Cell-Specific Regulation of Gene Expression in Mitochondria during Anther Development in Sunflower

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Mitochondrial gene expression was characterized during meiosis in sunflower anthers. In situ hybridization experiments showed that there was a marked accumulation of four mitochondrial gene transcripts (atpA, atp9, cob, and rrn26) in young meiotic cells. This pattern of transcript accumulation was only detected for mitochondrial genes and not for transcripts of two nuclear genes (atpB and ANT) encoding mitochondrial proteins or another nuclear gene transcript (25S rRNA). Immunolocalization studies showed that the pattern of accumulation of the protein product of the atpA gene, the F_1-ATP synthase α subunit, reflects that of the transcript. The expression of the novel mitochondrial orf522, which is associated with the cytoplasmic male-sterile (CMS) phenotype, was also studied by in situ hybridization. The orf522 transcripts were reduced in abundance in meiotic cells in the presence of fertility restorer genes. These results suggest that mitochondrial gene expression is regulated in a cell-specific fashion in developing anthers and that the restorer gene(s) may act cell specifically.

INTRODUCTION

In higher plants, mitochondrial function must be developmentally regulated to meet the changing respiratory demands of different tissues. In some tissues, high respiratory demand may be met by active mitochondrial biogenesis to produce sufficient numbers of mitochondria and therefore energy in the form of ATP (Warmke and Lee, 1978). Active biogenesis of mitochondria has been shown to occur in the root apical meristem of Arabidopsis and other species where mitochondrial numbers increase from 65 to 200 per cell and respiration rates are high (Yemm, 1965; Kuroiwa et al., 1992; Fujie et al., 1993). Mitochondrial biogenesis has also been reported to occur in meiotic and tapetal cells during early anther development in maize where numbers of mitochondria increase 20- to 40-fold (Lee and Warmke, 1979). This developmental control of mitochondrial biogenesis may be partly regulated at the level of mitochondrial gene expression, as has been suggested by experiments where mitochondria isolated from different tissues were found to synthesize different profiles of proteins (Newton and Walbot, 1985; Monéger et al., 1994). Finnegan and Brown (1990) proposed that this developmental regulation of mitochondrial gene expression occurs at the transcriptional and post-transcriptional levels.

In many biological systems, mutations that perturb the normal developmental pathway can be used to define the important processes occurring in that pathway. Unfortunately, our understanding of how mitochondrial function and gene expression are developmentally regulated in higher plants has been limited because only two mitochondrial mutations have been associated with nonlethal phenotypes: nonchromosomal stripe (NCS) in maize and cytoplasmic male sterility (CMS) in a range of higher plants (Newton, 1988; Hanson, 1991). Characterization of leaf development in NCS mutants has indicated that mitochondrial function is necessary for normal development of chloroplasts and acquisition of photosynthetic competence (Newton and Coe, 1986; Rousell et al., 1991). Similarly, investigation of CMS has shown that mitochondrial function is essential during pollen formation and therefore sexual reproduction in higher plants (Hanson, 1991).

CMS is a maternally inherited phenotype characterized by an inability to produce functional pollen (Laser and Lestern, 1972). Interestingly, it only disrupts pollen production; vegetative development and female fertility are apparently unaffected. In nature, CMS can arise spontaneously, where it may be maintained by an increase in female fertility (Couvet et al., 1990), but for commercial purposes it is often induced by interspecific and intraspecific crosses that introduce a nucleus into a foreign cytoplasm. In many species, the CMS phenotype is associated with mutations in the mitochondrial genome and can be suppressed by the action of nuclear-encoded fertility restorer genes (Hanson, 1991). Thus, the study of fertile, CMS, and restored fertile hybrid plants provides a good experimental system for the investigation of the developmental regulation of mitochondrial gene expression by nuclear genes.

A great deal of our present understanding of the molecular and biochemical basis of CMS comes from studies of male sterility in maize (CMS-T) and petunia. In these systems,

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polypeptide products of novel open reading frames (ORFs) have been found to be associated with CMS (T-*urf* 13 and the *pcf* gene, respectively) and are present at reduced levels in the restored hybrid plants (Dewey et al., 1987; Nivison and Hanson, 1989). However, our understanding of how these novel polypeptides might cause pollen forming cells to abort is still limited. It is also obvious from cytological studies of different types of CMS within the same species that the mechanisms of pollen abortion are diverse (Laveau et al., 1989). Therefore, analysis of different CMS systems will extend our knowledge of the important cytoplasmic processes occurring during pollen production.

Recently, molecular characterization of CMS in sunflower has shown that as in the maize CMS-T and petunia systems, a polypeptide that is the product of a novel ORF is associated with pollen cell abortion. The abundance of this protein is reduced specifically in male florets upon restoration of fertility (Monéger et al., 1994). CMS in sunflower provides an attractive system for further analysis because it is associated with the expression of a single novel ORF, and the timing of meiotic cell abortion has been characterized (Horner, 1977; Laveau et al., 1989; Laver et al., 1991). We have undertaken a cytological investigation of anther development in sunflower florets, coupled with further molecular analysis of CMS in sunflower, to characterize the nature of mitochondrial gene expression and function during anther development and the cause of meiotic cell (meiocyte) abortion in male sterile sunflowers.

CMS in sunflower was first identified by Leclercq (1969) in the progeny of an intraspecific cross between *Helianthus annuus* and *H. petiolaris* (PET1 type). Leclercq (1984) later identified two dominant restorer genes of the CMS phenotype in sunflower (Rf1 and Rf2) and postulated from restoration genetics that Rf2 is dominant in most sterile cytoplasms. In sunflower, the CMS phenotype is associated with a mutation in the mitochondrial genome of the sterile line that is thought to have been created by inversion/insertion events involving recombination across a small repeat (Siculella and Palmer, 1988; Köhler et al., 1991; Laver et al., 1991). The insertion of a novel fragment of DNA has led to the creation of a novel open reading frame (*orf522*) downstream of the *atpA* gene (Köhler et al., 1991; Laver et al., 1991). In sterile and restored hybrid lines, *orf522* is cotranscribed with *atpA* on a 3-kb transcript that is translated to produce a novel 15-kD polypeptide (ORF522), in addition to the α subunit of the mitochondrial F₁-ATP synthase, encoded by *atpA* (Monéger et al., 1994). The restorer gene(s) acts specifically in male florets to reduce the abundance of the *atpA-orf522* cotranscript and therefore the 15-kD ORF522 polypeptide, suggesting that the expression of *orf522* is probably causally related to the CMS phenotype (Monéger et al., 1994).

In this study, we have addressed the problem of why the expression of *orf522* only affects microsporogenesis by observing the expression of mitochondrial genes during anther development in sunflower. We showed that the *atpA* transcript and the α subunit protein are particularly abundant in young meiocytes of all lines, implying a cell-specific regulation of

expression of the mitochondrial genome. Our data indicated that this pattern of expression is not a general phenomenon but is only observed for mitochondrially encoded genes. Finally, we present evidence that suggests that the restorer gene(s) may act cell specifically in meiocytes of the restored hybrid line to reduce the abundance of the *atpA-orf522* transcript. These results provide compelling evidence for cell-specific regulation of the expression of the mitochondrial genome during anther development and demonstrate that nuclear genes can modify this expression.

RESULTS

Meiosis in Sunflower Anthers

To investigate the importance of mitochondrial gene expression during the early stages of anther development and its significance for the CMS phenotype, RNA in situ hybridization analysis was used to investigate the spatial distribution and abundance of mitochondrial gene transcripts. This first required a characterization of male meiosis in sunflower anthers.

Sunflower (*H. annuus*) is a member of the Compositae family, which is characterized by a single terminal inflorescence consisting of 700 to 3000 individual disc flowers or florets (Knowles, 1978). Sunflower florets are hermaphroditic, as they are composed of both male and female organs. The anthers are present in the upper part of the floret ("male floret," represented by shaded ellipses in Figure 1C; the ovaries are present in the lower part of the floret ("female floret," represented by open circles in Figure 1C). Sunflower florets exhibit protandry, that is, the male part of the flower (anthers) matures before the female. Therefore, under normal conditions, male meiosis occurs before the sunflower inflorescence opens when the inflorescence bud is between 2.5 and 4.5 cm in diameter (Figure 1A). Within the inflorescence, the florets develop sequentially in whorls from the periphery to the center at a rate of about one to four whorls a day (Hernández and Green, 1993). This sequential maturation of disc flowers is demonstrated in the open inflorescence shown in Figure 1B.

