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In vitro selection for methomyl resistance in CMS-T maize

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Summary. Many plants resistant to methomyl (Lannate), an insecticide which selectively damages maize with the Texas (T) type of cytoplasmic male sterility (CMS-T), were obtained by in vitro selection and also without selection. The selection procedure used 0.6–0.7 m*M* methomyl and callus from CMS-T versions of several field and sweet corn genotypes (W182BN, Wf9, P39, MDM1, SW1 and hybrids of SW1, IL766A1, IL766A2, and 442 with W182BN-N). Addition of 1 m*M* methomyl to the regeneration medium greatly reduced recovery of methomyl-sensitive escapes. Resistance was linked with reversion to male fertility and maternally inherited. Most progeny of resistant plants exhibited stable maternally inherited resistance for two generations in field tests. First-generation progeny of seven culture-derived plants segregated for resistance and sensitivity; this suggests that ears of these seven regenerants were cytoplasmically chimeral. Resistance to methomyl was associated with resistance to T toxin from *Helminthosporium maydis* race T and with changes in mitochondrial physiology. Prolonged culture (14–16 months versus 6–8 months) increased the frequency of resistance among both selected and non-selected regenerants. Little or no resistance was found among regenerants from certain genotypes. Selection with methomyl may be useful for production of improved sweet corn lines and as a source of mitochondrial mutants. This system is also convenient for studies of the effects of nuclear background and of culture and selection systems on the generation of cytoplasmic mutants.

Key words: Methomyl (Lannate) resistance – In vitro selection – Maize (*Zea mays* L.) tissue culture – Cytoplasmic male sterility (CMS) – Mitochondrial mutants

Introduction

The insecticidal compound methomyl (active ingredient of Lannate) damages maize with the Texas (T) male sterile cytoplasm (CMS-T) but not maize with other cytoplasms (Humaydan and Scott 1977). This selectivity is similar to that of T toxin, one of the major secondary metabolites produced by the fungus *Helminthosporium maydis (Drechslera maydis, Bipolaris maydis, Cochliobolus heterostrophus)* race T. *H. maydis* is responsible for the disease southern corn leaf blight (SCLB). After the devastating effects of this disease on hybrid corn produced on T cytoplasm during the early 1970s, use of CMS-T was largely discontinued in field corn. Nevertheless, growers have several reasons for wanting to continue use of CMS-T in some sweet corn lines: (1) early maturity permits these varieties to escape any serious threat of SCLB, which can be prolific later in the growing season; (2) some non-restored (Duvick 1958) as well as restored (T. Natti, personal communication) CMS-T genotypes perform better in hybrid combination than their N ("normal" fertile) counterparts; and (3) male sterility is still the most efficient method of producing hybrid corn.

CMS was recognized during the 1950s as a low-cost alternative to detasseling corn for the commercial production of F_1 hybrid seed. At the time that inbred lines were being converted into sterile cytoplasms, CMS-T was the most reliable of the cytoplasms available (reviewed by Duvick 1965); Corn Belt inbreds were not completely

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sterile in the alternate USDA (S) cytoplasm. T cytoplasm came to be used extensively in the USA following the identification of two codominant nuclear fertility-restorer genes, *Rf1* and *Rf2; Rf2* was already present in many of the popular breeding lines (Duvick 1965). These genes, as well as other unidentified fertility-modifier genes, could be introduced via the pollen parent to insure production of grain by the hybrid progeny. Maize with *Rf1* and *Rf2* is still susceptible to damage by Lannate, widely used throughout the growing season for the control of various pests including European corn borer (*Ostrinia nubilalis*), fall armyworm (*Spodoptera frugiperda*) and corn earworm (*Heliothis zea*).

Both methomyl, a carbamate with Mr = 162.2, and T toxin, thought to be a long-chain polyketol, Mr = 768 (Kono and Daly 1979), disrupt respiration of mitochondria isolated from cells containing T cytoplasm (Koeppe et al. 1978). The two compounds show about a six-order of magnitude difference in the concentration required to effect their minimal actions at the mitochondrial level (~ 10 n*M* toxin versus 10 m*M* methomyl). Normal cytoplasm mitochondria are not affected by these compounds, or are affected to a much lesser extent. Recently it has been shown that a hydrophobic 13-K polypeptide, encoded by *urf*13-T in mitochondria from T, but not N cytoplasm (Dewey et al. 1987; Wise et al. 1987a), confers susceptibility to both methomyl and T toxin when produced by *E. coli* transformed with the T-specific gene (Dewey et al. 1988). The differences in structure and activity suggest that the two compounds might have distinct binding domains on the 13-K protein.

Work by several researchers (Gengenbach and Green 1975; Gengenbach et al. 1977, 1981; Brettell et al. 1979, 1980; Umbeck and Gengenbach 1983) has demonstrated that tissue culture, without any supplementary mutagenesis, is a technique suitable for generation of, and selection for, T toxin resistance expressed at the callus and the whole plant levels. Resistance was shown to be maternally inherited, accompanied by a reversion to male fertility, and involved alterations of the mitochondrial physiology and genome.

This study examines the use of methomyl, rather than T toxin, as a selective agent. The goals of the work were: (1) to develop appropriate selection procedures, (2) to examine the effect of nuclear genotype and time in culture on recovery of resistant material, (3) to study the inheritance and basis of any resistance recovered, (4) to compare material obtained by selection with methomyl with that obtained via T toxin selection, (5) to generate new cytoplasmic mutants for molecular and biochemical studies. A second report (A. R. Kuehnle and E. D. Earle, in preparation) compares the effects of different selection pressures on recovery of methomyl-resistant callus and plants. Study of the mitochondrial DNA of resistant lines recovered from culture is in progress.

