A COMPARISON OF MITOCHONDRIAL PROTEINS FROM
DIVERSE CYTOPLASMS OF MAIZE BY
GEL ELECTROPHORESIS

BY

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1979

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to his major professor Dr. Daryl Pring, for his guidance and patience throughout this project. The author also wishes to thank the members of his committee Dr. J. R. Edwardson, Dr. R. J. Mans, Dr. E. Hiebert, and Dr. D. A. Roberts for their guidance and advice. A special appreciation goes to Dr. J. R. Laughnan and Dr. S. J. Gabay-Laughnan for the genetic stocks they provided which were an invaluable aid to this study.

Finally the author wishes to thank his parents, Dr. and Mrs. James H. Thornbury, for their faith and encouragement.

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Abstract of Dissertation Presented to the Graduate Council
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

A Comparison of Mitochondrial Proteins From
Diverse Cytoplasms of Maize by
Gel Electrophoresis

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June 1979

Chairman: Daryl R. Pring

Major Department: Plant Pathology

Male sterility is a phenomenon of higher plants usually expressed as a failure to produce functional pollen. Cytoplasmic male sterility (cms) is transmitted through the female parent only and its inheritance is non-mendelian. In maize (Zea mays, L.), cytoplasmic male sterility is economically useful in the production of hybrid seed. By 1970, the Texas male-sterile cytoplasm (Tcms) was predominant in the production of hybrid maize seed in the United States. The southern corn leaf blight epidemic of 1970 led to its abandonment, since Tcms maize lines were highly susceptible to race T of Bipolaris maydis (Nisikado) Shoemaker. This increased susceptibility resulted from the production by race T of a host-specific pathotoxin.

The cytoplasm-specific mitochondrial characteristics of a) differential sensitivity of Normal (N) and Tcms mitochondria to the pathotoxin from race T of B. maydis, b) differences in the endonuclease restriction fragment patterns between N and cms mitochondrial DNA, and c) differences among several N versus cms mitochondrial enzyme levels suggested a dual mitochondrial involvement in the susceptibility of Tcms lines and in male sterility. The objective

of this work was to investigate the hypothesis that these differences may be detected at the protein level. Therefore, mitochondria from isogenic lines of maize were examined for protein differences associated with male sterility and disease susceptibility. Soluble and membrane mitochondrial proteins were examined by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Two types of protein differences were detected in total mitochondrial protein preparations. Differences due to nuclear variation were seen among mitochondria from four maize lines in the USDA or S male sterile cytoplasm (Scms). One cytoplasm-specific protein was found in mitochondria from Scms maize lines. This S protein has an apparent molecular weight of 133,900 daltons, determined by SDS-PAGE. Examination of membrane fractions from purified mitochondria revealed no detectable cytoplasm-specific differences between N and Tcms maize.

Labeling of the S protein was detected in mitochondria from shoots germinated in the presence of ^{35}S . Emetine, an inhibitor of cytoplasmic ribosomes, was used to limit labeling of nuclear-coded mitochondrial proteins. Inhibition was incomplete, and specific labeling of mitochondrial gene products was not achieved. Analysis of mitochondrial proteins from other maize cytoplasms labeled with ^{35}S in detached shoots did not reveal protein differences that correlated with cytoplasmic male sterility or disease susceptibility.

The relationship of the S protein to male sterility was examined in restorer and non-restorer maize lines and in a line that carries a

cytoplasmic mutation to fertility. With the exception of the line carrying the cytoplasmic mutation to male fertility, all Scms lines tested contained the S protein in their mitochondria. Mitochondria from Scms maize contain two plasmid-like DNAs. Data from other workers suggested that these DNAs play a role in male sterility. The plasmid-like DNAs have also been shown to be absent in lines carrying this mutation. The presence of fertility restorer genes in the nucleus had no effect on either the S protein or the plasmid-like DNAs.

Data from this study indicate that cytoplasmically determined male sterility and disease susceptibility do not produce gross changes in the electrophoretic phenotype of maize mitochondria. However, one unique protein was detected in Scms lines by SDS-PAGE. Although its function is unknown, the presence of the protein correlates with the presence of plasmid-like DNAs in Scms maize, suggesting that the S protein may be a gene product of the plasmid-like DNAs.

INTRODUCTION

Male sterility in higher plants usually is expressed as a failure to produce functional pollen. Male sterility in maize (Zea mays, L.) can be determined by nuclear genes (Beadle, 1932) or by cytoplasmic factors (Rhoades, 1933; Duvick, 1965). Cytoplasmic male sterility (cms) is transmitted only through the female parent and the factors responsible for its expression are not known. In maize cytoplasmic male sterility is widely used in the production of hybrid seed. Cytoplasmic male sterility, used in conjunction with nuclear genes which restore pollen fertility, eliminates the need for detasseling in the production of hybrid seed.

Until 1970, the use of cytoplasmic male sterility was the dominant procedure for the production of maize seed in the United States (Tatum, 1971). Approximately 85 percent of all maize planted in the United States in that year had one type of male sterile cytoplasm, called Texas, or Tcms, cytoplasm (Ullstrup, 1972). This situation of cytoplasmic homogeneity was conducive to the development of the southern corn leaf blight epidemic of 1970. The epidemic was caused by a race of the causal agent, Bipolaris maydis (Nisikado) Shoemaker (formerly Helminthosporium), designated race T, which exhibited highly specific pathogenicity towards maize hybrids produced using Texas male sterile cytoplasm (Hooker et al., 1970; Scheifele et al., 1970). Prior to the epidemic, southern corn leaf blight had been of little

economic importance in the United States. In 1970, the disease caused an estimated one billion dollar loss in the maize crop in the United States (Tatum, 1971).

The specific pathogenicity of race T of B. maydis to Tcms maize lines raised questions about the nature of cytoplasmic male sterility and its role in disease susceptibility. Race T produces a host-specific toxin (Hooker et al., 1970) that inhibits root growth in seedlings having Tcms cytoplasm but not in those with normal (N) cytoplasm. Isolated mitochondria from Tcms maize were sensitive to T toxin. the toxin induced swelling of mitochondria, the uncoupling of oxidative phosphorylation, and other physiological effects on Tcms mitochondria (Miller and Koeppe, 1971; Peterson et al., 1975). Toxin did not have these deleterious effects on the mitochondria from N cytoplasm maize.

Cytological studies have indicated mitochondrial involvement in toxin sensitivity and male sterility. Ultrastructural studies by Aldrich et al. (1977) showed that toxin effects on Tcms mitochondria were rapid and preceded any other cellular changes. Involvement in male sterility was indicated by mitochondrial degeneration in Tcms anthers during microsporogenesis (Warmke and Lee, 1977).

In addition to physiological and cytological evidence for a mitochondrial role in cytoplasmic inheritance of male sterility and disease susceptibility, cytoplasm-specific differences have been found in mitochondrial DNA and proteins. Levings and Pring (1976) showed cytoplasm-specific differences in restriction endonuclease digest patterns of mitochondrial DNA from N and Tcms maize.