For the purposes of this study, six stages of meiosis were identified and investigated, beginning with the formation of the pollen mother cells prior to meiocyte abortion in sterile anthers and ending with microspore release from the tetrad in the fertile and restored hybrid lines (Figures 1C and 2). These phases of meiosis were characterized by using histological and 4',6-diamidino-2-phenylindole (DAPI) stains on fixed and embedded florets. It was found that the meiotic stage is correlated with the length of the male part of the floret bud (male floret), as shown in Figure 1C. These stages of sunflower microsporogenesis correspond to those described by Horner (1977).

The development of anthers in the fertile and sterile lines was compared at the six stages of meiosis described above. Development of anthers in florets of the sterile line appears
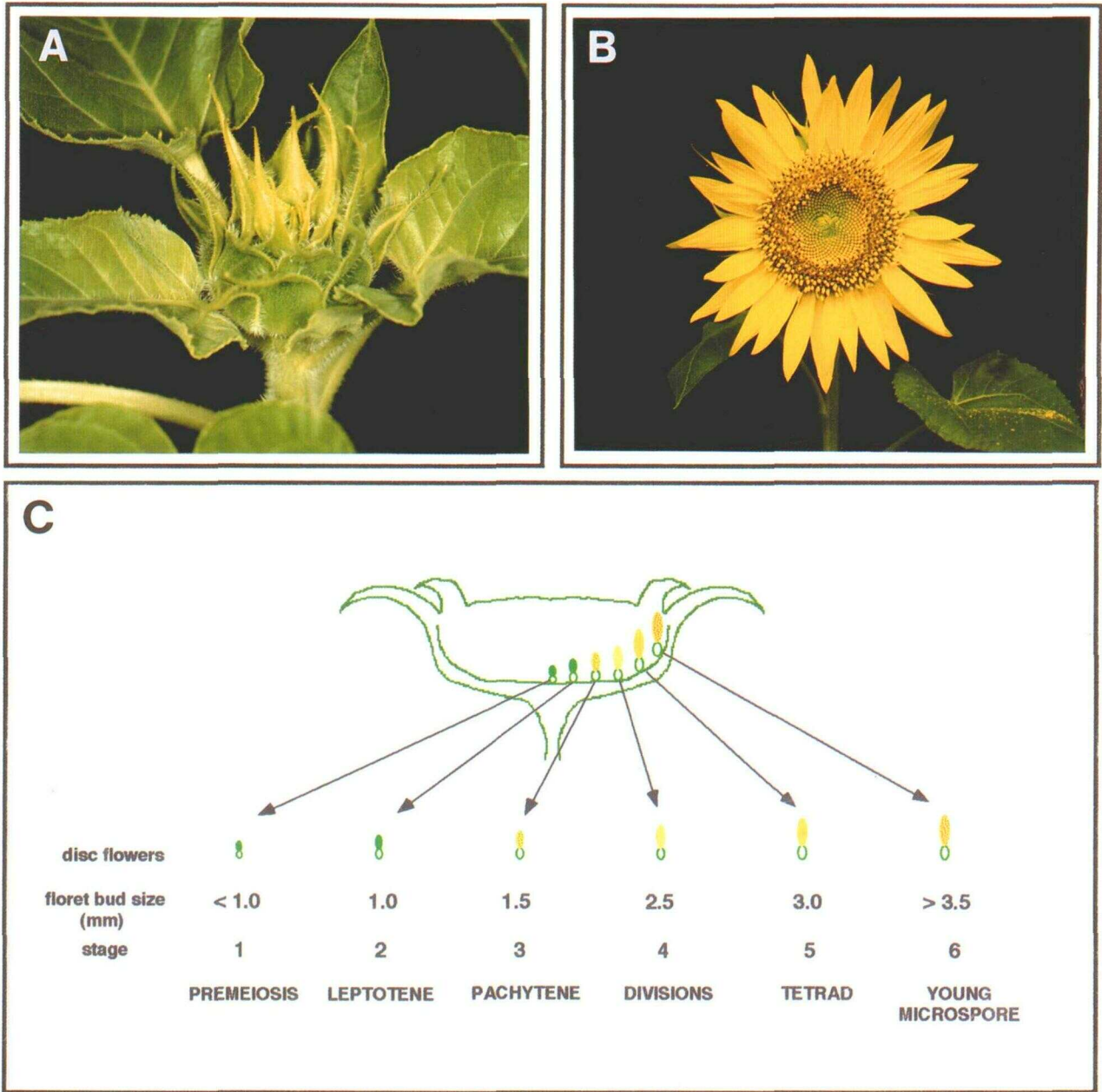
Figure 1. Sunflower Inflorescence Development.

(A) Male meiosis occurs in disc flower (floret) anthers prior to the opening of the inflorescence when the flower head is 2.5 to 4.5 cm in diameter (at 40 days after germination).
(B) Florets develop sequentially in whorls from the periphery of the inflorescence to the center. Shown here are mature anthers in the open fertile flower (50 days old).
(C) Diagram illustrating the six stages of meiosis characterized in this study (corresponding to those identified by Horner, 1977). During these stages, abortion of meiocytes in anthers of the male-sterile line occurs (see Figure 2). The upper part of the floret (shaded ovals) contains the anthers and can be described as the male floret. The lower part of the floret (open circles) represents the female floret. The length of the male floret bud increases in a linear fashion during development and can therefore be correlated with meiotic stage.
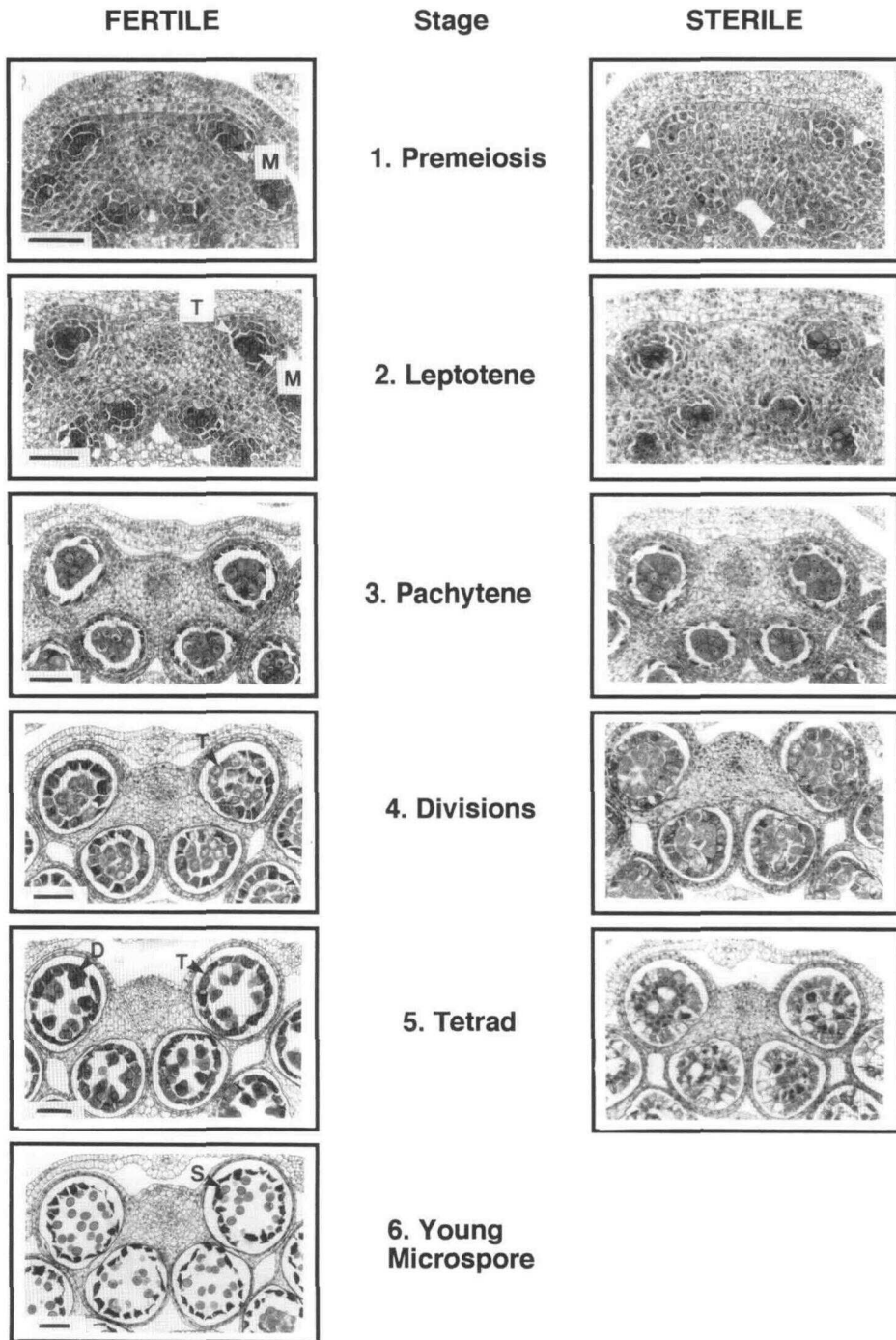
FERTILE	Stage	STERILE
	1. Premeiosis	
	2. Leptotene	
	3. Pachytene	
	4. Divisions	
	5. Tetrad	
	6. Young Microspore	

Figure 2. Anther Development in Florets of the Fertile and Sterile Sunflower Lines.

