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How Do Alterations in Plant Mitochondrial Genomes Disrupt Pollen Development?

Catharine A. Conley[1,2] and Maureen R. Hanson[1]

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Cytoplasmic male sterility arises when mitochondrial activities are disrupted that are essential for pollen development. Rearrangements in the mitochondrial genome that create novel open reading frames are strongly correlated with CMS phenotypes in a number of systems. The morphological aberrations which indicate CMS-associated degeneration are frequently restricted to the male sporogenous tissue and a limited number of vegetative tissues. In several cases, this tissue specificity may result from interactions between the mitochondrial genome and nuclear genes that regulate mitochondrial gene expression. A molecular mechanism by which CMS might be caused has not been conclusively demonstrated for any system. Several hypotheses for general mechanisms by which mitochondrial dysfunction might disrupt pollen development are discussed, based on similarities between the novel CMS-associated genes from a number of systems.

KEY WORDS: Mitochondria; respiration; pollen development; Petunia; cytoplasmic male sterility; tissue printing; fluorescence microscopy; meiosis.

INTRODUCTION

Male sterility in plants, characterized by flowers which can produce fertile seeds but do not produce viable pollen, is a common phenomenon in many species, and may be caused by mutations in either nuclear or mitochondrial genomes (reviewed in Hanson and Conde, 1985; Kaul, 1988; Chaudhury *et al.*, 1994; Vedel *et al.*, 1994). Additionally, male sterility resulting from nuclear-cytoplasmic incompatibility can be created by backcrossing a nuclear genome into a foreign cytoplasm from either the same species or a closely related species, although both parents are fully fertile (Kaul, 1988; Vedel *et al.*, 1994). Even within one species, several different defects in mitochondrial genomes may be present; for example, in maize, three distinct cytoplasms will confer male sterility (Levings, 1988).

In many species exhibiting cytoplasmic male sterility (CMS), individual nuclear genes or nuclear gene complexes exist that will restore male fertility to plants carrying a CMS cytoplasm (Hanson and Conde, 1985; Kaul, 1988; Vedel *et al.*, 1994). For example, in petunia, genetic studies have identified one single sterility-associated cytoplasm (Izhar and Frankel, 1976), which was the result of an interspecific cross onto an unidentified *Petunia* species with *P. hybrida* as the male parent (discussed in Edwardson and Warmke, 1967). Several nuclear gene systems can restore fertility to petunia plants carrying the sterile cytoplasm, including a single dominant gene *Rf* (Edwardson and Warmke, 1967) and a set of temperature-sensitive genes which interact to restore partial or full fertility (van Marrewijk, 1969). The associated phenotypes of male sterility and fertility restoration are of considerable agronomic importance, and have been studied extensively in a number of commercially significant species (reviewed in Hanson and Conde, 1985; Kaul, 1988; Levings, 1988; Chaudhury *et al.*, 1994; Vedel *et al.*, 1994).

[1] Section of Genetics and Development, Cornell University, Biotechnology Building, Ithaca, New York 14853-2703.

[2] Current address: The Scripps Research Institute, Department of Cell Biology, MB24, 10666 N. Torrey Pines Rd., La Jolla, California 92037.

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The widespread incidence of CMS in vegetatively normal plants from distantly related species (Laser and Lersten, 1972; Kaul, 1988) suggests that unique events occur during flower and pollen development which may be disrupted by mitochondrial defects. The aberrant development that culminates in pollen sterility may initiate at any stage during anther and pollen maturation, from the development of altered floral organs to the production of morphologically intact but nonviable pollen grains (Kaul, 1988; Trull and Malmberg, 1994). A large subset of CMS systems, including *Petunia* CMS, show aberrations in specific parental anther tissues just prior to male meiosis. This may indicate that the initial activity of these CMS genes occurs not in the pollen-forming tissue itself but in surrounding tissues, although the special roles that mitochondria play during male reproductive development are as yet unidentified. Some clues to mitochondrial function during pollen development can be gained by examining disruptions in floral morphology, anatomy, and biochemistry in CMS plants. Genes and gene products associated with CMS also provide hints to the processes occurring inside mitochondria during pollen development that might be disrupted by CMS mutations. Because our laboratory has focused on *Petunia* as a system to study CMS and pollen development, the following discussion concentrates on reproduction and mitochondrial function in this genus.

MORPHOLOGY OF FLORAL DEVELOPMENT IN NORMAL AND CMS PLANTS

The petunia flower is characteristic of many angiosperm flowers, containing four whorls of organs (Fig. 1), three of which are unaffected in CMS petunia plants. The outer two whorls of nonreproductive organs include five sepals, which are green and photosynthetic, and five petals, which become nonphotosynthetic as they mature and frequently are brightly colored. In the fourth and innermost whorl, the female reproductive organs, the carpels, are fused into a pistil, which includes the ovary, containing ovules that produce seeds, and an associated stigma and style, down which germinating pollen grows (Esau, 1977). In the third whorl, five male reproductive organs, the stamens, each are composed of a filament bearing the anther (Fig. 2), which contains four locules comprising support tissues and pollen mother cells (PMCs) that undergo meiosis and develop into pollen grains (Fig. 3). Around the PMCs are the tapetal cells and several middle cell layers, which extend outward to the epidermis (Fig. 2A). The epidermis, middle layers, and in mature anthers the endothecium, shield and contain the PMCs and developing microspores, and provide structural support in the mature anther (Fig. 2B). The four locules are held together by connective tissue surrounding a central vascular bundle, which connects the anther to the rest of the plant via the filament. Located on either side of the anther, at the junction between each pair of locules, is a small column of cells, termed stomial cells, that die as the anther matures. This permits a mature anther to split open along the resultant seam and release mature pollen grains (Fig. 2B). Water and nutrients enter the anther through the vascular bundle, that continues from the filament nearly to the tip of the anther. Materials are distributed from the vascular bundle through the connective tissue and enter the tapetum, where they are processed and transferred to the developing microspores (Albertini *et al.*, 1987; Pacini, 1994).

