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Mitochondrial genome diversity and cytoplasmic male sterility in higher plants

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Characteristic differences exist between the mitochondrial genome organization of fertile and cytoplasmic male-sterile (CMS) lines in a range of plant species. Current evidence suggests that these characteristic mitochondrial genotypes arose by aberrant recombination events, generating chimeric mitochondrial DNA sequences which have subsequently become stabilized, possibly by selective amplification.

An investigation of the variation in stoichiometry of the four *atp*A gene types in maize have suggested evolutionary mechanisms for the generation of mitochondrial genome diversity which are based on amplification of pre-existing, rare recombinant DNA molecules. As with a number of other well-documented examples of genome rearrangement, those involving the *atp*A gene appear to have no obvious phenotypic significance. However, in a number of cases, recombination events have resulted in either modification of existing mitochondrial genes, leading to the synthesis of a modified polypeptide, e.g. the *cox*I gene in the 9E sorghum cytoplasm, or the generation of novel open reading frames. In the latter case the unique open reading frame found in the mitochondrial DNA of CMS-T maize plants encodes a 13 kDa polypeptide, previously identified as a CMS-T-specific mitochondrial translation product. Current studies are directed towards establishing a causal link between the 13 kDa polypeptide, mitochondrial enzyme complexes, and the CMS phenotype.

Introduction

The mitochondrial genomes of higher plants are large and vary in size even between closely related species (218–2400 kilobases (kb)) (Ward *et al.* 1981). Mapping of the mitochondrial genomes of several higher plants has shown that they are circular and complex, owing to active recombination between pairs of repeated DNA sequences, which give rise to smaller subgenomic circles. This multipartite genome structure is generally stable and is inherited from generation to generation (Lonsdale *et al.* 1984; Palmer & Shields 1984; Oro *et al.* 1985). Despite the large variation in size between species the base composition is remarkably constant at *ca.* 47 % GC; this observation suggests the absence of extensive, non-coding AT-rich regions as found in fungal mitochondrial DNA (mtDNA).

Isolation of mitochondrially encoded genes, coupled with analysis of translation products labelled *in organello*, has shown that plant mtDNA encodes the 26*S*, 18*S* and 5*S* ribosomal RNAs (rRNAs), at least 20 transfer RNAs (tRNAs) and of the order of 18–20 polypeptides (Leaver & Gray 1982; Dawson *et al.* 1986). In addition to encoding subunits I, II, and III of the cytochrome *c* oxidase complex, apocytochrome *b*, subunits 6 and 9 of the F_0 ATPase, subunit I of the NADH dehydrogenase complex I and ribosomal protein S13, plant mitochondria are unusual in containing the gene for the α subunit of the F_1 ATPase complex (Isaac *et al.* 1985*a*),

which is encoded in the nucleus of animals and fungi. Mapping of these genes in maize (Dawson *et al.* 1986), spinach (Stern & Palmer 1986) and *Brassica campestris* (Markaroff & Palmer 1987) show that they are scattered throughout the genome and apparently independently transcribed. Gene order varies between even closely related species; this variation suggests that the order and arrangement has no functional significance and is a product of extensive genome reorganization.

Although the remaining mitochondrial genes which encode the additional mitochondrial translation products remain to be identified, it is obvious that the plant mitochondrial genome encodes at least 20 polypeptides, significantly more than are encoded by animal (13) or fungal genomes (*ca.* 15). However, this increased coding capacity cannot account for the large and variable size of the plant mitochondrial genome, and thus it is of considerable interest to investigate the origins and significance of mitochondrial genome diversity in higher plants.

Mitochondrial genome rearrangements and cytoplasmic male sterility

Genetic analysis of the plant mitochondrial genome has been hampered by the lack of identifiable mutations in the mitochondrial genome that result in an identifiable phenotype. This is not surprising because most mutations in mtDNA would either be 'silent' (owing to the large proportion of the genome that is not essential), or lethal, because higher plants cannot survive without functional mitochondria, in contrast to yeast. The best characterized exception is the maternally transmitted phenotype termed cytoplasmic male sterility (CMS), which is recognized by the failure of the mature plant to produce functional pollen. Cytoplasmic male sterility is found in a wide range of plant species and is used commercially in the production of F_1 hybrid seed varieties as it prevents self-fertilization of the seed parent. The large scale use of CMS lines as female parents in hybrid seed production was made possible by the discovery of male-fertile lines containing specific, dominant nuclear restorer genes (Rf genes). When these genes are introduced into a hybrid from the pollen parent, the male-sterile phenotype is suppressed and the F_1 hybrid progeny are fertile. In maize, for example, three different cytoplasms (CMS-T, S and C) that confer sterility have been identified and are distinguished from each other by the different nuclear genes required to restore fertility (Leaver & Gray 1982).

It is now becoming obvious in at least some cases that the CMS phenotype originated from mutation(s) in the mtDNA of the male-fertile progenitors as a result of aberrant recombination events. This resulted in different restriction endonuclease digestion patterns of mtDNA from fertile and male-sterile cytoplasms (Pring & Levings 1978; Dewey *et al.* 1986). These mutations may be phenotypically silent in the original nuclear background, which compensates for the mutation. However, if the cytoplasm containing the mutation is transferred by repeated backcrossing into a 'foreign' or non-fertility-restoring nuclear background, then the male-sterile phenotype is observed. Apparently, this is due to the inability of the foreign nucleus to suppress or compensate for the mutation at all stages of development (Leaver & Gray 1982).

We have observed that mitochondria isolated from CMS lines of maize (Forde *et al.* 1978; Forde & Leaver 1980) and sorghum (Dixon & Leaver 1982) synthesize characteristic variant polypeptides not found in mitochondria from normal (fertile) lines. In CMS-T lines of maize

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the synthesis of the variant polypeptide is suppressed in the presence of nuclear restorer alleles.

The primary aims of the work in our laboratory, and of that of others described in this volume, are to investigate the molecular events that generate mitochondrial genome diversity and, in particular, those causally related to the CMS phenotype. This involves establishing the relation between specific mitochondrial genome rearrangements, synthesis of variant polypeptides and the failure in mitochondrial biogenesis and/or function in the anther, tapetum or microspore during pollen formation.