Six stages of anther development in the fertile line and the corresponding development of anthers in the sterile line showing meiocyte cell abortion were identified by histological staining. Leptotene is the stage prior to meiocyte abortion that is used subsequently to compare all lines. Pachytene is the first stage at which cytological abnormalities become apparent in the meiocytes of anthers of the sterile line (as observed by electron micros-copy). The tapetum starts to degenerate during late pachytene in the anthers of the sterile line. M, meiocyte cells; T, tapetal cells; D, tetrads; S, microspores. Bars = 50 μm.

completely normal during the premeiosis and leptotene stages (Figure 2), but by pachytene (stage 3), abnormalities are detected in the meiocyte cells of the sterile line when observed by electron microscopy (Laveau et al., 1989; C.J. Smart, data not shown). By stage 4 (divisions), the tapetum shows abnormal development and has completely degenerated by the tetrad stage in the sterile line (Figure 2). Therefore, for the purposes of this study, comparisons of mitochondrial gene expression in fertile, sterile, and restored hybrid lines were made between male florets or anthers at the leptotene stage (stage 2) prior to meiocyte abortion in the sterile line.

Expression at the *atpA* Locus and the CMS Phenotype

Previously, we showed that the steady state levels of the chimeric *atpA–orf522* cotranscript and the sterile specific ORF522 protein are reduced in male florets from the restored hybrid line, indicating that the expression of *orf522* is correlated with the CMS phenotype (Monéger et al., 1994). Transcripts corresponding to the *orf873* (Figure 3A; previously called ORFb, Laver et al., 1991) were not detected in RNA from different tissues by RNA gel blot analysis (data not shown), suggesting that the disruption of *orf873* is probably not causally related to the CMS phenotype.

To further check that the reduction in the abundance of the 3-kb *atpA–orf522* transcript is specific to male floret tissue, an *atpA* gene–specific probe (Figure 3A) was hybridized to total RNA extracted from green cotyledons, etiolated cotyledons, root tips, and leptotene stage male florets of all lines in an RNA gel blot analysis. The results are shown in Figure 3B; in the fertile line, the *atpA* gene is transcribed as a 1.9-kb transcript, whereas sterile and restored hybrid lines contain an additional 3-kb *atpA–orf522* transcript (Laver et al., 1991). As shown previously, the 3-kb *atpA–orf522* cotranscript is specifically reduced in abundance in male florets of the restored hybrid line (Figure 3B; Monéger et al., 1994). In contrast, the steady state level of the 1.9-kb *atpA* gene transcript remains relatively constant between the sterile and restored hybrid lines. These results confirm that the reduction of the 3-kb *atpA–orf522* cotranscript is specific to restored hybrid male florets and does not occur in other tissues in which there is active mitochondrial biogenesis (Figure 3B).

To investigate at which stage of anther development the reduction of the chimeric transcript is most pronounced, an *orf522*-specific gene probe (Figure 3A) was hybridized to total RNA isolated from fertile, sterile, and restored hybrid male florets at different stages (Figure 3C). The reduction of the *atpA–orf522* (3 kb) transcript is apparent at all stages of floret development studied but is particularly pronounced in male florets at the leptotene stage (stage 2) and at the tetrad/microspore stage (stages 5 and 6). The filter shown in Figure 3C was reprobed with an *atp9* gene–specific probe (Table 1) to allow for correction of any differences in loading of RNA samples (data not shown). Densitometry was then carried out on
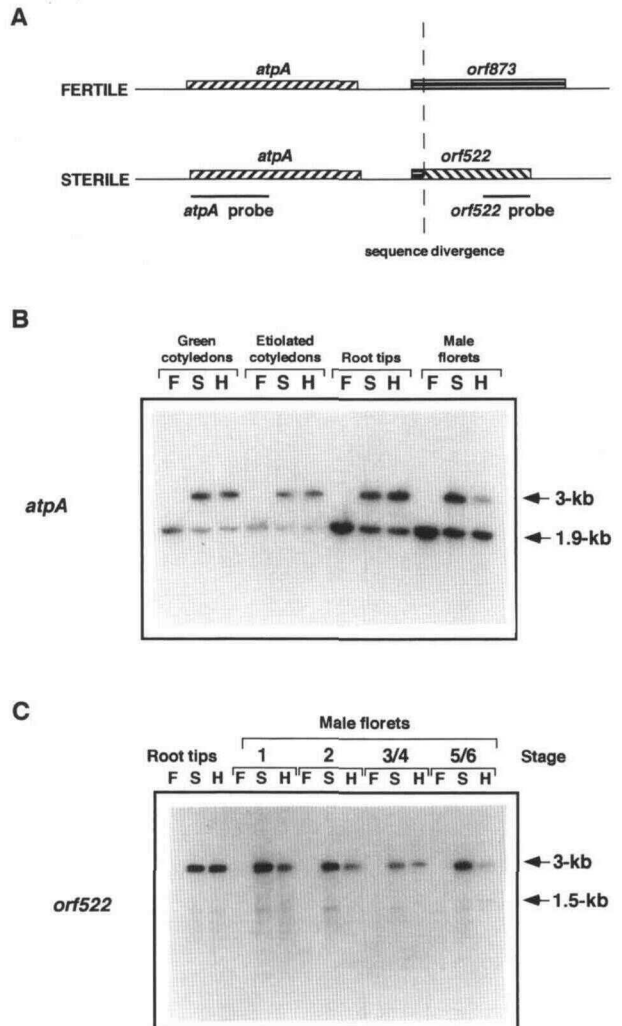

Figure 3. RNA Gel Blot Analysis of *atpA*- and *orf522*-Specific Gene Probes to Sunflower Total RNA.

(A) Diagram representing mitochondrial DNA of the fertile and sterile lines at the *atpA* locus and showing the origins of the *atpA*- and *orf522*-specific gene probes. The *orf873* (formerly ORFb in Laver et al., 1991) is apparently not expressed.

(B) Ten micrograms of total RNA from green cotyledons, etiolated cotyledons, root tips, and male florets (leptotene stage 2) of fertile (F), sterile (S), and restored hybrid (H) sunflowers was fractionated by electrophoresis, and RNA gel blot analysis was performed using an *atpA*-specific probe. The 1.9-kb (*atpA*) and 3-kb (*atpA–orf522*) transcripts are indicated by arrows.

(C) Ten micrograms of total RNA from root tips, premeiotic stage florets (stage 1), leptotene stage florets (stage 2), pachytene and divisions stage florets (stages 3/4), and tetrad and young microspore stage florets (stages 5/6) was fractionated by electrophoresis, blotted, and hybridized with the *orf522*-specific probe. The 3- and 1.5-kb transcripts are indicated by arrows.

Table 1. Origins of Homologous and Heterologous Gene Probes Used for RNA in Situ Hybridization Experiments

Gene	Gene Product	Species	Clone	Subclone	RNA length (kb) in Sunflower	Reference
atpA (atp1)	α Subunit mitochondrial F_1F_0-ATP synthase	H. annuus	Genomic	0.7 kb BamHI/EcoRI	1.9	Laver et al. (1991)
orf522	ORF522	H. annuus PET1	Genomic	0.25 kb BamHI/EcoRI	3.0 and 1.5	Laver et al. (1991)
atp9	Subunit 9 mitochondrial F_1F_0-ATP synthase	Z. mays	Genomic	0.94 kb XhoI/BamHI	0.8	Dewey et al. (1985)
cob	Apocytochrome b	Z. mays	Genomic	0.68 kb EcoRI/HindIII	2.7	Dawson et al. (1984)
rrn 26	Mitochondrial 26S rRNA	T. aestivum	Genomic	0.98 kb AvaI	3.3	Falconet et al. (1985)
atpB (atp2)	β Subunit mitochondrial F_1F_0-ATP synthase	Z. mays	cDNA	0.35 kb BamHI/SmaI	2.4	Winning et al. (1990)
ANT	Adenine nucleotide translocator	Z. mays	cDNA	0.63 kb ClaI/EcoRI	2.0	Winning et al. (1991)
Nuclear 25S rDNA	Cytoplasmic 25S rRNA	T. aestivum	Genomic	0.9 kb BamHI/EcoRI	3.6	Gerlach and Bedbrook (1979)

the autoradiograph to determine the extent of reduction of the orf522 transcripts relative to the abundance of the atp9 transcript. There was an ~75% reduction in the amount of the chimeric 3-kb atp A–orf522 cotranscript in male florets of the restored hybrid line at the leptotene stage of meiosis. The orf522 probe also hybridized to a low abundance transcript of 1.5 kb, which probably corresponds to the 1.2-kb orf522 transcript detected by Köhler et al. (1991). The steady state level of the low abundance, 1.5-kb orf522 transcript was also reduced in male florets upon restoration to fertility (Figure 3C).