Mitochondrial differences were also found in other male sterile cytoplasms (Pring and Levings, 1978). Chloroplast DNA from N, Tcms, and Ccms cytoplasms were indistinguishable using these techniques (Pring and Levings, 1978). Mitochondrial protein differences in N and Tcms maize were found in carboxymethylated acidic, chloroform/methanol extracts of submitochondrial particles and of the partially purified mitochondrial ATPase complex analyzed by isoelectric focusing in polyacrylamide gels (Barratt and Peterson, 1977). Forde et al. (1978) found that in vitro amino acid incorporation by isolated maize mitochondria yielded approximately twenty discrete polypeptides. They found Tcms maize mitochondria produced one polypeptide that was different from those of all other cytoplasms. Ccms mitochondria also produced a polypeptide that was distinct among the mitochondrially synthesized polypeptides.

Data on differential sensitivity of N and Tcms mitochondria to pathotoxin from B. maydis, race T and cytoplasm-specific mitochondrial DNA and protein differences suggested mitochondrial involvement in cytoplasmic male sterility and in susceptibility in Tcms lines. The objective of this work was to investigate the possibility of a dual role of mitochondria in these phenomena by examining mitochondria from isogenic lines of maize for cytoplasm-specific differences in protein components. To achieve this objective mitochondrial proteins were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using optical staining techniques as well as differential labeling with radioisotopes.

LITERATURE REVIEW

Cytoplasmic Male Sterility of Maize

Rhoades (1933) reported male sterility in maize supplied to him from Peru. He concluded that the male sterility was transmitted by cytoplasmic factors. Rhoades made no further studies and this source was lost. In the 1940's, two more sources of male sterility were found, one in Texas by J. S. Rogers (Rogers and Edwardson, 1952) termed "Texas" or "T" cytoplasm and the other, called "USDA" or "S" cytoplasm, by D. F. Jones (Jones et al., 1957).

The use of nuclear genes that restore fertility to cms maize (Jones et al., 1957) made cytoplasmic male sterility an economically useful trait. There have been many reported sources of male sterility in maize (Beckett, 1971), but most of these can be classed as either a T, S, or C-type according to patterns of fertility restoration. In the T-type two gene loci, called Rf1 and Rf2 for restoration factor, are involved. A heterozygous condition at both loci results in complete fertility. Therefore, it can be said that restoration is dependent upon the genotype of the sporophyte. In S-type a third locus (Rf3) is responsible for restoration. The heterozygous condition at this locus (Rf3 rf3) results in semifertile flowers. Half of the pollen carry rf3 and are sterile; half carry Rf3 and are fertile. The S-type restoration is thus dependent upon the genotype of the gametophyte

(Duvick, 1965). Beckett (1971) studied the restoration of thirty maize lines with male sterile cytoplasms. On the basis of restoration patterns Beckett was able to distinguish a third cytoplasm group, Ccms, in addition to T and S. As with Tcms, heterozygous restoration in C results in 100 percent fertility.

By 1970, the use of Tcms hybrids was the dominant procedure for the production of maize seed in the United States (Tatum, 1971). The advantages of complete fertility with heterozygous restoration and the fact that sterile and partially fertile plants can be distinguished by visual examination was responsible for this dependence upon one source. This led to a situation of cytoplasmic homogeneity which was conducive to the development of the southern corn leaf blight epidemic of 1970.

Association of Disease Susceptibility with Texas Cytoplasmic Male Sterility

The appearance of an extremely virulent race of B. maydis curtailed the use of Tcms hybrids. Race T was found to produce a host specific toxin (Hooker et al., 1970), and had increased virulence which is seen in the ability of race T to infect most parts of the plant. Infection by race O (old race) is restricted mainly to the leaves. Hooker and his co-workers (1970) found that small amounts of this pathotoxin caused severe inhibition of root growth in Tcms but not in normal (N) maize seedlings.

The site of T toxin action in Tcms maize has been investigated in isolated mitochondria and intact tissues. Miller and Koeppe's work (1971) indicated mitochondrial involvement in toxin sensitivity.

They observed that the pathotoxin caused immediate and irreversible swelling of Tcms mitochondria suspended in KCl medium. The pathotoxin also induced uncoupling of oxidative phosphorylation and inhibition of malate oxidation. Mitochondria from N cytoplasm shoots were unaffected by the pathotoxin. In addition, Peterson et al. (1975) showed that toxin induced activation of cytochrome oxidase and succinate-cytochrome c reductase in isolated Tcms mitochondria. Inhibition of the electron transport chain at a step prior to the entry of electrons from succinate dehydrogenase was also seen in susceptible mitochondria. From the inhibition of malate oxidation Flavell (1975) concluded that a site of toxin action is within the endogenous NADH dehydrogenase complex or the coupling of malate dehydrogenase to the complex.

Nine inbred maize lines showed differential sensitivity to toxin in both uncoupling of oxidative phosphorylation and inhibition of electron transport (Barratt and Flavell, 1975). It was suggested that interaction between Tcms mitochondria and nuclear restorer gene products produced structural changes in the mitochondria, which were responsible for modifications in mitochondrial response to the pathotoxin (Barratt and Flavell, 1975; Watrud et al., 1975a). Watrud et al. (1975b) found that removal of the outer mitochondrial membrane while having no effect on Tcms mitochondrial response, conferred sensitivity to normally resistant N mitochondria. They suggested that differences in outer membrane permeability to toxin affects the accessibility of a mitochondrial site to action of the toxin.

Association of Disease Susceptibility and Male Sterility with Mitochondria

The role of the mitochondrion as the primary site of pathotoxin action was questioned by Arntzen et al. (1973). They reported no significant changes in tissue respiration or phosphate uptake and release in intact roots treated with T toxin at high concentration or for ten minute exposures at lower concentrations. These results did not correlate well with toxin effects on isolated mitochondria. However, Bednarski et al. (1977) found that T toxin treated leaves and coleoptiles from susceptible plants exhibited up to 60 percent greater oxygen uptake than untreated controls; resistant plants showed no effects. An explanation of these conflicting data comes from work using 2-deoxyglucose to assay mitochondrial function in vivo. Mitochondrial contraction in response to addition of 2-deoxyglucose can be viewed ultrastructurally and indicates the phosphorylative capacity of mitochondria. Using this approach, Malone et al. (1978) found that T toxin penetrated root and leaf tissue slowly. However, once in the cells the toxin had rapid deleterious effect on mitochondrial function. In addition leaf studies showed that the toxin effect was only detectable in cells within 3.0 mm of the fungal lesions. They concluded that T toxin has in vivo effects similar to those reported from in vitro experiments, and that mitochondria are a primary site of toxin sensitivity. Cytological studies by Aldrich et al. (1977) also indicated that mitochondria are primary sites of toxin sensitivity. They found ultrastructural changes in root mitochondria of susceptible (but not resistant) maize treated in situ with T toxin. Mitochondrial effects were rapid and preceded any other detectable cellular changes.

A role for a plasmalemma target site for T toxin has been suggested by in vivo studies of Mertz and Arntzen (1977, 1978). They found selective inhibition of ion uptake and a depolarization of cell membrane potential in Tcms maize tissues treated with T toxin. Effects were seen after 10 to 30 minutes exposure to toxin; at higher toxin concentrations N maize tissues exhibited similar effects but to a lesser degree.

