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Mitochondrial DNA Variation in Maize Plants Regenerated During Tissue Culture Selection*

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Summary. Plants resistant to *Helminthosporium maydis* race T were obtained following selection for *H. maydis* pathotoxin resistance in tissue cultures of susceptible, Texas male-sterile (T) cytoplasm maize. The selected lines transmitted *H. maydis* resistance to their sexual progeny as an extranuclear trait. Of 167 resistant, regenerated plants, 97 were male fertile and 70 were classified male sterile for reasons that included abnormal plant, tassel, anther or pollen development. No progeny were obtained from these male-sterile, resistant plants. Male fertility and resistance to the *Phyllosticta maydis* pathotoxin that specifically affects T cytoplasm maize were co-transmitted with *H. maydis* resistance to progeny of male-fertile, resistant plants. These three traits previously were associated only with the normal (N) male-fertile cytoplasm condition in maize. Three generations of progeny testing provided no indication that the cytoplasmic association of male sterility and toxin susceptibility had been broken by this selection and regeneration procedure. Restriction endonuclease analysis of mitochondrial DNA (mtDNA) revealed that three selected, resistant lines had distinct mtDNA organization that distinguished them from each other, from T and from N cytoplasm maize. Restriction patterns of the selected resistant lines were similar to those from T cytoplasm mtDNA; these patterns had not been observed in any previous analyses of various sources of T cytoplasm. The mtDNA analyses indicated that the male-fertile, toxin-resistant lines did not originate from selection of N mitochondrial genomes coexisting previously with T genomes in the T cytoplasm line used for selection.

Key words: Extranuclear inheritance – Male sterile cytoplasm – *Helminthosporium maydis* resistance – Maize tissue culture – Mitochondrial DNA analysis

Introduction

Tissue culture selection techniques have been used to obtain maize (*Zea mays* L.) cultures (Gengenbach and Green 1975; Brettell et al. 1979) and regenerated plants and progeny (Gengenbach et al. 1977) that were resistant to the fungal pathotoxin produced by *Helminthosporium maydis* race T (Nisikado and Miyake). Maize lines used for tissue culture in these studies had Texas male-sterile cytoplasm (T cytoplasm) which conditions a highly susceptible interaction with *H. maydis* (Scheifele et al. 1970) and its toxin (Lim and Hooker 1972). Male fertile, or normal (N), cytoplasm maize plants and tissue cultures are highly resistant to *H. maydis* toxin (Hooker et al. 1970; Gengenbach and Green 1975; Brettell et al. 1979).

In the early stages of selection for toxin resistance, plants regenerated from the T cytoplasm cultures were both toxin-susceptible and male-sterile (Gengenbach et al. 1977). After additional selection cycles only toxin-resistant plants were obtained from the selected, toxin-resistant tissue cultures. Most regenerated, toxin-resistant plants also were male fertile; however, 13 of 65 plants failed to shed pollen and thus had been classified as 'male-sterile.' Three 'male-sterile' plants had normal tassel morphology and anthers that contained no starch-filled pollen grains. Only one seed, which was not viable, was obtained from pollinations of these three plants. Of the other 10 'male-sterile' plants, one had rudimentary florets on the

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tassel, six had pistilloid tassels, and three had tassels with single spikes and mostly aborted pollen within the anthers. Pollinations of ears of five of these plants produced no seed.

Both the toxin resistance and male fertility traits were transmitted only through the female parent in tests of the first generation progeny of the regenerated plants (Gengenbach et al. 1977). Mitochondria isolated from the toxin resistant cultures were shown to be resistent to the toxin and thus were unlike mitochondria from T cytoplasm maize. The previous study indicated that plants obtained following toxin selection had a phenotype and genetic behavior very similar to that of N cytoplasm plants. Similar results have been obtained by a group in France who selected for *H. maydis* resistance in seedlings and plants following mutagenic treatments of seeds (Berville 1978; Cassini et al. 1977; Cornu et al. 1977).