Cell-Specific Accumulation of Mitochondrial Gene Transcripts in Anthers of the Fertile Line

To investigate the spatial distribution of the atpA transcripts during normal anther development, the atpA gene probe (Figure 3A) was hybridized to anther sections of the fertile line in situ (Figure 4). During the early stages of meiotic prophase 1 (stages 1 and 2, premeiosis and leptotene, respectively), the atpA transcript is not distributed evenly throughout the anther tissue, but is highly localized to the meiocyte cells. The lack of hybridization to the leptotene stage anther sections with the sense strand probe indicates that this is a specific signal (Figure 4). By the divisions stage (stage 4), the tapetal cells have expanded and show high levels of accumulation of the atpA gene transcript, and in the later tetrad and microspore stages the atpA gene transcript is equally abundant in both cell types and less abundant in other anther cells (Figure 4). It is thought that the accumulation of atpA gene transcripts in the meiocyte and tapetal cells of developing anthers observed by RNA

in situ hybridization experiments accounts for the relatively high steady state levels of atpA gene transcripts in male floret tissue observed by RNA gel blot analysis (Figure 3B).

To further investigate whether the pattern of distribution of the atpA transcript in developing anthers is a general phenomenon for all cellular RNA or is specific to mitochondrially encoded genes, RNA in situ hybridization analysis was performed using various gene probes. Leptotene stage (stage 2) anther sections were hybridized with probes corresponding to other mitochondrial genes (atp9 encoding subunit 9 of the F_0-ATP synthase, cob encoding the apocytochrome b protein, and rrn26 encoding the mitochondrial 26S rRNA), nuclear genes encoding mitochondrial proteins (atpB, encoding the β subunit of the F_1-ATP synthase, and ANT, encoding the adenine nucleotide translocator), and a nonmitochondrial nuclear gene (nuclear rDNA 25S encoding the 25S rRNA; see Table 1 and Figure 5). All probes hybridized to single transcripts in total RNA (Table 1) and hybridized specifically to anther sections of the fertile line, as indicated by the lack of hybridization in the sense strand probe controls (Figure 5). The intensity of the signals with the different probes cannot be directly compared because the exposure time is different for each probe.

The results presented in Figure 5 show that the atp9, cob, and rrn26 mitochondrial gene transcripts exhibit the same meiocyte-specific accumulation at the leptotene stage of meiosis as the atpA gene transcript (Figure 4). In contrast, the atpB and ANT nuclear–encoded gene transcripts and the 25S rRNA do not accumulate in meiocyte cells but appear to be more equally abundant in all anther cell types. The mitochondrial atp9 and rrn26 gene transcripts have the same distribution pattern as the atpA transcript in the tapetum at the later meiotic

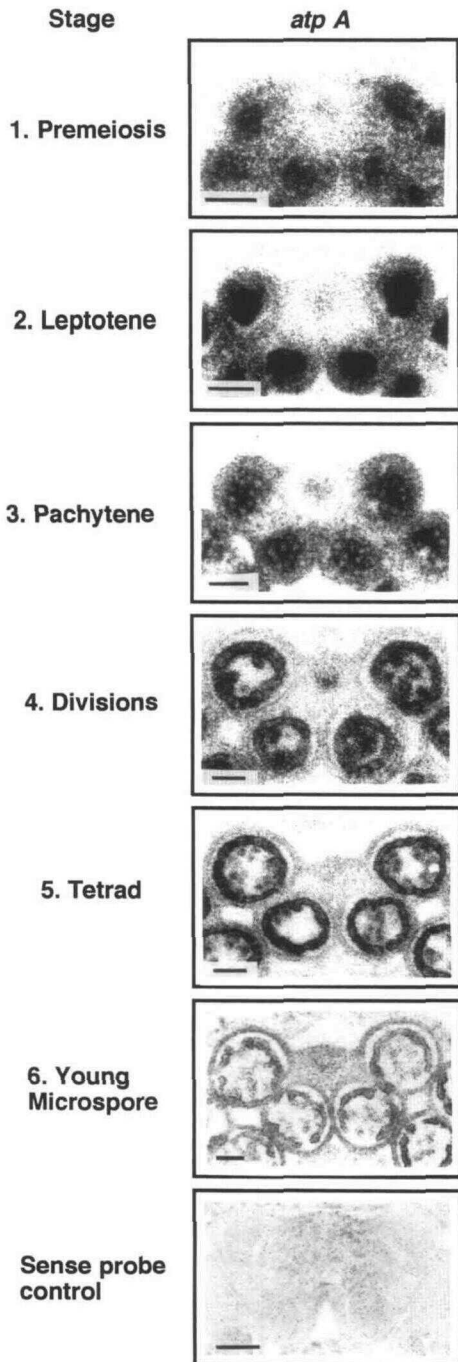| Stage	*atp A*
1. Premeiosis	
2. Leptotene	
3. Pachytene	
4. Divisions	
5. Tetrad	
6. Young Microspore	
Sense probe control	

Figure 4. Localization of *atpA* Gene Transcripts in Developing Anthers of the Fertile Sunflower Line by RNA in Situ Hybridization.

An *atpA*-specific, ^{35}S-labeled antisense strand RNA probe (see Figure 3A) was synthesized and hybridized to fertile anther sections at the six different stages of development. A sense strand probe failed to hybridize specifically to anther sections. Pachytene is the stage at which the meiocyte cells show the first signs of abnormal development in the anthers of the sterile line. Bars = 50 μm.

stages, whereas the nuclear-encoded RNAs do not (C.J. Smart, data not presented). These results suggest that this spatial pattern of transcript accumulation is characteristic of genes encoded by the mitochondrial genome and therefore is indicative of a cell-specific regulation of expression of these genes during male meiosis.

Reduction of the *atpA–orf522* Transcript Is Anther Specific

To determine whether the novel *orf522*, which is associated with CMS, exhibits the same pattern of expression as the other mitochondrial genes, *atpA*, *orf522*, and *atp9* gene transcripts were localized in anthers of the fertile, sterile, and restored hybrid lines by RNA in situ hybridization analysis. The *atpA* and *orf522* gene probes (Figure 3A) were hybridized to anther sections at the leptotene stage from the fertile, sterile, and restored hybrid lines, and an *atp9* gene probe was used as a control (Figure 6A). The *atpA* and *atp9* mitochondrial gene transcripts accumulate in the meiocyte cells of leptotene stage anthers of the sterile and restored hybrid lines in a similar way to that observed in the fertile line. In leptotene stage anthers of the sterile line, the *orf522* transcripts have the same pattern of distribution as the other mitochondrial gene transcripts studied (i.e., pronounced accumulation in the meiocyte cells). As expected, the *orf522* gene probe did not hybridize to the anther sections of the fertile line, and the *orf522* sense strand probe did not hybridize to anther sections of the restored hybrid line, indicating that this is a specific signal.

It is evident from the data shown in Figure 6A that the extent of hybridization of the *orf522* probe to anthers of the restored hybrid line is reduced relative to that in the anthers of the sterile line, particularly in meiocyte cells. The extent of hybridization of the *atpA* probe to anther sections of the restored hybrid line is also reduced, although the reduction is less marked than that observed with the *orf522* gene probe. In contrast, no reduction is observed for *atp9* gene transcripts, which are equally abundant in anthers of the fertile, sterile, and restored hybrid lines. A further control is shown in Figure 6B in which the *orf522* probe was hybridized to root tip sections from fertile, sterile, and restored hybrid lines. No difference in *orf522* transcript abundance is observed between sterile and hybrid root apical meristems, confirming that the reduction in the *orf522* transcript is specific to anthers of the restored hybrid lines (Figure 6B).

