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# Physical and gene organization of mitochondrial DNA in fertile and male sterile sunflower. CMS-associated alterations in structure and transcription of the *atp*A gene

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ABSTRACT

To study the molecular basis of cytoplasmic male sterility (CMS) in sunflower (Helianthus annuus), we compared the physical organization and transcriptional properties of mitochondrial DNAs (mtDNAs) from isonuclear fertile and CMS lines. Mapping studies revealed much greater similarity between the two mtDNAs than in previous comparisons of fertile and CMS lines from other plant species. The two sunflower mtDNAs 1) are nearly identical in size (300 kb and 305 kb); 2) contain the same 12 kb recombination repeat and associated tripartite structure; 3) have the same dispersed distribution of mitochondrial genes and chloroplast DNA-homologous sequences; 4) are greater than 99.9% identical in primary sequence; and 5) are colinear over a contiguous region encompassing 94% of the genome. Detectable alterations are limited to a 17 kb region of the genome and reflect as few as two mutations - a 12 kb inversion and a 5 kb insertion/deletion. One endpoint of both rearrangements is located within or near atpA, which is also the only mitochondrial gene whose transcripts differ between the fertile and CMS lines. Furthermore, a nuclear gene that restores fertility to CMS plants specifically influences the pattern of atpA transcripts. Rearrangements at the atpA locus may, therefore, be responsible for CMS in sunflower.

## INTRODUCTION

Cytoplasmic male sterility is a maternally inherited trait in which plants fail to produce normal pollen. CMS is used as a tool to generate F1 hybrid seed in such important crops as maize, rice, sorghum and sunflower. Indeed, in many crops several distinct male sterile cytoplasms are known (2). Alloplasmic male sterility, arising from interspecific or intergeneric crosses (1), is thought to be due to incompatibility between the nucleus and the cytoplasm (2). Several lines of evidence suggest that the CMS determinants reside on the mitochondrial genome (reviewed in refs. 2,3).

CMS was first reported in sunflower in 1969 by Leclercq (4), who discovered male sterile plants in the progeny of a cross between Helianthus annuus and Helianthus petiolaris. The subsequent discovery of fertility restoring lines has allowed use of this system in the commercial production of hybrid seed, thus obviating the expensive process of hand emasculation

(CMS in sunflower is reviewed in refs. 2,5). Little is known about the molecular determinants of CMS in sunflower. Early interest focused on a 1.45 kb mitochondrial plasmid found in several fertile sunflower lines but not in CMS lines (6,7). However, this correlation has recently broken down on the basis of a more comprehensive survey of sunflower CMS lines (8). As yet, there have been no detailed studies of the possible involvement of the main mitochondrial genome in CMS in sunflower.

To understand the molecular basis of CMS in sunflower we examined the physical organization and transcriptional properties of mitochondrial DNAs from a pair of isonuclear male sterile and male fertile sunflower lines. Our findings implicate DNA rearrangements located within or near the mitochondrial atpA locus as being candidate mutations for generating the male-sterile phenotype.

## MATERIALS AND METHODS

The four sunflower lines examined in this study are described in Table 1. Seed for Gloriasol was obtained from ISEA (Ancona, Italy). Seed for the three other lines were obtained from G. Seiler, USDA-ARS, Conservation & Product Research Laboratory, P.O. Drawer 10, Bushland, Texas 79012. MtDNA was purified from six day old etiolated shoots using the DNAase-I procedure (9). Methods used for restriction endonuclease analysis of sunflower mtDNA, agarose gel electrophoresis, bidirectional transfer from agarose gels to Zetabind filters, nick-translation of recombinant plasmids or gel-isolated fragments, and filter hybridization were as described (10). Mitochondrial gene clones used in gene mapping experiments are from Brassica, Oenothera and tobacco, and are described in (11).

Clone banks of mtDNA PstI and SalI fragments were constructed by ligating PstI digests or SalI digests of mtDNA with the appropriate digests of the vector pUC19 and transforming into E. coli strain JM105. Uncloned fragments used as hybridization probes were isolated from preparative low melting gels.

MtRNA was isolated from six day old etiolated shoots by the procedure of Stern and Newton (12), except that mitochondria were isolated from the 20%/30% interface of 20%/30%/52% sucrose step gradients. RNA was electrophoresed in 1.0% agarose gels containing 37% formaldehyde, 20 mM MOPS pH 7.0, 5 mM NaOAc, 1 mM EDTA and blotted to Zetabind filters in 20 x SSC. Hybridizations were performed at 60°C for 12 hrs in 1 M NaCl, 1% SDS, 5% Dextran Sulfate. Filters were washed extensively in 1 x SSC, 0.5% SDS at 65° prior to autoradiography.

