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Altered mitochondrial gene expression in the nonchromosomal stripe 2 mutant of maize

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The genetic and molecular analyses of higher plant mitochondria can be facilitated by studying maternally-inherited mutations, such as the nonchromosomal stripe (NCS) mutants of maize, that have deleterious effects on plant growth. We have previously demonstrated a correlation between specific alterations in mitochondrial DNA and the expression of NCS phenotypes. In the present studies, the effects of the NCS2 mutation on mitochondrial gene expression are evaluated. Proteins synthesized by mitochondria isolated from NCS2 mutants and from related plants with normal growth have been compared. NCS2 mitochondria synthesize much reduced amounts of a single polypeptide. Probes corresponding to the mitochondrial DNA region altered in NCS2 hybridize to an aberrant set of transcripts in NCS2 mitochondria. Transcripts homologous to several previously characterized plant mitochondrial genes are similar in NCS2 and related non-mutant mitochondria.

Key words: gene expression/mitochondrial mutants/*Zea mays*

Introduction

The maternally-inherited nonchromosomal stripe (NCS) mutants in maize are characterized by variable leaf striping, poor growth and decreased yields (Shumway and Bauman, 1967; Coe, 1983). It has been shown that two independently isolated and phenotypically distinguishable mutants, NCS2 and NCS3, have distinct alterations in their mitochondrial DNA (mtDNA) restriction enzyme profiles, relative to their common progenitor genome (Newton and Coe, 1986). NCS2 plants have a novel 21-kb *Xho*I mtDNA band and much reduced amounts of a progenitor 8-kb fragment. NCS3 plants have an extra 20-kb *Xho*I mtDNA band and a reduction in a 16-kb fragment. The goal of the work presented here was to determine whether the observed DNA alterations in such mutants are functionally significant. In this study, specific effects of the NCS2 mutation on mitochondrial gene expression are demonstrated.

Affected NCS2 plants have pale-green clonal sectors on the leaves and sectors of aborted kernels on the ears. Non-mutant individuals have been found among the progeny from affected plants. These normal derivatives from NCS2 lineages usually produce only normal progeny. They also lose the novel mtDNA restriction fragments characteristic of mutant plants, resembling instead the progenitor (cms-T) in mitochondrial genotype (Newton and Coe, 1986). This suggests that affected plants carry a mixture of defective and normal organelles and that the mutant and normal sectors result from sorting out of the two types of organelles during development.

It was further observed that more heavily striped NCS2 plants gave a higher incidence of ears with mostly aborted kernels (Newton and Coe, 1986). Because of this correlation, we chose ear shoots that should be enriched for mutant mitochondria, as source materials for our experiments. For comparison, ear shoots from normal derivatives were used. These plants have lost the genetic determinant responsible for the NCS2 phenotype.

We report that, in NCS2 mutants, the synthesis of one mitochondrial protein is reduced and that one set of transcripts, homologous to the NCS2-altered region of mtDNA, is also aberrant.

Results

Reduced synthesis of a polypeptide in NCS2 mitochondria

Mitochondria were purified from unpollinated ear shoots of NCS2 mutant- and normal-derivative plants. Proteins synthesized by these isolated mitochondria were labeled *in organello* with [^{35}S] methionine and analysed by polyacrylamide gel electrophoresis and fluorography (Figure 1). Despite slight sample-to-sample variation, the major, consistent difference in the polypeptide patterns was that a 24 000 M_r polypeptide synthesized by the non-mutant mitochondria (lanes 1, 2, 7 and 8) was either greatly reduced or not detectable in the mitochondrial translation products from the NCS2 mutants (lanes 3–6). Mitochondria isolated from the less phenotypically affected NCS2 sources synthesized a slight amount of the 24 000 M_r polypeptide (lanes 3 and 4). Its synthesis was reduced relative to the other polypeptides. Mitochondria isolated from the most phenotypically affected NCS2 mutants did not synthesize the 24 000 M_r protein to detectable levels (lanes 5 and 6).