Accumulation of the F_1-ATP Synthase α Subunit Protein Parallels That of the *atpA* Transcript

To further investigate the significance of the pattern of mitochondrial gene transcript accumulation at the protein level, the α subunit protein of the mitochondrial F_1-ATP synthase was localized in anthers of the fertile line by fluorescent immunolocalization.
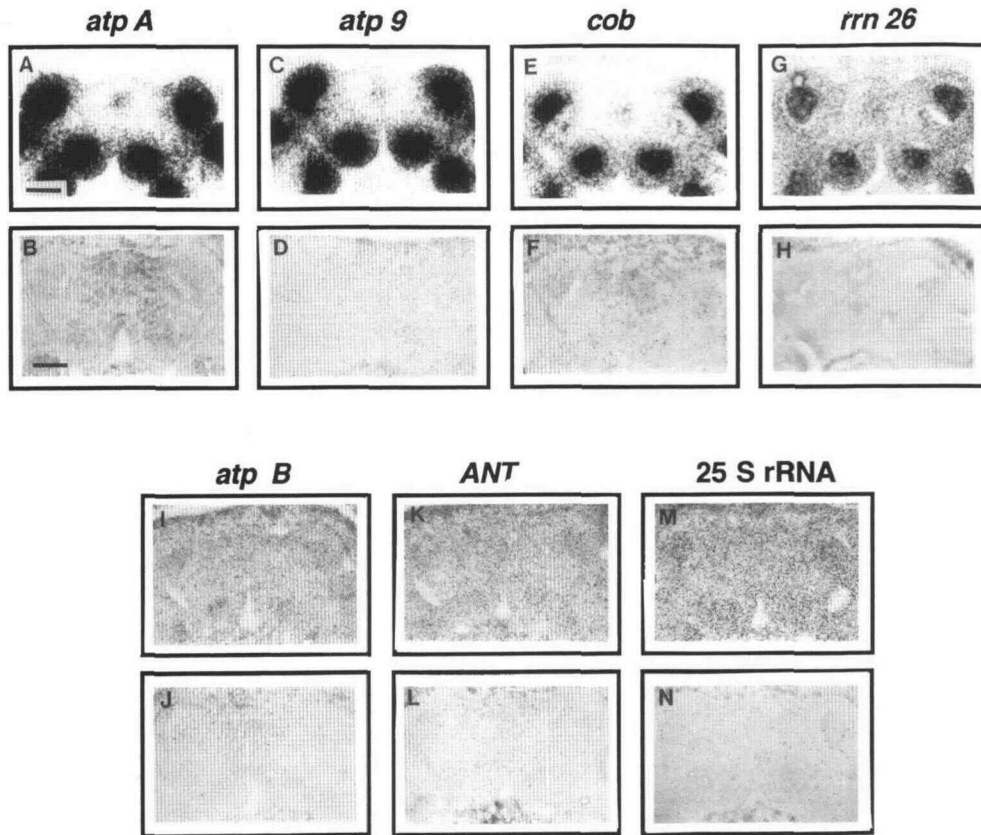
Figure 5. Localization of Gene Transcripts in Leptotene Stage Anthers of the Fertile Sunflower Line by RNA in Situ Hybridization.

[35]S-labeled antisense **(A)**, **(C)**, **(E)**, **(G)**, **(I)**, **(K)**, and **(M)** and sense **(B)**, **(D)**, **(F)**, **(H)**, **(J)**, **(L)**, and **(N)** strand probes were hybridized to leptotene stage (stage 2) anther sections from the fertile line in situ. All probes that were used hybridized to a single transcript in RNA gel blot hybridization experiments with total root RNA (data not presented). For the origins of gene-specific probes, see Table 1.

(A) and **(B)** *atpA* mitochondrial gene.
(C) and **(D)** *atp9* mitochondrial gene.
(E) and **(F)** *cob* mitochondrial gene.
(G) and **(H)** *rrn26* mitochondrial gene.
(I) and **(J)** *atpB* nuclear gene encoding a mitochondrial protein.
(K) and **(L)** *ANT* nuclear gene encoding a mitochondrial protein.
(M) and **(N)** 25S rRNA nuclear gene.
Bars in **(A)** and **(B)** = 50 μm.

A polyclonal antibody raised against the maize F_1-ATP synthase α subunit protein only recognized the 58-kD α subunit protein in a protein gel blot experiment on total male floret protein at the leptotene stage (Figure 7). This result also suggests that male florets of the fertile, sterile, and restored hybrid lines contain the same steady state levels of the α subunit protein.

The α subunit–specific antibody was then used to localize the α subunit protein in anther tissue and a tetramethylrhodamine isothiocyanate–labeled fluorescent secondary antibody was used to visualize the signal. Figures 8A to 8F show that the α subunit protein is localized in discrete spots, probably corresponding to individual mitochondria, in young meiocytes and developing tapetal cells of the fertile line. In addition, it

is evident that the distribution and cell-specific accumulation of the α subunit protein reflects that of the *atpA* gene transcript. Steady state levels of the α subunit protein are apparently highest in meiocyte cells at the leptotene stage and in tapetal cells at the tetrad and young microspore stages. The specificity of this signal is indicated by the lack of fluorescence in the anther sections in which the primary antibody was omitted (Figure 8H). These results suggest that the cell specificity of *atpA* gene expression is regulated prior to translation.

At the divisions stage of meiosis (stage 4), the α subunit protein is localized in tight rings around the dividing meiocyte nuclei (Figure 8D). These fluorescent rings probably correspond to the numerous mitochondria and vesicles that
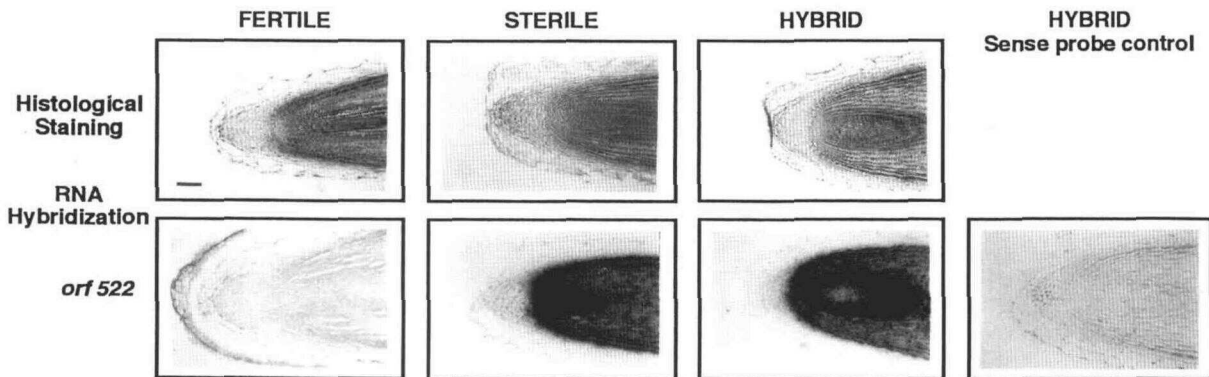
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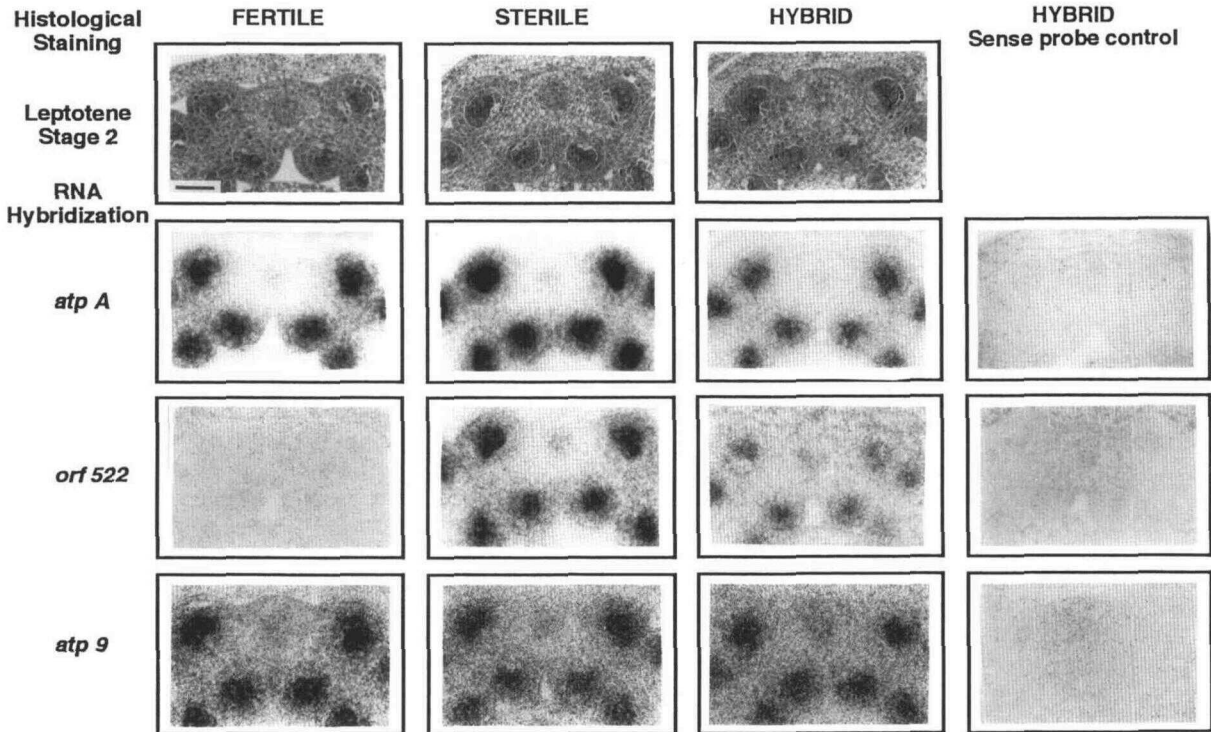
B

Figure 6. Abundance of the *atpA–orf522* Transcript Is Reduced Specifically in Anthers.