Materials and methods

Plant materials

Callus cultures (lines) were established in 1985 and 1986 from single immature embryos excised from nine different field-grown maize (*Zea mays* L.) genotypes, all carrying CMS-T (Table 1). The inbred W182BN was used as a pollen parent in several of the genotypes to aid in regenerability of callus; the sweet corn inbreds had not previously been established in culture, and crosses with W182BN often improve regeneration (E. D. Earle, unpublished results). Callus was increased over a period of 3 months (4 subcultures) before selection was initiated, and was selectively subcultured on a monthly basis thereafter (one selection cycle equals one month). Cultures of inbreds W182BN and Wf9 with N cytoplasm were also established for use in comparisons of N and CMS-T material. Callus was primarily type I (heterogeneous and compact) and pale yellow or white.

Inbreds W182BN (with N cytoplasm or CMS-T) and W64A (N cytoplasm, CMS-T or CMS-T*Rf*) were used to determine the effects of methomyl on elongation of excised root tips.

Media and culture conditions

Callus was established on two different nutrient media, MS2 and D, solidified with 0.88% Bacto-agar or 0.22% Gel-Rite (Kelco Corp.) and adjusted to pH 5.8 before autoclaving. MS2 medium contains MS salts (Murashige and Skoog 1962) supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 4% sucrose, 0.4 mg/l thiamine, 100 mg/l myo-inositol and 2.3 g/l proline. Medium D (Duncan et al. 1985), based on N6 salts

Table 1. Genotype and male fertility of sources for 1985 and 1986 CMS-T callus cultures

Genotype designation	Pedigree of embryo sources for callus cultures[a]	Fertility[b]
IL766A1 hybrid	(W182BN-T × IL766A1, F_1) × W182BN	sterile
IL766A2 hybrid	(W182BN-T/P39 × IL766A2, F_1) × W182BN	partially fertile or unrated
MDM1	(W182BN-T/MDM1, bc4 × MDM1) × MDM1	partially fertile
P39	(W182BN-T/P39, bc4 × P39) × P39	sterile
SW1	(W182BN-T/SW1, bc3 × SW1) × SW1	sterile
SW1 hybrid	(W182BN-T/SW1, bc × SW1) × W182BN	sterile
W182BN	(W182BN-N/ijij-Ev, bc5 × W182BN)[c] × W182BN	sterile
Wf9	(Wf9-T/Wf9,inbred) × Wf9	sterile
442 hybrid	(W182BN-N/442, bc5 × W182BN) × W182BN	sterile

[a] All parents except W182BN and Wf9 are sweet corn. All female parents are CMS-T

[b] All maternal parents show methomyl sensitivity regardless of male fertility

[c] A CMS-T line (CMS ij-2) derived from the cross of a fertile, normal cytoplasm W182BN with a homozygous source of the recessive gene, iojap (Lemke et al. 1988)

(Chu et al. 1975), contains 3.3 mg/l 3,6-dichloro-o-anisic acid (Dicamba). After two subcultures, callus was transferred either to MS5 (5 mg/l 2,4-D and no proline) or continued on medium D. Callus from W182BN and IL766A2 hybrid was of similar organization (hard, organogenic) and grew best on MS5; all other genotypes were plated on medium D. Cultures were incubated in the dark at 25 °C.

The media used for selection consisted of MS5 or D containing a range of methomyl concentrations (0.6, 0.65, or 0.7 m*M*). Maintenance media for the control callus did not contain methomyl.

Callus from both selective and non-selective media was induced to regenerate plants after 6–8 months and again after 14–16 months in culture. Regeneration was induced by transfer of tissue from either MS or D medium to MS medium lacking growth regulators (MS0) and containing 10% sucrose (MS0-10%). Methomyl was sometimes added to MS0-10% for a final concentration of 1 m*M*. After 1 month on MS0-10%, callus was transferred onto MS0, 4% sucrose and moved into light (16 h/day, 70–100 $\mu E/m^2/s$, mixed cool-white and Gro-lux bulbs). After roots were visible on developing shoots, plantlets were moved to Magenta GA-7 boxes or glass test tubes containing half-strength MS0, 2% sucrose. Vigorous plantlets were then transferred into a soil-vermiculite mix and placed in a growth chamber (12 h/day, 70–100 $\mu E/m^2/s$) for several weeks. After leaves were tested for methomyl sensitivity, plants were moved to the greenhouse and grown to maturity.

Methomyl powder (97% purity), a gift of Dupont Agricultural Chemicals (Wilmington/DE), was dissolved in warm, sterile, distilled and deionized water. Aliquots of fresh stock solution (0.3 *M*) were added without sterilization to autoclaved media, which were then poured into plastic petri dishes. Addition of methomyl did not affect the pH of the media.

Effect of methomyl on callus and root-tip growth

To determine the concentrations of methomyl to use for selection, the effect of methomyl on 2-month-old callus and on seedling root tips of several genotypes was tested. Callus growth was measured by the difference between initial and final fresh weights of callus pieces during 1 month of culture. The same embryo-derived callus lines, in the exponential growth phase, were used in each growth experiment.

For the root growth bioassay, corn seeds were surface sterilized in 30% Clorox bleach and 0.5% Pex detergent for 30 min, rinsed with 4 vol of sterile distilled H_2O, and germinated in the dark at room temperature between layers of moistened, sterile paper towels until 2-cm roots had emerged. Root tips (1 cm) were excised and placed in wells in a 6-well plate. Six to ten 1-cm root tips were used per treatment, with one replication. Each treatment consisted of MS0, 4% sucrose ± methomyl. The multiwell plates were sealed with Parafilm, wrapped together in aluminum foil and placed on a gyrotory shaker at 50 rpm for 3 days, following which root-tip lengths were measured.