Mitochondrial genome rearrangement and the synthesis of variant polypeptides

Most rearrangements of the mitochondrial genome found in CMS lines have no discernible effect on gene expression within the mitochondrion. For instance, we have described elsewhere (Isaac *et al.* 1985*b*) the rearrangement found near the *cox*I gene in the mtDNA of CMS-S maize, which acts as a substrate for subsequent recombination with the linear 'plasmid-like' DNAs (S1 and S2) found in this cytoplasm, resulting in the production of linear mitochondrial chromosomes with S1 or S2 attached to one end (Schardl *et al.* 1984). These recombination events result in the *cox*I gene's being located in multiple genomic environments, which do not appear to affect the expression of the *cox*I gene. In addition, revertants to fertility are known which maintain these rearrangements together with linear mitochondrial chromosomes and free S plasmids (Escote *et al.* 1986; Small *et al.* 1988). Thus, at most, only a subset of genomic rearrangements are causally linked to CMS.

However, a survey of variation in mitochondrial genome organization and expression between male-fertile and male-sterile nuclear–cytoplasmic combinations of sorghum (Bailey-Serres *et al.* 1986*a*) has revealed a mutant form of the *cox*I gene (see Bailey-Serres *et al.* 1986*b*) (figure 1). Mitochondria from lines containing the 9E cytoplasm synthesize a variant form of subunit I of cytochrome *c* oxidase with an apparent molecular mass of 42 kDa which replaces the usual 38 kDa subunit found in all other sorghum cytoplasms (see figure 1*c*). Isolation and analysis of the normal *cox*I gene from a Milo cytoplasm shows that it is located on a 4.3 kb *Eco*RI-generated mtDNA fragment and encodes a 530 amino acid polypeptide (58483 Da). In contrast the gene is located on a 10.4 kb *Eco*RI-generated mtDNA fragment from the 9E cytoplasm and encodes a 631 amino acid protein (70350 Da). The increased size of the *cox*I gene in 9E is due to a 3′-extension of the open reading frame (ORF), which results in the addition of 101 amino acids to the carboxyl terminus of the protein. The two genes are identical from position −100 base pairs (bp) 5′ to the presumptive AUG codon, to position +1579 bp within the *cox*I ORF (figure 1*a*). Thus the novel 9E *cox*I arises from at least two rearrangements, which lead to altered transcription (see figure 1*b*) and the synthesis of a significantly longer subunit I polypeptide. Our data suggest that the cytochrome *c* oxidase activity of seedling mitochondria is not affected by this modification of the subunit, although we have not carried out a definitive investigation of the structure and function of the cytochrome *c* oxidase holoenzyme at other development stages.

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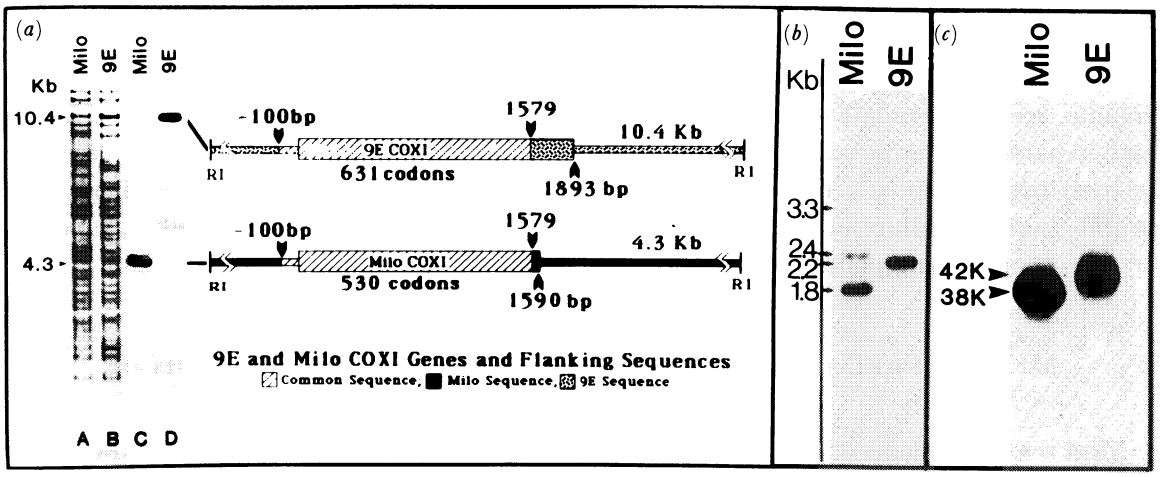

FIGURE 1. The location, structure and expression of the *cox*I gene in Milo and 9E cytoplasm sorghum. (*a*) Mitochondrial DNA from Milo and 9E cytoplasm was digested with *Eco*RI, fractionated by electrophoresis, stained with ethidium bromide and photographed. The mtDNA was transferred to nitrocellulose and hybridized with a ^{32}P-labelled clone containing an internal portion of the maize *cox*I gene. The radioautograph of the Southern blot shows that the *cox*I gene is located on a 4.3 kb *Eco*RI fragment in Milo and a 10.4 kb fragment in 9E. DNA sequence analysis of the *cox*I gene of Milo and 9E cytoplasm reveals that the Milo gene encodes a 530 amino acid polypeptide whereas the 9E gene encodes a 631 amino acid polypeptide. The diagram shows the location of the homologous *cox*I DNA sequences on the *Eco*RI fragments. The points of divergence between the two genes are indicated by arrows and non-homologous sequences are shaded. (*b*) Transcript analysis of *cox*I from Milo and 9E. mtRNA was fractionated by electrophoresis, transferred to nitrocellulose and probed with a ^{32}P-labelled M13 DNA probe containing a common portion of the *cox*I gene. The major Milo *cox*I transcript is 1.8 kb; in contrast, the major 9E *cox*I transcript is 2.2 kb. (*c*) Synthesis of the normal (Milo) and variant forms (9E) of cytochrome *c* oxidase subunit I in sorghum. [^{32}S]Methionine-labelled mitochondrial translation products were immunoprecipitated with an antibody raised against yeast cytochrome *c* oxidase subunit I, fractionated by SDS–polyacrylamide gel electrophoresis and radioautographed. In mitochondria from Milo cytoplasm a 38 kDa polypeptide was precipitated, whereas in 9E a 42 kDa polypeptide was identified.