An association of toxin sensitivity with Tcms mitochondria indicates a coupling of the susceptibility and sterility phenomena. Gengenbach and Green (1975) and Gengenbach et al. (1977) used toxin to select resistant cultures from T callus tissue culture. Mitochondria isolated from these cultures were unaffected by toxin. Plants differentiated from the calli were resistant to toxin and male fertile. Studies by Warmke and Lee (1977) also suggest the mitochondrion as the site of determinants conditioning male sterility in maize. They observed mitochondrial degeneration in the tapetum and middle layer of T anthers at the tetrad stage of microsporogenesis; no plastid changes were detectable until late in anther development. No mitochondria or plastid alterations were seen in N anthers.

The association of mitochondria with susceptibility and male sterility led to the examination of mitochondrial proteins for cytoplasm-specific differences. Examination of mitochondrial membranes using SDS-PAGE suggested quantitative differences between two low molecular weight species in inner membrane particles and one high molecular weight species in outer membrane fractions from N and Tcms maize (Watrud et al., 1975b). Neither Bolen (1975) nor Barratt and

Peterson (1977) were able to detect these differences. Bolen (1975) was unable to detect cytoplasm-specific protein differences among five cytoplasmic versions of six inbred lines. Using carboxy-methylated acidic, chloroform/methanol extracts of submitochondrial particles and partially purified mitochondrial ATPase complex, Barratt and Peterson (1977) were able to detect qualitative differences in mitochondrial protein patterns of both extracts from N and Tcms versions of the same line. The mitochondrial ATPase complex in yeast is known to have mitochondrially coded subunits (Schatz and Mason, 1974). Cytoplasm-specific protein differences in translation products from isolated mitochondria were reported by Forde et al. (1978). They found that isolated mitochondria from Tcms and Ccms maize each labeled one polypeptide not present in either N or Scms mitochondria. The additional Tcms and Ccms polypeptides had apparent molecular weights of 13,000 and 16,000 daltons respectively.

Levings and Pring (1976) isolated and characterized mitochondrial DNA from N and Tcms maize using restriction endonuclease fragment analysis on agarose gels. Their study revealed cytoplasm-specific DNA differences between N and Tcms mitochondria. Further studies of digestion fragment patterns from a number of inbreds and single crosses in N, Tcms, Scms, and Ccms cytoplasms revealed pattern differences between the cytoplasms (Levings and Pring, 1978). Pring et al. (1977) found two unique plasmid-like DNAs in addition to the high molecular weight mitochondrial DNA from Scms maize lines. These unique DNAs have molecular weights of 3.45 and 4.1×10^6 daltons, are not found in other cytoplasms and have not been isolated from Scms nuclear or chloroplast fractions.

Scms cytoplasm maize in some lines exhibits a lack of stability in its male sterility (Singh and Laughnan, 1972). This instability is seen as reversion to the male fertile condition. The existence of such cytoplasmic entities as the plasmid-like DNAs and a possible role for them in the instability of male sterility in Scms were predicted by Laughnan and Gabay (1975). Conversely, Duvick (1965) has noted that Tcms cytoplasm is extremely stable. Laughnan and his co-workers (Singh and Laughnan, 1972; Laughnan and Gabay, 1973) have found several hundred mutations where Scms male steriles have reverted to fertility. Two kinds of changes were observed; frequent cytoplasmic mutations from male sterility to male fertility and rare nuclear mutations giving rise to new fertility restorer genes (Laughnan and Gabay, 1975). From their studies of the genetics of newly arisen restorer genes, they proposed the existence of a male-fertility element which has the characteristics of an episome. They suggest that if the element is fixed in the cytoplasm the result is a cytoplasmic change from male sterility to male fertility; conversely, if it is fixed in the nucleus, it behaves as a new restorer gene. To date unequivocal evidence to identify the plasmid-like Scms DNAs as Laughnan's fertility element is not available.

The correlation between mitochondrial sensitivity to T toxin and susceptibility of Tcms maize lines to race T suggest a mitochondrial role in susceptibility of Tcms maize to race T, B. maydis. The interaction between the nuclear and cytoplasmic genomes in restoration (Duvick, 1965), modification of mitochondrial toxin response (Barratt and Flavell, 1975; Watrud et al., 1975b), and cytoplasm-specific differences in mitochondrial DNAs (Levings and Pring, 1976) would

justify study of mitochondrial gene products in the investigation of the mechanisms of cytoplasmic male sterility and the susceptibility of Tcms maize to race T of B. maydis.

Origin of Mitochondrial Constituents

Mitochondria are the product of two distinct genetic systems working cooperatively through separate protein synthesis systems. From the work on the biosynthesis of mitochondrial proteins in Ascomycete and animal mitochondria, it has been established that the majority of mitochondrial proteins are coded for by nuclear genes, translated on cytoplasmic ribosomes, and then imported into the mitochondria (Schatz and Mason, 1974). Subunits of F_1 ATPase, cytochrome b, and coenzyme QH_2 -cytochrome c reductase are products of mitochondrial protein synthesis in fungal and animal systems (Borst, 1977; Obbink et al., 1976). Evidence of mitochondrial origin for these proteins comes from studies using differential labeling in the presence of antibiotics (Schatz and Mason, 1974), cell-free synthesis of cytochrome-oxidase subunits from mitochondrial RNA (Moorman et al., 1978) and mapping of genes for mitochondrial proteins (Obbink et al., 1976). The mitochondrial subunits of cytochrome oxidase have been shown to be hydrophobic and are postulated to be responsible for the integration of the cytoplasmic subunits into the inner mitochondrial membrane.

Studies of plant mitochondrial protein synthesis have been hindered by technical difficulties in the preparation of coupled, uncontaminated plant mitochondria. Nevertheless, differences between plant and other eucaryotic mitochondria have been found, and they cast doubt on the direct applicability of conclusions drawn from other systems

to plant mitochondria. Animal mitochondrial DNA has a molecular weight of $9-12 \times 10^6$ daltons (Borst, 1977). Work with *Xenopus* (Dawid, 1970) and HeLa cells (Aloni and Attardi, 1971) suggest that at least 25-30 percent of the mitochondrial genome is involved in coding for mitochondrial rRNAs and tRNAs. This leaves enough genetic capacity to code for only about twenty small proteins. The molecular weight of higher plant mitochondrial DNA, although not unequivocally established, may be up to 165×10^6 daltons (Levings and Pring, 1978). Plant mitochondrial DNA could have the capacity to code for as many as 200 proteins assuming that the "additional" sequences are unique. Thus the possibility of greater autonomy exists for plant mitochondria as compared with that of other eukaryotes.

MATERIALS AND METHODS

Maize Genetic Stocks

Maize stocks used in this study are identified as normal (N) or male sterile cytoplasm (Tcms, Ccms, Scms, EKcms, Vgcms, CAcms). The Scms, EKcms, Vgcms, and CAcms cytoplasm are all members of the S group. Lines were furnished by J. R. Edwardson, University of Florida; J. R. Laughnan, University of Illinois; and Clyde Black and Sons, Ames, Iowa. Maize stocks not carrying dominant nuclear restorer alleles used were: W64A, B37 X NC236, WF9, FR37, Mol7, and N6 X W64A. Two maize stocks carrying the dominant restorer for Scms used were: Tr and Cl03. The line M825L/Oh07 in Vgcms cytoplasm frequently mutates to male fertility (Laughnan and Gabay, 1975) due to cytoplasmic mutation, indicated in this study by Vg_{mf} , or a nuclear mutation to new restorer genes, indicated here as the h, i, and m stocks of Laughnan (J. R. Laughnan and S. J. Gabay-Laughnan, personal communication).

