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A Rapid, Single Leaf Assay for Detecting the Presence of 'S'-Male-sterile Cytoplasm in Maize

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Summary. A simple, rapid, and reproducible assay is described for determining unambiguously the presence of S-type cytoplasm in male-sterile and male-fertile (restored) maize lines. Because the assay requires only 0.5 g leaf segment per sample, it is a single plant assay and the plant is not destroyed. Plants at any developmental stage can be used. The assay involves a 30 sec homogenization, 20 min centrifugation, one hour lysis, overnight agarose electrophoresis, 30 min gel staining, and photography of the gel to produce a result in much less than 24 hr. Many samples can be assayed simultaneously. The various assay methods available for classifying maize cytoplasms are compared and discussed.

Key words: *Zea mays* – Male-sterility – 'S'-Cytoplasm – Mitochondria – DNA

Introduction

Since the disastrous epidemic of *Helminthosporium maydis* race T in 1970 there has been concern to find a suitable alternate source of cytoplasmic male-sterility and to introduce greater cytoplasmic variation into cultivated maize (*Zea mays* L.) hybrids. There has therefore been a renewed emphasis on ways of recognizing and characterizing variation at both the molecular and plant phenotype level. So far, characterization at the molecular level has involved analysis of mitochondrial DNA species (Pring et al. 1977; Kemble and Bedbrook 1980), the use of restriction endonucleases on such DNAs (Levings and Pring 1976; Pring and Levings 1978), in vitro labelling of mitochondrial translation products (Forde et al. 1978; Forde and Leaver 1980), and studies on mitochondrial electron transport (Peterson et al. 1974). Characterization of the available cytoplasmic variation has been achieved at the plant phenotype level by scoring pollen fertility in progeny from crosses of cytoplasmic sources and tester stocks containing nuclear fertility restoration genes (Duvick 1965; Beckett 1971). These studies at both levels have resulted in the available cytoplasmic sources being classified into one of four groups, N male-fertile, or the male-sterile cytoplasms, T, S, and C.

Because some of the biochemical methods demand many seeds and are very time consuming, a rapid method for determining the presence of one of the cytoplasms, S, was recently developed (Kemble and Bedbrook 1979). This method required five or fewer etiolated shoots and was based on the finding of Pring et al. (1977) that mitochondrial DNA extracts from S-cytoplasmic sources possess two low molecular weight DNA species not found in mitochondrial DNA from N, T, or C cytoplasms. The present paper describes a modification of that assay (Kemble and Bedbrook 1979) that allows the presence or absence of S-cytoplasm to be unambiguously determined by analysing small leaf segments from growing plants. This simple, rapid method allows single plants to be assayed at any stage of development without destroying the plants under test. Since many samples can be assayed simultaneously it can be employed as a routine screening test for S-cytoplasmic types.

Materials and Methods

Maize Genotypes

The lines used in this study were B37 carrying N, S, and SRf cytoplasms, CO192 X WJ carrying N, S, R, CA, and TA-cytoplasms, WF9 carrying N and S-cytoplasms, and WM13 carrying S-cytoplasm. Plants were grown under normal summer daylight regimes in an unheated glasshouse.

Assay Procedure

All procedures were similar to those described previously (Kemble and Bedbrook, 1979) and were carried out at 4°C unless otherwise

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stated. One 0.5 g leaf segment was taken from each plant to be tested at various stages of development up to maturity. Each segment was cut into 0.5 cm^2 pieces prior to homogenization for 30 sec in a mortar and pestle containing 15 ml homogenization buffer consisting of 0.01M TES pH 7.2, 0.5M mannitol, 0.001M EGTA, 0.2% BSA, and 0.05% cysteine. Filtration of the homogenate through four layers of butter muslin and one layer of Miracloth (Calbiochem) was followed by centrifugation at 1,000 xg for 10 min. The resulting supernatant was carefully removed using pasteur pipettes and recentrifuged at 12,000 xg for 10 min. The pellet obtained was incubated at 37°C for 1 hr in 200 µl lysis buffer consisting of 0.05M Tris-HCl pH 8.0, 0.01M EDTA, 2% sarkosyl NL-97 (Ciba-Geigy) and 0.012% autodigested pronase (Calbiochem, B grade, autodigested by incubation at 37°C for 1 hr. Aliquots (50 µl) of the lysates were prepared for electrophoresis by adding glycerol and bromophenol blue to give final concentrations of 5% and 0.005% respectively. Electrophoresis was carried out at room temperature in 1% agarose horizontal slab gels (21 × 18 × 0.5 cm containing 14 sample wells) at 30mA constant current overnight until the bromophenol blue marker had reached the end of the gel. The electrophoresis buffer contained 0.04M Tris-HCl, 0.005M sodium acetate, 0.001M EDTA, pH 7.8 (Bedbrook and Bogorad 1976). Gels were stained in 0.5 µg/ml ethidium bromide for 30 min prior to illumination by an ultraviolet transilluminator (U.V. Products). DNA bands were not usually visible by eye above the background fluorescence but were clearly visible on positive and negative photographs of the gel. Either Polaroid type 47 high-speed land film or Kodak PXP film, both employing a Wratten 23A orange filter, were used. Greater resolution of DNA bands on gels was obtained by including a one hour water destain step after gel staining. However, DNA bands can be easily seen on photographs of gels which have not undergone this additional step, so unless high quality photographs are required this step only serves to increase the time taken for each assay.