TABLE 1. Genetic Constitution of Sunflower Lines

| Line | Cytoplasm | Restorer Gene | Fertility |
|---|---|---|---|
| CMS89 | petiolaris | rf1-rf1 | sterile |
| HA89 | annuus | rf1-rf1 | fertile |
| Gloriasol | petiolaris | Rf1-rf1 | fertile |
| RHA274 | petiolaris | Rf1-Rf1 | fertile |

CMS89 and HA89 are isonuclear lines described in (6). RHA274 is a restorer line. Gloriasol is a fertile commercial hybrid seed line based on CMS89.

## RESULTS

### Physical and Gene Organization of MtDNA in CMS Sunflower

To study the physical organization of sunflower mtDNA, we first constructed a clone bank containing 82% of the mitochondrial genome of the male sterile line CMS89 (see Table 1 for description of genetic stocks). To construct a restriction map of this genome, each mtDNA clone, as well as gel-isolated fragments covering uncloned regions of the genome, was nick-translated and hybridized to filters containing single and double digests of mtDNA with six restriction enzymes. The CMS89 mitochondrial genome is 305 kb in size and can be represented in the form of a single circular chromosome containing its entire sequence complexity (Fig. 1).

Only a single family of repeats larger than a few hundred base pairs was detected by the mapping hybridization experiments. This family consists of two, directly oriented 12 kb repeat elements separated by 52 kb and 229 kb regions of single copy DNA (Fig. 1). All six of the mapped enzymes cut within the 12 kb repeat, preventing detection of the products of possible recombination between the two repeat copies. Since high frequency recombination is invariably associated with large repeats in all plant mtDNAs studied to date (13-18), we screened with several additional enzymes and found two, NruI and BssHII, that do not cut within the 12 kb repeat. A cloned PstI fragment of 10.0 kb, which contains part of the repeat (Fig. 1) and lacks sites for both NruI and BssHII, hybridized to four large NruI-BssHII fragments (Fig. 2). Two of the four repeat-containing NruI fragments are located on the master circle shown in Fig. 1; these two interconvert with the other two via recombination across the repeats (Fig. 3). The direct orientation of the repeats on the master circle predicts two smaller circles, of 241 kb and 64 kb, as the outcome of this recombination (Fig. 3). The four repeat-