Because the NCS2 mutation arose in the male-sterile T cytoplasm, the translation products include a polypeptide (T) that is only synthesized by this type of mitochondrion (Forde *et al.*, 1978). Two of the normal-derivative plants represented in Figure 1 were male-fertile due to the segregation of nuclear restorer of fertility (Rf) genes, which had been introduced in previous crosses. In these cases (lanes 1 and 8), synthesis of the cms-T-specific polypeptide is reduced (Forde and Leaver, 1980), but the synthesis of the M_r 24 000 polypeptide is unaffected.

The addition of inhibitors that block the functioning of plastid and bacterial ribosomes (erythromycin, lanes 1–4) or cytosolic ribosomes (cycloheximide, lanes 5–8) did not alter the pattern of protein synthesis. Therefore, it can be concluded that contamination was minimal and the translation products obtained were synthesized on mitochondrial ribosomes. Thirteen different NCS2 sources and 12 different normal (NCS2-derived) mitochondrial preparations have been examined. The results were all consistent with those shown in Figure 1.

Nucleic acid hybridization experiments using previously cloned plant mitochondrial genes

The *in organello* labeling studies suggested that the NCS2 mutation affects a protein-coding mitochondrial gene. Hybridization experiments using previously cloned and characterized mitochondrial genes as probes on Northern blots of NCS and normal-derivative mtRNA were conducted in order to determine if cor-

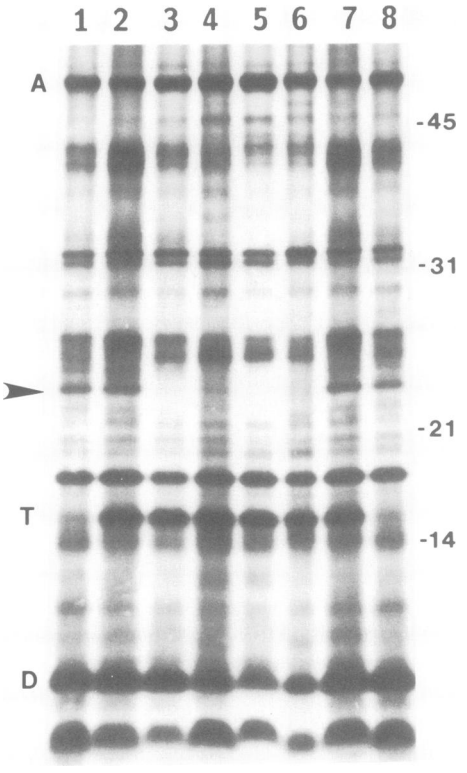

Fig. 1. Translation products of mitochondria isolated from NCS2 and normal-derivative plants. Fluorographs of [^{35}S]methionine-labeled polypeptides separated on a 12–18% polyacrylamide gradient gel. Lanes **1**, **2**, **7** and **8**: from NCS2-derived normal plants; Lanes **3–6**: from NCS2 mutant plants. In lanes **1–4**, erythromycin (40 μg/ml) was included in the labeling reaction. In lanes **5–8**, cycloheximide (40 μg/ml) was included in the reaction. The plants represented in lanes **1** and **8** were fertile due to the action of nuclear *Rf* genes. Positions of the T-polypeptide (**T**, reduced in fertile plants), subunits alpha (**A**) and 9 (**D**) of ATPase, and the 24 000 M_r polypeptide (arrow) are indicated on the left. Positions of protein standards are listed to the right in $M_r \times 10^{-3}$.

