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Mitochondrial DNA Analyses of Fertile and Sterile Maize Plants Derived from Tissue Culture with the Texas Male Sterile Cytoplasm

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Summary. Maize plants carrying Texas (T) cytoplasm are male-sterile and sensitive to *Drechslera maydis* race T toxin, whereas plants carrying Normal (N) cytoplasm are male-fertile and resistant to the toxin. Some plants regenerated from T cytoplasm tissue cultures exhibit a N cytoplasm-like phenotype with respect to male-fertility and toxin-resistance. Analysis of the high molecular weight mitochondrial DNA (mtDNA) of such regenerants has shown that the plants do not contain N cytoplasm mtDNA. However, their mtDNAs do show sequence differences from each other and from the mtDNA of plants possessing T cytoplasm. No single alteration detected correlates with the change to male-fertility or toxin resistance. Sequence alterations were also evident in high molecular weight mtDNA isolated from a plant regenerated from N cytoplasm callus. No changes in low molecular weight mtDNA molecules were observed in regenerants from N or T cytoplasm callus.

Key words: Maize tissue culture – *Drechslera maydis* race T toxin – Texas male-sterility – Mitochondrial DNA

Introduction

Maize (*Zea mays* L.) plants carrying T cytoplasm are characteristically male-sterile (in the absence of nuclear restorer genes) and highly susceptible to *Drechslera maydis* race T (=*Helminthosporium maydis,* race T) and to a toxin purified from the fungus (Scheifele et al. 1970; Lim and Hooker 1972). Maize plants carrying N cytoplasm are male-fertile and resistant to the fungus and fungal toxin (Hooker et al. 1970).

When plants are regenerated from T cytoplasm tissue cultures derived from immature embryos, some of them are fertile and resistant to *D. maydis* toxin (Gengenbach et al. 1977; Brettell et al. 1979, 1980). In the experiments of Gengenbach et al. the cultures were subcultured in the presence of increasing concentrations of *H. maydis* toxin. Plants regenerated after one to four subcultures retained the T cytoplasm phenotype with respect to male-sterility and toxin-sensitivity. However, plants regenerated after the fifth subculture were generally male-fertile and toxin-resistant. Genetic studies indicated that the phenotypic changes were inherited maternally. In the experiments of Brettell et al. (1980) some male-fertile, toxin-resistant plants were also regenerated from T cytoplasm cultures which had not been exposed to toxin.

To explain these findings it was suggested initially that in T cytoplasm there were some N type mitochondria or N mitochondrial genomes, and that in tissue culture these N type mitochondria or mitochondrial genomes were preferentially selected (Gengenbach et al. 1977; Brettell et al. 1979).

Recent electrophoretic analyses of restriction endonuclease digests of mitochondrial DNA (mtDNA) from the regenerated male-fertile, toxin resistant plants of Gengenbach et al. (1980) have not supported this suggestion because the restriction fragment patterns were very similar to those of T cytoplasm and not N cytoplasm (Gengenbach et al. 1981). In this paper we examine the mtDNAs of the lines regenerated by Brettell et al. (1980). We have studied not only the electrophoretic restriction endonuclease patterns but also the linear mtDNA species of about 2,000 to 2,400 bp which are different in mitochondria from T and N cytoplasms, and the 1940 bp supercoiled circular DNA (Kemble and Bedbrook 1980; Kemble et al. 1980). We have also characterised the mtDNAs for the sequences related to the linear mtDNAs S1 and S2 (6,200 and 5,200 bp respectively Pring et al. 1977) which are integrated in high molecular weight DNA in N mitochondrial genomes (Thompson et al. 1980; Lonsdale et al. 1981) but deleted from T mitochondrial

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genomes (Thompson et al. 1980). It is important to study these sequences because they are also absent from the main mitochondrial genome of two other cytoplasms conferring male-sterility (C and S). Their absence is therefore correlated with male sterility (Thompson et al. 1980).