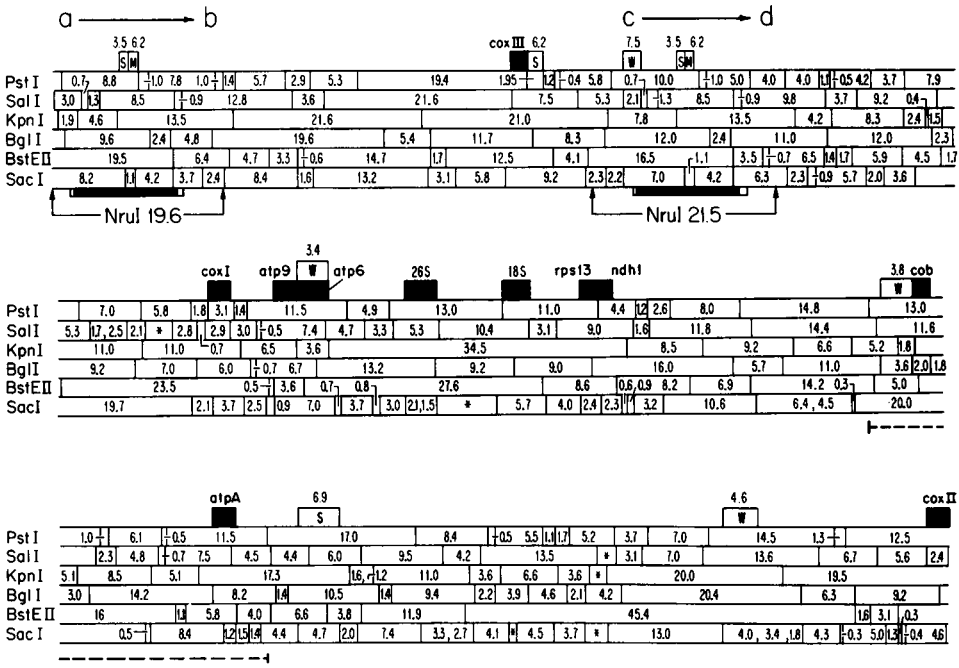


FIG. 1. Restriction map of the master chromosome of mtDNA from sterile sunflower (CMS89). The circular map is shown linearized at a SalI site separating fragments of 3.0 and 2.4 kb. Arrows indicate the approximate positions and relative orientation of the two copies of a 12 kb repeat. Letters flanking the arrows denote single copy sequences flanking the repeats. The solid bars below the repeat maps indicate the minimum extent of the repeat, as defined by common SalI sites, and the open extensions of the bars indicates its maximum possible size, as defined by unique KpnI and PstI sites. Sizes of the two repeat-containing NruI fragments located on the master chromosome are shown below the repeats (cf. Fig. 3). Closed boxes indicate mitochondrial genes and open boxes indicate cpDNA-homologous sequences. The letters in the open boxes denote a strong (s), medium (m), or weak (w) intensity of hybridization with cpDNA, while the numbers by the boxes give the sizes of the cross-hybridizing cpDNA fragments from lettuce (see Fig. 4). The dashed horizontal line indicates the rearranged region diagrammed in Fig. 6. Asterisks indicate unmapped fragments.

containing NruI-BssHII fragments are present in roughly equal levels relative to each other and at about half the level of unimolar fragments (Fig. 2). This indicates that the three mtDNA circles diagrammed in Fig. 3 are present in roughly equal amounts.

Cloned genes from Brassica, Oenothera and tobacco were hybridized to the same Southern filters used for restriction mapping in order to locate known mitochondrial genes on the sunflower physical map. Probes for all genes

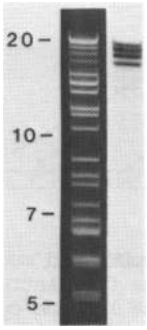


FIG. 2. Hybridization analysis of the 12 kb recombination repeat. Purified sunflower mtDNA from CMS89 was doubly digested with NruI and BssHII, electrophoresed in a 0.7% agarose gel (left panel), transferred to a Zetabind filter, and hybridized with nick-translated p871 (right panel). This pUC19 clone has a PstI insert of 10.0 kb that contains 6.5 kb of the recombination repeat (Fig. 1).

tested, five of which (coxI, coxII, coxIII, ndhI, cob) encode respiratory chain proteins, three of which (atp9, atp6, atpA) encode subunits of the ATPase complex (atp9, atp6, atpA), one (rps13) which encodes ribosomal protein S13, and two of which encode the 26S and 18S rRNAs, hybridized to a single region of the mitochondrial genome. These results suggest that each mitochondrial gene is present in one copy in the sunflower genome (Fig. 1). As in other plant mtDNAs (11,16,19), the genes are widely dispersed throughout the genome and are probably unlinked transcriptionally. Although the 18S and 26S rRNA genes map more closely (about 7 kb apart) in sunflower than in other plants (11,16,19,20), this probably reflects chance rearrangement and is unlikely to be of any functional significance.

Numerous sequence homologies between chloroplast DNA (cpDNA) and mtDNA have previously been reported in flowering plants (16,21-23). In order to determine whether inter-organellar sequence exchange has also occurred in sunflower, we hybridized cloned fragments covering 95% of the lettuce chloroplast genome (24) to Southern filters containing adjacent lanes of purified

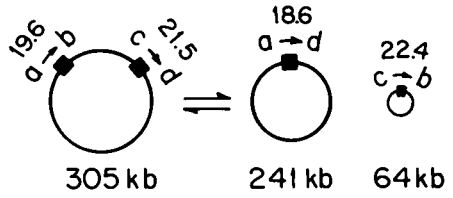


FIG. 3. Tripartite organization of sunflower mtDNA from CMS89. Filled boxes indicate the position and arrows the relative orientation of a 12 kb recombination repeat. Letters denote single copy sequences flanking the two repeat copies. Numbers indicate the sizes of four NruI fragments, each of which contains the entire repeat. Note that the four repeat-containing fragments shown in Fig. 2 are NruI-BssHII fragments and hence are smaller (17-20 kb) than the four NruI fragments (18.6-22.4 kb) from which they are derived.