These results have led to questions concerning the genetic relationship between *H. maydis* susceptibility and male sterility and to the nature of the mechanism that results in both resistance and male fertility when selecting only for toxin resistance. An extranuclear gene with pleiotropic effects could be postulated or individual genes for resistance and fertility could have been co-selected because they are linked. If correct, the latter theory would require that, at least, the gene for male fertility existed in the extranuclear DNA of T cytoplasm before selection began. It has been proposed that T cytoplasm could be heterogeneous for T and non-T genomes and that selection favors the non-T genome (Gengenbach et al. 1977; Berville 1978; Brettell et al. 1979). Electron microscopy of isolated mitochondrial DNA (mtDNA) recently has shown that T cytoplasm mtDNA consists of two major DNA species with contour lengths of 12 and 25 μm (Levings et al. 1979b). The mtDNA from N cytoplasm also exhibits heterogeneity of contour lengths, but the size classes are distinct from T cytoplasm mtDNA. The first evidence for recombination in mtDNA of a higher plant (tobacco) has been published recently (Belliard et al. 1979). This may indicate that, if separate genes for resistance and male fertility exist in T cytoplasm, recombination during selection could result in a toxin-resistant, male-sterile cytoplasm.

Here we report the characterization of additional regenerated plants and their progeny and further characterization of progeny from the previous selection work. Regenerated plants and their progeny were characterized for 1) resistance to *H. maydis* toxin, 2) resistance to *Phyllosticta maydis* (Arny and Nelson) toxin which also specifically affects T cytoplasm (Comstock et al. 1973), 3) male fertility and 4) restriction endonuclease digestion patterns of mtDNA. This latter analysis enables a distinction to be made between N and T cytoplasm maize (Levings and Pring 1976).

Materials and Methods

Tissue Culture and Plant Regeneration

Tissue cultures of Texas male-sterile (T) cytoplasm maize were initiated and maintained and plants were regenerated as described previously (Green and Phillips 1975; Green 1977; Gengenbach et al. 1977). The T genotype, designated BC_1 A188(T), was the first backcross by A188 onto the three-way hybrid (Wf9(T) X W22) X A188.

Selection for Toxin Resistance

Toxin preparations from *H. maydis* race T were incorporated into the tissue culture media at concentrations that caused slight to moderate growth inhibition (Gengenbach et al. 1977). A sublethal enrichment procedure was used to identify cultures capable of growing at successively higher toxin concentrations. Tissue cultures showing markedly improved growth in the presence of toxin usually were given a numerical designation to maintain the separate identity of the cell lines during further subculturing and plant regeneration. We regenerated plants after the fourth subculture on toxin media and continued until the 15th subculture (cultures were 12 to 30 months old).

Crossing Procedures

Regenerated plants were self-pollinated if possible or were pollinated with A188 pollen. Pollen from male-fertile, regenerated plants also was used in crosses to Wf9(T) and A619(T) X A188 susceptible, male-sterile tester females. Progeny from these crosses subsequently were backcrossed by an appropriate male parent to produce additional generations for testing.

Field Tests of Progeny

Leaf assays for toxin resistance were performed on plants grown in the field as described previously (Gengenbach et al. 1977) except that 1-cm pieces of absorbent cotton pipe cleaner saturated with toxin solution were used instead of filter paper to apply the toxin. Some plants were tested for resistance to both *H. maydis* toxin and to *P. maydis* toxin. The *P. maydis* toxin was prepared by the same method as that used for *H. maydis* toxin. Tassel morphology and pollen shed were observed regularly, and if it was not clear that a plant was fertile, anthers were collected for staining of pollen grains with potassium iodide-iodine solution.

Analysis of mitochondrial DNA

Mitochondrial DNA was isolated from leaf tissue of 4 week-old greenhouse plants using methods previously described (Pring and Levings 1978). Restriction endonucleases were purchased from Bethesda Research Labs, Rockville, MD. Enzyme digestion, agarose electrophoresis and photography were done as previously described (Pring and Levings 1978).