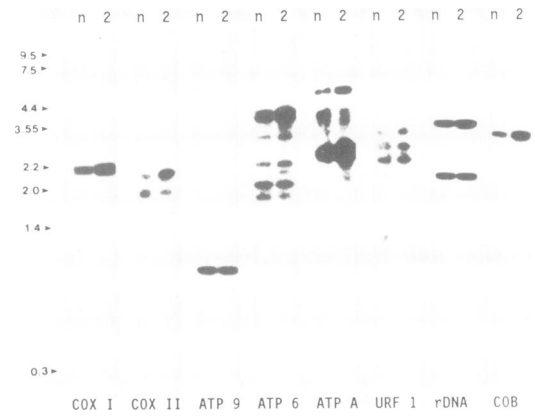

Fig. 2. Northern blot analysis of transcripts homologous to mitochondrial gene probes. NCS2 (**2**) and normal-derivative (**n**) mtRNAs were fractionated on agarose/formaldehyde gels, blotted onto nitrocellulose and hybridized with the ^{32}P-labeled DNA probes listed along the bottom of the autoradiographs. The positions of RNA size standards in nucleotides $\times 10^{-3}$ are listed to the left.

responding transcripts were present and correctly processed in the NCS2 mutant. The mitochondrial genes that were tested in this manner were those coding for cytochrome *c* oxidase subunits I (COXI; Isaac *et al.*, 1985) and II (COXII; Fox and Leaver, 1981), apocytochrome *b* (COB; Dawson *et al.*, 1984), ATPase subunits 6 (ATP6; Dewey *et al.*, 1985a), 9 (ATP9; Dewey *et al.*, 1985b), and alpha (ATPA; Braun and Levings, 1985), URF-1 (Stern *et al.*, 1986) and mt rRNAs (Figure 2).

Messages homologous to these DNA probes were present and the major hybridizing transcripts were similar in the NCS2 mutants and normal derivatives. Parallel Southern blot analyses also demonstrated that none of the above probes hybridized to the novel restriction fragments present in the NCS2 mutant mtDNA (not shown). They identified the same restriction fragments in NCS2 and normal-derivative samples. In addition, a COXIII probe (Hiesel *et al.*, 1987) does not hybridize to NCS2-novel mtDNA bands (not shown).

Cloning of a mtDNA restriction fragment altered in NCS2 mutants

Restriction enzyme analysis of mtDNA (Figure 3A) from NCS2 mutants have shown the reduction of an 8kb *Xho*I fragment correlated with the presence of a novel 21-kb band (Newton and Coe, 1986). A novel 11-kb *Hin*dIII fragment is also unique to NCS2 mtDNA and there is a corresponding reduction in a doublet band at 9.5 kb. *Pvu*II digests demonstrate the presence of an NCS2-unique 11.9-kb band and a reduction in a 7.8-kb band.

The NCS2-unique 11-kb *Hin*dIII fragment was cloned, after extracting it from an agarose gel, by ligation into pUC18 and transformation into HB101 cells, using standard procedures (Maniatis *et al.*, 1982). The 11-kb *Hin*dIII mtDNA fragment from the resulting clone, pMH1, was labeled with ^{32}P and used to probe Southern blots of digested mtDNA isolated from NCS2, NCS3 and normal derivative sources (Figure 3B).

The 11-kb *Hin*dIII fragment hybridized to the 21-kb *Xho*I band in digests of NCS2 mtDNA and to the 8-kb *Xho*I fragment in a matched normal derivative. This suggests that the novel 21-kb *Xho*I fragment includes sequences present in the 8-kb progenitor band. A small amount of hybridization to the 8-kb region is detected in the *Xho*I digests of NCS2 mtDNA, indicating that some non-mutant mitochondrial genomes are present in affected plants. The probe does not hybridize to the 20-kb *Xho*I band novel to NCS3, demonstrating that these two NCS mutants are distinguishable molecularly as well as phenotypically. This probe does, however, hybridize to another *Xho*I band (15.2 kb) in all the digests. This indicates that some of the DNA present in the 11-kb *Hin*dIII probe is normally present at another location in the mitochondrial genome.

The 11-kb *Hin*dIII fragment was digested with the restriction endonuclease *Kpn*I. The five *Kpn*I fragments were mapped by standard double digestion techniques. Two fragments at opposite ends of 11-kb *Hin*dIII region (Figure 4A), K-1 (1.3 kb) and K-5 (1.8 kb), as well as the central fragment K-3 (3.5 kb) were labeled and hybridized to digests of mtDNA isolated from NCS2, NCS3 and normal-derivative plants. Highly purified chloroplast DNA (c) was also included on the gels. None of the probes showed homology to chloroplast DNA.