Selection for methomyl resistance

In November of 1985, over 2,800 pieces of callus, representing eight genotypes, were plated onto media containing methomyl or onto control media. The initial fresh weight of each piece ranged from 30 to 40 mg. Approximately 15 pieces were cultured per 100 × 15 mm plastic petri dish and 5 per 60 × 15 mm dish. Callus pieces chosen for the initial selection were light yellow and appeared regenerable when viewed with a dissecting microscope. At each subculture regenerable tissue that was pale yellow, like the control callus, was selected for transfer. Growth per se was not a criterion for transfer. A similar procedure was used in 1986 with the 442 hybrid callus.

Three types of selection pressure (low, gradually increasing, and high) were used during 3 to 5 monthly selection cycles. Low selection pressure consisted of constant exposure to 0.6 m*M* methomyl; gradually increasing began at 0.6 m*M*, increased to 0.65 m*M* and then to 0.7 m*M*; high was constant at 0.7 m*M*. After the third to fifth selection cycle, some callus was transferred to regeneration medium; comparable callus was maintained on at least 0.65 m*M* methomyl for at least eight additional subcultures. Plants were again regenerated after 11 to 13 selection cycles.

Evaluation of regenerants and their progeny

Regenerated plantlets were tested for resistance to methomyl at the 3- to 5-leaf stage by swabbing a concentrated methomyl solution (0.3 *M*) onto the distal sections of an intact leaf with a Q-tip. Bleaching or necrosis of the treated regions of CMS-T plants was visible after about 3 days. No effects were seen on the treated leaves of plants with other cytoplasms (N, CMS-C, CMS-S). Regenerants which did not exhibit bleaching or necrosis were scored as resistant. In this assay, it was important to use leaves produced by the plantlets after transfer out of culture containers. In vitro testing or use of leaves formed in vitro did not distinguish between resistant and sensitive plants; even leaves treated with water (controls) showed a sensitive response.

Male-fertile regenerated plants were selfed when possible. Sterile ones, including plants with abnormal tassels, were pollinated by the sweet corn parent or W182BN, when possible. The male fertility of regenerated plants (R_0) and their first and second generation progeny (R_1 and R_2, respectively) was rated on a scale of 1 (fully sterile) to 5 (fully fertile) (Beckett 1971). Mature anthers were stained with acetocarmine and examined microscopically to evaluate pollen grain viability. Grains which appeared stained, rounded and full, rather than shrunken, were rated as normal. Plant height was recorded for the regenerants, their progeny, and non-regenerated checks.

Some fertile R_1 plants were grown in the greenhouse and crossed onto fully male-sterile plants (both tissue culture-derived R_1 and seed-grown plants) to use as testcrosses for detecting nuclear resistance or fertility-restorer genes. Testcrosses included 8 different R_1 × tester combinations for the Lannate ratings and 15 different combinations for the fertility ratings. Testcrosses were evaluated in 1987 in Ithaca/NY.

Field tests for methomyl resistance among R_1 and R_2 progeny were conducted in two locations during 1986–1987 using commercially available Lannate 90 (DuPont). In Homestead/FL plants were treated for 1 month with biweekly sprayings at the recommended rate of 0.56 kg/ha beginning 1 month prior to anthesis. In Ithaca/NY plants were treated with a biweekly spraying at the recommended rate followed by one application at slightly more than twice the rate recommended for corn (1.25 kg/ha), also approximately 1 month prior to anthesis. Those plants which did not show bleaching or necrosis 6 days after the final treatment were rated as resistant.

Progeny of regenerants were examined for sensitivity to T toxin by injection of diluted *H. maydis* race T culture filtrate (Earle et al. 1978) into leaf whorls. The activity and CMS-T specificity of the preparation was ascertained by injection of N and T cytoplasm checks. In the T cytoplasm checks, the filtrate caused several centimeters of bleached or necrotic tissue to develop from the injection sites. N cytoplasm checks showed only small chlorotic halos restricted to a few millimeters around the injection holes.

Assay of isolated mitochondria by phosphorylation reactions

Mitochondrial suspensions were prepared from 3-day-old etiolated shoots from N and T checks and from sensitive and resis-

tant R_1 and R_2 material, according to the procedure of Gregory et al. (1980). Mitochondrial phosphorylation reactions were carried out by adding 10 µl of suspension to a 0.5 ml volume containing 200 m*M* sucrose, 20 m*M* Tricine-KOH, 20 m*M* KH_2PO_4 (including 2 µCi of $^{32}PO_4$), 2.5 m*M* ADP, 1.0 mg BSA, and 3.75 m*M* NADH, as described by P.-Y. Bouthyette (personal communication). The pH of the ADP and the Tricine-KOH and KH_2PO_4 stock solutions was adjusted to 7.0 and 7.5, respectively, prior to addition to the reaction volume. Some reactions were carried out in the presence of 1 m*M* methomyl or 40 ng purified T toxin (gift of J. M. Daly). Phosphorylation was stopped after a 20 min incubation at 37 °C by the precipitation of inorganic phosphate following the addition of 1.8 ml of Sugino-Miyoshi reagent (Sugino and Miyoshi 1964). Following centrifugation for 5 min at 1,000 g, the supernatant was counted without liquid scintillant using a Beckmann 7,000 liquid scintillation counter. Mitochondrial protein was determined using a modified Bradford method (Pierce Chemical) with BSA standards. Phosphorylation activity was determined as the nmol ATP formed per minute per milligram of protein. Values were compared with those of the control mitochondria for each mitochondrial preparation.