Isolation and Purification of Mitochondria

Mitochondria were isolated from 6-day old Zea mays L. seedlings, germinated in vermiculite at 30° in the dark following the method of Pring (1974; Pring and Levings, 1978). Mesocotyl and coleoptile tissues (10-500 g) were harvested and homogenized in medium A, 0.5 M sucrose, 0.05 M Tris-HCl (pH 7.5), 5 mM Na₂EDTA, 0.1 % bovine serum albumin (Fraction V), and 5 mM 2-mercaptoethanol, in a chilled Waring blender for 5 sec at low speed. Batches of less than 50 g

were homogenized in a mortar and pestle or a Sorvall Omnimixer for 15 sec at a setting of 5. The homogenate was filtered successively through two layers of cheesecloth and two layers of Miracloth (Chicopee Mills, Inc., New York). All procedures were done at ice temperatures, unless otherwise noted. The homogenate was centrifuged for 15 min at 8,700 $\underline{g}$ (max) to pellet mitochondria. Mitochondria were resuspended in medium B, 0.3 M sucrose, 0.05 M Tris-HCl (pH 7.5), 5 mM Na_2EDTA , and 0.1 % bovine serum albumin, and purified on a discontinuous sodium diatrizoate gradient (Winthrop Labs) in medium B with a density of 1.19 g/cc overlaid with an equal volume of a 1.14 g/cc sodium diatrizoate solution. After centrifugation for 60 min at 72,000 $\underline{g}$ (avg), the mitochondria were collected from the 1.14-1.19 g/cc interface and diluted with three volumes of medium C, 0.3 M sucrose, 0.05M Tris-HCl (pH 7.5). Mitochondria were then pelleted for 10 min at 17,300 $\underline{g}$ (max), resuspended in medium C and repelleted.

Fractionation of Mitochondria

Mitochondrial membrane fractions were prepared by two methods. The first was a modification by Watrud et al. (1975b) of the Morreau and Lance (1972) procedure, and uses osmotic shock to rupture the outer membrane and ATP to contract the inner membrane cores to facilitate separation on sucrose gradients. Purified mitochondria were resuspended to 5 mg/ml mitochondrial protein, estimated by the method of Lowry et al. (1951), in 1 mM potassium phosphate, pH 7.2, and incubated for 15 min on ice. The suspension was then made 0.3 M in sucrose, 0.3 mM in MgSO_4 and 0.3 mM in ATP and allowed to stand 15 min on ice with occasional agitation. Treated mitochondria were then layered onto a discontinuous sucrose gradient of 4 ml 1.18 M and 4 ml 2M sucrose

in 1 mM KH_2PO_4 (pH 7.2) and centrifuged for 90 min at 90,000 $\underline{\text{g}}$ (avg). Outer membranes were collected from the 1.18 M sucrose step and inner membrane cores were caught on the 2 M shelf.

A second method used osmotic shock to rupture the outer membrane followed by sonication to separate outer and inner membranes. Membrane separation was assessed by observation of preparations negatively stained with 1 % potassium phosphotungstate in the electron microscope. Purified mitochondria were resuspended to 5 mg/ml protein in 1 mM potassium phosphate at pH 7.2, and incubated on ice for 30 min to allow osmotic swelling to rupture the outer membranes. The suspension was then sonicated for 30 sec at 20 % line voltage, using a Biosonic II sonicator with a microprobe. After sonication the inner membrane particles were pelleted at 17,300 $\underline{\text{g}}$ (max) for 10 min. The supernatant and resuspended pellet were then layered on separate 0.7 M, 1.18 M, 2 M sucrose step gradients and centrifuged for 90 min at 90,000 $\underline{\text{g}}$ (avg). Inner membranes were collected from the 1.18-2 M sucrose interface and outer membranes at the 0.7-1.18 M sucrose interface.

Membrane fractions from both methods were then diluted three-fold with 1 mM potassium phosphate (pH 7.2) and pelleted at 250,700 $\underline{\text{g}}$ (max) for 30 min. Pellets were resuspended in appropriate buffers for electrophoresis and stored at -20° .

Electrophoresis of Mitochondrial Proteins

Several electrophoresis systems were used in this study to examine mitochondrial proteins. Polyacrylamide gels using the Weber and Osborn (1969) buffer system, 0.1 M sodium phosphate (pH 7.0), were cast in 6 mm internal diameter X 125 mm long glass tubes coated with 0.5 % Kodak photoflo-200. A 7 cm, 10 % acrylamide, 0.27 % bisacrylamide,

0.1 % SDS, separating gel was polymerized with 1.5 ml of fresh 1.5 % ammonium persulfate and 45 μ l of TEMED per 30 ml. After polymerization the water overlay was removed and a 1.5 cm 5 % acrylamide, 0.135 % bisacrylamide, 0.1 % SDS stacking gel was cast using 0.05 M sodium phosphate (pH 7.0). The stacking gel was polymerized with 0.5 ml of fresh 1.5 % ammonium persulfate and 15 μ l of TEMED per 10 ml. Proteins were denatured by heating at 100^o for 5 min in 0.05 M sodium phosphate (pH 7.0), 3 % SDS, 3 % 2-mercapto-ethanol, and 10 % glycerol. Electrophoresis was conducted at constant current (8 ma/gel) until the bromphenol blue tracking dye reached the bottom of the tubes (anode). After electrophoresis, gels were fixed and stained in 0.1 % Coomassie brilliant blue R250, 50 % methanol, 10 % acetic acid for 2 hr. Gels were destained in 10 % methanol, 7.5 % acetic acid with frequent changes of solution.

A Tris-sulfate, discontinuous buffer system was used with an Ortec model 4200 slab gel electrophoresis tank and an Ortec model 4100 pulse power constant power supply. The molarity of this buffer refers to the molarity of the Tris with sufficient H₂SO₄ added to give a pH of 9.0 at 25^o. A two-step acrylamide gradient gel was cast using 0.22 M Tris-sulfate, pH 9.0, 0.1 % SDS. The first layer, 3.5 cm consisting of 12 % acrylamide, and 0.33 % bisacrylamide was overlayed with a 3.5 cm layer of 10 % acrylamide, 0.27 % bisacrylamide. Polymerization of each layer was initiated by the addition of 9.6 ml of 0.105 % ammonium persulfate and 12 μ l TEMED per 20 ml of solution. The separation gel was overlayed with H₂O and allowed to polymerize for 30 min. After the overlay was removed the unpolymerized acrylamide was washed off and the surface of the gel blotted with

filter paper. A 1 cm stacking gel, 5 % acrylamide, 0.135 % bisacrylamide, 0.1 % SDS, 0.0375 M Tris-sulfate, pH 9.0, was cast and polymerized with the addition of 4 ml of 0.21 % ammonium persulfate and 0.005 ml of TEMED per 8 ml. After 20 min polymerization time, the H₂O overlay was removed and an 8 % acrylamide, 0.22 % bisacrylamide well-forming gel was layered and a thirteen-tooth teflon comb inserted until it made contact with the stacking gel. Polymerization was initiated with 3.7 ml of 0.21 % ammonium persulfate and 0.005 ml of TEMED per 8 ml of solution. Buffer concentration was as in the stacking gel. Proteins were prepared for electrophoresis by heating for 5 min at 100° in 0.0375 M Tris-sulfate, pH 9.0, 3 % SDS, 3 % 2-mercaptoethanol, 10 % glycerol. Tris-borate electrophoresis buffer (0.065 M Tris, 0.1 % SDS) was prepared by adding 7.86 g Trizma base (Sigma), 0.09 g H₃BO₃, and 1 g SDS per liter of H₂O. Electrophoresis was carried out using pulsed power at a discharge capacitance of 1 µF, at 280 V. Pulses per second (pps) were increased in 75 pps increments every 5 min until a maximum of 300 pps was reached. Proteins were electrophoresed for 3 hr or until the bromphenol blue tracking dye reached the bottom of the gel.

