CLASSIFICATION OF NORMAL AND MALE-STERILE CYTOPLASMS IN MAIZE. II. ELECTROPHORETIC ANALYSIS OF DNA SPECIES IN MITOCHONDRIA

R. J. KEMBLE*, R. E. GUNN AND R. B. FLAVELL

Plant Breeding Institute, Maris Lane, Trumpington, Cambridge CB2 2LQ, England

Manuscript received November 5, 1979
Revised copy received February 27, 1980

ABSTRACT

Mitochondrial DNA preparations were made from 31 maize lines carrying different sources of cytoplasm in the same nuclear genetic background. The DNAs were analyzed by agarose gel electrophoresis. A number of discrete low molecular weight bands were present in all lines. However, only four different DNA banding patterns were observed. These were correlated with the N, T, S and C cytoplasms defined by nuclear fertility restorer genes. Of the 31 cytoplasmic sources examined, six possessed DNA species characteristic of N cytoplasms, four possessed DNA species characteristic of T cytoplasm, 19 possessed DNA species characteristic of S cytoplasm and two possessed DNA species characteristic of C cytoplasm. This classification is in complete agreement with that based on mitochondrial translation products reported in the accompanying paper. No within-group heterogeneity was observed in the DNA banding patterns, indicating a lack of cytoplasmic variation within the four cytoplasmic groups. Attributes of the various methods available for classifying maize cytoplasms are compared and discussed.

GENETIC variation that is inherited through the cytoplasm is well known in higher plants (SAGER 1972). Variation causing male sterility is one of the most studied examples because it has provided an efficient way of making hybrid seed in commercial agriculture. To extend the exploitation of cytoplasmically inherited male sterility and of cytoplasmic variation in general, there is a need to develop more rapid ways of recognizing and characterizing the variation and to understand it at the molecular level.

Because cytoplasmic variation is often expressed only in certain nuclear backgrounds, it is necessary to undertake extensive crossing programs to recognize and characterize such variation. In maize (*Zea mays* L.), cytoplasmic variation conferring male sterility has been recognized in breeding programs and has been classified into three groups (T, S and C) depending on the ability of nuclear genotypes in tester stocks to restore male fertility (DUVICK 1965; BECKETT 1971; GRACEN and GROGAN 1974).

Some years ago, attention was focussed on the mitochondria in cytoplasmic variants of maize because lines carrying the T-type cytoplasm were shown to have

* Present address: Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida.

mitochondria with an altered sensitivity to a pathotoxin from race T of the fungus *Helminthosporium maydis* (MILLER and KOEPPE 1971). Since then, research workers at several laboratories, including this one, have studied mitochondria from maize lines with different cytoplasms to identify and describe variation at the molecular level. FORDE, OLIVER and LEAVER (1978), FORDE and LEAVER (1980) and FORDE *et al.* (1980) have shown that mitochondria from lines carrying the T, S, C, or normal (N) cytoplasms can all be distinguished from one another by the *in vitro* synthesis of specific polypeptides. Mitochondrial DNAs from lines in each of the cytoplasmic groups have been distinguished by digestion with restriction endonucleases (LEVINGS and PRING 1976; PRING and LEVINGS 1978), but differences have also been found within the N and C groups (LEVINGS and PRING 1977; PRING, CONDE and LEVINGS 1979).

PRING *et al.* (1977) were the first to demonstrate that mitochondrial preparations (not subjected to restriction endonuclease digestion) from lines carrying *S* cytoplasm possessed two low molecular weight DNA species not found in mitochondria from N, T and C lines. More recently, a rapid mitochondrial DNA extraction procedure has been described (KEMBLE and BEDBROOK 1979) that permits the presence of these DNA molecules to be visualized using no more than five seeds. In later studies, other low molecular weight DNA differences have been found in mitochondrial preparations that specifically permit N, T and C cytoplasms to be distinguished from each other and from S cytoplasm (KEMBLE and BEDBROOK 1980).