K-1 hybridized to the NCS2-novel fragments (*Xho*I 21 kb and *Pvu*II 11.9 kb) and the corresponding progenitor bands (*Xho*I 8 kb and *Pvu*II 7.8 kb) as seen in Figure 4B. K-5 hybridized to the NCS2-unique fragments (21-kb *Xho*I and 11.9-kb *Pvu*II), as well as to the region common to the NCS2, NCS3 and normal-derivative mitochondrial genomes (15.2-kb *Xho*I and 6.5-kb *Pvu*II), originally identified with the 11-kb *Hin*dIII probe. The K-5 probe does not share homology with the progenitor fragments. The hybridization pattern for the central K-3 fragment was similar to that of the entire 11-kb *Hin*dIII probe. This indicates that one end of the NCS2 mtDNA alteration is located in the K-3 region of the 11-kb *Hin*dIII fragment.

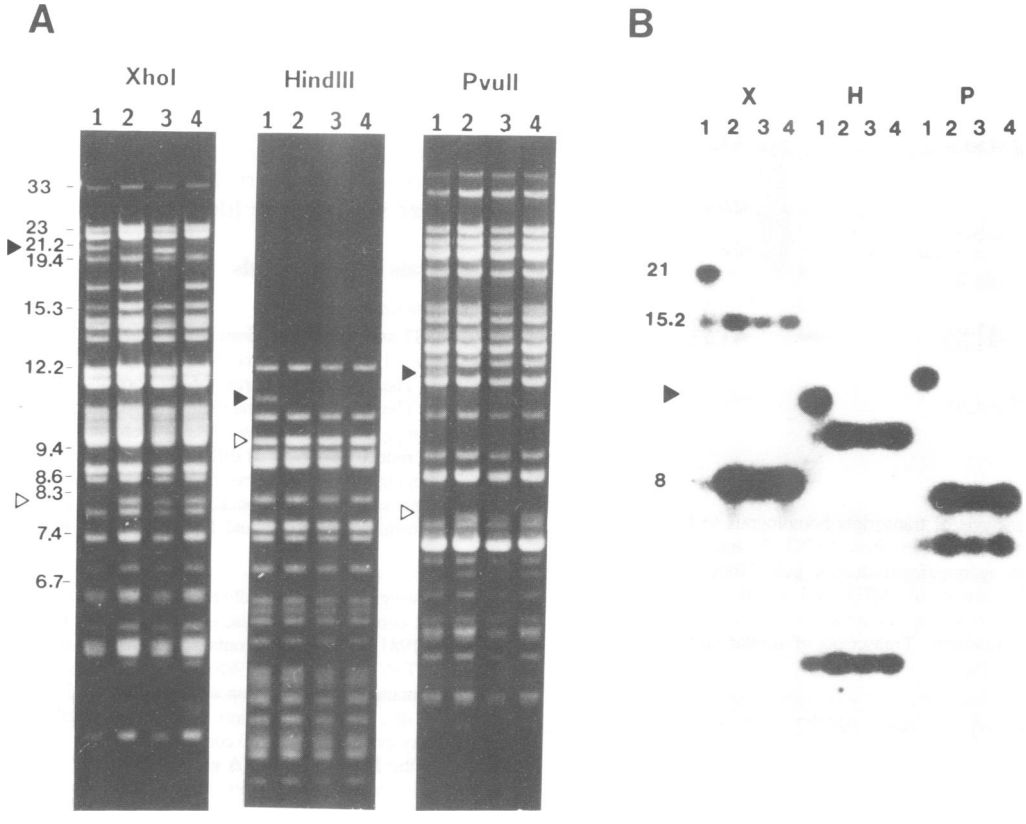

Fig. 3. Novel NCS2 restriction fragments. MtDNA digests from NCS2 (**1**), NCS2-derived normal (**2**), NCS3 (**3**) and NCS3-derived normal (**4**) sources. (**A**). Ethidium bromide-stained 0.62% agarose gel of the mtDNAs digested with *Xho*I, *Hin*dIII and *Pvu*II. The NCS2-unique fragments are indicated to the left of each digest (▶). Bands showing reduced staining in the NCS2 samples are also marked (▷). The positions of lambda size standards (in kb) are indicated on the left of the figure. (**B**). Southern blot analysis: autoradiograph resulting from hybridization with the NCS2-unique II-kb *Hin*dIII fragment of clone pMH1 to mtDNAs digested with *Xho*I (**X**), *Hin*dIII (**H**) and *Pvu*II (**P**). Sizes of the hybridizing *Xho*I fragments are indicated to the left. The position of the 11-kb *Hin*dIII fragment that was cloned and used as the probe is indicated by the arrowhead.

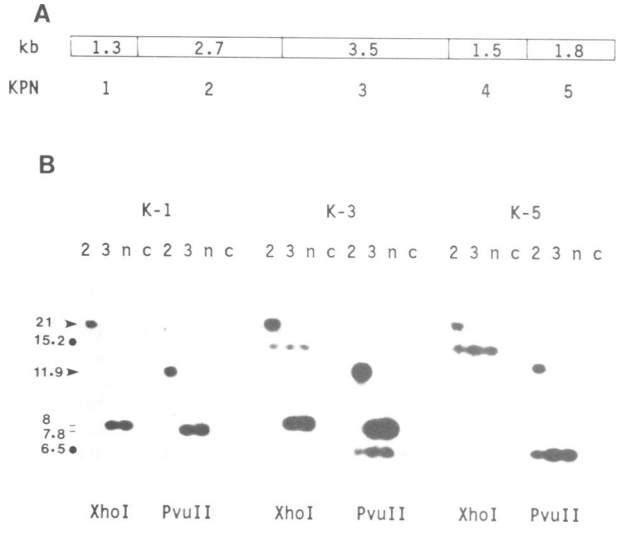