THE GENERATION AND EXPRESSION OF A NOVEL OPEN READING FRAME IN CMS-T MITOCHONDRIA

The work of Dewey *et al.* (1986; this symposium) has established that in CMS-T maize multiple recombination events have generated a chimeric DNA sequence (called TURF 2H3) containing portions of the flanking and coding regions of the 26*S* rRNA gene, the non-coding flank of the *atp*6 gene and a chloroplast tRNA^{Arg} gene. Analysis of this chimeric DNA fragment reveals that it contains two open reading frames, one of which is unique to CMS-T mtDNA and encodes a novel polypeptide of predicted molecular mass 12971 Da (13 kDa). We and others have recently shown that an antibody raised against a synthetic oligopeptide corresponding to part of the carboxy terminus of the 13 kDa ORF will immunoprecipitate the 13 kDa variant polypeptide that is synthesized by mitochondria from the T-cytoplasm (Forde *et al.* 1978; Forde & Leaver 1980). In contrast to the variant cytochrome *c* oxidase subunit I polypeptide in sorghum, generated by recombination events which alter a known protein-coding gene sequence, the information encoding the 13 kDa variant polypeptide found in the maize T-cytoplasm has been formed by recombination events involving sequences that do not normally encode a protein. The fragment containing this open reading frame has evolved as a product of at least seven rare or unique recombination events. The degree of sequence

conservation varies between the component sequences in this region and the homologous sequences in mtDNA from normal fertile plants. It can therefore be implied that the recombination events that generated the 13 kDa locus did not occur simultaneously, but happened sequentially over a prolonged period of time. Each recombination event presumably affected one DNA molecule in one mitochondrion in a single cell. In the following section we discuss some results which suggest how such novel organizations of the genome can be maintained in the mitochondrial population and subsequently evolve into a phenotypically significant DNA lesion.

Evolution of mitochondrial genome diversity

The maize mitochondrial *atp*A gene encoding the α subunit of the F_1 ATP synthase complex has been sequenced (Braun & Levings 1985; Isaac *et al.* 1985*a*) and located on the circular map of Wf9-N mtDNA (Dawson *et al.* 1986). In this fertile (N) cytoplasm of maize, the gene lies entirely within a 12 kb repeat, with the 3′ terminus of the gene positioned 650 bases from one end of the repeat (Isaac *et al.* 1985*a*). Consequently, two copies of the *atp*A gene can be represented, on the 570 kb 'master circle' form of the genome, which differ in their 3′ flanking sequences. Mitochondrial DNA from CMS-C cytoplasms contains a single *atp*A arrangement identical to one of those found in N mtDNA. However, novel DNA sequences are found 3′ to the gene in mtDNA from CMS-S and CMS-T cytoplasms (Braun & Levings 1985; Isaac *et al.* 1985*a*; Small *et al.* 1987). These four different flanking sequences can be distinguished by the DNA probe BLSC1, which covers, the 3′ end of the gene and the immediately adjacent flanking sequence (figure 2). We have designated these *atp*A arrangements as types 1–4 (Small *et al.* 1987).

We have screened mtDNA from a number of different maize cytoplasms in a variety of nuclear backgrounds with *atp*A-specific gene probes to examine differences in the organization

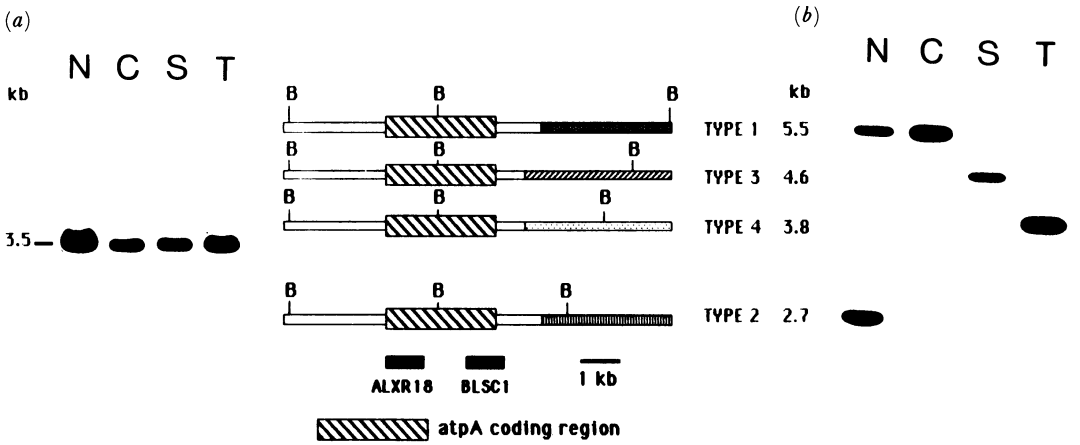

Figure 2. Location of the gene for the α subunit of the F_1-ATPase (*atp*A) in the mitochondrial DNA of N, C, S and T cytoplasms of maize. Mitochondrial DNA was isolated from N, C, S and T cytoplasms, digested with *Bam*H1, and probed with the DNA probe ALXR18 covering the 5′ end of the *atp*A gene (*a*) or (*b*) BLSC1 covering the 3′ end of the gene. The probe BLSC1 distinguishes the four major *atp*A gene arrangements, which we have designated types 1–4 as shown; the same DNA probed with ALXR18 shows that all four arrangements are identical at the 5′ end. B, *Bam*H1 restriction sites; filled boxes, region covered by probes. (Reprinted with permission from Small *et al.* (1987).)

of the 12 kb repeat region. The predominant types of the gene found varied even within the cytoplasmic groups N and S, whereas nuclear genotype did not appear to affect which of the *atp*A types were predominant in any cytoplasm (see figure 3). Figure 3 shows N cytoplasms with one or two *atp*A types, S cytoplasms with 1 or 2 *atp*A types, and an N cytoplasm with the type 4 arrangement previously thought to be unique to T cytoplasm.