Results

Effect of methomyl on callus and root tip growth

For all CMS-T genotypes examined, methomyl in the 0.6–0.7 m*M* range decreased callus growth, with greater inhibition at the higher levels (Fig. 1). Control callus growth rates as well as sensitivity to methomyl varied widely among genotypes. Callus organization and/or genotype may affect methomyl uptake or transport. Callus inhibited by methomyl turned pale or white (compared to control callus, which was usually light yellow), but retained its embryogenic or organogenic organization. Pale callus which failed to grow on high methomyl concentrations did not resume growth after transfer to methomyl-free medium.

The effect of methomyl on maize callus was cytoplasm-specific. Growth of W182BN callus with N cytoplasm was not significantly inhibited by 0.7 m*M* methomyl, a level which prevented growth of W182BN-T (Fig. 1). Growth of Wf9 callus carrying N cytoplasm was not inhibited even by methomyl levels of 1.0 m*M*, although growth of Wf9 callus with T cytoplasm was strongly inhibited at 0.6 m*M* (data not shown).

Growth of CMS-T root tips was also inhibited by methomyl (Fig. 2). Tips excised from W182BN-T roots (Fig. 2a) showed a significant reduction in length, compared with the controls, in the same range of methomyl concentrations that inhibited callus growth. Roots of the T*Rf* version of the inbred W64A were consistently less sensitive than T, but more sensitive than N roots in the intermediate range of methomyl concentration (Fig. 2b). Since the growth of callus and excised root tips was inhibited at similar methomyl concentrations, the root bioassay may be a useful first step to determine appropriate methomyl concentrations for in vitro selection.

Selection for methomyl resistance

Callus from five genotypes cultured in the presence of methomyl for 3–5 cycles (total of 6–8 months in vitro)

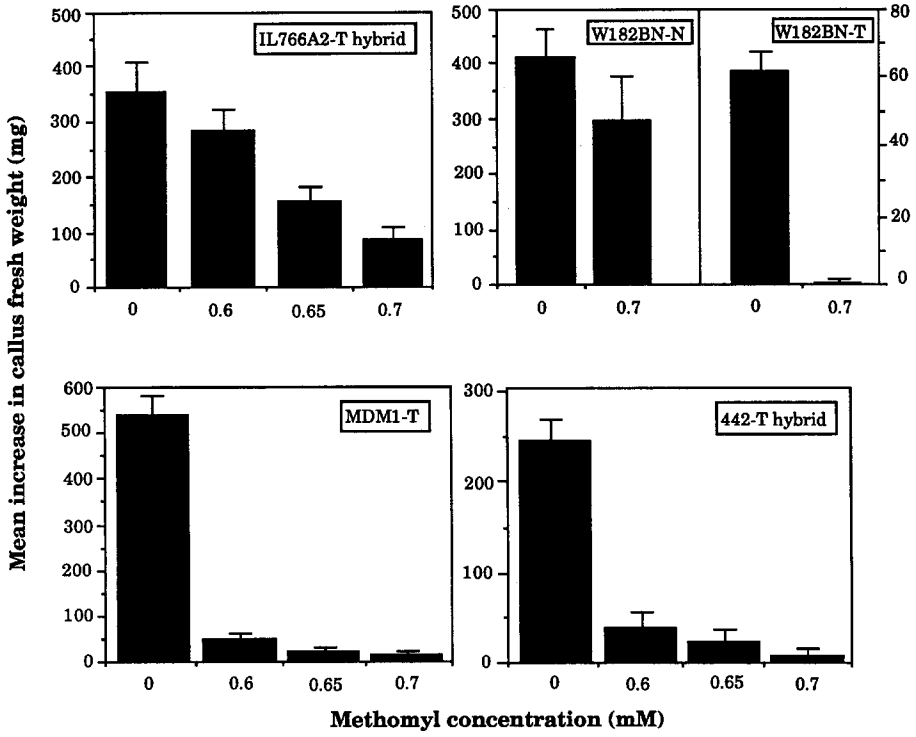

Fig. 1. Effect of methomyl on mean increase in fresh weight of callus from several maize genotypes following 1-month culture. IL766A2 hybrid and W182BN were grown on MS5 medium; MDM1 and 442 hybrid were grown on medium D. IL766A2-T, MDM1-T, and 442-T are CMS-T lines; for W182BN, results for both N cytoplasm (W182BN-N) and CMS-T (W182BN-T) callus are included. Growth of W182BN-T callus, but not W182BN-N callus, is strongly inhibited in the presence of 0.7 m*M* methomyl

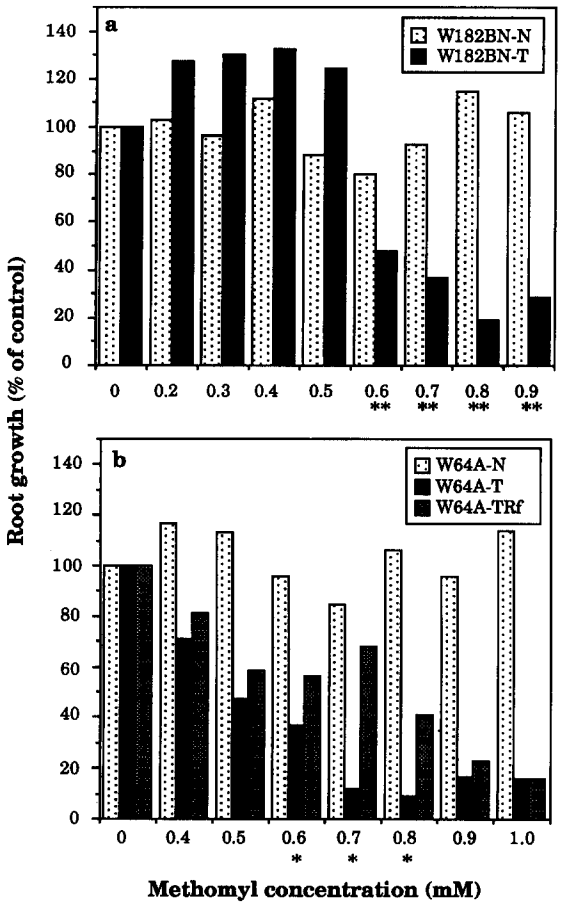

Fig. 2 a and b. Effect of methomyl on elongation of root tips excised from different cytoplasm versions of **a** W182BN and **b** W64A sprouted seed. **T growth is significantly lower than N growth ($p \leq 0.001$). *T growth is significantly lower than T*Rf* growth ($p \leq 0.03$); both are lower than N at all methomyl values ($p \leq 0.007$). Mean values were compared using the F-test (Snedecor and Cochran 1980)