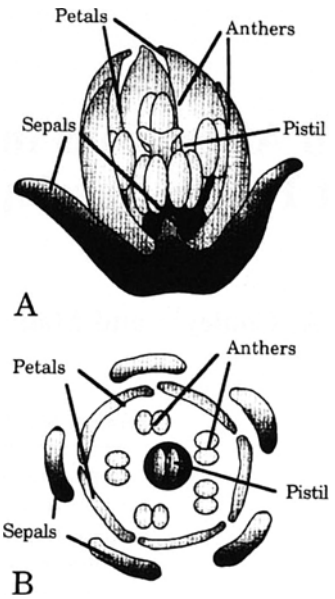

Fig. 1. Flower anatomy. (A) Side view of a flower bud showing the vegetative and reproductive organs; (B) top view of a flower bud showing the arrangement of whorls.

In order to characterize the morphology of the developmental disruption in *Petunia* CMS, anthers from fertile and sterile plants have been examined by light microscopy (*P. hybrida;* see Izhar and Frankel, 1971b; Bino, 1985a,b; *P. parodii;* see Conley *et al.*, 1994) and two-photon fluorescence microscopy, as well as scanning (*P. parodii;* see Conley *et al.*, 1994)

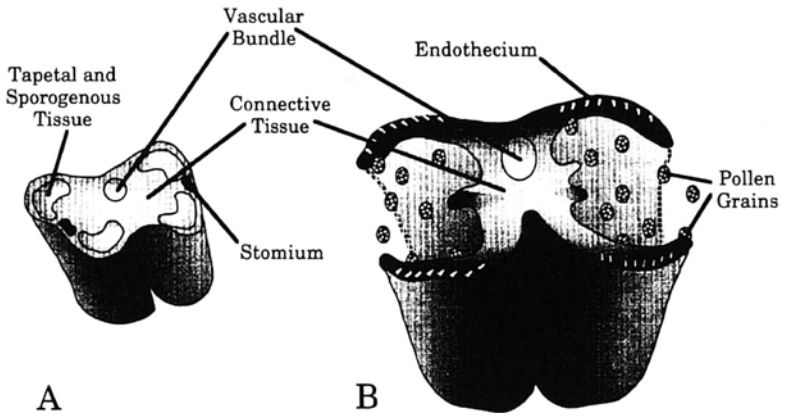

Fig. 2. Anther anatomy. (A) a premeiotic anther; (B) a mature anther.

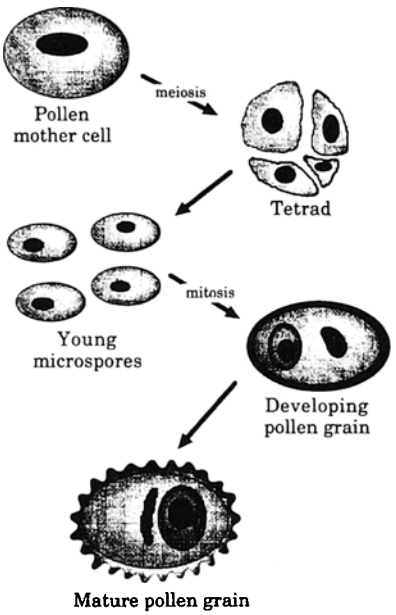

Fig. 3. Pollen development. A diagram of pollen development from a premeiotic pollen mother cell to a mature pollen grain.

and transmission electron microscopy (*P. hybrida;* see Bino, 1985b; Liu *et al.,* 1987). To be certain that the developmental defects being characterized are the result of the mitochondrial lesion and not simply due to nuclear genotypic differences, it is crucial to use material which is as similar as possible. In *Petunia,* repeated backcrosses with inbred lines have produced pairs of fertile and sterile plant lines which are isonuclear for either *P. parodii* or several *P. hybrida* nuclear genomes (Izhar and Frankel, 1976), as well as two near-isonuclear lines carrying the sterile cytoplasm and the dominant *Rf* fertility-restoration gene in the *P. parodii* and one *P. hybrida* nuclear background (Connett and Hanson, 1990; A. J. Calfee and M. R. Hanson, unpublished data).

The full developmental time course for a single *P. parodii* flower takes approximately two weeks. The stages of anther development are usually named according to the condition of the pollen-forming tissue within (Pacini, 1994); thus, an anther is termed "premeiotic," "meiotic," "tetrad-stage," or "postmeiotic." In flowers from both fertile and CMS plants, the development of the sepals, petals, and carpels is morphologically identical; only the development of certain anther tissues, beginning just before meiosis, is altered in CMS plants (Izhar and Frankel, 1971b; Bino, 1985a,b; Conley *et al.,* 1994).