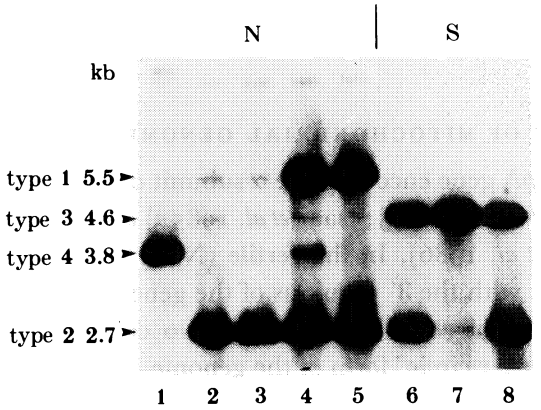

FIGURE 3. Substoichiometric restriction endonuclease fragments containing the *atp*A gene in maize mitochondrial DNA. One-day exposures of autoradiographs of *Bam*H1-digested maize mtDNA (nuclear genotype CO192 × WJ, 5 μg per track) probed with BLSC1 (see figure 2). Lane 1, 181 cytoplasm; lane 2, SG cytoplasm; lane 3, OY cytoplasm: lane 4, 234 cytoplasm; lane 5, N cytoplasm; lane 6, D cytoplasm; lane 7, J cytoplasm; lane 8, CA cytoplasm. Lanes 1–5 are fertile 'N-like' cytoplasms; 6–8 are CMS-S cytoplasms. Faintly hybridizing bands can be seen in all tracks, some of which correspond in size to predominant bands found in other cytoplasmic types. All the N cytoplasms shown, except 181, appear to contain *atp*A types 1, 2 and 3 in detectable amounts. The cytoplasm 234 contains only a low level of type 2; the other two S cytoplasms shown have equal amounts of types 2 and 3. Similar results were obtained with a wide range of other nuclear–cytoplasmic combinations (table 1). Special care was taken to avoid cross-contamination of samples, by preparing mitochondria and mtDNA from fertile and male-sterile lines on different days. (Reprinted with permission from Small *et al.* (1987).)

In those N and S cytoplasms containing only one predominant *atp*A type, the other expected type could always be detected, albeit at very low levels (see figure 3, lanes 2, 3, 7, and table 1). In addition, we could detect anomalous *atp*A gene arrangements in N mtDNA, not accounted for in the published map of the N mitochondrial genome, and not explicable as recombination products between any of the known repeats in the genome. Some of these rearrangements were characteristic of CMS lines, and were again present only at low stoichiometry (see figure 3 and table 1). It seemed unlikely that differences in hybridization of the same fragment in different cytoplasms were due to partial homology to the probe, because in many cases the cytoplasms were closely related. Later, we found that mitochondrial DNA cut with restriction enzymes other than *Bam*H1 gave similar results and that non-overlapping probes hybridized to the same fragments to similar extents; these results indicate that these faint hybridization signals are truly due to substoichiometric levels of the *atp*A types.

One possible artefactual explanation of these observations was that the seed stocks we had used for these experiments contained heterogeneous mixtures of cytoplasms, and the apparently substoichiometric fragments were due to a small subgroup of the seeds containing the anomalous *atp*A types at normal, abundant levels. We investigated this possibility by extracting mtDNA from immature cobs of individual plants and probing this mtDNA with BLSC1

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TABLE 1. STOICHIOMETRIES OF *atp*A GENES IN MAIZE CYTOPLASMS

(A summary of the relative abundance of different *atp*A types in a range of maize cytoplasms. Three groups of N-like cytoplasms have been identified, and two of S-like.

The number of pluses represents approximate relative abundance. ECU321, ECU398, CUN443 and PUN6 are male-fertile South American accessions with restriction enzyme cleavage profiles similar to inbred N cytoplasms. ECU321 and ECU398 contain RU episomes (Weissinger 1982).)

	*atp*A types detected			
	1	2	3	4
fertile cytoplasms				
N	+++++	+++++	+	+
LF	+++++	+++++	+	+
234	+++++	+++++	+	+
OY	+	+++++	+	·
SG	+	+++++	+	·
ECU321	+	+++++	+	·
ECU398	+	+++++	+	·
CUN443	+	+++++	+	·
PUN6	+	·	+	+++++
181	+	·	+	+++++
CMS-S cytoplasms				
S	·	+	+++++	·
J	·	+	+++++	·
VG	·	+++++	+++++	·
CA	·	+++++	+++++	·
MY	·	+++++	+++++	·
H	·	+++++	+++++	·
PS	·	+++++	+++++	·
G	·	+++++	+++++	·
I	·	+++++	+++++	·
D	·	+++++	+++++	·
CMS-C cytoplasms				
C	+++++	·	·	·
CMS-T cytoplasms				
T	·	·	·	+++++
Q	·	·	·	+++++

(figure 4). Such experiments revealed no significant differences between the *atp*A hybridization patterns of mtDNA from single plants and those from pooled seedling coleoptiles.

We have extended our observations to mtDNA from sorghum, sunflower and artichoke and have good evidence for substoichiometric arrangements of the *atp*A gene in all three species. Similar observations of substoichiometric organelle DNA organizations have been observed in chloroplasts of higher plants (Moon *et al.* 1987) and animal mitochondria (for reveiw, see Hauswirth & Laipis (1986)).

Using our observations of the variation in stoichiometry of the *atp*A gene, we can draw up a plausible evolutionary relationship between those maize cytoplasms that are available to us (see figure 5). We emphasize, however, that the proposed relationships are only based on existing maize cytoplasms, as the evolutionary progenitors of most of these cytoplasms are either extinct or unavailable.

The mechanisms responsible for the differences in stoichiometry of specific subgenomic molecules are currently unclear, but from a consideration of spontaneous reversion to fertility in CMS-S lines of maize it seems likely that stoichiometry changes may occur rapidly. During reversion, apparently novel recombinant molecules are fixed in the mitochondrial genome,