Results

A total of 167 plants were regenerated from 17 *H. maydis* toxin-resistant culture lines and were grown to maturity

(Table 1). This total includes 65 plants which were described previously (Gengenbach et al. 1977). All of the 167 plants were resistant according to the leaf bioassay tests. Ninety-seven regenerated plants were male fertile, but the remainder did not shed pollen and were classified male sterile (Table 1). Many of these male-sterile plants expressed morphological abnormalities in plant or tassel development such as pistilloidy or rudimentary tassels. A few resistant plants, however, developed normally and had male-sterile tassels very similar to those of T male-sterile plants. In about 20 crosses attempted with A188 males no viable seeds were obtained; therefore, the genetic basis for the observed sterility expression could not be determined. Most of the male-sterile plants (57/70) were obtained after the twelfth selection cycle when the cultures were 25 months old or older. Cell line 1, however, consistently produced male-sterile resistant plants after cycle seven and only 3 of 35 were male fertile (Table 1).

Progeny obtained from regenerated, resistant, male-fertile plants were tested for resistance to *H. maydis* race T and *P. maydis* toxins (Tables 2, 3). Some data from *H. maydis* tests of S_1, RBC_1, and TBC_1 progenies were reported earlier (Gengenbach et al. 1977). The results of additional testing reported here confirm the extranuclear inheritance of the resistance to *H. maydis* toxin (Table 2) and show that resistance to *P. maydis* toxin also was obtained (Table 3). Resistance to *P. maydis* was inherited only through the maternal parent and was not pollen transmitted.

Male fertility also was inherited as an extranuclear trait in crosses involving the regenerated, male-fertile plants (Table 4). Repeated backcrosses of male-fertile plants or their maternal progeny onto T females (TBC_1 to TBC_3) failed to reveal any paternal transmission of male fertility. Male-sterile segregates were obtained in 8 of 11 S_1 families produced by self-pollinating regenerated, male-fertile plants and in 4 of 20 RBC_1 families where A188 was the male parent (Table 4). These male-sterile plants usually were not morphologically abnormal, but most often they failed to shed pollen because of poor anther development and/or exsertion. When possible, the anther contents of these plants were examined by staining with an iodine solution. The frequency of starch-filled pollen grains varied from 0% to over 50%, and generally the anthers contained few pollen grains.

Male-sterile S_1 and RBC_1 segregates were pollinated

Table 1. Summary of phenotypes of maize plants regenerated during the fifth to the fifteenth cycle of selection for tissue cultures resistant to *Helminthosporium maydis* race T toxin[a]

Cell line	Number of plants	
	Resistant, male fertile	Resistant, male sterile[b]
Undesignated	14	5
1	3	32
2	43	8
5	1	3
6	6	1
7	1	0
9	1	0
10	7	1
14	1	0
16	1	0
17	3	6
18	3	0
20	3	1
21	5	1
22	1	3
23	2	3
27	1	5
29	1	1
Total	97	70

[a] Includes 65 plants reported previously (Gengenbach et al. 1977) from cycles 5 to 12

[b] The male sterile classification included any plant that failed to produce normal pollen for any reason including abnormal plant, tassel, anther or pollen development

Table 2. Reaction of progeny from regenerated, resistant, male-fertile (R) plants to *Helminthosporium maydis* race T toxin

Source of progeny	Number of families	Number of plants	
		Resistant	Susceptible
R selfed = S_1	13	311	0
R_1 selfed = S_2	55	743	0
R × A188 = RBC_1	20	332	0
RBC_1 × A188 = RBC_2	6	98	0
RBC_2 × A188 = RBC_3	1	149	0
T^a × R = TBC_1	45	0	2533
TBC_1 × R = TBC_2	23	0	467

[a] Wf9(T) and A619(T) × A188 were used as susceptible, male-sterile tester female plants in these crosses

Table 3. Reaction of progeny from regenerated, resistant, male-fertile (R) plants to *Phyllosticta maydis* toxin