Fig. 4. Hybridization of mtDNA *Xho*I and *Pvu*II fragments with ^{32}P-labeled *Kpn*I fragments from the pMH1 clone. (**A**). Order of *Kpn*I fragments of the NCS2 11-kb *Hin*dIII band of pMH1. (**B**). Hybridization with *Kpn*I fragments: K1-, K-3 and K-5. The mtDNAs in each lane are listed along the top: NCS2 (**2**), NCS3 (**3**), normal derivative (**n**) mtDNAs and highly purified chloroplast DNA (**c**). The enzymes used are listed below each panel. Indicated to the left: fragments unique to NCS2 (arrows), reduced in NCS2 (−), and the same in all mt DNA samples (●).

Aberrant mitochondrial transcripts identified in NCS2 mutants

NCS2 and normal derivative mtRNAs were electrophoresed on formaldehyde-agarose gels, blotted onto nitrocellulose and probed with the 11-kb *Hin*dIII fragment from the pMH1 clone. It hybridized to a complex set of transcripts in both samples, but larger transcripts were detected in NCS2 RNA (not shown). Probes consisting of *Kpn*I fragments from pMH1: K-1, K-3 and K-5, gave more defined results (Figure 5). K-1 hybridized to a number of transcripts from the normal-derivative mitochondria. These transcripts were approximately 3500, 3200, 3000, 2400, 1930, 1830 and 1370 nucleotides. The major transcripts detected in NCS2 mtRNA were much larger, ~15 700 and 10 600 nucleotides.

The central *Kpn*I fragment from pMH1, K-3, also hybridized to a complex pattern of transcripts (Figure 5). The transcripts identified in normal-derivative mtRNA were ~4400, 3000, 1930 and 1830 nucleotides. In NCS2 mtRNA, a series of transcripts between 7000 and 3000 nucleotides hybridized strongly, in addition to larger transcripts (~15 700 and 10 600 nucleotides).