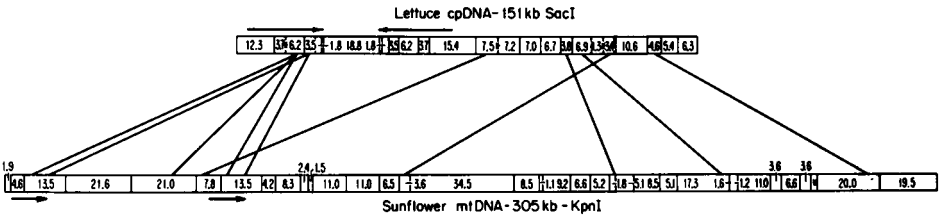


FIG. 4. Organization of homologous sequences in lettuce cpDNA and sunflower mtDNA (CMS89). Angled lines connect cloned cpDNA fragments from lettuce and the sunflower mtDNA fragments to which they hybridize. The lettuce cpDNA restriction map and clone bank are described in (24). All of the lettuce sites shown are SacI sites, except for the three indicated with asterisks. These are, from left to right, sites for PstI, HindIII, and SalI. The asterisk in the sunflower mtDNA map indicates a region unmapped for KpnI.

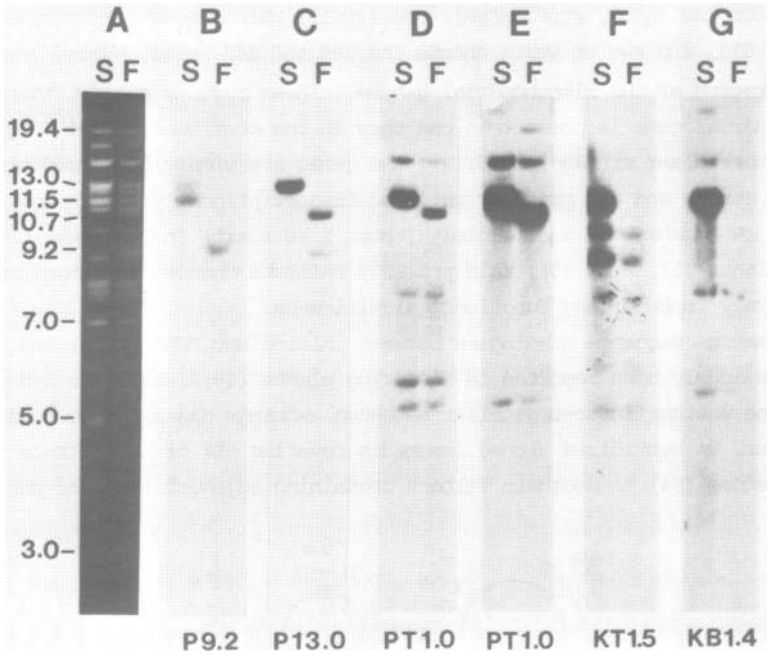


FIG. 5. Hybridization analysis of rearranged regions of mtDNAs from sterile (S; CMS89) and fertile (F; HA89) sunflower. DNAs were digested with PstI, electrophoresed in a 0.7% agarose gel (panel A), transferred to a Zetabind filter, and hybridized with the indicated fragments (panels B-G). P9.2 and P13.0 are PstI fragments from fertile and sterile mtDNAs, respectively, cloned into pUC19. The left PT1.0 probe is a 1.0 kb PstI-BstEII fragment isolated from a low melting gel-digest of clone P10.7 (fertile mtDNA). The rightmost three probes are 1.0 kb PstI-BstEII, 1.5 kb KpnI-BstEII, and 1.4 kb KpnI-BglI fragments isolated from a digest of clone P11.5 (sterile mtDNA). See Fig. 6 for the map locations of these six probe fragments.