Source of progeny	Number of families	Number of plants	
		Resistant	Susceptible
S_1	1	29	0
S_2	55	755	0
RBC_2	3	36	0
RBC_3	1	149	0
TBC_1	16	0	400

[a] See Table 2 for descriptions

Table 4. Fertility of progeny from regenerated, resistant, male-fertile (R) plants

Source of progeny	Number of families	Number of plants	
		Male fertile	Male sterile
S_1			
Non-seg.	3	62	0
Seg.	1	30	7
	1	24	5
	1	17	6
	1	10	2
	1	6	1
	1	5	1
	1	39	1
	1	21	20
Total seg.	8	152	43
S_2	7	97	0
RBC_1	20	290	5
RBC_2	6	98	0
RBC_3	2	299	0
TBC_1	45	0	4116
TBC_2	36	0	2436
TBC_3	13	0	872

[a] See Table 2 for descriptions

Table 5. Fertility of F_1 and F_2 progeny from crosses between resistant, male-sterile segregates and A188

Generation	Number of families	Number of plants	
		Male fertile	Male sterile
F_1	10	171	0
	1	11	2
Total	11	182	2
F_2	1	103	6
	1	44	3
	1	105	4
	1	130	5
Total	4	382	18

with A188 pollen to test whether the sterility had a cytoplasmic or nuclear origin. Although many crosses were made, only 11 gave viable F_1 seeds. All but 2 of the F_1 plants were male fertile (Table 5) indicating that the male sterility could not be attributed to a stable cytoplasmic factor. Four F_1 plants representing two families were selfed, and over 95% of the subsequent F_2 plants were male fertile (Table 5). The F_2 segregation ratios indicate that the sterility of the families tested was not due to a single nuclear male sterility gene but that it might be due to multiple nuclear genes or to cytological disturbances which infrequently were transmitted through the gametes. Further genetic tests and cytological analyses of such male-sterile plants have not been done.

Normal cytoplasm conditions male fertility in either the presence or absence of nuclear fertility restorer genes, provides plant resistance to *H. maydis* and *P. maydis* and their toxins, and renders isolated mitochondria insensitive to the toxins. In all cases where these parameters have been tested, the phenotype and genetic behavior of the toxin-resistant, male-fertile plants derived from T cytoplasm tissue culture selection were indistinguishable from that of N cytoplasm plants.

Restriction endonuclease analysis of mtDNA can distinguish between N and T cytoplasm maize (Levings and Pring 1976; Pring and Levings 1978). Mitochondrial DNA was isolated from four of the selected toxin-resistant lines and compared to mtDNA of N and T controls. Figure 1 shows the digestion of mtDNA with three restriction endonucleases, XhoI, BamHI and HindIII. Slots A and B of Fig. 1 compare A188(N) and W64A(T), respectively, and show that XhoI reveals N-specific bands at 26, 29 and 40 mm, T-specific bands at 22 and 30 mm, and many

