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Mitochondrial Sensitivity to *Drechslera maydis* T-toxin and the Synthesis of a Variant Mitochondrial Polypeptide in Plants Derived from Maize Tissue Cultures with Texas Male-sterile Cytoplasm

L. K. Dixon and C. J. Leaver
Department of Botany, University of Edinburgh, Edinburgh (Scotland)

R. I. S. Brettell
Friedrich Miescher-Institut, Basel (Switzerland)

B. G. Gengenbach
Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, Minn. (USA)

Summary. Tissue cultures of maize carrying cms-T cytoplasm have been found to regenerate fertile, T-toxin resistant plants, with and without a selective treatment with T-toxin. Progeny of these plants were tested for mitochondrial sensitivity to T-toxin and the translation products synthesised by isolated mitochondria were analysed. The results confirm previous indications of a close correlation between susceptibility to T-toxin and the synthesis of a variant 13,000 M_r mitochondrial polypeptide. Interestingly, there appeared to be a critical level at about 33% maximum synthesis of the 13,000 M_r polypeptide above which male sterility and sensitivity to T-toxin are jointly expressed. The possibility that there is a causal link between synthesis of this additional mitochondrial polypeptide, pollen abortion and sensitivity to T-toxin is discussed.

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

Introduction

Maize (*Zea mays* L.) plants carrying Texas male-sterile cytoplasm (cms-T) are particularly susceptible to the host specific fungal toxin (T-toxin) produced by race T of *Drechslera maydis* (Nisikado) Subram. and Jain (= *Helminthosporium maydis* Nisikado) (Turner and Martinson 1972), while fertile plants, with normal cytoplasm (N), are resistant. The toxic effects on the intact plant are associated with the sensitivity of isolated mitochondria (and not chloroplasts) which appear to be the primary site of action of the toxin (Gengenbach et al. 1973; Gregory et al. 1978). Thus, mitochondria from cms-T plants treated with low concentrations of T-toxin exhibit leakage of NAD^+ and are no longer capable of oxidative phosphorylation, suggesting a rapid effect on the permeability characteristics of the inner mitochondrial membrane (Mathews et al. 1979).

The introduction of nuclear fertility restorer alleles (*Rf* genes) into T-cytoplasm lines results in a decreased sensitivity of isolated mitochondria to the T-toxin (Barratt and Flavell 1975). Expression of the T-male sterile phenotype and susceptibility to *D. maydis*, race T have also been correlated with alterations in the organisation and expression of the mitochondrial genome. Restriction endonuclease analysis of mitochondrial DNA (mtDNA) from fertile, N and cms-T maize has revealed characteristic differences (Pring and Levings 1978). Analysis of the translation products synthesised by isolated mitochondria show that in addition to the 18–20 polypeptides synthesised by N mitochondria, cms-T mitochondria synthesise an additional, characteristic, 13,000 M_r polypeptide (Forde et al. 1978). In lines with T-cytoplasm which have been restored to fertility by nuclear restorer genes, synthesis of the 13,000 M_r polypeptide by isolated mitochondria is specifically suppressed (Forde and Leaver 1980). The correlation between pollen abortion in cms-T maize, susceptibility to T-toxin and the synthesis of the 13,000 M_r polypeptide is thus maintained when changes occur in both nuclear and mitochondrial genomes. That the observed link may be due to a single genetic defect in the mitochondrial genome is further supported by the results of two independent studies in which male-fertile plants, resistant to T-toxin were regenerated from tissue cultures derived from male-sterile, toxin-sensitive, cms-T lines. In one case selection for toxin resistance was employed (Gengenbach et al. 1977) while in the other fertile, toxin-

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resistant plants were also obtained from unselected cultures (Brettell et al. 1980). Restriction endonuclease analysis of mtDNA in both cases revealed that the toxin-resistant, male fertile regenerated plants had distinct mtDNA organisation that distinguished them from the parental T and from N cytoplasm maize (Gegenbach et al. 1981; Kemble et al. in preparation).