Very young premeiotic anthers exist as masses of undifferentiated meristematic tissue surrounded by an epidermis. A fully differentiated premeiotic or meiotic stage anther consists of four locules, each of which contains a core of sporogenous tissue surrounded by a single layer of tapetal cells. The tapetal layer is thought to nourish and protect the pollen-forming tissue during development, and all nutrients used by the PMCs and developing pollen grains must pass through the tapetal layer, which acts as a storage tissue and also synthesizes many components of the pollen wall (Albertini *et al.,* 1987; Pacini, 1994). In many species, during meiosis the tapetal cytoplasm is very dense and is thought to be synthesizing large amounts of nucleic acids, proteins, and lipids, and storing them for later transfer to the pollen grains (Albertini *et al.,* 1987; Pacini, 1994). In fertile *P. hybrida* plants, tapetal nuclei frequently undergo karyokinesis to become binucleate before male meiosis (Izhar and Frankel, 1971b; Bino, 1985a), although tapetal karyokinesis is less frequent in anthers from sterile *P. hybrida* (Bino, 1985a) plants.

The first identifiable developmental differences between anthers of fertile and sterile *P. hybrida* plants are found in the tapetal layer, appearing in the transmission electron microscope as abnormally swollen mitochondria (Bino, 1985b) and a reduced abundance of endoplasmic reticulum (Liu *et al.*, 1987). Male meiosis in fertile *P. hybrida* is very rapid, requiring only 12 hours from prophase of meiosis I to tetrad formation (Izhar and Frankel, 1971a). Meiosis in fertile *P. parodii* plants is also completed during a single day (Fig. 4; data not shown). The sporogenous tissue of sterile anthers from both species stops developing during early meiosis I (Izhar and Frankel, 1976). PMCs from CMS plants rarely complete anaphase I.

Pollen development in both fertile and restored *P. parodii* plants after meiosis is identical (Conley *et al.*, 1994). After the PMCs of fertile plants have undergone meiosis to produce microspores, the tapetal cells begin to degenerate (Conley *et al.*, 1994). In fertile plants, the microspores mature into pollen grains, expanding to crush the tapetum and connective tissue. In sterile anthers the connective tissue is not crushed, but accumulates materials that are retained until maturity (Conley *et al.*, 1994). Both the stomium and endothecium develop similarly in fertile and sterile plants, and sterile anthers do eventually split open (Conley *et al.*, 1994; Hanson *et al.*, 1995). The activity of esterases (van Marrewijk *et al.*, 1986) and cytochrome c oxidase (Bino *et al.*, 1986) has been found to be lower in anthers from sterile *P. hybrida* plants, but because these reductions occur after meiotic defects are apparent, they are likely to be secondary effects of the initial disruption.

In the majority of CMS systems, including petunia as described above, aberrant pollen development is first observed during meiosis or later. Interestingly, in a few systems, such as interspecific crosses (Spangenberg *et al.*, 1992) and somatic hybrids (Kofer *et al.*, 1991) between various *Nicotiana* species, development is disrupted even earlier, when floral organs are being specified and first differentiating. Flowers of the *Nicotiana* CMS plants demonstrate developmental aberrations in both anthers and petals, the two middle floral organ whorls. By analyzing somatic hybrids, Kofer *et al.* (1991) showed that a particular fragment of mitochondrial DNA cosegregated with a phenotype in which the petals did not fuse, but appeared rather threadlike, and the anthers developed tissue resembling petals. This set of characteristics is highly reminiscent of some floral homeotic transformations, which are caused by mutations in a number of nuclear genes. The existence of these early organ-specification CMS phenotypes as well as the later pollen-disrupting CMS phenotypes suggests that communication between the nucleus and mitochondria is necessary throughout floral development, and that CMS phenotypes may result from disruption of this communication.

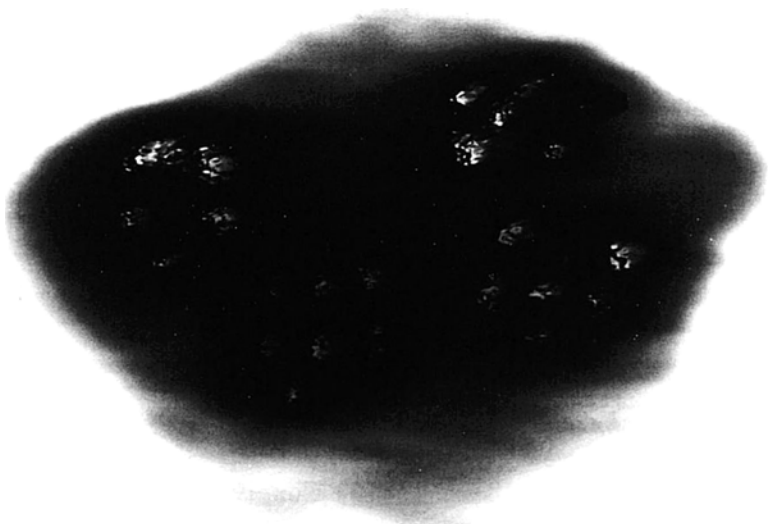

Fig. 4. Anaphase II of meiosis in a normal petunia ($n = 7$) anther. Chromosomes were stained with DAPI, visualized using a two-photon fluorescence microscope, and images were created by three-dimensional reconstruction with the IBM software package Data Explorer.