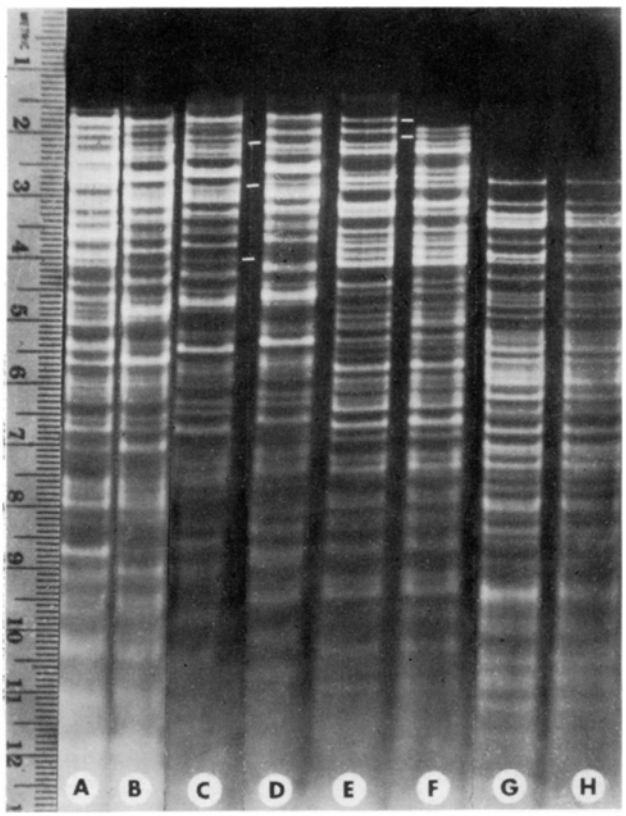

Fig. 1. Agarose gel electrophoretic patterns of maize mitochondrial DNA digested by XhoI (A-D), BamHI (E, F) and HindIII (G, H) restriction endonucleases. Slot A, A188(N); Slots B, C, E and G, W64A(T); Slots D, F and H, S_2 progeny line of a resistant, fertile plant from cell line 2

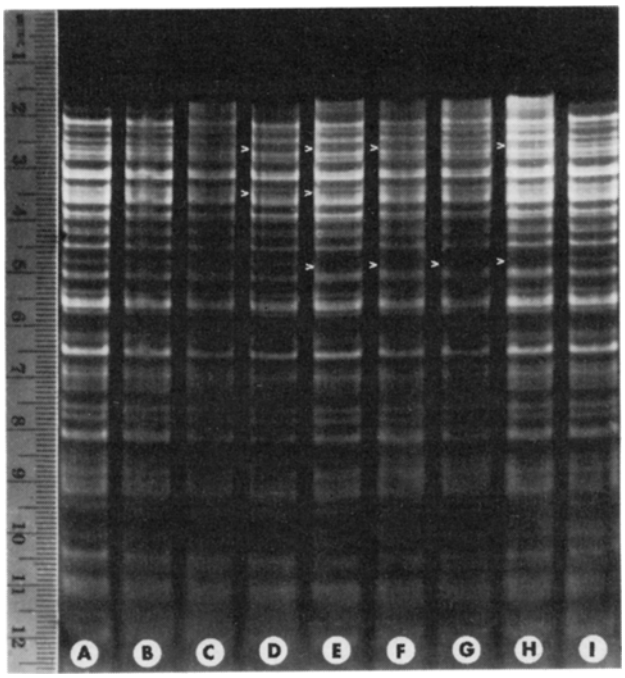

Fig. 2. Agarose gel electrophoretic patterns of XhoI digests of mtDNA from: A, W64A(T); B, BC_1 A188(T); C, A188(T); D, first generation progeny from a regenerated, susceptible, male-sterile plant; E, F, and G, S_2 or RBC_2 progeny lines of individual resistant, fertile plants from cell lines 2, 5 and 6, respectively; H, a second preparation of F; I, W64A(T)

common bands. A188(N) contributed either 75% (S_2 lines) or 87.5% (RBC_2 lines) of the nuclear genes to the progeny lines from regenerated plants. N cytoplasm S_2 or RBC_2 lines derived from (Wf9(N) x W22) x A188 would not have been exactly comparable to those from regenerated resistant plants because of segregation in these early generations; therefore, A188(N) was used for N cytoplasm comparisons. Slots C and D show W64A(T) and a toxin-resistant S_2 line from cell line 2 after digestion with XhoI. Three distinct differences were observed; the line 2 mtDNA lacked XhoI fragments at 21 mm, 28 mm and 39 mm compared to W64A(T). BamHI digests (Fig. 1 E, F) showed that line 2 lacked a band at 17 mm but had a new band at 21 mm. HindIII (Fig. 1 G, H) did not reveal any differences between W64A(T) and line 2.