Results

Leaf segments from maize plants carrying N male-fertile cytoplasm, S male-sterile cytoplasm, S-cytoplasm which had been restored to male-fertility by nuclear fertility restoration genes (SRf), and male-sterile cytoplasms which are members of the S-group were subjected to the rapid assay method described in Materials and Methods. Fig. 1 shows the results obtained for cytoplasms in B37 nuclear background. Track 1 contains lambda bacteriophage DNA digested with EcoRI restriction endonuclease to produce molecular weight markers of 13.7, 4.74, 3.73, 3.48, and 3.02 daltons (Thomas and Davis 1975). Lysates derived from N-cytoplasm are in tracks 2 and 5, S-cytoplasm in tracks 3 and 6, and SRf cytoplasm in Tracks 4 and 7. Sample volumes in Tracks 2, 3 and 4 were 50 µl (i.e. $\frac{1}{4}$ total lysate volume), those in tracks 5, 6, and 7 were 20 µl (i.e. $\frac{1}{10}$ total lysate volume). The presence of the two characteristic S-type DNA bands of about 4.1 and 3.4 × 10^6 daltons (Pring et al. 1977) are clearly visible in both the two S and the two SRf tracks. This indicates that the ability to visualize the S-type DNA bands is not dependent on the sample volume but simply depends on the fluorescence in the DNA bands being higher than the background fluorescence in the gel. It also shows that the assay can detect the presence of S-cytoplasm when the line under investigation has been restored to male-fertility by nuclear fertility restoration genes.

The S-type DNA bands were also detected in the other lines examined, namely CO192 × WJ, WF9, and WM13 carrying S-cytoplasm, indicating that the assay is independent of nuclear genetic backgrounds. These characteristic DNA bands were similarly found in CO192 × WJ lines carrying R, CA, and TA cytoplasms which are members of the S-group of cytoplasms (Beckett 1971; Gracen and Grogan 1974; Forde et al. 1980; Kemble et al. 1980).

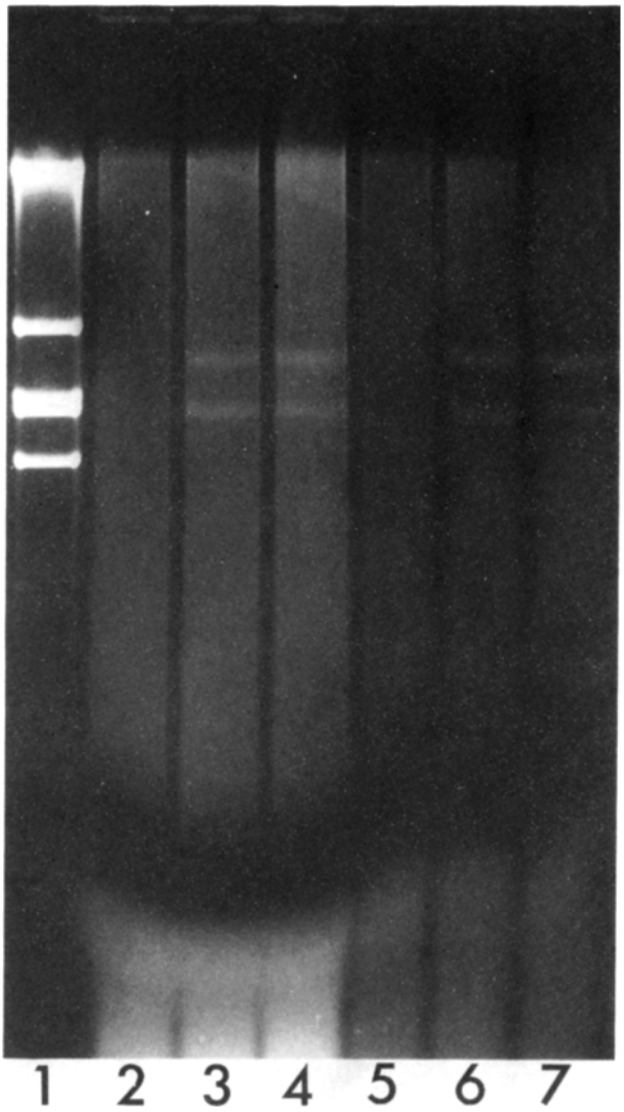

Fig. 1. Agarose electrophoresis of DNA in crude mitochondrial extracts from maize lines carrying N, S, and SRf cytoplasms. Track 1 = molecular weight markers; 2 and 5 = N cytoplasm; 3 and 6 = S cytoplasm; 4 and 7 = SRf cytoplasm

