The Effects of Nuclear Restorer Genes of Texas Male-Sterile Cytoplasm on Host Response to Helminthosporium maydis Race T

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ABSTRACT

The effects of the dominant nuclear restorer factors *Rf*1 and *Rf*2, which restore fertility to corn (*Zea mays*) with Texas male-sterile (Tms) cytoplasm, upon host response to *Helminthosporium maydis* (*Cochliobolus heterostrophus*) race T toxin(s) were determined. The effects of *H. maydis* toxin on isolated mitochondria, excised leaves, and seedling roots, were compared for several isogenic lines containing normal (N), Tms, and fertility-restored Texas male-sterile (TRf) cytoplasms. The effects of the toxin on TRf plants were intermediate to those on N and Tms plants. Toxin effects on isolated mitochondria were compared with field susceptibility data for the N, Tms, and TRf versions of the isogenic lines B37, A632, and C103D. A greater toxin effect was seen on mitochondria from B37 and A632 Tms cytoplasms, than on those isolated from their respective TRf shoots. The C103D data were the inverse, which correlated with field observations. The results suggest that the restoration of pollen fertility modifies the intensity of the toxin effect on leaves, roots, or isolated mitochondria.

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Additional key words: southern corn leaf blight, nucleo-cytoplasmic interactions, cytoplasmic inheritance.

Maternal transmission in corn (*Zea mays* L.) of both Texas male-sterility and the associated susceptibility to *Helminthosporium maydis* Nisikado and Miyake (*Cochliobolus heterostrophus* Drechs.), race T, the causal organism of southern corn leaf blight, strongly suggests a role for a cytoplasmically controlled factor in host response. The primary site of action of *H. maydis,* or of its toxin(s) (6), has not yet been clearly established. Of the major DNA-containing organelles in corn leaves, mitochondria and chloroplasts, only mitochondria have demonstrated differential in vitro reactions to race T toxin (1, 9) when normal (N) resistant sources were compared with Texas male-sterile (Tms) sources. The purpose of this study was to determine if the nuclear restorer factors, *Rf*1 and *Rf*2 (2, 5), which restore the ability to produce functional pollen to Tms cytoplasm, have any effects on the interaction of *H. maydis* race T toxin with isolated mitochondria. Comparisons were also made of leaf and root sensitivity to toxin and in vivo susceptibility to the fungus under field conditions.

MATERIALS AND METHODS.—Mitochondria were isolated from 30-40 etiolated shoots of corn that had germinated for 3 days at 28 C in glass trays on paper towelling moistened with 0.1 mM $CaCl_2$. A humid aerobic atmosphere was provided by cutting small slits in the plastic wrap covering the trays. After 3 days, the epicotyls were rinsed and ground in an ice-cold mortar in a grinding medium containing 0.5 M sucrose, 0.05 M tris-HCl (pH 7.5), 0.005 M EDTA, and 4 mM KH_2PO_4. The homogenate was filtered through cheesecloth and centrifuged at 1,500 *g* at 5 C for 10 minutes. The resulting supernatant was transferred to fresh tubes, underlayed with 8 ml of 0.6 M sucrose (pH 7.5) and centrifuged at 28,000 *g* at 5 C for 6 minutes. The resulting mitochondrial pellet was resuspended in 0.05 - 0.10 ml of 0.4 M sucrose, pH 7.5. Studies of mitochondrial respiration were made as described previously (8). Oxygen uptake was measured polarographically by means of a Clark oxygen electrode fitted into a reaction cell placed in the light path of a Bausch and Lomb Spectronic 70 spectrophotometer.