BIOCHEMISTRY OF MITOCHONDRIA DURING POLLEN DEVELOPMENT

Mitochondria play much more complex roles in plants than in nonphotosynthetic organisms. Plant mitochondria serve both as a major site of ATP synthesis and in the production of carbon skeletons and other substrates for extramitochondrial biosynthetic reactions (reviewed in Dennis, 1987; Douce and Neuburger, 1989; Siedow, 1990), and are involved in biosynthetic pathways that must proceed even when cellular energy reserves are being maintained by chloroplasts (Dennis, 1987; Raghavendra *et al.*, 1994). Plant mitochondria contain both a cytochrome oxidase pathway which is similar to that found in all eukaryotes (reviewed in Hatefi, 1985; Dennis, 1987; Douce and Neuberger, 1989; Diolez *et al.*, 1993), and an alternative pathway which is not present in animals (reviewed in Dennis, 1987; Siedow, 1990; Moore and Siedow, 1991; Moore *et al.*, 1993). The limiting effect of a high mitochondrial electrochemical gradient on the cytochrome oxidase pathway has led Diolez *et al.* (1993) to suggest that a major control point for plant respiration might be at the level of the ATP synthase, which would control release of the gradient and cap cytochrome oxidase pathway activity. Using the alternative oxidase, plants can continue electron transport, NAD^+/NADH cycling, and carbon skeleton production (reviewed in Siedow, 1990; Moore and Siedow, 1991; Moore *et al.*, 1993) even in the presence of high ATP concentrations, by bypassing the cytochrome *bc* and cytochrome oxidase proton translocating steps in the cytochrome oxidase pathway. This mechanism may be particularly important in tissues where high rates of biosynthetic activity must be maintained, such as in the tapetal layer and sporogenous tissue.

Moore *et al.* (e.g., 1992, reviewed in Moore and Siedow, 1991; Moore *et al.*, 1993) have found experimentally that the level of activation of the alternative oxidase is closely linked to the oxidation state of the ubiquinone pool, and thus propose that respiration is regulated at this level. Because the redox state of the ubiquinone pool reflects the input of the internal and external NADH dehydrogenases as well as the malate and succinate dehydrogenases on the one hand, and the reducing activity of the cytochrome oxidase and alternative oxidase pathways on the other, it might act as a central point regulating activity of both respiratory and biosynthetic activities of plant mitochondria. Recently it has been shown that the activity of the alternative oxidase is regulated by its dimerization state. In the reduced dimeric form, the alternative oxidase is more active per unit protein than when the dimer is oxidized (Umbach and Siedow, 1993), which could indicate yet another site for respiratory control in plant mitochondria.

Because of the difficulty of isolating quantities of mitochondria from young developing anthers, most studies of respiratory control in mitochondria from CMS vs. fertile plants have been performed with mitochondria from vegetative tissue. As CMS plants of most species are phenotypically normal except for disruption of microsporogenesis, mitochondria from vegetative tissues may not be appropriate material for analysis. On the other hand, perhaps mitochondria from vegetative tissue will exhibit small differences in function reflecting abnormalities that are amplified in reproductive tissue. One hypothesis concerning CMS is that microsporogenesis places a greater demand on a particular mitochondrial function that is disrupted in all tissues of CMS plants, such that the disruption is only exposed during reproductive development and not during vegetative development.

In petunia, initial oxygen electrode analyses of respiration in suspension cells and suspension cell mitochondria from CMS and fertile plants indicated lower alternative oxidase activity in sterile lines than in fertile lines (Connett and Hanson, 1990). The ubiquinone pool reduction state was unknown in these experiments. When the redox state of the ubiquinone pool was monitored along with oxygen consumption under state 4 conditions (Moore *et al.*, 1992; Moore, Ruth and Hanson, in preparation), isolated mitochondria from CMS lines showed respiratory activity over a smaller range of ubiquinone pool reduction levels than mitochondria from fertile lines. Under similar levels of ubiquinone reduction in state 4 conditions, inhibitors of the cytochrome oxidase did not affect mitochondrial respiration as severely as inhibitors of the alternative oxidase in isolated mitochondria from CMS relative to fertile plant cell lines. Whether this difference in partitioning of reducing equivalents through the two pathways is a primary or secondary effect of the petunia mitochondrial mutation is not known.

MITOCHONDRIAL GENE EXPRESSION IN CMS AND FERTILE PLANTS

Mitochondrial genes correlated with sterility have been cloned and sequenced in a number of different genera (e.g., CMS-T maize; see Dewey *et al.*, 1986;

petunia; see Young and Hanson, 1987; rice; see Kadowaki *et al.*, 1990; sunflower; see Koehler *et al.*, 1991; Polima *Brassica;* see Singh and Brown, 1991; bean; see Johns *et al.*, 1992; and Ogura radish; see Krishnasamy and Makaroff, 1992). Transcription of these novel CMS-associated genes can be regulated during development (e.g., Young and Hanson, 1987; Koehler *et al.*, 1991; Makaroff and Palmer, 1991), and reductions in specific transcripts are associated with the presence of nuclear fertility-restoration genes (Kennell *et al.*, 1987; Kadowaki *et al.*, 1990; Pruitt and Hanson, 1991; Singh and Brown, 1991; Krishnasamy and Makaroff, 1994; Moneger *et al.*, 1994).