Although, as previously stated, the sample volume loaded onto each gel track is not critical, other volumes quoted in the assay procedure are. If less than 15 ml homogenization buffer is used for each 0.5 g leaf segment, insufficient cellular disruption occurs resulting in an inability to detect the S-type DNA bands above background fluorescence on the gels. Similarly, if less than 200 μl lysis buffer is used, either the osmotic concentration of the homogenization buffer is not diluted sufficiently or the protein: detergent ratio is incorrect, resulting in inefficient mitochondrial rupture and insufficient DNA release to be detected on gels.

Crude mitochondrial pellets obtained after the 12,000 xg centrifugation, and consequently the lysates derived from them, are green in colour presumably due to contamination by chlorophyll containing chloroplast membrane fragments. However, this contamination does not interfere with the sensitivity of the assay. Indeed, if further purification steps are carried out to free the mitochondria from this contamination, or the DNA is purified by phenol-chloroform extractions and ethanol precipitations, no increase in sensitivity of the assay is observed; background fluorescence on gels is reduced but larger amounts of starting material are required.

Young or old leaves can be used from plants at any developmental stage. Clearer results are obtained with younger tissue but mature leaves can be used with success providing dead and chlorotic regions are removed. Experiments in which leaves were harvested and stored frozen at -80°C for several weeks before assaying were unsuccessful; fluorescence molecules smeared over the entire length of the gel and masked detection of the S-type DNA bands. However, lysates extracted from fresh tissue could be successfully assayed after being stored for several months at -20°C.

Discussion

In a previous report (Kemble and Bedbrook 1979) a rapid assay for detecting the presence of S-type cytoplasm in maize was described which required only five etiolated shoots per sample. Because multiple seedlings were used and destroyed, the procedure was useful only for assaying seed bulks. The assay reported here, which utilizes a portion of a leaf, has the advantage of using a single plant which is not destroyed. Individually tested plants can therefore be used in breeding programmes in total confidence that they carry S-type cytoplasm. Like the other method (Kemble and Bedbrook 1979) the leaf assay can detect the presence of S-cytoplasm in plants (i) regardless of nuclear genetic background (ii) which have been restored to full male-fertility by nuclear fertility restoring genes, and (iii) which have cytoplasms related to S-type cytoplasms but which often display different degrees of male-fertility in the same nuclear background. Other attributes of the leaf assay are that it is technically simple to carry out, is very rapid, and enables many samples to be tested simultaneously.

There are four other methods which as well as detecting S-cytoplasm are capable of differentiating between the other two male-sterile cytoplasms, T and C, and N male-fertile cytoplasm. However, all these methods require expensive equipment and chemicals and are of long duration. The classical field method (Duvick 1965; Beckett 1971; Gracen and Grogan 1974) is the most time consuming and involves crossing each cytoplasmic source with pollen from many inbred lines and backcrossing repeatedly. Restriction endonuclease digestion of mitochondrial DNA isolated from large quantities of etiolated shoots has been used (Levings and Pring 1976; Pring and Levings 1978). Mitochrondria isolated from 30 g etiolated shoots and incubated in vitro with ^{35}S-methionine produce radioactively labelled translation products characteristic of each cytoplasmic group (Forde et al. 1978; Forde and Leaver 1980; Forde et al. 1980). The fourth assay (Kemble et al. 1980) requires only 5 g of etiolated shoots, which can be obtained from 20 to 30 seeds, and is based on the finding of Kemble and Bedbrook (1980) that each cytoplasmic group has a characteristic mitochondrial DNA banding pattern on agarose gels.

In conclusion, unambiguous classification of maize cytoplasms into N, T, C, and S-groups is achieved most easily by the method of Kemble and Bedbrook (1980). Of the four methods capable of distinguishing between the four cytoplasmic groups, that assay is the least expensive, simplest, most rapid, and its success has been demonstrated (Kemble et al. 1980). When identification of T-types only is required the most rapid assay available is that which involves assaying mitochondrial electron transport in the presence of *Helminthosporium maydis* race T pathotoxin and an electron acceptor dye (Peterson et al. 1974; Flavell 1975). However, when identification of S-types is required the assay method reported here is, for reasons previously described, by far the simplest assay available. It is hoped that its many attributes will aid geneticists and breeders in their efforts to recognize and exploit cytoplasmic variation in maize.

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