(A) Localization of *atpA*, *orf522*, and *atp9* gene transcripts in leptotene (stage 2) anther sections from fertile, sterile, and restored fertile hybrid sunflowers by RNA in situ hybridization. ^{35}S-labeled antisense strand probes hybridized to anther sections from fertile, sterile, and restored hybrid lines at the leptotene stage (stage 2). Sense strand control hybridization to anthers of the restored hybrid line shows that the antisense signal is specific. Bar = 50 μm.
(B) Localization of *orf522* gene transcripts in root tip sections from fertile, sterile, and restored fertile hybrid sunflower lines by RNA in situ hybridization. The ^{35}S-labeled *orf522* antisense strand probe hybridized to root tips. The sense probe control is shown for root tip sections of the restored hybrid line. Bar = 100 μm.
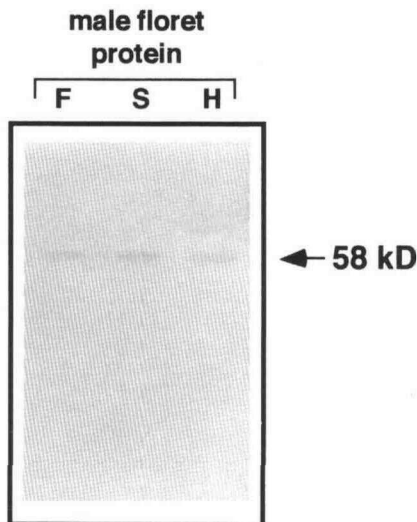
male floret
protein

Figure 7. Protein Gel Blot Analysis of the α Subunit Protein of the F₁-ATP Synthase in Sunflower Male Floret Protein.

Protein gel blot analysis was performed on 12 µg of total protein of male florets from fertile (F), sterile (S), and restored fertile hybrid (H) lines using a maize α subunit–specific polyclonal antibody. The 58-kD α subunit protein is indicated by an arrow.

surround the dividing nuclei (C.J. Smart, data not shown). Electron microscopic observations revealed that following meiotic divisions, mitochondria become closely associated with the nuclear envelope in tetrad and microspore cells (Figure 8G).

Immunolocalization of the α subunit protein of the F₁-ATP synthase was also performed on leptotene stage anther sections from fertile, sterile, and restored hybrid lines (Figures 8I to 8K). These results show that the α subunit protein accumulates in the meiocyte cells of the sterile and restored hybrid line at the leptotene stage in a similar punctate fashion to that observed in anthers of the fertile line. This result also confirms that there is no apparent difference in the abundance of the α subunit protein between fertile, sterile, and restored hybrid lines in spite of the reduced steady state level of the 3-kb atpA–orf522 transcript, as shown in Figure 7. These results suggest therefore that the abundance of the α subunit protein may be under translational or post-translational control.

DISCUSSION

In this study, we showed that mitochondrial gene expression is regulated during development of sunflower anthers. During the early stages of meiosis, mitochondrial gene transcripts and proteins (the α subunit protein of the F₁-ATP synthase) accumulate to relatively high steady state levels in the meiocyte cells of the anther. During the later stages of meiosis, mitochondrial transcripts and proteins appear to accumulate in tapetal cells (Figures 4 and 8). The high steady state levels of mitochondrial gene transcripts and proteins in meiocytes suggest that active mitochondrial biogenesis is occurring in these cells, as has been reported in maize sporocytes and tapetal cells (Lee and Warmke, 1979). Mitochondrial biogenesis in sunflower meiocyte cells may be the specific cellular function that is disrupted during meiosis in the male sterile line, leading to meiocyte cell abortion. Accumulation of atpA gene transcripts and the α subunit protein in tapetal cells of the fertile line occurring after the first signs of abnormal development in meiocyte cells was observed in the sterile line and is therefore unlikely to be related to the CMS phenotype (Figures 4 and 8).

In previous studies, Young and Hanson (1987) found a four- to fivefold increase in the abundance of a specific pcf transcript in the anthers of the CMS line of petunia, indicating that there is a differential developmental regulation of the level of expression of the CMS-associated pcf mitochondrial gene. In addition, a comparison of the translational profiles of proteins synthesized by mitochondria isolated from different tissues of maize (Newton and Walbot, 1985) and sunflower (Monéger et al., 1994) suggests that certain mitochondrial genes are differentially expressed during development. The developmental regulation of mitochondrial gene expression reported here may be particularly significant because it is cell specific to meiocyte and tapetal cells (Figures 4 and 8).

Our results demonstrated an accumulation of both mitochondrial gene transcripts and mitochondrial proteins in meiocyte and tapetal cells, implying that cell-specific mitochondrial gene expression is regulated prior to translation at the level of mitochondrial gene copy number or at the transcriptional or post-transcriptional levels (Figures 4 and 8). This increase in transcript and protein abundance in meiocytes could be due to a cell-specific replication of the mitochondrial genome (mitochondrial gene copy number). Alternatively, the rate of transcription of mitochondrial genes could be up-regulated cell specifically (transcriptional), or there may be cell-specific regulation of the stability of mitochondrial gene transcripts (post-transcriptional).

There is also apparently some degree of translational regulation of expression of the mitochondrially encoded genes. This is suggested by RNA gel blot analysis (Figure 3B), RNA in situ hybridization (Figure 6A), and protein gel blot analysis (Figure 7), which indicate that leptotene stage male florets of the restored hybrid line contain the same amount of the α subunit protein as male florets of the fertile and sterile lines, despite having only ∼66% of the steady state level of atpA gene transcripts. These results also suggest that a reduced amount of the α subunit protein of the F₁-ATP synthase in male florets of the sterile line is not responsible for meiocyte cell abortion.

If the accumulation of mitochondrially encoded gene transcripts in anther cells is associated with mitochondrial biogenesis, then one would expect there to be a coordinated cell-specific regulation of expression of nuclear genes encoding mitochondrial proteins. Nuclear gene transcripts of the F₁-ATP synthase β subunit and the adenine nucleotide
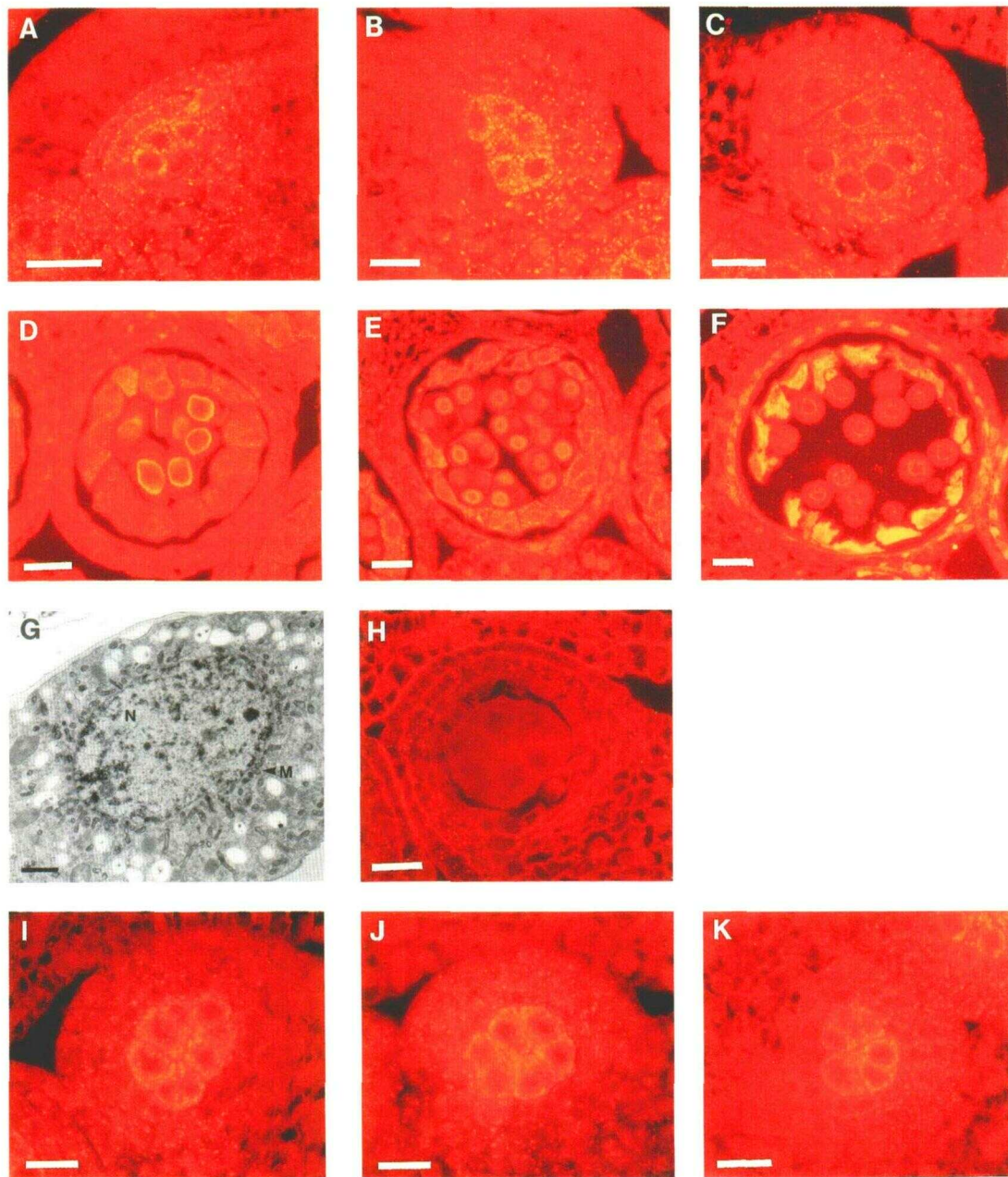
Figure 8. Immunolocalization of the Mitochondrial α Subunit Protein of the F₁-ATP Synthase in Sunflower Anther Loculi.