In this paper, we report a study on the low molecular weight mitochondrial DNAs in a collection of 31 separately discovered sources of cytoplasms, most of which have been classified by BECKETT (1971) and GRACEN and GROGAN (1974) into the N, T, S and C groups. This survey was undertaken for two reasons. First we wished to study the correlation between the low molecular weight DNA complement and the interaction of the cytoplasm with specific fertility restorer genes. Second, because the grouping on the basis of fertility restoration provided evidence for within-group heterogeneity, we wished to determine whether this was accompanied by within-group heterogeneity in the low molecular weight DNA complements. We have also included a survey of all the cytoplasms for sensitivity to *H. maydis* race T toxin, using the simple method previously described (PETERSON, FLAVELL and BARRATT 1974; FLAVELL 1975) to study the correlation between toxin sensitivity and low molecular weight DNA complement.

In the accompanying paper (FORDE *et al.* 1980), the same set of cytoplasms has been classified on the basis of the polypeptides synthesized *in vitro* by isolated mitochondria. Thus, two biochemical laboratory tests are now available, each of which is capable of distinguishing all four types of cytoplasmic variation in maize.

MATERIALS AND METHODS

Maize lines: The 31 different cytoplasms used were in the nuclear background of CO192 × WJ produced by the Eucarpia Northern Maize Committee (GUNN 1975) *via* CO192 cytoplasmic stocks obtained from V. E. GRACEN (Cornell University). Cytoplasms studied were: B, C, CA, D,

EK, F, G, H, HA, I, IA, J, L, LF, ME, MY, OY, PS, Q, R, RB, RS, S, SD, SG, T, TA, TC, 181, 234 and the normal maintainer line (nml).

Extraction and purification of mitochondria for DNA isolation: Seeds were surface sterilized with 2% v/v sodium hypochlorite for 15 min, washed and imbibed in running tap water overnight prior to germination on distilled-water-moistened paper towelling in the dark for 4 days at 27°.

Mitochondria were isolated at 0 to 4° by a modification of the method of DAY and HANSON (1977). Approximately 5 g of etiolated shoots were harvested from 20 to 30 seedlings and homogenized in 3 vols/g fresh weight 0.5 M mannitol, 0.01 M N-Tris (hydroxymethyl) methyl-2-amino ethane-sulphonic acid (TES), pH 7.2, 0.001 M EGTA, 0.2% BSA and 0.05% cysteine for 30 sec using a pestle and mortar, prior to filtration through 4 layers of butter muslin and 1 layer of Miracloth (Calbiochem). The filtrate was centrifuged at 1,000 × g for 10 min, the resulting supernatant being recentrifuged at 12,000 × g for 10 min. The mitochondrial pellet was resuspended in homogenization buffer and again centrifuged at 1,000 × g for 10 min. Magnesium chloride and DNAase were added to the supernatant to give final concentrations of 0.01 M and 10 μg/g tissue fresh weight respectively, followed by incubation at 4° for 1 hr. Mitochondria were centrifuged through a layer of 0.6 M sucrose, 0.01 M TES, pH 7.2 and 0.02 M EDTA at 10,000 × g for 20 min and washed twice in the same solution before being lysed in 0.05 M Tris-HCl, pH 8.0, 0.01 M EDTA, 2% sarkosyl NL-97 and 0.012% autodigested pronase at 37° for 1 hr. Lysates were made 0.2 M with respect to ammonium acetate and the DNA purified by three phenol-chloroform extractions, ethanol precipitation and ethanol washes prior to resuspension in 50 μl of 0.005 M Tris-HCl, pH 8.0 and 0.00025 M EDTA.

Electrophoretic analysis of mitochondrial DNA: The mitochondrial DNA was made 5% and 0.005% with respect to glycerol and bromophenol blue, respectively, and electrophoresed at room temperature in 1.5% agarose horizontal slab gels (21 × 18 × 0.5 cm containing 14 sample wells) at 30 mA overnight. Electrophoresis buffer was 0.04 M Tris, pH 7.8, 0.005 M sodium acetate, 0.001 M EDTA (BEDBROOK and BOGORAD 1976). After electrophoresis, the gels were stained in 0.5 μg/ml ethidium bromide for 30 min before visualization of the DNA bands on the gel using a UV transilluminator (U.V. Products). Gels were photographed with either Polaroid type 47 high-speed Land film or Kodak PXP film, using a Wratten 23A filter.