All of the mitochondrial DNA regions that correlate with CMS in various species appear to have resulted from recombination events (e.g., CMS-T maize; see Dewey *et al.*, 1986; petunia; see Young and Hanson, 1987; rice; see Kadowaki *et al.*, 1990; sunflower; see Koehler *et al.*, 1991; Polima *Brassica;* see Singh and Brown, 1991; bean; see Johns *et al.*, 1992; and Ogura radish; see Krishnasamy and Makaroff, 1992). The petunia CMS-associated region includes portions of mitochondrial respiratory complex genes, as well as an unidentified region not found elsewhere in petunia mitochondrial genomes (Young and Hanson, 1987; Pruitt and Hanson, 1991). This region of DNA was first identified by cosegregation of restriction fragments with the sterility phenotype in somatic hybrid plants regenerated from protoplast fusions of cells from fertile and sterile plants. Somatic hybrid plants from several independent protoplast fusions showed three distinct phenotypes; plants were either stably fertile, stably sterile, or produced fertile branches on otherwise sterile plants (Izhar *et al.*, 1983). Restriction digestion analyses of chloroplast and mitochondrial genomes demonstrated that no fragments of either chloroplast genome were specifically associated with the sterility phenotype (Clark *et al.*, 1985). However, two small mitochondrial DNA fragments from the sterile parent segregated exclusively with sterile somatic hybrid progeny (Boeshore *et al.*, 1985).

Cloning and sequencing of the petunia sterility-associated mtDNA fragments revealed the presence of a fused gene that was named *pcf* for *P*etunia *C*MS-associated *F*used gene (Fig. 5). The *pcf* gene is composed of the 5′ upstream sequences and the first membrane-spanning domain of ATP synthase subunit 9 (*atp9*), sequences coding for the first two membrane-spanning domains of cytochrome oxidase subunit II (*coxII*), and an unidentified region termed *urf-S* (Young and Hanson, 1987). Full-length and apparently functional copies of *atp9* and *coxII* exist at other sites in the CMS-associated mitochondrial genome (Young *et al.*, 1986; Pruitt and Hanson, 1991). No sequences homologous to *urf-S* have been found in database searches, and its codon bias is not characteristic of plant mitochondrial genes (Nivison *et al.*, 1994). Two additional cotranscribed genes are located downstream from the *pcf* gene, encoding the NADH dehydrogenase subunit 3 (*nad3*) and the small subunit ribosomal protein 12 (*rps12*), which are the only copies of these two genes present in the CMS-associated mitochondrial genome (Hanson *et al.*, 1988; Rasmussen and Hanson, 1989).

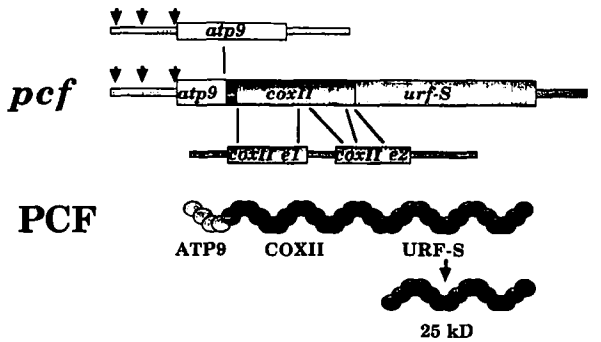

Fig. 5. The petunia CMS-associated fused gene and protein products. Portions of the normal mitochondrial genes that contributed to the *pcf* gene are indicated, and the transcription start sites are marked with arrowheads.

Cotranscription of other genes with a CMS-associated gene is not unique to petunia, but has been observed also in *Brassica* (L'Homme and Brown, 1993), maize CMS-T (Levings, 1993), and sunflower (Koehler *et al.*, 1991) CMS genotypes. One hypothesis for the disruption of pollen development in CMS plants suggests that normal mitochondrial genes cotranscribed with the novel CMS-associated open reading frames may be aberrantly expressed in CMS plants, and it is actually misexpression of these essential genes that causes male sterility. While this hypothesis has not been formally disproved for any system, it may not be a general mechanism causing CMS. CMS-associated alterations in expression of the cotranscribed genes from petunia, Ogura radish, and maize CMS-T have not been detected (R. Wilson, B. Lu, M. Mulligan, and M. Hanson, unpublished; Krishnasamy and Makaroff, 1992; Levings, 1993). In Polima CMS *Brassica,* however, reduced levels of ATP synthase subunit 6 (*atp6*) transcripts are found in floral tissues, which may explain the specificity of the CMS defect in this system (Singh and Brown, 1991).