Materials and Methods

Tissue Culture and Plant Regeneration

Initiation of tissue cultures, their maintenance, toxin selection and plant regeneration have been described previously (Brettell et al. 1979, 1980). Cultures carrying T cytoplasm were derived from the cross (WF9T/W22 × A188N) × W22. N cytoplasm cultures were derived from self-pollinated plants of A188N. Details of the specific lines used in this study together with their phenotypes with respect to male-fertility and toxin-sensitivity are given in Table 1. For four of the five lines studied second generation progeny of plants regenerated from tissue culture were used.

Isolation and Electrophoretic Analysis of Mitochondrial DNA

MtDNA was isolated from dark-grown coleoptiles as described previously (Kemble et al. 1980). The DNA was either subjected to electrophoresis without further treatment in 1.5% agarose gels or fragmented with restriction endonucleases before electrophoresis in 1% agarose gels (Kemble et al. 1980).

Detection of Sequence Homology between Mitochondrial DNAs

After electrophoresis in agarose gels the DNAs were transferred to nitrocellulose (Southern 1975) and prehybridized for 6 to 16 h at 65 °C in 0.3 M sodium citrate, 0.1% SDS, 0.2% each of Ficoll, BSA and PVP. Hybridizations were performed using fresh solution, adding 1×10^7 to 7×10^7 cpm freshly boiled, nick-translated (Maniatis et al. 1975), cloned DNA and incubated at 65 °C for 16 to 24 h. The nitrocellulose was washed in several changes of 0.3 M sodium chloride, 0.03 M sodium citrate, 0.1% SDS at 65 °C for 6 to 8 h, prior to drying and autoradiography. The recombinant plasmids used were pZmS 21, which contains DNA from the linear S1 species which also hybridises to S2 DNA (Thompson et al. 1980), and pZmpT 3.52, which contains sequences of the 1940 bp mitochondrial plasmid (Thompson et al. unpublished data). Both clones were constructed in pBR 322.

Results

Tissue culture regenerated plants subjected to mtDNA analysis are listed in Table 1. The morphological characteristics and toxin-sensitivity of these plants have been described previously (Brettell et al. 1980).

N and T cytoplasms possess characteristic mtDNA molecules which allow the two cytoplasms to be distinguished from one another (Kemble and Bedbrook 1980; Kemble et al. 1980). Mitochondria from N cytoplasms contain a linear DNA species of about 2,350 bp whereas the equivalent species found in T cytoplasm mitochondria has suffered a deletion of about 350 to 400 bp. Figure 1a shows an electrophoretic separation of the mtDNA species on an agarose gel. Lane 1 is mtDNA from N cytoplasm showing the 2,350 bp linear species at 117 mm and Lane 2 is T cytoplasm mtDNA with the characteristic band at 122 mm. If the hypothesis that male-fertile, toxin-resistant plants regenerated from T cytoplasm cultures result from a preferential amplification of a pre-existing mitochondrial N genome, these plants should show the characteristic N cytoplasm mtDNA pattern. However, regardless of the sterility/fertility and sensitivity/resistance traits expressed by plants regenerated from T cytoplasm callus, they all display the characteristic T cytoplasm mtDNA pattern (Fig. 1a, Lanes 3 to 6), arguing against this hypothesis.

Table 1. Details of tissue culture regenerated lines

Culture	T-toxin selection[a]	Phenotype of regenerated plant[b]	male parent and nomenclature of first generation progeny[c]	Lines analysed were progeny of the cross	Nomenclature of line analyzed
T cytoplasm	+	fertile-resistant	self-pollinated, V3	V3 × self-pollinated	V3 × V3
T cytoplasm	–	sterile-sensitive	fertile-resistant regenerant, V7	V7 × pollen from fertile-resistant plant (V3)	V7 × V3
T cytoplasm	–	fertile-resistant	self-pollinated, V18	V18 × self-pollinated	V18 × V18
T cytoplasm	+	fertile-resistant	self-pollinated, V25	V25, i.e. first generation progeny from a primary regenerant	V25
N cytoplasm	–	fertile-resistant	fertile-resistant regenerant, V16	V16 × self-pollinated	V16 × V16