In this present study mitochondria isolated from maize lines derived from comparable tissue cultures carrying the T-cytoplasm, were compared for both their sensitivity to a pure T-toxin sample (Kono and Daly 1979) and the synthesis of mitochondrial polypeptides.

Materials and Methods

Initiation of Maize Tissue Cultures and Regeneration of Plants

Tissue cultures of Texas male sterile cytoplasm maize were initiated, maintained and plants regenerated as described previously (Green and Phillips 1975; Gengenbach et al. 1977; Brettell et al. 1980). Regenerated plants and their progeny from the non-toxin selected tissue cultures were tested for pollen development and toxin sensitivity as described previously (Brettell et al. 1980).

Isolation of Mitochondria and Assay of Malate Oxidation

Maize seedlings were grown in the dark for 5–6 days at 25 °C and mitochondria isolated from 2.5–3 g of etiolated shoots as previously described (Brettell et al. 1979). Mitochondrial malate oxidation was assayed by following the reduction of 2,6 dichlorophenol indophenol (DCPIP) spectrophotometrically at 600 nm. The effect of T-toxin (a gift of Dr. J. M. Daly, University of Nebraska) was determined by adding a final concentration of 90 ng/ml to the assay mixture 90 seconds after the reaction was initiated by the addition of potassium malate.

Protein Synthesis by Isolated Mitochondria and Analysis of in Vitro Translation Products

Mitochondria were isolated from 5–10 g of 5 day old etiolated shoots and mitochondrial translation products labelled with [^{35}S]-methionine as described previously (Forde et al. 1978; Forde and Leaver 1980). Labelled polypeptides were fractionated by electrophoresis on 15–20% (w/v), SDS-polyacrylamide gradient gels (Laemmli 1970). The gels were stained with Coomassie brilliant blue, destained, dried onto Whatmann 3 MM paper and exposed for 7 days to Dupont Cronex X-ray film.

Results

Plant Regeneration from Tissue Cultures of Texas Male-sterile Cytoplasm (cms-T) Maintained Without Selection for T-toxin Resistance

The following describes the lines that were obtained without T-toxin selection from tissue cultures carrying cms-T cytoplasm. Four of the lines derived directly from a single plant regenerated from a tissue culture of genotype (WF9T/W22 × A188N *rf rf*) × A188N *rf rf*, Brettell et al. 1980. This plant was scored sensitive to T-toxin according to a leaf assay, yet produced fertile pollen. Four seeds were obtained by allowing this plant to self-pollinate. The resulting plants (A and B, toxin sensitive, C and D, toxin resistant) themselves all produced fertile pollen and were self-pollinated to give the lines 32A, 32B, 32C and 32D. Plants of line 32A and 32B have invariably given a sensitive toxin reaction, and those of 32C and 32D a resistant reaction when mature leaves were treated with T-toxin. A limited number of plants of these four lines were allowed to grow to maturity and scored for the percentage of anthers extruded and producing fertile pollen (Table 1). Progeny from line 32A were mostly completely sterile and those from 32C and 32D mostly fertile. In contrast of the progeny tested from line 32B, 6 were completely sterile, 6 partially fertile and 4 fertile. In addition, a line (denoted BC_3D) derived from pollinating a plant with cms-T cytoplasm and nuclear genotype *rf rf* with pollen from the fertile plant D obtained from the fertile and toxin-sensitive tissue culture regenerant described above was studied. Of 16 progeny tested from this line, 14 were completely sterile and 2 partly fertile, demonstrating that reversion of line 32D to fertility is not due to the presence of dominant nuclear restorer genes.