Transcriptional analysis of the *pcf* locus in CMS-associated mitochondria indicates that the genes of the *pcf* locus are cotranscribed. There are three 5′ ends in *pcf* transcripts that are found in transcripts of the normal *atp9* gene, as would be expected since the 5′ sequences for both regions are identical. The 3′ ends of the *pcf* transcripts also are found in normal transcripts of *nad3* and *rps12*, which are cotranscribed in normal mitochondria (Rasmussen and Hanson, 1989; Pruitt and Hanson, 1991). Transcripts of the *pcf* locus are regulated in a tissue-specific manner. Steady-state levels of *pcf* transcripts are 4–5 times more abundant in anthers relative to leaves, although the normal *atp9* gene is present at equal levels in both tissues (Young and Hanson, 1987). Analysis of mitochondrial transcripts from fertility-restored plants indicates that steady-state levels of the shortest *pcf* transcript are specifically reduced in the presence of the nuclear *Rf* restorer gene (Pruitt and Hanson, 1991).

A protein, termed the PCF protein, is translated from transcripts of the *pcf* gene (Fig. 5). No sterile-specific proteins are recognized on immunoblots by synthetic peptide antibodies raised against *coxII*-encoded sequences that are present in the *pcf* gene, although the COXII peptide antibodies do recognize normal COXII protein (Nivison and Hanson, 1989). Antibodies raised against a fusion protein expressed from a construct containing the *urf-S* portion of the *pcf* coding region or a synthetic peptide from that region recognize a band of apparent molecular weight 25 kD on immunoblots of isolated mitochondrial protein or total protein from sterile plants (Nivison and Hanson, 1989; Conley and Hanson, 1994; Nivison *et al.*, 1994). The peptide antibodies immunoprecipitate a protein of apparent molecular weight 25 kD from *in organello* labeled mitochondria (Nivison and Hanson, 1989), as well as a larger precursor protein of apparent molecular weight 55 kD. Amino-terminal protein sequencing of the immunoprecipitated apparent 25-kD protein identified a sequence internal to the *urf-S* portion of the *pcf* gene, which is located adjacent to a putative protease processing site (Nivison *et al.*, 1994). The 25-kD protein is found in both the membrane and soluble portions of fractionated mitochondria, a fractionation pattern characteristic of peripheral membrane proteins (Nivison and Hanson, 1989). Immunoprecipitation of protease-treated intact mitochondria labeled *in organello* with tritiated leucine demonstrated that most of the 25-kD band and a fraction of the full-length PCF protein were protected, which suggests that the apparent 25-kD protein may be associated with mitochondrial membranes, perhaps in a larger protein complex (Nivison *et al.*, 1994).

TISSUE-SPECIFIC GENE EXPRESSION DURING POLLEN DEVELOPMENT

The unique protein encoded by CMS-associated *pcf* gene of petunia is present in mitochondria of cells of both vegetative and reproductive tissue (Nivison and Hanson, 1989; Conley *et al.*, 1991; Conley and Hanson, 1994; Nivison *et al.*, 1994). Nevertheless, expression of PCF does vary in different tissues. The tissue-specific localization of the PCF protein has been examined using the technique of tissue-printing, in which unfixed plant tissues are blotted to nitrocellulose membrane, transferring cellular material while preserving the tissue structure (Conley and Hanson, submitted; Ye and Varner, 1991). In tissue-print immunoblots of vegetative tissues, polyclonal antibodies raised against the URF-S portion of the PCF protein localize specifically to vascular tissue in stems and leaves of sterile plants, but do not recognize antigens in tissue-prints of fertile plants, and bind only at very low levels to tissue-prints of restored plants (Conley *et al.*, 1991; data not shown). In developing floral buds, the anti-URFS antibodies bind most strongly to the sporogenous tissue and tapetal layer of premeiotic and meiotic CMS anthers, as well as binding to the anther vascular bundle (Conley and Hanson, 1994). Interestingly, anti-URFS antibodies also localize strongly to developing ovules in ovaries of CMS plants, although the plants are completely female-fertile (Conley and Hanson, 1994; data not shown).

Tissue-level immunolocalization of the 25-kD PCF protein and several mitochondrial respiratory complex subunits in immature floral buds from fertile and CMS plants demonstrates that plant cells control the relative expression of individual mitochondrial proteins in a tissue-specific manner (Fig. 6; Conley and Hanson, 1994). The PCF protein is localized to the vascular bundle, tapetal layer, and sporogenous tissue of premeiotic and meiotic anthers from CMS plants, which is very similar to the localization shown by the α subunit of the mitochondrial ATP synthase (ATPA) (Conley and Hanson, 1994). Subunit II of the cytochrome oxidase (COXII) is found predominantly in the subepidermal and connective tissue of similar-stage anthers from both fertile and CMS plants. The alternative oxidase (AO) is localized to the sporogenous tissue of premeiotic and meiotic stage anthers, and is found