Fluorescent immunolocalization of the α subunit protein was performed on anther sections using an α subunit–specific antibody. A tetramethyl-rhodamine isothiocyanate–labeled secondary antibody was used to reveal the position of the α subunit protein, which is indicated by the yellow fluorescent spots that decorate the mitochondria.

(A) Premeiosis stage.

(B) Leptotene stage.

(C) Pachytene stage.

(D) Divisions stage.

(E) Tetrad stage.

(F) Microspore stage in anthers of the fertile line.

(G) Electron micrograph of a tetrad cell of the fertile line showing mitochondrial association with the nuclear envelope. M, mitochondria; N, nucleus.

(H) Control immunolocalization in leptotene stage anthers of the fertile line where the α subunit primary antibody was omitted.

(I) Leptotene stage anther of the fertile line.

(J) Leptotene stage anther of the sterile line.

(K) Leptotene stage anther of the restored fertile hybrid line.

In **(G)**, bar = 1 μm; all other bars = 20 μm.

translocator (*atpB* and *ANT*, respectively) do not show the same cell-specific accumulation (at all meiotic stages) as observed for the mitochondrial gene transcripts (Figure 5). Therefore, if there was a coordinated regulation of expression of nuclear genes encoding mitochondrial proteins, it would have to occur at the translational or post-translational levels. It has recently been shown by tissue printing that the nuclear-encoded alternative oxidase protein accumulates in the tapetum of petunia anthers at the tetrad stage (Conley and Hanson, 1994). The authors also present evidence for the differential expression of mitochondrial proteins during the later stages of meiosis, insofar as the α subunit protein of the F_1-ATP synthase accumulates in the tapetal and sporogenous cells of tetrad stage anthers, whereas the cytochrome oxidase subunit II protein accumulates in the subepidermal anther tissue (Conley and Hanson, 1994). The pattern of accumulation of the α subunit protein at the tetrad stage of petunia anther development is in agreement with the results presented in this study. However, the authors only studied two stages of anther development, and the tissue-printing technique makes it difficult to distinguish between sporogenous (meiocyte) and tapetal cells.

In this study, we showed by electron microscopy that mitochondria become associated with the nucleus during the later stages of meiosis in sunflower meiocytes (Figure 8). Although this phenomenon has been described previously at the tetrad and young microspore stages (Simonenko, 1977; Dickinson and Potter, 1979; Laveau et al., 1989), our data suggest that the nuclear–mitochondrial association may begin at an earlier stage during meiotic divisions (stage 4). It is possible that this association may be a mechanism to ensure that mitochondria are distributed equally between the four microspores following meiotic divisions.

We have previously demonstrated that the restorer gene(s) in sunflower acts specifically in male florets resulting in a post-transcriptional reduction in the stability of the 3-kb chimeric *atpA–orf522* cotranscript and consequently a reduction in the abundance of the ORF522 protein (Monéger et al., 1994). Here, we show that the activity of the restorer gene(s) is specific to anthers in the restored hybrid line (Figure 6). Localization of the *orf522* transcripts by RNA in situ hybridization showed that the reduction in the abundance of the *atpA–orf522* transcript is particularly pronounced in meiocyte cells of the restored hybrid line. However, it is possible that the cells of the middle layer and developing tapetum, which surround the meiotic cells, also contained reduced levels of the *atpA–orf522* transcript (Figure 6A). The ORF522 protein has also been localized in anthers of the fertile, sterile, and restored hybrid lines (C.J. Smart, unpublished data), and preliminary immunolocalization data suggest that the reduction of the ORF522 protein in anthers of the restored hybrid line is meiocyte specific. In light of these results, we suggest that the restorer gene(s) acts specifically in meiocytes during the early stages of meiosis to reduce the stability of the chimeric *atpA–orf522* transcript and consequently the abundance of the ORF522 protein. However, the precise specificity and mode of action of the restorer gene(s)

will only be confirmed once the gene(s) has been cloned and its transcript and protein product are localized in situ.

Our data provide evidence that the dominant nuclear fertility restoration gene(s) acts anther specifically to reduce the abundance of a chimeric transcript and polypeptide thought to be causally related to the CMS phenotype. The only other report of a tissue-specific action of a nuclear restorer gene is in bean CMS where Johns et al. (1992) reported that the action of the Fr restorer gene causes the specific loss of the *pvs* region of the mitochondrial genome during pollen development.

A considerable body of evidence now exists linking mutations in the mitochondrial genome with the failure to produce pollen, yet why vegetative development and female fertility are unaffected remains to be determined (Hanson, 1991). This developmental specificity may be partly due to the fact that mutations in the mitochondrial genome, associated with CMS, apparently do not disrupt essential mitochondrial genes (Levings, 1993). Indeed, in NCS mutants, where essential mitochondrial genes are disrupted, the development of the whole plant is severely affected (Newton et al., 1990; Hunt and Newton, 1991; Rousell et al., 1991). The tissue specificity of phenotypes associated with mutations in human mitochondrial DNA is thought to be conferred by a combination of tissue-specific accumulation of mutant genomes and a tissue-specific sensitivity to reduced capacity for oxidative phosphorylation (Wallace, 1992, 1993). The tissue specificity of the CMS phenotype cannot be attributed to heteroplasmy, because male-sterile plants are apparently homoplasmic for the CMS mutations (Hanson, 1991). It is possible, however, that respiratory demand is higher in anther tissue than in any other tissue and that mutant mitochondria in the anthers of CMS lines cannot support these high demands (Singh and Brown, 1991; Levings, 1993).

Our findings provide an alternative explanation for the tissue specificity of the CMS phenotype and are consistent with the suggestion that a high level of mitochondrial gene expression and biogenesis is required in the meiocyte cells of the anther to produce sufficient mitochondria to sustain each of the four haploid microspore cells. The provision of mitochondria required for the development of microspore cells may be greater than that for female meiocyte cells or mitotic cells (e.g., in meristems) because the male meiocyte cell must divide equally and simultaneously into four cells, each of which becomes a male gametophyte that must exist independently of the parent anther tissue. The expression of *orf522* in male-sterile lines may disrupt or impair mitochondrial biogenesis in the meiocytes leading to cell abortion.

In the most extensively studied examples of CMS, sterile plants contain a chimeric mitochondrial ORF that is expressed as a novel polypeptide in all tissues studied (Dewey et al., 1987; Nivison and Hanson, 1989; Monéger et al., 1994). In these cases, the CMS phenotype may be directly caused by the presence of the novel polypeptide or indirectly by the effect of expression of the chimeric gene on expression of essential mitochondrial genes (Hanson et al., 1989). In sunflower CMS, the ORF522 protein may be directly deleterious to meiocyte

cells, or the cotranscription of orf522 with the atpA gene may indirectly affect the translation of the α subunit protein of the F₁-ATP synthase. The fact that the restorer gene(s) acts anther specifically to cause reduction of the orf522 transcript abundance of the ORF522 protein levels (Figure 6) suggests that the ORF522 protein itself may be directly toxic to meiocytes or that it interacts with a meiocyte-specific molecule to impair mitochondrial function (see Flavell, 1974). However, an indirect mechanism causing CMS cannot be ruled out. The requirement for high levels of mitochondrial gene expression and mitochondrial biogenesis (amplification) during meiocyte cell development may put increased demands on the processes of transcription and translation (Figures 4 and 8). It is possible that the cotranscription of the atpA gene with orf522 on the 3-kb transcript may disrupt the translation and subsequent assembly of the α subunit protein into the F₁-ATP synthase complex without affecting the abundance of the α subunit protein (Figure 7).