Another line was derived from a fertile, sensitive plant regenerated from a similar cms-T culture maintained in the absence of T-toxin. Of ten progeny tested all were toxin sensitive, 8 were sterile, 1 partly fertile and 1 fully fertile. This fully fertile, toxin sensitive plant was self-pollinated to give line 33-6 used in this study. In this case it is possible that the fertile phenotype is the result of contamination with nuclear restorer alleles as pollination was not controlled. The genotypes A188N

Table 1. Male-fertility of four lines obtained from a male fertile, toxin sensitive plant regenerated from tissue culture of cms-T maize[a] and used in this study

Percentage of anthers extruded and shedding pollen	Maize line			
	32A	32B	32C	32D
0 (plant male-sterile)	14	6	0	0
< 50% (plant partially male-fertile)	2	6	5	0
50 – 100% (plant male-fertile)	0	4	11	18
Total	16	16	16	18

[a] cms-T = (WF9T/W22 × A188N *rf rf*) × A188N *rf rf*

rf rf and (WF9T/W22)×A188N *rf rf* backcrossed 4 times with A188N *rf rf*, were used as N and cms-T cytoplasm controls respectively.

T-toxin Sensitivity of Mitochondria from Plants Derived from cms-T Maize Tissue Cultures Maintained Without Selection for T-toxin Resistance

T-toxin sensitivity of mitochondria isolated from 5–6 day old etiolated maize shoots was estimated by measuring the rate of malate oxidation in the presence and absence of T-toxin. Mitochondria isolated from shoots grown from seed of lines 32A and 32B were as susceptible to T-toxins as the control cms-T line whereas those from 32C and 32D were as resistant as the control N line (Table 2). Mitochondria from line BC_3D were also susceptible to T-toxin indicating that the reversion to toxin resistance of 32D is not due to the presence of dominant nuclear genes. Mitochondria from fertile progeny of 32B and from 33-6 gave a reaction intermediate between the control N and T lines and were significantly less sensitive than those from the sterile progeny of 32B.

Analysis of Mitochondrial Translation Products from Plants Regenerated from cms-T Maize Tissue Cultures Maintained Without Selection for T-toxin Resistance

Mitochondria were isolated from etiolated shoots of maize plants and allowed to synthesise protein in the presence of [^{35}S]-methionine. Mitochondrial translation products were analysed by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. As previously reported (Forde et al. 1978), mitochondria from plants with T cytoplasm (Fig. 1 B) can be distinguished from those with N cytoplasm (Fig. 1 A) by the synthesis of an additional 13,000 M_r polypeptide. Mitochondria from line 32D (Fig. 1 G) did not synthesise the 13,000 M_r polypeptide characteristic of T cytoplasm and those from line 32C (Fig. 1 F) synthesised it at a very low level. Thus reversion of T-toxin sensitive, male sterile cytoplasms to resistant male fertile cytoplasms is correlated with a dramatic decrease in the synthesis of the 13,000 M_r polypeptide. Mitochondria from shoots grown from seed of lines 32A (Fig. 1 C) and the progeny of 32B (Fig. 1 D and E) synthesised a form of the 13,000 M_r polypeptide with slightly faster electrophoretic mobility than the 13,000 M_r polypeptide of the control T cytoplasm. However, partial proteolysis (using chymotrypsin) of both forms of the 13,000 M_r polypeptide gave similar digestion patterns indicating that they are forms of the same polypeptide (data not shown).

Table 2. T-toxin sensitivity and synthesis of the 13,000 M_r polypeptide by mitochondria isolated from control and regenerated maize plants

Maize line	Fertility[a]	T-toxin sensitivity[b]	Relative synthesis of 13,000 M_r polypeptide[c]
A188N (*rf rf*) (N-Control)	MF	3	0
WF9T/W22×A188N (*rf rf*) (T-Control)	MS	91	100
32A	MS	100	58
32B	MS	95	95
32B	MF	86	33
32C	MF	4	10
32D	MF	6	0
BC_3D	MS	91	78
33-6	MF	78	28