TABLE 1. A comparison of the responses of isolated mitochondria from *Zea mays* to toxin produced by race T of *Helminthosporium maydis* with lesion size produced on excised corn leaves by toxin injection

Inbred corn line	Cytoplasm	Swelling (Δ%T/min/mg protein)	Stimulation of NADH[a] oxidation (%)	Lesion length (cm) 1 μliter toxin
W64A	N[b]	0	0	0
	Tms[c]	18	140	1.5
	TRf[d]	9	109	0.7
A632	N	0	0	0
	Tms	22	102	1.8
	TRf	11	93	0.8
SK2	N	0	0	0
	Tms	17	142	2.3
	TRf	6	155	0.8
N6	N	0	0	0
	Tms	26	171	4.6
	TRf	14	143	4.3
N28	N	0	0	0
	Tms	10	0	1.6
	TRf	4	0	0.7

[a]Reduced nicotine adenine dinucleotide.
[b]Normal cytoplasm.
[c]Texas male-sterile cytoplasm.
[d]Tms cytoplasm carrying dominant nuclear factors for restoration of pollen fertility.

Changes in the percent transmittance of light (swelling) at 520 nm were simultaneously monitored in the reaction cells. The reaction medium contained 0.2 M KCl, 1 mg/ml bovine serum albumin, 20 mM tris-HCl (pH 7.5), and 4 mM KH_2PO_4. The substrate consisted of 2 μmoles NADH (reduced nicotine adenine dinucleotide). Approximately 1.0 mg mitochondrial protein was used per run.

The effect of toxin on root elongation was determined by measuring the increase in length of the primary root of corn that had germinated for 24 hours on paper towels moistened with $CaCl_2$ (0.1 mM), and then transferred to dilutions of toxin in $CaCl_2$ (0.1 mM) ranging from 0-20% (v/v).

Excised leaf sections were inoculated with 1.0 μliter of undiluted toxin on either side of the midrib. The leaves were floated on a solution containing 0.146 M sucrose, 28 μM 3-indoleacetic acid, and 9.3 μM kinetin for 72 hours at room temperature and then scored for the occurrence and size of lesions.

Data on percentage leaf area infected was obtained on artificially inoculated adult plants grown in the field in 1970. The N, Tms, and TRf versions of each inbred or hybrid were grown in adjacent, but unreplicated, 12-plant plots. Percentage leaf area infected was estimated visually by three investigators 6 weeks after inoculation with *H. maydis* race T.

Toxin was obtained from culture filtrates of *H. maydis* race T grown in shake culture at 24 C for 9 days. The completely defined culture medium contained 11.1 mM glucose, 12.5 mM NH_4NO_3, 9.9 mM KNO_3, 7.3 mM KH_2PO_4, 2.0 mM $MgSO_4$, 1.7 mM NaCl, 0.88 mM $CaCl_2$, 3.6 μM $FeCl_3$, 3 μM $ZnSO_4$, 2 μM $MnSO_4$, and 1 μM $CuSO_4$. Desalting of the culture filtrate was achieved by mixing 10 g Amberlite MB-3 mixed-bed ion exchange resin per 100 ml of filtrate. The desalted filtrates were concentrated 10-fold by rotary evaporation at 40 C, prior to successive filtrations through 100, 12, and 3.6 nm