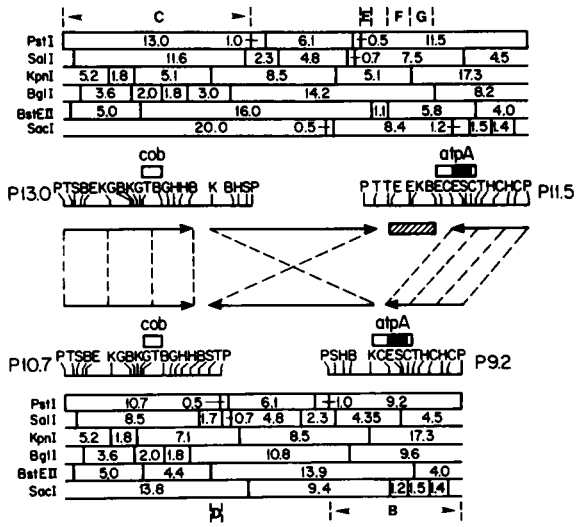


FIG. 6. Rearrangements in sunflower mtDNAs. Restriction maps are shown for the only altered region in mtDNAs from sterile (top; CMS89) and fertile (bottom; HA89) sunflower. Restriction sites shown: PstI (P), SacI (C), SalI (S), KpnI (K), BglI (B), BstEII (T), BglII (G), HindIII (H), EcoRI (E). Arrows indicate the position and relative orientation of blocks of sequences whose arrangement has been conserved between the two genomes. Crossing lines connect homologous sequence blocks. The striped box indicates sequences present only in sterile mtDNA (cf. Figs. 5F and 5G). Closed boxes indicate the minimum extent of the sunflower sequences that hybridize with heterologous gene probes, the open boxes indicate their maximum sizes. Letters (B-G) above and below the maps indicate the fragments used as hybridization probes in Fig. 5.

mtDNA and cpDNA from sunflower. Lettuce was chosen because of the availability of cpDNA clones (24), because it belongs to the same family (Asteraceae) as sunflower, and because its chloroplast genome is colinear with that of sunflower (25). Eight regions of the sunflower mitochondrial genome hybridized significantly, albeit with varying intensities, to lettuce cpDNA (Fig. 1). The linear arrangement of homologous sequences is significantly different in sunflower mitochondrial and chloroplast DNAs (Fig. 4; lettuce and sunflower cpDNAs are colinear; ref. 25)

Comparative Organization of MtDNA in Fertile and Male Sterile Sunflower

As a first step in identifying alterations potentially involved in CMS, we compared mtDNAs from the four sunflower lines described in Table 1. MtDNAs from each of the three lines containing H. petiolaris cytoplasm were indistinguishable with all restriction enzymes tested (data not shown). In

contrast, differences were observed in the mtDNAs of two isonuclear lines that are either male sterile (CMS89, containing cytoplasm derived from H. petiolaris) or male fertile (HA89, containing cytoplasm from H. annuus). These two mtDNAs have nearly identical restriction patterns with each of the six enzymes used to map the CMS89 genome (Fig. 5A and data not shown). Two of the enzymes we used, SalI and BglI, were also used by Leroy et al. (6) to compare the same two isonuclear sunflower lines; their published mtDNA restriction patterns appear identical to ours.

All but two cloned or gel-isolated fragments spanning the sunflower mitochondrial genome hybridized to fragments of identical size in the sterile and fertile mtDNAs (data not shown). The exceptions are cloned PstI fragments of 13.0 and 11.5 kb from CMS89 mtDNA, both of which hybridized to PstI fragments of 10.7 and 9.2 kb from HA89 mtDNA (Figs. 5B, 5C and 6). Large regions of restriction site identity (Fig. 6) reveal that sequences in P13.0 (sterile genome) are split between P10.7 and P9.2 (fertile genome), and that part of P11.5 (sterile) is contained on P9.2 (fertile). In addition, a shorter region of homology, not evident in the restriction maps, was observed by hybridization between one end of P11.5 (sterile) and an end of P10.7 (fertile) (Figs. 5D, 5E and 6). This pattern of rearrangement is most easily explained by an inversion of the central 12 kb segment of Fig. 6, with the inversion endpoints located within the four PstI fragments. In addition, at least one insertion/deletion must have occurred in the inversion region, as evidenced by the failure of two adjacent fragments from P11.5 (sterile) to hybridize to any significant extent to fertile mtDNA (Figs. 5F, 5G and 6). Thus, the sterile genome contains a segment approaching 5 kb in size that is absent from the fertile genome and which is located at one endpoint of the inversion.

Ignoring restriction sites located in the rearranged areas, 280 sites can be compared directly in the sterile and fertile sunflower mitochondrial genomes. Each of these 280 sites is conserved between the two genomes. Therefore, the two mtDNAs are at least 99.9% identical in primary sequence (no differences in 280 x 6 = 1680 bp compared).