[a] MS = Male sterile, MF = Male fertile

[b] Calculation of 'T-toxin sensitivity': The rate of malate oxidation at the time of addition of toxin (r_1) and the rate 90 seconds later (r_2) were measured, as were rates of reaction at the same times in control assay mixtures where no toxin was added, giving values r_3 and r_4 respectively. For each line T-toxin sensitivity was calculated as a percentage:

$$\frac{(r_4/r_3 - r_2/r_1)}{r_4/r_3} \times 100$$

[c] Mitochondrial translation products were labelled (no added toxin), fractionated by SDS-gel electrophoresis and autoradiographed as described in materials and methods. The relative amounts of the 13,000 M_r polypeptide synthesised under standard conditions was estimated from densitometric scans of the autoradiographs. Areas under the 13,000 M_r peak and under the peak of protein X (M_r = 18,000, a translation product whose synthesis does not alter in male sterile and male fertile linses) were estimated. The ratio 13,000 polypeptide/peak X was calculated for each line and compared with the 13,000/peak X ratio for the control T line (WF9T/W22×A188N). Synthesis of the 13,000 M_r polypeptide in any particular line relative to the T-control line (WF9T/W22×A188N) therefore =

$$\frac{\text{Area under 13,000 } M_r \text{ polypeptide peak}}{\text{Area under polypeptide 'peak X'}} \times \frac{\text{Area under polypeptide 'peak X' of T-control}}{\text{Area under 13,000 } M_r \text{ polypeptide of T-control}}$$

The relative proportion of the 13,000 M_r polypeptide synthesised in mitochondria from each line compared to those from the control line with cms-T cytoplasm was estimated as described in Table 2. The results indicate a correlation between the relative proportion of the 13,000 M_r polypeptide synthesised, relative sensitivity to T-toxin and the phenotypic expression of male sterility (Fig. 2). In mitochondria isolated from shoots grown from seed of the fertile progeny of 32B (Fig. 1 E) and from 33-6 (Fig. 1 I), the relative proportion of the 13,000 M_r polypeptide was approximately 30% of the rate in the control cms-T line

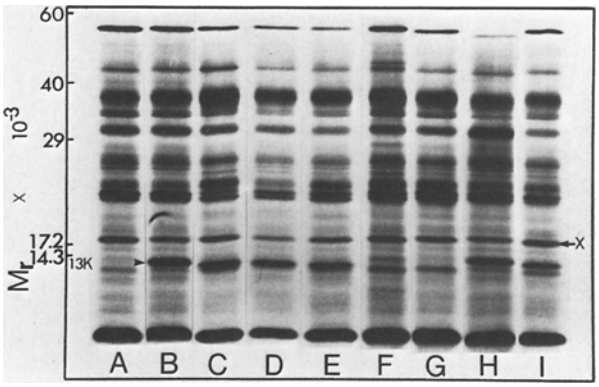

Fig. 1. Polyacrylamide gel electrophoresis of polypeptides synthesised by mitochondria isolated from control plants and maize lines derived from cms-T maize tissue cultures maintained without selection for T-toxin resistance. (A) A188N (*rf rf*) N-control; (B) WF9T/W22×A188N (*rf rf*), cms-T control; (C) line 32A, (D) line 32B – male sterile, (E) line 32B – male fertile, (F) line 32C, (G) line 32D, (H) line BC_3D, (I) line 33-6. 13K = 13,000 M_r polypeptide; 'X' = reference polypeptide, M_r = 18,000. Mitochondrial translation products were labelled with [^{35}S]-methionine, solubilised in SDS, electrophoresed on 15–20% (w/v) SDS-polyacrylamide slab gels and the dried gel autoradiographed

(Table 2). In these lines, as described in the previous section, T-toxin sensitivity of the isolated mitochondria was significantly less than the control cms-T line. Mitochondria from shoots of seed from sterile progeny of 32B (Fig. 1 D), from 32A (Fig. 1 C) and from BC_3D (Fig. 1 H) synthesised the 13,000 M_r polypeptide at over 60% of the level in the control cms-T line (Table 2).