Additional T and selected, toxin-resistant lines were compared with XhoI (Fig. 2). Slots A, B and C are patterns for W64A(T), BC_1 A188(T), and A188(T), respectively, and show that these nuclear genotypes in T cytoplasm were not different. BC_1 A188(T) was the genotype used in tissue culture selection. Slot D shows a line from a plant regenerated after four cycles of selection but which was male-sterile and toxin-susceptible (Gengenbach et al. 1977). The XhoI digest shows that this toxin-susceptible line also had altered mtDNA; on this gel the relative intensity of the band at 26 mm was reduced and a band at 34 mm was missing. Slots E, F and G show selected, toxin-resistant lines 2, 5 and 6, respectively. Each of these lines lacked the DNA fragment banding at 48 mm but the lines differed with respect to the bands at 26 and 34 mm. Line 2 plants (slot E) lacked the band at 34 mm and the 26 mm band was reduced in intensity; thus the resistant line 2 was quite similar to the regenerated, susceptible line (slot D) except for the band at 48 mm. Line 5 (slots F and H) had the 34 mm band and the 26 mm band was very faint. Line 6 (slot G) had no changes at 26 or 34 mm and, except for the missing 48 mm band, it was similar to unselected and nonregenerated T lines. A fourth regenerated, resistant fertile line (line 17, not shown) displayed a XhoI restrictionn pattern like that of line 2. Thus three of four resistant, fertile lines exhibited unique XhoI patterns.

The bands at 24-25 mm of slots D, E, F and H of Fig. 2 are slightly broader than corresponding bands in the other slots. Slots D, E, F and H show a reduction in the band at 26 mm. This result, although not conclusive, suggests that a DNA sequence normally cleaved by XhoI to give the 26 mm band is less subject to cleavage and results in a slightly larger DNA fragment which migrates to 24-25 mm.

Discussion

Resistant cell lines 1 and 2 both arose early in the toxin selection process (Gengenbach et al. 1977) and gave rise to the majority of the regenerated plants (Table 1). Because 4 of the first 5 plants from cell line 1 had been male-sterile, 30 additional cell line 1 plants were regenerated. All but 2 of these plants also were male sterile and, like the other male-sterile regenerated plants, no further progeny were obtained in crosses with them. The inability to recover progeny from the male-sterile regenerated plants precluded a genetic study of their sterility. Altered morphology, especially pistilloidy, has been observed among N cytoplasm plants regenerated under similar conditions, and their progeny were normal (Gengenbach, unpublished data).

Some male-sterile plants segregating in the S_1 and RBC_1 progeny of male-fertile, resistant, regenerated plants produced viable F_1 seeds in crosses with A188 although the seed set was low on most ears. The male fertility of the F_1 and F_2 plants from these crosses (Table 5) indicates that their male-sterile parents could not carry the T cytoplasm. Thus the regenerated, toxin-resistant, male-sterile plants were not likely to have been the result of a cytoplasmic separation of the toxin-resistance genetic factor from the male-fertility factor.

Tests of progeny from regenerated plants showed that resistance to *P. maydis* toxin had been obtained along

with male fertility and resistance to *H. maydis*. Each trait was transmitted only through the female and, except for the male-sterile plants discussed previously, the traits were inherited together. The phenotype of these plants, therefore, could not be distinguished from that of plants with N cytoplasm.

It was proposed previously that one mechanism by which toxin-resistant, male-sterile lines possibly could be selected in culture was by selecting in favor of N cytoplasm-like mitochondria existing within a heterogeneous population of mitochondria in T cytoplasm tissues. The mtDNA restriction endonuclease analyses have clearly shown that the normal cytoplasm phenotype of the selected, toxin-resistant lines is not due to an amplification of mitochondria with DNA organization similar to that of A188(N) mitochondria. If T cytoplasm contains heterogeneous mitochondria, toxin selection did not select for the N mitochondria type. Rather the toxin-resistant, selected lines exhibit either no (e.g. HindIII) or slight (e.g. XhoI and BamHI) restriction enzyme cleavage site differences from the T cytoplasm line originally put into culture.