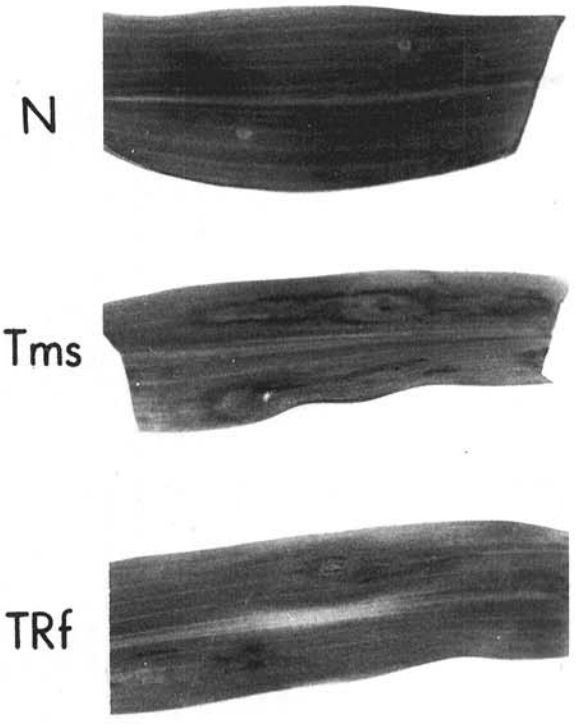

Fig. 1. Comparison of lesions produced by *Helminthosporium maydis* race T toxin in excised leaves of Normal (N), Texas male-sterile (Tms), and restored Tms (TRf) versions of inbred W64A of *Zea mays*. The lesions produced on Tms leaves by inoculation with race T toxin were generally larger than those produced on TRf leaves. The only symptom apparent on N leaves at the end of the 72-hour test period were necrotic spots at points of inoculation.

TABLE 2. Comparison of responses of mitochondria isolated from *Zea mays* to *Helminthosporium maydis* race T toxin with observed field susceptibility of *Zea mays* to *H. maydis* race T

Inbred Line	Cytoplasm	Swelling (Δ%T/min/mg protein)	Stimulation of NADH[a] oxidation (%)	Leaf area infected (%)
B37	Tms[b]	11	54	37
	TRf[c]	4	27	33
C103D	Tms	4	32	10
	TRf	27	106	13
A632	Tms	22	102	90
	TRf	11	93	80

[a]Reduced nicotine adenine dinucleotide.
[b]Texas male-sterile cytoplasm.
[c]Tms cytoplasm carrying dominant nuclear factors for restoration of pollen fertility.

filters (Sartorius 113-09, Amicon XM-100A and PM-10). Toxin solutions were kept frozen in small aliquots until use.

RESULTS.—The responses of mitochondria isolated from N, Tms, and TRf cytoplasm versions of six different inbred lines to the addition of 0.03 ml (0.75%) of race T toxin were compared by determining: (i) the stimulation of the state 4 rate of oxidation of exogenous NADH; and (ii) the swelling rate indicated by the percent transmittance of light through the mitochondrial suspension at 520 nm immediately after toxin addition. There was no detectable change in the rate of NADH oxidation or increase in swelling in any of the N cytoplasm versions of W64A (Clyde Black and Son, Ames, Iowa), A632, SK2, N6, or N28 (Table 1). The effect of toxin on mitochondria isolated from each of the respective Tms versions varied, with N28 showing the least (Δ 10% T/min) and N6 showing the greatest (Δ 26% T/min) swelling. Correspondingly, the greatest toxin-induced stimulation of NADH oxidation by isolated mitochondria was seen in N6 Tms, while no stimulation was detected in mitochondria isolated from N28 Tms (Table 1). The swelling response of each of the TRf versions as compared with its respective Tms version decreased approximately 50%. Three TRf versions (W64A, A632, and N6) demonstrated a decreased toxin stimulation of NADH oxidation when compared to their respective Tms versions (Table 1). The toxin-stimulated oxidation of NADH increased in the TRf version, SK2, but no detectable increase was observed in N28 Tms or N28 TRf (Table 1). The lesion size produced in excised leaves by toxin injection in the N, Tms, and TRf versions of W64A, N28, N6, A632, and SK2 was examined concomitantly with the isolated mitochondria experiments. The results suggest a decreased level of sensitivity in the TRf versions as compared with the Tms versions, comparable to the isolated mitochondrial results (Table 1). The only symptoms visible on the N leaves were necrotic spots at the points of toxin inoculation (Fig. 1).