yielded many resistant plants (Table 2). Resistance was recovered from all three selection regimes (low, increasing or high methomyl concentrations), with none of the regimes emerging as clearly superior for producing resistance. Only three resistant plants were obtained from non-selected callus. The difference in recovery of resistant plants from non-selected and selected cultures was not due to preferential regeneration of resistant plants. Ability of the callus lines listed in Table 2 to regenerate plants did not differ significantly among genotypes or between no exposure or exposure to methomyl (data from analysis of variance not shown).

Resistant plants were obtained from several different lines (each derived from a single embryo) within each genotype (Table 3). Most of the callus lines that produced resistant regenerants after 6–8 months also produced sensitive ones. The exception is IL766A1 hybrid, where two cultures produced only resistant plants and one produced both resistant and sensitive ones. Plant genotype may influence the success of selection for methomyl resistance (Tables 2 and 3). The genotypes IL766A1 hybrid, IL766A2 hybrid, SW1 hybrid, and W182BN responded very well to selection, with 60%–100% of their callus lines yielding some resistant plants (Table 3). In contrast, the genotype 442 hybrid was notably poor in producing resistance: none of the 54 plants regenerated from seven

Table 2. Resistance to methomyl among plants regenerated from callus after 6–8 months in culture[a]

Genotype designation	No selection	Methomyl selection[b]	
	non-selective regeneration[c]	non-selective regeneration	selective regeneration[c]
	No. resistant/No. regenerated		
IL766A1 hybrid	0/2	15/21	25/25
IL766A2 hybrid	3/97	25/44[d]	9/9
MDM1	0/7	0/1	–
P39	–	0/3	2/3[e]
SW1	0/1	–	–
SW1 hybrid	0/19	28/33	–
W182BN	0/3	8/8	6/6
Wf9	0/3	0/2	–
442 hybrid	0/22	0/54	–

[a] Ratings are based on leaf response to 0.3 *M* methomyl. All resistant regenerants with normal tassels were male fertile except where noted

[b] Includes low, gradually increasing and high selection intensities

[c] Non-selective regeneration: no methomyl in regeneration medium. Selective regeneration: 1 m*M* methomyl in the regeneration medium. With selective regeneration, no plants were recovered from control (no selection) callus

[d] Includes three resistant, male-sterile plants. Progeny were not stable for this combination of traits

[e] No seed was obtained from the sensitive regenerant

Table 3. Number of different callus lines from which methomyl resistant plants were regenerated after 6-8 months in culture

Genotype designation	Callus lines[a] which produced resistant plants[b]/lines used in selection (%)
IL766A1 hybrid	3[c]/3 (100)
IL766A2 hybrid	11/12 (92)
MDM1	0/3 (0)
P39	2/9 (22)
SW1	0/2 (0)
SW1 hybrid	3/5 (60)
W182BN	2/3 (67)
Wf9	0/8 (0)
442 hybrid	0/7 (0)

[a] Each callus line was derived from a single embryo

[b] Except where noted, each line also produced sensitive plants

[c] Only one line produced both sensitive and resistant plants

distinct embryo lines after selection were resistant to methomyl (Table 2). Three other CMS-T genotypes also produced no resistant regenerants. Callus from Wf9 barely survived selection, while MDM1 and SW1 had generally poor callus, including the controls. A fourth genotype, P39, also showed poor overall callus growth but yielded two resistant regenerants. In these particular cases it was difficult to assess the influence of genotype on the success of selection.

When 1 m*M* methomyl was included in the regeneration medium, only resistant plants were recovered; sensitive material was effectively eliminated (Table 2). At very high levels of methomyl (i.e. 6.85 m*M*), no plants were recovered, even from N cytoplasm lines.

Because many of the genotypes used in this study demonstrated long-term regeneration capacity, it was possible to examine the effects of time in culture on methomyl resistance (Tables 4 and 2). Two types of effects were seen. A higher proportion of plants from 14- to 16-month-old non-selected callus showed resistance than plants from 6- to 8-month-old non-selected callus. Moreover, almost all of the plants from selected older callus were resistant, even if the regeneration medium lacked methomyl.

All regenerated plants were examined for male fertility. Of the 455 R_0 plants, 44 produced no tassels and/or were not rated. Virtually all (263/270, 97%) of the methomyl resistant plants which produced tassels were fully male fertile. Plants with discordant ratings of leaves and tassel (i.e. methomyl-resistant leaves and male-sterile tassels or vice versa) were obtained from several different genotypes, but this combination of phenotypes was not stable in subsequent generations. Most methomyl-sensitive regenerants retained the fertility rating of their maternal parent (Table 1).

Most R_0 plants were as vigorous and normal-looking as seed-grown controls. However, abnormal plants (unusual phyllotaxy, stunting, feminization of tassels) also appeared occasionally.