Another interesting result is that four of the five culture-derived lines examined (four resistant and one susceptible) had unique XhoI restriction patterns. The resistant lines had one missing band in common, but otherwise the DNA changes noted for the resistant lines also had occurred in the susceptible, regenerated line. None of these four mtDNA patterns had been observed previously in other sources of T cytoplasm (Levings and Pring 1976; Pring and Levings 1978). These differences among the regenerated lines preclude any conclusion about the association of a particular mtDNA change with the phenotypic change to male fertility and toxin resistance. It is tempting to assume that the mtDNA band at 48 mm which the four resistant lines lack might have been the result of toxin selection; but, in fact, these results do not permit unequivocal separation of mtDNA changes caused by selection from those possibly caused or allowed by the tissue culture process alone.

An explanation is not apparent for the observed changes in mtDNA. Mitochondrial DNAs of different sizes have been isolated from maize (Levings et al. 1979b) and other plants (Quetier and Vedel 1977). The contour length of the largest circular mtDNA observed in maize is not sufficient to account for the sum of the molecular weights of mtDNA restriction fragments (Levings et al. 1979b). Heterogeneity could have arisen from mtDNA differences within individual mitochondria, cells, tissues and/or between tissues of a plant. There is no information about sequence homology of isolated mtDNA of different sizes either from restriction patterns or heteroduplex mapping. Differences in coding capacity are not known.

The mtDNA variants observed here could be due to the loss or rearrangement of a discrete part of this heterogeneous mtDNA. It is difficult, however, to reconcile a loss of DNA with the fact that HindIII did not show differences between T cytoplasm and the resistant line 2. In the BamHI and XhoI digests that showed missing high molecular weight bands, it is possible that new smaller fragments were hidden among other low molecular weight fragments on the gels. Another possible explanation for the apparent loss of bands is that methylation at certain restriction sites was altered. If normally methylated sites were close together and, as a result of selection and/or regeneration, methylation did not occur, then new cleavage sites also could result in new small fragments that may not be detected.

Belliard et al. (1979) recently have published the first report of possible recombination of mtDNA in higher plants. New mtDNA restriction patterns and new nonparental bands were obtained in progeny of tobacco plants produced from protoplast fusion. The hybrid plants also had mtDNA bands corresponding to bands from both parents and the molecular weights estimated for mtDNA in the hybrids were higher than for either of the parents. As yet there is no evidence for mtDNA recombination in corn and such an event cannot be inferred from the results of this study. Recombination possibly could be responsible for the altered corn mtDNA restriction patterns, but unlike the tobacco results, a new unique band was clearly evident only in the case of restriction with BamHI, and possibly with XhoI.

Another possible mechanism for obtaining resistant male-fertile lines during selection in T cytoplasm is analogous to the reversion of S cytoplasm male-sterile plants to male fertility (Singh and Laughnan 1972; Laughnan and Gabay 1973). Male-sterile S mitochondria have, in addition to the main mtDNA, two episome-like, low molecular weight DNAs (Pring et al. 1977). The reversion of S to male fertility is accompanied by a loss of the two episomal mtDNAs (Levings et al. 1979a) possibly by incorporation into the high molecular weight mtDNA. Low molecular weight episomal mtDNAs recently have been found in T, C and N cytoplasm maize (Kemble and Bedbrook 1980). The T cytoplasm-specific, low molecular weight DNA, however, is present in the mtDNA of lines from regenerated resistant plants.

The mtDNA analyses have identified new mtDNA restriction patterns but it is not known if mtDNA changes result in changes in expression of mitochondrial genes. Isolated N cytoplasm mitochondria have been shown to synthesize a 21,000 M_r polypeptide not synthesized by T cytoplasm mitochondria (Forde and Leaver 1980), while isolated T mitochondria synthesized a 13,000 M_r polypeptide not synthesized by N mitochondria (Forde et al. 1978). Nuclear restoration of T cytoplasm to fertility resulted in incomplete suppression of synthesis of the 13,000 M_r polypeptide, but the 21,000 M_r polypeptide was not synthesized (Forde and Leaver 1980). Similar in vitro analyses of mitochondria from the lines with altered T mtDNA are in progress and should help determine whether there are translatable as well as structural differences in the mtDNA.

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