CMS-Associated Transcriptional Alterations

Northern blot analyses were performed to identify any alterations in mitochondrial transcript patterns that might be associated with CMS. Cloned fragments covering 82% of the CMS89 mitochondrial genome and also heterologous clones for each of the genes mapped in Fig. 1 were hybridized to Northern blots containing mtRNA extracted from each of the four lines

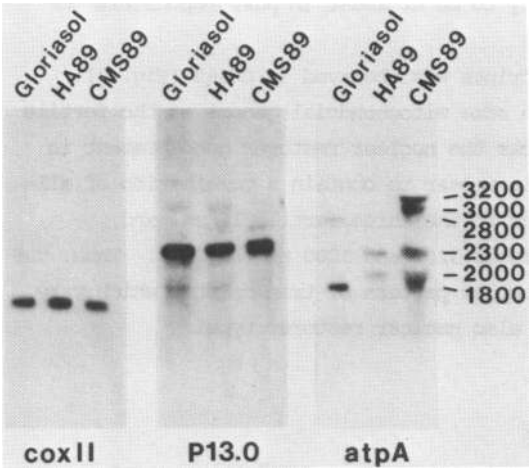


FIG. 7. CMS-associated transcriptional alterations of the atpA gene. MtRNAs from the indicated lines were electrophoresed on a 1.0% agarose-formaldehyde gel, transferred to a Zetabind filter, and hybridized with a cloned 4.8 PstI fragment containing the coxII gene from Brassica campestris (coxII; ref. 11), a cloned 13.0 kb PstI fragment from CMS89 mtDNA (P13.0), and a cloned 1.2 kb SacI fragment from CMS89 mtDNA (atpA). Transcript sizes are given in nucleotides.

described in Table 1. These include the isonuclear fertile and sterile lines (HA89 and CMS89) whose mtDNAs are compared in Fig. 6, and also fertile lines containing one (Gloriasol) and two (RHA274) copies of a nuclear gene that restores fertility to plants carrying the H. petiolaris cytoplasm. With one exception, none of the mitochondrial clones identified major alterations in the pattern of mitochondrial transcripts among the four lines compared. For example, transcript patterns are essentially identical for both coxII and P13.0 (Fig. 7; the transcript patterns for RHA274 were in all cases identical to those shown for Gloriasol). This PstI fragment contains one of the two rearranged regions of mtDNA and also at least one gene, cob (encoding cytochrome b; Fig. 6).

Major differences in mitochondrial transcripts were observed only with P11.5 (data not shown). This CMS89 fragment contains both a 5 kb insertion and also one inversion endpoint relative to the HA89 genome (Fig. 6). The mtDNA sequences specifying these altered transcripts were localized by hybridizing both gel-isolated and cloned fragments spanning P11.5 to Northern blots. One of these fragments, a 1.2 kb SacI fragment containing most of atpA (Fig. 6), gave the same pattern of hybridization to Northern blots as the parent clone, P11.5. The atpA transcripts are completely different in size in fertile lines containing the H. annuus mitochondrial genome (HA89; atpA transcripts are 2300 and 2000 nt in size) and the H. petiolaris genome (Gloriasol and RHA274; 2800 and 1800 nt transcripts). This suggests that the rearrangements located within or nearby atpA (Fig. 6) that distinguish the

two mitochondrial genomes are likely to be at least in part reponsible for these transcript differences.

A third pattern of atpA transcripts was observed in CMS89 (Fig. 7). This male-sterile line contains the same mitochondrial genome as the fertile lines Gloriasol and RHA274, but lacks the nuclear restorer gene present in these two lines. CMS89 mitochondria appear to contain a combination of all four of the atpA transcripts present in the three fertile lines, and, additionally, two larger transcripts of 3200 and 3000 nt (Fig. 7). Thus, the atpA locus is characterized by a complex pattern of transcripts, which vary in response to both mtDNA type and also nuclear restorer type.

## DISCUSSION

### General Features of Sunflower mtDNA

The general organization of mtDNA sequences in sunflower appears reminiscient of that in the typical flowering plant in four major respects. First, the sunflower mitochondrial genome is large, 300-305 kb, in comparison to all studied animal, fungal, and protist mtDNAs (26). At the same time, its size is unexceptional, indeed is near the small end of the size spectrum, for mtDNAs of flowering plants (27-29) and other major groups of vascular plants (J. Palmer, D. Soltis and P. Soltis, unpublished data).