Table 4. Resistance to methomyl among plants regenerated from callus after 14–16 months in culture[a]

Genotype designation	No selection	Methomyl selection[b]	
	non-selective regeneration[c]	non-selective regeneration	selective regeneration[c]
	No. resistant/No. regenerated		
IL766A1 hybrid	2/5	35/36[d]	34/34
IL766A2 hybrid	16/24	23/23[e]	18/18
P39	–	0/1	–
SW1 hybrid	0/1	1/1	4/4
W182BN	1/1	14/14	2/2

[a] Resistance ratings are based on leaf response to 0.3 *M* methomyl. All resistant regenerants are male fertile except where noted. The inbred genotypes MDM1, SW1, and Wf9 were not included in the 14–16 month regeneration study

[b] Callus was exposed to methomyl (at least 0.65 m*M*) for an additional eight subcultures following the regeneration of plants from some callus after 3–5 months selection

[c] After selective regeneration, five plants were recovered from unselected IL766A1, SW1 hybrid and W182BN callus. All were resistant

[d] Includes one resistant, partially fertile plant. No seed was obtained

[e] Includes one resistant, male sterile plant and two resistant, partially fertile plants. Seed was obtained from all three plants

Evaluation of progeny

Although quantitative ratings were not done in the 1986 winter nursery in Homestead, first-generation progeny of resistant regenerants clearly showed less leaf damage by Lannate than progeny of sensitive regenerants. Field tests conducted during the summer of 1987 showed that resistance to Lannate was stable through two generations and that the trait was maternally inherited. The plants evaluated in 1987 represented first- and/or second-generation progeny of 98 regenerated plants derived from 13 different embryo sources and five different genotypes. R_1 progeny of most of the regenerants (80/90, 89%) showed the same response to Lannate as the maternal parent (Table 5). Segregation ratios for the response to Lannate spray indicate maternal inheritance of Lannate resistance in the R_2 generation as well (Table 6).

Two types of deviations from the maternal response were observed among R_1 progeny of the other R_0s: segregation and non-maternal response. Segregation was seen in 7 of 90 rows, 5 of which came from discordant R_0 plants (Table 5). The segregating rows gave no familiar ratios of resistant to sensitive and were assumed to come from R_0s with chimeral cytoplasms. No segregation was seen among N and T cytoplasm controls. Progeny from 3 other discordant R_0s gave a uniform non-maternal response (Table 5). The R_2 generation showed no segregation but only clear-cut maternal inheritance; all 101 rows gave the maternal response (data not shown). The fact that no segregation was seen in the R_2 generation suggests that the chimeral cytoplasm of some of the original regenerants sorted out during the R_1 generation.

Lines that segregated for Lannate resistance in the R_1 generation also segregated for resistance to T toxin. All other Lannate-resistant progeny were also toxin-resistant, and all Lannate-sensitive progeny were toxin-sensitive.

None of the 270 plants derived from testcrosses (crosses of male-fertile R_1 onto sterile cms-T females) were male-fertile, indicating that fertility of regenerants did not involve novel or newly activated dominant nucle-

Table 5. Response to Lannate in ear-to-row plantings of first-generation (R_1) progeny from resistant and sensitive regenerated (R_0) plants[a]

R_0 rating	Pollination	n[b]	R_1 response to Lannate		
			Maternal	Segregating	Non-maternal
			No. of rows		
Resistant	self	26 (262)	25	1[c]	0
	cross as female	35 (344)	30	2[d]	3
	cross as male	8 (108)	8	0	0
Sensitive	self	2 (26)	0	2[e]	0
	cross as female	19 (174)	17	2[e]	0
Total		90 (914)	80	7[f]	3[g]

[a] Data taken in 1987 in Ithaca/NY. Lannate 90 was applied at 1.25 kg/ha, slightly more than twice the rate recommended for sweet corn

[b] Number of R_0 plants whose progeny were tested (total number of progeny scored)

[c] This row represents progeny of a resistant R_0 plant derived from non-selected IL766A2 hybrid callus after 6–8 months in culture

[d] One row represents progeny from a male-sterile methomyl-resistant (discordant) regenerant

[e] These rows represent progeny of fertile methomyl sensitive (discordant) R_0 plants obtained from callus selected on methomyl but regenerated on methomyl-free medium

[f] Unusual segregation ratios (not shown) suggest that the R_0 plants had chimeral cytoplasms. Five of these rows are from discordant regenerants. No segregation was observed among N- and T-cytoplasm controls

[g] A non-segregating, non-maternal rating suggests discordance between leaf and reproductive tissues of the R_0 plants. All three R_0 were methomyl-resistant but male sterile

Table 6. Response to Lannate among progeny from selected crosses using resistant first-generation plants (R_1) derived from resistant regenerants (R_0)[a]

Resistant R_1 line	× resistant N male	× sensitive CMS-T female
	Sensitive:resistant	Sensitive:resistant
SW1 hybrid		
2-13D	0:19	12:0
2-13F	0:16	11:0
2-13W	0:11	7:0
W182BN		
37-yr7	0:9	–
37-yr4	–	19:0
37-B4	–	12:0
IL766A1 hybrid		
66A	0:4	–
66C	0:7	18:0
66E	0:12	–
66G	0:5	–
66H	0:8	–
66J	0:10	–

[a] Data taken in 1987 in Ithaca/NY. Lannate 90 was applied at 1.25 kg/ha, slightly more than twice the rate recommended for sweet corn. Segregation ratios of Lannate sensitive: Lannate resistant are for individual rows representing each cross. R_1 lines within each genotype are closely related and share similar line designations – their R_0 parents were derived from the same callus culture. The plants scored are progeny from R_1 lines crossed with CMS-T or N cytoplasm plants available at the time of pollination

ar fertility-restorer genes. Furthermore, none of the 108 testcross progeny treated with Lannate were rated as resistant, i.e., Lannate resistance was not controlled by dominant nuclear genes. Field evaluation of N and T cytoplasm checks, representing the genotypes W182BN, IL766A1 hybrid, IL766A2 hybrid, SW1 hybrid, and P39, showed 173/173 N cytoplasm plants and 0/199 CMS-T plants were resistant to Lannate.