In conclusion, our results provide compelling evidence that the expression of the mitochondrial genome is regulated in a cell-specific manner during sunflower anther development. Furthermore, the fact that nuclear-encoded fertility restorer gene(s) can specifically modify the expression of a mitochondrially encoded gene (orf522) in meiocyte cells suggests that the nucleus is intimately involved in this developmental regulation. We propose that the action of the restorer gene(s) in meiocyte cells at a stage prior to abortion of these cells in the male-sterile lines is significant, because it suggests that expression of the CMS-associated orf522 is lethal to young meiotic cells during sunflower anther development.

METHODS

Plant Material

Sunflower seeds were provided by Rhône Poulenc Agrochimie (Lyon, France). Fertile (RPA842B) and sterile (RPA842A) lines are isonuclear containing the Helianthus annuus nuclear genotype; the fertile line carries the H. annuus cytoplasm, and the sterile line contains the H. petiolaris cytoplasm (Leclercq, 1969). The fertile restored hybrid (RPA844H) line results from a cross between the sterile line and a restorer line (RPA843R) carrying the dominant nuclear restorer genes. Seeds were germinated following surface sterilization with 5% (v/v) sodium hypochlorite, and plants were grown in a 16-hr 28°C day and 8-hr 24°C night regime. Cotyledons and root tips were harvested after 4 days, flower buds were harvested after ~40 days, and flowering occurred after 50 days (see Figure 1). Male florets consist of the upper part of the sunflower floret that contains the anthers and were dissected from flower buds for RNA and protein extractions.

RNA Gel Blot Analysis

Total RNA was extracted from root tips, etiolated cotyledons, green cotyledons, and male florets according to the method of Castresana et al. (1988). Ten micrograms of RNA was fractionated by electropho-

resis through a 3-(N-morpholino)propanesulfonic acid–buffered 1.3% (w/v) agarose gel containing 0.6 M formaldehyde and transferred to Hybond-N membrane (Amersham International, Buckinghamshire, U.K.) by capillary blotting overnight. Riboprobes were prepared by in vitro transcription according to the manufacturer's instructions using ³²P-UTP (Amersham) and a kit from Promega. Hybridization was performed overnight at 60°C in 50% (v/v) formamide, 0.75 M NaCl, 0.15 M Tris-HCl, pH 8.0, 10 mM EDTA, 0.1% (w/v) SDS, 1 × Denhardt's solution (see Sambrook et al., 1989), and 0.5 mg/mL denatured salmon sperm DNA. Washes were performed at 60°C in 2 × SSC (see Sambrook et al., 1989) and 0.1% (w/v) SDS for 10 min, 1 × SSC and 0.1% (w/v) SDS for 30 min, and 0.1 × SSC and 0.1% (w/v) SDS for 10 min. The membranes were then autoradiographed using Du Pont Cronex film.

Protein Gel Blot Analysis

Total protein was extracted from 1 g of male floret tissue by grinding in 3 mL of homogenization buffer (18% [w/v] sucrose, 10 mM MgCl₂, 100 mM Tris-HCl, pH 8.0, 40 mM β-mercaptoethanol, 0.1% [w/v] SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM amino caproic acid). The homogenate was then filtered and centrifuged at 10,000g for 15 min at 4°C. To each 500 μL of supernatant, 300 μL of stop dye was added (1% [w/v] SDS, 0.1% [w/v] bromophenol blue, 10 mM EDTA, 20% [w/v] Ficoll).

To produce the α subunit polyclonal antibody, the α subunit of the F₁F₀-ATP synthase was purified from Zea mays mitochondria according to the method of Hack and Leaver (1983) and electroeluted from an SDS–polyacrylamide gel, and antibodies were raised in rabbits.

Proteins were separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad) for 1 hr at 1 A in electrophoresis buffer containing 20% (v/v) methanol. The membrane was then incubated for 15 min in TBST-BSA buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% [w/v] Tween 20, and 1% [w/v] lyophilized BSA) and subsequently incubated for 3 hr in a 1:10,000 dilution of primary antibody. The filter was then washed in TBST and incubated for 1 hr ' with an alkaline phosphatase–conjugated secondary antibody and washed in TBST. The antibody was detected using an alkaline phosphatase (AP) color reaction, whereby the filter was equilibrated in 10 mL of AP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) and then incubated in AP color solution (10 mL AP buffer, 66 μL nitro blue tetrazolium [Sigma]), (0.5 g in 10 mL 70% [v/v] dimethylformamide, 33 μL 5-bromo-4-chloro-3-indolyl phosphate [Sigma]), and (0.5 g in 10 mL 100% [v/v] dimethylformamide). The reaction was stopped after the signal had appeared by washing the filter in water.

Preparation of Material for Light Microscopy

Plant tissues were dissected, fixed, embedded, and sectioned according to the method described by Langdale (1993).

Staging of Male Meiosis

The stage of meiosis was determined by the degree of condensation of chromatin after staining with 4,6-diamidino-2-phenylindole (DAPI) (Sigma). Paraplast wax was removed from sections by incubation in cnp 30 (Penetone, Cramlington, U.K.) for 2 × 10 min, and sections were rehydrated through an EtOH series. Sections were then incubated in 0.1% (w/v) BSA in PBS (10 mM sodium phosphate, pH 7.5, 150 mM

NaCl) for 10 min followed by 20 min in 200 μL of 0.1% (w/v) DAPI in 0.01% (v/v) Tween 20–PBS. The sections were then washed in BSA–PBS and mounted in Citifluor (Agar Scientific, Stansted, U.K.). Sections were viewed using a fluorescent microscope (Axiophot; Zeiss, Oberkochen, Germany) at an excitation wavelength of 365 nm.

Histological staining of sections was performed according to the method described by Langdale (1993).

RNA In Situ Hybridization

RNA in situ hybridization was performed using ^{35}S-UTP (Du Pont–New England Nuclear)–labeled RNA probes according to the method of Langdale (1993). All clones used were subcloned into pBluescript, pBS KS+, or pGEM vectors so that sense and antisense RNA probes could be synthesized by in vitro transcription. The origin and size of these probes are shown in Table 1. For each experiment, sections from florets at different stages or different lines were present on the same slide so that all sections were incubated with the same RNA probe and were exposed for the same length of time. Slides were viewed and photographed with a Dialux (20 EB; Leitz, Wetzlar, Germany) microscope on Ilford PAN-F film.

Immunolocalization

Tissue sections were dewaxed and dehydrated as described previously and incubated in 0.1% (w/v) BSA–PBS for 5 min at room temperature. Sections were then blocked using 0.1% (w/v) goat IgG (Sigma) for 15 min at room temperature and washed for 15 min in BSA–PBS. Sections were then incubated overnight with 100 μL of a 1:20 dilution of the α subunit antibody in BSA–PBS and then washed sequentially for 15 min in 0.5% (w/v) BSA–PBS, 0.01% (v/v) Tween 20–PBS, and 0.1% (w/v) BSA–PBS. Sections were then incubated for 1 hr at room temperature with tetramethylrhodamine isothiocyanate–conjugated goat anti-rabbit antisera (diluted in PBS as recommended by the manufacturer; Sigma). Slides were then washed in BSA–PBS and mounted in Citifluor. Control slides were treated identically except that sections were incubated overnight in BSA–PBS instead of primary antibody. All slides were viewed using a fluorescent microscope (Axiophot; Zeiss) at an excitation wavelength of 550 nm and photographed on transparency film (400; Fuji, Tokyo, Japan).

Preparation of Material for Electron Microscopy

Florets were dissected and fixed on ice according to Karnovsky (1965) and postfixed in 1% (v/v) osmium tetroxide (TAAB, Berkshire, U.K.). Samples were dehydrated through acetone and embedded in resin (TAAB). Sections (0.09 μm) were cut on an ultramicrotome (MT 5000; Sorvall, Stevenage, U.K.), stained with uranyl acetate followed by lead citrate (Reynolds, 1963), and observed with a transmisson electron microscope (JEM 2000 EX; Jeol, Ltd., Welwyn Garden City, U.K.).

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