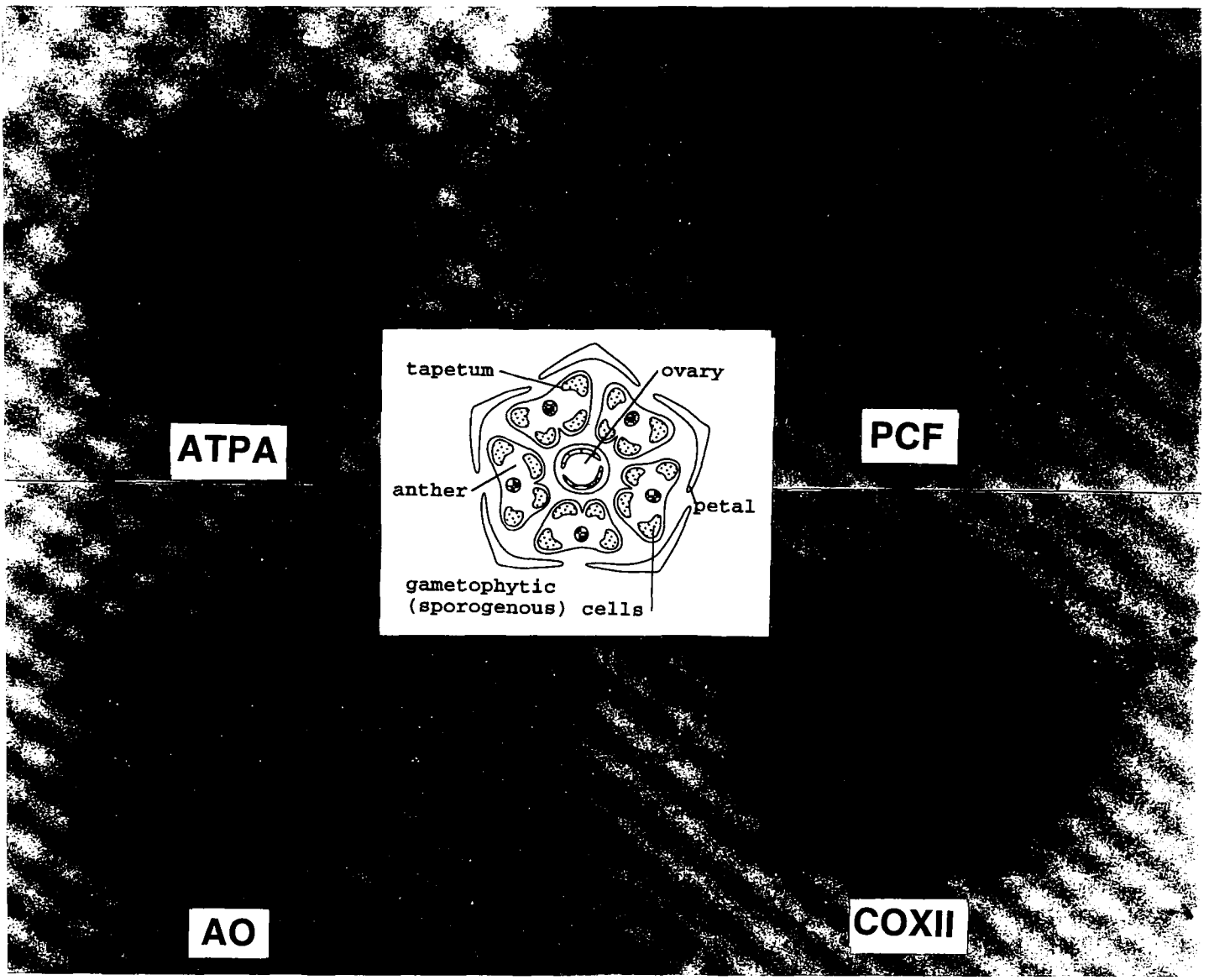

Fig. 6. Immunolocalization of mitochondrial proteins. Cryostat tissue prints of a sterile bud at a stage equivalent to tetrad stage in the fertile bud were probed with antibodies to the mitochondrial proteins indicated. The inset diagram shows the arrangement of bud tissues.

in the developing tetrads of fertile plants and the degenerating sporogenous tissue of anthers from CMS plants. The alternative oxidase is not present at high levels in developing ovules (Conley and Hanson, 1994; data not shown).

Expression of the *pcf* gene in early meiosis may be critical for the devastating action of the PCF protein. Transgenic plants expressing the 25-kD protein encoded by the *urf-S* portion of the *pcf* gene under control of the constitutive 35S promoter and the TA29 tapetal-specific promoter (Koltunow *et al.*, 1990) were male fertile (Wintz *et al.*, 1995). The 35S promoter and the TA29 promoter exhibit low activity in sporogenous tissue in early meiosis. These plants also did not fully mimic CMS plants in 25-kD protein expression, in that all of the transgenic protein was found in the soluble fraction while a portion of the genuine 25-kD protein can be found in the membrane fraction of anthers from CMS plants (Wintz *et al.*, 1995).

Tissue-specific expression of CMS-associated genes of other species has also been observed. Transcripts of the sunflower CMS-associated *orf522* (Koehler *et al.*, 1991) are found in meiotic cells from male florets of CMS plants, but at reduced abundance

only in male meiotic cells of plants carrying a nuclear restorer gene (Smart *et al.*, 1994). The PVS protein associated with CMS in bean is specifically localized to male and female reproductive tissue, and interferes with callose deposition (Abad *et al.*, 1995). Not only the protein but the entire region of mtDNA may be reduced by the presence of different nuclear fertility-restoration genes (He *et al.*, 1995). In fertility-restored Ogura radish plants, the CMS-associated ORF138 protein is reduced in leaves and flowers but not roots, although no effect on transcript levels was observed (Krishnasamy and Makaroff, 1994). Fertility restoration in Polima *Brassica* may occur by preventing the floral-specific reduction of *atp6* transcripts observed in CMS plants (Singh and Brown, 1991).