In order to prove that the intermediate levels of synthesis of the 13,000 M_r polypeptide obtained with certain seed lines were truly representative of an individual seedling and not an average derived from a mixed population in the 15–20 seedlings used in a normal mitochondrial preparation, the following comparison was made. Mitochondria were isolated from ten separate individual seedlings from each line as well as from the normal bulked preparation from 15–20 seedlings. In all cases the relative synthesis of the 13,000 M_r polypeptide was essentially the same (data not shown).

Analysis of Mitochondrial Translation Products from Plants Derived from Tissue Cultures of cms-T Maize Selected for T-toxin Resistance

One of us has previously demonstrated that it is also possible to regenerate male fertile plants, resistant to *D. maydis,* race T-toxin following selection for pathotoxin resistance in tissue cultures of susceptible cms-T maize (Gengenbach et al. 1977). Restriction endonuclease analysis of mtDNA revealed that three selected, resistant lines had distinct mtDNA organisation that distinguished them from each other, from the parental T, and from N cytoplasm maize (Gengenbach et al. 1981). In order to determine whether the differences in mtDNA restriction pattern are reflected in a change in mitochondrial translation products we have examined the proteins synthesised by mitochondria isolated from the same maize lines.

Tissue culture, selection for toxin resistance, and plant regeneration were carried out as previously described (Gengenbach et al. 1977). Mitochondria were isolated from 4 day old etiolated shoots of N (A188N *rf rf*), T (A188T), from regenerated, toxin-susceptible, male-sterile plants, and from individual resistant, male-fertile progeny lines regenerated from 6 different T-toxin resistant cell lines (designated 1, 2, 5, 6, 10 and 17 in Gegenbach et al. 1981). They were allowed to synthesise protein in the presence of [^{35}S]-methionine and the labelled translation products were fractionated by SDS-polyacrylamide gel electrophoresis, and visualised by autoradiography. In addition to the 18–20 labelled polypeptides synthesised by the N mitochondria (Fig. 3 A) T mitochondria synthesised an additional 13,000 M_r polypeptide (Fig. 3 B). The translation products of mitochondria from all the selected toxin-resistant lines (which were also male fertile) gave a pattern which was indistinguishable from N cytoplasm (Fig. 3 C for example). The mitochondrial translation products from a line regenerated after 4 cycles of toxin selection but which was male-sterile and toxin-susceptible, were indistinguishable from those synthesised by T-mitochondria (Fig. 3 D).

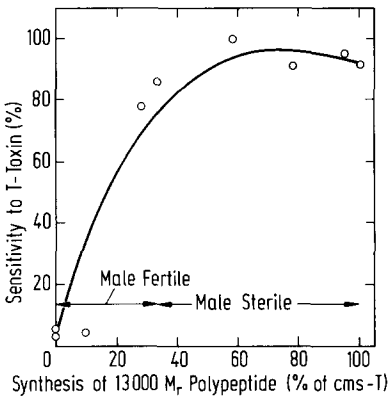

Fig. 2. Correlation between synthesis of the 13,000 M_r polypeptide by isolated mitochondria and their sensitivity to T-toxin. The data for mitochondria isolated from the maize lines listed in Table 2 are plotted to show the correlation between toxin sensitivity and relative synthesis of the 13,000 M_r polypeptide

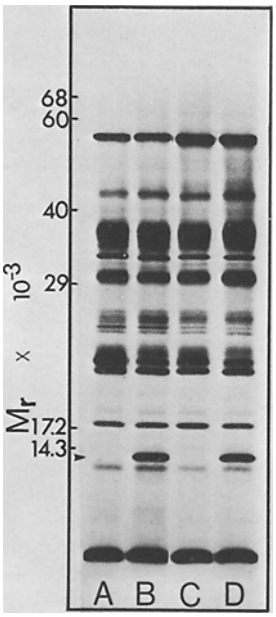

Fig. 3. Polyacrylamide gel electrophoresis of polypeptides synthesised by mitochondria from control plants and maize lines derived from cms-T, maize tissue cultures selected for T-toxin resistance. (A) A188N (*rf rf*), male fertile control, (B) A188T, male sterile control, (C) progeny line of a toxin-resistant, fertile plant from cell line 6 (D) progeny line from a regenerated, susceptible male-sterile plant. The experimental procedure was as outlined in the legend to Fig. 1

Discussion

This paper confirms and extends our earlier observations that male-fertile plants, resistant to *D. maydis,* race T-toxin can be regenerated from tissue cultures derived from T-toxin sensitive, Texas male-sterile cytoplasm maize. The fertile, toxin resistant plants can be obtained from either T-toxin selected or unselected tissue cultures and are phenotypically indistinguishable.