Assay for mitochondrial sensitivity to Helminthosporium maydis *race T pathotoxin*: Mitochondria were isolated from 4-day-old etiolated shoots as described by FLAVELL (1975). The effect of pathotoxin on mitochondria was assayed by recording the level of malate oxidation, using 2,6,dichlorophenol-indophenol (DCIP) as an electron acceptor. The reduction of DCIP was followed at 600 nm at 30° in 1.5 ml of assay medium consisting of 0.23 M mannitol, 0.003 M EGTA, 0.0076 M potassium phosphate, 0.0076 M TES, 0.0038 M $MgCl_2$, 0.008 M KCl and 0.15% BSA, 1.54×10^{-3} M sodium azide, 0.046 M malate and 0.6×10^{-5} M DCIP at pH 7.2. Concentrations of mitochondria were used that gave ΔOD_{600} 0.1/min. The pathotoxin preparation was a gift from CARL TIPTON, Iowa State University, *via* PETER PETERSON of Iowa State University. Concentrations of pathotoxin were chosen that completely inhibited malate oxidation in T type mitochondria (FLAVELL 1975).

RESULTS

Mitochondrial DNA preparations were made from seedlings of all 31 cytoplasms and fractionated on agarose gels. Only four different patterns of low molecular weight DNA bands were observed. These corresponded to the characteristic patterns previously found for N, T, S and C cytoplasms shown in Figure 1. T-type cytoplasms lack the band found in all the other cytoplasmic types that migrate like marker fragments approximately 2.35 kilobases long (KEMBLE and BEDBROOK 1980), S-types possess two unique DNAs of about 6.2 kilobases and 5.2 kilobases (PRING *et al.* 1977) and C-types possess two unique DNAs that migrate like marker fragments about 1.55 kilobases and 1.4 kilobases long (KEMBLE and

BEDBROOK 1980). The bands that are common to all cytoplasms represent supercoiled, open-circular and linear conformations of the same DNA species (KEMBLE and BEDBROOK 1980).

Table 1 summarizes the results for all 31 cytoplasms. The results of the patho-

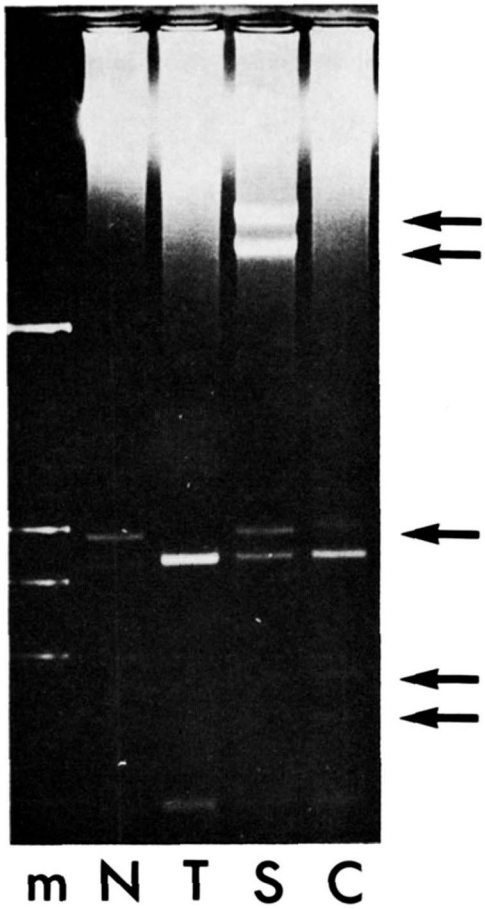

FIGURE 1.—Electrophoresis on 1.5% agarose gels of DNA isolated from the mitochondrial fraction of N, T, S and C cytoplasmic types of maize in the same nuclear background (CO192 × WJ). The N-, T-, S- and C-types shown here are represented by LF, HA, L and RB cytoplasms, respectively. The track labelled "m" is a collection of marker DNAs from lambda bacteriophage digested with the restriction endonuclease *Hae III*. The sizes of the marker DNA bands are: 3.9, 2.4, 2.1, 1.7 and 1.19 kilobases. The arrows indicate the DNA species that distinguish the four cytoplasmic types.