Increased abundance of a detrimental protein in reproductive tissues is one possible explanation for the specific disruption of sporogenesis by CMS-associated genes. However, comparisons of mitochondrial protein abundance from one tissue to the next can be difficult to interpret. What could seem to be a vast increase in abundance of a mitochondrial protein could instead be simply an increase in numbers of mitochondria. Lee and Warmke (1979) found a 20- to 40-fold increase of mitochondria in the tapetal layer and sporogenous tissue of maize during early anther development. Huang *et al.* (1994) suggest that higher levels of the Rieske iron-sulfur protein and both the α and β subunits of the mitochondrial ATP synthase found in tobacco floral tissue are due to increased numbers of mitochondria. In petunia, a simple increase in mitochondrion number cannot be the complete explanation for the increase in the PCF protein, since several normal mitochondrially encoded proteins show different localization patterns (Conley and Hanson, 1994).

An alternative explanation for the specific action of CMS genes in pollen abortion is to invoke the presence, only during sporogenesis, of an interacting compound or macromolecule that is needed to potentiate the detrimental effect of the CMS-associated protein. Perhaps specific nuclear-encoded mitochondrial proteins are expressed only in tapetal or pollen-forming tissue and interact with CMS-associated proteins to cause disruptions only in those tissues. Anther-specific isoforms of a number of nuclear genes that are expressed in vegetative tissue are now being identified, for example, the plasma membrane proton-translocating ATPase *aha9* of *Arabidopsis* (Houlne and Boutry, 1994), and the α-tubulin gene *tual* of *Arabidopsis* (Carpenter *et al.*, 1992). A pollen-specific isoform of the nuclear-encoded mitochondrial ATP synthase β subunit has been identified in petunia (DePaepe *et al.*, 1993).

A mitochondrially encoded gene product specifically expressed in reproductive tissue could also be the hypothetical factor that unmasks the detrimental effect of a CMS-associated protein. The different localization patterns observed for petunia COXII and ATPA demonstrate that the expression of individual normal mitochondrially encoded genes is controlled differentially in the same plant tissue (Conley and Hanson, 1994). Maize mitochondria isolated from vegetative or reproductive tissues and labeled *in organello* with radioactive amino acids were found to synthesize polypeptides specific for each tissue type (Newton and Walbot, 1985). In maize, transcripts of the mitochondrially encoded cytochrome b (*cob*), and the two ATP synthase subunits 6, and 9, increased in abundance until after the vacuolate microspore stage of pollen development, and then decreased to barely detectable levels in mature pollen grains (Moneger *et al.*, 1994).

Flavell (1974) was the first to suggest that a molecule similar to T-toxin might be expressed only in maize anthers, explaining why the maize *T-urf13* gene product is detrimental only in anthers and when T-toxin is present as a result of fungal infection. However, no developmentally regulated protein has been shown to interact with any CMS-associated protein as yet. We can only speculate about interactions based on the simultaneous presence of such proteins and CMS-associated proteins. The PCF protein is highly expressed in the tapetal layer of CMS anthers prior to male meiosis, at a stage when the ATP synthase subunit A, but neither COXII nor the alternative oxidase, are expressed in a tissue-specific manner. The colocalization of PCF and ATPA in petunia CMS anthers suggests that functioning of the ATP synthase in CMS systems may be a fruitful target for further analyses. Genes encoding ATP synthase subunits (e.g., petunia; see Young and Hanson, 1987; rice; see Kadowaki *et al.*, 1990; sunflower; see Koehler *et al.*, 1991; Polima *Brassica;* see Singh and Brown, 1991; wheat; see Pfeil *et al.*, 1994; and sugarbeet; see Xue *et al.*, 1994) are involved in many CMS-associated mitochondrial DNA rearrangements.

These differences in protein localization in fertile vs. CMS petunia anthers complement hypotheses suggested by the differences in partitioning between the cytochrome oxidase and alternative oxidase pathways identified by oxygen electrode analyses (Connett and Hanson, 1990; Moore *et al.*, 1992). High levels of the PCF protein in premeiotic anthers may accentuate the

interference with normal mitochondrial function. If the PCF protein were interfering with the ATP synthase of CMS plants, this could inhibit cytochrome oxidase activity by elevation of the mitochondrial electrochemical gradient, which might result in an increased contribution of alternative oxidase activity to the total respiration relative to fertile plants at similar ubiquinone reduction. If the PCF protein disrupts regulation of the ubiquinone reduction state, this might prevent the alternative oxidase from becoming engaged as fully in CMS plants as in fertile plants that are regulating redox poise effectively, which could appear as lower alternative oxidase activity at certain levels of ubiquinone reduction. Why disruption of these various complexes might specifically affect pollen development is unclear; however, it is likely that either tightly regulated energy production or synthesis of pollen-specific compounds, or both, would be required for proper development. Interestingly, maize plants heteroplasmic for normal mitochondria and mitochondria carrying a mutation in the *coxII* gene show severely aberrant anther and pollen development in sectors of plant tissue that have become homoplasmic or near-homoplasmic for the mutant mitochondria, suggesting that a threshhold level of cytochrome oxidase activity is required for proper development (K. Newton, personal communication). Further characterization of the physiological, morphological, and molecular alterations caused by CMS in petunia and other CMS systems will continue to reveal how the various functions of plant mitochondria influence plant development.

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