Both the K-1 and K-3 probes identify transcripts of ~1830 and 1930 nucleotides in normal-derivative mtRNA and these transcripts are not detected in the affected NCS2 sample. Both probes also demonstrate the accumulation of higher molecular weight transcripts (approximately 15 700 and 10 600 nucleotides) in NCS2 mtRNA.

The K-5 probe hybridized to one major 1760-nucleotide

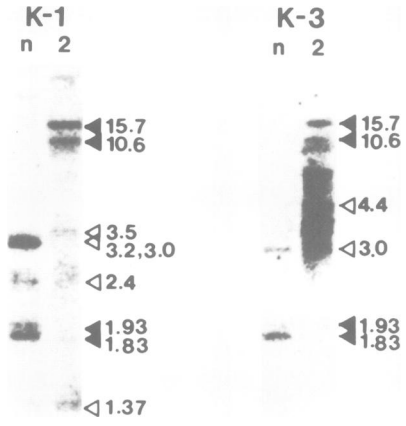

Fig. 5. Northern blot analysis of transcripts homologous to NCS2-altered regions of mtDNA. MtRNA samples from NCS2 (**2**) and normal derivatives (**n**) were fractionated on agarose/formaldehyde gels, blotted to nitrocellulose and probed with *Kpn*I fragments of pMH1: K-1 and K-3. The sizes (in nucleotides $\times 10^{-3}$) of hybridizing transcripts were estimated by comparison with RNA size standards. Transcripts of similar size identified by both probes are indicated (◀).

transcript (not shown), in both NCS2 and normal-derivative mtRNAs.

Discussion

Mitochondria from NCS2 plants synthesize reduced amounts of a single polypeptide, of approximately 24 000 M_r. Its synthesis was greatly reduced relative to other mitochondrial polypeptides even in those plants which showed only mild phenotypic expression in the leaves (slight striping). The biochemical properties and function of this polypeptide are unknown.

Northern blot analyses suggest that the defect conditioned by the NCS2 mutation does not greatly affect the expression of several previously characterized plant mitochondrial genes: those coding for ATPase subunits alpha, 6 and 9, cytochrome *c* oxidase subunits I and II, apocytochrome *b*, URF-1 or rRNAs. However, probes corresponding to the mtDNA region altered in NCS2 do identify grossly aberrant transcripts in NCS2 mtRNA.

These results correlate mitochondrial gene expression differences with previously reported mitochondrial genomic alterations in NCS2. This provides further evidence that the maternally-inherited NCS2 mutation is indeed mitochondrial. The DNA hybridization analyses also confirm that the NCS2 mutation is different from the phenotypically distinct NCS3 mutation.

Southern blot analyses using cloned probes indicate that the molecular alteration associated with NCS2 does not involve extensive rearrangements of the mitochondrial genome. The mutation appears to be limited to a specific region of mtDNA. Furthermore, the NCS2-novel restriction fragments have not been detected by hybridization in non-NCS samples, even after prolonged exposures. If NCS2 genomes exist in all cms-T mitochondria, they are present at very low levels. At this time, there is no evidence that the mutation arose by amplification of a pre-existing mitochondrial subgenome.

Although we have not provided direct evidence that the aberrant NCS2 transcripts correspond to those that are translated into a 24 000 M_r polypeptide in non-mutant mitochondria, the correlation between the alterations in one set of transcripts and one translation product is strong. We propose that an interruption in or near the gene coding for this protein leads to transcripts which are incorrectly processed.

Finally, our results suggest that the 24 000 M_r polypeptide is an essential gene product for normal functioning of maize cms-T mitochondria. Reduced synthesis of this protein is correlated with abortion during kernel maturation and defective growth during other stages of the life cycle.

