	18
1003	P6	105(100.0)	105	—	—	—	—	—	—	—
1168	P6	—	—	0	14(100.0)	0	14	0	3	3

* NK = Nonkiller; K = Killer; SEG = Segregating tetrad containing killer and nonkiller progeny.
† Number in parenthesis refers to per cent of total.

The results from ten strains crossed to the P6 killer conformed to earlier findings (Table 2). Killer progeny were recovered at frequencies ranging from 71% to 100%, and tetrad progenies were uniform, being either all killers or all nonkillers, the frequency of killer tetrads ranging from 83% to 100%. Diploids recovered as unreduced zygotes (DAY and ANAGNOSTAKIS 1971) were nearly all killer in phenotype. A similar pattern was found in crosses of five of these sensitive strains and one additional strain with the P4 killer (Table 2).

Totally different results were obtained with eight other sensitive strains (Table 3). Striking deviations from the expected proportions of killer progeny were noticed in crosses of some of these strains with all three killers (e.g., strain 66) and in others with only one or two of the killer strains (e.g., strains 18 and 70). The deviation was expressed in random samples as an unusually low frequency of killer progeny compared with the segregation observed in the crosses shown in Table 2 (38% to 58% *vs.* 71% to 100%). Among tetrads the frequency of killer tetrads ranged from 0%, in an extreme case in which killer function was totally abolished (strain 66 × P6), to 100%. In addition, a new type of tetrad was detected that was nonuniform and contained both killer and nonkiller progeny. These segregating tetrads are designated SEG in Table 3. The killers from segregating tetrads showed varying degrees of killer activity. Among the few diploids recovered from these crosses as unreduced zygotes more than 65% were nonkillers. Thus the suppression or dilution of killer determinants seen among meiotic products also occurs at the level of the zygote and maybe even prior to zygote formation.

TABLE 3

Suppression of killer phenotype in crosses between killer strains and sensitive strains

Parents		Random progeny		Tetrads				Diploids		
NK ×	K	Killers	Total	NK	K	SEG	Total	NK	K	Total
14	P6	32 (59.2)	54	5	3 (27.2)	3	11	4	2	6
18	P1	—	—	0	10(100.0)	0	10	—	—	—
18	P4	59 (58.4)	101	9	7 (41.1)	1	17	0	1	1
18	P6	39 (34.2)	114	5	6 (50.0)	1	12	4	2	6
36	P1	—	—	11	0 (0.0)	0	11	—	—	—
36	P4	—	—	0	14(100.0)	0	14	—	—	—
39	P4	—	—	0	12 (85.7)	2	14	—	—	—
39	P6	—	—	8	5 (33.0)	2	15	—	—	—
49	P1	—	—	0	14(100.0)	0	14	—	—	—
49	P4	—	—	4	12 (66.6)	2	18	—	—	—
49	P6	—	—	0	15(100.0)	0	15	—	—	—
54	P1	—	—	2	14 (87.5)	0	16	—	—	—
54	P4	—	—	2	12 (85.7)	0	14	—	—	—
54	P6	—	—	6	6 (50.0)	0	12	—	—	—
66	P1	—	—	1	7 (46.6)	7	15	—	—	—
66	P4	—	—	3	1 (6.6)	11	15	0	1	1
66	P6	—	—	12	0 (0.0)	0	12	5	0	5
70	P1	—	—	0	9(100.0)	0	9	—	—	—
70	P4	—	—	6	8 (53.3)	1	15	—	—	—

Comparisons of the effect of the four suppressive strains 18, 49, 54, and 66 on all 3 killers reveal some specificity in killer suppression. For example, strain 18 affects P4 and P6 but has no effect on P1; strain 66 abolishes P6 activity but its effects on P4 and P1 activities are less pronounced. Strains 49 and 54 affect only P4 and P6, respectively. Among the sensitive strains not completely tested, strain 36 abolishes killer P1 activity but has no effect on P4.