The Tris-sulfate buffer system was also used to examine proteins on a 12-16.5 % continuous gradient acrylamide gel in a Studier (1973) type apparatus. Gel solutions were prepared as described. The 12 % acrylamide and 16.5 % acrylamide, 0.45 % bisacrylamide solutions were placed in a gradient maker and poured by gravity feed into a 0.075 x 14.5 x 16.3 cm cell. The volume of the gel was 28 ml, and stir bars were used in both chambers of the gradient maker to inhibit polymerization and to mix the solutions. After layering, the gel was

overlayed with H_2O , allowed to polymerize for 30 min, the overlay was poured off, the gel surface rinsed with H_2O , and then blotted with filter paper. A 4.5 ml, 5 % stacking gel and an 8 % well-forming gel were cast as described. Since solubilization of membrane proteins is incomplete at protein concentrations greater than 10 mg/ml, proteins were resuspended to less than 10 mg/ml, and electrophoresed for 18-19 hr at 80 V constant voltage with Tris-borate buffers. Gels were stained with Coomassie brilliant blue R250 and photographed with Polaroid type 55 P/N film using a Wratten 9 filter.

Labeling and Fluorography of Mitochondrial Proteins

Four-day old etiolated maize seedlings were cut approximately 1 cm above the scutellar node and placed in individual wells of disposable microtiter plates (Cooke Laboratory Products, Alexandria, VA). Each well contained 150 μ l of 0.01 M sodium phosphate, pH 7.0, with 10 μ C³⁵S as carrier free H_2SO_4 in H_2O (New England Nuclear, Boston, MA). After incubating at 30° for 12 hr, mitochondria were isolated as described except that samples weighing less than 10 g were homogenized in a mortar and pestle. Labeled protein was assayed using the Mans and Novelli (1961) filter disc procedure and counted with a Beckman LS-150 liquid scintillation counter, in 6 g/l 2,5-diphenyloxazole (PPO) in Beckman Redisolve I.

Mitochondrial proteins were electrophoresed in the Tris-sulfate continuous gradient gel system and the gels prepared for fluorography according to the method of Bonner and Lasky (1974). Gels were soaked twice in twenty volumes of dimethyl sulfoxide (DMSO) for 20 min. Gels

were then impregnated with PPO by soaking for 3 hr in four volumes of 22 % w/v PPO in DMSO. The DMSO was removed by soaking the gel in twenty volumes of deionized H₂O for 1 hr with one change. Gels were dried onto chromatography paper in a Bio-Rad slab dryer for 1.5 hr under a vacuum of 68.6 cm of mercury. The dried gels were then overlaid with Kodak X-Omat RP X-ray film at -70⁰ for varying exposure times.

Isolation of Mitochondrial DNA

Mitochondrial DNA was isolated following the procedures of Pring and Levings (1978) . Mitochondria from the second cycle of differential centrifugation were resuspended in medium C and incubated in the presence of 0.05 g/ml deoxyribonuclease I (Worthington DPFF), 10 mM MgCl₂ for 60 min at room temperature. After incubation the mitochondria were diluted with seven volumes of medium B and pelleted for 10 min at 17,300 g(max). Pellets were resuspended in medium C and pelleted again at 17,300 g(max) for 10 min. Pellets were resuspended in 0.05 M Tris-HCl, pH 8.0, 0.02 M Na₂EDTA, lysed by the addition of 0.5 % SDS, and incubated for 60 min at room temperature in the presence of 0.2 g/ml Proteinase K (EM Laboratories, Inc., Elmsford, NY).

The mitochondrial DNAs were purified by CsCl/ethidium bromide preparative ultracentrifugation, as described by Pring and Levings (1978). Solid CsCl was added to the mitochondrial preparations to a density of 1.611 g/cc in the presence of 0.2 g/ml ethidium bromide. Gradients (10 ml) with the mitochondrial preparation from a maximum of 300 g of shoots were overlayed with mineral oil and centrifuged at

20° for 40 hr at 44,000 rpm in a Beckman Ti 75 rotor. Mitochondrial DNA was visualized using long wave UV-light and the DNA was collected by piercing the tubes with an 18 ga needle. Ethidium bromide was removed by three extractions with isopropanol and CsCl removed by dialysis against 36 mM Tris-HCl, 30 mM NaH₂PO₄, 2.5 mM Na₂EDTA (TPE) at pH 7.8. The DNA preparation was then made 1 M in NaClO₄ and then deproteinized by three extractions with chloroform/isoamyl alcohol (95:5, vol/vol). The aqueous layer was then dialysed against the appropriate buffer for agarose electrophoresis.

Agarose Electrophoresis of Mitochondrial DNA

DNA was electrophoresed at room temperature in 1 % agarose (Sea Kem, Bausch and Lomb) gels prepared according to the method of Pring and Levings (1978). Slab gels (0.38 x 14.5 x 14.3 cm) were run in a Studier type electrophoresis apparatus at 1.9 V/cm for 16 hr with the TPE buffer system. After electrophoresis gels were soaked in buffer containing 0.4 µg/ml ethidium bromide for 60 to 90 min. Gels were photographed over UV light with Polaroid type 55 P/N film using a Wratten 9 filter.

Restriction Endonuclease Fragment Analysis

Mitochondrial DNA was analysed using HindIII restriction endonuclease (Miles Laboratories, Inc.) following the method of Pring and Levings (1978). Approximately 0.001 g of DNA was digested in sterile plastic tubes containing enzyme in a 0.05 ml volume of 50 mM NaCl, 6 mM MgCl₂, 6 mM Tris-HCl, pH 7.5, and 0.005 g bovine serum albumin.

Reactions were terminated by heating at 60⁰ for 10 min in the presence of 1 % SDS. Bromphenol blue and glycerol were added to the digests and the samples electrophoresed on agarose as described above.

RESULTS

Purity of Mitochondrial Membrane Fractions

The relative purity of membrane fractions was assayed by the differential inhibition of NADH-cytochrome C reductase by 0.3 μ M antimycin A (Watrud et al., 1975b). The enzyme activity in the outer membrane is antimycin A insensitive, while the inner membrane activity is inhibited by antimycin A. Therefore, the percent inhibition of enzyme activity in treated membrane fractions over controls can be used to assess relative purity of the partially resolved fractions. A comparison of membrane fractions prepared by the osmotic shock method of Watrud et al. (1975b) and the sonication procedure is shown in Table 1. These data suggest that the sonication procedure gave better separation of both membrane fractions. However, neither procedure completely resolved the two membrane components nor gave highly reproducible fractions (note wide range among replications).