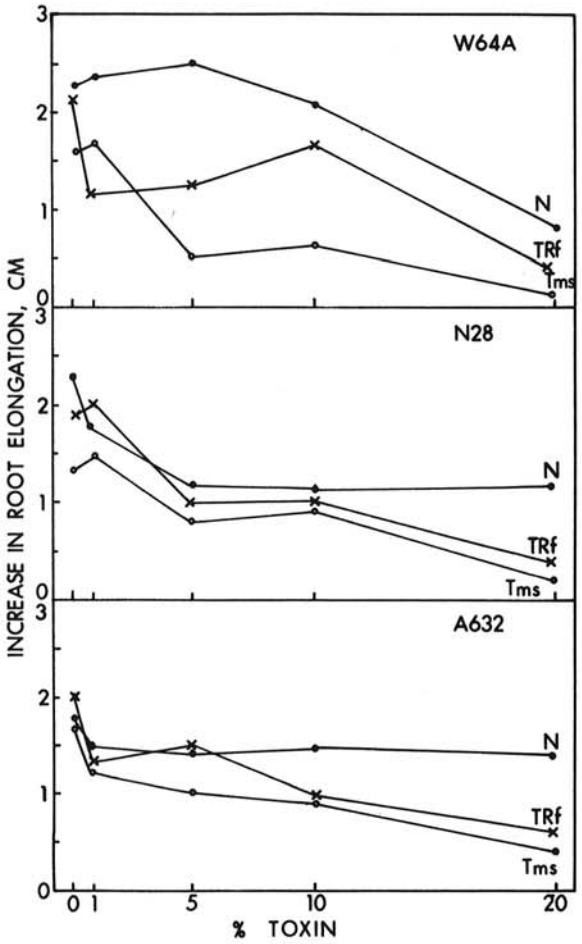

Fig. 2. Comparison of the inhibition of root elongation by *Helminthosporium maydis* race T toxin (0-20%, v/v) in Normal (N) (•—•), Texas male-sterile (Tms) (o—o), and restored Tms (TRf) (×—×) versions of inbreds W64A, N28, and A632 of *Zea mays*. The responses of TRf versions were intermediate to those of their respective N and Tms versions.

The response of isolated mitochondria to toxin from Tms and TRf cytoplasm versions of B37, C103D, and A632 was measured and compared with their susceptibility to race T in the field. Addition of toxin to mitochondria isolated from the Tms shoots increased the rate of oxidation of NADH and the concomitant swelling (Table 2). Addition of toxin to mitochondria isolated from shoots of B37 TRf and A632 TRf were less affected by toxin than were their Tms counterparts (Table 2). The effects of toxin on mitochondria isolated from C103D Tms shoots were less than mitochondria isolated from

A632 Tms or B37 Tms. Whereas decreases were observed in the TRf versions of B37 and A632, as compared with their Tms versions, a dramatic increase in the responses to toxin was observed in the C103D TRf mitochondria as compared with the C103D Tms mitochondria (Table 2).

Root elongation experiments compared the effects of toxin on N, Tms, and TRf versions of given inbreds. At 10 and 20% toxin concentrations, the toxin-affected inhibition of TRf root elongation was intermediate between N and Tms (Fig. 2). The effectiveness of toxin on root elongation varied among the inbreds tested, W64A, N28, and A632.

Further evidence for varied responses of Tms and TRf versions to *H. maydis* race T is presented in Table 3. Comparisons of these versions under field conditions showed 3 patterns: (i) No difference in the degree of infection by race T was detected in the inbred lines FR37 and C123 and the hybrids FR37 × FR14A, FR43 × FR177, and N28 × B14A; (ii) In the inbred lines Mo17, Oh43, A632, and B37, and the hybrid B37 × B14A, however, the TRf versions decreased in infection from 9 to 50% when compared with the Tms versions; and (iii) In C103D and FR14A the TRf versions exhibited possibly increased infection as compared to the Tms versions.

TABLE 3. Comparison of the degree of leaf area blighted in N, Tms, and TRf versions of given inbred lines of *Zea mays* 6 weeks after inoculation in the field with *Helminthosporium maydis* race T

Inbred line or hybrid	Leaf infection (%)			TRf/Tms infection (%)
	N[a]	TRf[b]	Tms[c]	
C123	1	16	16	100
FR37	1	20	20	100
FR37 × FR14A	3	20	20	100
FR43 × FR177	...	13	13	100
N28 × B14A	1	30	30	100
Mo17	1	5	10	50
Oh43	1	20	37	54
A632	1	80	90	89
B37	1	33	37	89
B37 × B14A	3	30	33	91
C103D	1	13	10	130
FR14A	1	15	14	107
		196*	231	

[a]Normal cytoplasm.
[b]Texas male-sterile cytoplasm carrying dominant nuclear factors for restoration of pollen fertility.
[c]Texas male-sterile cytoplasm.
*Chi-square test for goodness of fit of 1:1 ratio of TRf:Tms scores. Significant, $P = 0.025$.