toxin sensitivity assay and of the mitochondrial translation product assay from the accompanying paper (FORDE *et al.* 1980) have been included for completeness. On the basis of the DNA assay, six of the cytoplasmic sources carry N-type cytoplasm, four carry T-type cytoplasm, 19 carry S-type cytoplasm and two carry C-type cytoplasm. Cytoplasms B. D and LF, unclassified by the nuclear gene fer-

TABLE 1

Classification of 31 maize cytoplasms

Cytoplasm	Cytoplasmic grouping by Nuclear fertility restoration*	Mitochondrial translation products†	Specific DNA species	*H. maydis* Toxin assay
C	C	C	C	not T
RB	C	C	C	not T
HA	T	T	T	T
Q	T	T	T	T
RS	T	T	T	T
T	T	T	T	T
B	unclassified	S	S	not T
CA	S	S	S	not T
D	unclassified	S	S	not T
EK	S	S	S	not T
F	S	S	S	not T
G	S	S	S	not T
H	S	S	S	not T
I	S	S	S	not T
IA	S	S	S	not T
J	S	S	S	not T
L	S	S	S	not T
ME	S	S	S	not T
MY	S	S	S	not T
PS	S	S	S	not T
R	S	S	S	not T
S	S	S	S	not T
SD	S	S	S	not T
TA	S	S	S	not T
TC‡	probably S	S	S	not T
LF	unclassified	N	N	not T
OY	probably N	N	N	not T
SG	probably N	N	N	not T
181	probably N	N	N	not T
234	probably N	N	N	not T
nml	N	N	N	not T

* Data from BECKETT 1971; GRACEN and GROGAN 1974.
† Data from accompanying paper, FORDE *et al.* 1980.
‡ This source was an impure stock; see text.

tility resoration method (BECKETT 1971; GRACEN and GROGAN 1974), were found to belong to the S group (B and D) and N group (LF).

TC cytoplasm is an impure stock resulting from a mixture of T and S cytoplasm seed (BECKETT 1971). The parentage of the TC cytoplasmic source used in this study indicates that it is composed of S cytoplasm seed. This was verified by analysis of its mitochondrial DNA, which shows the presence of two DNA bands unique to S-types (PRING *et al.* 1977) and the presence of a band that *bona fide* T-types lack (KEMBLE and BEDBROOK 1980). Electron transport in mitochondria isolated from the TC cytoplasmic source was insentitive to the *Helminthosporium maydis* race T pathotoxin (Table 1). The only cytoplasmic sources in which mitochon-

drial electron transport was completely inhibited were HA, Q, RS and T (Table 1). These data agree with that obtained from mitochondrial DNA analysis, mitochondrial translation product analysis (FORDE *et al.* 1980) and nuclear gene fertility restoration analysis (BECKETT 1971; GRACEN and GROGAN 1974) in showing that only these four cytoplasms of the 31 assayed are members of the T group.

DISCUSSION

Analysis of mitochondrial DNA by agarose gel electrophoresis has been used in this study to classify unambiguously 31 cytoplasmic sources into one of four groups (Table 1). The results are in complete agreement with the classification according to nuclear gene fertility restoration data (BECKETT 1971; GRACEN and GROGAN 1974) and the variation seen in mitochondrial translation products reported in the accompanying paper (FORDE *et al.* 1980). The four cytoplasmic types found to give mitochondrial DNA banding patterns characteristic of the T group of cytoplasms were also verified as members of the T group by employing the *H. maydis* race T pathotoxin test (PETERSON, FLAVELL and BARRATT 1974; FLAVELL 1975). A study of the susceptibility of field-grown plants of 30 sources of male-sterile cytoplasms, 23 of which were similar to the sources used in the present study, to *H. maydis* race T was carried out by SMITH *et al.* (1971). Only HA, Q and T cytoplasms (RS cytoplasm was not used in their study) were severely infected by the fungus and were designated as T-types.