There were no significant differences in the height of field-grown resistant and sensitive progeny, within or among the genotypes (data not shown). Methomyl-resistant plants with fully fertile tassels had 70%–90% normal-appearing pollen grains, like N cytoplasm controls.

Assay of isolated mitochondria by phosphorylation reactions

Alterations in mitochondrial respiration are associated with methomyl resistance. Mitochondria from etiolated CMS-T shoots showed a significant inhibition of ADP phosphorylation in the presence of methomyl or T toxin, while those from N cytoplasm shoots did not. Like mitochondria from N cytoplasm, mitochondria from progeny of resistant regenerants were not inhibited, regardless of the cross made to obtain the progeny or the filial generation sampled (Table 7). Mitochondria from progeny of sensitive regenerants were equivalent to those from seed-grown CMS-T plants in their response.

Table 7. Effect of methomyl and T toxin on ADP phosphorylation by isolated mitochondria of culture-derived plants

Genotype and generation[a]	Entry[d]	Lannate response[b]	H_2O	Methomyl (1 m*M*)	T toxin[c] (40 ng/ml)
			nmol ATP/min/mg protein (% of H_2O control)		
IL766A1 hybrid					
R_1	a	S	20.5	2.6 (13)**	–
R_1	b	R	21.0	19.9 (95)	–
R_1	b	R	41.4	26.0 (87)	–
R_1	b	R	26.9	25.0 (93)	–
R_1	c	R	30.1	22.6 (75)	–
R_1	d	R	44.6	40.2 (90)	–
R_2	a	S	18.1	1.4 (19)**	1.4 (8)**
R_2	b	R	24.1	21.9 (91)	24.5 (102)
R_2	b	R	16.5	16.6 (101)	17.9 (108)
R_2	b	R	34.3	32.7 (95)	36.5 (106)
R_2	c	R	15.9	15.5 (98)	15.3 (99)
R_2	d	R	19.3	17.9 (93)	18.8 (97)
R_2	d	R	18.1	15.5 (86)	17.3 (96)
IL766A1-N	check	R	43.3	32.5 (75)	–
IL766A1-T	check	S	18.1	2.2 (12)**	–
IL766A2 hybrid					
R_1	e	S	16.7	7.3 (44)**	–
R_1	f	R	42.3	37.5 (89)	–
R_2	g	R	18.4	16.1 (88)	18.7 (102)
R_2	g	R	21.7	19.6 (90)	21.8 (100)
SW1 hybrid					
R_1	h	S	19.7	3.5 (18)**	–
R_1	i	R	31.2	27.1 (87)	–
R_1	j	R	30.3	26.1 (86)	–
R_2	h	S	28.3	9.3 (33)**	–
R_2	i	R	16.1	15.0 (93)	15.9 (99)
R_2	i	R	21.2	20.6 (97)	22.7 (107)
R_2	i	R	24.5	24.4 (100)	25.5 (104)
R_2	i	R	30.6	27.2 (89)	29.9 (98)
R_2	i	R	33.3	27.6 (83)	–
SW1-N	check	R	28.0	27.2 (97)	–
SW1-T	check	S	20.1	5.0 (25)**	–
W182BN-N	check	R	23.8	22.2 (93)	–
W182BN-T	check	S	24.8	2.2 (9)**	1.6 (6)**

[a] These plants are progeny from selfed or cross-pollinated tissue culture-derived lines. The genotypes are hybrids with W182BN

[b] Plants showed resistance (R) or sensitivity (S) to Lannate spray in the field

[c] Purified T toxin was obtained from J. M. Daly. The H_2O controls for the toxin samples are not shown

[d] Similar entry letters identify progeny arising from the same embryo-derived callus culture

** Phosphorylation rates of sensitive plants are significantly lower than those of methomyl-resistant plants ($p=0.00$). T, but not N cytoplasm checks show a similar response. Mean values of resistant and sensitive lines were compared using the student's *t* test (Snedecor and Cochran 1980)

Discussion

Resistance to methomyl in CMS-T maize was readily obtained by in vitro selection. The resistance trait appears to be identical to culture-derived T toxin resistance at the whole-plant and subcellular levels: it is maternally inherited, associated with reversion to male fertility, and expressed at the mitochondrial level.

Prolonged time in culture greatly increased the frequency at which resistance developed, with and without exposure to methomyl. With IL766A2 hybrid, 25/44 (57%) plants recovered from 6- to 8-month-old callus, with 3 months of selection and non-selective regeneration, were resistant, but only 3/97 (3%) plants recovered from similar callus not exposed to methomyl were resistant. After 14–16 months of culture, with 11–13 months of similar selection, 35/36 (97%) plants recovered from IL766A2 were resistant. Recovery of resistant plants from unselected callus was also greatly increased (16/24 or 67%). This indicates that the increase in resistance involves spontaneous changes in the culture and is not simply a result of prolonged selection.

Brettell and co-workers (1979, 1980) and Umbeck and Gengenbach (1983) recovered plants resistant to T toxin without selection using 11- to 12-month-old cultures of the genotype A188 or an A188 hybrid combination. Clear-cut evidence about the relation between time in culture and maternally inherited alterations in maize is available for W182BN CMS-S cultures (Earle et al. 1987). In that system, spontaneous cytoplasmic alterations occur more rapidly than in the CMS-T cultures; some reversion to male fertility was seen after only 3 months, and virtually all 1-year-old cultures produced only fertile plants.