DISCUSSION.—In experiments comparing N, Tms, and TRf versions of the same inbred or hybrid, comparable qualitative toxin effects were observed on isolated mitochondria, root elongation, and leaf lesions. Similar qualitative correlations can be drawn with toxin effects on isolated mitochondria from the inbreds B37, A632, and C103D with field susceptibility data to *H. maydis* race T. However, the role of mitochondria in the responses of whole plants to either *H. maydis* race T, or toxin(s) produced by it, is still hypothetical. It is difficult to explain, e.g., why higher toxin concentrations inhibit root elongation of N corn, but have no effect on isolated N mitochondria. The interesting point, however, is that isolated mitochondria treated with toxin do qualitatively mirror whole plant responses to the toxin, even in the intermediate susceptibility of TRf versions.

Texas male-sterile inbred lines of corn, which show maternal transmission of pollen sterility and which are susceptible to *H. maydis* race T, can have three possible nuclear genotypes, *rf*1*rf*1 *Rf*2*Rf*2, *Rf*1 *Rf*1*rf*2*rf*2, or *rf*1*rf*1*rf*2*rf*2 (5). The first of these is the most common (5). Restoration of fertility to given Tms lines may then involve the introduction of *Rf*1 or *Rf*2 or both *Rf*1 and *Rf*2 alleles. One explanation for the altered sensitivity (most often reduced) in the restored versions as compared with their respective Tms versions, is that other genes, closely linked to *Rf*1 and *Rf*2, were also introduced. Hence, a portion of a nuclear genome having a potentially different level of susceptibility to *H. maydis* race T could then occur in addition to the desired *Rf*1 and/or *Rf*2 contribution. Since the different versions of the inbreds studied were reasonably isogenic (backcrossed a minimum of six generations), the probability that the dominant nuclear restorers *Rf*1 and *Rf*2 modify the effects of the toxin on isolated mitochondria, leaves, and roots cannot be dismissed.

If nuclear genes *Rf*1 and *Rf*2 affect even minor alterations of conformation or composition in mitochondrial or plasma membranes, these changes could suffice to modify the solubility, passage, and action of toxin(s) (6). However, if the specificities of the fungus-leaf, toxin-leaf, toxin-root, or toxin-mitochondria interactions were controlled exclusively by cytoplasmic factors, we would expect that the nuclear restorers would not affect the parameters measured, i.e., that there would be no difference in the responses of Tms and TRf versions of given inbreds.

It is significant that a maternally transmitted trait, pollen sterility, can be restored by the dominant nuclear genes *Rf*1 and *Rf*2 (2, 5). The associated susceptibility of Tms corn in the field to *H. maydis* race T strongly suggests that a cytoplasmic factor, possibly the mitochondria (1, 9), may also play a role in disease reaction. If Tms cytoplasm of corn arose as a cytoplasmic mutation, the mutation might be expected to have occurred in the mitochondrial DNA. Although the bulk of mitochondrial proteins are believed to be coded by nuclear DNA (11), it is generally accepted that the interaction of both nuclear and mitochondrial genomes is involved in the production of essential components such as cytochrome oxidase (4, 7) and ATPase (10). Proteins coded exclusively by mitochondrial DNA are postulated to be generally of a structural nature (3). A slight compositional difference between N and Tms mitochondrial membranes due to a mutation of mitochondrial DNA could conceivably be the limiting factor in penetration of toxin(s). The modifying action of an *Rf* factor on mitochondrial sensitivity could then be due to a compositional change that alters the solubility and passage of the pathotoxin(s). It would be of interest in future studies to assess the individual contributions of *Rf*1 and *Rf*2 in given inbred lines of corn to host response to *H. maydis* race T toxin(s).

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