No heterogeneity was detected in the mitochondrial DNA banding patterns within any of the four cytoplasmic groups. Similarly, no within-group heterogeneity was detected employing the mitochondrial translation products assay reported in the accompanying paper (FORDE *et al.* 1980). We conclude that greater cytoplasmic variation is unlikely to be introduced into breeding programs by using sources of cytoplasm from the same group. However, restriction endonuclease digestion of mitochondrial DNA has illustrated variation within N and C groups (LEVINGS and PRING 1977; PRING, CONDE and LEVINGS, 1979).

Some attributes of the DNA assay for classification of cytoplasmic types described here are: (1) Only a small amount of starting tissue is required, *i.e.*, 5 g of etiolated shoots that can be harvested from 20 to 30 seeds if grown as described. (2) It is rapid. Seeds need be grown for only four days, followed by isolation and purification of mitochondrial DNA (one day) and an overnight agarose gel electrophoresis step. Many samples can be processed simultaneously. (3) Characteristic banding patterns of mitochondrial DNA are obtained regardless of nuclear background (PRING *et al.* 1977; KEMBLE and BEDBROOK 1979, 1980). (4) The presence of a normal, male-fertile cytoplasm can be unambiguously demonstrated (KEMBLE and BEDBROOK 1980). (5) Classification of cytoplasmic types can be performed even if the line has been restored to fertility by nuclear restoring genes (PRING *et al.* 1977; KEMBLE and BEDBROOK 1979; KEMBLE and BEDBROOK 1980). (6) It can classify cytoplasms that cannot be distinguished by the nuclear gene fertility restoration method.

These features give the method a considerable advantage over the classical field methods that depend on scoring male sterility in a range of nuclear back-

grounds (DUVICK 1965; BECKETT 1971; GRACEN and GROGAN 1974). The assay method detailed in the accompanying paper (FORDE *et al.* 1980), which is based upon *in vitro* mitochondrial protein synthesis, has many of the advantages of the DNA assay described here; however, the DNA assay will often be more convenient in that it requires fewer seeds (20 to 30), is more rapid and does not require expensive ^{35}S-methionine. When identification of S-types is required, the rapid DNA assays, which requires five or fewer etiolated shoots or 0.5 g of leaf material, is the simplest test available (KEMBLE and BEDBROOK 1979; KEMBLE 1980). When identification of T-types is required, the rapid electron transport assay using *H. maydis* race T toxin and an electron acceptor dye (dichlorophenol-indophenol) is the simplest assay method (PETERSON, FLAVELL and BARRATT 1974; FLAVELL 1975).

Although the assay of mitochondrial DNA species may be the simplest method available for classifying a range of cytoplasms, its relationship with the cytoplasmic variation expressed in specific plant genotypes is unknown. The DNA species that allow the four cytoplasmic types to be differentiated may not be transcribed or translated into protein products and may, therefore, have no functional significance. Clearly, the relationships between the DNA species, the mitochondrial protein products and plant phenotypic variation need to be investigated. However, we anticipate that the simplicity, rapidity and unambiguity of cytoplasm classification by the DNA assay described here will be of considerable help to maize breeders and geneticists interested in recognizing and exploiting cytoplasmic variation.

This research was supported by a Rank Prize Fund Fellowship to R. J. KEMBLE.

LITERATURE CITED

BECKETT, J. B., 1971 Classification of male-sterile cytoplasms in maize (*Zea mays* L.). Crop Sci. **11**: 724–727.

BEDBROOK, J. R. and L. BOGORAD, 1976 Endonuclease recognition sites mapped on *Zea mays* chloroplast DNA. Proc. Natl. Acad. Sci. U.S. **73**: 4309–4313.