Second, the multicircular organization of mtDNA in sunflower is a general and distinguishing feature of plant mtDNAs. The presence of a single pair of large, directly oriented repeats engaged in intramolecular recombination to generate a tripartite genome is characteristic not only of sunflower, but also four species of Brassica (13,14; J. Palmer and L. Herbon, unpublished data), radish (14,15), and spinach (16). A larger number of recombination repeats and hence a more complex multipartite organization is found in maize (17), wheat (18), and sugar beet (D. Lonsdale, personal communication). The only deviant from this theme of multicircular plant mtDNAs is white mustard (Brassica hirta), whose mtDNA lacks any large recombining repeats and exists as a single circle (30).

Third, the 11 mitochondrial genes mapped in this study are widely scattered throughout the sunflower genome and appear to be unlinked transcriptionally. Furthermore, the order of genes in sunflower differs markedly from that in maize (19), spinach (16), and turnip (11). Therefore, as noted in these earlier studies, the order of genes in plant mtDNAs is likely to have little effect on their proper function and therefore can and does vary widely from species to species.

Finally, we have shown that sunflower mtDNA, like those of maize (21-23), spinach (16,21), and pea and mung bean (21), contains a number of sequences with strong homology to cpDNA. According to the logic outlined earlier (21), it is likely that most or all of the cpDNA-homologous mtDNA sequences in sunflower represent duplicate copies of sequences that normally function in the chloroplast. Based on recent studies in Brassica (11), it is unlikely that these cpDNA-derived sequences are expressed in sunflower mtDNA. The scrambled arrangement of homologous sequences between mitochondrial and chloroplast DNAs of the same species observed here for sunflower was also reported in spinach (16). This implies independent movements of cpDNA into mtDNA and/or extensive mtDNA rearrangement following cpDNA integration.

Mitochondrial Alterations Associated with CMS in Sunflower

A growing body of evidence has established a correlation between defined mtDNA rearrangements, usually involving genes showing specific transcriptional alterations, and CMS in plants. The best studied case involves the Texas (T) male-sterile cytoplasm of maize, where multiple recombination events have created a novel mitochondrial gene (31) whose product appears to be related to the male-sterile phenotype (32-36). In addition, mitochondrial gene rearrangements and transcriptional alterations are associated with CMS in sorghum (37,38), petunia (39), and radish (15).

The situation described here for sunflower CMS conforms to the above pattern in several respects, but with one notable difference. While defined rearrangements at or near the atpA locus correlate with CMS-associated differences in its pattern of transcripts, the overall level of mtDNA divergence, in particular rearrangement, between CMS and fertile genomes is considerably less in sunflower than in other plants. Restriction patterns of CMS and fertile lines from the same species of plants have been shown to differ considerably in a number of plants (reviewed in 2). When examined further, these pattern differences have been shown to result from numerous rearrangements, only some of which are likely to be involved in CMS (15,31, 37,38,40). For example, a CMS mtDNA in radish differs from its fertile counterpart by a minimum of 11 large inversions and insertions/deletions, only some of which are even candidates for being causally related to the CMS phenotype (15). In sunflower, however, only two major rearrangements, a 12 kb inversion and a 5 kb insertion/deletion, distinguish the CMS and fertile mtDNAs, yet both of these mutations may be involved in CMS.

Plant mtDNAs are mostly noncoding (11) and hence can tolerate extensive rearrangment, most of which is unlikely to affect gene function and result in

mutations such as those giving rise to CMS. The somewhat aberrant situation described here for sunflower may simply reflect the chance probability that the first one or two rearrangements occurring following the divergence of two mitochondrial lineages from a common sunflower ancestor happen to have affected the same normal mitochondrial gene.

The pattern of atpA transcripts in sunflower depends on both mitochondrial and nuclear constitution. This suggests a complex interaction between mtDNA sequences and/or transcripts and nuclear gene products in determining the pathway of transcription and RNA processing at this locus. The fact that the CMS line appears to contain each of the pair of atpA transcripts found in fertile lines carrying either mtDNA type raises the possibility that CMS mitochondria may produce normal atpA polypeptide. This would not, however, preclude the alterations seen at the atpA locus being related to CMS. For example, the two additional, larger transcripts that are unique to the CMS line could specify altered forms of the atpA polypeptide whose interaction with the ATP synthase might be harmful. Alternatively, these transcripts could produce completely unrelated polypeptides of a harmful nature. To evaluate these possibilities and determine the exact involvement of the rearranged atpA locus in sunflower CMS will require a detailed DNA sequence analysis and the characterization of transcripts and polypeptides from this region.

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