The results presented show that mitochondria from the regenerated plants which have reverted to the male-fertile phenotype, are no longer sensitive to T-toxin and fail to synthesise significant levels of a 13,000 M_r polypeptide which is characteristically synthesised by mitochondria from cms-T maize plants. When considered with results showing that nuclear restorer genes specifically suppress synthesis of the 13,000 M_r polypeptide (Forde and Leaver 1980) and reduce sensitivity to T-toxin (Barratt and Flavell 1975) these results provide strong circumstantial evidence for a causal link between synthesis of this additional mitochondrial polypeptide, expression of the male-sterile phenotype and sensitivity to T-toxin. The inability to separate T-type cytoplasmic male sterility from disease susceptibility could be interpreted to mean that both phenomena are different expressions of a single genetic defect.

The explanation for this link remains obscure, although Flavell (1974) proposed that an altered structure of mitochondria from T cytoplasm might make them susceptible both to T-toxin and to an (hypothetical) anther-specific substance produced during normal pollen development. Based on this hypothesis Forde and Leaver (1979) suggested that integration of the 13,000 M_r polypeptide synthesised by mitochondria from T cytoplasm into the mitochondrial membrane might result in an alteration in membrane structure leading to the formation of a binding site for T-toxin and the anther specific substance. Binding of either substance would then result in a loss of mitochondrial function. The effects of T-toxin on isolated mitochondria are consistent with this hypothesis (Walton et al. 1979). However, an anther specific substance remains to be identified. Interestingly, in this study there appears to be a critical level at about 30% maximum synthesis to 13,000 M_r polypeptide above which male-sterility and mitochondrial sensitivity to T-toxin are jointly expressed (Fig. 2). This may reflect a threshold level of synthesis at which mitochondrial membrane structure is altered such that binding of T-toxin and the proposed anther substance can disrupt normal mitochondrial function. Our previous work has shown that the synthesis of the 13,000 M_r polypeptide can be suppressed by changes in the nuclear genome (Forde and Leaver 1980). In contrast, for plants derived from tissue culture, changes in their mitochondrial DNA are implicated. Recent experiments, with the same lines of plants as those used in this study, have shown by restriction endonuclease analysis of mtDNA, that rearrangement of the mitochondrial genome occurs in the tissue culture environment or during the regeneration of the plants from culture (Gengenbach et al. 1981; Kemble et al. in prep.). Such rearrangements generally do not seem to occur during a normal sexual cycle (Quetier and Vedel 1980) and might explain the relatively high frequency at which T-toxin resistance appears without selection in cultures carrying T cytoplasm (Brettell et al. 1980). These observations thus provide an example in which the synthesis of a specific mitochondrial polypeptide is under the control of both nuclear and cytoplasmic genes. They also support the hypothesis that changes in mtDNA organisation result in changes in gene expression as evidenced by differences in specific translation products (e.g. the 13,000 M_r polypeptide), which in turn may be causally associated with agronomically important phenotypic changes.

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Dr. L. K. Dixon
Dr. C. J. Leaver
Department of Botany
University of Edinburgh
The King's Buildings
Mayfield Road
Edinburgh EH9 3JH (Scotland)

Dr. R. I. S. Brettell
Institut de Biologie et de Physiologie Végétales
Université de Lausanne
1005 Lausanne (Switzerland)

Dr. B. G. Gengenbach
Department of Agronomy and Plant Genetics
University of Minnesota
1509 Gortner Avenue
St Paul, Minn. 55108 (USA)