Materials and methods

Plant materials

The NCS2 and NCS3 mutations originated in T-type cytoplasmic male sterile lines (Coe, 1983; Newton and Coe, 1986). Normal plants are found in NCS families. These were the sources of the normal-derivative plants used for comparisons. The mutant plants and their corresponding normal derivatives were propagated in parallel families by crossing them with pollen from the same inbred lines. To reduce the potential differential effects on mitochondrial gene expression by segregating nuclear genes (introduced in previous outcrosses), the materials used in this study had been backcrossed twice to the inbred lines A619 (NCS2 and its normal derivatives) and B37 (NCS3 and its normal derivatives).

Plasmids

The following clones were kindly provided by C.Leaver, C.S.Levings and D.Stern: pBN6601 containing COI (Isaac *et al.*, 1985); pZmE1 containing COII (Fox and Leaver, 1981); pZmEH680 containing COB (Dawson *et al.*, 1984); T25H containing ATP6 (Dewey *et al.*, 1985a); 15X containing ATP9 (Dewey *et al.*, 1985b); TA22 containing ATPA (Braun and Levings, 1985); pBH726 containing URF-1 (Stern *et al.*, 1986). In addition, probes for the mitochondrial ribosomal RNA genes were available: pMZ185 containing the 26S rRNA gene and pMZ206 which includes the 18S and 5S rRNA genes (Newton *et al.*, unpublished). A COXIII clone was provided by A.Brennicke (Hiesel *et al.*, 1987).

DNA isolation and analysis

MtDNAs were isolated from unfertilized ear shoots (cobs) of plants scored for the degree of mutant expression and were analysed by restriction endonuclease digestions and gel electrophoresis as previously described (Newton and Coe, 1986). Size standards included on each gel consisted of a mixture of lambda DNAs digested separately with *Sal*I, *Sma*I, *Hin*dIII and *Eco*RI. Duplicate nitrocellulose blots of each gel were obtained by the double dry blot technique (Smith and Summers, 1980).

RNA isolation and analysis

MtRNA was isolated from unfertilized ear shoots and analysed as previously described (Stern and Newton, 1986). The isolation procedure included two lithium chloride precipitations and aurintricarboxylic acid was used to inhibit RNase. Approximately 0.5 μg of RNA was loaded in each well of 1.2% agarose/6% formaldehyde gels and electrophoresis was conducted at 5 V/cm for ~5 h. RNA size standards from Bethesda Research Laboratories were included on the gels to give marker bands at 9500, 7500, 4400, 2400, 1400 and 300 nucleotides. Gels were double-blotted onto nitrocellulose (Thomas, 1980).

Southern and Northern blot hybridizations

The cloned DNAs were digested with appropriate restriction enzymes and the mtDNA fragments were excised from agarose gels. The DNAs in diluted gel slices were labeled with [α-^{32}P]dCTP by the techniques of Feinberg and Vogelstein (1983, 1984), using the oligolabelling kit supplied by Pharmacia. Southern blots were prehybridized at 68°C for ~12 h in 6 × SSC, 5 × Denhardt's, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA. The labeled DNA was then added and hybridization was continued for 16 h. Filters were rinsed in 2 × SSC and 0.5% SDS and washed twice for a total of 3 h at 68°C in 0.1 × SSC, 0.5% SDS (Maniatis *et al.*, 1982). ^{32}P-labeled lambda DNA was included in the hybridizations to facilitate localization of marker bands. Hybridizations of the DNA probes to Northern blots were as previously described (Stern and Newton, 1986).

Mitochondrial purification, protein labeling and analysis

Mitochondria were prepared from unfertilized ear shoots and labeled *in organello* with [^{35}S]methionine (Newton and Walbot, 1985). Mitochondrial proteins (approximately 100 000 c.p.m. of radioactivity per lane) were subjected to electrophoresis on 12–18% SDS-polyacrylamide gels and the gels were fluorographed. Protein standards were included as mol. wt markers on each gel: phosphorylase b (93 000 M_r), bovine serum albumin (66 000 M_r), ovalbumin (45 000 M_r), carbonic anhydrase (31 000 M_r), soybean trypsin inhibitor (21 000 M_r), lysozyme (14 400 M_r) and bovine trypsin inhibitor (6500 M_r).

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