Root tips and callus from different nuclear backgrounds vary in their sensitivity to methomyl. Two types of comparisons can be made, one concerning the presence of *RF1* and *Rf2* and the second concerning overall genotype. An example of the influence of the restorer genes on sensitivity to methomyl is seen with W64A root tips: the CMS-T*Rf* roots of this inbred were less sensitive than the CMS-T roots, though more sensitive than the N cytoplasm ones. A related observation has been reported by Watrud et al. (1975) with W64A-T*Rf*, -T and -N seedlings exposed to T toxin. Although the *Rf* genes suppress synthesis of the T-specific 13-K polypeptide (Forde and Leaver 1980) and may thereby lower sensitivity to T toxin or methomyl, fertility per se is not a clear predictor of resistance. Mitochondria isolated from tissue-culture-derived fertile revertant plants showed variable sensitivity depending on whether the 13-K polypeptide was produced at or below 33% maximum synthesis (full or intermediate sensitivity, respectively) (Dixon et al. 1982). At the callus level, the partially fertile line, MDM1, was extremely sensitive to methomyl concentrations at which callus from other fully male-sterile or fully male-fertile genotypes was only mildly inhibited.

The *Rf1* and *Rf2* status of the eight genotypes used in this original study is currently being determined. The *Rf1* allele has recently been shown to regulate the production of the 13-K protein in mitochondria from CMS-T plants by modification of *urf*13-T transcripts (Dewey et al. 1986, 1987). Regulation by *Rf1* does not involve alteration of the coding region's primary sequence (Stamper et al. 1987). The role, if any, of *Rf2* in sensitivity to methomyl and T toxin has not yet been elucidated. It would be informative to repeat the in vitro selection procedure with inbred genotypes for which both restored and sterile T cytoplasm versions are available.

The overall nuclear genotype also appears to influence sensitivity to methomyl. Callus growth data showed a clear difference in response among the various genotypes. These differences were not entirely unexpected since variable sensitivity to Lannate spray among different genotypes has been seen in the field (V. Gracen and J. Kelly, personal communication). Sensitivity to T toxin among mitochondria isolated from different CMS-T inbreds also varied significantly (Pham 1980). Nuclear background, independent of fertility restoration, has been reported to affect the abundance of the transcripts from *urf*13-T (Kennell et al. 1987).

Nuclear background may also affect the rate at which cultures become resistant to methomyl. For example, there were no resistant plants among the many regenerated from 442 hybrid, in spite of selection conditions which were successful for similar callus lines.

Nuclear genotype might mediate the response to methomyl and the development of resistance by influencing rearrangements of the mtDNA. Although the original source of all the T cytoplasms used in this study was the inbred W182BN [with the exception of CMS *ij*-2 (Table 1)], in vivo and/or in vitro changes in the mtDNA may have occurred under the influence of the diverse sweet corn and hybrid nuclear backgrounds used. There is mounting evidence that mtDNA alterations in CMS-S revertants are affected by nuclear background (Escote et al. 1985, 1988; Small et al. 1988). In the toxin-resistant CMS-T revertants, a similar recombination event has occurred among the majority of resistant lines; this event leads to the deletion of the gene *urf*13-T (Rottmann et al. 1987; Fauron et al. 1987; Wise et al. 1987b). The putative recombination site has been identified by several researchers (Fauron et al. 1987; Rottmann et al. 1987). If nuclear gene products of a particular genotype sequester or alter this or some other site, deletion of *urf*13-T may be hindered and sensitivity to methomyl would be retained.

Different nuclear genotypes may also show variation in relative stoichiometries of DNA fragments and in low molecular weight molecules among mitochondrial genomes isolated from the same class of cytoplasm (Levings and Pring 1977; Pring et al. 1979; McNay et al. 1983; Newton and Walbot 1985; Small et al. 1987). Other quantitative changes in mtDNA restriction fragments have been described for tissue cultures of wheat and maize (Rode et al. 1987; McNay et al. 1984).

Restriction fragment analysis of mtDNA from the methomyl-resistant lines has revealed changes from the original material (Kuehnle 1988), and further mtDNA comparisons of resistant lines from young and older cultures with their sensitive progenitors and with sensitive lines recovered from the same cultures are in progress (I. D. Small and C. J. Leaver, unpublished results). These studies should provide information about the molecular basis of the resistance and will reveal whether the mutants differ from each other or from those obtained from previous culture work using T toxin and/or different genotypes. Additional questions that remain to be answered include the following: (1) Are recombinant mtDNA molecules produced as a result of tissue culture and also amplified by selection and/or prolonged culture? or (2) Are low amounts of normal and/or recombinant

molecules already present in the cells of the original embryo explant and only amplified through successive cell generations in vitro, while T genomes are eliminated? The 25% lower specific activity of T-ATPase versus N-ATPase as shown by Bouthyette et al. (1985) may be a basis for elimination under the stress of tissue culture.

In vitro selection for methomyl resistance may have value for both maize breeders and cell and molecular biologists. In lines in which a particular T cytoplasm-nucleus combination is desirable and fertility changes are not significant, selection could provide resistance to Lannate damage as well as possible decreased susceptibility to SCLB without otherwise disturbing the variety genotype. This approach might be desirable for certain CMS-T sweet corn varieties, where T*Rf* versions have the best seed yields and/or when detasseling rather than CMS is already used for hybrid production. Methomyl is a more convenient selective agent than T toxin preparations, which may have undefined chemical components and which require more time and expense to produce. The disadvantages of this procedure for the maize breeder are that a plant regeneration system must be available for the desired varieties, and selection may not be successful for all genotypes.

For cell and molecular biologists, the results of this study raise further questions about the plasticity of the mitochondrial genome in vitro and the role of the nuclear genotype in cytoplasmic alterations. Selection with methomyl is also a simple way to generate mitochondrial mutants for molecular analysis of interesting biological phenomena including cytoplasmic male sterility.

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