[a] Cultures were grown on medium either containing *D. maydis* race T toxin (+) or not containing the toxin (–)
[b] Fertile-resistant and sterile-sensitive refers to male-fertile/sterile and toxin-resistant/sensitive
[c] In each case, the regenerated plant listed in the preceding column was used as the female parent

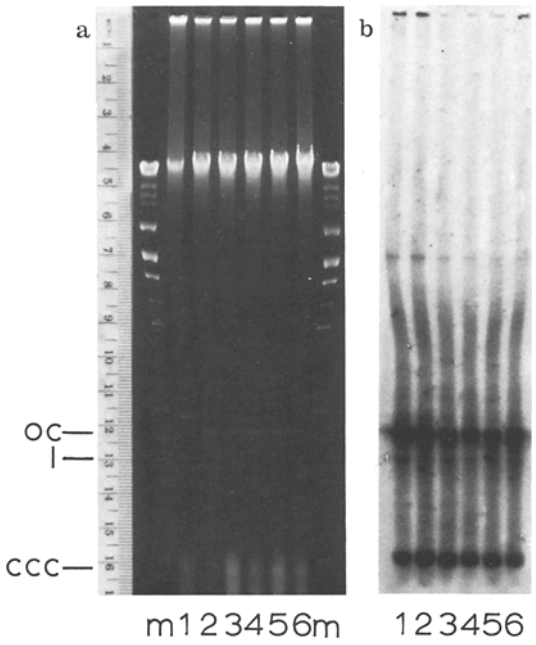

Fig. 1a and b. **a** Electrophoresis on 1.5% agarose gel of mtDNA isolated from: 1, W64A N; 2, W64A T; 3, V7×V3; 4, V25; 5, V3×V3; 6, V18×V18; m, bacteriophage lambda DNA digested with Eco RI and Hae III to act as size-markers. oc, l and ccc indicate the position of the open-circular, linear and supercoiled conformation of the 1,940 bp mtDNA plasmid respectively. **b** Autoradiograph of a southern blot of the gel after hybridization to pZmp T 3.52

All maize cytoplasms (N, T, S and C) have one low molecular weight mtDNA species in common. This is a supercoiled plasmid of approximately 1,940 bp (Kemble and Bedbrook 1980). During the isolation of mtDNA some molecules of this plasmid are usually nicked to produce open-circular (OC) and linear (l) conformations which are resolved on agarose gels (Fig. 1 a). The concentration of these molecules on the gel of Fig. 1 a is too low for easy detection of all three conformations by ethidium bromide staining. However, we investigated this plasmid in tissue culture regenerated plants by transferring the DNA in the gel to nitrocellulose (Southern 1975) and probing with radioactively labelled nick-translated (Maniatis et al. 1975) pZmp T 3.52. This plasmid contains a full length copy of the 1,940 bp mtDNA plasmid inserted into pBR 322 (Thompson et al. unpublished). Figure 1 b shows the autoradiograph obtained indicating that no alteration in size or conformation of this mtDNA plasmid occurs in plants derived from T cytoplasm cultures.