Electrophoresis of Mitochondrial Membrane Fractions

Mitochondrial membrane fractions from W64A in N, Tcms, and CAcms cytoplasms were examined for cytoplasm-specific protein differences on 10 % polyacrylamide cylindrical gels using the Weber and Osborn (1969) buffer system. Although protein pattern differences were seen

Table I. Inhibition of cytochrome c reductase by antimycin A in outer and inner maize mitochondrial membrane fractions.

Membrane Fraction	Method of Preparation	
	Osmotic %	Sonic %
<u>Outer</u>	17.6 (3.4-30) ^{1/}	11.3 (0.04-27)
<u>Inner</u>	60.3 (37-79)	65.2 (47-81)

^{1/} Numbers in parenthesis indicate the range from nine replications.

in individual preparations, there was a lack of reproducibility among experiments. Therefore, no consistent cytoplasm-specific protein differences were detected in inner or outer membrane fractions prepared by the sonication procedure (Figure 1). This conclusion was based on a minimum of five experiments. However, figures are only representative of these experiments, and include pattern variation unique to the individual experiment.

Greater resolution of proteins from these fractions was obtained using two-step acrylamide slab gels with a Tris-sulfate discontinuous buffer system. Membrane fractions from W64A in N, Tcms, and CACms cytoplasms were examined in this system. No reproducible cytoplasm-specific protein differences were detected in outer membrane (Figure 2) or inner membrane (Figure 3) fractions by Coomassie brilliant blue staining.

Electrophoresis of Total Mitochondrial Protein

Sodium diatrizoate gradient-purified mitochondria were solubilized as described and 150-200 µg protein samples were electrophoresed on 12 to 16.5 % continuous polyacrylamide gradient gels with the Tris-sulfate discontinuous buffer system.

The resolution attained with this system resulted in the detection of line specific protein differences as shown in figure 4. This figure shows electrophoresis of total mitochondrial proteins from four lines in Scms group cytoplasms. The sample of Mol7 exhibits one band at 53 mm not present in lines Wf9, FR37, and N6 X W64A; and is missing one band at 43 mm which is present in the latter lines.

Figure 1. SDS-polyacrylamide gel electrophoresis of Inner (A) and Outer (B) mitochondrial membrane fractions from W64A in a) Normal, b) Tcms, and c) CACms cytoplasms run in 10 % gels with 0.1 M sodium phosphate, pH 7.0, 0.1 % sodium dodecyl sulfate. Samples were denatured by heating at 100° for 5 min in 0.05 M NaH₂PO₄, pH 7.0, 3 % SDS, 3 % 2-mercaptoethanol.

Figure 2. SDS-polyacrylamide gel electrophoresis of maize outer mitochondrial membrane proteins run in 10 % - 12 % discontinuous acrylamide gradient gels with 0.22 M Tris-sulfate, pH 9.0, 0.1 % SDS. Fractions were prepared by the osmotic (A) and sonication (B) procedures from W64A in a) normal, b) Tcms, and c) CAcms cytoplasms. Samples were dissociated by heating at 100^o for 5 min in 0.11 M Tris-sulfate, pH 9.0, 3 % SDS, 3 % 2-mercaptoethanol.

Figure 3. SDS-polyacrylamide gel electrophoresis of maize inner mitochondrial membrane proteins run in 10 % - 12 % discontinuous acrylamide gradient gels with 0.22 M Tris-sulfate, pH 9.0, 0.1 % SDS. Fractions were prepared by the osmotic (A) and sonication (B) procedures from W64A in a) normal, b) Tcms, and c) CAcms cytoplasms. Samples were dissociated by heating at 100° for 5 min in 0.11 M Tris-sulfate, pH 9.0, 3 % SDS, 3 % 2-mercaptoethanol.

A

B

Figure 4. SDS-polyacrylamide gel electrophoresis of total maize mitochondrial protein from lines a) WF9, b) Mol7, c) FR37, and d) N6 X W64A in Scms cytoplasms. Samples were dissociated by heating at 100^o for 5 min in 0.11 M Tris-sulfate, pH 9.0, 3 % SDS, 3 % 2-mercaptoethanol and electrophoresed in 12 to 16.5 % continuous acrylamide gradient gels with 0.22 M Tris-sulfate, pH 9.0, 0.1 % SDS.

a b c d

A comparison of maize mitochondrial proteins from four cytoplasmic sources is shown in Figure 5A. No consistent cytoplasm-specific differences were detected in the mitochondrial proteins from N, Tcms, or Ccms cytoplasms. The Scms cytoplasm mitochondria contained a high molecular weight protein band at 12 mm which is distinct from the other cytoplasms tested. This protein, designated S protein, is present in all Scms cytoplasm maize lines tested. Figure 5B shows the presence of this protein in four maize lines and two versions of the Scms cytoplasm. The molecular weight (mw) of this protein was estimated by comparing the distance migrated of the S protein with the distance migrated by standards of known molecular weights on a 5 % acrylamide, 0.136 % bisacrylamide, Tris-sulfate slab gel. Myosin (mw 200,000 daltons), beta galactosidase (mw 116,394 daltons, Fowler and Zabin, 1978) and phosphorylase b (mw 94,000 daltons) were used as molecular weight standards. Linear regression analysis of the relationship of the log of molecular weight to the distance migrated of the standards indicated a mean molecular weight of $133,890 \pm 635$ daltons for the S protein (Table II).

Mitochondria were labeled with ^{35}S in detached seedlings and the proteins electrophoresed on gradient gels. Proteins were stained with Coomassie brilliant blue and labeled proteins were visualized using fluorography. Proteins present in concentrations too low to be seen using staining can be detected using fluorography; however, only those proteins synthesized during the labeling period will be seen. Figure 6A shows a comparison from the same set of stained and fluorographic protein patterns from N, Tcms and Scms maize mitochondria. Although the stained and fluorographic patterns were

Figure 5. SDS-polyacrylamide gel electrophoresis of total maize mitochondrial protein dissociated by heating at 100° for 5 min in 0.11 M Tris-sulfate, pH 9.0, 3 % SDS, 3 % 2-mercaptoethanol and electrophoresed in 12 to 16.5 % continuous acrylamide gradient gels with 0.22 M Tris-sulfate, pH 9.0, 0.1 % SDS.

- A. Patterns from a) normal, b) Tcms, c) Ccms, and d) Scms mitochondria.
- B. Patterns from Scms maize in a) WF9, b) Mol7 (EKcms), c) FR37, and d) N6 X W64A lines.

Table II. Determination of the S protein molecular weight by SDS-PAGE in 5 % acrylamide with 0.22 M Tris-sulfate, pH 9.0, 0.1 % SDS. Proteins were dissociated by heating at 100° for 5 min in 0.11 M Tris-sulfate pH 9.0, 3 % SDS, 3 % 2-mercaptoethanol.

Experiment	1	2	3	4	5	6
<u>Distance migrated (cm)</u>						
Myosin (200,000 MW)	5.0	5.3	5.23	5.1	5.28	5.2
Beta Galactosidase (116,394 MW)	9.4	9.6	9.52	9.35	9.5	9.4
Phosphorylase b (94,000 MW)	11.2	11.4	11.38	11.5	11.6	11.5
S protein	8.3	8.5	8.35	8.4	8.5	8.4
<u>Linear regression analysis</u>						
Slope	-0.53	-0.53	-0.53	-0.53	-0.53	-0.53
Correlation coefficient	-0.999 ^{1/}	-0.999	-0.998	-0.999	-0.999	-0.999
<u>Molecular weight of S protein</u>						
Mean molecular weight (daltons)	133,890					
Standard deviation of the mean	635					

^{1/} All correlations were significant at the 5 % level.

different, no consistent cytoplasm-specific protein differences were detected among labeled proteins. The S protein seen on stained gels was not detected under these labeling conditions. Therefore, Scms mitochondria were labeled using a 0-time labeling procedure. Scms maize seeds were imbibed in the presence of ^{35}S , and after four-day continuous labeling mitochondria were isolated as before and the protein patterns examined using Coomassie staining and fluorography (Figure 6B). Although not readily apparent in this photograph, a labeled band was detected in the same position (13 mm) as the stained S protein.