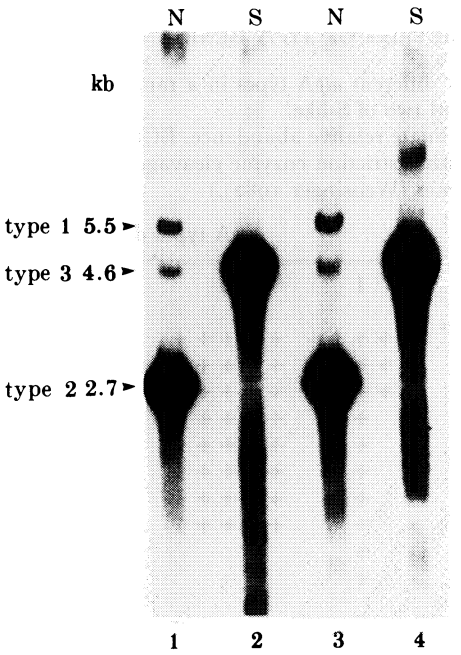

FIGURE 4. Substoichiometric *atp*A hybridizing fragments exist in the mitochondrial DNA from single plants. Three-day exposure of *Bam*H1-digested maize mtDNA probed with BLSC1 (see figure 2). Lanes 1 and 3, CO192 × WJ-SG; lanes 2 and 4, CO192 × WJ-J. Each lane contains mtDNA from a single immature cob. The SG mtDNA shows low levels of types 1 and 3 clearly, as predicted by the same experiment on pooled coleoptile mtDNA (figure 3, lane 2). The J mtDNA shows no clear faint bands, but the expected low levels of the type 2 fragment (figure 3, lane 7) may be obscured by the smear of degradation products from the abundant type 3 fragment. (Reprinted with permission from Small *et al.* (1987).)

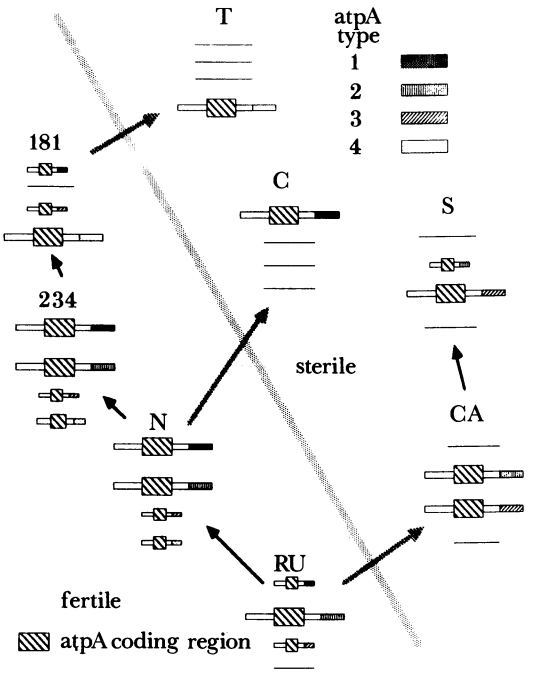

FIGURE 5. Possible interrelationships of maize cytoplasms based on *atp*A-type stoichiometries. The relative stoichiometries of different *atp*A gene types can be used to predict a possible evolutionary interrelationship between maize cytoplasms. The size of the boxes represents approximate relative abundance of the *atp*A types in the various genomes. The large arrows indicate differences in overall mitochondrial organization, as revealed by restriction endonuclease digestion patterns, separating fertile from sterile cytoplasms.

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sometimes within a single plant generation (Schardl *et al.* 1985). Many of these recombination products are undetectable in male-sterile plants, and so must be 'amplified' within this period to levels necessary for fixation in the progeny. The fact that these recombination products are often similar in independent revertants and yet are greatly influenced by the nuclear genotype (Schardl *et al.* 1985; Escote *et al.* 1985; Small *et al.* 1988) implies some degree of selectivity in either the initial recombination events or subsequent segregation–amplification or both. Reversion to fertility has also been reported in plants dérived from callus cultures of both CMS-T and CMS-S maize (Gengenbach *et al.* 1977; Brettell *et al.* 1980; E. D. Earle, personal communication). This shift to male fertility shows a maternal pattern of inheritance which is concomitant with alterations in mtDNA organization (Gengenbach *et al.* 1981; Lonsdale, this symposium; Pring, this symposium) and, in CMS-T, involves mutation or deletion of portions of the open reading frame coding for the CMS-T-specific 13 kDa polypeptide. The high frequency of reversion in plants regenerated from culture implies that there is a greater opportunity for segregation and/or selection in tissue culture, and further that there is heavy selection against the existence of the open reading frame encoding the 13 kDa polypeptide.

Speculation on the origin and significance of substoichiometric mitochondrial DNA *fragments (sublimons) in the generation of mitochondrial genome diversity*

From our observations we postulate that novel restriction fragments that are products of infrequent recombination events are sometimes maintained in the mitochondrial genome for many generations at low levels. These recombinant molecules, which we for convenience have termed sublimons, might be expected to show rapid molecular evolution for the following reasons.

(i) As these molecules are present at relatively low levels, further mutational events in one of these molecules will affect a relatively high proportion of the sublimon population. Mutations occurring under such circumstances can become more rapidly 'fixed' than mutations in highly abundant sequences.

(ii) There would be no selection pressure against mutations, as these sublimons cannot be expected to have any direct phenotypic significance. Consequently one would expect sublimons to accumulate mutational events progressively. Occasionally, owing to a specific mutation or recombination event, one of these sublimons might be amplified to a 'normal' stoichiometry and this amplification event would create a novel mtDNA restriction pattern. One prediction of such a hypothesis would be that the amplification event would lead to the formation of a novel repeat including the last sequence to be incorporated into the substoichiometric molecule.

A possible example of this phenomenon is the TURF2H3 region of the CMS-T genome (Dewey *et al.* 1986), which is the product of at least seven recombination events and encodes the unique 13 kDa polypeptide. The degree of conservation of the component sequences in this region with their homologous sequences in N mtDNA varies (this variation implies temporal separation of the formative recombination events) and yet no evolutionary intermediates have been discovered. The highest sequence conservation is seen for the 5′ flanking sequences of the *atp*6 gene (100 %), which now forms a large novel repeat in the T genome. It is therefore possible that this chimeric region of the T genome, encoding a variant polypeptide linked with the CMS phenotype, evolved as a low-abundance constitutent of the mtDNA by progressive and

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sequential recombination events, which resulted ultimately in the amplification and expression of the unique open reading frame.

This hypothesis relies on the speculation that molecules with very low abundance can be efficiently passed between generations. This requires that the 'unit of inheritance' for mtDNA be large (of the order of a few hundred genome equivalents). This would contrast with the better characterized mammalian systems, where the 'unit of inheritance' has been estimated to be as small as five mitochondrial genomes (Hauswirth & Laipis 1986). This small number of heritable units explains the rapid segregation of different mtDNA molecules observed in mammals (Hauswirth & Laipis 1986). In contrast, mtDNA restriction endonuclease patterns of siblings vary little in higher plants (Oro *et al.* 1985) considering the huge potential for change afforded by a large multipartite genome; this result implies that little segregation occurs.