The high molecular weight mtDNAs of tissue culture regenerated plants were also examined by fragmenting total mtDNA with Bam HI. Figure 2 a shows the electrophoretic separation obtained. Lanes 1 and 2 are control lanes of mtDNA isolated from A188 carrying N cytoplasm, and W64A carrying T cytoplasm respectively. Comparison of Lanes 1 and 2 show the characteristic differences in fragment patterns between N and T mtDNA (Levings and Pring 1976; Pring and Levings 1978). Lanes 3 to 7 contain Bam HI digests of mtDNA isolated from different plants derived from either N or T cytoplasm cultures. Lane 3 shows mtDNA isolated from male-sterile, toxin-sensitive V7×V3 which was regenerated from a T cytoplasm culture without toxin selection. There are no visible differences in restriction enzyme pattern between this sample and the control T cytoplasm sample (Lane 2) although they can be distinguished with Xho I (data not shown). Male-fertile, toxin-resistant V3×V3 was derived from a T cytoplasm culture selected and maintained for six months in the presence of toxin (Lane 6). The mtDNA pattern in the Bam HI digest does not differ from that of the control T cytoplasm, even though it has an altered sterility-sensitivity phenotype, but it does differ in Xho I digests (data not shown). However, another plant, V25, derived from a culture selected and maintained for twelve months in the presence of toxin does show an altered Bam HI restriction pattern (Lane 5). When compared to the control T cytoplasm, V25 possesses an additional fragment at about 9,500 bp but lacks one at about 7,500 bp which is also absent in the control N cytoplasm sample (Lane 1). Thus, although the plant phenotypes of V3×V3 and V25 are the same, they possess different mtDNAs. V18×V18 was derived from a T cytoplasm culture grown in the absence of toxin but, nevertheless, has a male-fertile, toxin-resistant phenotype. It has a restriction pattern (Lane 7) similar to control T cytoplasm (Lane 2) but possesses an additional fragment of about 16,000 bp and lacks a fragment of about 14,500 bp. V16×V16 was derived from an N cytoplasm culture maintained in the absence of toxin, and has retained its N cytoplasm-like male-fertile and toxin-resistant traits. However, a slight alteration in mtDNA pattern between it (Lane 4) and the control N cytoplasm mtDNA (Lane 1) is observed. It possesses an additional fragment of about 5,500 bp.

These results show that mtDNA changes have occurred in plants derived from both N and T cytoplasm cultures. The DNA in the gel of Fig. 2 a was transferred to nitrocellulose (Southern 1975) and probed with radioactively labelled, nick-translated (Maniatis et al. 1975) pZmS 21 (Thompson et al. 1980). This plasmid has high sequence homology to specific Bam HI mtDNA fragments from N cytoplasm and low sequence homology to other specific mtDNA fragments from T cytoplasm (Thompson et al. 1980; Lonsdale et al. 1981). The resulting autoradiograph (Fig. 2 b) indicates that only the control N cytoplasm sample (Lane 1) and V16×V16 (Lane 4), which was derived from an N cytoplasm culture, possess a hybridization

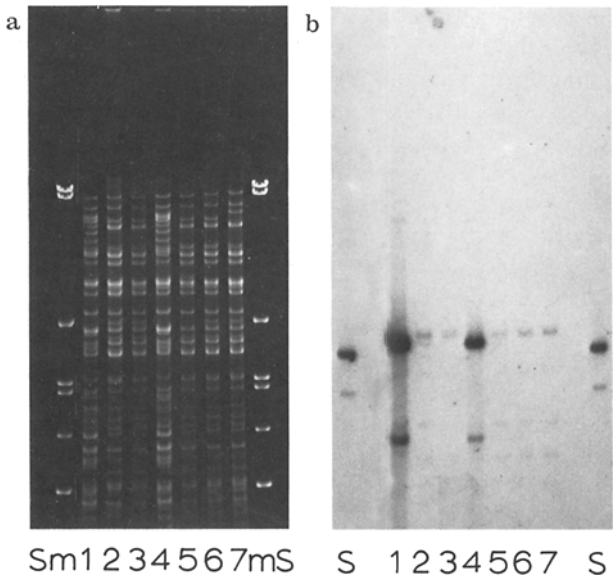

Fig. 2a and b. **a** Electrophoresis on 1% agarose gel of Bam HI digests of mtDNA from: 1, A188 N; 2, W64A T; 3, V7×V3; 4, V16×V16; 5, V25; 6, V3×V3; 7, V18×V18; S, low concentration of S cytoplasm mtDNA as a marker for the radioactive probe; m, Eco RI digest of bacteriophage lambda DNA as size-markers. The sizes of the fragments are 21,569 bp (a doublet is seen on the gel due to head-tail dimers), 7,536 bp, 5,901 bp, 5,681 bp, 4,845 bp and 3,601 bp (Daniels et al. 1980). **b** Autoradiograph of a Southern blot of the gel after hybridization to pZmS 21

pattern characteristic of N cytoplasm. The other samples (Lanes 3, 5, 6 and 7) give a pattern characteristic of T cytoplasm (Lane 2) regardless of their sterility/fertility and sensitivity/resistance traits.