An attempt was made to differentially inhibit cytoplasmic protein synthesis by enhancing the labeling of mitochondrial gene products. Emetine, an inhibitor of cytoplasmic protein synthesis (Peska, 1971), at 300 $\mu\text{g/ml}$ (saturating level) inhibited the incorporation of ^{35}S into maize mitochondrial protein 85 % (Figure 7). A comparison of maize mitochondrial proteins labeled with and without emetine is shown in Figure 8. Consistent cytoplasm-specific protein differences were not detected under these conditions of incomplete inhibition. Figure 8 shows an example of the inconsistent results obtained using emetine. All samples were labeled simultaneously; however, in sample b the antibiotic appears to have stimulated rather than inhibited incorporation. This effect was not limited to any one cytoplasm and was not seen consistently in all experiments.

Scms Cytoplasm Instability and the S Protein

The effects of nuclear restoration and of nuclear and cytoplasmic

Figure 6. SDS-polyacrylamide gradient gel electrophoresis of total maize mitochondrial protein.

- A. Comparison of the stained and fluorographic patterns of proteins from maize mitochondria labeled in detached shoots with ^{35}S . a) Normal cytoplasm mitochondrial proteins stained with Coomassie brilliant blue, b) fluorograph of the same gel; c) stained Tcms mitochondrial proteins, d) fluorograph of the same gel; e) stained Scms mitochondrial proteins, f) fluorograph of the same gel.
- B. Comparison of the stained and fluorographic patterns of proteins from Scms mitochondrial proteins labeled by imbibition of maize seed in the presence of ^{35}S (0-time labeling). a) Proteins stained with Coomassie brilliant blue, b) fluorograph of the same gel. S protein is indicated by arrow.

Figure 7. Inhibition by emetine of incorporation of ^{35}S into mitochondrial protein. Four day-old etiolated seedlings were cut approximately 1 cm above the scutellar node, pretreated in the presence of emetine for 30 min, and placed in individual wells of disposable microtiter plates and allowed to take up 150 μl of 0.01 M sodium phosphate, pH 7.0 with 10 $\mu\text{C}^{35}\text{S}$ as carrier-free H_2SO_4 with various concentrations of emetine. After incubation at 30° for 12 hr mitochondria were isolated and incorporation assayed using the Mans and Novelli (1961) filter disc procedure. Inhibition was calculated by comparing incorporation by emetine treated seedlings with that of untreated controls. The average specific activity of controls was 2000 cpm/ μg mitochondrial protein.

Figure 8. Fluorograph of ^{35}S labeled maize mitochondrial proteins electrophoresed on SDS-polyacrylamide gradient gels. Mitochondrial proteins were labeled in detached four day-old etiolated shoots with $10\ \mu\text{C}/\text{shoot}$ ^{35}S as carrier-free H_2SO_4 . Fluorograph was prepared by exposing Kodak X-Omat RP X-ray for 24 hrs at -70° to dried gels impregnated with PPO. Normal (a), Tcms (c), and Scms (e) proteins labeled without antibiotic treatment. Normal (b), Tcms (d), and Scms (f) labeled in the presence of emetine.

mutation to male fertility on the presence of the S protein in Vgcms cytoplasm maize were examined by gradient gel electrophoresis.

Lines Tr and Cl03 carry the dominant nuclear restorer genes (Rf3 Rf3) for Scms cytoplasm. A comparison of mitochondrial proteins from these lines in N, Scms, and Vgcms cytoplasms showed that nuclear restoration had no detectable effect on the S protein (Figure 9A). This figure is overdeveloped to emphasize the S protein.

Line M825L/Oh07 does not carry the nuclear gene for fertility restoration. Laughnan and his co-workers have identified several nuclear mutations to male fertility in the Vgcms version of this line. These mutations have been characterized as the formation of new restorer loci. Three of these were examined for the presence of the S protein. As with lines Tr and Cl03 the new restorer genes h, i, and m had no effect on the presence of the S protein (Figure 9B). Although not readily apparent in Figure 9B, slot a, M825L/Oh07 with the new restorer h did yield the S protein.

Cytoplasmic mutation to male fertility in the Vgcms version of line M825L/Oh07 was also examined for the presence of the S protein. Figure 9B (Slots d, e, f) shows a comparison of this line in N and Vgcms cytoplasms and in a mutant cytoplasm Vg_{mf} . No S protein was detectable in the cytoplasmic mutant.

Restriction Endonuclease Analysis of Mitochondrial DNA

The identity of Vgcms cytoplasm as a member of the Scms group was substantiated by an examination of the mitochondrial DNA from the h, i, and m versions of M825L/Oh07 in Vgcms. Two criteria were

Figure 9. SDS-PAGE of total mitochondrial protein solubilized by heating at 100° for 5 minutes in the presence of 3 % SDS, 3 % 2-mercaptoethanol. Photograph is overdeveloped to emphasize the S protein.

- A. Mitochondrial proteins from restorer lines Tr in
a) normal, and b) Scms cytoplasms and C103 in c) normal,
b) Scms, and Vgcms cytoplasms.
- B. Proteins from M825L/Oh07 with new restorer genes in
Vg cytoplasm a) h, b) i, c) m, and in d) normal,
e) Vgcms, and f) Vg_{mf} cytoplasmic versions.

used: the presence of the two unique DNAs described by Pring et al. (1977) and a comparison of restriction endonuclease fragment patterns of mitochondrial DNA with that of a known Scms cytoplasm. Figure 10 is a comparison of mitochondrial DNA and HindIII restriction endonuclease digests from W64A in CAcms cytoplasm and h, i, and m in Vgcms cytoplasm. All four mitochondrial DNAs contain the two plasmid-like DNAs characteristic of the Scms cytoplasm. DNA digest patterns from h and i are identical to that of W64A. The digest of mitochondrial DNA from m was incomplete, which resulted in a smeared pattern. However, the similarities in this pattern and the patterns from h and i in addition to the presence of the two plasmid-like DNAs, provide sufficient data to verify Vgcms as having Scms type DNAs.

Figure 10. Agarose electrophoresis of undigested (b, d, f, h) and HindIII-digested (a, c, e, d) mitochondrial DNAs from W64A CACms (a, b) and from M825L/Oh07 m (c, d), M825L/Oh07 i (e, f), and M825L/Oh07 h (g, h) lines in Vgcms.

DISCUSSION

The objective of this study was to search for aberrant mitochondrial gene products from male sterile cytoplasms of maize. Information on cytoplasm-specific toxin effects on Tcms mitochondria, and specific mitochondrial DNA differences among the cytoplasms of maize indicated that such a search may be useful in the elucidation of the role of nuclear-mitochondrial interaction in Tcms maize susceptibility and in cytoplasmic male sterility.