Expression and localization of the 13 kDa variant protein synthesized by mitochondria from CMS-T maize

Our earlier studies demonstrated that mitochondria isolated from CMS cytoplasms of both maize and sorghum synthesized characteristic variant polypeptides (Forde & Leaver 1980; Dixon & Leaver 1982).

Although considerable progress is being made in our understanding of the molecular basis of CMS, a major challenge is to establish how reorganization of the mitochondrial genome is related to the synthesis of these variant polypeptides, and causally related to the CMS phenotype. We have therefore sought to localize and assign a functional role to the 13 kDa polypeptide that is characteristic of the CMS-T cytoplasm.

This variant 13 kDa translation product is, together with the majority of the mitochondrially encoded translation products, targeted to the inner mitochondrial membrane. The specific localization of this polypeptide to an individual enzyme complex has been investigated by immunoprecipitation with specific antibodies. In an analysis of *in organello* translation products, the 13 kDa variant polypeptide coprecipitated with cytochrome *c* oxidase subunit I, in membrane preparations solubilized with Triton X-100, by an antibody against yeast cytochrome *c* oxidase subunit I. This coprecipitation was not seen with any of a wide range of other antibodies tested (including anti-yeast cytochrome *c* oxidase subunit II and anti-holo plant cytochrome *c* oxidase), nor was it observed in SDS-solubilized membranes. In preparations, solubilized with Triton X-100, from both N (fertile) and CMS-T cytoplasms, ATPase subunit 9 also coprecipitated with the cytochrome *c* oxidase subunit I. The precipitation of cytochrome *c* oxidase subunit I was inhibited by the addition of purified maize cytochrome oxidase; the precipitation of the 13 kDa polypeptide was unaffected. This result suggested that the immunoprecipitation of the 13 kDa polypeptide by the yeast cytochrome *c* oxidase subunit I antibody preparation was both independent of cytochrome *c* oxidase precipitation, and also that it was not due to a shared epitope but was more probably due to the presence of an antibody to a contaminating protein. In support of this idea, the anti-cytochrome *c* oxidase subunit I antibody preparation recognizes the ATPase subunit 9 (immunoprecipitates this polypeptide), and may also recognize other additional as yet unidentified polypeptides.

An antibody to a synthetic oligopeptide corresponding to 11 amino acids in the carboxy-terminal region of the predicted 13 kDa ORF (kindly provided by W. H. Rottmann and D. M.

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Lonsdale) precipitated both the 13 kDa and the ATPase subunit 9 *in organello* translation products in Triton X-100 solubilized membrane preparations of T-sterile mitochondria. However, a number of problems exist with the *in organello* translation system. It is likely that assembly of the radioactively labelled translation products into fully mature oligomeric complexes is incomplete, as nuclear-encoded subunits may be limiting. It is also possible that there could be non-specific aggregation phenomena between unassembled hydrophobic translation products; for example, between the 13 kDa polypeptide and ATPase subunit 9. To avoid these problems, mature, fully assembled complexes within the membrane were radiolabelled with either Bolton and Hunter reagent or with DCCD (a reagent found to label the 13 kDa polypeptide particularly efficiently) before solubilization and immunoprecipitation. The anti-cytochrome *c* oxidase subunit 1 immunoprecipitated cytochrome *c* oxidase subunit 1 and ATPase subunit 9 in mitochondrial extracts from both fertile (N) and T cytoplasms and the 13 kDa polypeptide in the T-sterile mitochondria. The anti-13 kDa polypeptide antibody immunoprecipitated specifically the 13 kDa polypeptide and not the ATPase subunit 9. Contrary to the results obtained after *in organello* labelling, these data suggest that there are no specific interactions between these polypeptides. It is, however, impossible to exclude an interaction between the 13 kDa polypeptide and nascent ATPase subunit 9 and to hypothesize that such interactions may interfere with mitochondrial biogenesis. It has not been possible, as yet, to investigate precipitation of cytochrome *c* oxidase subunits with the anti-13 kDa polypeptide antibody. Separation of mitochondrial proteins by size exclusion chromatography has shown that both the labelled translation product and the bulk pool of the 13 kDa variant polypeptide (detected by using the anti-13 kDa antibody) fractionate at a molecular mass of more than 200 kDa. This result suggests that the 13 kDa polypeptide assembles rapidly into either a discrete aggregate or possibly into an as yet unidentified oligomeric complex in the inner membrane. It therefore seems unlikely that there is a specific association between cytochrome *c* oxidase subunit I or ATPase subunit 9 and the 13 kDa polypeptide in the T-sterile mitochondria. Further work is in progress to determine the localization and functional significance of the 13 kDa polypeptide.