DAY, D. A. and J. B. HANSON, 1977 On methods for the isolation of mitochondria from etiolated corn shoots. Plant Sci. Lett. **11**: 99–104.

DUVICK, D. N., 1965 Cytoplasmic pollen sterility in corn. Advan. Genet. **13**: 1–56.

FLAVELL, R., 1975 Inhibition of electron transport in maize mitochondria by *Helminthosporium maydis* race T pathotoxin. Physiol. Plant Path. **6**: 107–116.

FORDE, B. G. and C. J. LEAVER, 1980 Nuclear and cytoplasmic genes controlling synthesis of variant mitochondrial polypeptides in male-sterile maize. Proc. Natl. Acad. Sci. U.S. **77**: 418–422.

FORDE, B. G., R. J. C. OLIVER and C. J. LEAVER, 1978 Variation in mitochondrial translation products associated with male-sterile cytoplasms in maize. Proc. Natl. Acad. Sci. U.S. **75**: 3841–3845.

FORDE, B. G., R. J. C. OLIVER, C. J. LEAVER, R. E. GUNN and R. J. KEMBLE, 1980 Classification of normal and male-sterile cytoplasms in maize. I. Electrophoretic analysis of variation in mitochondrially synthesized proteins. Genetics **95**: 443–450.

GRACEN, V. E. and C. O. GROGAN, 1974 Diversity and suitability for hybrid production of different sources of cytoplasmic male-sterility in maize. Agron. J. **65**: 654–657.

GUNN, R. E., 1975 Report on the activities of the Northern Maize Committee (1974–75). pp. 22–29. *Eucarpia, Eighth meeting of the maize and sorghum section held in Paris-Versailles, 15–17 September.*

KEMBLE, R. J., 1980 A rapid, single leaf assay for detecting the presence of 'S'-type male-sterile cytoplasm in maize. Theor. Appl. Genet. (in press).

KEMBLE, R. J. and J. R. BEDBROOK, 1979 A rapid assay for detecting male-sterile maize lines possessing 'S'-type cytoplasm. Maydica **24**: 175–180. ——, 1980 Low molecular weight circular and linear DNA molecules in mitochondria from normal and male-sterile cytoplasms of *Zea mays*. Nature **284**: 565–566.

LEVINGS, C. S., III and D. R. PRING, 1976 Restriction endonuclease analysis of mitochondrial DNA from normal and Texas cytoplasmic male-sterile maize. Science **193**: 158–160. ——, 1977 Diversity of mitochondrial genomes among normal cytoplasms of maize. J. Hered. **68**: 350–354.

MILLER, R. J. and D. E. KOEPPE, 1971 Southern corn leaf blight: susceptible and resistant mitochondria. Science **173**: 67–69.

PETERSON, P. A., R. B. FLAVELL and D. H. P. BARRATT, 1974 A simple biochemical assay for "Texas" cytoplasm in corn by use of *Helminthosporium maydis*, race T pathotoxin. Plant Dis. Reptr. **58**: 777–780.

PRING, D. R., M. F. CONDE and C. S. LEVINGS, III, 1979 Heterogeneity within the C group of male-sterile cytoplasms. Maize Genet. Coop. News Lett. **53**: 42.

PRING, D. R. and C. S. LEVINGS, III, 1978 Heterogeneity of maize cytoplasmic genomes among male-sterile cytoplasms. Genetics **89**: 121–136.

PRING, D. R., C. S. LEVINGS, III, W. W. L. HU and D. H. TIMOTHY, 1977 Unique DNA associated with mitochondria in the 'S'-type cytoplasm of male sterile maize. Proc. Natl. Acad. Sci. U.S. **74**: 2904–2908.

SAGER, R., 1972 Cytoplasmic genes and organelles. Academic Press, New York.

SMITH, D. R., A. L. HOOKER, S. M. LIM and J. B. BECKETT, 1971 Disease reaction of thirty sources of cytoplasmic male-sterile corn to *Helminthosporium maydis* race T. Crop Sci. **11**: 772–773.

Corresponding editor: R. L. PHILLIPS