Three types of protein differences could be expected: line specific differences, cytoplasm-specific differences associated with disease susceptibility, and cytoplasm-specific differences associated with cytoplasmic male sterility. Line specific differences due to line variation in nuclear genes coding for mitochondrial proteins may be seen as protein differences from individual lines in the same cytoplasm. Susceptibility associated differences may be seen as variation in protein patterns which distinguish susceptible Tcms mitochondria from resistant N, Scms, and Ccms mitochondria. Differences associated with cytoplasmic male sterility may be represented by patterns which differentiate the male sterile cytoplasms from the normal fertile cytoplasms. Protein differences which could be associated with line variation and cytoplasmic male sterility were detected in Scms maize. However, no mitochondrial protein differences were detected which could be correlated with disease susceptibility in Tcms maize.

No mitochondrial protein difference between N, Tcms, or Scms maize were detected in mitochondrial fractions prepared using either the method of Watrud et al. (1975b) or the sonication procedure. The Watrud procedure was followed exactly; however, mitochondria used in this study were purified on gradients and have been shown to be free of microsomal contamination when assayed for the presence of cytoplasmic ribosomal RNA (rRNA) (Pring, 1974). Membrane fractions prepared by the sonication procedure exhibited less cross-contamination, using the cytochrome c reductase assay, than those prepared by the procedure of Watrud et al. (1975b). Protein differences in mitochondrial fractions reported by Watrud et al. (1975b) have not been verified by this work or that of Barratt and Peterson (1977), who looked at submitochondrial particles prepared by sonication. In all three cases, mitochondrial fractions were examined on 10 % polyacrylamide gels using the Weber and Osborn (1969) SDS system. In addition, cytoplasm-specific protein differences were not apparent in Tris-sulfate step gradient gels which gave improved resolution of protein species.

Mitochondrial protein differences were resolved with continuous polyacrylamide-gradient gels. Line specific differences were detected in four nuclear lines in Scms cytoplasm. Bolen (1975), using polyacrylamide-gradient gels and isoelectric focusing, also found line specific protein differences; however, his results are not directly comparable to those in this study due to differences in the methods used. These line specific proteins probably represent variants of nuclear-coded mitochondrial proteins. Contrary to Bolen's work, one

cytoplasm-specific mitochondrial protein was detected in mitochondria from Scms maize. This high molecular weight protein, 133,900 daltons, was present in all Scms lines tested with the exception of the line with a cytoplasmic mutation to fertility. Absence of detectable amounts of this protein in the cytoplasmic mutant and other maize cytoplasms suggests that it is associated with male sterility in Scms maize. Failure of Bolen to detect this protein is probably due to differences in mitochondrial preparation or sample solubilization procedures. The protein was not detected when sample concentrations exceeded 10 mg/ml mitochondrial protein, estimated by the method of Lowry et al. (1951). Protein aggregation at higher sample concentrations with failure of the aggregated protein to enter the gel may result in failure to detect the S protein.

In vivo labeling with radioisotopes has been used in animal and fungal systems to identify mitochondrial gene products. The inconsistency and failure of labeling in detached shoots to reveal cytoplasm-specific proteins is probably indicative of technical difficulties rather than absence of such proteins. Limited inhibition of labeling by emetine (85 %) probably results from incomplete translocation of the antibiotic to all cells. The most successful examples of this type of experiment have come from studies in yeast. Therefore, it seems likely that use of cell suspension culture or protoplast systems would be more efficient for this type of study in plants.

The results, in this study, of labeling experiments in the absence of protein synthesis inhibitors revealed that labeling of the S protein occurred only in shoots germinated in the presence of

isotopes. Forde et al. (1978) examined protein synthesis in isolated maize mitochondria. Their results showed that approximately twenty polypeptides were labeled and detectable by SDS-PAGE. Although mitochondria from Tcms and Ccms maize were found to synthesize a single additional polypeptide; no differences in mitochondrial translation products were detected between N and Scms mitochondria. The results from Scms mitochondria in these studies may indicate that the S protein is made early in the germination process and has a relatively slow rate of synthesis or degradation.

An important part of this study is the investigation of the S protein and its relationship to the instability of male sterility in Scms maize. Genetic studies by Laughnan and his co-workers (Laughnan and Gabay, 1975) indicated that this instability was due to an episome-like male fertility element. Fixed in the nucleus, it behaves as a new restorer gene; if it is fixed in the cytoplasm, the result is seen as a cytoplasmic mutation to male fertility. The plasmid-like DNAs described by Pring et al. (1977) are possible candidates for the role of Laughnan's fertility elements. Pring et al. (personal communication) have found that the plasmid-like DNAs are not present in Vgcms lines which have a cytoplasmic mutation to male fertility.

Agarose gel electrophoresis and restriction endonuclease fragment analysis of mitochondrial DNA verified the presence of the plasmid-like DNAs in three nuclear mutants and confirmed that Vgcms has Scms type DNA. Examination of mutant stock supplied by Laughnan showed that the presence of the S protein is coincident with the plasmid-like DNAs (Table III). These data suggest that the S

protein may be a gene product of these DNAs. This can be proved by showing that a gene product from one or both of these DNAs is identical to the S protein found in this study. Identification of the plasmid-like DNAs as Laughnan and Gabay's (1975) fertility element depends on evidence which shows the fate and function of these DNAs in the two types of mutation to fertility.

Data from this study indicate that cytoplasmically determined male sterility and disease susceptibility do not produce gross changes in the electrophoretic phenotype of maize mitochondria when examined on one dimensional gels. The gametophytic type of fertility restoration, mitochondrial DNA content, and the presence of a cytoplasm-specific protein make Scms distinct among male sterile cytoplasms. These differences suggest that the mechanism of male sterility may be different among male sterile cytoplasms. In Scms maize the association of the plasmid-like DNAs and the S protein with male sterility provide supportive evidence that the mitochondrial genome is involved in cytoplasmic male sterility.

Table III. Relationship of S protein to restoration, male sterility and the presence of plasmid-like DNAs in Scms maize.

		Cytoplasm	Male Sterile	Plasmid-like DNAs	S-Protein
<u>Restored lines</u>					
Tr	N		-	-	-
Tr	Scms		-	+	+
Tr	Vgcms		-	+	+
C 103	N		-	-	-
C 103	Scms		-	+	+
C 103	Vgcms		-	+	+
<u>New restorer genes</u>					
M825L/Oh07 <u>h</u>	Vgcms		-	+	+
M825L/Oh07 <u>i</u>	Vgcms		-	+	+
M825L/Oh07 <u>m</u>	Vgcms		-	+	+
<u>Non-restored lines</u>					
M825L/Oh07	N		-	-	-
M825L/Oh07	Vgcms		+	+	+
M825L/Oh07	Vg _{mf}		-	-	-

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David Walter Thornbury was born in Charleston, West Virginia, on May 4, 1948. He spent his childhood in Belle, West Virginia, where he attended Belle Elementary School. He graduated from DuPont High School in May of 1966. He received his undergraduate training at West Virginia University, Morgantown, West Virginia, where he was awarded a Bachelor of Arts in zoology in 1970. He continued his education at West Virginia University and received a Master of Science in biology in 1973. In September, 1973, he entered the University of Florida to commence work towards a Doctor of Philosophy in plant pathology.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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