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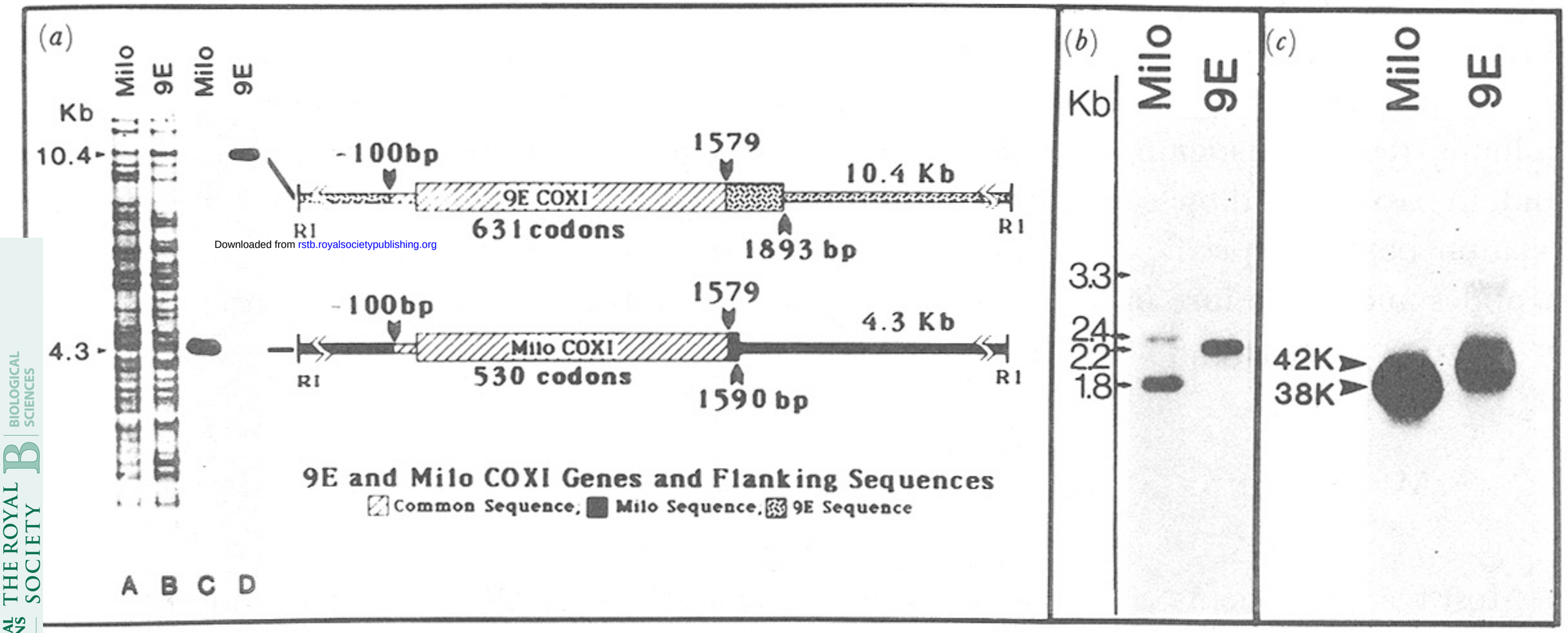
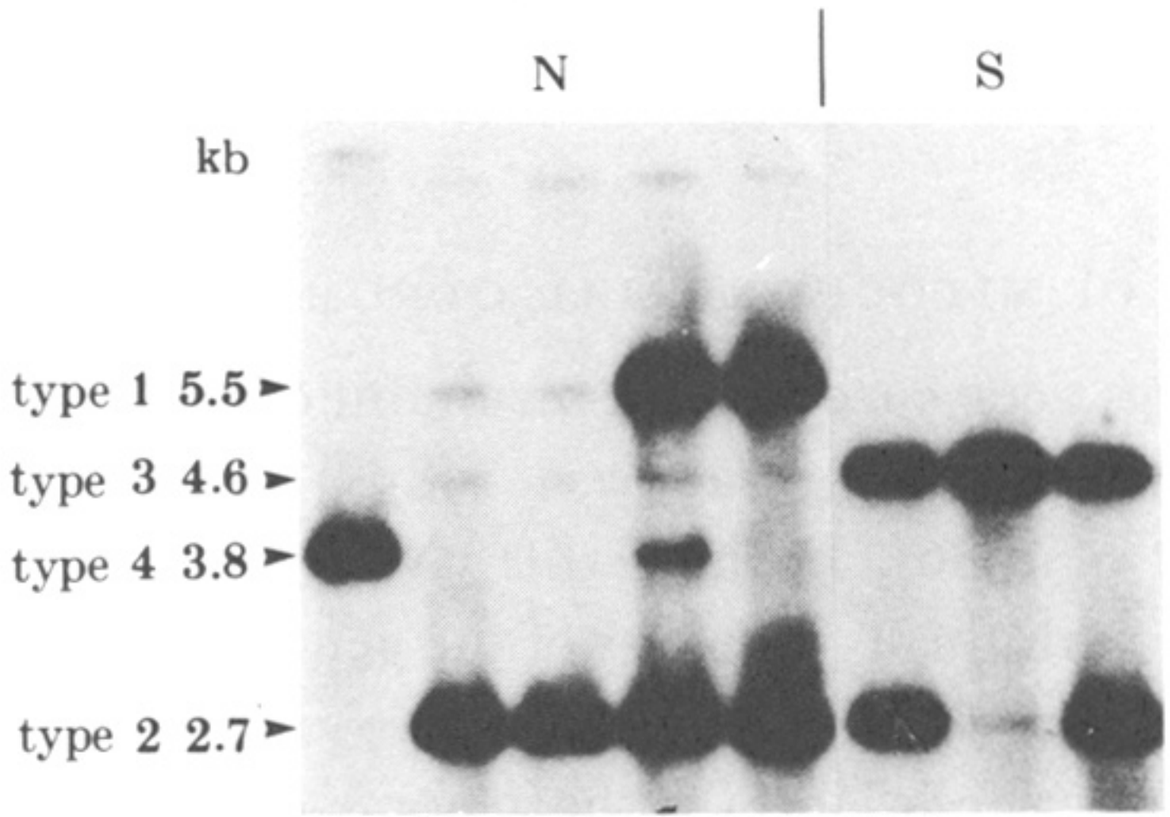


FIGURE 1. The location, structure and expression of the *cox*I gene in Milo and 9E cytoplasm sorghum. (*a*) Mitochondrial DNA from Milo and 9E cytoplasm was digested with *Eco*RI, fractionated by electrophoresis, stained with ethidium bromide and photographed. The mtDNA was transferred to nitrocellulose and hybridized with a ^{32}P-labelled clone containing an internal portion of the maize *cox*I gene. The radioautograph of the Southern blot shows that the *cox*I gene is located on a 4.3 kb *Eco*RI fragment in Milo and a 10.4 kb fragment in 9E. DNA sequence analysis of the *cox*I gene of Milo and 9E cytoplasm reveals that the Milo gene encodes a 530 amino acid polypeptide whereas the 9E gene encodes a 631 amino acid polypeptide. The diagram shows the location of the homologous *cox*I DNA sequences on the *Eco*RI fragments. The points of divergence between the two genes are indicated by arrows and non-homologous sequences are shaded. (*b*) Transcript analysis of *cox*I from Milo and 9E. mtRNA was fractionated by electrophoresis, transferred to nitrocellulose and probed with a ^{32}P-labelled M13 DNA probe containing a common portion of the *cox*I gene. The major Milo *cox*I transcript is 1.8 kb; in contrast, the major 9E *cox*I transcript is 2.2 kb. (*c*) Synthesis of the normal (Milo) and variant forms (9E) of cytochrome *c* oxidase subunit I in sorghum. [^{32}S]Methionine-labelled mitochondrial translation products were immunoprecipitated with an antibody raised against yeast cytochrome *c* oxidase subunit I, fractionated by SDS–polyacrylamide gel electrophoresis and radioautographed. In mitochondria from Milo cytoplasm a 38 kDa polypeptide was precipitated, whereas in 9E a 42 kDa polypeptide was identified.