Figures 2a and b confirm that male-fertile, toxin-resistant plants derived from T cytoplasm cultures, regardless of toxin selection pressure, do not possess an N type mtDNA genome. Some of these plants possess a Bam HI mtDNA restriction fragment pattern identical to that of the control T cytoplasm, whereas other plants do not. Those that differ do not do so with respect to the same fragments, which suggests but certainly does not prove that not all the visible fragment alterations are involved in the fertility-resistance traits. A plant derived from an N cytoplasm culture also exhibited mtDNA changes when compared to the N cytoplasm control.

Discussion

The ability to generate phenotypically different plants from tissue culture systems is undoubtedly an attractive addition to traditional plant breeding techniques. This is especially so in maize since attempts to regenerate plants from single cells such as protoplasts have, to date, been unsuccessful. However, to exploit fully this technology it is important that we understand the nature and the source of the variation in tissue culture regenerated plants. Many studies have implicated mitochondria as determinants of male-sterility and *D. maydis* race T toxin sensitivity in maize. To assess the relationship, if any, between these traits and mtDNA, studies employing a limited number of tissue culture regenerated plants have been described (Kemble and Bedbrook 1980; Gengenbach et al. 1981). These studies cast doubt on the earlier hypothesis (Gengenbach et al. 1977; Brettell et al. 1979) that the regeneration of male-fertile, toxin-resistant plants from T cytoplasm (male-sterile, toxin-sensitive) cultures involved amplification of a pre-existing N cytoplasm (male-fertile, toxin-resistant) mtDNA genome. Of the four toxin-selected fertile-resistant plants of Gengenbach et al. analyzed with Xho I, two produced identical fragment patterns, the other two produced different fragment patterns and all four were unlike the N and T cytoplasm controls. The one male-sterile, toxin-sensitive regenerated plant analyzed possessed a different restriction fragment pattern from all the others (Gengenbach et al. 1981).

The results reported in this paper also refute the hypothesis that male-fertile, toxin-resistant plants derived from T cytoplasm cultures result from the selective amplification of a pre-existing N cytoplasm mtDNA genome. All such regenerated plants analysed had characteristics of T cytoplasm mtDNA regardless of their sterility/fertility and sensitivity/resistance traits. However it is possible that another T-like pre-existing mitochondrial genome which does not confer sterility could have been selectively amplified.

It is not possible to correlate specific mtDNA fragments with male-fertility or toxin-resistance from the analyses of the plants derived from tissue culture (Fig. 2). Although V25 (toxin selected; male-fertile, toxin-resistant) and V18×V18 (unselected; male-fertile, toxin-resistant) both produced mtDNA fragment patterns different from the N and T cytoplasm controls they also differed from each other. This was not due to the presence of the toxin during growth of the V25 callus because V3×V3 was selected in the same way and had the same phenotype as V25 but, nevertheless, produced a different mtDNA fragment pattern.

These findings, together with the observation that a plant regenerated from N cytoplasm callus (V16×V16) produced a different fragment pattern from the N cytoplasm control, indicate that changes in mtDNA can occur readily during the culture and/or regeneration of cells and relatively few of them are likely to be functionally related to the fertility-resistance traits. This conclusion is very important because a similar frequency of changes in mtDNA does not occur from one life cycle to the next during conventional plant propagation.

Previously we noted the correlation between male sterility and the deletion of sequences related to S1 and S2 from the main mitochondrial genome of S, T and C cytoplasms (Thompson et al. 1980). The observation here, using pZmS 21 (Fig. 2 b), that the fertile revertants from T cytoplasm cultures also lack the sequences related to S1 and S2, implies that these sequences are not indispensable for normal pollen development in the absence of nuclear restorer genes and that male sterility in T, C and S cytoplasms is not due to the simple deletion of these sequences.

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