Another feature of progeny from the crosses between killer and suppressive strains was that many killers were unstable. The stability of killer expression in the original P6 killer strain and progeny from the cross between a P6 killer and strain 18 is shown in Table 4. As was shown earlier for P1 (DAY and ANAGNOSTAKIS 1973) P6 very rarely (<.1%) loses the killer phenotype during vegetative replication. However, 1.5% and 5.5% of cells from tetrads that were uniformly killers in the cross with strain 18 became nonkillers. In the segregating tetrads the proportion of nonkiller cells among the descendents of individual basidiospores was 60%–70%. This proportion varied continuously and eventually the killer phenotype was no longer expressed after a number of cell replications. Nonkiller progeny were never observed to revert to the killer state.

The loss of killer expression can also be followed in diploids from crosses between suppressive and killer strains. Some diploids that upon isolation were killer in phenotype yielded, by mitotic division, nonkiller descendents. Ultimately only nonkiller descendents were recovered from these diploids.

The segregations observed in the crosses between killer and suppressive strains are unlike those expected for segregation of a nuclear gene(s) since not all tetrads show segregation. In crosses where they occurred the frequency of segregating tetrads was ca. 24.6%. In many cases the killer phenotype in these tetrads was transitory.

A test of the genetic basis for suppression was performed with progeny from the cross between strain 18 and P6. The test was based on the assumption that if 18 carried a nuclear gene affecting the maintenance of the killer determinant, leading to its dilution in the dikaryon and zygotes, then among the progeny of the cross between 18 and a killer, a tetratype tetrad that lacks killer activity

TABLE 4

Stability of killer phenotype in replication of single meiotic products from two killer tetrads and one segregating tetrad from the cross 18 × P6

Type of tetrad	Progeny number	No. tested	Killers	Nonkillers
Killer	4–1*	90	85	5 (5.5)
Killer	5–1	65	64	1 (1.5)
SEG	8–1	99	39	60 (60.6)
	8–2	91	24	67 (73.6)
	8–3	96	22	74 (77.0)
P6 Parent	1371	600	600	0 (0.0)
Nonkiller	6–2	92	0	92 (100.0)

* Tetrad and meiotic product identifying numbers.

should include meiotic products with and without the gene. Its presence or absence can be tested by crossing with the killer parent. If a single nuclear gene was segregating, some crosses would yield a normal cytoplasmic pattern of segregation while others would display the suppressive pattern. If, however, all the crosses display the suppressive pattern it can be assumed that the suppression results either from the action of a dominant cytoplasmic suppressor similar to the suppressive sensitive strains in *S. cerevisiae* (SOMERS 1973) or from several duplicate genes.

A tetrad from the cross 18 × P6 that was tetratype for mating type and lacked killing activity was analyzed by backcrossing to P6. Only three products were examined since no P6 stock compatible with the fourth product was available. Each cross yielded segregating tetrads and with one exception a lower frequency of killer tetrads (Table 5). None yielded only uniform tetrads. Diploids recovered as unreduced zygotes from these crosses displayed similar characteristics to the diploids recovered from the original cross of strain 18 with the P6 killer. In one of the crosses, of 31 diploids detected, 20 were killers and 11 nonkillers. Upon subsequent subculturing of the diploids 2 additional diploids lost the killing function and others displayed weak killing activity, which was found earlier to be a first indication of the accumulation of nonkiller cells in the colony.

The simplest explanation for these results is that 18 carries a dominant cytoplasmic suppressor. Further evidence supporting this view is given below. Alternative explanations such as a single semi-dominant chromosomal suppressor gene or several duplicate suppressor genes appear unlikely since from 5.5% to 28.5% of the tetrads from each backcross were uniformly killer (Table 5). We were unable to transfer the suppressor determinant from 18 to P6 by heterokaryon transfer because of the sensitivity of 18 to P6 on agar media. Crosses to produce meiotic products and diploids were made in maize seedlings where killing appears not to take place.

TABLE 5

Segregation of "killer" suppressor within a tetrad from 18 × P6

Progeny crossed with P6			Number of tetrads			
Number	Mating type		NK	K	SEG	Total
9–1	a1bC*		12	1	5	18
9–5	a1bC		10	2	4	16
9–11	a1bC		11	1	6	18
9–9	a1bG		0	2	5	7
9–12	a1bG		10	1	4	15
9–13	a1bG		5	1	7	13
9–3	a2bG		8	4	4	16
9–4	a2bG		8	4	2	14
9–6	a2bG		3	3	10	16
		Total	67	19	47	133
		Per cent	49.6	14.4	36.0	

* Fourth product (a2bC) was incompatible with available P6 stocks.