GURE 3. Substoichiometric restriction endonuclease fragments containing the *atp*A gene in maize mitochondrial DNA. One-day exposures of autoradiographs of *Bam*H1-digested maize mtDNA (nuclear genotype CO192 × WJ, 5 μg per track) probed with BLSC1 (see figure 2). Lane 1, 181 cytoplasm; lane 2, SG cytoplasm; lane 3, OY cytoplasm: lane 4, 234 cytoplasm; lane 5, N cytoplasm; lane 6, D cytoplasm; lane 7, J cytoplasm; lane 8, CA cytoplasm. Lanes 1–5 are fertile 'N-like' cytoplasms; 6–8 are CMS-S cytoplasms. Faintly hybridizing bands can be seen in all tracks, some of which correspond in size to predominant bands found in other cytoplasmic types. All the N cytoplasms shown, except 181, appear to contain *atp*A types 1, 2 and 3 in detectable amounts. The cytoplasm 234 contains only a low level of type 2; the other two S cytoplasms shown have equal amounts of types 2 and 3. Similar results were obtained with a wide range of other nuclear–cytoplasmic combinations (table 1). Special care was taken to avoid cross-contamination of samples, by preparing mitochondria and mtDNA from fertile and male-sterile lines on different days. (Reprinted with permission from Small *et al.* (1987).)

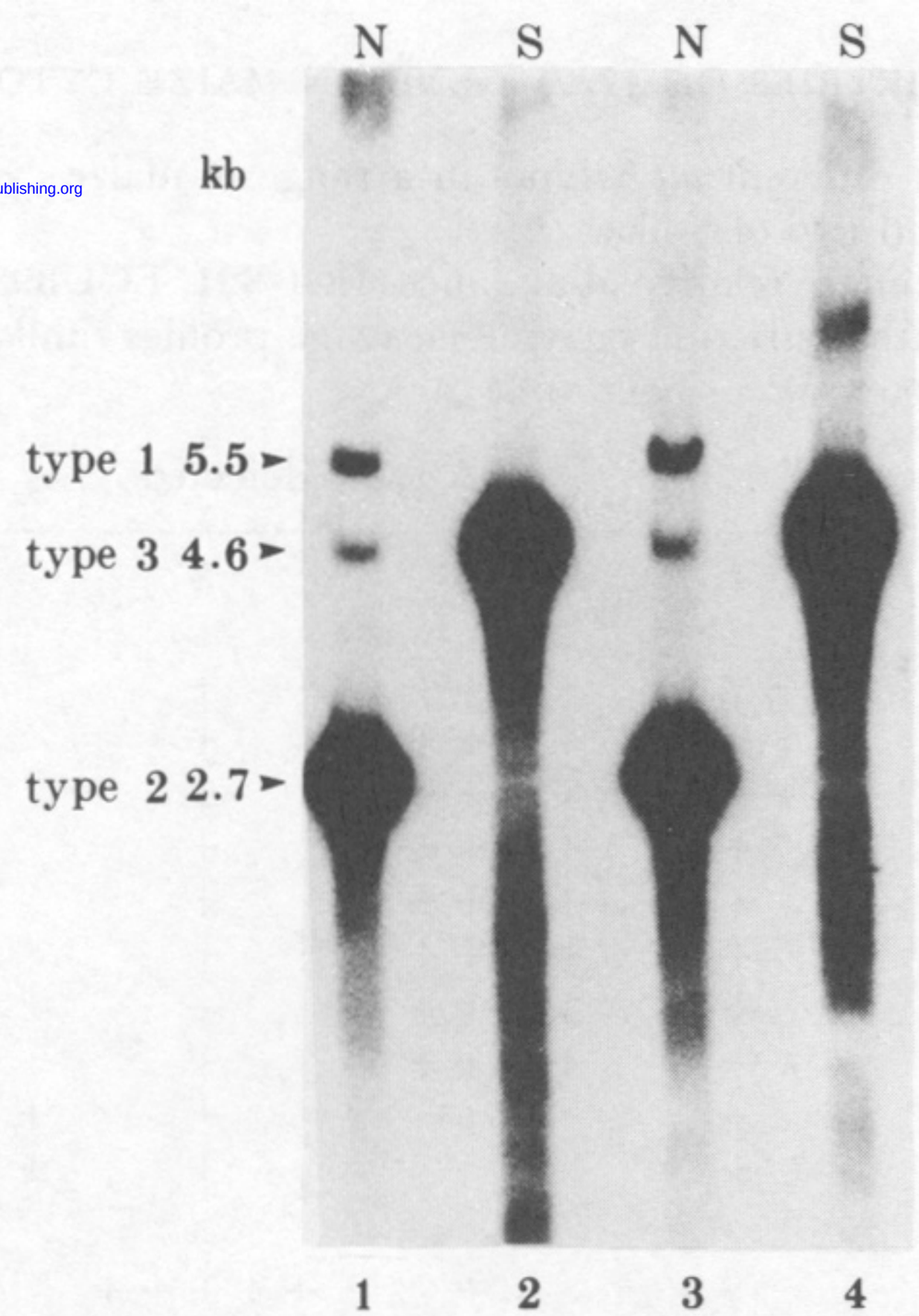


FIGURE 4. Substoichiometric *atp*A hybridizing fragments exist in the mitochondrial DNA from single plants. Three-day exposure of *Bam*H1-digested maize mtDNA probed with BLSC1 (see figure 2). Lanes 1 and 3, CO192 × WJ-SG; lanes 2 and 4, CO192 × WJ-J. Each lane contains mtDNA from a single immature cob. The SG mtDNA shows low levels of types 1 and 3 clearly, as predicted by the same experiment on pooled coleoptile mtDNA (figure 3, lane 2). The J mtDNA shows no clear faint bands, but the expected low levels of the type 2 fragment (figure 3, lane 7) may be obscured by the smear of degradation products from the abundant type 3 fragment. (Reprinted with permission from Small *et al.* (1987).)