In spite of the fact that the information for killer function and immunity may reside in different dsRNA fractions (KOLTIN and DAY 1976), the suppressive action operates on the immunity function as well. Crosses between P3(6), a strain with immunity to P6 but lacking the P6 killer function, and 2 nonkillers from the cross 18 × P6 both yielded tetrads segregating for immunity. The instability of the immune phenotype was shown by the formation of turbid zones of inhibition around killer inoculum spotted on the tested progeny. A test of mitotic descendents from such colonies revealed the presence of both immune and sensitive cells. These colonies were derived from single meiotic products and showed a continuous loss of immunity during growth. Thus, it is suggested that the suppressive factor of strain 18 operates in a way that makes no distinction between the P6 killer function and the P3(6) immunity function.

Seven sensitive strains were examined for the presence of dsRNA. Three (5, 79, and 1003) were not suppressive in crosses (Table 2) and contained no detectable dsRNA. The remaining four strains (14, 18, 39, and 66) were suppressive (Table 3) and two of them contained dsRNA. Strain 18 carried 2.1 and 1.8×10^6 dalton fractions that were comparable to the species of dsRNA of these molecular weights found in P4, P3(4), and P3(6) (KOLTIN and DAY 1976). Strain 39 carried 6 dsRNA fractions indistinguishable from the 6 dsRNA fractions found in P4 (KOLTIN and DAY 1976) (Figure 1). No dsRNA was detected in strains 14 and 66. However, our procedure did not detect fractions smaller than 0.3×10^6 daltons, and WOOD and BOZARTH (1973) reported a fraction of $.06 \times 10^6$ daltons from P1. Strains 18 and 39 were examined for VLPs and none was found. We have already reported that no VLPs were found in the non-suppressive strains 5 and 1003 (KOLTIN and DAY 1976).

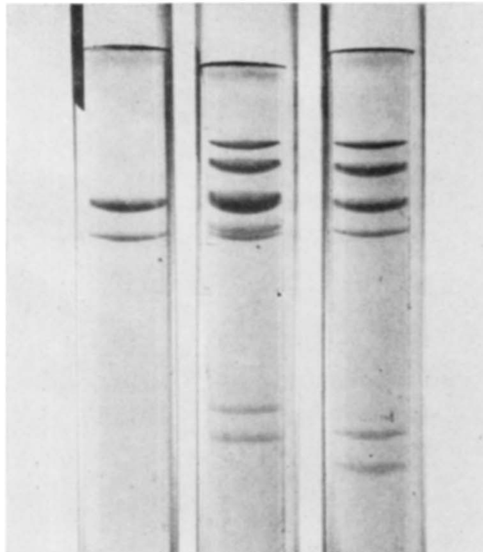

FIGURE 1.—dsRNA components of the suppressive strains 18 (left), 39 (right), and 18 +39 (center), separated by electrophoresis on 5% acrylamide gel (8 hrs).

In none of the 34 crosses detailed in Tables 2 and 3 was there evidence that any of the 18 sensitive strains carried nuclear genes that restricted replication, maintenance or expression of a killer determinant. In all cases of suppression (Table 3) the segregation patterns observed were most easily explained on the basis of a cytoplasmically inherited suppressive factor.

Some sensitive strains of *U. maydis* clearly possess genetic information that can suppress the replication and/or expression of specific killer determinants. If the different molecular weights of the dsRNA species that typify each killer reflect differences in viral genotypes, then specific suppression may occur through some kind of recognition mechanism that permits replication of some species of dsRNA but interferes with others. For strain 18 suppression appears to be cytoplasmically inherited. Since 18 carries dsRNA, even though it does not appear to be packaged in VLPs, the basis for its suppressiveness may well be a restriction system like that which prevents the occurrence of more than one killer specificity in a cell (Koltin and Day 1976). Strain 39 is expected to show a similar inheritance of suppressiveness. Although no dsRNA was found in strains 14 and 66, our experiments do not rule out the possibility that small molecular weight dsRNA may be involved.

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